Occurrence of perfluorinated alkylated substances (PFASs) and evaluation of potential genotoxic effects in small rodents from a skiing area, Granåsen, Norway

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Abstract

Perfluorinated alkylated substances (PFASs) are man-made chemicals widely used in the last few decades due to their physico-chemical properties, which make them suitable for industrial and commercial purposes. PFASs are found in several consumer products, such as waterproof clothing and ski waxes. High concentrations of PFASs have been reported recently in biotic and abiotic samples in skiing areas. Few studies have investigated the genotoxicity of these chemicals and conflicting results have been found.

In the present study, wild small rodents inhabiting Granåsen were sampled for evaluating the PFASs levels in liver; as a reference area, the woods next to the lake Jonsvatnet were chosen. 57 small rodents were sampled in total (n = 25 in Granåsen and n = 32 in Jonsvatnet). The concentration of chemicals in liver was analysed by HPLC-MS. Results showed that the concentration of PFOS, PFTrDA, PFTeDA, PFDoDA, PFDA, PFHxS and PFBS was statistically higher in rodents inhabiting Granåsen compared to the reference area. Contrarily, the chemicals PFHpA and PFHxA were found in higher concentrations in the reference area, where PFNA and PFOS showed high concentrations as well. The concentration of chemicals was not related to age, weight or gender in individuals from Granåsen, while individuals from Jonsvatnet showed a significant positive correlation of PFOS concentration with age ($p = 0.007$) and weight ($p = 0.001$).

Additionaly, the incidence of chromosomal aberrations (CAs) in lymphocytes was analysed. Blood was sampled by cardiac puncture and lymphocytes were isolated by density barrier. The percentage of cells in metaphase with aberrations was higher in the individuals analysed from Granåsen (n = 3) compared to Jonsvatnet (n = 8) (81 and 31% respectively). Nevertheless, few individuals could be included in the scoring of CAs and the total number of well spread metaphases for each individual was too low (ranging from 3 to 21 scorable metaphases per individual) to draw any conclusions.

Further studies are needed to clarify whether the small rodents of Granåsen suffer from a higher incidence of chromosomal aberrations due to PFASs exposure.

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Abbreviations

ANCOVA	Analysis of covariance
AS	Anonymous society
BBB	Blood brain barrier
BrdU	Bromodeoxyuridine
C	Carbon
Co	Corporation
CA	Chromosomal Aberration
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EFSA	European Food Safety Authority
F	Fluorine
FBS	Foetal bovine serum
FOSA	Perfluorooctane sulfonamide
GLM	General linear model
G1	Gap 1 phase
G2	Gap 2 phase
H	Hydrogen
HPLC-MS	High-performance liquid chromatography-mass spectrometry
KCl	Potassium chloride
LOD	Limit of detection
Log	Logarithm
M phase	Mitotic phase
M.I.	Mitotic Index
MN	Micronucleus
Na-citrate	Sodium citrate
NaCl	Sodium chloride
n.d.	Not detected
N.EtFOSA	N-Ethyl perfluorooctane sulfonamide
N.EtFOSE	N-Ethyl perfluorooctane sulfonamido ethanol
NMBU	Norwegian University of Life Sciences

N.MeFOSE	N-Methyl perfluorooctane sulfonamido ethanol
NTNU	Norwegian University of Science and Technology
PASF	Perfluoroalkyl sulfonamides
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PFAS	Per- and polyfluoroalkylated substance
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonate
PFCA	Perfluorinated carboxylic acid
PFDA	Perfluorodecanoic acid
PFDoDA	Perfluorododecanoic acid
PFHpA	Perfluoroheptanoic acid
PFHxA	Perfluorohexanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFSA	Perfluoroalkyl sulfonate
PFTeDA	Perfluorotetradecanoic acid
PFTrDA	Perfluorotridecanoic acid
PFUdA	Perfluoroundecanoic acid
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator-activated receptor
PS	Penicillin streptomycin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 medium
S phase	Synthesis phase
ssDNA	Single-strand DNA
w.w.	Wet weigh

1 Introduction

1.1 PFASs

Per- and polyfluorinated alkylated substances (PFASs) constitute a large group of fluorinated chemicals that includes both oligomers and polymers (Hekster et al., 2002). These chemicals have been produced since the 1950s due to their numerous industrial and commercial applications (Kissa, 2001) and are used around the globe in industrialized and urbanized zones (Hekster et al., 2003). Several PFAS congeners are known to be toxic pollutants (Ahrens and Bundschuh, 2014) and two of them, perfluorooctanesulfonic acid (PFOS) and perfluorooctanesulfonyl fluoride (PFOSF), as well as their related precursor substances, were added to the Stockholm Convention list in 2009, becoming the first fluorinated persistent organic pollutants (POPs) (Stockholm Convention, 2009).

PFASs are highly fluorinated aliphatic substances that contain one or more carbon (C) atoms where all the hydrogen (H) substituents are replaced by fluorine (F) atoms, forming the perfluoroalkyl moiety C_nF_{2n+1} (Buck et al., 2011). PFASs have been formally divided into two groups according to their C-chain length: long PFASs are those perfluoroalkyl carboxylates (PFCAs) with seven or more fluorinated carbons and perfluoroalkyl sulfonates (PFSAs) with six or more fluorinated carbons (see Table 1). Long-C-chain PFASs have been confirmed to be persistent, ubiquitous and bioaccumulative. Short-C-chain PFASs are those PFCAs with six or less fluorinated carbons and PFSAs with five or less fluorinated carbons (Krafft and Riess, 2015). Table 1 presents some of the most renowned PFAS congeners, split into the chemical groups carboxylic and sulfonic acids.

As a consequence of the widespread use of PFASs and their emissions, these substances have been recently detected in wildlife (Falk et al., 2012), humans (Brantsæter et al., 2013), dust (Yao et al., 2016) and even in food (Papadopoulou et al., 2017) and drinking water (Hu et al., 2016). Due to the strong binding between the carbon and the fluorine atoms, these compounds are extremely persistent and virtually indestructible (Buck et al., 2011). In addition, PFASs have been detected in remote locations far away from direct sources, such as the Arctic, indicating that long-range transportation of these substances occurs (Giesy and Kanan, 2001).

Table 1 Some of the environmentally relevant PFASs. The table includes the acronym, name, chemical structure and classification according to chain length and chemical group of the congeners.

Acronym	Name	Chemical structure	Classification
PFBS	Perfluorobutane sulfonic acid		Short-chained sulfonic acid
PFHxS	Perfluorohexane sulfonic acid		Long-chained sulfonic acid
PFOS	Perfluorooctane sulfonic acid		Long-chained sulfonic acid
PFHpA	7H-Dodecadluoro-heptanoic acid		Short-chained carboxylic acid
PFOA	Perfluorooctanoic acid		Long-chained carboxylic acid
PFNA	Perfluorononanoic acid		Long-chained carboxylic acid
PFTeDA	Perfluorotetradecanoic acid		Long-chained carboxylic acid

1.1.1 PFASs: properties and applications

PFASs have unique physicochemical properties that are determined mainly by the length of the perfluoroalkyl chain and the functional group. PFASs are regarded as amphiphilic because they possess both, lipophobic and hydrophobic nature. These chemicals are also characterised by thermal and chemical stability, low surface energy and special surface-active properties. These compounds are incorporated to surfactants and polymers for increasing their resistance. Surfactant applications include processing aid for fluoropolymer manufacture, coating, and aqueous film-forming foams used to extinguish fires in presence of highly flammable liquids. Polymer applications of the substances include textile stain, soil repellents and grease-proof food-contact paper (Buck et al., 2011). Thus, one can find PFASs in a wide range of consumer products such outdoor textiles, carpets, cleaning and impregnating agents, baking and sandwich papers, paper baking forms and ski waxes (Kotthoff et al., 2015).

1.1.2 Ski products

While some consumer products as cleaning agents or baking papers might have low or negligible PFASs content, others contain high levels, such as ski waxes, which were found to have up to 2000 μg/kg of PFOA (Kotthoff et al., 2015). To ensure a better performance of the skiers, various products are used in the preparation of the skis, as glide waxes, which can be found as powders or solid blocks; or grip waxes (Freberg et al., 2014). Ski waxing ensures a better performance of the athletes because it gives water and dirt repellence to the skis, increasing the glide; or in case of the grip waxes, increasing the friction between the snow and the skis.

During the last decade, the production and use of ski waxing products has increased considerably, and the chemical composition of these products has been continuously evolving (Axell, 2010). Grip waxes are usually mixtures of paraffin waxes, synthetic rubber and oils. Glide waxes instead, contain petroleum-derived hydrocarbons (HC), where all the H atoms have been replaced by F atoms, i.e. PFASs (Axell, 2010; Freberg et al., 2014). Glide waxes can contain nearly all PFASs spectrum and accordingly, several PFASs have been found in snow and soil samples from skiing areas (Plasmann and Berger, 2003; Chropenová et al., 2016).

1.1.3 PFASs in the Nordic environment

Kallenborn et al. (2004) investigated the occurrence, distribution and fate of PFASs and related chemicals in six Nordic countries: Norway, Sweden, Finland, Denmark, Iceland and Faroe Islands. Measurable amounts of PFASs were found in all samples, indicating that this group of contaminants is widely distributed in the Nordic environment. The main sources were found to be sewage sludge and landfill effluents, where PFOS was the predominant PFAS congener found. However, even though the main source of PFASs in the environment is known to be primarily anthropogenic, sources in anadromous sites indicated that long-range transport in air and/or precipitation also occurs (Kallenborn et al., 2004).

The samples from Nordic biota showed species dependent distribution, the highest levels were found in the top predating Danish harbour seal (*Phoca vitulina).* PFASs levels in marine mammals from Nordic countries (grey-/ harbour seals, pilot whales and minke whales) were found in similar concentration ranges as reported for PCBs, HCB and γ-HCH in marine mammals from Norwegian coastal waters (Kallenborn et al., 2004).

Kallenborn et al. (2004) assessed that in Norway half of the PFASs used in the country come from firefighting foams, while protective coating and textiles constitute nearly the other half. The usage of ski waxes also poses an important source of PFASs in Norway as well as in the other Nordic countries, where winter sports are really popular.

NILU/NINA made a report on environmental contaminants in terrestrial and urban regions for various organic and inorganic pollutants (Herzke et al., 2015). High levels of PFASs were reported on earthworms inhabiting the skiing area Voksenkollen (Oslo), and follow-up studies were recommended in order to elucidate possible sources of PFASs in skiing areas.

1.1.4 Exposure and toxicokinetics of PFASs

In the lower trophic levels, PFASs enter the food web via plants (Blaine et al., 2014), drinking water (Dai et al., 2013) or soil (Rick et al., 2015). Toxicokinetic studies showed that PFASs have high dietary absorption efficiencies (Martin et al., 2003a; Martin et al., 2003b), most likely due to the strong affinity to plasma proteins that PFASs exhibit, together with enterohepatic circulation processes (Goecke-Flora and Reo, 1996). Maternal transfer to foetuses and breastfeeding constitute an additional exposure pathway of PFASs in mammals (Grønnestad et al., 2016). Moreover,

humans are further exposed to PFASs by contact with consumer products, as food packaging treated with water and grease-resistant coatings or non-stick wares (Herzke et al., 2012).

As mentioned, oral PFASs absorption is generally fast. Once in the blood, PFASs bind to albumin and their distribution occurs to the organs with higher protein content, such as the liver and kidney. Perfluoroalkyl acids (PFAA) are not metabolised in animal bodies (Kudo, 2015) but metabolism of precursor compounds to PFAAs occurs. That is, fluorotelomer-based polymers, phosphates and alcohols are degraded by microorganisms, animals and atmospheric oxidation to PFAAs, therefore, exposure to precursor chemicals results in accumulation of PFAAs (Kudo, 2015). The pathway of metabolism of precursor chemicals depends on the matrix in which the transformation takes place. This process has been well studied *in vivo,* where fluorotelomer alcohol (FTOH) has been administered in rats and PFOA, PFNA and PFHxA have been identified as metabolites (Buck et al., 2011). The primary route of excretion of PFASs is in urine via kidneys, this process though, displays large species and gender differences; excretion through faeces also occurs but to a lesser extent. PFASs excreted in bile undergo faecal elimination or enterohepatic circulation (Galatius et al., 2013). The biological half-life ($T_{1/2}$) of PFASs depend on the species, the sex and the length of the carbon chain of the compound; for example, PFHxA (C6) in female rat exhibits $T_{1/2}$ = 0.5h (Chengelis et al., 2009), PFOA (C8) in male monkey has $T_{1/2}$ = 21 days (Butenhoff et al., 2004), while in humans the $T_{1/2}$ of PFOA is 3.5 years (Olsen et al., 2007).

1.1.5 Toxic properties of PFASs

After the turn of the century, around 3000 papers about PFASs have been published (DeWitt, 2015). To date, several biological toxic effects of PFASs have been characterised; below there is a brief description of them.

Even though PFASs are metabolically inert compounds, they can interfere with the metabolic processes of living organisms. PFASs have the ability to bind certain receptors by mimicking endogenous ligands, creating agonizing or antagonizing effects (Jiang et al., 2015). The main molecular target of PFASs is the peroxisome proliferator-activated receptor (PPAR). Interaction of PFASs with endogenous receptors can disrupt several metabolic pathways and induce various biochemical and physiological changes. Moreover, by disrupting metabolic pathways, they might interfere with detoxifying processes of other substances (Jiang et al., 2015).

Neurotoxic effects have also been reported. Physicochemical properties of PFASs allow them to modulate the membrane fluidity of the cells, entering the brain blood barrier (BBB). In laboratory animals, increased exposure levels of these chemicals during the gestation produced in the offspring an increased locomotor activity and inability to habituate to new conditions (Viberg and Mariussen, 2015).

In rodents, PFOS and PFOA have shown to produce many immunotoxic effects as suppression of adaptive immunity, disruption of antigen-specific antibody production, and reduced number of B and T cells (Yang et al., 2002; Qazi et al., 2012).

Some studies reported endocrine-related effects of PFOA in rodents, humans and other species; and there is a wide variety of congeners that could exert potential endocrine effects. Although the exact mechanism behind the action of PFASs is still unknown, the mammary gland, pancreas, ovaries, thyroid and adipose tissue are known to be endocrine targets in females (mainly but not exclusively) of both humans and rodents (Reed and Fenton, 2015).

1.2 Genetic toxicology

Genetic toxicology is "a branch of the field of toxicology that assesses the effects of chemical and physical agents on the hereditary material (DNA) and on the genetic processes of living cells" (Preston and Hoffman, 2013). Agents are classified as genotoxic when they are able to produce alterations in nucleic acids at sub-lethal exposure concentrations, resulting in changes in the DNA. Populations exposed to genotoxic contaminants experience increased mutation rates (Bickham et al., 2000), which might enhance accumulation of genetic load and eventually a decrease in the average fitness of the population (Crow, 2001). Reproductive disruption at a population level is one of the most concerning sublethal effects of genotoxic pollutants, since it might challenge the conservation of population (Anderson et al., 1994).

1.2.1 Cell cycle

The cell cycle consists of a number of complex processes -all coordinated to start at a precise time and defined order- that result in the division of the cells (Elledge, 1996). During the cell cycle, the DNA is duplicated and then is segregated into two genetically identical daughter cells. This occurs in two phases, *S phase* (S from synthesis), where the single-stranded DNA is duplicated in about 10-12 hours (in a typical mammalian cell); and *M phase* (M from mitosis), where the chromosomes

are segregated and cell division occurs through a set of events that receive the name of mitosis. For allowing more time to grow, after S phase there is a gap phase called G1, and after M phase there is another called G2. G1, S and G2 altogether receive the name of interphase, which represents most of the time in the cycle of the cell (Figure 1) (Alberts et al., 2015a). Mitosis starts with the condensation of the chromosomes, where the duplicated DNA strands compress into much more compact chromosomes. The nuclear envelope breaks down at prometaphase and the replicated chromosomes, each consisting of a pair of sister chromatids, get attached by their centromeres to the microtubules of the mitotic spindle. The next stage is metaphase, when the chromosomes are aligned at the equator of the mitotic spindle, forming the metaphase plate. Anaphase comes after, when the sister chromatids are separated and pulled to opposite poles of the cell. During telophase, the mitotic spindle is broken, and the two sets of chromatids reform each one a nucleus. The process where the cytoplasm is divided to form two new cells is called cytokinesis and it overlaps with the final stages of mitosis (Alberts et al., 2015a).

