Agenda Item 3.4

Review of New Information on Threats to Small Cetaceans

Pollution and its Effects

Document 3.4

Final Draft
Proceedings of the ECS Workshop *Chemical Pollution and Marine Mammals*

Action Requested

- Take note

Submitted by

European Cetacean Society

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DELEGATES ARE KINDLY REMINDED TO BRING THEIR OWN COPIES OF DOCUMENTS TO THE MEETING

Secretariat's Note

This report is being made available to the Advisory Committee as a final draft for information. It is currently with the authors for approval of the edits made on their submissions. The final version will be made available in the ECS Special Publication Series, as well as on the ASCOBANS website.

PROCEEDINGS OF THE ECS WORKSHOP

CHEMICAL POLLUTION AND MARINE MAMMALS

Held at the
European Cetacean Society's 25th Annual Conference,
Cadiz, Spain, 20th March 2011

Editor:

Peter G.H. Evans

ECS SPECIAL PUBLICATION SERIES NO. 55
AUG 2013

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Editor:
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AUG 2013

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INTRODUCTION

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Around fifty persons specialising in marine mammal toxicology and ecology from twelve countries participated in a workshop on the subject of chemical pollution and its effects upon cetaceans and seals, reviewing current levels of pollutants across Europe, long-term trends, and biological effects upon marine mammals at the individual and population level. The workshop was held in Cadiz at the 25th Annual Conference of the European Cetacean Society, with support from ASCOBANS and ACCOBAMS, and was co-organised by Peter Evans and Mark Simmonds.

The main purpose of the workshop was: *to make an assessment of the current level of understanding of the risks posed by chemical pollution to cetaceans and seals in the North East Atlantic, Baltic, Mediterranean and Black Sea regions, and to make recommendation for future investigations and appropriate actions to address threats.*

There were eleven contributions to the workshop, addressing two main themes: Insights from long-term datasets, and Pollutant trends and effects. Extended summaries of these papers are presented below. Some of the invited contributors were unable to attend the workshop in person but nevertheless generously submitted papers subsequently.

I am very grateful to UNEP/ASCOBANS for their funding support to enable the proceedings to be published, and in particular to Heidrun Frisch and Ana Berta García for their efforts, as well as to Peter Reijnders for kindly writing a Preface to the volume.

EUROPEAN CETACEANS AND POLLUTION – AN HISTORICAL PERSPECTIVE

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AN ISSUE EMERGES

Since the mid-1940s, large numbers of industrial chemicals and pesticides have been released into the environment and the final resting place for many is the marine environment (Colborn & Smolen, 2003). Concerns for cetacean health emerged when new technology started to show remarkably high concentrations of some substances in certain populations, and these same substances were associated with problems being seen in other species. Two classes of pollutants became the primary focus of concerns: the heavy metals, especially mercury (in its various forms), and the organochlorines, in particular the PCBs. This latter group of industrial compounds had been produced since the 1930s and were used as insulating and coolant fluids for industrial transformers and capacitors, hydraulic fluids, surface coatings for copy paper, lubricating oils, flame retardants, pesticide extenders and as additives in rubber, plastics, paints, and waxes. They were banned in many countries, along with some other compounds of concern, in the late 1970s and early 1980s. However, in some countries, particularly developing countries, they were still used in so-called closed systems.

Concerns were voiced both about impacts on cetaceans (e.g. O'Shea *et al.*, 1980) and also their human consumers when it became apparent that levels in some tissues far exceeded human health advisory limits (e.g. Simmonds *et al.*, 1994; and Simmonds & Johnston, 1994). Of particular concern were the endocrine disrupting effects of PCBs (e.g. Jacobson & Jacobson, 2003). By the early 1990s, a range of problems in marine mammals had been linked to tissue residues of pollutants (Reijnders, 1996, 2003). These included problems in Baltic and Wadden Sea seals, Californian sealions, Atlantic bottlenose dolphins in the USA, Mediterranean striped dolphins, and beluga whales in the St Lawrence estuary. Whilst the mechanisms linking effects to cause remained elusive, it was clear that bioaccumulation led to increases in body burdens of various xenobiotics, and that the primary exposure mechanism was diet. Other factors affected the observed body burdens including age and sex. In cetaceans, levels generally increased with age, until sexual maturity was reached when, typically, females started to show lower levels as lipophilic contaminants were passed to offspring during pregnancy and lactation.

A spate of epizootics in marine mammal populations around the world in the latter part of the twentieth century was also linked to their contaminant burdens – both in terms of their severity and occurrence (Simmonds & Mayer, 1997) - leading to much research and debate on this topic (e.g. Kennedy, 1999). In 1996, Peter Reijnders commented that "the existing epidemiological and experimental data... plus the possible contributing role of contaminants in some recent epizootics, warrant the conclusion that contaminants are a potential threat to cetaceans. Given the prolonged existence of the persistent pollutants already present in the oceans, there are certainly reasons to be concerned for cetaceans, and to establish a

comprehensive monitoring scheme" (Reijnders, 1996). By this time it was known that contaminants exerted their toxicity in a range of ways: for example, either as the parent compound or as metabolites or breakdown products. Different marine mammals and even different cetaceans were found to have differing capacities to metabolise xenobiotics, and it was suggested that cetaceans might be even more vulnerable than pinnipeds to certain pollutants, particularly the dioxin-type of PCB congeners (Reijnders, 1996).

POLLUTION CONTROLS ARE INITIATED

Key contaminant levels were seen to fall in many marine mammal populations, especially in the most highly polluted areas, as a result of improved pollution controls in the late 1970s, with the exception of mercury in the Arctic (Borrell & Reijnders, 1999; Colborn & Smolen, 2003; Reijnders & Simmonds, 2003). Borrell and Reijnders (1999) commented that whilst this trend was likely to continue for DDTs, PCBs would stabilise as degradation was compensated by new inputs. This has since been shown to be the case for a large sample of harbour porpoises (Law *et al.*, 2010). Nonetheless, the idea that pollution controls were being effective and levels were falling seems to have significantly drawn attention away from pollution as a threat to these species. Some key matters were unresolved, including whether existing levels in wild cetaceans could still be harmful.

PROBLEMS REMAIN AND NEW ONES THREATEN

Paul Jepson and his co-workers have relatively recently reported a correlation between poor health status (mortality due to infectious disease) and chemical contamination, for a large sample of UK-stranded harbour porpoises (Jepson *et al.*, 2005). This association exists for blubber concentrations above 17 ppm total PCBs lipid weight. Bottlenose dolphins (*Tursiops truncatus*) from the same region show even higher levels of contamination (up to one order of magnitude higher PCB levels in blubber), and, historically, levels are likely to have been even higher. Indeed, the available evidence shows that a decline in bottlenose dolphins around Europe coincides with the peak time for PCB exposure (Jepson *et al.*, 2008), and a study on population consequences of PCB exposure in bottlenose dolphins has revealed that the then current accumulation rate of PCBs might depress the potential growth rate through lowered calf survival (Hall *et al.*, 2006).

Whilst attention has continued to focus on the same classes of compounds that have become well known as environmental contaminants, new pollutants are also entering aquatic systems. Simmonds *et al.* (2002) drew attention to some of these novel xenobiotics, including the brominated flame retardants; the polyaromatic hydrocarbons and the organotins. To this as substances of potential concern for cetaceans now would certainly be added certain plasticisers, the perfluorinated compounds, nanoparticles, perchlorate (ammonium perchlorate (NH_4ClO_4), an inorganic chemical widely used as an oxidizer in solid propellants for rockets, missiles, fireworks and explosives), and pharmaceuticals and personal care products (medicines, insect repellents, sunscreens, perfumes, soaps, fragrances and lotions).

In the late 1990s, The International Whaling Commission (IWC) initiated a major collaborative research programme known as "Pollution 2000+" (Reijnders *et al.*, 1999). Annual reviews of its progress have been made, and in 2007 a major review meeting was held and the following goals established:

(1) develop an integrated modelling and risk assessment framework to assess cause-effect relationships between pollutants and cetaceans at the population level, using modification of a tiered risk assessment paradigm;

(2) extend the work to new species and contaminants as appropriate; and

(3) validate further biopsy sampling techniques for use in addressing issues related to pollution, including legacy contaminants and new contaminants of concern, and associated indicators of exposure or effects.

Most recently the IWC pollution initiative has been renamed "Pollution 2020", and is now focused on assessing the toxicity of microplastics and polycyclic aromatic hydrocarbons and dispersants in cetaceans (IWC, 2013).

Certainly much progress has been made in cetacean pollution studies over this period, and this includes work on non-lethal evaluation techniques and stress (e.g. Fossi *et al.*, 2003).

CONCLUSIONS

The latest evidence shows that there is still a significant pollution issue for at least some species in European waters. This might be particularly significant for our remaining small inshore populations of bottlenose dolphins and for the harbour porpoises generally, and especially in the Baltic. Monitoring and investigation will continue to be important, and research in this field should not remain locked on 'old' compounds or focused too narrowly at the individual level. Other new issues that deserve some careful consideration include geno-toxic effects (Reijnders *pers. comm*.), including epigenetics (see e.g. Baccarelli & Bollati, 2009).

Some of the key questions for the coming few years include:

- Why have the PCBs not continued to decline and what does this mean for the conservation and welfare of affected populations and how can this be addressed?
- What is the toxicological significance of the reported levels of novel xenobiotics?
- Are the monitoring schemes in place across Europe adequate to the task of monitoring the range of contaminants and potential effects present in cetaceans/marine wildlife?

ACKNOWLEDGEMENTS

With thanks to Peter Reijnders, Paul Jepson, and Peter Evans for their insights into this topic.

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LONG-TERM TIME-TRENDS IN ORGANOHALOGEN CONCENTRATIONS IN BLUBBER OF PORPOISES FROM THE UK

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INTRODUCTION

The UK Cetacean Strandings Investigation Programme (CSIP) began in 1990, following the 1988 seal phocine distemper virus outbreak. One of its aims was to gain an understanding of current contaminant levels in UK marine mammals, following a period in which few data had been gathered (Falconer *et al.*, 1983; Law *et al.*, 1989; Morris *et al.*, 1989), alongside the main aims of establishing the major causes of death and to investigate possible links between contaminants and disease. The latter because of expressed views, particularly from NGOs, that pollution had played a part in the disease outbreak. The initial focus was on trace elements, organochlorine pesticides and PCBs, and butyltin compounds. More recently, brominated flame retardants have been an additional focus of attention, as significant emerging contaminants. The use of banked tissue samples collected and archived within the CSIP also allowed retrospective trend analysis to be undertaken for some of these chemicals. We have recently reviewed and assessed time trend data available for polychlorinated biphenyls (PCBs; sum of 25 CB congeners during 1991-2005), polybrominated diphenyl ethers (PBDEs; sum of 9 BDE congeners during 1992-2008), and hexabromocyclododecane (HBCDs; sum of three isomers during 1994-2006).

METHODS

The analytical methods used are described in the original publications (Law *et al.*, 2008a,b; 2010a,b). Details of the statistical methods used are given in Law *et al.* (2010a), and will be summarised here. Data presented in Law *et al.* (2008a and 2010b) were originally treated in a slightly different manner, but have been reanalysed for this paper using the same method as that used in Law *et al.* (2010a). In short, for statistical treatment, congener/isomer concentrations below the limit of quantification (LOQ) were set to one-half of the LOQ. Congener/isomer concentrations for the three compound groups were summed following normalisation to the lipid content of individual blubber samples. Natural log transformation of summed concentrations was undertaken so as to stabilise the variance. We then investigated the effects of days since the 1st January of the initial year of each time-trend dataset on the summed lipid-normalised concentrations of the compound group. Potential confounding factors (area, season, nutritional status, whether individuals were stranded or bycaught, and age class) were also studied. In order to assess possible trends, we fitted a generalised additive model to the data using the function *gam* in the R package mcgv (R development core team, 2007), which smoothed the data as a function of days (Law *et al.*, 2010a).

RESULTS

PBDEs

PBDEs have been widely used as additive flame retardants in textiles, thermoplastics, polyurethane foams, and electrical products. Three main PBDE products have been used for

different applications, known as the penta-, octa- and deca-mixes. EU risk assessments led to controls on production and use of the penta- and octa-mixes, and they were withdrawn from the European market prior to August 2004. Use of the deca-mix in electrical and electronic goods was also banned from July 2008 (Law *et al.*, 2010a). The trend plot for Ln S9BDEs is shown below in Figure 1 (after Law *et al.*, 2010a).

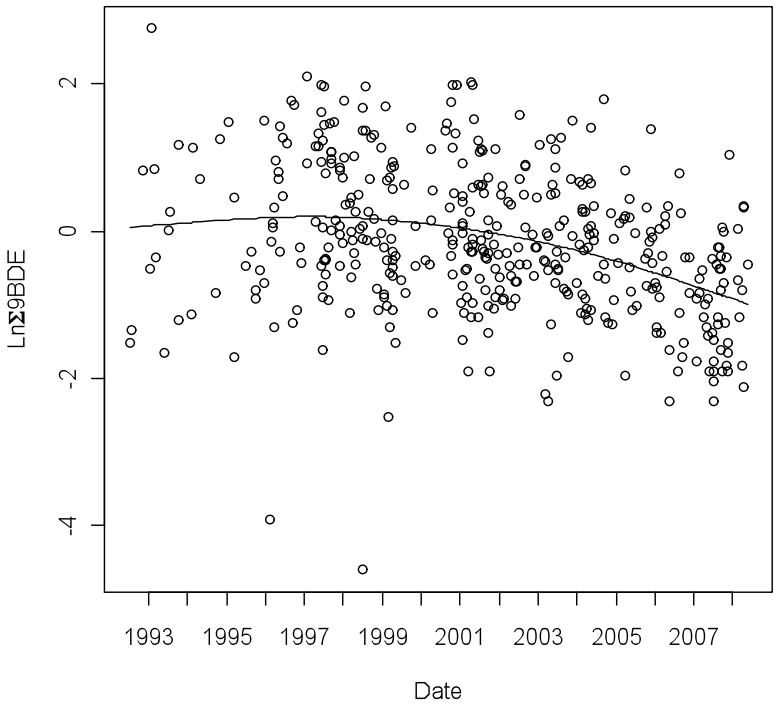

Figure 1. Ln Σ9BDE concentrations in porpoise blubber on a lipid basis by year. The continuous line represents the smoothed values from a Generalized Additive Model fitted to the data

It can be seen from Figure 1 that PBDE levels are now declining, with a reduction in concentrations of approximately two-thirds from 1997-2007. All the BDE congeners found in UK porpoises arise from the penta-mix PBDE product. In addition to the EU ban on the production and use of these products, a UK plant manufacturing PBDEs closed during the study period.

HBCDs

The trend plot for Ln SHBCDs is shown in Figure 2 below (after Law *et al.*, 2008a).

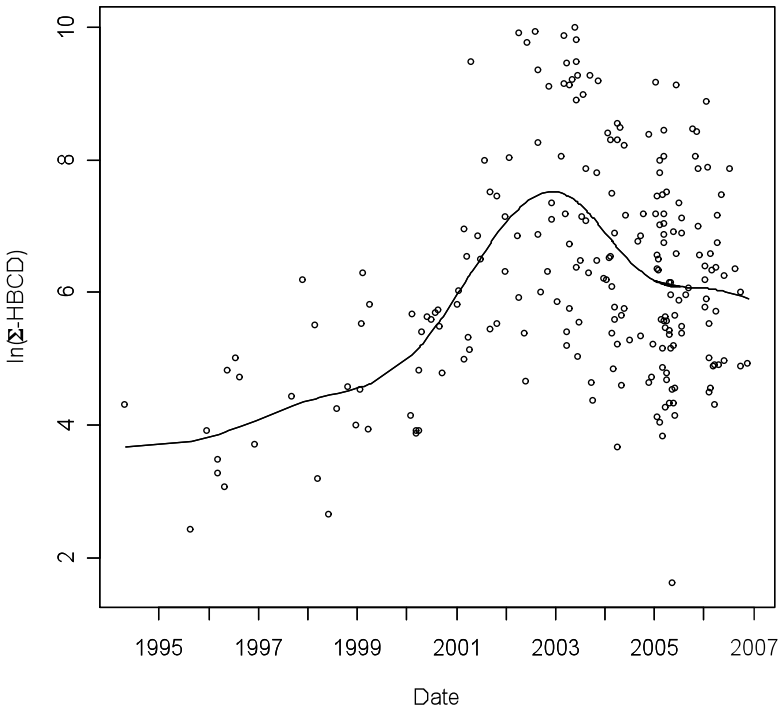

Figure 2. Ln SHBCD concentrations in porpoise blubber on a lipid basis by year

HBCD is an additive flame retardant used primarily in expanded and extruded polystyrene applied as thermal insulation in buildings. Following an earlier steep rise (reported in Law *et al.*, 2006), HBCD levels are now declining. HBCD has undergone risk assessment within the EU but remains in use. Two UK plants closed in 2002-2003: one manufacturing HBCD, and another using it in textile treatment. Improvements in industry practice may also have reduced emissions, although these schemes did not begin formally until 2006.

The removal from the marketplace of the penta- and octa-PBDE formulations and the restrictions on the use of the deca-PBDE product has led to an increase in use of alternative, "novel" brominated flame retardants, and these are also beginning to be found in environmental samples, including marine mammals (Covaci *et al.*, 2011). There is a wide range of these compounds now in use, and we currently have a project, which will screen UK porpoise blubber samples for around 20 of the more prominent of these alternatives. Results are expected during early 2012, and the data will then be reported in the literature.

PCBs

The trend plot for Ln S25CBs is shown below in Figure 3 (after Law *et al.*, 2010b).

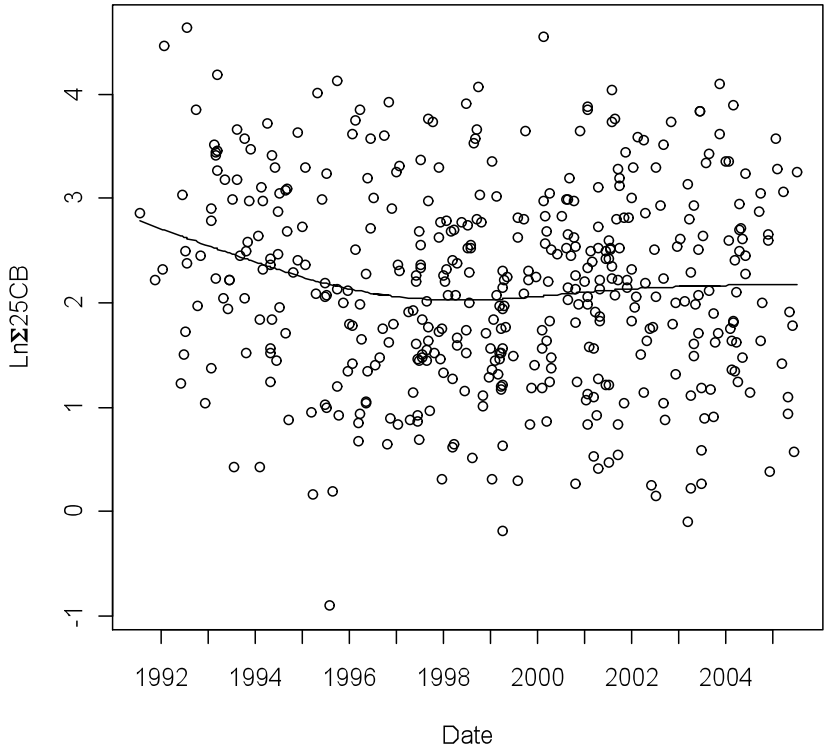

Figure 3. Ln S25CB concentrations in porpoise blubber on a lipid basis by year

PCBs were extensively used in a number of applications, particularly in electrical equipment. Since controls on the use of PCBs in the UK were initiated in 1981, it seems that concentrations declined during the early 1990s, but that this decline plateaued around 1997, and that only a slow decline can be expected in the future. Current levels are likely to represent those maintained by historic contamination of the marine environment and ongoing diffuse inputs. These concentrations are still at toxicologically significant levels in UK harbour porpoises (Jepson *et al.*, 2005; Hall *et al.*, 2006), and further efforts to reduce or limit discharges are needed. PCBs used in joint sealants in buildings built between the 1950s and the 1980s have been identified as a significant source of diffuse contamination (Kohler *et al.*, 2005). In Norway, Sweden and Switzerland, programmes are in place to tackle this problem. The PCB-containing joint sealants have been inventoried in buildings, and when buildings with such materials are demolished or remodelled, these materials are removed to secure landfill or high-temperature incineration. Removal from and replacement in other buildings is also either underway or proposed.

Geographical differences were observed in trends in S25CB concentrations (Law *et al.*, 2010b). In the East of England and the West of England and Wales, concentrations have stabilised, but in Scotland a slow increase has been apparent since 1995. As the concentrations in Scotland were initially lower than those in the other two regions, it is thought that this may reflect short-range atmospheric transport of PCBs – essentially a "levelling-up" of concentrations across the country.

DISCUSSION

One major group of organohalogen compounds not studied in this paper is the organochlorine pesticides. We have data for hexachlorocyclohexanes (including lindane), hexachlorobenzene, DDT and its metabolites, and dieldrin, but as these have not been analysed in all years, then the trend data are not as numerous. Our intention is to add some more data and analyse time-trends for these compounds during 2012. In other studies, steep declines have been noted in both marine mammals and birds of prey (Henny *et al.*, 2009a, b; Leonel *et al.*, 2010; Vorkamp *et al.*, 2008, 2009), and we wish to establish trends in UK porpoises in relation to these studies.

We have also studied another group of persistent organohalogens, the perfluorinated compounds, in a single study in UK porpoises, but have no trend information (Law *et al.*, 2008b). These compounds have many applications, including making textiles water-resistant, in carpets and papers, in metal plating, in the photographic and semiconductor industries, in hydraulic fluids, in fire-fighting foams, in medical applications and as mining and oil surfactants. As a result of this widespread use, they are globally distributed, and are also bioaccumulative. Two of these compounds, PFOS and PFOA, were determined in the liver tissue (they bind to proteins, unlike lipophilic compounds which accumulate in blubber) of 58 animals stranded or bycaught between 1992-2003. No PFOA was detected; concentrations of PFOS were <16 to 2,420 µg/kg wet weight, comparable to those seen in other European studies. The highest concentration was observed in a juvenile female porpoise from Swansea (South Wales) in 2002.

From the trends presented here, it is clear that restrictions on production and use, and improved industry housekeeping, have led to significant declines in the concentrations of both of the brominated flame retardants, but that reductions in concentrations of PCBs are proving more problematic. This is despite the fact that regulatory controls were initially set in place 30 years ago. This highlights the problems encountered following unrestricted or poorly restricted use and release to the environment of persistent, bioaccumulative and toxic organic compounds. Hopefully, this is something that the EU REACH (Regulation, Evaluation, Authorisation & restriction of CHemicals) legislation will be able to redress (Kemmlein *et al.*, 2009).

ACKNOWLEDGEMENTS

The authors would like to thank the UK Department for Environment, Food and Rural Affairs for funding this work.

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PERSISTENT ORGANIC POLLUTANTS IN UK-STRANDED CETACEANS (1990-2008): LINKING EXPOSURE WITH TOXIC EFFECTS

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ABSTRACT

Between 1990 and 2008, a close collaboration between the Centre for Environment, Fisheries and Aquaculture Science (Cefas) and the UK Cetacean Strandings Investigation Programme (CSIP) has generated one of the largest time-series datasets on chemical contaminants in a marine mammal species - the UK-stranded harbour porpoise (*Phocoena phocoena*). This dataset shows that some organochlorine pesticides like summed DDTs (n=368) and dieldrin (n=429), have gradually declined over time since 1990. In contrast, summed 25 individual chlorobiphenyl levels (sum25CBs) (n=540) have remained relatively stable in UK-stranded harbour porpoises since the mid-1990s, and levels in many individual porpoises often exceed all known or proposed toxicity thresholds for marine mammals. Brominated flame retardants including summed brominated diphenylethers (sumBDEs) (n=415) and hexabromocyclododecane (HBCD) (n=223) emerged in the 1990s with periods of increasing blubber concentrations in UK-stranded harbour porpoises towards the late 1990s-early 2000s, before mean levels peaked and subsequently declined. Brominated diphenylethers trends were linked to anticipated and actual EU-wide bans (e.g. penta-mix PBDE). Using relatively large sample sizes, sum25CBs levels are statistically linked here to susceptibility to fatal infectious diseases (n=182) in case-control epidemiological studies using "healthy" acute trauma cases as controls (n=276). Although using much smaller sample sizes, mean sum25CBs levels in UK-stranded bottlenose dolphins (*Tursiops truncatus*)(n=15) and killer whales (*Orcinus orca*)(n=5) were up to one order of magnitude higher than mean levels found in UK-stranded harbour porpoises. Such high PCB exposure poses a significant (but very cryptic) threat to the conservation status of at least some bottlenose dolphin and killer whale populations/ecotypes in the NE Atlantic region.

