

EXPOSURE AND EFFECT ASSESSMENT OF PERFLUORINATED ALKYLATED SUBSTANCES IN MARINE AND ESTUARINE ORGANISMS

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Since several decades, interest has grown by scientists as well as policy makers, that environmental pollution threatens the biological structure and function of estuarine and coastline ecosystems. One of the most crucial and urgent aspects of the environmental monitoring policy is to unravel 'new chemicals' and to document the risks caused by these new substances, which often circulate already for decades in the environment. Persistent chemicals, like the halogenated organochemicals, have been studied very intensely, however, within this important group of substances, perfluorinated alkylated substances (PFAS) have escaped to the attention of environmental toxicologists.

Nevertheless, PFAS such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), as well as their related products, are being produced over 50 years and have a broad application spectrum in industry and households. These compounds are stable and chemically inert and therefore, always considered as being safe for humans and environment. The presence of fluorine in human blood has been reported for the first time in 1968. However, it took several decades before other research studies reported on the occurrence of alarmingly high levels of PFAS in the aquatic and terrestrial environment. In addition, they tend to accumulate along the food chain. Among the effects known are induction of peroxisomal β -oxidation, induction of microsomal liver carboxylesterase, membrane-related effects, and developmental problems in Rodentia and fish.

Nowadays, public concerns about the persistence and the toxicological effects of PFAS have increased. However, a detailed quantitatively description of their distribution in the European aquatic environment is lacking.

This doctoral thesis tried to give a better insight in the following research questions:

- 1) what are the actual concentrations of perfluorinated alkylated substances occurring in biota from the European estuarine and marine environment and is there a difference in accumulation pattern between various tissues,
- 2) is there an influence of trophic position and/or feeding ecology on the levels of perfluorinated compounds in marine mammals, and
- 3) what are the biochemical and molecular effects of water exposure to PFOS?

We focused mainly on the European marine and estuarine environment, namely the southern North Sea and the Western Scheldt Estuary, the northern North Sea and Baltic area and the Black Sea.

With the exception of Chapter 2 in which invertebrates [starfish (*Asterias rubens*), crab (*Carcinus maenas*) and shrimp (*Crangon crangon*)] were used, and Chapter 7 in which the European sea bass (*Dicentrarchus labrax*) was used, all chapters studied marine mammals, because they are situated at the higher levels of the food chain and will therefore most probably suffer most from these new types of pollutants. PFAS levels have been quantified using high performance liquid chromatography combined with electrospray tandem mass spectrometry (HPLC-MS/MS).

The first two questions addressed in this study were answered in Chapters 2 to 6. Results of a biomonitoring study performed in the Western Scheldt Estuary and the Southern North Sea, showed concentrations on a wet weight basis in soft tissues of shrimp, crab and starfish that ranged from 19 to 520 ng.g⁻¹, from 24 to 877 ng.g⁻¹ and from 9 to 176 ng.g⁻¹, respectively. These concentrations were higher compared to invertebrate studies using bivalves and studies determining PFOS in shrimp and zooplankton from the Canadian Arctic. Furthermore, the study results demonstrated a clear PFOS pollution gradient along the Western Scheldt, with higher levels in crab and shrimp near Antwerp (Chapter 2). This might be explained by the presence of a major fluorochemical manufacturing facility near the harbour of Antwerp, and the large domestic and industrial wastewater discharges further upstream.

The highest individual PFOS concentrations were determined in tracheo-bronchial muscle from a harbour seal stranded along the Dutch coast of the Wadden Sea (2724 ng.g⁻¹ wet wt; Chapter 5) and in liver tissue of a harbour porpoise from the Ukrainian Black Sea (1790 ng.g⁻¹ wet wt; Chapter 6). The levels measured in marine mammals from the North Sea and adjacent areas are comparable to PFOS concentrations found in marine mammals from all over the world. Concentration differences are mainly due to spatial and temporal variation (e.g. the proximity of possible pollution sources) and to inter-species differences in uptake rate, metabolism and excretion potential.

