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Bioaccumulation and effects of parent and alkylated PAHs in an Arctic marine amphipod (*Gammarus setosus*) originating from pristine and historically contaminated sites in Svalbard: implications for sensitivity and adaptation.

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Marina Vázquez Alonso
Master thesis in International Fisheries Management (30 ECTS)
September 2016

UNIVERSITY OF TROMSØ

Caminante, son tus huellas

el camino y nada más;

Caminante, no hay camino,

se hace camino al andar.

Al andar se hace el camino,

y al volver la vista atrás

se ve la senda que nunca

se ha de volver a pisar.

Caminante no hay camino

sino estelas en la mar.

Antonio Machado (1875 - 1939)

Marina Vázquez Alonso

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Supervisors: Maria Granberd and Jasmine Nahrgang

The Arctic University of Norway

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ABSTRACT

Reduction in the sea-ice cover will potentially lead to an increase in the trans-Arctic ship transportation routes as well as the petroleum extraction activities in the Arctic. The major threats of these activities are considered to be oils pills or leaks or accidental releases. The release of oil related pollutants in the Arctic could lead to acute and long term consequences for the marine environment. Ships using heavy fuel oil in Svalbard are banned in the natural reserves and national parks, and only ships using marine diesel fuel are allowed to navigate these areas. PAHs and their alkylated forms are organic compounds found in both coal deposits and petroleum derivates. Petrogenic PAHs contain mainly alkylated forms, which are considered more toxic and harmful to biota than their parent congeners. Their classification is in function of their physico-chemical properties being the heavier compounds more hydrophobic and less bioavailable than the lighter ones.

The objectives of the study were to assess the bioaccumulation and genotoxic effects of PAHs and their alkylated PAHs from marine diesel fuel and coal polluted sediments in two populations of *Gammarus setosus*. Testing whether the alkylated forms are bioaccumulating more than their parent compounds, the degree of oxidative stress and ulterior DNA SSBs in the amphipods, as well as possible adaptation to toxicity of the pollutants in the two populations. The two populations were exposed during a period of 28 days to three treatments, MDF spiked sediment, naturally coal contaminated sediment and control sediment from a pristine area. PAHs concentrations in both biota and sediment, oxidative stress and DNA damage were quantified at the start of the exposure and during 5 sampling times. A conservative concentration of MDF was spiked (900 mg kg^{-1} sediment) simulating a non-acute oil spill. Coal samples from the abandoned mining area in Ny-Alesund and Thiisbukta were collected for PAHs quantification.

Total Alkylated PAHs (95% of total ΣPAHs) were detected in coal and sediment samples from Kongsfjorden. The sediment from Thiisbukta contained the highest concentration of total ΣPAHs 323 mg kg^{-1}, and Krossfjod sediment was under the detection limit. Both populations of amphipods from MDF spiked treatment, bioaccumulated alkylated PAHs, 2702 ng g^{-1} in Kongsfjorden amphipods and 1579 ng g^{-1} in Krossfjoden amphipods. Results from the lipid peroxidation were inconclusive since no significant difference was detected between the

treatments. DNA damage was measured as "% tail intensity" of DNA fragments extracted from single blood cell of exposed animals. In the two populations there was no significant difference at the start of the exposure but an oscillating pattern was observed after 4 and 16 days of exposure with an opposite pattern in the two populations. DNA damage in Kongsfjorden amphipods decreased to 0.10 % at ST4 after an initial increase   9.89 % ST2 and, while Krossfjoden was 0.68 % at ST2 and 3.98 % at ST4 for the MDF spiked treatment. For the Thiisbukta treatment DNA damage in Kongsfjorden amphipods was 10.16 % at ST2 and 3.95 at ST4, while the DNA damage in Krossfjoden amphipods was 0.59 % at ST2 and 3.08 % at ST4.The two populations thus react differently to PAH stress, where the DNA repair system appears to act faster in amphipods from Krossfjoden than in naïve amphipods from Krossfjoden. This may indicate adaptive differences in the two populations related to historical PAH exposure from unburnt coal.

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LIST OF ABREVIATIONS

AhR	Aryl hydrocarbon receptors
ANOVA	Analysis of variance
BSAF	Biota Sediment accumulation factor
B(a)P	Benzo(a)pyrene
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DSWs	Double strandbreak
DWS	Dry weight sediment
EqP	Equilibrium partitioning Theory
HFO	Heavy fuel oil
HPAH	High-molecular weight polycyclic aromatic hydrocarbon
K_{ow}	Octanol-water partitioning coefficient
LPAH	Low-molecular weight polycyclic aromatic hydrocarbon
MAH	Monocyclic aromatic hydrocarbon
MDF	Marine diesel fuel
PAHs	Polycyclic aromatic hydrocarbon
PLE	Pressurized liquid extraction
PBS	Phosphate buffered saline
POP	Persistent Organic Pollutant
SD	Standard deviation
ST	Sampling time
SSB	Single strandbreak
TOC	Total organic carbon
US EPA	USA´s Environmental Protection Agency
WSF	Water soluble fraction
WW	Wet weight

