

Contract no. 508967

PERFORCE

PERFLUORINATED ORGANIC COMPOUNDS
IN THE EUROPEAN ENVIRONMENT

FP6-NEST

INSIGHT

Specific Targeted Research Project

**PERFLUORINATED ORGANIC COMPOUNDS
IN THE EUROPEAN ENVIRONMENT
SCIENTIFIC REPORT**

Period covered: from 1 July 2004 to 30 June 2006

Date of preparation: 15 September 2006

Start date of project: 1 July 2004

Duration: 24 months

Project coordinator name: Dr. Pim de Voogt

Project coordinator organisation name:

Institute for Biodiversity and Ecosystem Dynamics,
Universiteit van Amsterdam

[draft]

Disclaimer

This report was prepared in good faith by the PERFORCE work-package leaders. At the time of writing not all results had been published in peer-reviewed journals, nor had a formal peer review by the work-package leaders taken place on each work item executed in the individual workpackages. Hence, once they are available, the peer reviewed papers should be consulted and used as the reference

Authors of the final report:

Pim de Voogt

Urs Berger

Wim de Coen

Watze de Wolf

Eldboerg Heimstad

Michael McLachlan

Stefan van Leeuwen

André van Roon.

Executive summary

The PERFORCE project started in July 2004 and covered a period of two years. Its principal aim was to make an exposure assessment of perfluorinated organic compounds (PFCs) in the European environment. Perfluorinated chemicals is a general term used to describe chemical substances which are largely comprised of or contain a perfluorinated or polyfluorinated carbon chain moiety such as $F(CF_2)_n-$ or $F(CF_2)_nC_2H_4-$. At the start of the project it was realised that Europe was lagging behind North America with respect to the collection of knowledge and data on these compounds. Several tools were considered necessary to fulfil the task indicated above. These included the development of chemical analytical and bioanalytical tools for identification and quantification of the compounds, the validation of these methods, and the collection of relevant physicochemical compound property data that would serve to understand their fate and to model and explain their behaviour in environmental compartments. The tools were then used in a Europe wide monitoring campaign that included sampling of surface waters, air, sediments, biota, and wastewater treatment plants. The campaign was aimed at identifying possible sources of PFCs, and establishing spatial and temporal trends in Europe.

The Perforce project selected some representative PFCs from the many that are manufactured or observed in the environment. The chemical analytical and quality assurance work packages showed that blank contamination is an item of paramount importance in the analysis. Analytical methods for four different matrices were developed and validated; these include water, sediment, air, and biota. For these matrices analytical protocols were developed that are deliverables of the project. The analytical methods developed showed good accuracies on the matrices included in the validation, demonstrating that these methods are fit-for-purpose. A worldwide interlaboratory study was organised using a fish tissue, fish liver extract and a water sample. The results revealed large variations in the between-laboratory results, showing that participating laboratories were not yet able to generate comparable results.

Specific bio-assays were developed that were able to quantify individual compounds (PFOA and PFOS) but were less promising when applied to extracts from environmental matrices due to cytotoxic side effects. Perfluoralkyl compounds show distinct toxicological modes of action *in vitro* that include estrogen-like, mitogen-like properties, membrane and DNA interference, and oxidative stress.

The physicochemical data collected in this work confirm that atmospheric transport may be important for certain PFCs, notably the fluorotelomer alcohols. In addition they showed that the two major representatives of the PFCs, viz. PFOA and PFOS, do not accumulate in sediments, and that sorption to sediment does not strongly affect water-mediated transport of these PFCs. Sediment is probably not a major sink for PFOS, PFOA and shorter chain homologues. Sorption does increase with carbon chain length, however, and thus becomes more important in the environmental fate of longer chain PFAS. Anaerobic and aerobic degradation of PFCs was tested and did not occur under the test conditions used.

The results of the sampling campaigns show that PFCs are ubiquitously present in the European environment. Sewage treatment plants probably serve as sources of PFAS

both for the aquatic ecosystems (through effluent discharges) and the terrestrial environment (through application of sewage sludge in agriculture). Levels of PFOS in sediments have increased from 1990 to 2005, whereas for PFOSA an initial increase was followed by a possible decreases after 2000.

The annual loading to the European environment of PFHxA, PFHpA, and PFOA from rivers is estimated to be of the order of 10, 2, and 20 tonnes. The Danube and Rhine watersheds are particularly important source regions, whereby the Elbe and Po also make a significant contribution for PFHxA and PFHpA/PFOA, respectively. In European air, PFOA was often the predominant PFC found in the particulate phase, while 6:2 FTOH and 8:2 FTOH were the prevailing analytes found in the gas phase. Many other compounds were also present in air.

Spatial differences were observed particularly in biota. PFOS and PFOSA concentrations were higher in North Sea cod liver than in cod liver from the Kattegat and the Baltic. In marine mammals concentrations of PFOS are higher in species feeding close to the shore or in estuaries than in off shore feeders. A relationship appears to exist between concentrations of PFOS and trophic levels in marine mammals. In these mammals perfluorinated carboxylic acids are relatively low in all species and tissues analysed. PFOS, PFDA and PFUnA bioaccumulate in a simple estuarine food chain, PFOA accumulates significantly less.

The PERFORCE study has obviously taken away some of the important knowledge gaps that existed several years ago with regard to the occurrence of the PFCs in the European environment. In particular the exposure levels in Europe are much better known, as a result of the project. Yet, it is obvious that further work is necessary to identify unknown origins, e.g. of PFHxA, to assess the fluxes to the environment from STPs, and to quantify loadings of river water and identify sources. There is also a need to improve our understanding of PFC transfer to as well as removal from the atmosphere, oceanic transport routes, and the mechanisms of bioaccumulation / biomagnification / bioelimination of PFC.

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1. INTRODUCTION

1.1 *Project objectives*

The PERFORCE project was initiated in July 2004 after a successful application to the NEST-INSIGHT instrument of the FP6 programme. PERFORCE addresses a newly emerging group of chemical contaminants which are likely to pose problems in the European environment, and for which a significant gap of knowledge exists. Because of very low levels of contamination and limited knowledge on the possible sources and pathways of perfluorinated organic compounds (PFCs), as well as on their key compound properties, at the stage of the project's initiation it was difficult to estimate the real risk of fluorinated compounds to man and the environment.

The project aimed to establish Europe as international scientific leader in environmental research and exposure assessment of PFCs. The lack of pertinent data and information on deviating properties of these newly emerging persistent hazardous substances presents urgent needs for elucidation on both the socio-economical and the scientific level. In addition, PFCs present interesting and challenging possibilities to gain new knowledge on environmental chemical mechanisms. By understanding their behaviour and distribution in the environment the sources and routes of PFCs detected in remote areas may be elucidated.

The major objective of the project was to introduce and evaluate new chemical and biological techniques and tools in order to assess the occurrence and distribution of PFCs in the European ecosystems. This exposure assessment will, together with ongoing hazard assessment (see e.g. ISEA 2003, UK Environment Agency 2006) and toxicity testing elsewhere enable a proper environmental risk assessment of PFCs to be made in the nearby future.

The project covered a full array of investigations required to correctly assess and model the fate and impact of a new series of anthropogenic compounds. The project objectives correspond to providing tools for developing a rapid assessment of new substances, which may lead to emerging risks or high importance to the European society. This assessment will enable a better understanding of the problem and can contribute to understand potential dangers.

This report summarizes the main findings, results, conclusions and recommendations of the project. The project has resulted in many scientific publications and contributions, as well as other dissemination deliverables. These products have been listed in the Annexes.

1.2 The consortium

Six partner institutes constituted the PERFORCE consortium. Their identities and roles are presented in Table 1-1. In the course of the project several interested parties applied for an associate membership of the PERFORCE project.

Representatives of the partners met at regular progress meetings, as well as informally at e.g., scientific conferences during the course of the project. In total, a kick-off meeting (Amsterdam, July 2004), four progress meetings (Tromsø, December 2004; Antwerp June 2005; Stockholm, December 2005; The Hague May 2006) and a report editorial meeting (Amsterdam, September 2006) were organized. In addition, three knowledge transfer workshops were organized in conjunction with progress meeting, in Tromsø, Antwerp and Stockholm, at which representatives from partner institutes as well as from associated members were participating.

Table 1.1. List of partners in the PERFORCE Consortium

Partic. Role*	Partic no.	Participant name	Participant short name	Country	Date enter project	Date exit project
CO	1	Universiteit van Amsterdam, Environmental and Toxicological Chemistry-IBED	UvA	Netherlands	1	24
CR	2	Norwegian Institute for Air Research, The Polar Environmental Centre	NILU	Norway	1	24
CR	3	Netherlands Institute for Fisheries Research	RIVO	Netherlands	1	24
CR	4	Institute for Applied Environmental Chemistry, Stockholm University	ITM	Sweden	1	24
CR	5	University of Antwerp-RUCA	UA	Belgium	1	24
CR	6	DuPont Coordination Center CVA	DuPont	Belgium	1	24
AM	-	Plastics Europe	-	Belgium		
AM	-	3M Company	-	Belgium		
AM	-	Lancaster University, Environmental Science	-	UK		
AM	-	Department of Environmental Chemistry, GKSS Research Centre	GKSS	Germany		

*CO = Coordinator, CR = Contractor, AM = Associated member

1.3 PFCs prioritised within PERFORCE

Several different PFCs were selected within the project as target compounds for the work packages. The selection criteria for these PFCs were:

- * At least 6 compounds should be selected.
- * Include PFOS and PFOA to allow comparison with previous work.
- * The compounds should be available (synthesis was not an option).
- * The compounds (the standards) should be of sufficient quality.
- * The selection depends on the compartments selected in the monitoring program, the questions we wish to answer (e.g. on sources, pathways, transformation, long range transport).
- * Detection with the same technique. This criterion was not used as a starting point in the selection, but was applied later.

A total of nine PFCs were thus selected:

Sulfonates (PFOS type):	C4	C8	6:2FTS (THPFOS)
Carboxylic acids (PFC _n A):	C4	C8	C9 C11
Telomer alcohols (FTOH):	6:2	8:2	(one of the two)
Other :	PFOSA		

Sulfonates: C4-PFS was included in the selection because it has been proposed as a substitute for PFOS. C8 (“PFOS”) is the most abundant PFC and was included to allow comparison with previous studies. The fluorotelomer sulfonate (6:2FTS, also known as THPFOS) is a representative of the telomer manufacturing process and has been found in the abiotic environment.

Carboxylic acids: The most relevant range to consider is from C4 to C14. C6, C8 and C9 are the three most important PFCA in the abiotic environment. C4-PFCA has been measured in biota. The source of C4-PFCA is unknown. It was decided to include a low, intermediate and high molecular weight (or chain length) PFCA. C13 was not available at the start of the project and C5 is not available within the consortium. C4-PFCA was selected therefore as the short chain representative. C8 and C9 were eventually selected to represent intermediate chain length congeners, and C11 was selected to represent the long chain PFCA. Longer chain PFCA (C14) may not pass all quality assurance requirements.

Telomer alcohols: Either 6:2 or 8:2 FTOH will be included. For these compounds analytical method development in WP1 is required. Based on optimisation experiences a final selection of the telomer to be included will be made.

Other compound: PFOSA has been observed in biota. It is very lipophilic, comparable to classical POP. It is not charged, similar to FTOH. PFOSA was also selected because its abundant use and because it has been shown to be a precursor of PF-sulfonates.

Linear and branched PFCs will behave differently in the environment. No standards are available to discriminate between the two types. Therefore, the project focuses on the sum of isomers.

The several work packages were not solely dedicated to the nine selected compounds. Whenever possible, in particular in the monitoring program, other related compounds were included in the work. In some cases, however, some of the selected compounds could not be included in (part of) a work package, e.g. due to particular analytical problems. Where this is the case, the pertinent chapter will address the problems in detail.

2. INTRODUCTION TO THE CHEMICALS

Perfluoroalkylated substances is a general term used to describe chemical substances which are largely comprised of or contain a perfluorinated or polyfluorinated carbon chain moiety such as $F(CF_2)_n-$ or $F(CF_2)_n-(C_2H_4)_n-$. Table 2-1 provides further information on terminology and definitions used.

Table 2-1: Terminology and Definitions

Fluorochemical	A general, non-specific, term used to describe broadly all chemicals containing the element fluorine; specifically, the term is used most commonly to describe small (1-8 carbon length) fluorinated molecules which are most often used for refrigeration, as fire suppression agents and as specialty solvents.
Fluorinated chemical	A general, non-specific, term used synonymously with “fluorochemical”
Fluorotelomer	A specific term used to describe an oligomer created by reaction of tetrafluoroethylene (TFE) with perfluoroethyl iodide CF_3CF_2I to produce $F(CF_2CF_2)_n-I$ [$n = 3-6$, avg. 4], a linear, even carbon number chain length oligomer; the term “telomer” is often used synonymously with fluorotelomer.
Fluoropolymer	A general term used to describe a polymer which has fluorine attached to the majority of carbon atoms which comprise the polymer chain backbone [common fluoropolymers are: polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF), fluorinated ethylene-propylene (FEP), etc.]; these are typically high molecular weight polymers used in high performance applications where chemical resistance and thermal stability are essential.
Fluorinated organic polymer	A general term used to describe a polymer which has a hydrocarbon backbone (polyamide, polyester, polyurethane, etc.) to which is appended a fluorinated carbon chain, also known as a fluorinated alkyl chain; an example would be a polymer such as $-[CH_2CH(C(O)OCH_2CH_2(CF_2)_8F)]_n-$
Perfluoro- / perfluorinated	Describes specifically a substance where all hydrogen atoms attached to carbon atoms are replaced with fluorine atoms – CF_n- where $n = 1 - 4$.
Perfluoroalkylated substance	A general term which describes a substance which bears a perfluorocarbon unit, also known as a perfluoroalkyl, functional group. $F(CF_2)_n-R$ where n is an integer and R is not a halogen, or hydrogen. Examples include $F(CF_2)_6CH_2CH_2OH$, $F(CF_2)_6SO_2N(CH_3)CH_2CH_2OH$, and $p-F(CF_2)_6-C_6H_4OH$
Fluorosurfactant	A non-specific, general term used to describe a surface active, low molecular weight (<1000), substance where carbons bear fluorine in place of hydrogen. Examples would include $CF_3(CF_2)_7SO_3^-K^+$, $H(CF_2)_7COO^-NH_4^+$, $F(CF_2CF_2)_3CH_2CH_2SO_3^-NH_4^+$, $CH_3CH_2CF_2CF_2CH_2COO^-NH_4^+$, etc.
Fluorinated organic surfactant	A term to describe a surface active, low molecular weight (<1000), substance which contains fluorinated carbons; the term fluorosurfactant is non-specific but often used synonymously; an example is $F(CF_2)_6CH_2CH_2SO_3^-NH_4^+$
Perfluorinated surfactant	A term used to describe a surface active, low molecular weight (<1000), substance where all carbons bear fluorine in place of hydrogen; the term fluorosurfactant is less specific but used synonymously; an example is $F(CF_2)_6SO_3^-NH_4^+$

The PERFORCE consortium selected a subset of perfluorinated substances manufactured or occurring in the environment as the main study objects for this project. These materials are presented in Table 2-2.

Table 2-2. Perfluorinated chemicals included in PERFORCE project

Name	Chemical Formula	Acronym	CAS# (§)
<i>Sulfonates</i>			
Perfluorobutane sulfonate	$F(CF_2)_4SO_3^-$	PFBS	375-73-5
Perfluorooctane sulfonate	$F(CF_2)_8SO_3^-$	PFOS	1763-23-1
<i>Carboxylates</i>			
Perfluorobutanoate	$F(CF_2)_3CO_2^-$	PFB	375-22-4
Perfluorooctanoate	$F(CF_2)_7CO_2^-$	PFO	335-67-1
Perfluorononanoate	$F(CF_2)_8CO_2^-$	PFN	375-95-1
Perfluoroundecanoate	$F(CF_2)_{10}CO_2^-$	PFUn	2058-94-8
<i>Alcohols</i>			
Perfluorohexyl ethanol	$F(CF_2)_6 CH_2CH_2OH$	8-2 FTOH	678-39-7
Perfluorooctyl ethanol	$F(CF_2)_8 CH_2CH_2OH$	6-2 FTOH	647-42-7
<i>Other</i>			
1H,1H,2H,2H-perfluorooctane sulfonate	$F(CF_2)_6 CH_2CH_2SO_3^-$	6-2 FTS	27619-97-2
Perfluorooctyl sulfonamide	$F(CF_2)_8SO_3NH_2$	PFOSA	754-91-6

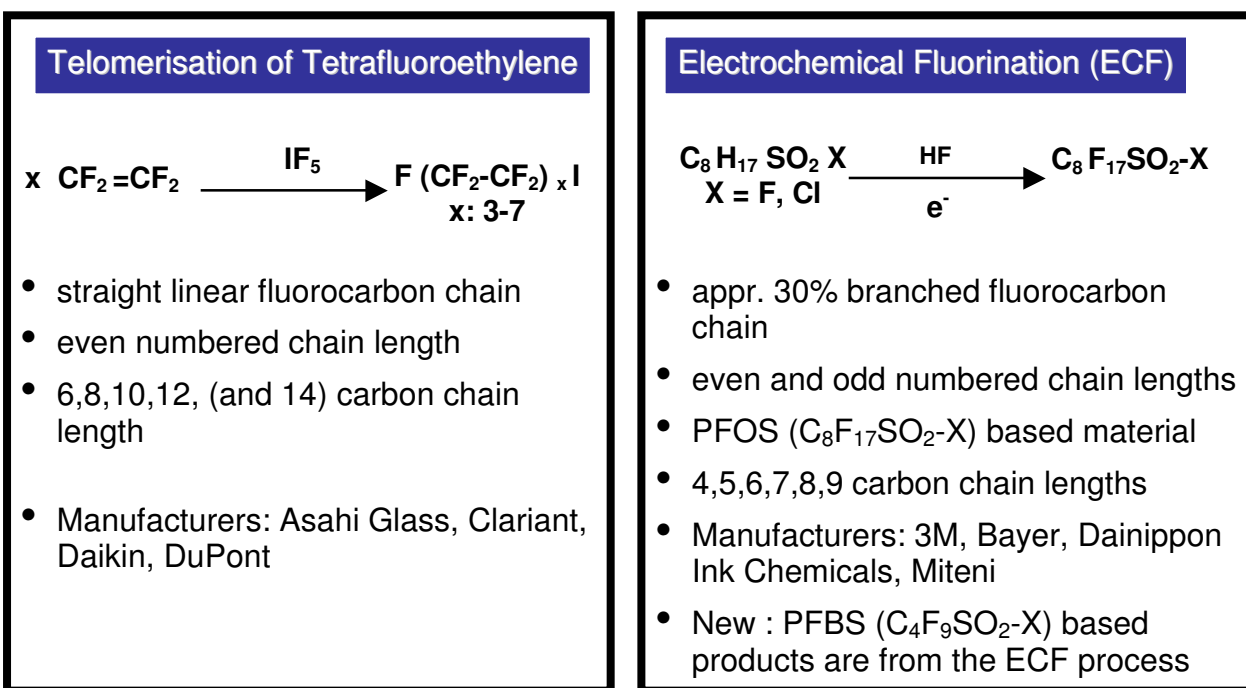
§ for the acids the CAS number is for the protonated acid form, e.g. $F(CF_2)_3COOH$ (=PFBA)

2.1 Manufacturing Routes

Chemical substances containing perfluorinated chains (e.g. four carbons or greater) are commercially manufactured primarily by two synthesis routes, electrochemical fluorination (ECF) and telomerisation (Kissa, 2001). The two routes are shown in Figure 2-1. The significant differences between the two approaches are that ECF, being a free-radical process, yields a product mixture of many chain lengths (e.g. an eight carbon starting material gives product ranging from four to nine carbons) including up to 30% product with branching on the fluorinated chain while telomerisation as practiced commercially yields only linear fluorinated chains of even carbon number consisting mainly of six, eight, ten and twelve carbon perfluorinated chains with smaller amounts of greater than twelve. For both processes, refinement by distillation is practiced to tailor

mixtures to specific applications. While trifluoroacetic acid has been shown to be a natural substance (Frank et al, 2002), no substances containing more than one perfluorinated carbon have been identified as naturally-occurring.

Figure 2-1. Electrochemical fluorination and telomerisation processes: general description



2.2 Perfluorinated sulfonates and sulfonamide

2.2.1 Perfluorobutane Sulfonate (PFBS) $\text{F}(\text{CF}_2)_4\text{SO}_3^-$

Perfluorobutane sulfonate is manufactured by the electrochemical fluorination (ECF) process. PFBS is chemically and biologically stable and not expected to degrade in the environment.

Currently, perfluorobutyl sulfonyl fluoride (PBSF) is being produced as a raw material for the commercial manufacture of a wide array of surfactant and polymeric products including PFBS that are being offered as replacements for the withdrawn POSF-based products. These products are expected to contain PFBS as a manufacturing by-product.

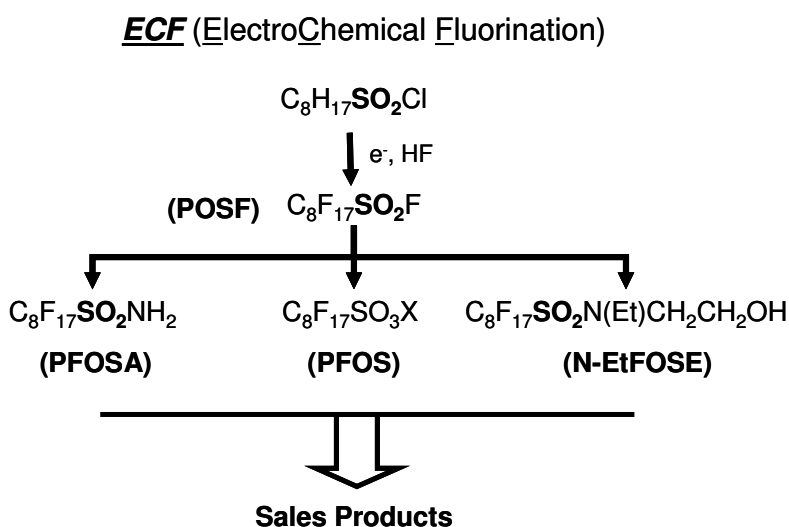
Before 2002, PFBS was largely a low level impurity or present as a perfluorobutyl sulfonyl component in perfluorooctyl sulfonyl fluoride (POSF)-based products whose manufacture was terminated by the major global producer in 2002 (3M, 2000a).

PBSF is manufactured at one site in North America and possibly two sites in Europe. The past and current global production tonnage of PFBS is unknown. The distribution, use and environmental releases for POSF-based products have been reported (3M, 1999). No comparable use, distribution and environmental release information is available for PBSF-based products or PFBS.

2.2.2 Perfluorooctane Sulfonate (PFOS) $F(CF_2)_8SO_3^-$

Perfluorooctane sulfonate is manufactured by the ECF process (See Fig. 2-2). PFOS is chemically and biologically stable and not expected to degrade in the environment.

Figure 2-2. Electrochemical fluorination (ECF) process schematic



The production (3M, 2000a,b), use, distribution and environmental releases (3M, 1999) of PFOS and perfluorooctyl sulfonyl fluoride (POSF)-based substances has been well documented by the major global producer, who terminated manufacture in 2002, and by global regulatory agencies (OECD, 2002; Brooke et al., 2004).

An Environmental Risk Assessment on PFOS was performed by the UK Environment Agency (Brooke et al, 2004) in the context of the EU Existing Chemicals Legislation. Excerpts from this assessment are reproduced below:

“The starting feedstock for the production of PFOS is 1-octanesulphonyl fluoride, and the initial product is perfluorooctanesulphonyl fluoride (POSF). This product

is mainly used as an intermediate in the production of other substances. The simplest of these is PFOS itself, produced by hydrolysis of POSF. The various salts are then produced from this.

The majority of POSF is reacted first with either methylamine or ethylamine to give either N-methyl- or N-ethyl perfluorooctane sulphonamide. These intermediates can be used to make various commercialized products such as amides, oxazolidinones, silanes, carboxylates and alkoxylates which are available commercially.

The sulphonamide derivatives can be reacted with ethyl carbonate to form either N-methyl- or N-ethylperfluorooctanesulphonamidoethanol (N-MeFOSE and N-EtFOSE). These intermediates can be used to make various commercialized products such as adipates, phosphate esters, fatty acid esters, urethanes, copolymers and acrylates. The majority of the POSF-related products made by 3M were from this group of products.

Although (for the production process for PFOS-related substances) the starting material is n-octane sulphonyl fluoride, this will contain some non-linear C₈ compounds. The fluorination process is expected to lead to some fragmentation of the chain. Thus the product of the fluorination step will contain linear and non-linear chains, mostly C₈ but with other chain lengths present. RIKZ (2002) quote 3M as reporting a final product (as PFOSF, see Section 2) of approximately 70% n-PFOSF and 30% branched impurities including odd and even chain lengths. An alternative description of the content is 90% of C₈ molecules, of which 25% are branched, with 5-10% C₆ compounds and the remainder C₇ (2-5%) and C₅ compounds. A similar distribution is assumed to apply to all products based on the ECF process (see Section 2.1.1), whether produced by 3M or by other companies. No specific information on other companies' products has been identified.

It should be noted that the secondary reactions producing the various products are single or sequential batch reactions, and do not necessarily lead to pure products. There may be varying amounts of fluorochemical residuals (unreacted or partially reacted starting materials or intermediate products) carried forward into the final product. According to 3M these residues are present at around 1% or less in the final commercial products.

The major uses for the PFOS-related substances were in providing grease, oil and water resistance to materials such as textiles, carpets, paper and in general coatings. The substances used in these areas were largely PFOS-polymers for fabrics and PFOS-substances for paper treatment and coatings. Other smaller volume uses, which are continuing for the present, are in chromium plating, photolithography, photography and in hydraulic fluids for aviation. The inclusion of PFOS-related substance in new fire-fighting foams has ceased, but stockpiles of foams containing PFOS still exist and may be used."

Perfluorooctane sulfonate (PFOS), the primary impurity in and degradation product of POSF-based products has been found ubiquitously present in the environment and has been classified as a persistent, bioaccumulative and toxic, PBT, chemical (OECD, 2002; ECB, 2005)

PFOS does not degrade chemically or biologically.

2.2.3 Perfluorooctane Sulfonamide (PFOSA) $F(CF_2)_8SO_2NH_2$

The production (3M, 2000a,b), use, distribution and environmental releases (3M, 1999) of PFOSA and perfluorooctyl sulfonyl fluoride (POSF)-based substances has been well documented by the major global producer, who terminated manufacture in 2002, and by global regulatory agencies (3M, 1999; 3M 2000a,b; OECD, 2004).

2.3 Perfluorinated carboxylates

2.3.1 Perfluorobutanoate (PFB) $F(CF_2)_3CO_2^-$

There are no known commercial manufacturers of PFB, although it is available as a research chemical. It is a known by-product from electrochemical fluorination processes used to manufacture perfluorohexanoate and perfluorohexane sulfonate. There is one manufacturer each in North America, Europe and Japan using the ECF process to manufacture perfluorinated substances. No information is known about the chemical or biological stability of PFB.

2.3.2 Perfluorooctanoate (PFO) $F(CF_2)_7CO_2^-$

Commercial Manufacturing Processes: Ammonium Perfluorooctanoate (APFO) $F(CF_2)_7COONH_4$

- Electrochemical Fluorination (ECF): $H(CH_2)_7COF + e^- + HF$ (Branched & Linear Isomers)
- Perfluorooctyl Iodide Oxidation: $F(CF_2)_8I + [O]$ (Linear Isomers Only)

PFO was first manufactured in 1947 by the electrochemical fluorination process and has been used for over fifty years. The ammonium salt (APFO) is the most widely produced form used as an essential surfactant for the manufacture of fluoropolymers such as polytetrafluoroethylene (PTFE). The ECF process for the manufacture of PFO yields a complex mixture containing fluorinated carbon chain lengths from four to nine comprised of linear ($\geq 70\%$) and branched ($\leq 30\%$) isomers. The branched isomers are numerous and arise due to the free-radical nature of the ECF process. The perfluorooctyl iodide process utilizes high purity starting material yielding only linear PFOA of high chemical purity ($\leq 99\%$). A recent critical review article provides significant details on the

production, use, environmental releases and physical-chemical properties of PFO as well as other potential sources of PFO (Prevedouros et al, 2006).

The largest historic production sites for APFO were in the U.S. and Belgium, the next largest in Italy and small scale producers in Japan. The remaining 10-20% of APFO was manufactured from about 1975 to present by direct oxidation of perfluorooctyl iodide (Grottenmuller et al, 2002) at one site in Germany and at least one site in Japan. Solid APFO was used in making fluoropolymers (e.g. Fluorad™ FC-143) (3M, 1995). An aqueous solution (e.g. Fluorad™ FC-118) has been used in recent years because solid APFO readily sublimates and proved difficult to handle. Additional production, use and disposal of limited research quantities of PFCAs has taken place in numerous academic and industrial locations worldwide over the past fifty years as indicated by patents and papers in the scientific literature. In 1999, global annual APFO production was approximately 260 tonnes (FMG, 2002). PFO emissions from the largest ECF production plant, located in the U.S., were reported to be approximately 20 tonnes (5-10% of total annual production) in 2000, roughly 5% discharged to air and 95% to water (3M, AR226-0595, 2000). The estimated historical (1951-2004) industry-wide global emissions from APFO manufacture are between 400 - 700 tonnes (Prevedouros et al, 2006).

By 2002, the principal worldwide APFO manufacturer by the ECF process discontinued external sales and ceased production leaving only a number of relatively small producers in Europe and in Asia (OECD 2004). New APFO production capacity based on >99% pure perfluorooctyl iodide commenced in the U.S. in late 2002 with reported annual releases of approximately 50 kilograms per year to air (DuPont, 2005). With the termination of U.S. ECF-based manufacture, current and future U.S. releases from APFO manufacture have been dramatically reduced from many tonnes per year to kilograms per year. As a result, global APFO manufacturing emissions have decreased from about 45 tonnes in 1999 to about 15 tonnes in 2004 and to an expected 7 tonnes in 2006 (FMG, 2002). Recently, a number of global companies who manufacture or use PFOA have committed to a voluntary stewardship program to reduce manufacturing emissions and product content (US EPA, 2006).

2.3.3 Perfluorononanoate (PFN) $F(CF_2)_8CO_2^-$

Commercial Manufacturing Processes: Ammonium Perfluorononanoate (APFN WG, 2003): $F(CF_2)_nCOONH_4$ n = 8, 10, 12

- Fluorotelomer Olefin Ozonolysis: $F(CF_2)_nCH=CH_2 + [O_3]$ (Linear)
- Perfluorooctyl Iodide Carbonylation: $F(CF_2)_8I + [CO_2]$ (Linear)

PFN has been manufactured since about 1975 by the ozonolysis of fluorotelomer olefins and has been used principally in the form of the ammonium salt as an essential surfactant for the manufacture of fluoropolymers such as polyvinylidene fluoride (PVDF). A recent review article provides significant details on the production, use, environmental releases from PFN manufacture and use in the manufacture of fluoropolymers (Prevedouros et al, 2006). The only known commercial manufacturer of PFN is located in Japan.

Ammonium perfluorononanoate (APFN WG, 2003) is manufactured primarily in Japan by oxidation of a mixture of linear fluorotelomer olefins (FTOs) to the corresponding odd-numbered PFCAs (Asahi Glass, 1975; Daikin, 1998). The principal raw material is 8-2 fluorotelomer olefin (8-2 FTO). Surflon[®] S-111, a commercial product (CAS # 72968-3-88), is described as “Fatty acids, C7-13, perfluoro, ammonium salts” a mixture of PFCAs between seven and thirteen carbons in length (APFN WG, 2003). Patent citations also indicate fluorotelomer iodide carboxylation as a process for APFN production (Tosoh, 1990). The starting fluorotelomer olefin or iodide dictates the resulting PFCA composition. APFN production is believed to have started in about 1975 and continues today. APFN is primarily used as a processing aid in fluoropolymer manufacture, most notably polyvinylidene fluoride (PVDF). We estimate annual APFN production in 2004 to be between 15-75 tonnes. Estimated emissions to air and water from APFN production to be 10% of the amount produced (Prevedouros et al, 2006). Based upon APFN production from 1975 to 2004, estimated historical global emissions from APFN manufacture are between 70 - 200 tonnes. Recently, a number of global companies who manufacture or use PFOA have committed to a voluntary stewardship program to reduce manufacturing emissions and product content (US EPA, 2006).

2.3.4 Perfluoroundecanoate (PFUn) $F(CF_2)_{10}CO_2^-$

PFUn has been manufactured as a principal component of commercially available perfluorononanoate since about 1975 by the ozonolysis of fluorotelomer olefins and has been used principally in the form of the ammonium salt as an essential surfactant for the manufacture of fluoropolymers such as polyvinylidene fluoride (PVDF). A recent review article provides significant details on the production, use, environmental releases from PFN (Prevedouros et al, 2006). The only commercial manufacturer of PFN is located in Japan.

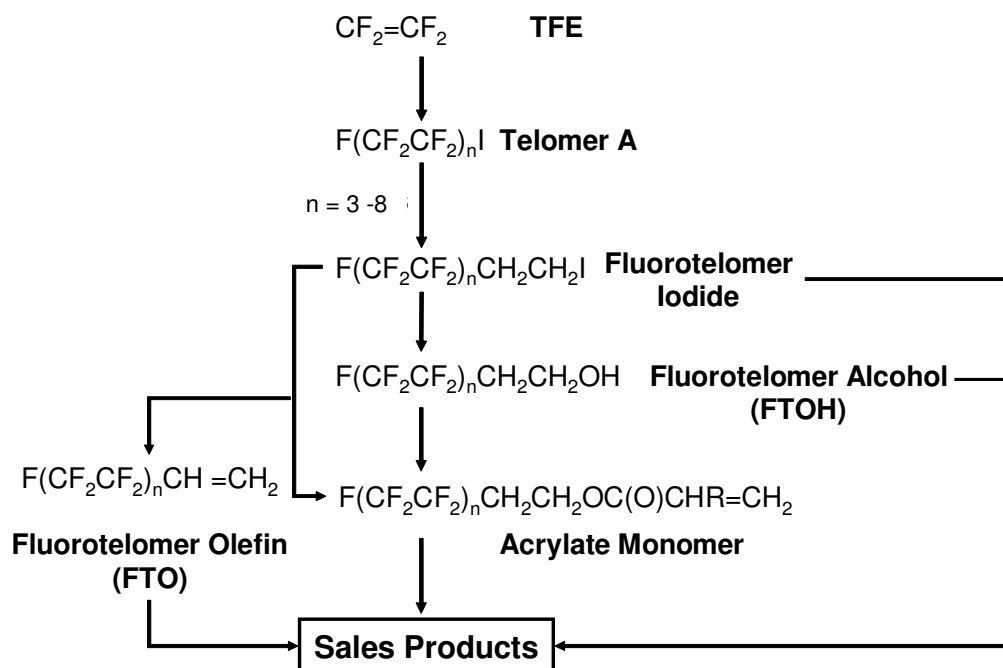
2.4 Fluorotelomer alcohol and fluorotelomer sulfonate

2.4.1 6-2 and 8-2 Fluorotelomer Alcohol (FTOH) $F(CF_2)_nCH_2CH_2OH$ (n= 6 or 8)

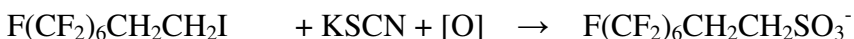
Fluorotelomer alcohols are manufactured as a raw material used in the synthesis of fluorotelomer-based surfactant and polymeric products (See Figure 2-1) (Kissa, 2001; Rao and Baker, 1994). They are produced as a mixture comprised principally of six to twelve fluorinated carbon congeners. (e.g. n = 6,8,10,12) Fluorotelomer alcohols are present in these products as residual raw materials (Larsen et al, 2006). There are one North American, one European and two Japanese manufacturers of fluorotelomer-based raw materials. No global production data is available for fluorotelomer alcohols. The annual production of perfluoroalkyl iodide (aka Telomer A), the starting raw material made in the telomerisation process, was reported to be in the range of five to six million kilograms per year from 2000-2002 by the Telomer Research Program (TRP, 2002). The

majority, at least 50%, of the perfluoroalkyl iodide is processed to fluorotelomer alcohols. However, a significant amount, 30-40%, is not. This is because there are two routes commercially used to make the fluorotelomer acrylate monomer. One uses fluorotelomer alcohol esterification, while the alternative uses fluorotelomer iodide (Telomer B). Total historic production, environmental releases and degradation of fluorotelomer-based raw materials, including fluorotelomer alcohols, has recently been reviewed (Prevedouros et al, 2006). Fluorotelomer alcohols have been shown to degrade biologically (Wang et al, 2005a,b; Fasano, 2005; Martin et al, 2005; Kudo et al, 2005; Fasano et al, 2006) and abiotically (Ellis et al, 2004; Gauthier and Mabury, 2005) to form low yields of perfluorocarboxylates. Fluorotelomer-based products are the subject of ongoing studies to determine whether and to what extent they may degrade to form fluorotelomer alcohols.

