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Persistent organic pollutants (POPs) and per- and polyfluoroalkyl substances (PFASs) present in human breast milk, and maternal sea food intake as a potential source of PFOS in breast milk.

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Abstract

Breast milk is both an important and uniquely composed source of nutrition to babies and an elimination route of chemical contaminants for the maternal body. It may therefore contain chemicals to which the mother has been exposed; chemicals which may be associated with unfavourable health outcomes in the nursing children. In a public health perspective, it is of great interest to gain more knowledge about associations both between maternal exposure to chemicals and presence of chemicals in breast milk, and between presence of chemicals in breast milk and potential health outcomes in breastfed children in order to give recommendations regarding breastfeeding and to apply other preventive measures.

Sea food is known to be an important source of chemical contaminants, and in this study, concentrations for a broad selection of lipid soluble POPs and PFAS in human breast milk samples were measured in a laboratory. In addition, statistical analysis was performed to look for an association between maternal sea food intake and PFOS levels in breast milk. Several different POPs and PFAS were found in all samples. A statistically significant positive association with maternal sea food consumption and PFOS levels in breast milk was found in a bivariate analysis for crabs, with breaking point at 2 annually crab meals ($p = 0.0114$), but not for lean fish, fatty fish and total fish consumption. This positive association was also seen in the regression analysis adjusted for confounders, however not statistically significant ($\beta = 0.49$, $p = 0.341$) Statistically significant associations were also found for the confounders maternal age, education and birth year.

Preface

I would like to thank, first of all, my two supervisors Jan Ludvig Lyche at NMBU and Merete Åse Eggesbø at NIPH for accepting me as a master student and helping me to find a suitable project within the field of environmental contaminants and health which included laboratory work, as I wished for.

The engineers Gabrielle Haddad-Weiser and Mariana Paz at NMBU have been guiding me safely through the laboratory analyses, and PhD student Erik Magnus Ræder helped me do the HPLC analysis of PFAS. Hein Stigum at FHI has been indispensable in doing statistical analysis. A thank is also directed to Anuschka Polder for sharing from her long time acquired knowledge about breast milk studies, and to everyone else in the MT lab for welcoming and helping me with this master thesis.

I appreciate the support, patience and encouragement from my family which made it possible for me to start and complete this master degree in Public Health Science. How adorable aren't the kids when they say that mummy needs peace and quiet in order to do her homework:)

I would also like to thank Jordmorforeningen for financial support and interest in my project.

"Something will grow from all you are going through, and it will be you"

To all the people I have met on my way, how grateful I am to have you all in my life! How you bear out with my prejudices and questions, my weird and unconventional thoughts, my crazy ideas. How you inspire me, challenge me and my beliefs, break me down and build me up, how you help me see and understand the parts in their context, to broaden my view.

What an amazing journey it has been to study again as an adult, to combine a realistic mindset with a humanistic in order to see the world with a more holistic approach. May I never stop eager for more knowledge and understanding, may I inspire others to seek the same and may I be able to use my knowledge and wisdom to make this world a better place.

Stabekk, 14. august 2021

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Innholdsfortegnelse

Abbreviations

BFR	Brominated flame retardants
CHX:AC	Cyclohexane: acetone mix
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
EFSA	European Food Safety Authority
GC(-MS)	Gas chromatography (- mass spectrometry)
HBDC	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HPLC(-MS)	High prerformance liquid chromatography (- mass spectrometry)
HUMIS	The Norwegian breast milk study
LB	Lower bound concentration (in the EFSA report by using zero for non-detected levels)
LC	Liquid chromaytography
MS	Mass spectrometry
MT	“Miljøtokslabben”, Laboratory of environmental toxicology at NMBU
NIPH	Norwegian Institute of Public Health
NMBU	Norwegian University of Life Science
PBB	Polybrominated biphenyls
PBDE /BDE	Polybrominated diphenyl ethers / Brominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PFASs	Per- and polyfluoroalkyl substances
PFOA	Perfluorooctanoic acid

PFOS	Perfluorooctanesulfonic acid
POPs	Persistent Organic Pollutants
PP	Polypropylene
UB	Upper bound concentration (in the EFSA report by using 93 LOQ/LOD in case of non-detected levels)
WHO	World Health Organisation

1 Introduction

“A good childhood will last for a lifetime. We are formed the most in our youngest years. To contribute to give all children a good start in life, is the most important we can do as a community.” (Det Kongelige Kunnskapsdepartement, 2013). This is how the Proposition to the Parliament about kindergartens starts. It is reasonable to think that it’s not only the social and physical environment in which the children spend their time that is important for their development and health, but also their exposure to chemical agents.

It is a general public opinion that “breastfeeding is best for the baby” and contributes to give the child a good start in life. Both WHO and Norwegian health authorities recommend to exclusively feed infants with breast milk for the first six months and then continue breastfeeding as a supplement until the child is one year or more (Meltzer et al., 2016; WHO).

Breast milk is an important and uniquely composed source of nutrition to newborns and babies. The milk is highly customized to meet the needs of the baby, both nutritional and developmental. Unfortunately, since breast milk is an elimination route for chemical contaminants, the milk will also contain pollutants to which the mother has been exposed. Of special concern are the persistent toxicants (persistent organic pollutants, POPs) which accumulate in maternal tissues throughout the woman’s lifetime and then are being transferred from mother to child during pregnancy and breastfeeding (Meltzer et al., 2016).

Several different POPs have been detected in human breast milk in studies from all over the world (Aerts et al., 2019; Forns et al., 2020; Kang et al., 2016; Müller et al., 2017; Someya et al., 2010). The presence of POPs in breast milk can be linked to both observed health outcomes in children and to maternal exposure from diet and other sources. Recently, EFSA published a scientific evaluation on the risks to human health related to the presence of PFASs in food. The report concludes that “fish and other sea food” are the most important contributors to the mean lower bound (LB) exposure of PFOA and PFOS, followed by ”eggs and egg products”, “meat and meat products”, “fruit and fruit products” (Schrenk et al., 2020). However, due to beneficial compounds in fish, especially the long chain ω-3 fatty acids, the Norwegian authorities generally recommend fish for dinner 2-3 times per week and as bread spread several times per week. It should be consumed as a mix of different fish species, both oily and lean fish (Matportalen, 17.03.2011).

Children are known to be particularly susceptible when it comes to adverse health outcomes as a result of chemical exposure. Their behaviour and the fact that they breathe more air, eat

more food and drink more water per unit body weight than adults often lead to a higher exposure. Due to developing organs and immature biological defences they may be less able to withstand exposure effects. In addition, exposure to chemical agents may interfere with normal development and growth, and with many years left to live, delayed toxic reactions are more likely to occur in people exposed in early life (Frumkin, 2016).

Rachel Carson argued in her book *Silent Spring* (2002, first edition 1963) for public health and environment, humans and nature, being inseparable, and called for a public debate about the risks of hazardous technologies. The book states that bodies are not boundaries – humans too are permeable. Chemical corruption of the globe affects us from conception to death; "Like the constant dripping of water that in turn wears away the hardest stone, this birth-to-death contact with dangerous chemicals may in the end prove disastrous. Each of these recurrent exposures, no matter how slight, contributes to the progressive build-up of chemicals in our bodies and so to cumulative poisoning."

This book, *Silent Spring*, has its main focus on pesticides, but it's reasonable to apply this mindset also to other chemicals we are exposed to. Paracelcus' famous statement "All things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing poison", should remind us to act carefully. We should also keep in mind the fact that we are exposed to a cocktail of chemicals which may interact and amplify the consequences in our bodies. Still, what we see, is a constant entry of new and modified compounds into the market, often with little or no knowledge about how they affect ecosystems and human health (Meltzer et al., 2016). Also, due to persistency, previously used compounds and their metabolites are still present in our environment. As Rachel Carson called for in her book *Silent Spring* (2002), chemicals like DDT are found "in fish in mountain lakes, in earthworms in soil, in eggs of birds and in humans, including the mother's milk and probably the tissues of the unborn child".

As a response to this global problem of persistent organic pollutants, the Stockholm Convention was adopted in 2001 (entered into force in 2004). The aim of the convention is to "protect human health and the environment from chemicals that remain intact in the environment for long periods, become widely distributed geographically, accumulate in the fatty tissue of humans and wildlife, and have harmful impacts on human health or on the environment." The parties are committed to take measures to eliminate or reduce the release of POPs into the environment (UN Stockholm Convention, 2019).

Knowledge about associations between exposure/sources to chemical pollutants and health outcomes is important in a public health context when it comes to assessing preventive measures, and it may provide basis for recommendations regarding breastfeeding (Meltzer et al., 2016), like maternal diet and duration of breast feeding. On an individual level, however, there are many other factors which affect concentrations of chemicals in breast milk of a mother and health outcomes in the child.

In this master thesis work, 90 samples with human breast milk from 60 mothers in the HUMIS study run by Norwegian Institute of Public Health (NIPH) were analysed with respect to fat soluble POPs (30 milk samples) and protein binding PFAS (60 milk samples). It was decided to proceed with statistical analyses on PFOS results only, and also include data from 1383 milk samples analysed previously with respect to PFOS (in total 1428 after removal of duplicates). In the statistical analyses, 544 samples were selected to look for potential associations between maternal sea food intake and PFOS concentrations in the milk by combining laboratory findings and information gained from questionnaires (Attachment 1).

In this thesis, some material from my own term paper "*Associations between exposure to persistent organic pollutants (POPs) through breast milk and health outcomes in children*" and the project plan associated with this master thesis has been reused.

As a part of this master thesis, an attempt will be made to have an article published in a scientific publication. A draft for this article, with the preliminary title *Association between maternal sea food consumption and PFOS concentration in human breast milk* can be found in attachment B.

2 Aim

Several studies show that sea food is a common source of many environmental toxicants and that there is or may be an association between maternal sea food intake and health outcomes in their nursed children (Stratakis et al., 2016). Still more knowledge is being requested, and the recently published EFSA report (Schrenk et al., 2020), which concludes that “fish and other sea food” is the main contributor to several PFAS, draws even more attention into the field of environmental pollutants from human activity and how it hits back on us.

The aim of this work is to contribute to increase the knowledge regarding levels of and sources to environmental pollutants in breast milk with the specific approach being to investigate whether or not maternal sea food consumption can be linked to observed PFOS levels. Milk samples and questionnaires from Norwegians mothers participating in the HUMIS study form the study material. As stated in the Introduction, this is important knowledge in a public health perspective in order to create a scientific basis for recommendations regarding breastfeeding.

3 Background

3.1 Breast milk

Breast milk is an important and uniquely composed source of nutrition to newborns and babies. The milk is highly customized to meet the needs in the baby, both nutritional and developmental (Meltzer et al., 2016).

In Norway, 80% of the infants are breastfed at six months. Norwegian authorities recommend to exclusively feed infants with breast milk for the first six months and a total duration of twelve months (Meltzer et al., 2016).

In addition to nutrition, breast milk also contains components like growth hormones, factors with anti-microbial and ani-inflammatory properties, and components which stimulate maturation of the immune system in the infants. These components are not present in infant formula milk (Meltzer et al., 2016), making breast milk a unique source of both nutrition and factors aiding child's development.

But breast milk is also an elimination route for chemicals from the maternal body. Therefore, milk will contain pollutants to which the mother has been exposed, and their metabolites. Of special concern are the persistent toxicants (persistent organic pollutants, POPs) which are stored in maternal tissues throughout the woman's lifetime and then transferred from mother to child during breastfeeding. A recent breast milk study found that PFAS contamination of breast milk is widely spread in the US, and the co-author Erica Schreder stated to the Guardian that "these harmful chemicals are contaminating what should be nature's perfect food" (Perkins, 2021; Zheng et al., 2021).

3.1.1 Chemical composition of breast milk

To explain why certain compounds are found in breast milk, the composition of the milk, the physio-chemical properties of the compounds and the toxicokinetic of the compounds need to be understood. The milk composition varies both inter- and intra-individually. The postpartum milk contains a high level of proteins (10%) and a low level of lipids (1%). In mature milk the lipid content will increase (4%) and the protein content will decrease (1%). As a consequence, the transfer of highly lipophilic chemicals is less pronounced in early lactation while transfer of protein binding chemicals may be more pronounced at the same time. In later lactation, when lipid content increase and protein content decrease, the situation will be the opposite. Also, in each individual, the first milk in every nursing will contain less lipids and therefore

less lipophilic substances compared to the milk later in the nursing (Clewell & Gearhart, 2002).

The transfer of a compound to the milk is dependent on its lipid solubility, degree of ionization, molecular weight and ability to bind to maternal blood and / or milk components. Maternal characteristics like degree of exposure, physiology, adipose tissue level, nursing routine, age and parity (number of pregnancies) are also of importance. Lipophilic substances will be partitioned into fatty tissue, where they can be stored for a long time due to low blood flow and limited tissue mass turnover. In case of weight loss, the stored chemicals can be released back to the blood stream, from where they can be taken up into fat in the mammary glands and therefrom be transferred to the milk (Clewell & Gearhart, 2002).

3.2 Persistent organic pollutants (POPs)

POPs are synthetic organic compounds which, due to their physical-chemical properties, are resistant to metabolism and elimination from the body (Jansen et al., 2020). They contain carbon atoms which are able to form stable bonds with each other to create long chains and ring molecules, thereby making persistent compounds. Stable bonds can also be formed with nitrogen, oxygen and hydrogen. The properties of organic molecules depend upon molecular structure, size and shape, and the presence of functional groups. The latter is of great importance when it comes to metabolism and toxicity as functional groups like OH, HCO and NO_2 will make the compound more polar and thereby more chemical reactive (Walker, 2012).

Unfortunately, POPs have found and find their way into the natural environment and can be found all over the earth, including places far away from site of origin and human habitation. Typical sources are pesticides, by-products of industrial processes, industrial chemicals, paint, combustion of fossil fuel and waste, and plastic products. They may evaporate and be transported with air currents to cold areas, where they precipitate and thereby end up in places like Arctic and Antarctic. POPs may also be transported with water systems and water streams and with migrating animals like fish, birds and fish eating mammals (Bernes, 1998).

3.2.1 Classic fat-soluble POPs: PCBs, chlorinated pesticides (DDTs, HCHs, chlordane) and bromated flame retardants (BRFs)

The fat-soluble POPs include a wide range of different compounds which are all fat soluble and will bind to fat in the human body and other organisms. This leads to bioaccumulation and biomagnification in the food chain (Frumkin, 2016).

PCBs (polychlorinated biphenyls) are stable, unreactive, viscous liquids of low volatility. They are used as coolants, plasticizers in paint, insulation fluids in transformers and hydraulic fluids. PCB mixtures are highly soluble in oils and organic solvents of low polarity and poorly soluble in water. The production and use of PCBs are internationally banned according to the Stockholm Convention (UN Stockholm Convention, 2019) and careless disposal and dumping are the main current sources of spread of PCBs to the nature (Walker, 2012).

There are 209 different PCB congeners (isomers), of which 13 exhibit a dioxin like (dl) toxicity. According to the Stockholm Convention, PCBs are toxic to fish, and acute effects such as increased mortality occur at high doses whereas chronic effects such as spawning failures occur at lower doses. In wild animals, like seal and mink, PCBs are linked to reproductive failure and immune system suppression. Their degree of persistence increases with the degree of chlorination and half-lives vary from 10 days to 1 ½ years (UN Stockholm Convention, 2019). The position of the chlorine atoms are also of importance when it comes to toxic properties and persistence (Bernes, 1998).

Further, the convention states that human exposure to high doses of PCBs through food contamination has caused acute toxic effects such as pigmentation of nails and mucous membranes, swelling of eye lids, fatigue, nausea and womiting. Children born by mothers exposed to high amounts of PCBs showed choric effects such as poor short-term memory function and other delayed behavioral problems (UN Stockholm Convention, 2019), increased prevalence of infection and lesions in reproductive organs (Guo et al., 1995).

