Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
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Supervisor: Bjørn Munro Jenssen
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Abstract

Grey seal (*Halichoerus grypus*) is a marine mammal that has breeding grounds at Froan in Norway. The number of newborn pups each year has decreased during the past two decades in this area. As a marine top predator, the accumulation of persistent organic pollutants poses a potential threat to their health and fitness. Per- and polyfluoroalkyl substances (PFAS) are a group of contaminants that are recently suspected to have harmful effects on biota, and one of these suggested effects is endocrine disruption. Thyroid hormones (TH), that is thyroxine (T4) and triiodothyronine (T3), are important for development in young mammals, regulating growth and development. A pollutant accumulation *in utero*, or by transfer through mothers-milk can increase the pollutant burden. If PFAS alters hormone homeostasis, it can pose a threat to young grey seal pups.

In this study plasma samples were obtained from 12 grey seal pups sampled at Froan to determine the concentration of 46 different PFAS, and the concentration of TH (tT4, fT4, tT3 and fT3). In addition, the relationship between tT4 and tT3 (tT4:tT3), and fT4 and fT3 (fT4:fT3) was determined. The correlations between the contaminants and the THs as well as the correlation between the biometric variables and the contaminants were determined.

Biometric variables had significant positive correlations with PFOA, PFNA and PFDA, and significant negative correlations with PFHxS. There were significant negative correlations between biometric variables and THs. Thus, biometric variables were used as a covariate for correlation analysis between THs and contaminants. When this analysis was done, only one significant relationship was identified: a positive correlation between PFECHS and fT4:fT3. The support of these results is divided in literature.

In conclusion, it is difficult to determine whether PFAS contamination profoundly affects thyroid homeostasis in grey seals. Since the identified PFECHS-fT4:fT3 correlation is not evidence of a causal relationship, the correlation found does not necessarily mean that the contaminants directly affect the hormones. There might be other co-occurring contaminants present in the seals that were not considered in this study. More research on the matter should be conducted to support or contradict the current result and increase the weight of evidence, improving the knowledge of the possible harmful effect of the contaminant.

Sammendrag

Havert (*Halichoerus grypus*) er et marint pattedyr som har kasteplass på Froan langs Norskekysten. De siste årene har antallet selunger født på disse øyene sunket. Siden havert er et rovdyr på toppen av næringskjeden, er det en større risiko for at akkumulering av persistente organiske miljøgifter kan påvirke dyrenes overlevelsesevne. Per- og polyflouroalkyl substanser (PFAS) er en gruppe miljøgifter som i senere tid har blitt mistenkt for å ha skadelige effekter på levende organismer, og en av de mistenkte mekanismene er hormonforstyrrelse. Skjoldbruskkjertelhormoner (TH), tyroksin (T4) og trijodtyronin (T3), er viktig for utviklingen til unge pattedyr, siden de regulerer vekst og utvikling. Akkumulering av forurensing i dyret mens det er i livmor, eller overførsel via morsmelk kan øke forurensningsbelastningen. Dersom PFAS kan påvirke hormonhomeostase, kan det derfor være en trussel for selunger.

I denne studien ble det tatt prøver av 12 selunger fra Froan, og vekt, lengde og omkrets ble målt, samt at alder ble bestemt. Plasmaprøver ble tatt for å bestemme konsentrasjonen av 46 forskjellige PFAS, og konsentrasjonen av skjoldbruskkjertelhormoner (tT4, fT4, tT3 og fT3). I tillegg ble forholdet mellom tT4 og tT3 (tT4:tT3) og fT4 og fT3 (fT4:fT3) bestemt. Korrelasjonene mellom PFAS og TH, samt korrelasjonen mellom PFAS og biometriske variabler ble undersøkt.

De biometriske variablene hadde signifikante positive korrelasjoner med PFOA, PFNA og PFDA, og signifikante negative korrelasjoner med PFHxS. Mellom TH og PFAS var det flere signifikante korrelasjoner som ble oppdaget, men siden korrelasjonen mellom hormoner og biometriske variabler var så tydelige, ble biometriske variabler ble brukt som en kovariat mellom PFAS og hormoner. Etter denne analysen var det bare en positive korrelasjon igjen, den positive sammenhengen mellom PFECHS og fT4:fT3, men korrelasjonen er antakelig drevet av en avvikende observasjon.

Konklusjonen er at det ikke er mulig å bestemme om forurensninger fra PFAS har en viktig effekt på hormonhomeostase i havert. Korrelasjonene betyr ikke at PFAS direkte påvirker hormonene, og det kan være andre forurensninger til stede som ikke ble regnet med i denne studien. Flere forsøk bør bli gjennomført for å støtte eller motbevise gjeldene resultat, for å øke bevistyngden, og øke kunnskapen om mulige skadelige effekter fra PFAS.

Abbreviations

4:2 FTS	1H,2H-Perfluorohexan sulfonate (4:2)
6:2 FTS	1H,2H-Perfluorooctane sulfonate (6:2)
6:2FTS-^{13}C2	1H,2H-Perfluorooctane sulfonate (6:2) -^{13}C2
6:6PFPi	Bis(tridecafluorohexyl)phosphinic acid
6:8PFPi	Perfluorohexylperfluorooctyl phosphinate
8:2 FTS	1H,2H-Perfluorodecan sulfonate (8:2)
8:8PFPi	Bis(perfluorooctyl) phosphinate
10:2 FTS	1H,2H-Perfluorododecan sulfonate (10:2)
7H-PFHpA	7H-dodecafluoroheptanoic acid
9Cl-PF3ONS	9-chlorohexadecafluoro-3-oxanonane-1-sulfonate
ADONA	dodecafluoro-3H-4,8-dioxanonanoate
ANCOVA	Analysis of covariance
ATP	Adenosine triphosphate
BMD10	Benchmark dose limit 10%
BMR	Basal metabolic rate
C	Celsius
cm	centimetre
CV	Coefficient of variation
DecaS	Sodium 1-decasulfonate
diSAMPAP	bis[2-(N-ethyl perfluoro octane-1-sulfonamido)ethyl] phosphate
dL	deciliter
E	East
EFSA	European food safety authority
ELISA	Enzyme-linked immunosorbent assay
EtFOSA	Sulfuramide
EtFOSAA	N-ethylPerfluoro-1-octanesulfonamide acetic acid
EtFOSE	N-ethyl-N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide
EtOH	Ethanol
FOSAA	Perfluoro-1-octanesulfonamidoacetic acid
fT3	Free triiodothyronine

fT4	Free thyroxine
GenX	2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy) propanoate
h	Hours
HPLC	High pressure liquid chromatography
Hybrid SPE	Hybrid solid phase extraction
I_2	Iodine
IS	internal standard
kg	Kilogram
km	kilometer
LOD	Limit of detection
LOQ	Limit of quantification
m	meter
MeFOSA	N-methylPerfluoro-1-octanesulfonamide
MeFOSAA	2-(N-methylPerfluoro-1-octansulfonamido) acetic acid
MeFOSE	N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide
mm	Millimeter
mg	Milligram
min	minutes
mL	Milliliter
N	North
n	Number of observations
ng	Nanogram
NH_4COOH	Ammonium formate
NTNU	Norwegian University of Science and Technology
p	Probability of rejecting the hypothesis
P37DMOA	Perfluoro-3,7-dimethyl octanoic acid
PC	Principal component
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PFAS	Per- and polyfluorinated substances
PFBA	Perfluorobutanoic acid

PFBS(NonaFBS)	Perfluorobutanic acid sulfonate
PFCA	Perfluorinated carboxylates
PFDA	Perfluorodecanoic acid
PFDS	Perfluorodecane sulfonic acid
PFDoDA (TricoFDoDeA)	Perfluorododecanoic acid
PFDoDS	Perfluorododecane sulfonic acid
PFECHS	Perfluoroethylcyclohexane sulfonic acid
PFHpA	Perfluoroheptanoic acis
PFHpS	Perfluoro-1-heptanesulfonate
PFHxA (UnFHxA)	Perflourohexanoic acid
PFHxDA	Perfluoro-n-hexadecanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFNA (PFNonDeA)	Perfluorononanoic acid
PFNS	Perfluorononane sulfonic acid
PFOA	Perfluorooctanoic acid
PFOA-^{13}C8	Perfluorooctanoic acid -^{13}C2
PFODcA	Perfluorooctadecanoic acid
PFOS	Perfluorooctane sulfonic acid
PFOS-^{13}C8	Perfluorooctane sulfonic acid -^{13}C2
PFOSA	Perfluorooctane sulfonamide
PFPeA	Perfluoropentanoic acid
PFPeS	Perfluoropentane sulfonic acid
PFSA	Perfluoroalkyl sulfonates
PFTE	Polytetrafluoroethylene
PFTDA(PFTetDeA)	Perfluorotetradecanoic acis
PFTriDA	Perfluorotridecanoic acid
PFUnA	Perfluoroundecanoic acid
pg	picogram
POP	Persistent Organic Pollutant
ppm	parts per million

ppb	parts per billion
r	Correlation coefficient
RIA	Radioimmuno assay
rpm	Reveloutions per minute
SAMPAP	2-(N-ethylperfluorooctane-1-sulfonamido) ethyl phosphate
SD	Standard deviation
sec	seconds
T3	Triiodothyronine
T4	Thyroxine
TA	Target analytes
TH	Thyroid hormone
TRH	Thyroid releasing hormone
TriDeFHxSA/ PFHSK	Tridecafluorohexane-1-sulfonic acid potassium salt
TSH	Thyrotropin hormone
tT3	Total triiodothyronine
tT4	Total thyroxine
UHPLC-MS/MS	Ultrahigh-pressure liquid chromatography-tandem mass spectrometry
µl	Microliter

Table of contents

1 Introduction

1.1 Per- and polyfluoroalkyl substances

Per- and polyfluoroalkyl substances (PFAS) are a collective term for a group of chemicals containing a fully fluorinated methyl or methylene group in an alkyl chain (Buck et al., 2011; Kissa, 2001). Figure 1.1 shows the structure of a common PFAS, perfluorooctane sulfonate (PFOS) (Bangma et al., 2022). PFAS are man-made synthetic chemicals, produced commercially and used in a variety of products. The result of their chemical composition is compounds that are both hydrophobic and lipophobic (Kissa, 2001), and that can withstand a broad temperature range and are stable (Kissa, 2001). An example of application PFAS exhibit is one of the first commercially produced PFAS compounds, polytetrafluoroethylene (PFTE), which is commonly known by the DuPont trademark Teflon. It was discovered in 1938 by Roy J. Plunkett in his work for DuPont (Dams and Hintzer, 2016; Gaines, 2022). PFTE is used as a non-stick coating for pans, cookware, and water-resistant clothing because of its hydrophobic and lipophilic properties. Because of its non-reactive properties, it is also used for containing reactive chemicals, and the low friction of the compound makes it a great lubricant (Dams and Hintzer, 2016).

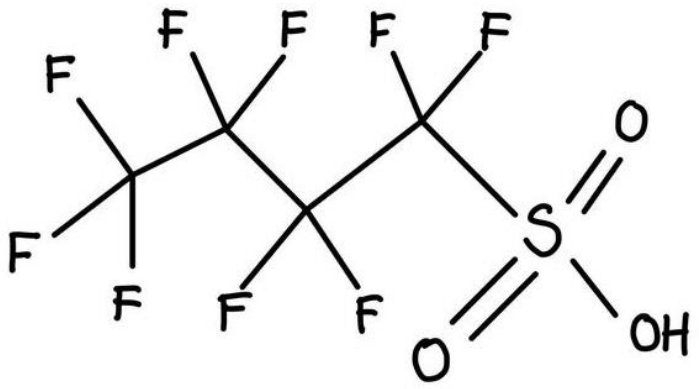

Figure 1.1: Perflourooctane sulfonate structure, by Anette Hornnæs.

After introduction of the compound group, several other PFAS have been produced and their area of use is wide. It includes firefighting foam, cosmetics, photo imaging, semi-conductor or hydraulic fluids used in aviation, and the previously mentioned usages (Gaines, 2022). There are more than 6,000 distinct PFASs known, depending on the source (De Silva et al., 2021). Release into the environment is mainly through industry and manufacturing for example primary and secondary manufacturing sources, where PFAS is synthesized or where PFAS materials are being used. In addition, construction, firefighting foam, wiring, and metal finishing and plating can be

sources of PFAS in the environment (De Silva et al., 2021; Gaines, 2022). Organismal exposure is higher closer to the release site, but because of environmental transport, PFAS is ubiquitous in the environment, and exposure will happen even if an organism is far from the exposure site (De Silva et al., 2021).

PFASs were considered inert for a long time, because of their stability, but possible health risk was proposed in the 1990s. Since then, there has been an increasing interest in their effect on humans and wildlife, resulting in the discovery of several profound effects. Well-known substances such as PFOS and perfluorooctanoic acid (PFOA) are shown to disturb the mammalian immune system, reducing the production of antibodies after exposure to a foreign substance in humans (Grandjean et al., 2012; Grandjean et al., 2017; Granum et al., 2013), and compromising the immune responses in marine mammals (Routti et al., 2019; Soloff et al., 2017). This results in increased susceptibility to infection and repetitive infection of the same infectant. Human foetal development is affected by both PFOS and PFOA, resulting in lower birth weight (Haug et al., 2018). Other PFASs exposure correlate with miscarriages (Rogers, 2019). Furthermore, the effects on thyroid hormones by PFAS have been determined in several mammalian studies. In hooded seals (*Cystophora cristata*) PFAS is known to alter the ratio between thyroxine (T4) and triiodothyronine (T3) so that the free T4: free T3 ratio increases (Grønnestad et al., 2018). Free T3 is negatively related to PFAS in polar bears (*Ursus maritimus*) during spring (Bourgeon et al., 2017), in addition to showing thyroid homeostasis disruption in Alaskan natives (Byrne et al., 2018). Pregnant human women with high blood concentrations of perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnA) had lower T3 levels than the rest of the sample population (Berg et al., 2015). The changes in T3 were too low to cause clinical effects in the women exposed to PFAS but may affect the health of the foetus (Berg et al., 2015). For humans, a tolerable weekly intake of four main PFAS (PFOA, PFOS, perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHxS)) is determined to 4.4 ng/kg body weight, established by the European food safety authority (EFSA) in 2020 (EFSA, 2020).

Even though several studies on the effect of PFAS on organismal health has been conducted, there is a lack of knowledge on the harmful effects of PFAS on wildlife and humans. Several new PFASs are produced resulting in lower weight of evidence, and unknown long-term effects for these new compounds. Because of their stability in the environment, the chemicals accumulate in organisms,

resulting in exposure to a higher concentration over a longer period (Bangma et al., 2022; Houde et al., 2011). All time release of the chemicals will therefore affect the exposure.