INTRODUCTION

Marine mammals are exposed to a variety of anthropogenic contaminants mainly through their diet. This group of chemicals includes the organohalogenated compounds (such as the polychlorinated biphenyls - PCBs), the dichlorodiphenyltrichloroethanes (DDTs), chlordane, toxaphene, the cyclodienes (such as aldrin and dieldrin), and polychlorinated terphenyls (PCTs), polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDEs). Such compounds are typically highly lipophilic and hydrophobic, and bioaccumulate sometimes to high concentrations in lipid-rich tissues like marine mammal blubber (Tanabe *et al.*, 1994). They can have many different isomers and congeners, and comprise hundreds of different chemical formulations, which may have different behaviours and toxicities. They are chemically very stable and persistent, many compounds being resistant to metabolic degradation. Top predators are at particular risk from biomagnification of persistent organic pollutants (POPs) due to the greater abundance, resistance to environmental degradation, and known toxicity of these compounds. In marine and terrestrial mammals these

compounds can have a range of reproductive, immunosuppressive and other effects (Reijnders, 1994; Tanabe *et al.*, 1994; Colborn & Smolen, 1996).

A number of factors can affect the occurrence and distribution of POPs in marine mammals including diet, foraging strategy, age, species, sex and nutritional condition (Aguilar *et al.* 1999). This is particularly the case for animals that do not feed during the breeding season, and adult females that can offload a large proportion of their pollutant burdens to their offspring (Debier *et al.*, 2003). The production of PCBs and DDTs has been limited or completely banned since the 1970s in most developed countries (1981 for full PCBs ban in the UK). Nearly 97% of the historical use of PCBs was estimated to have occurred in the Northern Hemisphere (Breivik *et al.*, 2007), and only 30% of the produced PCBs have dispersed in the environment (Tanabe *et al.*, 1988). PCBs have an environmental half-life of 20–40 years but are declining only very slowly in most ecosystems globally (Erickson, 1986). Several factors including improper storage, accidental release, inadequate disposal and ongoing use in materials and products contribute to their continuing presence in the marine environment (Tanabe *et al.*, 1994; Aguilar *et al.*, 2002; Breivik *et al.*, 2007). Predictions of global trends in PCBs suggest that PCB levels will not decline until around 2050 onwards (Breivik *et al.*, 2007). The use of PCBs was banned progressively from open and closed uses in the UK, beginning in 1981. The other two compounds are both flame retardants. Polybrominated diphenyl ethers (PBDEs) comprise three technical products, known as the penta-mix, octa-mix and deca-mix formulations, of different degrees of bromination. The structure of BDEs is similar to that of chlorinated biphenyls (CBs), and 209 congeners are possible in both cases. The penta- and octa-mix products were withdrawn from the European market prior to August 2004 and the deca-mix product was banned from use in electrical and electronic goods within the EU from July 2008. HBCD has been subject to an EU risk assessment of continued production and use and currently no restrictions have been placed upon that compound.

The main objective of this study was to use available toxicological and pathological data from UK-stranded cetaceans to assess trends in contaminant exposure and to assess risk of toxicological effect. Statistical analyses were conducted to test the hypothesis that chronic PCB exposure may predispose UK-stranded harbour porpoises (*Phocoena phocoena*) to mortality associated with infectious disease using a case-control study approach. Specifically, summed blubber concentrations of 25 individual CB congeners (sum25CBs) in apparently healthy harbour porpoises that died due to acute physical trauma (e.g. by-catch) were compared statistically with summed blubber concentrations in animals that died due to a variety of infectious diseases. These levels were also compared to mean levels in selected other species including UK-stranded bottlenose dolphins (*Tursiops truncatus*) and killer whales (*Orcinus orca*).

MATERIALS AND METHODS

Between January 1990 and December 2010, 2884 cetacean carcasses stranded in the UK or caught in commercial fishing nets were retrieved for necropsy using a standard necropsy protocol (Jepson, 2006; Law, 2006c). These included harbour porpoises (n=1804), bottlenose dolphins (n=124) and killer whales (n=5). Microbiological examination consisted of a routine tissue sample or swab taken aseptically for aerobic and anaerobic

bacteriological analyses and tissue samples for histological examination which were preserved in neutral buffered 10% formalin, embedded in paraffin, sectioned at 2-6 µm and routinely stained with haematoxylin and eosin, and occasionally with periodic acid-Schiff (Jepson *et al.*, 2005; Jepson, 2006). The diagnosis of entanglement in fishing gear (by-catch) and a range of other causes of death were determined according to specific diagnostic criteria (Jepson, 2006; Deaville & Jepson, 2011). Individuals that were freshly dead or in slightly decomposed condition (Jepson, 2006), and for which a cause of death could be identified, were prioritised for chemical analysis to minimise changes in blubber organochlorine levels associated with postmortem decomposition (Borrell & Aguilar, 1990). Toxicological examinations were conducted in harbour porpoises (n=540), bottlenose dolphins (n=15), and killer whales (n=5).

Analyses of contaminants in UK cetaceans were conducted according to standardised necropsies (Jepson, 2006) and analyses of all chemical contaminants in blubber/liver samples were conducted using internationally standardised methodologies (e.g. Jepson, 2006; Law *et al.*, 2006c; Law *et al.*, 2010a; Law *et al.*, 2010b). The methods used for determining concentrations of contaminants in blubber were: CBs – gas chromatography with electron-capture detection; BDEs – gas chromatography/electron capture negative ion mass spectrometry; HBCD – high performance liquid chromatography/electrospray negative ion mass spectrometry. Full analytical quality control procedures were applied in all cases, including the analysis within each batch of a certified or laboratory reference material used to track the day-to-day performance of the method, and participation in both a laboratory proficiency scheme and inter-laboratory studies as available. A non-parametric statistical method was used, because it avoids making assumptions about the distribution of the *S*-values, and, more importantly, does not assume any particular functional form for the trend (e.g. linear, exponential). Potential confounding factors (area, season, by-caught or stranded, age class, sex, blubber thickness and lipid content) were investigated and found not to confound any of the trends identified. In studying possible time-trends for CBs, data were available for harbour porpoises (n=540) (Figure 1), bottlenose dolphins (n=15) (Figure 2) and killer whales (n=5) during 1991-2005. In this case, the same suite of 25 CB congeners (Σ25CBs) was determined throughout the study period, comprising CB18, CB28, CB31, CB44, CB47, CB49, CB52, CB66, CB101, CB105, CB110, CB118, CB128, CB138, CBc 2006).

RESULTS

The results of these temporal trend assessments have been published in the scientific literature (Law *et al.*, 2008; Law *et al.*, 2010a; Law *et al.*, 2010b). Summed 25 CBs (Σ25CBs) concentrations in UK harbour porpoises declined only slowly in the 1990s and levelled off in the 2000s as a result of a ban on the use of PCBs which began more than two decades ago (Figure 1) (Law *et al.*, 2010a). In contrast, organochlorine pesticides such as DDTs and dieldrin have declined much faster in UK-stranded harbour porpoises (Law *et al.*, 2010a). There are also regional differences in PCB and OC pesticide levels within UK waters (e.g. lower levels in strandings in Scotland) (Law *et al.*, 2010a). PCB exposure data also exist for UK-stranded bottlenose dolphins (n=15) and killer whales (n=5) for the same period (1991-2005). The mean level for PCBs in UK-stranded bottlenose dolphins was almost 100 mg/g lipid weight (Jepson *et al.*, 2008) (Figure 2), and 225mg/g lipid weight for the killer whales (Law, 2006c; CEFAS, unpublished data).

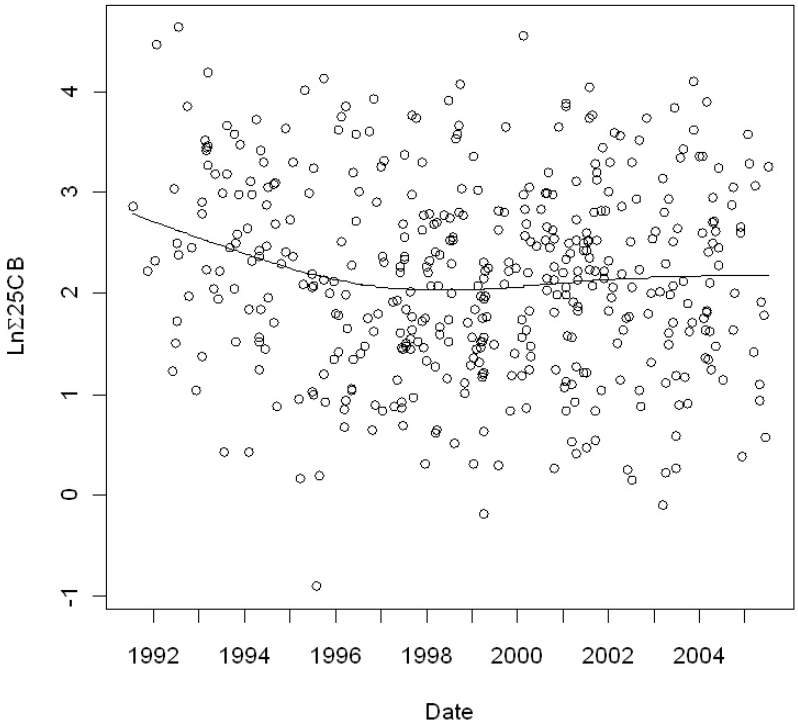

Figure 1. Ln Σ25CB (the natural logarithm of the sum of 25 CB congeners determined) concentrations on a lipid basis by year for 440 harbour porpoises (*Phocoena phocoena*) stranded in the UK from 1991-2005 (based on Law *et al.* 2010; Marine Pollution Bulletin 60: 470-473)

In the period 1994-2003, a sharp increase in blubber concentrations of hexabromocyclododecane (HBCD) from about 2001 onwards, was reported in UK-stranded harbour porpoises (Law *et al.*, 2006). The maximum HBCD concentration observed was 21.4 $mgkg^{-1}$ lipid wt in a porpoise which died in 2003. A further study of UK-stranded harbour porpoises (n=223) showed a statistically significant decrease in HBCD levels from 2003 to 2006 (Law *et al.*, 2008). For the investigation of BDE time-trends, data were available for 415 porpoises necropsied between 1992 and 2008. Nine congeners were determined throughout and the sum of the concentrations of these was used for the time-trend assessment. The congeners were: BDE28, BDE47, BDE66, BDE85, BDE99, BDE100, BDE138, BDE153 and BDE154. The maximum summed BDE concentration observed was 15.7 $mgkg^{-1}$ lipid wt in an animal that died in 1993. The analysis indicates that the median concentrations peaked around 1998 and had reduced by between 55% and 76% by 2008. The best point estimate is 66% ($p < 0.001$). This finding was not confounded by a range of other factors, which were also considered (area, season, nutritional status, bycaught/stranded and age class) (Law *et al.*, 2010b).

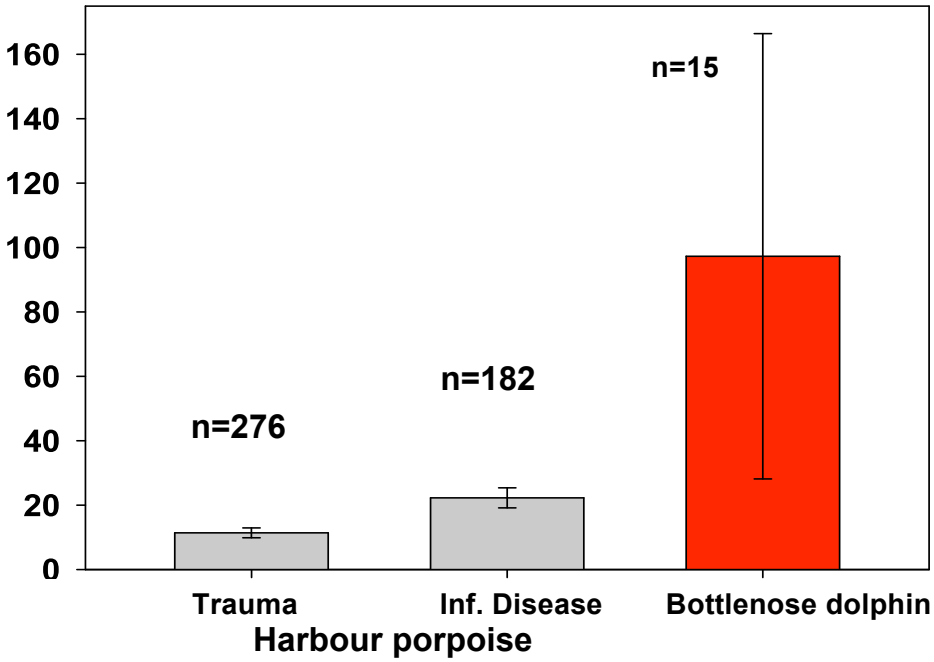

Figure 2. Comparison of mean summed 25CBs concentrations in UK-stranded harbour porpoises (trauma and infectious disease cases) and bottlenose dolphins (1991-2005). Bars=2SE. Taken from 2010 annual report for ICES Working Group on Marine Mammal Ecology (ICES 2010)

CONCLUSIONS

Summed 25 CBs (Σ25CBs) concentrations in UK-stranded harbour porpoises declined only slowly in the 1990s and have levelled off since 1997 following a ban on the use of PCBs which began more than two decades ago (Law *et al.*, 2010a). In contrast, organochlorine pesticides such as HCHs and dieldrin have declined much more quickly, and are often now at low levels (Law *et al.*, 2010a). There are also regional differences in PCB and OC pesticide levels within UK waters (lower levels in Scotland), possibly reflecting differences in diffuse inputs and transfer between regions. The reason for the slow decline in PCBs is not known with certainty but is likely to be due to both continuing diffuse inputs from e.g. PCB-containing materials in storage and in landfills, where these were disposed of prior to the more stringent requirements for such sites being enacted, and to the substantial reservoir of PCBs already in the marine environment.

Potential effects of PCBs in individual harbour porpoises in European waters have been identified, including immunosuppression (Beineke *et al.*, 2005) and mortality due to infectious disease (Jepson *et al.*, 2005; Hall *et al.*, 2006a; Deaville & Jepson, 2011). Statistical associations between high PCB exposure and high parasite burdens have also been found in

UK-stranded harbour porpoises (Bull *et al.*, 2006). Similar or even higher PCB levels have been recorded in stranded bottlenose dolphins and killer whales in UK waters (Jepson *et al.*, 2008, ICES, 2010; Davison *et al.*, 2011; Deaville & Jepson, 2011). These PCB levels greatly exceed those associated with infectious disease mortality in case-control studies on UK-stranded harbour porpoises (Jepson *et al.*, 2005; Hall *et al.*, 2006a). It is difficult to obtain sufficient sample sizes to conduct case-control studies in bottlenose dolphins or killer whales, partly because stranding rates of both these species are low. However, one study has recently associated high blubber PCB levels with *Brucella ceti* infection in UK-stranded bottlenose dolphins in southwest England (Davison *et al.*, 2011). Although these data are from stranded animals, they show that PCB exposures in UK-stranded bottlenose dolphins are often greater than levels in biopsied bottlenose dolphins in the SW Atlantic such as Indian River Lagoon (Florida, USA), Sarasota Bay (Florida, USA) and Charleston (North Carolina, USA) (Schwacke *et al.*, 2002; Wells *et al.*, 2005; Hall *et al.*, 2006b; Fair *et al.*, 2010). Current abundance estimates for bottlenose dolphins in the NE Atlantic and Mediterranean Sea often show small or contracting populations in coastal waters (e.g. North Sea, Biscay, Mediterranean Sea) (Hammond *et al.*, 2002; ICES, 2010).

PCB blubber levels in UK-stranded killer whales are also similar to the very highest PCB levels recorded in adult transient male killer whale blubber in British Columbia, Canada (Ross *et al.*, 2000; McHugh *et al.*, 2007). There is a scarcity of data on PCB levels from stranded or biopsied killer whales but the few studies that have been conducted show extremely high levels in killer whales in the North-east Atlantic, Arctic waters (Law, 2006c, McHugh *et al.*, 2007; Wolkers *et al.*, 2007) and British Columbia, Canada (Ross *et al.*, 2000), which typically exceed proposed thresholds for PCB toxicity (Kannan *et al.*, 2000; Jepson *et al.*, 2005). Most killer whale populations that have been assessed for abundance and population trends (mainly in the Pacific) are stable or declining (e.g. COSEWIC, 2008) and concerns must exist about the very high exposure to PCBs in North-east Atlantic waters where no reliable population estimates are available. Killer whales in the North-east Atlantic could be negatively impacted by PCBs at the population level throughout their entire range. Due to the lack of abundance estimates for killer whales in the North-east Atlantic, any population level declines due to PCBs, and other factors, would be largely undetected.

For the brominated flame retardants (BDEs), the median concentrations peaked around 1998 and then reduced by between 55% and 76% to 2008, primarily due to the anticipation of a commercial ban on the penta-mix PBDE product by the EU in 2004 (Law *et al.*, 2010b). Other PBDE commercial products were subsequently banned by the EU after 2004, following further risk assessments. One particular flame retardant, hexabromo-cyclododecane (HBCD), was found at relatively high levels in the blubber of harbour porpoises stranded along the Irish Sea coast, where levels were an order of magnitude higher (~3 μg g^{-1} lipid) than elsewhere except on the north-west coast of Scotland where levels were ~5 μg/g lipid (Zegers *et al.*, 2005). Possible contributory factors to the observed decrease include the closure in 2003 of an HBCD manufacturing plant in NE England, which had considerable emissions up to 2003, and the closure in 2002 of a plant in NW England using HBCD in the manufacture of expanded polystyrene. Two voluntary schemes intended to reduce emissions of HBCD to the environment from industry may also have had some impact, although they did not formally begin until 2006.

SUMMARY

In summary, although polychlorinated biphenyls (PCBs) levels have stabilised in UK and other waters (e.g. harbour porpoises in UK waters), they undoubtedly continue to pose the greatest toxicological threat to some marine mammal species within UK and adjacent European waters. In several UK-stranded species, including bottlenose dolphins and killer whales, blubber PCB concentrations still regularly exceed all known or proposed thresholds for mammalian toxicity (e.g. Kannan *et al.*, 2000; Jepson *et al.*, 2005). Despite being banned for two to three decades, further steps to reduce high PCB exposure in marine top predators like bottlenose dolphins and killer whales, are still undoubtedly needed (Law *et al.*, 2010a).

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LONG-TERM RESEARCH ON PERSISTENT ORGANIC POLLUTANTS IN BOTTLENOSE DOLPHINS FROM SARASOTA BAY, FLORIDA, USA

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Research initiated in 1970 has identified a long-term, year-round resident community of about 160 bottlenose dolphins (*Tursiops truncatus*) spanning up to five generations in Sarasota Bay, Florida, USA, providing unparalleled opportunities to investigate the status, dynamics, and potential impacts of persistent organic pollutants (POPs) on small cetaceans (Wells, 2009). Regular observational monitoring, combined with occasional capture, sample, and release operations for health assessments provide direct measures of contaminant concentrations, health and body condition, and endpoints including survival and reproductive success (Wells, 2003; Wells *et al.*, 2004, 2005). The research in Sarasota Bay has also contributed to the development of techniques for investigation of contaminant exposure and effects, and has provided reference data for interpreting findings from other sites.

The long-term monitoring of the Sarasota Bay dolphin community has served as a valuable resource for understanding the biological factors that influence contaminant exposure in cetaceans. Wells *et al.* (2005) examined relationships between POP residues and life-history and reproductive parameters, taking advantage of the availability of data from identifiable individual dolphins of known age, sex, and maternal lineage, as well as information on spatial and temporal occurrence, births and fates of calves, and birth-order. POP concentrations in blubber and plasma were examined relative to age, sex, lipid content, and birth-order. Wells *et al.* (2005) analysed 47 blubber samples collected during June 2000 and 2001 for concentrations of 22 polychlorinated biphenyl (PCB) congeners. Classical patterns of accumulation with age were identified in males, but not in females. Before reaching sexual maturity, males and females exhibited similar concentrations (range, 15–50 ppm lipid wt). Following the onset of maturity, males continued to accumulate PCBs throughout their lives (range 50 to 843 ppm lipid wt). In contrast, females began to depurate with the birth of their first calf, reaching a balance between contaminant intake and lactational loss (maximum <15 ppm lipid wt). Primiparous mothers exhibited both higher PCB concentrations in blubber and plasma, and higher rates of first-born calf mortality, than more experienced mothers. Correspondingly, first-born calves had higher PCB concentrations than subsequent calves of similar age (>25 ppm vs. <25 ppm). Maternal tissue concentrations increased from early lactation to the time when calves approached nutritional independence. Wells *et al.* (2005) demonstrated that long-term observational monitoring and periodic biological sampling provide a powerful, non-lethal approach to understanding relationships between POP concentrations in tissues, life history traits and reproductive parameters for inshore dolphins.

Additional work by Yordy *et al.* (2010a) found that significant variations in POP mixtures existed within the Sarasota Bay population, and that life history traits such as age and sex may drive individual differences. A full suite of 66 PCBs, 14 organochlorine pesticides (OCP) and 5 polybrominated diphenyl ether (PBDE) congeners were measured in 104 blubber samples and 20 milk samples from Sarasota Bay dolphins. Principal components analysis (PCA) identified significant variations in individual POP mixtures related to age, sex and reproductive maturity. POP mixtures in juvenile dolphin blubber initially resembled patterns in milk, their primary food source, and were dominated by lower-halogenated PCBs and PBDEs. With age, juvenile POP patterns gradually shifted away from the milk-like pattern. POP patterns continued to change gradually with age in mature males, a likely result of the selective accumulation of persistent POP congeners. In contrast, mature females exhibited dramatic changes in POP profiles upon reaching reproductive maturity. Congener-specific blubber/milk partition coefficients indicated that lower-halogenated POPs were selectively offloaded into milk and changes in adult female contaminant profiles likely resulted from depuration of these compounds during birth and lactation, with gradual re-accumulation thereafter. Taken together, data from Wells *et al*. (2005) and Yordy *et al.* (2010a) suggest POP exposure is highly variable among individuals within a population, strongly related to biological factors and an important consideration for assessing risks to individuals.

Regular health assessments of Sarasota Bay dolphins have facilitated direct comparisons between contaminant exposure and health parameters. Early work by Lahvis *et al.* (1995) suggested that levels of POPs measured in Sarasota Bay male bottlenose dolphins were sufficiently high to warrant concern. Blood was sampled from five Sarasota Bay male dolphins and concurrently assessed for immune function and contaminant concentrations. The *in vitro* responses of peripheral blood lymphocytes to two T-cell mitogens (concanavalin A (Con A), phytohemagglutinin (PHA)) were used as indicators of immune function. When compared with contaminant concentrations using regression analysis, a reduced immune response was found to be significantly correlated with increasing whole blood concentrations of several contaminants, specifically, the tetra-chlorinated to octa-chlorinated biphenyls and p,p' DDT, o,p'- DDE, and p,p'- DDE, indicating a potential risk of immune suppression for Sarasota Bay dolphins at their current level of exposure.

Risk assessment models have also suggested that the current level of POP exposure may impact the reproductive success of Sarasota Bay female dolphins. Hall *et al.* (2006) examined the effect of different PCB accumulation scenarios on potential population growth rates. Through the development of an individual-based model framework simulating the accumulation of PCBs in the population and modifying first-year calf survival based on concentrations of PCBs in the mothers' blubber, Hall *et al.* determined that the estimated annual PCB accumulation rate for the Sarasota Bay dolphin population might be depressing the potential population growth rate, but cautioned that their predictions might be limited to some extent by model naivety and parameter uncertainty. Schwacke *et al.* (2002) included Sarasota Bay females in their probabilistic risk assessment approach that integrated measured tissue concentrations of PCBs with a surrogate dose–response relationship, leading to predictions of detrimental reproductive effects in female dolphins. The outcome of risk analyses for Sarasota Bay females, as well as populations near Beaufort, NC and Matagorda Bay, TX, indicated a high probability that reproductive success, primarily in primiparous females, is being severely impaired by chronic exposure to PCBs. Excess risk of

reproductive failure, measured in terms of stillbirth or neonatal mortality, was estimated as 79% for primiparous Sarasota Bay females, higher than the other sites. Experienced mothers, having previously depurated much of their PCB burden, exhibited a much lower risk.

