Fluorinated, brominated and chlorinated contaminants in fish for human consumption

Methods and measurements

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VRIJE UNIVERSITEIT

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Aard- en Levenswetenschappen
op woensdag 9 september 2009 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

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geboren te Boxtel

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copromotor: dr. P.E.G. Leonards

Voor mijn liefsten

Jolanda, Pien en Sam

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Chapter 1

Introduction and objectives

Introduction

Since the industrial revolution in the 19th century, the knowledge on chemistry has developed rapidly. Together with the strong development of several industries (i.e. petrochemical, pharmaceutical, etc.), the number of chemicals being applied grew to large numbers. In the framework of the EU REACH programme, over 100,000 compounds have been pre-registered[1] for use within the European Union (*1*). Chemicals find their way in all kinds of industrial applications and consumer products. They play an important role in the convenience, safety and wealth we enjoy everyday. Chemistry is everywhere around.

Most of the compounds and products are harmless. However, several compounds can do harm to wildlife and humans, some already at very low concentrations. Well known examples are polychlorinated biphenyls (PCBs), lindane, aldrin, dieldrin, endrin and DDT (*2-6*). These were produced and applied in the past, because of their useful properties as e.g. flame retardant or insecticide. However, their presence in the environment and humans is undesired because of specific toxic effects at very low concentrations. Therefore, and because of their persistent character, these compounds classified as "persistent organic pollutants" (POPs).

Persistent organic pollutants (POPs)

POPs were defined under the Stockholm Convention that entered into force in 2004 (*7*). POPs are compounds that are persistent, bioaccumulate, show long-range transportation and are toxic. The definitions are mentioned in the grey box below. POPs are resistant to degradation processes such as photolytic, chemical and biological degradation (their structure is not easy accessible for micro-organisms). Consequently, they remain intact and do not degrade under environmental conditions. Most POPs are lipophilic and their uptake rates in organisms are higher than the rate of depuration. This results in an accumulation in aquatic and terrestrial organisms and in humans (bioaccumulation). Further transfer up in the food chain can lead to elevated levels in top predators (biomagnification). These properties lead to continuous exposure to POPs. Their toxic properties can cause serious health effects such as certain cancers, birth defects, dysfunctional immune and reproductive systems, greater susceptibility to diseases and even diminished intelligence (*2,3,5,6,8-11*). Because their widespread use and aerial transport, the contamination with POPs has become a world-wide problem.

[1] Pre-registration in the framework of the EU Reach program (Registration, Evaluation, Authorisation and restriction of Chemicals) is required for chemicals that are *substances* on their own, in preparations and those which are intentionally released from articles. Pre-registration stopped at 1 December 2008.

The group of POPs currently consist of the following 12 compounds: (i) eight chlorinated pesticides (dieldrin, endrin, aldrin, chlordane, heptachlor, DDT, mirex and toxaphene), (ii) two industrial chemicals (hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs)) and (iii) two unintentionally produced compounds (polychlorinated dibenzo-p-dioxins (PCDDs)), also abbreviated as dioxins, and polychlorinated dibenzofurans (PCDFs), also abbreviated as furans) (*12*). The structures are shown in Figure 1.1. These compounds (except dioxins and furans) were produced intentionally between the 1930s to the 1980s for use as insecticide or fungicide and as flame retardants in heat capacitors (see chapter 2.1 for more information on applications). POPs entered the environment during their production, use and after disposal. Dioxins and furans have never been produced intentionally, but result from incomplete combustions and are by-products of the production of certain pesticides and some other specific chemicals.

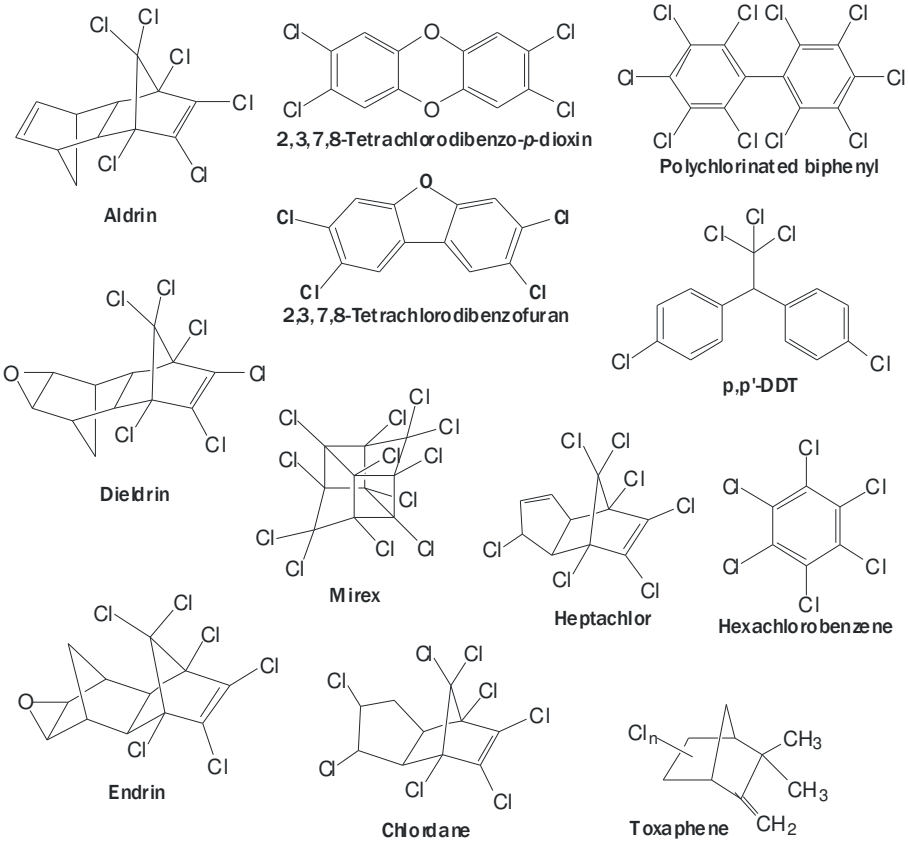

Figure 1.1 Chemical structures of the 12 persistent organic pollutants (POPs).

Stockholm Convention criteria for POPs (Annex D of (*7*)).

Persistence:

(i) Evidence that the half-life of the chemical in water is greater than two months, or that its half-life in soil is greater than six months, or that its half-life in sediment is greater than six months; or

(ii) Evidence that the chemical is otherwise sufficiently persistent to justify its consideration within the scope of this Convention;

Bio-accumulation:

(i) Evidence that the bio-concentration factor or bio-accumulation factor in aquatic species for the chemical is greater than 5,000 or, in the absence of such data, that the log Kow is greater than 5;

(ii) Evidence that a chemical presents other reasons for concern, such as high bio-accumulation in other species, high toxicity or ecotoxicity; or

(iii) Monitoring data in biota indicating that the bio-accumulation potential of the chemical is sufficient to justify its consideration within the scope of this Convention;

Potential for long-range environmental transport:

(i) Measured levels of the chemical in locations distant from the sources of its release that are of potential concern;

(ii) Monitoring data showing that long-range environmental transport of the chemical, with the potential for transfer to a receiving environment, may have occurred via air, water or migratory species; or

(iii) Environmental fate properties and/or model results that demonstrate that the chemical has a potential for long-range environmental transport through air, water or migratory species, with the potential for transfer to a receiving environment in locations distant from the sources of its release. For a chemical that migrates significantly through the air, its half-life in air should be greater than two days; and

Adverse effects:

(i) Evidence of adverse effects to human health or to the environment that justifies consideration of the chemical within the scope of this Convention; or

(ii) Toxicity or ecotoxicity data that indicate the potential for damage to human health or to the environment.

Countries and organisations that are bound to the Stockholm Convention take measures to reduce the environmental presence of these POPs. The reduction of emissions of these contaminants is achieved by two means: (i) reducing the emissions of *intentionally produced compounds* (aldrin, dieldrin, endrin, chlordane, DDT, heptachlor, hexachlorobenzene (HCB), mirex, PCBs and toxaphene), by terminating their production and use and (ii) by reducing the emissions of *unintentionally produced contaminants* (dioxins and furans) (*7*).

POPs in the environment and human food chain

POPs enter the environment through different routes. Figure 1.2 shows an example of the contamination of the environment with contaminants from point sources (e.g. production) and diffuse sources, and subsequent aerial, terrestrial and aquatic distribution.

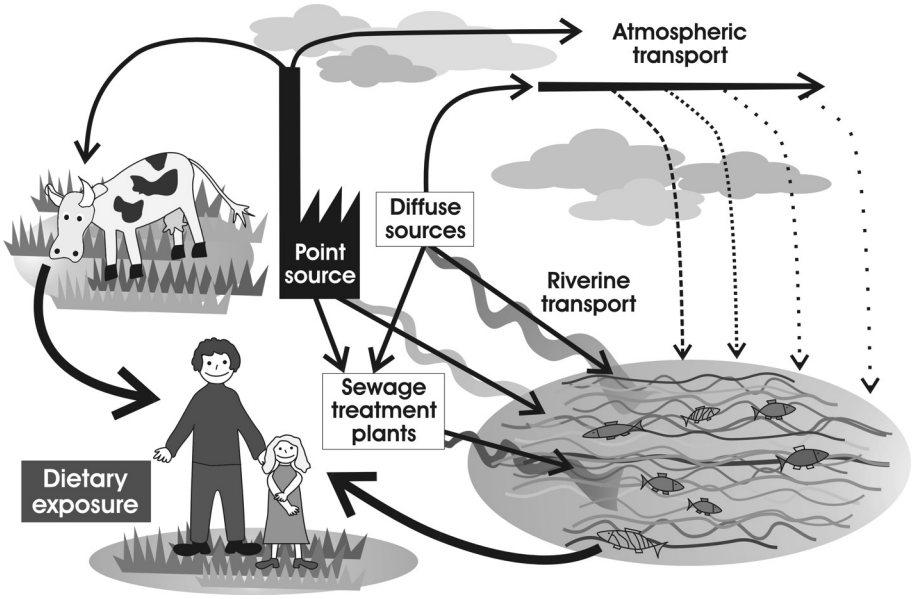

Figure 1.2 Contaminant emissions and typical aerial, terrestrial and aquatic distribution routes. The arrows pointing towards the humans indicate their dietary exposure through food from animal origin. Copyright A. Jahnke.

The emission routes that can be distinguished are:

- *Production or synthesis:* during the production (i.e. synthesis), POPs were emitted from the POP manufacturing plant through spillage or evaporation.

- *Application or product formulation:* POPs were never applied in the pure form, but were applied as an active ingredient in a formulated product. For example, PCBs were added to transformer oil to provide heat resistance to the oil (*2*). DDTs Technical DDT has been formulated in very diverse forms (e.g. emulsifiable concentrates, granules, smoke candles, charges for vaporizers, and lotions) (*6*). During this product formulation stage, POPs may have leached to the environment.
- *In-service life:* during the life-time of a product small amounts may have leached from the material to which they were applied. For example, small amounts of PCB containing transformer oil may have leaked from transformers (*2*). Because of snowfall and rainfall, DDT leached from the crops (and leafs) into surface water, soil and ground water (*6*).
- *Disposal:* after the in-service life has finished, products are disposed to waste incinerations and landfills. In some cases, PCBs have emitted from landfills through evaporation and leaching into soil and groundwater (*2*).

New POPs

Following the ratification of the Stockholm Convention, parties took action in order to reduce the emissions of the 12 POPs. The production and use of POPs have substantially decreased (DDTs) or even completely stopped (most other POPs) in most countries. Also, the emission of dioxins was reduced in several countries e.g. by removing them from the flue gasses emitted from waste incinerators (*13*).

Unfortunately, several compounds have been produced in the last decades that also meet (some of) the persistency, bioaccumulative, long range transportation and toxicity criteria. In some cases, these have been produced as an alternative for a phased-out POP. Examples of these potential new POPs are brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD). They are produced because there is a need (and legal obligation) to make materials flame retardant. Another example is the surfactant perfluorooctane sulfonate (PFOS), which was applied in e.g. aqueous fire fighting foams (AFFF).

Because the Stockholm Convention aims at the decrease of the environmental and human exposure to POPs, new substances that fulfil the POP criteria can be proposed for inclusion in the POP list. After a process of evidence gathering and recommendation, the parties decide by voting on the inclusion of these "candidate POPs" in the official POP list. Other international organisations such as the United States Environmental Protection Agency (USEPA), Environment Canada and the European Chemicals Agency (ECHA) are also actively evaluating chemical substances. Some examples of compounds currently being evaluated are:

- *Polybrominated diphenyl ethers (PBDEs)* – This is an additive flame retardant that was applied in e.g polyurethane foams for cars and furniture, textiles, building materials and packaging (*14,15*). In the framework of the Stockholm Convention, the commercial *octabromo diphenyl ether* mixture was recently proposed for the risk management evaluation process (RMEP). This will result in a positive or negative recommendation for inclusion in the POP list (*16*). The commercial *pentabromo diphenyl ether* mixture is recommended for inclusion in the POP list aiming at elimination of its use (*17*).
- *Hexabromocyclododecane (HBCD)* – HBCD is an additive flame retardant applied (mostly) in polystyrene, but also in textile and upholstery (*18,19*). The European Chemicals Agency (ECHA) is evaluating HBCD as a substance of very high concern (SVHC). It was recently concluded that HBCD is a PBT substance (*20*). HBCD was recently proposed by Norway for inclusion in Annex A (elimination of production and use) under the Stockholm Convention (*21,22*).
- *Perfluorooctane sulfonate (PFOS)* – PFOS has excellent surfactant properties and was applied in aqueous fire fighting foams (AFFFs), as mist suppressant and as a water oil and stain repellent (*23*). In the early 2000's, a major PFOS producer voluntarily phased out the production of PFOS (*24*). Recently, PFOS is recommended for inclusion in the POP list of the Stockholm Convention, aiming at elimination (Annex A) or restriction of the use (Annex B) (*25*). The application of PFOS is restricted by 2008 in major applications, effectively resulting in a ban of its use in most applications (although in some cases (e.g. AFFFs) the use of PFOS is still allowed until 2011) (*26*).

Structures of PFOS, HBCD and PBDEs are shown in Figure 1.3.

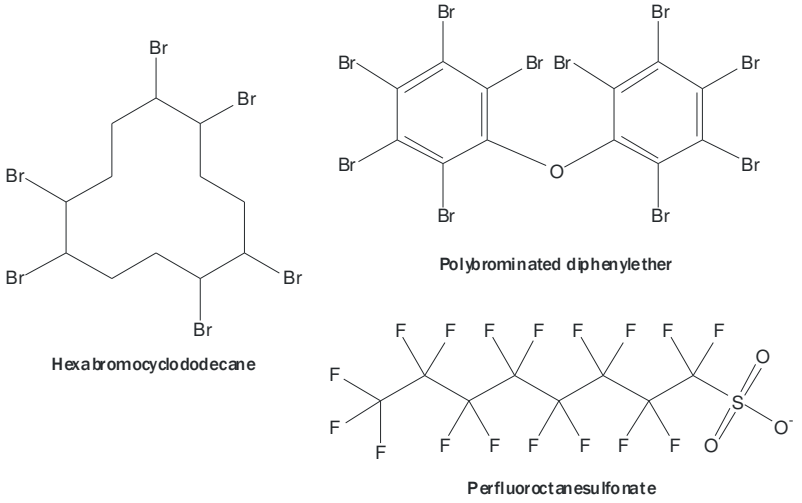

Figure 1.3 Chemical structures of HBCD, PBDE and PFOS.

Dilemmas

The evaluation of POPs and candidate POPs and the restriction of their use are difficult processes. All pro's and con's of a substance should be carefully balanced. Clearly, on the one hand, emissions should be reduced as much as possible and production may have to be terminated. On the other hand, some of these chemicals help to save lives or have other important functionalities that cannot at once be replaced. For example, some BFRs that enter the environment result in environmental and human exposure and accumulation. This is an undesirable side-effect of the use of these compounds and could on the long term lead to health effects on organisms and humans (*27*). On the other hand, BFRs save over hundreds of lives world-wide each year by preventing products from rapidly catching fire (*28*). Another example is the dilemma of DDT. Every 30 seconds a child dies of malaria (*29*). DDT very effectively kills the malaria carrying mosquitoes and saves thousands of lives in Africa where malaria still isn't under control (*30*). On the other hand, DDT is found in every hidden corner of the world and has caused e.g. egg shell thinning of birds of prey (*31*). Furthermore, DDT showed to have *in vivo* and *in vitro* effects on the female reproductive tract of mammals, and was associated with e.g. pancreatic cancer and neuropsychological dysfunction (*32,33*).

As a final example, PFOS was applied as a surfactant in e.g. AFFFs (*23*). Due to the perfluorination of the molecule, PFOS has outstanding surface tension lowering properties. This makes the AFFF spread very rapidly over a fuel, thereby rapidly diminishing a fire by disclosing oxygen from the system. Alternatives have been developed, but these less effectively extinct liquid fires. PFOS enters the environment from fire events where the foam/water mixture leaches to the surface waters or sewage systems. Furthermore accidental and uncontrolled releases may result in environmental exposure. At Schiphol airport, PFOS was recently (summer 2008) released into the environment due to an accidental initiation of a fire sprinkler system (*34*). As a result, consumption of certain fish species that were caught in the receiving waters had to be restricted (*35*). Unfortunately, PFOS has several adverse effects, such as developmental effects (*36*), changes in thyroid hormone system (*37*) and high density lipoprotein levels (*36*). The liver is the major target organ for most effects (*36,38*).

The cases of PFOS, HBCD and PBDEs show that although a simple ban of the compounds would result in a rapid decrease of emissions to the environment, the needs for effective flame retardants, fire fighting foams and malaria insecticides remain. It is therefore very important that industries, policy makers, academia and research institutes jointly search for alternatives that are less harmful to the environment and humans, but offer an equal safety level as the original use substances. This dilemma was recognised by the European Commission. Recently, the ENFIRO project was launched in which industry, academia and research institutes together look for non-halogenated

alternatives for BFRs. These alternatives will be evaluated in terms of flame retardency effectiveness and persistency, bioaccumulative and toxicological properties. In addition, (economic) feasibility of production will be regarded.

Human risk assessment of (candidate) POPs

POPs and candidate POPs are found in humans around the world. They have been detected in blood, organs, lipid depots, cord blood and mothers milk (*36,39-45*). A major route of exposure for POPs discussed above is through food (*38,46-52*). Other pathways are exposure from air, through dust ingestion and drinking water (*53-63*). For the traditional lipophilic POPs such as PCBs, organochlorinated pesticides and dioxins, fish is a dominant contributor to dietary exposure in most parts of Europe (*64,65*). In the Netherlands, because of the high consumption, dairy products are the predominant source. Fish is also in important source of dietary exposure (*47,51,52*). Because of the POP emission restriction measures, the emission of most POPs has been reduced substantially, which in turn led to a decrease of the levels in food, and decreased exposures (*51,52*). However, more than 30 years after the ban on PCB production in Europe, eel from the rivers Meuse and Rhine still contain PCB concentrations (*66,67*) that exceed Dutch maximum levels (MLs) (*68*). This shows the enormous impact of these POPs, once they have reached the environment.

The risks of exposure to POPs are evaluated in risk assessment processes. These typically consist of a hazard identification followed by hazard characterisation, exposure assessment and, finally, a risk characterisation (*69*). The *hazard identification* typically involves the identification of the contaminant and of the effects that are considered as adverse (*69*). The identification traditionally follows from in vivo (animal) experiments. However, with the aim of reducing animal experiments, other approaches gain more importance such as computational toxicology and in-vitro toxicology evaluation (*70,71*). The *hazard characterisation* describes the process of quantification of the relevant adverse effects. This is often referred to as the dose-response relationship. This results in benchmarks such as the no-observed-adverse-effect-level (NOAEL), which is the level of exposure of which the effects in the treated animals do not differ significantly from those in the untreated (control) animals. The *exposure assessment* aims at characterisation of the nature and size of the human population exposed to an emission source and the magnitude, frequency and duration of that exposure (*69*). Finally, *risk characterisation* relates to the estimation of the probability of the occurrence and the severity of adverse effects in a certain human population, based on the previous three stages by comparing the estimated exposure and the hazard characterisation (*69*).

Analytical challenges for the analysis of (candidate) POPs

For a reliable risk assessment, accurate exposure data are needed. The quality of an exposure assessment is determined by the quality of two groups of experimental input data: (i) the food item consumption data and (ii) the contaminant concentrations in these food items. In both cases, care should be taken that the data is representative. As regards food consumption, several Dutch National Food Consumption Surveys (DNFCS) have been carried out in which consumers were asked to record in detail their food and beverages consumed at two consecutive days. The DNFCS-3 (1997/1998) focussed on the population of 7-69 years, the DNFCS-Young adults (2003) focussed on the age of 19 to 30 years and the DNFCS-Young children (2005/2006) focussed on the age of 2-6 years (*72*).

For some POPs, accurate methods are available and various quality assurance tools are in place. This is the case for e.g. dioxins and dl-PCBs. Accurate methods are available as well as annually organised interlaboratory studies (*73-75*). Some certified reference materials (CRMs) are available, although there is a need for matrices with relevant concentration ranges (*76*). Because of the need for monitoring dioxins and dl-PCBs in food, the EU has established criteria for accurate analysis (*77*). The drawback of the analysis of dioxins and dl-PCBs is the extensive sample extraction and clean-up and the use of expensive equipment for analysis such as gas chromatography – high resolution mass spectrometry (GC-HRMS) (*78*). The major challenge for the analysis of these compounds is to reduce costs per analysis by improving the speed of extraction and clean-up and by introducing less expensive alternatives to GC-HRMS, while maintaining the same level of performance. The EU projects DIFFERENCE and DIAC (*79*) have shown that alternative methods (e.g. GC-ion trap MS/MS; comprehensive multidimensional GCxGC and CALUX bioassay) are available and can produce reliable results.

For PBDEs, many methods for analysis of fish have become available since the early 2000's (*78*). Methods for PBDEs in other food items are also available (*80,81*). Although the analysis of PBDEs may seem as 'straightforward' as that of PCBs, there are several issues may complicate the determination of some of these compounds. This includes problems with blanks, contamination of samples and degradation of higher brominated BDE congeners (*80*).

The analysis of HBCD is also challenging. HBCD consists of 3 (major) diastereomers (α-, β- and γ-HBCD). Initially, HBCD was analysed by GC, but the accuracy of the results was limited by HBCD degradation in the injector and column, different response factors of each diastereomer and the inability to separate the diastereomers on any GC column (*46,80*).

The class of poly- or perfluorinated compounds (PFCs) only received attention as a food contaminant during the last 3-4 years. PFCs are surfactants and accumulation is not lipid driven. Because they are not stored in lipids, they require different analytical techniques than the lipophilic compounds like dioxins, PCBs, PBDEs and HBCD. Further complicating factors are the diversity

of this group of compounds due to different chain lengths of the apolar tail, different degrees of fluorination of the tail and different (polar) functional heads of the molecules (*82*). These differences result in a broad range of aqueous solubilities, which should preferably all be covered by a single method. Complicating factors are the absence of good quality (well-defined) standards, the absence of suitable internal standards, the presence of interferences, matrix effects and the lack of CRMs and interlaboratory studies (*83*). Although these issues have been solved partly in recent years (*84,85*) many analytical issues remain.

Scope and outline of this thesis

The work in this thesis focuses on human exposure assessment aspects, i.e. the assessment of the levels of environmental contaminants in foods. The focus is placed on fish, as in the past fish proved to an important contributor to the exposure to lipophilic compounds (*51,52,64,86-89*). Assessment of contaminant levels in fish requires the following steps to be taken:

- Development of specific, robust, precise and accurate methods of analysis;
- In-house and between laboratory validation of the analytical method;
- Sampling relevant fish species for chemical analysis;
- Determination of the contaminant levels in the fish samples.

In addition to the development of sound methods and the assessment of the contaminant levels in fish, the final part of this thesis deals with an estimation of human exposure to a broad suite of contaminants from wild fish and farmed fish in order to determine the relative importance of specific contaminants and fish species.

In other words, we have tried to answer the following questions:

(i) Can we develop methods for a suite of candidate POPs, which are reliable and sufficiently accurate to produce data for human exposure assessment?

(ii) Which contaminant (group) contributes predominantly to the exposure of the general Dutch population?

(iii) Which fish species contributes most to the exposure and which alternatives are available in order to reduce exposure?

This may provide answers to risk managers on where to put their focus on.

When breaking down to chapters, the reader will find the following information: In chapter 2, an overview is presented on *the current state-of-the-art of methods* for chemical analysis of contaminants. This includes both the traditional lipophilic contaminants (generally analysed by GC) as well as candidate POPs such as several BFRs and the more recently discovered surfactant type of contaminants (analysed by liquid chromatography). In chapter 3, *methods are described that were developed and validated* for the determination of PCDD/Fs, (dl-)PCBs, PBDEs, HBCD diastereomers and

PFCs in fish. In chapter 4, *contaminant levels in a wide range of wild fish, farmed fish, crustacea and shellfish* samples are presented. In addition, the *relevance of these contaminants for human exposure* is discussed. In chapter 5, *concluding remarks* are presented.

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Chapter 2

State of the art of analysis

2.1 Advances in the gas chromatographic analysis of chlorinated and brominated contaminants[2]

Abstract

Environmental chemists have been challenged for over 30 years for analysis of complex mixtures of halogenated organic contaminants like polychlorinated biphenyls (PCBs), polychlorinated alkanes (PCAs), polybrominated diphenyl ethers (PBDEs) and polychlorinated dibenzo-*p*-dioxins and polychlorinated furans (PCDD/Fs). Gas chromatography (GC) often proved to be the method of choice because of its' high resolution. The recent developments in the field of comprehensive two-dimensional GC (GCxGC) show that this technique can provide much more information than conventional (single-column) GC. Large volume injection (e.g. by programmed temperature vaporiser, or on-column injection) can be employed for injection of tens of microliters of sample extract, in that way substantially improving the detection limits. Electron-capture detection (ECD) is a sensitive detection method but unambiguous identification is not possible and misidentification easily occurs. Mass spectrometric detection (MS) substantially improves the identification, and the better the resolution (as with MS/MS, time-of-flight (TOF) MS and high-resolution (HR)MS), the lower the chances of misidentification are. Unfortunately, this comes only with substantially higher investments and maintenance costs.

Co-extracted lipids, sulphur and other interferences can disturb the GC separation and detection leading to unreliable results. Extraction, and more so, sample clean-up and fractionation, are crucial steps prior to the GC analysis of these contaminants. Recent developments in sample extraction and clean-up show that selective pressurised liquid extraction (PLE) is an effective and efficient extraction *and* clean-up technique that enables processing of multiple samples in less than 1 hour.

Quality assurance tools such as interlaboratory studies and reference materials are very well established for PCDD/Fs and PCBs but improvement of that infrastructure is needed for brominated flame retardants, PCAs and toxaphene.

Introduction

Since the 1950s, persistent organic pollutants (POPs) have been produced in large volumes. During production, use and disposal, these POPs have entered the environment. The so-called 'dirty dozen' are POPs that are toxic,

[2] Based on S.P.J. van Leeuwen and J. de Boer (2008) Advances in the gas chromatographic determination of persistent organic pollutants in the aquatic environment, *Journal of Chromatography A*, 1184, 161-182

bioaccumulate in fatty tissues of animals and humans and do not easily degrade. These contaminants are officially registered by the United Nations Environmental Programme (UNEP) under the Stockholm Convention (*1*). They can be sub-divided as (i) eight chlorinated pesticides (dieldrin, endrin, aldrin, chlordane, heptachlor, DDT, mirex and toxaphene), (ii) two industrial chemicals (hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs)) and (iii) two unintentionally produced compounds (polychlorinated dibenzo-*p*-dioxins (PCDDs)), also abbreviated as dioxins, and polychlorinated dibenzofurans (PCDFs), also abbreviated as furans) (*2*). Although production of most POPs has ceased for over 20 years, we are still facing considerable POP levels in the environment. Apart from the aforementioned POPs, other contaminants have been proposed as candidates for addition to the POP list, e.g. hexachlorobutadiene (HCBD), polybrominated diphenyl ethers (PBDE: penta, octa and deca technical mixtures), pentachlorobenzene (QCB), polychlorinated naphthalenes (PCNs), short-chain polychlorinated alkanes (PCAs), dicofol and perfluoroctane sulfonate (PFOS). Furthermore, hexabromocyclododecane (HBCD) was recently proposed by Norway for elimination of production and use (Annex A) under the Stockholm Convention (*3*,*4*), and therefore, and a future ban on the use of HBCD is not unlikely.

Dioxins and furans have never been produced intentionally for use in industrial or consumer products or processes. However, they are generated in waste combustion processes. Other recorded sources are paper production, fuel burning and as by-products in pesticide/herbicide production (*5-7*). They have also been produced as undesired by-products in the production of technical mixtures of PCBs (*7*). PCDD/Fs are very persistent and accumulate in the lipid phase of biota or bind to the organic matter fraction of abiotic samples like sediment and soil (*5*). Table 2.1 shows the theoretical number of congeners possible. The 2,3,7,8-substituted dioxins and furans are among the most toxic contaminants known. Apart from the PCDD/Fs, 12 PCBs with a non-ortho or mono-ortho chlorine substitution (so called dioxin-like PCBs or dl-PCBs) have a similar toxic mode of action. Because of these toxic similarities, seventeen PCDD/Fs and 12 dl-PCBs were appointed a TCDD (tetraCDD) equivalency factor (TEF). All seventeen dioxins, furans and twelve PCBs have been compared to 2,3,7,8-TCDD, the most toxic congener with a TEF of 1. The other congeners are less toxic and therefore received a TEF lower than 1. The TEFs for humans and mammals were recently updated by WHO (*8*). Multiplying the concentration of a congener in a sample with their respective TEF will result in a TCDD equivalent (TEQ). Accumulation of all TEQs in a sample, the sum-TEQ is obtained. For more information on the individual TEFs and the TEF concept, please refer to van den Berg et al. (*8*). The high toxicity of PCDD/Fs and dl-PCBs and the low concentrations in aquatic samples (fg/g-pg/g range) calls for very sensitive, accurate, precise and selective detection techniques. Gas chromatography (GC) coupled with high resolution mass

spectrometry (HRMS) has served as the 'golden standard' for this analysis since the mid-1970s (*9*).

PCBs have been used for a number of decades e.g. as a dielectric in transformers and capacitors, as plasticizers and as fire resistant liquid in closed systems (*10*). PCBs are synthesized with different chlorination degrees. Although theoretically 209 congeners are possible (Table 2.1), the actual number of congeners found in the environment is much lower. PCBs are ubiquitously distributed in the (aquatic) environment (*10*). The dl-PCBs are discussed in detail together with the dioxins. The analysis of the other PCBs is often limited to a selection of 6 or 7 CBs, the so-called 'ICES-7' or 'indicator PCBs'. This selection consists of the CBs 28, 52, 101, 118, 138, 153 and 180 and covers a wide range of chlorination degrees (tri- to hepta-chlorination) and boiling points. Some specialized laboratories analyse 20-40 PCB congeners.

PCNs have been synthesised from melted naphthalene and chlorine in the presence of a catalyst. The application of PCNs is similar to that of PCBs and includes application as dielectrics for flameproofing and insulation in various industries, additives to rubber products, flame retardant and in lubricants (*11*). PCNs are also found as impurities in PCB technical mixtures and can be formed in thermal processes (e.g. solid waste burning) (*11*). PCNs can be potent inducers of ethoxyresorufin-O-deethylase (EROD) and the aryl hydrocarbon (Ah) receptor, and relative potencies (REPs, relative to TCDD) were derived for some tetra-hepta-PCN congeners (*11-13*). PCNs have been found in the environment world-wide, mostly at concentrations lower than those of other POPs (*14-16*).

PCAs have found their application as extreme pressure additives in lubricants and cutting oils, as plasticizers and flame retardants. They were also used as replacements for e.g. for PCBs (*17*). The terminology of chlorinated paraffins (CPs) is commonly used and therefore, in this paper we will use CPs rather than PCAs. Commercial CP products are classified according to their carbon chain length in short chain CPs (SCCPs, C10-C13), medium chain CPs (MCCPs, C14-C17) and long-chain CPs (LCCPs, >C17). CPs are produced by chlorination of n-paraffin or paraffin wax. Their widespread use has resulted in an ubiquitous distribution in the environment (*18-20*). Technical CP mixtures are among the most complex halogenated mixtures encountered in the environment. The carbon chain length may vary (C10-C22) and isomerisation of the carbon chain occurs. Furthermore, the different chlorination degree (30-70%) of the carbon chain leads to numerous possible contaminants (*21*). Methods for analysis of CPs are developed for the determination of either the SCCPs, MCCPs or the LCCPs, and the focus in recent years has been on SCCPs mainly (probably reflecting the continued production and use) (*18,20,22-26*). The complex nature of the technical mixtures has challenged several scientists trying to obtain accurate data.

Toxaphene is a very complex mixture of chlorinated bornanes, bornenes, camphenes and dihydrocamphenes with an average elemental composition

of $C_{10}H_{10}Cl_8$ (*27*). It consists of theoretically 32,768 possible congeners (Table 2.1). It was produced in volumes estimated to be larger than those of PCBs (per year) and marketed under a wide variety of trade names (Table 2.1) (*28*). It was used as pesticide on cotton, fruits and crops and for controlling ticks and mites on livestock (*29*). Several nomenclature systems were developed in the past (see (*28*) for an overview), but the system developed by Parlar is mostly used (*30*). In environmental samples a limited number of congeners, ca. 50-100, is found. Kimmel et al. determined that P26, 40, 41, 44, 50 and 62 were the predominant congeners in fish oil (*31*).

Organochlorine pesticides (OCPs) are a diverse group of chlorinated contaminants that have been used as pesticides. Well-known examples are (see Table 2.1) DDT, dieldrin, endrin, aldrin, lindane, HCB and chlordane. Most OCPs were very effective, broad spectrum pesticides, resulting in extensive use (Table 2.1). Examples of the insecticide use are on wood and structures (dieldrin, aldrin), crops (chlordane), animals (chlordane, lindane), seed and soil treatment (lindane) and protection of humans (mainly against malaria, typhus, and certain other vector borne diseases) (*32-36*). Hexachlorobenzene (HCB) was used as fungicide mainly (*37*). The production of OCPs was diminished in North America, Europe and Japan since the (late) 1970s, but production may have continued in other regions. The use of DDT in Africa is still supported by WHO as a cost effective way of reducing deaths caused by the malaria carrying mosquito (*38*).

Brominated flame retardants (BFRs) constitute a diverse group of contaminants that are added to a variety of materials in order to reduce, delay or even prevent them from catching fire. A substantial part of flame retardants consists of brominated compounds. The most frequently used BFRs are tetrabromobisphenol-A (TBBP-A), hexabromocyclododecane (HBCD) and polybrominated diphenylethers (PBDEs). BFRs are used at relatively high concentrations in various materials and polymers, such as polyurethane and polystyrene foams, in a wide range of products, such as printed circuit boards, television sets and computers and other electronic household equipment, cars and construction materials (*47*). Information on BFR usage figures (from 2003) can be found elsewhere (*48*). BFRs can be released into the environment through production, use, and especially from disposal of the flame retarded products. Various BFRs are present in biota (*49-51*) due to their lipophilicity and persistence. Although theoretically, 209 BDE congeners exist (Table 2.1), only a subset is commonly found in the environment, and therefore analysed. This subset consists of the BDEs 28, 47, 99, 100, 153, 154, 183 and 209, and maybe ca. 50 other BDEs present in much lower concentrations. Deca-BDE is predominantly found in sediments but nearly not in aquatic biota (*48*), although Eljarrat et al. found levels up to 707 ng/g lipid weight in fish downstream a deca-BDE discharging industrial park (*52*).

Table 2.1 Contaminant groups, abbreviations and theoretical number of possible congeners (or isomers) and a selection of trade names. Table edited from (39).

Name	N[a]	Production Volume	Typical trade names of technical mixtures	Ref
Polychlorinated dibenzo-p-dioxins (PCDDs)	135	Na	Na	(*7*)
Polychlorinated dibenzofurans (PCDFs)	75	Na	Na	(*7*)
Polychlorinated biphenyls (PCBs)	209	1,000,000 tonnes (ww, cumulative 1930-1980)	Aroclor (1242, 1254 or 1260), Pyranol, Pyroclor, Phenochlor, Pyralene, Clophen, Elaol, Kanechlor, Santotherm, Fenchlor, Apirolio, Sovol	(10,40)
Polychlorinated naphthalenes (PCNs)	75	150,000 tonnes (ww)	Halowax (1014, 1051), Nibren wax, Seekay Wax, Clonacire wax, N-oil, N-Wax, Cerifal Matarials	(*11*)
Chlorinated paraffins (CPs)	Unkn.	300,000 tonnes/yr (ww, currently)	SCCP: Cereclor 50LV, PCA 60, PCA 70, Witachlor149 and Witachlor 171P, Chlorawax, Chlorafin	(*21*)
Toxaphene (chlorinated bornanes, CHBs, PCCs[b])	32,768	34,200 tonnes (USA in 1974)	Alltex, Alltox, Attac 4 2, Attac 4 4, Attac 6, Attac 6 3, Attac 8, Camphechlor, Camphochlor, Camphoclor, Chemphene M5055, chlorinated camphene, Chloro camphene, Clor chem T 590, Compound 3956, Huilex, Kamfochlor, Melipax, Motox, Octachlorocamphene, Penphene, Phenacide, Phenatox, Phenphane, Polychlorocamphene, Strobane T, Strobane T 90, Texadust, Toxakil, Toxon 63, Toxyphen, Vertac 90%	(*40*)
Organochlorine pesticides (OCPs)	na	DDT: 60,000 tonnes (ww in 1974) Endrin: 2,300-4,500 tonnes (sales USA in 1962) Aldrin + dieldrin 13,000 tonnes (ww in 1972) Chlordane: 9,500 tonnes (USA in 1974) HCB: 10,000 tonnes/yr (ww 1978-1981)	DDT: Agritan, Anofex, Arkotine, Azotox, Bosan Supra, Bovidermol, Chlorophenothan, Chloropenothane, Clorophenotoxum, Citox, Clofenotane, Dedelo, Deoval, Detox, Detoxan, Dibovan, Dicophane, Didigam, Didimac, Dodat, Dykol, Estonate, Genitox, Gesafid, Gesapon, Gesarex, Gesarol, Guesapon, Gyron, Havero extra, Ivotan, Ixodex, Kopsol, Mutoxin, Neocid, Parachlorocidum, Pentachlorin, Pentech, PPzeidan, Rudseam, Santobane, Zeidane, Zerdane. Dieldrin: Dieldrite, Dieldrix, Illoxol, Panoram D 31. ENT 16 225 (compound 497), HEOD, Alvit, Octalox, OMS 18, Quintox Endrin: Endrex, Experimental Insecticide 269, Hexadrin, Nendrin, NCI-COO157, ENT17251, OMS 197, and Mendrin Aldrin: Aldrec, Aldrex, Aldrex 30, Aldrite, Aldrosol, Altox, Drinox, Seedrin. ENT 15 949 (compound 118), HHDN, Octalene, OMS 194 Chlordane: Aspon, Belt, CD 68, Chlorindan, Chlorkil, Chlordane, Corodan, Cortilan-neu, Dowchlor, HCS 3260,Kypchlor, M140, Niran, Octachlor, Octaterr, Ortho-Klor, Synklor, Tat Chlor 4, Topichlor, Toxichlor, Velsicol-1068 HCB: Amaticin, Anticarie, Bunt cure, Bunt no more, Co op hexa, Granox, No bunt, Sanocide, Smut go, Sniecotox	(32-36,40)

Table 2.1 Continued

Name	N[a]	Production Volume	Typical trade names of technical mixtures	Ref
Polybrominated diphenyl ethers (PBDEs)	209	Penta: 4,000 tonnes (ww in 1994) Octa: 6,000 tonnes (ww in 1992) Deca: 55,100 tonnes (sales in 2001)[c]	Penta-BDE: DE 60FTM, Planelon PB 501, Saytex 125, Bromkal 70 DE, Great Lakes DE-60 F (85% PeBDE), Saytex 115, Tardex 50 DE 71; Bromkal 70-5 DE; FR 1205/1215; Bromkal 70; Bromkal G1; Pentabromprop; Hexa-BDE: BR 33N Octa-BDE: Bromkal 79-8 DE; DE-79™; FR 143; Tardex 80; FR 1208; Adine 404; Saytex 111 Deca-BDE: FR-300 BA; DE-83-RTM; Saytex 102; Saytex 102E; FR-1210; Adine 505; AFR 1021; Berkflam B10E; BR55N; Bromkal 81; Bromkal 82-ODE; Bromkal 83-10 DE; Caliban F/R-P 39P; Caliban F/R-P 44; Chemflam 011; DE 83; DP 10F; EB 10FP; EBR 700; Flame CutBR 100; FR 300BA; FR P-39; FRP 53; FR-PE; FR-PE(H); Planelon DB 100; Tardex 100; NC-1085; HFO-102; Hexcel PF1; NCI-C55287	(41-43)
Hexabromocyclododecanes (HBCD)	10	16,700 tonnes (sales in 2001)[c]	HBCD	(44,45)
Tetrabromobisphenol-A (TBBP-A)	1	130,000 tonnes (sales in 2002) [c]	Derakane	(46)

[a] *Theoretical no. of congeners. Possible enantiomers not included. Number of congeners does not reflect the number of compounds generally encountered in environmental samples*

[b] *None of the abbreviations chlorobornanes, polychlorinated camphenes cover the complete mixture*

[c] *In USA, Europe and Asia*

Analytical chemists have been working for over 40 years to develop a wide range of analytical methodologies for the often complex mixtures of halogenated contaminants, trying to meet the ongoing requests from policy makers, risk assessors, and environmental scientists for accurate data on the presence of these contaminants in the environment and humans. Most halogenated contaminants are relatively volatile, non-polar and thermally stable compounds that can perfectly be determined by GC. Capillary GC offers a high number of theoretical plates, resulting in a high resolution. When used in a multidimensional (MD) mode (heart-cut MDGC or GCxGC), the resolution increases substantially. MS detection strongly contributes to the overall selectivity. Several excellent dedicated reviews have been produced in recent years on PCDD/Fs and dl-PCBs (*9,53*), CPs (*54,55*), PCBs and OCPs (*56*), toxaphene (*28,57*), BFRs (*58-61*) and PCNs (*57*). The aim of this work is to review recent developments in GC methods for halogenated contaminants and to provide an overview of the applicability of methods for these contaminants. In addition to injection, gas chromatographic separation and detection, attention is being paid to sample pre-treatment (extraction and clean-up), as this is recognised as a critical step in the whole analytical procedure. Finally, quality assurance issues are discussed. This review focuses

on biota (fish, shellfish and crustaceans) and sediment only, as these matrices have been successfully used for several years to monitor the aquatic environmental exposure to these contaminants. The analysis of these contaminants in water is not considered here, because, due to the extremely low levels of POPs in water significant errors are easily made.

Sample extraction

The determination of target contaminants typically starts with extracting them from the sample matrix. Halogenated contaminants are lipophilic and stored in the body lipids in biota. In lipid-rich biota, the majority of contaminants may be stored in the depot lipids, whereas in lean biota (<1% lipids), the contaminants are also stored in the phospholipids. By extraction, the contaminants are liberated from the matrix and made available for further analysis. Several parameters influence the extraction efficiency, e.g. choice of extraction medium (solvents), duration, temperature of extraction medium, pressure in extraction chamber and the possibility of the solvent to penetrate the matrix. These parameters should be optimised to exhaustively extract the contaminants from the matrix.

Soxhlet

Soxhlet extraction is the classical method for extraction of POPs from a variety of matrices. It has widely been used in the past and still is an important technique disregarding the appearance of various instrumental extraction techniques. There are several benefits connected with Soxhlet extraction. Due to the simplicity of the method, no sophisticated (and expensive) equipment is needed. The method is simple to operate under routine conditions and multiple samples can be extracted at the same time. The method requires long extraction times (approx. 6-24 h), but performing the extractions overnight can circumvent that drawback. Another benefit is that Soxhlet can be employed on a wide variety of matrices and a wide range of contaminants such as PCBs, OCPs, PCDD/Fs and BFRs. Extraction of lipid-rich materials (mainly triglycerides) may be performed using a non-polar solvent only (e.g. n-hexane, n-pentane), but lean biological tissues require the use of medium polar (binary) solvent mixtures (e.g. pentane-dichloromethane (DCM) or hexane-acetone) to extract the POPs with the phospholipids (*62*). De Boer et al. evaluated several binary solvent mixtures for the extraction of BDEs from fish tissue and sediment and concluded that mixtures of hexane-acetone (1:1 or 3:1) were suitable for quantitative extraction of the target analytes (*59*). Both sediments and biota need to be dried before they are Soxhlet extracted, as the presence of water disturbs the extraction process. Drying prior to extraction can be done by mixing them with sodium sulphate and allow some drying time (1-2 h) or by freeze-drying (or air drying for sediment). When freeze-drying, attention should be paid to avoid cross-

contamination and losses of volatile compounds. It should be noted that when using a Dean Stark adaptor combined with Soxhlet extraction set-up, drying of the sample prior to extraction is not required.

Pressurised liquid extraction

Pressurised Liquid Extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) has gained considerable interest over the last decade. It is a powerful technique and reduces extraction times. Even more time is saved when extraction and clean-up are combined in one run within the extraction cell. PLE is employed for the extraction of PCBs and OCPs from biota and sediment samples (*63*). Extraction of PCBs and OCPs from a fish sample showed that extraction efficiencies and precision of the PLE extraction (hexane-acetone 4:1, 3 cycles) were similar to Soxhlet extraction (*63*). Josefsson et al. (*64*) tested the exhaustiveness of a 2x5 min extraction of PCBs from sediments using a hexane-acetone mixture (1:1). Extraction efficiencies were 96-99% using this approach. They found correlations between extraction efficiency, the water content and the carbon/nitrogen ratio, but, surprisingly, no (significant) relation was found with total organic carbon, soot carbon or amorphous carbon content. PLE is increasingly used for the analysis of BFRs with e.g. DCM or a DCM-hexane (1:1) mixture (*65-68*). Using PLE, recoveries were low (<60%) for the lower (mono to tri) brominated BDEs in fish and sediment, but increase up to 103% for the higher brominated ones (*66,68*). PLE was also used for PCDD/Fs (*69-71*). The combination of within-cell extraction and clean-up will be discussed later. A drawback of PLE is that the cells should be cleaned thoroughly to prevent cross contamination. Because the cell contains more parts than a typical Soxhlet extraction thimble, this requires special attention. It is recommended to select a set of cells for highly contaminated samples and another set for low contaminated samples.

Microwave-assisted extraction

Microwave-assisted extraction (MAE) is a very simple extraction technique. The requirements are the microwave equipment with vessels. This technique allows for simultaneous extraction of several (e.g. 6) samples but requires solvents that can absorb the microwave radiation (due to their dielectric nature) such as dichlorobenzene, methanol, ethanol and, to a lesser extent, acetone, ethyl acetate and chloroform. Alternatively, microwave transformers (e.g. Weflon discs, (*72*)) can be used, which transform the radiation to heat, which is transferred to the solvents that poorly absorb microwave radiation (e.g. n-hexane, dichloromethane or chloroform). The extraction solvent, temperature and time are typical conditions that require optimisation. Care should be taken to avoid degradation of labile contaminants at the elevated extraction temperatures. MAE was used in several studies to extract PCBs and OCPs (*73-77*), PCNs (*78*), PBDEs (*78,79*), and SCCPs (*25*) from biota and sediments. Although MAE should in principle

be applicable for PCDD/Fs, a comprehensive evaluation was not found. Extraction efficiencies and precision (<5%) were good for PCBs in cod livers (*75*). Good average recoveries and precision were also obtained (89 +/- 8% to 95 +/- 14%) for extraction of BDEs 47, 99 and 100 from various fish samples with 8 ml ethyl acetate-cyclohexane mixture at 115°C (*79*). These extraction efficiencies were only slightly lower than those of Soxhlet. Yusa et al. (*78*) optimised conditions (extraction time, temperature and solvent volume) for the extraction of PCNs, PBDEs and PBBs from spiked marine sediments. At optimum conditions (24 min, 152°C, 48 mL 1:1 v/v hexane-acetone mixture), recoveries were 74-93% with a precision of 4-13%, being comparable to the results of the reference method (Soxhlet). Good extraction efficiencies (>90%) and run-to-run precision (<10%) were obtained by Perera et al., who extracted PCBs and SCCPs from 5 g river sediment sample using 30 ml hexane-acetone mixture (1:1, 15 min, 115°C) (*25*). OCPs were successfully isolated from oyster samples by MAE combined with mild saponification. At optimised conditions, results were comparable to Soxhlet and no degradation of labile contaminants was observed (*80*). This shows that MAE is a viable extraction method for most of the POPs and candidate POPs.

Other extraction techniques

Matrix solid-phase dispersion (MSPD) is an extraction technique in which the sample is dispersed in a solid phase material of choice (e.g. silica or C18) until a free flowing powder is obtained. Subsequently, the dispersed material is loaded into a syringe tube. The contaminants are then eluted by e.g. hexane, dichloromethane or acetonitrile. MSPD has successfully been employed for the extraction of PCBs, OCPs and BFRs in fish samples (*81-85*). The benefit of this method is the ease of operation, low solvent consumption and no investments in (expensive) equipment are required. The application is limited to fish samples and cannot be applied to sediments (as with e.g. Soxhlet and PLE) due to the strong adsorption of the contaminants to the sediment, which may be a drawback for laboratories aiming at both matrices. Recoveries of PCBs in fish samples were 81-106% (*86*). Sample intakes were as low as 0.5 g (*86*). Care should be taken to ensure the homogeneity at such low sample intake levels.

Supercritical fluid extraction (SFE) has been employed for extraction of environmental samples (*87-89*), but has never found a broad application. Zougagh et al. recently reviewed the application of SFE extraction (*90*). Benefits of the technique are the short extraction times (<1 h) and low solvent consumption (< 5 mL), but the major drawback is the labour-intensive method development. Different sample matrices require specific method development and therefore, contrary to Soxhlet, PLE and MAE, universal methods cannot be applied.

Clean-up of sample extracts

Clean-up is a very important and critical step in the analysis of halogenated contaminants. The extremely low concentrations of POPs in environmental samples (e.g. sub-pg/g concentrations for PCDD/Fs) demand a thorough clean-up of the extracts in order to remove co-extracted substances (e.g. lipids, fatty acids, elemental sulphur) that are normally present at concentrations that are several orders of magnitude higher than those of the target contaminants.

Generally, the crude extract is concentrated by e.g. rotary evaporation or a Kuderna Danish method (*91,92*), in order to remove the excess solvents. We experienced that of these two the Kuderna Danish method is the least sensitive for cross contamination and offers somewhat better recoveries than rotary evaporation. It also allows more extracts to be handled at the same time with less attention (*92*). The first step in the clean-up of biological extracts is the removal of bulk lipids (triglycerides), which can be performed by destructive or non-destructive methods. Destructive methods (sulphuric acid treatment or saponification) efficiently remove the bulk lipids. However, some contaminants (e.g. dieldrin and endrin) degrade under the strong acidic conditions. Saponification can cause dechlorination of higher PCBs and HCB (*93*). Efficient non-destructive removal of lipids can be obtained by adsorption on alumina (*53*). Dependent on the desired fat capacity, glass columns can be used with different alumina amounts can be used (e.g. 15 g in a 24 mm i.d. 23 cm glass column). Gel permeation chromatography (GPC) may serve as an alternative fat separation method. The most commonly applied are polystyrene–divinylbenzene copolymeric columns (e.g. Bio-beads SX-3) (*31,78,94*), although nowadays rigid PLgel columns (from Polymer Laboratories) appear to be more efficient (*59*). GPC is not capable of removing all lipid-related substances (e.g. sterols) and therefore, additional clean –up or repeated GPC (e.g. up to 4 GPC columns in series) is required. Lipids may also be removed by freezing them out the extract and subsequent filtration. This very simple method allowed for 90% lipid removal from a mackerel extract (*95*). However, residue lipids and fatty acids that remain in the extract require additional clean-up.

For clean-up of sediment extracts, alumina columns can be applied for removal of non-volatile co-extractants (*96*). Elemental sulphur is a major co-extractant from sediments. It should be removed as it will heavily disturb the GC analysis by a broad peak somewhere half way a regular PCB chromatogram. Sulphur is not removed by alumina or silica gel column chromatography but can be removed by several other methods, i.e. GPC, reaction with copper (curls, beads, rods, powder) (formation of CuS) or by complexation with tetrabutyl ammonium sulfite (*96*).

After lipid or sulphur removal, pre-fractionation is carried out in order to separate the target contaminants from other contaminants that may interfere during GC separation. Silicagel or Florisil columns are frequently used for that

purpose, sometimes in combination with an additional step for isolation of planar contaminants such as PCDD/Fs, dl-PCBs and PCNs. Adsorption characteristics of the silicagel can be adjusted by heating the silicagel and subsequent addition of water. Batches prepared in the desired way need to be stored in a desiccator as SiO_2 is sensitive for moisture present in the air.

PCDD/Fs and dl-PCBs require (additional) clean-up by application of porous graphitic carbon (*53,71,97,98*) or 2-(1-pyrenyl)ethyldimethylsilyl (PYE) column chromatography (*53,98-100*) to separate them from the non-planar compounds (e.g. bulk of PCBs). When using carbon columns, typically the non-planar contaminants are eluted by a non-polar solvent (e.g. n-hexane), whereas the target contaminants are back flush eluted from the column using toluene. Immuno chromatographic LC columns may be used for separation of OCPs and dioxin-like compounds (*101*).

PCBs and OCPs Clean-up of PCBs is often combined with that of OCPs. After removal of lipids or sulphur, the PCBs are separated from the more polar OCPs by silica fractionation. PCBs are eluted from the column by a non-polar solvent (e.g. n-pentane or n-hexane), whereas elution of most OCPs requires a more polar solvent or solvent mixture (e.g. 15% diethyl ether: 85% n-pentane). Some OCPs (trans-nonachlor, cis- and trans-chlordane, hexachlorobutadiene (HCBD), QCB, HCB, OCS, p,p'-DDE, o,p'-DDE, pentachloroanisol and pentachlorothioanisol) may partially elute in the 1st fraction, together with the PCBs, depending on the elution volume and polarity of the solvents used (*39*). Several authors applied multilayer silica columns, typically containing a combination of acid impregnated, base impregnated and regular (deactivated) silica gel (*102,103*). These columns effectively remove potentially disturbing matrix constituents. More details on clean-up for PCBs and OCPs can be found elsewhere (*56,104*).

PCNs The clean-up of biota and sediment extracts can be achieved by lipid removal by e.g. GPC or alumina and subsequent silicagel fractionation. Further removal of interferences is achieved using porous graphitic carbon or PYE columns because the molecular planarity allows to selectively separate PCNs from interferences (similar to PCDD/Fs) (*57*). Because environmental levels are higher compared to PCDD/Fs, a less complex clean-up is required.

CPs The complete removal of interfering contaminants (e.g. toxaphene) is essential when using short GC columns combined with GC electron-capture negative ionisation (ECNI)-MS (*26*). Florisil can be used to effectively separate interferences (PCBs, toxaphene, o,p'-DDT and α-HCH) from the SCCPs as demonstrated by Reth et al. (*20*). Apart from contaminant class separation, silicagel and florisil also trap polar interferences that are not removed in earlier clean-up steps. Sometimes, a final clean-up step may be required such as treatment with sulphuric acid. Photolysis was effective for (partial) removal of interfering contaminants like PCBs, chlordanes and DDTs (*105*).

Toxaphene For the analysis of toxaphene, removal of PCBs from the extract can be achieved on silicagel but some losses of the lower chlorinated

toxaphene congeners may occur (depending on the deactivation of the silica) and care should be taken to avoid this, or correction for the losses should be made (*106*). Krock et al. found that 8 g activated silica eluted with 48 mL of n-hexane efficiently separated toxaphene from most of the interferences (PCBs, PCNs, HCB, p,p'-DDE and octachlorostyrene) (*107*).

BFRs The clean-up of PBDEs is similar to that used for PCBs. GPC, alumina, silica and concentrated sulphuric acid have all been successfully used for clean-up of extracts, as showed in detail in a recent review by Covaci et al. (*108*). Clean-up of HBCD and dimethyl-TBBP-A is partially similar to the clean-up for PBDEs, but due to its polar character dissociation should be avoided. The pKa_1 and pKa_2 values of TBBP-A is estimated at 7.5 and 8.5, respectively (*109*), which means that in neutral environments, a substantial part of the TBBP-A is present in it's dissociated state. This causes losses in the clean-up steps when a neutral environment combined with polar solvent is maintained (the polar solvent could just be a little bit of co-extracted water from the sample). Care should be taken to avoid these losses and a possible solution is to treat the raw extract with acidified water. This results in associated TBBP-A only, which is driven almost quantitatively towards the organic phase. Concerning HBCD, care should be taken with the silica elution. HBCD consists of several diastereomers (α-, β, and γ-HBCD are the major ones) and β-HBCD requires a larger volume of solvent for complete elution from silica columns as compared to PBDEs, me-TBBP-A and α- and γ-HBCD (*110*)). Because of these specific requirements it is ambitious (but feasible) to combine the clean-up of extracts for PBDEs, HBCD, dimethyl-TBBP-A and TBBP-A analysis.

The final step prior to GC injection is the concentration of the sample extract. This is achieved by solvent evaporation (N_2 blow down, Kuderna-Danish or Turbovap). Care should be taken to avoid losses (of volatile) compounds during this process. Larger losses were reported for OCPs using the Turbovap as compared to Kuderna-Danish (but nearly no losses for PCBs) (*91*). A conical Kuderna-Danish receiving flask is preferred over a cylindrical flask for reducing extract volumes to below 100 µl (*92*). Another way of preventing losses is the addition of iso-octane or nonane prior to the concentration step as a so-called keeper, and these solvents are suitable for injection in the GC.

Several of the aforementioned clean-up steps may be combined in one step. The advantage of doing so is that between the various clean-up steps no concentration steps are required which reduces the risk of losses due to evaporation and contamination due to the use of glassware in several steps. Also, the volume of solvent and the amount of labour are reduced in that way. One option is the combination of several clean-up steps in a single glass column (multi-layer column) loaded with e.g. alumina oxide, anhydrous sodium sulphate, acidified silica, basic silica, neutral silica and porous graphitic carbon. The set-up of the method (number and type of layers) varies among the studies. The multi-layer clean-up was successfully applied for BFRs, PCBs, OCPs, PCDD/Fs, dl-PCBs and brominated dioxins and furans

(*70,71,111-117*). In recent years, complete clean-up systems (e.g. PowerPrep, Fluid Management Systems, USA) were developed for environmental analyses, which combine and automate several clean-up steps in a modular system using disposable columns. After sample extraction, the extract is loaded in this system and automatically processed resulting in the final extract, ready for injection. For PCDD/Fs and dl-PCBs, the used columns are a multilayer silica column, followed by alumina and finally porous graphitic carbon (*118*). In a parallel system, multiple samples can be processed in 1 hour. Although labour reduction is considerable, the initial investments for such system are substantial. Moreover, the system requires large amounts of high purity solvents and also the consumables (e.g. clean-up columns) are more costly compared to home-made multilayer columns. Therefore, these systems may fit perfectly in a commercial routine laboratory for obtaining high throughput at low labour costs, whereas flexibility may be too low for research laboratories. So far, they have only been applied for PCDD/F analysis (*119-121*) and PBDEs (*122,123*), but application to other contaminants should also be feasible. Recently, the concept of coupling PLE in-line with the PowerPrep system was presented (*124*). This method potentially further reduces the sample handling time, but a thorough evaluation of the system is not yet presented.

Combined extraction and clean-up by selective PLE

Recently, several studies have explored the possibilities of combined automated extraction and clean-up of environmental samples by means of PLE with within-cell clean-up. Such approach can substantially reduce the labour spent on extraction and clean-up of environmental samples. This method is sometimes referred to as selective PLE, or SPLE (*81*). In selective PLE the extraction cell is filled with sample material and the sorbents that perform the clean-up (whereas in conventional PLE the extraction cell is only filled with sample material, sometimes mixed with anhydrous Na_2SO_4 for binding moisture from the matrix). Recently, the potential of selective PLE was reviewed by Björklund et al. (*125*). Within the DIFFERENCE project (funded by the European Community), considerable improvements were obtained on selective PLE for extraction and clean-up of PCDD/Fs and dl-PCBs from feed and food (e.g. fish) (*69,125-128*). Silica (or florisil in (*81*)) was employed for lipid rich samples (such as herring) in order to retain the co-extracted lipids in the extraction cell (extraction with n-hexane). The optimum fat-fat retainer ratio was 1:40 (*126*). Porous graphitic carbon was applied in a specially designed cell inlay to retain the planar compounds (Figure 2.1). In the forward flush mode, the lipids and non-planar compounds are extracted and eluted by n-heptane (fraction 1) and 1:1 DCM:n-heptane (fraction 2), whereas the PCDD/Fs and non-ortho-PCBs are retained on the porous graphite. The latter are than backflush eluted by toluene (fraction 3). A subsequent miniaturised

multilayer clean-up of fraction 3 was sufficient for accurate determination by GC-HRMS afterwards. Results obtained by above methods were very well comparable (accuracy and precision) to traditional extraction and clean-up techniques. Selective PLE was also developed for PBDEs in sediment (*65,66*). For PBDE extraction in sediment, 1 gram of sediment was mixed with alumina and cupper (1:2:2, w/w) and extracted with hexane-DCM (1:1, v/v, 100 °C). Compared to Soxhlet, recoveries of a spiked sediment were slightly lower for mono-BDEs to tri-BDEs, but comparable for tetra-hepta-BDEs (*66*).
Extraction cells for commercial PLE systems (Dionex) are available up to 100 mL. The largest cell volume is large enough to accommodate either a lipid rich fish sample mixed in the proper ratios with silica, or the fish sample and the carbon cell inlay (Figure 2.1). Considering that, especially for PCDD/F analysis, 2-6 g of lipids are needed to obtain sufficient sensitivity, the cell volume is too small for lean fish samples (1-5% of lipids). Therefore, larger extraction cells are required. Splitting the sample over two or three extraction cells can circumvent this. Method development of selective PLE is somewhat more laborious than conventional PLE, but the benefit is the strongly reduced sample handling time once the method is established. At the moment selective PLE is one of the few techniques that offer a substantial reduction of labour time of the pre-treatment of sediments and biota samples for POP analysis. Within the DIFFERENCE project, selective PLE was evaluated for PCDD/Fs and dl-PCBs. The costs breakdown (Figure 2.2) shows that the costs of extraction and clean-up (indicated in horizontal black lines) were similar to traditional extraction. Only a miniaturised additional multi-layer clean-up for removal of residual interferences (primarily lipids) was required prior to injection (*127*). It may be expected that due to pressures of authorities to reduce costs of analyses more labour-reducing methods may be developed in the near future.

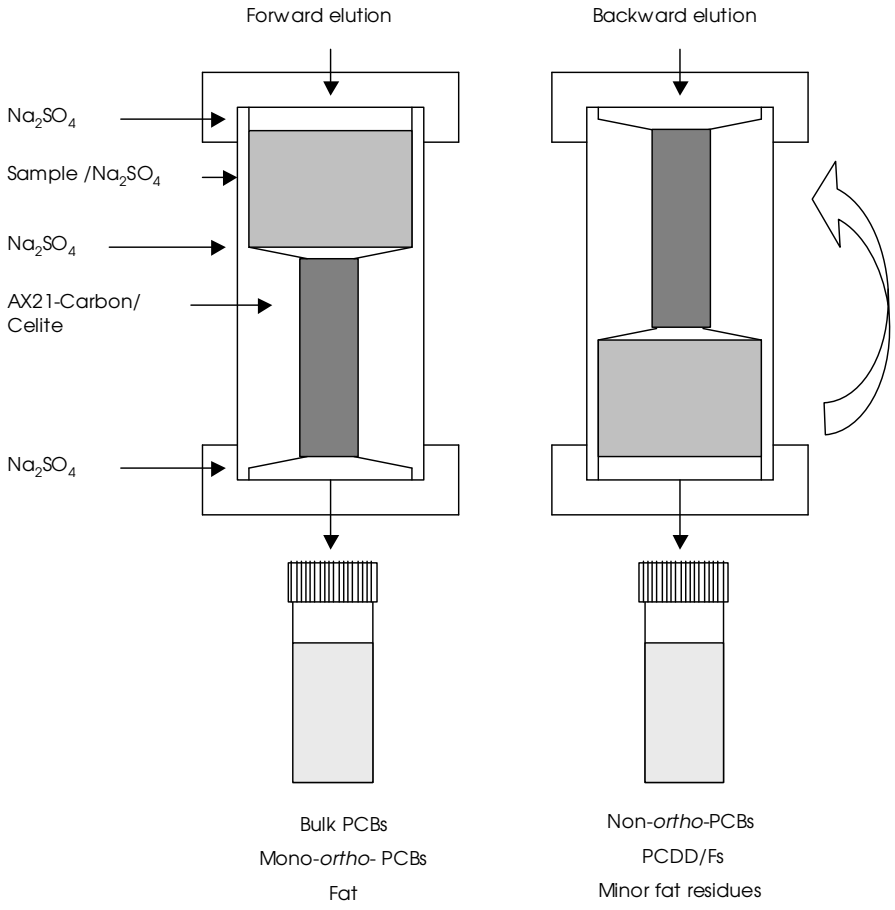

Figure 2.1 Selective PLE set-up for within-cell extraction and fractionation of PCDD/Fs and dl-PCBs. Fraction 1: n-heptane (forward elution); fraction 2: DCM/n-heptane (1:1 (v/v) forward elution); and, fraction 3: toluene (backward elution). In backward elution mode, the cell had been turned upside down. From ref. (125).

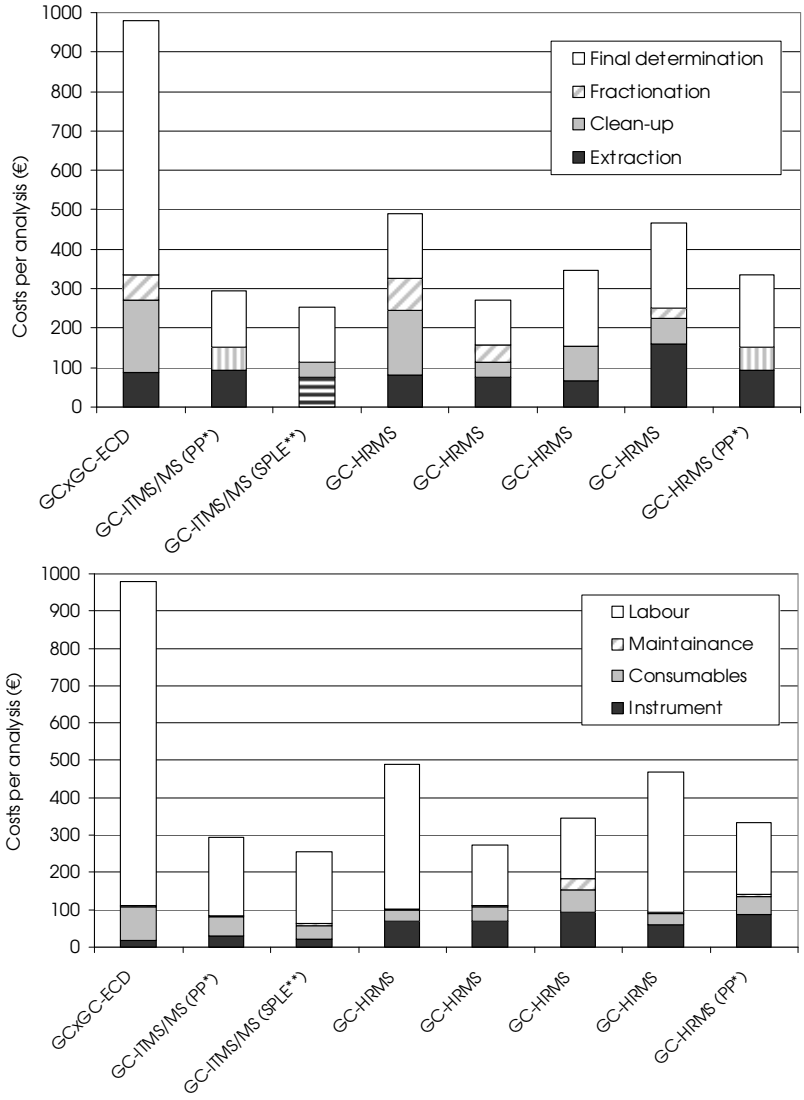

*Figure 2.2 Cost per analysis for GC-HRMS and alternative techniques (GC-ITMS/MS, GCxGC-ECD) for the analysis of dioxins and dl-PCBs (edited from (129)). Top: cost expressed per stage of analysis; bottom: cost expressed per item. The costs per technique are calculated on the basis of labour (in man hours x €75/hr) in each step of analysis, consumable use and costs, the costs involved with instrument investment and depreciation and instrument maintenance costs (service costs). Costs for QA/QC (20-40%, including purchase and analysis of reference materials, blanks, replicates etc.) and analytical standards (ca 3%) were not taken into account. *PP: Powerprep™ (automated extraction and clean-up, indicated in vertical grey lines (top graph only)); **SPLE: selective PLE (extraction and clean-up partially integrated, indicated in horizontal black lines (top graph only)). Prices are of 2005.*

Injection

GC is the method of choice for analysis of complex mixtures of halogenated contaminants for its unsurpassed resolution offered by capillary columns. The three parts of the GC analysis, injection, separation and detection all need to be optimized and validated to guarantee a high-quality analysis.

Injection of POP containing extracts can be performed by various automatic injection systems. The most commonly applied system is splitless injection (*16,17,94,105,112,114,130-136*), but alternative techniques such as by programmed temperature vaporizer (PTV) and on-column injection can be applied as well (*137,138*). In splitless injection, 1-2 µL extract is injected in a glass liner. The liner serves as the evaporation chamber where the liquid extract is rapidly volatilised at elevated temperatures (150-250°C). The liner may be open, or partially filled with a plug of glass wool or other surface area increasing materials (*139*). Open liners are generally preferred as glass wool or other materials can easily cause thermal degradation. This process is enhanced by active sites at elevated temperatures in the injector. Active sites also occur in the liner due to accumulation of dirt, typically after multiple injections of dirty sample extracts. Several studies reported the degradation of contaminants due to dirty liners and the high injection temperatures, including DDT (*140*), toxaphene (standard mix of 22 congeners) (*141,142*), HBCD (*60*), and higher brominated BDEs (octa- to deca-BDE) (*60,143,144*). Thermal degradation in the injector can be minimised by frequently replacing the liner by a clean one. Furthermore, the residence time of the contaminants in the injector can be minimised by application of a pressure pulse. This pulse rapidly transfers the volatilised contaminants to the column. Pressure pulse injection was applied e.g. for PBDEs (*60*). Another undesired phenomenon is discrimination of contaminants. This is the fractionation of the sample in the injector whereby the least volatile contaminants are (partly) splitted rather than being introduced in the GC column, resulting in a non quantitative introduction of these heavy compounds in the column. This phenomenon occurs when using non optimised splitless times and was reported for higher chlorinated toxaphene homologous (nona- and decachloro congeners) (*141*) and BDE 209 (*143*).

With (cold) on-column injection, the complete extract is introduced directly in the first part of the GC column at room temperature. In that way no losses can occur. The vaporisation of the sample extract takes place in the column at a temperature just above the solvent boiling point. The instrumental setup is simple as well as the operation and maintenance. However, the sample extracts should be very clean to prevent introduction of dirt from the sample matrix. The accumulation of dirt in the first part of the column leads to deterioration of the GC column and can lead to active sites. These active sites may catalytically degrade labile contaminants. These phenomena can be reduced by application of an uncoated, deactivated retention gap. The accumulation of dirt from the sample extract then occurs at the retention

gap. However, after multiple injections system performance can decrease (*116*) and therefore, the retention gap should be changed regularly. Extracts should be as clean as possible, even when using this retention gap system. On-column injection was successfully applied for PBDEs (*60,116,143*) and toxaphene (*141*). On-column injection can also be used for injection of large volumes (large volume injection, LVI) (*145,146*). Björklund et al. explored this principle for the analysis of PBDEs and injected 50 µL into a 10 m retention gap. They evaporated the solvent through the GC column and ECD prior to GC analysis (*147*). Large quantities of solvent cannot be evaporated through an MS. In that case, an early solvent vapour exit is required. The PTV injector is a generic injector, some of which can be used in several modes (e.g. split-splitless injector). The more interesting application of PTV is that of LVI for increasing the sensitivity. Using this technique, volumes of 10 – 50 µL have been injected. This injector was used for PCDD/Fs and dl-PCBs (*148,149*), PBDEs (*78,150,151*), and PCNs (*78*). Typically, the solvent is introduced in the 'cold' PTV injector, which is set at a temperature just below the solvent boiling point. Then, the solvent is removed by heating to above the boiling point and evaporating the solvent by a split flow through the opened split valve. Finally, the split valve is closed (splitless mode) and by rapidly raising the injector temperature, the target contaminants are transferred to the analytical column. Eppe et al. injected 10 µL of the final extract (in toluene) in order to compensate for the lower sensitivity of their detection method (Ion-trapMS/MS). Doing so, they arrived at an instrumental LOD (iLOD) of 200 fg/ µL (S/N=5:1) (*148*). Using LVI, sample handling can be reduced by leaving out the final extract concentration step.

Gas chromatographic separation

Column selection

The heart of the GC is the capillary column. The selection of stationary phase, column dimensions and carrier gas (velocity) determines the separation characteristics. Given the complex composition of most POP mixtures, most studies have tried to increase and optimise the resolution of the chromatography in order to separate a maximum number of contaminants. Hydrogen and helium are generally used carrier gasses and (especially hydrogen) provide optimum resolution at highest carrier gas velocities. Table 2.2 shows stationary phases used in GC of halogenated contaminants. The most widely used are non-polar to slightly polar stationary phases, such as DB-1, DB-5, BPX-5, HT-8, CP-Sil8CB-MS or CP-Sil-19 (*12,16,53,60,112,133,151-153*). The addition of MS to a column type name means that suppliers have have slightly adjusted the chemistry of the stationary phase and/or minimised the bleeding of the stationary phase, which is necessary when using MS

detection. Column bleed may show up in the MS spectra and complicate identification of the target contaminants. Film thickness is typically 0.25 µm. Column lengths are 30-60 meter, but shorter columns are beneficial in certain cases. The column diameter is directly proportional to the resolution. Typical column diameters are in the range of 0.25-0.32 mm, but narrow bore columns (0.10-0.15 mm) provide substantially more theoretical plates at the same column length. These small dimensions require high gas pressures. Nowadays, GC pneumatics are equipped to accurately deliver carrier gas at these high pressures (up to 150 psi), which enables the use of narrow bore columns.

Co-elution of contaminants with other contaminants of interest or with interferences is a common problem in GC separation. No single analytical column is able to separate all PCBs (*152*), PBDEs (*154*), HBCDs (*51*) or even the 17 WHO PCDD/Fs (*114,130,148*). The identity of co-eluting contaminants can be determined by elution over a secondary column with a different stationary phase (either or not in a dual-column system). Polar phases like CP-Sil 88 have been employed for that reason. Furthermore, liquid crystalline columns show distinct separation characteristics based on molecular structure rather then on boiling point (*155*). Unfortunately, these columns suffer from high column bleed (*9,114*). *PCDD/Fs and dl-PCBs* Column manufacturers have developed dedicated columns for a number of applications to resolve critical contaminants, e.g. for dioxin analysis (e.g. DB-dioxin, BPX-DXN, RTX-Dioxin2). These columns have a more polar stationary phase (e.g. DB-Dioxin: 44% methyl–28% phenyl–20% cyanopropyl polysiloxane) and enable the separation and quantification of critical pairs (e.g. 2,3,7,8-TCDD being separated from 2,3,4,7,8-PentaCDF, 2,3,4,6,7,8-HexaCDF). However, incomplete separation of all 17 WHO PCDD/F congeners remains. Details on co-elution of PCDD/Fs can be found elsewhere (*156*).

PCBs and OCPs PCBs and OCPs are typically separated on non polar (e.g. BPX-5, HP-5MS, DB-5MS, VF-5MS) or slightly polar (e.g. CP-Sil 8CB) stationary phases with dimensions of 30-60 m x 0.25 mm i.d. x 0.25 µm film thickness (*84,112,140,157-159*). These columns are not able to separate the complete set of 209 PCBs, but the indicator-PCBs can nearly all be separated from other PCBs. Well known co-elutions on non-polar phases are PCB 28 and 31 and 138 and 163 (*56,96*). The OCP fraction may contain many interferences that preferably are removed by clean-up of the extract as they may lead to inaccurate results, especially when using non-selective ECD detection. In these cases, a column length of 50-60 m is recommended for maximum separation. Furthermore, confirmation may be required by analysis on a second column with different (polar) stationary phase. De Boer et al. investigated the separation of PCBs on several narrow bore columns (0.15 mm i.d.) and although the resolution further improves using these smaller dimensions (*160*), narrow bore columns have not found a wide application.

Table 2.2 Selection of popular stationary phases used in GC analysis of halogenated contaminants.

Polarity scale*	Stationary Phase	Brand and type names
5	100% Dimethyl polysiloxane	ZB-1(ms), CP-Sil5CB, DB-1, HP-1(ms), PE-1, Rtx-1, BP(x)-1, Ultra-1
8	5 % Phenyl-(arylene-) 95 % methyl polysiloxane	ZB-5(ms), CP-Sil8CB, DB-5(ms), HP-5(ms), PE-5, Rtx-5(ms), BPX-5, Ultra-2
17	50 % Phenyl-50 % methyl polysiloxane	ZB-50, DB17(ms), HP-50+, HP-17, PE-17, RTx-50, BPX-50, OV-17, Optima 17
24	75 % Phenyl-25 % methyl polysiloxane	ZB-50, CP Sil 24 CB
43	50 % 3-Cyanopropyl-50 % phenylmethyl polysiloxane	007-225, CP-Sil 43 CB, AT-225, BP-225
52	Polyethylene glycol	ZB-WAX, ZB-WAXplus, DB-WAX, CP-Wax 52 CB
88	100% 3-Cyanopropylpolysiloxane	BPX70, CP-Sil 88 CB, DB23, HP23, PE-23, Rtx-2330, VF-23MS
Non polar**	50% n-octyl-50% dimethyl siloxane	SB-Octyl 50
Moderately polar**	65% Phenyl-35% methyl polysiloxane	007-65HT
moderately polar**	Cross-linked methyl-phenyl-polysiloxane block polymers	Optima delta-3
Polar**	Polysilphenylene phase	BPX-DXN, Rtx-Dioxin2, SP-2331, 007-23, Rtx-2332, DB-dioxin
Polar**	44% Methyl–28% phenyl–20% cyanopropyl polysiloxane	DB-Dioxin
***	Biphenylcarboxylate ester methylpolysiloxane	SB-Smectic
***	Dimethyl (50% liquid crystal) polysiloxane	LC-50
****	α-Cyclodextrin	α-DEX 120
****	β-Cyclodextrin	CP-Chirasil-Dex CB, β-DEX 120, Cyclodex-B, HP-Chiral- β, Rt- βDEX
****	γ-Cyclodextrin	BGB-176SE, BGB-172, Rt- γDEX

* *Relative polarity as determined by McReynolds and Kováts indices*
** *Qualitative classification. No quantitative figure on the polarity scale available.*
*** *Shape selective*
**** *On basis of chirality*

PCNs Järnberg et al. determined the retention behaviour of a PCN standard mixture on 6 capillary columns: Ultra 1, Ultra 2; HT-5 (5% phenyl-dimethylpolysiloxane on carborane); CP-Sil-88, SB-octyl 50 and SB-smectic (*161*). None of the columns was able to resolve all congeners, but Ultra 1 and -2 were able to separate 44 out of 75 possible congeners. On these columns,

the different homologue groups eluted as distinct clusters, whereas on CP-Sil 88 an overlap between clusters was found because of a higher resolution within each homologue group (*161*). The SB-octyl 50 and SB-Smectic columns were able to resolve specific pairs, although resolution was very temperature dependent. Specific hexa-CN pairs can be separated on alpha-cyclodextrin and beta-cyclodextrin (α-DEX 120, β-DEX 120, Supelco) columns (*135*).

CPs The technical mixtures of SCCPs are so complex that current state-of-the-art capillary GC does not provide a solution for the separation of all congeners. CPs are generally separated on non-polar columns (DB-5MS, HP-1) with lengths of approx. 15-30 meter (*17,20*). Complete separation has to date not been feasible and is not likely to be achieved in the near future. Complete separation may sometimes even not be a desirable goal, as such separations would generate extensive amounts of data that are not easy to handle, and not very informative for authorities. Instead, it is desirable to focus on determination of a representative selection of compounds (as with toxaphene) or on the toxicological relevant isomers (similar to PCDD/Fs and dl-PCBs). It should be noted that such selection of 'relevant' isomers has not yet been proposed.

Toxaphene In the case of toxaphene, not all congeners in technical mixtures are present in environmental samples and only a small selection is typically observed (i.e. P26, 39, 40, 41, 44, 50, 62). These congeners can be separated chromatographically. Baycan-Keller and Oehme have reviewed the GC separation of toxaphene on capillary columns (*142*). Non-polar stationary phases like DB 5, CP-Sil 8, HP 5, Ultra 2 are commonly used and allow for separation of P26, 50 and 62 (*28,94,142,162*). The congeners P39-44 are generally difficult to resolve, but medium polar columns like Optima delta-3 and HT-8 successfully resolved these congeners (in a standard mixture of 23 congeners) (*142*). Polar columns should be used with caution, as considerable toxaphene degradation may take place (see (*142*) for details). Oehme and Baycan-Keller conducted temperature programming experiments and found the best resolution with 10°C/min temperature ramping as compared to 1°C/min (*163*).

BFRs Korytar et al. created an extensive PBDE retention-time database for 126 PBDEs, HBCD, TBBP-A and PBBs on 7 GC columns (17-30 m) i.e. DB-1, DB-5, HT-5, HT-8, DB-17, DB-XLB and CP-Sil 19 (*154*). None of the columns was able to separate all major PBDEs, but the most abundant BDEs (47, 99 and 100) were baseline separated on the DB-1, DB-5, DB-XLB, HT-8 columns. BB 153 and me-TBBP-A co-elute with BDE 154 on a DB-1 and DB-5 column (*154*). This could result in inaccuracies because BB 153 and me-TBBP-A can be found in environmental samples at significant concentrations (*60*). Technical HBCD consists predominantly of 3 diastereomers (α, β and γ) and each of those has 2 enantiomers (*44,164,165*). These cannot be separated by GC. Furthermore, at temperatures >160°C, the diastereomer composition changes (*60*) and considering the different response factors of the diastereomers (*166*), this may

result in a response that does not represent the actual concentrations in the extract. HBCD and TBBP-A can be determined by HPLC Electrospray Ionisation (ESI) MS(/MS) as well (*110*). The benefit of this technique is the chromatographic separation of the individual diastereomers and thus, diastereomer profile information can be obtained. Furthermore, LC-ESI-MS/MS does not suffer from thermal degradation in the injection system and isomerisation of the diastereomers in the column. Considerable differences, up to a factor 5, are sometimes observed between GC- and HPLC-generated results (*166*), whereas Goemans et al. found a smaller difference (<2) between GC and LC results (*167*). This calls for further exploration of the underlying reasons, but the application of LC-ESI-MS/MS using clean extracts and $^{13}C_{12}$ labelled standards (for all three diastereomers) appears to be the best road to accurate results. An additional drawback of GC is that TBBP-A needs to be derivatised to enable GC-analysis, whereas this is not needed for analysis of TBBP-A by LC.

Chiral compounds can be separated using columns with beta-cyclodextrin stationary phases specifically developed for that purpose. Chiral PCBs were separated on Chirasil-Dex, BGB-176SE and BGB-172 (*132,168*) columns and Bordajani et al. were able to separate nine out of the nineteen enantiomeric PCBs (PCB 84, 91, 95, 132, 135, 136, 149, 174, and 176) on the Chirasil-Dex phase. Cyclodextrin stationary phases have also been employed for enantioselective separation of α-HCH, chlordanes, and DDTs (*169-172*). Vetter and Luckas (*173*) studied the enantioselective separation of toxaphene congeners on a tert-butyldimethylsilylated beta-cyclodextrin column (β-BSCD; 30 m x 0.25 mm id x 0.25 µm film) and on a permethylated β-cyclodextrin column (β-PMCD; dimensions not reported). They separated several enantiomeric pairs and doing so, they were able to determine that selective enantiomeric enrichment took place at high throphic level biota.

The use of shorter columns (5-15 m) for analysis of halogenated contaminants has not (yet) found a wide application. However, short columns enable rapid analysis of compounds and without sacrificing resolution, provided small internal diameters are being used. Faster analysis also means shorter residue time in the column at elevated oven temperatures, which is beneficial for thermo-labile compounds such as BDE 209 for which minimised column residence times are crucial (*58,59*). Binelli et al. determined the response of BDE 209, BB 209 and BDE 183 and found a 50-fold response increase for BDE 209 when shortening the column from 16 to 6 meters (Rtx-5 MS, 0.25 mm id, 0.25 µm film), and optimisation of the carrier gas flow (*116*). The authors concluded that interactions with active sites in the column were the cause for poor chromatography on the longer column lengths they tested. Björklund et al. tested discrimination on analytical columns (15 m x 0.25 mm i.d.) and found that severe discrimination occurred for the higher brominated BDEs (BDE 203, 209) on DB-XLB, HP-1 and RTX-500 stationary phases (*143*). They also found that a small film thickness of 0.1 µm (instead of the commonly used 0.25

μm) was beneficial for the yield of the hepta to deca-BDEs. Finally, they determined that for the temperature program a final oven temperature of 300°C was a good compromise between degradation and band-broadening (*143*). Stejnarova et al. used a very short column without stationary phase (1.3 m, 0.15 mm i.d. quartz column) coupled to ECNI-MS for the determination of SCCPs (*26*). Coelhan et al used even a shorter column of 0.65 m only (*174*). Short columns provide condensed chromatograms and narrow peaks (few seconds only), resulting in increased sensitivity (*174*), but care should be taken in operating the detector at sufficiently high frequency to record 10-12 datapoints over the whole peak (for modern ECD and MS detectors, that should not cause any problem). When using narrow bore columns, the amount of sample that can be loaded on the column decreases, which counteracts the sensitivity improvement discussed above.

