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Development of physiologically based pharmacokinetic models for the bioaccumulation of persistent organic pollutants in marine mammals

Ontwikkeling van fysiologisch gebaseerde farmacokinetische modellen voor de bioaccumulatie van persistente organische polluenten in zeezoogdieren

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Wetenschappen: Biologie aan de Universiteit Antwerpen

Te verdedigen door **Liesbeth WEIJS**

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LIST OF ABBREVIATIONS

PCB	-	Polychlorinated biphenyl
PBDE	-	Polybrominated diphenyl ether
$MeSO_2$-PCB	-	Methylsulfon-PCB
HO-PCB	-	Hydroxylated PCB
HO-PBDE	-	Hydroxylated PBDE
MeO-PBDE	-	Methoxylated PBDE
HBCD	-	Hexabromocyclododecane
HCB	-	Hexachlorobenzene
CHL	-	Chlordanes
POP	-	Persistent organic pollutant
DDT	-	Dichlorodiphenyltrichloroethane
DDE	-	Dichlorodiphenyldichloroethylene
DDD	-	Dichlorodiphenyldichloroethane
o	-	*Ortho*
p	-	*Para*
DDX	-	Sum of *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDD, *p,p'*-DDD, unless otherwise specified in the respective paper
PDV	-	Phocine Distemper Virus (same as PMV or Phocine MorbilliVirus)
DDV	-	Delphinoid Distemper Virus (same as DMV or Delphinoid MorbilliVirus)
ADME	-	Absorption, Distribution, Metabolism, Excretion
MCMC	-	Markov chain Monte Carlo
MLE	-	Maximum Likelihood Estimation
DENSF	-	Density of the blubber
DENSB	-	Density of the brain
DENSL	-	Density of the liver
DENSK	-	Density of the kidneys
DENSBlood	-	Density of blood
DENSR	-	Density of the muscle (rest of the body)
FATPERCF	-	Lipid percentage in the blubber
FATPERCK	-	Lipid percentage in the kidneys
FATPERCL	-	Lipid percentage in the liver
FATPERCB	-	Lipid percentage in the brain
FATPERCR	-	Lipid percentage in the muscle (rest of the body)
FATPERCBlood	-	Lipid percentage in the blood
QFC	-	Fractional blood flow to the blubber
QLC	-	Fractional blood flow to the liver
QBC	-	Fractional blood flow to the brain
QKC	-	Fractional blood flow to the kidney
QRC	-	Fractional blood flow to the muscle (rest of the body)
PF	-	Blubber/blood partition coefficient
PL	-	Liver/blood partition coefficient
PB	-	Brain/blood partition coefficient
PK	-	Kidney/blood partition coefficient
PR	-	Rest of the body or muscle/blood partition coefficient
TOTDIET	-	Concentration in the fish diet
AE1	-	Assimilation efficiency from the fish diet
AE2	-	Assimilation efficiency from the milk diet

FATPERCMilk	-	Lipid percentage of the milk
CMILK	-	Concentration in the milk diet
K_{fp}	-	Adipose tissue/plasma partition coefficient
AUC	-	Area under the curve
F/M	-	Fetus/mother ratio
IN	-	Assimilation efficiency
DCMILK	-	Daily consumption of milk
CFoetusF	-	Concentration in blubber of foetus
CFoetusL	-	Concentration in liver of foetus
CFoetusK	-	Concentration in kidney of foetus
CFoetusB	-	Concentration in brain of foetus
CFoetusBlood	-	Concentration in blood of foetus
X	-	Rate constant for elimination through feces
$t_{1/2}$	-	Metabolic half life
CFoetusR	-	Concentration in rest of the body of the foetus
NIST	-	National Institute for Standards and Technology
AMAP	-	Arctic Monitoring and Assessment Program
SD/StD	-	Standard deviation
SE	-	Standard error
A_t	-	Amount of chemical in a specific compartment or tissue t
Q_t	-	Blood flow to compartment t
P_t	-	Partition coefficient between the blood and tissue t
C_b	-	Concentration of the chemical in blood
C_t	-	Concentration of the chemical in tissue t
A_m	-	Amount of the chemical that has been metabolically transformed into something else
Q_l	-	Blood flow to the liver
E_h	-	Hepatic extraction rate
$C_{l,int_{c}}$	-	Intrinsic clearance per kilogram of liver at the age of half-life (HL_h; expressed in hours, h)
V_d	-	Volume of distribution
V_t	-	Volume of tissue
V_b	-	Volume of blood
OxC	-	Oxychlordane
TN	-	*Trans*-nonachlor
CN	-	*Cis*-nonachlor
TC	-	*Trans*-chlordane
SC	-	Sandy Cape, Tasmania
S	-	Stanley, Tasmania
RI	-	Robbins Island, Tasmania
BB	-	Butlers Beach, Tasmania
GSA	-	Global sensitivity analysis
LSA	-	Local sensitivity analysis

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'...no model can be said to be 'correct'. The role of any model is to provide a framework for viewing known facts and to suggest experiments.'

-- Suresh Moolgavkar

'All models are preliminary. There is no such thing as a final model.'
-- Raymond S. H. Yang

Chapter 1

Introduction

1.1.1. Chemical pollution

1.1.1.1 Background

Over the last decades, the chemical industry has undergone a large evolution. This is not only due to an increasing number of people and thus a greater demand for products, but also to an extensive industrial modernization and expansion. These factors are responsible for large amounts of chemicals in the environment (Tanabe et al., 1994) with often different physicochemical properties (Walker et al., 2006). For several decades, only the benefits of the chemicals were seen because of a lack of knowledge about these properties. As a result, they were produced on a global scale (Iwata et al., 1994; Simonich and Hites, 1995; Breivik et al., 2002; Fernandez and Grimalt, 2003) and used in a variety of products and equipment ranging from insecticides to flame retardants (ATSDR, 2001, 2002a, 2002b, 2004). However, the toxic impact of several chemicals eventually outweighed the benefits (Chen and Rogan, 2003; Birnbaum and Staskal, 2004). Some time after the discovery of the toxic effects of the chemicals on humans, wildlife and environment, scientists slowly became aware of the fact that the negative effects of chemicals had to be distributed worldwide as well. This has lead to a growing number of studies investigating the presence of pollutants in all layers of the food chains in all corners of the world and their effects on several systems (e.g. endocrine system, immune system, reproductive system) of all kinds of organisms (e.g. Chen and Rogan, 2003; Law et al., 2003; Schantz et al., 2003; Fritsche et al., 2005; Athanasiadou et al., 2008; Shaw and Kannan, 2009; Sonne et al., 2009; Zhang et al., 2009).
On a regulatory scale, the awareness of the harmful effects of several chemical classes resulted in the establishment of the Stockholm Convention which aims to eliminate or restrict the production and use of several persistent organic pollutants (POPs). At first, this international treaty focused on 12 pollutants or pollutant classes. Another 9 pollutant(s) (classes) were added to the list in 2009. The latest addition was endosulfan and its related isomers which were added to the list in 2011 (www.pops.int). Obviously, the Stockholm Convention develops in time and tries to keep up with the latest and newest chemicals. Evidence for that are hexabromocyclododecane (HBCD), short-chained chlorinated paraffins, chlorinated naphtalenes, hexachlorobutadiene and pentachlorophenol which are currently under review and potential additions to the Convention in the near future.

Nowadays, new chemicals are produced all the time as a consequence of the continuous search for more and more innovative products. The Stockholm Convention on POPs is therefore an ongoing initiative although it has a more retrospective nature than the European REACH legislation. REACH (Registration, Evaluation and Authorisation of Chemicals) aims to 'improve the protection of human health and the environment from the risks of chemicals while enhancing the competitiveness of the chemical industry of the European Union' (REACH, 2007). Improving the protection of human health and the environment is achieved through a better and earlier identification of the intrinsic properties of chemicals making REACH a more prospective initiative

than the Stockholm Convention. Before commencing a global scale production of chemicals, REACH forces the industry to test and classify their chemicals, preferably through non-animal testing. Although *in silico* or computer-based research does not use animals in theory, it needs the input of datasets acquired from biomonitoring efforts and may benefit from knowledge gathered during exposure experiments. It is therefore connected to some extent to studies that involve animal testing and is not a stand-alone approach. However, *in silico* models can fill missing gaps in biomonitoring datasets and provide ideas for experimental set-ups. As such, they can minimize the number of organisms sampled in biomonitoring studies or restrict the number of (animals used in) exposure experiments. Consequently, computer-based models are definitely approved by REACH (Bouvier d'Yvoire et al., 2007).

This work investigates the bioaccumulation of several Persistent Organic Pollutants (POPs) in marine mammal species by using computerized models. The targeted POPs are polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides and their associated products, e.g. (hydroxylated) metabolites and isomers, which are all included in the Stockholm Convention. The computerized models are **p**hysiologically **b**ased **p**harmaco**k**inetic (PBPK) models, a type of models that fits in the REACH legislation.

1.1.1.2 Classes of chemicals

PCBs

Polychlorinated biphenyls (PCBs) are a type of POPs that exist of 209 congeners. Each congener differs from the other in the number and the position of the chlorine-atoms on the phenyl rings ($C_{12}H_{10-n}Cl_n$ or $C_{12}H_{10-n}Br_nO$ with $1 < n < 10$) (Fig 1A) (ATSDR, 2001).

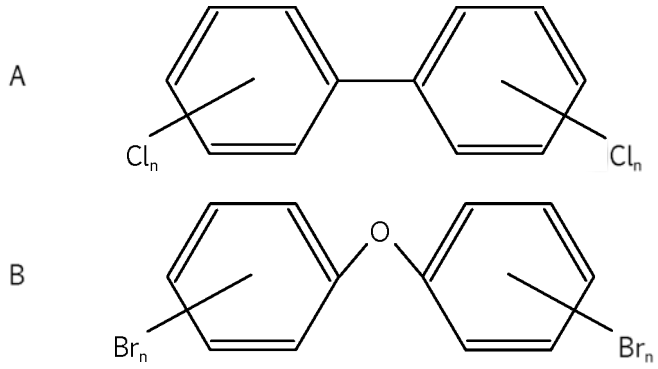


Fig 1. Chemical structure of PCBs (A) and PBDEs (B).

Most congeners are stable and resistent against chemical reactions. As such, they have been used as fire retardants and coolants in all kinds of equipment ranging from furniture to textile, paint, glue and transformers. PCBs are lipophilic and dissolve readily in organic solvents, fats and oils. Because of their stability and persistence, these compounds bioaccumulate in the environment, wildlife and humans. Although organisms have various pathways to (slowly) eliminate PCBs (e.g. metabolic breakdown, fecal or urinary excretion), these processes depend on the degree of chlorination of the compounds and on the species of interest, but are limited overall (Letcher et al., 2000; Houde et al., 2005; McKinney et al., 2006a). As a result,

PCBs are capable of biomagnifying in the food chain (i.e. the transfer of pollutants from a lower trophic level to a higher trophic level reaching higher concentrations with each step up) (Ruus et al., 1999; Wolkers et al., 2006; Voorspoels et al., 2007).

In theory, PCBs can be transformed into two different types of metabolites: methylsulfon-PCBs ($MeSO_2$-PCBs) or hydroxylated PCBs (HO-PCBs) (Letcher et al., 2000). The enzyme-mediated insertion of sulfur or oxygen is a common way for metabolic breakdown of POPs and tends to give more polar compounds. These polar compounds should, in theory, be excreted more efficiently than the parent compounds. However, elevated levels of PCB metabolites found in several species suggest otherwise (Sandala et al., 2004; McKinney et al., 2006b; Dirtu et al., 2010). The retention of both $MeSO_2$-PCBs and HO-PCBs mostly in liver and blood, respectively, can be explained by their greater affinity for proteins. Compared to the parent PCBs, this may result in higher elimination half-lives for some of the metabolites. So from this perspective, the metabolism of PCBs only leads to a next generation of persistent pollutants rather than a way for eliminating the initial problem (Letcher et al., 2000).

PBDEs

Polybrominated diphenyl ethers (PBDEs) have a similar structure to PCBs as they also have two phenyl rings and exist in 209 different congeners (Darnerud et al., 2001). Compared to the PCBs, PBDEs differ in the number and the position of the bromine-atom instead of the chlorine-atom for PCBs. Additionally, the two phenyl rings are connected with an oxygen-atom in contrast to the PCB phenyl rings (Fig 1B) (ATSDR, 2004). PBDEs have been used mostly as flame retardants (Birnbaum and Staskal, 2004; ATSDR, 2004). Similar as for PCBs, PBDEs are, depending on the number of bromine atoms, persistent and bioaccumulative (Wolkers et al., 2004a, 2006; Sørmo et al., 2006; Kelly et al., 2008).

For several years, anthropogenically produced PBDEs were assumed to be broken down into lower brominated compounds or metabolically biotransformed into hydroxylated metabolites (HO-PBDEs) (Stapleton et al., 2004; McKinney et al., 2006b; Stapleton et al., 2009) whereas many methoxylated PBDEs (MeO-PBDEs) were considered as a separate class of chemicals because of their natural origin. Evidence for the natural origin of MeO-PBDEs were provided by several studies. Teuten et al. (2005) found two MeO-PBDEs in whale blubber (True's beaked whale; *Mesoplodon mirus*) and concluded that these compounds were naturally produced by analyzing the ^{14}C-content. In another study, Teuten and Reddy (2007) measured MeO-PBDEs in pre-industrial whale oil from 1921 which was a time when PBDEs were not produced yet. Furthermore, organisms such as algae and sponges were identified as natural producers of MeO-PBDEs (Vetter et al., 2002; Malmvärn et al., 2008). However, studies conducted recently *in vitro* with fish, chicken and rat microsomes have suggested that there can be an interconversion of HO-PBDEs and MeO-PBDEs (Wan et al., 2009; Wan et al., 2010). These findings indicate that MeO-PBDEs might not be solely naturally produced after all. Nevertheless, further research is required to shed some light on the possible conversion pathways and eventually on the origin of HO-PBDEs and MeO-PBDEs.

Organochlorine pesticides

Hexachlorobenzene (HCB) is a by-product of several processes (e.g. combustion or burning of waste, production of solvents or pesticides) which has been used mostly as a fungicide until its ban in the 1970s in Europe (Fig 2A). As for PCBs and PBDEs, HCB is hydrophobic and accumulates in organisms (Borgå et al., 2001; ATSDR, 2002b). Known metabolites of HCB in rodents and humans are various phenolic derivatives such as pentachlorophenol (PCP) as well as tri- and tetrachlorophenol (To-Figueras et al., 1997).

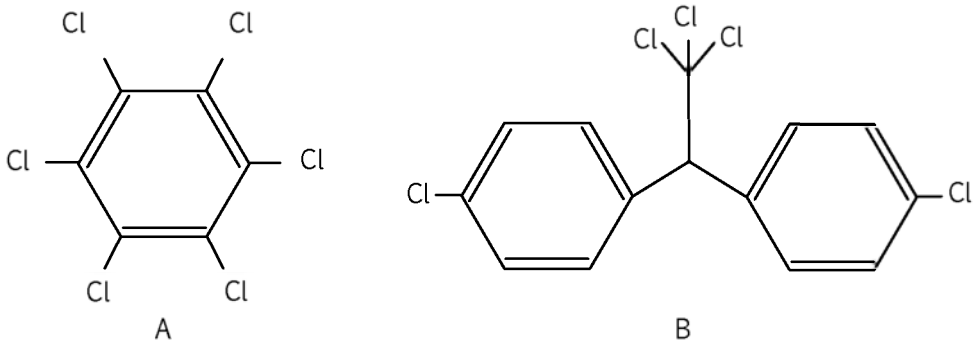


Fig 2. Chemical structure of HCB (A) and DDT (B).

Dichlorodiphenyltrichloroethane (DDT) is one of the best known pesticides worldwide (Fig 2B). The commercial product exists of different isomers (*p,p'*- and *o,p'*-isomers) and metabolites (dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyldichloroethane (DDD)) (ATSDR, 2002a). The composition of the commercially available mixture can be an explanation for a part of the DDD and DDE concentrations detected in wildlife. In some organisms, however, DDE and DDD can also be the result of the metabolic biotransformation capacities of the organism. So, whereas DDT is entirely human-made, the origin of DDE and DDD is not always very clear (ATSDR, 2002a). Its efficient use as poison against several arthropod species has been demonstrated in the past in agriculture and disease prevention (e.g. malaria; Roberts et al., 1997). Since the discovery of the environmental impact, the use of DDT mixtures has been banned or restricted (Roberts et al., 1997).

Chlordane as a pesticide is actually a mixture of related compounds such as *trans*-nonachlor, *cis*-chlordane and *trans*-chlordane (ATSDR, 1995). Chlordane has been used in agriculture and for fighting termites in houses. As for other lipophilic compounds, chlordane does not dissolve in water easily and tends to accumulate in lipid-rich tissues of organisms. This pesticide has been associated with the presence of various cancers and is known to be persistent in the environment (ATSDR, 1995). Chlordane is one of the 12 initial POPs included in the Stockholm Convention.

1.1.2. Persistent chemicals in marine wildlife

Due to their extensive use in the past, PCBs, PBDEs and organochlorine pesticides are ubiquitous in the environment and levels of these contaminants have been described in a variety of marine wildlife species

ranging from zooplankton and deepsea fish to polar bears (Law et al., 2003; Covaci et al., 2008; Kelly et al., 2008; Gebbink et al., 2008a).
As top predators in aquatic ecosystems, marine mammals are endpoints for compounds that biomagnify up in the food webs (Mössner and Ballschmiter, 1997; Ross, 2000). Levels of pollutants are generally higher in marine mammals than in their prey. To date, the highest concentrations were found in polar bears and killer whales both of which also eat smaller marine mammal species such as porpoises, dolphins, seals and sea lions (Ross, 2006; Wolkers et al., 2007; Letcher et al., 2009). In contrast, Mysticeti or baleen whales have generally the lowest concentrations because they consume only planktonic organisms (Houde et al., 2005). These organisms are situated at the basis of the food webs and accumulate chemicals as well, but not relatively as much as fish or any other organism at higher stages of the food web.

1.1.3. Impact of chemical pollution on the health of marine mammals

In the wild, it is difficult to assign an outcome or effect to the presence of a specific chemical because of the high number of chemicals to which marine mammals are simultaneously exposed and the potential interactions among the chemicals (Hall et al., 2007; Mori et al., 2008). Nevertheless, direct or indirect evidences of contaminant induced health effects in marine mammals can be found in the literature. Significantly higher PCB concentrations were measured in harbour porpoises that have died of infectious diseases than in animals that have died of acute physical trauma (Jepson et al., 2005). Das et al. (2006) suggested a relationship between PCBs, PBDEs, DDE and DDT and interfollicular fibrosis in harbour porpoise thyroids. Mos et al. (2007), Vanden Berghe et al. (2010 and 2013) and Debier et al. (2005) reported an influence of organochlorines on the vitamin A homeostasis in harbour seals (Mos et al., 2007), grey seals (Vanden Berghe et al., 2010 and 2013) and sea lions (Debier et al., 2005). *In vitro* experiments using marine mammal cells exposed to selected POPs were also conducted and revealed reduced cell proliferations in beluga whale splenocytes (De Guise, 1998), affected reactive oxygen species (ROS) levels, thiol levels and phagocytosis in harbour seal granulocytes (Frouin et al., 2010), increased mitogen-induced lymphocyte proliferation in blood cells of harbour seals (Levin et al., 2005) and other marine mammal species (Mori et al., 2006 and 2008) and reduced phagocytosis of bottlenose dolphin and beluga whale leukocytes (Levin et al., 2004). Other effects include reproductive failure for harbour seals (Reijnders, 1986) and other seal species (Bergman, 1999) as well as claw lesions, colonic ulcers, occlusions, stenoses and tumours in the uterus (Bergman, 1999) and bone-related effects (Routti et al., 2008) in grey and ringed seals. In a unique feeding study in which harbour seals were fed a contaminant-rich diet or a contaminant-poor diet over a longer period, the animals showed impaired immune systems (de Swart et al., 1996). Thus, marine mammals do not only accumulate high concentrations of pollutants, they also experience the negative effects of these chemical loads on multiple systems thereby affecting their overall development and survival (Houde et al., 2005; Gulland and Hall, 2007).

1.2 Physiologically based pharmacokinetic modeling: scientific background

1.2.1. Kinetics of chemicals

In pharmacological research and the pharmaceutical industry, PBPK models are routinely used to investigate the kinetics of a pharmaceutical in an organism. The efficacy of a drug depends not only on whether the drug is capable of acting upon the desired target site, but also on whether the administered concentration is appropriate for the purpose. All this requires basic knowledge about the kinetics of the drug which can be divided into Absorption, Distribution, Metabolic biotransformation and Excretion (ADME) processes (Clewell and Clewell III, 2008). However, pharmaceuticals are not the only chemicals that are the focus of PBPK models. PBPK models also exist for environmentally relevant chemicals giving the perhaps more reasonable term 'physiologically based toxicokinetic' (PBTK) models. One can argue that there are differences between the two models, but in essence, they are the same as they have both the power to unravel the kinetics of a chemical in an organism.

1.2.1.1 Absorption

Chemicals can get into the body of an organism through several general routes. Inhalation, dermal and oral exposure scenarios depend largely on the properties of the chemical and on the body features of the organism. Airborne PCBs are thought to have only minor contributions to the overall body load of PCBs in humans (Duarte-Davidson and Jones, 1994) and in wildlife such as marine mammals (Hickie et al., 1999), whereas inhalation exposure to DDT in occupational settings can exceed by far the oral/dermal exposure (Sereda et al., 2009). Regarding marine mammals, oral exposure represents the major source of intake of lipophilic compounds. Although a significant water flux across the skin of fasting dolphins has been reported (Hui, 1981). The levels of lipophilic compounds are very low in water making the skin a minor route for absorption.
The amount of a chemical that is administered is not necessarily the same as the amount that is effectively absorbed. Chemicals that are not absorbed are not bioavailable for the organism and are thus useless (for pharmaceuticals) or harmless (for toxic compounds) for the organism. Depending on the administration route, the chemical has to pass through some barriers to get into the blood. Consequently, the administration route, the dimensions and features of those barriers and of the chemical determine to a great extent the percentage of the administered concentration that ends up in the bloodstream. In PBPK models, this percentage is often called the 'assimilation efficiency'. In case the assimilation efficiency is lower than 100%, the internal concentration to which the model organism is actually exposed to is lower than the administered concentration. It is important to distinguish between the internal dose or concentration and the administered dose or concentration as the former is a much more reliable dose metric for assessing drug efficacy or toxicity of a chemical.

1.2.1.2 Distribution

Once a chemical has entered the blood, it is subject to distribution processes as well as other processes such as metabolism and elimination. In due course, these processes lead to the transport of the chemical to the target sites. The proportion of the absorbed chemical concentration ending up in a certain tissue depends on the physicochemical properties of the chemical (an environmental contaminant in this work), on the composition of that specific tissue (e.g. lipid content) compared to the blood and on the amount of blood received by the tissue (i.e. the blood flow rate to the tissue). The first and second can be deduced from octanol/water partition coefficients (usually log K_{ow} > 3 for lipophilic compounds) and can be translated into partition or permeability coefficients (Parham et al., 1997), the third is a perfusion rate. The perfusion rate is calculated using the cardiac output, which is body weight dependent and species specific, multiplied by the percentage of the cardiac output that goes to the tissue of interest (Brown et al., 1997). For environmental contaminants, log K_{ow} values are often available in the literature (Parham et al., 1997; Svendsgaard et al., 1997; Braekevelt et al., 2003; Hayward et al., 2006). These values show the preference of a contaminant for lipids (represented by octanol) or for water. Considering the low lipid percentage of blood (on average 0.5%) and the higher lipid percentages of tissues, the log K_{ow} values have been used to calculate blood/tissue partition coefficients (Parham et al., 1997). In a specific tissue of interest, the kinetics of a chemical can be described by two types of processes: 'diffusion-limited' and 'flow-limited'. In the former case, the blood flows freely and rapidly to the tissue so the rate-limiting process is the trans-membrane movement. In the latter case, the blood flow to the tissue is slow enough to be rate-limiting on itself.

1.2.1.3 Metabolic biotransformation

Metabolic biotransformation of lipophilic compounds, such as PCBs and PBDEs, can be a way to render hydrophilic metabolites which should be easier to eliminate from the body (Letcher et al., 2000). However, in reality, metabolites of PCBs or PBDEs can be toxic and quite lipophilic too and are, therefore, retained in the body (Letcher et al., 2000). In terms of modeling, metabolic biotransformation can be seen as a clearance or elimination pathway of the chemical of interest as it has been transformed into something else. That 'something else' is invisible for the model as long as it excludes the kinetics of the chemical's metabolites which is the case in most models to date. In general, metabolic biotransformation can be described in several ways. If sufficient information is available, equations describing first and/or zero order kinetics or Michaelis-Menten kinetics can be used. Without sufficient information or for compounds that convert into multiple metabolites like PCBs, metabolic biotransformation rates can be deduced from first order kinetics (Redding et al., 2008) or from elimination half-life values of the chemicals (Verner et al., 2008). Elimination half-lives are not fixed constants. They differ from one species to another and can even change within the lifetime of an organism (Miniero et al., 2001). Consequently, there is a wide range of possible elimination half-life values for a specific chemical available in the literature (e.g. ATSDR, 2001 for PCBs).

1.2.1.4 Excretion

Excretion processes eliminate the chemical from the body. Depending on the characteristics of the compound of interest, this may occur in mammals through different pathways of which the lungs, kidneys, digestive tract and skin are probably the best known. In marine mammals, excretion through exhalation is not considered as having a major influence on their body burdens (Hickie et al., 1999). However, health assessments using exhaled breath condensate from bottlenose dolphins are currently underway giving the opportunity to quantify the exact amounts of compounds exhaled in the future (http://sarasotadolphin.org/2012/01/17/using-exhaled-breath-condensate-for-marine-mammal-health-assessment/). Dermal excretion can be a significant route of elimination. Seals as well as Cetaceans, undergo molting or sloughing on a regular basis so there definitely is a movement of cells from the inside to the outside. Corneocytes are keratinocytes in the last stage of differentiation located in the outermost skin layer. They are surrounded by a lipid rich matrix and also contain lipids. The sloughing or molting could therefore be a way for marine mammals to excrete lipophilic compounds. However, there is no information on whether the keratinocyte movements actually involve lipophilic compounds or on exchange rates that include the growth of the organism. Because of this lack of information, Hickie et al. (1999) has mentioned that dermal transfer can be ignored for most purposes. Excretions through the kidneys (urine) or digestive tract (feces) are other ways to eliminate chemicals. Urinary or fecal samples are relatively easy to collect for terrestrial animals and humans, but unfortunately not for marine mammals. For terrestrial animals and humans, these losses can be described by the levels detected in a fecal/urinary sample multiplied by an elimination rate. For marine mammals, urinary and fecal losses are often lumped together with the metabolic biotransformation (which is described by an elimination half-life value) or as discussed above sometimes one of the two pathways may be completely ignored (Hickie et al., 1999).

1.2.2. PBPK models, sensitivity analyses, Bayesian approach and MCMC simulations

Information regarding the ADME processes can be integrated in mathematical equations and put into a broader context for example to simulate the chemical kinetics in the entire lifetime of the animals. The system created with these equations is called a physiologically based pharmacokinetic (PBPK) model. PBPK models have been developed for multiple reasons ranging from designing experiments to testing several hypotheses and are therefore gaining in popularity for risk assessment purposes (Andersen, 1995; Andersen, 2003; Barton et al., 2007). PBPK models can reconstruct previous exposure scenarios, provide a framework for a thorough investigation of current exposures and give the opportunity to predict the kinetics of chemicals in future situations (Chiu et al. 2007).
For typical model organisms, such as rodents, there is usually a fair amount of ADME information available. These models are often developed following an exposure experiment in which the organism has been exposed to a certain chemical for a limited duration of time under specific conditions (e.g. Lee et al., 2002; Emond et al., 2010). For less typical or even atypical model

organisms such as humans (Lyons et al., 2008; Redding et al., 2008; Verner et al., 2008 and 2009) or marine mammals (Hickie et al., 1999; Hickie et al., 2005), models frequently span their entire life. For humans as well as for marine mammals, information about the ADME processes is scarce.
There usually is a great deal of uncertainty and variability associated with PBPK models concerning the level of confidence in model predictions and the degree to which predictions may differ from one individual to the other in a population (Barton et al., 2007; Bernillon and Bois, 2000). These uncertainty and variability are due to either measurement errors which are possible under any circumstances or to genetic polymorphism in the whole population or in a given species (Bernillon and Bois, 2000). For typical model organisms, there would be uncertainty about the translation of the model results from a controlled experiment in a laboratory to uncontrolled scenarios in the wild. For atypical model organisms, there would be uncertainty about a lot of (individual) parameter values because these values are often taken from the literature, even from other species. The Bayesian approach executed using Markov chain Monte Carlo (MCMC) simulations is a statistical way to assess the uncertainty and variability associated with PBPK models (Bernillon and Bois, 2000; Hack, 2006; Barton et al., 2007). Therefore, the modeling work in this study can be divided into two parts: the structural model and the statistical model. The details of both types of models are given below.

1.2.2.1 Structural model: physiologically based pharmacokinetic (PBPK) model

The first step in developing a PBPK model is determining the level of complexity and the number of (homogenous) compartments which all depends on the questions the model has to solve and on the availability of information or data. The amount of a chemical in each compartment is than described by a mass-balanced differential equation:

$$\frac{dA_t}{dt} = Q_t \times \left(C_b - \frac{C_t}{P_t} \right) \quad (1)$$

In this equation, A_t is the amount of chemical in a specific compartment or tissue, Q_t is the blood flow to that compartment, P_t is the partition coefficient between the blood and the tissue and C_b and C_t are the concentrations of the chemical in blood and in the tissue, respectively. C_b and C_t are expressed per unit of volume (V) of the respective tissue, giving:

$$C_b = \frac{A_b}{V_b} \quad \text{and} \quad C_t = \frac{A_t}{V_t} \quad (2)$$

Input concentrations will be added to (1) whereas equations about the excretion will be subtracted from equation (1). In general, all input and excretion factors can be described by rates. However, since that kind of information for elimination pathways is hardly available for marine mammals for example, the elimination can be described by using an elimination half-life value which can be incorporated in the models by the equations from Verner et al. (2008):

$$\frac{dA_m}{dt} = Q_l \times E_h \times C_b \quad (3)$$

A_m is the amount of the chemical that has been metabolically transformed into something else, Q_l is the blood flow to the liver and E_h is the hepatic extraction rate that can be calculated as follows:

$$E_h = \frac{C_{l,\mathrm{int}_C} \times V_l}{C_{l,\mathrm{int}_C} \times V_l + Q_l} \quad (4)$$

C_{l,int_C} is the intrinsic clearance per kilogram of liver at the age of half-life (HL_h expressed in hours, h), given by:

$$C_{l,\mathrm{int}_C} = \frac{\left(\frac{E_{h,age} \times Q_{l,age}}{1 - E_{h,age}}\right)}{V_{l,age}} \quad (5)$$

in which,

$$E_{h,age} = \frac{C_{l,age}}{Q_{l,age}} \quad (6)$$

$$C_{l,age} = \left(\frac{\ln 2}{HL_h}\right) \times V_{d,age} \quad (7)$$

$$V_{d,age} = \left(\sum P_{age} \times V_{t,age}\right) + V_{b,age} \quad (8)$$

V_d is the volume of distribution, V_t and V_b are the volumes of tissue and blood, respectively, and the subscript *age* means that the parameters are calculated at the age of the individual sampled for the half-life value (Verner et al., 2008).

After integrating the mass-balanced differential equations from each compartment in the model, values need to be assigned to all parameters in the model. There are three types of parameters: the physiological (e.g. tissue volumes), physicochemical (e.g. tissue/blood partition coefficients) and biochemical parameters (e.g. metabolic biotransformation rates) (Chiu et al., 2007). For humans and laboratory animals such as rodents, these values can be found in the literature (Brown et al., 1997) if not taken directly, in part, from a certain exposure experiment (Lee et al., 2002; Emond et al., 2010). For marine mammals, parameter values are taken from the literature, from humans or rodents if not available for marine mammals, or fitted using the available marine mammal datasets.
To make sure that the model predictions are representing the situation in reality, the model needs to be evaluated using real life datasets. In case refinements are required, it is useful to know which parameters are the most important in terms of model changes. Sensitivity analyses are often performed to have an idea about how sensitive each parameter is with respect to the outcome of a specific endpoint in model simulation to pre-defined changes in parameter value, to assess where the sources of

uncertainty are in the models and to know which parameter would benefit from further optimization. For the latter reason, sensitivity analyses can be regarded as the connection between the structural model and the statistical model although they are not a requirement for developing statistical models. Sensitivity analyses can be 'local' or 'global'. During a local sensitivity analysis (LSA), the model predictions are judged against pre-defined changes of for example 1, 2 or 5% in each separate parameter value and expressed in, for instance, area under the curve (AUC) values (Emond et al., 2010). In a global sensitivity analysis (GSA), however, all model parameters vary in pre-defined ranges and their relative influence on the model output is assessed (McNally et al., 2011). LSA generally works fine for simple and linear models in which there are no interactions between the parameters. However, interactions between parameters are usually unavoidable in more complex models and this issue highlights the need for methods that are more global or randomized compared to LSA (McNally et al., 2011).

1.2.2.2 Statistical model: Bayes' theorem and Markov chain Monte Carlo simulations

The goal of a model is to show a simplified version of a sometimes very complex reality. Clearly, such reality can cause some uncertainty and variability that should be addressed in a statistically sound manner (Bernillon and Bois, 2000). The Bayesian approach using Markov chain Monte Carlo simulations is a way to explore the parameter probability distribution that is created by the variation in parameter values around a certain parameter distribution (Bernillon and Bois, 2000). Especially in uncontrolled environments like situations with wild animal populations, parameters tend to have ranges of potential values instead of single and fixed values. The role of the Bayesian approach with MCMC is to test all possible parameter values in the overall parameter space in order to come up with parameter values or (smaller) ranges. These posterior values or ranges are more meaningful to describe a specific dataset and by extension also the population where the dataset was taken from. The Bayesian approach allows the inclusion of prior knowledge of the parameters and is therefore totally different from any frequentist approach (Hack, 2006). This prior knowledge can relate to specific parameter values or parameter ranges taken from the literature, even from other species, or updated results of previous model runs. Bayes' theorem is based on:

$$p(\theta|d) \propto p(\theta) \bullet p(d|\theta)$$

Where d stands for the data, θ for the parameter values, $p(\theta|d)$ for the posterior distribution of the parameters given the data, $p(\theta)$ for the prior distribution of the parameters and $p(d|\theta)$ the likelihood of the data given the parameters (Hack, 2006). If the prior distribution of the parameters is uninformative, this method will give the same parameter means as the maximum likelihood estimation (MLE) which is a frequentist approach.

Monte Carlo is an efficient numerical way to repeatedly draw samples from a (prior) distribution in order to estimate averages and variances, thus posterior parameter distributions. Combined with Bayes' theorem, this prior distribution is formed by the likelihood of the data given the parameters and the prior knowledge of the data. Markov chains are applied to optimize the 'repeatedly

drawing samples' part inasmuch that every drawing depends on the immediately preceding one thereby creating chains. The impact of Markov chains on the Monte Carlo method is thus that the posterior parameter distribution is going to be different and narrower if the prior parameter knowledge is sufficiently informative (Fig 3).

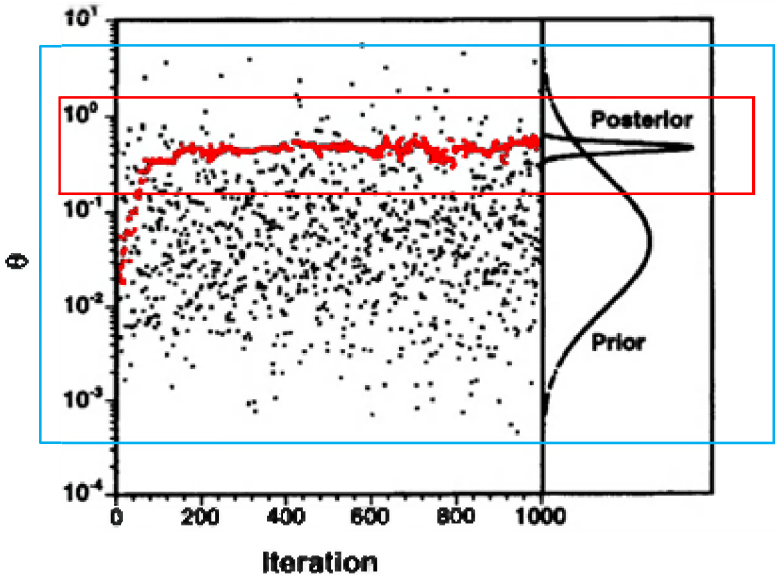


Fig 3. Illustration showing the difference between MCMC sampling (□) and simple Monte Carlo sampling (□). Y-axis represents the parameter values (θ), X-axis represents the number of iterations (parameter drawings or model runs). Figure adjusted from Bernillon & Bois (2000).

This approach, Bayes' theorem combined with MCMC simulations, can be applied for multiple datasets. Prior values of parameters from the literature (even from other species) and dataset A can yield posterior distributions of the initial, prior parameter values. These posterior distributions can be used as prior parameter distributions in the next successive parameter estimation approach using dataset B. Repeating this process for datasets C, D and E will finally result in parameter estimates that are suitable for datasets A-E. Assuming that datasets are different populations from the same animal species as in this work, the parameter estimates will be representative for the entire population.

1.3 Harbour seals, harbour porpoises and long-finned pilot whales

1.3.1. Protected status of marine mammals

There are several organisations, guidelines and laws that have been set up worldwide to protect marine mammals. Marine mammals are key predators in aquatic ecosystems and are referred to as sentinals of the health of the oceans (Ross, 2000). From a toxicological point of view, they accumulate considerable amounts of pollutants in their tissues through the biomagnification process thereby compromising their survival in the wild. All together, they are vulnerable to changes in their life history at the individual,

population and species level both in the wild as in captivity. To protect them from extinction, guidelines and laws have been made both nationally and internationally. With these guidelines and laws, trading of marine mammal parts requires permits and killing of marine mammals is prohibited. Furthermore, deliberately exposing (wild) marine mammals to toxic substances is unacceptable, although some countries are exceptions. This work meets all these guidelines and laws as no animal has been killed or deliberately exposed to pollutants for any study or chapter published within the framework of this research.

1.3.2. Harbour seals

Harbour seals (*Phoca vitulina*) belong to the family Phocidae (earless seals) which is part of the order Carnivora. As such, they are not directly related to other marine mammals like whales, dolphins or porpoises and they have their own unique physiological and ecological characteristics. Harbour seals are distributed in coastal waters around the Northern Hemisphere (Fig 4), but can be found upstream in rivers as well. They prefer coastal regions because they come on land on a daily basis to rest, to digest their food, to give birth or to nurse their pups. Harbour seals are basically generalists that eat small or medium-sized fish (± 30 cm) which are easy to catch and readily available. Because of this, their diet may vary on a spatial, seasonal and yearly basis and consists mainly of fish, cephalopods, crustaceans and occasionally seaweed (Hall et al., 1998; Meininger et al., 2003; Andersen et al., 2004; Brasseur et al., 2006; Rae, 1973). Harbour seals can reach a maximum age of 35 years, although few animals live that long. Reproduction occurs typically once a year (1 pup/year), mostly in spring. Gestation lasts for approximately 10-11 months (8 months plus embryonic diapause), followed by a lactation period of 4-6 weeks (Burns, 2009).

Fig 4. Left: Harbour seal (*Phoca vitulina*). Right: The blue colour shows the worldwide distribution range of the harbour seals.

Harbour seals spend considerable time on land which brings them in close proximity of humans and their activities. Hunting, accidental by-catch, disturbance of haulout-sites or pollution of their food and habitat are factors that influence the survival of this species to a great extent. Hunting is commonly done in order to reduce population sizes in places where they are in competition with fishermen for food, such as some areas in Canada and Japan. The last decades, however, disease-caused mortality has had a major impact on harbour seals throughout their entire distribution range. In this context, the best known mortality events are the Phocine Distemper Virus (PDV) outbreaks in European populations. PDV outbreaks in 1988, 1992 and 2008 have diminished the harbour seal stocks to nearly half (Müller et al.,

2004; Hall et al., 2006b). Over 20 years, more than 20,000 animals died in European waters because of PDV (Müller et al., 2004; Hall et al., 2006b).
An impaired immune system can be an explanation for the occurrence of PDV. Likewise, pollution can be linked to impaired immune systems. Harbour seals are therefore a very interesting species to study in an ecotoxicological and immunological context. Since these animals spend a considerable amount of time on land, it is also easier to obtain blood, milk and blubber samples in a non-destructive way for a thorough health assessment. Additionally, harbour seals inhabit the coastal zones around the Northern hemisphere and are as such giant biomarkers or sentinel species for the condition of these zones which can be of great economical importance for humans.

1.3.3. Harbour porpoises

Harbour porpoises (*Phocoena phocoena*) are one of the smallest cetaceans that inhabit the coastal waters in the Northern Hemisphere (Fig 5), although they occasionally also visit more open waters (Tolley et al., 2001). Harbour porpoises share a large part of their habitat with the harbour seals and, as for the harbour seals, cannot swallow large (> 30 cm) prey. There are thus major similarities in their diet concerning the fish species they eat. Similar to harbour seals, there is also a spatial, seasonal and yearly variation in the prey species consumed by the harbour porpoises (Rae, 1965; Aarefjord et al., 1995; Lawson et al., 1998; Börjesson et al., 2003; Santos et al., 2004). Most harbour porpoises reach a maximum age of about 15 years, but there are studies reporting animals of 20 years and older (Kuiken et al., 1993; Law et al., 2002; Strand et al., 2005). Reproduction occurs once a year with a gestation period of 10.5 months and a lactation period of about 4 months although this can go on for several months more.

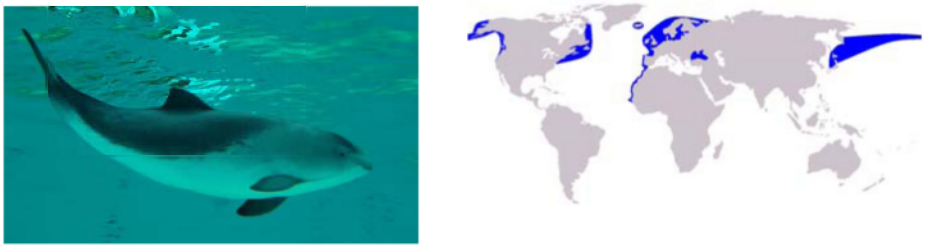


Fig 5. Left: Harbour porpoise (*Phocoena phocoena*). Right: The blue colour shows the worldwide distribution range of the harbour porpoises.

Although they are distributed over the coastal areas in the northern hemisphere, the highest density of harbour porpoises can be found in the European North Sea where there are currently over 200,000 animals (Camphuysen and Siemensma, 2011). Harbour porpoises are sensitive to DDV (delphinoid distemper virus) which is a Morbillivirus genetically distinct from the PDV viruses found in seals (Van Bressem et al., 1999). DDV infections have killed bottlenose dolphins in US waters and striped dolphins in the Mediterranean (Raga et al., 2008), but has not yet led to a major increased mortality among the harbour porpoise population. Taking into account the impact that PDV and DDV has had on the European seal population and delphinid populations, respectively, the high density of harbour porpoises in

the European North Sea area and their movements towards more southern regions (Alfonsi et al., 2012), it is desirable to keep the habour porpoise immune system as well-developed and active as possible. From this perspective, pollution is a key detrimental factor that definitely has to be controlled and understood as harbour porpoises are already experiencing the toxic effects of pollutants (Beineke et al., 2005; Hall et al., 2006a).

1.3.4. Long-finned pilot whales

Long-finned pilot whales (*Globicephala melas*) are toothed whales which can be found in open oceans, as well as in coastal waters antitropically (Fig 6). As for the killer whales, pilot whales are very social animals and tend to form groups of up to 100 animals. They can be distinguished from the short-finned pilot whales (*Globicephala macrorhynchus*) by their skull and pectoral fins while morphological differences exist between long-finned pilot whales from the northern hemisphere (*G. melas melas*) and long-finned pilot whales from the southern hemisphere (*G. melas edwardii*). The diet of pilot whales consists primarily and preferably of squid, although they can also eat several fish species whenever the occasion calls for it. Long-finned pilot whale females reach sexual maturity around the age of 8 years, whereas males reach sexual maturity around the age of 12 years. Females produce a single calf in multi-year intervals as the gestation lasts approximately 12 months and lactation can go on for at least three years. Females may live up to 60 years, males can reach the age of 40 years.
DDV has been detected in long-finned pilot whales, but most individuals are thought to be immune because virus neutralizing antibodies were found in long-finned and short-finned pilot whales. Pilot whales, however, have a nomadic lifestyle and have been observed in mixed species aggregations, so it is likely that they act as a vector for morbilliviruses in other cetacean species. Pilot whales are often involved in mass stranding events which can have both anthropogenical or natural causes. Due to the very tight group bonds, illnesses in some individuals of the pod can already be sufficient to lead the entire group to the beach. In a mass stranding, it is often difficult to select the leader(s) when all animals are laying on the beach, so focusing on a certain specific cause for the mass stranding is usually impossible. However, considering the effect that pollution can have on immune systems or on the overall development of marine mammals, the level of exposure and bioaccumulation is definitely worthwhile to investigate in this species.

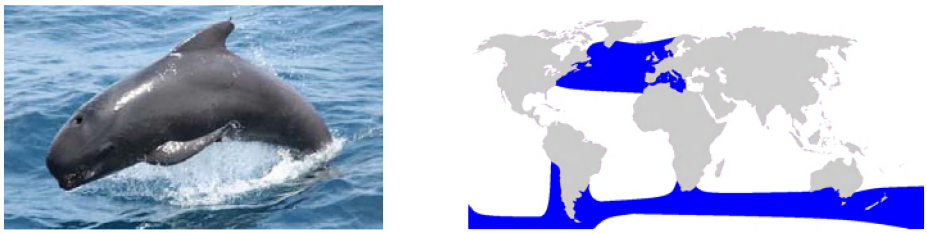


Fig 6. Left: Long-finned pilot whale (*Globicephala melas*). Right: The blue colour shows the worldwide distribution range of the long-finned pilot whales.

1.4 Objectives

In the last few decades, studies have reported levels of chemicals in tissues of several marine mammal species. However, these biomonitoring studies offer only a 'one time' view or snapshot on the level of pollutant accumulation in these animals, whereas they are exposed to chemicals for an entire lifetime. In order to investigate the impact of pollution on the health of marine mammals, it is very important to know which pollutants are going where inside their body, the concentrations of each pollutant at the target sites and the kinetics of the accumulated chemicals. All this knowledge as a function of the age of the animals, is essential for the understanding of the toxicology of environmental pollutants.
For ongoing biomonitoring studies, this work provides a framework to assess current exposures in animals from all ages. For *in vitro* studies, this work might help to select reasonable exposure scenarios for cells (animals) from a certain age and to interpret the outcome of the exposure experiment in a much broader and more realistic manner.
The present work emphasizes the complementarity between the biomonitoring and modeling approaches. Its major objective is to provide a more and better mechanistic understanding of what is observed at the biomonitoring level. This mechanistic understanding can than be very useful for risk assessment purposes and for the development of conservation and protection strategies. The consolidation between the biomonitoring and modeling efforts is reflected in the structure of this work that is divided into two big parts, each part with their own goals:

1_ Bioaccumulation studies

The goals of this part were more precisely to:

- Investigate the occurrence and levels of various pollutants in marine mammals
- Unravel the specific profiles of POPs
- Compare the findings to results of the same species from other locations and to results of other marine mammal species around the globe
- Examine the tissue distribution of POPs
- Explore the potential influences of factors like age, gender, pathology or health condition on the presence of POPs in marine mammals

2_ PBPK models as *in silico* tools to assess bioaccumulation results

The goals of this part were more precisely to:

- Develop PBPK models to assess the kinetics of predominant POPs in marine mammals
- Evaluate the model predictions compared to the bioaccumulation results
- Study the trends in the levels of selected POPs with age as well as investigate temporal and spatial trends
- Locate the model parameters that are the most sensitive to changes in parameter value
- Apply a Bayesian approach executed with MCMC simulations in order to provide more robust parameter estimations and reduce model uncertainty and variability

1.5 Structure of the thesis

Chapter 1 provides general background information about the pollutants of interest, biomagnification, effects of pollution on marine mammals, PBPK modeling with Bayesian approach, Markov chain Monte Carlo simulations and sensitivity analyses.

Chapter 2 deals with the differences in bioaccumulation of persistent organic pollutants (POPs) between two marine mammal species, namely harbour seals and harbour porpoises. Harbour seals and harbour porpoises may have some features in common such as their diet, habitat and size, but they also differ in life history characteristics like their reproductive cycle. All these characteristics can have an impact on how these animals accumulate pollutants.

In physiologically based pharmacokinetic models, organisms are considered to be a collection of tissues or compartments connected to each other via a circulation medium. In marine mammals, this circulation medium is obviously represented by blood. **Chapter 3** investigates if and how the concentrations of POPs in blood of harbour seals and harbour porpoises are influenced by time of sampling, health status or age of the animals.

Chapter 4 provides a thorough description of POPs in tissues of harbour porpoises from the Black Sea and the North Sea and in long-finned pilot whales from Australia. Results of some of the most persistent PCBs and PBDEs from these species were used to parameterize and evaluate the PBPK models in further chapters.

Starting with the most persistent PCB in marine mammals worldwide, **Chapter 5** gives the first PBPK models for the lifetime bioaccumulation of PCB 153 in male and female harbour porpoises. Next, PBPK models are developed and evaluated for PCB and PBDE congeners other than PCB 153 in male harbour porpoises. The selected PCBs and PBDEs are either environmentally relevant or (theoretically) metabolized by different enzymatic subsystems.

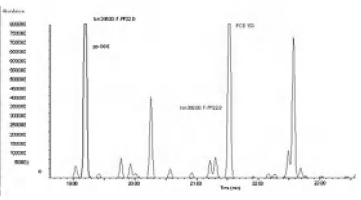
Following the example of the PCB 153 model for harbour porpoises, a PBPK model was also developed for PCB 153 in male long-finned pilot whales in **Chapter 6**. Instead of using local sensitivity analyses as in the PCB and PBDE models for harbour porpoises in Chapter 5, the most influential parameters in the pilot whale models were selected by global sensitivity analyses. Assessing uncertainty and variability with regard to those most influential parameters has been done using Bayesian techniques and Markov Chain Monte Carlo simulations. The same approach was used for a model for pesticides in harbour porpoises.

Conclusions and future perspectives are given in **Chapter 7**.

Note: Papers published or submitted for publication in this thesis were not re-written and are given as published in or submitted to their respective journal. Also, numbers of tables and figures were not adjusted according to the chapter, but are given with the same number as used in their respective paper.

Part 1

Bioaccumulation studies


Chapter 2

Inter-species differences in bioaccumulation of POPs

2.1 Paper I

Inter-species differences for polychlorinated biphenyls and polybrominated diphenyl ethers in marine top predators from the Southern North Sea:

Part 1. Accumulation patterns in harbour seals and harbour porpoises

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Abstract

Harbour porpoises and harbour seals are two representative top predator species of the North Sea ecosystem. The median values of sum of 21 PCB congeners and sum of 10 PBDE congeners were 23.1 mg/g lw and 0.33 mg/g lw in blubber of harbour seals (n = 28) and 12.4 mg/g lw and 0.76 mg/g lw in blubber of harbour porpoises (n = 35), respectively. For both species, the highest PCB concentrations were observed in adult males indicating bioaccumulation. On the contrary, the highest PBDE concentrations were measured in juveniles, likely due to better-developed metabolic capacities with age in adults. A higher contribution of lower chlorinated and non-persistent congeners, such as CB 52, CB 95, CB 101, and CB 149, together with higher contributions of other PBDE congeners than BDE 47, indicated that harbour porpoises are unable to metabolize these compounds. Harbour seals showed a higher ability to metabolize PCBs and PBDEs.

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1. Introduction

Since several decades, it has been shown that pollution puts a great pressure on the marine environment. Local input through rivers and runoff, together with (long-range) atmospheric transport are major factors governing the presence and distribution of anthropogenic contaminants, such as PCBs and PBDEs in the aquatic environment, including seas and oceans (Tanabe et al., 1994; AMAP, 2004; Law et al., 2003, 2006a). Due to their physical and chemical properties, these contaminants are capable of entering aquatic ecosystems and as a consequence, they can be a threat to organisms in every trophic level (Tanabe et al., 1994; Boon et al., 2002). Among them, PCBs are the most monitored contaminants in marine mammals (Duinker et al., 1989; Hutchinson and Simmonds, 1994; Vetter et al., 1996; Severinsen et al., 2000; Kajiwara et al., 2001).

PCBs have been used for a variety of applications including dielectric fluids for transformers, plasticisers, or components in glue and paint. Although their production was banned since the end of the 1970s, PCBs can still be found in wildlife. Recently, attention has been drawn towards the accumulation and effects of new persistent contaminants, such as PBDEs, in marine mammals.

The PBDE commercial mixtures contain fewer congeners than the corresponding PCB mixtures. PBDEs are used as flame retardants in textiles, furniture, and plastics (de Boer et al., 2000; Birnbaum and Staskal, 2004). The use of the penta- and octa-BDE technical mixtures is currently banned in Europe (EU-directive 2002/95/EC). Several adverse effects observed in wildlife, such as endocrine dysfunction, reproductive failure, immunological impairment, developmental stress and genotoxic disorders have been linked to the presence of these contaminants (Reijnders, 1986; Gauthier et al., 1999; Fair and Becker, 2000; Damstra et al., 2002; Beineke et al., 2005; Das et al., 2006).

Marine mammals are top predators in aquatic food chains and are, thus, particularly vulnerable and sensitive to contaminants which are persistent in the environment and which can accumulate in high concentrations. In marine mammals, uptake of organic contaminants occurs mainly through their diet (Borgå et al., 2004), while routes such as placental transfer and lactation may affect the offspring at a critical stage of their development (Duinker and Hillebrand, 1979; Debier et al., 2003a,b; Wolkers et al., 2004b). Harbour seals and harbour porpoises are two representative top predator species for the North Sea ecosystem. Their long life spans and population density make them suitable for monitoring pollution in the North Sea. These two species share an extensive part of their diets, such as benthic and pelagic fish species (Hall et al., 1998; Santos and Pierce, 2003). However, comparisons between the harbour seals and porpoises in the accumulation of contaminants must be made with caution. Harbour seals are more sedentary, while porpoises seem to move over larger distances and, as a consequence, concentrations of contaminants in these two species may reflect contamination on a different spatial scale (Vetter et al., 1996; Law et al., 2002; Das et al., 2004b; Fontaine et al., 2007a).

The movement of lipophilic contaminants in marine mammals is strongly influenced by the lipid dynamics inside the body. The investigation of the presence of PCBs and PBDEs in blubber, the subcutaneous fat layer, is therefore important to assess the overall contamination status of the animals. Blubber provides insulation for the body and acts as a metabolic energy storage site (Dunkin et al., 2005). This latter role is important in the

mobilization of lipids and lipophilic contaminants, depending on the animal's condition.

In the present study, we have investigated the accumulation and biomagnification of PCB and PBDE congeners in blubber of harbour seals and harbour porpoises from the Southern North Sea. An overall objective of this study was to gain knowledge about the metabolic capacities of both harbour seals and porpoises. The first part involves the study of PCB and PBDE concentrations and profiles and their species-dependent relationship with age and gender. In

the second part (Weijs et al., 2009b), biomagnification factors for individual PCB and PBDE congeners were calculated and the influence of various factors, such as octanol–water partition coefficients and trophic position assessed through measurements of 15N stable isotopes, was discussed.

2. Materials and methods

2.1. Samples

Necropsy was carried out at the Department of Veterinary Pathology (Liège University) and at the IMARES Research Center at Texel (The Netherlands). Blubber samples were collected from 35 harbour porpoises and 28 harbour seals stranded or bycaught in the Southern North Sea between 1999 and 2004. The animals were dissected and tissues were archived at the Laboratory of Oceanography, University of Liège (Belgium) at -20°C. Biological parameters, such as age, gender, weight and blubber thickness, were also recorded (standard procedure in Jauniaux et al., 2002 and Das et al., 2004b) and given in Table 1. Age classification (< 3 years for juveniles and > 3 years for adults) was based upon the length of the animals (for harbour porpoises; T. Jauniaux, personal communication) and the development of their gonads (for harbour seals; T. Jauniaux, personal communication).

2.2. Targeted compounds

The following PBDE congeners (IUPAC numbers) were targeted for analysis: 28, 47, 66, 85, 99, 100, 153, 154, 183, and 209. BDE 77 was used as internal standard (IS) for tetra- and penta-BDE congeners, while BDE 128 was used as IS for hexa- and hepta-BDE congeners. For BDE 209, 13C-labelled BDE 209 was used as IS. The following 21 PCB congeners (IUPAC numbers) were targeted: 28, 31, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194 and 199. Internal standards used were CB 46 and CB 143. Individual standards for PBDEs (Wellington Laboratories, Guelph, ON, Canada) and PCBs (Dr. Ehrenstorfer Laboratories, Augsburg, Germany) were used for identification and quantification.

2.3. Chemicals

All solvents used for the analysis (n-hexane, acetone, dichloromethane, isooctane) were of pesticide-grade (Merck, Darmstadt, Germany). Sodium sulphate and silica were pre-washed with n-hexane before use. Extraction thimbles were pre-extracted for 1h with the extraction mixture used for the samples and dried at 100°C for 1h.

2.4. Sample preparation and clean up

The method used for the sample extraction and clean up has been previously described and validated (Covaci et al., 2002; Voorspoels et al., 2003), and is briefly presented below. Between 0.3 and 0.5 g blubber was dried with ~8 g anhydrous Na_2SO_4, spiked with internal standards BDE

77/BDE 128 (25 ng), CB 46/CB 143 (75 ng) and ^{13}C-BDE 209 (7.5 ng) and extracted for 2h by hot Soxhlet with 100 ml hexane/acetone (3/1; v/v). After lipid determination (performed on an aliquot of the extract), the extract was cleaned on 8 g of acidified silica. After elution of analytes with 15 ml hexane and 10 ml dichloromethane, the cleaned extract was concentrated to 200 ml.

2.5. Analysis

PBDEs were measured with an Agilent 6890-5973 gas chromatograph coupled with a mass spectrometer system (GC–MS). The GC was equipped with a 20m x 0.18 mm x 0.20 mm AT-5 capillary column (Alltech, Lokeren, Belgium) and the MS was operated in electron capture negative ionisation (ECNI) mode. Methane was used as reagent gas and the ion source, quadrupole and interface temperatures were set at 230, 150 and 300°C, respectively. The MS was used in the selected ionmonitoring (SIM) mode with ions m/z = 79 and 81 (for tri- to hepta-BDEs) and m/z = 484.7/486.7 and 494.7/496.7 (for BDE 209 and ^{13}C-BDE 209, respectively) monitored during the entire run. Dwell times were set at 40 ms. One microlitre of the cleaned extract was injected in solvent vent mode (injector temperature: 90°C, held for 0.05 min, then with 700°C/min to 305°C and kept for 25 min; vent flow was set at 75 ml/min and the purge vent opened at 1.5 min). Helium was used as carrier gas at constant flow (0.8 ml/min). The temperature of the AT-5 column was kept at 90°C for 1.50 min, then increased to 200°C at a rate of 20°C/min, further increased to 300°C at a rate of 5°C/min, kept for 15 min.

PCBs were measured with the same GC–MS system as for the PBDE determination, operated in electron ionisation (EI) mode and equipped with a 25m x 0.22 mm x 0.25 mm HT-8 capillary column (SGE, Zulte, Belgium). The ion source, quadrupole and interface temperatures were set at 230, 150 and 300°C, respectively. The MS was used in the SIM mode with two ions monitored for each PCB homologue group. One microlitre of the cleaned extract was injected in cold pulsed splitless mode (injector temperature 90°C (0.03 min) then to 300°C with 700°C/min), pressure pulse 25 psi, pulse time 1.50 min. The splitless time was 1.50 min. Helium was used as carrier gas at constant flow (1 ml/min). The temperature of the HT-8 column was kept at 90°C for 1.50 min, then increased to 180°C at a rate of 15°C/min (kept for 2.0 min), further increased to 280°C at a rate of 5°C/min and finally raised to 300°C at a rate of 40°C/min, kept for 12 min.

2.6. Quality assurance/quality control (QA/QC)

Multi-level calibration curves were created for the quantification and good correlation (r^2> 0.999) was achieved. The identification of each target analyte was based on their relative retention times (RRTs) to the internal standard used for quantification, ion chromatograms and intensity ratios of the monitored ions. A deviation of the ion intensity ratios within 20% of the mean values of the calibration standards was considered acceptable. Recoveries for individual PBDE congeners were between 87 and 104% (RSD < 12%), while recoveries of PCBs ranged between 75 and 90% (RSD < 10%). For each analyte, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at three times the standard deviation of the procedural blank, which ensures > 99% certainty that the reported value is originating from the sample. For analytes that were not detected in procedural blanks, LOQs were calculated for a signal-to-noise ratio equal to 10. LOQs depended on the sample intake and on the analyte and ranged between 1 and 4 ng/g lw. QC was performed by

regular analyses of procedural blanks, by random injection of standards and solvent blanks. A standard reference material SRM 1945 (PCBs and PBDEs in whale blubber) was used to test the method accuracy. Obtained values were not deviating more than 10% from the certified values. The QC scheme is also assessed through regular participation to interlaboratory comparison exercises organised by the Arctic Monitoring Assessment Programme (AMAP) and the National Institute of Standards and Technology (NIST).

2.7. Statistical analysis

Statistical analyses were conducted using the SPSS 14.0 statistical package. The level of statistical significance was defined at $p < 0.05$. Outliers in all groups, detected using Grubbs' test, were removed before further calculations. Differences in the concentrations and profiles of PCBs and PBDEs were compared between the groups (adult males, adult females, juvenile males and juvenile females) using one-way ANOVA, followed by Tukey's post hoc test. Correlation coefficients between PCBs and PBDEs were calculated using GraphPad Prism 4 (GraphPad Software, Inc.).

3. Results and discussion

3.1. PCB concentrations

Of the 21 congeners analyzed, only congener CB 31 was detected in less than 50% of the blubber samples from harbour seals and porpoises and therefore this congener was removed from the following statistical interpretation. The remaining PCBs were measured in all samples. PCB concentrations (sum of 21 congeners) in blubber tissue ranged between 2.2–172 mg/g lw and 1.3–126 mg/g lw for harbour seals and porpoises, respectively. These minimum and maximum values represent a large range, underlying the numerous biotic factors involved in PCB lipid accumulation (e.g. age, gender and body condition). Therefore, the samples from both species were divided into four groups according to their age and gender: adult male (AM), adult female (AF), juvenile male (JM) and juvenile female (JF). The results for the ∑PCBs for harbour seals and porpoises are given in Table 1. Results for CB 153, the most persistent PCB congener in marine mammals are also shown in Table 1 to allow comparisons with other studies. Almost all conclusions drawn for the ∑PCBs were similar for CB 153 (although with other F and p-values).

For both species, the AM group contained the highest PCB concentrations probably due to bioaccumulation of these contaminants in time. Contrarily, the AF group displayed the lowest concentrations linked to the well-described transfer during gestation and lactation (Covaci et al., 2002; Wolkers et al., 2004b; Shaw et al., 2005). PCB concentrations were similar between JM and JF ($p > 0.05$) suggesting that the accumulation pattern is comparable between males and females until sexual maturity.

Harbour porpoises from the AM group tend to have higher, although not statistically significant, concentrations of ∑PCBs than harbour seals ($F_{1,14} = 0.200$; $p = 0.662$), while the concentrations in the other groups (JM, JF and AF) were lower compared with the corresponding group of the harbour seals ($F_{1,17} = 1.520$; $p = 0.234$ for JM, $F_{1,16} = 2.539$; $p = 0.131$ for JF and $F_{1,4} = 0.907$; $p = 0.395$ for AF, respectively). An explanation can be found in differences in age, body size or in the blubber thickness. Differences in the concentrations

Table 1. Arithmetic means, standard deviations (SD) and range of biological data (length, weight and blubber thickness), concentrations of CB 153, sum PCBs, BDE 47 and sum PBDEs (µg/g lw) measured in blubber of harbour seals and harbour porpoises from the Southern North Sea.

	Harbour seals			
	AM	**JM**	**AF**	**JF**
N	8	9	2	9
Length (cm)				
Mean (SD)	139.4 (12.1)	106.6 (9.0)	153.0 (24.0)	112.2 (11.9)
Range	128 - 163	93 - 120	136 - 170	94 - 130
Weight (kg)				
Mean (SD)	46.6 (8.9)	23.3 (5.1)	69.5 (41.7)	25.0 (8.5)
Range	34 - 58	17 - 33	40 - 99	10 - 36
Blubber thickness	(N = 6)	(N = 5)	(N = 1)	(N = 7)
Mean (SD)	20.5 (9.5)	14.6 (6.6)	50	10.9 (2.8)
Range	11 - 35	7 - 20	-	6 - 15
N	8	8*	2	8
CB 153 (µg/g lw)				
Mean (SD)	28.9 (23.3)	7.2 (2.4)	4.3 (4.3)	10.3 (10.8)
Range	0.8 - 65.9	4.7 - 11.8	1.3 - 7.3	2.2 - 35.2
Σ PCBs (µg/g lw)				
Mean (SD)	72.4 (58.2)	20.7 (6.7)	12.5 (12.2)	28.3 (27.6)
Range	2.2 - 171.7	12.7-33.8	3.9 - 21.5	6.5 - 91.5
N	8	9	2	9
BDE 47 (µg/g lw)				
Mean (SD)	0.21 (0.11)	0.35 (0.21)	0.12 (0.05)	0.42 (0.31)
Range	0.07 - 0.40	0.11 - 0.73	0.08 - 0.15	0.07 - 0.82
Σ PBDEs (µg/g lw)				
Mean (SD)	0.30 (0.14)	0.44 (0.27)	0.18 (0.09)	0.54 (0.40)
Range	0.11 - 0.52	0.13 - 0.87	0.11 - 0.24	0.09 - 1.15
	Harbour porpoises			
	AM	**JM**	**AF**	**JF**
N	8	12	5	10
Length (cm)				
Mean (SD)	145.5 (7.9)	107.3 (7.2)	149.4 (5.4)	111.8 (9.4)
Range	137 - 160	96 - 117	144 - 158	94 - 127
Weight (kg)				
Mean (SD)	41.6 (7.1)	19.2 (4.9)	47.6 (10.0)	22.8 (4.9)
Range	36 - 58	11.3-26.5	36 - 60	15 - 30
Blubber thickness				
Mean (SD)	9.1 (5.1)	10.8 (9.6)	15.6 (6.9)	14.2 (5.9)
Range	1.7 - 18	4 - 40	10 - 26	3 - 22
N	8	11*	4*	10
CB 153 (µg/g lw)				
Mean (SD)	28.7 (12.0)	3.9 (3.0)	1.7 (0.6)	3.7 (4.1)
Range	11.6 - 46.0	1.2 - 11.5	1.0 - 2.3	0.2 - 13.4
Σ PCBs (µg/g lw)				
Mean (SD)	82.9 (31.8)	15.4 (10.7)	7.3 (2.0)	12.9 (11.9)
Range	38.7-125.5	5.3 - 39.8	4.4 - 8.9	1.3 - 39.3
N	8	12	5	9*
BDE 47 (µg/g lw)				
Mean (SD)	0.69 (0.46)	1.11 (1.16)	0.43 (0.30)	0.45 (0.27)
Range	0.11 - 1.43	0.27 - 3.88	0.15 - 0.79	0.16 - 0.99
Σ PBDEs (µg/g lw)				
Mean (SD)	1.54 (0.96)	1.73 (1.77)	0.85 (0.60)	0.70 (0.41)
Range	0.28 - 3.10	0.50 - 5.93	0.32 - 1.56	0.22 - 1.48

J-juvenile (< 3 yr); A-adult (> 3 yr); F-female; M-male
* - one outlier was excluded from the data set of the respective age-gender group
Blubber thickness is expressed in mm

of PCBs between the outer and inner blubber layers, with the outer layers having significantly higher concentrations, have been reported previously in grey seals (*Halichoerus grypus*) (Debier et al., 2003a), in harp seals (*Phoca*

groenlandica) (Lydersen et al., 2002), in ringed seals (*Phoca hispida*) (Severinsen et al., 2000) and in bottlenose dolphins (*Tursiops truncatus*) (Montie et al., 2008). The mean blubber thickness in the AM group was 9.1 mm for harbour porpoises and 20.5 mm for harbour seals. Therefore, the probability of having samples from the outer blubber layer is greater for porpoises than for seals, with an overestimation of the reported PCB concentrations as a consequence (Fig. 1A). The difference in the body size, which influences the food intake and therefore the contaminant uptake, could be another possible explanation for variation between species (reviewed by Borgå et al., 2004). However, in the present study, no influence of body size could be detected, because there were no significant differences in body size between the same age groups of both species (all $p > 0.1$).

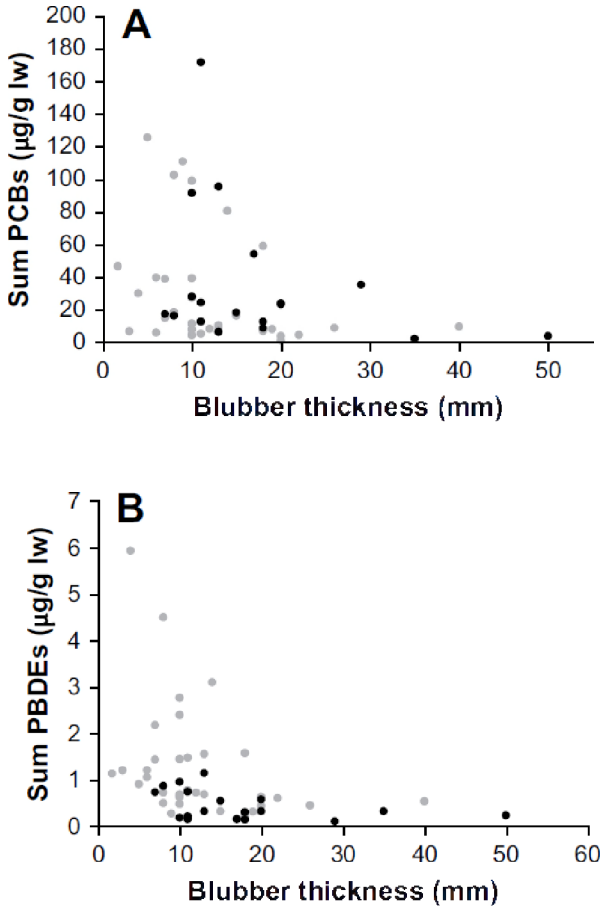

Fig 1. Relationship between the blubber thickness (mm) and (A) the ∑PCB concentrations (µg/g lw) and (B) the ∑PBDE concentrations (µg/g lw) in blubber of harbour porpoises (●) and harbour seals (●).

Due to the number and position of chlorine atoms, PCB congeners do not follow the same metabolic pathways which result in the formation of different metabolites (Letcher et al., 2000) and in differences in accumulation patterns and persistence of PCBs. This has resulted in the classification of PCBs in several groups as introduced by Bruhn et al. (1995) and Boon et al., 1997 and recently further developed by Wolkers et al. (2007) (Table 2). The most persistent congeners from the metabolic groups I and IIIB reached the highest proportions in harbour seals and porpoises, with percentages between 90–95% and 67–81%, respectively (Fig. 2). Less persistent congeners

(metabolic groups IIB, IIC and IIIA) had higher contributions in the blubber of harbour porpoises (especially CB 149), but were less important in harbour seals.

Table 2. Classification of the PCB congeners analysed in the present study according to Bruhn et al. (1995), Boon et al. (1997) and Wolkers et al. (2007).

Metabolic group	Description	Cytochrome P450 induction	PCB congeners in the present study
I	No vicinal *o,m* or *m,p* H-atoms	2B (*)	153, 180, 183, 187, 194,
IIA	Vicinal *m,p* H-atoms and ≤ 1 *o*-	2B/3A and 1A (maximum	None
IIB	Vicinal *m,p* H-atoms and 2 *o*-Cls	1 *o*-Cl)	52, 101, 110
IIC	Vicinal *m,p* H-atoms and 3 *o*-Cls		95, 149
IIIA	No vicinal *m,p* H-atoms and ≤ 1	1A and 2B (*)	28, 31, 74, 105, 118, 156
IIIB	No vicinal *m,p* H-atoms and ≥ 2	2B/1A (*)	99, 128, 138, 170

(*) Boon et al., 1997; *o-ortho, m-meta, p-para*

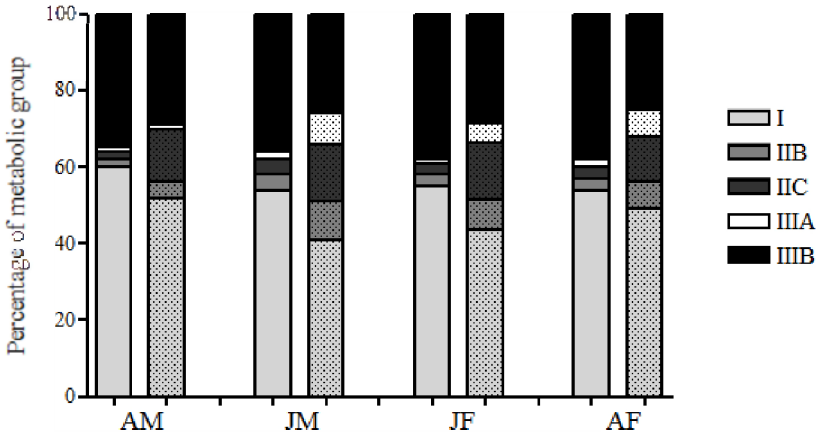

Fig 2. Percentages of the metabolic groups (see Table 2) in the four age-gender groups of harbour seals (colums without dots) and porpoises (columns with dots).

Concentrations of CB 153 were higher in the present study than in similar species from other seas and oceans, indicating that the Southern part of the North Sea is still highly contaminated with PCBs, in agreement with previous published studies. Reijnders (1986) measured high PCB levels causing reproductive failures in harbour seals from the Wadden Sea. Vetter et al. (1996) found the highest PCB levels in harbour seals from the Dutch Wadden Sea and concluded that this area is the major source of input of PCBs into the North Sea and North Atlantic. The same study also found decreasing PCB levels along the continental line from the North Sea to Germany, Denmark and Norway (Table 3). Similar trends have been observed in harbour porpoise with decreasing PCB and PBDE concentrations from German Baltic and North Sea to Iceland (Das et al., 2006). Covaci et al. (2002) also found that concentrations of PCBs in harbour porpoises from the Southern North Sea were higher than in porpoises from the English or Scottish coast of the North Sea.

3.2. General PCB profiles

CB 153 was the dominant PCB congener in all individuals of both marine mammal species. Profiles for harbour seals (CB 153 > CB 138 > CB 187 > CB 180 > CB 99) and harbour porpoises (CB 153 > CB 138 > CB 149 > CB

187 > CB 180) were similar for all age groups, except for AF porpoises (Fig. 3A and B). These results confirm the PCB profiles reported in the literature and reflect the differences in the accumulation of certain PCB congeners (e.g. CB 101 and CB 149) between pinnipeds (seals) and cetaceans (porpoises) (Hutchinson and Simmonds, 1994; Vetter et al., 1996; Boon et al., 1997).

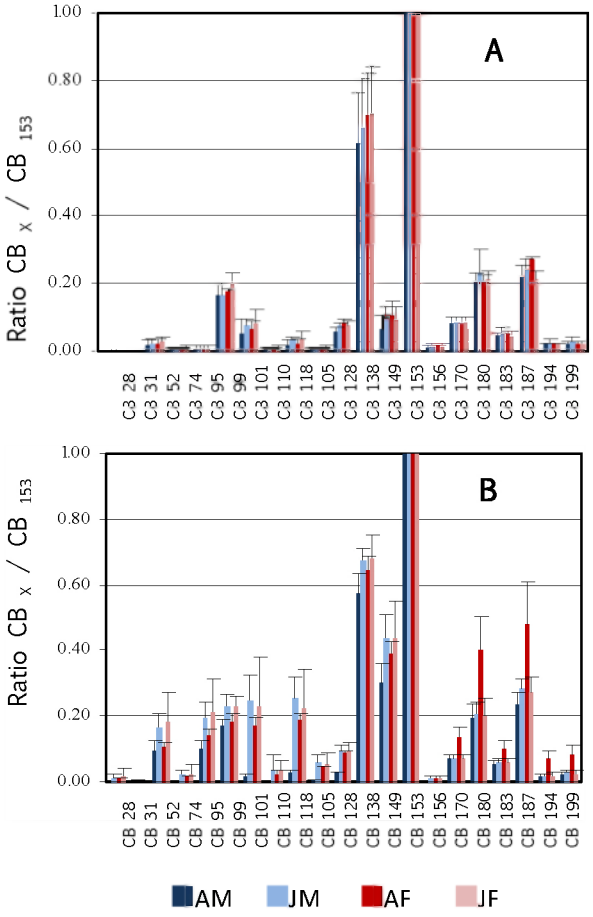

Fig 3. Ratios between mean concentrations of each individual PCB congener and CB 153 (R_{153} (CB_X)) in harbour seals (A) and harbour porpoises (B). Error bars represent SD.

Ratios between the concentrations of individual PCB congeners and concentration of CB 153 for each animal within the four age-gender groups were used to construct relative PCB profiles in order to be able to make comparisons between the two species:

$$R_{153}(CB_x) = \frac{[CB_x]}{[CB_{153}]}$$

For harbour seals, the JM, JF and AF groups showed similar and higher ratios for all PCBs than the AM group (Fig. 3A), suggesting a better-developed metabolic capacity with age or an increased metabolism with higher blubber concentrations for adult males.
For harbour porpoises, a higher contribution of higher chlorinated congeners, such as CB 170, CB 180, CB 183, CB 187, CB 194 and CB 199, was observed for the AF group (Fig. 3B). This might be the result of a selective transport of lower chlorinated PCB congeners to their offspring and, as a consequence, an enrichment of the higher chlorinated PCB congeners in the blubber of AF individuals. Similar to our observations, Debier et al. (2003b)

reported higher contributions of higher chlorinated PCBs in blubber of adult female grey seals and higher proportions of lower chlorinated PCBs in milk. The same study also assumed a higher contribution of lower chlorinated PCBs in blubber of pups. Indeed, juvenile porpoises from our study had higher ratios for lower chlorinated congeners (CB 28, CB 52, CB 74, CB 95, CB 99, CB 101, CB 118, CB 110 and CB 105), probably due to their limited capacity for metabolic breakdown and as a result of selective transfer of PCB lower chlorinated congeners during lactation. For all congeners, the AM group showed the lowest ratios in harbour porpoises suggesting a better-developed capacity for PCB metabolism for this group.
Compared to harbour seals, harbour porpoises had a higher proportion of lower chlorinated (less persistent) congeners, such as CB 52, CB 95, CB 101, CB 118 and CB 149. In both species, persistent PCB congeners (CB 138, CB 170, CB 180 and CB 187) had a similar
contribution. All together, this means that harbour seals are able to metabolize lower chlorinated PCB congeners in a more efficient way than harbour porpoises. This finding, namely a distinction between lower and higher chlorinated compounds for harbour seals, agrees with findings of Boon et al. (1997) and Hobbs et al. (2002).

3.3. PBDE concentrations

Of the 10 congeners analyzed, congeners BDE 85 and BDE 183 were detected in less than 50% of the blubber samples from both harbour seals and porpoises. BDE 66 was measured in all samples from harbour porpoises, but in less than 50% of harbour seals. BDE 209 could not be detected in any investigated sample at concentrations higher than 10 ng/g lw (LOQ). This agrees with previous reports which could not detect BDE 209 in marine mammals (Boon et al., 2002) or which have infrequently measured BDE 209 at concentrations between 1 and 8 ng/g lw in seals (Thomas et al., 2005; Shaw et al., 2007). Since very low or not detectable concentrations of BDE 209 were found in fish species which are prey for the two studied marine mammal species (Voorspoels et al., 2003) and the half-life of BDE 209 in blood of grey seals was estimated between 8.5 and 13 days (Thomas et al., 2005), it is plausible to assume that BDE 209 does not bioaccumulate in aquatic biota.
However, this congener is of particular concern, because it debrominates (in fish) to lower brominated PBDE congeners (such as BDE 154 and BDE 155), which are more water soluble and probably more persistent in biota (Stapleton et al., 2004). The remaining congeners were measured in all samples from both species. Results for PPBDEs and BDE 47, the most persistent PBDE congener in marine mammals, are given in Table 1. Statistical comparisons for PPBDEs and BDE 47 were similar, although with different F and p-values.
The highest concentrations of ∑PBDEs for all age–gender groups were observed in harbour porpoises (range 0.22–5.93 mg/g lw) compared with harbour seals (range 0.09–1.15 mg/g lw) (Table 1).
For harbour porpoises, males were more contaminated than females ($F_{1,32}$ = 4.942; p = 0.033) with the JM group having the highest (1.73-1.77 mg/g lw) and the group JF the lowest (0.70-0.41 mg/g lw) mean concentrations. For harbour seals, juveniles tended to have higher ∑PBDE concentrations than adults suggesting that the capacity for metabolic breakdown increases with age or with higher body burdens. Yet these findings were not statistically significant. This finding contrasts with the higher concentrations measured in

adult (age > 5 years) ringed seals compared to subadult specimens (age < 5 years) from East Greenland (Vorkamp et al., 2004), but agrees with results from harbour seals from UK waters (Law et al., 2006b; MAFF, 1994) (Table 4).
Similar to PCBs, PBDE concentrations in the AF group were lower than the other age–gender groups, supporting the hypothesis that adult female animals reduce their contaminant loads through gestation and lactation (Covaci et al., 2002; Law et al., 2002).
Similar to PCBs (see above), a decrease in the PBDE concentrations was observed with the increase in the blubber thickness of harbour porpoises, but not of harbour seals (Fig. 1B). This, together with the fact that the blubber thickness was lower for harbour porpoises, suggests that the probability of having samples from the outer blubber layer was greater for porpoises than for seals. As a consequence, a higher frequency of high PBDE concentrations was observed for low blubber thickness values (Fig. 1B).

The PBDE concentrations measured in the present study are similar or slightly higher than in other studies, though information about PBDEs in marine mammals (reviewed by Law et al., 2003, 2006a; Das et al., 2006) is scarce compared to PCBs (Table 4). Kajiwara et al. (2006) investigated PBDEs in several small male cetaceans from Asian waters and reported concentrations between 0.006 mg/g lw in blubber of spinner dolphins (*Stenella longirostris*) from India (between 1990 and 1992) and 6 mg/g lw in Indo-Pacific humpback dolphins (*Sousa chinensis*) from Hong Kong (between 1997 and 2001). The same study found PBDE concentrations (sum of 10 congeners) ranging from 0.024 to 0.100 mg/g lw in male harbour porpoises (n = 3) from Japan. Despite the small sample size, these results are an order of magnitude lower than these for harbour porpoises from the present study. Shaw et al. (2007)
reported mean PBDE concentrations in blubber tissue of harbour seals stranded in the North-Western Atlantic between 1991 and 2005 and found concentrations almost 10 times higher as in present study (3.65 mg/g lw for harbour seal pups (n = 13), 2.94 mg/g lw for juveniles (n = 14), 1.39 mg/g lw for AM (n = 7) and 0.33 mg/g lw for AF (n = 8)). This is probably a reflection of the higher usage of the penta-BDE technical mixture in North America compared to Europe (Law et al., 2003).

3.4. General PBDE profiles
BDE 47 was the most abundant congener in all analyzed samples of both species similar to previous findings for the same species (Boon et al., 2002; Covaci et al., 2002; Shaw et al., 2007) and other marine mammals (sperm whales *Physeter macrocephalus*, de Boer et al., 1998; ringed seals and beluga whales *Delphinapterus leucas*, Wolkers et al., 2004a; bottlenose dolphins, Johnson-Restrepo et al., 2005; Californian sea lions *Zalophus californianus*, Stapleton et al., 2006).
Profiles for JF and JM harbour porpoise were similar, namely BDE 47 > BDE 100 > BDE 99 > BDE 154 > BDE 153. For AF and AM harbour porpoises, this pattern changed into BDE 47 > BDE 99 > BDE 100 > BDE 154 > BDE 153. These profiles are comparable with results from Boon et al. (2002) for harbour porpoises, but differ from bottlenose dolphins (Johnson-Restrepo et al., 2005).
Profiles for harbour seals are different, with the AM harbour seal showing the following pattern, BDE 47 > BDE 153 > BDE 99, BDE 154 > BDE 100. The

profiles for other age–gender groups are different from that of the AM group BDE 47 > BDE 99 > BDE 100 ~ BDE 153 > BDE 154. Similar results were also found by Shaw et al. (2007). Ratios between the concentrations of individual PBDE congeners and the concentration of BDE 47, the most persistent and dominant PBDE, for each animal within the four age–gender groups were used to construct relative PBDE profiles for harbour seals and porpoises (Fig. 4A and B):

$$R_{47}(BDE_x) = \frac{[BDE_x]}{[BDE_{47}]}$$

For harbour seals (Fig. 4A), the JM and JF groups showed lower proportions of all measured PBDEs, while the AM and AF groups had slightly higher contributions of BDE 99, BDE 100, BDE 153 and BDE 154, combined with lower concentrations of this congeners, which is a reflection of the higher concentrations of BDE 47 in juveniles compared to adults.

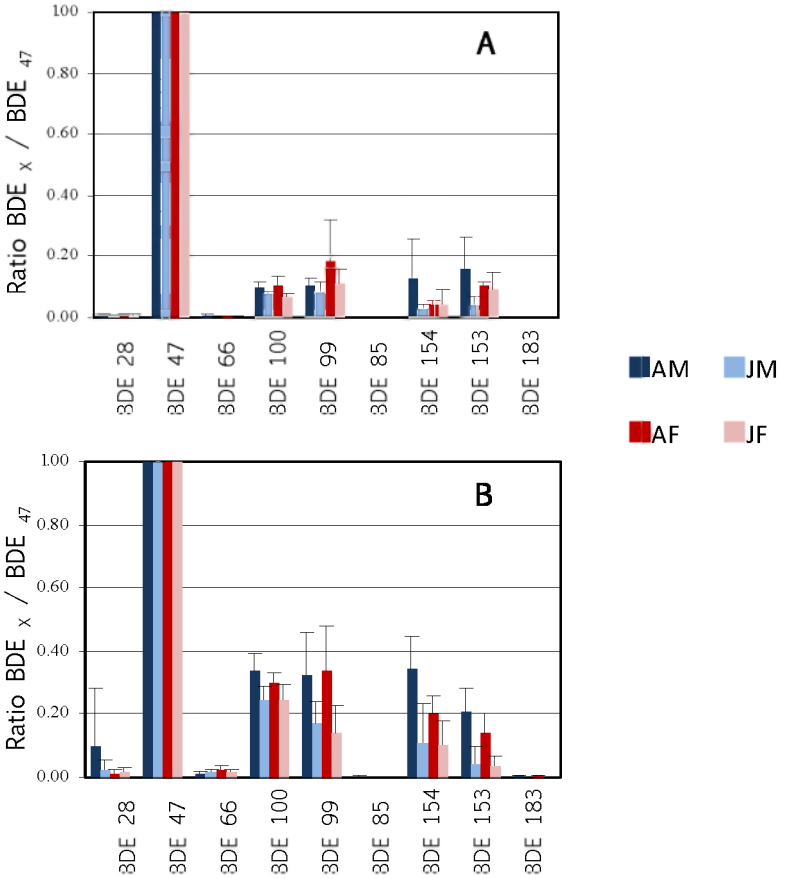

Fig 4. Ratios between mean concentrations of each individual PBDE congener and BDE 47 (R_{47} (BDE_X)) in harbour seals (A) and harbour porpoises (B). Error bars represent standard deviations (SD).

For harbour porpoises (Fig. 4B), the same trends as found for harbour seals were observed. Juveniles seemed to have lower contributions of all measured PBDE congeners than adults, probably because of a higher 'start concentration' of BDE 47 from lactation and gestation (assuming that similar to PCBs, the less lipophilic congeners will be mostly abundant in milk), together with a minimal metabolism at that age. In general, harbour porpoises had a higher contribution of congeners BDE 99, BDE 100, BDE 153

and BDE 154 compared to harbour seals. Although congeners BDE 28, BDE 66 and BDE 183 were infrequently detected, their concentrations were higher in porpoises than in seals, indicating that harbour porpoises have difficulties with metabolizing PBDEs.

3.5. Relationship between PCBs and PBDEs

Johnson-Restrepo et al. (2005) reported a significant correlation ($r = 0.83$, $p < 0.01$) between PCB and PBDE concentrations in fish and a higher correlation coefficient for the relationship between PCBs and PBDEs in dolphins and sharks from coastal Florida. Shaw et al. (2007) found a highly significant correlation ($r = 0.82$, $p < 0.01$) between PCBs and PBDEs in harbour seals from the North-Western Atlantic coast. In the present study, no significant correlations between PCBs and PBDEs or between CB 153 and BDE 47 in harbour seals or harbour porpoises could be found (all $p > 0.05$), all age groups together or separate. This could be an indication for a different accumulation and biomagnification through the food chain, but it may also reflect the variation in accumulation within each age–gender group.

3.6. Adverse effects

The PCB and PBDE concentrations, found in the present study, can be a serious threat for harbour seals and porpoises. Mean concentrations for PCBs and PBDEs in harbour porpoises in the present study (Table 1) are more than 2 (for AF) to 20 (for AM) times higher for PCBs and about 10 times higher for PBDEs compared to concentrations from stranded or bycaught harbour porpoises from European coasts which are associated with interfollicular fibrosis, splenic depletion and thymic atrophy (Beineke et al., 2005; Das et al., 2006). Furthermore, PCB concentrations in almost all age–gender groups of both species are more than an order of magnitude higher than levels of PCBs negatively associated with vitamin A (a dietary hormone essential to growth, development, reproduction and immune function) concentrations in plasma and blubber of free-ranging harbour seals from British Columbia (Canada) and Washington State (USA) (Mos et al., 2007).

4. Conclusions

Harbour porpoises and harbour seals, two representative top predator species for the North Sea ecosystem, are good indicators of coastal pollution, because they have long life spans, feed high in the food chain and do not present large-scale migration. We found that factors, such as age and gender, among others, are important for the bioaccumulation of PCBs and PBDEs in marine mammals. The AM group had the highest concentrations of PCBs, but not of PBDEs, probably because of an increased metabolism with age or body burden. The AF group could eliminate considerable amounts of PCBs and PBDEs by gestation and lactation resulting in low concentrations. However, the transfer of PBDEs to the offspring needs more attention in the future. Juvenile animals had mixed trends in concentrations with the lowest concentrations for PCBs, but the highest for PBDEs. Harbour seals, members of the pinnipeds, and harbour porpoises, members of the Cetacea, are from an evolutionary point of view different and have therefore a different ability for metabolic breakdown reflected by the different PCB or PBDE profiles. Harbour porpoises have more difficulties of metabolizing lower halogenated and less persistent PCB and PBDE congeners than harbour seals probably due to less efficient cytochrome P450 enzymes. This could lead to bioaccumulation of these

contaminants to a greater extent in harbour porpoises and subsequently to possible adverse effects.

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Table 3. Mean concentrations and standard deviations (between brackets) in µg/g lw of CB 153 in blubber tissue of harbour seals and porpoises.

Species	Location	Year	n	CB 153 (µg/g lipid weight)				Reference
				AM	JM	AF	JF	
Harbour seal	Canada	1996-2000	8	10.6 (5.1)				Hobbs et al., 2002
	Norway		6-4	0.61		0.12		Wolkers et al., 2004b
	North Sea*	1999-2004	8-8-2-8	28.9 (23.3)	7.2 (2.4)	4.3 (4.3)	10.3 (10.8)	Present study
Harbour porpoise	Baltic Sea	1985-1993	4-13	20 (13)	6.6 (3.6)			Berggrena et al., 1999
	Kattegat-	1988-1990	7-10	5.7 (2.3)	4.8 (2.5)			Berggrena et al., 1999
	Skagerrak	1978-1981	5	19 (12)				Berggrena et al., 1999
	Norway	1988-1990	8	5.6 (4.6)				Berggrena et al., 1999
	UK	1999-2004	16-18-8-15	3.7 (3.2)	4.4 (7.4)	2.2 (1.5)	2.9 (1.6)	Law et al., 2006 (a)
	North Sea*	1999-2004	8-11-4-10	28.7 (12.0)	3.9 (3.0)	1.7 (0.6)	3.7 (4.1)	Present study

Table 4. Mean concentrations and standard deviations (between brackets) in µg/g lipid weight of BDE 47 in blubber tissue of harbour seals and porpoises.

Species	Location	Year	n	BDE 47 (µg/g lipid weight)				Reference
				AM	JM	AF	JF	
Harbour seal	California	1989-1998	6-4	2.04 (2.41)		0.20 (0.15)		She et al., 2002
	North Sea		9			1.24		Boon et al., 2002
	North Sea*	1999-2004	8-9-2-9	0.21 (0.11)	0.35 (0.21)	0.12 (0.05)	0.42 (0.31)	Present study
Harbour porpoise	North Sea		9			0.86		Boon et al., 2002
	UK	1992-2004	31-31-24-22	0.52 (0.53)	1.02 (1.23)	0.74 (1.07)	1.23 (1.21)	MAFF, 1994; Law et
	North Sea*	1999-2004	8-12-5-9	0.69 (0.46)	1.11 (1.16)	0.43 (0.30)	0.45 (0.27)	Present study

*Southern North Sea

II

Inter-species differences for polychlorinated biphenyls and polybrominated diphenyl ethers in marine top predators from the Southern North Sea:

Part 2. Biomagnification in harbour seals and harbour porpoises

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Abstract

Harbour porpoises and harbour seals were found to differ in the ability to metabolize PCBs and PBDEs. Biomagnification factors (BMFs), calculated between both predators and their prey (sole – *Solea solea* and whiting – *Merlangius merlangus*), had a large range of variation (between 0.5 and 91 for PCBs and between 0.6 and 53 for PBDEs). For the higher chlorinated PCBs and the highest brominated PBDEs, the BMF values in adult males were significantly higher than in the juvenile individuals of both species. BMF values of hexa- to octa-PCBs were the highest, suggesting reduced ability to degrade these congeners. Harbour porpoises had higher BMFs for lower chlorinated PCBs and for all PBDEs compared to harbour seals. Other factors, which may influence biomagnification, such as the octanol–water partition coefficients and the trophic level position measured through stable isotope ($\delta^{15}N$) analysis, were found to be of lesser importance to predict biomagnification in the studied food chain.

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1. Introduction

Due to their chemical stability and other properties, such as low water solubility and vapour pressure, PCBs biomagnify in aquatic food webs, leading to increased concentrations throughout all trophic levels (Borgå et al., 2004; Burreau et al., 2006; Persson et al., 2007). Marine mammals occupy the top of these aquatic food chains and accumulate considerable amounts of PCBs in their tissues compared with their prey (Ruus et al., 1999; Fraser et al., 2002; Johnson-Restrepo et al., 2005; Wolkers et al., 2007), causing adverse health effects (De Swart et al., 1996; Reijnders, 1986; Mos et al., 2007).

Harbour seals and harbour porpoises are two representative top predators for the North Sea ecosystem. They do not migrate on a larger scale and spend their entire life in the North Sea, which makes them suitable for pollution monitoring. Due to its limited depth, high traffic and function as an endpoint of pollutants through runoff via land and rivers, the North Sea is a highly contaminated area. This is reflected in the amounts of chemicals, such as PCBs and PBDEs, which were measured in species representative for each trophic level of the North Sea food chain (Boon et al., 2002; Voorspoels et al., 2003, 2004).

Although the behaviour of PCBs in marine food webs is relatively well studied, the biomagnification power of PBDEs in aquatic food chains is less documented (Law et al., 2006c; Ramu et al., 2006). Moreover, information about biomagnification involving marine mammals is still scarce (Wolkers et al., 2004a; Johnson-Restrepo et al., 2005). While harbour seals and porpoises may reduce high PCB and PBDE concentrations by metabolism, there are species-specific differences in the ability for metabolic breakdown of PCBs and PBDEs, leading to different accumulation patterns (Weijs et al., 2009a). Since the uptake of contaminants in marine mammals depends on the diet, any comparison of metabolic capacities between harbour seals and porpoises would be incomplete without taking into account the concentrations and patterns of contaminants in their prey. Both species share an extensive part of their diets because they feed on benthic as well as pelagic fish species from the same area and have similar requirements regarding energy content and prey size (Hall et al., 1998; Santos and Pierce, 2003; Santos et al., 2004).

The aim of the second part of the present study was to investigate metabolic capacities of PCBs and PBDEs in harbour seals and porpoises from the Southern North Sea by assessing the biomagnification between these predators and their prey. Calculating biomagnification factors (BMFs) generally implies awareness that several biological, such as age, gender, biotransformation, trophic position according to stable isotopes ($\delta^{15}N$) and chemical factors, such as octanol–water partition coefficients (K_{ow}), are important in bioaccumulation and biomagnification processes (Borgå et al., 2004). The influence of these factors on BMFs has therefore been investigated.

2. Materials and methods

2.1. Sample preparation and analysis

Complete details for the sample collection, sample preparation and analysis by gas chromatography coupled with mass spectrometry (GC–MS) are given in the first part of this study (Weijs et al., 2009a) and are briefly presented below. Blubber samples were collected from 35 harbour porpoises and 28 harbour seals stranded or bycaught in the Southern North Sea between 1999 and 2004 and necropsied by T. Jauniaux (University of Liège). Age

classification (< 3 years for juveniles and > 3 years for adults) was based upon the length of the animals (for harbour porpoises; T. Jauniaux, personal communication and Lockyer et al., 1995) and the development of their gonads (for harbour seals; T. Jauniaux, personal communication). The fish samples (sole – *Solea solea* and whiting – *Merlangius merlangus*) used for the calculation of BMFs were previously analysed and discussed by Voorspoels et al. (2003, 2004).