In vitro assays are proving to be an important tool with which to assess relationships between exposure and effects in protected species such as bottlenose dolphins. By utilizing an estrogen-responsive human cell culture model (E-SCREEN), Yordy *et al.* (2010b) contributed to the understanding of how POPs may interact in mixture to influence the magnitude and type of endocrine effects observed in bottlenose dolphins. A comparative approach was used to assess whether the complex contaminant mixtures in bottlenose dolphins are estrogenic or antiestrogenic in nature. Interactions of antiestrogenic and estrogenic compounds were first investigated with the E-SCREEN assay using a mixture of four POPs commonly found in dolphin blubber. Estrogenic/antiestrogenic activity was determined for the individual compounds and for their binary, tertiary, and quaternary combinations. Variations in POP mixture composition led to significantly different responses, including enhanced estrogenic and antiestrogenic effects and antagonistic interactions. Yordy *et al.* (2010b) then compared these results to the concentrations and estrogenic/antiestrogenic activity of POP mixtures isolated directly from the blubber of 15 bottlenose dolphins from five Atlantic and Gulf of Mexico sites. Significant estrogenic activity was detected in male dolphin blubber from Cape May, NJ and from Bermuda, and positive correlations between blubber estrogenicity and select POPs were also found, suggesting, but not necessarily proving, that some populations may be exposed to POPs that synergistically yield estrogenic effects at concentrations of biological relevance.

The ability to monitor exposure and effects of POPs on bottlenose dolphins has been aided by new methodology, some of which was developed through work with Sarasota Bay dolphins. Wilson *et al.* (2007) assessed the utility of cytochrome P450 1A1 (CYP1A1) induction as a biomarker of POP exposure in integument biopsies from Sarasota Bay dolphins. CYP1A1 expression was observed in endothelial cells, vascular smooth muscle, and nerve cells in the dermis, similar to what has been found for other cetaceans. Total PCB and toxic equivalent quotient (TEQ) concentrations in blubber were positively correlated with dermal endothelial CYP1A1 expression (but mono-ortho PCBs concentrations did not show this relationship), suggesting that POP concentrations may be stronger determinants of CYP1A1 expression than are age, sex, or reproductive status. Tornero *et al.* (2005) alternatively assessed the use of blubber retinoids as biomarkers of POP exposure. Blubber samples were collected from 47 individuals and relationships between age, sex, blubber lipid content, retinoid concentrations and POP concentrations were assessed. Blubber lipid content was low overall and decreased significantly with age in adult males. Retinoid blubber concentrations were strongly related to lipid content, therefore, as a result, retinoid concentrations appeared to decline with age in adult males. This effect could not be dissociated statistically from the negative correlation between levels of POPs and retinoid blubber concentrations, so it could not be determined whether high POP loads lowered retinoid concentrations or, conversely, whether depleted lipid reserves were responsible for the high POP concentrations and the low blubber retinoid levels.

By facilitating the collection of blood and other tissue samples from free-ranging dolphins, health assessments of the Sarasota Bay dolphin population have led to the development of less invasive sampling techniques for assessing POP exposure. Yordy *et al.* (2010c) determined the relationship between blubber and blood POP concentrations by analyzing matched blubber and plasma samples from 56 dolphins for 61 PCB congeners, 5 PBDE congeners, and 13 organochlorine pesticides. With the exception of PCB 209, lipid-normalised concentrations of the major POPs in blubber and plasma were positively and significantly correlated, suggesting blubber may be used to estimate blood concentrations, and vice versa.

The analysis of tissue samples collected from stranded dolphins at necropsy can also provide valuable information on the toxicodynamics and tissue distribution of POPs, an important consideration for assessing health risks from exposure. Yordy *et al.* (2010d) provided a detailed description of the distribution of POPs in dolphin tissues and assessed the role of lipid dynamics in mediating POP distribution. Thirteen tissues were sampled during necropsies of four bottlenose dolphins and analyzed for lipid and 85 POP congeners, including PCBs, OCPs, and PBDEs. The distribution of POPs was found to be generally but not entirely, related to tissue lipid content. Blubber was determined to be the primary site for POP accumulation, contributing >90% to the whole-body burdens. The melon, a metabolically inert lipid-rich structure, was also identified as an alternate POP depot unique to cetaceans that is potentially capable of reducing POP availability to other tissues. Yordy *et al.* (2010c,d) also found that as lipid mobilises from blubber, contaminants may redistribute within the body, resulting in elevated concentrations in blood and other tissues. As a consequence, individuals with reduced blubber lipid may be at increased risk for exposure-related health effects.

The extended monitoring of Sarasota Bay dolphin population has resulted in a comprehensive collection of life history, health and contaminant exposure data that has served as a basis for establishing spatial trends in bottlenose dolphin exposure and health effects. Houde *et al.* (2006) measured PCBs and hydroxylated metabolic products (OH-PCBs) in plasma collected from bottlenose dolphins from five different locations in the Western Atlantic and the Gulf of Mexico. Dolphins sampled off Charleston, SC exhibited significantly higher plasma PCB concentrations compared with dolphins from Indian River Lagoon, FL and Sarasota Bay. OH-PCB concentrations were also significantly higher in plasma of Charleston dolphins than animals from Florida and Bermuda, but were not different from dolphins in Delaware Bay, NJ. OH-PCBs made up about 2-68% of the total plasma PCB concentrations, suggesting that these hydroxylated metabolites are important contaminants and, similar to PCBs, remain as threats of concern to wildlife.

In an effort to evaluate large-scale spatial trends in bottlenose dolphin contaminant exposure, Kucklick *et al.* (2011) recently compiled POP data (including PCB, OCP and PBDE concentrations) measured in 480 blubber biopsies sampled from dolphins at 14 sites along the U.S. East Coast and Gulf of Mexico coast. Sites included a range of urban and rural estuaries, and a site contaminated with Aroclor 1268. Not surprisingly, POP concentrations varied significantly by site for all classes of compounds, with blubber POP concentrations being generally higher for dolphins from urban areas or from areas with known contamination. For example, a dolphin sampled near an EPA Aroclor 1268 Superfund site

near Brunswick, GA, had the highest total blubber PCB concentration. Other legacy POPs, in particular the pesticides mirex and chlordanes, varied in accordance with past usage patterns. Dolphins in Sarasota Bay had among the highest concentrations of chlordanes, for example. In contrast, current-use contaminants, such as PBDEs, did not show obvious relationships with sample site.

Fine-scale spatial trends in contamination may also serve as a source of variation for exposure in dolphins. Scientists from the Sarasota Dolphin Research Program, working in collaboration with researchers from a variety of institutions in the southeastern U.S., examined POP concentrations in 102 bottlenose dolphin blubber samples in relation to individual ranging patterns and specifically, distance of sightings from the PCB (Aroclor 1268) point source Superfund site near Brunswick, GA (Balmer *et al.*, 2011). Dolphin ranging patterns were based upon five years of photo-identification data from two field sites approximately 40 km apart: (1) Brunswick, including the Turtle/Brunswick River Estuary (TBRE), and (2) Sapelo including the Sapelo Island National Estuarine Research Reserve (SINERR). Dolphins with sighting histories exclusively within one of the two field sites were designated as having either Brunswick or Sapelo ranging patterns, whereas individuals observed at both sites were designated as having a mixed ranging pattern. Brunswick males had the highest PCB concentrations reported for any marine mammal. Sapelo male PCB concentrations were lower than Brunswick males, but similar to the highest levels at other sites in the southeastern U.S. Overall, females had higher Aroclor 1268 proportions than males, suggesting that the highly chlorinated congeners of the Aroclor 1268 mixture may not be depurated as readily as less halogenated POPs. Not surprisingly, dolphins inhabiting waters farther from the Superfund site had lower Aroclor 1268 proportions, indicating that fine scale movements may be an important determinant of exposure in areas where point-sources of pollution exist.

In comparison to other sites along the US Atlantic and Gulf of Mexico coasts, Sarasota Bay dolphins generally exhibit low concentrations of the majority of POPs measured to-date, making this population an ideal reference site for assessing relationships between contaminant exposure and health effects. By utilizing Sarasota Bay dolphins as a basis for comparison, Schwacke *et al.* (2011) demonstrated that highly exposed dolphins are vulnerable to the immune and endocrine disrupting effects of PCBs. Including scientists from the Sarasota Dolphin Research Program, Schwacke *et al.*, conducted a 2009 health assessment of bottlenose dolphins near the Aroclor 1268 Superfund site at Brunswick, GA, previously determined to exhibit elevated PCB concentrations (Kucklick *et al.*, 2011; Balmer *et al.*, 2011). A high proportion of the dolphins sampled exhibited anaemia, a finding that had been reported previously from primate laboratory studies involving high doses of a more common PCB mixture, Aroclor 1254. Furthermore, Georgia dolphins also showed reduced thyroid hormone levels and total thyroxine, free thyroxine and triiodothyronine correlated negatively with blubber PCB concentration, potentially indicating endocrine disruption. Similarly, T-lymphocyte proliferation and innate immunity indices decreased with blubber PCB concentration, suggesting immune suppression and an increased susceptibility to infectious disease. Concentrations of other POPs such as DDT, which could potentially confound results were similar to reference sites, and therefore probably did not contribute to the observed correlations. According to Schwacke *et al.* (2011), the severity of the effects

suggests that the PCB mixture to which the Georgia dolphins were exposed has substantial toxic potential.

Research on POP exposure and effects in Sarasota Bay dolphins is continuing. In particular, long time series of data from specific individuals for which contaminant, health and reproductive data are available, will permit more detailed examination of relationships, and tracking of changes in POPs in the environment, and their effects, over time.

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NEW INSIGHTS IN THE TOXICOLOGY AND HEALTH STATUS OF MARINE MAMMALS: SAMPLES OF FREE-RANGING HARBOUR SEALS FROM THE WADDEN SEA

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INTRODUCTION

The harbour seal (*Phoca vitulina*) from the North Sea has experienced various abundance variations these last decades due to habitat loss, prey fluctuation, offshore constructions, and pollution of the marine environment. Since the last epizootics in 1989 and 2002, the population has displayed a positive sign of recovery with more than 20,000 individuals counted recently (OSPAR, 2009). However, there are still some signs of instability including a high birth rate, signs of infectious diseases, and outbreaks of unknown viruses (OSPAR, 2009). Analyses of organic and inorganic contaminants in tissues of stranded and bycaught individuals displayed worrying levels of organic and inorganic contaminants such as polychlorobiphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and total mercury (T-Hg). PCBs in blubber of stranded seals exceeded adverse effects thresholds (Weijs *et al*., 2009a, b), proposed by Jepson *et al.* (2005) for marine mammals. Due to potential biases associated with stranded individuals, a complementary strategy has been developed over the last few years allowing the development of monitoring programmes and non-invasive sampling techniques to assess health and ecotoxicological status of seals in the North Sea.

STRATEGY

Seal sampling

Harbour seals in the German Wadden Sea are captured twice a year as part of a monitoring programme allowing collection of biological samples: blood, hair and saliva. At the laboratory of the Institute of Terrestrial and Aquatic Wildlife Research (ITAW), several tests are run on blood and serum while the other samples are preserved and either shipped to specific laboratories or stored for further analyses.

Blood cell populations in free-ranging seals

Blood samples allow haematology profiles (Hasselmeier *et al*., 2008).

1. High levels of white blood cells indicate current infection while low levels indicate chronic infection. They are also susceptible to changes when the animals are stressed;
2. The amount of eosinophils provides information on potential parasitic burdens;
3. Haematology profiles also underlie seasonality;
4. The MCV gives some information about diving ability.

Pollutant monitoring

Essential (Se, Zn, Cu, Fe) and non-essential elements (T-Hg, MeHg, Cd, Pb), PCBs, and PBDEs were measured in blood (for selected compounds) of stranded, bycaught and free-ranging individuals from Belgian and German coasts (Covaci *et al*., 2002; Chu *et al.,* 2003; Das *et al*., 2003, 2004; Weijs *et al*., 2008, 2009a, b, c, d). In contrast to Cd and Pb, T-Hg can reach concentrations as high as 2.1 $\mu g.g^{-1}$ dry weight but depending on several factors including position in the trophic chain (inferred from $\partial^{13}C$ and $\partial^{15}N$ values), age group and body mass (Das *et al*., 2008). Organic compound analysis (PCBs, PBDEs) revealed widespread dispersion of contaminants in the marine environment with higher concentrations in harbour seals from the German Wadden Sea compared to grey seals from the Island of May (Scotland) (Van den Berghe *et al*., 2012; Habran *et al*., submitted). T-Hg, PCBs and PBDEs were detected in pups, confirming maternal transfer to offspring (Van den Berghe *et al*., 2012; Habran *et al*., submitted).

Development of biomarkers

In recent decades, the harbour seal population in the North Sea experienced high prevalences of parasitism and two PDV outbreaks. The grey seal population in the Baltic Sea experienced reproductive problems and osteoporosis as well as intestinal parasitism and colon ulcers. Environmental contaminants were suspected as causal or contributing factors in both species. To assess the impact of anthropogenic stressors on seals, the development of non-invasive methods to detect changes in the health status of these marine top predators is vital. mRNA expression levels of relevant proteins for immuno-modulatory functions and physiological mechanisms in the seal metabolism were measured in blood samples. Our aim was to establish new potential biomarkers (like AhR, ARNT & PPARα) for effects of pollution on seals that can be used as early warning signs in coastal ecosystems. In 2010, blood from 16 free ranging harbour seals from the North Sea and eight rehabilitated grey seals from the Baltic was analysed, and the mRNA expression levels of several potential biomarkers were measured: cytokines IL10; Il2; heat shock protein HSP70 and established AhR, ARNT and PPARα. Significant changes in expression levels between harbour seals from the North Sea and grey seals from the Baltic were found. Expression levels changed significantly in harbour seals between years and with season. Analogous analysis of pollutant manipulated liver cells will allow the comparison with baseline data from the wild and show if expression levels are pollutant-induced.

***In vitro* exposure of lymphocytes and hepatocytes**

1. *In vitro* exposure of lymphocytes

   The aims of the present study were to examine the link between methylmercury *in vitro* exposure and immune functions using seal and human mitogen-stimulated peripheral blood mononuclear cells (T-lymphocytes) (Das *et al*., 2008). Mononuclear cells of peripheral blood were isolated from seals (n = 11). Stimulated lymphocytes were exposed to functional tests (proliferation, metabolic activity, radioactive precursor incorporation) under increasing doses of methylmercury (0.1 to 10 µM).

   The results showed that the number of seal lymphocytes, viability, metabolic activity, DNA and RNA synthesis were reduced *in vitro*, suggesting deleterious effects of

methylmercury concentrations naturally encountered in free-ranging seals. Functional tests showed that a 1 µM concentration was the critical concentration above which lymphocyte activity, proliferation, and survival were compromised. The expression of IL-2 and TGF-β mRNA was weaker in exposed seal lymphocytes compared to control cells (0.2 and 1 µM). Proteomics showed some variation in the protein expression profile (*e.g*. vimentin). MeHg could be an additional cofactor in the immunosuppressive pollutant cocktail generally described in the blood of seals and this therefore raises the possibility of additional additive effects on the marine mammal immune system.

2. Hepatocytes *in vitro* exposure

Currently, marine mammals are heavily polluted even years after the reduction or cessation of the emission of persistent organic pollutants (POP) (Hellwig, 2011). For understanding and predicting toxic effects of such contaminants also at a sublethal level, an experimental set up is needed, which allows one to study subtle changes in protein patterns from chemical stress. The establishment of isolation of primary hepatocytes from harbour seals enables *in vitro* exposition studies, which should reflect the *in vivo* response in the hepatic system. This should offer an alternative to extensive *in vivo* exposition studies, and provides a means to examine species-specific effects at a cellular as well as biomolecular level.

For isolation of primary seal hepatocytes, liver tissue material was obtained from wild – ranging individuals, which had died or had to be euthanised by a designated seal ranger or a veterinarian due to severe illness. Methods like *in situ* perfusion, which are used to obtain hepatocytes from anesthetised lab animals, are not suitable for marine mammals. Here we established a two-step biopsy perfusion method to isolate viable hepatocytes (Clement *et al*., 2001; Wargel *et al*., 2009). Intact parts of the liver lobes were perfused with collagenase-containing perfusion buffer for tissue dissection reaching a cell yield of approx 1-2 x 10^6 cells/g perfused liver tissue with a viability greater than 70%.

To verify the applicability of the cells for the subsequent toxicoproteomic experiments focusing on sublethal effects, cellular damage must be excluded and a functioning metabolism confirmed.

Three parameters of cell viability were assessed to evaluate potential cytotoxic effects: activity of mitochondrial dehydrogenases (XTT assay), membrane integrity (LDH release), and maintenance of hepatospecific metabolism (urea synthesis). Cell viability was not significantly reduced after pollutant exposure with 1 or 100 µM PCBs.

Behr *et al.* (2008) described the *in vitro* approach comprising the comparison of protein expression levels in cells incubated with a technical mixture of polychlorinated biophenyls (Aroclor 1254,1260,1262) in environmentally relevant concentrations.

CONCLUSIONS

These pollutants are strongly suspected to affect the immune and endocrine systems, which as a consequence, raises concern about exposure-related health effects, especially in younger individuals.

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POLLUTANTS EXPOSURE AND EFFECTS IN SOUTHERN EUROPE: THE CASE STUDY OF STRIPED DOLPHIN IN THE MEDITERRANEAN SEA

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Background on pollutants exposure and effects in Mediterranean Sea

Stress due to chemical compounds in Mediterranean cetaceans is higher than in the same species for other marine environments (Aguilar *et al.*, 2002). Since the Mediterranean Sea is a semi-enclosed basin with limited exchange of water with the Atlantic Ocean and surrounded by heavily industrialised countries, the anthropogenic pressure on long-living and top predator species, such as cetacean odontocetes, is elevated. Xenobiotic compounds, such as organochlorines (OCs), and polybrominated diphenylethers (PBDEs), are widespread in the environment and can affect animal health at different levels of biological organisation because they are resistant to environmental and biological degradation. Polycyclic aromatic hydrocarbons (PAHs) are abundant and ubiquitous in the Mediterranean basin. Because of their lipophilic and persistent nature, several of these compounds and their metabolites bioaccumulate and biomagnify and, therefore, top predators are threatened by both processes (Fossi & Marsili, 2003; Corsolini *et al.*, 2008; Leonards *et al.*, 2008). The high levels of OCs and PBDEs in cetaceans (Petterson *et al.*, 2004; Aguilar & Borrell, 2005) also suggest that top predators are at risk of endocrine disruption (Porte *et al.*, 2006).

The striped dolphin (*Stenella coeruleoalba*) is the most abundant cetacean species in Mediterranean Sea and are estimated to number about 117,880 individuals (from line-transect surveys in 1991 and 1992) measured after the massive die-off caused by morbillivirus infection throughout the basin in the early 1990s (Forcada *et al.*, 1994). Since striped dolphins have a pelagic distribution throughout the basin, feed on pelagic and bathypelagic species of teleosts and cephalopods, have abundant fatty tissue and a limited capacity to metabolise certain PCB congeners (Norstrom *et al.*, 1992), as other small odontocetes, they show the highest levels of OCs of all marine mammals sharing the same habitat (Storelli & Macrotigiano, 2003; Fossi *et al.*, 2004; Aguilar & Borrell, 2005). A geographical trend of OC contamination in the Mediterranean Sea was observed by measuring their accumulation in striped dolphin skin biopsies and CYP1A and CYP2B induction (Fossi *et al.*, 2003). Little is known about the effects of PBDE exposure on Mediterranean cetaceans. PBDEs analysed in five species of Mediterranean cetaceans showed the same congener pattern recorded in the Atlantic Ocean and other seas (Petterson *et al.,* 2004; Isobe *et al.*, 2009).

Biomonitoring of the health status of the Mediterranean striped dolphin is therefore warranted. In the assessment of ecotoxicological hazard and stress exposure of animal species, biomarkers are powerful tools in the prognostic and diagnostic phases. In this study we investigate gene expression by quantitative Real-Time PCR (qRT-PCR) in cetacean skin biopsies and skin biopsy slices exposed to increasing doses of contaminants in order to obtain an early warning of the toxicological hazard to which Mediterranean striped dolphins

are exposed. These diagnostic signals were used to identify hot spots of contamination stress across the basin.

The case study of striped dolphin: field study

Three sampling areas were selected to have an overview of the status of striped dolphin populations in the western part of the Mediterranean basin, including the contiguous area of the Strait of Gibraltar. These areas are geographically distinct with different geographical characteristics, levels and classes of contaminants, and types of anthropogenic pressure. The Pelagos Sanctuary has been a Marine Protected Area (MPA) since 2002, and extends from SE France to northwestern Italy (Notarbartolo di Sciara *et al.*, 2008). It is the largest European pelagic protected area and contains an abundance of cetaceans (Panigada *et al.*, 2011). However, they are exposed to high anthropogenic pressure due to maritime traffic, high levels of POPs and trace elements, and heavy exploitation in the coastal zone. The Ionian Sea sampling area lies between eastern Sicily and southwestern Calabria. The Strait of Gibraltar sampling area includes Spanish and Moroccan waters where the Mediterranean meets the Atlantic. Human activities in the area are mainly due to its strategic position and include maritime traffic, which also produce noise pollution and collisions.

In this study, we tested five putative “gene-expression biomarkers” in cetacean skin biopsies (Panti *et al.*, 2011). Each biomarker is involved in responses to different environmental stresses, providing a broad spectrum of toxicological health status assessment of the species. Biomarkers at the molecular level (by quantitative Real-Time PCR) indicate any variation linked to chemical, ecotoxicological or other environmental stresses at an early stage, increasing and integrating the specificity and sensitivity of conventional biomarker responses. Detection of an early warning signal using a small amount of tissue sampled in a non-destructive way (as the skin biopsy from free-ranging animals) was perfectly coherent with the choice of validating biomarkers at the molecular level. Furthermore, detection of variations in mRNA levels can be integrated with protein expression responses to obtain insights into the mechanisms of the action of mixtures of known and unknown contaminants in organisms, and enables a wide range of simultaneous analyses, integrating the responses of several genes involved in different physiological and metabolic pathways, from specific to generic stress. The five genes represent different exposure signals. In this regard, two of the genes, heat shock protein 70 (HSP70) and E2F-1 transcription factor (E2F-1), are involved in responses to “generic stress”; cytochrome P450 1A (CYP1A) and aryl hydrocarbon receptor (AHR), are involved in more specific pathways such as activating metabolism of planar fat-soluble compounds (e.g. PAHs and PHAHs); the fifth gene, estrogen receptor 1 (ESR1), is involved in some ligand-inducible regulation processes of the reproductive system.

Experimental design

- Field study: sampling area and biopsy procedure

Skin biopsies (epidermis, dermis, and blubber) from free-ranging striped dolphin were obtained in the three areas: Pelagos Sanctuary (F=8, M=6), Ionian Sea (F=5; M=8), and Strait of Gibraltar (F=8; M=7) on several sampling expeditions (summer 2006-2007). Striped dolphins were sampled using an aluminium pole, as previously described (Fossi *et al.*, 2000; CITES permit: Int. IT007, Nat. IT025IS). The gender of the dolphins was determined according to Bérubé and Palsbøll (1996).

- In vitro study: slices exposure

Immediately after collection, biopsy slices (about 2 mm thick spanning the epidermis, dermis, and blubber) were subjected to three different classes of contaminants: OCs, PBDEs, PAHs and mixture of the three classes. Slices were incubated 24 hours in cell culture media with different mixtures of contaminants as reported below. OCs mixture of Arochlor 1260 (Chem Service), pp'DDT (Chem Service) and pp'DDE (Chem Service) in Dimethyl sulfoxide (0.05%, DMSO) at three doses: 0.01 µg/ml (+), 0.1 µg/ml (++) and 1 µg/ml (+++), plus a DMSO (0.05%) control (-). The PBDEs mixture containing 27 congeners (BDE-MXE, Wellington Laboratories), from mono- to deca-brominated, in nonane (0.01 µg/ml; Merck) at three doses: 0.01 µg/ml (+), 0.05 µg/ml (++) and 0.1 µg/ml (+++), plus a nonane (0.01 µg/ml) control (-). The PAHs mixture contains benzo(a)pyrene (Sigma; 1 mM) and beta-naphthoflavone (ChemTrec; 20 mM) in acetone (BDH, 0.1%) at three doses: 0.5 µM BaP + 10 µM BnF (+), 2.5 µM BaP + 50 µM BnF (++), and 12.5 µM BaP + 250 µM (+++) BnF plus an acetone (0.1%) control (-). The fourth was a mixture combining the other mixtures, in a mix of carriers (DMSO 0.005%, nonane 0.01% and acetone 0.1%) at two doses: Low Dose (+, OCs 0.01 µg/ml + PBDEs 0.01 µg/ml + PAHs 0.5 µM) and High Dose (++, OCs 1 µg/ml + PBDEs 0.1 µg/ml + PAHs 12.5 µM) plus a control (-, mix of carriers only). Incubation was carried out at room temperatures ranging from 24°C to 28°C. After 24 hours incubation in media, untreated and treated slices were placed in liquid nitrogen.

- Methods: total RNA, cDNA synthesis, and qRT-PCR assays

Sub-samples of the biopsies (about 30 mg) were homogenised using a tissue lyser (Qiagen). Total RNA was extracted from homogenised material using the Aurum™ Total Fatty and Fibrous Tissue kit (Bio-Rad) following the manufacturer's instructions. Genomic DNA was eliminated by DNase-on-column treatment of each sample. DNA and RNA were quantified by Nano-Drop® ND-100 UV-vis spectrophotometer (NanoDrop Technologies). Reverse transcription reactions were performed using the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The amount of initial total retrotranscribed RNA was 500 ng.