Multi dimensional GC

Heart-cut multi dimensional GC was used in the 1990s for analysis of e.g. toxaphene and (chiral) PCBs making use of the Deans switch for transfer of the heart-cut to the 2nd dimension column (*132,168,175,176*). With the introduction of modulators such as the sweeper (*177,178*), and later, the cryogenic modulators, the field of GCxGC has made a breakthrough in the analysis of halogenated contaminants in recent years. A GCxGC system consists of two GC columns connected by a connector (e.g. press fit). The first column is often a traditional 30 to 50 meter column with a non-polar phase, which separates the contaminants of interest based on their boiling points. The second column is a very short (0.5 to 1.5 m) column with a different stationary phase (e.g. polar or shape-selective). The contaminants eluting from the 1st column are trapped (often cryogenically) for a short period of time (modulator time) and subsequently released by heating for separation on the second column (a visualisation of this process can be found elsewhere (*179*)). The modulation causes a focussing of the peaks, which improves the sensitivity of the system. Where traditionally a two-dimensional plot is obtained (retention time and response), here a three dimensional plot is obtained. The 1st dimension (x-axis) is similar to a conventional chromatogram (with retention times of typically 40 to 90 min) and the 2nd dimension with very short retention times (typically 6-9 sec) is plotted on the y-axis. The peak response rises from this two-dimensional plane (along the z-axis). For a graphic explanation of the resulting chromatogram, please refer to Adahchour et al. (*180*). More details on the principles of this technique can be found elsewhere (*175,179-181,181*). The interpretation of the chromatograms requires specific GCxGC imaging software and several GCxGC suppliers provide software with their instruments (e.g. Thermo Finnigan and Leco). Generic GCxGC software is available from Zoex Corporation (Texas, USA). A three dimensional peak is composed of several individual 2nd dimension chromatograms in which the compound of

interest elutes. The quantification of peaks is thus based on summarising the peak areas of the individual 2nd dimension peaks. The optimisation of the GCxGC separation is more laborious than traditional GC separation and involves a proper selection of the column combination, temperature programming of one or two ovens, carrier gas velocity and modulating time.
GCxGC provides a very strong separation method, and has been used for the separation of complex mixtures of PCDD/Fs and (dl-)PCBs (*113,114,132,182-185*), BFRs (*113,186*), toxaphene (*113,176,187*), CPs (*113,188*), OCPs (*113,189*) and PCNs (*113*). The DIFFERENCE and DIAC projects (*129,190*) have given the development of GCxGC for analysis of PCDD/Fs and dl-PCBs a considerable push forward by evaluating crucial parameters such as column selection, modulator type and detection method (*113,114,182,184,191,192*). Korytar et al. investigated a range of column combinations and obtained complete separation of all 29 WHO dioxins and dl-PCBs on a DB-XLB column (1st dimension) combined with a 007-65HT, VF-23MS or LC-50 columns (2nd dimension) (*114*). Liquid crystalline phases (e.g. LC-50) in combination with a non-polar column also allowed for separation of the 29 contaminants from matrix constituents. Unfortunately, the LC-50 column is not widely available due to column bleed of this column type. However, as long as it is used as the 2nd dimension column the bleeding does not play a role because of the short length and thin film thickness. Focant et al. achieved separation of all 12 PCDD/Fs and 4 dl-PCBs on a RTX-500 x BPX-500 combination (*149*). Modulators using CO_2 as cryogenic coolant are preferred over other types (e.g. thermal modulation (sweeper) and liquid nitrogen cooled jets) for producing narrow peaks and a broad application range (*193*). In the framework of the DIFFERENCE project, an extensive validation took place of the DR-CALUX bioassay, GC-ITMS/MS and GCxGC-ECD vs. GC-HRMS for the detection of PCDD/Fs and dl-PCBs in food and feed samples (*192*). Three datasets on GCxGC-ECD were obtained and these showed that performance compared to GC-HRMS was comparable for a cleaned fish extract, a fish oil, a spiked vegetable oil and a herring sample (*129*). Some overestimation that was found could easily be explained by the somewhat higher detection levels of the GCxGC-ECD system that resulted in higher numbers when applying the upper-bound approach (*129*).
Harju et al. and Focant et al. studied the separation of all 209 CBs in a standard mixture on a DB-XLB, DB-1, HT-8 (1st dimension, 30-60 m) combined with a HT-8, BPX-50, BPX-70, SP-2340 or LC-50 column (2nd dimension, 0.9-2.3 m) (*183,185*). The DB-XLB x BPX-70 combination provided the best resolution with only 15 co-elutions but at the cost of a 240 min runtime, whereas in approx. 144 min, nearly similar results were obtained when using BPX-50 as the 2nd dimension column (see (*181*) for an overview table). With the DB-XLB x SP-2340, HT-8 x BPX-50 and HP-1 x HT-8 combinations, group separation information was obtained (*183-185*). Application of GCxGC using column combinations like Chirasil-Dex/SUPELCOWAX-10 and Chirasil-Dex/VF-23ms

provided enantiomeric separation as well as separation from the non-enantiomeric PCBs and matrix contaminants (*132*). Korytar et al. evaluated column combinations for GCxGC of 125 PBDEs, some BBs, HBCD and (me-) TBBP-A (*186*). On a DB-1 (1st dimension) x 007-65HT (2nd dimension) combination, they resolved 90 out of 125 PBDEs, including the environmentally relevant BDEs (i.e. BDE 28, 47, 99, 100, 153, 209). In addition, the 2nd dimension column was able to separate meTBBP-A, TBBP-A, BB 169 and two metabolites of BDE 47 which interfere in the 1st dimension (*186*). The potential of GCxGC was also investigated for technical toxaphene (*187*). At optimised conditions (30 m x 0.25 mm x 0.25 µm HP-1 1st dimension and 1 m x 0.1 mm x 0.1 µm HT-8 2nd dimension column, GCxGC-µECD) over 1000 individual toxaphene congeners could separately be determined in the technical mixture. In the same study, a standard containing 23 individual congeners was analysed (GCxGC-TOF-MS, 1st dimension column 10 m x 0.25 mm x 0.25 µm DB-1; 2nd dimension column: 1 m x 0.1 mm x 0.1 µm HT-8). Nearly all congeners were baseline separated and group separation of the chlorinated bornanes and camphenes was obtained based on the number of chlorine substitutions). Using this method, they were able to confirm that the technical mixture consists primarily (97%) of hexa- to nona-chlorinated compounds. Korytar et al. evaluated GCxGC for CPs (*188*). They evaluated six column combinations and found that DB-1 x 007-65HT provided most information on group separation (homologue groups). No complete separation of congeners was obtained, but the technique proved to be a strong additional tool for profiling CPs in environmental samples. Finally, Korytar et al. challenged the GCxGC separation by trying to achieve group separation of several contaminant classes in a single column combination (*113*). The DB-1 (1st dimension) x LC-50 (2nd dimension) column set provides group separation based on planarity and planar compounds such as PCDD/Fs, polychlorinated dibenzothiophenes (PCDTs) and PCNs are more retained on the 2nd dimension LC-50 column than non-planar analytes. The DB-1 (1st dimension) x 007-65HT (2nd dimension) column set effectively separates PCAs and PBDEs from all other compound classes, and provides a good separation of brominated and chlorinated analogue classes from each other (*113*). This column set was the most efficient one for within-class separation of OCPs and PCNs.

Comprehensive GCxGC has proven to be a strong technique for separating complex mixtures and provides considerably more information on the contaminant profile when compared to traditional GC. GCxGC is excellent for identification of unknown compounds appearing (or interfering) in the chromatogram. For example, unknown PCBs in a sample can be identified based on the number of chlorines (*184*) or based on the number of ortho substituted chlorines (*183*). Similar characterisations can be achieved for toxaphene (*113*) and PCAs (*188*). The use of selective detectors such as TOF-MS further increases the identification possibilities. A current drawback is the interpretation of the complex chromatograms, in particular when quantitative

analyses are needed of low concentrations. This is currently a very labour intensive task as software is not yet capable of automatic accurate identification and integration of peaks close to the limit of quantification (LOQ). Instrument suppliers put much effort in software development and it is therefore expected that this is only a temporary problem. Furthermore, at very low concentrations, as with PCDD/Fs in selected food, fish and sediment samples, more effort should be put in clean-up. It should be noted that this is less of a problem when contaminant concentrations in the samples are higher. Finally, the GCxGC optimisation and maintenance is less straightforward than the traditional GC set-up. Presumably these issues have prevented up-to-date a wide acceptance of GCxGC as a routine instrument.

Table 2.3 Qualitative scoring of the various methods for the analysis of trace levels of halogenated contaminants in aquatic sediment and biota (Valuing: -: not recommended, +/- intermediate, +: good and ++: excellent choice)

	Sample extraction and clean-up				**Injection method**			**GC method**		**Detection**			
	Soxhlet	MAE	PLE	Power-prep	Split-splitless	On-column	PTV	GC	GCx GC	ECD	LRMS	MS/MS	HRMS
Ease of method development	++	++	+	+	+	+	+/-	++	+/-	++	+	+	+/-
Robustness	++	++	++	+	++	+	+	++	+/-	++	++	+	+
Sensitivity					+	+	+ (++)**	+	++	++	ECNI:+, EI:-	+	++
Selectivity			(+)*	++				+	++	+/-	+	+	++
Labour***	+	+	+ (++)*	++	++	++	++	++	+	+	+	+	++
Speed/ throughput	+/-	+	+ (++)*	++	+	+	+ (++)**	++	+/-	++	++	++	++
Costs: investment	++	+	+/-	+/-	++	++	+	++	+	++	+	+/-	+/-
Costs, other****	++	+	+	+	++	+	+	++	+	++	+	+	+/-

* *Between brackets: combined extraction and clean-up*

** *Between brackets: when used in LVI mode*

*** *- is very laborious, +/- is intermediate, + is not very laborious, ++ is not laborious at all*

**** *Other costs: Consumables and maintenance*

When comparing costs per analysis (Figure 2.2), the costs for a GCxGC-ECD determination of PCDD/Fs and dl-PCBs (ca 900 Euro) are much higher than for GC-HRMS (275-500 Euro). This is mainly caused by the low contaminant concentrations combined with the considerable data treatment as discussed above. Furthermore, more emphasis was put on clean-up for removal of interferences from extracts. Investment costs per sample are, as expected, much lower (see also Table 2.3). It is believed that the price difference will become less in the future when software developments allow for rapid data treatment. It should be noted that in total, the cost data of five GC-HRMS laboratories were obtained, whereas the cost estimation for the other

techniques is based on a lower number of laboratories that participated in the DIFFERENCE project (*129*). Therefore, for GC-HRMS the range of costs among laboratories can be determined whereas this is not feasible for GCxGC-ECD for which only the data of one laboratory was obtained.

Detection

The third step in the GC analysis of halogenated contaminants is the detection. The predominantly used detectors are ECD, LRMS, ITMS/MS, TOF-MS and HRMS. Triple quadrupole MS/MS has not found a wide application (*194*). MS techniques can be used either with electron impact (EI) or ECNI ionisation. Table 2.3 shows a qualitative evaluation of the pro's and cons of the various detectors. ECD has found its application mainly in the analysis of PCBs and OCPs (*107,195-201*), toxaphene (*31,107,202*) PBDEs (*203*) and as a detector for GCxGC applications on PCDD/Fs and dl-PCBs and PBDEs (*182,186,193*). The use of ECD detection is straightforward; it is sensitive and provides fairly simple chromatograms. However, ECD detection is sensitive to electronegative interferences. $^{13}C_{12}$ labelled standards cannot be used and coelutions (often present in aquatic samples) can cause biased results. When not successfully resolved chromatographically, such compounds complicate the interpretation of the chromatograms and may result in inaccurate quantification. Therefore, much effort should be put in the GC separation and in the removal of interferences by clean-up of the extract, as discussed earlier. Compared to normal ECD, µECD is equipped with a small volume detection cell (e.g. 150 µl), which is essential for maintaining narrow peaks after GC separation, especially with applications that produce narrow peaks (e.g. GCxGC and narrow bore short column separations). The benefit of MS techniques is the improved identification compared to ECD. In the MS, compounds are being ionised and subsequently separated based on their mass-to-charge ratio (m/z). There are two ionisation techniques: EI and ECNI. The separation of ions takes place in an electromagnetic field induced by a quadrupole, a magnetic field or based on the time it takes the ions to arrive at the detector in an electromagnetic field (time-of-flight, TOF). With low-resolution instruments, the mass resolution is unit mass generally, whereas with high-resolution instruments mass resolution of over 10,000 are achieved. LRMS detection (EI or ECNI combined with single quadrupole separation of the resulting ions) has been used in a variety of studies for its sensitivity, selectivity and the fact that this type of instrument is widely available and fairly easy to operate and optimise. Applications of LRMS include analysis of BFRs (*59,60,108*), CPs, toxaphene (*204*) and PCNs (*12,14,15,135*). Ion trap MS/MS (ITMS/MS) has been used in several studies on toxaphene (*94,162,205,206*) and on PCDD/Fs (*121,133,207*) and CPs (*208,209*). The benefit of ITMS/MS is its higher selectivity and sensitivity when used in the MS/MS mode and the confirmation possibilities by recording full scan spectra of product ions (*194*).

TOF-MS is a very strong MS technique that is increasingly used in environmental analysis. It provides excellent resolution and mass accuracy (*194*). Full scans are being generated continuously (i.e. throughout the chromatogram), which allows for unambiguous identification. Chromatographically unresolved and interferences may be separated using the deconvolution software, a feature that is only available for TOF analysers. Prices of TOF-MS instruments are considerably higher than those of LRMS. This limits the broad application of the technique (Table 2.3). Finally, HRMS has widely been applied for the analysis of PCDD/Fs and dl-PCBs (*121,210*), toxaphene (*31,211*) and PBDEs (*123*). The technique provides excellent sensitivity (down to 100 fg for 2,3,7,8-TCDD) and mass resolution (*194*). Unfortunately, the investment and maintenance costs are high (Table 2.3) and Figure 2.2), which also has limited its broad application. It should be noted that prices of GC-HRMS equipment continue to drop resulting in increased access to this equipment in the future. Please refer to the review by Santos and Galceran for MS techniques applied in environmental analysis (*194*).

Because of the narrow peaks provided by GCxGC (100-600 ms at the baseline), high data-acquisition rates are required in order to obtain sufficient data points accurately describing the eluting peak (*181,212*). Its high operating speed (up to 500 spectra/sec) makes TOF-MS the ideal detector for the narrow peaks from GCxGC (*118,149*). In spite of its relatively low frequency, LRMS (ECNI mode) has been successfully applied as detector for GCxGC (*191*). Other detectors used in GCxGC studies, including µECD (*114,182*) and ITMS/MS (*149*).

PCDD/Fs and dl-PCBs. In the detection of PCDD/Fs and dl-PCBs, EI-HRMS is currently the golden standard. Alternative techniques are ECD (for mono-ortho CBs, but with co-elution risks (*156*)), ECNI-LRMS (for non-ortho CBs) (*53,97*), ITMS/MS (see below) and GCxGC-TOF-MS (*149*). Grabic et al. introduced GC-ITMS/MS as s sensitive and selective method for the detection of 17 WHO PCDD/Fs in human and fish tissue (*213*). Kemmochi et al. optimised collision characteristics in ITMS/MS and thereby improved the mass resolution. As a result, the iLOD for 2,3,7,8-TCDD decreased from 100 to 50 fg (*214*). Within the framework of the DIFFERENCE project (*129*), GCxGC-µECD, GC-ITMS/MS were further developed, optimised and subjected to an extensive validation against the GC-HRMS technique. For real samples, accuracy, precision and LOQs were in the same range (fish oil, fish), or slightly less (milk, pork) compared to GC-HRMS results (*121,129,149,192*), confirming the potential of these alternative techniques. However, it should be noted that although the GCxGC-µECD and GC-ITMS/MS techniques require lower investments, the samples may require more labour due to additional clean-up, more frequent maintenance of the instrument (GC-ITMS/MS) or more data treatment time to evaluate the complex chromatograms (GCxGC-µECD) (*149*). The overall costs for the ITMS/MS analysis of PCDD/Fs and dl-PCBs (Figure 2.2) are

comparable to the lower range of GC-HRMS and much lower than for those of GCxGC-μECD. For GCxGC analysis, ECNI-LRMS is a suitable detector for most PCDD/Fs (iLOD=10-110 fg injected), except for the important 2,3,7,8-TCDD congener and OCDD, for which ECNI provided not enough sensitivity (430-710 fg injected) to compete with HRMS (*191*).

PCBs and OCPs. ECD and MS may both be used for the detection of PCBs and OCPs. ECD detectors are attractive because of their low costs and high sensitivity. However, their selectivity is limited. $^{13}C_{12}$ labelled standards cannot be used and coelutions and other interferences can cause biased results, as is experienced in PCB-OCP interlaboratory studies (*215*). μECDs are even more sensitive (5 to 10-fold) due to the smaller cell volume. MS techniques are preferred for accurate determination, because of the unambiguous identification and the possibility to use $^{13}C_{12}$ labelled standards (*215*), although it should be noted that for PCB homologues the spectra are identical (which limits the selectivity gain compared to ECD). EI-LRMS is less sensitive (low pg) than ECNI-LRMS (low fg). The latter technique is especially sensitive to higher chlorinated compounds (*56*). Gomara et al. evaluated EI-ITMS/MS. They isolated the $(M+2)^{\cdot+}$ and $(M+4)^{\cdot+}$ as precursor ions and the resulting daughter ions were obtained through loss of 2 chlorine ions ($(M-2^{35}Cl)^+$ and $(M-^{35}Cl^{37}Cl)^+$) (*112*). Verenitch et al. selected slightly different precursor and daughter ions (*133*). The iLOQ in both studies was approx. 0.1-1.2 pg injected, being somewhat higher than that of EI-HRMS (but this may be compensated by using LVI). Depending on the PCB of interest, sensitivity was only slightly better or worse compared to μECD. EI-ITMS/MS and EI-HRMS results were comparable for marine biota extracts (*133*). EI-HRMS was also used in some studies and provides excellent selectivity (*112,133*). The most abundant isotope ions monitored are M^+, $(M+2)^+$ and $(M+4)^+$ (*133*). In GCxGC analysis, TOF-MS (*216*) and μECD (*113*) were used for detection of OCPs and μECD (*114,132,184*), EI-LRMS (*217*) and TOF-MS (*113,183*) were used as detectors for PCBs.

PCNs. ECD and MS may both be used for the detection of PCNs, although the latter technique is more selective. Mostly applied are EI-LRMS and ECNI-LRMS (*12,15,153,218*). SIM is used for quantification of the individual congeners and homologue groups. Ion trap-MS was used by Wiedman et al. (*219*), using molar responses for quantification. Wang et al. reported the use of ITMS/MS detection (*16*), but without reporting the MS/MS transitions used. ITMS/MS provides good sensitivity and improved selectivity compared to single MS techniques. EI-HRMS has also been used for detection by several labs providing excellent sensitivity and selectivity (*14,153,220*). For GCxGC detection of PCNs, μECD has been used (*113*).

CPs. The problem of CP analysis is the extreme complexity of the technical mixture and of the patterns in the environmental samples. When using ECD the chromatogram shows one huge hump, which can of course be quantified, but which at the same time lacks any accuracy because of

differences between the technical standards and the samples (*105*). In ECNI-MS, the main ions produced are $(M\text{-}Cl)^-$, $(M\text{-}HCl)^-$ and $(M+Cl)^-$. ECNI response factors vary with chlorination degree: 3-4 chlorines are not detected whereas congeners with 7 or more chlorine atoms are overestimated (*20*). ECNI-LRMS suffers from some mass interferences from ions with 5 carbon atoms less and 2 chlorine atoms more (*17*). Many of such 'pairs' exist (e.g. $C_{10}H_{14}Cl_8$ and $C_{15}H_{26}C_{l6}$, see (*17*) for an overview table) when both SCCPs and MCCPs are present in environmental samples. The determination of isotope ratios can be used for tracking possible interferences. Identification is often performed by summarising the possible isomer ions per number of carbons (e.g. $C_{11}Cl_{15}$ to $C_{11}Cl_{19}$) (*20,26*). This results in a total response per chain length, which can be compared with the total response per chain length obtained from a selected technical mixture. Another means to determine the carbon chain length profile is by carbon skeleton reaction GC, in which the CPs are dechlorinated in the injector with a palladium catalyst (*221*), but this method has to our knowledge not been applied to environmental samples. For GCxGC detection of CPs, µECD has been used (*113*).

Toxaphene. ECD has yet been used for detection of toxaphene in several studies (*31,202*). ECD response factors for the predominant congeners vary from 0.6-2.0 (*31*). ECD is much less sensitive for toxaphene than for e.g. PCBs, due to the aliphatic character of toxaphene. Again, ECD lacks specificity and OCPs in particular can interfere. A selective clean-up can minimise but not omit these interferences (for details see the sample clean-up section). Therefore, ECD results in environmental samples tend to be higher than results obtained by MS detection. With the introduction of ECNI-MS, more reports on toxaphene in the European environment became available. The higher selectivity of MS provides, information on homologue groups (hexa- through decachlorobornanes and –bornenes). EI is less sensitive than ECNI, except for the lower chlorinated congeners where EI provides best sensitivity (*28*). With ECNI, the $(M)^-$ and $(M\text{-}Cl)^-$ ions can be monitored (*28,106,134,141,202*). ECNI-HRMS used at a resolution of 10,000 is a very selective method of detection, virtually free of interferences (*31,134*). Gouteux et al. (*94*) evaluated EI-ITMS/MS for detection of individual congeners. The EI mass spectra are rich of ions that can be chosen as parent ion in MS/MS experiments. They tested several transitions and concluded that for P26, 40, 41, 44 transition of m/z 125→89 was most sensitive, whereas for P50 it was 279→243 and 305→267 for P62. Their detection limits were 0.08 and 0.37 ng/g ww. A similar EI-ITMS/MS method was used by Bernardo et al. (*162*). For additional information, one should consult the comprehensive reviews available (*28,57,106*). For GCxGC detection of toxaphene, µECD and ECNI-TOF-MS have been used (*113,187*).

BFRs EI-HRMS and ECNI-LRMS are the detection techniques most commonly applied (*59,60*). Other techniques used are EI-LRMS and EI-ITMS/MS (*151*). ECD (*116*) has been used as well. ECNI-MS provides a good sensitivity and selectivity for the detection of BFRs. The most commonly monitored ions are

m/z 79 and 81, representing the two bromine isotopes (*59*). These ions are not very specific, but the molecular ions are only produced at low yields, resulting in insufficient sensitivity. For BDE 209, the m/z 484.7 and 486.7 can be monitored as well and for HBCD m/z 561 can be used as qualifier ion (but due to the low yield, it's not suitable for quantitation when aiming for low level samples). In EI-MS, the most commonly monitored ions are $(M\text{-}Br_2)^+$ and $(M)^+$. They provide a good selectivity, but a lower sensitivity, especially for the higher brominated PBDE congeners (hepta- to deca-BDE) (*60*). This can be overcome by LVI of larger volumes (e.g. 20 µl (*222*)). EI-MS enables the use of $^{13}C_{12}$-labelled standards, which is important for a reliable quantification. HRMS provides good sensitivity and selectivity, but at higher instrumentation investment and maintenance costs. In EI-ITMS/MS the molecular BDE ion fragmented (using CID) in the $(M\text{-}Br_2)^+$ or $(M\text{-}COBr)^+$ ion. The instrumental LOD was 0.1-1.3 pg/µl (4 µl injected) (*151*). TOF-MS may be used for detection of PBDEs at a sensitivity comparable to other MS techniques (*189,223*). Due to the limited linear range of the instrument, samples with large variation in concentration of PBDEs often require re-analysis (*223*), which hampers a broad application of the instrument. An overview of detection techniques, benefits, drawbacks and ions monitored in MS detection can be found elsewhere (*60,108*). For GCxGC detection of BFRs, TOF-MS (*186,216*) and µECD (*113,186*) have been used.

Quality assurance

The analysis of organic contaminants is laborious and complex and involves many steps. Errors are easily made in extraction, clean-up, GC determination and quantification, as discussed earlier. Accurate analysis of halogenated contaminants is important for scientists and policy makers who rely on the data produced in environmental laboratories. To minimise the chance of errors, steps should be taken to improve the analysis and quality control systems should be established and routinely applied (e.g. according to ISO-17025). This includes the use of high quality standards and internal standards, blank tests, replicate analysis, recovery experiments, plotting quality control charts, participation in interlaboratory studies and the analysis of certified or standard reference materials (CRM, SRM) and laboratory reference materials (LRMs) (*224*). When these measures are implemented appropriately, performance of different laboratories can be comparable and contaminant data produced by them can be used for successful policy making.

High quality standards are commercially available from various suppliers. Internal standards should preferably be mass labelled and used in combination with MS detection. Mass labelled standards are available e.g. for PCDD/Fs, PCBs, OCPs and BFRs. Most compounds can be quantified individually, but in case of total-toxaphene and SCCPs, quantification is

based on available technical mixtures. Because of that, large inaccuracies result as will be discussed below.

Table 2.4 Overview of frequently operated interlaboratory schemes for halogenated contaminants.

Compound	Interlaboratory study matrices	Organisation*
PCDD/Fs and dl-PCBs	Fish, shellfish, sediment	QUASIMEME, Folkehelsa
PCBs, OCPs	Fish, shellfish, sediment	QUASIMEME, Fapas
PCNs	Na	Na
Toxaphene	Fish, shellfish	QUASIMEME
CPs	Na	Na
BFRs	Fish, shellfish, sediment	QUASIMEME, Folkehelsa, Fapas

* *Further information can be found at www.quasimeme.org; www.folkehelsa.no; www.fapas.com*

Participation in interlaboratory studies and analysis of CRMs and SRMs on a regular basis provides a performance test compared with external sources. An overview of frequently organised interlaboratory studies can be found in Table 2.4. Unfortunately, no frequent interlaboratory studies are available for SCCPs and PCNs. Fish and sediment CRMs and SRMs are available for PCBs, OCPs, PCDD/Fs, PBDEs and toxaphene (*225-229*). Wet, sterilised matrix-type CRMs produced by the Community Bureau of Reference of the European Commission (BCR) are favourite over non matrix-type CRMs for their very close matrix resemblance (*230*). These CRMs are available through the Institute for Reference Materials and Measurements, Geel, Belgium. Feasibility studies showed that a successful certification of low level CRMs is possible for BFRs, PCDD/Fs, PCBs and OCPs, but unfortunately, these materials have not become available (*229*). Again, no RMs are available for PCNs and SCCPs. This is surprising as SCCPs are produced by far more than other compounds (Table 2.1) and the especially SCCP analysis is vulnerable for large inaccuracies.

PCDD/Fs and dl-PCBs Because of the low concentration levels, laboratories should take care of cross contamination between high and low contaminated samples. Specialised laboratories often use separate glassware and clean rooms for low level samples. Laboratories are often accredited for this method and although the analysis is very laborious and complicated, the results within one laboratory can be very accurate (with repeatability as low as 5% for individual congeners), mainly because of the application of mass labelled internal standards. The agreement between data on individual congeners, expressed as relative standard deviation, ranged from 21-200%, with the 'difficult' congener, OCDD, showing the least accuracy (*231*). Interlaboratory studies (ILS) and RMs are available.

PCBs and OCPs High quality standards (individual OCPs and PCB congeners) are widely available and several CRMs for PCBs and OCPs in biota (fish oils,

whale blubber, mussel tissues and fish tissues) and sediment are available. ILS are available (Table 2.4) and they show that laboratories have generally more difficulties in producing good quality data for OCPs than for PCBs. ECD is a commonly used detector, but inaccuracies due to interferences often occur. At recent dedicated QUASIMEME workshop it was concluded that MS detection is preferred over ECD (*215*). This also allows the use of mass labelled standards, further improving the accuracy.

PCNs Several PCN congeners are commercially available as standards (*232*). Wiedmann and Ballschmiter developed a GC-MS quantification method using molar responses of electron-impact ionisation. They were able to quantify all congeners on the basis of a small set of PCNs (*233*). Due to a lack of standards, response factors for homologue groups have repeatedly been used (*153*). In a PCN ILS, nine laboratories quantified homologue groups and individual congeners in test solutions derived from Halowax 1014. The variability in homologue quantification was slightly better (11-43% RSD) than for the individual congeners (18-51%, excl. CN-29) (*153*). The results of the announced 2nd phase ILS (including real environmental samples) are not reported yet. To our knowledge no CRM is available.

CPs Quantification of CPs is mainly done by calibration with technical mixtures. Recently, individual congeners have been produced and are commercially available (*188*). Coelhan et al. (*23*) have quantified SCCPs in fish samples using C_{10}, C_{11}, C_{12} and C_{13} CPs with different chlorination degrees (47-68%) as well as quantification against Cerechlor SCCP technical mixture (63% chlorination). Differences were as high as 1,100% when fish samples were quantified against a low chlorinated or a highly chlorinated standard. The authors recommended the use of single-chain length standards for quantification in order to meet the specific profiles found in the fish samples (*23*). The use of technical mixtures that do not match the pattern as observed in the sample decrease the accuracy of data reported (*23*). The quality of reported data is also decreased by interference of other chlorinated contaminants in the extract (e.g. OCPs, toxaphene) when using Total Ion Current-MS. More specific information on individual formula and homologue groups is obtained by detection with high-resolution (HR)-MS (*234*). Further details on MS detection and quantification can be found elsewhere (*55*). Tomy et al. (*235*) organised an interlaboratory study on the quantification of SCCPs. The data from the 7 participating laboratories showed that the true value of standard solutions was overestimated up to 150%. The coefficient of variation for the fish extracts was 27 and 47%, which is reasonably good taking into account the lack of reliable standards. Given the lack of accuracy, it is highly surprising that many studies report data with high level of suggested accuracy (e.g. reporting in several decimals). The reporting should be adjusted so as to really represent the level of accuracy from the methods applied. Currently, no CRMs are available for PCAs and although PCAs were detected in standard reference materials (SRMs) from the National Institute for

Standards and Technology (NIST) (*234*), these SRMs are not certified for CPs. There is a clear need for further method development and ILS for CPs also because they are included in the target contaminants list of the European Water Framework Programme.

Toxaphene For the quantification of toxaphene often technical mixtures are used as standards and levels are reported as total-toxaphene resulting in a mismatch between the congener profile present in the sample and the technical mixture (*106,211*). A change in the composition of technical toxaphene may for example occur in split/splitless injection (*141*), leading to biased results when quantifying against technical toxaphene mixtures. Although there is a lack of standards for individual congeners, a limited number is commercially available (e.g. Parlar nos. 26, 32, 40, 41, 44, 50 and 62) (*106*). Currently, CRM is available (*225*). The NIST SRM 1588a and 1945 have indicative values for toxaphene (*106,236*). ILS is available from QUASIMEME (Table 2.4).

BFRs Methods for PBDE analysis have been developed by a vast number of laboratories world-wide in the last 5 years. Recently, over 170 PBDE congeners, individual HBCD diastereomers, (me-)TBBP-A and others became commercially available. For many of those, isotope labelled and fluorinated internal standards are available. A number of ILS have been organised for sediment and biota samples. Improvement was seen over the years for most PBDEs, although BDE 183 and 209 remain problematic (*58*). De Boer and Wells provided several analytical solutions for these problems (*58*). Recently, SRMs for sediment and fish were analysed (and certified for fish only) for PBDEs (*237*). Blank tests are important, because of the presence of organohalogen contaminants in dust, electric equipment and building materials (*238-241*) present in laboratories. De Boer et al. presented an overview of blank problems in the BFR analysis (*58*). Special care should be taken to avoid contamination of samples and extracts by BDE 209 from dust and air. Furthermore, it is essential to use separate sets of glassware and extraction and clean-up equipment for high contaminated samples and low contaminated samples.

Conclusions

Extraction, and more so, clean-up and fractionation, are crucial steps prior to the GC analysis of halogenated contaminants because co-extracted compounds such as lipids and sulphur have a major negative effect on their detectability at the trace levels at which they normally occur in the environment. Selective PLE provides an effective and efficient extraction *and* clean-up technique that enables processing of multiple samples in a short time (less than 1 h). Developments in injection have been somewhat limited over the last years. Large volume injection (e.g. by PTV, or cold on-column) is interesting for obtaining better LOQs. Septumless injection has been introduced to avoid septum particles to enter the column. A wide choice of

autosamplers is now available, both for on-column and splitless injection. GCxGC is a strong technique for unravelling complex mixtures. By selecting the right column combinations, structural information can be obtained. The narrow peaks offer a better sensitivity compared to single-column GC, which even enables the determination of low (pg/g) dioxin concentrations. Mass spectrometry in various set-ups is the preferred detection technique. QA tools such as interlaboratory studies, use of LRMs and CRMs are very well established for PCDD/Fs, OCPs and PCBs but improvement of that infrastructure is needed for BFRs, CPs, PCNs and toxaphene.

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2.2 Extraction and clean-up methods for perfluorinated contaminants[3]

Abstract

The rapidly expanding field of per- and polyfluorinated compounds (PFCs) research has resulted in a wide range of analytical methodologies to determine the human and environmental exposure to PFCs. This paper reviews the currently applied techniques for sample pre-treatment, extraction and clean-up for the analysis of ionic and non-ionic PFCs in human and environmental matrices. Solid phase extraction (SPE) is the method of choice for liquid samples (e.g. water, blood, serum, plasma), and may be automated in an on-line set-up for (large volume) sample enrichment and sample clean-up. Prior to SPE, sample pre-treatment (filtration or centrifugation for water or protein precipitation for blood) may be required. Liquid-liquid extraction can also be used for liquid sample extraction (and does not require above mentioned sample pretreatment). Solid-liquid extraction is the commonly applied method for solid matrices (biota, sludge, soil, sediment), but automation options are limited due to contamination from polytetrafluorethylene tubings and parts applied in extraction equipment. Air is generally preconcentrated on XAD-resins sandwiched between polyurethane foam plugs. Clean-up of crude extracts is essential for destruction and removal of lipids and other co-extractives that may interfere in the instrumental determination. SPE, (fluorous) silica column chromatography, dispersive graphitized carbon and destructive methods such as sulphuric acid or KOH treatment can be applied for clean-up of extracts. Care should be taken to avoid contamination (e.g. from sample bottles, filters, equipment) and losses of PFCAs (e.g. adsorption, volatilization) during sampling, extraction and clean-up. Storage at -20°C is generally appropriate for conservation of samples.

Introduction

Poly- and perflourinated compounds (PFCs) have been and are still being used widely for their surface tension lowering properties in a variety of domestic and industrial applications such as polymerization aid for production of fluorinated polymers, for metal plating, in photographic industry, in the semi-conductor industry, in the aviation industry (hydraulic fluids), in fire fighting foams and as fat, and water repellents for textiles, paper and leather

[3] Based on S.P.J. van Leeuwen and J. de Boer (2007) Extraction and clean-up strategies for the analysis of poly- and perfluoroalkyl substances in environmental and human matrices, *Journal of Chromatography A*, 1153, 172-185.

(*1,2*). These compounds have entered the environment from all stages: the production of PFC, application to products and use and disposal of these products. Historic emissions of perfluorocarboxylates (PFCAs) were estimated at 3200-7300 tonnes (1951-2004) (*2*). The OECD estimated the historic production volume of perfluoroctanesulfonate (PFOS) and related compounds at 4500 tonnes/year (*1*).
A selection of PFCs reported in environmental and human matrices are shown in Table 2.5. PFCs can enter the environment in the chemical form as they have been produced and applied or as a precursor. Precursors such as fluorotelomer alcohols (FTOHs) or perfluorosulfonamide (PFOSA) can transform (a)biotically to their stable end products like perfluorocarboxylic acids (PFCAs) (*3-6*) or PFOS (*7*).
After initial reports on the presence of PFCs in the environment (*8-10*) in the past five years many researchers have started to investigate this class of compounds. Initial studies focussed on PFOS and perfluorooctanoic acid (PFOA) and those have received most attention. However, a range of other PFCs receive increasing attention because they are produced as alternatives for PFOS and PFOA, as intermediates in PFC production, as by-products or as (bio)degradation products. This includes PFCAs and perfluorosulfonates (PFSAs) with different chain lengths (typically between C4-C14), fluorotelomer carboxylic acids (FTCAs) and non-ionic (volatile) compounds such as fluorotelomer alcohols (FTOHs), FFOSA and N-substituted sulfonamides.
Initially, laboratories analysed biota samples employing an extraction method according to Hansen *et al.* (*11*), which is based on ion pairing of the ionic PFCs with tetra-*n*-butylammonium hydrogensulfate (TBA), followed by a liquid-solid extraction (LSE) with methyl-*tert*-butylether (MTBE), filtration of the extract and instrumental determination by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS). This method is often referred to as the ion pair extraction method (IPE). The IPE method was applied to a wide range of biological matrices such as animal (liver) tissue (*8,12,13*) and serum (*14,15*). Some of the limitations of the IPE method are that the method is laborious, time consuming and difficult to automate. With the rapid expansion of the PFCs research area, there was a need for more dedicated methods which enable efficient and accurate analysis. In recent years, new extraction and clean-up methods have been introduced that enable the analysis of a variety of PFCs in wide range of matrices (e.g. sewage treatment samples, air, sediment, soil, blood and milk). These methods include solid phase extraction (SPE) of fluid samples (*16-18*) and accelerated solvent extraction, here referred to as pressurized liquid extraction (PLE) and liquid solid extraction (LSE) of solid materials (*16,19*). Furthermore, (fully) automated methods have been introduced that allow for on-line extraction and clean-up of samples with a minimum of labour involved (*17,20-22*).

Table 2.5 Full names, abbreviations and chemical formulas of a selection of PFC.

Full name	Abbreviation	Chemical formula
Poly- and perfluorinated acids	PFCAs	
Perfluorobutanoic acid	PFBA	$CF_3(CF_2)_2COOH$
Perfluorohexanoic acid	PFHxA	$CF_3(CF_2)_4COOH$
Perfluoroheptanoic acid	PFHpA	$CF_3(CF_2)_5COOH$
Perfluorooctanoic acid	PFOA	$CF_3(CF_2)_6COOH$
Perfluorononanoic acid	PFNA	$CF_3(CF_2)_7COOH$
Perfluorodecanoic acid	PFDA	$CF_3(CF_2)_8COOH$
Perfluoroundecanoic acid	PFUnA	$CF_3(CF_2)_9COOH$
Perfluorododecanoic acid	PFDoA	$CF_3(CF_2)_{10}COOH$
Perfluorotridecanoic acid	PFTrA	$CF_3(CF_2)_{11}COOH$
Perfluorotetradecanoic acid	PFTA	$CF_3(CF_2)_{12}COOH$
Perfluoropentadecanoic acid	PFPA	$CF_3(CF_2)_{13}COOH$
6:2 Fluorotelomer carboxylic acid	6:2 FTCA	$CF_3(CF_2)_5CH_2COOH$
8:2 Fluorotelomer carboxylic acid	8:2 FTCA	$CF_3(CF_2)_7CH_2COOH$
10:2 Fluorotelomer carboxylic acid	10:2 FTCA	$CF_3(CF_2)_9CH_2COOH$
6:2 Fluorotelomer unsaturated carboxylic acid	6:2 FTUCA	$CF_3(CF_2)_4CF{=}CHCOOH$
8:2 Fluorotelomer unsaturated carboxylic acid	8:2 FTUCA	$CF_3(CF_2)_6CF{=}CHCOOH$
10:2 Fluorotelomer unsaturated carboxylic acid	10:2 FTUCA	$CF_3(CF_2)_8CF{=}CHCOOH$
Poly and perfluorinated sulfonates	PFSAs	
Perfluorobutane sulfonate	PFBS	$CF_3(CF_2)_3SO_3^-$
Perfluorohexane sulfonate	PFHxS	$CF_3(CF_2)_5SO_3^-$
Perfluorooctane sulfonate	PFOS	$CF_3(CF_2)_7SO_3^-$
Perfluorodecane sulfonate	PFDS	$CF_3(CF_2)_9SO_3^-$
6:2 Fluorotelomersulfonate	6:2 FTS	$CF_3(CF_2)_5CH_2CH_2SO_3^-$
Non-ionic PFCs		
4:2 Fluorotelomer alcohol	4:2 FTOH	$CF_3(CF_2)_3CH_2CH_2OH$
6:2 Fluorotelomer alcohol	6:2 FTOH	$CF_3(CF_2)_5CH_2CH_2OH$
8:2 Fluorotelomer alcohol	8:2 FTOH	$CF_3(CF_2)_7CH_2CH_2OH$
10:2 Fluorotelomer alcohol	10:2 FTOH	$CF_3(CF_2)_9CH_2CH_2OH$
Perfluorosulfonamide	PFOSA	$CF_3(CF_2)_7SO_2NH_2$
N-ethyl perfluorooctane sulfonamidoethanol	NEtFOSE	$CF_3(CF_2)_7SO_2N(CH_2CH_3)CH_2CH_2OH$
N-methyl perfluorooctane sulfonamidoethanol	NMeFOSE	$CF_3(CF_2)_7SO_2N(CH_3)CH_2CH_2OH$
N-ethyl perfluorooctane sulfonamide	NEtFOSA	$CF_3(CF_2)_7SO_2NHCH_2CH_3$
N-methyl perfluorooctane sulfonamide	NMeFOSA	$CF_3(CF_2)_7SO_2NHCH_3$

Recently, two comprehensive reviews were published that focused predominantly on the instrumental determination and quantification of PFCs (*23,24*). This paper aims at reviewing the sample pre-treatment, extraction and clean-up strategies for PFCs environmental and human samples. Furthermore, some attention has been paid to sample storage.

Storage of samples and conservation

Storage and conservation of samples for PFCs analysis is critical because during these stages losses and contamination can easily occur. Substantial attention in the literature has been paid to the contamination of samples with PFCs during analysis, and suggestions have been made to eliminate, circumvent or to control the contamination (*25,26*). Several authors pre-cleaned the sampling bottles prior to sampling by rinsing with (semi-)polar solvents such as de-ionised water, acetone, methanol or MTBE (*27,28*). In one study, it was shown that polypropylene sample bottles contained traces of PFOA (*18*) and pre-cleaning is therefore important when scientists target low concentrations in water samples (sub-ng/L to low ng/L). Less attention has been paid to the potential losses during storage of samples. Preferably, samples should be analysed directly after sampling, but this may not be feasible for several reasons. Alternatively, samples can be stored prior to analysis under conditions that prevent changes in composition of the sample matrix and the concentrations of PFCs (by contamination or losses).

Adsorption to sample containers. There has been some debate on whether and which (ionic) PFCs adsorb to glass surfaces (*25,29*). Although this may happen at low concentrations in analytical standards (*21*), it is expected that this will not happen in samples which contain large amounts of matrix components (such as biota, serum and blood) that can shield the active sites at the glass surface. Karrman *et al.* stored whole blood in glass containers and analysed the sample several times during 4 months multiple freezing and thawing cycles. They did not find any indication for losses of perfluorohexane sulfonate (PFHxS), PFOS, PFOA and perfluorononanoic acid (PFNA) (*30*). On the other hand, for water samples, irreversible adsorption of PFCs to the sample container surface (polypropylene-PP or high density poly ethylene-HDPE) was reported for long chain PFCAs (>C10), PFOSA and NEtFOSA (*28,31*) and PFOS and PFOA in acidified water (*32*). Shorter chain PFC are sufficiently water soluble to remain in solution (at neutral pH), as was –apart from above studies- also shown by the good recoveries obtained after analysis of a transportation-recovery sample (stored in PP bottles) as part of a QA/QC program in a water study by Rostkowski *et al.* (*33*).

Volatilisation. FTOHs are volatile compounds (*34,35*), therefore, losses through volatilization are readily encountered during sampling, sample storage and analysis (*31*). A likely way to minimize these losses during sample storage is not to allow for headspaces in sample bottles. Liu and Lee limited the headspace

in their 8:2 FTOH solubility experiments to limit evaporative losses (*36*). In another study, it was found that 8:2 FTOH remains in aqueous solution when stored at -20°C in a glass vial, sealed with a septum lined with alumina foil (see below) (*37*). To the best of our knowledge, no (other) information is available on the evaluation of sample storage for potential losses of FTOHs and other volatile PFCs.

Conservation of samples. Conservation of samples is often done by storage in freezers or refrigerators. As mentioned above, for whole blood this was shown to be safe (*30*). Water samples are stored in various ways, including at room temperature, freezing, storage in refrigerators and acidification with formic acid in combination with storage in a refrigerator (*38,39*). The latter method lowers the pH to approx. 2. At decreased pH, PFCAs become increasingly associated with the available protons, changing the physicochemical properties of the acids. Kaiser *et al.* determined ca. 20% PFOA loss in 300 hrs from a pH 4 buffered aqueous solution, possibly increased by the constant air flow that was applied over the test solution (*40*). In real samples, loss of ionic character (due to the proton association) may also lead to adsorption to the sample container (*32*). Therefore, acidification of water samples for storage purpose should be avoided. Storage of water samples in a refrigerator or freezer, similar to blood samples, is presumably the best alternative. Szostek *et al.* investigated the stability of FTOHs in water and water samples mixed with acetonitrile during storage. From the different storage conditions tested over a 7-day period, they concluded that aqueous samples can safely be stored in the freezer in a glass vial, sealed with a septum lined with alumina foil (*37*).

Biotransformation of PFCs may occur in biologically active samples such as sewage sludge. Dinglasan *et al.* and Wang *et al.* reported on microbial transformation of FTOHs yielding unsaturated and saturated PFCAs (*5,6,41*). This potentially changes the composition of the PFCs in the sample if not stored in a way that prevents biodegradation. PFCAs were stable for at least 6 months in an acetonitrile sewage sludge extract in a freezer (*6*). Schultz *et al.* tried formalin for inhibition of biological activity in wastewater samples but it was found to suppress the PFCs MS responses (*42*). They have frozen the samples at -20°C instead. Scott *et al.* found no deterioration of the 8:2 and 10:2 FTCAs stored in aqueous (calibration) solutions after a 9-month period (*43*) (the storage conditions were not reported).

Sample pre-treatment

The aims of extraction and clean-up are (i) to transfer the analytes to the physical state that enables the analysis and final detection, (ii) to enrich the analytes of interest and (iii) to purify the extract prior to instrumental determination. Prior to the extraction of the PFCs from the matrix, often a certain degree of pre-treatment of the sample is required to facilitate extraction or to remove matrix constituents that will disturb the extraction or

the instrumental determination. Several modes of sample pre-treatment are mentioned in Figure 2.3.

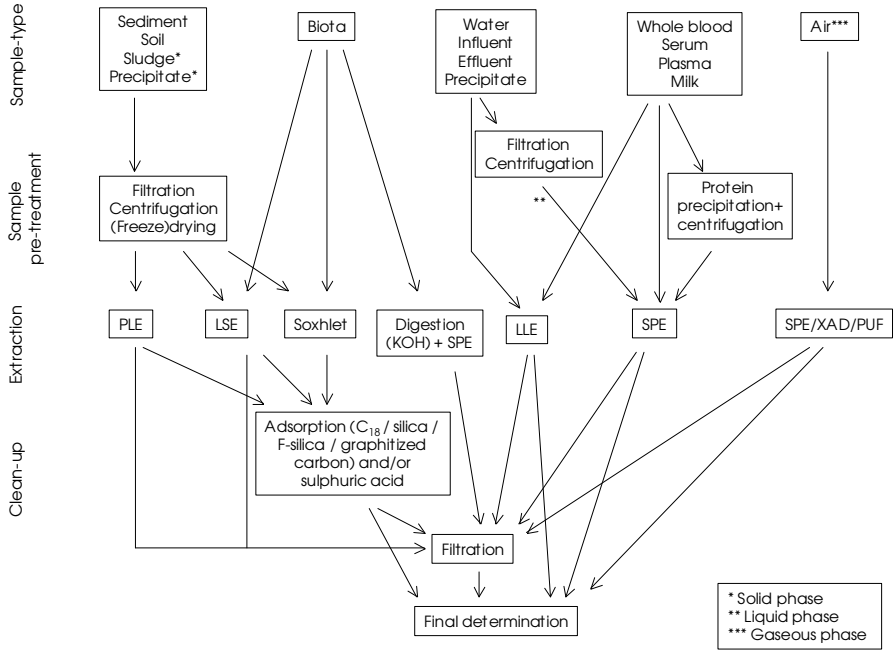

Figure 2.3 Extraction and clean-up methods for the analysis of PFCs in human and environmental matrices.

Water samples and sewage treatment plant influent and effluents may be filtered (e.g. on glass fiber filters (GFF) (*42,44*)) to separate solids from the liquid phase. However, filtration can result in losses by adsorption of PFCs on the filters. On the other hand, levels can increase by PFCs contamination originating from the filters, as was found by Schultz *et al.* for four types of filters (namely glass fiber, nylon, cellulose acetate, and polyethersulfone filters) (*42*). They applied centrifugation as alternative for separation the liquid from the solids.

For human matrices such as plasma, serum or whole blood, trichloroacetic acid, formic acid or acetonitrile need to be added to the sample for precipitation of the red blood cells in whole blood in order to prevent clogging of the SPE column (*45*), or for precipitation of the proteins present in serum and plasma (*20,21*). This step is typically followed by centrifugation to separate the precipitates from the liquid phase (see Figure 2.3) (*20,21,45*). Kukyenlik *et al.* designed an on-line SPE-LC-MS/MS method in which no precipitation step was required, thereby greatly reducing sample handling

time. Only the addition of formic acid to the sample in the vial prior to analysis was required (*22,46*). Flaherty *et al.* designed a automated high-throughput acetonitrile method based on protein precipitation arrayed in a 96 wells setup (*20*). Column eluates were analysed directly without further pre-treatment or concentration of the sample. The various precipitation methods may have specific (dis)advantages (*21,45*) and scientists should carefully evaluate the selected method. Karrman *et al.* found that formic acid was more effective for red blood cell precipitation than acetonitrile and trichloroacetic acid (*45*). Acetonitrile increases the elution strength of the sample and should be removed as otherwise it and may cause breakthrough in a SPE sample enrichment procedure. Trichloroacetic acid can co-precipitate hydrophobic acids and can result in loss of sensitivity in the instrumental determination due to abundant cluster formation in the mass spectra (*21*).

Extraction of PFCs

Due to their different polarities, the PFCs mentioned in Table 2.5 require different extraction strategies. The *ionic* PFCAs and PFSAs require moderately polar media (Oasis WAX SPE or methanol and acetonitrile) for efficiently trapping of water soluble short-chain (C4-C6) compounds. For longer chains, less polar or non-polar SPE phases (C18 and Oasis HLB) may be applied. When an ion-pairing agent is used that decreases the polarity of the ion pair complex, a non-polar solvent (MTBE) may be used. *Non-ionic* PFCs may be extracted from the matrix by non-polar media (C18 SPE or hexane). Moderate polar media (Oasis HLB and Oasis WAX SPE, a hexane-acetone mixture or acetonitrile) have also been applied for extraction of non-ionic PFCs.

Water (including wastewater)

PFCs concentrations reported in water cover a range of several orders of magnitude. Schultz *et al.* found concentrations up to 32 µg/L in groundwater contaminated with aqueous fire fighting foams using a direct injection technique without sample enrichment (*47*). In most cases, lower concentrations (ng-pg/L (*26*)) are found, requiring enrichment of the sample. Both LLE and SPE are suitable for this purpose. A wide variety of SPE methods have been reported for sample extraction and clean-up of water samples (Table 2.6). Different column types have been used, including purely hydrophobic (C18) (*48-50*), mixed hydrophobic/polar (e.g. Oasis HLB) (*18,31*) and WAX-type phases (*31*). Taniyashu *et al.* evaluated Oasis HLB and Oasis-WAX columns for the extraction of PFCs. In general, the performance of these columns was comparable. Recoveries were good (70-100%) for most compounds.

Table 2.6 Typical extraction and clean-up techniques for water, wastewater, influent and effluents.

PFCA	PFSA	Other	Sample Type	Pre-treatment	Extraction	SPE elution solvent	Instrumental determination	Sample intake (mL) / LOQ[a] (ng/L)	Ref.
8,9	4,6,8	PFOSA, 6:2 FTS	Seawater	Filtration	SPE: Oasis HLB and C18	Methanol	LC-ESI-MS/MS	1000 / 0.4-5.2 pg/L (LOD)	(*18*)
6-12	8,10	PFOSA, NEtFOSA	Waste-water, river water	None	LLE with MTBE after addition of 50 g/L NaCl and sulphuric acid (pH=4)	Na	LC-ESI-MS/MS	900 / 0.94-16	(*28*)
4-12, 14,16,18 8:2 FTCA 8:2 FTUCA	8,6,4	10:1, 7:1 FTOH, PFOSA, NEtFOSA	Water	Filtration	SPE: Oasis HLB and Oasis WAX	0.1% NH_4OH/ methanol	LC-ESI-MS/MS	100-200 / 0.004-4 (LOD)	(*31*)
Na	Na	6:2, 8:2 and 10:2 FTOH	Water	Na	LLE (acetonitrile or MTBE)	Na	LC-ESI-MS(/MS)	3 / 2-3	(*37*)
4,6,8,10	6-10	PFOSA, 6:2 FTS	Municipal waste-water	Centrifu-gation	On-line SPE: C18	Methanol/ water/NH_4Ac	LC-ESI-MS/MS	0.5 / 0.5	(42)
3-10	Na	Na	Rainwater	Meth. 1. Concen-tration (rotary evapora-tor) and adjust-ment pH 2	Meth 1. Difluoroanilide derivatisation, followed by LLE (ethylacetate) Meth 2. Oasis HLB Meth 3. XAD-7 resin	Meth 2/3. Methanol	Meth 1. GC-MS Meth 2/3. LC-ESI-MS/MS	Meth 1. 500?/0,15-0,3 Meth 2. 300 / 0.5 ng/l (OASIS HLB) Meth 3. 34 L / 0.01	(*43*)
6-8	4-8	4:2, 6:2, 8:2 FTS	Military base ground-water	Centrifu-gation	Na	Na	Direct injection, LC-ESI-MS/MS	Na / 0.5-0.6 µg/L	(*47*)
Na	8	Na	River water	Centrifu-gation	On-line turbulent flow chromatography on C18 column	Na	LC-APPI-MS	1 / 18	(*48*)
6:2, 8:2, 10:2 FTCA, 6:2, 8:2, 10:2 FTUCA	8	Na	Rainwater	None	SPE: C18	Methanol	LC-ESI-MS/MS	4 L / 0.08-7.2 (LOD)	(*50*)
8-11, 13	Na	Na	Water	Centrifuga tion	SPE: C18	Methanol	LC-ESI-MS/MS	40 / 25	(*52*)
4-8	3,4,6, 8	Na	Water	Nr	SPE styrene-divinylbenzene copolymer)	Methanol	LC-conductivity	100 / ~50 µg/L (LOD)	(*53*)
7,8,10	Na	Na	Aqueous	Na	SPME (PDMS), derivatisation using TBA	Na	GC-NCI-MS	5 / 20	(*54*)

Na, not applicable; Nr, not reported

[a] Sample intake in mL and LOQ in ng/mL unless otherwise specified

Short chain PFCAs (C4-C6) were only efficiently trapped by an Oasis-WAX column. The recoveries for the long chain PFCAs acids (≥ C11), PFOSA, NEtFOSA and the 7:1 and 10:1 FTOH were <70% (spiked water sample) on both column types (*31*). Losses due to evaporation during analysis, and adsorption to the PP sample container surface as discussed earlier were suggested causes for the lower recoveries. Yamashita *et al.* developed a very sensitive SPE method (pg/L level) for 7 PFCs based on Oasis HLB SPE for seawater samples (*18*). In order to improve the sensitivity, they extensively identified and eliminated blank contributions from filters, chemicals, sample vials and septa, resulting in detection limits of 0.4-5.2 pg/L (1000 ml sample intake). Yet very sensitive, the method was somewhat less accurate than the above method by Taniyashu *et al.*. Recoveries (spiked HPLC-grade water) were good for PFNA, PFBS, PFHxS and PFOS, but high for PFOA (147%) and 6:2 FTS (137%) and low for PFOSA (61%). Gonzalez-Barreiro *et al.* developed a liquid-liquid extraction (LLE) method for extraction of 7 PFCAs (C6-C12), PFOS, PFDS, PFOSA and NEtFOSA from tap water and waste water (*28*). They have extracted 900 mL water samples within the PP sample bottle, allowing for total-water extraction (water soluble and particle associated PFCs). This method may furthermore be capable for extraction of PFCs that may have adsorbed on the sample bottle interior during transportation and storage. They evaluated the extraction solvents *n*-hexane, MTBE and trichloromethane. Trichloromethane and *n*-hexane yielded very low recoveries except for PFDS and PFOSA. MTBE was the solvent of choice and the extraction accuracies of all compounds was improved by addition of sodium chloride (NaCl, 50 g/L) and acidification of the sample to pH=4 (sulfuric acid). Recoveries (based on spikes to waste water and tap water) were ca 40-70% for PFHxA and PFHpA, whereas for the other compounds (C8-12 PFCAs; PFOS, PFDS, PFOSA and NEtFOSA), recoveries were approx. 70-100%, all obtained with good precision. Compared to the performance of Oasis-HLB or WAX columns (*31*), the method was slightly less efficient in trapping short chain PFCAs but longer chains (C11-12) were better trapped. LLE with acetonitrile or MTBE was employed by Szostek *et al.* for the extraction of FTOHs (*37*). After extraction, the water-acetonitrile extract or the MTBE extract was transferred to the LC-vial for analysis. The MTBE extract was mixed 1:1 (v/v) with MeOH-water (1:1 v/v) to obtain a single phase suitable for injection in the LC-system. Seawater was best extracted with MTBE, as acetonitrile resulted in problems with chromatography and detection due to matrix effects. Using this simple method, detection limits on a single-quad MS system were ca 1 ng/mL, being approx. 10 times less sensitive compared to a triple-quad MS system. Recoveries in spiked water samples were good (71-120%) and precision was generally less than 6%, showing the suitability of this method for analysis of FTOHs. Takino *et al.* (*48*) used an automated on-line extraction technique using turbulent flow chromatography (TFC) for the determination of PFOS in river water. PFOS was trapped on the TFC column and subsequently

backflushed in the LC-atmospheric pressure photo ionization (APPI)-MS. With a sample intake of only 1 ml river water, they achieved an LOQ of 18 ng/L (recovery of spiked river waters 94-97%), making the method a rapid technique for analysis when no pg/L sensitivity is required. Schultz *et al.* recently reported on a large volume injection (LVI) method for the analysis of 11 PFCs in municipal wastewaters (*51*). Using a PEEK tubing loop, they loaded 500 µl sample on a C18 guard cartridge. By switching to the methanol – aqueous NH_4Ac mobile phase, the compounds were eluted onto a C18 analytical column and separated. The LOQ of this simple and rapid method was good (0.5 ng/L). Recoveries (based on spiked influent and effluent) were good (82-100%) for PFBS to PFDS, PFHxA to PFDA, 6:2 FTS and PFOSA. Whether or not this method would be suitable for short chain PFCAs was not reported.
Although many papers report on the SPE enrichment of the samples after transportation of the water sample to the laboratory, enrichment can already take place in the field. Scott *et al.* employed a large volume sampler based on an XAD-7 resin, being capable of enriching ca 35 liters of Lake Superior water (*43*). After derivatisation of the PFCAs with 2,4-difluoroanilide derivate, the derivates were analysed by GC-MS. Doing so, he obtained detection limits for PFOA of as low as <0.01 ng/l (recoveries 105-140%). They also reported on another method, which was capable of analyzing very short chain PFCAs (trifluoroacetic acid, C2, to C9) by extracting 1 liter of water sample, derivatisation and detection as mentioned above.

Whole blood, serum, plasma and milk

Typical PFCs concentrations found in human blood matrices are in the range of 0.01-100 ng/mL, with PFOS in the highest concentrations observed (*22,30,55,56*). A selection of methods applied can be found in Figure 2.3 and Table 2.7. Kubwabo et al. (*15*) and Sottani and Minoia (*14*) applied LLE in combination with ion-pairing agent of Hansen *et al.* (*11*) for the extraction PFOA, PFOS and PFOSA from the serum of (non)-occupationally exposed people. Although the sensitivity is not high (3.6-10 ng/ml at a 1 ml sample intake), it was sufficient for detection of PFOS and PFOA in most samples. Yet labor intensive, this method has a certain advantage of a very simple extraction and clean-up not requiring any sample pre-treatment such as protein precipitation. LLE can also be used for extraction of FTOHs from plasma. Szostek and Pricket extracted 8:2 FTOH from rat plasma with MTBE (*57*). The extract was analysed without further clean-up. Recoveries were 86-113% and the LOD was estimated at 5 ng/ml.
When using SPE extraction, most of the until now reported methods require sample pre-treatment to prevent clogging of columns or for removal of e.g. proteins, which was discussed earlier in this paper. Karrman *et al.* developed a SPE/LC-MS method for PFCs in whole blood (*45*). They tested ten SPE sorbent materials ranging from non-polar (C18) to medium polar (phenyl). Acceptable to good extraction efficiencies (69-112%) were obtained on C18

SPE columns for PFCAs (C6-14), PFHxS, PFOS, PFDS and PFOSA, whereas recoveries for PFBS were <30% (*45*). This method did not suffer from matrix effects, which emphasises the potential of this simple method. Holm *et al.* reported on a LVI-capillary column-LC-ESI-MS/MS for determination of PFOS and PFOA in plasma (*21*). The sample is enriched on-line on a Kromasil C18 packed capillary column and back-flush eluted to the analytical column. They obtained sub-ng/ml LOQs at a 250 µl sample intake level. The recovery was estimated at ca 75%. Similar results were obtained by Inoue *et al.* using a column switching method for PFOS, PFOA and PFOSA in human plasma (recoveries 82-99%) (*17*). Kuklenyik *et al.* (*46*) developed an automated high throughput SPE method for serum and milk, capable of pre-treatment of 100 samples per 4 hours. They used Oasis-HLB columns for retention of a wide range of compounds and found that addition of formic acid (3 ml, 0.1 M) to the original sample was required for retention of the PFCs on the column. By washing the concentrated sample on the column with 3 mL 0.1M formic acid/methanol (1:1), they got rid of matrix components such as proteins. Thorough washing of the SPE column after sample application resulted in considerable losses of short chain PFCAs (C5: 20% and C6: 60% recovery) as well as long chains (C10: 70%, C11: 72% and C12: 30% recovery) due to insufficient binding to the SPE column. The precision of the complete method was 10-29% (n=30). The same group later reported on an improved and automated method for serum analysis, based on-line SPE-LC-negative ion turbo ion spray-MS/MS (*22*). The improvements included a wider range of compounds, better recoveries for short and longer chain PFCAs (improved yields of 75-114%) and improved precision (75% of the CVs were ≤15%). Although they used C18 SPE, they were able to efficiently trap the short chain compounds, contrary to the earlier discussed methods (see also the water section). Possibly, the addition of 0.1M formic acid to the matrix has improved the retention of the compounds on the non-polar SPE column. Furthermore, the improved recoveries for the short chain PFCAs and the N-substituted sulfonamides was obtained by elimination of the solvent evaporation step. This method stands out from before mentioned methods for it's 10-fold better LOD (0.05-0.8 ng/ml), while consuming only 100 µl serum. The required sample pre-treatment was sonication with 0.1M formic acid.

The results of the first international interlaboratory study on PFCs in human samples showed a good comparibility of the different methods applied by the participants as 61-73% of the participants had satisfactory z-scores for PFOS and PFOA in blood and plasma (*38*). This shows that the methods for these matrices generally include accurate extraction and clean-up of samples.

Table 2.7 Typical extraction and clean-up techniques for whole blood, serum, plasma and milk.

PFCA	PFSA	Other	Sample Type	Pre-treatment	Extraction and clean-up	SPE elution solvent	Instru-mental determi-nation	Sample intake (mL) / LOQ (ng/mL)[a]	Ref.
8	8	PFOSA	Serum	None	IPE (MTBE), filtration	na	LC-ESI - MS/MS	1 / 3.6-4.8	(*15*)
8	na	na	Serum	None	IPE (MTBE)	na	LC-APCI-MS/MS	1 / 25	(*14*)
8	8	PFOSA	Plasma	Acetonitrile PP[b], centrifugation	On-line SPE	water/methanol (90/10, v/v)	LC-ESI - MS/MS	0.3 / 0.5-1	(*17*)
8	na	APFO	Serum, plasma	Acetonitrile PP on PP columns in a 96 wells design	n.a.	na	direct injection, LC-ESI-MS/MS	0.05 / 0.5	(*20*)
8	8	na	Plasma	Acetonitrile PP, 1:1 dilution	On-line SPE	ACN/H_2O (10 mM NH_4Ac) gradient	LC-ESI-ion trap MS	0.25 / 0.2-0.5 (LOD)	(*21*)
5-12	6,8	PFOSA, NMeFOSA, -AcOH[c], NEtPFOSA, and – AcOH[d], NMeFOSE, NEtPFOSE	Serum	Sonication with 0.1M formic acid	On-line (column switching) SPE (C18)	0.2% NH_4OH:in water	LC-TIS[e]-MS/MS	0.1 / 0.05-0.8 (LOD)	(*22*)
8-12,14	4,6,9, 10	PFOSA	Whole blood	Formic acid, centrifugation	SPE (C18)	Methanol	LC-ESI-MS	0.5 / 0.4-3	(*45*)
5-12	6,8	PFOSA, NMeFOSA-AcOH, NEtFOSA-AcOH	Serum, milk	Sonication with 0.1M formic acid	SPE (Oasis HLB)	NH_4OH:ACN (1:99)	LC-TIS-MS/MS	1 / 0.1-1 (LOD)	(*46*)
na	na	8:2 FTOH	Rat plasma	None	LLE (MTBE), vortexing, centrifugation	na	GC-EI-MS	0.25 / 5 (LOD)	(*57*)
6-12, 14	na	na	Blood, plasma, serum	Acetonitrile PP, centrifugation	LSE (acetonitrile) and dispersive Envi-carb, centrifugation	na	LC-ESI-MS/MS	0.1 / 2 ppb	(*58*)

Na, not applicable; Nr, not recorded

[a] Sample intake in mL and LOQ presented (in ng/mL) unless otherwise specified

[b] Protein precipitation

[c] N-methyl perfluorooctane sulfonamido acetic acid

[d] N-ethyl perfluorooctane sulfonamido acetic acid

[e] Turbo Ion Spray

Sewage sludge, sediment, soil and suspended matter

Typical PFCs concentrations in sediments range from approx. 10 pg/g to the mid-ng/g range (*39,59-61*), whereas concentrations in sewage sludge may be much higher ranging from low ng/g to low µg/g range (*16,39*).

Figure 2.3 and Table 2.8 show a selection of methods developed for abiotic solid matrices. Powley *et al.* developed a virtually matrix-effect free LSE extraction and clean-up method for C6 to C14 PFCAs in soil, sediment and sludge matrices (*62*). The target compounds are extracted from the matrix by soaking the sample with water under basic conditions (200 mM NaOH). After addition of MeOH to the soaked sample, the extraction was performed by shaking for 30 minutes. The extract was afterwards neutralized by addition of HCl.

Table 2.8 Typical extraction and clean-up techniques for sediment, soil and sewage sludge.

PFCA	PFSA	Other	Sample type	Pre-treat-ment	Extraction	Clean-up	Instrumental determina-tion	Sample intake (g) / LOQ (ng/g)[a]	Ref
8-12, 14	6,8,10	PFOSAA[b], Me-FOSAA[c], Et-PFOSAA[d]	Sediment and sludge	Drying	2 acid washes and extractions	SPE C18, eluted with MeOH-acetic acid (1%)	LC-ESI-MS/MS	Sediment 1 / 0.01-0.25 Sludge 0.1 / 0.6-2.2	(*16*)
8	8	Various others	Sludge	Drying	PLE, Soxhlet and steam distillation, various solvent compositions	na	LC-APCI-MS or Flow injection analysis-MS	2 / 10-20 µg/g dw	(*19*)
Na	Na	8:2 FTOH	Soil	Na	LSE (Acetone or 20 mM NaOH (90/10 v/v) acetonitrile/ water)	Dispersive Envi-carb	LC-ESI-MS/MS	Nr	(*36*)
6-9	4,6,8, 10	PFOSA	Sediment, sludge	Drying	PLE (methanol)	Na	LC-ESI-TOF-MS	5 / 7-200	(*39*)
7,8,10, 12	Na	Na	Sediment	Drying, sieving	PLE (Acetone, MeOH 1:3), derivatisation (alkyl ester)	SPME (PDMS)	GC-NCI-MS	3 / 1.3-2.6	(*60*)
6-12, 14	Na	Na	Soil, sediment, sludge	Soaking with NaOH in water	LSE (methanol), HCl neutralisation	Dispersive Envi-carb, centrifugation	LC-ESI-MS/MS	5 / 1 ppb	(*62*)

Na, not applicable

Nr, not recorded

[a] Sample intake in g and LOQ presented (in ng/g) unless otherwise specified

[b] Perfluorooctane sulfonamido acetic acid

[c] N-methyl perfluorooctane sulfonamido acetic acid

[d] N-ethyl perfluorooctane sulfonamido acetic acid

After clean-up using active carbon, the extract was ready for analysis. Although extraction and clean-up involves several steps, the method is sensitive (1 ng/g) and method recoveries were good for all chain lengths (75-120%) and various tests showed that the method did not suffer from matrix effects. The method, initially developed for PFCAs, was later adapted for inclusion of the PFSAs and PFOSA within the framework of the EU funded PERFORCE project (www.science.uva.nl/perforce). A slightly different method was applied by Higgins et al. (*16*) for extraction of ionic and non-ionic PFCs from sediment and sludge. The sample was washed with acetic acid and subsequently extracted with a 90:10 (v/v) methanol-acetic acid (1%) mixture. Clean-up was performed with C18 SPE. They showed that 2-3 repetitive extractions were sufficient for obtaining all extractable PFCs (>95%) from the matrix. The method recoveries ranged from 73-98% (dry sediment) and 56-93% (reconstituted wet sediment) 41-91 (digested sludge) and 37-98% (primary sludge). The lowest recoveries were obtained for PFHxS and >C10 PFCAs. The authors indicated that analyte losses may have occurred due to inefficient extraction from the environmental solids, insufficient retention and/or elution during the SPE clean-up, and suppression of signal due to matrix effects during LC/MS/MS analysis (matrix effects were most pronounced for the long chain PFCAs). Although the method suffered from some uncontrolled accuracies, it was very sensitive (0.01-0.25 ng/g for sediment). A LSE method was also applied for extraction of 8:2 FTOH that was applied to soils in a partitioning experiment (*36*). They found extraction efficiencies up to 95% (of the applied 8:2 FTOH) when 10-24% acetone was added to the solvent mixture.

PLE has widely been employed for extraction (and clean-up) of classical POPs, metals, oils, natural toxins (*63-65*) and linear alkylbenzene sulfonates (LAS) and alkylphenol type surfactants (*65*). The benefits of PLE are automation, short extraction times, high throughput and possibilities for efficiently combining extraction and clean-up within the extraction cell (*65,66*). Despite it's potential, PLE was only used in limited number of PFCs studies (*19,60,67*). A wider application of PLE is hindered by the considerable amounts of PTFE tubing in the instrument, resulting in unacceptable blank contributions for several PFCAs. PLE can therefore only be applied to highly contaminated samples unless the PFCA contribution originating from the PTFE tubing is dramatically reduced (e.g. by replacement of the tubing by stainless steel). Schröder investigated the efficiency of Soxhlet extraction, PLE and steam distillation in combination with a wide range of extraction solvents for the extraction of PFOS and PFOA from sewage sludge (*19*). He found that sequential application of solvent mixtures (PLE with ethylacetate/ dimethylformamide and methanol/phosphoric acid) was essential for the exhaustive extraction of the sludge samples. With slight changes in the extraction parameters, large variance was observed in the recoveries (17-319%), indication that the method was not robust yet. Alzaga et al. (*60*)

analysed PFCAs (C7, 8, 10 and 12) in harbour sediments by extraction of the acids using PLE (acetone-methanol 1:3 mixture), subsequent derivatisation to alkylesters, concentration of the volatile derivatives on an SPME fibre and analysis by GC-NCI-MS. Extraction efficiencies increased with increasing chain length (C7 to C10, ca 70 to 100%) and the optimum temperature was found at 100°C. The extraction solvent mixture may not be suitable for the very short chain PFCAs. It is, however, not likely that these polar short chains sorb to sediments to a large degree. Procedural blanks were reported to be below the method LOQ (0.5-0.8 ng/g). A drawback of this method is that many analytical steps are required, especially after the extraction, making the method rather laborious. Kallenborn *et al.* (*39*) applied PLE to sewage sludge and sediment samples using methanol (3 cycles, 17 min/cycle, 150°C, 2000 psi). The extracts were analysed without further purification. The LOQ was highest for PFOA (200 ng/g), based on 5 times the highest PFOA concentration in a field blank. The possible source for that high background was not recorded, but may be associated with contamination from PTFE parts in the PLE instrument.
Higgins and Luthy investigated the sorption of anionic PFCs to sediments (*68*). They found that adsorption increases with decreasing solution pH and increasing Ca^{2+} cation concentration. Furthermore, sorption was positively correlated with organic carbon content. Lui and Lee also found indications that sorption of 8:2 FTOH was related to the organic carbon content in soil (through hydrophobic interactions) (*36*). In a sediment-solvent system, spiked 8:2 FTOH sorbed to the soil (irreversibly), increasing with time. Less than 50% of the FTOH could be recovered after a 72 hour period. These studies show that hydrophobic interactions with organic carbon and electrostatic interactions should not be neglected when developing exhaustive extraction procedures for soils, sediments and sludges.

Biota
The IPE method of Hansen *et al.*(*29*) is widely applied in the past. The method (see Table 2.9) is based on ion-pairing of the target compounds with TBA and subsequent extraction with MTBE (3 times for exhaustive extraction). A simple filtration step was included prior to LC-ESI-MS/MS injection to remove solids from the extract. This method has been applied for the extraction of a wide range of biota (*8,9,69*), such as seal blubber, fish liver and polar bear. However, this method has shown to have some disadvantages, such as (i) co-extraction of lipids and other (disturbing) matrix constituents and the absence of a clean-up step to overcome the effects of matrix compounds and (ii) the wide variety of recoveries observed, typically ranging from <50% to >200%. The latter is believed to be related to matrix effects mentioned above.
To avoid excessive sample handling, Berger and Haukas developed a straightforward and time-efficient screening technique based on extraction of the PFCs by the HPLC eluent (methanol/water 50:50; 2 mM ammonium

acetate). Vortex mixing and sonication for 30 minutes (*70*). After filtration, the extracts were analysed by LC-ESI-TOF-MS. The results were comparable to those of the IPE method. Drawbacks of the method are the limited applicability for less polar PFCs (such as PFDcS, PFUnA to PFTeA and PFOSA), which were not efficiently extracted by the polar solvent mixture, resulting in recoveries of <50%. Furthermore, considerable matrix-effects were observed (by means of electrospray ionisation suppression or enhancement), but this was circumvented by matrix matched calibration. Powley *et al.* adapted their before mentioned soil, sludge and sediment method (*62*) for the extraction of biota (whole blood, plasma, serum, liver and plant tissue samples). Basically, the method was simplified as the NaOH treatment and HCl neutralizing step were left out. Recoveries were in the 80-110% range (*58*). Tittlemier *et al.* reported upon an analytical method for the analysis of PFOSA, N-EtPFOSA and N,N-Et_2PFOSA in fish and mammal liver samples (*71*). These lipid-soluble non-ionic PFCs can be extracted with similar methods as applied

Table 2.9 Typical extraction and clean-up techniques for biological tissues.

PFCA	PFSA	Other	Sample Type	Pre-treat-ment	Extraction and clean-up	Clean-up	Instrumental determina-tion	Sample intake (g) / LOQ (ng/g)[a]	Ref
8	6,8	PFOSA	Rabbit and rat liver	1:5 mixing with Milli-Q	IPE (with TBA, MTBE extraction)	Na	LC-ESI-MS/MS	1 / 1-9	(*29*)
Na	Na	8:2 FTOH	Rat liver, kidney and adipose tissue	Na	LSE (hexane)	Silica column	GC-EI-MS	0.5 / 4-12 (LOD)	(*57*)
6-12, 14	Na	Na	Liver, plant tissue	Na	LSE (methanol)	Dispersive Envi-carb, centrifugation	LC-ESI-MS/MS	1 / 2-5 ppb	(*58*)
6-13	4,6,8, 10	PFOSA, 6:2 FTS	Fish and bird liver	Na	LSE (1:1 methanol: water (2mM NH_4OAc)	Filtration	LC-ESI-TOF-MS	1 / 0.04-10 (LOD)	(*70*)
Na	Na	PFOSA, *N*-EtPFOSA, *N,N*-Et_2PFOSA[b]	Fast food, fish fillet and marine mammal liver tissue	Na	Soxhlet extraction (hexane-acetone 2:1)	H_2SO_4 washing, silica column (elution with dichloro-methane)	GC-PCI-MS (He)	10 / 0.33-0.83	(*71*)
8-12	6,8	Na	Fish, fish liver	Na	IPE (with TBA, MTBE extraction)	Silica column (elution with acetone)	LC-ESI-ion trapMS(/MS)	10 / 1-6	(*72*)

Na, not applicable

[a] Sample intake in g and LOQ presented (in ng/g) unless otherwise specified

[b] N,N-diethyl perfluorooctane sulfonamide

for the classical POPs. The authors applied Soxhlet extraction with a hexane-acetone solvent mixture (2:1 (v/v)). The crude extract was dried over sodium sulfate (*71*), prior to further clean-up and detection with GC-PCI-MS. Average recoveries for the 3 target compounds were 83-89%, which was better than the results they obtained by the IPE method. Szostek *et al.* developed a simple determination of 8:2 FTOH for analysis of rat tissues and plasma. 8:2 FTOH was extracted from biological tissues with hexane and perchloric acid, followed by silica column clean-up. Recoveries obtained at different spiking concentrations were 90-102% for plasma and 63-113% for tissues. LODs were 4-12 ng/g for tissues and 5 ng/ml for plasma. Polytron extraction should be avoided to prevent losses of analyte (*57*).

Air

The aerial transportation of volatile PFCs may play an important role in the translocation of these chemicals to e.g. the Arctic. Several studies have been dedicated to the atmospheric chemistry of volatile PFCs (*3,73-75*) in order to investigate this route of environmental exposure. Furthermore, knowledge on the PFCs levels in (indoor) air is of importance for human exposure studies.

Two recent studies showed that FTOHs preferentially partitioning in the gaseous phase, whereas FOSE/A compounds can be found in the particulate phase to a much larger extent (up to 90%) (*76,77*). The analytical chemistry discussion here will be limited to gaseous air phase. Sampling is mostly based on flow through large volume samplers. Typical PFC concentrations in air are in the low-mid pg/m^3 range. The sorbents in the flow through samplers retaining the volatile PFCs are XAD resin sandwiched between polyurethane (PUF) plugs (*76,78,79*) or just PUF plugs (*80*). Jahnke *et al.* extensively validated the performance of their sampling and analytical procedures (*76*). Breakthrough experiments showed that the very volatile FTOHs were not completely trapped in their PUF/XAD train, with largest losses encountered for 4:2 FTOH. A variety of recovery tests were performed, showing that recoveries were around 100% for 6:2 perfluorooctyl acrylate, 6:2-10:2 FTOHs and FOSAs, whereas losses were found for 4:2 FTOH and PFOSA. Considerable recoveries (up to 300%) were found for the FOSEs. These elevated recoveries were recently also found in another study (*77*). Jahnke *et al.* (*81*) evaluated sampling by the use of SPE cartridges (Isolute Env+) in a flow-through set-up. No break-through was found for all compounds, but recoveries were more variable that in the PUF/XAD setup (*76*). Shoeib *et al.* evaluated the use of passive air samplers (*82*). Although this set-up is simple to handle and operate in the field, the parallel operation of flow-through samplers was required to calibrate the passive air samplers.

Extraction of the XAD resins and/or PUF plugs is done by (a combination of) medium polar organic solvents (see Table 2.10) like methanol, petroleumether and ethylacetate. An Env+ SPE column was eluted with ethylacetate. After (purification,) simple filtration and concentration steps, the final extracts were

analysed by GC-MS with EI, NCI and PCI ionisation. Prior to the sampling, the XAD resins and PUF plugs require thorough precleaning. Several procedures have been applied, including washes with ultrapure water and NaOH and multiple day Soxhlet extractions with different organic solvents (*78-80*).
Particulate matter in flow through samplers is mostly collected by quartz or glass fiber filters (GFF). Air particles can be analysed in the same way as solid samples (e.g. extraction with methanol (*83*) or ethylacetate (*76*)). Barton *et al.* sampled air next to a manufacturing facility using a high volume cascade impactor which fractionates the particulate matter in different particle sizes. Using this device, they determined that approx. 60% of the PFOA was associated with the <0.28 µm particulate size (*84*).

Table 2.10 Typical sampling, extraction and clean-up techniques for air.

FTOHs	Other	Sampling (extraction of air)	Extraction from the sampler and clean-up	Instrumental determination	Sample intake (m^3) / LOQ (pg/m^3) [a]	Ref.
4:2, 6:2, 8:2, 10:2	NEtFOSA/E, NMeFOSA/E, PFOSA, 6:2 PFOAc[b]	PUF, XAD-2 and glass fiber filters	Ethylacetate	GC-PCI-MS	1160 / 0.2-2.5	(*76*)
4:2, 6:2, 8:2, 10:2	NEtFOSE, NMeFOSE, NMeFOSEA[c]	PUF, XAD and glass fiber filters	Petroleumether-acetone 50/50%, alumina column clean-up	GC-PCI-MS	300, 0.3-3.5 (LOD)	(*77*)
4:2, 6:2, 8:2, 10:2	NEtFOSA/E, NMeFOSE, PFOSF[d]	PUF, XAD and quartz fiber filters	Methanol, ethylacetate Glass wool filtration	GC-NCI/PCI-MS	600-850 / 0.15-6.2	(*78*)
6:2, 8:2, 10:2	NEtFOSA/E, NMeFOSE,	PUF, XAD and quartz fiber filters	Nylon filtration	GC-NCI/PCI-MS	500-1600 / 2-14	(*79*)
Na	NEtFOSE, NMeFOSE, NMeFOSEA	PUF plugs and glass fiber filters	Soxhlet extraction (petroleumether), filtration	GC-EI/NCI-MS	100-600 / <0.3-20	(*80*)
4:2, 6:2, 8:2, 10:2	10:2 FT-olefin[e], NEtFOSA/E, NMeFOSA/E	Isolute Env+ SPE	Elution with ethylacetate	GC-PCI/ NCI-MS/MS	50 / 3-189 (LOD)	(*81*)
Na	NEtFOSA, NMeFOSA/E, NMe-FOSEA	Passive sampler (PUF disk)	Soxhlet extraction (petroleumether), filtration	GC-EI/NCI-MS	Na	(*82*)

Na, not applicable
[a] Sample intake in m^3 and LOQ presented (in pg/m^3) unless otherwise specified
[b] 1H,1H,2H,2H-perfluoro octyl acrylate
[c] N-methylperfluorooctane sulfonamido ethylacrylate
[d] perfluorosulfonylfluoride
[e] 1H,1H,2H,2H-perfluoro-1-dodecene

Clean-up strategies

For various sample types (e.g. fish liver, lipid rich samples, sediments, sewage sludge samples) extracts require further clean-up to remove co-extracted lipids and other matrix constituents. Without further clean-up, this may lead to enhancement or suppression of the electrospray ionization, resulting in inaccuracies (*70*). The first international interlaboratory study on PFCs in environmental matrices showed that these matrix effects may cause large inaccuracies (*38*).

Clean-up of water samples is generally performed by a washing step after sample enrichment on the SPE cartridge. Simcik *et al.* performed additional clean-up by fluorous silica column chromatography for purification of surface water extracts, prior to LC-ESI-MS (*85*). Flourous silica can selectively isolate PFCs from a matrix with potential interferences. After washing the column with 20% methanol in MTBE, they eluted the PFCs with tetrahydrofuran and methanol. With recoveries on 98% (spiked after clean-up), they showed that this method adequately removed interfering compounds.

Abiotic matrices (soil, sediment, sewage sludge) can be cleaned-up by addition of Envi-carb (graphitized carbon) and 50 µl glacial acetic acid (*58*). Higgins *et al.* cleaned their sediment extracts by C18-SPE. After loading the crude sample extract, the cartridge was washed with Milli-Q water and the target compounds were eluted with 4 mL MeOH.

As a final clean-up step, extracts may be filtrated over e.g. nylon filters to remove solids from the final extract (see Figure 2.3), but care should be taken to avoid PFC losses or contamination of the sample extract. Yamashita *et al.* tested several nylon filter types used for removal of solids from the final extract and found that some filters contained trace amounts of PFOS and PFOA (*86*). A simple methanol washing step reduced the filter-originating PFOS and PFOA to <LOQ. A nylon syringe filter is commonly applied for water filtration (*18,31*).

The co-extraction of lipids from biological matrices can be reduced by the use of medium polar extraction solvents such as methanol and acetonitrile (*58,70*). Powley *et al.* developed a clean-up strategy for biological matrices (blood, plasma, serum, liver and plant tissue) by purification of the crude extract with 25 mg Envi-carb and 50 µl glacial acetic acid (*58*). The IPE extraction method co-extracts lipids from biological matrices. Strategies for removal of these lipids include sulfuric acid washing and subsequent silica-column chromatography (combination of acidic and neutral silica, and elution with dichloromethane), as demonstrated by Tittlemier *et al.* (*71*). Van Leeuwen *et al.* developed a direct silica column clean-up for lipid rich fish samples such as herring and eel. They eluted the lipids with dichloromethane, while the target compounds (PFCAs and PFSAs) were eluted with acetone. PFOSA was not recovered using this method as it co-eluted with the lipids. Silica column chromatography was also used for clean-up of other matrices such as of rat liver and kidney tissues (*57*) and sample extracts (after derivatisation of PFCAs to difluoroaniline derivates) (*43,87*). Finally, proteins

and lipids can be destroyed by KOH digestion of the biota sample prior to SPE sample enrichment (*31,88*).
For air samples, clean-up of extracts is not commonly applied, although a recent study indicated that matrix effects may play a role in the instrumental determination of FOSA/Es (*81*). Shoeib *et al.* performed an alumina column clean-up for their air sample extracts for improvement of the chromatograms.
During sample manipulation care should be taken to avoid losses of sulfonamides (*22*) and TH-PFOS (*46*), FTOHs (*57,76*) and short chain PFCAs (*46*) when extracts are concentrated by evaporation. Losses may be avoided by using a keeper solvent and by not blowing the extract down to dryness.

Instrumental determination by LC-MS(/MS) or GC-MS(/MS)

Most studies focus on the analysis of PFCAs, PFSAs and PFOSA and employed LC-MS/MS for final determination. LC-ESI-MS(/MS) combined with a selective extraction and clean-up provides a sensitive and selective method for detection of PFCAs and PFSAs. Furthermore, LC-ESI-MS(/MS) can also be employed for detection of PFOSA, N-EtFOSA, N-MeFOSA, N-EtFOSE, N-MeFOSE and the FTOHs. Therefore, broad multi-PFC detection methods can be developed using LC-ESI-MS(/MS) detection. Some disadvantages of LC-ESI-MS(/MS) are (i) electrospray ionization enhancement or suppression (matrix effects) (*25*) and (ii) poor ionization yields for non-ionic FOSA/FOSE type PFCs, reducing the sensitivity when detected by ESI-MS (*25*). The matrix effects can be overcome by using a selective clean-up step that removes the interferences. Furthermore, the use of mass labeled standards corrects for matrix effects. A mass labeled internal standard should be used for every compound of interest (*89*). The chromatography is mostly performed on reversed phase (C18) columns (*23*), although some studies used a fluorinated reversed phase column for additional selectivity (*19*). Because of sample interferences (e.g. in liver) (*25*), the MS detection should also be selective. Therefore, most laboratories employ triple quadrupole MS/MS, time-of-flight (TOF) MS or ion trap MS(/MS). Several transitions have been reported and allow for selective MS/MS (*24*). But even then, interferences may occur as was recently reported by (*90*). They reporte taurodeoxycholate isomers as interference on the 499→80 transition (for PFOS) and pregnancy hormones interfere on the PFHxS transitions (5-pregnan-3,20-diol-3-sulfate interferes on the 399→80 transition and the natural isotope 34S-3-hydroxy-5-pregnan-20-one sulfate on the 399→99 transition). For these reasons, care should be taken to minimize the influence of interferences. Single quadrupole MS should not be used unless it has been ensured that no interferences occur, or their effect is minimised. Generally, the sum of linear and branched PFOS isomers is determined and reported. However, isomer separation is feasible and with the recent availability of the individual PFOS isomers (www.well-labs.com), it is now possible to quantitatively determine isomer profiles (*90*).

GC-MS in combination with positive or negative chemical ionization (PCI, NCI) has limited applicability for PFC analysis. It is mainly used for sensitive analysis of the non-ionic (volatile) PFCs like PFOSA and N-ethyl-FOSA, N-methyl-FOSA, N-ethyl-FOSE, N-methyl-FOSE and the 6:2, 8:2 and 10:2 FTOHs (*57,71,78*). Detection of PFCAs is also feasible, but only after derivatisating to methylesters, butylesters or 2,4-difluoroanilides (recently reviewed by De Voogt and Saez (*23*)). Derivatisation techniques improve the selectivity of the analytical method, thereby reducing disturbing matrix effects. Selectivity is further improved with the application of CI-MS detection. With GC it is also possible to separate branched PFCA isomers (*87*), but no standards are available for quantitative determination. PFSAs cannot be analysed by GC-MS as it is difficult to create sufficiently stable PFSA derivates, suitable for GC analysis.

Conclusions

In less than a decade of PFC research, a wide variety of methods have been developed that enable the extraction and clean-up of all relevant environmental matrices. Solid phase extraction (SPE) is the method of choice for liquid samples (e.g. water, blood, serum, plasma), and may be automated in an on-line set-up for (large volume) sample enrichment and sample clean-up. Prior to SPE, sample pre-treatment (filtration or centrifugation for water or protein precipitation for blood) may be required. Liquid-liquid extraction can also be used for liquid sample extraction (and does not require above mentioned sample pretreatment). Solid-liquid extraction is the commonly applied method for solid matrices (biota, sludge, soil, sediment), but automation options are limited due to contamination from polytetrafluorethylene tubings and parts applied in extraction equipment. Air is generally preconcentrated on XAD-resins sandwiched between polyurethane foam plugs. Clean-up of crude extracts is essential for destruction and removal of lipids and other co-extractives that may interfere in the instrumental determination. SPE, (fluorous) silica column chromatography, dispersive graphitized carbon and destructive methods such as sulphuric acid or KOH treatment can be applied for clean-up of extracts. Care should be taken to avoid contamination (e.g. from sample bottles, filters, equipment) and losses of PFCAs (e.g. adsorption, volatilization) during sampling, extraction and clean-up. Storage at -20°C is generally appropriate for conservation of samples.

Although many methods are currently already available, it is not yet conclusive which method performs best in a specific matrix. In many cases, methods can be developed further to improve accuracy (i.e. reduce matrix interferences, reduce losses of target analytes) and to enlarge the scope (i.e. number of target analytes and sample types). Further automation of methods will improve the applicability in routine laboratories.