The GreenFacts initiative fact sheet about PCBs states that animal testing has shown that PCBs are readily absorbed through the digestive tract after being swallowed and to a less extent through the skin. Although PCB levels in food have decreased since the late 70's, the main source of human exposure is consumption of contaminated foods, particularly meat, fish, and poultry. The compounds are lipid-soluble and will diffuse across cell membranes to enter the blood stream and lymphatic system. In the body, the compounds will undergo transformations to persistent metabolites or to water-soluble substances depending on the structure of the mother compound. PCBs and metabolites, and especially those with a greater number of chlorine atoms, are readily soluble in fat and tend to accumulate in tissues with high fat content, like liver, brain and skin. Water-soluble metabolites may combine with glutathione and glucuronic acid forming substances which are excreted into urine and faeces. PCBs pass into the placenta, umbilical cord and breast milk (Greenfacts, 2003) thereby exposing the fetus via the placenta and the suckling baby through mother's milk.

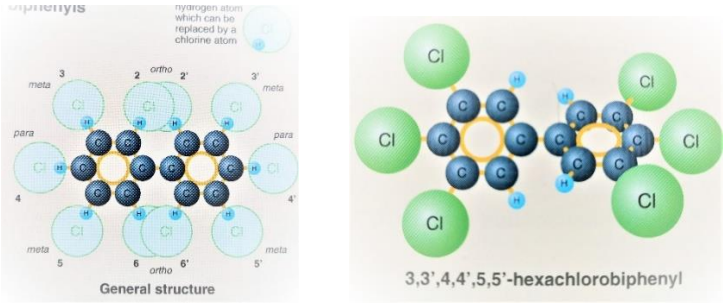


Figure 1 General structure for PCBs and an example, PCB-169 (Bernes, 1998)

DDT (dichlorodiphenyltrichloroethane) is an insecticide which came into large scale use after the second world war (Bernes, 1998). Similar to PCBs, it was also discovered that DDT was resistant to degradation in nature and living organisms, accumulated in animal and human tissues, and had toxic properties (Frumkin, 2016). DDT was banned in several countries in the 1970's, and internationally in 2004 when it was listed in the Stockholm convention, but are still in use in some countries to fight malaria (UN Stockholm Convention, 2019). In living organisms, DDT is gradually metabolized to **DDD** and **DDE**. DDD is relatively rapidly excreted from the body via urine, while DDE degrades very slowly and accumulates in body fat for years. DDD and DDE exhibits toxic effects similar to its mother compound. In areas where DDT is banned, DDE is the most common form of DDT to be found in wildlife and the human population (Bernes, 1998).

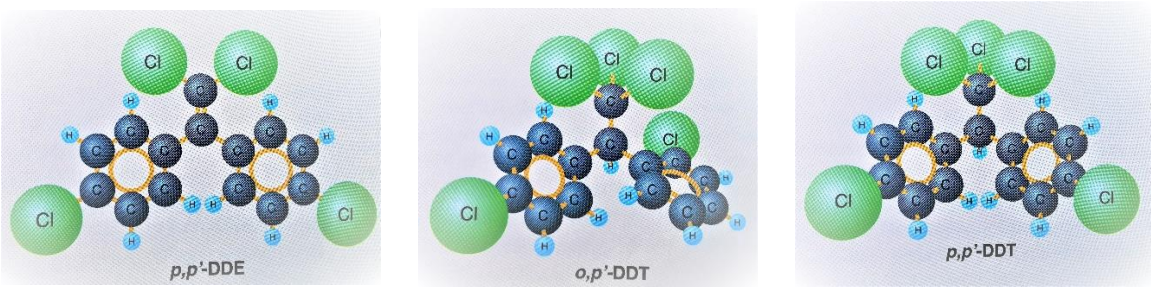


Figure 2 DDT exists in the form of different isomers (Bernes, 1998)

Chlordane is an insecticide which consists of a mixture of more than 140 different components, ***cis*- and *trans*-chlordane**, **heptachlor** and **trans-chlordane** being some of them. ***Trans*-nonachlor** is particularly persistent while heptachlor and the chlordane compounds are metabolized more readily, including to heptachlor-*exo*-epoxide and oxychlordane, respectively. These metabolites are more persistent, and in some cases also more toxic (Bernes, 1998). Chlordane may enter the body through the skin, respiratory system

and orally through diet and accumulate in fat, and the pharmacologist Dr. Lehman described in 1950 chlordane as "one of the most toxic of insecticides – anyone handling it could be poisoned" (Carson, 2002). According to the Stockholm Convention, this broad-spectrum insecticide may have effects on the human immune system and is classified as a possible human carcinogen. Lethal effects on animals vary among the species, but tests have shown that it can kill mallard ducks, bobwhite quail and pink shrimp (UN Stockholm Convention, 2019).

HCH (hexachlorocyclohexane) is another widely spread insecticide. It exists as several isomers, of which **α-HBH**, **β-HCH** and **γ-HCH** (lindane) are the most significant ones. γ-HCH is less persistent, but more toxic to insects compared to the two other isomers. Still, it is toxic enough to induce undesirable effects also in aquatic organisms and terrestrial animals, so effects on environment and health are to be considered (Bernes, 1998). The three HCH isomers are all in the list of the Stockholm Convention (UN Stockholm Convention, 2019). According to this convention, the production of γ-HCH has decreased rapidly, but a few countries still produce the compound as a pharmaceutical to control head lice and scabies. Further, HCH is persistent and biomagnify in the food chain. Toxic effects are seen on the immunological, reproductive and developmental systems. α-HCH and β-HCH are nowadays produced only unintentionally as a by-product in γ-HCH production. Both are subject to long range transport and are highly persistent in water in colder areas, where they accumulate and biomagnify in arctic food webs. α-HCH and β-HCH are classified as potentially carcinogenic to humans and both compounds also adversely affect wildlife (UN Stockholm Convention, 2019).

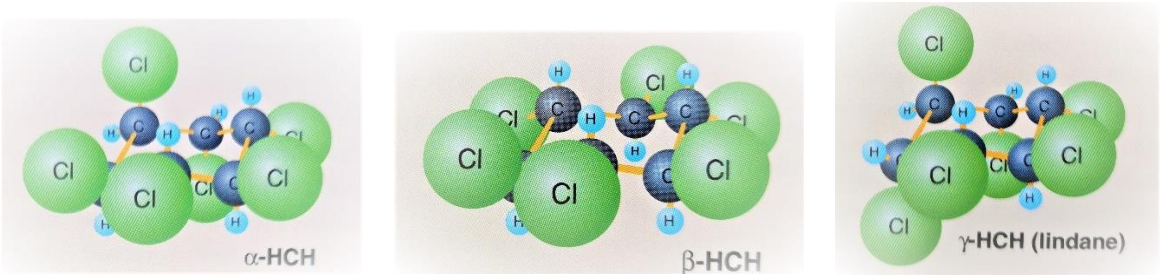


Figure 3 The three HCH isoforms (Bernes, 1998)

HCB (hexachlorobenzene) is a pesticide introduced in 1945 as a fungicide for seeds, now regulated by the Stockholm Convention. According to this convention, HCB is also a by-product of the manufacture of certain industrial chemicals and an impurity in some pesticide

formulations. The compound has been found in any kind of food and is transferred from mother to the fetus through placenta and to infant from breast milk. In high doses, HCB is lethal to animals, and in lower doses it may impair the reproductive ability. From Turkey (1954-59), there is reported a variety of symptoms like skin lesions photosensitivity, colic, debilitation, the metabolic disorder porphyria turcica and death (14%) after eating HCB treated grain (UN Stockholm Convention, 2019).

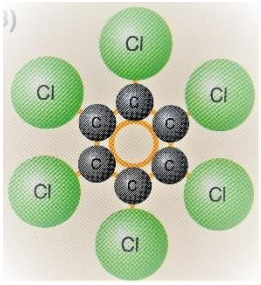


Figure 4 Structure of HCB (Bernes, 1998)

Mirex is an insecticide under regulation by the Stockholm Convention, used mainly to combat fire ants, but also other ants and termites. Other areas of use are as fire retardant in plastics, rubber and electrical goods. Laboratory studies with animals indicate that the compound might be carcinogenic to humans, but direct exposure to Mirex does not appear to cause injury to humans. It proved toxic to several plant species, fish and crustaceans. With a half-life up to 10 years, Mirex is one of the most stable and persistent pesticides. Food like meat, fish and wild game is the main route for exposure for humans (UN Stockholm Convention, 2019).

BFRs (brominated flame retardants) are compounds used to reduce the risk of ignition and spread of fire (Abbasi et al., 2015), and may make up 10 - 30% of the plastic in products like computers and other electronic equipment (Bernes, 1998). Furnitures like couches and chairs, and curtains are other products commonly to contain BFRs (Abbasi et al., 2015). The use of these chemicals significantly increased in the 1970s and 1980s in line with the growing production of computers.

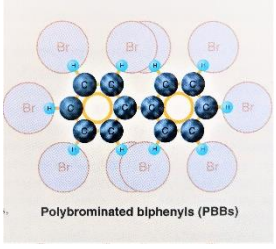


Figure 5 General structure for the BRF subgroup PBBs

The first BFRs to be introduced were the **PBBs**, in which hydrogen atoms are changed for bromine, similar as for chlorine in PCBs. These compounds may escape into the environment where they behave in a similar way as the PCBs (Bernes, 1998).

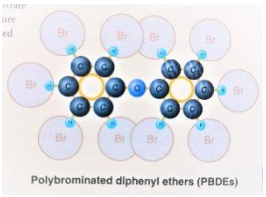


Figure 6 General structure for the BFR subgroup PBDE

PBDE (often shortened to BDE) is another group of BFRs which consists of 209 possible congeners, with the highly brominated ones being the most commonly used. PBDEs with four and five bromine atoms tend to accumulate the easiest in living organisms, while the more brominated ones are less taken up. They may however degrade to molecules with less bromine atoms to become more bioaccumulating (Bernes, 1998). The isomers most commonly found in humans are tetra-, penta-, and hexa-BDEs (Linares et al., 2015), all being regulated by the Stockholm Convention which states that the compounds are highly persistent in the environment, bioaccumulate and have a high potential for long-range environmental transport. There is evidence for their potential toxic effects in wildlife, including mammals (UN Stockholm Convention, 2019). A review study looking into human exposure to PBDE and critical health hazards found that animal studies suggests that exposure to PBDEs is likely to induce thyroid homeostasis disruption, neurodevelopmental deficits, reproductive changes, and even cancer. Results from experimental animal studies and epidemiological observations in humans suggest that PBDEs may be developmental neurotoxicants. It also states that exposure to PBDEs during pregnancy and early life, when organs and biological systems are developing, may cause long-lasting behavioural abnormalities, particularly on motor activity and cognition. Body burden of PBDEs is three- to ninefold higher in infants and toddlers compared to adults, this may be due to exposure via maternal milk and through dust (Linares et al., 2015).

3.2.2 Per- and polyfluoroalkyl substances (PFAS)

PFAS is a group of more than 9000 distinct chemicals compounds of anthropogenic origin detected in higher levels in populations in industrialized and urbanized areas compared to rural areas without industrial activity. Structurally, PFAS are characterized by a fully fluorinated carbon chain attached to a polar tail consisting of a functional group like carboxylate, sulfonate and sulphonamide. Due to their amphiphilic nature (both lipophilic and hydrophilic), PFAS have surface active properties which make them suitable for industrial use as surfactants, emulsifiers, food packaging, non-stick pan coatings, fire extinguishers, water-, stain- and grease repellents, in textiles, electronic equipment, cosmetics, cleaning agents and paper (Antignac et al., 2013; Forns et al., 2015; Zheng et al., 2021).

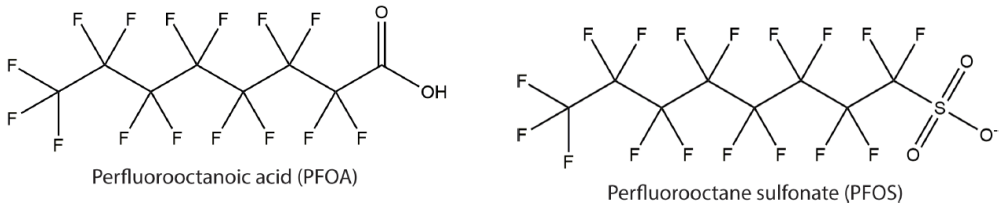


Figure 7 Chemical structure of PFOA and PFOS (Post et al., 2017)

The strength in the C-F bond makes PFAS persistent in nature and biota, thereby making them subject to bioaccumulation and toxicity to wildlife and humans (Antignac et al., 2013; Zheng et al., 2021). Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are two of the most studied compounds in this group, both being listed in the Stockholm convention, in 2009 and 2019, respectively (UN Stockholm Convention, 2019). Decreased body burdens of PFOA and PFOS is reported in the following years (Haug, Line S. et al., 2009). However, due to scientific data showing long half-lives of PFOS and PFAS combined with present use of less studied unregulated fluorinated compounds, the potential harmful exposure to PFASs will continue in the future and remain an unsolved global concern (Forns et al., 2015).

PFASs is absorbed easily after oral exposure and eliminated slowly from the body. The main routes of exposure to PFASs are via inhalation of contaminated air or by ingestion of contaminated water or food. In contrast to other classical lipophilic POPs, PFASs do not typically accumulate in lipids. Instead, they bind to serum proteins and proteins in tissues, thereby accumulating in serum and liver. They are metabolised only to a small extent. PFOS is excreted predominantly in urine and to a lesser extent to faeces, while renal clearance of PFOA is almost negligible. Both PFOS and PFOA are being detected in human milk. Estimated half-lives for PFOS and PFOA in humans are 8.7 and 3.8 - 4.4 years respectively (Forns et al., 2015; PHE Centre for Radiation Chemical and Environmental Hazards, 2017).

3.3 Studies on POPs and human breast milk

A vast scientific literature has documented that human breast milk contains potentially harmful chemicals (Colles et al., 2008; Haraguchi et al., 2009; Zheng et al., 2021), consequently exposing children to POPs already at an early life stage through breastfeeding (Cariou et al., 2015).

Epidemiological studies have shown associations between PFAS exposure and a variety of health effects. Altered immune functions, like reduced antibody response after child vaccination (Abraham et al., 2020; Grandjean et al., 2012; Granum et al., 2013) and increased prevalence of child infections, asthma, rhinitis and atopic dermatitis (Goudarzi et al., 2017; Kvalem et al., 2020), has been seen in association with prenatal and postnatal exposure to PFAS. Positive associations between prevalence and incidence of chronic autoimmune outcomes and PFAS exposure have been found for ulcerative colitis and rheumatoid arthritis (Steenland et al., 2015) and for a variety of thyroid function alterations (Jain, 2013; Melzer et al., 2010; Webster et al., 2014). As the liver is a primary target organ for storage of PFASs, it should be no surprise that toxic liver effects like altered levels of enzymes and other biomarkers, oxidative stress and apoptosis, hepatocellular adenomas and carcinomas, disrupted fatty acid transport and steatosis are seen across species (Bassler et al., 2019; Filgo et al., 2015; Guillette et al., 2020; Huang et al., 2013; Hui et al., 2017; Pérez et al., 2013; Xu et al., 2016). In the article *Exposure to Perfluorinated Alkyl Substances and Health Outcomes in Children: A Systematic Review of the Epidemiologic Literature*, the authors have reviewed 64 studies on PFAS exposure and health outcomes in children. Although there were a limited number of studies for any particular health outcome, it was documented evidence for positive associations between PFAS and dyslipidaemia, changes in immune response (including vaccine response and asthma), renal function and age at menarche (Rappazzo et al., 2017).

For lipid soluble POPs in breast milk, to mention a few, associations have been found between PCB-153 and reduced growth (Iszatt et al., 2015), several POPs and changes in gut bacterial flora (Iszatt et al., 2019) , β-HCH and slowed growth (Criswell R1, 2017) and increased risk for ADHD (Lenters et al., 2019). A review study on the status of PBDE found that early childhood exposure may induce neurobehavioral, developmental and reproductive effects (Linares et al., 2015).