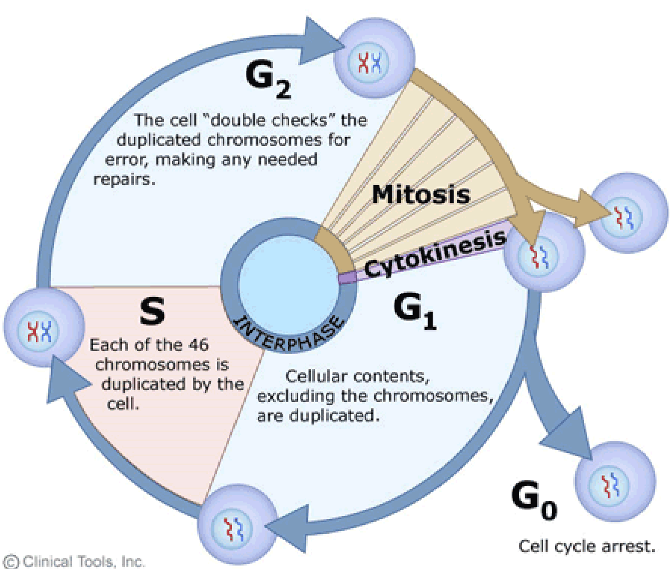

Figure 1. Stages of the cell cycle. Retrieved 14.04.2018, from le.ac.uk.

1.2.2 DNA injury and repair

Genetic stability is a condition *sine qua nom* for the survival of individuals. In order to achieve this, an accurate mechanism for replicating DNA is required, as well as effective mechanisms for repairing any accidental lesions that may occur. DNA repair is the onset of processes by which spontaneous changes in DNA are corrected; these processes are extraordinarily efficient, to the extent that less than one in 1000 accidental base changes end up being a permanent mutation. The inactivation of DNA repairing genes results in an increased rate of mutation. In fact, defects in human DNA repair machinery can lead to a higher predisposition to certain cancers (Alberts et al., 2015b).

DNA is a highly stable material, nevertheless, it is susceptible to spontaneous changes that lead to mutations if these are unrepaired. When unrepaired DNA is replicated, these changes lead either to the deletion or addition of one or more base pairs or to a base-pair substitution in the daughter DNA chain. As cell divides, the mutations are propagated throughout the next generation of cells, enhancing adverse consequences for the organism if a high rate of random changes is present (Alberts et al., 2015b).

DNA can be repaired through multiple pathways by enzymes that specifically act upon different kinds of lesions. The major pathway by which DNA is repaired is base excision repair, in this process the DNA glycolase, an enzyme that is specific for each kind of altered base, recognizes the altered base in the DNA and catalyse its hydrolytic removal. This is followed by the restoration by the DNA polymerase, which uses the undamaged strand as a template to copy and insert the base pair missing (Alberts et al., 2015b).

Alternatively, cell death might occur when unrepairable damage appears. Apoptosis is a regulated cell death mechanism by which a series of coordinated and energy-dependent processes activate a group of cysteine proteases that enhance protein degradation (Nowsheen and Yang, 2012). Apoptosis is a crucial event in the maintenance of the cell population of organisms. When apoptosis is unregulated, cells will either not be able to die, as it occurs in cancer, or die excessively, as happens in neurodegenerative disorders and AIDS (Turk and Stoka, 2007).

1.2.3 Genotoxic compounds

DNA damage can be caused by both endogenous processes and exogenous compounds. Endogenous processes cause several hundred DNA damages per cell per day, an example is the formation of reactive oxygen species (ROS) as by-products in the mitochondrial respiratory chain (Preston and Hoffmann, 2013). DNA can also be damaged by genotoxic compounds, which are either physical agents like ionizing radiation and ultraviolet light; or chemical agents, capable of inducing mutations and related genetic changes in living cells (Würgler and Kramers, 1992).

Chemicals can produce DNA alterations either reacting with the DNA directly by forming adducts, or indirectly by intercalating between the base pairs (Preston and Hoffmann, 2013). These chemicals are also known as environmental mutagens. A well-known example is polycyclic aromatic hydrocarbons (PAH), which after being bioactivated bind to nucleotides forming adducts (Sato and Aoki, 2002). On the other hand, there are chemicals that are non-genotoxic by themselves but can exert genotoxic effects by the indirect formation of ROS, producing cytotoxicity, or causing inflammation or infection (Klaunig et al., 2012). ROS are generated by the cytochrome P450 isoenzymes during the detoxification of chemicals, lipid peroxidation and other intracellular processes. ROS are genotoxic in principle, they oxidize lipid and protein molecules generating intermediates that can react with DNA forming adducts or can attack directly the DNA producing strand breaks (Kakehashi et al., 2013).

1.2.4 Genetic toxicity of PFASs

Relatively few studies have been performed to investigate the genotoxicity of PFASs, finding conflicting results. Zheng et al. (2016) exposed the earthworm *E. fetida* to PFOS and PFOA for 14 days and an increased mortality and DNA damage was detected. Wielsøe et al. (2015) exposed *in vitro* human hepatoma cell line (HepG2) to seven different kinds of PFASs (PFHxS, PFOA, PFOS, PFNA, PFDoA, PFUnA, PEDA) and investigated ROS production and chromosomal damage by comet assay. Significant dose-dependent DNA damage was observed for PFHxS, PFOS, PFOA, and PFNA, showing that these compounds can produce DNA strand breaks. This article suggests that ROS production might be the mechanism by which PFAS are genotoxic, however, they theorize that there are other mechanisms involved. Nevertheless, the study by Wielsøe et al. (2015) used relatively high concentrations compared to the levels found in humans.

Contrarily, the European Food Safety Authority (EFSA, 2008) concluded that PFOS and its salts do not possess genotoxic properties based on several assessments. Negative results were found for PFOS in the reverse mutation assay, with strains of *Salmonella* and *E. coli,* at different chemical concentrations (Litton Bionetics, Inc., 1978; Mecchi, 1999). PFOS did not induce chromosomal aberrations in culture human lymphocytes, nor unscheduled DNA synthesis in primary cultured rat liver cells (Cifone, 1999). In an *in vivo* study on mouse, negative results in the micronucleus assay were also found after a single oral high dose (237, 450 and 950 mg/kg) (Corning Hazleton, Inc., 1993).

1.2.5 Double strand breaks and chromosomal aberrations

The types of DNA damages produced by physical and chemical agents are many and varied, including single- and double- strand breaks in the backbone of the DNA, cross-links between DNA bases or between DNA bases and proteins, and chemical addition to the DNA bases (adducts) (Preston and Hoffmann, 2013). Figure 2 shows the spectrum of DNA damages induced by physical and chemical agents.

Estimations say that each human cell is subjected to approximately 70,000 lesions per day (Lindahl and Barnes, 2000). The majority of lesions (75%) are single-strand DNA (ssDNA) breaks, which can also be converted to DNA double-strand breaks (DSBs). DSBs occur when both strands of the double helix are severed (Schwab, 2011). DBSs are critical for the cells because although they occur at a lower frequency than single strand breaks, they are considered the main lesions leading to chromosomal aberrations (CAs) when they are un- or missrepaired (Tubbs and Nussenzweig, 2017; Obe and Durante, 2010).

According to the Enclyclopedia of cancer (Schwab, 2011), CAs are any damage caused to the chromosomes of a cell by a genotoxic agent in a way that the shape of the chromosome is changed. Examples include breaks, fragmentation, dicentric chromosome formation, chromosomal translocation or chromosomal rearrangement. Chromosomal aberrations have been long recognized as a biomarker to human exposure to ionizing and genotoxic chemicals. CAs are an important endpoint in genetic toxicology testing because it has been seen that specific CAs are present in cancer cells (Natarajan et al., 2008)

When cells with CAs undergo mitosis, the aberrant chromosomes or chromosome fragments might not be distributed properly to the poles and might end up surrounding themselves with a nuclear membrane forming micronuclei (MN). MN sometimes also contain whole chromosomes that were not correctly distributed (Obe and Durante, 2010).

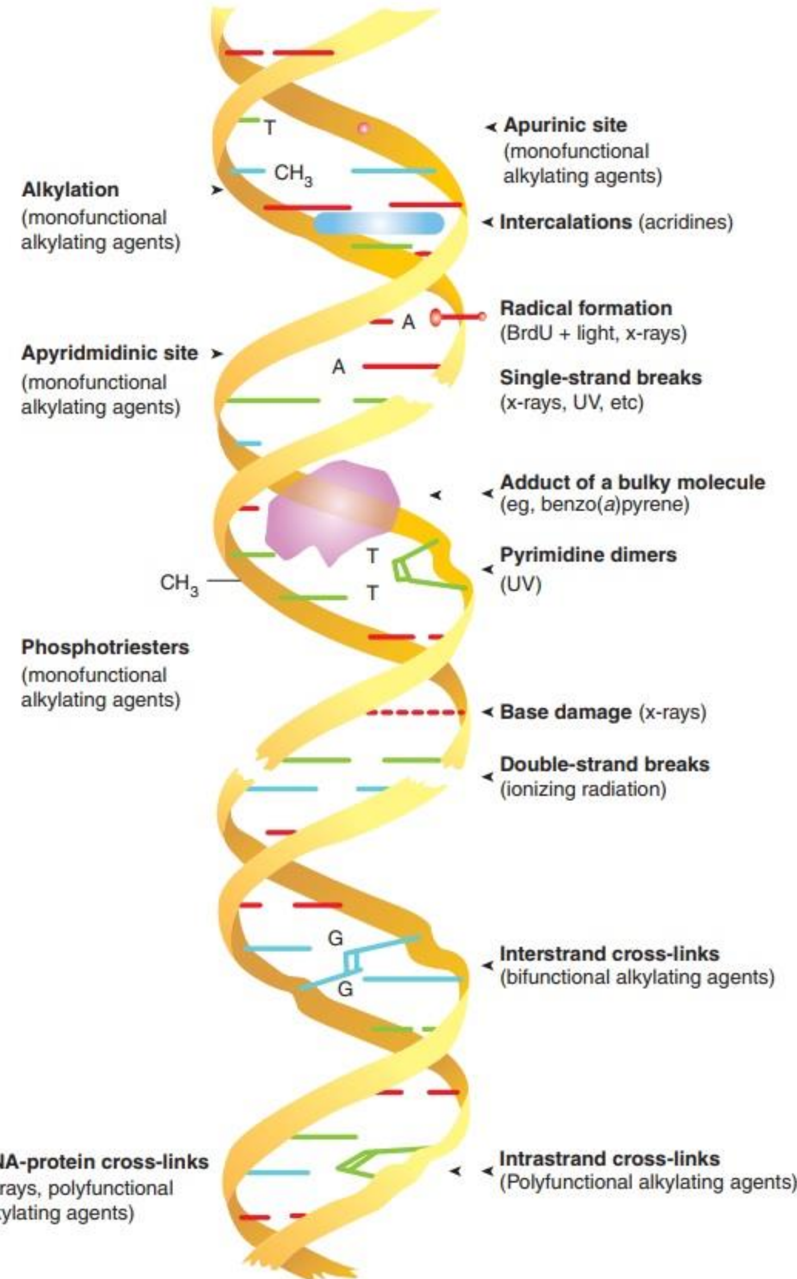

Figure 2. Types of DNA damage produced by chemical and physical agents. Extracted 14.05.2018, from Preston and Hoffman (2013).

1.3 Study animal: rodents

The term rodents refers to animals in the order Rodentia, which encompasses more than 2000 species and constitutes 40% of mammal species. The common characteristics that all individuals of this order share are the possession of a pair chisel-shaped incisors and the lack of canine teeth, which allow them to gnaw essentially everything. Rodent species are usually relatively small, but the order is varied and their size ranges from 7 g, which is the average weight of the pygmy mouse, to 50 kg, weight that a South American capybara can achieve (Kay and Hoekstra, 2008). Rodents have a wide and not-so-specific diet; they can feed on grasses, seeds, insects, fish and even scorpions. In addition, these animals have a prolific reproduction. Because of these characteristics, rodents have adapted to practically all habitats (with exception to the oceanic ones) and are successful colonizers of almost every existent niche (Kay and Hoekstra, 2008).

1.3.1 Small rodents as bioindicators

Chemical analyses of soil, air and water are useful for studying the concentration of contaminants in the environment. However, these studies do not provide information about the bioavailability and potential toxicity of the environmental contaminants in wildlife. Animals instead, can be indicator species that can tell us about the environmental conditions of habitats (Reif, 2011). Within all animal orders, small rodents are considered useful for pollution biomonitoring and hazard assessment given that they are intermediates between the lowest trophic levels (because they feed on seeds and insects) and high trophic levels (carnivorous birds and mammals feed on them). In addition, given their small size, these animals are easy to handle and possess a high metabolic rate, therefore are subjected to a higher degree of exposure to contaminants comparing to larger herbivorous mammals (Sheffield et al., 2001). Lastly, their home range is reduced to 897 m^2 for mature females and 1753 m^2 for mature males (Bujalska and Grüm, 1989), so they offer a reliable picture of the contamination of a specific area.

1.4 Aims and hypotheses

Aim 1: Quantify the levels of PFASs in small rodents inhabiting the study area, Granåsen; and compare them to the population of the reference area chosen, Jonsvatnet.

H1: Since high PFASs levels have been reported in skiing areas, the small rodents inhabiting Granåsen will have higher PFASs concentrations in liver than individuals from Jonsvatnet.

H2: Studies show that perfluorooctane sulfonate (PFOS) has the highest concentrations among all PFAS congeners in wildlife. The small rodents sampled will therefore show higher concentrations of PFOS.

H3: Older individuals will have higher concentration of long-chain PFASs due to bioaccumulation.

Aim 2. Study the chromosomal aberrations in the lymphocytes of small rodents from Granåsen and compare them to the population of small rodents inhabiting Jonsvatnet.

H4: A higher incidence of chromosomal aberrations is expected in the population of Granåsen due the possible genotoxic properties of PFASs.

2 Materials and methods

2.1 Study area

The study area is “Granåsen skisenter” (63º 22’N, 10º18’E), located approximately 10 km away from Trondheim’s city centre (Norway). Granåsen is Trondheim’s main arena for winter sports, and is where the annual World Cup of ski jumping takes place; the facilities were built in 1997 for the World Championship in skiing. Granåsen offers several cross-country ski tracks, used in competitions as well as by amateur skiers. During summer, Granåsen is visited for field hiking and other recreation activities.

As a reference site, the woods in the vicinity of an ecological farm next to the lake Jonsvatnet were chosen (63º20’N, 10º33’E), at approximately 15 km away of Trondheim’s city centre. The lake lays in the municipality of Trondheim and Malvik and supplies drinking water to Trondheim (Figure 3).

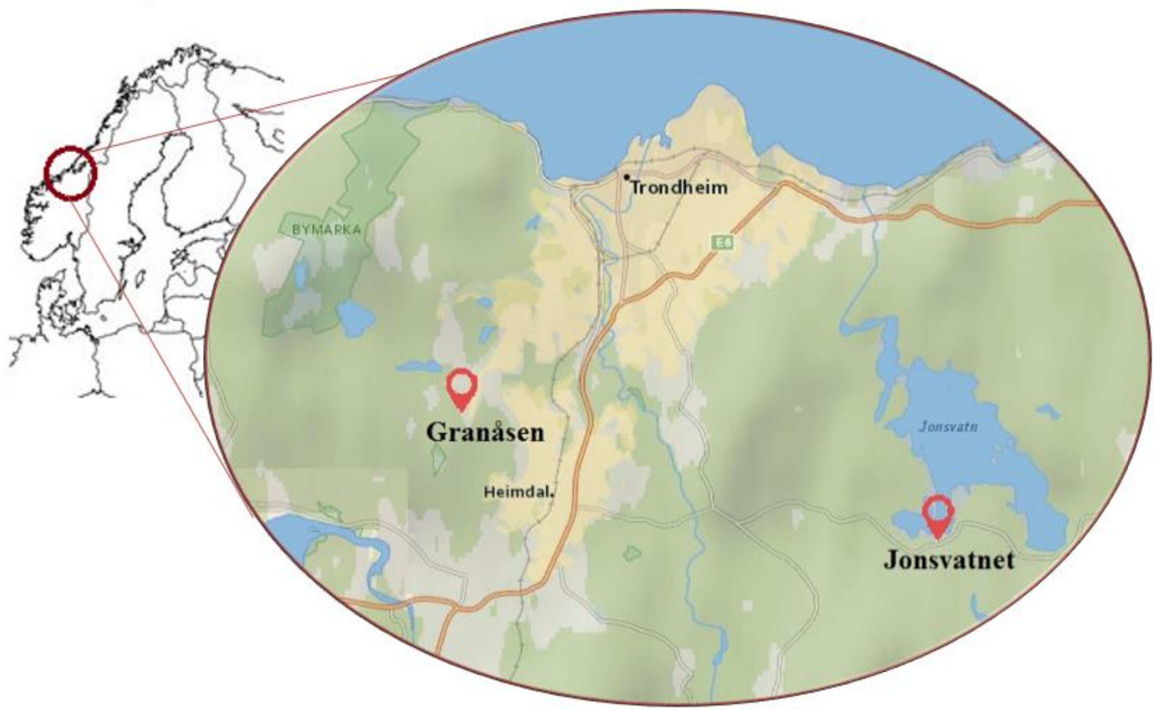

Figure 3 Map showing the two areas where the small rodents were sampled. Granåsen is the study area and Jonsvatnet the reference area. Modified figure retrieved 15.04.2018, from ArcGis.