The qRT-PCR assays were carried out in 96-well reaction plates with an iCycler iQ5 (Bio-Rad) using SYBR® Green detection chemistry as described in Spinsanti *et al.* (2006). Amplicon lengths ranged from 111 bp to 234 bp to guarantee high efficiency during the reaction. Amplification efficiency (E), slope (s) and correlation coefficient (R^2) of each primer pair in the qRT-PCR were calculated using 1:5 serial dilutions of cDNA as template on a iQ5 machine (Bio-Rad) (Panti *et al.*, 2011). The five genes of interest (GOIs) and two housekeeping genes (HKGs) for the normalisation procedure were amplified for each of the 42 skin biopsies. The housekeeping genes were selected in a previous study of *S. coeruleoalba* skin biopsies and

cell cultures (Spinsanti *et al.*, 2006, 2008). Each reaction was run in triplicate, as well as the no-template control. Raw threshold cycles (Ct) were converted to quantities by the comparative ΔΔCt method (Livak & Schimttgen, 2001).

- Statistical data analysis

Gene expression levels in the skin biopsies were calculated using *GenEx* v. 4.3.8 Software (MultiD Analyses AB). Input Ct values were pre-processed by efficiency correction to indicate technical repeats. Normalisation to reference genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide), SDHA (succinate dehydrogenasecomplex subunit A) and to sample amount was applied. Normal distribution of the data was checked by the one-sample Kolmogorov-Smirnov test. For variables not normally distributed, the data were expressed as natural logarithms. Two-way analysis of variance was then performed to verify whether sampling area and sex significantly affected expression of the selected genes, and whether any significant effect was due to interaction of experimental factors. Multiple *post-hoc* analysis of variance was also used to consider the comparisons between areas. Specifically, Dunnett's T3 test was applied (variances not homogeneous) as well as the Student-Newman-Keuls test (variances homogeneous). Hierarchical cluster analysis by the minimum energy (E) distance method was used to define clusters on the basis of areas, and canonical discriminant analysis on PCA factors was performed to reveal clustering variables. All statistical analyses were performed using SPSS 12.0 Software (IBM® SPSS® Statistics).

RESULTS

Gene expression as a diagnostic signal

Five genes of interest were selected tested as biomarker responses in the 42 biopsies quantifying the mRNA expression levels of the target genes (AHR, CYP1A, E2F-1, HSP70, ESR1). The expression levels of the five genes were compared among areas and between males and females (F= 22, M=20). The mRNA expression levels were normalised to GAPDH and YWHAZ reference genes.

Levels of mRNA expression for the genes AHR, CYP1A and E2F-1 reflected a similar trend in the three areas, suggesting exposure to different toxicological stressors. Gene expression levels were highest in specimens from the Pelagos Sanctuary and lowest in those from Gibraltar Strait (Figure 1). The responses of the other two genes, ESR1 and HSP70, did not reflect the same trend but the Ionian samples showed the highest levels of mRNA expression, followed by the Pelagos Sanctuary and finally the Gibraltar Strait specimens (Figure 1). Statistical analysis showed normal distributions for the genes AHR (K-S Z=0.060; two tailed t-test p=0.857), CYP1A (K-S Z=1.260; two tailed t-test p=0.084), E2F1 (K-S Z=1.167; two tailed t-test p=0.131) and HSP70 (K-S Z=0.997; two tailed t-test p=0.273), whereas ESR1 showed a normal distribution (K-S Z=0.815; two tailed t-test p=0.520) after log-transformation.

CYP1A response allowed the discrimination of areas/sub-populations exposed to different levels of lipophilic contaminants (Figure 2a). Expression of the CYP1A gene in skin biopsies collected in the three areas showed differentials, indicating that the striped dolphins were exposed to potential toxicological risk. Comparison of the presumably most polluted (Pelagos Sanctuary) and least polluted areas (Strait of Gibraltar) showed 3.24-fold induction

of mRNA levels of CYP1A (Fig 1). It is well known that the Pelagos Sanctuary is broadly contaminated by lipophilic compounds, such as PAHs and OCs (Fossi *et al.*, 2004). Interaction of AHR with PHAHs and dioxins is widely documented, as is its role in the activation of CYP1A transcription. Gene expression values of AHR in our data set again reflected a regional response trend. Since males and females were homogeneously distributed, a *post-hoc* analysis of variance was applied, independent of sex (Levene test $F(2, 39)=8.31$, $p=0.001$) and Dunnett's T3 test underlined a significant difference between specimens from the Strait of Gibraltar and the Ionian Sea ($p=0.016$) and the Strait of Gibraltar and Pelagos Sanctuary ($p<0.001$; Figure 1). On the contrary, expression of ESR1 (applying *post-hoc* comparison of variance, independent of sex: Levene test $F(2, 39)=0.81$, not significant) did not follow the same geographical trend, but individuals from the Ionian Sea showed higher levels of mRNA than those from Pelagos Sanctuary (Test S-N-K $p<0.05$,1.65-fold) and the Strait of Gibraltar (Test S-N-K $p<0.05$; 4.75-fold; Figure 1). This probably indicates higher exposure of the Pelagos and especially Ionian populations to xeno-estrogens than dolphins from Gibraltar, and suggests the hypothetical presence of different EDCs in different areas. However, since the estrogen receptor signaling pathway is complex, a more detailed functional assessment is warranted. The ligand (for instance dioxin-like compounds) that activates ESR1 seems to activate AHR as well, suggesting competitive binding (Othake *et al.*, 2003) and inhibition of AHR induction.

These findings may explain the low levels of induction of AHR compared to ESR1, but further investigation of this mechanism in the species investigated, is necessary. With regard to the E2F-1 gene, little is known about the effects of contaminants on its expression. Its role in regulation of the cell cycle and apoptosis and its response to stress led us to propose it as a possible biomarker of exposure to general stress. The formation of complexes composed of AHR/ARNT and E2F-1 have been demonstrated, indicating that AHR ligands, such as dioxins, are involved in activation of E2F-1 and, therefore, in induction of apoptosis (Watabe *et al.*, 2010). The response in skin biopsies showed higher induction of mRNA levels in specimens from Pelagos Sanctuary than in striped dolphins from the Strait of Gibraltar. Comparing the Pelagos Sanctuary with Ionian Sea and Strait of Gibraltar specimens, the gene proved upregulated (1.85-fold and 2.00-fold, respectively), but the differences did not appear to be statistically significant (Figure 1). Finally, the stress-related HSP70 gene showed greater up-regulation of expression in Ionian Sea specimens than in those from the other two sites (1.55-fold and 1.68-fold versus Pelagos Sanctuary and Strait of Gibraltar, respectively (Dunnett's T3 test, $p=0.027$). The ability of HSP70 to respond to multiple stressors, does not give a clear and specific cause-effect response, but underlines the exposure of dolphins to general stress that may be chemical or otherwise (Figure 1).

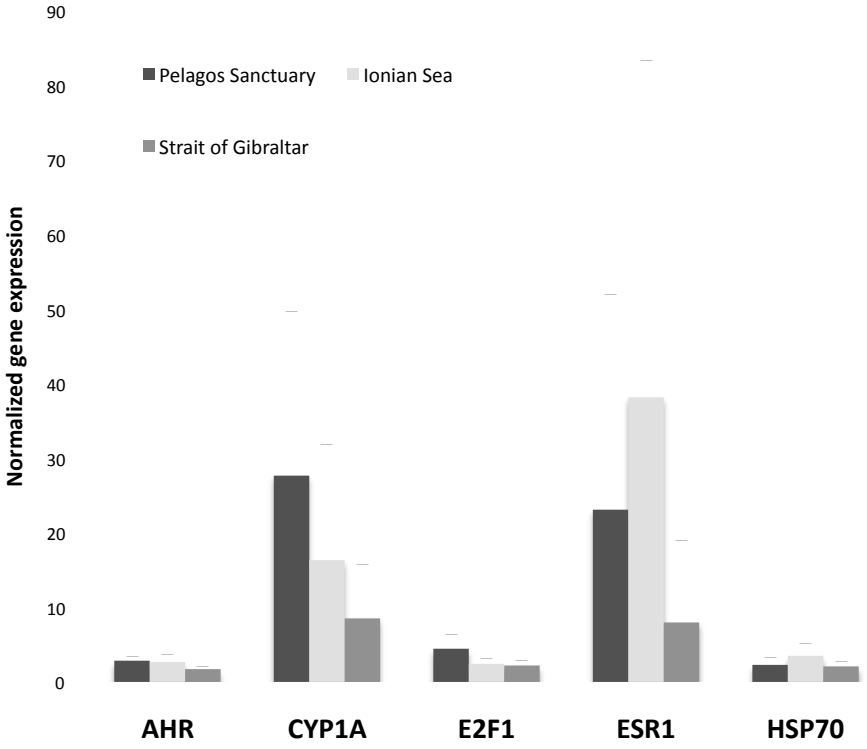

Figure 1. Gene expression profile in skin biopsies of the five GOIs in the three areas. Bars show the mean of the gene expression for each gene ±SD. The expression was normalised to YWHAZ and GAPDH and calculated using *GenEx* v. 4.3.8 Software (MultiD Analyses AB)

Effects of the area on gene expression responses

The geographically different response exhibited by at least three genes (AHR, CYP1A, E2F-1) is a clue that dolphins from Pelagos Sanctuary and the Ionian Sea are more exposed to toxicological hazard than those inhabiting the Strait of Gibraltar. Since no clear genetic distinction exists between these three populations (also demonstrated in Gaspari *et al.* 2007), the responses to exposure to a wide range of other toxic compounds did not depend on intra-species variability but on the different levels of contamination of geographical area where the animals live, breed and feed, even if the striped dolphin is known to range widely. On this point, further analysis was performed to verify whether the differences among the proposed suite of gene-expression biomarkers could help distinguish responses on the basis of the geographical distribution of populations and which parameter (gene) contributed most to separation by areas.

Cluster and discriminant analysis of the PCA factors was performed (Figure 2). Cluster analysis indicated that specimens sampled in the Strait of Gibraltar area were significantly distinct from those from Pelagos Sanctuary and the Ionian Sea (Figure 2), allowing the populations to be clearly distinguished by our variables. This revealed the greater potential ecotoxicological risk of the two Mediterranean sub-populations compared to dolphins living in the contiguous Mediterranean area (Gibraltar). Comparison of the correlation between the discriminant variable plot, the discriminant function, and the ellipsoid plot showed that specimens from the Ionian Sea had high values in terms of canonical weights of HSP70 and ESR1, while those from Pelagos Sanctuary had high levels of CYP1A, E2F1 and AHR (Figs.

2a,b). This evidence suggests exposure to planar lipophilic compounds and compound with dioxin-like activity. Specimens sampled in the Strait of Gibraltar had low canonical weights of the genes E2F1, CYP1A and AHR (Figure 2a,b). Discriminant analysis (Monte Carlo Test based on 999 permutations RV=0.201, p=0.001) confirmed that the three group-areas were significantly distinct (Figure 2c).

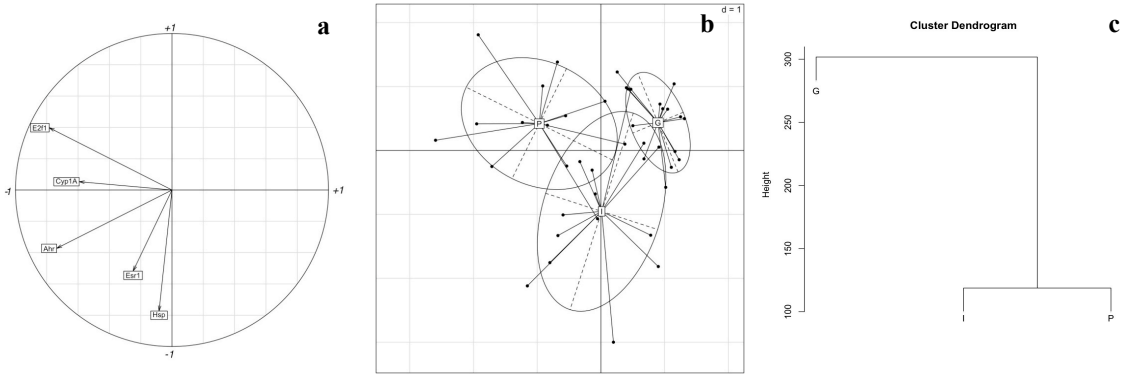

Figure 2. Discriminant analysis applied on the Principal Components Analyisis (PCA): a) plot of the canonical weights for the five variables (genes), b) correlations between the discriminant variables and the discriminant functions plotted on the correlation circle, c) dendrogram of the cluster analysis for the three areas (PelagosSanctuary = P, Ionian Sea = I, Strait of Gibraltar = G) clearly distinguished by the variables

2.4 Results: Slices exposure as an alternative in vitro *approach*

In this *in vitro* experiment the quantitative qRT-PCR was applied to measure expression of CYP1A1, E2F-1, HSP70 and ERα (Figure 3) mRNA levels in biopsy slice samples. Regarding the OCs treatment, the upregulation of CYP1A and ERα was detected at the lowest dose, which increased 17- and 15-fold as compared to the control vehicle, respectively. A “bell-shaped” induction phenomenon was shown for both genes at medium and high doses of OC mixtures. A moderate upregulation was reported for HSP70 mRNA, with a 4-fold increase at the medium dose with respect to the control vehicle. No variations were reported for E2F-1 (Fig 3a).

The slices exposed to PAHs show an upregulation of the CYP1A gene levels increasing doses of PAH mixtures (Figure 3b), increasing respectively 1.5-, 6- and 9.5-fold at the different doses with respect to the control vehicle. No upregulation was observed for HSP70 and no variation was reported for E2F-1. Moderate upregulation phenomena were observed for HSP70 and E2F-1 with respect to the control vehicle (Figure 3c) in the slices exposed to PDBEs mixture, as well as a slight inhibition of CYP1A at the lowest dose mixture was measured for ERα, for all the three doses, suggesting a toxic effect or an inhibiting action due to the mixture, with respect to the control vehicle, suggesting a potential influence of the nonane on gene expression responses. Finally, the exposure to the mixture of OCs, PBDEs and PAHs cause an upregulation of CYP1A mRNA levels at the medium dose of contaminant mixture (Figure 3d), showing a “bell-shaped” dose-response curve. Slight upregulation of HSP70 was detected in skin slice samples with increasing doses of contaminants. No induction phenomena were observed for E2F-1. The high potential of a

mixture of multiple contaminants (ecotoxicologically relevant to Mediterranean areas) to induce CYP1A mRNA was documented by these *in vitro* data, confirming the potential use of CYP1A induction in cetacean skin samples as a "diagnostic" biomarker of exposure to lipophilic contaminants.

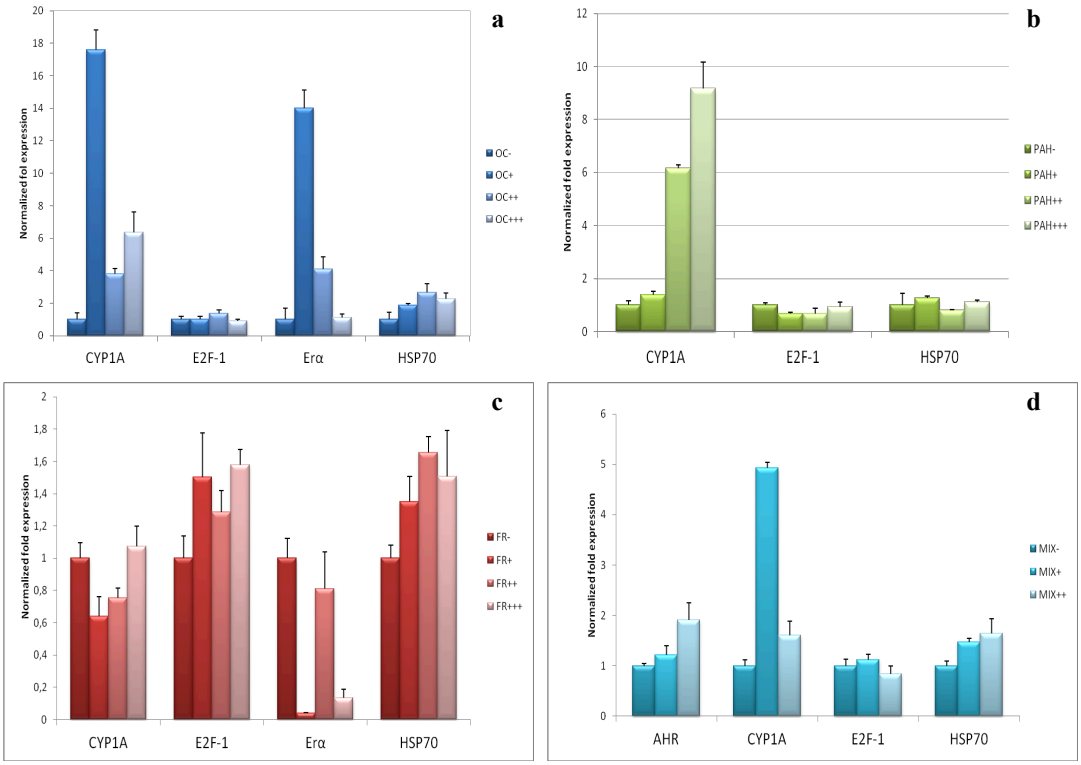

Figure 3. Gene expression profile of slices of skin biopsies exposed to increasing doses of contaminants: a) organochlorines (OCs), b) polycyclic aromatic hydrocarbons (PAHs), c) brominated flame retardants (FR), d) mixture of OCs, PAHs, and PBDEs (MIX). The symbols (+, ++, +++, -) indicate the increasing doses of chemicals plus control (see text for detailed doses and treatment composition). The bars show the mean values of normalised gene expression to YWHAZ, GAPDH, and SDHA gene. The values were calculated using GenEx v. 4.3.8 Software (MultiD Analyses AB)

CONCLUSIONS

In conclusion, clear evidence of geographical variability in the responses of the diagnostic biomarkers in striped dolphins suggests different exposure to mixture of various classes of contaminants and varying levels of hazard in different areas of the Mediterranean basin. All five genes proved to be modulated in the skin biopsies and they can therefore be proposed as biomarkers for assessing the toxicological status of Mediterranean striped dolphins and other cetacean species and areas. The simultaneous analysis of genes involved in different signaling pathways, including the *in vitro* predictive experiments, combined with proper multivariate statistical analysis, makes it possible to assess whether animals are exposed to stress, and is a more powerful tool than analysis of single biomarkers and/or contamination levels. Moreover, the *in vitro* experiment allowed us to identify, among the various selected gene expression biomarkers, the biomarkers and/or a series of biomarkers that best allow us to diagnose the presence of a specific class of pollutants, or a mixture of them, in future investigations in field studies.

Finally, striped dolphins from the northwestern Tyrrhenian Sea (Pelagos Sanctuary) are evidently more exposed to ecotoxicological hazard than those inhabiting the Ionian Sea and the Strait of Gibraltar. This evidence focuses attention on the potential risk to cetaceans inhabiting the largest pelagic MPA in Europe, and underlines the importance of far-sighted management of protected areas in order to preserve species in their habitats.

ACKNOWLEDGEMENTS

We thank the Italian Ministry for the Environment for financing this research within the ACCOBAMS Agreement. We also thank Dr. Maria Grazia Finoia (ISPRA) for her fundamental support in statistical analysis.

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NEW EVIDENCE OF RELATIONSHIP BETWEEN PCBS AND THE CAUSE OF DEATH OF NORTH SEA HARBOUR PORPOISES

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ABSTRACT

Since 1990, the Marine Animals Research & Intervention Network (MARIN) investigates marine mammal strandings along the coastlines of Belgium and northern France. In harbour porpoises (*Phocoena phocoena*), death appeared to be caused by infectious diseases (37%), characterised by severe parasitism and pneumonia, and trauma (23%), mostly due to by-catch in fishing gear. For about 40% of the stranded individuals, no cause of death could be determined. Most porpoise strandings occurred in winter, with a peak of bycaught animals in March and April. More males stranded than females. Most bycaught animals were juveniles. Compared to the bycaught porpoises, the animals that had died of an infectious process had a thinner blubber layer (emaciation) and the histological investigation showed a marked lymphoid depletion (spleen, thymus and lymph nodes). The total PCB concentration was assessed in the blubber of selected individuals. The concentration was higher in adult males. Additionally, the animals that had died of infectious disease were more contaminated than bycaught porpoises.

INTRODUCTION

The multidisciplinary research group MARIN (Marine Animals Research and Intervention Network) investigates marine mammal strandings along the coasts of Belgium and northern France (in collaboration with the Marine Mammal Research Centre, La Rochelle, France). Its aims are to identify the causes of death and monitor the health status of marine mammals. This work includes determining concentrations of selected chemical contaminants in tissues, and relating lesions to toxicological data and human activities. Harbour porpoise (*Phocoena phocoena*) is the most abundant cetacean in the North Sea (Hammond *et al.*, 2002). In the southern North Sea, from 1990 to 2000, the most frequent causes of death of harbour porpoises were trauma, mostly accidental capture in fishing gear, and infectious diseases, mostly pneumonia (Jauniaux *et al.*, 2002).

Recently, evidence has been demonstrated between infectious diseases and high levels of pollutants, mostly polychlorinated biphenyls (PCBs) (Jepson *et al.*, 1999; Jepson *et al.*, 2005). It has been shown that PCB levels in the blubber are significantly higher in animals dying of an infectious disease compared to porpoises dying of a trauma. Similar associations were suspected for morbillivirus and *Brucella* sp. infections (Kennedy *et al.,* 1991, 1992a; Davison *et al.*, 2009; Jauniaux *et al.*, 2010).

The present study reports the most frequent lesions, and compares biological, pathological and toxicological parameters involved in the main causes of death (traumatic or infectious) of harbour porpoises stranded along the Belgian and northern France coastlines between 1990 and 2010.

MATERIALS AND METHODS

Between 1990 and 2010, porpoises stranded on the Belgian and northern French coastlines have been collected. A total of 654 individuals were necropsied by a standard procedure (Jauniaux and Coignoul, 2002). During necropsies, samples were collected for histology, bacteriology, virology, parasitology and life history investigations (Jauniaux *et al.*, 2002). In addition, on selected animals, histological investigations were performed and lymphoid tissue depletion index –LTDI- was determined on the spleen, thymus and lymph node (Wünschmann *et al.*, 2001; Beineke *et al.*, 2007), from 0 (no depletion) up to 3 (severe depletion). Finally, PCB concentrations (21 polychlorinated biphenyl congeners) were measured in the blubber of 18 individuals (Weijs *et al.*, 2009; Weijs *et al.*, 2010).

RESULTS AND DISCUSSION

The temporal distribution of strandings is very heterogeneous with a continuous increase, particularly since 2000. Indeed, between 1990 and 2000, the average of porpoise strandings was less than 5.5/yr. Since 2000, there is a significant increase of strandings with an average of 54,4/yr. and a peak of 96 porpoises in 2006 (Figure 1). A similar increase has been reported for the coast of the Netherlands (Camphuysen *et al.*, 2008; Haelters & Camphuysen, 2009) and the United Kingdom (Leeney *et al.*, 2008). The increase of strandings could potentially be linked with the southward shift of the distribution of harbour porpoises in the North Sea (Hammond *et al.*, 2002; Hammond, 2008).

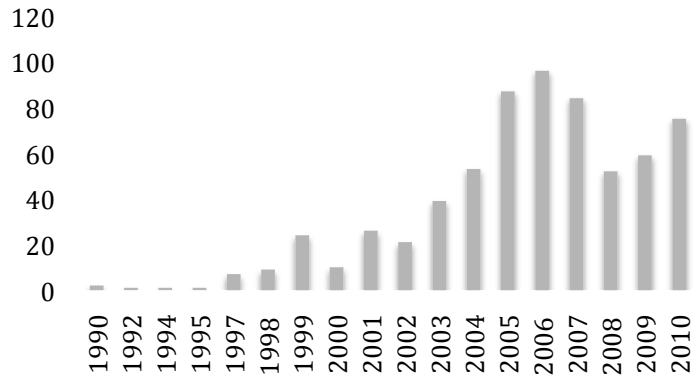

Figure 1. Annual number of harbour porpoise strandings along the coasts of Belgium and northern France between 1990 and 2010

Among all strandings, there were more males than females and more juveniles than adults. Furthermore, most strandings occurred at the end of the winter with a second peak in the mid of the summer (Figure 2). The end-winter peak was associated with a higher abundance of harbour porpoises in the region in combination with a higher fishing effort (Haelters & Camphuysen, 2009).