3.3.1 Associations between fish intake and POPs concentration in breast milk

Previous research concerning POPs in human breast milk and fish intake, indicates an association between the two variables. In milk samples from women in Kolkata, POPs were found in all of them, in concentrations significantly increasing with residence time. For dioxin-like PCBs and total PCBs, the concentrations were significantly higher in site samples compared to reference samples. The study also revealed that dioxin-like PCBs occurred at a notably higher concentration in fish from the sample site compared to from the reference site,

indicating fish to be a potential source of PCB for people living near the dumpster place in Kolkata (Someya et al., 2010).

Another study showed that women from Mohawk Nation-territory, US giving birth between 1986 and 1989 had significant higher concentrations of total PCBs in their milk compared to women in the control group. From 1990 this phenomenon was no longer seen, and the observation may be explained by reduced intake of local fish from the heavily polluted St. Lawrence River after advices given to reduce the consumption by pregnant and nursing women (Fitzgerald et al., 1998).

In a Belgian study aimed to identify maternal characteristics that determined POPs concentration in human milk, consumption of oily fish and fish oil supplements were associated with higher concentrations of DDT and DDE in the milk. Consumption of fatty fish and being breast fed during childhood were associated with higher concentrations HCB and β-HCH, and fish oil supplements was associated with higher concentrations of BDEs (Aerts et al., 2019).

Studies have also been performed for sea food intake and PFAS in breast milk, for many of them the conclusion is inconsistent and inconclusive. Some of these are handled in the Discussion part of this document.

4 Material and Methods

4.1 Collection of data material

Data used in the statistical analysis were obtained from laboratory analysis of milk samples collected in the multi-center birth cohort study HUMIS. The milk samples were all collected between 2002 and 2009, from a total of 2606 mothers from six counties in Norway. The mothers were recruited through routine postnatal care home visits, except from in Østfold where mothers were recruited from the maternity ward by a paediatrician. Two consecutive term births for every preterm birth were enrolled. Each of the mothers was asked to collect and donate 25 ml of breast milk per day for eight following days, preferably in the morning and between third and eighth week postpartum, but milk sampled otherwise was also accepted. Date and time of collection was written on each sample, as well as whether a breast pump was used or not, and the milk samples were kept frozen. The samples have since collection been kept frozen at -20°C at the Norwegian Institute of Public Health (IPH). Breast milk was collected at a median 33 (interquartile range [QR]: 26-43) days after birth.

In addition, the mothers were asked to complete questionnaires when the baby was one month, six months, one year, two years and ten to twelve years old. The aim of these questionnaires was mapping of factors which might be of relevance for levels in breast milk and the health and development of the child. Some information was also collected from the Medical Birth Register of Norway (Eggesbø et al., 2009; Vollset et al., 2019).

In the present study, 60 new milk samples were randomly chosen from the "milk stock" and analysed with respect to PFAS (N = 60) and lipid soluble POPs (N = 30). For the statistical analysis, results from another 1383 previously analysed samples from HUMIS milk samples were included.

4.2 Laboratory analyses at NMBU

The laboratory analysis included in this thesis work were performed in "MT-labben" at Adamstuen, NMBU with milk samples provided by NIPH.

The samples were analysed with respect to a selection of PFAS (2 x 30 samples) and a selection of lipid-soluble POPs (2 x 15 samples). After sample preparation, HPLC-MS was used to detect PFAS and GC-MS was used to detect lipid-soluble POPs.

The analytical method used to detect PFASs has been used and described previously (Grønnestad et al., 2017). A total of 60 human breast milk samples (split into two series) were analysed with respect to different PFASs, following the NMBU procedure M-MT.2.7 (NMBU Veterinærhøgskolen, 2020a). All samples and six controls (one blind, two recoveries, three blank) were added a standard to ensure accuracy of the analysis. Fluor compounds tend to stick to glass, therefore plastic equipment had to be used.

The method used for detecting POPs is based on the method *Gas Chromatographic Method for the Determination of Organochlorine Pesticides in Human Milk* which was developed and published by Brevik (Brevik, 1978). 30 milk samples (split into two series) were analysed with respect to a wide selection of lipid soluble POPs, following the NMBU procedure M-MT.2.2 (NMBU Veterinærhøgskolen, 2020b). All milk samples and control samples were added a standard to ensure accuracy of the analysis. All multiple use glass equipment had to be washed with a cyclohexane:acetone mix (CHX:ACH) before use to avoid POPs contamination from the environment in the samples.

4.2.1 Sample preparation

Before analysis and identification of present pollutants could be performed, the milk samples were prepared in order to make them suitable for the detection instruments.

4.2.1.1.PFAS

Sample preparation of samples to be analysed for PFASs consists of in-weigh of samples, addition of standards, extraction with methanol and purification with Envi-Carb.

Table 1 Materials used in PFASs sample preparation

Materials	Number of samples	Batch/serial number	Manufacturer
Human breast milk, 1g	60	Available in MT lab journal	From HUMIS study
Cow milk from supermarket, «lettmelk»	1		Tine
Methanol		10953935 + I1072218005	Supelco
Envi-Carb		127937	Supelco

Table 2 Equipment used in PFASs sample preparation

Equipments	Model/material/spec.	Manufacturer
Centrifugation vials (tubes)	PP kon. 17x118 mm 15 ml	VWR
Pasteurpipette	PE grade 3 ml 150 mm	VWR
Automatic pipettes	5-50 µl, 10-100 µl, 20-200 µl, 50-200 µl, 10-500 µl, 100-1000 µl, 50-1200 µl, 1-5 ml	Sartorius
LC vials	Plastic with plastic inlets (PP)	VWR

Table 3 Analytical standards used in PFASs sample preparation

Standards	Concentration in st. solution	Added volume	Concentration in samples	Manufacturer	Added to
PFAS I.S.	125 ng/m	80 µl	10 ng/l	Laboratory's own	30 milk samples 2 recovery samples 3 blank samples
PFAS	250 ng/m	40 µl	10 ng/l	Laboratory's own	2 recovery samples
Novel PFAS mix	500 ng/m	20 µl	10 ng/l	Laboratory's own	2 recovery samples
6=2 FTS (new)	250 ng/m	40 µl	10 ng/l	Laboratory's own	2 recovery samples

Table 4 Instruments used in PFASs sample preparation

Instruments	Specification	Manufacturer
Scales	3 decimals (MT-34) and 4 decimals (MT-36)	Mettler Toledo
Table mixer		
Shaker	Vibrax VXR basic (MT-146)	IKA
Centrifuge	X-12 Series Centrifuges (MT-140)	Allegra
Evaporation Unit	Turbovap LV Evaporator	Zymark

Procedure:

1) Weigh-in, standards and liquid-liquid extraction with methanol

1.1 Weigh-in of 1g of each breast milk sample and the cow milk sample (blind) into a tube

1.2 Addition of internal standards to each milk sample, two recovery samples and three blank samples

1.3 Addition of analyte standards to the recovery samples

1.4 Addition of 5 ml methanol to each tube

1.5 Mixing and shaking at 2000 rpm for 30 minutes

1.6 Centrifugation at 3000 rpm for 10 minutes

1.7 Transfer of the supernatant into a new 15 ml tube

1.8 Addition of 3 ml methanol to each tube

1.9 Mixing and shaking at 2000 rpm for 30 minutes

1.10 Centrifugation at 3000 rpm for 10 minutes

1.11 Transfer of the supernatant into the same tubes as in 1.7

1.12 Evaporate the volume of the extract down to 2 ml

2) Purification of fat and other pollutants

2.1 Addition of a small amount of Envi-Carb (0.1 – 0.3g) to each tube

2.2 Mixing

2.3 Centrifugation at 3000 rpm for 10 minutes

2.4 Transfer of the supernatant into a new 15 ml tube

2.5 Addition of 1 ml methanol to each tube

2.6 Mixing

2.7 Centrifugation at 3000 rpm for 10 minutes

2.8 Transfer of the supernatant into the same tubes as in 2.4

3) Preparation to HPLC analysis

3.1 Transfer of the solution to plastic LC vials with inlet and cap them

3.2 Stored in freezer until the analysis was to be performed

4.2.1.2 Lipid soluble POPs

The principles of the method include extraction of lipids and lipophilic compounds with water and CHX:AC, purification with concentrated sulphuric acid, and derivatization.

Table 5 Materials used in POPs sample preparation

Materials	Number of samples	Batch/serial number	Manufacturer
Human breast milk, 5 g	30	Unique number for each	From HUMIS study
Cow milk from supermarket, «lettmelk»	1	Unknown	Tine
Seal, 0.25 g	1	MTref01	NA
Water, grade 1	NA	NA	Direct-Q purification system
Cyclohexane (CHX)	NA	200716A002	Pestinorm, VWR
Acetone (AC)	NA	200420A008	Pestinorm, VWR
Sulphuric acid, conc. (97.5% H_2S0_4)	NA	Emsure Iso K51949631 947	Sigma-Aldrich
Keeper – 2 % decan in CHX	NA	200716A002 (CHX) Decan unknown	VWR

Table 6 Analytical standards used in POPs sample preparation

Standards	Concentration in st. solution	Added volume	Concentration in samples	Manufacturer	Added to
I.S. x 3	1 µg/ml	25 µl	50 ng/ml	Ultra Scientific	Samples, recovery, blank
BFR I.S. mix	500 mg/ml	50 µl	50 ng/ml	Cambridge Isotope Laboratories	
I.S. x 3	10 µg/ml	25 µl	50 ng/ml	Ultra Scientific	Seal
BFR I.S. mix	500 mg/ml	50 µl		Cambridge Isotope Laboratories	Samples, recovery, blank, seal
34 PCB x 5		25 µl	1:4	Laboratory's own	Recovery
CPM x 5		25 µl	1:4	Sigma	Recovery
Chlordane mix	100 µg/ml	15 µl	3 ng/ml	Laboratory's own	Recovery
BFR mix	50 µg/ml	20 µl	2 ng/ml	Cambridge Isotope Laboratories	Recovery
BFR, new	50 µg/ml	20 µl	2 ng/ml	Laboratory's own	Recovery
Octa-decan	50 µg/ml	20 µl	2 ng/ml	Cambridge Isotope Laboratories	Recovery

Table 7 Equipment used in POPs sample preparation

Equipments	Model/material/spec.	Manufacturer
Centrifugation glass vials (tubes)	80 ml	Unknown
Zymark evaporation glass	50 ml	Zymark
Glas Pasteur pipette	230 ml, min 250 ml	VWR
Automatic pipettes	5-50 µl, 10-100 µl, 20-200 µl, 50-200 µl, 10-500 µl, 100-1000 µl, 50-1200 µl, 1-5 ml	Sartorious
Pipette tips	100 µl, 300 µl, 1250 µl	Biotix
pH paper		Supelco
Measuring flasks	5, 10, 20, 50, 100, 200 ml	VWR
Test tubes with slip and glass plugs	Round bottom, 10 ml	Unknown
Test tubes with slip and glass plugs	Conic bottom, 10 ml	Unknown
8-dram glass with plastic lid	20 ml	Unknown
Beakers	Different sizes	VWR
Ola beaker	PP	Häger, Rjukan
GC glass vials with inlet and metal cap	Blanc and brown (BFR)	VWR

Table 8 Instruments used in POPs sample preparation

Instruments	Specification	Manufacturer
Scales	3 decimals (MT-34) and 4 decimals (MT-36)	Mettler Toledo
Shaker	IKA Vibrax VXR basic (MT-146)	IKA
Ultrasound sonicator	MT-82 and MT-121	Cole Parmer
Centrifuge	X-12 Series Centrifuges (MT-140)	Allegra
Evaporation Unit	Turbovap LV Evaporator	Zymark

Procedure

1) Weigh-in, standards and liquid-liquid extraction with acetone

1.1 Washing with CHX:AC 1:1 to remove POPs contamination from the tubes

1.2 Weigh-in 5g of each sample and 0,25g of seal fat

1.3 Addition of standards to milk samples and control samples

1.4 Addition of solvents to each tube, from most to least polar: 2ml NaCl 6%, 5ml H_20, 15ml acetone and 20ml cyclohexane

1.5 Sonicate for 1 minute

1.6 Centrifugation for 10 minutes at 300 rpm

1.7 Transfer of upper layer to Zymark tubes

1.8 Addition of solvents to each tube, from most to least polar: 5ml acetone and 10ml cyclohexane

1.9 Sonicate for 30 seconds

1.10 Centrifugation for 10 minutes at 300 rpm

1.11 Transfer of upper layer to same Zymark tubes as in vii.

1.12 Evaporate on water bath under N_2 flow until approximately 1 ml

1.13 Transfer of sample into a volumetric flask

1.14 Washing of Zymark tubes with CHX, add the washing solution to the volumetric flasks and adjust with CHX to 5ml

2) Determination of fat content

2.1 Weight of empty drum glasses

2.2 Transfer of 1ml solution from the volumetric flasks

2.3 Leave overnight on metal sand bath to dry

2.4 After cooling, measure and note the weight of the drum glasses again

2.5 Return to metal sand bath under N2 flow for 15 minutes

2.6 After cooling, measure and note the weight of the drum glasses again

2.7 Calculate content of fat obtained from 1 ml sample and decide of much acid is needed to break down the fat cells.

3) Purification from fat and other contaminants

3.1 Add an excess of sulphuric acid to make sure all fat cells and other contaminants are degraded.

3.2 Leave the tubes for one hour in the dark

3.3 Centrifugation for 10 minutes at 300 rpm

3.4 Place the tubes in the freezer overnight to allow a better phase separation.

3.5 Centrifugation for 10 minutes at 300 rpm after thawing

3.6 Transfer of 0.2 ml from the seal sample to a clear GC vial. Check for acidity by addition of one drop to a pH strip

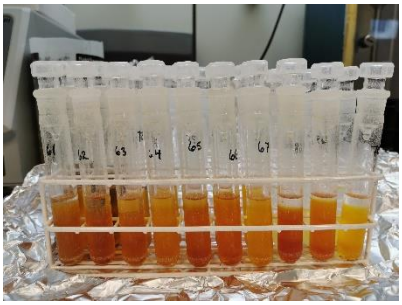


Figure 8 Samples after addition of sulphuric acid to degrade fat and other contaminants.

3.7 Transfer of upper layer to cone tubes. Check for acidity by addition of one drop to a pH strip before each transfer.

4.2.2 Separation and detection

After sample preparation, separation and detection were performed with HPLC-MS and GC-MS for PFAS and POPs, respectively

4.2.2.1 HPLC-MS

High performance liquid chromatography (HPLC) is a column chromatographic method where separation of compounds is based on the principle of different retention times, and where the mobile phase is a liquid. An HPLC instrument consists of a reservoir of mobile phase, a pump, an injector, a column, a detector and a printer integrator. Most commonly, a column based on reverse phase chromatography with chemically bound stationary phase. Separation is achieved due to different retention times which make the different compounds in the sample pass through the column at different times. Those that are readily soluble in the mobile phase will elute out first while those with great affinity to the column material will elute out in the end (Greibrokk et al., 1998).

Mass Spectrometer (MS) is a mass sensitive detector which may give both qualitative and quantitative information about the compounds presented in the sample. A mass spectrometer consists of three main components: an ion source, a mass analyser and a detector. The molecules are ionized, and the ionized molecules may be fragmented. In the analyser, the

ionized molecules and fragments are separated according to mass / charge ratio before the detector detects every mass as they are separated out (Greibrokk et al., 1998).