The method used for the sample extraction and clean up has been previously described and validated (Covaci et al., 2002; Voorspoels et al., 2003) and involves the Soxhlet extraction of blubber dried with anhydrous Na_2SO_4 and clean up of the extract by acidified silica. The following 21 PCB congeners (IUPAC numbers) were targeted: 28, 31, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187,194 and 199. Moreover, the following 10 PBDE congeners (IUPAC numbers) were targeted for analysis: 28, 47, 66, 85, 99, 100, 153, 154, 183 and 209. The analysis was done by GC–MS operated in electron impact (for PCBs) and electron-capture negative ionization mode (for PBDEs).

2.2. Stable isotope analysis

Measurements of $\delta^{13}C$ and $\delta^{15}N$ in muscle of harbour seals and porpoises were used to investigate the influence of trophic position on biomagnification of PCBs and PBDEs. Procedure and results for $\delta^{13}C$ and $\delta^{15}N$ were presented elsewhere (Das et al., 2003, 2004a,b, 2007). Briefly, after drying at 50°C (48h), muscle samples were ground into a homogeneous powder and treated with a 2:1 chloroform:methanol solution to remove lipids. CO_2 and N_2 gas were analyzed on a VG Optima (Micromass) IR-MS coupled to an N–C–S elemental analyzer (Carlo Erba) for automated analyses. Routine measurements are precise to 0.3‰ for both ^{13}C and ^{15}N. Stable isotope ratios were expressed in δ notation according to the following equation:

$$dX = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio ($^{13}C/^{12}C$ or $^{15}N/^{14}N$). Carbon and nitrogen ratios are expressed relative to the Vienna Pee Dee Belemnite standard and to atmospheric nitrogen, respectively. Reference materials were IAEA CH-6 (sucrose) ($\delta^{13}C$= -10.4 ± 0.2‰) and IAEA-N1 ($\delta^{15}N$ = +0.4 ± 0.2‰), respectively.

2.3. Calculation of biomagnification factors

The BMF was defined as the ratio between the lipid-normalized contaminant concentrations in predator and prey (Ruus et al., 1999; Mackay and Fraser, 2000; Borgå et al., 2004).

$$BMF = \frac{C_{predator}}{C_{prey}}$$

Biomagnification occurs when BMFs are greater than 1, indicating that predators are less capable of metabolizing these compounds compared with their prey. BMFs are usually calculated relative to only one prey species (Ruus et al., 1999; Wolkers et al., 2007), but using a diet of mixed prey

items is a more realistic approach (Fraser et al., 2002; Ramu et al., 2006; Borgå et al., 2007). Harbour seals and porpoises have a diet consisting of pelagic fish species, as well as benthic fish. Recent information about the diet of harbour seals and porpoises from the Southern North Sea is scarce. Studies reported previously that specific contributions of pelagic and benthic fish to the overall diet of harbour seals and porpoises may vary from season to season (more pelagic fish in winter, more benthic fish in summer for harbour seals in the Wash, UK; Hall et al., 1998) and from location to location (more pelagic fish in the Netherlands, while more than 50% benthic fish in the diet of harbour porpoises in German waters) (reviewed by Meininger et al., 2003). All animals from the present study were bycaught or found stranded in different seasons between 1999 and 2004.
Also, it is impossible to know the exact diet of an individual since they do not have to be resident in exactly one place of the Southern North Sea, an area consisting of the Southern Bight (coasts of Belgium and The Netherlands) and the German Bight (German coast). Therefore, pelagic and benthic fish species were assumed to have an equal contribution (Leonards et al., 2008). In the present study, sole and whiting, both present in the diet of harbour seals and porpoises, were taken as representative prey for benthic and pelagic prey, respectively (Hall et al., 1998; Meininger et al., 2003; Santos and Pierce, 2003; Santos et al., 2004; Brasseur et al., 2006). The size of the fish was not taken into consideration here. Lipid-normalized concentrations of PCBs and PBDEs in sole and whiting from the Southern North Sea were previously reported (Voorspoels et al., 2003, 2004). The fish samples were collected during the same time period as the marine mammal samples, though for different purposes, and were from the same location (the Southern North Sea). Since reproduction reduces through lactation and gestation the concentrations of hydrophobic contaminants (Covaci et al., 2002; Shaw et al., 2005; Weijs et al., 2009a), BMFs were not calculated for the AF group of harbour seals and porpoises.

2.4. Statistical analysis

Statistical analyses were conducted using the SPSS 14.0 statistical package. The level of statistical significancewas defined at $p < 0.05$. Outliers in all groups, detected using Grubbs' test, were removed for further calculations. BMFs of individual PCB and PBDE congeners were compared between the four age–gender groups (adult males-AM, adult females-AF, juvenile males-JM and juvenile females-JF) using one way analysis of variance (ANOVA), followed by Tukey's post hoc test, to determine differences between groups. Pearson's r and corresponding p-values for relationships between log concentrations of individual PCBs and PBDEs were obtained using GraphPad Prism 4 (GraphPad Software, Inc.).

3. Results and discussion

Concentrations and profiles of PCBs and PBDEs are discussed in detail in the first part of this study (Weijs et al., 2009a) and therefore are only briefly presented in Table 1. These results were used for further calculations.

Table 1. Arithmetic mean concentrations and standard deviations (between brackets) expressed in µg/g lw (lipid weight) of CB 153, sum PCBs, BDE 47 and sum PBDEs in harbour seals and porpoises from the Southern North Sea.

	Harbour seal			
	AM	**JM**	**AF**	**JF**
N	8	8*	2	8
CB 153	28.9 (23.3)	7.2 (2.4)	4.3 (4.3)	10.3 (10.8)
Σ PCBs	72.4 (58.2)	20.7 (6.7)	12.5 (12.2)	28.3 (27.6)
N	8	9	2	9
BDE 47	0.21 (0.11)	0.35 (0.21)	0.12 (0.05)	0.42 (0.31)
Σ PBDEs	0.30 (0.14)	0.44 (0.27)	0.18 (0.09)	0.54 (0.40)
	Harbour porpoise			
	AM	**JM**	**AF**	**JF**
N	8	11*	4*	10
CB 153	28.7 (12.0)	3.9 (3.0)	1.7 (0.6)	3.7 (4.1)
Σ PCBs	82.9 (31.8)	15.4 (10.7)	7.3 (2.0)	12.9 (11.9)
N	8	12	5	9*
BDE 47	0.69 (0.46)	1.11 (1.16)	0.43 (0.30)	0.45 (0.27)
Σ PBDEs	1.54 (0.96)	1.73 (1.77)	0.85 (0.60)	0.70 (0.41)

Complete details are given in Weijs et al. (2009a).
J-juvenile (< 3 yr); A-adult (> 3 yr); F-female; M-male
* - one outlier was excluded from the respective age-gender group

3.1. Biomagnification factors

3.1.1. Harbour seals

As there were no significant differences in BMFs, calculated for each individual PCB and PBDE congener, between JF and JM (all $p > 0.05$), both groups were pooled and further referred to as 'juvenile seals' or 'juveniles'. For juvenile seals, as well as for the AM group, BMFs were generally lower for lower chlorinated PCBs (tri- to penta-CBs) and higher for higher chlorinated PCBs (hexa- to octa-CBs) as a consequence of dietary exposure (Voorspoels et al., 2004). The fact that there is no statistical difference in the BMFs of lower chlorinated compounds (such as CB 28, 52, 74 and 95) between juvenile and AM seals (all $p > 0.05$) shows that this metabolic capacity does not decline with age or higher body burdens (Table 2). In contrast, higher BMFs for most hexa- to octa-CB congeners indicate lower metabolic activities in harbour seals causing therefore bioaccumulation. Indeed, BMFs of these higher chlorinated PCB congeners were statistically higher in the AM group (all $p < 0.05$; Table 2), as a result from longer life-spans and limited biotransformation capacities for several PCB congeners. The BMFs obtained in the present study were difficult to compare across the studies due to the unclear definition of juvenile individuals and to the use of PPCBs for the calculation of BMFs. Ruus et al. (1999) found BMF values of 8.7 and 28.0 for cod (*Gadus morhua*) and sandeel (*Ammodytes marinus*) to harbour seal, respectively, and 10.0 and 32.2 for cod and sandeel to grey seal (*Halichoerus grypus*), respectively. However, BMF values were calculated only for PPCBs and for juvenile and adult animals of both genders taken together, thus making any comparison difficult. Fraser et al. (2002) calculated BMFs of individual PCB congeners for JM and JF harp seals (*Phoca groenlandica*) with a diet consisting of 50% polar cod (*Boreogadus saida*) and 50% crustaceans (*Themisto libellula*).

Except for some PCB congeners (e.g. CB 105 and CB 118), the BMF values found by Fraser et al. (2002) compare favourably with the present study (Table 2), suggesting that bioaccumulative capacities are comparable between pinnipeds. BMFs of PBDEs in seals and in other marine mammals are not well established. Boon et al. (2002) reported that the biggest biomagnification

step in the North Sea food web occurs from gadoid fish (cod and whiting) to marine mammals (seals and porpoises). Wolkers et al. (2004a) found metabolic indices (MI) (which expressed BMFs relative to CB 153) greater than 1 for BDE 47 and BDE 99 and MI below 1 for BDE 100 in ringed seals (*Phoca hispida*), suggesting some metabolism for BDE 100. In the present study (Table 3), BMFs of lower brominated congeners were higher (although not significant) for juveniles than for AM harbour seals. This might be due to an increased metabolism with the animal's body burden or a better developed metabolic capacity with age. Contrarily, BMFs of higher brominated congeners, such as BDE 153 and BDE 154, were lower for juveniles compared with the AM group.

3.1.2. Harbour porpoises

There were significant differences between the JM (n = 11) and JF (n = 10) porpoises for BMF values of CB 74 ($p = 0.010$), CB 101 ($p = 0.026$), CB 105 ($p = 0.033$) and CB 156 ($p = 0.043$). For these congeners, BMFs for the JM group alone were compared with the values for the AM group. For all other PCBs, JM+JF porpoises were compared with the AM group (Table 2). Except for CB 110, BMFs of PCBs in the AM group were greater than 1 (Table 2). For this congener, the lower BMF value in the AM group may be a reflection of the fact that the prey species were able to accumulate it directly from the sea water (bioconcentration), while the AM porpoises have better developed metabolizing capacities compared with juveniles (Weijs et al., 2009a). The AM group had significantly higher BMFs for the most PCB (including persistent and non-persistent) congeners compared with juveniles (Table 2). Juveniles (JM) showed higher (although not statistical significant) BMFs for congeners 28, 101, 110, 105 and 156 than the AM group, reflecting increased metabolism for these congeners in adults (Table 2). The BMFs obtained in the present study were difficult to be compared across the studies and between different cetacean species, due to the (sometimes ambiguous) definition of juvenile individuals and the frequent use of ∑PCBs (and not individual PCB congeners) for the calculation of BMFs. Ramu et al. (2006) reported BMFs for ∑PCBs of 2.4 and 2.2 for two adult male finless porpoises (*Neophocaena phocaenoides*) stranded along the Hong Kong coast (2000–2001) using their stomach contents. Johnson-Restrepo et al. (2005) found that the mean BMFs for ∑PCBs ranged from 148 to 863 for bottlenose dolphins (*Tursiops truncatus*) using a wide variety of prey, such as silver perch (*Bairdiella chrysoura*) spotted seatrout (*Cynoscion nebulosus*), striped mullet (*Mugil cephalus*), and Atlantic stingray (*Dasyatis sabina*). Wolkers et al. (2007) calculated MI-values (BMFs relative to CB 153) in killer whales, which revealed biomagnification of persistent PCBs from groups I and IIIB, similar to BMFs calculated in our study.

Since there were no significant differences in the BMFs of individual PBDE congeners (all $p > 0.05$) between the JF and JM groups, both groups were pooled and further used as "juveniles". Increasing BMFs with higher number of bromine atoms were seen in the AM harbour porpoises (Table 3) indicating lower ability to metabolise higher brominated PBDEs. The lack of this trend for juveniles and the significant differences between AM and juveniles for the highest brominated compounds, might be an indication for comparable metabolic capacities for lower and higher brominated PBDEs in juvenile harbour porpoises (Table 3). Information in literature about BMFs of PBDEs in marine mammals is scarce. Wolkers et al. (2004a) reported MI close to 1 for BDE 47, BDE 99 and BDE 100 in beluga whales (*Delphinapterus*

leucas), which differ from the harbour porpoises in the present study. Johnson-Restrepo et al. (2005) found BMFs of sum PBDEs ranging from 29 to 150 for bottlenose dolphins and a variety of prey (see above).

Table 3. Biomagnification factors (BMFs) for individual PBDE congeners for adult male (n = 8) and juveniles (n = 18) harbour seals and for adult male (n = 8) and juveniles (n = 21) harbour porpoises.

PBDE congener	Log K_{ow}	Harbour seals				
		Juveniles (n = 18)		Adult males (n = 8)		
		Mean	SD	Mean	SD	p-value
28	5.94	0.9	0.6	0.6	0.2	n.s.
47	6.81	8.4	5.7	4.6	2.4	n.s.
100	7.24	1.7	1.1	1.3	0.7	n.s.
99	7.32	5.9	6.1	2.9	1.2	n.s.
154	7.82	2.2	1.5	4.8	3.6	**0.014**
153	7.90	15	13	15	7.8	n.s.
PBDE congener	**Log K_{ow}**	**Harbour porpoises**				
		Juveniles (n = 21)		**Adult males (n = 8)**		
		Mean	SD	Mean	SD	p-value
28	5.94	7.7	6.1	11	15	n.s.
47	6.81	18	21	15	10	n.s.
100	7.24	13	15	15	9.7	n.s.
99	7.32	19	24	33	23	n.s.
154	7.82	16	17	50	32	**0.001**
153	7.90	17	21	77	53	**< 0.001**

One-way ANOVA was used to test differences in the BMF for juveniles and adults, values in bold are significant at the $p < 0.05$ level. Log K_{ow} values for PBDEs were taken from Braekevelt et al. (2003). n.s. – Not significant

3.1.3. Inter-species comparison

Harbour porpoises seem to have difficulties with metabolizing lower chlorinated PCBs, such as CB 52 and 95, but show slightly lower BMFs for higher chlorinated PCBs, e.g. CB 194 and 199. The difference between harbour seals (pinnipeds) and porpoises (cetaceans) ($p = 0.014$ for comparison between juveniles and $p < 0.001$ between AM) for CB 149 has been described before (Hutchinson and Simmonds, 1994; Vetter et al., 1996). For most hexa- to octa-CBs, juvenile harbour seals have higher BMFs compared with juvenile harbour porpoises (all $p < 0.05$). Taking into consideration that these differences are diminished for AM seals and porpoises (all $p > 0.1$), this probably means that the capacity to metabolize PCBs is higher for AM harbour seals than for AM porpoises. Significant differences were obtained for CB 156 between juvenile seals and porpoises ($p < 0.001$) and AM of both species ($p = 0.003$).

There were large inter-species differences between harbour seals and porpoises for the biomagnification of PBDEs. In general, biomagnification occurs mainly in harbour porpoises. The AM porpoises had higher BMFs (all $p < 0.05$, except for BDE 28, $p = 0.058$) than AM seals. BMFs in juvenile porpoises and seals were similar for BDE 47 and 153 ($p = 0.059$ and 0.717, respectively) and different for other PBDEs (all $p < 0.05$).

3.2. Influence of K_{ow} on the biomagnification factors

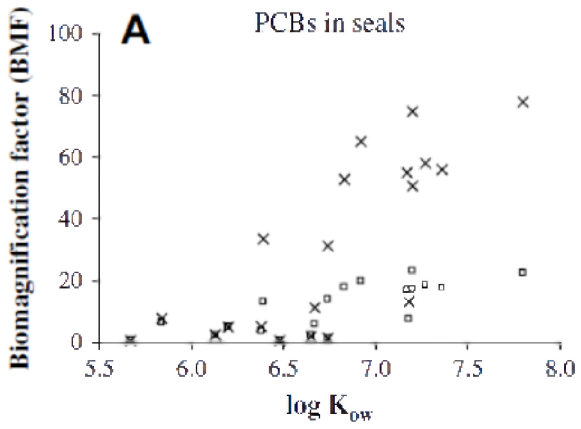
Physical factors, such as the octanol–water partition coefficient (K_{ow}), might predict to a certain extent the biomagnification of lipophilic contaminants, such as PCBs and PBDEs (Fisk et al., 1998; Borgå et al., 2004). Relationships between BMFs and log Kow of individual PCB or PBDE congeners were investigated for the juveniles and AM in harbour seals and porpoises, but no clear trends could be established (Fig. 1).

For PCBs in harbour seals, significant regressions ($p < 0.001$) were found for the AM group ($R^2 = 0.600$) and the juvenile group ($R^2 = 0.563$) (Fig. 1A). Interestingly, the regression coefficients improved substantially ($R^2 = 0.815$ and 0.774 for adult males and juveniles, respectively) when several PCB congeners (CB 99, CB 118, CB 105, CB 149 and CB 156) were manually removed. CB 99 has a higher potential for biomagnification than the other penta-CB congeners, probably as a result of the position of the chlorine atoms which makes this congener as persistent as CB 138 (Boon et al., 1997). Congeners CB 118 and CB 105 (penta-CBs) and CB 149 and CB 156 (hexa-CBs) biomagnify to a lesser extent due to their molecular structure, which suggests faster metabolisation (Boon et al., 1997).

In contrast to PCBs, there is no clear relationship between BMFs of PBDEs and log K_{ow} values in harbour seals ($R^2 = 0.218$; $p = 0.351$ for juveniles and $R^2 = 0.406$; $p = 0.173$ for adult males) (Fig. 1B).

In harbour porpoises, there was no significant linear relationship between BMFs of PCBs and log K_{ow} ($R^2 = 0.029$, $p = 0.48$ and $R^2 = 0.033$, $p = 0.44$, for juveniles and adult males, respectively) (Fig. 1C). In contrast, a borderline significant linear relationship was found between BMFs of PBDEs and log K_{ow} in the AM group ($R^2 = 0.64$; $p = 0.056$), not for juveniles ($R^2 = 0.47$; $p = 0.135$) (Fig. 1D). A similar increase in the biomagnification power and the number of bromine atoms (n = 3–6) for PBDE congeners (directly correlated to log K_{ow}) was observed by Burreau et al. (2006) in marine food webs from the Baltic Sea and the Atlantic Ocean.

From our results, it appears that the K_{ow} values can only partly predict BMFs for PCB and PBDE congeners. For a better understanding of the biomagnification processes for individual congeners, several other parameters, such as the molecular structure, molecular weight and the number and position of the halogen atoms, should be taken into account as well.



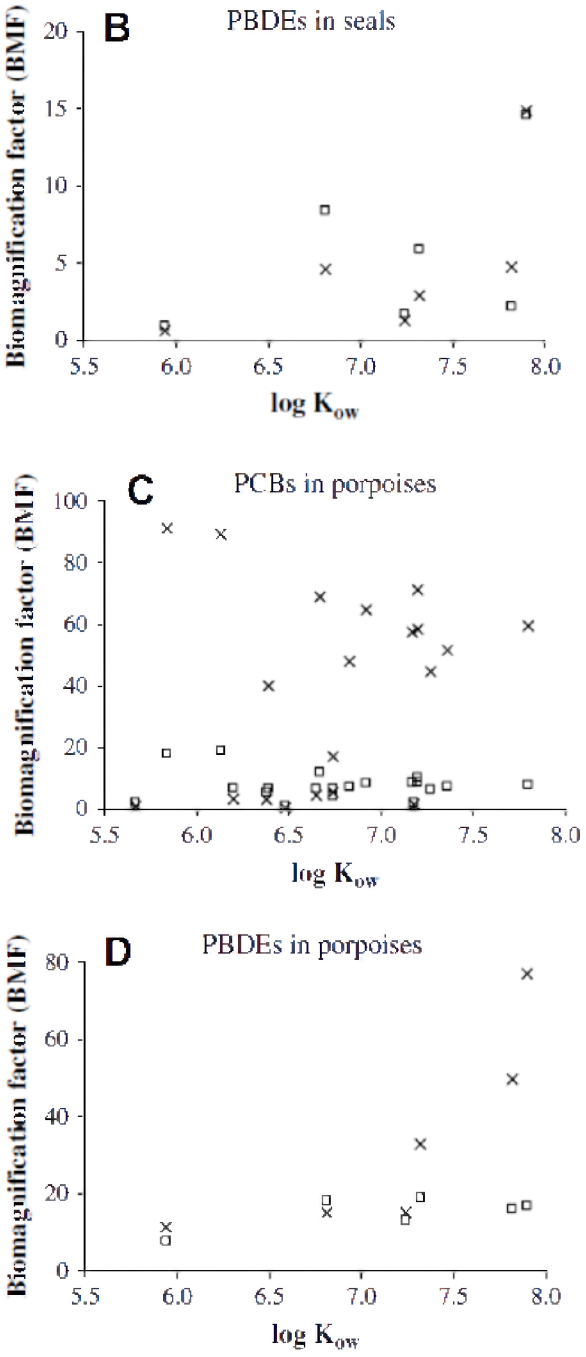


Fig. 1. Influence of K_{ow} coefficients on the biomagnification of individual PCB and PBDE congeners in adult males (crosses) and juveniles (squares). (A) Individual PCB congeners in harbour seals, (B) individual PBDE congeners in harbour seals, (C) individual PCB congeners in harbour porpoises (for juveniles n = 11 for PCB 74, 101, 105 and 156, n = 21 for all other congeners), (D) individual PBDE congeners in harbour porpoises. Log K_{ow} values of PCBs were taken from Svendsgaard et al. (1997), while log K_{ow} values of PBDEs were taken from Braekevelt et al. (2003).

3.3. Influence of stable isotope on the biomagnification factors

When investigating biomagnification in a food web, information about food sources (feeding ecology) and the position in the food chain can be obtained using measurements of stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes (Kelly, 2000; Das et al., 2003). While stable isotopes of ^{15}N provide a continuous measure of trophic level position, the ^{13}C stable isotopes give information on the carbon sources, with isotopic signals characteristic for location of prey. Thus, relationships between concentrations of lipophilic

contaminants, which are expected to biomagnify in the food chain, and the trophic levels can be established (Fisk et al., 2001).
Fig. 2 shows the relationship between the $\delta^{13}C$ and $\delta^{15}N$ stable isotopes of juveniles and adult males in the two investigated species from the Southern North Sea. Harbour seals were found to have significantly higher $\delta^{15}N$ values than the harbour porpoises ($F_{1,48}$ = 91.831; $p < 0.001$). Moreover, the higher $\delta^{13}C$ values for seals indicated also preferential in-shore feeding compared to the off-shore feeding of porpoises. These differences in the stable isotope signatures between harbour seals and porpoises are more likely caused by differences in their prey's isotopic signature and not by different diets, since harbour seal and porpoise diets contain largely the same prey species (Hall et al., 1998; Meininger et al., 2003; Santos and Pierce, 2003; Santos et al., 2004; Brasseur et al., 2006).

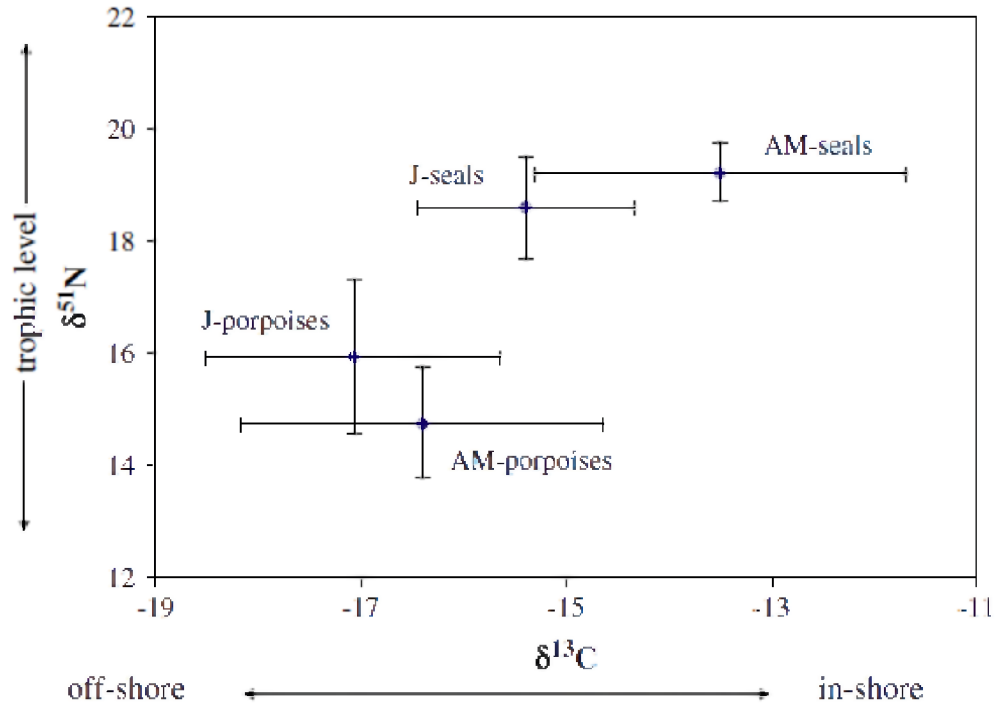


Fig. 2. Relationship of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$, average and standard deviations) of juvenile (males + females) and adult male (AM) harbour seals and harbour porpoises from the southern North Sea.

The $\delta^{15}N$ and $\delta^{13}C$ values in marine predator tissues are determined initially by the isotopic composition of the baseline phyto- and zooplankton sources which is measured in the particulate organic matter (POM). A mean $\delta^{15}N$ value of 5‰ was found for off-shore POM, increasing up to 9‰ at the North Sea coasts (Das et al., 2003). In-shore feeding of harbour seals, as revealed by the $\delta^{13}C$ values, is therefore responsible for higher $\delta^{15}N$ values, even with the same prey species as for the harbour porpoises. The two species had also inverse positions for juveniles and adult males, probably due to a change in the diet with age. There was no difference in the $\delta^{15}N$ values ($F_{1,23}$ = 2.832; p = 0.106) between juveniles (n = 18) and adult male (n = 7) harbour seals (Das et al., 2007). No difference could be detected in trophic status, assessed through $\delta^{15}N$ analysis between juvenile (n = 21) and adult male (n = 7) harbour porpoises ($F_{1,26}$ = 1.772; p = 0.195). Only few correlations between the logarithmic concentrations of individual PCB or PBDE congeners and the $\delta^{15}N$ were found to be significant at $p < 0.05$ (Table 4).

Trophic position, an important biological factor in the bioaccumulation process as mentioned earlier (Borgå et al., 2004), is therefore probably not sufficient to explain the variation in the BMFs between juveniles and adult males. However, the low number of significant correlations between BMF and $\delta^{15}N$ values is not surprising since the present study did not investigate different levels in the trophic web, but instead looked closer to only one position in the food chain.

4. Conclusions

The biomagnification of lipophilic contaminants, such as PCBs and PBDEs, in marine mammals is influenced by several factors from which age and gender seem to be the most important. Furthermore, the present study evidenced inter-species specific abilities for metabolic breakdown of contaminants. Harbour porpoises have more difficulties for metabolizing several PCB and PBDE congeners than harbour seals, probably due to a less efficient cytochrome P450 system. Biomagnification factors, calculated between predators and their prey (sole – *S. solea* and whiting – *M. merlangus*), confirm this hypothesis. However, factors which may influence biomagnification, such as K_{ow} and the trophic position measured through stable isotope analysis $\delta^{15}N$, could only partially predict biomagnification for these two species.

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Table 2. Biomagnification factors (BMFs) for individual PCB congeners for adult male (n = 8) and juveniles (n = 16) harbour seals and for adult male (n = 8) and juveniles (n = 21) harbour porpoises.

PCB congener	Log K_{ow}	Harbour seals						Harbour porpoises					
		Juveniles (n = 16)		Adult males (n = 8)					Juveniles (n = 21)		Adult males (n = 8)		
		Mean	SD	Mean	SD	p-value	BMF **	PCB	Mean	SD	Mean	SD	p-value
28	5.67	0.7	0.2	0.6	0.3	n.s.	0.5	28	2.2	2.9	1.1	0.33	n.s.
52	5.84	6.4	2.5	7.7	8.0	n.s.	4.1	52	18	12.3	91	34	**< 0.001**
74	6.20	5.0	1.4	5.0	3.4	n.s.	4.6	74 (*)	6.9	3.7	3.4	0.9	**0.018**
95	6.13	2.4	1.0	2.5	2.0	n.s.	n.a.	95	19	12.7	89	39	**< 0.001**
99	6.39	13	11	34	25	**0.009**	20	99	6.7	5.6	40	17	**< 0.001**
101	6.38	3.7	1.5	5.1	3.9	n.s.	5.7	101 (*)	5.5	2.9	3.2	1.1	n.s.
110	6.48	0.6	0.3	0.5	0.3	n.s.	n.a.	110	1.2	1.6	0.5	0.3	n.s.
118	6.74	1.4	0.5	1.4	0.8	n.s.	9.7	118	4.3	2.7	5.7	2.2	n.s.
105	6.65	2.3	0.8	1.9	1.2	n.s.	6.2	105 (*)	6.7	3.4	4.6	1.5	n.s.
128	6.74	14	9	31	23	**0.013**	20	128	6.7	4.8	17	6.4	**< 0.001**
138	6.83	18	15.9	53	49	**0.015**	22	138	7.4	6.4	48	18	**< 0.001**
149	6.67	5.8	2.6	11	8.7	**0.030**	2.5	149	12	9.5	69	29	**< 0.001**
153	6.92	20	17.4	65	53	**0.004**	22	153	8.6	7.8	65	27	**< 0.001**
156	7.18	7.5	3.5	13	9.1	**0.039**	14	156 (*)	2.2	1.4	1.7	0.6	n.s.
170	7.27	19	18.4	58	51	**0.010**	20	170	6.5	5.8	45	18	**< 0.001**
180	7.36	18	15.4	56	47	**0.006**	18	180	7.5	6.8	52	21	**< 0.001**
183	7.20	17	13.7	51	41	**0.007**	n.a.	183	8.9	7.7	59	21	**< 0.001**
187	7.17	17	15.0	55	44	**0.004**	12	187	8.8	7.3	58	21	**< 0.001**
194	7.80	23	22.0	78	70	**0.007**	n.a.	194	8.1	7.4	60	25	**< 0.001**
199	7.20	23	20.0	75	64	**0.006**	n.a.	199	11	9.8	71	30	**< 0.001**

For harbour porpoises, congeners with (*) do not include the JF group, but only the JM group (n = 11). One-way ANOVA was used to test differences in the BMF for juveniles and adults, values in bold are significant at the $p < 0.05$ level. For comparative purposes, BMFs (**) of individual PCB congeners for JM + JF harp seals (n = 13) from Fraser et al. (2002) are also given. Log K_{ow} values of PCBs were taken from Svendsgaard et al. (1997).

n.a. – Not available; n.s. – not significant;

Table 4. Pearson's correlation coefficients (r) and p values for correlations between log concentration of individual PCB or PBDE congeners and the $\delta^{15}N$ values for juvenile and adult male harbour seals and harbour porpoises. The significance level was set at $p < 0.05$.

	Harbour seals				Harbour porpoises			
	Juveniles (n=16)		AM (n=7)		Juveniles (n=20)		AM (n=7)	
PCB	r	p	r	P	r	p	r	p
28	- 0.294	n.s.	- 0.300	n.s.	- 0.036	n.s.	- 0.553	n.s.
52	0.617	**0.011**	0.355	n.s.	0.022	n.s.	- 0.646	n.s.
74	0.093	n.s.	0.448	n.s.	0.157	n.s.	- 0.336	n.s.
95	0.538	**0.032**	0.157	n.s.	0.049	n.s.	- 0.676	n.s.
99	0.589	**0.016**	0.644	n.s.	0.089	n.s.	- 0.673	n.s.
101	0.558	**0.025**	0.409	n.s.	0.212	n.s.	- 0.043	n.s.
110	0.192	n.s.	- 0.097	n.s.	0.249	n.s.	- 0.241	n.s.
118	0.278	n.s.	0.300	n.s.	0.132	n.s.	- 0.657	n.s.
105	0.033	n.s.	0.221	n.s.	0.073	n.s.	- 0.505	n.s.
128	0.555	**0.026**	0.654	n.s.	0.105	n.s.	- 0.582	n.s.
138	0.581	**0.018**	0.700	n.s.	0.060	n.s.	- 0.494	n.s.
149	0.666	**0.005**	0.424	n.s.	0.060	n.s.	- 0.654	n.s.
153	0.581	**0.018**	0.586	n.s.	0.066	n.s.	- 0.553	n.s.
156	0.437	n.s.	0.698	n.s.	0.115	n.s.	- 0.407	n.s.
170	0.505	**0.046**	0.643	n.s.	0.076	n.s.	- 0.303	n.s.
180	0.411	n.s.	0.577	n.s.	0.090	n.s.	- 0.229	n.s.
183	0.402	n.s.	0.541	n.s.	0.093	n.s.	- 0.134	n.s.
187	0.635	**0.008**	0.638	n.s.	0.101	n.s.	- 0.481	n.s.
194	0.301	n.s.	0.588	n.s.	0.169	n.s.	0.029	n.s.
199	0.285	n.s.	0.566	n.s.	0.136	n.s.	- 0.095	n.s.
PBDE	r	p	r	P	r	p	r	p
28	- 0.552	**0.022**	0.287	n.s.	-0.030	n.s.	- 0.532	n.s.
47	- 0.531	**0.028**	- 0.229	n.s.	0.016	n.s.	0.672	n.s.
66	n.a.	n.a.	n.a.	n.a.	-0.044	n.s.	0.619	n.s.
100	- 0.440	n.s.	- 0.085	n.s.	0.101	n.s.	0.712	n.s.
99	- 0.572	**0.016**	- 0.485	n.s.	0.078	n.s.	0.548	n.s.
154	- 0.156	n.s.	0.485	n.s.	0.168	n.s.	0.825	**0.022**
153	- 0.572	**0.017**	- 0.295	n.s.	0.152	n.s.	0.789	**0.035**

n.a. – not available; n.s. – not significant ($p > 0.05$)

Chapter 3

POPs in blood of marine mammals

3.1 Paper III

Concentrations of chlorinated and brominated contaminants and their metabolites in serum of harbour seals and harbour porpoises

Liesbeth Weijs, Krishna Das, Ursula Siebert, Niels van Elk, Thierry Jauniaux, Hugo Neels, Ronny Blust, Adrian Covaci

Abstract

Harbour seals and harbour porpoises are top predators in the North Sea and consequently accumulate a variety of pollutants in their tissues. Concentrations of PCBs, PBDEs and their hydroxylated metabolites (HO-PCBs and HO-PBDEs) were measured in serum of wild harbour seals (n = 47) and captive harbour porpoises (n = 21). Both species exhibit long life spans and do not have extreme situations, such as complete fasting during periods of lactation, in their annual cycles. For PCBs, concentrations in adult males were slightly higher than in juveniles and lowest in juvenile females. For PBDEs, juveniles have higher levels than adult males and females, probably as a consequence of lactational transfer. However, differences between these age–gender groups were not statistical significant, indicating that individual variation was limited within each species, even without knowing the feeding status of the animals. Body condition, particularly emaciation, has a major influence on the levels of chlorinated and brominated contaminants in serum. Profiles of PCBs were CB 153 > CB 138 > CB 187 > CB 180 and CB 153 > CB 138 > CB 149 > CB 187 > CB 180 for harbour seals and porpoises respectively. For PBDEs, BDE 47 was the predominant congener followed by BDE 100 and 99 in both species. In harbour seals, concentrations of sum PCBs (median: 39,200 pg/ml) were more than 200 times higher than levels of sum PBDEs (median: 130 pg/ml) and almost 10 times higher than concentrations of sum HO-PCBs (4350 pg/ml). In harbour porpoises, concentrations of sum PCBs (median: 24,300 pg/ml) were about 20 times higher than concentrations of PBDEs (median: 1300 pg/ml). HO-PCBs were detected in only 4 harbour porpoises and this at very low concentrations. Naturally-produced MeO-PBDEs were only found in harbour porpoises at concentrations ranging from 120 to 810 pg/ml. HO-PBDEs were not found in any species. In general, harbour seals accumulate a lower number of compounds and have mostly lower concentrations than harbour porpoises possibly as a result of a better developed metabolism.

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1. Introduction

The bioaccumulative potential and toxicity of PCBs and PBDEs as well as pesticides (HCB, DDT and metabolites) in marine mammals have been the focus of numerous papers worldwide (Tanabe et al., 1994; Bruhn et al., 1995; Reijnders and Aguilar, 2002; Reijnders and Simmonds, 2003; Thron et al., 2004; Ross, 2006). These types of chlorinated and brominated contaminants have been associated with immunological, reproductive and mostly endocrine/cytotoxic (e.g. thyroid hormone action) effects in various marine mammal species and, due to their persistence in the environment, are still a threat to the health condition of aquatic organisms in general (Damstra et al., 2002; Beineke et al., 2005; Das et al., 2006; Bossart, 2007). Among these, PCBs and PBDEs are assumed to have comparable toxic action mechanisms since they have similar chemical properties (de Boer et al., 1998; Birnbaum and Staskal, 2004). Despite their ban in Europe (PCBs in 1970s, most PBDE congeners in 2004), both types of contaminants can still be found at all levels of the aquatic food chains (Ruus et al., 1999; Boon et al., 2002).

PCBs and PBDEs may undergo metabolic/enzymatic breakdown resulting in methylsulfone and hydroxylated PCB and PBDE metabolites (Letcher et al., 2000) or lower brominated PBDE congeners (Letcher et al., 2000; Birnbaum and Staskal, 2004). Debromination of PBDEs into lower brominated congeners has been shown to occur in a few terrestrial and aquatic organisms such as birds (Pirard and De Pauw, 2007; Van den Steen et al., 2007), rats (Huwe and Smith, 2007) and fish (Stapleton et al., 2004; Benedict et al., 2007). Although methylsulfone and hydroxylated metabolites are more polar and consequently easier to eliminate from the body than their parent compounds, considerable amounts of these metabolites are retained in the body of several species (Sandala et al., 2004; Gebbink et al., 2008b; Jaspers et al., 2008).

Hydroxylated metabolites can be formed by direct insertion of a HO-group or by formation of an arene oxide intermediate that rearranges to a HO-group. Both ways are possible, but the extent to which each pathway occurs is probably highly dependent of the species (Letcher et al., 2000). Effects of these metabolites are mostly related to disturbances of hormonal and endocrine systems as they can bind to and interact with several hormone receptors and transport proteins (Cheek et al., 1999; Birnbaum and Staskal, 2004; Shimokawa et al., 2006). As a result, toxic effects can have a great impact on the health condition of organisms in general. Hydroxylated metabolites are not particularly associated with lipids as can be seen for the parent compounds, but have a high affinity for plasma proteins. Therefore, they can primarily be found in blood (Gebbink et al., 2008b).

Harbour seals and harbour porpoises are common marine mammals in West-European waters (Burns, 2002; Hammond et al., 2002). They are known to accumulate high contaminant concentrations in their tissues because of their longer life spans and top-position in aquatic food chains (Shaw et al., 2005, 2007). Although seasonal changes in blubber thickness may occur, both species do not have extreme fasting periods in their annual cycles as both species continue eating during their reproductive and lactational periods (Kastelein et al.,1997b; Burns, 2002; Lockyer, 2007). Weijs et al. (2009a) suggested a higher capacity in harbour seals for metabolizing PCBs and PBDEs compared to harbour porpoises. However, considering the assumed toxicity of the resulting metabolites (Meerts et al., 2000; Birnbaum and Staskal, 2004) and their presence in blood, concerns have been raised about the higher metabolic capacity of harbour seals in terms of their global health

and the conservation of marine mammals on a longer term. While extensive studies described PCBs and PBDEs in blubber and other tissues of caught or stranded marine mammals, fewer data were documented in blood of free-ranging seals and harbour porpoises (Bang et al., 2001; Sørmo et al., 2003; Sørmo, 2005). Levels of persistent organic pollutants (POPs) in blood depend not only on environmental contamination; but also numerous biotic factors are suspected to modulate concentrations: gender, diet, age, pregnancy, lactation and weaning (Debier et al., 2006).
The objective of the present study was to investigate the occurrence and distribution of PCBs, PBDEs, their hydroxylated metabolites (HO-PCBs and HO-PBDEs), HCB and DDTs (p,p′-DDE, p,p′-DDT and p,p′-DDD) in blood of free-ranging harbour seals, harbour porpoises held in captivity and a stranded harbour porpoise in order to elucidate the metabolism of these compounds. Several factors including species, age class, gender and year of sampling were apprehended to get further understanding of PCB and PBDE kinetic in harbour seals and harbour porpoises.

2. Materials and methods

2.1. Samples, chemicals and target compounds

Serum samples of 21 harbour porpoises in captivity from 2006–2008 were provided by SOS Dolfijn, Dolfinarium Harderwijk (The Netherlands), and were taken for regular medical purposes from the tail fluke. Information about the medical situation of these animals at the time of sampling can be found in Table 1. Serum was isolated by centrifugation at 4000 rpm during 15 min (Hettich EBA-20) and kept at −20 °C until further analysis. A serum sample of an adult harbour porpoise, stranded on the North Sea coast in 2003 was also analyzed. Serum samples of free-ranging harbour seals were collected from 47 animals caught in the frame of monitoring programs for the health assessment organized on Helgoland and Lorenzenplate (North Sea, Germany) in 2006–2008 and in Rømø (Denmark) in 2008. Seals were physically restrained and blood was drawn from the extradural venous sinus into sterile evacuated blood collection tubes (serum tubes Monovette®, Germany) and kept at −20 °C. Serum was isolated by centrifugation at 1500 g during 20 min at 20 °C (Multifuge 3S-R, Kendro) (Hasselmeier et al., 2008).
In all samples, target compounds were PCBs (IUPAC-numbers: 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194, 199), PBDEs (IUPAC-numbers: 28, 47, 99, 100, 153, 154), HCB, and pesticides (p,p′-DDE, p,p′-DDT and p,p′-DDD). The following 22 HO-PCB congeners were investigated: 3-HO-CBs (numbers 118, 153, 138 and 180), 4-HO-CBs (numbers 79, 120, 107, 146, 127, 130, 163, 187, 162, 202, 177, 172, 193, 198, 199 and 208), and 4,4′-diHO-CB 202. The following 8 HO-PBDEs were also targeted: 2′-HO-BDE 68, 3-HO-BDE 47, 5-HO-BDE 47, 6-HO-BDE 47, 4-HO-BDE 42, 4′-HO-BDE 49, 6-HO-BDE 99 and 4-HO-BDE 90. Standards were from Accustandard (HO-PBDEs) or from Wellington Laboratories (HO-PCBs).

2.2. Sample preparation

The method for serum analysis was adapted from the methods described by Covaci and Voorspoels (2005) for the determination of neutral compounds in serum and by Weiss et al. (2006) for the determination of phenolic compounds. An accurate volume of serum (typically 1.5 ml) was spiked with internal standards (CB 143 and BDE 77 for neutrals and 4′-HO-CB 159 for phenolics), diluted 1:1 with Milli Q water, mixed with formic acid, sonicated for 20 min and extracted using solid-phase extraction (SPE) cartridges (6

ml/500mg Oasis HLB, Waters). Elution was done by 10 ml of MeOH:DCM (1:1, v/v). After evaporation to near dryness, the analytes were reconstituted in 500 µl hexane:DCM (1:1, v/v) and fractionated on silica SPE cartridges (3 ml/500 mg, Varian). A first fraction containing PCBs and PBDEs was eluted with 5 ml hexane, while the phenolic compounds were eluted with 6ml MeOH:DCM (1:1, v/v). Both fractions were evaporated to dryness. The first fraction (neutrals) was cleaned-up on 500 mg acid silica (44%, w/w) and the analytes were eluted with 8 ml hexane:DCM (1:1, v/v). The cleaned extract was evaporated to dryness under a gentle nitrogen stream and reconstituted in 100 µl iso-octane. The second fraction (phenolics) was derivatized for 30 min with diazomethane when MeO-PCBs and MeO-PBDEs were formed. After solvent evaporation, the dried residue was reconstituted in 200 µl DCM and further cleaned-up on 500 mg acid silica (25%, w/w). Methoxylated compounds were eluted with 10 ml hexane:DCM (1:1, v/v), the extract was evaporated to dryness under a gentle nitrogen stream and reconstituted in 100 µl iso-octane.

2.3. Analysis

For the analysis of methoxylated derivatives and of PBDEs, a GC–MS operated in electron capture negative ionisation (ECNI) mode was equipped with a 30 m × 0.25 mm × 0.25 µm DB-5 capillary column (J&W Scientific). The ion source temperature was 170 °C. The MS was used in the SIM mode with two ions monitored for each MeO-PCB congener in specific windows, while ions m/z=79 and 81 were monitored for MeO-PBDEs and for PBDEs during the entire run. Two µl of the extract was injected in cold pulsed splitless mode, splitless time
1.50 min. Helium was used at constant flow (1.0 ml/min). For the PCB, HCB and DDT (p,p′-DDT, p,p′-DDE and p,p′-DDD) analysis, a GC–MS operated in electron impact ionisation (EI) mode was equipped with a 25 m×0.22 mm×0.25 µm HT-8 capillary column (SGE). The ion source temperature was 230 °C. The MS was used in the SIM mode with two ions monitored for each PCB homologue group in specific windows. Two µl of the extract was injected in cold pulsed splitless mode, splitless time 1.50 min. Helium was used at constant flow (1.0 ml/min).

2.4. Quality Assurance/Quality Control (QA/QC)

Multi-level calibration curves (r^2 = 0.999) in the linear response interval of the detector were created for the quantification. QC was performed by regular analyses of procedural blanks, by random injection of standards, spiked samples and solvent blanks. The Quality Control scheme is also assessed through regular participation to interlaboratory comparison exercises organized by AMAP (POPs in serum). Obtained values deviated with no more than 20% from the consensus values. The mean recovery of internal standard 4′-HO-CB 159 in serum was 96±2%. Recoveries assessed through spiking experiments at 25 and 125 pg/ml ranged between 90 and 93% with precision (RSD) < 2%. For analytes detected in the procedural blanks, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the procedural blank. For analytes that were not detected in the procedural blanks (all HO-PCBs and HO-PBDEs), LOQs were calculated for S/N=10.

2.5. Statistical analysis

Statistical analyses were conducted using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Data were log-transformed and a value 1/2 LOQ was used for concentrations below LOQ. Outliers were detected using boxplots and were removed for further statistical analysis. The influence of age, gender, location (only for harbour seals) and year of sampling was investigated with a two-way ANOVA test followed by a Tukey's post hoc test. Age (juvenile—J and Adult—A) and gender (Male—M and Female—F) were used as fixed variables, location (Germany—G and Denmark—D) and year of sampling (2006, 2007 and 2008) as random variables. The level of statistical significance was defined at $p < 0.05$.

3. Results

Median values and ranges (minimum and maximum) of all compounds measured in this study in serum of harbour seals and harbour porpoises are presented in Table 2.

3.1. Levels and profiles in harbour seals

BDE 28 and HCB were not detected in any investigated sample, while p,p′-DDD and congeners CB 110, BDE 99, BDE 154 and BDE 153 were found in less than 50% of all samples. In general, values of sum PCBs were more than 200 times higher compared to concentrations of sum PBDEs and about 15 times higher than concentrations of the sum DDTs (sum of p,p′-DDT, p,p′-DDE and p,p′-DDD). Values ranged from 9180 pg/ml to 194 000 pg/ml for sum PCBs, 772 pg/ml to 9140 pg/ml for the sum DDTs and 37 pg/ml to 726 pg/ml for sum PBDEs. For PCBs, CB 153 was the predominant congener in all samples, followed by respectively CB 138, CB 187 and CB 180 (Fig.1A). p,p′-DDE was the most dominant pesticide, followed by p,p′-DDT and p,p′-DDD. For PBDEs, BDE 47 and BDE 100 were the most dominant congeners. Other congeners, such as BDE 99, BDE 153 and BDE 154 (Fig.1B), were of reduced importance. HCB was not detected in any serum sample in the present study.

No HO-PBDEs were found in any serum sample of harbour seals in the present study. Some HO-PCBs (3-HO-CB 118 and 4-HO-CB 127) were not found in any sample, while 4-HO-CB 199, 3-HO-CB 180 and 4,4′-diHO-CB 202 were detected in less than 50% of all samples. The highest concentrations were found for 4-HO-CB 107, a lower chlorinated compound, while higher chlorinated compounds showed lower values (Fig. 2). In all individuals, 4-HO-CB 107 was followed by 4-HO-CB 162 and 4-HO-CB 146.

Table 1: Medical information of the harbour porpoises, held in captivity during rehabilitation, at the time of sampling.

Code	Days in rehabilitation	Gender	Estimated age at time of sampling (years)	Length (cm)	Weight (kg)	Condition at time of sampling
P1	3247	M	9	132.5	40.25	Healthy
P2	1946	M	6	120	29.55	Slightly anaemic due to blood loss associated with a urogenital lesion/inflammation
P3	571	F	2	140	35.8	Healthy
P4	117	M	1	116.5	27.8	Healthy
P5	0	M	Adult	145	44	
P6	5	M	Adult	149	42.3	Sample on day of death, very severe inflammatory reaction probably due to pneumonia
P7	434	F	2	126	38.05	Anaemic
P8	37	F	1	113	26.46	Healthy, on antibiotics after recent stranding
P9	0	M	Adult	142	41.3	Sample shortly after stranding, inflammatory reaction in blood
P10	128	F	1	122	29.9	Healthy (animal at the end of treatment with antibiotics)
P11	42	M	1	110-114	22.85	Severe anaemia and inflammation
P12	9	F	1	118	19.6	Pneumonia, sepsis and gastric impaction, emaciation
P13	7	F	Adult	146	48.8	Anaemic and pregnant. Animal dies a month later due to acute hepatic lipidosis
P14	30	M	1	108-112.5	22.25	Severe anemia, antiparasitic treatment
P15	174	F	2	136	34	Healthy (animal at the end of treatment)
P16	355	F	3	133-134.5	39.04	Healthy
P17	34	M	2	117-123	26.15	Laryngitis
P18	183	F	1	105	29.24	Anaemic due to lungworm infection (at time of sampling only on antibiotics after lungworm treatment)
P19	1	M	2	116	29.55	Healthy
P20	77	F	1	108.5	26.95	Chronic hepatitis of unclear significance
P21	32	F	2	116	27.2	Healthy

Table 2: Medians and range (minimum – maximum) in pg/ml of all compounds measured in the present study in serum of harbour seals and harbour porpoises.

	Harbour seals	Harbour porpoises
n	47	19
CB 52	180 (< 40 – 689)	759 (471 – 6040)
CB 74	89 (< 40 – 333)	332 (123 – 711)
CB 95	47 (< 30 – 178)	889 (535 – 6330)
CB 99	1970 (456 – 8490)	1030 (503 – 9360)
CB 101	523 (173 – 1960)	1280 (606 – 2890)
CB 105	60 (< 30 – 160)	199 (119 – 564)
CB 110	< 30 (< 30 – 111)	144 (< 30 – 288)
CB 118	250 (73 – 689)	1200 (786 – 2900)
CB 128	1170 (256 – 5020)	610 (254 – 1510)
CB 138	7670 (1700 – 34200)	3390 (1770 – 25300)
CB 149	830 (327 – 3870)	2960 (1670 – 17600)
CB 153	16000 (2930 – 79800)	6880 (3330 – 51300)
CB 156	175 (< 20 – 622)	< 20
CB 170	1230 (200 – 8110)	428 (175 – 2690)
CB 180	3890 (626 – 22400)	1400 (703 – 8190)
CB 183	720 (127 – 3890)	399 (206 – 2520)
CB 187	4030 (1047 – 19000)	1820 (889 – 12200)
CB 194	307 (67 – 2310)	173 (93 – 754)
CB 199	353 (60 – 2380)	266 (126 – 1110)
BDE 28	< 10	14 (< 10 – 33)
BDE 47	59 (11 – 348)	668 (271 – 1670)
BDE 99	< 10 (< 10 – 35)	155 (29 – 352)
BDE 100	57 (11 – 315)	334 (116 – 710)
BDE 153	< 10 (< 10 – 57)	31 (10 – 413)
BDE 154	< 10 (< 10 – 21)	78 (16 – 766)
4-HO-CB 79	147 (< 20 – 467)	< 20
4-HO-CB 107	1840 (301 – 6440)	< 20 (< 20 – 28)
3-HO-CB 118	< 20	< 20
4-HO-CB 120	69 (< 20 – 241)	< 20
4-HO-CB 127	< 15 (< 15 – 15)	< 15
4-HO-CB 130	66 (< 15 – 474)	< 15 (< 15 – 19)
3-HO-CB 138	117 (16 – 529)	< 15
4-HO-CB 146	491 (108 – 2340)	< 15
3-HO-CB 153	24 (< 15 – 101)	< 15
4-HO-CB 162	657 (191 – 1940)	< 10
4-HO-CB 163	169 (25 – 985)	< 10
4-HO-CB 172	22 (< 10 – 136)	< 10
4-HO-CB 177	151 (31 – 697)	< 10
3-HO-CB 180	< 10 (< 10 – 32)	< 10
4-HO-CB 187	253 (96 – 870)	< 10
4-HO-CB 193	16 (< 10 – 56)	< 10
4-HO-CB 198	12 (< 10 – 56)	< 10
4-HO-CB 199	< 10 (< 10 – 28)	< 10
4-HO-CB 202	257 (87 – 695)	< 10
4-diHO-CB 202	< 10 (< 10 – 11)	< 10
4-HO-CB 208	64 (23 – 200)	< 10
HCB	< 20	641 (343 – 1650)
p,p'-DDE	2750 (722 – 8440)	3860 (1590 – 15600)
p,p'-DDD	< 50 (< 50 – 107)	636 (269 – 3320)
p,p'-DDT	213 (< 50 – 678)	510 (197 – 2330)
6-MeO-BDE 47	< 10	195 (100 – 732)
2'-MeO-BDE 68	< 10	34 (10 – 95)

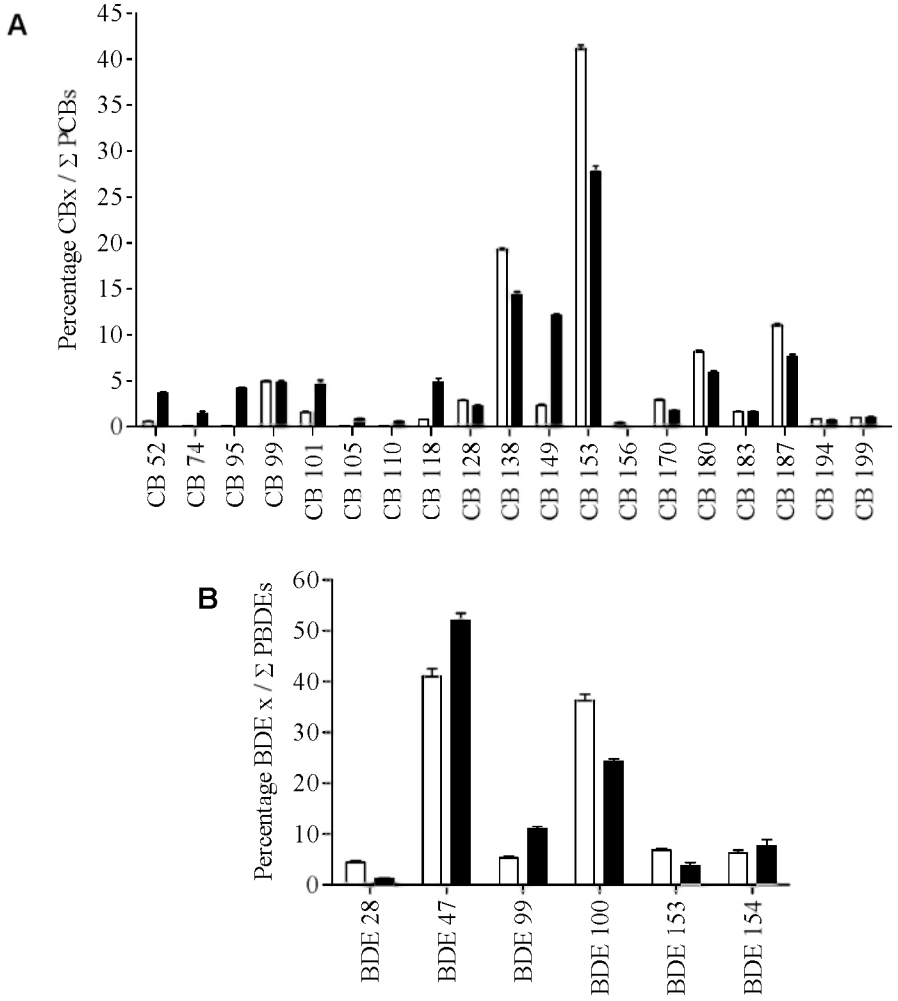

Fig. 1. Mean percentages of individual PCB and PBDE congeners in the sum of PCBs (A) and PBDEs (B) in serum of harbour seals (white bars; n=47) and harbour porpoises (black bars; n=19). Error bars represent standard errors (SE).

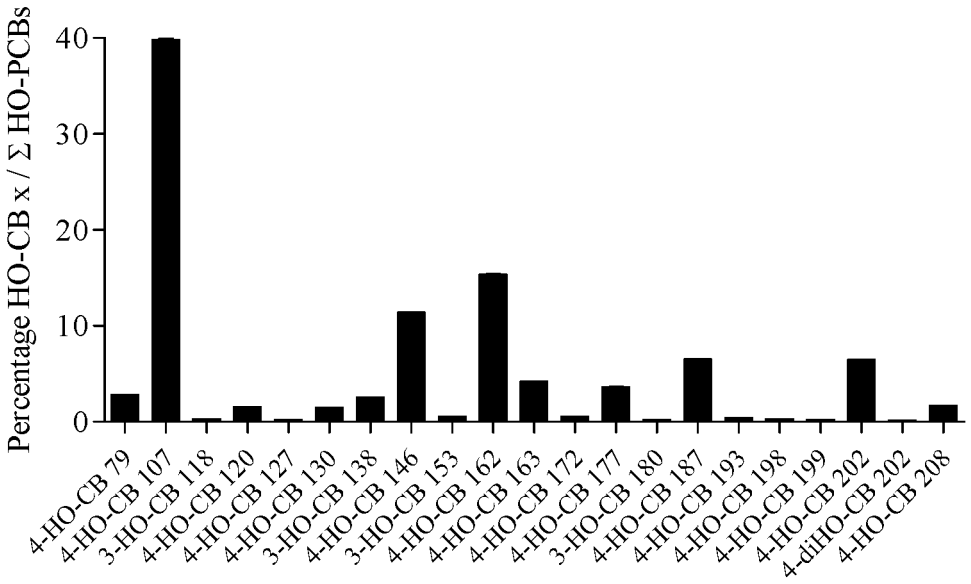

Fig. 2. Mean percentages of HO-PCB congeners in sum of HO-PCBs in serum of harbour seals. Error bars represent standard errors (SE).

When divided into 4 groups (AM—adult males, JM—juvenile males, JF—juvenile females and AF—adult females), distribution patterns of sum PBCs and sum HO-PCBs were AM > JM, JF > AF. Patterns of sum PBDEs and sum DDTs were JM, JF > AM > AF suggesting that AM have better developed metabolic capacities than juveniles. Unfortunately, differences between the age–gender groups were too small to be statistically significant (all $p > 0.05$). Location and year of sampling were not important for sum PCBs and sum DDTs, while only the year of sampling had a minor effect on concentrations of sum HO-PCBs and of sum PBDEs. For these latter two, the highest concentrations of sum HO-PCBs and sum PBDEs were found in 2006 followed by 2008 and 2007, respectively. However, these differences were considered to be a consequence of the different sample sizes (n = 21, 10 and 16 for 2006, 2007 and 2008 respectively). In general, levels of sum PCBs were approximately 11 times higher than levels of their metabolites (ratio ΣHO-PCBs/ΣPCBs = 0.086). In this study, a good correlation ($r^2 = 0.80$; $p < 0.0001$) was found between the sum of HO-PCBs and their possible precursor congeners (Fig. 3).

HO-compounds	PCB precursors	
	direct insertion	NIH-shift
4-HO-CB107	CB 107	**CB 118, 105**
3-HO-CB118	**CB 118**	107,126
4-HO-CB120	CB 120	**CB 118**
4-HO-CB130	CB 130	**CB 128, 138**
3-HO-CB138	**CB 138**	CB 130, 157
4-HO-CB146	CB 146	**CB 138, 153**
3-HO-CB153	**CB 153**	CB 146, **128**
4-HO-CB172	CB 172	**CB 170, 180**
3-HO-CB180	**CB 180**	CB 172
4-HO-CB187	**CB 187**	**CB 183**
4-HO-CB-199	**CB 199**	CB 204
4-HO-CB202	CB 202	**CB 199**
4,4'-diHO-CB202	CB 202 (*)	**CB 199**

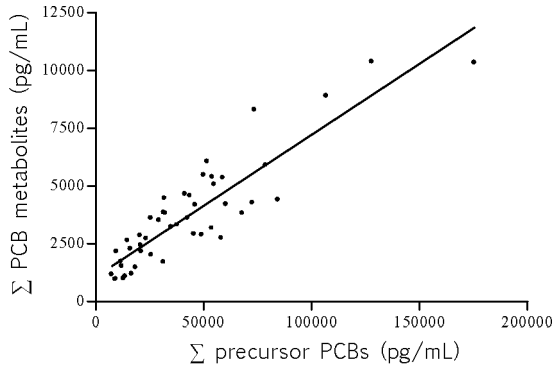

Fig. 3. Relationship between HO-PCB metabolites and their possible precursor congeners in serum of harbour seals ($r^2 = 0.80$; $p < 0.0001$; n = 47). Only congeners in bold were measured in the present study and were therefore included in the calculations. The table was made according to Jaspers et al. (2008). (*) Double insertion.

3.2. Levels and profiles in harbour porpoises

CB 156 was found in less than 50% of all samples. The levels of sum PCBs (range: 13,300–148,300 pg/ml) were higher than sum DDTs (range: 2150–20,900 pg/ml) followed by sum PBDEs (range: 495–2900 pg/ml) and HCB (range: 343–1650 pg/ml). In general, PCB and PBDE profiles were: CB 153 >

CB 138 > CB 149 > CB 187 > CB 180 for PCBs and BDE 47 > BDE 100 > BDE 99 > BDE 154 > BDE 153 for PBDEs (Fig.1A and B). p,p′-DDE was the most dominant compound among the DDTs, followed by p,p′-DDD and p,p′-DDT. Only 4-HO-CB 107 and 4-HO-CB 130 could be measured in serum samples of harbour porpoises at very low levels and in a limited number of samples (4 and 1 sample, respectively). No other HO-PCBs or HO-PBDEs were found in any serum sample of harbour porpoises in the present study. The only AF harbour porpoise analyzed in this study was pregnant at the time of sampling (animal P13, Table 1) and had very low concentrations of sum PCBs, sum PBDEs, HCB and sum DDTs compared to all other individuals. Also, there was an outlier in the JF-group with concentrations 10–20 times higher than the average of the JF-group (animal P12, Table 1). Both samples were excluded from statistical analysis and further calculations (see further). In all other samples (AM, JM and JF), no significant effects of age, gender or time of sampling were found on concentrations of sum PCBs, sum PBDEs, sum DDTs and HCB. For all these compounds, AM had higher concentrations (although not significant) compared to the juveniles.

3.3. Influence of body condition on concentrations in serum

In addition to the 21 harbour porpoises in captivity, serum of a wild adult male harbour porpoise (stranded at the Belgian coast in 2003) was also analyzed. This animal was emaciated and necropsy revealed lung pneumonia. The outlier in the JF-group (animal P12, Table 1), which was excluded from further statistical analysis as mentioned earlier, was also emaciated. Both animals have concentrations for sum PCBs, sum PBDEs, HCB and sum DDTs that exceed by far the median values of the harbour porpoises in captivity (Fig. 4).

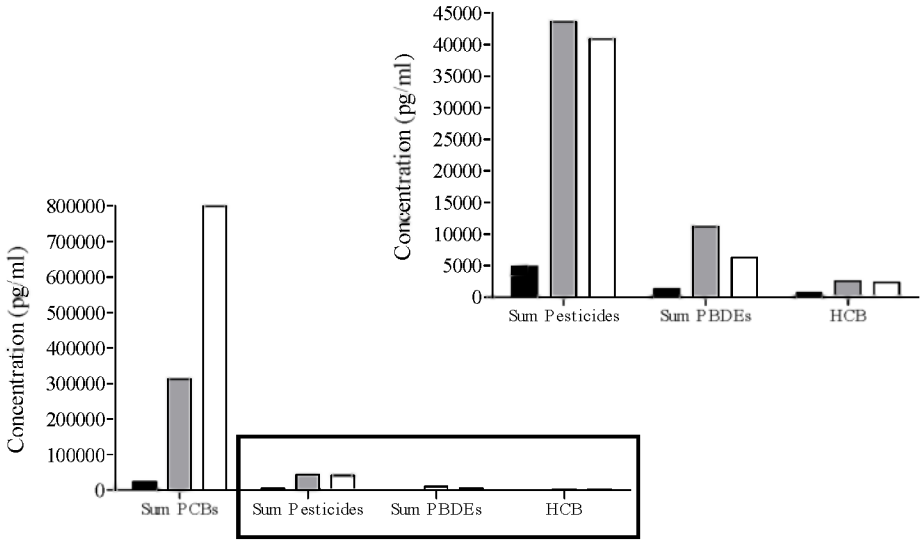

Fig. 4. Influence of body condition on concentrations of sum PCBs, sum pesticides (p,p′-DDT, p,p′-DDE and p,p′-DDD), sum PBDEs and HCB in serum samples of harbour porpoises (■=median of harbour porpoises in captivity (n=19), ■= concentrations in serum of the outlier in the JF-group, □=concentrations in serum of the wild adult male).

3.4. Naturally-produced MeO-PBDEs in harbour seals and harbour porpoises

Two naturally-produced MeO-PBDEs, 2′-MeO-BDE 68 and 6-MeO-BDE 47, were analyzed as well. Both compounds were not detected in any serum sample of the harbour seals. In harbour porpoises, no influence of age, gender and

location of sampling was found and a median concentration of 221 pg/ml, with 6-MeO-BDE 47 accounting for more than 84% of the total sum of MeO-PBDEs was calculated.

4. Discussion

This is the first study to report the simultaneous measurement of PCBs, PBDEs, HO-PCBs, HO-PBDEs, DDTs and naturally-produced MeO-PBDEs in blood of living harbour seals and harbour porpoises from the North Sea.

4.1. Levels

Information on the levels of PBDEs and HO-PCBs in blood of marine mammals is scarce. A comparison with results from other areas is complicated because contaminants are reported in other tissues than serum (e.g. liver, adipose tissue), because concentrations are expressed in different ways (pg/ml, pg/g wet weight (ww), pg/g lipid weight (lw)) and because not always the same congeners are analyzed. Concentrations of PCBs in serum of harbour seals in the present study (sum of 19 congeners) were approximately 3 to 6 times higher than concentrations of Scottish adult grey seal (*Halichoerus grypus*) females in late lactation when lipid mobilization is highest (sum of 26 congeners; Debier et al., 2003a). Levels of PCBs are also higher than the concentrations reported in whole blood of ringed seals (*Phoca hispida*) and bearded seals (*Erignathus barbatus*) from Svalbard (sum of 33 congeners; Bang et al., 2001) and in whole blood of harbour seals from California (sum of 20 congeners; Young et al., 1998). In an experiment where harbour seals were fed fish from a contaminated area, the Baltic Sea, blood (fraction III containing mostly high density particles) concentrations of PCBs were associated with immune disorders and reproductive impairment (Boon et al., 1987).

Since the results from the present study are more than an order of magnitude higher compared to these results, serious concerns have been raised about the health condition of the harbour seals in the North Sea at this moment. It is difficult to discuss differences in levels in serum between harbour seals and harbour porpoises, since it is unknown how (and if) living in captivity for a few months affects the contaminant concentrations. Although several PCBs and PBDEs are stable and persistent in marine mammals and the porpoises in rehabilitation from the present study received amounts of fish caught in the North Sea, it remains unclear how captivity may influence the concentrations of these pollutants. A number of 7 out of 20 (the emaciated animal excluded) porpoises were healthy at the time of sampling, while the other 13 were ill in some way (Table 1). Yet, no part of the individual variation could be assigned to the health status. Concentrations of PCBs in harbour porpoises are in general somewhat lower compared to the concentrations found in harbour seals, but still exceed the levels mentioned above in other marine mammal species.

4.2. Patterns

Profiles of PCBs and DDTs in serum of harbour seals were similar to the patterns found in whole blood samples (Young et al., 1998) and in blubber or liver (Kajiwara et al., 2001; Shaw et al., 2005; Weijs et al., 2009a), suggesting that the contaminant's profiles are well conserved in this species, regardless of the tissue (Hutchinson and Simmonds, 1994; Vetter et al., 1996; Boon et al., 1997). For PBDEs, patterns in blood differed from patterns in

blubber (Shaw et al., 2007; Weijs et al., 2009a) probably due to a selective retention of some congeners in other tissues than blood. HCB was not detected in any serum sample but seems to be a minor contaminant in blubber of several other pinnipeds from other areas as well (Ruus et al., 1999; Kajiwara et al., 2001; Hobbs et al., 2002; Shaw et al., 2005). Formation of PCB metabolites may occur via direct insertion and/or NIH shift (Letcher et al., 2000). Since HO-PCBs were not detected in liver of fish species caught in the North Sea in 2008 (Covaci, unpublished data), concentrations found in harbour seal serum are most probably the result of intrinsic metabolic breakdown of PCBs into HO-PCBs in the marine mammals. ΣHO-PCBs/ΣPCBs ratios smaller than 1 were also found for bottlenose dolphins (*Tursiops truncatus*) from the Indian River Lagoon (Florida, USA) and Charleston (Montie et al., 2008), for ringed seals from Québec, Canada (Sandau et al., 2000), and for bowhead whales (*Balaena mysticetus*) from Alaska (Hoekstra et al., 2003a). In contrary, ratios greater than 1 were detected in blood of polar bears (*Ursus maritimus*) from Canada (Sandau et al., 2000) and Greenland (Sandala et al., 2004; Gebbink et al., 2008) as a result of a high capacity to form HO-PCBs (Table 3). The pattern found in the present study was dominated by 4-HO-CB 107 followed by 4-HO-CB 162 and 4-HO-CB 146. Although these results agree well with results from Løken et al. (2008), they are different from patterns reported in liver, brain, blood and adipose tissue of polar bears (Sandala et al., 2004; Gebbink et al., 2008b), in plasma of bowhead whales (Hoekstra et al., 2003a) and in bottlenose dolphins (Montie et al., 2008).

PCBs can be divided into several metabolic groups according to their structure and affinity for Phase 1 cytochrome P450 enzyme subgroups. Different patterns are therefore caused by the presence and activity of these enzyme subgroups and are considered to be species specific. Meijer et al. (2008) measured HO-PCBs in maternal and cord serum of humans and concluded that HO-PCBs can be transferred to the offspring. Debier et al. (2003a) found that young animals have a lower ability to detoxify contaminants compared to adults because their metabolism is primarily focused on their growth and overall development. As a consequence, regardless of their metabolic capacities, young animals are probably exposed to HO-PCBs and may experience the possible effects of these compounds as well. The PCB, PBDE and pesticide patterns found in serum of harbour porpoises agree well with profiles found in liver and blubber (Covaci et al., 2002; Weijs et al., 2009a) and seem also to be highly species specific. In contrast to harbour seals, HO-PCBs were only occasionally measured in serum of harbour porpoises. Houde et al. (2006) detected HO-PCBs in plasma of bottlenose dolphins, while Hoekstra et al. (2003a) found HO-PCBs in plasma of bowhead whales. The difficulties of harbour porpoises to form HO-PCBs can therefore not be extrapolated to other cetaceans.

When comparing harbour seals and harbour porpoises, it is very clear that the porpoises have difficulties in forming HO-PCBs and that they accumulate a higher number of PCB and PBDE congeners compared to the seals. Troisi et al. (2001) analyzed PCB and DDE methyl sulfones in lung and uterus of a cetacean (striped dolphins — *Stenella coeruleoalba*) and a pinniped species (harbour seal) (all morbillivirus epizootic victims) and found higher concentrations of methylsulfones in both tissues of harbour seals compared to striped dolphins. Further, it seems that harbour porpoises have only one possible way for metabolic breakdown of PCBs, namely formation of $MeSO_2$-PCBs (Chu et al., 2003). In contrast, harbour seals can form HO-PCBs and

$MeSO_2$-PCBs and both metabolites to a greater extent than harbour porpoises. Both classes of PCB metabolites including the precursor PCBs are potential endocrine-disruptors and are associated with endocrine-related effects, such as cytotoxicity, competitive binding with several receptors and disruption of hormone homeostasis (Letcher et al., 2000; Sandala et al., 2004). So far, no classification regarding toxicity of PCBs, HO-PCBs and $MeSO_2$-PCBs is available. Therefore, it remains debatable whether metabolic transformation capacities can improve the overall health condition of an organism. Further toxicity tests with PCBs, HO-PCBs and $MeSO_2$-PCBs are needed to assess and to compare the condition of harbour seals and harbour porpoises at this moment.

Table 3. Means and standard deviations (SD) of the concentrations (ng/g ww) of HO-PCBs in tissues of four marine species.

Species	n	Mean ± SD	Tissue	HO-PCB/PCB	Reference
Bowhead	10	1.52 ± 0.31 [a]	Plasma	0.547	Hoekstra et al.
Bottlenose dolphins	10	3.9 ± 3.2	Plasma	0.016	Houde et al. (2006)
	42	18 ± 21	Plasma	0.082	
	32	209 ± 211	Plasma	0.679	
	5	94 ± 103	Plasma	0.470	
	12	33 ± 21	Plasma	0.236	
	35	64 ± 53	Plasma	0.435	
	21	168 ± 110	Plasma	0.644	
Polar bears	20	60 ± 8 [a]	Adipose	0.011	Gebbink et al. (2008b)
		1020 ± 132 [a]	Blood	25.5	
		18 ± 3 [a]	Brain	0.122	
		355 ± 36 [a]	Liver	0.114	
Harbour seals	19	182.3 ± 72.1	Blood	8.30	Sandala et al. (2004)
	5	0.49 [b]	Liver	0.009	Løken et al. (2008)
		2.37 [b]	Plasma	0.344	
	47	4.35 [b]	Serum	0.086	Present study [c]

[a] – standard error or SE
[b] – median values
[c] – expressed in ng/ml

Although biotransformation of PBDEs in beluga whales (*Delphinapterus leucas*) was earlier shown to occur (McKinney et al., 2006a), no HO-PBDEs were found in the investigated blood samples of harbour seals and harbour porpoises. This is a confirmation of the results of a recent study (Meijer et al., 2008), performed in blood of pregnant women and their infants in The Netherlands, which was also unable to detect a HO-PBDE (6-HO-BDE 47). Moreover, no HO-PBDEs were found in ringed seal blubber and beluga whale blood and liver (Kelly et al., 2008). In contrast, detectable but not quantifiable HO-PBDE concentrations were reported in beluga whale livers (McKinney et al., 2006b) and very low yet measurable concentrations of 0.01 to 0.1 ng/g lipid equivalent were found in blubber and milk of beluga whales (Kelly et al., 2008). However, since concentrations of PBDEs in harbour seals are lower than levels in harbour porpoises, a greater capacity for debromination of PBDEs in harbour seals is assumed as previously shown for BDE 209 in grey seals (Thomas et al., 2005).

4.3. Naturally-produced MeO-PBDEs

Vetter (2006) raised the hypothesis that higher contributions of 2'-MeO-BDE 68 are caused by sponges or associated organisms, whereas higher proportions of 6-MeO-BDE 47 are an indication of the presence of algae or associated organisms. The dominance of 6-MeO-BDE 47 in the present study

was also found in blubber of minke whales (*Balaenoptera acutorostrata*) (Marsh et al., 2005), in male ringed seals and beluga whales (Kelly et al., 2008), in grey seals and ringed seals (Haglund et al., 1997) and in pre-industrial whale oil (Teuten and Reddy, 2007). Reversed patterns were found in blubber of striped dolphins (Marsh et al., 2005) and several marine mammal species from Oceania (Melcher et al., 2005) and Brazil (Dorneles et al., 2010). A possible explanation for the presence of MeO-PBDEs only in harbour porpoises (21 porpoises in rehabilitation and the wild emaciated porpoise) in the present study might be that they feed on offshore prey coming within the southern part of the North Sea, while harbour seals feed more inshore as evidenced by their stable isotope signatures (Das et al., 2003; Weijs et al., 2009b). Higher concentrations of MeO-PBDEs in cetaceans in continental shelf and oceanic environments compared to species from estuarine areas were also recently found in Brazilian waters (Dorneles et al., 2010).

4.4. Blood as biomonitoring tool

In marine mammals, or in all mammals for that matter, the blood is responsible for the transport of all kinds of molecules, such as lipids and proteins, from one organ to the other inside the body. However, the concentrations of these compounds in blood may change as a result of several factors like the feeding status, metabolism and the overall health condition of the animal. These factors are also important for explaining the variation of pollutants in blood due to the (high) lipophilic nature of, for example, PCBs, PBDEs and the affinity of HO-PCBs for proteins. To date, no information about the concentrations of pollutants before and
after a meal in marine mammal blood is available. However, although the feeding status of the animals at the moment of blood sampling was unknown, it cannot be ruled out for explaining the individual variation among the animals. It is impossible to discuss the influence of the other two factors, namely metabolism and health condition, without taking the role of blubber into account. In general, blubber has a double function. It provides insulation for the body (Dunkin et al., 2005) and it also stores energy in the form of lipids (Koopman et al., 1996; Kastelein et al., 1997b). A depletion of the blubber, caused by seasonal changes in blubber thickness or more extreme cases such as emaciation or complete fasting during lactation, may lead to lipid mobilization throughout the body (Debier et al., 2003a,b; 2006), reflected in higher levels of lipids/lipophilic pollutants in the blood. Harbour seals and porpoises do not fast during lactation or other periods of their reproductive cycle (Burns, 2002), but have a seasonally variation in blubber thickness (Lockyer, 2007). In the present study however, for the harbour seals, sampling was only done once every year, making it impossible to compare between seasons of the same year. For the harbour porpoises, being in rehabilitation may suppress this seasonality. The very high concentrations found in serum of the two porpoises (one from a rehabilitation centre, one found stranded), both suffering from emaciation and lung pneumonia, were considered to be a reflection of the depletion of the blubber. These conditions, however, are more exceptional than general (Siebert et al., 2001) and can be seen relatively easy for harbour porpoises as these animals develop a 'neck' after a few days without feeding (Kastelein et al., 1997b). So far, biomonitoring of PCBs and PBDEs was mainly done in blubber of marine mammals. As concentrations in blubber were found to be correlated with

blubber thickness (Debier et al., 2003a; Montie et al., 2008; Weijs et al., 2009a), these results are equally dependent on the blubber thickness as concentrations in blood. It was not possible to correlate concentrations in blood to concentrations in blubber in the present study. In contrast to blubber, blood has the advantage that sampling can more easily occur in living animals which is always more realistic.

5. Conclusions

HO-PCBs are particularly bound to proteins, so that blood is the ideal substrate for detecting HO-PCBs. The presence of HO-PCBs in serum of harbour seals suggests that these animals are capable of metabolizing PCBs, while harbour porpoises are not. In general, higher number of compounds (PCBs, PBDEs, HCB, 2'-MeO-BDE 68, 6-MeOBDE 47 and DDTs) were detected in serum of harbour porpoises. Despite the fact that correlations between levels in serum and blubber, as storage compartment for the lipophilic compounds, could not be made, profiles of PCBs and PBDEs in serum were comparable with profiles in blubber. Within each species, variation between individuals remained limited, even without knowing the feeding status (time between feeding and sampling, amount of food ingested, etc.) of each individual and without taking the season of sampling into account. Concentrations in serum were assumed to be correlated with the body condition, in particular the emaciation, of the animals.

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Chapter 4

POPs in animals from the Black Sea, North Sea and Australia

4.1 Paper IV

Occurrence of anthropogenic and naturally-produced organohalogenated compounds in tissues of Black Sea harbour porpoises

Liesbeth Weijs, Krishna Das, Hugo Neels, Ronny Blust, Adrian Covaci

IV

Abstract

Harbour porpoises are one of the three cetacean species inhabiting the Black Sea. This is the first study to report on PBDEs and naturally-produced compounds, methoxylated PBDEs (MeO-PBDEs) and polybrominated hexahydroxanthene derivatives (PBHDs), in tissues (kidney, brain, blubber, liver, muscle) of male harbour porpoises (11 adults, 9 juveniles) from the Black Sea. Lipid-normalized concentrations decreased from muscle > blubber > liver > kidney > brain for the sum of polychlorinated biphenyls (PCBs) and for the sum of PBDEs. Among the naturally-produced compounds, levels of PBHDs were higher than of MeO-PBDEs, with tri-BHD and 6-MeO-BDE 47 being the dominant compounds for both groups, respectively. Concentrations of naturally-produced compounds decreased from blubber to brain, similarly to the sum of DDT and metabolites (DDXs). Concentrations of DDXs were highest, followed by PCBs, HCB, PBHDs, PBDEs and MeO-PBDEs. Levels of PCBs and PBDEs in blubber were lower than concentrations reported for harbour porpoises from the North Sea, while concentrations of DDXs were higher.

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1. Introduction

PCBs and PBDEs are two groups of lipophilic anthropogenic compounds which are banned in Europe since the 1970s (PCBs) and in 2004 and 2008 (PBDEs). PCBs and PBDEs were used in electrical devices, textiles, furniture and other household products, foams and plastics (ATSDR, 2001, 2004; Birnbaum and Staskal, 2004). Both groups of pollutants consist of 209 different congeners, which differ in the number and the position of the chlorine or bromine atoms, respectively. The biochemical and physical characteristics are therefore different for each congener, thereby influencing their fate, distribution and toxicity in wildlife and in the environment. As a result, PCBs and PBDEs are still present in ecosystems (Ruus et al., 1999; Boon et al., 2002; Borgå et al., 2004; Johnson-Restrepo et al., 2005; Burreau et al., 2006). In these systems, concentrations of PCBs and PBDEs tend to biomagnify throughout food chains, ultimately reaching high levels in tissues of top predators (Ruus et al., 1999; Boon et al., 2002; Johnson-Restrepo et al., 2005). Marine mammals are situated at the top of their food chains and accumulate considerable amounts of chemicals in their bodies causing adverse or toxic effects.

Besides PCBs and PBDEs, hexachlorobenzene (HCB) and pesticides (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE and *o,p'*-DDD) are well known anthropogenic pollutants. HCB was formerly used as a fungicide, but its production and use have been banned globally after the discovery of its carcinogenic nature and its high toxicity in aquatic organisms (ATSDR, 2002b). Organochlorine pesticides, such as DDT and its metabolites (DDE and DDD), are proven to be beneficial for controlling insects in agriculture and also for preventing malaria. Although OCPs were once widely used, nowadays, they are banned in most regions of Europe and North-America. Yet, some OCPs are still in use in a number of countries surrounding the Black Sea (East-Europe) (Fillmann et al., 2002), in Asia and in South-America.

Recently, attention has been drawn towards the presence of naturally-produced compounds, such as methoxylated PBDEs (MeO-PBDEs) and polybrominated hexahydroxanthene derivatives
(PBHDs) in aquatic ecosystems (Melcher et al., 2007; Covaci et al., 2008; Weijs et al., 2009c; Pena-Abaurrea et al., 2009). MeO-PBDEs can biomagnify in the food chains, acting in the same way as the anthropogenic PBDEs (Kelly et al., 2008; Losada et al., 2009; Weijs et al., 2009c).

Next to short-beaked common dolphins (*Delphinus delphis ponticus*) and bottlenose dolphins (*Tursiops truncatus ponticus*), harbour porpoises (*Phocoena phocoena relicta*) are one of three cetaceans inhabiting the Black Sea (Birkun, 2003; Tonay et al., 2007). Porpoises from the Black Sea are currently classified as 'endangered' (IUCN Red List of Threatened Species, 2008) as they suffer from habitat loss, accidental entanglement in fishing gear and pollution with metals and organic compounds (Madhusree et al., 1997; Tanabe et al., 1997a,b; Joiris et al., 2001; Birkun, 2003; Das et al., 2004a). The Black Sea acts as a sink for chemicals as it receives high loads of pollutants through run-off from the surrounding countries and because it is linked to the Mediterranean Sea only through the Marmara Sea (Tanabe et al., 1997a). As a consequence, porpoises from the Black Sea are isolated from their counterparts in other European waters, migrate only on a small scale and spend their entire life in the same area (Fontaine et al., 2007b; Viaud-Martinez et al., 2007).

To our knowledge, this is the first study to report on anthropogenic and naturally-produced brominated compounds in tissues of harbour porpoises from the Black Sea. The aims of this study were to discuss levels and profiles of anthropogenic and naturally-produced compounds in harbour porpoises from the Black Sea and to investigate the distribution of these pollutants between tissues in these animals.

2. Materials and methods

2.1. Samples, chemicals and target compounds

Blubber, liver, muscle, kidney and brain samples were collected from 20 male harbour porpoises (9 juveniles and 11 adults) stranded or bycaught in the Black Sea in 1998 (Fig. 1). In all samples, 39 PCB congeners (IUPAC numbers: CB 18, 28, 31, 44, 47, 49, 52, 74, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 146, 149, 151, 153, 156, 158, 170, 171, 172, 174, 177, 180, 183, 187, 194, 195, 196/203, 199, 203, 205, 209), 8 PBDEs (IUPAC numbers: BDE 28, 47, 49, 99, 100, 153, 154, 183), DDXs (*o,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT) and HCB were targeted. In addition, two naturally-produced methoxylated PBDEs (2'-MeO-BDE 68 and 6-MeO-BDE 47), together with tri-BHD and tetra-BHD were investigated. Standards were from Wellington Laboratories (PBDEs and MeO-PBDEs), from Dr. Ehrenstorfer Laboratories (PCBs) and a gift from Walter Vetter (PBHDs).

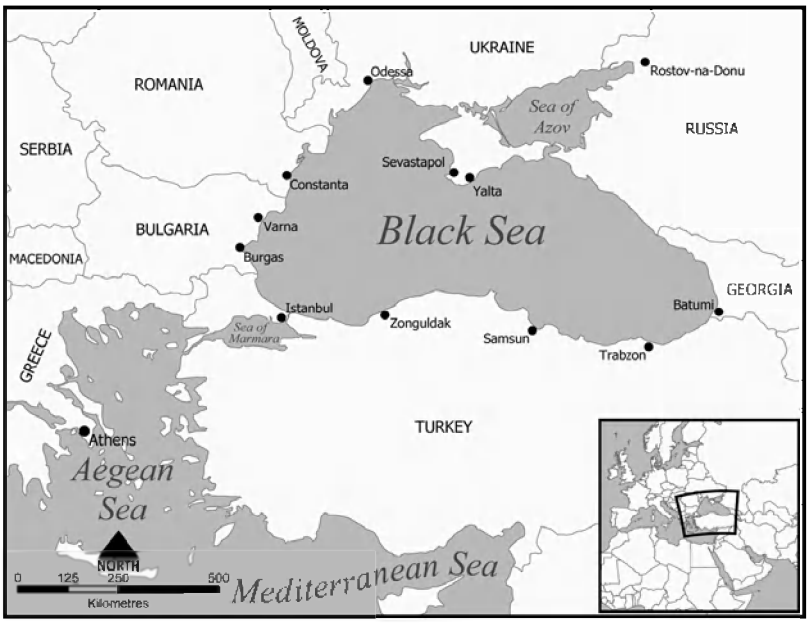

Fig. 1. The Black Sea – map of the study area. Source: climatelab.org/Bucharest_Convention

2.2. Sample preparation

The method used for the sample extraction and clean-up has been previously described (Covaci et al., 2008) and is briefly presented below. Approximately 2 g of liver and brain, 0.2 g of blubber and 3 g of muscle and kidney were dried with ~8 g anhydrous Na_2SO, spiked with internal standards BDE 77/BDE 128 (25 ng) and CB 143 (100 ng) and extracted for 2 h by hot Soxhlet with 100 ml hexane/acetone (3/1; v/v). After lipid determination (performed on an aliquot of the extract), the extract was cleaned on 8 g of acidified silica. After elution of analytes with 20 ml hexane and 15 ml dichloromethane, the cleaned extract was evaporated to dryness and reconstituted in 150 µl iso-octane.

2.3. Analysis

PBDEs, MeO-PBDEs and PBHDs were measured with an Agilent 6890 gas chromatograph coupled with a 5973 mass spectrometer system (GC-MS). The GC was equipped with a 30 m x 0.25 mm x 0.25 µm DB-5 capillary column. The MS was operated in electron capture negative ionisation (ECNI) mode and was used in the selected ion-monitoring (SIM) mode with ions m/z = 79 and 81 monitored during the entire run. PCBs, HCB and DDXs were measured with a similar GC-MS system as for the PBDE determination, operated in electron ionisation (EI) mode and equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium). The MS was used in the SIM mode with two ions monitored for each PCB homologue group. The latter system (GC-EI/MS) was also used for confirmation of organobromine compounds.

2.4. Quality assurance/quality control (QA/QC)

Recoveries for individual PBDE congeners were between 87% and 104% (RSD < 12%), while recoveries of PCBs ranged between 75% and 90% (RSD < 10%). For each analyte, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at three times the standard deviation of the procedural blank, which ensures > 99% certainty that the reported value is originating from the sample. For analytes that were not detected in procedural blanks, LOQs were calculated for a ratio S/N equal to 10. LOQs depended on the sample intake and on the analyte and ranged between 1 and 4 ng/g lw. QC was performed by regular analyses of procedural blanks, by random injection of standards and solvent blanks. A standard reference material SRM 1945 (PCBs and PBDEs in whale blubber) was used to test the method accuracy. Obtained values were not deviating more than 10% from the certified values. The QC scheme is also assessed through regular participation to interlaboratory comparison exercises organised by the US NIST.

2.5. Statistical analysis

Statistical analyses were conducted using the SPSS 15.0 statistical package. The level of statistical significance was defined at $p < 0.05$. For concentrations below the LOQ, a value of ½ LOQ was used. Outliers in all groups were detected by making boxplots. Non-parametric statistical tests were used since the data were found to have a non-normal distribution (Shapiro Wilk's statistical test). Differences in the concentrations and profiles of PCBs and PBDEs were compared between the two age-groups (adult males and juvenile males) using Kruskal–Wallis. Spearman's correlation coefficients were calculated using GraphPad Prism 4 (GraphPad Software, Inc.).

3. Results

3.1. Lipids

Lipid percentages ranged in general from 1.97–4.93% for kidney, 7.80–18.5% for brain, 2.64– 5.54% for liver, 0.22–7.33% for muscle and 87.6–97.3% for blubber in the present study. Concentrations discussed here are all lipid-normalized.

3.2. Levels and profiles of PBDEs

BDE 183 could not be detected in any sample, while BDE 47 was the most dominant PBDE congener in all samples representing between 50% and 72% of total PBDEs. Profiles were consistent among adults, with BDE 47 > BDE 100 > BDE 99 > BDE 154 in all tissues. In contrast, there was some variation in juveniles with BDE 100 and 99 switching places depending on the type of tissue. Similar to sum PCBs, levels of sum PBDEs decreased from muscle to blubber, liver, kidney and brain (Table 1). No age influence was found as there were no significant differences in sum PBDEs (or any individual PBDE congener) between adults and juveniles in all tissues (all $p > 0.05$).

Table 1. Medians (range) expressed in ng/g lw for sum PCBs, sum DDXs, HCB, sum PBDEs, sum MeO-PBDEs and sum PBHDs in tissues of harbour porpoises from the Black Sea.

		N	Adults	N	Juveniles
Sum PCBs	Kidney	10[a]	4044 (2723 - 8739)	9	3384 (1851 - 7429)
	Brain	10[a]	1614 (897 - 2282)	9	1009 (758 - 1575)
	Liver	10[a]	8450 (4161 - 20131)	8[a]	6508 (5311 - 13532)
	Muscle	9[b]	17420 (9908 - 24956)	8[a]	11707 (8479 - 15022)
	Blubber	11	13215 (8810 - 24875)	9	6956 (4896 - 13665)
Sum PBDEs	Kidney	10[a]	15.9 (10.6 - 27.1)	9	18.4 (11.0 - 63.2)
	Brain	10[a]	3.1 (2.4 - 5.5)	9	3.9 (2.1 - 6.2)
	Liver	10[a]	44.7 (20.0 - 59.9)	8[a]	44.0 (30.8 - 66.3)
	Muscle	9[b]	80.8 (54.1 - 115)	8[a]	80.4 (48.0 - 102)
	Blubber	11	65.6 (43.2 - 85.1)	9	57.0 (47.5 - 72.8)
Sum DDXs	Kidney	10[a]	13643 (9085 - 35706)	9	10987 (9474 - 20116)
	Brain	10[a]	4821 (2682 - 9149)	9	2921 (1803 - 4401)
	Liver	10[a]	28022 (14808 - 85864)	8[a]	20053 (16055 - 48934)
	Muscle	9[b]	75821 (42258 - 100858)	8[a]	42964 (31675 - 54377)
	Blubber	11	77329 (54993 - 156750)	9	40891 (27387 - 81709)
HCB	Kidney	10[a]	521 (408 - 572)	9	393 (275 - 655)
	Brain	10[a]	185 (144 - 217)	9	114 (93 - 226)
	Liver	10[a]	1456 (976 - 1848)	8[a]	972 (507 - 2107)
	Muscle	9[b]	823 (633 - 1356)	8[a]	518 (332 - 875)
	Blubber	11	575 (487 - 926)	9	394 (298 - 774)
Sum MeO-PBDEs	Kidney	10[a]	10.6 (7.6 - 19.9)	9	11.7 (9.9 - 44.9)
	Brain	10[a]	2.3 (0.6 - 5.6)	9	3.1 (2.2 - 7.0)
	Liver	10[a]	18.2 (11.6 - 35.7)	8[a]	21.7 (16.9 - 61.6)
	Muscle	9[b]	39.0 (20.8 - 72.8)	8[a]	45.7 (21.4 - 122)
	Blubber	11	46.6 (30.7 - 73.1)	9	52.9 (44.9 - 79.9)
Sum PBHDs	Kidney	10[a]	212 (139 - 445)	9	230 (95 - 618)
	Brain	10[a]	36.2 (16.9 - 53.7)	9	32.5 (20.8 - 55.9)
	Liver	10[a]	743 (318 - 1444)	8[a]	599 (504 - 1324)
	Muscle	9[b]	1502 (1003 - 2497)	8[a]	1341 (831 - 1963)
	Blubber	11	2204 (1535 - 3538)	9	1509 (945 - 2206)

[a] One sample was not available

[b] Two samples were not available

3.3. Occurrence of naturally-produced organobromines

Tri-BHD, tetra-BHD, 2'-MeO-BDE 68 and 6-MeO-BDE 47 were targeted in the present study. Among MeO-PBDEs, 6-MeO-BDE 47 was the most dominant compound. Tri-BHD had the highest concentrations for PBHDs and for all naturally-produced organobromines in total. Although concentrations of sum MeO-PBDEs were lower in adults compared to juveniles, no statistical significant differences were found for the sum MeO-PBDEs between adults and juveniles in all tissues (all $p > 0.05$), except for brain (median concentrations of 2.3 and 3.1 ng/g lw for adults and juveniles, respectively ($p = 0.041$)). Levels of sum PBHDs were higher in adults than in juveniles, although only they were statistically different for concentrations in blubber

(median concentrations of 2204 and 1509 ng/g lw for adults and juveniles, respectively ($p = 0.004$)). In addition, the highest concentrations of sum PBHDs and of sum MeO-PBDEs were found in blubber, while the lowest concentrations were in brain.

3.4. Levels and profiles of PCBs

Concentrations of sum PCBs decreased from muscle > blubber > liver > kidney > brain (Table 1). In all tissues and in both age-groups (adult males and juvenile males), CB 153 was the predominant congener (17–21% of sum PCBs), followed by CB 138 and CB 149. For adults, CB 99 was the fourth congener as abundance, replaced by CB 118 in juveniles. The next congeners were CB 95 or CB 180, depending on the age-group and on the tissue. CB 31 could only be detected in some brain samples. No differences were found for the sum PCBs between the adults and the juveniles in kidney, brain and liver (all $p > 0.05$), while significant differences were found in blubber (medians of 13,215 and 6956 ng/g lw in adults and juveniles, respectively ($p = 0.003$)) and muscle (medians of 17,420 and 11,707 ng/g lw in adults and juveniles, respectively ($p = 0.016$)).

3.5. Levels and profiles of DDXs and HCB

Results for HCB and sum DDXs (*o,p'*-DDT, -DDE, -DDD and *p,p'*-DDT, -DDE, -DDD) show that, for both age-groups, HCB levels were highest in liver, while sum DDXs were highest in blubber. Brain had the lowest concentrations of HCB and DDXs (Table 1). Significant age-related differences for HCB were found in all tissues, except in kidney, while concentrations of sum DDXs differed significantly between adults and juveniles in brain (medians of 4821 and 2921 ng/g lw in adults and juveniles, respectively ($p = 0.024$)), in muscle (medians of 75,821 and 42,964 ng/g lw in adults and juveniles, respectively ($p = 0.003$)) and in blubber (medians of 77,329 and 40,891 ng/g lw in adults and juveniles, respectively ($p = 0.002$)). Among DDXs, *p,p'*-DDE and *p,p'*-DDD were the most dominant compounds, contributing to more than 90% to the sum DDXs. o,p'-DDE represented the DDX compound with the lowest concentration in all animals.

4. Discussion

To our knowledge, the present study is the first to report on the levels of PBDEs and naturally-produced brominated compounds (MeO-PBDEs and PBHDs) in marine mammals from the Black Sea. The Black Sea represents a semi-enclosed area, linked to the Mediterranean Sea only through the Marmara Sea (Fig. 1). The Black Sea receives high loads of pollutants through run-off from the surrounding countries, from which some are still using banned/restricted POPs, and is of great economical importance for the people inhabiting these countries (Fillmann et al., 2002).