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Chapter 3

Method development

3.1 Validation of bioanalytical and chemical screening methods for dioxins and dioxin-like PCBs[4]

Abstract

The European research project DIFFERENCE focused on the development, optimisation and validation of screening methods for dioxin analysis, including bio-analytical and chemical techniques (Chemical Activated Luciferase Gene Expression (CALUX); GC- Low Resolution Mass Spectrometry (GC-LRMS/MS) and comprehensive multi-dimensional GC with Electron Capture Detection (GCxGC-ECD) and on the optimisation and validation of new extraction and clean-up procedures. The performance of these techniques was assessed in an international validation study and the results were compared with the reference technique GC- high resolution (HR)MS. This study was set up in three rounds and was in accordance with the International Harmonized Protocol for Proficiency Studies and the ISO 5725 standard. The results are very promising, in particular for GC-LRMS/MS. The results obtained with this technique were as accurate and precise as the results reported by the labs using GC-HRMS. A major advantage of the MS techniques (over GCxGC-ECD and CALUX) is the use of ^{13}C-labeled analogues as internal standards. The initial results reported for GCxGC-ECD overestimate the dioxin concentrations in the samples (quality control spiked vegetable oil (QC-oil), milk and fish oil), probably due to insufficiently low quantification limits for dioxins combined with reporting upperbound values. GCxGC-ECD z-scores of a herring tissue sample were well below $\leq |21|$. The results reported by the labs using the CALUX technique underestimate the total TEQ concentrations in a spiked vegetable oil sample, but CALUX overestimated results in milk, fish oil and herring samples. Application of a recovery correction improved the accuracy, but a considerable overestimation remained. The repeatability of the CALUX is significantly worse than that of the other screening techniques.

It was shown that Accelerated Solvent Extraction (ASE) is a valid alternative extraction and clean-up procedure for fish oil and vegetable oil. The results obtained with CALUX and GC-HRMS after ASE were equivalent to those obtained with classical extraction and purification procedures. GC-HRMS results of a herring tissue extracted with ASE showed a considerable

[4] Based on J. van Loco, S.P.J. van Leeuwen, P. Roos, S. Carbonnelle, J. de Boer, L. Goeyens & H. Beernaert (2004) The international validation of bio- and chemical- analytical screening methods for dioxins and dioxin-like PCBs: the DIFFERENCE project rounds 1 and 2. *Talanta*, 63, 1169-1182 and additional data from the DIFFERENCE final project report (round 3).

underestimation (z=-3.1). More research is needed to find the possible causes of this underestimation.

Introduction

The European research project DIFFERENCE (Dioxins in Food and Feed Reference Methods and New Certified Reference Materials) aimed at the development, optimisation and validation of screening methods for dioxin analysis in food and feed, including bio-analytical and chemical techniques (Chemical Activated Luciferase Gene Expression (CALUX); GC- Low Resolution Mass Spectrometry (GC-LRMS/MS) and multi-dimensional GC with Electron Capture Detection (GCxGC-ECD). In addition, it aimed at the optimisation and validation of new extraction and clean-up procedures such as Accelerated Solvent Extraction (ASE) (also referred to as Pressurised Liquid Extraction (PLE)). Furthermore the project focussed on the feasibility testing of the production and certification of five high quality certified reference materials (CRMs) for dioxins, furans, indicator PCBs and dioxin-like PCBs (dl-PCBs) in food and animal feed.

The purpose of the validation protocol in the DIFFERENCE project was to ensure that the bio-analytical and chemical analytical screening methods for dioxins and dl-PCBs respond to the EU criteria. Screening methods are used to distinguish between compliant and non-compliant samples. The requirements for analytical methods for the official control of dioxins and dioxin-like PCBs in food and feeding stuffs are laid down in the EU commission regulation 1883/2006 and 152/2009 (*1,2*). The analytical procedures must have a high sensitivity, a low limit of detection and a high accuracy.

This international validation protocol, which is based on the International Harmonized Protocol for Proficiency Testing (*3*), provides information about the accuracy (trueness and precision), ruggedness, detection capability and selectivity of the biological and chemical analytical screening methods. This was carried out in three rounds. The first round focussed on the goodness-of-fit of the calibration curve and on the accuracy of the methods. In round 2 the detection capability and selectivity were assessed. The robustness and the accuracy of the methods were evaluated in round 3.

Materials and methods

Validation protocol

The first round primarily focussed on the goodness-of-fit of the calibration curve and provided the first data concerning repeatability and reproducibility of the screening methods. The objective of the second round was to assess the detection capability and selectivity of the method. Information about the detection capability of the methods was obtained with the procedures

described in the ISO 11843-2 (*4*). Furthermore the accuracy of the results obtained with the methods applied could be investigated, because the exact amount added to the samples is known. Round three provided more data on the precision and robustness of the methods. The ISO 5725 "Accuracy (trueness and precision) of measurement methods and results" (*5*) was used as guidance to evaluate the accuracy of the bio and chemical analytical screening methods. In particular the repeatability, within-lab reproducibility and the reproducibility of the methods were assessed. The protocol is shown in detail in Table 3.1. The information obtained during the three rounds was used to gauge the ruggedness of the methods. During the whole validation process a quality control oil spiked with dioxins and dioxin-like PCBS (QC-oil) is used to assure the validity of the data.

Table 3.1 Validation protocol of bioanalytical and chemical screening methods. In brackets the number of runs and replicates per run are mentioned. The aims of the tests are mentioned per sample set.

Round 1	
Aim 1: goodness- of-fit Standard A (1x2) Standard B (1x2) Standard C (1x2) Standard D (1x2) Aim 2: between-round reproducibility Quality control oil 3 pg dioxin + 3 pg PCB TEQ/g (1x1)	Aim 3: repeatability and robustness Clean fish extract (1x2) Fish oil (3x2) Milk (3x2)
Round 2	
Aim 4: detection capability Blank vegetable oil (veg. oil) (4x1) Veg. oil + spike 0.2 pg dioxin TEQ/g and 0.2 pg PCB TEQ/g (4x1) Veg. oil + spike 0.75 pg dioxin TEQ/g and 0.75 pg PCB TEQ/g (4x1) Veg. oil + spike 1.5 pg dioxin TEQ/g and 1.5 pg PCB TEQ/g (4x1) Veg. oil + spike 3 pg dioxin TEQ/g and 3 pg PCB TEQ/g (4x1)* Veg. oil + spike 6 pg dioxin TEQ/g and 6 pg PCB TEQ/g (4x1)	Aim 5: selectivity Veg. oil + 3 pg dioxin TEQ/g + 3 pg PCB TEQ/g + PCB-spike (4x1) Veg. oil + 3 pg dioxin TEQ/g + 3 pg PCB TEQ/g + PCN spike (4x1) Veg. oil + 3 pg dioxin TEQ/g + 3 pg PCB TEQ/g + PCDE spike (4x1)
Round 3	
Aim 6: between-round reproducibility Quality control oil 3 pg dioxin + 3 pg PCB TEQ/g (1x1)	Aim 7: selectivity, repeatability, robustness Chicken tissue (1x2) Egg yolk (1x2) Sepiolithic Clay (1x2) Fish Tissue (1x2) Pork Tissue (3 x 2) Chicken compound feed (3 x 2)

** Sample result is also used for between-round reproducibility aim (QC-oil)*

Preparation of test materials

The materials that have been prepared for each round are mentioned in Table 3.2. The preparation is described in detail below for the materials 1-28. Materials 29-33 are nut further discussed as they fall outside the scope of this thesis. Detailed information can be found elsewhere (*6*).

Table 3.2 Materials used in round 1 (1 to 17), round 2 (18 to 26) and round 3 (27-33) for the evaluation of the screening techniques.

No.	Container	Material	Volume/ weight	Solvent
1	Ampoule	Blank solvent	1 ml	DMSO
2	Ampoule	Standard 2,3,7,8-TCDD: 0.04 ng-TEQ/ml	1 ml	DMSO
3	Ampoule	Standard 2,3,7,8-TCDD: 0.1 ng-TEQ/ml	1 ml	DMSO
4	Ampoule	Standard 2,3,7,8-TCDD: 0.4 ng-TEQ/ml	1 ml	DMSO
5	Ampoule	Standard 2,3,7,8-TCDD: 1.6 ng-TEQ/ml	1 ml	DMSO
6	Ampoule	Standard 2,3,7,8-TCDD: 6.25 ng-TEQ/ml	1 ml	DMSO
7	Ampoule	Blank solvent	1 ml	Nonane
8	Ampoule	Standard 2,3,7,8-TCDD: 0.1 ng-TEQ/ml	1 ml	Nonane
9	Ampoule	Standard 2,3,7,8-TCDD: 0.5 ng-TEQ/ml	1 ml	Nonane
10	Ampoule	Standard 2,3,7,8-TCDD: 5 ng-TEQ/ml	1 ml	Nonane
11	Ampoule	Standard 2,3,7,8-TCDD: 50 ng-TEQ/ml	1 ml	Nonane
12	Ampoule	Standard 2,3,7,8-TCDD: 100 ng-TEQ/ml	1 ml	Nonane
13	Ampoule	Standard 2,3,7,8-TCDD: 200 ng-TEQ/ml	1 ml	Nonane
14	Ampoule	Quality Control Oil (QC-Oil), 3 pg dioxin and 3 pg PCB-TEQ/g oil	5g	Veg. oil
15	Glass jar	Milk sample	250 ml	Na
16	Ampoule	Fish oil (herring, close to 4 pg dioxin-TEQ/g oil)	7 ml	Na
17	Ampoule	Clean fish extract of fatty fish (fat removed), equivalent of 5 g lipid intake	5ml	Pentane
18	Ampoule	Blank vegetable oil	5g	Veg. oil
19	Ampoule	Veg. oil + 0.2 pg dioxin- and 0.2 pg PCB-TEQ/g oil	5g	Veg. oil
20	Ampoule	Veg. oil + 0.75 pg dioxin- and 0.75 pg PCB-TEQ/g oil	5g	Veg. oil
21	Ampoule	Veg. oil + 1.5 pg dioxin- and 1.5 pg PCB-TEQ/g oil	5g	Veg. oil
22	Ampoule	Veg. oil + 3.0 pg dioxin- and 3.0 pg PCB-TEQ/g oil (QC-Oil)	5g	Veg. oil
23	Ampoule	Veg. oil + 6.0 pg dioxin- and 6.0 pg PCB-TEQ/g oil	5g	Veg. oil
24	Ampoule	Veg. oil (see mat. 22) + PCB-spike (200 ng/g oil)	5g	Veg. oil
25	Ampoule	Veg. oil (see mat. 22) + PCN-spike (10 ng/g oil)	5g	Veg. oil
26	Ampoule	Veg. oil (see mat. 22) + PCDE-spike (20 ng/g oil)	5g	Veg. oil
27	Ampoule	Veg. oil + 3.0 pg dioxin- and 3.0 pg PCB-TEQ/g oil (QC-Oil)	5g	Na
28	Tin	Herring tissue	70 g	Na
29	Tin	Chicken from RIKILT feed experiment	70g	Na
30	Glass jar	Feed additive (Sepiolitic clay)	100 g	Na
31	Glass jar	Egg yolk and white homogenate from RIKILT feed experiment	100g	Na
32	Tin	Pork tissue	70 g	Na
33	Plastic jar	Compound feed from RIKILT feed experiment	100 g	Na

All solvent and oil-based materials were ampouled. The amber coloured ampoules (Nederlandse Ampullen Fabriek, Nijmegen, The Netherlands) were used without prior cleaning, which has been demonstrated to be a safe approach for PCBs and other POPs for the QUASIMEME interlaboratory studies (*7*).
Material 1 is pure DMSO (Acros, Geel, Belgium). Materials 2 to 6 were produced by gravimetrical dilution of a standard of 2,3,7,8-TCDD (Cambridge Isotope Laboratories, Andover, MA, USA) with DMSO. Material 7 is pure nonane (Merck, Darmstadt, Germany). Materials 8 to 13 were produced by gravimetrical dilution of a standard of 2,3,7,8-TCDD (Wellington, Guelph, Ontario, Canada) with nonane. Material 14 is a vegetable oil (corn oil), which was purchased in a local super market in The Netherlands (Deka Markt, IJmuiden). Prior to spiking, the levels of dioxins and dl-PCBs have been determined in the oil by RIKILT – Institute for Food Safety, Wageningen, The Netherlands. The oil was spiked based with a profile of PCDD/Fs and dl-PCBs that is normally seen in North Sea in herring. A commercial mixture containing all WHO dioxins and furans was used and additionally 2,3,4,7,8-PeCDF, 1,2,3,4,6,7,8-HpCDF, OCDF, 1,2,3,4,6,7,8-HpCDD and OCDD (all obtained from Wellington Laboratories, Guelph, Ontario, Canada) were spiked to resemble the herring profile. The non-ortho PCBs (PCB 77, 81, 126 and 169) were all individually spiked and the mono-ortho PCBs were spiked using a standard solution, obtained from RIKILT (containing PCB 105, 114, 118, 123, 156, 157, 169 and 189), with additional spiking of PCB 105, 118 and 156 (Ultra Scientific, North Kingstown, RI, USA). The spiked milk sample (material 15) was produced by spiking dioxin and dl-PCB congeners to 20 L of sterilized whole milk, which had been purchased from a local supermarket in The Netherlands (Deka Markt, IJmuiden). The spiking-profile of the dioxins and dl-PCBs was obtained from Dutch raw milk monitoring data (RIKILT). All 17 WHO congeners were spiked at the level of interest using a standard solution containing all congeners (Wellington). Furthermore, the following individual congeners were added to approach the milk congener profile: 2,3,4,7,8-PeCDF, OCDF, 1,2,3,4,6,7,8-HpCDD, OCDD. The non-ortho PCBs were spiked from standard solutions of the individual congeners (obtained from RIKILT). The mono-ortho PCBs were spiked using a mixture of these PCBs (RIKILT standard solution). Furthermore, the indicator PCBs (PCB 28, 101, 118, 138, 153 and 180 (all obtained from Ultra Scientific, North Kingstown, RI, USA) were added to the milk for the homogeneity study. To enable quantification, the indicator PCBs were spiked at higher concentration. Due to this fact, PCB 118 had been added twice: once as a mono-ortho in the RIKILT standard solution and again as an indicator PCB at higher level. Therefore, the second addition resulted in a somewhat unbalanced mono-ortho PCB TEQ and a total TEQ with PCB 118 as the predominant congener (with a concentration of 4.7 pg TEQ/g lipid for PCB-118 on a total of 5.1 pg PCB TEQ/g lipid). The crude fish oil sample (material 16) was obtained as a remainder of a project on the upgrading

herring by-products (e.g. heads) (*8*). The herring was caught in May 2000, west of the Shetland Islands (60.50° N/03.00 W). The oil was filtered over 0.45 µm paper filter (Schleicher & Schuell, Dassel-Relliehausen, Germany) to remove solid particles and subsequently ampouled.
The clean fish extract (CFE) (material 17) was produced by extracting a pooled eel sample from various Dutch freshwater locations. After extraction, portions of 5 g lipid were cleaned over acidic silica columns (48 g silica per column). The solvent was evaporated and the residue was redissolved in n-heptane (Promochem, Wesel, Germany, Picograde purity). Twenty-five ampoules were produced containing 5 ml of clean fish extract (CFE) which is equivalent to 4 g of lipid. The blank vegetable oil (material 18) is of the same origin as material 14 but without dioxins and dl-PCBs spike. The spiked vegetable oils (materials 19-23) were prepared as material 14. Their spiking levels are given in Table 3.1. The materials 24-26 have also been prepared from material 14. An in-house standard solution of 29 PCBs (including the mono-ortho PCBs 105, 118 and 156) was used to spike to the required level of 200 ng/g oil (material 24). Material 25 was prepared by additional spiking of polychlorinated naphthalenes (PCNs) 27, 28, 36, 52, 54, 67, 68, 71, 53, 66, 73 and 74 (Wellington Laboratories) to a total level of 10 ng/g oil. Material 26 was prepared by spiking with a polychlorinated diphenyl ether (PCDE) standard solution (Cambridge Isotope Laboratories). PCDEs were reported as interferences in the GC-HRMS analysis (*9*) and show weak dioxin like response in bioassays (*10*). The standard contained native and ^{13}C-labeled mono-decaCDEs at a level of 20 ng/g oil (sum of all PCDEs). The preparation of the herring muscle material (material 32) was carried out by the Netherlands Institute for Fisheries Research (RIVO). Herring originating from the North Sea was filleted until ca. 5 kg of fillet remained. The fillets were frozen at –20 °C until further treatment. After thawing, the material was minced using a mincer (Finis Machinefabriek, Ulft, The Netherlands) in combination with a Fryma mill equipped with toothed rotary knives (Fryma Maschinen AG, Rheinfelden, Switzerland) to a final size of 3.5 mm^2. Subsequently, ca. 25 kg minced material was homogenised for three minutes, after adding 0.02% butylhydroxytoluene (BHT), in a Stephan cutter (Stephan Machines, Almelo, The Netherlands), type UMM/SK25 (made in 1979). Subsequently, coated tins (Eurocan Food, Mechelen, Belgium, volume ca. 75 ml) were filled to the brim with tissue homogenate (ca. 65 g) using a manual dosing machine (Machinenfabrik Engler, Vienna, Switzerland). The tins were sealed by a Lanico TVM 335 sealing machine (Thomassen and Drijver, Deventer, The Netherlands). The tins were sterilised in a Muvero-Mat sterilizer (type 90E) for 45 minutes at 122 °C (pressure 1.4 bar, heating-time: 90 minutes, cooling time: 20 minutes). The tins were stored at RIVO at room temperature.
A homogeneity test was carried out in the herring samples spiked milk sample and the fish oil sample in order to determine whether the materials are homogeneous (within a lot and between lot). The standard solutions, the

quality control oil (QC-oil) and the clean fish extract of round 1 and also the spiked vegetable oils of round 2 were assumed to be homogeneous in all the ampoules. The homogeneity testing was carried out according to guidelines of the Community Bureau of Reference (BCR) and International Standardisation Organisation (ISO) (*11*). For between-lot homogeneity testing, the indicator PCBs (CB 28, 52, 101, 118, 138, 153 and 180) were analysed in ten lots out of the complete batch. The within-lot homogeneity testing was carried out by 5 replicate analyses of the indicator PCBs in one lot. The reasoning behind using indicator PCBs for the homogeneity tests is based on the fact that at very low levels of PCDD/Fs it is likely that one would rather determine the within-laboratory method variance (typically 5-20% using isotope dilution), reflecting the competence of the laboratory to analyse at very low levels instead of the (in)homogeneity of the sample. A possible intrinsic heterogeneity will therefore possibly not be detected (*12*). However, at the concentration level of the PCBs, the within-laboratory method variance can be very low (<3-5%), which improves the potential of the method to detect heterogeneity in the material. Furthermore, it is expected that the PCDD/Fs will behave physically similar to PCBs and are therefore similarly distributed in the sample, also at lower levels, compared to PCBs. Moreover, the homogeneity study was based on a lipid intake of 250 mg, whereas typical lipid intakes for PCDD/F analysis are typically 20-fold higher (5-6 g), thereby reducing possible effects of inhomogeneity, which might have been detectable at very low sample intakes. The samples were analysed with GC-ECD (*13*). The analysis of the indicator PCBs consisted of Soxhlet extraction (n-hexane/ dichloromethane, 1:1) and subsequent removal of the lipids using alumina column chromatography. The organochlorine pesticides were removed from the extract by silica column fractionation prior to analysis by GC-ECD. The instrumental variance, tested by replicate analysis of a standard solution, was 0.6 - 2.7%.

According to the ANOVA approach used previously in similar studies (*14*), the coefficient of variation of the between-unit experiment (CV_b) represents a combined uncertainty; contributing factors are the variation of the measurements itself and the uncertainty due to the between-unit inhomogeneity of the material (u_{hom}). In the present study, the coefficient of variation of the within-unit experiment (CV_w) was considered to be the best available estimate of the variation of the measurements itself; it includes the complete analysis (including sample pretreatment and extraction) whereas the separate determination of the method variance could start only from the cleanup stage. The uncertainty contribution for inhomogeneity was thus quantified as:

$$u_{hom} = \sqrt{CV_b^2 - CV_w^2}$$

When CV_w is equal or larger than CV_b, u_{hom} cannot be assessed by the above formula. In such case an estimate for a maximum between-unit variability that could be masked by method variations is given, by establishing a kind of "upper detection limit for inhomogeneity" as follows (*14*).

$$u^{*}_{\text{hom}} = CV_w \cdot \sqrt[4]{\frac{2}{\nu_w}}$$

in which ν_w is the degrees of freedom for the determination of this coefficient of variation.

Furthermore, F tests were carried out in order to determine if variance of the within lot homogeneity results deviate significantly from the variance obtained from the between lot homogeneity test (at 95% level), which can show inhomogeneity of the material.

For both the milk sample and the herring oil sample the variances were not significantly different between the between-homogeneity samples and the within-homogeneity samples. The RSDs were all below 6%, except for the PCBs 101 and 138 in herring oil which showed a RSD from 7.2 - 20.2%. The reason for these elevated RSD values is not known. Table 3.3 shows a summary of the herring homogeneity tests. The herring tissue shows a very good homogeneity with a variance due to inhomogeneity (u_{inh}) below 6%. The CVs are rather low compared to the variance normally observed in interlaboratory studies for dioxins and dioxin-like PCBs. The F-test, carried out for all PCBs showed no significant difference between the within-unit and between-unit variance. It was therefore concluded that the materials were sufficiently homogeneous and suitable for the interlaboratory study. Finally, it should be noted that the homogeneity has been demonstrated at a sample intake corresponding to approx. 250 mg lipids. As the RSDs of the indicator PCBs were almost all below 6%, it is unlikely that possible inhomogeneity (at the higher sample intakes for dioxins) will contribute to the variance resulting from the interlaboratory studies.

Table 3.3 Summary of the herring homogeneity test results.

Material	**Tested PCB congeners**	**GC-ECD variance: 0.6-2.7 %**		
		u_{inh} (%)	$CV_{between}$ (%)	CV_{within} (%)
Herring tissue	52, 101, 118, 138, 153, 180	1.6-5.6	2.3-6.7	2.6-7.0

Proficiency testing scoring techniques

The results were evaluated according to the international harmonized protocol for proficiency testing of chemical analytical laboratories (*3*). It determines that for the quantitative results of the laboratories the z-scores are calculated according to the following equation:

$$z = \frac{x - X}{\sigma_p}$$

With:
x: lab result
X: assigned value
σ_p: target value for the standard deviation

The target value for the standard deviation can be determined via the (modified) Horwitz function (*15*), but preference is given to the use of the acceptance criteria in the Commission Regulation 1883/2006 and 152/2009 (*1,2*). The standard deviation is therefore derived from:

$$\sigma_p = \frac{CV_{max}}{100} \times X$$

With CV_{max} = 30%

The CV_{max} is based on the acceptance criteria for screening methods as laid down in the Commission Regulation 1883/2006 (*1*). This approach is in close agreement with the (modified) Horwitz function as presented by Thompson (*15*). In this case σ_p is defined as: $\sigma_p = 0.22X$

The assigned value (X) is calculated using the added concentration (standard solutions and QC-oil) or using the median of the results obtained from the 3 laboratories using the GC-HRMS.

The sum of the squared z-scores (SSZ) is calculated to give a composite score of the individual results for each laboratory.

$$SSZ = \sum z^2$$

The SSZ is evaluated by comparing it with critical χ^2 values with n degrees of freedom (where n is the number of scores) and a probability of 0.95 and 0.997, which corresponds, with z-scores of 2 and 3. Z-scores: $|z| < 2$ is satisfactory, $2 < |z| < 3$ is questionable and $|z| > 3$ is unsatisfactory.

Method validation parameters
The repeatability (r), the within-lab reproducibility (W) and the reproducibility (R) are calculated using a two factor nested ANOVA as explained in the ISO 5725-3 (*5*). The sources of variation are given in the Table 3.4.

Table 3.4 ANOVA-table explaining the contribution of the variance of the laboratories, the analytical runs and the replicate measurements to the total variance.

Source	Sum of squares (SS)	Degrees of freedom (df)*	Mean square (MS)	Expected mean square (EMS)
Lab	SS_L	n_{Lab}-1	MS_{Lab}	$\sigma^2 + 2.\ \sigma_1^2 + 6.\ \sigma_0^2$
Run	SS_R	$n_{Lab}.n_{Run} - n_{Lab}$	MS_{Run}	$\sigma^2 + 2\ \sigma_1^2$
Replicate	SS_E	$n - n_{Lab}.n_{Run}$	MS_{repl}	σ^2
Total	SS_{TOT}	$n - 1 = n_{Lab}.n_{Run}.n_{repl} - 1$	MS_{TOT}	

* *n_{Lab}: number of participating laboratories; n_{Run}: number of analytical runs (=3); n_{repl}: number of replicates per run (=2)*

The repeatability-, the within-lab reproducibility- and the reproducibility variance are as follows for a balanced nested design:

$S_r^2 = MS_{repl}$
$S_W^2 = S_r^2 + S_1^2$
$S_R^2 = S_r^2 + S_1^2 + S_0^2$

with

$S_0^2 = 1/6\ (MS_{Lab} - MS_{Run})$
$S_1^2 = ½\ (MS_{Run} - MS_{repl})$

The repeatability and the within-lab reproducibility variance for each laboratory are analogously derived using a single factor design. The apparent recovery (*16*) is estimated by dividing the mean of the lab results through the reference value and the coefficient of variation (CV) is obtained by dividing the respectively S_r, S_W and S_R through the mean of the lab results. The method bias is calculated by comparing the result (X_i) with the median of the results of the GC-HRMS labs (X_{ref}): bias = $(X_i - X_{ref})/X_{ref}$*100.

Detection Capability

The methodology for the determination of the minimum detectable value (MDV) in the case of a linear regression model (LRM), has been extensively described in the ISO 11843-2 (*4*). Under the assumption of linearity, normality, independence and homoscedasticity, the MDV (= x_d) is given by:

$$MDV = x_d = \delta \frac{Sy}{b} \sqrt{\frac{1}{K} + \frac{1}{I.J} + \frac{\overline{x}^2}{J.\sum (x_i - \overline{x})^2}}$$

In case of weighted linear regression models (WLRM), the MDV is given by:

$$MDV = x_d = \frac{\delta}{b} \sqrt{S_{x_d}^2 + \left[\left(\frac{1}{J.\sum w_i} \right) + \frac{\overline{x}_w^2}{J.\sum w_i (x_i - \overline{x}_w)^2} \right] S_y^2}$$

with b : estimate of the slope; δ : non-centrality parameter; I : number of reference states (= number of replicates per concentration for the spiked or reference samples), i = 1, 2, …, I; J : number of preparations for the reference states (= number of concentrations for the spiked or reference samples); Sy : standard error of the estimate; Sx_d : residual standard deviation at $x = x_d$; w_i : applied weights ($w_i = 1$ in the case of unweighted regression); x_i : spiked concentration; $\overline{x}$: mean of the concentrations and

$$\overline{x}_w = \frac{\sum w_i x_i}{\sum w_i}$$

The weights are calculated by taking the reciprocal of the variance function. The variance function ($VAR_i = (c + dx_i)^2$) is estimated by a linear regression of the standard deviations versus the concentration.

Laboratories and their analytical methods

Table 3.5 gives an overview of the participating laboratories and the techniques used. Accelerated Solvent Extraction (ASE) (*17*) is evaluated as a combined extraction and clean-up technique. Details on the principles of the methods can be found elsewhere (*18,19*).

Table 3.5 Overview of the participating laboratories and the used techniques.

LAB	Method	Remarks round 1 & 2	Remarks round 3
A	CALUX		
A*	CALUX		Data corrected for recovery
B	ASE+GC-HRMS		
B'	ASE+GC-HRMS		Samples reanalysed
C	GC-HRMS		
D	CALUX		
E	CALUX	TCCD calibration curve used for quantification	TCCD calibration curve used for quantification
E*	CALUX	Results quantified by comparison with the value of a reference sample	Results quantified by comparison with the value of a reference sample
F	GC-HRMS		
G	GC-LRMS/MS		
H	ASE+CALUX		
I	GCxGC-ECD		
I*	GCxGC-ECD	Reprocessed data after the initial presentation of the results of the validation study in Brussels, February 2003	
J	GC-HRMS		
K	GCxGC-ECD		Data not in duplicate

In round 3, partners 4, 7, 9 have reported corrected data for some samples. These data are treated separately for the partners 4 and 9, because they were submitted after the evaluation meeting where all data was undisclosed. The corrected data of partner 7 were already included in the statistical analysis prior to that meeting and are therefore not separately reported. Partners 2 and 7 have reported corrected CALUX data. An asterisk indicates these corrected results.

Results and discussion

The aim of the first round was to test the goodness-of-fit of the calibration curve by analysis of standards with undisclosed concentrations of TCDD in n-nonane or DMSO. Furthermore, information on repeatability and reproducibility was obtained from the analysis of fish oil and a spiked milk sample. A quality control sample was analysed each round to check the performance of the methods.

Standard Solutions

The aim of the standard solutions with undisclosed concentrations of 2,3,7,8-TCDD (further referred to as TCDD) was to test the goodness-of-fit of the calibration curve. The relative deviations from the assigned value of the GC-method results are presented in Figure 3.1. The standards were analysed in duplicate by direct injection in the GC (some labs have diluted the standard solutions). Five different concentrations of TCDD in DMSO and a blank DMSO solution were prepared at RIVO. The standards were analysed in duplicate by direct addition to the cell medium. The CALUX bio assay results are all, except one, positively biased. A graphical representation of the relative deviation of the results for the different standard solutions can be found in Figure 3.2.
It can be concluded that the GC-method results perform better than the CALUX bioassays. However, it should be noted that the dataset was limited as, only one GCxGC-ECD lab (I) and one GC-LRMS/MS lab (G) have provided results. The other GC labs have used the GC-HRMS reference technique.

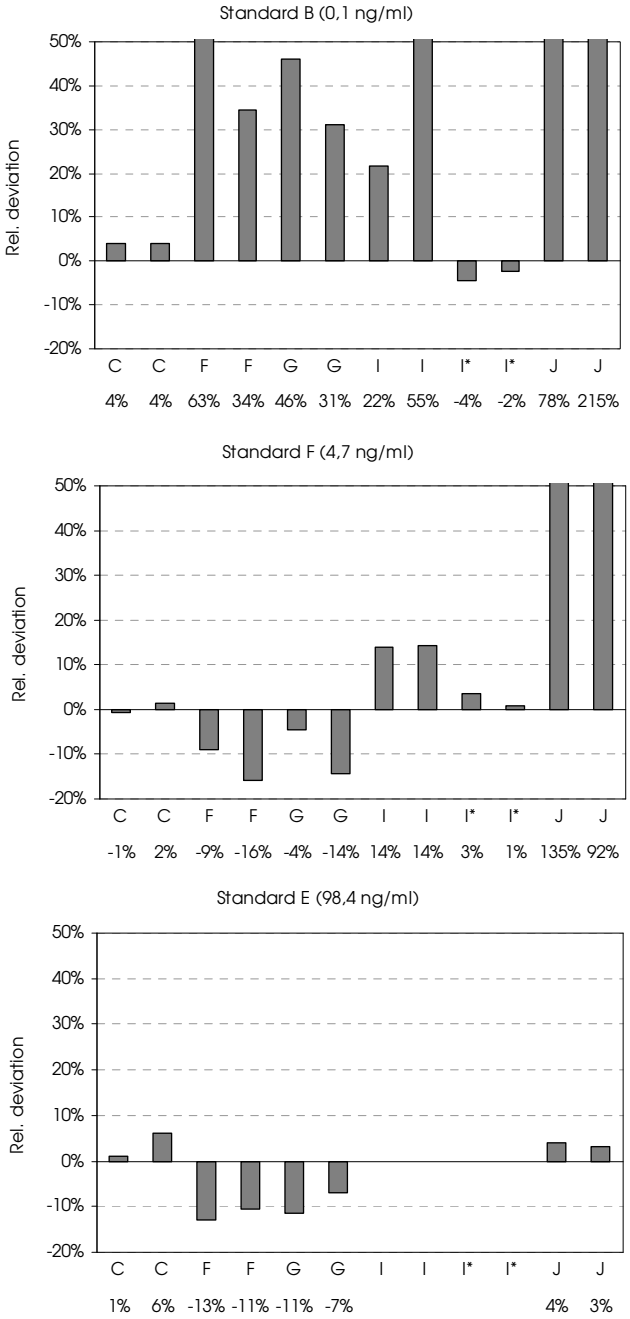

Figure 3.1 Relative deviation between measured (GC screening methods, I and G) and the assigned value for the standard solutions in nonane (B, C, F, A, E and G). The assigned value is shown on top of each graph, in brackets. On the x-axys, the laboratory code and the value of the relative deviation (corresponding to the bar) are mentioned.

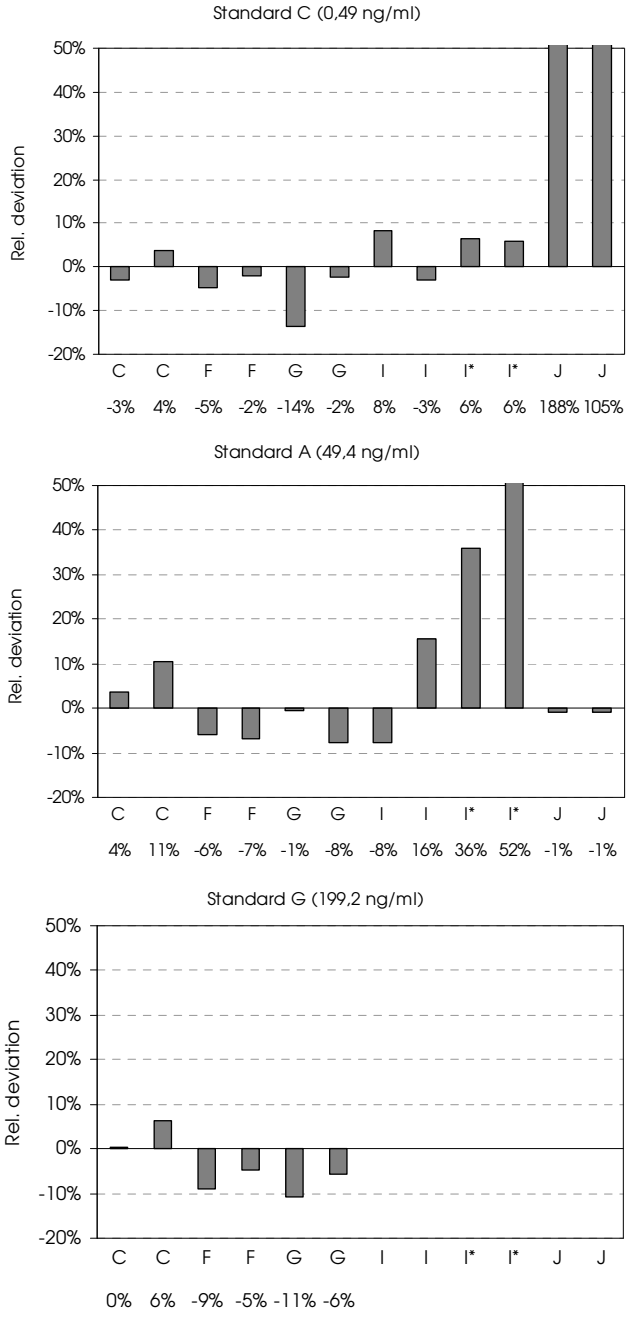

Figure 3.1 Continued

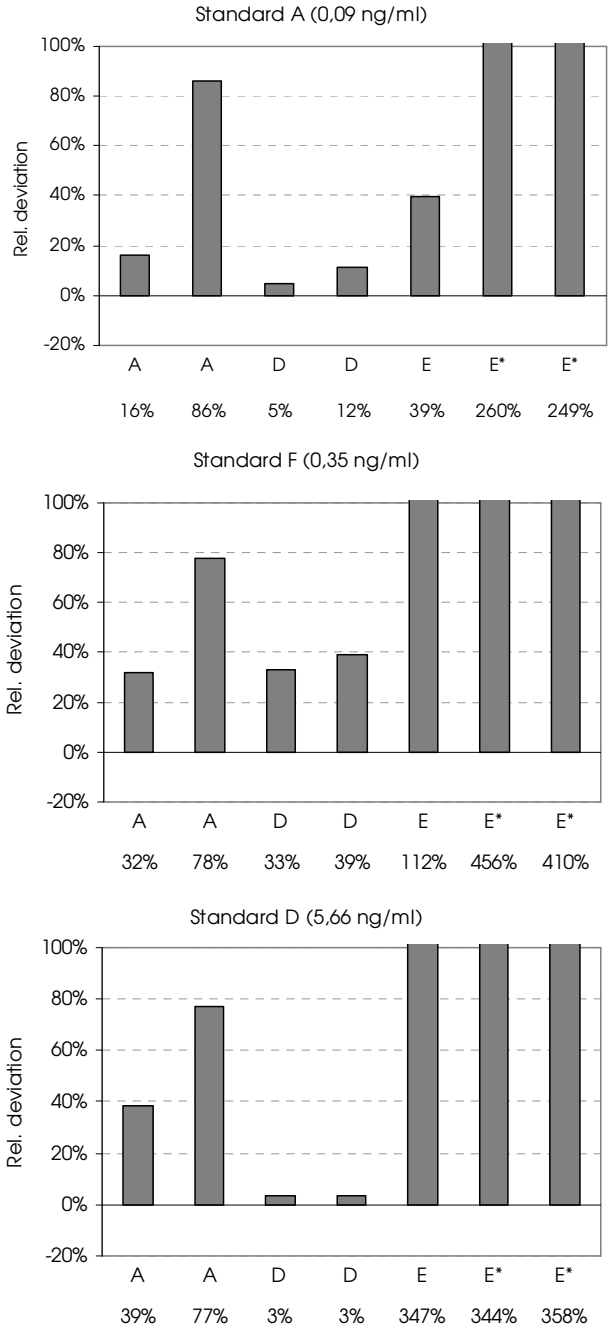

Figure 3.2 Relative deviation between measured (CALUX-methods) and the assigned value for the standard solutions in DMSO (A, E, F, C, D). The assigned value is shown on top of each graph, in brackets. On the x-axys, the laboratory code and the value of the relative deviation (corresponding to the bar) are mentioned.

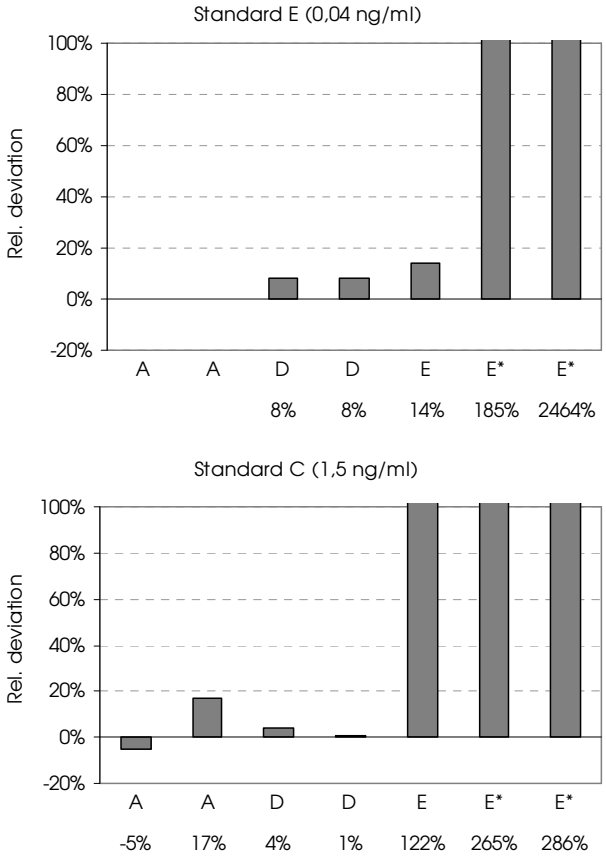

Figure 3.2 Continued

Detection capability and selectivity

The aim of round 2 was to determine the detection capability and selectivity of the methods. During this round vegetable oil samples spiked with a mixture of dioxins and dl-PCBs at a concentration of 0 - 0.4 - 1.5 - 3 - 6 and 12 pg total TEQ/g lipid are analysed under within-lab reproducibility conditions. This means that they are analysed once during 4 independent analytical runs, by different operators and using different equipment whenever feasible. The procedures described in the ISO 11843-2 (*4*) were used to gauge the detection capability of the analytical techniques. It was shown by Van Loco *et al.* (*20*), that heteroscedasticity of the data has a major impact on the detection capability. Therefore, heteroscedasticity of the variance was evaluated and corrected for by assuming that the standard deviation is linearly dependent on the concentration. The variance function $VAR_i = (c + dX_i)^2$ is estimated by a linear regression of the standard deviations versus the concentration.

The detection capability data, here expressed as MDV, are summarized in Table 3.6. Results below the lowest spiked concentration are expressed as "<", because the variance function below this concentration is obtained by extrapolation. This is the case for labs C and G. Their MDV is lower than the 0.367 pg total TEQ/g oil concentration in the lowest spike. One should not conclude from Table 3.6 that the detection capability of the GC-LRMS/MS is better than that of GC-HRMS since the experiments on the GC-LRMS/MS were performed under optimal conditions, while the GC-HRMS was used under routine conditions. In addition, only one laboratory used GC-LRMS/MS and therefore, these data need to be confirmed by other laboratories. Nevertheless, the low MDV of GC-LRMS/MS does show the potential of the technique. The lowest MDV of the CALUX methods is 0.9 pg total TEQ/g oil, which is close to the highest MDV of the GC-HRMS laboratories.

Table 3.6 Detection capabilities of the methods for dioxins and dl-PCB's in vegetable oil.

Laboratory	Method	MDV (pg TEQ/g oil)
A	CALUX	3.83
B	ASE+GC-HRMS	0.57
C	GC-HRMS	< 0.37
D	CALUX	7.79*
E	CALUX	0.90
E*	CALUX	1.04
F	GC-HRMS	0.50
G	GC-LRMS	< 0.37
H	ASE+CALUX	4.86*
I	GCxGC-ECD	< 1.42
J	GC-HRMS	0.88

* *The correlation coefficient of the dose-response curves is lower than 0.85*

It was not possible to provide a good estimate for the MDV for the labs D and H. Both labs are using the CALUX technique. These data are marked with asterisks. An explanation can be found in the low correlation coefficient for their calibration lines: respectively 0.837 and 0.811. The correlation coefficients of the other laboratories were all above the required 0.95 (*1,2*).
The apparent recovery of the CALUX methods was evaluated. The apparent recovery is defined as the observed value derived from an analytical procedure by means of a calibration graph divided by the reference value (*16*). The apparent recovery for the CALUX labs D, E and H is very low (18 - 44%). The apparent recovery of the CALUX lab A is function of the concentration, since the calibration graph does not pass through zero. At the lower concentrations the recovery is larger than 100%. At concentrations around 4.5 pg TEQ/g the recovery is 100% and at higher concentrations the recovery is lower than 100%. Hence, the method bias is positive at lower concentrations and negative at the higher concentrations. The same tendency can be seen for some other CALUX labs. However, this has not been statistically confirmed. The apparent recovery for the GCxGC-ECD lab I is around 108%. The apparent recoveries for the GC-LRMS/MS and GC-HRMS are approximately 100%. At very low concentrations the GC-methods are positively biased. This is probably caused by the presence of traces of PCDD/Fs and dl-PCBs in the blank vegetable oil, since the vegetable oil is off-the-shelf purchased without further purification.

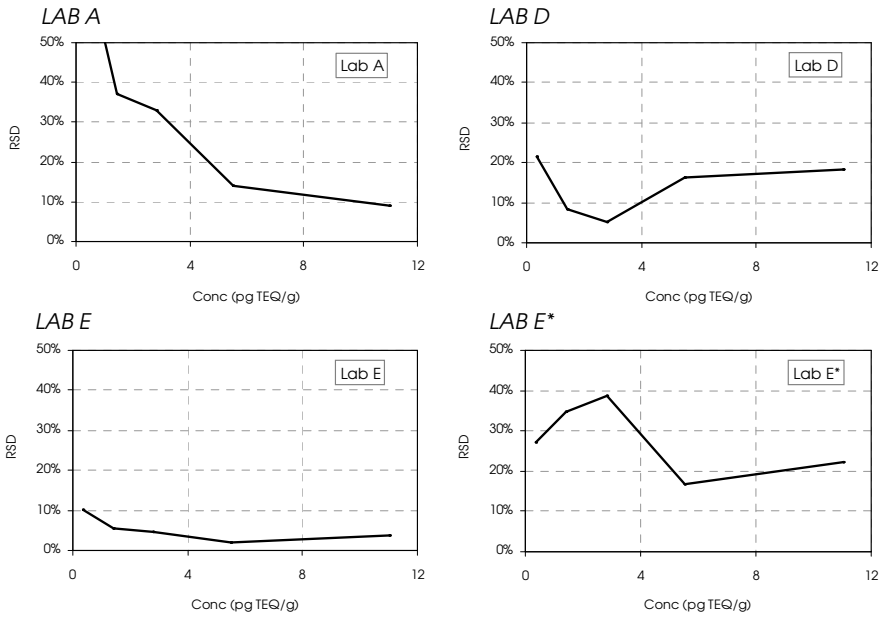

Figure 3.3 Within-lab reproducibility of the CALUX labs as function of the concentration. Results based on 4 analysis of the spiked vegetable oils (Table 3.1).

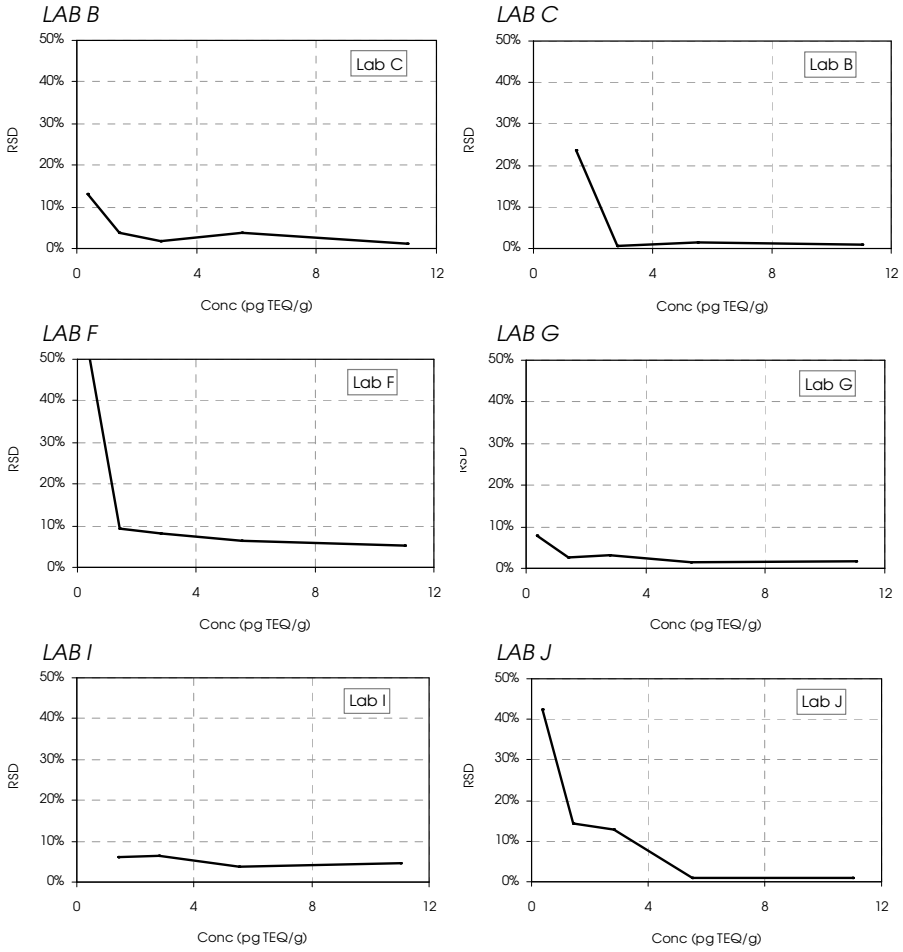

Figure 3.4 Within-lab reproducibility for the GC labs as function of the concentration. Results based on 4 analysis of the spiked vegetable oils (Table 3.1).

The precision of the method as function of the concentration is shown Figures 3 and 4. In all cases the relative standard deviation (RSD) decreases at higher concentrations. The RSD of the CALUX technique is higher than that of the GC screening methods (GCxGC and GC-LRMS/MS). The RSD of the CALUX labs is around 20% at the higher concentration range. The RSD of the GC-labs is below 10% at the higher concentration range.
The selectivity of the screening methods was evaluated by spiking PCB, PCN and PCDE to the 6 pg/g total TEQ vegetable oil. The influence of possible interferences was evaluated with ANOVA (*21*). No interferences were detected. However due to an error during the preparation of the interference samples, the PCB interference spike contained an additional 2.7 pg TEQ/g oil

of mono-ortho PCBs (PCB 105, 118 and 156) in comparison with the reference spike. The CALUX methods could not detect this additional amount of PCB TEQ in the sample. This confirms that the CALUX technique has a weakness in detecting and quantifying mono-ortho PCBs. This can be easily explained by the very low REP (Relative Potency) values of the mono-ortho PCBs (*22,23*). When the REP-values are taken into account instead of the TEF-values, the additional amount of PCB TEQ is only 0.07 pg PCB TEQ/g. The GC-screening methods all detected the additional amount of PCBs in the sample. However, the results reported by the labs B and I were significantly higher than those of the other GC labs. There was also a slight, but statistically significant, increase found by lab C (using GC-HRMS) for the total TEQ concentration of the PCDE interference sample. No explanation was found for this PCDE interference.

Table 3.7 QC-oil validation data (the oil was spiked at a concentration of 5.52 pg total TEQ / g oil).

Lab	Method	N	Mean concentration (pg total TEQ/g oil)	Bias (%)	CV_W (%)	CV_R (%)
A	CALUX	6	5.05	-8.5	15.1	54.9
D	CALUX	6	3.97	-28.2	22.8	
E	CALUX	6	1.6	-71.0	89.0	
A*	CALUX*	6	5.68	2.8	15.1	24.7
E*	CALUX*	5	7.64	38.4	18.2	
I	GCxGC-ECD	6	6.56	18.8	21.8	22.0
K	GCxGC-ECD	1	6.1	10.5	-	
C	GC-HRMS	6	5.73	3.7	15.8	8.2
F	GC-HRMS	6	5.82	5.3	6.0	
J	GC-HRMS	7	5.24	-5.0	11.3	
G	GC-LRMS/MS	6	5.68	2.8	2.2	-
H	ASE+CALUX	5	3.06	-5.0	127	-

The QC-oil was analysed during all the 3 rounds of the project: vegetable oil was spiked with a mixture of dioxins and dl-PCB at a concentration of 5.52 pg total TEQ / g oil. The mean found concentration, the bias, the within-lab reproducibility coefficient of variation (CV_W) and the reproducibility coefficient of variation (CV_R) are summarized in Table 3.7.
The CV_W for the biological and chemical screening methods are all, except labs E and H (both CALUX), lower than 30%. The European directives (*1,2*) require that the variation of screening methods is below 30%. A very small variation (CV_W < 3%) for the GC-LRMS/MS screening method is noticed. Furthermore, the results of the CALUX laboratories underestimate the total TEQ concentration in the sample. However, the CALUX results are not corrected for recovery. Two CALUX laboratories (A and E) have also reported results with applying recovery correction (see Table 3.5). These labs are marked with an asterisk "*". The CV_W of the CALUX*-labs is below 20% and the concentration

of the samples is not underestimated anymore. On the contrary, lab E* do overestimate the concentration with 38%.
The variation of the chemical screening methods GCxGC-ECD and GC-LRMS/MS are below the required 30%. The CV_W of the GC-LRMS/MS is even below the 3% and is the lowest of all the participating labs. This may be caused by the optimal conditions under which this instrument was run, whereas the GC-HRMS labs ran their instruments under routine conditions. The GCxGC-ECD has slightly overestimated the concentration in the sample.
For each reported result (total TEQ) z-scores are calculated. The squared z-scores are presented in Figure 3.5. Only the results of lab E and H are unsatisfactory. However, it cannot be concluded that the ASE+CALUX (extraction and clean-up followed by analysis with CALUX) is unsuitable, since the CALUX part was performed by the same lab E and the results were not corrected for recovery. The SSZ-scores for the recovery corrected CALUX results are satisfactory. The results reported with GCxGC-ECD (1 outlier removed), with GC-LRMS/MS and with ASE+GC-HRMS (extraction and clean-up followed by GC-HRMS) are satisfactory.

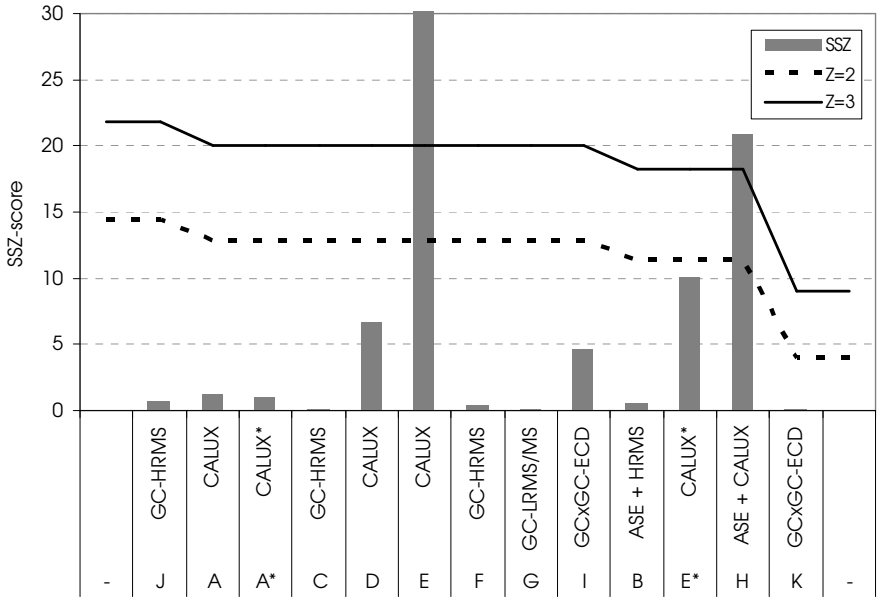

Figure 3.5 The sum-of-the squared Z-scores (SSZ) for the QC-oil sample (total TEQ). The results were obtained during the three rounds of the validation study. The interpretation of the SSZ-scores is performed by the full and the dotted line, which represents the acceptance criteria with the same probability of z = 3, respectively z = 2.

The selectivity and robustness of the screening methods is evaluated by spiking potentially interfering compounds (PCBs, PCNs and PCDEs) to a 6 pg total TEQ/g vegetable oil (Table 3.2, material 11-13). The influence of these possible interferences is evaluated with ANOVA, followed by Tukey's HSD method. The compounds did not significantly interfere in the CALUX and GC determinations (data not shown). However due to an error by the preparation of the interference samples, the PCB interference spike was spiked with an additional amount of 2.7 pg PCB TEQ/g mono-ortho PCBs (PCB 105, 118 and 156). The CALUX methods could not detect this additional amount of PCB TEQ in the sample. This confirms that the CALUX technique has a weakness in detecting and quantifying mono-ortho PCBs. This can be easily explained by the very low REP values of the mono-ortho PCBs (*23*). When the REP-values are taken into account instead of the TEF-values, the additional amount of PCB TEQ is 0,07 pg PCB TEQ/g. The CALUX method will not be able to detect this addition of mono-ortho PCBs

The GC-screening methods could all detect the additional amount of PCBs in the sample. However, the results reported by the labs B and I are significantly higher than the results of the other GC labs. It was the same partner that performed the chromatography on the ASE extracts of lab B and GC*GC-ECD results from lab I. Hence, one might not conclude that ASE causes the overestimating of the results. There was also a slight, but statistically significant, increase found by lab C (using GC-HRMS) for the total TEQ concentration of the PCDE interference sample. No explanation was found for this PCDE interference.

Milk sample

The aim of the milk sample was to provide data on the within-laboratory reproducibility and repeatability of a real matrix sample. The milk samples were prepared by spiking with a mixture of dioxins and dl-PCBs at a concentration of 10.23 pg total TEQ/g lipid. The milk samples were analysed by the participants in duplicate in 3 different analytical runs with different equipment and different operators whenever feasible. The data were obtained with CALUX (3 labs), GC-HRMS (3 labs), GC-LRMS/MS (1 lab), GCxGC-ECD (2 labs). A large variation in the reported results was observed. The mean results reported by the GC-HRMS labs vary from 8.7 to 14.1 pg total TEQ/g lipid. The milk sample is a spiked sample and it appeared that the PCB-118 congener was spiked in an unusually high concentration of 4.7 pg TEQ/g lipid. This resulted in calibration problems for most of the GC methods.

The CALUX labs (A, D and E) have reported the lowest total TEQ concentrations for the milk sample. However, the CALUX results were not corrected for recovery whereas the GC methods were. All GC methods used internal standards or isotopic dilution to correct for the extraction yield. The highest results were reported by the labs using GCxGC-ECD. The SSZ-scores are visualized in Figure 3.6. (The SSZ-score is a combination score of the 6

individual z-scores.) The SSZ-scores for the CALUX labs D and E and the GCxGC-ECD labs I and K are unsatisfactory.

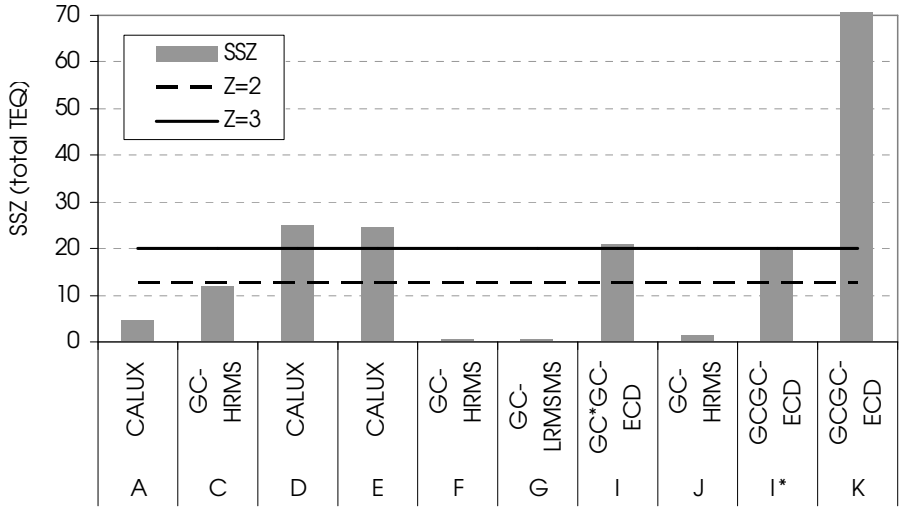

Figure 3.6 Total TEQ SSZ-scores for the milk samples.

A summary of the statistical evaluation of the laboratory results is given in Table 3.8. In this table the mean, repeatability and within-lab reproducibility standard deviation (S_r and S_W) and coefficient of variation are given. Normality of the results for each lab was evaluated with χ^2 goodness-of-fit and Shapiro-Wilks W tests. Normality was not rejected and outliers were not detected with Grubbs' test at the 99% confidence level.

Table 3.8 Statistical summary of the total TEQ results (upperbound) for the milk sample.

Lab	Method	Number	Average (pg TEQ/ g lipid)	S_r (pg TEQ/ g lipid)	CV_r (%)	S_W (pg TEQ/ g lipid)	CV_W (%)
A	CALUX	6	7.61	1.14	15.0	1.35	17.7
C	GC-HRMS	6	14.06	0.44	3.1	0.44	3.1
D	CALUX	6	3.95	0.59	14.9	1.52	38.5
E	CALUX	6	3.93	0.66	16.7	1.08	27.6
F	GC-HRMS	6	9.61	0.61	6.4	1.03	10.7
G	GC-LR MS/MS	6	10.83	0.54	5.0	0.54	5.0
I	GCxGC-ECD	6	15.32	1.05	6.9	1.2	7.8
I*	GCxGC-ECD	6	15.17	1.28	8.4	1.28	8.4
J	GC-HRMS	6	8.71	0.62	7.1	0.64	7.3
K	GCxGC-ECD	6	19.89	1.99	10.0	2.83	14.2

The precision of the analytical methods was assessed by evaluating the repeatability and within-lab reproducibility standard deviation and CV. Note that the CVs for the CALUX methods (lab A, D and E) are significantly higher than the CV's for the GC screening methods (labs G, I and K). One might expect that the repeatability CV (CV_r) is between 1/2 and 2/3 of the Horwitz CV (*24*). Using the modified Horwitz equation (*15*) the CV_r should be between 11 and 14.7 %. All CALUX labs have reported higher CV_r's. The criterion of CV < 30% for screening methods (*1*) is violated by the CALUX Lab D.

Fish Oil

To assess the within-lab repeatability and reproducibility the fish oil samples were analysed in duplicate in three different analytical runs. The analyses were performed using different equipment and different operators whenever feasible (Lab H has only reported 5 results and Lab K only 3 results). The data were obtained with CALUX (3 labs), GC-HRMS (3 labs), GC-LRMS (1 lab), GCxGC-ECD (2 labs). The samples were also analysed by accelerated solvent extraction (ASE) followed by a detection and quantification of the results with GC-HRMS (B) and CALUX (H).

Box and whisker plots of the total TEQ upperbound results for the samples are presented in Figure 3.7. The values range between 1.94 and 15.5 pg total TEQ/g lipid. It was observed that the results obtained with CALUX (labs A, D, E, H) are significantly lower than the results reported by the GC screening labs (G, I, I* and K), except for lab A. Lab A has a much larger variance than the other laboratories.

The labs D, E and H, all using CALUX, have z values <2 for some of the total TEQ results. The z-scores for lab A, which was also applying the CALUX methodology, are satisfactory for the total TEQ results, but not for the dioxin or PCB TEQ results. Lab K (GCxGC-ECD) has reported too high values for the total TEQ and the dioxin TEQ results. The other laboratories have all satisfactory results. The ASE is a valid dioxin and PCB extraction and purification alternative, because the z-scores of lab B are all satisfactory. An overall score of the labs for this sample is given by the SSZ (Figure 3.8). The overall score for the labs D, E, H and K is unsatisfactory. The labs D, E and H are all using the CALUX methodology. As explained earlier in the text, the results of the CALUX labs were not corrected for recovery, while the GC methods were. Assuming that these labs have a recovery of 70%, an SSZ of 21.2 for lab D and 13.4 for lab E would be obtained. These results would still not be satisfactory. So, apparently, recovery is not the only factor that influences the CALUX results. It is also known (*22*) that CALUX underestimates the PCB TEQ in a sample. This phenomenon is illustrated by the PCB TEQ results of lab A, another CALUX lab. The PCB TEQ results are, compared to the GC-HRMS results, significantly lower.

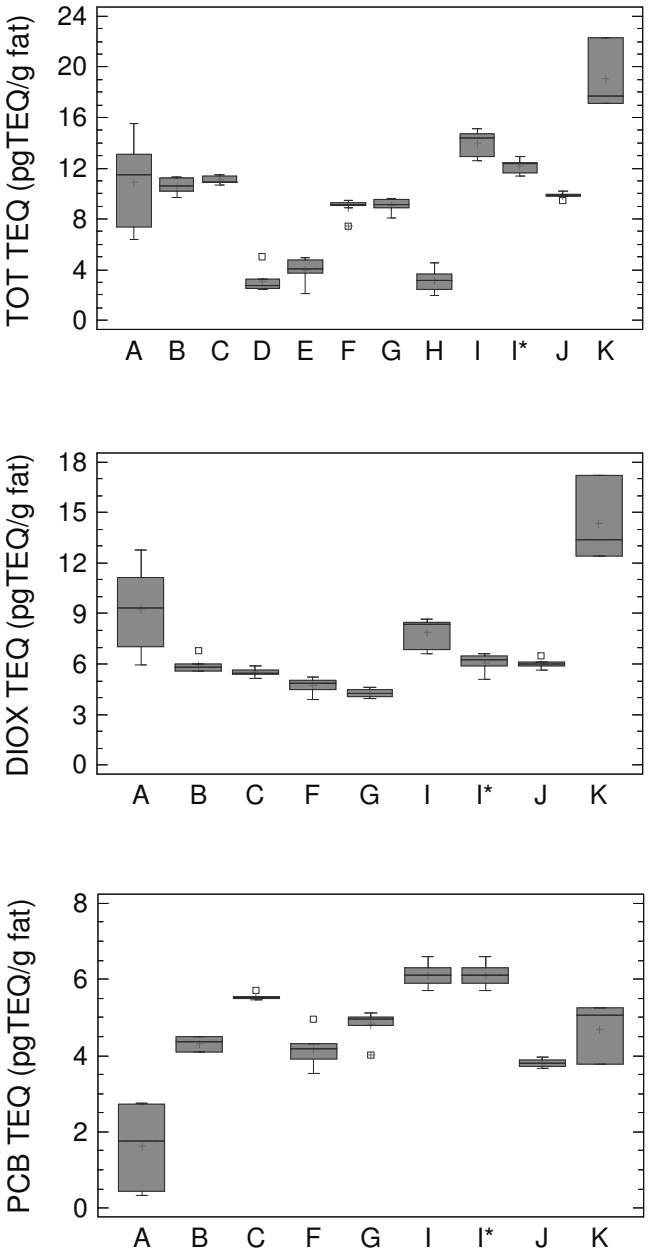

Figure 3.7 Visual representation of the fish oil (upperbound) data in Box-and-Whisker plots. See Table 3.5 for laboratory codes.

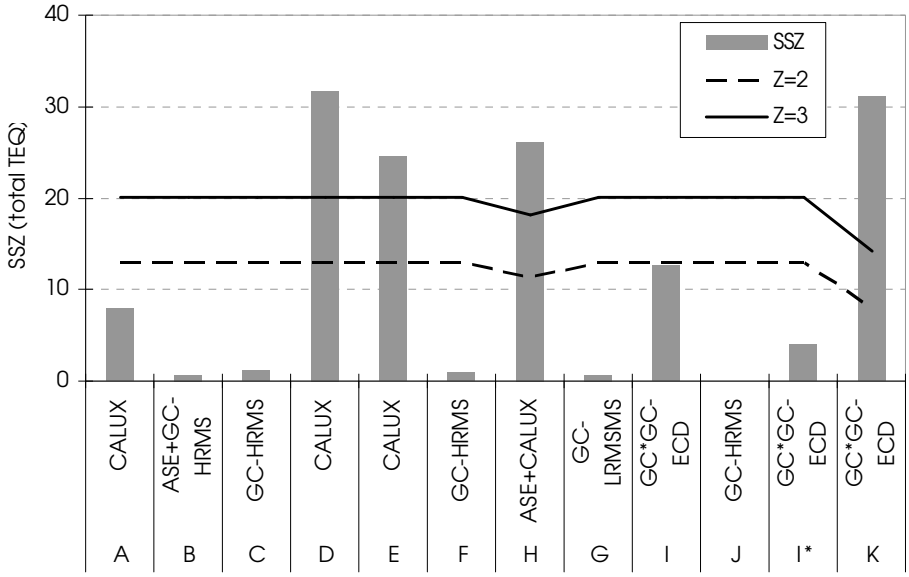

Figure 3.8 Total TEQ SSZ-scores for the fish oil.

Table 3.9 Statistical summary of the total TEQ results (upperbound) for the Fish Oil.

Lab	Method	No.	Average (pg TEQ/ g lipid)	S (pg TEQ/ g lipid)	CV (%)	S_r (pg TEQ/ g lipid)	CV_r (%)	S_w (pg TEQ/ g lipid	CV_w (%)
A	CALUX	6	10.89	3.53	32.4	1.35	12.4	3.89	35.7
B	ASE + HRMS	6	10.25	0.6	5.8	0.58	5.6	0.6	5.9
C	GC-HRMS	6	11.04	0.31	2.8	0.3	2.7	0.32	2.9
D	CALUX	6	3.1	0.97	31.3	0.95	30.7	0.98	31.5
E	CALUX	6	3.93	1.01	25.8	0.66	16.7	1.08	27.6
F	GC-HRMS	6	8.91	0.74	8.3	0.83	9.3	0.83	9.3
G	GC-LRMS/MS	6	9.08	0.57	6.3	0.56	6.1	0.57	6.3
H	ASE + CALUX	5	3.14	1.01	32.0	0.49	15.5	1.1	34.9
I	GCxGC-ECD	6	14	1.02	7.3	0.34	2.4	1.13	8.1
I*	GCxGC-ECD	6	12.18	0.59	4.8	0.25	2.1	0.64	5.3
J	GC-HRMS	6	9.84	0.25	2.5	0.27	2.8	0.27	2.8
K	GCxGC-ECD	3	19.03	2.82	14.8	-	-	2.82	14.8

A summary of the statistical evaluation of the lab results is given in Table 3.9. Normality of the results for each lab was evaluated and was not rejected at the 99% confidence level. Three labs (A, D and H) have a CV_W > 30%. These are all CALUX labs. The maximum CV_r (= 14.7%) (*24*) was exceeded by the labs D, E and H. A more extensive variance analysis of the CALUX screening method (labs A, D and E) was performed according to the ISO 5725 standard. The method ASE + CALUX (lab H) was not included in the evaluation. The between-lab reproducibility and repeatability CV of the CALUX method were 79.0% and 17.2%, respectively.
The differences between the participating laboratories were statistically evaluated by applying a one-way ANOVA on the total TEQ results followed by the Bonferroni test (*21*). The ANOVA decomposes the variance of total TEQ (pg TEQ/g lipid) into two components: a between-lab component and a within-lab component. In ANOVA the between-lab and the within-lab component are compared via an F-test (F= 50.2). Since the P-value of the F-test is <0.05, there is a statistically significant difference between the mean total TEQ (pg TEQ/g lipid) from one lab to another at the 95.0% confidence level. The ASE extraction/purification technique can also be evaluated with this analysis. The results of lab H (ASE+CALUX) are not significantly different from the results of lab E (CALUX) and the results of ASE+GC-HRMS (lab B) are not significantly different from the results of the labs F, G, J and C (GC-HRMS and GC-LRMS/MS). Since the results are situated within the same homogeneous group (Bonferroni test) it can be concluded that ASE is equivalent to the classic extraction/purification techniques for fish oil, although it should be stressed that fish oil is a very simple matrix for extraction. In the classical extraction/purification procedures fish oil is normally analysed without an extraction step.

Herring tissue
A summary of the statistical results of the herring sample is shown in Table 3.10 and the graphical representation is shown in Figure 3.9. The ASE + GC-HRMS results show a severe underestimation of the dioxin-TEQ as well as of the PCB-TEQ. Re-analysis of the sample (B') resulted in a better agreement. More research is needed to find out if the extraction from the herring matrix is incomplete or if the within cell clean-up is not (yet) robust. The CALUX labs D and E also underestimate the result, even when a recovery correction (lab E*) is made. Lab A overestimates the result. The GC-LRMS/MS results and the GCxGC-ECD results of lab K are very close to the GC-HRMS reference values, whereas lab K (GCxGC) somewhat underestimates the result (z = -1.4). This is a much better performance than in the round 1 and 2 samples (QC-oil, milk and fish oil), most likely because method performance improvements were made between rounds 1 / 2 and round 3.

Table 3.10 Summary of statistical results of the herring sample. Concentrations ('Conc.') in (pg TEQ/g).

		Total TEQ			Dioxin TEQ			PCB TEQ		
Lab	**Method**	**Conc.**	**Bias (%)**	**Z-score**	**Conc.**	**Bias (%)**	**Z-score**	**Conc.**	**Bias (%)**	**Z-score**
B	ASE+GC-HRMS	0.15	-93	-3.1	0.09	-90	-3.0	0.05	-96	-3.2
B	ASE+GC-HRMS	0.17	-92	-3.1	0.08	-91	-3.0	0.07	-94	-3.1
B'	ASE+GC-HRMS	0.89	-56	-1.9	0.45	-49	-1.6	0.44	-61	-2.0
A	CALUX	2.35	17	0.6	2.18	146	-	0.17	-85	
A	CALUX	1.57	-22	-0.7	1.49	68	-	0.08	-93	-
D	CALUX	0.47	-77	-2.6	-	-	-	-	-	-
D	CALUX	0.42	-79	-2.6	-	-	-	-	-	-
E	CALUX	0.49	-76	-2.5	-	-	-	-	-	-
E	CALUX	0.45	-78	-2.6	-	-	-	-	-	-
A*	CALUX*	3.22	60	2.0	-	-	-	-	-	-
A*	CALUX*	2.15	7	0.2	-	-	-	-	-	-
E*	CALUX*	1.45	-28	-0.9	-	-	-	-	-	-
E*	CALUX*	1.29	-36	-1.2	-	-	-	-	-	-
C	GC-HRMS	2.04	2	0.1	0.92	4	0.1	1.12	0	0.0
C	GC-HRMS	1.99	-1	0.0	0.88	-1	0.0	1.11	0	0.0
F	GC-HRMS	2.07	3	0.1	0.89	1	0.0	1.18	5	0.2
F	GC-HRMS	2.03	1	0.0	0.88	-1	0.0	1.15	3	0.1
J	GC-HRMS	1.56	-22	-0.7	0.88	-1	0.0	0.68	-39	-1.3
J	GC-HRMS	1.57	-22	-0.7	0.90	1	0.0	0.67	-40	-1.3
G	GC-LRMS/MS	2.09	4	0.1	1.00	13	0.4	1.09	-2	-0.1
G	GC-LRMS/MS	2.13	6	0.2	0.98	11	0.4	1.15	3	0.1
I	GCxGC-ECD	2.03	1	0.0	1.05	19	0.6	0.98	-12	-0.4
I	GCxGC-ECD	2.14	7	0.2	1.14	29	1.0	1.00	-10	-0.3
K	GCxGC-ECD	1.17	-42	-1.4	0.66	-25	-0.8	0.51	-55	-1.8

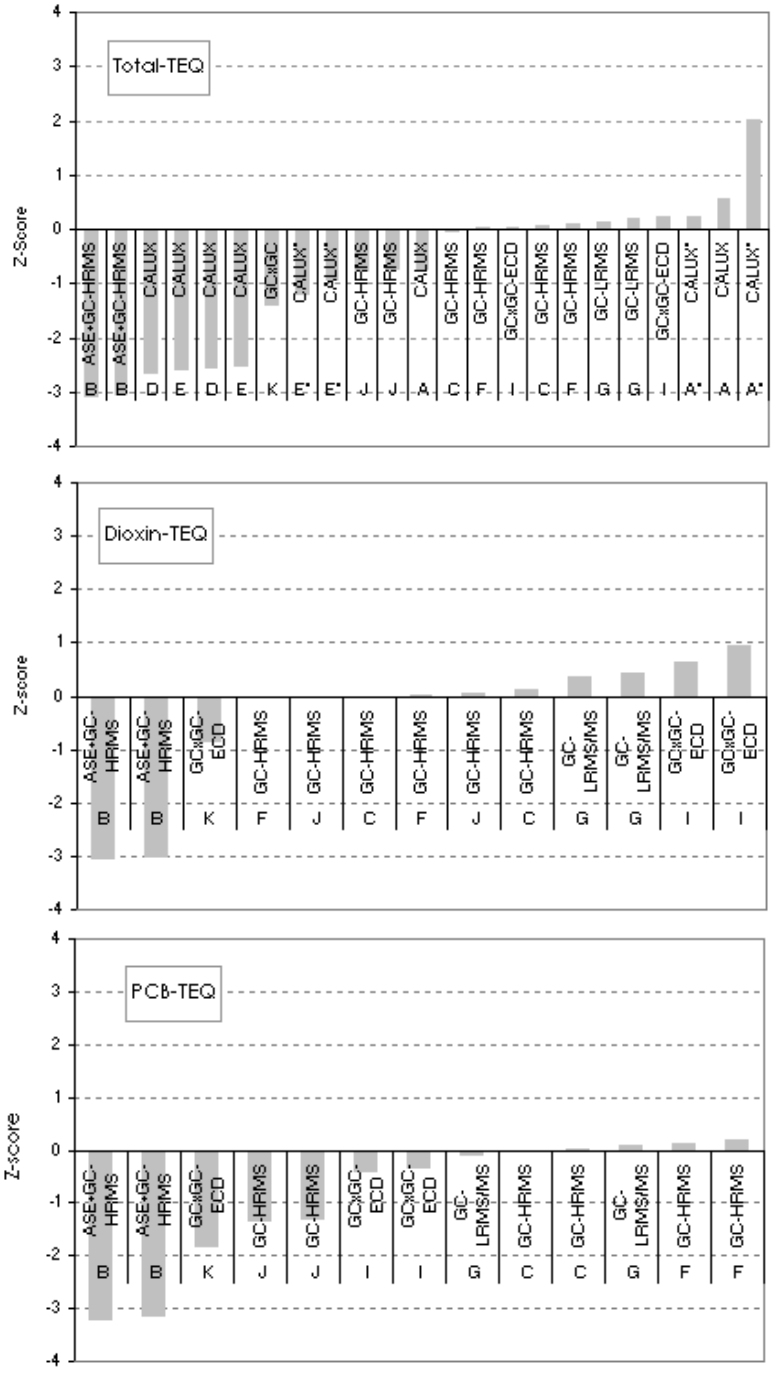

Figure 3.9 The Z-scores (total, dioxin and PCB TEQ) of the duplicate analysis of the herring tissue sample.

Conclusions

Excellent results were reported with the GC-LRMS/MS method. Based on these results, this method would be a good candidate screening method for the analysis of dioxins and PCBs. The results obtained with this technique were as accurate as the results reported by the labs using the GC-HRMS technique. All the z-scores are satisfactory. The repeatability and the within-lab reproducibility of the method are below 7%. The MDV is below 0.36 pgTEQ/g oil. It should be noted that only one GC-LRM/MS lab joined the study, and that a lot of effort was done to keep the instruments under optimal conditions. Confirmation of these promising results is therefore desirable.

Table 3.11 Overview of the performance of the different screening techniques for dioxins and dl-PCBs.

Parameter	GC-HRMS	CALUX	GC-LRMS/MS	GCxGC-ECD
Goodness-of-fit	++	+	++	+
Repeatability	++	+	++	+
Within Lab Reproducibility	++	+/-	++	+
Accuracy	++	+/-	++	+/-
Detection capability	+	+/-	++	+/-
Selectivity	++	+/-	++	+

The CALUX results were also promising, taken into consideration that the results of the CALUX technique were not corrected for recovery, while all results obtained by the GC-labs were corrected for recovery by internal standards or isotopic dilution. CALUX labs D and E had accuracy problems with the total TEQ determination in the milk and fish oil sample. The repeatability of the CALUX technique is around 15% for the milk and the fish oil samples. The within-lab reproducibility is higher (up to 38%). The CALUX technique has all features to become an excellent screening technique if a solution could be found for a correction for the matrix-dependent bias and if the variability of the results could be reduced. A reduced variation would automatically lead to a much lower minimum detectable value. Lab E had an MDV (= 0.9 pg TEQ/g oil), close to the MDV of one of the GC-HRMS labs. A second issue that should be addressed by the CALUX labs is the sensitivity of the method for dl-PCBs. The low REP values for the dl-PCBs cause an underestimation of the PCB TEQ compared to the GC-HRMS reference method. This is most pronounced for the mono-ortho PCBs (*23*). It should be noted that CALUX was predominantly developed for use as a screening technique enabling rapid analysis of samples. As such, it has proved it's value during crises, such as the citrus pulp contamination affair (1998) and the Belgian dioxin crisis (1999) (*25,26*).

The initial results reported by lab I and K (GCxGC-ECD) were not very good. The results submitted by these labs tend to overestimate the dioxin concentration in the samples. After the initial presentation of the results in

Brussels, lab I reprocessed their data resulting in satisfactory z-scores. The MDV of lab I is <1.4 pg TEQ/g oil. Results of the herring tissue analysis (round 3) were good and comparable to the GC-HRMS results.
Accelerated solvent extraction (ASE) is a viable alternative extraction and clean-up procedure for fish oil and vegetable oil. ASE extraction combined with CALUX and GC-HRMS detection provides results equivalent to those obtained with the classical extraction and purification procedures. However, ASE + GC-HRMS results of the herring tissue showed a serious underestimation. This calls for further investigation on the possible causes.
The results of the present study are regarded from an analytical perspective. When the economic aspects (i.e. costs per analysis) are evaluated (*6,27*), GCxGC-ECD turns out – for the moment – to be an expensive choice due to the labour-intensive manual integration of the complex chromatograms. GCxGC data handling software develops rapidly and it is likely that labour costs will drop, making the technique more competitive with e.g. GC-HRMS. The GC-LRMS/MS and CALUX analysis price is on the low end of the range of prices for GC based analysis, making it attractive for large-scale sample screening under routine conditions. Obviously, above mentioned accuracy issues and limited precision need to be resolved in order to maintain this low price level. Selective ASE is a promising approach that potentially decreases the costs of traditional extraction and clean-up by performing these two steps in a single step, within the extraction cell.

Acknowledgements

The European Commission is gratefully acknowledged for funding the DIFFERENCE project (contract G6RD-CT-2001-00623). The following partners and their co-workers and subcontractors are gratefully acknowledged for their hard work and stimulating contribution to the project: D. Fraise (CARSO, Lyon, France), R. van Cleuvenbergen and G. Schoeters (VITO, Mol, Belgium), E. Björklund (Lund University, Sweden), J. Rivera (IIQAB-CSIC, Barcelona, Spain), J. Santos (University of Barcelona, Spain), R. Hoogenboom and W. Traag (RIKILT, Wageningen, The Netherlands), P. Haglund (Umeå University, Sweden), and P. Korytar and J. van Hesselingen, RIVO, IJmuiden, The Netherlands).

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3.2 Analysis of hexabromocyclododecane *pitfalls and method comparisons*[5]

Abstract

Gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) are both used for the analysis of HBCD. An important advantage of LC-MS is the separation of individual diastereomers. This allows the use of mass labelled internal standards, which improves the accuracy of the results. The advantage of GC-MS is the simultaneous analysis with other brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs). However, until now severe discrepancies were found between GC-MS and LC-MS results. Several experiments were conducted to evaluate performance of both methods: (i) check of degradation of HBCD in the GC; (ii) measuring of HBCD response factors in the GC-MS; (iii) application of a rapid resolution column for LC separation, (iv) sensitivity test of different instruments and (v) the evaluation of GC vs. LC for the analysis of fish samples. This provided the following insights:

- In GC, degradation of HBCD diastereomers occurs, resulting in the formation of the degradation products pentabromocyclododecene and tetrabromocyclododecadiene. This leads to erroneous HBCD results. In addition, the degradation products can disturb the analysis of major PBDEs (e.g. BDE 49 and 99).
- The GC response factors of the diastereomers are different when using electron capture negative ion MS. This leads to serious quantification errors as the diastereomer profiles in standards and samples are different.
- In LC-MS, the use of a rapid LC-column (Zorbax SB-C18, 2.1 x 30 mm x 3.5 µm) reduced the run-time (5-fold) and improved sensitivity (4-fold) to 20 pg absolute.

Comparative studies in fish showed that GC results are (on average) 4.4-fold higher than the LC results. Research is not conclusive which of the methods delivers biased results (GC-MS or LC-MS or both), and this should be further elucidated. However, because of several advantages of the LC-MS method (i.e. determination of individual diastereomers, the use of mass labelled standards, good sensitivity, no thermal degradation, and simultaneous analysis with tetrabromobisphenol-A) LC-MS is currently the preferred method of analysis.

[5]Based on S.P.J. van Leeuwen and J. de Boer (2008) Brominated Flame Retardants in Fish and Shellfish – Levels and Contribution of Fish Consumption to Dietary Exposure of Dutch citizens to HBCD. *Molecular Nutrition and Food Research* 52, 204-216, and on a presentation by P.E.G. Leonards and discussions at the QUASIMEME Workshop on Brominated Flame Retardants of 23-24 April 2007 (IVM, VU-University, Amsterdam, The Netherlands).

Introduction

Hexabromocyclododecane (HBCD) is applied as an additive brominated flame retardant (BFR) used in polystyrene. In 2001, the world production totaled 16,700 ton (*1*). HBCD predominantly consists of 3 diastereomers (α-, β- and γ-HBCD), of which γ-HBCD predominates in the technical product (*1*). Apart from these three, two (very) minor other diastereomers may also be present in the technical product (δ- and ε-HBCD) (*1,2*). In addition, the diastereomers consist of enantiomeric pairs (*3*).

HBCD can leach to the environment during production, application in products, in-service life (use of the product) and after disposal. HBCD is omnipresent in the environment and it was found in sediments, biota and humans world-wide (*4*). Initially, HBCD was analysed by gas chromatography, combined with mass spectrometric detection (GC-MS) (*4*). Later, the analysis by liquid chromatography (LC) combined with MS was introduced (LC-MS) (*5,6*).

In GC-MS, electron chemical negative ionization (ECNI) is generally used as ionization method for BFRs. This ionization method provides significantly better sensitivity compared with electron impact (EI) ionization, although at the cost of less selectivity (*4,7*). With ECNI-MS, the $(Br)^-$ isotopes are generally monitored (m/z 79 and 81). With EI $(M-Br)^-$ can be monitored resulting in higher selectivity (*8*). The latter provides the possibility to use mass labeled standards, but as the sensitivity is too low for many environmental samples, this ionization technique is not often used.

LC-MS detection is mostly performed on triple-quad instruments (MS/MS) using the electrospray (ESI) source. ESI-ion trap MS instruments (ITMS) have been used as well as atmospheric pressure chemical ionisation (APCI) (please refer to Covaci et al. (*7*) for an extensive overview of methods used). ESI was preferred over APCI by Budakowski and Tomy (*6*), but Suzuki and Hasegawa preferred APCI because of better S/N ratios in leachate samples (*9*). In the ESI source, the formation of $(M-H)^-$ takes place. The MS spectrum results in bromine clusters because of the two bromine isotopes m/z 79 and m/z 81 present. The most intense peak in the cluster is m/z 640.7 ($^{12}C_{12}{}^{1}H_{17}{}^{79}Br_3{}^{81}Br_3$). Triple quadrupole MS/MS instruments allow for selective detection by isolation of the mother ion $(M-H)^-$ (m/z 640.6) in the first quadrupole, followed by detection of the bromine isotope $(Br)^-$ (m/z 79 and/or 81) in the 3rd quadrupole. Because of the low-mass cut-off of ITMS instruments, detection of the bromine isotope is not feasible. Adding ammoniumchlorine to the mobile phase can solve this selectivity drawback. This promotes (and stabilises) the chlorine adduct formation ($(M+Cl)^-$ ion of m/z 676.6), which allowed for MS/MS analyses by monitoring the 676.6→640.7 transition ($(M+Cl)^-$→ $(M-H)^-$), in that way creating a selective method.

A major advantage of LC-ESI-MS(/MS) over GC-ECNI-MS is the option of using ^{13}C labelled internal standards. These standards allow correction for losses during extraction and clean up. Furthermore, several studies showed that

these labelled standards effectively correct for matrix suppression or enhancement occurring in the ESI source (*10-12*).
The results obtained by both techniques for biota samples showed large discrepancies. GC results were on average 4.4-fold higher than LC results (*13*). To explore the causes of these differences, the following experiments were conducted: (i) determination of GC-MS response factors of individual diastereomers; (ii) determination of degradation in the GC-MS. In addition, a short LC column was tested for improving speed and sensitivity of the LC-MS system. The results were discussed at the QUASIMEME workshop on the analysis of BFRs (23-24 April 2007, VU University, IVM, Amsterdam, The Netherlands).

Table 3.12 Advantages and disadvantages of HBCD analysis by LC-MS and GC-ECNI-MS (6,7,12,14,15).

	Advantage	**Drawback**
LC-ESI-MS(/MS)	- Determination of individual diastereomers - Determination of enantiomers - Use of $^{13}C_{12}$ and/or $^{2}H_{18}$ mass labeled internal standards - Simultaneous analysis with TBBP-A - Selective detection (MS/MS)	- Matrix effects may occur in the ESI source, leading to erroneous results[1] - Ion suppression reduces sensitivity - Lower sensitivity compared to GC
GC-ECNI-MS	- Simplicity (single value) - Sensitivity - Simultaneous analysis of other BFRs (e.g. PBDEs)	- Semi-selective detection ($(Br)^-$, m/z 79 and 81) - No separation of diastereomers - Interconversion of diastereomers and degradation in injector and oven >160°C

[1] *Matrix effects can be controlled by using mass labeled internal standards*

Degradation of HBCD in the GC-MS
The GC analysis of HBCD, the HBCD degradation products and the polybrominated diphenylethers (PBDEs) were performed on a GC-ECNI-MS (Agilent 6890, Wilmington, USA) (Instrument 1, Table 3.13). The following samples were run: (A) standard solution of a technical HBCD mixture (500 ng/mL); (B) a BDE standard solution containing BDE 47, 49, 58 (internal standard), 66, 71, 75, 77, 85, 99, 100, 119, 138, 153, 154, 183 and the methylderivative of TBBP-A (500 ng/mL) and (C) a Western Scheldt sediment extract. Extraction and clean-up was performed according to (16).

Different GC-ECNI-MS response factors for each HBCD diastereomer
This experiment was also performed on the aforementioned GC-ECNI-MS system. The HBCD diastereomer response factors were determined by injection of 0.53 ng (α-HBCD), 0.54 ng (β-HBCD) and 0.55 ng (γ-HBCD). The responses were corrected for the slight differences in injected amounts.