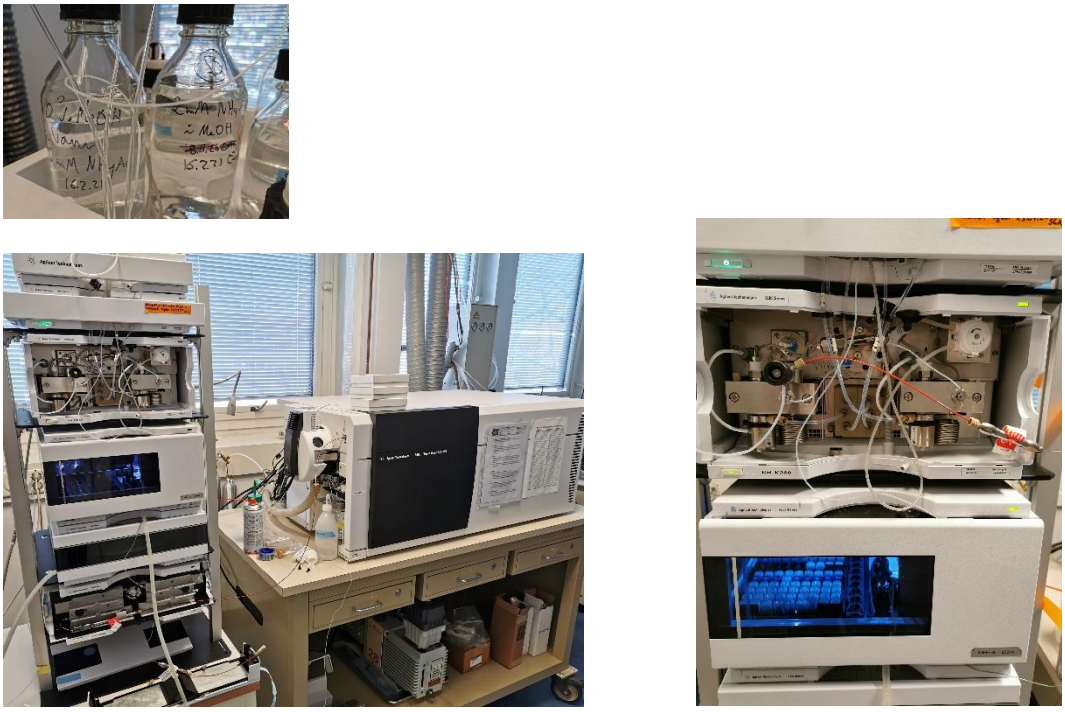


Figure 9 Agilent HPLC (left) system with MS detector (right). Bottles with mobile phases (over)

Table 9 Instruments used in PFASs separation and detection

Instruments	Specification	Manufacturer
Liquid chromatograph	Agilent 1200 HPLC	Agilent Technologies
Injector	HiP model G1367C	Agilent Technologies
MS detector	Agilent 6460 quadrupole MS with ESI ion source	
Column	Luna Omega C18 column (10 cm x 4,6 mm x, 3 µm) with "delay column" Luna Omega C18 column (5 cm x 4,6 mm x, 3 µm);	Phenomenex

Table 10 Mobile phases used in PFASs separation and detection

Mobile phases	Specification	Manufacturer
Mobile phase A	2 Mm ammonium acetate (NH_4Ac) in 90% water (H2O) and 10% methanol (MeOH)	Methanol, VWR, pestinorm grade
Mobile phase B	2 Mm ammonium acetate (NH_4Ac) in methanol (MeOH)	Ammoniumacetat, VWR, Chromanorm

Table 11 Conditions for PFASs HPLC system

Conditions	Specification		
Pump	Flow:	0.300 ml/min	
Injector	Mode:	Injection with needle wash	
	Draw and eject speed: 200.0 µl/min		
	Injection vol.:	20.00 µl	
Column	Valve position:	2 (port 1->6)	
	Temperature:	40°C	
MS detector	Mode: MRM (multiple reaction monitoring)		
	Ion source:	Temperature:	300°C
		Gas flow:	5 l/min
		Nebulizer:	25 psi
		SheatGasHeater:	400
		SheatGasFlow:	8
		Capillary:	2000 (+), 2500 (-) V
		Charging:	2000 (+), 500 (-) V

Procedure:

1. Programming and set up were performed using the software Masshunter.
2. Standards and samples were placed in correct order on the injecting tray.
3. The injecting syringe was washed outside with 1:1 methanol:water.
4. The two columns were mounted on and the mobile phases connected to the system.
5. After starting, the program ran automatically until it's end. A gradient program was used, in which the percentage of methanol (mobile phase B) increases from 10% after injection to 100% after 9 minutes. After another 8 minutes it is decreased to 10% and held at this concentration for 10 minutes before next injection. Mobile phase A is adjusted accordingly so that the two of them make up 100% in total.

4.2.2.2 GC-MS

Gas chromatography (GC) is a column chromatographic method where separation of compounds is based on the principle of different retention times, and where the mobile phase is a gas. The stationary phase (column) may be either a solid (GSC) or a liquid (GLC) material. Different compounds exhibit different characteristics when it comes to volatility and solvability in or affinity to the stationary phase. The separation is also dependent on the temperature. An increase in temperature will lead to a decrease in retention time. The compounds to be analysed must be volatile and stable at the temperatures used. The gas used as mobile phase must be inert to both the stationary phase and the sample. The stationary phase used must consist of a material which gives the desired separation, is thermically stable with a minimum of "bleeding" and do not react irreversibly with the sample. After injection, the sample will evaporate and be transported through the column by the carrier gas for thereafter to have its components detected by a mass spectrometer (Greibrokk et al., 1998).

As in the HPLC procedure (see 4.2.2.1 HPLC-MS), a mass spectrometer (MS) was used as detector.

Figure 10 GC-MS instrument for separation and detection of lipid soluble POPs

Table 12 Instruments used in POPs separation and detection

Instruments	**Specification**	**Manufacturer**
Gas chromatograph	OCPs and PCBs: HRGC-ECD, Agilent 6890 Series BFRs: HRGC, Agilent 6890 Series	Agilent Technologies
Injector	7683 Series injector and autosampler	Agilent Technologies
MS detector	OCPs and PCBs: Agilent 5975C BFRs: Agilent 5973	Agilent Technologies
Column	OCPs and PCBs: DB-5 MS (60m, 0.25mm i.d., 0.25mm film thickness) BFRs: DB-5 MS (30m, 0.25mm i.d., 0.25mm film thickness	J&W Scientific
Sampler	Auto-sampler, Agilent 7683 Series	Agilent Technologies
Carrier gas:	Helium (He)	Linde

Table 13 Conditions for POPs - BFR GC-MS system

BFRs	**Conditions**
Injector	Mode: Pulsed splitless Temperature: 250°C Puls pressure: 50 psi Puls time: 1 min Purge flow: 50 ml/min Purge time: 1 min Injection vol.: 2 µl
Column	1.6 ml/min constant flow
MS detector	Mode: NCI, SIM Temperature: ion source 250°C, quadrupole 150°C Dwell time: 100 ms
Temperature program (31.6 min run time)	25°C/min 2.5°C/min 20°C/min 90°C 1 min ------→180°C 0 min ------→220°C 1 min ------→ 320°C 5 min

Table 14 Conditions for POPs - PCB and OC GC-MS system

OCPs and PCBs	Conditions	
Injector	Mode: HP PTV	
	Temperature:	270°C
	Pressure:	29.13 psi
	Purge flow:	54.8 ml/min
	Purge time:	1.50 min
	Total flow:	54.8 ml/ml
	Injection vol.:	2 µl
Column	1.3 mL/min constant flow	
MS detector	Mode:	NCI, SIM
	Temperature:	ion source 150°C, quadrupole 150°C
	Dwell time:	50 / 70 / 80 / 100 ms, dependens on compound
Detector	Mode ^{63}Ni ECD	
	Temperature:	300°C
Makeup gas	Argon + 5% methane, 60 ml/min, 6ml/min anode flow	
Temperature program (71.6 min run time)	25°C/min	1.5°C/min 3°C/min
	90°C 2 min -----→180°C 2 min -----→220°C 2 min -----→ 275°C 12 min	

Procedure

1. Programming and set up were performed using the software ChemStation.
2. Standards and samples were placed in correct order on the injecting tray.
3. The injecting syringe was washed in the written order with dichloromethane, acetone and cyclohexane.
4. After starting, the program ran automatically until its end. The sequence includes running of cyclohexane before and after the GC standards, before and after seal control and between all samples. For every 10th sample, a GC standard («drift») is run to enable measuring of reproducibility.

4.3 Laboratory analysis at NIPH

The previously analysed samples which were included for statistical analysis went through a different procedure (Forns et al., 2015). In brief, 200 µl of each breast milk sample were transferred to a centrifugation tube after thawing and homogenisation in a thermoshake incubator at 37 °C. Internal standards and acetonitrile were added until a total volume of 600 µl for thorough precipitation of proteins, then the solution was mixed using a whirl mixer. After centrifugation, the supernatant was transferred to a glass autosampler vial and added 500 µl of 0.1 formic acid. 400 µl of the extract was then injected into a column switching LC-MS/MS system. Calibration solutions were made from cow's milk. PFOS was detected and quantified using high performance liquid chromatography / tandem mass spectrometry (LC-MS/MS) according to a described method (Haug, Line Småstuen et al., 2009)

4.4 Data handling / statistics / Stata

4.4.1 Qualification and quantification

After separation and detection, software programs were used to identify and calculate the amount of each substance present in the milk samples. For POPS, MSD ChemStation was used, for PFAS, MassHunter was used.

4.4.2 Statistical analysis

For statistical analysis it was decided to continue with PFOS only, and to also include data on PFOS obtained from 1383 milk samples from HUMIS analysed previously. That resulted in a transfer of concentration data from a total of 1428 samples (1383 (FHI) + 60 (NMBU) – 15 ("clean up")) to a statistical software program (STATA). In addition, a selection of variables obtained from the HUMIS questionnaires (see attachment 1) were imported into the software program. To explore the association between sea food intake (meals per year of fatty fish, lean fish, total fish and crabs) and the concentration of PFOS in breast milk, bivariate analysis and linear regression models were used. Both crude and adjusted models were run. Different kinds of sea food intake (as reported in the questionnaire) were set as exposure variables, and the concentration of PFOS (obtained in the laboratory analysis) was set as outcome variable. Potential confounders were identified and are represented by a directed acyclic graph (DAG). Obesity (BMI), maternal age, smoking status, education level, self-catch and ethnicity, as well as child birth year and preterm birth were included in the adjusted models. To avoid

uncertainty connected to total breast feeding before current child and all potential factors tied to this, only data from 1st parity mothers were included in the final report. That resulted in 544 samples to proceed with in the statistical analysis.

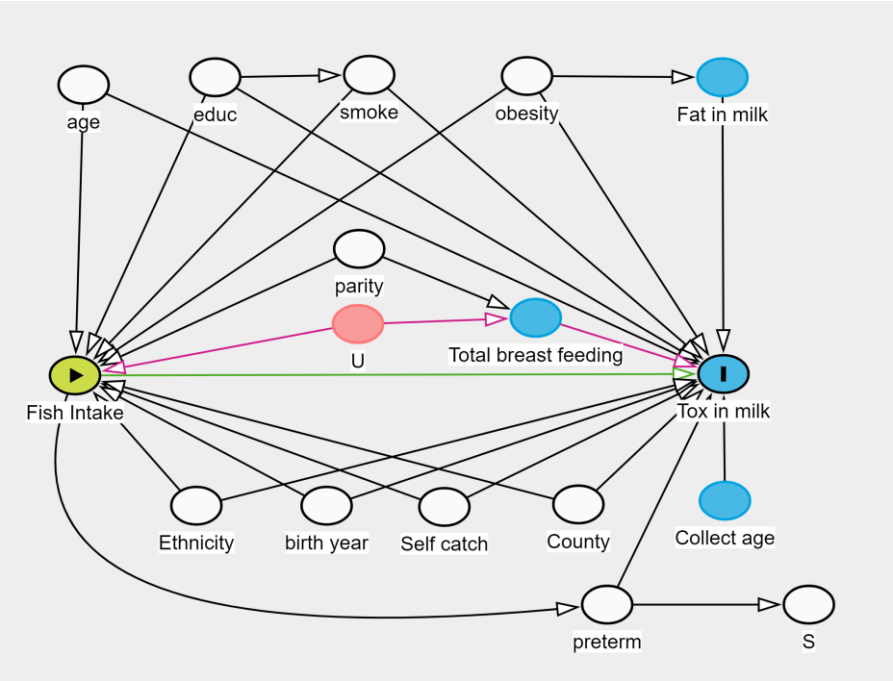


Figure 11 DAG showing exposure (Fish intake) and outcome (Tox in milk) variable, and potential confounders

5 Results

5.2 Laboratory findings – levels of POPs and PFAS in breast milk

All results obtained from laboratory analysis are given in table 15 and 16. For the lipid soluble POPs, concentrations are given as ng/g lipid weight. For the more water soluble PFAS, the concentrations are given as ng/g wet weight. 30 samples were analysed with respect to lipid soluble POPs and 60 samples were analysed with respect to PFASs.

The ∑ values given has emerged from simply adding the concentrations measured for the analysed compounds within each group. In Table 15, **∑PCB** includes PCB-28, PCB-52, PCB-74, PCB-99, PCB-101, PCB-105, PCB-114, PCB-118, PCB-138, PCB-149, PCB-153, PCB-156, PCB-157, PCB-170, PCB-180, PCB-183, PCB-187, PCB-189, PCB-194, PCB-196, PCB-206 and PCB-209, **∑HCH** includes α-HCH, β-HCH and γ-HCH, **∑Chlordanes** includes oxychlordan, trans-chlordan, cis-chlordan, trans-nonachlor and cis-nonachlor, **∑DDT** includes DDT and it's metabolites p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT and p,p'-DDT, **∑BDE** includes BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183, **∑PFAS** includes PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFHxS, L-PFOS and FOSA.

Table 15 Concentrations of POPs, summarized for each group, in each of the analysed human breast milk samples (ng/g lipid weight)

Sample number	∑PCB	HCB	∑HCH	∑CHL	∑DDT	Mirex	∑BDE	HBCD
1	117,998	6,342	4,364	19,904	39,801	0,456	1,535	0,000
2	128,586	7,117	7,637	14,659	40,154	0,359	1,114	6,893
3	78,130	4,648	11,057	11,457	26,228	0,285	0,317	0,000
4	112,685	6,628	5,331	14,196	50,799	0,389	1,510	0,000
5	103,314	8,225	4,051	17,361	31,335	0,482	0,618	0,000
6	103,659	6,770	5,055	9,575	33,360	0,421	0,860	0,000
7	108,836	4,612	6,575	5,537	43,079	0,342	1,973	0,000
8	106,906	6,963	5,117	8,862	43,228	0,300	3,355	0,000
9	54,092	5,226	5,626	9,009	40,367	0,106	1,549	0,000
10	122,397	8,154	7,314	16,439	73,788	0,537	4,000	0,000
11	64,901	5,256	3,031	7,892	23,031	0,255	1,803	0,000

Sample number	∑PCB	HCB	∑HCH	∑CHL	∑DDT	Mirex	∑BDE	HBCD
12	123,466	5,138	4,079	10,572	33,822	0,578	2,628	0,000
13	50,765	4,371	2,041	6,220	17,839	0,281	1,883	0,000
14	77,915	7,496	8,363	11,162	22,451	0,247	0,780	4,389
15	47,981	2,222	1,174	7,645	12,505	0,516	1,033	0,000
16	142,777	12,387	9,226	15,384	50,677	0,273	2,850	0,000
17	96,913	5,268	3,717	7,344	32,985	0,217	15,996	0,000
18	127,215	7,239	3,534	14,270	36,487	0,397	2,106	0,000
19	81,947	8,440	3,235	7,194	15,309	0,333	1,278	7,900
20	93,940	6,855	5,883	7,835	48,889	0,199	2,518	0,000
21	168,037	8,052	10,466	28,380	37,357	0,849	0,736	0,000
22	107,658	7,602	6,266	8,857	53,586	0,338	2,190	0,000
23	119,823	6,254	5,285	7,895	29,178	0,255	3,415	0,000
24	27,596	2,336	1,586	2,783	8,299	0,153	2,322	0,000
25	85,778	6,692	6,508	6,917	22,441	0,138	1,412	0,000
26	78,236	5,621	4,141	12,950	27,209	0,301	0,997	0,000
27	258,068	13,854	10,219	38,728	64,864	0,841	1,544	0,000
28	113,836	5,005	5,464	9,866	34,203	0,320	2,607	0,000
29	119,151	6,071	4,893	12,745	47,835	0,381	3,989	0,000
30	190,105	10,069	13,129	24,614	47,248	0,519	9,032	0,000

Table 16 Concentration of summarized PFASs in each of the analysed human breastmilk samples (ng/g wet weight)

Sample number	∑PFAS	Sample number	∑PFAS	Sample number	∑PFAS
1	0,051	21	0,190	41	0,070
2	0,124	22	0,131	42	0,039
3	0,050	23	0,167	43	0,070
4	0,000	24	0,079	44	0,121
5	0,109	25	0,111	45	0,158
6	0,082	26	0,060	46	0,135
7	0,085	27	0,140	47	0,096

Sample number	∑PFAS	Sample number	∑PFAS	Sample number	∑PFAS
8	0,158	28	0,106	48	0,135
9	0,102	29	0,278	49	0,093
10	0,119	30	0,134	50	0,236
11	0,050	31	0,031	51	0,139
12	0,103	32	0,059	52	0,107
13	0,049	33	0,112	53	0,142
14	0,078	34	0,123	54	0,132
15	0,065	35	0,131	55	0,264
16	0,066	36	0,055	56	0,126
17	0,105	37	0,023	57	0,098
18	0,117	38	0,062	58	0,173
19	0,246	39	0,091	59	0,161
20	0,057	40	0,129	60	0,112

All breast milk samples did contain several of the chemicals analysed for. Values for mean, min, max, median, 25th percentile, 75th percentile and 90th percentile for each group of the lipid soluble POPs are given in table 17.