4.1. Distribution between tissues

The highest lipid percentages were consistently found in blubber, while the lowest percentages could be detected in kidney and muscle. The lipid percentages of each tissue seemed to be consistent, since no statistically significant differences were found between adults and juveniles. Lipid results from the present study were also comparable to those from other studies. Lipid percentages in porpoises analysed by Kannan et al. (1993), Tanabe et al. (1997a) and Berge et al. (2004) fall, in general, well in the ranges

reported in the present study. Duinker et al. (1989) analyzed harbour porpoises from the North Sea between 1977 and 1979 and found percentages ranging from 1.4% to 6.7% for kidney, 5.3–13.9% for brain, 2.1–8.6% for liver, 0.5–7.9% for muscle and 54.2–93.5% for blubber. Due to differences in the function and in the lipid percentage between tissues and in the biochemical properties of the pollutants, the distribution of the chemicals may vary significantly in the animal's body.
For sum PCBs and sum PBDEs, concentrations were higher in muscle, followed by blubber, liver, kidney and brain, respectively. Some PCB and PBDE congeners are highly lipophilic and are therefore primarily stored in blubber, which explains the high levels of PCBs in blubber. For similar reasons, PCB and PBDE concentrations in kidney were relatively low. Brain tissue often has a higher lipid percentage than muscle, liver and kidney. However, most likely due to the blood–brain barrier, concentrations of chemicals are usually lower than levels in any other tissue investigated in the present study (Bernhoft and Skaare, 1994).
For the sum MeO-PBDEs, sum PBHDs and the sum DDXs, blubber had the highest concentrations, followed by muscle, liver, kidney and brain, respectively. These compounds are lipophilic, naturally-produced (MeO-PBDEs and PBHDs) or still in use (DDT), which increases their bioaccumulative potential. For HCB, a different pattern was found with the highest concentrations in liver followed by muscle, blubber, kidney and brain respectively. The distribution of all investigated compounds remained the same in both age-groups (adults and juveniles), suggesting that partitioning between tissues is not changing over time or with age.

4.2. Levels and profiles

CB 153 and BDE 47 were predominant among PCBs and PBDEs, respectively. This is a typical and well known finding, not only in several marine mammal species (e.g. Covaci et al., 2002; Johnson-Restrepo et al., 2005; Ikonomou and Addison, 2008; Shaw et al., 2008, 2009; Montie et al., 2009; Weijs et al., 2009a), but in various fish species (Voorspoels et al., 2003, 2004; Johnson-Restrepo et al., 2005; Shaw et al., 2009) as well. CB 153 was followed by CB 138 and CB 149, which is different from PCB profiles in pinnipeds (Weijs et al., 2009a), but similar to the profile in harbour porpoises from the North Sea (Weijs et al., 2009a) and again very specific for harbour porpoises. Due to bioaccumulation with age and a reduced metabolism of some PCB congeners, levels of sum PCBs were consistently higher for adults than for juveniles in each tissue, although these differences were only statistically significant for muscle and blubber. In contrast, levels of sum PBDEs in kidney and brain of juveniles were higher compared to the levels in adults. The higher concentrations of sum PBDEs in brain in particular, are of concern, since the juvenile stage is an important time period in the development of the brain and for the animal in general. Profiles of PCB and PBDE congeners may differ in both age classes, probably due to an age-related potential for metabolism or a slow elimination of some congeners received from the mother during gestation or lactation (Debier et al., 2003a,b).
To enable comparisons with previous reports, the ratio of each of the seven PCBs (IUPAC numbers 28, 52, 101, 118, 138, 153 and 180), proposed by the International Council for the Exploration of the Sea (ICES) to CB 153 was calculated for the liver and blubber of harbour porpoises from the Black Sea and the North Sea (data of liver and blubber from Covaci et al. (2002) and Weijs et al. (2009a), respectively; Fig. 2). Higher ratios of lower chlorinated

PCBs for both liver and blubber were found in the Black Sea porpoises, compared to the North Sea counterparts. This might be due to the historical use of different Aroclor mixtures in both areas. CB 180 is present in a higher proportion in Aroclor 1260 whereas CBs 101, 118 and 138 are indicative for Aroclor 1254 (Thompson et al., 1996). Although CB 153 is the predominant congener in this study and in other studies conducted in the North Sea (Covaci et al., 2002; Weijs et al., 2009a) and the ratios of CB 180 and CB 138 to CB 153 are similar in both areas, the higher ratio of CB 101 and CB 118 to CB 153 in the Black Sea might be indicative for a higher use of PCB mixture Aroclor 1254 in the Black Sea area compared to the North Sea.

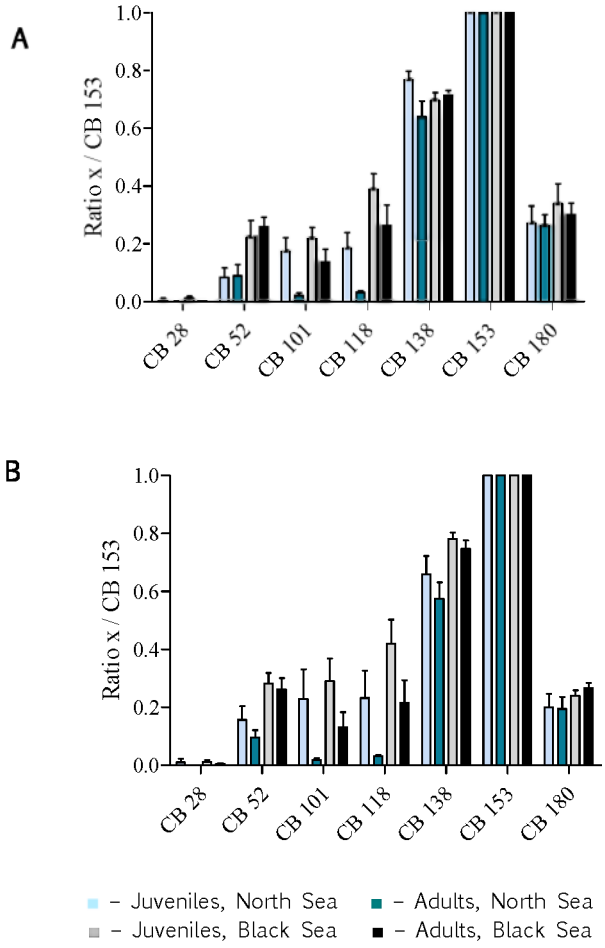

Fig. 2. Ratio of the seven PCBs proposed by the ICES (International Council for the Exploration of the Sea) to CB 153 in: (A) liver and (B) blubber of adult and juvenile male harbour porpoises from the North Sea (data of liver and blubber from Covaci et al. (2002) and Weijs et al. (2009a), respectively) and Black Sea (present study). Error bars represent SD.

Levels of PCBs and PBDEs in blubber and liver were lower than concentrations reported for harbour porpoises from the North Sea (Covaci et al., 2002; Weijs et al., 2009a) and were also lower than levels of PCBs measured in blubber of adult males from the Black Sea in 1993 (Tanabe et al., 1997a). This is the first study to report on PBDEs in harbour porpoises from the Black Sea, therefore, there are no results of PBDEs in Black Sea marine mammals to compare with. Overall, PBDEs had only a minor contribution in the animals (Fig. 3), suggesting that PBDEs were not consistently used before 1998 in countries surrounding the Black Sea.

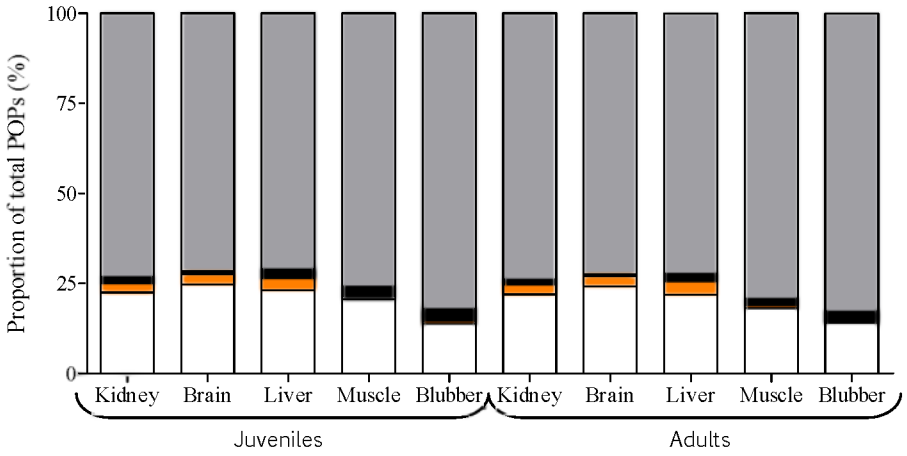

Fig. 3. Overview of the proportions of sum PCBs (□), HCB (■), sum PBDEs (■), sum DDXs (■) and sum of naturally-produced organobromines (■) in tissues of harbour porpoises from the Black Sea.

For DDXs, the *p,p'*-metabolites (*p,p'*-DDE and *p,p'*-DDD) had the highest concentrations among all DDXs and in all tissues, while *o,p'*-DDE had the lowest levels. Higher contributions of the *p,p'*-DDX metabolites were previously also found by Montie et al. (2009) in cerebellum grey matter and cerebrospinal fluid of Atlantic white-sided dolphins, by Covaci et al. (2002) in liver of harbour porpoises, and by Hoekstra et al. (2003b) in blubber of bowhead whales, beluga whales, ringed seals and bearded seals. Increasing concentrations of *p,p'*-DDE and decreasing concentrations of *p,p'*-DDT can be explained by a higher metabolism of DDT in marine mammals relative to their prey together with the bioaccumulation of DDT metabolites, as suggested by Hoekstra et al. (2003b). Concentrations in adults were, in general, higher than in juveniles, indicating bioaccumulation over time. The highest concentrations found by Hoekstra et al. (2003b) for sum DDXs were reported in blubber of beluga whales with an average of 1979 ± 231 ng/g lw (mean and standard error, SE), which is more than 39 and 21 times lower than levels in blubber of adult and juvenile harbour porpoises from the present study. Also, concentrations of sum DDXs exceed by far the concentrations found in liver of harbour porpoises from the North Sea (3.4 ± 2.3 lg/g lw (mean ± SD; Covaci et al., 2002). Considering an average lipid percentage of 90% in blubber of harbour porpoises from the Black Sea, concentrations reported in Tanabe et al. (1997b) were 77,800 ng/g lw for male porpoises (adult and juvenile) from the Black Sea and 5200 ng/g lw for male porpoises (adult and juvenile) from Hokkaido (Japan). Results from the present study were about 10–15 times higher than concentrations from Hokkaido, however, they were lower than levels reported for male porpoises from the Black Sea in 1993 indicating that DDX concentrations decreased slightly in a 5 year period.

MeO-PBDEs and PBHDs have been suggested to be produced by marine organisms, such as algae and sponges (Vetter et al., 2002; Malmvärn et al., 2008). These compounds have been reported previously in whale and fish oil (Teuten and Reddy, 2007; Covaci et al., 2007), in fish and in marine mammals (Vetter et al., 2002; Pettersson et al., 2004; Melcher et al., 2005; Losada et al., 2009; Weijs et al., 2009c). To date, no reports on their presence of algae and sponges in the Black Sea are available. Among MeO-

PBDEs, 6-MeO-BDE 47 was the most dominant compound, as indicated for marine mammals from the Northern hemisphere in general (Weijs et al., 2009c). Although not statistically significant, adults had lower concentrations of sum MeO-PBDEs compared to the juveniles, suggesting that they are more capable of metabolic breakdown or elimination of these naturally-produced compounds than juveniles or that the juveniles have a high 'start' concentration due to maternal placental transfer to offspring. This finding was also reported for adult male and juvenile male harbour seals and porpoises from the North Sea (Weijs et al., 2009c). Concentrations of MeO-PBDEs were lower compared to those in cetaceans from Canada, Japan, Australia and Europe (Kelly et al., 2008; Marsh et al., 2005; Melcher et al., 2005; Weijs et al., 2009c), but higher than concentrations in pinnipeds from Canada and Europe (Kelly et al., 2008; Weijs et al., 2009c) (Table 2).

Table 2. Inter-species comparison for MeO-PBDEs (sum of 2'-MeO-BDE 68 and 6-MeO-BDE 47). All concentrations are expressed in ng/g lw.

Species	Tissue	Concentration (ng/g lw)	Reference
Ringed seal[a]	blubber	6.3	Kelly et al., 2008
Beluga whale[a]	blubber	298	
	liver	302	
Striped dolphin	Fresh blubber	52	Marsh et al., 2008
	Cooked liver	540	
Bottlenose dolphin	Sliced bacon	2910	
Minke whale	Fresh blubber	48	
Baird's beaked whale	Shredded bacon	58	
Common dolphin	Blubber	5435	Melcher et al., 2005
Bottlenose dolphin	Blubber	13145	
Melonhead whale	Blubber	2005	
Pygmy sperm whale	Blubber	2888	
Humpback dolphin	Blubber	2795	
	Brain	151	
Harbour porpoise[a]	Blubber	113.7 – 143[b]	Weijs et al., 2009c
Harbour seal[a]	Blubber	3.2 – 7[b]	
Harbour porpoise[a]	Kidney	10.6 – 11.7[b]	Present study
	Brain	2.3 – 3.1[b]	
	Liver	18.2 – 21.7[b]	
	Muscle	39.0 – 45.7[b]	
	Blubber	46.6 – 52.9[b]	

Harbour porpoise-*Phocoena phocoena*; harbour seal-*Phoca vitulina*; humpback dolphin-*Sousa chinensis*; bottlenose dolphin-*Tursiops truncatus*; ringed seal-*Phoca hispida*; Beluga whale-*Delphinapterus leucas*; common dolphin-*Delphinus delphis*; striped dolphin-*Stenella coeruleoalba*; minke whale-*Balaenoptera acutorostrata*; Baird's beaked whale-*Berardius bairdii*; melonhead whale-*Peponocephala electra*; pygmy sperm whale-*Kogia breviceps*

[a] - only results from males

[b] - concentration in adult males – concentration in juvenile males (values are medians)

Tri-BHD (2,7-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthene) had the highest concentration among all naturally-produced compounds. Concentrations of sum PBHDs were more than 10 times higher than concentrations in blubber of harbour porpoises from the North Sea (Weijs et al., 2009c). Except for kidney, concentrations were higher (only statistically significant for blubber) with age, again indicating bioaccumulation over time or a reduced metabolism with age. For both age-groups, sum DDXs > sum PCBs > HCB > sum naturally-produced organobromines > sum PBDEs for kidney, brain and liver. For muscle and blubber, the contribution of sum naturally-produced organobromines was higher compared to HCB (Fig. 3). A higher presence of DDXs compared to PCBs was reported previously for harbour porpoises from the Black Sea, being different from the North Sea (Tanabe et al., 1997b). To check for possible relationships between the

concentrations of sum PCBs, sum PBDEs, sum DDXs, sum naturally-produced organobromines and HCB and the feeding ecology (assessed through stable isotope analyses, $\delta^{13}C$ and $\delta^{15}N$), correlations were established. Measurements of $\delta^{13}C$ and $\delta^{15}N$ in muscle of 17 out of 20 harbour porpoises (reported by Das et al., 2003, 2004a) were used to investigate the influence of trophic position on the levels of the organohalogenated compounds in the present study. However, none of these correlations were significant (Spearman; all $p > 0.05$).

5. Conclusions

The Black Sea is a region that is linked to the Mediterranean only through the Marmara Sea and thus the exchange of seawater is limited. This means that the input of anthropogenic pollutants in the Black Sea is largely influenced by the run-off from the surrounding countries. Harbour porpoises from the Black Sea spend their entire life in this area and are exposed to a typical mixture of pollutants. To our knowledge, the present study is the first to characterize the presence of anthropogenic PBDEs and naturally-produced compounds (MeO-PBDEs and PBHDs) in tissues of adult and juvenile male harbour porpoises sampled in 1998 from the Black Sea. Profiles and levels of anthropogenic and naturally-produced compounds varied with age and between different tissues. However, our results show that concentrations of PBDEs were low, indicating that PBDEs were not commonly used in the 1990s in the countries surrounding the Black Sea. In contrast, DDXs and especially p,p'-DDE, were dominant in the overall profiles in all tissues, suggesting that DDT was still used at least in some countries around the Black Sea.

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4.2 Paper V

Persistent organic pollutants and methoxylated PBDEs in harbour porpoises from the North Sea from 1990 until 2008: Young wildlife at risk?

Liesbeth Weijs, Cornelis van Elk, Krishna Das, Ronny Blust, Adrian Covaci

V

Abstract

In the European North Sea, harbour porpoises are top predators with relatively long life spans and a limited capacity for metabolic biotransformation of contaminants compared to some other marine mammal species. As such, they are exposed to a mixture of persistent pollutants, such as PCBs, PBDEs, DDT and isomers/metabolites (DDXs), HCB and chlordanes (CHLs) that bioaccumulate in their tissues. We report here on the levels of persistent organic pollutants and of the naturally-produced methoxylated PBDEs (MeO-PBDEs) in blubber, liver and kidney of harbour porpoise neonates (n = 3), calves (n = 15), juveniles (n = 6) and adults (n = 4) of the southern North Sea. Concentrations of almost all contaminant classes decrease slightly in all age groups over the period 1990–2008. For some classes (e.g. PCBs and DDXs) however, levels increase slightly in harbour porpoise calves. In all animals, blubber had the highest concentrations, followed by liver and kidney, whereas liver and kidney were the preferred tissues for several compounds, such as octa- and deca-PCBs. Our data suggest that harbour porpoises calves are exposed to higher or comparable concentrations of POPs and of MeO-PBDEs and somewhat different patterns of selected POPs than adults, potentially placing them, and the entire population, at a disproportionate risk for exposure-related health effects.

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1. Introduction

In the past, POPs, such as PCBs, DDT and isomers/metabolites, PBDEs, HCB and chlordanes (CHLs) were extensively used worldwide. PCBs were widely used for many applications, such as insulating and cooling in electrical equipment due to their flame retarding properties (ATSDR, 2001). Although PBDEs have a different chemical structure compared to PCBs, they also do not burn easily. Therefore, PBDEs were used often in a lot of products ranging from household products to textiles (ATSDR, 2004). PBDEs and PCBs are both a group of 209 congeners and each congener differs from the other in the number and position of the bromine or chlorine atoms, respectively.

DDXs are a mixture of isomers (*p,p'*- and *o,p'*-DDT) and metabolites (DDE and DDD). These pollutants have been proven effective in controlling pests and preventing diseases and had a main function in agriculture (ATSDR, 2002a). HCB and chlordane related compounds, such as cis-nonachlor and trans-nonachlor, and metabolites, such as oxychlordane, were also used as pesticides (ATSDR, 1995, 2002b). All contaminant classes mentioned so far are persistent, bioaccumulative and toxic. As a consequence, they are all banned globally: for PCBs, DDXs and HCB since the 1970s, for chlordane related compounds since the 1980s and for most PBDEs since 2004. Unfortunately, because of their stability in the environment, they can still be found at all levels of the terrestrial and aquatic food chains where they can be toxic to all organisms in these food webs (e.g. Hoffman et al., 1996; Michielsen et al., 1999; Ruus et al., 1999; Bondy et al., 2003; Birnbaum and Staskal, 2004; Johnson-Restrepo et al., 2005; Kelly et al., 2008; Sonne et al., 2009).

MeO-PBDEs differ from all contaminants mentioned so far because they have a different origin. PCBs, PBDEs, CHLs and metabolites and HCB are all intentionally or unintentionally (metabolites or as byproducts) produced by humans. In contrast, MeO-PBDEs are produced by natural sources, such as algae or sponges (Vetter et al., 2002; Kelly et al., 2008; Malmvärn et al., 2008; Weijs et al., 2009a, b, c).

The marine environment acts as a sink for pollutants as it receives a lot of chemicals through run-off from the mainland or atmospheric deposition. Most compounds have the capacity to biomagnify in marine food webs and can thus be found at high levels in marine top predators, such as marine mammals (e.g. Blasius and Goodmanlowe, 2008; Kelly et al., 2008; Weijs et al., 2009b; Ylitalo et al., 2009; Dorneles et al., 2010).

Harbour porpoises are small cetaceans that live in waters of the Northern Hemisphere. Although all harbour porpoises have, as top predators, high levels of contaminants in their tissues (Zegers et al., 2005; Pierce et al., 2008; Weijs et al., 2009a), results of Weijs et al. (2010b) for PCB 153 suggest that calves are possibly the most vulnerable age class among all other age classes because they already have a 'start' concentration from birth due to the transfer of chemicals through the placenta and via the lipid-rich milk during lactation as found previously (Debier et al., 2003a; Yordy et al., 2010). In addition, their metabolism is probably not fully developed yet, which makes it difficult for them to eliminate these compounds (Sly and Flack, 2008). Several studies have investigated the impact of pre- or post-natal exposure to environmental pollutants on the overall health and development of an organism. Tiedeken and Ramsdell (2009) have shown that fetal exposure to *p,p'*-DDE in zebrafish increases the sensitivity to domoic

acid-induced seizures at later stages. The same study found comparable *p,p'*-DDE levels in California sea lion fetuses and suggests a link between the DDE exposure and domoic acid toxicity in these animals. Rice (1999) found that monkeys, exposed to PCBs during lactation, experience a behavioral impairment later, whereas brominated and chlorinated dioxins may influence the fear memory of male mice after *in utero* and lactational exposure (Haijima et al., 2010).

The aim of the present study was to evaluate the bioaccumulation of PCBs, PBDEs, CHLs, DDXs, HCB and MeO-PBDEs in harbour porpoises from the North Sea. Until now, blubber has mainly been used as the ideal matrix for the biomonitoring of lipophilic compounds such as PCBs or PBDEs. However, the distribution of chemicals inside the body of harbour porpoises depends on the biochemical properties of the compound rather than its lipophilicity alone. Therefore, we aimed at investigating the presence of these contaminants in various tissues of harbour porpoises to determine the distribution or preference of the compounds for a specific tissue. The animals investigated in the present study died from 1990 until 2008 covering a period of 18 years. Therefore, an attempt was made to look for possible time trends within these 18 years with factors such as age or gender taken into account.

2. Materials and methods

2.1. Samples, chemicals and target compounds

Blubber samples were collected from 28 harbour porpoises (3 neonates, 15 calves, 6 juveniles and 4 adults; Supporting Information Table S1). In addition, 6 kidney samples and 18 liver samples of these animals were analysed to assess the distribution of pollutants between tissues. In the present study, calves are animals that are still drinking milk as part of their diet and that are younger than 1 year old. Juveniles are animals older than 1 year, but younger than 3 years, while adults are animals older than 3 years (Lockyer, 1995). All porpoises stranded alive on the North Sea coasts, but died during rehabilitation at the SOS Dolfijn rehabilitation center, Dolfinarium Harderwijk, The Netherlands between 1990 and 2008 (Supporting Information Table S1). In all samples, 35 PCB congeners (IUPAC numbers: CB 18, 28, 44, 47, 49, 52, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 146, 149, 151, 153, 156, 170, 171, 172, 174, 177, 180, 183, 187, 194, 195, 199, 205, 206, and 209), 7 PBDEs (IUPAC numbers: BDE 28, 47, 49, 99, 100, 153, and 154), 6 DDXs (*o,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, and *p,p'*-DDT), 3 chlordanes (oxychlordane (OxC), trans-nonachlor (TN), and cis-nonachlor (CN)) and HCB were targeted. Also, 2 naturally-produced methoxylated PBDEs (2'-MeO-BDE 68 and 6-MeO-BDE 47) were investigated. PBDE standards were from Wellington Laboratories (Guelph, ON, Canada) and all other POPs were from Accustandard (New Haven, CT, USA).

2.2. Sample preparation

The method used for the sample extraction and clean-up has been previously described (Covaci et al., 2008) and is briefly presented in the following discussion. Approximately 2 g of liver, 0.2 g of blubber and 3 g of kidney were dried with ~8 g anhydrous Na_2SO_4, spiked with internal standards BDE 77/BDE 128 (25 ng) and CB 143 (100 ng) and extracted for 2 h by hot Soxhlet with 100 ml hexane/acetone (3/1; v/v). After lipid determination (gravimetrically, performed on an aliquot of the extract, typically 1/8), the extract was cleaned on 8 g of acidified silica. After elution

of analytes with 20ml hexane and 15 ml dichloromethane, the cleaned extract was evaporated to dryness and reconstituted in 150 µl iso-octane.

2.3. Analysis

PBDEs, MeO-PBDEs and CHL were measured with an Agilent 6890 gas chromatograph coupled with a 5973 mass spectrometer system (GC–MS). The GC was equipped with a 30 m × 0.25 mm × 0.25 µm DB-5 capillary column. The MS was operated in electron capture negative ionisation (ECNI) mode and was used in the selected ion-monitoring (SIM) mode with ions m/z=79 and 81 monitored during the entire run for PBDEs and MeO-PBDEs and two specific ions for each CHL compound. PCBs, HCB and DDXs were measured with a GC–MS system operated in electron ionisation (EI) mode and equipped with a 25 m × 0.22 mm × 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium). The MS was used in the SIM mode with 2 ions monitored for each PCB homologue group. The latter system (GC–EI/MS) was also used for confirmation of organobromine compounds.

2.4. Quality assurance/quality control (QA/QC)

Recoveries for individual PBDE congeners were between 87 and 104% (RSD < 12%), while recoveries of PCBs ranged between 75 and 90% (RSD < 10%). For each analyte, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the procedural blank, which ensures > 99% certainty that the reported value is originating from the sample. For analytes that were not detected in procedural blanks, LOQs were calculated for a S/N ratio equal to 10. LOQs depended on the sample intake and on the analyte and ranged between 1 and 4 ng/g lw. QC was performed by regular analyses of procedural blanks, by random injection of standards and solvent blanks. A standard reference material SRM 1945 from the National Institute of Standards and Technology (PCBs, PBDEs and OCPs in whale blubber) was used to test the accuracy of the method. Obtained values were not deviating more than 10% from the certified values. The QC scheme was also assessed through regular participation to interlaboratory comparison exercises organised by the US NIST.

2.5. Statistical analysis

Statistical analyses were conducted using the SPSS 18.0 statistical package (PASW Statistics 18). The level of statistical significance was set at $p < 0.05$. Because the animals are of different age classes (neonate, calf, juvenile, and adult), different genders (male and female) and from different years (1990 until 2008), sample sizes of paired age–gender groups are sometimes very small. Therefore, non-parametric statistical tests (Kruskal–Wallis tests) were performed only for calves. To assess temporal trends between the calves, we divided the individuals in two groups: group 'calf 1', with calves from 1990 until 1998, and group 'calf 2' group, with calves from 2000 until 2008. Because gender differences are often only apparent for juveniles and adults, there was no division made between male or female neonates and between male or female calves (Mos et al., 2006; Weijs et al., 2010b). Where possible, Kruskal–Wallis was used to test the differences in lipid percentages and pollutant concentrations between the groups. Outliers (one adult female and one calf of 1990) were detected by making boxplots and removed from further statistical analyses.

3. Results

PCBs, PBDEs, naturally-produced MeO-PBDEs, DDXs, CHLs and HCB were measured in 28 blubber samples, 18 liver samples and 6 kidney samples of harbour porpoises (3 neonates, 15 calves, 6 juveniles and 4 adults) from the North Sea. All animals were stranded alive between 1990 and 2008, but died during rehabilitation. The only adult female of this dataset was excluded from all statistical analyses. Levels of all compounds in this individual were much lower compared to the concentrations measured in all other animals of the present study and can be found for comparison reasons in Table 1. Although the low levels were probably caused by elimination of compounds through gestation/lactation during previous pregnancies, this animal was anaemic, pregnant and died at the rehabilitation center SOS Dolfijn (Harderwijk, the Netherlands) in 2007 due to acute hepatic lipidosis.

Table 1. Median concentrations and range of sum PCBs and of sum DDXs (expressed in µg/g lipid weight) in blubber, liver and kidney of harbour porpoises from the North Sea between 1990 – 1998 and 2000 – 2008.

Age class	Tissue	N[a]	∑ PCBs[b]	
			1990 - 1998	2000 - 2008
Neonate	Blubber	1 / 2	13.7	16.8 (4.7-29.0)
Calf	Blubber	3[d] / 11	10.0 (8.2-11.6)	12.8 (4.0-25.2)
	Kidney	0 / 2	-	4.0 (2.8-5.2)
	Liver	3 / 8	9.1 (3.0-10.5)	11.2 (3.8-23.4)
Juvenile	Blubber	1 / 5	19.1	9.9 (1.1-68.2)
	Kidney	0 / 1	-	16.5
	Liver	0 / 3	-	8.3 (6.8-36.7)
Adult	Blubber	1 / 2	81.5	24.9 (15.3 – 34.5)
	Kidney	0 / 2	-	6.1 (4.1-8.2)
	Liver	1 / 2	66.1	11.3 (10.0-12.5)
Outlier[e]	Blubber	0 / 1	-	1.1
	Kidney	0 / 1	-	0.5
	Liver	0 / 1	-	0.7
Age class	**Tissue**	**N[a]**	**∑ DDXs[c]**	
			1990 - 1999	**2000 – 2008**
Neonate	Blubber	1 / 2	1.9	1.8 (0.5-3.0)
Calf	Blubber	3[d] / 11	2.2 (1.9-4.7)	2.4 (0.8-3.6)
	Kidney	0 / 2	-	0.5 (0.4-0.5)
	Liver	3 / 8	0.7 (0.5-2.8)	0.9 (0.7-2.9)
Juvenile	Blubber	1 / 5	4.5	1.7 (0.4-6.4)
	Kidney	0 / 1	-	1.3
	Liver	0 / 3	-	0.8 (0.7-3.2)
Adult	Blubber	1 / 2	22.9	3.4 (2.3-4.4)
	Kidney	0 / 2	-	0.7 (0.5-0.9)
	Liver	1 / 2	9.9	1.3 (1.2-1.4)
Outlier[e]	Blubber	0 / 1	-	> 0.1
	Kidney	0 / 1	-	< 0.1
	Liver	0 / 1	-	< 0.1

[a] Number before '/' is sample size of time period 1990-1998, number after '/' is sample size of time period 2000-2008.

[b] Sum of 35 PCB congeners: IUPAC numbers: CB 18, 28, 44, 47, 49, 52, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 146, 149, 151, 153, 156, 170, 171, 172, 174, 177, 180, 183, 187, 194, 195, 199, 205, 206, 209

[c] Sum of 6 DDXs: *o,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT

[d] Considered as an outlier, calf from 1990. Sum of PCBs is 79.9 µg/g lw, sum of DDXs is 34.6 µg/g lw.

[e] Adult female considered as an outlier and excluded from further statistical analyses. Animal was anaemic and pregnant and died due to acute hepatic lipidosis.

3.1. Lipid percentages

While there were no statistically significant differences between the lipid percentages in blubber of calves (n=15), juveniles (n=6) and adults (n=3) (median: 89.6%; range: 60.1–95.1%; p=0.115), lipid percentages of neonates (n=3) were significantly lower compared to these 3 groups (median: 79.5%; range: 73.9–82.5%; p=0.019). There were no statistically significant differences between the lipid percentages of the kidney and liver between calves (n=2 for kidney, n=11 for liver), juveniles (n=1 for kidney, n=3 for liver) and adults (n=2 for kidney, n=3 for liver) (median: 3.3%; range: 3.0–4.9%; p=0.497 for kidney, median: 5.2%; range: 4.5–30.5%; p=0.637 for liver). Samples of kidney and liver of neonates were not analysed.

3.2. Comparison between life history groups and temporal trends

3.2.1. PCBs

PCBs and DDXs had the highest concentrations in all tissues of all animals with PCBs being predominant (Table 1). Levels of PCBs and of DDXs depended on the age class, the tissue and the period of time. Due to a sample size of only one animal for the juvenile- and adult group for 1990–1998, results for PCBs of the calves of that time period were difficult to compare to these two older age classes. For animals from 2000 to 2008 however, concentrations of the sum of PCBs in blubber decreased from neonates (median: 16.8 µg/g lw) to juveniles (median: 9.9 µg/g lw) after which they increased to higher levels in adults (median: 24.9 µg/g lw). Levels of the sum of PCBs in liver were also higher in calves (median: 11.2 µg/g lw) compared to juveniles (median: 8.3 µg/g lw), but lower in calves compared to adults (median: 11.3 µg/g lw). Trends of the sum of PCBs in kidneys were the opposite compared to those observed in liver or blubber, because these concentrations seem to increase from calves (median: 4.0 µg/g lw) to juveniles (median: 16.5 µg/g lw), but were lower in adults (median: 6.1 µg/g lw) than in juveniles. PCB 153 had the highest concentrations in all samples followed by PCB 138. For most samples, PCB 149 was the third congener in the PCB pattern. In 6 samples (1 kidney, 2 blubber and 3 liver samples) however, PCB 149 was replaced by PCB 187 as third congener. Percentages of these four congeners ranged from 16.3 to 28.3% for PCB 153, from 11.9 to 16.3% for PCB 138, from 7.8 to 12.3% for PCB 149 and from 4.2 to 10.7% for PCB 187. To assess temporal trends, age groups were divided into 2 time groups (1990–1998 and 2000–2008) each covering 8 years. However, only blubber samples of calves could be tested statistically because sample sizes of all other age groups, especially when they are divided into 2 time groups, were too small. Statistically, there were only differences in PCB percentages between calves from 1990 to 1999 and calves from 2000 to 2008 for PCB 151, PCB 187, PCB 177, PCB 174, PCB 180, PCB 199 and PCB 194 (all $p < 0.05$) with a decrease in PCB 174, PCB 180 and PCB 194 percentages and an increase in PCB 151, PCB 187, PCB 177 and PCB 199 percentages over time. However, when all PCBs were grouped according to the number of chlorine atoms (Fig. 1), these differences for calves were no longer apparent so that there was no division in calves from 1990 to 1999 and calves from 2000 to 2008 needed. Percentages of PCB groups differed only slightly between the three different tissues (Fig. 1). In blubber or liver, percentages of lower chlorinated penta-PCBs decreased with age, whereas percentages of higher chlorinated hepta- and octa-PCBs increased slightly with age. These trends were not clear in the kidney, but this might be due to the limited sample size for the kidney. Overall, the proportion of hexa-

PCBs to total PCBs remained preserved in all tissues and all age classes. Octa- and deca-PCBs were better represented in kidney and liver than in blubber.

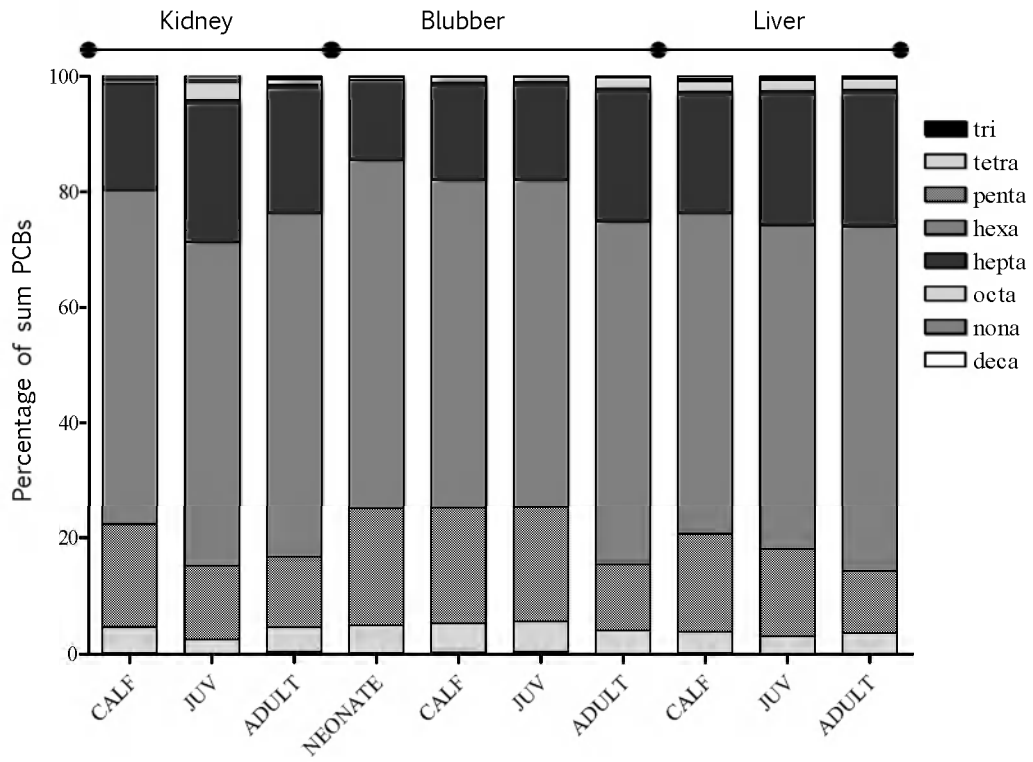

Fig 1. Proportions of the different PCB groups in kidney, blubber and liver of neonates, calves, juveniles and adult harbour porpoises from the North Sea (1990-2008). PCB groups are structural homologs listed by degree of chlorination.

3.2.2. DDXs

Within the time period 2000–2008, there was a decrease in the sum of DDXs from calves to juveniles, followed by an increase from juveniles to adults for liver and blubber (Table 1). Similar to the sum of PCBs, the sum of DDXs was highest in the kidney of the only juvenile harbour porpoise analysed compared to the kidneys of calves and adults. When comparing blubber of neonates and calves for both time periods, concentrations of the sum of PCBs were lower in blubber of the calves compared to neonates, while levels of the sum of DDXs were lower in blubber of the neonates than those in blubber of calves. For DDXs, the dominant DDX was *p,p'*-DDE with concentrations ranging from 58.5 to 88.4% of the total sum of DDXs. Both *o,p'*-DDT and *p,p'*-DDT were not present in samples of kidneys and were only sporadically found in some samples of liver, but were consistently detected in all samples of blubber. Congener *o,p'*-DDE was only detected in 3 blubber samples and 1 liver sample.

3.2.3. PBDEs and MeO-PBDEs

Sample sizes of juveniles and adults of the time period 1990–1998 were too small to compare the results to the calves of the same time period. For the sum of PBDEs in animals of 2000–2008, concentrations of PBDEs in blubber and liver were highest in calves and adults while juveniles had the highest levels of the sum of PBDEs in their kidneys (Table 2). For the sum of MeO-PBDEs, the highest levels for all tissues (liver, kidney and blubber) were found in juveniles, whereas the lowest concentrations were present in adults. PBDE

patterns were dominated by BDE 47, covering more than 30% of the sum of PBDEs, in all tissues and in all animals (Fig. 2). In all samples, BDE 47 was followed by BDE 100, although the differences between percentages of BDE 47 and BDE 100 were small in the kidney. The next congeners were BDE 99 and BDE 154, but the order depended on the tissue and the age class. Overall, the contribution of BDE 47 to the total sum of PBDEs was highest in blubber, followed by liver and kidney. In all tissues however, the percentage of BDE 47 decreased with age. Together with declining BDE 47 percentages with age, there was a slight decline in percentages of lower brominated PBDEs, e.g. BDE 28. To compensate for these decreasing BDE 47 and BDE 28 contributions, the percentages of higher brominated compounds such as BDE 100, BDE 153 and BDE 154 increased with age in each tissue. To investigate possible time trends, differences in the percentages of each PBDE congener were tested between calves of 1990–1998 and calves of 2000–2008. Although the percentages were not statistically different for all PBDE congeners, there were some statistical significant differences for BDE 47, BDE 100 and BDE 154 ($p=0.006$; $p=0.006$ and $p=0.026$) in blubber and for BDE 154 ($p=0.034$) in liver. For consistency, the calves were divided into calf 1 (calves of 1990–1998) and calf 2 (calves of 2000–2008) for all PBDE congeners (Fig. 2).

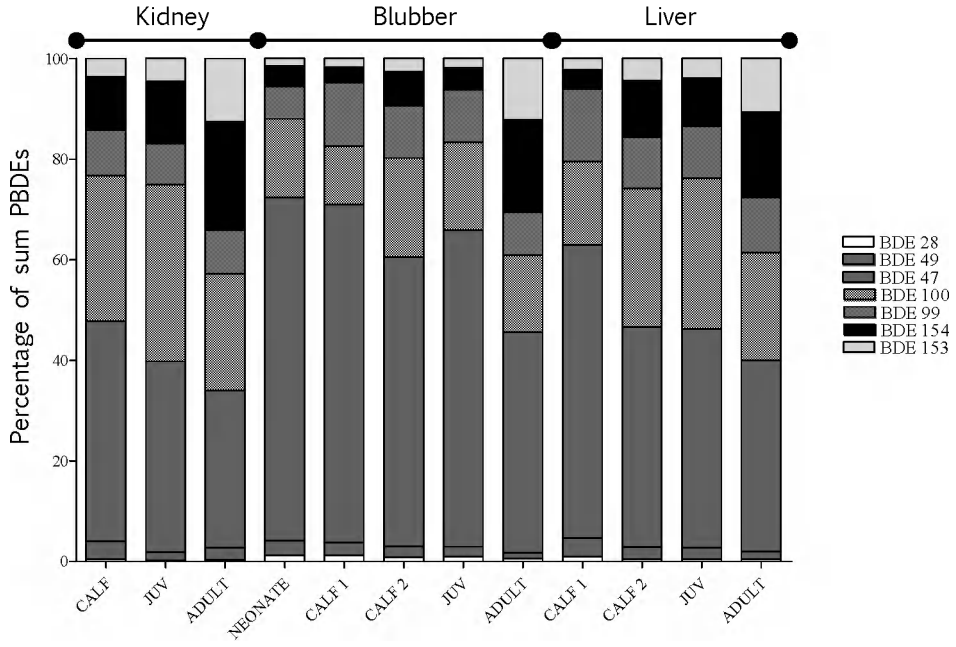

Fig 2. Proportions of the different PBDE congeners in kidney, blubber and liver of neonates, calves, juveniles and adult harbour porpoises from the North Sea (1990-2008). The calves are divided into calf 1 (1990-1998) and calf 2 (2000-2008) because there are statistical significant differences for select PBDE congeners between these two groups.

Table 2. Median concentrations and range of sum PBDEs, HCB, sum CHLOR and sum MeO-PBDE (expressed in ng/g lipid weight) in blubber, liver and kidney of harbour porpoises from the North Sea between 1990 – 1998 and 2000 – 2008.

Age class	Tissue	N[a]	∑ PBDEs[b]		HCB		∑ CHLs[c]		∑ MeO-PBDEs[f]	
			1990-1998	2000-2008	1990-1998	2000-2008	1990-1998	2000-2008	1990-1998	2000-2008
Neonate	Blubber	1 / 2	132	462 (82-841)	143	104 (25-184)	150	186 (24-349)	20	97 (13-180)
Calf	Blubber	4/ 11	2586 (1479-4062)	552 (229-1457)	117[d] (88-135)	100 (54-195)	250[d] (216-269)	268 (72-382)	149[d] (136-236)	138 (32-293)
	Kidney	- / 2	-	119 (86-152)	-	75 (55-94)	-	50 (42-59)	-	26 (18-33)
	Liver	3 / 8	485 (89-1157)	337 (135-1419)	153 (80-195)	184 (67-313)	77 (59-159)	83 (58-245)	43 (15-173)	70 (29-210)
Juveniles	Blubber	1 / 5	4771	494 (280-1501)	191	141 (53-214)	680	194 (68-551)	118	224 (67-405)
	Kidney	- / 1	-	575	-	129	-	129	-	111
	Liver	- / 3	-	318 (290-1305)	-	141 (114-238)	-	90 (56-289)	-	120 (77-265)
Adult	Blubber	1 / 2	1900	1194 (563-1825)	350	90 (88-91)	3611	688 (371-1004)	156	80 (67-94)
	Kidney	- / 2	-	221 (114-328)	-	56 (54-58)	-	151 (78-225)	-	19 (14-24)
	Liver	1 / 2	1552	426 (329-522)	515	97 (96-97)	1808	270 (199-341)	100	43 (39-47)
Outlier[e]	Blubber	- / 1	-	42	-	5	-	9	-	11
	Kidney	- / 1	-	21	-	5	-	3	-	5
	Liver	- / 1	-	37	-	8	-	7	-	9

[a] Number before '/' is sample size of time period 1990-1998, number after '/' is sample size of time period 2000-2008.
[b] Sum of 7 PBDEs: IUPAC numbers: BDE 28, 47, 49, 99, 100, 153, 154
[c] Sum of 3 CHLs (cis-nonachlor, trans-nonachlor, oxychlordane)
[d] Sample size of 3 animals. One animal considered as an outlier, calf from 1990. HCB is 2288 ng/g lw, sum of CHLs is 5223 ng/g lw, sum of MeO-PBDEs is 599 ng/g lw.
[e] Adult female considered as an outlier and excluded from further statistical analyses. Animal was anaemic and pregnant and died due to acute hepatic lipidosis.
[f] Sum of 2 congeners: 2'-MeO-BDE 68 and 6-MeO-BDE 47

Ratios of the naturally-produced 6-MeO-BDE 47 reached up to 90% of total MeO-PBDEs, while 2'-MeO-BDE 68 only had a minor contribution (Fig. 3). Time trends were not apparent, since differences between the calf 1 and calf 2 groups were not statistically significant for blubber or liver. In each tissue type, the contribution of 6-MeO-BDE 47 was highest in juveniles and lowest in adults.

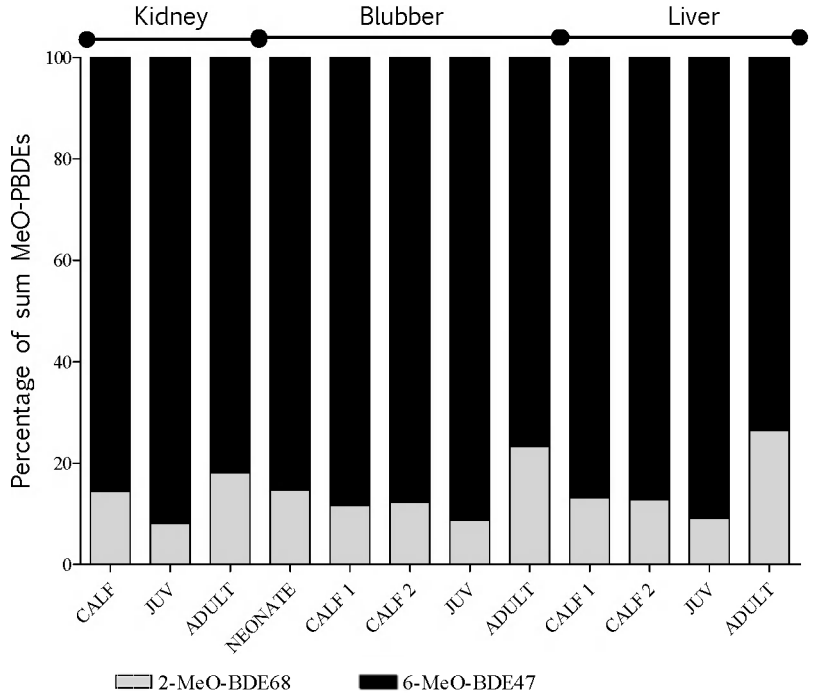

Fig 3. Proportions of the two MeO-PBDEs, 6-MeO-BDE 47 (■) and 2'-MeO-BDE 68 (■) in kidney, blubber and liver of neonates, calves, juveniles and adult harbour porpoises from the North Sea (1990-2008). The calves are divided into calf 1 (1990-1998) and calf 2 (2000-2008).

3.2.4. HCB and CHLs

Concentrations of these pollutants depended on the tissue, the time group and the age class (Table 2). Due to the low sample sizes in the time period 1990–1998, comparisons were only made for the animals from 2000 to 2008. For this time group, the lowest and highest concentrations of HCB and of the sum of the CHLs (sum of trans-nonachlor, cis-nonachlor and oxychlordane), respectively, were found in all tissues of adult harbour porpoises. Trans-nonachlor had the highest levels among all CHLs with percentages of more than 50% of total CHLs.

3.3. Comparison between tissues

The ratios of pollutant concentrations in liver and blubber were calculated for 17 animals (11 calves, 3 juveniles, and 3 adults) and kidney/blubber ratios were calculated for 5 animals (2 calves, 1 juvenile, and 2 adults) for each individual compound (Supporting Information, Table S2). Ratios > 1 were indicative for a preferential accumulation of the compound in liver or kidney, and ratios < 1 indicated preferential accumulation of the compound in blubber. Roughly, higher chlorinated PCBs, higher brominated PBDEs and HCB were preferentially stored in liver, whereas all other compounds were found mainly in blubber. However, the preference of these compounds for the liver disappeared when the animals grew older as liver/blubber ratios become smaller than 1. The only exception was HCB which was present in the liver

rather than in blubber regardless of the age. In contrast, only higher chlorinated PCBs were present in the kidneys more than in blubber. CHLs and MeO-PBDEs were preferentially stored in blubber in all animals investigated in the present study.

3.4. Metabolic biotransformation of dominant compounds

To investigate their capacity for metabolic biotransformation of some compounds, ratios between the most dominant compounds (p,p'-DDE and BDE 47) and PCB 153 were calculated (Fig. 4). PCB 153 was chosen due to its low, if any, metabolic breakdown in marine mammals. Results show that p,p'-DDE/PCB 153 ratios were only higher than 1 in blubber of five calves (two animals of 1990–1998 and three animals of 2000–2008) and in blubber of one juvenile harbour porpoise. Ratios were lower than 1 in all other animals. Ratios of p,p'-DDE/PCB 153 did not differ statistically between calves of 1990–1998 and calves of 2000–2008 (p=0.151 for blubber; p=0.059 for liver). Ratios of BDE 47/PCB 153 were only higher than 1 in one calf and in one juvenile animal. These ratios were statistically different between the two groups of calves in blubber (p=0.039), but not in liver (p=0.099).

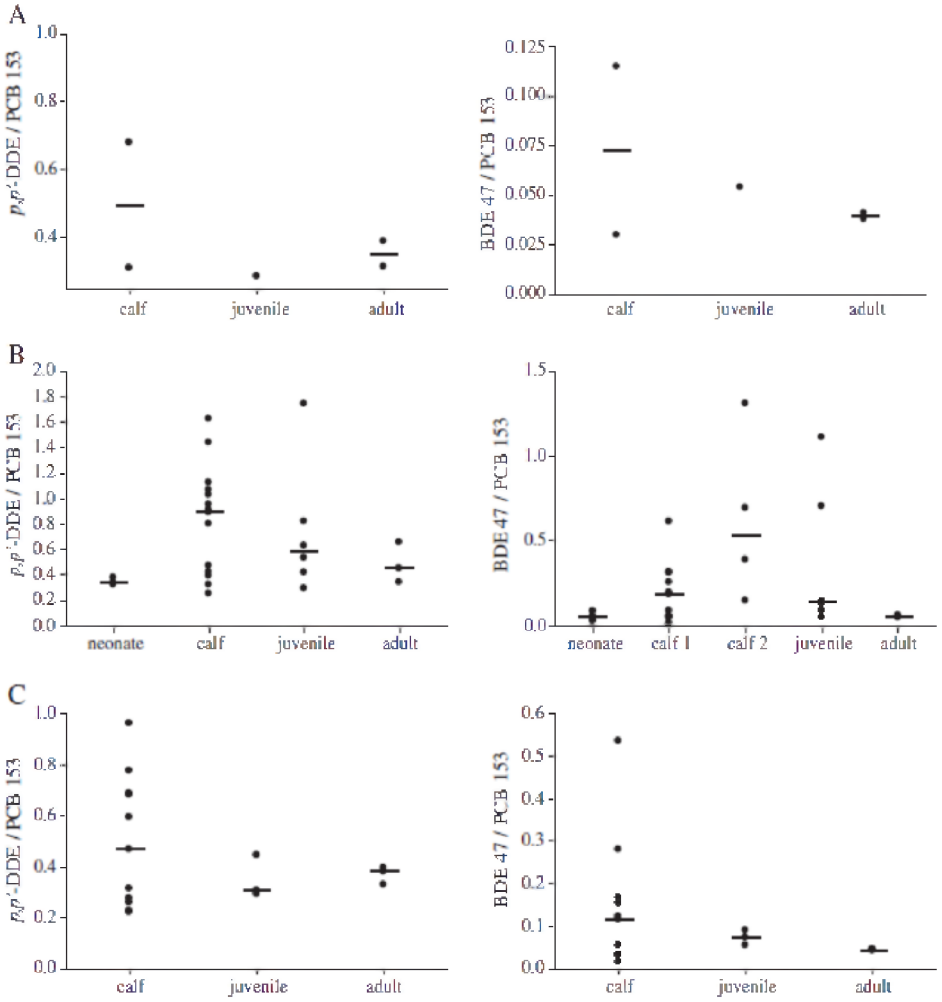

Fig 4. Ratios of p,p'-DDE/PCB 153 and BDE 47/PCB 153 in (A) kidney, (B) blubber and (C) liver of harbour porpoises from the North Sea from 1990 until 2008. BDE 47/PCB 153 ratios in blubber of calves from 1990-1998 differed significantly from ratios of calves from 2000-2008 (p = 0.039). Therefore the 'calf-group' was divided into calf 1 (1990-1998) and calf 2 (2000-2008). ● = individual data, ▬ = median value.

Table 3. Summary of temporal and spatial trends of POPs and MeO-PBDEs (expressed in ng/g lw) previously reported for harbour porpoise blubber, including results of the present study.

Year	ΣPCBs	ΣDDXs	ΣPBDEs	ΣCHLs	ΣMeO-PBDEs	HCB	Location, Reference
1971	94943[d]						
1972	96552[d]						Bay of Fundy, Canada
1973	74598[d]						
1975	83908[d]						Gaskin et al. (1983)[e]
1976	87471[d]						
1977	103103[d]						
1989-1991	12329[b]	6071[b]		4024[b]			Bay of Fundy, Gulf of Maine Westgate et al. (1997)[f]
	14753[c*]	8012[c*]		5741[c*]			
	15776[c]	7376[c]		5624[c]			
	28400[d]	12188[d]		9906[d]			
1990-1998	13700[a]	1900[a]	132[a]	150[a]	20[a]	143[a]	North Sea
	10000[b]	2200[b]	2586[b]	250[b]	149[b]	117[b]	
	19100[c]	4500[c]	4771[c]	680[c]	118[c]	191[c]	Present study[g]
	81500[d]	22900[d]	1900[d]	3611[d]	156[d]	350[d]	
1993	13122[c]	54981[c]		689[c]		368[c]	Black Sea[e] ; Tanabe et al. (1997b)
	23524[d]	101878[d]		1212[d]		498[d]	
1993	5087[c]	3372[c]		840[c]		419[c]	Japan[e] ; Tanabe et al. (1997b)
	12360[d]	9326[d]		1612[d]		433[d]	
1998	6956[c]	40891[c]	57[c]		53[c]	394[c]	Black Sea[g] ; Weijs et al. (2010)
	13215[d]	77329[d]	66[d]		47[d]	575[d]	
1999-2004	12900[c*]	2496[c*]	700[c*]			249[c*]	North Sea[g] ; Weijs et al. (2009a)
	15400[c]	3517[c]	1730[c]			375[c]	
	82900[d]	8387[d]	1450[d]			312[d]	
2000-2008	16800[a]	1800[a]	462[a]	186[a]	97[a]	104[a]	North Sea
	12800[b]	2400[b]	552[b]	268[b]	138[b]	100[b]	
	9900[c]	1700[c]	494[c]	194[c]	224[c]	141[c]	Present study[g]
	24900[d]	3400[d]	1194[d]	688[d]	80[d]	90[d]	

[a] neonate, [b] calf, [c] juvenile, [d] adult, [*] juvenile female, [e] mean values, [f] mean values, calculated with lipid percentage of 85% for blubber, [g] median values.

4. Discussion

This study investigated the distribution of several POP classes and MeO-PBDEs in blubber, kidney and liver of harbour porpoises from the North Sea and focused particularly on the youngest members of the population, namely the neonates and calves. All animals were found alive, but died during rehabilitation in SOS Dolfijn, Dolfinarium Harderwijk, the Netherlands between 1990 and 2008. Except for the outlier (* in Table 1 and Table 2), all adults were males. For juveniles and neonates, a division according to gender was not taken into account because it would have limited the sample sizes even further. Calves were not divided in groups according to their gender either, because gender differences only become more apparent at older stages.

4.1. Levels and time trends

Because all animals died in different years, from 1990 until 2008, and belong to different age classes (neonate, calf, juvenile, and adult), levels and patterns of POPs were difficult to compare to the literature, especially when other studies included different compounds and congeners in the sums of the chemicals. Moreover, all animals in the present study (Table S1) died at the rehabilitation center, so possible interference of exhaustion, starvation or diseases on the POP results cannot be ruled out, although the influence of diseases on POP levels in marine mammals is not fully understood yet. Jepson et al. (2005) and Hall et al. (2006a) found higher levels of PCBs in blubber of harbour porpoises that died due to infectious diseases compared to harbour porpoises that died because of physical trauma. On the other

hand, Weijs et al. (2009d) analysed serum samples of harbour porpoises in rehabilitation, of which some animals were sick and others were healthy, but found that only starvation had an influence on the contaminant levels.

4.1.1. PCBs and PBDEs

Compared to the levels of the sum of PCBs in blubber of harbour porpoises from the Bay of Fundy, Canada, concentrations of the sum of PCBs in blubber of harbour porpoises from the present study, with respect to their age classes, were relatively low (Gaskin et al., 1983; Table 3). However, compared to the levels of PCBs in blubber of harbour porpoises from the Black Sea (Tanabe et al., 1997b; Weijs et al., 2010), concentrations found in the present study were high (Table 3). Within the North Sea, results of PCBs in adult males from 1999 to 2004
(82,900 ng/g lw; Weijs et al., 2009a and Table 3) did not differ much from the results of adult males from 1990 to 1998 (81,500 ng/g lw) from the present study. Whereas Law et al. (2010), who investigated PCBs in blubber of harbour porpoises from the UK from 1991 until 2005, found that concentrations were only decreasing slowly over that time period, the results of the present study are not that clear. Here, there is a decline in levels of PCBs and PBDEs for animals from 1990 until 2008, except for the calves. Basically, only levels of the sum of PBDEs were lower in calves from 2000 to 2008 than in calves from 1990 to 1998. It has been observed that concentrations of all other contaminant types in calves decreased slightly or were even increasing over the time period from 1990 until 2008 (Table 1 and Table 2). Since all contaminant types, except for the PBDEs and MeO-PBDEs were banned before 1990, the reported decline in juveniles and adults can be considered as expected. Although these molecules are stable and persistent in the environment, their levels will decrease eventually as they are phasing out very slowly. However, this would also be true for calves in theory and this is in contrast with the slight increases over time that were found for several contaminant types in this particular age class. A possible explanation for this might be that calves are essentially feeding at a trophic position higher than their mothers, so they are actually 'consuming' the tissues of their mothers. This has been reported for a range of cetaceans and pinnipeds (e.g. for bottlenose dolphins in Knoff et al., 2008 and for fur seals in Hobson et al., 1997). Higher concentrations of several chemicals in marine mammal-eating killer whales and polar bears, which are two predators feeding at a higher trophic level as well, compared to other marine mammals have been found previously (Ross et al., 2000; Kelly et al., 2007). Due to this higher trophic level, the phasing out step might go even slower. The production and use of PBDEs have been restricted in 2004/2008 for most congeners which are more at the end of the sampling design of the present study. As such, higher concentrations in 2008 compared to 1990 would seem normal. Nevertheless, concentrations of PBDEs are decreasing in calves, juveniles and adults. This can be an artifact from the low sample sizes for juveniles and adults in this study, but that would not explain the decrease in calves. Compared to PCBs, PBDEs accumulate less in marine mammals from the North Sea and on top of that, levels of PBDEs are found to decrease with age, not only in females, but also in males (e.g. Shaw et al., 2008; Weijs et al., 2009a) indicating that the capacity for metabolic breakdown of PBDEs might increase with age. As a consequence, the transfer of PBDEs from mother to offspring will reduce with each pregnancy even more than for PCBs.

4.1.2. CHLs and HCB

Concentrations of CHLs found in the present study were, for the two time periods in the North Sea, slightly higher for the youngest animals (neonates and calves), but lower in 2000–2008 than in 1990–1998 for juveniles and adults (Table 2). CHL levels were also lower in 2000–2008 for juveniles and adults compared to concentrations in juveniles and adults from the Black Sea or Japan (Tanabe et al., 1997b; Table 3). The highest concentrations were found in 1989–1991 in harbour porpoises from the Bay of Fundy/Gulf of Maine (Westgate et al., 1997; Table 3). HCB levels experienced a decreasing trend in the North Sea from 1990 until 2008, but were in both time periods lower compared to HCB in harbour porpoises from the Black Sea (Tanabe et al., 1997b; Weijs et al., 2010a; Table 3).

4.1.3. MeO-PBDEs

This paper is the first to report on MeO-PBDEs in blubber, liver and kidney of harbour porpoise calves. Results are therefore difficult to compare. Weijs et al. (2009c) measured MeO-PBDEs in juvenile and adult harbour porpoises, but not in calves or neonates while Weijs et al. (2009d) analysed MeO-PBDEs in serum of harbour porpoises. In addition, MeO-PBDEs have a natural origin and were not banned from production or use like PCBs or PBDEs. Temporal trends for MeO-PBDEs depend therefore only on the presence of algae. To our knowledge, information about the presence of MeO-PBDEs producing algae in the North Sea from 1990 until 2008 does not exist. So, although no conclusions can be drawn about the presence of MeO-PBDEs in harbour porpoises over time, the data suggest that these compounds are present in liver, blubber and kidney of harbour porpoises of all age classes from neonates to adults. For neonates, this would mean that MeO-PBDEs can be transferred from the mother to the offspring through the placenta similar as for anthropogenically produced PBDEs. The levels in calves also suggest that MeO-PBDEs are available for bioaccumulation in calves through their milk diet. For the juveniles and adults however, concentrations depend largely on the presence of these sources in their habitat.

4.2. Patterns

Patterns or percentages are, for some POP classes such as PCBs, DDXs, PBDEs and CHLs, difficult to compare to the literature, because they depend on the number of compounds (e.g. for CHLs) or congeners (e.g. for PCBs and PBDEs) that were analysed. Overall, the most dominant compounds were PCB 153 for PCBs, p,p'-DDE for DDXs, 6-MeO-BDE 47 for MeO-PBDEs and BDE 47 for PBDEs. PCB 153, BDE 47 and p,p'-DDE were commonly found as the most persistent congeners among PCBs, PBDEs and DDXs respectively, not only in harbour porpoises from the North Sea, but also in harbour porpoises from other areas (Weijs et al., 2010a) or even other marine mammal species around the world (e.g. Johnson-Restrepo et al., 2005; Shaw et al., 2008; Weijs et al., 2009a; Dorneles et al., 2010). Among MeO-PBDEs, 6-MeO-BDE 47 was the most dominant congener. In contrast to PBDEs, PCBs and DDXs, MeO-PBDEs are not anthropogenic chemical mixtures with fixed contributions of each congener. MeO-PBDEs have a natural origin and as a consequence, they depend on the geographic position of the natural sources, such as sponges or algae (Vetter et al., 2002; Malmvärn et al., 2008). In the Northern Hemisphere, 6-MeO-BDE 47 is often found in marine mammals as the main MeO-PBDE congener (Kelly et al., 2008; Weijs et al., 2009c), whereas 2'-MeO-

BDE 68 is dominant in the Southern Hemisphere. Because the harbour porpoises only inhabit waters of the Northern Hemisphere, the 6-MeO-BDE 47 is always found at higher concentrations in harbour porpoises than the other congener, 2'-MeO-BDE 68. Overall, and especially compared to MeO-PBDE concentrations in cetaceans from Brazil (Dorneles et al., 2010), levels reported in the present study were low.
In humans, trans-nonachlor and oxychlordane are the predominant compounds among all CHLs measured (Bondy et al., 2003). However, in the present study, trans-nonachlor had consistently higher concentrations than cis-nonachlor or oxychlordane in blubber, liver and kidney of all animals, while there was little difference between cis-nonachlor and oxychlordane.

4.3. Metabolism

Possible metabolic biotransformation was investigated for the most dominant compounds by calculating the ratios of BDE 47/PCB 153 and *p,p'*-DDE/PCB 153. PCB 153 is considered to be highly persistent, not only in harbour porpoises, but also in other marine mammals. Weijs et al. (2010) suggested that there is littlemetabolic biotransformation of PCB 153 in harbour porpoises, if any, and probably only at higher age. With the limited sample sizes for each age–gender group in mind, medians of BDE 47/PCB 153 and *p,p'*-DDE/PCB 153 ratios were, highest in calves, regardless of the tissue, while the lowest ratios were found in the adults (Fig. 4). The only exception was the *p,p'*-DDE/PCB 153 ratio in the liver, where ratios of juveniles were lower compared to ratios of adults. Again, this could mean that the calves have no or a lesser developed ability formetabolic biotransformation of BDE 47 or *p,p'*-DDE compared to juveniles and adults. It is not certain whether this ability for metabolic biotransformation develops with age or is induced by higher concentrations. However, similar or even higher concentrations of chemicals in some calves compared to adults (Tables 1 and 2) together with the higher BDE47/PCB 153 and *p,p'*-DDE/PCB 153 ratios in calves in general (Fig. 4), lead to the preliminary conclusion that higher concentrations, at least for BDE 417 and *p,p'*-DDE, alone are not capable of inducing metabolic biotransformation in harbour porpoises although this should be investigated with a larger dataset.
Next to an absence of or a lesser developed capacity for metabolic breakdown of some chemicals in calves, the higher ratios in calves could also indicate a more recent exposure. It has been established that POPs are selectively offloaded into milk (e.g. Debier et al., 2003a; Yordy et al., 2010). Higher levels of BDE 47 and of *p,p'*-DDE compared to PCB 153 in milk would therefore also lead to higher BDE 47/PCB 153 and *p,p'*-DDE/PCB 153 ratios. In the Black Sea, harbour porpoises from all age–gender groups have higher concentrations of DDXs compared to PCBs and much lower levels of PBDEs than PCBs (Weijs et al., 2010a). These patterns were also been found in milk samples obtained from adult females from the Black Sea (Weijs et al., 2010b). In harbour porpoises from the North Sea however, levels of DDXs (or *p,p'*-DDE) and of PBDEs (or BDE 47) are typically lower compared to PCBs (or PCB 153). There are no milk samples available for harbour porpoises from the North Sea. Nevertheless, assuming the same pattern in milk as in the animals as found for milk and harbour porpoises from the Black Sea, a lesser developed metabolism for calves seems a more plausible explanation for the observed higher BDE 47/PCB 153 and *p,p'*-DDE/PCB 153 ratios in calves.

4.4. Implications for monitoring and toxicity

Although blubber is the most convenient matrix to analyse for lipophilic compounds, such as PCBs or PBDEs, not all congeners have the same properties or molecule sizes. Both factors determine to a large extent where the specific congeners or compounds will accumulate inside the body. The ratios of concentrations of pollutants from liver to blubber and from kidney to blubber, calculated in this study (Supporting information, Table S2), showed that HCB, higher brominated PBDEs and higher chlorinated PCBs accumulate selectively in the liver or the kidneys rather than in blubber. Not because these compounds are less lipophilic, but probably because theirmolecule sizes are too large so that they cannot pass through the membranes (Kannan et al., 1998; Ikonomou et al., 2002). For monitoring purposes, these preferences should be taken into account as the levels of some compounds in blubber might be an underestimation of the concentrations to which the animal is actually exposed to.

Concentrations of PCBs in blubber from this study fall within the range of 10.0 and 81.5 µg/g lw (Table 1). This range is comparable with or higher than levels in blubber of harbour seal pups which are associated with depressed vitamin A levels (Mos et al., 2007). These concentrations are also much higher than concentrations from harbour porpoises from the German and Danish North Sea and from Iceland and Norway, which are linked to interfollicular fibrosis in harbour porpoise thyroids, splenic depletion and thymic atrophy (Beineke et al., 2005; Das et al., 2006). There is no doubt that the concentrations measured here are a threat to all animals of this study. However, the high concentrations found in the calves are of particular concern. It has been suggested already that exposure to a mixture of POPs, such as PCBs and PBDEs, might have an impact on the central nervous system (Montie et al., 2009). The first months of the animals' lives are critical and important for their further development. Tiedeken and Ramsdell (2009) have found that fetal exposure of zebrafish to p,p′-DDE can increase the sensitivity towards domoic acid in fish. That study also suggested a possible link between fetal p,p′-DDE exposure of California sea lions and a higher domoic toxicity in these animals. Relationships between pre- or post-natal exposure to pollutants and developmental impairment or health problems have been found in monkeys and mice as well (Rice, 1999; Haijima et al., 2010). Calves continue to assimilate organic pollutants through milk, but are possibly not capable of dealing with these burdens as their ability for metabolic breakdown is not yet fully operational according to the BDE 47/PCB 153 and *p,p′*-DDE/PCB 153 ratios. If exposure to pollutants at very young ages negatively influences the animals' development with respect to sensitivity for diseases at older stages, than neonates and calves are the most vulnerable age classes of the entire population.

5. Conclusions

Because of the temporal variation of POPs in marine mammals and due to the different age classes of the harbour porpoises, sample sizes in the present study are often too small to perform statistical analyses. Some conclusions in this study may therefore have only a preliminary character and deserve further investigations with larger sample sizes. Overall, biomonitoring studies should keep in mind that not all lipophilic compounds prefer blubber as main storage place. Some compounds accumulate readily in kidneys or liver so that these tissues give a more accurate view of the

actual concentrations the animals are exposed to than blubber. In general, concentrations of all pollutants decreased in all groups from 1990 until 2008 except in calves where only levels of PBDEs decreased. Higher concentrations of pollutants alone are possibly not enough to induce metabolic breakdown because these higher levels are also present in calves and these animals do not seem to have a well developed mechanism for metabolic biotransformation of contaminants. This leaves the younger members of the population, the calves, as probably the most vulnerable animals.

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Supplementary data

Table S1: Life history of the harbour porpoises that were analysed in the present study.

Code	Days in rehabilitation	Gender	Age class	Length (cm)	Weight (kg)	Year of death	Condition at time of sampling
22	5	F	Calf	85	8.8	1990	Sepsis – poor nutritional condition
23	1	M	Adult	151	35.8	1990	Pneumonia – poor nutritional condition
24	8	F	Calf	101	21.4	1991	Pneumonia – good nutritional condition
33	3	M	Juvenile	119	25.8	1993	Unknown – moderate nutritional condition
38	4	M	Neonate	77	6.6	1994	Unknown – poor nutritional condition
42	7	M	Calf	98	12	1995	Unknown – poor nutritional condition
52		M	Calf	125	29.4	1998	Sepsis – good nutritinal condition
66	9	M	Neonate	85	8.9	2001	Unknown – poor nutritional condition
67	1	F	Neonate	72.5	6.85	2001	Pneumonia – good nutritional condition
69	2	F	Juvenile	116	NM	2002	Idiopathic ileus – moderate nutritional condition
80	1	M	Calf	NM	NM	2003	Unknown – emaciation
98	1	M	Calf	NM	NM	2005	Unknown – good nutritinal condition
102	8	F	Juvenile	110.5	19.55	2005	Pneumonia – poor nutritional condition
105	2	M	Calf	103	20.95	2006	Unknown – good nutritional condition
106	16	M	Adult	145.5	47.8	2006	Unknown – good nutritional condition
108	6	F	Calf	108	16.25	2006	Unknown – emaciation
109	5	M	Adult	146.5	42.3	2006	Pneumonia – good nutritional condition
114	1	F	Calf	115	NM	2006	Trauma followed by euthanasia – good nutritional condition
115	1	M	Calf	111	19.1	2006	Pneumonia – poor nutritional condition
117	9	F	Calf	108	21.4	2006	Unknown – unknown
118	13	F	Juvenile	118	19.9	2006	Sepsis - emaciated
119	1	F	Juvenile	119	26	2006	Unknown – good nutritional condition
120	1	F	Calf	93.5	15.15	2006	Unknown – unknown
121	11	M	Juvenile	116	20.05	2006	Pneumonia – poor nutritional condition
123	37	F	Adult	146	48.35	2007	Hepatic necrosis – good nutritional condition
125	71	M	Calf	112.5	24.4	2007	Unknown – unknown
130	1	M	Calf	84	13.5	2007	Pneumonia – moderate nutritional condition
134		F	Calf	106	22.45	2008	Unknown – good nutritional condition

Table S2. Mean congener-specific concentration ratios for POPs and MeO-PBDEs in liver/blubber and kidney/blubber of harbour porpoises from the North Sea from 1990-2008. Ratios in bold are > 1.00. The calves are divided into calf 1 (1990-1998) and calf 2 (2000-2008). Ratios were calculated using lipid normalized concentrations.

Compound	Liver/Blubber ratio				Kidney/Blubber ratio		
	Calf 1	Calf 2	Juvenile	Adult	Calf 2	Juvenile	Adult
N	9	2	3	3	2	1	2
PCB 18	0.72	0.79	0.73	0.50	0.47	0.38	0.94
PCB 28	0.33						
PCB 52	0.58	0.59	0.58	0.52	0.28	0.31	0.28
PCB 49	0.64	0.57	0.56	0.62	0.36	0.32	0.41
PCB 47	0.62	0.58	0.58	0.55	0.30	0.33	0.30
PCB 44	0.40	0.55	0.56				
PCB 95	0.62	0.59	0.61	0.54	0.27	0.32	0.28
PCB 101	0.68	0.63	0.67	0.63	0.29	0.33	0.35
PCB 99	0.66	0.60	0.66	0.55	0.27	0.34	0.26
PCB 87	0.61	0.55	0.60	0.62	0.30	0.32	0.39
PCB 110	0.83	0.62	0.63	0.67	0.62	0.29	0.21
PCB 118	0.68	0.64	0.69	0.62	0.28	0.34	0.32
PCB 105	0.63	0.56	0.61	0.59	0.27	0.32	0.34
PCB 151	0.71	0.59	0.72	0.56	0.27	0.38	0.26
PCB 149	0.79	0.69	0.82	0.62	0.30	0.39	0.28
PCB 146	0.81	0.63	0.83	0.61	0.28	0.40	0.26
PCB 132	0.67	0.61	0.70	0.56	0.27	0.35	0.28
PCB 153	0.81	0.67	0.89	0.63	0.29	0.42	0.25
PCB 138	0.70	0.58	0.77	0.60	0.26	0.39	0.24
PCB 128	0.73	0.60	0.78	0.62	0.25	0.38	0.27
PCB 156	0.79	0.53	0.61	0.68	0.38	0.28	0.16
PCB 187	**1.00**	0.66	**1.08**	0.65	0.33	0.54	0.23
PCB 183	**1.00**	0.64	**1.10**	0.67	0.36	0.57	0.23
PCB 174	**1.06**	0.81	**1.16**	0.75	0.41	0.55	0.30
PCB 177	0.94	0.67	0.96	0.62	0.31	0.50	0.24
PCB 171	0.94	0.65	**1.02**	0.66	0.33	0.51	0.25
PCB 172	**1.02**	0.60	**1.04**	0.62	0.34	0.54	0.24
PCB 180	**1.07**	0.67	**1.19**	0.66	0.37	0.59	0.23
PCB 170	0.89	0.56	0.89	0.55	0.28	0.44	0.20
PCB 199	**1.54**	0.70	**1.52**	0.68	0.48	0.78	0.20
PCB 195	**1.47**	0.70	**1.26**	0.44		0.63	0.13
PCB 194	**1.46**	0.57	**1.55**	0.46		**1.09**	
PCB 205	**1.86**	**1.03**	**1.90**	**6.09**		**12.23**	
PCB 206	**3.92**	**1,14**	**3.37**	0.96	**1.55**	**2.48**	0.39
PCB 209	**14.51**	**3.80**	**8.36**	**1.49**	**4.55**	**6.83**	0.51
HCB	**1.40**	**1.29**	**1.25**	**1.21**	0.65	0.60	0.63
o,p'-DDE				0.50			
p,p'-DDE	0.54	0.47	0.58	0.48	0.20	0.29	0.22
o,p'-DDD	**1.00**	0.90	0.96	**1.47**	0.32	0.54	0.60
o,p'-DDT				< 0.01			
p,p'-DDD	0.58	0.49	0.61	0.61	0.19	0.30	0.23
p,p'-DDT	0.02	0.04					
OxC	0.54	0.52	0.53	0.98	0.28	0.34	0.88
TN	0.48	0.44	0.52	0.44	0.18	0.26	0.18
CN	0.51	0.45	0.53	0.46	0.19	0.27	0.21
BDE 28	0.42	0.44	0.44	0.49	0.24	0.22	0.20
BDE 49	0.74	0.61	0.76	0.71	0.42	0.44	0.45
BDE 47	0.49	0.49	0.52	0.49	0.18	0.25	0.19
BDE 100	0.87	0.71	**1.12**	0.84	0.34	0.62	0.26
BDE 99	0.65	0.61	0.79	0.73	0.19	0.36	0.20
BDE 154	**1.03**	0.56	**1.27**	0.72	0.36	0.67	0.17
BDE 153	**1.16**	0.60	**1.38**	0.71	0.37	0.73	0.14
2'-MeO-BDE68	0.61	0.53	0.58	0.67	0.24	0.29	0.21
6-MeO-BDE47	0.56	0.53	0.59	0.56	0.22	0.29	0.25

OxC – oxychlordane, TN – trans-nonachlor and CN – cis-nonachlor.
Cells are left blank when the specific compound was below LOQ or not detected in one of the paired tissues

4.3 Paper VI

Assessing pollution in victims of mass-stranding events: Long-finned pilot whales from Australia

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Abstract

Pollution is a threat to the health of marine mammals worldwide. Mass-strandings are poorly understood, but often involve pilot whales. However, there is limited information regarding pollution in long-finned pilot whales from Australia. Consequently, the profiles and levels of several pollutant classes were investigated in blubber of Tasmanian long-finned pilot whales. DDX levels were highest in all groups, followed by PCBs or MeO-PBDEs and lowest for PBDEs. The concentrations of all pollutants differed little between locations and time periods, but decreased with age in males. This is at least partly due to the growth dilution effect although it might also be caused by decreasing levels of PCBs, PBDEs, DDXs, HCB and CHLs in the environment. Fetus/mother ratios of higher chlorinated PCBs increased with the duration of pregnancy suggesting a preference for offloading via gestation rather than through lactation. Overall, the highest pollutant levels were found in the youngest animals.

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1. Introduction

Processes such as run-off, atmospheric deposition and oceanic currents ensure that pollutants, like PCBs or PBDEs become a part of the aquatic food chains. Once these persistent chemicals are in the environment, they move into the food chain via uptake from water and diet thereby increasing in concentration with every step. These biomagnification processes for persistent chemicals together with the relatively long life spans of most top predator species can lead to elevated levels of pollutants in top predators like marine mammals (Burreau et al., 2006; Kelly et al., 2008; Weijs et al., 2009b). Over the years, high levels of pollutants have been linked to various health effects potentially affecting the overall survival of these animals (e.g. Ross et al., 1996; Hall and Thomas, 2007; Mos et al., 2007; Sonne et al., 2009; Beineke et al., 2010; Frouin et al., 2010).

PCBs, PBDEs and other halogenated compounds have been produced and used for years until their impact on the environment and wildlife became apparent. Since then, the production of these chemicals has been banned or limited and studies have been performed to measure the damage done to environment and biota. There are studies reporting the levels of pollutants and their effects in a wide range of species other than marine mammals (Law et al., 2006a; Shaw and Kannan, 2009). The latter species, however, deserve some special attention as they can be considered to be sentinels of the ocean's health (Ross, 2000; Bossart, 2011).

In Tasmania (Australia), mass stranding events involving long-finned pilot whale (*Globicephala melas*) are quite common and perhaps even more frequent than in other places (Rudolph and Smeenk, 2009; DPIPWE, Unpublished). Mass stranding events are defined as events in which groups of cetaceans come ashore alive (Geraci and Lounsbury, 2009). Social cetaceans are more likely to be involved in mass-stranding events as the tight group or family bonds force the animals to follow one or more individuals without questioning (Geraci and Lounsbury, 2009). These mass stranding events often result in a large number of mortalities. Several plausible explanations for the mass stranding events have been explored so far: Animals appear to be disorientated or ill which can have both natural (e.g. escaping from predators, anomalous magnetic fields, naturally occurring biotoxins) or anthropogenic (e.g. military sonar exercises, pollution) causes (Hall and Harwood, 2009). Previous studies have suggested that pollution can lead to impaired immune systems in marine mammals (Ross et al., 1996; Beineke et al., 2010), thereby resulting in a higher susceptibility for infectious diseases or an increased sensitivity towards the harmful effects of pollutant and biotoxin (domoic acid) exposure. Mass stranding events are not common for all cetaceans, but seem to occur mostly for Odontocetes or toothed whales (Hall and Harwood, 2009) which often have the highest concentrations of pollutants among all cetaceans (Houde et al., 2005). However, in any case, mass-stranding events provide fantastic opportunities for investigating pollution in these animals.

Long-finned pilot whales can live in pods of up to one hundred individuals or occasionally even larger associations. They are distributed antitropically in contrast to the short-finned pilot whales (*Globicephala macrorhynchus*) which can be found in tropical and subtropical regions (Olson, 2009). Little information is available about levels of pollutants in southern hemisphere long-finned pilot whales (Gaus et al., 2005). They live too far away from their northern hemisphere counterparts to reasonably assume a similar diet and/or exposure. Moreover, it is unknown if and how much the long-finned

pilot whales resemble the short-finned ones in terms of metabolic biotransformation capacities for pollutants. Studies reporting levels of pollutants in short-finned pilot whales from any region or in long-finned pilot whales from the northern hemisphere are, therefore, probably not applicable for long-finned pilot whales from the southern hemisphere.
Because of the limited information available about pollution in long-finned pilot whales from Australia, there is a clear interest to investigate and quantify pollution in Australian long-finned pilot whales. The objective of the present study was, therefore, to investigate the bioaccumulation of persistent organic pollutants (POPs) in these animals.

2. Materials and methods

2.1. Samples.

Blubber samples were collected from 55 long-finned pilot whales from Sandy Cape (SC), Tasmania and 53 long-finned pilot whales from Stanley (S), Tasmania (Fig 1). Body sizes were recorded for all animals, except for a fetus and its mother from SC. The age of the animals could not be assessed through counting dentine layers, but was estimated via the recorded body size of each animal and growth equations for long-finned pilot whales (Bloch et al., 1993). The animals were divided in groups according to their estimated age, gender and lactation status (for females). All animals were victims of the mass-stranding events at Stanley and Sandy Cape, Tasmania in November and December 2008, respectively. In addition, two long-finned pilot whale males from Robbins Island (RI), found stranded in January 2011, and five male long-finned pilot whales from Butlers Beach (BB; Bruny Island), found stranded in March 2011, were analysed as well (Fig 1). In all samples, 37 PCB congeners, 7 PBDEs, 6 DDXs, HCB, chlordanes (CHLs) and 5 MeO-PBDEs were targeted.

2.2. Sample preparation and analysis.

The method used for the sample extraction and clean-up has been previously described (Weijs et al., 2010a) and is briefly presented below. Approximately 0.2 g of blubber was spiked with internal standards BDE 77, BDE 128 and CB 143 and extracted by hot Soxhlet for 2h with hexane/acetone (3/1; *v/v*). After lipid determination (performed on an aliquot of the extract), the extract was cleaned on ~8 g of acidified silica and analytes eluted with 20 mL hexane and 15 mL dichloromethane. The cleaned extract was evaporated to dryness and reconstituted in 150 µL iso-octane. PBDEs, MeO-PBDEs and CHLs were measured by GC-ECNI/MS on a 30 m x 0.25 mm x 0.25 µm DB-5 column by monitoring ions m/z = 79 and 81 (for PBDEs and MeO-PBDEs) and two specific ions for each CHL. PCBs and DDXs were measured by GC-EI/MS on a 25 m x 0.22 mm x 0.25 µm HT-8 column by monitoring two ions for each homologue group. Th system was also used to confirm MeO-PBDEs.

2.3. Quality assurance/quality control (QA/QC).

Recoveries for individual PCB and PBDE congeners ranged between 75 and 104 % (RSD < 12 %). For each analyte, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the procedural blank. For analytes that were not detected in procedural blanks, LOQs were calculated for a ratio S/N equal to 10. LOQs depended on the sample intake and on the analyte and ranged between 1 and 4 ng/g lw. QC was performed by

regular analyses of procedural blanks and by random injection of standards and solvent blanks. A standard reference material SRM 1945 (whale blubber) was used to test the method accuracy. Obtained values did not deviate more than 10 % from the certified values.

2.4. Statistical analysis.

Statistical analyses were conducted using the SPSS 18.0 statistical package (PASW Statistics 18). The level of statistical significance was defined at $p < 0.05$. For compounds detected in more than 50% of the samples, concentrations below LOQ were replaced by a value of f (frequency of detection) * LOQ. Only groups with samples sizes > 2 animals were included in the statistical analysis. Results were log-transformed in order to fit a normal distribution. The parametric ANOVA test was used to test the differences in lipid percentages, contaminant percentages and concentrations between the groups. Tests were also limited to the sums of PCBs, PBDEs, DDXs, CHLs and MeO-PBDEs, the most dominant compound of each contaminant class (PCB 153, PBDE 47, p,p'-DDE, TN and 6-MeO-PBDE 47, respectively) and the PCB groups (tetra, penta, hexa, hepta, octa, nona, and deca).

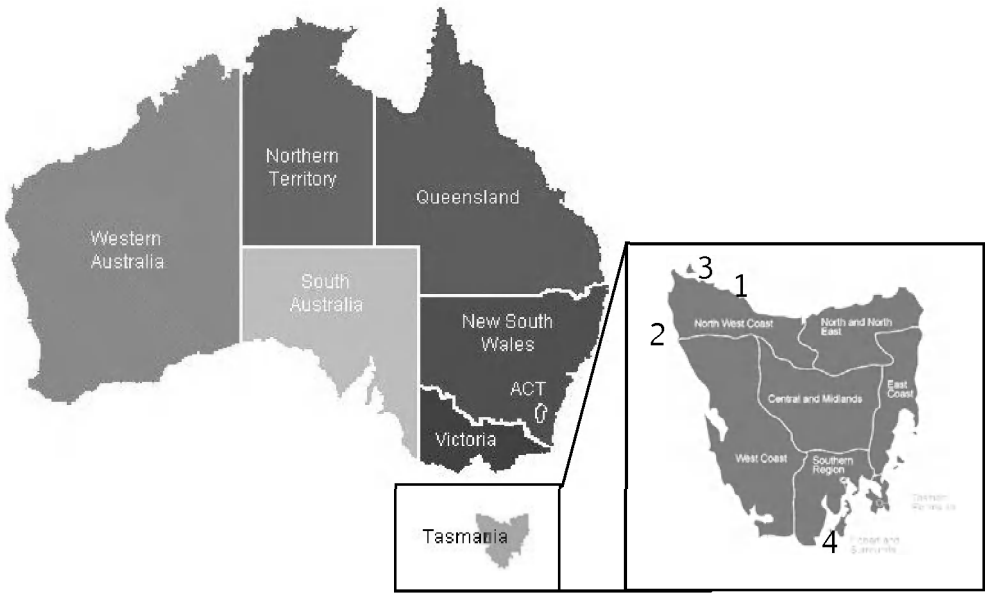

Fig 1. Map of Australia and Tasmania showing the location of the long-finned pilot whale strandings. 1) Stanley, 2) Sandy Cape, 3) Robbins Island and 4) Butlers Beach.

2.5. Groups.

According to Olson (2009), long-finned pilot whale females reach sexual maturity at 8 years, males at about 12 years and lactation can go on for at least 3 years. The animals in the present study were therefore divided into groups following these life history guidelines. Females (F) or males (M) younger than 3 years were called juvenile (J) as they were probably drinking milk from their mothers, but also eating fish or squid occasionally. Sexually immature (I) females (age between 3-8 years) were those not drinking milk anymore, but reproducing. Sexually mature (A) females were females older than 8 years and this group can be divided into two subgroups, namely the animals that were lactating (L) at the time of death and those that were not (NL). Males with estimated ages between 3 and 12 years were the sexually immature (I) animals, whereas males older than 12 years were those

considered to be sexually mature (A). Animals from RI and BB were pooled taking into account their respective age/gender group as the group sample sizes would otherwise have been too small to allow statistical comparisons with similar groups from other locations.

3. Results

PCB 18 was not detected in any sample, whereas PCB 28, PCB 47, PCB 44 and TC (trans-chlordane) were detected in less than 50% of SC and S samples. Furthermore, PCB 209 and BDE 28 were detected in less than 50% of all SC samples, while PCB 49 and PBDE 49 were detected in less than 50% of all S samples. In addition to PCB 18, the congeners PCB 49, PCB 47 and TC were not detected in any animal from RI and BB and PCB 44 was only found in 1 out of 7 blubber samples of animals from RI and BB. PBDE 49 was detected in all 7 samples of animals from RI and BB in contrast to samples from SC and S, but was excluded from any further calculation or statistics to allow consistency between the number and type of compounds included in the mean and standard deviation calculations.

3.1. Lipid percentage

For animals from Sandy Cape, the lipid percentage of the blubber averaged 84.2 ± 4.0 % and ranged from 74.9 % for an adult male to 96.9 % for a non-lactating adult female. The results for the fetus (73.7 %) and the lactating sexually immature female (84.8 %) could not be tested statistically due to low sample size (n = 1), but there were no statistically significant differences in lipid percentage of the blubber between all other SC groups. For animals from Stanley, there was a significant statistical difference between the lipid percentage of the blubber in the fetuses (mean ± SD: 61.3 ± 14.9 %, min-max: 42.6 – 75.0 %) compared to the lipid percentage in most other groups, but not between all other groups (mean ± SD: 78.7 ± 7.5 %, min-max: 59.7 – 89.0 %). The lipid percentages of a juvenile male (87.3 %) and a non-lactating immature female (67.1 %) were excluded from statistical tests because of their low sample size (n = 1). There were no statistical significant differences in lipid percentages in the blubber of animals from RI/BB (mean ± SD: 79.4 ± 3.7 %, min-max: 74.4 – 83.2 %). Comparisons between lipid percentages by location revealed that there were statistical significant differences between S and SC ($p < 0.001$) but not between RI/BB and S or SC ($p = 0.943$ and 0.080, respectively).

3.2. Overall contamination profiles

Figures 2A (Sandy Cape) and 2B (Stanley and Robbins Island/Butlers Beach) show the overall contamination profile in all age-gender groups of the long-finned pilot whales from the present study. In all groups from all locations, DDXs represented the greatest proportion of the overall contamination profile (41-67 %), whereas the proportions of PCBs and of MeO-PBDEs were often comparable. For tetra- and penta-PCBs, there were no differences in percentage between the groups. Statistically significant differences were found for percentages of hexa-PCBs between juvenile females and lactating adult females from S ($p = 0.012$), between non-lactating adult females from S and SC ($p < 0.001$) and between juvenile and immature males from SC ($p = 0.009$). Percentages of hepta-PCBs in lactating adult females from S differed significantly from percentages in adult males ($p < 0.001$), juvenile females ($p < 0.001$), non-lactating adult females ($p < 0.001$) and pregnant adult females ($p = 0.001$) from S. There were also significant differences in hepta-PCBs

between juvenile and non-lactating adult females from S ($p = 0.024$), between pregnant adult females and their fetuses from S ($p = 0.027$) and between juvenile and non-lactating adult females from SC ($p < 0.001$). The same differences found for hepta-PCBs were found for octa-PCBs and nona-PCBs, although with different p-values. In contrast to the hepta-PCBs, there was also a difference in octa-PCBs and nona-PCBs between lactating adult females from S and SC ($p = 0.001$). Percentages of HCB only differed significantly between juvenile and sexually immature males from SC ($p = 0.002$). For DDXs, differences were found between adult males and lactating adult females from S ($p < 0.001$) and between non-lactating adult females from S and SC ($p = 0.012$). CHL and PBDE percentages in adult males from SC differed from those in the same group of RI/BB and S ($p = 0.005$ and 0.001, respectively, for CHLs and $p = < 0.001$ and < 0.001, respectively, for PBDEs). Similar results were found for sexually immature males from RI/BB and SC ($p < 0.001$ for CHLs and PBDEs) and between non-lactating adult females from S and SC for CHLs only ($p = 0.001$). MeO-PBDE percentages only differed between adult males from RI/BB and S ($p = 0.012$). Contributions of PBDEs to the overall profile were < 1%, except for the immature and mature males from RI/BB.

3.3. Levels of PCBs, PBDEs and MeO-PBDEs

Levels of PCBs, PBDEs and MeO-PBDEs for long-finned pilot whales from RI/BB are given in Table 3. When comparing the sexually immature and adult males, there was a statistical significant difference only in the levels of tetra-PCBs ($p = 0.005$). For animals from S (Table 2), the most notable differences were between the non-lactating and lactating adult females which had differences in tetra-PCBs ($p = 0.039$), PBDE 47 ($p < 0.001$), sum of PBDEs ($p = 0.001$) and sum of MeO-PBDEs ($p = 0.004$). The penta PCB mixture only differed significantly between the juvenile females and the non-lactating adult females. Levels of nona- and deca-PCB were different between the adult males and non-lactating adult females, between juvenile females and non-lactating adult females and between pregnant adult females and their fetuses. The octa-PCB concentrations in the fetuses were also significantly lower compared to their mothers ($p < 0.001$). MeO-PBDEs were found to differ significantly between the juvenile females and non-lactating adult females and between adult males and lactating adult females. For animals from SC (Table 1), there were statistically significant differences in PCB 153 levels between the juvenile females and the lactating adult females ($p = 0.027$) and between the immature females and the lactating adult females ($p = 0.028$). Levels of hepta-and octa-PCBs did not differ between the groups. However, there were differences between all other groups for all other compounds or sums tested. These differences could be attributed to the very low concentrations of contaminants in the lactating adult females.

To assess spatial trends, concentrations of PCBs, PBDEs and MeO-PBDEs were compared between adult males and the only statistically significant differences were found for tetra-PCBs ($p = 0.005$), BDE 47 ($p < 0.001$) and sum of PBDEs ($p = 0.001$). Levels of MeO-PBDEs were higher than sum of PCBs in the youngest animals compared to older animals, but MeO-PBDEs and PCBs reversed for the older animals from S and SC. Levels of PBDEs were lowest in all animals regardless of their location. At all locations, levels of PCBs, PBDEs and MeO-PBDEs were highest in the juvenile animals (male or female) and lowest in adult males and lactating adult females.

3.4. Levels of DDXs, CHLs and HCB

Regardless of the location, concentrations of DDXs were highest, followed by CHLs and HCB. Furthermore, concentrations of all compound classes were highest in the youngest animals and lowest in the oldest animals with emphasis on the lactating adult females. Despite this decrease in concentrations with age, no statistical significant differences were found between the groups for *p,p'*-DDE or sum of DDXs in the animals from S or RI/BB. The statistically significant differences between the DDXs in SC animals were all due to the very low concentrations in the lactating adult females. Furthermore, levels of DDXs did not differ between the three locations for the adult males. In contrast to DDXs, levels of HCB differed between the adult males and non-lactating adult females ($p = 0.005$), between juvenile females and non-lactating adult females ($p < 0.001$), between non-lactating adult females and lactating adult females ($p < 0.001$) and between lactating adult females and pregnant adult females ($p = 0.015$) from S. The low levels in lactating adult females from SC can be used as an explanation for the differences in HCB and CHL levels in animals from the SC. There were no differences in CHL levels between the groups from RI/BB whereas significant differences were found between juvenile females and non-lactating females ($p = 0.022$) and between non-lactating adult females and lactating adult females ($p = 0.029$) from S.

3.5. Gestational transfer of contaminants

There were 4 mother/fetus pairs of which the fetuses were, based on their body sizes, all in different stages of development (Table S1). In order to assess the transfer of pollutants from mothers to their offspring during gestation, fetus/mother ratios were calculated for all compounds studied. These ratios are shown in Fig 3 for HCB and the sums of PCBs, PBDEs, DDXs, CHLs, MeO-PBDEs, but are given for each compounds separately in Table S1 (Supporting Information). Ratios for HCB were all > 1 indicating a higher potential for bioaccumulation in the fetus compared to its mother (Fig 3). In contrast, ratios of PBDEs were lowest (Fig 3). If the body sizes of the fetuses are a good measure for their stage of development, it is worthwhile to note that ratios of PCB 101, 149, 132, 153, 138, 156, 183, 174, 177 and *o,p*-DDT follow the order of D (smallest fetus) < B < A < C (tallest fetus) (Table S1). In contrast, ratios of PCB 172, HCB, *o,p*-DDD, *p,p'*-DDD, OxC, CC, CN, PBDE 100, PBDE 99, 2-MeO-PBDE 68 and 6-MeO-PBDE 47 are lower in C than in D (Table S1).