Figure 2-3. Fluorotelomer-Based Product Manufacture Schematic



2.4.2 6-2 Fluorotelomer Sulfonate (6-2 FTS) $\text{F}(\text{CF}_2)_6\text{CH}_2\text{CH}_2\text{SO}_3^-$



Fluorotelomer Iodide

6-2 Fluorotelomer Sulfonate

Fluorotelomer sulfonates are commercial surfactants useful in aqueous formulations to lower surface tension and improve wetting and leveling. They are generally manufactured from fluorotelomer iodide as shown above. The commercial

production tonnage is not available. In addition, fluorotelomer sulfonates may be formed from the degradation of fluorotelomer mercaptans (e.g. $F(CF_2)_mCH_2CH_2SH$), fluorotelomer mercaptan-based products or fluorotelomer-based sulfone products. A specific example is aqueous fire-fighting foams synthesized from fluorotelomer mercaptan- or fluorotelomer sulfone-based surfactants (Schultz et al, 2003; Moody et al, 2003; Moody and Field, 2000).

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3 Chemical method development

3.1 Analytical method development and validation

The main objective of the chemical analytical work package was to develop and validate trace analytical methods for quantification of PFCs in biota, sediment, water and air. Main target compounds were perfluorinated sulfonates C4 (PFBS) and C8 (PFOS), perfluorocarboxylates C4 (PFBA), C8 (PFOA), C9 (PFNA) and C11 (PFUnA), perfluorooctane sulfonamide (PFOSA), 6:2 fluorotelomer sulfonate (6:2 FTS) as well as 6:2 and 8:2 fluorotelomer alcohols (FTOH).

Martin et al. (2004) summarized the key challenges in environmental trace analysis of the target compounds. They include blank contamination issues, purity of reference standards and matrix effects in the ionisation process of the mass spectrometer. Special attention was paid to these challenges during the development and validation of the methods described below. The following general conclusions can be drawn from the analytical experiments performed (for further literature see also Powley et al. 2006; de Voogt and Sáez, 2006):

Blank contamination is most problematic for perfluorinated carboxylates, especially PFOA. It is associated with fluoropolymer materials used in the laboratory (e.g. PTFE) or in the analytical instrument, rather than field contamination. These materials must be avoided in trace analysis of perfluorinated carboxylates. Solvents (from the PTFE-lining in the cap) and nitrogen blow down were identified as additional sources. High concentration factors of solvent volumes should therefore be avoided and nitrogen gas should be cleaned by passing through a charcoal cartridge. In terms of environmental matrices, the biggest challenges with blank values are encountered when analysing water. This is due to the very low levels of perfluorinated compounds in environmental water samples (low ppt to ppq) relatively to biological matrices and hence the need for a high concentration factor during sample preparation. Furthermore, water samples are usually extracted using solid phase extraction and a vacuum manifold, which commercially contains PTFE parts. Another challenge is the need of water free from the target compounds that could be used as blank control water. “Blank” water should therefore be cleaned using the water sample extraction setup, or blank experiments should be performed without using a water matrix.

Purity of reference standards used as internal standards or in external calibration solutions is still an issue. These standards may contain homologues of different chain lengths or branched isomers. Response factors of different isomers of a given compound vary greatly in MS/MS (see Martin et al. 2004; Powley et al. 2006). In single MS, response factors of isomers are more uniform, but must still be considered for quantification (for a comparison of MS instruments see Berger et al. 2004). Commercial standards must therefore be carefully characterised before use, and uncertainties in analytical results have to be reported also considering standard purities.

Matrix effects are known to be present especially when applying weak ionisation techniques, such as electrospray ionisation used in mass spectrometry of perfluorinated compounds. Furthermore, due to the amphiphilic properties of the target compounds and due to blank problems, a short and crude clean-up is usually performed, leaving many matrix compounds in the final extract. Measures have to be taken to control matrix effects in MS. For example matrix extract dissolved external calibration standards, matrix spike experiments and determination of suppression/enhancement factors, standard addition methods or the use of authentic mass labelled internal standards for all analytes of interest. Some of the methods developed in this project proved to be virtually free from matrix effects (Powley et al. 2005). However, these methods do not normally contain a concentration step and are therefore not very sensitive. Increasing the sensitivity by extract concentration may again lead to matrix effects and/or blank contamination. This has to be considered for all methods described below containing an additional concentration step.

3.2 Methods for biota (including eggs)

A quick and easy-to-use screening method for biological samples was developed and validated. The method is described and discussed in detail in Berger and Haukås (2005). The method was also compared to the ion pair extraction method (Hansen et al. 2001), which is used by most analytical laboratories.

Furthermore, a method developed by Powley and Buck (2005) for analysis of perfluorocarboxylates in biological matrices was extended to also cover perfluorosulfonates, PFOSA and 6:2 FTS. Additionally, a concentration step was introduced, to increase sensitivity. Method protocols are given in Annex 1. Recoveries for the target analytes extracted from fish liver and brain tissues were typically between 80 and 100%, when no concentration step was applied. For fish spleen tissues, recoveries ranged between 50 and 70%. Using an additional concentration step, the recoveries from liver were typically 60 – 80%. Method detection limits with and without concentration step were typically around 0.5 and 0.05 ng/g wet weight, respectively. This method was also compared to both the screening and the ion pair extraction method and results were given in Berger et al. (2005). The modified Powley method is recommended as the method of choice for trace analysis of perfluorinated compounds in biological samples.

3.3 Sediment and compost

A method developed by Powley et al. (2005) for analysis of perfluorocarboxylates in sediment, sludge and soil was extended to also cover perfluorosulfonates, PFOSA and 6:2 FTS. Again, an additional concentration step was introduced to achieve better sensitivity. The method was also successfully applied to compost samples. Protocols are given in Annex 2. This method is recommended for analysis of solid abiotic environmental samples. Recoveries from spiked sediment (without concentration) were

85 – 105% and typical detection limits 0.05 ng/g dry weight. Recoveries from compost samples (no concentration step) were between 70 and 100% and typical method detection limits around 0.1 ng/g dry weight. Repeatability was tested by analysing a non-spiked compost sample in triplicate. The obtained concentration values varied <18% for all of six detected perfluorinated compounds.

3.4 Water

A method for analysis of up to 500 ml water samples was developed. The method protocol is given in Annex 3. Challenges in water analysis are discussed above. Furthermore, the water method does not always perform properly for the perfluorinated sulfonates. This might be due to irreversible adsorption of these compounds to surfaces like polyethylene. However, this phenomenon is under investigation, and the water method has to be considered as still under development. For water samples with high particle content, the particle phase has to be analysed separately, due to the tendency of PFOSA and long-chain perfluorosulfonates and –carboxylates to bind to particles. As much as 20% of the extracted PFOS and 30% of the extracted PFNA from a sewage water sample were found in the particle phase. Recoveries for the water and the particle phase are 60 – 100% (lower for long-chain compounds) and 60 – 90%, respectively. Method detection limits range between 20 and 200 pg/L for the water and the particle phase, but are often elevated for dissolved PFHxA and PFOA due to blank contamination. Repeatability depends on the concentration of the analytes and the matrix in the water (purity of the water), but is usually excellent.

In Annex 4 the general LC/MS method developed for analysis of extracts from biota, sediment, compost and water samples is given. Further analytical details for different MS instruments can be found in Berger et al. (2004).

3.5 Air samples

An analytical method for airborne polyfluorinated compounds was developed in cooperation with GKSS research centre in Geesthacht, Germany and Lancaster University, UK. It is based on the method published by Martin et al. (2002) and described in detail by Jahnke et al. (submitted). Also in the GC/MS method used for air analysis, attention has to be paid to matrix effects. Furthermore, the chromatographic performance depends strongly on the applied column (especially for the FTOHs) and the purity of the injection liner. The liner has to be changed quite often to maintain acceptable instrument performance. Method detection limits for the developed method are typically around 0.1 to 1 pg/m³ and recoveries vary strongly between analytes. For 6:2 and 8:2 FTOH recoveries of around 60 and 95% were obtained, respectively. Due to varying recoveries and matrix effects, authentic mass labelled standards have to be employed for all target analytes to ensure accuracy of results.

Additionally, first experiments were performed to test the applicability of commercial solid phase extraction cartridges for sampling of fluorinated compounds in air. Results are given in Jahnke et al. (2006). This method gave very promising results and will be developed further. Compared with the method above, the solid phase extraction cartridges are much easier to use and thus less time and solvent consuming and less susceptible to blank contamination.

3.6 Conclusions

- Due to the presence of various PTFE materials in laboratory equipment and solvents, blank contamination is a challenge when analysing per- and polyfluorinated compounds, especially for water samples with relatively low levels of the compounds of interest.
- Purity of internal standards is an important issue. Commercial standards must therefore be carefully characterised before use and uncertainties in analytical results have to be reported.
- Matrix effects and blank contamination is potential when increasing the sensitivity by extract concentration
- Modified Powley methods with additional concentration step is recommended as the method of choice for trace analysis of per- and polyfluorinated compound in biological samples and solid abiotic environmental samples.
- Method for water analysis is still under development. For water samples with high particle content, particle phase should be analysed separately due to the tendency of PFOSA and long-chain perfluorosulfonates and –carboxylates to bind/adsorb to particles.
- A promising easy to use, less time and solvent consuming method for analysing air-samples with the use of commercial solid phase extraction cartridges is under development. This method is also less susceptible to blank contamination.

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4. Quality assurance

The need for quality improvements in PFC analysis was already recognised a few years ago. Several error sources were identified that required attention, such as (Martin et al. 2004) the lack of internal standards, the lack of well-characterised commercially available authentic standards, occurrence of matrix effects (due to e.g. insufficient sample clean-up), blank problems originating from PTFE parts in the instrumentation and considerable inaccuracies (recoveries largely outside the generally applied acceptable ranges of 70-115%).

Many developments have taken place in the past few years that support improvements of PFC analysis. A wide range of authentic standards became commercially available (especially saturated and unsaturated PFCAs, PFSA's and FTOHs), as well as mass-labeled standards for internal standard use (e.g. $^{13}\text{C}_2$ -PFCAs, $^{13}\text{C}_4$ -PFOA, $^{18}\text{O}_2$ - and $^{13}\text{C}_4$ -PFOS, $^{18}\text{O}_2$ -PFOSA, $^{13}\text{C}_2$ -FTOHs). Furthermore, more knowledge on the physicochemical properties of the different compounds became available (see chapter 5 and Hekster et al., 2003), helping the analytical chemists to design appropriate extraction and clean-up procedures (see chapter 3). Whereas early reports on PFCs in environmental samples reported limited quality assurance/quality control (QA/QC) details or poor accuracy data (Giesy and Kannan, 2001; Kannan et al. 2002), recent publications show that substantial attention is paid to QA/QC and increasingly good validation data (precision, accuracy) is shown (Martin et al. 2003; Bossi et al. 2005).

In the PERFORCE project, considerable attention was paid to QA/QC. QA was based on: (i) in-house QA by the participating laboratories, (ii) interlaboratory comparison by analysis of selected samples by two or more PERFORCE laboratories (referred to as co-analysis) and (iii) a worldwide interlaboratory study (ILS) in which a wide range of labs (including the PERFORCE laboratories) participated.

4.1 Interlaboratory comparisons

Several tools exist for determination of the accuracy of a laboratory's test method, including analysis of certified reference materials (CRMs), spiking experiments, analysis of laboratory reference materials, participation in ILS, and so on (Thompson et al. 1995). Certified reference materials are valuable as they allow checking a test method's bias against a certified value. However, in the case of the PFCs, no CRMs are available yet. Alternatively, ILS enable labs to crosscheck their performance with other laboratories, which stimulates laboratories to further improve their analytical methods.

Prior to the 1st worldwide ILS on PFCs, NILU provided a PFC standard solution to the PERFORCE consortium for evaluating their calibration (using their in-house methods and calibration solutions). The results can be found in Table 4-1. For most compounds, 4 to 6 values were submitted (except 7H-PFHpA and TH-PFOS). Basic statistics show that all values are between 15 and 31 % relative standard deviation (except for PFHpA since this compound was not meant to be in the standard and hence was present at a very low concentration). The analysed solution was a mixture of standards, some of which

contained shorter chained homologues as impurities. This has led to a bias in the theoretical values, especially an underestimation of PFHxS (impurities from PFOS) and PFHxA and PFHpA (impurities from PFOA, PFNA and PFDcA). The results of this study were promising, and showed that most PERFORCE laboratories control their calibration.

Table 4-1. Calibration performance of PERFORCE laboratories

	7H-PFHpA	TH-PFOS	PFOSA	PFBS	PFHxS	PFOS	PFHxA	PFHpA	PFOA	PFNA	PFDcA
Theoretical value	10.4	2.6	121	47.4	5.3	33.3	22.9	0.0	12.5	83.2	21.6
Mean (ng/ml)	10.8	2.1	106	49.9	6.9	28.7	30.7	0.4	14.0	91.3	25.8
Std. dev. (ng/ml)	2.4	0.2	33.2	13.9	1.2	4.2	9.6	0.3	2.7	17.5	4.1
Rel. std. dev. (%)	(22)	(10)	31	28	18	15	31	(70)	20	19	16
N	2	2	6	4	4	6	6	4	6	6	5

In the framework of PERFORCE and with additional support of Plastics Europe, RIVO (in collaboration with Örebro University, Örebro, Sweden) organised in 2004/05 the 1st worldwide ILS on the determination of PFCs in environmental and human matrices. The main objective was to assess the between-laboratory reproducibility for various PFCs in a number of matrices: fish muscle tissue (FT), a purified fish liver extract (FLE), a brackish water sample, human plasma (HP) and human whole blood (HB). In addition, a standard solution (SS) was included in the study to evaluate instrumental and quantitation performance. Thirty-eight laboratories from 13 countries participated in the analysis of one or several matrices, including 5 PERFORCE laboratories.

The participating laboratory's ability to estimate a true value was evaluated (Table 4-2). The submitted data of this study were evaluated by determination of z-scores according to the Cofino model (Wells and Cofino, 1997). In general, the level of agreement between the participating laboratories decreased with increasing complexity of the matrix. Relatively good agreement between the laboratories was obtained for the study standard, the whole blood and the plasma sample. On the other hand little agreement was obtained for the fish tissue. For the more difficult matrices extraction and clean-up showed large effects on the results. Concerning the water sample, the ranges in the data submitted are large. Sources of this variation are unknown, although PFC adsorption to the container inner wall surface may have played a role. In a follow-up study on the first worldwide ILS, Powley et al. (2006) showed that adsorption of (protonated) PFCs to the high-density polyethylene (HDPE) or polypropylene (PP) bottle wall leads to analyte losses. Only 14% of the original PFOS spike was recovered after one week of storage time. These findings show that attention should be paid to sampling and sample storage conditions. Detailed reports on the set-up of the study and results have been produced elsewhere (van Leeuwen et al. 2005; van Leeuwen et al. 2006).

TABLE 4-2. Summary of PFOS and PFOA results of the 1st world-wide PFC interlaboratory study for all test materials using Cofino statistics and descriptive statistics

	PFOS						PFOA					
	SS	FLE	FT	Water	HB	HP	SS	FLE	FT	Water	HB	HP
Satisfactory $ z < 2$ (%)	76	55	17	31	67	63	64	40	25	22	73	61
Questionable $2 < z < 3$ (%)	3	18	-	13	11	-	6	20	30	6	-	11
Unsatisfactory $ z > 3$ (%)	21	27	83	56	22	37	30	40	45	72	27	28
N obs > LOQ	29	22	18	16	9	16	33	25	20	18	11	18
N obs < LOQ	1	1	-	1	-	-	-	1	1	-	-	-
Min ^a	6.2	2.7	2.8	4.7	1.8	7.1	4.1	4.5	0.54	3.4	1.4	0.50
Max ^a	65	62	295	112	24	35	46	77	204	190	4.1	5.2
Spiked amount ^a	33	45	4.4	-	-	-	8.4	11	9.7	-	-	-
Assigned value ^a	29	19	37	20	9.8	22	7.8	12	10	19	1.8	2.0
Average ^a	32	22	55	34	10	23	11	18	22	41	2.2	2.1
Median ^a	29	19	40	23	10	23	8.2	13	13	25	1.9	2.0
Standard deviation ^a	12	13	69	32	5.9	7.2	9.0	15	44	49	0.91	1.1
Relative standard deviation (%)	38	57	125	95	56	32	83	79	201	118	42	51

^a Units: SS in ng/ml, FLE in ng/ml, FT in ng/g ww, water in ng/L, HB in ng/ml and HP in ng/ml

4.2 Interlaboratory comparison on selected samples

Within PERFORCE, to evaluate the performance of the consortium, a range of samples were analysed in duplicate or triplicate by different partners. Samples were homogenised, split in sub-samples and sent to the co-analysis partner. Laboratories were asked to handle the samples using their in-house protocols for sample storage, pretreatment, extraction, clean-up and final determination. By doing so, all steps in the analytical process were included, which allowed for an insight in results obtained on the same sample using independent methods.

The partners reported their data on the target compounds (Table 4-3). The experiment was set-up in a way that a wide range of matrices was included, with a limited number of replicates. As a result, much information was obtained on these matrices, but with only duplicates or triplicates, the abilities for thorough statistical evaluation were limited. Therefore, the results were compared qualitatively (i.e. the factor difference between results). The ratio of the co-analysis laboratory result to the main laboratory result were taken to obtain the factor (e.g. for PFOS in common tern: result lab1/result lab 2 = 0.9). Unfortunately, for the FTOHs, PFBS, PFBA and PFOSA, the data was very limited.

The following stands out from the dataset presented in table 4-3:

- Many samples were contaminated at a low level, resulting in a number of <LOQ values. Sometimes these values (if in the same range) may confirm partner's findings, but often the <LOQ values are of different concentration ranges so that no conclusions can be drawn. The values are not mentioned in table 4-3.

Most data was comparison data obtained for PFOS, followed by PFOA.

Table 4-3. Lab-to-lab ratios (comparison factors) from co-analysis results.

Results obtained were divided to obtain the factor (e.g. for PFOS in common tern: result lab1/result lab 2 = 0.9). Where two data appear, two laboratories had co-analysed the sample and were compared to the main analysing laboratory. No comparison factors were obtained for PFHxS, PFBA, PFHxA, PFHpA, PFDA, PFDoA, 6:2 FTS, 6:2 FTOH and 8:2 FTOH

Matrix	Origin	PFBS	PFOS	PFHpA	PFOA	PFNA	PFUnA	PFOSA
Water	Rhine		1.1		2.0			
Water	Lago Trasimeno			1.7	0.5	19		
Common Tern	Saeftinge		0.9				2.2	
Common Tern	Terneuzen		0.4				14	
Eel	Sloehaven	0.6	1.1		1.8			
Eel	Hansweert	0.2	0.7		0.8			
Shorthorn sculpin	Western Scheldt		0.9		0.5		1.1	
Whiting	Western Scheldt		0.8		0.8			
Lugworm	Western Scheldt		0.4		2.2			
Flounder	Western Scheldt		0.4		0.1		0.9	
Shrimp	Western Scheldt		0.9				0.3	
Seal liver 1	UK		0.9			0.9	1.1	
Seal liver 2	UK		1.2			0.6	0.5	
Cod liver 1	Baltic		0.4					1.1
Cod liver 2	Baltic							0.4
Cod liver 3	Baltic		0.9					0.4
Sediment 1	Vlissingen*		1.8, 1.8					
Sediment 2	Hansweert		1.0				3.0	
Sew sludge 1	Nijverdal*		16.3, 0.02		3.4, 1.6	0.4	0.07	
Sew sludge 2	Amsterdam		0.1					
Influent 1**	Nijverdal*	40	2.5, 0.8		0.3			
Influent 2**	Amsterdam		0.8					
Effluent 1	Nijverdal*	0.9	0.8, 0.01***		0.03, 0.003***	0.01	0.08	
Effluent 2	Amsterdam		0.8					

* Sample analysed in triplicate, resulting in two comparison factors.

** One laboratory analysed total influent, whereas two labs separated solids from the liquid phase by filtration or centrifugation. For calculation of the comparison factor, the data of the liquid phase were used.

*** Low factor due to low concentrations reported by one lab, possibly due to storage at room temperature for some weeks (see below).

- PFC data for sewage sludge, influent and effluent showed large differences between partners (factor 0.003 to 40). The low factors (0.01 and 0.003) reported by one lab for the Nijverdal effluent sample may be due to losses as a result of unintended storage for some weeks at room temperature (due to delays at customs). Powley et al (2006) showed losses in a water sample due to storage for

- two weeks in HDPE bottles at room temperature. Freezing of water samples during storage may limit losses due to adsorption or volatilisation (see below) to a large extent.
- PFOS data in fish samples and common tern samples were closer (factor 1-3). This also holds for most PFOA (except flounder) and PFUnA data (except shrimps and common tern eggs from Terneuzen). PFOSA data in cod livers is within factor 1-3.
 - Seal liver results were within a factor 2.
 - The water data is scarce. The differences are within a factor 2, except for PFNA in the Lago Trasimeno sample.

It may appear surprising that the results of co-analyses show ratios substantially different from unity, revealing a considerable variability. Although analytical procedures were developed for different matrices (see chapter 3), partners have applied small deviations from those protocols (see Annexes to chapter 3), e.g., different final determination techniques were used (triple-quadrupole MS, ion-trap MS or TOF-MS). Furthermore sample pretreatment differed among partners. One laboratory separated influent SPM from the water phase, whereas others extracted whole water. It is believed that the above situation does reflect a conservative estimate of intercomparability between laboratories. Improvement of this situation is likely to occur in the future.

4.3 Needs for future improvements

The interlaboratory comparisons have showed that future efforts are needed to improve accuracy of the results reported. Those improvements should focus on the sampling as well as on the analytical determination.

Concerning classical POPs, the effect on sampling, sample storage and final determination on the accuracy of the final result has been evaluated thoroughly. However, these principles do not apply to the PFCs due to their distinct physicochemical behaviour such as the relatively high water solubility of the perfluorocarboxylic acids and short chain perfluorosulfonates. More attention should be directed to the physicochemical properties in relation to sampling and sample pre-treatment, as discussed below:

- In a laboratory study Kaiser et al. (2006) showed that at pH 4, representing acid rain conditions, a fraction of the PFOA is released from the water phase to the air. In principle, this may lead to a complete removal of PFOA (and other acids) from the water phase. In field situations, when microbial degradation of a water sample is reduced as a result of acidification, there is a risk of losses of the perfluorocarboxylic acids due to volatilisation. The PFOA will then move to the headspace of the sample bottle. Therefore, acidification is not the method of choice for sample conservation. Also, samples from acidified water bodies risk to lose perfluorocarboxylic acids due to this phenomenon if no precautions are taken.
- PFC containing pore water may influence the final results of matrices such as soil and sediment. The PFCs in the pore water are included in the sample when (freeze)drying is applied as sample pretreatment, whereas it is (partly) lost when

centrifugation is applied and supernatants are discarded. These differences in sample pretreatment may lead to deviating results for the relative water soluble PFCs and may lead to biased conclusions on sediment contamination.

- Evaporative techniques for sample pretreatment (e.g. freeze-drying) and concentration of final extracts should be applied only when the target compounds are not susceptible to volatilisation (de Voogt et al. 2000 . Szostek and Prickett (2004) discussed this in relation to analysis of telomer alcohols, and Kuklenyik et al. (2004) observed losses of sulfonamides and TH-PFOS when extracts are evaporated to dryness.
- Spike experiments are good ways of evaluating the accuracy of the applied analytical methods. Preferably, the spiked sample should not be analysed directly after spiking as the added PFC contaminants will be easily available to the extraction system (e.g solvents and/or SPE sorbents) rather than interact with the sample matrix in a similar way as the PFCs originally present in the sample. Therefore, spiked compounds should be left to equilibrate with the matrix for a period of time so that the spiked compounds can interact with matrix. No experiments were conducted within the Perforce project, but the spiking procedures may need more attention in the future.

4.4 Concluding remarks

- Analytical methods for four different matrices were developed and validated; these include water, sediment, air, and biota.
- The analytical methods developed showed good accuracies on the matrices included in the validation, demonstrating that these methods are fit-for-purpose.
- For specific matrices such as cod liver, where matrix effects were observed it should be noted that the methods are not yet sufficiently robust to provide accurate results.
- Interlaboratory comparison by co-analysis of selected samples within the consortium showed that the comparability and sample pre-treatment and analytical determination is reasonably good for the biota and sediment matrices, but poor in some sewage sludge and water samples.
- The worldwide interlaboratory study on a fish tissue, fish liver extract and a water sample showed large variation in the between-laboratory results, showing that participating laboratories were not yet able to generate comparable results. Poor accuracy of individual laboratories is most likely caused by improper choice of (internal) standards, non-selective extraction methods and non-selective final detection.
- QA/QC should be carefully considered when generating and interpreting the results of PFAS analyses.

4.5 Recommendations

- The behaviour of PFAS during sample pre-treatment and storage should be studied in more detail to further reduce within- and between-laboratory variability.
- The accuracy and robustness of methods should be improved to support policy makers, industries and academia.
- Follow up interlaboratory comparisons are necessary to assess improvements in the accuracy of individual laboratories performing PFAS analysis.

4.6 References to chapter 4

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5. Environmental fate parameters of PFCs

5.1 Introduction

Partitioning and reactivity properties of chemicals are important to understand and model their environmental behaviour. At the start of the project, such data were largely unknown for PFCs, or the available data were contradictory. An obvious starting point would be to estimate properties using available estimation methods. However, due to the unique properties of the perfluorinated chain, PFCs are outside the scope of available estimation methods. An efficient way to fill the large data gap is by the measurement of key properties for some PFCs, to use these data to develop new (mechanistic) estimation methods, and to apply these to other PFCs. Mechanistic estimation methods have the advantage that they require less experimental data in their development. The focus of this work-package was on the measurement of selected properties and on the elucidation of the mechanisms. The selected parameters were (i) the liquid vapour pressure, (ii) the sediment-water distribution coefficient, (iii) biodegradation rates and (iv) membrane-water distribution coefficients:

(i) The liquid vapour pressure

The vapour pressures and related thermodynamic properties were measured for perfluorinated telomer alcohols using a method based on gas-chromatographic retention. In addition, molecular mechanics force field (FF) simulations were used to investigate the presence of intramolecular hydrogen bonds in fluorotelomer alcohol liquids. A high vapour pressure may result in long-range atmospheric transport.

(ii) The sediment-water distribution coefficient

The sediment-water distribution coefficients (K_d) were measured in batch experiments for several sediments, a wide range of PFCs and as a function of concentration, acidity and temperature. The distribution between sediment and water affects the mobility of PFCs in surface and ground water.

(iii) Biodegradation rates

Biodegradation studies were carried out with sludge and sediment under aerobic and anaerobic conditions. Biodegradation processes could potentially affect the long-term environmental fate of PFCs, particularly in environmental compartments with long residence times, such as soil and sediment.

(iv) Membrane-water distribution coefficients

Molecular mechanics FF simulations were used to calculate the energy of water to (biological) membrane transfer energies. These energies give information about the partitioning mechanism of PFCs in membranes and can be used as organism specific descriptors for the bio-concentration factor and toxicity parameters. This is particularly important because the widely used n-octanol-water partition constant is not a reliable descriptor for the estimation of properties of perfluorinated surfactants. The study is also an illustration of the application of highly mechanistic FF models to the estimation of chemical properties as well as to the elucidation of (e.g. partitioning) mechanisms.

5.2 Vapour pressures of fluorotelomer alcohols

5.2.1 Introduction

The long-range transport properties of fluorotelomer alcohols (FTOHs) have recently become of interest because they are a potential precursor for perfluoroalkyl carboxylates that have been observed in the arctic food chain (see e.g. Stock et al. 2004 and references cited therein). The vapour pressure of compounds is an important determinant for their long-range transport potential. Experimental vapour pressure data in the literature are contradictory (Stock et al. 2004; Lei et al. 2004; Krusic et al. 2005; Cobranchi et al. 2006). For example, the reported vapour pressure values for 8:2 FTOH range from 2 (Krusic et al. 2005) to 254 Pa (Stock et al. 2004). The variation in reported values is caused by the different experimental methods used, which all make use of different assumptions. Because of the unique properties of perfluorinated compounds, the validity of these assumptions is not always obvious. Recently, Goss et al. (2006) showed that the trend for the vapour pressure of the homologous series obtained by a boiling point method (Stock et al. 2004) and a method based on gas-chromatographic retention (Lei et al. 2004) were not consistent with the trend derived from homologous series of perfluorinated alkanes. The expected trend did apply to the data obtained from nonchromatographic measurements (Krusic et al. 2005). Based on these observations and thermodynamic theory, Goss et al. (2006) concluded that the high vapour pressure values reported by Stock et al. (2004) and Lei et al. (2004) must be flawed. It was further explained that the method based on GC retention overestimates vapour pressure of FTOHs because hydrogen bonding to the column stationary phase, as occurs in the pure liquid, is not possible. On the other side, Stock et al. (2004) explained the high vapour pressures mechanistically by the presence of an intramolecular hydrogen bond, which may reduce the extent to which the hydroxyl group can form intermolecular hydrogen bonds. Quantum chemical gas phase calculations, however, suggest that this intramolecular hydrogen bond is not significant (Krusic et al. 2005).

The ongoing discussion summarized above illustrates that there is no consensus on the values of FTOH vapour pressure. We would like to contribute to this discussion by (i) generating new vapour pressure data using a method based on GC, resulting in further insight in the applicability of GC based methods to FTOHs, and (ii) by investigating the presence of an intramolecular hydrogen bond using molecular modeling.

With respect to (i): In our laboratory a gas chromatography (GC) based retention index method (GC-VAP) (e.g. Van Roon et al. 2002) has been developed, different from the one referred to above, for the estimation of liquid vapour pressures and the thermodynamically related enthalpy and heat capacity differences. The GC-VAP method has been applied successfully to several classes of compounds (see e.g. Van Roon et al. 2002 and references cited therein), including polar and hydrogen bonding compounds. We will apply the method to FTOHs, and will focus specifically on the choice of reference compounds.

With respect to (ii): The intramolecular hydrogen bond affects the vapour pressure when it competes with the intermolecular hydrogen in the pure liquid phase. However, in the quantum chemical calculations FTOH was in the gas phase rather than in the liquid phase (Krusic et al. 2005). Quantum chemical calculations on FTOHs in their liquid phase would be too time-consuming. Therefore, we will use faster molecular mechanical calculations on FTOH liquids. By calculating their dynamic behaviour, the presence of intramolecular hydrogen bonds can be studied.

5.2.2 Methods

Vapour pressure and related thermodynamic properties were measured using a method based on gas-chromatographic retention (GC-VAP). See Annex 5.2.1 for a description of the GC-VAP method. The presence of intramolecular hydrogen bonds was studied by simulating liquid phases of selected compounds using molecular mechanical force fields. See Annex 5.2.2 for a description of relevant theory of force field calculations.

The compounds included in the study are summarized in Table 5-1. The X:1 FTOHs were included because they are structurally (very) similar to the X:2 FTOHs, but the effect intramolecular hydrogen bond is expected to be reduced due to steric effects. n-Alcohols are structurally similar to the FTOHs but are unable to form intramolecular hydrogen bonds. In the GC-VAP work, the n-alcohols and the perfluorinated n-alkanes were included as reference compounds, i.e. to determine the ratio γ_z/γ_i .

Table 5-1 FTOHs and reference compounds included in the GC-VAP measurements (A) and force field calculations (B)

#C	perfluorinated n-alkanes	n-alcohols	X:2 FTOHs	X:1 FTOHs
5		A		
6	A	A,B	A,B (X=4)	
7		A,B	B (X=5)	A (X=6)
8	A	A,B	A,B (X=6)	A (X=7)
9				A (X=8)
10		B	A,B (X=8)	A (X=9)

The torsional angle shown in Figure 5-1 was monitored in the force field simulations. When this angle is within 90° from linear (angle = 0°), we assume an intramolecular hydrogen bond to be possible. Calculations were repeated with zero atomic charges on all atoms. In these calculations, hydrogen bonding is not possible because these interactions are part of the electrostatic energy (charge-charge interactions).

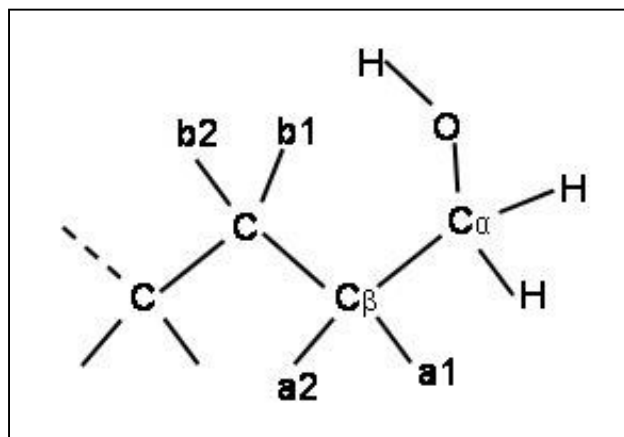


Figure 5-1. When in the plane of the paper, the torsional angle O-C α -C β -C is 0°. We assume an intramolecular hydrogen bond between H and fluor atoms b1 or b2 to be possible when O-C α -C β -C is within $\pm 90^\circ$.

5.2.3 Results

Vapour pressure and related thermodynamic properties

Annex 5.2.1 provides intermediate results of calculations (e.g. Kováts indices). The vapour pressures obtained at 25°C are listed in Table 5-2, together with literature data. Boiling points at atmospheric pressure, heats of vapourization and gas-liquid differences in heat capacity are listed Annex 5.2.1, together with literature data. The uncorrected GC-VAP vapour pressures are the values without correcting for the ratio γ_z/γ_i . Using the GC-VAP and literature values for the n-alcohols, the ratio γ_z/γ_i was calculated. The values for n-pentanol, n-hexanol, n-heptanol and n-octanol were 8, 6, 7 and 6, respectively. The average value of 7 was used to correct the GC-VAP data. The n-perfluoroalkanes were not used to correct the GC-VAP data because they did not show significant retention in the GC column.

Intramolecular hydrogen bonding

Annex 5.2.2 provides the results of the force field calibration, construction of liquid boxes, and so on. The monitoring data for the intramolecular hydrogen bond, as obtained from the liquid phase molecular dynamics simulations, are summarized in Table 5-3. The torsional angles in the n-alcohol liquids were within 90° during approximately 50% of the simulated time. This suggests no preference for a certain conformation, and is in agreement with the fact that n-alcohols cannot form intramolecular hydrogen bonds. If intramolecular hydrogen bonding occurs in FTOH liquids, we would expect the values in Table 5-3 to be higher than observed for the n-alcohols. The results suggest that intramolecular hydrogen bonding is not significant. In fact, the results suggest the opposite, i.e. that the conformation required for intramolecular hydrogen bonding is energetically unfavourable. In agreement with our expectations, the values for the X:1 FTOHs were lower. The results obtained with zero atomic charges suggest that in X:2

FTOH liquids the conformation of the O-C α -C β -C is determined largely by electrostatic interactions. For the X:1 FTOHs also other types of interactions are important. This is in agreement with the hypothesis that in X:1 FTOH liquids intramolecular hydrogen bonding is less important due to steric effects.

Table 5-2. Uncorrected and corrected (γ_z/γ_i , based on the n-alcohol data) GC-VAP and experimental literature data for vapour pressures (P, Pa) at 298.15 K unless indicated otherwise.

compound	GC-VAP uncorrected	GC-VAP + correction	Literature						
			^a	NMR ^b	DM ^c	BP ^d	HS ^e	GC ^g	GC ^h
n-pentanol	2398 $\pm$ 353	351	293						
n-hexanol	682 $\pm$ 132	100	124						
n-heptanol	215 $\pm$ 29	31	29						
n-octanol	66 $\pm$ 10	10	11						
6:1 FTOH	1790 $\pm$ 168	262							
7:1 FTOH	814 $\pm$ 73	119							
8:1 FTOH	373 $\pm$ 38	55							
9:1 FTOH	168 $\pm$ 18	25							
4:2 FTOH	3116 $\pm$ 401	456		216		992			1670
6:2 FTOH	617 $\pm$ 85	90		18	44	713			876
8:2 FTOH	136 $\pm$ 20	20		4	7	254	29 ^f	31	227

a) SRC Physprop Database. b) Method based on NMR (Krusic et al. 2005). c) Dynamic measurement procedure (Krusic et al. 2005). d) Boiling point method (Stock et al. 2004). e) Method based headspace GC and atomic emission detection (Cobrianchi et al. 2006). f) T = 45°C. g) Method based on GC retention (Cobrianchi et al. 2006). h) Method based on GC retention (Lei et al. 2004).

Table 5-3. Results of the liquid phase simulation. See Figure 5-1 and the text for details.