Table 17 Mean, min, max, median, 25th, 75th and 90th percentile for each of the groups of lipid soluble POPs (ng/g)

Compound group	Mean	Min	25th	Median	75th	90th	Max
∑PCB lw	107.09	27.60	79.16	107.28	121.75	145.30	258.07
HCB lw	6.70	2.22	5.23	6.66	7.58	8.60	13.85
∑HCH lw	5.81	1.17	4.06	5.31	7.13	10.24	13.13
∑Chlordanes lw	12.54	2.78	7.85	10.22	14.56	20.38	38.73
∑DDT lw	36.28	8.30	26.47	35.35	46.24	51.08	73.79
Mirex lw	0.37	0.11	0.26	0.34	0.45	0.54	0.85
∑BDE lw	2.60	0.32	1.16	1.84	2.62	3.99	16.00
HBDC lw	0.64	0	0	0	0	0.44	7.9

PFOA and PFOS are the only PFAS detected in this analysis. Due to the method and column used, only L-PFOS (and not Br-PFOS / total PFOS) was possible to quantify. Values for mean, min, max, median, 25th percentile, 75th and 90th percentile are given in Table 18 and Table 19. The concentrations were originally given as weigh/weight (Table 18) and then converted into weight/volume (Table 19) by multiplying with the reference value of 1.03 g/ml for density of breast milk (Hamosh & Pitkin, 1991).

Table 18 Mean, median, min, max and 25th, 75th and 90th percentiles for PFAS concentrations in human breastmilk (µg/g)

Compound	N	Mean	Min	25th	Median	75th	90th	Max
PFOA	48 (of 60)	32.99	0	23.83	33.05	44.77	54.54	168.63
L-PFOS	59 (of 60)	77.61	0	54.28	74.19	91.70	120.15	190.85
∑PFAS	59 (of 60)	110.60	0	68.81	108.09	134.02	167.86	278.43

L-PFOS was detected in all samples except from one, while PFOA was detected in 48 milk samples. Concentrations varied from 0 to 196.57 ng/l for L-PFOS and to 173.69 ng/l for PFOA, with a mean at 79.93 ng/l for L-PFOS and 33.98 ng/l for PFOA, and a median at 76.41 ng/l for L-PFOS and 34.04 ng/l for PFOA (Table 19).

Table 19 Mean, median, min, max and 25th, 75th and 90th percentiles for PFAS concentrations in human breastmilk (ng/l)

Compound	N	Min	25th	Mean	Median	75th	90th	Max
PFOA	48 (of 60)	0	24.54	33.98	34.04	46.12	56.18	173.69
L-PFOS	59 (of 60)	0	55.90	79.93	76.41	94.45	123.76	196.57
∑PFAS	50 (of 60)	0	70.87	113.92	111.33	138.04	172.90	286.78

5.2 Statistical findings – seafood consumption and PFOS levels

5.2.1 Level of PFOS in the analysed breast milk samples

Even though only L-PFOS was detected and quantified in the samples analysed at NMBU while total PFOS value (L-PFOS and Br-PFOS) is reported for the samples analysed at FHI, the results were emerged and the term PFOS is used for the combined dataset (se section Discussion) used in the statistical analysis. Concentrations varied from 0 to 560 ng/l in the samples, with a mean at 119.15 ng/l and a median at 116.43 ng/l (see Table 20). For 109 of the samples, no PFOS was detected.

Table 20 Concentration details for PFOS (ng/l) in the 544 breastmilk samples from 1st parity mothers which were used in the statistical analysis

	N	Min	25th	Mean	Median	75th	90th	Max
PFOS	544	0	61.90	119.15	116.43	170	230	560

5.2.2 Consumption details about exposure variables (seafood)

Details about mean intake and distribution are shown in Table 21. For all variables, there seems to be a skewness in that a few mothers have a very high consumption.

Table 21 Sea food consumption frequency (number of meals per year) among the 544 1st parity breast feeding mothers

Seafood	N	Mean + SD	Min	25th	Median	75th	90th	Max
Lean fish	523	33 + 30	0	12	24	52	60	180
Crab	505	3 + 8	0	0	0	2	6	100
Fatty fish	510	32 + 37	0	9	22	46	104	401
Total fish	507	64 + 54	0	26	52	84	126	413

5.2.3 Factors associated with the concentration of PFOS

A separate bivariate analysis (t-test) was performed for all identified variables; exposure variables (sea food consumption) and the confounders. The results (Table 22) show statistically significant differences ($p \leq 0.05$) between the two groups tested towards each other for crab consumption ($p = 0.0114$), maternal age ($p = 0.0274$) and education (<12 years vs 12 years) ($p = 0.0521$). Higher levels of PFOS are seen in the group with smokers compared to non-smokers and in the group eating self-catched seafood vs not. Maternal obesity (BMI>25), being ethnically Caucasian (vs not) and giving birth preterm (vs on time) all show lower PFOS levels, but not statistically significant. The variable birth year looks "messy", but from 2003 to 2009 there is a significant decreasing trend, indicating that birth year is negatively associated.

Table 22 Concentrations of L-PFOS (ng/l) in breast milk from 1st parity mothers, according to maternal sea food consumption and confounding variables

Exposures	N	Mean	delta-mean	p-verdi	95 % CI
Overall	544	119			
Seafood type consumption					
Lean fish*	523				
<52	466	120			113-128
>52	57	117	-3	0.7739	84-149
Fatty fish*	510				
<46	379	120			112-129
>46	131	117	-3	0.7728	99-136
Fotal fish*	507				
<84	379	129			111-128
>84	128	119	-1	0.9127	100-138
Crab*	505				
≤2	371	114			105-122
>2	134	136	22	0.0114	118-155
Maternal age	544				
≤ 30 år	404	114.2			105.8-122.6
> 30 år	140	133.3	19.2	0.0274	117.2-149.6
Education	542				
<12 years	40	95.2			67.2-123.2
12 years	58	126.8	31.6	0.0521	108.1-145.5
>12 years	444	120.8	25.7	0.0868	112.4-129.3
Smoking status	539				
Never smoked	321	117.0			107.7-126.3
Former smoker	147	124.4	7.3	0.4165	107.7-141.0
Smoking at pregnancy start	71	119.4	2.4	0.8259	100.1-138.8
BMI	544				
≤25	358	121,1			111.9-130.4
>25	186	115,4	-5,8	0.4735	102.5-128.2
Ethnicity - caucasian	544				

Exposures	N	Mean	delta-mean	p-verdi	95 % CI
No	70	123,5			100.4-146.5
Yes	474	118,5	-4,9	0.6655	110.6-126.5
Birth year	544				
2002	8	82.8			4.4-161.2
2003	193	139.2	56.4	0.0603	127.5-150.9
2004	161	131.4	48.6	0.1458	117.1-145.7
2005	105	94.0	11.2	0.7529	75.2-112.8
2006	28	60.9	-21.9	0.4813	33.2-88.6
2007	13	110.2	28.2	0.4654	63.8-158.1
2008	23	87.6	4.8	0.8423	70.0-105.1
2009	13	84.2	1.5	0.9657	48.1-120.4
Self catch	544				
No	317	117.0			107.8-126.1
Yes	227	122.2	5.3	0.4962	109.4-135.0
Preterm	544				
No	475	120.3			112.2-128.3
Yes	69	111.6	-8.7	0.4501	90.4-132.8

**For sea food, values are given based on 75th percentile values for number of dinners per year (see Table 21)*

Neither crude nor adjusted data from linear regression did show any statistically significant associations between maternal consumption of any of the four sea food categories and concentration of PFOS in breast milk (Table 23). However, a positive contribution was seen for crabs.

Table 23 Crude and adjusted values from linear regression (ng/l)

	Crude values	Adjusted for confounders*
Lean fish	-0.061 (p = 0.636)	-0.008 (p = 0.953)
Fatty fish	-0.111 (p = 0.302)	-0.109 (p = 0.332)
Total fish	-0.072 (p = 0.325)	-0.060 (p = 0.427)
Crab	0.705 (p = 0.167)	0.503 (p = 0.324)

**adjusted for the following confounders: Obesity (BMI), maternal age, smoking status, education level, self-catch, ethnicity, child birth year and preterm birth. ** Total fish includes lean fish and fatty fish.*

Results from the multiple regression analysis are shown in Table 24. Among the exposure variables, only crab consumption shows a positive association with PFOS concentration in the milk (0.49, [0.52-1.49]), however not statistically significant (p = 0.341). Maternal age, education, smoking and self-catch of fish are the confounding variables associated with increased levels of PFOS in breast milk. For maternal age, there is a statistically significant association, and an increase of one year in age accounts for an increase in PFOS concentration of 2.25 ng/l. For education, the values given in the table are relative to less than 12 years of education. A statistically significant association is seen for 12 years of education compared to less than 12 years. For longer education (>12 years), a positive association is also seen (22.60 ng/l), but it's not statistically significant. A 12-year long education will in this model contribute with 39.15 ng/l of PFOS (p = 0.050), relative to less than 12 years of education. When it comes to smoking and self-catch of sea food, the associations are not statistically significant.

On the other hand, BMI, being ethnic Caucasian, birth year and preterm birth are variables negatively associated with PFOS levels. Higher BMI is associated with lower levels of PFOS in the milk, a BMI >25 explains -0.98 ng/l of the PFOS concentration in milk from those who are overweight, relative to those who have a BMI value < 25. To be ethnic Caucasian is associated with 13.40 ng/l lower level of PFOS compared to other ethnicities. The samples were collected between 2002 and 2009, and the regression analysis shows that it's a statistically significant negative association between giving birth later and PFOS concentration in the mothers' milk. However, in the bivariate analysis this is not clear as the level in samples from 2002, which is used as reference, is quite low (Table 8). But from 2003 to 2009 there is a significant downward trend in PFOS concentration. Giving birth prematurely (before day 259 / complete week 37) is associated with a reduction of 9.40 ng/l PFOS in the milk compared to giving birth on time, but the difference is not statistically significant.

Table 24 Factors associated with PFOS levels in human breast milk (ng/l) and the estimate for how they contribute to the total PFOS level.

Explanatory variable	Coefficient *(β)**	95 % CI	p
Exposure variables			
Lean fish	-0.00006	0.27-0.27	1.000
Fatty fish	-0.11	0.33-0.11	0.341
Crab	0.49	0.52-1.49	0.341
Confounding variables			
Maternal age	2.25	0.39-4.11	0.018
Education level 12 years	39.15	0.007-78.23	0.050
Education level > 12 years	22.60	-9.34-54.53	0.165
Former smoker	6.14	-11.54-23.82	0.495
Smoking at pregnancy start	-3.00	-28.29-22.28	0.815
BMI	-0.98	-2.80-0.83	0.288
Caucasian	-13.40	-38.66-11.86	0.298
Birth year	-15.25	-20.69-9.82	0.000
Self-catch of sea food	12.83	-3.17-28.84	0.116
Preterm birth	-9.40	-34.25-15.45	0.458

** Explanation of the coefficient (β), representing the contribution estimate to the PFOS level for each variable: For the exposure variables, it's per meal per year. For maternal age, it's for each year, for education the values are relative to less than 12 years of education, for smoking the values are relative to never-smokers, for BMI it's per unit on the BMI scale, for Caucasian it's relative to non-Caucasian, for birth year it's a mean reduction per year from 2002-2009, for self-catch it's relative to not eating self catched sea food and for preterm it's relative to giving birth on time.*

6 Discussion

6.1 Laboratory analysis

6.1.1 Evaluation of methods used in the NMBU laboratory

The methods used to analyse the milk samples are standard methods set up and tested by others. Still the results may be affected by operator related factors like technique, experience and accuracy, the laboratory environment and factors related to equipment and chemicals. However, the Laboratory of Environmental Toxicology at NMBU is nationally and internationally accredited according to the requirements of NS-EN ISO/TEC 17025. To ensure accuracy, the critical step of adding standards was performed by an approved operator. Also, a total of 6 controls were used to be able to adjust for pollutants from other sources than the milk sample itself.

The levels of detection (LOD) for all the compounds are given in Table 25. Among the PFASs analysed, only L-PFOS and PFOA were detected in the breast milk samples. The fact that the samples were collected between 2002 and 2009 may explain the relative high concentration of these two substances to the "newer" ones, which are now replacing PFOS and PFOA according to the Stockholm Convention (UN Stockholm Convention, 2019). For PFHxS, there were some issues with the analysis method giving a relatively high LOD, which may explain why this compound was not detected. Recovery values have been calculated from the two spiked recovery samples, and are given in Table 26, varying from 66 to 187%. Usually, acceptable levels are 80-120%, or 60-140% if a component is present at a very low level. Commonly "difficult" components are DDT/DDD/DDE, HBCD, oxochlordane and PCB-52, known to be unstable in the column. Detected POPs and PFASs concentrations are not adjusted according to recovery values unless specified.

Among the breast milk samples analysed at NIPH, PFHxS was detected in addition to PFOS and PFOA (Forns et al., 2015; Haug, Line Småstuen et al., 2009).