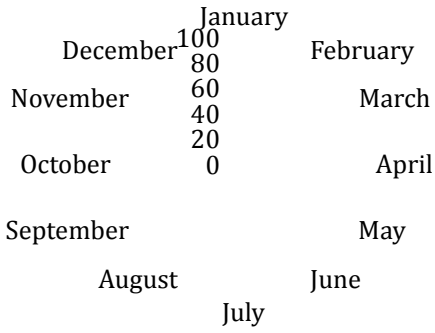

Figure 2. Monthly distribution of porpoise strandings along the coasts of Belgium and Northern France between 1990 and 2010

Post-mortem examinations of porpoises revealed two main causes of death: infectious diseases and emaciation for 191 porpoises, and net capture (by-catch) for 120 porpoises. For the remaining 209 porpoises examined, the cause of death was ascribed to another cause such as a tumour, starvation or stillborn (not associated with an infectious process or a capture), or could not be determined. For 191 porpoises, observations specific to infection were multisystemic severe parasitism, acute pneumonia and emaciation. The blubber thickness was usually less than 13 mm and the remains of recent feeding were present in the gastro-intestinal tract in less than 38% of individuals. For 120 porpoises, observations specific for capture were skin laceration, net marks and flipper or fluke amputation. Other observations were bruises, lung edema and congestion. The blubber thickness was 18 mm and the remains of recent feeding were present in the gastro-intestinal tract in more than 76% of individuals. By-catches were more pronounced in winter and for juveniles. Similar causes of death were previously reported for the coast of Belgium from 1990 to 2000 (Jauniaux *et al.*, 2002), the United Kingdom (Kirkwood *et al.*, 1997) and Germany (Siebert *et al.*, 2001). The only difference concerns the distribution of the two categories, with by-catch being more frequently reported for other countries, even if for the Belgium coastline, the average of capture was 20% in the period 1990-2000 and it was 42% in the period 2001-2010.

The LTDI (all lymphoid tissues) revealed that animals that had died of an infectious disease were more immunodepleted (LTDI: 2) than bycaught porpoises (LTDI: 1.5). A similar observation has been reported previously by Beineke *et al.* (2007).

Total PCB concentration in the blubber (Figure 3) were higher in males (61.7 ±44.1 µg/g lw) than in females (15.2 ±12.6 µg/g lw), p= 0.013, and higher in adults (66.2 ±44.0 µg/g lw) than in juveniles (15.5 ±10.4 µg/g lw), p= 0.034. Similar ranges of concentration were reported for the Belgian coastline by Weijs *et al.* (2009, 2010).

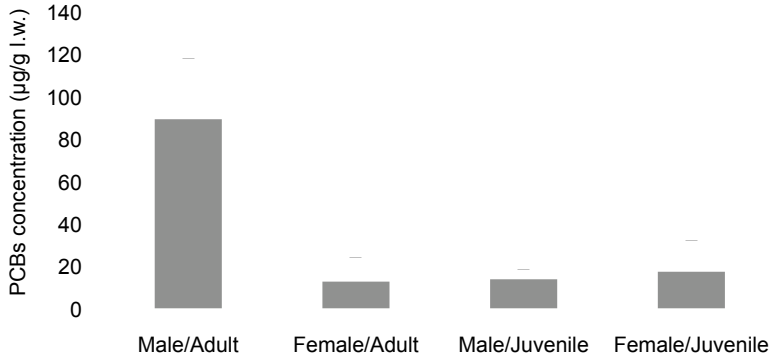

Figure 3. Mean (and SD) total blubber PCB concentration by gender and age

The process of biomagnification can explain the age variation, while the sex variation can be caused by mother-calf PCB transfer during gestation and lactation. Finally, the animals that had died of infectious disease (49.9 ± 43.2 µg/g lw) were more contaminated than bycaught porpoises (12.4 ± 4.1 µg/g lw) although this difference could not be evaluated statistically due to the small sample size. A similar association between PCB concentration and death was reported in porpoises as well as other marine mammal species of the North Sea (Jepson *et al.*, 1999; Jepson *et al.*, 2005; Hall *et al.*, 2006). Jepson *et al.* (2005) reported PCB concentrations of 13.6 mg/kg in porpoises dying of physical trauma and 27.6 mg/kg in case of infectious disease. It is supposed that PCBs predisposed to infectious diseases, and a blubber total PCB threshold of 17 mg/kg lipid has been proposed for adverse health effects in marine mammals such as immunosuppression. Therefore, in the present study, porpoises dying of an infectious process had a more severe immunosuppression than the others and showed the highest PCB concentration, suggesting that there is indeed a relationship between the health status of harbour porpoises and pollution.

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ASSESSING THE RELATIONSHIP BETWEEN MORBILLIVIRUS EPIZOOTIC VIRULENCE AND ORGANOCHLORINE LEVELS IN MEDITERRANEAN STRIPED DOLPHINS

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INTRODUCTION

Organochlorine compounds (OCs), especially polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethanes (DDTs), have been of great concern due to their occurrence in high concentrations, even in remote ecosystems, despite bans on their production and usage (Iwata *et al.*, 1994). Because of their chemical stability and the slow biodegradation of many of their forms, these compounds have become ubiquitous, particularly in marine environments. Top predators, such as dolphins, have been proposed as potential bioindicators to monitor OC contamination in marine ecosystems (Aguilar & Borrell, 2005). Dolphins are particularly susceptible to the impact of OCs because they have a high metabolic rate, feed at high trophic levels, and their bodies contain a large proportion of fat (up to 40%), and thus, they accrue large loads of OC compounds (Borrell *et al.*, 1996). These characteristics make dolphins suitable indicators of ecosystem contamination.

Starting in the summer of 1990 and continuing through 1991 and 1992, an epizootic that was identified as an infection produced by a morbillivirus, similar to that which produces distemper in carnivores (Domingo *et al.*, 1990), caused a massive die-off of striped dolphins in the Mediterranean Sea. Several hundred dolphin carcasses reached the coast (Aguilar & Raga, 1993) It is difficult to know how many total individuals were affected because this species inhabits offshore waters, and it is rarely found closer than 10 miles from the coast. Because of this, only a small proportion of the affected dolphins were stranded, but it is speculated that the total population declined to just one third of its initial level (Aguilar & Raga, 1993).

Considering that the number of dead dolphins found was around one thousand, the magnitude of the epizootic was huge. It has been known for a long time that the striped dolphin population in the Mediterranean waters has been exposed to high levels of OCs pollutants and that PCB levels in their tissues are extremely high. However, it was not expected for the levels of OCs pollutants found in the dolphins examined during the epizootic to be between two and three fold the levels commonly found in healthy populations (Aguilar and Borrell, 1994). Based on these high levels and on the knowledge that organochlorine compounds are depressors of the immune system of mammals (Loose *et al.*, 1977; Brouwer *et al.*, 1989; De Swart *et al.*, 1996; Busbee *et al.*, 1999), Aguilar and Borrell (1994) suggested that PCBs caused higher susceptibility to the viral disease and played an important role in the development of the epizootic.

In 2007 and 2008, 17 years after the 1990 epizootic, dozens of striped dolphins appeared dead in the Mediterranean beaches with the same symptoms of the epizootic in 1990, taking place what seemed to be a new bud of the virus.

OBJECTIVES

The objective of this study was to establish whether the dolphins that died in 2007–2008 had OCs concentrations that were high enough to depress the immune systems of Mediterranean striped dolphins and would enhance their mortality in response to infection by morbillivirus.

OC levels were compared with those measured in the dolphins from the 1990 epizootic, and with those of presumably healthy members of the contemporary population. In addition, the trend in OC levels during the period 1988–2009 was explored.

MATERIALS AND METHODS

Blubber samples from the period 1988-2009 were obtained from 1) striped dolphins that stranded in Catalunya, Valencia and the Balearic Islands; and 2) biopsies collected from free ranging dolphins. OCS were analysed with a GC/MS system, as described in Castrillon *et al.*, (2010).

RESULTS & DISCUSSION

Comparison between outbreaks

The values of the PCB and DDT levels that we measured in 2007–2008 were much lower than the values that were found in the 1990 outbreak by Aguilar and Borrell (1994). In the 2007–2008 outbreak, the tPCB mean was 57 ppm, with a range of 7–153 ppm, and the tDDT mean was 73 ppm with a range of 8–258 ppm. In contrast, individuals from the 1990 outbreak had mean levels of 788 ppm for tPCB and of 392 ppm for tDDT. Data were highly variable, with some of the diseased dolphins exhibiting levels of tPCB and tDDT in excess of 1,000 ppm.

It is well known that high levels of PCBs and other organochlorine compounds depress the immunological system, increasing susceptibility to diseases (Koller & Thigpen, 1973; Imanishi *et al.*, 1980; Wu *et al.*, 1984; Dean *et al.*, 1989; Busbee *et al.*, 1999; Beckmen *et al.*, 2003). The OC levels that were detected in many of the dolphins in the 1990 epizootic in the Mediterranean were extremely high (Kannan *et al.*, 1993; Aguilar & Borrell, 1994; Borrell *et al.*, 1996). Both tDDT and tPCB blubber concentrations of these dolphins were much higher than those in the presumably healthy population sampled before or after the event. Aguilar and Borrell (1994) suggested that when the virus reached the population, those individuals with the highest PCB levels and with depressed immune function were more susceptible to the disease, and suffered a higher mortality rate than the other dolphins.

Between both outbreaks, significant differences were found in the concentrations of tPCB, for each PCB congener individually (Student's t-test $p < 0.001$) (Figure 1), and tDDT and individual metabolites (Student's t-test $p < 0.001$) (Figure 2). Furthermore, the OC levels in the 1990 epizootic dolphins were more than 10 times higher for tPCB and six times higher for tDDT than the levels in 2007– 2008 outbreak.

Additionally, we found that the mortality in the 1990 epizootic was much higher in numbers than in 2007–2008. In 1990, approximately 150 carcasses reached Catalonian coasts, while in 2007–2008 only approximately 30 carcasses were found in the same area. Part of this decrease in the number of deaths could be due to the fact that some of the oldest

individuals might have generated antibodies against the morbillivirus during the first outbreak. However, only a very small portion of the population is likely to have survived 17 years later.

Alternatively, this could be caused by the much lower severity of the 2007–2008 infection and the viral morbidity. Another, more likely, explanation would be that the population in the years preceding the 2007–2008 epizootic had not been immunodepressed by OCs because they were at much lower concentrations, and as a result, the dolphins were better prepared to recover from morbillivirus infection than their 1990 counterparts. To explore this last hypothesis, we decided to analyse the trend in OC levels in the period 1988–2009, and to compare the levels of OCs in the presumably healthy population with the levels in individuals that died in the 2007–2008 epizootic.

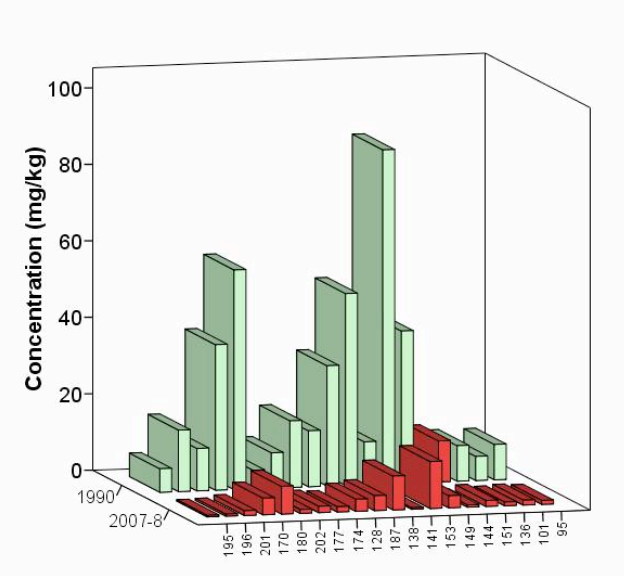

Figure 1. Comparison of PCB-congener levels between the 1990 (n = 33) and 2007– 2008 (n = 20) Mediterranean epizootics

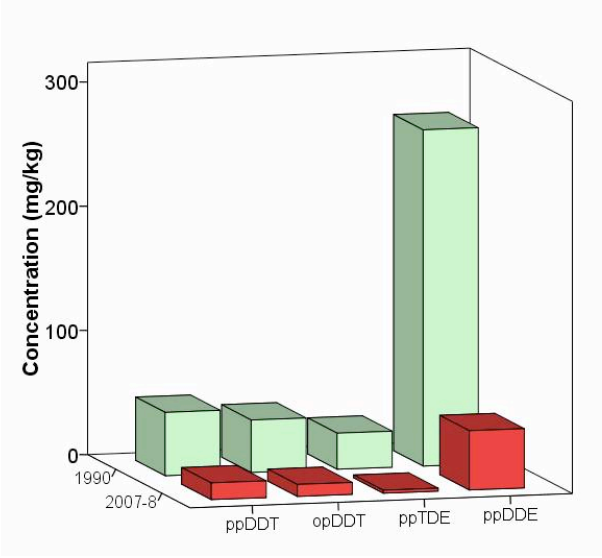

Figure 2. Comparison of DDT-compound levels between the 1990 (n = 33) and 2007–2008 (n = 20) Mediterranean epizootics

Organochlorine trends in the population

The trend in OC levels in the population between 1988 and 2009 (excluding individuals from the outbursts) showed an exponential decline for both tPCB and tDDT, as shown in Figure 3 ($r = 0.71$, and $p < 0.0001$; and $r = 0.6$, and $p < 0.0001$, respectively). These pollutants have levelled off over the last 10 years.

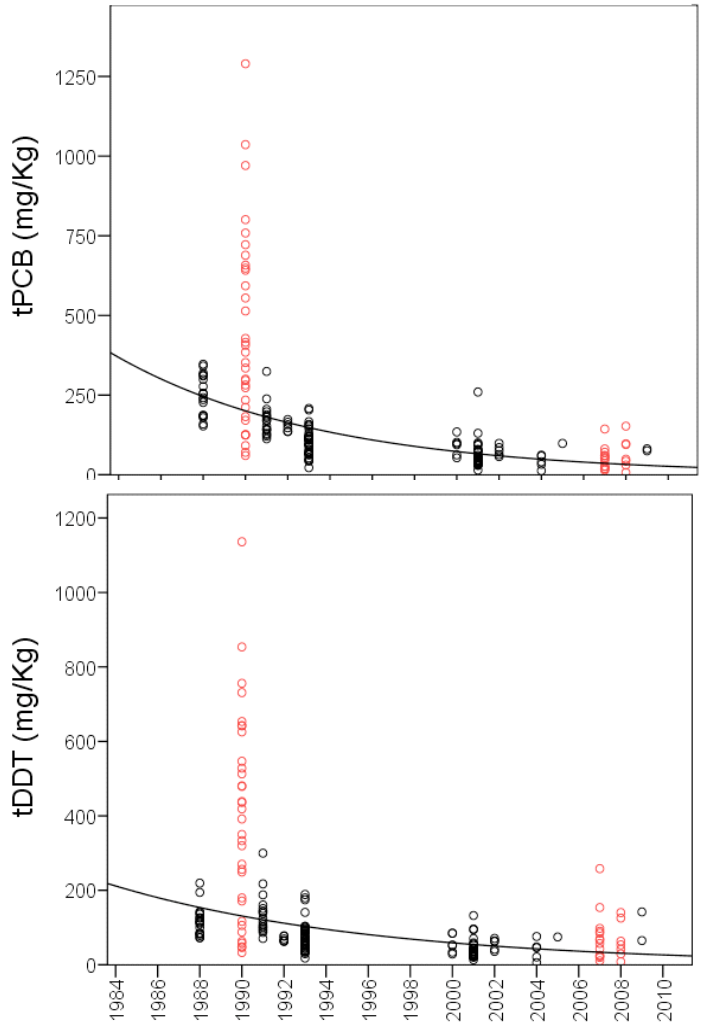

Figure 3. Trends in concentrations of tPCB and tDDT in Mediterranean striped dolphins from 1988 to 2009. The figure shows that the 1990 outburst data (in red) deviate from the general trend, while 2007–2008 outburst data (in red) fit well within the trend

The observed negative trends are in accordance with the chronology of the manufacture of these types of compounds. The amount of DDT and PCB manufactured increased exponentially until the 1970s, when its use ceased in the western world, due to restrictive regulations caused by environmental problems associated with bioaccumulation through the food chain. Consequently, a general reduction of tDDT, PCB, and the subsequent decomposition and dispersion processes that the pollutants have undergone, was observed in different Sea matrices as mussels, sediment, and vertebrates in the Northwestern Mediterranean

(Solé *et al.*, 1994; Tolosa *et al.*, 1997; Fasola *et al.*, 1998; Villeneuve *et al.*, 1999; Storelli *et al.*, 2004; Aguilar and Borrell, 2005; Borrell and Aguilar, 2007; Calic *et al.*, 2007; Gómez-Gutiérrez *et al.*, 2007). Studies by Aguilar and Borrell (2005) of *Stenella coeruleoalba* have shown that the concentrations of OCs in the Mediterranean Sea have exhibited a significant decline in the last 15 years, although this decline has ceased in the latter years.

The variability in OC concentrations among different dolphins in any given year (Figure 3) is attributable to the specific biological traits of the individuals sampled. In particular, age, nutritive condition, and sex are known to significantly affect blubber tissue OC levels in cetaceans (Aguilar *et al.*, 1999; O'Shea and Aguilar, 2001). In general, mature males present higher levels of OCs than mature females, due to the reproductive transfer of lipids and the substances contained in them, from females to their offspring (Borrell *et al.*, 1996; Aguilar *et al.*, 1999).

We could observe two important results when considering individuals from both epizootics (Figure 3): the 1990 OC data clearly exceeded the mean of the dolphin population at that time, whereas the data from the 2007 to 2008 outbreak fell within the mean of the population. This means that OC concentrations of the dolphins that died in the 2007 outbreak were similar to overall population levels of OCs found in the previous and succeeding years (Figure 3). Therefore, this leads us to suppose that these levels of OC did not have a determining effect on their immune systems, otherwise the dolphins that succumbed to the epizootic would have presented higher concentrations, similar to what was seen in 1990. In support of this hypothesis, when the tDDT and tPCB levels of deceased dolphins between 2000 and 2009 (excluding the 2007–2008 individuals) were compared with those from 2007 to 2008 with a t-test, no significant differences were found between means of tDDT and tPCB in these two groups of dolphins ($p = 0.075$ and $p = 0.46$ respectively), indicating that animals that died during the second outbreak did not present higher levels of tPCB or tDDT than the rest of the population.

CONCLUSIONS

The results of this study indicate that the epizootic event that occurred in 2007–2008 in Mediterranean striped dolphins was unlikely to be enhanced by the OC concentrations in these dolphins, which, despite being high, do not seem to have affected the immune systems of the dolphins.

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TRACE ELEMENT TRENDS & EFFECTS FOR SMALL CETACEANS IN EUROPEAN WATERS

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INTRODUCTION

The marine environment is contaminated by a lot of different chemicals regarded as pollutants, and since pollutants are capable of movements over considerable distances, all the marine food webs are of concern. Pollutants range from simple inorganic ions to complex organic molecules, which exhibit completely different properties. The organic pollutants are mainly man-made ("anthropogenic") compounds, which have appeared in the natural environment only during the 19th Century. The consequence is a very short time in evolutionary terms, and there has been only limited opportunity for the evolution of protective mechanisms against their toxic effects (e.g. detoxication by enzymes) (Walker *et al.*, 1996).

By contrast, trace elements (as metals and metalloids) are natural substances and have been present on the earth since its formation. Thus marine mammals and other marine organisms have developed mechanisms either to control or to mitigate the toxic effects. The mechanisms consist of hiding active metal ions within a protein such as metallothionein, or depositing them in an insoluble form in intra cellular granules for long-term storage or excretion in the faeces. However, in most cases, biogeochemical cycles are modified by human activities, which are mainly responsible for their releases through mining and smelting from the rocks. As a consequence, the extent to which human activity contributes to global cycles of trace elements can be described by the Anthropogenic Enrichment Factor (AEF) defined as the ratio between anthropogenic sources and natural sources expressed as a percentage (Walker *et al.*, 1996).

Cadmium (Cd), mercury (Hg) and lead (Pb) are the main non-essential metals (which in contrast to essential trace elements do not exhibit deficiency effects) that can give rise to pollution problems in the environment. Their AEF ranges from 66 to 97% (Walker *et al.*, 1996), showing the high contribution of human activities to their global cycles. Such an increase of environmental concentrations raises the question of the detoxification rates and success in marine mammals that are mainly exposed to trace elements *via* feeding and thus subject to long term exposure and chronic toxicity. Moreover, marine mammals are exposed to several types of pollutants in the environment, and not only trace elements. Finally, several factors affect rates of uptake, elimination and bioaccumulation of trace elements in marine mammals, which induce a high inter- and intra-specific variation in concentrations (Wagemann & Muir, 1984; Bowles, 1999; Das *et al.*, 2003).

These different characteristics (natural character of trace elements, exposure of marine mammals to a mixture of pollutants, the detoxication processes, and the large variation in concentrations) make the direct link between trace elements contamination and effect very difficult to show both at an individual level and population level (Figure 1). It is the role of toxicology that has been referred to as the science of poisons and their adverse effects on living systems. Its functions have expanded from identifying poisons and searching for treatments to include forensic toxicology and testing and detection of a fast-growing number of new potentially toxic substances used in workplaces, in agriculture (e.g., insecticides, other pesticides, fertilizers), in cosmetics, as food additives, and as drugs. It also implies epidemiological studies and the determination and research of an endpoint, which would reveal a transitional and reversible effect in order to preserve individuals of pathology and population from decline (Figure 1).

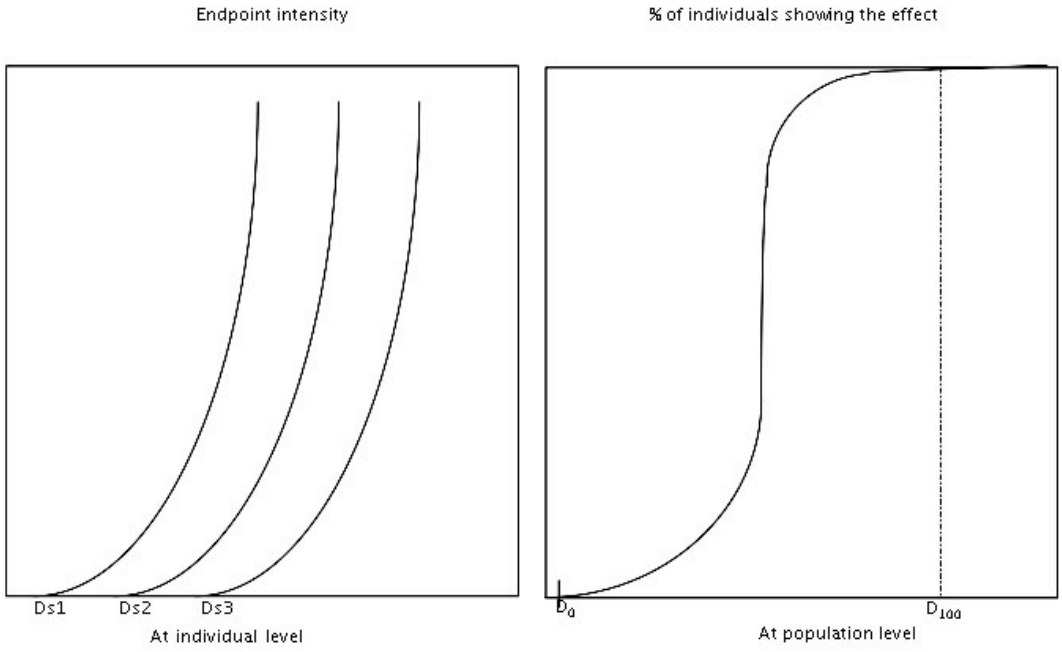

Figure 1. Dose-relationship between the dose of compound and the effect at individual and population level. The determination and the research of the endpoint imply skills in biochemistry, histology, pharmacology, pathology, depending on the known effect of the metal. It also implies to evaluate this effect in one population in order to estimate the % of the individuals exhibiting this effect in the population. In order to preserve species of pathology and population of decline, it is necessary to research and study an endpoint, which has a minimal and reversible effect

But the necessary epidemiological studies are very difficult to realise in wild animals. This is why, although many investigations on trace elements in marine mammals have been carried out during the past few decades, they mainly focus on concentrations in species and factors affecting bioaccumulation (see Law, 1996; Das *et al.*, 2003 for reviews). Thus, to what extent trace elements constitute a threat to marine mammals and to what extent they are responsible for the decline of some populations has not been established so far.

The objective of this short contribution is to show through some examples how difficult it is to evaluate the potential direct impact of two metals (cadmium and mercury) on marine mammals, and the probable very low direct effect of cadmium and mercury on small cetaceans off European waters.

Cadmium (Cd)

As with other metals, cadmium toxicology has mainly been studied in humans and small rodents (WHO, 1992). With the exception of the occupational exposure, food is the major source of cadmium exposure for the non-smoking general population (EFSA, 2011) and thus the Threshold Limit Value (TLV) and the Provisional Tolerable Weekly Intake (PTWI) refer to humans. The PTWI of 7 µg/kg body weight previously established by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA, 2003) was not maintained, and a considerably lower TWI of 2.5 µg/kg body weight was recently established by the Panel on Contaminants in the Food Chain (EFSA, 2011).