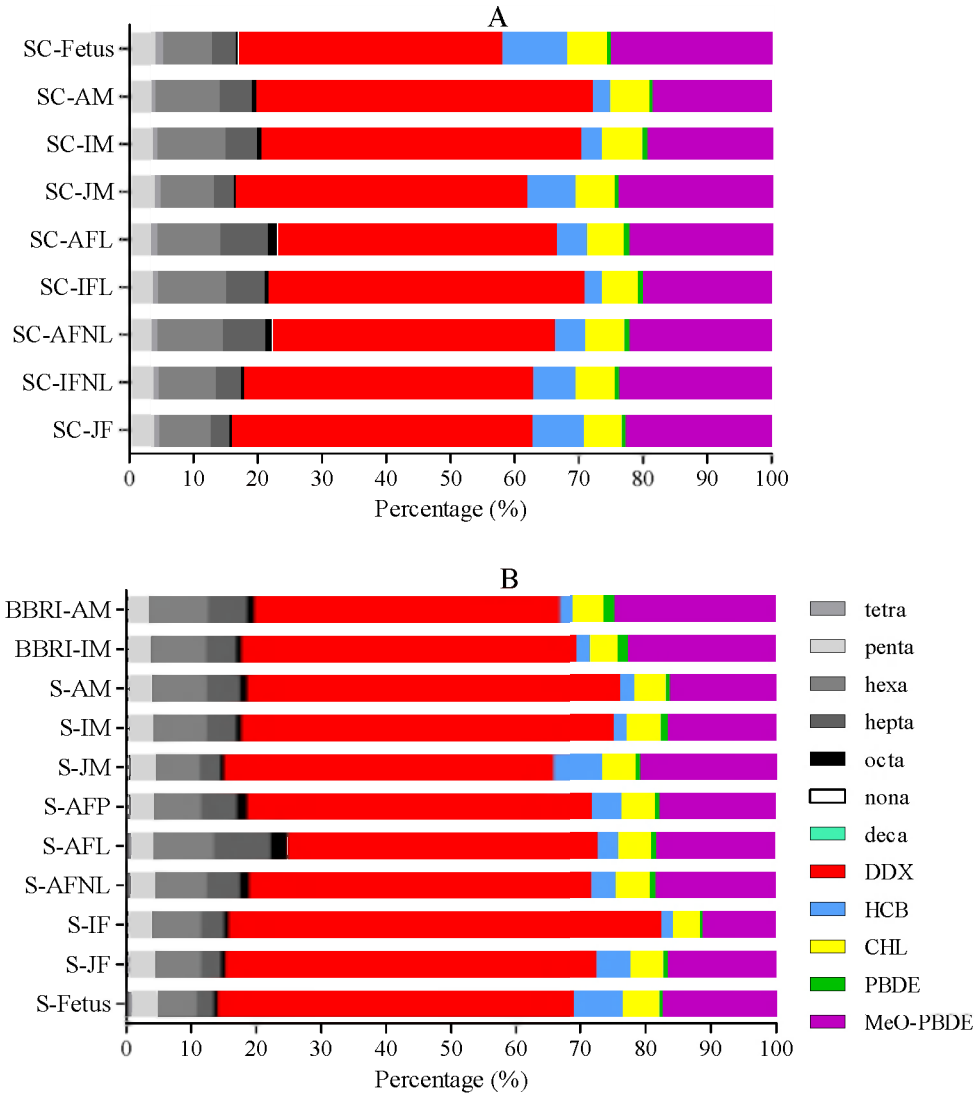

Fig 2. Percentages of PCBs, PBDEs, MeO-PBDEs, DDXs, HCB and CHLs in blubber of A) 55 pilot whales from Sandy Cape (SC) and B) 53 pilot whales from Stanley (S), 2 pilot whales from Robbins Island (RI) and 5 pilot whales from Butlers Beach (BB). Animals from RI and BB were pooled according to age. I-sexually immature, A-sexually mature (adult), J-juvenile, F-female, M-male, NL-non-lactating, L-lactating, P-pregnant.

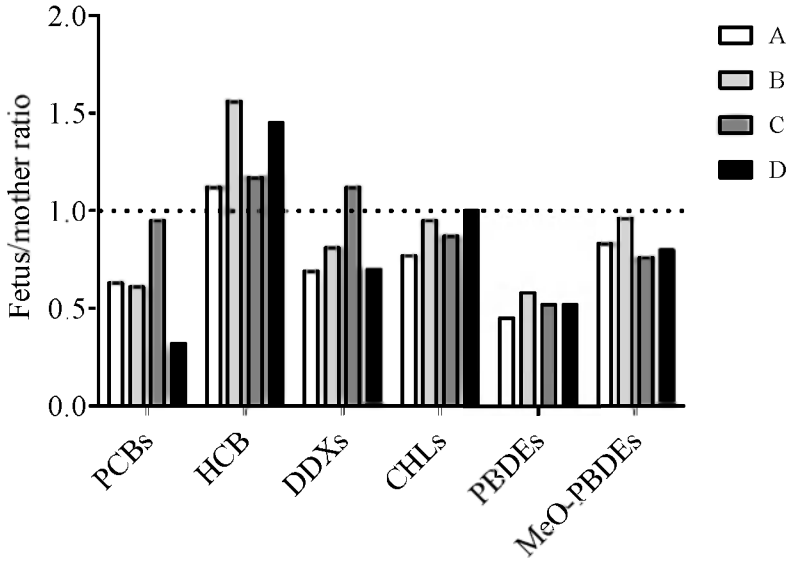

Figure 3. Fetus/mother (F/M) ratios of PCBs, PBDEs, MeO-PBDEs, DDXs, HCB and CHLs in blubber of 4 fetus/mother long-finned pilot whales from Stanley, Tasmania. Gender of fetus, body size of mother and fetus as well as F/M ratios for all individual compounds are given in Table S1 (Supporting Information). A, B, C and D refer to the respective fetus/mother pair with D < B < A < C representing the development of the fetus based on its body size.

4. Discussion and conclusions

To the best of our knowledge, this is the first study to report on several pollutant classes in victims of two mass-stranding events in Australia. Mass stranding events provide unique opportunities to study contaminant levels in a population in which all animals have been exposed to contaminants under similar environmental conditions.

In the present study, there were almost no statistically significant differences for individual PCBs, PCB groups, the sum PCBs or other pollutant classes, between juvenile males and females and between sexually immature males and females indicating that there is an equal bioaccumulation process in all animals as long as they are not reproducing (Weijs et al., 2009a). Compared to other marine mammal species (Weijs et al., 2010a and c), the concentrations of the sum of PCBs in these pilot whales were more than 10 to 25 times lower. In contrast, they were higher than the concentrations of the sum PCBs in several other marine mammals from Australia (Gaus et al., 2005).

For PBDEs, BDE 47 was predominant in all animals from the present study. Similar to the sum of PCBs, the sum of PBDEs in the pilot whales was much lower than those in marine mammals worldwide (Lindström et al., 1999; Weijs et al., 2010 a and c). Considering the naturally produced MeO-PBDEs, levels of 6-MeO-BDE 47 were predominant, followed by 2-MeO-BDE 68 which is in contrast with previous reports for animals from the southern hemisphere (Melcher et al., 2005; Dorneles et al., 2010). Most differences among the groups were caused by the lower concentrations found in the group of the lactating adult females in this study (Table 1). The levels of sum MeO-PBDEs in the pilot whales from Tasmania (Table 1) were higher than those in harbour porpoises and harbour seals (Weijs et al., 2010a) from the Northern Hemisphere, similar to the levels in several marine mammal species from Australia (Melcher et al., 2005) and lower than the levels in marine mammal species inhabiting the continental shelf and open ocean near Brazil (Dorneles et al., 2010).

p,p'-DDE had the highest concentration of the DDXs and most statistically significant differences involved the lactating adult female group. The lactating female group was significantly different for *p,p'*-DDE and the sum DDXs from all other female groups and from all male groups. With DDXs being detected in the fetus (Table 1) and in the milk of marine mammals (e.g. harbour porpoises; Weijs et al., 2010b), this finding was not a surprise. Compared to other marine mammal species and other areas around the globe, the pilot whales from the present study have lower concentrations of sum DDXs than harbour porpoises (Weijs et al., 2010a) and pilot whales from the Faroe Islands (Dam and Bloch, 2000), but similar concentrations to harbour porpoises from the North Sea (Weijs et al., 2010c) and other parts of Europe (Das et al., 2006) and several marine mammal species from Australia (Melcher et al., 2005).
Trans-nonachlor (TN) was the most dominant compound among the CHLs. For TN as well as for CHLs and HCB, the largest differences were found between the lactating adult females and the other groups. Information about CHLs and HCB in pilot whales is scarce. Levels of HCB in these pilot whales were lower than in harbour porpoises from the Black Sea (Weijs et al., 2010a), but comparable to levels in harbour porpoises from the North Sea (Weijs et al., 2010c) and in pilot whales from the Faroe Islands (Dam and Bloch, 2000). Levels of CHLs were lower in the Tasmanian pilot whales than in the pilot whales from the Faroe Islands (Dam and Bloch, 2000), whereas they were comparable to levels in harbour porpoises from the North Sea (Weijs et al., 2010c).

4.1. Female pilot whales and reproduction

Adult females from Sandy Cape had similar profiles regardless of whether they were lactating or not, whereas adult females from Stanley differed only in the proportion of hepta-PCBs which was higher in the lactating adult females. The estimated ages for the adult females ranged from 7.5 to about 45 years, with the non-lactating adult females being the oldest. The similarity in contamination profiles independent of the age or lactation status is quite remarkable and may point towards a stable bioaccumulation profile coupled to a consistent and regular offloading of pollutants to their offspring throughout their lives. Long-finned pilot whales seem to reproduce more often than short-finned pilot whales (Kasuya and Marsh, 1984). Moreover, for several Odontocetes species, such as short-finned pilot whales and killer whales, it is not uncommon to find that non-reproductive older females are still producing milk for their offspring (Martin and Rothery, 1993). Throughout their pregnancy, long-finned pilot whale mothers seem to pass on increasing levels of higher chlorinated and persistent PCBs, such as PCB 153, 183 and 149, to their fetus during gestation. As a result, the fetus ends up with higher levels of those PCB congeners compared to its mother upon birth. According to Bloch et al. (1993), it is hard to draw a firm line between the body sizes of calves and fetuses. That study reported body sizes ranging between 163 and 191 cm for 49 fetuses and 39 calves of both genders and suggested a body size of 177.6 cm as the best estimate of the length at birth. The fetus of mother D (Table S1) is therefore definitely at the end of pregnancy. In contrast to the transfer of higher chlorinated and persistent PCBs during pregnancy, marine mammals seem to transfer more lower chlorinated PCBs via milk during lactation. In agreement with our findings, Debier et al. (2003b) reported higher proportions of lower chlorinated PCBs than higher chlorinated PCBs in the milk of grey seals. A recent study also

confirmed this and found stable percentages of hexa-PCBs in milk of grey seals (Vanden Berghe et al., 2012).
Milk samples of long-finned pilot whales were not available in the present study. Nevertheless, it looks like the conserved contamination profile in the long-finned pilot whale females, at least for PCBs, is a result of a balanced, selective and combined offloading of compounds during gestation and lactation. In both mass-stranding events, the lowest concentrations were found in lactating adult females. Multiple pregnancies with associated lactation periods are therefore capable of decreasing the levels of pollutants in the mothers, but are not capable of changing the contaminant profiles. This opportunity for contaminant transfer to offspring probably stops as soon as the females are too old to reproduce. As mentioned previously, the non-lactating adult females were the oldest according to their body sizes and estimated age. In Tables 1 and 2, however, the levels of all contaminant groups are higher in the non-lactating adult females than in the lactating adult females although there were no changes in contaminant profile. Because of the higher levels, adult females might, therefore, face an increasing environmental stress later in life which is in contrast to the adult males of the same population.
There was a difference in contaminant profiles when comparing the adult females to the juvenile females and the sexually immature female groups. For the juvenile females, there was more similarity with the profile of the fetuses, whereas the profiles of the sexually immature females were intermediate between the profiles of the juveniles and the adults. The changes in contaminant profiles are generally characterized by an increase in PCB proportions and a decrease in HCB proportions, whereas percentages of DDXs and CHLs remain similar. With respect to the levels of all contaminant classes, all levels decreased from juvenile females to adult females in both populations because of the gestational/lactational transfer at older age as well as the growth dilution effect at a younger age. Also, there were no differences between the same groups of the Stanley and Sandy Cape populations. The highest levels in the youngest animals of both populations are results of concern, since these animals are still in a critical stage of development. Nevertheless, it is unknown what impact this might have on their development or survival on the longer term.

4.2. Bioaccumulation in males

In terms of bioaccumulation in males, concentrations of PCBs, PBDEs, MeO-PBDEs, CHLs, DDXs and HCB decreased with age. Elimination pathways, such as gestation and lactation do not exist for males. For these animals, there are a number of explanations as to why the concentrations of these chemicals decreased with age. For sexually immature animals, the growth dilution effect plays an important role as the animals experience a rather steep growth curve until the age of 10-15 years (Bloch et al., 1993). After that age, a possible explanation is the change in ability to metabolise and eliminate these chemicals that occurs across a lifespan. This would mean that juveniles have a limited capacity to eliminate the chemicals while adults may have much more capacity which is a hypothesis that deserves some more investigation. Another explanation would be the worldwide bans and control actions on several of these chemicals that have been introduced over the last decennia which may have lowered the input in the environment and decreased the exposure of these animals.

4.3. Influence of location and time

Overall, there were little differences between the locations for the same groups. Furthermore, levels of pollutants between 2008 (Stanley and Sandy Cape) and 2011 (Robbins Island/Butlers Beach) were comparable leading to the conclusion that contaminant exposure does not seem to decline over a period of 3 years. But, regarding the long life spans of the animals and the decreasing levels with age in males, it would take longer than 3 years to see any decrease in pollutant levels.

4.4. Implications for health and survival

The levels of DDE found in these animals are similar to those found in harbour porpoises from various European waters which were shown to have adverse effects on the thyroids of the porpoises (Das et al., 2006). In laboratory animals, PCBs and other POPs also have an endocrine-disrupting potential even at low concentrations (Arsenescu et al., 2008; Lee et al., 2011). Although the toxicological understanding of dose response is limited in marine mammals and average concentrations of chemicals in the present study seem lower than concentrations reported worldwide, it is of concern that the highest levels of POPs were found in the youngest animals. It is unknown whether these levels are toxic for this particular group or whether these levels induce changes that are compromising the well-being of pilot whales in the longer term.

Acknowledgements

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Table 1. Concentrations, expressed in ng/g lw in blubber of 55 long-finned pilot whales from Sandy Cape, Tasmania. Values are mean ± SD and minimum – maximum. I-sexually immature, J-juvenile, F-female, NL-non-lactating, L-lactating, A-sexually mature or adult, M-male.

	Fetus	**JF**	**IFNL**	**AFNL**	**IFL***	**AFL**	**JM**	**IM**	**AM**
n	1	6	9	9	1	8	6	10	5
∑ PCBs	80	676 ± 562 67 – 1382	418 ± 208 242 – 933	244 ± 150 77 – 472	306	167 ± 114 59 – 399	446 ± 223 97 – 709	404 ± 53 320 – 504	380 ± 88 287 – 512
∑ PBDEs	3	25 ± 19 3 - 54	16 ± 7 10 – 31	8 ± 4 3 – 14	11	6 ± 4 2 – 15	17 ± 8 4 – 26	13 ± 3 10 – 20	10 ± 3 7 – 13
∑ MeO-PBDEs	117	843 ± 561 105 – 1635	553 ± 262 292 - 1100	237 ± 142 82 – 431	280	157 ± 110 59 – 410	628 ± 297 157 – 1007	389 ± 72 267 – 492	353 ± 73 278 – 434
∑ DDXs	191	2123 ± 1965 174 – 4748	1072 ± 623 543 – 2613	528 ± 408 133 – 1255	687	347 ± 311 92 – 1036	1229 ± 635 246 – 2071	983 ± 141 769 – 1177	997 ± 223 810 – 1366
∑ CHLs	29	246 ± 205 27 – 530	144 ± 81 83 – 344	71 ± 51 20 – 152	78	43 ± 33 12 – 116	164 ± 88 38 – 287	125 ± 19 98 – 153	116 ± 19 92 – 139
HCB	47	244 ± 110 45 - 375	149 ± 69 78 - 254	47 ± 26 18 - 90	38	32 ± 24 15 - 86	171 ± 66 75 - 258	64 ± 24 23 - 113	52 ± 13 40 - 72

* In theory, this animal was not long enough to be an adult according to the growth equations from Bloch et al. (1993), but she was producing milk at the time of death.

Table 2. Concentrations, expressed in ng/g lw in blubber of 53 long-finned pilot whales from Stanley, Tasmania. Values are mean ± SD and minimum – maximum. I-sexually immature, J-juvenile, F-female, NL-non-lactating, L-lactating, A-sexually mature or adult, M-male, P-pregnant.

	Fetus	**JF**	**IFNL**	**AFNL**	**AFL**	**AFP**	**JM**	**IM**	**AM**
n	4	6	1	16	6	5	1	3	11
∑ PCBs	196 ± 154 45 – 368	830 ± 604 412 – 2005	942	393 ± 237 140 – 996	193 ± 69 119 – 316	292 ± 215 95 – 580	249	388 ± 239 199 – 657	320 ± 93 210 – 509
∑ PBDEs	5 ± 3 2 - 9	31 ± 13 18 - 53	25	17 ± 7 5 – 27	6 ± 4 4 – 13	10 ± 6 5 – 16	12	19 ± 7 13 - 27	10 ± 2 7 – 13
∑ MeO-PBDEs	218 ± 138 98 – 357	856 ± 462 392 – 1676	680	359 ± 161 134 – 653	144 ± 62 106 – 269	242 ± 127 123 – 381	356	332 ± 122 242 – 471	272 ± 48 207 - 372
∑ DDXs	813 ± 655 201 – 1414	2123 ± 1965 174 – 4748	4061	1187 ± 901 353 – 3661	397 ± 225 245 – 808	949 ± 838 261 – 2038	862	1338 ± 984 591 – 2452	1020 ± 402 640 – 1931
∑ CHLs	78 ± 59 26 – 135	246 ± 205 27 – 530	258	109 ± 63 37 – 214	41 ± 22 28 – 84	84 ± 66 26 – 176	87	111 ± 60 61 – 177	83 ± 18 58 – 107
HCB	77 ± 35 46 - 121	244 ± 110 45 - 375	102	67 ± 26 27 - 120	23 ± 5 17 - 31	56 ± 20 37 - 78	129	36 ± 7 27 - 42	35 ± 8 27 - 58

Table 3. Concentrations, expressed in ng/g lw in blubber of long-finned pilot whales from Butlers Beach and Robbins Island, Tasmania. Values are mean ± SD and minimum – maximum. I-sexually immature, A-sexually mature or adult, M-male.

	IM	AM
n	3	4
∑ PCBs	408 ± 136 252 – 499	286 ± 67 222 – 366
∑ PBDEs	32 ± 12 18 - 41	26 ± 11 11 – 37
∑ MeO-PBDEs	471 ± 119 366 – 601	355 ± 67 261 - 413
∑ DDXs	1249 ± 628 633 – 1889	683 ± 137 486 – 788
∑ CHLs	99 ± 35 66 – 135	68 ± 10 54 – 75
HCB	40 ± 21 19 - 62	29 ± 1 28 - 31

Supporting information

Table S1. Fetus/mother ratio for all individual compounds. All animals were involved in the mass-stranding of Stanley, Tasmania, in 2008. Results in bold are shown in Figure 3. Ratios of the compounds highlighted in dark grey follow the order D < B < A < C, which mimics the fetus development based on the body size of the fetus. Ratios of the compounds highlighted in black are lower for C (tallest fetus) than for D (smallest fetus).

	A	B	C	D
Mother	4280 mm	4500 mm	4250 mm	4420 mm
Fetus	1340 mm, male	935 mm, female	1660 mm, female	660 mm, female
PCB 52	1.09	0.97	1.54	1.04
PCB 74	0.80	0.85	1.16	1.00
PCB 66	0.82	0.82	0.72	0.67
PCB 95	0.84	0.92	1.54	0.82
PCB 101	0.87	0.76	1.31	0.74
PCB 99	0.67	0.80	0.92	0.77
PCB 87	1.10	0.83	1.07	0.81
PCB 110	5.98	0.40	5.34	0.86
PCB 118	0.76	0.81	0.98	0.61
PCB 105	0.63	0.83	0.33	0.31
PCB 149	0.88	0.60	1.32	0.37
PCB 146	0.57	0.66	1.01	0.43
PCB 132	1.04	0.69	2.33	0.32
PCB 153	0.64	0.56	1.12	0.28
PCB 138	0.62	0.60	1.14	0.36
PCB 128	0.71	0.76	1.02	0.52
PCB 167	0.55	0.99	0.98	0.45
PCB 156	0.54	0.53	0.90	0.33
PCB 187	0.40	0.43	0.66	0.18
PCB 183	0.45	0.30	0.64	0.18
PCB 174	0.71	0.64	1.11	0.40
PCB 177	0.45	0.41	0.60	0.23
PCB 171	0.55	0.61	0.96	0.57
PCB 172	0.52	0.16	0.53	0.55
PCB 180	0.37	0.48	0.48	0.10
PCB 170	0.41	0.65	1.71	0.16
PCB 199	0.21	0.36	0.20	0.10
PCB 196/203	0.21	0.29	0.18	0.12
PCB 194	0.14	0.24	0.23	0.15
PCB 206	0.24	0.29	0.38	0.24
PCB 209	0.40	0.31	0.37	0.21
∑ PCBs	**0.63**	**0.61**	**0.95**	**0.32**
HCB	**1.12**	**1.56**	**1.17**	**1.45**
op-DDE	0.70	1.17	1.74	0.90
pp-DDE	0.69	0.83	1.23	0.70
op-DDD	0.93	1.08	0.52	1.09
op-DDT	0.72	0.69	1.64	0.67
pp-DDD	0.81	0.91	0.60	0.99
pp-DDT	0.63	0.65	1.14	0.62
∑ DDXs	**0.69**	**0.81**	**1.12**	**0.70**
OxC	1.01	1.28	0.43	1.00

CC	1.03	1.09	0.76	1.35
TN	0.69	0.87	0.97	0.86
CN	0.84	0.99	0.87	1.11
∑ CHLs	**0.77**	**0.95**	**0.87**	**1.00**
PBDE 28	0.82	0.99	1.00	1.00
PBDE 47	0.77	1.03	0.74	0.71
PBDE 100	0.07	0.08	0.29	1.00
PBDE 99	0.25	0.18	0.37	0.43
PBDE 154	0.28	0.45	0.30	0.23
PBDE 153	0.22	0.23	0.61	0.53
∑ PBDEs	**0.45**	**0.58**	**0.52**	**0.52**
6-MeO-PBDE49	0.85	1.06	0.61	0.51
2-MeO-PBDE68	0.85	0.99	0.70	0.76
6-MeO-PBDE47	0.83	0.95	0.77	0.82
5-MeO-PBDE47+4-MeO-PBDE49	1.00	0.23	1.00	0.32
∑ MeO-PBDEs	**0.83**	**0.96**	**0.76**	**0.80**

ABC News

Mass stranding: All whales found dead

Updated Sun Nov 30, 2008 9:10pm AEDT

There are no survivors from the latest mass whale stranding on Tasmania's west coast.

Authorities were alerted to the stranding of between 80 and 100 long-finned pilot whales on a rocky stretch of Sandy Cape yesterday.

A parks and wildlife team sent to investigate by helicopter yesterday found around 12 survivors, but most of them were already badly injured by the rocky shoreline.

A larger rescue team arrived at the remote beach this morning by four-wheel-drive to find all the whales dead.

Chris Arthur from Parks and Wildlife says he was not surprised given the condition of the survivors yesterday.

"It's an a extremely difficult site," he said.

"It is different from last weekend where the animals came ashore in sand.

"Where they are is in a rocky shore with a lot of multiple reefs and small channels.

"The animals were quite badly battered."

A group of around 16 whales believed to be from the same pod are still milling around off shore.

Rescuers will now focus their attention on trying to prevent them also becoming beached.

The deaths come only a week after than 60 whales of the same species became stranded at Anthony's Beach on the state's north-west coast.

(Source: http://www.abc.net.au/news/2008-11-30/mass-stranding-all-whales-found-dead/223904)

Stranded whales rescued in Australia

23 November 2008, 08:29

11 pilot whales were successfully returned to sea on Saturday following a mass stranding on a beach in Tasmania, Australia. 64 whales in total stranded themselves on one of Tasmania's northern beach, prompting a day-long rescue effort. Environmentalists said that it was unusual to save any animals after such a large scale stranding.

A day-long rescue effort returned 11 stranded whales to the sea between Tasmania and the Australian mainland Saturday evening
Australian wildlife rescuers on Sunday said they successfully returned a small number of pilot whales to the ocean after a mass stranding in Tasmania
Australia Environment Wildlife Pilot Whales Whales Stranding Rescue Environmental Chris Arthur, who coordinated the rescue effort, said 11 of the 64 animals found stranded on the island's north coast on Saturday were released after a day-long effort which involved relocating them by road to another beach.

"We have successfully released 11 animals out to sea," Arthur told Reuters by telephone. "The last one went out less than 20 minutes ago."

While the possibility that the animals would strand themselves again could not be ruled out, he said, the hope was that they would instead join up with other pilot whales in the ocean. Some the whales have been tagged and aerial reconnaissance is planned to check on their progress.

"We have had a reasonable outcome. They will form a small pod. We have given them the best chance they have got," said Arthur, a regional officer with the Tasmanian state parks and wildlife service.

This maternal pod of 64 long-finned pilot whales, around one-third of them juveniles, were found stranded on Saturday along a stretch of Anthony's Beach at Stanley on the island's northwest coast, a site where repeated strandings have occurred in the past.
Pilot whales are among the smaller whales, typically up to about five metres in length and dark with a grey underbelly. Their relatively small size may have helped rescuers save them, environmentalists said.
Although most of the pod could not be saved, a team of around 65 people battled throughout much of Sunday to move 12 survivors, including both adults and juveniles, 17 kilometres by road in trailers to nearby Godfrey's Beach to try to return them to the sea. One whale died during the operation.

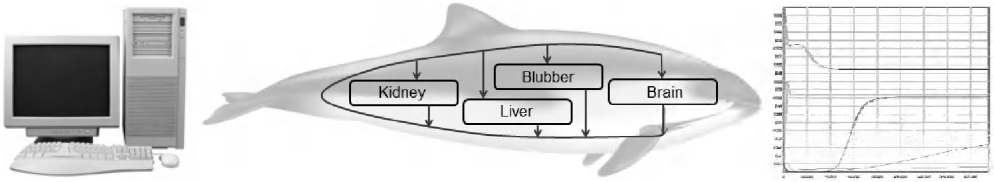
Mass strandings of whales occur periodically in Australia and New Zealand for reasons that are not entirely understood. Theories include disturbance of echo-location, possibly by interference from sound produced by human activities at sea, a spokeswoman for the environmental group Greenpeace told Reuters.

In a statement, the state government said satellite trackers had been placed on some of the released whales and a reconnaissance plane would fly over the area on Monday to check on the whales' progress. Samples are to be taken from the dead whales and a mass burial organised.
Although wildlife officials and volunteers have often tried to save stranded whales, relatively few attempts have been successful.

(Source: http://www.liveleak.com/view?i=01a_1227509876)

Part 2

PBPK models as *in silico* tools to assess bioaccumulation results



Chapter 5

PBPK modeling

5.1 Paper VII

Physiologically based pharmacokinetic (PBPK) models for lifetime exposure to PCB 153 in male and female harbor porpoises (*Phocoena phocoena*): Model development and evaluation

Liesbeth Weijs, Raymond S.H. Yang, Adrian Covaci, Krishna Das, Ronny Blust

Abstract

PBPK models were developed for the most persistent polychlorinated biphenyl (PCB 153) in male and female harbor porpoises to elucidate processes such as uptake, distribution, and elimination. Due to its limited metabolic capacities, long life span, and top position in marine food chains, this species is highly sensitive to pollution. The models consist of 5 compartments, liver, blubber, kidney, brain, and a compartment which accounts for the rest of the body, all connected through blood. All physiological and biochemical parameters were extracted from the literature, except for the brain/blood partition coefficient and rate of excretion, which were both fitted to data sets used for validation of the models. These data sets were compiled from our own analyses performed with GC/MS on tissue samples of harbor porpoises. The intake of PCB 153 was from milk from birth to 4 months, and after weaning fish was the main food source. Overall, these models reveal that concentrations of PCB 153 in males increase with age but suggest that, as the animals grow older, metabolic transformation can be a possible pathway for elimination as well. In contrast, the model for females confirms that gestation and lactation are key processes for eliminating PCB 153 as body burdens decrease with age. These PBPK models are capable of simulating the bioaccumulation of PCB 153 during the entire life span of approximately 20 years of the harbor porpoises.

Published in: Environmental Science & Technology (2010), ***44****: 7023-7030*

1. Introduction

Although PCBs have been banned since the 1970s, their levels in the environment and biota are decreasing slowly. In the past, PCBs have been used extensively in transformers, capacitors, or other electrical equipment due to their insulating properties and chemical stability (ATSDR, 2001). Because of their stable characteristics, PCBs are still persistent in the environment and can be found in all levels of terrestrial and aquatic food webs (Johnson-Restrepo et al., 2005; Burreau et al., 2006) causing toxic effects in wildlife. As marine mammals occupy top positions in aquatic ecosystems, they accumulate considerable amounts of these lipophilic compounds (Johnson-Restrepo et al., 2005; Shaw et al., 2005; Weijs et al., 2009a), and they are considered to be particularly vulnerable to the effects of PCBs on the reproductive, endocrine, and immunological system (Ross et al., 1996; Das et al., 2006; Schnitzler et al., 2008).

PBPK models are a mathematical simplification of reality. Based upon the physiology of the organism and the chemical properties of the compound, they provide insight into the kinetics of the compound which includes the uptake, distribution, metabolism, and excretion (Yang et al., 2004; Reddy et al., 2005). PBPK models can be used as a tool to describe and predict real situations and can be valuable in risk assessment (Andersen, 1995; Chiu et al., 2007).

Traditionally, PBPK models were designed to explain the fate and kinetics of drugs or pharmaceutical products in rodents (Reddy et al., 2005). However, an increasing number of studies show that this approach can also be applied to understand and predict the kinetics of accumulation and effects of environmental contaminants in other species including humans (Lee et al., 2002; Reddy et al., 2005; Redding et al., 2008; Verner et al., 2008; Sonne et al., 2009). For marine mammals, PBPK models are rather scarce although they would be an ideal target species for modeling due to the high contaminant loads, the limited information on tissue residues with respect to blubber or the unresolved questions about maternal transfer of pollutants. PBPK models have been used to interpret the presence and behavior of several chemicals in ringed seals (*Phoca hispida* (Hickie et al., 2005)) and beluga whales (*Delphinapterus leucas* (Hickie et al., 1999)). However, to date, no models are available for any chemical in harbor porpoises.

Harbor porpoises are small cetaceans inhabiting coastal areas in the Northern Hemisphere. They feed high in the aquatic food web, have relatively long life spans, and are assumed to have a limited metabolism for several pollutants compared to other marine mammal species, such as harbor seals (Weijs et al., 2009a; Weijs et al., 2009d). Consequently, they are considered to be particularly vulnerable to pollution and sensitive for toxic effects.

The present study aims to develop and validate PBPK models for the bioaccumulation of PCB 153, as the most persistent PCB congener in male and female harbor porpoises. An important goal is to extrapolate these models in future studies to other PCBs and halogenated pollutants, such as polybrominated diphenyl ethers (PBDEs), to understand the dynamics and fate of these pollutants in harbor porpoises and to make predictions in function of space and time.

2. Model Development.

The development of the PBPK models for lifetime PCB 153 exposure in male and female harbor porpoises can be separated into three parts: 1)

specification, 2) calibration, and 3) evaluation, which are given briefly below. These sections are explained in detail in the Supporting Information.

1) In the present study, harbor porpoises are described as consisting of five tissue compartments perfused by blood (Figure 1). All compartments are selected because of their relevance for exposure to and bioaccumulation of PCB 153 and because of the data availability in the literature.

2) The uptake, distribution, and elimination were included. The uptake is characterized by milk for younger animals and fish for animals after weaning. Distribution is modeled through the blood flow going to each compartment and partition coefficients between blood and each compartment. Elimination processes considered are the dilution effect due to growth, metabolic biotransformation, biliary excretion, and the influence of gestation and lactation (only for the female model). Equations and parameters used for the models for males and females are given in Table 1 and were taken from the literature or fitted to the data when clearly indicated. The models are coded using Berkeley Madonna 8.3.14 (Berkeley Madonna Inc.) and are available on request to the corresponding author.

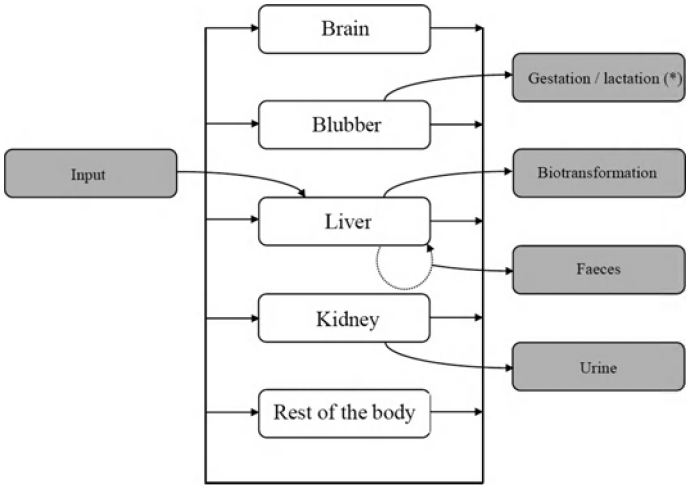


Fig 1. Conceptual diagram of the PBPK model for PCB 153 in male and female ((*) included) harbor porpoises.

3) Real life data of harbor porpoises were used to evaluate the models. PCB 153 was measured in liver, kidney, blubber, and brain samples of 1 fetus and 17 females (1 to 12 years) (Table S5) and in liver, kidney, blubber, muscle, and brain samples of 20 males (1 to 10 years) (Weijs et al., 2010a) and in milk samples (Table S6). All animals were from the Black Sea from 1998 to restrict spatial and temporal variation. The sensitivity of the parameters was tested for the female and male model by assessing the change in area under the curve (AUC) of the blood curve for a 5% increase and decrease of each original parameter value. Parameters were considered sensitive when the absolute Sc (sensitivity coefficient) > 1%.

Table 1. Parameters used for PBPK model calibration of male and female harbour porpoises.

Parameter	Male	Female
Body Weight (BW; g)[a]	$1.387 \times BS^{2.076}$	$1.677 \times BS^{2.051}$
Body Size (BS; cm)[b]	$142.4 \times (1-0.3751\ e^{(-0.000068 \times age)})$	$156.0 \times (1-0.3570\ e^{(-0.000040 \times age)})$
Cardiac output (L/hr)[c]	$0.1017 \times BW^{0.9988}$	
Compartment mass (g):		
blubber (V_F)[d]	$18.41 \times BW^{0.607}$	$22.59 \times BW^{0.589}$
brain (V_B)[d]	$49.20 \times BW^{0.211}$	$36.48 \times BW^{0.236}$
liver (V_L)[d]	$0.060 \times BW^{0.932}$	$0.000154 \times BW^{1.498}$
kidney (V_K)[d]	$0.002 \times BW^{1.137}$	$0.002 \times BW^{1.134}$
muscle (V_R)	$(0.99 \times BW) - (V_F + V_B + V_L + V_K + V_{Blood})$	
blood (V_{Blood})[e]	$0.143 \times BW$	
Density (g/L):		
Blubber[f]	920	
Brain[f]	1050	
Liver[f]	1040	
Kidney[f]	1050	
Muscle[f]	1040	
Blood[g]	1068	
Fractional blood flow (%)[h]:		
to blubber (Q_FC)	5	8.5
to brain (Q_BC)	12	12
to liver (Q_LC)	25	27
to kidney (Q_KC)	19	17
to muscle	$100 - (Q_FC + Q_BC + Q_LC + Q_KC)$	
Partition coefficient[i]:		
blood/blubber	331.6	331.6
blood/liver	7.9	11.3
blood/brain[j]	6.3	6.3
blood/kidney	4.6	4.7
blood/muscle	6.8	6.8
Lipid percentage (%)[k]:		
blubber	92.85	92.68
liver	3.73	5.33
kidney	3.24	3.31
brain	11.49	15.04
blood	0.45	0.45
muscle	2.27	
Input fish (ng/g ww)[l]	1.1	
Input milk (ng/g ww)[m]	127.6	
Fat percentage milk (%)[m]	29.94	
Assimilation efficiency (%)[n]	90	
Daily consumption of milk (g/day)[o]	540	
Daily consumption of fish (g/day)[p]	$0.123 \times BW^{0.80}$	
Extra daily input during gestation (%)[q]		12.5
Extra daily input during lactation (%)[q]		68.7
Metabolic half life (years)[r]	27.5	

[a]-correlations developed using existing data from the literature; [b]-Von Bertalanffy age dependent growth-curves developed using existing data from the literature; [c]-Altman and Dittmer (1971); [d]-McLellan et al. (2002); [e]-Reed et al. (2000); [f]-Maruyama et al. (2002); [g]-Dolfinarium Harderwijk, The Netherlands, personal communication; [h]-Williams and Leggett (1989), Brown et al. (1997); i-calculated according to the principle of Parham et al. (1997) and lipid percentages from Weijs et al. (2010a) and supporting information; [j]-fitted to the data of the brain; [k]-Weijs et al. (2010a) for males and supporting information for females; [l]-Tanabe et al. (1997a); [m]-average of table S6; [n]-Hickie et al. (2005); [o]-Oftedal (1997); [p]-Innes et al. (1987); [q]-Dolfinarium Harderwijk, The Netherlands, personal communication; [r]-Verner et al. (2008).

3. Results

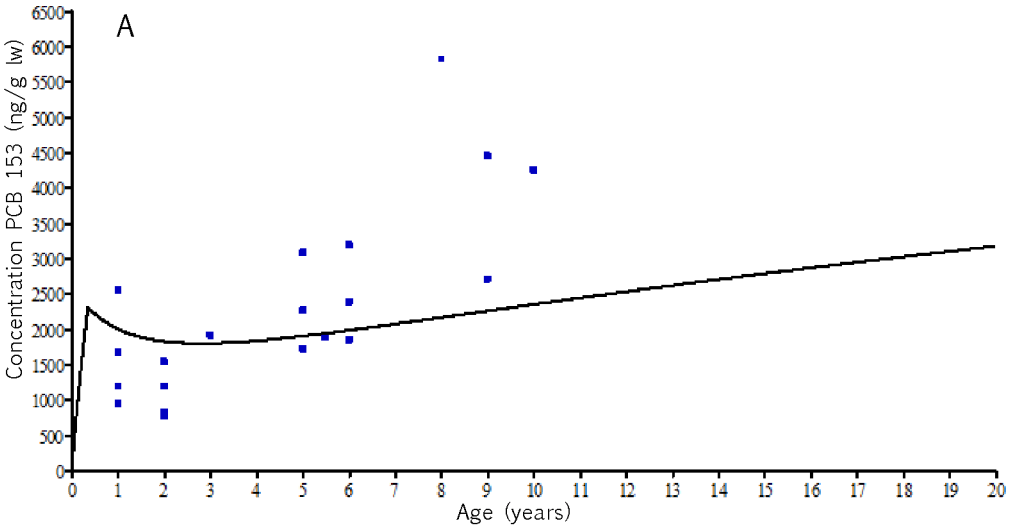
3.1. General Trends.

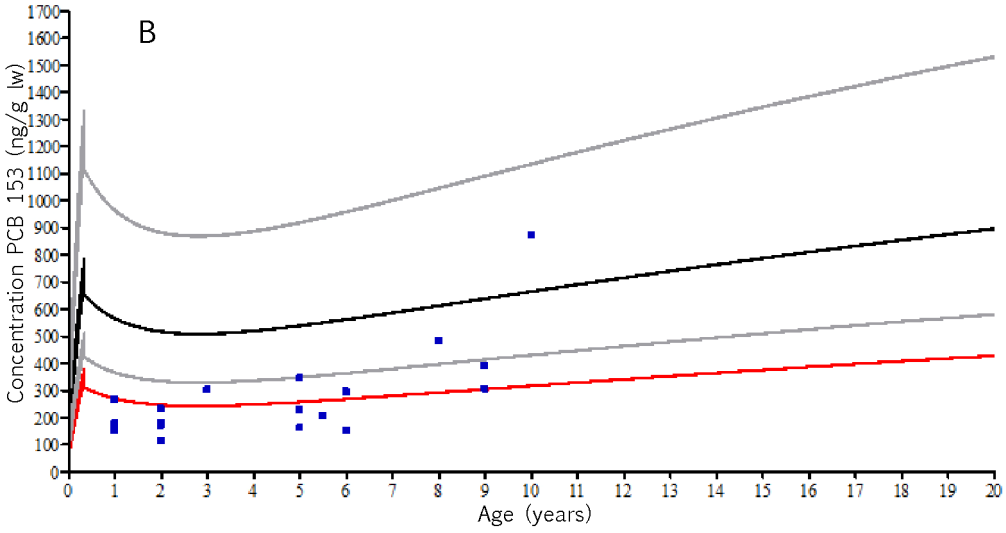
Concentrations of PCB 153, starting from the levels found in the tissues of a fetus from the Black Sea at birth (0 years), increase rapidly during the first months in the male and female model. They reach a peak at 4 months, and, after switching from 100% milk to a 100% fish diet, concentrations start declining until adulthood. From that time, the differences between the male and female model become clear. The male model shows a slow increase, whereas the female model gives a stepwise decrease in PCB 153 levels. This pattern is consistent for all tissues of the respective models. In males and females, concentrations in blubber were highest followed by the 'rest of the body'-compartment, blood, liver, kidney, and brain. PCB 153 concentrations in

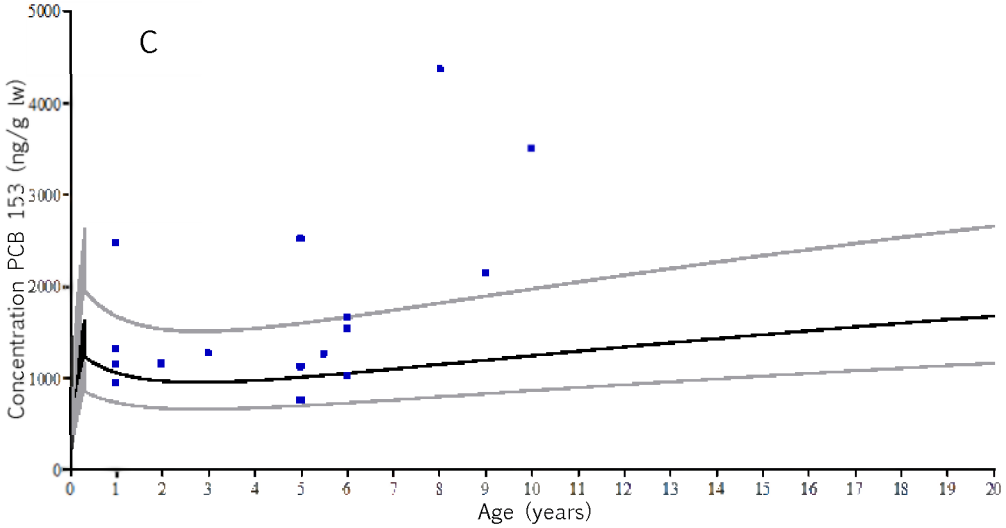
male (Figure 2) or female (Figure 3) harbor porpoises do not reach a steady-state condition during the entire life of the animals.

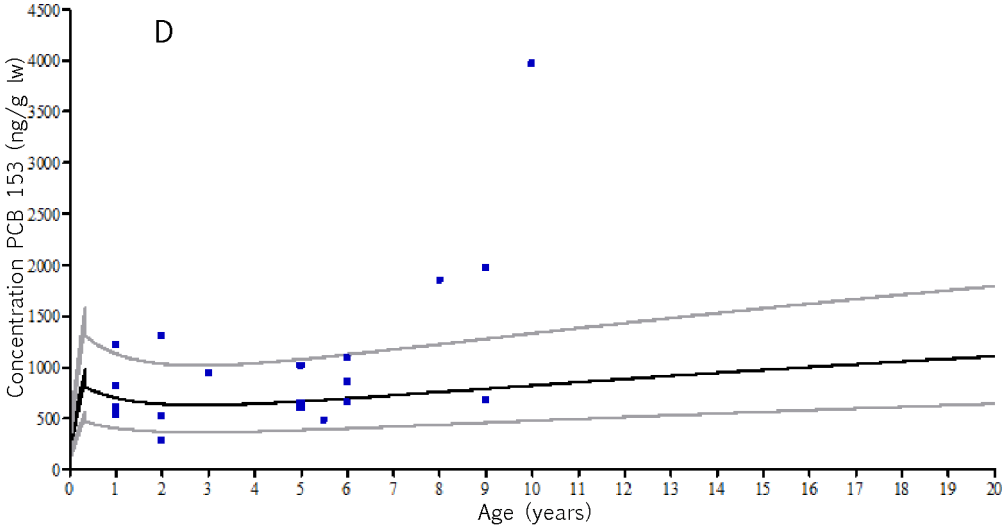
3.2. Metabolic Transformation and Partition Coefficients in Males.

The blood-brain partition coefficient PB, calculated with the adjustment factor based on the average lipid content of the brain (Weijs et al., 2010a), was found to provide simulation results that were inconsistent compared to the data set used for validation (Figure 2B - black line). Considering the range of brain lipid content (7.80-18.5% (Weijs et al., 2010a)), the adjustment factor was recalculated for the minimum and maximum values of the range, but simulation results were still found to be inadequate (Figure 2B - gray). To look for an appropriate blood-brain partition coefficient, the model was fitted to the data of the brain and revealed a partition coefficient of 6.3 (Figure 2B - red). For liver (PL) and kidney (PK), the simulations with partition coefficients calculated with the average lipid content of liver and kidney, respectively, were a good approach compared to the data set for validation (Figure 2C,D - black). Moreover, model simulations with PL and PK calculated with the minimum and maximum lipid contents from Weijs et al. (2010a) for liver and kidney were able to capture over 70% of the data points from the data set for validation (Figure 2C,D - gray). For the 'rest of the body'-compartment, the average (Figure 2E - black) and minimum/maximum (Figure 2E - gray) lipid contents of muscle were used to calculate the partition coefficients. Although the model with a partition coefficient using the average lipid content of muscle does not seem to be consistent with the muscle data, the models with partition coefficients based on the minimum and maximum lipid contents are capable of covering 90% of the muscle data from the Black Sea data set.




B
Concentration PCB 153 (ng/g lw)
Age (years)


C
Concentration PCB 153 (ng/g lw)
Age (years)


D
Concentration PCB 153 (ng/g lw)
Age (years)

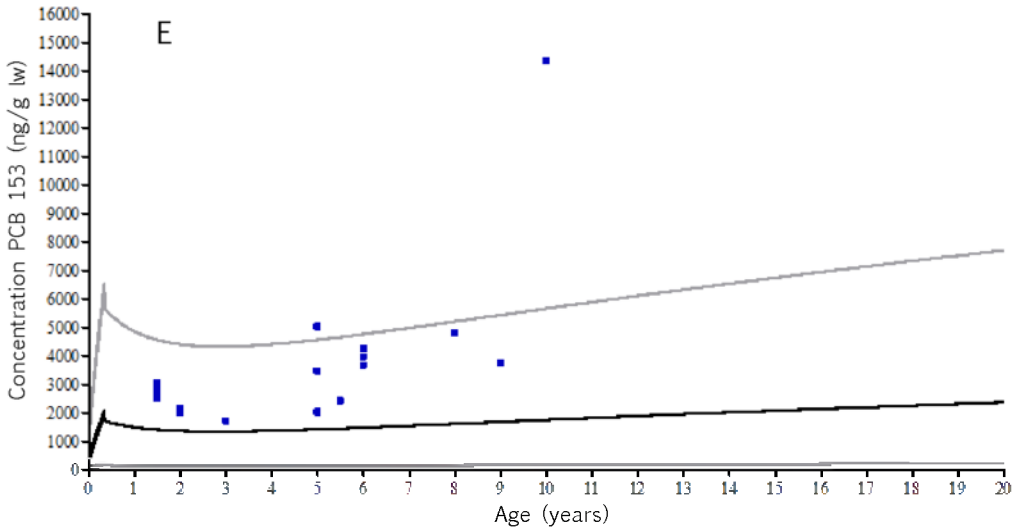
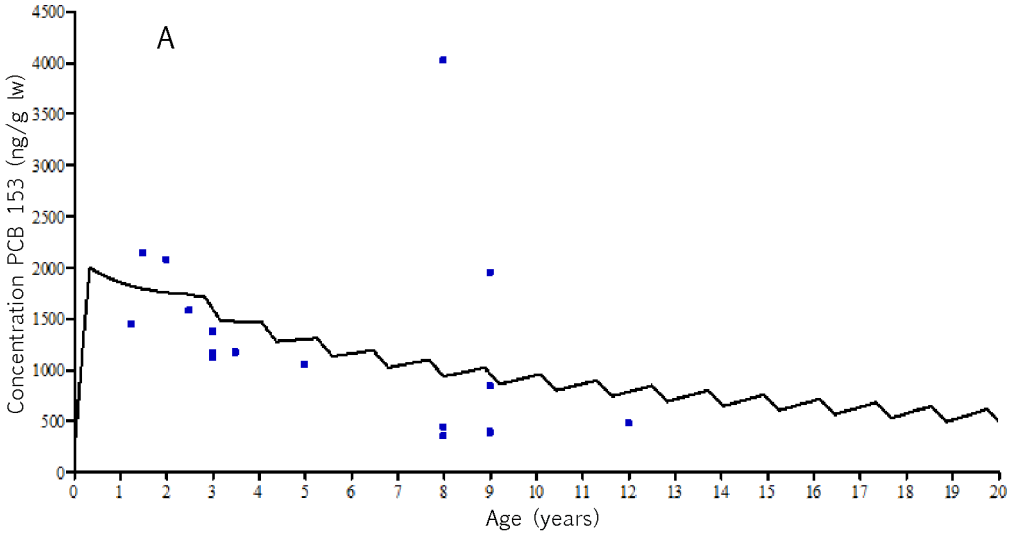


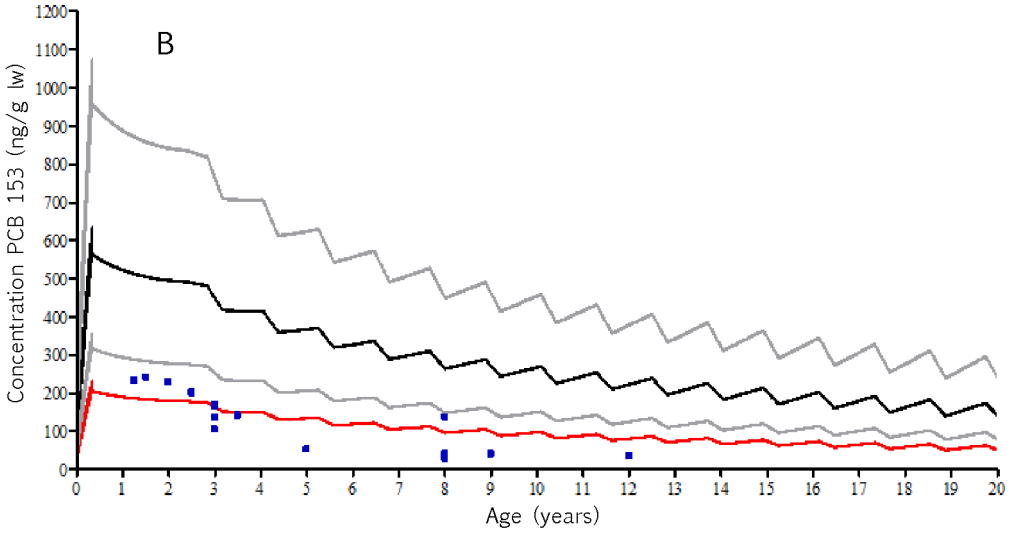
Fig 2. Age-dependent bioaccumulation of PCB 153 (in ng/g lw) in blubber (A), brain (B), liver (C), kidney (D), and the rest of the body (E) of male harbor porpoises. — (black) = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — (gray) = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid contents of the respective tissue, ■ = individual data of male harbor porpoises from the Black Sea (data set for model validation), — (red) = model prediction with fitted tissue/blood partition coefficient.

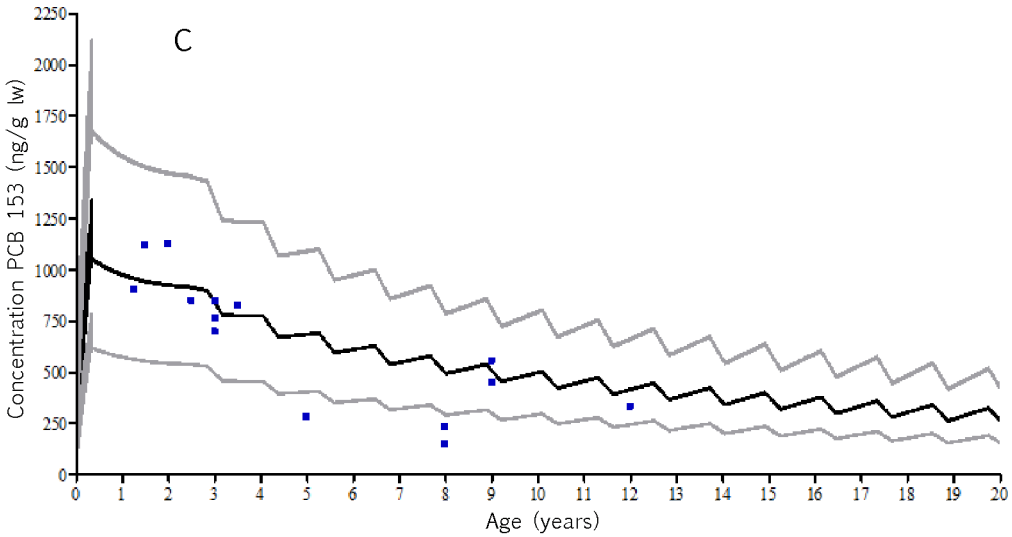
The metabolic half-life of 27.5 years of PCB 153 in humans (Verner et al., 2008), used to calculate the metabolic rate in the present study, exceeds the maximum age of the harbor porpoises, which suggests that this chemical is not eliminated by metabolism in harbor porpoises. This raises the question whether it is useful to include possible metabolic transformation in the models. Therefore, the model was run again without taking the metabolism of PCB 153 into account. Results reveal that possible metabolism has apparently a minor impact on the outcome of the model for animals younger than 5-6 years but gains in importance with higher age (Figure S3). It seems that addition of metabolism results in a worse fit with the data at higher age. However, there are more possibilities than metabolism to describe the high concentrations found at age 8-10 years, but these were not taken into account in the present models due to the lack of information. For instance, it is possible that older porpoises forage larger and thus higher contaminated fish. However, higher exposures in the past or higher 'start' concentrations of the fetus are also possible explanations. Although there are no data of animals older than 15 years to determine whether metabolic transformation occurs, it cannot be ruled out that older harbor porpoises are capable of eliminating PCB 153 through metabolic transformation. Elimination through feces was fitted for the model with possible metabolism and without metabolism and was considered to be comparable and negligible in both scenarios.

3.3. Metabolic Transformation and Partition Coefficients in Females.

Similar to the males, PB calculated with the average lipid content or the minimum and maximum lipid percentages resulted in an overestimation of the model compared to the data set for validation, whereas the use of 6.3 from the male model gave good results (Figure 3B). The two situations, namely a metabolism with a metabolic half-life of 27.5 years (verner et al., 2008) and no metabolism, revealed that metabolism is only of minor importance over the entire lifetime of females.


A
Concentration PCB 153 (ng/g lw)
Age (years)


B
Concentration PCB 153 (ng/g lw)
Age (years)


C
Concentration PCB 153 (ng/g lw)
Age (years)

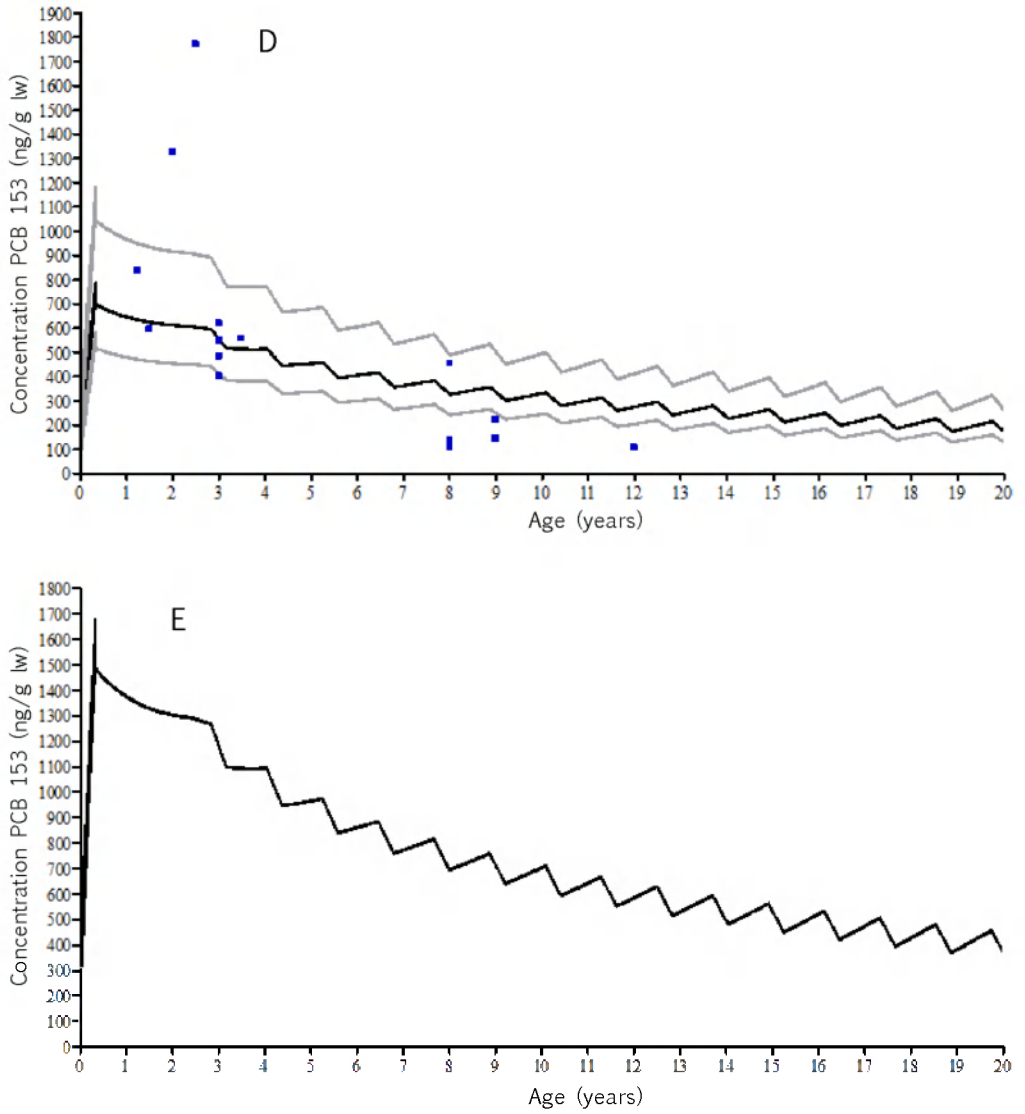


Fig 3. Age-dependent bioaccumulation of PCB 153 (in ng/g lw) in blubber (A), brain (B), liver (C), kidney (D), and the rest of the body (E) of female harbor porpoises. — (black) = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — (gray) = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid contents of the respective tissue, ■ = individual data of female harbor porpoises from the Black Sea (data set for model validation), — (red) = model prediction with fitted tissue/blood partition coefficient. Muscle data for the 'rest of the body'-compartment were not available for females.

3.4. Sensitivity Analyses.

Results of the sensitivity analysis are found in the Supporting Information (Table S3 for males and Table S4 for females). Next to the blood parameters (density of blood and fat percentage of blood), the parameters of the blubber compartment (density of blubber and blubber/blood partition coefficient) seem to have the greatest impact on the outcome of the model. As expected, parameters dealing with the input (e.g., concentrations in the fish and milk, assimilation efficiency, or the percentage absorbed from the amount of PCB 153 that is ingested) are also sensitive. All other parameters have little or no influence on the models.

4. Discussion

Our PBPK models explain the accumulation of PCB 153 in blubber, kidney, brain, blood, liver, and the rest of the body of harbor porpoises from the

Black Sea until a maximum age of 20 years. Longevity in harbor porpoises is normally around 12 years, although some may reach higher ages (Lockyer, 2007). Harbor porpoises from the Black Sea were used for model evaluation to restrict variation in space (limited migration in the Black Sea) and time (all samples were from 1998). Due to an additional elimination pathway (gestation and lactation) in females, models of males and females are different.

4.1. From Birth until 4 Months.

There is a sharp increase in concentration of PCB 153 in the models of both genders during the first 4 months of their lives. The literature reports lactation periods ranging from 4 months to 1 year (Tinker, 1988; Oftedal, 1997; Evans and Stirling, 2001). According to Read (2001), calves depend entirely on milk with a lipid percentage of up to 30% for the first 3 months after which they start to forage and have a mixed diet of milk and fish for several months. Although the lipid percentage of harbor porpoise milk may seem high compared to human or cow milk, it is comparable with lipid percentages of milk in other marine mammal species (Table S8). Due to modeling issues, the mixed diet was not taken into account in the present models. As a compensation, lactation (100% milk) was increased to 4 months followed by a 100% fish diet for the rest of their lives. Ridgway and Reddy (1995) reported a variation in total PCB concentration in milk throughout lactation in bottlenose dolphins, whereas Debier et al. (2003b) found that levels of PCB 153 remained constant in early and late lactation in fasting gray seals. Results of milk samples from the Black Sea (Table S2) were from females of different ages and probably also in different stages of lactation. Since there is no preference for one of these results, the average has been used in the female model. Oftedal (1997) estimated the daily milk intake of harbor porpoises by investigating the mammary mass, since the calf can only drink as much as the mother can produce. Despite that calves of 1 month old probably drink less than calves of 4 months old, the reported average of 540 g milk on a daily basis (Oftedal, 1997) is most likely a reliable approximation over the entire lactation of 4 months.

4.2. From 4 Months until 3-4 Years.

After 4 months, the models switch to a 100% fish diet. From this time until the age of approximately 3-4 years, PCB 153 levels decrease slowly due to a dilution effect induced by a temporarily higher growth rate (Figure S1A for males and Figure S1B for females). The concentration of PCB 153 in fish is kept constant, while the daily intake is body weight dependent. To our knowledge, information about PCB 153 in fish of the Black Sea in 1998 is non existent. Therefore the diet concentration (1.1 ng/g wet weight of PCB 153) from Tanabe et al. (1997a) for Black Sea harbor porpoises in 1993 is used. Since this concentration is low compared to concentrations in fish from other areas, the male and female model were run with a diet concentration of 11 ng/g ww (10x higher) and of 110 ng/g ww (100x higher) (Figure S4A for males and Figure S4B for females; shown for the blubber compartment only). Results of both options, however, exceed by far the body burdens from the data set for validation.

The differences between the male and female models become apparent from the age of 3-4 years which is the time that the growth levels off and the animals have reached sexual maturity. The age of sexual maturity depends on the population and time but has been found to range from 3-5 years for

females and 3-4 years for males (Lockyer, 1995; Lockyer et al., 2001). Nevertheless, the age of sexual maturity for Black Sea harbor porpoises is unknown, although the data set for validation suggest that this would be between the age of 2 and 3 years for females as used in the female model.

4.3. Male Adults.

Males start to accumulate PCB 153 from the age of 3-4 years. The dilution effect of growth has a minor impact at this stage due to a lower growth rate (Figure S1A). Metabolism, as calculated in the present study, is both a function of the body weight (thus age) and of the concentration of PCB 153 in the porpoises. A metabolism induced only by the PCB 153 concentrations in the animals would not be as efficient since calves would then need to have very high metabolic capacities. The ratio of ΣHO-PCBs/ΣPCBs in liver of adult harbor seals is two times higher than the ratio in harbor seal pups (Park et al., 2009) indicating that adults are capable of metabolizing more PCBs compared to pups. For PCB 153, the male model with metabolism does not differ much from the male model without metabolic elimination. The metabolic breakdown of PCB 153 in harbor porpoises, even for the older animals, seems thus to be a minor elimination pathway. This confirms the conclusions of McKinney et al. (2006a) who found no depletion of higher chlorinated PCBs in an *in vitro* assay using hepatic microsomes of beluga whales, and it is supported by Yordy et al. (2010) where recalcitrant PCBs (e.g., PCB 153) increased with age in bottlenose dolphins while metabolically labile congeners did not increase as much. Next to metabolic breakdown, elimination of PCB 153 through excretion seems also a process of minor importance which is in contrast with results in other cetacean species from the literature. Weisbrod et al. (2000) reports that PCB 153 levels in feces of right whales are about 4 times lower than concentrations in blubber. On the other hand, Marsili et al. (1995) found that concentrations in feces of bottlenose dolphins are between 2.4 and 7.3 times higher than in blood. However, these scenarios are not possible in the current models. An increase in dietary input (through higher concentrations in the diet, a higher daily consumption or higher assimilation efficiency) would primarily lead to an increase in blubber, because levels in blubber are only indirectly influenced by elimination pathways in liver. Eventually, the distribution of PCB 153 between the tissues would change entirely which is not in accordance with the validation data set of harbor porpoises from the Black Sea used for validation. As a result, there is an age-dependent bioaccumulation of PCB 153 in all compartments, although not always in the same way. The combination of different blood flow rates to each compartment and blood/tissue partition coefficients leads to differences in concentrations of PCB 153 in compartments with a high turnover rate, such as the liver, and compartments with a low turnover rate such as blubber or fat. The high blood flow rate to the liver and a relatively low affinity for PCB 153 cause a peak in liver concentrations during feeding immediately followed by a steady decline (Figure S5A). On the other hand, the low blood flow to the blubber and the high blubber/blood partition coefficient facilitate the storage of PCB 153 in this compartment (Figure S5B). In accordance with the mass-balance principle and a low metabolism, the off-loading of PCB 153 from the liver results in a redistribution of PCB 153 in the entire body ultimately ending in a compartment with a high affinity for PCBs, such as the blubber. According to the brain/blood partition coefficients calculated with the method described in Parham et al. (1997) and using the average or minimum/maximum brain

lipid percentages, concentrations of PCB 153 in the brain should be higher. However, the equations from Parham et al. (1997) do not take into account the blood-brain barrier or a different lipid composition of the brain, thereby possibly overestimating the actual PCB 153 levels in the brain. This is in accordance with Yordy et al. (2010b) who suggested that contaminant distribution might not be entirely dependent on lipid content. The data set for validation suggests that the current model output does not apply for animals of older ages as concentrations seem to increase more from the age of 8 years. An increased input (more fatty or larger fish) or a higher 'start' concentration might be responsible for this, rather than excluding the limited capacity for metabolic breakdown. However, because there is no information available about diet preferences at older age for harbor porpoises or historical data about concentrations in harbor porpoises from the Black Sea before the 1990s, these situations could not be taken into account in the present models.

4.4. Female Adults and the Influence of Pregnancy and Lactation.

Models for PCB 153 accumulation in females are more difficult to develop than models for males. Because of the losses due to gestation and lactation, the number of pregnancies is of major importance in these models, and such background information is seldom known for wild marine mammals. Female harbor porpoises are capable of reproducing every year, and sexual maturity has been reported between 3 and 5 years (Lockyer, 1995; Lockyer et al., 2001). Yet, there is a great deal of individual variation involved, impossible to include accurately in a model. In the present model, non overlapping reproductive cycles were repeated every 14.5 months (10.5 months gestation, 4 months lactation) with the first pregnancy starting from the age of 2 years, because concentrations of PCB 153 in females from the Black Sea started to decline earlier than 3 years. Although this is theoretically not the only possibility, it seems to be reliable enough to explain the PCB 153 concentrations in the majority of the animals in the female model. Exceptions or outliers are assumed to follow a different pattern. They have probably experienced a pregnancy only once or twice in their lives and apply therefore more to the male model. For females, the 14.5 month cycles represent a great advantage for off-loading their PCB body burdens as found for other species as well (e.g., Duinker et al., 1979; Lee et al., 2002; Debier et al., 2003a). Although they continue eating, increasing their intake during gestation and even more during lactation, there is a substantial transfer of PCB 153 to the offspring. With each cycle, the females can lower their levels of PCB 153 without having time between two cycles to build up higher concentrations. According to Table S9, females pass about 50% of the PCB 153 levels in blubber on to their offspring regardless of the age of the mothers or the time and place of sampling. Assuming that the mothers give the same amount of nutrients and milk in each cycle, the concentrations in milk will decrease. As a result, firstborns will be contaminated significantly more compared to lastborns. Combined with an insufficient metabolism at younger ages, this leaves the calves as a very vulnerable group.

4.5. Sensitivity Analysis.

Overall, the models are sensitive to changes in blood parameters (density of blood and lipid percentage of blood). However, since blood concentrations were monitored during sensitivity analysis, these are the only parameters that can influence the blood concentrations directly. Indirectly, the blood

concentrations are sensitive to parameters dealing with the PCB 153 intake and with the blubber, as expected. These models will not give an output without an input and are therefore highly dependent on the input parameters (concentration in milk, concentration in fish, assimilation efficiency). The higher the affinity of blubber for PCB 153, the lower its presence in the blood, thereby confirming again the function of blubber as storage compartment for lipophilic compounds.

4.6. Biomonitoring Tool.

Despite the lack of detailed information of some parameters in harbor porpoises, or even in marine mammals, the PBPK models presented here describe sufficiently the bioaccumulation and kinetics of PCB 153 in male and female harbor porpoises from the Black Sea. For both genders, concentrations in all tissues increased rapidly during the nursing period of four months and declined until the animals have reached sexual maturity. From that time, male and female models were different with a steady bioaccumulation of PCB 153 for males and decreasing body burdens due to reproduction for females. Metabolic breakdown of PCB 153 is a negligible elimination pathway for harbor porpoises in general, although adult males are assumed to have a slightly better developed metabolism compared to calves or juveniles. Calves receive considerable amounts of PCB 153 from their respective mothers without having the capacity to eliminate this compound. The models that were developed in this study show that there is a specific distribution of PCB 153 in the body of harbor porpoises. Blubber and blood are the only matrices that can be (relatively) easily obtained in a nondestructive way from wild harbor porpoises, whereas current toxicity tests are mainly based upon hepatic concentrations. With respect to the specific distribution between the tissues, such models can predict concentrations in liver, kidney, or brain by using measured levels in blubber or blood, thereby paving the way for the biomonitoring of wild marine mammals.

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Supporting Information

1. Model development

Model specification

In the present study, harbour porpoises are described as consisting of five tissue compartments perfused by blood (Fig 1, manuscript). All compartments are selected because of their relevance for exposure to and bioaccumulation of PCB 153 and because of the data availability in the literature. Due to the high lipophilic nature of PCB 153, blubber is chosen as a compartment for storage. Kidney represents the tissue where possible excretion of the parent compound may occur via urine. The uptake of PCB 153 occurs via the liver as this is the only tissue of the gastrointestinal system included in the model. Two elimination pathways, biliary clearance (elimination through feces) and possible metabolic biotransformation, are also set in the liver. The brain is included too due to the possible neurotoxic effects of PCB 153. A compartment which accounts for the rest of the body is included as well to meet mass balance principles. All tissues in the model are considered to be flow-limited, consistent with a PBPK model for lactational transfer of PCB 153 in human (Redding et al., 2008).

Model calibration

Uptake. The uptake of PCB 153 in marine mammals occurs mainly through food intake. From birth to the age of approximately four months, porpoises are exclusively fed with milk from their mothers. After weaning, the major exposure route is through feeding on fish. PCB 153 intake is assumed to be directly into the liver, due to the lack of information about the intestinal uptake of chemicals in marine mammals and similar to PBPK models of PCB 153 in humans (Redding et al., 2008; Verner et al., 2008). A transition period between the milk diet and the fish diet is not taken into account for practical reasons. For both milk and fish diets, the average daily input (ADI), expressed in ng PCB 153 per day, was calculated by the following equation:

$$ADI = DC \times TOTDIET \times IN$$

with DC as the daily consumption (g/day), TOTDIET as the concentration of PCB 153 in the diet (milk or fish; ng/g wet weight) and IN as assimilation efficiency. An IN of 90 % of the total amount of PCB 153 ingested is used for the actual uptake of PCB 153 (Hickie et al., 2005). Foraging of harbour porpoises in the wild might be related to the tidal cycle (Johnston et al., 2005). Therefore, milk or fish are given twice a day in the current PBPK models.

Milk diet. In harbour porpoises, a variation in duration of weaning has been reported. According to Read (2001), calves depend on lipid-rich milk for the first three months after birth, whereas lactation might occur until the age of 8-12 months (Oftedal, 1997). In the present models, weaning (diet consisting of 100 % milk) is estimated to last for four months. This includes the reported three months (Read, 2001) and one additional month as a compensation for the months after, in which the porpoises partly rely on milk for their daily food intake. During these four months, DC is kept constant in the models at 540 g milk/day (Oftedal, 1997). The concentration of PCB 153 in the milk (TOTDIET for milk or CMILK) was measured using 6 milk samples of harbour porpoises from the Black Sea (Table S6).

Fish diet. From weaning to adulthood, DC (= 0.123 x $BW^{0.80}$ (kg/day)) depends on the body weight and is taken from Innes et al. (1987) and Read and Brownstein (2003). TOTDIET(fish) is taken from Tanabe et al. (1997a) who investigated PCBs in harbour porpoises and their prey from the Black Sea.

Reproductive cost. Harbour porpoises do not fast in any period of their lives. Because of their large body surface to body volume ratios, harbour porpoises in general need a high amount of energy to maintain a constant body temperature and their relatively small sizes limit the amount of energy that they can store (Kastelein et al., 1997a; MacLeod et al., 2007; Lockyer, 2007). Kastelein et al. (1997b) report that these animals can only survive as little as 3 to 5 days without food. To survive, they have to continue hunting and eating, even during gestation or lactation. Despite this, there is good evidence that there still is a reproductive cost for the porpoises (Read, 2001; Lockyer, 2007) with an increase in the blubber lipid content in pregnancy and a decrease during lactation. To meet their own energetic requirements, females have to increase their intake of fish, and thus PCB 153, during gestation and lactation (Read, 2001) which is primarily stored in their blubber. In 2009, a harbour porpoise calf was born in Harderwijk, the Netherlands. Because the mother was not capable of surviving in the wild, both mother and calf were held in captivity, which made it possible to monitor the actual daily food requirements of the mother. The dietary intake of the mother increased slightly by about 0.5 kg fish on a daily basis during gestation (normally between 3.5 and 4 kg per day), whereas an extra amount of 2.75 kg of fish was given each day during lactation (Harderwijk, personal communication). Because there is no information available in the literature concerning the increases in dietary intake during gestation and lactation in wild harbour porpoises, these results were used. Therefore, increases of fish intake of 12.5% (0.5 kg divided by 4 kg) and 68.7% (2.75 kg divided by 4 kg) during gestation and lactation respectively, are included in the female model.

Distribution. The distribution of PCB 153 is determined by the blood flow to the four compartments and the partition coefficients between the blood and each compartment or tissue. The distribution processes are coded using the following mass balanced differential equations:

$$\frac{dA_t}{dt} = Q_t \times \left(C_{Blood} - \frac{C_t}{P_t} \right) - elimination + uptake$$

With A_t the amount of PCB 153 in tissue t, Q_t the blood flow to tissue t, C_{Blood} the concentration of PCB 153 in arterial blood, C_t the concentration of PCB 153 in tissue t and P_t the partition coefficient between tissue t and blood. The ratio of C_t and P_t equals C_{vt} which is the concentration in venous blood of tissue t.

Blood flow rates. The cardiac output is defined as the volume of blood pumped per unit time from the ventricle. It can be found by multiplying the heart rate with stroke volume which is the volume of blood ejected from the ventricle with every heart beat. Marine mammals have, as part of their aquatic lifestyle, several adjustments, such as a heart rate that changes

during submersion, that allow them to dive (Kastelein and Meijler, 1989). Few studies have investigated the heart function in marine mammals and respiratory arrhythmia has been reported in some cases. Kastelein and Meijler (1989) found respiratory arrhythmia in harbour porpoises on land and in water, while Kanwisher and Ridgway (1983) came to the same conclusion for common dolphins (*Delphinus delphis*), bottlenose dolphins (*Tursiops truncatus*), and beluga whales (*Delphinapterus leucas*). Overall, the heart rate and stroke volume can differ from one activity to the other. In a lifetime based model, it is impossible to take the number of dives per hour, the duration of the dives and the possible changes in stroke volume or heart rate during the dive into account. For this reason, the following equation which expresses the change in cardiac output (QC; in liters per minute) in function of body weight (BW; in kg) for mammals in general (Altman & Dittmer, 1971), was used:

$$QC = 0.1017 \times BW^{0.9988}$$

Compared to data from Thornton et al. (2005), the cardiac outputs calculated with this equation correspond well to predictions using Stahl's equation mentioned in the same study.
Blood flow rates to various compartments were achieved by multiplying the cardiac output (QC) with the percentage of blood going to each compartment. These percentages were derived from humans (Williams and Leggett, 1989; Brown et al., 1997) and recalculated for the 'rest of the body'-compartment to fulfill the mass-balance principle of the model.

Partition coefficients. The partitioning of pollutants between blood and a specific target tissue can be calculated by dividing the concentration in tissue by the concentration in blood under steady-state conditions. For marine mammals however, this approach leads to some practical problems. In general, blood samples are more reliable when taken from living animals. On the other hand, due to the endangered and protected status of most marine mammal species, samples of tissues (e.g., liver, kidneys, brain) are from dead animals. Parham et al. (1997) found a poor relationship (r^2=0.38) between octanol/water partition coefficients and adipose tissue/plasma partition coefficients, but were able to predict adipose tissue/plasma partition coefficients based on several structural parameters of PCBs (e.g., number and position of chlorines). The same study used the blood composition of rats and humans to calculate adipose tissue/blood partition coefficients from adipose tissue/plasma partition coefficients which were successfully used by Redding et al. (2008). In the present study, the transformation of adipose tissue-plasma partition coefficients to adipose tissue-blood partition coefficients (P_F) was done by creating equations using the blood composition and specific blood parameters of bottlenose dolphins (Bossart et al., 2001). Results do not differ much from P_F values for PCB 153 or other highly lipophilic compounds from the literature (Table S1). Similar to Parham et al. (1997), partition coefficients for other tissues are derived by multiplying the P_F with an adjustment factor based on the average lipid content of the tissues of harbour porpoises from the Black Sea (Weijs et al., 2010 for males and in Supporting Information for females). Although the lipid contents of the tissues, and thus the partition coefficients as well, might change due to circumstances, such as the nutritional status, the partition coefficients were considered as constants.

Elimination. Processes considered in the present study are the dilution effect of growth on the bioaccumulation of contaminants, metabolic transformation and excretion via the production of feces and urine. In females, gestation and lactation was included as an additional pathway for elimination.

Growth. Gol'din (2004) investigated a growth pattern of harbour porpoises in two phases with a fast growth rate at first followed by a slower growth rate with higher age, but could not find a statistical significance. Moreover, the beginning of the second phase remains unclear: in the first year of life, between the first and second year, between the third and fourth year or between the fourth and fifth year (Gol'din, 2004). Lockyer et al. (2001) recommended a growth pattern in only one phase. Such relationship between the age of marine mammals and their body length was previously found by using von Bertalanffy or Gompertz growth models (*e.g.* for bottlenose dolphins in Stolen et al., 2002; for harbour porpoises in Galatius, 2005; for harbour seals (*Phoca vitulina*) in Hauksson, 2006) where L_∞ represents the asymptotic body length, b is a growth constant, t is age (years) and k is the growth rate constant. In the present study, the growth of the animals during their entire lifetime was modeled with a Von Bertalanffy age-dependent growth equation fitted to data from Gaskin et al. (1983), Duinker et al. (1989), Kuiken et al. (1993), Szefer et al. (2002), Ciesielski et al. (2004), Strand et al. (2005), Law et al. (2006b) and Weijs et al. (2010a and unpublished data) (Supporting Information, Fig S1 A (males), Fig S1 B (females)). Values compare favorably with results from harbour porpoises from West Greenland (Lockyer et al., 2001) or other areas mentioned in the same study.

Due to the body weight-dependency of some parameters (e.g., cardiac output, compartment volumes), a relationship between the body size and the body weight was established. Therefore, a simple allometric $Y=a \times X^b$ equation with Y=body weight (BW) in grams (g) and X=body size (BS) in centimeters (cm) was plotted using data from Duinker et al. (1989), Kannan et al. (1993), Strandberg et al. (1998), Covaci et al. (2002), Szefer et al. (2002), Ciesielski et al. (2004), Strand et al. (2005) and Weijs et al. (2010a and unpublished data) (Supporting Information, Fig S2 A (males), Fig S2 B (females)).

Compartment volumes. The volumes of organs are increasing in relation to the growth of the organism. The equations needed to account for this organ-specific growth depend on the body weight (McLellan et al., 2002) and these equations are given in Table S2 for males and females. The respective tissue densities are 920, 1050, 1040, 1050 and 1040 g/L for blubber, brain, liver, kidneys and the rest of the body respectively and are taken from Maruyama et al. (2002) in humans. The tissue density of muscle was used for 'the rest of the body'-compartment because the muscles represent the highest proportion of this compartment. The density of blood is 1068 g/L and was measured in blood of bottlenose dolphins (*Tursiops truncatus*) (Dolfinarium Harderwijk; Personal communication) and does not differ much from the density of blood of 1060 g/L as reported by Maruyama et al. (2002).

Metabolism. PCB 153 is the most persistent PCB congener in marine mammals and is therefore considered to be poorly metabolized. To the best

of our knowledge, there is no information available regarding the metabolic transformation rate of PCB 153 in harbour porpoises or other cetaceans. Therefore, the metabolic transformation rate was estimated according to Verner et al. (2008) by multiplying the hepatic extraction ratio, the liver blood flow and the blood concentration. The hepatic extraction ratio is calculated with a log K_{ow} value of 6.72 and a metabolic half-life of 27.5 years in humans with the same equations as shown in Verner et al. (2008).

Urine. Although urine is an important pathway for elimination of xenobiotics, it is not a suitable matrix for measuring the highly lipophilic PCB 153 or for PCB 153 elimination, as it will not accumulate in a water-rich environment such as the urine. As PCB metabolites are more water soluble than the parent compounds, these metabolites may be present in the urine. However, PCB 153 is considered to be poorly metabolized and any possible metabolic biotransformation of this compound is already taken into account in the previously described 'metabolism' paragraph. Therefore, the elimination of PCB 153 through urine was not included in the models.

Feces. The enterohepatic circulation is responsible for eliminating POPs through the production of feces (Moser and McLachlan, 1999; Meijer et al., 2006). For cetaceans, collecting feces is not easy because they are not solid and spread out very fast in water. As a result, it is likely that a great portion of sea water would be included in the fecal sample. Due to the presence of pollutants in surface water and even deeper waters (Lohmann et al., 2007), it might bias the results of pollutant analyses. Hickie et al. (1999) found that excretion accounts for 67.5% of total losses of PCBs in beluga whales. However, that study did not focus on PCB 153, but rather on total PCBs. Concentrations of PCB 153 in feces of harbour porpoises are unknown. As a result, the elimination through feces was estimated by fitting the model output for the liver compartment to the liver data from the Black Sea.

Gestation/lactation. Studies in the literature have shown that pregnant females have clearly greater body fat deposits than other reproductive classes such as juvenile females (Lockyer, 2007); it is believed for other cetaceans that most of the blubber of the mothers is converted to milk lipids during lactation (Oftedal, 1997). Thus, PCB 153 is mobilized from the blubber. Processes for elimination of PCB 153 through gestation and lactation were therefore set in the blubber. The age of sexual maturity has been reported between 3-5 years (Lockyer, 1995 and 2007), after which the animals produce a new calf every year. The same studies also found a gestation period of 10-11 months (Lockyer, 1995 and 2007; Read, 2001). Together with the duration of lactation discussed previously, this means that females might be pregnant and lactating at the same time. Due to modeling issues, cycles of non-overlapping successive gestation and lactation events were simulated. Little information is available on the exact amount of PCB 153 which is transferred to the offspring during gestation. However, the Black Sea dataset contains 1 mother-fetus pair (Supporting Information, Table S5). The ratio of the PCB 153 concentration in blubber of the mother and fetus (51 %; Table S9) was therefore used as estimation. This percentage was divided by the duration of the entire gestational period (taken as 10.5 months), resulting in a loss of 0.006654% of PCB 153 on an hourly basis for the mother during gestation. The loss of PCB 153 due to lactation was modeled in the same way as the milk diet, meaning that adult females were

assumed to give 540 g of milk to their calf on a daily basis (Oftedal, 1997) with a concentration of PCB 153 of 127.6 ng/g ww (Table S6).

Model evaluation

Samples. Samples of liver, kidney, blubber and brain of 1 neonate, 17 female (11 adults, 6 juveniles) and 20 male (11 adults, 9 juveniles) harbour porpoises from the Black Sea were used to validate the theoretical model. Together with these tissue samples, 8 milk samples from lactating female harbour porpoises from the Black Sea were included in the analysis as well to assess dietary input for the youngest animals. For males, samples of muscles were included as well to compare to the model predictions for the 'rest of the body'-compartment. All animals were found stranded or bycaught in the Black Sea in 1998. Because migration within the Black Sea is limited and since there is no contact or exchange between harbour porpoises from the Black Sea and from other European waters, it was believed that this dataset is the most homogeneous (limited temporal and spatial individual variation) and therefore the most suitable for validation purposes. The procedure for the analysis of PCB 153 in the samples can be found in Supporting Information (section 2) together with the results of the females, the neonate and the milk samples. Results for the males were discussed thoroughly in Weijs et al. (2010a).

Sensitivity analysis. Sensitivity analysis was performed to test the impact of some physiological parameters independent of the body weight on the outcome of the model. For each parameter, 3 runs (a batch run) were set simultaneously using the original value of the parameter and a coefficient of variation of 5%, resulting in a run with the original parameter, a run with the original parameter increased with 5% and a run with the original parameter decreased with 5%. The impact of the parameter changes on the concentration of PCB 153 in blood was determined by calculating sensitivity coefficients (%) according to the following equation (modified from Mörk and Johanson (2006)):

$$S_c = \left(\frac{AUC_5}{AUC_{Orig}} - 1 \right) 100$$

With AUC_{Orig} the area under the blood concentration curve with the original parameter value and AUC_5 the areas under the blood concentration curves with the original parameter value increased and decreased with 5%. The blood concentration curves were used because, as blood is the circulation medium between all tissue compartments (liver, brain, blubber, kidney and rest of the body), changes in one or more of these compartments would be reflected in the blood.

2. Dataset for evaluation of female model: Females from the Black Sea

Description of sample clean-up, analyses and quality assurance

Note: Methods and results of male harbour porpoises can be found in Weijs et al. (2010a).

Samples, chemicals and target compounds. Blubber, liver, kidney and brain samples were collected from 17 female harbour porpoises (6 juveniles and 11 adults) and 1 neonate stranded or by-caught in the Black Sea in 1998.

Together with these samples, 8 milk samples from female harbour porpoises from the Black Sea were analysed as well. In all samples, 39 PCB congeners (IUPAC numbers: CB 18, 28, 31, 44, 47, 49, 52, 74, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 146, 149, 151, 153, 156, 158, 170, 171, 172, 174, 177, 180, 183, 187, 194, 195, 196/203, 199, 203, 205, 209), 8 PBDEs (IUPAC numbers: BDE 28, 47, 49, 99, 100, 153, 154, 183), DDXs (*o,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT) and 2 naturally-produced methoxylated PBDEs (2'-MeO-BDE 68 and 6-MeO-BDE 47) were targeted. Standards were from Wellington Laboratories (PBDEs and MeO-PBDEs), from Dr. Ehrenstorfer Laboratories (PCBs).

Sample preparation. The method used for the sample extraction and clean-up has been previously described (Covaci et al., 2008) and is briefly presented below. Approximately 2 g of liver and brain, 0.2 g of blubber, 3 g of kidney and 0.6 g of milk was dried with ~8 g anhydrous Na_2SO_4, spiked with internal standards BDE 77/BDE 128 (25 ng) and CB 143 (100 ng) and extracted for 2 h by hot Soxhlet with 100 ml hexane/acetone (3/1; *v/v*). After lipid determination (performed on an aliquot of the extract), the extract was cleaned on 8 g of acidified silica. After elution of analytes with 20 ml hexane and 15 ml dichloromethane, the cleaned extract was evaporated to dryness and reconstituted in 150 µl iso-octane.

Analysis. PBDEs and MeO-PBDEs were measured with an Agilent 6890 gas chromatograph coupled with a 5973 mass spectrometer system (GC-MS). The GC was equipped with a 30 m x 0.25 mm x 0.25 µm DB-5 capillary column. The MS was operated in electron capture negative ionisation (ECNI) mode and was used in the selected ion-monitoring (SIM) mode with ions *m/z* = 79 and 81 monitored during the entire run. PCBs and DDXs were measured with a similar GC-MS system as for the PBDE determination, operated in electron ionisation (EI) mode and equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium). The MS was used in the SIM mode with 2 ions monitored for each PCB homologue group. The latter system (GC-EI/MS) was also used for confirmation of organobromine compounds.

Quality assurance/quality control (QA/QC). Recoveries for individual PBDE congeners were between 87 and 104 % (RSD < 12 %), while recoveries of PCBs ranged between 75 and 90 % (RSD < 10 %). For each analyte, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the procedural blank, which ensures > 99 % certainty that the reported value is originating from the sample. For analytes that were not detected in procedural blanks, LOQs were calculated for a ratio S/N equal to 10. LOQs depended on the sample intake and on the analyte and ranged between 1 and 4 ng/g lipid weight (lw). QC was performed by regular analyses of procedural blanks, by random injection of standards and solvent blanks. A standard reference material SRM 1945 (PCBs and PBDEs in whale blubber) was used to test the method accuracy. Obtained values were not deviating more than 10 % from the certified values. The QC scheme is also assessed through regular participation to interlaboratory comparison exercises organised by the US National Institute of Standards and Technology.

Results of 17 female, 1 neonate harbour porpoise and 8 milk samples

Lipids

Lipid percentages ranged in general from 2.53 – 4.54% for kidney, 8.74 – 23.54% for brain, 3.25 – 7.84% for liver and 85.35 - 96.06% for blubber for the female harbour porpoises. Lipid percentages for the neonate were 6.51%

for brain, 85.78% for blubber, 5.83% for kidney and 2.36% for liver. Lipid percentages for the milk samples varied from 22.43 to 51.79%. Concentrations and levels discussed here are all lipid-normalized.

Levels of PCBs

Individual data of PCB 153, used for validation of the female PBPK model, are given in Table S5 together with the individual data of sum PCBs in parentheses and the age of the animals. Results for sum of PCBs and of PCB 153 of the milk samples are given in Table S6. All females, except for the animals marked with (#), were by-caught and were therefore considered to be in a good health at time of death. For the females with (#) no information regarding situation of death or illnesses or health was available.

For all samples, regardless of age or tissue, PCB 153 was between 14 and 23 % of the sum of PCBs and was therefore the most dominant congener. In all tissues, PCB 153 was followed by PCB 138, PCB 149, PCB 180, PCB 187 or PCB 118 although the order of these congeners changed according to the tissue and probably also to the age of the animals and stage of pregnancy (if pregnant). PCB 205 was detected in less than 50% of the brain, liver, kidney and blubber samples. PCB 28 was detected in less than 50% of the liver and kidney samples. PCB 44, PCB 47, PCB 49, PCB 110, PCB 156, PCB 172, PCB 195, PCB 194, PCB 206 and PCB 209 were found in less than 50% of all brain samples.

Patterns in milk samples were the same as in the tissue samples: PCB 153 was predominant followed by PCB 138, PCB 149, PCB 180, PCB 118, and PCB 187. The order of the PCB congeners however, was not the same for all milk samples analyzed as it probably depends on the stage of lactation or the number of pregnancies.

Levels of PBDEs, DDXs and naturally produced MeO-PBDEs

Individual data of PBDE 47, sum of PBDEs, sum of DDXs and sum of MeO-PBDEs are given in Table S7. Although not needed for validation of the model, these data were given for the reader's information in order to give more insight into the bioaccumulation of other contaminants in Black Sea females. Similar to the males (Weijs et al., 2010a), concentrations of pesticides are mostly higher than sum of PCBs indicating that these compounds are still widely used in countries surrounding the Black Sea in the 90s. Levels of PBDEs and naturally produced MeO-PBDEs are low compared to PCBs and DDXs. As indicated by the concentrations found in the neonate and in the milk samples, all contaminant groups can be transferred during gestation and lactation to the offspring, regardless of whether the contaminants are anthropogenically- or naturally-produced.

3. Additional tables

Table S1. Adipose tissue-blood partition coefficients (P_F) for PCB 153 or other lipophilic compounds in the literature.

P_f	Compound	Species	Reference
55 - 1466	PCDDs	human	Maruyama et al. (2002)
303	CB 153	human	Redding et al. (2008)
269.8	CB 153	human	Wolff et al. (1982)
227.8	CB 153	rat	predicted in Parham et al. (1997)
350	TCDD	rat	Evans and Andersen (2000)
331.6	CB 153	harbour porpoise	Present study

Table S2. Body weight dependent compartment masses (expressed in g) for male and female harbour porpoises.

Tissue/Compartment	Males	Females	Reference
Blubber	$18.41 \times BW^{0.607}$	$22.59 \times BW^{0.589}$	McLellan et al. (2002)
Brain	$49.20 \times BW^{0.211}$	$36.48 \times BW^{0.236}$	
Liver	$0.060 \times BW^{0.932}$	$1.54 \times 10^{-4} \times BW^{1.498}$	
Kidneys	$0.002 \times BW^{1.137}$	$0.002 \times BW^{1.134}$	
Blood	$0.143 \times BW$		Reed et al. (2000)

Note: the compartment mass of the 'rest of the body'-compartment was calculated as the difference of the entire body weight and the mass of blubber, brain, liver, kidneys and blood to meet mass-balance.

Table S3. Sensitivity coefficients (S_c) indicating how much (in %) the area under the curve of blood concentration curves with the original parameter value ± 5% is different from the area under the curve of the blood concentration curve with the original parameter value in the model for **male** harbour porpoises.

Parameter			S_c	
Name	Original value	Reference	- 5 %	+ 5 %
DENSF	920 g/L	Maruyama et al. (2002)	**-3.96**	**3.88**
DENSB	1050 g/L		0.01	-0.01
DENSL	1040 g/L		-0.98	0.89
DENSK	1050 g/L		0.02	0.01
DENSR[1]	1040 g/L		-0.13	0.12
DENSBlood	1068 g/L	Harderwijk, Pers Comm	**5.27**	**-4.75**
FATPERCF	92.85 %	Weijs et al. (2010a)	< 0.01	< 0.01
FATPERCK	3.24 %		< 0.01	< 0.01
FATPERCL	3.73 %		< 0.01	< 0.01
FATPERCB	11.49 %		< 0.01	< 0.01
FATPERCR[1]	2.27 %		< 0.01	< 0.01
FATPERCBlood	0.45 %	Maruyama et al. (2002)	**5.26**	**-4.76**
QFC	5 %	Williams & Leggett (1989) and Brown et al. (1997)[2]	0.03	< 0.01
QLC	25 %		< -0.01	< -0.01
QBC	12 %		< 0.01	< 0.01
QKC	19 %		0.01	< 0.01
PF	331.6	Parham et al. (1997), Bossart et al. (2001) and Weijs et al. (2010a)	**4.05**	**-3.78**
PL	7.9		0.01	-0.01
PB	6.3		-0.03	< -0.01
PK	4.6		< -0.01	< -0.01
PR[1]	6.8		0.13	-0.11
TOTDIET	1.1 ng/g ww	Tanabe et al. (1997a)	**-2.49**	**2.49**
IN	90 %	Hickie et al. (2005)	**-4.85**	**4.84**
DCMILK	540 g/day	Oftedal (1997)	**-2.34**	**2.35**
FATPERCMilk	29.94 %	Table S6; Present study	< 0.01	< 0.01
CMILK	127.6 ng/g ww		**-2.34**	**2.35**
CFoetusF	168.99 ng/g ww	Table S5; Present study	-0.17	0.16
CFoetus L	2.04 ng/g ww		< -0.01	-0.01
CFoetusK	6.20 ng/g ww		0.01	-0.01
CFoetusB	1.65 ng/g ww		-0.01	< 0.01
CFoetusR	0.01 ng/g ww	Estimated[3]	-0.01	0.01
CFoetusBlood	0.01 ng/g ww		0.01	< -0.01
X	$3.22e^{-7}$	Fitted	-0.01	-0.02
$t_{1/2}$	27.5 years	Verner et al. (2008)	-0.93	0.92

[1] – Parameters taken from muscle since muscles are the biggest part of the 'rest of the body'
[2] – QRC or fractional blood flow to 'rest of the body' was calculated as 100-QFC-QLC-QKC-QBC
[3] – Samples not available, 0.01 was used for modeling reasons

Table S4. Sensitivity coefficients (S_c) indicating how much (in %) the area under the curve of blood concentration curves with the original parameter value ± 5% is different from the area under the curve of the blood concentration curve with the original parameter value in the model for **female** harbour porpoises.

Parameter			S_c	
Name	Original value	Reference	- 5 %	+ 5 %
DENSF	920 g/L	Maruyama et al. (2002)	**-3.21**	**3.10**
DENSB	1050 g/L		< 0.01	< 0.01
DENSL	1040 g/L		-0.88	0.82
DENSK	1050 g/L		< 0.01	< 0.01
DENSR[1]	1040 g/L		-0.11	0.11
DENSBlood	1068 g/L	Harderwijk, Pers Comm	**5.27**	**-4.76**
FATPERCF	92.68 %	Supporting Information, present study	< 0.01	< 0.01
FATPERCK	3.31 %		< 0.01	< 0.01
FATPERCL	5.33 %		< 0.01	< 0.01
FATPERCB	15.04 %		< 0.01	< 0.01
FATPERCR[1]	2.27 %	Weijs et al. (2010a)	< 0.01	< 0.01
FATPERCBlood	0.45 %	Maruyama et al. (2002)	**5.26**	**-4.76**
QFC	8.5 %	Williams & Leggett (1989) and Brown et al. (1997)[2]	0.03	-0.02
QLC	27 %		0.01	< 0.01
QBC	12 %		< 0.01	< -0.01
QKC	17 %		< -0.01	< -0.01
PF	331.6	Parham et al. (1997), Bossart et al. (2001), Weijs et al. (2010a) and supporting information	**4.18**	**-3.85**
PL	11.3		0.01	< -0.01
PB	6.3		< 0.01	< -0.01
PK	4.7		< -0.01	< 0.01
PR[1]	6.8		0.11	-0.10
TOTDIET	1.1 ng/g ww	Tanabe et al. (1997a)	**-5.30**	**5.31**
IN[3]	90 %	Hickie et al. (2005)	**-9.40**	**9.40**
DCMILK	540 g/day	Oftedal (1997)	0.68	-0.67
FATPERCMilk	29.94 %	Table S6; Present study	< 0.01	< 0.01
CMILK	127.6 ng/g ww		0.68	-0.67
CFoetusF	168.99 ng/g ww	Table S5; Present study	-0.37	0.38
CFoetus L	2.04 ng/g ww		< 0.01	< 0.01
CFoetusK	6.20 ng/g ww		< 0.01	< 0.01
CFoetusB	1.65 ng/g ww		< 0.01	< 0.01
CFoetusR	0.01 ng/g ww	Estimated[4]	< 0.01	< -0.01
CFoetusBlood	0.01 ng/g ww		< -0.01	< 0.01
X	$3.22e^{-7}$	Fitted	< 0.01	< -0.01
$t_{1/2}$	27.5 years	Verner et al. (2008)	-0.84	0.85

[1] – Parameters taken from muscle since muscles are the biggest part of the 'rest of the body'
[2] – QRC or fractional blood flow to 'rest of the body' was calculated as 100-QFC-QLC-QKC-QBC
[3] – IN has been used twice: for the 'normal' daily input and the 'additional' input during reproduction. A change in IN has thus a double impact on the model output.
[4] – Samples not available, 0.01 was used for modeling reasons

Table S5. Concentrations of PCB 153 and (sum PCBs), expressed in ng/g lipid weight (lw) in female harbour porpoises and 1 fetus from the Black Sea. All samples were from 1998.