Table 25 Detection levels (LOD) for all POPs and PFASs analysed for (ng/g)

Compound, POPs	LOD	Compound, PFASs	LOD
HCB	0,002	PFHxA	0,079
α-HCH	0,002	PFHpA	0,017
β-HCH	0,003	PFOA	0,020
γ-HCH	0,003	PFNA	0,017
Chlordanes	0,002	PFDA	0,028
p,p'-DDE	0,006	PFUnDA	0,041
o,p'-DDD, corrected	0,006	PFDoDA	0,038
p,p'-DDD, corrected	0,019	PFTrDA	0,065
o,p'-DDT	0,012	PFHxS	0,091
p,p'-DDT, corrected	0,006	L-PFOS	0,022
Mirex	0,002	FOSA	0,056
PCB-74	0,006		
PCB-99	0,007		
PCB-101	0,004		
PCB-28, PCB-52, PCB-105, PCB-114, PCB-118, PCB-138, PCB-149, PCB-153, PCB-156, PCB-157, PCB-170, PCB-180, PCB-183, PCB-187, PCB-189, PCB-194, PCB-196, PCB-206, PCB-209	0,002		
BDE-28, BDE-100, BDE-153, BDE-154	0,003		
BDE-47, BDE-99	0,002		
BDE-183	0,004		
HBCD	0,122		

Table 26 Recovery values for all POPs and PFASs analysed for

Analyte	Recovery %	Analyte	Recovery %	Analyte	Recovery %
HCB	66	PCB-28	81	PFHxA	86
α-HCH	94	PCB-52	53	PFHpA	88
β-HCH	127	PCB-74	109	PFOA	85
γ-HCH	109	PCB-99	105	PFNA	88
Oksyklordan	137	PCB-101	104	PFDA	88
trans-Klordan	108	PCB-105	110	PFUnDA	87
cis-Klordan	106	PCB-114	113	PFDoDA	89
trans-Nonaklor	107	PCB-118	107	PFTrDA	67
cis-Nonaklor	106	PCB-138	106		
p,p'-DDE	101	PCB-149	109	PFHxS	86
o,p'-DDD,corr	187	PCB-153	99	L-PFOS	80
p,p'-DDD, corr	149	PCB-156	107		
o,p'-DDT	113	PCB-157	113		
p,p'-DDT, corr	158	PCB-170	106		
Mirex	104	PCB-180	101		
BDE-28	79	PCB-183	98		
BDE-47	88	PCB-187	97		
BDE-99	86	PCB-189	106		
BDE-100	94	PCB-194	104		
BDE-153	93	PCB-196	117		
BDE-154	79	PCB-206	105		
BDE-183	97	PCB-209	101		
HBCD	84				

6.1.2 Comparison with laboratory findings from similar studies

There are several studies which have analysed POPs and PFASs in human breast milk. However, it is not always straightforward to compare them because of differences in study design. In addition to the wide range of chemicals to study, factors like sampling year and period, inclusion criteria, sampling size, local variations in exposure and differences in analytical procedure give different conditions. Also, some studies report concentration in weight per weight while other in weight per volume, this must be taken into account when comparing results. The reference value for the density of human breast milk, which is 1.03 g/ml, may be used for conversion in order to do the comparison (Hamosh & Pitkin, 1991). Table 27 shows the median, minimum and maximum levels of POPs detected in the present study compared with results from a selection of other studies which have detected POPs in breast milk. In general, there are individual variations within each study and country, but some geographically differences can be seen. For pesticides like DDT and HCH, milk

concentrations are significantly higher in China, Japan and Korea compared to the European countries. This may be explained by reduced use of these pesticides in Europe due to earlier bans and restrictions than in Asian countries (Fång et al., 2015). It is interesting to notice that PCB levels are generally higher in European countries compared to African and Asian, while the situation is the other way when it comes to the pesticides, for which the levels are higher in the three Asian countries. A reasonable explanation to this is that European countries consumed more industrial products containing PCBs than Asian and African countries before the international ban of PCBs (UN Stockholm Convention, 2019). The relatively high levels of PCBs detected in European breastmilk 40-50 years after the ban, illustrates the persistency of this group of chemical contaminants in the human body. The higher levels of pesticides in Asian and African breast milk samples are suggested to be caused by more recent production ban than in Europe and US (20-30 years), illegal use in agriculture and for the control of diseases such as malaria, typhus and cholera (Wong et al., 2005).

*Table 27 Comparison of results from the present study with findings from other POPs studies. Median** (min - max) concentrations (ng/g) are given*

Country	N	∑PCB	HCB	∑HCH	∑CHL	∑DDT	Mirex	∑BDE	∑HBCD	Study reference
Norway	30	107.28 (27.6-258.07)	6.66 (2.22-13.85)	5.31 (1.17-13.13)	10.22 (2.78-38.73)	35.35 (8.30-73.79)	0.34 (0.11-0.85)	1.84 (0.32-16.00)	0 (0-7.9)	Present work
Norway	377/46*	103/110* (30-450)	11/13* (0.88-255)	4.7/8.1* (0,88-255) (only β-HCH)	2.8/3.2* (0.54-30) (only oxy-chlordane)	41/61* (0.78-1280) (only DDE)	NA	NA	NA	(Polder et al., 2009)
Tanzania	150	(<LOD-157)	(<LOD-29.8)	<LOD-24.5	<LOD-12.9	26.3-2490	NA	NA	NA	(Müller et al., 2017)
Belgium	197	122.7	15.5	ND	ND	95.9 (only DDE)	NA	NA	NA	(Colles et al., 2008)
UK		180 (26-530)	18 (ND-180)	18 (1.2-1500)	ND (ND-1.4)	160 (24-2300)	NA	6.3 (0.3-69)	NA	(Kalantzi et al., 2004)
Japan**	60	110 (14-360)	13 (8.1-21)	140 (9-1200)	31 (8.6-140)	170 (23-970)	NA	1.5 (0.36-4.7)	NA	(Haraguchi et al., 2009)
China**	25	56 (26-130)	86 (48-150)	570 (67-3000)	3.8 (1.4-11)	1300 (430-3000)	NA	1.9 (0.88-7.7)	NA	
Korea**	29	61 (20-128)	13 (8.1-21)	110 (17-830)	14 (6.4-31)	180 (49-580)	NA	3.7 (0.82-24)	NA	

* Norwegian/Non-Norwegian

** Mean concentrations for Japan, China and Korea

Table 28 shows the results from some studies looking into PFAS levels in breast milk. Most of these studies include a quite small number of participants, so precaution should be made to generalize. It seems to be reasonable to think that results from Norway and Sweden would be similar, but in general the levels in the Norwegian samples are significantly higher. One explanation may be found in the year of sampling. Milk samples from Sweden were collected in 2016 while the samples from Norway were collected 2002-2009. The lower levels seen in the Norwegian samples may reflect a lower environmental exposure due to phasing out of PFOS and PFOA (UN Stockholm Convention, 2019). Also, levels in the US (2019) and the "42 countries" from around the world (2016-2019) are significantly lower than in the Norwegian samples, possibly related to sampling year. Still the US values are about 2000 times higher than the value recommended as safe for drinking water according to an article in the Guardian in which the authors say that "the findings are cause for concern and highlight a potential threat to newborns' health" (Perkins, 2021; Zheng et al., 2021).
However, in the samples from China (2010-2016), higher levels were found, indicating a more extensive use of PFOS and PFAS in these areas. The samples from France, collected in the same period of time as the Norwegian ones, show higher values for mean, min and max values.

Table 28 Comparison of results from the present study with findings from other PFASs studies from other studies. L means L isomer and Br means Br isomer. Concentrations are given as ng/l

Population	Sample year	Sample size	PFOS	PFOA	Reference
Norway	2002-2009	60	Detected in 98%	Detected in 80%	Present work
mean			77.61	32.99	
median			74.19	33.05	
min, max			0, 190.85	0, 168.63	
France	2007	48	Detected in 90%	Detected in 98%	(Antignac et al., 2013)
mean			92	82	
median			79	75	
min, max			<50, 330	<50, 224	
Korea	2011	293	Detected in 100%	Detected in 88%	(Lee et al., 2018)
mean			57.3	55.6	
median			47.8	40.1	
25^{th} ,75^{th}			35.1, 67.5	22.3, 73.2	
min, max			14.8, 380	10, 657	
US	2019	50	Detected in 100%	Detected in 86%	(Zheng et al., 2021)
mean			30.4	13.9	
median			16.8, 63.0	10.6, 23.5	
25^{th} ,75^{th}			6.35, 187	<16, 50.7	
min, max					
42 countries*	2016-2019	44	L-PFOS detected in 82 % and Br-PFOS in 68 %	Detected in 100 %	(Fiedler & Sadia, 2021)
Africa		14			
mean			9.55	12.7	
median			10.3	12.5	
min, max			0, 21.9	6.2, 18.1	
Asia-PAC		13			
mean			32.0	16.9	
median			17.2	14.6	
min, max			0, 212	9.98, 31.8	

Population	Sample year	Sample size	PFOS	PFOA	Reference
GRULAC		9			
mean			12.5	14.3	
median			11.8	15.9	
min, max			0, 40.5	7.81, 19.0	
WEOG+CEE		8			
mean			22.3	28.6	
median			17.8	31.0	
min, max			12.2, 51.4	17.7, 37.4	
China					(Awad et al., 2020)
Shanghai	2015-2016	10			
mean			65 (L), 8 (Br)	139 (L)	
min, max			16, 177 (L), 2.22 (Br)	64, 308 (L), <2, 3 (Br)	
Jiaxing	2015-2016	10			
mean			119 (L), 12 (Br)	266 (L), 4 (Br)	
min, max			21,321 (L), 3,26(Br	177,411(L), <2,8(Br)	
Shaoxing	2010	10			
mean			77 (L), 7 (Br)	94 (L), 2 (Br)	
min, max			7,308 (L), 1,27 (Br)	33,151(L), <2,5 (Br)	
Sweden	2016	10			
Stockholm					
mean			39 (L), 7 (Br)	42 (L), 2 (Br)	
min,max			23,58 (L), 1,14 (Br)	<2, 81 (L), <2, 6 (Br)	

6.2 Statistical analysis

6.2.1 Sea food consumption and PFOS in breast milk

Based on the results from the EFSA report (Schrenk et al., 2020), showing that fish and other sea food is the most important contributor to mean LB exposure of PFOS, it was expected to find a positive association between maternal sea food intake and concentration of PFOS in the breast milk. As the highest levels are to be found in lean predatory species (Table 29), it was especially looked for an association between lean fish consumption and PFOS levels. However, no significant association were found between fish consumption and PFOS levels in

breast milk, whereas a positive association was found for crabs in the bivariate analysis shown in Table 22 (p = 0.0114). In the multiple regression analysis (Table 24), a possible contribution from crab consumption was seen, but it was not statistically significant (p = 0.341).

In should be kept in mind the fact that the EFSA report considers levels of PFOS in different food products, and not human milk. It could be expected that the exposure situation would be reflected in the milk concentration, but several factors related to the compound, the maternal body and maternal lifestyle may come into account. This may be part of the explanation why in this thesis work it was not found an equivalent positive association between maternal sea food consumption (except for crabs in the bivariate analysis, Table 22) and PFOS content in the milk.

Possible explanations for our findings could be that the EFSA report does not mirror the conditions in Norway when it comes to PFOS levels in the sea food products consumed by Norwegians. Table 29 ("Table 5" from the EFSA report) shows measured mean values for PFOS in several fish species. According to Norsk Sjømatråd, the most commonly consumed fish species in Norway are salmon and trout (laks og ørret), and then white fish (hvitfisk) like cod (Table 30) (Norges Sjømatråd, 2018), while the species with highest PFOS levels were found to be carp and eel (Schrenk et al., 2020). The results from the EFSA report show that PFOS is present in 88% of analysed salmon and trout samples, and in 67% of analysed cod and whiting samples. However, the levels are significantly lower than in species like carp and eel. Another explanation could be uncertainty related to the self-reported fish intake and confounders which have not been identified and adjusted for.

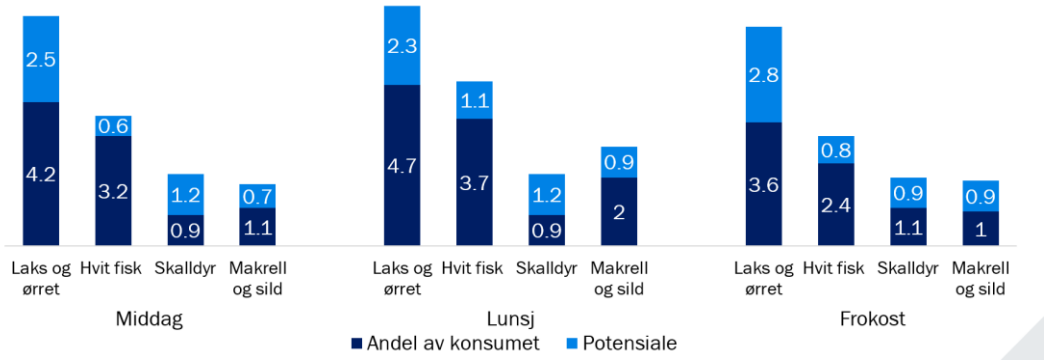
Table 29 Mean levels of PFOS, PFOA, PFNA and PFHxS in selected fish species analysed by EFSA (Schrenk et al., 2020)

Table 5: Mean levels (µg/kg) of PFOS, PFOA, PFNA and PFHxS in selected fish species and fish offal

Fish species	PFOS				PFOA				PFNA				PFHxS			
	N	%LC	LB	UB	N	%LC	LB	UB	N	%LC	LB	UB	N	%LC	LB	UB
Herring (Clupea)	288	74%	0.32	0.62	290	96%	0.016	0.38	243	90%	0.023	0.38	237	99%	0.000	0.38
Sardine and pilchard (Sardina)	14	0%	4.73	4.73	28	64%	0.101	0.37	14	57%	0.084	0.53	14	64%	0.014	0.45
Anchovy (Engraulis)	5	0%	0.58	0.98	13	62%	0.044	0.12	–	–	–	–	–	–	–	–
Salmon and trout (*Salmo* spp.)	574	88%	0.31	0.83	521	95%	0.13	0.63	522	100%	0.003	0.70	365	100%	0.000	0.63
Mackerel (Scomber)	125	79%	0.36	0.93	136	81%	0.31	0.88	129	96%	0.004	0.74	122	99%	0.001	0.74
Tuna (Thunnus)	21	39%	0.16	0.26	34	100%	0.000	0.12	17	100%	0.000	0.13	17	100%	0.000	0.11
Cod and whiting (*Gadus* spp.)	174	67%	0.47	1.05	145	93%	0.012	0.74	130	92%	0.016	0.78	27	100%	0.000	0.53
Halibut (*Hippoglossus* spp.)	487	71%	0.26	0.81	106	99%	0.003	0.30	487	100%	0.000	0.77	487	100%	0.002	0.69
Carp (Cyprinus)	145	14%	14.12	14.21	149	32%	4.10	4.33	125	65%	0.84	1.47	126	97%	0.066	1.01
Eels (Apodes)	164	35%	9.23	9.44	177	96%	0.071	0.68	54	91%	0.98	1.66	58	98%	0.017	0.73
Fish offal	208	83%	3.38	4.99	208	100%	0.010	3.51	204	99%	0.011	2.41	202	100%	0.031	1.65

N: number; %LC: Percentage left-censored; LB: Lower bound; UB: upper bound; –: no data provided to EFSA; PFOS: perfluoroheptane sulfonate; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFHxS: perfluorohexane sulfonic acid.

Table 30 Consumption of sea food in the Norwegian population. The dark blue column shows intake of salmon and trout, white fish, shell fish, and mackerel and herring. The numbers given show how big share the fish species make up of total dinner, lunch and breakfast meals (Norges Sjømatråd, 2018)



6.2.2 Factors influencing the level of PFOS in breastmilk

Several possible confounders were identified. Factors influencing concentration of PFOS that are to be found in breast milk can be grouped into maternal, environmental and compound related factors. In this study, the main focus was on maternal sea food consumption. Among the sea food groups studied, a statistically significant association was found in the bivariate analysis for crabs only ($p = 0.0114$). In addition, the identified confounders maternal age and education level were found to have a statistically significant positive impact on PFOS level in human breast milk.

For maternal age, the bivariate analysis shows an estimated mean increased level of 19.2 ng/l ($p = 0.0274$), in milk from mothers above 30 years compared to younger mothers. In the regression analysis, the confounding variable maternal age is associated with a contribution of 2.25 ng/l per year ($p = 0.018$). These findings may be explained by older mothers having accumulated PFOS over a longer period of time and have lived a longer part of their life before restrictions were applied to the compound, thereby being exposed to higher levels in their environment.

When it comes to education level, a statistically significant difference in mean values (31.6 ng/l, $p = 0.0521$) is seen in the bivariate analysis between less than 12 years of education compared to 12 years of education. According to the regression analysis, to have 12 years of education will contribute with 39.15 ng/l ($p = 0.0050$) relative to less than 12 years of education. An explanation to this may be that more educated people tend to eat more fish as part of a generally healthier lifestyle. For some reason a statistically significant contribution is

not seen for the group with more than 12 years of education, but the estimated impact on the PFOS level is 22.60 ng/l (p = 0.165). Other factors may come into play.

The higher, but not statistically significant levels (p = 0.116 in the multivariate regression analysis and p = 0.4962 in the bivariate analysis) of PFOS seen in the group eating self-catched seafood versus not, may reflect that sea food in lakes and fjords tend to contain more pollutants than sea food in the open ocean (Augustsson et al., 2021).