The same can be concluded concerning the different tissue patterns. In Chapter 5 and 6, tissue distribution of perfluorinated compounds in seals and porpoises was investigated. The difference in tissue concentrations might be a consequence of spatial (Wadden Sea versus Black Sea) and inter-species variation. Both species share some of their prey species, but differ in trophic position and metabolism of organic pollutants, which might explain the difference in toxic levels.

The influence of gender and age was investigated if sample size allowed the comparison between respectively male and females, and juveniles and adults. Only in a few cases, significant differences ($p < 0.05$) were obtained. The lack of clear age or sex-related differences in concentrations of PFOS is a general observation in all biomonitoring surveys. Apparently, PFAS have a very different accumulation pattern than other persistent organic pollutants which decrease with age in female organisms because of the transfer to their young. Although there is no observation that adult females have lower PFAS levels like with DDTs and PCBs, there might be another transfer from mother to offspring as foetuses from different species showed much higher PFOS concentrations than their mothers (Chapter 4). However, this potential transfer still has to be determined.

Concentrations of other perfluorinated compounds like the perfluorinated carboxylates (PFCAs) were fairly low in all species and all tissues analysed. In general, PFCAs were present at detectable concentrations in those samples with the highest PFOS concentrations. Concentrations generally decreased for all PFCA homologues with increasing chain length. A remarkable difference between harbour porpoises and seals was that the dominant PFCAs in all porpoise samples were PFDA and PFUA, and PFNA could only be detected in 41% of the samples. In the seal samples, however, PFNA was the major compound, followed by PFDA. Probably there is a difference in metabolism between the two species, although no clear explanation can be given.

Only in spleen tissue from harbour seals, perfluorobutane sulfonate (PFBS) could be detected at concentrations ranging from 1.74 to 3.28 ng.g⁻¹ ww. Until now, studies on PFBS, showed that PFBS did not bioconcentrate in organisms to levels greater than the concentrations to which they were exposed and that this compound was rapidly cleared from the body, with an elimination half-life of hours in rats and a few days in primates. So far, no clear explanation can be given to the fact that PFBS was detected, although at very low concentrations, in spleen tissue and no one knows what the possible consequences might be for the animal.

In order to provide an answer to the second question, we have considered that trophic position and feeding ecology might be important factors with which we could explain some of the variation in concentrations of perfluorinated compounds seen in marine mammals. Therefore, we have tried to link the obtained PFAS concentrations to stable nitrogen (¹⁵N/¹⁴N) and carbon (¹³C/¹²C) isotope ratios. The use of stable isotope ratios has evolved to a powerful tool to study element cycles and various biological, physical and chemical processes in different research areas. In marine mammals, this technique has been used to study migrations, trophic ecology and pollution. Briefly, the higher the trophic position of an animal the higher the $\delta^{15}\text{N}$ value will be. In addition, $\delta^{13}\text{C}$ values indicate if animals are offshore and/or pelagic feeders (low $\delta^{13}\text{C}$ values), or more inshore and/or benthic feeders (high $\delta^{13}\text{C}$ values).

Our results indicate that the ¹⁵N/¹⁴N ratio was strongly associated with PFOS concentrations in livers of marine mammals, with animals that displayed the highest trophic positions, namely harbour and grey seals and white-beaked dolphins, having the highest PFOS levels. Offshore feeders originating from the southern North Sea such as sperm whales, fin whales, striped dolphin and white-sided dolphin showed low ¹³C/¹²C ratios and were also having lower PFOS concentrations than inshore species (Chapter 3). PFOS levels up to 50 ng.g⁻¹ wet wt in sperm whales which feed mainly on abyssal cephalopods, but also on bottom-dwelling organisms, suggest that fluorinated organic compounds have not only reached remote Arctic regions, but also deeper water layers.