1 INTRODUCTION.

1.1 RISK OF ENVIRONMENTAL EXPOSURE TO OIL RELATED POLLUTANTS IN THE ARCTIC OCEAN

1.1.1 Increase of shipping activities lead the increase of oil spills risk

Global warming has been affecting the Arctic sea-ice covering since the last half of the 20th century, inducing its retreat and decreasing the thickness (AMSA, 2009). Consequences are also visible in the permafrost melt and the increase of seasonal variability in temperature. Climate prediction models show a continuous decrease in both the permanent and winter ice covered areas with a permanent sea-ice free cover (less than 1m km^{-2} in September) during summer seasons. Since the 70´s, the periphery of the Arctic Ocean and part of the ice covered area has been the all-year round route for transportation. However, more recent changes in the ice cover have modified the trans-Arctic ship transportation routes in areas impossible to navigate through decades ago, so new routes have been explored and established and navigation periods have been extended during the year. Countries with higher vessel activity in the Arctic (figure 1.1) are Northwest Russia, Norway, Greenland, Iceland and U.S.A to which activities such as nature resource exploitations (hydrocarbons, hard minerals and fisheries) as well as regional trade, depend on coastal and regional shipping (AMSA, 2009; Smith et at., 2012).
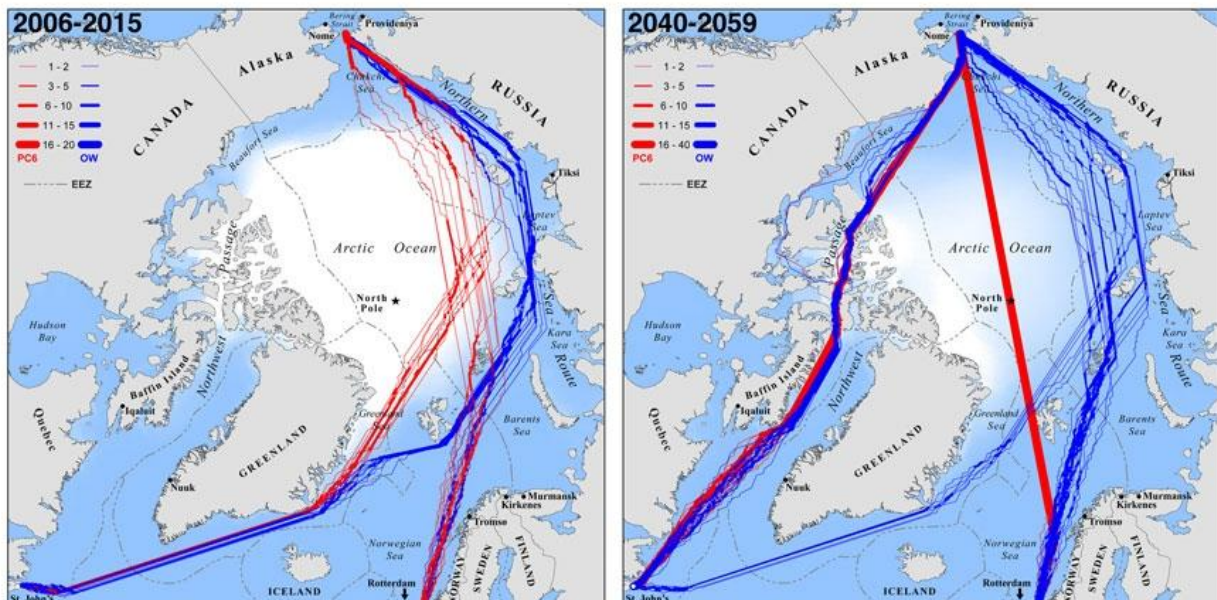
Figure 1.1 Trans Arctic shipping traffic routs during September, from 2006 to 2015 and 2040-2059 prediction models for hypothetical ships crossing the Arctic Ocean. Blue lines show fastest routes for common ships and red lines shows routes for icebreakers. Smith and Stephenson, (2013)

Shipping is considered to be the factor with greatest impact on the Arctic environment (AMSA, 2009). Shipping oil and gas volume transportation is predicted to reach 40 million tons per year by 2020. Thus the major environmental threats are considered to be oil spills accidental or illegal and waste discharge or leaks from the vessels during the transport of fossil fuels (AMSA, 2009). The release of oil related pollutants in the Arctic could lead to acute and long term consequences for the marine environment. Coastal environments, which are ice-covered during long periods, are low energy shores where the wave action is highly reduced. Pollutants originating from a spill may be concentrated and enclosed in such low energy areas, persisting for most of the year (Brown et al., 2016). The consequences of an oil spill can be worsened by the lack of Arctic marine infrastructures and emergency response capacity for pollution mitigation, as well as by incomplete hydrological and meteorological data for primary shipping routes that support safe navigation and satellite communications and monitoring control of ships movements. Although the possibility of an accident is small, the consequences may be severe due to the assumed fragility and sensitivity of the Arctic ecosystems (AMSA, 2009).