Sample ID	Age[a]	Brain	Liver	Kidney	Blubber
U 48	8	28 (193)	149 (1037)	104 (734)	349 (2038)
U 49	9	40 (239)	NA	NA	840 (4193)
U 50 (*)	9	41 (239)	450 (2583)	223 (1319)	387 (2299)
U 51	3	170 (898)	NA	484 (2615)	1371 (7723)
U 56	5	52 (287)	281 (1597)	NA	1051 (4952)
U 58	3	105 (579)	764 (4358)	401 (2321)	1150 (6500)
U 60	1.5	233 (1280)	902 (5018)	838 (4561)	1439 (8517)
U 61	3	165 (874)	845 (4515)	549 (2900)	1124 (6167)
U 67	12	35 (236)	333 (2166)	105 (710)	471 (3036)
U 74	1.5	242 (1268)	1116 (5743)	594 (3280)	2138 (11279)
U 75	3.5	141 (782)	825 (4628)	557 (3070)	1169 (6691)
U 76	2.5	201 (1078)	848 (4600)	1772 (8994)	1574 (8538)
U 78 (#)	2	228 (1188)	1125 (6017)	1326 (6821)	2074 (10975)
U 80 (#)	3	136 (736)	698 (3903)	619 (3517)	1160 (6694)
U 81 (#)	8	41 (258)	232 (1486)	137 (887)	441 (2856)
U 90 (#)	9	41 (234)	556 (3387)	142 (870)	1945 (11460)
U 94 (#)	8	137 (666)	NA	454 (2286)	4021 (17495)
U 50 NN (*)	Fetus	106 (149)	87 (550)	25 (522)	197 (1291)

(*) mother-fetus pair
(#) no information available regarding the situation of death (stranded or by-caught)
NA – sample not available
[a] – Age in years

Table S6. Concentrations of PCB 153 and (sum of PCBs) (expressed in ng/g lw) and lipid percentages of milk samples of female harbour porpoises from the Black Sea in 1998 unless stated otherwise.

Sample ID	% Lipid	Concentration	Comments
U 31 Milk (°)	51.8	4075 (19310)	Female (7yr), 1997
U 39 Milk (#)	36.9	199 (1241)	Female (>1.5yr), 1997
U 40 Milk (°)	36.6	1683 (8094)	Female (>1.5yr), 1997
U 48 Milk	24.7	176 (1233)	From female U 48 (Table S1)
U 67 Milk	22.4	140 (966)	From female U 67 (Table S1)
U 89 Milk (#)	26.1	1119 (5873)	No information available
U 90 Milk (#)	36.9	301 (1797)	From female U 90 (Table S1)
U 94 Milk (#)	26.0	824 (4099)	From female U 94 (Table S1)

(°) Outlier; Excluded from further calculations of the average milk concentration
(#) no information available regarding the situation of death (stranded or by-caught)

Table S7. Concentrations of BDE 47, sum of PBDEs, sum of DDXs and sum of MeO-PBDEs, expressed in ng/g lipid weight (lw) in female harbour porpoises, 1 fetus and 8 milk samples. All samples were from 1998 from the Black Sea (unless stated otherwise in Table S6 for the milk samples).

Sample ID	Tissue	BDE 47	Sum PBDEs	Sum DDXs	Sum MeO-PBDEs
U 48	Brain	0.8	2.3	544	0.8
	Liver	5.7	11.4	3065	6.1
	Blubber	12.0	21.0	9836	9.3
	Kidney	4.1	7.3	2322	2.7
U 49	Brain	0.7	1.7	579	2.2
	Liver	NA	NA	NA	NA
	Blubber	17.3	31.2	13500	16.8
	Kidney	NA	NA	NA	NA
U 50 (*)	Brain	0.8	2.2	689	1.5
	Liver	16.1	27.6	8285	13.2
	Blubber	13.8	23.0	8285	10.4
	Kidney	7.3	13.1	4064	5.2
U 51	Brain	2.2	4.2	2639	1.8
	Liver	NA	NA	NA	NA
	Blubber	48.9	65.3	25352	26.8
	Kidney	9.0	14.5	8132	4.7
U 56	Brain	0.8	1.8	860	1.3
	Liver	6.9	13.4	4713	7.1
	Blubber	19.5	33.1	18548	15.8
	Kidney	NA	NA	NA	NA
U 58	Brain	1.5	2.6	1630	ND
	Liver	20.4	33.2	14558	11.1
	Blubber	41.7	57.3	24943	19.0
	Kidney	9.1	15.6	8276	4.5
U 60	Brain	3.8	7.5	3771	2.2
	Liver	22.7	36.7	16013	10.9
	Blubber	56.8	74.2	33754	24.8
	Kidney	17.4	29.0	14418	8.9
U 61	Brain	2.6	5.0	2874	2.6
	Liver	18.9	33.6	14503	11.7
	Blubber	36.3	50.0	28216	19.6
	Kidney	11.8	19.5	10658	6.9
U 67	Brain	0.8	2.4	675	1.3
	Liver	13.1	24.9	6679	13.4
	Blubber	18.2	30.5	11236	15.4
	Kidney	3.7	7.6	2087	3.8
U 74	Brain	2.8	5.7	4266	1.7
	Liver	20.9	33.5	21270	9.8
	Blubber	55.3	76.4	47772	23.7
	Kidney	10.7	17.7	11719	5.3
U 75	Brain	2.2	4.2	2432	2.0
	Liver	19.9	32.5	14502	13.3
	Blubber	41.2	56.3	25854	24.7
	Kidney	12.4	19.1	10142	8.0
U 76	Brain	3.3	6.5	3496	2.3
	Liver	19.5	31.1	15204	11.0
	Blubber	51.3	69.7	40290	24.6
	Kidney	37.0	60.7	34094	20.4
U 78 (#)	Brain	2.8	4.7	3850	1.7
	Liver	22.8	36.0	21784	11.8
	Blubber	53.1	74.1	37677	22.6
	Kidney	26.1	41.4	26800	13.6
U 80 (#)	Brain	2.4	4.4	2269	1.2
	Liver	18.6	29.8	12517	9.6
	Blubber	47.2	62.2	26032	20.8
	Kidney	14.7	30.0	11305	8.3
U 81 (#)	Brain	0.6	1.6	746	ND
	Liver	7.9	14.2	4345	7.5

	Blubber	16.7	27.2	10472	13.0
	Kidney	5.4	9.8	2793	4.7
U 90 (#)	Brain	0.6	1.4	704	0.2
	Liver	12.6	36.7	9559	16.0
	Blubber	77.1	101.1	28228	33.3
	Kidney	3.8	8.7	2371	3.9
U 94 (#)	Brain	1.0	2.8	2150	1.1
	Liver	NA	NA	NA	NA
	Blubber	54.1	83.3	43618	24.5
	Kidney	4.9	10.7	8027	4.3
U 50 NN (*)	Brain	1.0	2.0	654	4.1
	Liver	5.5	12.7	24560	14.9
	Blubber	14.1	21.8	8040	42.3
	Kidney	6.3	24.1	2630	15.6
U 31 (°)	Milk	99.4	163.5	101376	227.0
U 39 (#)	Milk	11.8	19.2	6476	47.4
U 40	Milk	32.7	55.2	42790	69.8
U 48	Milk	13.8	22.6	7626	44.4
U 67	Milk	10.9	18.2	5649	38.0
U 89 (#)	Milk	39.0	60.1	36256	93.1
U 90 (#)	Milk	16.6	26.3	10734	51.8
U 94 (#)	Milk	19.3	32.0	27940	51.8

ND – Compound not detected
NA – Sample not available
(°) Outlier; Excluded from further calculations of the average milk concentration
(#) no information available regarding the situation of death (stranded or by-caught)
(*) mother-fetus pair

Table S8. Reported lipid percentages of milk in the literature.

Lipid percentage	Species	Reference
4	Cow	Kierkegaard et al. (2007)
0.9 – 10.4	Human	Polder et al. (2008)
1.9 – 6.1	Human	She et al. (2007)
13.2 ± 4.1	Bottlenose dolphin	Yordy et al. (2010)
26.2 ± 7.5	Northern fur seal	Beckmen et al. (1999)
22.4 – 51.8	Harbour porpoise	Table S6, Present study
38.5 ± 6.4	Grey seal (early lactation)	Vanden Berghe et al. (2010)
56.7 ± 3.4	Grey seal (late lactation)	

Table S9. Levels of PCB 153 in fetus/mother pairs (in ng/g lw) from the literature.

	Age (yr)	Place and time	Brain	Liver	Kidney	Blubber	Reference
Fetus		Black Sea	106.4	86.6	25.4	197.0	Present study
Mother	9		40.8	449.6	223.2	387.0	
F/M		1998	2.61	0.19	0.11	0.51	
Fetus		UK				379.7	Law et al. (2006b)
Mother	6					549.5	
F/M		1997				0.69	
Fetus		UK				256.4	Law et al. (2006b)
Mother	Unknown					468.1	
F/M		1997				0.55	
Fetus		Black Sea				413.3	Tanabe et al. (1997a)
Mother	7					961.0	
F/M		1993				0.48	

4. Additional figures

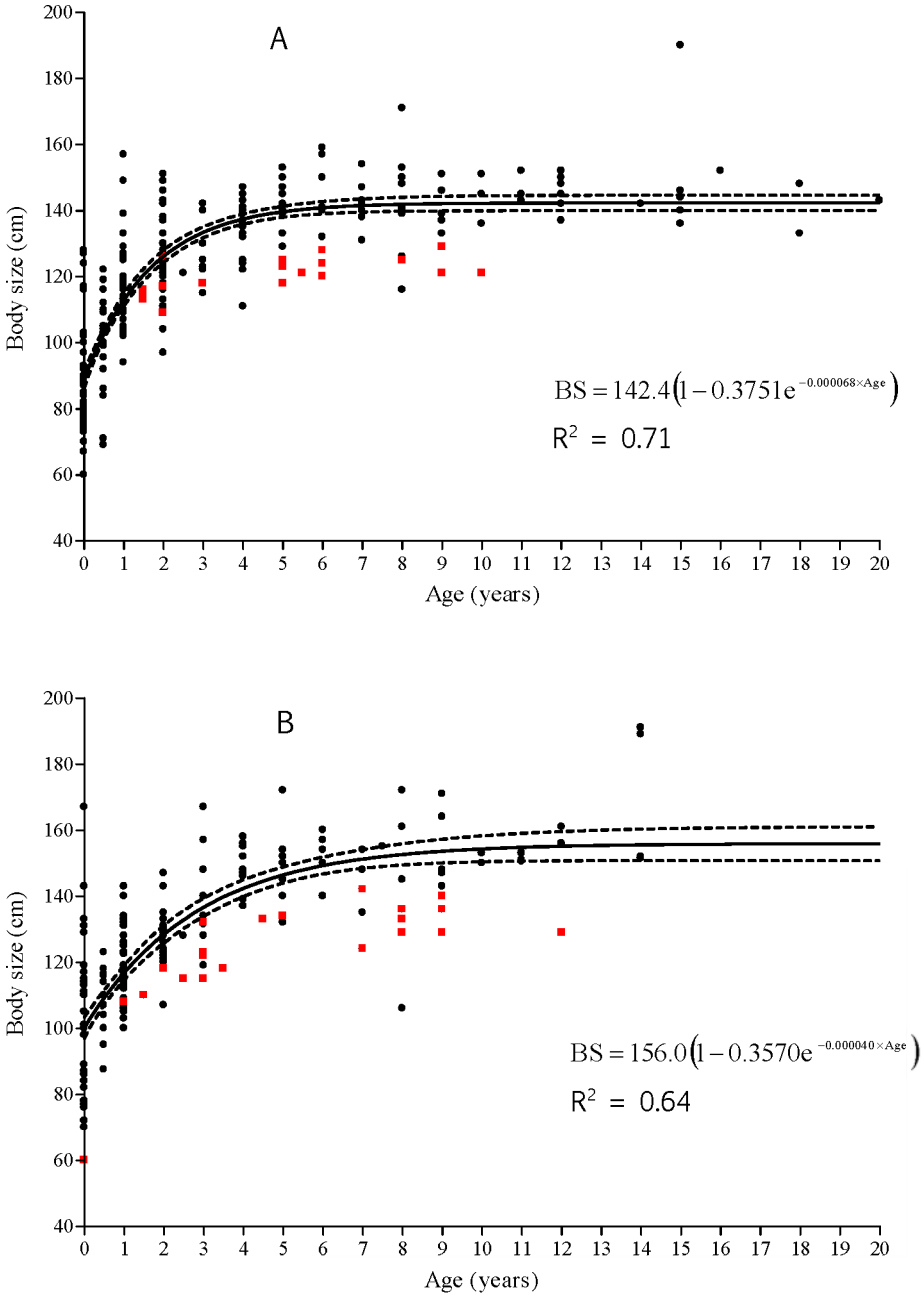

Fig S1. Von Bertalanffy age dependent growth-curves for male (A) and female (B) harbour porpoises. ● = Data for both curves were taken from Gaskin et al. (1983), Duinker et al. (1989), Kuiken et al. (1993), Szefer et al. (2002), Ciesielski et al. (2004), Strand et al. (2005), Law et al. (2006b) and Weijs et al. (2010a and unpublished data). ■ = Weijs et al. (2010a and unpublished data; data of animals from the Black Sea in 1998); — = Von Bertalanffy growth curve; ... = 95% confidence interval.

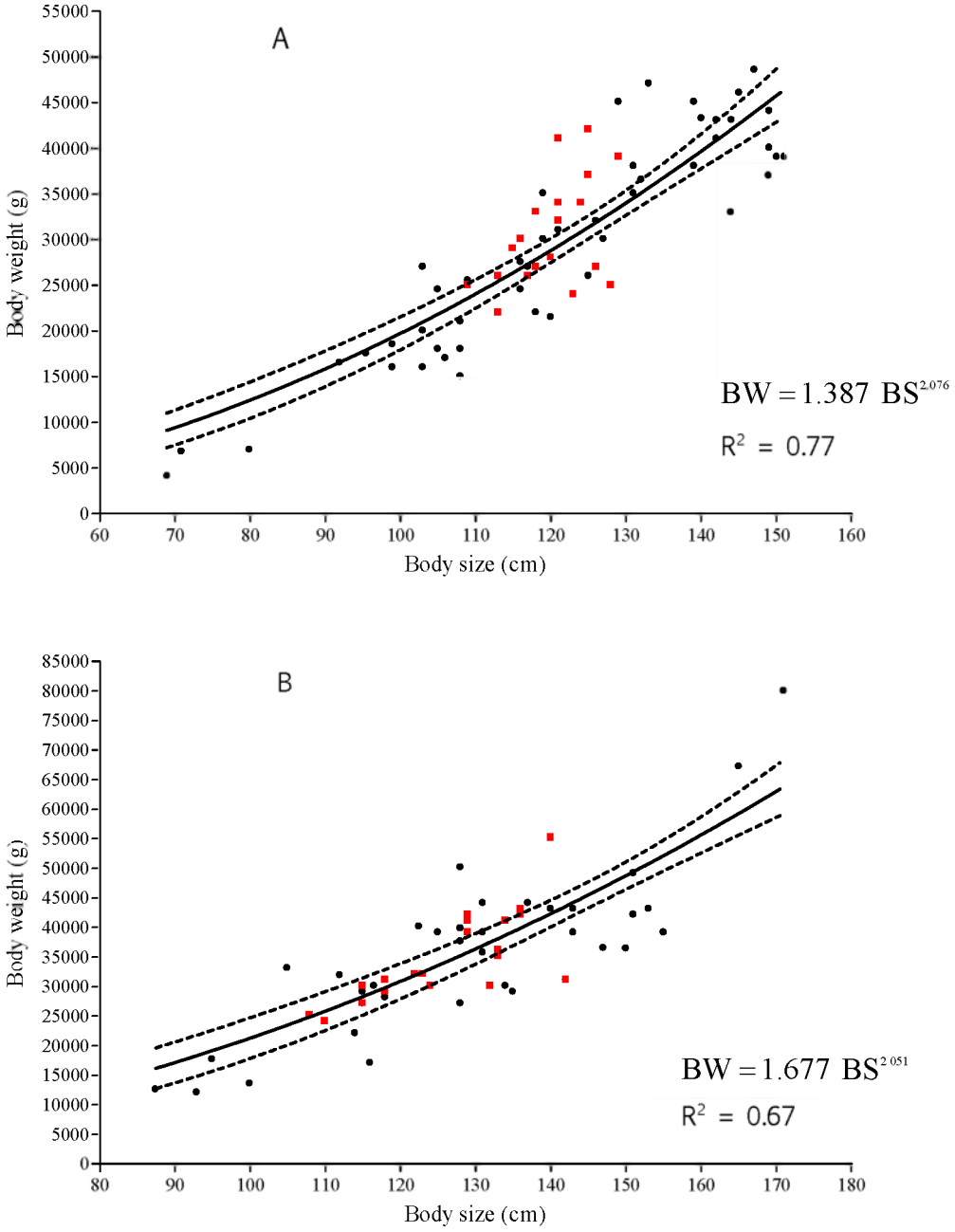

Fig S2. Correlation between body weight (BW) and body size (BS) in (A) male and (B) female harbour porpoises. ● = data from Duinker et al. (1989), Kannan et al. (1993), Strandberg et al. (1998), Covaci et al. (2002), Szefer et al. (2002), Ciesielski et al. (2004), Strand et al. (2005), ■ = data from Weijs et al. (2010a and unpublished data), — = allometric growth curve, ... = 95% confidence intervals.

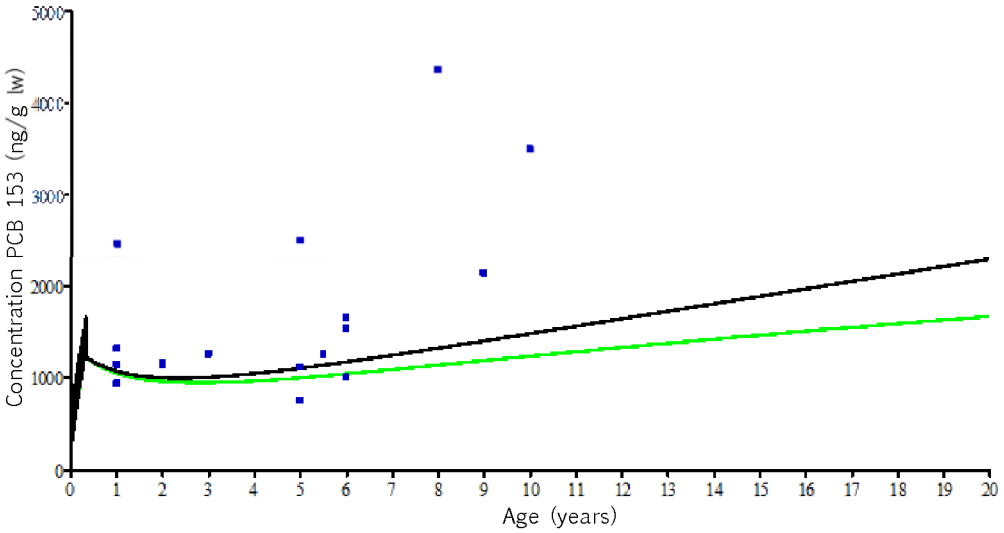

Fig S3. Role of metabolic breakdown in the bioaccumulation process of PCB 153 in the liver of male harbour porpoises. — = Model prediction without possible metabolism, — = Model prediction with a metabolism with PCB 153 half life of 27.5 years, ■ = individual data of male harbour porpoises from the Black Sea (dataset for model validation).

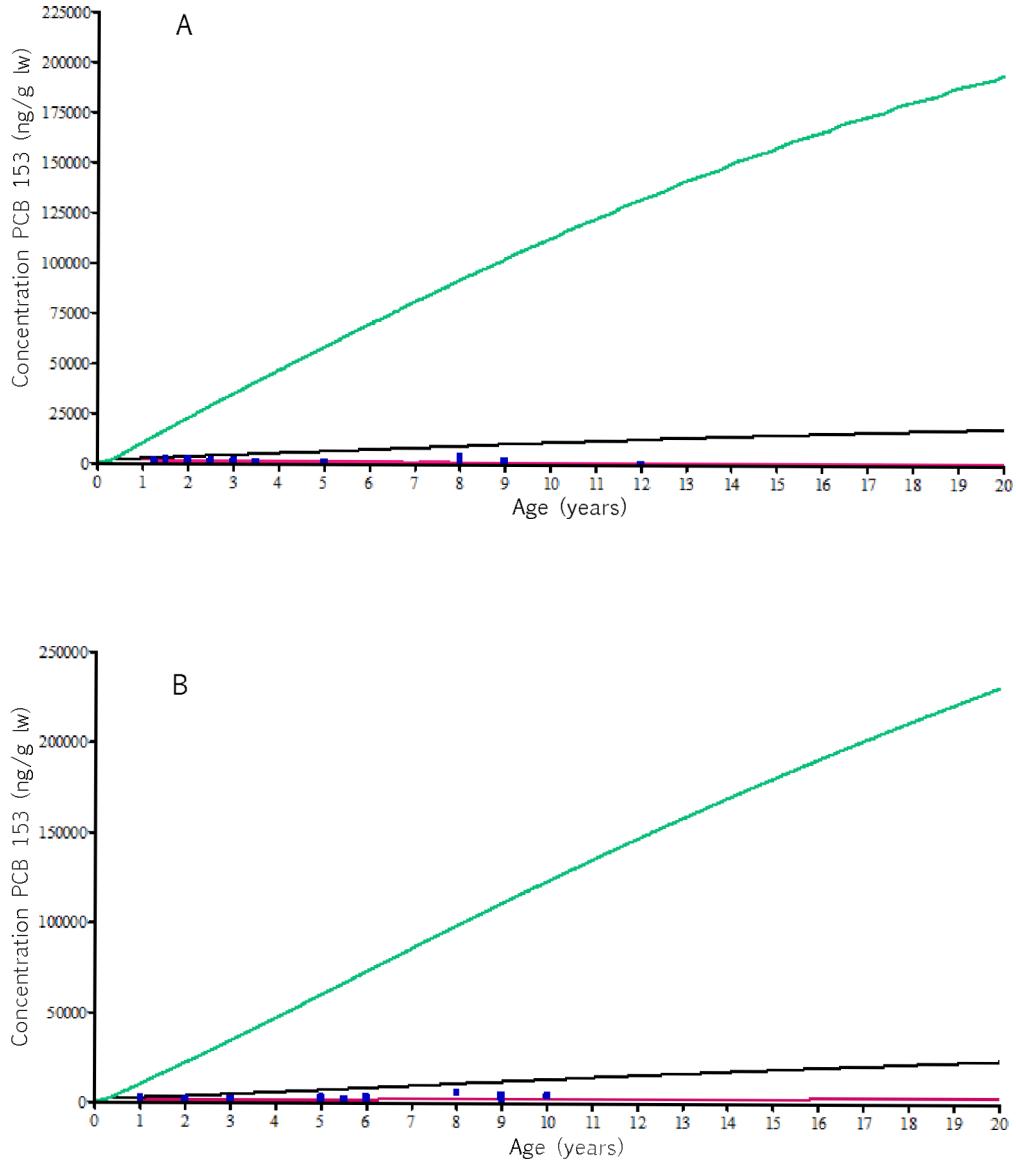

Fig S4. Influence of a higher PCB 153 concentration of the diet (fish) on the bioaccumulation of PCB 153 in blubber of female (A) and male (B) harbour porpoises. — = PCB 153 concentration in the diet of 1.1 ng/g wet weight (Tanabe et al., 1997a), — = 10x, — = 100x.

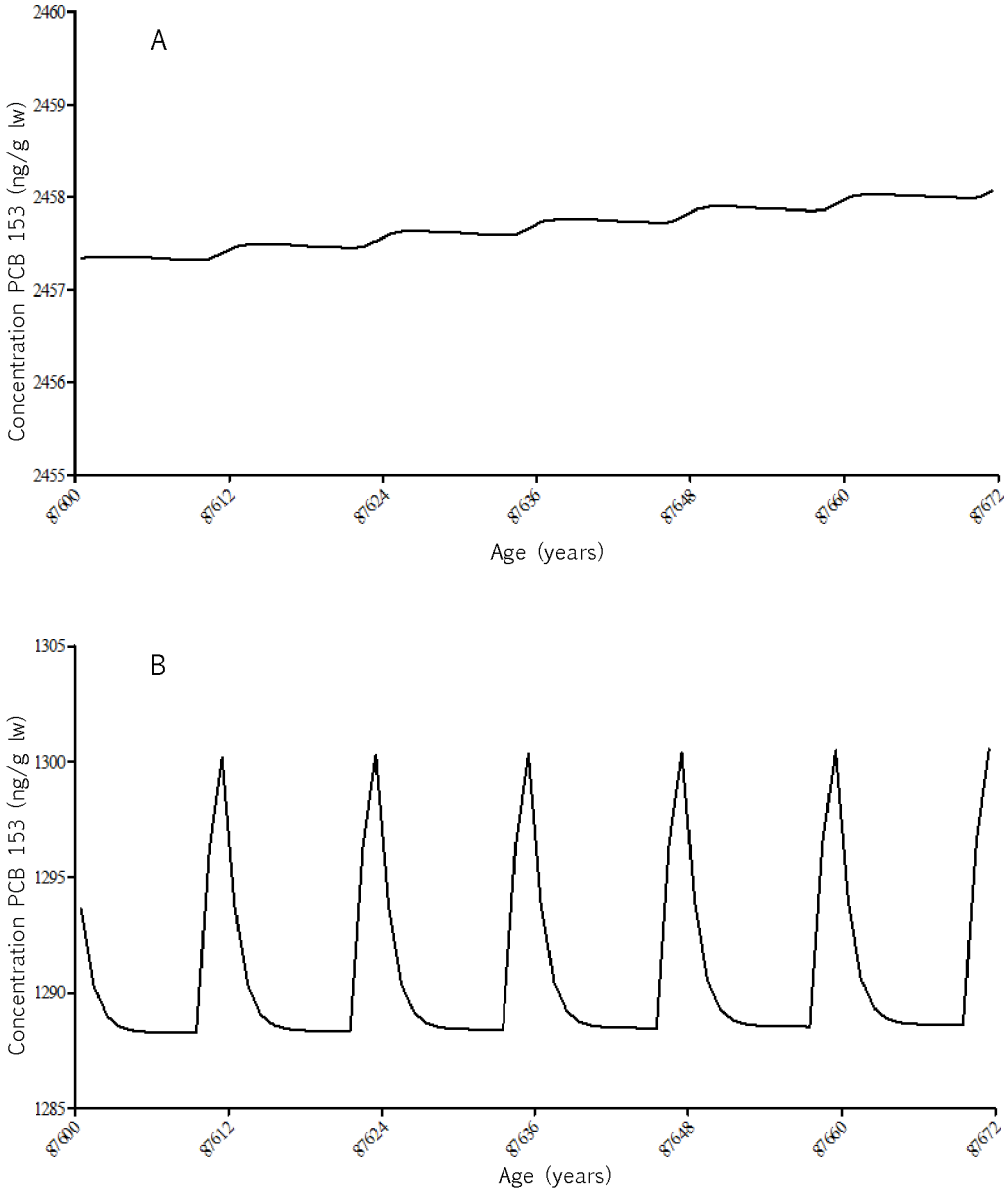

Fig S5. 72-hour detail at the age of 10 years (87600 hours) from the bioaccumulation model of PCB 153 in blubber (A) and liver (B) of male harbour porpoises.

5.2 Paper VIII

A non-invasive approach to study lifetime exposure and bioaccumulation of PCBs in protected marine mammals:

PBPK modeling in harbor porpoises

Liesbeth Weijs, Adrian Covaci, Raymond S.H. Yang, Krishna Das, Ronny Blust

Abstract

In the last decade, PBPK models have increasingly been developed to explain the kinetics of environmental pollutants in wildlife. For marine mammals specifically, these models provide a new, non-destructive tool that enables the integration of biomonitoring activities and *in vitro* studies. The goals of the present study were firstly to develop PBPK models for several environmental relevant PCB congeners in harbor porpoises, a species that is sensitive to pollution because of its limited metabolic capacity for pollutant transformation. These models were tested using tissue data of porpoises from the Black Sea. Secondly, the predictive power of the models was investigated for time trends in the PCB concentrations in North Sea harbor porpoises between 1990 and 2008. Thirdly, attempts were made to assess metabolic capacities of harbor porpoises for the investigated PCBs. In general, results show that parameter values from other species (rodents, humans) are not always suitable in marine mammal models, most probably due to differences in physiology and exposure. The PCB 149 levels decrease the fastest in male harbor porpoises from the North Sea in a time period of 18 years, whereas the PCB 101 levels decrease the slowest. According to the models, metabolic breakdown of PCB 118 is probably of lesser importance compared to other elimination pathways. For PCB 101 and 149 however, the presence of their metabolites can be attributed to bioaccumulation of metabolites from the prey and to metabolic breakdown of the parent compounds in the harbor porpoises.

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1. Introduction

The global awareness of the major impact of anthropogenically produced pollutants on the environment makes risk assessment in wildlife a topic of great interest. Assessing toxicity in organisms requires correlations between the concentration of a chemical in an organism and the response induced by that specific pollutant (Walker et al., 2006). Typically, this approach leads to some practical problems for marine mammals. Marine mammals are long-lived mammals that occupy the top positions in aquatic food webs around the world (Ross, 2000) and that can transfer considerable amounts of pollutants to their offspring through lactation because of their lipid rich milk (Debier et al., 2003b). They have limited capacities for eliminating pollutants and have experienced effects on several health endpoints (e.g. Mos et al., 2007; Reijnders, 1986; Ross et al., 1996). Because of this, marine mammals are sensitive to pollution and therefore relevant study organisms (Ross, 2000).

However, due to their protected status, *in vivo* toxicological research or exposure experiments in marine mammals are undesirable and prohibited. As a consequence, due to the more advanced techniques and procedures, risk assessment in marine mammals occurs more and more through *in vitro* studies (e.g. Dufresne et al., 2010; Li et al., 2003; McKinney et al., 2006a), probably the only ethical possibility to investigate the effects of pollution in these animals. However, such work focuses mainly on one target tissue or type of cells. It thus fails to provide an integrative picture and to understand the interactions between several tissues. Computer models or *in silico* studies may be able to provide a solution. The type of models used depends on the questions that need to be addressed or on the availability of data required to develop the models. PBPK models give information about the absorption, elimination and distribution of a pollutant in an organism by integrating physiology of the organism and biochemistry of the specific pollutant (Clewell and Clewell, 2008; Reddy et al., 2005). Traditionally, these models were used to describe the kinetics of chemicals in rodents (Corley et al., 1990; Reitz et al., 1988). Recently, the bioaccumulation of environmental pollutants, such as polychlorinated biphenyls (PCBs) in wildlife and humans has received increasing attention (Hickie et al., 1999; Maruyama and Aoki, 2006; Redding et al., 2008; Sonne et al., 2009; Verner et al., 2008; Weijs et al., 2010b).

Harbor porpoises are small cetaceans living in the Northern Hemisphere. They are apex predators, have long life spans and are assumed to have limitedmetabolic capacities for the breakdown of pollutants compared to other marinemammals, such as harbor seals (Weijs et al., 2009a, 2009b). During the last decade, populations of harbor porpoises in northern Europe were moving more south, probably following the fish migrations, towards the relatively smaller and more land locked North Sea (SCANS II, 2006), thereby exposing themselves to higher levels of pollutants that are present in that area due to run-off of the highly industrialized surrounding countries. Together with their potential limited metabolic capacities, there is a clear need for information regarding the kinetics and effects of pollutants in their bodies to ensure proper protection and viable populations worldwide.

Recently, models were developed for the lifetime distribution and kinetics of PCB 153 in harbor porpoises which is the most persistent PCB in marine mammals (Weijs et al., 2010b). However, PCB 153 is not the only threat for these animals as it is only one congener in the PCB mixtures (Aroclor) which were commercially available and widely used before their ban in the 1970s. The goals of the present study were therefore, 1) to develop PBPK models

for PCBs other than PCB 153 in harbor porpoises, 2) to investigate the temporal trends of PCBs using these models, 3) to gather more information about the metabolic breakdown of some PCBs. The PCBs other than PCB 153 were selected according to the chlorine substitution pattern on the ortho, meta and para positions (Wolkers et al., 1998) implying that they are all metabolized by different subsystems of the cytochrome P450 enzyme complex. Congeners PCB 180 (group I), PCB 101 (group II), PCB 118 (group IIIa) and PCB 170 and PCB 99 (group IIIb) were chosen because of their persistence in marine mammals. A model for PCB 149 (group II) was developed as well because of its typically higher concentrations in cetaceans compared to pinnipeds (Boon et al., 1997).

2. Materials and methods

PBPK models were constructed for six PCB congeners and were based on our earlier published model for PCB 153 in male harbor porpoises (Weijs et al., 2010b). Accordingly, all models consist of 5 compartments: liver, blubber, kidneys, brain and rest of the body (Fig. 1), all connected through blood. For the 'rest of the body'-compartment, parameters and data of muscle tissue of harbor porpoises were used. All tissues were considered to be flow-limited similar as in Weijs et al. (2010b) and for humans in Redding et al. (2008). Exposure was assumed to be through fish and milk consumption only. Dermal uptake was neglected because lipophilic compounds do not dissolve readily in sea water. Moreover, Hickie et al. (1999) found that dermal exposure only played a negligible role for the bioaccumulation of PCBs in beluga whales. The uptake of PCBs through the fish or milk diet was set to the liver as this compartment was the only tissue of the gastrointestinal tract represented in the models. All models were coded using Berkeley Madonna (version 8.3.14) and are available on request to the corresponding author.

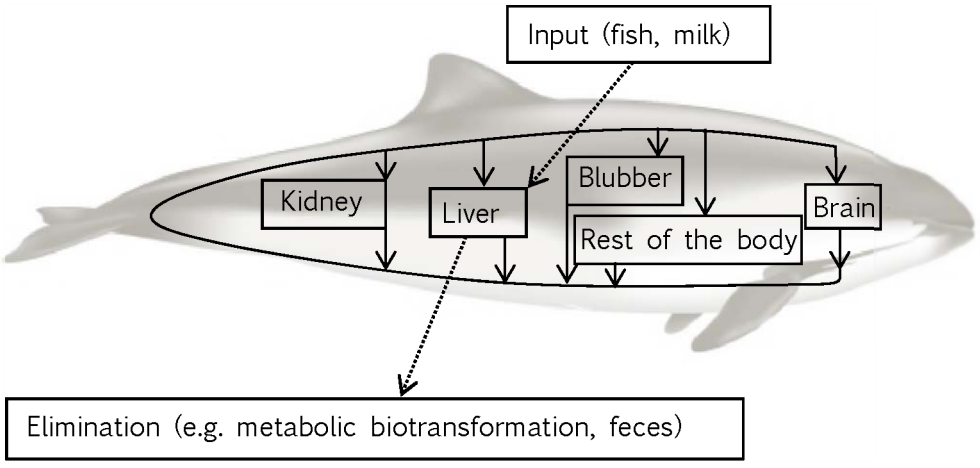


Fig 1. Conceptual representation of the PBPK models for selected PCBs in male harbor porpoises.

2.1. Parameters.

All models were developed using parameters from the literature or obtained through fitting to the data if clearly indicated. The same physiological parameters and equations of the male harbor porpoises as in Weijs et al. (2010b) were used. Biochemical parameters were adjusted according to the specific PCB (Table 1). Blood/tissue partition coefficients were calculated using the approach from Parham et al. (1997) and the average lipid percentage of the tissues which can be found in Weijs et al. (2010a).

However, blood/brain partition coefficients (PB) calculated with this approach overestimated the levels of the PCBs in the brain of the Black Sea harbor porpoises and were therefore fitted to the data of the brain. Both calculated and predicted PBs can be found in Table 1. Other parameters that were fitted (elimination half-lives and assimilation efficiency for the milk or AE2; see Table 1) were chosen upon visual inspection of the position and shape of the curves compared to the real-life data from animals from the Black Sea. In general, the following order in the fitting process was followed: Elimination half-lives were fitted first as this parameter affects the slope of the curve in each compartment, AE2 was fitted after that as it determined only the concentrations in all compartments for animals b1 year and PBs only had an impact on the curve of the brain compartment and were therefore fitted last. Overall, fitting or estimating several parameter values through modeling generally reduces the reliability of the model outcome as these parameters are more mathematically justified than biologically inspired. Therefore, this procedure was kept to a minimum and only employed in cases where there were no parameters available in the literature or where the parameters (the models) did not match the real-life data from the harbor porpoises.

Table 1. Compound specific parameters for several PCBs. PCB congeners were selected based on the groups from Wolkers et al. (1998). The original values of the parameters are given between brackets for parameters that were fitted to the data.

	PCB 180	PCB 101	PCB 149	PCB 118	PCB 99	PCB 170
Group[a]	I	II	II	IIIa	IIIb	IIIb
log (K_{fp})[b]	2.41682	1.91643	1.85712	2.23748	2.41682	2.41682
PF[c]	380.2	101.4	88.5	251.6	380.2	380.2
PL[c]	9.0	2.4	2.1	6.0	9.0	9.0
PK[c]	5.3	1.4	1.2	3.5	5.3	5.3
PB[c]	6.3 (15.2)	1.3 (4.0)	1.4 (3.5)	3.5 (10.0)	6.3 (15.2)	6.1 (15.2)
PR[c]	9.2	2.5	2.2	6.1	9.2	9.2
AE 1 (%)[d]	91	98	90	99	90	90
AE 2 (%)[e]	54	66	54	70	55	46
CFoetusF[f]	53.6	87.1	99.3	113.1	61.0	18.0
CFoetusL[f]	33.0	37.4	49.4	43.8	26.8	10.4
CFoetusK[f]	32.9	48.6	58.2	52.1	36.0	ND
CFoetusB[f]	8.5	11.7	13.6	13.7	8.2	ND
Half life[g]	521 (9.9)	6.1 (5.7)	80.00 (5.7)	9.6	334 (5.7)	21.0 (3.9)

K_{fp} – adipose tissue/plasma partition coefficient, PF – adipose tissue/blood partition coefficient, PL – liver/blood partition coefficient, PK – kidney/blood partition coefficient, PB – brain/blood partition coefficient, PR – muscle/blood partition coefficient, AE – assimilation efficiency

[a] – Groups based on the chlorine substitution pattern on the *ortho*, *meta* and *para* positions according to Wolkers et al. (1998).

[b] – Adipose tissue to plasma partition coefficients from Parham et al. (1997).

[c] – Equations from Parham et al. (1997) were transformed to equations for bottlenose dolphins (blood composition from Bossart et al. (2001)). For partition coefficients of tissues (liver, kidneys, brain) as given in Table 1, the average lipid content was used (Weijs et al., 2010a). For the 'rest of the body'-compartment, the average lipid content of muscle was used (Weijs et al., 2010a).

[d] – Assimilation efficiency for the fish diet or the percentage of PCB absorbed by the juveniles and adults after ingestion of the fish prey. Values taken from Thomas et al. (2005), average net absorption for all congeners measured was > 89%, so for PCB 99, PCB 149 and PCB 170, an assimilation efficiency of 90% was assumed.

[e] – Assimilation efficiency for the milk diet or the percentage of PCB absorbed by the calves after milk ingestion. Values were fitted to the Black Sea dataset.

[f] – Results from own analyses (Weijs, unpublished data) and expressed in ng/g lipid weight (lw). For modeling purposes, values of 0.01 ng/g lw were used for concentrations below limit of detection (ND). Muscle tissue of the fetus was not available, so a value of 0.01 ng/g lw was used in the models as well.
[g] – Values between brackets are the original elimination half-lives used in the first modeling attempt. Other values are fitted to the dataset for validation and are used in all other models (models for goals 2 and 3) of this study.

2.2. Datasets. Physiological parameters were kept rather general so that the models would be species-specific instead of population-specific. In that way, it is assumed that the models can be used for all male harbor porpoises. The models include also five compartments which allows to use other data than only PCB levels in blubber. All this is reflected here as there are several datasets used for the different applications:

Black Sea. This dataset was used to parameterize the PCB models (goal 1), similar to the PCB 153 model in male harbor porpoises (Weijs et al., 2010b). All animals (9 juveniles, 11 adults) were bycaught (n=17) or found stranded (n=3) in 1998 in the Black Sea. Carcasses were in good condition or only moderately composed and none of the animals was severely emaciated. Levels in blubber, liver, kidney, brain andmuscle are discussed in Weijs et al. (2010a). Levels of one neonate/fetus (Table 1) were used as well together with the levels of PCBs in milk of Black Sea harbor porpoises (Table S1).

North Sea. This dataset consists of blubber and liver PCB concentrations. The blubber data were used to investigate the predictability of the PBPK model for the PCBs in time (goal 2), the liver data were used to better understand the importance of metabolic breakdown of some PCB parent compounds (goal 3). One part of the animals included in this dataset were found stranded or were by-caught on the Belgian coast of the North Sea in 1999–2004. The PCB results in blubber (n=20) were used for goal 2 and can be found in Weijs et al. (2009a and b). The PCB, methylsulfone-PCB ($MeSO_2$-PCB) and hydroxylated PCB metabolites (HO-PCB) levels in liver (n=10) were used for goal 3 and were discussed in Covaci et al. (2002) (PCBs), in Chu et al. (2003) ($MeSO_2$-PCBs) and were from Weijs (unpublished data) (HO-PCBs) (Table 2). The other part of the animals included in this dataset (n=26) were found alive on the coasts of Belgium and The Netherlands, but died during rehabilitation in SOS Dolfijn, Harderwijk, The Netherlands in 1990–2006. Levels (sum of PCBs) can be found in Weijs et al. (2010c). Data of PCBs in blubber were used for goal 2 whereas data of PCBs in liver were not used for goal 3 since PCB metabolites were not targeted.

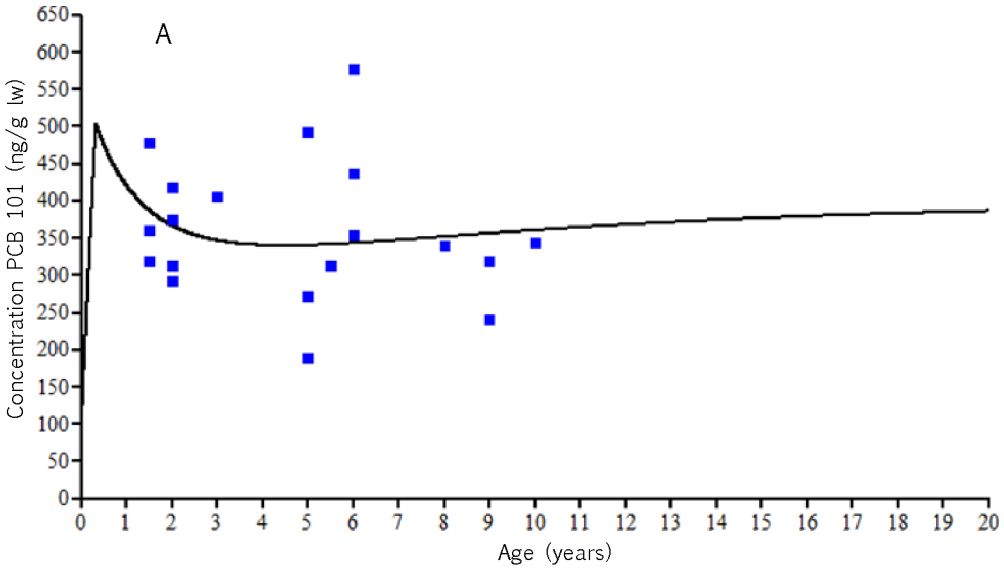
3. Results

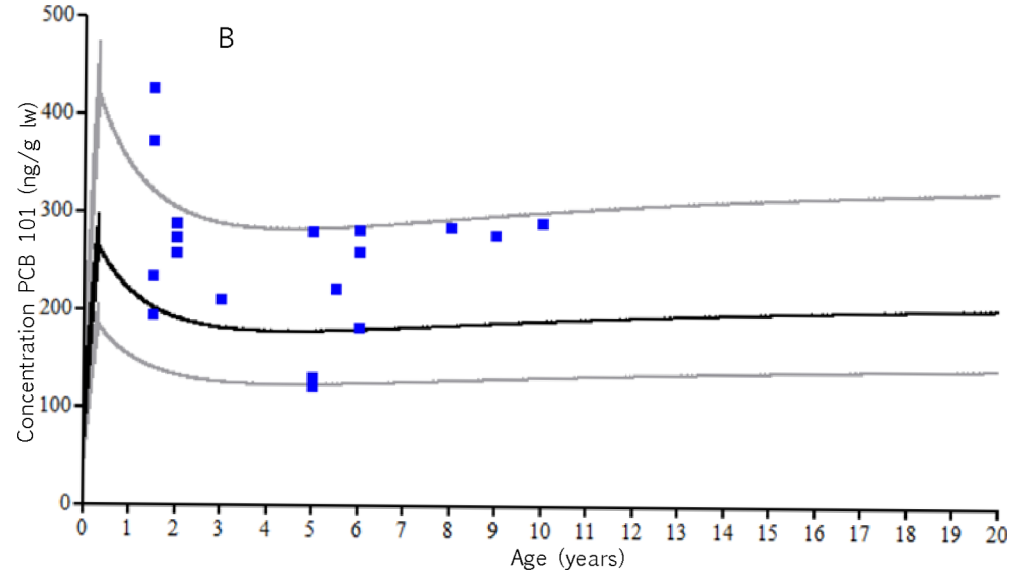
Of the 6 PCBs selected, only PCBs 101, 118 and 149 were involved in all goals. Of these three PCBs, PCB 101 was randomly selected to be shown in the manuscript (Figs. 2, 4) whereas figures of all other PCBs can be found in Supporting Information (Fig S1–S7). PCBs 180, 99 and 170 could not be used for goal 3 because metabolites of those congeners were not targeted (for PCB 99) or were only detected in low concentrations in 1 out of 10 samples (for PCBs 180 and 170) (Table 2).

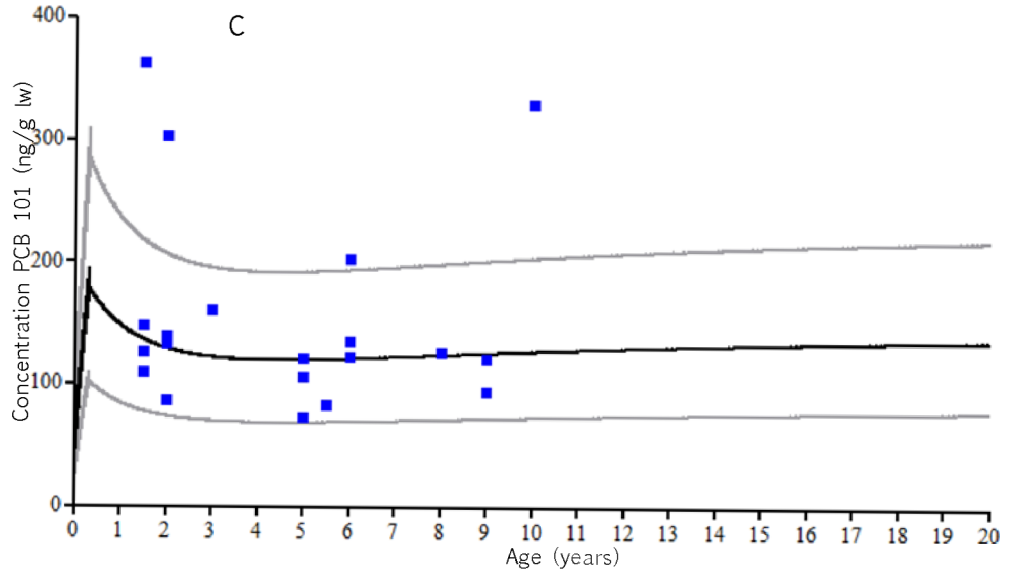
3.1. Goal 1: Models for PCBs other than PCB 153

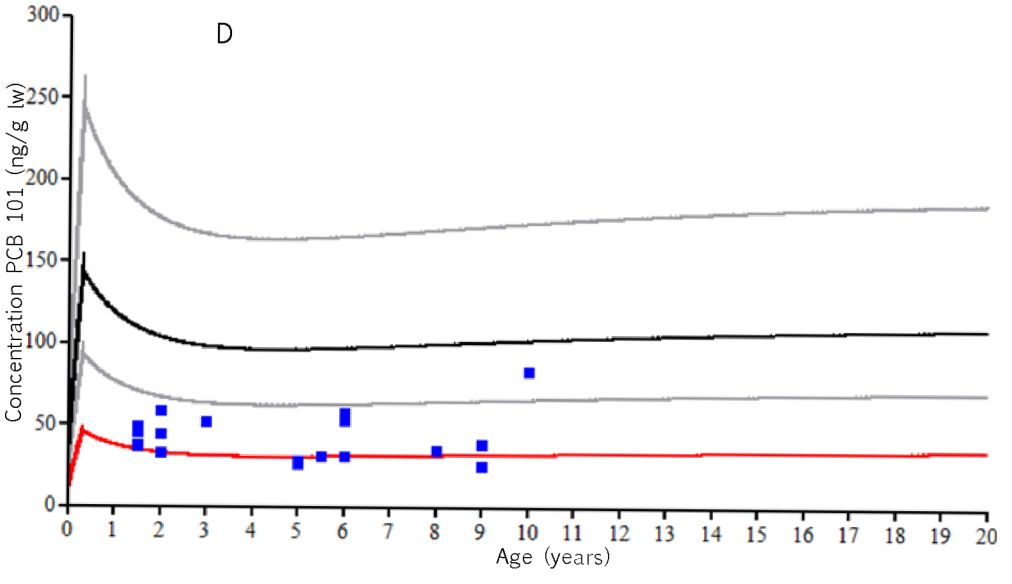
Elimination half-lives, in the present study defined as the time at which 50% of the chemical is eliminated (e.g. by metabolic transformation, fecal

excretion) from the body, are important parameters. For most PCBs, the literature provides several half-lives dependent on the investigated species (e.g. humans, rodents) and circumstances (e.g. occupational exposure, long term exposure). Initially, all models were run with the longest elimination half-lives available in the literature (see elimination half-lives between brackets in Table 1). However, because the curves did not reflect the Black Sea dataset for 5 out of 6 PCBs, new elimination half-lives (Table 1) were estimated by fitting themodels to the Black Sea data (i.e. model parameterization). Models for all 6 PCB congeners had two things in common: 1) the blood/brain partition coefficients (PB), originally calculated according to Parham et al. (1997), were not consistent with the results of the Black Sea dataset and were therefore fitted to the brain data, 2) an additional assimilation coefficient for the milk diet (AE_2) was added to the models of all PCB congeners because initial modeling attempts suggested that the models overestimated the validation dataset from the Black Sea for the youngest animals when using the same assimilation efficiency as the fish diet (Table 1). Tanabe et al. (1997) reported concentrations of PCB 118, 170 and 180, but not of PCB 101, 99 and 149, in fish prey of harbor porpoises from the Black Sea from 1993. Therefore, levels of PCB 101, 99 and 149 in the fish diet were estimated using the average concentration of the respective congener in the milk diet (Table S1). In the PBPK model for PCB 153 bioaccumulation in harbor porpoises, there was a 116 times difference between the fish diet (1.1 ng/g ww; Tanabe et al., 1997a) and milk diet (127.6 ng/g ww; Weijs et al., 2010b). The factor 116 has been used here as an estimate leading to a fish diet of 0.3 ng/g ww for PCB 101, of 0.5 ng/g ww for PCB 99 and of 0.7 ng/g ww for PCB 149.




B
Concentration PCB 101 (ng/g lw)
Age (years)


C
Concentration PCB 101 (ng/g lw)
Age (years)


D
Concentration PCB 101 (ng/g lw)
Age (years)

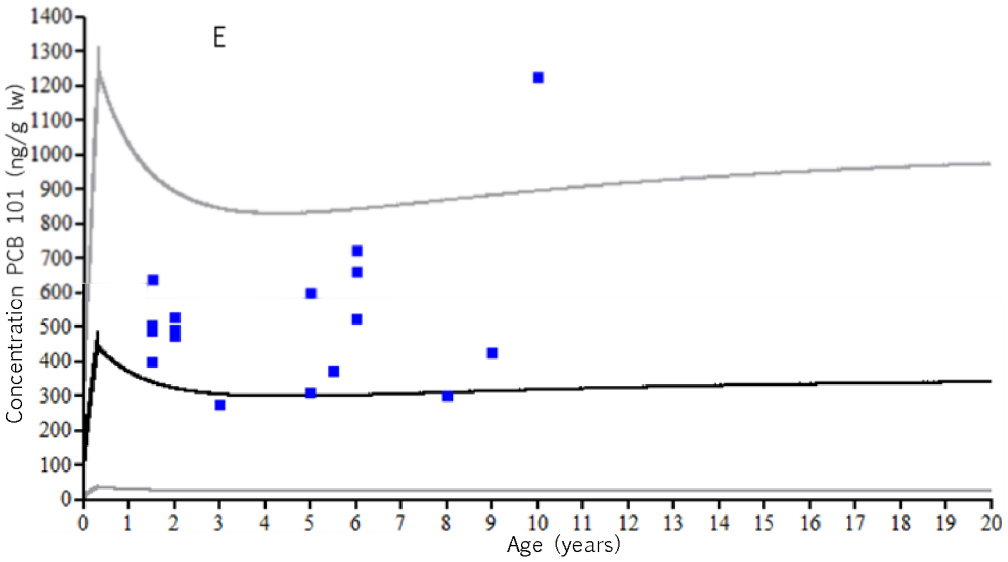


Fig 2. Age-dependent bioaccumulation of PCB 101 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.

PCB 101

Levels of PCB 101 in all compartments increase little with age. In each tissue, the highest concentrations can be found for animals younger than 1 year, especially the animals that are drinking milk or animals that have just switched from a milk diet to a fish diet. The elimination half-life, PB and AE_2 that fitted best to the dataset of the harbor porpoises from the Black Sea are given in Table 1 and model results can be found in Fig. 2 (A–E).

PCB 149

Because metabolic half-lives for PCB 149 are scarce in the literature, even for typical model species such as rodents, the first modeling exercise used a metabolic half life of 5.7 years, similar as the metabolic half life of PCB 101 (also group II; Wolkers et al., 1998) which resulted in model predictions that underestimated the Black Sea dataset by far. Therefore, the Black Sea data were used to find a better fit of the curves, resulting in an estimated elimination half-life value of about 80 years (Table 1; Fig S1 A–E).

PCB 99

The longest elimination half-lives for PCB 99 in the literature are not compatible with the Black Sea dataset for validation, so the software was asked again to find an elimination half-life so that the curves would fit to the Black Sea dataset, resulting in an estimated elimination half-life of 334 years (Table 1; Fig S2 A–E).

PCB 118

In contrast with all other PCBs in the present study, the Black Sea data are more scattered for PCB 118 than for all other PCBs considered so far. Nevertheless, the model, developed with the parameters from Table 1 and a concentration of PCB 118 in the fish diet as measured by Tanabe et al.

(1997a), is a fairly good reflection of the real life data from the Black Sea (Fig S3 A–E).

PCB 170

The curves were again underestimating the Black Sea data by far using the elimination half-life from the literature. In contrast to PCB 99, levels of PCB 170 were measured in fish prey of harbor porpoises from the Black Sea at a concentration of 0.18 ng/g ww (Tanabe et al., 1997a). The only parameter that can be adjusted was thus the elimination half-life. This was again fitted to the Black Sea data by the model giving a value of 21 years (Table 1; Fig S4 A–E).

PCB 180

The concentration of PCB 180 in the fish diet from Tanabe et al. (1997a) and an elimination half-life of 9.9 years, gives slightly increasing curves with age for all compartments, whereas they should be increasing much more according to the Black Sea data. As for PCB 99, the model was used to find a value for the elimination half-life so that the curves would fit better to the Black Sea dataset. Similar to PCB 99, the resulting elimination half-life was very high, namely 521 years (Fig S5 A–E).

3.2. Goal 2: Assessing temporal trends for PCBs

Using the blubber data from the North Sea dataset, temporal trends were investigated. The entire dataset (n=46) covers data from 1990 until 2008. The models used are exactly the same as developed and parameterized in goal 1, except for the input parameters. These input parameters, namely the concentration of the specific PCB in the fish and milk diet, were found by Reverse Dosimetry Modeling meaning that they were adjusted in order to find curves that would fit to the North Sea data (Redding et al., 2008). The ratio of the concentration in milk to the concentration in fish was kept the same as in the models that were parameterized using Black Sea data (so 116 times difference for PCB 101, 99 and 149; 84 times difference for PCB 118, 107 times difference for PCB 170 and 203 times difference for PCB 180; see Goal 1). Overall, although the data of each year were added to the models separately, results revealed that they could easily be divided into 2 groups, from 1990 until 2000 and from 2001 until 2008. Within each group, the levels of the PCBs increased insignificantly in time. Between the two groups, there was a difference between the groups with lower concentrations in the second group (2001–2008) compared to the first group (1990–2001). The difference in concentrations in the diet ranged from a factor 1.9 to 3.5 with 1.9, 2.5, 2.8, 2.8, 3.3 and 3.5 for PCB 101, PCB 99, PCB 118, PCB 180, PCB 170 and PCB 149, respectively (Fig. 3 A–F).

3.3. Goal 3: Metabolism and elimination of PCBs

Attempts were made to link the concentrations of PCB metabolites to the models of the parent PCB congeners. This was done using data of parent PCBs (Covaci et al., 2002), $MeSO_2$-PCBs (Chu et al., 2003) and HO-PCBs (Weijs, unpublished data) in liver of harbor porpoises from the North Sea (n=10). This method was applied for PCB 101, 118 and 149, but not for PCB 99, 170 and 180 because the metabolites were not targeted or were present in low concentrations in only 1 out of 10 samples (Table 2). Theoretically, under the assumption that the diet or prey (and not seawater or air) is the only source of PCBs and/or metabolites for marine mammals, there are

three different possible situations (Letcher et al., 1998): 1) the precursor PCB is present in the prey, the metabolites are not, 2) the metabolites are present in the prey, the precursor PCB is not, and 3) the precursor PCB and its metabolites are present in the prey. PCB 118, 101 and 149 were all present in the prey (Table 2), making the second situation impossible. PCB 118 belongs to the first situation as potential HO-metabolites of PCB 118 were targeted, but not detected in the prey (Table 2; Weijs, unpublished data). The presence of HO-metabolites of PCB 118 is thus solely due to PCB metabolism in the harbor porpoises. PCB 101 and 149 as well as their $MeSO_2$-metabolites were found in the prey, so both PCB 101 and 149 can be assigned to the third situation.

Table 2. Hydroxylated (HO) and methylsulfon ($MeSO_2$)-metabolites of the parent PCB compounds in liver of harbour porpoises from the North Sea (n = 10; expressed in ng/g lw). Parent PCB compounds are discussed in Covaci et al. (2002), $MeSO_2$-PCBs in Chu et al. (2003), HO-PCBs from Weijs, unpublished data.

	PCB 180	PCB 101	PCB 149	PCB 118	PCB 99	PCB 170
Harbour porpoises						
HO-PCB	ND-3.8[a]	NT	NT	ND-5.9[b]	NT	ND-0.3[a]
$MeSO_2$-PCB	NT	21.7-1176.1	7.6-618.9	NT	NT	NT
Fish						
HO-PCB[c]	ND	NT	NT	ND	NT	ND
$MeSO_2$-PCB[d]	NT	0.9-1.4	1.0-2.7	NT	NT	NT

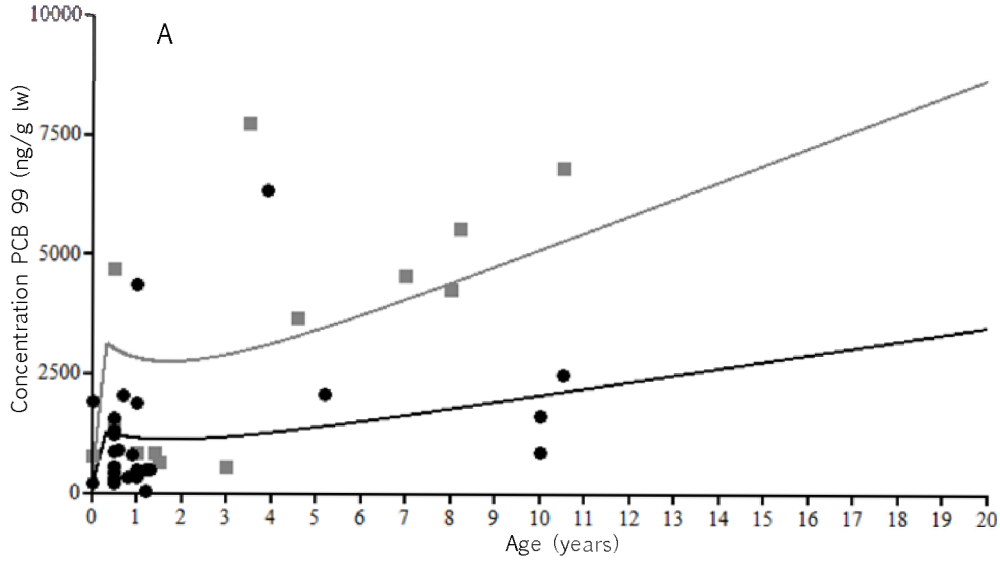
ND – Not Detected; NT – Not Targeted
HO-PCBs for PCB 180 is the sum of 3-HO-PCB 180 and 4-HO-PCB 172; HO-PCBs for PCB 118 is the sum of 4-HO-PCB 120 and 3-HO-PCB 118; HO-PCBs for PCB 170 is only 4-HO-PCB 172
$MeSO_2$-PCBs for PCB 101 is the sum of 3-$MeSO_2$-PCB 101 and 4-$MeSO_2$-PCB 101; $MeSO_2$-PCBs for PCB 149 is the sum of 3-$MeSO_2$-PCB 149 and 4-$MeSO_2$-PCB 149
[a] – ND in 9 out of 10 samples
[b] – ND in 5 out of 10 samples
[c] – Investigated in flounder (*Platichthys flesus*), cod (*Gadus morhua*), dab (*Limanda limanda*), whiting (*Merlangius merlangus*) caught in 2008 in the North Sea (Weijs, unpublished data)
[d] – Investigated in plaice (*Pleuronectes platessa*), sole (*Solea solea*), pout (*Trisopterus luscus*) and whiting (*Merlangius merlangus*) caught in 2001 in the North Sea (Voorspoels et al., 2003)

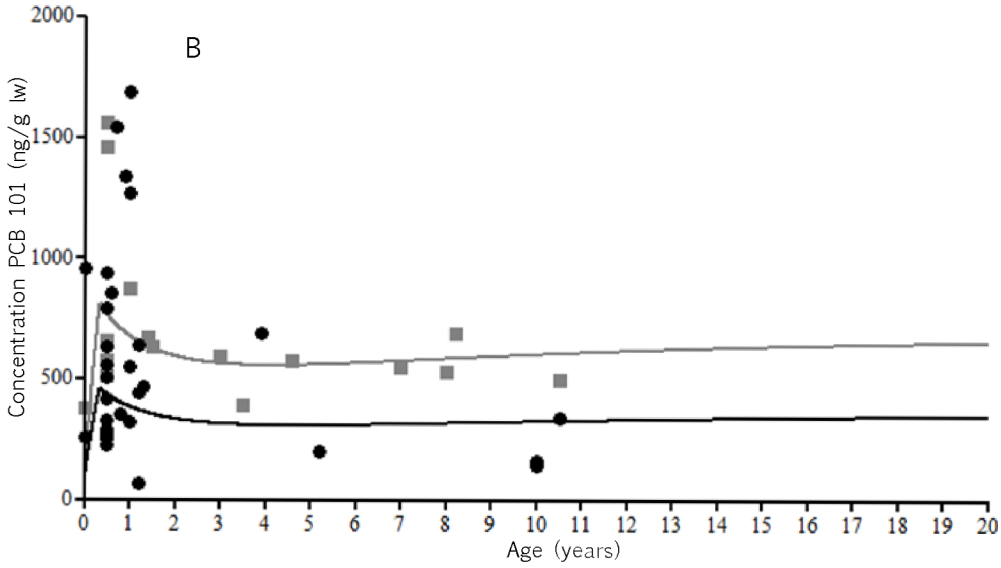
PCB 118

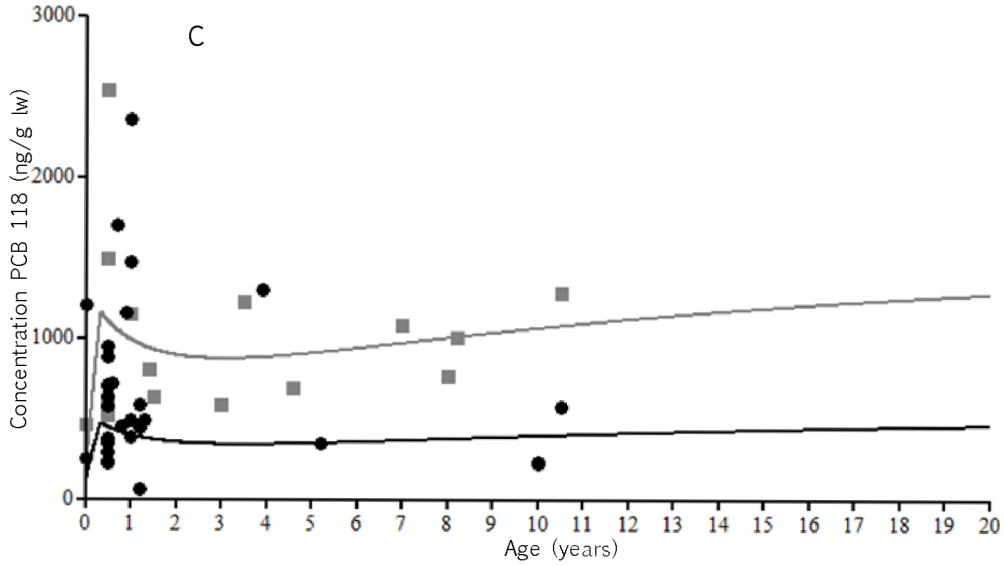
The difference between themodelwithout elimination (green curve; Fig S6) and the model with elimination (characterized by an elimination half-life of 9.6 years) (Table 1; black curve; Fig S6) gives the concentration of PCB 118 eliminated from the body by metabolic breakdown or fecal excretion (gray curve; Fig S6). The levels of potential HO-metabolites of PCB 118 are only a minor fraction of this, indicating that metabolic breakdown is of lesser importance compared to fecal excretion of PCB 118.

PCB 149

There is an increase in concentrations of metabolites of PCB 149, similar as predicted by the model (gray curve; Fig S7). However, the origin of the metabolites in this study, either from the prey through bioaccumulation or from metabolic breakdown in harbor porpoises, remains unknown.


A
Concentration PCB 99 (ng/g lw)
10000
7500
5000
2500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)


B
Concentration PCB 101 (ng/g lw)
2000
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)


C
Concentration PCB 118 (ng/g lw)
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

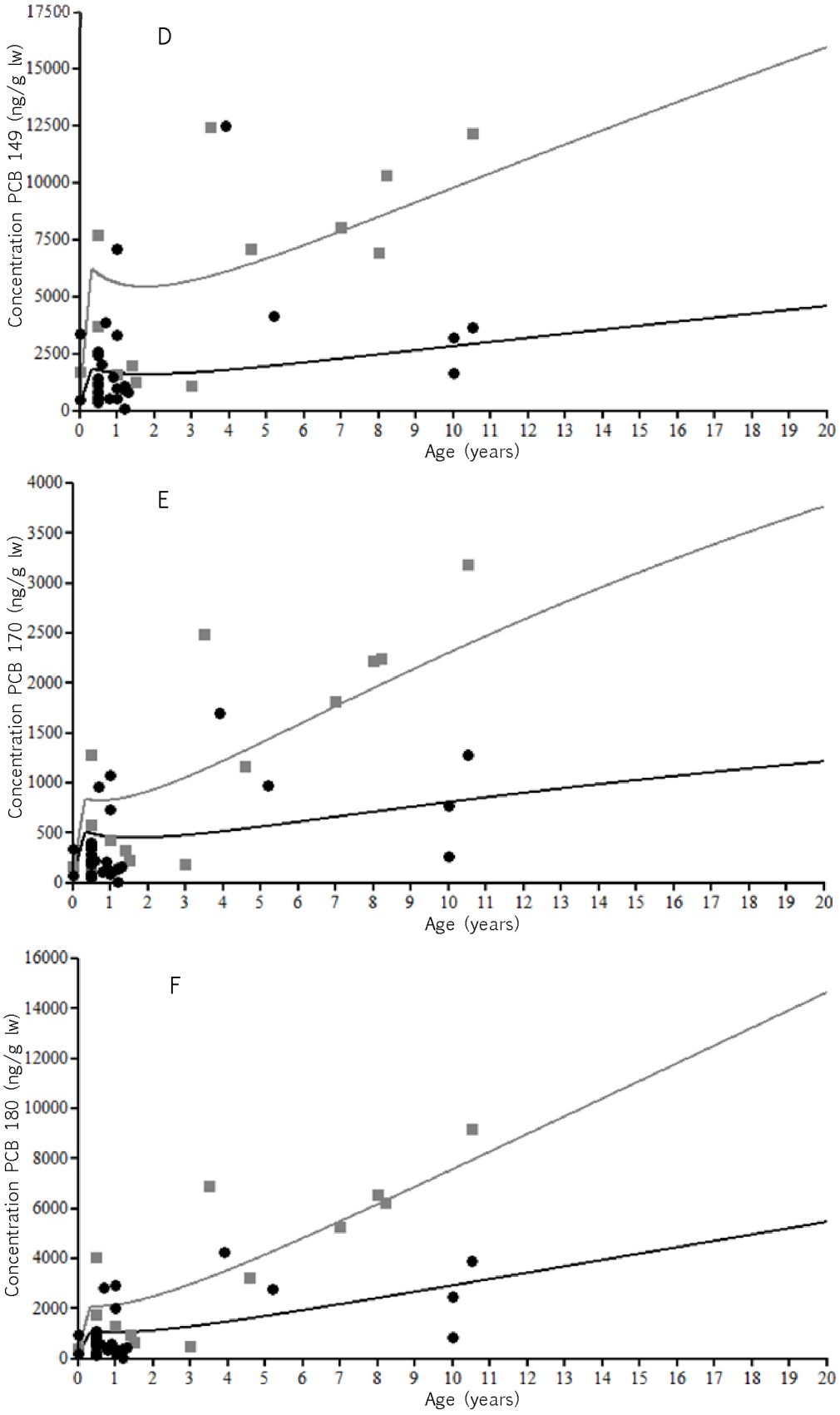


Fig 3. Time trends in age-dependent bioaccumulation of (A) PCB 99, (B) PCB 101, (C) PCB 118, (D) PCB 149, (E) PCB 170 and (F) PCB 180 in blubber of male harbour porpoises from the North Sea. All concentrations are expressed in ng/g lw. ■ = individual data for male harbour porpoises from the North Sea from 1990-2000, ● = individual data for male harbour porpoises from the North Sea from 2001-2008, — = model prediction for male harbour porpoises from 1990-2000, — = model prediction for male harbour porpoises from 2001-2008.

PCB 101

Similar as PCB 149 and as predicted by the model (gray curve; Fig. 4), there is also an increase in concentrations of metabolites of PCB 101. For the animals younger than 3 years, the concentrations of metabolites are situated under the gray curve, so for these animals, the origin of the metabolites remains unknown. For the animals older than 3 years, there is a sudden increase in concentrations of the metabolites as well as in the metabolites/parent compound percentages. At higher age, these animals clearly accumulate more metabolites as can be produced through metabolic breakdown. The difference in metabolites/parent compound percentages between the younger animals (< 3 years) and the older animals (> 3 years) suggests that the capacity for metabolic breakdown of PCB 101 is greatly enhanced or induced at higher ages as it would be difficult to explain this steep increase simply by bioaccumulation.

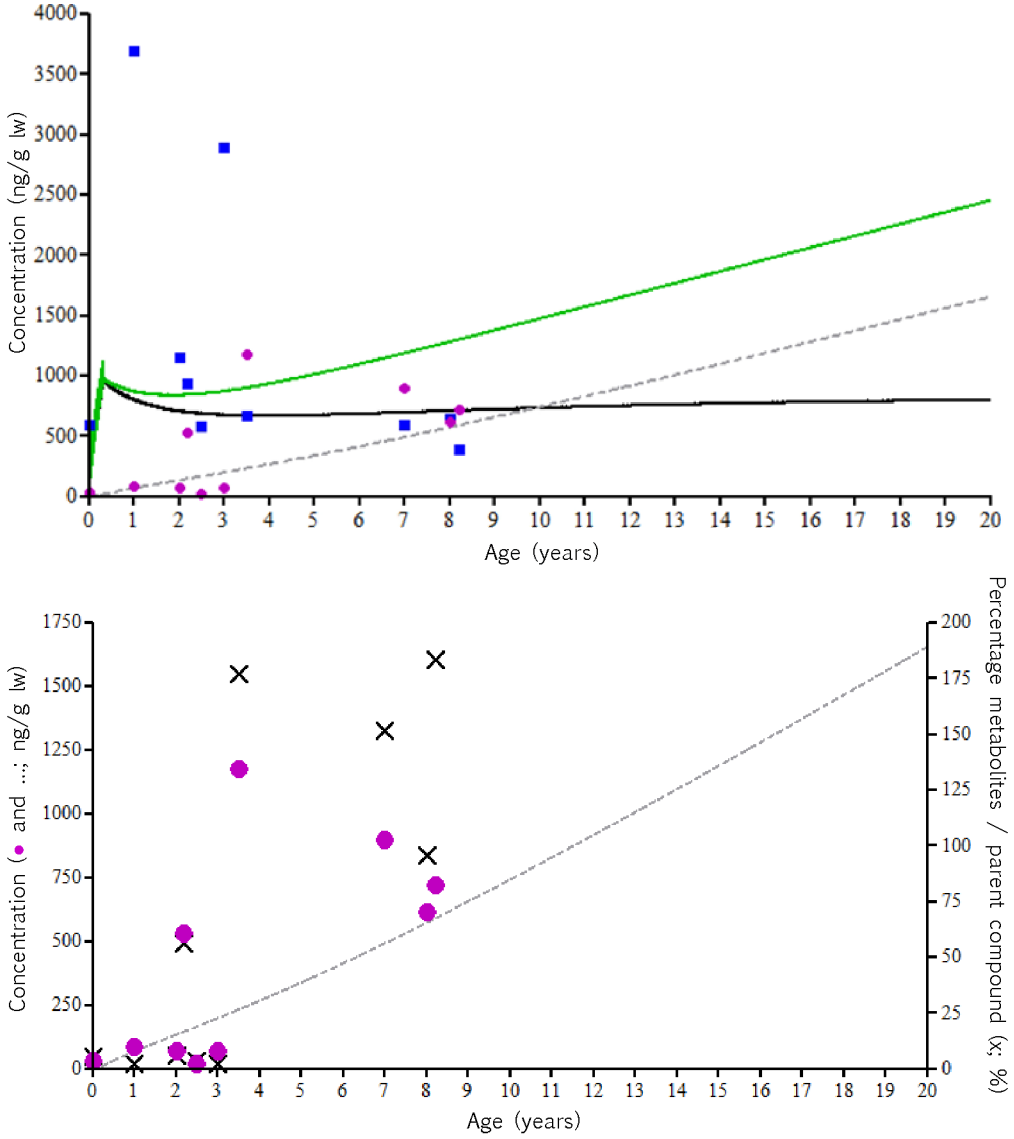


Fig 4. Age-dependent elimination of PCB 101 in liver of male harbour porpoises from the North Sea. All concentrations are expressed in ng/g lw. ■ = individual data of PCB 101 in livers of male harbour porpoises from the North Sea, • = individual data of $MeSO_2$-PCB metabolites of PCB 101 (Table 2), — = model predictions with elimination half-life of 6.1 years (Table 1), — = model predictions without elimination, — = difference between — and — (thus the concentration that is eliminated), x = percentage of concentration of PCB 101-metabolites/concentration of PCB 101.

4. Discussion

The significance of this work is the development and demonstration of a workable, non-invasive, computational approach to study lifetime exposure and bioaccumulation of PCBs in protected marine mammals, such as the harbor porpoises. For protected marine mammals, biomonitoring data on stranded or dead animals are generally the only experimental information available. In the past, such data were reported as survey results in the literature and that was the end of the study. With the application of PBPK modeling, however, the utility of such biomonitoring results is greatly expanded to provide further insights into the pharmacokinetics of the interested chemicals in these marine mammals and to help with the interpretation of their toxic effects as revealed by results of in vitro studies. This approach is certainly in line with the spirit of reducing or eliminating animal experimentation advanced by REACH (Registration, Evaluation, Authorization, and restriction of Chemical substances) in the European Union (EU).

PBPK modeling integrates computational technology and all the physiological and biochemical information on the chemical(s) of interest in the species of interest. Thus, it can be used as well in other species in which in vivo experiments are often unethical, such as humans. This report, as well as other similar work (Weijs et al., 2010a; Weijs et al., 2012), demonstrates that lifetime modeling including special physiological states can be effectively implemented for an entire class of chemicals. Although PBPK models for some selected chemicals in humans already exist, only few studies compare models for several PCBs. It is through comparisons between highly comparable and similar compounds, such as PCBs, that the knowledge about the kinetics of these compounds and the influence of their biochemical properties on the kinetics can progress. This is the first study that compares the kinetics of several PCBs, assesses their temporal trends and attempts to unravel metabolic pathways in a marine mammal species through PBPK modeling. Although the use of more datasets in the future will make the models stronger and more robust, the datasets used now take nothing away from the quality of the models, nor from their applications (goals 2 and 3) or conclusions.

4.1. Goal 1: Usefulness of models for other PCBs

PBPK models in the present study rely partly on biochemical parameters found in the literature from in vitro or in vivo experiments with other species and partly on parameters estimated by using data of PCBs in relevant tissues of harbor porpoises from the Black Sea. In the wild, the health condition of marine mammals ranges from healthy to emaciated and severely ill which can influence the kinetics of pollutants in their body as such. However, the Black Sea data used to evaluate the models consisted of more by-caught (considered healthy) animals than stranded (potentially sick) ones. In addition, as the models are not entirely based on the datasets in the present study, the model outcomes or conclusions drawn from the models are not influenced by including data of some ill animals.

Elimination half-life

In Weijs et al. (2010b), the models with a metabolic half-life of 27.5 years for bioaccumulation of PCB 153 in male and female harbor porpoises reflected nicely the Black Sea data. The half-life of 27.5 years was taken from a

human model (Verner et al., 2008) and was among the highest for PCB 153 available in the literature. Because of the usefulness of the human elimination half-life in the PBPK model of PCB 153 in harbor porpoises (Weijs et al., 2010b) and the resemblance of porpoises and humans (in body size, body weight and body composition), the same approach was followed in the present study. Thus, the highest elimination half-life values from the literature were used first, but the model was asked to estimate new half-lives in cases where the curves initially differed from the Black Sea data. Except for PCB 118, these new values were slightly higher (for PCB 101) to much higher (for PCB 170, 149). For PCB 180 and 99, the new half-lives even exceeded 300 years (Table 1). Although these extremely high half-lives may seem impossible, they are most likely an artifact caused by the relatively short life-spans of the harbor porpoises and thus the limited number of data points that were available in the fitting-process. For animals that live longer, such as killer whales, elimination half-lives for PCB 180 and 99 would probably be lower because of the higher number of data points at higher ages that can be included in the fitting-process. Nevertheless, for harbor porpoises that only live for about 20 years, the elimination half-lives of PCB 180 and 99 mean that both PCBs are barely eliminated during their entire life spans. In the present study, the elimination half-life only deals with chemicals absorbed by the organism. Assuming that 46% (for calves that drink milk) and 9% (for porpoises that eat fish) of the ingested amount of PCB 180 and 45% (for calves that drink milk) and 10% (for porpoises that eat fish) of the ingested amount of PCB 99 are not part of the model because these percentages were not absorbed by the animals (Table 1), it is noteworthy that it is still possible to find PCB 180 and PCB 99 in feces of harbor porpoises as also found in feces of bottlenose dolphins (Marsili et al., 1995) and right whales (Weisbrod et al., 2000).
It has been suggested that the amount of fat influences the elimination half-life value of chemicals in an organism as the chemical is no longer available for metabolic transformation or fecal/urinary elimination (Grandjean et al., 2008). Compared to rodents for example, marine mammals have much larger fat deposits to be able to maintain a constant body temperature in cold waters, to enhance locomotion and to provide energy in the form of lipids in times of food scarcity (Koopman, 2007). Obviously, this has a major impact on the elimination half-lives which were found to be much higher than those reported in the literature for humans or rodents. Elimination half-lives in the literature are often calculated after experiments in which the organisms were exposed to high doses of pollutants for only a short term (e.g. Lee et al., 2002; Maruyama and Aoki, 2006; Reitz et al., 1988). However, information about elimination half-lives after continuous exposure for years is scarce. Collectively, therefore, larger fat reserves and lifetime exposure, may lead to half-lives that are expected to be much higher as found in exposure experiments in the literature.

In the models, changes in the input (concentration of the compound in fish or milk) or elimination half-life can give the same model results. Concentrations of all PCBs were measured in seven milk samples from animals from the Black Sea and concentrations of PCB 118, PCB 170 and PCB 180 in their prey were taken from Tanabe et al. (1997a), both of which were reliable sources for intake levels of the respective PCB congeners. For PCB 118, 170 and 180, the only appropriate parameter to adjust in the models is thus the elimination half-life. For PCB 99, 101 and 149, the levels

in the fish prey were not available in Tanabe et al. (1997a), so these levels were estimated. Since milk concentrations were available for all PCBs, there were actually two different ways to estimate the fish levels for PCB 99, 101 and 149: 1) The first way was to use the milk-to-fish ratio of PCB 153 (Weijs et al., 2010b), PCB 118, 170 or 180 and the milk levels of PCB 99, 101 and 149; 2) The second way was to look at the PCB patterns in fish in general and the fish data that we have for PCB 153, 118, 170 and 180 from Tanabe et al. (1997a), e.g. the proportion of PCB 149 to PCB 153 in North Sea fish is probably the same in Black Sea fish (and the levels of PCB 153 in Black Sea fish can be found in Tanabe et al., 1997a).
In the present study, both ways were used: the first way was used to estimate the fish levels and the second way to check whether these fish levels were realistic. The following discussion provides more specifics to the estimations of the fish levels of PCB 99, 101 and 149. In the PCB 153 model (Weijs et al., 2010b), there is a 116 times difference between the PCB 153 concentration in fish and milk which was used to estimate the levels in fish of PCB 99, 101 and 149. This factor differs little from the 84, 107 and 203 times differences between the concentration of PCB 118, 170 and 180, respectively, in fish and milk in the current PCB 118, 170 and 180 models. To test whether the calculated fish levels of PCB 99, 101 and 149 were comparable with real concentrations in fish, the patterns of PCBs reported in fish were taken into account as well. In the North Sea, levels of PCB 149 in several fish species may reach relatively high concentrations which are between 30 and 50% of the levels of PCB 153 (Voorspoels et al., 2004). The concentration used in the present study for PCB 149, which is 0.7 ng/g ww or 64% of 1.1 ng/g ww (input used in the PCB 153 model (Weijs et al., 2010b), seems therefore already high enough. Consequently, an increase in elimination half-life in the PCB 149 model was assumed to be more logic than an increase in dietary input of fish. Similar, in fish species from the North Sea, PCB 99 is between 24 and 35% of the concentration of PCB 153 (Voorspoels et al., 2004). This would give a concentration of PCB 99 between 0.26 and 0.39 ng/g ww. Therefore, the value of 0.51 ng/g ww, calculated as a 116 times difference compared to the value in milk, is already high enough and should not be any higher just to get a steep increase in the curves. So also for PCB 99, a higher elimination half-life is preferred rather than an increase in input concentration. In the present study, concentrations in fish were used as a constant thereby probably neglecting a potential decrease in fish levels over time. Tanabe et al. (1997a) report on PCBs in harbor porpoises and their prey (European anchovy and whiting) in the Black Sea from 1993. Since there are no available prey or fish data for harbor porpoises in the current dataset from 1998, the fish data from 1993 were used for further calculations. To our knowledge, there are no reports of gradual decreases in fish concentrations from the Black Sea. However, as concentrations of PCBs in fish probably change over time, it is worthwhile to mention that future models would benefit from having fish data reflecting these changes.

Blood/brain partition coefficients

Tissue/blood partition coefficients were calculated with the method of Parham et al. (1997). This method is based on the lipid percentage of the respective tissues (Weijs et al., 2010a) which is the most logical background for the partitioning of lipophilic compounds. All calculated tissue/blood partition coefficients worked nicely for all PCBs, except for the blood/brain

partition coefficients. Unlike the blood–brain barriers (BBB) in fish, mammalian BBBs have been suggested to act as a potential protective shield by blocking some chemicals from entering the brain. Bachour et al. (1998) found a uniformly distribution of PCBs in several tissues, including the brain, of fish (rainbow trout), whereas there were significantly lower concentrations of PCBs in the brain of mammals (fox, human) compared to all other tissues investigated. However, next to the presence of a BBB, there is also the possibility that the lipid composition of the brain affects the accumulation of PCBs. The brain is mainly made up of polar phospholipids and sphingolipids whereas it has only a minor portion of triglycerides in contrast with other tissues. In an exposure experiment with chicken embryos, Maervoet et al. (2005) found that concentrations of PCBs (PCB 77, 153 and 180) remained relatively stable in the brain at the latest stage before hatching, while the levels increased exponentially in other tissues such as the liver. Considering that the BBB was still incomplete at that time (the BBB is only fully completed after hatching), the same study concluded that the specific lipid composition of the brain is less attractive for lipophilic compounds such as PCBs and thus responsible for the lower concentrations of PCBs found in the brain. In vitro studies have also proven that PCBs have amuch higher affinity for triglycerides than for phospholipids which is indicative for a lesser bioaccumulation in the brain (Sandermann, 2003). Therefore, a different lipid composition might indeed be the reason why concentrations of PCBs are lower in the brain than in any other tissue. However, it is probably not the only explanation. The lipid composition of the brain of fish (Atlantic herring) is comparable to the lipid composition of the brain of mammals with a higher proportion of polar lipids in mature animals (Mourente and Tocher, 1992). Together with the results of Bachour et al. (1998), this would mean that there is indeed something like a BBB that results in lower concentrations in the brain of mammals compared to other tissues. In the present study, lipids were determined gravimetrically and a hexane/acetone mixture was used for extraction (Weijs et al., 2010a). This method allows to measure triglycerides, cholesterol and less polar phospholipids, but not polar phospholipids or sphingolipids. The lipid percentages were then used to calculate the blood/tissue partition coefficients (Parham et al., 1997a) which were for the blood/brain partition coefficients, in all models, too high compared to the Black Sea dataset.

The adjusted (fitted) blood/brain partition coefficients are, for all PCB congeners, 2.5 to 3 times lower than the partition coefficients originally calculated with the method of Parham et al. (1997a). The fact that this is independent of the molecular sizes of the molecules (PCB 99 has a comparable blood/brain partition coefficient as PCB 180), is in favor of the theory that the lipid composition of the brain determines the accumulation of PCBs in the brain. This is also supported by the lower concentrations of the PCBs found in the brain of the fetus compared to blubber, liver or kidney (Table 1). In humans, the BBB is incompletely developed at birth (Anthony et al., 1996). If this is true for marine mammals as well, than the lower PCB concentrations in the brain of the fetus can only be caused by the different lipid composition. However, the influence of the BBB cannot be ruled out as there are studies that have reported that the BBB excludes effectively substances with molecular weights greater than 180 Da (Doolittle et al., 1998), while molecular weights of penta-PCBs (such as PCB 99) and hepta-PCBs (such as PCB 180) are between 325 and 400 Da. Lower concentrations of PCBs in the brains of pilot whales and harbor porpoises compared to

levels in other tissues were also reported by Tilbury et al. (1999) and Tilbury et al. (1997).

Assimilation efficiency for calves

Debier et al. (2003b) and Beckmen et al. (1999) reported that there is a selective gastrointestinal uptake of PCBs from milk, but assimilation efficiencies for individual PCBs from milk remained unknown. Hickie et al. (1999) used higher assimilation efficiency from milk than from fish in the models of beluga whales, but did not discuss PCBs separately. In the present study, the model outputs for the youngest animals overestimated the dataset used for validation when using identical assimilation efficiencies as for the fish. There are basically two parameters that can influence the input of PCBs through milk, namely the assimilation efficiency for milk and the concentration of the PCB in the milk. The concentrations of the individual PCBs in milk were analyzed in seven milk samples. The only parameter that could be changedwas thus the assimilation efficiency from the milk. The resulting assimilation efficiencies for the milk range from almost 50 to 70%, which differs from to the assimilation efficiencies for the fish (90–99%). As unabsorbed PCBs would end up in the feces, concentrations of PCBs in feces of calves should be higher relative to PCBs in feces of older animals. However, fecal samples of harbor porpoises were not available, so this explanation could not be checked.

4.2. Goal 2: Assessing temporal trends for parent PCBs

Concentrations of all PCBs investigated in harbor porpoises from the North Sea decreased over a time period of 18 years (1990–2008) although not at the same rate (Fig. 3 A–F). Considering the levels of the PCB congeners in liver of harbor porpoises of five years of age for example, levels of PCB 149 decreased the fastest (from 6675 ng/g lw in 1990–2000 to 1943 ng/g lw in 2001–2008; Fig. 3D) whereas levels of PCB 101 decreased the slowest (from 561 ng/g lw in 1990–2000 to 309 ng/g lw in 2001–2008; Fig. 3B). This decrease in PCB levels in harbor porpoises over time is most likely due to a decline in PCBs in the fish, thus a decline in input concentrations for the porpoises over time. In Europe, declining PCB levels over time were reported in several fish species (Skåre et al., 1985; Szlinder-Richert et al., 2009). Decreasing levels over time were also reported for several marine mammal species (harbor porpoises-Law et al., 2010; beluga whales-Lebeuf et al., 2007; Baikal and Caspian seals-Tanabe et al., 2003; polar bears-Dietz et al., 2004) although there were also reports about increasing PCB concentrations over time (sea lions-Borrell et al., 2010; northern fur seals-Kajiwara et al., 2004). Despite these decreasing PCB trends in the North Sea, the area is still a highly polluted area for several years to come.

4.3. Goal 3: Metabolism/elimination

Metabolic breakdown and/or elimination pathways are typically difficult to study in living marine mammals, however, in vitro studies in these animals exist. In an in vitro hepatic microsomal assay with cells from beluga whales, McKinney et al. (2006a) found a slow, but significant metabolic biotransformation of PCB 118 (98 ± 1% remaining), whereas PCB 101 and PCB 105 were not depleted. For PCB 118, the depletion of 2% is roughly 10 times higher than the ratio between PCB 118 metabolites and the parent compound which ranged from 0 to 0.26% (Fig S6) in the present study. This discrepancy might be due to the duration of exposure; the assay was only

90 min whereas animals in the wild are continuously exposed. PCB 118 is a molecule without vicinal meta, para H-atoms, and is as such metabolized by the cytochrome P450 monooxygenase isoform CYP1A, which is present in marine mammals (Hirakawa et al., 2007; Miller et al., 2005; Routti et al., 2008b; Tilley et al., 2002; Wilson et al., 2010). Yordy et al. (2010) found that concentrations of PCBs relying on CYP1A metabolism did not increase with age in bottlenose dolphins, thus assuming a metabolic pathway for these congeners in cetaceans. In the present study, HO-metabolites of PCB 118 were detected in 2 out of 6 young animals (age b3 years) and in 3 out of 4 older animals (age N3 years). This might be an indication for an age-dependent induction of the CYP1A system although this needs further investigation with a higher sample size. In contrast to what was found by McKinney et al. (2006a), PCB 101 is probably metabolized in liver of harbor porpoises as has been reported for gray seals (Li et al., 2003). PCB 101 has vicinal meta, para H-atoms, 2 Cl-atoms at both ortho-positions and is metabolized by CYP2B and 3A family enzymes which are present in marine mammals (Li et al., 2003; Routti et al., 2008b). On the other hand, metabolites of this compound have been found in the prey of the harbor porpoises as well (Table 2). According to the PCB 101 model in the present study and the PCB 101-metabolites detected in the harbor porpoises, the animals, especially at older ages, have more PCB 101-metabolites than they can produce through metabolic breakdown (Fig. 4). It is also apparent that the levels of the PCB 101-metabolites are much higher for older animals (>3 years) than for younger animals (<3 years). Dietary accumulation of PCB 101-metabolites should be a continuous process shown as a gradual increase in metabolite levels. Fig. 4, however, shows a more sudden increase in metabolites for older animals compared to younger animals suggesting that there might be an age-dependent biotransformation of PCB 101 additional to a continuous bioaccumulation of PCB 101 metabolites from the fish with age. The increase in levels of PCB 149 metabolites is more gradual or continuous (Fig S7) than for the PCB 101 metabolites (Fig. 4). In the past, PCB 149 has not been the focus of many studies as it is not very common in marine mammals other than cetaceans. In harbor porpoises from the North Sea, PCB 149 is consistently measured as the third congener after PCBs 153 and 138 (Weijs et al., 2009a). In other cetacean species, PCB 149 is also considered as one of the most persistent PCB congeners. In theory, PCB 149 is metabolized by CYP2B/3A enzyme systems, but that probably does not play an important role taken the persistence of PCB 149 in cetaceans into account. Yordy et al. (2010) found a significant relationship between the age of bottlenose dolphins and the concentration of PCBs subjected to CYP2B/3A enzymes. In the present study, the continuous increase in PCB 149-metabolites (Fig S7) can be explained by dietary bioaccumulation, although possible metabolic biotransformation of PCB 149 cannot be completely excluded based on the PCB 149 model alone.

5. Conclusions

Understanding the bioaccumulation of pollutants and associated risks in marine mammals is a challenging problem. In silico or computer-based models take the animal as a whole, but often lack important, detailed information about distribution or uptake processes. This information can be taken from studies with other organisms such as rodents, but that does not always seem to work because of the physiological differences between marine mammals and rodents. In the present study, the estimated elimination

half-lives of PCBs in marine mammals were higher to much higher compared to the elimination half-lives of PCBs taken from the literature, probably because of the greater lipid deposits in marine mammals. For the brain, not only the lipid percentage plays an important role in the distribution of chemicals, also the lipid composition, together with a potential influence of the blood–brain barrier, are determining factors for the bioaccumulation of lipophilic contaminants. Although more physiological background information of marine mammals (e.g. lipid composition of the tissues, enzyme-mediated metabolic transformation) would enhance the extrapolation of the models from one chemical to the other, PBPK models are certainly useful. Once they have been developed, these models can be used for a broad range of applications, such as visualizing temporal trends or shedding some light on possible metabolic breakdown capacities and as such, they go further than simply describing the results of typical biomonitoring studies. As developed and used in the present study, PBPK models are non-invasive and non-destructive which is the only possible approach to study the kinetics of chemicals in marine mammals.

Acknowledgments

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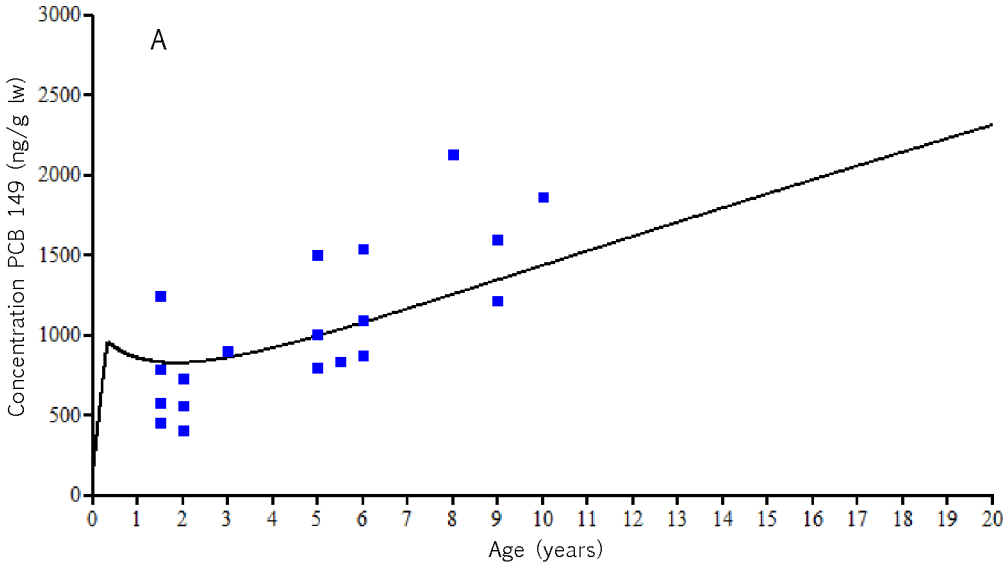
Supporting Information

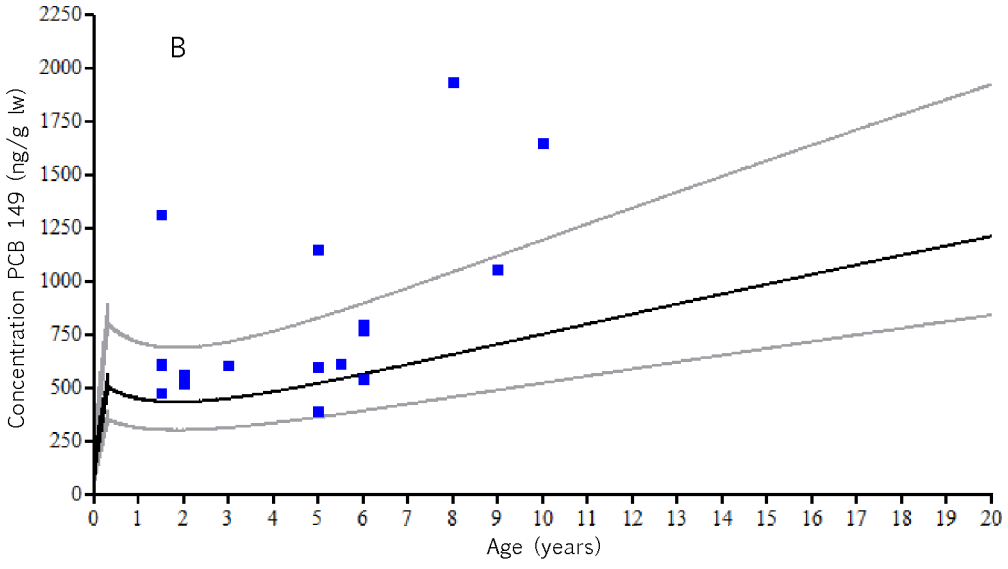
Table S1. Concentrations of selected PCBs in milk samples of adult females from the Black Sea (Weijs et al., 2010a). All results are given in ng/g lipid weight (lw). Biological data of the females (age, cause of death) can be found in Weijs et al. (2010b).