Selected PFASs are considered a persistent organic pollutant (POP) because of their resistance against physical, chemical and biological degradation, their ability to long-range transport and biomagnification, as well as their harmful effects. However, while legacy POPs often are lipophilic and are associated to the lipids in fat tissue, the properties that make PFASs good surfactants, keep them from binding to fat tissue (Gaines, 2022; Sørmo et al., 2003). PFAS are rather associated to proteins, causing them to be accumulated in protein-rich tissues, and more readily present in blood and liver (De Silva et al., 2021). This means research done on exposure, transport and accumulation of other lipophilic POPs cannot be applied to PFAS (Gaines, 2022; Kelly et al., 2009; Meylan et al., 1999). While some lipophilic POPs are stored in fat tissue, protecting the organism from exposure until an event causes weight loss (Jansen et al., 2017; Sørmo et al., 2003), PFAS is mainly stored in protein-rich tissue and will cause effects independent of weight loss (Jansen et al., 2017), unless protein stores are catabolized.

There is a need for empirical data concerning the bioaccumulation of PFAS, although there are some studies indicating bioaccumulation in arctic mammals (Boisvert et al., 2019). Several studies have shown that there is an increase in contaminant concentration depending on the trophic level of an organism, but it is harder to determine the reason for PFAS accumulation than it is for lipophilic compounds, as it is possible to control for lipid content in lipid binding pollutants, but not for protein binding sites in the tissues (Grønnestad et al., 2018; Haukås et al., 2007). Some studies show PFAS biomagnify, and that the ability increases with the length of the alkyl chain, which will result in a higher burden for organisms on top of the food chain (Haukås et al., 2007; Kelly et al., 2009).

Organisms are mainly exposed to PFAS through diet, although a major source can be drinking water in some exposed areas (De Silva et al., 2021). Younger individuals are often more susceptible to harmful effects after chemical exposure (Rogers, 2019). For mammals, lipophilic contaminants are transferred from the mother to their young through the mother's milk, which is rich in lipids (Sørmo et al., 2003). The result of this is a high concentration of pollutants in the young individual. For PFAS this transfer is expected to be different, as the pollutant does not so readily bind to the lipids in milk, and therefore the transfer from mother to young is relatively low

compared to lipophilic compounds (Grønnestad et al., 2017; Kelly et al., 2009). On the other hand, it is probable that during the pregnancy, the foetus is exposed to a higher concentration of PFAS than it is of present lipophilic pollutants, relative to their presence in the mother (Grønnestad et al., 2017).

Some animals, like seals, are precocial, meaning that they are close to fully developed when they are born (Kovacs and Lavigne, 1986). Their protein-rich organs are fully developed, because the proteins are transferred from mother to cub *in utero*, and this can cause a high PFAS burden, especially when the mother is a marine top predator (Houde et al., 2011; Kelly et al., 2009; Kovacs and Lavigne, 1986). The foetus will be exposed to protein-associated contaminants at a higher rate, which may cause impaired development (Grønnestad et al., 2017). Foetuses are more susceptible to damage by contaminants than newborn pups as most of the development happens before birth. Small changes in hormone homeostasis can have large effects on thethightly regulated development (Grandjean et al., 2008). Exposure during gestation can affect early physical and mental development and, depending on the developmental stage, have profound effects on survival in later life stages (Rogers, 2019).

Observed harmful effects caused by PFASs such as PFOS and PFOA have led to a ban on some of the compounds. The PFAS PFHxS is banned in the Stockholm convention, and all use and production of the compound are banned. PFOA is also banned by the convention, but as there are some areas of use where there is no replacement chemical, there is still some usage of the chemical. PFOS is partially banned but is allowed in some cases in developmental countries where no replacement is available, or alternatives must be phased in. Several of the other PFAS compounds are under investigation for profound effects and might be regulated by the Stockholm convention in the future (StockholmConvention, 2019).

1.2 Thyroid hormones

Thyroid hormones (TH) are a collective term for several closely related hormones. The two most important are thyroxine (T4) and triiodothyronine (T3). They are both produced in the thyroid gland, which is located in the front of the neck in mammals. The hormones are mainly responsible for the regulation of metabolism and for development of young individuals (Halse and Berg, 2022).

Production of T3 and T4 starts with the follicle cells producing thyroglobulin, which is a glycoprotein containing about 140 tyrosine units. This is released into the colloids, which also

contain iodine (I_2). The tyrosine units bind iodine, creating mono-iodine tyrosine and diiodine tyrosine, which combine and create T3 and T4. Thyroid hormones are lipid soluble and they are stored in colloid islands in the thyroid gland after production until they are released into the blood through capillaries. In the blood they are mostly bound to plasma proteins, with 99,95% of T4 and 99,5% of T3 bound to proteins (Halse and Berg, 2022). The T3 and T4 that are not bound are called free T3 and free T4 (fT3 and fT4) (Woldstad and Jenssen, 1999). The thyroid gland produces and releases more T4 than T3 (Halse and Berg, 2022). The structure of T4 and T3 is shown in Figure 1.2. They both consist of a phenyl ring bound to a tyrosine molecule by an ether linkage (Hulbert, 2000; PubChem, 2011).

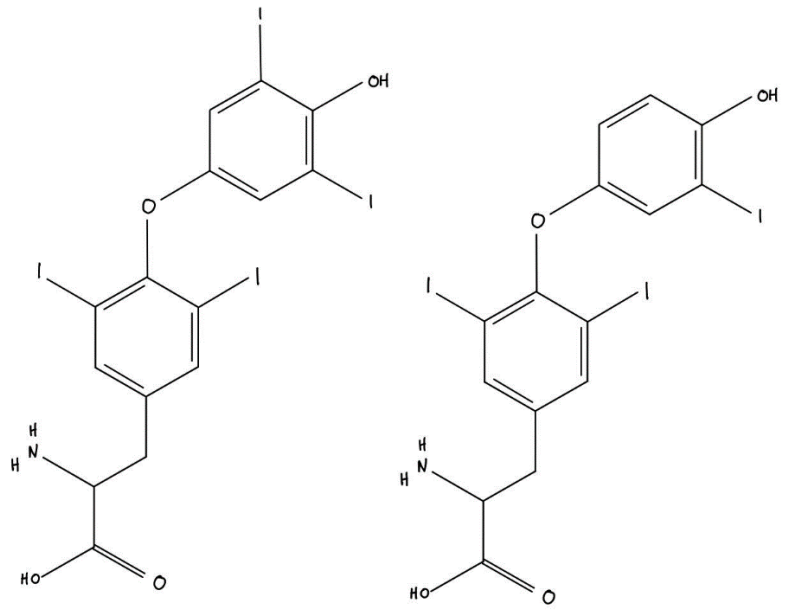

Figure 1.2: Structure of thyroxine (T4) and triiodothyronine (T3) (PubChem, 2011). Figure by Anette Hornnæs

The release of thyroid hormones is regulated by the hypothalamus and hypophysis through negative feedback. Blood levels of the two hormones are precisely supervised, and if they drop too low, the hypothalamus will release thyroid-releasing hormone (TRH) into the anterior pituitary gland, stimulating the release of thyrotropin hormone (TSH) which binds to follicle cells in the thyroid gland, causing the release of T4 and T3. Normal or high levels of T4 and T3 will decrease the release of TSH to make sure the hormone concentration in the blood does not surpass normal concentrations. In this way, the central nervous system can regulate the concentration of thyroid

hormones, adjusting to current needs based on factors such as developmental stage and sex (Halse and Berg, 2022).

The thyroid gland releases more T4 than T3, but T4 is often considered the inactive form of the hormone, as the potency of T3 is up to four times as high (Hulbert, 2000). Cells in the liver, thyroid, muscles, and kidney can deiodinate T4 to T3, "activating" the hormone, but the half-life of T3 is much lower than the half-life of T4 (Leboeuf et al., 2007). Because of this, T4 can be present in the blood for a longer period, and when the hormone action is needed, deiodination of T4 to T3 will increase the hormone action for a shorter period until T3 is broken down (Halse and Berg, 2022).

Thyroid hormone receptors are located at the cellular nucleus, and as thyroid hormones are lipophilic, they diffuse through cell membranes and bind to the receptors. Their mode of action is to alter gene expression by activating transcription and translation of target genes (Pitt-Rivers and Tata, 2013). Thyroid hormones are key regulators of metabolism. They increase the basal metabolic rate (BMR) by generating and maintaining Na^+/Ka^+ and Ca^{2+} ion gradients and increasing adenosine triphosphate (ATP) production. In addition, thyroid hormones stimulate the metabolic cycles for lipids, glucose and proteins. This results in the production of heat and the use of oxygen (Mullur et al., 2014). Brain development is also dependent on regulation by thyroid hormones. They affect the neurons throughout the brain, regulating the size, packing density and dendritic morphology of the cells. For proper brain development, the presence of thyroid hormones is important before birth and in the neonatal period (Thompson and Potter, 2000). Lack of thyroid hormones will also affect the birth weight and cause growth defects in newborns. By increasing the release of growth hormone from the pituitary gland, thyroid hormones also affect growth in older individuals. In addition, T4 work synergistically with growth hormone and enhances its effect. Normal physiological levels of thyroid hormones increase the synthesis of proteins, but thyroid hormone excess will cause protein breakdown and hypermetabolism (Gelfand et al., 1987; Halse and Berg, 2022; Pitt-Rivers and Tata, 2013; Sokoloff et al., 1968).

Careful regulation of thyroid hormones is important for organismal survival, especially in younger individuals. Levels of thyroid hormones that are outside of normal ranges can cause a disadvantage leading to lower general fitness of the organism (Halse and Berg, 2022). Pollutants in the

environment can work as endocrine disruptors, either by working as a TH, inhibiting or altering the level of thyroid hormones and causing harm to organisms.

Pollutant effects on thyroid hormone levels have been studied. Polychlorinated biphenyls (PCB) and other organochlorine compounds have been shown to reduce the levels of thyroid hormones in seals (Bennett et al., 2021; Brouwer et al., 1989). PFAS is also considered to affect the levels of thyroid hormones. In a mixture, PFAS is shown to disrupt the fT4:fT3 ratio in seals, where increasing concentration of PFAS increases the ratio (Grønnestad et al., 2018). There are shown thyroid-disrupting properties of PFAS in birds as well, increasing the concentration of tT3 with increasing PFAS concentration (Sebastiano et al., 2021). In addition, PFOS and PFOA have been shown to reduce the activity of one of the most important enzymes in thyroid synthesis in an *in vitro* study (Song et al., 2012). The increasing presence of PFAS in the environment can therefore be a threat, causing a lower survival rate of offspring.

1.3 Grey seals

Grey seal (*Halichoerus grypus*) is a phocid seal widely distributed around the Atlantic in the northern hemisphere. There are populations in the western Atlantic, eastern Atlantic and in the Baltic Sea, with a total abundance of about 650 000 individuals (Haug, 2021). Along the Norwegian coast, there are three major populations. One of the populations can be found along the southern coast of Norway, and is considered a part of the English population (Wiig, 1986). There is one population in the north of Norway which is considered a part of the Russian population. Along mid-Norway, there is a population (Fiskeri-ogKystdepartementet, 2010) that may be considered as the original Norwegian population (B.M. Jenssen, pers.comm).

With adult sizes around 2m and 200-300kg grey seals are a relatively large species of phocid seal. Males are bigger than females, and their face grows longer, resulting in a horse-like appearance in adults. Grey seals live to be 35-40 years, and they mature after 5-6 years. They live in solitude and breed on remote islands and skerries. Female seals give birth to one pup each year, and the lactation period is 15-20 days. Grey seals are considered generalist predators, but they feed mainly on benthic species (Haug, 2021).

As the grey seals are marine top predators, they are vulnerable to pollution by contaminants that bioaccumulate and biomagnify. Biomagnification cause a high concentration of pollutants higher up in the trophic levels. During their lifespan, adult individuals may accumulate high

concentrations of pollutants because of this (Houde et al., 2011; Kelly et al., 2009). As the females are lactating and feeding the young, lipophilic pollutants may be transferred from the mother to the pup in the milk, which is rich in lipids (Addison and Brodie, 1977, 1987). Newborn pups are still under development, and high exposure to pollutants during the weaning period may be harmful and decrease the chance of survival. In addition, they are less capable of metabolizing toxicants during this period, and this can result in high toxicant burdens in the pups (Bearer, 1995; Nickerson, 2006).

All grey seals from the population in mid-Norway breed and give birth in the same area, the nature reserve outside of Frøya in Trøndelag called Froan (Røv et al., 1990). While the total abundance of grey seals in Norway has increased over the last few years, the population breeding at Froan is decreasing. In 1996 and 2001 the numbers of pups were 262 and 283 respectively, but in 2014 the number was down to 77, and only 60 in 2018 (Nilssen et al., 2018). The reason for this is unknown, but it might be a connection between the release of toxicants and the resulting harmful effects on reproduction or survival.

1.4 Froan

Froan is a nature reserve outside Frøya in Trøndelag, Norway, 63°59′34″N 9°03′13″E. The reserve consists of hundreds of small islands and skerries, a total size of 400 km^2. In addition, there is an 80km^2 landscape protection area southeast of the nature reserve. The area is important for a lot of bird species, as there is no mink there and they can nest more safely. There is a large population of harbour seal (*Phoca vitulina*), and the area is the most important breeding ground for grey seal in Norway (Frøya-kommune, 2022; Røv et al., 1990).

Froan is named after the foaming of the sea as the waves hit land, as the area is exposed to a lot of harsh weather conditions (Thorsnæs, 2019). It is remote, and therefore exposed to waves from the Atlantic Ocean, as there is no land that stops the winds. Because of this it is not much precipitation, as rain clouds drift past the area. The tides have large fluctuations, with high tides two meters higher than the low tides (Kartverket, 2023b).

Although Froan is a nature reserve, there is nothing stopping pollutants from nearby areas from drifting into the area. Because of currents, plastic- and other waste accumulate on the islands (Cyvin et al., 2021). In addition, there is nothing stopping chemicals and toxicants from entering the remote area by air, through water or inside migrating organisms. Considering PFAS is often

released into the water, ocean currents are the most important transport route, and they can transport PFAS over long distances (Wania, 2007; Zhao et al., 2012). Water currents can transport discharges from cities and urban areas along the coast of Norway, as well as pollutants from the petroleum installations south of Froan. Furthermore, PFAS can be transported inside organisms that migrate, like birds or fish (Roscales et al., 2019). Concentrations of chemicals are always higher closer to the source of release, and even though Froan is in a remote area, there is infrastructure close by that can cause higher levels of pollutants.

The Norwegian army has an aeroplane base close to Froan, at Ørland, and there is activity at Ørland connected to military aeroplane operations. This include aeroplane fuel use and storage, firefighting exercises releasing firefighting foam, known to increase the PFAS burden in the environment (Skaar et al., 2019), as well as release of de-icing liquid into the waters only 43km southeast of Froan (Mørch et al., 2018). In addition, there is release of pollutants in the air and particle fallout (Forsvarsbygg, 2013). On the opposite side, 80km northwest of Froan, there are active oilfields with large oil platforms pumping up oil and gas (Kartverket, 2023a). From this area, there is a release of organic pollutants, with a substantial risk of affecting the life in the nature reserve. Previous studies have found external oil contamination on the fur of grey seal pups lying on the skerries at Froan (Jenssen, 1996). This shows that the grey seal pups at Froan might be at risk of exposure to anthropogenic pollutants, reducing their overall fitness and causing a decline in the grey seal population.