Table 3.13 Instruments used.

	Instrument 1	**Instrument 2**
Method	GC-ECNI-MS	LC-ESI-MS/MS
Instrument	Agilent 6890 GC Agilent 5973 MSD	Agilent 1200 HPLC pump Agilent 6410 QQQMS
Column	CP-Sil-8CB (50 m x 0.25 mm id x 0.25 µm film)	Zorbax Eclipse (150 mm*2.1 mm ID, 3.5 µm)
Ionisation	ECNI	ESI$^-$
Ions	$(Br)^-$ m/z 79/81	$(M-H)^-$>$(Br)^-$ m/z 640.7>79
	Instrument 3	**Instrument 4**
Method	LC-ESI-MS/MS	LC-ESI-ion trapMS/MS
Instrument	Shimadzu LC-10 AD Micromass Quattro 2000 QQQMS	ThermoFinnigan LCQ Advantage
Column	Zorbax Eclipse (150 mm*2.1 mm ID, 3.5 µm)	Zorbax XDB-C18 (150 mm x 2.1 mm ID, 3.5 µm)
Ionisation	ESI$^-$	ESI$^-$
Ions	$(M+Cl)^-$>$(M-H)^-$ m/z 676.7>640.7	$(M+Cl)^-$>$(M-H)^-$ m/z 676.7>640.7

Evaluating LC separation on a rapid resolution column and sensitivity of different instruments

The sensitivity of different instruments (Table 3.13) was evaluated by injection of α-HBCD standard solutions. The detection limit was set at a signal-to-noise ratio of 3:1. In addition to the column mentioned in Table 3.13 (instrument 2), the separation and sensitivity of a rapid resolution column (Zorbax SB-C18 column (Agilent, Wilmington, USA, 2.1 mm x 30 mm x 3.5 µm particles)) was evaluated. In this case, a mixture of 3 diastereomers was injected.

Comparison of GC versus LC results

The comparison of GC and LC was carried out during a survey on BFRs in Dutch fish samples (chapter 4.2). Briefly, samples were extracted and cleaned-up by gel permeation chromatography. After additional clean up over a silica column, the extracts were concentrated and analysed by GC-ECNI-MS monitoring $(Br)^-$ m/z 79 and 81 (Table 3.13, instrument 1). To enable LC analysis, the GC extract was evaporated to dryness, followed by a solvent change to methanol. At that stage ^{13}C-HBCD internal standards (for all 3 diastereomers) were added and the extract was subsequently analysed by LC-ion trap MS/MS (Table 3.13, instrument 4) monitoring m/z 676.7 $(M+Cl)^-$ (chlorine adduct) → m/z 640.7 $(M-H)^-$. More details on the analytical approach can be found in chapter 4.2.

Results and discussion

Degradation of HBCD in the GC-MS

Although there are some benefits of the GC analysis (see Table 3.12), several serious disadvantages are connected to GC-MS. An important issue is the instability of HBCD at elevated temperatures applied in the GC injector and oven. At these temperatures (>160°C), the thermally labile HBCD diastereomers can rearrange and this may considerably affect the results (4). Even more important, at elevated injector and oven temperatures HBCD may degrade to pentabromocyclododecene (PBCDe) and tetrabromo-cyclododecadiene (TBCDe). This is demonstrated for a standard solution of α-, β- and γ-HBCD diastereomers (Figure 3.10, top) and was also recently reported by Abdallah et al. (15). Figure 3.10 shows an example of the degradation of HBCD on a CP-Sil-8 column (50 m x 0.25 mm x 0.25 µm) that was extensively used (hundreds of environmental samples had been analysed before on this column). The degradation is most likely caused by the active sites in the column. Furthermore, active sites in a dirty GC liner may cause degradation as well (17). The HBCD degradation results in serious errors in the HBCD determination, because of the reduced peak area of HBCD in the standard solution. It is not known if HBCD degrades to the same extent in sample extracts where other co-extracted interferences are present. Poole recently reviewed 'matrix induced signal enhancement' in GC analysis (18). He showed that thermolabile compounds could be degraded in hot vaporizing injectors. However, in a sample extract, matrix components can also 'shield' the compound, minimizing degradation. Possibly, this plays a role in the HBCD analysis as well, which requires further investigations. Obviously, degradation on the GC column should be avoided, and liners should be replaced regularly.

The degradation products (PCBDe and TBCDe) can disturb the determination of BDEs when using ECNI-MS for detection (monitoring the m/z 79 and 81 ions). On a CP-Sil 8 column, TBCDe interferes with BDEs 49, 71 and 75 and PBCDe interferes with the important congener BDE 99 (Figure 3.10, bottom). This hinders an accurate determination of these BDEs. Obviously, the co-elutions may be omitted when selecting a GC column with different polarity. However, given the broad PBCDe and TBCDe peaks, it may be very difficult to reduce their influence. In addition, more polar columns will lead to an increase of HBCD degradation. Abdallah et al. distinguished four TBCD isomers[6] in dust samples (by LC-MS) (*15*). This may explain the broad peak, which is observed in the GC chromatogram in Figure 3.10. In addition, they found two PCBDe isomers. Possibly, the actual number of isomers is even higher, than the six they determined.

[6] Although the definition of "isomers" was used to differentiate between the structures, it may be more correct (just as with the mother compound) to use the definition of 'diastereomer'.

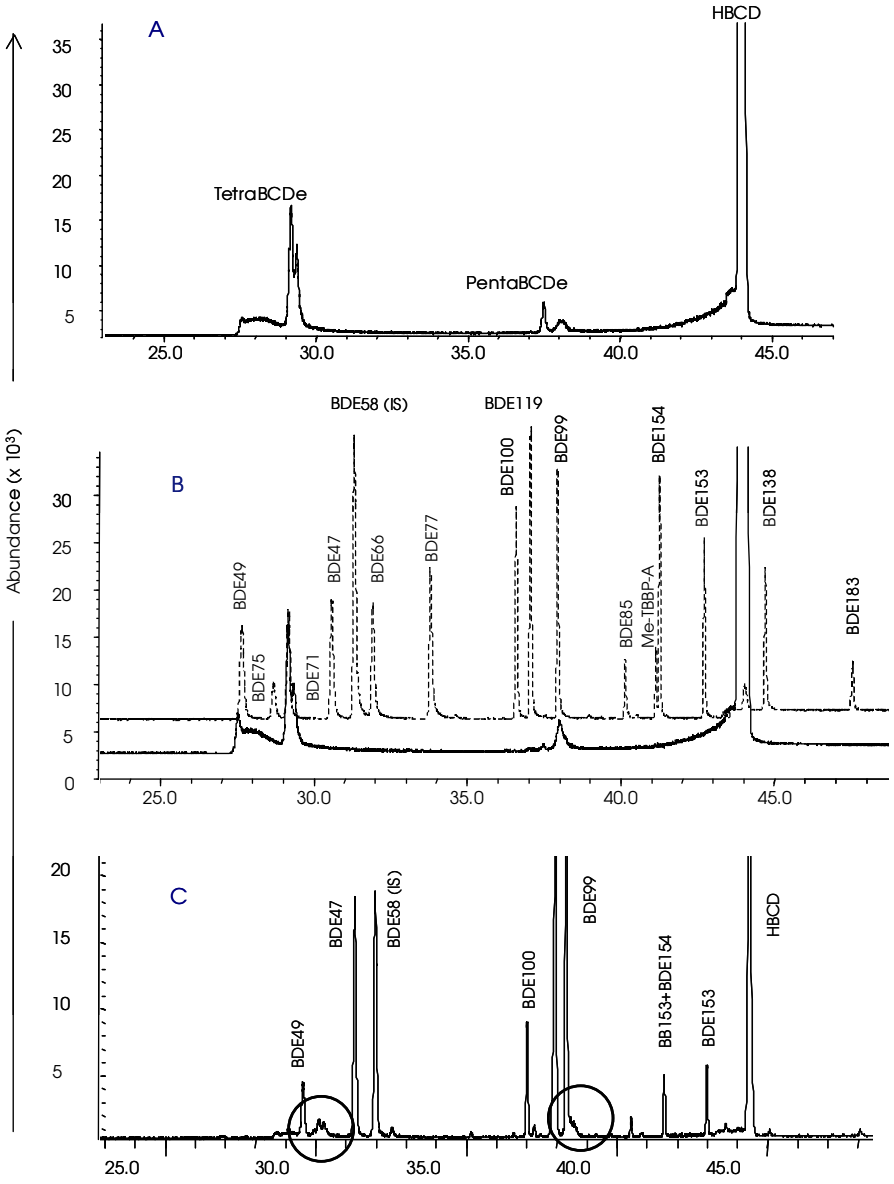

Figure 3.10 Degradation of HBCD to pentabromocyclododecene (PBCDe) and tetrabromocyclododecadiene (TBCDe) on a GC- column (CP-Sil-8CB, 50 m x 0.25 mm x 0.25 µm). A: standard solution of HBCD with TBCDe and PBCDe degradation products. B: TBCDe and PBCDe degradation products superimposed on a PBDE standard solution (dotted line), showing the co-elutions. C: co-elutions with BDE 49 and 99 in a Western Scheldt sediment extract. See text for instrumental conditions.

Apart from degradation in the GC, PBCDe and TBCDe may also be present as impurities in technical mixtures (*19*). Furthermore, PBCDe was reported as a metabolite/degradation product by Hiebl and Vetter (*20*). This shows that PBCDe and TBCDe may be target compounds themselves, which is an additional reason to avoid degradation of HBCD.

Different GC-ECNI-MS response factors for each HBCD diastereomer

GC-ECNI-MS response factors are different for the different diastereomers. After injection of equal amounts of the individual diastereomers on the GC-ECNI-MS column, the responses for α-, β- and γ-HBCD were 100, 71 and 73%, respectively (see Figure 3.11). When determining total HBCD in biota (mainly α-HBCD) while using a standard consisting of equal concentrations of α-, β- and γ-HBCD, errors of 10-20% in the final result can be made. Errors become even more pronounced (up to 40%) when using a technical HBCD mixture (containing mainly γ-HBCD) as a standard. It is therefore recommended, when doing GC analysis, to match the diastereomer profile in the sample with the profile in the standard.

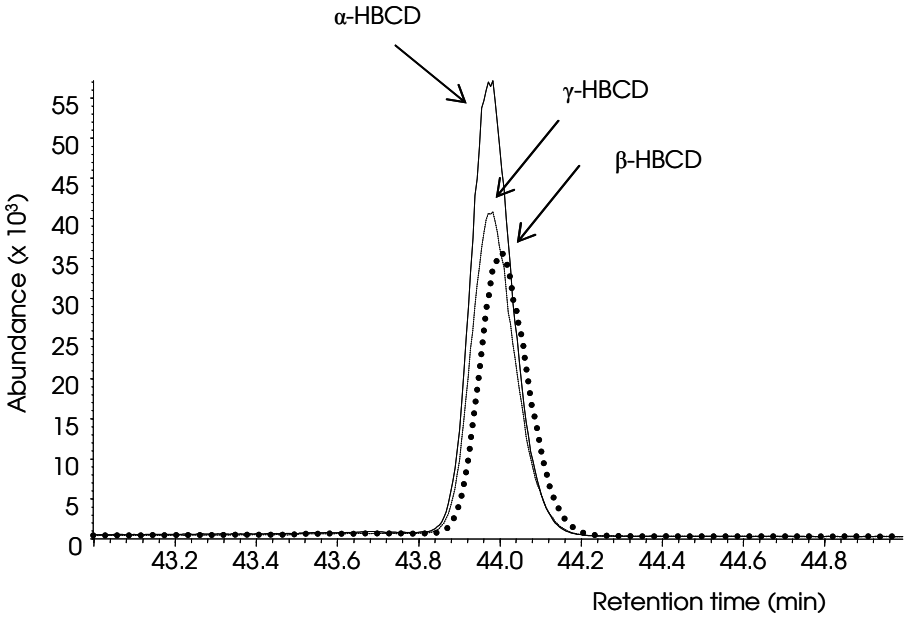

Figure 3.11 GC-ECNI-MS response of α-, β-, γ-HBCD diastereomers. Injected amounts were 0.53 ng (α-HBCD), 0.54 ng (β-HBCD) and 0.55 ng (γ-HBCD). Relative responses are 100, 71 and 73% for α-, β-, γ-HBCD.

Evaluation of a rapid resolution column

The separation of HBCD diastereomers is typically achieved by a gradient of water and a modifier, typically methanol or acetonitrile (or a combination of both), using a reversed phase column (C18) (*21*). Some authors have used ammonium acetate or chloride in the water phase to aid the ionization (*5,12,22,23*). A typical run takes 20 to 30 minutes in order to complete the gradient elution and to return to the initial conditions. Leonards (*24*) explored the possibilities for improving sensitivity by focusing the peaks on a short column. He successfully separated the diastereomers on a 30 mm column (Zorbax SB-C18, 2.1 x 30 mm x 3.5 µm) using isocratic elution. The optimal solvent composition was found to be methanol-acetonitrile-ammonium acetate (0.01 mM) mixture (38:38:24%). This resulted in a reduced analytical runtime, from 11 to 6.5 minutes for elution of the three diastereomers (Figure 3.12).

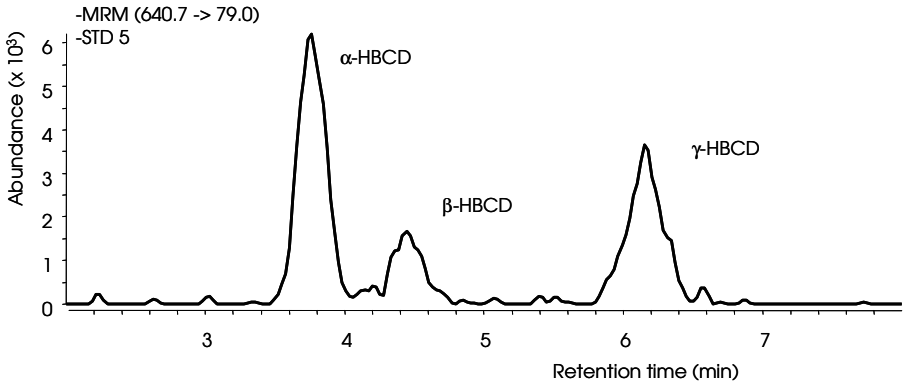

Figure 3.12 Analysis of HBCD diastereomers using LC-ESI-MS/MS (Agilent 6410) by isocratic elution on a rapid resolution column (Zorbax SB-C18, 2.1 x 30 mm x 3.5 µm, 100 pg of each diastereomer injected). Experimental details mentioned in the text.

Because no conditioning of the column is required, the next sample can be injected directly after elution of the last diastereomer, which results in an additional gain in time. It should be noted that with real samples, slowly eluting matrix components could accumulate on the column, leading to a decreased chromatographic performance. However, these matrix components can be removed by running a gradient to e.g. 100% acetonitrile after multiple sample injections.

In addition to the reduced runtime, the limit of detection (LOD) improved 5-fold compared to that of the 150 mm column. This resulted in a LOD of 20 pg absolute (Table 3.14), which is one order of magnitude less sensitive than GC-ECNI-MS (Table 3.13, instrument 1). The sensitivity of other instruments (Table 3.13, instrument 2-4) was evaluated by analysis of HBCD standard solutions.

The LODs were based on a minimum signal-to-noise ratio of 3. The LOD of the Agilent 6410 instrument (instrument 2) is only slightly better (using a 150 mm column) compared to the other LC-MS instruments (instrument 3 and 4). The main reason is that m/z 640.7>79 (instrument 2) is a low yield transition, whereas the yield of the 676.7>640.7 transition (instrument 3 and 4) is higher, resulting in a higher sensitivity. The most efficient transition is instrument dependent. Although small amounts of the chlorine adduct could be detected on the Agilent 6410 instrument (instrument 1), it was too low for producing a sensitive 676.7>640.7 transition. Therefore, the m/z 640.7>79 was used for detection and confirmation on this instrument.

Table 3.14 Sensitivity differences for the α-HBCD diastereomer (pg absolute)

Method **Ions detected**	**Agilent GC-ECNI-MS (Br)⁻ m/z 79/81**	**LCQ Advantage LC-ITMS/MS (M+Cl)⁻ >(M-H)⁻ m/z 676.7>640.7**	**Quattro 2000 LC-QQQ (M+Cl)⁻ >(M-H)⁻ m/z 676.7>640.7**	**Agilent 6410 LC-QQQ (M-H)⁻ >(Br)⁻ m/z 640.7>79**
HBCD per isomer	2[1]	100	100	75/20[2]
TBBP-A	1	100	200	n.a.

[1] Sum of 3 diastereomers
[2] LOD 75 pg for the 150 mm column and 20 pg for the rapid resolution (30 mm) column

Evaluation of HBCD concentrations measured by GC and LC

A small number of studies have compared results obtained by GC and LC based methods. In chapter 4.2. α-, β- and γ-HBCD were determined by LC-ESI-MS/MS, whereas total-HBCD was measured by GC-ECNI-MS. The LC results show that α-HBCD is the predominant isomer in the samples analysed, followed by γ- and β-HBCD (Figure 3.13). The samples plotted in this figure only include those for which the LC-ESI-MS/MS result was above the limit of quantification.

Figure 3.13 shows concentrations of total-HBCD determined by GC-ECNI-MS (printed on top of the bars) and the sum of the three diastereomers determined by LC-ESI-MS/MS (indicated by the bars). In most fish samples, the GC results were higher than the LC results. The correlation line of the results of both methods shows a slope of 0.23 meaning that the LC-result = 0.23 x GC-result. Or, the GC-based results are 4.4-fold higher compared to the LC based results. There can be several causes for this phenomenon. On the GC-side, the thermally labile HBCD diastereomers can rearrange above oven temperatures of 160°C (*22*), resulting in different response. Furthermore, HBCD can degrade (Figure 3.10). It's important to note that the magnitude of these phenomena may be different in a real sample as compared to a standard solution. On the LC-side, the issue of different response factors is not relevant because the diastereomers are separated chromatographically.

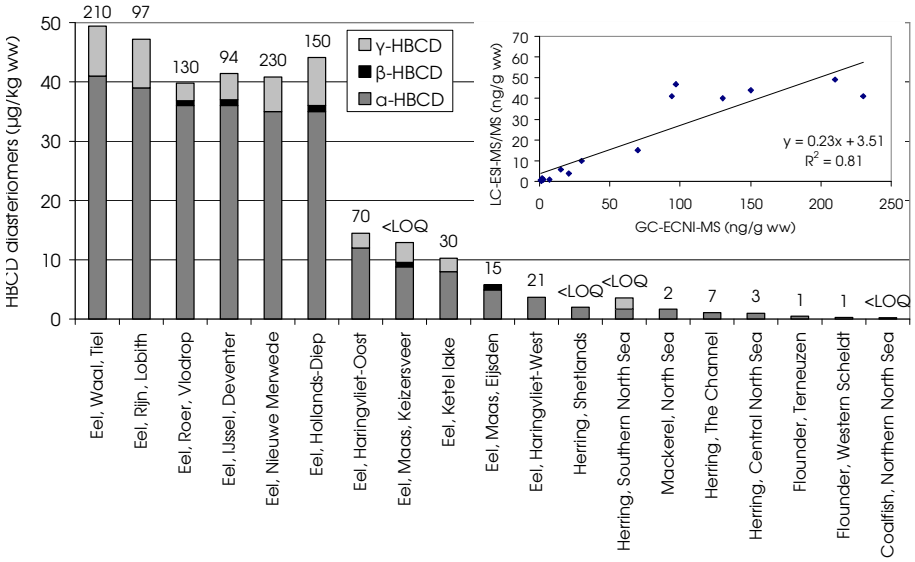

Figure 3.13 HBCD diastereomers (α, β, and γ) profile as determined by LC-ESI-MS/MS. For comparison, the value determined by GC-ECNI-MS is plotted at the top of each bar. Only the fish samples with at least 1 diastereomer concentration above LOQ are plotted. The regression plot (inlay) shows the correlation between the GC and LC data.

However, signal suppression may occur in the electrospray ionisation due to co-eluting matrix constituents. These effects were found to be insignificant by Dodder *et al.* (*21*) although Tomy et al. did report matrix effects occurring in the LC-ESI-MS/MS (which were overcome by using ^{13}C-labeled internal standards) (*12*). In the present study compensation for matrix effects was achieved by using a ^{13}C-labeled HBCD internal standard for each diastereomer. Also, the samples have been submitted to a very thorough clean up, which strongly reduces the chance of ion suppression in the MS. For these reasons, the LC results are regarded the most accurate ones. Obviously, the additional diastereomer profile information is beneficial and crucial when assessing the fate and behaviour of HBCD. Roosens et al. recently presented a comparison of five different methods (LC and GC based) for analyzing HBCD in eel from the river Scheldt basin (*8*). The concentrations in those samples were high (400–1400 ng/g wet weight). The high concentrations enabled the detection of a more specific ion (i.e. $(M\text{-}Br)^-$) for GC-MS quantification, and this enabled the use of ^{13}C-labeled α-HBCD for correcting the GC results. They concluded that the results obtained were very well comparable between methods. In fact, their study shows that these methods were capable of producing data that lie within a 2-fold difference from each other, and this is better than the results from our study (Figure 3.13). However, a few remarks should be made here:

- Their methods 3 (GC-EI-MS), 4 (GC-ECNI-MS), 5 (LC-ITMS) and 6 (LC-ESI-MS/MS) all used ^{13}C-HBCD as internal standard. Nevertheless, differences between these methods were up to 53% (minimum vs. maximum) for the six eel samples analysed. It is therefore surprising that the results were not closer under these conditions.
- The differences between methods were not consistent. For example, compared to the other methods, LC-ESI-MS/MS (method 6) produced the lowest HBCD concentration in eel sample L5p1, whereas it produced the nearly highest result in eel sample L5.
- Because the levels were high in these eel samples, they were able to report data within a 2-fold difference. However, in the majority of the biota samples, concentrations are 1-3 orders of magnitude lower. In those cases, mass labelled standards can no longer be used for GC-MS quantification and most likely the same level of agreement between the GC and LC methods can not be maintained.

Haug et al. (*25*) recently reported on a comparison of GC-MS and LC-MS results from two interlaboratory studies (2005 and 2007). The test materials provided to the laboratories were (in the order of decreasing concentrations) a cod liver oil, Baltic Sea herring filet, salmon fillet, butter and chicken meat. They observed that in the cod liver oil and herring fillet the mean and median GC-MS results were 10-40% higher as compared to LC-MS results. Although the differences were statistically insignificant, this is also an indication that GC based results (in fish) can be higher compared to LC based results. In salmon, this difference was less pronounced. Judging from the higher GC values, the authors concluded that thermal degradation in the GC did not influence the results. However, this statement is debatable as a multitude of experimental and instrumental factors can have influenced the final result, both in GC and LC (as discussed above). Unfortunately, the LC datasets and GC datasets originated from different laboratories, and none of the labs used both techniques, which hampered an in-depth analysis of the different techniques. For the butter and chicken sample insufficient LC results were obtained to allow a comparison with GC. The method sensitivities of most LC laboratories were insufficient for the low HBCD levels in the butter and chicken sample. These studies show that in fish samples (mostly) higher signals are observed with GC-MS as compared to LC-MS. Possibly, on the GC side, a matrix effect occurs leading to elevated results. Poole recently reviewed 'matrix induced signal enhancement' in GC analysis (*18*). He showed that thermolabile compounds can be degraded in hot vaporizing injectors. However, in a sample extract, matrix components can 'shield' the compound, minimizing degradation.

In a study on house-dust, Abdallah et al. (*15*) compared LC-ESI-MS/MS of diastereomers with GC-ECNI-MS. He evaluated the use of different internal standards and daughter ions by the following methods:

1. Quantifying on the m/z 561 (daughter) and using $^{13}C_{12}$-HBCD diastereomers as internal standards (m/z 573)
2. Quantifying on the m/z 561 (daughter) and using BDE 128 as internal standard
3. Quantifying on m/z 79 (daughter) and using BDE 128 as the internal standard.

In house dust, both α- and γ-HBCD may be present in substantial amounts. They detected 14-67% α-HBCD of total-HBCD in dust samples (*26*). Therefore, they also explored the above-mentioned three methods in combination with quantification using α-HBCD or γ-HBCD response factors. The closest match between GC-ECNI-MS and LC-ESI-MS/MS was obtained with method 1 using the α-HBCD response factor and method 3 using the γ-HBCD response factor. The GC vs. LC slope values were 1.05 and 0.97, respectively. This shows that the match between GC-MS and LC-MS may depend on analytical factors on the GC side (i.e. choice of native standard, choice of internal standard) and may also be dependent of the composition of individual samples. The authors recommended LC-ESI-MS/MS as the method of choice and proposed the use of GC-ECNI-MS for screening of samples with very low HBCD concentrations (e.g. human samples). This avoids extensive GC method optimisations and comparison studies.

Issues for further research

Recently, new GC stationary phases were introduced based on ionic liquids with completely different retention characteristics (*27*). Possibly, these phases enable separation of the HBCD diastereomers allowing further study on diastereomer quantification by GC-ECNI-MS. Nevertheless, GC should be used with caution due to possible degradation and because no mass labeled internal standards can be used.

The LC-ESI-MS methods would benefit from further lowering of LODs. However, the m/z 640.7>79 transition has low yields, resulting in reduced sensitivity as compared to the chlorine adduct transition (676.6>640.7). More research is needed to find out if the yield of m/z 640.7>79 can be improved. Alternatively, the chlorine adduct formation may be used. However, as the formation varies from instrument to instrument, more efforts are needed to find out what instrument (and eluent) conditions determine the adduct formation and how they could be influenced for improving instrument sensitivity. The study by Roosens et al. (*8*) showed that even when using ^{13}C-labeled internal standards for the analysis of eel samples, considerable different results were obtained between different LC-ESI-MS instruments. For a full acceptance of LC-ESI-MS, more efforts are needed to explain the experimental differences between the LC- and GC-MS. A further acceptance of LC-ESI-MS is supported by future method comparison (interlaboratory) studies that unambiguously

show that different methods and LC-MS detection techniques provide comparable answers.

Conclusions

This study showed that different diastereomer response factors and degradation of HBCD to PBCDe and TBCDe can significantly reduce the accuracy of the GC based results for HBCD. In addition, the degradation products can also add to the m/z 79 signals of major BDE congeners like 49 and 99. Because no mass labelled standards can be used, especially in low contaminated samples, corrections for inaccuracies are difficult. An LC-MS method overcomes these problems as mass labelled internal standards can be used that allow accurate quantification. Furthermore, the use of a short LC column provides additional sensitivity and speed. This results in a LOD of 20 pg for our LC-MS method which is only 10-fold higher than for GC-ECNI-MS. Therefore, LC-MS is currently the preferred method for determination of HBCD.

Acknowledgements

Sicco Brandsma, Ike van der Veen, Pim Leonards and Jacob de Boer are acknowledged for their experimental work and input in the discussions. The participants of the QUASIMEME Workshop on Brominated Flame Retardants of 23-24 April 2007 (IVM, VU-University, Amsterdam, The Netherlands) are gratefully acknowledged for their stimulating discussions.

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3.3 Struggle for quality in the determination of perfluorinated contaminants[7]

Abstract

The first worldwide interlaboratory study on the analyses of 13 perfluorinated compounds (PFCs) in three environmental and two human samples points at a varying degree of accuracy in relation to the matrix or analyte determined. The ability of 38 participating laboratories from 13 countries to determine the analytes in the various matrices was evaluated by calculation of z-scores according to the Cofino model. The PFCs which were reported most frequently by the laboratories, and assessed with the most satisfactory agreement, were perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). In general, the level of agreement between the participating laboratories decreased in the following order: PFC standard solution (76% satisfactory z-scores of $<|2|$ for PFOS)<human blood (67%)<human plasma (63%)<fish liver extract (55%)<water (31%)<fish tissue (17%). This shows that relative good agreement between laboratories was obtained for the study standard and human matrices. For the fish extract most laboratories underestimated the actual PFOS concentration due to matrix effects. The results for the fish tissue and water are also poor, indicating that the extraction and clean-up steps require further improvement. It was concluded that the PFC determinations in various matrices are not fully mastered yet.

Introduction

The ubiquitous occurrence of perfluorinated compounds (PFCs) in the environment as well as in humans has in recent years been confirmed in an increasing number of studies conducted by laboratories worldwide (*1-5*). Assessments of PFC levels, trends, environmental distributions and human exposures are undertaken by laboratories with varying degrees of experience in analysis of these relatively new environmental pollutants. Compared to other well known organic pollutants, such as persistent organochlorines, the environmental fate and risks associated with PFCs are much less understood. To understand the potential threats of PFCs more data is needed. Thus, the accurate and reproducible determination of various PFCs in environmental and human samples is a necessity. However, this is a challenge in many ways (*6*). The lack of analytical standards, the distinctive physical-chemical properties of the PFCs, and matrix effects resulting in ionisation problems

[7] Based on S.P.J. van Leeuwen, A. Kärrman, B. van Bavel, J. de Boer and G. Lindström (2006) Struggle for quality in determination of perfluorinated contaminants in environmental and human samples, *Environmental Science and Technology*, 40, 7854-7860

during mass spectrometric detection may all contribute to the uncertainty of the analytical data. On top of that, contamination, at every step of the analysis from sampling to detection, is a common problem. Since no certified reference materials are available, only in-house validation of methods has taken place. Laboratories in the field clearly apprehended the need for an interlaboratory study to assess the accuracy and levels of agreement, and thus to further enhance the current state-of-art in PFC analyses (*6*). The first worldwide interlaboratory study on PFCs in human and environmental matrices, organized in 2005, was coordinated by The Netherlands Institute for Fisheries Research (RIVO) and the Man-Technology-Environment (MTM) Research Centre, Örebro University, Sweden. The objectives of this study were to determine the current levels of interlaboratory agreement between determinations of different PFCs in different matrices.

The analysis of PFCs in environmental and human samples includes extraction and clean-up steps followed by the final determination, normally by liquid chromatography-mass spectrometry (LC-MS). The interlaboratory study was designed in such a way that enables the laboratory to determine possible sources of error in each step of the analyses. A study standard was provided to check solely the calibration of the LC-MS instrument, a fish liver extract was provided to check (possible) clean-up and final determination. Human and environmental matrices were provided to evaluate the complete analytical method (i.e. extraction, clean-up and final determination). This approach was shown to work well for laboratories in previous interlaboratory studies on emerging contaminants such as brominated flame retardants (*7*), and has also been advocated by the former EU Community Bureau of Reference (BCR) (*8*). The statistical evaluation was based on the Cofino Model (*9*), which was developed in the early 2000's. This model was chosen as it can handle non-normally distributed datasets (i.e. datasets with extreme (outlying) values and bimodal data distributions). The model evaluates extreme values objectively rather then selecting data from the dataset on subjective criteria. Other techniques, such as robust statistics can handle only 5-7% extreme values (*10*), which is insufficient for this study as typically interlaboratory studies on emerging contaminants deal with higher proportions of extreme values (*11*).

Materials and methods

Study materials

The human matrices selected for the current study were human plasma (HP) and whole blood (HB), and the environmental test materials were fish muscle tissue (FT), fish liver extract (FLE) and water. In addition, a study standard mixture (SS) consisting of undisclosed amounts of ten PFCs was included. Table 3.15 shows details of the test materials provided to the participants.

The environmental samples (SS, FLE, FT and water) were prepared at the Netherlands Institute for Fisheries Research (RIVO) and the human samples (HP and HB) were prepared at MTM. The laboratories were asked to analyze the samples using their in-house methods and their own standards and were given 3 months to analyze the samples and to submit their results and details of the analytical methods to the coordinator for statistical analysis. The following PFCs were included in this study (selection based on often reported PFCs as well as some less commonly reported PFCs): perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorodecane sulfonate (PFDS) and perfluorooctane sulfonamide (PFOSA).
The SS consisted of a methanol solution with the target compounds in undisclosed concentrations as given in Table 3.15. After homogenization, 4 mL aliquots of SS were ampouled in amber glass. The FLE was prepared from flounder (*Platichthys flesus*) livers from the Western Scheldt in The Netherlands. After mincing and homogenization of a pooled sample of 75 g flounder liver tissue, the homogenate was batchwise extracted according to an up-scaled method first published by Hansen et al. (*12*). Subsequently, the lipids were removed from the extract by silica adsorption chromatography (1.8 g column, Merck, Darmstadt, Germany). The MTBE was evaporated from the extract after addition of 1 ml dichloromethane (DCM) and the extract was loaded on the silica column. The lipids (including PFOSA) were eluted by 15 mL DCM, whereas the perfluorinated carboxylates (PFCAs) and perfluorinated sulfonates (PFAS) were retained on the column. The latter compounds were then eluted by acetone (30 mL). The acetone was subsequently replaced by methanol and the extract was analysed to determine the concentrations of the target compounds. Finally the extract was fortified with target compounds (Table 3.15), homogenized and amber glass ampoules with 3 mL of extract (equivalent to 1.5 g fish liver) were prepared.
The FT sample was prepared from fillets of pike perch (*Stizostedion lucioperca*) caught in the IJssel lake, The Netherlands. Muscle tissue was minced and thoroughly homogenized in a Stephan cutter (after addition of butylhydroxytoluene as an antioxidant). Details on the preparation process of similar materials can be found elsewhere (*13*). About 55 g of homogenate was packed in a glass jar tightly closed to prevent leakage. The material was sterilized at 121°C and 3 bar for 30 minutes. Because of the very low levels of some of the target compounds in the fish material, some compounds were spiked (solution in methanol) to the FT prior to the homogenization step (Table 3.15).

Table 3.15 Spiked and assigned concentrations in the test materials included in the interlaboratory study on PFCs. Assigned value was calculated according to the Cofino model. PFDS was not spiked and insufficient data was submitted for calculation of assigned or average value.

Materials/	**SS (ng/ml)**		**FLE (ng/ml)**		**FT (ng/g ww)**		**Water (ng/L)**	**HP (ng/ml)**	**HB (ng/ml)**
Analytes	spiked	assigned	spiked	assigned	spiked	assigned	assigned	assigned	assigned
PFBA	4.3	***7.1***[a]	18	***15.5***	50	-[b]	-	-	-
PFHxA	17.8	9.0	2.7	1.7	-	1.0	5.0	-	-
PFHpA	-	***0.76***	-	***2.2***	-	***2.8***	***4.8***	-	-
PFOA	8.4	7.8	11	12.3	9.7	10.2	19.4	2.0	1.8
PFNA	-	***5.2***	-.	***1.1***	-	***33.3***	***8.7***	***0.62***	***0.47***
PFDA	13.5	7.7	14	8.9	-	2.2	0.42	***0.43***	-
PFUnA	17.3	***17***	2.1	***5.4***	-	***13.1***	***10.4***	***0.23***	-
PFDoA	3.8	4.1	17	18.1	40	19.6	-	-	-
PFBS	47.3	***35***	6.9	***6.9***	21	***13.2***	***17.2***	-	-
PFHxS	24	23.6	19	17.0	22	13.2	6.3	1.2	1.4
PFOS	33.3	28.6	45	19.3	4.4	36.5	19.5	22.2	9.8
PFOSA	60.9	51.5	6.1	5.6	49	20.3	1.0	-	0.39

[a] *Numbers in italic-bold are arithmetic average values (based on datasets with ≥5 submitted results) and given when the assigned value was not calculated*

[b] *No assigned/average value calculated because <5 results reported*

[c] *Not spiked*

Homogeneity analysis was carried out by duplicate analysis of 10 lots out of the complete batch. The compounds determined were PFOS, PFOA (using the method by Hansen et al. (*12*) with additional silica clean-up as mentioned earlier) and the moisture content. The relative standard deviation (rsd) of these determinations was 7.9%, 3.5% and 0.24% respectively. ANOVA statistics and Snedecor F-test revealed no significant difference (95 and 99% level) between the lots and within a lot. The rsd due to inhomogeneity is low compared to the overall rsd (based on all submitted results for the fish tissue) for PFOS and PFOA found in this interlaboratory study. This confirms the homogeneous distribution over the pike perch sample material.

The water sample was prepared from 100 L of naturally contaminated brackish water sampled from the North Sea Canal, just outside the locks of IJmuiden, The Netherlands. The water was filtrated over 0.45 µm paper filter to remove particulate matter and stored in a 100 liter high density polyethylene (HDPE) tank. Microbial activity was reduced by lowering the pH to ca. 2 by addition of 0.5% (v/v) formic acid. The water was thoroughly homogenized and was continuously homogenized during dispersion into 1 L brown high density polyethylene (HDPE) bottles. No homogeneity evaluation was performed as this procedure was considered to result in homogeneous samples (both within the bottle as well as between the bottles). Between

bottling and dispatch, the samples were stored at 4°C. The participants were advised to, prior to subsampling, re-homogenize the content of the bottle by gentle manual shaking.
Authentic human plasma and whole blood samples, without addition of PFCs, from the Swedish general population were used in this study. The samples were provided and administrated by the University Hospital of Örebro (USÖ), Sweden, and released for medical use according to the regulations by the Swedish National Board for Health and Welfare. This includes negative test results for HIV1/2, HBs-Ag, HCV-Ak and Syphilis (VDRL). Blood was taken at one occasion by venipuncture from two individuals. The blood from one donor was centrifuged in order to isolate the plasma, which was collected in a citrate treated 1L blood bag. The plasma material was homogenized and distributed over polypropylene tubes in approximately 7 mL portions and thereafter kept at -20°C before shipment to the participants. The whole blood from the second donor was collected in a heparin treated 1L blood bag. The material was homogenized and divided into polypropylene tubes in approximately 3 mL portions and thereafter kept at -20°C before shipment to the participants. The within and between-tube homogeneity (n=3-8) varied between 1-4 % for PFOS in the whole blood and plasma samples.

Analytical techniques applied by participants
The laboratories were asked to analyze the samples using their in-house methods and their own standards and were given 3 months to analyze the samples and to submit their results and details of the analytical methods to the coordinator for statistical analysis.
Method information of the analytical techniques applied for the analysis of the FT, water and HP samples, as supplied by the participants is condensed in Figure 3.14 and further details can be found in the Annexes of chapter 3.3. Most laboratories applied the ion-pair extraction for the FT sample by Hansen *et al.* (*12*), in some cases slightly adjusted for e.g. higher sample intake. Four laboratories applied methanol or acetonitrile extraction, and the latter was followed by a clean-up step with dispersive solid-phase extraction. Concerning the water sample, 15 out of 21 labs applied solid phase extraction (SPE) extraction and 2 labs applied liquid-liquid extraction (LLE) with MTBE as the organic solvent. For the HP sample the extraction techniques applied are SPE, acetonitrile precipitation and ion-pair extraction (Figure 3.14)
Regarding separation and detection most laboratories used LC coupled to a tandem quadrupole mass spectrometer (MS/MS). One laboratory used LC with high resolution time-of-flight mass spectrometry (TOF-MS) and a few laboratories used single quadrupole mass spectrometry (MS) or ion-trap MS (IT-MS). One laboratory analysed PFOA and PFNA (HP sample) after derivatization by gas chromatography coupled to a mass spectrometer in negative chemical ionisation mode (GC-NCI-MS).

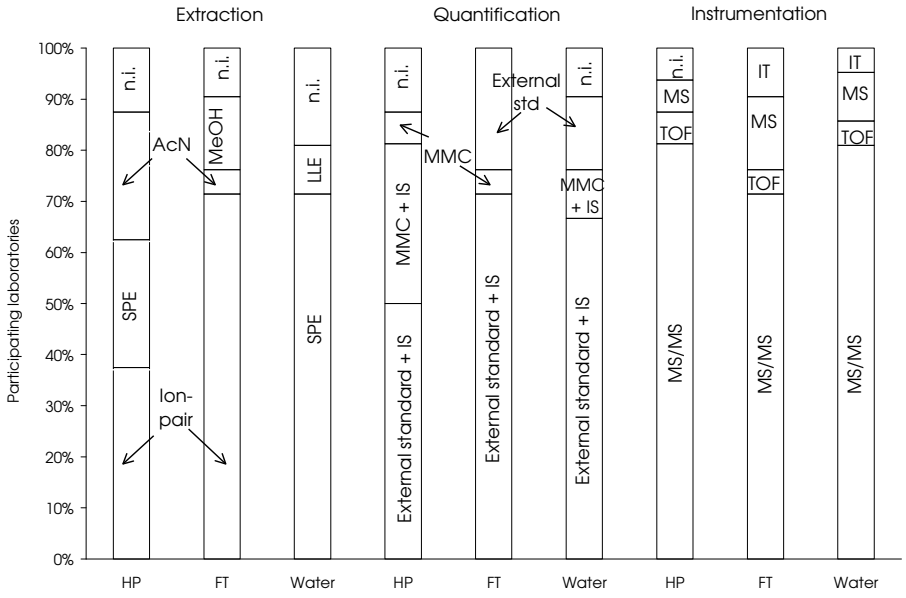

Figure 3.14 The analytical methods used by the participants in the interlaboratory study on PFCs for the analysis of the human plasma (HP) and fish tissue (FT) samples. n.i. = no information available, SPE = solid phase extraction, LLE = Liquid-liquid extraction, AcN = acetonitrile extraction, MeOH = methanol extraction, MMC = matrix match calibration, IS = internal standard, TOF = time of flight MS, IT = ion-trap MS.

For the environmental samples, most laboratories quantified the levels of the target compounds by application of external (solvent based) calibration curves, and the majority used additional internal standards. Only one laboratory applied matrix matched calibration curves. For the HP sample, matrix matched or surrogate matrix (rabbit serum) calibration was used by 6 out of 16 laboratories.

Many laboratories used one or a combination of isotopically labeled and deuterated PFCs such as $^{13}C_2$-PFOA, $^{13}C_2$-PFDA, $^{18}O_2$-labelled PFOS or d5-N-ethyl-PFOSA as procedural internal standard (added prior to extraction) and used this standard in most cases for correction of all compounds analysed. Some laboratories used alternative PFCs such as 7H-perfluoroheptanoic acid (7H-PFHpA), PFHpA, PFNA, PFDoA, perfluoro-3,7-dimethyloctanoic acid and 1H,1H,2H,2H-perfluorooctanesulfonic acid (TH-PFOS). Another internal standard used was 2-(4-chloro-2-methylphenoxy)propanoic acid (meco-prop). For the environmental samples and the human samples, most laboratories reported the sum of the branched and linear isomers, whereas a limited number of laboratories reported the linear isomer only (see also the Annexes of chapter 3.3). The results were taken into account as they were reported by the participants.

Statistical evaluation of reported data

The data of this study is evaluated by determination of z-scores. Z-scores provide descriptive information on the performance of one laboratory in an interlaboratory study. In order to calculate a z-score, the *true* value (defined as the exact concentration of a PFC in the sample) should be obtained. A *spiked* value in the fortified samples is not suitable as true value as the current samples already contained PFCs prior to additional spiking, so that the true value cannot be determined. In practice, the true value is not known and therefore, the *assigned* value is obtained from the dataset as the best estimate. In this study, we also used the assigned value, which was determined as mentioned below.

Population characteristics (the distribution of the PFC data) were obtained by application of Cofino statistics (*9*). This model derives concepts and mathematical procedures from quantum chemistry. Each laboratory is represented by a probability density function (pdf) and a normal distribution is chosen as pdf for individual laboratories. A probability measurement function, PMF, with the mean and standard deviation (sd) are calculated and a probability factor (λ) is obtained which describes how well the PMF describes the dataset. The PMF_1 is chosen as the assigned value as it shows the highest probability and represents the underlying data in the best way. Figure 3.15 shows the PMF1, 2 and 3 for PFOS in the FLE sample and for PFOA in the FT sample. Apart from considering the sd, it is important to include λ in the final judgement of the data. A λ value of <50% would point to a bimodal or multimodal distribution for which the sd of PMF would not be representative.

The assigned value is used for calculation of the z-scores and was calculated for PFOS, PFHxS, PFOA, PFHxA, PFDA, PFDoA and PFOSA, when more than 5 observations from laboratories were reported. The z-score is calculated as the deviation of the individual laboratory result from the assigned value (sd_a) divided by the desired (target) standard deviation (sd_t). The sd_t is set at 0.125 being a measure for target performance. Z- scores of $-2<z<2$ are considered satisfactory. For example for the FLE, a laboratory reported 2.1 ng/ml for PFHxA, whereas the assigned value was 1.66 ng/ml. The z-score is then calculated as (laboratory result-assigned value) / (sd_t*assigned value) = (2.1-1.66) / (0.125*1.66) = 2.14.

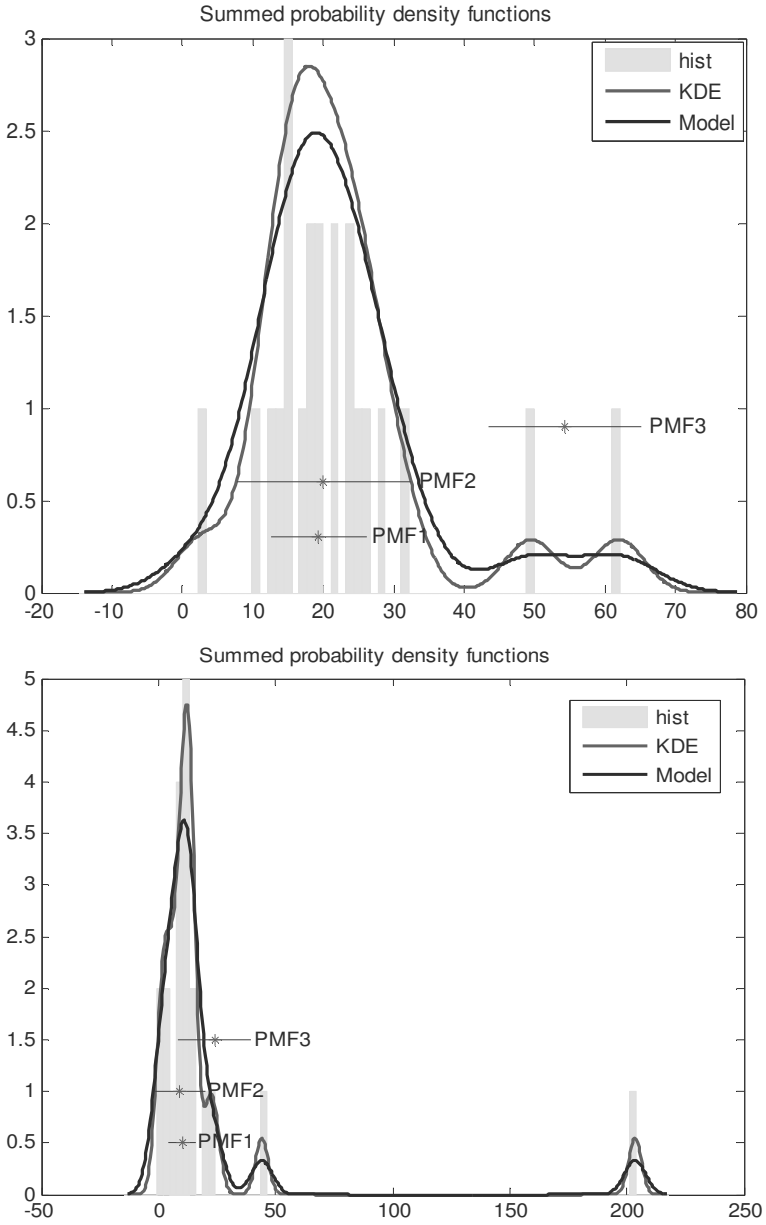

Figure 3.15 Summed probability density functions for calculation of the assigned value (PMF 1) for PFOS in the fish liver extract (left) and PFOA in the fish tissue (right). X-axis: concentration range of submitted results, Y-axis: probability of submitted results (1 stands for 1 submitted result at that concentration, 2 stands for 2 submitted results etcetera). Each bar represents the result for PFOS, submitted by a participating laboratory. Legend: hist = histogram; KDE = Kernel density estimator and Model = Cofino model fit.

Results and discussion

Thirty-eight laboratories initially entered the interlaboratory study. Registrations for participation were from laboratories in Austria, Belgium, Canada, Denmark, Germany, Italy, Japan, Norway, Sweden, Switzerland, The Netherlands, U.K. and USA. In total 31 laboratories signed up for the environmental matrices and 19 for the human matrices. Out of these, 27, respectively 17 laboratories submitted results, often including details on methods used.

PFOS

A summary of the results for PFOS is shown in Table 3.16. The percentage of satisfactory z-scores decreases in the following order: PFC standard solution (76% satisfactory z-scores of $<|2|$) < human blood (67%) < human plasma (63%) < fish liver extract (55%) < water (31%) < fish tissue (17%). The difference between the minimum and maximum reported values ranges from a factor of 5 (HP) up to 100 (FT). This is further illustrated in Figure 3.16, where the distribution of the reported results around the assigned value is shown. Most of the SS results are close to the assigned value whereas especially for the FLE, the FT and the water samples the data is distributed along the entire y-axis, and outside the scale of the figure for a few laboratories.

Calibration Most laboratories employed standard solution curves (or solvent based calibration), and no matrix was used for the calibration (Figure 3.14). Evidently, this works well for the SS, where 76% of the laboratories obtained good calibration results for PFOS (Table 3.16). The coherence of the data can be seen in Figure 3.16, where the majority of the submitted results are close to the assigned value. The assigned value (28.6 ng/mL) is slightly lower than the spiked concentration (33.3 ng/mL) of the SS and the majority of the laboratories are close to the spiked value. However, individual laboratories (see Figure 3.16) such as lab 9 and 6 show a considerable negative bias, whereas labs 26, 10, 3 and 5 show a positive bias. A different isomer pattern for PFOS in the laboratory's standard compared to the pattern in the SS may be an explanation for the deviating results, although this could not be confirmed as no pattern information was available. Furthermore, some laboratories reported only the linear PFOS isomer (e.g. lab 3, 23 and 24 for the HP sample). Although it is likely that their results are lower compared to those reporting the sum of all isomers, this does not show from their submitted results, meaning that other variables play a role as well (e.g. matrix matched calibration (MMC) for lab 23). Laboratory 29 consistently obtained negative z-scores in all matrices, between -1.4 and -6.5, which may be an indication for a systematic calibration error. Also, laboratory 9 and (to a minor extent) 6 consequently show low z-scores for the SS, FLE and FT samples. Apart from general analytical variance due to instrument performance, dilution or concentration errors, other explanations for these z-scores might be the choice or absence of internal standards or the usage of external standards only.

Table 3.16 Summary of PFOS and PFOA results in all test materials using Cofino statistics and descriptive statistics.

	PFOS					
	SS	FLE	FT	Water	HB	HP
Satisfactory |z|<2 (%)	76	55	17	31	67	63
Questionable 2<|z|<3 (%)	3	18	-	13	11	-
Unsatisfactory |z|>3 (%)	21	27	83	56	22	37
N obs > LOQ	29	22	18	16	9	16
N obs < LOQ	1	1	-	1	-	-
Min[a]	6.2	2.7	2.8	4.7	1.8	7.1
Max[a]	65	62	295	112	24	35
Spiked amount[a]	33	45	4.4	-	-	-
Assigned value[a]	29	19	37	20	9.8	22
Average[a]	32	22	55	34	10	23
Median[a]	29	19	40	23	10	23
Standard deviation[a]	12	13	69	32	5.9	7.2
Relative standard deviation (%)	38	57	125	95	56	32

	PFOA					
	SS	FLE	FT	Water	HB	HP
Satisfactory |z|<2 (%)	64	40	25	22	73	61
Questionable 2<|z|<3 (%)	6	20	30	6	-	11
Unsatisfactory |z|>3 (%)	30	40	45	72	27	28
N obs > LOQ	33	25	20	18	11	18
N obs < LOQ		-	1	1	-	-
Min[a]	4.1	4.5	0.54	3.4	1.4	0.50
Max[a]	46	77	204	190	4.1	5.2
Spiked amount[a]	8.4	11	9.7	-	-	-
Assigned value[a]	7.8	12	10	19	1.8	2.0
Average[a]	11	18	22	41	2.2	2.1
Median[a]	8.2	13	13	25	1.9	2.0
Standard deviation[a]	9.0	15	44	49	0.91	1.1
Relative standard deviation (%)	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*

MMC instead of standard solution curves can be used for an accurate quantification when matrix effects due to ion suppression can occur using LC-MS/MS. This is probably the reason why for the FLE sample the assigned value (19.3 ng/mL) is considerably lower than the spiked addition of PFOS (44.7 ng/mL). It should be noted that the actual level of PFOS is probably even higher because the FLE sample already contained PFOS prior to the additional added amount of 44.7 ng/mL. This indicates that the majority of

the laboratories have not been capable to accurately quantify PFOS (using solvent based calibration curves) in the FLE due to matrix effects. Indications that matrix effects can cause inaccuracy was also demonstrated in other studies (*14*).

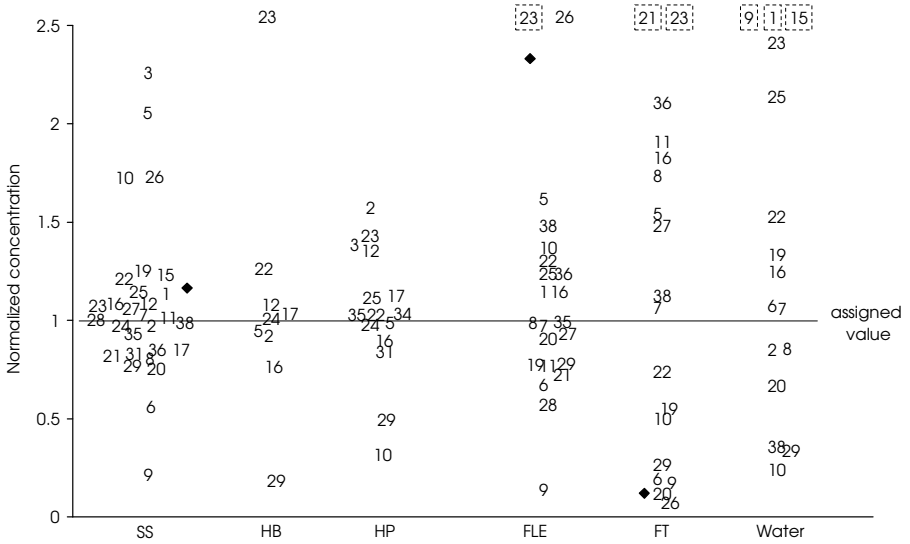

Figure 3.16 PFOS concentrations reported by participating laboratories (lab code 1-38) for the study standard (SS), human whole blood sample (HB), human plasma sample (HP), fish liver extract (FLE), fish tissue (FT) sample and the water sample. Concentrations are normalized to the assigned concentration (continuous line) obtained in each matrix. For example the SS conc. from lab 6 (16 ng/mL) divided by the assigned conc. in the SS (28.6 ng/mL) gives the normalized conc. 0.6. Filled diamonds are the spiked target concentrations, lab codes in dotted squares are out of scale.

Laboratory 23 applied MMC and the PFOS level reported by this lab in the FLE sample (66 ng/mL) is relatively close to the spiked level, although their reported PFOS level in the FT (applying MMC) was ca. 3 times higher than assigned value, suggesting that possible other factors than MMC may have contributed to their elevated results. Concerning the HP and HB samples, the results for those laboratories that applied MMC are scattered. For example, lab 23 reported high PFOS values for both matrices, whereas laboratory 2 had a high z-score for the HP sample but a negative z-score for the HB sample. Laboratories 3 and 35 used surrogate rabbit matrix for the plasma determination resulting in a high z-score for laboratory 3 and a z-score close to zero for laboratory 35. These MMC results are inconsistent, but together with the overall relatively good agreement between the laboratories (compared to the FLE), this may indicate that human blood is less likely to cause ion-

suppression in electrospray LC-MS. Furthermore, the extraction procedures applied may be sufficient of removing matrix interferences originating from HB and HP (*15*).

Use of internal standards. Concerning the use of internal standards, ^{13}C-labelled perfluorinated carboxylic acids (PFCAs) were used by 12 out of 18 participants that submitted PFOS results for the FT, 10 out of 21 for the water and 10 out of 16 participants for the HP. From the z-scores, it cannot be concluded that these laboratories have obtained more accurate PFOS results than other laboratories. Due to different physico-chemical properties, a labeled PFCA standard can behave differently during extraction and clean-up compared to PFOS. Two laboratories used $^{18}O_2$-labeled PFOS for analysis of the HP and HB. Their results for PFOS in the HP (19.9 and 22.7 ng/ml) were close to the assigned value (22.2 ng/ml). Therefore, the application of the currently commercially available isotopically labeled standards including $^{13}C_4$- or $^{18}O_2$-labelled PFOS is recommended to minimize the analytical bias. It should be noted however, that some electrospray ionisation suppression may still occur by application of such an internal standard at high concentrations. Also native PFCs like TH-PFOS, PFDoA, PFNA and PFHpA were used as internal standards. However, these compounds can be found in real environmental samples (*3,16*) and consequently, the application of these internal standards can result in considerable errors. TH-PFOS was detected at considerable amounts in the water sample, resulting in the negative bias of the laboratory that used TH-PFOS as internal standard. Great care should thus be taken when using native PFCs as internal standards and samples should be checked for the absence of these natives prior to application as internal standard. An increasing suite of ^{13}C or ^{18}O-mass labeled PFCs has become commercially available which are strongly recommended for use as internal standards.

Extraction and clean-up. Concerning the FT sample, most laboratories have applied the ion-pairing method originally published by Hansen *et al.* (*12*). Although individual laboratories may have obtained good recoveries in their internal validation experiments, the agreement between the participating laboratories for PFOS in FT is relatively poor. Only 27% of the laboratories obtained a satisfactory z-score of less than |2|. This is merely an effect of the extraction efficiency and the absence of a clean-up procedure since the overall performance for only the instrument calibration is much better (75% satisfactory z-scores). For the human matrices ca. 60% satisfactory z-scores were obtained, although three different techniques were used. Two out of the six laboratories that used ion-pair extraction had satisfactory results for PFOS, four out of five using solid phase extraction, and 2 out of three using acetonitrile extraction methods had satisfactory PFOS results.

The agreement between the submitted water results (16 results for PFOS) is relatively poor. Although it may appear to be an easy matrix to extract (mainly using SPE), the high rsd values may be caused by difficulties arising from the low concentration level (<20 ng/l for all PFCs). Although laboratories

can encounter blank problems from various sources (*17*) at these low levels, only few laboratories reported blank value-corrected results. The reported concentration range for PFOS is considerable: from 6.6 to 112 ng/l. The microbial activity in the water sample was stopped by addition of formic acid to pH 2. Analytical methods for water are developed for samples with pH 7-8, meaning that a low sample pH may have effected the analytical method. Some laboratories have adjusted the pH back to 7, prior to analysis.

Level of experience. The participants were asked to provide information on the number of years of experience in this field (recently started, 1-3 years of >3 years of experience). The laboratories participating in the HM part were more experienced (56% had >3 years experience) compared to the laboratories analyzing the environmental matrices (30% had >3 years experience). This may be the key reason why better results were obtained in the human samples dataset as compared to the environmental samples, although the different nature of the samples and the different analytical methodology should not be neglected. Some laboratories indicated that they just started with method development and used this opportunity to evaluate their progress. Their methods were not fully validated which may have added substantially to the high variance in the datasets. However, principal component analysis (PCA) of the environmental data did not reveal a relation between the level of experience and satisfactory z-scores in FLE, FT or water samples.

MS/MS versus MS. Most laboratories used MS/MS for analysis of PFOS, which enables the detection of daughter-ions, of which m/z 80 and 99 were the most commonly measured. IT-MS can be used in MS/MS mode for perfluorinated carboxylic acids whereas for perfluorinated sulfonates the technique can only be applied in single MS mode. Single quadrupole MS laboratories (labs 20, 21, 24 and 35) and those using ion-trap MS (labs 15, 27 and 36) lack the ability to detect a daughter ion and may therefore suffer from mass interferences. However, from Figure 3.16 it cannot be concluded that there is a systematic bias from laboratories using single quadrupole compared to MS/MS laboratories as they are distributed over the dataset just as the MS/MS laboratories are. This does not *exclude* the occurrence of a mass interference, but other error sources may have a stronger effect on the total analytical error. Several MS/MS laboratories show a considerable bias from the assigned value, which presumably is due to the matrix effect discussed above. Laboratory 22 applied TOF-MS with sufficient resolution to avoid mass interferences, although overestimation occurred for PFOA, PFDA, PFUnA and PFDoA. However, this is not likely to be associated with the TOF-MS resolution. Generally, care should be taken to avoid interferences when using less sophisticated techniques, e.g. single quadrupole MS.

PFOA

The agreement between the laboratories was less good for more complex matrices (fish tissue, fish liver extract and water, Table 3.16). Also, fewer reports were submitted for these matrices. As with PFOS, the majority of the laboratories achieved good results for the SS (Table 3.16) and should not have serious problems with their calibration. Concerning the FLE and the FT samples, assigned and median values are close to the spiked values, although it should be noted that the true value is not exactly known since small amounts of PFOA were expected to be present in the FLE and FT samples prior to the fortification. Compared to PFOS, more laboratories produced satisfactory PFOA results for the FT sample (Table 3.16). This may be associated with the use of a perfluorinated carboxylic acid-type of internal standard ($^{13}C_2$-PFOA, $^{13}C_2$-PFDA, PFDoA or 7H-PFHpA) by most of the laboratories. However, laboratories using no internal standard showed a performance similar to those using internal standards. Some of these laboratories have applied other corrections like e.g. such as recovery data from spiking experiments. The agreement of the 19 submitted results for the water sample was also poor for PFOA (min-max range of 3.4-190 ng/l). The low sample pH of 2 presumably led to a partial protonation the anion PFOA. This may have effected the solubility in the sample as well as the extraction/clean-up of the sample. Further research is underway exploring the effect of storage conditions of the water sample.

The agreement between laboratories for the HP and HB samples was good with a percentage of satisfactory z-scores similar or even better than for the SS (Table 3.16). The min-max range is less than a factor of 10, which is better than for the environmental matrices included in this study. This indicates that currently applied extraction and clean-up methods for human blood are satisfactory as opposed to the methods used for environmental samples.

Other PFC compounds

A considerable number of laboratories submitted data on other PFCs such as PFOSA, PFHxS, PFHxA, PFNA, PFDA and PFDoA. For all PFCs with >5 reported results basic statistical analyses were performed. Limited data was provided for PFBA and PFDS. For PFDS no fully characterized standard was commercially available at the time of the study, which hampered the quantification of this compound. Figure 3.17 shows the rsd values based on all submitted results (no outliers have been removed) for seven PFCs in all matrices included in this study.

Regarding the SS results of PFHxS and PFHxA there is reasonable agreement between the individual laboratories. For PFDoA the situation is not as good, probably due to the low concentration of 3.8 ng/mL in the SS. The high rsd value of PFOSA may be caused by the difficult deprotonation of this neutral compound in the LC-MS electrospray and therefore, GC-MS is suggested as an alternative method (*6*). Concerning the FLE and the FT samples, the rsd

values increase as the matrix becomes more complex. This was also observed for PFOS and PFOA and is confirmed by the PFHxS, PFHxA and PFNA results (Figure 3.17). The results for the water sample are poor: besides the reasons already discussed in the PFOS and PFOA section, the high rsd values may also result from the very low concentration levels in the sample.

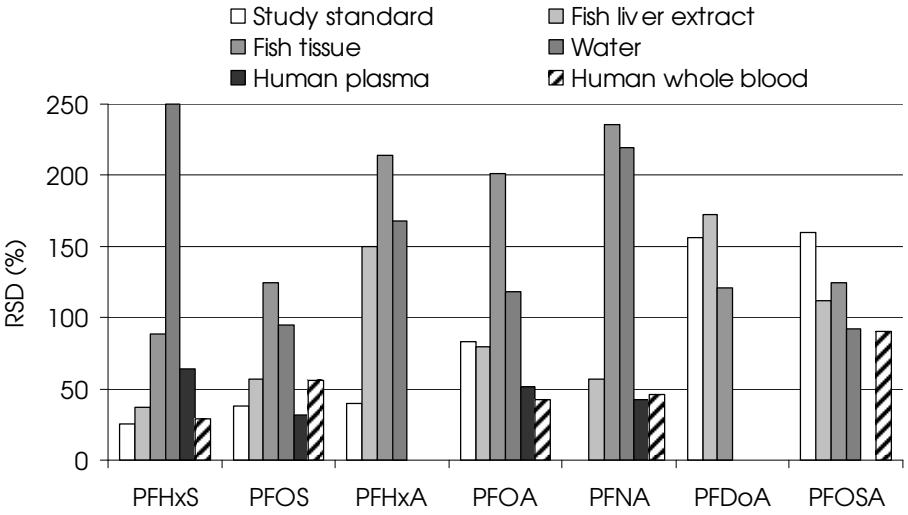

Figure 3.17 Relative standard deviations (RSD) of all reported results for 7 PFCs in all tested matrices. RSD values were only calculated for datasets with >5 submitted results (no bar present in the graph means that <5 datasets were available).

The results for human samples are generally in better agreement compared to the environmental samples. The rsd values of the HP and HB data range from 29-64% (excluding PFOSA), and are often close or even below the SS rsd values. The levels of PFHxA were below the limit of quantification (LOQ) in both human samples. The level of PFOSA was below LOQ for the HP sample.

Summarizing, this study showed that the pool of participating laboratories were not able to produce consistent data, although individual laboratories may have long experience in the field and applied their very well validated methods. In some cases, interlaboratory results can highlight a relation between use of unoptimised techniques or methods and poor results. However, in many cases, laboratories may suffer from multiple difficulties, which hinder clear identification of the error sources. This has also been observed in interlaboratory studies on other emerging contaminants such as BFRs (*11*).

The laboratories are recommended to critically assess their analytical procedures aiming at reducing possible sources of error. Issues to be assessed are (poor) extraction efficiency, suitability of external (or solvent) calibration,

suitability of native PFCs as internal standards, quality of (internal) standards used, matrix effects (and need for clean-up steps to remove those), and the selectivity of MS(/MS) technique. These issues have been discussed here and more information on quality issues in PFC analysis can be found elsewhere (*6*).

Acknowledgements

The authors would like to thank the participants for their contribution to this study. MD Olle Berséus at the University Hospital of Örebro is acknowledged for supplying the blood and plasma sample material. Prof. dr. Wim Cofino is thanked for his support in the statistical evaluation of the data. Ike van der Veen and former collegues of RIVO (Marco Lohman, Evert van Barneveld, Christiaan Kwadijk) are thanked for their skilful contributions to this study. The European Commission is thanked for the financial support of the Perforce project (www.science.uva.nl/perforce, NEST contract 508967). Plastics Europe is acknowledged for their financial support. QUANAS are acknowledged for supporting mrs. Astrid Zammit's stay at RIVO.

Annexes

Detailed method information and participant results (including basic statistics) are available in the Annexes of chapter 3.3.

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Annexes of 3.3

Additional information for the Tables with submitted data

Average, median and standard deviation was calculated after removing "non detect" values and with as many digits the laboratories reported. Distribution figures are given.

NObs > LOQ	Number of observations above limit of quantification
NObs < LOQ	Number of observations below limit of quantification
Assigned value	True value obtained using Cofino statistics
-	Not analysed
ND	Not detected
NQ	Not quantified
<	Less than

Table A-1 Method information for the analysis of the fish tissue (FT)

Lab no	Experience level (years)	Correction for possible blank	Linear and branched together	Internal standard (covering the complete procedure)	Syringe standard	Sample extraction	Mass separation mode	Calibration mode
1*	1-3	No	Sum	N.a.	N.a.	N.a.	LC-ESI-MS/MS	Unextracted
5	?	?	?	^{13}C-PFOA	?	?	LC-ESI-MS/MS	Unextracted
6	Recently	No	Sum	7H-PFHpA	^{13}C-PFOA, ^{13}C-PFDA	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
7	1-3	No	?	^{13}C-PFDA	-	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
8	1-3	Only PFOS	Sum	Mecoprop (deuterated)	-	Confidential	LC-ESI-MS/MS	Unextracted
9	>3	No	Sum	-	-	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
10	>3	No	?	TH-PFOS	-	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted?
11	Recently	No	?	-	-	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
13	1-3	No	Sum	-	-	MeOH extraction, divinyl-benzene SPE clean-up	LC-ESI-MS	Unextracted
15*	Recently	No	Sum	N.a.	N.a.	N.a.	LC-ESI-ITMS(/MS)	Unextracted
16	>3	No	Sum	^{13}C-PFOA	-	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
19	1-3	No	Lin?	^{13}C-PFOA	-	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
20	1-3	No	Sum	-	-	Ion-pair, MTBE	LC-ESI-MS	Unextracted
21	1-3	No	Sum	^{13}C-PFOA	-	Ion-pair, MTBE	LC-ESI-MS	Unextracted
22	>3	No	Sum	7H-PFHpA	$^{13}C_2$-DH-PFOA	Ion-pair, MTBE	LC-ESI-TOF-MS	Unextracted
23	>3	Partly	Lin (PFOS), sum (others)	-?	7H-PFHpA	Ion-pair, MTBE	LC-ESI-MS/MS	MMC**
25*	1-3		Sum	N.a.	N.a.	N.a.	LC-ESI-MS/MS	Unextracted
26	1-3	No	Sum	?	-?	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
27	1-3	No	Sum	^{13}C-PFDA	-	Ion-pair, MTBE	LC-ESI-ITMS(/MS)	Unextracted
28*	Recently		Sum	N.a.	N.a.	N.a.	LC-ESI-MS/MS	Unextracted
29	Recently	No	Sum	PFDoA	PFNA	Ion-pair, MTBE	LC-ESI-MS/MS	Unextracted
30	1-3	No	Sum	^{13}C-PFOA	-	AcN extraction, Envi-Carb clean-up	LC-ESI-MS/MS	Unextracted
33	Recently	No	Lin?	PFNA, ^{13}C-PFOA	-	MeOH extraction	LC-ESI-MS/MS	Unextracted
35*	>3	No	Sum	N.a.	N.a.	N.a.	LC-ESI-MS	Unextracted
36	1-3	No	Sum	^{13}C-PFOA for PFCAs and 7H-PFHpA for PFSAs	-	Ion-pair, MTBE, Silicagel clean-up	LC-ESI-ITMS(/MS)	Unextracted
38	>3		Sum	TH-PFOS, ^{13}C-PFDA and d5-N-ethyl-PFOSA	d3-N-ethyl-PFOSA for PFOSA	MeOH extraction	LC-ESI-MS/MS	Unextracted

* *Participant has not analysed fish tissue sample, (limited) information shown here concerns the fish liver extract method. ** MMC by standard addition to the FT matrix at 3 concentration levels.*

Table A-2 Method information for the analysis of the water sample

no	Experience level (years)	Correction for possible blank	Linear and branched together	Internal standard (covering the complete procedure)	Syringe standard	Sample extraction	Mass separation mode	Calibration mode
1	1-3	Yes	Sum	-*	1H,1H,10H,10H-Hexadecafluor 1,10-decandiol	Confidential	LC-ESI-MS/MS	Unextracted
2	>3	?	Sum	13C-PFOA and 13C-PFDA	13C-PFHEA	SPE (styrene divinylbeneze)	LC-ESI-MS/MS	MMC**
5	?	?	?	13C-PFOA	?	?	LC-ESI-MS/MS	Unextracted
6	Recently	No	Sum	-	13C-PFOA, 13C-PFDA	Confidential	LC-ESI-MS/MS	Unextracted
7	1-3	No	?	PFHpA	-	SPE (C18)	LC-ESI-MS/MS	Unextracted
8	1-3	No	Sum	Mecoprop (deuterated)	-	SPE Lab	LC-ESI-MS/MS	Unextracted
9	>3	No	Sum	-	-	SPE (Oasis HLB?)	LC-ESI-MS (TSQ700)	Unextracted
10	>3	No	?	TH-PFOS	-	SPE (Oasis HLB)	LC-ESI-MS/MS	Unextracted?
13	1-3	No	Sum	-	-	SPE (divinylbenzene)	LC-ESI-MS	Unextracted
15	Recently	No	Sum	13C-PFDA	13C-PFOA	LLE with MTBE	LC-ESI-ITMS(/MS)	Unextracted
16	>3	No	Sum	13C-PFOA	-	SPE (Oasis Wax)	LC-ESI-MS/MS	Unextracted
19	1-3	No	Lin?	13C-PFOA	-	SPE (Oasis HLB)	LC-ESI-MS/MS	Unextracted
20	1-3	No	Sum	-	-	SPE (styrene divinylbenzene polymethacrylate)	LC-ESI-MS	Unextracted
22	>3	No	Sum	7H-PFHpA	13C2-DH-PFOA	SPE (Oasis HLB)	LC-ESI-TOF-MS	Unextracted
23	>3	Partly	Lin (PFOS), sum (others)	-	7H-PFHpA	SPE(porous graphitic carbon)	LC-ESI-MS/MS	MMC***
25	1-3	Yes	Sum	13C2-PFOA and TH-PFOS	11H-PFUnA	SPE (Oasis HLB)	LC-ESI-MS/MS	Unextracted
28	Recently	No	Sum	13C-PFDA	-	-	LC-ESI-MS/MS	Unextracted?
29	Recently	No	Sum	PFDoA	PFNA	LLE with MTBE	LC-ESI-MS/MS	Unextracted
30	1-3	No	Sum	13C-PFOA	-	SPE (Oasis HLB)	LC-ESI-MS/MS	Unextracted
33	Recently	No	Lin?	PFNA, 13C-PFOA	-	SPE (C18)	LC-ESI-MS/MS	Unextracted
38	>3	Partly	Sum	TH-PFOS, 13C-PFDA and (?) d5-N-ethyl-PFOSA	d3-N-ethyl-PFOSA?	SPE (Oasis HLB)	LC-ESI-MS/MS	Unextracted

* *means not applied (in this case no internal standard was applied)*

** *MMC = matrix match calibration (not specified)*

*** *MMC by standard addition to the water matrix at 2 concentration levels*

Table A-3. Study standard (SS) results (ng/ml)

Lab. code	PFOS	PFOA	PFNA	PFBS	PFOSA	PFHxS	PFHxA	PFHpA	PFDA	PFUnA	PFDoA	PFBA	PFDS
1	32.70	9	<1	-	64.2	-	21.60	3	8.3	17	4.3	-	<2
2	28.4	8.18	<0.130	37.0	79.2	25.1	11.0	<0.105	7.70	14.9	3.23	-	-
3	65	6.4	ND	-	-	-	15	ND	6.8	16	4.5	-	-
5	59.1	8.3	ND	-	8.4	-	-	-	10.9	21.7	6.6	-	-
6	16	6.5	< 0.4	-	40	15	-	< 0.4	7.5	8.0	3.0	-	-
7	29.8	7.7	ND	-	54.4	23.9	-	ND	7.3	13.1	-	-	-
8	23.02	12.05	ND	-	55.44	22.22	8.67	ND	8.86	-	-	-	-
9	6.2	12.7	2.7	15.3	13.5	16.5	11.9	-	7.1	12.3	7.3	-	0.5
10	49.8	9.7	-	-	30.9	17.3	11.8	-	-	-	-	-	-
11	29.43	4.05	-	-	-	-	-	-	-	-	-	-	-
12	31.2	8.4	<0.2	35.2	-	-	-	-	-	-	-	-	-
13	NQ	12.5	ND	-	-	-	-	-	ND	ND	ND	-	-
15	35	7.2	< LOQ	53	37	-	4.2	< LOQ	9.4	-	3.5	-	-
16	30.85	7.37	0.08	35.15	54.97	25.21	8.49	0.13	6.41	10.54	3.08	4.97	-
17	24.4	7.4	ND	-	35.3	-	-	-	7.1	-	-	-	-
19	36	46	-	7.1	690	31	-	-	-	-	5.1	-	-
20	21.5	7.1	0.1	33.8	40.9	21.5	5.0	0.2	7.2	10.8	3.5	2.1	-
21	24	5	<1.6	25	52	26	7	1.4	7	11	3	-	-
22	34.7	12.7	0.11	42.9	59.1	26.2	15.4	0.21	13.2	24.7	6.74	5.07	<0.01
23	31.5	7.5	<0.5	48	87.5	24	9	<0.5	10	17	6.3	-	0.8
24	28	7	0.3	36	73	20	7	-	8	11	<0.3	-	<0.5
25	32.9	7.88	0.18	44.1	65.4	23.6	8.69	-	9.53	18.4	8.2	-	-
26	49.7	40.8	38	38.2	58.2	34.4	15	LOD	13.8	56.6	19.9	17.8	9.2
27	29.9	6.5	-	35.7	55.5	23.3	10.4	-	4.7	8.1	12.3	5.3	-
28	29	9	<2	38	387	32	8	<2	9	46	65	-	-
29	22	9.2	-	53	-	30	12	0.58	6.4	15	-	-	-
30	-	10	ND	-	-	-	6.6	-	9.3	14	2.8	-	-
31	23.8	7.3	<0.1	22.1	35	10.5	-	<0.4	6.8	14.5	4.3	-	-
32	-	12.27	-	-	-	-	-	-	-	-	-	-	-
33	-	9.0	-	-	-	-	-	-	-	-	-	-	-
35	26.7	7.73	<0.50	-	-	-	-	-	-	-	-	-	-
36	24	4	<3.7	37.2	29.8	21.4	8	-	5.9	6	<6.5	-	-
38	28.7	20.8	ND	-	81.8	-	-	-	10.6	6	4.5	-	-
NObs > LOQ	29	33	7	18	24	20	20	6	25	22	20	5	3
NObs < LOQ	1		19					10	1	1	3		3
Spiked amount	33.3	8.4	-	47.3	60.9	24	17.8	-	13.5	17.3	3.8	4.3	-
Assigned value	28.6	7.8	-	-	51.5	23.6	9.0	-	7.7	-	4.1	-	-
Min	6.2	4.1	0.08	7.1	8.4	10.5	4.2	0.13	4.7	5.8	2.7	2.1	0.5
max	65	46	38	53	690	34	22	2.8	14	57	65	18	9
Average	32	11	5.2	35	91	23	10	0.76	8.3	17	8.8	7.1	
Median	29	8.2	0.15	37	55	24	8.9	0.21	7.7	14	4.5	5.1	
St. dev.	12	9.0	13	12	146	5.9	4.2	1.0	2.2	12	14	8.2	
RSD (%)	38	83	256	34	160	25	40	134	26	72	156	87	

Table A-4. Fish liver extract results (ng/ml)

Lab code	PFBA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFBS	PFHxS	PFOS	PFDS	PFOSA
1	-	17	11	18	1.7	9.8	4.8	20	-	-	22	17	8.8
5c*	-	-	-	8.9	0.79	9.7	4	17.2			31.2		3
6	-	-	< 0.4	10	0.6	8.8	1.5	16	-	13	13	-	4.6
7	-	-	<LOD	13.1	<LOD	8.8	3.8	-	-	17.4	18.8	-	3
8	-	1.36	0	16.07	0.66	11.77	-	-	-	17.74	19.18	-	5.87
9	-	-	-	4.5	0.7	2.4	1	5.5	4	5.4	2.7	0.5	1
10	-	3	-	13.2	-	-	-	-	-	12.3	26.4	-	3.3
11	-	-	-	8.32	-	-	-	-	-	-	14.83	-	-
15c	-	-	-	26.1	-	< LOD	-	-	< LOD	-	< LOD	-	6.1
16	22.06	1.3	0.11	12.18	0.81	8.19	2.48	12.75	5.4	19.16	22.16	-	4.67
19	-	-	-	77	-	-	-	22	<0.39	13	15	-	53
20	9.3	1.6	0.2	11.7	0.4	9.2	2.3	18.2	5.3	17.3	17.5	-	5
21	-	<7	1.4	8.7	<1.6	5.8	1.5	13	<15	20	14	-	27
22	18.6	2.58	0.23	22.9	2.48	21.3	6.64	34.2	7.01	19	25.1	0.24	6.69
23	-	0.63	<0.5	21	1.6	20	4.8	27	11	27	62	1.2	11
25	-	0.84	-	13.7	1.08	28.6	11.2	54	3.61	16.6	23.9	-	10.4
26	9.3	2.1	LOD	29.1	-	31.9	12.4	95.7	13.7	36.6	49.5	<LOD	9.7
27	18.1	ND	-	12.4	-	6.2	14.5	15.1	<LOD	18.2	17.8	-	<LOD
28	-	<2	<2	11	<2	11	11	337	<5	15	11	-	24
29	-	1.6	<0.2	10	VS**	7.6	1.9	IS**	5.5	16	15	-	-
30c		2.1	-	24.6	1.4	20.6	5.4	19	-	-	-	-	-
33	-	-	-	19	-	-	-	-	-	-	-	-	-
35	-	-	-	15.6	0.61	-	-	-	-	-	19.2	-	-
36	-	<3.5	-	12.2	<3.7	15.7	<3.5	24.1	<3.0	16.6	23.9	-	-
38c	-	-	-	40.6	-	8.4	3.2	38.8	-	-	28.6	-	14.7
NObs > LOQ	5	11	6	25	12	19	17	17	8	17	22	4	18
NObs < LOQ	-	4	6	-	4	1	1	-	6	-	1	1	1
Spiked amount	18	2.7	-	11	-	14	2.1	17	6.9	19	45	-	6.1
Assigned value	-	1.7	-	12.3	-	8.9	-	18.1	-	17.0	19.3	-	5.6
Min	9.3	0.63	0	4.5	0.4	2.4	1	5.5	3.61	5.4	2.7	0.24	1
Max	22.06	17	11	77	2.48	31.9	14.5	337	13.7	36.6	62	17.325	53
Average	15.5	3.1	2.2	18.4	1.1	12.9	5.4	45.3	6.9	17.7	22.4		11.2
Median	18.1	1.6	0.2	13.2	0.8	9.7	4.0	20.4	5.5	17.3	19.2		6.4
St. dev.	5.8	4.7	4.4	14.6	0.6	8.0	4.2	78.1	3.6	6.6	12.7		12.6
RSD	38	150	202	79	57	62	78	172	51	37	57		112

* *Submitted data converted to ng/ml, ** VS: volume standard, IS: internal standard*

Table A-5. Fish tissue results (ng/g ww)

Lab code	PFBA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFBS	PFHxS	PFOS	PFDS	PFOSA
5	-	-	-	9.9	0.9	1.5	1.6	5.7	-	-	56.3	-	31.5
6	-	-	<0.4	5.3	0.6	1.3	0.4	14	-	0.6	7.1	-	20
7	-	-	-	1.7	<LOD	<LOD	<LOD	-	-	12.6	38.8	-	21.8
8	-	0.18	0.24	13.85	0.68	2.99	-	-	-	22.01	63.32	-	30.2
9	-	36.2	-	44.2	48.2	44.1	42.6	53.2	8.1	7.1	5.9	35.3	3.4
10c*	-	4.2	-	13	-	-	-	-	-	4.7	18.3	-	17.0
11	-	-	-	14.02	-	-	-	-	-	-	69.64	-	-
13	-	-	-	12.5	<LOD	<LOD	<LOD	<LOD	-	-	-	-	-
16	-	<0.25	<0.25	7.78	0.99	1.9	1.09	26.11	16.46	16.36	66.76	-	29.83
19	-	-	-	13	-	-	-	17	<0.54	3.0	20	-	230
20	1.91	0.01	0.06	0.54	0.15	0.19	0.07	1.50	0.95	1.13	4.41	-	1.97
21	-	337	13	204	272	<0.5	90	54	23	54	295	-	112
22	29.7	<0.67	0.32	9.85	1.17	2.64	1.03	18.3	16.0	13.0	27.0	0.08	26.6
23	-	<3	1.3	9.2	1.4	3.8	2	191	22	26	128	<0.5	207
26	-	<LOD	1.9	2.7	70.8	0.63	0.39	7.9	2.3	29.5	2.8	<LOD	3.6
27	41	<LOD	-		-	29	<LOD	74	<LOD	21	54	-	<LOD
29	-	1	3.1	3.8	VS**	<1.0	<0.5	IS**	7.7	6.3	9.63	-	-
30	-	<LOD	-	13	1.8	3.7	2	47	-	-	-	-	-
33	-	-	-	24	-	-	-	-	-	-	-	-	-
36	-	<1.7	-	13	<1.9	4.0	<1.8	21.0	22	24	77	-	-
38	-	-	-	21.3	0.9	1.5	2.1	26.9	-	-	41.1	-	110.2
NObs > LOQ	3	6	7	20	12	13	11	14	9	15	18	2	14
NObs < LOQ	-	7	2	1	3	4	5	1	2	-	-	2	1
Spiked amount	50	-	-	9.7	-	-	-	40	21	22	4.4	-	49
Assigned value		1.0		10.2		2.2	-	19.6	-	13.2	36.5	-	20.3
Min	1.91	0.01	0.06	0.54	0.15	0.19	0.07	1.5	0.95	0.6	2.8	0.08	1.97
Max	41	337	13	204	272	44.1	90	191	23	54	295	35.3	230
Average		63.2	2.8	21.8	33.3	7.5	13.1	39.9	13.2	16.1	54.7		60.4
Median		2.6	1.3	12.8	1.1	2.6	1.6	23.6	16.0	13.0	40.0		28.2
St. dev.		135.1	4.5	43.9	78.7	13.3	28.5	48.4	8.6	14.2	68.5		75.5
RSD %		214	161	201	236	178	218	121	65	88	125		125

* *Converted to ng/g ww, ** VS: volume standard IS: internal standard*

Table A-6. Water sample results (ng/l)

Lab code	PFBA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFBS	PFHxS	PFOS	PFDS	PFOSA	TH-PFOS
1c*	-	94.40	17.10	44.10	9.40	6.40	14.10	6.50	-	-	112.00	16.40	8.10	
2	-	4.02	2.48	20.0	1.45	1.09	<0.506	<0.361	16.3	5.02	16.6	-	1.46	
6	-	-	< 4	11	< 2	< 2	< 2	< 2	-	< 4	21	-	< 1	
7	-	-	IS**	15.2	n.d.	0.3	0.3	-	-	5.6	20.7	-	0.8	
8	-	4.2	2.11	18.75	0.55	0.78	-	-	-	4.45	16.66	-	1.42	
9	-	233.5	-	117.5	62.0	29.6	37.0	43.8	-	232.0	105.0	39.4	2.31	
10	-	< 0,5	-	3.4	-	-	-	-	-	< 0,5	4.7	-	< 0,5	
13	-	-	-	10	<LOD	<LOD	<LOD	<LOD	-	-	-	-	-	
15	-	-	-	31	-	< LOD	-	-	16	-	56	-	< LOD	
16	22.86	5.04	2.09	17.1	0.46	0.31	<0.04	<0.04	13.18	6.63	24.3	-	0.8	
19	-	-	-	190	-	-	-	LQL	8.4	7.6	26	-	4.0	
20	1.2	10.7	1.9	10.8	0.5	0.4	<LOD	<LOD	30.6	4.3	13.0	-	1.2	
22	<0.02	8.23	3.74	29.9	2.76	1.50	0.13	<0.03	17.0	8.01	29.8	0.23	0.94	186
23		-	-	30	0.9	2.5	0.5	<0,2	-	7.7	47	3.1	4	100
25	-	44.7	-	44.5	5.9	10.7	< LOD	< LOD	27.4	10.7	41.7	-	< LOD	
28c	-	<2000	<2000	<2000	<2000	<2000	<2000	<2000	<5000	<5000	<5000	-	<20000	
29	-	6.1	4.2	13	VS**	<1.0	<1.0	IS**	8.7	6.8	6.6	-	-	
30	-	-	-	-	<LOD	<LOD	<LOD	<LOD	-	-	-	-	-	
33c	-	-	-	110	-	-	-	-	-	-	-	-	-	
38	-	-	-	29.5	2.6	<LOD	<LOD	<LOD	-	-	6.9	-	-	
NObs > LOQ	2	9	7	18	10	10	5	2	8	11	16	4	10	2
NObs < LOQ	1	2	2	1	4	7	10	11	1	3	1	-	5	-
Spiked amount	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Assigned value	-	5.0	-	19.4	-	0.42	-	-	-	6.3	19.5	-	1.0	-
Min	1.2	4.02	1.9	3.4	0.46	0.3	0.13	6.5	8.4	4.3	4.7	0.23	0.8	100
Max	22.86	233.5	17.1	190	62	29.6	37	43.8	30.6	232	112	39.4	8.1	186
Average		45.7	4.8	41	8.7	5.4	10.4		17.2	27.2	34.2		2.5	
Median		8.2	2.5	24.8	2.0	1.3	0.5		16.2	6.8	22.7		1.4	
St. dev.		76.6	5.5	49	19.0	9.2	16.0		8.0	68.0	32.4		2.3	
RSD		168	114	118	219	171	154		47	250	95		92	

* *Submitted data converted to ng/l, ** VS: volume standard, IS: internal standard*

3.4 Significant improvements in the determination of perfluorinated contaminants[8]

Abstract

The 2nd international interlaboratory study (ILS) on perfluorinated compounds (PFCs) in environmental samples was organized to assess the performance of 21 North American and European laboratories on the analysis of PFCs in water and fish. A study protocol was provided to assess accuracy, precision, matrix effects and to study the use of in-house standards. The participants used shared native and mass-labelled standards that were provided for this study to quantify the PFC concentrations in the samples. Matrix effects in the determination of PFCs can be considerable and can decrease the sensitivity, the accuracy and internal standard recoveries. Therefore, two quantification methods were evaluated by all laboratories: standard addition quantification (SAQ) and solvent based calibration curve quantification (SBCCQ; using mass-labelled internal standards (IS)). The between laboratory reproducibility (i.e. coefficient of variance) was smaller for the SBCCQ results (except for PFBS and PFHxS for which no mass-labelled analogues were available) compared to those obtained by the SAQ method. The within laboratory precision of individual laboratories is good (mean for all PFCs in water 12% and 6.8% in fish). The good performance is partially attributable to the use of well-defined native and mass-labelled standards. Therefore, the SBCCQ method is recommended. The results show that analytical methods for PFCs in water and fish have improved considerably. Critical steps identified in this study are (i) the use of well-defined native standards for quantification, (ii) the use of mass-labelled internal standards (preferably one for each target compound) and (iii) minimization of matrix effects by a better clean up.