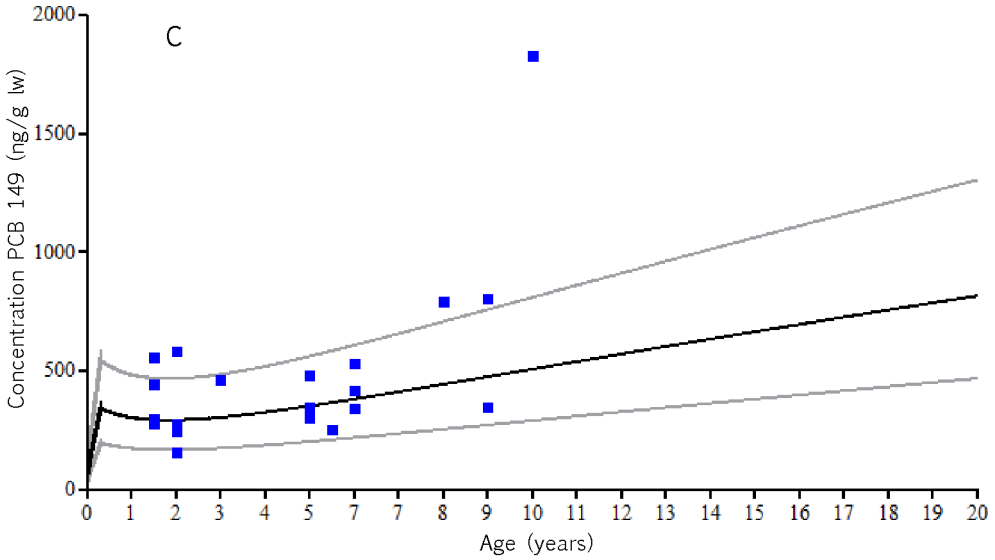
Sample ID	Lipid %	PCB 99	PCB 101	PCB 118	PCB 149	PCB 170	PCB 180
U 31 (*)	51.8	1140.2	565.5	994.9	1729.6	333.3	1124.9
U 39	36.9	54.0	77.1	102.0	86.5	21.1	63.9
U 40	36.6	450.4	174.7	358.6	695.4	181.9	603.0
U 48	24.7	60.1	85.0	111.9	93.6	15.9	48.8
U 67	22.4	46.5	67.6	88.2	72.8	14.8	41.0
U 89	26.1	359.2	225.5	367.4	515.0	95.4	288.5
U 90	36.9	90.4	97.0	141.6	142.6	28.6	92.1
U 94	26.0	294.6	108.6	178.3	359.6	65.4	200.8

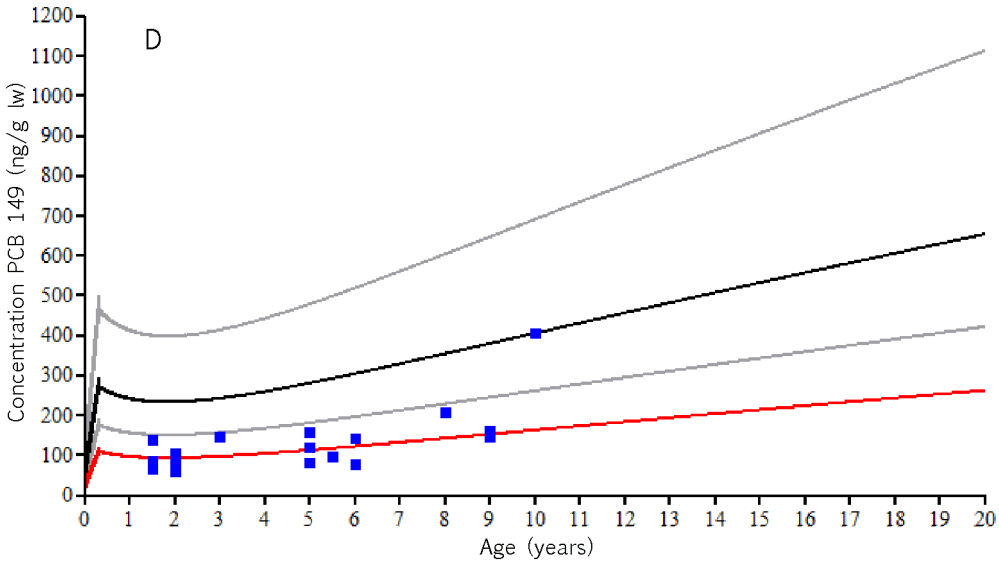
(*) Considered as outlier and excluded from the calculation of the average concentration in the milk.

Fig S1. Age-dependent bioaccumulation of PCB 149 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.






C
Concentration PCB 149 (ng/g lw)
2000
1500
1000
500
0
0 1 2 3 4 5 7 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)


D
Concentration PCB 149 (ng/g lw)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

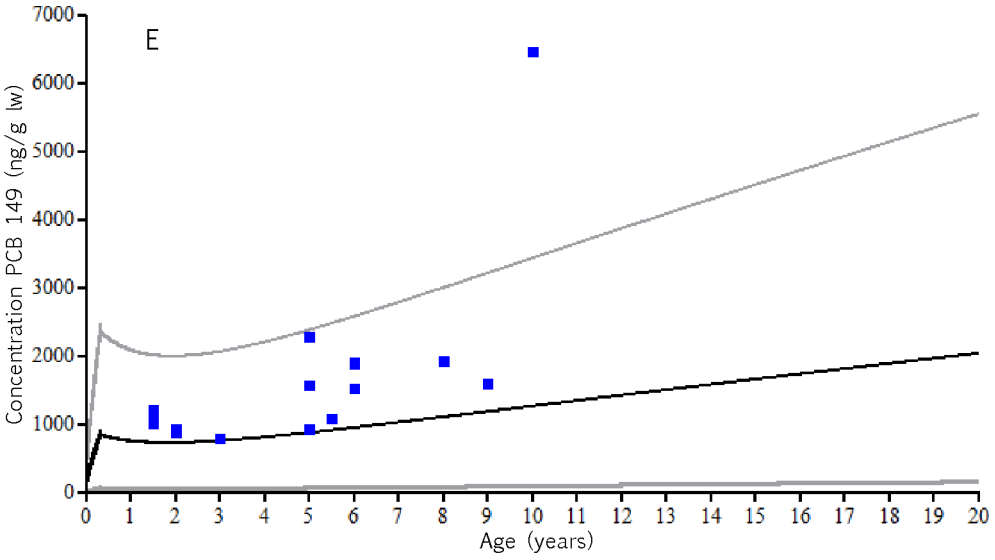
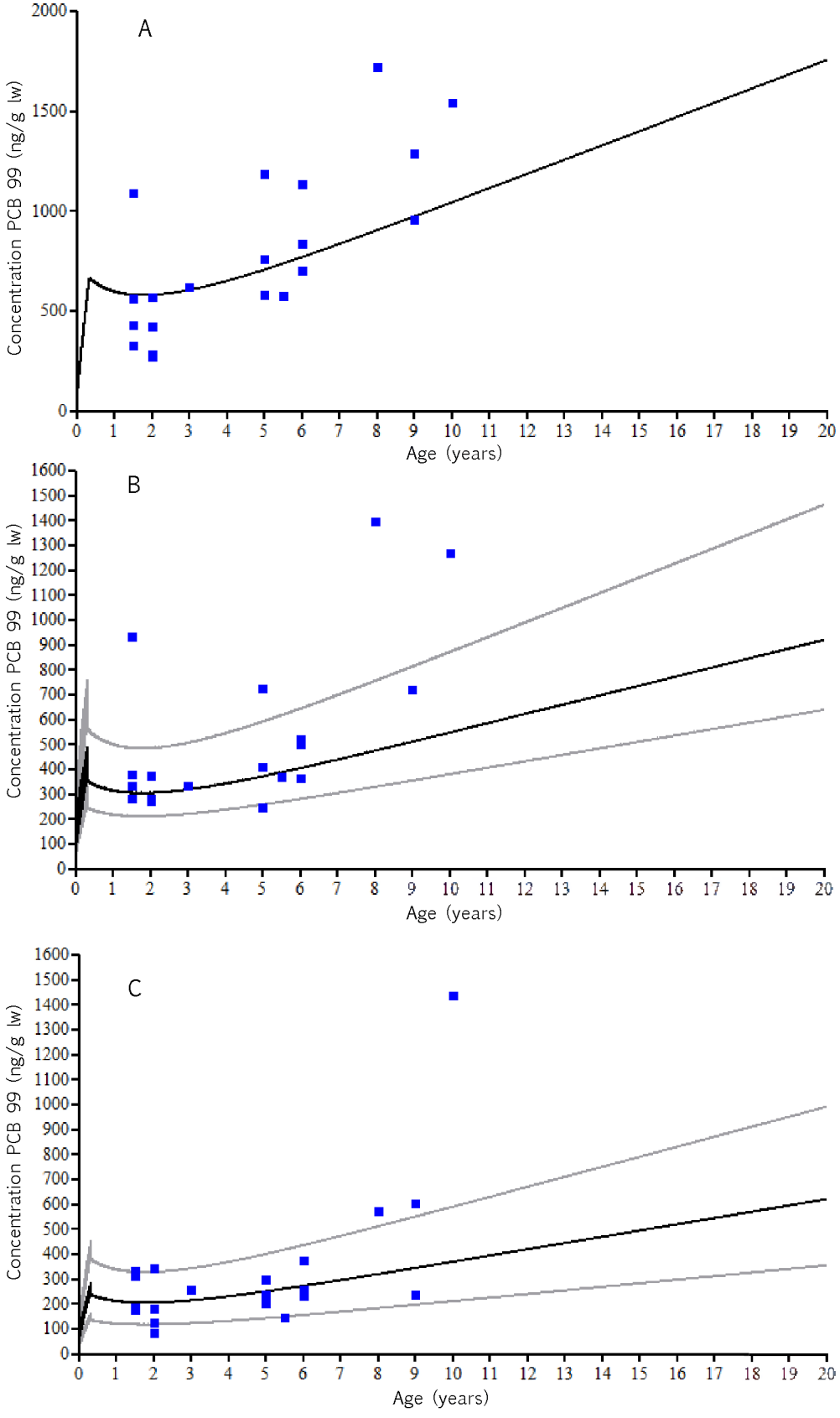
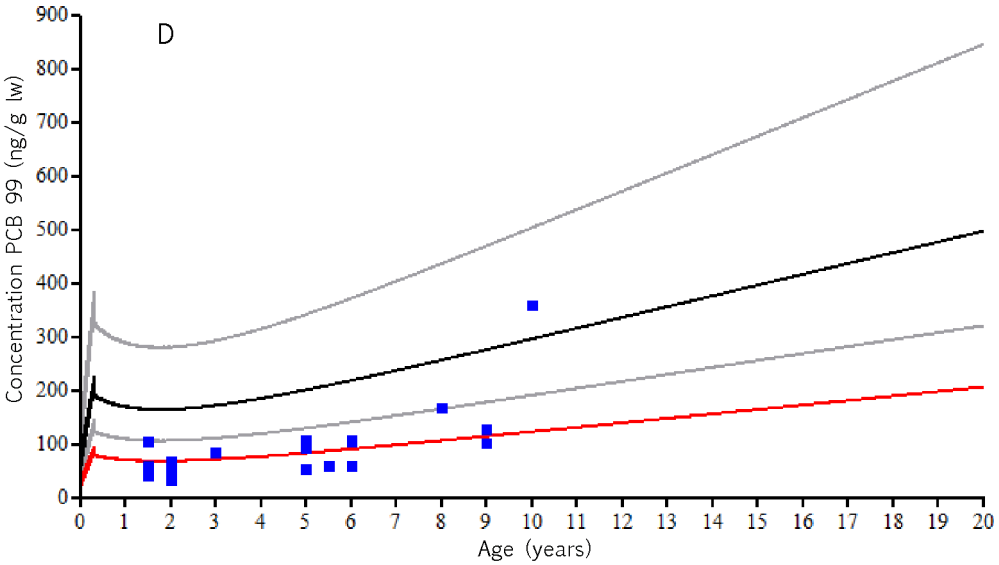

E
Concentration PCB 149 (ng/g lw)
7000
6000
5000
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

Fig S2. Age-dependent bioaccumulation of PCB 99 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.




D
Concentration PCB 99 (ng/g lw)
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

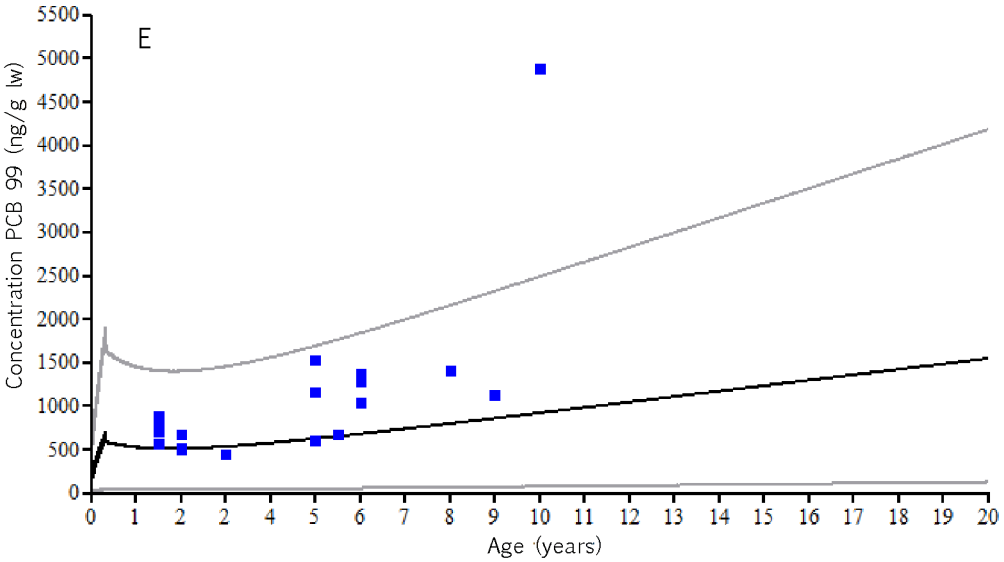
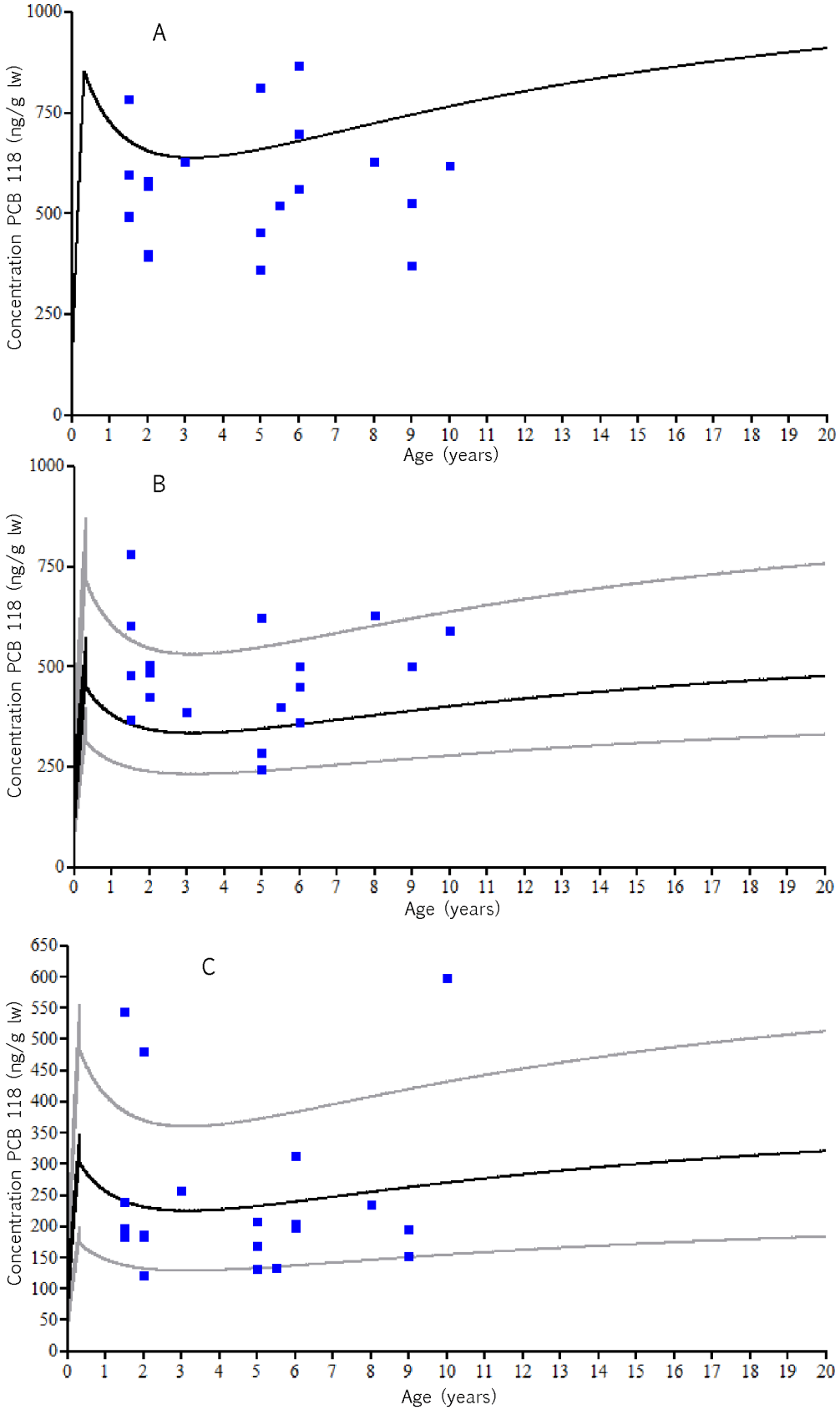
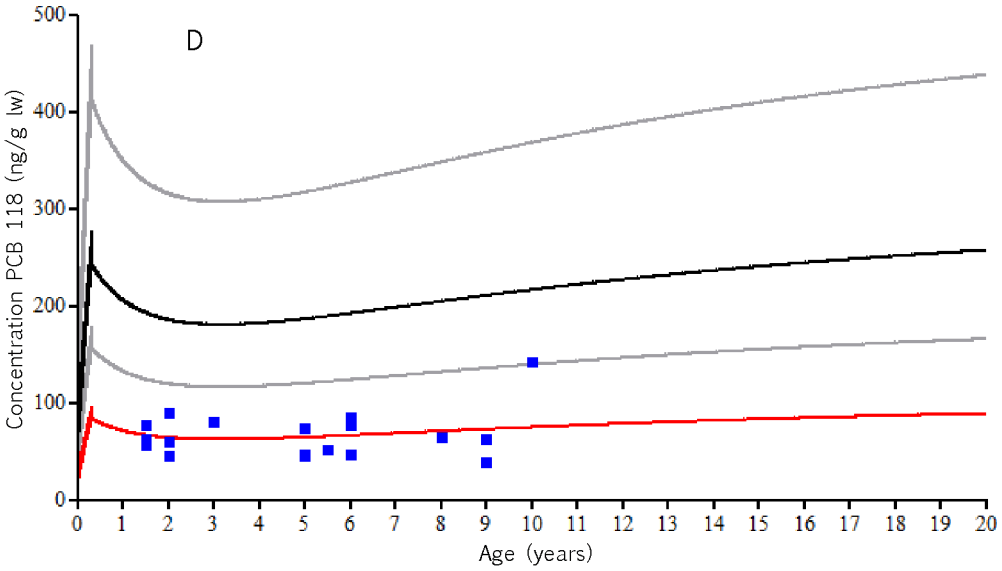

E
Concentration PCB 99 (ng/g lw)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0 1 2 2 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

Fig S3. Age-dependent bioaccumulation of PCB 118 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.




D
Concentration PCB 118 (ng/g lw)
0
100
200
300
400
500
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

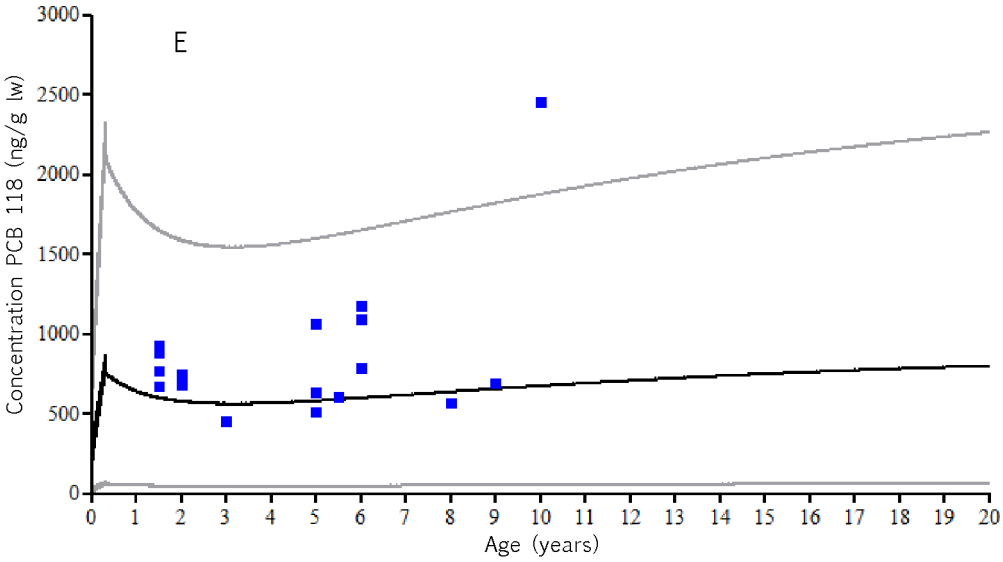
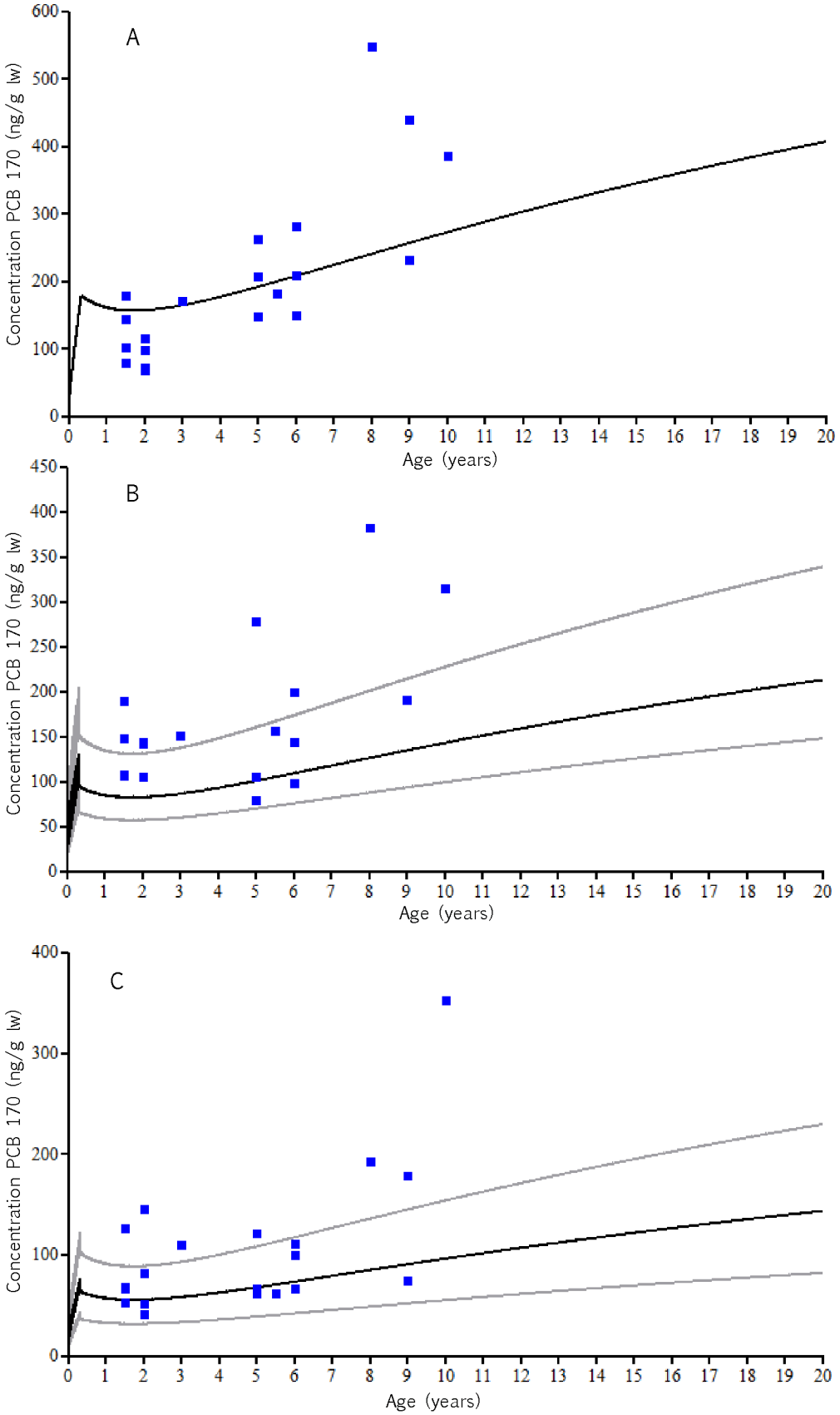
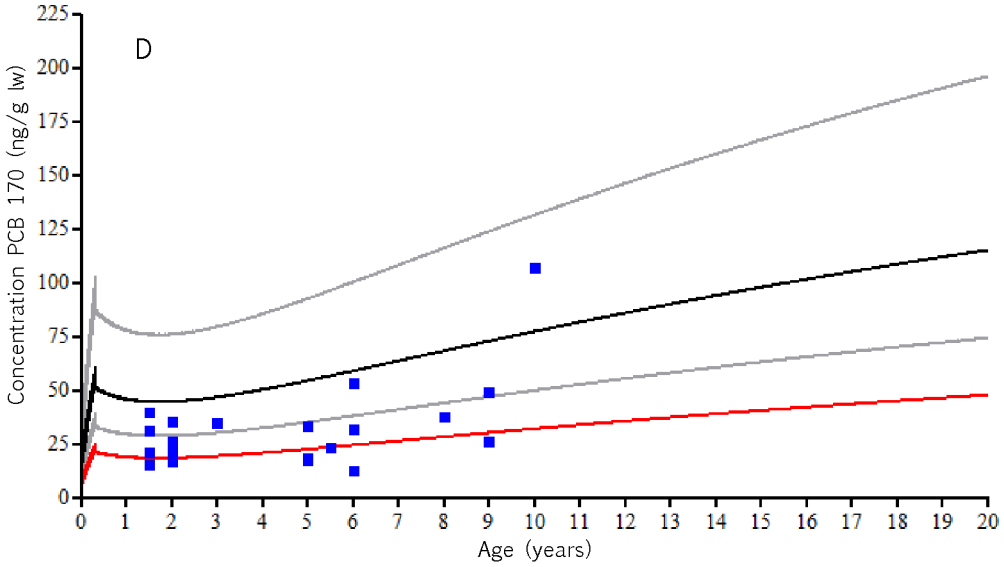

E
Concentration PCB 118 (ng/g lw)
0
500
1000
1500
2000
2500
3000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

Fig S4. Age-dependent bioaccumulation of PCB 170 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.




D
Concentration PCB 170 (ng/g lw)
225
200
175
150
125
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

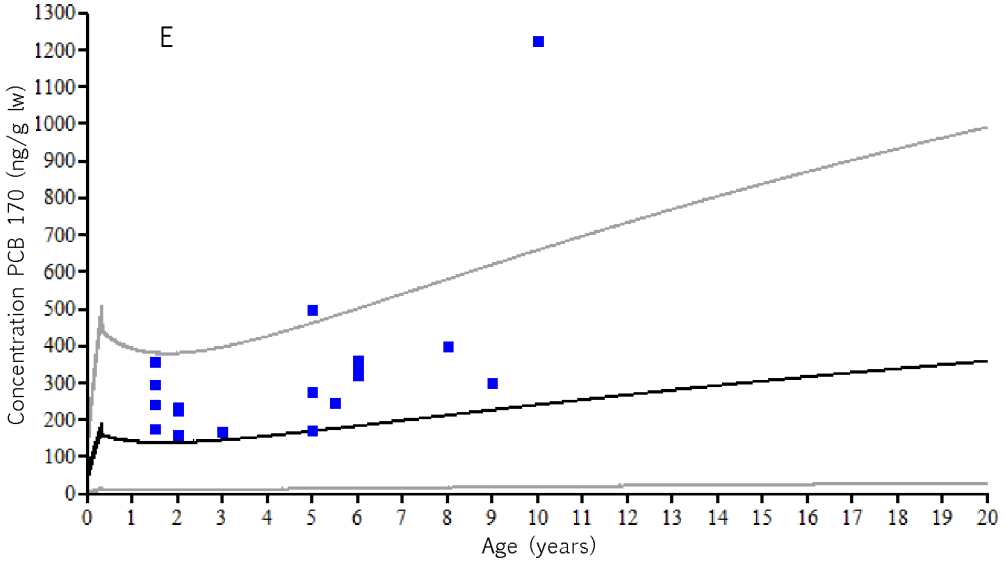
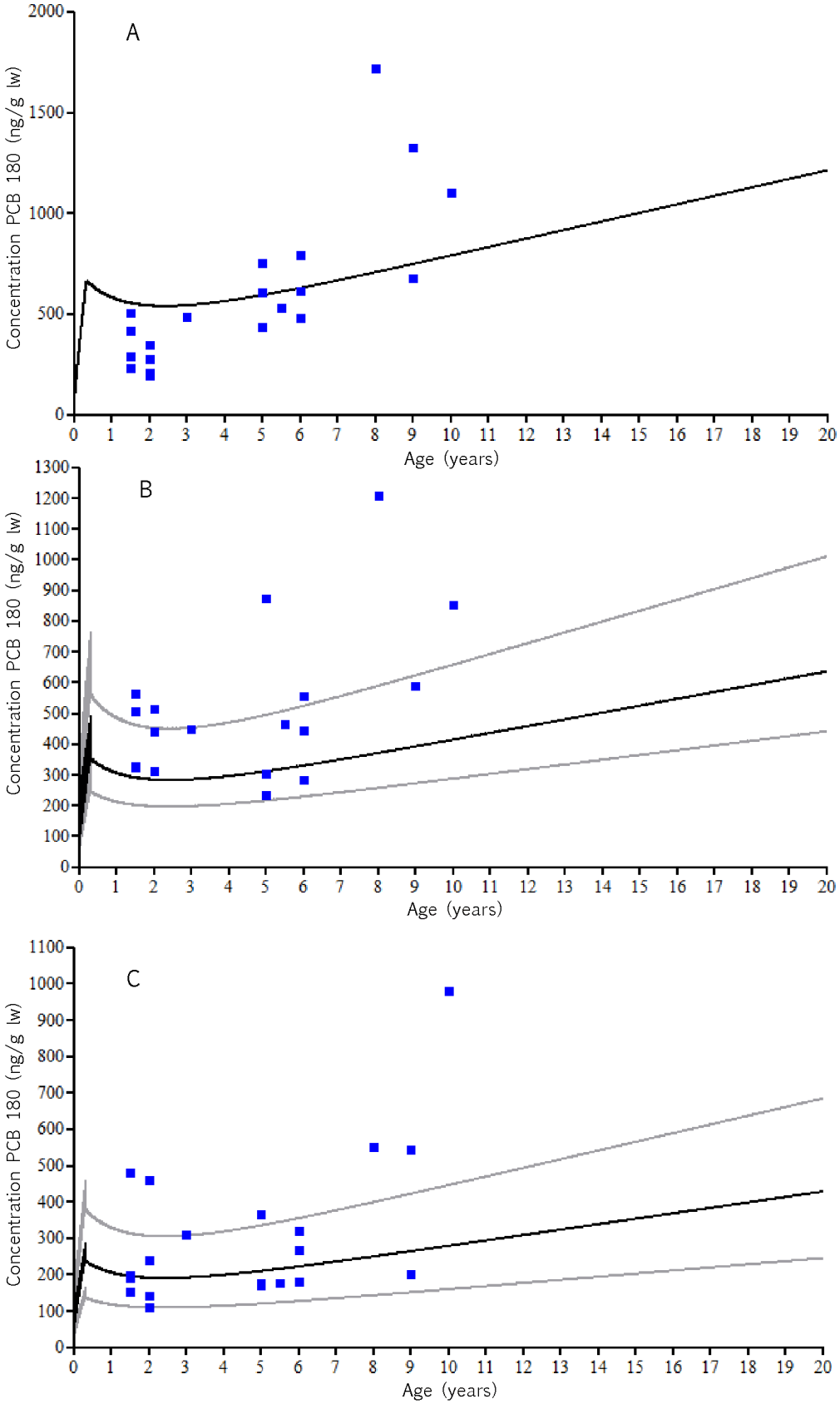
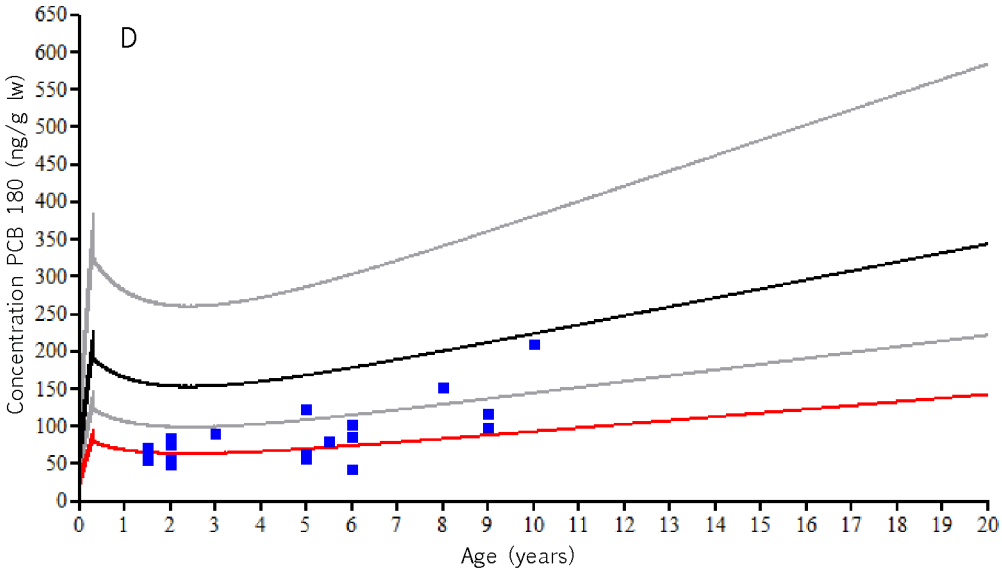

E
Concentration PCB 170 (ng/g lw)
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

Fig S5. Age-dependent bioaccumulation of PCB 180 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.




D
Concentration PCB 180 (ng/g lw)
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

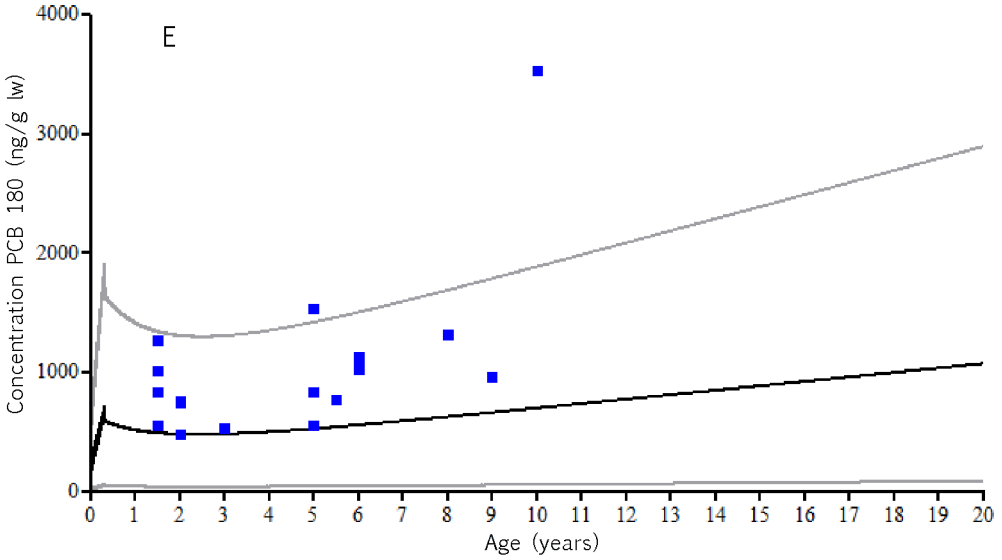

E
Concentration PCB 180 (ng/g lw)
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

Fig S6. Assessing elimination of PCB 118 in liver of male harbour porpoises from the North Sea. All concentrations are expressed in ng/g lw. ■ = individual data of PCB 118 in livers of male harbour porpoises from the North Sea, ● = individual data of HO-PCB metabolites of PCB 118 (Table 2), — = model predictions with elimination half-life of 9.6 years (Table 1), — = model predictions without elimination, --- and --- = difference between — and — (thus the concentration that is eliminated), x = concentration of PCB 118-metabolites/concentration of PCB 118.

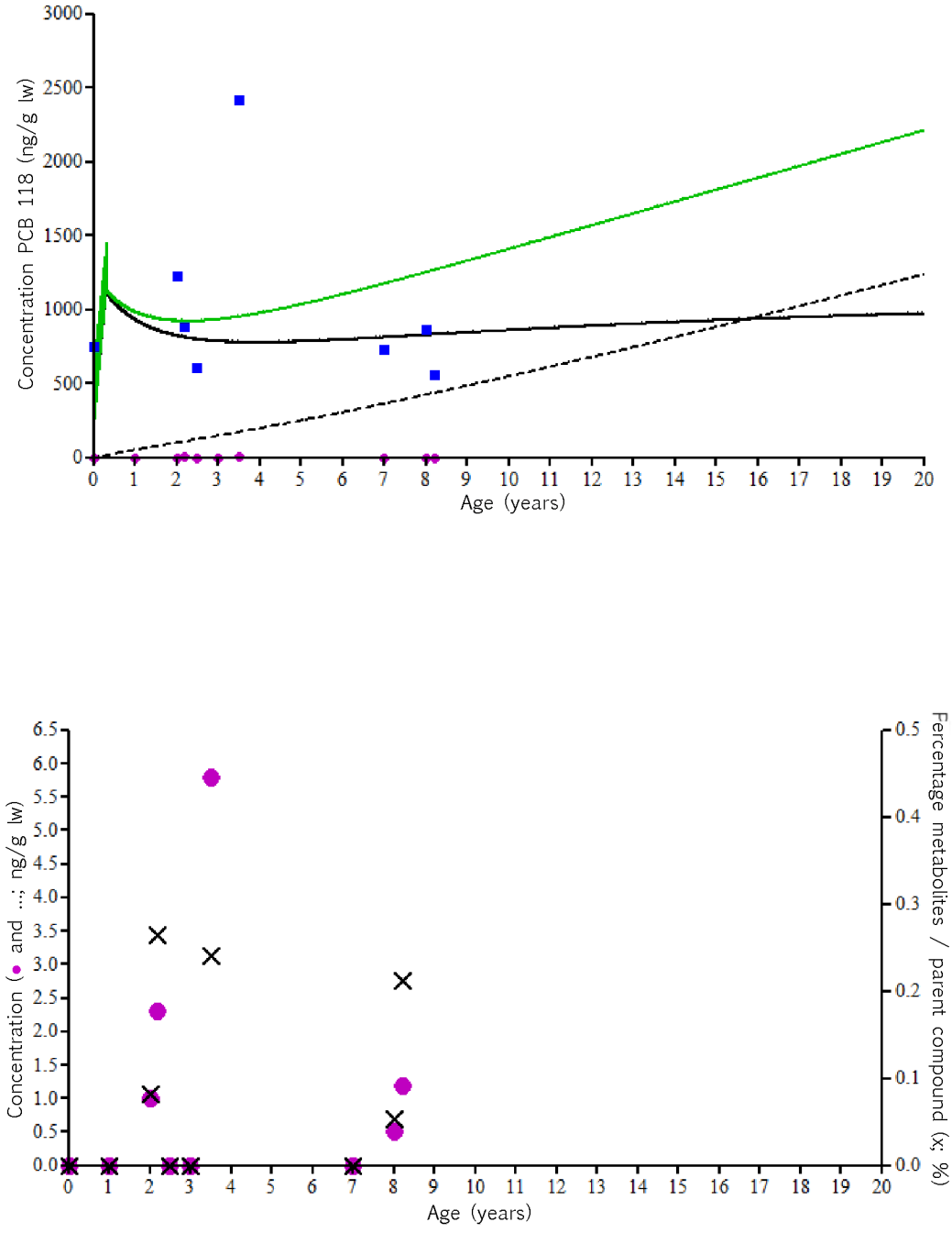
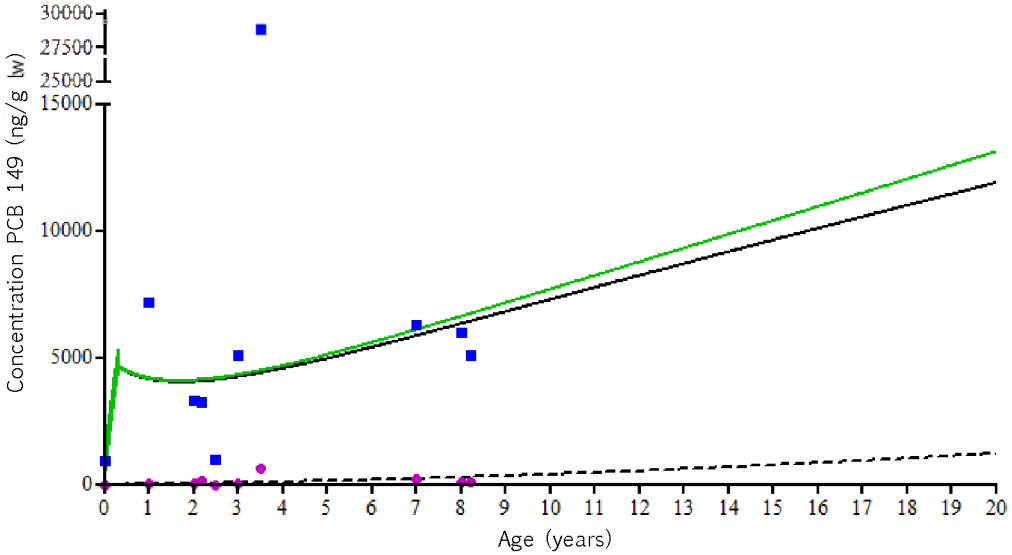
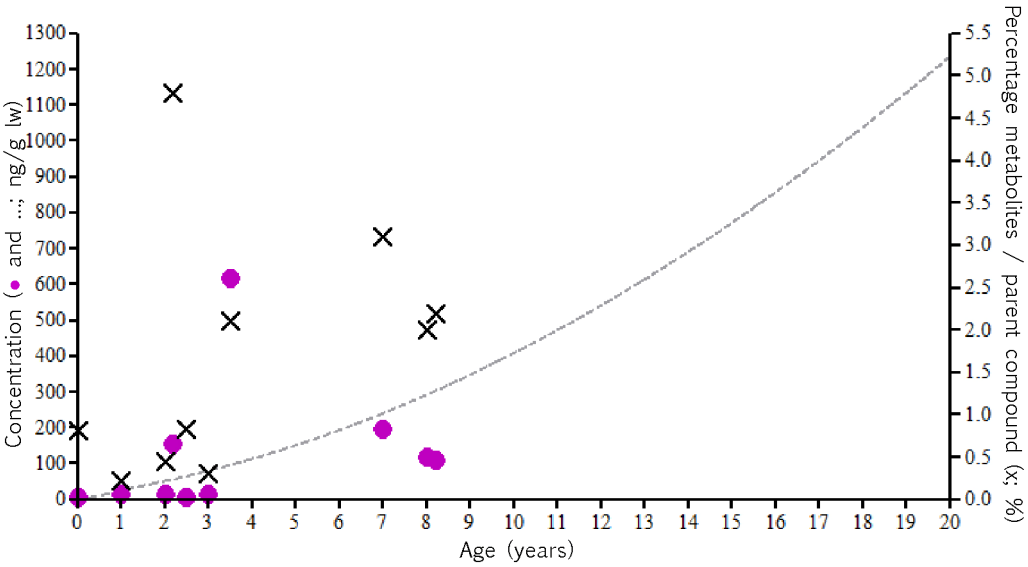


Fig S7. Assessing elimination of PCB 149 in liver of male harbour porpoises from the North Sea. All concentrations are expressed in ng/g lw. ■ = individual data of PCB 149 in livers of male harbour porpoises from the North Sea, ● = individual data of $MeSO_2$-PCB metabolites of PCB 149 (Table 2), ▬ = model predictions with elimination half-life of 80.0 years (Table 1), ▬ = model predictions without elimination, ··· and --- = difference between ▬ and ▬ (thus the concentration that is eliminated), x = concentration of PCB 149-metabolites/concentration of PCB 149.





5.3 Paper IX

Computational toxicology: Physiologically based pharmacokinetic models (PBPK) for lifetime exposure and bioaccumulation of polybrominated diphenyl ethers (PBDEs) in marine mammals

Liesbeth Weijs, Adrian Covaci, Raymond S.H. Yang, Krishna Das, Ronny Blust

Abstract

Due to migration of harbour porpoises towards more polluted areas like the North Sea and their sensitivity towards pollution, there is a need for proper conservation measures for this species. As a consequence, knowledge about the pollutant's kinetics is required. The present study is the first to investigate the kinetics of PBDEs in marine mammals using PBPK modeling as a non-destructive tool for describing the chemical's kinetics in a protected animal species. The models were developed and parameterized using data from the literature and Black Sea harbour porpoises through computer optimization. The predictability of these models in time was assessed by reverse dosimetry modeling using data from North Sea porpoises (1990-2008). From these predictions, PBDE 99 levels were found to decrease the fastest, followed by PBDE 153, 47 and 100. Results show that the PBPK models can be applied for harbour porpoises from different regions and also simulate time trends.

IX

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1. Introduction

PBDEs have been used as flame retardants in various products and equipment ranging from textiles to electronics. They have been banned in many countries (ATSDR, 2004); however, they are still ubiquitous in the environment due to their stability and persistence (Birnbaum and Staskal, 2004). Some PBDEs have the potential to biomagnify in terrestrial and aquatic food webs (Kelly et al., 2007; Voorspoels et al., 2007). Biomagnification is of particular concern for organisms at the top of the food chains, such as marine mammals, as they receive high concentrations of PBDEs from their prey. In addition, lactational transfer of chemicals through the lipid rich milk (up to 40% fat) represents also a major source of contaminants for these animals (Debier et al., 2003b; Hickie et al., 2007; Weijs et al., 2009a). Several PBDEs were shown to be toxic on the reproductive, immune and endocrine systems of marine mammals (Sonne et al., 2009; Beineke et al., 2010; Frouin et al., 2010).

PBPK models represent a mass-balanced system of compartments where the pollutant's distribution is regulated according to the physiological properties of the compartments or tissues and the biochemistry of the pollutants (Reddy et al., 2005; Chiu et al., 2007). Recently, PBPK modeling has been applied for showing the kinetics of specific environmental pollutants in marine mammals (Hickie et al., 1999, 2005; Weijs et al., 2010b). PBPK models for marine mammals are very helpful since these animals are endangered and protected, so that in vivo exposure experiments cannot be undertaken. *In vitro* exposure tests can be performed in marine mammal derived cell and tissue cultures of blood and liver (McKinney et al., 2006a; Das et al., 2008; Frouin et al., 2010). However, the sampling of liver biopsies or blood from the same animal of a wild marine mammal species is too invasive. Therefore, blubber was the most investigated tissue and is still the best known source of biomonitoring results of lipophilic compounds to date. PBPK models have the potential to integrate in vitro experimental data in liver or blood with biomonitoring information from blubber, so that a non-destructive, *in silico* approach can be developed. With the help of PBPK models, blubber concentrations can be used to predict levels in liver, blood and other tissues thus giving more realistic exposure scenarios than working with hepatic or blood cell lines alone. Since blubber samples can be taken in a non-destructive manner from dead and living marine mammals, the PBPK models would, in addition to the in vitro tests done with liver cells, allow the assessment of toxicity in a more rapid and instantaneous way. Furthermore, PBPK models are important to describe the dynamics of the exposure-accumulation process. This can simulate the impact of releases of pollutants and remedial measures on their long-term fate and accumulation in marine mammals.

Harbour porpoises have relatively long life spans and feed at the top of the aquatic food chains, resulting in high body burdens of pollutants (Weijs et al., 2009a, 2010b). Previous studies have suggested that harbour porpoises are less able to metabolize several pollutants compared to harbour seals. They were found to have higher proportions of PBDEs in their blubber compared to harbour seals even though both species had a comparable diet (Weijs et al., 2009a,b). In addition, hydroxylated PBDE metabolites (HO-PBDEs) could not be detected in serum of harbour porpoises (Weijs et al., 2009d). During the last decade, movements of harbour porpoises from northern areas to the more polluted Southern North Sea have raised concerns about the viability of the population. These movements and their sensitivity to pollution

justify the need for proper conservation measures for these animals. Similarly to models developed for polychlorinated biphenyls (PCBs; Weijs et al., 2011), the goals of the current study were 1) to develop a non-destructive computational toxicology approach to study protected marine mammals such as harbour porpoises by formulating PBPK models for PBDEs, and 2) to assess temporal trends of lifetime exposure to PBDEs in North Sea harbour porpoises using data from 1990 to 2008.

2. Materials and methods

The models developed for PBDEs are based on the PBPK model for the bioaccumulation of PCB 153 in male harbour porpoises (Weijs et al., 2010b). In general, PBPK models for females include processes (e.g. lactation, gestation) which are responsible for the transfer of contaminants to the offspring thereby reducing the overall concentrations of the chemicals in the females. PBPK models for males lack these processes, so the models developed in the present study are suitable for males only. Similar as in Weijs et al. (2010b), all models consisted of 5 compartments, liver, blubber, kidneys, brain and rest of the body. All tissues were considered to be flow limited similar as in Weijs et al. (2010b). Dietary uptake, either through the consumption of fish or milk, was set directly to the liver compartment as the liver was the only tissue of the gastrointestinal tract used in the models. Models were developed and parameterized using Berkeley Madonna software version 8.3.14 (Berkeley Madonna Inc) and model codes are available on request to the corresponding author.

2.1. Parameters

All models were developed using parameters taken from the literature or fitted to the data if clearly indicated. The same physiological parameters and equations of the male harbour porpoises (such as the relationships between body size, age and daily intake) as in Weijs et al. (2010b) were used. Biochemical parameters were adjusted according to the specific PBDE congener (Table 1). The parameters that were fitted (elimination half-lives, assimilation efficiency for the milk or AE2 and brain/blood partition coefficients or PB) were chosen upon visual inspection of the position and shape of the curves compared to the real-life data from animals from the Black Sea. Of these three parameters, elimination half-lives were fitted first as this parameter affects the slope of the curve in each compartment. AE2 was fitted after that as it determined only the concentrations in all compartments for animals < 1 year. PBs only had an impact on the curve of the brain compartment and were therefore fitted last.

Exposure was assumed to be through fish and milk consumption only which were both set in the liver as this compartment was the only tissue of the gastrointestinal tract represented in the models. Lipophilic compounds like PBDEs do not dissolve easily in sea water and dermal exposure was earlier found to play only a negligible role for the bioaccumulation of PCBs in beluga whales (Hickie et al., 1999). Tanabe et al. (1997a) reported on the concentrations of several PCBs, but not of PBDEs, in the fish prey of the harbour porpoises from the Black Sea. The lack of PBDE data in marine mammals and their prey appears to be the norm, especially for Black Sea porpoises. Thus, we relied on PBPK modeling using the best available data to determine the validity of the model. An assumption was made that PBDEs share similar physical and chemical properties (e.g. two phenyl rings with

halogenated atoms, comparable log Kow values for matched congeners) with PCB congeners. This assumption was applied to estimate the dietary fish input of PBDEs using the concentrations of PBDEs in milk samples (n = 7) of Black Sea porpoises. In the PCB 153 model, there was a 116 times difference between the concentration in the milk (127.6 ng/g ww) and the concentration in the fish (1.1 ng/g ww; Tanabe et al., 1997a; Weijs et al., 2010b). This factor, together with the results of the milk samples (Supporting Information Table S1), was used to calculate the concentrations in the fish prey of the Black Sea porpoises leading to a concentration in fish of 0.054, 0.008, 0.009 and 0.002 ng/g ww for PBDE 47, 99, 100 and 153, respectively. Physiological parameters were kept as general as possible by using preferentially physiological information of harbour porpoises in general and not only from Black Sea or North Sea harbour porpoises. This was done to make the models species specific rather than population-specific. As such, the models should allow comparisons between harbour porpoises from different areas (e.g. Black Sea and North Sea).

Table 1. Compound specific parameters for several PBDEs. The original values of the parameters are given between brackets for parameters that were fitted to the data.

	PBDE 47	PBDE 99	PBDE 100	PBDE 153
Log (K_{fp})[a]	2.35750	2.41682	2.41682	2.35750
PF[b]	331.6	380.2	380.2	331.6
PL[b]	7.9	9.0	9.0	7.9
PK[b]	4.6	5.3	5.3	4.6
PB[b]	1.6 (13.3)	2.2 (15.2)	8.2 (15.2)	6.3 (13.3)
PR[b]	8.1	9.2	9.2	8.1
AE1 (%)[c]	95	98	99	97
AE2 (%)[d]	40	31	25	25
CFoetusF[e]	14.1	1.8	2.0	0.3
CFoetusL[e]	5.5	0.8	1.1	0.7
CFoetusK[e]	6.3	5.6	0.5	ND
CFoetusB[e]	1.0	ND	0.2	ND
Half-life (yr)	4.24 (3.1[f])	5.17 (2.9[g])	4.63 (1.6[g])	9.43 (6.5[g])

[a] – Adipose tissue to plasma partition coefficients (K_{fp}) from Parham et al. (1997) are actually for PCBs, but are used here for PBDEs as well; e.g. the log (K_{fp}) for PBDE 47 is actually for PCB 47. Parham et al. (1997) does not report on PCB 100, so for PBDE 100 the same value as for PBDE 99 (or PCB 99) was used.

[b] – Equations from Parham et al. (1997) were transformed to equations for bottlenose dolphins (blood composition from Bossart et al. (2001)). For partition coefficients of tissues (liver, kidneys, brain) as given in Table 1, the average lipid content was used (Weijs et al., 2010a). For the 'rest of the body'-compartment, the average lipid content of muscle was used (Weijs et al., 2010a).

[c] – Assimilation efficiency for the fish diet or the percentage of PBDE absorbed by the juveniles and adults after ingestion of the fish prey. Values taken from Thomas et al. (2005).

[d] – Assimilation efficiency for the milk diet or the percentage of PBDE absorbed by the calves after milk ingestion. Values were fitted to the Black Sea data.

[e] – Results from own analyses (Weijs, unpublished data) and expressed in ng/g lipid weight (lw). For modeling reasons, values of 0.01 ng/g lw were used for concentrations below limit of detection (ND). Muscle tissue of the fetus was not available, so a value of 0.01 ng/g lw was used here for the 'rest of the body' compartment as well.

[f] – Staskal et al. (2005) found a terminal half-life of PBDE 47 in mice of 23 days after a single exposure, which is comparable with the half-life values of TCDD in mice (Miniero et al., 2001). Therefore, the elimination half-life for PBDE 47 in harbour porpoises was derived from the half-life of TCDD which is body weight-dependent (Miniero et al., 2001). The body weight of the harbour porpoises from the Black Sea in the present study varies from 4.1 to 48.5 kg, resulting in half-life values for TCDD (and thus PBDE 47) ranging from 330 days to 5.4 years with an average of 3.1 years.

[g] – Geyer et al. (2004) expressed in years.

2.2. Datasets

The datasets used include results of PBDEs in tissues of harbour porpoises from the Black Sea (milk, liver, blubber, kidney, brain and muscle) for goal 1 and blubber results from the North Sea for goal 2. For goal 1, results of one neonate were also used (Table 1).

- Black Sea dataset

This dataset was used to parameterize the PBDE models (goal 1), similar to the PCB models in male harbour porpoises (Weijs et al., 2010b, 2011). The dataset contains results for 8 PBDEs in 20 male harbour porpoises (9 juveniles, 11 adults). All animals were by-caught or found stranded in 1998 in the Black Sea and results of PBDEs in blubber, liver, kidney, brain and muscle are discussed thoroughly in Weijs et al. (2010a).

- North Sea dataset

The blubber data were used to investigate the usefulness of the PBPK models to predict PBDE accumulation in time (goal 2), as done for PCBs (Weijs et al., 2011). A part of the animals included in this dataset were found stranded or were by-caught along the Belgian coast of the North Sea. These animals were from 1999-2004, the PBDE results in blubber can be found in Weijs et al. (2009a,b). The other part of the animals from this dataset were found alive on the coasts of Belgium and The Netherlands, but died during rehabilitation in SOS Dolfijn, Harderwijk, The Netherlands. These animals were sampled between 1990 and 2008 and data of PBDEs in blubber can be found in Weijs et al. (2010c).

2.3. Sensitivity analysis

To assess the impact of some physiological parameters independent of the body weight on the model outcome, sensitivity analyses were performed as was done previously for PCB 153 (Weijs et al., 2010b). For each parameter, 3 runs (a batch run) were set simultaneously using the original value of the parameter and a coefficient of variation of 5%, resulting in a run with the original parameter, a run with the original parameter increased with 5% and a run with the original parameter decreased with 5%. The impact of the parameter changes on the concentration of PBDE 47, PBDE 99, PBDE 100 and PBDE 153, respectively, in blood was determined by calculating sensitivity coefficients (%) according to the following equation (modified from Mörk and Johanson, 2006):

$$S_c = \left(\frac{AUC_5}{AUC_{Orig}} - 1 \right) 100$$

With AUC_{Orig} the area under the blood concentration curve with the original parameter value and AUC_5 the areas under the blood concentration curves with the original parameter value increased and decreased with 5%. Blood is the circulation medium between all tissue compartments (liver, brain, blubber, kidney and rest of the body), so changes in one or more of these compartments are reflected in the blood. Therefore, the blood concentration curves were used for the sensitivity analyses.

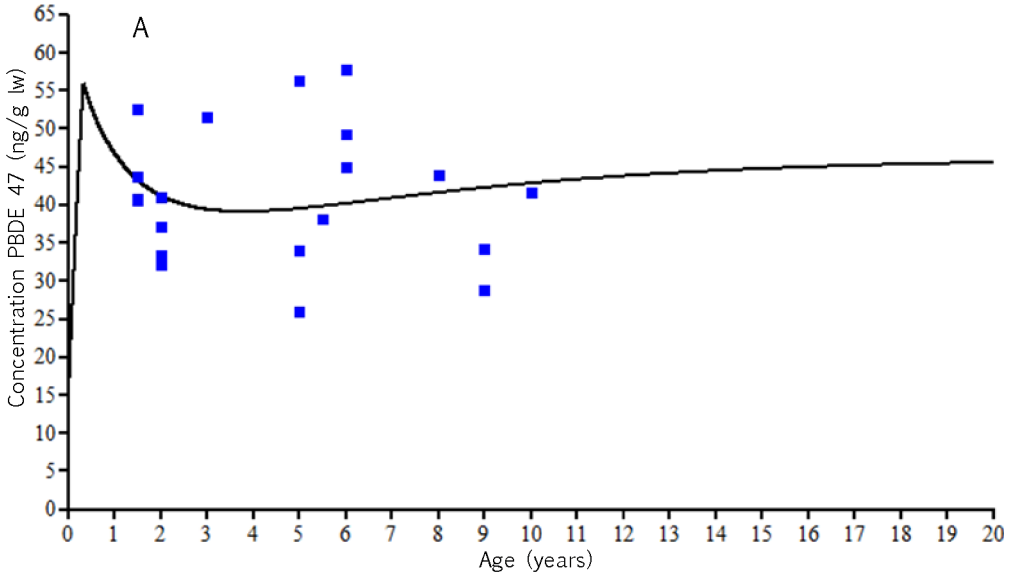
3.Results

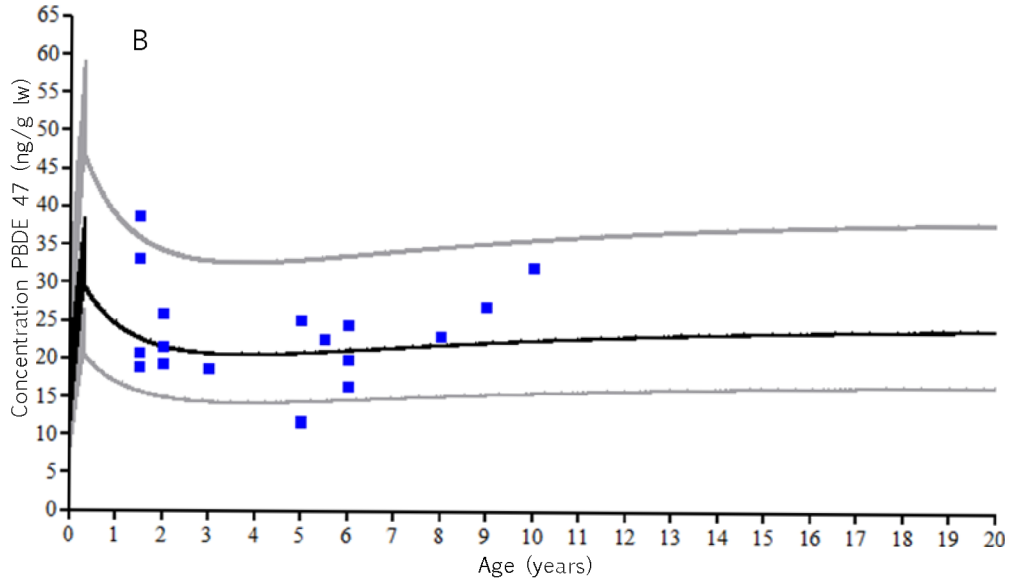
3.1. Goal 1: computational toxicology approach: development of PBPK models for PBDEs

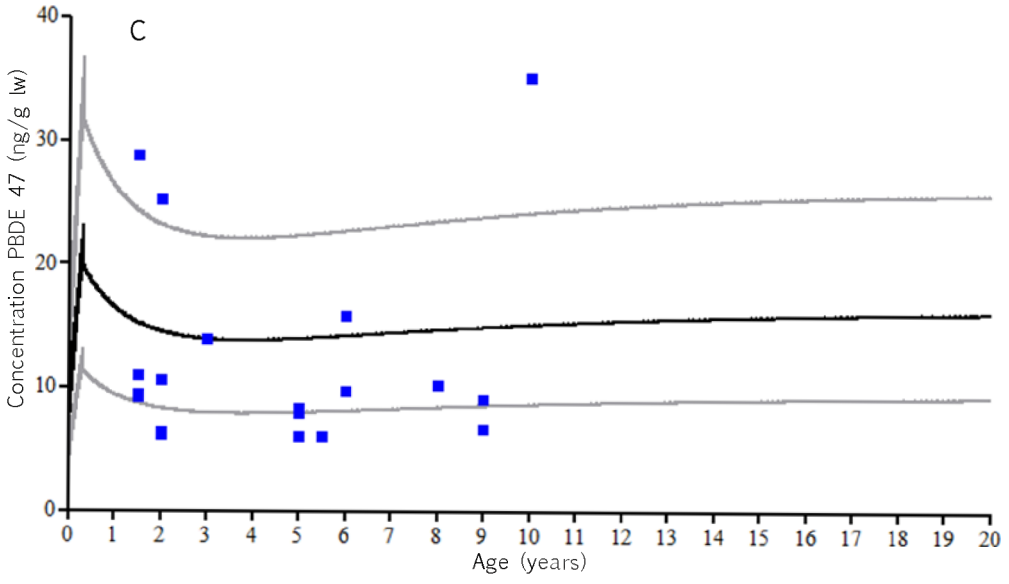
Elimination half-lives of PBDEs, or the time at which 50% of the chemical is eliminated from the body by metabolic biotransformation, fecal or urinary excretion, are scarce in the literature. Geyer et al. (2004) estimated elimination half-lives for several PBDEs in humans (Table 1) which are higher than PBDE half-lives in rodents (Staskal et al., 2005). Nevertheless, considering the continuous exposure and the higher lipid deposits of marine mammals compared to rodents, the human values were here preferred. However, when the human PBDE elimination half-lives were incorporated into the PBPK models, the resulting simulation results were not consistent with the tissue concentration dataset from Black Sea harbour porpoises. Therefore, optimization for suitable elimination half-lives for each PBDE congener was obtained by using the Black Sea dataset (Table 1).

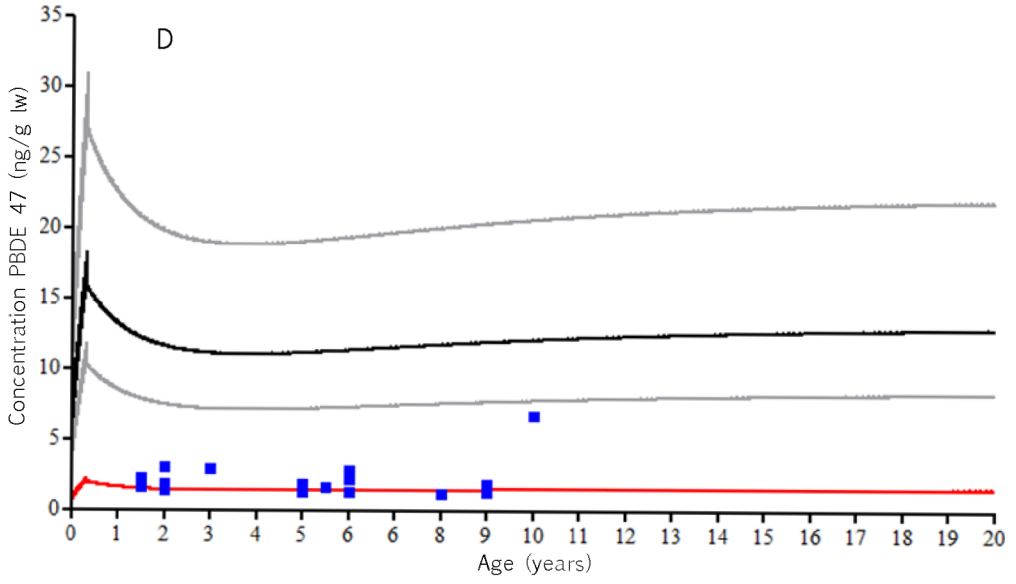
- PBDE 47

When the optimized half-life for PBDE 47 of 4.24 years was incorporated into the PBPK model, the computer simulation results seemed more appropriate, not only for all compartments, but also for all ages (Fig. 1A-E). In all compartments, the model reached a peak in concentrations at the end of lactation followed by a steady decline until the age of about 3 years. After that, levels of PBDE 47 increased slightly in the males until the end of their lives. Despite this increase, the calves at the end of the nursing period were predicted to have the highest concentrations of all ages, as the concentrations of PBDE 47 never reach the same level again according to the model predictions (Fig. 1A-E). Typically, concentrations of PBDE 47 were highest in the blubber, followed by the muscles (rest of the body), liver, kidney and brain (Fig. 1A-E).




B
Concentration PBDE 47 (ng/g lw)
Age (years)


C
Concentration PBDE 47 (ng/g lw)
Age (years)


D
Concentration PBDE 47 (ng/g lw)
Age (years)

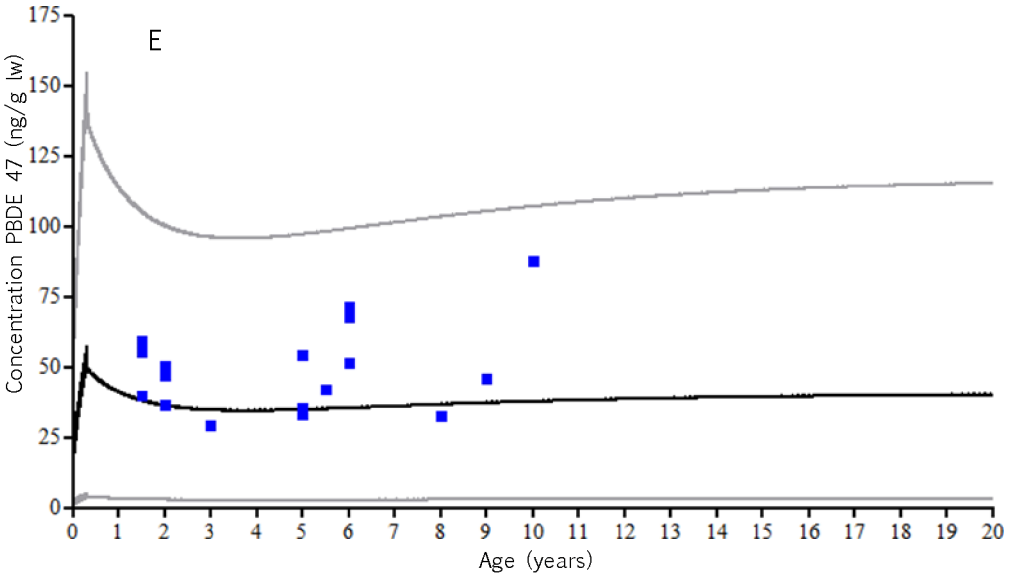


Fig 1. Age-dependent bioaccumulation of PBDE 47 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.

- PBDE 99

According to the model simulations, the half-lives from Geyer et al. (2004) needed to be almost doubled to provide a better fit compared to the Black Sea data (Table 1). The resulting curves reached a peak after the first 4 months due to lactational transfer, followed by a decline until the age of 3 years. The increase after the age of about 3 years was however steeper compared to the increase seen in the PBDE 47 model. Consequently, the predicted concentrations of PBDE 99 from the age of 9 years onward were higher than those in calves at the end of the nursing period (Supporting Information Fig. S1A-E). This pattern was observed in all compartments with the highest concentrations in blubber, followed by the muscle (rest of the body), liver, kidney and brain (Supporting Information Fig. S1A-E).

- PBDE 100

Although the individual data from the Black Sea porpoises were more scattered for PBDE 100 (for example in the liver, Supporting Information Fig. S2B), there was an increase in concentrations of PBDE 100 in all compartments. The final value for the elimination half-life of 4.63 years, fitted by the model, was almost three times higher than the original value of 1.6 years for humans (Geyer et al., 2004). The model predicted a peak concentration at the end of lactation, a decrease in concentrations from the end of lactation until the age of about 3 years and a steep increase after that, resulting in higher concentrations of PBDE 100 in adults than in calves (Supporting Information Fig. S2A-E). Again, higher levels of PBDE 100 were found in blubber, muscle (rest of the body), liver, kidney and brain, respectively (Supporting Information Fig. S2A-E).

- PBDE 153

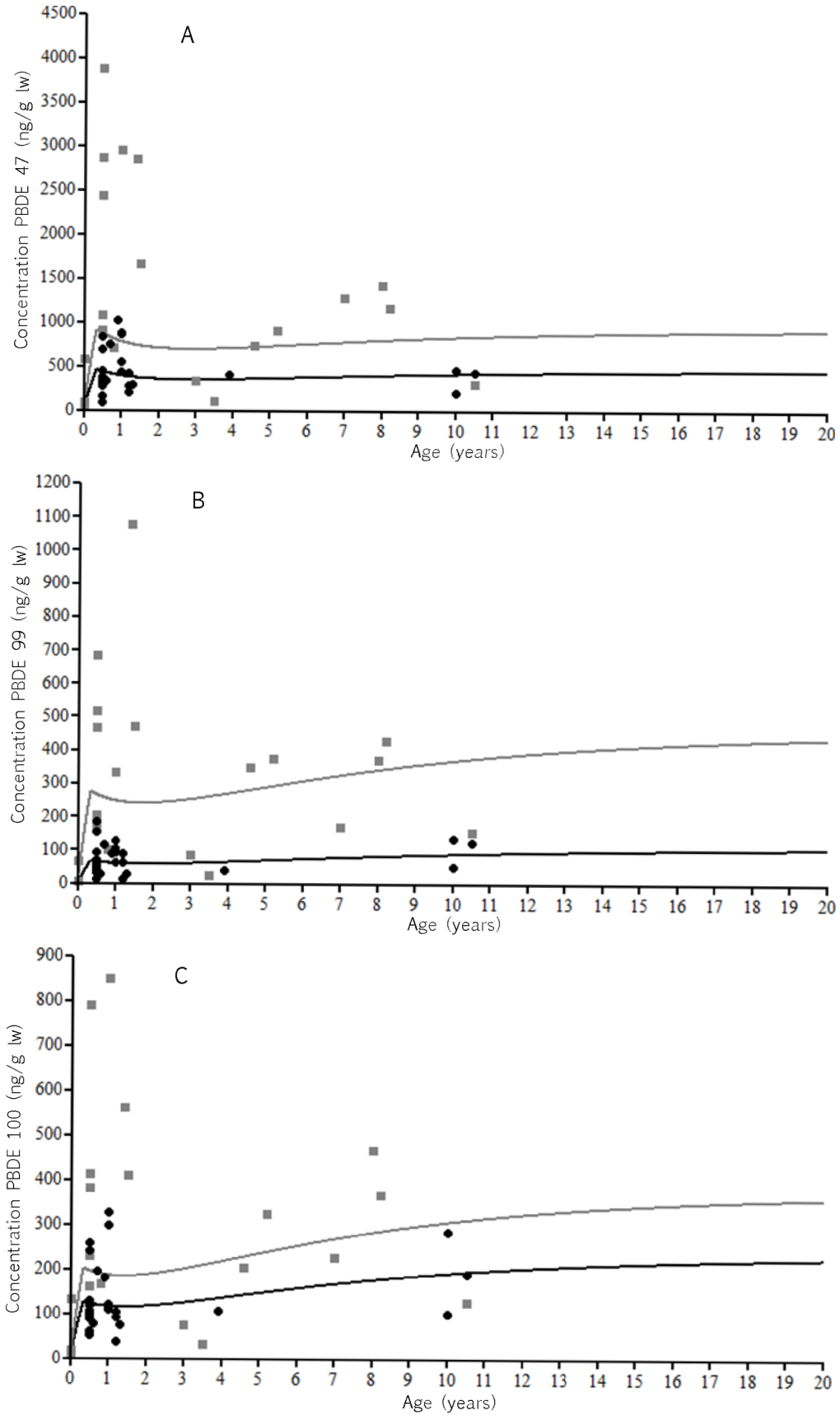
The Black Sea animals lacked a general trend for PBDE 153 in the liver compartment compared to all other tissues (Supporting Information Fig. S3B), which could not be solved by other values for the blood/liver partition coefficient because of the high degree of scattering. In accordance with PBDE 99 and 100, the concentrations in the adult animals after the age of 3 years were higher than the levels in the calves. The final value for the elimination half-life of 9.43 years, fitted by the model, was 50% greater than the literature value of 6.5 years (Geyer et al., 2004). As for the other PBDE congeners, the general profile was conserved also for concentrations of PBDE 153 in all compartments (Supporting Information Fig. S3A-E).

- Sensitivity analysis

Sensitivity coefficients are available in Tables S2-S5 in Supporting Information for the model of PBDE 47, 99, 100 and 153, respectively. In all models, the most sensitive parameters are the parameters related to the blood (e.g. density of blood, fat percentage of blood), followed
by the parameters that are responsible for the intake of fish (e.g. concentration in the fish and assimilation efficiency for the fish diet), the elimination half-life values and some tissue-related parameters (e.g. blood/blubber partition coefficient, density of the liver and density of the blubber. Parameters that are responsible for the intake ofmilk have an impact on the bioaccumulation of PBDE 47 (Table S2), but not on PBDE 99, 100 and 153 (Tables S3-S5).

3.2. Goal 2: assessing temporal trends for PBDEs

Using the PBDE results in blubber of North Sea harbour porpoises from 1990 until 2008 (n = 46) (Weijs et al., 2009a, 2010c), changes over time were investigated. The models used for the time trends were those described in goal 1, except for the specific input values for PBDE concentrations in the fish and in the milk. Although the input parameters changed with reverse dosimetry modeling (PBDE 99: 0.42 to 0.10 ng/g; PBDE 100: 0.38 to 0.24 ng/g; PBDE 47: 1.08 to 0.54 ng/g; PBDE 153: 0.43 to 0.17 ng/g) meaning that they were adjusted in order to find curves that would fit to the North Sea data, the proportion between the concentrations in milk and fish remained unchanged (116 times difference) throughout this modeling exercise. As in Weijs et al. (2011), the North Sea data were initially added to the models per year, but since the input parameters were very similar for data from 1990-2001 and from 2002-2008, all data were pooled in these two groups (Fig. 2A-D). Between the two groups, there were differences with the highest concentrations of PBDEs in the older data (1990-2001). Results reveal that, in a period of about 18 years, levels of PBDE 99 decreased the fastest with 4.2 times lower levels in 2002-2008 than in 1990-2001 (Fig. 2B). Levels of PBDE 100 decreased the slowest with only a 1.6 times difference between concentrations in 1990-2001 compared to 2002-2008 (Fig. 2C). For PBDE 47 and 153, there was a difference of 2.0 and a 2.5 times, respectively, between the levels in 1990-2001 and 2002-2008 (Fig. 2A, D).


A
Concentration PBDE 47 (ng/g lw)
Age (years)
B
Concentration PBDE 99 (ng/g lw)
Age (years)
C
Concentration PBDE 100 (ng/g lw)
Age (years)

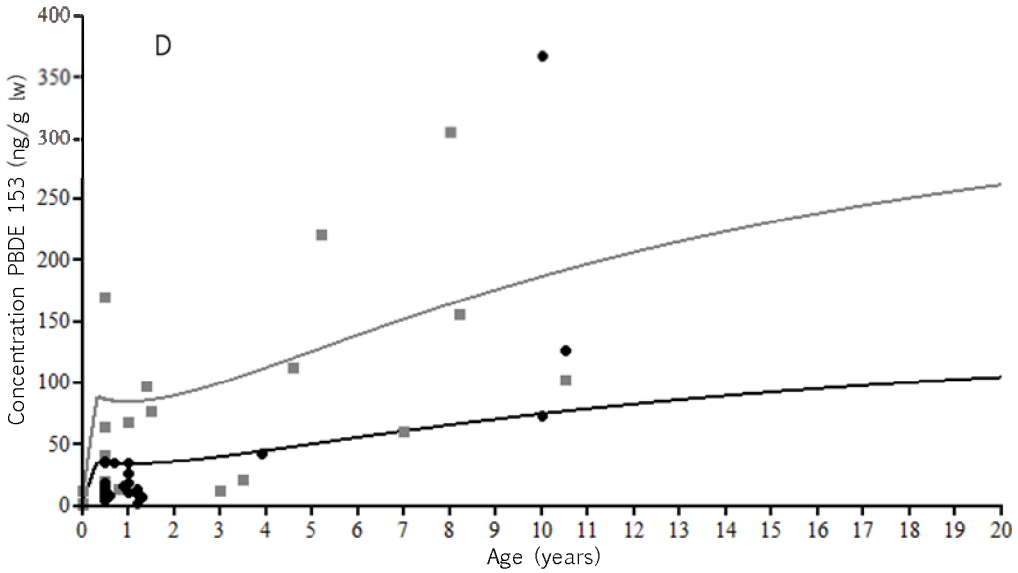


Fig 2. Time trends in age-dependent bioaccumulation of (A) PBDE 47, (B) PBDE 99, (C) PBDE 100 and (D) PBDE 153 in blubber of male harbour porpoises from the North Sea. All concentrations are expressed in ng/g lw. ■ = individual data for male harbour porpoises from the North Sea from 1990-2001, ● = individual data for male harbour porpoises from the North Sea from 2002-2008, — = model prediction for male harbour porpoises from 1990-2001, ▬ = model prediction for male harbour porpoises from 2002-2008.

4. Discussion

Although PBDEs have been banned in the EU in 2004 and 2008, their presence and high levels in wildlife can still be toxic. For decades, risk assessment of pollutants in marine mammals has mainly been performed through biomonitoring on blubber from dead animals. Marine mammals are protected species, so these blubber data are generally the only experimental data available. Recently, in vitro exposure studies in hepatic or blood cells of marine mammals have gained more attention (McKinney et al., 2006a; Das et al., 2008; Frouin et al., 2010). Unfortunately, the connection between reported levels of pollutants in the blubber (from dead or live animals) and the effects found in liver (from dead animals) or blood (from live animals) is often blurred or even non-existent. This can be solved through PBPKmodeling as a non-destructive approach in marine mammal toxicology. It combines computational technology, physiology of the organism and biochemistry of the compound of interest. As such, PBPK modeling is certainly in line with the spirit of reducing or eliminating animal experimentation by the REACH (Registration, Evaluation, Authorization, and Restriction of Chemical substances) legislation in the EU.

4.1. Goal 1: computational toxicology approach: development of PBPK models for PBDEs

The search for reliable parameters necessary to develop PBPK models for marine mammals is a challenging task. Typical experimental model organisms, such as rodents, often have a different physiology from marine mammals and thus the available parameters from other species are not always suitable for marine mammals.

- Elimination half-lives

In PBPK models for PCBs in harbour porpoises, the elimination half-lives were much higher than those reported in the literature, probably due to their larger lipid stores of marine mammals compared to rodents or humans. Because of the size differences between humans and rodents, the longer human elimination half lives were preferred at the first modeling attempt (Geyer et al., 2004). However, model simulations with these half-lives underestimated the Black Sea dataset. To solve this, the fish intake rate (e.g. PBDE concentration in the prey) or the elimination half-lives had to be increased. The fish intake rate was calculated based on the PBDE concentrations in milk of Black Sea porpoises. The difference in PCB 153 concentrations in fish and milk (116 times; Weijs et al., 2010b) was used to estimate the fish PBDE concentrations. Although the validity of these input concentrations cannot be checked, the calculated milk:fish factor of 116 is similar to the milk:fish ratios of several PCBs (Weijs et al., 2011) which have comparable tissue/blood partition coefficients as the PBDEs in the present study (Table 1; Weijs et al., 2011).

Since there was no further available information to calculate new fish intake concentrations, the only solution was thus to increase the elimination half-lives. The elimination half-lives were originally lowest, so faster elimination, for PBDE 100 followed by PBDE 99, 47 and 153, respectively. The elimination half-lives found by the software after fitting to the Black Sea datawere lowest for PBDE 47, followed by PBDE 100, 99 and 153 (Table 1). These findings are in accordance with the results of Lupton et al. (2009) who reported that PBDE 153 was not metabolized by human liver microsomes, but are in contrast with the same study which found that the relative rate of PBDE 99 metabolism was slightly faster than that of PBDE 47 in humans. Lupton et al. (2009) investigated only the metabolic biotransformation of PBDEs, whereas the elimination half-lives in the current models also include fecal and urinary excretion. According to our results, PBDE 153 is more persistent than PBDE 47 in marine mammals, although the concentrations of PBDE 47 are much higher than of PBDE 153 due to a higher input (higher concentrations in prey fish) caused by a higher solubility and bioavailability of PBDE 47. The differences in behavior of PBDEs among species (e.g. rodents, humans) and the lack of information about elimination of PBDEs in marine mammals complicate the search for appropriate elimination half-lives. Nevertheless, within the limits of the PBPK models and the input concentrations used, the current elimination half-lives are the most viable values. The elimination half-life in this study includes elimination through metabolic breakdown and fecal/urinary excretion although the urinary excretion will be very low due to the high lipophilic nature of the four PBDEs. As an overall elimination half-life, it is impossible to distinguish between these three processes in the models. Nevertheless, with the currently available information, the three processes are not equally important and are highly species specific. Since there were no fecal or urine samples of Black Sea porpoises available for analysis, it is impossible to estimate the levels of PBDEs in their feces or urine. Urinary elimination of the parent PBDE 47, one of the most persistent PBDEs, has been described in mice as an important elimination pathway (Örn and Klasson-Wehler, 1998; Staskal et al., 2005). However, other studies have shown that urinary elimination of PBDEs plays only aminor role in rats (Hakk and Letcher, 2003; Emond et al., 2010). The lack of consistency between elimination pathways in mice and rats makes the extrapolation to marine mammals difficult.

PBDEs can be metabolically biotransformed into HO-PBDEs metabolites, but can also be debrominated into lower brominated PBDEs or form bromophenols through a cleavage of the ether bridge (Qiu et al., 2007). Debromination of PBDEs in common sole was more important than the formation of HO-PBDEs (Munschy et al., 2010). This was also confirmed for PBDE 99 for common carp and Chinook salmon, but the end product of debromination differed among species (PBDE 47 for common carp and PBDE 49 for Chinook salmon) (Browne et al., 2009). Contrastingly, human liver cells metabolized PBDEs in vitro primarily to oxidative metabolites rather than through reductive debromination (Lupton et al., 2009; Stapleton et al., 2009). McKinney et al. (2006a) reported insignificant metabolic biotransformation of PBDE 47, 99, 100 and 153 in beluga whales, whereas these compounds were significantly depleted by rat microsomes. De Boer et al. (1998) found no indication for biotransformation of PBDE 47 and 99 in a white beaked dolphin, a sperm whale and a harbour seal. In addition, Weijs et al. (2009d) showed that HO-PBDEs were not detected in serum of harbour porpoises, even though blood is the preferred storage medium because of their higher affinity for plasma proteins than for lipids. This suggests that metabolic biotransformation of PBDEs into HO-PBDEs is probably not an important elimination pathway in marine mammals. Model simulations excluding elimination processes predicted curves with much steeper upward slopes which are obviously not consistent with the dataset from the Black Sea (models not shown). Differences between those models without elimination half-lives and the current models indicate the existence of elimination processes in porpoises. However, the models are not capable of pointing at a specific elimination route.

- Brain PBDE concentrations in relation to lipid composition and blood/brain barrier

Similar to the PBPK models for PCBs in porpoises (Weijs et al., 2011), the blood/brain partition coefficients (PB) calculated with themethod from Parham et al. (1997) and the lipid percentages of the tissues (Weijs et al., 2010 a,b) were too high, leading to overestimations of the real data (Fig. 1D and Supporting Information Fig. S1-3D). In vitro studies have shown that the affinity of PCBs for triglycerides, the major group of lipids in the blubber, is higher than for phospholipids which are more abundant in the brain (Sandermann, 2003). Therefore, the lower PBs for PBDEs can be due to the different lipid composition of the brain compared to all other tissues. On the other hand, it has also been reported that the blood/brain barrier (BBB) is capable of blocking molecules larger than 180 Da (Doolittle et al., 1998) from entering the brain. Since the investigated PBDEs have molecular weights higher than 180 Da (Burreau et al., 1997), both options can be used to explain the lower PBs. In the PBPK models for PCBs, the PB of PCB 99 was comparable to PCB 180 despite their different molecular sizes indicating a higher influence of the lipid composition of the brain (Weijs et al., 2011). However, the role of the brain lipid composition or the presence of a BBB on the uptake of PBDEs in the brain is less predictable, as the PBs are different for each PBDE congener. PBDE 47 has a lower PB than PBDE 153, although their partitioning is similar in other tissues (Table 1). The lower PB of PBDE 47 suggests that the brain is much more efficient in blocking PBDE 47 (molecular weight: 485, Effective Cross Section (ECS): 8.1 Å) from entering the brain than PBDE 153 (molecular weight: 646, ECS: 9.6 Å; Burreau et al., 1997). On the other hand, PCB 153 is also a smaller molecule than PBDE

153 (Burreau et al.,1997), but has the same PB as PBDE 153 (Table 1; Weijs et al., 2010b). Therefore, it appears that the brain lipid composition and not the BBB, is important to explain the distribution of PBDEs in the brain. Contrastingly, PBDE 99 has a lower PB than PCB 99 (Weijs et al., 2011), but the same partition coefficients for the other tissues.
The lower concentrations of PBDEs in the brain compared to blubber or other tissues of porpoises are not uncommon and they have been reported as well in polar bears (Letcher et al., 2009) and striped dolphins (Isobe et al., 2009). However, none of these studies included enough information on the lipid composition of the brain to present this as an alternative explanation next to the presence of a BBB.

- Assimilation efficiency of milk

Levels of PBDEs in harbour porpoise calves depend on the concentrations of the PBDEs in the milk and on the assimilation efficiencies. The concentrations of the individual PBDEs used in the present study were the average of seven milk samples of harbour porpoise mothers from the Black Sea in 1997-1998. The assimilation efficiencies for the uptake of PBDEs through milk in harbour porpoise calves are not reported in the literature so these parameters were fitted in order to achieve good model predictions. The resulting assimilation efficiencies for milk were lower than the assimilation efficiencies for fish. This can indicate that calves are somewhat protected against the PBDEs delivered by the milk of their mothers as they seem to absorb on average only a third of the PBDEs present in the ingested milk (Table 1). To investigate this explanation, PBDEs in feces or urine samples of harbour porpoise calves should be analyzed as the concentrations are expected to be proportionally much higher than in feces or urine samples of juvenile or adult harbour porpoises. Unfortunately, samples of feces and urine were not available for this study in any animal. Nevertheless, even though the calves only absorb a third of the PBDEs present in the ingested milk, the concentrations in the milk are still high enough to cause elevated levels in the body of the calves.

- Sensitivity analysis

For the sensitivity analysis, the changes in blood curves were considered. These changes are directly influenced by the blood parameters which explain the high sensitivity coefficients found for parameters like the density of blood and the lipid percentage of blood. Obviously, the combination of input and output has a large impact on the bioaccumulation of a chemical in an organism. However, as mentioned earlier, the calculated fish concentrations and fitted elimination half-lives are the best approach available as the information about both parameters in the literature is nonexistent. The blubber in marine mammals is the main compartment for storage of lipophilic compounds. Anything that can compromise that, such as the blood/blubber partition coefficient, has logically consequences for the distribution of the chemical in the entire body. The concentrations of PBDE 47 in tissues of the fetus are much higher than the concentrations of the other three PBDEs in the fetus which might explainwhy the PBDE 47 model is more sensitive to small changes in dietary milk input than PBDE 99, 100 and 153.

4.2. Goal 2: assessing temporal trends for PBDEs

Concentrations of PBDEs investigated for North Sea porpoises decreased from 1990 until 2008, but not at the same rate. There was a 4.2 times difference between levels of PBDE 99 in porpoises in 1990-2001 and levels

of PBDE 99 in animals from 2002-2008 (Fig. 2B). For PBDE 153, there is a 2.5 times difference between the two time periods (Fig. 2D) followed by a 2.0 and 1.6 times difference for PBDE 47 and 100, respectively (Fig. 2A, C). The two time periods were not the same as for PCBs where the groups were divided from 1990-2000 and from 2001-2008 (Weijs et al., 2011). Since the production of the Penta-BDE mixture containing the investigated congeners has been banned since 2004, a difference between PBDE levels in animals from 1990-2004 and from 2005-2008 would seem plausible. However, harbour porpoises are top predators in the aquatic food chains and it takes some time to see the result of changes in the production or release of chemicals throughout the food chain. The results show that PBDE concentrations were already decreasing before PBDEs were banned. However, this conclusion should be taken with caution because it might be that the two groups are divided differently with larger sample sizes per year. Although there are reports of increasing PBDE trends in fish and marine mammals (She et al., 2002; Lebeuf et al., 2004), there are also studies reporting the absence of temporal trends (Stapleton et al., 2006) or even decreasing trends in guillemot eggs (Sellström et al., 2003) and pike (Kierkegaard et al., 2004). Harbour porpoises live for about 20 years and are capable of producing a calf each year. Because of the high lipid percentage in the milk, the calves receive high loads of pollutants. In addition, the low ability for eliminating these compounds in calves, juveniles and adults ensure that these high loads are retained in the body of the individual animals and in the population in general. Over 18 years, PBDE levels have decreased only slowly in harbour porpoises, which has obviously health implications for the viability of the population.

5. Conclusions

This is the first study to assess the kinetics of PBDEs in a marine mammal species through PBPK modeling. PBPK models combine physiological information of the species and biochemical information of the pollutant and can connect the typical biomonitoring data with the results of in vitro studies in a non-invasive manner. The parameterization and validation of the PBPK models were executed using PBDE data found in the literature from harbour porpoises from the Black Sea and North Sea. Although some parameters from other species were proven to be inadequate for these models, the final parameterization was carried out by performing computer optimization using data from Black Sea porpoises. Since the models with the optimized parameters were capable of visualizing the slow decrease in PBDE levels in North Sea porpoises from 1990 until 2008, it proves that the use of these parameters is justified for all harbour porpoises. From this perspective, the PBPK models for PBDEs can be used as a framework to improve our knowledge about the kinetics of PBDEs in harbour porpoises and to test future exposure scenarios.

Acknowledgements

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Supplementary Information

1. Results of PBDE 47, PBDE 99, PBDE 100 and PBDE 153 in milk samples of Black Sea harbour porpoises (Table S1)

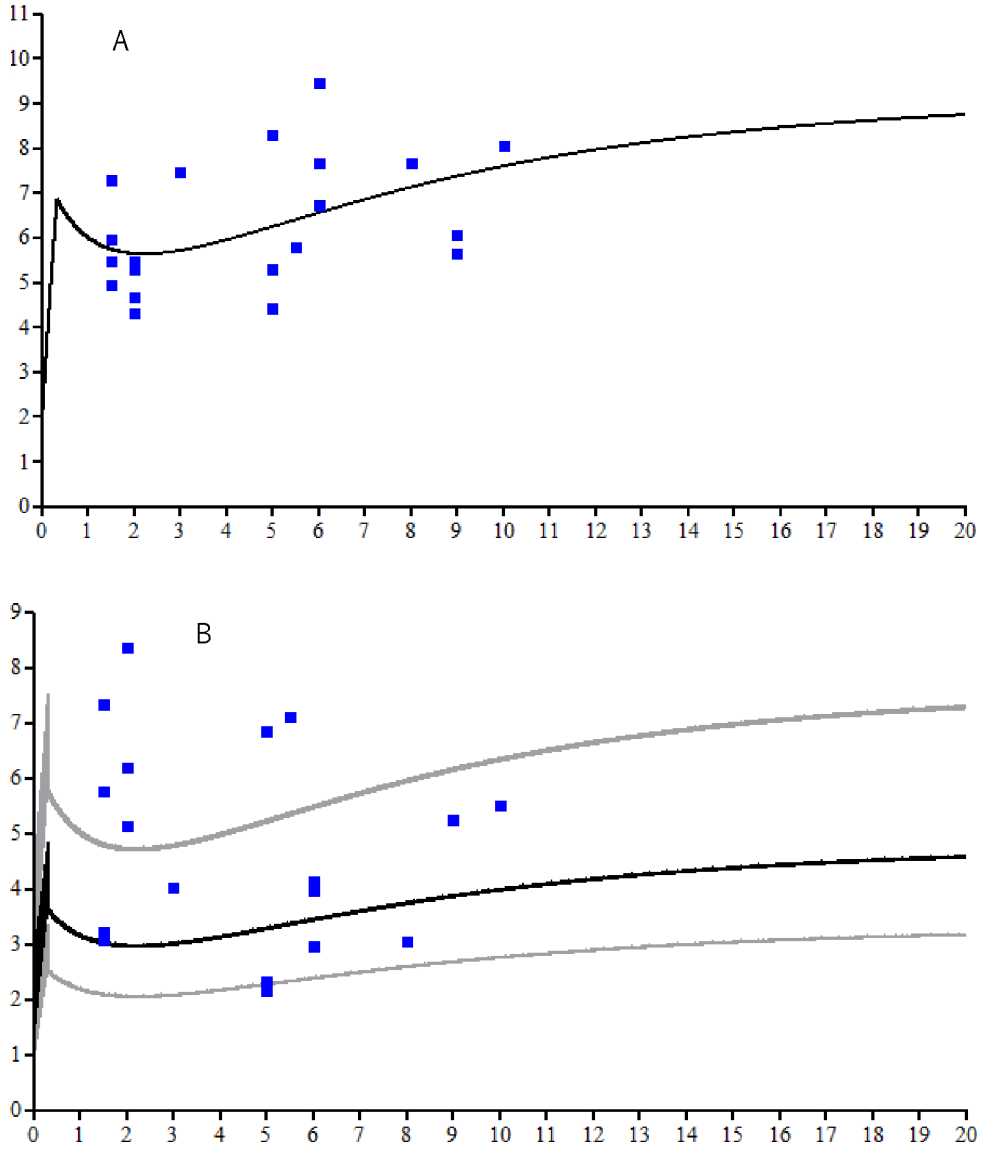
Table S1.
Concentrations of selected PBDEs in milk samples of adult females from the Black Sea. All results are given in ng/g lipid weight (lw). Biological data of the females (age, cause of death) can be found in Weijs et al. (2010b)

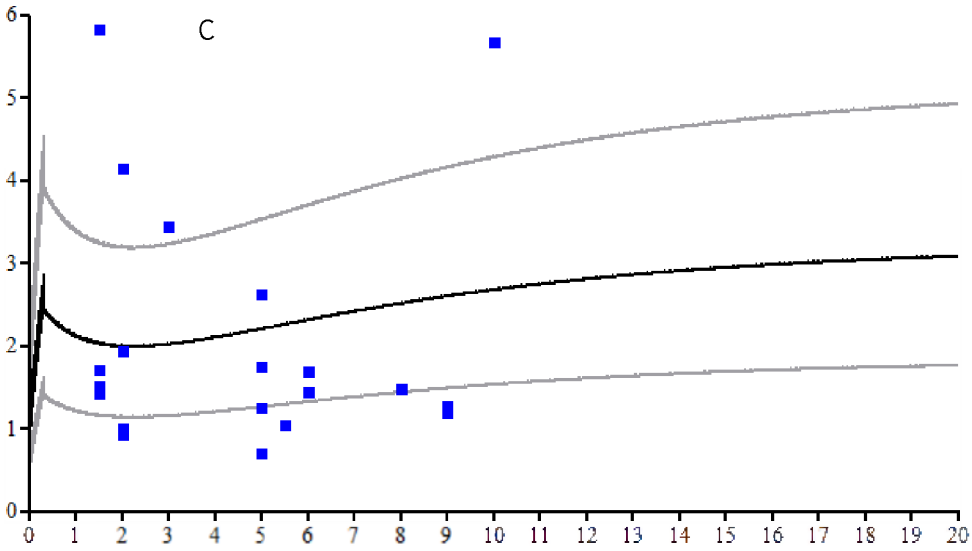
Sample ID	Lipid %	PBDE 47	PBDE 99	PBDE 100	PBDE 153
U 31 (*)	51.8	99.4	10.6	21.8	5.4
U 39	36.9	11.8	1.8	1.7	0.3
U 40	36.6	32.7	5.4	7.2	2.2
U 48	24.7	13.8	2.5	1.8	0.3
U 67	22.4	10.9	2.0	1.6	0.3
U 89	26.1	39.0	5.4	7.4	1.2
U 90	36.9	16.6	2.6	2.7	0.5
U 94	26.0	19.3	3.1	3.7	0.8

(*) Considered as outlier and excluded from the calculation of the average concentration in the milk.