1.5 Aim of the study

The aim of the study is to investigate the PFAS burden in grey seal pups sampled from Froan, and the relationship between the contaminant and thyroid hormones in relation to the growth of the individuals. This is to determine whether the contaminants affect the development of the pups via thyroid hormone disruption, and if this can be one of the reasons for a decline in the population size at Froan. In addition, the aim of the study is to determine if PFAS is transferred from mother to pup during and after gestation.

2 Methods

2.1 Sampling

Sampling was done at the nature reserve area at Froan, which is an island group outside the coast of Trondheim at 63°59′34″N 9°03′13″E. The study animal, the grey seal, has one main breeding population in Mid-Norway. This population breeds and gives birth at Froan. Sampling of blood, hair and blubber biopsy from the pups was done between 29. September and 18. October 2022, which is in the breeding period of the population. The pups are born on small islands and skerry, often areas laying bare during low tide, and covered during high tide. The sampling area is exposed to wind and ocean waves, and because the sampling method involves going ashore from a boat (19 feet), the days available for sampling are restricted by weather conditions.

The grey seal pups (n=12) were captured in a net, and pups that were on land were sampled. It was done during low tide. A boat was driven to the skerry where a pup was present, and then the pup was approached and caught in a net. Blood samples were drawn with heparinized vacutainers, preferably from the hind flipper. However, if this was not possible, the blood sample was taken from the lumbar vein. Hair samples were taken with scissors, one sample was stored in aluminum foil to analyze for organic pollutants, and another was stored in a plastic bag for elemental analysis (These samples were not included in the present study). A blubber biopsy was taken from the back of the pup. To do this, the area was sanitized with ethanol (EtOH) before and after the biopsy was taken with a biopsy punch (5.0 mm). The tissue was stored in aluminum foil (These samples were not included in the present study). Sex was determined, as well as age, using developmental stage classification shown in Table 2.1, as described by Ekker et. al. (Ekker et al., 1995). The size of the seal pup was determined by three factors, length, girth, and weight. Length was measured from head to tail end along the curve of the animal. The girth was measured along the broadest part of the animal. Weight was determined using a Salter hanging scale (Dalton Engineering, Melmerby, UK) while the seal was in the net.

The samples were stored in a thermobox (5 ℃) during fieldwork (2-8 h). Once when returning from the field to the field station at Sørburøy, blood samples were centrifuged (3000 rpm for 10 min) and plasma was transferred to sterile cryopure tubes (1.8 mL, Sartsedt AG & Co, Nümbrecht, Germany), for further analysis. White blood cells were transferred to a vial for genetic analysis (analysis not included in the present study). All biological samples were then frozen at -20 ℃ and stored in a freezer (-20 ℃) until further analysis.

Table 2.1: Definition of developmental stages used as a determination of age (Ekker et al., 1995).

Developmental stage	Morphological description	Approximate age (days) mean±SD
0	Newborn, traces of birth.	0
1	Thin, defined neck and loose skin.	2±1
1+	Body as stage 1, umbilicus dried but present.	5±1
2	Body similar to stage 1 but smoother and with no umbilicus.	8±4
2+	Even smother body, still neck, but shoulder to hip filled out.	10±4
3	No visible neck, barrel-shaped body.	12±4
3+	Body as stage 3, some loss of lanugo in the facial region.	16±5
4	Body as stage 3, less than 50% lanugo shed, exposed juvenile pelage.	17±5
4+	50-95% lanugo moulted	22±7
5	Moulted, less than 5% lanugo remains.	25±7

2.2 Thyroid hormones

2.2.1 Analysis

Determination of plasma tT4, fT4, tT3 and fT3 concentrations in the 12 grey seal samples were conducted using enzyme-linked immunosorbent assay (ELISA) kits. The kits used were AccuBind T4, Free T4, T3 and Free T3 (Monobind Inc., Salt Lake, USA). The absorbance was measured using Cytation 5 Cell Imaging Multimode Reader (BioTek, Santa Clara, USA).

The methods used were given by Monobind Inc. specifically for the kits and are summarized shortly below.

2.2.1.1 T4

Samples (25 µL) and working reagent A (T4-enzyme conjugate: Total T3/T4 conjugate buffer, 1:11) (100 µL) were added to each well of the microwell strips in the 96-well plate. Then the plate was shaken (100 rpm, 20 sec), covered and incubated (room temperature, 60 min). After incubation, the solution was decanted, and wells were washed with wash buffer (wash concentrate diluted to 1000 mL with distilled water) (350 µL) and decanted. Washing was done three times. Working substrate (mix of solution A and Solution B) (100 µL) was added, and the plate was incubated (room temperature, 15 min) before stop solution (50 µL) was added and the plate shaken (20 sec). Absorbance was read at 450nm and 625nm after 5 min and 20 min.

2.2.1.2 Free T4

Samples (50 µL) and fT4 enzyme reagent (100 µL) were added to each well of the microwell strips in the 96-well plate. Then the plate was shaken (100 rpm, 20 sec), covered and incubated (room temperature, 60 min). After incubation, the solution was decanted, and wells were washed with wash buffer (wash concentrate diluted to 1000 mL with distilled water) (350 µL) and decanted. Washing was done three times. Working substrate (mix of solution A and Solution B) (100 µL) was added, and the plate was incubated (room temperature, 15 min) before stop solution (50 µL) was added and the plate shaken (20 sec). Absorbance was read at 450 nm and 625 nm after 5 min and 20 min.

2.2.1.3 T3

Samples (50 µL) and working reagent A (T3-enzyme conjugate: Total T3/T4 conjugate buffer, 1:11) (100 µL) were added to each well of the microwell strips in the 96-well plate. Then the plate was shaken (100 rpm, 20 sec), covered and incubated (room temperature, 60 min). After incubation, the solution was decanted, and wells were washed with wash buffer (wash concentrate diluted to 1000mL with distilled water) (350 µL) and decanted. Washing was done three times. Working substrate (mix of solution A and Solution B) (100 µL) was added, and the plate was incubated (room temperature, 15 min) before stop solution (50 µL) was added and the plate shaken (20 sec). Absorbance was read at 450 nm and 625 nm after 5 min and 20 min.

2.2.1.4 Free T3

Samples (50 µL) and fT3 enzyme reagent (100 µL) were added to each well of the microwell strips in the 96-well plate. Then the plate was shaken (100 rpm, 20 sec), covered and incubated (room temperature, 60 min). After incubation, the solution was decanted, and wells were washed with wash buffer (wash concentrate diluted to 1000 mL with distilled water) (350 µL) and decanted. Washing was done three times. Working substrate (mix of solution A and Solution B) (100 µL) was added, and the plate was incubated (room temperature, 15 min) before stop solution (50 µL) was added and the plate shaken (20 sec). Absorbance was read at 450 nm and 625 nm after 5 min and 20 min.

2.2.2 Quality control

For external control, Lyphochek Immunoassay Plus Contol 2 (Bio-Rad, Irvine, USA) (tT4: 10.2 (6.84-13.6) µg/dl, tT3: 2.47 (1.65-3.28) ng/mL, fT4: 2.35 (1.58-3.13) ng/dL and fT3: 5.48 (3.67-7.29) pg/mL) was used.

Each kit was tested before sample concentration determination by using 4 microwell strips. Here, the 6 standards, external control, and three samples from grey seal pups were added in triplicate. In addition, a duplicate of one of the three seal samples was added as an internal control. This was to test the kits and to determine whether the sample concentrations were within the range of the standard curve or if they needed dilution. This test showed that the concentration of all THs in the tested grey seal plasma samples were within the range of the standard curve.

After testing, sample concentrations were determined with the remaining 8 microwell strips from each of the four kits. To conduct this testing the 12 samples were analyzed in triplicate. In addition, external control and the 6 standards were added. These were also analyzed in triplicate. The samples that had previously been added during the testing of the kits were added additionally in duplicate as internal control between the two tests, for T4 this was sample #8 and for T3 this was sample #1 (and for fT3 one of the three was sample #4). The number given to identify the samples corresponds to the number of each sample in the sample ID, which can be observed in table C1 in Appendix C. Two samples were added in duplicate again as internal control, for T4 this was samples #11 and #3 and for T3 this was samples #10 and #3. After centrifugation in the field, samples were divided into different cryovials. Mostly, all the samples added to the plates were taken for the same cryovial, but because of low volume in some of the cryovials, on two instances the sample that was added both during the test run, and during the determination run, had to be taken from two vials. For TT4 and FT4, one cryovial was used to add samples in the test run and as control during the determination run, and the sample added in the determination run were from another cryovial. The plate setup for the 96-well plates is given in Appendix A, where tT4 is in Table A1, fT4 in Table A2, tT3 in Table A3 and fT3 in Table A4.

Because samples were mostly added in triplicate, outliers were only removed where possible mistakes were recorded during the procedure. This was the case for one well where the added sample volume was too low, and the results reflected this. For samples that were added both in the test run and the determination of all samples, the result was compiled. This was to increase in replicates and statistical power, and to reduce the effect of possible differences in method between the test run and determination run.

The coefficient of variation (CV) from the analysis of the different thyroid hormone concentrations was for tT4 4.67±3.48% with a range of 0.56%-11.06%. For fT4 it was 6.46±4.25% with a range

of 1.11%-13.23%. The CV for the tT3 analysis were 8.46±5.68% (0.57%-19.34%) and 6.85±3.55% (3.07%-15.15%) for fT3.

2.2.3 Calculations

Data was obtained using microplate imaging using Cytation 5 Cell Imaging Multimode Reader (BioTek, Santa Clara, USA) and data analysis software Gen5 (BioTek, Santa Clara, USA). The concentration of the samples was determined based on the absorbance and the standards that were included in the kit. Subsequentrly, the results were calculated by Gen5 before the data was transferred to Microsoft Excel (version 2301) (Microsoft Corporation, Redmond, USA). In Microsoft Excel, further data processing was completed. Average concentration and standard deviation were calculated for each sample, and total average concentration and standard deviation of hormones in all animals. The collective median and range for all animals were also presented. In addition, the relationship between T4 and T3 was determined. This was done by dividing the concentration of fT4 with the concentration of fT3 in each sample, and the same was done for tT4 and tT3. The absorbance were read at 5 min and 20 min, and the values read at 20 min was used for further calculations.

2.3 PFAS

2.3.1 Analysis

Plasma samples were analyzed for 46 PFASs listed in Table 2.2, by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) at the Norwegian University of Science and Technology (NTNU).

Table 2.2: Per- and polyfluoroalkyl substances (PFAS) analyzed in plasma samples from grey seal pups (n=12) at Froan, autumn 2022.

PFAS compounds acronym	PFAS compound
DecaS	Sodium 1-decasulfonate
GenX	2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy) propanoate
PFPeA	Perfluoropentanoic acid
PFHxA(UnFHxA)	Perflourohexanoic acid
4:2 FTS	1H,2H-Perfluorohexan sulfonate (4:2)
7H-PFHpA	7H-dodecafluoroheptanoic acid
ADONA	dodecafluoro-3H-4,8-dioxanonanoate
TriDeFHxSA/ PFHSK	Tridecafluorohexane-1-sulfonic acid potassium salt
PFOA	Perfluorooctanoic acid
6:2 FTS	1H,2H-Perfluorooctane sulfonate (6:2)

PFHpS	Perfluoro-1-heptanesulfonate
PFNA(PFNonDeA)	Perfluorononanoic acid
P37DMOA	Perfluoro-3,7-dimethyl octanoic acid
PFOSA	Perfluorooctane sulfonamide
PFOS	Perfluorooctano sulfonic acid
MeFOSA	N-methylPerfluoro-1-octanesulfonamide
PFDA	Perfluorodecanoic acid
EtFOSA	Sulfuramide
EtFOSAA	N-ethylPerfluoro-1-octanesulfonamide acetic acid
8:2FTS	1H,2H-Perfluorodecan sulfonate (8:2)
9Cl-PF3ONS	9-chlorohexadecafluoro-3-oxanonane-1-sulfonate
FOSAA	Perfluoro-1-octanesulfonamidoacetic acid
PFUnA	Perfluoroundecanoic acid
MeFOSAA	2-(N-methylPerfluoro-1-octansulfonamido) acetic acid
EtFOSE	N-ethyl-N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide
PFDoDA(TricoFDoDeA)	Perfluorododecanoic acid
MeFOSE	N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide
10:2FTS	1H,2H-Perfluorododecan sulfonate (10:2)
PFTriDA	Perfluorotridecanoic acid
diSAMPAP	bis[2-(N-ethyl perfluoro octane-1-sulfonamido)ethyl] phosphate
PFTDA(PFTetDeA)	Perfluorotetradecanoic acid
PFHxDA	Perfluoro-n-hexadecanoic acid
PFBA	Perfluorobutanoic acid
PFBS(NonaFBS)	Perfluorobutanic acid sulfonate
PFPeS	Perfluoropentane sulfonic acid
PFHpA	Perfluoroheptanoic acid
PFHxS	Perfluorohexane sulfonic acid
PFECHS	Perfluoroethylcyclohexane sulfonic acid
PFNS	Perfluorononane sulfonic acid
PFDS	Perfluorodecane sulfonic acid
SAMPAP	2-(N-ethylperfluorooctane-1-sulfonamido) ethyl phosphate
PFDoDS	Perfluorododecane sulfonic acid
PFOcDA	Perfluorooctadecanoic acid
6:6PFPi	Bis(tridecafluorohexyl)phosphinic acid
6:8PFPi	Perfluorohexylperfluorooctyl phosphinate
8:8PFPi	Bis(perfluorooctyl) phosphinate

All PFAS standards, both internal standards and target analytes were purchased from Chiron AS (Trondheim, Norway). Hybrid solid phase extraction (Hybrid SPE) cartridges were Hybrid® SPE-Phospholipid (30mg, 1mL) cartridges purchased from Sigma-Aldrich (Darmstadt, Germany)).

Ammonium formate (NH_4COOH) was purchased from Sigma-Aldrich (Darmstadt, Germany) and Methanol (MeOH) (HPLC gradient grade) was purchased from VWR (Radnor, USA).

The preparation of the samples before analysis and the analysis was performed according to Trimmel et al. (2021) (Trimmel et al., 2021). In brief, 150 uL of plasma was spiked with 10 uL 1 ppm internal standard (IS) (PFOS-$^{13}C8$, PFOA-$^{13}C8$ and 6:2FTS-$^{13}C2$). Proteins were precipitated with 450 μL MeOH with 0.1% NH_4COOH, and samples were vortexed (30 sec) and centrifuged (4000 g, 10 min). The supernatant was transferred to the pre-conditioned (1 mL MeOH with 0.1% NH_4COOH) Hybrid SPE cartridges. The eluent was collected in LC vials and stored at -20°C before analysis.

Separation with UHPLC was conducted on an Acquity UPLC system (Waters, Milford, USA), and detection with MS/MS analysis was performed on a Xevo TQ-S, Triple Quadrupole Mass analyzer (Waters, Milford, USA).

2.3.2 Quality control

Quality control was conducted by pooling 7 samples. Several pooled samples were analyzed, spiked with IS (10 μL) pre-extraction, spiked with the IS and target analytes (TA) at three different concentrations (5 ppb, 10 ppb and 20 ppb) pre-extraction, or spiked with IS and TA at three different concentrations (5 ppb, 10 ppb, and 20 ppb) post-extraction. All quality control samples were analyzed in triplicates. 4 method blanks were added, spiked with internal standard, but no plasma was added, only MeOH with 0.1% NH_4COOH. In addition, a calibration curve was created using the TA, consisting of 14 concentrations (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 ppb).