O-C α -C β -C < $\pm$ 90° (% of simulation time)		
	PM3 charges	all charges set to zero
FTOH42	21	38
FTOH62	27	47
FTOH82	21	49
average	23	45
FTOH61	6	17
FTOH71	4	20
FTOH81	7	23
FTOH91	5	20
average	6	21
n-hexanol	58	46
n-pentanol	51	47
n-octanol	56	43
n-decanol	44	39
average	50	43

5.2.4 Discussion and conclusions

The GC-VAP method has been applied before to several classes of compounds with relatively low vapour pressures (see Van Roon et al. 2002 and references cited therein). Although the compounds were structurally different from the reference n-alkanes, the differences with vapour pressures obtained by non-chromatographic methods were typically less than a factor of two. The success of the GC-VAP method can be explained by the following two points: (i) The activity coefficient in the stationary phase had only a small effect on the retention time at the high chromatographic temperatures that were applied. (ii) Because the vapour pressures of the reference n-alkanes are accurately known over a large temperature range, accurate extrapolations to environmentally relevant temperatures were possible. We have also applied the GC-VAP method to monoterpenes (Van Roon et al. 2002), a structurally diverse group of relatively volatile compounds (vapour pressure range: 3 – 184 Pa). Although this group contains compounds which are structurally very different from n-alkanes and includes compounds which can form intermolecular hydrogen bonds (e.g. carvacrol), the agreement with literature data was again within a factor of 2. The GC-VAP ΔH_{VAP} values for the monoterpenes were in agreement with ΔH_{VAP} values estimated with the Hildebrand rule, with the exception of the monoterpenes, which can form intermolecular hydrogen bonds (Van Roon, 2006). This was expected because the Hildebrand rule does not take into account hydrogen bonding. Interestingly, the GC-VAP and Hildebrand rule ΔH_{VAP} values were similar for eugenol; a monoterpene for which intramolecular hydrogen bonding may be important. These results suggest that the GC-VAP method can be used to measure vapour pressures of volatile compounds, which are structurally very different from n-alkanes, including compounds that are able to form intra- and intermolecular hydrogen bonds. Despite that the GC-VAP equations were derived by assuming ‘infinite dilution’ conditions, the results can only be explained by compound-compound interactions during the chromatography.

In contrast to the results obtained for the monoterpenes, we found in the current study that the GC-VAP method overestimates the vapour pressures of the n-alcohols at $T = 25^\circ\text{C}$ by a factor of 7. Together with GC-VAP ΔH_{VAP} values, which were lower than the literature data, this resulted in boiling point temperatures reasonably close to the literature values (data in Table 5-2). In previous work on the GC-VAP method (Spieksma et al. 1994), we found that for n-butanol at $T = 100^\circ\text{C}$ a ratio of activity coefficients ($\log \gamma_z/\gamma_i$) of -0.284, suggesting an overestimation of the vapour pressure of less than a factor 2. These findings could be in agreement with the discussion above, i.e. the activity coefficient in the stationary phase becomes more important at lower chromatographic temperatures. Because we found no evidence for intramolecular hydrogen bonding in FTOH liquids, we concluded that n-alcohols can be used as reference compounds for FTOHs, and applied the factor of 7 to lower the GC-VAP FTOH vapour pressures.

In the applied GC settings n-perfluorohexane and n-perfluorooctane showed little retention. They eluted just after methane and earlier than n-pentane. It was not possible to obtain Kováts indices with reasonable accuracy. Assuming n-perfluorooctane to have the

same retention time as n-butane or n-pentane, the GC-VAP method would overestimate its vapour pressure by a factor of 150 – 500 (calculated with vapour pressure data listed in Stock et al. 2004). Applying this correction to the GC-VAP vapour pressures of FTOHs, results in values that are somewhat lower than those obtained by non-chromatographic methods. The question, however, is how to combine the correction factors obtained from the n-alcohols and n-perfluoroalkanes. Applying the product of the correction factors would lead to vapour pressures an order of magnitude lower than those obtained by non-chromatographic methods. We have no previous experience with combining correction factors.

The results suggest that after proper corrections, the GC-VAP vapour pressures of FTOHs will be in the same range or even lower than the values obtained by non-chromatographic methods. This indicates that intramolecular hydrogen bonding is not important in FTOH liquids. Our simulation results also suggest intramolecular hydrogen bonding to be unimportant.

A critical discussion on the vapour pressures obtained by other chromatographic and non-chromatographic methods is required but not presented here. The other chromatographic methods are largely based on the same assumptions as used in the GC-VAP method. So their results depend strongly on the selection of reference compounds. Other (non-chromatographic) methods may be more direct, but are also based on certain (perhaps even unknown) assumptions that are not necessarily valid for perfluorinated compounds. The main case for trusting the relatively low FTOH vapour pressures in Table 5-2 is that they were obtained by *different types* of non-chromatographic methods.

Finally it should be noted that the relatively low vapour pressures of FTOHs in Table 5-2 are still high enough to explain significant atmospheric transport of these compounds.

5.3 Partitioning of perfluorinated chemicals between water and sediment

5.3.1 Introduction

Although perfluorinated chemicals (PFCs) are measured in remote areas such as the Arctic (Hekster et al. 2003), the sources of PFCs in the environment, their physical-chemical properties, fate and transport are not well understood or described. It was recently suggested that a significant route for perfluorinated carboxylates to the Arctic is discharge of the chemicals in surface waters and subsequent transport through river and ocean water (Prevedouros et al. 2006). Less information is available on perfluorinated sulfonates, e.g. on emission data, but their environmental behaviour is probably similar to that of the perfluorinated carboxylates. The partitioning of chemicals into non-mobile phases such as sediment reduces their transport rate in surface waters. The partitioning of chemicals between sediment and water is typically expressed as the sediment-water distribution coefficient K_d (L/kg). Only very few experimental K_d values for PFCs are available, and most of them are published in the grey literature (Dupont, 2003; Beach et al. 2006). A reliable estimation of K_d values is currently not possible because PFCs are, due to their unique properties, outside the scope of available prediction methods. In addition, the mechanism of PFC sorption is unclear. This makes it difficult to extrapolate the few available experimental data to other PFCs and sediments.

Obviously, there is a need for experimental K_d values as well as a mechanistic understanding of the sorption process. Therefore, the aims of this study were (i) to measure K_d values for PFCs in controlled laboratory experiments and to compare them to K_d values observed in the field, and (ii) to elucidate the sorption mechanism.

With respect to (i): The K_d values were measured for three sediments originating from the Rhine and Scheldt estuaries and the North Sea by fitting the sorption isotherms with a linear (Henry's) equation. These values were compared to the distribution of PFCs between water and sediment in field samples collected from various locations in the Rhine and Scheldt estuaries and the North Sea. This comparison is not strictly required for the aim of this study, but gives additional information on the prevailing conditions (equilibrium or non-equilibrium) in the field.

With respect to (ii): The K_d values of PFCs were measured as a function of carbon chain length ($6 \leq \#C \leq 14$), functional group (carboxylates versus sulfonates and sulfonamide), sediment properties (three different sediments differing in e.g. organic carbon contents), temperature ($T = 4, 10, 20^\circ\text{C}$) and acidity ($\text{pH} = 5, 7, 9$). In this way, we attempt to identify the contributions of hydrophobic, specific chemical and electrostatic interactions between the PFCs and sediment.

Sorption isotherms were obtained by plotting the concentration in the sediment (C_{sed} , $\mu\text{g/kg}$) against the concentration in the water (C_{wat} , $\mu\text{g/L}$). The field distribution coefficients were calculated as: $K_d \text{ (L/kg)} = C_{sed} / C_{wat}$. Because PFC concentrations were often below detection limits, the K_d values could be calculated only for PFOS and PFOA for a few locations. Average values for Rhine and Scheldt estuaries and North Sea will be presented here, concentrations for specific locations will be published elsewhere.

5.3.3 Results

Laboratory experiments: Mass balance

The mass balance was calculated for the experiments in which both the water and sediment concentrations were measured. The results are summarized in Figure 5-3. The recoveries are all within 75-100% with the exception of PFNA and PFDA. The reason for the high values for these PFCs is unclear at the moment. Considering the high amounts spiked, it is unlikely that this is the result of background problems. Because the (K_d) results for PFNA and PFDA were in agreement with the general trends observed for PFCs, we believe that the mass balance approach for calculation sediment concentrations (see Annex 5.3) is valid.

Laboratory experiments: Sorption isotherms

Figure 5-4 shows the sorption isotherm for PFAS obtained for the Rhine sediment at $\text{pH}=7$ and $T=4^\circ\text{C}$. The curved line is the nonlinear fit of the Freundlich isotherm ($K_F = 18 \pm 6$, $n = 0.8 \pm 0.3$; 95% confidence interval). Because n is not significantly different from 1, the isotherm is not significantly different from linear. The straight line is the linear Henry's isotherm fit ($K_d = 11 \pm 3 \text{ L/kg}$; 95% confidence interval). Because the scatter in other isotherms was typically larger, we applied the Henry's fit to all isotherms. When the scatter in the data was very high, the K_d values were not included in the results. In these cases the sorption was probably too low to result in a correlation between water and sediment concentrations.

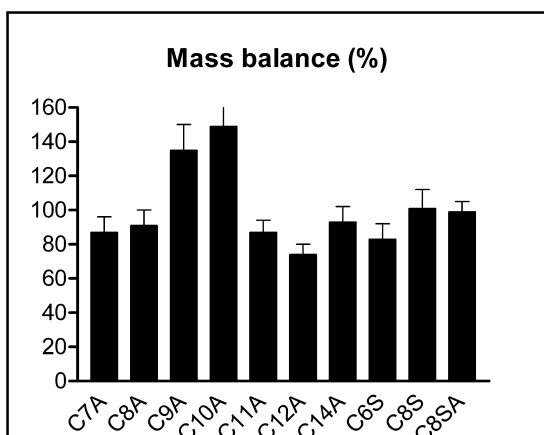


Figure 5-3. Mass balance obtained for the selected PFCs. Percentage of the spiked amount.

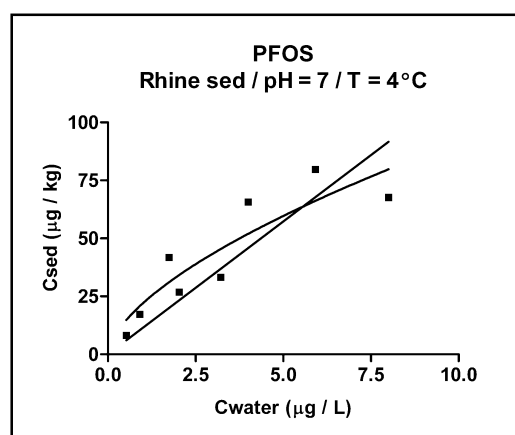


Figure 5-4. Sorption isotherm for PFAS fitted with the Freundlich and Henry's isotherm.

Sediment-water distribution coefficients K_d

The K_d values at pH = 7 and T = 4°C for both series of experiments and for the three soils are summarized in Figure 5-5. The K_d values increased with the carbon chain length of the PFC. The K_d values decreased in the order sulfonamide > sulfonate > carboxylate. In this comparison, it was taken into account that C8S and C8A have 17 and 15 F atoms, respectively. The K_d values were not correlated with organic carbon content. The K_d values obtained at 'low concentration' levels were lower than at high test levels. For the North Sea sediment in the 'high concentration' experiments the concentrations were often close to the detection limit, and the recoveries of the internal standards were low. These data are included in Figure 5-5, but will not be used for interpretation purposes.

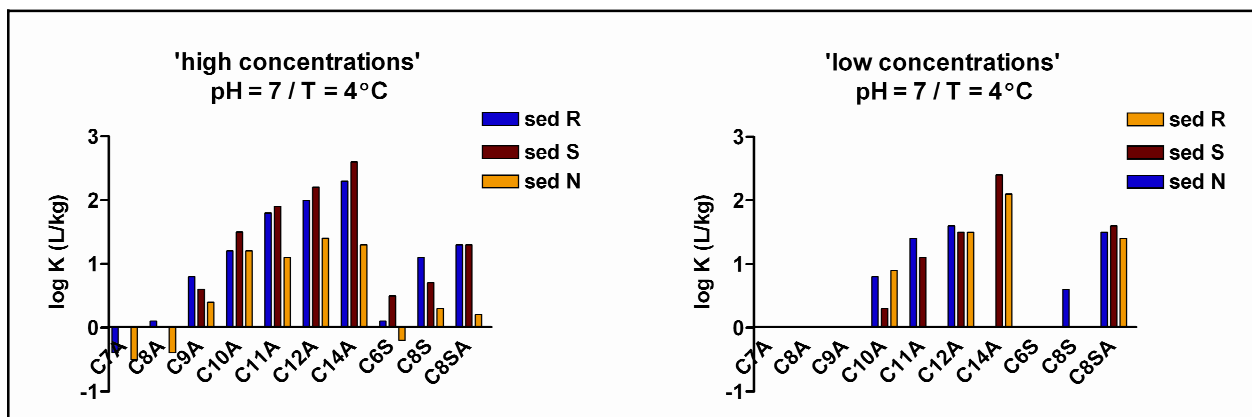


Figure 5-5. The K_d values obtained from the series with high concentrations and low concentrations for the three different sediments.

The effects of pH and temperature are shown in Figure 5-6. For the effect of pH, the results for the Rhine and North Sea sediments were similar to that of the Western Scheldt sediment. It seems that the K_d values increase at higher pH values, but the trend was not consistent. For C8SA, the K_d decreased at higher pH values for all three sediments. No clear trend was observed for the effect of temperature.

Comparison with field and literature data

The K_d values measured in this study are summarized in Table 5-5, together with the field distribution coefficients and the K_d values found in the literature. The values measured at pH = 5 and pH = 9 are not included in the Table. Note that only for PFOS and PFOA we could calculate field K_d values.

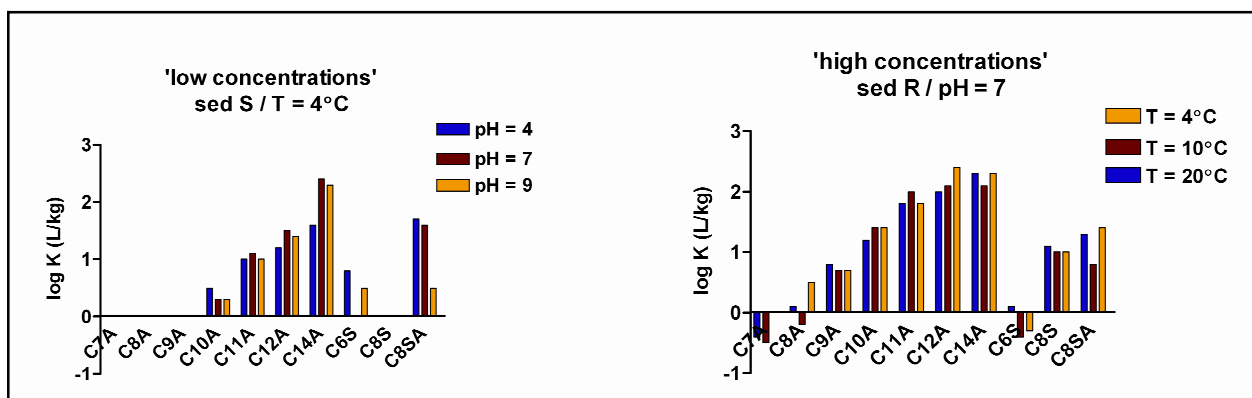


Figure 5-6. The K_d values at different pH values (left) and temperatures (right). Note that the concentration levels were different in these experiments, and that results for different sediments are shown.

Table 5-5. Summary of K_d values. Only values obtained at pH = 7 are included. Data are missing (-) when either sediment or water concentrations were below limit of detection (Field, Lab.) or when the K_d was not significantly different from zero (Lab.).

	Rhine		Scheldt		North Sea		Literature	
	Lab.	Field	Lab.	Field	Lab.	Field	Lab	
C7A	0.4 - 1.1	-	-	-	0.4	-	0.41 - 8.86 ^a	12.6 - 36.8 ^b
C8A	0.6 - 3.4	0.4 - 2	-	-	0.4	-		
C9A	4.5 - 5.9	-	4.3	-	2.6	-		
C10A	6.6 - 26	-	2.0 - 31	-	7.5 - 16	-		
C11A	23 - 103	-	12 - 77	-	12	-		
C12A	44 - 269	-	30 - 172	-	24 - 32	-		
C14A	123 - 215	-	231 - 424	-	18 - 115	-	9.72 - 35.3 ^c	7.42 ^d
C6S	0.4 - 1.2	-	3.2	-	0.6	-		
C8S	4.3 - 11	3 - 10	5.2	8 - 97	2.1	-		
C8SA	7.1 - 32	-	2.1 - 36	-	1.7 - 28	-		

a) Different soils (Dupont, 2003). b) Sludge (Dupont, 2003). c) Different soils (values listed in Beach et al. 2006). d) River sediment (value listed in Beach et al. 2006).

5.3.4 Discussion and conclusions

Origin, consequences and solution to the scatter in the data

The experimental design was based on an existing OECD protocol. However, the PFCs are different from other compounds to which this protocol has been applied in previous studies. PFCs pose new challenges with respect to e.g. sample extraction, measurement and background levels. For example, the mass balances for PFNA and PFDA (>100%) could not be explained. In addition, the scatter in the sorption isotherms was often high. This scatter could have masked the possible non-linearity of the sorption isotherms and the effects of e.g. pH and temperature. Part of this scatter can be explained

by the relatively low values of K_d , which makes random variations in sediment concentrations significant compared to systemic (mechanistically driven) changes. The scatter can be reduced by further optimizing the extraction and analysis of PFCs, and by using labeled PFCs in the sorption experiments. Despite these issues, the observed trends in the data (e.g. carbon chain length versus K_d) suggest that the results are useful.

Parameters are not independent

An important aim of this study was to understand the sorption of PFCs to sediment in terms of the hydrophobic, specific chemical, and electrostatic interactions. We approached this objective by designing experiments in which one parameter at a time is varied while the others are held constant. As discussed by Westal et al. (1999), this is impossible to realize in practice. For example, a change in pH also changes the free Ca^{2+} concentration and the properties of the organic matter. Thus, it is often difficult to distinguish among several possible causes for any particular effect.

General sorption mechanism

The results in Figure 5-5 suggest that the sorption mechanism of PFCs is similar to other surfactants (Krop and de Voogt, 2006). Thus, the interaction between sediments and PFCs is electrostatic in character. Increasing the carbon chain length increases the sorption strength. This is not caused by absorption into organic matter, but by the general property of an increase in additional entropy of the surfactant molecule. Specific chemical interactions between the PFC and sediment surface cannot be excluded, but would probably have resulted in higher K_d values. Below this is discussed in more detail.

Effect of functional group and carbon chain length

The observation that the sorption strength depends on the functional group of the PFCs (see Figure 5-5) suggests that the functional group interacts with the sediment surface. The increase of the sorption strength with carbon chain length could point at an *absorption* mechanism. However, in that case we would have expected a correlation with organic carbon, i.e. the K_d values obtained with the Rhine sediment should have been much higher compared to the other sediments. It is more likely that the increase in K_d with carbon chain length is caused by an increase in entropy of the surfactant molecule. The K_d values in the experiments performed at relatively low concentrations were lower than those obtained at the higher concentrations (see Figure 5-5). This is interesting because, in case of a linear fit of nonlinear sorption (possibly hidden in the scatter), we would expect the reverse, i.e. higher K_d values at lower concentrations. Two explanations are possible. First, because the sediments used in the 'low concentration' experiments were not washed before use, we expect the concentration of dissolved organic matter (DOM) to be higher in those experiments. Sorption to DOM would result in higher water phase concentrations and to lower K_d values. However, because we did not find evidence for *absorption* into organic matter in the sediments, we expect the effect of sorption to DOM to be small. Second, in the 'low concentration' experiments a phosphate buffer was used. It is possible that the phosphate ions compete with the surfactants for *adsorption* sites on the sediment. This process is in agreement with the proposed sorption mechanism.

Effect of pH

The effect of pH was unexpected (see Figure 5-6). Soil surfaces are mostly negatively charged. Through various possible mechanisms, soil surfaces become more positively charged at lower pH values. Therefore, we would expect the sorption strength of the anionic surfactants to increase at lower pH values. This was not observed in our experiments. A possible explanation for these results is again competition with phosphate ions. This competition is in agreement with the proposed sorption mechanism, but could have masked the effect of pH.

Effect of temperature

No trend for the effect of temperature was observed (Figure 5-6). This may not be surprising since the effect of temperature on sorption is typically small. The effect of temperature was studied because it could have provided further confirmation of the proposed sorption mechanism: In principle, a plot of $\ln(K_d)$ against $1/T$ should give a linear relationship with the slope and intercept equal to $-\Delta H/R$ and $\Delta S/R$, respectively. If the proposed sorption mechanism is correct, the slopes would be equal for PFCs with the same functional group, and the intercept would depend on the carbon chain length.

Hydrophobic interactions

For longer chain hydrocarbon surfactants, it has been hypothesised that the carbon chain bends and interacts with hydrophobic patches on the sediment (see Krop and de Voogt 2006). Because the perfluorinated chain is rigid, this mechanism is unlikely to play a role in PFC sorption. For the nonionic surfactant C8SA it is possible that both electrostatic and hydrophobic interactions play a role.

K_d values for soils and sediments

Only few literature data, mostly on the sorption of PFCs to soils, could be found (see Table 5-5). One value for the sorption of PFOS to a river sediment was found (Beach et al. 2006), and this value is in good agreement with our values. The sorption to soils was typically stronger than to the sediments in our work. Many explanations are possible for these differences. For example, different ion strengths applied in the studied could significantly have affected the measured K_d values. We did not include a comparison with K_d values of other chemicals, but the K_d values for PFCs are in the same range as observed for other anionic surfactants (see e.g. Westal et al. 1999; Krop and de Voogt 2006).

Correlation between K_d and soil organic matter

Interestingly, in a study by Dupont (2003) a strong linear correlation ($R^2=0.9465$) was found between K_d and the fraction of organic carbon. It was hypothesized that PFOA sorbs to soil through an *absorption* mechanism. This is not in agreement with our results and proposed mechanism. The reported high correlation was based on the data of four soils only. It is therefore possible that the correlation was a coincidence (Note: A correlation coefficient alone is not sufficient to judge the significance of a trend.). Using their data, we found high inverse correlations between K_d and both potassium

($R^2=0.7715$) and sodium ($R^2=0.8218$) (expressed as % of cation exchange capacity). This may point at the importance of electrostatic interactions.

The PFC concentrations tested in the Dupont study were relatively high ($> 100 \mu\text{g/L}$). It is possible that at these high concentrations positive charges become saturated with PFCs and *absorption* into organic matter becomes an important process. Because of the possibility that different sorption mechanisms are dominant at different concentration levels, it is important that environmentally relevant concentrations are used in sorption experiments. The concentrations used in the Dupont study are much higher than typical field concentrations.

Comparison with field data

The field K_d values for the Rhine estuary were similar to the experimental data observed in the laboratory. This suggests equilibrium or steady state conditions in the field. The field K_d values for PFOS in the Scheldt estuary were considerably higher than the laboratory data. A difference between laboratory and field K_d values points at the non-equilibrium conditions which typically prevail in the environment. A possible explanation for the higher field K_d values for PFOS are reduced water concentrations (caused by e.g. reduced emissions) and a relatively slow desorption of PFOS. We should add that the sediments used in the laboratory are not representative for entire water systems. Thus, the discussion above on the comparison between laboratory and field distribution coefficients is somewhat speculative.

5.4 Persistence of PFAS in sludge and sediment

5.4.1 Introduction

Despite the inherent stability of perfluorinated compounds, microbial and other degradation processes could potentially affect their long-term environmental fate. This could be particularly important in environmental compartments with long residence times, such as soil and sediment. In order to gain more insights into the potential for degradation of PFAS, we carried out biodegradation studies with sludge and sediment under aerobic and anaerobic conditions. Sludge from wastewater treatment plants was chosen since it is a potential source of PFAS contamination in soil and sediment since is a potential sink in the aquatic environment.

5.4.2 Materials and methods

The biodegradation experiments with PFAS were performed with activated sludge and with sediment, according to the OECD guideline 301D (closed bottle test, OECD 1992) with slight modifications. The activated sludge was taken from a STP in the Northwest of the city of Amsterdam (The Netherlands). The sediment was sampled from the vicinity of a company that manufactures fluorinated compounds, in an area where the microbiota is expected to be adapted to PFAS due to the high levels found there. Experimental details are presented in Annex 5.4.

5.4.3 Results

Aerobic experiments with sludge

Figure 5-7 shows the evolution of the PFAS during the biodegradation experiments performed with sludge under aerobic conditions. No significant decrease is observed for all the PFAS tested, with the exception of PFHxA and the fluorotelomers, 6:2 FTOH and 8:2 FTOH. But in these cases the concentration in the control is also reduced, which can be due to a degradation process different from biological activity or to non-sterile conditions of the control bottles. Therefore, there is no evidence that biodegradation is taking place. No biodegradation has been reported for perfluorocarboxylic acids, so the decrease in the PFHxA concentration observed in this experiment is not likely to be due to microbial activity. The reduction of the FTOH is sharper and aerobic degradation of the 8:2 FTOH has been reported in sludge by Dinglasan et al. (2004) and by Wang et al. (2005a).

Aerobic experiments with sediment

Sediment is a more heterogeneous medium than sludge, which leads to higher differences between replicates, as Figure 5-8 shows, making a bit more difficult to clearly see the evolution of the concentrations. In general no significant decrease is observed, with exception of the 8:2 FTOH. THPFOS also shows a slight decrease in the concentration. THPFOS is also a fluorotelomer, with hydrogen substitutions in the alkyl chain. Therefore is expectable to go under same degradation as the FTOH.

Ethanol was added as extra carbon source after 3 weeks, in order to enhance the aerobic biodegradation of PFAS in the experiments, as described in the literature (Wang et al. 2005a). Figure 5-8 shows almost no difference between concentrations in week 3 and 4, with exception of THPFOS. The concentration of 8:2 FTOH was already quite small in week 3 to observe a reduction.

Anaerobic experiments with sludge

The batches of experiments performed with sludge under anaerobic conditions are presented in Figure 5-9, showing again a good reproducibility of the data. Under these conditions and time intervals (9 or 15 weeks) none of the tested compounds experienced any decrease in the concentrations, which is expected since anaerobic biodegradation is less favourable than aerobic and a slower process. These results are consistent with the literature, in which there is no evidence for anaerobic degradation of FTOHs.

Anaerobic experiments with sediment

In the experiments performed under anaerobic conditions, the experimental time was elongated until 40 weeks in order to have time enough for anaerobic biodegradation, if any, to take place. It is known that sometimes microorganisms need long exposure times to certain compounds before anaerobic biodegradation could take place. This acclimation period was supposed not to be needed for the sediment used in the experiments since came from a contaminated site and the bacteria present in it should be already acclimatised to this type of chemicals. In general no decrease trend is observed for any of the PFAS tested with sediment under anaerobic conditions (Figure 5-10), with the exception of the FTOHs showed a decrease, sharper in the 6:2 FTOH in this case. Ethanol was added after 3 weeks as in the other sediment biodegradation experiments, but again no improvement was observed between weeks 3 and 4.

In order to discern if the decrease observed in the concentrations was due to degradation (also in the controls, meaning non sterile conditions) or to a non-biological process, the concentration of methane gas was determined in the headspace of bottles from experiments An S 1 and An S 2 before the extraction of the samples at 40 weeks. Methane is a product of anaerobic degradation, and it was not observed in the controls, confirming that they were sterile. Therefore the concentration decrease observed in these experiments cannot be related to biodegradation.

5.4.4 Discussion

The results presented here show that the PFAS tested in these experiments are not biodegradable under the experimental conditions used. The persistence of the tested compounds is consistent with the stability of the C-F bond of PFAS. Although the non-totally fluorinated PFAS, such as the FTOH have previously been observed to be aerobically biodegradable in sludge (Wang et al. 2005a; Dinlgasan et al. 2004) and sediment (Wang et al. 2005b), this could not be demonstrated in the present experiments. Obviously more research is needed in this field in order to elucidate which PFAS are susceptible to biodegradation, the conditions under which this can occur and the possible degradation routes followed.

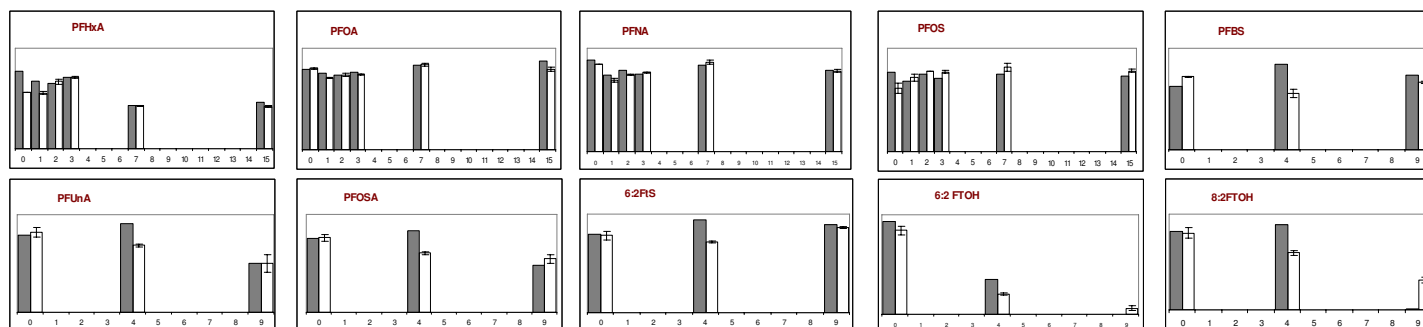


Figure 5-7 (Top). Temporal trend (in weeks) of the concentration (mg/L) of PFAS during the biodegradation experiments with sludge under aerobic conditions, corresponding to batches A L 1 and A L 2. Grey bars correspond to controls (sterile bottles) and white bars to the experiments (average).

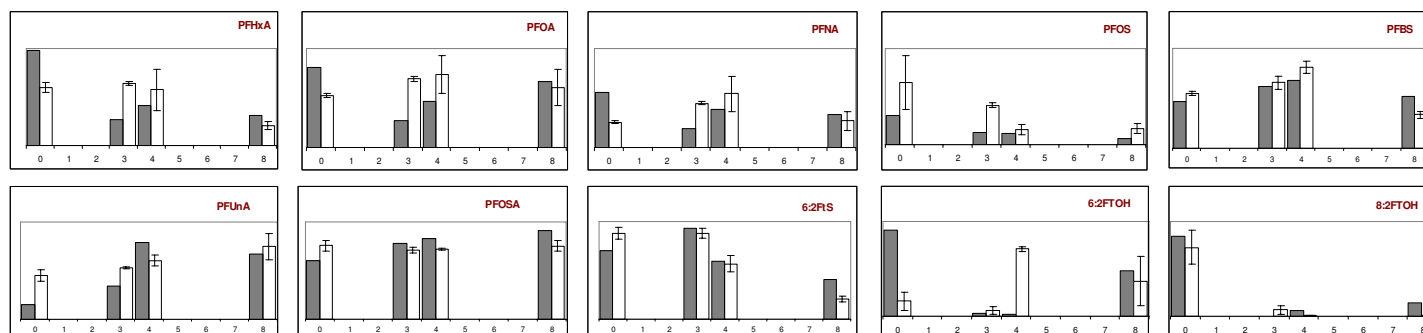


Figure 5-8 (Bottom). Temporal trend (in weeks) of the concentration (mg/L) of PFAS during the biodegradation experiments with sediment under aerobic condition corresponding to batches A S 1 and A S 2. Grey bars correspond to controls (sterile bottles) and white bars to the experiments (average).

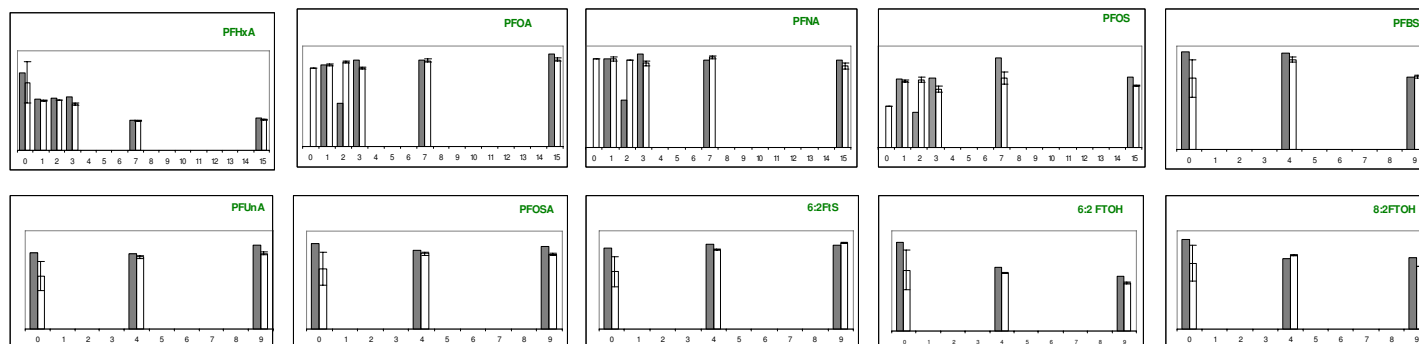


Figure 5-9. Temporal trend (in weeks) of the concentration (mg/L) of PFAS during the biodegradation experiments with under anaerobic conditions, corresponding to batches An L 1 and An L 2. Grey bars correspond to controls (sterile bottles) and white bars to the experiments (average).

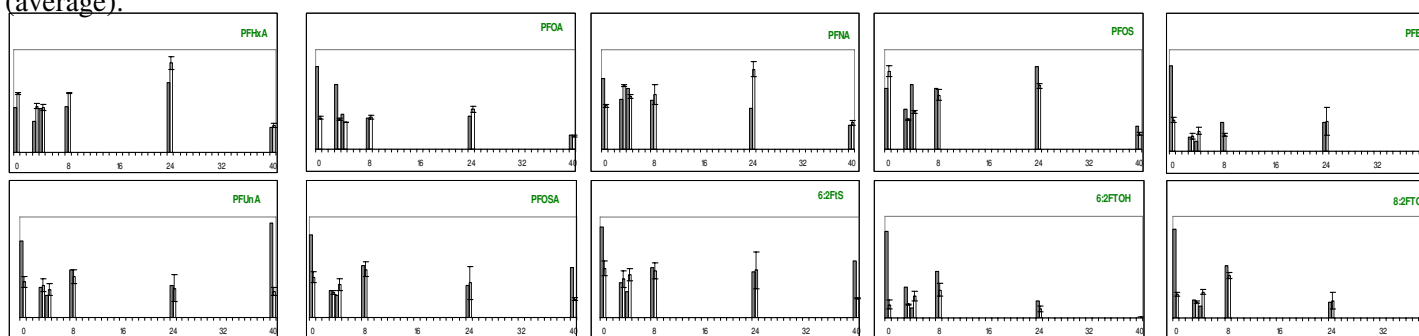


Figure 5-10. Temporal trend (in weeks) of the concentration (mg/L) of PFAS during the biodegradation experiments with sediment under aerobic conditions, corresponding to batches An S 1 and An S 2. Grey bars correspond to controls (sterile bottles) and white bars to the experiments (average).

5.5 Partitioning of PFCs between water and a bi-layer membrane: a theoretical study

5.5.1 Introduction

Although many studies have been carried out on the toxicity of perfluorinated alkylated substances (PFAS) (Hekster et al. 2003; Beach et al. 2006; Hu et al. 2003), little do we know about the mechanisms underlying their modes of action. One possible mechanism is the partitioning of PFAS in biological membranes, which causes a change in membrane structure and function (Sikkema et al. 1995). Hu et al. (2003) demonstrated by in vitro assays that perfluorooctane sulfonic acid (PFOS) increases membrane fluidity and permeability, while perfluorohexane sulfonic acid (PFHxS) and perfluorobutane sulfonic acid (PFBS) had no effect in the concentration range tested. Hu et al. (2003) explained that their results are suggestive of lipid/PFOS interactions. However, they also note that, given the highly hydrophobic nature of the fluorocarbon chain of PFOS, it is probable that this compound may be most active at the lipid/protein interfaces within membranes.

The aim of this study was to obtain further insight into the interactions between PFAS and biological membranes. As demonstrated before (Govers et al. 2002), molecular mechanics force field calculations can be used to obtain water to membrane transfer energies and the sites in the membrane, which are preferred by contaminants from an energetic point of view. In this study the molecular modeling approach was applied to the partitioning of PFAS between water and a 1,2-Dilauroyl-DL-phosphatidyl ethanolamine (DLPE) membrane model. The PFAS included in the study were the protonated and ionized forms of perfluorobutanoic sulfonic acid (PFBSH and PFBS⁻), perfluorohexane sulfonic acid (PFHxSH and PFHxS⁻), perfluorooctane sulfonic acid (PFOSH and PFOS⁻), and the corresponding carboxylic acids (PFBAH, PFBA⁻, PFHxAH, PFHxA⁻, PFOAH, PFOA⁻). Thus, the influence of carbon chain length, functional group and protonation state of the functional group were studied. Note that in the abbreviations we distinguish between the protonated and ionized states of the functional group. The relation between the commonly applied abbreviations is straightforward, e.g. PFOS = PFOSH + PFOS⁻.

The AMBER force field and parameters were used (Weiner et al. 1986; Cornell et al. 1995). FF parameters for perfluorinated compounds were taken from the literature (Watkins et al. 2001) or were derived by analogy to similar structures. The force field was calibrated by reproducing the experimentally known enthalpy of vapourization (ΔH_{VAP}) values for n-perfluorohexane and n-perfluorooctane. Control calculations were carried out to verify if the force field and setting reproduced the expected helical structure of the perfluorinated tail. In addition, the structures obtained by the force field calculations were compared to structures obtained by quantum chemical calculations. The DLPE membrane was constructed previously (Govers et al. 2002) from available crystal structure data (Elder et al. 1977). To save computational time, simulated annealing instead of full molecular dynamics was applied to selections within the large DLPE structure.