2. Figure S1: PBDE 99 (goal 1)

Fig S1. Age-dependent bioaccumulation of PBDE 99 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.

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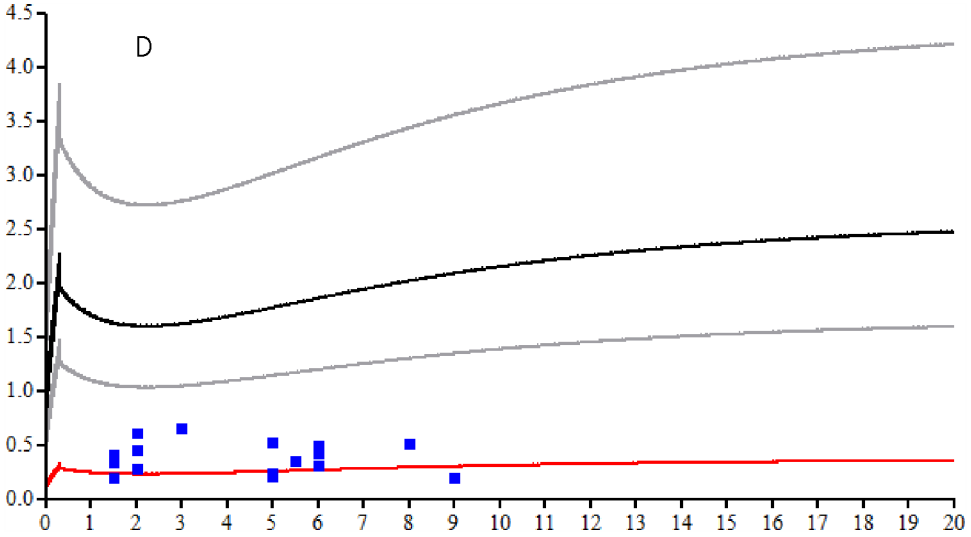
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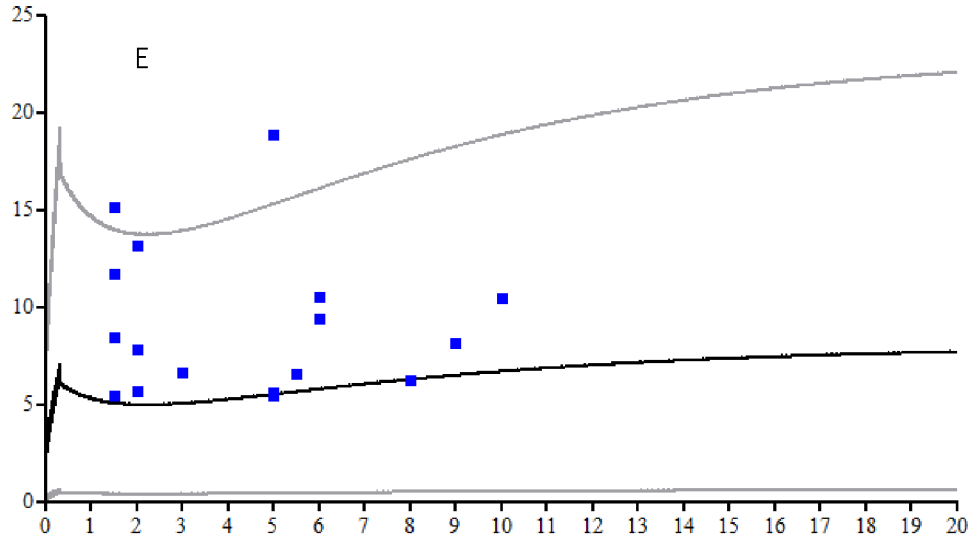
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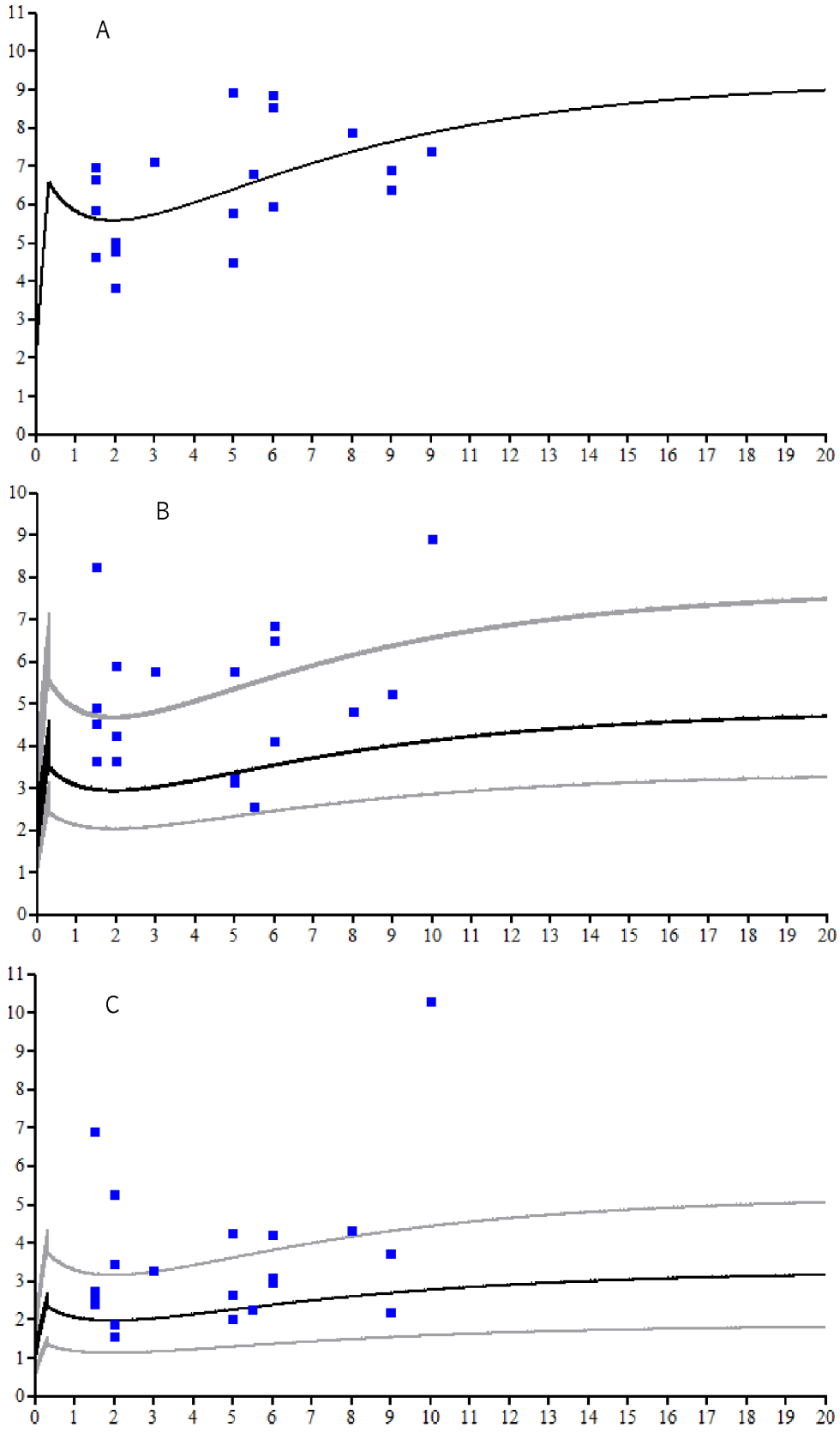
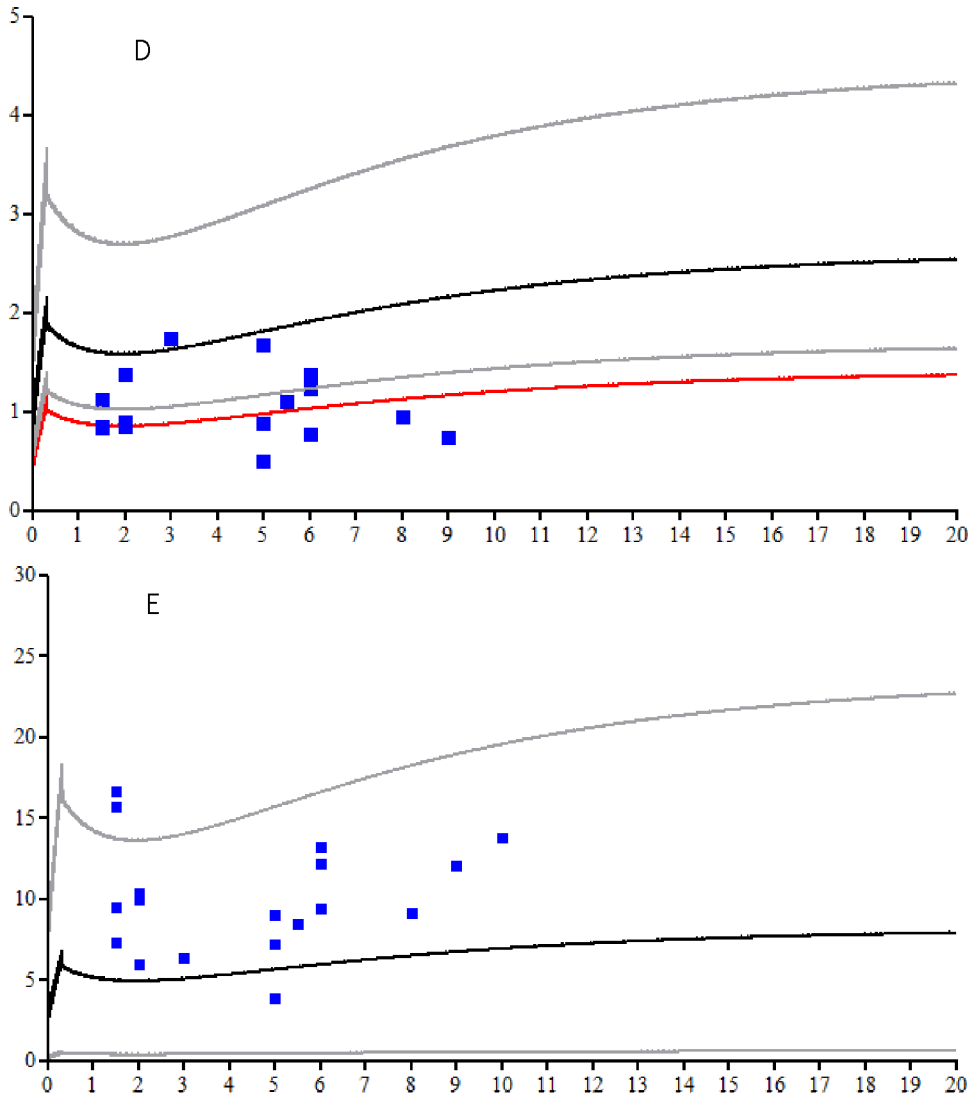
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

3. Figure S2: PBDE 100 (goal 1)

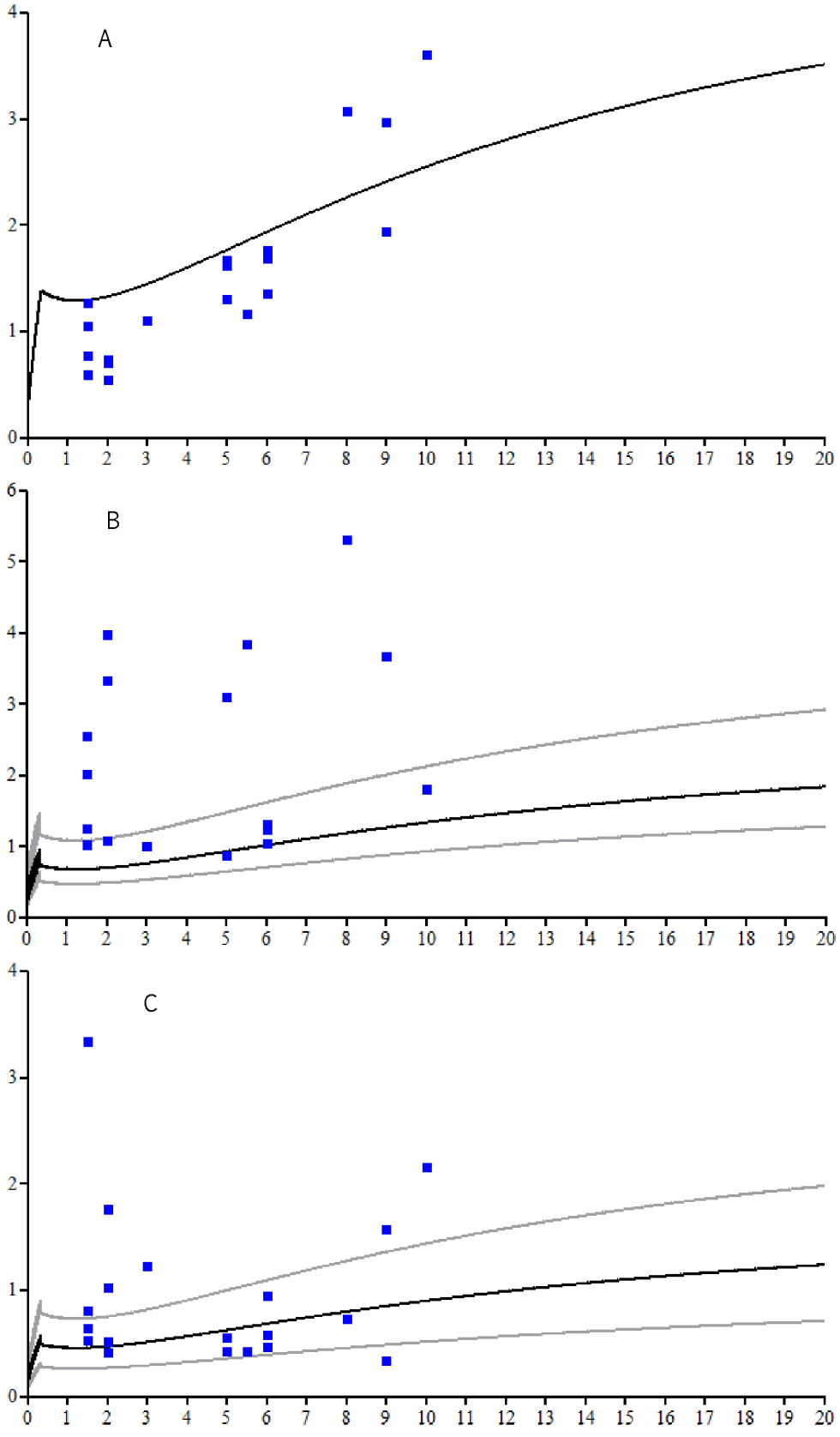
Fig S2. Age-dependent bioaccumulation of PBDE 100 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.

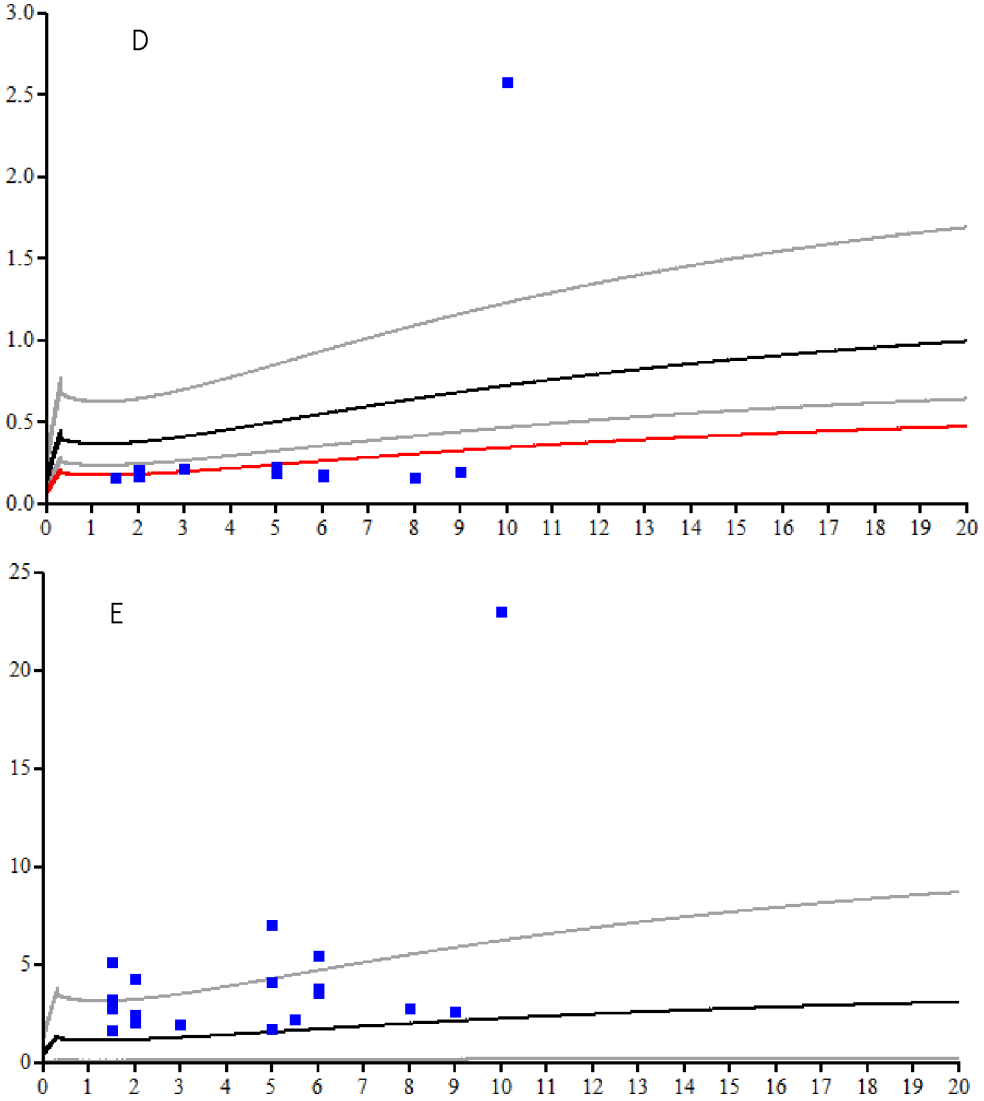



D
E

4. Figure S3: PBDE 153 (goal 1)

Fig S3. Age-dependent bioaccumulation of PBDE 153 (expressed in ng/g lw) in (A) blubber, (B) liver, (C) kidney, (D) brain and (E) muscle (rest of the body) of male harbour porpoises from the Black Sea. ■ = Individual data from male harbour porpoises from the Black Sea from 1998 (Weijs et al., 2010a), — = model prediction with tissue/blood partition coefficient calculated with the average lipid content of the respective tissue, — = model predictions with tissue/blood partition coefficients calculated with the minimum and maximum lipid percentage of the respective tissue, — = model prediction with fitted tissue/blood partition coefficient.





5. Sensitivity analysis for the PBPK models of PBDE 47 (Table S2), PBDE 99 (Table S3), PBDE 100 (Table S4) and PBDE 153 (Table S5)

To assess the impact of some physiological parameters independent of the body weight on the model outcome, sensitivity analyses were performed as was done previously for PCB 153 (Weijs et al., 2010b). For each parameter, 3 runs (a batch run) were set simultaneously using the original value of the parameter and a coefficient of variation of 5%, resulting in a run with the original parameter, a run with the original parameter increased with 5% and a run with the original parameter decreased with 5%. The impact of the parameter changes on the concentration of PBDE 47, PBDE 99, PBDE 100 and PBDE 153, respectively, in blood was determined by calculating sensitivity coefficients (%) according to the following equation (modified from Mörk and Johanson (2006)):

$$S_c = \left(\frac{AUC_5}{AUC_{Orig}} - 1 \right) 100$$

With AUC_{Orig} the area under the blood concentration curve with the original parameter value and AUC_5 the areas under the blood concentration curves with the original parameter value increased and decreased with 5%. Blood is the circulation medium between all tissue compartments (liver, brain, blubber, kidney and rest of the body), so changes in one or more of these compartments are reflected in the blood. Therefore, the blood concentration curves were used for the sensitivity analyses.

Table S2.
Sensitivity coefficients (S_c) indicating how much (in %) the area under the curve of blood concentration curves with the original parameter value ± 5% is different from the area under the curve of the blood concentration curve with the original parameter value in the model for PBDE 47 in male harbour porpoises.

Parameter			S_c	
Name	**Original value**	**Reference**	**- 5 %**	**+ 5 %**
DENSF	920 g/L	Maruyama et al. (2002)	**-1.68**	**1.54**
DENSB	1050 g/L		-0.01	-0.02
DENSL	1040 g/L		**-3.39**	**3.26**
DENSK	1050 g/L		-0.02	0.01
DENSR[1]	1040 g/L		-0.08	0.06
DENSBlood	1068 g/L	Harderwijk, Pers Comm	**5.26**	**-4.75**
FATPERCF	92.85 %	Weijs et al. (2010a)	< 0.01	< 0.01
FATPERCK	3.24 %		< 0.01	< 0.01
FATPERCL	3.73 %		< 0.01	< 0.01
FATPERCB	11.49 %		< 0.01	< 0.01
FATPERCR[1]	2.27 %		< 0.01	< 0.01
FATPERCBlood	0.45 %	Maruyama et al. (2002)	**5.24**	**-4.77**
QFC	5 %	Williams & Leggett (1989) and Brown et al. (1997)[2]	< 0.01	-0.01
QLC	25 %		< 0.01	-0.02
QBC	12 %		< 0.01	-0.01
QKC	19 %		-0.02	-0.02
PF	331.6	Parham et al. (1997), Bossart et al. (2001) and Weijs et al. (2010a)	**1.60**	**-1.58**
PL	7.9		0.01	< 0.01
PB[3]	1.6		0.01	< 0.01
PK	4.6		< 0.01	< 0.01
PR[1]	8.1		0.07	-0.06
TOTDIET	0.054 ng/g ww	Calculated via CMILK	**-3.60**	**3.61**
AE1	95 %	Thomas et al. (2005)	**-3.60**	**3.61**
AE2	40 %	Fitted	**-1.12**	**1.14**
FATPERCMilk	29.94 %	Weijs et al. (2010b)	< 0.01	< 0.01
CMILK	6.21 ng/g ww	Table S1; Present study	**-1.13**	**1.11**
HALF-LIFE	4.24 years	Fitted	**-3.38**	**3.65**

1 - Parameters taken from muscle since muscles are the biggest part of the 'rest of the body'
2 - QRC or fractional blood flow to 'rest of the body' was calculated as 100-QFC-QLC-QKC-QBC
3 - Originally calculated as for PF, PL, PK and PR, but eventually fitted to the data

Table S3.
Sensitivity coefficients (S_c) indicating how much (in %) the area under the curve of blood concentration curves with the original parameter value ± 5% is different from the area under the curve of the blood concentration curve with the original parameter value in the model for PBDE 99 in male harbour porpoises.

Parameter			S_c	
Name	**Original value**	**Reference**	**- 5 %**	**+ 5 %**
DENSF	920 g/L	Maruyama et al. (2002)	**-1.98**	**1.84**
DENSB	1050 g/L		-0.01	< 0.01
DENSL	1040 g/L		**-3.08**	**2.92**
DENSK	1050 g/L		< 0.01	-0.01
DENSR	1040 g/L		-0.08	0.06
DENSBlood	1068 g/L	Harderwijk, Pers Comm	**5.27**	**-4.77**
FATPERCF	92.85 %	Weijs et al. (2010a)	< 0.01	< 0.01
FATPERCK	3.24 %		< 0.01	< 0.01
FATPERCL	3.73 %		< 0.01	< 0.01
FATPERCB	11.49 %		< 0.01	< 0.01
FATPERCR	2.27 %		< 0.01	< 0.01
FATPERCBlood	0.45 %	Maruyama et al. (2002)	**5.28**	**-4.77**
QFC	5 %	Williams & Leggett (1989) and Brown et al. (1997)	< 0.01	-0.02
QLC	25 %		0.01	< 0.01
QBC	12 %		< 0.01	< 0.01
QKC	19 %		-0.01	-0.01
PF	380.2	Parham et al. (1997), Bossart et al. (2001) and Weijs et al. (2010a)	**1.95**	**-1.89**
PL	9.0		< 0.01	< 0.01
PB*	2.2		< 0.01	< 0.01
PK	5.3		< 0.01	< 0.01
PR	9.2		0.07	-0.08
TOTDIET	0.0084 ng/g ww	Calculated via CMILK	**-3.84**	**3.84**
AE1	98 %	Thomas et al. (2005)	**-3.84**	**3.84**
AE2	31 %	Fitted	-0.93	0.95
FATPERCMilk	29.94 %	Weijs et al. (2010b)	< 0.01	< 0.01
CMILK	0.98 ng/g ww	Table S1; Present study	-0.93	0.92
HALF-LIFE	5.17 years	Fitted	**-3.04**	**3.21**

1 - Parameters taken from muscle since muscles are the biggest part of the 'rest of the body'
2 - QRC or fractional blood flow to 'rest of the body' was calculated as 100-QFC-QLC-QKC-QBC
3 - Originally calculated as for PF, PL, PK and PR, but eventually fitted to the data

Table S4.
Sensitivity coefficients (S_c) indicating how much (in %) the area under the curve of blood concentration curves with the original parameter value ± 5% is different from the area under the curve of the blood concentration curve with the original parameter value in the model for PBDE 100 in male harbour porpoises.

Parameter			S_c	
Name	**Original value**	**Reference**	**- 5 %**	**+ 5 %**
DENSF	920 g/L	Maruyama et al. (2002)	**-1.83**	**1.69**
DENSB	1050 g/L		< 0.01	-0.01
DENSL	1040 g/L		**-3.20**	**3.09**
DENSK	1050 g/L		-0.01	< 0.01
DENSR	1040 g/L		-0.08	0.05
DENSBlood	1068 g/L	Harderwijk, Pers Comm	**5.25**	**-4.75**
FATPERCF	92.85 %	Weijs et al. (2010a)	< 0.01	< 0.01
FATPERCK	3.24 %		< 0.01	< 0.01
FATPERCL	3.73 %		< 0.01	< 0.01
FATPERCB	11.49 %		< 0.01	< 0.01
FATPERCR	2.27 %		< 0.01	< 0.01
FATPERCBlood	0.45 %	Maruyama et al. (2002)	**5.28**	**-4.73**
QFC	5 %	Williams & Leggett (1989) and Brown et al. (1997)	0.01	-0.02
QLC	25 %		< 0.01	< 0.01
QBC	12 %		-0.01	-0.01
QKC	19 %		< 0.01	-0.01
PF	380.2	Parham et al. (1997), Bossart et al. (2001) and Weijs et al. (2010a)	**1.77**	**-1.74**
PL	9.0		< 0.01	-0.01
PB*	8.2		< 0.01	< 0.01
PK	5.3		0.01	0.01
PR	9.2		0.06	-0.08
TOTDIET	0.0095 ng/g ww	Calculated via CMILK	**-3.99**	**3.99**
AE1	99 %	Thomas et al. (2005)	**-3.99**	**3.99**
AE2	25 %	Fitted	-0.75	0.76
FATPERCMilk	29.94 %	Weijs et al. (2010b)	< 0.01	< 0.01
CMILK	1.1 ng/g ww	Table S1; Present study	-0.77	0.75
HALF-LIFE	4.63 years	Fitted	**-3.19**	**3.39**

1 - Parameters taken from muscle since muscles are the biggest part of the 'rest of the body'
2 - QRC or fractional blood flow to 'rest of the body' was calculated as 100-QFC-QLC-QKC-QBC
3 - Originally calculated as for PF, PL, PK and PR, but eventually fitted to the data

Table S5.
Sensitivity coefficients (S_c) indicating how much (in %) the area under the curve of blood concentration curves with the original parameter value ± 5% is different from the area under the curve of the blood concentration curve with the original parameter value in the model for PBDE 153 in male harbour porpoises.

Parameter			S_c	
Name	**Original value**	**Reference**	**- 5 %**	**+ 5 %**
DENSF	920 g/L	Maruyama et al. (2002)	**-2.92**	**2.78**
DENSB	1050 g/L		0.01	0.01
DENSL	1040 g/L		**-2.08**	**1.95**
DENSK	1050 g/L		< 0.01	< 0.01
DENSR	1040 g/L		-0.12	0.11
DENSBlood	1068 g/L	Harderwijk, Pers Comm	**5.26**	**-4.75**
FATPERCF	92.85 %	Weijs et al. (2010a)	< 0.01	< 0.01
FATPERCK	3.24 %		< 0.01	< 0.01
FATPERCL	3.73 %		< 0.01	< 0.01
FATPERCB	11.49 %		< 0.01	< 0.01
FATPERCR	2.27 %		< 0.01	< 0.01
FATPERCBlood	0.45 %	Maruyama et al. (2002)	**5.27**	**-4.76**
QFC	5 %	Williams & Leggett (1989) and Brown et al. (1997)	0.02	-0.01
QLC	25 %		< 0.01	0.01
QBC	12 %		< 0.01	< 0.01
QKC	19 %		< 0.01	< 0.01
PF	331.6	Parham et al. (1997), Bossart et al. (2001) and Weijs et al. (2010a)	**2.93**	**-2.77**
PL	7.9		< 0.01	< 0.01
PB*	6.3		0.02	< 0.01
PK	4.6		< 0.01	< 0.01
PR	8.1		0.12	-0.10
TOTDIET	0.0022 ng/g ww	Calculated via CMILK	**-3.99**	**3.99**
AE1	97 %	Thomas et al. (2005)	**-3.99**	**3.99**
AE2	25 %	Fitted	-0.85	0.85
FATPERCMilk	29.94 %	Weijs et al. (2010b)	< 0.01	< 0.01
CMILK	0.253 ng/g ww	Table S1; Present study	-0.86	0.86
HALF-LIFE	9.43 years	Fitted	**-2.01**	**2.08**

1 - Parameters taken from muscle since muscles are the biggest part of the 'rest of the body'
2 - QRC or fractional blood flow to 'rest of the body' was calculated as 100-QFC-QLC-QKC-QBC
3 - Originally calculated as for PF, PL, PK and PR, but eventually fitted to the data

Chapter 6

Bayesian approach and Markov chain Monte Carlo simulations

6.1 Paper X

Application of Bayesian population PBPK modeling and Markov chain Monte Carlo simulations to pesticide kinetics studies in protected marine mammals: DDT, DDE, DDD in harbour porpoises

Liesbeth Weijs, Raymond SH Yang, Krishna Das, Adrian Covaci, Ronny Blust

Abstract

Physiologically based pharmacokinetic (PBPK) modeling in marine mammals is a challenge because of the lack of parameter information and the ban on exposure experiments. To minimize uncertainty and variability, parameter estimation methods are required for the development of reliable PBPK models. The present study is the first to develop PBPK models for the lifetime bioaccumulation of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD in harbour porpoises. In addition, this study is also the first to apply the Bayesian approach executed with Markov chain Monte Carlo simulations using two datasets of harbour porpoises from the Black and North Sea. Parameters from the literature were used as priors for the first "model update" using the Black Sea dataset, the resulting posterior parameters were then used as priors for the second "model update" using the North Sea dataset. As such, PBPK models with parameters specific for harbour porpoises could be strengthened with more robust probability distributions. As the science and biomonitoring effort progress in this area, more datasets will become available to further strengthen and update the parameters in the PBPK models for harbour porpoises as a species anywhere in the world. Further, such an approach could very well be extended to other protected marine mammals.

X

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1. Introduction

p,p'-Dichlorodiphenyltrichloroethane (DDT) has been used for decades as an insecticide. It was used a lot in the second World War during which it helped to protect soldiers from diseases transferred by insects like malaria and it has later played a major role in agriculture (ATSDR, 2002a). The use of DDT in agriculture has been banned in several countries starting in the 1970s in the US and has been included in the Stockholm Convention in 2001. In the 1980s, DDT was replaced by various pyrethroids, but returned eventually as it was proven to be more efficient for mosquito control than pyrethroids. As such, DDT is still used in some countries and continues to be a threat for (marine) wildlife (Mandavilli, 2006).

The technical mixtures are mainly composed of *p,p'*-DDT and *o,p*-DDT, but also include trace amounts of the *o,p* and *p,p'*- isomers of the metabolites, e.g., dichlorodiphenyl-dichloroethylene (DDE) and dichlorodiphenyldichloro ethane (DDD) (ATSDR, 2002a). Metabolically however, DDT can also be transformed into DDE and DDD (ATSDR, 2002a; Kitamura et al., 2002; Tebourbi et al., 2006). Therefore, the profiles or concentrations of DDT and its metabolites found in wildlife are not solely the result of the intrinsic metabolic capacities of the organisms. DDXs can act as endocrine disruptors (Patisaul and Adewale, 2009). As a result, they can interfere with several systems such as the immune system and eventually be a threat to the overall development and survival of the organism (You et al., 1998; Jaga, 2000; Lopez-Espinosa et al., 2009; Torres-Sanchez et al., 2012).

Harbour porpoises are small cetaceans that inhabit the northern hemisphere. They live in relatively shallow waters, such as the North Sea or Black Sea which are both sinks for pollutants coming from intense ship traffic, harbour activities and land run-offs. Consequently, these animals are exposed to large amounts of pollutants that affect many systems, such as the immune or endocrine system (Beineke et al., 2005; Das et al., 2006). In marine mammals, DDT and its metabolites have often been reported (Das et al., 2006; Tilbury et al., 1997; Troisi et al., 2001; Thomas et al., 2005; Wolkers et al., 2008; Weijs et al., 2010a and 2010c). However, since marine mammals are protected from *in vivo* experiments and since the metabolic capacities of marine mammals regarding the biotransformation of environmental pollutants are poorly understood, there is little or no knowledge about the kinetics of DDT and its metabolites in marine mammals. At present, high numbers of harbour porpoises are present in the European North Sea, making them an important part of the food web. As a result, following up on their current situation in terms of toxicology is definitely worthwhile, especially when the compounds of interest are not totally banned.

In silico toxicology, represented by physiologically-based pharmacokinetic (PBPK) modeling in this case, enables us to investigate the different metabolic pathways of DDT, DDE and DDD, as well as the kinetics of each compound separately inside the body of the organism via computer simulation. For pharmaceuticals, PBPK models are usually linked to pharmacokinetic experiments with laboratory animals or humans. For these compounds, PBPK models are often a necessary part of the approval process that a pharmaceutical product has to undergo before being put on the market. For environmentally relevant chemicals, however, the goal of most PBPK models has been to elucidate the kinetics of a chemical in organisms. In that way, these models can be helpful to understand the potential damage that has been done already if they are linked to effect studies, but can also be helpful for risk assessment in future exposure scenarios. The PBPK models

can be linked to experimental work (Lee et al., 2002; Emond et al., 2010), but have also been developed based solely on biomonitoring data (Redding et al., 2008; Weijs et al., 2010b). In the latter case, variable input concentrations, individual variation or scattered data lead to a substantial amount of uncertainty to the overall model. This is where the Bayesian statistical approach and Markov chain Monte Carlo (MCMC) simulations are most useful, with 'prior knowledge', 'randomized parameter sampling from a distribution', and 'updated posterior parameter values and distributions', being the key elements in this technique (Bernillon and Bois, 2000). The 'prior knowledge' provides the opportunity to update the model as it refers to knowledge found in the literature, as well as to knowledge resulting from previous model runs. The 'randomized parameter sampling' gives a more objective estimate for a specific parameter based on an available dataset and its probability distribution where each data point is assumed to have an equal weight of importance. This process can be repeated with each available dataset in order to update or optimize posterior parameter values. Because harbor porpoises and other marine mammals are protected species and experimental work is impossible to conduct in these animals, we are left with only scattered data from dead animals due to stranding or accidental deaths with little or no available knowledge on life history and feeding habits of the animals. Under these limitations, the Bayesian Approach and MCMC analyses offer the only means to minimize the uncertainty associated with the parameter values and probability distributions in our PBPK models. In that sense, Bayesian Population PBPK Modeling utilizing MCMC analyses becomes an ideal and non-invasive approach to assess the toxicology and health risk of environmental pollutants to marine mammals. It also serves as an illustration of the utility and power of *in silico* or computational toxicology.
The objectives of the present study were therefore 1) to elucidate the kinetics of *p,p'*-DDT and its metabolites (*p,p'*-DDD and *p,p'*-DDE) in harbour porpoises using PBPK modeling and 2) to estimate or update species specific parameters with the Bayesian approach and MCMC simulations. To do this, *p,p'*-DDT, *p,p'*-DDD, and *p,p'*-DDE, concentrations in male harbour porpoises reported in previously published studies (Weijs et al., 2009a, 2010a and 2010c) were used.

2. Materials and methods

The development of the PBPK model for lifetime exposure to DDT, DDD and DDE in male harbour porpoises can be separated into two phases: 1) the development of the "Structural PBPK Model" and 2) the estimation/update of some parameters using the Bayesian approach and the MCMC simulations in a "Statistical Model." These "Models" are explained below together with a description of the Black Sea and North Sea datasets which were used to evaluate the models. In this study, the DDT, DDD and DDE mentioned are the *p,p'*-isomers only. Levels of the *o,p*-isomers are much lower in harbour porpoises than the *p,p'*-isomers and were thus not taken into account. The term 'DDXs' is used at some places to represent collectively different forms of DDT, DDE, and DDD.

Black Sea and North Sea datasets. DDE, DDD and DDT concentrations in blubber, brain, kidney, muscle and liver samples from 20 males and one fetus from the Black Sea were used as well as milk samples (Table 1 for milk; Table 2 for fetus and Weijs et al. (2010a) for all other results). These data are referred to as the 'Black Sea dataset' in the present study. In

addition, DDE, DDD and DDT results in blubber from 9 males and 1 neonate from the North Sea were used (animals from 2000-2008 from Weijs et al. (2010c)), as well as blubber samples from 20 males from the North Sea (unpublished data, but same animals from 1999-2004 as discussed in Weijs et al. (2009a)). All results in the animals from the North Sea were pooled and referred to as the North Sea dataset. Animals from the Black Sea and North Sea were victims of accidental by-catch or were found stranded. Extraction and clean-up procedures, details about the GC/MS analysis and QA/QC results are given in (Weijs et al., 2009a, 2010a and 2010c).

Table 1. Concentrations of DDT, DDD, DDE and (sum of DDXs) (expressed in ng/g lw) and lipid percentages of milk samples of female harbour porpoises from the Black Sea in 1998 unless stated otherwise. Sum of DDXs is the sum of all *o,p*-DDXs and *p,p'*-DDXs.

Sample ID	% Lipid	DDT	DDD	DDE	∑ DDXs	Comments
U 31 Milk (°)	51.8	12541	33327	51243	101376	Female (7yr), 1997
U 39 Milk (#)	36.9	917	2164	3152	6476	Female (>1.5yr), 1997
U 40 Milk (°)	36.6	5845	15711	19386	42790	Female (>1.5yr), 1997
U 48 Milk	24.7	899	3027	3391	7626	From female U 48 ($)
U 67 Milk	22.4	467	2294	2710	5649	From female U 67 ($)
U 89 Milk (#)	26.1	4039	13834	17101	36256	No information
U 90 Milk (#)	36.9	1350	3941	5044	10734	From female U 90 ($)
U 94 Milk (#)	26.0	4218	10158	12041	27940	From female U 94 ($)

(°) Outlier; Excluded from further calculations of the average milk concentration

(#) no information available regarding the situation of death (stranded or by-caught)

($) Weijs et al. (2010b)

Table 2. Levels of DDT, DDD and DDE expressed in ng/g lw in tissues of a Black Sea harbour porpoise fetus. Sum of DDXs is the sum of all *o,p*-DDXs and *p,p'*-DDXs.

Tissue	% Lipid	DDT	DDD	DDE	∑ DDXs
Brain	6.5	33.7	240.2	365.4	654.3
Blubber	85.8	989.6	3170.5	3609.7	8039.9
Kidney	5.8	143.9	1097.2	1336.1	2630.0
Liver	2.4	ND	1008.4	1354.0	2455.9

ND – not detected

Structural PBPK Model. The structural PBPK model for the kinetics of DDT, DDE, and DDD is similar to the ones developed for lifetime bioaccumulation of PCBs and PBDEs in male harbour porpoises (Weijs et al., 2010b, 2011 and 2012). For each of the three compounds, the model consisted of 5 compartments [liver, blubber, kidneys, brain and muscle (rest of the body)]. The three models for DDT, DDE, and DDD are inter-connected at the level of the liver compartment, thus the overall model has 15 compartments in total (Fig 1). The entire model includes metabolic biotransformation pathways from the DDT model to the DDD and DDE models, and from the DDD model to the DDE model. While each model is capable of working on its own, the models of DDE and DDD will not give realistic results without being connected to the DDT model. Physiological parameters and equations, such as the growth, the blood flow, cardiac output and daily fish consumption were taken from the PBPK model for PCB 153 in male harbour porpoises (given in Table S1) (Weijs et al., 2010b). Compound specific parameters were either taken from the literature (e.g. assimilation efficiencies or average net absorptions from Thomas et al. (2005) which were 98% for DDT, 99% for DDD and 97% for DDE) or were estimated using the Bayesian/MCMC

approach (Tables 3 and 4). The structural model was developed initially with Berkeley Madonna software; it was later transformed with AcslX/Libero software (AEgis Technologies, Orlando, FL).

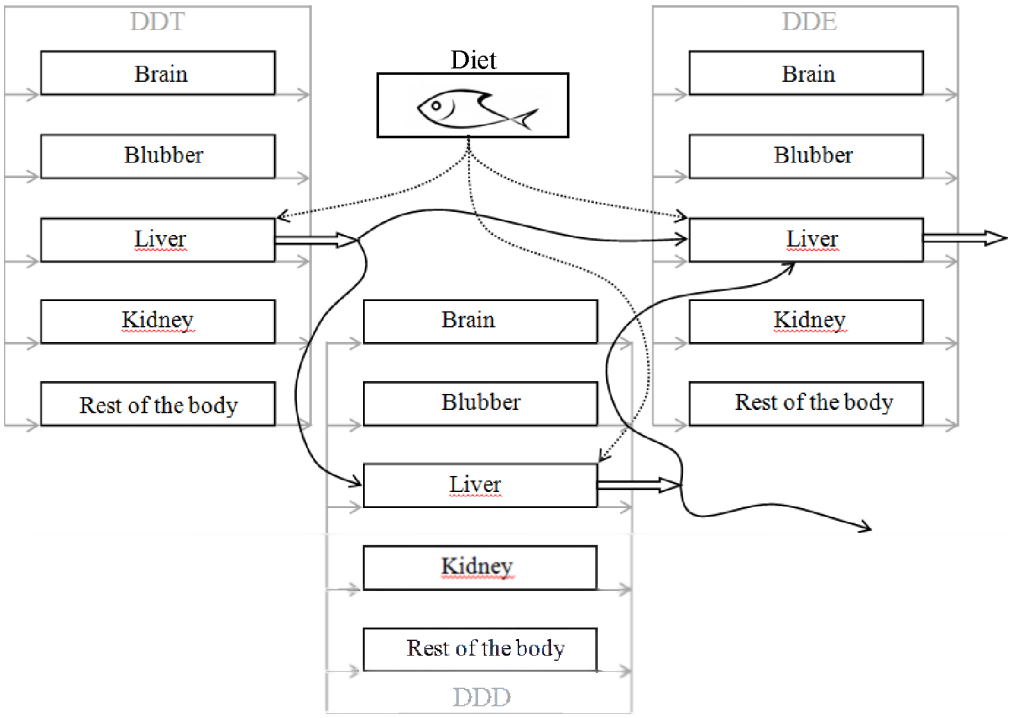


Fig 1. Conceptual diagram of the PBPK model for DDT/DDD/DDE in male harbour porpoises. Solid lines and arrows represent the elimination pathways characterized by their respective elimination half-lives, dotted lines represent the input pathways. DDXs in the milk for the pups and fish diet for the adults are direct inputs into the liver.

Statistical Model. Since there were 49 parameters in the PBPK model for DDT, DDE, and DDD, only a selection of those parameters that were shown to be sensitive in the global sensitivity analyses (GSA) were included in the statistical model. The GSA (Table S2 and Figures S1, S2 and S3 in Supporting Information) followed the scheme of McNally et al. (2011) with the Morris test to de-select the least sensitive parameters followed by the eFAST (extended Fourier amplitude sensitivity) test to quantitatively test the influence of the most sensitive parameters on the model output throughout the entire lifetime of the animals. The Bayesian approach with MCMC simulations allows updating the parameters in a model with every dataset that becomes available. In the present study, two datasets were used; one from male harbour porpoises from the Black Sea, the other from male harbour porpoises from the North Sea. Since the Black Sea dataset was much more extensive compared to the North Sea dataset, the statistical model was run using the Black Sea data first, followed by a second run using the North Sea data. The initial values (priors) of the parameters with their probability distribution characterized by a mean and standard deviation are therefore different for the two model runs (Tables 3 and 4). To allow the MCMC simulation to converge and to calculate convergence factors (R-values), three chains of 15,000 iterations each were used for the statistical model with the Black Sea data and for the statistical model with the North Sea data.

3. Results

3.1. Sensitivity analyses

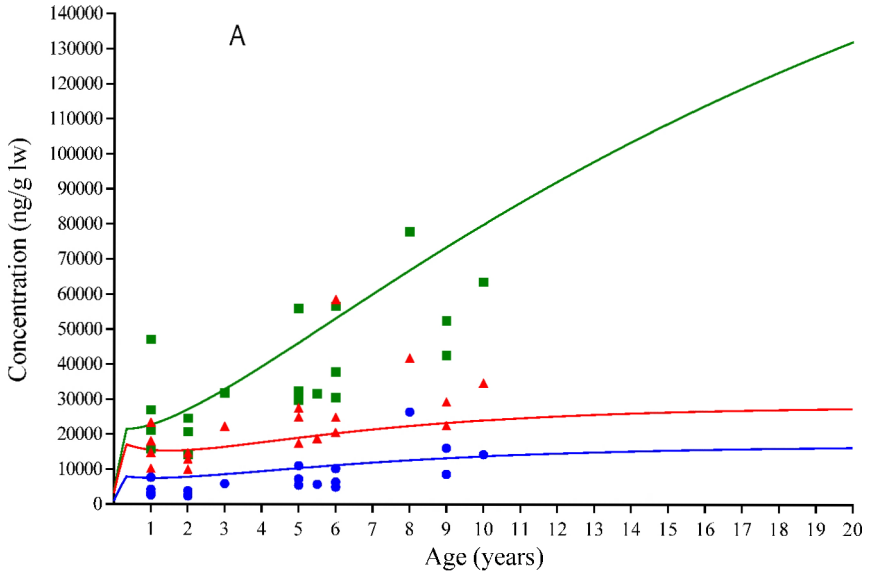
Morris sensitivity tests were performed with the entire set of parameters (49 parameters; Table S2), whereas the eFAST tests were only performed with a subset of parameters (19 parameters) selected based on the availability of parameter information in the literature and the results of the Morris test. Percentages of DDT transforming into DDD and DDE, percentages of DDD transforming into DDE and tissue/blood partition coefficients of DDT and DDD were selected because there was no information about these parameter values in the literature (for DDE in ATSDR, 2002a). Therefore, these parameters needed to be estimated regardless of their sensitivity in the model. Partition coefficients between liver, kidney and blood for the DDD model (PL_DDD and PK_DDD) were not included in the eFAST test exclusively due to practical constraints since the eFAST test in AcslX/Libero does not work with more than 20 parameters. However, they were included in the statistical model and were estimated using the Bayesian approach and MCMC simulations. Concentrations in the fish or milk diet (TOTDIET and CMILK parameters) were selected, because these parameters are generally unknown for most harbour porpoise populations, but were known for the Black Sea dataset. Hepatic elimination rates of DDT, DDE and DDD were initially calculated using elimination half-life values from Verner et al. (2009), so these values were included in the eFAST tests because of their sensitivity in the models.

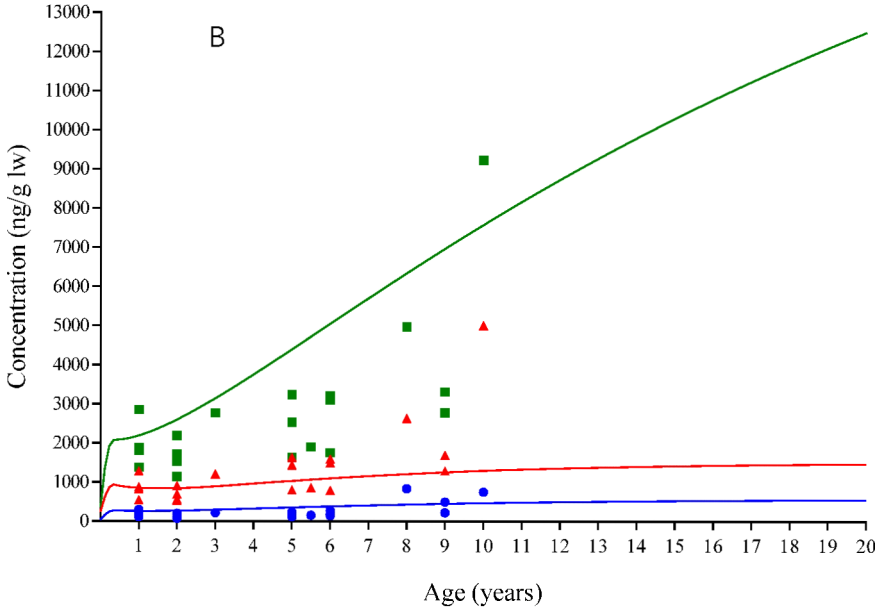
3.2. Selection of parameters in the statistical models for MCMC analyses

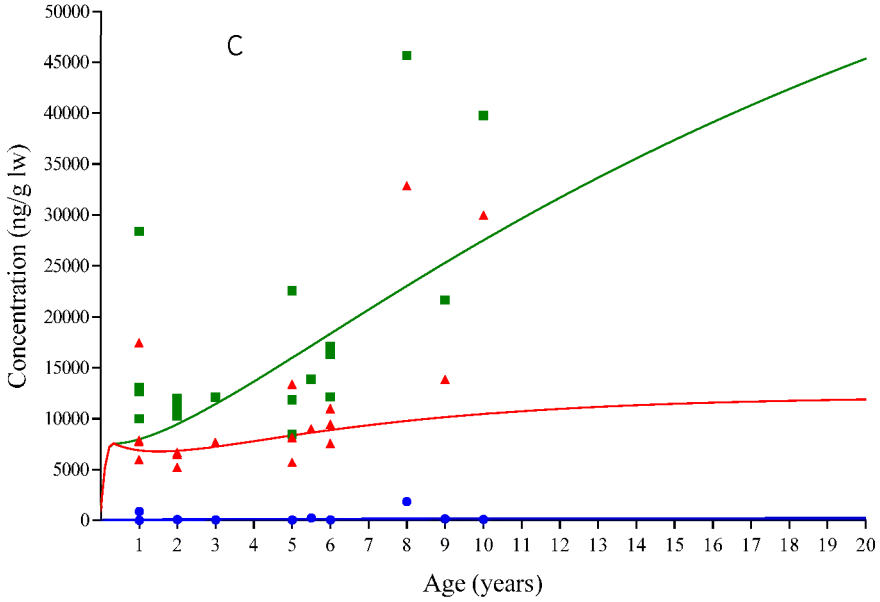
Black Sea: For the Black Sea dataset, the fish diet was assumed to be consisting of European anchovy and whiting (Tanabe et al., 1997a). The levels of total DDXs in these two fish species from the Black Sea were taken from Tanabe et al. (1997b). Concentrations of DDT, DDD and DDE in the fish diet were recalculated according to the composition of DDT (15%), DDD (31%) and DDE (52%) in the total DDTs (Tanabe et al., 1997b) resulting in input concentrations of 33.9, 117.4 and 70.0 ng/g ww, respectively. The concentrations of DDT, DDD and DDE in the milk of Black Sea harbour porpoises were measured in milk samples of female porpoises and the average values (Table 1) were used in the model. Partition coefficients for DDE in various tissues of pregnant rats were available in the literature (ATSDR, 2002a), but partition coefficients for DDD and DDT were not. Likewise, percentages of DDT metabolically transformed into DDD or DDE and of DDD transformed into DDE were scarce in the literature. These parameters were therefore included in the statistical model and estimated using the Bayesian approach. The prior values for these parameters were initially taken from our earlier study on harbour porpoises (Weijs et al., 2010b), but were updated in subsequent range finding model runs (executed with the Bayesian method and MCMC simulations, 5000 iterations). The posterior values of those range finding model runs were then included in the statistical model characterized as a normal distribution with their randomly chosen standard deviation (SD) of 15% and a range of ± 25%. Elimination half-life values for DDE of 15 years and for DDT of 5 years in humans were taken from Verner et al. (2009). Both parameters were also included in the statistical model for further optimization using the Bayesian approach which was also used to estimate the elimination half-life value of DDD. In total, there were 15 parameters included in the statistical model (Table 3). As noted in Table 3, the posterior means were very similar to the prior values. This is not

unexpected since the prior ranges of 10 out of 15 parameters were already updated once with the Bayesian approach/MCMC under the assumption of uniform distributions because of lack of information. The posterior means for the elimination half-lives did not differ much from the prior values either, but had relatively greater SDs compared to all other parameters.

Figure 2 shows all model predictions (curves) and data points in all five compartments male harbour porpoises from the Black Sea. In all compartments or tissues, DDE levels were highest followed by DDD and DDT. Although the trend was less clear for DDT, all levels, as well as the differences in levels of DDE, DDD and DDT appear to increase with age.






C
Concentration (ng/g lw)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
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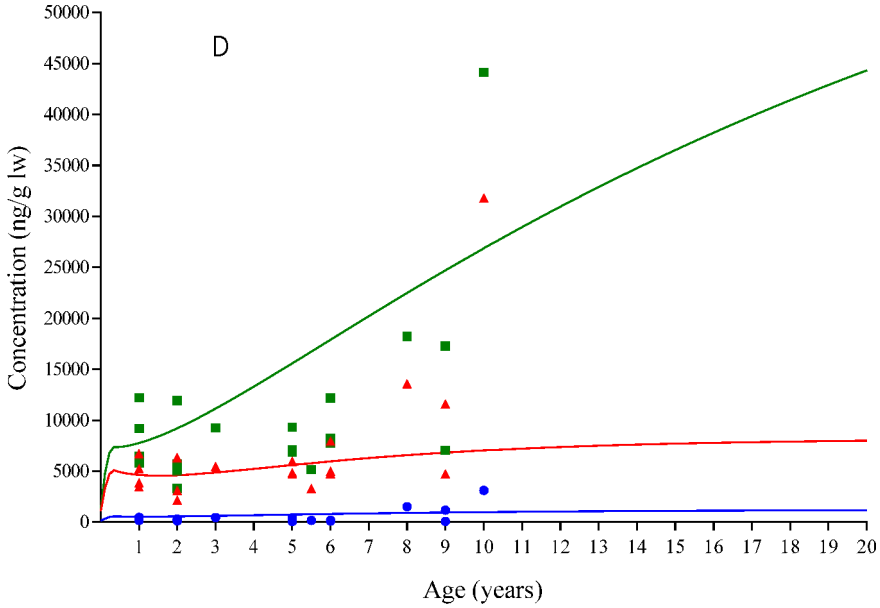
Age (years)


D
Concentration (ng/g lw)
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45000
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Age (years)

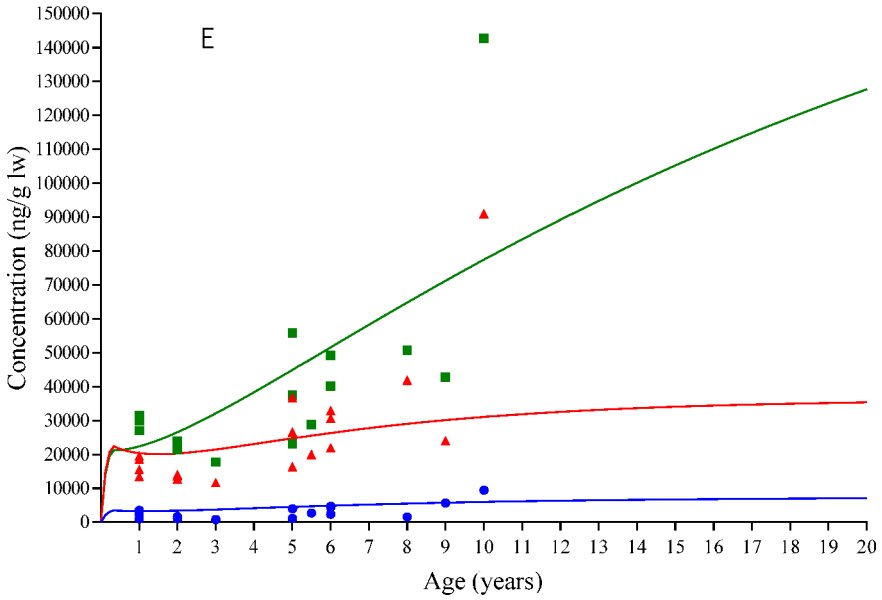

Fig 2. Concentrations of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD in A) blubber, B) brain, C) liver, D) kidney, E) rest of the body (muscle) of Black Sea harbour porpoises. Concentrations are expressed in ng/g lipid weight (lw), the age of the animals in years. __= model predictions with posterior mean values of the parameters of Table 3, ■/▲/● = Black Sea dataset (Weijs et al., 2010a). Green, red and blue curves/data points represent *p,p'*-DDE, *p,p'*- DDD, *p,p'*-DDT, respectively.

Table 3. Prior parameter values, posteriors, updated probability distributions and R-values (convergence factors) of the parameters that were estimated through Bayesian PBPK modeling and MCMC simulations using the Black Sea dataset. For prior information of parameters, an SD of 15% of the Prior Value was included in the statistical model as well which is not given in the table. Prior Ranges were set at ± 25% of the Prior Value.

	Prior			Posterior		
Parameter	Value	Range	Distribution	Mean	SD	R-value
PF_DDT	331.6	248.7-414.5	Normal	331.0	44.2	1.0003
PL_DDT	0.2	0.15-0.25	Normal	0.2	0.0	1.0006
PR_DDT	3.5	2.625-4.375	Normal	3.5	0.5	1.0000
PK_DDT	0.9	0.675-1.125	Normal	0.9	0.1	1.0010
PB_DDT	1.5	1.125-1.875	Normal	1.5	0.2	1.0007
PF_DDD	400.0	300.0-500.0	Normal	400.8	52.9	1.0006
PL_DDD	7.9	5.925-9.875	Normal	7.9	1.1	1.0009
PR_DDD	15.0	11.25-18.75	Normal	15.0	2.0	1.0013
PK_DDD	4.6	3.45-5.75	Normal	4.6	0.6	1.0007
PB_DDD	3.0	2.25-3.75	Normal	3.0	0.4	1.0012
ElimHL_DDE	15.0	11.25-18.75	Normal	11.8	5.7	1.0002
ElimHL_DDD	3.5	2.625-4.375	Normal	3.6	1.4	1.0001
ElimHL_DDT	5.0	3.75-6.25	Normal	4.8	1.8	1.0016
PercDDD	10.0	7.5-12.5	Normal	10.0	1.3	1.0011
PercDDT	50.0	37.5-62.5	Normal	50.2	6.6	1.0003

PF – blood/adipose tissue partition coefficient, PL – blood/liver partition coefficient, PR – blood/muscle (rest of the body) partition coefficient, PK – blood/kidney partition coefficient, PB – blood/brain partition coefficient, ElimHL – elimination half-life value, PercDDT – percentage of DDT that metabolically biotransforms into DDD, PercDDD – percentage of DDD that metabolically biotransforms into DDE.

North Sea: In total, 12 parameters were included in the statistical model (Table 4) which were: TOTALDIET_DDT, TOTALDIET_DDD, TOTALDIET_DDE (concentrations of DDT, DDD and DDE in the fish diet), CMILK_DDT, CMILK_DDD, CMILK_DDE (concentrations of DDT, DDD and DDE in the milk diet), PF_DDT, PF_DDD, PF_DDE (blubber/blood partition coefficients for DDT,

DDD and DDE), PL_DDT, PL_DDD and PL_DDE (liver/blood partition coefficients for DDT, DDD and DDE). These parameters were selected based on the following rationale:

1) The PF and PL parameters were important because they were very "sensitive" to the changes in parameter value according to the results of Morris sensitivity analyses (Table S2). Also, liver and blubber samples of marine mammals are more easily selected for POP analyses compared to muscle samples. Therefore, updating liver or blubber related parameters was given priority.

2) The TOTDIET and CMILK parameters were selected because it was impossible to find any information in the literature. Therefore, the best option was to make assumptions of the priors (parameter values) and their respective probability distributions and let MCMC analyses optimize the parameter values based on the newly available dataset.

For the partition coefficients of DDT and DDD, the prior values (Table 4) were the posterior values (Table 3) from the model runs with the Black Sea data, whereas the range was, similar as for the previous run with the Black Sea dataset, set at ± 25% of the prior parameter value. Partition coefficients for DDE taken from the literature (ATSDR, 2002a) got a SD of 15% and a range of ± 25%. Concentrations in the fish diet (TOTDIET parameters) were assigned a range taken into account the proportion of DDT, DDD and DDE in North Sea fish species (Voorspoels et al., 2004). Concentrations in the milk diet (CMILK parameters) were higher in the Black Sea milk samples than in the Black Sea fish input parameters. Therefore, ranges for CMILK in the North Sea dataset were also assumed to be higher than the input parameters. The DDD, DDE and DDT proportions were also taken into account as was done for the TOTDIET parameters of the North Sea model.

Figure 3 gives all model predictions (curves) and datapoints (squares) in blubber of male harbour porpoises from the North Sea. Similar to the Black Sea dataset, DDE levels were highest followed by DDD and DDT. Although not that clear for DDT, all levels of DDE, DDD and DDT increased with age.

Table 4. Prior parameter values, posteriors, updated probability distributions and R-values (convergence factors) of the parameters that were estimated through Bayesian PBPK modeling and MCMC simulations using the North Sea dataset.

	Prior			Posterior		
	Value	**Range**	**Distribution***	**Mean**	**SD**	**R-value**
TOTDIET_DDE		15-40	Uniform	15.2	0.4	1.0039
TOTDIET_DDT		2-10	Uniform	2.8	0.7	1.0000
TOTDIET_DDD		5-20	Uniform	8.5	2.9	1.0003
CMILK_DDE		50-150	Uniform	63.8	12.4	1.0018
CMILK_DDT		0-50	Uniform	22.0	13.0	1.0003
CMILK_DDD		40-100	Uniform	69.2	15.9	1.0003
PF_DDT	331.0	248.2-413.7	Normal	338.3	34.6	1.0002
PL_DDT	0.2	0.15-0.25	Normal	0.2	0.0	1.0039
PF_DDE	450.0	225.0-562.5	Normal	232.9	9.8	1.0003
PL_DDE	7.0	3.5-14.0	Normal	7.1	1.0	1.0014
PF_DDD	400.8	347.9-543.7	Normal	406.8	41.9	1.0004
PL_DDD	7.9	5.9-9.9	Normal	7.9	0.8	1.0020

* Prior Value, SD (Table 3 for PFs and PLs of DDD and DDT, but not given in this table) and Range (Prior Value ± 25% of Prior Value) are specified for normal distributions, whereas only a Range is specified for uniform distributions.

TOTDIET – concentration of DDE, DDT or DDD in the fish diet of the harbour porpoises, CMILK – concentration of DDE, DDT or DDD in the milk diet of the harbour porpoises, for description of PF, PL see Table 3.

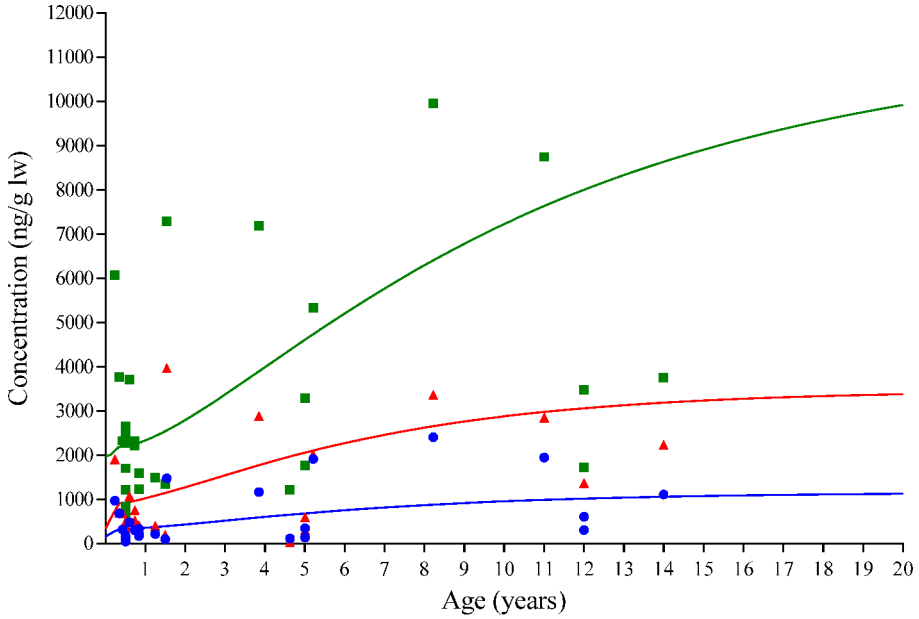

Fig 3. Concentrations of *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD in blubber of North Sea harbour porpoises. Concentrations are expressed in ng/g lw, the age of the animals in years. __= model predictions with posterior mean values of the parameters of Table 4, ■/▲/● = North Sea dataset (Weijs et al., 2010c). Green, red and blue curves/data points represent *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, respectively.

4. Discussion and conclusions

The present study is the first to describe the kinetics of *p,p'*-DDT and its metabolites *p,p'*-DDE and *p,p'*-DDD in multiple tissues of a marine mammal species using Bayesian Population PBPK modeling with MCMC analyses. The importance of this work lies in the fact that DDXs are currently still produced and used as they are more useful for malaria control than alternative substances (pyrethroids). The present models are therefore not only suitable for explaining past exposures but can also be used in a pro-active way for future exposure scenarios.

The presence of DDT, DDD, and DDE in the environment and biota can partly be explained by the composition of the technical mixture and partly by the metabolic biotransformation of the organism. A realistic model should therefore reflect both issues. From the literature it is known that DDT can be converted metabolically into DDE and DDD in mammals, including humans (ATSDR, 2002a) and in birds (Scollon et al., 2012). In these studies, the pathways suggested are from DDT to DDD and from DDT to DDE. In sheep, however, DDE was suggested to appear as a metabolite of both DDT and DDD (Hunnego and Harrison, 1971) and this finding was confirmed later in rats (Fox et al., 1998; Kitamura et al., 2002). In the past, DDXs have been found in marine mammal species, but the origin of DDD and DDE could not be determined. Given the above literature evidence, we believe that two major sources for DDD and DDE in harbor porpoises are through direct food intake and from biotransformation of DDT. Accordingly, the DDT model has only one input factor, the presence of DDT in the fish/milk diet of the porpoises while the DDD model has two input factors, the presence of DDD in the fish/milk diet of the porpoises as well as a percentage of the metabolism of the DDT model. Similarly, the DDE model has three input factors, the presence of DDE in the fish/milk diet of the porpoises, a percentage of the

metabolic endproducts of the DDT model and a percentage of the metabolism of the DDD model. Because of this, the three models are metabolically connected through the liver compartment.

Parameter estimation through the Bayesian approach/MCMC analyses are such that all succeeding available datasets, as well as all the biological and toxicological information associated with the PBPK models, are taken into consideration. Theoretically, during the optimization process, numerous possible values for a given parameter may be suitable for the simulation outcome to be consistent with the available data. However, the Bayesian approach/MCMC analyses narrow down these numerous possible values for the given parameter because all datasets and other biological/toxicological information including the inter-dependence of all parameters are taken into consideration. The resulting final "posteriors" for the updated parameters and their respective probability distributions are therefore the most robust estimates based on the presently available information. It is conceivable, as the science in this domain continues to advance, more and more data will be available for further updates/refinements of the parameters by the Bayesian Approach/MCMC analyses.

4.1. DDX PBPK model for harbour porpoises from the Black Sea

The Black Sea dataset is useful to work with from a modelling perspective. All individuals died in the same region, had limited migration and were at different ages. Furthermore, several tissues, and the subsequent analytical data, are available for each individual; this, plus the dietary information from Tanabe et al. (1997a and 1997b) constitutes a more suitable database than any other harbour porpoise dataset to date. For that reason, the Black Sea dataset was chosen as the first step in the whole modelling exercise. In the Black Sea dataset, only 3 out of 20 animals were found stranded. Victims of by-catch are often considered healthy in contrast to stranded animals. However, the three stranded animals did not visibly stand out compared to the concentrations of the 17 victims of by-catch so all data were pooled. In the model, prior information of the parameters was taken from the literature (e.g. for the elimination half-lives of DDE and DDD) and estimated in short and simple model runs using uniform parameter distributions if necessary (e.g. for some of the partition coefficients). Later, the parameter values were all estimated together in the entire model as the short and simple model runs included only maximum 3 parameters at the same time thereby ignoring potential co-variations between parameters.

The posterior parameter values estimated through the Bayesian approach and MCMC simulations (Table 3) were for most parameters close to the prior parameter values. A potential explanation for this could be that the prior values were, unknowingly, already good enough to represent the Black Sea dataset. On the other hand, there is also the possibility that the Black Sea dataset was not informative enough for some of the parameters to be updated. In both cases, adding more datasets will provide a bigger challenge for the parameters to be updated or optimized.

Compared to the PCB 153 model (Weijs et al., 2010b) or other PCB models (Weijs et al., 2011), the growth dilution effect in the present PBPK models for DDT, DDE, DDD did not seem to play a large role because of the high concentrations in the fish diet for all three compounds. For PCB 153 and other PCB congeners, the growth dilution effect represented a time period in

the entire lifetime of the harbour porpoises where the animals were exposed to relatively lower concentrations of PCBs. The same cannot be found for the PBPK models for DDT, DDD and DDE. Concentrations of DDT were consistently lower than those of DDE and DDD, whereas levels of DDE were highest, a common pattern in marine mammals (Tilbury et al., 1997; Das et al., 2006). In the present study, the DDE levels in blubber were higher than those found in male ringed seals from Svalbard (300 ng/g lw) (Wolkers et al., 2008), in blubber of harbour seals (∑DDXs = 1780 ng/g lw; Troisi et al., 2001) and in blubber of striped dolphins (∑DDXs = 61 100 ng/g lw; Troisi et al., 2001). Das et al. (2006) suggested that the levels of PCBs, DDT, DDE and PBDEs in porpoises from European coasts potentially interfere with the thyroid functions in these animals. Since the levels of DDE found in the younger Black Sea harbour porpoises (the present study) were much higher than those reported by Das et al. (2006), it is possible that even the lowest concentrations in the Black Sea animals are already toxic to the animals. Such risk undoubtedly increases with age as the Black Sea harbour porpoises accumulate higher levels of these pollutants through feeding.

4.2. Further Update of DDX PBPK models Using North Sea Dataset

The sample size of the North Sea dataset was larger than the Black Sea dataset (29 animals versus 20 animals), but smaller in several other aspects. Blubber was the only tissue in the North Sea dataset, animals were from a longer time span (1999-2008) and information about the concentrations of DDXs in the animals' diet was not available in the literature. However, the model with the North Sea dataset could benefit from the posterior parameter information deduced via the model with the Black Sea dataset. For historical samples of marine mammals where the diet was not simultaneously analysed, the pollutant input parameters will always need to be estimated from little or no background information. An alternative for those samples is the possible use of the existing age-dependent bioaccumulation models for the respective chemical (Weijs et al., 2010b, 2011 and 2012) even if input parameters are unknown (reverse dosimetry modelling; Redding et al., 2008). Information regarding the health status was not available for all 29 North Sea animals: 12 were reported as victims of accidental by-catch whereas 4 were found on the beach. Similar as for the Black Sea dataset, concentrations in those 4 animals did not differ from the others upon visual inspection. All data were therefore pooled. For all parameters other than the input parameters, the posterior values of the model with the North Sea dataset were, where possible, based on and estimated with the posterior values of the Black Sea data. Consequently, the model with the North Sea dataset covers the Black Sea data as well. There was a big difference between the prior blubber/blood partition coefficient value of 450 for DDE and the posterior value of 233 even though the prior value worked fine for the model with the Black Sea dataset. This low partition coefficient could be due to the North Sea dataset where data are more scattered than in the Black Sea dataset. The Bayesian approach does not distinguish between these data and does not recognize outliers. By excluding outliers in the North Sea dataset, the value of this parameter will change. However, outliers or exceptions are undoubtedly part of any dataset in wild populations and will perhaps be more 'normal' in datasets with larger sample sizes or when more datasets are included in the model. Therefore, the decision was made to keep the parameter value of 233 and test it in the future whenever more, bigger and/or better datasets become available.

With respect to the North Sea data, it is important to note that there were no data of DDXs in tissues other than blubber. Thus, parameters for liver, brain, kidney, and 'rest of the body' could not be updated in this iteration. The accuracy of these other parameters could therefore benefit from further modelling with more elaborate datasets. Similar to the Black Sea model predictions, the DDE concentrations were highest, followed by DDD and DDT. For all three compounds, concentrations were increasing over the entire lifetime of the porpoises with no growth dilution effect. Levels of DDE from the present study were about 5 times higher compared to DDE levels reported in Das et al. (2006), but approximately 13 times lower than the levels in the Black Sea dataset.

4.3. Future Perspectives

In this work, we demonstrated the utility of refined and updated parameter values of PBPK models for DDXs using Bayesian Population PBPK modelling and MCMC analyses. As indicated earlier, such an approach may be repeated when new data become available. Assuming that all future datasets are sufficiently informative, the posterior distribution ranges will become narrower than the prior ones and all resulting updates will encompass all earlier datasets. Having narrower posterior distribution ranges for the parameter values with every update has two advantages for the entire model:

First, the model uncertainty will be reduced until, in theory, only the model variability is left. As the model variability reflects the differences between individuals, this is an intrinsic source of variation in the model that cannot be reduced by the Bayesian approach. However, since marine mammals in general are not typical organisms used in experiments, there is a great deal of variability between wild individuals which we do not know. Consequently, the variability in the model parameters will present a challenge for all future investigators of marine mammals.

Second, by using knowledge from previous studies to get posterior parameter distributions, the updated PBPK model and parameters not only reflect the situation in the current dataset, but also inclusive of the datasets from previous studies. Such an integration of computational technology and chemical, physiological and toxicological information reflects the essence of systems biology. Altogether, the Bayesian Population PBPK modelling and MCMC analyses approach applied here may lead to a useful, reliable, species-specific model or tool with parameter ranges that mimic the variability between individuals in reality. This is a very powerful and efficient way to assess pollution and risk in these protected wild marine mammal species.

Acknowledgements

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Supporting Information

Table S1. Parameters used for PBPK model calibration of male harbour porpoises. Table modified from Weijs et al. (2010b).

Parameter	Value/equation
Body Weight (BW; g)[a]	$1.387 \times BS^{2.076}$
Body Size (BS; cm)[b]	$142.4 \times (1-0.3751\ e^{(-0.000068 \times age)})$
Cardiac output (QC; L/hr)[c]	$(0.1017 \times (BW/1000)^{0.9988}) \times 60$
Compartment mass (g):	
blubber (V_F)[d]	$18.41 \times BW^{0.607}$
brain (V_B)[d]	$49.20 \times BW^{0.211}$
liver (V_L)[d]	$0.060 \times BW^{0.932}$
kidney (V_K)[d]	$0.002 \times BW^{1.137}$
muscle (V_R)	$(0.99 \times BW) - (V_F + V_B + V_L + V_K + V_{Blood})$
blood (V_{Blood})[e]	$0.143 \times BW$
Density (DENS; g/L):	
Blubber (F)[f]	920
Brain (B)[f]	1050
Liver (L)[f]	1040
Kidney (K)[f]	1050
Muscle (R)[f]	1040
Blood[g]	1068
Fractional blood flow (%)[h]:	
to blubber (Q_FC)	5
to brain (Q_BC)	12
to liver (Q_LC)	25
to kidney (Q_KC)	19
to muscle (Q_RC)	$100 - (Q_FC + Q_BC + Q_LC + Q_KC)$
Lipid percentage (FATPERC; %)[i]:	
blubber	92.85
liver	3.73
kidney	3.24
brain	11.49
blood	0.45
muscle	2.27
Daily consumption of milk (DCMILK; g/day)[k]	540
Daily consumption of fish (DC; g/day)[l]	$0.123 \times BW^{0.80}$

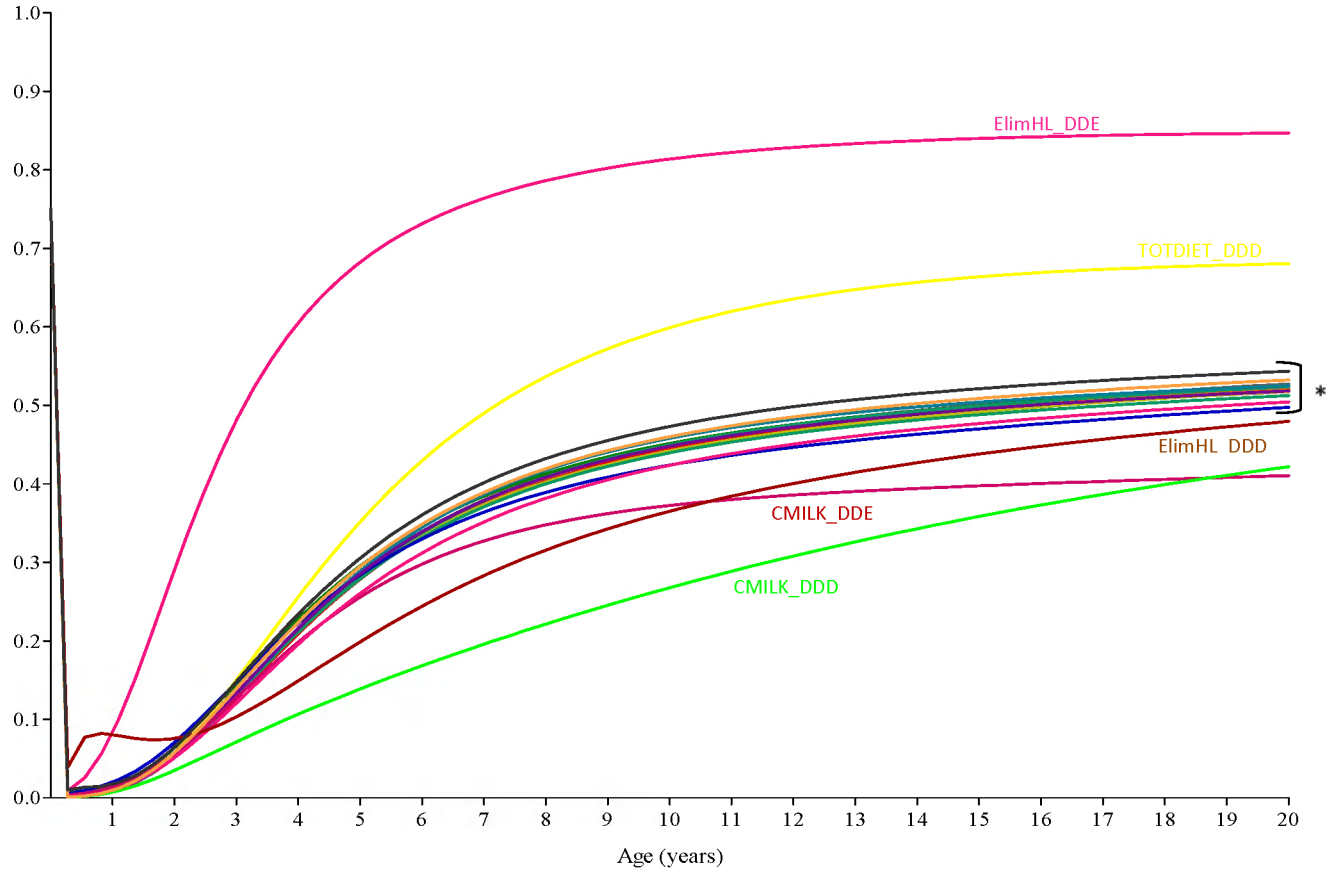
a-correlations developed using existing data from male harbour porpoises from the literature; b-Von Bertalanffy age dependent growth-curves developed using existing data male harbour porpoises from the literature; c-Altman and Dittmer (1971); d-McLellan et al. (2002); e-Reed et al. (2000); f-Maruyama et al. (2002); g-Dolfinarium Harderwijk, The Netherlands, personal communication; h-Williams and Leggett (1989), Brown et al. (1997); i-Weijs et al. (2010a); k-Oftedal (1997); l-Innes et al. (1987)

Table S2. Results of the Morris sensitivity test. The influence of changes in all 49 parameters was tested on the concentration of DDT, DDE and DDD in the blood, respectively, as this medium connects all compartments. Parameter ranges were broad, but arbitrarily chosen. The Morris test yields two sensitivity measures for each parameter: μ as an overall sensitivity measure and σ as an indication for a parameter interacting with other parameters or a parameter with a non-linear effect (McNally et al., 2011). According to the values of μ and σ, parameters were divided into 3 categories: I) *very sensitive* (values for μ and σ), II) *intermediate sensitive* (values for μ but σ values equal to zero) and III) *not sensitive* (extremely low values for μ or μ values equal to zero, σ values equal to zero). Parameters in grey were de-selected for the eFAST tests.

Parameter	Range	cBlood_DDT	cBlood_DDE	cBlood_DDD
PF_DDT	200 - 500	I	II	III
PR_DDT	0 - 15	I	I	I
PL_DDT	0.1 – 10	II	III	III
PB_DDT	0.1 – 20	II	III	III
PK_DDT	0.1 – 10	II	III	III
PF_DDE	200 - 500	III	II	III
PR_DDE	0 - 15	II	II	I
PL_DDE	0.1 – 10	III	II	III
PB_DDE	0.1 – 20	III	III	III
PK_DDE	0.1 – 10	III	II	III
PF_DDD	200 - 500	III	III	I
PR_DDD	0 - 15	II	II	II
PL_DDD	0.1 – 10	III	III	III
PB_DDD	0.1 – 20	III	III	III
PK_DDD	0.1 – 10	III	III	III
TOTDIET_DDT	10 - 60	I	I	II
CMILK_DDT	400 – 12 000	I	II	III
DCMILK	300 - 800	I	I	I
IN_DIET_DDT	0.5 – 1	I	I	I
IN_MILK_DDT	0.5 – 1	II	I	II
TOTDIET_DDE	50 - 150	III	II	III
CMILK_DDE	2700 – 51 200	III	II	III
IN_DIET_DDE	0.5 – 1	III	I	III
IN_MILK_DDE	0.5 – 1	III	II	III
TOTDIET_DDD	30 - 120	III	II	I
CMILK_DDD	2300 – 33 000	III	II	I
IN_DIET_DDD	0.5 – 1	III	II	I
IN_MILK_DDD	0.5 – 1	III	II	I
QLC	0.2 - 0.4	II	III	II
QFC	0.01 – 0.2	II	III	III
QBC	0.1 - 0.2	III	III	III
QKC	0.1 - 0.2	III	III	III
DENSL	1000 - 1200	II	II	II
FATPERCL	0.01 – 0.2	III	III	III
DENSF	850 - 1100	II	II	I
FATPERCF	0.65 - 0.99	III	III	III
DENSB	1000 - 1200	III	III	III
FATPERCB	0.03 – 0.3	III	III	III
DENSK	1000 - 1200	III	III	III
FATPERCK	0.01 - 0.15	III	III	III
DENSR	1000 - 1200	II	III	III
FATPERCR	0.01 - 0.15	III	III	III
DENSBlood	1000 - 1200	II	II	I
FATPERCBlood	0.0001 – 0.01	I	I	I
ElimHL_DDT	0.0001 – 7	I	II	I
ElimHL_DDE	0.0001 – 7	III	I	III
ElimHL_DDD	0.0001 – 7	III	III	I
PercDDT	0.1 - 0.7	III	II	I
PercDDD	0.01 – 0.5	III	II	III

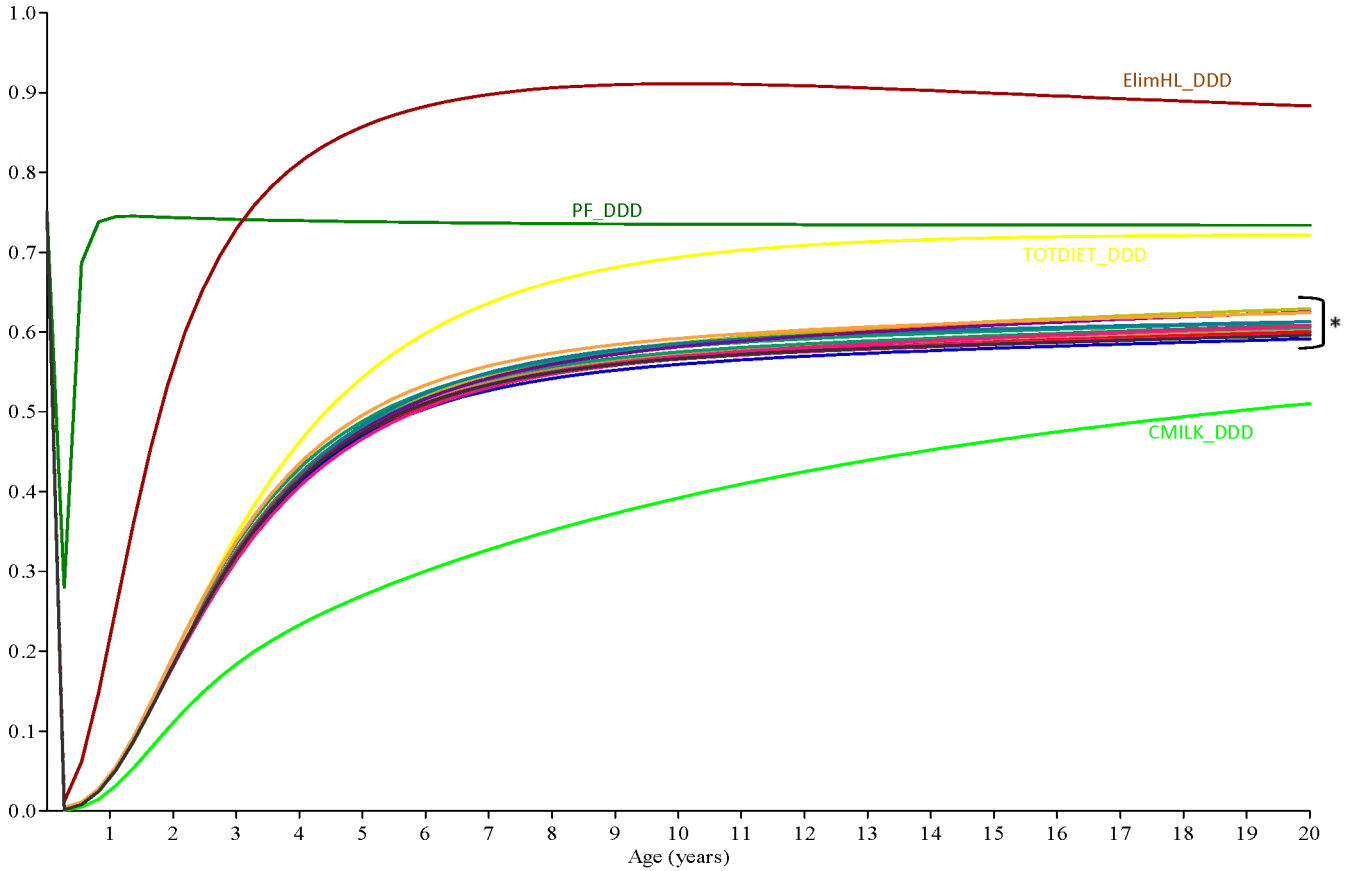
P – partition coefficient between blood and tissue; ElimHL – elimination half-life value; TOTDIET – concentration in the fish diet; CMILK – concentration in the milk diet

Figure S1. Results of the global sensitivity eFAST test on the concentration of *p,p'*-DDE in blood over the entire lifetime of the animals. X-axis represents the age of the animals expressed in years. Y-axis represents the sensitivity coefficients.



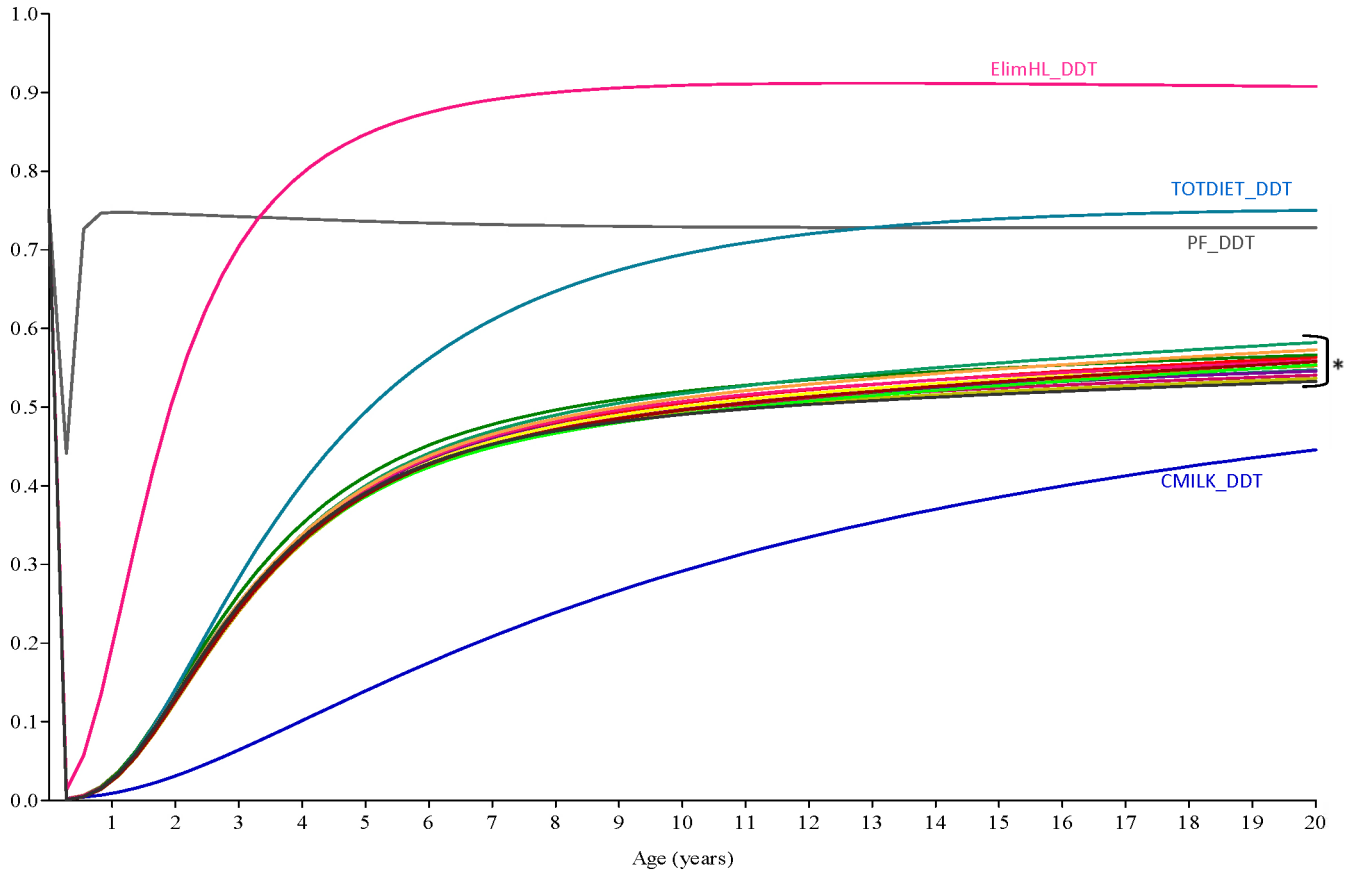
* - PF_DDT, PL_DDT, PR_DDT, PK_DDT, PB_DDT, PF_DDD, PR_DDD, PB_DDD, CMILK_DDT, TOTDIET_DDT, TOTDIET_DDE, ElimHL_DDT, PercDDD, PercDDT

Figure S2. Results of the global sensitivity eFAST test on the concentration of *p,p'*-DDD in blood over the entire lifetime of the animals. X-axis represents the age of the animals expressed years. Y-axis represents the sensitivity coefficients.



* - PF_DDT, PL_DDT, PR_DDT, PK_DDT, PB_DDT, PR_DDD, PB_DDD, CMILK_DDE, CMILK_DDT, TOTDIET_DDT, TOTDIET_DDE, ElimHL_DDT, ElimHL_DDE, PercDDD, PercDDT

Figure S3. Results of the global sensitivity eFAST test on the concentration of *p,p'*-DDT in blood over the entire lifetime of the animals. X-axis represents the age of the animals expressed in years. Y-axis represents the sensitivity coefficients.



* - PL_DDT, PR_DDT, PK_DDT, PB_DDT, PF_DDD, PR_DDD, PB_DDD, CMILK_DDE, CMILK_DDD, TOTDIET_DDD, TOTDIET_DDE, ElimHL_DDT, ElimHL_DDE, PercDDD, PercDDT

Codes of structural model (Berkeley Madonna)

NOTE: Parameters with the following description 'UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS' can be found in Table 3 in the main manuscript.