In their review, Godt *et al.* (2006) discuss historic and more recent developments of cadmium toxicological and epidemiological questions, including damage processes. The main organ for long-term cadmium accumulation is the kidney where the half-life is approximately ten years but can be even longer (up to 38 years), according to Friberg (1974) and Kjellstrøm & Nordberg (1978). In the case of chronic exposure, the kidney has been described as the main target organ of damage. One of the primary markers of kidney damage is the urinary excreted β2-microglobulin, which has been shown to appear at the TLV of 50 µg Cd/g wet weight in kidney cortex (Elinder & Jarup, 1996). In humans, both environmentally exposed populations in general, and elderly persons in particular (because of the cumulative nature of cadmium), have displayed evidence of cadmium-induced renal dysfunction at a critical urinary excretion of the order of 5 $\mu g.g^{-1}$ creatinine.

Marine mammals that exhibit cadmium concentrations likely to induce pathological effects are mainly squid-eating odontocetes from sub polar or polar areas (see AMAP 2005, for review). Thus cephalopods have been shown to be an important vector of cadmium to top marine predators in the North-East Atlantic and especially in high latitude areas (Bustamante *et al.*, 1998). As an example, cadmium levels have been determined in the kidneys of 114 pilot whales (*Globicephala melas*) belonging to three schools (Caurant *et al.*, 1994). Levels were >100 $\mu g.g^{-l}$ in the whole kidney in 31.6% samples and in three pregnant females, concentrations as high as 500-960 $\mu g.g^{-l}$ have been shown. Cadmium concentrations in urine of seventeen individuals varied between 1.4 and 13.9 $\mu g.g^{-1}$creatinine with two values >10 $\mu g.g^{-1}$ and five ranging between 5 and 10 $\mu g.g^{-1}$ (Caurant & Amiard-Triquet, 1995). However, cadmium impact at the population level was not obvious. Thus, although a long series of data does exist in the Faroe Islands, they give no indication that the stock size has been affected (IWC, 1992). The toxic effect of cadmium would be mitigated by the induction of metallothionein a protein that has been shown to participate in this metal detoxification (see Das, 2000 for review). Thus in pilot whales the percentage of cytosolic cadmium bound to metallothionein varied between 6% and 51% according to cadmium concentrations in kidney.

The Atlantic white-sided dolphin (*Lagenorhynchus acutus*) was another species investigated in that area (Gallien *et al.*, 2001). One individual with renal cadmium concentrations of 35 µg.g^{-1} (which means that the concentrations in the cortex would be approximately 50% higher than the values cited here) exhibited electron dense mineral concretions in the basal membranes of the proximal tubules. They were spherocrystals made up of numerous strata mineral deposits of calcium and phosphorus together with cadmium. Whether these granules could constitute a way of immobilisation and detoxification of the metal or a sign of the perturbation of calcium metabolism as a consequence of Cd effect is still an issue (Gallien *et al.*, 2001).

Another example concerns ringed seals (*Phoca hispida*) from Northwest Greenland studied by Dietz *et al.* (1998). Kidney samples were selected for microscopic examination from three concentration ranges, these being 1.63-5.19, 86.5-91.3 and 259-581 µg.g^{-1} wet weight respectively. Although a quite high incidence of renal tubular proteinurea could be expected at this level, light microscopy showed that the renal cortical and medullary zones appeared normal in all three groups, resembling typical seal kidneys (Dietz *et al.*, 1998).

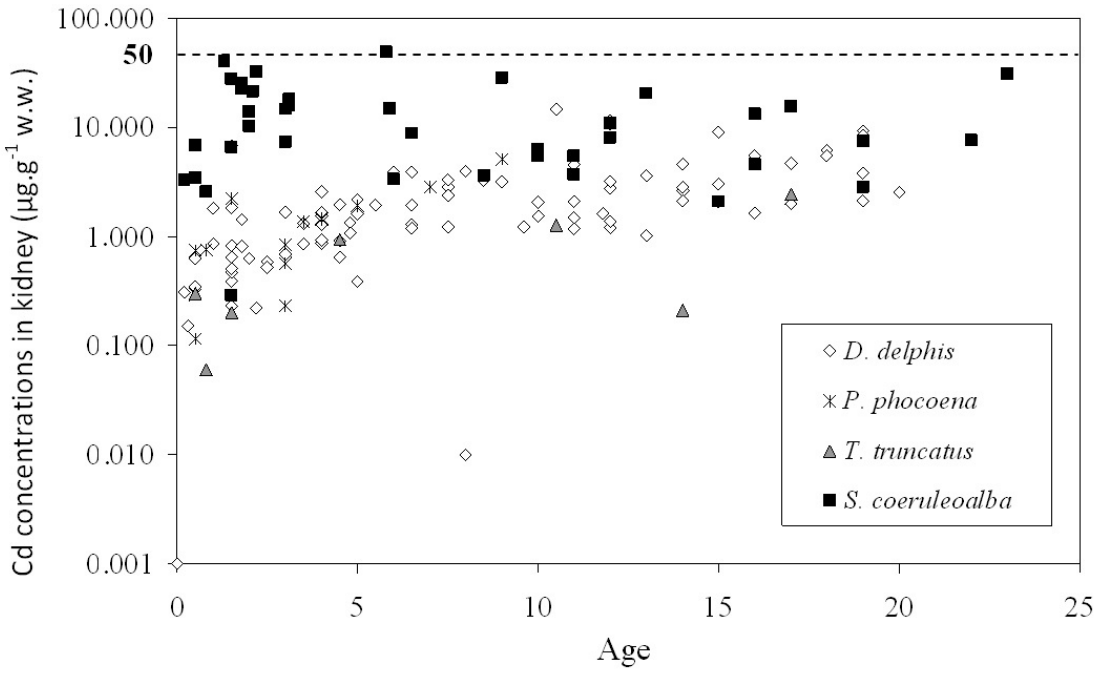

Figure 2: Bioaccumulation of cadmium in kidney (µg/g wt) with age (years) in small cetaceans from the Bay of Biscay. The Threshold Limit Value of 50 µg.g^{-1} w.w is indicated (see text). (modified from Lahaye *et al.*, 2004; Lahaye, 2006)

Compared to arctic marine mammals, species from the temperate waters exhibit much lower Cd concentrations (Figs. 2 & 3). In these temperate areas, Lahaye *et al.* (2004) and Lahaye (2006) have shown that cephalopods also contributed to

cadmium exposure of their predators. Although the level of exposure for the most exposed species, the oceanic striped dolphin (*Stenella coeruleoalba*), was underestimated, it was in the range of the PTWI defined for humans (Figure 4). This species also exhibited the higher Cd concentrations (Figure 2). But like the oceanic by-caught common dolphins (*Delphinus delphis*), which exhibited about four times higher Cd concentrations (16.3±14.0 $\mu g.g^{-1}$ wet weight) than neritic ones (Lahaye *et al.*, 2005), concentrations were rather lower than the TLV.

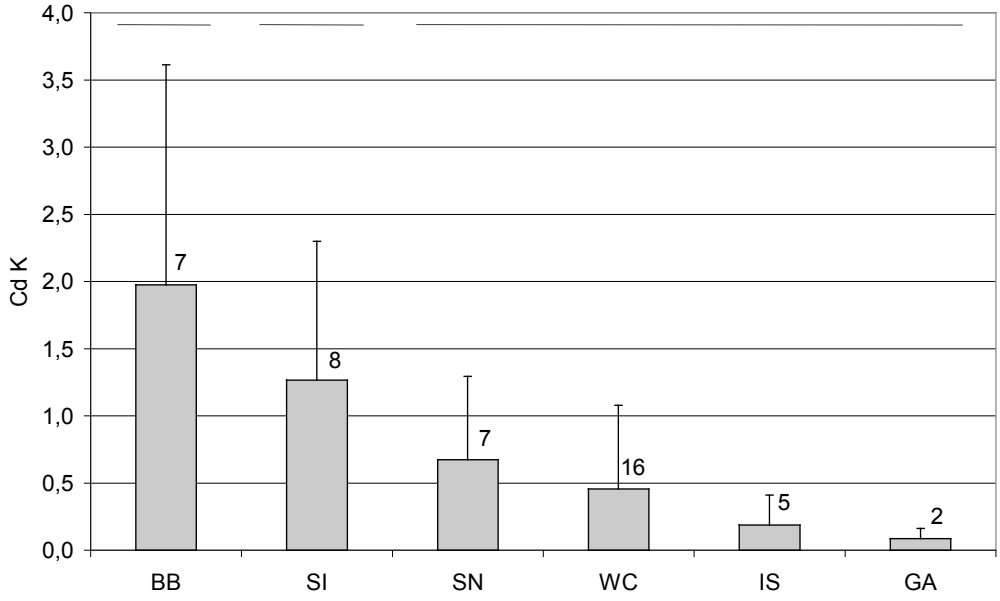

Figure 3. Mean cadmium concentrations in kidney (µg/g w.wt) of harbour porpoises from the different areas in the European waters. Key: BB, Bay of Biscay; SI, South of Ireland; SN, Southern North Sea; WC, West Channel; IS, Irish Sea; GA, Galicia. Homogeneous groups are indicated with bars. The number of individuals analysed in each area are indicated above the histograms. (from Lahaye *et al.*, 2004)

Mercury (Hg)

Mercury appears as different chemical forms in the environment. In 1967, Jensen & Jernelov demonstrated the mercury methylation by microorganisms in sediments. Thus, MeHg enters the aquatic food chain where it is biomagnified, inducing its uptake by top predators or humans through fish and seafood consumption. Moreover, MeHg is probably by far the most toxic form of mercury in food.

The study of MeHg effects in humans is a long story (see Grandjean *et al.*, 2010). In their synthesis, the authors catalogue key publications related to human MeHg toxicity, and underline the different scientific and legislative gaps that led to a delay affecting the recognition of MeHg as a cause of serious human poisoning, despite the early warning since 1865. The first seafood-related disease was discovered in Minamata (Japan) in 1956. Then a series of food poisoning incidents following expanded use of MeHg fungicides were also reported in Iraq, Pakistan and Guatemala in 1955-1972. The neurotoxic effects observed in adults (paresthesia, ataxia, gait disturbance, sensory disturbances, tremors, visual and hearing impairments) and the impaired neurodevelopment of the children induced by the

mother's exposure (even if the exposed mother was virtually unaffected) are thus known as Minamata disease. Current evidence suggests that concentrations above 200 µg.L^{-1} in blood are associated with early manifestations of neurotoxicity in adults (WHO, 1990). Since then, studies have shown that the developing nervous system is a very sensitive target for MeHg and that depending on the dose and timing of exposure during gestation, the effects may be severe and immediately obvious (such as deafness and blindness, cerebral paralysis) or subtle and delayed (such as low cardiac rate variability, memory problems, language disorders, deficits in fine motor function, etc) (Clarkson & Magos, 2006; Choi & Grandjean, 2008; Grandjean *et al.*, 2010). Thus, more recently, most attention has been paid to three major epidemiological studies involving a large number of infant-mother pairs in fish-eating populations: a New Zealand study, Faroese study;, and Seychelles study (see Clarkson & Magos, 2006; Choi & Grandjean, 2008 for reviews).

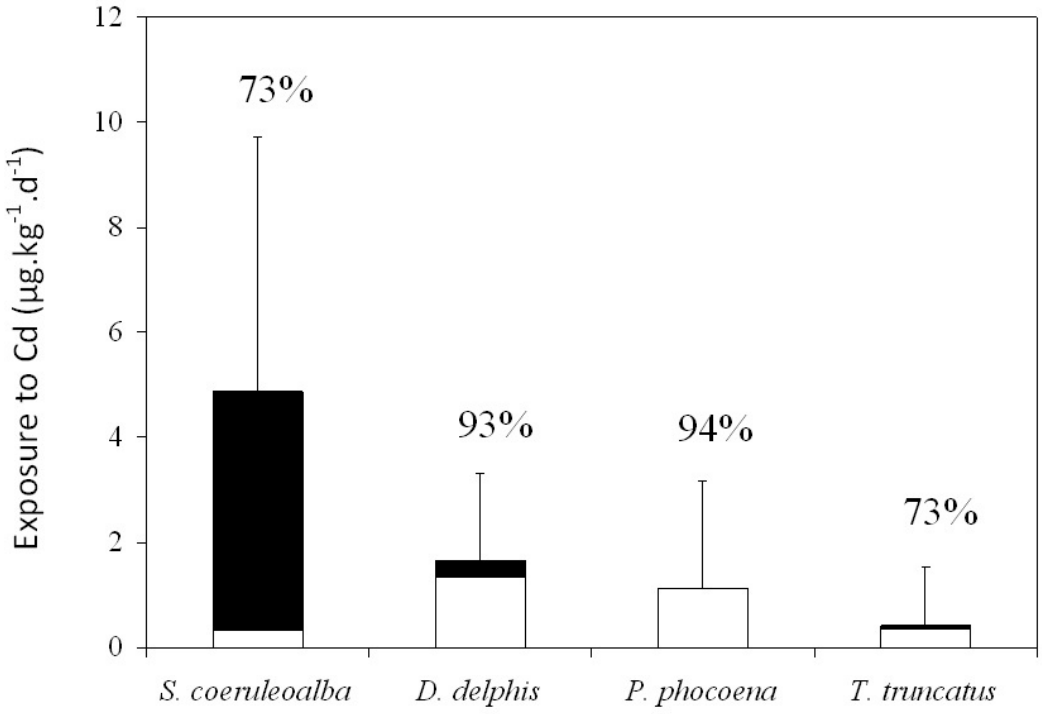

Figure 4. Exposure to Cd (µg/day/kg) through the diet for small cetaceans from France (Bay of Biscay). Percentage at the top of bars indicates the biomass proportion of diet considered for calculations. (from Lahaye *et al.*, 2004 ; Lahaye, 2006)

These three studies aimed at relating prenatal exposure (from fish or marine mammal diet of the mothers) to developmental neurotoxicity in children. If MeHg risks may have been underestimated in the past (Choi & Grandjean, 2008), the significant MeHg effect in New Zealand and Faroese studies provide no doubt that the developing human brain is particularly susceptible to injury caused by this compound. However, the No Observed Adverse Effect Level and the calculation of Benchmark Dose Level are still the subject of debate. Maternal hair served as the primary biomarkers of *in utero* exposure and considering only the Faroes and Seychelles studies, the Joint FAO/WHO expert committee on food additives (JEFCA,

2003) settled on a NOAEL of 14 µg.g^{-1} for maternal hair corresponding to 56 µg.L^{-1} for blood (considering a hair:blood ratio of 250). They also settled on a PTWI of 1.6 µg.kg^{-1} body weight. However, Grandjean *et al.* (2007, 2010) considered that deficits in several brain functions in school-age children were apparently well below a maternal hair mercury concentration of 10 µg.kg^{-1}. But highlighting the dose-response relationship required subtle endpoints through a test battery related to neuropsychology and neurophysiology. Sophisticated techniques are susceptible to show adverse effects at even lower Hg exposure (see Figure 5, & Grandjean *et al.*, 2010).

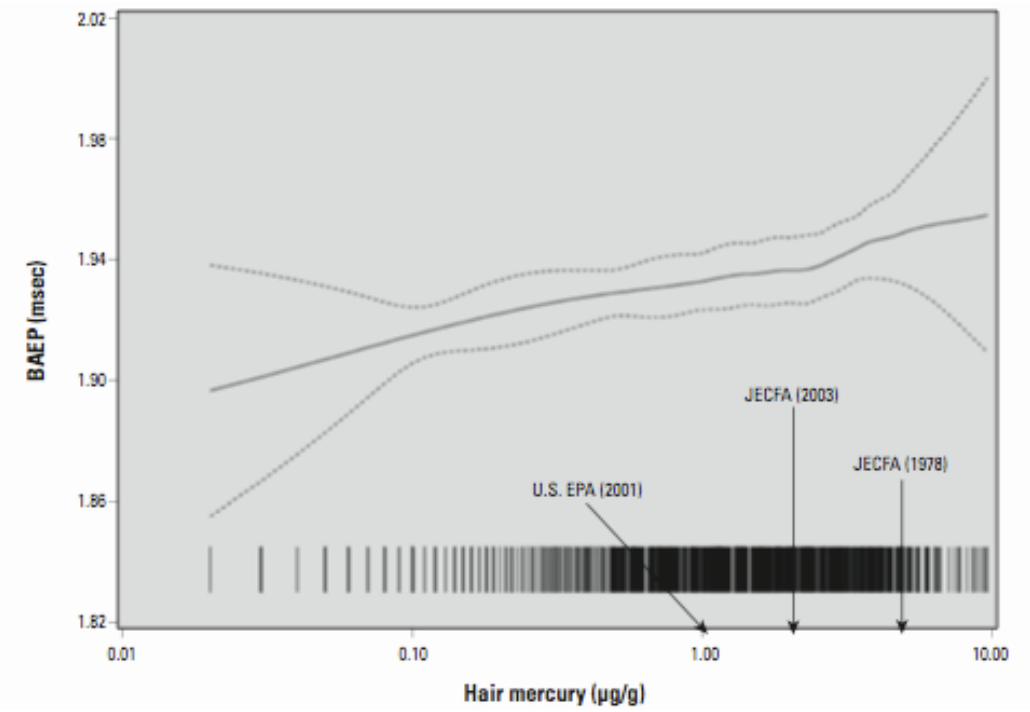

Figure 5. Association between brainstem auditory evoked potential (BAEP) latency (interpeak III–IV at 20 Hz) and dietary exposure to methylmercury (reflected by hair mercury concentration) in 14-year-old Faroese children. Data are from examinations of a Faroese cohort of 878 subjects at 14 years of age. Each vertical line represents one subject, dotted lines indicate the 95% confidence limits, and arrows represent three methylmercury exposure limits. Modified from Murata *et al.* (2004), in Grandjean *et al.*, 2010)

Some examples of wildlife poisonings were shown in marine organisms at Minamata (floating seabass), and also in predatory birds and fish-eating birds exhibiting reproductive failure in relation to the contamination of their food chain because of the use of MeHg as treatment of seed grain (Grandjean *et al.*, 2010).

Coming back to marine mammals and especially odontocetes or pinnipeds at high levels in the food chain, there is no doubt that they are exposed to high levels of MeHg *via* their diet. Thus, whatever the type of prey (fish or squid), MeHg is the main chemical form to which they are exposed (Ahmed *et al.*, 1988; Bustamante *et al.*, 2006). However, in contrast to humans, the exposure of marine mammals to MeHg has occurred throughout their evolutionary history, during which they have

developed detoxification mechanisms. The main detoxification process is related to the correlation of Hg with Selenium (Se) in their liver, first shown by Koeman *et al.* (1973).

A few years later, Martoja & Berry (1980) demonstrated the presence of granules of tiemmanite (Hg:Se) in the liver of two species (*Ziphius cavirostris* and *Tursiops truncatus*) as the result of the demethylation of MeHg by Se. This Hg-Se complex would be the last stage of the detoxification process leading to the fossilization of mercury and selenium in the form of non-biodegradable compounds. As a consequence of this mechanism, the major part of Hg in marine mammals accumulates in the liver under inorganic form and despite high Hg concentrations (Wagemann & Muir, 1984; Bowles, 1999; O'Shea, 1999), no obvious signs of Hg poisoning were ever shown. Nevertheless, Wagemann & Muir (1984) admitted a TLV as a wide range between 100 and 400 $\mu g.g^{-1}$ fresh weight of total mercury in the liver of marine mammals in the northern hemisphere, lowered later on to 60 $\mu g.g^{-1}$ fresh weight (Law, 1996). However, no epidemiological studies have helped define these values. And this is quite different from humans, since in Minamata, concentrations of total mercury in the livers of people with the disease ranged between 22 and 70 $\mu g.g^{-1}$ but mainly in the form of methyl mercury (Britt & Howard, 1983). Since then, numerous studies have shown the correlation between Hg and Se, and the ratio Hg:Se of 1 in the liver of marine mammals (Figure 6).

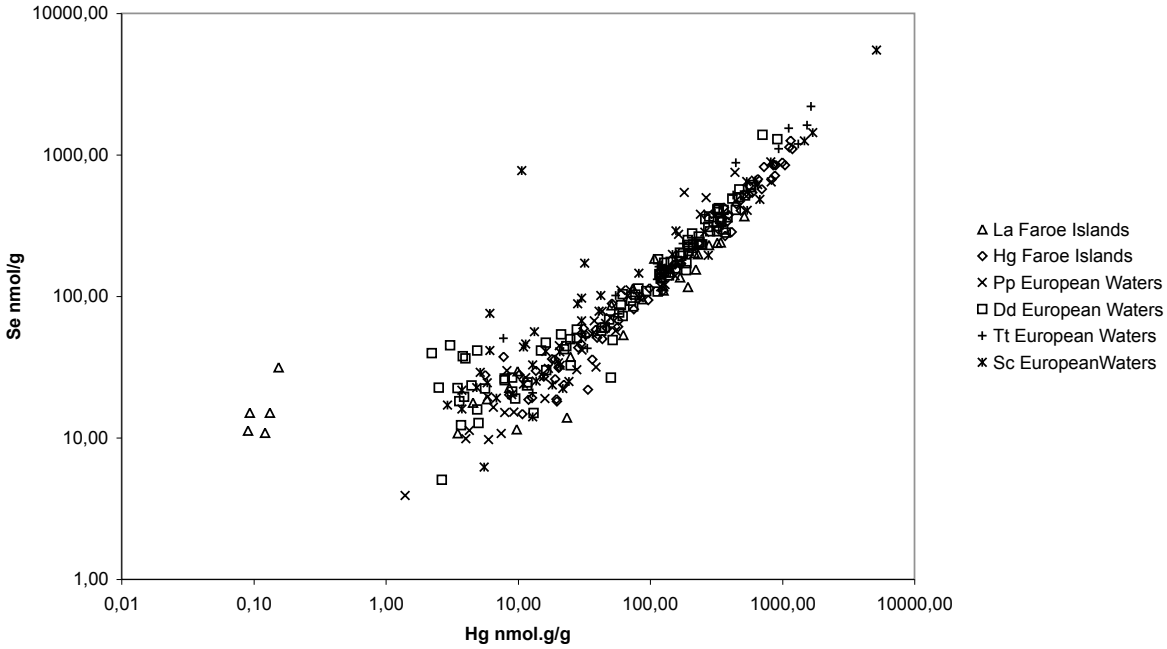

Figure 6. Molar selenium and molar mercury concentrations in liver tissue of marine mammals from the European waters and Faroe Islands. Key: La, *Lagenorhyncus acutus* ; Hg, *Halichoerus grypus*, Pp, *Phocoena phocoe*na ; Dd, *Delphinus delphis* ; Tt, *Tursiops truncatus*; Sc, *Stenella coeruleoalba*. Data included personal data and data from Lahaye *et al.* (2004), Lahaye (2006)

The process of demethylation seems to be dependent on the mean concentrations in the species or on the concentrations encountered in individuals of the same species. Thus, the mechanism of demethylation has evolved in response to

biomagnification of MeHg in species at high levels in the food chain. Moreover, the formation of this complex in the liver would protect sensitive organs from the toxic effects of methyl mercury (Cuvin-Aralar & Furness, 1991).

A few studies of mercury levels in the blood of marine mammals are available in the literature. Species of concern were pilot whale and sperm whale (*Physeter macrocephalus*) (Nielsen *et al.*, 2000), bottlenose dolphin (*Tursiops truncatus*) (Woshner *et al.*, 2008, Stavros *et al.*, 2008), and northern elephant seal (*Mirounga angustirostris*) (Habran *et al.*, 2011). They all reported MeHg or TotHg in some individuals above the TLV (200 $\mu g.L^{-1}$), giving rise either to a mercury poisoning effect in adults or a potential effect in juveniles because of the concentrations encountered in mature and potentially reproducing females. However, high levels of Se were also reported, highlighting again the possibility of interactions between both elements, inducing mitigation of the toxic effects. Then in most of these studies, blood was collected on live and apparently healthy individuals. In the same way, pinnipeds may exhibit Hg concentrations in fur above the TLV determined in human hair, a similar tissue (Aubail *et al.*, 2011). But direct comparison of dose-response relationships with humans may be inappropriate as the toxicodynamics and toxicodistribution of mercury are probably distinct between humans and marine mammals. And considering the detoxification process, the TLV in hair and blood reported for humans probably does not make much sense for marine mammals.

Nevertheless, some signs of a limited detoxification process exist in marine mammals. This was the case for the lactating females of pilot whales studied in the Faroe Islands which exhibited a molar ratio Hg:Se of 1.93 significantly higher than 1 (Caurant *et al.*, 1996). This has been attributed to a change in the diet inducing a lower availability of efficient forms of Se counteracting Hg toxicity. The question has also been raised In the Arctic, where an increase in mercury concentrations in the upper layers of sediment was found (Dietz *et al.* 1998; Asmund & Nielsen 2000), showing the changes in the biogeochemical cycling of mercury through anthropogenic enrichment. As a result, some species such as polar bears and ringed seals that have the highest concentrations of mercury and Hg:Se greater than or equal to 1, would be most at risk if mercury enrichment continued (Dietz *et al.* 2000).