1.1.2   Oil activities in the Arctic.

Norwegian oil activities have already reached the Arctic and in the near future both Iceland and Greenland will be exploited in search for oil (Jörundsdóttir et al., 2014).

Produced waters from oil and gas activities contain the same type of organic pollutants, polycyclic aromatic hydrocarbons (PAHs) as crude oil, being dominated by compounds such as naphtalenes, phenanthrenes and fluoranthene (Hylland, 2006). The increasing use of fossil fuels has led to the increasing concentration of PAHs in sea water and organisms (Carrasco-Navarro, 2013). Carrasco-Navarro (2015) stated the need for the evaluation of effects of fossil fuel compounds in Arctic marine organisms due to the general lack of information about the topic in this region and the predictions related to climate change related ecosystem changes.

1.2 NORWEGIAN REGULATIONS ON OIL POLLUTION

On June 2007 the Norwegian government introduced a ban on the use of heavy fuel oils (HFO) in Svalbard for vessels entering both nature reserves and national parks, which represents more than 85% of the total area of the Svalbard archipelago. The objective of this restriction was to avoid HFO spills in such vulnerable coastal environments as the archipelago. Thus only ships with very high quality of light fuel oil, marine diesel fuel (MDF) are allowed to enter the nature reserves (Ministry of the Environment of Norway, 2009)

1.3 POLYCYCLIC AROMATIC HYDROCARBONS POLLUTANT SOURCES IN ARCTIC WATERS.

The major contributors to pollution in marine coastal environments as a result of the shipping and oil exploitation activities are PAHs. Due to their resilience in nature, PAHs are considered to have long-term effects in the environment, and PAHs are the petroleum compounds that are longer retained in marine environments due to their stable structures (Jörundsdóttir et al. 2014; Douben, 2003).

Oil retention times can vary as function of the coastal morphology and wave energy, for tidal flats and cobble shorelines it can be for more than 10 years. In cold environments with sea ice, low chemical and microbial activity and a lower wave energy can lead oil related pollutants to be preserved in the coast during longer periods (Albers, 2003). PAHs and alkylated PAHs are natural compounds of petroleum and coal deposits in which diagenesis of organic matter in sediments during geological time scales at low temperatures are precursors of those compounds (Albers, 2003). But its level in marine environments has been increased by anthropogenic activities derived from fuel fossil combustion processes (Hylland, 2006).

1.3.1 Physico-chemical properties of PAHs

Aromatic hydrocarbons include monocyclic aromatic hydrocarbons (MAHs), e.g. benzene, toluene, xylene, alkylated benzenes, and PAHs. Polycyclic aromatic hydrocarbons are a wide class of organic compounds, ubiquitous in sediments since they are originated by different natural and antrophogenic processes (Konovalov et al., 2010). They are formed by two or more benzene rings and classified as persistent organic pollutants (POPs) (Pampanin, 2013). They can

represent between 0.2% to more than 7 % of crude oil composition, resulting the most toxic fraction (Martínez-Gómez, 2010).

Pyrogenic PAHs are formed by natural or anthropogenic combustion processes (thermal decomposition) of fossil fuels or organic matter at high temperatures and further restructuration of organic molecules, resulting in non-alkylated molecules. They enter the marine environment as exhaust or solid residues. Petrogenic PAHs are formed by diagenesis of plant material at low temperature (100-300^{0}C) under geological time scales, resulting in the formation of fossil fuels which contains mainly alkylated PAHs. They are released by natural seepage, erosion of col laden bedrock, accidental oil spills, or tanker operations.
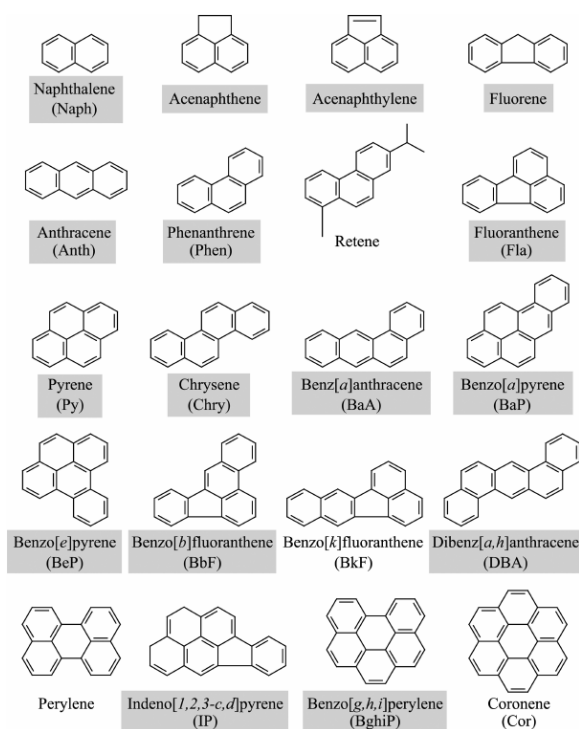
Figure 1.2 Polycyclic aromatic hydrocarbons structures. In gray box the 16 US Environmental Protection Agency listed priority pollutants, plus retene, perylene and coronene structures (Lima et al., 2003)