The vegetation of Granåsen and Jonsvatnet are similar, dominated by the same species. The forests differ notably in density, Jonsvatnet is denser and wilder while Granåsen is more scattered, with several tracks fragmenting the forest. Both forest floors are covered nearly completely by moss; the schrub *Vaccinium* spp. also abounds in the two areas. The trees that predominate in these coniferous woods are the spruce, the pine and the birch.

2.2 Small rodents sampling

The sampling of small rodents started the 30/05/2017 and finished the 04/07/2017. The population of small rodents in Granåsen was first sampled: 68 traps were set in the two main hills that surround the ski centre. The traps (Figure 4) were live trap of type "Ugglan". They were placed strategically according to the habitats preferred by the small rodents, few meters away from the cross-country ski tracks. Small pieces of rye bread soaked with sunflower oil and additive-free peanut butter were placed in the traps (all food products were sold as ecological), together with some moss for avoiding additional stress to the individuals. The trapps were arranged in stations (16 stations in total) of four traps per station. The trapping stations were spaced out with a minimum of 300 m in between to avoid depletion of the populations. All traps were controlled every day. The population of small rodents from Jonsvatnet was sampled following the same procedure as in Granåsen. The size of the areas sampled was estimated by using the tool 'area calculator' of Google maps.

Figure 4. Cage trap in Granåsen. Picture taken by Randi Grønnestad

All individuals were brought to the Department of Biology at the Norwegian University of Science and Technology (NTNU). Each rodent was first anesthetized with Ketamine (100mg/kg) and Xylazine (10mg/kg) injected intraperitoneally with a 3 ml syringe attached to a 23 gauge / 0.6 mm needle. Specimens were then weighted, and their length was measured. Blood samples were taken by cardiac puncture with a heparinized 3 ml syringe attached to a 20 gauge / 0.9 mm needle. The animals were then sacrificed by neck dislocation and dissected right after, separating the liver, brain, gonads, heart, kidneys and a sample of muscle tissue. Samples were frozen straight away and will be analysed by the PhD student Randi Grønnestad. Eyes were dissected and stored in 10% formalin.

All animal handling was performed following the principles and guidelines and by permit from the Norwegian Animal Research Authority (Mattilsynet, ID 10237) and the Norwegian Environmental Agency.

2.3 Age determination of the small rodents

The weight of the eye lenses was used as an age indicator of the rodents. The lenses were dried in an incubator to constant weight, which was completed after 24 h at 80 °C. Weight was measured with accuracy to 0.1 mg as described by Nabaglo and Pachinger (1979). Different formulas were then applied for estimating the age of the different species. *C. glareolus* age was estimated by applying the formulas developed by Kozakiewicz (1976), which found out that the lenses of *C. glareolus* grow at higher rate during the first three months of life and slow down with age. Hence, the formula $y = 0.0063x + 1.050$ was used for individuals with eye lenses average weight lower than 5 mg, and the formula $y = 0.013x + 4.610$ for individuals with heavier lenses (where y is the average weight of the two lenses). The age of *A. flacivollis* specimens was calculated with the formula $y = 2.2869x + 10.0208$ developed by Nabaglo and Pachinger (1979). For *A. sylvaticus*, the formula $\log.age = 0.0059x + 0.907$ was applied (Quere and Vincent, 1989). Since a formula for determining the age with eye lenses weight was not found for *Microtus agrestis*, the formula $y = 0.8821 - 1.9565x$, developed by Gourley and Jannett (1975), for *Microtus pinetorum* was used.

Each rodent was classified within an age class, for doing that, the age classification proposed by Steinar (1968) and discussed by Nabaglo and Pachinger (1978) was used. According to this, individuals are classified within seven different groups: I, for individuals around 1 month old; II

for individuals around 2 months old; III, from 2 to 4 months; IV, from 4 to 6, V, from 6 to 8; and VI, older than 8 months.

2.4 Chemical analysis

The concentration of PFASs in the liver of the small rodents sampled was analysed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) at the Laboratory of Environmental Toxicology at the Norwegian University of Life Sciences (NMBU), these analyses were carried out by the PhD student Randi Grønnestad and engineers of the laboratory. An overview of the PFAS contaminants analysed is shown in table 2.

Table 2. PFAS contaminants included in the chemical analysis of the small rodents livers

PFAS group	Congener	Chain length
Perfluoroalkyl carboxylates (PFCAs)	Perfluorobutanoic acid (PFBA)	C4
	Perfluorohexanoic acid (PFHxA)	C6
	Perfluoroheptanoic acid (PFHpA)	C7
	Perfluorooctanoic acid (PFOA)	C8
	Perfluorononanoic acid (PFNA)	C9
	Perfluorodecanoic acid (PFDA)	C10
	Perfluoroundecanoic acid (PFUdA)	C11
	Perfluorododecanoic acid (PFDoDA)	C12
	Perfluorotridecanoic acid (PFTrDA)	C13
	Perfluorotetradecanoic acid (PFTeDA)	C14
Perfluoroalkyl sulfonates (PFSAs)	Perfluorobutane sulfonate (PFBS)	C4
	Perfluorohexane sulfonate (PFHxS)	C6
	Perfluorooctane sulfonate (PFOS)	C8
Perfluoroalkyl sulfonamides (PASFs)	Perfluorooctane sulfonamide (FOSA)	C8
	N-Methyl perfluorooctane sulfonamido ethanol (N.MeFOSE)	C8
	N-Ethyl perfluorooctane sulfonamido ethanol (N.EtFOSE)	C8
	N-Ethyl perfluorooctane sulfonamide (N.EtFOSA)	C8

2.5 Chromosome analysis

2.5.1 Chemicals and equipment

Chemicals	Product number	Producer
Acetic acid (CH_3COOH, 100%)	1.00063.1000	Merck
CO_2-Independent Medium (1x)	I8045-054	Gibco BRL
Concavavalin A	C5275	Sigma-Aldrich Co
Eukitt (xylene 30-60%)		Chemi-Teknik AS
FBS (Fetal Bovine Serum)	F9665	Sigma-Aldrich Co
Gimsa, Chroma		Chemi-Teknik AS
KaryoMax Colcemid Solution	I008995	Gibco BRL
L-glutamine 200 mM (100x)	712923A	Gibco BRL
Methanol for analysis (CH_3OH, 100%)	1.06009.2500	Merck
OptiPrep	LYS 3782	Axis Shield PoC AS
PBS (phosphate buffered saline)	10010023	Gibco BRL
Potassium chloride (KCl)	P9541	Sigma-Aldrich Co
PS (Penicillin streptomycin)	15070-063	Gibco BRL
RPMI Medium 1640 (1x)	42402-016	Gibco BRL
Sodium chloride (NaCl)	S3014	Sigma-Aldrich Co
Sodium citrate tribasic dihydrate ($C_6H_5O_7 \cdot 2H_2O \cdot 3Na$)	S-4641	Sigma-Aldrich Co
Tricine	T0377	Sigma-Aldrich Co

Equipment and consumption material	Product number	Producer
Centrifugator (Labozentrifugen)		Sigma-Aldrich Co
Eppendorf tubes	L25788G	Eppendorf
Falcon tubes	2057	Falcon
Infrared CO_2 incubator		Forma Scientific
Microscope glass cover slips 24x50 mm		Sigma-Aldrich Co
Microscope slides cut edges frosted	ECN 631-1551	VWR
Pasteur Pipette 250 mm	D812	Volac
Vortex mixer Vibrofix VF1 electronic	659073	Janke & Kunnel

2.5.2 Lymphocyte isolation

The lymphocytes were separated from the whole blood following the OptiPrep™ Application C43 protocol (Alere technologies AS, 2016). Before starting, the three following solutions were prepared: stock solution of Tricine 100Mm (kept at 4 ºC); Solution B (buffered saline): in 100 ml: 0.85 g NaCl, 10ml of Tricine stock solution and distilled water (pH 7.0); and density barrier: solution B and water in a proportion 2.5:0.5 respectively, and then this solution was mixed with OptiPrep ™ in a ratio 9.3:2.7 respectively.

The blood was mixed with an equal amount of solution B in a 15ml Falcon round bottomed culture tube. First, the density barrier was added, and the diluted blood was gently poured on top taking care that the two phases did not mix. The tube was centrifuged at 700 g for 20 min. The layer of lymphocytes (second layer starting from the top in Figure 5) was taken up by using a Pasteur pipette and placed carefully in another centrifugation tube. The lymphocytes were then washed by adding 10 ml of phosphate buffered saline (PBS) and centrifuging at 400 g for 10 min and the supernatant was taken out. 10 more ml of PBS were added to the cell pellet for a second washing.

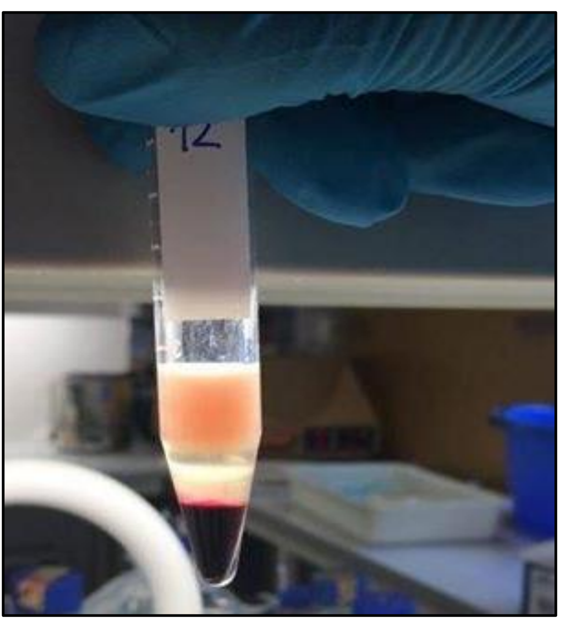

Figure 5. Falcon tube with the different layers formed after centrifugation of the whole blood with the density barrier. Four layers are observed in the picture, starting from above: 1) plasma and dilution buffer, 2) mononuclear cells, 3) OptiPrep and solution B and 4) erythrocytes, granulocytes and dead cells.

2.5.3 Lymphocyte culturing

The culture medium was prepared before getting the first blood samples by mixing 10ml FBS, 0.5ml of L-glutamine, 0.5ml of PS, 20ml of RPMI and 20ml of CO_2-IND.

1 ml of culture medium and the mitogen Concavavalin A (10µg/ml) were added to the washed lymphocytes, the tubes were incubated during 24 h at 37 ºC. After 24 h incubation, a further 1 ml of complete medium containing 4 µl of 5 µM BrdU (preheated to 37 ºC) was added, this step had to be done fast not to allow the culture temperature to drop below 37 ºC. The culture was placed in the incubator for 3.5 h. After 44.5 h (of the conavalin starting point), 100 µl of Colcemid was added with a syringe and the cultures were placed back in the incubator for 3.5 h. After 48 h total incubation, the cultured was first transferred to another Falcon tube and washed twice in 10 ml of PBS, about 200 µl of PBS was left in each tube.

2.5 ml of hypotonic treatment (prepared with KCl (75 mM) and Na-citrate (30 mM) in a proportion volume of 2:1 respectively) were poured gradually under gentle mixing (using a vortex mixer) to the 200 µl cell suspension and left for 8 min. The cells were then fixated with 2.5 ml of methanol and acetic acid (3:1) poured under gentle mixing (using a vortex mixer). The cells were pelleted afterwards by 10 min of centrifugation at 400 g. The supernatant was taken out and 5 ml of fixative were added. The cells were stored at -20 ºC until the slides preparation started.

2.5.4 Cell dropping and staining

Staining was performed by combination of the techniques of Edelman and Lin (1986), Erexson and Kligerman (1987), and Østby (1998). The cells were first washed twice with fresh fixative (400 g, 5 min) (following the same procedure as in 2.5.2) and transferred to Eppendorf tubes with 100 µl of fixative. Each sample was dropped with a 20 µl pipette to a wet chilled slide that had been previously washed with ethanol and distilled water. Several heights were tried and 1.5-2 m was proven to be the best for the proper spreading of the cells and chromosomes. Three to four drops were put on each slide and slides were let air dry overnight.

The next day, the slides were treated with Gimsa staining for 15 min, few drops of Gurr R66 Gimsa 4% (pH = 7) were added on each slide and covered with coverslips. After 15 min, coverslips were removed and slides were washed with distilled water and dried on a bench warmer at 40 ºC. Once

slides had dried, two small drops of glue Eukitt were put on them and the final coverslips were mounted.

2.5.5 Scoring of the slides

The prepared slides were randomly number coded and were examined at random order to ensure that the origin of the specimen was unknown while chromosomal aberrations were scored. For each slide, the total number of lymphocytes was recorded, as well as the mitotic cycle stage of the cells. The chromosomal aberrations were scored with the 60x and 100x magnification (100x was an oil-immersion objective). An optical microscope Nikon Eclipse E400 was used. Some pictures were taken with a Nikon camera Coolpix 900 with a MDC lens.

The CAs were scored according to the classification made by Savage (1975). According to this classification, there are two main types of CAs: *chromosome-type,* where both sister-chromaids are affected, and *chromatid-type,* affecting only one of the sister-chromatids.

2.6 Statistical methods

The limit of detection (LOD) was set at 3*background noise levels for each contaminant (Jian-Li et al., 2015). The samples with concentrations below the LOD were substituted by LOD/2, since it is a common way for treating data (Schlabach et al., 2017). The congeners that had less than 60% of the values above the LOD in each sampling area were not included in the statistical analyses.

All statistical analyses were performed with IBM SPSS Statistics 25 and graphs drawn with SigmaPlot 13.0. To determine if the data followed a normal distribution, the Shapiro-Wilk test was used. Since data was not normally distributed, the nonparametric Mann-Whitney U test was used to analyse if the concentrations of each chemical congener were statistically different between the two sampling areas.

Prior to further analysis, data were log-transformed to assure a normal distribution. Non-parametric tests were used for the chemical concentrations that did not follow a normal distribution after log-transformed. The Pearson's correlation coefficient was used to examine the correlation among the log-transformed chemicals. It was considered that the correlations were statistically significant at a 95% confidence interval ($p < 0.05$).

Before performing an analysis of the covariance (ANCOVA), a new variable called 'winter' was created, in which individuals born before winter (older than 6 months) were sorted in one group and the individuals born after (younger than 6 months) in another. This variable was created to analyse if small rodents born before winter, i.e. before the ski season, had a different chemical burden than the younger individuals born after the ski season. The stations where the small rodents were captured were sorted into two groups. ANCOVA was then performed with the variables age class, 'winter' gender and station number as independent variables; age in days, weight and total length as covariates and the chemicals congeners as dependent variables. The species of the small rodents could not be added as a variable because of few individuals other than bank vole were captured. For the chemicals that did not follow a normal distribution, the non-parametric tests Kruskal-Wallis H test and the Spearman rank correlation coefficient were used.

To get a better visualisation of the relationship between variables that showed a significant correlation, data was subjected to Principal Component Analysis (PCA).

3 Results

3.1 Animals captured

Four different species of wild small rodents were captured in the two sampling areas. The species found were the bank vole (*Clethrionomys glareolus*), the field vole (*Microtus agrestis*), the yellow-necked mouse (*Apodemus flavicollis*) and the small wooden mouse (*Apodemus sylvaticus*). High number of shrew (family Soricidae) were also trapped, but were not included in the chemical and cytogenetic analyses. The bank vole was the predominant small rodents species sampled, representing 80% of the total small rodents caught in Granåsen and 96.8% in Jonsvatnet.

An overview of the rodents captured in each sampling area together with an approximate size of the sampling areas (estimated with the tool 'area calculator' of Google maps) is given in table 3. The biological data recorded is presented in tables 1 and 2 of the appendix A.