Introduction

Perfluorinated compounds (PFCs) are omnipresent in the environment (*1-3*). To study the distribution of these chemicals in the environment and to assess the environmental and human exposure, many laboratories have developed methods for analysis of PFCs in environmental matrices. For several years, the quality of data obtained was a major issue of concern (*4*). Identified problems in the quantification were the limited availability of high quality standards and mass-labelled standards, severe matrix effects and interferences, the occurrence of branched isomers and blank problems due to contamination

[8] Based on S.P.J. van Leeuwen, C.P. Swart, I. van der Veen and J. de Boer (2009) Significant improvements in the analysis of perfluorinated compounds in water and fish: results from an interlaboratory method evaluation study *Journal of Chromatography A* 1216, 401-409

from labware and instrumentation. This was reflected in the 1st interlaboratory study (ILS) conducted in 2004/2005 and organized within the framework of the European Perforce project (http://www.science.uva.nl/PERFORCE/index.htm). The between laboratory coefficients of variation for environmental samples amounted to 95% for perfluorooctane sulfonate (PFOS) in water and 125% for PFOS in a fish sample (*5*). This illustrated that improvement of method performance was required in order to obtain reliable analytical results.
Meanwhile, a larger number of high quality standards have become commercially available, and the list of these standards continues to expand. Furthermore, a wide range of mass-labelled standards is available for use as internal standards. Earlier on, many laboratories used ion-pair extraction for biota often leading to inaccurate results. Nowadays, more diverse extraction, clean-up and quantification approaches exist (*6,7*) with good performance characteristics. Yamashita et al. reported on a method evaluation study of PFOS and perfluorooctanoic acid (PFOA) in water (performed in the framework of an ISO technical working group) (*8*). Good performance (23-32% RSDs for PFOS and 27-30% RSDs for PFOA) in seawater was reported, but this study limited to PFOS and PFOA. The present study was initiated and aimed at evaluation of the following analytical aspects:

(i) Analysis of 11 perfluoroalkyl carboxylates, 4 perfluoroalkyl sulfonates and perfluorinated sulfonamide (PFOSA)
(ii) Comparison of results obtained by standard addition quantification (SAQ) and solvent based calibration curve quantification (SBCCQ)
(iii) Determination of the precision of individual laboratories
(iv) Determination of the influence of in-house standards on the quantification
(v) Quantification of the matrix effect

This work was performed on a fish sample and a freshwater sample.

Materials and methods

The PFCs included in this study were perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrA), perfluorotetradecanoic acid (PFTeA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), PFOS, perfluorodecane sulfonate (PFDS) and PFOSA.

Design of the study

This study was designed to evaluate the performance of the current state-of-the art methods in terms of quantification principles, use of standards, accuracy, precision and matrix effects. The participants were invited to a 2-day workshop to stimulate exchange of expertise and receive instructions on

how to conduct the experiments according to the exercise protocol. At this workshop, specialists from industries and research institutes presented their technical insights in the extraction, clean-up and analysis of PFCs in water and biota. From these discussions, the following critical factors were identified: the use of well-characterized native and mass-labelled standards, different responses of branched and linear compounds, control of method and instrumental blanks and the occurrence and influence of matrix effects. Based on these discussions, a study was designed that is shown in Table 3.17. A protocol was developed to provide guidance to the participants for performing the study. It included suggestions on how to improve the critical analytical aspects to obtain proper data on accuracy and precision, while avoiding e.g. blank problems.
This design enabled the determination of performance characteristics of the two quantification methods used. SBCCQ was chosen as this is a commonly applied (routine) method in most laboratories, whereas SAQ is very suitable for unknown matrices (and matrix effects) as it intrinsically takes matrix effects into account. The SAQ method was derived from general guidelines on method validation (*9,10*). Furthermore, the design of the study enabled the determination of factors contributing to method accuracy and precision (e.g. use of in-house standards and matrix interferences).

Table 3.17 Study design. In-house methods and instruments were used for all experiments.

Study aspect	Experimental execution
Variance due to in-house standard	Quantification of a 50-ng/mL in-house standard against the shared standard (50 ng/mL). No mass-labelled standards.
Accuracy by two different quantification methods: SBCCQ and SAQ*	SBCCQ: analysis of the sample using shared native and mass-labelled standards. SAQ: analysis of the sample by standard addition of 1, 2 and 4 times the PFC levels already present in the sample. No mass-labelled standards are used.
Precision	Triplicate analysis of the sample by SBCCQ using shared native and mass-labelled standards.
Matrix interferences in ESI-MS(/MS)	Preparation of an extract and fortification by 50 ng/mL of the shared standard. Net peak areas compared to a 50-ng/mL standard. No mass-labelled standards are used.

* *SBCCQ: solvent based calibration curve quantification; SAQ: standard addition quantification*

Study material preparation

Water sample - The water sample was taken in April 2007 from the North Sea Canal (which connects Amsterdam with the North Sea) close to the Assendelft-Spaarndam ferry (The Netherlands). The water here is mainly freshwater, with a slightly elevated salinity due to the inflow of seawater from the IJmuiden locks. Five 30 L high-density polyethylene (HDPE) containers

were filled with water and after transport to the laboratory they were stored at 4°C. Residuals were allowed to settle and after 1 week, the water was slowly decanted in a large 150 L container while filtering over 3 stainless steel sieves with (top to bottom) 1.0, 0.53 and 0.22 µm pore sizes for removal of residual particles. The large container containing approx. 150 liter of water sample was kept at 4°C under continuous mixing using a stainless steel stirring device. All materials that came in contact with the water were rinsed three times with ultra pure methanol (JT-Baker, HPLC Analyzed) prior to use. The container and 30 L containers were all tested for blank contributions. The water sample was characterized by Omegam Laboratories (Amsterdam, The Netherlands) and the results show a typical freshwater composition (pH 6.4, conductivity 1529 mS/m, calcium 160 mg/L, magnesium 320 mg/L, dissolved organic carbon 14 mg C/L, total organic carbon 14 mg C/L and hardness 35 mequiv./L). A preliminary PFC analysis was carried out, and based on the low PFCs concentrations detected (<1 for PFNA to 20 ng/L for PFBA), it was decided to spike the water sample with relevant PFCs mentioned in Table 3.18. This was done so as to facilitate the detection of the target compounds by all laboratories. A stock solution of the target PFCs was made in methanol. This solution was added slowly (under continuous mixing) to the large water container containing 150 L water sample. The sample was homogenized for 48 hrs. Subsequently, 500 mL HDPE bottles were filled under continuous homogenization of the bulk sample. The bottles were stored at 4°C until transportation.

Table 3.18 PFCs spiked to the fish and water sample. Concentrations refer to the spiked amount.

Compound	Fish (ng/g ww)	Water (ng/L)
PFBA	N.s.	25
PFPeA	N.s.	5.0
PFHxA	N.s.	5.0
PFHpA	N.s.	5.0
PFOA	22.6	25
PFNA	17.2	5.0
PFDA	21.9	5.0
PFUnA	17.8	5.0
PFDoA	20.1	5.0
PFBS	N.s.	17.7
PFHxS	N.s.	9.5
PFOS	145	23.2
PFOSA	3.2	5

N.s. Not spiked

Fish sample - The preparation of the fish sample was sub-contracted to IMARES (IJmuiden, The Netherlands). The sample was prepared from fillets of flounder (*Pleuronectes platessa*) from the North Sea (52°55'N – 03°30'E (52.916667, 3.500000)), which were caught (ship TX43) on June 15, 2007. The fish was transported to the laboratory and filleted. Approx. 25 kg of fillets were minced thoroughly and homogenized in a Stephan cutter (after addition of 0.02% butylhydroxytoluene as an antioxidant) for 1 hour. Details on the preparation process of similar materials can be found elsewhere (*11*). About 55 g of homogenate was packed in a glass jar that was tightly closed to prevent leakage. Approx. 250 lots were produced. The jars were sterilized at 121°C and 3 bar for 45 minutes. Because of the very low concentrations of some of the target compounds, the fish was spiked with a selection of PFCs (from a solution in methanol). The spike was performed between the mincing and homogenization step by adding the PFC spike solution to the minced fish material. The between-jar and within-jar homogeneity was tested to ensure that all jars contained a homogeneous material and to determine the variety due to the production of the material. These tests were carried out by duplicate analysis of 9 lots out of the complete batch. The compounds determined were PFOS and PFOA, using a method according to Powley et al. (*12*). The relative standard deviations (RSD) were 5.9% and 3.6% (n=18 determinations), respectively. The data was analysed by ANOVA using SoftCRM software, which was developed for producers of certified reference materials (CRMs) according to EU guidelines (*13*). In this study, the software was used for statistical evaluation of the homogeneity of the fish sample. The different lots were not significantly different, indicating that the material was homogeneous.

Shared analytical standards of the target PFCs

At the kick-off workshop (IVM, Amsterdam, The Netherlands, 18-19 March, 2007) the use of standards for quantification was discussed. As it was anticipated that the use of standards from different sources and stocks would significantly influence the results, it was decided to use analytical standards from the same source (so called "shared standards") so as to rule out this source of analytical variance. The analytical standards contained (1st ampoule) PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA and PFTeA all at 2.0 ng/mL and (2nd ampoule) PFBS-K, PFHxS-Na, PFDS-Na and PFOSA at 2.0 ng/mL and PFOS-K at 20 ng/mL. The anion concentrations were 1.77 (PFBS), 1.89 (PFHxS), 18.5 (PFOS) and 1.93 (PFDS) ng/mL. Wellington Laboratories (Guelph, Ontario, Canada) kindly supplied these analytical standards free of charge. Furthermore, Wellington supplied mixtures of mass-labelled standards. These contained (3rd ampoule) $^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-PFDoA at 2.0 ng/mL and (4th ampoule) $^{13}C_4$-PFOS-Na and D3-N-MePFOSA at 2.0 ng/mL.

All individual compounds were >99% linear and >98% pure. Isotopic purity was >99%.

Blank tests of sample containers

Blank tests were carried out on all sample containers in order to rule out possible contributions from the sampling, storage and shipment containers. For the water sample, the sampling containers (five equal containers of 30 L each, 1 storage container of 150 L and a valve, all made of HDPE) were rinsed and left overnight with 500 mL methanol. The methanol was concentrated to a final volume of approx. 0.7 mL and the PFCs were quantified by HPLC-ESI-MS/MS. The bottles used for transport of the samples to the participants (4 different types of 0.5 L each, all HDPE) were rinsed with 100 ml methanol. The methanol was subsequently concentrated and analysed. A blank of 500 mL methanol was concentrated and analysed to account for possible contributions from the solvent and the procedure.

The results of the container blanks in Figure 3.18 show that PFCs were present in all sample, storage and shipment containers. Although absolute concentrations in the 30 and 150 L containers were higher than in the 0.5 L containers, they were lower when expressed on a pg/L basis, taking into account the volume sample material stored in the container (being 30, 150 and 0.5 L, respectively). Shipment container 1 showed elevated concentrations compared to the other types. It was found out after the experiment that the lid of that specific bottle contained a poly(tetrafluoroethylene) (PTFE) lined inlay. Presumably, this has caused the elevated concentrations of some perfluoroalkyl acids, but we have no explanation for the presence of the perfluoroalkyl sulfonates.

All equipment used for preparation of the fish sample was made of stainless steel and cleaned thoroughly prior to use. The transportation jars were made of glass (Carnaud Metal Box in France and delivered through Eurocan Food, Mechelen, Belgium, (volume 70 mL) and a steel lid (lined with a synthetic coating of epoxyphenol/epoxyphenyl lacquer). A blank test was performed on the jars and lids. Four jars (4 replicates) were filled with methanol, closed with the lids and tumbled for 24 hours. The methanol was concentrated and analysed by LC-ESI-Ion Trap MS(/MS). Single MS was used for detection of PFBS, PFHxS and PFOS, whereas MS/MS was used for detection of the perfluoroalkyl carboxylates and PFOSA. The methanol concentrates appeared white (semi-transparant). The results showed no presence of PFCs in the jars and lids (<2.5 ng absolute per jar and lid). When related to the amount of fish sample in the jar (approx. 55 g), the PFC contamination would, if present, be <0.05 ng/g sample. This is far below the concentrations of the target compounds (see Table 3.20).

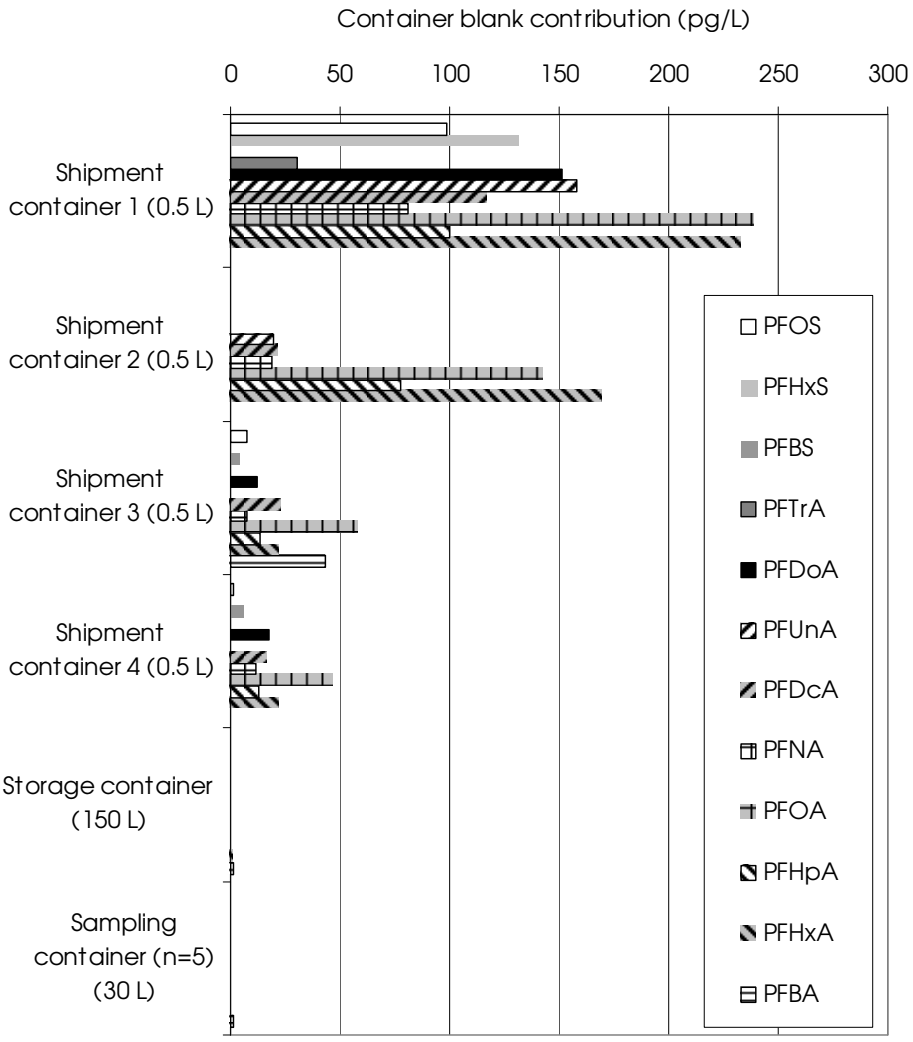

Figure 3.18 Overview of blanks from the water sampling containers (30 L, mean of 5 identical containers, all HDPE), storage tank (150 L, HDPE) and 4 different types of shipment container (500 mL, HDPE and PP containers). Shipment container nr 4 showed the lowest contribution and was selected for shipment of the samples.

Methods used by the participants

In this study, a variety of methods were used by the participants. For the water study, most laboratories concentrated the sample by solid phase extraction (SPE) using Oasis HLB, Oasis WAX and C18 columns or by liquid-liquid extraction (LLE) with methyl-tert-butyl ether (MTBE) (see Table 3.18).

Table 3.18 Analytical methods used by participants in this study[a].

Lab	Fish		Water		Instrumental
	Extraction	Clean-up	Extraction (SPE)	Clean-up	determination
1	IPE (TBA)	Filtration (Nylon)	C_{18}	Wash: water	LC-ESI-QQQMS
2	-	-	Oasis-HLB	No clean-up	LC-ESI-QQQMS
3	LSE (Acn)	Envicarb	-	-	LC-ESI-QQQMS
4	LSE (MeOH)	Active carbon	C_{18}	No clean-up	LC-ESI-QQQMS
5	LSE (MeOH)	SPE (washing unkn, MeOH-type elution)	SPE (type confidential)	Confidential	LC-ESI-QQQMS
6	-	-	Oasis-HLB	Wash: water	LC-ESI-QQQMS
8	LSE (Acn)	Envicarb	Oasis-WAX	Wash: water	LC-ESI-QQQMS
9	LSE (Acn)[b]	SPE (see left)	C_{18}	No clean-up	LC-ESI-QQQMS
10	LSE (Acn)	Envicarb	-	-	FIA-ESI-QQQMS
11	LSE (Acn)	Envicarb	Oasis HLB	Wash: MeOH:H_2O (60:40)	LC-ESI-QTOFMS
12	LSE (MeOH)	Reconstitution in H_2O	Styrene-divinylbenzene SPE, use of IPA	Wash: MeOH:H_2O (20:80)	LC-ESI-QMS
13	LSE (Acn)	Envicarb	-	-	LC-ESI-ITMS(MS)
14	LSE (Acn)	Envicarb	Oasis HLB	Wash: MeOH:H_2O (40:60)	LC-ESI-QQQMS
15	LSE (Acn)	Envicarb	Oasis HLB	Wash: Acn:H_2O (40:60)	LC-ESI-QQQMS
17	LSE (Acn)	Envicarb	LLE (MTBE)	No clean-up	LC-ESI-QQQMS
19	LSE (Acn)	Envicarb, filtration 0.2 µm	Oasis HLB	Wash: acetate buffer	LC-ESI-QQQMS
20	-	-	n.a.[c]	n.a.	LC-ESI-QQQMS
21	LSE (MeOH)	Freezing, centrifugation	SPE (Chromabond HR-P)	Wash: water	LC-ESI-QQQMS
22	-	-	Oasis-HLB	No clean-up	LC-ESI-QQQMS
23	-	-	LLE (MTBE)	No clean-up	LC-ESI-QQQMS
24	-	-	LLE (MTBE)	No clean-up	LC-ESI-QQQMS
	n=15		n=17		

[a] *Abbreviations: TBA: tetrabutylammonium hydrogen sulfate; LSE: liquid solid extraction; Acn: acetonitrile; MeOH: methanol; SPE: solid phase extraction; IPA: ion pairing agent; LLE: liquid liquid extraction; MTBE: methyl-tert-butyl ether; LC: liquid chromatography; ESI: electrospray ionization; QQQ: triple quadrupole; MS: mass spectrometry; FIA: flow injection analysis; QTOF: quadrupole time of flight; Q: single quadrupole; IT: ion trap.*

[b] *After LSE extraction, the following clean-up was performed: freezing out, centrifugation, decanting in 2% H_3PO_4, SPE (Oasis-HLB, wash with 5% MeOH), elution with MeOH (+5% NH_4OH)*

[c] *Not applicable, because 1 mL water was directly injected (large volume injection with preconcentration on the analytical column)*

SPE methods limit to the dissolved fraction of a water sample, whereas the LLE method allows extracting the dissolved and particle-associated fraction of a water sample. It should be noted that, during the sample production, the particles >0.22 µm have been removed from the sample. After loading the sample in the SPE cartridge, clean-up consists of a simple wash step after

loading the sample on the cartridge. The laboratories applying LLE have not used any clean-up.
For the extraction of biota, many laboratories adopted the liquid-solid extraction (LSE) method by Powley et al. (*12*) using a medium polar solvent (methanol or acetonitrile), whereas in the first ILS (*5*), most laboratories used the ion pair extraction (IPE) method, initially published by Hansen et al. (*14*). The latter method was used by one laboratory only. Clean-up of fish extracts is often performed by suspending with Envicarb (or active carbon) and glacial acetic acid. Other clean-up methods included freezing out of matrix constituents, SPE (Oasis HLB) clean-up and centrifugation or filtration for removal of solids.
The analyses were often performed by LC-ESI-MS/MS using a triple quadrupole MS system. Other systems included LC-ESI-(single quadruple)MS, LC-ESI-time-of-flight (TOF)MS/MS, flow injection analysis (FIA)-ESI-MS/MS and LC-ESI-ITMS(/MS).

Results and discussion

The data was collected using report forms specially designed for this study. Participants were asked to provide details on their extraction and clean-up methods, the chromatographic and mass spectrometric conditions and the results of the individual experiments. After submission by the laboratories, the results were collected and statistical evaluation was performed. Afterwards, the results were critically assessed at a technical workshop (18/19 October 2007, IVM, Amsterdam). All participants contributed to a thorough technical discussion on their data. The quality of the data was judged and discussed. Some of the submitted data were removed from the dataset, but only in case of clear technical reasons, such as: (i) high blank values; (ii) SBCCQ calibration curve having a r^2 value <0.99 (iii) detector response of sample being considerably above the response of the highest calibration point; (iv) SAQ curve having a r^2 value <0.95; (v) SAQ spiking levels deviating significantly from the agreed spiking levels (see Table 3.17); (vi) other reasons for lacking confidence (e.g. low performance of the MS instrument). This resulted in a revised dataset, on which the discussion below is based.

Impact of different in-house standards from a variety of (commercial) suppliers

The in-house standards were tested against the shared standards to determine the impact of the in house standards as these originate from a variety of (commercial) suppliers, have different purities and isomeric composition. The participants analysed their in house standard (approx. 50 ng/mL) and the shared standard (dilution, approx. 50 ng/mL), both in triplicate. The standards were analysed on the same day and therefore under repeatability conditions, but no mass-labelled internal standards were used

for quantification. The mean comparability (see Figure 3.19) was in the range of 95-105%, which is good. Results of individual laboratories showed more variance and comparability values as low as 46% (PFOS) and 185% (PFUnA) were found. A similar experiment conducted in the first interlaboratory study (*5*). In that case the solution distributed to the participants contained PFCs from a variety of sources, purities and isomeric composition and was quantified against their in house PFC standards (also with PFCs from a variety of sources, purities and isomeric composition). These data are also plotted in Figure 3.19 and they show that the differences in relative response were larger (from 80% for PFDoA to 202% for PFHxA) than in the current study. Also the error bars were larger. This demonstrates that the use of high quality standards (in terms of purity and isomeric composition) can considerably improve the comparability between laboratories. In this study, the shared standards originated from a single source. Therefore, it is useful to agree on the use of well-defined (shared) standards when comparability of data is required between environmental compartments, between regions, from year to year etcetera (e.g. in the case of monitoring programs). However, isomer profiles may be different in environmental samples and it may be desirable to match the profile observed in the sample with that of the standard. Therefore, the use of linear or isomeric profile standards should be judged case by case. In any case the type of standard used should be clearly reported.

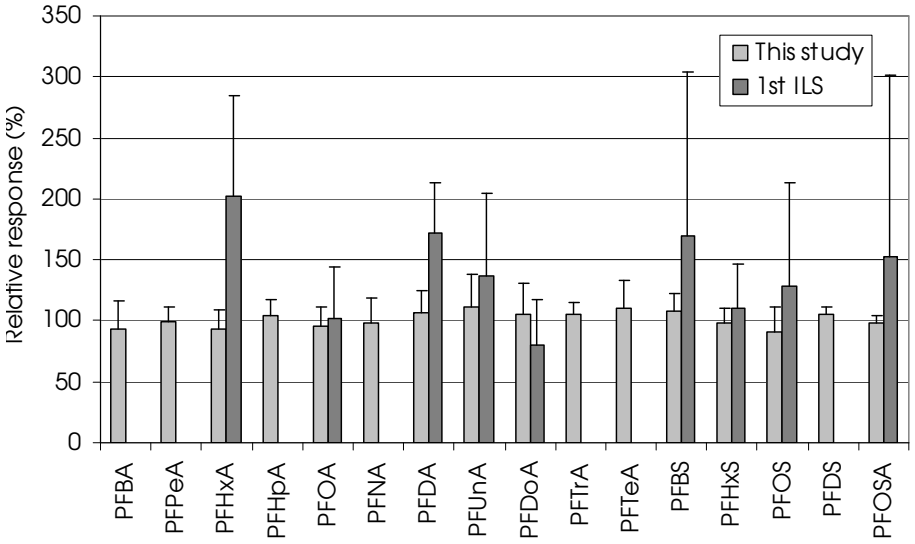

Figure 3.19 Relative responses of in-house PFC standards (from participants) as compared to the shared standards. The shared standards were set at 100%. For comparison reasons, the results from a similar experiment conducted in the first world wide PFC interlaboratory study (5) are shown in the Figure as well (see text for details).

Accuracy evaluation: standard addition quantification versus solvent based calibration curve quantification

Participants were asked to quantify the PFC concentrations in the samples by two methods: SBCCQ and SAQ. The first method is common practice (routine) in many laboratories, whereas the latter method is less often applied. The SBCCQ method is a simpler approach than the SAQ, but matrix effects may occur. The use of a mass-labelled analogue for each target compound allows accounting for these effects. The SAQ method is especially suitable to quantify concentrations in samples with unknown matrix effects as it corrects for these effects. SAQ also accounts for possible losses that may occur at several stages of analysis (extraction, clean-up, electrospray ionization).

The SAQ method used in this study was derived from validation guidelines (*9,10*). Participants were asked to analyse the samples after spiking the target compounds at 0, 1, 2 and 4 times the concentrations already present in the sample (the 0-level). Mass-labelled standards were not used in this case. For the SBCCQ method, the participants were asked to spike the mass-labelled standards prior to analysis (or, in the case of the water sample, directly after receipt of the samples) and to equilibrate the spike in the sample overnight.

The results are presented in Table 3.19 and 3.20. Results with less than five data points (e.g. PFTrA and PFTeA in both water and fish) are not presented. The SAQ results showed more variation than those obtained by the SBCCQ method (Figure 3.20). This is concluded from the higher ranges for nearly all compounds. This was more pronounced in the water sample. Exceptions are PFBS, PFHxS and PFOSA in water. For PFBS and PFHxS, no mass-labelled internal standards were available and the results of these have been corrected using the mass-labelled PFOS internal standard. In these cases the SAQ method provides an equal or better between-laboratory reproducibility than the SBCCQ method. For PFOSA, the D3-N-MeFOSA internal standard is not suitable (as will be discussed below). Furthermore, the mean SAQ results (and often also the median) are higher than the SBCCQ results (except PFOSA and PFHxS in water and PFDoA in fish). For PFOS in fish, the mean SAQ result was 33% higher, whereas for other results, this was less pronounced. Underlying reasons for the overestimation and the larger variance in the SAQ dataset could be:

- The intrinsic uncertainty of the SAQ method contributes to the variance of the SAQ dataset. This uncertainty is due to the fact that the concentration is derived by extrapolation of a regression curve beyond (i.e. below) the actual quantified range. This can partly be solved by performing replicate spikes at each level, which then narrows down the 95% confidence interval of the standard addition curve. However, this is laborious and in this study, no replicate spiking was performed.
- R^2 values of the standard addition curves of individual laboratory datasets were often far below R^2 >0.99, whereas this value was met in most cases with the SBCCQ, due to the use of mass-labelled standards for many compounds. For SAQ, the criterium was lowered to R^2 >0.95 in order not to

reject large amounts of SAQ as this would have hampered a reasonable comparison with the SBCCQ results. Obviously, this resulted in a larger variance of the SAQ results.

- In several cases, the SAQ spiking levels did not match the 1, 2 and 4x level as mentioned in the protocol. At the evaluation meeting, these protocol deviations were assessed and extreme deviations have been removed from the dataset.
- SAQ is not employed on a routine basis in laboratories, resulting in a higher chance of errors being made in performing the analytical work and interpretation and calculation of results.

Table 3.19 Water sample: summary statistics of the SBCCQ and the SAQ quantification methods.

	Spike	**SBCCQ**							**SAQ**						
	(ng/L)	Mean (ng/L)	STDEV (ng/L)	RSD* %	Min (ng/L)	Max (ng/L)	n >LOQ	n <LOQ	Mean (ng/L)	STDEV (ng/L)	RSD %	Min (ng/L)	Max (ng/L)	n>LOQ	n <LOQ
PFBA	25	44.8	7.0	16	33.7	51.6	5	-	59.1	23.3	39	29.6	89.9	5	-
PFPeA	5.0	6.8	3.7	55	2.3	14.7	9	1	8.0	4.7	59	2.0	17.3	10	1
PFHxA	5.0	9.5	4.8	50	4.2	23.8	13	-	13.5	11.3	83	4.1	44.7	13	-
PFHpA	5.0	5.9	2.0	34	0.8	8.4	12	-	6.5	5.0	77	0.1	19.1	12	-
PFOA	25	41.1	13.1	32	20.5	83.2	18	-	52.9	35.1	66	30.3	181.4	18	-
PFNA	5.0	5.3	2.0	37	2.9	10.4	15	1	7.1	4.4	63	1.9	17.4	14	-
PFDA	5.0	4.8	1.8	38	2.9	9.9	13	2	6.1	3.1	52	2.3	11.3	13	-
PFUnA	5.0	3.3	1.3	39	1.5	5.7	12	1	4.6	4.1	87	0.9	15.9	11	1
PFDoA	5.0	2.8	1.2	44	1.2	4.5	10	1	5.0	5.7	114	0.7	19.6	9	2
PFBS	17.7	27.5	18.2	66	0.9	54.4	12	-	33.7	14.7	43	7.8	59.3	11	-
PFHxS	9.5	14.4	5.5	38	5.5	24.3	14	-	11.4	4.5	40	1.2	18.6	13	-
PFOS	23.2	34.4	9.9	29	19.9	60.3	18	-	42.3	43.1	102	10.0	196.8	18	-
PFOSA	5	3.5	2.4	69	1.6	10.1	11	2	2.2	1.6	72	0.5	5.2	11	-

* *Underlined: the compounds for which no ^{13}C-labelled internal standard was available in this study. For PFOSA, the internal standard was D_3-N-MeFOSA.*

Table 3.20 Fish sample: summary statistics of the SBCCQ and the SAQ quantification methods. All concentrations are expressed as ng/g ww.

Spike		**SBCCQ**							**SAQ**						
	(ng/g)	Mean (ng/g)	STDEV (ng/g)	RSD %	Min (ng/g)	Max (ng/g)	n >LOQ	n <LOQ	Mean (ng/g)	STDEV (ng/g)	RSD %	Min (ng/g)	Max (ng/g)	n >LOQ	N <LOQ
PFOA	22.6	18.0	4.1	23	9.2	23.6	14	-	21.5	8.3	39	8.6	41.5	13	-
PFNA	17.2	17.5	4.6	26	8.9	27.3	12	-	23.9	13.2	55	12.6	57.2	12	-
PFDA	21.9	21.1	4.6	22	12.9	26.7	12	-	22.9	5.3	23	14.2	30.6	12	-
PFUnA	17.8	15.9	4.1	26	9.0	21.0	11	-	20.2	5.3	26	11.9	28.6	11	-
PFDoA	20.1	17.3	5.2	30	8.5	23.6	11	-	16.8	5.2	31	6.2	23.4	11	-
PFOS	145	150	44.0	29	49.9	230	14	-	200	93.1	47	34.5	388	11	-
PFOSA	3.2	3.6	1.7	47	1.5	7.5	10	-	4.3	2.5	57	1.2	9.8	9	1

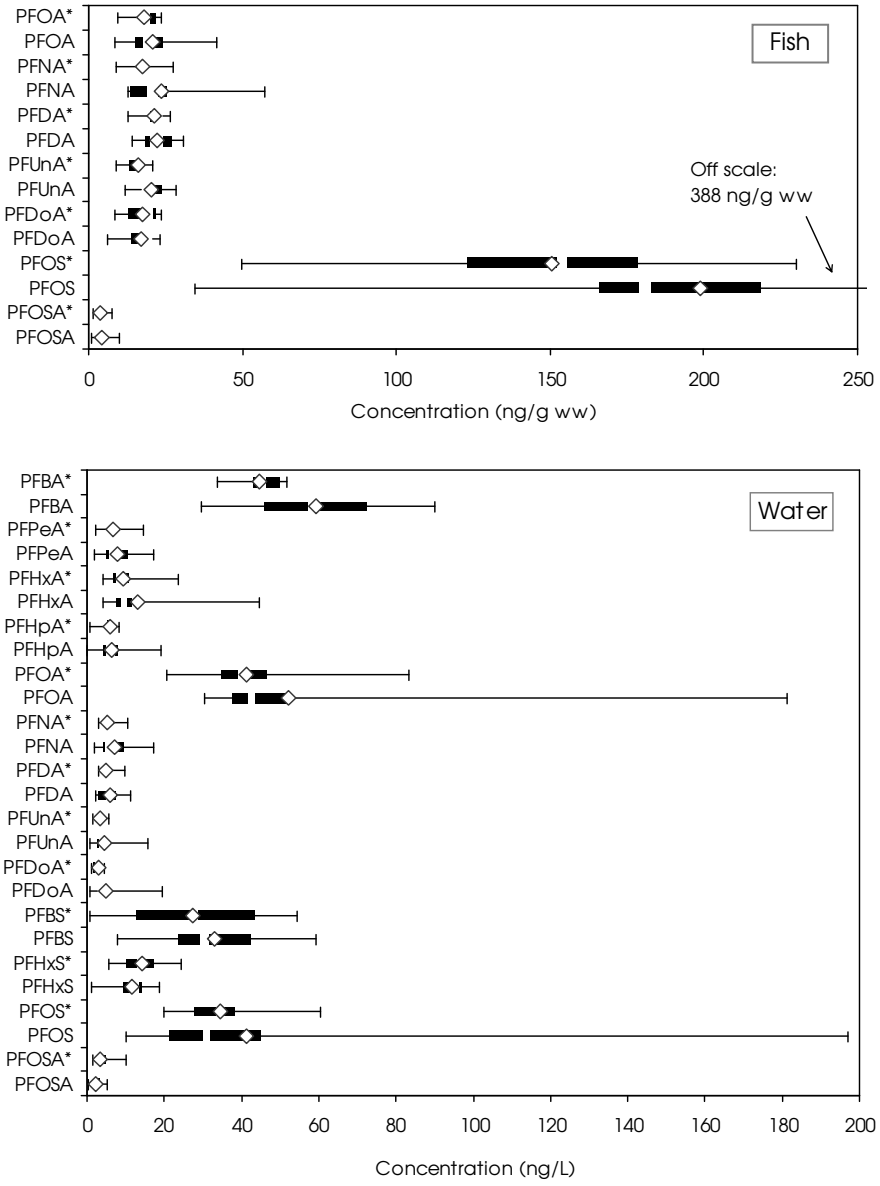

*Figure 3.20 Data distribution for the SBCCQ data (marked by *) and the SAQ data. The top Figure shows the data for the fish sample and the bottom Figure shows the data for the water sample. The number of data points included is indicated in Table 3.19 and 3.20. Bars represent lower quartile, median (blank interruption of bar) and upper quartile. The diamond represents the mean of the dataset.*

It should be noted that the mean SBCCQ results were in some cases (PFUnA, PFDoA and PFOSA in water and PFOA, PFUnA, PFDoA in fish) lower than the spiked amount. The reason for this is not known. For the water sample, possibly adsorption on the transportation bottle wall has taken place. In the fish sample, inefficient extraction the matrix may have played a role. Both possible causes require further investigation.
D3-N-MeFOSA was used as the internal standard for PFOSA, but turned out not to be suitable because several laboratories have observed considerable losses of this internal standard. Therefore, some laboratories decided not to report the PFOSA value (e.g. lab 19), whereas others decided to use the ^{13}C-PFOS is internal standard. This uncertainty is also reflected in the higher RSD values for PFOSA. The losses are possibly due to the low solubility of D3-N-MeFOSA in an aqueous solution. An additional experiment by the coordinating laboratory showed that losses of 80% of D3-N-MeFOSA are observed over a 46 hours period after spiking to ultra-pure water (Figure 3.21). After 46 hours, the remaining water was discarded and replaced by methanol. After vigorous homogenization, the D3-N-MeFOSA was partially recovered again. This indicates that D3-N-MeFOSA was adsorbed on the container surface (wall) is not a suitable internal standard for correction of results of PFOSA in water.

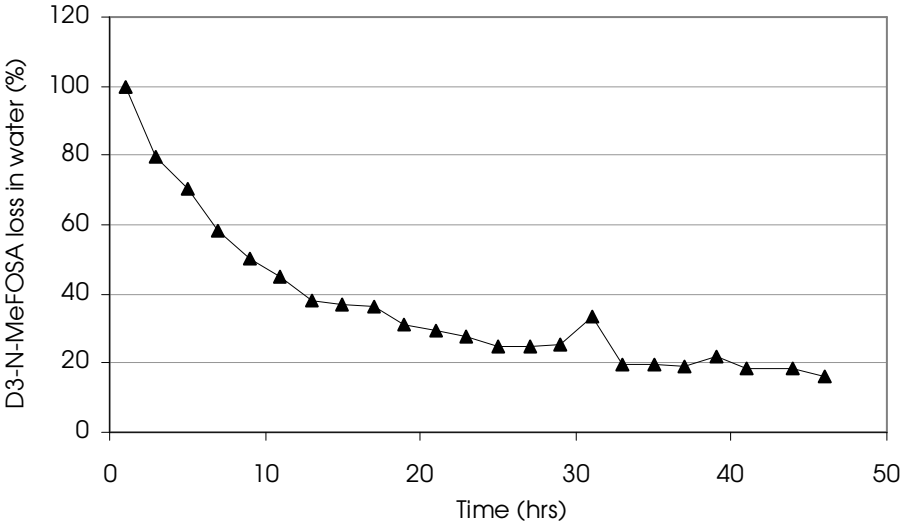

Figure 3.21 Losses of D3-N-MeFOSA in water over time. ^{13}C-PFOS was used as internal standard.

As compared to the first world-wide interlaboratory study, the performance of the participants has improved considerably. RSD values in this study are 16-69% and 22-47% for water and fish (SBCCQ), whereas they were 47-250% and 65-235%, respectively in the first world-wide interlaboratory study (excluding values close to the LOQ) (*5*). The underlying reasons for this are the improved knowledge on the behavior of PFCs (and therefore better design and control of the methods) and the use of well-defined (mass labelled) standards, as discussed below. The between laboratory variances in the water sample are similar to the results obtained in a study by Yamashita et al. (*8*). He reported on good performance (23-32% RSDs for PFOS and 27-30% RSDs for PFOA) in seawater. It should be noted that, different from the Yamashita et al. study, in the current study participants were allowed to use their in-house methods. This shows that mass-labelled standards are capable of correcting results, even when obtained with the different methods applied in this study.

Precision

The study included an evaluation of the precision of individual laboratories. The laboratories were asked to perform the SBCCQ in triplicate. The precision criterion in a FDA validation guideline is 15% for at least 5 replicates (*9*). When taking the mean precision values (mean of all compounds) per lab into account, most laboratories meet this criterion for the water sample (except Labs 4, 9, 20 and 24 which had higher values) and for the fish sample (Figure 3.22). Limited precision is either caused by very low concentrations (e.g. PFTeA in water and PFPeA and PFTrA in fish, data not shown) or, in the case of

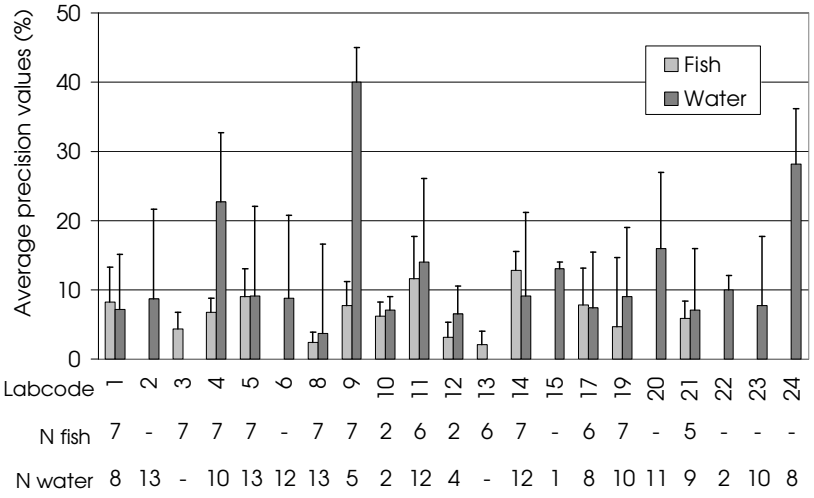

Figure 3.22 Mean precision (as RSD, or %) of the different laboratories for water and fish (SBCCQ results). The values concern the mean of all precision values (of all PFCs) reported by a single laboratory for the PFCs they analysed. Error bars indicate the standard deviation of the mean. N indicates the number of PFCs analysed by a laboratory (and therefore, the number of precision values included).

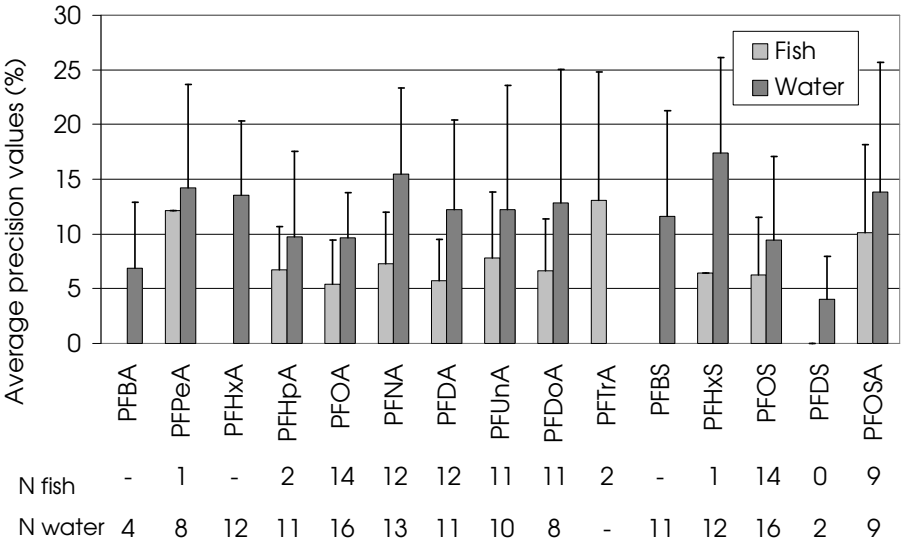

Figure 3.23 Mean precision (as RSD, or %) of the different laboratories in water and fish (SBCCQ results). The values concern the mean of each PFC of all precision values reported by all laboratories for a specific PFC. Error bars indicate the standard deviation of the mean. N indicates the number of precision values included.

PFOSA, by a poor performance of the D3-N-MeFOSA internal standard (as explained earlier). When expressed per PFC (Figure 3.23), the precision for the fish sample was better (<7.5% for PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFHxS, PFOS) than for the water sample, suggesting that the analysis of fish is somewhat better controlled than the water analysis. The explanation for the overall good precision data is the use of a broad range of mass-labelled internal standards.

Matrix effects

Ion suppression or ion enhancement in the electrospray ("matrix effects") were tested in order to determine to what extent clean up strategies remove potential interferences from the extracts. For the water sample, no specific clean-up was applied. Most laboratories pre-concentrated the sample by SPE and after sample loading a simple wash step (water, water/methanol or water/acetonitrile mixture) was applied for the removal of salts and other interferences. For the fish sample, the majority of the laboratories applied an Envirocarb clean-up step as published by Powley et al. (*12*). Other methods included freezing-out matrix components (e.g. lipids) and reconstitution of the extract in water and subsequent concentration and clean-up by SPE (Oasis HLB) (Table 3.18).

The experiments were carried out by spiking an extract (just before injection: "pre-injection") with (in most cases) 50 ng/mL PFCs. The resulting response was compared to the response of a 50 ng/mL standard solution (after correction for the response of an unspiked extract). The mean matrix effect per PFC and matrix is plotted in Figure 3.24. Predominantly ion suppression was found. However, ion enhancement was also observed by several laboratories for individual compounds in the same samples (as indicated by the error bars).

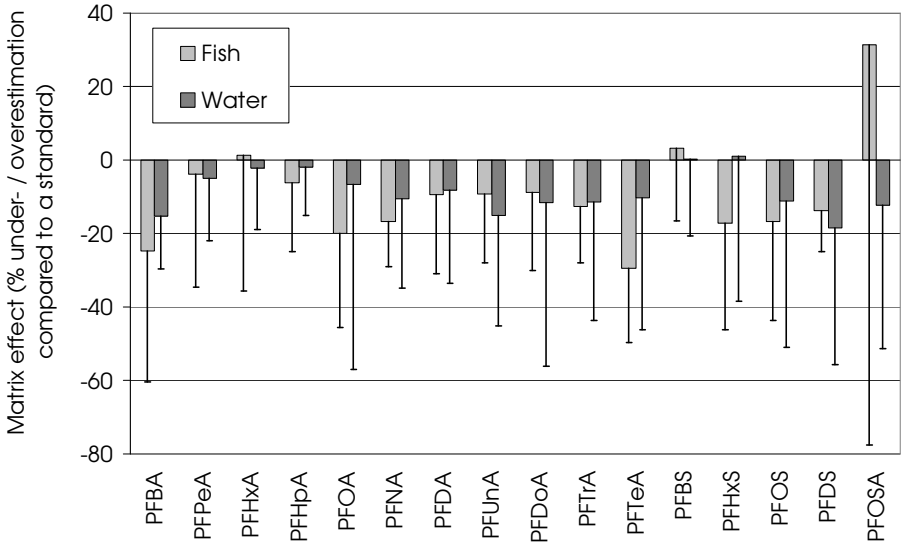

Figure 3.24 LC-ESI-MS(/MS) matrix effects determined by comparing the response of an extract spiked prior to instrumental analysis and comparison of response with a standard solution (details provided in the text and Table 3.17). The response of the standard solution was set at 100%. Mean values (of all labs for a specific PFC) are plotted together with standard deviation.

For the fish sample, the range of matrix effects (mean values) was +31% (PFOSA) to -30% (PFTeA). PFBA and PFTeA showed the strongest suppression and PFOSA the strongest enhancement, due to extreme values of individual laboratories. Individual laboratory data showed suppressions down to -80% (lab 9, PFBA) and enhancements up to +330% (Lab 1, PFOSA). Lab 10 used flow injection analysis (instead of HPLC), in which the complete extract is injected at once in the MS without chromatographic separation. They found a suppression of approx. -80%. For the water sample, the matrix effect ranged from –18% to +1%, but in this matrix extreme values were also observed: -91% (Lab 10, PFOA) and +142% (lab 24, PFOA). Some laboratories (5, 6 and 14) found a suppression for PFBA to PFHpA in the water sample (decreasing with increasing chain length). Possibly, co-extracted and early eluting organic acid complexes (e.g. humic acid) have suppressed their responses, but this

was not confirmed by other laboratories. It should be noted that the results depend on the design and execution of the experiment. For example, the matrix effect as it is defined here is PFC concentration dependant. The amount spiked prior to injection was in most cases 50 ng/mL per PFC, which is relatively high compared to concentrations encountered in the environment (e.g. seawater). The matrix effect becomes more pronounced when lower concentrations are spiked (e.g. 5 ng/mL). Therefore, in low contaminated environmental samples, matrix effects are likely to be larger. In principle, mass-labelled analogues correct for these effects and therefore enable an accurate determination of their native analogues. However, for PFHpA, PFTrA, PFHxS, PFBS and PFOSA no ^{13}C analogues were available at the time of the study and therefore no correction for matrix effects could be made.

Conclusions

This study has shown that an accurate and precise analysis of PFCs in freshwater and fish samples is feasible if several critical factors in the analysis are addressed. These are i) the use of well-defined native standards, ii) the use of mass-labelled internal standards (preferably one for each target compound) and iii) minimization of matrix effects (e.g. by a proper clean up of extracts). Apart from these, it's important to control and minimize the background contamination (blanks). SAQ is a useful technique for analysis of matrices with unknown matrix effects or for compounds for which no mass-labelled standards are available. SBCCQ combined with mass-labelled standards facilitating the analysis of PFCs is very suitable for routine analysis. For reasons of comparability among laboratories (e.g. in monitoring programs), it can be useful to standardize the selection of native, and internal standards.

In this study, several PFCs were spiked to the samples to facilitate detection. In the environment, however, lower concentrations are often found e.g. sub-ng/g and sub-ng/L in fish and marine water, respectively). In this study, we have seen that precision decreased at concentrations close to the LOQ. It will therefore be challenging to maintain the same level of performance at low concentrations. It is therefore recommended to conduct further interlaboratory studies at these low concentrations. Finally, there is a need for certified reference materials for PFCs that will aid laboratories to improve their methods.

Acknowledgements

Plastics Europe is gratefully acknowledged for funding this study in the framework of the PERFORCE-2 project (Coordinated by the University of Amsterdam, dr. P. de Voogt). Wellington Laboratories is gratefully acknowledged for making the native and mass-labelled standards available. Dr W. Reagen (3M Environmental Laboratory), Dr. F. Morandi (Solvay-Solexis), Dr. C. Powley (Dupont Haskell laboratories) and Dr. U. Berger (ITM, Stockholm University) are gratefully acknowledged for their comments and advice on the study design. Mrs. S. So is acknowledged for the D3-N-MeFOSA solubility experiments. Finally, all participants are gratefully acknowledged for their active participation in the study: Rossana Bossi (National Environmental Research Institute-Dept. Atmospheric Environment, Roskilde, Denmark); Robert Loos (JRC, Ispra, Italy); Bruno Veyrand (Ecole Nationale Veterinaire de Nantes – LABERCA, Nantes, France); Gail DeRuzzo (TestAmerica, Arvada CO, USA); Dale Hoover (AXYS Analytical Services Ltd, Sidney, BC, Canada); Ab Borburgh (VITO, Mol, Belgium); Lutz Ahrens (GKSS Research Centre Geesthacht GmbH, Geesthacht, Germany); William Reagen (3M Environmental Laboratory, St. Paul, Minnesota, USA); Monica Saez (Dpt. Instrumental Analysis and Environmental Chemistry. Institute of Organic Chemistry. CSIC, Madrid, Spain); Dorte Herzke (Norwegian Institute for Air Research, Tromsø, Norway): Francesco Morandi (Solvay-Solexis -Research & Technology - Lab HPLC MS, Bollate, Milano, Italy); Christiaan Kwadijk (IMARES, IJmuiden, The Netherlands); Urs Berger (Dept. of Applied Environ. Sciences (ITM), Stockholm University, Stockholm, Sweden); Johan Meyer (Ecophysiology, Biochemistry and Toxicology, University of Antwerp, Antwerp, Belgium); Stefan Weiß (Umweltbundesamt GmbH, Abt. Organische Analytik, Wien, Austria); Heinz Rupp (Bundesamt für Gesundheit - Sektion Chemische Risiken, Bern, Switzerland); Wolfgang Gerwinski (Bundesamt für Seeschifffahrt und Hydrographie, Hamburg, Germany); Barbara Le Bot (ENSP/LERES, Rennes, France); Sonia Wanner (C.A.R., Dpt Hydrologie, Illkirch, France); Nizar Benismail (IPL Santé Environnement Durables, Maxéville, France).

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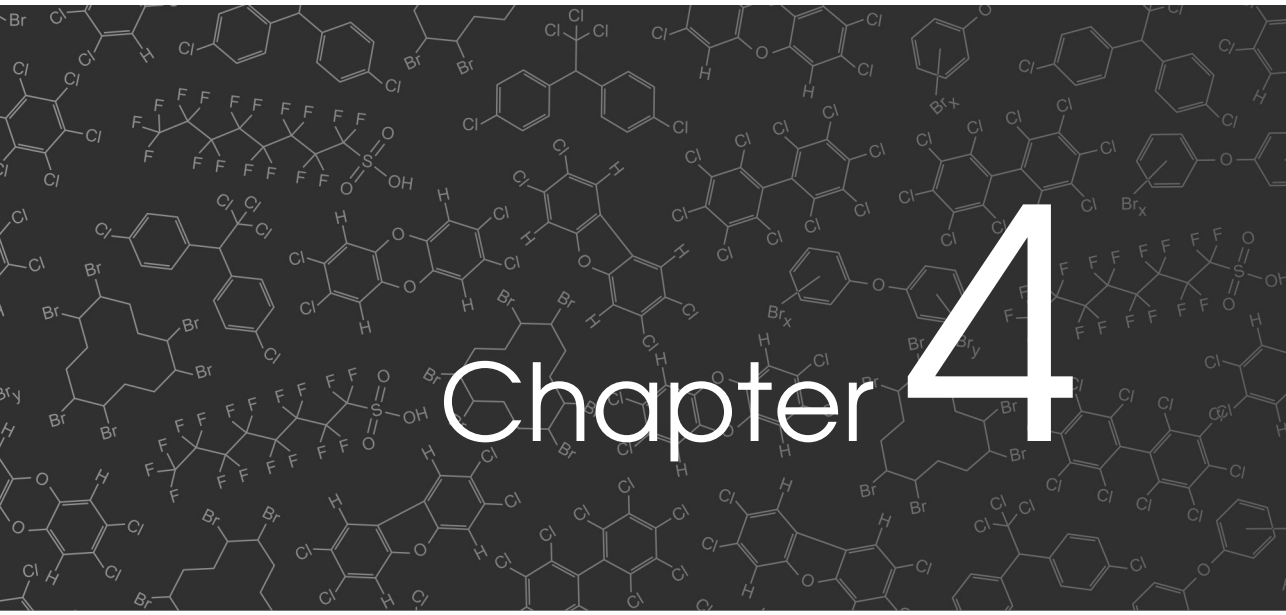
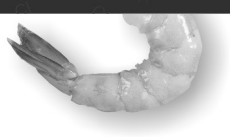


Chapter 4

Contaminants in fish, shellfish and shrimp from The Netherlands



4.1 Dioxins and dioxin like-PCBs
concentrations, profiles and DR-CALUX bioassay results[9]

Fish from Dutch markets were analysed for concentrations of polychloro-*p*-dibenzodioxins, dibenzofurans (PCDD/F) and dioxin-like polychlorinated biphenyls (dl-PCB) and compared with the new European maximum levels (MLs), set in 2006. In a first study on eleven different fish and shellfish from various locations, concentrations of PCDD/Fs were nearly all below the ML for PCDD/Fs (4 pg TEQ (Toxic-EQuivalents)/g wet weight (ww) and nearly all below 8 pg total-TEQ/g ww, being the new ML for the sum of PCDD/Fs and dl-PCBs. Some samples exceeded the total-TEQ ML, such as anchovy, tuna and sea bass. Furthermore, 20 (out of 39) wild eel samples exceeded the specific ML for eel (12 pg TEQ/g ww), as the study revealed PCDD/F-TEQ levels of 0.2-7.9 pg TEQ/g ww and total-TEQ values of 0.9 to 52 pg/g ww. TEQ levels in farmed and imported eel were lower and complied to the MLs.
Smoking eel, a popular tradition in The Netherlands, only had marginal effects on PCDD/F and dl-PCB concentrations. Due to volatilization, concentrations of lower chlorinated PCBs were reduced to below the limit of quantification after smoking.
Dl-PCBs contributed 61-97% to the total-TEQ in all eel samples. This also holds for other fish and shellfish (except shrimps): dl-PCB contributed (on average) from 53 to 83% to the total TEQ, for herring and tuna, respectively. Principle component analysis revealed distinctive congener profiles for PCDD/Fs and non-ortho PCBs for mussels, pike-perch, herring and various Mediterranean fish.
The application of new TCDD equivalency factors (TEFs) set by the World Health Organisation (WHO) in 2005 (to replace the 1998 TEFs) resulted in lower TEQ values (10-20%), mainly due to a substantially lower mono-ortho PCB contribution. This decrease is more pronounced for wild eel (40%), due to the relative high mono-ortho PCB concentrations in eel. Consequently, a larger number of samples would comply to the MLs when the new TEFs would be applied.
The DR CALUX® assay may be used for screening total-TEQ levels in eel, in combination with GC-HRMS confirmation of suspected samples. An almost 1:1 correlation was found when the 1998 TEFs were applied, but, surprisingly, a 1.4 fold overestimation occurred with application of the 2005 TEFs.

[9] Based on S.P.J. van Leeuwen, P.E.G. Leonards, W.A. Traag, L.A.P. Hoogenboom and J. de Boer (2007) Polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls in fish from The Netherlands: concentrations, profiles and comparison with DR CALUX® bioassay results. *Analytical and Bioanalytical Chemistry* 389, 321-333

Introduction

Following the Belgium dioxin crisis in 1999, polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) in food have received considerable attention from the European Commission (EC), leading to new maximum levels (MLs) for various food items. For fish, an ML of 4 pg TEQ/g wet weight (ww), expressed as equivalents of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Toxic EQuivalents, or TEQ) was set in July 2002, temporarily excluding the contribution of the dioxin-like PCBs (dl-PCBs) *(1)*. In 2006, a new ML for the sum of PCDD/Fs and dl-PCBs was set at 8 pg TEQ/g ww, and a specific one for eel (12 g TEQ/g) *(2)*. PCDD/Fs and dl-PCBs are persistent contaminants that are ubiquitous in the aquatic environment. Compared to other food items, fish can bioaccumulate considerable amounts of these contaminants (*3*). The presence of PCDD/Fs and dl-PCBs in regularly consumed fish or in fish originating from point sources has been demonstrated (*4-8*). The Dutch rivers Meuse, Rhine, Waal and IJssel are highly polluted with PCBs and PCDD/Fs due to a high degree of urbanization and industrialization along these rivers in The Netherlands, Germany, France and Belgium. Total-TEQ (sum of PCDD/F and dl-PCB) values in marine and freshwater fish fillets from 1991 ranged from 0.8 (cod) to 22 (eel) pg TEQ/g ww and up to 504 pg TEQ/g ww for fish livers have been reported (*9*). In order to determine the current contamination of Dutch edible fish and shellfish, two studies were undertaken. The specific aims of these studies were:

(i) To determine PCDD/F and PCB concentrations in popular fish from the Dutch market and to evaluate the compliance with current EC legislation. Because of the high contaminant concentrations found in earlier studies, eel was investigated in more detail in a separate study;

(ii) To determine a possible removal of contaminants by smoking of eel;

(iii) To evaluate the PCDD/F and PCB profiles in the fish samples;

(iv) To evaluate the increase or decrease of TEQ concentrations as a result of application of the new WHO TEFs from 2005 (TEF_{2005}) versus the 1998 TEFs (TEF_{1998});

(v) To evaluate the suitability of the DR CALUX® bioassay (*10*) for screening eel samples in combination with gas chromatography-high resolution mass spectrometry (GC-HRMS).

(vi) To evaluate the risk for human consumption of highly contaminated eel samples.

Materials and methods

Sampling and sample preparation

Fish survey - Fish samples were purchased in 1999 and 2000 from fishermen and commercial traders. The set of samples included coalfish and cod (liver) from the North Sea, eel from the IJssel Lake, herring from the North Sea and

the English Channel, mackerel from the Celtic Sea, Skagerrak and Atlantic Ocean, mussels from the Dutch Wadden Sea and the Eastern Scheldt, pike-perch from various Dutch fresh water locations, farmed salmon from Scotland and Norway, shrimps from Norway and the Dutch coast and tuna from the Mediterranean and Sri Lanka. In addition, a limited number of fish samples were purchased in 2003 and 2004 in order to monitor contaminant concentrations over time. The complete list of fish samples can be found in the Table A-1 in the Annex of chapter 4.1.

Eel study - The wild eel (yellow eel – *Anguilla anguilla*) survey included eel from 39 freshwater locations originating from main river systems (e.g. Meuse, Rhine, Lake IJssel), small rivers, canals and lakes. The eel was caught in May and June 2001 by using electrical fishing equipment. Eel hibernates in The Netherlands in the sediments during the winter season and cannot be caught between November and April. In addition, during the end of the summer and in the autumn, some eels build up higher lipid contents and start to migrate to sea to begin their journey to the Sargasso Sea (*11*). Although those 'silver eels' can be distinguished by their grey colour, in contrast with the regular 'yellow eel', mistakes in this identification can be made (*12*). Therefore, May and June are the best months for sampling of wild eel. Farmed eel was purchased from eleven Dutch eel farmers. Fourteen samples of imported eel were purchased from different commercial traders (3 wild eel and 11 farmed eel samples). The influence of smoking on the contaminant concentrations in eel was determined by smoking 2 batches of farmed eel, either traditionally (n=3) or industrially (n=1) and analysing the raw material and the smoked material.
For both studies, pooled samples were prepared for the fish and shellfish samples. All fish and shellfish samples were market-size. Lengths and weights of each individual fish were recorded. For all fish, pooled samples were prepared from equal amounts of fillets of 25 individuals per location or origin. For the pooled mussel samples, approximately 5 kg of mussels were cooked for 5 minutes, the shells were removed and the meat (1 kg) was collected. Concerning shrimps, approximately 1 kg was cooked for 5 minutes and peeled. All fish and shellfish samples were homogenised using a Waring blender and stored in glass jars at –20°C until analysis.

Analytical procedure
PCDD/Fs and dl-PCBs - The complete WHO set of 17 PCDD/Fs and 12 dl-PCBs (*13*) were analyzed in all fish samples. The lipid fraction, including the indicator PCBs, dl-PCBs and PCDD/Fs in the survey study was extracted by chloroform/methanol according to a modified method of Bligh and Dyer (*14*). In the eel study the lipids were extracted for 12 h by Soxhlet extraction with a dichloromethane/n-pentane mixture (1:1 v/v) (picograde, LGC Promochem GmbH, Wesel, Germany). The solvent was evaporated from the extracts and the remaining concentrates were sent to RIKILT (Wageningen, The

Netherlands) for analysis by GC-HRMS of PCDD/Fs and dl-PCBs. Levels of PCDD/Fs, non-ortho PCBs (NO-PCBs) and mono-ortho PCBs (MO-PCBs) were determined by GC-HRMS, according to Tuinstra et al. (*15*). Separation of the target compounds from the fat was carried out using gel permeation chromatography (GPC). The system consisted of a high performance liquid chromatography (HPLC) pump (Gilson, model 305), an autosampler (Gilson, model 231) equipped to inject 12.5 ml, and a fraction collector (Gilson, model 202) adapted to collect 300-ml fractions in 500-ml glass flasks. The glass GPC column (Spectrum, 62.5 cm) was packed with Biobeads SX 3. An additional clean-up was performed with activated Al_2O_3 in an automatic sample preparation system using solid phase extraction columns (ASPEC, Gilson). The columns were packed with 1.0 g deactivated Al_2O_3 (7% water) shortly before use. The separation between planar (dioxin like) and non-planar compounds (di-ortho PCBs) was carried out with porous graphitized carbon (Hypercarb 1006, 4.6 mm, Shandon, Runcorn, UK). The column was successively eluted with cyclohexane-dichloromethane (1:1 v/v) and toluene, both at a flow rate of 2 ml/min. This resulted in one fraction with mono-ortho and di-ortho PCBs. The second fraction contained PCDD/Fs and NO-PCBs. The final extracts were concentrated to 10 µl and analyzed with GC-HRMS (Autospec, Micromass, operated at 10.000 resolution), equipped with a DB-5-MS capillary column (60 m, 0.25 mm i.d., 0.25 µm film thickness). The MS method to determine the tetra-octa PCDD/Fs is based on United States Environmental Protection Agency protocol 1613.

Indicator PCBs - The indicator PCBs (IUPAC nos. 28, 52, 101, 118, 138, 153 and 180) (*16*) were Soxhlet extracted for 6 hrs with dichloromethane/n-hexane (1:1, v/v) (picograde, LGC Promochem GmbH, Wesel, Germany) (*14*). The lipids were removed by Al_2O_3 column chromatography (20 x 2 cm i.d., 15 g deactivated Al_2O_3). After concentration, the eluate was further cleaned using silica column chromatography on a 40 cm x 6 mm i.d. glass column, containing 1.8 g SiO_2 Kieselgel (Merck, Darmstadt, Germany). After treatment with concentrated sulphuric acid (95-98%, Merck, Darmstadt, Germany) the first fractions of 10 ml iso-octane were analysed using a HP-6890 Hewlett-Packard GC, equipped with splitless injection (250°C) and electron capture detection (ECD, 300°C), and a CP-Sil-8 capillary column (50 m, 0.15 mm i.d., 0.30 µm film thickness). The standards used were, whenever possible, >99% pure and certified and were obtained from Promochem GmbH (Wesel, Germany), Ultra Scientific, Cambridge Isotope Laboratories (Andover, USA), BCR (Brussels, Belgium) and TNO (The Netherlands).
Lipid content. The fat contents of all fish samples except eel were determined using an adapted method of Bligh and Dyer (*14*), whereas for eel, in which the contribution of phospholipids is negligible, the total fat content was determined gravimetrically from the Soxhlet extract as described above.

DR CALUX® screening - In the eel study, the total-TEQ levels in eel were also estimated by the DR CALUX® assay. Samples of 0.25 g fish oil (kindly supplied by Nutreco, Boxmeer, The Netherlands) were purified on columns containing 10 g acid silica (33% H_2SO_4), as described by Bovee e*t al.* (*17*). Before total evaporation of the hexane/diethylether extract in a SpeedVac, 100 µl of dimethylsulfoxide (DMSO) was added as a keeper. An aliquot of 20 µl was added to 2 ml incubation medium and 250 µL added in triplicate to three different wells of a 48-well plate containing p-GudLuc 1.1-transfected H4IIE cells. After 24 h, the medium was aspirated, the cells washed and lysed and an aliquot used for determining the luciferase response in a Luminoskan (Labsystems). Total-TEQ sample in the oil was estimated from a calibration curve, prepared from cleaned fish oil samples spiked with the 17 PCDD/Fs, 3 NO-PCBs (nos 77, 126 and 169) and 2 MO-PCBs (118 and 156) at 30, 60, 120 and 203 pg TEQ/g oil, as confirmed with GC-HRMS (relative contribution to TEQ 15, 40 and 45%). These samples were included in each analytical series. The level in the fat was subsequently transferred to a wet weight level based on the amount of fat.

Quality assurance - The analysis of the PCDD/Fs and (dl-)PCBs is accredited according to ISO 17025. The quality of the analysis was assured routinely by including blanks, duplicate samples, recovery experiments and the analysis of internal reference materials in each series of samples. Furthermore, certified reference materials (CRM) are analysed frequently, and both the laboratories participate in the proficiency testing schemes of QUASIMEME (www.quasimeme.org) and Folkehelsa (www.fhi.no). The DR CALUX® method participated in several interlaboratory studies, such as from the EU DIFFERENCE project (www.dioxins.nl) (*18,19*).

Calculations and statistical evaluation

The TEQ calculations are based on the WHO TEF values from 1998 (*13*). In the first fish study, the dl-PCBs included the NO-PCBs 77, 126 and 169 and the MO-PCBs 105, 118 and 156. For an eel sample from the river Rhine it was calculated that when using this limited set of dl-PCBs and the $TEFs_{1998}$, an underestimation of 11% was found for the MO-PCBs but no significant under- or overestimation was seen for the NO-PCBs. This somewhat affected the total-TEQ (6% underestimation). For other fish, this effect will be smaller because the MO-PCB contribution to the total-TEQ is smaller. In the eel study, all dl-PCBs were included, i.e. the NO-PCBs 77, 81, 126 and 169 and the MO-PCBs 105, 114, 118, 123, 156, 157, 167 and 189. All TEQ concentrations values are reported according to the lower bound principle, which means that individual congeners below the detection limit or non-quantifiable peaks due to interferences were not taken into account in the calculation of the TEQ concentrations. In this chapter, a comparison is made between the results

calculated with either the 1998 TEF values and the 2005 revised values (*20*) (see below).

Results and discussion

Fish survey

PCDD/Fs - The results in Table 4.1 show that PCDD/F-TEQ values in all samples were below the European ML of 4 pg TEQ/g ww *(2)*. Median PCDD/F-TEQs in other species like herring, salmon, pike-perch and mussel were intermediate between the highly contaminated eel and the low concentrations found in cod, coalfish, mackerel, tuna, shrimp (where concentrations were below 1 pg TEQ/g ww). Concentrations in individual samples can be found in the Table A-1 in the Annex of chapter 4.1. For comparison of our results with those obtained in other European regions, a selection of concentration data from other studies is shown in Table 4.2. In some other studies, different TEF systems were used *(21-23)*. These TEFs are listed in the Table A-3 in the Annex of chapter 4.1. The median concentrations reported here are very similar to those reported in Dutch fish from 1991 (*9*), although the use of a different TEF system in that study (from a Dutch working group) led to an underestimation of ca 10% for the PCDD/F-TEQ as compared to the TEF_{1998} (data not shown). The lower TEF of 1,2,3,7,8-PeCDD (0.5 instead of the TEF_{1998} of 1.0) accounted primarily for this difference. The PCDD/F concentrations in mussels, shrimps, mackerel and cod are in the same range comparable to those found in North-West European fish, but higher than the levels reported for in the Mediterranean fish (Table 4.2). Levels in herring in this study were much lower than those reported for the Bothnian Sea and Grenland fjord. The latter location was highly polluted by PCDD/Fs from historical industrial activity. A study on the correlation of age and size of herring versus PCDD/F-TEQ concentrations in herring from the Bothnian Sea revealed that herring aging over 4 years exceeded the EC ML for PCDD/F-TEQ *(24)*. Consequently, that herring would not be suitable for consumption, whereas herring from a similar size in The Netherlands still complies with the EU-ML. PCDD/F-TEQ levels in salmon from the 2000 study were higher than those from 2004. The 2004 samples agreed nicely with the levels observed in other studies *(24,25)*.

dl-PCBs - Median dl-PCB-TEQ concentrations showed a wide variation (Table 4.1). Median concentrations in herring, tuna, mackerel, mussels, pike-perch and salmon were intermediate, whereas median concentrations in cod, coalfish and shrimps were below 1 pg TEQ/g. DI-PCB-TEQ concentrations in Dutch fish from 1991 were considerably higher for mussels, shrimps and mackerel (*9*).

Table 4.1 Concentration ranges and medians (in brackets) of PCDD/Fs, dl-PCBs (based on TEFs$_{1998}$) and indicator PCBs in various fish.

Species	Origin	n	Lipids (%)	NO-PCBs	MO-PCBs	dl-PCBs	PCDD/Fs (pg TEQ/g ww)	dl-PCBs + PCDD/Fs	ΣPCB$_7$[2] (ng/g ww)
EC-ML[1]	-	-	-	-	-	-	4	8 (12[3])	-
Anchovy	Mediterranean	1	4.4	6.1	1.9		0.6	8.5	
Coalfish	North Sea	2	0.9-1.7	0.2-0.7 (0.4)	0.01-0.2 (0.1)	0.2-0.8 (0.50)	0.1 (0.1)	0.3-1.0 (0.6)	1.0-6.6 (3.8)
Cod	North Sea	2	0.8-1.0	0.2-0.4 (0.3)	0.05-0.07 (0.06)	0.2-0.5 (0.36)	0.07-0.3 (0.2)	0.3-0.8 (0.6)	1.6-2.9 (2.3)
Eel farmed	The Netherlands, Italy	4	25-34	2.2-6.2 (4.8)	0.9-2.7 (1.2)	3.1-8.2 (6.3)	0.7-2.5 (1.5)	3.9-10.7 (7.8)	43-69 (62)
Eel wild	IJssel lake	5	21-25	3.7-13.9 (7.0)	3.6-18.9 (7.6)	7.3-33 (15)	1.4-3.9 (3.1)	8.7-37 (18)	129-602 (150)
Herring	The English Channel, North Sea	4	1.8-15	0.8-3.6 (1.5)	0.2-0.5 (0.3)	1.1-3.9 (1.9)	1.3-2.1 (1.6)	2.4-5.7 (3.7)	9.5-16 (16)
	The English Channel, North Sea, Shetland Islands[4]	4	14-20	0.5-0.9 (0.7)	0.2-0.3 (0.2)	0.6-1.2 (1.0)	0.7-1.2 (1.0)	1.3-2.4 (2.0)	NA
	The English Channel, North Sea, Skagerrak[5]	3	2.6-16	0.7-1.8 (0.8)	0.1-0.6 (0.2)	0.8-2.5 (1.0)	0.7-1.6 (1.1)	1.6-4.0 (2.1)	5.6-22 (8.5)
Mackerel	Skagerrak, Atlantic Ocean, Celtic Sea	3	17-24	1.0-1.3 (1.0)	0.3-0.3 (0.3)	1.0-1.6 (1.0)	0.3-0.6 (0.3)	1.3-2.2 (1.3)	1.1-19 (1.6)
	North Sea, Shetland Islands, Atlantic Ocean[4]	3	3.3-17	0.5-1.5 (0.5)	0.1-0.6 (0.2)	0.6-2.0 (0.6)	0.2-0.4 (0.2)	0.8-2.4 (0.8)	NA
	North Sea, Shetland Islands[5]	2	4.8-5.4	0.9-1.1 (1.0)	0.2-0.3 (0.3)	1.1-1.4 (1.3)	0.3-0.3 (0.3)	1.4-1.7 (1.6)	8.3-13
Mussel	Eastern Scheldt, Wadden Sea	2	1.5-1.7	0.9-1.7 (1.3)	0.3-0.3 (0.3)	1.2-2.0 (1.6)	1.1-1.5 (1.3)	2.3-3.5 (2.9)	12.7-14.5 (14)
Pike-perch	Nieuwe Merwede, Rivers Lek, Amer, Rhine, Waal	5	0.9-1.3	1.1-2.1 (1.4)	0.8-1.9 (1.1)	1.8-4.0 (2.5)	0.8-1.5 (1.1)	2.7-5.5 (3.7)	37-87 (49)
Salmon	Norway, Scotland	4	15-24	1.8-2.2 (2.2)	0.3-0.8 (0.5)	2.0-2.9 (2.7)	1.1-1.4 (1.3)	3.3-4.3 (4.0)	16.6-37.7 (26)
	Norway, Scotland[5]	2	12-17	1.0-1.2 (1.1)	0.3-0.5 (0.4)		0.3-0.6 (0.5)	1.9-2.0 (2.0)	12-19
Seabass	Mediterranean	1	3.6	10	3.6		1	15	
Shrimp	Norway, Western Scheldt, Wadden Sea	4	1.2-2.1	0.1-0.8 (0.4)	0.09-0.2 (0.1)	0.1-0.9 (0.5)	0.1-1.0 (0.68)	0.2-2.0 (1.2)	0.2-2.2 (1.9)
Tuna	Sri Lanka, Mediterranean	3	0.6-4.0	0.02-7.4 (3.0)	0.5-1.7 (1.1)	0.02-9 (3.5)	0.01-0.7 (0.60)	0.03-9.8 (4.2)	0.8-63 (15)

[1] *Maximum Level set by the EC (2006)*

[2] *Sum of PCB 28, 52, 101, 118, 138, 153 and 180*

[3] *For eel only the ML for the sum of PCDD/Fs and dl-PCBs is 12 pg TEQ/g ww.*

[4] *Sampled in 2003*

[5] *Sampled in 2004*

Table 4.2 Selection of literature data on concentrations of PCDD/Fs and dl-PCBs (pg TEQ/g ww) in edible fish and shellfish from various European origins.

Species	Origin	Year	TEFs[1]	dl-PCBs	PCDD/Fs	Sum	Ref
				TEQ pg/g ww			
Eel	Freshwater, Netherlands	1991	DWG[2]	1.7-19	0.32-4.2	2.0-22	(9)
Pike-perch				2.6	0.79	3.4	
Herring	North Sea/Shetland Islands			1.5-4.4	0.84-1.9	2.3-6.3	
Mackerel				3.7-4.2	0.66-0.77	4.4-5.0	
Shrimp/mussel				1.7-4.4	0.93-1.9	2.6-6.3	
Cod/Sole				0.66-2.0	0.14-0.77	0.8-2.0	
Herring	Bothnian Sea		WHO-98	1.3-10	2.5-25	3.8-35	(24)
Herring	Northern Europe	1995-1998	WHO-98		0-57-1.9		(25)
Mackerel					0.32-0.33		
Cod		1995-1997			0.04-0.05		
Plaice		1996-1998			0.29-0.36		
Salmon, farmed	Norway	1997			0.35-0.50		
Mussel	Denmark	1998			0.39		
Herring	Grenland Fjords, Norway	2000/2001	WHO-98	2.1	9.6	12	(7)
Mackerel				2.6	4.9	7.4	
Cod				0.67	0.85	1.5	
Eel				1.4-3.9	5-23	6.4-24	
Shrimps				0.23	8.2	8.4	
Mussel				0.27-0.34	1.3-2.6	1.6-3.0	
Eel	Amsterdam area, Netherlands		DWG		1.9-71		(21)
Eel	Havel, Oder, Germany	1996	I-TEQ	0-170[1]	2-15[1]		(22)
Anchovy	Adriatic Sea, Italy	1997-1998	I-TEQ		0.23-0.47		(23)
Mussel					0.11-0.24		
Mackerel					0.59-1.1		
Prawns	Retail samples, Belgium	2000-2001	WHO-98	-	0.65		(6)
Mackerel					6.2		
Trout					0.04		
Salmon	Market fish, Spain	2001-2003	WHO-98	0.97-5.1	0.45-0.54	1.4-5.6	(27)
Sardines				1.7-2.1	0.39-0.50	2.1-2.6	
Tuna				0.68-6.9	0.01-0.66	0.69-7.5	
Oyster				0.16-0.98	0.37-0.73	0.53-1.7	
Clams				0.01-0.38	0.001-0.16	0.01-0.54	
Mussels				0.07-1.2	0.05-0.15	0.12-1.3	

[1] See Table A-3 in the Annex of chapter 4.1 for the actual TEF values
[2] Dutch Working Group
[3] On lipid weight basis

This is partly explained by different TEFs used in that study (DWG-TEFs, see Table A-3 in the Annex of chapter 4.1) which led to an overestimation of the PCB-TEQ of 10-40% in the 1991 study, depending on the species and origin (data not shown). Furthermore, a steep decrease of PCB contamination in Dutch river systems was observed from the late 1970's to present *(26)*. DI-PCB-

TEQ concentrations in cod and pike-perch were in the same range in present (Table 4.1) and past (Table 4.2) Dutch studies.

Eel study

PCDD/Fs - A second study was initiated entirely focusing on eel from Dutch markets. As regards the freshwater locations, emphasis was placed on sampling of eel from polluted areas, but imported and farmed eel were included as well. The PCDD/F-TEQ concentrations in eel from different freshwater locations ranged from 0.2 to 7.9 pg/g ww (Table 4.3, see Table A-2 in the Annex of chapter 4.1 for concentrations in the individual samples). The highest PCDD/F-TEQ concentrations were found in IJssel Lake and the main rivers Meuse, Rhine and their respective deltas in the western part of the Netherlands. PCDD/F-TEQs in six out of 39 (15%) eel samples were above the ML of 4 pg/g ww. Dutch farmed eel and imported eel (also mainly consisting of farmed eel) showed lower PCDD/F-TEQ concentrations and were all below the EC-ML. The PCDD/F-TEQ concentrations in this study correspond with data of 1991 (*9*). A study on wild eel from the south coast of Norway showed PCDD/F-TEQ concentrations of 0.4 to 2.7 pg TEQ/g ww, which are within the range of the PCDD/F-TEQ concentrations in the current study (*28*).

dl-PCBs and indicator-PCBs - In all wild eel samples PCB-TEQ values were much higher than PCDD/F-TEQ values, ranging from 0.7-44 pg TEQ/g ww. A higher contribution of the dl-PCBs to the total-TEQ was also observed for farmed and imported eel. Due to the high dl-PCB contamination, a large fraction (53%) of the wild eel samples does not comply with the current EC-ML of 12 pg TEQ/g ww (sum of dl-PCBs and PCDD/Fs). Dl-PCB-TEQ values in the early 1990s study were higher (by factor 1.2 to 7.7) than those from the same locations in this study (data not shown) (*9*). This is mainly explained by the downward trend of PCB concentrations *(26)*. However, the TEF values used at that time (originating from the DWG) resulted in ca 10-20% lower TEQ compared to the $TEFs_{1998}$ (mainly due to a lower TEF_{DWG} value for CB 118). NO-PCB-TEQs in Norwegian south coast eel amounted 0.8 to 3.6 pg/g ww, which is within the range of the current study *(28)*.

The highest indicator-PCB concentrations observed in this study (Table 4.3), ranging between 13 and 1739 ng/g ww correspond to PCB concentrations observed in Finnish eel (852-1722 ng/g ww) originating from lakes with a known PCB contamination due to presence of a paper mill upstream (*8*). The wide range of concentrations in the current study is similar to the wide range observed in American eel from the Delaware River and coastal tributaries (ΣPCBs 80-1600 ng/g ww), but (much) lower than PCB concentrations observed in the Hudson river (1800-7730 ng/g ww) *(29)*. Swedish eel samples contained (on average) a ΣPCB_7 concentration of 193 ng/g ww for eel caught in the Baltic (*5*), which is below the median ΣPCB_7 concentration in wild eel in the present study. The aforementioned downward PCB trend is

confirmed by much lower ΣPCB_7 concentrations compared to those found in the late 1970's *(26)*.

Table 4.3 Ranges and medians (in brackets) of PCDD/Fs, dl-PCBs (based on $TEFs_{1998}$) and indicator PCBs in a wide range of eel samples. Levels above the current ML are printed bold.

Species	n	Lipids	NO-PCBs	MO-PCBs	dl-PCBs	PCDD/Fs	dl-PCBs + PCDD/Fs	ΣPCB_7[2]
		(%)				(pg TEQ/ g ww)		(ng/ g ww)
EC-ML[1]	-	-	-	-	-	4	12	-
Wild	39	3.7-23	0.3-14 (3.8)	0.4-30 (6.0)	0.7-44 (10)	0.2-7.9 (1.8)	0.9-52 (13)	13-1739 (296)
Farmed	11	29-38	1.2-6.1 (3.9)	0.6-2.0 (1.3)	1.8-7.7 (5.2)	0.8-3.3 (2.2)	2.6-11 (7.6)	18-70 (45)
Imported	14	20-34	0.3-5.2 (1.5).	0.1-1.8 (0.7)	0.3-7.1 (2.1)	0.2-2.9 (0.9)	0.5-9.8 (3.2)	LOQ-65 (23)

[1] *Maximum Level set by the EC (2006)*
[2] *Sum of PCB 28, 52, 101, 118, 138, 153 and 180*

Effect of smoking of eel on contaminant concentrations

An experiment was conducted in order to determine if smoking of eel would result in a reduction of contaminants. The results presented Figure 4.1 show that both traditional and industrial smoking influences the contaminant concentrations in eel. In the first experiment a concentration effect can be seen: as a result of decreasing moisture content due to the evaporation of water during the smoking process, the fat content and the concentrations of contaminants (on a wet weight basis) have increased. The contaminant concentrations in experiment 2 remained almost the same after smoking. The results, expressed as chemical mass (as determined by the weight of fish multiplied by the concentration of contaminants) show a decrease of PCDD/F-TEQ of 12-45% (data not shown) for all smoked samples compared with the raw material. This decrease corresponds to the PCBs reduction of 40% found by Zabik *et al. (30)* after smoking of Great Lakes lake trout fillets. A 2,3,7,8-TCDD reduction of 100% was determined in an other study on smoking of Great Lakes lake trout *(31)*. It should be noted that in our study the chemical mass was calculated based on weight of the *whole fish* multiplied by the concentration of contaminants in the fillet, whereas in the other studies the weight of the *fillet only* was multiplied by the concentration of contaminants in the fillet, which is more correct because it takes only the edible parts into account.

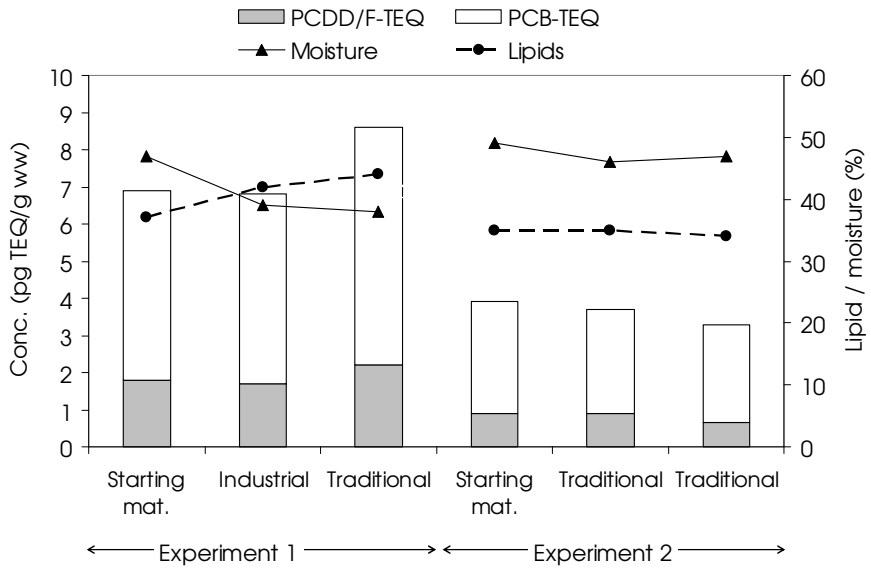


Figure 4.1 Influence of smoking on the PCDD/F-TEQs, dl-PCB-TEQs and lipid and moisture content in smoked eel. Starting mat. = source material prior to smoking. Traditional and industrial depict the type of smoking process.

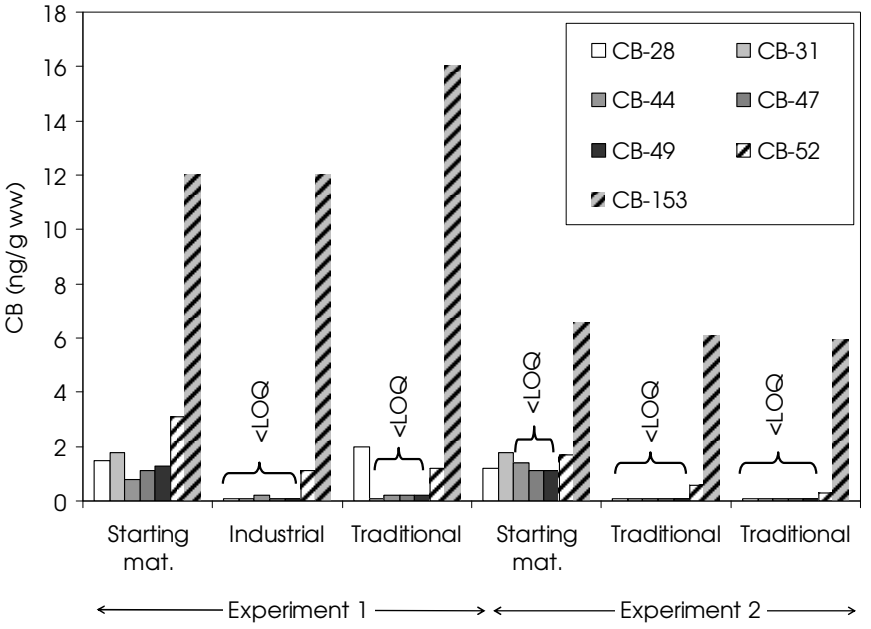


Figure 4.2 Loss of lower chlorinated PCBs from the fillets as a result of smoking of eel. Starting mat. = source material prior to smoking. Traditional and industrial depict the type of smoking process.

Lower chlorinated PCBs (up to CB-52) largely disappeared from the eel due to volatilization. The concentrations were mostly below the LOQ after smoking (Figure 4.2). To our knowledge, no other results on reduction of lower chlorinated PCBs in fish by smoking have been reported earlier.

Contaminant profile information

Contribution of dl-PCBs to the total TEQ - The contribution of PCBs and PCDD/Fs to the TEQ is shown Figure 4.3. In all samples (except shrimps), a prevalence of dl-PCBs (on TEQ basis) is found ranging from 53% for herring to 83% for tuna. This corresponds nicely to data reported by Focant et al. (*6*) but is somewhat lower than in data from Dutch fish from 1991 (*9*), which most likely is caused by the decrease of the (dl-)PCB concentrations since 1991.