Considering small cetaceans in European waters, contaminant levels appeared not to be particularly high, except for 20 individuals (7% of sampling) with concentrations of Hg in the liver higher than the TLV. These individuals were mainly common dolphins from the Western Channel and Bay of Biscay, and striped dolphins from the Bay of Biscay (Figure 7) The evaluation of efficiency of detoxification process through the pattern of Se:Hg ratios revealed that whatever the species and the geographical area, the Hg:Se is less than 1 up to age 5 years and then tends to approach the threshold 1:1 with some variability. Indeed, beyond 5 years, 60% of individuals have Hg-Se ratios below 0.9, and 30% of individuals have a ratio close to 1, i.e. between 0.9 and 1.1. Finally, 8% of cetaceans present Hg-Se ratios >1.1 and the extreme ratios of 6:1 are only observed in two individuals (Figure 8).

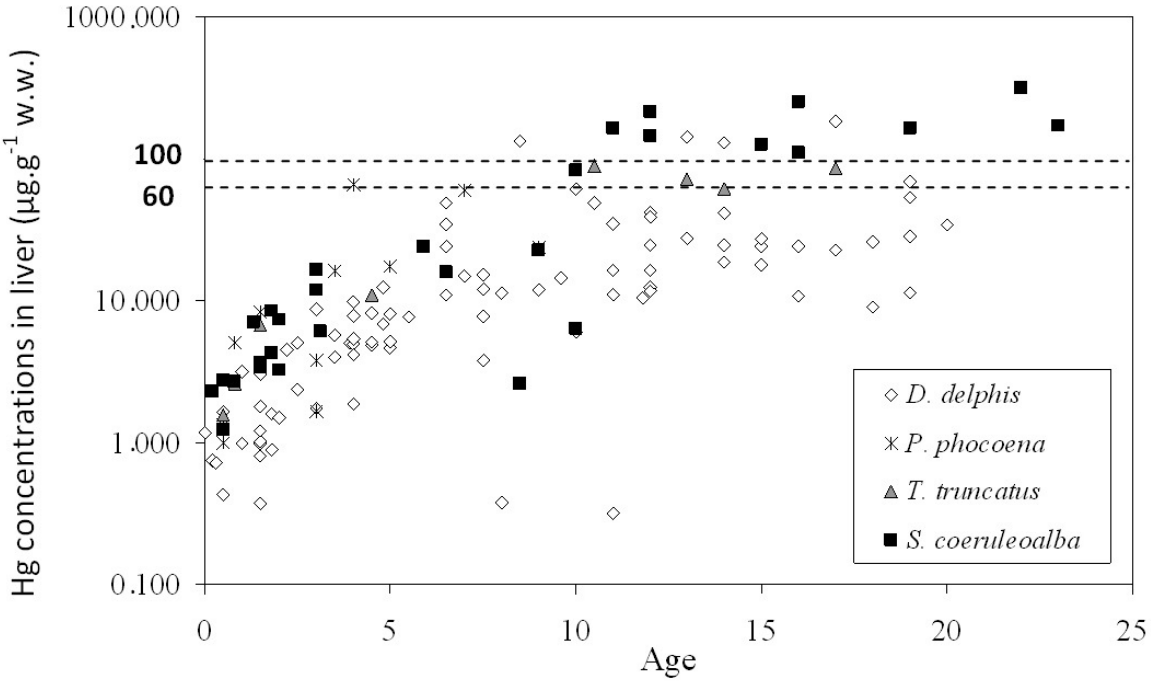

Figure 7. Bioaccumulation of mercury in liver (µg/g wt) with age (years) in small cetaceans from the Bay of Biscay. The Threshold Limit Value of 60 $\mu g.g^{-1}$ w.w and 100 $\mu g.g^{-1}$ are indicated (see text). (modified from Lahaye *et al.*, 2004; Lahaye 2006).

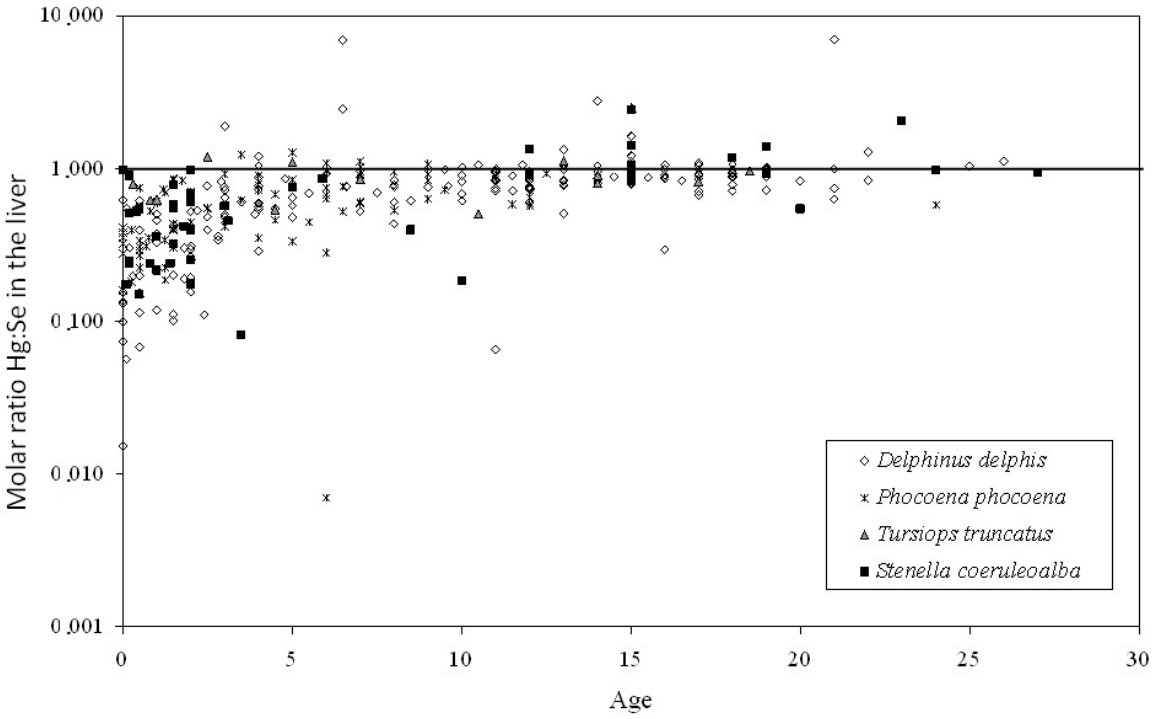

Figure 8. Relationship between molar ratio Hg:Se in liver tissue and age in small cetaceans from the European Waters. The line indicates 1:1 molar ratio. (from Lahaye, 2006)

Since the individuals exhibiting the high Hg:Se ratio belonged to different species and different geographical areas (common dolphin from Galicia, striped dolphin from the Bay of Biscay, and harbour porpoise from the Irish Sea), it is difficult to consider that any populations are particularly vulnerable.

CONCLUSIONS

The marine environment is contaminated or polluted by a wide variety of organic chemicals and metals. Most of these compounds exhibit adverse effects on health, demonstrated either through toxicological studies on small rodents or through epidemiological studies in humans. Thus, marine mammals are likely to develop diseases related to the exposure to these contaminants and especially organic ones to which they are exposed for a shorter period than the inorganic ones. Direct effects of only metals are not obvious and marine mammals have to face to other factors that obviously impact populations such as by-catch in fisheries, degradation of habitat and other ecosystem changes induced by human activities. Moreover, because of the detoxification processes, determining a direct link between metal concentrations and effects in marine mammals through dose-response relationships may require a search for endpoints that would be liminal effects. It would necessitate many epidemiological studies, assuming comparisons between populations exposed and unexposed to only metals, which is impossible.

Nevertheless, Indirect effects of trace elements probably include immunodeficiency, altered physiological or metabolic functions that can lead to decrease reproductive success. They can also act as confounding factors in synergistic processes and thus increase the response to organic pollutants.

In the context of a rapid and great variability of the marine environment induced by anthropogenic activities and including biogeochemical enrichment and modifications of the links between prey and predators in food webs, will the detoxification processes acquired by marine mammals during evolution still be efficient? What would be the cost of this detoxification processes in populations that have to face a very variable environment, and that can be vulnerable from other points of view?

So trace elements that should not be a problem probably constitute an additional stress to marine mammals exposed nowadays to multiple chemicals in a highly variable marine environment.

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BIOACCUMULATION OF POPS AND TOXIC ELEMENTS IN SMALL CETACEANS ALONG EUROPEAN ATLANTIC COASTS

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ABSTRACT

Results on persistent organic and toxic element concentrations in tissues of small cetaceans from five northeast Atlantic European coastal regions in 2001-03 are summarised. PCB concentrations in blubber frequently exceeded the threshold at which effects on reproduction might be expected, notably in harbour porpoises from the southern North Sea (reflecting high concentrations in prey) and common dolphins from France. Concentrations were often higher in males than females, as expected since females transfer lipophilic pollutants to their offspring during pregnancy and lactation. Highest concentrations were recorded in blubber of bottlenose dolphins. The regional distributions of PBDE and HBCD concentrations differed from that of PCBs. Mercury concentrations in liver were generally below the threshold for toxic effects. Cadmium levels never exceed the threshold for toxic effects and were higher in species feeding more on cephalopods. While strandings data may result in samples biased towards animals in a poor state of health, dangerously high concentrations of PCBs were nonetheless frequently recorded in small cetacean blubber during 2001-03.

INTRODUCTION

In marine mammals, since the main route of entry for contaminants is the food, differences in bioaccumulation will arise from differences in diet and trophic level, and from feeding in different food chains. Boon *et al.* (1987) showed that PCB levels were higher in seals fed on fish from the Waddensea than in seals fed on fish from the Atlantic. Species that feed primarily on cephalopods may be expected to accumulate higher levels of cadmium than those feeding on fish (Bustamante *et al.*, 1998). In addition, contaminant concentrations in cetacean tissues may be affected by body size (which affects excretion rate, activity of detoxifying enzymes and metabolic rate), body composition (especially, in the case of lipophilic POPs, the mass of blubber), nutritive condition, disease, age, sex and duration of lactation (Aguilar *et al.*, 1999).

Harmful consequences of bioaccumulation of POPs in marine mammals include suppression of the immune system (e.g. de Swart, 1995; Ross, 1995) and reproductive failure (Helle *et al.*, 1976; Reijnders, 1986). High mercury concentrations have been linked to a prevalence of parasitic infection and pneumonia (Siebert *et al.*, 1999). As top predators that eat many of the same prey species as marine mammals, humans are potentially subject to similar risks.

Even though production and use of some harmful organic compounds has decreased or even ceased, they may persist in the marine environment. Meanwhile, new classes of chemicals continue to come into use, e.g. brominated flame retardants, and are also a cause for concern (de Boer *et al.*, 1998). Hexabromocyclododecane (HBCD) is a brominated flame retardant in polystyrene foams used in the building industry, and has been recognised as a priority pollutant by the EU.

Cadmium (Cd), mercury (Hg) and lead (Pb) are generally considered to be the heavy metals most likely to give rise to pollution problems in the marine ecosystem (Bryan, 1984). Indeed, even if they are naturally present in the environment, their important anthropogenic enrichment is likely to induce a further contamination in industrialised areas (Walker *et al.*, 1996). Cadmium appears to be particularly bioaccumulated in the kidney while highest mercury levels are found in the liver (Dietz *et al.*, 1998). Marine mammals have evolved mechanisms to counteract the action of such trace elements, e.g. demethylation of mercury through the formation of granules, which associate selenium and mercury in the liver, although some limits to this detoxification process have been identified (Caurant *et al.*, 1994, 1996).

The EU-funded project BIOCET (2001-04) aimed to quantify and model contaminant bioaccumulation and its consequences for small cetaceans in European Atlantic waters. The main focus were the two most frequently stranded species, common dolphin *Delphinus delphis* and harbour porpoise *Phocoena phocoena*, but data were also collected on several other species, notably bottlenose dolphin *Tursiops truncatus* and striped dolphins *Stenella coeruleoalba.* Some results on POPs and toxic elements, and their effects, have been previously published in several papers (e.g. Zegers *et al.*, 2005; Lahaye *et al.*, 2007; Pierce *et al.*, 2008; Murphy *et al.*, 2010; see also Caurant, this volume). The present report summarises results on POP, Hg and Cd concentrations in small cetaceans, highlighting geographical and species differences and possible causes, and the prevalence of concentrations above thresholds for toxic effects.

METHODS

During 2001-03, in collaboration with national strandings schemes, stranded harbour porpoises and common dolphins were sampled from Scottish (UK), Irish, Dutch, Belgian, French and Galician (NW Spain) Atlantic coasts (see Figure 1). Priority was given to females recovered in good condition, from which all necessary samples could be obtained, but data and samples were collected from other animals when possible. Samples obtained from France included 52 common dolphins from a mass live stranding of a nursery group of females and calves in February 2002 at Pleubian, Brittany. Small numbers of other species, notably striped dolphins and bottlenose dolphins, were also sampled during the study. Animals sampled ranged in decomposition state from extremely fresh (2a) to moderately decomposed (3). Post-mortem examination and tissue sampling followed European Cetacean Society guidelines (Kuiken & Hartmann, 1991). Basic data collected from each animal included stranding location, date, species, sex, total length and blubber thickness (measured immediately in front of the dorsal fin in dorsal, midline and ventral positions). Samples collected included teeth and gonads. In females, the entire

reproductive tract was collected, the uterus was examined for presence of a foetus, and milk glands were examined for evidence of lactation. Blubber was sampled for POP and fatty acid analyses, from the left side in front of the dorsal fin. Samples of liver and kidney were taken for trace element analysis. Pathological and histopathological analyses were routinely carried out in Scotland, The Netherlands, Belgium, and Galicia, and for some samples from France and Ireland. Samples were also collected of some of the main prey species of common dolphins and harbour porpoises in each region, to allow measurement of POPs, trace elements, and fatty acid profiles in prey tissues.

Full details of the analytical methodology for determination of POPs and trace elements appear in Lahaye *et al.* (2005, 2006, 2007), Zegers *et al.* (2005), and Pierce *et al.* (2008), so only a reduced account appears here. POP analysis was focused on the largest sample sets, concentrating on those females for which most data were available on other variables. Thus, for porpoises, analysis focused on samples from Ireland, Scotland, and the southern North Sea (The Netherlands, Belgium and northern France). For common dolphins, analysis focused on samples from Ireland, France and Galicia.

POPs measurements were made on 70 female common dolphins and 67 female porpoises (out of 531 common dolphins and 243 porpoises collected, the latter figures including individuals both sexes and all decomposition states). Five bottlenose dolphins and eight striped dolphins were sampled for POPs. Analysis of POP concentrations in cetacean and prey samples was carried out at the Royal Netherlands Institute for Sea Research (NIOZ), with some Scottish cetacean samples analysed at the Centre for Environment, Fisheries and Aquaculture Science (Cefas). Organochlorines were determined by gas chromatography with electron capture detection. Since concentrations of many compounds were often below the limit of detection, we selected eighteen PCB congeners for further analysis (CB28, CB49, CB52, CB99, CB101, CB118, CB128, CB138; CB141, CB149, CB151, CB153, CB170, CB177, CB180, CB183, CB187 and CB194). Data available from Cefas (for Scottish cetaceans) excluded values for CB99 and CB177, which were therefore dropped from analyses of data for porpoises. Other OCs analysed were *p,p'*-DDE, hexachlorobenzene (HCB), and pentachlorobenzene (PeCBz). Brominated flame retardants were determined by gas chromatography with electron-capture negative ion mass spectrometry. Again, many compounds were often below limits of detection, and we selected five PBDE congeners (BDE47, BDE99, BDE100, BDE153 and BDE154) for further analysis. Additional funding became available to measure levels of hexabromocyclododecane (HBCD) in some of the samples. Both participating laboratories regularly tested the analytical protocols used, and performed up to expected standards.

A Σ-PCB level of 17 $\mu g\ g^{-1}$ lipid has been reported as a threshold level for effects in the bottlenose dolphin (*Tursiops truncatus*) (Kannan *et al.*, 2000; Schwacke *et al.*, 2002). For comparison with this figure, which was based on the commercial PCB mixture Aroclor 1254, we also derived the "ICES7" value (the sum of concentrations

of CB28, CB52, CB101, CB118, CB138, CB153, CB180), since three times this value is equivalent to the Aroclor 1254 value (Jepson *et al.*, 2005).

Total Hg in liver was determined using a mercury analyser. Cadmium in the kidney was analysed using either a graphite furnace or flame absorption spectrophotometer according to levels exhibited in each sample. Determination of Zn in the liver was carried out by flame atomic absorption spectrometry. Since the Hg:Se ratio may provide an indicator of detoxification of mercury, Se in liver was determined by graphite furnace atomic absorption spectrometry. Quality control was assured by analysis of reference materials. Hg was reported to produce toxic effects at liver concentrations of 100-400 µg/g wet weight (Wagemann & Muir, 1984), while Law (1996) indicated that a threshold of 60 µg/g wet weight for toxic effects. Toxic effects of cadmium are reported above 50 µg/g w.wt in kidney (Dietz *et al*., 1998). Our results were therefore compared with these values.

Patterns and trends in the data were investigated using principal component analysis (PCA), redundancy analysis (RDA) and generalised additive models (GAM) as implemented in BRODGAR (Highland Statistics Ltd).

RESULTS

PCA results indicate a positive association between blubber concentrations of most categories of POPs, as seen in the data for female common dolphins (Figure 1; angles between vectors of $<90^{o}$ imply a positive association). There is also clustering of the compounds into three broad groups: (1) the HBCDs (Hexabromocycloodecanes), (2) PBDEs (polybrominated diphenyl ethers), HCB (Hexachlorobenzene) and PeCBz (Pentachlorobenzene), and (3) PCBs (polychlorinated biphenyl congeners) and DDE.

Results for summed PCB concentrations are summarised in Table 1. Highlighted values in the table show where the threshold for effects on reproduction is most frequently exceeded. In females, porpoises in the Netherlands (74% above critical) and common dolphins in France (50%) appear to be most at risk, although where data are available for males, values are also very high, e.g. for common dolphins in Galicia (85%). The lowest PCB concentrations were generally seen in Ireland. There was no consistent difference in concentrations between porpoises and common dolphins but the highest value of all (almost 15 times the threshold) was seen in a bottlenose dolphin from Scotland. In prey samples, the highest PCB concentrations were recorded in fish from the Netherlands.

Geographical patterns of PBDEs and HBCD differed from those of PCBs: highest PBDE concentrations were seen in common dolphins in Ireland, and harbour porpoises in Scotland and France. Concentrations were usually higher in porpoises than in common dolphins although, as for PCBs, the highest concentrations were seen in bottlenose dolphins from Scotland. Highest concentrations of HBCD were seen in Ireland, where they were around three times higher in porpoises than in common dolphins (Table 1).

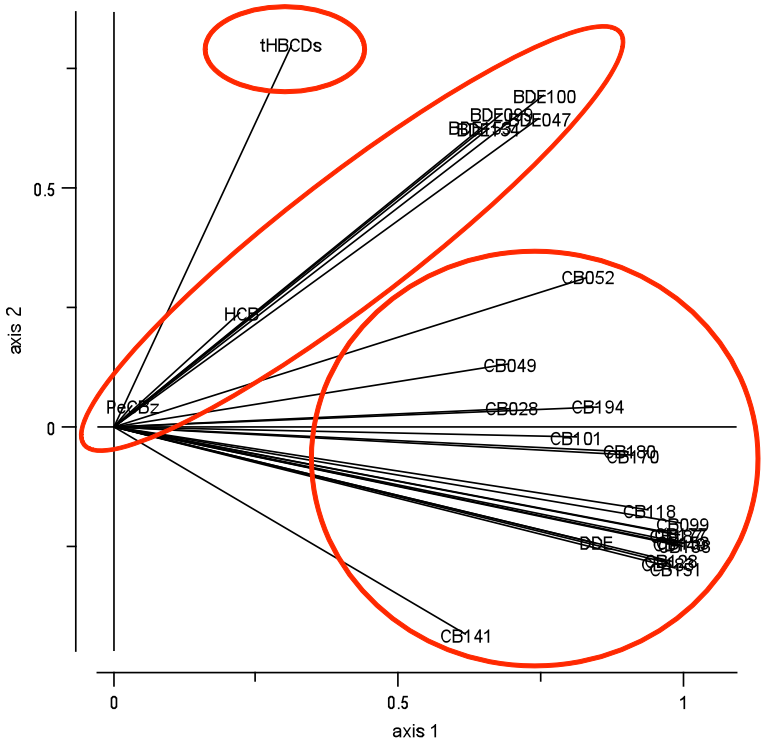


Figure 1. PCA results for POPs in female common dolphins (N=60)

Further analysis of PCB concentrations in female common dolphins based on RDA and GAMs highlighted regional differences (high values in France), a dietary effect (as shown by a strong association with the fatty acid profile), an effect of maturity (values fall, presumably due to transfer of PCBs to calves during pregnancy and lactation), as well as seasonal differences. Regional differences were also confirmed for female harbour porpoises, in which there was also a weakly significant association between blubber PCB concentration and zinc concentration in the liver, a possible indication of poor health; see Pierce *et al.* (2008) for full details. There was no clear relationship between PCB concentration and age. POP data were available for four mother-foetus pairs from Ireland, one common dolphin, two porpoises and a pygmy sperm whale. In the common dolphin, a 50 cm foetus already had higher PCB and PBDE concentrations in the blubber than did the mother, while in all the other pairs, concentrations were considerably higher in the mother (Table 2).

Concentrations of mercury in the liver and cadmium in the kidney are summarised in Table 3. Both geographical and species differences are apparent but age is a confounding factor, since both metals bio-accumulate with age (Figure 2). Nevertheless, in common dolphins, it is clear that levels of cadmium and mercury were generally low in Galicia and cadmium bioaccumulation was highest in Ireland. In harbour porpoises, the highest mercury levels were recorded in the Netherlands and the highest cadmium levels in Scotland. The Galician sample consisted of three very young animals. Comparing across species, cadmium is highest in striped dolphin and common dolphin, presumably reflecting the relatively high importance of cephalopods in their diets. Bottlenose dolphins seem to have the highest mercury levels.

Table 1. PBDE, PCB and HBCD concentrations in blubber (ng/g lipid) of small cetaceans, based on summed values for the most important congeners (mean and standard deviation). Sample sizes are recorded separately for PBDEs (N), PCBs (N1) and HBCD (N2). For PCBs, the table also gives the Aroclor 1254-equivalent value (3 times the sum of the "ICES 7" values) and the percentage of individuals with blubber PCB concentrations above the threshold of 17,000 ng/g lipid at which effects on reproduction are predicted (%>T). High average concentrations are highlighted in yellow. "Netherlands" samples include some Belgian animals.