On the other hand, BMI, being Caucasian, birth year and preterm birth are variables negatively associated with PFOS levels, only birth year showing statistically significance. In the regression analysis, birth year contributes with 15.25 ng/l less PFOS per year (p = 0.000). In the bivariate analysis, birth year 2002 was set as reference group, and all other groups were compared to that one. The 2002 group consists of only 8 individuals, giving birth at the end of the year as that was when the study recruitment started. The PFOS level is low in this group, but the low number of individuals ties an uncertainty to the value. That may be the reason why this analysis does not show a reduction from birth year 2002. The drop in PFOS level with later birth year may be explained by the phase out of PFOS under the Stockholm Convention (Land et al., 2018)

The difference seen for ethnicity (Caucasian versus other) may reflect differences in diet and other life style factors, genetic variance or a general higher exposure in the upbringing environment. As PFOS is mainly protein bound it is maybe not clear that higher BMI would negatively affect the level in breast milk, but the results from this study indicate that BMI may play a role. However, the difference or contribution is not statistically significant neither in the bivariate nor in the regression analysis (p = 0.4735 and 0.288, respectively).

6.2.3 Comparison with other studies

Several studies have been performed earlier about PFOS and human breast milk. The results are inconsistent and inconclusive when it comes to the association between maternal intake of sea food and PFOS concentrations in breast milk. As stated previously, it's not always straightforward to compare results. In addition to already motioned factors, some studies differentiate between isomers (L (linear) and Br (branched)) while others do not. Some studies look at only concentrations while others also look at associations between PFOS levels and health outcomes or possible sources. PFOS levels in human breast milk from some studies are shown in Table 28. In the study on breast milk from French women (Antignac et al., 2013), no statistically significant associations between PFOS levels and various socio-

geographic parameters including seafood consumption were found. Nor did it show statistically significant associations with maternal age or parity. The first finding is similar to the present study which shows an association with the sea food group crabs only while the second is contradictory when it comes to maternal age. As only data from 1st parity mothers were included in the present study, no comparison can be made with that variable. In the Korean study (Lee et al., 2018), significant associations between PFOS levels in breast milk and maternal age, maternal BMI, parity, snack consumption, milk intake and eating out were found. The findings regarding maternal age and BMI are in line with findings in the present study, the other variables were not considered here. The findings in the US study (Zheng et al., 2021) indicate that both legacy and current-use PFOS contaminates breast milk. A statistically significant correlation was found between PFOS concentration in milk samples and duration of breast feeding, estimated from the age of the infant at sampling time ($\beta = -0.411$, $p = 0.047$), indicating that lactation is an important elimination way of this chemical. The present study did not look into baby age and PFOS concentration, but all samples were collected early in the breastfeeding period (between third and eighth week postpartum).

L-PFOS and PFOS

In this study, data from two different data sets were merged. In the 60 samples analysed at NMBU, only L-PFOS was detected. In the samples analysed by FHI, total PFOS was detected. Due to a low number of samples with L-PFOS compared to the number with total PFOS and the fact that Br-PFOS is generally low compared to L-PFOS, it was considered to be acceptable to merge the data sets.

7 Conclusion

The chemical analysis in the laboratory revealed that all milk samples contained POPs and/or PFASs. For PFAS, only PFOA and L-PFOS were detected at quantifiable levels. When comparing with results from other studies, it was noted that the content of PCBs was higher in Norwegian samples than in samples from Asian countries, possibly reflecting a high use of products (like clothes, electronics, furniture) containing PCBs before the ban in Europe in the 1970's. For pesticides / insecticides, the relation was the opposite which may be explained by continued illegal use of these chemicals in agriculture and insect protection as well as more recent regulation than in Europe and US. For PFAS, the concentration of PFOS and PFAS were higher in the Norwegian samples compared to samples from Swedish, US and a range of other countries sampled from 2016 to 2019, indicating that levels are decreasing due to phase out of these compounds. Compared to results from three Chinese cities, the levels were lower, indicating a more extensive use in Asian countries.

In the statistical analysis, the expected positive association between maternal sea food intake and concentration of PFOS in the breast milk from first parity Norwegian mothers was not seen, except for crabs. However, the study gave several interesting findings related to defined confounders, indicating that the association of interest is strongly biased. This, together with uncertainty in the self-reported sea food consumption data and the possibility that the fish eaten by Norwegians may contain less PFOS than samples analysed in the EFSA food study, may explain our results. There may also be other confounding factors than the ones defined in this study.

Quite a few studies have been performed on persistent pollutants in breast milk. However, many of them are very limited when it comes population size and geographical area, and results are often inconclusive and inconsistent. The finding in the EFSA report, that fish and other sea food is a source to PFAS, is of interest to explore further – why was this not reflected in the milk samples analysed? In addition, compounds enter and leave the market continuously, therefore a continuously research in this area is necessary to keep up to date.

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Attachment A Questionnaire (extract translated into English) HUMIS Study

. Your diet through life

83. Approximately how many times <u>in your life</u> have you had:

	Never	1-10	11-100	>100
Pike ……	☐	☐	☐	☐
Codliver +roe+ oil(*mølje*)	☐	☐	☐	☐
Seagull eggs……..	☐	☐	☐	☐
Liver/kidney from game ……..	☐	☐	☐	☐
Codliver *(not mølje)*..	☐	☐	☐	☐
Crabs ……..	☐	☐	☐	☐
	1	2	3	4

16. Your diet during the last year

Note: *Choose whether you want to reply for week, month OR year for each food choice!*

84. <u>Breadmeals:</u>	**<u>Please mark:</u>** Never	***<u>Give number of meals</u>*** Week	Month	Year
Cheese …..	☐	☐☐	☐☐	☐☐
Eggs *(not dinners)* ……	☐	☐☐	☐☐	☐☐
Milk/yoghurt *(number glass)*	☐	☐☐	☐☐	☐☐
Peanuts ……..	☐	☐☐	☐☐	☐☐
Cod liver oil……..	☐	☐☐	☐☐	☐☐
Liver pâté ……..	☐	☐☐	☐☐	☐☐
Mackerel/salmon(breadsmears)	☐	☐☐	☐☐	☐☐
Crab breadsmear ……	☐	☐☐	☐☐	☐☐
Tuna fish (canned)……..	☐	☐☐	☐☐	☐☐
Fishliver/roe pâté……	☐	☐☐	☐☐	☐☐

⊥

	Please mark:	***Give number of meals***		
85. Dinnerportion:	Never	Week	Month	Year
White meat *(chicken,turkey)*	☐	☐☐	☐☐	☐☐
Pork ……	☐	☐☐	☐☐	☐☐
Red meat (…….	☐	☐☐	☐☐	☐☐
Lamb ……..	☐	☐☐	☐☐	☐☐
Processed meat …….	☐	☐☐	☐☐	☐☐
Eggs …….. *(omeletts, pancakes etc)*	☐	☐☐	☐☐	☐☐
Liver/kidney game ……	☐	☐☐	☐☐	☐☐
Liver/kidney , ……. Pork, lamb,	☐	☐☐	☐☐	☐☐
Mackerel, salmon, herring	☐	☐☐	☐☐	☐☐
Cod, flounder, coalfish, …. haddoc	☐	☐☐	☐☐	☐☐
Halibut ……	☐	☐☐	☐☐	☐☐
Pikee and perch ……	☐	☐☐	☐☐	☐☐
Processed fish ……	☐	☐☐	☐☐	☐☐
Fish liver ….	☐	☐☐	☐☐	☐☐
Mussel	☐	☐☐	☐☐	☐☐
Shrimp ……	☐	☐☐	☐☐	☐☐
Crab ……..	☐	☐☐	☐☐	☐☐
Vegeterian dinner *(no fish, eggs, meat)*	☐	☐☐	☐☐	☐☐
		⊥		

Attachment B Draft for a scientific article with the preliminary title

Association between maternal sea food consumption and PFOS concentration in human breast milk

Association between maternal sea food consumption and PFOS concentration in human breast milk

ABSTRACT

Breast milk is both an important and uniquely composed source of nutrition to babies and an elimination route for the maternal body. It may therefore contain chemicals to which the mother has been exposed. Sea food is known to be an important source of pollutants like PFOS, and this study looked at the association between maternal sea food intake and PFOS levels in breast milk. A statistically significant positive association was found for crab consumption, but not for lean fish, fatty fish and total fish consumption. Statistically significant associations were also found for the confounders maternal age, education, smoking and birth year.

1 Introduction

Breast milk is both an important and uniquely composed source of nutrition to babies and an elimination route for the maternal body. That means, even though it is a general public opinion that "breastfeeding is best for the baby", the milk may also contain unfavourable pollutants to which the mother has been exposed. Of special concern are the persistent chemicals which are stored in maternal tissues throughout the woman's lifetime and then transferred from mother to child during breastfeeding. Due to developing organs and immature biological defences children may be less able to withstand exposure effects. In addition, exposure to chemical agents may interfere with normal development and growth, and with many years left to live, delayed toxic reactions are more likely to occur in people exposed in early life (Frumkin, 2016).

PFAS (perfluorooctanoic acid) is a group of chemically and thermally stable amphiphilic anthropogenic compounds widely used in fire-fighting foams, as non-stick coatings, in water- and stain-repellent textiles and food packaging (Antignac et al., 2013; Stratakis et al., 2020). They are absorbed easily after oral exposure, protein-binding and slowly eliminated from the body. One of the most highly detected PFAS is perfluorooctanesulfonic acid (PFOS) which distributes mainly to serum and liver. The main routes of exposure to PFOS are via inhalation of contaminated air and by ingestion of contaminated water or food (PHE Centre for Radiation Chemical and Environmental Hazards, 2017).

PFOS is covered for by the Stockholm Convention, which aims to reduce and replace the use of a selection of chemicals. However, PFOS is still present in our environment due to slow degradation of previous use (Bernes, 1998; Carson, 2002) and continued use (UN Stockholm Convention, 2019). Studies have shown that PFOS exist in our environment (Kunacheva et al., 2012), are taken up in the food chain and presented in human food, especially fish and other seafood (Schrenk et al., 2020) and that there are possible associations between diet and PFOS concentrations in humans (Skuladottir et al., 2015). There are also studies suggesting an association between PFOS early childhood exposure and unfavourable health outcomes like increased risk of ADHD (Lenters et al., 2019), reduced antibody response after child vaccination (Abraham

et al., 2020; Grandjean et al., 2012; Granum et al., 2013), increased prevalence of child infections, asthma, rhinitis and atopic dermatitis (Goudarzi et al., 2017; Kvalem et al., 2020), less microbiome diversity (Iszatt et al., 2019), liver disease (Stratakis et al., 2020) and lipid dysregulation (Blomberg et al., 2021).

2 Material and methods

2.1 Study population and sampling

Data used in statistical analysis were obtained from laboratory analysis of milk samples collected in the multi-center birth cohort study HUMIS. The milk samples were all collected between 2002 and 2009, from a total of 2606 mothers from six counties in Norway. The mothers were recruited through routine postnatal care home visits, except from in Østfold where mothers were recruited from the maternity ward by a paediatrician. Two consecutive term births for every preterm birth were enrolled. Each of the mothers was asked to collect and donate 25 ml of breast milk per day for eight following days, preferably in the morning and between third and eighth week postpartum. Date and time of collection was written on each sample, as well as whether a breast pump was used or not, and they were kept frozen. The samples have since collection been kept frozen at -20°C at the Norwegian Institute of Public Health (IPH). Breast milk was collected at a median 33 (interquartile range [QR]: 26-43) days after birth.

In addition, the mothers were asked to complete questionnaires at the baby age of one month, six months, one year, two years and ten to twelve years. The aim of these questionnaires was mapping of factors which might be of relevance for levels in breast milk and the health and development of the child (Eggesbø et al., 2009; Vollset et al., 2019).

For the present study, a total of 544 milk samples were used in the statistical analysis. These samples emerged from a "clean up" and by including 1st parity mothers only from dataset consisting of results from 60 randomly selected samples analysed at NMBU for this work and 1383 randomly selected samples previously analysed at IPH.

2.2 Ethics and confidentiality

The mothers signed an informed consent before the milk samples were collected. Samples used in this work were labelled with a code and thereby appeared as anonymous to the participants. The HUMIS study is approved by the Norwegian Data Inspectorate (Ref: 2002/1398-2 and 02/01398-7) and the Regional Committees for Medical and Health Research Ethics (REK) (Norwegian Institute of Public Health, 2017).

2.3 Chemical analyses of breast milk

The chemical analysis was performed either at the Laboratory of Environmental Toxicology, Norwegian University of Life Sciences (NMBU) (N=60) or at Institute of Public Health (IPH) (N=1383), Oslo, Norway. For the samples analysed at NMBU, only L-PFOS was detected, while for the samples analysed at FHI, total PFOS was detected.

2.3.1 Sample preparation at NMBU laboratory

After defrosting, 1g (3 decimal scale, Mettler Toledo) of breast milk from each sample was transferred into a polypropylene centrifugation tube (VWR). After addition of 5 ml methanol (Supelco) to each tube, the samples were mixed, shaked (IKA Vibrax) and centrifugated (X-12 Series Centrifuges, Allegra). The supernatant was transferred into a new tube, and the methanol procedure was repeated with the precipitate. The total supernatant was evaporated (Turbovap LV Evaporator, Zymark) down to a volume of 2 ml. To clean the samples from fat and other pollutants, a small amount (0.1 – 0.3 g) of Envi-Carb (Supelco) was added to each tube. After mixing and centrifugation, the supernatant was transferred to a new centrifugation tube and the precipitate was added 1 ml of methanol. Another mixing and centrifugation were performed, and the second supernatant was added to the first. The solution was then transferred to plastic LC vials with inlet and capped (VWR). Thereafter stored in the freezer until HPLC analysis could be performed.

2.3.2 Instrumental analysis at NMBU laboratory

Separation and detection of L-PFOS were performed on a HPLC-MS (Agilent 1200 HPLC, Agilent 6460 quadrupole MS with ESI ion source; Agilent Technologies) configured with a HiP model G1367C injector. L-PFOS was monitored using electron capture negative ionization (ECNI) in multiple reaction monitoring (MRM) mode. Pump flow was 0.300 mL/min, injected volume was 20.00 µl and draw and eject speed 200.0 µl/min. The column had valve position 2 (port 1->6) and a constant temperature of 40°C. At the ion source, the temperature was 300°C, the gas flow 5 L/min, nebulizer pressure 25 psi, SheatGasHeater was 400, SheatGasFlow 8, Capillary: 2000 (+), 2500 (-) V and charging 2000 (+), 500 (-). The results were quantified using the software Masshunter.

2.3.3 QA/QC at NMBU laboratory

Three blank samples were included to adjust for contamination of reagents and equipment used. To ensure accuracy of the analysis, all samples and six controls (one blind (cow milk, Tine), two recoveries, three blanks) were added standard PFAS solutions. The recoveries were also added analyte standards (laboratory's own).

2.3.4 Analysis at FHI laboratory

The previously analysed samples went through a different procedure (Forns et al., 2015). In brief, 200 µl of each breast milk sample was transferred to a centrifugation tube after thawing and homogenisation in a thermoshake incubator at 37 °C. Internal standards and acetonitrile were added until a total volume of 600 µl for thorough precipitation of proteins, then the solution was mixed using a whirl mixer. After centrifugation, the supernatant was transferred to a glass autosampler vial and added 500 µl of 0.1 formic acid. 400 µl of the extract was then injected into a column switching LC-MS/MS system. Calibration solutions were made from cow's milk. PFOS was detected and quantified using high performance liquid chromatography / tandem mass spectrometry (LC-MS/MS) according to a described method (Haug et al., 2009)

2.4 Statistical analysis

Concentration data from a total of 1428 samples (1383 (FHI) + 60 (NMBU) – 15 ("clean up")) were transferred to a statistical software program (STATA), together with a selection of exposure variables obtained from the HUMIS questionnaires. To explore the association between sea food intake (meals per year of fatty fish, lean fish, total fish and crabs) and the concentration of PFOS in breast milk, bivariate analysis and linear regression models were used. Both crude and adjusted models were run. Different kinds of sea food intake (as reported in the questionnaire) were set as exposure variables, and the concentrations of PFOS (obtained in the laboratory analysis) were set as outcome variable. Potential confounders were identified and are represented by a directed acyclic graph (DAG). Obesity (BMI), maternal age, smoking status, education level, self-catch and ethnicity, as well as child birth year and preterm birth were included in the adjusted models. To avoid uncertainty connected to total breast feeding, as there are many other factors than siblings that affects breast feeding, only data from 1st parity mothers were included in the final report. That resulted in 544 samples to proceed with in the statistical analysis.