COMMENT: Codes of the structural and statistical model (AcslX/Libero) are available upon request to the corresponding author.

```
{----------PHYSIOLOGICAL PARAMETERS----------}

BW=1.387*BS**2.076
{body weight (g), body size-dependent; Weijs et al., 2010b}

BS=142.4*(1-0.3751*exp(-0.000068*TIME))
{body size or body length (cm), age-dependent; Weijs et al., 2010b}

VF=18.41*BW**0.607
{Mass of fat (g), body weight-dependent; McLellan et al., 2002}
VFL=VF/DENSF
{Volume of fat (L)}
DENSF=920
{density of fat (human) =0.92 g/mL, Maruyama et al., 2002}

VB=49.20*BW**0.211
{Mass of brain (g), body weight-dependent; McLellan et al., 2002}
VBL=VB/DENSB
{Volume of brain (L)}
DENSB=1050
{density of brain (human) =1.05 g/mL; Maruyama et al., 2002}

VL=0.060*BW**0.932
{Mass of liver (g), body weight-dependent; McLellan et al., 2002}
VLL=VL/DENSL
{Volume of liver (L)}
DENSL=1040
{density of liver (human) =1.04 g/mL; Maruyama et al., 2002}

VK=0.002*BW**1.137
{Mass of kidney (g), body weight-dependent; McLellan et al., 2002}
VKL=VK/DENSK
{Volume of kidney (L)}
DENSK=1050
{density of kidney (human)=1.05 g/mL; Maruyama et al., 2002}

VBlood=0.143*BW
{Mass of blood (g), body weight dependent; Reed et al., 2000}
VBloodL=VBlood/DENSBlood
{Volume of blood (L)}
DENSBlood=1068
{density of blood (bottlenose dolphin; n=3; Harderwijk, personal
communication)=1.068 g/mL}
```

VR=(0.99*BW)-VBlood-VK-VL-VB-VF
{Compartment for 'Rest of the body'; under the assumption that 1% of the body is pharmacokinetically inactive}
VRL=VR/DENSR
{Volume of rest of the body (muscle) (L)}
DENSR=1040
{Density of muscle (human)=1.04 g/mL, Maruyama et al., 2002. The 'muscle' is the biggest part of 'the rest of the body' compartment and muscle parameters are therefore used when needed}

FATPERCF=0.9285
{average fatpercentage of blubber; data from male porpoises Black Sea; Weijs et al., 2010a }
FATPERCK=0.0324
{average fatpercentage of kidney; data from male porpoises Black Sea; Weijs et al., 2010a}
FATPERCL=0.0373
{average fatpercentage of liver; data from male porpoises Black Sea; Weijs et al., 2010a}
FATPERCB=0.1149
{average fatpercentage of brain; data from male porpoises Black Sea; Weijs et al., 2010a}
FATPERCBlood=0.0045
{fatpercentage of blood; Maruyama et al., 2002}
FATPERCR=0.0227
{average fatpercentage of muscle; data from male porpoises Black Sea; Weijs et al., 2010a}

QC=(0.1017*(BW/1000)**0.9988)*60
{Cardiac output (L/hr); Equation from Altman and Dittmer, 1971}

QFC=0.05
{ fat blood flow (human) - portion of total, fractional blood flow to fat, Williams and Leggett, 1989 and Brown et al., 1997; percentage }
QF=QFC*QC
{L/hr}

QLC=0.25
{ liver blood flow (human) - portion of total, fractional blood flow to liver, Williams and Leggett, 1989 and Brown et al., 1997; percentage }
QL=QLC*QC
{L/hr}

QBC=0.12
{ brain blood flow (human) - portion of total, fractional blood flow to brain, Williams and Leggett, 1989 and Brown et al., 1997; percentage }
QB=QBC*QC
{L/hr}

QKC=0.19
{ kidney blood flow (human) - portion of total, fractional blood flow to kidney, Williams and Leggett, 1989 and Brown et al., 1997; percentage }
QK=QKC*QC {L/hr}

QRC=100-QFC-QLC-QBC-QKC
{muscle blood flow (human) - portion of total, fractional blood from to muscle, Williams and Leggett, 1089 and Brown et al., 1997. Constant is 17%, however recalculated to meet assumptions of mass balance; percentage}
QR=QRC*QC
{L/hr}

PF_DDT=331.0
{Fat/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PL_DDT=0.2
{Liver/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PB_DDT=1.5
{Brain/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PK_DDT=0.9
{Kidney/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PR_DDT=3.5
{Rest of the body/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS; Rest of the body here is actually 'muscle'}

PF_DDD=400.8
{Fat/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PL_DDD=7.9
{Liver/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PB_DDD=3.0
{Brain/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PK_DDD=4.6
{Kidney/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
PR_DDD=15.0
{Rest of the body/blood partition coefficient; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS; Rest of the body here is actually 'muscle'}

PF_DDE=450.0
{Fat/blood partition coefficient;ATSDR, 2002a}
PL_DDE=7.0
{Liver/blood partition coefficient; ATSDR, 2002a}
PB_DDE=6.0
{Brain/blood partition coefficient; ATSDR, 2002a}
PK_DDE=6.0
{Kidney/blood partition coefficient; ATSDR, 2002a}
PR_DDE=12.0
{Rest of the body/blood partition coefficient; ATSDR, 2002a; Rest of the body here is actually 'muscle'}

```
{--------------------DOSING--------------------}

TOTDIET_DDT=33.88
{Tanabe et al., 1997a and b; unit ng/g ww (wet weight)}
IN_DDT=0.98
{Assimilation efficiency; Thomas et al., 2005}
DC=(0.123*(BW/1000)**0.80)*1000
{Daily Consumption (g/day) for porpoises according to Innes et al. (1987)}
DIET_DDT=(DC*IN_DDT*TOTDIET_DDT)/24
{ng/hr ///// no unit*g/day*ng/g= ng/day divided by 24 hrs=ng/hr}
LACTATION_DDT=(IN_DDT*DCMILK*CMILK_DDT)/24
{the same unit as DIET = ng/hr}
DCMILK=540
{Daily Milk Consumption (g/day) for porpoises during lactation according to
Oftedal (1997)}
FATPERCMILK=0.2994
{Fat percentage of milk of harbour porpoises; own analyses of Black Sea
animals; Table 1 (main manuscript)}
CMILK_DDT=779.3
{ng/g; own analyses of Black Sea animals}
DOSE_DDT=IF TIME<=2920 THEN LACTATION_DDT ELSE DIET_DDT
{switch from milk-diet to fish-diet after 4 months (2920 hours) lactation}

TOTDIET_DDD=68.87
{Tanabe et al., 1997a and b; unit ng/g ww (wet weight)}
IN_DDD=0.99
{Assimilation efficiency; Thomas et al., 2005; this is a percentage }
DIET_DDD=(DC*IN_DDD*TOTDIET_DDD)/24
{ng/hr ///// no unit*g/day*ng/g= ng/day divided by 24 hrs=ng/hr}
LACTATION_DDD=(IN_DDD*DCMILK*CMILK_DDD)/24
{the same unit as DIET = ng/hr}
CMILK_DDD=1628.6
{ng/g; own analyses of Black Sea animals}
DOSE_DDD=IF TIME<=2920 THEN LACTATION_DDD ELSE DIET_DDD
{switch from milk-diet to fish-diet after 4 months (2920 hours) lactation}

TOTDIET_DDE=114.79
{Tanabe et al., 1997a and b; unit ng/g ww (wet weight)}
IN_DDE=0.97
{Assimilation efficiency; Thomas et al., 2005; this is a percentage, so no unit}
DIET_DDE=(DC*IN_DDE*TOTDIET_DDE)/24
{ng/hr ///// no unit*g/day*ng/g= ng/day divided by 24 hrs=ng/hr}
LACTATION_DDE=(IN_DDE*DCMILK*CMILK_DDE)/24
{the same unit as DIET = ng/hr}
CMILK_DDE=2011.7
{ng/g; own analyses of Black Sea animals}
DOSE_DDE=IF TIME<=2920 THEN LACTATION_DDE ELSE DIET_DDE
{switch from milk-diet to fish-diet after 4 months (2920 hours) lactation}

{--------------------MASS-BALANCE EQUATIONS DDT--------------------}

AF_DDT'=QF*(CBlood_DDT-CVF_DDT)
CVF_DDT=CF_DDT/PF_DDT
```

```
CF_DDT=AF_DDT/VFL
CFG_DDT=CF_DDT/(DENSF*FATPERCF)
init AF_DDT=CFoetusF_DDT*DENSF*VFL
CFoetusF_DDT=918.8

AR_DDT'=QR*(CBlood_DDT-CVR_DDT)
CVR_DDT=CR_DDT/PR_DDT
CR_DDT=AR_DDT/VRL
CRG_DDT=CR_DDT/(DENSR*FATPERCR)
init AR_DDT=CFoetusR_DDT*DENSR*VRL
CFoetusR_DDT=0.000001

AL_DDT'=(QL*(CBlood_DDT-CVL_DDT)-HepMet_DDT+DOSE_DDT)
CVL_DDT=CL_DDT/PL_DDT
CL_DDT=AL_DDT/VLL
CLG_DDT=CL_DDT/(DENSL*FATPERCL)
init AL_DDT=CFoetusL_DDT*DENSL*VLL
CFoetusL_DDT=0.000001

CLint_DDT=0.06315
{Biotransformation in liver; calculated according Verner et al. (2009) using an elimination half life of 4.8 years; UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}
Eh_DDT=(CLint_DDT*VLL)/(CLint_DDT*VLL+QL)
HepMet_DDT=QL*Eh_DDT*CBlood_DDT

AK_DDT'=QK*(CBlood_DDT-CVK_DDT)
CVK_DDT=CK_DDT/PK_DDT
CK_DDT=AK_DDT/VKL
CKG_DDT=CK_DDT/(DENSK*FATPERCK)
init AK_DDT=CFoetusK_DDT*DENSK*VKL
CFoetusK_DDT=4.7

AB_DDT'=QB*(CBlood_DDT-CVB_DDT)
CVB_DDT=CB_DDT/PB_DDT
CB_DDT=AB_DDT/VBL
CBG_DDT=CB_DDT/(DENSB*FATPERCB)
init AB_DDT=CFoetusB_DDT*DENSB*VBL
CFoetusB_DDT=3.9

ABlood_DDT'=QF*CVF_DDT+QR*CVR_DDT+QL*CVL_DDT+QK*CVK_DDT+QB*CVB_
DDT-(QF+QR+QL+QK+QB)*CBlood_DDT
CBlood_DDT=ABlood_DDT/VBloodL
CBloodG_DDT=CBlood_DDT/(DENSBlood*FATPERCBlood)
init ABlood_DDT=CFoetusBlood_DDT*DENSBlood*VBloodL
CFoetusBlood_DDT=0.000001

ABioaccumulation_DDT'=Dose_DDT-HepMet_DDT
init
ABioaccumulation_DDT=CFoetusF_DDT*DENSF*VFL+CFoetusR_DDT*DENSR*VRL+
CFoetusL_DDT*DENSL*VLL+CFoetusK_DDT*DENSK*VKL+CFoetusB_DDT*DENSB*V
BL+CFoetusBlood_DDT*DENSBlood*VBloodL
```

```
{--------------------MASS BALANCE--------------------}

TOTAL_DDT=ABioaccumulation_DDT
CALCULATION_DDT=AF_DDT+AR_DDT+AL_DDT+AB_DDT+AK_DDT+ABlood_DDT
MASSBALANCE_DDT=(TOTAL_DDT-CALCULATION_DDT)/(TOTAL_DDT+1E-
30)*100

{--------------------MASS-BALANCE EQUATIONS DDD--------------------}

AF_DDT'=QF*(CBlood_DDD-CVF_DDD)
CVF_DDD=CF_DDD/PF_DDD
CF_DDD=AF_DDD/VFL
CFG_DDD=CF_DDD/(DENSF*FATPERCF)
init AF_DDD=CFoetusF_DDD*DENSF*VFL
CFoetusF_DDD=2943.8

AR_DDD'=QR*(CBlood_DDD-CVR_DDD)
CVR_DDD=CR_DDD/PR_DDD
CR_DDD=AR_DDD/VRL
CRG_DDD=CR_DDD/(DENSR*FATPERCR)
init AR_DDD=CFoetusR_DDD*DENSR*VRL
CFoetusR_DDD=0.000001

AL_DDD'=(QL*(CBlood_DDD-CVL_DDD)-
HepMet_DDD+DOSE_DDD+PercDDT*HepMet_DDT)
CVL_DDD=CL_DDD/PL_DDD
CL_DDD=AL_DDD/VLL
CLG_DDD=CL_DDD/(DENSL*FATPERCL)
init AL_DDD=CFoetusL_DDD*DENSL*VLL
CFoetusL_DDD=37.6

PercDDT=0.502
{UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}

CLint_DDD=0.111919
{Biotransformation in liver; calculated according Verner et al. (2009) using an
elimination half life of 3.6 years; UPDATED WITH BAYESIAN APPROACH AND
MCMC SIMULATIONS}
Eh_DDD=(CLint_DDD*VLL)/(CLint_DDD*VLL+QL)
HepMet_DDD=QL*Eh_DDD*CBlood_DDD

AK_DDD'=QK*(CBlood_DDD-CVK_DDD)
CVK_DDD=CK_DDD/PK_DDD
CK_DDD=AK_DDD/VKL
CKG_DDD=CK_DDD/(DENSK*FATPERCK)
init AK_DDD=CFoetusK_DDD*DENSK*VKL
CFoetusK_DDD=35.5

AB_DDD'=QB*(CBlood_DDD-CVB_DDD)
CVB_DDD=CB_DDD/PB_DDD
CB_DDD=AB_DDD/VBL
CBG_DDD=CB_DDD/(DENSB*FATPERCB)
init AB_DDD=CFoetusB_DDD*DENSB*VBL
```

```
CFoetusB_DDD=27.6

ABlood_DDD'=QF*CVF_DDD+QR*CVR_DDD+QL*CVL_DDD+QK*CVK_DDD+QB*CVB
_DDD-(QF+QR+QL+QK+QB)*CBlood_DDD
CBlood_DDD=ABlood_DDD/VBloodL
CBloodG_DDD=CBlood_DDD/(DENSBlood*FATPERCBlood)
init ABlood_DDD=CFoetusBlood_DDD*DENSBlood*VBloodL
CFoetusBlood_DDD=0.000001

ABioaccumulation_DDD'=Dose_DDD-HepMet_DDD+PercDDT*HepMet_DDT
init
ABioaccumulation_DDD=CFoetusF_DDD*DENSF*VFL+CFoetusR_DDD*DENSR*VRL+
CFoetusL_DDD*DENSL*VLL+CFoetusK_DDD*DENSK*VKL+CFoetusB_DDD*DENSB*V
BL+CFoetusBlood_DDD*DENSBlood*VBloodL

{--------------------MASS BALANCE--------------------}

TOTAL_DDD=ABioaccumulation_DDD
CALCULATION_DDD=AF_DDD+AR_DDD+AL_DDD+AB_DDD+AK_DDD+ABlood_DDD
MASSBALANCE_DDD=(TOTAL_DDD-CALCULATION_DDD)/(TOTAL_DDD+1E-
30)*100

{--------------------MASS-BALANCE EQUATIONS DDE--------------------}

AF_DDE'=QF*(CBlood_DDE-CVF_DDE)
CVF_DDE=CF_DDE/PF_DDE
CF_DDE=AF_DDE/VFL
CFG_DDE=CF_DDE/(DENSF*FATPERCF)
init AF_DDE=CFoetusF_DDE*DENSF*VFL
CFoetusF_DDE=3351.6

AR_DDE'=QR*(CBlood_DDE-CVR_DDE)
CVR_DDE=CR_DDE/PR_DDE
CR_DDE=AR_DDE/VRL
CRG_DDE=CR_DDE/(DENSR*FATPERCR)
init AR_DDE=CFoetusR_DDE*DENSR*VRL
CFoetusR_DDE=0.000001

AL_DDE'=QL*(CBlood_DDE-CVL_DDE)-HepMet_DDE+DOSE_DDE+((1.0-
PercDDT)*HepMet_DDT)+(PercDDD*HepMet_DDD)
CVL_DDE=CL_DDE/PL_DDE
CL_DDE=AL_DDE/VLL
CLG_DDE=CL_DDE/(DENSL*FATPERCL)
init AL_DDE=CFoetusL_DDE*DENSL*VLL
CFoetusL_DDE=50.5

PercDDD=0.10
{UPDATED WITH BAYESIAN APPROACH AND MCMC SIMULATIONS}

CLint_DDE=0.036037
{Biotransformation in liver; calculated according Verner et al. (2009) using an
elimination half life of 11.8 years; UPDATED WITH BAYESIAN APPROACH AND
MCMC SIMULATIONS}
```

```
Eh_DDE=(CLint_DDE*VLL)/(CLint_DDE*VLL+QL)
HepMet_DDE=QL*Eh_DDE*CBlood_DDE

AK_DDE'=QK*(CBlood_DDE-CVK_DDE)
CVK_DDE=CK_DDE/PK_DDE
CK_DDE=AK_DDE/VKL
CKG_DDE=CK_DDE/(DENSK*FATPERCK)
init  AK_DDE=CFoetusK_DDE*DENSK*VKL
CFoetusK_DDE=43.3

AB_DDE'=QB*(CBlood_DDE-CVB_DDE)
CVB_DDE=CB_DDE/PB_DDE
CB_DDE=AB_DDE/VBL
CBG_DDE=CB_DDE/(DENSB*FATPERCB)
init  AB_DDE=CFoetusB_DDE*DENSB*VBL
CFoetusB_DDE=42.0

ABlood_DDE'=QF*CVF_DDE+QR*CVR_DDE+QL*CVL_DDE+QK*CVK_DDE+QB*CVB_
DDE-(QF+QR+QL+QK+QB)*CBlood_DDE
CBlood_DDE=ABlood_DDE/VBloodL
CBloodG_DDE=CBlood_DDE/(DENSBlood*FATPERCBlood)
init  ABlood_DDE=CFoetusBlood_DDE*DENSBlood*VBloodL
CFoetusBlood_DDE=0.000001

ABioaccumulation_DDE'=Dose_DDE-HepMet_DDE+((1.0-PercDDT)*HepMet_DDT)+
(PercDDD*HepMet_DDD)
init
ABioaccumulation_DDE=CFoetusF_DDE*DENSF*VFL+CFoetusR_DDE*DENSR*VRL+
CFoetusL_DDE*DENSL*VLL+CFoetusK_DDE*DENSK*VKL+CFoetusB_DDE*DENSB*V
BL+CFoetusBlood_DDE*DENSBlood*VBloodL

{--------------------MASS  BALANCE--------------------}

TOTAL_DDE=ABioaccumulation_DDE
CALCULATION_DDE=AF_DDE+AR_DDE+AL_DDE+AB_DDE+AK_DDE+ABlood_DDE
MASSBALANCE_DDE=(TOTAL_DDE-CALCULATION_DDE)/(TOTAL_DDE+1E-30)*100
```

6.2 Paper XI

Lifetime PCB 153 bioaccumulation and pharmacokinetics in pilot whales: Bayesian population PBPK modeling and Markov chain Monte Carlo simulations

Liesbeth Weijs, Anthony C Roach, Raymond SH Yang, Robin McDougall, Michael Lyons, Conrad Housand, Detlef Tibax, Therese M Manning, John C Chapman, Katelyn Edge, Adrian Covaci, Ronny Blust

Abstract

PBPK models for wild animal populations such as marine mammals typically have a high degree of model uncertainty and variability due to the scarcity of information and the embryonic nature of this field. Parameters values used in marine mammals models are usually taken from other mammalian species (e.g. rats or mice) and might not be entirely suitable to properly explain the kinetics of pollutants in marine mammals. Therefore, several parameters for a PBPK model for the bioaccumulation and pharmacokinetics of PCB 153 in long-finned pilot whales were estimated in the present study using the Bayesian approach executed with Markov chain Monte Carlo (MCMC) simulations. This method uses prior information of the parameters, either from the literature or from previous model runs, and available datasets to estimate posterior parameter values that are useful to explain the bioaccumulation in the dataset(s) involved in the model. The datasets used were PCB 153 concentrations in blubber of long-finned pilot whales from Sandy Cape and Stanley, Tasmania, Australia. The model predictions showed an overall decrease in PCB 153 levels in blubber of the pilot whales. All parameters from the Sandy Cape model were updated using the Stanley dataset, except for the concentration of PCB 153 in the milk. The model presented here is a promising and preliminary start to PBPK modeling in long-finned pilot whales that would provide a basis for non-invasive studies in these protected marine mammals.

XI

Submitted for publication to Chemosphere

1. Introduction

PBPK models are mathematical representations of the uptake, distribution, metabolism, and elimination processes of chemicals in organisms integrating physiology, biochemistry, pharmacology and toxicology of the chemicals of interest (Bernillon and Bois, 2000; Andersen, 2003; Chiu et al., 2007; Clewell and Clewell III, 2008). Such models have been used to describe and predict the kinetics of drugs in rodents and humans, but have gained importance and prominence in environmental toxicology (Ramsey and Andersen, 1984; Andersen et al., 1987; Lee et al., 2002; Andersen, 2003; Allen et al., 2007; Verner et al., 2008; Redding et al., 2008; Emond et al., 2010). The development of PBPK models is often associated with exposure/ pharmacokinetic experiments in which the models relate the external, administered concentration to the internal concentrations in the target tissue(s) (Lee et al., 2002; Simmons et al., 2002; Emond et al., 2010). These experiments usually employ a small number of animals with similar ages, fixed input concentrations and administration/sampling times and an overall controlled environment. None of this applies to situations in the wild where populations are composed of organisms of all ages, where input concentrations vary on a temporal, local and global scale and where environmental factors are beyond control. Under these circumstances, proper computer modeling becomes a real challenge. Even though PBPK modeling for wild populations may not be as detailed as the ones involving exposure/pharmacokinetic experiments in laboratory animals, such an approach is definitely valuable as it provides an integrated systems biology overview of the bioaccumulation/pharmacokinetics of environmental chemicals by utilizing biomonitoring without resorting to experimental work on these protected marine mammals.

PBPK modeling is of particular importance for assessing pollution in wild marine mammal species, since toxicological experiments are prohibited or ethically undesirable in these animals as is the case with humans (Verner et al., 2008; Redding et al., 2008). Marine mammals typically accumulate considerable amounts of persistent environmental chemicals such as PCBs and pesticides in their tissues because of their long life spans and top positions in the aquatic food webs (Ross, 2000). So far, parameter values for PBPK models of marine mammals have been estimated either using those from laboratory animals, or optimized from data in tissue samples from dead, stranded marine mammals (Hickie et al., 2005; Hall et al., 2006; Weijs et al., 2010b, 2011 and 2012). However, to improve the robustness of the PBPK model, two issues, uncertainty and variability (Bernillon and Bois, 2000; Barton et al., 2007), must also be addressed. Model uncertainty refers mostly to technical issues, such as errors in measurements or in model specification. With appropriate analytical methods, optimized model designs or a proficient knowledge about the processes involved in the models, this type of model uncertainty can be reduced. Model variability is caused by the intrinsic variation in a population such as genetic polymorphism, life history, etc., which play undeniably a large role in the wild. As a consequence, parameter variability cannot be reduced. The Bayesian-based Markov chain Monte Carlo (MCMC) approach is currently one of the best available statistical techniques to study these uncertainty and variability issues (Bernillon and Bois, 2000; Hack, 2006). This method provides posterior probability distributions for the model parameters based on prior information and informative datasets (Bernillon and Bois, 2000; Lyons et al., 2008). The prior information can be taken from the literature, but also from previous

model runs. In the latter case, the model parameters can be refined and updated with each new available dataset resulting in more robust model parameters that are specific for several populations instead of one single population.
Long-finned pilot whales are very social cetaceans with tight family bonds that are often involved in mass-stranding events (Olson, 2009). Having entire pods at the same time offers a unique opportunity to assess chemical pollution in a group of animals of which all individuals have been exposed to the same conditions at least for a certain part of their lives. Mass-stranding events can have anthropogenic or natural causes, but are still poorly understood (Hall and Harwood, 2009). The unique opportunity for sampling offered by those mass-strandings, combined with the need to assess the role of pollution in the occurrence of mass-strandings, are the reasons why PBPK models for bioaccumulation in long-finned pilot whales are useful and needed.
Therefore, the goal of the present study was to develop a PBPK model for PCB 153, one of the most persistent and prevalent pollutants in marine mammals, in long-finned pilot whales and to estimate and refine the unknown model parameters using a Bayesian-based approach via MCMC.

2. Materials and methods

The development of the PBPK model for lifetime exposure to PCB 153 in male pilot whales can be separated into two phases: the development of the 'Structural PBPK Model' and the evaluation/estimation of its parameter values using the Bayesian approach and MCMC simulations in a 'Statistical Model'.

2.1. Structural PBPK model.

Since only blubber samples and related analytical data were available (Weijs et al., submitted 1), the structural PBPK model for lifetime accumulation of PCB 153 in male pilot whales consisted of only two compartments, the blubber and a compartment that accounts for the rest of the body. In this way, the blubber compartment could be considered as a storage depot for lipophilic compounds, whereas intake and elimination processes (metabolic biotransformation and excretion) were set in the 'rest of the body'-compartment. All compartments were considered to be flow-limited, similar to the PBPK models for lifetime accumulation of PCB 153 in harbour porpoises (Weijs et al., 2010b). The uptake of PCB 153 occurs in marine mammals mainly through milk and food intake. Long-finned pilot whale (*Globicephala melas*) calves may nurse from their mothers for up to 3 years (Kasuya and Marsh, 1984), but switch gradually to a fish/cephalopod diet after one year of suckling. However, for modeling purposes, the animals were assumed to drink only milk during their first year of life after which a fish/cephalopod diet was taken as the only source for intake. For both fish/cephalopod and milk, an assimilation efficiency of 90% was used (Hickie et al., 2005; Weijs et al., 2010b). The daily intake for the milk diet was set at 4 kg (Oftedal, 1997), whereas the daily intake for the fish/cephalopod diet was body weight-dependent (Innes et al., 1987). *In vitro* studies with pilot whale hepatic cells have, to our knowledge, never been performed; thus, we have no knowledge regarding their metabolic biotransformation capacity. Furthermore, pilot whale feces and urine samples were not available. Therefore, all these elimination pathways were lumped together, and a clearance constant was derived from an estimated elimination half-life of 27.5 years for PCB 153 in humans and harbour porpoises (Verner et al., 2008; Weijs et al., 2010b). The "growth

dilution effect" for PCB 153 was taken into account by incorporating the growth equations from Bloch et al. (1993) into the model. An equation for the body weight dependent cardiac output together with the portions of the blood flow going to each compartment were taken from Altman and Dittmer (1971) and from Brown et al. (1997), respectively. The proportion of blubber in long-finned pilot whales was set at 25% of body weight (Lockyer, 1993). A lipid percentage of 84.4 % for blubber was used to express the final outcome of the model in PCB 153 concentration per lw (Weijs et al., submitted 1). The lipid percentage for the 'rest of the body'-compartment was 5.2 % and was the average of the lipid percentages of liver, kidney, brain and muscle of male harbour porpoises (Weijs et al., 2010b). Input concentrations, being the levels of PCB 153 in the milk diet and in the fish/cephalopods diet, were not available. However, since these input parameters typically have a high impact on the model outcome, they were automatically included in the statistical model and estimated through MCMC simulations (Table 1) (Allen et al., 2007).

2.2. Statistical model.

A Bayesian approach executed using Markov chain Monte Carlo (MCMC) analysis, was applied in order to evaluate or update the parameters taken from the literature (priors) with regard to the current pilot whale datasets. The entire structural model contained more than 15 different parameters, however, not all parameters were included in the statistical model for MCMC analyses. Parameters with the highest influence on the concentration of PCB 153 in the PBPK model outcome were selected by performing a global sensitivity analysis (GSA) first (McNally et al., 2011). For the first dataset (Sandy Cape), the priors were taken from the literature – the harbour porpoise model (Weijs et al., 2010b) (Table 1). For the second dataset (Stanley), the priors were taken from the posterior values found in the Sandy Cape MCMC analyses. In order to allow the curves to converge and to be able to calculate convergence factors (R-values; Gilks et al. (1996)), three chains of 10,000 iterations each were used. The development of the PBPK model, the GSA and all simulations were performed using acslX/Libero software (AEgis Technologies, Orlando, FL).

2.3. Pilot whale data.

The Bayesian approach with MCMC simulations gives the opportunity to use multiple datasets making the model or model parameters estimates more robust and informative with the incorporation of each succeeding dataset. In the present study, two datasets were used (Weijs et al., submitted 1). PCB 153 results in blubber samples from 21 male pilot whales and one fetus from Sandy Cape, Tasmania, were used as well as PCB 153 results in blubber samples from 15 male pilot whales and 1 fetus from Stanley, Tasmania. Males were chosen because they better represent the overall pollution status of the populations than females who regularly pass on pollutants to their offspring during reproduction. There were 4 fetuses in the Stanley dataset, but according to their body sizes, only one of them was an end-term fetus thereby being more representative for a neonate. Body sizes were recorded for all animals, except for the fetus in the Sandy Cape dataset. The age of the animals could not be assessed through counting dentine layers, but was estimated via the recorded body size of each animal and the growth equations for long-finned pilot whales from Bloch et al. (1993). All animals were victims of two mass-stranding events - one at Sandy

Cape, Tasmania, Australia and one at Stanley, Tasmania, Australia. Both events occurred in 2008. In all samples, 37 PCB congeners, 6 PBDEs, 6 DDXs, HCB, chlordanes (CHLs) and 5 MeO-PBDEs were targeted of which only the PCB 153 results were used in the present study. As discussed in earlier studies (Redding et al., 2008; Weijs et al., 2010b), since PCB 153 usually represents over 20% of PCBs in biological samples, it is considered as an indicator chemical for all PCBs. Information about the sample preparation and GC-MS analyses as well as the results of all targeted chemicals have been described previously (Weijs et al., submitted 1).

3. Results and discussion

This PBPK model for bioaccumulation and pharmacokinetics of PCB 153 in male pilot whales should be seen as a preliminary model for two reasons: First, the model has the potential to be transformed into a multi-compartment model as soon as information about levels of PCBs in tissues other than blubber becomes available; and, second, the Bayesian approach allows progressive updates of the current model with new datasets.
Despite its preliminary nature, the model in this study is a useful starting point to evaluate the PCB 153 bioaccumulation and pharmacokinetics in pilot whales specifically, and provides insights into environmental pollution in other large marine mammals where experimental work is usually impossible to conduct.

After performing global sensitivity analyses (eFAST, Figure S1 and S2), 4 parameters (TOTDIET, CMILK, PF, and PR) were selected to be included in the statistical model for MCMC analyses. TOTDIET or the concentration of PCB 153 in the fish/cephalopod diet of the whales and CMILK or the concentration of PCB 153 in the milk diet of the animals are obviously sensitive as input parameters. Literature values or even approximations were not available for these two parameters for Sandy Cape or Stanley animals, so the prior values for these parameters were estimated in previous simple model runs using the Sandy Cape dataset. The blood/blubber partition coefficient (PF) and the blood/rest of the body partition coefficient (PR) were taken from the respective partition coefficients for PCB 153 in harbour porpoises (Weijs et al., 2010b). In the statistical model for the Sandy Cape dataset, all four parameters were allowed to vary over a broad range of potential values in order to cover all possible combinations of parameter values (Table 1). In the statistical model for the Stanley dataset, the ranges of the parameters were restricted to, arbitrarily chosen, ± 25 % of the prior parameter values (Table 3).

Table 1. Prior parameter values, posterior probability distributions and R-values (convergence factors) updated in a 'flat' PBPK model with the Bayesian approach with MCMC simulations using data from male long-finned pilot whales from Sandy Cape, Tasmania. The prior parameter ranges were broad, but arbitrarily chosen.

	Prior			Posterior		
Parameter	Value	Range	Distribution	Mean	StD	R-value
PF	331.6*	0 – 800	normal	325.2	40.5	1.0087
PR	7.9$	0 – 40	normal	8.7	3.35	1.0003
TOTDIET	0.3	$1x10^{-10}$ – 1	normal	0.3	0.04	1.0114
CMILK	0.006	$1x10^{-10}$ – 1	normal	0.0049	0.0008	1.0003

PF-Blood/fat partition coefficient (dimensionless), PR-Blood/rest of the body partition coefficient (dimensionless), TOTDIET-concentration of PCB 153 in the fish/cephalopods diet of pilot whales (ng/g ww), CMILK-concentration of PCB 153 in the milk of pilot whales (ng/g ww), *-based on Weijs et al. (2010b) and Parham et al. (1997), $-average of PL, PB, PK and PR from Weijs et al. (2010b).

3.1. 'Flat' models versus hierarchical models

With the Bayesian approach, PBPK models can have several levels. In the present study, a 'flat' model with only one level, the population level, and a hierarchical model with two levels, the population and individual level, were developed for the Sandy Cape dataset. Both types of models have exactly the same structure, but differ in their respective statistical models (codes for Bayesian population PBPK models and for MCMC analyses are given in Supporting Information). In 'flat' models, all data points are assumed to be from the same individual whereas all data points are assumed to be from different animals in a hierarchical model. From a biological point of view, the hierarchical models are the best choice in the present study as the data points were from different animals. A hierarchical model not only provides a population mean and standard deviation for each parameter included in the statistical model, but also probability distributions for all individuals separately. A 'flat' model, on the other hand, provides only a population mean and standard deviation (Clewell III and Andersen, 1996; Bernillon and Bois, 2000). Comparing the results on the population level for the 'flat' model (Table 1) and the hierarchical model (Table 2) shows the posterior values were very similar. Since the goal is to assess the bioaccumulation of PCB 153 in the Sandy Cape population instead of in a single individual, those individual probability distributions were not needed. Therefore, further modeling efforts in the present study were done with the 'flat' statistical model and the posterior parameter values from Table 1.

Table 2. Prior parameter values and posterior means and probability distributions updated in a hierarchical PBPK model with the Bayesian approach with MCMC simulations using the data from male long-finned pilot whales from Sandy Cape, Tasmania. The prior parameter ranges were the same as in (Table 1).

	Prior			Posterior	
Parameter	Value	Range	Distribution	Mean	StD
PF	331.6*	0 – 800	normal	313.2	38.5
PR	7.9$	0 – 40	normal	12.1	3.0
TOTDIET	0.3	$1x10^{-10}$ - 1	normal	0.3	0.03
CMILK	0.006	$1x10^{-10}$ - 1	normal	0.0058	0.0012

Table 3. Prior parameter values, posterior means and probability distributions and R-values (convergence factors) updated in the Bayesian PBPK modeling with MCMC simulations exercise using data from male long-finned pilot whales from Stanley, Tasmania. The prior parameter values were the posterior values from Table 1. The prior parameter ranges were calculated as prior value ± 25% of the prior value.

	Prior			Posterior		
Parameter	Value	Range	Distribution	Mean	StD	R-value
PF	325.2	244 – 407	normal	309.3	29.2	1.0000
PR	8.7	7 – 11	normal	9.0	1.0	1.0017
TOTDIET	0.3	0.2 – 0.4	normal	0.3	0.03	1.0002
CMILK	0.0049	0.004 – 0.006	normal	0.0049	0.0005	1.0001

3.2. PCB 153 levels in male pilot whales

Figure 1 shows the concentration of PCB 153 in blubber of male long-finned pilot whales from different ages from Sandy Cape, Tasmania. There is a steep increase in PCB 153 level from birth until the age of approximately 1.5 years followed by a rapid decline until the age of about 5 years. A similar pattern was also found for the PCB 153 concentrations in harbour porpoises

(Weijs et al., 2010b). In that case, the steep increase and rapid decline were mostly due to the large difference in PCB 153 concentrations in the milk diet and fish diet of the harbour porpoises. However, the PCB 153 level in the milk (CMILK, Table 1) of pilot whales appears to be lower than the concentration in the fish/cephalopod diet (TOTDIET, Table 1). The lipid percentage in marine mammal milk can be up to 60% (Vanden Berghe et al., 2012) which is much higher than in milk of cows. In theory, the milk should contain high levels of PCB 153. Indeed, milk samples of harbour porpoises (Weijs et al., 2010b), grey seals (*Halichoerus grypus*; Vanden Berghe et al., 2012) and bottlenose dolphins (*Tursiops truncatus*; Ridgway and Reddy, 1995) were found to have considerable concentrations of PCB 153.
In the current study, the input of milk in the pilot whales was calculated from the concentration in the milk multiplied by the daily consumption of milk and the assimilation efficiency which is the proportion of absorbed amount of PCB 153 from the ingested amount of PCB 153. Data on environmental pollutants in milk samples of pilot whales were not available in this study or in the literature which was also the reason why this parameter was included in the MCMC simulations. The daily consumption of milk of 4 kg of milk/day, on the other hand, was taken from Oftedal (1997), and that study estimated this value based on the mass of the mammary gland and the growth rate of the suckling animals. As the mothers can nurse more than one offspring at the same time (Kasuya and Marsh, 1984; Olson, 2009), the amount of milk used in the current study is probably higher than any calf receives in reality. The assimilation efficiency was set as 90 % which is similar to that in harbour porpoises (Weijs et al., 2010b), and consistent with the 89 - 93 % range found by Thomas et al. (2005) for PCB 153 absorption in grey seals. Overestimations of either the daily consumption of milk or the assimilation efficiency from milk could lead to underestimations of the concentrations of PCB 153 in milk. Since the assimilation efficiency was high when compared to the harbour porpoises (Weijs et al., 2010b) and grey seals (Thomas et al., 2005), the low PCB 153 levels in milk as predicted by the model are probably due to a relatively high value for the daily consumption of milk. Unfortunately, there are no other studies than Oftedal (1997) reporting the daily milk consumption in long-finned pilot whales. The low level for the PCB 153 level in milk should, therefore, be considered as a preliminary conclusion with regard to the current model formulation based on available information. Male pilot whales grow fast from birth to adulthood (around 12 years of age; Bloch et al., 1993) and this “growth dilution effect” of PCB 153 in tissues due to body mass increase definitely plays a role in the initial rapid growth phase (Figure 1) for the overall decrease of PCB 153 levels in blubber. After that age, the growth slowly levels off according to the growth equations from Bloch et al. (1993) thereby causing only a slight decline in PCB 153 concentrations in the older animals.

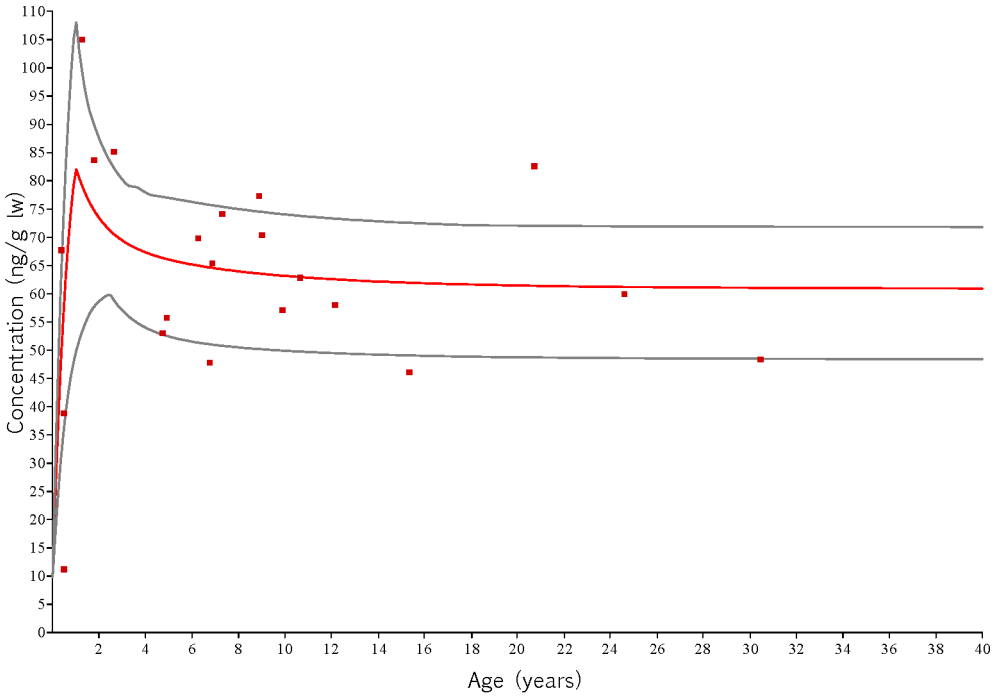


Fig 1. Concentration of PCB 153 (expressed in ng/g lw) with age in blubber of male pilot whales from Sandy Cape, Tasmania. ___ = curves for mean (red curve) ± 3σ with 3σ including about 99.7% of the population based on the marginal priors (grey curves), ■ = individual data-points of male pilot whales (n = 21) from the mass-stranding event in Sandy Cape, Tasmania in 2008 (Weijs et al., submitted 1).

From Figure 2 it is clear that the same findings as for Figure 1 apply. The Stanley dataset did not contain as many young animals as the Sandy Cape dataset, so there is no cause for estimating a different CMILK value (Table 3 versus Table 1). PF and TOTDIET were lower in the Stanley model (Table 3) than in the Sandy Cape model (Table 1), whereas PR was higher. Since there were no data to check the model predictions in the rest of the body-compartment, the PR estimate would probably benefit from more thorough datasets in the future. The biggest difference, however, between the parameter estimates from the Sandy Cape model (Table 1) and those from the Stanley model (Table 3), is that the posterior parameter estimates (Table 3) are suitable to explain the bioaccumulation of PCB 153 in 36 long-finned pilot whales whereas those from Table 1 only work for 21 long-finned pilot whales.

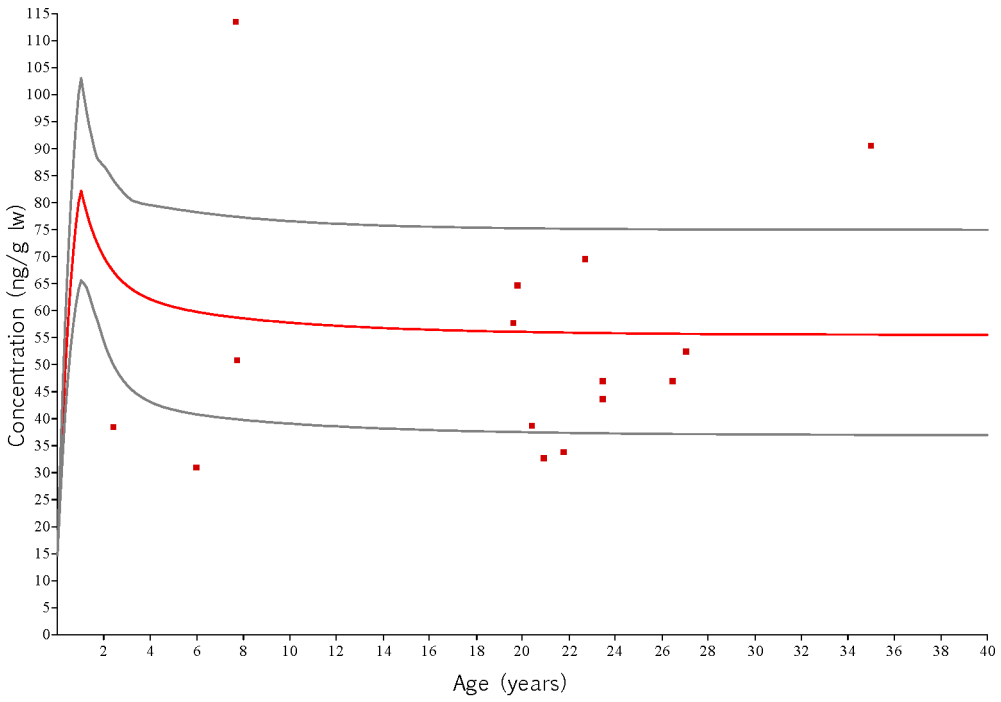


Fig 2. Concentration of PCB 153 (expressed in ng/g lw) with age in blubber of male pilot whales from Stanley, Tasmania. ___ = curves for mean (red curve) ± 3σ with 3σ including about 99.7% of the population based on the marginal priors (grey curves), ■ = individual data-points of male pilot whales (n = 15) from the mass-stranding event in Stanley, Tasmania in 2008 (Weijs et al., submitted 1).

4. Conclusions

Understanding the bioaccumulation and pharmacokinetics of pollutants in pilot whales, or marine mammals, in general, is a challenging task. Their protected status does not allow real-life exposure experiments nor would such studies be easy to undertake should they be permitted. As a consequence, parameters from typical organisms such as rodents are often used in marine mammal PBPK modeling work (Weijs et al., 2010b). Consequently, the PBPK model is usually capable of explaining only a part of the marine mammal dataset and contains a fair amount of model uncertainty. The Bayesian method applied in the present study started with parameter values from the literature and used a dataset (Sandy Cape) to derive new parameter values through MCMC simulations. These new parameter values were accordingly used further as 'priors' with the next available dataset (Stanley) resulting in more updated parameter values. Accordingly, such a Bayesian approach provides progressively more robust results so that each set of updated parameter values better represent a more general result that is applicable across populations. From this perspective, the present study is the first step towards the optimization of reliable PBPK model parameter values for long-finned pilot whales. Even though the posterior parameter values in this study are by no means final, they are more credible for pilot whales than any existing information so far. However, the model and its parameters would benefit greatly from more complete datasets in order to more fully understand the impact of environmental pollution on long finned pilot whale specifically, and marine mammals at large. As such, a better understanding of the bioaccumulation of

chemicals in marine mammals can be helpful to assess their survival or health condition now and in the future.

Acknowledgements
This paper is dedicated to Tony (AC) Roach who initiated this study, but passed away unexpectedly in May 2011. LW is financially supported by a PhD scholarship from the Scientific Research Foundation (FWO) in Belgium. AC acknowledges financial support from FWO and the University of Antwerp (UA-BOF). The present study was funded by a VOCATIO grant awarded to LW; partial funding by the Australian Department of Sustainability, Environment, Water, Population and Communities, under the Chemical Monitoring Initiative, is also acknowledged. Assistance from Dr Karrie Rose (Zoological Parks Board of NSW, Taronga Conservation Society Australia) and from the Princess Melikoff Marine Mammal Conservation Program, Tasmanian Department of Primary Industries, Parks, Water and Environment for transporting and sourcing the samples is appreciated.

Supporting Information

Figure S1. Results of the global sensitivity eFAST test on the concentration of PCB 153 in blood over the entire lifetime of 40 years of the long-finned pilot whales. X-axis represents the age of the animals expressed in years. Y-axis represents the sensitivity coefficients.

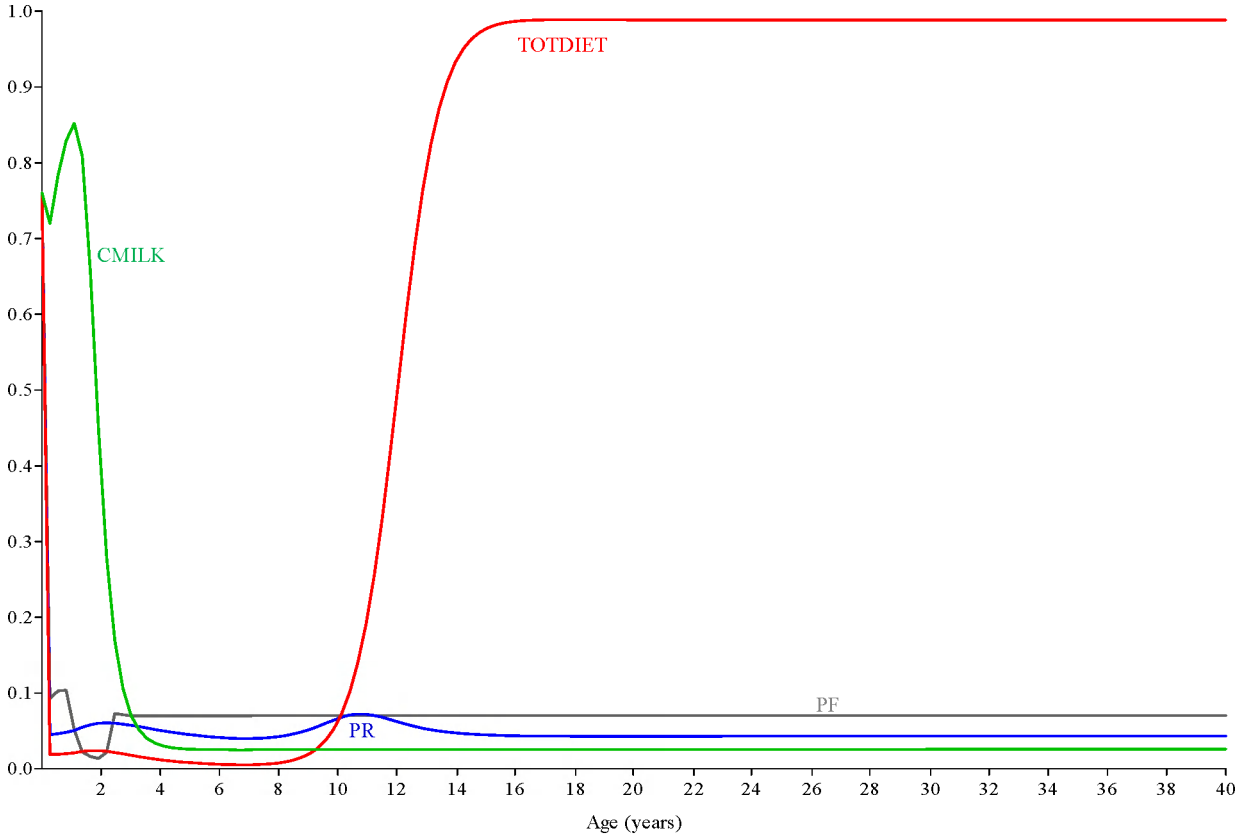
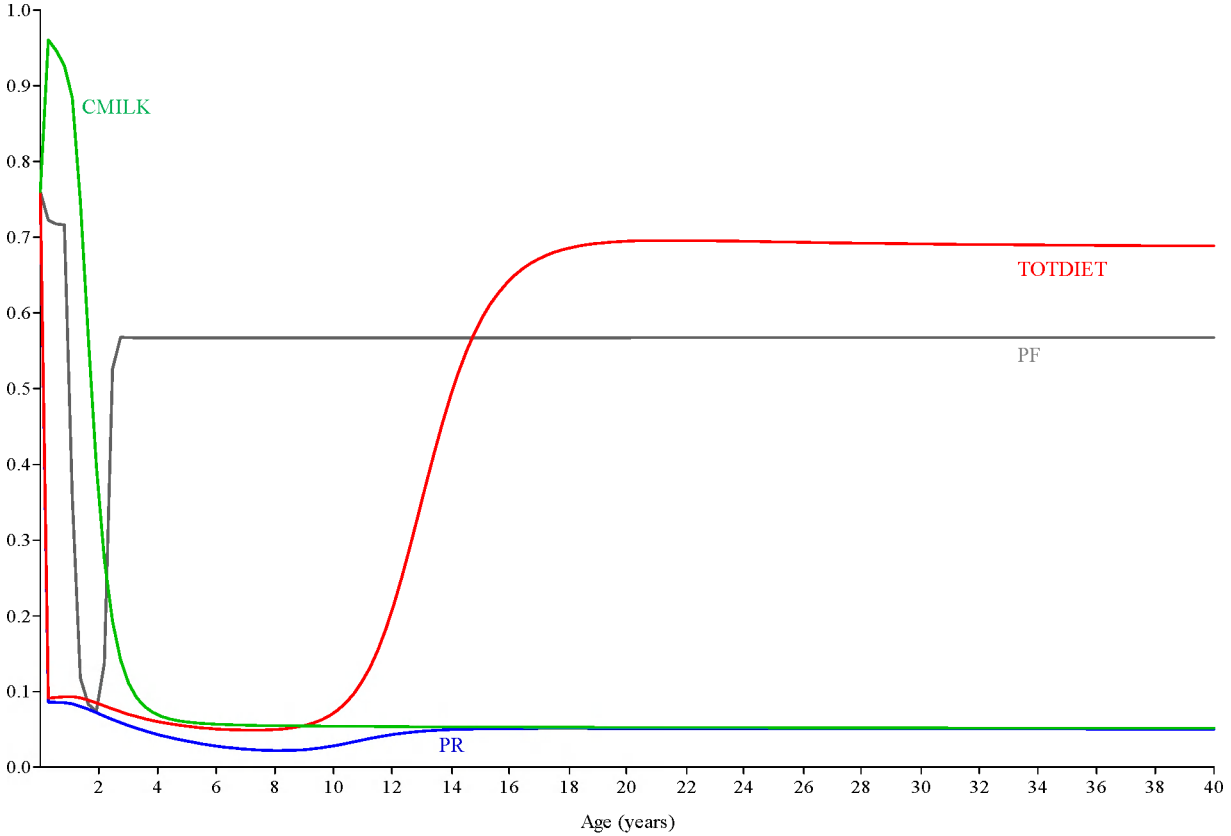


Figure S2. Results of the global sensitivity eFAST test on the concentration of PCB 153 in the blubber compartment over the entire lifetime of 40 years of the long-finned pilot whales. X-axis represents the age of the animals expressed in years. Y-axis represents the sensitivity coefficients.



AcslX/Libero codes (Italics) for the statistical (A) 'flat' model and (B) hierarchical model.

(A) 'flat' model

- file with prior parameter information (Bayesian approach), 'Blubber' is the name given to the results of the 21 male pilot whales from Sandy Cape.

```
observed Blubber

PF ~ norm(331.6, 47.4) I(0, 800)
PR~ norm(7.9, 3.5) I(0, 40)
TOTDIET ~ norm(3e-1, 6e-2) I(1e-10, 1)
CMILK ~ norm(6e-3, 1.8e-3) I(1e-10, 1)
s1 ~ unif(0,100)

preds = getpreds (PF, PR, TOTDIET, CMILK)
for i=1:21
 Blubber(i) ~ norm(preds(i,1),s1)
end
```

- file with which the MCMC simulations were done using the prior parameter information and Sandy Cape dataset specified in the previous file.

```
clear @all

seedrnd(123456)

use dataBlubber.m

data @clear
data ("Ages",["T"], Ages');
prepare @clear
prepare T CFG CRG

WEDITG = 0;
WESITG = 0;
TSTOP = 340500;
CINT = 20000;
start @nocallback

global _t
global _cfg
global _crg

function preds = getpreds (PF,PR, TOTDIET, CMILK)
        global _t
        global _cfg
        global _crg
        global Ages

 setmdl("PF", PF);
 setmdl("PR", PR);
```

```
 setmdl("TOTDIET", TOTDIET);
 setmdl("CMILK", CMILK);

start @nocallback

        idxs = [];
        for i = Ages
                        idx = find(_t == i);
                        idxs = [idxs idx];
        end

        preds =[ _cfg(idxs) _crg(idxs)];

end

use MCmodeler_10000runs_Pilotwhales_SCflat.m

chains = runmcmc([])
```

(B) hierarchical model

- file with prior parameter information (Bayesian approach), 'Blubber' is the name given to the results of the 21 male pilot whales from Sandy Cape.

```
observed Blubber
dimension iPF(21), iPR(21), iTOTDIET (21), iCMILK(21)

mPF ~ norm(331.6, 47.4) I(0, 800)
mPR~ norm(7.9, 3.5) I(0, 40)
mTOTDIET ~ norm(3e-1, 6e-2) I(1e-10, 1)
mCMILK ~ norm(6e-3, 1.8e-3) I(1e-10, 1)

sPF ~ norm(47.4, 47.4) I(0, 800)
sPR~ norm(3.5, 3.5) I(0, 40)
sTOTDIET ~ norm(6e-2, 6e-2) I(1e-10, 1)
sCMILK ~ norm(1.8e-3, 1.8e-3) I(1e-10, 1)

s1 ~ unif(0,100)

for i=1:21
iPF(i) ~ norm(mPF, sPF) I(0, 800)
iPR(i)~ norm(mPR, sPR) I(0, 40)
iTOTDIET(i) ~ norm(mTOTDIET, sTOTDIET) I(1e-10, 1)
iCMILK(i) ~ norm(mCMILK, sCMILK) I(1e-10, 1)

preds = getpreds (iPF(i), iPR(i), iTOTDIET(i), iCMILK(i), i)
 Blubber(i) ~ norm(preds,s1)
end
```

- file with which the MCMC simulations were done using the prior parameter information and Sandy Cape dataset specified in the previous file.

```
clear @all
```

```
seedrnd(123456)

use dataBlubber.m

data @clear
data ("Ages",["T"], Ages');
prepare @clear
prepare T CFG CRG

WEDITG = 0;
WESITG = 0;
TSTOP = 340500;
CINT = 20000;
start @nocallback

global _t
global _cfg
global _crg
preds_cache = NaN*ones(21,1);
parms_cache = NaN*ones(21,4);

global parms_cache
global preds_cache

function cache_store(id,parms,preds)
global parms_cache
global preds_cache
parms_cache(id,:)=parms;
preds_cache(id,:)=preds;
end

function preds=cache_fetch(id,parms)
global parms_cache
global preds_cache
if (any(parms_cache(id,:)~=parms));
return
else
preds=preds_cache(id,:);
end
end

function preds = getpreds (PF,PR, TOTDIET, CMILK, id)
        global _t
        global _cfg
        global _crg
        global Ages

        parms=[PF, PR, TOTDIET, CMILK];
        preds = cache_fetch(id, parms);
if preds ~=[]
return
end
```

```
preds=cache_fetch(id, parms);
setmdl("PF", PF);
setmdl("PR", PR);
setmdl("TOTDIET", TOTDIET);
setmdl("CMILK", CMILK);
setmdl("TSTOP", Ages(id));

start @nocallback

idx = find(_t == Ages(id));
preds =[ _cfg(idx)];
cache_store(id, parms, preds);

end

use MCmodeler_10000runs_Pilotwhales_hierarchical.m

chains = runmcmc([])
```

Chapter 7

General discussion and future perspectives

The objectives of this study were to investigate the bioaccumulation and kinetics of organohalogenated compounds in marine mammals by developing PBPK models. PBPK models are *in silico* tools that integrate available knowledge about the organism and the compound(s) of interest in order to explain their behavior and kinetics inside the organism's body. Over the years, there has been an increasing interest in the application of these models in an environmental context. The usefulness of these models to marine mammals is especially of interest given the ecological importance of these animals. The modeling approaches can explain or reveal much about the underlying mechanisms and kinetics of chemicals that would otherwise be impossible to explore in protected species such as marine mammals.
Each chapter/paper presented in this work has several highlights and results on its own. However, across the chapters and papers, there are findings that keep on coming back over and over again. In addition, the power and usefulness of modeling is emphasized several times, but in terms of possible applications, there is more than meets the eye. This chapter is therefore devoted to a general discussion of the most important findings of this work and of some interesting model applications.

<<< PBPK models are species specific >>>

Until now, PBPK models for wild animals such as marine mammals are scarce. Consequently, this work basically started from scratch. One of the most basic questions to begin with was whether making one model would be enough to represent the bioaccumulation process in two (or more) marine mammal species that are similar in diet, share the same habitat and have comparable life history traits. This question was investigated in Chapter 2, in which the bioaccumulation of several lipophilic organic contaminants in harbour seals from the North Sea was compared to the bioaccumulation in harbour porpoises from the same area. Both species consume similar amounts of fish on a daily basis, have a preference for fish smaller than 30 cm, have comparable life spans and share the relatively small and shallow North Sea. However, despite all these similarities, there were undoubtedly differences in bioaccumulation between both species. Harbour porpoises were shown to accumulate higher proportions of higher chlorinated and brominated compounds compared to harbour seals. Although PCB 153 and PBDE 47 were the most persistent among PCBs and PBDEs, respectively, in both species, patterns of most PCB and PBDE congeners were different. Since these differences could not be caused by significant differences in diet, they were a clear indication of species-specific metabolism and elimination capacities. This was also supported by the findings of Chapter 3 in which HO-PCBs, metabolites of PCBs, were measured in relatively high concentrations in serum of harbour seals, but only in trace amounts, if at all, in serum of harbour porpoises. These differences in metabolic capacities are too important to ignore in PBPK models. Indeed, in the PBPK models developed for harbour porpoises (Chapter 5 and 6), the metabolic half-life value, as a measure for the elimination capacity of a species for a certain compound, was often found to be a sensitive parameter. As a result, the conclusion was that models need to be species-specific in order to accurately assess the bioaccumulation process. Of course, for a certain

compound, elimination half-life values for a species in particular can be used as *prior* information in the models for another species. But since the Bayesian approach also focuses on the available dataset(s), the resulting *posterior* value will likely be different compared to the prior one.

‹‹‹ POPs in marine mammal serum can be used in PBPK models ›››

In PBPK models, chemicals circulate and move from one body compartment to the other. This circulation medium is obviously blood in marine mammals. In contrast to the blubber for instance, blood samples are difficult to obtain and concentrations of chemicals in blood are thus sparsely presented and discussed in the literature. As the blood compartment is an important part of the models, more knowledge was needed regarding the factors that might have an influence on the levels of lipophilic compounds in the blood. Therefore, blood samples of harbour seals and harbour porpoises were analysed and discussed in Chapter 3. Samples were obtained from animals in captivity and in the wild, from animals from different ages and gender and from animals in different health conditions. Results showed that only starvation had a noticeable impact on the levels of contaminants in the blood. Starving animals use their lipid reserves thereby mobilizing the lipophilic compounds present in those lipid reserves. Other than starvation, the levels in the blood were relatively 'stable' which is a very useful finding from a modeling point of view.
So far, only results of tissues sampled from dead animals were used to evaluate the model predictions in Chapters 5 and 6. The reason for that is that datasets with levels of POPs in five compartments were assumed to be more reliable and useful for the evaluation of multi-compartment model predictions than datasets with only blood levels of POPs. For marine mammals, it will be a very difficult task to sample blood, liver, blubber, brain, kidney and muscle at the same time. The first tissue, blood, is mostly sampled in animals that are still alive. The other tissues, blubber, liver, kidney, brain and muscle, are sampled in animals that are dead. Using the tissues of dead animals was therefore considered as a more thorough way to evaluate the model outcomes in this work.

‹‹‹ Males and females accumulate POPs differently ›››

In the first part as well as in the second part of this work, males and females were separated. In this entire work, for all compounds analysed, concentrations in adult females were lower than those in adult males. Regardless of whether the concentrations of POPs increased or decreased in adult males, the levels in adult females were always declining. Reproductive females have the advantage that they can transfer a large percentage of their body burdens to the offspring, while males do not have such elimination pathway. The levels measured in males are, therefore, more indicative for environmental exposure. As long as the animals are not sexually active yet, there is hardly any difference in concentrations of POPs. For these groups, pooling can be an option. However, upon adulthood, males and females have to be separated. Of course, the choice for modeling bioaccumulation of POPs in males or females depends on the type of study. Models for females can give information of the body burdens in fetuses,

neonates, pups and calves. These burdens may be important to understand what happens on a longer term in terms of health effects since those four age classes are all in a vulnerable developmental stage of their lives. Models for males, on the other hand, can give information of the age-dependent bioaccumulation of POPs and are more reliable when addressing temporal or spatial trends. This latter is perhaps the most reasonable approach for legislation and conservation purposes.

<<< Offloading of POPs in adult females happens in all tissues simultaneously >>>

First and foremost, it is important to note that this conclusion only applies for sexually active adult females of species that do not fast during lactation. In fasting seals, there are numerous studies that have shown a mobilization of lipids and associated lipophilic compounds primarily from the blubber during lactation. However, harbour porpoises are known to show signs of starvation after only a few days without nutrition. With a lactation period that can go on for months, this species definitely does not fast during lactation. As a result, there is a gradual decrease in concentrations of PCB 153 in all tissues simultaneously as has been shown in the PBPK model in Chapter 5 (paper VII). However, it obviously does not matter for the calf whether the compound is coming from the liver, kidney, brain or blubber of the mother.

<<< 'What goes in, pretty much stays in' >>>

Marine mammals have relatively long life spans compared to other organisms. Unfortunately, such long life spans do not help much in terms of elimination of several PCBs, especially not for males. In male harbour porpoise models, elimination half-lives for several PCBs such as PCB 153 and PCB 180 are higher than the maximum life span of the animal. For these models, it is possible to completely ignore the elimination pathway through metabolic biotransformation, since it would not yield visible changes in the model predictions. Despite that, it is not correct to say, from a biological point of view, that there is no metabolic biotransformation of PCBs at all in these animals. There is some metabolic biotransformation, but this is simply too little relative to the animal's body burdens that it is almost not noteworthy. For these very persistent PCB congeners, the only elimination pathway that has a clear and visible impact on the model outputs, is the growth dilution effect. This dilution effect is, however, more a re-scaling of the POPs already present in the body rather than a way to actually remove the chemical from the body. Thus, for some of the most persistent compounds, it is not entirely wrong to assume that whatever goes into the body of the animal hardly ever comes out.

<<< Tough life to be a young marine mammal >>>

Males can experience increasing concentrations over their lifetime and have for several POPs analysed in this work the highest levels compared to subadults or even younger animals. For several PCBs, however, levels can reach a peak in the youngest members of the population. This has been

found for harbour porpoises as well as long-finned pilot whales. For young animals, the effects of these peak levels are unknown, but consequences may be acute as well as chronic.
Firstly, these young animals might not have proper metabolic capacities yet to be able to deal with high levels of POPs and are likely to experience the impact of pollution on their health condition to the maximum. Secondly, they are still in crucial developmental stages. Disturbances of normal cell functions and signaling might have consequences on the longer run, thereby affecting a normal development. Thirdly, in combination with the long elimination half-life values for several PCB congeners in marine mammals, these high peak levels can largely be conserved across several generations. In general, such peak levels are of concern and may compromise the survival of a population for years and even decades.

<<< PBPK models go a long way... >>>

It takes time, effort and a large amount of datasets to make models that run and that provide realistic outcomes. However, once these models exist, they can be applied for several situations and scenarios. In this work, the focus has been on assessing bioaccumulation in past and in more recent populations of marine mammals. The power of PBPK models, however, stretches also beyond past and current situations. PBPK models can be applied for several purposes to predict into the future. Two of these purposes are illustrated below for harbour porpoises since the PBPK models for this species are currently the most advanced and more datasets are available.

Predicting the end of the PCB 153 threat to harbour porpoises from the North Sea

There are reports in the literature stating that toxic effects (different endpoints) occur in marine mammals at certain levels of a specific chemical. According to Das et al. (2006), harbour porpoises from German and Norwegian coasts have higher percentages of connective tissue and higher levels of ∑PCBs compared to harbour porpoises from the Icelandic coast. A high percentage of connective tissue in the thyroid gland is seen as an indication for a severe dysfunction of the thyroid gland. In that study, the ∑PCBs was the sum of PCB 99, 138, 149, 153, 180 and 187. In blubber of harbour porpoises from the Black Sea, PCB 153 represents on average 33.3% of the sum of these 6 PCBs. A percentage of 33.3% of the ∑PCB levels reported in Das et al. (2006) for harbour porpoises from the Icelandic and Norwegian coast (516 and 1568 ng/g lw, respectively) was thus used as thresholds in the harbour porpoise PCB 153 PBPK model in Fig 1. This PBPK model for PCB 153 (Chapter 5) was used as a template for this exercise together with the data from harbour porpoises from the North Sea (Chapter 4).

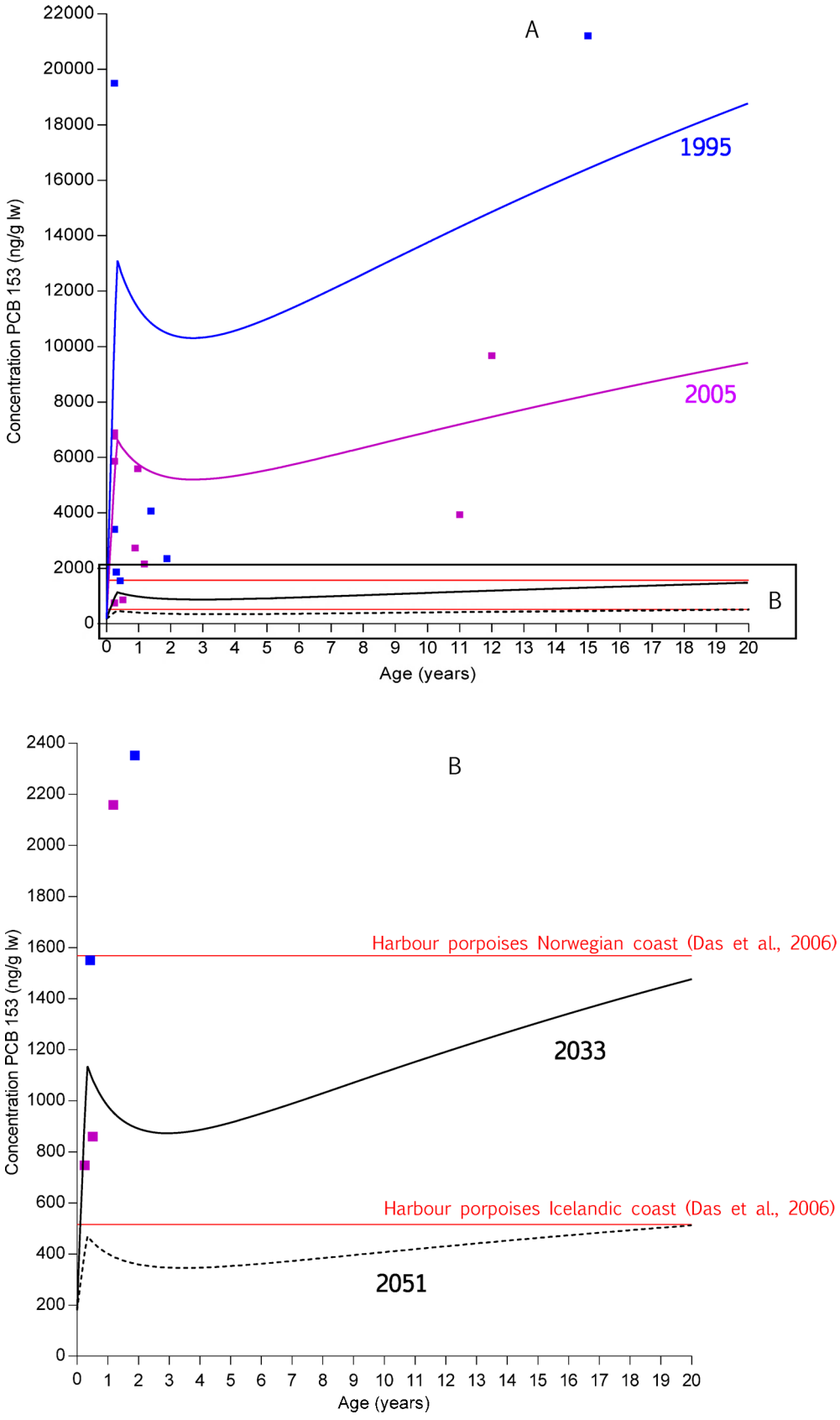


Fig 1. Predictions of the decrease in PCB 153 in blubber of harbour porpoises from the North Sea using the effect concentrations for harbour porpoises from the Norwegian coast and Icelandic coast from Das et al. (2006). **A)** Situation in 1995 and 2005 (using data from North Sea harbour porpoises from 1990-1998 and 2000-2008, respectively, from Chapter 4), **B)** close-up from the bottom part of A showing the levels from Das et al. (2006) in animals from Norway

and Iceland (red lines) and the associated bioaccumulation curves for PCB 153: ▬ = situation that will be reached in 2033, --- = situation that will be reached in 2051.

The data from Chapter 4 were from animals from 1990-1998 and 2000-2008. The change in PCB 153 input levels (diet of fish and milk) used to make PBPK models for these two situations was taken as a reference to calculate the new input values for the two scenarios in the future.
According to the models, levels of PCB 153 in blubber of harbour porpoises from European waters are predicted to be low enough in 2051. Of course, this exercise has only focused on the thyroid effects reported by Das et al. (2006), so it would not be correct to state that these animals will be out of danger in 2051. Harbour porpoises experience immunological effects as well and are exposed to a mixture of PCBs and other POPs simultaneously. However, this exercise was done to show three things: 1) PCB 153 is highly persistent in the environment. The production and use of PCB 153 has been banned since the 1970s, but even without a 'fresh' input in the environment, the animals are still experiencing the toxic effects for many years to come. 2) PBPK models are not a stand-alone topic. They need data from bioaccumulation studies (Chapter 4) and from effect studies (Das et al., 2006) to get the most information out of them. However, 3) bioaccumulation studies and effect studies alone are not capable of performing similar predictions. Therefore, all three disciplines are required to provide useful tools in risk assessment.

Creating a harbour porpoise-friendly chemical?
PBPK models provide the opportunity to test how a certain species reacts in terms of bioaccumulation to chemicals with different properties. Exercises with PBPK models show that there is, regardless of the level of a specific chemical in the environment, hardly any bioaccumulation of that chemical in harbour porpoises if assimilation efficiencies are low, elimination half-life values are in days or minutes instead of years/decades or log K_{fp} (adipose tissue/plasma) partition coefficients are low. These things depend partly on the species and partly on the chemical. Large chemicals for example, are unlikely to be absorbed as they are not able to pass through the membranes. These chemicals will not be a threat to the animals. The PBPK models developed in this work are primarily for lipophilic compounds and may not be helpful to assess proteinophilic compounds. Still, models were developed for several PCBs, PBDEs and OCPs which are chemicals with different properties. New chemicals in the future might therefore be more marine mammal friendly if the models of this work as well as their results are used as a reference framework to learn from.

<<< ...but there is also still a long way to go towards models that work across chemicals >>>

PBPK models for PCB 153 were not automatically suitable for explaining the bioaccumulation of PBDE 153 in this work. On first sight, these molecules only differ in the type of atom (chlorine for PCB 153 and bromine for PBDE 153) and the presence of an ether-bridge. However, this difference has consequences on multiple levels (e.g. compound size) which are not directly represented in the models. In a way, the models use more 'superficial' characteristics of the chemical without taking into account the causes for

these characteristics. It would be better if, for example, the size of a molecule is used in a model instead of an assimilation efficiency. Unfortunately, such detailed descriptions of a chemical are hard to find, especially in relation to information about the pore sizes or limits for intestinal absorption of the marine mammals. This information is, certainly for marine mammals, difficult to obtain. So far, there has been little interest for models that work across chemicals. Models are usually developed for a specific chemical only. Models that work across chemicals would be useful in terms of risk assessment in environmental toxicology. Since REACH demands more and better information from the industry regarding the chemicals they produce, such models might be a possibility in the future.

The work done so far is fairly new in the field of marine mammal toxicology. As such, the PBPK models for marine mammal species presented here can and should be expanded in the future in order to facilitate our understanding of the health situation of marine mammals in the past, present and future. In this regard, the word 'expand' refers to:

- Adding more datasets. With the Bayesian approach and MCMC simulations, parameter information from previous model runs can be updated with each new dataset. With each added dataset, the parameter ranges get more narrow until the model variability is minimized and only the model uncertainty is left. PBPK models in which the parameter values are attached to a single dataset, are basically only population-specific. PBPK models in which the parameter values are attached to a series of datasets, are suitable for several populations and are therefore more species-specific. For risk assessment purposes, these species-specific models are more attractive than population-specific models as scenarios from the past, present and future can be judged for much larger groups of animals.

- Adding more compartments. Models should reflect the reality as good as possible. In reality, a long-finned pilot whale for instance is composed of more tissues than currently involved in the pilot whale models. An organism is a complex system in which each cell or tissue has a function and perhaps an impact on the kinetics of a chemical inside the body. Of course, depending on the chemical and on the availability of information about the tissues in a specific species, simple, even one-compartment models can fit the purpose. Yet, to fully understand the kinetics of a chemical and its effect on an organism, one-compartment models are not helpful as they actually only give the deposition of a chemical in that compartment instead of its kinetics in the body.

- Coupling an effects section. This means that PBPK models can be transformed into PBPK/PD (physiologically based pharmacokinetic/ pharmacodynamic) models. To be able to do this, pharmacodynamic components or biomarkers and their mechanisms should be added to the model. Examples could be the expression and levels of a specific type of cytokines, lipids, hormones, enzymes or receptors as biomarkers for the immune, endocrine, cognitive or reproductive system.

- Extrapolating to other species/chemicals. Next to harbour porpoises and long-finned pilot whales, there are more marine mammal species threatened by contaminated environments. Ideal would be to develop models for each species separately. Unfortunately, considering the number of different chemicals in the environment, making separate models per species and chemical would be a time consuming and unmanageable process. Therefore, it would be worthwhile to investigate whether and how models for example for long-finned pilot whales can be extrapolated to closely related species such as short-finned pilot whales or how models for the bioaccumulation of PCB 153 could be used for explaining the bioaccumulation of PBDE 153.

By expanding the current PBPK models in all possible directions, they can be used and explored to the maximum of their potential. As shown earlier, PBPK

models greatly benefit from inputs from biomonitoring studies as well as effect studies. They are not limited in space or time as they can provide information about past, current and future exposures in species worldwide. A tool that integrates knowledge from different fields of research and that can anticipate the potential impact of new chemicals, can only be beneficial for risk assessment and conservation of marine mammal species.

SUMMARY

Factors, such as a greater demand for products and more sophisticated industrial techniques, have caused a growing number of chemicals in the environment. Due to a lack of efficient metabolic breakdown or elimination processes in organisms and in the environment, these chemicals can be passed on in the aquatic and terrestrial food webs leading to higher levels in the predator compared to its prey. Marine mammals are apex predators in marine ecosystems and as such, they accumulate considerable amounts of chemicals through their diet. Evidence in the literature has shown that these amounts can have a major negative impact on their immune, endocrine and reproductive system or even on their survival in general. Marine mammals do not seem to have the appropriate enzyme systems to be able to deal with chemicals. They experience the negative effects of pollution themselves and at the same time, pass the chemicals on to their offspring. As a consequence, levels of contaminants in these animals decrease only slowly even though the industrial production of several chemicals has been restricted or banned in the past.
In order to prevent repetitions of past situations, it is important to gain knowledge about the absorption, distribution and elimination of known or 'old' chemicals in marine mammals. Understanding the kinetics and effects of old chemicals can be useful to assess the impact of new chemicals with comparable properties compared to the old ones before these new chemicals are being manufactured. From this point of view, studying known, old chemicals is undoubtedly useful for the risk assessment of new compounds.

In this thesis, the kinetics of known or traditional chemicals such as PCBs and PBDEs, was investigated in harbour porpoises and long-finned pilot whales by using physiologically based pharmacokinetic (PBPK) models. These computerized models combine physiological information of the organism of interest and chemical properties of the chemical of interest to reflect the kinetics of that compound in the body of the organism. Similar to exposure experiments in which all factors are controlled to minimize the large degree of variability, models for wild populations are also more reliable if the datasets used for evaluation of the models are somewhat 'robust' or 'uniform'. For practical reasons, trends are easier to visualize and parameters are easier to estimate if the number of interfering, external factors are reduced to a minimum. For theoretical reasons, scattered data can lead to parameter estimates that are not reflecting the intrinsic, physiological capabilities of the species.

Consequently, it was important to investigate first which factors were influencing the levels of pollutants in the blood since blood is the only circulation medium in the models. Blood samples of harbour seals and porpoises of different health condition, origin (captivity versus wild), gender and age were analysed. Results showed that only emaciated animals had deviating concentrations and profiles of PCBs and PBDEs in their blood compared to animals that were not emaciated. Of course, starvation can occur in wild populations, but is definitely not common for all wild animals. The conclusion here was thus that datasets of blood could be used in the bioaccumulation models. However, blood is never sampled at the same time as tissues in marine mammals. In multi-compartmental models as the ones developed in this work, data of more tissues was preferred to evaluate the model predictions for as much compartments as possible simultaneously.

Because the Black Sea harbour porpoise dataset was both restricted in time (animals were from 1997-1998) and space (animals from the Black Sea do not leave the Black Sea area), these results were preferably used to evaluate the very first preliminary harbour porpoise model predictions. These models were developed to explain the bioaccumulation of several PCB congeners (PCB 153, PCB 180, PCB 101, PCB 149, PCB 118, PCB 99, PCB 170) and PBDE congeners (PBDE 47, PBDE 99, PBDE 100 and PBDE 153) in male harbour porpoises. Model outputs showed that levels of all PCBs and PBDEs reached high levels at the end of lactation period (e.g. first year of life) after which the growth dilution effect and a change in diet caused a decline in concentrations followed by an increase in concentrations for the rest of the lives of the Black Sea harbour porpoises. The models were than applied to assess temporal trends by using the dataset of harbour porpoises from the North Sea. During this modeling exercise, levels of PCBs and PBDEs were found to decrease from 1990 until 2008, although not at the same rate for all PCB and PBDE congeners. For some PCB congeners, the PBPK models were also used to test the metabolic biotransformation capacity for PCB 118, PCB 149 and PCB 101. Results suggested a fairly weak metabolic breakdown of PCB 118 and an enhanced capacity for metabolic breakdown of PCB 101 with higher age. In contrast, results were inconclusive about the metabolic capacities for PCB 149. So far, all attempts to estimate parameters were performed manually and the sensitivity of the parameters on the model output was tested by a "one-at-a-time" or local sensitivity analysis. However, this type of sensitivity analysis ignores potential correlations between the parameters. Hence, more statistically sound parameter estimation methods and global sensitivity tests that take into account potential interactions between the parameters were needed in order to improve the robustness of the models.

Applying new methods for parameter estimation and sensitivity analyses was, thus, the next step. So, in the most recent PBPK model for bioaccumulation of pesticides (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD) in harbour porpoises, parameters were estimated using Bayes' theorem executed with Markov chain Monte Carlo (MCMC) simulations. In addition, the influence of changes in parameter values on the model output was tested using global sensitivity analyses. Compared to all previous PBPK models, this model for bioaccumulation of pesticides differed not only in the statistical techniques, but also in its complexity. Whereas all previous models showed the kinetics of a single compound, the pesticide model showed the kinetics of *p,p'*-DDT and its two metabolites *p,p'*-DDE and *p,p'*-DDD at the same time ensuring a high connectivity between the kinetics of these three compounds. Similar to the previous harbour porpoise models, the structural model was first evaluated using a dataset of harbour porpoises from the Black Sea after which the parameter range estimates were further optimized using the dataset of harbour porpoises from the North Sea.
The same techniques (Bayesian PBPK modeling and MCMC simulations) were also used for a PBPK model for the lifetime bioaccumulation of PCB 153 in long-finned pilot whales. For this species, two datasets were available from two mass stranding events. Long-finned pilot whales have tight family group bonds so whenever an individual ends up on the beach, all other members of the group follow. For the animals, these mass strandings are traumatic, but they are a great opportunity for monitoring and modeling as a dataset cannot possibly be more 'homogeneous'. In contrast to the dataset of the

Black Sea harbour porpoises, only blubber samples were available for the long-finned pilot whales. The PBPK models for the bioaccumulation of PCB 153 in pilot whales are therefore smaller than the models in harbour porpoises. Nevertheless, parameters were estimated with the most suitable technique for this type of models, making the pilot whale model already a useful framework for evaluating similar or more elaborate datasets in the future.

This work provides new ideas and innovative approaches to study biomonitoring data. The bioaccumulation models developed here can already be used as a framework to compare to new datasets, but can also be further optimized and expanded in the future. These results are, therefore, not only a useful addition to existing knowledge, but provide also new perspectives to assess pollution and its effects in marine mammals. Such an integrated approach is required to set up guidelines for conservation of a species. As a result, the models developed in this work are undoubtedly useful tools for risk assessment purposes.

SAMENVATTING

Factoren zoals een grotere vraag naar producten en meer doorgedreven industriële technieken hebben gezorgd voor een groeiend aantal chemicaliën in het milieu. Door een gebrek aan efficiënte metabolische afbraak- of eliminatieprocessen in veel organismen en in het milieu zelf, kunnen chemicaliën in de aquatische en terrestrische voedselketens doorgegeven worden van de prooi naar de predator waarbij de predator uiteindelijk hogere concentraties chemische stoffen heeft dan zijn prooi. Zeezoogdieren zijn toppredatoren in mariene ecosystemen en accumuleren als zodanig enorme hoeveelheden chemische stoffen via hun voedsel. Het is reeds eerder gebleken dat die hoeveelheden een negatieve impact kunnen hebben op hun immuunsysteem, endocrien en voortplantingssysteem of zelfs gewoon op de overleving van de dieren. Zeezoogdieren hebben over het algemeen niet de nodige enzymesystemen om met deze stoffen om te gaan. Ze ervaren dus niet alleen zelf de negatieve effecten van de stoffen, maar geven ze ook door van de ene generatie naar de andere. Als gevolg hiervan dalen de concentraties van de chemische stoffen in deze dieren maar traag ook al werd de productie van verschillende chemicaliën in het verleden al aan banden gelegd of zelfs volledig verboden.
Om herhalingen te voorkomen in de toekomst is het belangrijk om meer kennis te vergaren over de opname, verspreiding en eliminatie van 'oude', reeds bestaande chemische stoffen in zeezoogdieren. Een beter begrip van de kinetiek en effecten van meer traditionele chemische stoffen kan nuttig zijn om de impact in the schatten van nieuwe stoffen met vergelijkbare eigenschappen als de oude stoffen. Vanuit dit perspectief is het bestuderen van gekende, oude chemische stoffen dus zonder twijfel nuttig voor de risico-analyse van nieuwere stoffen.

In dit werk werd de kinetiek van reeds bestaande stoffen zoals PCB's en PBDE's in gewone bruinvissen en grienden onderzocht door gebruik te maken van fysiologisch gebaseerde farmacokinetische (PBPK) modellen. Dit zijn computermodellen die fysiologische informatie van het organisme combineren met chemische eigenschappen van de chemische stof om zo de kinetiek van die bepaalde stof in kaart te brengen in het lichaam van het organisme. Zoals in blootstellingsexperimenten waarin zoveel mogelijk factoren gecontroleerd worden om variabiliteit te vermijden, zijn modellen voor wilde dieren ook betrouwbaarder als ze opgebouwd worden met behulp van robuuste of uniforme datasets. Eventuele trends zijn gemakkelijker te observeren en parameters zijn gemakkelijker te schatten als de invloed van externe factoren tot een minimum kan gereduceerd worden.

Bijgevolg was het belangrijk om eerst op zoek te gaan naar geschikte datasets. Daarvoor werd eerst onderzocht welke factoren een invloed kunnen hebben op de concentraties van chemische stoffen in het bloed, als enige circulatiemedium in de modellen, van zeezoogdieren. Bloedstalen van gewone zeehonden en bruinvissen met verschillende gezondheidstoestanden, oorsprong (gevangenschap versus wild), geslacht en leeftijd werden dus geanalyseerd. De resultaten hiervan toonden dat enkel uitgehongerde dieren afwijkende concentraties en profielen van PCB's en PBDE's hadden in hun bloed vergeleken met dieren die niet uitgehongerd waren. Uitgehongerd zijn kan uiteraard voorkomen in populaties in het wild, maar is daar zeker niet de norm. De conclusie hier was dus dat datasets van bloed gebruikt mogen worden voor het evalueren van bioaccumulatiemodellen. In zeezoogdieren

worden bloedstalen echter nooit genomen tegelijkertijd met stalen van andere weefsels omdat de dieren levend moeten zijn voor bloedafname, maar dood voor het verzamelen van stalen van andere weefsels. Voor modellen met meerdere compartimenten, zoals de modellen ontwikkeld in dit werk, werd echter de voorkeur gegeven aan het gebruik van resultaten uit andere weefsels dan bloed omdat op deze manier meerdere compartimenten tegelijkertijd geëvalueerd kunnen worden.
De dataset (vet, lever, nieren, spier, hersenen) van bruinvissen uit de Zwarte Zee werd het meest robuust of uniform geacht. Deze dataset was immers beperkt in tijd (stalen waren van dieren die allemaal gestorven waren in 1997-1998) en in ruimte (dieren van de Zwarte Zee blijven in de Zwarte Zee). Omwille van die redenen werd de voorkeur gegeven aan de dataset van de Zwarte Zee voor het evalueren van de allereerste bruinvismodellen. Deze modellen werden ontwikkeld om meer uitleg te kunnen geven over de bioaccumulatie van verschillende PCB's (PCB 153, 180, 101, 149, 118, 99, 170) en PBDE's (PBDE 47, 99, 100, 153) in mannelijke bruinvissen. De resultaten van deze modellen lieten zien dat concentraties van alle PCB's en PBDE's een piek bereikten op het einde van de lactatieperiode waarna ze daalden door het verdunningseffect tijdens de groei en een verandering in dieet (vis versus melk). Deze daling werd dan op iets latere leeftijd gevolgd door een stijging in concentraties die aanhield tot op het einde van het leven van de dieren. De modellen werden daarna gebruikt om tijdtrends bloot te leggen bij bruinvissen van de Noordzee. Deze oefening liet zien dat de PCB en PBDE concentraties daalden van 1990 tot 2008 al gebeurde dat niet aan dezelfde snelheid voor alle PCB's en PBDE's. De PBPK modellen werden eveneens gebruikt om de metabolische biotransformatie capaciteit van de bruinvissen te onderzoeken voor PCB 118, 149 en 101. Hieruit bleek dat er een relatief zwakke capaciteit was voor metabolische biotransformatie van PCB 118 en een capaciteit voor metabolische biotransformatie van PCB 101 die verbeterde met de leeftijd van de dieren. De resultaten lieten echter niet toe om besluiten te kunnen trekken voor PCB 149. Tot hiertoe werden alle parameters manueel geschat en werd de gevoeligheid van de parameters in de modellen getest via lokale gevoeligheidsanalyse. Dit type van gevoeligheidsanalyse houdt geen rekening met potentiële correlaties tussen de parameters. Daarom waren er andere, meer statistisch onderbouwde, methoden nodig voor het schatten van de parameters en voor globale gevoeligheidsanalyse.

Het toepassen van deze meer statistisch onderbouwde methoden voor de gevoeligheidsanalyse en het schatten van parameters was dus een logische volgende stap. In de meest recente PBPK modellen voor de bioaccumulatie van pesticiden (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD) in bruinvissen werden de parameters dus geschat met behulp van de theorie van Bayes uitgevoerd met Markov keten Monte Carlo (MCMC) simulaties. Daarbovenop werd de invloed van veranderingen in parameterwaarden op de output van de modellen getest met globale gevoeligheidsanalyse. Vergeleken met alle vorige PBPK modellen, verschilde het pesticide-model niet enkel in statistische technieken, maar ook in complexiteit. Het pesticide-model liet immers de kinetiek zien van drie stoffen (DDT en de metabolieten DDD en DDE) tegelijkertijd in plaats van een enkele stof. De kinetieken van die drie stoffen zijn dus onderling sterk verbonden. Net zoals de vorige modellen, werd dit pesticide-model eerste geëvalueerd met de dataset van bruinvissen van de Zwarte Zee

waarna de geschatte parameters verder werden geoptimaliseerd met behulp van de dataset van bruinvissen van de Noordzee.
Dezelfde technieken (theorie van Bayes en MCMC simulaties) werd ook toegepast in een PBPK model voor de bioaccumulatie van PCB 153 in grienden van Australië. Hiervoor waren er twee datasets beschikbaar, afkomstig van twee verschillende massa-strandingen. Grienden hebben sterke familieverbanden, dus wanneer er een individu terecht komt op het strand, volgen alle andere dieren zonder zich daar vragen bij te stellen. Voor de dieren zijn deze massa-strandingen uiteraard traumatische ervaringen. Voor onderzoekers zijn het echter mooie kansen omdat een dataset van zeezoogdieren bijna niet meer homogener kan zijn. In tegenstelling tot de bruinvismodellen, waren er echter enkel vetstalen beschikbaar van de grienden. De PBPK modellen van deze dieren zijn daarom natuurlijk kleiner dan de modellen van de bruinvissen. Desalniettemin werden de parameters geschat met de meest betrouwbare technieken zodat de modellen van de grienden eveneens reeds een nuttig en bruikbaar werkmiddel zijn voor het evalueren van gelijkaardige of meer uitgebreide datasets van grienden in de toekomst.

Dit werk zorgt voor nieuwe ideeën en innovatieve benaderingen om datasets van biomonitoring studies te bestuderen. De bioacumulatiemodellen die in het kader van dit werk werden ontwikkeld kunnen reeds gebruikt worden als referentiekader voor nieuwe datasets, maar kunnen eveneens verder geoptimaliseerd en uitgebreid worden in de toekomst. Deze resultaten zijn daarom niet alleen een nuttige aanvulling op reeds bestaande kennis, maar bieden ook nieuwe perspectieven om pollutie en effecten van pollutie in zeezoogdieren onder de loep te nemen. Zulke geïntegreerde benaderingen zijn noodzakelijk om beschermingsmaatregelen op te stellen voor een soort. De modellen ontworpen in dit werk zijn op deze manier dus nuttige tools voor risico-analyse.

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Weijs L. Yang RSH, Covaci A, Das K, Blust R, 2009. Physiologically based pharmacokinetic models for lifetime exposure to persistent organic pollutants by harbour porpoises (*Phocoena phocoena*). SMM (Society for Marine Mammalogy) Biennial Meeting, Quebec, Canada, 12-16 October 2009. (Oral presentation)

Shaw SD, Berger ML, Kannan K, Paepke O, Weijs L, Roosens L, Covaci A, 2009. Brominated flame retardants pose increasing health risks to marine mammals. SMM (Society for Marine Mammalogy) Biennial Meeting, Quebec, Canada, 12-16 October 2009. (Co-author)

Weijs L, Yang RSH, Covaci A, Das K, Blust R, 2009. Physiologically based pharmacokinetic models for lifetime exposure to persistent organic

pollutants by harbour porpoises (Phocoena phocoena): Data from the past, models for the future? VLIZ 10 years, special edition of the Jongerencontactdag, Oostende, 26-27 November 2009. (Poster presentation)

Weijs L, Jauniaux T, Das K, Neels H, Blust R, Covaci A, 2009. The fin whale from Antwerp: A toxicological perspective. VLIZ 10 years, special edition of the Jongerencontactdag, Oostende, 26-27 November 2009. (Poster presentation)

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Weijs L. van Elk N, Das K, Blust R, Covaci A, 2010. Persistent organic pollutants in harbour porpoise calves from 1990 until 2008: Young wildlife at risk? 30th International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2010, San Antonio, Texas, USA, 12-17 September 2010. (Poster presentation)

Weijs L. Covaci A, Das K, Blust R, 2010. Physiologically based pharmacokinetic (PBPK) models for the bioaccumulation of PBDEs in male harbour porpoises. 30th International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2010, San Antonio, Texas, USA, 12-17 September 2010. (Oral presentation)

Covaci A, Weijs L. Roosens L, Berger ML, Shaw SD, 2010. MeO-PBDEs, HO-PBDEs and HO-PCBs in liver samples of harbour seals from the Northwest Atlantic. 30th International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2010, San Antonio, Texas, USA, 12-17 September 2010. (Co-author)

Weijs L. Yang RSH, van Elk N, Jauniaux T, Das K, Covaci A, Blust R, 2010. Use of physiologically based pharmacokinetic models in marine mammal toxicology. 17th BENELUX Congress of Zoology (Classic Biology in Modern Times), Gent, Belgium, 22-23 October 2010. (Oral presentation)

Weijs L. van Elk N, Das K, Blust R, Covaci A, 2011. Persistent organic pollutants in harbour porpoise calves from 1990 until 2008: Young wildlife at risk? VLIZ 2011 Jongerencontactdag, Brugge, 25 February 2011. (Poster presentation)

Jauniaux T, Das K, Haelters J, Jacques T, Kiszka J, Pezeril S, Stekké V, Weijs L, Coignoul F, 2011. New evidence of PCB implication on the death of North Sea harbour porpoises. ECS (European Cetacean Society) meeting (22-24 March 2011). (Co-author)

Das K, Weijs L. Habran S, Gillet S, Dupont A, Lepoint G, Jauniaux T, Blust R, Covaci A, Debier C, Siebert U, 2011. The harbor seal and the harbor porpoise from the North Sea: Review of their ecotoxicological status based on stranded and free-ranging individuals and potential threats to the population. PRIMO meeting, Long Beach, California, USA (15-18 May 2011). (Co-author)

Weijs L. Yang RSH, Das K, Blust R, Covaci A, 2011. PCBs versus PBDEs: How similar compounds can behave differently in harbour porpoises. 31st International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2011, Brussels, Belgium, 21-25 August 2011. (Oral presentation)

Vanden Berghe M, Weijs L. Habran S, Das K, Pomeroy P, Covaci A, Debier C, 2011. Relationships between PCBs, PBDEs, their hydroxylated metabolites and vitamin A in grey seals during lactation. 31st

International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2011, Brussels, Belgium, 21-25 August 2011. (Co-author)

Vanden Berghe M, Weijs L. Habran S, Das K, Pomeroy P, Covaci A, Debier C, 2011. Maternal transfer of PCBs, PBDEs and their hydroxylated metabolites in grey seals (*Halichoerus grypus*) from the Isle of May, Scotland. 31st International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2011, Brussels, Belgium, 21-25 August 2011. (Co-author)

Dupont A, Weijs L. Siebert U, Hasselmeier I, Covaci A, Debier C, De Pauw-Gillet MC, Das K, 2011. Potential effects of blood contaminants on immune responses in harbour seals (*Phoca vitulina)*. 31st International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2011, Brussels, Belgium, 21-25 August 2011. (Co-author)

Dorneles PR, Lailson-Brito J, Secchi ER, Dirtu AC, Weijs L. Dalla Rosa L, Bassoi M, Cunha HA, Azevedo AF, Covaci A, 2011. Anthropogenic and naturally-produced organobrominated compounds in Antarctic humpback whales, *Megaptera novaeangliae*. 31st International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2011, Brussels, Belgium, 21-25 August 2011. (Co-author)

Weijs L. Roach AC, Manning TM, Chapman JC, Edge K, Blust R, Pemberton D, Covaci A, 2012. Is there a connection between pollution, mass-strandings and pilot whales from Australia? 32nd International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2012, Cairns, Australia, 26-31 August 2012. (Oral presentation)

Weijs L. Roach AC, Yang RSH, McDougall R, Lyons M, Housand C, Manning TM, Chapman JC, Edge K, Pemberton D, Covaci A, Blust R, 2012. Lifetime PCB 153 bioaccumulation and pharmacokinetics in pilot whales: Bayesian population PBPK modeling and Markov chain Monte Carlo simulations. 32nd International Symposium on Halogenated Persistent Organic Pollutants, Dioxin 2012, Cairns, Australia, 26-31 August 2012. (Oral presentation)

Weijs L. Briels N, Adams D, Blust R, Covaci A, 2013. Shark fin soup from a toxicological perspective: Eat it or leave it? VLIZ Jongerencontactdag 2013, Brugge, Belgium, 15 February 2013 (Poster presentation)

Weijs L. Tibax D, Roach AC, Manning TM, Chapman JC, Edge K, Blust R, Covaci A, 2013. Is there a connection between pollution, mass-strandings and pilot whales from Australia? 27th European Cetacean Society conference, Setubal, Portugal, 8-10 April 2013. (Poster presentation)

Weijs L. Yang RSH, Das K, Covaci A, Blust R, 2013. Bayesian population PBPK modeling and Markov chain Monte Carlo simulations as a computational approach to assess the bioaccumulation and kinetics of pollutants in marine mammals. PRIMO 17, Pollutant Responses In Marine Organisms, Faro, Portugal, 5-8 May 2013. (Poster presentation)

Weijs L. Yang RSH, Das K, Covaci A, Blust R, 2013. Kinetics of DDT, DDE and DDD in harbour porpoises: Application of Bayesian population PBPK modeling and Markov chain Monte Carlo simulations. PRIMO 17, Pollutant Responses In Marine Organisms, Faro, Portugal, 5-8 May 2013. (Oral presentation)

Weijs L. Tibax D, Roach AC, Yang RSH, McDougall R, Lyons M, Housand C, Manning TM, Chapman JC, Edge K, Pemberton D, Covaci A, Blust R, 2013. Lifetime PCB 153 bioaccumulation and pharmacokinetics in pilot whales: Bayesian population PBPK modeling and Markov chain Monte

Carlo simulations. 23rd SETAC Europe Annual Meeting, Glasgow, UK, 12-16 May 2013. (Oral presentation)

INVITED SPEAKER

2011: Oral presentation at the 'International Symposium on Advanced Studies by Young Scientists on Environmental Pollution and Ecotoxicology' held at Ehime University, Japan, August 4-7, 2011.

AWARDS

- 2007: Jacques Kets-award (Platform presentation; KMDA; Zoo Antwerpen; Parc Paradisio)
- 2008: Student Bursary Award (Best Student Paper in the 'POPs in Marine Mammals' session; Dioxin 2008; Birmingham, UK)
- 2009: Award for Best Student Presentation (Platform presentation; March 2009 at EAAM-meeting; Malta) + Student Participation Grant + Student Travel Grant
- 2009: Student Travel Grant to attend the biennial meeting of the Society for Marine Mammalogy in Quebec, Canada (12-16 October 2009)
- 2009: Honorable Mention (by the Jury) and First Runner-up (by the audience) at the VLIZ 10 years – meeting (Platform presentation, Oostende, November 2009)
- 2010: Best PhD Oral Presentation, 17th Benelux Congress of Zoology: Classic Biology in Modern Times (22-23 October 2010, Gent)
- 2012: Laureate of VOCATIO-award
- 2013: 3rd place in poster competition, VLIZ meeting (Brugge, 15 February 2013)
- 2013: Student Award, PRIMO 17: Pollutant Responses In Marine Organisms (Faro, Portugal, 5-8 May 2013)