Table 3. Overview of the species of small rodents captured in each sampling area in June 2017. The total number of shrew (family Soricidae) is indicated but not included in the total number of small rodents. An estimation of the sampling areas (km^2) is also given

Sampling area	Approx. area (km^2)	*A. flavicollis*	*M. agrestis*	*C. glareolus*	*A. sylvaticus*	Total small rodents	Soricidae
Granåsen	0.22	4	1	20	0	25	34
Jonsvatnet	0.04	0	0	31	1	32	58

Table 4. Number of small rodents captured in each area, number of traps and nights of catching are indicated

	Granåsen	Jonsvatnet
Small rodents captured	25	32
Number of traps	68	66
Number of nights	30	7
Catching effort[1]	2040	462
Trapping success (%) [2]	1.23	6.9

[1] Catching effort is calculated as the product of the number of traps and nights (nº traps * nº nights).
[2] The trapping success is calculated as (nº of small rodents captured / catching effort) *100.

The catching success was considerably higher in Jonsvatnet than in Granåsen (6.9% and 1.23% respectively). While in Jonsvatnet 32 small rodents individuals were caught in 7 nights, 25 individuals were trapped in 30 nights in Granåsen (table 4).

The sex ratio of the trapped rodents showed an unbalanced proportion of males and females, being skewed towards males (1:2.3 in Granåsen and 1:2 in Jonsvatnet for *C. glareolus*). Neither males nor females of *C. glareolus* showed statistically significant differences in their total length, tail's length or weight among both sampling areas (T test, $p > 0.05$). Table 5 presents the main physical traits recorded (total length, tail's length and weight) for each gender of species captured in the two sampling areas.

Table 5. Differentiated for genders, male (M) and female (F): total length, tail's length and weight (average ± SD) for different species in each sampling area.

Area	Species	Gender	Number	Total length (cm, average ± SD)	Tail length (cm, average ± SD)	Weight (g, average ± SD)
Granåsen	***C. glareolus***	M	14	13.5 ± 0.96	4.53 ± 0.64	21.19 ± 3.98
		F	6	12.45 ± 1.49	4.5 ± 0.65	20.54 ± 8.13
	A. flavicollis	M	3	15.33 ± 2.01	6.83 ± 2.01	22.4 ± 2.0
		F	1	15.00	7.00	16.60
	M. agrestis	M	1	12.50	4.00	15.21
Jonsvatnet	***C. glareolus***	M	20	12.72 ± 1.56	4.33 ± 0.66	21.86 ± 5.90
		F	11	13.33 ± 1.76	4.61 ± 0.65	23.16 ± 6.31
	A. sylvaticus	M	1	14.00	7.00	15.70

3.2 Age of the small rodents

The age in days of the small rodents was estimated using the formulas developed in different studies (Nabaglo and Pachinger, 1979; Kozakiewicz, 1976; Quere and Vincent, 1989; Gourley and Jannett 1975) and each rodents was classified within an age class (Steinar, 1968; Nabaglo and Pachinger, 1978). Table 6 summarizes the age classes of the small rodents sampled in the two areas. Many individuals belong to class I and II, these small rodents were born in May and April respectively. There are relatively fewer small rodents of the class III and IV (born between January and March). Since the small rodents classified in age class V and VI are estimated to be older than 6 months, we consider that they were born in 2016, before the winter (and ski season) started.

Table 6. Number of infividuals in each age class (total number of individuals from Granåsen n = 24, and from Jonsvatnet n = 31).

	I	II	III	IV	V	VI
Granåsen	8	4	0	1	9	2
Jonsvatnet	12	4	3	1	6	5

3.3 PFAS concentrations

18 PFAS congeners were analysed in the liver samples of the small rodents captured in Granåsen and Jonsvatnet. Out of the 18 PFASs, 10 were detected above LOD in Granåsen and 7 in Jonsvatnet. The perfluoroalkyl sulphonamides analysed (FOSA, N-MeFOSE, N-EtFOSE, N-EtFOSA) and the carboxylate PFOA were either not detected (n.d.) or detected below the limit of detection (<LOD) in the majority of individuals from the two sampling areas.

Out of the PFAS congeners analysed in the small rodents livers, PFOS was the predominant PFAS. In Granåsen PFOS was detected above LOD in 92% of the samples and in Jonsvatnet in 85%. Moreover, PFOS had the highest average concentration (ng/g ww) among all PFAS congeners in the two sampling areas (3.27 ± 0.68 and 1.67 ± 0.25 in Granåsen and Jonsvatnet respectively) (mean ± SE).

In the Mann-Whitney U test (results presented in the appendix C, table 5), 7 congeners: PFOS, PFTrDA, PFTeDA, PFDoDA, PFDA, PFHxS and PFBS, showed significantly higher concentrations ($p < 0.05$) in the liver of the small rodents sampled in Granåsen compared to the

reference area. Since the congeners PFTrDA, PFTeDA, PFHxS and PFBS were not detected above LOD in Jonsvatnet, the values LOD/2 were used instead. The perfluoroalkyl carboxylates PFHxA and PFHpA showed higher concentrations in Jonsvatnet (Figure 6), compared to Granåsen. PFNA did not show statistical differences in the concentrations between the two sampling areas.

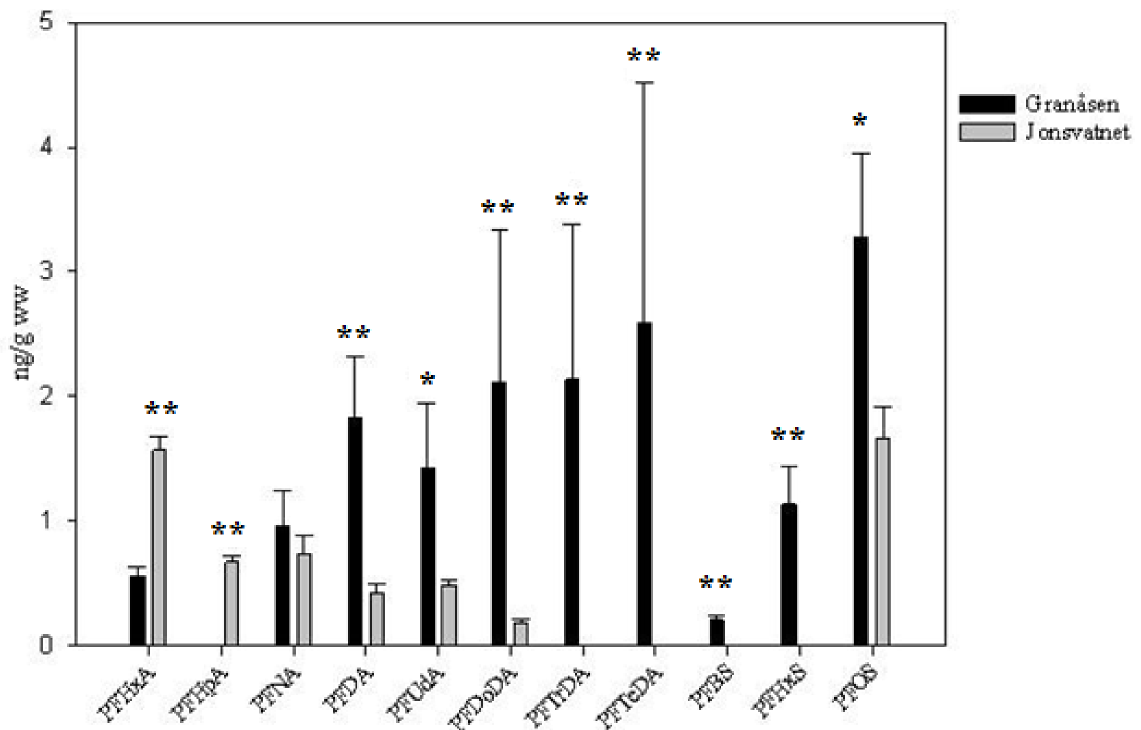

Figure 6. PFAS concentrations (ng/g ww) mean and SE in wild small rodents sampled in Granåsen (n = 24) and Jonsvatnet (n = 32) in June 2017. * indicates $p < 0.05$, ** indicates $p < 0.01$.

3.3.1 Multivariate, correlation and ANCOVA analyses

The PCA loading plot (Figure 7) indicates how the different PFAS congeners in the small rodents inhabiting Granåsen and Jonsvatnet are related to each other. The principal component (PC) 1 explains 52% of the total variance, in which the long-chain carboxylic acids PFDoDA, PFTeDA, PFTrDA, PFDA, and PFUdA contribute to the greatest extent, and these compounds are strongly correlated with each other. PFOS, PFBS and PFHxS are, to a lesser extent, also positively correlated with the PC1, but are not correlated with each other. PFHpA is strongly associated with PFHxA and both short-chain carboxylic acids show a strong negative loading in PC1. PC2 explains 17% of the total variance. In PC2, PFOS and PFNA have the biggest weight, and are negatively correlated with it. Note that PFOS has a strong weight in both PCs.

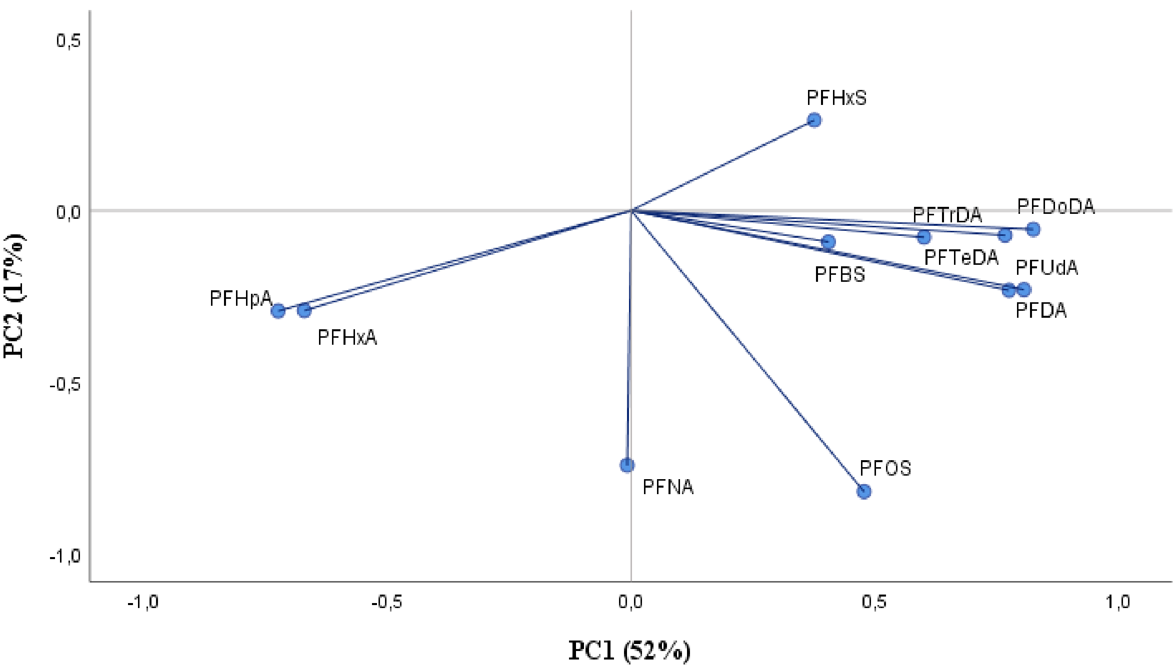

Figure 7. PCA loading plot of the overall PFAS congeners in small rodents from the two sampling areas (n = 24 in Granåsen and n = 32 in Jonsvatnet, June 2017).

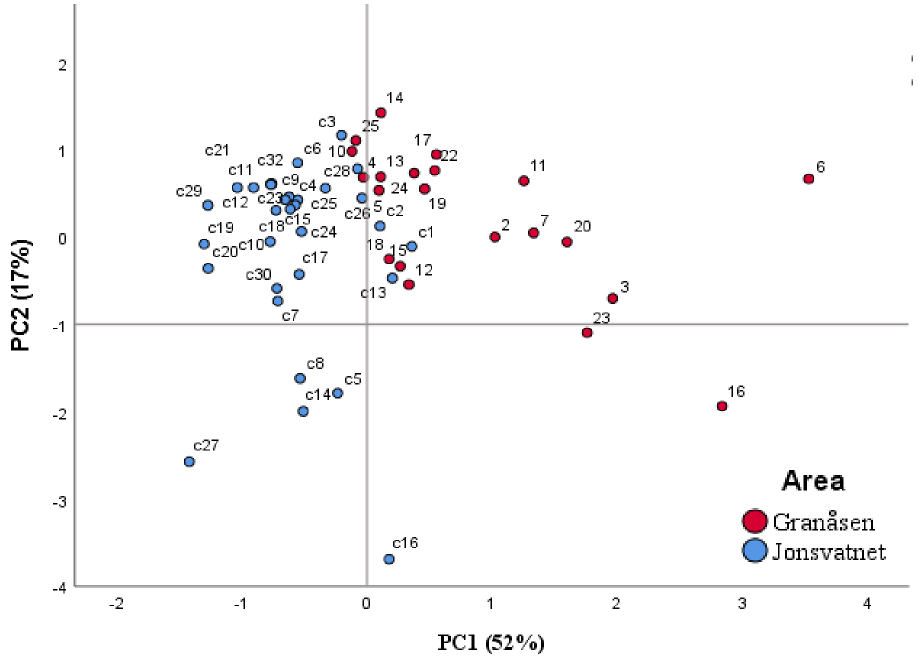

Figure 8. Score plot for the first two principal components (PCs), small rodents sampled in Granåsen are represented with red dots and small rodents from Jonsvatnet with blue dots (n = 24 in Granåsen and n = 32 in Jonsvatnet, June 2017).

The PCA score plot (Figure 8) indicates how the different individuals are related to each other when comparing the concentrations of PFASs. The PCA score plot showed a partial separation of the individuals according to sampling areas. Individuals from Granåsen are more spread in the graph in both axis, but mainly through PC1, while individuals from Jonsvatnet are less spread and they are distributed primarily along the PC2. Most individuals from Jonsvatnet are correlated with PFHpA and PFHxA, even though we find some exceptions of individuals distributed in the negative axis of PC2, thus being dominated by PFOS and PFNA. Specimens from Granåsen instead, are characterised by different concentrations of the long chained carboxylic acids.

Table 7. Results of the Pearson's correlation test among the log-transformed concentration of PFASs analysed in individuals from Granåsen (n = 24, June 2017). * Indicates significance P < 0.005 (two-tailed), ** indicates significance P < 0.001 (two-tailed).

	PFHxA	PFDA	PFDoDA	PFTrDA	PFBS	PFHxS
PFDA	0.088					
PFDoDA	-0.175	**0.586****				
PFTrDA	0.053	0.38	**0.770****			
PFBS	0.348	0.238	0.108	-0.103		
PFHxS	**0.557****	0.059	-0.266	-0.281	0.329	
PFOS	-0.13	**0.619****	**0.516****	0.367	0.393	-0.056

The Pearson's correlation among the PFAS chemical congeners in Granåsen is summarized in table 7. PFOS is strongly and positively correlated with the congeners PFDA and PFDoDA. One can see that the acids PFHxA and PFHxS are positively correlated with each other and not with other congeners. PFDoDA and PFTrDA are strongly positively correlated with each other, as the PCA (Figure 7) shows. The congener PFBS, which is the one with the lowest concentration, is not correlated with any other chemical. The chemicals PFNA, PFUdA and PFTeDA could not be included in this correlation because the concentrations did not follow a normal distribution. In the Pearson's correlation test made with the chemicals found in small rodents inhabiting Jonsvatnet (results not shown), the two chemicals with higher concentrations in Jonvatnet than in Granåsen, PFHxA and PFHpA, are strongly and positively correlated with each other ($r^2 = 0{,}719$, $p < 0.01$). PFOS, PFDA and PFDoDA could not be included in this analysis due to the non-normal distribution of the data.

ANCOVA was performed to study the possible effects of the different variables on the PFASs concentrations. Since some chemicals concentrations did not follow a normal distribution after log-transformed, the nonparametric analysis Kruskal-Wallis test and the Spearman's rank correlation coefficient were used.

Several variables were included in the analyses: weight of the individuals, total length, age (both in days and age class) and gender. The created variables called 'station' and 'winter were also added in the analyses. Differences between species could not be analysed since few individuals of other species than bank vole were captured. The results of these analyses are presented in the appendix D (tables 6 and 7) for individuals from Granåsen and appendix E (tables 8 and 9) for individuals from Jonsvatnet.