The proportion of alkylated PAHs or parent compounds, especially high molecular weight PAHs (HPAHs), can be used to identify the petrogenic or pyrogenic origin of the compounds due to its higher resistance to weathering processes (Pampanin, 2013; Douben, 2003; Meador et al., 1995; Albers, 2003; Stogiannidis and Laane, 2015). Meador et al. (1995) identified the pyrogenic origin of PAHs by the predominance of HPAHs and the petrogenic origin of PAHs by the high predominance of low molecular weight PAHs (LPAHs) in tidal sediments of Washington D.C. Hylland (2006) remarked the difference in bioavailability between pyrogenic and petrogenic PAHs since pyrogenic PAHs can be aggregated to the carbon structure, inhibiting its bioavailability unlike petrogenic PAHs, which remains available.

PAHs are classified according to structure, LPHAs contain two or three rings and HPHAs contain more than four fused benzene rings (figure 1.2). The hydrophobicity of PAHs increases with increasing molecular weight and degree of alkylation, thus the differentiation in physicochemical properties of both groups will influence the bioavailability, bioaccumulation and toxicity in marine environments (Meador, 2008).

1.3.2 Alkylated PAHs

Alkylated PAHs consist of basic ring structures of the parent compounds with alkyl (methyl) side groups. Each parent compound can have several alkylated homologues, which increases in number with the number of rings. Their denomination follows the number and position of methyl moieties (Meador, 2008). The molecular weight of the alkylated forms of LPAHs is higher than non-substituted HPAHs thus its properties will be similar to the HPAHs parent compounds. Alkylated forms are considered more toxic than their parent homologs due to its increase in hydrophobicity and bioaccumulation, in fact PAHs become more hydrophobic with increasing alkylation. For that reason, considering only parent compounds in assessment lead to underestimate the alkylated forms potential (Meador, 2008). Alkylated PAHs are dominant compounds in fossil fuels, and increase in dominance over un-substituted PAHs during weathering processes (Wang et al., 2003).

1.3.3   Marine diesel fuel (MDF)

After the distillation (boiling process for crude oil fraction separation) five fractions are obtained, the distillated fuels are the fraction which boils at higher temperatures. The last fraction called residuum does not boil without thermal decomposition. Marine diesel fuel (MDF) is an intermediate type of fuel distillate made by a mixture of distilled and residual oil (US EPA, 1999)

After being released into sea water, marine diesel dispersion would differ from bunker and crude oil in terms of behavior. Composed by more volatile compounds, its low viscosity confers a higher grade of dispersion and quick evaporation. Hydrocarbons contained in marine diesel oil are short-chained (Fingas, 2011). Hansen et al. (2013) analyzed the composition of the water soluble fraction (WSF) before testing marine diesel (1:40) on two copepods species, obtaining a higher relative proportion of light PAHs (44,3 ug/L) compared with heavy PAHs (0,80 ug/L). The toxicity of marine diesel may be related to the polycyclic aromatic compounds content (Sagerup et al., 2016).

1.3.4   Natural coal

Coal is mainly composed by carbon, hydrogen, sulfur, oxygen and nitrogen. It is a sedimentary rock formed as coal seams or layers. It can range from peat, lignite, bituminous coal, steam coal, anthracite to graphite depending on the maturity and contents of volatile organic compounds. Bituminous coal is a black sedimentary rock used as fuel in electric power generators. PAHs originating from coal have recently been considered in the scientific literature since unburnt coals may contain PAHs up to hundred mg kg^{-1} (Ahrens and Morrisey, 2005). Studied compounds include the 16 US Enviromental Protection Agency (US EPA) PAHs and its alkylated homologs in different coal ranks (Barrick et al. 1984). The increase of the coal maturity may lead the increase of PAHs and its number of ring concentrations due to the higher condensation, carbon concentration and aromatization of the coal, thus higher concentrations of napthalenes and its alkylated forms may shift towards 4-6 ring PAHs (Ahrens and Morrisey, 2005). Bituminous coals contain the higher amount of PAHs compared with lower or higher mature coals (Meyer et al., 2013). Also, alkylated PAHs decrease while parent compounds increase with increasing coal maturity, from low volatile bituminous coal to anthracite.