5.5.2 Methods

Force field calculations

See Annex 5.5 for relevant background on force field calculations. Annex 5.5 also includes information on the force field and parameters used, their derivation and validation and the applied software and hardware.

Structure of membrane and PFCs

The construction of the isolated molecules and liquids of perfluorinated sulfonic and carboxylic acids and the DLPE membrane is described in Annex 5.5. Both the protonated and ionized state of the PFCs were included in the calculations. To reduce computational time, three selections (A, B and C) were made in the membrane structure. Selection A was used to model PFOSH/PFOS⁻ and PFOAH/PFOA⁻ in the apolar part of the membrane. The apolar part is the area within the membrane occupied mostly by the hydrophobic membrane lipid tails. Selection B was used to model PFOSH/PFOS⁻ and PFOAH/PFOA⁻ in the polar part of the membrane. The polar part is the outer region of the membrane occupied mostly by polar functional groups of the membrane lipids. Selection C was applied to model all selected PFCs in both the polar and apolar parts of the membrane. Selection C is smaller than selections A and B and was included to further reduce computational time. The comparability of the results obtained for PFOSH/PFOS⁻ and PFOAH/PFOA⁻ in the large selections (A and B) and in selection C was used as a validation for the use of selection C. See Annex 5.5 for details on selections A, B and C.

Force field approach

A PFC was placed in either the apolar (selection A or C) or polar part (selection B or C) of the membrane, followed by a simulated annealing calculation. This resulted in several positions and orientations for each PFC in the membrane. The position and orientation with the lowest energy was selected as the preferred one. The water to membrane transfer energies of a PFC was calculated by subtracting the energy of the PFC in the membrane from the energy of the PFC in water. See Annex 5.5 for details on the starting positions and orientations and on computational settings.

5.5.3 Results

Energy of partitioning over DLPE membrane and water

Figure 5-11 shows the thermodynamic cycles for partitioning of PFOA⁻/PFOAH and PFOS⁻/PFOSH between water and the membrane. PFOS and PFOA partition mainly to water. In water, the energy of the ionized state is lower. This was found for all studied PFAS (see Table 5-6).

For the energy in the membrane, we selected the lowest energy after simulated annealing. The results in Figure 5-11 are based on the results obtained with the larger selections A and B. For PFOS, the energy of PFOSH was lower than the energy of PFOS⁻. For PFOA, the opposite was found, i.e. the energy of PFOA⁻ was lower than the energy of PFOAH. When we include all the data in Table 5-6, so also the data obtained with the smaller selection C, we find that the energy of PFASH in the membrane is always lower than the energy of PFAS⁻.

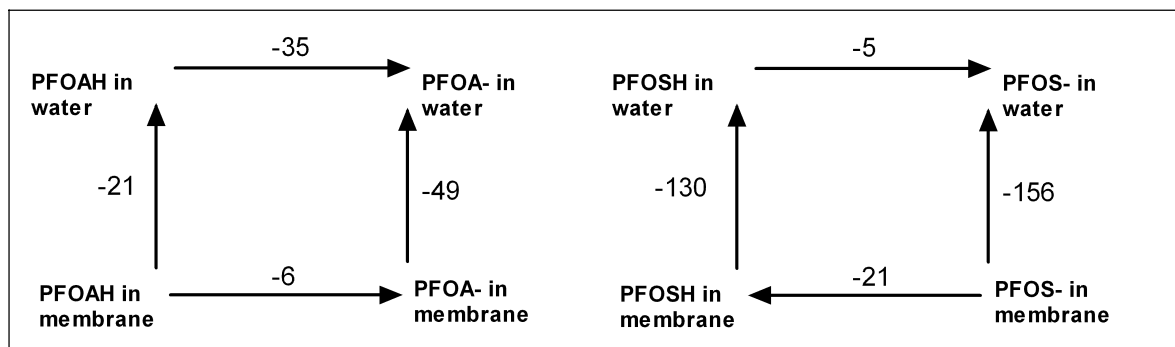


Figure 5-11. Thermodynamic cycles for PFOA⁻/PFOAH and PFOS⁻/PFOSH. Energies in kcal/mol.

Preferred position of PFAS in the membrane

Figure 5-12 shows the orientations of PFOA⁻/PFOAH and PFOS⁻/PFOSH after simulated annealing in selections A and B. For PFOS, we found that the polar starting orientations stayed at their original position during simulated annealing. The PFOS in the apolar starting orientations moved to an orientation in between the tails of the membrane. The energy of PFOSH was lower than of PFOS⁻. Similar results were obtained with the smaller selection C. A difference is that the energy of PFOS⁻ in selection C (the apolar starting position) was much lower than found for the larger selection. A closer inspection of the data revealed that in the larger selection, the sulfonate group of PFOS⁻ was directed inwards (see Figure 5-12). From these observations, we concluded that the results obtained with the smaller selection could be included safely in the study. When we include all the results from Table 5-6, we find that all PFAS follow the same trend as PFOS. However, the apolar starting positions of the shorter chain PFAS stayed close to their original position instead of moving in between the lipid tails. PFOA follows the same trend. The deviating behaviour in Figure 5-12 is caused by the relatively high energy of PFOAH in the polar part of the membrane. A lower energy was found in the smaller selection.

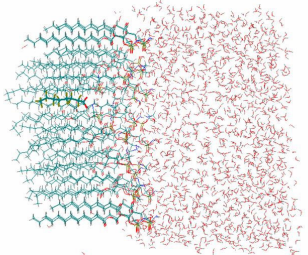
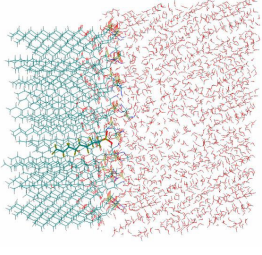
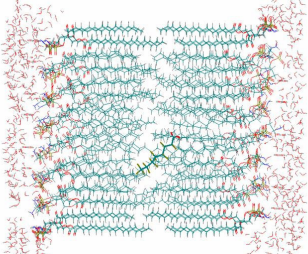
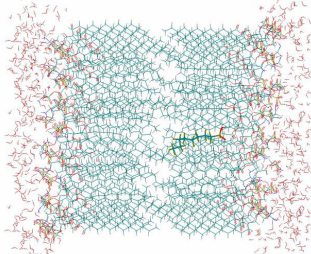
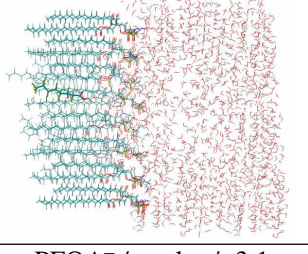
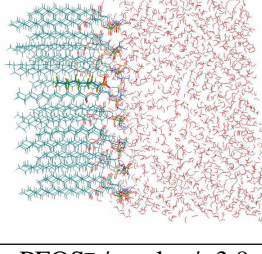
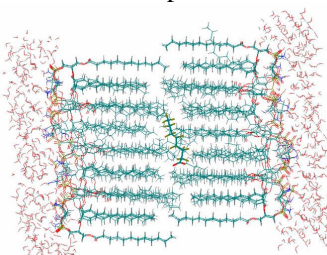
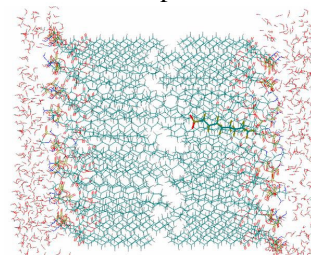
PFOA ⁻ / PFOAH	PFOS ⁻ / PFOSH
PFOA ⁻ / polar / -38.4 	PFOSH / polar / -68.3 
PFOAH / apolar / -32.2 	PFOSH / apolar / -52.7 
PFOAH / polar / -30.4 	PFOS ⁻ / polar / -47.6 
PFOA ⁻ / apolar / -3.1 	PFOS ⁻ / apolar / -3.9 

Figure 5-12. Orientations and energies of PFOAH, PFOA⁻, PFOSH and PFOS⁻ after simulated annealing. The lowest (most favourable) energy orientation is shown on top. The information with each orientation reads: ‘compound’ / starting orientation / energy (kcal/mol).

Table 5-6. Summary of the results obtained by the simulated annealing calculations of PFAS in the membrane. The results obtained with the small selection and large selections are compared. The results for the larger selections are the same as in Figure 5-12. Energy of the PFAS in the membrane (E_m) and water to membrane transfer energies (ΔE_{mw}) are in kcal/mol.

Compound	Starting orientation in		Orientation after simulated annealing	E_m	ΔE_{mw}
	large selection	small selection			
PFBS ⁻		apolar	Reorientated at starting position	-10	140
PFBS ⁻		polar	Reorientated at starting position	-20	130
PFBSH		apolar	Reorientated at starting position	-32	118
PFBSH		polar	Reorientated at starting position	-43	108
PFHxS ⁻		apolar	Reorientated at starting position	-15	175
PFHxS ⁻		polar	Reorientated at starting position	-20	170
PFHxSH		apolar	Reorientated at starting position	-44	143
PFHxSH		polar	Reorientated at starting position	-52	135
PFOS ⁻		apolar	Reorientated and moved to polar part	-32	172
PFOS ⁻	apolar		Reorientated and moved to polar part, sulfonic group directed to middle of membrane	-4	200
PFOS ⁻		polar	Reorientated at starting position	-32	172
PFOS ⁻	polar		Reorientated at starting position	-48	156
PFOSH		apolar	Reorientated at starting position	-52	146
PFOSH	apolar		Reorientated and moved to polar part	-53	146
PFOSH		polar	Reorientated at starting position	-57	142
PFOSH	polar		Reorientated at starting position	-68	130
PFBA ⁻		apolar	Reorientated at starting position	-4	72
PFBA ⁻		polar	Reorientated at starting position	-15	61
PFBAH		apolar	Reorientated at starting position	-16	12
PFBAH		polar	Reorientated at starting position	-20	9
PFHxA ⁻		apolar	Reorientated and moved to polar part	-14	56
PFHxA ⁻		polar	Reorientated at starting position	-18	52
PFHxAH		apolar	Reorientated and moved to polar part	-25	18
PFHxAH		polar	Reorientated at starting position	-26	18
PFOA ⁻		apolar	Reorientated and moved to polar part	-16	71
PFOA ⁻	apolar		Reorientated at starting position	-3	85
PFOA ⁻		polar	Reorientated at starting position	-28	60
PFOA ⁻	polar		Reorientated at starting position	-38	49
PFOAH		apolar	Reorientated at starting position	-30	23
PFOAH	apolar		Reorientated at starting position	-32	21
PFOAH		polar	Reorientated at starting position	-40	13
PFOAH	polar		Reorientated at starting position	-30	23

5.5.4 Discussion and conclusions

Partitioning of PFAS between water and sediment

The results of the calculations suggested that PFAS dissolve preferentially in water, where they are mostly ionized. This agrees with their low pK_a values (Hekster

et al. 2003). The transfer of PFAS from water into the membrane was energetically unfavourable, but less unfavourable for the (neutral) protonated states. In the membrane, PFAS tended to align with the lipids in their protonated state. So the functional group of the PFAS interacted with the polar head group of the membrane lipids and the fluorinated chain interacted with the hydrophobic lipid tails. Longer chain PFAS were bound stronger to the membrane, the water to membrane energies showed a reverse trend with chain length.

The calculated behaviour of PFAS in the membrane agrees with the discussion by Hu et al. (2003) of their experimental data (see Introduction). Although we believe that our force field calculations are reliable, there are fundamental differences between the calculated and experimental data. These issues are discussed below.

Reliability of the force field calculations

The results of this study depend on the quality of the force field molecular model. A serious problem is that experimental data for a direct comparison with the calculated data was not available. For a thorough validation of the calculations, membrane-PFAS interaction enthalpies should be known, at least for a few compounds. Nevertheless, we believe that the results obtained in this study are reasonable and for the following reasons: In a similar study, Govers et al. (2002) found reasonable agreement between calculated and experimental water to membrane transfer energies of chlorinated compounds. Our calculations are probably more reliable because we used parameters derived specifically for perfluorinated compounds and found a perfect agreement between calculated and experimental energies of vapourization for perfluorinated n-alkanes. In addition, we used simulated annealing (instead of geometry optimizations) on larger membrane selections. This means that our simulations are less likely to converge in local (high) energy minima. Also, the results obtained in our study agree with what you would expect based on chemical intuition. This holds for the preferred orientation of PFAS in the membrane and the trend observed for different PFAS chain lengths.

Limitations of the approach

Next to the technical (computational) limitations discussed above, there are more fundamental issues:

- Entropy effects were ignored in the calculation. It has been argued, however, that entropy might strongly influence the water-membrane partitioning process (Dulfer, 1996). Direct free energy calculations (including both enthalpy and entropy effects) are possible but require much more computational time. Alternatively, free energy may be calculated in a QSAR approach using the calculated enthalpy as one of the descriptors.
- The calculations were carried out for non-buffered pure water and the DLPE membrane, whereas in reality both water and the membrane will be different. Although we do not expect this to change the conclusions of the current calculations, the calculations should be repeated using different types of bi-layered membranes. In addition, mono-layered membranes as found in liver should be included in the study, because PFOS distributes mainly into the liver (Hekster et al. 2003, Beach et al. 2006).
- The focus of our study was on the position of PFAS in the lipid bilayer. Another mechanism in membrane toxicity is the interaction of contaminant with functional

proteins in the membrane. The interaction between contaminants and enzymes can also be studied using force field calculations (Van Roon et al. 2005).

5.6 References to chapter 5

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6. Exposure assessment of the European environment

6.1 Introduction

The purposes of the PERFORCE sampling campaign were to:

- Describe the discharge of PFCs into the European aquatic environment;
- Study the wind driven distribution of PFC over the European continent;
- Establish baseline concentrations for PFCs in the aquatic environment;
- Assess PFC near point sources using a combination of chemical and biological tools;
- Assess the exposure to PFCs in Europe.

To approach these goals with the limited resources available, a targeted sampling program was designed with the following foci: PFC sources, time trends of PFCs in the aquatic environment, spatial distribution of PFCs in the aquatic environment, PFC bioaccumulation in aquatic food chains, and PFC contamination around a point source. The investigation of PFCs in air was undertaken in cooperation with scientists outside of the PERFORCE consortium, and the results from these studies will also be briefly summarised in this report.

The source characterisation sampling effort focused on sewage treatment plants (STPs). This strategy was adopted as STPs are expected to be the major vector of diffuse releases of PFCs into the aquatic environment. Influent water, effluent water, and sewage sludge were analysed in order to assess the diffuse emissions of PFCs by European urban societies, the effectiveness of the STPs at eliminating the PFCs, and the discharge of PFCs into European waterways and – via agricultural use of sewage sludge – to land.

The purpose of the time trend studies was to assess the historical exposure of the European aquatic environment to PFCs. Two appropriate sample sets were identified and could be accessed. The first was an archive of cod liver oil collected from North Sea cod. The second was a dated sediment core from the Baltic Sea. Hence, two important European marine environments that could be expected to have been particularly affected by PFC releases were studied. In addition, two different sampling matrices based on different PFC accumulation principles were employed, one biotic and one abiotic.

To determine the baseline concentrations of PFCs in the aquatic European environment, careful consideration was given to the most appropriate matrices. It was decided to focus on water, since model assessments of PFC behaviour in the environment suggest that these chemicals are largely associated with water. Major European rivers were sampled as close to the river estuary as possible, under the assumption that the rivers would collect and integrate a large fraction of the emissions of PFCs within the drainage basin. In this way, it was possible to generate information on both the emissions of PFCs for a large portion of Europe as well as to assess the PFC levels in major European waterways. Additional water samples from the major European seas were also collected. Furthermore, sediment samples were collected from a number of sites. In addition, PFCs were analysed in biota, both fish and marine mammals. Together, these data constitute a broad assessment of exposure to PFCs in Europe.

The wind driven distribution of PFCs over the European continent was studied with the help of PERFORCE associated partners at the GKSS Research Institute in Germany and Lancaster University in the UK. They developed suitable sampling and analytical methods together with PERFORCE partners and employed these methods to sample for the PERFORCE chemicals at a range of land-based and marine locations. Thanks to the efforts of the associated partners, outstanding progress was made in this field and the PERFORCE partners were able to focus their efforts on other project tasks.

The bioaccumulation of PFCs was assessed in detail in the point source case study (see below). This advantage of this approach was that the higher PFC levels in this environment facilitated the collection of high quality data and thereby the assessment of the bioaccumulation behaviour. Some biota samples from the European seas were also analysed to assess exposure of marine organisms to PFCs.

The case study of a point source was conducted in the Western Scheldt estuary. The Western Scheldt is a well described study area and an example of a well mixed (non-stratified) estuary. Aqueous discharges from a major PFC manufacturer using the electrochemical fluorination process enter the estuary from an industrial area west of the city of Antwerp. PFCs were studied in the physical environment (water and sediment) and in biota, as well as using the bioanalytical methods developed in the project. In the interest of a process oriented perspective, the results of the case study are incorporated into several parts of the report including the sub-chapters on sediments and bioaccumulation below and the chapter on bioanalytical methods.

All samples were analysed for the PFCs prioritised within PERFORCE, with the exception of the telomer alcohols which were only analysed in air. As a rule, not all PFCs could be quantified in a given matrix. This depended on the specific tendency of each chemical to accumulate in the matrix and the sensitivity of the method for the chemical in the matrix.

6.2 Sources of PFCs in Europe: Sewage treatment plants

6.2.1 Sampling and methodology

The source characterisation sampling effort in the Perforce monitoring program focused on sewage treatment plants (STPs). This strategy was adopted as STPs are expected to be the major vector of diffuse releases of PFCs into the aquatic environment. Six STPs were selected from 4 countries giving geographical coverage of Europe (Spain, Sweden, The Netherlands, United Kingdom). Four of the STPs (one in each country) served large urban centres (Cadiz, Stockholm, Amsterdam, Lancaster). A second STP in Sweden (Alingsås) served only households and was a control for the influence of commercial and industrial activity on PFCs in STPs. The Nijverdal STP in The Netherlands was introduced as it receives a large part of its wastewater from textile industries (advanced textiles/outdoor fabrics), including those that apply 'fluor' type finishing. Releases to the sewage system may occur during application/washing steps in the manufacturing process.

The concentrations in the influents and effluents were determined after filtration. 100 ml of the filtrate was extracted on C18 cartridges and cleaned up using the Powley method (Powley et al., 2004). The filters were treated according to the Powley method modified for sediment analysis (Powley et al., 2005). Separation,

identification and quantification was performed using LC-MS/MS. As the chapter on QA/QC illustrates, the analysis of sewage sludge and wastewater is particularly challenging. Therefore, further study of the comparability of the results is necessary in order to evaluate the possible uncertainty associated with the absolute magnitude of the data produced. However, since they were all produced by one lab, the data should be internally consistent.

6.2.2. Concentrations in influents and effluents

As can be seen from Figure 6-1, STP influents contain relatively high levels of PFCs. The reference STP (Alingsås, S) in general exhibited the lowest levels of PFCs, but detectable levels of several PFCs were found in both the influent and the effluent of this STP.

PFOS (range: 10-200 ng/L), PFOA (20-65 ng/L), PFNA (8-45 ng/L) and FTS (15-300 ng/L) were most abundant in the dissolved phase of the STP influents. The particulate phase of the influents contributed significantly to the overall influent concentration in the case of the carboxylic acids, but less for the sulfonates and for PFOSA. In general, within a specific STP the order of abundance of the sulfonates in (the dissolved phase of) influents was PFOS>6:2FTOS>PFBS>PFHxS. For the carboxylic acids this order was PFOA>PFNA>PFHpA>PFDA>PFUA>PFDoA. Samples from the Nijverdal STP contained the highest levels of C7-C12 acids.

The concentrations in the STP effluents are shown in Figure 6-2, with relatively high concentrations of sulfonates (C4: 2-50 ng/L, C6: 2-50 ng/L, PFOS: 15-200 ng/L) and PFHpA (4-18 ng/L), PFOA (20-110) and PFNA (2-19 ng/L). Very high concentrations (560 ng/L of PFOA, 380 ng/L of PFNA, 200 ng/L of PFDA) of PFCAs (C7-C12) were observed in the effluent of the Nijverdal STP.

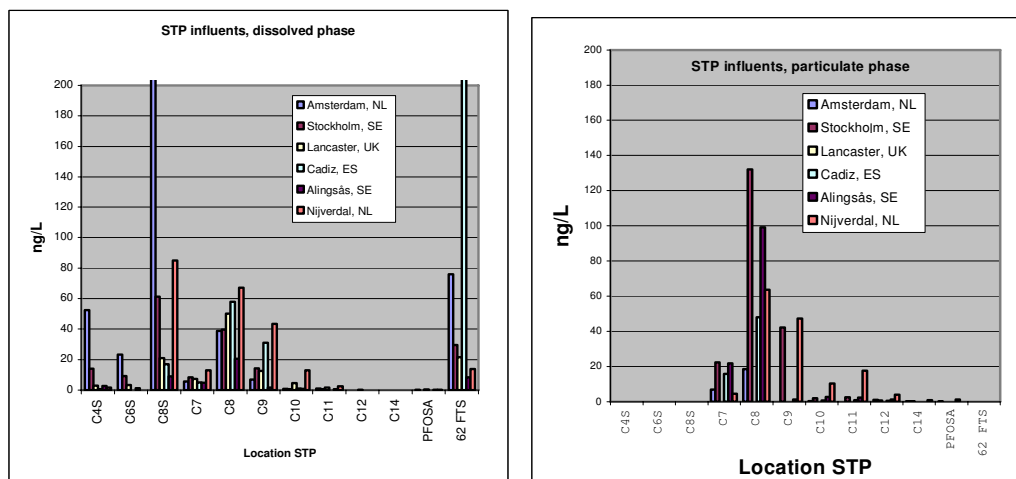


Figure 6-1. Concentrations of perfluorinated compounds in influents from six European sewage treatment plants

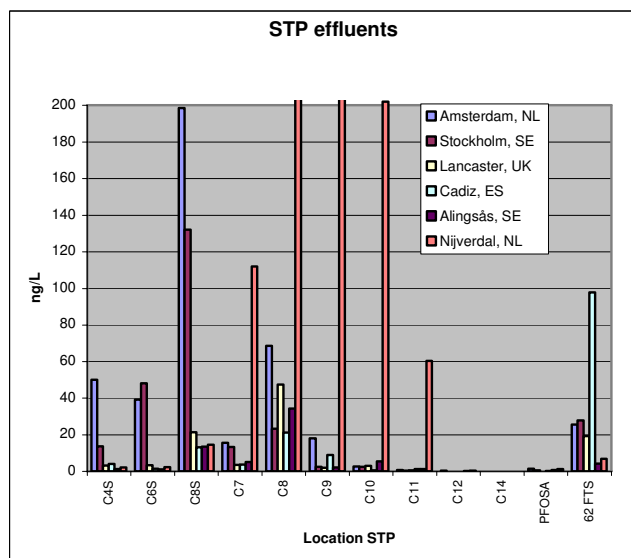


Figure 6-2. Concentrations of perfluorinated compounds in effluents from six European sewage treatment plants

6.2.3 Removal efficiency

Comparison of the concentrations in the effluent and the influent provides insight into the efficiency of removal of the PFCs in the treatment process. In the Nijverdal STP, which, contrary to the other STPs in this study, was strongly influenced by influents from textile industries, the effluent contained much higher concentrations of PFCAs than the influent. Concomitantly with this finding relatively high concentrations of PFCAs were found to be present in the STP sludge (see below). No clear explanation is available for this phenomenon yet. Possibly the plant uses PFCA containing materials in its treatment process. The pH of both the influent and effluent samples from this STP was much higher (pH~9) than in other STPs. The possible influence of this condition remains unclear so far.

The removal efficiency, E , of the STP can be expressed as:

$$E_j = \left(1 - \frac{C_e}{C_i}\right) * 100\%$$

where C_e is the total concentration in the effluent of STP j , and C_i is the total concentration in the STP influent. The total concentration is calculated from the contributions of the dissolved phase and the particulate phase.

Two special cases can be distinguished. First, in the case that the concentrations in the effluent were below the limit of detection (LOD), the LOD was used to calculate the value of E . If both the influent and effluent concentrations were below the LOD, then no value of E was calculated. Second, in the case that the concentration in the effluent was above that in the influent, the corresponding value of E_j was set to zero.

The removal efficiencies are tabulated in Table 6-1. The STP Nijverdal was considered as a special case because, with a relative high input of industrial sewage, the high alkalinity of the samples (pH 9), and the high concentrations observed in the

effluent. The mean removal efficiencies were therefore evaluated for all STPs, and also for all excluding the Nijverdal STP. In general, large differences in removal efficiencies were observed between the STPs. The removal efficiencies for the fully fluorinated sulfonic acids was poor, with mean values ranging between 0 and 47%. For 6:2FTS a somewhat higher removal efficiency was observed (~40%). For the carboxylic acids the removal efficacy of STPs was higher, ranging from 20-80%, showing the following order: C12>C9=C7>C8>C11>C10. For PFOSA large differences in efficiency were observed between STPs, with a mean value of 28%. The general observation is that PFC removal efficacy of STPs is poor to moderate compared to e.g. non-ionic surfactants or estrogenic compounds (de Voogt et al., 2006).

Table 6-1. PFC removal efficiencies of sewage treatment plants ^a

	C4S	C6S	C8S	C7	C8	C9	C10	C11	C12	C14	PFOSA	62 FTS
Amsterdam	4	0	3	0	0	0.	0	11	70	na	0	66
Nijverdal	0	na	83	0	0	0	00	0	59	na	0	50
Stockholm	3	0	0	56	86	96	8	90	88	na	0	6
UK	0	2	0	51	5	84	34	55	71	na	85	11
Alingsas, Nohaga	47	0	0	81	71	23	0	54	85	na	54	49
Cadiz	0	na	23	82	80	71	56	0	86	na	0	64
Mean	9	na	18	45	40	46	16	35	76	na	23	41
Mean-Nijverdal ^b	11	1	5	54	49	55	20	42	80	na	28	39
Std-Nvd ^b	20	1	10	33	42	41	25	37	9	na	40	29

^a na, could not be evaluated; ^b excluding the STP Nijverdal values

6.2.4 Concentrations in sewage sludge

The concentrations of PFCs in sewage sludge from the STPs are shown in Figure 6-3. The highest concentrations were observed in the Nijverdal STP, with levels up to 150 ng/g dry weight for PFDA. With the exception of the Nijverdal STP, levels of the PFCAs ranged between 0 and 25 ng/g, with the following general order:

C9>C8=C10>C11>C12>C14.

PFOS and 6:2FTS concentrations were the most abundant of all PFCs, ranging between <LOD and 110 ng/g for PFOS and <LOD and 80 ng/g for FTS.

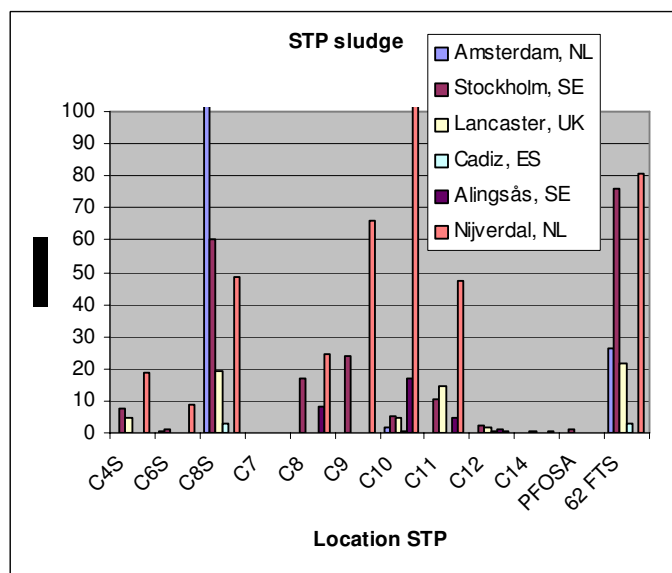


Figure 6-3. Concentrations of perfluorinated compounds in sludge from six European sewage treatment plants

6.2.5. Distribution of PFCs between the particulate and dissolved phases

The distribution of PFCs between the dissolved and particulate phases in the influents is depicted in Figure 6-4. The mean fraction in the particle phase of the effluent was calculated for each compound in each STP. If a value of one of the phases was below the LOD and this LOD was also below the value observed for the corresponding other phase, half of the LOD value was used for the calculation. If the value was below the LOD in both phases, or if the value for one phase was below the LOD but this LOD was higher than the value measured in the corresponding other phase, then no fractional distribution was calculated.

For carboxylic acids with higher carbon chain lengths (above C10), the fraction bound to particles increased with increasing number of carbon atoms. Below C10 no statistically significant trend was observed for the PFCAs. For the sulfonic acids, the scatter in the data was too large to draw statistically sound conclusions. The PFOSA appears to sorb more to particulate matter ($\phi = 0.56$) than PFOS ($\phi = 0.16$).

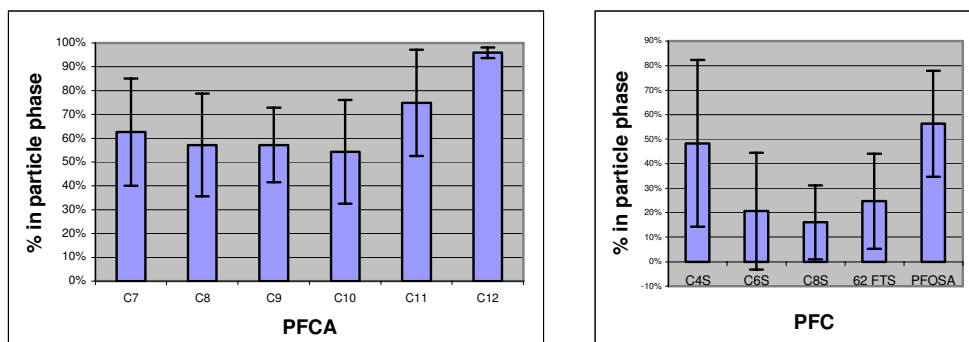


Figure 6-4. Distribution of PFCs between the dissolved phase and the particulate phase in STP influents. Left hand panel: PFCAs; right hand panel: Sulfonates and sulfonamide.

6.2.6 Conclusions

The results of the monitoring of several STPs in Europe show that PFCs are present in domestic sewage and that STPs are insufficiently effective in removing these. Hence, STPs probably serve as point sources of PFCs both for the aquatic ecosystems (through effluent discharges) and the terrestrial environment (through application of sewage sludge in agriculture) in Europe.

6.3 Time trends of PFC levels in the European environment

The time trends of PFCs in the European environment focused on the aquatic environment. Two sets of samples were analysed, namely a sediment core from the Baltic Sea and archived cod livers collected from cod from the North Sea.

6.3.1. Time trend from a sediment core from the Baltic Sea

A sediment core was collected from the outer reaches of the Stockholm Archipelago the spring of 2006 (N59.20.7; E18.39.4; depth 36m). This is an area of sediment accumulation, with an accumulation rate of $\sim 1 \text{ cm yr}^{-1}$. Due to the anoxic bottom conditions, the sediments are laminated.

The sediment core was transported to the laboratory, dated via counting of the well-defined varves, and sliced into segments representing single years. The sediment slices representing the years 1958, 1965, 1968, 1975, 1980, 1985, 1990, 1995, 2000, and 2005 were then subjected to analysis for PFCs using the method described in Annex 3-2.

Neither PFBS, 6:2-FTOH, nor any PFCAs were detected in the sediments. This was expected, as these compounds either do not readily sorb to sediments or are present in low concentrations in the water column.

PFOS and PFOSA, on the other hand, were detected in all of the more recent sediment layers, starting in 1990. The results are shown in Figure 6-5. For PFOS, an increase with time was observed, whereby the functional form was well fitted by both a linear regression ($C_s = 0.07004 y - 139.14$, $r^2 = 0.998$, where C_s is the sediment concentration in ng/g and y is the year) and a log-linear regression ($\ln C_s = 0.1097 y - 219.5$, $r^2 = 0.962$). For PFOSA a curvilinear relationship was obtained, with increasing concentration through the later 80s and early 90s, a plateau in the late 90s, and a lower concentration in 2005. The levels in the sediment layers deposited before 1990 were below the LOQ.

The time trend for PFOS in Baltic Sea sediments can be compared with the time trend measured in guillemot eggs collected in the northern Baltic Proper (Hölmström et al., 2005). This study also reported increasing concentrations, whereby it was possible to follow this further back in time to the late 1960s. The doubling time for the PFOS levels in guillemot eggs was 7-10 years, which agrees quite well with the 6.3 years obtained from the log-linear regression of the sediment core data. However, the guillemot egg data showed a decrease in PFOS levels between 1997 and 2003. This was not reflected in the sediment core and it is not consistent with the production pattern of PFOS. Holmström et al. suggested that this aspect of their time trend may have been related to changes in the feeding behaviour of the guillemots.

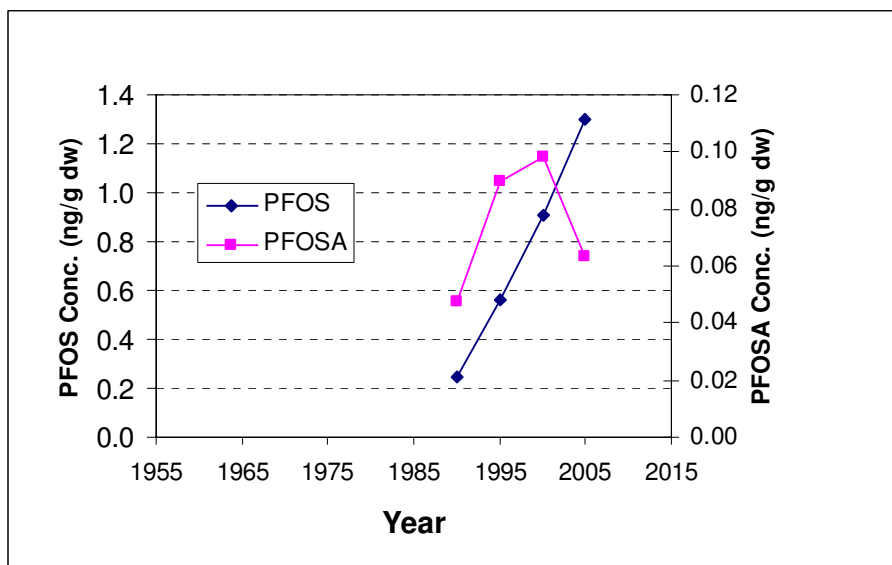


Figure 6-5: Time trend of PFOS and PFOSA in a sediment core from the Stockholm Archipelago

The only other time trend data available for PFCs is for the liver of polar bears from the North American Arctic (Smithwick et al., 2006). These data show an exponential increase in PFOS levels between 1972 and 2002 with a doubling time of 10-13 years. This is consistent with the observations in the Baltic Sea sediment core. The shorter doubling time in the Baltic Sea may be related to differences in historical release patterns in the Baltic watershed compared to the sources impacting the Arctic, or to differences in the relative contributions of PFOS and volatile PFOS precursors to levels at the two locations.

The only time trend data for PFOSA reported in the literature are for the polar bear livers described above (Smithwick et al., 2006). Over the time period 1972-2002 the PFOSA levels decreased, and in one of the two polar bear populations this decrease was significant. This stands in contrast to the results of this study, which show a marked increase through much of the study period. Nevertheless, both studies show that the time trends of PFOS and PFOSA are clearly different, suggesting that there is no correlation between the historical emissions of PFOS and its precursors on the one hand and the precursors of PFOSA on the other.

In undertaking this study, uncertainty existed about the usefulness of sediments as archives of environmental levels of PFCs. It was questioned whether the sediment:water partition coefficients would be high enough to conserve the contaminant concentration over many years under ongoing vertical movement of porewater. Little was known about the sorption behaviour of PFCs, and this continues to be the case. However, recent work has shown that significant sorption of perfluoroalkyl sulfonates occurs for PFOS, while sorption of PFOA and shorter chained perfluorinated carboxylic acids is insignificant (Higgins and Luthy, 2005). The fact that the PFOS time trend derived from this sediment core is consistent with time trend data from archived biological samples suggests that sediments can be used to study historical trends of PFOS in the environment. In the case of PFOSA, a consistent temporal signal can also be measured in sediment, but it would be prudent

to further explore whether this signal is modified by transformation processes resulting in either the formation or the loss of PFOSA in the sub-surface sediment.

6.3.2 Time trend from archived cod livers from the North Sea

The cods were sampled annually in the Southern North Sea for a Dutch national monitoring program on POPs. Each sample consisted of 25 individual cod livers (when available) in order to reduce the impact of natural variations on the final result. Upon arrival in the laboratory, the length and weight of each individual fish was recorded. The livers were removed and pooled. The samples were stored at – 20°C. The cod liver samples represented the years 1979, 1981, 1982, 1988, and every year from 1990-2005 with the exceptions of 1995, 1996, 1998, and 2004.

The samples were analysed for the project PFCs using the method described in Annex 3-1. The performance of the analytical method was not satisfactory. The high lipid content of the cod liver resulted in matrix problems which led to a broad range of standard recoveries (38-181 % for PFOS and 41-81 % for PFOA) as well as high limits of quantification. As a consequence, only PFOS and PFOSA could be quantified, and not in all samples. An interlaboratory comparison of 3 other cod liver samples between 2 project partners revealed differences in PFOS and PFOSA levels of up to a factor of 2.5 (see Chapter 4). This indicates that the matrix also had a deleterious effect on the accuracy of the results.