Generally, the variables included in the ANCOVA showed no significant effect on the PFASs concentrations. Nonetheless, there are some exceptions, as the concentration of PFOS in specimens from Jonsvatnet, which is positively correlated with age (in days $P = 0.848$; $p = 0.007$; and age class $P = 0,468$; $p = 0.009$), weight ($P = 0.58$; $p = 0.001$) and total length ($P = 0,429$; $p = 0,018$). Individuals born before winter showed statistically higher PFOS concentrations compared to individuals born after winter ($H = 3.02$; $p = 0.002$). Weight and length were correlated with age (for weight: $Z = 0.901$, $p < 0.001$; for length: $Z = 0.631$, $p < 0.001$). The loading plot (Figure 9) indicates how the variables PFOS, weight of individuals, length and age (in days and age class) are related to each other. The PC1 explains 76% of the variance: PFOS, the two age variables and weight of individuals are strongly and positively correlated to it. Length is, to a lesser extent, positively correlated to the PC2, which explains 10% of the variance. In addition, PFHxA showed statistically higher concentration in females than males in specimens from Granåsen ($F = 21.633$, $p = 0.000$) (appendix F, Figure 1).

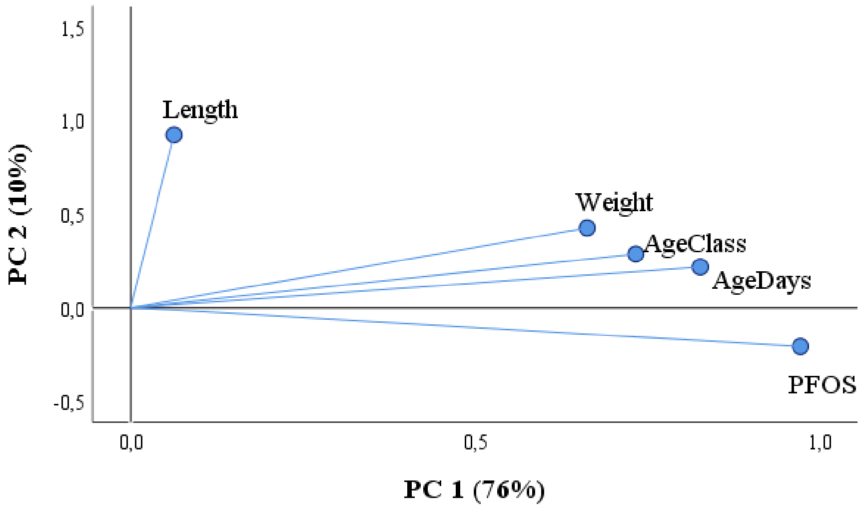

Figure 9. PCA loading plot for small rodents sampled in Jonsvatnet (n = 32) in June 2017. The PCA includes PFOS concentration, age in days, age class, weight and total length of the individuals (including the tail).

With the exception of the concentration of PFOS in rodents from Jonsvatnet, the concentration of the contaminants was not different between the individuals born before and after ski season (> 6 months and < 6 months respectively) (Figure 10). Nevertheless, the individuals older than 6 months sampled in Granåsen displayed varied sum PFAS concentrations, as is reflected with a large standard deviation in the figure 10.

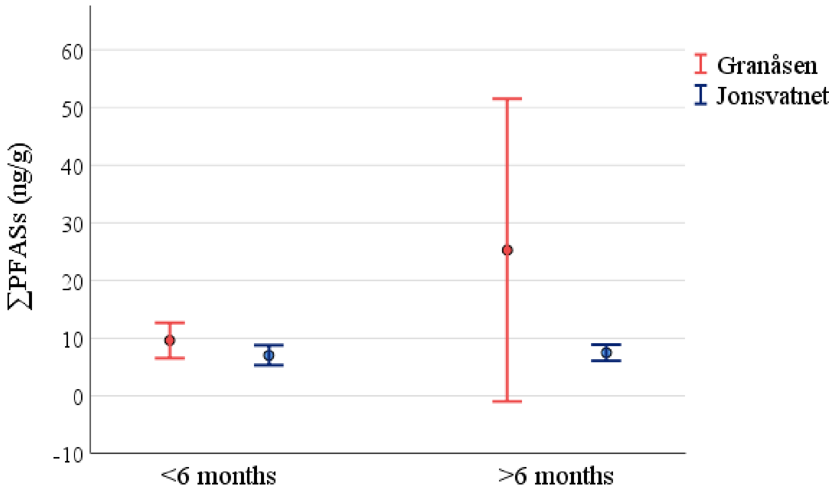

Figure 10. Average sum of PFASs (ng/g) for the individuals sampled in June 2017 grouped into two age classes, younger than 6 months (< 6 months) and older (> 6 months). Individuals younger than 6 months: n = 13 in Granåsen and n = 18 in Jonsvatnet. Individuals older than 6 months: n = 12 in Granåsen and n = 13 in Jonsvatnet.

3.4 Cytogenetic results

The outcome of all the processes involved in the isolation of lymphocytes and the preparation of the slides for scoring chromosomal aberrations was satisfactory in a small proportion of the total samples prepared. The lymphocytes separation and culture of cells succeeded in few individuals (15 out of 49), and the number of scorable metaphases finally obtained was also too low to be able to perform a statistical analysis of the data. An overview of the cytogenetic results is shown in table 8, indicating the number of individuals used for preparing samples, the samples obtained where the mitotic index could be analysed, the samples that had well-spread metaphases, and the total number of scorable metaphases (note that each sample comes from a different individual).

Table 8. Overview of the number of samples prepared, samples used for analysis of the mitotic index (MI), samples used for chromosomal aberrations (CA) and the total number of scorable metaphases.

Area	N samples prepared[1]	Samples for MI[1]	Samples for CA[1]	Scorable metaphases[2]
Granåsen	23	4	3	16
Jonsvatnet	26	11	8	69
Total	49	15	11	85

[1]Each sample belongs to a different individual
[2]Total scorable metaphases from all individuals

3.4.1 Mitotic Index

The mitotic index (MI) was calculated for each individual as the percentage of dividing lymphocytes out of the total number of lymphocytes. The MI differs considerably between individuals of the same samling area. In Granåsen the MI of the individuals (n = 4) varies from 0.5 - 8.5% and in Jonsvatnet (n = 12) from 1.76 - 21.08% (including an outlier) (see figure 11). The average MI of the individuals from Granåsen is 3.83 ± 3.0, slightly lower than in Jonsvatnet, which is 6.41 ± 4.9. The MI of the individuals is not statistically different between the two areas (Mann Whitney U-test, $z = 32$, $p = 0.379$, $n = 16$).

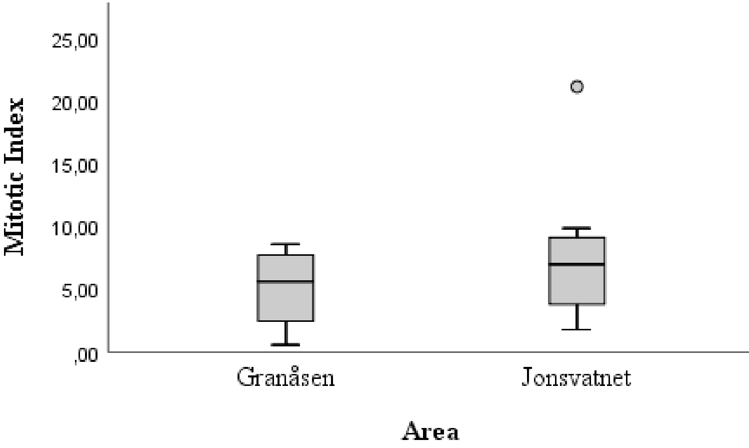

Figure 11. Boxplot showing the distribution of the mitotic index of all individuals from the two sampling areas. The medians for each area are marked in the boxes. The dot in the column of Jonsvatnet represents an outlier. The number of individuals for Granåsen is n = 4 and for Jonsvatnet, n = 12.

3.4.2 Mitotic cycle state

The distribution on the mitotic cycle of the cells in division is summarised in the table 9. Most of the mitotic cells, approximately 65%, are in metaphase; 12% in prophase, 2% in anaphase, and 4.7% in telophase.

Table 9. Total counted lymphocytes for all individuals, number of lymphocyte in division and which phase of the mitosis is presented.

	Individuals	Total lymphocytes	Lymphocytes in division	Prophase	Metaphase	Anaphase	Telophase
Granåsen	4	3257	125	6	105	2	12
Jonsvatnet	12	13707	879	121	547	19	35
Total	16	16964	1004	127	652	21	47

3.4.3 Chromosomal aberrations

The maximum number of cells in metaphase was scored for each individual. The results of the scoring of chromosomal aberrations include few individuals because high proportion of the slides could not be scored because they did not have lymphocytes on them, but cell debris instead. On the other hand, out of the total number of metaphase cells encountered, a high rate showed poor quality of the spread cells, where the chromosomes were either overlapping or too short and thick without distinguishable arms. In total, 85 metaphase cells could be scored, 16 from Granåsen specimens and 69 from Jonsvatnet, the number of metaphases scored for each individual ranged from 3-8 in Granåsen and 2-21 in Jonsvatnet.

An overview of the CAs scored is given in table 10. Out of 16 cells scored in individuals from Granåsen, 14 showed at least one aberration; for the population of Jonsvatnet, aberrations were detected in 27 cells out of 69. The most common aberration found was acentric fragments (n = 34), followed by chromatid breaks (n = 13). An extremely aberrant cell with several interchanges and intra-arm intrachanges was detected in an individual from Granåsen with ID 17 (marked in the table with **, Figure 12.A).

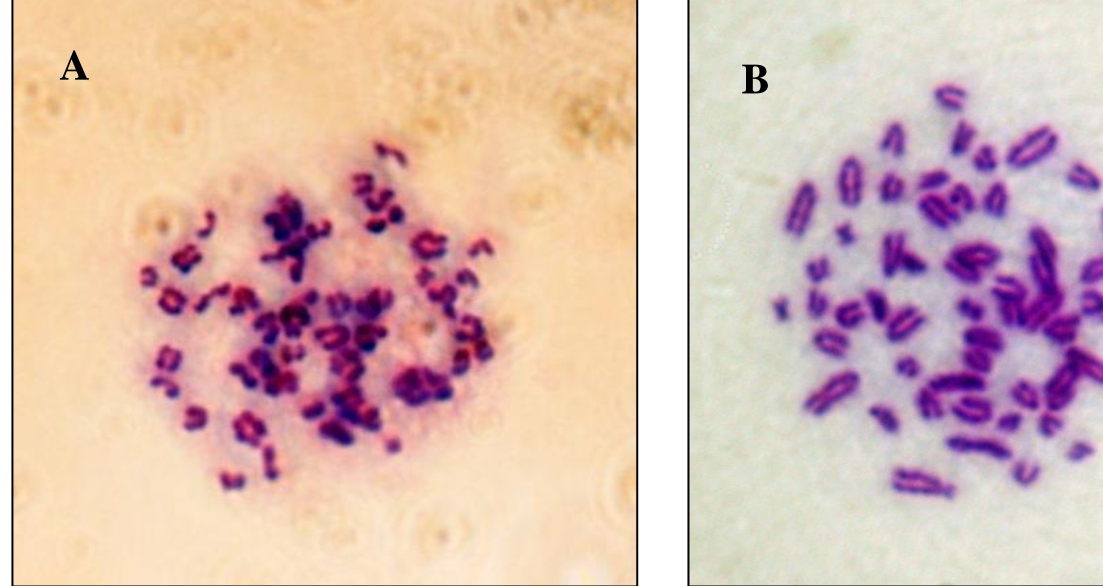

Figure 12. Comparison of an extremely aberrant cell with several interchanges and intra-arm intrachanges detected in an individual of *C. glareolus* sampled in Granåsen with ID 17 (A) with a non-aberrant cell of a *C. glareolus* from Jonsvatnet with ID C23 (B).

Table 10. Number of small rodents, Mitotic Index (M.I), number of metaphase cells scored, number of aberrations ecountered, and aberration type: acentric fragments (ac), rings (r), chromatid breaks (ctb), other kind of aberrations and multiple aberrations (mult.) are presented.

Area	ID	M.I	Metaph. Scored (n)	Aberrations (n)	Aberration type				
					ac	r	ctb	Other	Mult.
Granåsen	17	0.58	5	5	4		2		1**
	22	8.55	3	3	2	1			
	23	4.34	8	6	11	1		2	
Total	3	4.49	16	14	17	2	2	2	1
Jonsvatnet	C4	9.28	7	3	1		2		
	C6	7.65	9	2	1		2		
	C7	2.75	4	2	3				
	C12	4.51	2	0					
	C18	6.99	3	0					
	C23	4.45	12	7	8	2			
	C25	21.09	21	11	7	3	7		
	C26	1.76	11	2	1	1			
Total	8	7.31	69	27	21	6	11	0	0

Whether the damage was found in autosomal or sex chromosomes could not be determined given that the X chromosome in *C. glareolus* has similar size and shape as the first large acrocentric pairs of the karyotype.

The total number of metaphase cells were totalled for each sampling area and the percentage of cells with chromosomal aberrations was calculated (table 11). In Granåsen 81.82% of the cells in metaphase showed at least one kind of aberration while in Jonsvatnet the percentage was lower, where 39.13% of the cells had aberrations.

Table 11. Number of individuals, Mitotic Index (M.I) and percent of damaged cells in metaphase of the total metaphase cells scored for the individuals of each sampling location.

	Number of individuals	**M.I**	**Percent damaged metaphases**
Granåsen	3	3.8 ± 3.0	81.8
Jonsvatnet	8	6.41 ± 4.9	39.1

4 Discussion

4.1 Animals captured

Small rodents were captured in Granåsen ski centre and in the woods next to the lake Jonsvatnet. The bank vole (*C. glareolus*) was the predominant species trapped, representing 96% and 80% of individuals caught in Jonsvatnet and Granåsen respectively. *C. glareolus* has a wide geographical distribution in Europe and adjusts easily to habitats of different characteristics (Hansson 1985, Johannesen and Mauritzen, 1999). The predominance of this species in both sampling areas could therefore be explained by their ability to adapt, which benefits them in comparison to other species of wild small rodents.

The catching success was considerably higher in Jonsvatnet compared to Granåsen (6.9% and 1.23%), indicating that the population of small rodents in Jonsvatnet might have a higher density. The woods of Granåsen are scattered by several ski tracks frequented by hikers and skiers; the woods of Jonsvatnet instead, are less frequented. The woods of Jonsvatnet are dense, and practically all the soil is covered with shrubs, which offers protection against predation and creates micro-climatic conditions that benefit small rodents during winter. Moreover, *Vaccinium* spp., which constitutes a substantial part of the diet of the bank vole in Northern Fennoscandia (Hansson, 1985), is especially abundant in the woods of Jonsvatnet.

The females of *C. glareolus* show territoriality, where each mature female establishes a certain range and these ranges do not overlap with other mature females. On the other hand, males do share the same territory and show a type of social hierarchy (Bujalska, 1970; Wiger, 1979). Concurring with this social organisation, in our findings male gender predominated over females; thus, in the sampled areas, several male individuals cohabit with fewer females.

Considering that the lifespan of *C. glareolus* is 18 months (Torre and Arrizabalaga, 2008), the individuals found in both sampling areas were generally young. 33 of the specimens caught were born after winter 2017, so their age ranged from <1 to 6 months old. This fact could partly explain their low weight. The other 22 specimens sampled were born before winter, being from 6 to 8 months old.

Since regions with long and snowy winters do not offer much food, small rodents adapt by lowering their daily food requirement, experiencing a decrease in their body weight during the coldest

months (Wiger, 1979). In summer, the bank vole can achieve a weight of over 30 g (Raczynski, 1983). In the present study, sampling was done in early summer, when the weather in Trondheim was still cold, with an average temperature of 12.5 °C in June 2017 (Norwegian Meteorological Institute, 2018). Due to the cold conditions, the individuals sampled were not large, moreover, animals were probably in poor condition given that during the cold season the thermogenic energy requirements are high and food is scarce. Probably starting the sampling in the height of summer would have resulted in a higher catching success of individuals of larger size and better health condition.

4. 2 Chemical analysis

4.2.1 Granåsen

The concentration of PFASs was statistically higher for the congeners PFOS, PFDA, PFUdA, PFDoDA, PFTrDA, PFTeDA, PFBS and PFHxS in small rodents from Granåsen compared to Jonsvatnet, the area chosen as a reference site. Higher contamination levels of PFASs was expected in Granåsen (H1) because of the skiing activities that take place in the area (Trondheim Kommune, 2016). Our findings are in concurrence with several other studies (e.g. Plasmann and Berger, 2003; Herzke et al., 2015; Chropenová et al., 2016) that have reported high PFASs concentrations nearby ski areas.