Open pit mining, abandoned coal mounds and sedimentary strata are sources of coal particles and PAHs compounds into the marine environment by erosion and transportation. Their behavior in sea water is similar to PAHs from oil derivates, thus binding to particles to form colloids and being reallocated in sediments (Stout et al. 2002; Hofmann, 2002). Thus unburnt coal is a PAHs source in coastal environments. In reported oils spills such as the Exxon Valdez, the uncertainty about the PAHs origin, from coal or oil, was raised (Boehm et al. 2001). Hydrocarbons fingerprints can be used in that purpose to differentiate pyrogenic and petrogenic PAHs but it may not be so evident and a lack of information about native PAHs in sediments may remain (Alimi et al., 2003). In native PAHs from coals, naphthalene, phenantere and their alkylated PAH may dominate but this pattern can be also found in petrogenic oils, even though naphtalenes have higher rate of evaporation in oils compared with coals due to it is a strong sorbent.

1.3.5   Environmental relevance of the PAHs and alkylated PAHs.

The 16 US Environmental Protection Agency (EPA) priority pollutants were established in 1976 assessing human food and water health and they were classified as relevant pollutants by its carcinogenic and toxic effects in organisms (Keith, 2015). Since then, different scientific fields have based the research mainly on the listed components and the list or part of it has been implemented by governmental organizations and institutions. But a review of the list has been claimed due to the limited compounds included and its non-useful application in some areas due to the higher toxicity of non-listed compounds and the lack of toxicological concern of some others (Anderson and Achten, 2015). For environmental purposes the list may underestimate the potential toxicity of petroleum pollutant sources, for example, when only the 16 priority pollutants are analyzed, being non-representative of the potential harmful effects. The absence of compounds such as highly toxic benzo(c)fluorene o dibenzo(a,h)pyrene which are 20 times more carcinogenic than benzo(a)pyrene (the most studied compound), alkylated homologs or heterocyclic aromatic compounds will induce to a misinterpretation of the information (Anderson and Achten, 2015, 2015; Achten and Anderson, 2015).

Alkylated PAHs are an essential tool for environmental forensic chemistry. Due to the different physicochemical properties of petrogenic and pyrogenic origin pollutants, alkylated PAHs are used to recognize the source of the pollution, since they are source-specific the relative amounts will depend on the specific oil/coal (Stout et al., 2015; Stogiannidis and Laane, 2015).

1.4 FATE OF PAHs IN AQUATIC SYSTEMS

Coastal marine environments and especially the intertidal are sensitive areas to effects of oil related pollutants released during spills which reach the coastal line and are deposited on the sediments. In marine environments, the sediment is the main depositional compartment for persistent hydrophobic pollutants, affecting sediment associated organisms (Webster and Tronczynski, 2009; Gunnarsson et al., 1999). Once PAHs enter the water column they bind to particulate and dissolved organic matter, due to their lipophilic properties exerting a strong affinity to organic coated particles and solid surfaces, being deposited on the sediment by sinking of the particles (Clément, 2012; Granberg, 2004; Meador et al. 2015). The distribution of the PAHs between different phases (water, sediment and organism) is represented by the partitioning coefficient of PAHs, which is considered to be in equilibrium when the exchange between the different compartments equals zero (Equilibrium partitioning theory (EqP)). This dynamic equilibrium depends on the factors affecting all the phases and leads PAHs to sorb or desorb from phases (Burgess et al. 2003). The concentration of organic carbon in pore water or sediments will influence the partitioning coefficient (K_{oc}) (formula 1.1) of the PAHs.

Formula 1.1 $$\mathrm{Koc} = \frac{\text{concentration PAH g}^{-1} \text{ organic carbon in sediment}}{\text{PAH concentration instertitial water}}$$

Hydrophobic PAHs show more affinity to non-polar particles such as lipids or organic carbon than polar molecules (water phase). Water solubility of PAHs range in the lower parts per million (Eriksson, 2003). Hydrophobicity can be expressed in terms of octanol-water partitioning coefficient (K_{ow}) (formula 1.2) which is the parameter that defines the distribution of compounds between, water and solid phases.

Formula 1.2 $$\mathrm{Kow} = \frac{Concentration\ in\ octanol\ phase}{Concentration\ in\ aqueous\ pahse}$$

Compounds with a low K_{ow} are less hydrophobic and thus more bioavailable from the pore water due to the low sorption to particles, also the transfer through membranes and assimilation efficiency of low K_{ow} PAHs results higher compared with high K_{ow} PAHs. These lighter compounds results more volatile and its persistence can vary from hours to days (Meador, 1995; 2008; Landrum and Robbins, 1990; French-McCay, *2004*). For high K_{ow} values, the partitioning of PAHs to sediment increases and the strong sorption leads difficult assimilation efficiencies PAHs bound to ingested particles ingested particles. Sorption is also

affected by the sediment chemistry, particle size, clay content, cation exchange and pH (Landrum and Robbins, 1990).