The time trends derived from these data are illustrated in Figures 6-6 and 6-7. For both PFOS and PFOSA, a trend of increasing concentrations with time was observed. However, the trends were not consistent, but instead were characterised by large year to year variability. This may be attributable to the analytical difficulties outlined above. In any case, it can be concluded that, without further improvements in the analytical method, cod liver is not a useful matrix for time trend analysis of PFCs.

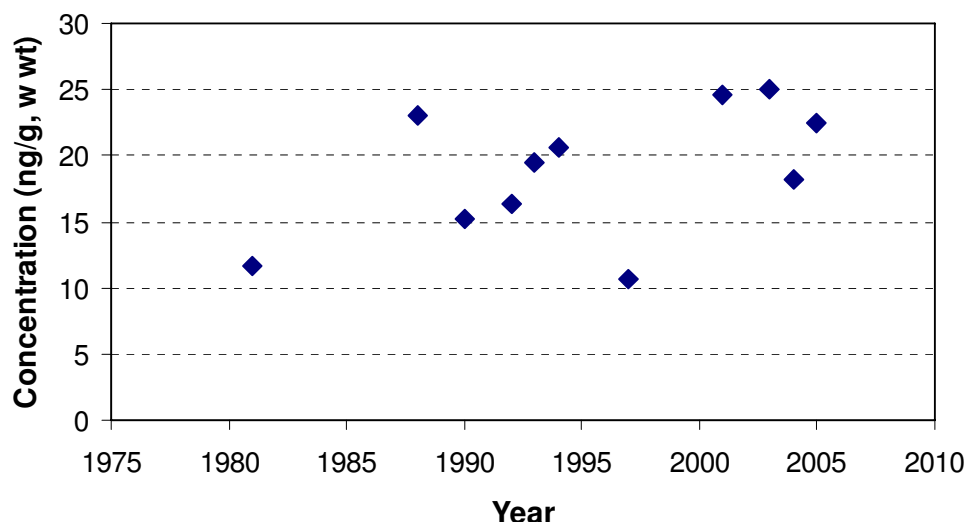


Figure 6-6: PFOS concentrations in archived cod liver samples from the North Sea

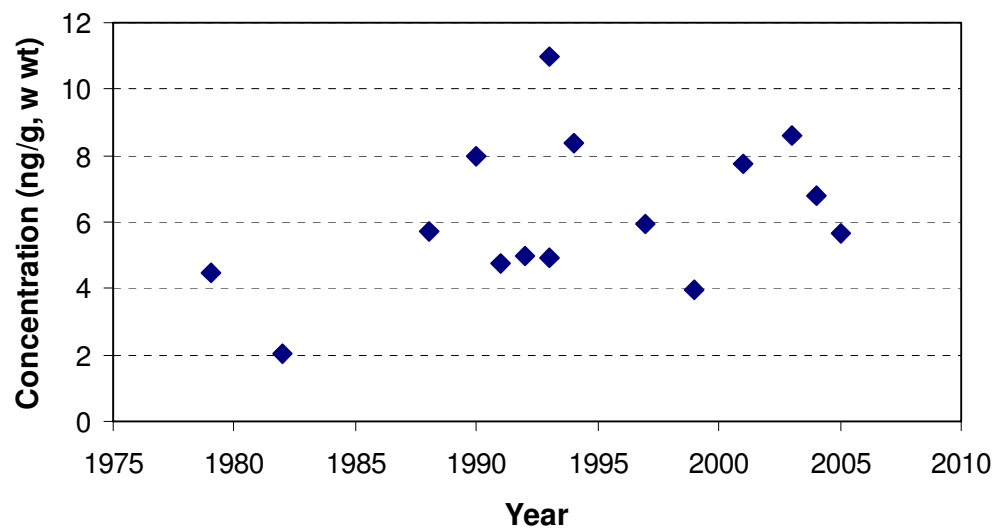


Figure 6-7: PFOSA concentrations in archived cod liver samples from the North Sea

6.4 Baseline concentrations of PFCs in the European environment

6.4.1 Levels in Water

To study the spatial distribution of PFCs, water samples were collected from major European rivers. The rivers sampled and their watersheds are shown in Figure 6-8. The samples were collected either by PERFORCE members or by qualified scientific personnel contacted by project members. All samplers were provided with empty sample bottles, a 2 sample bottles filled with laboratory water as transport and field blanks, a sampling protocol, and detailed instructions on where and how to collect, store, and ship the samples, as well as how to complete the sampling protocol (see Annex 3-5). Sampling was carried out during the autumn of 2005 (for the Baltic rivers as well as the Elbe, Po, and Danube) or the winter of 2006. Periods of unusually high or low river flow were avoided. The samples were collected as close to the river mouth as possible to capture the PFC load from as large a drainage basin as possible. At the same time, sampling of estuarine water was avoided as a rule to preclude dilution of the river water with saline water. The samples were not collected immediately downstream of potential sources, as far as these could be identified. The water was taken from the sub-surface but well above the river bottom. 500 mL PP or PE bottles were filled to the top, sealed, stored in a refrigerator, and sent by courier to the laboratory. Field blanks were collected by filling the laboratory water from the field blank bottle into the sampling device, letting it stand for 5 minutes, and then transferring this water back to the field blank bottle.

In addition to the rivers, samples were collected from several European marine waters including the Black Sea, the Mediterranean Sea, the Gulf of Riga, the Bothnian Sea, and the Baltic Proper. The sampling procedure was similar. The samples were either collected near the shore (Black Sea, Mediterranean) or from research vessels (Baltic Sea). In the latter case, the samples were collected from bottles on the rosette.

In the laboratory the samples were extracted and analysed using the procedure described in Annex 3-3. The interlaboratory comparison carried out with this work yielded no useful information as one of the comparison laboratories had trouble with the method and the second had LODs that lay above the concentrations in the samples. Consequently, there is uncertainty associated with the accuracy of the data produced, although the dataset can be expected to be internally consistent. In addition, issues relating to possible losses of PFCs during sample storage have been reported recently. Work has been initiated to constrain the uncertainties in the data reported here, but this work was not complete at the time of reporting.

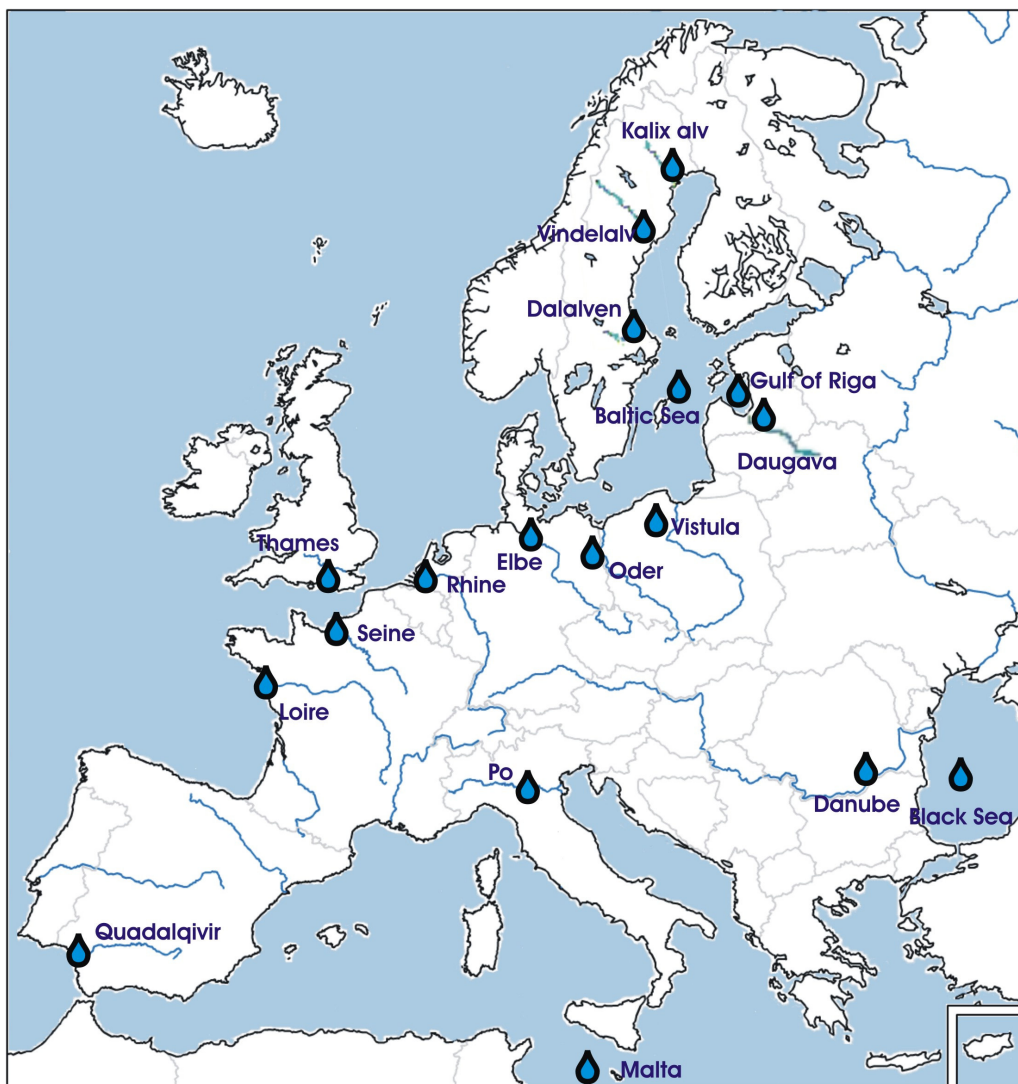


Figure 6-8: Map showing the water sampling sites and the watersheds captured by the PERFORCE river water sampling program

Several PFCAs (PFHxA, PFHpA, PFOA, and PFNA) could be quantified in most samples. The recovery of the labelled PFOA internal standard ranged from 33-98 %, whereby it was >50 % for 80 % of the samples. The transport blanks and field blanks were well above the laboratory blanks due to contamination of the laboratory water used in these blanks. Nevertheless, the field blanks and transport blanks were lower than the levels of the PFCAs in most of the water samples. The levels in the Scandinavian rivers and the Daugava were below the limit of detection (set to 3 x the laboratory blank). The data were, however, included in the interpretation below for illustrative purposes. For many of the rivers, several samples were taken close to each other (e.g. on a transect across the river, or from opposite banks). The PFCA concentrations in these samples frequently differed by <20 % and the difference seldom exceeded a factor of 2. The PFCA concentrations in the marine samples were close to or below the limit of detection.

The PFOSs could not be quantified in the water samples due to poor and highly variable recoveries of the labelled PFOS internal standard. Despite extensive experience in analysing water with this method in 2 PERFORCE laboratories, this study showed that the method is not sufficiently robust to be applied to a wide range of different waters.

The PFCA concentrations are summarised in Table 6-2. Mean values are given for each river. PFHxA and PFOA had the highest level of contamination. The concentrations ranged over a factor of >30, with very low levels in the 3 Scandinavian rivers samples, comparatively low levels in Eastern European rivers, and higher levels in Western European rivers. The PFOA concentrations were mostly in the range observed in urban surface waters in Minnesota and Japan (Simcik and Dorweiler, 2005; Saito et al., 2004), but lower than reported for the lower Great Lakes and surface waters in New York State (Boulanger et al., 2004; Sinclair et al., 2006). The ratio of PFHpA:PFOA levels in this study were lower (0.06-0.4 vs. 0.53-0.9 in Minnesota) (Simcik and Dorweiler, 2005).

Table 6-2: Concentrations of PFCAs in European River Water (ng/L)

	PFHxA	PFHpA	PFOA	PFNA
Dalälven	<0.94	0.36	<0.97	<0.14
Vindelälven	<0.58	0.20	<0.65	0.22
Kalix Älv	<0.58	0.26	<0.85	<0.14
Elbe	15.3	2.7	7.6	0.27
Oder	2.2	0.73	3.8	0.73
Vistula	2.3	0.48	3.0	0.36
Po	<0.30	2.5	21	0.18
Danube	3.0	0.95	16.4	0.27
Daugava	<0.90	0.53	<1.37	0.22
Seine	14.5	3.9	10.6	1.43
Loire	3.2	0.87	3.3	0.42
Thames	32	4.1	23	0.79
Rhine	18.2	1.80	11.6	0.55
Quadalquivir	6.2	1.58	4.6	1.02

While several samples were analysed for most of the rivers, only one sample was available for one of the major rivers, the Rhine. However, it was possible to corroborate the PFOA results with water samples that were collected somewhat further downstream and analysed by a different PERFORCE laboratory within a separate program (see Table 6-3). These data indicate that there are similar levels of PFOA in the Rhine, Meuse, and Scheldt, and that PFOS concentrations are similar to PFOA concentrations in these rivers.

Table 6-3: Concentrations of PFOA and PFOS in several rivers sampled in the Netherlands (ng/L)

Code	PFOA	PFOS
Meuse 1	19	n.d.
Meuse 2	22	22
Meuse 3	57	20
Meuse 4	25	56
Meuse 5	33	16
Rhine 1	17	25
Rhine 2	24	30
Rhine 3	16	30
Scheldt 1	29	43
Scheldt 2	25	38
LOD	12	10

The only other data found on PFC concentrations in European rivers were also for the Rhine. Skutlarek et al. (2006) reported 8 ng/L for PFOA in the Rhine close to the German/Dutch border, which compares favourably with the PERFORCE data (11.6 ng/L), while their data for PFHxA were considerably lower (3 vs. 18.2 ng/L).

The data in Table 6-2 were used to estimate the annual flux of the PFCAs. The average concentration was multiplied by the mean annual river discharge as reported in the EEA water quality and water quantity databases. While the limitations of calculating an annual flux based on one sampling time point are recognized, this approach does provide interesting insights into PFCA fluxes and potential sources in Europe.

The riverine flux is a particularly informative quantity for PFCAs. Given the strong tendency of PFCAs to associate with water and the negligible retention of PFOA on solids (Kaiser et al., 2005), it can be expected that PFCAs will not be retained to any great extent in terrestrial or benthic environments, with the exception of ground-/porewater. They will move with water. Most of the PFCA emissions are to water (Prevedouros et al., 2006), and some of the emissions to air and land will be rapidly transferred to surface water or shallow groundwater and thereby move rapidly through the hydrographic system. Therefore, the river flux of PFCAs should give a rough (lower) estimate for total emissions of PFCAs in the watershed.

The total fluxes obtained by adding the fluxes in all of the studied rivers are given in Table 6-4. These fluxes can be compared with the global emissions estimates for PFCAs given by Prevedouros et al. (2006). They estimated a global PFOA emission of ~75 tonnes/year for the year 2005. While they give no information on the spatial distribution of these emissions, one might expect that Europe could account for ~20 tonnes/year. The drainage basin of the rivers sampled in this study accounted for ~35 % of the European continent (excluding Iceland, Russia, the Ukraine, and the Belarus). Although it included a greater proportion of the more industrialized areas, the sampling program did not account for sources in estuaries or on the coast (e.g. Hamburg, London, and The Netherlands were not captured in the sampling of the Elbe, Thames, and Rhine, respectively). Thus, the PFOA flux of 5.6 tonnes/year from this study are consistent with the emissions estimates of Prevedouros et al..

Table 6-4: Total flux (t/y) of PFCAs in the rivers studied (see Table 6-2)

	PFHxA	PFHpA	PFOA	PFNA
Flux	2.6	0.58	5.6	0.16

For PFHxA, agreement was poor. Prevedouros estimated total global emissions of 1 tonne/year for the year 2000, while the riverine flux from this study was 2.6 tonnes/year. The emissions of PFHxA were estimated to be 0.5 % of the emissions of PFOA, while the riverine flux was approximately 50 %. The disagreement between the emissions estimates and the observations is further emphasized by the fact that the concentrations of PFHxA were greater than the concentrations of PFOA in several of the rivers studied (see Table 6-2). Since there is virtually no retention of PFOA in soils (see above), it is unlikely that this discrepancy can be explained by a strong retention of PFOA in the watershed. Rather, it suggests that the sources of PFHxA were underestimated or that there are other sources that were not accounted for in the work of Prevedouros et al..

The distribution of the riverine flux between the different watersheds yields further insight (see Figure 6-9). The Scandinavian rivers and the Daugava made a negligible contribution to the total flux as a result of their low levels. The contribution of the French, Polish, and Spanish rivers was also small due to the moderate concentrations and/or water discharge rates. The PFCA flux was dominated by the Danube, Rhine, Po, and Elbe, whereby there were marked differences in the relative contribution for the different acids. PFHpA and PFOA had largely similar patterns, with a large contribution from each of the Danube, Rhine, and Po. It would appear reasonable that these chemicals had common major sources.

The distribution of PFNA also showed similarities to PFHpA and PFOA. The smaller rivers made a more predominant contribution, which may be a reflection of the proximity of the measured concentrations to the limit of detection. The other major difference was the smaller contribution of the Po to the PFNA flux, which may suggest that there is a strong PFOA source in the Po watershed, one that has a comparatively low level of PFNA.

The distribution of PFHxA was clearly different than for the other chain lengths. The flux in the Po River was negligible, also compared to smaller rivers such as the Seine, Loire, and Vistula, which further highlights the unusual situation in the Po. The PFHxA flux was dominated by the Rhine, with a large contribution from the Elbe, particularly compared with PFOA and PFNA. This suggests the presence of strong PFHxA sources in these two watersheds.

In summary, the study of the spatial distribution of PFCAs in river water indicates that the emissions of PFHxA, PFHpA, and PFOA in Europe are of the order of 10, 2, and 20 tonnes annually. The Danube and Rhine watersheds are particularly important sources, whereby the Elbe and Po also make a significant contribution for PFHxA and PFHpA/PFOA, respectively.

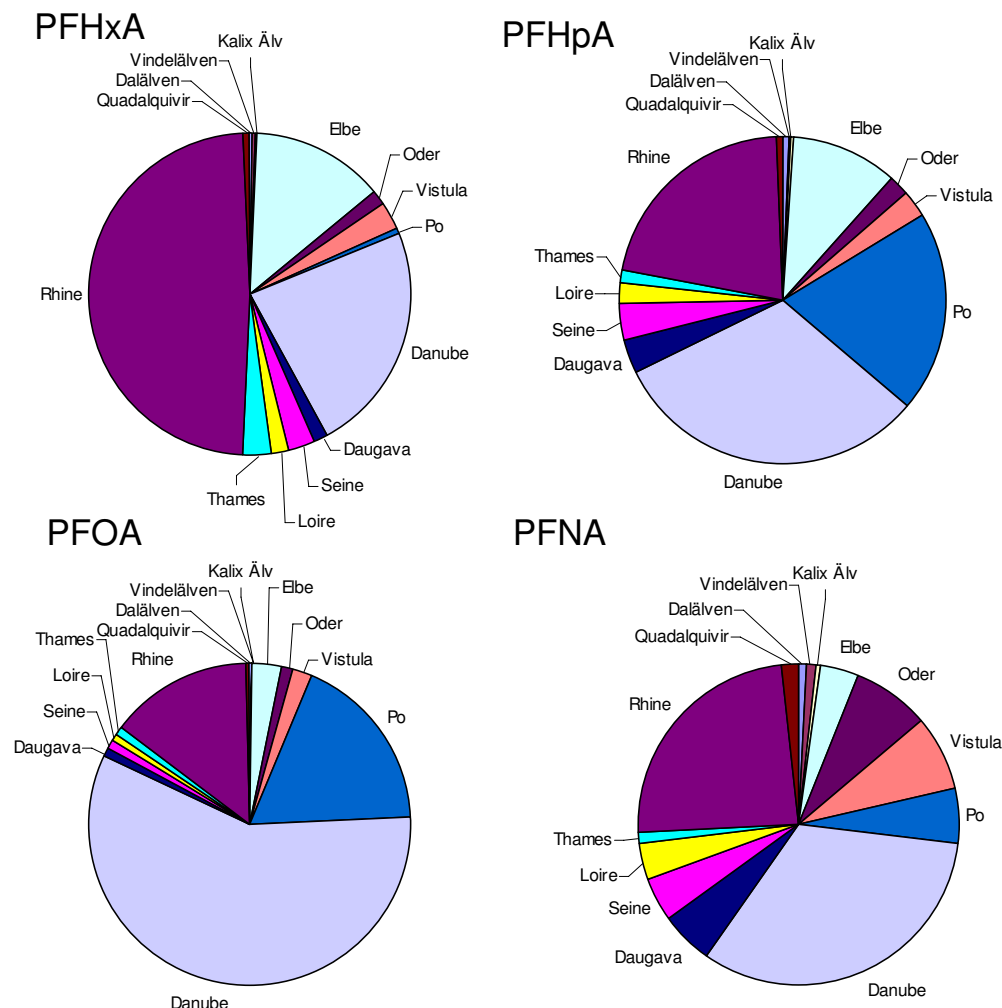


Figure 6-9: Distribution of the PFCA fluxes amongst the different rivers sampled.

6.4.2 Levels in sediments

A total of 14 sediment samples were collected from five different locations in Europe: the Western Scheldt estuary (border Belgium-Netherlands), the river Rhine at Rotterdam and IJmuiden, both in The Netherlands, Tago Bay in Portugal, the river Danube in Austria and the river Po in Italy (see Figure 6-10). Sampling was carried out in 2004 and 2005 by qualified personnel from Perforce partners or scientific colleagues. Samples were sent cooled or frozen to the analyzing laboratories and stored until analysis in the freezer. The sediments were extracted, purified and analysed according to the modification of the method of Powley et al. (2005) described in Annex 3-2.

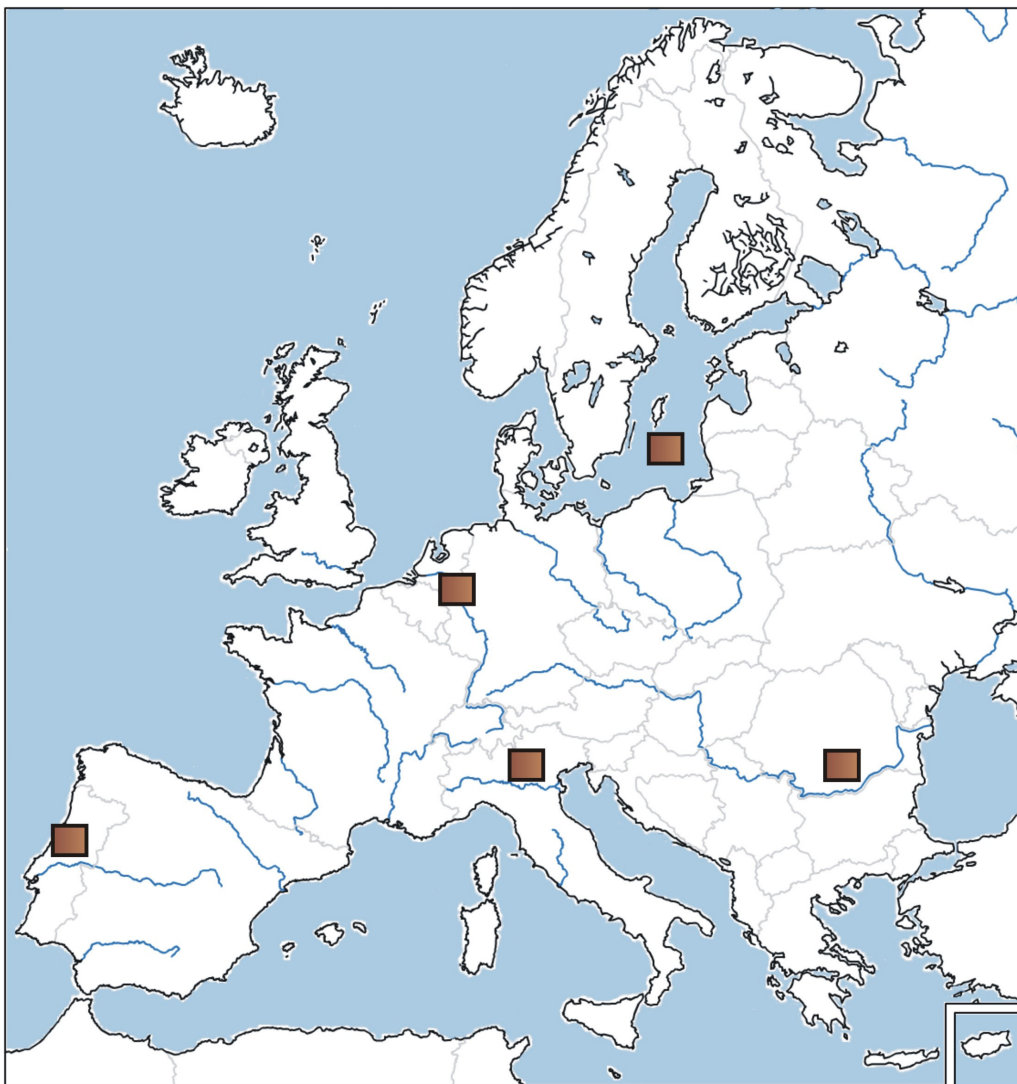


Figure 6-10: Sediment sampling sites.

For QA purposes, two additional sediments were obtained from the Perforce partner coordinating the QA/QC work package and analysed according to the same protocol. The recovery of the labelled PFOA and PFOS internal standards ranged from %, whereby it was >.. % for .. % of the samples[MM1].

The results of the sediment analysis are shown in Table 6-5. The river sediments from eastern and southern Europe appear to have lower concentrations of PFCs than those from the river sedimentation areas in The Netherlands (see Figures 6-11 and 6-12). The levels in the Scheldt estuary increased from the mouth (S01, ZL08) to the upstream station (S20). The concentrations of the sulfonates varied between levels below the LOD to up to 2 ng/g dry weight. Of the sulfonates, 6:2FTS was the only compound found to be present at levels above the LOD in most sediment samples (see Fig. 6-11). PFOS was generally not found above the LOD of 0.5 ng/g dw, with the exception of two samples from the Scheldt estuary (0.6 and 1.5 ng/g) and one sample from IJmuiden containing a relatively high concentration of 14 ng PFOS/g dw. Carboxylic acids were generally not found at levels above the lod, with some notable

exceptions. In several samples from the Scheldt estuary, C7-C11 acids were found to be present, with PFOA and PFNA being the most abundant, and levels ranging up to 20 ng/g dw. C12A and C14A were sporadically observed in Dutch sediments, in particular from two locations in the Scheldt close to industrial sites (S4 and S20). At these sites the levels of sulfonates were not elevated. In sediment from the Po river a relatively high concentration of C11A (0.7 ng/g) was observed.

The concentrations observed are in agreement with data reported in the literature. Higgins et al. (2005) reported sulfonate concentrations ranging between <0.06 and 5.5 ng/g dw and total carboxylate concentrations between <0.05 and 6.5 ng/g for San Francisco Bay. In a screening study along the Dutch coast, the levels of PFOA and PFOS ranged between <0.4 and 3.0, and <0.4 and 4.6 ng/g, respectively (Schrapp et al., 2004; de Voogt et al., 2005).

Considering the concentrations reported in water samples from this monitoring study, it appears that for the Po, Danube and Rhine rivers field sorption coefficients for PFOA vary roughly between 20 and 80, indicating that sediment is probably not a major sink for these types of compounds. Unfortunately, this coefficient could not be evaluated for other PFCs because the levels in sediment were generally below the LOD.

Table 6-5: Concentrations of PFCs in sediments collected from Scheldt, Rhine, Po, Danube and Tague rivers

Location	Sulfonates					Carboxylates						
	C4	C6	C8	62 FTS	PFOSA	C7	C8	C9	C10	C11	C12	C14
Western Scheldt, ZL08	0.35	<0.10	<0.54	1.07	0.01	<0.22	<1.34	<2.54	0.01	0.08	0.01	0.03
Western Scheldt, S01	<0.39	<0.11	<0.62	0.39	0.25	<0.25	<1.55	<2.94	<0.01	<0.06	<0.02	<0.10
Western Scheldt, S4	0.06	<0.10	<0.57	<0.86	<0.01	0.86	14.51	4.15	0.21	0.08	0.01	0.02
Western Scheldt, S07	<0.33	<0.09	<0.53	0.18	0.00	<0.21	<1.32	<2.50	0.01	<0.05	0.02	<0.09
Western Scheldt, S20	<0.34	<0.10	<0.54	<0.82	<0.04	0.48	16.75	18.28	0.85	0.69	0.08	0.03
Western Scheldt, <90 um, si no. 1144 (BROC)	0.26	0.24	0.59	0.71	0.32	0.10	0.60	<4.17	<0.02	<0.08	0.00	0.01
Western Scheldt 2005/1128	<0.55	<0.16	1.46	2.85	0.05	<0.36	<2.19	<4.14	0.29	0.20	0.06	0.14
Ijmuiden, 200610311	0.73	0.09	13.96	1.40	0.12	<0.36	<2.21	<4.18	0.29	0.22	0.48	0.09
Netherlands, Nieuwe Waterweg,	<0.31	<0.09	<0.50	<0.75	0.02	<0.20	<1.25	<2.36	0.03	<0.05	0.01	0.00
Danube 1	0.09	<0.12	<0.67	0.92	<0.01	<0.27	0.20	<3.19	<0.01	0.10	0.01	<0.11
Danube 2	<0.30	<0.09	<0.48	0.60	<0.01	<0.20	<1.21	<2.28	<0.01	<0.05	0.00	<0.08
Italy, Po	0.15	<0.13	<0.71	2.05	0.05	<0.29	<1.77	<3.34	0.02	0.68	0.02	0.02
Portugal, Lisbon, Tajo Bay #16	<0.57	<0.16	<0.91	<1.37	<0.01	<0.37	<2.28	<4.31	<0.02	<0.09	0.01	0.00
Portugal, Lisbon, Tajo Bay #33	<0.54	<0.15	<0.86	<1.30	<0.01	<0.35	<2.16	<4.08	<0.02	<0.08	<0.03	0.04

6.4.3 PFC concentrations in cod liver

One focus of the biota sampling in the Perforce project was cod liver (*Gadus morhua*). Samples were collected from the Baltic Sea, Fladen (in the Kattegat), and the Southern North Sea. Sampling information on the North Sea samples is provided in Chapter 6-3. The Fladen and Baltic samples were collected in 1997 by the Swedish Museum of Natural History.

Cod liver is a lipid rich (ca 50% lipids) matrix which is difficult to analyze with the methods that were available. Due to matrix effects, PFNA and PFUnA could not be determined in the North Sea samples, and PFHxA was not determined in any of the samples. PFBS concentrations were all <LOQ.

PFOS and PFOSA concentrations were highest in the North Sea samples (see Figure 6-11). PFCAs were only present in a small number of Fladen and Baltic samples, and then at low concentrations (typically 20-30% of the PFOS concentrations). The cod liver PFOS results agree with concentrations in cod livers from the Baltic and flounder livers from the Copenhagen area reported earlier (Kallenborn et al., 2004). As expected, the levels were considerably lower as compared to fish livers from freshwaters or highly contaminated sites (Hoff et al., 2005). However, the PFC concentrations in cod were also low compared to other fish liver samples (see Annex 6-3). This may be explained by the low PFCA levels in the water samples from the Baltic Sea which were among the lowest encountered in the Perforce water monitoring program (PFSAs were not determined), which is surprising considering the fact that the Baltic waters are influenced by large contaminant discharges from the surrounding countries. Cod feeds on lower organisms such as shrimps, crabs, mollusks, small fish, polychaetes and cephalopods, which are presumably the determinant for the low cod liver contamination levels. However, the importance of the various prey for cod liver contamination remains to be determined.

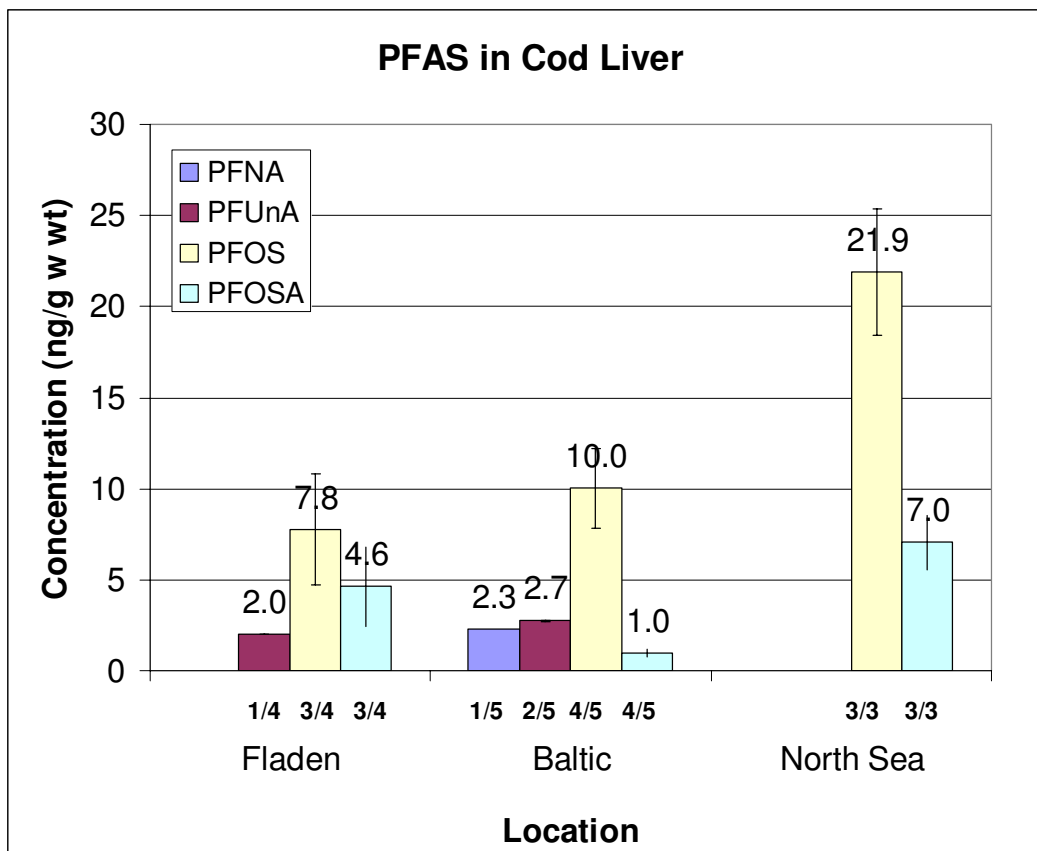


Figure 6-11. Average PFC concentrations in cod liver samples. North Sea samples are the average of samples collected in 2003-2005, whereas the Fladen and Baltic samples were taken in 1997. PFNA and PFUnA were not determined in the North Sea samples due to matrix effects. Numbers below the bars represent the number of values above LOQ/total number of samples. Error bars represent standard deviation of the means.

6.4.4. PFC concentrations in marine mammals

Marine mammals occupy the highest trophic positions in the marine food web and may therefore be more affected by pollutants in comparison to other animals. Different research studies have focussed on perfluorinated alkylated compounds in marine mammals like seals, dolphins, whales and polar bears, from a wide range of geographical regions: from remote regions such as the Canadian and Norwegian Arctic, to more industrialized, coastal areas like Florida and the Baltic Sea (Kannan et al., 2001, Kannan et al., 2002a, Martin et al., 2004, Bossi et al., 2005). However, information on the distribution of PFAS in marine mammals from Western and Eastern Europe is very scarce. Therefore, we assessed the PFAS levels in different marine mammal species, from harbour seals and harbour porpoises to sperm whales, originating from the coastline of Western and Northern Europe (Belgium, France, the Netherlands and Scandinavia). We also investigated harbour porpoises from the Black Sea (Ukraine).

The samples in the present study were analysed according to the method of Hansen et al. (2001). The analysis of biological tissue yielded the best results in the interlaboratory comparisons (see Chapter 4), so one can assume that quality of the

data is likely high. However, the laboratory that analysed the marine mammal samples did not participate in the worldwide ILS.

Perfluorooctane sulfonic acid (PFOS) was the predominant compound in all organisms and in all tissues. An overview of all hepatic PFOS concentrations measured in PERFORCE is given in Table 6-6 for each species and each location analyzed. Almost all PFOS concentrations were below 1000 ng/g wet wt.

Table 6-6: Hepatic PFOS concentrations (ng/g wet wt) in various marine mammals from the European marine and estuarine environment.