Kotthoff et al. (2015) studied the presence of PFASs in consumer products. 13 ski waxes were included in the study and showed to contain the same congeners that were found in the small rodents sampled in Granåsen. High concentrations, over 100 µg/kg, were reached in these products. PFHpA and PFBA, chemicals not detected over the LOD in our study area, were additionally found. Figure 14 includes the data presented by Kotthoff et al. (2015) and our data, comparing the contribution of the PFAS congeners analysed to the total median concentration of PFASs. The pattern of contribution of each PFAS congener in ski waxes (Kotthoff et al., 2015) differs slightly from the pattern found in small rodents sampled in Granåsen. The main differences rely on PFOS, which represents one of the main contributors in small rodents sampled in Granåsen while in ski waxes has the lowest contribution; and PFOA, which is a high contributor in ski waxes but not in small rodents.

PFOS usage has been restricted since 2009 (Stockholm Convention, 2009), but their persistence makes them present in the environment at certainly high concentrations (Haukås et al., 2007). The high PFOS levels in small rodents from Granåsen reflect the old use of ski products, which used to contain this chemical compound. Regarding PFOA, restrictions were made under REACH legislation in Europe in 2017 (European Comission, 2017), while in Norway were made in 2014. Since the study of Kotthoff et al. (2015) is connected to German institutions (therefore we assume that the ski waxes analysed were produced in Germany) in 2015, restrictions were not in force and ski waxes produced in the country could contain PFOA.

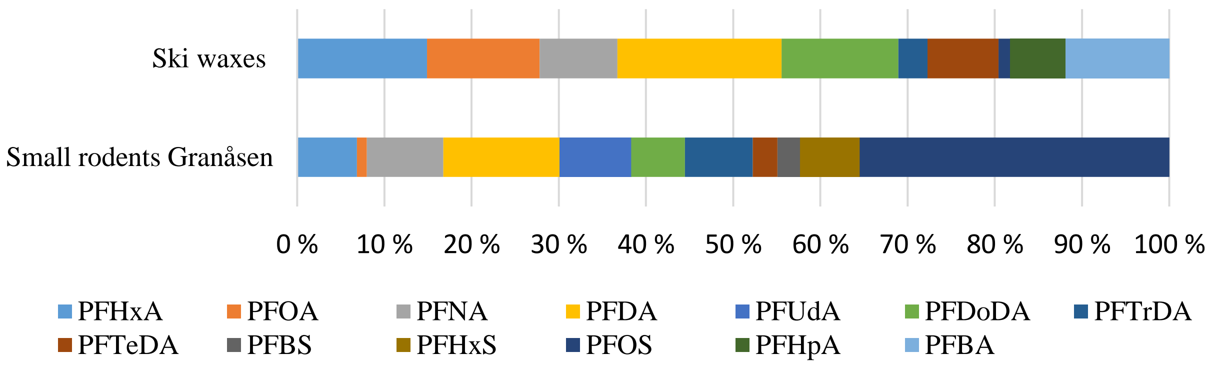

Figure 13. Comparison of the contribution each PFAS congener to the total median concentration of PFASs in ski waxes analysed by Kotthoff et al., (2015) and in liver of small rodents sampled in Granåsen.

NILU/NINA made a report on environmental contaminants in terrestrial and urban environment (Herzke et al., 2015). High PFAS levels were reported in earthworms (∑PFAS 34.85 ng/g ww, standard deviation not indicated) from a skiing area in Oslo, Voksenkollen, compared to a reference site; and follow-up sampling in skiing areas was recommended. In the present study, the small rodents sampled in the skiing area displayed lower average sum of PFASs (17.08 ± 28.71 ng/g ww) compared to earthworms from Voksenkollen. Small rodents and earthworms share a low trophic level in the food web, nonetheless, earthworms are more exposed to PFASs because they interact with chemicals in the soil by direct dermal contact and ingestion of soil (Lanno et al., 2004). Red foxes were also included in the NILU/NINA report, and showed a lower average sum of PFASs than small rodents from Granåsen (∑PFAS 3.76ng/g ww, standard deviation not indicated). The differences in ∑PFAS that these species display might reflect particular exposure levels and interspecific toxicokinetic differences.

The present study, altogether with the studies above mentioned, shows that ski wax usage poses a local source of almost all congeners integrated in the PFAS family. The production of ski waxes is estimated to be, at a global scale, several tonnes per year and even though the application of ski waxes is not the main source of PFASs to the environment, it contributes considerably to the contamination at a local scale (Plassmann and Berger, 2013). In addition to this, outdoor waterproof clothing may pose a supplementary source of perfluorinated compounds in skiing areas (Lu et al., 2014).

In accordance with expectations (H2), the average concentration of PFOS in the small rodents livers was the highest among all PFAS congeners detected for both sampling areas. This is in concurrence with previous studies on the exposure of PFASs on wild animals, where PFOS occurred at highest concentrations compared to other PFAS congeners (Falk et al., 2012; Norwegian Environmental Agency, 2013; Herzke et al., 2015). PFOS have also exhibited considerably high concentrations in farm animals (Zafeiraki et al., 2016) and in humans Brantsæter et al. (2013), found that the median plasma concentration of PFOS in Norwegian pregnant women was 12.8ng/ml, exhibiting the highest concentrations of the PFASs considered in the study.

In 2000, the Environmental Protection Agency of the United States of America (EPA) negotiated for the phase-out of PFOS with 3M, the main global producer company of this compound. 3M announced that they would voluntarily stop the production of PFOS and seek for other substitute chemicals (EPA, 2000). In 2009, PFOS and related compounds were listed as POPs under the Annex B of the Stockholm Convention and their use got restricted to certain applications (Stockholm Convention, 2009). PFOS have been produced in Asia since 2000, but generally they have been displaced by other fluorinated compounds with different carbon chain lengths (Wang et al., 2017). Thus, the global production of PFOS has been decreasing since the beginning of the century. Nevertheless, under environmental conditions, PFOS does not degrade appreciably due to the strength of the carbon-fluorine bonds (Wang et al., 2015). Moreover, it has been suggested that some precursors such as perfluorooctanesulfonyl fluoride (POSF) based compounds (Xu et al., 2004) and perfluorooctane sulfonamido alcohols and acrylates (OECD, 2002) degrade to PFOS. These facts, together with the possibility of skiers using old ski waxes containing PFOS, could explain why the environmental levels of this compound are still high despite the global manufacture reduction.

Concentrations of PFOS in the liver of wood small rodents (*Apodemus sylvaticus*) in the vicinity of a fluorochemical plant in Blokkersdijk (Belgium) were up to 178.55 µg/g wet weight (Hoff et al., 2004), becoming the highest concentration of PFOS ever reported in wild animals (Harrad, 2010). Another study made few years after on the same species in the same area reported lower average concentrations (ranging from 787 to 22355 ng/g ww), but they still constituted, with exception of the ones found by Hoff et al. (2004), the highest ever recorded (D'Hollander et al., 2014). PFOS exposure levels found in Blokkersdijk are several orders of magnitude higher than the levels found in our study, ranging from 0.55 to 16.04 ng/g ww.

The Canadian Environmental Protection Act (1999) set the liver PFOS critical toxicity value at 14.4 µg/g, based on laboratory studies in rats. Hoff et al. (2004), extrapolated the environmental toxicity value for mammals at 0.144 µg/g. In the present study, the concentrations of PFOS in wild rodents did not exceed this value in any specimen (the maximum PFOS concentration found was 0.016 µg/g). Accordingly, the liver levels of PFOS detected in the population of Granåsen might not suppose a toxicological risk by themselves, but we must consider that specimens are subjected to a complex mixture of the whole spectrum of PFASs, in which PFOS only represents about 30% (Figure 12).

While most of the research has focused on effects of single compounds, targeting specially PFOS, several hundreds of other per- and polyfluorinated compounds are currently in use (Lindstrom et al., 2011; Krafft and Riess, 2015) and the potetntial toxicological effects of PFASs considered as a mixture are generally unknown (Wang et al., 2017).

PFOA, together with PFOS, is frequently detected as predominant PFAAs in the environment (Wang et al., 2014).This compound was not detected above LOD in more than 60% of the samples from Granåsen and Jonsvatnet. These results might indicate that the ban of PFOA in consumer products in Norway (Miljødirektoratet, 2013 June 28) effectively reduced the release of this chemical in the Norwegian environment. None of the sulfonamides were found above LOD in the sampling areas. Since these compounds are not included in other studies related to ski waxes usage (Kotthoff et al., 2015; Nilsson et al., 2010; Herzke et al., 2015), we hypothesise that they are not used in the production of ski products.

The longed-chain carbon chemicals PFDoDA (C12), PFTrDA (C13), and PFTeDA (C14) were found in high concentrations in Granåsen. PFDA (C10) and PFUdA (C11) were, to a lesser extent, also detected at high concentrations. The Pearson's correlation and the PCA (Table 7 and Figure 7) showed that these chemicals are strongly correlated with each other, indicating a common source. These compounds are present in ski waxes (Kotthoff et al., 2015) and have been detected in the serum of professional ski waxers at higher concentrations than the general population in Norway (Ferberg et al., 2010).

The PFASs with shorter chain lengths, PFBS (C4), PFHxS (C6), PFHxA (C6) and PFNA (C9), were found at lower concentrations than PFASs with longer C-chains. Generally, increasing concentrations of PFASs congeners with increasing C-chain length could be observed in individuals from Granåsen. This could be explained by two different facts, the first is that PFASs with shorter C-chains are excreted easier and therefore have shorter half-lives than the longer ones (Brendel et al., 2018). The second, is that PFASs with longer chain (C10-17) have low water solubility and sorbe in the soil from snow, while C6-9 PFASs have higher water solubility and are washed out the surface layer of snow with rain and melt water (Plassmann and Berger, 2013).

For most of the specimens sampled in Granåsen, the chemical burden was not correlated with any of the variables recorded: age, weight, sex or station (results presented in the appendices D and E). Since some studies have found that PFOS and other long-chained PFASs are able to bioaccumulate (Krafft and Riess, 2015; Hoff et al., 2004; Valdersnes et al. 2017), higher concentrations were expected in older individuals (H3). Individuals from Granåsen born before the ski season (>6 months old) showed a higher sum PFASs average compared to the younger individuals (<6 months) (Figure 9). Nevertheless, the chemical concentrations varied greatly between older individuals, especially for the compounds PFOS, PFDoDA, PFTrDA and PFTeDA (appendix A, table 1 and appendix B, table 3). Differences in food intake, together with different excretion rates between individuals might be the main traits influencing the concentration patterns of PFASs in individuals from Granåsen. Plasmann and Berger (2013) investigated the presence of PFCAs in a ski track after a skiing competition and found that the pattern of the chemical's concentration changed along the route. Higher concentrations of PFCAs with longer chain were detected at the start of route. Similarly, different concentrations of PFASs might occur in Granåsen, therefore individuals might be exposed to different concentrations depending on their diet, even sharing the same habitat.

Further studies are needed to understand individual differences in PFASs exposure and absorption through food intake, as well as intraspecific differences in excretion rates.

In the present study, females from Granåsen displayed higher concentrations of PFHxA compared to males. The rate of urinary excretion and half-life of PFHxA have been studied in rats under laboratory conditions, finding no difference between genders (Chengelis et al., 2009). On the other hand, gender differences in PFAAs clearance have been observed in rats, where females showed 20 to 50 times shorter half-lives of PFNA and PFOA. These results are not in accordance with our findings in Granåsen, where males seem to have a higher clearance rate of PFHxA.

4.2.2 Jonsvatnet

PFOS showed the highest concentration of all PFASs analysed in Jonsvatnet, followed by PFHxA, PFNA and PFHpA (figure 5). All these compounds are used in the production of ski waxes (Nilsson et al., 2010; Kotthoff et al., 2015) but to the writer's knowledge, skiing activities do not take place in the reference area. PFOS is a widespread contaminant and has been found in urbanized and remote locations (Giesy and Kannan, 2001), our findings support thus its ubiquity. The concentrations of PFOS and PFNA could be explained by the theory discussed by Armitage et al. (2009), in which long-range transportation of precursor volatile compounds such as FTOHs, together with their atmospheric oxidation to PFAS compounds, contribute to the ubiquity of some PFAS chemicals.

Unexpectedly, PFHxA and PFHpA showed statistically higher concentrations in small rodents from Jonsvatnet than in Granåsen. Experimental studies with mammalian specimens showed a fast elimination of PFHxA (Chengelis et al., 2009; Gannon et al., 2011) and PFHpA (Ohmori et al., 2003) after exposure. Thus, we estimate that these chemicals were released to the reference area shortly before the sampling took place. High concentration levels of PFHpA and PFHxA could also be explained by toxicokinetic processes, since these are two of the major metabolites of FTOH biotransformation in rats and small rodents.

In contrast to Granåsen, the results of the statistical analyses showed that PFOS was correlated with age, weight and length of the small rodents sampled in Jonsvatnet. A plausible explanation of the differences between the two sampling areas could be the distribution of PFOS, we hypothesise that this chemical is spread evenly in Jonsvatnet as a result of long-range transportation. Thus, small

rodents in Jonsvatnet are exposed to similar levels of pollution regardless of their food intake and since PFOS are able to bioaccumulate (Hoff et al., 2004), older individuals have higher concentrations. In Granåsen, bioaccumulation was not observed due to the uneven distribution of PFOS in the area. The PCA showed that PFOS was also correlated with weight of individuals sampled in Jonsvatnet, and this is because older individuals are usually heavier than the young ones. Regarding length, the PCA does not correlate it with the other variables, and most likely due to the variable length included the tail, which varies from individual to individual of similar age and weight.

4.3 Cytogenetic results

4.3.1 Chromosomal aberrations

Relatively few studies assessing the genotoxic effects of PFASs have been published, in addition, conflicting results have been reported. Liu et al. (2014) found that exposure to PFNA, PFOS, PFOA and PFDA damaged the genetic material of mussels; observing DNA strand breaks and fragmentation, chromosomal breaks and apoptosis. Wilsøe et al. (2015) tested several long-chain PFASs on hepatome cell line and found DNA damage and increased ROS production. On the other hand, EFSA (2008) classified PFOS and PFOA as non-genotoxic based on the results of some *in vitro* and *in vivo* studies (Litton Bionetics, Inc., 1978; Corning Hazleton, Inc., 1993; Mecchi, 1999; Cifone, 1999).

Given that PFASs might have genotoxic properties, the present study intended to evaluate whether the small rodents inhabiting a PFASs *hotspot* area had higher number of chromosomal aberrations compared to a reference area. Some metaphase cells were evaluated and chromosomal aberrations were encountered, nevertheless, the number of successful slides prepared was too low to be able to compare the individuals from the two sampling areas.

Blood samples were taken from 49 small rodents found alive in the two sampling areas and lymphocytes were separated and cultured. Nevertheless, only slides from 16 individuals could be used for scoring the mitotic index, and out of them, only slides from 11 individuals (n = 3 from Granåsen and n = 8 from Jonsvatnet) could be used for studying the chromosomal aberrations. For the proper comparison of the chromosomal aberrations held by individuals, at least 100 cells in metaphase must be analysed for each animal (OECD, 1997); in the current study, the maximum number of metaphase cells scored for one individual was 21 (table 10), far less than the

precondition for this method. To see if the obtained results could indicate any trend in the two populations, the total number of cells in metaphase was pooled for each population of small rodents. The percentage of cells in metaphase with at least one kind of aberration was lower for Jonsvatnet (39%) compared to Granåsen (81%). These results might indicate a higher level of CAs in the population of small rodents from Granåsen, nevertheless, the results are very limited and further studies should be performed in orther to clarify whether the populations of wild animals suffer from CAs due to environmental concentrations of PFASs.

4.3.2 Choice of methodology and successfulness

The most important prerequisite for cytogenetic studies of mitotic cells is to get well spread cells in metaphase with the least number of overlapping chromosomes possible (Akeson and Davisson, 2000). In the present study, few well spread metaphases were obtained, and this precluded the aim of comparing the chromosomal aberrations held by individuals from the two different populations.

First of all, there are many factors that might influence the results of the separation and culture of cells, such as pH, cell content, volume, osmolality, and dilution of the sample (Denman, 1973; Blaxhall, 1981). An obstacle in the present study was the volume of blood sampled from each individual, which was certainly small (ranging from 0.3 ml to 1 ml) due to the small size of the animals. Considering that white blood cells represent about 1% of the volume of the blood (Alberts et al., 2002), most likely the initial volume of lymphocytes was not adequate for the culture of the cells. In addition, the protocol followed for separating the lymphocytes of the whole blood was made for higher initial volumes of blood, therefore the relation between the solutions was not optimal and probably not all the lymphocytes could be separated.