2.3.3 Calculations

All data from the analyses were acquired using MassLynx (Waters, Milford, USA). Microsoft Excel (version 2301) (Microsoft Corporation, Redmond, USA) was used for the rest of the data processing. Calculations of relative response, relative retention time, relative- and absolute recovery, matrix effects and concentration of the samples were conducted. The limits of quantification (LOQ) were determined for all compounds using the calibration curve. LOQ was determined to be either where the concentration of the calibration curve started to deviate from linearity, or where the noise in the calibration samples was too high to confidently determine concentration. The limit of detection (LOD) was determined to be LOQ divided by 3.3.

Calculations of the average concentration and standard deviation of contaminants in all seals, as well as median and range were conducted. As many contaminants had several samples below LOD, it was determined that only contaminants with concentrations higher than LOD in more than 50% of the samples should be analyzed further. By this determination there were 16 samples included for further analysis, and 30 that were not, as shown in Table 2.3.

Table 2.3: Summary of which 16 samples were included in further calculations in this study because more than 50% of the samples had concentrations was over LOD, and which 30 was not included because more than 50% of the samples had concentrations below LOD.

Contaminants included (>50% >LOD)		Contaminants excluded (<50% >LOD)		
PFOA	PFDoDA	Gen X	PFOSA	6:2 FTS
PFHpS	PFTriDA	PFPeA	MeFOSA	8:2 FTS
PFNA	PFTDA	PFHxA (UnFHxA)	EtFOSAA	PFBA
PFOS	PFHxS	4:2 FTS	PFHxDA	PFPeS
PFDA	PFECHS	7H-PFHpA	9Cl-PF3ONS	PFHpA
FOSAA	SAMPAP	EtFOSA	MeFOSAA	PFOcDA
PFNS		NaDONA	EtFOSE	6:6PFPi
PFDS		TriDeFHxSA	MeFOSE	PFDoDS
DecaS		PFBS(NonaFBS)	10:2 FTS	6:8PFPi
PFUnA		P37DMOA	diSAMPAP	8:8PFPi

Some of the contaminants still had samples with concentrations below LOD, and for further calculations with these values, concentration was assumed to be distributed normally and set to LOD/2 for all samples when the statistical analysis was conducted.

2.4 Statistics

2.4.1 Principal component analysis

A principal component analysis (PCA) was conducted using SIMCA17 (version 17.0.0.24543) (Sartorius Stedim Data Analytics AB, Göttingen, Germany). This was to investigate for relationships between contaminant concentrations, biometric variables, thyroid hormone concentrations and the relationship between T4 and T3. Loadings were used to determine the relationships between the variables. The score plot from the same analysis was also used to determine if there were any outliers in the sampled animals, and if there was a relationship between the contaminants and sex of pups.

2.4.2 Correlation analysis

To further determine whether the sex and biometry of the pups are important factors for the observed relationships, ANCOVA analysis was conducted in R. The differences in length, weight and girth between males and females were determined, with age as a covariate, as the goal was to determine if the males and the females developed at different rates and if this could affect the results. The significance level was set to $p<0.05$.

To examine the possible relationships between the variables, correlation analyses were conducted. This investigated the relationship between the contaminant and the thyroid hormones, and the relationship between the contaminants and the biological parameters (Age, Length, Weight and Girth). Firstly, it was determined whether the data were normally distributed, to determine which correlation analysis was appropriate to use. All statistical analysis was performed in R and RStudio (version 2023.03.0+386) (Posit Software PBC, Boston, USA). Determination of normality was done using the Shapiro-Wilks method, and p-value≤0,05 was set as the statistically significant limit for normality.

Pearson correlation analysis was applied for all normally distributed data, and Spearman rank correlation analysis was applied for all data that showed a non-normal distribution. Statistical significance was set to p-value≤0,05. Because the loading plot in the PCA analysis suggested a strong negative connection between thyroid hormones and biometric parameters, the analysis of all significant results between TH and PFAS was repeated, but with the biological variables as covariates to check if the correlation was mainly driven by these parameters. Here, Pearson correlation analysis with covariates was used for normally distributed data, and Spearman rank correlation with covariates was used for non-normally distributed data. Weight was considered the most important covariate, as age was stepwise determined, and the length and girth data were more biased than weight due to high mobility of some individuals during the measurements. All code used in R for calculations is described in Appendix B.

3 Results

3.1 Biometric variables

Age, weight, length and girth from sampling 12 grey seal pups are reported in Table 3.1. There were 4 females and 8 males, and no difference between the sexes were found. ANCOVA conducted for biometric variables and sex, with age as a covariate showed no significant relationships between the sexes. Because of this, the samples from both sexes were pooled in the further analyses. Some seals were sampled close to birth day, and some many days later, resulting in an age stage range from 1-4. The median and average were close for all parameters, indicating a normal distribution. Individual measurements are reported in Appendix C in Table C1.

Table 3.1: Average, standard deviation (SD), median and range for the age, weight, length and girth measured in the 12 grey seal pups sampled at Froan autumn 2022.

	Average ± SD	Median	Range
Age	2.59 ± 0.10	3	1-4
Weight [kg]	37.01 ± 14.57	38.6	15-64.2
Length [cm]	113.75 ± 14.63	117.5	83-132
Girth [cm]	90.29 ± 14.15	99	70-110

3.2 Thyroid hormones

The concentration of tT4, fT4, tT3 and fT3 were determined from serum samples from 12 grey seal pup sampled at Froan Fall 2022. In addition, the ratio between fT4 and fT3 (fT4:fT3), as well as tT4 and tT3 (tT4:tT3) were determined. The measured absorbance in the 96-well plates is reported in Appendix D, where tT4 is in Table D1, fT4 in Table D2, tT3 in Table D3 and fT3 in Table D4. The collective results for all individuals are reported in Table 3.2, which includes the average, SD, median and range. Individual results are reported in Table E1 in Appendix E. There was a broad range, resulting in a high SD for all parameters. The concentration ranges are at different orders of magnitude, with total tT4 being more than 10 times as high as tT3. The concentration of fT4 is one order of magnitude higher than the concentration of fT3. This results in a positive relationship between fT4:fT3, and tT4:tT3.

Table 3.2: Average, SD, median and range of concentrations of tT4, fT4, tT3 and fT3 in serum samples from seal pups at Froan, autumn 2022, and the ratio between fT4 and fT3, and tT4 and tT3.

Sample type	Average ± SD [ng/mL]	Median [ng/mL]	Range [ng/mL]
tT4	57.62 ± 22.49	44.66	37.95-102.04
fT4	0.0234 ± 0.0076	0.0201	0.0130-0.384
tT3	1.528 ±0.669	1.319	0.857-3.135
fT3	0.004052 ± 0.000627	0.003922	0.003280-0.005467
fT4:fT3	5.69 ± 1.28	5.80	3.67-8.69
tT4:tT3	38.93 ± 7.74	36.81	30.30-53.78

3.3 Contaminants

Concentrations of 45 different PFASs and PFAS precursors were determined in serum samples of the 12 grey seal pups sampled at Froan autumn 2022. The absolute and relative recovery of the PFAS standards in this analysis is given in Table F1 in Appendix F. Only contaminants where more than 50% of samples had a concentration level higher than the LOD were reported and used for further calculations. Which contaminants that were included and excluded are shown in Table 2.3 in materials and methods. The measured concentrations for all contaminants and all individuals are reported in Table G1 in Appendix G.

The concentration of the remaining contaminants is reported in Table 3.3, with the average, SD, median and range of the compounds. The range was broad for all the compounds, in some cases, like for PFOA and DecaS there were samples with concentrations below LOD. This results in a high SD for most of the compounds. The compounds with the highest concentrations were PFOS and the PFOS precursors FOSAA and SAMPAP, with concentration averages of 47.41 ng/mL, 261.41 ng/mL and 298.04 ng/mL respectively. All the other contaminants had averages below 10 ng/mL. The lowest average concentrations were for DecaS, with an average of 0.08 ± 0.13 ng/mL. Other compounds with low averages were 4 perfluorinated carboxylates (PFCA) (PFHpS, PFECHS, PFNS and PFDS) and one perfluoroalkyl sulfonate (PFCA) (PFOA) with averages below 1 ng/mL.

Table 3.3: Average, SD, median and range of concentration of PFAS in plasma samples taken from 12 grey seal pups at Froan autumn 2022. All contaminants had more than 50% of sample concentrations higher than LOD.

Contaminant	Average ± SD [ng/mL]	Median [ng/mL]	Range [ng/mL]	Detection
DecaS	0.08 ± 0.13	0.02	<0.02-0.42	7/12
PFOA	0.56 ± 0.28	0.57	<0.02-1.18	11/12
PFHpS	0.84 ± 0.48	0.64	0.32-1.85	12/12
PFNA(PFNonDeA)	3.53 ± 1.22	3.80	0.50-6.26	12/12
PFOS	47.99 ± 19.04	41.64	26.76-89.50	12/12
PFDA	2.68 ± 1.09	2.54	1.13-5.34	12/12
FOSAA	261.41 ± 140.59	258.83	23.35-537.17	12/12
PFUnA	8.11 ± 3.74	6.84	5.28-17.97	12/12
PFDoDA			1.32-4.29	12/12
(TricoFDoDeA)	2.28 ± 0.97	2.18		
PFTriDA	4.89 ± 1.98	4.28	2.79-9.36	12/12
PFTDA(PFTetDeA)	1.18 ± 0.54	0.98	0.59-2.37	12/12
PFHxS	4.28 ± 2.54	3.44	1.83-10.48	12/12
PFECHS	0.50 ± 0.44	0.36	0.25-1.88	12/12
PFNS	0.11 ± 0.09	0.09	<0.02-0.32	9/12
PFDS	0.30 ± 0.17	0.33	<0.10-0.66	11/12
SAMPAP	298.04 ± 275.59	324.50	<20.20-651.97	7/12

3.4 Principal component analysis

A PCA was performed with all variables reported previously in Table 3.1-3.3. The loadings are reported in Figure 3.1, showing that PC1 and PC2 explained 38.8% and 25.9%, respectively, of the variation in the data. There was also a third significant component explaining 9.7% of the variation. For the component explaining most of the variation, PC1, the thyroid hormones are the main driving force in the negative direction, and the physiological parameters are the main force in the positive direction. The analysis indicates that there is a negative correlation between some PFAS and thyroid hormones, but probably, the biological variables are a confounding effect. There is also a possible correlation between the individual PFAS, but this seems to have no connection to the biological variables or the thyroid hormones. The score plot of the same analysis is shown in Figure H1 in Appendix H.

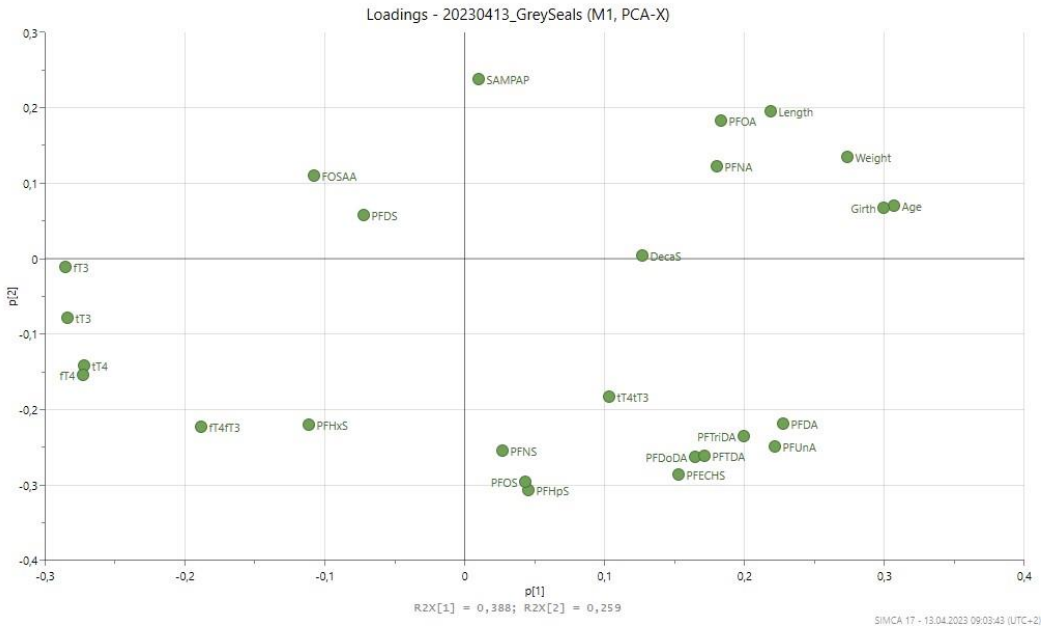

Figure 3.1: Principal component analysis loading plot including physiological parameters, thyroid hormone concentrations and contaminant concentrations. PC1 and PC2 explained 38.8% and 25.9% of the variation, respectively.

3.5 Correlation

Using the Shapiro-Wilks method, 8 contaminants were determined to be normally distributed ($p>0.05$: PFOA, PFHpS, PFNA, PFOS, PFDA, FOSAA, PFNS and PFDS), and 8 were determined to be non-normally distributed ($p<0.05$: DecaS, PFUnA, PFDoDA, PFTriDA, PFTDA, PFHxS, PFECHS and SAMPAP). Of the biological variables and thyroid hormones, all were normally distributed except for girth and tT4. Correlations were done using the Pearson correlation coefficient for all normally distributed data, and the Spearman rank correlation when the data was non-normally distributed. Correlation values are shown in Figure 3.4, with the background color indicating the correlation coefficient, the higher number over 0 the darker red, and the lower number below 0 the stronger blue. Data correlated using Spearman rank correlations is marked with green and data correlated with Pearson correlations has no color. Significant values are marked in the table by square borders. Figure 3.5 shows the p-values from the same analysis, without correlation color, but the significant numbers are marked with square borders.

Table 3.4: Correlation coefficients from the correlation analysis of the contaminants with the different physiological parameters, concentrations of thyroid hormones and the relationship between T4 and T3, and the correlation between biological parameters and thyroid hormones. Values are marked with a color map of red for high values and blue for low values. Significant values are indicated with square borders. Analysis done with the Pearson correlation is not marked, but an analysis done with the Spearman rank correlation has a green background.