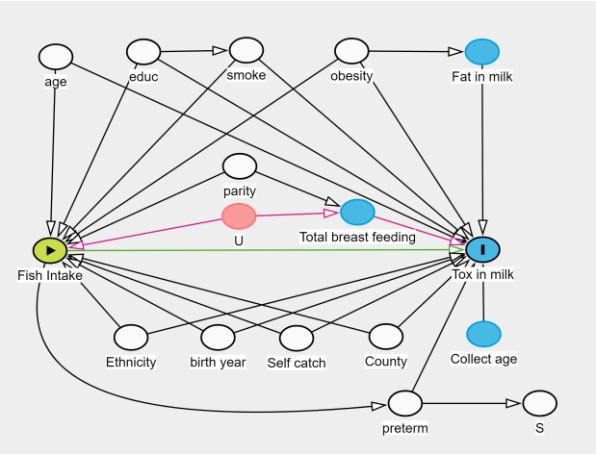

Figure 1 DAG showing exposure (Fish intake) and outcome (Tox in milk) variable, and potential confounders

3Results

3.1. Level of PFOS in the analysed breast milk samples

Even though only L-PFOS was detected and quantified in the samples analysed at NMBU while total PFOS value (L-PFOS and Br-PFOS) is reported for the samples analysed at FHI, the results were emerged and the term PFOS is used for the combined dataset (se section Discussion). Concentrations varied from 0 to 560 ng/l in the samples, with a mean at 119.15 ng/l and a median at 116.43 ng/l (see Table 1). For 109 of the samples, no PFOS was detected. The concentrations from the NMBU laboratory were originally given as

ng/g and then converted into ng/l by multiplying with the reference value of 1.03 g/ml for density of breast milk (Hamosh & Pitkin, 1991).

Table 1 Concentration details for PFOS (ng/l) in breast milk samples used in the statistical analysis

	N	Mean	Min	25th	Median	75th	90th	Max
PFOS	544	119.15	0	61.90	116.43	170	230	560

3.2 Consumption details about exposure variables (sea food)

Details about mean intake and distribution for different sea food categories are shown in Table 2. For all variables, there seems to be a skewness in that a few mothers have a very high consumption.

Table 2 Seafood consumption frequency (number of meals per year) among breast feeding mothers

Seafood	N	Mean ± SD	Min	25th	Median	75th	90th	Max
Lean fish	523	33 ± 30	0	12	24	52	60	180
Crab	505	3 ± 8	0	0	0	2	6	100
Fatty fish	510	32 ± 37	0	9	22	46	104	401
Total fish	507	64 ± 54	0	26	52	84	126	413

3.3 Factors associated with the concentration of PFOS

A separate bivariate analysis (t-test) was performed for all identified variables; exposure variables (sea food consumption) and the confounders. The results (Table 3) show statistically significant differences ($p \leq 0.05$) between the two groups tested towards each other for crab consumption ($p = 0.0114$), maternal age ($p = 0.0274$) and education (<12 years vs 12 years) ($p = 0.0521$). Higher levels of PFOS are seen in the group with smokers compared to non-smokers and in the group eating self-catched seafood vs not. Maternal obesity (BMI>25), being ethnically Caucasian (vs not) and giving birth preterm (vs on time) all show lower PFOS levels, but not statistically significant. The variable birth year looks “messy”, but from 2003 to 2009 there is a significant decreasing trend, indicating that birth year is negatively associated.

Table 3 Concentrations of PFOS (ng/l) in breast milk from Norwegian mothers, according to maternal sea food consumption and confounding variables

Exposures	N	Mean	delta-mean	p-verdi	95 % CI
Overall	544	119			
Seafood type consumption					
Lean fish*	523				
<52	466	120			113-128
>52	57	117	-3	0.7739	84-149
Fatty fish*	510				
<46	379	120			112-129
>46	131	117	-3	0.7728	99-136
Fotal fish*	507				
<84	379	129			111-128
>84	128	119	-1	0.9127	100-138
Crab*	505				
<2	371	114			105-122
>2	134	136	22	0.0114	118-155
Maternal age	544				
< 30 år	404	114.2			105.8-122.6
> 30 år	140	133.3	19.2	0.0274	117.2-149.6
Education	542				
<12 years	40	95.2			67.2-123.2
12 years	58	126.8	31.6	0.0521	108.1-145.5
>12 years	444	120.8	25.7	0.0868	112.4-129.3
Smoking status	539				
Never smoked	321	117.0			107.7-126.3
Former smoker	147	124.4	7.3	0.4165	107.7-141.0
Smoking at pregnancy start	71	119.4	2.4	0.8259	100.1-138.8
BMI	544				
<25	358	121,1			111.9-130.4
>25	186	115,4	-5,8	0.4735	102.5-128.2
Ethnicity - caucasian	544				
No	70	123,5			100.4-146.5
Yes	474	118,5	-4,9	0.6655	110.6-126.5
Birth year	544				
2002	8	82,8			4.4-161.2
2003	193	139,2	56,4	0.0603	127.5-150.9
2004	161	131,4	48,6	0.1458	117.1-145.7
2005	105	94,0	11,2	0.7529	75.2-112.8
2006	28	60,9	-21,9	0.4813	33.2-88.6
2007	13	110,2	28,2	0.4654	63.8-158.1
2008	23	87,6	4,8	0.8423	70.0-105.1
2009	13	84,2	1,5	0.9657	48.1-120.4
Self catch	544				
No	317	117.0			107.8-126.1
Yes	227	122.2	5,3	0.4962	109.4-135.0
Preterm	544				
No	475	120.3			112.2-128.3
Yes	69	111.6	-8,7	0.4501	90.4-132.8

*For sea food, values are given based on 75th percentile values for dinners per year (see table 2)

Neither crude nor adjusted data from linear regression did show any statistically significant associations between maternal consumption of any of the four sea food categories and concentration of PFOS in breast milk (Table 4). However, a positive contribution was seen for crabs.

Table 4 Crude and adjusted values from linear regression

	Crude values	**Adjusted for confounders***
Lean fish	-0.061 (p = 0.636)	-0.008 (p = 0.953)
Fatty fish	-0.111 (p = 0.302)	-0.109 (p = 0.332)
Total fish	-0.072 (p = 0.325)	-0.060 (p = 0.427)
Crab	0.705 (p = 0.167)	0.503 (p = 0.324)

*adjusted for the following confounders: Obesity (BMI), maternal age, smoking status, education level, self-catch, ethnicity, child birth year and preterm birth. ** Total fish includes lean fish and fatty fish.

Results from the multiple regression analysis are shown in Table 5. Among the exposure variables, only crab consumption shows a positive association with PFOS concentration in the milk (0.49, [0.52-1.49]), however not statistically significant (p = 0.341). Maternal age, education, smoking and self-catch of fish are the confounding variables associated with increased levels of PFOS in breast milk. For maternal age, there is a statistically significant association, and an increase of one year in age accounts for an increase in PFOS concentration of 2.25 ng/l. For education, the values given in the table are relative to less than 12 years of education. A statistically significant association is seen for 12 years long education compared to less than 12 years. For longer education (>12 years), a positive association is also seen (22.60ng/l), but it's not statistically significant. A 12-year long education will in this model contribute with 39.15 ng/l of PFOS, relative to less than 12 years of education. When it comes to smoking and self-catch of sea food, the associations are not statistically significant.

On the other hand, BMI, being ethnic Caucasian, birth year and preterm birth are variables negatively associated with PFOS levels. Higher BMI is associated with lower levels of PFOS in the milk, a BMI >25 explains -0.98 ng/l of the PFOS concentration in milk from those who are overweight, relative to those who have a BMI value < 25. To be ethnic Caucasian is associated with 13.40 ng/l lower level of PFOS compared to other ethnicities. The samples were collected between 2002 and 2009, and the regression analysis shows that it's a statistically significant negative association between giving birth later and PFOS concentration in the mothers' milk. However, in the bivariate analysis this is not clear as the level in samples from 2002, which is used as reference, is quite low (Table 3). But from 2003 to 2009 there is a significant downward trend in PFOS concentration. Giving birth prematurely (before day 259 / complete week 37) is associated with a reduction of 9.40 ng/l PFOS in the milk compared to giving birth on time, but the difference is not statistically significant.

Tabell 5 Table 24 Factors associated with PFOS levels in human breast milk (ng/l) and the estimate for how they contribute to the total PFOS level.

Explanatory variable	Coefficient (β)*	95 % KI	p
Exposure variables			
Lean fish	-0.00006	0.27-0.27	1.000
Fatty fish	-0.11	0.33-0.11	0.341
Crab	0.49	0.52-1.49	0.341
Confounding variables			
Maternal age	2.25	0.39-4.11	0.018
Education level 12 years	39.15	0.007-78.23	0.050
Education level > 12 years	22.60	-9.34-54.53	0.165
Former smoker	6.14	-11.54-23.82	0.495
Smoking at pregnancy start	-3.00	-28.29-22.28	0.815
BMI	-0.98	-2.80-0.83	0.288
Caucasian	-13.40	-38.66-11.86	0.298
Birth year	-15.25	-20.69-9.82	0.000
Self-catch of sea food	12.83	-3.17-28.84	0.116
Preterm birth	-9.40	-34.25-15.45	0.458

** Explanation of the coefficient (β), representing the contribution estimate to the PFOS level for each variable: For the exposure variables, it's per meal per year. For maternal age, it's for each year, for education the values are relative to less than 12 years of education, for smoking the values are relative to never-smokers, for BMI it's per unit on the BMI scale, for Caucasian it's relative to non-Caucasian, for birth year it's a mean reduction per year from 2002-2009, for self-catch it's relative to not eating self catched sea food and for preterm it's relative to giving birth on time.*

4 Discussion

4.1 Factors associated with concentration of PFOS

Breast milk is a route of elimination of unwanted substances from the mother's body. Factors influencing this process can be grouped into maternal, environmental and compound related factors. In this study, the main focus was on sea food consumption. Among the sea food groups studied, a statistically significant association was found for crabs only (p=0.0114). In addition, the identified confounders maternal age, and education level were found to have a statistically significant positive impact on PFOS level in human breast milk. For maternal age, the bivariate analysis shows a difference in mean value of 19.2 ng/l (p = 0.0274), in favour of age >30 years. In the regression analysis, the confounding variable maternal age is associated with a contribution of 2.25 ng/l per year (p = 0.018). These findings may be explained by older mothers having accumulated PFOS over a longer period of time and have lived a longer part of their life before restrictions were applied to the compound, thereby being exposed to higher levels in their environment. When it comes to education level, a statistically significant difference in mean values (31.6 ng/l, p = 0.0521) is seen in the bivariate analysis between less than 12 years of education compared to 12 years of education. According to the regression analysis, to have 12 years of education will contribute with 39.15 ng/l (p = 0.0050) relative to

less than 12 years of education. An explanation to this may be that more educated people tend to eat more fish as part of a generally healthier lifestyle. For some reason a statistically significant contribution is not seen for the group with more than 12 years of education, but the estimated impact on the PFOS level is 22.60 ng/l (p =0.165). Other factors may come into play.

The higher, but not statistically significant levels (p = 0.116 in the multivariate regression analysis and p = 0.4962 in the bivariate analysis) of PFOS seen in the group eating self-catched seafood vs not, may reflect that sea food in lakes and fjords tend to contain more pollutants than in the open ocean (Augustsson et al., 2021).

On the other hand, BMI, being Caucasian, birth year and preterm birth are variables negatively associated with PFOS levels, of which only birth years showing statistically significance. In the regression analysis birth year contributes with 15.25 ng/l less PFOS per year (p = 0.000). In the bivariate analysis, birth year 2002 was set as reference group, and all other groups were compared to that one. The 2002 group consists of only 8 individuals, giving birth at the end of the year as that was when the study recruitment started. The PFOS level is low in this group, but the low number of individuals ties an uncertainty to the value. That may be the reason why this analysis does not show a reduction form birth year 2002 to 2009. The drop in PFOS level with later birth year may be explained by the phase out of PFOS under the Stockholm Convention (Land et al., 2018)

The difference seen for ethnicity (Caucasian versus other) may reflect differences in diet and other life style factors, genetic variance or a general higher exposure in the upbringing environment. As PFOS is mainly protein bound it is maybe not clear that higher BMI would negatively affect the level in breast milk, but the results from this study indicate that BMI may play a role. However, the difference or contribution is not statistically significant neither in the bivariate nor in the regression analysis (p = 0.4735 and 0.288, respectively).

4.2 Comparison with other studies

Based on the results from the EFSA report (Schrenk et al., 2020), saying that fish and other sea food is the most important contributor to mean LB exposure of PFOS, it was expected to find a positive association between maternal sea food intake and concentration of PFOS in the breast milk. However, this was found for crabs only. Many factors, like characteristics related to the compound, the maternal body and maternal lifestyle, may come into account. There may therefore not be a direct association between concentration in food and concentration in breast milk. Possible explanations for our findings could be that the EFSA report does not mirror the conditions in Norway when it comes to PFOS levels in the sea food products consumed e.g. that Norwegians eat mostly cod and salmon (Norges Sjømatråd, 2018) while the species with highest

PFOS levels were found to be carp and eel (EFSA), uncertainty related to self-reported fish intake and confounders which have not been identified.

Several studies have been performed earlier about PFOS and human breast milk. The results are inconsistent and inconclusive when it comes to the association between maternal intake of sea food and PFOS concentrations in breast milk. It's not always straightforward to compare results as some report the results in g/l (or pg/ml) while others report in ng/g. Also, some studies differentiate between isomers (L (linear) and Br (branched)) while others do not. In addition, sampling year, inclusion criteria, sampling size, local variations in PFOS exposure and differences in analytical procedure may affect the results. Some studies only look at concentrations while others also look at associations between PFOS levels and health outcomes or possible sources. In the study on breast milk from French women (Antignac et al., 2013) no statistically significant associations between PFOS levels and various socio-geographic parameters including seafood consumption were found. Nor did it show statistically significant associations with maternal age or parity. The first finding is similar to the present study which shows an association with the sea food group crabs only while the second is contradictory when it comes to maternal age. As only data from 1st parity mothers were included in the present study, no comparison can be made with that variable. In the Korean study (Lee et al., 2018), significant associations between PFOS levels in breast milk and maternal age, maternal BMI, parity, snack consumption, milk intake and eating out were found. The findings regarding maternal age and BMI are in line with the findings in the present study, the other variables were not considered here. The findings in the US study (Zheng et al., 2021) indicate that both legacy and current-use PFOS contaminates breast milk. A statistically significant correlation was found between PFOS concentration in milk samples and duration of breast feeding, estimated from the age of the infant sampling time ($\beta = -0.411$, $p = 0.047$), indicating that lactation is an important elimination way of this chemical. The present study did not look into baby age and PFOS concentration, but all samples were collected early in the breastfeeding period (between third and eighth week).

4.3 L-PFOS and PFOS

In this study, data from two different data sets were merged. In the 60 samples analysed at NMBU, only L-PFOS was detected. In the samples analysed by FHI, total PFOS was detected. Due to a low number of samples with L-PFOS compared to the number with total PFOS and the fact that Br-PFOS is generally low compared to L-PFOS, it was considered to be acceptable to merge the data sets.

4.5 Strengths and limitations of the study

Strengths of this study include a relatively large sample size with participants from different parts of Norway. Only 1st parity mothers were included in order to exclude uncertainty related to total breast feeding, as the number of siblings alone is not a good measure for this, and diet changes related to having kids in the household. A limitation with the study is that sea food consumption is self-reported and based on the memory of the participants.

5 Conclusion

The expected positive association between maternal sea food intake and concentration of PFOS in the breast milk from 1st parity Norwegian mothers was not seen, except from with crabs. However, the study gave several interesting findings related to defined confounders, indicating that the association of interest is strongly biased. This, together with uncertainty in the self-reported sea food consumption data and the possibility that the fish eaten by Norwegians may contain less PFOS than samples analysed in the EFSA food study, may explain our results. There may also be other confounding factors than the ones defined in this study.

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Conflict of interest

The authors declare no conflicts of interest

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