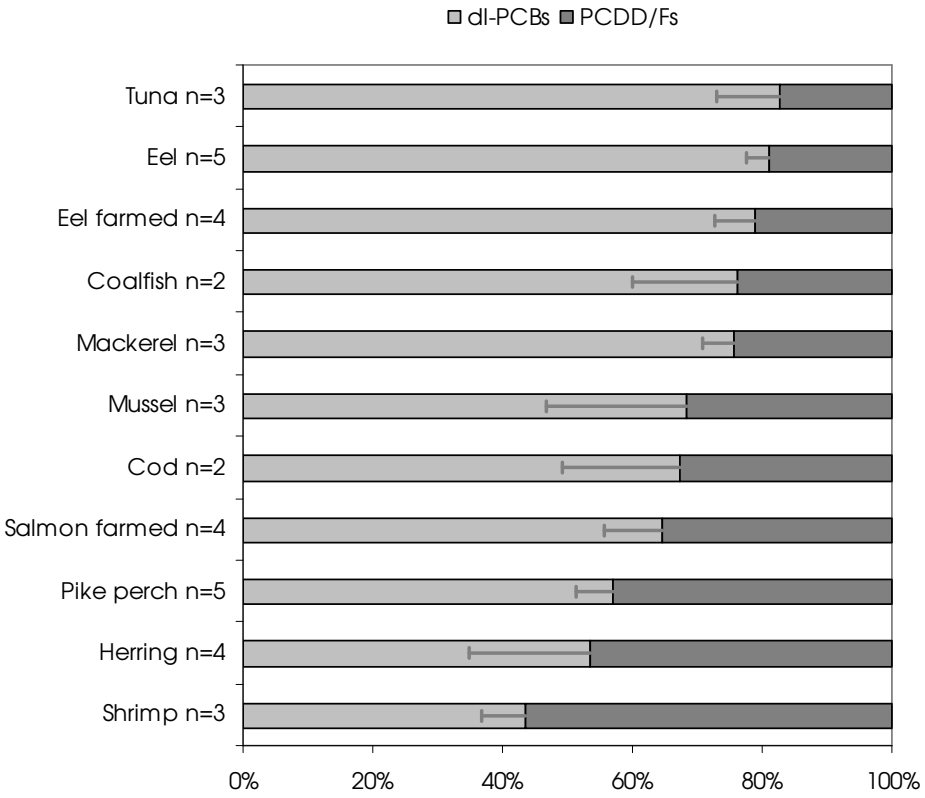


Figure 4.3 Average contribution of PCDD/Fs and dl-PCBs to the total TEQ for various fish (see Table 4.1 for the fish data) using TEFs1998. Bars represent the standard deviation of the average value.

Eel shows a high PCB contribution to the total-TEQ, which is caused by the relatively high PCB contamination of the Dutch freshwater systems. In wild eel, dl-PCBs contributed 69-98% to the total-TEQ (Table 4.3), and within the dl-PCBs, the MO-PCBs generally predominate (contribution of 49 to 58% to the PCB-TEQ), while in other fish the NO-PCBs predominate (average 80%). This could be caused by the ability of eel to metabolise CBs 77 and 126, leading to a lower NO-PCB contribution (9). However, in farmed eel, the contribution of NO-PCBs to the PCB-TEQ is predominant (67-86%). This is presumably caused by a different contamination pattern of their feed (typically composed feed with marine fish oil). Furthermore, farmed eel is less active than wild eel, which may influence their metabolic activity as well.

Congener profile analysis - A principal component analysis (PCA) was carried out to investigate differences in congener profiles of marine fish, shellfish and farmed fish (salmon). The PCA was carried out for CB-77, CB-126, CB-169 and all PCDD/F congeners except those for which the majority of the samples were below the LOQ (1,2,3,7,8,9-HxCDF and 1,2,3,4,7,8,9-HpCDF). Furthermore, highly contaminated samples like eel and fish livers were removed from the dataset. PC1 is not shown because observed clustering was based on absolute contaminant concentrations (high concentrations were clustered as well as low concentrations). The PCA (PC2 and PC3) plots are shown Figure 4.4. The profiles of four identified clusters, are shown in Figure 4.5. The columns in the histograms are relative to the sum of the absolute (*not* multiplied with TEFs) concentrations of all compounds. The histograms concern averages of the clustered samples and standard deviations are indicated by bars. Concentrations below LOQ were set at zero.
The four identified congener profiles are distinctive. Cluster 1 (fish from the Mediterranean, n=3) stands out from the other clusters as they contain relatively high concentrations of NO-PCBs (making up over 98% of the sum of the absolute concentrations i.e. sum of the concentrations of PCDD/Fs and CB-77, CB-126, CB-169). Similar studies on Mediterranean fish confirm the relatively high NO-PCB concentrations *(4,23,32,33)*. The PCDD/F profile from the present study is very similar to the profiles for Mediterranean fish published by Bayarri *et al. (23)* with 2,3,4,7,8-PeCDF being the predominant congener which makes up over 40% of the sum of PCDD/Fs. Together with 2,3,7,8-TCDF, 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD they make up nearly 80% of the sum of PCDD/Fs (present study). As NO-PCBs almost exclusively determine the total-TEQ in Mediterranean fish, monitoring of these three PCBs could be sufficient for food safety purposes. Cluster 2 concerns pike-perch, profile of which is also dominated by the NO-CBs (97% of the sum of PCDD/Fs and no-CBs). The enrichment of CB-77, found by de Boer *et al.* (*9*), was confirmed in the present study. Among the PCDD/Fs, 2,3,7,8-TCDD, 2,3,7,8-TCDF and OCDD dominate the profile. Cluster 3 shows one salmon and two mussel samples.

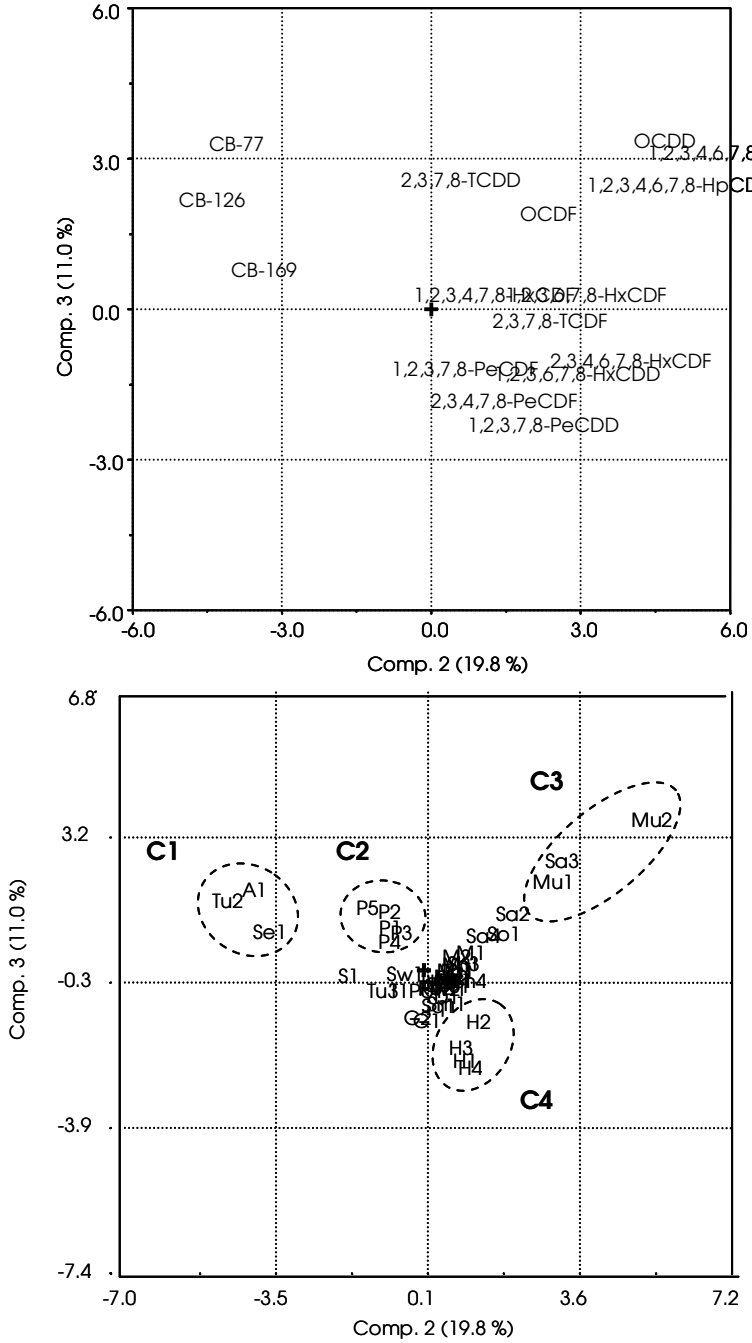


Figure 4.4 Principal-component score and loading plot of PC2 and PC3 of fish samples from the year 2000 fish samples. The circles represent the different fish groups and have no statistical significance. C1 = Mediterranean fish, C2 = Pike-perch, C3 = Mussel, C4 = Herring.

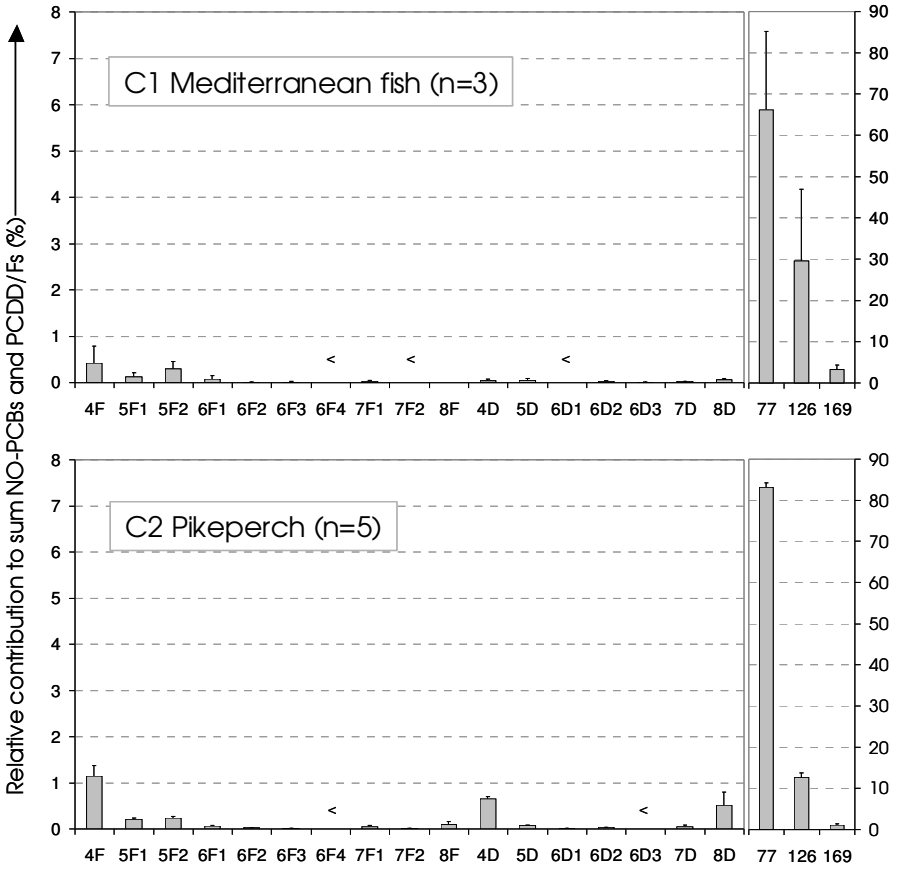


Figure 4.5 Congener profile information of pike-perch, mussels, herring and Mediterranean fish, relative to the sum of PCDD/F and NO-CB concentrations. < indicates that this congener was <LOQ in all samples. Abbreviations: 4F=2,3,7,8-TCDF; 5F1=1,2,3,7,8-PeCDF; 5F2=2,3,4,7,8-PeCDF; 6F1=1,2,3,4,7,8-HxCDF; F2=1,2,3,6,7,8-HxCDF; 6F3=2,3,4,6,7,8-HxCDF; 6F4=1,2,3,7,8,9-HxCDF; 7F1=1,2,3,4,6,7,8-HpCDF; F2=1,2,3,4,7,8,9-HpCDF; 8F=OCDF; 4D=2,3,7,8-TCDD; 5D=1,2,3,7,8-PeCDD; 6D1=1,2,3,4,7,8-HxCDD; 6D2=1,2,3,6,7,8-HxCDD; 6D3=1,2,3,7,8,9-HxCDD; 7D=1,2,3,4,6,7,8-HpCDD; 8D=OCDD; 77=CB-77; 126=CB-126; 169=CB-169

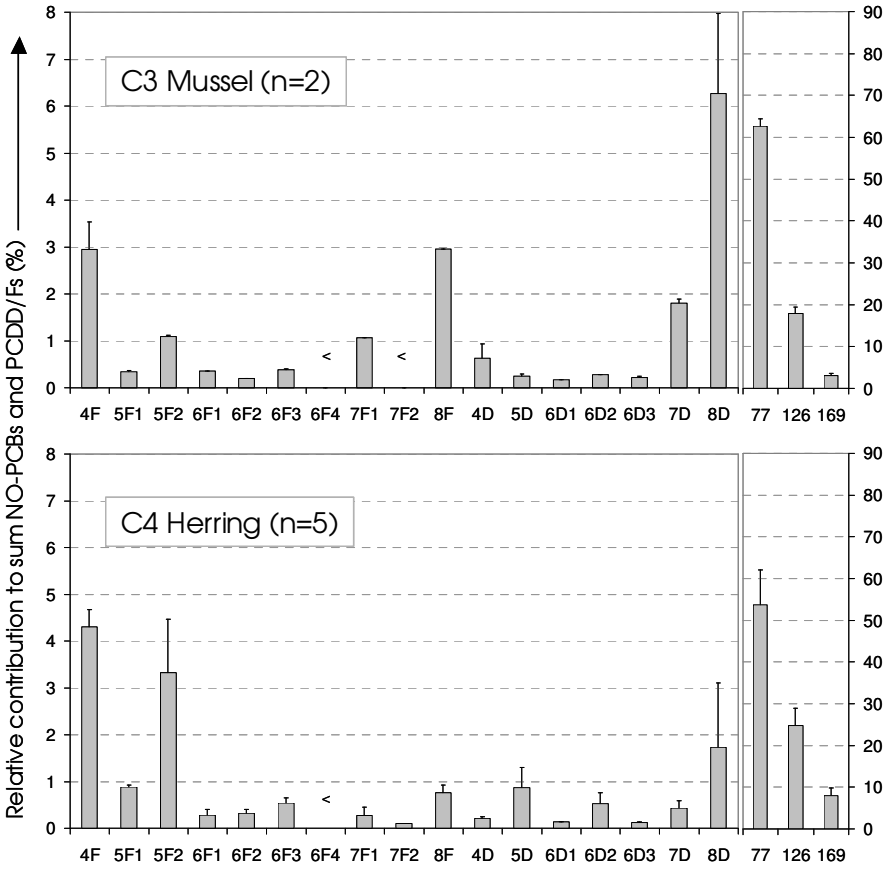


Figure 4.5 Continued

The salmon sample within the mussel cluster could not be confirmed by the other three salmon samples. The low number of samples and a different origin probably explain these differences. Concerning the mussel samples, NO-PCBs make up ca. 84% of the sum of PCDD/Fs and NO-PCBs. Within the group of PCDD/Fs, OCDD is the predominant congener, followed by 2,3,7,8-TCDD, 1,2,3,4,6,7,8-HpCDD and OCDF. Burgess and McKinney *(34)* showed that the PCB profile in the tissue of a filter feeding marine bivalve species (*M. Lateralis*) is very similar to the profile observed in the sediment and overlying water. This suggests that the profile observed in our samples is caused by the profile in the sediment and particulate matter at the harvest location of the mussels, although there is no data available to confirm this. The profile reported by Gomara *et al.* on mussel samples from the Spanish market *(27)* was very similar to the current data. Bayarri *et al.* reported a different pattern (2,3,4,7,8-PeCDF and 2,3,7,8-TCDF as almost the only PCDD/F congeners present) in mussels from the Adriatic Sea *(23)*, which is characteristic for that location as it was also observed in other fish. Concerning the herring cluster (cluster 4), the predominance of 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF and 1,2,3,7,8-PeCDD determine the deviating profile as compared to other fish from the English Channel and the North Sea. Different feeding habbits (and therefore bioaccumulation) and a stronger migration pattern compared to other species could be the cause for these deviating profiles. The PCDD/F profile in herring from the present study was similar to the profile of herring and herring products from North-West Europe (including the Baltic Sea *(25)*). A food web study of Baltic herring showed that the contamination profile for PCBs was similar to the profile in the diet of herring (mysis and zooplankton) *(35)*. It's likely that the North Sea herring contamination profile is also dictated by its diet, but contamination data of the diet are not available at the moment.

Table 4.4 Average concentrations of PCDD/Fs and dl-PCBs calculated with $TEFs_{2005}$. A selection of fish samples is taken from Table 4.1 and 4.3. Between brackets the relative concentrations (%) calculated with $TEFs_{1998}$ are given.

Species	Origin	n	NO-PCBs	MO-PCBs	dl-PCBs	PCDD/Fs	dl-PCBs + PCDD/Fs
			(pg TEQ/g ww)				
EC-ML[1]	-	-	-	-	-	4	8 (12 for eel)
Cod	North Sea	2	0.3 (104)	0.01 (22)	0.3 (89)	0.2 (95)	0.5 (91)
Wild eel	Dutch fresh water systems	39	5.1 (105)	1.7 (20)	6.8 (51)	2.0 (90)	8.9 (57)
Farmed eel	Various Dutch farmers	11	4.1 (103)	0.3 (22)	4.4 (84)	1.6 (77)	5.9 (82)
Herring	The English Channel, North Sea	4	1.4 (106)	0.1 (24)	1.5 (89)	1.3 (80)	2.8 (85)
Salmon	*Norway, Scotland*	*4*	*2.2 (104)*	*0.2 (30)*	*2.3 (90)*	*1.1 (84)*	*3.4 (88)*

[1] *Maximum Level set by the EC (2006)*

Evaluation of new TEFs

Following revision of the TEF values from 1998, the WHO has accepted new human TEFs (www.who.org and van den Berg *et al.* (*20*)) in 2005 ($TEFs_{2005}$). The changed TEFs comprise (1998→2005) for 1,2,3,7,8-PeCDF (0.05→0.03), 2,3,4,7,8-PeCDF (0.5→0.3), OCDD (0.0001→0.0003), OCDF (0.0001→0.0003), CB 81 (0.0001→0.0003), CB 169 (0.01→0.03), CB 105, 118, 123 and 189 (0.0001→ 0.00003), CB 114, 156, 157 (0.0005→0.00003). For CB 167, the TEF was slightly increased from 0.00001 to 0.00003.

Table 4.4 shows that the application of the 2005 TEFs results in a slight increase of the NO-CB-TEQ, mainly resulting from an increased CB-169 contribution. However, the MO-PCB contribution to the TEQ dramatically decreased by 70-80%, largely caused by a combination of the reduced CB-156 and CB-118 TEFs and the relative high concentrations of these congeners in fish samples. The reduction of the PCDD/F-TEQs is mainly a result of the reduced 2,3,4,7,8-PeCDF TEF. The total-TEQ reduction caused by use of the new TEFs is in the range of 10-20%. Wild eel is the exception to this, due to the relatively high level of MO-PCBs with an average reduction of the total-TEQ of 43%. The current EC-MLs, based on the $TEFs_{1998}$ would result in 51% of the eel samples not complying with the ML. Using the $TEFs_{2005,}$ non-compliance is reduced to 28%.

Screening samples using the DR CALUX® assay

The DR-CALUX® (*10*) assay was used to estimate the total-TEQ levels and in particular to select samples requiring further analysis by GC-HRMS. Considering the high concentrations of MO-PCBs in eel samples and the relatively poor response of these PCBs in the assay, concentrations were estimated by comparison with a set of fish oil samples spiked with PCDD/Fs, NO-PCBs and MO-PCBs in a representative composition of 15/40/45 in terms of contribution to the TEQ-level. The PCDD/F mixture used for spiking contained equal amounts of all congeners, the NO-PCB mix equal amounts of PCBs 77, 126 and 169, and the MO-PCBs mix contained PCBs 118 and 156 in a composition of 7 to 1. Based on the $TEFs_{1998}$ the levels in oil were 200, 120, 60, 30 and 0 pg TEQ/g. Concentrations were 30% lower with the TEF_{2005}. Samples that were below the DR-CALUX LOQ (10 pg TEQ/g lipid weight) were removed from the dataset (13% of the datapoints, see Figure 4.6), because the CALUX sensitivity was not optimized for the very low concentrations at the time of the study. A further improvement in sensitivity was obtained by dissolving the extract in smaller amount of DMSO (*10*). Figure 4.6 compares the levels based on both TEF systems, showing a good relation between DR-CALUX® and GC-HRMS with an almost 1:1 relationship when the $TEFs_{1998}$ are used. Based on the $TEFs_{2005}$ the correlation has improved (R^2=0.91) but in this case the DR-CALUX® overestimated the levels 1.4 fold. This was somewhat unexpected since the new TEF values are much closer to the relative potency (REP)-values *(10,36)* in

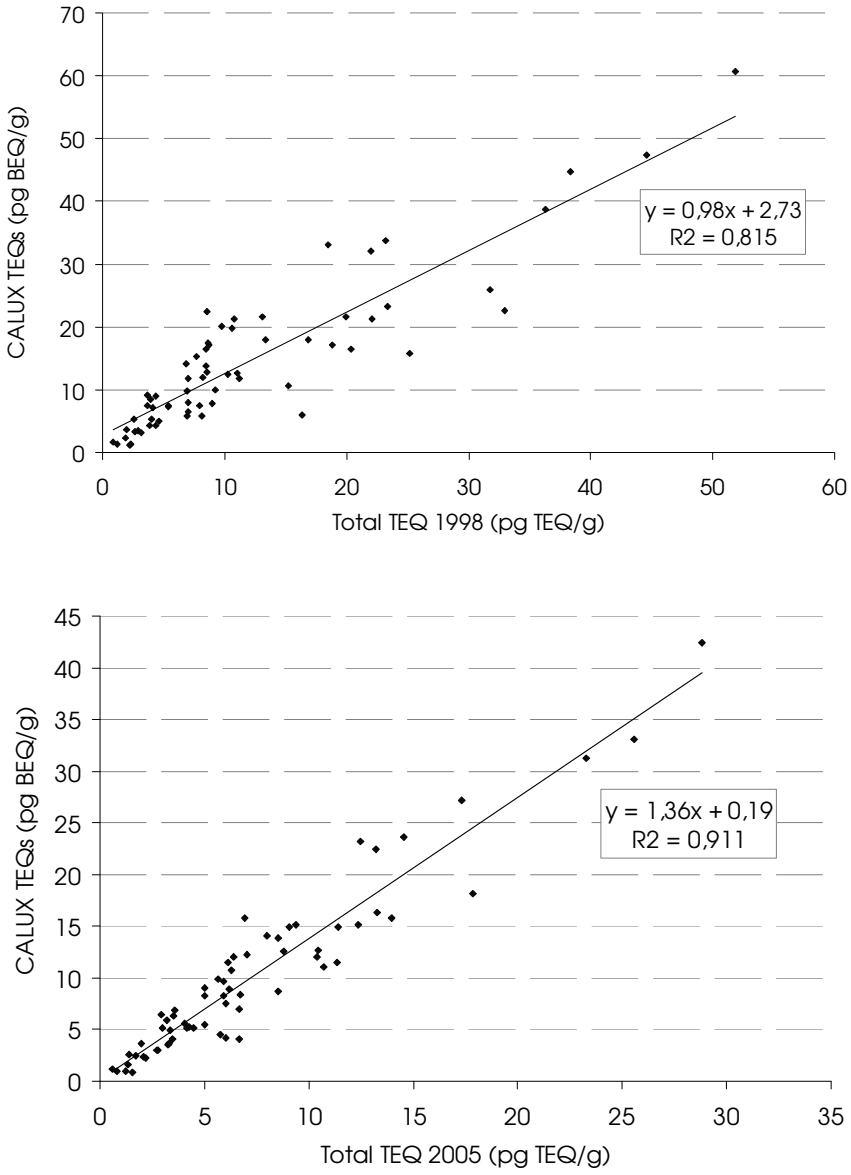


Figure 4.6 Comparison of total-TEQ results obtained by GC-HRMS and CALUX. GC-HRMS results were calculated using two different TEF systems (1998 top and 2005 bottom). Lower bound results are shown for GC-HRMS and CALUX data below the LOQ (10 pg TEQ/g lipids) excluded.

the bioassay. Although this was overcome by the use of reference samples, a better correlation was particularly expected (using the new TEFs) for samples with a composition deviating from that of the reference samples. The intercept of the regression line almost goes through the origin (intercept = 0.2), meaning that the use of TEF_{2005} results in an improved relation of the data in the lower parts of the curve. One explanation for the overestimation (1.4-fold) by the bioassay may be that the DR-CALUX® assay actually detects other dioxin-like compounds present in eel. Several contaminants (such as polybrominated diphenyl ethers) have shown agonistic or antagonistic behaviour *(37,38),* which may have influenced the DR-CALUX signal. However, this requires further investigation.

Implications for eel consumption.
The high PCDD/F and PCB levels found in eel may pose a risk to consumers in case of preference consumption. In order to determine this, we calculated the amount of eel that safely could be eaten without exceeding the tolerable weekly intake (TWI) of 14 pg total-TEQ/kg bodyweight (bw) as set by the EC Scientific Committee for Food (SCF) *(39)*. Baars *et al. (40)* determined a life-long average Dutch intake of 1.2 pg total-TEQ/kg bw per day (median), being equivalent to 8.4 pg/kg bw per week. As a result, the average intake is 5.6 pg/kg bw per week below the TWI of 14. With a median eel total-TEQ of 13 pg TEQ/g ww, the average consumer is allowed to consume only 28 g of the median contaminated eel per week (life-long). The consumption of the most contaminated eel (52 pg total-TEQ/g ww) drops down to 7 g/week (life-long). Although this is a very small amount, and portion sizes are in the order of 100-200 g, the average consumption of eel in the Netherlands is only 0.9 gram/week *(41)*. Therefore, the average Dutch consumer will presumably not be at risk when consuming Dutch wild eel, even from the most polluted sites. Only a small group of frequent consumers such as sports anglers, professional fishermen and consumers preferring wild eel may be at risk when frequently consuming the most contaminated eels.

Acknowledgements

The Dutch Ministry of Agriculture, Nature and Food Quality is acknowledged for their financial support to this project. The colleagues of IMARES, IJmuiden, The Netherlands are acknowledged for their skillful contributions to this project.

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Annexes of 4.1

Table A-1 Individual fish sample results (PCDD/Fs, dl-PCBs and indicator-PCBs)

Species	Name (Latin)	Location	TEQ-concentrations (pg TEQ/g product)					Σ 7PCBs	Lipids
			NO-PCBs	MO-PCBs	Σdl-PCBs	PCDD/Fs	ΣPCB+PCDD/Fs	(ng/g ww)	(%)
Anchovy	*Engraulis encrasicolus*	Italy	6.0	1.9	7.9	0.6	8.5	71	4.4
Blue Whiting	*Micromesistius poutassou*	Atlantic Ocean	0.36	0.16	0.52	0.11	0.63	4.4	0.65
Trout, farmed	*Oncorhynchus mykiss*	Farmed	0.6	0.1	0.7	0.2	0.9	6.7	5.4
Grey Gurnard	*Eutrigla gurnardus*	North Sea	1.6	0.3	1.9	1.4	3.3	15	7.4
Herring	*Clupea harengus*	The Canal	1.2	0.6	1.8	1.5	3.2	16	7.1
Herring	*Clupea harengus*	North Sea	0.8	0.3	1.1	1.3	2.4	9.5	1.8
Herring	*Clupea harengus*	The Canal	3.6	0.3	3.9	1.8	5.7	16	9.1
Herring	*Clupea harengus*	The Canal	1.8	0.3	2.1	2.1	4.2	16	15
Hake	*Merluccius merluccius*	Celtic Sea	0.3	0.1	0.4	0.05	0.4	3.8	1.2
Halibut	*Hippoglossus hippoglosus*	Farmed	0.3	0.2	0.5	0.2	0.7	6.3	2.2
Horse mackerel	*Trachurus trachurus*	Celtic Sea	1.4	0.7	2.1	0.57	2.7	21	7.7
Cod	*Gadus morhua*	Silverpit	0.17	0.05	0.22	0.07	0.29	1.6	0.77
Cod	*Gadus morhua*	North Sea	0.4	0.1	0.5	0.3	0.8	2.9	1.0
Cod liver	*Gadus morhua*	North Sea	57	15	72	17	89	555	58
Coalfish	*Pollachius virens*	North Sea	0.6	0.2	0.8	0.1	1.0	6.6	0.94
Coalfish (black)	*Rachycentron canadum*	Northern North Sea	0.2	0.01	0.2	0.1	0.3	1.0	1.7
Dab	*Limanda limanda*	North Sea	0.66	0.17	0.84	0.54	1.4	7.1	2.0
Eel	*Anguilla anguilla*	Yssel Lake (Enkhuizen)	14	19	33	3.9	37	602	23
Eel	*Anguilla anguilla*	Yssel Lake (Staveren)	7.0	7.6	15	3.1	18	263	24
Eel	*Anguilla anguilla*	Yssel Lake (Medemblik)	3.8	3.7	7.5	2.1	9.6	136	25
Eel	*Anguilla anguilla*	Yssel Lake (Urkerhoek)	8.3	10.2	19	3.6	22	351	22
Eel	*Anguilla anguilla*	Yssel Lake (Staveren)	3.7	3.6	7.3	1.4	8.7	129	21
Eel, farmed	*Anguilla anguilla*	Netherlands	5.5	2.3	8.2	2.5	11	69	34
Eel, farmed	*Anguilla anguilla*	Italy	2.3	0.9	3.2	0.8	3.9	43	25
Eel, farmed	*Anguilla anguilla*	Netherlands	6.2	1.0	7.2	2.2	9.4	57	33
Eel, farmed	*Anguilla anguilla*	Italy	4.1	1.3	5.4	0.7	6.1	68	27
Haddock	*Melanogrammus aeglefinus*	North Sea	0.1	0.01	0.1	0.1	0.2	0.5	0.7
Haddock liver	*Melanogrammus aeglefinus*	North Sea	23	3	26	18	43	150	48
Mackerel	*Scomber scombrus*	South Ireland	1.3	0.3	1.6	0.63	2.2	19	17

Table A-1 Continued

Species	Name (Latin)	Location	TEQ-concentrations (pg TEQ/g product)					Σ 7PCBs	Lipids
			NO-PCBs	MO-PCBs	ΣdI-PCBs	PCDD/Fs	ΣPCB+PCDD/Fs	(ng/g ww)	(%)
Mackerel	*Scomber scombrus*	Skagerrak	1.0	<0.7	1.0	0.3	1.3	1.6	24
Mackerel	*Scomber scombrus*	Atlantic Ocean	1.0	<0.7	1.0	0.3	1.3	1.1	24
Mussel	*Mytulis edulis*	Eastern Scheldt	1.7	0.3	2.0	1.5	3.5	15	1.7
Mussel	*Mytulis edulis*	Wadden Sea West	0.9	0.3	1.2	1.1	2.3	13	1.5
Pike-perch	*Stizostedion Lucioperca*	Nieuwe Merwede	1.5	1.2	2.7	1.1	3.8	63	0.94
Pike-perch	*Stizostedion Lucioperca*	Lek, Hagestein	1.4	1.1	2.5	1.3	3.7	49	0.97
Pike-perch	*Stizostedion Lucioperca*	Amer, Drimmelen	1.0	0.8	1.8	0.9	2.7	37	0.91
Pike-perch	*Stizostedion Lucioperca*	River Rhine	1.3	1.0	2.3	0.8	3.1	47	0.95
Pike-perch	*Stizostedion Lucioperca*	River Waal	2.1	1.9	4.0	1.5	5.5	87	1.3
Pilchard	*Sardina Pilchardus*	The Canal	5.0	1.3	6.3	1.6	7.9	50	5.3
Plaice	*Pleuronectes platessa*	North Sea	0.18	0.05	0.23	0.25	0.48	1.3	0.9
Redfish	*Sebastes marinus*	Northern North Sea	1.4	0.2	1.6	0.8	2.4	7.4	3.0
Salmon (Norwegian)	*Salmo salar*	Norway, farmed	1.7	0.3	2.0	1.3	3.3	17	16
Salmon (Norwegian)	*Salmo salar*	Norway, farmed	2.1	0.8	2.9	1.4	4.3	38	24
Salmon (Scottish)	*Salmo salar*	Scotland, farmed	2.2	0.6	2.8	1.1	3.9	29	15
Salmon (Scottish)	*Salmo salar*	Scotland, farmed	2.2	0.4	2.6	1.4	4.1	23	19
Sardinella	*Sardinella uarita*	Afrika	0.07	0.01	0.08	0.02	0.10	1.2	2.7
Sea bass	*Dicentrarchus labrax*	France	10	4.0	14	1.0	15	138	3.6
Sea devil	*Lophius piscatorius*	Northern North Sea	0.2	<0.07	0.2	0.1	0.3	0.2	0.47
Shrimps (Dutch)	*Crangon crangon*	Western Wadden Sea	0.7	0.2	0.9	1.0	2.0	3.0	2.0
Shrimps (Dutch)	*Crangon crangon*	Wadden Sea (Sylt)	0.49	0.07	0.57	0.76	1.3	1.2	2.1
Shrimps (Dutch)	*Crangon crangon*	Western Scheldt	0.4	0.09	0.5	0.6	1.1	2.6	1.8
Shrimps (Norwegian)	*Pantalus borealis*	Norway	0.1	0.006	0.1	0.1	0.2	0.9	1.2
Sole	*Solea solea*	North Sea	0.11	0.07	0.18	0.15	0.32	1.9	1.2
Sprat	*Sprattus sprattus*	North Sea	2.7	0.4	3.1	2.5	5.6	26	10
Squid	*Loligo spp.*	Dutch coast	1.7	0.3	2.0	1.2	3.1	14	2.4
Swordfish	*Xiphias gladius*	Italy	2.6	0.8	3.4	0.5	3.9	37	6.1
Tuna	*Thunnus thynnus*	Sri Lanka	0.02	<0.07	0.02	0.01	0.03	0.2	0.6
Tuna (Bonito)	*Thunnus thynnus*	Italy	7.3	1.7	9.0	0.7	9.8	63	1.4

Table A-1 Continued

Species	Name (Latin)	Location	TEQ-concentrations (pg TEQ/g product)					Σ 7PCBs	Lipids
			NO-PCBs	MO-PCBs	Σdl-PCBs	PCDD/Fs	ΣPCB+PCDD/Fs	(ng/g ww)	(%)
Tuna	*Thunnus thynnus*	France	3.0	0.5	3.5	0.6	4.2	15	4.0
Turbot	*Psetta Maxima*	Dutch coast	1.9	0.5	2.4	0.8	3.3	17	2.8
Whiting	*Merlangius merlangus*	North Sea	0.14	0.02	0.17	0.07	0.24	1.3	1.0
Yellow Gurnard	*Trigla lucerna*	North Sea	2.0	0.4	2.4	1.5	4.0	17	4.2
Silversmelt	*Argentina silus*	Atlantic Ocean	0.47	0.08	0.54	0.40	0.94	2.4	1.1

Table A-2 Individual eel (Anguilla anguilla) sample results (PCDD/Fs, dl-PCBs and indicator-PCBs).

	TEQ-concentrations (pg TEQ/g product)						Σ7PCBs	lipids
Origin / sample i.d.	**NO-PCBs**	**MO-PCBs**	**Σdl-PCBs**	**PCDD/Fs**	**ΣPCB+PCDD/Fs**	**CALUX[1]**	**(ng/g ww)**	**(%)**
Wild eel								
New Merwede	14	30	44	7.9	52	61	1739	23
Hollands Deep	13	24	38	6.6	45	47	1560	22
Haringvliet West (near locks)	13	19	32	6.3	38	45	1057	19
Meuse, Keizersveer	10	24	34	2.4	36	39	1583	21
Haringvliet-East	9.4	18	27	4.7	32	26	1031	12
Meuse, Venlo	6.3	18	24	1.1	25	16	1164	10
Lek, Culemborg	7.1	13	20	3.3	23	23	651	14
IJssel Lake, monding Ketelmeer	8.0	11	19	4.2	23	34	551	18
Roer, Vlodrop	4.7	17	21	1.1	22	15	635	7.2
Meuse-Waal canal, Malden	6.7	14	20	1.8	22	21	827	11
Waal, Tiel (Hagestein)	8.1	11	19	2.8	22	32	510	17
Rhine, Lobith	6.7	11	18	2.3	20	16	539	12
Ketel Lake Schokkerhav. K12	7.4	9.7	17	2.9	20	22	438	18
Amsterdam-Rhine canal	5.1	11	16	2.9	19	17	437	18
IJssel Lake, Urk	6.7	7.5	14	4.3	18	33	369	21
Volkerak VZ3	5.1	7.8	13	4.0	17	18	356	13
Meuse, Eijsden	3.2	13	16	0.41	16	6.0	944	6.0
Amstel, Uithoorn	2.4	11	14	1.4	15	11	553	19
Zuid-Willems canal, Veghel	6.0	6.0	12	1.4	13	18	385	18
IJssel Lake, Enkhuizen	5.3	4.7	10	3.1	13	22	190	18
IJssel, Deventer	3.3	6.7	10	1.2	11	12	296	11
IJssel Lake, Afsluitdijk	3.9	3.1	7.0	2.3	9.3	10	130	19
Aar canal, Ter Aar	2.5	5.1	7.6	1.4	9.0	7.8	217	14
IJssel Lake, Stavoren	3.8	2.6	6.5	2.3	8.7	17	121	19
IJssel Lake, Medemblik	3.3	2.9	6.2	2.3	8.5	16	137	22
Marker Lake, Enkhuizen	3.4	3.1	6.5	2.0	8.5	14	125	15
North Sea canal, Kruithaven	1.7	4.4	6.2	1.8	8.0	7.5	166	9.0

[1] *CALUX-TEQ determined by CALUX bioassay*

Table A-2 Continued

Origin / sample i.d.	TEQ-concentrations (pg TEQ/g product)						Σ7PCBs	lipids
	NO-PCBs	MO-PCBs	Σdl-PCBs	PCDD/Fs	ΣPCB+PCDD/Fs	CALUX[1]	(ng/g ww)	(%)
Zoom Lake	2.7	2.1	4.9	2.2	7.0	12	86	16
North Sea canal, Velsen/IJmuiden	1.7	4.3	6.0	0.95	6.9	5.9	161	5.9
Twente canal., Hengelo	2.1	4.2	6.3	0.60	6.9	10	155	7.1
Marker Lake, kuil van Marken	2.4	1.4	3.8	1.6	5.4	7.3	56	18
Lauwers Lake	1.8	1.7	3.5	1.1	4.6	5.0	67	20
Veluwe Lake, Harderwijk	1.9	2.0	3.8	0.52	4.3	4.3	55	11
Gooi Lake, Naarden	1.6	1.2	2.8	1.0	3.8	4.4	47	17
Princess Margriet canal, Suawoude	1.2	1.2	2.5	0.69	3.2	3.2	48	15
Vecht, Ommen	1.0	1.6	2.6	0.37	2.9	3.5	59	6.6
Linge, Rhenoy	0.65	1.4	2.0	0.28	2.3	1.4	63	3.9
Wolderwijd (WOL6)	0.95	0.85	1.8	0.46	2.3	1.2	26	7.1
North Holland canal.,Akersloot	0.28	0.39	0.7	0.21	0.89	1.7	13	3.7
Farmed eel								
Sample 4	6.1	1.6	7.7	3.1	11	21	57	37
Sample 5	5.3	2.0	7.3	3.3	11	20	70	34
Sample 9	5.6	1.6	7.2	3.1	10	12	58	38
Sample 8	4.3	1.3	5.6	2.7	8.2	12	47	33
Sample 3	3.9	1.3	5.2	2.5	7.7	15	48	36
Sample 6	4.0	1.3	5.4	2.2	7.6	1.9	45	37
Sample 1	3.9	1.2	5.1	1.8	6.9	14	40	37
Sample 7	3.3	0.98	4.3	1.1	5.4	7.4	31	34
Sample 10	2.6	0.92	3.5	0.9	4.4	9.0	31	37
Sample 11	2.1	0.65	2.8	1.2	4.0	5.3	23	29
Sample 2	2.3	0.75	3.0	0.90	3.9	8.5	21	35

[1] *CALUX-TEQ determined by CALUX bioassay*

Table A-2 Continued

Origin / sample i.d.	TEQ-concentrations (pg TEQ/g product) NO-PCBs	MO-PCBs	Σdl-PCBs	PCDD/Fs	ΣPCB+PCDD/Fs	CALUX[1]	Σ7PCBs (ng/g ww)	lipids (%)
Imported eel								
Hongary (farmed eel)	5.1	1.7	6.8	2.9	9.8	20	62	34
Sweden 2 (farmed eel)	5.2	1.8	7.1	1.5	8.6	17	65	33
Denmark (farmed eel)	4.6	1.4	6.0	2.1	8.1	5.8	53	34
Sweden 1	4.1	1.5	5.6	1.4	7.0	6.4	51	31
Israel	2.3	0.75	3.0	1.1	4.1	7.1	25	30
Northern Ireland Laugh-neagh	1.5	0.73	2.2	1.4	3.7	7.4	21	31
Italy 4 (Milano)	1.5	1.0	2.5	0.30	2.8	1.8	33	27
Ierland (2)	1.2	0.63	1.8	0.79	2.6	3.4	18	24
Italy 2	1.3	0.60	1.9	0.65	2.5	5.3	25	26
Italy 1	0.88	0.48	1.4	0.57	1.9	3.6	13	28
Italy 3 (Sardinie)	0.80	0.33	1.1	0.16	1.3	0.0	11	23
Ireland (wild eel)	0.41	0.18	0.6	0.37	0.96	0.2	2.3	25
Ireland 1	0.35	0.24	0.6	0.22	0.82	0.7	7.1	20
Turkey (wild eel)	0.25	0.06	0.3	0.19	0.51	1.9	0.0	19

[1] *CALUX-TEQ determined by CALUX bioassay*

Table A-3. TEF-values for PCDD/Fs and dl-PCBs

	DWG-TEF	I-TEF [1]	WHO-TEF 1997[2]	WHO-TEF 2005[3]
PCDDs/Fs				
2,3,7,8-TCDD	1[4]	1	1	1
1,2,3,7,8-PeCDD	0.5[4]	0.5	1	1
1,2,3,4,7,8-HxCDD	0.1[4]	0.1	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1[4]	0.1	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1[4]	0,1	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01[4]	0.01	0.01	0.01
OCDD	0.001[4]	0.001	0.0001	0.0003
2,3,7,8-TCDF	0.1[4]	0.1	0.1	0.1
1,2,3,7,8-PeCDF	0.05[4]	0.05	0.05	0.03
2,3,4,7,8-PeCDF	0.5[4]	0.5	0.5	0.3
1,2,3,4,7,8-HxCDF	0.1[4]	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1[4]	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1[4]	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1[4]	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01[4]	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01[4]	0.01	0.01	0.01
OCDF	0.001[4]	0.001	0.0001	0.0003
dl-PCBs				
CB 77	0.01	0.0005	0.0001	0.0001
CB 81	-	-	0.0001	0.0003
CB 126	0.1	0.1	0.1	0.1
CB 169	0.005	0.01	0.01	0.03
CB 105	0.0001	0.0001	0.0001	0.00003
CB 114	-	0.0005	0.0005	0.00003
CB 118	0.00005	0.0001	0.0001	0.00003
CB 123	-	0.0001	0.0001	0.00003
CB 156	0.0005	0.0005	0.0005	0.00003
CB 157	-	0.0005	0.0005	0.00003
CB 167	-	0.00001	0.00001	0.00003
CB 189	-	0.0001	0.0001	0.00003
Other CBs				
CB 180	-	0.00001	-	-

[1] *Nato/CCMS, 1988,* [2] *Van den Berg et al., 1998,* [3] *Van den Berg et al. 2006,* [4] *Van Zorge et al. 1989.*

4.2 Brominated flame retardants
concentrations and dietary exposure to HBCD from fish products[10]

Abstract
In order to determine the contamination with brominated flame retardants (BFRs) in fish regularly consumed by Dutch citizens, 44 samples of freshwater fish, marine fish, and shellfish were analysed for polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol-A (TBBP-A) and its methylated derivative (me-TBBP-A), and hexabromocyclododecane (HBCD), including its α, β and γ-diastereomers. The highest BFR concentrations were found in pike-perch and eel from the highly industrialised and urbanised rivers Rhine and Meuse. The sum concentrations of BDEs 28, 47, 99, 100, 153, 154, 183, 209, and brominated biphenyl (BB) 153 and HBCD (selection based on an EFSA monitoring recommendation) ranged from below quantification limits to 17 ng/g ww in marine fish and in freshwater fish from 0.6 ng/g ww in pike-perch to 380 ng/g ww in eel. The BDE congener profile in all fish and shellfish samples is dominated by BDE 47, followed by BDE 99, except for eel in which BDE 100 is higher than BDE 99. BDE 209 was detected in two mussel samples, most likely due to BDE 209 contaminated particulate matter in their intestines. Total-HBCD (as determined by GC-ECNI-MS) was detected in 22 out of the 44 samples in concentrations between 0.20 ng/g in marine fish and 230 ng/g ww in eel. Three HBCD diastereomers were determined by HPLC-ESI-MS/MS. α-HBCD was the prevalent congener in most fish samples, followed by γ-HBCD. β-HBCD, TBBP-A and Me-TBBP-A were only detected in a few samples and at low concentrations. A considerable difference was found between HBCD results obtained from GC-ECNI-MS and HPLC-ESI-MS/MS: the GC-ECNI-MS results were 4.4 times higher, based on regression analysis. There are strong indications for LC-ESI-MS/MS delivering the best data quality but further research is needed to underpin this.
There is hardly any data on human dietary exposure to HBCD. We have estimated the fish related dietary exposure of HBCD for the average Dutch population. The medium bound intake was estimated at 8.3 ng/day for a 70 kg person (0.12 ng/kg bodyweight/day). For this estimation we relied mostly on HPLC-ESI-MS/MS data as we argue that these results are more accurate than those obtained by GC-ECNI-MS.

[10] Based on S.P.J. van Leeuwen and J. de Boer (2008) Brominated Flame Retardants in Fish and Shellfish – Levels and Contribution of Fish Consumption to Dietary Exposure of Dutch citizens to HBCD. *Molecular Nutrition and Food Research* 52, 204-216

Introduction

Flame retardants constitute a diverse group of compounds that are added to materials in order to reduce, delay or even prevent them from catching fire. A substantial part of flame retardants consists of brominated compounds. The most frequently used brominated flame retardants (BFRs) are tetrabromobisphenol-A (TBBP-A), hexabromocyclododecane (HBCD) and polybrominated diphenylethers (PBDEs). The BFRs are used at relatively high concentrations in various materials and polymers, such as polyurethane and polystyrene foams, in a wide range of products, such as printed circuit boards, television sets and computers and other electronic household equipment, cars and construction materials. Information on BFR usage figures (from 2001) can be found elsewhere (*1*). BFRs can be released into the environment through production, use, and especially from disposal of the flame retarded products. Various BFRs are present in biota due to their lipophilicity and persistence. Several of the PBDEs and HBCD have shown potential for biomagnification in the food chain. Extensive information on the environmental concentrations of PBDEs and HBCDs can be found in recent comprehensive reviews by Law et al. (*1*) and Covaci et al. (*2*).

BFRs have been found in several human samples (*3-5*) showing that also humans are exposed to these chemicals. An important exposure route of the European general human population to PBDEs is through the diet. This is confirmed in an UK study showing estimates of median PBDE exposures (sum of 9 PBDEs) for UK adults of 98.7; 0.9 and 0.4% for food, air and house dust, respectively (*6*). The exposure route for BDE 209 is presumably different as this is in most cases the predominant congener in dust (up to ca 90%) (*7-9*). Within the food group as such, local consumption habits determine if e.g. fish (*10*) or dairy products (*11*) predominate the exposure to PBDEs. HBCD has been detected in breast milk and human blood (*2*) in the range of 0.08-7.0 ng/g lipid weight. The human exposure to HBCD remains to be quantified, as virtually no data is available on the relevance of different exposure routes such as dietary exposure, dust ingestion, air inhalation, and other routes. A study by Lind *et al.* (*12*) showed a median dietary exposure of 141 ng/day, being dominated by fish. Also TBBP-A was found in human blood samples (*13*).

The European Food Safety Authority (EFSA) has recognized the concern for the contamination of food and feed with BFRs (*14*). In 2006, EFSA has adopted an opinion in which the monitoring of the BDEs 28, 47, 99, 100, 153, 154, 183, 209, and brominated biphenyl (BB) 153 and HBCD is recommended. This baseline study, conducted in 2003, aimed at the determination of 16 BDEs, HBCD, TBBP-A and me-TBBP-A in a broad selection of edible fish and shellfish in order to investigate the importance of fish consumption for human exposure to BFRs. More specifically, the human exposure to HBCD in relation to fish consumption was studied. In a separate contribution to this issue, the dietary exposure to PBDEs is reported, combining the fish contamination data with BDE data in other food commodities (*11*).

Materials and methods

Sampling and sample preparation

The choice of samples was based on i) origin from Dutch marine and freshwaters or ii) regular consumption by the Dutch population. The analysed samples and their origins are mentioned in Table 4.5. A wide variety of marine species were included, as these are important fish in The Netherlands from a consumption perspective. Two popular farmed fish species, eel and salmon, have been included for the same reason. Furthermore, emphasis was placed on eel by sampling a large number of freshwater locations in order to assess the contamination of Dutch fresh waters. Eel has proven to be a valuable indicator of the contamination of Dutch freshwaters with persistent organic pollutants (POPs) like polychlorinated biphenyls (PCBs) and chlorinated dioxins and furans (*15,16*). Finally, two samples of flounder were obtained from the Western Scheldt to monitor i) the production of BFRs (by a chemical industry) in Terneuzen, The Netherlands, and ii) the effects of the utilization of BFRs in the textile industry in Antwerp and further upstream the river Scheldt in Belgium.

The sample number in Table 4.5 concerns the number of pooled samples analysed. Each pooled sample consisted of 16-25 individual fishes (except farmed salmon which samples contained 7-9 individuals). The majority of locations were sampled between September and December 2003. Sea fish was mostly sampled during surveys of the research vessel Tridens. Remaining samples were obtained directly from fishermen, from the auction or from wholesale traders (farmed fish). Eel was caught between May and June 2003 by electric fishery. After transportation to the laboratory, lengths and weights of the individual fishes were measured. All fishes were within market size. Subsequently, fishes were filleted and equal amounts of filet per fish were pooled. The mussel sample was obtained by taking the whole organism out of the shells after cooking the mussels for 5 minutes in tap water at 100°C. After rinsing the cooked mussels with water, approx. 100 g mussel meat was pooled. For shrimps, a pooled sample was prepared from approx. 500 g unpeeled and uncooked whole organisms. The pooled samples were homogenized in a Waring Blender and stored at -20°C until analysis. Sampling data, including date of sampling, sampling coordinates, number of individuals per fish, sizes and weights can be found in Table 4.5.

Table 4.5 Sample overview.

Sample species	Scientific name	Catching area	Sample date	Number	Weight			Length		
					Min	Mean	Max	Min	Mean	Max
		Marine								
Mussels	*Mytilus edulis*	Eastern Scheldt	-	500 g[1]	-	-	-	-	-	-
Mussels	*Mytilus edulis*	Western Wadden Sea	-	500 g	-	-	-	-	-	-
Mussels	*Mytilus edulis*	Eastern Wadden Sea	-	500 g	-	-	-	-	-	-
Shrimps	*Crangon crangon*	Rijnmond	-	500 g[2]	-	-	-	-	-	-
Shrimps	*Crangon crangon*	Wadden Sea	-	500 g	-	-	-	-	-	-
Cod	*Gadus morhua*	Central North Sea	28-10-2003	23	588	1564	2444	40.5	53.3	63.5
Cod	*Gadus morhua*	Southern North Sea	7-11-2003	22	794	1457	2343	40.8	51.6	63.8
Haddock	*Melanogrammus aeglefinus*	Central North Sea	10-11-2003	25	331	401	550	34.8	36.8	40.2
Haddock	*Melanogrammus aeglefinus*	Northern North Sea	18-6-2003	25	223	366	590	29.0	33.2	40.0
Coalfish	*Pollachius virens*	Central North Sea	26-8-2003	20	588	816	1209	40.5	44.5	49.0
Coalfish	*Pollachius virens*	Northern North Sea	20-6-2003	23	578	885	1631	42.0	46.1	57.0
Plaice	*Pleuronectes platessa*	Central North Sea	15-9-2003	25	247	443	814	29.3	35.5	43.4
Plaice	*Pleuronectes platessa*	Southern North Sea	7-11-2003	25	334	481	713	32.0	36.0	42.0
Sole	*Solea solea*	Central North Sea	15-9-2003	20	167	230	301	27.0	29.5	31.8
Sole	*Solea solea*	Southern North Sea	19-9-2003	24	122	298	554	24.0	31.1	37.5
Herring	*Clupea harengus*	Central North Sea	26-8-2003	25	69	99	148	20.0	22.1	25.0
Herring	*Clupea harengus*	Southern North Sea	26-8-2003	25	61	136	224	20.0	24.0	28.0
Herring	*Clupea harengus*	Shetlands	2-6-2003	25	94	105	120	22.0	22.7	24.0
Herring	*Clupea harengus*	The Channel	24-10-2003	25	104	162	241	22.5	25.5	28.5
Mackerel	*Scomber scombrus*	North Sea	26-8-2003	24	252	357	632	30.5	33.3	41.0
Mackerel	*Scomber scombrus*	South-west of Ireland	22-8-2003	25	243	385	543	30.5	36.3	41.5
Mackerel	*Scomber scombrus*	Shetlands	18-6-2003	25	167	329	486	29.0	34.9	41.5
Flounder	*Platichthys flesus*	Western Scheldt (Terneuzen)	8-9-2003	25	92	139.6	224	20.0	23	27.5
Flounder	*Platichthys flesus*	Western Scheldt	25-9-2003	18	87	178.9	401	20.0	23.9	32.5
		Dutch freshwater								
Eel	*Anguilla anguilla*	Haringvliet-East	12-6-2003	25	49	109.6	168	30	36.6	40
Eel	*Anguilla anguilla*	Hollands Deep	28-5-2003	25	52	107.8	187	31	37.4	40
Eel	*Anguilla anguilla*	Meuse, Eijsden	21-5-2003	16	48	81.4	127	31	35.2	40
Eel	*Anguilla anguilla*	Roer, Vlodrop	22-5-2003	19	64	109.9	164	33	38.2	40
Eel	*Anguilla anguilla*	North-Hollands canal, Akersloot	13-6-2003	18	55	82.4	123	30.5	34.6	39

-: not recorded

[1] Pooled sample of 500 g mussels, resulting in ca 100 g meat after removal from the shells

[2] Pooled sample of 500 g uncooked and unpeeled shrimps

Table 4.5 Continued.

Sample species	Scientific name	Catching area	Sample date	Number	Weight			Length		
					Min	Mean	Max	Min	Mean	Max
Eel	*Anguilla anguilla*	Pr. Margrietkanaal, Suawoude	5-6-2003	25	44	83.4	149	30	34.6	39
Eel	*Anguilla anguilla*	Waal, Tiel	30-5-2003	24	39	95.7	159	30	35.8	40
Eel	*Anguilla anguilla*	IJssel, Deventer	3-6-2003	22	48	101.3	156	31	36.7	40
Eel	*Anguilla anguilla*	Ketel Lake	4-6-2003	25	57	92.4	163	31	36.2	40
Eel	*Anguilla anguilla*	New Merwede	28-5-2003	25	64	96	133	31.0	35.8	39.0
Eel	*Anguilla anguilla*	Meuse, Keizersveer	11-6-2003	25	64	93	132	32.5	35.7	39.5
Eel	*Anguilla anguilla*	Haringvliet-West	16-6-2003	25	49	110	168	30.0	36.6	40.0
Eel	*Anguilla anguilla*	Rijn, Lobith	17-6-2003	16	57	97	159	31.0	36.3	40.0
Eel	*Anguilla anguilla*	IJssel Lake, Medemblik	12-5-2003	25	60	92	131	31.5	35.3	39.0
Pike-perch	*Sander lucioperca*	Holland's Deep	29-9-2003	20	650	956	1500	42.9	49.1	58.3
Pike-perch	*Sander lucioperca*	IJssel Lake	1-10-2003	25	743	1096	1814	45.8	50.6	59.5
		Farmed fish								
Salmon	*Salmo salar*	Fishtrade Norway	12-9-2003	9	2360	2678	2989	62.0	66.8	70.0
Salmon	*Salmo salar*	Fishtrade Schotland	12-9-2003	7	3128	3493	4107	69.5	72.2	75.5
Eel	*Anguilla anguilla*	Italian Fish Farm	9-10-2003	20	236	297	342	47.0	53.2	59.0
Eel	*Anguilla anguilla*	Dutch Fish Farm	9-10-2003	20	174	222	322	41.5	46.5	54.0

Analytical methods and QA/QC

The concentrations of the following BFRs were determined: BDEs 28, 47, 99, 100, 154 (+BB 153), 183 and 209; HBCD, TBBP-A, and me-TBBP-A. The method for the extraction, clean-up and GC analysis of BDEs, HBCD, TBBP-A, and me-TBBP-A is described in detail elsewhere (*17*). Briefly, the samples were Soxhlet extracted with hexane / acetone (3:1). The crude extract was treated with acidified water so as to protonate TBBP-A and thereby force it into the organic extraction solvent. After removal of the aqueous layer, the co-extracted fat and other contaminants were removed by gel permeation chromatography (GPC). The BFRs were separated from other contaminants by silica column chromatography (Merck, Darmstadt, Germany) and the target fraction was treated with concentrated sulphuric acid (Merck, Darmstadt, Germany) prior to GC-electron capture negative ion (ECNI)-MS analysis (HP-6890 GC and HP5973 MSD, Agilent, USA), monitoring the $(Br)^-$ ions at m/z 79 and 81 (*17*). BDE 154 is reported as the sum of this BDE and BB 153 as these BFRs are not separated on the GC-column used (CP-Sil-8, 50m, 0.25 mm id., 0.25 µm film thickness; Chrompack, Middelburg, Netherlands). However, given the low production and application volumes of PBBs compared to the PBDEs (*18,19*), it is unlikely that BB 153 will be found at significant concentrations in the analysed fish samples and will therefore presumably not add significantly to the BDE 154 signal. Deca-BDE (BDE 209) was determined

using a shorter column (DB-5, 15m, 0.25 mm id., 0.25 µm film thickness; J&W Scientific, USA) in order to reduce possible thermal degradation of BDE 209 due to long residence times in the heated GC column (*20*). The internal standard (IS) covering the complete method was BDE 116. Furthermore, $^{13}C_{12}$-BDE 209 was added as IS for BDE 209. HBCD was analyzed by both GC-ECNI-MS and LC-ESI (electrospray ionisation interface)-MS/MS method to enable a method comparison as both methods have their strengths and weaknesses. The GC-ECNI-MS method is very sensitive but provides the HBCD concentration only as a sum, whereas the individual diastereomers (α-, β- and γ-HBCD) can be separated and determined individually by LC-ESI-MS/MS but at the cost of higher limits of quantification (LOQs). The LC separation of the compounds was performed on a Zorbax column (XDB-C18 150 mm*2.1 mm ID, 3.5 µm, Agilent, USA), kept at 20°C, using an acetonitrile (A)-0.01 mM ammoniumchloride (B) gradient. The gradient was programmed as follows: 0-4 min kept 70% A, 4-4.1 min quick ramping to 90% A, 4.1-8 min kept at 90% A and then returning to the initial solvent composition again.

A LCQ-advantage mass-spectrometer with an ESI (Thermo-Finnigan, USA) was used for detection of the HBCD diastereomers and TBBP-A. The optimised settings were as follows: sheath gas: 46 arbitrary units; capillary spray voltage -4.5 kV; capillary temperature 160°C; capillary voltage -4 V; radio frequency voltage: 500 Vpp and helium was used as collision gas (40% energy for TBBP-A and 20% for HBCD). Initial experiments showed that the $(M-H)^-$ ion response was too variable for quantitative analysis. A small signal was observed at m/z 676.7 being most likely the chlorine adducts of HBCD. By addition of ammonium chloride to the LC-eluens, we forced the formation of the chlorine adducts of the HBCD diastereomers, leading to increased response (and sensitivity). The adduct ions of m/z 676.7 $(M+Cl)^-$ +/- 5 Da were isolated and fragmented to $(M-H)^-$ of m/z 640.7 (and m/z 688.7 and 652.7, respectively for $^{13}C_{12}$-HBCD). For TBBP-A the settings were m/z 555 (parent ion) and m/z 543 (product ion). LC methods for determination of the individual HBCD diastereomers were recently reviewed by Morris *et al.* (*21*).

After analysis of the fish extracts by GC-ECNI-MS, the solvent (hexane) was blown down almost to dryness and the residue was redissolved in methanol for LC analysis. Blowing down extracts was tested and was found safe for the 3 diastereomers. At that stage, ^{13}C-HBCD diastereomers (α-, β- and γ-HBCD) were added to the extract. They could only be added after GC-ECNI-MS analysis of the extracts as otherwise during GC analysis, the $(Br)^-$ originating from the ^{13}C-HBCD diastereomers would add to the signal originating from the native HBCD in the sample, making quantification impossible. The ^{13}C-HBCD diastereomers were used to correct for the LC-ESI-MS/MS analysis performance only. Possible losses during extraction, clean-up and GC injection and needle wash were corrected using an empirically determined correction factor (multiplying by 1.25 to correct for losses of extract volume (estimate)). The native standards were obtained from Cambridge Isotope

Laboratories (Andover, Canada). The ^{13}C-labelled HBCD diastereomers were obtained from Wellington Laboratories (Guelph, Ontario, Canada).
QA/QC. The PBDE, TBBP-A, Me-TBBP-A and HBCD analyses were performed at the Netherlands Institute for Fisheries Research, accredited under ISO17025 lab no. L097 (see www.RvA.nl). The quality was assured by the analysis of laboratory reference materials, regular duplicate analyse, high numbers of blanks, recovery tests, the use of internal standards, and by an annual participation in interlaboratory studies organized by QUASIMEME (*20*) with satisfactory results (i.e. most results showing z-scores of <|2|).

Table 4.6 BDE and HBCD concentrations in fish and shellfish samples consumed by Dutch citizens (by GC-ECNI-MS, ng/g ww).

Species	Source area	Lipids (%)	BDE 28	BDE 47	BDE 99	BDE 100	BDE 154 + BB 153	BDE 183	BDE 209	Total HBCD	Sum (EFSA)*
	Marine										
Mussels	Eastern Scheldt	2.2	<0.1	0.2	<0.1	<0.1	<0.1	<0.1	0.1	0.9	1.2
Mussels	Western Wadden Sea	2.1	<0.1	0.1	0.1	<0.1	<0.1	<0.1	0.8	0.2	1.2
Mussels	Eastern Wadden Sea	2.3	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1
Shrimps	Wadden Sea	2.1	<1.0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.7	<0.1	<2.3
Shrimps	Rijnmond	2.2	<0.1	0.7	0.8	<0.1	0.2	<0.1	<0.3	<0.1	1.7
Cod	Central North Sea	0.7	0.2	<0.1	<0.1	0.4	<0.1	<0.1	<0.5	<0.1	0.6
Cod	Southern North Sea	0.9	<0.1	0.4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.4
Haddock	Central North Sea	0.7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1
Haddock	Northern North Sea	0.8	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.1	<1.0
Coalfish	Central North Sea	1	<0.1	0.2	<0.1	0.1	<0.1	<0.1	<0.1	0.2	0.5
Coalfish	Northern North Sea	1.1	<0.1	0.2	<0.1	<0.1	<0.1	<0.1	<0.2	<0.1	0.2
Plaice	Central North Sea	0.9	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.8
Plaice	Southern North Sea	1.3	<0.1	0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.2
Sole	Central North Sea	1.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.8
Sole	Southern North Sea	1.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.8
Herring	Central North Sea	20	0.2	2.8	0.9	0.8	0.2	<0.1	<0.2	2.7	7.6
Herring	Southern North Sea	18	<0.1	3.2	0.9	1.2	<0.1	<0.1	<0.5	<0.1	5.3
Herring	Shetlands	16	0.1	1.8	<0.5	0.5	<0.2	<0.1	<0.7	<1.2	2.4
Herring	The English Channel	14	0.2	3.8	0.9	1.2	0.2	<0.1	<0.5	7.3	13.6
Mackerel	North Sea	17	<0.1	<0.8	0.7	0.1	0.1	<0.1	<0.3	2.1	3.0
Mackerel	South-west of Ireland	13	<0.1	1	0.7	0.2	0.2	<0.1	<1	2	4.1
Mackerel	Shetlands	3.3	<0.1	0.6	<0.3	0.1	<0.1	<0.1	<0.3	<0.5	0.7
Flounder	Western Scheldt (Terneuzen)	1.1	0.2	4.4	0.3	1.1	0.4	<0.1	<0.1	0.9	7.3
Flounder	Western Scheldt	1.7	0.3	11	1.0	2.1	0.7	<0.1	<0.1	1.3	16.4
	Dutch freshwater										
Eel	Haringvliet-East	17	0.6	20	1.4	8.1	2.1	0.2	<0.7	70	102.4
Eel	Hollands-Diep	16	0.7	36	<1.3	24	2.8	<0.1	<0.4	150	214
Eel	Maas, Eijsden	5.3	<1.9	<4.5	<0.2	2	<0.2	<0.1	<0.5	15	17.0

* *Sum based on selection from EFSA recommendation (14). Results <LOQ are considered 0 and have not been added to the sum.*

Table 4.6 Continued.

Species	Source area	Lipids (%)	BDE 28	BDE 47	BDE 99	BDE 100	BDE 154 + BB 153	BDE 183	BDE 209	Total HBCD	Sum (EFSA)*
Eel	Roer, Vlodrop	14	0.3	26	<1.7	11	<0.8	<0.1	<0.5	130	167
Eel	Noord-Hollands kanaal, Akersloot	4.1	<0.1	0.4	0.2	0.1	<0.1	<0.1	<0.5	0.7	1.4
Eel	Pr. Margrietkanaal, Suawoude	16	<0.1	< 7.4	< 7.3	< 1.6	0.1	0.1	<4.5	2.5	2.7
Eel	Waal, Tiel	16	0.4	43	<2.6	22	2.3	<0.1	<0.5	210	278
Eel	IJssel, Deventer	8.7	<1.9	17	<1.0	7.3	<0.9	<0.1	<0.6	94	118
Eel	Ketel lake	21	0.2	<15	<0.7	4.8	1.1	<0.1	<0.5	30	36.1
Eel	Nieuwe Merwede	22	1.7	81	<3.2	61	5.7	<0.1	<0.4	230	3794
Eel	Maas, Keizersveer	24	0.3	16	<0.6	8.5	2.4	0.1	<0.6	<0.1	27.3
Eel	Haringvliet-West	12	0.1	5.8	<0.6	3.7	0.7	<0.1	<1.0	21	31.3
Eel	Rijn, Lobith	9.8	0.2	21	<1.7	7.6	<0.9	<0.1	<0.7	97	126
Eel	IJssel Lake, Medemblik	23	0.4	6.8	0.9	1.7	0.7	<0.1	<0.6	<3.4	10.5
Pike-perch	Hollands Diep	0.9	<0.1	0.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.6
Pike-perch	IJssel Lake	1	<0.1	0.3	0.2	0.1	0.1	<0.1	<0.1	<0.1	0.7
	Farmed										
Salmon	Fishtrade Norway	12	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.8
Salmon	Fishtrade Schotland	12	0.1	1.6	0.7	0.3	0.1	<0.1	<0.2	1.3	4.1
Eel	Italian Fish Farm	22	<0.1	1.1	0.2	0.2	0.1	<0.1	<0.5	<0.4	1.6
Eel	Dutch Fish farm	36	<0.1	1.3	0.3	0.2	0.2	0.2	<0.6	<0.5	2.2

* *Sum based on selection from EFSA recommendation (14). Results <LOQ are considered 0 and have not been added to the sum.*

Table 4.7 Concentrations of HBCD isomers, ΣHBCD, TBBP-A and MeTBBP-A in fish and shellfish samples (ng/g ww).

Sample species	Catching area	α-HBCD[1]	β-HBCD[1]	γ-HBCD[1]	ΣHBCD[1,2]	Total HBCD[3]	TBBP-A	Me-TBBPA	Lipids (%)
	Marine								
Mussels	Easter Scheldt	<0.1	<0.1	<0.2	-	0.9	<0.6	<0.1	2.2
Mussels	Western Wadden Sea	nd	nd	nd	-	0.2	nd	<0.1	2.1
Mussels	Eastern Wadden Sea	<0.1	<0.1	<0.2	-	<0.1	<0.6	0.3	2.3
Shrimps	Wadden Sea	<0.5	<0.5	<1.0	-	<0.1	<2.5	<0.1	2.1
Shrimps	Rijnmond	<0.5	<0.5	<0.9	-	<0.1	<2.4	<0.1	2.2
Cod	Central North Sea	<0.1	<0.1	<0.2	-	<0.1	<0.4	<0.1	0.7
Cod	Southern North Sea	<0.2	<0.2	<0.2	-	<0.1	<0.1	<0.1	0.9
Haddock	Central North Sea	<0.2	<0.2	<0.2	-	<0.1	<0.1	<0.1	0.7
Haddock	Northern North Sea	<0.1	<0.1	<0.2	-	<0.1	<0.4	<0.1	0.8
Coalfish	Central North Sea	<0.1	<0.1	<0.2	-	0.2	<0.5	<0.1	1

[1] *By LC-ESI-MS/MS*

[2] *<LOQ values were regarded as 0 (i.e. not added to the ΣHBCD)*

[3] *By GC-ECNI-MS. The data from Table 4.6 is reproduced again for easiness of comparison of GC and LC data.*

Table 4.7 Continued.

Sample species	Catching area	α-HBCD[1]	β-HBCD[1]	γ-HBCD[1]	ΣHBCD[1,2]	Total HBCD[3]	TBBP-A	Me-TBBPA	Lipids (%)
Coalfish	Northern North Sea	0.2	<0.1	<0.2	0.2	<0.1	<0.5	<0.1	1.1
Plaice	Central North Sea	<0.1	<0.1	<0.2	-	<0.1	<0.5	<0.1	0.9
Plaice	Southern North Sea	<0.2	<0.2	<0.2	-	<0.1	<0.1	<0.1	1.3
Sole	Central North Sea	<0.1	<0.1	<0.2	-	<0.1	<0.6	<0.1	1.1
Sole	Southern North Sea	<0.1	<0.1	<0.2	-	<0.1	<0.6	<0.1	1.2
Herring	Central North Sea	1	<0.8	<1.6	1	2.7	<4.1	<0.1	20
Herring	Southern North Sea	1.7	<0.9	1.9	3.6	<0.1	<4.9	<0.1	18
Herring	Shetlands	2	<0.8	<1.7	2	<1.2	<4.6	<0.1	16
Herring	The Channel	1.1	<0.8	<1.6	1.1	7.3	<4.1	<0.1	14
Mackerel	North Sea	1.7	<1.0	<2.1	1.7	2.1	1.9	<0.1	17
Mackerel	South-west of Ireland	<0.9	<0.9	<1.9	-	2	<4.8	<0.1	13
Mackerel	Shetlands	<0.5	<0.5	<1.1	-	<0.5	<2.7	<0.1	3.3
Flounder	Terneuzen	0.5	<0.1	<0.2	0.5	0.9	<0.6	<0.1	1.1
Flounder	Western Scheldt	0.3	<0.1	<0.2	0.3	1.3	0.2	<0.1	1.7
	Dutch freshwater								
Eel	Haringvliet-East	12	<1.0	2.5	15	70	<5.2	0.1	1
Eel	Hollands Diep	35	1.0	8.1	44	150	<5.1	0.6	16
Eel	Maas, Eijsden	4.9	0.9	<2.0	5.8	15	<5.0	<0.1	5.3
Eel	Roer, Vlodrop	36	0.8	3	40	130	<5.2	0.3	14
Eel	Noord-Hollands kanaal, Akersloot	<0.7	<0.7	<1.5	-	0.7	<3.8	<0.1	4.1
Eel	Pr. Margrietkanaal, Suawoude	<0.8	<0.8	<1.5	-	2.5	<3.9	<0.1	16
Eel	Waal, Tiel	41	<0.9	8.4	49	210	<4.8	0.3	16
Eel	IJssel, Deventer	36	1.0	4.4	41	94	<4.9	<0.1	8.7
Eel	Ketel Lake	8	<1.6	2.3	10	30	<5.1	0.3	21
Eel	Nieuwe Merwede	35	<0.9	5.8	41	230	<4.7	1.2	22
Eel	Maas, Keizersveer	8.8	0.8	3.3	13	<0.1	<5.2	0.2	24
Eel	Haringvliet-West	3.7	<1.0	<2.1	3.7	21	<5.3	0.2	12
Eel	Rijn, Lobith	39	<0.9	8.2	47	97	<4.8	<0.1	9.8
Eel	IJssel lake, Medemblik	nd	nd	nd	-	<3.4	nd	0.4	23
Pike-perch	Hollands Diep	<0.1	<0.1	<0.2	-	<0.1	<0.5	0.01	0.9
Pike-perch	IJssel Lake	<0.1	<0.1	<0.2	-	<0.1	<0.6	<0.1	1
	Farmed fish								
Salmon	Fishtrade Norway	<0.4	<0.4	<0.9	-	<0.1	<2.2	<0.1	12
Salmon	Fishtrade Schotland	<0.5	<0.5	<1.0	-	1.3	<2.7	0.1	12
Eel	Italian Fish farm	<0.7	<0.7	<1.4	-	<0.4	<3.7	<0.1	22
Eel	Dutch Fish farm	<0.9	<0.9	<1.8	-	<0.5	<4.5	<0.1	36

[1] *By LC-ESI-MS/MS*

[2] *<LOQ values were regarded as 0 (i.e. not added to the ΣHBCD)*

[3] *By GC-ECNI-MS. The data from Table 4.6 is reproduced again for easiness of comparison of GC and LC data.*

Results and discussion

Concentrations of BDEs and HBCD in eel

Table 4.6 shows the results of the GC-ECNI-MS analysis of eight BFRs in the fish samples. This is the selection of BFRs recently recommended by EFSA for monitoring *(14)* and includes the BDEs 28, 47, 99, 100, 154 (+BB 153), 183, 209 and total-HBCD (by GC). The results clearly show that eel samples from various freshwater locations contain high BFR concentrations (up to approx 379 ng/g ww) compared to the other samples. In addition to the main rivers Meuse and Rhine, various other freshwater locations are also contaminated with BFRs. In these eel samples, HBCD, BDE 47 and BDE 100 predominate, accounting for on average 95% of the sum of the EFSA selection, while the other PBDEs were only found at very low concentrations. HBCD concentrations are higher than any of the BDE concentrations in the eel samples. The absence of BDE 99 in nearly all eel samples (contrary to most other fish samples) suggests a specific elimination/metabolism in eel for this congener. The two analysed pike-perch samples from the same locations could not confirm this specific phenomenon. In another eel study PCBs 77 and 126 were found to be metabolised by eel *(22)*. It should be noted that the molecular structure of these PCBs are different from BDE 99. It should be noted that relative low BDE 99 concentrations were also found in other studies on eel, and this was attributed to the metabolic capacity of European eel (*Anguilla anguilla*) *(23)* and American eel (*Anguilla rostrata*) *(24)*. The BDE 47 and 99 concentrations in eel are comparable to those reported earlier in eel from the same locations *(25)*. Levels in large mouth bass and Detroit river two US rivers were lower (2.2-18 ng/g ww for the sum of 8 BDEs) *(26)*. Levels in Swiss lake whitefish varied from 1.6-7.4 ng/g ww for the sum of 7 BDEs *(27)*. Janak *et al*. determined HBCD diastereomers in 2 eel samples from the Western Scheldt estuary and found α-HBCD to be the dominating diastereomer (1.8-7.0 ng/g ww) *(28)*. Levels of γ-HBCD were 0.5-0.8 ng/g ww). These data are in the same range as our observations, although eel from some major Dutch river systems showed considerably higher levels (see Table 4.7 for total HBCD and diastereomer specific information). Eels from UK rivers Skerne and Tees also showed much higher levels (up to 10275 ng/g ww), being related to a HBCD production plant *(29)*. Roosens et al determined HBCD in a variety of fish samples from the river Scheldt (Oudenaarde area) *(23)*. Average concentrations ranged from 3000 to 10000 ng/g lipid weight (lw) in eel (corresponding to 440-7770 ng/g ww, LC-ESI-MS/MS data). This is much higher than the concentrations in our study. However, the Oudenaarde area is a polluted area because of intense industrial activity *(23)*.

Concentrations of BDEs and HBCD in other fish species

The ΣBFR (EFSA selection as specified earlier) concentrations in the fish species other than eel ranged from <LOQ for all congeners to 16.5 ng/g ww. The six samples with <LOQ values were shrimps from the Wadden Sea, haddock from the northern North Sea, plaice and sole from the central North Sea and sole from the southern North Sea. All these species feed on organisms that are relatively low in the food chain. In addition, they have low fat contents (≤ 2.1%). Low ΣBFR concentrations were found in other lean marine fish (up to 1.7 ng/g ww for Rijnmond shrimps). These concentrations are lower than those observed in the Belgian part of the North Sea *(30)*. ΣBFR concentrations in flounder from the Western Scheldt (7.3, 16.5 ng/g ww) were somewhat higher than in various fish fillets from the Western Scheldt by Voorspoels *et al. (30)*. Herring and mackerel showed ΣBFR concentrations of 0.7-13.6 ng/g ww. Paepke and Herrmann determined several BDEs in herring from the North Sea and North East Atlantic and found concentrations ranging from 6.7-14 ng/g lipid weight (lw) for the sum of 11 BDEs, which is lower than our findings (when expressed on a wet weight basis) *(31)*. Farmed salmon from Norway did not contain detectable BFR concentrations whereas the Scottish salmon sample showed ΣBFR concentrations of approx. 4 ng/g ww. This is consistent with findings in a study by Hites *et al. (32)*. They found approx. 0.1 to 4 ng/g ww for the sum of 43 BDEs *(32)* in farmed salmon from North-west Europe, with concentrations of salmon from Scotland being among the highest. HBCD concentrations in our herring samples were much lower than those reported by Remberger et al. in Swedish fish (21-180 ng/g lw) *(33)*. Janak *et al.* determined HBCD diastereomers in various biota samples (shrimp whole body and muscle tissue of bib, plaice, sole and whiting) from the Western Scheldt estuary and found 0.2-0.3 ng/g ww for the sum of α- and γ-HBCD (except for sole which contained higher levels of 1.2-11 ng/g ww) *(28)*. Roosens et al determined HBCD in a variety of fish samples from the river Scheldt (Oudenaarde area) *(23)*. Average concentrations in various fish samples ranges from 3000 to 6000 ng/g lw.

BDE 209 was not at all detected in any of the samples except for 2 mussel samples. This is most likely caused by the fact that the mussels were analysed as harvested, without depuration. Sediment particles (known to contain high concentrations of BDE 209 e.g. in the Western Scheldt *(34)*) may have remained in the mussel stomachs, thereby contaminating the meat. Typically, BDE 209 is, if detected at all, only observed at low concentrations in European fish samples (*1*). Furthermore, it should be noted that various pitfalls may influence the BDE 209 result (*20*). Although we took great care in reducing potential error sources, the BDE 209 results should be treated with care.

The BFR patterns vary with type and origin of the samples. In the Western Scheldt flounder, BDE 47 predominates, whereas HBCD is more prominent in some of the North Sea fish samples.

TBBP-A and me-TBBP-A

TBBP-A and me-TBBP-A have been analysed in all samples. In nearly all samples TBBP-A was below the LOQ. Me-TBBP-A was detected in some marine fish, shellfish and farmed fish samples but at concentrations close to the LOQ. Me-TBBP-A concentrations in eel and pike-perch samples range from <0.1 to 1.4 ng/g ww, which is lower than the BDE and HBCD concentrations. The low concentrations of TBBP-A can be explained by its relatively low Log K_{ow} value of 4.5-5.3 *(35)*. Furthermore, TBBP-A is often applied as flame retardant in printed circuit boards. In this application, TBBP-A is covalently bound to the epoxy resin *(35)* and, therefore, leaching into the environment during the life-time cycle of the product is less likely.

Differences in HBCD results obtained by GC-ECNI-MS and LC-ESI-MS/MS

HBCD consists of three diastereomers, α-, β- and γ-HBCD. These diastereomers, were determined by LC-ESI-MS/MS, whereas total-HBCD was measured by GC-ECNI-MS. The LC results show that α-HBCD is the predominant isomer in the samples analysed, followed by γ- and β-HBCD (see Table 4.7). The results allow a comparison of the performance of GC and LC. This is limited to the samples for which results >LOQ were obtained (herring, mackerel, flounder and eel). In most cases, the GC results are higher than the LC results (up to 8-fold higher concentration for eel from Haringvliet-west). A correlation analysis shows a 4.4 fold higher result by GC. There are indications that the LC results are most accurate for the following reasons:

- The benefits if LC-ESI-MS/MS are the use of ^{13}C internal standards (in this study they were used for the correction of LC-ESI-MS/MS variability) and the separation of the diastereomers, resulting in specific information and deeper insights
- In GC-ECNI-MS no diastereomer separation can be achieved, the diastereomers have different response factors and degradation and interconversion of HBCD diastereomers takes place at higher temperatures.

A more thorough discussion on this subject and experimental results can be found in Chapter 3.2. This discussion is not yet conclusive and more research is needed to underpin if LC-MS delivers the most accurate results.

Estimates of dietary exposure to HBCD from fish consumption

Virtually no information is available on dietary human exposure to HBCD, although studies reporting HBCD in human blood and breast milk (*2*) show that HBCD can enter the human body. We have therefore made an estimation of the fish-related dietary exposure of the average Dutch citizen. This estimation is based on HBCD data from the present study combined with consumption data from the Dutch National Food Consumption survey (DNFCS) of 1997/1998 *(36)*. The DNCSF contains files of 6250 people in the age of 1 to 90 years, who recorded the food they consumed during two

consecutive days in a food diary. The HBCD exposure calculation is based on the daily fish consumption multiplied by the average lowerbound concentrations (per species) as in $DE=\Sigma(Q_a \cdot C_a+Q_b \cdot C_b+....Q_z \cdot C_z)$, in which DE = Daily Exposure, Q_a = Quantity of daily consumption of fish species a and C_a = average lowerbound concentration of HBCD in fish species a (measured in this study). The HBCD data used originated from the LC-ESI-MS method (sum of α-, β- and γ-diastereomers), but for 4 species, being coalfish, mackerel, mussels and farmed salmon, we have used the GC-ECNI-MS data available because the LC data of *all* diastereomers was <LOQ (due to a low sensitivity as discussed earlier). These GC-ECNI-MS data were divided by a factor 4.4 to account for the difference between GC-ECNI-MS and LC-ESI-MS/MS results, as discussed earlier. Concentrations per fish species were averaged. Results of HBCD diastereomers that were <LOQ were assumed to be 0 ("lower bound" approach, LB), 0.5xLOQ ("medium bound", MB) and equal to LOQ ("upper bound", UB). The fish samples included in this study covered 88% of the Dutch daily fish intake, thereby representing very well the fish consumed by Dutch citizens.

Table 4.8 Average dietary exposure of Dutch population to HBCD resulting from fish consumption.

Species	**Average dietary exposure (ng/day)**		
	Lowerbound (<LOQ = 0)	**Medium bound (<LOQ = 0.5xLOQ)**	**Upper bound (<LOQ = LOQ)**
Herring	3.5	5.4	7.2
Cod	0	0.69	1.4
Farmed salmon	0.16	0.61	1.1
Mackerel	0.20	0.44	0.59
Shrimps	0	0.33	0.65
Coalfish	0.22	0.35	0.48
Farmed eel	0	0.17	0.27
Wild eel	0.14	0.14	0.14
Mussels	0.04	0.08	0.11
Plaice	0	0.13	0.25
Total	*4.3*	*8.3*	*12.2*

The results are shown in Table 4.8. The average Dutch exposure varied from 4.3-12 ng/day from LB to UB (0.06-0.17 ng/kg bw/day for a 70 kg individual). Figure 4.7 shows the contribution of the various fish species to the exposure. This figure shows that herring is responsible for approx. 65% of the exposure (MB), followed by cod, farmed salmon and mackerel. Although high BFR concentrations were found in wild eel, this species hardly contributes to the exposure as the consumption of eel is low. Furthermore, approx. 95% of the consumed eel consists of farmed eel, and the HBCD concentrations in both farmed eel samples were <LOQ. There is a considerable difference between

the LB and UP result (factor 2.8). This is mainly caused by the low levels in some popular fish (e.g. cod, coalfish and farmed salmon) resulting in a large number of <LOQ values. By increasing the sensitivity of the analytical method, this situation can be improved.

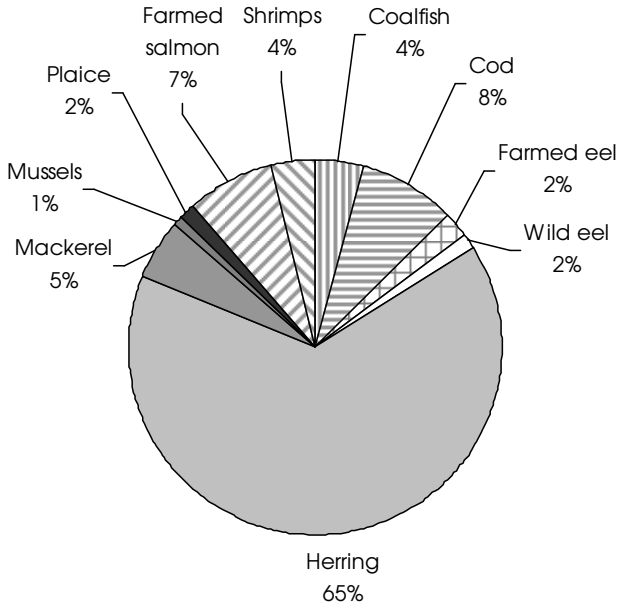

Figure 4.7 Relative contribution of various fish species to the exposure of Dutch consumers to α-, β-, and γ-HBCD (determined by LC-ESI-MS/MS) For coalfish, mackerel, mussels and farmed salmon samples, corrected GC-ECNI-MS data (see text for explanation) was used in case all diastereomers determined by LC were <LOQ. In other cases, diastereomer values <LOQ were considered 0.5xLOQ.

Lind et al. (*12*) found mean dietary exposures of 2.5 ng/kg bw/day for Swedish females (age 17-74 years). About 1.8 ng/kg bw/day could be attributed to fish consumption, being considerably higher than our observation. Presumably, this is due to a combination of higher HBCD concentrations in their fish samples (Remberger et al. found HBCD levels of 21-180 ng/g lw in Swedish herring samples *(33)*), combined with a higher daily intake of fish (although the authors did not report the underlying data). De Bakker et al. (*11*) determined the dietary exposure for BDEs for Dutch citizens, in which they used the BDE dataset of the fish samples in this study. They found a long term median dietary exposure of 0.79 ng/kg bw/day (MB) for the sum of BDE 47, 99, 100, 153 and 154. Fish accounted for 28%, being 0.22 ng/kg bw/day, being higher than the 0.12 ng/kg bw/day that we found for HBCD (MB in both studies). It should be noted that in this study, we calculated the average

exposure based in a two-day average fish consumption pattern, whereas de Bakker et al. used different (statistical) approaches to determine the long term dietary exposure for a wide range of food commodities. Roosens et al. determined the exposure related to the consumption of the very polluted eel from the Oudenaarde area *(23)*. For the most polluted location (L5), the average daily consumption of eel (2.9 g) would result in an exposure of 4350 ng/day, being much higher that our findings. In case of local fishermen eating their own caught eel, this situation can be even worse. However, it should be noted that this is a worst case scenario for a local situation and it does not hold for the general Belgian population.
From a 28 days endocrine effects toxicity study with Wistar rats a benchmark dose (based on 10% thyroid weight) of 1.6 mg/kg bw/day was derived for HBCD *(37)*. Germer et al. *(38)* found significant induction of drug metabolizing enzymes in female Wistar rats in a 28 day oral exposure study at a concentration of 3.0 mg/kg bw and higher (technical HBCD mixture). In both cases, the effect level is approx. 10,000,000 times higher than the intake calculated in this study, suggesting a large margin of safety.
The above estimation is only a first attempt towards human risk characterization due to dietary exposure to HBCD in fish. Apart from fish, other sources will contribute to the human exposure as well, including other food commodities (e.g. dairy and meat products), exposure through dust and air (*2*), and dermal exposure. The increasing environmental HBCD concentrations call for more efforts on this contaminant, preferably by evaluating the individual diastereomers from an exposure and toxicity point of view.