Species	Location	n	mean $\pm$ SD (range)
<i>Phocoena phocoena</i> (harbour porpoise)	Southern North Sea	48	93 $\pm$ 86 (12 – 395)
<i>Phoca vitulina</i> (harbour seal)	Southern North Sea	24	155 $\pm$ 145 (<10 – 530)
<i>Lagenorhynchus albirostris</i> (white-beaked dolphin)	Southern North Sea	7	130 $\pm$ 150 (14 – 445)
<i>Physeter macrocephalus</i> (sperm whale)	Southern North Sea	6	36 $\pm$ 13 (19 – 52)
<i>Balaenoptera physalus</i> (fin whale)	Southern North Sea	1	<10
<i>Lagenorhynchus acutus</i> (white-sided dolphin)	Southern North Sea	2	16 $\pm$ 13 (7.0 – 26)
<i>Halichoerus grypus</i> (grey seal)	Southern North Sea	6	88 $\pm$ 82 (11 – 230)
<i>Cytophora cristata</i> (hooded seal)	Southern North Sea	1	<10
<i>Stenella coerulealba</i> (striped dolphin)	Southern North Sea	2	11 $\pm$ 0.33 10.8 – 11.2
<i>Phoca vitulina</i>	Wadden Sea (The Netherlands)	24	175 $\pm$ 105 (46 – 490)
<i>Phocoena phocoena</i>	Iceland	8	38 $\pm$ 14 (26 – 67)
<i>Phocoena phocoena</i>	Denmark	7	270 $\pm$ 170 (130 – 620)
<i>Phocoena phocoena</i>	Baltic Sea (Germany)	7	535 $\pm$ 355 (230 – 1150)
<i>Phocoena phocoena</i>	Finnmark (Norway)	3	123 $\pm$ 39 (87 – 165)
<i>Phocoena phocoena</i>	Nordland (Norway)	8	115 $\pm$ 49 (71 – 225)
<i>Phocoena phocoena</i>	Southwest coast (Norway)	8	345 $\pm$ 250 (80 – 750)
<i>Phocoena phocoena</i>	Black Sea (Ukraine)	31	330 $\pm$ 350 (33– 1800)

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 wet wt; Van de Vijver et al., 2005) and in liver tissue of a harbour porpoise from the Ukrainian Black Sea (1790 ng/g wet wt; Van de Vijver et al., in press). 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 (Kannan et al., 2001, Giesy and Kannan, 2001). Annex 6-4 allows a comparison between the data found in this study and hepatic PFOS concentrations in

marine mammals from other locations. 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.

The influence of gender and age was also investigated. Only in a few cases were significant differences ($p < 0.05$) obtained. Mean hepatic levels in female harbour porpoises and seals from the southern North Sea were significantly higher than in males (Van de Vijver et al., 2003). A similar trend, although not significant, was seen in porpoises from the Black Sea. Juvenile harbour porpoises from the southern North Sea showed higher hepatic PFOS concentrations than adults ($p < 0.05$). Similarly, juvenile porpoises from the Black Sea had higher levels in liver, kidney and blubber, but lower in brain tissue ($p > 0.05$). The lack of clear age or sex-related differences in concentrations of PFOS is a general observation in all biomonitoring surveys (Kannan et al., 2001, 2002b). Only a few studies have reported significant differences in some species like the polar bear (Smithwick et al., 2005). 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 than males, there might be potential transfer from mother to offspring as foetuses from different species showed much higher PFOS concentrations than their mothers. The hepatic concentration in a porpoise foetus from the Norwegian Barents Sea contained 224 ng/g wet wt, the highest concentration of all samples analyzed from that region, while its mother had only 87 ng PFOS/g wet wt (Van de Vijver et al., 2004). A female porpoise foetus from the Black Sea region, contained high PFOS concentrations in both kidney (1371 ng/g wet wt) and brain (92 ng/g wet wt). The mother had strikingly low PFOS concentrations in all tissues, with levels of respectively 17 and 21 ng PFOS/g wet wt in kidney and brain. These observations point out the need for a better understanding on how these chemicals behave.

Concentrations of other perfluorinated compounds like the perfluorinated carboxylates (PFCAs) were fairly low in all species and all tissues analyzed. In general, PFCAs were present at detectable concentrations in those samples with the highest PFOS concentrations. Concentrations generally decreased with increasing chain length for all PFCA homologues. A remarkable difference between harbour porpoises and seal 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.

Perfluorobutane sulfonate could only be detected in spleen tissue from harbour seals, at concentrations ranging from 1.74 to 3.28 ng/g ww. So far, no clear explanation can be given to the fact that PFBS was detected, although at very low concentrations, in spleen tissue, and it is not known what the possible consequences might be for the animal.

Trophic position and feeding ecology might be factors which could explain some of the variation in concentrations of perfluorinated compounds seen in marine mammals. Therefore, possible relationships between PFAS concentrations and stable nitrogen ($^{15}\text{N}/^{14}\text{N}$) and carbon ($^{13}\text{C}/^{12}\text{C}$) isotope ratios were explored. The use of stable isotope ratios has evolved into a powerful tool to study element cycles and various biological, physical and chemical processes in different research areas (De

Niro et al., 1981; Kelly, 2000). 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. 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).

The results indicate that the $^{15}\text{N}/^{14}\text{N}$ 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 such as sperm whales, fin whales, striped dolphin and white-sided dolphin showed low $^{13}\text{C}/^{12}\text{C}$ ratios and also had lower PFOS concentrations than inshore species (Figure 6-12). A significant difference in the mean $\delta^{13}\text{C}$ values between the two Norwegian populations of harbour porpoises was also detected in the PFOS concentrations. Apparently, porpoises feeding in more inshore areas had higher hepatic PFOS levels. These results are consistent with previous data suggesting that coastal regions are more polluted than offshore areas (Kannan et al., 2001).

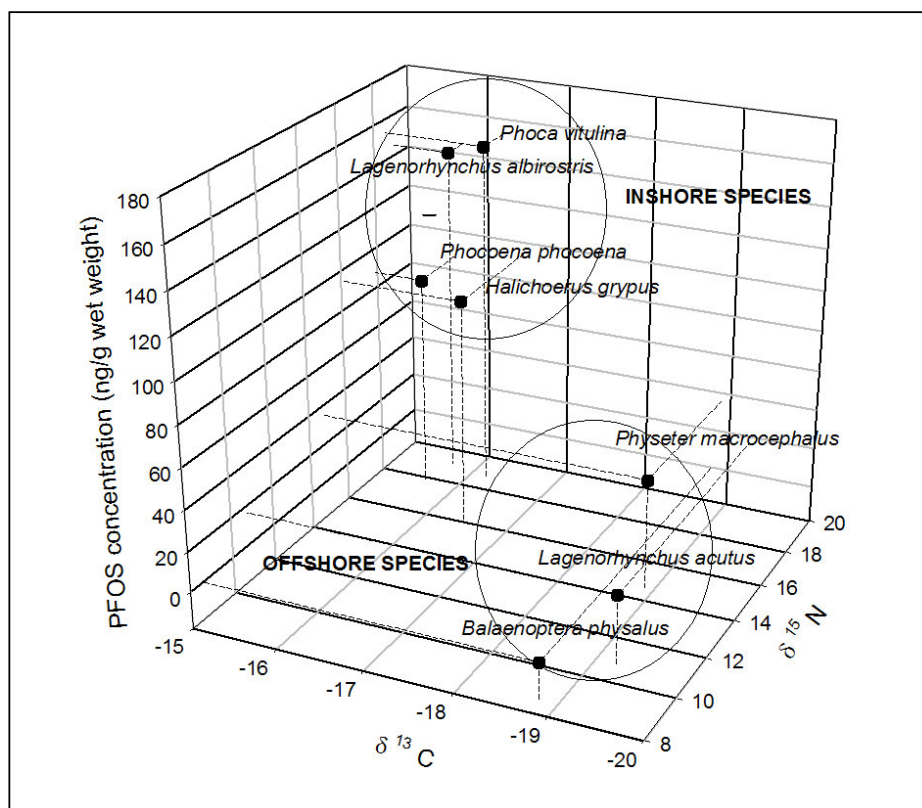


Figure 6-12: Stable isotope ratios ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) versus mean hepatic PFOS concentrations in seven marine mammal species from the southern North Sea.

6.4.5 PFCs in European air

One of the main hypotheses for the widespread detection of PFCs in remote locations is the long-range transport of neutral, volatile ‘precursor’ PFCs in the atmosphere, followed by deposition and transformation into less volatile ionic species such as PFOA and PFOS, which are not expected to travel atmospherically (Ellis et al., 2004). Therefore, measurement of PFAS in air is important to the understanding of their transport and environmental fate. Prior to the PERFORCE project, there were no data for PFAS levels in European air. A few data were available for North America and Japan, as shown in Annex 6-5, Tables 1 (neutral PFAS) and 2 (ionic PFAS). A number of air measurement studies were performed by the associated PERFORCE members, Lancaster University and GKSS Research Centre Geesthacht, in order to fill this data gap.

Air samples were collected using high-volume air samplers employing sampling modules containing glass-fibre filters (GFFs, particle phase), and glass columns with a polyurethane foam (PUF)/XAD-2/PUF sandwich (gaseous phase; Jahnke et al., 2006a). Typical outdoor air volumes required for analysis range from 600-1500 m³. GFFs and PUF/XAD2/PUF columns were analyzed separately to obtain information on phase partitioning.

All air samples were analyzed for volatile, neutral PFCs, with a selection of GFF samples halved for analysis of both neutral and ionic PFCs. Volatile PFCs were extracted from air samples by cold-column immersion with ethyl acetate, and were analyzed by gas chromatography-mass spectrometry in the positive chemical ionisation mode (GC/PCI-MS) using single ion monitoring (SIM), with subsequent analysis in negative chemical ionisation (NCI) mode for confirmation (Jahnke et al., 2006a). Ionic PFAS were extracted from GFFs by sonication in methanol, and were analyzed by liquid chromatography/time-of-flight mass spectrometry (LC-TOF-MS) using electrospray ionisation in the negative ion mode (ESI-) (Berger and Haukas, 2005).

Air samples were collected from several field sites in Europe, including 2 urban and 3 rural locations: 2 sites from the UK: Hazelrigg (54.050N, 2.800W, semi-rural) and Manchester (53.500N, 2.220W, urban); 2 sites from Germany: Waldhof (49.530N, 8.480E, rural) and Hamburg (53.550N, 9.970W, urban); 1 site from Norway: Kjeller (59.980N, 11.050E, rural). Additionally, air samples were taken from indoor locations in Tromsø, Norway (69.660N, 18.950E).

The mean concentrations of the individual analytes at the air sampling locations are listed in Tables 6-7 (neutral PFCs) and 6-8 (ionic PFCs), with minimum and maximum concentrations given in round brackets. These data are reported in more detail elsewhere (Jahnke et al., 2006b; Berger et al., 2005; Barber et al., in preparation). The ratio of analyte detection to the number of samples is shown in square brackets. In Table 3, Σ FTOH and Σ FOSA/FOSE concentrations are calculated as the sums of individual analytes in each sample.

Considering the data for the neutral ‘precursor’ PFCs at outdoor sites, Σ FTOH concentrations were a factor of 4-10 times higher than Σ FOSA/FOSE concentrations, with 8:2 FTOH as the dominant analyte followed by 6:2 FTOH and 10:2 FTOH. This distribution pattern was the same at all outdoor European sampling sites, with the exception of the rural Norwegian site with lower Σ FTOH and higher Σ FOSA/FOSE, resulting in a ratio of 1. The predominant FOSA/FOSE was typically N-Me-FOSE, followed by N-Et-FOSE. 10:2 FT olefin was also detected but could not be accurately quantified using the current analytical method. Generally levels of PFAS were slightly

higher in urban areas than rural sites. Compared to data published for North America, outdoor FTOH levels are slightly higher in Europe, whilst levels of FOSAs / FOSEs are much lower. This may relate to different use patterns in the two different markets, or may be an artifact of comparing more recent European data with older North American data, since the production of PFOS-based chemicals, including FOSA/FOSEs, has been phased out since 2002. The results from the limited Norwegian indoor air study showed much higher concentrations of both Σ FTOHs and Σ FOSA/FOSEs than were found in outdoor air, and the Σ FOSA/FOSE concentrations exceeded those reported from a larger Canadian study.

In addition to land-based measurements, a sampling campaign was performed on board of the German research vessel POLARSTERN. The first samples during expedition ANTXXIII-1 in October / November, 2005 were taken between Bremerhaven, Germany and the Biscay area (channel between the European mainland and the UK). They showed high FTOH concentrations with up to 176 pg/m³ for 8:2 FTOH. This compares with the mean value of 119 pg/m³ measured in Hamburg city centre (max. 275 pg/m³). Levels of 6:2 FTOH (157 pg/m³) even exceeded those determined in the Hamburg city center where mean concentrations were 66 pg/m³ (max. 149 pg/m³). 10:2 FTOH was found in comparable concentrations in Hamburg and on board of the POLARSTERN. Analogous to the German sampling campaign, NMeFOSE and NMeFOSA were the dominating FOSAs / FOSEs. The ship-based measurements and levels of FOSAs / FOSEs determined in Hamburg were in good agreement. Concentrations of airborne PFAS rapidly decreased along the latitudinal gradient between Germany and Capetown, South Africa (Jahnke et al., 2006c).

Considering ionic PFCs, several compounds were present in the particle phase of UK air samples including PFBS, PFOS, 6:2 FTS, and 6-10 carbon chain length PFCAs. Therefore it is possible that non-volatile ionic compounds might directly undergo atmospheric transport on particles from source regions. Indeed, the highest concentration of all analyzed compounds, including the precursors, was found for PFOA. The concentrations of PFCAs were found to be higher at the semi-rural Hazelrigg site than at Manchester. The Hazelrigg site may have been affected by a fluoropolymer production plant within 40 km of the Hazelrigg site. However, the concentrations measured at Hazelrigg were orders of magnitude lower than those observed at a fluoropolymer production facility in North America. The levels of ionic PFAS at the rural Norwegian site were significantly lower than found in the UK. Compared to data published from Japan and North America, the concentrations of PFOA in the UK were similar to one location in Japan, and the concentrations in Norway were similar to other much lower reported values. The levels of PFOS at Manchester were the highest reported anywhere to date. A number of ionic PFCs were also detected in indoor air samples, including 6-10 carbon chain length PFCAs, although the levels were not significantly elevated above outdoor levels.

In summary, in the first studies of PFCs in European air samples, PFOA was often the predominant analyte found in the particulate phase, and 6:2 FTOH and 8:2 FTOH were the prevailing analytes found in the gas phase, with many other compounds also present. The levels of individual analytes were in the 10-100 pg/m³ range. Further monitoring of levels of PFCs in air is required.

Table 6-7a. Concentrations of neutral PFAS measured in European air

	Hazelrigg, UK (Feb-March 2005)	Manchester, UK (Feb-March 2005)	Hamburg, Germany (April-May 2005)			
Air Volume (in m ³)	1436 (1426-1446)	1419 (1202-1635)	1167 (840-1496)			
	Measured air concentrations (in pg/m ³)					
	Gas	Particle	Gas	Particle	Gas	Particle
	n=2	n=2	n=2	n=2	n=14	n=14
10:2 FT olefin *	0.2 [1/2] (<0.2 -0.2)	<0.4	0.7 [2/2] (0.4-1.0)	0.3 [2/2] (0.2-0.3)	N/A	N/A
4:2 FTOH	56.5 [1/2] (<50 -56.5)	n.d., 0.7	38 [2/2] (14.4-59.3)	n.d., 2.1	54 [14/14] (22-117)	n.d.
6:2 FTOH	81 [2/2] (15.9-147)	<1.1	187 [2/2] (58.2-315)	1.8 [1/2] (<1.2 -1.8)	66 [14/14] (33-149)	n.d.
8:2 FTOH	102 [2/2] (9.0-196)	<1.1	237 [2/2] (148-326)	5.8 [1/2] (<1.2 -5.8)	119 [14/14] (62-275)	<1.0
10:2 FTOH	75 [2/2] (25.2-125)	<1.1	65 [2/2] (40.7-88.8)	2.7 [2/2] (0.8-4.6)	35 [14/14] (16-93)	<0.7
Σ FTOH	269 [2/2] (50-489)	n.q.	527 [2/2] (265-789)	7.6 [2/2] (2.9-12)	288 [14/14] (150-546)	n.q.
N-Me-FOSA	5.5 [2/2] (2.1-8.9)	<1.1	6.1 [1/2] (<0.6 -6.1)	1.5 [1/2] (<1.2 -1.5)	9.0 [14/14] (3.4-20)	<0.2
N-Et-FOSA	7.9 [2/2] (1.6-14.2)	0.4 [2/2] (0.3-0.4)	9.6 [2/2] (7.9-11.2)	0.7 [2/2] (0.4-0.9)	3.1 [14/14] (1.3-5.9)	n.d.
N-Me-FOSE	24 [2/2] (19.2-29.1)	12.1 [1/2] (<4.2 -12.1)	23.7 [2/2] (20.0-27.4)	24 [2/2] (14.6-33.3)	30 [14/14] (3.0-89)	11 [14/14] (2.3-18)
N-Et-FOSE	9.2 [2/2] (5.1-13.3)	6.9 [1/2] (<3.2 -6.9)	6.4 [1/2] (<3.2 -6.4)	11 [2/2] (4.6-18.0)	7.6 [13/14] (0.5-27)	6.7 [14/14] (2.4-12)
Σ FOSA/Es	47 [2/2] (36-57)	10 [2/2] (0.4-19)	39 [2/2] (31-48)	30 [2/2] (6.5-54)	50 [14/14] (9-142)	18 [14/14] (4-29)

* 10:2 FT olefin had a very poor recovery (1-13%) in extraction tests, so the concentrations may have been considerably higher.

Numbers in Table represent arithmetic mean, calculated from values above LOD;
[#/#] = number of samples above LOD/number of samples; (#-#) = min. – max.
§ Due to the large concentration range observed in Tromsø indoor air, geometric means are given.

N/A = not analysed; n.d. = not detected; n.q. = not quantifiable

Table 6-7b. Concentrations of neutral PFAS measured in European air

	Waldhof, Germany (May-June 2005)	indoor Tromsø, Norway (May-June 2005)	Kjeller, Norway (Nov-Dec 2005)			
Air Volume (in m ³)	1165 (846-1573)	(49-81)	1411 (1398-1423)			
	Measured air concentrations (in pg/m ³)					
	Gas	Particle	Gas	Particle	Gas	Particle
	n=8	n=8	n=4	n=4	n=2	n=2
10:2 FT olefin *	N/A	N/A	199 [2/4] (<5 -365)	6 [1/4] (<5 -6)	N/A	N/A
4:2 FTOH	19 [8/8] (3.3-45)	n.d.	114 [1/4] (<100 -114)	<20	n.d.	n.d.
6:2 FTOH	64 [8/8] (17-125)	n.d.	2990 [§] [4/4] (352-62970)	<40	11.7 [2/2] (11.5-11.9)	n.d.
8:2 FTOH	75 [8/8] (33-112)	<1.0	3424 [§] [4/4] (295-1584300)	<10	34.4 [2/2] (34.4, 34.4)	n.d., <0.27
10:2 FTOH	23 [8/8] (10-32)	<0.7	3559 [§] [4/4] (<50 -120950)	13 [1/4] (<5 -13)	17.2 [2/2] (16.5-17.8)	n.d., <0.40
Σ FTOH	181 [8/8] (64-311)	n.q.	11075[§] [4/4] (844-369470)	n.q.	63.3 [2/2] (62.8-63.8)	n.q.
N-Me-FOSA	7.0 [8/8] (3.8-11)	n.d.- <0.2	6608 [1/4] (<120 -6608)	6 [1/4] (<5 -6)	5.5 [2/2] (3.1-7.8)	<0.43
N-Et-FOSA	2.6 [8/8] (1.5-3.4)	n.d.	6626 [1/4] (<100 -6626)	7 [3/4] (<5 -8)	5.0 [2/2] (4.3-5.6)	<0.71
N-Me-FOSE	6.5 [8/8] (0.5-11)	2.4 [8/8] (0.9-4.7)	6018 [§] [4/4] (232-83045)	363 [4/4] (35-1289)	48.9 [2/2] (47.3-50.5)	3.6 [2/2] (3.2-4.0)
N-Et-FOSE	11 [7/8] (<0.3 -23)	5.9 [8/8] (1.2-15)	5755 [§] [4/4] (2231-29340)	76 [3/4] (<30 -124)	29.5 [2/2] (28.2-30.8)	3.4 [2/2] (3.1-3.7)
Σ FOSA/Es	26 [8/8] (6-48)	8 [8/8] (2-20)	14909[§] [4/4] (2466-125617)	427 [4/4] (35-1418)	88.9 [2/2] (85.6-92.2)	7.0 [2/2] (6.3-7.8)

* 10:2 FT olefin had a very poor recovery (1-13%) in extraction tests, so the concentrations may have been considerably higher.

Numbers in Table represent arithmetic mean, calculated from values above LOD;

[#/#] = number of samples above LOD/number of samples; (#-#) = min. – max.

§ Due to the large concentration range observed in Tromsø indoor air, geometric means are given.

N/A = not analysed; n.d. = not detected; n.q. = not quantifiable

Table 6-8. Concentrations of ionic PFAS measured in the particulate phase of European air

	Hazelrigg, UK (Feb-March 2005)*	Manchester, UK (Feb-March 2005)	indoor Tromsø, Norway (May-June 2005)	Hazelrigg, UK (Nov 2005- Feb 2006)	Manchester, UK (Nov-Dec 2005)	Kjeller, Norway (Nov-Dec 2005)
Air Volume (in m ³)	718 (713-723)	710 (602-818)	(49-81)	444 (329-913)	527	705 (699--711)
	n=2	n=2	N=4	n=10	n=1	n=2
Measured air concentrations (in pg/m ³)						
6:2FT(U)CA	N/A	N/A	18.8 [2/4] (n.d.-30.5)	n.d.	n.d.	n.d.
8:2FT(U)CA	N/A	N/A	20.7 [2/4] (n.d.-33.1)	n.d.	n.d.	n.d.
PFOSA	<2.1	<1.6	2.8 [3/4] (<1.0-3.5)	0.2 [1/10] (n.d.-0.2)	n.d.	0.78 [2/2] (0.76-0.80)
6:2 FTS	n.d.	9.4 [2/2] (9.2-9.7)	<1.5	1.9 [1/10] (n.d.-1.9)	1.2	0.18 [2/2] (0.14-0.22)
8:2 FTS	N/A	N/A	3.1 [4/4] (1.4-6.7)	N/A	N/A	N/A
PFBS	<3.2	2.2 [2/2] (2.0-2.5)	<0.5	0.3 [3/10] (n.d.-0.4)	n.d.	n.d.
PFHxS	<5.9	1.0 [2/2] (0.9-1.0)	<4.1	0.04 [10/10] (0.01-0.06)	0.1	0.05, 0.05
PFOS	<44.5	46 [2/2] (41-51)	<47.4	1.6 [10/10] (0.9-2.6)	7.1	1.0 [2/2] (0.89-1.13)
PFDcS	<0.45	0.8 [1/2] (<0.4-0.8)	2.6 [1/4] (<0.4-2.6)	n.d.	n.d.	0.06 [2/2] (0.04-0.08)
PFHxA	<55.6	<26.0	17.1 [4/4] (11.7-21.5)	107 [2/10] (<34.4-113)	<34.4	0.50 [2/2] (0.45-0.54)
PFHpA	14.4 [1/2] (<6.27-14.4)	8.2 [2/2] (4.9-11.6)	0.8 [1/4] (<0.6-0.8)	1.6 [10/10] (0.5-3.2)	0.2	0.87 [2/2] (0.35-0.52)
PFOA	552 [2/2] (276-828)	341 [2/2] (226-455)	4.4 [4/4] (3.4-6.9)	101 [10/10] (6.3-222)	15.7	1.54 [2/2] (1.42-1.67)
PFNA	<13.8	<26.6	2.7 [4/4] (0.9-4.7)	0.9 [9/10] (n.d.-1.7)	0.8	0.12 [2/2] (0.10-0.13)
PFDcA	8.3 [2/2] (2.4-14.3)	5.4 [1/2] (n.d.-5.4)	3.4 [2/4] (n.d.-5.0)	1.0 [6/10] (n.d.-1.1)	<0.8	<0.15
PFUnA	<4.6	n.d.	n.d.	0.7 [5/10] (n.d.-1.1)	n.d.	n.d.
PFDcA	n.d.	n.d.	1.2 [1/4] (n.d.-1.2)	n.d.	n.d.	n.d.
PFTeA	<9.3	n.d.	n.d.	n.d.	n.d.	n.d.

*Note: the Hazelrigg Feb-March 2005 GFFs were analysed in a batch of samples which showed contamination problems, so the high associated blank value used to calculate the detection limit put most analytes below LOD.

Numbers in Table represent arithmetic mean, calculated from values above LOD; [##/#] = number of samples above LOD/number of samples; (#-#) = min. – max.
N/A = not analysed; n.d. = not detected; n.q. = not quantifiable

6.5 Bioaccumulation of PFCs

6.5.1. Bioaccumulation

Bioaccumulation is the generic term expressing the concentration elevation of a chemical in an organism as compared to its immediate environment. *Bioconcentration* is the process where chemicals enrich in (aquatic) organisms from the surrounding water. Finally, *biomagnification* describes the concentration of chemicals in (higher) organisms resulting from exposure via their diet. Bioaccumulation is the net result of uptake and elimination processes. Elimination processes include direct elimination of the chemical as well as biotransformation and elimination of the transformation product. Several processes influence the bioaccumulation, biotransformation and elimination processes (van den Berg, 1995).

The octanol-water partitioning constant (K_{OW}) is valuable for estimation of the bioaccumulation potential of contaminants and modelling their behaviour. In general, the higher the K_{OW} value, the higher the bioaccumulation potential of a contaminant is. This concept, however, cannot be applied to PFCs due to their surfactant nature. The bioaccumulation data that have been reported so far are based on laboratory experiments with model animals (Martin, Mabury et al. 2003; Martin, Mabury et al. 2003) or field data (Martin, Whittle et al. 2004; Tomy, Budakowski et al. 2004; Kannan, Tao et al. 2005).

In this study, bioaccumulation was determined in field samples from The Western Scheldt. The biota samples were collected in 2004 and 2005. Common tern eggs from two common tern nesting locations (Saeftinge and Terneuzen, each 10 eggs) were sampled and individually analysed. In addition, relevant organisms from the common tern food chain were sampled at the Terneuzen location. The food web is shown in Figure 6-13. Furthermore, the eel food chain organisms, as well as water samples were sampled and analysed according to the methods described in chapter 3.

6.5.2. QA/QC of the dataset

The batch-to-batch precision (or reproducibility), based on analysis of a laboratory reference material (spiked pike-perch) with every series, was 25-40%. Although there's room for improvement, this is satisfactory. Accuracy was also determined by spiking several samples (sediment, seabass, cuckels and eel) with surrogates. The recoveries were on average 67, 117, 104 and 118%, suggesting satisfactory accuracy (although sediment accuracy was low). However, some low/high values were found (e.g. 137% for PFUnA in eel and 51% for PFOA in sediment), showing that the accuracy is not yet controlled for every compound-matrix combination. These results were produced using the ion-pair extraction method mainly, with a limited set of isotope-labeled standards. It is expected that the accuracy will improve with the currently applied acetonitrile and methanol based extraction method.

A selection of samples (eel, whiting, lugworm, flounder and shrimp) were co-analysed by a partner with independent extraction, clean-up, calibration and final determination methods (see Chapter 4). Out of 16 duplicate observations, ca 50% differed

by a factor 0.7 to 1.1, whereas the other half the differences were larger, showing that there's room for improvement in specific cases.

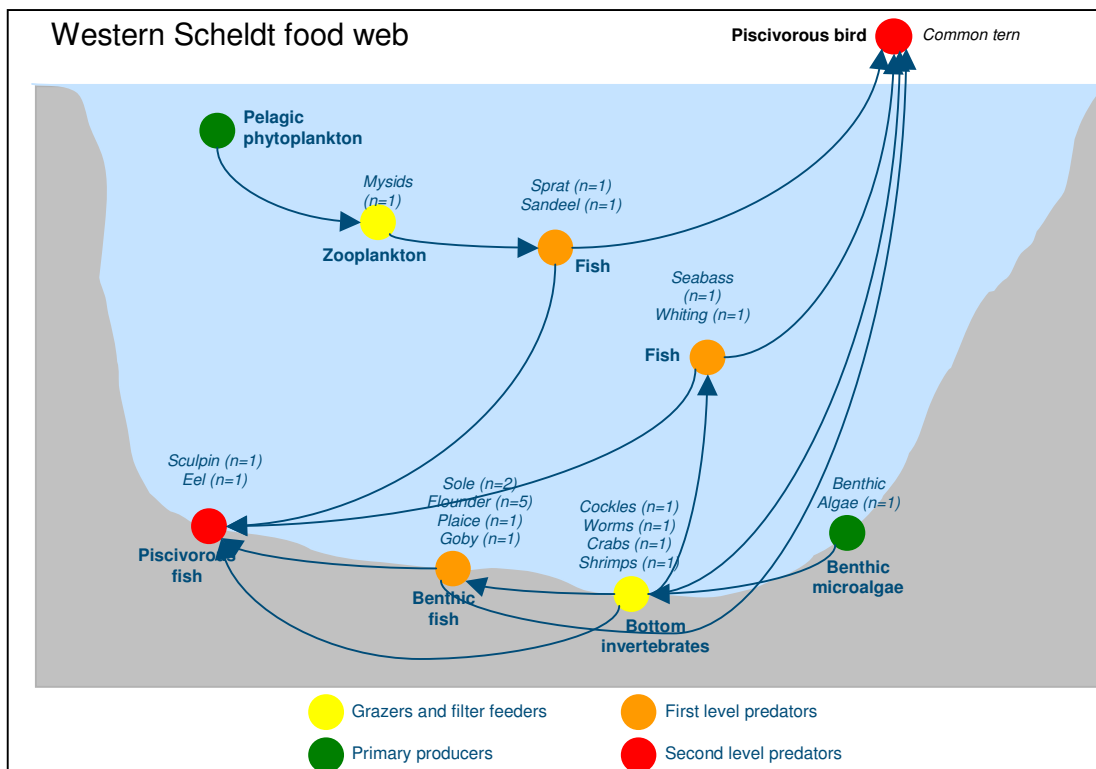


Figure 6-13. Western Scheldt food web of eel and common tern showing the number of samples taken.

6.5.3 Bioconcentration

Bioconcentration factors (BCFs) were determined based on single organisms/single determinations Table 6-9 provides an overview of the available bioaccumulation data.

Table 6-9. BAFs determined in Western Scheldt biota samples*

Organism	PFOA	PFDA	PFUnA	PFOS
Seabass n=1	44	2300	5000	5200
Shorthorn sculpin, n=1	49	2900	6400	3000
Invertebrate (lugworm, n=1)	410	21	17	170

*BCFs could not be determined because the concentration in the aquatic organisms were below the limit of quantification.

BCFs were calculated for PFOA, PFDA, PFUnA and PFOS. For PFHxS, PFHxA, PFNA, PFDoA, the values in the biotic samples were generally below the LOQ and no BCF could be determined. PFOSA, 6:2 FTS and 6:2 and 8:2 FTOH were not determined in the biota samples.

BCFs in the fish increased with increasing PFCA chain length (except PFDA/PFUnA in flounder). Furthermore, PFOS (C8 chain) BCFs were between those of PFDA (C10) and PFUnA (C11), which was also observed by Martin et al. (2003). This shows that both the length of the perfluorinated chain and the acid group influence bioconcentration behaviour. PFOS BCFs determined in a study on round gobies in the Great Lakes and in a laboratory experiment on rainbow trout were lower (factor 1-5) than those observed in our study. A surprising finding is that the lugworm PFOA BCF was higher compared to the higher chain length acids and PFOS, whereas this was not found for the other invertebrates (shrimps, crabs and cuckels).

6.5.4 Biomagnification in a common tern food web

High concentrations of PFCs were determined in the common tern egg samples. Figure 6-14 shows the concentrations in 10 individual eggs for the Terneuzen and Saeftinghe location. There is limited data on PFOS levels in bird eggs. Levels in glaucus gull eggs from the Norwegian Arctic (Verreault et al., 2005) were 104 µg/kg ww, being in the same order of magnitude as the levels in liver (100) and plasma (134). Some other groups reported levels in bird eggs of 20 (glaucus gull, Eastern Arctic) (Tomy et al., 2004), 29 (fulmar, Faroe Island) (Bossi et al., 2005) and 1055 µg/kg ww (bleu tit, Blokkersdijk, Belgium). The latter birds originated from a nature reserve nearby a PFC manufacturing plant in Antwerp (Hoff et al., 2005), which may explain the high findings.

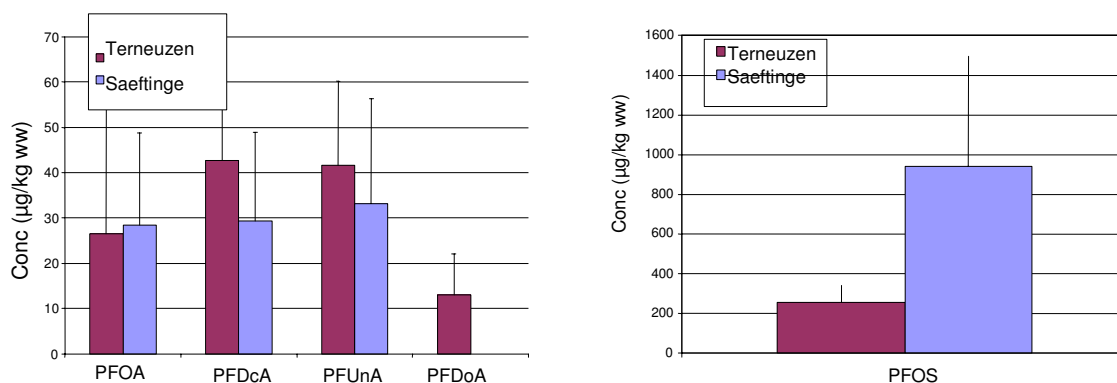


Figure 6-14: Average PFC concentrations in common tern eggs from Terneuzen and Saeftinge (n=10). The bars represent 1 standard deviation. Note the difference in y-axis scale of the PFOS and PFCA graphs.

Newsted et al. (2005) reported toxicity reference values (reproduction) for mallards and northern bobwhites. The no observed adverse effect level (NOAEL) of PFOS in the bird food was 17.6 mg/kg ww, corresponding to egg yolk concentrations of 33-53 µg/ml. This is considerably higher than the levels we observed in our field data (up to 2.3 µg/g ww in individual eggs) and therefore no adverse effect on reproduction are expected from PFOS only. Obviously, inter-species variations, other modes of action and the potential effect of a mixture of contaminants in these eggs should not be neglected when making a thorough toxicity evaluation.

Biomagnification of PFOS was assessed in the common tern food web. Although considerable amounts of PFCs were determined in the common tern (CT) egg samples, the prey fish samples (sprat, sandeel) only contained PFOS at detectable levels. The PFCAs were all <1.9-<3.2 µg/kg ww. PFOSA, 6:2 FTS and 6:2 and 8:2 FTOH were not analysed in the samples.

The PFOS BMF_{CT whole eggs: sprat, sandeel} was 6.9. The BMF_{eel:shrimp} was 0.3, 0.4 and 3.0 for PFDA, PFUnA and PFOS, respectively. This shows that these perfluorinated carboxylic acids did not biomagnify. A similar observation was made by Tomy and co-workers (Tomy et al., 2004), who found a BMF_{Glaucous gull liver: cod whole body} of 0.6 (corrected for trophic level). Houde et al. (2006) assembled a substantial BMF dataset for bottlenose dolphin food chains from two US locations. The BMFs for the PFCs were variable, meaning that no consistent trend (PFC chain length, trophic level) could be determined (e.g. location- dependent BMFs were determined). Whether the observations from both studies hold for more organisms at different trophic levels and locations remains to be determined.

The PFOS BCF_{eel:shrimp} in this study (3.0) is similar to the BMF_{lake trout:prey (weighted)} of 2.9 reported by Martin et al. (2004), although it should be noted that feeding habits differ for eel and lake trout. Concerning the PFCAs, Martin and co-workers found no biomagnification for PFOA (0.41), but PFNA through to PFTrA were found to biomagnify (BMFs of 1.6-3.4) in the lake trout's diet. Trophic magnification factors

(TMFs) (integrated over 3 organisms covering 1.5 trophic levels) increased from PFTrA (2.45) < PFDA (3.67) < PFUnA (4.71) < PFOS (5.88). Although not significant, PFDoA and PFNA showed a weak increasing trend, whereas PFOSA and PFOA showed a decreasing trend, suggesting that these substances are not accumulated in the food web.

In a laboratory study, Martin and co-workers (2003) determined dietary accumulation of PFCs in juvenile rainbow trout. The growth corrected BMFs were 0.038 (PFOA)-1.0 (PFOS). Sulfonates bioaccumulated more strongly than the carboxylates, showing the role of the acid group in bioaccumulation, as discussed in section 6.5.4.

6.5.6. Bioaccumulation in Western Scheldt biota

The results of the bioaccumulation study in the Western Scheldt are summarized in Figure 6-15. PFOSA, 6:2 FTS and FTOHs were not determined. PFBS did not pass the QA/QC criteria. No PFCs were found at detectable levels (<1-2 ng/g ww) in low level organisms such as benthic algae, cuckels and mysids. The PFC profile in all biota samples except crabs and lugworm is dominated by PFOS. Some samples contained low amounts of PFOA, PFNA and PFUnA. On the other hand, the profile of the water samples is dominated by PFBS>PFOA>PFOS>PFHxS. As expected, this is different from the profile found in biota, as PFBS and PFOA are more water-soluble than PFOS. With liver being a target organ, the highest PFOS concentrations were detected in the flounder liver samples. Flounder feeds mainly on benthic fauna (small fish and invertebrates), including shrimps, crab and lugworm. Sprat shows low PFOS concentrations, which may be explained by it feeding mainly on mysids (which showed values below the LOD). Seabass, shorthorn sculpin and eel whole body concentrations ranged from 100-280 ng PFOS/g ww. These relative high concentrations may be explained by their foraging behavior. Being predatory fish, they feed on small (benthic) fish and invertebrates (the latter contributing to a lesser extent to their uptake).