	Age	Weight	Lenght	Girth	DecaS	PFOA	PFHpS	PFNA	PFOS	PFDA	FOSAA	PFUnA	PFDoDA	PFTriDA	PFTDA	PFHxS	PFECHS	PFNS	PFDS	SAMPAP
Age	-	0.90	0.76	0.95	0.18	0.61	-0.03	0.59	-0.02	0.60	-0.36	0.41	0.32	0.33	0.26	-0.31	-0.15	0.01	-0.21	0.05
Weight	0.90	-	0.73	0.95	0.33	0.55	-0.12	0.70	-0.15	0.47	-0.30	0.12	0.09	0.11	-0.03	-0.49	-0.26	-0.13	-0.32	0.13
Length	0.76	0.73	-	0.59	0.21	0.64	-0.25	0.64	-0.19	0.16	0.19	-0.02	-0.19	-0.25	-0.07	-0.70	-0.33	-0.16	-0.24	0.39
Girth	0.95	0.95	0.59	-	0.29	0.61	-0.02	0.61	0.07	0.59	-0.37	0.31	0.28	0.26	0.20	-0.32	-0.21	-0.12	-0.31	0.01
tT4	-0.76	-0.78	-0.56	-0.73	-0.02	-0.61	0.16	-0.47	0.01	-0.49	0.36	-0.16	-0.07	-0.01	0.12	0.42	0.31	0.32	-0.03	-0.46
fT4	-0.93	-0.90	-0.79	-0.85	-0.09	-0.63	0.13	-0.75	0.06	-0.41	0.14	-0.07	0.06	-0.01	0.06	0.48	0.50	0.21	0.13	-0.12
tT3	-0.89	-0.86	-0.75	-0.90	-0.20	-0.56	0.01	-0.45	0.04	-0.55	0.24	-0.41	-0.41	-0.40	-0.24	0.34	0.11	0.13	0.03	-0.24
fT3	-0.87	-0.81	-0.68	-0.87	-0.09	-0.47	-0.17	-0.43	-0.16	-0.64	0.26	-0.55	-0.54	-0.43	-0.40	0.15	-0.02	0.04	0.01	-0.17
fT4:fT3	-0.72	-0.74	-0.64	-0.66	0.01	-0.58	0.35	-0.80	0.24	-0.12	0.03	0.02	0.19	0.10	0.18	0.57	0.60	0.30	0.18	-0.24
tT4:tT3	0.17	0.22	-0.04	0.40	-0.08	-0.01	0.30	-0.14	0.17	0.42	-0.34	0.20	0.55	0.54	0.39	-0.15	0.04	0.14	-0.19	0.06

Table 3.5: P-values from the correlation analysis of the contaminants with the different physiological parameters, concentrations of thyroid hormones and the relationship between T4 and T3, and the correlation between biological parameters and thyroid hormones. Significant values are marked by square borders. Analysis done with the Pearson correlation is not marked, but an analysis done with the Spearman rank correlation has a green background.

	Age	Weight	Length	Girth	DecaS	PFOA	PFHpS	PFNA	PFOS	PFDA	FOSAA	PFUnA	PFDoDA	PFTriDA	PFTDA	PFHxS	PFECHS	PFNS	PFDS	SAMPAP
Age	-	0.00	0.00	0.00	0.57	0.04	0.92	0.04	0.96	0.04	0.25	0.18	0.31	0.30	0.41	0.32	0.63	0.98	0.51	0.62
Weight	0.00	-	0.01	0.00	0.29	0.06	0.71	0.01	0.64	0.12	0.35	0.71	0.78	0.73	0.93	0.11	0.42	0.69	0.31	0.51
Length	0.00	0.01	-	0.04	0.51	0.02	0.43	0.03	0.56	0.61	0.55	0.94	0.56	0.44	0.84	0.01	0.30	0.62	0.46	0.23
Girth	0.00	0.00	0.04	-	0.36	0.03	0.94	0.03	0.84	0.04	0.24	0.33	0.39	0.42	0.54	0.31	0.51	0.71	0.33	0.96
tT4	0.00	0.00	0.06	0.01	0.95	0.04	0.62	0.12	0.97	0.11	0.25	0.62	0.83	0.98	0.71	0.17	0.32	0.32	0.91	0.13
fT4	0.0	0.0	0.0	0.00	0.77	0.03	0.69	<0.01	0.84	0.18	0.66	0.83	0.85	0.98	0.85	0.11	0.10	0.52	0.70	0.61
tT3	0.0	0.0	0.0	0.00	0.53	0.06	0.97	0.15	0.91	0.06	0.45	0.18	0.18	0.20	0.44	0.28	0.73	0.68	0.94	0.28
fT3	0.0	0.0	0.0	0.00	0.77	0.13	0.60	0.16	0.62	0.03	0.41	0.07	0.07	0.16	0.20	0.63	0.95	0.90	0.98	0.49
fT4:fT3	0.0	0.0	0.0	0.02	0.96	0.05	0.27	<0.01	0.45	0.71	0.92	0.95	0.56	0.76	0.57	0.05	0.04	0.34	0.57	0.82
tT4:tT3	0.6	0.5	0.9	0.20	0.81	0.98	0.34	0.67	0.60	0.17	0.27	0.53	0.06	0.07	0.21	0.65	0.90	0.66	0.56	0.80

The only significant correlations between contaminants and the thyroid hormone or biological variables were for PFOA, PFNA, PFDA, PFHxS and PFECHS. However, as indicated in the PCA and Table 3.4, it appears to be confounding effects between the biological variables, the PFAS concentrations and the thyroid hormones. Thus, covariates were introduced to the correlation analyses to correct for the possible relationships between THs and biological variables. The correlation between TH and PFAS was analyzed with age, weight, length and girth as covariates. This was to determine if the significance observed between the THs and PFAS were caused by correlation between THs and the biological variables. The p-value of the correlation analysis after introduction of covariates is shown in Table 3. The values are determined with weight as covariate, and the significant value is marked with yellow. Following the correction for covariates, the analysis showed that only PFECHS correlated significantly with fT4:fT3. PFNA correlated significantly with fT4:fT3 following correction for all biological variables, except weight. Normally distributed values were analyzed with the Pearson correlation, while non-normally distributed values were analyzed with the Spearman rank correlation.

Table 3.6: P-values for correlation between TH and PFAS when weight was introduced as a covariate. Significant values are marked with a yellow background.

	PFOA	PFNA	PFDA	PFECHS
tT4	0.56	0.93	-	-
fT4	0.26	0.24	-	-
fT3	-	-	0.12	-
fT4:fT3	0.37	0.06	-	0.04

The raw data for the correlation between PFECHS concentration and fT4:fT3 is plotted in Figure 3.2. Since the relationship between the variables was found with Spearman rank correlation there is no line to represent the correlation, but the correlation- and p-value from the correlation with weight as a covariate is presented in Figure 3.2 as well.

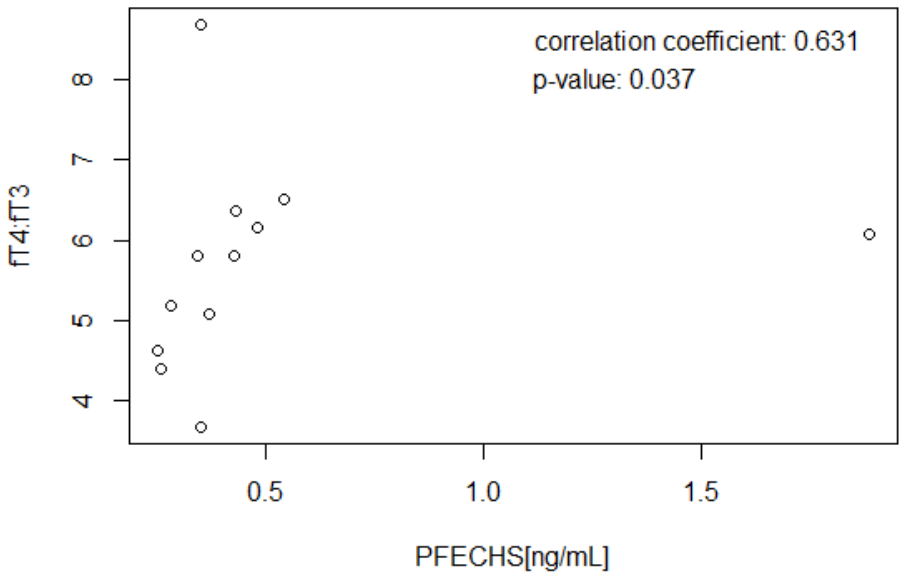

Figure 3.2: Plot of PFECHS concentration and fT4:fT3. The correlation coefficient and p-value from the Spearman rank correlation done between the two variables is presented in the figure.

There is an outlier in the data with a higher PFECHS concentration than the other samples. When this outlier was removed from the data the correlation coefficient changes and is no longer significant. The correlation was done with Pearson correlation as the data was normally distributed after removing the outlier. This is shown in Figure 3.3, which is a plot of the raw data, with the correlation coefficient (0.186) and p-value (0.96).

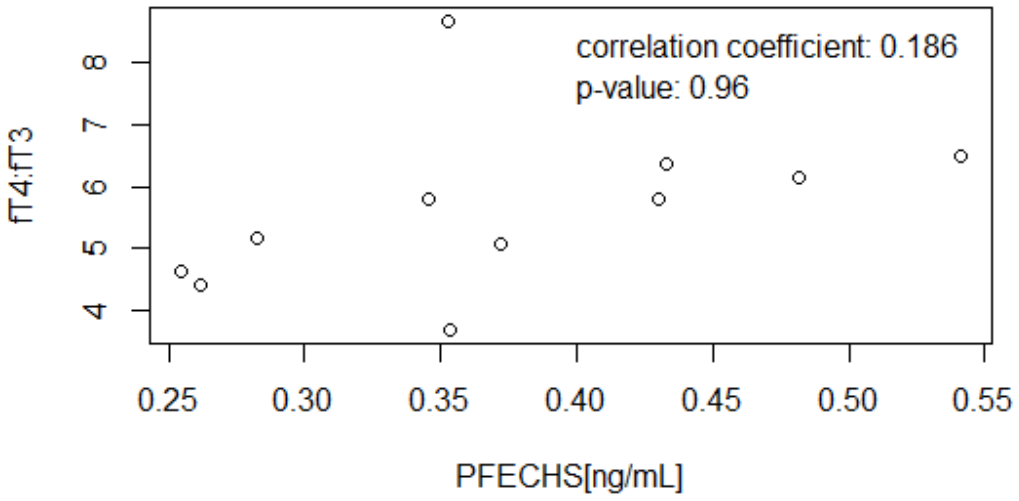

Figure 3.3: Plot of PFECHS concentration and fT4:fT3 with an outlier removed. The correlation coefficient and p-value from the Pearson correlation done between the two variables is presented in the figure.

4 Discussion

4.1 Biometric variables

The pups were at different ages when they were sampled, from approximately 2 days of age (age stage 1) to circa 17 days of age (age stage 4) (Jenssen et al., 2010). The pups usually weigh 13-16kg at birth, and they will add about 2kg of weight each day, resulting in a weaning weight of 40-50kg (Haug, 2021). Therefore, it is not surprising that there were differences in the biometric variables sampled. The smallest seals were also the youngest, with fresh umbilical cords and traces of birth. The largest seals also showed signs of being the oldest, with partly shed lanugo. The largest weighed 64.4kg, which is high, but this is not surprising; it might have been that this specimen was already weaned. The smallest sampled specimen weighed 15kg. This is in accordance with other studies on the species, where the birth mass of pups was determined to be 14,42kg and 13,67kg on average for two different years (Bennett et al., 2021), or where pups sampled at an average age of 3,4 and 3,1 days of age weighed 19,5kg and 19,7kg on average (Jenssen et al., 2010).

The age of the animals was determined in stages. This is because it is difficult to determine the exact age without knowing the time of birth. It is a standard method, that is used in several studies to determine the age of grey seal pups (Ekker et al., 1995; Woldstad and Jenssen, 1999). Still, there might be some uncertainties regarding the age determination, as the stages span several days, and some of the characteristics used to determine the age stage are not absolutes.

4.2 Thyroid hormones

Thyroid hormones are important for development, and because of their regulatory function, they will be at different concentrations during early life. Thyroid hormone levels are often high directly after birth, but then the concentration rapidly decreases after a short period (Segni, 2015). This pattern is observable in samples from this study, where the older individuals have a lower concentration of thyroid hormones than the younger individuals. This is in accordance with previous reports on T4 in grey seals from Svalbard (Woldstad and Jenssen, 1999).

The thyroid hormone concentrations from the current study are within the ranges of previously reported concentrations, as shown in Table 4.1. The concentration of fT3 is higher than reported in the other studies, but in the same order of magnitude. All data shown in Table 4.1 are from grey seal pups, except from Grønnestad et.al, 2018 which was done on hooded seal pups. As shown in

Table 4.1 the values from the present study and from Hall et. al., 1998 and Bennet et al., 2021 are similar for T4 values, which are in accordance with the result in the current study and is not surprising as the sampling is done on pups of the same species at a similar age. Even so, the concentration of tT3 and fT3 is slightly higher in the present study than those reported in Hall et al., 1998, and Bennet et al., 2021. Since those previous studies did not include individuals as old as the present study, this result is opposite of what could be expected if concentrations decrease with age. This can possibly be explained by the study done by Woldstad and Jenssen, 1999, which found that while T4 decreases with age, tT3 increases, findings that supports the possibility of lower T3 concentrations in the present younger animals sampled in Hall et al., 1998 and Bennet et al., 2021 (Woldstad and Jenssen, 1999).

The decrease of T4 during early development is what might explain the difference in concentrations between the current study and Woldstad and Jenssen, 1999. In Woldstad and Jenssen, 1999, the sampled animals are divided into two age groups, 0-2 days and 3-23 days old. The mean TH concentrations from pups aged 3-23 days, are slightly lower than in the current study, and the one measured TH concentration from the 0-2 days old group, tT4, is higher. If the two age groups in Woldstad and Jenssen, 1999, were combined, as they are in the current study, the TH concentrations in the present study and Woldstad and Jenssen, 1999, might be more similar. The changes in thyroid hormone concentrations in blood are age dependent, resulting in an importance of knowing the age of the sampled animal.

The last study included in Table 4.1, Grønnestad et al., 2018, is done on hooded seal, in the same age group as the current study. The concentrations of thyroid hormones are higher and this might be because of the difference between the species. As the grey seal pups weans for about 18 days (Reilly, 1991), hooded seal weans for approximately 4 days (Bowen et al., 1985). Thus, they have to gain weight at a much higher rate than the grey seal pups. This might explain the higher concentration of thyroid hormones in Grønnestad et al., 2018 and shows that even if the species are similar and of the same age there might be a pronounced difference in the thyroid hormone concentrations.

Table 4.1: Concentration of thyroid hormones in seal pup samples from the current study and other studies done on species of phocid seals of similar age.

Source	tT4 [ng/mL]	fT4[ng/mL]	tT3[ng/mL]	fT3[ng/mL]	Method	Note
Present study	57.62	0.0234	1.528	0.004052	ELISA	
(Hall et al., 1998)	48.32	0.0256	0.4687	0.0009	ELISA	
(Bennett et al., 2021)	56.8 & 47.2[1]		0.88 & 0.84[1]		ELISA	
(Woldstad and Jenssen, 1999)	99.5 & 37.7[2]	0.0180	0.996		RIA	
(Grønnestad et al., 2018)	61.37	0.0202	1.4285	0.0012	RIA	Hooded seal

[1]samples taken different years, 2016 and 2017 respectively.

[2]samples taken in the age group 0-2 days and 3-23 days old respectively, fT4, tT3 and fT3 concentrations from the study are from the 3-23 days old age group.