Table 1.1 Physico-chemical, toxicological values for PAHs.

Table 1
US EPA's 16 priority-pollutant PAHs and selected physical–chemical properties (ATSDR, 2005)

Polycyclic aromatic hydrocarbons[a]	Structure (# of rings)	Molecular weight (g/mole)	Solubility (mg/L)	Vapor pressure (mm Hg)
Naphthalene	2	128.17	31	8.89E−02
Acenaphthene	3	154.21	3.8	3.75E−03
Acenaphthylene	3	152.20	16.1	2.90E−02
Anthracene	3	178.23	0.045	2.55E−05
Phenanthrene	3	178.23	1.1	6.80E−04
Fluorene	3	166.22	1.9	3.24E−03
Fluoranthene	4	202.26	0.26	8.13E−06
Benzo(a)anthracene	4	228.29	0.011	1.54E−07
Chrysene	4	228.29	0.0015	7.80E−09
Pyrene	4	202.26	0.132	4.25E−06
Benzo(a)pyrene	5	252.32	0.0038	4.89E−09
Benzo(b)fluoranthene	5	252.32	0.0015	8.06E−08
Benzo(k)fluoranthene	5	252.32	0.0008	9.59E−11
Dibenz(a,h)anthracene	6	278.35	0.0005	2.10E−11
Benzo(g,h,i)perylene	6	276.34	0.00026	1.00E−10
Indeno[1,2,3-cd] pyrene	6	276.34	0.062	1.40E−10

[a] US EPA has classified PAHs in italics as probable human carcinogens (NTP, 2005).

Degradation of PAHs is driven by chemicals (UV light breakdown) and biological processes such as microbial degradation (Hylland, 2006). Wilcock et al. (1996) showed the fast removal of LPAHs in intertidal sediments due to microbial degradation processes. In Arctic and temperate regions, sea bottom can remain at 10 ^{0}C or even lower and few publications have been focused on the low-temperature PAH degradation. Factors affecting PAH persistence are nutrients, oxygen and degrading microorganisms (Eriksson, 2003). Increased levels of oxygenation in sediments carried out by bioturbation biota result in high levels of microbial degradation of PAHs (Malmquist et al., 2013).

1.5 BIOAVAILABILITY OF SEDIMENT ASSOCIATED PAHs IN BENTHIC ECOSYSTEMS

Bioavailability results from the potential transfer of a pollutant from the deposition compartment to the biota, being the precursor of both bioaccumulation and toxicity. Thus the total concentration of pollutants will not determine the toxicity of PAHs, rather than the relative bioavailable fraction of bulk PAHs, which is controlled by the physico-chemical properties of the sediment such as carbon content, particle size, the interstitial water and the microbiological degradation. PAHs body burden in organisms will depend on the pollutants bioavailability and the physiology of the organisms (Clément, 2012; Meador et al. 2015). Total PAHs concentrations variations in tissues will be influenced by environmental concentrations, time of exposure and metabolization efficiency of organisms (Meador et al, 2003).

1.5.1   Exposure pathways and uptake routes by benthic organisms

Biological factors that control pollutants bioavailability depend on the feeding mode and the uptake or exposure route. Food selection, digestion and assimilation also influence the bioavailability once PAHs enter the body organism (Burgess and McKinney, 1999). PAH bioaccumulation is controlled by physiological factors such as total lipids in the organisms and uptake-elimination equilibrium rates. The uptake route must be considered when determining the steady-state body burden when there is no equilibrium conditions between water, sediment and prey (Meador et al., 1995).

Benthic amphipods, having a rigid cuticle, may acquire pollutants by two main routes, water ventilation by gills and food-sediment particle ingestion (Gross-Sorokin et al., 2003). Although the rate of water ventilation is considered higher than food intake rates and the bioavailability of pore water dissolved compound is also higher, the ingestion route becomes more relevant for high lipophilic compounds since HPAHs strongly adsorb to sediment particles and organic matter in particular. Thus interstitial water is a source of uptake and bioaccumulation for the LPAHs compounds with lower hydrophobic properties while sediment is the important source for HPAHs and hydrophobic compounds (Richter et al. 2006; CCME, 1999; NCR, 2003). Selective feeder feeding on the finest particles (< 63µm) may take up higher organic contaminant concentrations since smaller particles have a higher surface to volume ratio and thus sorb more of the contaminants (Harkey et al. 1994). Meador et al. (1995) showed experimentally the accumulation of HPAHs (>3 rings) in polychaetes by direct ingestion of PAHs bound to

sediments while accumulation of LPAHs (2-3 rings) in both polychaetes and amphipods resulted from PAHs associated with interstitial water.