Acknowledgements

The Dutch Ministry of Agriculture, Nature Management and Food Quality is acknowledged for their financial support. Dr G. Arsenault of Wellington Laboratories is acknowledged for the generous gift of ^{13}C-HBCD diastereomers. The colleagues at the IMARES institute are acknowledged for their skillful contributions to the project. Dr. M. Bakker (RIVM) is acknowledged for providing detailed fish consumption data.

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4.3 Perfluorinated compounds
concentrations and dietary exposure to PFOS from fish products[11]

Abstract
Seven perfluorinated compounds (PFCs), including perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) were determined in a variety of fish and shellfish that are frequently consumed in The Netherlands. PFOS was detected in 35 of the 45 analysed samples (78%) at concentrations from 2 to 230 ng/g ww (in edible parts). Concentrations in livers were higher, up to 730 ng/g ww in flounder livers from the river Western Scheldt. PFOA was found in ten samples, although at lower concentrations, 2-53 ng/g ww. In several samples longer chain PFCs (perfluoroundecanoic and –dodecanoic acid) were also found. Generally, PFC concentrations increase in the following order: open sea and ocean ≈ coastal water < freshwater. The highest concentrations were found in samples from the Western Scheldt, which may be related to use and former production along the river Scheldt.
The fish related human exposure to PFOS was estimated at 73 ng/day. Herring was the major contributor to the exposure (52%), followed by fish from the Gadidae family (incl. cod and haddock) (17%) and plaice (14%). This is substantially lower than the recent estimate by EFSA for human exposure to PFOS in The Netherlands (3410 ng/day). The difference is explained by lower fish consumption figures and lower PFOS concentrations in Dutch fish species.

Introduction
Perfluorinated compounds (PFCs) have been found in a wide variety of environmental matrices including fish (*1-4*), marine mammals, polar bears (*5-7*), and bird (eggs) (*1,4,5,8-10*). Until recently, the focus was on evaluation of ecotoxicological effects of PFCs. The human exposure to PFCs received little attention apart from several studies on PFCs in human blood and serum (*11-14*). The body burden results from several exposure pathways, including air, drinking water and food. Little attention was paid to characterization of exposure through food. Perfluoroctane sulfonate (PFOS) has several adverse effects, such as developmental effects, changes in the thyroid hormone regulation and high lipoprotein concentrations, while the liver is the major target organ for most effects *(15-17)*. Perfluoroctanoic acid (PFOA) may show e.g. developmental, reproductive and carcinogenic effects *(16,17)*.

[11] Adapted from S.P.J. van Leeuwen, I. van der Veen, P.E.G. Leonards and J. de Boer (2006), Perfluorinated compounds in edible Dutch fish: a source for human exposure *Organohalogen compound* 68, 535-538, 2006.

The European Food Safety Authority (EFSA) recently published a human risk evaluation of PFOS and PFOA. They determined that the mean Dutch population is exposed (diet) to 58 ng PFOS/kg body weight (bw) per day *(17)*. This estimate was based on fish and drinking water. Other foods were not included in that exposure assessment due to the lack of data. The exposure (58 ng PFOS/kg bw per day) was slightly below the tolerable daily intake (TDI) of 150 ng PFOS/kg bw per day *(17)*, leaving only a small margin of safety. High fish consumers exceed the PFOS TDI. Fish was the main contributor whereas drinking water contributed less than 0.5% (14.2 ng/day). The mean dietary exposure to PFOA is 2 ng/kg bw per day, which is well below the TDI of 1.5 µg/kg bw per day *(17)*. For evaluation of human exposure in relation to fish consumption, there is a need for data on PFC concentrations in edible fish tissues. This study presents PFC concentrations in fish tissues of popular fish and shellfish consumed in The Netherlands.

Materials and methods

The investigated species are given in Table 4.9. The majority of fish and shellfish were sampled between April and October 2004. Marine fish was mostly sampled during surveys of the research vessel Tridens. Remaining samples were obtained directly from fishermen, from fish auctions or from wholesale traders. Eel was caught by electric fishery.

After transportation to the laboratory, lengths and weights of the individual fishes were measured (except for tuna, mussels, oysters and shrimps). Each sample consisted of multiple individuals (17-25 in the case of wild fish, 5-22 for farmed fish, 22 for the oyster sample; 500 grams of shrimps and 3 kg of mussels). The fish was filleted (except for farmed salmon and tuna which were purchased as parts of fillets). Each sample consisted of equal amounts of fillet per individual fish. The pooled samples were homogenized in a Waring blender. Each mussel sample was prepared by taking the meat from the shells of a 3 kg sample. Subsequently, 100 g mussel meat was pooled and homogenised. The oyster sample was prepared by pooling the meat from the individual oysters and subsequent homogenisation. Pooled shrimp samples were prepared by homogenization of approx. 500 g unpeeled and uncooked whole shrimps.

The seven compounds analysed in this study are PFOS, perfluorohexane sulfonate (PFHxS), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDcA), perfluoroundecanoic acid (PFUnA) and perfluorododecanoic acid (PFDoA). PFCs were extracted according to an adapted method by Hansen *et al. (18)*. Briefly, the method is as follows: 10 g of homogenised sample was extracted 3 times with methyl-*tert*-butylether (MTBE) in the presence of the ion pairing agent tetrabutylammonium hydrogen sulfate (TBA). The extracts were pooled and MTBE was concentrated to a final volume of 1 ml. Clean up was performed by neutral silica column

chromatography (0.6 mm internal diameter glass column with 1.8 gram, 1.5% deactivated silica). The lipids were removed from the extract by (elution by 15 ml dichloromethane), followed by 30 ml acetone for elution of the target compounds. The acetone was removed by evaporation and replaced by 0.7 ml methanol, after which the extracts were ready for analysis. The extracts were injected on a Thermo Electron Surveyer high pressure liquid chromatography (HPLC) system, coupled with an LCQ-Advantage ion trap mass spectrometric system (MS) and electrospray ionisation interface (ESI).

Table 4.9 PFC concentrations in fish and shellfish in ng/g wet weight (data taken from (21)).

	Species name	PFOA	PFNA	PFDcA	PFUnA	PFDoA	PFHxS	PFOS
Shellfish and crustaceans								
Mussels Eastern Scheldt	*Mytilus edulis*	<2	<2	<2	<2	<4	<3	<2
Mussels, Wadden Sea (East)	*Mytilus edulis*	<2	<2	<2	<2	<4	<4	4
Mussels, Wadden Sea (West)	*Mytilus edulis*	<2	<2	<2	<2	<3	<3	<2
Shrimps, North Sea (Rijnmond)	*Crangon crangon*	<2	<2	<2	<2	<3	<6	8
Shrimps, Wadden Sea	*Crangon crangon*	<2	<2	<2	<2	<3	<6	30
Oysters, Eastern Scheldt (Yerseke)	*Ostrea edulis*	<2	<2	<2	<2	<4	<4	2
Western Scheldt and freshwater								
Flounder A liver, Western Scheldt	*Platichthys flesus*	15	5	62	33	4	<3	730
Flounder A, Western Scheldt	*Platichthys flesus*	2	<2	<2	<2	<3	<6	230
Flounder B liver, Western Scheldt	*Platichthys flesus*	53	6	50	52	<3	27	540
Flounder B, Western Scheldt	*Platichthys flesus*	3	<2	<2	8	<3	<6	93
Pike-perch liver, Hollands Diep	*Stizostedion lucioperca*	<2	<2	27	15	<3	<3	270
Pike-perch, Hollands Diep	*Stizostedion lucioperca*	<2	<2	2	3	<4	<3	40
Pike-perch, IJssel Lake	*Stizostedion lucioperca*	2	<2	<2	<2	<4	<4	150
Eel, Nieuwe Merwede	*Anguilla anguilla*	<2	<2	<2	<2	<3	<3	30
Eel, Ketel Lake	*Anguilla anguilla*	<2	3	30	57	<3	<3	57
Eel, Haringvliet (West)	*Anguilla anguilla*	<2	<2	6	8	<3	<3	37
Eel, IJssel Lake (Medemblik)	*Anguilla anguilla*	<2	<2	<2	<2	<3	<3	52
Eel, Meuse (Keizersveer)	*Anguilla anguilla*	<2	<2	<2	<2	<3	<3	5.9
Eel, Rhine (Lobith)	*Anguilla anguilla*	<2	<2	4	5	<3	<3	44
Marine fish								
Herring liver, Southern North Sea	*Clupea harengus*	<2	<2	<2	<2	<3	<3	67
Herring, Southern North Sea	*Clupea harengus*	<1	<1	<2	<1	<3	<5	8
Herring, English Channel	*Clupea harengus*	<2	<2	<2	<2	<3	<6	<1
Herring, central North Sea	*Clupea harengus*	<2	<2	<2	<2	<3	<6	51
Plaice liver, Southern North Sea	*Pleuronectes platessa*	<2	<2	2	<2	<3	<3	35
Plaice, Southern North Sea	*Pleuronectes platessa*	<2	<2	<2	<2	<4	<4	20
Mackerel, North Sea	*Scomber scombrus*	<2	<2	<2	<2	<3	<5	7
Cod, Central North Sea	*Gadus morhua*	<1	<1	<2	<1	<3	<5	<1

Table 4.9 Continued.

	Species name	PFOA	PFNA	PFDcA	PFUnA	PFDoA	PFHxS	PFOS
Haddock, Central North Sea	*Melanogrammus aeglefinus*	<1	<2	<2	<1	<3	<5	5
Sole liver, Southern North Sea	*Solea solea*	3	4	6	<2	<3	<3	130
Sole, Southern North Sea	*Solea solea*	3	<2	<2	<2	<4	<4	45
Sole, Mouth Western Scheldt	*Solea solea*	<2	<2	<2	<2	<4	<4	10
Sole, Dutch coast, Hoek van Holland	*Solea solea*	2	<2	<3	<2	<4	<4	13
Sole, Dutch coast, IJmuiden	*Solea solea*	2	<2	<2	<2	<4	<4	12
Sole, Dutch coast, Egmond	*Solea solea*	<2	<2	<2	<2	<4	<4	<2
Sole, Dutch coast, Texel	*Solea solea*	<2	<2	<2	<2	<4	<4	<2
Herring, Skagerak	*Clupea harengus*	<2	<2	<2	<2	<3	<6	23
Herring, Shetland Islands	*Clupea harengus*	<2	<2	<2	<2	<3	<6	23
Mackerel, Shetland Islands	*Scomber scombrus*	<2	<2	<2	1	<3	<5	22
Tuna, Mediterranean	*Thunnus thynus*	<2	2	<2	2	<4	<3	<2
Farmed fish								
Salmon, Farmed, Scotland	*Salmo salar*	<2	<2	<2	<2	<4	<4	<2
Salmon, Farmed, Norway	*Salmo salar*	1	<2	<2	<2	<4	<4	<2
Eel liver, Farmed, Italy	*Anguilla anguilla*	<2	<2	<2	<2	<3	<3	14
Eel, Farmed, Italy	*Anguilla anguilla*	<2	<2	<2	<2	<3	<6	<1
Eel liver, Farmed, Netherlands	*Anguilla anguilla*	<2	<2	<2	<2	<3	<3	23
Eel, Farmed, Netherlands	*Anguilla anguilla*	<2	<2	<2	<2	<3	<5	10

For PFOS and PFHxS, 7H-perfluorinated heptanoic acid (7H-PFHpA) was used as internal standard, whereas for PFOA and other perfluorinated acids $^{13}C_2$-labeled PFOA has been used. The sensitivity of 7H-PFHpA was limited at the MS conditions used for PFOS. Therefore, in some cases, $^{13}C_2$-labeled PFOA was used as internal standard for the calculation of PFOS. Admittedly, this may not be the optimum internal standard, but at the time of the study (2004) no mass-labeled PFOS was available.

Results and discussion

Table 4.9 shows that fish from nearly all origins is contaminated with PFOS. PFOA is also found in fish from different origins, although at lower concentrations and in fewer samples. Generally, concentrations increase in the following order: open sea and ocean ≈ coastal water < freshwater. The highest PFC concentrations were found in the river Western Scheldt. This is related to the (historic) production of PFCs in Antwerp and the industrial and domestic use of PFCs in the river Scheldt basin.

PFCs preferentially accumulate in fish liver as compared to muscle tissue, which can be seen in all samples of which both liver and muscle tissue were analysed (farmed eel, herring, sole, plaice and pike perch). Liver concentrations were ca. 5-fold higher than muscle tissue concentrations (mean of 8 values). PFC concentrations in flounder liver from the Western

Scheldt compare well to those reported by de Vijver *et al. (19)* and Hoff *et al. (20)* for this location.
Freshwater fish (eel and pike perch) show in this study the next highest concentrations as compared to the other locations. Hoff *et al.* found very high PFOS concentrations in eel liver samples (up to 9 µg/g ww) from the leperlee canal at Boezinge (Belgium) (*2*), being much higher than the levels in eel (incl. farmed eel livers) and pike-perch in our study. The authors suggested that nearby industrial and household discharges may have caused these high concentrations (*2*).
The PFOS concentrations for sole and plaice from the Southern North Sea in our study compare well to those reported for plaice and bib caught off the Belgian North Sea coast *(20)*. PFOS concentrations in other marine fish are in the same order of magnitude as those reported by Kallenborn *et al. (22)*
Other PFCs detected were e.g. PFDcA and PFUnA (up to 60 ng/g ww) in flounder liver from the Western Scheldt. PFDcA and PFUnA were also found in eel from the river Rhine and the Ketel Lake. PFNA was found in four samples (up to 6 ng/g ww) and PFDoA was found in only one flounder liver from the Western Scheldt (4 ng/g ww). PFHxS was detected in one Western Scheldt flounder liver (27 ng/g ww).
Concentrations in Chinese seafood (wild fish) were in the same range as those observed in the present study (PFOS: 0.4-2.9 ng/g ww (fish) and 1.8-14 ng/g ww (shrimp), PFOA: 0.42-0.45 ng/g ww (shrimp) and <LOD (fish), PFNA: all <LOD, PFDcA: 0.3 ng/g ww (shrimp) and <LOD in fish, PFUnA: 0.35-0.65 ng/g ww (fish) and 0.42-0.93 ng/g ww (shrimps) *(23)*). PFOS concentrations in seafood (wild fish) from Cataluna, Spain were lower (0.65 ng/g ww) and PFOA was <LOQ *(24)*. The concentrations in wild fish are higher than those in farmed fish (see Chapter 4.4). PFCs were only detected in approx. half of the farmed fish samples analysed at concentrations from 20 (PFUnA) to 600 pg/g ww *(25)*.

Human exposure

Virtually no detailed information is available on human exposure to PFCs from fish consumption. Therefore, an estimation was made of the fish-related dietary exposure of the mean Dutch citizen. This estimation was limited to PFOS as only for this PFC considerable data on individual species is available (see Table 4.9). This data was combined with consumption data from the Dutch National Food Consumption survey (DNFCS) of 1997/1998 *(26)*. The DNCSF contains files of 6250 people in the age of 1 to 99 years, who recorded the food they consumed during two consecutive days in a food diary. The PFOS exposure calculation is based on the daily fish consumption multiplied with the mean lower bound concentrations (per species): DE = (Qa x Ca + Qb x Cb +…Qz x Cz), in which DE = Daily Exposure, Q = Quantity of daily consumption of fish species and C = mean lower bound concentration of

PFOS in fish species (measured in this study). A to z indicate different fish species.
These estimations are compared with an estimation made by EFSA (Table 4.10) for which the Concise European Food Consumption Database (CEFCD) for the exposure assessment was used *(17,27)*. This database contains concise food consumption data from a variety of European countries for people from 16-64 years. The food consumption data used in the EFSA estimate concerns *consumers-only* data, meaning only data of people that actually consumed fish during the survey were used. The PFOS concentration data used for this estimation is the mean of PFOS concentrations in fish from various European countries *(17)*. The dietary exposure estimated by EFSA therefore results from DE = (Q_{mean} x C_{mean}) in which DE = Daily Exposure, Q_{mean} = mean quantity of daily consumed fish species (*consumers-only*) and C_{mean} = mean PFOS concentration in fish from Europe. The results are presented in Table 4.10.

Table 4.10 Dietary exposure estimations for the Dutch population according to two scenarios.

	EFSA[1]	**This study**
Food consumption data base:		
Name abbreviation	CEFCD[2]	DNFCS[3]
Population included[4]	Consumers only	Total population
No. individuals / age range	901, 16-64	6250, 1-99
Exposure input data:		
Fish consumption (g/day)	50 (median)	10.4 (mean)[5]
PFOS concentration in fish (ng/g ww)	68.1 (mean)	Range: <1-57 (see Table 4.9)
Results:		
Exposure (ng/day)	3410	72.5
Exposure (ng/ kg bw per day) for a 60 kg person	56.8	1.2
Margin of exposure:	2.6	125

1 *Scenario taken from (17);*
2 *Concise European Food Consumption Database (agregated fish consumption data);*
3 *Dutch National Food Consumption Survey (individual fish consumption data);*
4 *Consumers only: data only based on persons that actually consumed fish during the survey; total population: data based on the entire survey population, also including persons who did not consume fish at all during the survey;*
5 *This mean is based on fish species of which both consumption data (DNFCS) and contaminant data (this study) were available. Other species were left out. The included fish species represent 92% of all consumed species.*

The EFSA estimates of the PFOS exposure from fish and drinking water consumption for Italy, The Netherlands, Sweden and the UK are 58, 57, 45 and 49 ng PFOS/kg bw per day, respectively *(17)*. Fish dominates this exposure. Drinking water contributed only <0.5%, and can, therefore, be neglected. These exposure estimates are much higher than estimates resulting from the present study (1.2 ng/kg bw per day, or 73 ng PFOS per day for a 60 kg person). This is partly due to the high fish consumption estimate used by EFSA

(50 g/day) for the Dutch adult population. This estimate was based on *consumers-only*, which results in a conservative, higher exposure estimate. The data from the DNFCS were taken as *total population* data, which is 5-fold lower (10 g/day), because the frequency (and therefore volume) of fish consumption in The Netherlands is much lower than the *consumers-only* data would suggest. Furthermore, the PFOS concentrations in fish in the EFSA dataset (covering whole Europe) covered 3 to 4 orders of magnitude and contained higher PFC concentrations than are being found in The Netherlands (excluding fish from the Western Scheldt area) *(17)*. The resulting mean value of 68.1 ng/g ww was used for the exposure estimation for the four aforementioned countries, leading to a higher exposure estimate. The lower fish consumption data and the lower PFOS concentration data in this study resulted in a 50-fold lower exposure (72.5 ng/d) and a 50-fold larger margin of exposure compared to EFSA. In case we would consider Dutch consumers-only, the difference with the EFSA would have been less pronounced.

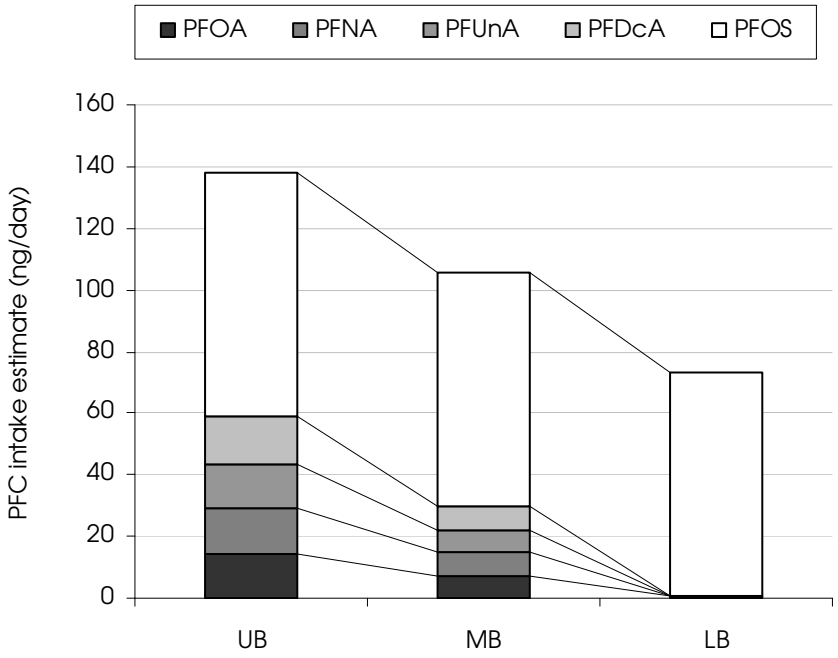

Figure 4.8 PFC exposure estimate for the Dutch population from fish consumption. UB = upperbound, MB = middle bound, LB = lower bound

The mean dietary intake for the general population of Bavaria, Germany was estimated at 1.8 ng/kg bw per day (duplicate diet study, 31 individuals, aged 16-45 years) *(28)*. This is slightly higher than in our study, possibly because Fromme et al. included all food items and beverages whereas the present study only took fish into account. The dietary exposure of the Catalan citizens to PFOS was estimated to be 63 ng/d for food, to which fish contributed 34 ng/d *(24)*. No beverages (except for milk) or drinking water were included. The intake from drinking water in Catalonia was estimated at 0.8-1.7 ng/d *(29)* being 10-20 times lower than the EFSA estimate (14 ng/d) *(17)*.
The human exposure is predominated by PFOS in all three scenarios (lower bound, LB; medium bound, MB and upper bound, UB) (see Figure 4.8). Because PFOS was detected in almost all samples, there is only a small difference between the UB and LB scenario for this PFC (79 vs. 73 ng/d). For the other PFCs, the differences are much larger because of the low detection frequency. Focussing on PFOS, herring contributes most to the intake (52%, Figure 4.9), followed by fish from the Gadidae family (cod, haddock), plaice and shrimps.

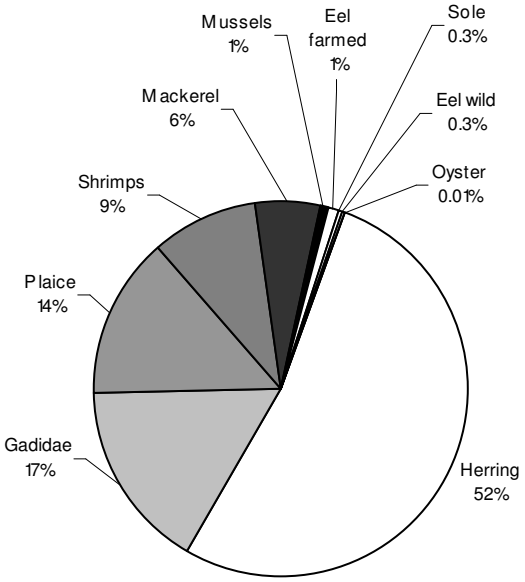

Figure 4.9 Relative contribution of fish species to the fish related human exposure to PFOS (lowerbound). The contribution of salmon and tuna was 0% (not shown in graph).

Acknowledgements

The Dutch Ministry of Agriculture, Nature Management and Food Quality is gratefully acknowledged for providing funding. Furthermore, Dr. M.I. Bakker is acknowledged for making the DNFCS fish consumption data available.

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4.4 Halogenated contaminants in farmed salmon, trout, tilapia, pangasius and shrimp[12]

Abstract

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and –furans (PCDD/Fs), organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane diastereomers (HBCDs) and perfluorinated compounds (PFCs) were analysed in popular farmed fish such as salmon, trout, tilapia, pangasius and in farmed shrimp. The samples originated from Southeast Asia, Europe and South America.

Results show that (i) carnivorous species contained higher contaminant concentrations than omnivorous species; (ii) contaminant concentrations generally decreased per species in the order: salmon > trout >> tilapia ≈ pangasius ≈ shrimp; (iii) most contaminant concentrations decreased in the following order PCBs ≈ DDTs >> hexachlorobenzene ≈ pentachlorobenzene ≈ dieldrin ≈ PBDEs ≈ α-HBCD ≈ PFOS >> WHO-TEQ (PCDD/Fs and dioxin-like (dl)-PCBs); (iv) the contaminant concentrations were very low (mostly <1 ng/g wet weight), and far below the European and Dutch legislative limits and (v) contaminant concentrations in farmed shrimp, pangasius and tilapia were lower than in wild fish from other studies, whereas farmed salmon and trout were higher than lean wild marine fish (from other studies).

From the five species investigated, salmon is predominantly responsible (97%) for the human exposure to the sum of investigated contaminants. The contribution of trout, tilapia, pangasius and shrimp is small (3%) because contaminant concentrations and consumption volumes were (much) lower.

Introduction

During the last decades the world production of aquaculture has grown considerably (*1*). In relation with that, the human consumption of farmed fish and crustaceans is also increasing. This is true for both well-known species such as salmon, trout and shrimp as well as for new species like pangasius and tilapia (*1*). Recent reports have shown that commonly consumed farmed salmon and trout can be contaminated with a range of contaminants including polychlorinated dibenzo-*p*-dioxins and –furans (PCDD/Fs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs) (*2-6*). However, almost no information is available on the contamination of farmed shrimp and new species like

12 Based on S.P.J. van Leeuwen, M.J.M. van Velzen, C.P. Swart, I. van der Veen, W.A. Traag and J. de Boer (2009) Halogenated contaminants in farmed salmon, trout, tilapia, pangasius and shrimp. *Environmental Science and Technology*, 43, 4009–4015

tilapia and pangasius, whereas this is urgently needed because of the rapidly growing consumption of these species world-wide. Pangasius (*Pangasius hypopthalmus*, also known as Swai, Tra, Basa, Sutchi catfish, Siamese shark, Striped catfish, Shark catfish and Iridescent shark) is an omnivorous fish which is primarily farmed in the Mekong delta in Vietnam. Virtually all fish is processed (filleted) in Vietnam and is shipped deep-frozen to Russia, North America and Europe (*1,7*). The fish is sold frozen or 'refreshed' (meaning that the fillets are thawed and sold as such). Tilapia is the common name for *Oreochromis mossambicus* and *Oreochromis niloticus*. Tilapia is farmed in South-East Asia and South America (*1*) in rice-fields, floating net cages and ponds. However, tilapia is also successfully (commercially) farmed in recirculation systems (e.g. in The Netherlands). Tilapia is offered fresh and deep-frozen. Both pangasius and tilapia are omnivorous fish, and their diet is dominated by proteins and lipids from vegetable sources, which may suggest that their contaminant concentrations are low. However, there is no comprehensive data to confirm this hypothesis, and therefore, together with the Dutch Food and Consumer Product Safety Authority (VWA), the following objectives were identified for this study: (i) to determine the contamination of new farmed species (tilapia and pangasius) with PCBs, OCPs, PCDD/Fs and dl-PCBs; (ii) to determine the contamination of all investigated species (salmon, trout, shrimp, tilapia and pangasius) with PBDEs, PFCs and HBCDs.

Materials and methods

Sampling and sample pre-treatment

The fish species selected for sampling were based on information on trade flows of farmed fish in The Netherlands (*7*). From that study, it became clear that the current top-5 farmed fishery product species consumed in The Netherlands are salmon, trout, shrimp, tilapia and pangasius. Consumption volumes are given in Table 4.10.

The investigated species, number of samples and contaminants are shown in Table 4.10. PCDD/Fs and dioxin-like PCBs (dl-PCBs) were not investigated in salmon and trout because there is already a substantial amount of information available in the literature (*2,6,8-10*) and the concentrations commonly observed are below the maximum levels (MLs) of the European Union (EU) (*11*).

The fish and shrimp samples were purchased between October 2007 and January 2008 from various suppliers from different places in The Netherlands. These included supermarkets, fish stores, week markets and suppliers for restaurants. One shrimp sample was obtained directly from a farm in The Netherlands. The fish samples were purchased fresh (cooled) or frozen. Detailed sample information, including Laboratory Information Management System (LIMS) identities, sample weights, physical state at purchase etc. was

recorded and can be found in Table 4.11. All samples were stored at –20°C in their original packaging. Pangasius and tilapia samples were purchased as whole fillets. Salmon was purchased as parts of the whole fillets. Trout was purchased as whole fish but with intestines being removed (degutted). The trout samples were filleted. One trout sample was bought as fillet. For each sample, generally 10 or more fillets were purchased, and these were pooled. Shrimp were purchased in a variety of physical states (cooked, raw, frozen, cooled, decapitated etc. – see Table 4.11). In case where the heads were still on, these were removed prior to pooling the individuals. Twenty-five or more individuals were pooled per sample. Each pooled sample was ground using a kitchen machine (Type AL2-3, Krefft Gmbh, Gevelsberg, Germany) equipped with a rotary knife and sieve with 10 mm diameter holes. Subsequently, the samples were further ground (to reduce particle size) and homogenised (Warring blender) and stored in glass containers at –20°C until analysis.

Table 4.10 Investigated farmed fish and shrimp samples and species.

Investigated species			**Number of samples investigated**					
Common names	**Dutch Consumption estimate (tons/yr)***	**Origin**	**PFC**	**PBDE**	**HBCD**	**WHO-TEQ**	**PCB**	**OCP**
Salmon, Atlantic Salmon	8700	Norway, Scotland, Chile	7	7	7	-	7	7
Pangasius, Swai, Sutchi catfish, Striped catfish, Iridescent shark	1700	Vietnam	7	7	7	5	7	7
Tilapia	1200	China, Ecuador, Indonesia, Netherlands	7	7	6	5	7	7
Trout	900	Denmark, Italy, Turkey	5	5	5	-	5	5
Shrimp	1500	Bangladesh, Mixed-Asia**, Netherlands	6	6	6	5	6	6
Totals	14 000		32	32	31	15	32	32

* *Dutch consumption of farmed fish in 2006 (ton/yr), representing approx. 18% of the total fish and shellfish consumption in the Netherlands (7).*

** *Mixed origins were declared on the package label (Bangladesh/India, Indonesia/China or Thailand/Malaysia/ Indonesia)*

Table 4.11 Sample details.

IVM LIMS code	Sample name, origin and number	Latin name	Country of origin	No individuals in pooled sample	Weight of pooled sample (gram)	Whole fish, whole fillet or piece of a fillet*	Physical state at purchase	Packaging material
07/860	Salmon NO 3**	*Salmo salar*	Norway	2	1107	Fillet (piece)	4°C, raw	Polystyrene tray
07/790	Salmon NO 1	*Salmo salar*	Norway	10	941	Fillet (piece), skin removed	4°C, raw	XPS*** tray
07/804	Salmon NO 2	*Salmo salar*	Norway	5	491	Fillet (piece)	4°C, raw	Transparent plastic box
07/870	Salmon NO 4	*Salmo salar*	Norway	10	939	Fillet (piece)	4°C, smoked	Plastic foil (vacumised)
07/805	Salmon UK 2	*Salmo salar*	UK (Schotland)	5	480	Fillet (piece)	4°C, raw	Transparent plastic box
07/785	Salmon UK 1	*Salmo salar*	UK (Schotland)	10	1441	Fillet (piece)	4°C, raw	Transparent plastic box
07/810	Salmon CL	*Salmo salar*	Chile	10	1213	Fillet (piece)	-20°C, raw	Plastic foil (vacumised)
07/862	Trout DK 3	*N.r.*	Denmark	10	1359	Whole fish, filleted	4°C, raw	N.r.
07/861	Trout DK 2	*N.r.*	Denmark	10	1208	Fillet	4°C, raw	Plastic bag
07/807	Trout DK 1	*Onchorhynchus mykiss*	Denmark	5	296	Fillet	4°C, raw	Transparent plastic box
07/793	Trout IT	*Salmo trutta*	Italy	10	1081	Whole fish, filleted	4°C, raw	XPS tray
07/869	Trout TR	*Onchorhynchus mykiss*	Turkey	10	949	Fillet (whole)	4°C, smoked	Plastic foil (vacumised
07/864	Pangasius VN 5	*Pangasius hypophthalmus*	Vietnam	10	1383	Fillet (whole)	4°C, raw	Plastic bag
08/002	Pangasius VN 7	*Pangasius hypophthalmus*	Vietnam	6	Nr.	Fillet (whole)	4°C, raw	Polystyrene tray
07/868	Pangasius VN 6	*Pangasius hypophthalmus*	Vietnam	8	1013	Fillet (whole)	-20°C, raw	Transparent plastic bag
07/791	Pangasius VN 2	*Pangasius hypophthalmus*	Vietnam	10	1187	Fillet (whole)	4°C, raw	XPS tray
07/796	Pangasius VN 3	*Pangasius hypophthalmus*	Vietnam	Approx. 12	1594	Fillet (whole)	-20°C, raw	Transparent plastic bag
07/806	Pangasius VN4	*Pangasius hypophthalmus*	Vietnam	5	870	Fillet (whole)	-20°C, raw	Transparent plastic bag
07/786	Pangasius VN 1	*Pangasius hypophthalmus*	Vietnam	9	372	Fillet (whole)	4°C, raw	N.r.

N.a.: not applicable; N.r.: not recorded.

* *Underlined: additional pre-treatment prior to grinding and homogenisation of pooled sample*

** *Sample name, abbreviation of country of origin (e.g. Norway = NO) and sample number*

*** *XPS: Extruded polystyrene*

**** *Declared sizes (e.g. in no of individuals/kg)*

Table 4.11 Continued.

IVM LIMS code	Sample name, origin and number	Latin name	Country of origin	No individuals in pooled sample	Weight of pooled sample (gram)	Whole fish, whole fillet or piece of a fillet*	Physical state at purchase	Packaging material
07/809	Shrimp BG 2	*Penaeus monoden*	Bangladesh	41/50****	827	N.a.	-20°C, raw	Plastic bag
07/797	Shrimp BG 1	*Penaeus monoden*	Bangladesh	21-30/ kg****	867	Whole, heads removed	-20°C, raw, unpeeled	Cartoon box, lined with plastic
07/811	Shrimp M-AS 3	*Penaeus vannamei*	Indonesia/ China	45/50****	1275	Whole	-20°C, cooked	Cartoon box
07/794	Shrimp M-AS 2	*Penaeus monoden*	Bangladesh / India	N.r.	1444	N.a.	4°C, blanched	Transparent plastic box
07/789	Shrimp M-AS 1	*Litopenaeus vannamei*	Thailand, Malaysia en Indonesia	N.r.	787	N.a.	4°C, cooked	Transparent plastic box
07/871	Shrimp NL	*N.r.*	Netherlands	30/kg****	871	Whole, heads removed	4C, raw, unpeeled	Polystyrene box
07/863	Tilapia*****	*Oreochromis spp*	N.r.	10	1654	Fillet (whole)	4°C, raw	N.r.
07/792	Tilapia CN 1	*Oreochromis mossambica*	China	10	1149	Fillet (whole)	4°C, raw	XPS tray
07/808	Tilapia CN 2	*Oreochromis nilloticus*	China	6	956	Fillet (whole)	-20°C, raw	Plastic foil (vacumised)
07/788	Tilapia EC	*Oreochromis nilloticus*	Ecuador	10	985	Fillet (whole)	4°C, raw	Transparent plastic box
07/795	Tilapia ID 1	*Oreochromis spp*	Indonesia	Approx. 18	1649	Fillet (whole)	-20°C, raw	Transparent plastic bag
08/001	Tilapia NL	*Oreochromis niloticus*	Netherlands	6	N.r.	Fillet (whole)	4°C, raw	Polystyrene tray
07/867	Tilapia ID 2	*Oreochromis spp*	Indonesia	Approx. 18	1578	Fillet (whole)	-20°C, raw	Transparent plastic bag

N.a.: not applicable; N.r.: not recorded.

* *Underlined: additional pre-treatment prior to grinding and homogenisation of pooled sample*

** *Sample name, abbreviation of country of origin (e.g. Norway = NO) and sample number*

*** *XPS: Extruded polystyrene*

**** *Declared sizes (e.g. in no of individuals/kg)*

***** *Country of origin not recorded*

Contaminants investigated and chemical analysis
The contaminants analysed in the samples are shown in Table 4.12.

Table 4.12 Contaminants analysed in the samples.

Compound class	Individual compounds
Polychlorinated dibenzo-*p*-dioxins and –furans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (dl-PCBs)	All 29 WHO PCDD/F and dl-PCB congeners (*12*)
Polychlorinated biphenyls (PCBs)	Congeners 28, 52, 101, 118, 138, 153 and 180
Organochlorine pesticides (OCPs)	Hexachlorobutadiene (HCBD), pentachlorobenzene (QCB), hexachlorobenzene (HCB), α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH, heptachlor, trans-heptachlor epoxide, cis-heptachlor epoxide, aldrin, telodrin, isodrin, dieldrin, endrin, α-endosulfan, o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT and p,p'-DDT
Polybrominated diphenyl ethers (PBDEs)	Congeners 28, 49, 71, 47, 66, 77, 100, 119, 99, 85, 126, 154*, 153, 138, 156, 184, 183, 191, 197, 196, 208, 206, 209
Hexabromocyclododecane diastereomers (HBCDs)	α-, β- and γ-diastereomers
Perfluorinated compounds (PFCs)	Perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS) and perfluorinated sulfonamide (PFOSA)

** Sum of BDE-154 and bromobiphenyl (BB)153*

Lipid determination – The lipid determination was performed according to a modified Bligh and Dyer method (*13*). This method determines both the triglycerides as well as the more polar lipid compounds such as phospholipids and sterols, and is therefore suitable for both lean and lipid-rich fish.
*PCDD/Fs and dl-PCBs – A*ll samples were extracted using an accelerated solvent extraction system (ASE 200 Dionex). In order to obtain 2.5 gram of lipids from the sample, the relating amount of sample material was extracted. Prior to extraction sixteen ^{13}C labelled PCDD/Fs, four ^{13}C labelled non-ortho PCBs, eight ^{13}C labelled mono-ortho PCBs internal standard were added to the samples. The samples were extracted three times with hexane/acetone (7:3, v/v) at 100°C and 1500 PSI during 10 minutes. Extracts were concentrated down to < 0.1 ml and after addition of the ^{37}Cl-2,3,7,8-TCDD (clean up standard) extracts were made up with hexane to 25 ml. Extracts were purified by a comprehensive automated system, the so called Power-

Prep™ (Fluid Management Systems, Waltham, USA). Extracts were transferred to the Power-Prep™ system and purified on an acid silica column, a neutral silica column, a basic alumina column and an activated carbon/celite column. Custom made solvents and mixtures were used for elution; hexane, hexane/dichloromethane (1:1, v/v) ethylacetate/toluene (1:1, v/v) and toluene. The volume of the final extract was reduced to 0.5 ml using a turbovap. The recovery standards ^{13}C 1,2,3,4-TCDD and ^{13}C 2,3,4,6,7,8-HxCDF were added and the volume of the extract was again reduced to 0.5 ml using a turbovap. PCDD/Fs and PCBs analyses were performed by gas chromatograph coupled with high resolution mass spectrometer (GC-HRMS) using an Agilent (Wilmington, USA) 6890 Series GC and an AutoSpec Ultima HRMS (Waters, Milford, USA) (resolution 10 000). The GC column was a DB5 MS (60 m, 0.25 mm i.d., 0.25 µm; J&W, Folson, USA). The mass spectrometer was operated in the electron ionization mode (EI), using selected-ion monitoring (SIM). Of the fraction containing the mono-ortho PCBs a splitless injection of 2 µl is used to introduce the sample on the GC. The PCDD/Fs and non-ortho-PCB containing fraction (100 µl) was injected by a CIS-3 PTV injector in the solvent-vent mode with a vent flow of 100 ml/min. and a vent pressure of 100 Pa. The initial temperature of the PTV was 70 °C. After injection the temperature of the PTV was raised to 280 °C with 720 °C/min.

PCBs and OCPs – The PCBs and OCPs (mentioned in Table 4.12) were Soxhlet-extracted (dichloromethane (DCM)-acetone 3:1 v/v, 16 h) from the sample. Internal standard CB 103 was added after extraction. The co-extracted lipids were removed by Al_2O_3-column chromatography (15 g, 8% w/w H_2O, eluted with 170 ml n-pentane) and subsequently fractionated over a silica column (1.8 g, 1.5% w/w H_2O, PCB fraction eluted with 14 ml n-hexane and the OCP fraction eluted with 10 ml n-hexane-diethylether (DEE) 85:15 v/v). After a final concentration step (to approx. 500 µl), both fractions were analysed on a dual column GC-electron capture detection (ECD) system. 1 µl of the extract was injected in a split-splitless injector operated in the pulsed splitless mode (injector operated at 250°C). A pressure pulse (280 kPa, 1.5 min) was used for rapid transfer of the analytes to the columns. The columns used were CP-Sil-8 CB (50 m x 0.2 mm id x 0.33 µm film) and CP-Sil-19 CB (custom made, 50 m x 0.2 mm id x 0.33 µm film). They were both inserted in the injector using a 2-hole ferrule. The column flow was 1 ml/min (helium). Because the OCP fraction of the pangasius and tilapia samples contained interferences, they were treated with concentrated sulphuric acid (H_2SO_4) and re-analysed. In these cases, the drins were quantified from the untreated fraction, whereas the other OCPs were quantified in the treated fraction. CB 103 was added as internal standard after extraction to correct for the clean up and GC analysis procedure.

PBDEs and HBCDs - PBDEs and HBCDs were Soxhlet-extracted (16 hrs) from the matrix using a 3:1 dichloromethane (DCM):acetone (v/v) mixture. After extraction, the following IS were spiked to the sample extract: $^{13}C_{12}$-α-, β- and

γ-HBCD, $^{13}C_{12}$-BDE 209 and BDE 58. These IS correct for the complete analysis except the extraction. The lipids were removed from the crude extract by acid-silica column chromatography (20 g, 40% w/w H_2SO_4, elution with 150 ml DCM:n-hexane 3:7 v/v). The eluate was fractionated over a silica column (1.8 g, 1.5% w/w H_2O, 1st fraction eluted with 14 ml n-hexane and subsequently with 25 ml n-hexane-diethylether 85:15 v/v for the PBDEs and α- and γ-HBCD, and with 10 ml DEE (2nd fraction) for β-HBCD. After concentration of the purified extract (fraction 1 only) to 500 µl, the PBDEs were analysed by GC-ECNI-MS (Agilent 6890, Wilmington, USA). The column used was CP-Sil-8 CB (50 m x 0.25 mm id x 0.25 µm film). BDE 209 was analysed on a short column (DB-5, J&W, Folson, USA, 15 m x 0.25 mm id x 0.25 µm film) in order to prevent degradation due to long residence times in the GC oven at high temperatures (*14*). The PBDEs were detected using the bromine isotope (m/z 79 and 81) except for BDE 209 that was quantified based on the molecular ion (m/z 486, and m/z 494 for the ^{13}C internal standard). After analysis of the PBDEs by GC, fraction 1 was combined again with fraction 2 (containing β-HBCD), carefully evaporated to dryness and redissolved in 100 µl acetonitrile:water 75:25 (v/v). The HBCD diastereomers were analysed by HPLC-ESI-MS/MS using a Zorbax eclipse 2.1 x 30 mm, 3.5 µm particles analytical column and a Zorbax eclipse 2.1 x 12.5 mm, 5 µm particles (both from Agilent, Wilmington, USA). The diastereomers were quantified using MRMs.

PFCs – Prior to extraction, $^{13}C_n$-analogues of PFHxA, PFOA, PFNA, PFDA, PFUnA and PFOS were added (all Wellington Laboratories, Guelph, Ontario, Canada) as well as $^{18}O_2$-PFOSA (RTI International, NC, USA). Prior to extraction, the samples were dried by mixing with Kieselguhr. This improved the extraction efficiency, especially of the longer chain perfluorinated acids from 30-40% to nearly 80% (PFDA and PFUnA). By binding the moisture from the sample, this cannot mix with the extraction solvent (methanol, MeOH). Therefore, the solvent strength of the MeOH is maintained, resulting in a more efficient extraction. The sample extraction was performed with 10 ml MeOH and shaking for 30 minutes. This was repeated once with 5 ml fresh MeOH. After combining the extracts and reducing the volume under N_2 stream, a clean-up was performed according to the method first published by Powley et al. (*15*). The PFCs were chromatographically separated on a Symmetry C18 (50 x 2.1 mm, 5 µm particle size, kept at 20°C), which was preceded by a Symmetry C18 (20 x 3.9 mm, 5 µm particle size) (Waters, USA). The eluent consisted of (A) 2 mM ammonium acetate in water and (B) methanol. After dilution of the methanol extract 1:1 with ultrapure water, the extracts were analysed on an Agilent 1200 HPLC coupled with an Agilent 6410 ESI-MS/MS system. The system was equipped with a degasser and an autosampler. The injection volume was 20 µL. The capillary voltage was set at 1000 V, the nebuliser at 25 PSI, the gas flow at 6 L/min and the gas temperature was set at 325°C. The samples were quantified using MRMs.

Quality Assurance - The quality of the analysis was assured routinely by analysis of procedural blanks, duplicate analysis of selected samples, internal reference materials, certified reference materials (CRMs) (mussel tissue standard reference material (SRM) 2978 for PCBs and OCPs and the candidate CRM BROC-01 for the PBDEs, the use of (mass labelled) internal standards (as mentioned above). The recoveries for mass labeled PFC internal standards were 72% (PFOA), 81% (PFNA), 77% (PFDcA), 79% (PFUnA) and 51% (PFOS). Specifically for BDE 209, the blanks were in the very low pg/g ww range and the level was stable between the analysed sample batches. The reported concentrations were all 2-4 times higher than the blank concentrations. The BDE-209 concentration in the trout sample from Turkey was far above the blank value. The recovery of the ^{13}C-BDE 209 internal standard ranged from 58-78%. The recovery of the $^{13}C_{12}$-HBCD internal standard (α-diastereomer) was 86% (mean). The laboratories participate in various interlaboratory studies (e.g. Folkehelsa (www.fhi.no), QUASIMEME (www.quasimeme.org), and the 2nd world-wide PFC interlaboratory study (*16*) with satisfactory results. For example, in the PBDE exercise of QUASIMEME (round 54), the majority of the z-scores obtained from analysis of two fish samples were scored 'satisfactory' (i.e. $-2 \leq z \leq 2$). For BDE-209 specifically, the z-scores in both fish samples were satisfactory. For PCBs, two fish samples were analysed in the framework of QUASIMEME round 52 and the majority of the results had satisfactory z-scores. For the OCPs (p,p'-DDTs, HCB and dieldrin) all results were satisfactory.

Results and discussion

Contaminants in farmed fish and shrimp

There is a clear distinction in the contaminant concentrations between the fish and shrimp samples, with concentrations decreasing in the following order: salmon>trout>>tilapia≈shrimp≈pangasius (Figure 4.10). For example, ∑7-PCB concentrations (the sum of CB 28, 52, 101, 118, 138, 153 and 180) in salmon are 3-fold higher than in trout and 100-200-fold higher than in the other species. The median concentrations were (pg/g ww): salmon: 10 860; trout: 3480; shrimp: 117; tilapia: 47 and pangasius: 47). This generally also holds for the other contaminants.

∑9-PBDE represents the sum of the BDEs 28, 47, 99, 100, 153, 154, 183 and 209, which were recently recommended for monitoring by the European Food Safety Authority (EFSA) (*17*). BDE 49 was added because this congener was detected frequently at concentrations similar to those of BDE 100 (this study). ∑4-DDT represents the sum of p,p'-DDD, p,p'-DDE, p,p'-DDT, o,p'-DDT. ∑7-PCB, OCP, ∑9-PBDE concentrations in salmon and ∑7-PCB and ∑4-DDT concentrations in trout were >1 ng/g ww. Contaminant concentrations in all other species were generally <1 ng/g ww. The higher concentrations in the

carnivorous fish (salmon, trout) are believed to be related to their diets, which consists for a substantial part of fish oil and meal. Although the diets of these fish were not analysed, it is known that fish oil and meal are contaminated with a range of contaminants (*4*). Farmed tilapia and pangasius feed on a higher proportion of vegetable lipids and proteins, resulting in lower contamination concentrations. Lipid contents of the samples are: salmon 14.5±3.7%; trout 6.6±1.4%; shrimp 1.2±0.5%; pangasius 1.9±0.9% and tilapia 3.1±1.2% (see Table 4.13). In case the contaminant concentrations are expressed on lipid weight basis, the concentrations in different fish samples will be closer to each other (data not shown), but salmon and trout remain the samples with highest contaminant concentrations.

Per contaminant, the concentrations generally decrease in the following order ∑7-PCB ≈ ∑4-DDT >> hexachlorobenzene (HCB) ≈ pentachlorobenzene (QCB) ≈ dieldrin ≈ ∑9-PBDE ≈ α-HBCD >> WHO-TEQ (PCDD/Fs and dl-PCBs). This order is observed in nearly all species. The ∑4-DDT concentrations were in the same range as the ∑7-PCB concentrations. Dieldrin, QCB and HCB concentrations were lower. α-HCH, β-HCH, γ-HCH, HCBD, aldrin, telodrin, isodrin, endrin, o,p'-DDE, o,p'-DDD, α-endosulfan, cis-heptachlorepoxide and trans-heptachlorepoxide were below the LOD in all samples (or detected infrequently) and were therefore not included in Figure 4.10. Median ∑9-PBDE concentrations range from 12 pg/g ww (tilapia) to 1164 pg/g ww (salmon). The following PBDEs were not detected in any of the samples or only at low frequency (BDEs 17, 66, 71, 77, 85, 119, 126, 138, 156, 184, 191, 196 and 197) and were therefore not included in Figure 4.10. ∑9-PBDE accounted for approx. 90% of the sum of all analysed congeners. BDE 209 was observed in many samples and was the predominant congener in shrimp and most pangasius samples. The predominance of BDE 209 in farmed fish has not been shown before. The PBDE congener profile will be discussed later.

HBCD was detected in 16 samples ranging from 6 to 1200 pg/g ww for the sum of the 3 diastereomers. In all cases, α-HBCD was the predominant diastereomer, which is commonly observed in fish samples (*18*). β- and γ-HBCD were hardly detected in any of the samples, and are therefore not presented in Figure 4.10. No α-HBCD was detected in all tilapia, most pangasius, some shrimp and salmon from Chile (LODs ranging from 10 (pangasius) to 100 pg/g ww (salmon)). The Dutch shrimp sample had a surprisingly high α-HBCD concentration (710 pg/g ww), as well as β-HBCD concentration (520 pg/g). This was not observed in any of the other fish or shrimp samples and it is unclear what could have caused this. These shrimp are farmed in a closed recirculation system rather than in natural ponds as for the Asian shrimp samples. Possibly, contaminants arise from construction materials of that recirculation system. In addition, ingredients for their feed may originate from other sources than of the Asian shrimp feed.

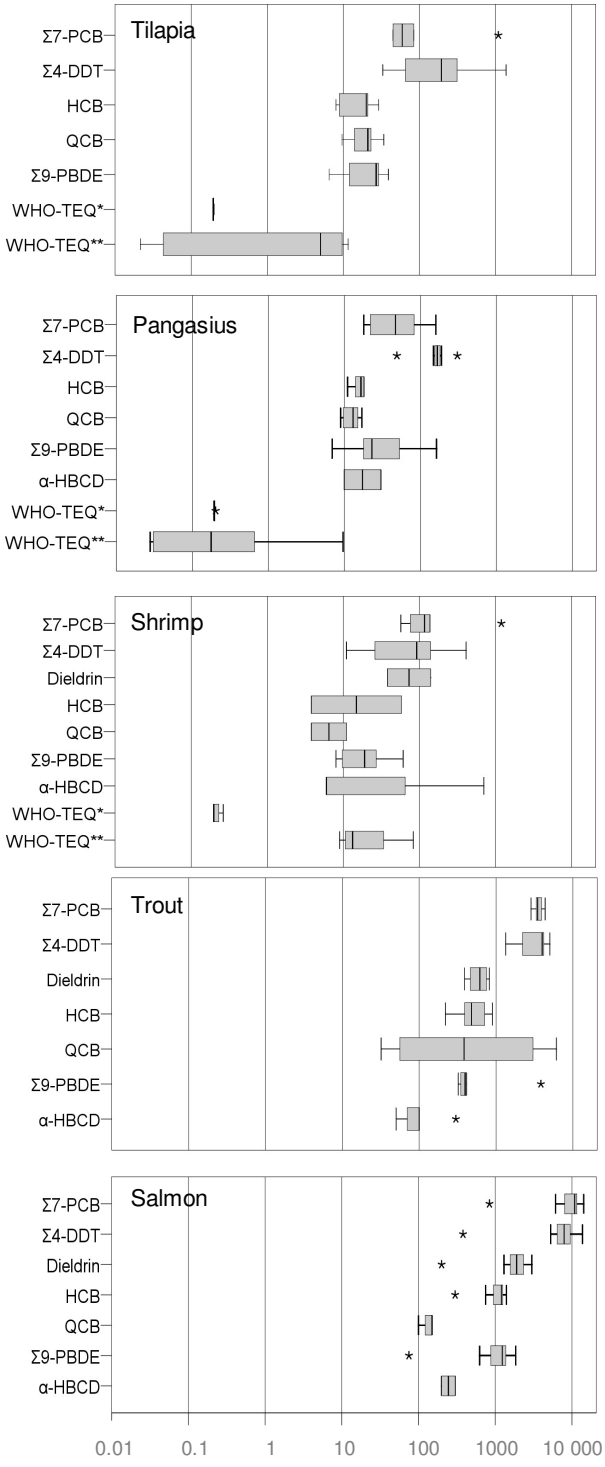

Figure 4.10
Contaminant concentrations in farmed fish and shrimp. Data for Σ7-PCB, Σ4-DDT, dieldrin, HCB, QCB, Σ9-PBDE, and α-HBCD is presented in pg/g ww). WHO-TEQ data is presented as upperbound data (in pg/g ww). WHO-TEQ** data presented as lowerbound data (in fg/g ww). Σ7-PCB values represent the sum of the indicator PCBs, Σ9-PBDE values are the sum of EFSA8+ (see text for details), and Σ4-DDT values represent the sum of p,p'-DDD, p,p'-DDE, p,p'-DDT, o,p'-DDT). Vertical bars in the boxes represent the median. Outlying values are indicated in the plot by *.*

These suggestions on possible causes require further study. The PCDD/F and dl-PCB concentrations (as 2,4,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (WHO-TEQ) concentrations (*12*)) in pangasius, tilapia and shrimp were extremely low (all approx. 0.2 pg WHO-TEQ/g ww). On a lowerbound basis (as presented in Figure 4.10 as WHO-TEQ**), concentrations ranged from <1 to 82 fg WHO-TEQ/g ww, and except for e.g. OCDD, CB 77 and CB 126, nearly all congeners were <LOQ in most samples. The upperbound concentrations (WHO-TEQ*, Figure 4.10) were approx. 0.2 pg/g ww (Figure 4.10). Salmon and trout were not analysed for PCDD/Fs and dl-PCBs in this study, as a substantial amount of data shows that these species meet the EU ML of 8 pg total-TEQ/g ww.

PFCs were not detected in most of the samples at all. Out of all PFC observations (33 samples x 13 PFCs analysed), 41 values were above the LODs (Figure 4.11). When detected, PFC values ranged from 10 (e.g. PFNA in shrimp M-AS 1 (Mixed-Asia 1)) to 600 pg/g ww (PFOS in shrimp from the Netherlands, see Figure 4.11). Highest concentrations were found for PFOS. All other PFC concentrations were (much) lower. PFUnA and PFTrA were detected at higher frequencies than PFOS, which is not commonly observed. Possibly, the higher LOD for PFOS (0.2-0.8 ng/g ww) caused this uncharacteristic observation, as this may have resulted in more non-detects compared to PFUnA and PFTrA (LOD of 0.01-0.1 ng/g ww). PFUnA and PFTrA concentrations were not associated with a specific species: salmon and trout do not show the highest concentrations compared to other species (as was found for PCBs, OCPs, PBDEs and α-HBCD). The reasons of the specific accumulation patterns require further study. Accumulation of PFCs in biota may be comparable to that of short and medium chain length fatty acids (*19*), being different from the accumulation of neutral lipophilic contaminants such as PCBs.

Table 4.13 Lipid contents (%) of the samples.

	Salmon		Trout		Tilapia		Shrimp		Pangasius	
	Origin*	**Lipids**	**Origin**	**Lipids**	**Origin**	**Lipids**	**Origin**	**Lipids**	**Origin**	**Lipids**
	CL	9.5	DK 1	5.5	CN 1	2.5	BG 1	1.3	VN 1	3.5
	NO 1	14.5	DK 2	6.2	CN 2	3.3	BG 2	0.5	VN 2	2.5
	NO 2	17.3	DK 3	7.3	EC	3.5	M-AS 1	1.5	VN 3	1.2
	NO 3	20.1	IT	8.5	ID 1	1.7	M-AS 2	0.7	VN 4	1.3
	NO 4	12.3	TR	5.3	ID 2	1.9	M-AS 3	1.2	VN 5	1.7
	UK 1	11.5	-	-	NL	5.2	NL	1.8	VN 6	1.3
	UK 2	16.5	-	-	N.r.	3.3	-	-	VN 7	5.8**
Mean		14.5		6.6		3.1		1.2		1.9
St. dev.		3.7		1.4		1.2		0.5		0.9

N.r. Not recorded

* *See Table 4.11 for country codes*

** *This sample was treated with a marinade (94% fish, 6% marinade). The marinade primarily consisted of vegetable oil. This explains the higher lipid level as compared to the other pangasius samples. Therefore, this sample is not included in the calculation of the mean and standard deviation.*

Comparison to other (farmed) fish

The ∑7-PCB and OCP concentrations in salmon in this study are at the lower end of the range reported by Hites et al. (*2*). The Hites et al. results were based on the sum of 197 congeners. The ∑7-PCBs account for approx. 30-35% of the sum of 197 congeners. Accounting for this difference, the PCB results in our study are comparable to those of Hites et al. (*2*). Also in agreement with the Hites study, PCB and OCP concentrations in salmon from Chile were lower (10 to 20-fold) than those from Europe (see Figure 4.10, the low outlying values in the salmon dataset). The ∑7-PCB and OCP results are lower than those reported by Jacobs et al.(*4*). Orban et al. (*20*) recently reported the first data on PCBs and OCPs in two pangasius samples. Although they reported many <LOQ values, the PCB and DDT concentrations of the lowest contaminated sample were similar to our results. The concentrations in the other sample were higher (820 pg/g ww for sum PCBs and 2610 pg/g ww for the sum of DDTs) (*20*).

The PBDE concentrations in salmon in the present study are at the low end of those reported earlier for European salmon (*4,21*). The PBDE concentrations in trout of the present study were much lower than those reported in farmed trout by Zenneg et al. (740-1300 pg/g ww) (*3*), except for the trout sample from Turkey that had a high BDE 209 concentration (3500 pg/g ww, see outlying ∑9-PBDE value in Figure 4.10 and for discussion the PBDE profile section).

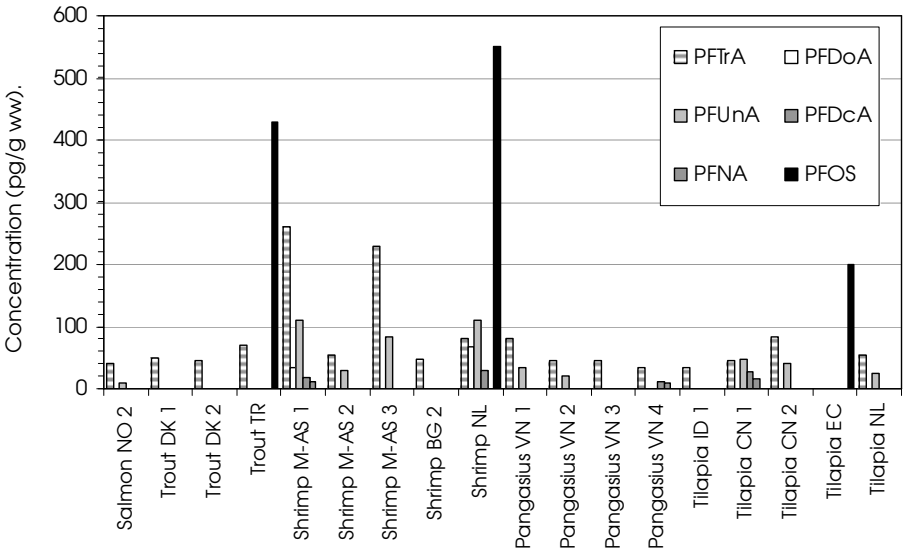

Figure 4.11 PFCs in farmed fish and shrimp. Country codes: NO = Norway, DK = Denmark, TR = Turkey, M-AS = Asia, mixed origins, BG = Bangladesh, NL = Netherlands, VN = Vietnam, EC = Ecuador, ID = Indonesia and CN = China. In case no bars are shown, concentrations were below LOD.

In an earlier Dutch study, total-HBCD was analysed in two farmed salmon samples (by GC-ECNI-MS). In that study Norwegian salmon showed lower concentrations, whereas the Scottish sample had approx. 3-fold higher HBCD concentrations (1.3 ng/g ww) (*22*). To our knowledge, no information has been published to date on the α-HBCD contamination of farmed shrimp, trout, tilapia and pangasius.
Apart from farmed fish, the Dutch population consumes wild fish and shellfish like herring, cod, sole, pike-perch, mussels and shrimp. WHO-TEQ concentrations in farmed fish are lower than those earlier reported by van Leeuwen and de Boer (*22*) in Dutch wild marine and freshwater fish (1.5-fold lower than cod and coalfish to 260-fold lower than wild eel). This is even more pronounced when the concentrations of farmed fish are expressed in lowerbound basis (see Figure 4.10). The concentrations of ∑7-PCBs in that earlier study (*23*) ranged from 0.2 (shrimp) to 1739 ng/g ww. The farmed fish samples in this study are at the low end of that range. The same holds for PBDEs. In an earlier Dutch study on wild fish (*22*) the concentrations ranged from 0.1 (haddock, mussels) to 149 ng/g ww (eel) for the sum of 7 BDEs, whereas the concentrations in the current study are at the low end of that range (see Figure 4.10).
HBCD concentrations in the present study (0.006 (shrimp) to 1.2 ng/g ww (shrimp) for the sum of 3 diastereomers) are at the low endof those in wild fish (0.2 (coalfish, mussels) to 230 (wild eel) ng/g ww) (*22*). An earlier survey on PFCs in wild Dutch fish showed PFOS concentrations of 5.9 – 150 ng/g ww (eel and pike-perch) and <1 to 51 ng/g ww in marine fish (*24*), which are all substantially higher than the concentrations in the present study. Similar to the present study, PFNA, PFUnA and PFDoA were also detected in Dutch wild fish (livers) (*24*).
Summarising, the PBDE, WHO-TEQ, PCB and OCP concentrations for salmon and trout in this study are generally lower than those reported in earlier studies. In addition, contaminant concentrations in farmed shrimp, pangasius and tilapia were lower than in wild fish, whereas farmed salmon and trout were higher than lean wild marine fish.

PBDE profiles

BDE 47 was predominant in salmon, trout and tilapia. BDEs 28, 49 (salmon and trout), 99, 100, 153 and 154 were also detected frequently. Meng et al. found a similar PBDE profile in farmed tilapia from China (*25*). BDE 209 was detected in a limited number of salmon and trout samples (4 out of 12). In all tilapia samples BDE 209 was below LOD. The most remarkable observations were the BDE 209 frequent detection (in 12 out of 13 shrimp and pangasius samples) and the relative high BDE 209 contribution in shrimp and pangasius (56 and 50 %, respectively). Such a high BDE 209 contribution has not been reported in fish before. One trout sample showed a high BDE 209 concentration (3500 pg/g ww) compared to other trout samples in which BDE 209 was close to the

LOD (<19 to 22 pg/g ww). Re-analysis of the sample (starting from sample extraction onwards) confirmed the high concentration. The BDE 209 concentration was uncharacteristically high compared to BDE 47, 99 or 100. This high observation was excluded from Figure 4.12 as it would have strongly affected the profile. The trout sample is nearly equal to the salmon profile, possibly because salmon and trout are closely related species (both belonging to the Salmoninae subfamily).

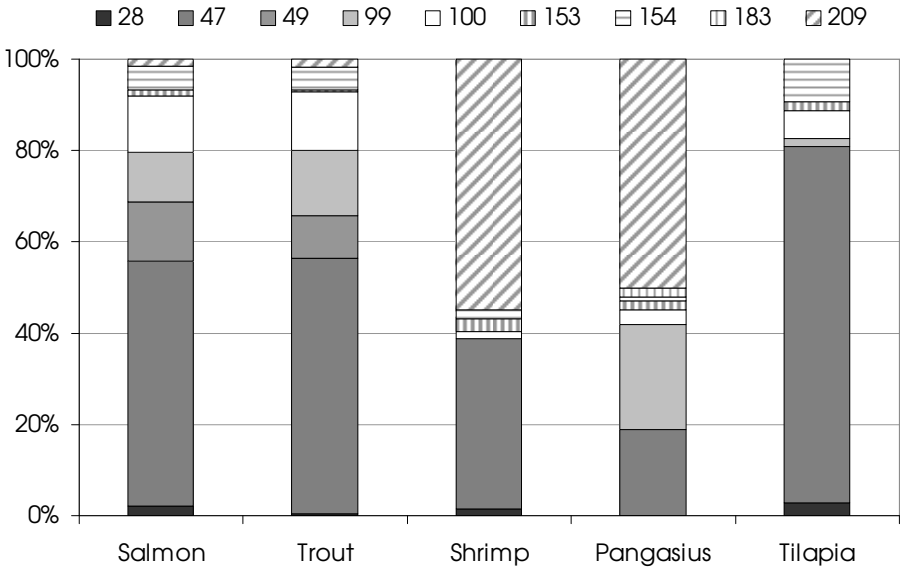

Figure 4.12 ∑9-PBDE profiles. The profile is based on mean lowerbound concentrations of each species. The ∑9-BDE concentrations were 1045 (salmon), 333 (trout), 21 (shrimp), 50 (pangasius) and 16 (tilapia) pg/g ww. The outlying high value of BDE 209 in trout from Turkey was left out to prevent an uncharacteristically high BDE 209 contribution to the PBDE profile of trout (see text).

It is not clear what caused the contamination of shrimp, pangasius and the trout sample from Turkey with BDE 209. Ashizuka et al. (*26*) recently also reported BDE 209 as the predominant congener in two out of three wild shrimp samples. They speculated that BDE 209 in particulate matter present in the digestive tract could have caused these elevated BDE 209 concentrations. Possibly, this plays a role in this study as well as the digestive tracts were (whenever present) not removed from the shrimp. Shrimp are generally farmed in ponds (except the Dutch sample which is farmed in a recirculation system) and particulate matter may have been present in the digestive tract. The reason of the predominance of BDE 209 in pangasius is unknown. No other data on PBDEs in pangasius is available in literature. In a recent study on halogenated contaminants in compound fish feed from

China by Guo et al. *(27)*, BDE 209 was detected in nearly all compound feeds analysed. In fact, BDE 209 (together with BDE 196 and 206) contributed 75% (mean) to the sum of BDEs. This may be an explanation for the predominance of BDE 209 in several samples in the current study. On the other hand, BDE 209 is a high production chemical that is used in many polymer applications *(28)*, and possibly, the contamination of the samples originates from direct contact with polymer materials (or recycled polymers) during harvesting, processing, packaging and transportation. The analysis of BDE 209 is complex, and errors easily occur *(14,29)*. We have made considerable efforts to control the BDE 209 analysis (e.g. blank contribution) and to reach low detection limits. We therefore believe that the data discussed above is attributable to the fish samples themselves rather than being laboratory artefacts. Obviously, these (high) BDE 209 concentrations require further study, e.g. by analysis of BDE 209 in feeds, farming conditions and possible post-harvesting contamination of the samples. Another remarkable observation was the absence of BDE 99 in all shrimp samples. Voorspoels et al. suggested that wild shrimp (*Crangon crangon*) lack the possibility for BDE 99 metabolisation *(30)*. The species in the present study (Table 4.10) are different and may have contaminant metabolisation capacities, but we have found no information on his matter. BDE 183, although recommended for monitoring by EFSA because this and the earlier mentioned congeners are "predominantly found in feed, food and human samples" (*17*), was below the LODs in all samples, including salmon and trout. Possibly, BDE 183 is not bioavailable for accumulation, or may be debrominated in the intestinal tract *(31)*.

Human exposure

WHO-TEQ concentrations found in this study are far below the EU-ML of 8 pg total-TEQ/g ww (*11*). The Dutch ML for indicator PCBs in fish (40-120 ng/g ww per congener) *(32)* are easily met by the samples from this study. For the other contaminants, no EU or Dutch MLs are available.

Recent studies have shown that fish contributes 28 and 12 % to the exposure of Dutch citizens to PBDEs and PCDD/Fs and dl-PCBs *(33,34)*. In these studies both wild fish (e.g. herring, eel, plaice, sole and cod) and farmed fish (i.e. salmon) were included. Herring, salmon and eel showed the highest contaminant concentrations. Herring is a popular fish in The Netherlands and is an important contributor to contaminant exposure. However, in this study, we focus on the top-five of farmed fish consumed in The Netherlands. With new farmed species being consumed in increasing amounts, one might expect a change of the human exposure to contaminants. We have investigated this for WHO-TEQs, ∑7-PCBs, OCPs, ∑9-PBDEs and α-HBCD from the five species in this study.

Preferably, one uses food consumption survey data to determine the contaminant exposure. However, the data covering the Dutch population originates from 1997/1998 *(35)* and does not include consumption data of the

new species like pangasius and tilapia. Therefore, we used the estimates of a recent inventory on Dutch sales volumes per species as sold in supermarkets, weekly markets, fish shops, etcetera (Table 4.10) (*7*). For pangasius, tilapia and salmon, this concerns almost exclusively edible parts. Trout is partly sold as whole fish (degutted). In order to arrive at the estimate for the edible parts, it was assumed 30% of whole trout concerned fillets. The sales volumes from Table 4.10 were corrected for this. For shrimp, the edible parts were assumed to be 50%. We summarized the concentrations of WHO-TEQs, PCBs, OCPs, PBDEs and α-HBCD per fish sample (sum concentration), and calculated the means per species. By multiplying these mean sum concentrations with the (corrected) sales volumes per species (Figure 4.13), an estimate of the human exposure is obtained per species. Salmon dominates the contaminant exposure from the investigated species (approx. 97%, data not shown), whereas trout, tilapia, pangasius and shrimp together contribute approx 3%. This is caused by: (i) salmon is (still) consumed in highest quantities and (ii) the contaminant concentrations in salmon are much higher, approx. 100-fold compared to pangasius, tilapia and shrimp, see Figure 4.13).

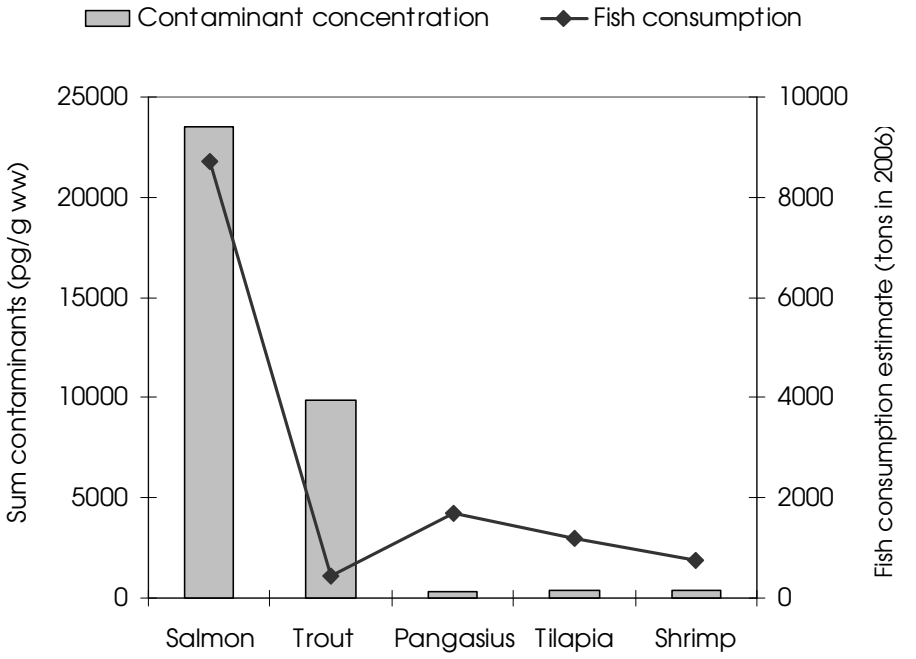

Figure 4.13 Total contaminant concentrations (WHO-TEQ, PCBs, OCPs, PBDEs and α-HBCD) in relation to the Dutch sales volumes of salmon, trout, pangasius, tilapia and shrimp (data of 2006). The sales volumes of trout and shrimp were corrected for non-edible parts.

This is only a first estimation of the relative importance of contaminant exposure originating from new species like pangasius and tilapia, but it clearly shows that the contribution from these species to the exposure is still minimal, compared to farmed salmon. Obviously, these results should also be viewed in relation to the degree of contamination and consumption of wild fish, and further investigations are needed to determine the relative importance the different fish. In addition, cooking fish can decrease contaminant concentrations by a few to >50% *(36-38)* depending on fish species, type of contaminant, cooking conditions etc. This may lead to lower exposure to these contaminants.

Acknowledgements

The Dutch Ministry for Agriculture, Nature Management and Food Quality and the Food and Consumer Product Safety Authority (VWA, dr. M.J.B. Mengelers) are acknowledged for funding and guiding this study. Dr M. van Dongen (Innotact Consulting) and Mrs. W.M.C. van Leeuwen are thanked for their assistance in fish sampling. Mrs. S. So is acknowledged for fish and shrimp sample pre-treatment. Happy Shrimp (mr. J. Munt) is thanked for the generous gift of sample material. Wellington Laboratories are acknowledged for the generous gift of the mass labelled PFOS.

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4.5 Estimation of human exposure to halogenated contaminants from wild and farmed fish, shellfish and shrimp

Abstract

Fish and fish products contribute substantially to the human exposure to organohalogenated contaminants. In recent years, several organohalogen contaminant surveys on wild and farmed fish consumed in The Netherlands were carried out. This resulted in a substantial amount of data on the concentrations of polychlorinated dibenzo-*p*-dioxins and –furans (PCDD/Fs), polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), p,p'-DDT, -DDD and -DDE, polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and perfluorooctane sulfonate (PFOS). These datasets are integrated and combined contaminant exposures are estimated. The objective of this study was to specify (i) the main contributing fish species to the human exposure in The Netherlands; (ii) the main contributing contaminants, and (iii) to determine the contribution of recently introduced farmed species like pangasius and tilapia. The exposure is dominated by PCBs (sum of seven indicator congeners) and PFOS. PFOS shows a distinct exposure pattern as compared to the other contaminants (e.g. because there is no contribution from salmon). From a species point of view, herring and farmed salmon are the main contributors to the contaminant exposure from fish, followed by cod, plaice and mussels. The contribution of farmed tilapia, pangasius and shrimp was very low (<1% for all species).

Introduction

The human exposure to organohalogenated contaminants has been the subject of several studies. Often individual contaminant groups (e.g. polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/Fs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs)) were studied, but in some cases multiple contaminant groups were investigated in food basket studies and studies on individual food items (e.g. shellfish). Many exposure studies point towards fish and shellfish as the important sources for human exposure to contaminants (*1,2*). In The Netherlands, dairy is the predominant source for exposure to PBDEs and PCDD/Fs and dioxin-like PCBs (dl-PCBs) because dairy consumption is high (*3,4*). Fish is also an important source of exposure. However, in spite of the presence of these contaminants, fish consumption is promoted because it contains selenium and unsaturated (omega-3 and 6) fatty acids which are believed to be beneficial for human health (*5,6*).

The fish and shellfish we consume is a very heterogeneous group. They originate from different waters (e.g. freshwater, brackish, marine), have different positions in an aquatic food chain (pelagic or beenthic prey or predator) or may be farmed. Fish feeds may contain high amounts of marine proteins and lipids, or may consist of vegetable ingredients. All these variables determine to what extent fish is contaminated with organohalogenated contaminants. In several studies, exposure assessments were carried out on single contaminants (e.g PBDEs or hexachlorobenzene (HCB) or PCDD/Fs and dl-PCBs (*2,4,7,8*), or a few contaminant groups (*1,9*). In a Swedish study the exposure from fish decreased in the following order: PCBs (349 ng/day), DDTs (256 ng/day), chlordanes (87 ng/day), HCB (36 ng/day), hexachlorocyclohexane (35 ng/day), PBDEs (23 ng/day) and total-TEQ (31 pg/day) (*1*). These exposure calculations were based on 13 fish samples. In the present study the dietary exposure from fish to multiple contaminant groups (i.e. PCDD/Fs, PCBs, HCB, DDTS, PBDEs, hexabromocyclododecane (HBCD) and perfluorooctane sulfonate (PFOS)) was estimated for The Netherlands. In recent Dutch surveys and monitoring studies edible wild fish species such as eel, herring, mussels, shrimp, cod, plaice, tuna, pike-perch and sole and several farmed species including salmon, eel, trout, pangasius, tilapia and shrimp were investigated for the above mentioned contaminants. The availability of these data allows an integrated exposure analysis of several contaminants based on a diverse group of fish species.

Materials and methods

The human exposure to contaminants is determined by combining information on contaminant concentrations in fish with information on the consumed quantities of fish. The contaminant data was taken from several studies (see Table 4.14). In most studies the same species were analysed. Therefore, we have collected data for all contaminants in cod and related species (*Gadidae* family), plaice, sole, wild eel and farmed eel, mussels, wild and farmed shrimp, herring, mackerel, sole, plaice, salmon, trout, pangasius and tilapia. Pike-perch and tuna data were not available for all contaminants and were therefore left out in this study. An overview of the contaminants studied is presented in Table 4.14. As many contaminant concentrations were rather low, detection and quantification limits (LOD and LOQ, respectively) could have a substantial influence on the final result. Therefore, the selection of contaminants in this study was limited to those of which considerable numbers of concentrations were above the LOD/LOQ. For the OCPs, the study was limited to HCB (86% >LOD). p,p'-DDE was present in all samples (100% >LOD) and p,p'-DDT and -DDD in the majority of the samples. The selection of the other contaminants was made in a similar way. Additional criteria were applied for PCDD/Fs, PCBs, PBDEs and PFOS (see Table 4.14). This study was limited to the edible parts (i.e. data on livers was excluded). Data on Western Scheldt fish were not considered because only very little fish from

that region is consumed, whereas contaminant concentrations are high due to local pollution. This would have led to an unrealistically high contribution of fish from this area to the contaminant exposure (i.e. PFOS, PBDEs and HBCD).

Table 4.14 Overview of contaminants included in this study.

Contaminant class	Individual compounds included	Rationale for selection and source of data
PCDD/Fs and dl-PCBs	All 17 WHO PCDD/Fs and 12 WHO dl-PCBs	TEF[1] values and TDI[2] values available based on these congeners (*10*). Source: (*11,12*).
PCBs	CB 28, 52, 101, 118, 138, 153 and 180 (7 indicator PCBs)	Selection commonly reported. Source: (*11,12*).
HCB	N.a.	In 86% of the samples HCB >LOD. Source: (*12,13*).
DDTs	Sum of p,p'-DDT, p,p'-DDD and p,p'DDE	In 100% of the samples the sum-DDTs >LOD. Source: (*12,13*).
PBDEs	BDE 28, 47, 49, 99, 100, 153, 154 and 209	Eight congeners selected by EFSA[3] for monitoring (*14*). BDE 183 was excluded because nearly all samples were <LOD. On the contrary, BDE 49 was included because in nearly all samples >LOD. Source: (*12,15*).
HBCD	α-HBCD	A large proportion of samples (47%) >LOD. β-HBCD and γ-HBCD were excluded because in nearly all samples <LOD. Source: (*12,15*).
Perfluorinated compounds	Perfluorooctane sulfonate (PFOS)	Emerging contaminant and recent risk assessment by EFSA (*16*). A large proportion of samples (39%) >LOD. Other PFCs were <LOD in nearly all samples. Source: (*12,17,18*).

N.a Not applicable

1 *TEF: 2,3,7,8-Tetrachlorodibenzodioxin Equivalency Factor*

2 *TDI: Tolerable daily intake*

3 *EFSA: European Food Safety Authority*

The daily food consumption data was taken from the Dutch National Food Consumption Survey (DNFCS), which specifies the diet of 6,250 individuals (male, female, age 1-97) over two consecutive days (*19*). The DNFCS was performed in 1998, and does not include recently introduced species like pangasius and tilapia. In order to come to a reasonable inclusion of these species, sales volumes of these fishes were taken from a recent inventory (*20*). Based on interviews with commercial fisheries representatives, an estimate was made of the volumes of these fish being sold in The Netherlands to consumers in 2006. Taking into account the number of Dutch citizens (assumed 16,000,000), these data can be transferred into daily consumption data (in g/day). For trout, a correction of this consumption was required as the majority of trout is sold as whole fish (degutted), whereas only the fillets are consumed. It was assumed that the fillets represent 30% of the eight of a whole degutted fish, and therefore a 0.3 correction factor was applied. For farmed shrimp, a 0.5 correction factor was applied as shrimp are often sold with non-edible parts (e.g. tails, heads, and legs) still on (*20*). It is recognised that the DNFCS data and the fish sales data are obtained through different methods and represent fish *consumption* (DNFCS) or fish *sales* to consumers

(*20*). In the latter case, this concerns mostly raw (uncooked) products, and this may differ somewhat from the consumed products. Furthermore, both studies are from different periods (1998 vs. 2006) and changes in consumption patterns may not be represented very well. However, these differences were accepted considering the aim to compare contaminant exposure from newly farmed species (tilapia, pangasius) and wild species (e.g. herring, eel and plaice).
The contaminant data of all species of the same *Gadidae* family were combined in one dataset. This included cod, haddock, pollock and hake. These have similar feeding habits and their contaminant levels were similar. Also the consumption data on *Gadidae* were combined. Consumption data on processed products like e.g. fish fingers, 'lekkerbekje' and 'kibbeling' (battered and fried *Gadidae*) were added to the *Gadidae* category.
Two categories of shrimp were distinguished, i.e. farmed shrimp (*Penaeus monoden, Penaeus vannamei, Litopenaeus vannamei*) and wild shrimp (*Crangon crangon*). For farmed shrimp, consumption data of the 2006 sales inventory was used, whereas for wild shrimp (mainly North Sea), the DNFCS consumption data was used. The DNFCS data of wild shrimp was corrected by a factor 0.2 as farmed shrimp have become much more popular in recent years than wild shrimp. All contaminant concentrations are expressed on a wet weight (ww) basis as the objective of the study is to study the dietary exposure and to enable comparison with e.g. TDI values.
The exposure calculation is based on the daily fish consumption multiplied by the mean lowerbound concentrations (per species) as in $DE=\Sigma(Q_a \cdot C_a+Q_b \cdot C_b+\ldots.Q_z \cdot C_z)$, in which DE = Daily Exposure, Q = Quantity of daily consumption of fish species a, b etc. and C = mean lowerbound contaminant concentration in fish species a, b etc.. The mean contaminant concentrations are based on lowerbound data, except for total-TEQ values.

Results and discussion

The bottom panel of Figure 4.14 shows that herring and salmon are the predominant contributors to the exposure of the lipophilic contaminants like PCDD/Fs, PCBs, DDTs, HCB, PBDEs and HBCD. This is caused by the relative high daily consumption (Figure 4.14, top panel) combined with the relatively high contaminant levels in these species (Figure 4.14, middle panel). Wild eel has high contaminant levels, but due to the low consumption, the contribution to the exposure is minimal. The farmed species tilapia and pangasius had very low exposure levels because of their low contaminant concentrations, being approx. 100-fold lower (mean) than those in e.g. salmon and herring (Figure 4.14, top). Cod (and other *Gadidae*) show concentrations in between salmon/herring and sole/ plaice/ pangasius etc.

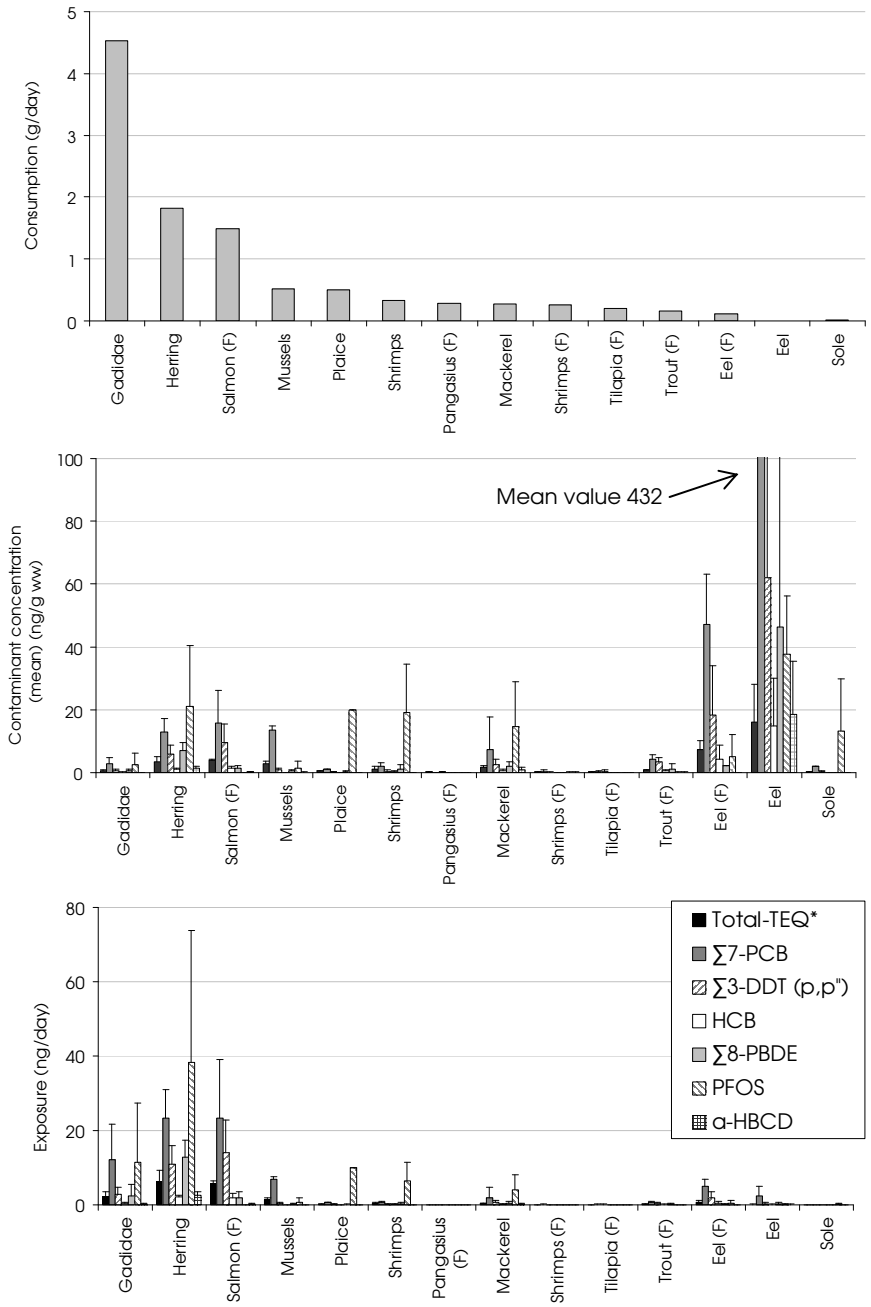

*Figure 4.14 Fish consumption / sales (top panel), the mean contaminant concentrations (middle panel), and mean dietary exposure (third panel). *Data for Total-TEQ is expressed in pg TEQ/g ww (second panel) and pg TEQ / day (bottom panel). The legend in the bottom panel also applies to the middle panel.*

The contaminant concentrations were low but the exposure from *Gadidae* is nevertheless substantial (i.e. for PCBs and PFOS) due to the high *Gadidae* consumption (both from fish fillet and popular processed fish such as fish fingers, "kibbeling" and "lekkerbekje"). Exposure from wild and farmed shrimp and from mussels is low (except for PFOS in wild shrimp).
PFOS shows a different exposure pattern. PFOS is a surfactant and because it mostly occurs in the ionic state, it has a different accumulation mechanism compared to the neutral lipophilic contaminants (*21*). PFOS was not found in any of the salmon samples (<0.6 ng/g ww). This is somewhat surprising as salmons are fed with feeds containing fish meal and fish oil. There is no information on PFOS levels in fish meal, nor in fish oil. Herring stands out for the high contribution to the PFOS exposure. Feeding habits may play a role. Herring feeds predominantly on zooplankton and in other studies it was shown that zooplankton contains PFOS in the low ng/g ww range (*22*). Finally, plaice stands out for the relatively high PFOS exposure. This is caused by the relatively high PFOS concentrations (higher than lipophilic compounds), in combination with the consumption data. Sole showed PFOS concentrations comparable to those in plaice, which is explained by similar feeding habits of these species, but due to a lower consumption, the exposure contribution of sole is low.
The data is presented as lowerbound data (except for PCDD/Fs and dl-PCBs), in order to minimise the role of LODs on the result. Upperbound data did not differ much (data not presented), because only contaminants were selected for which a large proportion of the samples had >LOD concentrations. Means of all contaminant concentrations per species were calculated. For comparison, the median was also calculated, but the results showed no large differences (data not shown).
The total exposure per contaminant (Figure 4.15) shows that the seven indicator PCBs and PFOS represent approx. 70% of the exposure to all contaminants. The exposure of the general Dutch citizens (i.e. exposure averaged over all Dutch citizens, including non-consumers) to the seven indicator-PCBs was 77 ng/day (1.1 ng/kg bw per day). No TDI exists for the indicator PCBs. A re-evaluation of the toxicity of non dioxin-like PCBs (ndl-PCBs) is currently ongoing in the framework of the EU project Athon (*23*). The ndl-PCB concentrations in all fish samples were (far) below the current Dutch maximum level (ML) (see (*11*)). A Swedish study showed higher PCB exposures (349 ng/day, sum of 23 PCBs, 79 ng/day for CB 153 only) (*1*).
The PFOS exposure is 72 ng/day (1.0 ng PFOS/kg bw per day). The EFSA recently published a human risk evaluation of PFOS. They determined a TDI of 150 ng PFOS/kg bw per day (*16*). In that study, an exposure of 58 ng PFOS/kg bw per day was determined for the Dutch population (from fish). This is considerably higher than found in the present study because EFSA (i) used much higher PFOS concentrations in fish (68 ng/g ww for all fish) and (ii) they used *consumer only* fish consumption data, meaning a 5-fold overestimation of the fish consumption compared to the general Dutch population. A more

thorough discussion on the causes of these differences can be found in Chapter 4.3. Based on the exposure estimated in the present study, it is assumed that the average consumer will not exceed the PFOS-TDI from fish consumption only. Data on other food items should also be considered, but data is still lacking. Within the framework of the EU Perfood project (2009-2012) data will be produced EU-wide that will enable an exposure assessment including several food items, drinking water, beverages and other exposure pathways.