The present study aims to determine the correlation between thyroid hormone concentration, PFAS and biometric measures. To achieve this, it is important that the relationship between hormone concentrations is correct, and not necessarily the concentrations themselves. Nevertheless, to enhance the weight of knowledge, the determined thyroid hormone concentrations must be correct, so that they can be compared with other studies. In the ELISA method used, the variation between sampled animals was great. The average thyroid hormone concentrations have, as shown in Table 3.2, high SD. The reason for this may, be the large difference between sampled animals or that the kit is developed for humans, this might affect the preciseness of the results. Nevertheless, the CV% was between 0.56% and 19.34%, which is acceptable for ELISA.

4.3 Contaminants

Grey seals have an efficient maternal-foeatal transfer of proteinophilic PFAS *in utero* because they give birth to well-developed pups. Therefore, in this study the concentration of PFAS in the pup can be related to the concentration of contaminant in the mother, as well as the absorption rate and elimination rate in the pup. PFAS is known to not eliminate easily and accumulates in the body (Bangma et al., 2022; Houde et al., 2011). PFAS are often accumulated in the liver, and many studies use the liver to measure concentrations in the animal (Androulakakis et al., 2022; De Wit et al., 2020; Trimmel et al., 2021). Because of accumulation, this will give a detailed picture of the PFAS burden in the organism, but the animal must be sacrificed to use the liver for analysing levels of PFAS. An alternative is to sample animals after planned hunts, or sample animals that have otherwise died from other causes, such as bycatch in fishing nets or natural deaths (Ahrens et al.,

2009; Routti et al., 2016). In the latter case, it is not possible to be certain of the freshness of the samples, and there might be some time after the animal has died until the sample can be taken. The advantage of using plasma samples is that they can be obtained while the animal is alive, and they will not cause a reduction in fitness after sampling. The result of these different methods is that it might be difficult to compare results from several other studies.

PFASs have different properties that make them behave differently in an organism. They are known for having an affinity to proteins, but they may also bind to water or lipids, depending on their structure (Bangma et al., 2022; Grønnestad et al., 2017). In plasma samples the concentrations of proteins may be different depending on the organisms, their health, age, sex or if they are hydrated or not (Bangma et al., 2022). Because of this, the concentration of PFAS may vary, and the relationship between PFAS concentration and other factors in an organism might be harder to examine. Determining the concentration in the plasma will give valuable information about how much PFAS is in the blood at that exact point in time, and how much that can affect organs that are not normally target organs for PFAS. If you are determining i.e., PCB in the plasma, the concentration can be very different than the concentration of PCB in the blubber, but the PCB in the plasma is what is biologically available in all organs except the fat. To standardize the variation, the concentration of proteins in plasma can be determined, and how much PFAS is present dependent on proteins can be calculated (Grønnestad et al., 2017). This might give a better estimation for the PFAS body burden, and it can be a better method to compare across animals. The problem with this approach is that it can give a false sense of security in the results. As PFAS have different affinity to proteins, it might still be differences in concentrations that are not detected by total protein contents (Smeltz et al., 2023).

The concentrations of PFAS measured in the present study varied between individuals. The contaminants with the highest concentrations were SAMPAP and FOSAA. Their concentrations were several times as high as the concentration of PFOS, which is known to be a main contributor to the PFAS load (Table 4.2). It is probable that the reason for this is that the method of detection is not optimal for SAMPAP and FOSAA, and this has been previously reported (Lona, 2022). The real concentration of these contaminants is probably lower than what was reported in the present study. Because of this, the concentration of SAMPAP and FOSAA are removed from Table 4.2 which shows the total PFAS load along with the concentration of PFOS in different studies.

Table 4.2: The concentration of total PFAS and PFOS in samples of different phocid seals, and this study. The concentration of SAMPAP and FOSAA in the present study has been excluded from the table. Assuming a density of 1,03g/mL plasma.

Source	∑PFAS [ng/g]	PFOS [ng/g]	Notes
Present study	79.65	49.43	
(Ahrens et al., 2009)	380	349	Harbor seal adult, Germany
(Routti et al., 2014)	61.92	38	Harbor seal adult, Svalbard
(Grønnestad et al., 2017)	65.44	30.4	Hooded seal pups, Greenland
(Routti et al., 2016)	75.31	48	Ringed seal adult, Svalbard

The concentration both of total PFAS and of PFOS is similar in the present study and Routti et al., 2014, Routti et al., 2016 and Grønnestad et.al.,2017, although the concentration is somewhat higher in the present study as shown in Table 4.2. All sampled animals are phocid seals, however there are still some differences between the species and individuals that could explain the observed concentration patterns. Age might be a factor, as the longer an animal lives, the more time they have, to accumulate pollutants. Only the current study and the one by Grønnestad et al., 2017 are performed on pups, and the concentration of pollutants in these studies is higher than some of the others. Because the main source of pollutants for the pups is the placental transfer and milk from the mother, this shows that there is a transfer of pollutants from mother to pups. This might be harmful for the pups as they are more prone to the negative health effects by pollutants as they are still growing and developing.

Another reason for the higher concentration of PFAS and PFOS in this study compared to Routti et al., 2014, Routti et al., 2016 and Grønnestad et.al., 2017 might be the location of sampling. While the present study is done outside Trondheim in Norway, and close to two known sources for PFAS contamination, oil platform and a military airplane base, the three other studies have sampled from what is considered more remote areas, which is Svalbard and Greenland. The distance between the location of sampling and the source of contamination may explain why there is a higher concentration of PFAS in the present study. The only study shown in Table 4.2 which have a higher concentration of contaminants than the current study is the study on harbour seals by Ahrens et al., 2009. The concentration of PFOS is the main contributor to this difference, and while the relationship between total PFAS and PFOS in the other shown in Table 4.2 is relatively similar, the concentration of PFOS in the Ahrens et al., 2009 study is much higher. The sampling in this study was done on four animals and many tissues, but all tissues showed a high

concentration of PFOS. In this case it is also likely that the sampling was done close to a source of contamination. While sampling in the current study was done on the Norwegian coast, sampling from Ahrens et al., 2009 was done in the German Blight, a much more polluted area. In addition, in Ahrens et al., 2009 the sampling was done by euthanizing sick animals that had stranded on the beaches, which might create a biased sample group of individuals exposed to pollutants, including PFAS, which is previously reported to reduce function of the immune system.

4.4 Principal component analysis

There is a strong connection between the thyroid hormones and the biometric variables. This is not surprising as all the biometric variables are associated with age, and as the animals grow older there is a decrease in thyroid hormone concentration (Segni, 2015; Woldstad and Jenssen, 1999). This connection makes it difficult to determine if there is any connection between the thyroid hormones and the contaminants, as the main driver of thyroid differences is age. The PC1 explained 38,8% of the variation observed in the variables. This is probably biologically driven, by the growth and developement of the individual. It has been known for a long time that the thyroid hormones decrease in concentration after the birth of the individual (Segni, 2015; Woldstad and Jenssen, 1999), and the same mechanisms do not apply to the differences found in concentrations of the contaminants. The decrease of thyroid hormones in the body during growth and development is an active mechanism. In comparison, the increase or decrease of PFAS in the organism during growth is a passive mechanism, driven by the difference in transfer of the contaminants and how they are absorbed or excreted from the body.

4.5 Correlations

Some studies have previously investigated the differences between males and females, mainly regarding the thyroid hormones. This is because there could be a difference in the production and release of hormones and uptake of contaminants depending on the sex. This was not the case in this study because the PCA scores showed no differences between the sexes, in addition to the ANCOVA analysis done to compare the sexes, which showed no significant results. It should, however, be noted that the sample size in the present study was low.

During the correlation analysis, several significant correlations between PFAS and either thyroid hormones or biometric variables were found. The significant correlations between thyroid hormones and PFAS were mainly negative (PFOA, PFNA and PFDA), except for the correlation

between fT4:fT3 and PFECHS which had a correlation coefficient of 0.60 ($p<0.04$). For the biometric variables, all significant correlations were positive (PFOA, PFNA and PFDA) except for the correlation between PFHxS and length, which had a correlation coefficient of -0.70 ($p<0.01$). The other compounds that had significant correlations between PFAS and thyroid hormones or biometric variables (PFOA, PFNA and PFDA) are all PFCA. PFOA was negatively correlated with tT4, fT4 and fT4:fT3, and positively correlated with age, length and girth. PFNA was negatively correlated with fT4 and fT4:fT3, and positively correlated with all biometric variables. PFDA had only three significant correlations, that is positive with age and girth, and negative with fT3.

The negative correlation between the sulfonate PFHxS and length can be caused by biodilution, where a possible mechanism is the transfer of contaminant through the placenta *in utero*. When the pups are developing in the uterus, there might be a transfer of contaminants over the placenta into the animal, causing accumulation of the contaminant in the animal (Grønnestad, 2015). When the pup is born, this transfer will stop, and the concentration of contaminants in the body of the animal will not change. As the animal feeds and grows, increasing both length, weight and girth, the contaminant will dilute, causing a decreased concentration, although the amount of contaminant in the body stays the same. That is an opposite pattern than what has been observed in previous studies on mother pup pairs in hooded seals and in harbour seals, where a more efficient transfer of sulfonates has been observed, by comparing concentrations in mothers plasma and milk to the concentration in pup plasma (Grønnestad et al., 2017; Shaw et al., 2009). Nevertheless, the findings of lower transfer of the sulfonate PFHxS are supported by Bischel et al., 2011, which shows that sulfonates have stronger binding affinity than carboxylates to proteins (Bischel et al., 2011). The result of this is that PFHxS will not be transferred from the body of the mother and into the lipid-rich milk. In addition, the difference between Grønnestad et al., 2017 and the current study may have been because the findings in this experiment were determined by age, and not by concentrations in the mother.

For the contaminants that increase in concentration during growth and development, PFOA, PFNA and PFDA, the transfer may work differently. Since the concentration is increasing the contaminants in the body are still accumulating after birth, and this means there must be a source of contaminants that can be absorbed by the body. The pup feeds on milk, so it is probable that

some of the contaminants that the mother has accumulated during her lifespan can be more lipid soluble than others, and therefore can be transferred from the mother to the pup via the milk (Grønnestad et al., 2017). As the animal uses milk to grow and develop, the concentration of contaminants in the body might increase because of accumulation and transfer of contaminant by milk.

It is not surprising that the compounds that were positively correlated with biometric variables are negatively correlated with thyroid hormones, as the correlation between biometric variables and thyroid hormones are so strongly negative. This is observable from the PCA analysis, and the correlation analysis in this study.

Therefore, it is important to determine whether the correlation observed between PFAS, and biometric variables or thyroid hormones is driven by their correlation, or if the main component is the correlation between biometric variables and thyroid hormones. To investigate this, correlations between TH and PFAS were determined again but with weight was as a covariate. The reason for this is that the age was determined by stages, thus this parameter has an intrinsic bias due to differences in time gaps, so an animal could be at one stage for a longer time than other stages. It is previously determined that seal length is better correlated with age than weight (Wilman et al., 2023). As the seal grows it might add weight dependent on several factors, but the length may be less affected by these changes. The problem with the length is that it is more difficult to measure precisely than the weight. The movement of the animal could alter the measured length, also because it was measured along the back of the animal, so it was different depending on how the animal was moving at the point of measurement. The weight would be the same every time the measurement was done.

The only correlation that was still significant after the usage of covariates was the positive correlation between PFECHS and fT4:fT3. This means that the concentration of fT4 increases relative to fT3 as the plasma PFECHS concentration increases. This may indicate an inhibition of deiodinasaes that convert T4 to T3. Previously, correlation between PFAS and the ratio fT4:fT3 has been reported in hooded seal pups, both for PFCA and PFSA (Grønnestad et al., 2018). In addition, it has been reported a positive correlation between the tT4:tT3 ratio and PFTriDA, also in hooded seals (Grønnestad, 2015). Although in none of these analyses, age was included, the results are similar to the findings that PFECHS has a positive correlation with the fT4:fT3 ratio.

As can be seen in Figure 3.2 there is an outlier with a high concentration and where the fT4:fT3 ratio is not as high. The other samples have a low concentration of PFECHS, and detection of specific concentrations in the lower ranges is less precise than at higher concentrations. It is then important to be aware that this may be affecting the results, but even so the correlation is significant.

The outlier was removed to see how it would affect the result, and the result was changes in both the correlation coefficient and the p-value, resulting in a non-significant correlation. Thus, it is probable that the outlier caused the observed correlation. Nevertheless, the changes could also be a result of the lower number of observations. As the concentration of the outlier is different than the other samples, it is not a difference that is biologically improbable, and there were no events during the lab or field work that would imply a different result. Because of this it is relevant to still include the value in the analysis, but important to take note of the lack of significance when the outlier is removed.

Nevertheless, the research on how PFAS affects thyroid hormones and thyroid homeostasis in mammals had had varying results. PFOS exposure to pregnant rats has caused reduced foetal weight, and exposure during gestation and after birth has caused and decreased level of tT4 in rat pups (Thibodeaux et al., 2003; Yu et al., 2009). In human in vitro studies PFOS and PFOA reduce the activity of one of the most important glycoproteins in thyroid synthesis, thyroperoxidase (Song et al., 2012), but in human cohort studies, there is found no significant relationship between PFAS and thyroid stimulating hormone, even though the concentrations of PFOA in plasma where much higher than recommended levels, and higher than in individuals living in PFOA contaminated water districts in Sweden and the United States of America (Liu et al., 2023). Moreover, in a study on walruses, there was found no significant relationship between thyroid hormones and PFAS (Routti et al., 2019).

In 2020 EFSA reported a benchmark dose limit 10% (BMDL10) of 17.5 ng/mL for the sum of PFAS (PFOA, PFNA, PFHXS and PFOS) in serum of 1-year-old children related to immune-response (Schrenk et al., 2020). To be certain that the level of PFAS present in 1-year-old children is not affecting the immune-response, the concentration in serum should be below 17.5 ng/mL. In the present study, the average sum of these four PFAS in the plasma of the grey seals was 56.41 ng/mL. Assuming that PFAS concentrations in serum and plasma are relatively similar (Uges,

1988), this indicates that the present level of these four PFAS may affect the immune system of grey seal pups in Froan.

One of the problems with many of these experiments is that there is no way of knowing whether the correlations is a result of causations, especially in the *in vivo* studies, where there can be a lot of confounding factors. In this study, only the concentrations of PFAS were examined, but there may have been other pollutants present that disrupted thyroid hormones. Areas with high pollution can have many types of pollutants present together, so the correlations observed may be caused by contaminates other than PFAS. In addition, the sample size was rather small. Because of this, it can be hard to determine whether PFAS influences the growth and development of grey seal pups at Froan, and if the contaminant reduces their fitness. The contaminant likely has some effect on the thyroid hormones, but it might be dependent on the body burden, and it is not certain that it will affect survival.

5 Conclusion

In the present study, concentrations of 46 PFAS and thyroid hormone concentrations of tT4, fT4, tT3 and fT3 were determined in 12 grey seal pup plasma samples collected at Froan Autumn 2022, alongside a collection of biometric information. Of the 46 samples of PFAS, 16 had concentrations higher than LOD, and of those, PFOS, SAMPAP and FOSAA had the highest concentrations, although the high concentrations of SAMPAP and FOSAA were considered to be overestimated due to the method being unsuitable to quantify these compounds. The concentrations of thyroid hormones were well within the expected ranges considered previously measured thyroid concentrations.