Separation of cells by density gradient (Bøyum, 1968) is a widely used technique that relies on the physical characteristics of the cells such as size and density. These characteristics determine the sedimentation rate that the cells will experience during centrifugation, resulting in differential migration and the subsequent formation of layers containing different cell types (Bøyum et al., 2002). Erythrocytes and granulocytes are the densest cells and end up forming the bottom layer, below the density barrier layer. Lymphocytes, monocytes and platelets have too low density for penetrating the density barrier and therefore end up in the layer above it. The uppermost layer is plasma (Bøyum, 1968).

Lysis methods that eliminate erythrocytes are also used for leukocyte enrichment. This method has a higher production of leukocytes and is quicker than the gradient separation method. On the other hand, the density gradient method has its advantages, as it removes most granulocytes from the sample, which often comprise over 60% of the leukocytes in peripheral blood (Dagur and McCoy, 2015). Since the gradient separation method had been previously used in our laboratory, obtaining satisfactory results (Østby, 1998), this method was chosen over the lysis method. Nonetheless, in the present study a new product was used as density barrier (OptiPrep) due to the cessation of the production of NycoPrep, the solution used as density barrier in the study by Østby (1998).

During the separation of the lymphocytes process, there are other factors that might lead to low viability of the desired cells in the final sample. Examples are the presence of cytotoxic agents that are sometimes used as preservatives in the chemical reagents, harsh treatment of the cells or inappropiate temperature (Dagur and McCoy, 2015).

As a general rule, it is better to disregard a cell in metaphase where the chromosomes cannot be seen clearly than counting it as a cell without aberrations because it raises the risk of error (Brøgger et al., 1984). In many of our samples the spread metaphases could not be analysed due to poor quality of the spread of the cells, where the chromosomes were either overlapping or too short and thick without distinguishable arms. It is known that the inappropriate application of hypotonic treatment can lead to overlapping chromosomes (Brøgger et al., 1984), this could be the case because the hypotonic treatment used in this study was at room temperature instead of being warmed to 37 °C, as Howe et al. (2014) suggest in their publication. Other studies used hypotonic treatment at room temperature with successful results (Østby, 1998).

Cells were incubated for 3 and a half hours with Colcemid, which is a drug used to arrest the mitotic cycle in metaphase by interfering with the mitotic spindle (Liu and Tsai, 2017). The period of time of incubation with Colcemid affects the size and shape of the chromosomes: short time incubation makes long and thin chromosomes while with longer incubation time chromosomes get short and thick. In the protocol made by Howe et al. (2014), the incubation lasts 45 min, but in our study we employed 3 and a half hours and indeed, many chromosomes were too thick and short and did not have distinguishable arms. Nevertheless, other studies have used Colcemid or other chemicals for arresting mitotic cycle as demecolcine during 3 h, obtaining satisfactory results (Erexson and Kligerman, 1987; Østby, 1998).

5 Conclusions

The present study reports higher levels of PFASs in wild small rodents inhabiting a skiing area (Granåsen), compared to a reference area (Jonsvatnet), confirming that the usage of ski products with PFAS chemicals causes contamination in skiing areas.

PFOS was the most predominant PFAS in the two sampling areas, as expected from other studies in wildlife. In addition, the chemical congeners with longer C-chain showed higher concentrations, most likely due to their longer half-life in comparison to PFASs with shorter C-chains, and due to their low water solubility, which makes them sorbe in the soil from the snow.

PFHxA and PFHpA were found at higher concentrations in Jonsvatnet, these chemicals were possibly related to a local and recent source. PFNA and PFOS levels were also high in Jonsvatnet, most likely as a consequence of long-range transportation of precursor compounds.

The ANCOVA test revealed that the concentration of the different chemicals analysed was not related to age, weight or gender of the individuals sampled in Granåsen. Contrarily, the concentration of PFOS was positively correlated with age in individuals from Jonsvatnet, supporting the publications about the bioaccumulative properties of this compound.

Chromosomal aberrations in lymphocytes were analysed in the individuals sampled. Differences in CAs at individual level could not be registered, but by pooling the metaphase cells for each population, we could observe a higher percentage of cells with aberrations in Granåsen (89 %) compared to the reference area (31 %). Nevertheless, few individuals could be included in the scoring and the total number of scorable metaphases obtained was too low to draw any conclusion. To gain more knowledge about the possible genotoxic effects exerted by PFASs, future studies should be performed on wild species, or on laboratory animals at environmentally relevant concentrations.

6 References

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Appendices

Appendix A. Biological data of the small rodents sampled

Table 1. Biological data of the small rodents sampled in Granåsen

ID	Species	Date found	Station nr	Sex	Total length (cm)	Tail length (cm)	Weight (g)	Dead/alive	Pregnant
1	*A. flavicollis*	30.05.2017	8	M	16.5	8	23.0	Alive	
2	*A. flavicollis*	31.05.2017	14	F	15	7	16.6	Alive	X
3	*C. glareolus*	03.06.2017	2	F	14	5.5	35.3	Alive	X
4	*C. glareolus*	05.06.2017	9	M	15	5.5	26.0	Alive	
5	*C. glareolus*	06.06.2017	3	F	10.2	3.5	11.9	Dead	
6	*A. flavicollis*	07.06.2017	3	M	12.5	4	19.7	Alive	
7	*C. glareolus*	08.06.2017	2	M	11	3.5	14.4	Alive	
8	*A. flavicollis*	08.06.2017	14	M	17	8.5	24.5	Alive	
9	*C. glareolus*	08.06.2017	1	M	14	5.5	23.4	Dead	
10	*C. glareolus*	13.06.2017	14	M	14	5	22.5	Alive	
11	*C. glareolus*	14.06.2017	9	F	12	4.5	17.5	Alive	
12	*C. glareolus*	14.06.2017	16	M	14	5	24.6	Alive	
13	*C. glareolus*	14.06.2017	15	M	14	5	23.8	Alive	
14	*C. glareolus*	14.06.2017	16	F	11	4	12.6	Dead	
15	*C. glareolus*	18.06.2017	5	M	14	4.5	25.5	Alive	
16	*C. glareolus*	18.06.2017	6	M	14	5	21.8	Alive	
17	*C. glareolus*	28.06.2017	3	M	12.5	4	15.9	Alive	
18	*C. glareolus*	29.06.2017	6	M	13	4	19.8	Alive	
19	*C. agrestis*	03.07.2017	1	M	12.5	4	15.2	Alive	
20	*C. glareolus*	03.07.2017	1	F	14	5	26.2	Alive	
21	*C. glareolus*	04.07.2017	9	M	14	4	15.4	Alive	
22	*C. glareolus*	04.07.2017	1	M	13	4	26.5	Alive	
23	*C. glareolus*	05.07.2017	1	M	12.5	4.5	17.4	Alive	
24	*C. glareolus*	06.07.2017	7	F	13.5	4.5	19.7	Alive	
25	*C. glareolus*	17.06.2017	6	M	14	4	19.6	Dead	

Table 2. Biological data of the small rodents sampled in Jonsvatnet.

ID	Station nr	Date found	Species	Sex	Total length (cm)	Tail length (cm)	Weight (g)	Dead/alive	Pregnant
C1	3	20.06.2017	*C. glareolus*	F	16.5	5.5	30.9	Alive	X
C2	5	20.06.2017	*C. glareolus*	M	15	5.5	25.0	Alive	
C3	14	20.06.2017	*C. glareolus*	M	10	4.5	11.3	Alive	
C4	15	20.06.2017	*C. glareolus*	F	15	5.5	29.4	Alive	
C5	6	20.06.2017	*C. glareolus*	M	14	4.5	29.0	Alive	
C6	1	21.06.2017	*C. glareolus*	M	11.5	3.5	15.9	Alive	
C7	5	21.06.2017	*C. glareolus*	M	14	5	26.7	Alive	
C8	5	21.06.2017	*C. glareolus*	M	15	5	31.5	Alive	
C9	11	21.06.2017	*C. glareolus*	M	13	5	27.0	Alive	
C10	15	21.06.2017	*C. glareolus*	M	11.5	4	15.8	Alive	
C11	5	22.06.2017	*C. glareolus*	M	13	4	18.5	Alive	
C12	4	22.06.2017	*C. glareolus*	F	12.5	4	19.4	Alive	X
C13	13	23.06.2017	*C. glareolus*	F	15	5.5	33.6	Alive	X
C14	9	23.06.2017	*C. glareolus*	M	14.5	4.5	28.9	Alive	
C15	16	23.06.2017	*C. glareolus*	M	10.5	4	14.0	Alive	
C16	10	23.06.2017	*C. glareolus*	F	12	4.5	17.3	Alive	
C17	7	26.06.2017	*C. glareolus*	M	10	2.5	27.4	Alive	
C18	4	26.06.2017	*C. glareolus*	M	12	4	17.0	Alive	
C19	15	26.06.2017	*C. glareolus*	M	13.5	4.5	24.7	Alive	
C20	3	26.06.2017	*C. glareolus*	M	12	4	15.0	Alive	
C21	11	26.06.2017	*C. glareolus*	F	12.5	4.5	16.9	Alive	
C22	10	26.06.2017	*C. glareolus*	M	13.5	4.5	21.9	Alive	
C23	10	26.06.2017	*C. glareolus*	F	11.5	4	16.9	Alive	
C24	15	26.06.2017	*C. glareolus*	M	12	4	19.7	Alive	
C25	15	26.06.2017	*C. glareolus*	F	11	4	18.7	Alive	
C26	8	26.06.2017	*A. sylvaticus*	M	14	7	15.7	Alive	
C27	12	21.06.2017	*C. glareolus*	M	14	5	23.9	Dead	
C28	1	22.06.2017	*C. glareolus*	F	14	4	25.2	Dead	
C29	2	21.06.2017	*C. glareolus*	M	13.5	5	23.2	Dead	
C30	10	20.06.2017	*C. glareolus*	M	14	5	25.4	Dead	
C31	10	21.06.2017	*C. glareolus*	F	11	4	18.9	Dead	
C32	8	21.06.2017	*C. glareolus*	F	14	6	24.6	Dead	

Appendix B. Results chemical analysis by HPLC-MS

Table 3. Concentrations (ng/g ww) of PFAS contaminants in liver of small rodents from Granåsen (note that the table is split into two pages). <LOD = Below limit of detection; n.d. = not detected.

ID	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoDA	PFTrDA
LOD	0.25	0.11	0.32	0.14	0.20	0.41	0.18	0.26
1	0.88	n.d.	1.47	7.15	11.29	13.59	30.40	31.52
2	0.84	n.d.	0.35	0.48	0.65	1.63	1.03	2.45
3	0.49	<LOD	n.d.	<LOD	2.00	1.37	0.48	0.47
4	<LOD	0.12	<LOD	0.34	<LOD	<LOD	0.26	<LOD
5	1.30	n.d.	0.52	0.68	0.98	0.59	0.35	0.61
6	0.59	n.d.	0.63	1.18	5.05	2.12	3.00	1.32
7	0.70	<LOD	n.d.	0.87	1.48	1.09	1.10	1.20
8	<LOD	n.d.	0.59	1.41	2.01	2.87	7.36	6.50
9	0.38	0.25	0.46	2.21	6.23	1.46	0.26	0.29
10	0.91	0.11	n.d.	<LOD	<LOD	<LOD	<LOD	0.30
11	0.88	<LOD	<LOD	0.31	2.01	1.13	0.92	0.84
12	0.50	n.d.	<LOD	0.74	0.99	0.54	<LOD	0.29
13	0.50	n.d.	n.d	0.50	0.55	0.62	0.19	0.29
14	0.84	0.22	n.d.	<LOD	0.36	<LOD	<LOD	0.57
15	<LOD	<LOD	0.37	0.36	0.35	0.44	0.18	0.41
16	0.32	<LOD	<LOD	1.31	1.79	2.15	2.39	1.87
17	<LOD	n.d.	n.d.	0.76	1.26	0.52	0.39	<LOD
18	0.72	n.d.	n.d.	0.64	0.83	<LOD	0.28	<LOD
19	0.32	n.d.	<LOD	0.47	0.74	0.60	0.46	0.51
20	1.07	n.d.	n.d.	1.31	2.13	1.26	0.98	0.76
21	0.69	n.d.	<LOD	0.30	0.29	<LOD	0.45	0.63
22	0.27	n.d.	n.d.	0.54	0.67	0.58	0.66	0.63
23	<LOD	n.d.	<LOD	0.84	2.47	1.17	0.96	0.61
24	1.07	0.14	n.d.	0.84	0.91	0.58	0.24	0.54
25	<LOD	0.20	0.50	0.64	0.31	<LOD	0.20	0.37

ID	PFTeDA	PFBA	PFBS	PFHxS	PFOS	FOSA	N-MeFOSA	N-EtFOSA	N-MeFOSE	N-EtFOSE
LOD	0.20	0.56	0.07	0.22	0.79	0.05	0.17	0.23	0.85	0.20
1	48.37	n.d	0.12	<LOD	5.83	0.16	n.d	<LOD	<LOD	n.d
2	0.60	<LOD	<LOD	<LOD	2.61	0.09	<LOD	n.d	<LOD	n.d
3	<LOD	<LOD	0.19	2.91	4.98	n.d	n.d	n.d	<LOD	<LOD
4	<LOD	<LOD	0.30	0.37	1.29	<LOD	n.d	n.d	n.d	<LOD
5	<LOD	n.d	0.09	0.81	1.15	n.d	n.d	n.d	n.d	n.d
6	1.93	n.d	0.57	0.37	2.30	n.d	n.d	n.d	4.09	n.d
7	1.14	n.d	0.17	0.86	2.65	<LOD	n.d	n.d	<LOD	<LOD
8	5.09	n.d	0.17	<LOD	9.17	2.07	n.d	n.d	n.d	n.d
9	<LOD	n.d	0.44	3.99	16.04	<LOD	n.d	<LOD	1.55	<LOD
10	<LOD	n.d	0.21	1.32	0.89	0.05	n.d	n.d	n.d	n.d
11	0.42	n.d	0.40	1.13	1.85	<LOD	n.d	n.d	<LOD	<LOD
12	0.28	n.d	0.18	<LOD	3.05	n.d	n.d	n.d	<LOD	<LOD
13	<LOD	n.d	0.27	0.50	1.16	n.d	n.d	n.d	n.d	<LOD
14	<LOD	n.d	<LOD	1.93	<LOD	n.d	n.d	<LOD	n.d	n.d
15	<LOD	n.d	0.23	<LOD	3.02	n.d	n.d	n.d	n.d	<LOD
16	2.84	n.d	0.22	<LOD	6.40	n.d	n.d	n.d	<LOD	n.d
17	<LOD	n.d	<LOD	0.88	0.98	n.d	n.d	<LOD	<LOD	n.d
18	<LOD	n.d	0.35	0.52	2.60	n.d	n.d	n.d	1.59	n.d
19	0.73	n.d	<LOD	<LOD	1.56	n.d	n.d	n.d	n.d	n.d
20	0.48	n.d	0.37	2.69	2.94	n.d	n.d	n.d	n.d	<LOD
21	0.21	n.d	0.23	6.24	3.45	<LOD	n.d	n.d	n.d	n.d
22	0.82	n.d	<LOD	<LOD	1.28	<LOD	n.d	n.d	n.d	<LOD
23	0.61	n.d	<LOD	0.31	4.68	<LOD	n.d	n.d	n.d	n.d
24	<LOD	n.d	0.34	2.13	1.21	n.d	n.d	n.d	<LOD	0.21
25	<LOD	n.d	<LOD	0.39	<LOD	n.d	n.d	n.d	<LOD	<LOD

Table 4. Concentrations (ng/g ww) of PFAS contaminants in liver of small rodents from Jonsvatnet (note that the table is split into two pages). <LOD = Below limit of detection; n.d. = not detected.