After ingestion, the gut processes such as hydrolysis and solubilization of PAH compounds determine its internal bioavailability (Mayer et al., 2001). Deposit feeding invertebrates have an efficient retention in the digestive system which leads to optimize nutrients from poor sedimentary diets (Andresen and Kristensen, 2002; Mayer et al., 2001). Jørgensen et al. (2008) suggested that digestive solubilization of pollutans by polychaetes determines the bioavailability level, when the amount of solubilized contaminant is correlated with the amount of absorbed contaminant during digestion (Ganberg and Forbes, 2006).

1.6 BIOACCUMULATION OF PAHs BY ORGANISMS.

The bioaccumulation of PAHs by an organism is determined by the difference between the uptake by organisms and the elimination or biotransformation and is described by the steady-state model. The model assumes that the distribution of the PAHs between compartments is regulated by the total organic carbon (TOC) on the sediment and the lipid content of the organism. Biota-sediment accumulation factors (BASF) values are obtained when the system is thermodynamically in equilibrium, when the balance between compartments equals zero. BASF are specified in terms of exposure time (Granberg, 2004; Burgess et al., 2003). Lee et al. (1993) estimated a BSAF value about 1,7, but Thomann et al., (1992) established field BSAF about 0,8-1 for PAHs with $logK_{ow}$ 2 - 5 and 1 - 10 BASF for PAH with $logK_{ow}$ 5 – 8.

1.7 BIOTRANSFORMATION IN MARINE INVERTEBRATES

Detoxification activities performed by marine invertebrates include the biotransformation, bioactivation, excretion of the pollutant molecules (Shugart, 2000). Invertebrates can biotransform PAHs at different degrees due to the variability in the cytochrome P450 oxygenase system (Granberg, 2004; Meador, 2003) thus both parent and metabolite compounds should be assessed in order to avoid underestimation of organism´s efficacy during metabolization (Jørgensen et al., 2008). Enzymatic biotransformation activity in invertebrates take place in tissues related with food digestion and absorption (Livingstone, 1998).

In some groups of invertebrates the metabolizing and excretion processes are initiated by PAHs binding to the aryl hydrocarbon receptors (AhR). The ligand-receptor complexes that are formed lead to the activation of the transcription gene CYP1A, resulting in the synthesis of CYP1A proteins which metabolize and transform the PAHs parent compounds into more hydrophilic excretable forms (Dupuis, 2015). Hahn (2002) listed a series of mollusk such as *Mya arenaria* and *Mytilus edulis* in which AhR homologues were found. The binding of PAHs to AhR have ulterior toxic effects by three main processes such as inappropriate gene expression after the binding, oxidative stress by the production of ROS species during the metabolization process and reactive metabolites formed as products, which damage DNA. Also alkylated PAHs are involved in the production of reactive metabolites via CYP1A enzymes (Dupuis, 2015). Hodson et al. (2007) related the alkylated PAHs toxicity with the AhR and further metabolization by CYP1A enzymes and Turcotte et al. (2011) suggested that higher toxicity of alkylated PAHs would result from its specific AhR affinity.

1.7.1 Phase I and phase II of PAHs transformation

Both vertebrates and invertebrates have similar PAH biotransformation pathways with similar catalytic enzymes and resulting metabolites (Jørgensen et al., 2008). Invertebrate biotransformation systems are, however, considered much less efficient than that of vertebrates. Biotransformation of pollutants by invertebrates involves two steps in order to decrease the lipophilic character of the molecules which are transformed into hydrophilic water soluble metabolites or ionic product, which become easily excretable (Granberg, 2004). Polychaetes such as *Nereis virens* have been proved to efficiently biotransform PAHs, which after 5 days of Pyrene exposure the 80% of metabolites compounds were related to phase II transformation (Jørgensen et al., 2008).

During the phase I, the enzyme Cytochrome P450 (CYP P450) mixed function oxygenase catalyzes the binding of a functional group (-OH, -COOH, -NO2) into the PAHs structure becoming slightly water soluble. During the phase II, a polar group (glutathione, sulphate, glucuronide, amino acid...) is covalently bound to the previous structure by enzymatic activity, which largely increases the water soluble character of the molecule.
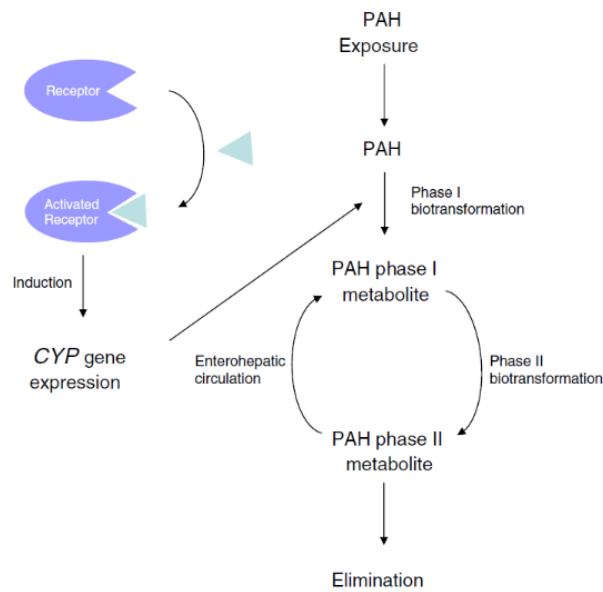
Figure 1.3. Biotransformation process of PAHs involving phase I and phase II (Jørgensen et al. 2008).