Relationships between the contaminants and thyroid hormones, as well as the relationship between contaminants and biometric variables, were determined. It was discovered that there were several significant relationships between contaminants and both biometric variables and thyroid hormones. Biological variables had significant positive correlation with PFOA, PFNA and PFDA, and a significant negative correlation with PFHxS. The positive correlation was identified as accumulation and transfer of PFAS into the pups because of mothers milk. The negative correlation was identified as transfer of PFAS *in utero* but not as much via mothers milk. Because of a strong relationship between biometric variables and TH the correlation between TH and PFAS was done again, but with weight as a covariate. After this, only the positive correlation between PFECHS and fT4:fT3 was still significant. The correlation between PFECHS and fT4:fT3 have not been supported by previous research specifically, although some reports show a positive relationship between other PFAS and the fT4:fT3 ratio. Although other factors may play a part in the observed results, they support the possibility of PFAS being a hormone disruptor. If high concentrations of PFAS can affect the thyroid hormone levels in young seal pups, this may reduce their fitness. In addition, the concentrations of PFAS (PFOA, PFNA, PFHxS and PFOS) detected in the grey seal pup samples are higher than the BMDL10 limit related to immune response in 1-year-old children, and may affect immune response. Even so, more research on the matter should be conducted to increase the weight of evidence, or possibly discover the underlying mechanisms behind the possible effect.

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Appendix A

The thyroid hormone plate setup is given in the following 4 tables, Table A1, A2, A3 and A4.

Table A1: Plate setup for the test run and the sample run for the tT4 samples. Abbreviations used are standard reference material (SRM), standard (St) and quality control (QC).

tT4 test					tT4 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	St A	St A	St A	01	St A	St A	St A	01	03	06	09	11
B	St B	St B	St B	01	St B	St B	St B	01	04	06	09	12
C	St C	St C	St C	01	St C	St C	St C	01	04	07	09	12
D	St D	St D	St D	06	St D	St D	St D	02	04	07	10	12
E	St E	St E	St E	06	St E	St E	St E	02	05	07	10	11QC
F	St F	St F	St F	06	St F	St F	St F	02	05	08	10	11QC
G	SRM2	SRM2	SRM2	08QC	SRM2	SRM2	SRM2	03	05	08	11	03QC
H	08	08	08	08QC	08QC	08QC	08QC	03	06	08	11	03QC

Table A2: Plate setup for the test run and the sample run for the fT4 samples. Abbreviations used are standard reference material (SRM), standard (St) and quality control (QC).

fT4 test					fT4 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	St A	St A	St A	01	St A	St A	St A	01	03	06	09	11
B	St B	St B	St B	01	St B	St B	St B	01	04	06	09	12
C	St C	St C	St C	01	St C	St C	St C	01	04	07	09	12
D	St D	St D	St D	06	St D	St D	St D	02	04	07	10	12
E	St E	St E	St E	06	St E	St E	St E	02	05	07	10	11QC
F	St F	St F	St F	06	St F	St F	St F	02	05	08	10	11QC
G	SRM2	SRM2	SRM2	08QC	SRM2	SRM2	SRM2	03	05	08	11	03QC
H	08	08	08	08QC	08QC	08QC	08QC	03	06	08	11	03QC

Table A3: Plate setup for the test run and the sample run for the tT3 samples. Abbreviations used are standard reference material (SRM), standard (St) and quality control (QC).

tT3 test					tT3 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	St A	St A	St A	03	St A	St A	St A	01	03	06	09	11
B	St B	St B	St B	03	St B	St B	St B	01	04	06	09	12
C	St C	St C	St C	03	St C	St C	St C	01	04	07	09	12
D	St D	St D	St D	09	St D	St D	St D	02	04	07	10	12
E	St E	St E	St E	09	St E	St E	St E	02	05	07	10	10QC
F	St F	St F	St F	09	St F	St F	St F	02	05	08	10	10QC
G	SRM2	SRM2	SRM2	01QC	SRM2	SRM2	SRM2	03	05	08	11	03QC
H	01	01	01	01QC	01QC	01QC	01QC	03	06	08	11	03QC

Table A4: Plate setup for the test run and the sample run for the fT3 samples. Abbreviations used are standard reference material (SRM), standard (St) and quality control (QC).

fT3 test					fT3 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	St A	St A	St A	03	St A	St A	St A	01	03	06	09	11
B	St B	St B	St B	03	St B	St B	St B	01	04	06	09	12
C	St C	St C	St C	03	St C	St C	St C	01	04	07	09	12
D	St D	St D	St D	09	St D	St D	St D	02	04	07	10	12
E	St E	St E	St E	09	St E	St E	St E	02	05	07	10	10QC
F	St F	St F	St F	09	St F	St F	St F	02	05	08	10	10QC
G	SRM2	SRM2	SRM2	01QC	SRM2	SRM2	SRM2	03	05	08	11	03QC
H	01	01	01	01QC	01QC	01QC	04QC	03	06	08	11	03QC

Appendix B

The code used to determine if there was a relationship between the sexes was as described below.

```
ancova_model<- aov(x ~ y + z, data = GreySeals)
Anova(ancova_model, type="3")
```

In this model, "x" was the biometric variable where a difference was to be determined, "y" was the sex, and "z" was age used as a covariate. The "GreySeals" data was all information gathered about the grey seals.

The following code was used to determine the Person correlation values and the p-values for the correlation.

```
cor(GreySeals)
rcorr(as.matrix(GreySeals))
```

A similar code was used to determine Spearman rank correlation, but with defining that the correlation used was Spearman.

```
cor(GreySeals, method"spearman")
rcorr(as.matrix(GreySeals), type="spearman")
```

When the covariate was introduced, a different code was used. For Pearson correlation, this was as followed.

```
summary(lm(a~b+c, data=GreySeals))
```

Here, "a" was the hormone or the biometric variable, "b" was the pollutant and "c" was the chosen covariate. This code gave both the correlation value and the p-value.

For the Spearman rank correlation, the code used for getting the same values was as described below, where "a", "b" and "c" are the same as above.

```
pcor.test(GreySeals$b,GreySeals$a,GreySeals$c, method = c("spearman"))
```

Appendix C

The individual biometric measures for all animals are given in Table B1.

Table C1: Individual biometric measures for all seal pup samples.

Seal	Age [stage]	Sex	Weight [kg]	Length [cm]	Girth [cm]
F_22_01	3	M	42	106	99
F_22_02	3	M	44.5	128	99.5
F_22_03	2	M	27.5	116	83
F_22_04	1.5	M	22.2	100	71
F_22_05	4	F	51.2	119	110
F_22_06	3	M	49	120	100
F_22_07	1	F	15	83	70
F_22_08	3	M	35.2	132	99
F_22_09	1	M	19.7	98	71
F_22_10	2.5	F	31.2	120	81
F_22_11	3.5	M	64.2	130	100
F_22_12	3.5	F	43.2	113	100

Appendix D

The measured absorbance for the thyroid hormone analysis is given in the four tables, Table D1, D2, D3 and D4. It is important to note that the test run, and the sample run were at different times, so the standard for the respective run must be used for calculations.

Table D1: Measured absorbance at 450nm in the tT4 thyroid hormone analysis. The run of the test and the samples was done at different times. The setup is described in Appendix A, Table A1.

tT4 test					tT4 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.946	2.85	2.699	1.744	2.873	2.63	2.954	1.485	0.925	1.457	0.606	1.54
B	2.213	1.919	2.005	1.598	2.163	2.171	2.305	1.557	0.915	1.431	0.646	1.471
C	1.24	1.204	1.141	1.527	1.24	1.233	1.197	1.52	0.923	0.707	0.653	1.509
D	0.627	0.618	0.603	1.56	0.621	0.626	0.659	1.413	0.931	0.67	1.057	1.504
E	0.497	0.425	0.466	1.43	0.465	0.461	0.453	1.455	1.363	0.706	1.109	1.555
F	0.29	0.242	0.273	1.513	0.295	0.288	0.292	1.432	1.375	1.492	1.11	1.439
G	0.579	0.52	0.541	1.499	0.537	0.646	0.624	1.051	1.359	1.397	1.528	1.083
H	1.404	1.427	1.284	1.615	1.435	1.461	1.425	1.076	1.479	1.487	1.652	1.071

Table D2: Measured absorbance at 450nm in the fT4 thyroid hormone analysis. The run of the test and the samples was done at different times. The setup is described in Appendix A, Table A2.

fT4 test					fT4 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.271	2.189	2.299	0.802	2.13	2.077	2.242	0.817	0.613	0.906	0.375	1.187
B	1.733	1.53	1.718	0.824	1.576	1.684	1.611	0.792	0.542	0.827	0.365	1.009
C	1.217	1.159	1.191	0.781	1.217	1.147	1.198	0.835	0.58	0.448	0.356	1.013
D	0.785	0.713	0.728	0.818	0.636	0.743	0.759	0.807	0.537	0.408	0.64	1.074
E	0.268	0.273	0.274	0.732	0.26	0.267	0.273	0.896	0.79	0.466	0.631	1.136
F	0.122	0.113	0.123	0.74	0.138	0.121	0.139	0.832	0.835	0.956	0.685	1.148
G	0.714	0.67	0.746	0.737	0.743	0.757	0.77	0.569	1.425	0.932	1.064	0.686
H	0.806	0.756	0.72	0.814	0.855	0.881	0.965	0.584	1.01	0.975	1.213	0.77

Table D3: Measured absorbance at 450nm in the tT3 thyroid hormone analysis. The run of the test and the samples was done at different times. The setup is described in Appendix A, Table A3.

tT3 test					tT3 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.978	2.031	2.083	0.942	1.974	2.126	2.121	1.238	0.909	1.152	0.883	1.272
B	1.392	1.56	1.532	0.955	1.565	1.596	1.556	1.223	0.89	1.21	0.974	1.24
C	1.044	1.191	1.223	1.065	1.25	1.303	1.232	1.21	0.889	0.723	1.009	1.265
D	0.675	0.786	0.746	0.935	0.822	0.795	0.836	1.189	0.883	0.718	1.001	1.239
E	0.427	0.427	0.443	0.942	0.493	0.469	0.507	1.165	1.345	0.763	1.05	1.067
F	0.322	0.314	0.514	0.948	0.381	0.333	0.372	1.196	1.398	1.178	1.05	1.09
G	0.694	0.654	0.631	1.295	0.696	0.74	0.759	1.025	1.307	1.173	1.285	1.073
H	1.172	1.269	1.268	1.243	1.346	1.368	1.357	1.018	1.308	1.102	1.288	1.082

Table D4: Measured absorbance at 450nm in the fT3 thyroid hormone analysis. The run of the test and the samples was done at different times. The setup is described in Appendix A, Table A4.

fT3 test					fT3 samples							
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.278	2.109	2.293	1.498	2.266	2.333	2.225	1.415	1.406	1.463	1.226	1.599
B	1.808	1.915	1.876	1.535	1.954	2.097	2.178	1.445	1.249	1.608	1.381	1.621
C	1.618	1.527	1.59	1.48	1.768	1.747	1.75	1.498	1.233	1.099	1.426	1.595
D	1.041	0.992	1.142	1.328	1.157	1.166	1.115	1.431	1.323	1.167	1.494	1.728
E	0.701	0.675	0.763	1.335	0.766	0.793	0.74	1.456	1.75	1.174	1.479	1.39
F	0.336	0.381	0.376	1.338	0.393	0.398	0.388	1.531	1.689	1.516	1.443	1.395
G	0.99	1.082	1.171	1.58	1.232	1.273	1.218	1.449	1.683	1.561	1.641	1.481
H	1.546	1.589	1.562	1.52	1.739	1.746	1.411	1.44	1.627	1.84	1.669	1.512

Appendix E

The calculated thyroid hormone concentrations and the fT4:fT3 and tT4:tT3 ratios are given in Table E1.

Table E1: The individual thyroid hormone concentrations calculated in each pup sample, and the fT4:fT3 and tT4:tT3 ratios from each individual.

SEAL	tT3[ng/mL]	fT3[pg/mL]	tT4[µg/dL]	fT4[ng/dL]	fT4:fT3	tT4:tT3
F_22_01	1.13	4.24	4.10	1.99	5.81	40.86
F_22_02	1.26	4.17	4.36	1.93	4.63	34.50
F_22_03	1.94	4.32	6.75	2.79	6.50	37.84
F_22_04	2.26	4.94	7.55	2.95	6.15	33.38
F_22_05	0.86	3.28	4.57	1.62	6.08	53.29
F_22_06	1.16	3.81	4.30	1.78	5.17	35.54
F_22_07	3.14	5.47	9.50	3.48	6.37	30.30
F_22_08	1.37	3.50	4.29	1.70	5.07	30.42
F_22_09	2.03	4.65	10.20	3.84	8.69	53.78
F_22_10	1.77	4.17	6.12	2.49	5.80	35.78
F_22_11	0.97	3.55	3.94	1.29	3.67	41.63
F_22_12	1.05	3.50	4.18	1.54	4.40	39.87

Appendix F

The absolute recovery and relative recovery for the PFAS standards are given in Table F1.

Table F1: The absolute and relative recovery for the PFAS standards at three different concentrations, 5ppb, 10ppb and 20ppb given as a percent.