ID	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoDA	PFTrDA	PFTeDA
LOD	0.31	0.21	0.15	0.17	0.18	0.27	0.17	0.40	0.33
c1	0.59	0.08	<LOD	0.23	<LOD	0.66	0.25	0.79	<LOD
c2	<LOD	0.08	<LOD	1.00	0.36	0.68	0.23	<LOD	n.d
c3	1.61	0.35	<LOD	0.20	0.24	0.46	0.22	<LOD	n.d
c4	1.38	0.63	<LOD	0.25	0.18	0.36	0.20	0.40	n.d
c5	1.40	0.51	0.54	1.36	0.73	0.70	0.23	<LOD	n.d
c6	1.47	0.48	<LOD	<LOD	0.32	0.33	0.18	0.58	n.d
c7	2.00	0.46	0.16	0.60	0.34	0.46	0.21	<LOD	<LOD
c8	2.06	0.51	0.23	0.96	0.61	0.59	0.18	0.45	n.d
c9	0.94	0.60	<LOD	0.49	<LOD	0.30	0.20	<LOD	n.d
c10	1.80	0.82	0.17	<LOD	0.38	0.48	<LOD	<LOD	n.d
c11	2.28	0.60	<LOD	<LOD	0.99	<LOD	0.19	<LOD	n.d
c12	1.58	0.42	0.17	0.61	<LOD	0.24	<LOD	<LOD	n.d
c13	0.91	0.32	<LOD	0.39	0.73	0.89	0.25	<LOD	<LOD
c14	1.92	0.83	0.17	0.97	0.52	0.53	0.33	<LOD	<LOD
c15	1.09	0.79	0.28	0.38	<LOD	0.62	<LOD	<LOD	<LOD
c16	1.33	0.72	0.65	3.78	2.14	0.66	<LOD	0.42	n.d
c17	1.25	0.76	0.16	0.41	<LOD	0.74	<LOD	<LOD	<LOD
c18	1.53	0.73	<LOD	0.20	0.36	0.37	<LOD	<LOD	<LOD
c19	2.61	0.51	0.23	0.57	<LOD	<LOD	0.25	<LOD	n.d
c20	2.31	1.21	0.21	1.13	0.21	0.53	0.29	<LOD	n.d
c21	1.76	0.92	<LOD	0.19	<LOD	0.31	<LOD	<LOD	n.d
c22	1.10	0.52	<LOD	0.36	<LOD	0.27	0.24	<LOD	<LOD
c23	1.50	0.48	<LOD	0.56	0.49	<LOD	<LOD	<LOD	n.d
c24	0.80	0.70	<LOD	0.61	0.37	0.19	<LOD	<LOD	n.d
c25	1.12	0.51	<LOD	<LOD	0.48	0.50	<LOD	<LOD	n.d
c26	1.84	0.62	0.20	0.30	0.56	1.22	0.65	1.67	0.55
c27	3.41	1.61	0.34	4.03	1.51	0.54	0.39	0.77	n.d
c28	1.05	0.84	<LOD	0.34	0.27	0.46	0.20	<LOD	<LOD
c29	2.47	1.14	0.31	0.30	<LOD	<LOD	<LOD	<LOD	<LOD
c30	1.86	0.62	0.64	1.82	0.62	0.90	<LOD	<LOD	<LOD
c31	1.36	1.01	0.21	0.36	0.30	0.55	<LOD	0.49	<LOD
c32	1.67	0.97	<LOD	0.57	<LOD	0.64	0.26	0.50	n.d

ID	PFBA	PFBS	PFHxS	PFOS	FOSA	N.MeFOSA	N.EtFOSA	N.MeFOSE	N.EtFOSE
LOD	0.56	0.37	0.55	0.86	0.18	0.92	1.29	0.72	0.61
c1	n.d	n.d	1.17	2.88	n.d	n.d	n.d	n.d	<LOD
c2	n.d	<LOD	<LOD	1.90	n.d	n.d	<LOD	<LOD	n.d
c3	n.d	<LOD	2.24	<LOD	<LOD	n.d	n.d	1.58	<LOD
c4	n.d	<LOD	<LOD	1.08	n.d	<LOD	n.d	n.d	<LOD
c5	n.d	<LOD	<LOD	4.18	n.d	n.d	n.d	<LOD	<LOD
c6	n.d	<LOD	<LOD	<LOD	<LOD	n.d	n.d	n.d	<LOD
c7	n.d	<LOD	<LOD	2.46	<LOD	n.d	n.d	<LOD	n.d
c8	n.d	n.d	n.d	3.76	<LOD	n.d	n.d	1.30	n.d
c9	n.d	n.d	n.d	0.90	<LOD	n.d	n.d	<LOD	n.d
c10	n.d	n.d	n.d	1.56	<LOD	n.d	n.d	<LOD	n.d
c11	n.d	n.d	n.d	<LOD	<LOD	n.d	n.d	1.32	n.d
c12	n.d	<LOD	<LOD	<LOD	<LOD	n.d	n.d	<LOD	n.d
c13	n.d	<LOD	<LOD	2.94	n.d	n.d	n.d	1.44	n.d
c14	n.d	n.d	n.d	4.39	n.d	n.d	n.d	<LOD	n.d
c15	n.d	<LOD	<LOD	1.13	n.d	n.d	n.d	n.d	n.d
c16	n.d	n.d	0.57	6.22	<LOD	n.d	n.d	n.d	n.d
c17	n.d	<LOD	n.d	2.30	<LOD	n.d	n.d	n.d	n.d
c18	n.d	<LOD	n.d	1.04	<LOD	n.d	n.d	<LOD	n.d
c19	n.d	n.d	n.d	0.96	<LOD	n.d	n.d	<LOD	n.d
c20	n.d	n.d	n.d	1.11	<LOD	n.d	n.d	<LOD	<LOD
c21	n.d	<LOD	<LOD	<LOD	<LOD	n.d	<LOD	<LOD	<LOD
c22	n.d	<LOD	<LOD	0.97	n.d	n.d	n.d	<LOD	n.d
c23	n.d	<LOD	<LOD	<LOD	0.13	n.d	n.d	1.09	n.d
c24	n.d	<LOD	<LOD	1.55	n.d	n.d	n.d	n.d	n.d
c25	n.d	n.d	<LOD	1.08	n.d	n.d	n.d	<LOD	n.d
c26	n.d	n.d	n.d	1.05	<LOD	n.d	<LOD	n.d	n.d
c27	n.d	0.68	<LOD	2.73	<LOD	n.d	n.d	<LOD	<LOD
c28	n.d	n.d	1.92	1.06	n.d	n.d	n.d	n.d	n.d
c29	n.d	<LOD	<LOD	<LOD	n.d	n.d	n.d	n.d	n.d
c30	n.d	n.d	<LOD	1.64	<LOD	n.d	n.d	<LOD	n.d
c31	n.d	n.d	<LOD	1.05	<LOD	n.d	<LOD	n.d	n.d
c32	n.d	<LOD	<LOD	<LOD	<LOD	n.d	n.d	n.d	n.d

Appendix C. Results of the Mann-Whitney U test

Table 5. Results of the Mann-Whitney U test comparing PFAS levels in liver of small rodents sampled in Granåsen and Jonsvatnet.

Compound	**Z**	**p**
PFHxA	150.00	0.000
PFHpA	788.00	0.000
PFNA	318.00	0.187
PFDA	150.00	0.000
PFUdA	260.00	0.024
PFDoDA	132.00	0.000
PFTrDA	175.00	0.000
PFTeDA	64.00	0.000
PFHxS	219.00	0.003
PFOS	244.00	0.012

Appendix D. Results of ANCOVA and non-parametric tests for individuals from Granåsen

Table 6. Results of ANCOVA for determining the possible effect of the variables recorded on the PFASs concentrations in the individuals from Granåsen. Significant p-values are in bold and marked with (*) when $p < 0.05$ and (**) when $p < 0.01$.

	Sex		Weight		Length		AgeClass		Age (days)		Winter		Station	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
PFHxA	21.633	**0.000 ****	2.015	0.17	3.938	0.06	2.396	0.086	1.433	0.244	1.283	0.27	0.485	0.494
PFDA	0.333	0.570	0.049	0.827	0.432	0.518	1.594	0.217	0.481	0.495	0.105	0.749	5.828	**0.025***
PFDoDA	0.019	0.893	0.02	0.89	0.649	0.429	1.142	0.367	1.439	0.243	0.077	0.784	0.686	0.416
PFTrDA	1.049	0.317	0.217	0.646	0.54	0.47	1.184	0.349	0.702	0.411	0.029	0.866	0.019	0.893
PFTeDA	0.838	0.37	0	0.994	0.511	0.482	0.871	0.499	0.314	0.581	0.029	0.867	0.254	0.619
PFBS	0.007	0.934	2.885	0.104	0.488	0.492	1.386	0.276	0.497	0.488	0.808	0.378	0.404	0.532
PFHxS	3.639	0.07	0.194	0.664	1.384	0.252	1.088	0.39	0.283	0.6	0.432	0.518	0.011	0.917
PFOS	0.711	0.408	3.6	0.071	4.187	0.053	2.187	0.109	1.061	0.314	3.579	0.072	0.042	0.84

Table 7. Results of the non-parametric tests Kruskal-Wallis H test and the Spearman's rank correlation coefficient (P)

	Sex		Weight		AgeClass		Age (days)		Winter		Station	
	H	p	P	p	H	p	P	p	H	p	H	p
PFNA	1.169	0.28	0.025	0.805	1.467	0.832	0.025	0.906	0.102	0.75	31.0	**0.029***
PFUdA	0.692	0.405	0.136	0.527	6.49	0.165	-0.097	0.651	0.621	0.431	53.0	0.384
PFTeDA	1.072	0.301	-0.024	0.912	3.787	0.436	-0.214	0.316	0.008	0.927	67.0	0.975

Appendix E. Results of ANCOVA and non-parametric tests for individuals from Jonsvatnet

Table 8. Results of ANCOVA for determining the possible effect of the variables recorded on the PFASs concentrations in the individuals from Jonsvatnet. Significant p-values are in bold and marked with (*) when p < 0.05 and (**) when p < 0.01.

	Sex		**Weight**		**Length**		**AgeClass**		**Age (days)**		**Station**	
	F	p	F	p	F	p	F	p	F	p	F	p
PFHxA	0,603	0,447	0,901	0,354	1.421	0.242	0,882	0,524	0,18	0,676	0	0,989
PFHpA	0,827	0,375	1,37	0,256	0.565	0.461	0,764	0,605	0,249	0,624	0,224	0,641
PFUdA	0,355	0,559	0,091	0,767	0.342	0.566	1,399	0,257	0,059	0,811	0,15	0,703
PFNA	1,525	0,232	0,026	0,873	1.275	0.273	2,002	0,107	0,208	0,653	0,903	0,354

Table 9. Results of the non-parametric tests Kruskal-Wallis H test and the Spearman's rank correlation coefficient (P)

	Sex		**Weight**		**Length**		**AgeClass**		**Age (days)**		**Station**	
	H	p	P	p	P	p	P	p	P	p	H	p
PFDA	1,021	0,328	0,092	0,627	0,201	0,288	-0,165	0,385	-0,13	0,495	0,142	0,909
PFOS	0,896	0,397	0,58	**0,001****	0,429	**0,018****	0,468	**0,009****	0,484	**0,007****	0,454	0,662
PFDoDA	0,568	0,588	0,255	0,173	0,578	0,001**	0,307	0,099	0,29	0,12	0,242	0,836

Appendix F. PFHxA concentration in females and males sampled in Granåsen

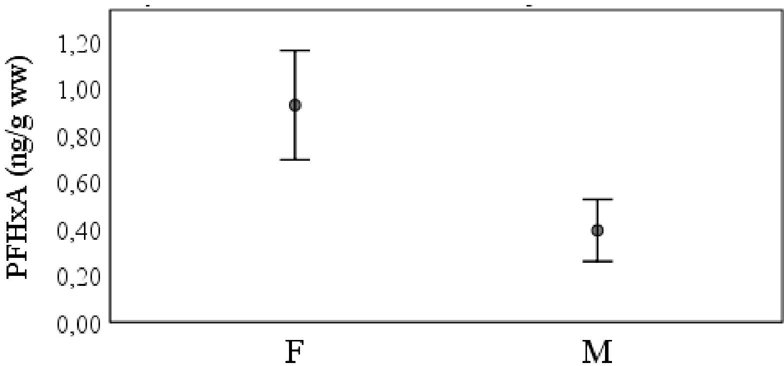

Figure 1. Average PFHxA concentration (ng/g ww) for females and males of small rodents sampled in Granåsen.

Appendix G. Pictures of chromosomal aberrations

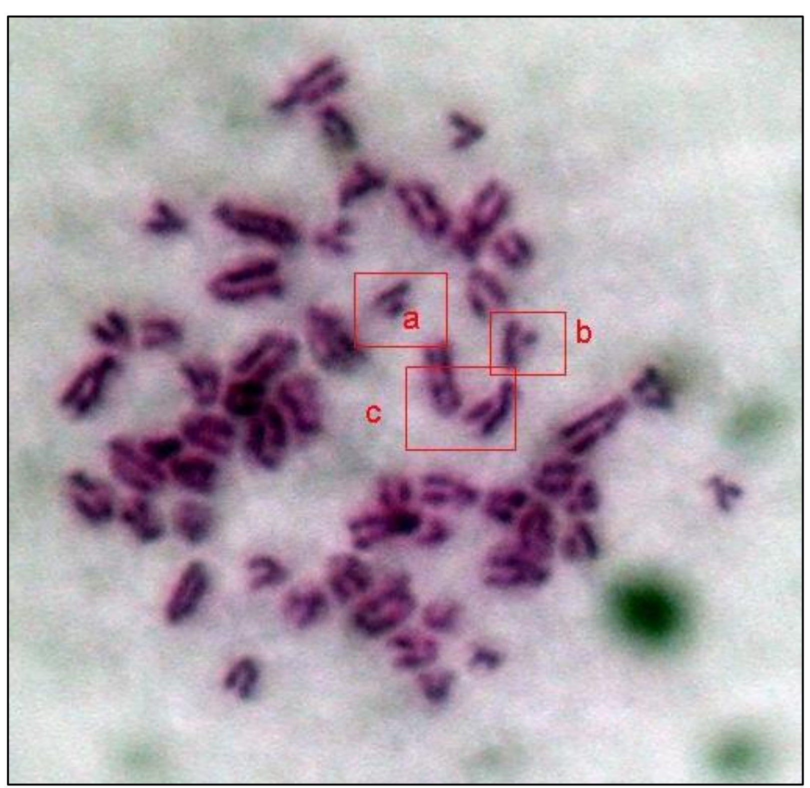

Figure 2. Chromosomal aberrations detected in a cell of an individual of *C. glareolus* from Jonsvatnet with ID C25. A: acentric fragment, B: chromatid break, C: gaps (not considered chromosomal aberrations)

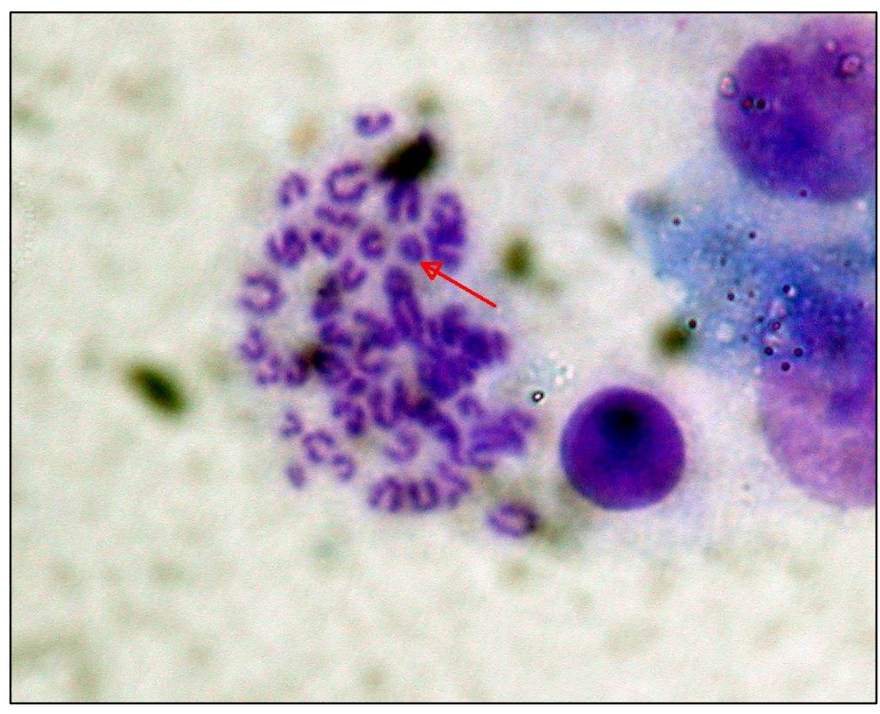

Figure 3. Ring (r) detected in an individual of *C. glareolus* sampled in Jonsvatnet (ID C23).