Stable isotope ratios were also applied in Chapter 4 where the significant difference in mean $\delta^{13}\text{C}$ values between two distinct Norwegian populations of harbour porpoises could also be detected in the PFOS concentrations. Apparently, porpoises feeding in more inshore areas, were having higher hepatic PFOS levels. In addition to this last observation, shrimp, starfish and crabs collected at marine locations closer to the Belgian shore had higher PFOS levels than those collected at open sea locations (Chapter 2). Discharges of PFOS from the coastal zone into the aquatic environment

might be the reason why concentrations near the coast are slightly higher than further into sea.

In Chapter 7, we tried to characterize the toxicological mechanism of PFOS in the European sea bass, *Dicentrarchus labrax* (Moronidae) using selected biochemical endpoints (e.g. serum alanine aminotransferase activity (ALT), serum electrolyte levels and hematocrit) and molecular responses (using cDNA microarrays). For the latter purpose, a recently established technique for differential gene expression, Suppression Subtractive Hybridization-Polymerase Chain Reaction (SSH-PCR), has been used in combination with cDNA microarrays.

Juvenile fish were exposed to four different PFOS concentrations (0.1; 1; 10 and 100 mg.l⁻¹) via water and after 14 days effects were assessed in liver and serum. It was remarkable that within a rather short space of time there was already a very high and rapid accumulation, even at the lowest exposure doses. The liver PFOS concentration was positively and significantly related to both ALT activity and total serum protein content, and significant negative relations were found with the serum Na⁺ and K⁺ concentrations and the hematocrit values.

Multiple gene expression profiles were examined and we were able to isolate several genes that were up or down regulated after a short-term exposure. A total of 195 cDNAs were differentially expressed in liver tissue of sea bass after exposure to PFOS. Although DNA sequence data are poorly represented in the public data base for non-model species, 28 cDNAs could be identified by homology. Major functional classes of differentially expressed genes included oxidative stress related genes, genes involved in energy or iron/heme metabolism, genes which play a role in the immune system, translation and/or transcription processes and signal transduction. Interesting in our data was the influence on genes which are known to respond to organic pollutants and are involved in detoxification (28% of the genes). Eight of the identified clones corresponded to genes of the cytochrome P450 family (subfamilies 1, 2 and 3). The majority of these genes were up regulated after PFOS exposure. The induction of hepatic cytochrome P450 isomers can lead to DNA-adduct formation and carcinogenesis. The data obtained in this study identified several genes whose mRNA was regulated after PFOS exposure suggesting an array of hypotheses which could be tested to reveal the toxicological mode of action of perfluorinated chemicals.

At the end of this thesis we have generated a better insight into the hazard that is caused by perfluorinated chemicals for marine and estuarine organisms. This is crucial to ensure ecosystem health and to minimize the exposure towards these highly persistent and potentially hazardous chemicals. Results from biomonitoring and toxicological studies like the ones in this doctoral thesis might have additional value for producers and policy makers in order to set up some regulations. In 2000, 3M, one of the biggest producers of PFAS in the United States, has announced the phase out of the production of PFOS-based chemicals from December 2000 and to stop external sale of PFOA. New studies on the accumulation of PFOS and related chemicals in biota, should be performed in the near future to investigate if the phase-out has led indeed to a reduction of PFOS in the environment as was expected. In addition, due to the introduction of a Significant New Use Rule (SNUR), there is now the possibility for the government of the US to evaluate every new application in which PFAS are used.

To our knowledge, there are still no clear European or Belgian regulations on PFAS. For the moment, the OECD (Organisation for Economic Co-operation and Development) is trying to map the production and uses of PFAS in the entire European Union. Great Britain and Sweden have made some proposals for their own national legislation, but the European Commission has frozen these regulations as the European Commission is working on a draft concerning the production or use of PFAS within the framework of Directive 76/769/EEC. Other regulation processes within Europe are the nomination of PFOS under the UNECE POP LRTAP protocol (United Nations Economic Commission for Europe, Convention on Long-range Transboundary Air Pollution concerning persistent organic pollutants) and the UNEP (United Nations of Europe) POPs protocol of the Stockholm Convention.