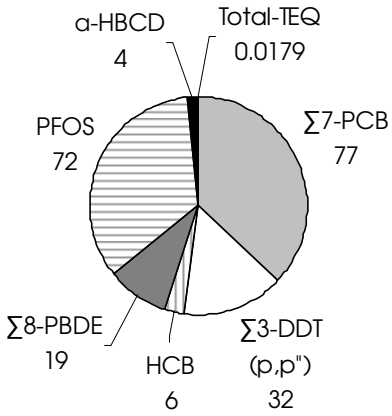

Figure 4.15
Summarised exposure to different contaminants (ng/day) from all fish, and relative importance of different contaminant groups. Legenda: Σ denotes sum (e.g. Σ7-PCB means the sum of 7 PCB congeners).

The exposure to DDTs in this study is lower than in Sweden (256 ng/day for the sum of four DDTs, 164 ng/day for p,p'-DDE) (*1*). The dietary exposure to DDT (sum of 6 isomers) of secondary school students in Hong Kong amounted 145-291 ng/kg bw per day and was predominantly caused by seafood consumption (*24*). This is much higher than found in the Swedish study and in this study.

The exposure to PBDEs is lower than in Sweden (fish only, 23 ng/day for 5 PBDEs, 17 ng/day for BDE 47) (*1*). The exposure for a 70 kg man from Catalonia, Spain was estimated at 27 ng/day (fish only, sum of six PBDEs) (*2*). In both studies, fish was the predominant contributor to the exposure.

The exposure to α-HBCD is low (4 ng/day) and far below benchmark doses (1.6-3.0 mg/kg bw per day) obtained from toxicity evaluations (*25,26*).

The mean total-TEQ was 18 pg/day, which corresponds to an exposure of 0.26 pg/kg bw per day. This is in the same order of magnitude as reported by de Mul et al. (*4*). This contribution is well below the TDI of 2 pg/kg bw per day as set by the Scientific Committee on Food (*27*). When other foods are included, the exposure approaches the TDI (0.8 pg/kg bw per day) (*4*). The exposure used in this study is lower than reported by Darnerud et al. (2006) for Sweden (30 pg/day) (*1*).

The HCB exposure is well below the suggested guidance value of 160 ng/kg bw per day (*28*) and was 6-fold lower than in Sweden (*1*).

Conclusions

Herring and salmon are the most important fish species that contribute to the exposure of Dutch consumers when considering all contaminants studied. The exposure resulting from wild and farmed shrimp, sole and trout and the newly farmed species pangasius and tilapia was low.

The exposure was dominated by PFOS and the sum of the 7 indicator PCBs (approx. 70% of the total). PFOS showed a deviating exposure pattern mainly because no exposure through salmon was observed, whereas salmon is an important contributor for other contaminants. PFOS exposure through plaice was substantial, whereas plaice was not relevant for the other contaminants. Exposure to HCB and α-HBCD was low due to low contaminant concentrations in all fish species.

The exposure to dioxins and dl-PCBs was the low (18 pg total-TEQ/day, or 0.26 pg total-TEQ/kg bw per day). Nevertheless, the exposure was only 8-fold below the TDI, which is a small margin. Safety margins were higher for the other contaminants.

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Chapter 5

Conclusions and future perspectives

Conclusions and future perspectives

In the last decade, considerable public attention was spent on the safety of our food. A major concern was the presence of environmental contaminants in food. In 1999 the Belgian dioxin crisis (which in fact was a polychlorinated biphenyl (PCB) crisis) showed the world that contaminants might still be present in our food at threatening levels. This crisis showed the vulnerability of our food supply when contaminants accidentally or by means of illegal practices enter the food supply chains. Other dioxin and PCB crises have occurred such as the dioxin contamination of butter and milk related to contaminated citrus pulp (1997-1998) (*1*), dioxin contaminated mozzarella from Campania, Italy (2008) (*2*) and the PCB contamination of Irish pork meat which entered the human food chain (2008). With food chains becoming more complex due to the globalisation of the production and a more intensive transportation of foods, food safety problems have become a global issue. This was clearly demonstrated in the autumn of 2008 when illegal practices of Chinese dairy producers resulted in an extensive melamine contamination (*3,4*). On a Chinese national level the melamine contamination of baby foods resulted in several deaths. In addition, contaminated ingredients were sold worldwide and used in a wide range of foods. As a result, considerable food recall actions were initiated resulting in substantial economic loss and consumer deception.

These crises show that food safety remains an important issue and continuous attention is needed to secure it. A food safety crisis characteristically runs over a relatively short period of time but results in elevated contaminant concentrations in food that approach or sometimes even exceed health safety limits. Some contaminants are continuously present in food. For example, mycotoxins can be produced by e.g. molds and yeasts in vegetable products (e.g. fruits, vegetables, cereals, nuts) during production, storage and transportation. In such cases, continuous monitoring of these products and ingredients is needed. The same holds for the contamination of foods with halogenated contaminants such as DDT, lindane and PCBs. The production of these compounds started before World War II. The production and use have been terminated after adverse effects were discovered. Following this phase out, the release to the environment decreased to a large extent. This is visible from the levels in the environment: long-term trends show a substantial decrease of concentrations (e.g. PCBs, DDT, lindane) in e.g. fish (*5-7*). Exposure to e.g. dioxins and dl-PCBs through food has also decreased (*8*). However, new environmental contaminants were discovered in the last 10-15 years such as brominated flame retardants (BFRs), including polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) (*9-11*). Furthermore, perfluorinated compounds (PFCs) were discovered as environmental contaminants in the early 2000's (*12-15*). Information on human exposure to these contaminants through food consumption is scarce. In addition, e.g. house dust (BFRs and PFCs) (*16-19*),

drinking water (PFCs) (*20-22*) food cookware materials (PFCs) (*23,24*) and food packaging (PFCs) (*24-26*) were identified as routes that can also contribute to human BFR and PFC exposure.
The risks of exposure to environmental contaminants are evaluated in risk assessment processes. One important aspect of this process is the exposure assessment, which requires information of food consumption as well as the concentration of contaminants in foods. The generation of reliable food contaminant data requires the development of methods being capable to deliver accurate and precise data at the low concentration levels observed in foods.

Analytical method development

In the last decade, considerable developments have taken place on the analysis of halogenated contaminants. The PFCs were discovered as environmental pollutants and they are present in edible fish (*27,28*). Initial methods for fish and other biota relied on ion pair extraction (IPE), followed by detection with liquid chromatography (LC) coupled with electrospray ionisation (ESI) and tandem mass spectrometry (MS/MS) (*29,30*). No clean-up of extracts was applied. In addition, the quality of native and internal standards was very poor (*29*). This led to poor accuracies of the reported data, which was reflected by a poor performance in the 1st world-wide interlaboratory study (ILS). This situation improved largely with (i) the availability of a wide range of high quality native standards, (ii) the availability of many mass labelled standards, especially for important PFCs such as PFOS and PFOA and (iii) the improved knowledge on the physico-chemical behaviour of these compounds, which aided analytical chemists in developing their methods. In the 2nd ILS, large improvements were observed (Figure 5.1). Very precise and comparable results were obtained between laboratories. Even different analytical approaches provided comparable results, which underlines the progress being made in the field. It was concluded that accurate and precise analysis of PFCs in water and fish is feasible if a mass labelled analogue is used for each of the target compounds. The case of PFCs shows that the performance of the analytical community can reach maturity in 5-7 years when chemists are motivated to deliver high quality data and with good instrumentation and the support of suppliers of high quality native and mass labelled standards.
Also in the field of BFRs large developments have taken place. Many laboratories have embarked on the analysis of PBDEs. The analysis may appear similar to the PCBs and, therefore, rather straightforward, but analytical issues such as positive blanks for the major congeners (BDE 47, 99, 100 and 209) negatively affect the accuracy and precision. The analysis of BDE 209 is a challenge in itself with – apart from blank problems – possible de-

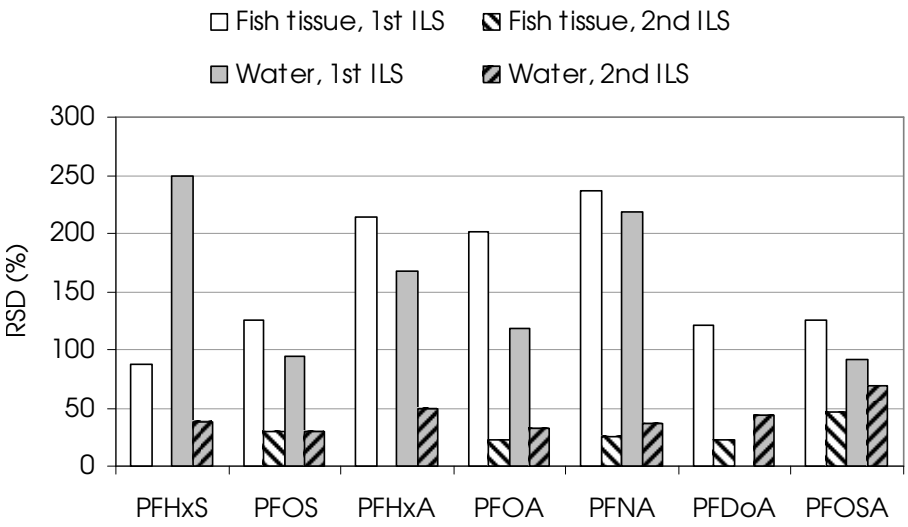

Figure 5.1 Progress being made in two world-wide PFC interlaboratory studies, judged from the relative standard deviations (RSDs) of the submitted pool of data.

gradation in the injector of the gas chromatograph (GC) and on the GC column. To complicate things further, laboratories try to analyse PBDEs, tetrabromobisphenol-A (TBBP-A), the methyl derivative of TBBP-A (me-TBBP-A) and HBCD simultaneously, from extraction to clean-up and finally in a single GC run. This is not the best approach. HBCD degrades in the GC (column and injector), which negatively affects the accuracy. In addition, degradation products interfere with important BDEs like 49 and 99, and this seriously hampers the determination of these important BDE congeners when using GC-ECNI-MS. Analysis of HBCD by LC-MS/MS overcomes these problems and has additional advantages over GC such as the separation of individual diastereomers and the use of mass labelled internal standards. A study in fish samples showed that GC delivered nearly 5-fold higher HBCD values than LC. Although research is not yet conclusive, it is believed that LC-ESI-MS/MS currently delivers highest quality data. GC-electron capture negative ionisation (ECNI)-MS detection limits are still 10-fold lower than in LC-MS/MS, but at the price of delivering inaccurate data, that cannot be considered a great advantage.

For dioxins and dl-PCBs, GC coupled with high-resolution mass spectrometry (HRMS) is the current reference method. Unfortunately, GC-HRMS equipment and maintenance is expensive, and is therefore only applied in a limited number of laboratories. Alternative detection techniques were developed, optimised and in the framework of this study, they were validated. These methods are GC-ion trap (IT)MS/MS, comprehensive multidimensional GC (GCxGC) with electron capture detection (ECD) and the CALUX bioassay. CALUX is simple and straightforward but suffered both from a positive and

negative bias and limited precision. GCxGC-ECD was precise and accurate but suffered from a time-consuming manual integration of peaks, especially at the low concentrations relevant for food. GC-ITMS/MS showed a very good performance and has the potential to become a true alternative to the current reference method GC-High Resolution MS (HRMS), provided utmost attention is paid to the maintenance (source cleaning, etc.). Commission Regulation 1883/2006 specifies performance criteria for screening methods, and all three methods meet these requirements. Apart from the final analysis, sample extraction and clean-up are time consuming as well and therefore add substantially to the costs. Integrated sample extraction and clean-up methods were developed based on accelerated solvent extraction (ASE) and combined with within-cell clean-up and fractionation of the extracts using silica to reduce sample clean-up time (*31*,*32*). The results for a herring sample were lower to those obtained by classical extraction and clean-up, showing that further development of this approach is needed. When estimating the costs per analysis, it is clear that labour costs make up half, or even more of the total costs per sample. These costs are mostly related to laborious extraction and clean-up processes and the largest improvements can be obtained there. For that reason, further efforts are needed to integrate extraction and clean-up methods, thereby resulting in a lower price per sample. Also, instrumental developments (e.g. sensitivity, specificity, and accuracy) are ongoing, and it is expected that in the next decade low cost alternatives to GC-HRMS will become available that deliver the accuracy and precision needed for this complicated analysis. On the other hand the prices of GC-HRMS instruments are dropping and these instruments will come within reach of more laboratories. Either way, it is expected that the capacity on the analysis of dioxins and dl-PCBs in food will increase considerably, which will support a stronger monitoring network globally.

Contaminant concentrations, exposure and risks

Fish is the predominant contributor to the exposure of halogenated contaminants in several European diets. However, it was not fully understood to what extent this also holds for Dutch citizens. It was also not clear which fish contributes predominantly to the Dutch exposure and to what extent newly farmed species contribute. Finally, emerging contaminants like PBDEs, HBCD and PFCs had not been investigated before and no knowledge was available on the exposure of Dutch citizens to these compounds. This study has filled several of these knowledge gaps as we have used the aforementioned analytical methods for analysis of contaminants in a wide range of fish commonly consumed in The Netherlands. The resulting data was used as input for exposure calculations.

Many of the investigated contaminants were present in the wild and farmed fish species. PFOS was present in many samples, and to a lesser extent also

the longer chain acids (perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrA). PFOA and other shorter chain PFCs were only found in a few samples. This is caused by high water-solubility of these compounds, which makes them hardly bioaccumulate. α-HBCD is the predominant BFR present in fish. The PBDE profiles are dominated by the BDEs 47, 49, 99 and 100. However, in farmed shrimp and pangasius BDE 209 was the dominant contributor. It is not clear if this is caused by a different accumulation mechanism or by contamination during harvesting, processing and packaging. PCBs and OCPs were also present in all samples, with CB 153 as the dominant congener in all samples. This shows again how persistent these compounds are and how long it takes before they disappear from our environment and food, even after a complete ban on their production and use. Several OCPs were found in the samples (DDTs, drins, HCHs, HCB etc.), but only HCB and the DDTs (p,p'-DDT, p,p'-DDD and p,p'-DDE) were found in the majority of the samples. Dioxins and dl-PCBs were also present in all samples. When comparing contaminant concentrations in wild and farmed fish, shellfish and shrimp samples, they (generally) decrease in the following order sum 7 indicator PCB > PFOS ≈ sum 3 DDTs > sum 8 PBDEs > HCB ≈ HBCD >> total-TEQ (the sum of 2,3,7,8-tetrachloro-*p*-dioxin equivalents (TEQ)). When expressing the results per fish species, these lipophilic contaminants decreased in the following order: wild eel >> farmed eel > herring ≈ salmon > mussel ≈ mackerel > others. For PFOS, the situation was slightly different: wild eel > herring > plaice ≈ wild shrimp > sole ≈ mackerel > other fish, underpinning the different bioaccumulation process of PFOS. Highest total-TEQ concentrations were found in wild eel from the Dutch river deltas (e.g. New Merwede and Hollands Diep). Eel from these locations exceeded the EC maximum limit (ML) of 12 pg TEQ/g ww. Very low concentrations were observed in farmed fish species such as the recently introduced tilapia and pangasius, and in farmed shrimp (~0.2 pg TEQ/g ww, upperbound). These concentration differences (high in wild eel, low in pangasius, tilapia and farmed shrimp) were also found for indicator PCBs, PBDEs, HBCD, DDTs and HCBs. Eel is a carnivorous species, requiring large amounts of animal proteins. Eel is a lipid-rich fish and accumulates large amounts of organic contaminants when its habitat (and therefore also the prey animals) is polluted. These contaminants are stored in the lipids. Pangasius and tilapia feed on a higher proportion of vegetable proteins and lipids and these are less contaminated, resulting in much lower contaminant concentrations. The differences between concentrations of the lowest contaminated fish (pangasius, tilapia) and the highest contaminated samples (eel) were large, spanning several orders of magnitude: 250-fold (total-TEQ, upperbound) and 50,000-fold (indicator PCBs).

These contaminants found in fish result in exposure of consumers. In order to assess the exposure, detailed data on fish consumption and fish sales (farmed shrimp, pangasius and tilapia) were combined with detailed contaminant

data. The consumption data was used as 'average consumer', meaning that the results describe the mean Dutch exposure from fish consumption. High-consumers (e.g. sports-fisherman that consume own caught fish) were not considered. The consumption of fish by Dutch consumers (mean) decreases in the following order: Gadidae (e.g. cod, haddock, fish fingers, kibbeling) > herring > salmon > mussels = plaice > other fish and shellfish. The consumption of other fish is much lower than these species. When looking at the dietary exposure to the total of contaminants investigated the order changes (Figure 5.2). Herring predominates (41%), followed by salmon (21%) and Gadidae (14%) (total 76%). The other fish species together contribute 24%. The contribution of pangasius, tilapia and farmed shrimp is less than 1%. This order only slightly changes when looking at individual contaminant groups rather than at the totals. These data are relevant for other European countries also. The newly farmed species are introduced in other countries as well and will become part of our diet. In addition, herring, salmon and cod are also popular species for other countries (e.g. Scandinavia) and will probably contribute substantially to the exposure in those countries as well.

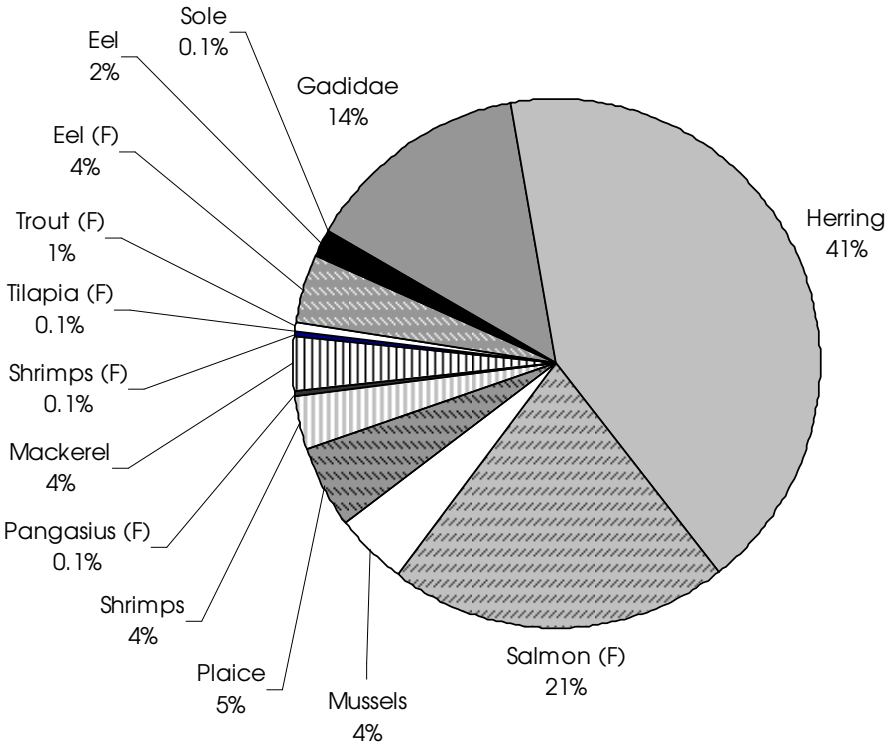

Figure 5.2 Contribution of different fish species to the dietary exposure of the sum of investigated contaminants. Farmed species are indicated by F between brackets.

The absolute exposure per contaminant group decreases in the following order: ∑indicator-PCBs (1.1 ng/kg bw per day) > PFOS (1.0 ng/kg bw per day) > ∑3DDTs (0.45 ng/kg bw per day) > ∑8PBDEs (0.27 ng/kg bw per day) > HCB (0.09 ng/kg bw per day) ≈ α-HBCD (0.06 ng/kg bw per day) >> dioxins and dl-PCBs (0.26 pg TEQ/kg bw per day). The exposure for high fish consumers is not considered, but may be substantially higher. The margin of safety (MOS) is smallest for dioxins and dl-PCBs, as there is only an 8-fold difference between the fish-related exposure and the WHO tolerable daily intake of 2 pg/kg bw per day). When also dairy, pork and other food items would be taken into account, this margin becomes even smaller. The MOS for the other contaminants are larger (e.g. 150 for PFOS, 2,400 for HCB and $3.7x10^7$ for HBCD). The MOS for PFOS as derived by EFSA was much lower (MOS of 3), which was caused by the fact that (i) EFSA used a PFOS concentration which was the average of all European data available, (ii) EFSA used a high estimate of the fish consumption (consumers only) and regarded the sum of fish without specifying per species. This resulted in a very conservative exposure estimate. In the present study, it is shown that with fish-specific consumption data and fish-specific PFOS contamination data, the exposure is much lower. This information will be helpful to risk assessors and policy makers when setting their priorities on the human exposure to PFCs.

To summarise, the present unique and detailed study created a large database on contaminant levels in many wild fish species that are important in the Dutch diet (herring, salmon, Gadidae) and in newly farmed fish species like tilapia and pangasius. This dataset allowed the determination of the relative contribution of different contaminants and different fish species to the exposure of Dutch citizens and provided the following answers: (i) the exposure is dominated by the indicator PCBs and by PFOS; (ii) herring and salmon are the predominant contributors whereas contribution from farmed shrimp, pangasius and tilapia in negligible, (iii) the exposure to dioxins and dl-PCBs (as WHO-TEQ) is lowest but due to the high toxicity of these compounds, the margins of safety are smallest, and (iv) detailed PFOS data provided a more accurate exposure estimate than the recently calculated exposure by EFSA, resulting in a substantially larger margin of safety.

Future perspectives

Analytical methods

After the ban of PCBs, history repeated itself with e.g. PBDEs, and several undesired (and adverse) side-effects were found again such as widespread environmental pollution and accumulation in aquatic and human food chains (*33*,*34*). Due to measures agreed in the Stockholm Convention, the use and emissions of the current POPs were successfully decreased. The process

of assessing potential bioaccumulation, persistency, adverse effects and long range transportation in the framework of the Stockholm convention continues. PBDEs, HBCD and PFOS were proposed for inclusion in the convention. In addition, the EU REACH program (Registration, Evaluation, Authorisation and restriction of Chemicals) is initiated for registration and evaluation (and ultimately authorisation) of chemicals in the EU (http://echa.europa.eu/). One of the aims is to prevent the emission of chemicals that can do harm to humans, animals and the environment and as such, REACH intends to prevent that e.g. the history of PCBs and PBDEs repeats again with other chemicals. Detailed testing programs should be undertaken and completed with satisfactory results before allowing a chemical to be used (in products) on the European market. However, because of the growing global population, the use and presence of chemicals in our daily life will increase. Chemicals will be emitted to the environment and may end up in human food chains. Safety aspects of these chemicals and their doses are important with regard to a safe environment and safe food and feed. Analytical chemistry will continue to play an important role in food safety research. Continuous development of methods is needed for (i) identification of unknown chemicals that enter the food chain, (ii) very sensitive detection of contaminants at the parts-per-trillion and parts-per-quadrillion range and (iii) development of methods that allow rapid and high-throughput analysis of food and feed. Developments of versatile instruments continue. Mass spectrometry, once a hardly available technique, has become more robust and affordable, and is nowadays routinely applied for identification and quantification (e.g. quadrupole and ion trap instruments and to a certain degree also time-of-flight instruments). With extraction and clean-up of samples, the emphasis will be on minimizing sample handling and increasing speed and high throughput. Further integration of extraction and clean-up will take place (e.g. by accelerated solvent extraction (ASE) and within-cell or in-line clean-up). Improved instruments will enable detection at the required low levels and reduce the influence of matrix constituents. This helps to deliver accurate data to scientists, policy makers and food industry, and food safety will benefit from this.

Bioanalytical methods are increasingly used in food safety research. They have been applied on organohalogenated contaminants such as the dioxin receptor (DR)-CALUX (*35-37*) and immunoassays using surface plasmon resonance (SPR) techniques for the detection of dioxins and dl-PCBs in food (*38*).

Examples of other biosensors based on antibody binding are multi-analyte ELISA and lateral flow assays. These type of sensors can be used on broad range of food contaminants, proteins, allergens whenever a suitable immunoassay is developed (e.g. (*39,40*). The method offers sensitivity, specificity, speed (high degree of automation) and multi-analyte detection in complex analytical matrices. Drawbacks of bioanalytical tools are the lack of

identification and cross reactivity with other compounds and matrix constituents and, therefore, the higher degree of uncertainty associated with the test result (as compared to chemical analytical techniques). For that reason, they are often applied as screening techniques and complemented with a chemo analytical confirmatory technique (e.g. GC-HRMS).
The increasing number of halogenated (and non-halogenated) industrial contaminants that are being found in our environment may further stimulate bioassay analyses. Effect-directed analysis (EDA) combines bioassays with chemical analysis for compound identification. Extracts that show a specific response in a bioassay are fractionated and the compounds responsible for the bioassay response in a fraction are identified by chemical analytical analysis. This approach combines the advanced analytical methods described above with toxicological information (*41,42*). The EDA approach has hardly been applied in food safety testing, but certainly provides a powerful approach for the identification of unknown biologically active contaminants. Bioassays such as the DR-, estrogen receptor (ER)- (*43,44*), androgen receptor (AR)-CALUX (*45*) can be used for that purpose. This approach was successfully applied for the identification of endocrine disrupting potency of chlorinated contaminants in fish which were responsible for estrogenic activity (*46,47*).

Human exposure
The exposure part of the present study was focussed on fish only. Several other pathways, such as exposure from other foods (e.g. diary, cereals, meat) were not taken into account. For lipophilic compounds, food is the most significant pathway, with dairy, fish and meat being predominant contributors (*8,48*). This may be different for more water soluble PFCs such as perfluorbutane sulfonate and perfluorbutanoic acid. For water-soluble PFCs, drinking water (*20-22*), beverages and milk (*49*) may be relevant contributors. In addition, uptake of these contaminants by plants from groundwater or contaminated agricultural land (by sludge) may be a relevant source as well (*50*). PFCs were detected in vegetable products (*51,52*) and there is a need to determine the mechanisms behind the plant uptake.
Apart from dietary exposure, exposure from air and dust also contributes to the overall human exposure to e.g. PBDEs, HBCD and PFCs (*16-18,53,54*). To evaluate this, one should analyse representative samples from each of the pathways. This would provide in-depth insights, allowing a total-exposure assessment. Obviously, this is a time consuming task. On the other hand the analysis of human samples (e.g. blood, plasma) provides an integrated descriptor of exposure from all pathways. This approach has some benefits: (i) absorption through membranes (e.g. dermal and intestines) has been accounted for, (ii) the distribution over body tissues can be estimated by relating it to animal experiments and toxicokinetic models, and (iii) possible

degradation in the human body can be determined by analysis of metabolites in blood or excreted body fluids (e.g. urine).
PFOSA is shown to be a precursor of PFOS. Biodegradation occurs in fish livers and liver microsomes, thereby contributing to the PFOS body burden (*55*). Several PFOSA type compounds exist with methyl, ethyl or even more complex groups covalently bound to the nitrogen atom (e.g. N-MeFOSA, N-EtFOSA) to which we are exposed. N-substituted PFOSAs can also biodegrade to PFOS (*56*). The same holds for fluorotelomer alcohols and polyfluoroalkyl phosphate surfactants (PAPs) which showed to biodegrade to PFOA in vivo (*57*). PAPs are applied in food packaging and this is presumably a relevant source of PFOA after ingestion. These precursors may result in a continued exposure to their stable degradation products (e.g.. PFOS and PFOA). On the other hand, Haug et al. recently demonstrated in a time-trend study in human serum samples from Norwegian people (1976-2006) that PFOS and PFOA concentrations increased and reached a plateau in the mid 1990s. From 2001 onwards, the concentrations were decreasing. Longer chain acids (perfluornonanoic acid and perfluordecanoic acid) did not decrease, suggesting different sources or/and longer half-lives (*58*). Clearly, the potential risks of PFCs in relation to exposure are not completely understood yet.
Apart from the contaminant classes studied in this thesis, there are several others that were discovered in recent years as environmental contaminants that (may) contaminate foods. These include phosphorus flame retardants (*59-61*), siloxane compounds (*62-64*), and nano particles (*65*). Concerning the latter, the EFSA recently published an opinion on the nano particles in food and feed (*65*). EFSA recommends that research is needed to address the multitude of uncertainties in this field. Specific recommendations include (i) the investigation of the interaction and stability of nano particles in food and feed, in the gastro-intestinal tract and in biological tissues, (ii) the development and validation of routine methods to detect, characterise and quantify nano particles in food contact materials, food and feed, and (iii) the development, improvement and validation of test methodologies to assess toxicity of nano particles (including reliability and relevance of test methods). This calls for further development of analytical approaches that help to address these questions.

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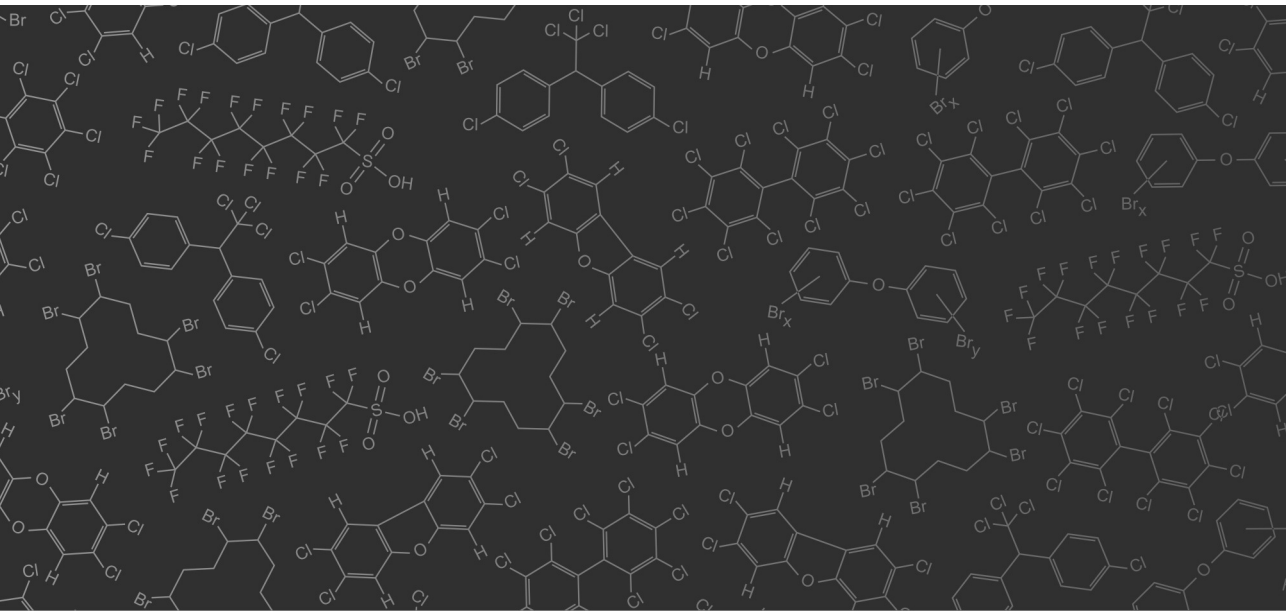

Final

Summary

Fish is an important part of our food. In addition to its general nutritional value fish contains essential nutrients like omega-3 and omega-6 fatty acids, which are believed to be beneficial for our health. Fish is also an important dietary source of selenium. Unfortunately, several contaminants are present in fish, such as heavy metals and organohalogen contaminants. Well-known examples of the latter are polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and –furans (dioxins) and DDT. Production of PCBs and DDT has started before World War II and were used in industrial and consumer applications as well as in agriculture. PCBs and DDT have entered the environment during production, product formulation, application and disposal or (for dioxins) as a result of combustion processes or as a by-product of chemical synthesis *(chapter 1)*. Environmental residues of these xenobiotic compounds have been found since the late 1960s. Extensive research is carried out to these contaminants since then. This showed that these contaminants are persistent, bioaccumulate, have adverse effects and are transported globally over large distances. They are therefore qualified as persistent organic pollutants (POPs) in the framework of the Stockholm Convention. More recently, new contaminants were found in fish, such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and perfluorinated compounds (PFCs). These compounds are still being produced for application in consumer products and industrial processes. There is toxicological concern on these contaminants. The toxicity of dioxins and dioxin-like (dl)-PCBs received much attention, and these are among the most toxic substances known. Non-dioxin-like (ndl) PCBs are less toxic, although they can cause neurotoxic effects. The toxicity of PFCs, PBDEs and HBCD is under investigation, and their complete toxic profile is not yet elucidated, although several adverse effects of these compounds were reported. Through the consumption of fish, these contaminants are ingested and may potentially harm consumer's health. Several studies showed that fish is an important contributor to the dietary exposure to e.g. dioxins, PCBs, organochlorine pesticides (OCPs) and PBDEs. Information on the dietary exposure to HBCD and PFCs is urgently needed to allow adequate exposure assessments. Unfortunately, validated methods for the analysis of HBCD and PFCs were not readily available. To overcome this problem, methods needed to be developed and validated. Apart from that, for dioxins and dioxin-like (dl-)PCBs less expensive and complicated methods were needed to enlarge food safety monitoring capacity.

This study was undertaken with the following aims:
- Development of reliable methods of analysis, including in-house and between laboratory validations;
- Determination of the contaminant concentrations in fish, shellfish and shrimp species relevant for human exposure;
- Estimation of the dietary exposure to a broad suite of contaminants from wild and farmed fish.

This allows determining the possible health risks and the relative importance of specific contaminants and fish species, and may help policy makes and scientists to determine where to put their focus on.

Analytical method development

Current methods for halogenated lipophilic contaminants in fish often start with extraction based on Soxhlet, although accelerated solvent extraction (ASE) becomes more popular (*chapter 2.1*). Clean-up of the sample consists of lipid removal (e.g. by gel permeation chromatography, sulphuric acid treatment of alumina oxide column chromatography). Fractionation of the extract is often achieved by silica column chromatography after which the extract is ready for analysis by gas chromatographic (GC) techniques. Electron capture detection (ECD) is still used as an inexpensive and simple detector, but mass spectrometric (MS) techniques are increasingly being used because of unambiguous identification and for their sensitivity. In recent years, comprehensive multidimensional GC (GCxGC) gained popularity as it enables the separation of very complex mixtures in a single run. Because of the low concentrations in fish samples, dioxins and dl-PCBs require additional clean-up over e.g. graphitised carbon columns. GC-high resolution (HR)MS is being used in order to reduce interferences in the detection. Unfortunately, GC-HRMS equipment requires large investments and, therefore, it is only applied in a limited number of specialized laboratories. In addition, the sample extraction and clean-up is time consuming, further adding to the costs. Within the framework of the EU DIFFERENCE project, integrated sample extraction and clean-up methods were developed based on ASE extraction and combined with within-cell clean-up and fractionation of the extract. For the final determination, alternative detection techniques were developed and optimised (i.e. GC-ion trap MS/MS, GCxGC with ECD and the DR-CALUX bioassay). These techniques were validated in an international framework, including sensitivity, accuracy and precision tests *(chapter 3.1)*. GC-ion trap MS/MS was most promising in terms of performance and costs. GCxGC-ECD performed also well, but a drawback (for the moment) is the time-consuming manual integration of peaks, especially at low concentrations. DR-CALUX is sensitive but suffered from a bias and limited precision. Nevertheless, DR-CALUX can serve as an excellent screening technique during crises, as well as

for finding unknown compounds that exhibit an Ah receptor response. It should be noted that GC-LRMS/MS, GCxGC-ECD and DR-CALUX all three met the EU requirements of screening methods for dioxins and dl-PCBs in food. Extraction by ASE and sample clean-up within the extraction cell is promising, but further method optimisation is needed.
For HBCD, both GC- and liquid chromatography (LC-)MS/MS techniques were available. However, discrepancies were observed between results from both techniques, with the GC results being 4.4-fold higher (on average) than the LC based results *(chapter 3.2)*. Although this difference could not be explained completely, the LC method is preferred for a number of reasons: (i) the specific detection of the three major HBCD diastereomers; (ii) the use of ^{13}C-labelled standards allowing a more accurate analysis; (iii) no thermal degradation of HBCD or interconversion of individual diastereomers.
For PFCs, the methods initially developed internationally were based on ion pair extraction (IPE) and no further clean-up. In recent years, the number of PFCs included in methods increased and more diverse methods were developed for fish, such as extraction by methanol or acetonitrile and clean-up by suspended graphitised carbon, or saponification of the sample followed by solid phase extraction (SPE) for concentration and clean-up (*chapter 2.2*). Chromatographic separation is typically achieved over reversed phase (C18) columns and the best detection method is MS/MS or time-of-flight (TOF) MS, which both provide selectivity and allow unambiguous identification. The lack of high quality standards, mass labelled internal standards, suitable clean-up methods and the presence of interferences has put a pressure on the accuracy of the data produced world-wide. This was reflected in the first worldwide PFC interlaboratory study (ILS), organised within the framework of the EU Perforce project (*chapter 3.3*). The comparability of results for water and fish was very poor, showing the need for improvement of methods and availability of high quality standards (native and mass labelled). Large developments took place on both aspects in recent years. A broad suite of high quality standards and mass labelled standards became commercially available. Furthermore, a variety of analytical approaches were developed delivering accurate data *(chapter 2.2)*. In a follow-up study the between laboratory comparability improved considerably (*chapter 3.4)* as a result of the use of high quality native and internal standards by all participants. Furthermore, the routinely applied method of using solvent based calibration curves (i.e. a calibration curve in solvent) delivers very precise and reproducible (between labs) results in case it is combined with a mass labelled analogue for each single target compound. Results were more precise and reproducible than those based on standard addition quantification (i.e. a calibration curve in the matrix), although this technique is highly suitable for quantification of those compounds that have no mass labelled analogue in samples with substantial matrix effects.

Contaminant concentrations in fish

A broad selection of contaminants (dioxins, dl-PCBs, indicator-PCBs, OCPs, PBDEs, HBCD and PFCs) was determined in a wide range of fish, shellfish and shrimp species. Emphasis was put on species, which are regularly consumed by Dutch citizens or which – from previous research – were known to contain high PCB concentrations (eel). This study included salmon (farmed), eel (wild and farmed), trout (farmed), pikeperch, herring, mackerel, cod, coalfish, haddock, flounder, sole, shrimp (wild shrimp from the Dutch coast and farmed shrimp, mainly from Asia) and mussels. In addition, recently introduced farmed species like tilapia and pangasius were investigated. Most fish originated from Dutch freshwaters (eel, pikeperch), the North Sea and the Atlantic Ocean. Farmed species like salmon, eel and trout originated mainly from Europe while tilapia, pangasius and farmed shrimp originated mainly from Asia.

Dioxins and dl-PCBs were highest in wild eel samples from the river Meuse and Rhine deltas *(chapter 4.1)*. Total-TEQ concentrations (i.e. the sum of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity equivalents) were up to 4.5 times above the EU maximum level (ML) for eel (12 pg TEQ/g ww). The concentrations in fish from other Dutch freshwater locations were (much) lower. Concentrations in marine fish such as herring, flounder, mackerel, salmon etc. were also lower and below the ML of 8 pg TEQ/g ww (for other fish). The farmed species pangasius and tilapia stand out because of their extremely low total-TEQ concentrations *(chapter 4.4)*. In most pangasius and tilapia samples nearly all dioxin congeners and PCB congeners were below the limit of quantification (LOQ). A 250-fold difference was observed between the concentrations in the pangasius and tilapia samples and the highest contaminated eel samples. This difference would be much larger (2,400,000 fold) when the concentrations would be expressed on a lower bound basis. The revised World Health Organisation (WHO) toxic equivalency factors (TEFs) of 2005 result in 10-20% lower TEQ values compared to the 1998 TEFs because mono-ortho PCBs were assigned lower TEFs in the 2005 TEF revision *(chapter 4.1)*. This effect is most pronounced in eel (40% lower TEQ values) as these have relative high mono-ortho PCB concentrations.

The indicator PCBs follow a similar pattern as discussed above, with eel being the highest contaminated fish species (sum of indicator PCBs 1,740 ng/g ww for eel from the river New Merwede, *chapter 4.1*) and tilapia and pangasius being the lowest contaminated species *(chapter 4.4)*. The lowest ΣPCB concentration was found in pangasius from Vietnam with as little as 0.034 ng/g ww. This is 50,000 times lower than in the New Merwede eel sample. Some of the eel samples (New Merwede, Hollands Diep, Meuse at Keizersveer) exceeded the Dutch MLs for indicator PCBs, but no other species exceeded these MLs. CB 153 was in all cases the predominant congener.

The analysis of PBDEs was limited to the congeners proposed by the European Food Safety Agency (EFSA) (BDE 28, 47, 99, 100, 153, 154, 183 and 209). BDE 49 was added to the selection as it was in most samples detected in

concentrations comparable to BDE 99. In nearly all samples, these BDEs were detected. BDE 183, also recommended by EFSA, was not detected in most samples. The sum of BDEs 28, 47, 49, 99, 100, 153, 154, 183 and 209 was 0.01-0.15 ng/g ww in farmed shrimps, pangasius and tilapia, 0.34-3.9 ng/g ww in farmed trout, salmon and eel, 0.1-9.3 ng/g ww in mussels and marine fish and 0.2-220 ng/g ww in wild eel (*chapter 4.2 and 4.4*). BDE 209 was not detected in most of the wild fish species. For long it was believed that BDE 209 would not accumulate in fish, or that reported values were debatable because of analytical problems (e.g. blank contributions). Due to a very well controlled low blank contamination, BDE 209 could be detected in most farmed shrimp samples (8-17 pg/g ww) and pangasius samples (7-70 pg/g ww) *(chapter 4.4)*. It was not detected in any of the tilapia samples, and only in two salmon samples (45 and 59 pg/g ww) and in one trout sample (3600 pg/g ww). The cause of this high concentration in trout is unknown. Re-analysis confirmed the concentration. The predominant congeners were BDE 47>49≈99≈100, except for pangasius and farmed shrimps in which BDE 209 contributed approx. 50% to the sum. It is not clear what explains the presence of BDE 209 in several samples. Possibly, it is ingested through the diet. On the other hand, it may be a contamination that occurred during processing, packaging, storage and transportation of these farmed fish samples.

HBCD was detected in approx. 50% of the samples (*chapter 4.2 and 4.4*). In nearly all of these samples, α-HBCD was the only diastereomer present at concentrations from 0.01 ng/g ww (pangasius, farmed shrimps) to 41 ng/g ww (eel), spanning four orders of magnitude. β- and γ-HBCD were present in eel, but at lower concentrations than α-HBCD (e.g. γ-HBCD was approx. 21% of α-HBCD). In other species, these differences were much larger and β- and γ-HBCD were only detected in a few trout and shrimp samples (0.01-0.05 ng/g ww).

Within the group of perfluorinated compounds, PFOS was the dominating contaminant (*chapter 4.3*). Short chain PFCs (C4 to C7) hardly accumulate in fish and also perfluoroctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoA) were only found in a 1-2 samples of the 70 samples analysed. Perfluordecanoic acid (PFDcA) and the odd chain length perfluorundecanoic acid (PFUnA) and perfluortridecanoic acid (PFTrA) were detected more frequently (in 10-20% of the samples). There was no clear relationship with species or origin of the sample. It is not known why these two stand out, as no widespread use was reported. Possibly, these are stable end products of (bio)degraded precursors. In wild fish (marine and freshwater), 2-150 ng/g ww PFOS was found. Concentrations in Western Scheldt flounder samples were higher due to (historic) contamination caused by PFC manufacturing plant and PFC users. PFOS concentrations in livers were on average 5.3-fold higher as in the fillets from the corresponding fish. PFOS was only detected in 4 out of the 37 farmed fish species (*chapter 4.3 and 4.4*). Surprisingly, PFOS was not detected in any of the salmon samples, whereas

the other (earlier mentioned) contaminants often are found in salmon. This may be partly due to the higher LODs for PFOS (approx. 0.5 ng/g ww) as compared to e.g. PCBs (LOD of approx. 0.005 to 0.1 ng/g ww). On the other hand, the distinct accumulation behaviour of PFCs may play a role here as well.
When comparing contaminant concentrations in the investigated samples, they (generally) decrease in the following order ∑indicator PCB > PFOS ≈ ∑3 DDTs (i.e. p,p'-DDT, p,p'-DDD and p,p'-DDE) > ∑8 PBDEs > HCB ≈ α-HBCD >> total-TEQ. This is only a general indication, as the order may vary between species and per contaminant.

Human exposure estimation from fish consumption

The human exposure from fish consumption was estimated for dioxins and dl-PCBs, ∑indicator-PCBs, ∑3 DDTs, HCB, ∑8PBDEs, α-HBCD and PFOS. The exposure was calculated from fish consumption figures from the Dutch National Food Consumption Survey (DNFCS), and multiplied with mean contaminant data. The DNFCS database of 1997/1998 contains mean fish consumption data for a variety of fish including herring, salmon, cod, eel, mackerel, mussels and wild shrimp. New farmed fish species like pangasius and tilapia are not represented in the DNFCS. Because consumption of these species increases rapidly, recent estimates of sales to consumers (2006) were taken and the consumption was derived from these figures. The same holds for farmed shrimp and trout. Contaminant data that consisted mainly of <LOD values was not taken into account. This holds e.g. for BDE 183 and PFOA. On the other hand, BDE 49 was included as it was present in nearly every fish sample.
The exposure estimates are based on fish only, meaning that other sources of dietary exposure (e.g. dairy, cereals and pork) were not taken into account. The absolute exposure amounts decrease in the following order *(chapter 4.5)*: ∑indicator-PCBs (1.1 ng/kg bw per day) > PFOS (1.0 ng/kg bw per day) > ∑3 DDTs (0.45 ng/kg bw per day) > ∑8PBDEs (0.27 ng/kg bw per day) > HCB (0.09 ng/kg bw per day) ≈ α-HBCD (0.06 ng/kg bw per day) >> Dioxins and dl-PCBs (0.26 pg total-TEQ/kg bw per day). The exposure of dioxins and dioxin-like PCBs (as compared to other contaminants) is closest is to the WHO tolerable daily intake (TDI) of 2 pg/kg bw per day, leaving only a small margin of exposure. This margin will even be smaller when exposure to other food items will be included in the estimation. The human toxicity data of non-dioxin-like PCBs is currently revised, and therefore no comparison is made with reference values. The PFOS exposure is 150-fold lower than the estimate made by the EFSA (2008). There are two underlying reasons for this difference (*chapter 4.3*). First, EFSA used 5-fold higher fish consumption estimates, based on consumer-only data, meaning that it was assumed that every citizen consumes fish every day (which is a conservative approach). Secondly, EFSA used a high

estimate for the PFOS concentration in fish in general (68 ng/g ww), which is much higher than was shown in this study. In this study, species-specific PFOS contamination data was available, allowing a more accurate estimation of the exposure of the Dutch population. When looking at the exposure from all contaminants (summarised), herring predominates (41%), followed by salmon (21%) and Gadidae (14%) (total 76%). The other fish species together contribute 24%. The contribution of pangasius, tilapia and farmed shrimp is less than 1%.

Conclusions and future perspectives

The results of this study show that promising alternative techniques (GC-ITMS/MS) to GC-HRMS are available for the detection of dioxins and dl-PCBs in food and feed. DR-CALUX and GCxGC-ECD are valuable screening tools, but they would benefit from further development in terms of accuracy (DR-CALUX) and reduction of labour involved in peak integration (GCxGC-ECD). For the analysis of HBCD, LC-ESI-MS/MS is preferred over GC-ECNI-MS for its better accuracy and because separation of individual diastereomers is feasible. The quality of the analysis of PFCs in fish and water benefited to a large extent from the improved knowledge on the behaviour of PFCs, a broader range of analytical approaches and the availability of a high quality native and mass labelled standards.

Contaminant concentrations were highest in wild eel from polluted areas (e.g. Haringvliet) and lowest in farmed fish samples. From a contaminant point of view, concentrations decreased as follows: ∑7indicatorPCB > PFOS ≈ ∑3 DDTs > ∑8PBDEs > HCB ≈ α-HBCD >> total-TEQ. Although contaminant concentrations in wild eel were the highest, herring and salmon dominate the human exposure from fish consumption. The contribution from newly farmed species (pangasius, tilapia and shrimp) is <1%. The exposure to PFOS was different as compared to that of the more lipophilic contaminants.

Future analytical developments will focus on (i) reduction of time and labour needed for sample pre-treatment (sample extraction and clean-up), (ii) further lowering of detection limits of MS instruments (iii) miniaturisation of analytical methods (iv), improving speed and throughput of analytical methods. This will enable the analysis of hundreds of compounds in a single run. On the other hand, bio-analytical approaches are complementary to chemo-analytical methods. These will develop further allowing rapid analysis of contaminants with a comparable biological activity and the identification of unknown contaminants.

New contaminants are discovered continuously. Recent examples of new contaminants that drew attention of scientists and policy makers are PFCs, nano particles and siloxanes. Whereas exposure to traditional lipophilic contaminants is dominated by the diet (i.e. foods form animal origin), these contaminants have different properties that require the development of new

analytical approaches. In addition, the different properties require a broader exposure assessment including diet, drinking water, beverages, dust and air. Characterisation of these different routes is laborious. The analysis human body fluids (e.g. blood, milk) can provide an integrated picture of all exposure routes and is a valuable complementary approach.

Samenvatting

Vis is een belangrijk onderdeel van onze voeding. Het bevat waardevolle voedingsstoffen en is daarnaast een belangrijke bron voor bijvoorbeeld selenium en omega-3 en omega-6 vetzuren. Dit betreffen essentiele nutrienten, en daarvan wordt aangenomen dat ze gezondheidsbevorderende eigenschappen hebben.
Helaas kunnen ook diverse contaminanten in vis voorkomen. Bekende voorbeelden hiervan zijn zware metalen en gehalogeneerde (organische) contaminanten zoals polychloorbifenylen (PCBs), polychloordibenzo-*p*-dioxines en –furanen (‘dioxines’) en DDT. Productie van PCBs en DDT is (ver) voor de 2e wereld oorlog gestart en ze zijn in het verleden in grote hoeveelheden gebruikt in o.a. industriële en consumenten toepassingen en in de landbouw. PCBs en DDT zijn op diverse wijzen in het milieu gekomen (tijdens de productie van deze stoffen, product formulering, product toepassing en als afval) en ze worden verspreid over de hele wereld aangetroffen. Dioxines zijn nooit geproduceerd met een specifiek gebruik als doel, maar het zijn (ongewenste) bijproducten van verbrandingsprocessen en chemische synthese (*hoofdstuk 1*). Residuen van deze stoffen zijn in de late 1960-er jaren aangetroffen in het milieu. Sindsdien is er veel onderzoek verricht naar deze contaminanten. Deze stoffen zijn toxisch, persistent en bioaccumuleren en zijn daarom in het kader van de Stockholm Conventie aangemerkt als persistente organische verontreinigingen (Persistent Organic Pollutants, POPs). Deze stoffen hopen zich op in vis en daarom is de consumptie van vis een belangrijke blootstellingsroute voor mensen.
Recent zijn er nieuwe contaminanten gevonden in het milieu (o.a. vis), zoals polybroom difenylethers (PBDEs), hexabroomcyclododecaan (HBCD) en perfluoroctaansulfonaat (PFOS). Deze stoffen worden nog steeds geproduceerd en toegepast in diverse industriële en consumenten toepassingen. Er is reden voor bezorgdheid vanwege de toxische eigenschappen van deze stoffen. Er is inmiddels bekend dat deze stoffen diverse toxische eigenschappen hebben. Hun eigenschappen lijken sterk op die van de huidige POPs. Zo zijn deze stoffen persistent en accumuleren ze in vissen. Door consumptie van gecontamineerde vis worden mensen blootgesteld aan deze contaminanten, hetgeen een mogelijke bedreiging kan vormen voor de humane gezondheid. Diverse studies hebben reeds aangetoond dat vis een belangrijke bijdrage levert aan de humane blootstelling aan bijvoorbeeld dioxines, PCBs, organochloor pesticiden (OCPs) en PBDEs. Er is nauwelijks informatie beschikbaar over de blootstelling aan HBCD en PFCs vanuit voeding. Die informatie is snel nodig om een accurate risicoinschatting te kunnen maken. Helaas waren er nog geen goed gevalideerde meetmethodes beschikbaar om HBCD en PFCs te kunnen meten. Daarom moesten methodes ontwikkeld en gevalideerd worden. Voor dioxines en dioxine-achtige PCBs (dl-PCBs) zijn wel goede methodes

beschikbaar, maar deze zijn ingewikkeld en duur. Daarom is er de noodzaak om goedkopere en eenvoudigere methodes te ontwikkelen.

Deze studie is ondernomen met de volgende doelen:
- De ontwikkeling van betrouwbare methodes voor analyse van contaminanten, inclusief de validatie (binnen het laboratorium en tussen laboratoria onderling);
- Het meten van contaminanten in, voor de humane consumptie relevante soorten, wilde en gekweekte vis, schaal en schelpdieren;
- Het schatten van de blootstelling van mensen aan een brede reeks contaminanten.

Aan de hand van de resultaten van deze studie kunnen mogelijke gezondheidsrisicos als gevolg van blootstelling ingeschat worden alsmede het relatieve belang van de diverse contaminanten en de diverse soorten vis. Dit kan beleidsmakers en wetenschappers helpen te bepalen waar hun focus op gericht moet worden.

Analytische methodeontwikkeling

De huidige methodes voor de extractie van gehalogeneerde lipofiele (vetminnende) contaminanten in vis starten vaak met de klassieke Soxhlet extractie, hoewel nieuwe technieken zoals vloeistofextractie onder verhoogde druk (Pressurised Liquid Extraction, PLE) steeds populairder worden (*hoofdstuk 2.1*). De typische opzuivering van het extract bestaat uit het verwijderen van de geëxtraheerde lipiden (bv. door Gel Permeation Chromatography, GPC), zwavelzuur behandeling of chromatografie over een kolom met alumina oxide). Verdere opzuivering van het extract (fractionering) gebeurt vaak met chromatografie over een silica kolom, waarna het extract gereed is voor analyse met gaschromatografische technieken (GC). Electronenvangst detectie (Electron Capture Detection, ECD) wordt nog steeds veelvuldig gebruikt omdat het goedkoop en eenvoudig in gebruik is, maar interferenties kunnen identificatie en goede kwantificering bemoeilijken. Massaspectrometrische (MS) technieken worden in toenemende mate gebruikt vanwege de ondubbelzinnige identificatie en vanwege de goede gevoeligheid. In recente jaren is multidimensionele GC (GCxGC) ontwikkeld. De kracht van deze techniek is dat het erg complexe mengsels in één enkele analyse kan scheiden en meten. Vanwege de lage gehalten in vis is voor de analyse van dioxines en dl-PCBs nog een extra opzuivering nodig over een kolom met bv. actieve kool. Voor de analyse van dioxines en dl-PCBs is tot op heden GC gekoppeld met hoge resolutie MS (GC-HRMS) de gouden standaard omdat het de invloed van interferenties kan minimaliseren, de gevoeligheid hoog en de detectie ondubbelzinnig is. Helaas is de investering in deze apparatuur hoog en daarom wordt GC-HRMS

vooralsnog in een beperkt aantal laboratoria gebruikt. Daarnaast is ook de extractie en opschoning bewerkelijk hetgeen de kosten per analyse verder verhoogt. In het kader van het EU-DIFFERENCE project zijn de mogelijkheden voor een geïntegreerde extractie en opschoning onderzocht. Hiervoor is gekeken naar extractie op basis van PLE, aangevuld met verwijdering van lipiden en fractionering in de extractiecel. Voor de meting van de dioxines en dl-PCBs in het extract zijn eveneens alternatieven ontwikkeld en gevalideerd (GC-ion trap MS/MS, GCxGC gekoppeld met ECD en de DR-CALUX bioassay). Deze technieken zijn internationaal gevalideerd, waarbij gevoeligheid, juistheid en precisie onderzocht zijn (*hoofdstuk 3.1*). GC-ion trap MS/MS is de meest veelbelovende techniek vanwege goede prestaties en de lage kosten per analyse. GCxGC-ECD presteerde ook goed, maar een belangrijk minpunt op dit moment is de nog bewerkelijke handmatige integratie van pieken, in het bijzonder vanwege de erg lage contaminantgehalten die normaliter in voedsel gevonden worden. DR-CALUX is gevoelig maar de juistheid en precisie waren beperkt. Niettemin kan DR-CALUX een goede screeningstechniek zijn om hoog gecontamineerde monsters op te sporen (bv. tijdens een crisis). Daarnaast kunnen met DR-CALUX onbekende stoffen opgespoord worden die (net als dioxines) aangrijpen op de Ah receptor. Niettemin voldeden zowel de DR-CALUX als GC-ion trap MS/MS en GCxGC-ECD aan de criteria die door de EU zijn gesteld aan screeningstechnieken voor dioxines en dl-PCBs. Extractie met PLE en opschoning en fractionering in de extractiecel is veelbelovend, maar verdere ontwikkeling en optimalisatie zijn vereist.

Voor de analyse van HBCD zijn GC technieken beschikbaar, alsmede technieken gebaseerd op vloeistofchromatografie (LC) gekoppeld aan MS (bv. tandem massa spectrometrie, MS/MS). Echter, behoorlijke verschillen zijn gevonden tussen de resultaten verkregen met GC-electronen vangst negatieve ionisatie (Electron Capture Negative Ionisation, ECNI)-MS en die met LC-Electrospray Ionisatie (ESI)-MS/MS, waarbij de resultaten van GC-ECNI-MS gemiddeld een factor 4.4 hoger waren dan die van LC-ESI-MS/MS (*hoofdstuk 3.2*). Hoewel dit verschil niet volledig kan worden verklaard, heeft LC-ESI-MS/MS de voorkeur vanwege (i) de specifieke detectie van de drie belangrijkste diastereomeren; (ii) de mogelijkheid tot gebruik van ^{13}C-gelabelde interne standaarden resulterend in een accuratere analyse en (iii) geen thermische degradatie of interconversie van individuele diastereomeren plaats vindt.

Voor PFCs waren de methodes aanvankelijk gebaseerd op ion paar extractie (Ion Pair Extraction, IPE) zonder verdere opschoning. In recente jaren is het aantal gemeten PFCs toegenomen en zijn er diverse methodes ontwikkeld. Zo zijn er voor vis methodes beschikbaar gebaseerd op extractie met methanol of acetonitril en een opschoning met gesuspendeerd actief kool, of verzeping van het monster gevolgd door vaste fase extractie (Solid Phase Extraction, SPE) voor preconcentratie en opschoning (*hoofdstuk 2.2*).

Chromatografische scheiding wordt meestal verkregen met een reversed phase kolom (bv C18) en de beste detectiemethode is MS/MS of time-of-flight MS, welke beide ondubbelzinnige identificatie en detectie mogelijk maken. Het gebrek aan goede kwaliteit standaarden, massa gelabelde interne standaarden, geschikte opzuiveringsmethodes en de aanwezigheid van interferenties heeft de kwaliteit (juistheid) van de wereldwijd gerapporteerde data onder druk gezet. Dit werd duidelijk uit de eerste wereldwijde interlaboratorium studie (ILS) die georganiseerd is in het kader van het EU PERFORCE project (*hoofdstuk 3.3*). De onderlinge vergelijkbaarheid van de resultaten van de deelnemende laboratoria voor een monster vis en water was slecht. Dit toonde de noodzaak voor verbetering van de methodes en de noodzaak voor de beschikbaarheid van goede kwaliteit (interne) standaarden. Op beide terreinen is de laatste jaren veel vooruitgang geboekt. Een brede selectie goede kwaliteit (interne) standaarden is (commercieel) beschikbaar gekomen. Daarnaast zijn er diverse analytische methodes beschikbaar gekomen die ieder goede kwaliteit data opleveren (*hoofdstuk 2.2*). Dit bleek ook uit de resultaten van een tweede wereldwijde ILS, waarbij de vergelijkbaarheid tussen de laboratoria aanzienlijk is verbeterd (*hoofdstuk 3.4*). De reden hiervoor is het gebruik van goede kwaliteit (interne) standaarden door alle deelnemers. Uit deze studie bleek ook dat de meestal routinematig toegepaste calibratie (i.e. de calibratiecurve in een oplosmiddel) herhaalbare en reproduceerbare (tussen-lab) resultaten oplevert indien het wordt gecombineerd met een massa gelabelde analoog (interne standaard) voor ieder te kwantificeren PFC. Daarnaast is een kwantificering ook onderzocht met behulp van standaard additie, maar de resultaten hiervan hadden een grotere spreiding (i.e. een slechtere tussen-lab reproduceerbaarheid). De standaardadditie methode is wel bijzonder geschikt voor kwantificering van de PFCs waarvoor geen massa gelabelde analoog beschikbaar is in monsters met aanzienlijke matrix effecten.

Contaminantgehalten in vis

Diverse contaminanten (dioxines, dl-PCBs, OCPs, PBDEs, HBCD en PFCs) zijn gemeten in een brede selectie vis, schelpdieren en garnalen. De nadruk is gelegd op de soorten die regelmatig door de Nederlandse consument worden geconsumeerd, of op soorten waarvan uit eerder onderzoek bekend is dat deze hoge PCB-gehalten hadden (bv aal uit de benedenstroomse gebieden). De volgende soorten zijn in deze studie onderzocht: zalm (gekweekt), aal (wild en gekweekt), forel (gekweekt), snoekbaars, haring, makreel, kabeljauw, koolvis, schelvis, bot, tong, garnalen (van de Nederlandse kust en gekweekt uit Azië) en mosselen. Daarnaast zijn ook recent geïntroduceerde nieuwe gekweekte soorten zoals tilapia en pangasius in het onderzoek betrokken. De meeste vis is gevangen in de

Nederlandse binnenwateren (aal, snoekbaars), de Noordzee (o.a. tong, bot, garnalen) of de Atlantische oceaan. De gekweekte soorten zoals forel, zalm en aal waren voornamelijk afkomstig uit Europa, pangasius uit Vietnam (Mekong delta) en tilapia en garnalen voornamelijk uit Azië. Gehalten van dioxines en dl-PCBs waren het hoogst in aal uit de delta van de Maas en de Rijn. Het totaal van dioxines en dl-PCBs (uitgedrukt als het totaal van 2,3,7,8-tetrachloordibenzo-*p*-dioxine toxiciteits equivalenten, totaal-TEQ) lag in deze monsters tot 4.5 keer de door de EU toegestane maximum gehalte (maximum level, ML) in aal van 12 pg totaal-TEQ/g natgewicht (wet weight, ww) (*hoofdstuk 4.1*). De gehalten in vis van andere locaties waren (veel) lager en lager dan de EU ML van 8 pg totaal-TEQ/g ww. Gehalten in mariene soorten zoals haring, bot, makreel, tong, zalm, garnalen etc. waren ook lager en altijd beneden de EU ML. Pangasius en tilapia onderscheidden zich vanwege hun bijzonder lage totaal-TEQ-gehalten (*hoofdstuk 4.4*). In bijna alle pangasius en tilapia monsters waren de meeste dioxine en dl-PCB congeneren onder de kwantificeringslimiet (limit of quantification, LOQ). Het verschil tussen deze monsters en de hoogst gecontamineerde aal monsters bedroeg een factor 250. Deze factor zou nog veel groter zijn (ca. 2.400.000) wanneer de gehalten op lowerbound basis zouden worden uitgedrukt (i.e. gehalten lager dan de LOQ worden op nul gesteld). De door de wereld gezondheidsorganisatie (World Health Organisation, WHO) herziene toxiciteits equivalentie factoren (TEFs) van 2005 resulteren in 10-20% lagere TEQ-waarden in vergelijking met de TEFs van 1998 omdat de mono-ortho PCBs lagere TEF-waarden toegekend hebben gekregen in de 2005 TEF herziening. Dit effect is het meest uitgesproken in aal (40% lagere TEQs) vanwege de relatief hoge mono-ortho PCB-gehalten in aal (*hoofdstuk 4.1*).

De indicator-PCBs volgen een vergelijkbaar patroon zoals hierboven besproken voor de dioxines en dl-PCBs. Aal uit de Nieuwe Merwede is het hoogst gecontamineerd (1740 ng/g ww voor de som van 7 PCBs) (*hoofdstuk 4.1*) terwijl de gehalten in tilapia en pangasius wederom het laagst waren (*hoofdstuk 4.4*). Het laagste gehalte bedroeg 0.034 ng/g ww en is gemeten in een pangasius, hetgeen 50.000 keer lager is dan het gehalte in aal uit de Nieuwe Merwede. De aalmonsters uit de Nieuwe Merwede, Hollands-Diep en de Maas bij Keizersveer kwamen boven de Nederlandse ML voor PCBs in aal. Aal van andere locaties en andere soorten vis lagen (ver) onder deze norm. In alle gevallen is PCB 153 de dominante congeneer.

De keuze van de geanalyseerde PBDEs is gebaseerd op de selectie die voor monitoring is voorgesteld door de Europese autoriteit voor de voedselveiligheid (European Food Safety Authority, EFSA): BDE 28, 47, 99, 100, 153, 154, 183 en 209. BDE 49 is toegevoegd aan de selectie omdat deze regelmatig in monsters is aangetroffen in gehalten vergelijkbaar met BDE 99. Deze selectie BDEs is in bijna alle monsters aangetroffen. BDE 183 is ook voorgesteld door de EFSA, maar deze is in bijna geen van de monsters aangetroffen. De som van BDEs (28, 47, 49, 99, 100, 153, 154, 183 en 209) is

0.01-0.15 ng/g ww in gekweekte garnalen, pangasius en tilapia, 0.34-3.9 ng/g ww in gekweekte forel, zalm en aal, 0.1-9.3 ng/g ww in mosselen en mariene soorten en 0.2-220 ng/g ww in wilde aal (*hoofdstuk 4.2 en 4.4*). BDE 209 is niet gedetecteerd in de meeste wilde vissoorten. Lange tijd werd het als niet aannemelijk beschouwd dat BDE 209 kon accumuleren. Anderzijds waren de gerapporteerde gehalten lange tijd twijfelachtig vanwege diverse analytische problemen bij de analyse van BDE 209 (bv. blanco problemen). Vanwege een erg lage en goed gecontroleerde blanco is het mogelijk om BDE 209 te detecteren in de meeste gekweekte garnalen (8-17 pg/g ww) en pangasius (7-70 pg/g ww). BDE 209 is niet aangetroffen in de tilapia monsters, slechts in twee zalm monsters (45 en 59 pg/g ww). Het is niet bekend of BDE 209 in het vlees aanwezig is vanwege opname vanuit de voeding, of dat het vlees gecontamineerd is geraakt tijdens verwerking, transport en opslag. Een hoog BDE 209 gehalte is gevonden in een forelmonster (3600 pg/g ww) (*hoofdstuk 4.4*). De reden van deze hoge concentratie in dit monster is onbekend. Heranalyse bevestigde de bevinding en het gehalte wordt daarom toegeschreven aan het monster en niet aan een mogelijke analysefout. De meest dominante congeneren betroffen BDE 47>49≈99≈100, behalve voor pangasius en gekweekte garnalen waar BDE 209 ca. 50% van het totaal bedroeg.

HBCD is aangetroffen in ca. 50% van de monsters (*hoofdstuk 4.2 en 4.4*). In bijna alle monsters is α-HBCD de enige diastereomer die is aangetroffen, in gehalten van 0.01 ng/g (pangasius, gekweekte garnalen) tot 41 ng/g ww (aal), hetgeen een factor 4000 verschil is. β- en γ-HBCD zijn aangetroffen in aal, maar op veel lagere gehalten (bv. γ-HBCD is ongeveer 21% van α-HBCD). In andere soorten is dit verschil veel groter: β- en γ-HBCD zijn alleen aangetroffen in enkele gekweekte forel en garnalen monsters (0.01-0.05 ng/g ww).

Binnen de groep van PFCs is PFOS de dominante contaminant (*hoofdstuk 4.3*). Korte keten PFCs (C4 t/m C7) accumuleren nauwelijks in vis en ook perfluoroctanoaat (PFOA), perfluornonanoaat (PFNA) en perfluordodecanoaat (PFDoA) zijn maar in 1-2 van de 70 geanalyseerde monsters gevonden. Perfluordecanoaat (PFDcA) en de oneven ketenlengtes perfluorundecanoaat (PFUnA) en perfluortridecanoaat (PFTrA) zijn wat vaker aangetroffen (10-20% van de monsters). Er is geen duidelijke relatie tussen de gevonden gehalten enerzijds en de soort of herkomst van het monster anderzijds. Het is niet duidelijk waarom PFUnA en PFTrA zo regelmatig zijn aangetroffen, omdat er geen gebruik of toepassing van deze stoffen bekend is. Mogelijk speelt de degradatie van precursors tot deze stabiele eindproducten een rol. In wilde vis (marien en zoetwater) zijn PFOS gehalten aangetroffen van 2-150 ng/g ww. Gehalten in bot uit de Westerschelde waren hoger, wat veroorzaakt kan zijn door een (historische) PFOS productie en toepassing van PFOS op producten in het Schelde stroomgebied. PFOS-gehalten in levers waren gemiddeld 5.3 keer hoger dan in de filets van de

bijbehorende vissen. In gekweekte vis is PFOS slechts gedetecteerd in 4 van de 37 onderzochte monsters (*hoofdstuk 4.3 en 4.4*). Verrassenderwijs is PFOS niet aangetroffen in zalm, terwijl in deze vis juist de meeste andere contaminanten wel waren aangetroffen (in vergelijking tot de andere gekweekte soorten). Mogelijk houdt dit verband met de hogere detectielimiet (limit of detection, LOD) voor PFOS (ca. 0.5 ng/g ww) in vergelijking tot bv. de PCBs (LOD 0.005 tot 0.1 ng/g ww). Echter, de specifieke milieuvervuiling en accumulatie van PFOS spelen hier waarschijnlijk ook een rol.
Wanneer de gehalten van de diverse contaminanten met elkaar vergeleken worden, dan lopen de gehalten af in de volgorde: som van de indicator PCBs > PFOS ≈ som van 3 DDTs (p,p'-DDT, p,p'-DDD en p,p'-DDE) > som van 8 PBDEs > hexachloorbenzeen (HCB) ≈ HBCD >> totaal TEQ. Dit is slechts een indicatieve volgorde omdat dit kan afwijken per soort en per contaminant.

Humane blootstelling door visconsumptie

Er is een inschatting gemaakt van de humane blootstelling (door vis consumptie) aan dioxines en dl-PCBs, de som van de indicator PCBs, som van 3 DDTs, HCB, som van 8 PBDEs, α-HBCD en PFOS. De blootstelling is berekend aan de hand van vis consumptie gegevens uit de Nederlandse voedselconsumptiepeiling, vermenigvuldigd met de gemiddeldes van de contaminantgehalten per soort. De voedselconsumptiepeiling stamt uit 1997/1998 en is enigszins gedateerd. Het bevat wel de consumptiegegevens van o.a. haring, zalm, kabeljauw, aal, makreel, mosselen en wilde garnalen, maar gegevens van de nieuwere soorten als pangasius en tilapia zijn niet beschikbaar in deze peiling. Omdat de consumptie van deze soorten snel toeneemt, zijn verkoopgegevens uit 2006 gebruikt om toch een redelijke inschatting te kunnen maken van de consumptie van deze soorten. Hetzelfde geldt voor gekweekte garnalen en forel. Contaminant data die voornamelijk uit <LOD/LOQ waarden bestond is niet in beschouwing genomen. Dit geldt voor bv BDE 183 (wel opgenomen in de EFSA selectie, maar nauwelijks aangetroffen – zie boven), PFOA en β- en γ-HBCD. Aan de andere kant is BDE 49 wel meegenomen omdat het in deze studie in bijna elk vismonster is aangetroffen.
De in deze studie gemaakte schattingen zijn gebaseerd op de blootstelling door consumptie van vis. Andere blootstellingsroutes (bv. zuivel, vlees, groenten etc) zijn buiten beschouwing gelaten. De absolute blootsteling neemt af in de volgende volgorde: som van de indicator PCBs (1.1 ng/kg lichaamsgewicht (bodyweight, bw) per dag > PFOS (1.0 ng/kg bw per dag) > som van 3 DDTs (0.45 ng/kg bw per dag) > som van 8 PBDEs (0.27 ng/kg bw per dag) > HCB (0.09 ng/kg bw per dag) ≈ α-HBCD (0.06 ng/kg bw per dag) >> dioxines en dl-PCBs (0.26 pg totaal-TEQ/kg bw per dag) (*hoofdstuk 4.5*). Hoewel de blootstelling aan dioxines en dl-PCBs het laagst is komt deze wel

het dichtst bij de door de WHO afgeleide toelaatbare dagelijkse inname (tolerable daily intake, TDI) van 2 pg/kg bw per dag. De veiligheidsmarge (als margin of exposure, MOS) is dus erg klein. Deze marge wordt zelfs kleiner wanneer blootstelling uit andere voedingsmiddelen eveneens worden meegenomen in deze inschatting. De humane toxiciteit van niet dl-PCBs wordt momenteel geëvalueerd, en daarom is geen vergelijking met een referentiewaarde gemaakt. De blootstelling aan PFOS is 150 keer lager dan een recente inschatting gemaakt door EFSA voor de Nederlandse bevolking (2008). De twee redenen voor deze lagere inschatting zijn (*hoofdstuk 4.3*): (i) EFSA gebruikte hogere visconsumptie schattingen (5-voud hoger) en (ii) EFSA gebruikte een generieke en hoge inschatting van het PFOS-gehalte in vis in het algemeen (68 ng/g ww), hetgeen veel hoger is dan de gehalten gevonden in deze studie. In de onderhavige studie is specifieke data beschikbaar voor zowel de visconsumptie alsook de PFOS-gehalten per vis, waardoor een meer accurate inschatting gemaakt kon worden van de blootstelling van de Nederlandse bevolking. Wanneer per soort naar de blootstelling van het totaal van contaminanten wordt gekeken, dan domineert haring (41%), gevolgd door zalm (21%) en kabeljauw en soortgelijken (Gadidae) (14%). De andere soorten tezamen dragen 24% bij.

Conclusies en vooruitblik

Deze studie laat zien dat er veelbelovende technieken voor de detectie van dioxines en dl-PCBs beschikbaar zijn (GC-ion trapMS/MS) die een alternatief kunnen vormen voor de huidige gouden standaard GC-HRMS. DR-CALUX en GCxGC-ECD zijn waardevolle screeningstechnieken, maar verdere ontwikkeling is nodig zoals verbetering van juistheid (DR-CALUX) en vermindering van de benodigde tijd voor de manuele piek integratie (GCxGC-ECD). Voor de analyse van HBCD heeft LC-ESI-MS/MS de voorkeur boven GC-ECNI-MS vanwege de betere juistheid en omdat met deze techniek de diastereomeren van elkaar gescheiden kunnen worden. De kwaliteit van de PFC analyses in water en vis heeft erg veel baat gehad bij de groeiende kennis van het gedrag van de PFCs, de grotere verscheidenheid van analytische methodes en de sterk gegroeide (commerciële) beschikbaarheid van goede kwaliteit standaarden en interne standaarden.
Contaminantgehalten gemeten in deze studie waren het hoogst in aal uit vervuilde locaties (bv Haringvliet en Nieuwe Merwede) en het laagst in gekweekte vis monsters (o.a. tilapia en pangasius). Wanneer de contaminanten gerangschikt worden, dan nemen de gehalten als volgt af: som van 7 PCBs > PFOS ≈ som van 3 DDTs > som van 8 PBDEs > HCB ≈ HBCD >> totaal-TEQ. Hoewel de gehalten in aal het hoogst waren, wordt de humane blootstelling gedomineerd door haring en zalm. De bijdrage van nieuwe gekweekte soorten als pangasius en tilapia en garnaal is minder dan 1%. De blootstelling van PFOS wijkt af van die van lipofiele contaminanten.

Toekomstige analytische ontwikkelingen richten zich op (i) de reductie van tijd en arbeid die nodig is voor monstervoorbewerking (extractie en opzuivering van het extract), (ii) het verder verbeteren van de gevoeligheid en selectiviteit van detectietechnieken (veelal gebaseerd op MS), (iii) miniaturisering van analytische technieken, en (iv) verhogen van de snelheid en capaciteit van analytische methodes. Op deze wijze kunnen honderden componenten in een enkele analyse gemeten worden. Aan de andere kant zullen bio-analytische technieken verder ontwikkeld worden omdat deze complementair zijn aan de chemisch analytische technieken. Met deze technieken kunnen onbekende contaminanten opgespoord worden.
Nieuwe contaminanten worden continu ontdekt. De PFCs, nano materialen en siloxanen zijn een recent voorbeeld van contaminanten die de aandacht van wetenschappers en beleidsmakers hebben gekregen. De blootstelling aan traditionele lipofiele contaminanten verloopt voornamelijk via de voeding (i.e. voedsel van dierlijke oorsprong). Deze nieuwe stoffen hebben dermate afwijkende eigenschappen in vergelijking tot de hier behandelde stoffen dat het vraagt om andere analytische benaderwijzen. De afwijkende eigenschappen betekenen ook dat humane blootstelling via diversere wegen kan verlopen zoals voedsel, drinkwater, lucht, stof en via de huid. Dit maakt dat de karakterisering van blootstelling via al deze routes erg bewerkelijk is. De analyse van humane vloeistoffen (bv bloed, melk, urine) kan een geïntegreerd beeld geven van alle blootstellingsroutes en is een waardevolle complementaire aanpak.

Abbreviations

2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
2,3,7,8-TCDF	2,3,7,8-tetrachlorodibenzofuran
AcN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
ASE	Accelerated solvent extraction
BCR	Community bureau of reference
BFR	Brominated flame retardant
Bw	Bodyweight
CE	Collision energy
CID	Collision induced dissociation
CP	Chlorinated paraffin
CRM	Certified reference material
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulfoxide
DNFCS	Dutch national food consumption survey
dl-PCB	Dioxin-like PCB
ECD	Electron capture detection
ECF	Electrochemical fluorination
ECNI	Electron capture negative ionisation
EFSA	European food safety authority
EI	Electron ionization
ESI	Electrospray ionization
FTOH	Fluorotelomer alcohol
GC	Gas chromatography
GCxGC	Comprehensive multidimensional gas chromatography
GFF	Glass fiber filter
GPC	Gel permeation chromatography
HBCD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCBD	Hexachlorobutadiene
HCH	Hexachlorocyclohexane
HxCDD	Hexachlorodibenzo-*p*-dioxin
HxCDF	Hexachlorodibenzofuran
HpCDD	Heptachlorodibenzo-*p*-dioxin
HpCDF	Heptachlorodibenzofuran
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
ILS	Interlaboratory study
IPE	Ion pair extraction
IS	Internal standard
ITMS	Ion trap mass spectrometry

LC	Liquid chromatography
LCCP	Long chain chlorinated paraffin
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LRM	Laboratory reference material
LSE	Liquid solid extraction
LVI	Large volume injection
MCCP	Medium chain chlorinated paraffin
MeOH	Methanol
ML	Maximum level
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl-*tert*-butylether
m/z	Mass-charge-ratio
NCI	Negative chemical ionisation
NOAEL	No observable adverse effect level
OCDD	Octachlorodibenzo-*p*-dioxin
OCDF	Octachlorodibenzofuran
OCP	Organochlorine pesticide
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ether
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PCI	Positive chemical ionisation
PCN	Polychlorinated naphthalene
PeCDD	Pentachlorodibenzo-*p*-dioxin
PeCDF	Pentachlorodibenzofuran
PFAS	Polyfluoroalkylated substance
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonate
PFC	Perfluorinated compound
PFCA	Perfluorocarboxylic acid
PFDA	Perfluorodecanoic acid
PFDS	Perfluorodecane sulfonate
PFDoA	Perfluorododecanoic acid
PFHpA	Perfluoroheptanoic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluoroctane sulfonate
PFOSA	Perfluoroctane sulfonamide

PFPeA	Perfluoropentanoic acid
PFSA	Perfluorinated sufonates
PFTeA	Perfluorotetradecanoic acid
PFTrA	Perfluorotridecanoic acid
PFUnA	Perfluoroundecanoic acid
PLE	Pressurised liquid extraction
POP	Persistent organic pollutant
PUF	Polyurethane foam
QCB	Pentachlorobenzene
QQQ	Triple quadrupole
RSD	Relative standard deviation
SAQ	Standard addition quantification
SBCCQ	Solvent based calibration curve quantification
SCCP	Short chain chlorinated paraffin
SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid phase extraction
SRM	Standard reference material
SS	Standard solution
TBBP-A	Tetrabromobisphenol-A
TCDD	tetrachlorodibenzo-*p*-dioxin
TCDF	tetrachlorodibenzofuran
TDI	Tolerable Daily Intake
TEF	Toxic equivalency factors
TEQ	Toxic equivalents
TH-PFOS	Tetrahydropolyfluorooctanesulfonate
TOFMS	Time of flight mass spectrometry
WHO	World Health Organisation
ww	Wet weight

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Dankwoord

Het is af. Hier ligt het dan. Na veel lange avonden en soms korte nachten is mijn proefschrift klaar. Het avontuur begon drie jaar geleden met de gedachte om de resultaten van een aantal studies bij elkaar te brengen. Om het een mooi geheel te maken is het uitgebreid met nog enkele studies, en nog wat, en nog wat…. En dat alles ligt nu hier voor me.

Ook ik heb het niet alleen gedaan. Ik heb met veel mensen van binnen en buiten het instituut samengewerkt. Het is een genoegen geweest om met al deze getalenteerde en gedreven collega's te werken aan een breed scala van onderwerpen. Al die mensen verdienen een bedankje.

Allereerst Jacob, een groot dank-je-wel voor jou. We werken nu zo'n negen jaar samen en ik heb erg veel van je geleerd. Of het nu de analyse van contaminanten is, het schrijven van een goed onderzoeksvoorstel of publicatie, of enkele voetstappen op het managementpad, ik heb steeds bij je binnen kunnen lopen voor advies. Je weet goede wetenschap te combineren met zakelijkheid, en daarbij creëer je ook nog een prettige omgeving om in te werken. Voor mij is het erg stimulerend om met je samen te werken. Ondanks je drukke bestaan heb jij hier ook flink wat uren in gestopt om de stukken van commentaar te voorzien en met suggesties te komen om het geheel te verbeteren. Nogmaals dank!!

En Pim, ook jij natuurlijk heel veel dank. Het is een plezier om met je samen te werken. Altijd kan ik even binnen lopen en meestal loop ik dan weer met een paar ideeën naar buiten. Van een paar stukken in dit proefschrift kunnen we nog mooie publicaties maken. Daarnaast weet je ook erg goed je weg te vinden in grote kluwen data. Na een uurtje stoeien met de gegevens rollen er dan opeens mooie profielen en PCA plots uit. Bedankt!!

En dan mijn andere C&B collega's. Ike, met jou heb ik al lang samengewerkt. Je bent op dezelfde dag begonnen op het RIVO en samen naar het IVM gegaan. Ik kan blind vertrouwen op de getallen die ik van je krijg. Aan een half woord heb je vaak al genoeg. En het is plezierig samenwerken met jou, maar dat kan ook niet anders want jij bent ook zo'n Myers-Briggs ESTP typetje (of was het nou ESTJ?).

En natuurlijk veel dank voor labhoofd Bert die zorgt dat alles altijd voor elkaar komt, Kees (de SMF tornado op de fiets), Sicco en Martin (samen sleutelen aan een lekkende GC injector in Nairobi – yes!! eindelijk scherpe pieken), Petra, Gerda, Riet, Jacco, de biojongens Peter en Jorke, de PhD's Eszter, Thijs en Adriana en alle stagiaires en gastmedewerkers. Het is altijd leuk om het lab binnen te lopen of om 3 uur met z'n allen koffie te drinken.

En niet te vergeten de collega wetenschappers. Marja zullen we binnenkort weer een voorstel in elkaar draaien? Jammer dat we het niet gehaald hadden, maar het ging als een trein. Timo en Juliette (de andere wijzen uit het Oosten), op vrijdagnacht 00.30 uur met z'n allen on-line om aan het Obelix voorstel te werken, en het was het waard. En ook Jan Willem, Bert, Heather en Harry. Dank jullie allemaal voor de lol, de inspiratie, het samenwerken en nog veel meer!!

Goede herinneringen bewaar ik ook aan de ex-collega's van IMARES. Judith, Quy, Marco, Michiel, Peter, Christiaan, Gerda, Evert en alle anderen. Zonder jullie goede inzet was een deel van dit werk niet mogelijk geweest. En het was erg leuk met jullie samen te werken. Daar doet een scheiding niets aan af.

I had the luck and pleasure to collaborate with several great (inter)national colleagues. Some parts of this work would not have been possible without your contributions. Special thanks to all (inter)national co-authors. I enjoyed the stimulating discussions at several meetings. These meetings brought me to great places like Lyon, Toronto, Tromsø, Stockholm, Barcelona, Nairobi etc. I hope to meet you all again soon.

Dit proefschrift had er niet zo mooi uitgezien als Rachel er niet bij betrokken was geraakt. Je hebt er een mooi boekje van gemaakt!! And Annika, thanks for the creating the wonderful dietary exposure picture.

Gelukkig is er naast werk ook nog een privé. Dat heb ik wel een beetje verwaarloosd de laatste tijd, maar dat halen we wel weer in. Ik begin bij mijn vrienden. Samen op vakantie, Oerol, naar het strand, eten, naar de kermis, borrelen en wandelen. Goede gesprekken over hobbies, werk, het leven en de laatste jaren vooral ook over de kroost. De WHW vakantie is elk jaar een mannen-onder-elkaar hoogtepuntje. Dat gaan we natuurlijk gewoon blijven volhouden tot we 85 zijn! Als kersverse Arnhemmer ga ik jullie -Haarlemmers- natuurlijk niet vergeten. De plannen voor korte vakanties in Haarlem zijn al gemaakt!

De Eindhovenseweg is een belangrijke plek. In een ruim en warm nest ben ik opgegroeid en gevormd, tussen de koeien, het gras en de immer volle theepot. Pap en mam, heel veel dank voor die goeie tijd op de Eindhovenseweg en het vertrouwen dat jullie in me hebben gehad. Ik beloof dat ik weer vaker een tas thee kom drinken. Heel veel plezier heb ik ook gehad met al mijn broers en zussen. Frans, Adelien, Gerie, Carla, René (ik kom weer langs voor een rondje in de duinen), Sjanette, Wilma (zet hem op he!!) en 's Jeroen. En jullie aanhang en kroost niet te vergeten. Dank!!

Maar het allerbelangrijkste, het allermooiste en het allerliefste zijn die drie lieve meiden. Lieve Jolanda, heel veel dank voor je aanmoedigingen, je steun en je geduld. Je bent een enorme lieverd!! Samen kunnen we de hele wereld aan. Ik ben nu klaar, mijn laptop gaat uit. Nog een paar dagen en dan komt er weer wat rust in mijn hoofd. Dan ben ik weer wat vaker ook geestelijk aanwezig in plaats van af en toe op te kijken vanachter het schermpje. We gaan nog heel lang genieten van elkaar en van onze twee möpkes, Pieneke en Sammie. Jullie zijn echt de allerleukste hobby die ik ooit heb gehad. Wat een feest om met jullie te spelen, daar kan echt niks tegenop. Dikke kus!!

Stefan

Colophon

ISBN:
978-90-8891-108-8

Cover design and lay-out:
Rachel van Esschoten (Divingduck, www.divingduck.nl)

Photography:
Sieto Verver (p. 9 bottom, p. 29 bottom, p. 301 bottom, p. 319 bottom)
www.Istockphoto.com (all other photos)

Printed by:
BOX Press, Oisterwijk

The printing of this thesis was financially supported by Wellington Laboratories.

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