Martin and co-workers (2004) found surprisingly high concentrations in Lake Ontario diporeia of 280, 180 and 90 ng/g ww for PFOS, PFOSA and PFOA respectively. PFOS concentrations in higher level organisms were in the same range as we observed (46-450 ng/g ww, whole body homogenates). Tomy et al. (2004) determined PFC concentrations in an arctic food web. Their PFOS and PFOA results were lower than we observed, which can be explained by the remoteness of their sampling location, whereas the Western Scheldt is directly influenced by industrial activity including a PFC production plant.

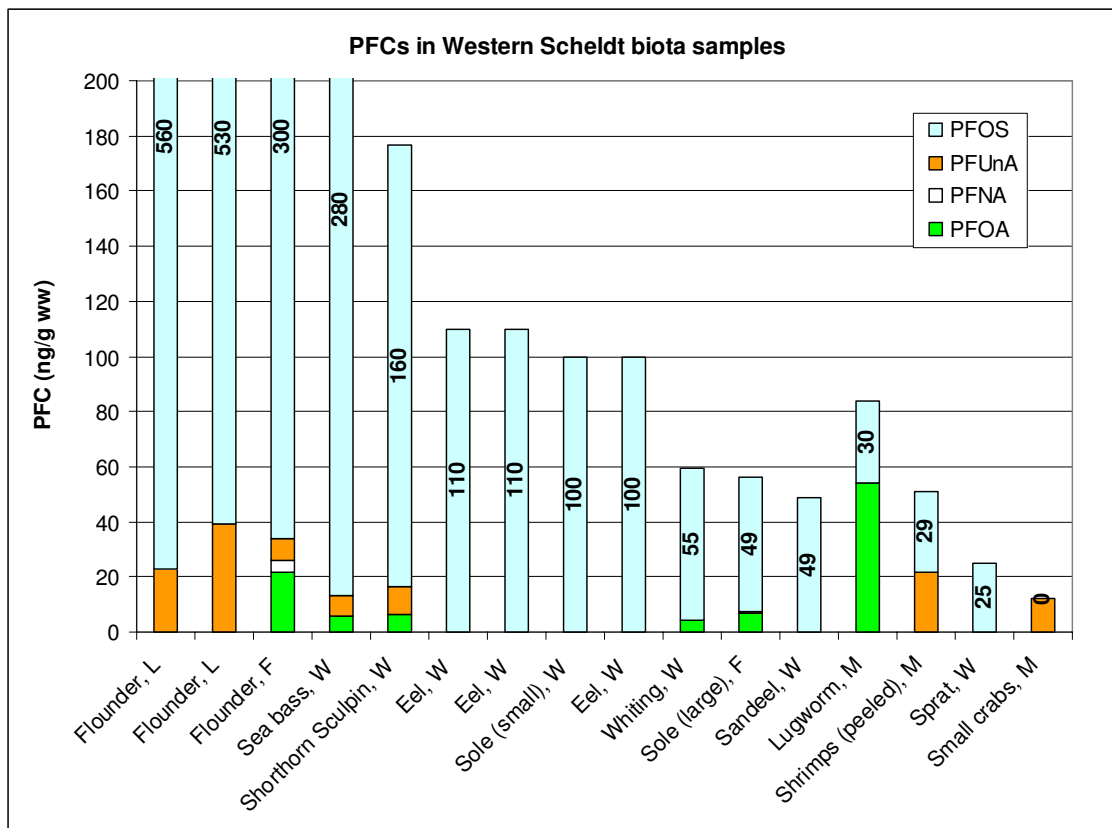


Figure 6-15: PFC concentrations in biota samples from the Western Scheldt. L stands for liver tissue, W is whole body, F is fillet and M is meat.

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7 Biological and bio-analytical assessment

7.1 Introduction

Cell-based assays provide rapid and sensitive alternatives to chemical detection of contaminants. Gene regulatory elements involved in the primary toxicological interaction of contaminants (such as for example Dioxin Responsive Elements for PCDDs, PCDFs, and PCBs) have been fused to various reporter systems (such as luciferase) in order to quantify specific contaminants in a fairly simple manner. Such constructs (e.g. DR-Calux for dioxins, Yeast assay for Estrogens) have been used extensively in the past for rapid evaluation of the presence of chemicals in various environmental matrices (Murk et al. 1996).

At present no clear-cut bioassay exists for the detection of PFOCs, mainly due to the fact that the exact toxicological mechanism is not yet revealed. Nevertheless several studies have already suggested specific genes as targets for PFOCs interaction. For perfluorinated straight chain monocarboxylic fatty acids such as PFOA it is for example known that they are able to induce peroxisomal proliferation in rodents, through interaction with the nuclear PPAR receptor, a process intimately linked to carcinogenesis (Ikeda et al, 1987). Other studies have documented the potential of perfluorinated chemicals to induce specific growth factors in liver cells (Van de Vijver et al, 2003), and to disturb cell-cell communication (Upham et al, 1998) and Cytochrome P450 2B (Giesy et al, 2003) and 2G (Van de Vijver et al, 2003). Hu et al (2005) used micro-array analysis on rats exposed in vivo and rat cells exposed in vitro. The general outcome of both exposure types resulted in the conclusion that the genes related to the peroxisomal fatty acid metabolism were the most strongly induced. These results suggests that if important gene regulatory elements of these genes are selected and fused to a reporter system, a PFOC specific cellular assay can be developed which can be used to rapidly screen pure compounds and environmental samples for the presence of these compounds.

In order to derive such PFOC-specific bio-assay, specific information was needed on the induction of key-genes that are related to the overall toxicological mechanisms of these compounds. Therefore a strategy was chosen that combined analysis of targeted genes (related to peroxisomal metabolism) and genes derived from whole-genome assessments. Due to this search for specific genes related to exposure to perfluorinated chemicals we were also able to generate generic insights into the mode of action of these compounds. As a result, certain interesting aspects of the toxicity of these compounds could be highlighted.

7.2 Gene expression analysis in transgenic rat hepatoma cells containing the ACOX promotor

7.2.1 Aim

Evaluating relevance and usefulness of existing rat hepatoma cell line, using the acyl-CoA oxidase (ACOX) promotor: Establishing Toxicity Equivalence Factors for a family of PFOCs

7.2.2 Introduction

An existing rat hepatoma cell line containing the AcylCoA oxidase (ACOX) promotor was evaluated in its responsiveness towards perfluorinated chemicals. Previous studies had shown that PFOA was able to induce the transcriptional activity of this promotor (Hu et al, 2003). The acyl-CoA oxidase (ACOX) gene is responsible for the oxidation of the CoA esters of straight chain fatty acids and donates electrons directly to molecular oxygen, thereby producing H₂O₂. The inducibility of this H₂O₂-generating ACOX in rat and mouse liver by peroxisome proliferators and the postulated role of the resulting oxidative stress in hepatocarcinogenesis have generated a wide interest in this gene. In animal cells, mitochondria as well as peroxisomes oxidize fatty acids via β -oxidation. In general, fatty acids up to 18 carbons in length are oxidized mainly by the mitochondrial β -oxidation system, whereas the long and very long chain fatty acids are processed predominantly in peroxisomes (Reddy and Mannaerts, 1994). The peroxisomal β -oxidation system consists of several genes among which the H₂O₂-generating fatty acyl-CoA oxidase (Hashimoto, 1992). The rat liver contains three forms of peroxisomal fatty acyl-CoA oxidase: (a) a palmitoyl-CoA oxidase (ACOX), inducible by peroxisome proliferators, that oxidizes esters of medium, long, and very long chain fatty acids; (b) a noninducible pristanoyl-CoA oxidase, which oxidizes the CoA esters of 2-methyl-branched fatty acids such as pristanic acid; and (c) a noninducible trihydroxycoprostanoyl-CoA oxidase, which oxidizes the CoA esters of bile acid intermediates di- and trihydroxycoprostanic acids (Reddy and Mannaerts, 1994). Among these three oxidases, the inducible ACOX is well characterized and is implicated in the oxidative DNA damage and hepatocarcinogenesis resulting from exposure to peroxisome proliferators (Reddy and Rao, 1992). In contrast to rat, peroxisomes in human liver and kidney contain only two forms of acyl-CoA oxidases

In the present study a transgenic cell system based on an existing construct ACOX::CAT was used to evaluate the responsiveness of this gene to detect perfluorinated chemicals. The experimental procedure was described in Annex 7.1.

7.2.3 Results

Figure 7-1 shows the results of the cytotoxicity assays. For both PFOS and PFOA, significant cytotoxicity was measured at the highest concentration tested ($p < 0.05$). However, still sufficient viability was measured at these PFOC levels to allow the

application of the reporter assay (i.e. the cells need to be in sufficient good condition to allow measurement of the reporter gene).

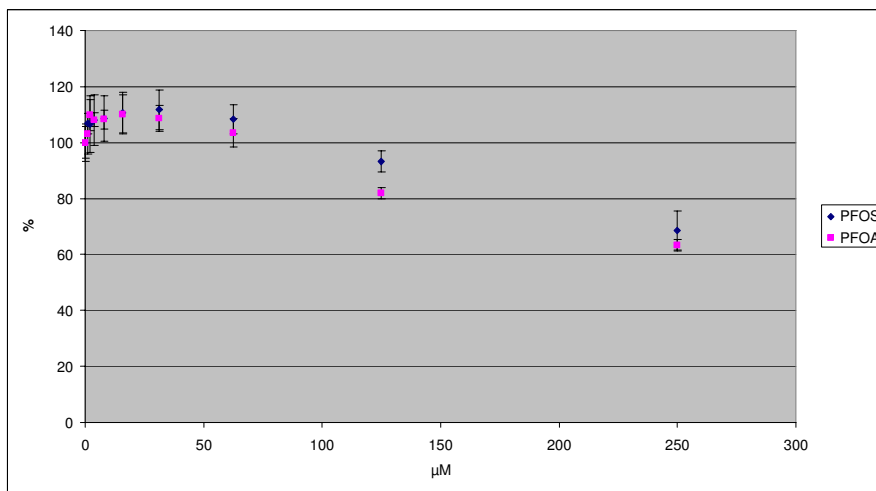


Figure 7-1. Cell viability of rat hepatoma cells exposed to PFOS and PFOA (expressed as % relative to control)

Figure 7-2 shows the CAT reporter protein induction due to PFOS and PFOA exposure, respectively. From these figures can be concluded that the induction of the reporter gene (CAT) after PFOS and PFOA exposure is rather limited. At the highest PFOS concentration tested, a 40% increase in reporter protein was present compared to the control. For PFOA, a 50% increase in CAT protein was measured.

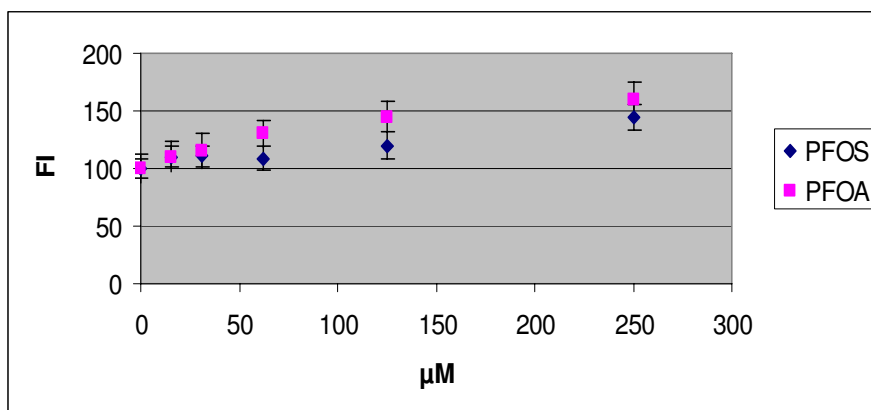


Figure 7-2. CAT reporter activity of the rat hepatoma cells exposed to PFOS and PFOA (FI= fold induction relative to control)

7.2.4 Discussion

The results of these initial reporter activity studies showed that the CAT response was too low to allow future bio-analytical applications of this cell line for PFOC detection. A 50% induction of the reporter signal is not sufficiently high to quantify perfluorinated chemicals in a wide range of concentrations. Lee et al (1997) showed that a wide variety of peroxisomal proliferators was able to up-regulate ACOX::CAT (WY 14,643, clofibrate, di(2-ethylhexyl) pthalhate, and acetylsalicylic acid e.d.). Although this assay was able to detect peroxisome proliferators in a short exposure period, due to the limited response of the cell system no further work was conducted with this cell line.

As a consequence a much wider set of genes was selected for specific detection of perfluorinated chemicals. Rather than one single gene, a set of key-genes was selected and their induction was quantified using PCR-based methods.

7.3 Gene expression analysis in rat hepatoma cells exposed to perfluorinated chemicals using real time PCR

7.3.1 Aim

Selection of genes that are highly and specifically induced by perfluorinated chemicals.

7.3.2 Introduction

Based on literature study a selection of relevant genes related to the presumed mode of action of perfluorinated chemicals was made. Studies with rats for instance clearly demonstrated that PFOA and PFOS behave as peroxisome proliferators, i.e. that they increase the number of peroxisomes in liver and induce peroxisomal β oxidation of fatty acids. More detail of the mode of action of perfluorinated compounds will be given in section 7.3. The experimental setup of this part of the work is described in Annex 7-2. Based on this information a selection of genes was made which is given in the Figure 7-3.

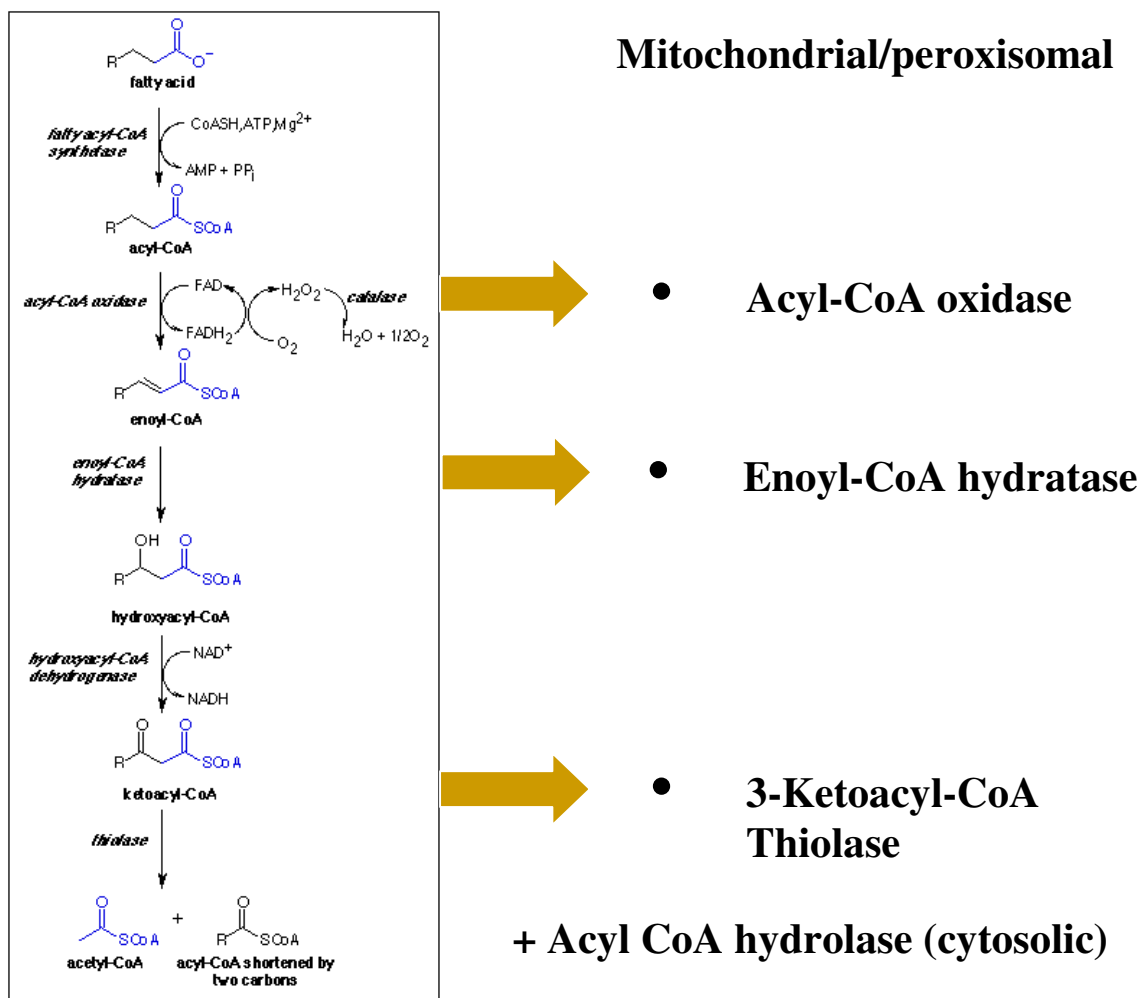


Figure 7-3. Selection of mitochondrial and peroxisomal genes for Real Time PCR quantification.

7.3.3 Results

The results of the differential gene expression analysis are presented in table 7-1.

Table 7-1 Gene expression analysis of selected genes related to (mitochondrial and peroxisomal) β -oxidation in rat hepatoma cells exposed to model chemicals. (Average value of minimum 3 replicates, + means upregulation relative to control, - means downregulation relative to control, standard deviation between brackets)

Chemical tested	Gene name – Fold induction			
	Acyl CoA hydrolase	Acyl CoA oxidase	Enoyl-CoA hydratase	3-Ketoacyl-CoA Thiolase
PFOS	+ 5,3 (0.13)	+ 1,53 (0.24)	- 1,83 (0.12)	- 1,9 (0.08)
PFNA	+ 6,2 (0.03)	+ 4,35 (0.02)	+ 2,3 (0.06)	- 1,24 (0.04)
6:2FTOH	+ 1,2 (0.39)	+ 1,57 (0.51)	+ 1,64 (0.17)	+ 1,35 (0.08)
PFHS	+ 5,74 (0.03)	+ 1,68 (0.01)	+ 1,19 (0.06)	- 1,26 (0.06)
Pirinixic acid	+ 5,86 (0.22)	+ 3,3 (0.19)	+ 7,6 (0.26)	+ 1,5 (0.91)
Phenobarbital	+ 1,08 (0.08)	+ 1,45 (0.17)	+ 1,2 (0.07)	+ 1,1 (0.33)

7.3.4 Discussion

From table 7-1 it can be seen that different PFOC chemicals show a different induction profile for the selected genes. These genes should represent the most relevant ones that can be used to describe the phenomenon of peroxisomal proliferation. These results demonstrate that there is not a single gene that specifically or individually responds to all different model chemicals. Pirinixic acid (a typical peroxisomal proliferator) shows a 5,86, 3,3 and 7,6 fold upregulation of Acyl CoA hydrolase, Acyl CoA oxidase and Enoyl-CoA hydratase, respectively. PFOS on the other hand also showed a 5,3 upregulation for Acyl CoA hydrolase, but a downregulation (- 1,83 fold relative to control) for the Enoyl-CoA hydratase. From all perfluorinated chemicals, only PFNA shows the most “peroxisomal inducer”-like properties.

Expression analysis of these mitochondrial and peroxisomal genes using Real Time PCR, showed that each of the perfluoralkyl compounds tested showed a different response profile. From this we can conclude that not all perfluoralkyl compounds show the same toxicological impact at the level of β oxidation of fatty acids. Clearly this chemical family exhibits distinct differences at the level of toxicological mode of action. There is not a single gene that specifically or individually responds to all different model chemicals.

Based on this targeted gene list, the likelihood of finding a “generically” responsive gene for a wide family of perfluorinated chemicals is low, we decided to broaden the analysis and evaluate the whole-genome response of the rat cells after exposure to perfluorinated chemicals.

7.4 Gene expression analysis in rat hepatoma cells exposed to perfluorinated chemicals using micro arrays: selection of ‘biomarker genes’

7.4.1 Aim

Molecular biological research was done in order to find ‘biomarkers’ genes that are highly and specifically induced by perfluorinated compounds

7.4.2 Introduction

Rat hepatoma cells were exposed to perfluorinated compounds followed by studying gene expression changes in the exposed cells. PFOS, PFOA and PFNA were chosen as the test compounds for this study. The transcriptome was analyzed by means of microarray analyses. Differential gene lists were compared in order to find genes that were commonly up-regulated by perfluorinated chemicals. In addition, gene expression profiles were also compared to that from cells that had been exposed to WY 14,643, since the latter compound is a prototype peroxisome proliferator and activator of PPAR alpha. This decision was made due to the appearance of publications of ‘in vivo’ studies (with rat) in which perfluorinated compounds are suggested to behave as ‘peroxisome proliferator-like’. Details concerning the experimental set-up of these experiments are given in Annex 7-3. By means of diverse bio-informatics tools, functional analyses of the transcriptome were done, so that better insights were gained concerning the mode of action of the perfluorinated compounds in rat hepatoma cells. These insights are needed for proper selection of candidate biomarker genes.

7.4.3 Results

The results of the 20.000 gene expression analysis is shown based on a Gene Ontology approach. Figure 7-4 summarizes the effect of the different model chemicals on the most important biological processes. Figure 7-5 summarizes the effect of the different model chemicals on the most important cellular compartment.

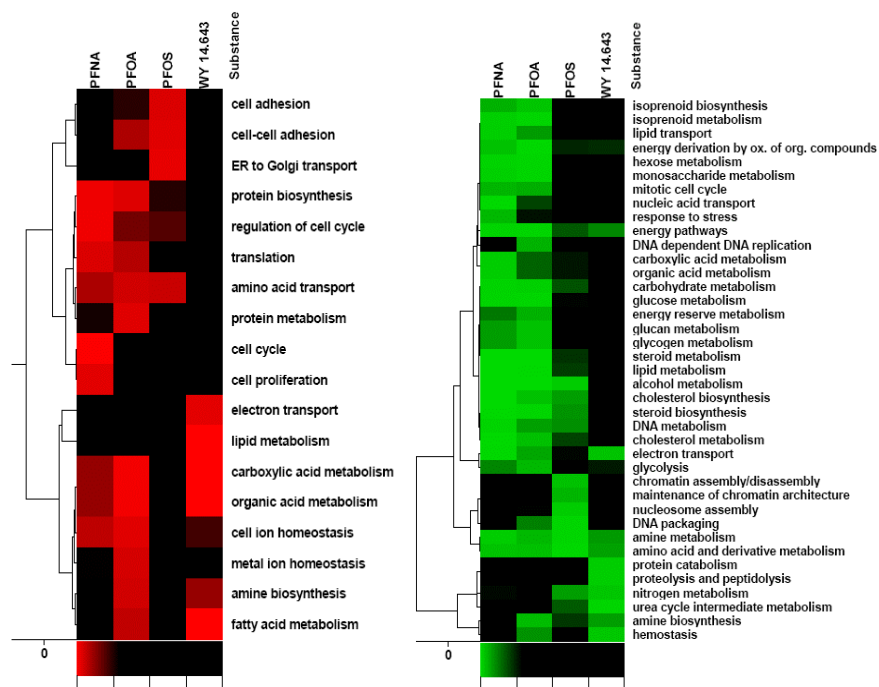


Figure 7-4 Effect of 4 model chemicals on the biological process in the exposed rat hepatoma cell. (Red = significant upregulation of the activity in this process, green = significant upregulation of the activity in this process).

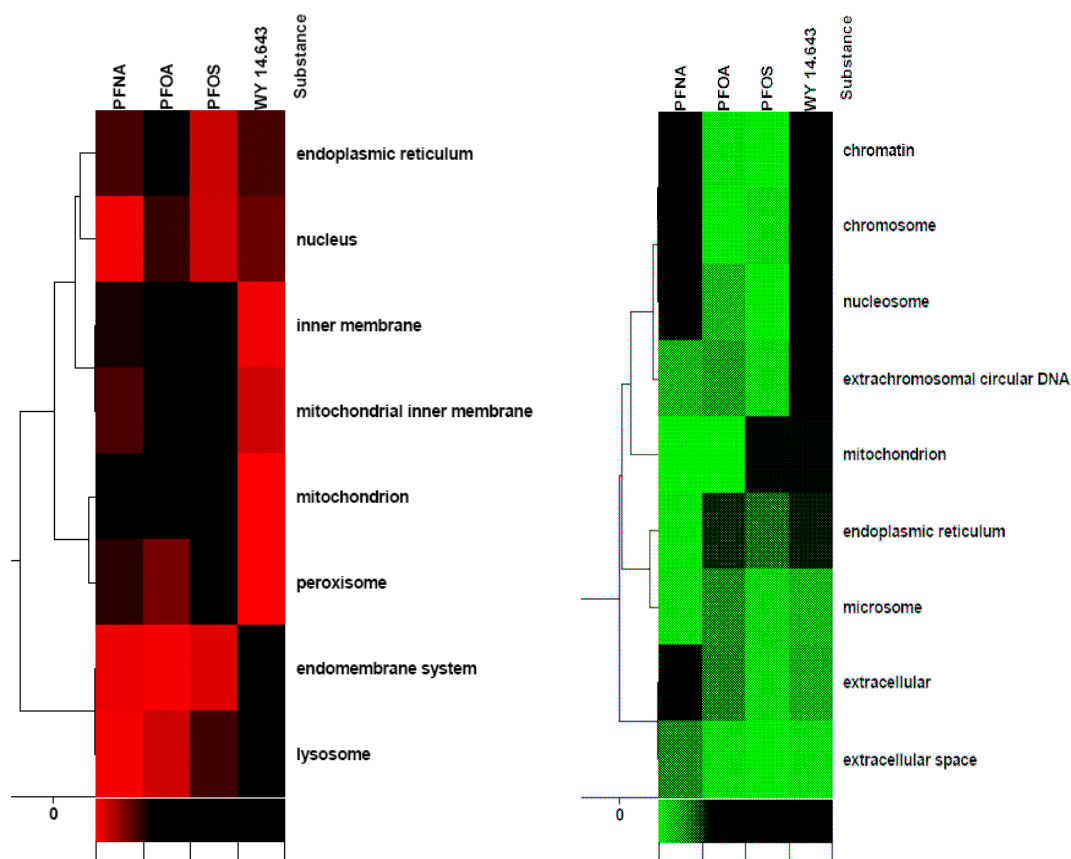
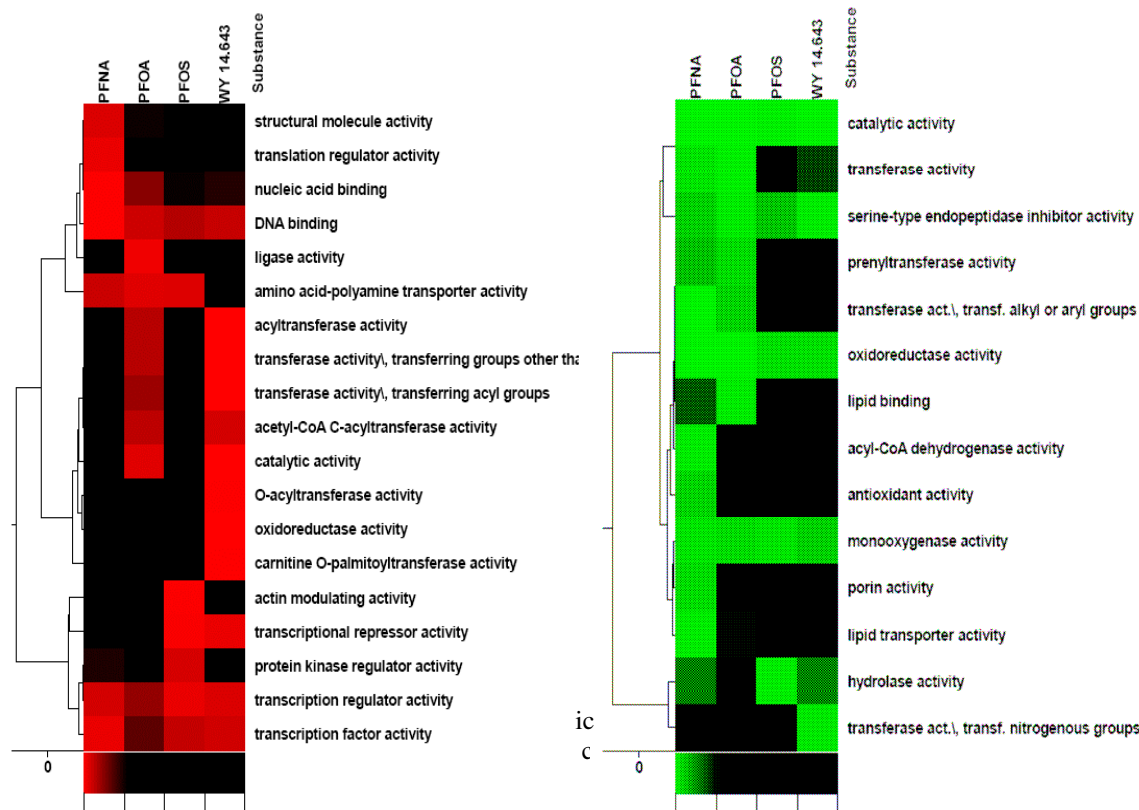


Figure 7-5 Effect of 4 model chemicals on the cellular compartment in the exposed rat hepatoma cell. (Red = significant upregulation of the activity in this compartment, green = significant upregulation of the activity in this compartment).

Figure 7-6 summarizes the effect of the different model chemicals on the most important biological function.



Based on this analysis, a more detailed analysis is shown of the following pathways: Fatty acid oxidation (Fig. 7-7), Mitochondrial catalytic & transport activity (Fig. 7-8) and Lipid transport & metabolism (Fig. 7-9).

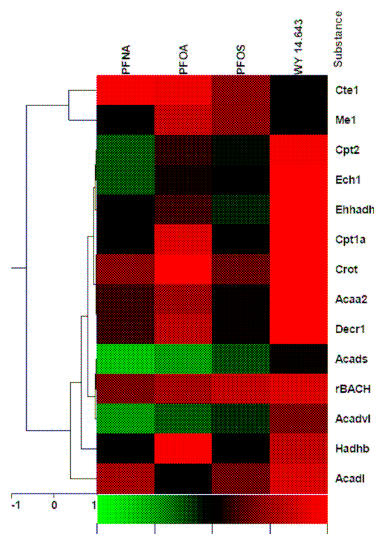


Figure 7-7. Effect of the 4 model chemicals on genes of the Fatty acid oxidation pathway. (Red = gene expression up regulated, green = gene expression down regulated, black= no effect on gene expression)

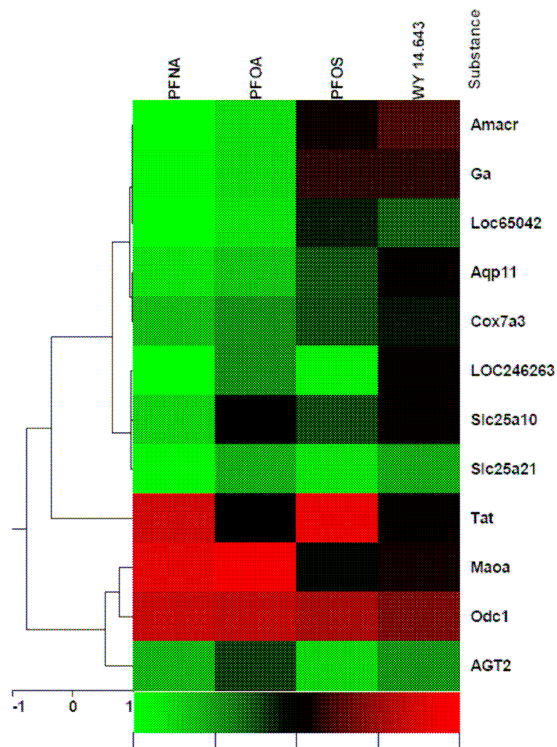


Figure 7-8. Effect of the 4 model chemicals on genes related to the Mitochondrial catalytic & transport activity. (Red = gene expression up regulated, green = gene expression down regulated, black= no effect on gene expression)

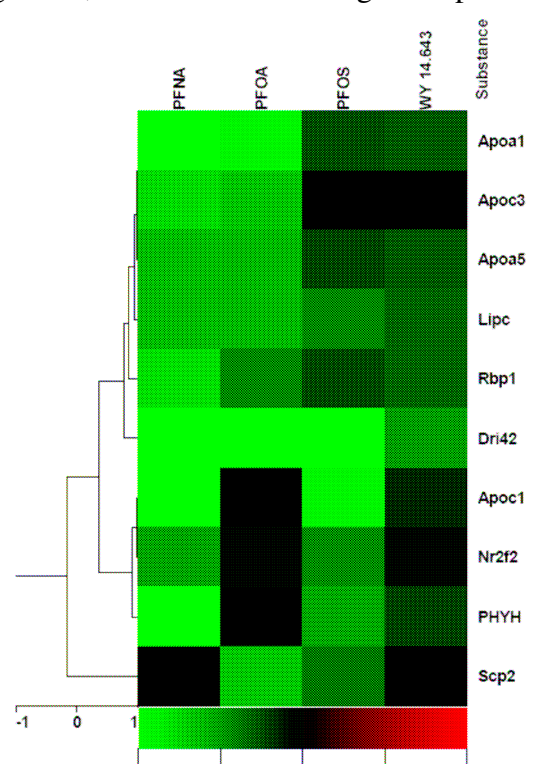


Figure 7-9. Effect of the 4 model chemicals on genes related to Lipid transport & metabolism. (Red = gene expression up regulated, green = gene expression down regulated, black= no effect on gene expression)

7.4.4 Discussion

This study demonstrates that in vitro cell lines can be used to discriminate chemicals based on their cellular mode of action. Wyeth 14,643 clearly increased mitochondrial and peroxisomal beta-oxidation of fatty acids and showed significant up-regulations of well-known PPAR responsive cytochromes involved in omega-oxidation of fatty acids. The perfluorinated chemicals induced mitochondrial or peroxisomal beta-oxidation of fatty acids only slightly (which confirms our previous results, section 7.3). On the other hand, down-regulations of lipid transport genes coding for several (PPAR-dependent) apolipoproteins were clearly observed with the perfluorinated carboxylic acids. It became clear that the perfluorinated chemicals previously catalogued as ‘peroxisome proliferators’ (PPs) are able to provoke very different (i.e. more pleiotropic) transcriptional responses from the “conventional” PPs.

PFOS mainly downregulated gene expression in pathways related to cholesterol and steroid biosynthesis whereas functions related to protein kinase regulator activity were upregulated. The endomembrane system (responsible for intracellular interaction between organelles) was highly affected.

Perfluorinated carboxylic acids (PFOA and PFNA) showed a clear downregulation of genes related to apolipoprotein synthesis, whereas processes related to cell cycle and cell proliferation were upregulated. Like PFOS, they also downregulated the expression in pathways related to cholesterol and steroid biosynthesis and their impact was clearly seen at the endomembrane system. .

Compared to a known peroxisomal proliferator (Wy 16,463) the effect of the perfluorinated chemicals was more pleiotropic in nature: rather than specifically affecting the β oxidation of fatty acids, a wide range of other relevant pathways was affected.

Based on these analyses we selected 10 genes that were submitted to further analyses by real time PCR. Using the latter techniques, we were able to corroborate the finding from microarray analyses. In addition, biomarker genes were selected for the construction of transgenic cell lines. A manuscript describing this experimental work is in preparation.

7.5 Construction of transgenic reporter cell lines for detection of perfluorinated compounds

7.5.1 Aim

1. construct transgenic cell lines based on the biomarker genes, in which chemiluminescent signals are synthesized in response to perfluorinated chemicals.
2. use the transgenic cell lines for assessment and quantitation of perfluorinated compounds in samples extracted from the environment.

7.5.2 Introduction

The promoters of previously selected biomarker genes were studied, amplified and cloned in front of luciferase reporter gene, which allows us to study reporter activity in a transient, fast and sensitive way. Two reporter constructs were found to be successful tools for the assessment of perfluorinated chemicals (the promotor of the caspase 11 and the pyruvate dehydrogenase kinase gene). Experimental details are given in Annex 7.4.

7.5.3 Results

Upon transfection by electroporation, rat hepatoma cells were exposed to increasing concentrations of PFOA and PFOS, during 24 hours. Next, cells were lysed and submitted to the Dual-Glo luciferase assay (supplied by Promega) to assess reporter activity. As shown in figures 7-10 and 7-11, concentration of PFOA and PFOS were found that increase reporter activity in a linear way. Hence, transfected rat hepatoma cells containing these vectors offer us a tool to detect and to quantify perfluorinated compounds, at least if they are in a pure form.