			Sum5 PBDEs			Sum 18 PCBs		3x ICES7 PCBs				t HBCDs	
Country	**Sex**	**N**	**Mean**	**StDev**	**N1**	**Mean**	**StDev**	**Mean**	**StDev**	**%>T**	**N2**	**Mean**	**StDev**
Common dolphin													
Ireland	M	2	653	756	2	4447	4796	8233	9124	0	0	*	*
France	M	1	501	*	1	17232	*	29314	*	100	1	201	*
Galicia	M	13	480	250	13	25408	25972	45065	45298	85	13	236	131
Ireland	F	11	758	505	11	3649	3394	6919	6404	9	7	1086	1137
France	F	36	612	413	36	13692	12721	24644	22938	50	31	433	210
Galicia	F	23	422	182	23	10955	11563	19875	20797	39	23	185	101
Harbour porpoise													
Scotland	M	19	1426	1758	20	10318	10120	20811	19366	50	0	*	*
Ireland	M	4	1000	920	4	6507	5617	14154	12363	50	3	3519	4147
Netherlands	M	5	807	346	5	45145	33895	90320	67426	100	5	1290	577
Scotland	F	31	1369	1352	31	10525	13152	20320	25243	39	20	2236	2562
Ireland	F	12	656	492	12	5347	4750	10492	9451	25	7	2961	2716
Netherlands	F	19	1056	803	19	15021	8574	30599	17794	74	12	1080	354
France	F	2	1398	939	2	13809	10582	27601	20872	50	2	1533	1101
Galicia	F	3	284	43	3	5306	4199	10266	7972	33	3	121	37
Bottlenose dolphin													
Scotland	Both	2	4144	2989	2	75651	82263	149843	161574	100	0	*	*
Ireland	F	3	1142	901	3	10549	13862	19923	26167	33	3	914	656
Striped dolphin													
Ireland	F	3	628	454	3	5815	4918	11183	9263	33	3	325	193
France	Both	2	397	46	2	12514	1032	21480	1412	100	2	112	5
Galicia	Both	3	350	219	3	10917	10314	19709	19187	33	3	221	127

Table 2. POPs data (blubber concentrations in ng/g lipid) from mother-foetus pairs in Ireland

Species	**ID**	**Who**	**Length cm**	**% lipid**	**PBDEs**	**PCBs**
Common	151	Mother	185.5	82.00%	106.9	960.0
dolphin	152	Foetus	50.5	13.66%	117.6	1055.2
Harbour	224	Mother	171.0	88.32%	353.5	3795.8
porpoise	225	Foetus	63.5	54.10%	171.4	1790.2
Harbour	226	Mother	150.6	86.17%	609.1	2558.6
porpoise	227	Foetus	40.0	29.64%	421.9	1773.2
Pygmy	243	Mother	305.0	56.23%	96.2	2852.6
sperm whale	244	Foetus	99.5	51.85%	40.6	1375.0

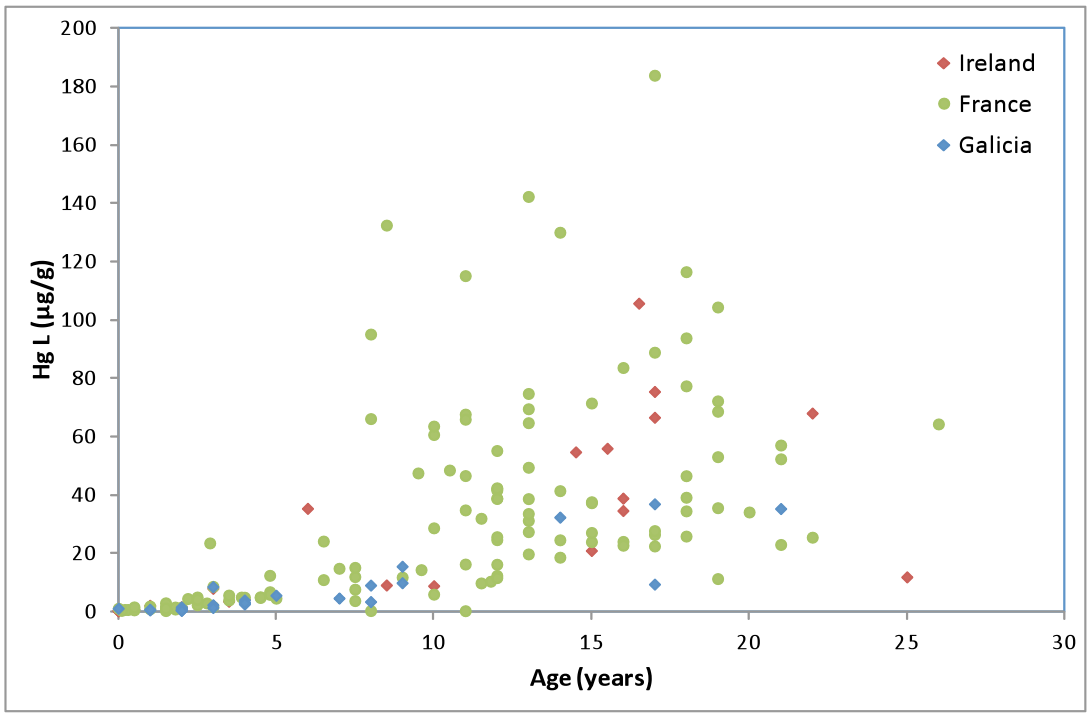
Across the four main species, 21 individuals (7% of the sampled animals) showed concentrations of hepatic Hg higher than 100 µg/g w.wt, while 53 animals had concentrations above 60 µg/g w.wt. These individuals comprised mainly common

dolphins from the Western English Channel and Bay of Biscay, bottlenose dolphins from the Bay of Biscay,and striped dolphins from the Bay of Biscay and Galicia. However, the highest value of all was recorded in a bottlenose dolphin from Scotland (480 µg/g w.wt). Concerning cadmium in the kidney, the maximum level recorded (30 µg/g w.wt in a striped dolphin) was lower than the level known to produce toxic effects (50 µg/g w.wt, Dietz *et al.*, 1998).

Table 3. Toxic element concentrations in small cetaceans: mercury in liver and cadmium in kidney (sample size, mean, standard deviation). High average concentrations are highlighted. "Netherlands" samples include some Belgian animals.

		Hg L (µg/g)			Cd K (µg/g)	
Country	**N1**	**Mean**	**StDev**	**N2**	**Mean**	**StDev**
Common dolphin						
Ireland	31	26.41	29.80	29	9.11	9.06
France	143	28.27	34.32	144	3.92	5.28
Galicia	31	8.19	12.93	31	1.58	1.69
Harbour porpoise						
Scotland	37	14.84	28.57	26	2.36	2.79
Ireland	22	14.14	20.78	26	0.81	0.73
Netherlands	19	28.78	35.24	19	0.92	0.88
France	21	20.49	24.32	20	1.01	1.26
Galicia	3	1.20	0.33	2	0.09	0.08
Bottlenose dolphin						
Scotland	2	303.50	249.61	0	*	*
Ireland	2	95.74	30.08	2	1.71	2.06
France	17	60.24	88.70	16	0.61	0.62
Galicia	4	42.78	24.94	4	2.97	1.57
Striped dolphin						
Ireland	3	51.09	74.40	2	3.86	2.38
France	17	32.51	58.86	17	9.47	8.35
Galicia	6	60.91	69.66	7	15.44	8.48

a



b

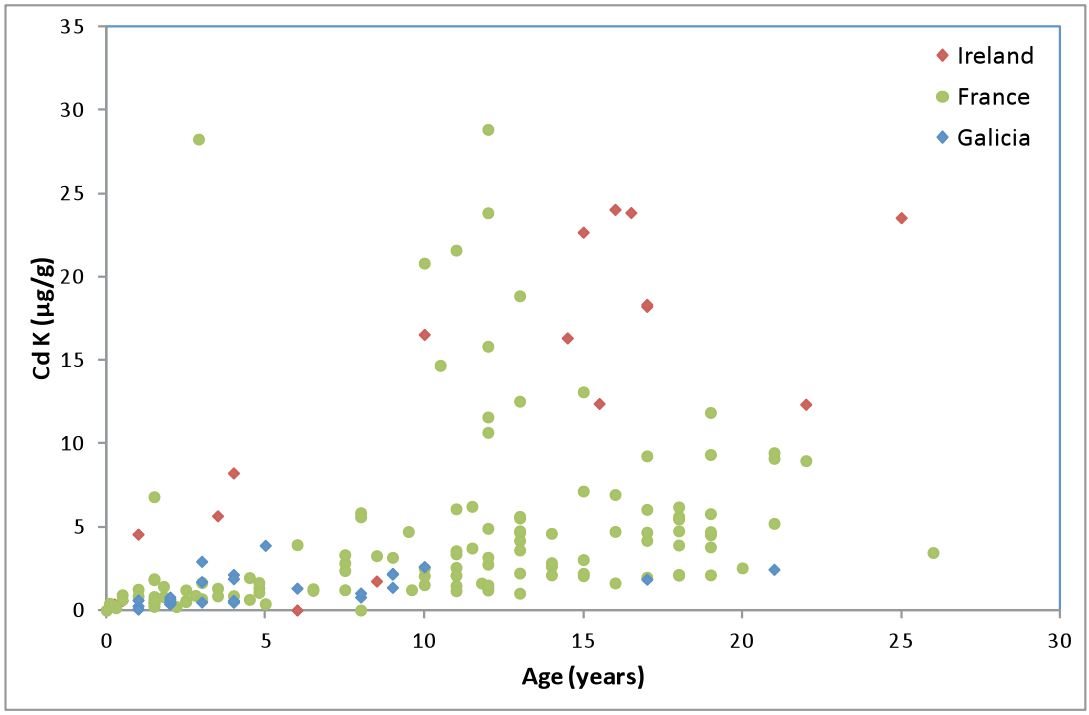


Figure 2. Mercury (liver) and cadmium (kidney) concentrations in common dolphins as a function of age and country of origin

DISCUSSION

Substantial geographic variation was recorded in POP concentrations in small cetacean blubber. In most areas, some females had blubber PCB concentrations above the threshold at which effects on reproduction could be expected, notably 74% of female porpoises sampled from the southern North Sea, where high levels of PCBs are also recorded from the prey, but also in 50% of female common dolphins from France. As expected, higher concentrations were recorded in males, which have no opportunity to offload POPs to their offspring via pregnancy and lactation, and the highest concentration of all was seen in a male bottlenose dolphin in Scotland. It is not clear whether males are affected by PCBs in the same way as females but there is no reason to assume they would be unaffected. Very high concentrations of PCBs in bottlenose dolphins may be a particular concern.

Given the reported link between PCB bioaccumulation and ill-health in porpoise (Jepson *et al.*, 2005), and the obvious association between ill health and death, it is important to note that the average PCB levels in blubber of stranded animals may be unrepresentative of the living population. More representative data on contaminant levels can be derived from animals whose cause of death was independent of health state, in particular those dying from physical trauma (e.g. fishery by-catch) or live stranded. Careful interpretation is still needed as by-catch mortalities may for example be recorded disproportionately among younger animals. In general, in the BIOCET study, trauma deaths seemed to be more common among stranded common dolphins and less so in harbour porpoises (except in Scotland, where bottlenose dolphin attacks are a major cause of porpoise mortality) (Pierce *et al.*, 2008).

While high PCB concentrations in porpoises are associated with low estimated pregnancy rates (see Pierce *et al.*, 2008), again there is likely to be a bias in estimates due to the high proportion of ill-health related deaths among strandings, and some caution is therefore needed in extrapolating from these results. Nevertheless a high incidence of potentially harmful concentrations of PCBs in the blubber of all the studied species during the study period (2001-03) seems to be undeniable. More recent analysis suggests that there has been no discernible temporal trend in PCB concentrations in blubber of stranded porpoises in the UK, at least since the mid-1990s and that further efforts to limit or eliminate PCB discharges to the marine environment are still needed (Law et al., 2010a; Law & Barry, this volume).

The geographical distribution of other persistent organic pollutants differs from that of PCBs. Thus, it can be concluded that the PBDEs occur more in the seas around the UK than in seas to the south of it. The largest regional differences were found for the brominated flame retardant HBCD, with highest concentrations recorded in cetaceans from Ireland.

Due to the co-occurrence of different types of POPs in cetacean tissues it can be difficult to isolate the effects of each one when making inferences from analysis of stranded animals. Beineke *et al.* (2005) highlighted the link between PCBs, PBDEs and immunosuppression, finding that high concentrations of PCBs and PBDEs in porpoises

were associated with thymic atrophy and splenic depletion. Recent studies on belugas suggest that PBDEs have a negative influence on thyroid hormone levels (Villanger *et al.*, 2011). Some controls have been placed on the production and use of PBDE in the European Union (e.g. under Directive 2002/95/EC, implemented in 2006). Penta- and octa-PBDE are banned but restricted use of deca-PBDE continues (the European Court of Justice banned its use in electrical and electronic goods from July 2008) (Kemmlein *et al.*, 2009). There is evidence that concentrations of PBDEs in harbour porpoises from both the UK and the (more polluted) southern North Sea have declined since the late 1990s, although there is wide between-individual variation in concentrations (Law *et al*., 2010b; Weijs *et al.*, 2012).

HBCD has attracted increasing attention since the first studies on their appearance in aquatic food chains in the mid-2000s (Morris *et al.*, 2004; Zegers *et al.*, 2005). HBCD concentrations in harbour porpoises in the UK increased sharply after 2001 but decreased again after 2003, the latter decline perhaps relating to closure of a manufacturing plant in NE England (Law *et al.*, 2006, 2008). The effects of HBCD in marine mammals appear to remain unknown. Indeed Arnot *et al.* (2009) concluded that "evaluations do not support the classification of HBCD as a POP since there are no indications that significant adverse effects are likely to occur in organisms living in remote areas distant from known point-source emissions of HBCD". However, laboratory studies have shown that HBCD is capable of producing adverse effects in a variety of organisms, including disturbances in thyroid hormone system and effects on the thyroid in rats (U.S. Environmental Protection Agency, 2010).

Toxic element levels were determined in the four most abundant small cetacean species of European waters from five regions, which provided an important contribution to knowledge of both impregnation levels and factors influencing variations of concentrations.

Thus, impregnation levels of mercury appeared not to be particularly high, although 21 individuals had concentrations of hepatic Hg above the threshold for toxic effects (Wagemann & Muir, 1984). Concerning cadmium in kidney, levels were not a matter of great concern since the maximal level obtained in striped dolphins (30 µg/g w.wt) was lower than the level known to produce toxic effect - 50 µg/g w.wt (Dietz *et al.*, 1998).

Whatever the metal or compound, age was the most important factor accounting for variations in toxic element concentrations within groups, which agreed with previous studies (reviewed by Aguilar *et al.*, 1999).

Diet appeared to be the main factor explaining interspecific variation in cadmium levels. Indeed, the main difference occurring in the diet of the four species considered was the proportion of cephalopods consumed. Given that cephalopods exhibit high levels of cadmium in a biologically available form (Bustamante *et al.*, 2002), it is not surprising that the teuthophagous striped dolphins also displayed the highest cadmium concentrations.

ACKNOWLEDGEMENTS

The results presented here derive from data collected during the EC-funded BIOCET project (*Bioaccumulation of persistent organic pollutants in small cetaceans in European waters: transport pathways and impact on reproduction*, EVK3-CT-2000-00027. As such they represent the work of a large team of project members and collaborators, including: Begoña Santos, Jennifer Learmonth, Jamie Banks, Sarah Bannon, Fiona Read, Gabrielle Stowasser, Jianjun Wang, Lindsay Brown, Bill Edwards (University of Aberdeen, Aberdeen, UK); Emer Rogan, Sinead Murphy (University College, Cork, Ireland); Vincent Ridoux, Paco Bustamante, Virginie Lahaye, Willy Dabin, Olivier Van Canneyt, Laureline Meynier, Jérôme Spitz, Ghislain Dorémus, Cecile Vincent (University of La Rochelle / CRMM, France); Marjan Addink, Chris Smeenk, Manuel García-Hartmann (National Museum of Natural History, Leiden, The Netherlands); Jan Boon, Bart Zegers, Anchelique Mets (Netherlands Institute of Sea Research, The Netherlands); Angel González, Angel Guerra, Maria Teresa Fernandez, Camino Gestal (Instituto de Investigaciones Marinas, Vigo, Spain); Alain Zuur (Highland Statistics, UK); Colin Moffat (FRS Marine Laboratory, Aberdeen, UK); Christina Lockyer (Age Dynamics, Denmark); Bob Reid, Tony Patterson (SAC Veterinary Science Division, UK); Alfredo López, Josep Alonso (CEMMA, Spain) and other members of CEMMA; members of the Irish Whale and Dolphin Group; Thierry Jauniaux, Jean-Marie Bouquegneau (University of Liège, Belgium); Claude Joiris, Ludo Holsbeek (Free University of Belgium); and Paul Jepson (Institute of Zoology, London, UK).

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A BRIEF REVIEW ON THE CHEMICAL POLLUTION IN THE BLACK SEA CETACEANS

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INTRODUCTION

The Black Sea is the world's most isolated sea from the major oceans and the largest anoxic body on the planet (87% of its volume is anoxic). It is surrounded by only six countries, i.e. Bulgaria, Georgia, Romania, Russia, Turkey, and Ukraine, but its catchment area is over 2 million km^2, covering 22 countries (Figure 1). Freshwater, nutrients as well as pollutants are transported to the sea by various rivers, such as the Danube, Dniestr, Dniepr, Don, Coruh, Kizilirmak, and Yesilirmak.

Figure1. The Black Sea and its catchment area. (Paleari *et al.*, 2005)

There are three cetacean species found in the Black Sea. Those are common dolphin *Delpninus delphis ponticus*, bottlenose dolphin *Tursiops truncates ponticus*, and harbour porpoise *Phocoena phocoena relicta*. The Black Sea bottlenose dolphin and harbour porpoise are currently recognised as Endangered, while the Black Sea common dolphin is recognised as Vulnerable in IUCN Red List of Threatened Species (IUCN, 2013).

All three species had been hunted for centuries in the Black Sea until the fishery was finally banned in Turkey in 1983. Although the direct catch is not a serious problem

anymore in the region, except for some live capture of bottlenose dolphins, there are other threats for these cetaceans. Notarbartolo di Sciara and Birkun (2010) summarised them as prey depletion due to overfishing or ecological catastrophe, i.e. the outbreak of the alien ctenophore *Mnemiopsis leidyi,* (Zaitsev and Ozturk 2001), habitat degradation due to various kinds of pollution, epizootics, and by-catch in bottom set gillnets.

Chemical pollution is one of the reasons for habitat degradation and it directly affects cetaceans through the food intake and bioaccumulation. There have not been many studies on chemical pollution of the Black Sea cetaceans, although the potential threat is considerable, considering the fact that various rivers flow into the Black Sea through the eastern European countries and ex-Soviet Union countries. This paper reviews studies published in the last two decades, to understand the present status of the Black Sea cetaceans in terms of chemical pollution.

Organochlorine and organobromine compounds

Very high levels of pesticides (DDTs and HCHs) were recorded in bottom sediments sampled between 1995 and 2005 in some areas in the Black Sea (BSC, 2008). High concentration of trace metals were also recorded in the sediments sampled in 1995-2007 in Ukraine, Russia, Georgia, and Romania (BSC, 2008). Illegal dumping or discharge of chemicals was recognised as a particular transboundary problem, which makes it very difficult to reduce the amount of these chemicals in the water column.

In the 1990s, some studies were made on such contamination in Black Sea cetaceans. Tanabe *et al.* (1997a) reported various levels of organochlorine contamination in the blubber of harbour porpoises collected in 1993 on the Turkish coast of the Black Sea; the concentrations of DDTs (8.3–180 μg g^{-1} wet weight) were the highest, followed by PCBs (1.6–39 μg g^{-1}), HCHs (1.5–17 μg g^{-1}), CHLs (0.11 – 2.4, μg g^{-1}), and HCB (0.057 – 0.61 μg g^{-1}). They also suggested that relatively higher concentrations of *p,p'*-DDD were due to the poor condition of the Black Sea resulting from organic waste pollution. They concluded that contamination by DDTs and HCHs in the Black Sea harbour porpoises was at a relatively high level in a worldwide comparison of organochlorine residues of the same species.

Using the same samples, Tanabe *et al.* (1997b) determined the highly toxic coplanar, PCBs, and other isomers in harbour porpoises and fish (porpoise diet), suggesting long-term accumulation and possible toxic effects of PCBs in this species.

Since these studies, however, few papers have been published on organochlorine contamination. Malakhova and Ostapchuk (2004) reported organochlorine levels in two harbour porpoises and one bottlenose dolphin. They found residual amounts of such chloropesticides as isomers of hexachlorcyclohexane, DDT – p,p'-DDD and p,p'-DDE, and also polychlorinated biphenyls. The concentrations of the sum total of α- and γ-isomers of HCCH varied from 2 to 150 μg/kg fresh weight. No DDT pesticide was found in the samples. However, its metabolites p,p'- DDD and p,p'-DDE were found in large amounts (from 4 to 245 μ/kg of fresh weight) which were still lower than the contamination level in Risso's dolphins *Grampus griseus*, of the

Mediterranean (Storelli & Marcotrigiano, 2000). The absence of DDT and the high content of its metabolites indicate the "old" pollution of the organs of dolphins by the given pesticide.

The contamination by organobromine compounds was only recently reported by Weijs *et al.* (2010). They studied the contamination of polybrominated diphenyl ethers (PBDEs) and naturally-produced compounds, methoxylated PBDEs (MeO-PBDEs) and polybrominated hexahydroxanthene derivatives (PBHDs), in male Black Sea harbour porpoises. 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.

Perfluorochemical surfactants

Perfluorooctanesulfonic acid or perfluorooctane sulfonate (PFOS) is a man-made fluorosurfactant used on fabrics and paper products. Although it is globally distributed, environmentally persistent, bioaccumulative and potentially harmful, its distribution and effect are yet to be investigated widely (Giesy & Kannan, 2002). In Black Sea cetaceans, only one study is known so far. Van de Vijver *et al.* (2007) determined the PFOS and other perfluorinated alkylated substances (PFAS) in 31 harbour porpoises stranded along the Ukrainian coast of the Black Sea, and concluded that the contamination by PFOS in Black Sea harbour porpoises is comparable to levels found in porpoises from the German Baltic Sea and from coastal areas near Denmark and, therefore, might pose a threat to this population.

Organometals

Butyltins, such as tributyltin (TBT), a representative group of organotin compounds, have been used in antifouling paints on ship hulls and fishing nets, known to cause various abnormalities in marine invertebrates. Madhusree *et al.* (1997) measured the concentrations of butyltin compounds in Black Sea harbour porpoises. Referring to that study, Tanabe (1999) reviewed the contamination of butyltin compounds in the liver of cetaceans from various regions of the world. In terms of the butyltin concentration, the Black Sea harbour porpoises are placed between the cetaceans from the industrially developed countries and those from the developing countries. However, this study was carried out on samples taken in 1993, and the regulations on TBTs have been changed both in developed and developing countries, which may result in a totally different order of the cetaceans from various water bodies.

Joiris *et al.* (2001) reported total Hg and organic Hg (MeHg) mercury concentrations in harbour porpoises in the Black Sea between 1997 and 1999, mainly bycaught in fishing nets (n=79). In older adults, liver concentrations reached 35µg g^{-1} dry weight (`ppm') total Hg and 3µ g g^{-1} dw MeHg. A geographical comparison with other regions showed a generally low Hg contamination of Black Sea porpoises, one order of magnitude lower than, e.g. in the North Sea.

CONCLUSIONS

Very few studies have been carried out during the last two decades on chemical pollution within Black Sea cetaceans. The contamination in other media and organisms in the Black Sea, however, has been demonstrated regularly and widely. The difficulty in sampling cetaceans and the low interest in these animals as an indicator of the health status of the Black Sea is one of the reasons for this gap.

The use of such chemicals may have decreased but not totally stopped in some riparian countries. Other anthropogenic factors may act synergistically with chemical pollution to make an adverse negative effect on cetaceans in this highly polluted sea. In order to understand the potential threat level of such pollutants in Black Sea cetaceans, it is necessary to carry out more systematic research. Moreover, international collaboration should be continued where it already exists and promoted where it has been rather scarce. Collection of specimens needs to be more organised, using stranding networks, following internationally accepted protocols.

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CONCLUSIONS

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The following recommendations were made by the workshop for priority areas of research:

1) *Research on understudied contaminants or those of particular concern*
• PCBs, brominated flame retardants (BFRs) and perfluorinated compounds (PFCs) are priority compounds for further research
• Trace elements also require study (e.g. for understanding detoxification processes in methyl-mercury and cadmium)
• All chemicals found at high concentrations in estuaries & coastal waters should be considered for study offshore (in accordance with EU Water Framework Directive assessments)

2) *Research on effects at individual level*
• Risk assessment, including predictive modelling should be undertaken
• *In vitro* tests should be conducted to explore effects of emerging contaminants
• Diagnostic biomarkers should be further developed

3) *Research on effects at population level*
• Risk assessment, including predictive modelling
• Inshore and offshore populations or fragmented populations should each be studied and compared
• Transients and their pollutant levels should be examined
• Possible ecotypes should be compared
• Parallel ecological and dietary studies should be undertaken
• Dose-response curves (e.g. harbour porpoise) should be established
• Screening for tumours

4) *Research in geographic areas*
• Greater focus upon Baltic, Mediterranean, and Black Sea where pollutant levels have generally been higher than elsewhere
• Comparisons of high *vs* low exposure area studies (involving collaborative studies between countries)
• More detailed examination of the role of diet (seasonal and temporal variation)

5) *Research on particular species*
• Continue current monitoring programs (e.g. harbour porpoise, seals)
• Focus upon bottlenose dolphins throughout the region
• Harbour porpoise in the Baltic & Black Sea
• Orcas throughout the region (sample sizes remain very low)

• Common dolphins, long-finned pilot whales, and fin whales should be targeted in the Mediterranean
• Grey, harbour, ringed and monk seals throughout their range

6*) Studies using Biomarkers including gene expression analyses*
• Investigate hydrocarbon receptor binding affinity (focusing upon common dolphins in the Mediterranean, bottlenose dolphins, and orcas)
• Develop biomarkers on non-threatened species so that they can be applied later to other rarer species
• Develop diagnostic biomarkers
• Use immune histochemistry techniques
• Undertake stress/sex hormone assays

Biomarker studies of POPs in blood that are related to genotoxic (epigenetic) effects enable one to investigate if and to what extent toxic effects are actually occurring, and to what extent blood parameters are indicative of effects upon organisms. The reasoning for this approach is that many environmental compounds can turn genes on and off, and this can, for example, lead to endocrine and reproductive disruption and, ultimately, reduced population viability.

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