	Absolute recovery			Relative recovery		
	5ppb	10ppb	20ppb	5ppb	10ppb	20ppb
DecaS	50.32 %	55.80 %	58.46 %	34.46 %	108.78 %	233.29 %
GenX	32.93 %	55.56 %	60.80 %	1.99 %	7.78 %	21.24 %
PFPeA	49.93 %	52.95 %	57.36 %	28.69 %	79.72 %	175.95 %
PFHxA (UnFHxA)	52.62 %	56.85 %	61.40 %	53.41 %	143.56 %	302.12 %
4:2 FTS	47.93 %	57.30 %	60.09 %	40.43 %	125.45 %	271.65 %
7H-PFHpA	45.26 %	57.94 %	62.67 %	63.86 %	210.19 %	446.29 %
NaDONA	48.21 %	54.77 %	62.86 %	327.89 %	935.49 %	2049.50 %
TriDeFHxSA	86.69 %	144.85 %	164.83 %	3.57 %	5.33 %	6.27 %
PFOA	48.44 %	55.76 %	62.68 %	47.23 %	138.97 %	304.18 %
PFOA-13C8	82.53 %	64.82 %	57.30 %	288.30 %	277.59 %	263.80 %
6:2 FTS	46.44 %	56.08 %	71.31 %	2.94 %	8.62 %	21.79 %
PFHpS	49.84 %	56.66 %	62.23 %	72.79 %	214.99 %	456.34 %
PFNA (PFNonDeA)	56.75 %	59.49 %	67.09 %	178.89 %	444.54 %	924.43 %
P37DMOA	48.74 %	54.93 %	61.85 %	254.03 %	747.80 %	1629.75 %
PFOSA	43.04 %	52.76 %	62.91 %	198.52 %	608.85 %	1412.87 %
PFOS	89.40 %	81.47 %	81.26 %	349.12 %	469.89 %	699.44 %
PFOS-13C8	66.55 %	56.11 %	49.04 %	100.00 %	100.00 %	100.00 %
MeFOSA	45.85 %	53.30 %	59.62 %	77.12 %	230.73 %	540.41 %
PFDA	55.60 %	62.85 %	66.20 %	26.92 %	73.06 %	145.15 %
EtFOSA (Sulfuramide)	47.96 %	51.21 %	59.36 %	122.32 %	348.34 %	824.13 %
EtFOSAA	47.94 %	52.47 %	57.54 %	104.82 %	299.08 %	688.30 %
8:2FTS	42.31 %	49.12 %	55.51 %	24.77 %	75.10 %	181.36 %
9Cl-PF3ONS	45.99 %	54.74 %	61.62 %	214.96 %	676.98 %	1517.07 %
FOSAA	30.20 %	15.20 %	9.75 %	102.79 %	102.80 %	124.23 %
PFUnA	60.21 %	61.40 %	64.72 %	256.71 %	559.07 %	1076.58 %
MeFOSAA	5.06 %	4.70 %	12.53 %	13.16 %	32.07 %	178.93 %
EtFOSE	44.17 %	49.26 %	59.17 %	4.07 %	12.39 %	30.15 %
PFDoDA (TricoFDoDeA)	54.45 %	57.00 %	59.78 %	248.37 %	638.10 %	1317.10 %
MeFOSE	44.49 %	48.90 %	60.75 %	2.67 %	8.42 %	22.66 %

10:2FTS	48.26 %	54.18 %	57.30 %	27.07 %	78.62 %	172.55 %
PFTriDA	59.05 %	59.86 %	61.42 %	223.41 %	524.19 %	1074.42 %
diSAMPAP	0.54 %	0.94 %	9.37 %	0.54 %	2.50 %	52.33 %
PFTDA (PFTetDeA)	48.54 %	55.48 %	57.84 %	104.33 %	301.55 %	644.40 %
PFHxDA	34.05 %	40.01 %	53.54 %	19.50 %	61.99 %	167.21 %
6:2FTS 13C2	72.82 %	61.79 %	54.67 %	100.00 %	100.00 %	100.00 %
PFBA	16.67 %	10.00 %	10.22 %	2.59 %	3.79 %	8.35 %
PFBS (NonaFBS)	46.39 %	53.63 %	62.36 %	69.80 %	208.70 %	501.17 %
PFPeS	46.63 %	56.64 %	64.47 %	71.84 %	209.25 %	482.03 %
PFHpA	46.37 %	53.32 %	64.44 %	23.91 %	67.10 %	148.73 %
PFHxS	52.16 %	59.59 %	63.59 %	77.63 %	203.72 %	423.36 %
PFECHS	46.93 %	54.25 %	61.48 %	354.86 %	1071.98 %	2424.24 %
PFNS	46.31 %	52.17 %	61.80 %	69.10 %	208.79 %	474.99 %
PFDS	47.64 %	53.51 %	60.99 %	70.16 %	209.30 %	481.02 %
SAMPAP	6.20 %	0.85 %	0.66 %	0.95 %	0.39 %	0.70 %
PFDoDS	46.55 %	54.83 %	58.92 %	33.39 %	100.33 %	225.19 %
PFOcDA	26.41 %	31.29 %	43.76 %	6.22 %	17.86 %	52.39 %
6:6PFPi	48.74 %	43.58 %	57.27 %	3.04 %	7.63 %	22.21 %
6:8PFPi	44.54 %	25.02 %	46.05 %	0.51 %	0.84 %	2.96 %
8:8PFPi	24.07 %	30.87 %	45.10 %	0.36 %	1.14 %	3.77 %

Appendix G

Individual PFAS concentrations in the samples from the 12 seal pups are given in Table G1.

Table G1: Individual PFAS concentrations in ng/mL in the different individuals sampled. <LOD in samples where the values were lower than the limit of detection.

SEAL	F_22_01	F_22_02	F_22_03	F_22_04	F_22_05	F_22_06	F_22_07	F_22_08	F_22_09	F_22_10	F_22_11	F_22_12
DecaS	0.02	0.42	0.03	0.05	0.25	0.06	<LOD	<LOD	<LOD	0.03	<LOD	<LOD
GenX	<LOD	<LOD	<LOD	<LOD	3.06	<LOD	2.47	<LOD	<LOD	<LOD	<LOD	<LOD
PFPeA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.89	<LOD	<LOD	<LOD
PFHxA (UnFHxA)	<LOD	<LOD	<LOD	0.41	0.82	<LOD	0.30	0.72	<LOD	<LOD	<LOD	0.67
4:2 FTS	<LOD	<LOD	<LOD	<LOD	1.14	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
7H-PFHpA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
NaDONA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.05	<LOD
TriDeFHxSA	<LOD	<LOD	<LOD	<LOD	31.71	<LOD	28.60	<LOD	<LOD	<LOD	22.41	<LOD
PFOA	0.62	1.18	0.55	<LOD	0.58	0.33	0.29	0.55	0.30	0.68	0.74	0.76
6:2 FTS	<LOD	<LOD	<LOD	<LOD	173.5	0.62	<LOD	<LOD	<LOD	<LOD	<LOD	0.66
PFHpS	1.17	0.45	1.85	0.98	1.59	0.65	0.88	0.41	0.63	0.32	0.47	0.62
PFNA (PFNonDeA)	2.64	4.47	3.89	3.77	3.83	3.10	2.45	3.87	1.50	2.45	6.26	4.06
P37DMOA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOSA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOS	56.37	26.76	89.50	63.75	73.16	34.41	45.22	38.06	32.49	34.40	36.14	45.66
MeFOSA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFDA	3.78	2.28	2.88	2.59	5.35	2.94	1.81	2.49	1.62	1.13	2.39	2.84
EtFOSA (Sulfuramide)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EtFOSAA	0.05	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.03	0.01	<LOD	0.04
8:2FTS	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
9Cl-PF3ONS	<LOD	<LOD	<LOD	<LOD	0.15	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
FOSAA	100.00	447.91	537.17	329.3	23.35	167.8	186.1	306.4	297.5	253.5	264.1	223.5

PFUnA	9.79	6.42	7.44	7.15	17.97	5.55	5.32	7.88	5.61	6.52	5.28	12.37
MeFOSAA	<LOD	<LOD	<LOD	<LOD	0.60	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
EtFOSE	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFDoDA (TricoFDoDeA)	2.57	1.36	2.55	2.14	4.29	1.42	1.32	1.79	2.21	2.41	1.36	3.90
MeFOSE	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
10:2FTS	<LOD	<LOD	<LOD	0.08	0.03	<LOD	<LOD	<LOD	0.02	0.02	<LOD	0.04
PFTriDA	6.32	4.35	4.25	4.32	9.36	2.79	3.20	4.13	4.64	4.09	3.32	7.94
diSAMPAP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFTDA (PFTetDeA)	0.99	0.96	1.73	0.87	2.37	0.59	0.86	1.06	1.01	0.97	0.76	1.99
PFHxDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBA	<LOD	<LOD	<LOD	<LOD	71.14	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFBS (NonaFBS)	<LOD	0.03	<LOD	<LOD	0.01	<LOD	0.09	<LOD	0.06	<LOD	<LOD	0.02
PFPeS	<LOD	0.19	<LOD	0.18	<LOD	0.07	0.07	0.10	<LOD	<LOD	<LOD	0.15
PFHpA	<LOD	<LOD	<LOD	<LOD	0.82	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFHxS	3.44	2.12	7.86	10.48	3.98	3.12	4.99	3.37	3.43	1.83	2.06	4.68
PFECHS	0.43	0.25	0.54	0.48	1.88	0.28	0.43	0.37	0.35	0.35	0.35	0.26
PFNS	0.07	<LOD	0.10	0.16	0.32	0.16	0.17	0.20	0.08	0.02	<LOD	<LOD
PFDS	0.66	0.31	0.41	0.15	<LOD	0.24	0.31	0.34	0.36	0.34	0.08	0.42
SAMPAP	651.97	291.66	<LOD	<LOD	<LOD	555.66	<LOD	628.59	426.62	614.09	357.35	<LOD
PFDoDS	<LOD	<LOD	0.21	0.03	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PFOcDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6:6PFPi	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6:8PFPi	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
8:8PFPi	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Appendix H

The score plot for the PCA analysis is given in Figure H1. Males and females are noted with a different colour in the score plot.

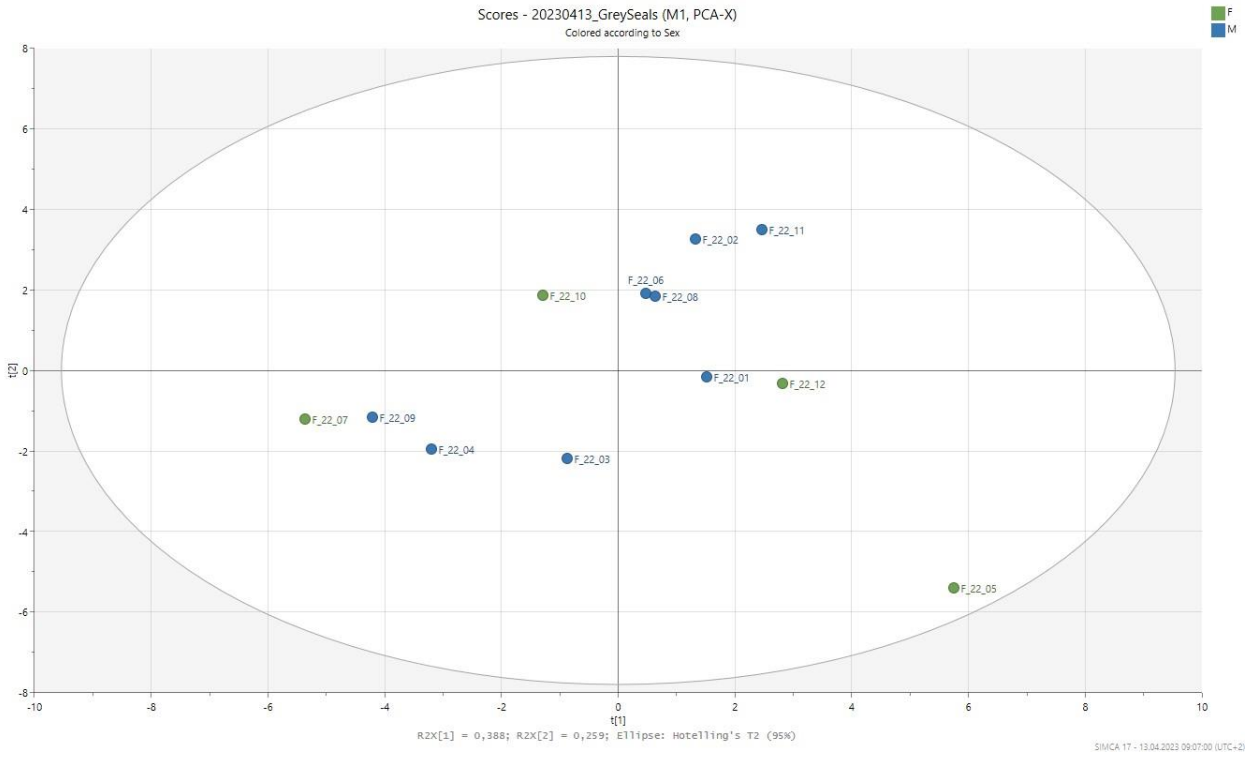
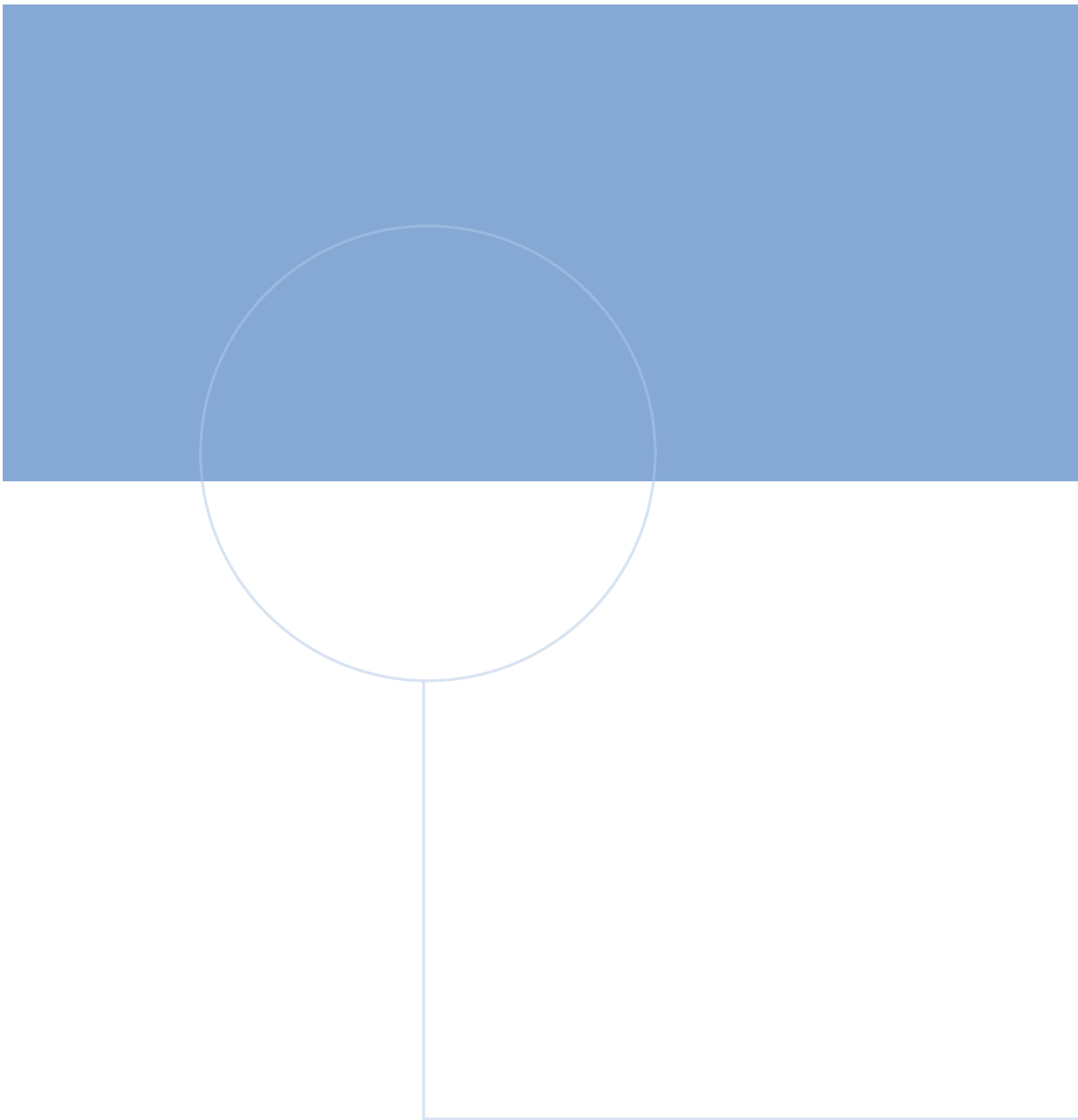


Figure H1: Score plot form PCA analysis for the different variables from the samples. Males are marked with a blue color, and females with green.



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