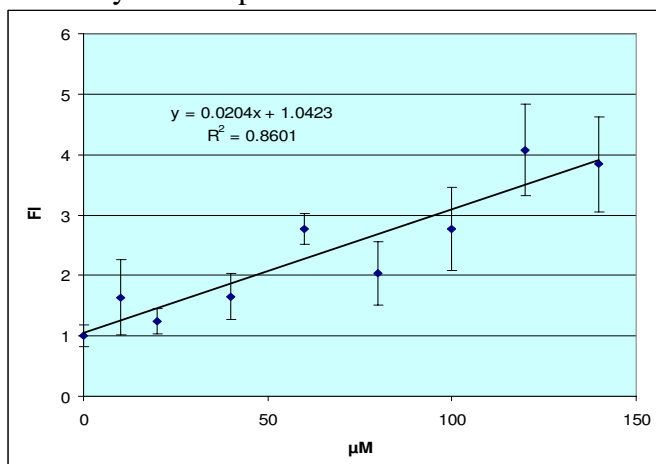


Fig 7-10 Effect of PFOA exposure on the Caspase11 promotor activity measured through luciferase quantification (n=6) (FI = fold induction relative to control)

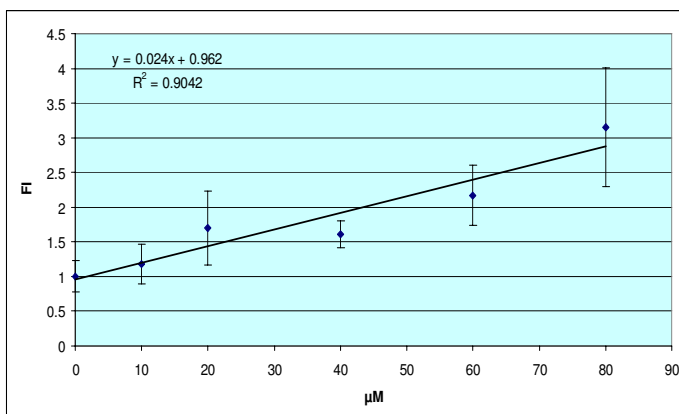


Fig 7-11 Effect of PFOS exposure on the Caspase11 promotor activity measured through luciferase quantification (n=6). (FI = fold induction relative to control)

The Caspase11 promotor generated the most linear results (compared to the Pdk4 promotor). Therefore we have chosen to evaluate the effect of the model compounds on this reporter system. The lowest concentration that significantly differed from the control response (based on ANOVA testing ($p < 0.05$)) was considered as the Limit of Detection. For both PFOA and PFOS this LOD varied between 20 - 40 μ M.

While these reporter constructs were successful in responding to concentration ranges of perfluorinated compounds individually, testing extracts from biological samples resulted in significant problems to evaluate this bio-analytical response. Most often the highest concentrations tested, resulted in significant mortality of the cells, suggesting toxic conditions that interfered with the reporter response at the highest concentration tested. In certain samples, only at lower concentrations (higher dilutions of the original extract) a significant response of the luciferase reporter gene could be measured. To assess the relevance of the biological signal a cut-off value of 1.5 FI (50% increased activity of the reporter gene compared to the control) was used.

Practically, extracts from diverse matrices were tested (see Chapter 6). The extracts were lyophilized in order to remove the solvent and re-dissolved in growth medium for the rat hepatoma cells, in order to submit them to testing using transient transfected cell lines. The results of the highest reporter activity that was measured without cytotoxic effects are shown in figure 7-12.

From these data it can be seen that with certain samples, positive results were obtained. In Fig 7-13 a comparison is made between the PFOS total equivalent that was detected in these extracts and compared with the actual PFOS concentrations determined by analytical chemistry.

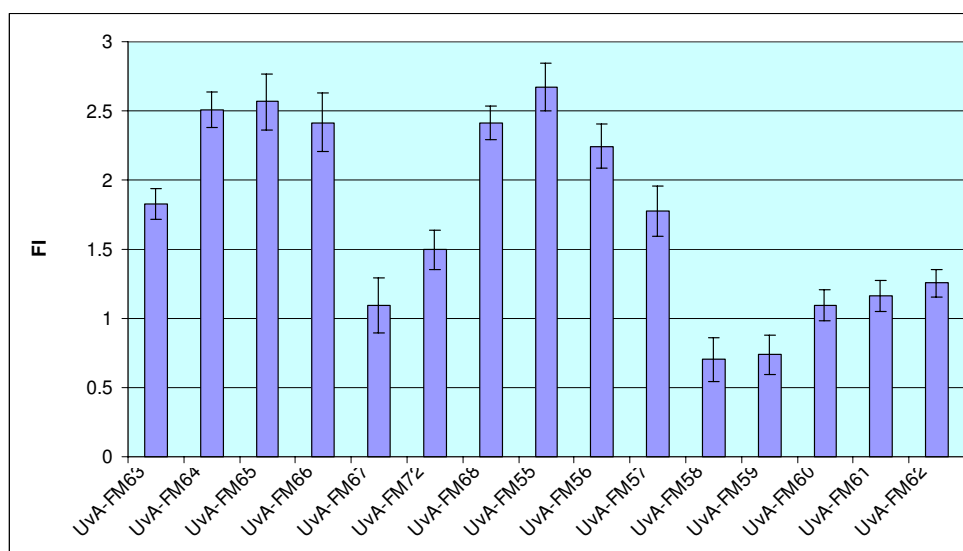


Fig 7-12 Fold inductions (FI) of the Caspase 11 promoter-luciferase construct after exposure to biological extracts.

As can be seen from Fig. 7-12, for most biological samples analysed an increased transcription activity could be measured. We used a cut-off value of 1.5 FI to calculate the bio-analytical PFC content based on our calibration curves for PFOA and PFOS. As can be seen from Fig 7-13 however a poor correlation between both types of methods was observed. An overestimation was made of the total PFOS/PFOA like activity present in the samples, compared to the result of the LC-MS² based methods.

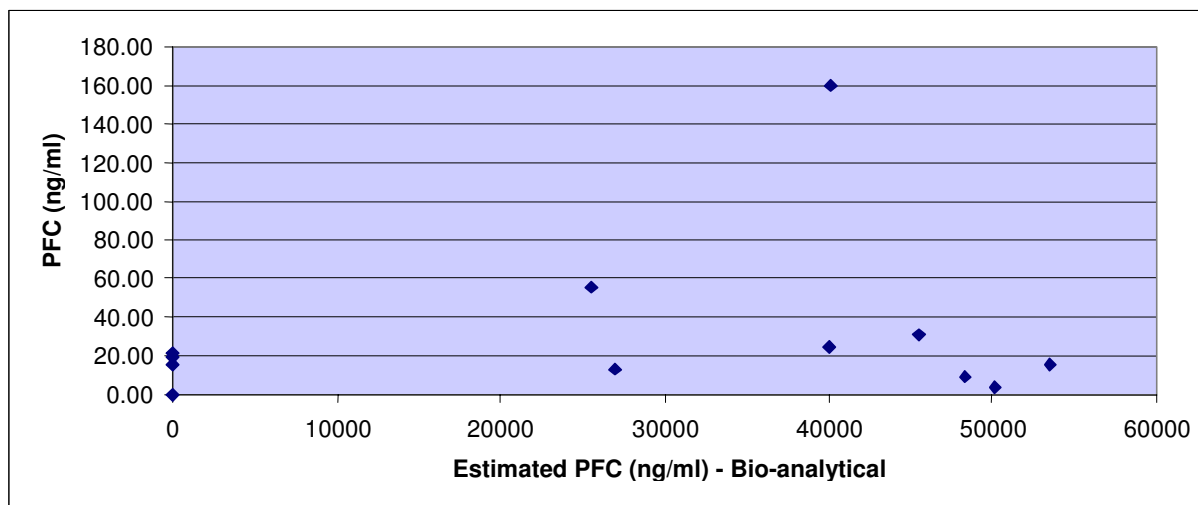


Fig 7-13 Comparison between the predicted total PFOS equivalents present in the samples and the PFOS content measured using chemical methods. (X axis-total PFC content based on bio analytical measurements; Y-axis-total PFC content as determined by chemical methods described higher in the report)

7.5.4 Discussion and conclusions

By means of microarray analyses, the transcriptomes of ‘in vitro’ rat hepatoma cells exposed to diverse perfluorinated compounds were studied. These studies allowed us to select a specific set of candidate ‘biomarker’ genes for the assessment of perfluorinated compounds in samples from diverse origins. The promoters of two of these biomarker genes were successfully cloned in front of the firefly luciferase gene, which resulted in the construction of a chemiluminescent reporter construct allowing us to measure the concentrations of compounds such as PFOA and PFOS. While successful measurement over a specific concentration range of mentioned compounds was possible with the compounds in their pure form, this is not always the case with environmental samples.

The 2 promoters used (Casp11 and Pdk4) are not related to a direct receptor-mediated event, but are rather linked to the overall stress or energy metabolism of the cell. Caspase11 is a key-enzyme of the apoptotic events (i.e. programmed cell death), and

is also linked to control of cell proliferation and inflammation. PdK4 is a key enzyme that is linked to the intermediary metabolism (i.e. energy metabolism). In contrast to other examples from the past such as dioxins (Murk et al, 1998), our study has so far demonstrated that perfluorinated chemicals cannot be directly linked to such a single receptor-mediated event. The “perfluorinated genes” that were used in the present study, are linked to stress responses and the specificity of the responses needs further investigation. Additional experiments with other chemicals (for example for kepone - a chlorinated insecticide) showed that this non-perfluoralkyl compound to a certain extent is also able to induce the caspase11 - promotor. Care should be taken that when testing complex extracts, specific extraction methods for perfluoralkyl chemicals are used. Such extracts (e.g. from biological samples) possibly contain other compounds besides perfluoralkyl chemicals that may affect the viability of the tested cells, as suggested from the decrease in luminescence values with many of these samples. A potential solution for the future application of these bio-analytical methods, is to combine them with a better fractionation methodology that prevent such interfering compounds to disturb the cellular metabolism. Future activities should be focused on the development of such complementary fractionation methods. Furthermore, the testing conditions (e.g. exposure period) should be investigated, as it is likely that by prolonging the exposure period from 24 to 48h or longer, a more sensitive biological signal could be detected. Presently the LOD is 40µM which is higher than the concentrations of perfluorinated chemicals in most matrices.

7.6 Mechanisms of toxicity in human cell lines

7.6.1 Aim

In our search towards specific genes or pathways that specifically responded to perfluorinated chemicals, we have also tested the response of two human in vitro systems: the breast cancer cells (MCF7) and the human liver cell line (HepG2).

7.6.2 Endocrine disrupting properties - Results & discussion

The estrogen like properties of perfluorinated chemicals was investigated using three different in vitro assays using the human breast cancer cell line. Practical details can be found in Annex 7.5.

Estrogen-like properties of five perfluorinated compounds were investigated by a combination of three in vitro assays. By means of an E-screen assay, the proliferation promoting capacity of the fluorotelomer alcohols 1H,1H,2H,2H-perfluorooctan-1-ol (6:2 FTOH) and 1H,1H,2H,2H-perfluorodecan-1-ol (8:2 FTOH) was detected. The more widely environmentally distributed compounds perfluoro-1-octan sulfonate, perfluorooctanoic acid and perfluorononanoic acid did not seem to possess this hormonal-dependent proliferation capacity. Cell cycle dynamics was investigated by flow cytometric analyses of the DNA content of the breast cancer cell nuclei. Exposure to both fluorotelomer alcohols stimulated resting MCF-7 cells to re-enter the synthesis phase (S-phase) of the cell cycle. Already after 24 hours treatment, significant increases in the

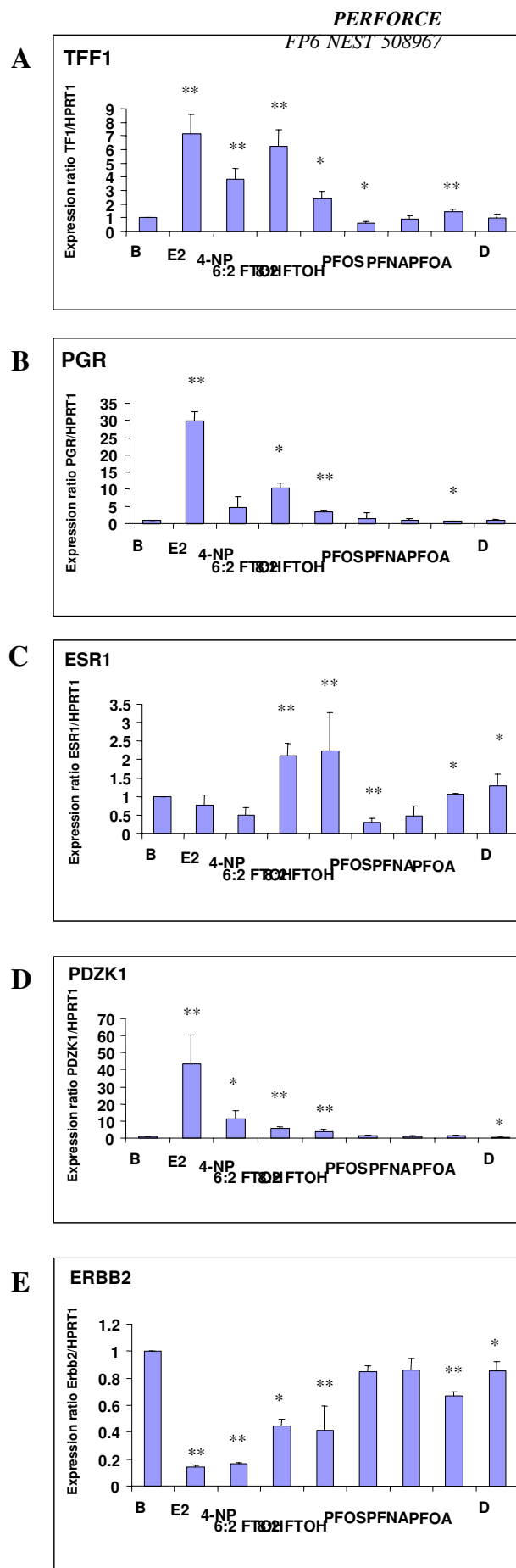
percentage of cells in the S(ynthesis) phase were observed (Table 7-2). In order to further investigate the resemblance of the newly detected xeno-estrogens to the reference compound 17 β -estradiol, gene expression analysis of a number of estrogen-responsive genes was analysed by real time polymerase chain reaction. With 17 β -estradiol as well as with 4-nonylphenol and the fluorotelomer alcohols upregulation of trefoil factor 1, progesterone receptor and PDZK1 was noted, while downregulation of ERBB2 gene expression was observed. Small but relevant upregulation of the estrogen receptor was observed as a consequence of exposures to 6:2 FTOH or 8:2 FTOH. The latter finding suggests an alternative mode of action of the fluorotelomer alcohols compared to that of 17 β -estradiol. This study clearly underlines the need for future in vivo testing for specific endocrine related endpoints.

Table 7-2. Cell cycle analyses of MCF-7 cells exposed to different perfluorinated compounds. The higher the % of cells in the S-phase, the more cell division is stimulated

	G1/G0 phase (%)	S phase (%)	G2/M phase (%)
Treatments			
Solvent control (0.1 % DMSO)	90.43 +/- 1.01	6.00 +/- 1.00	3.57 +/- 1.64
Estradiol 1 nM	63.14 +/- 1.61	34.84 +/- 2.48	2.03 +/- 0.88
4-Nonylphenol 10 µM	63.71 +/- 1.86	34.64 +/- 0.47	1.64 +/- 1.41
6:2 FTOH 30 µM	66.98 +/- 4.09	30.83 +/- 3.23	2.19 +/- 0.87
8:2 FTOH 10 µM	68.53 +/- 1.48	29.36 +/- 1.78	2.11 +/- 0.49
PFOS 50 µM	85.63 +/- 0.94	10.49 +/- 0.71	3.87 +/- 0.73
PFNA 50 µM	85.53 +/- 1.64	9.89 +/- 1.53	4.57 +/- 0.42
PFOA 50 µM	83.57 +/- 1.04	9.17 +/- 0.57	6.83 +/- 0.59
2,3,7,8-TCDD 10 nM	87.46 +/- 0.30	7.96 +/- 0.37	4.58 +/- 0.45

Cell cycle analysis by flow cytometry followed by analysis using ModFit LT 3.0 software resulted in percentages of cells in G1/G0, S or G2/M phase. The data are mean values of three measurements per treatment. During all measurements, Cv values of the G0/G1 peak were below 3.6. (n=3)

Figure 7-14. Effect of perfluorinated chemicals on mRNA expression of estrogen-responsive genes. MCF-7 cells were treated with 0.1 % DMSO (B = blanc) 1 nM 17 β -estradiol (E2), 10 mM 4-nonylphenol (4-NP), 30 mM 1H,1H,2H,2H-perfluorooctan-1-ol (6:2 FTOH), 10 mM 1H,1H,2H,2H-perfluorodecan-1-ol (8:2 FTOH), 50 mM perfluoro-1-octan sulfonate (PFOS), 50 mM perfluorononanoic acid (PFNA), 50 μ M perfluorooctanoic acid (PFOA), 10 nM dioxin (D). Upon exposure to the test compounds during 48 hours, mRNA levels of (A) trefoil factor 1 *TFF1*, (B) Progesterone receptor *PGR*, (C) estrogen receptor- α *ESR1*, (D) *PDZK1* and of (E) *ERBB2* were measured by real time-PCR and normalized using hypoxanthine phosphotransferase 1 *HPRT1* as internal control. Results are means from 3 replicate measurements and are expressed as fold relative to 0.1% DMSO. * $p < 0.05$; ** $P < 0.001$



7.6.3 Mode of action study of PFOS and PFOA with a human gene profiling assay (Cat-tox)

Aim

To gain insight in the mode of action of PFOS and PFOA by means of genetically modified human hepatocarcinoma cells (HepG2).

Introduction

Gene profiling assays make use of a battery of stressgene promoters fused to a reporter gene that is easily detectable. In this assay promoter::chloramphenicol acetyltransferase (CAT) constructs were used. The promoters used are sensitive to a whole variety of toxicological endpoints DNA damage, oxidative stress, osmotic stress, protein perturbation, tumor induction, ... (Table 7-3). Methods are described in Annex 7.6.

Table 7-3. Cat-tox promoters (HepG2, promoter::cat)

Abbreviation	Description	Sensitivity
CYP1A1	Cytochrome p450 1A1	PAH, dioxins
GSTY _a	Glutathione S transferase	PAH, phenolic antioxidants
XRE	Xenobiotic response element	PAH
HMTIIA	Metallothioneine IIA	Heavy metals, glucocorticoids
FOS	c-fos proto-oncogene	Tumor induction, oxidative stress, DNA damage
NFκBRE	Nuclear factor κ binding site	Oxidative damage, inflammation
HSP70	Heat shock protein	Protein perturbation, temperature
CRE	C-AMP response element	Increased c-AMP level
P53RE	P53 tumor suppressor binding site	DNA damage
RARE	Retinoic acid responsive element	Retinoids
GADD153	Growth arrest/DNA damage	DNA damage, not to X-ray or phorbol esters
GADD45	Growth arrest/DNA damage	DNA damage and growth arrest
GRP78	Glucose regulated protein	Protein misfolding

Results & Discussion

After exposure to PFOS (Fig. 7-15) significant inductions of FOS (responsive to DNA damage, oxidative stress and cell cycle) and GRP78 (responsive to effects occurring at the endoplasmatic reticulum, e.g. protein folding) were found. Only Gadd45 was significantly induced at PFOA concentrations of 125 and 250 µM (Fig. 7-16).

When comparing the results for both perfluorinated compounds, we clearly see that the upregulation of the different stress genes is much higher after exposure to PFOS than after exposure to PFOA (although similar concentrations were used). The results for the human gene expression profiling assay show that after PFOS exposure the stress genes related to DNA damage and cell cycle events were affected together with disturbance of the endoplasmatic reticulum (i.e. part of the endomembrane system – see higher), even at the lowest concentration tested (62,5 µM).

For PFOA only an effect on the Gadd45 Gene was noticed, a stress gene that can be linked to growth arrest and DNA damage.

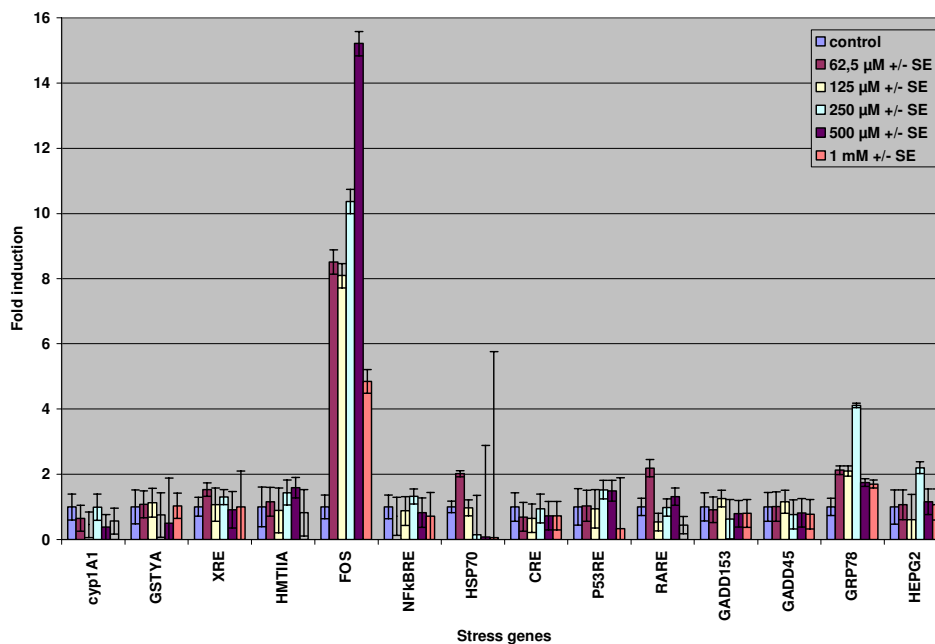


Figure 7-15: Dose response curves after exposure to PFOS. Results are represented as mean (n = 3) $\pm$ SE.

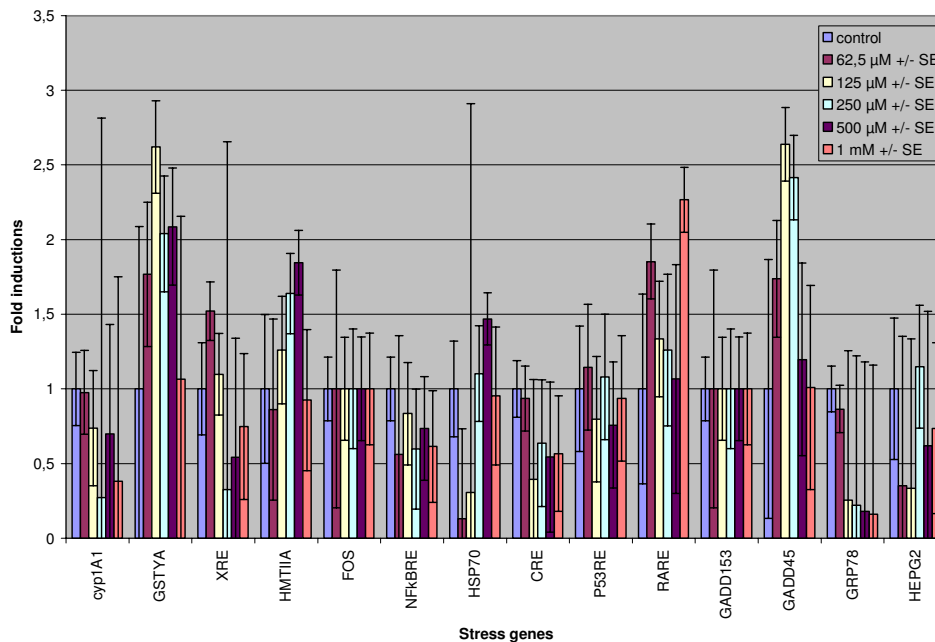


Figure 7-16: Dose response curves after exposure to PFOA. Results are represented as mean (n = 3) $\pm$ SE.

7.7 Mode of action study of PFOS and PFOA with a bacterial gene expression profiling assay (protox)

7.7.1 Aim

Using *Eschericia coli* gene expression profiles to gain insight in the mode of action of PFOS and PFOA.

7.7.2 Introduction

The complete protox assay consists of 13 transgenic *E. coli* strains with single copy chromosomal inserts of different *promoter:lacZ* fusions, originally published by (Orser et al. 1995) complemented with the PQ37 strain described by (Quillardet et al. 1985) which harbors an equivalent fusion, i.e. the SOS chromotest. The promoters belong to different toxicological endpoint classes, e.g. oxidative stress, DNA damage, membrane integrity and respond in a dose dependent manner to their respective inducers (Table 7-4). The integration of 14 endpoints in one test enables a much more complete characterization of the mode of action of the toxicants or mixtures compared to one endpoint tests. The *lacZ* gene, encoding the bacterial β -galactosidase is widely used as a reporter gene and allows for fast and easy detection through colorimetric measurement. Experimental details are given in Annex 7.6.

Table 7-4: stress gene promoters (*E. coli*, promoter::*lacZ*)

Promoter	Gene product/Function	Responsive to
KatG	Hydrogen peroxidase I	Oxidative damage
MicF	Antisense RNA to 5' OmpF	Membrane integrity, osmotic stress
OsmY	Periplasmic Protein	Osmotic stress
UspA	Universal stress protein	Growth arrest
RecA	General recombination and DNA repair	SOS response
Zwf	Glucose-6-phosphate dehydrogenase	Oxidative stress
ClpB	Proteolytic activation of ClpP	Protein perturbation
UmuDC	DNA repair	Radiation and/or chemically induced DNA damage
MerR	Regulation of the mercury resistance operon (mer)	Heavy metals
Ada	Adaptive response to alkylation	DNA damage, mainly methyl adducts
DinD	Unknown function within the DNA damage inducible response	DNA damage
Soi28	Superoxide inducible gene	Superoxide radical generating agents
Nfo	Endonuclease IV	ss and dsDNA breaks, oxidative DNA damage
PQ37	Inhibitor of cell division	SOS response

7.7.3 Results & Discussion

Figure 7-17 shows the results of the bacterial gene expression profile after exposure to PFOS, different stress genes are significantly induced: OsmY, UspA, RecA, Zwf, ClpB, Ada, DinD and PQ37. This suggests that PFOS is targeting the membrane, is causing oxidative damage and results in interference with DNA metabolism. The membrane related stress is most probably a direct consequence of the detergent like nature of the compound.

Toxicity results for the exposure experiment with PFOA (Figure 7-18) illustrate that the markers for osmotic damage (MicF and OsmY), the markers for oxidative damage (KatG, Zwf and Nfo), the marker for protein damage (ClpB) and the markers for DNA damage (UspA, , DinD and PQ37) are significantly induced. The overall PFOA toxic mechanisms for bacteria is rather similar to that of PFOS although other genes are affected (MicF – membrane related damage; KatG and Nfo – oxidative damage). Also here the observed membrane damage could be linked to the oleophobic properties of the chemical.

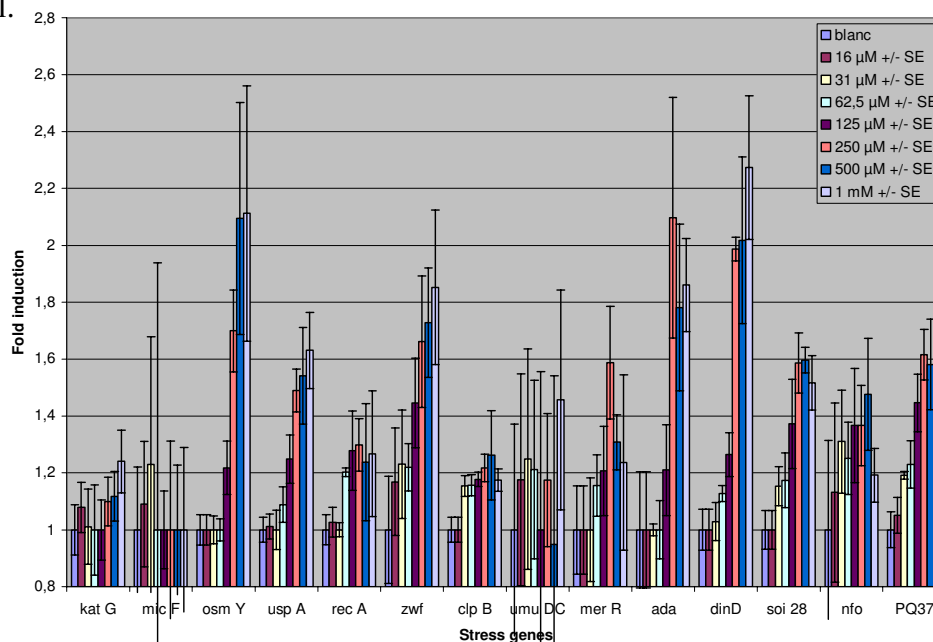


Figure 7-17. Bacterial gene expression profile after exposure to PFOS. Results are represented as average ($n = 3$) $\pm$ standard error.

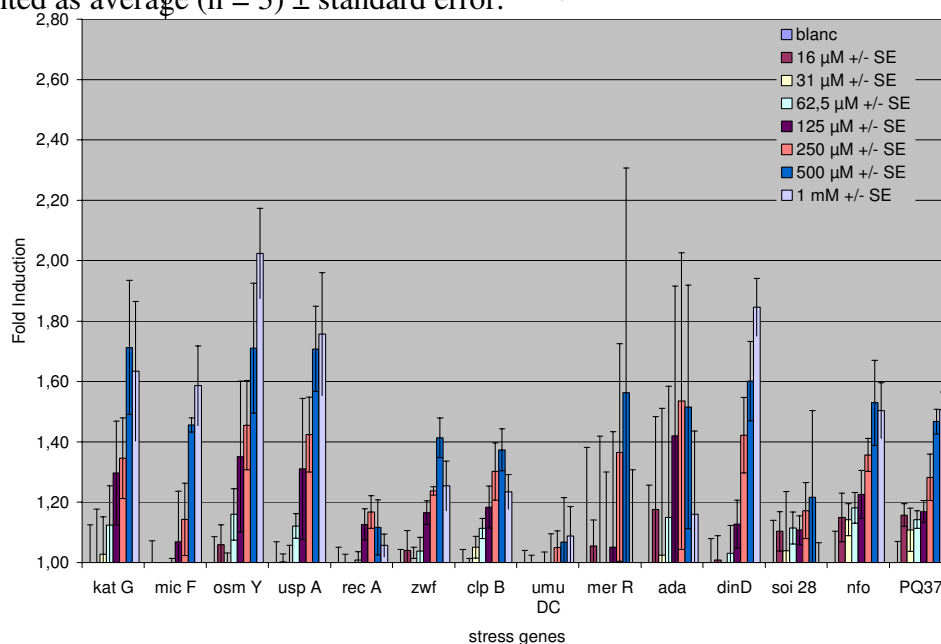


Figure 7-18. Bacterial gene expression profile after exposure to PFOA. Results are represented as average ($n = 3$) $\pm$ standard error.

7.8 References to chapter 7

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8. Conclusions and recommendations

8.1 Conclusions

Perfluorinated chemicals is a general term used to describe chemical substances which are largely comprised of or contain a perfluorinated or polyfluorinated carbon chain moiety such as $F(CF_2)_n-$ or $F(CF_2)_nC_2H_4-$.

Two different major production processes exist, giving rise to linear and branched materials, depending on the production process. The Perforce project selected some representative PFAS from the many that are manufactured or observed in the environment.

Due to the presence of various PTFE materials in laboratory equipment and solvents, blank contamination is a challenge when analysing per- and polyfluorinated compounds, especially for water samples with relatively low levels of the compounds of interest.

Purity of internal standards is an important issue. Commercial standards must therefore be carefully characterised before use and uncertainties in analytical results have to be reported.

Matrix effects are specifically inherent to PFAS analysis because of their amphiphilic character and existing methods lack robustness to cope with a broad range of different matrices

A modification of the Powley method with additional concentration step was developed and validated successfully.

Analytical methods for four different matrices were developed and validated; these include water, sediment, air, and biota.

The analytical methods developed showed good accuracies on the matrices included in the validation, demonstrating that these methods are fit-for-purpose.

For specific matrices such as cod liver, where matrix effects were observed it should be noted that the methods are not yet sufficiently robust to provide accurate results.

Interlaboratory comparison by co-analysis of selected samples within the consortium showed that the comparability and sample pre-treatment and analytical determination is reasonably good for the biota and sediment matrices, but poor in some sewage sludge and water samples.

The worldwide interlaboratory study on a fish tissue, fish liver extract and a water sample showed large variation in the between-laboratory results, showing that participating

laboratories were not yet able to generate comparable results. Poor accuracy of individual laboratories is most likely caused by improper choice of (internal) standards, non-selective extraction methods and non-selective final detection.

QA/QC should be carefully considered when generating and interpreting the results of PFAS analyses.

The vapour pressure values of 4:2 FTOH, 6:2 FTOH and 8:2 FTOH suggest that atmospheric transport may be important for FTOHs.

The measurements suggest that intra-molecular hydrogen bonding is not important in X:2 FTOH liquids. Molecular simulations also indicate that intra-molecular hydrogen bonding in X:2 FTOH liquids is not significant.

The sediment-water distribution coefficients (K_d , L/kg) of PFAS obtained in equilibrium batch experiments suggest that PFOA and PFOS do not accumulate in sediments, and that sorption to sediment does not strongly affect water-mediated transport of these PFAS.

Sorption increases with carbon chain length and thus becomes more important in the environmental fate of longer chain PFAS. Sediment organic matter does not appear to affect the sorption of PFAS. Ion strength strongly affects the sorption of PFAS to sediment. The results agree with the general sorption mechanism of anionic surfactants.

Degradation of PFAS was tested in long-term (2-10 months) aerobic and anaerobic closed bottle tests. No degradation was observed under the test conditions used

Molecular simulations indicate that water to biological membrane transfer of PFAS is energetically unfavorable. In a membrane, PFAS tend to align with the lipids. PFC – membrane interaction energies are stronger for longer chain PFCs.

PFAS are ubiquitously present in the European environment.

Sewage treatment plants probably serve as sources of PFAS both for the aquatic ecosystems (through effluent discharges) and the terrestrial environment (through application of sewage sludge in agriculture).

Analysis of a dated sediment core from the Baltic Sea indicates that levels of PFOS increased from 1990 to 2005, whereas PFOSA showed a different trend over the same period.

The annual loading to the European environment of PFHxA, PFHpA, and PFOA is estimated to be of the order of 10, 2, and 20 tonnes. The Danube and Rhine watersheds are particularly important source regions, whereby the Elbe and Po also make a significant contribution for PFHxA and PFHpA/PFOA, respectively.

Sediment is probably not a major sink for PFOS, PFOA and shorter chain homologues.

PFOS and PFOSA concentrations were higher in North Sea cod liver than in the Kattegat and the Baltic.

In European air, PFOA was often the predominant PFAS found in the particulate phase, while 6:2 FTOH and 8:2 FTOH were the prevailing analytes found in the gas phase. Many other compounds were also present.

In marine mammals several PFAS are found. PFCAs are relatively low in all species and tissues analysed. A relationship appears to exist between levels of PFOS on the one hand and trophic levels and on shore/offshore feeding on the other.

PFOS, PFDA and PFUnA bioaccumulate in a simple estuarine food chain, PFOA accumulates significantly less.

Perfluoralkyl compounds show distinct differences in toxicological mode of action *in vitro*. They do not act through similar pathways as a classical “peroxisomal proliferator” in rat a hepatoma cell line.

Transgenic reporter bio-assays with rat hepatoma cells were developed. These cell lines were able to quantify individual compounds (PFOA and PFOS) but were less promising when applied to extracts from environmental matrices due to cytotoxic side effects.

In vitro toxicological effects of perfluorinated compounds were tested on human cell lines. Estrogen-like (breast cancer cells, MCF7) and mitogen-like properties (liver hepatoma cells, HepG2) could be demonstrated for certain compounds.

In vitro toxicological effects of perfluorinated compounds were tested on transgenic bacteria (*E. coli*). Both PFOS and PFOA affected membrane integrity, caused oxidative stress and interfered with DNA metabolism.

8.2 Recommendations and future work

The modified Powley method is recommended as the method of choice for trace analysis of per- and polyfluorinated compounds in biological samples and solid abiotic environmental samples.

The method for water analysis is still under development and needs further validation. For water samples with high particle contents, the particle phase should be analysed separately

A promising easy to use, less time and solvent consuming method for analysing air-samples with the use of commercial SPE cartridges is under development. This method is also less susceptible to blank contamination.

The behaviour of PFAS during sample pre-treatment and storage should be studied in more detail to further reduce within- and between-laboratory variability.

The accuracy and robustness of methods should be improved to support policy makers, industries and academia.

Follow up interlaboratory comparisons are necessary to assess improvements in the accuracy of individual laboratories performing PFAS analysis.

The *n*-octanol/water partition coefficient as a descriptor for bioaccumulation may be replaceable by a descriptor for partitioning into biological membranes calculated from water to membrane transfer energies (enthalpies).

Pending the improvements in analytical methodologies, assessment of the fluxes to the environment from STPs requires further work on levels of PFAS in STP matrices.

Further sampling of river water is required to quantify loadings and identify sources.

There is a need to improve our understanding of PFAS transfer to the atmosphere and removal from the atmosphere.

Specific efforts, both of analytical and source inventory kind, are necessary to identify unknown origins of PFHxA.

A better characterizing of industrial uses for 6:2FTS is necessary.

There is a need for more detailed studies of the mechanisms of bioaccumulation / biomagnifications / bioelimination of PFAS

More work is needed on the specificity of the *in vitro* transgenic cell constructs and possibly more specific extraction methods should be performed to allow the use of the cell lines.

More mechanistic *in vivo* work would be needed to unravel the toxicological modes of action of PFAS.