Phase II conjugation pathway in some invertebrates is glycosylation instead of glucuronosylation which is related as more important pathway in vertebrates (Jørgensen et al. 2008; Livingstone, 2001; Malquist et al. 2013)

Both pyrene and benzo(a)pyrene (B(a)P) are commonly used in experiments as model substances since they are found in all mixtures of PAHs. Pyrene is metabolized by some invertebrates to 1-hydroxy-pyrene during phase I and later conjugated to 1-hydroxy-pyrene during phase II (Carrasco Navarro, 2015). B(a)P metabolites after phase II have been identified as quinones in molluscs, but in crustaceans and echinoderms they were identified as phenols and diols. Fernández et al. (2012) exposed *Mytilus edulis* to B(a)P, showing the formation of DNA strand breaks after the metabolization to B(a)P quinones.

1.7.2 Oxidative stress – Reactive oxygen species

During the metabolization of PAHs, the enzymatic activity of CYP1A proteins may lead the production of reactive oxygen species (ROS) by reduction reactions of oxygen, which are involved in cellular oxidative damage (Dupuis, 2015; Lushchak, 2015). ROS are cellular and extracellular free radical species such as superoxide anion (O_2^-), hydroxyl radical ($^.OH$), peroxyl radical, alcoxyl radical ($RO^.$) and hydroperoxyl radical ($HO_2^.$) but also no radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (González et al., 2015; Livingstone, 2001). Oxidative stress results from the imbalance between the ROS production and antioxidant repair leading to oxidative damage of DNA, lipids and proteins. This situation results either from a decrease of antioxidants or an increase in ROS production. DNA damage includes 8-hydroxydeoxyguanosine and oxidized bases; lipids peroxidation by the formation of aldehydes such as malonaldehyde species or 4-hydroxyalkenals and protein oxidation of non-peptide carbonyl groups (Livingstone, 2001). Lipid peroxidation is the degradation of the lipids by oxidative reactions which decreases the membranes fluidity modifying the membranes characteristics and thus disrupting the protective barrier function of the biological membranes (Devasagayam, et al. 2003)

Both invertebrates and mammal organisms share the same oxidant and antioxidant processes and aerobic cells contain a minimum amount of ROS since they regulate physiological processes such as hormonals and signal transducers and are also products of aerobic metabolism (González et al., 2015; Lushchak, 2011; 2015). The aerobic ROS production in non-stressed cells take place in the mitochondria, which consumes about 90% of the cellular oxygen and the microsomal system in the endoplasmic reticulum (Abele and Puntarulo, 2004). ROS are naturally produced by the reduction reactions of molecular O_2 in the aerobic metabolism (Camus and Gulliksen, 2005).

When referring to ectothermic organisms, temperature is a parameter to consider. Since physiological processes are temperature dependent, in general an increase of environmental temperature may lead to an increase in the metabolism, which means more oxygen consumption and thus higher oxidative stress (González et al., 2015).

Also environmental changes may cause oxidative stress in organisms inhabiting certain niches (González et al., 2015) or experiencing seasonal fluctuations. Krapp et al. (2009) reported a

higher ROO· and HO· scavenging capacity in the Arctic *Gammarus wilkitzkii* (amphipoda) during summer compared with winter. Both estuarine and intertidal environments are considered the most complex areas due to its cyclical variations of physical parameters: temperature, salinity, dissolved oxygen and pH. Also UV radiation, wave action and desiccation affect benthic invertebrates during low tide (Freire et at., 2012). Thermal stress in polar areas can be derived from the low tide exposure to both high and freezing temperatures in summer and winter (Freire et at., 2012). But Malek et al. (2004) and Niyori et al. (2001) reported that a decrease in temperature leads an increase in ROS production in fish and barnacles may be due to the reduction of ROS elimination system (Lushchak et al. 2015)

Organisms inhabiting polar areas are adapted to extremely cold environments. Lower metabolic rates at low temperatures may limit the production of mitochondrial ROS in ectotherms, but those organisms contain a higher level of polyunsaturated fatty acids (PUFAs) in fat deposits and membrane lipids in order to maintain the structure and function of biological membranes. Higher PUFA contents are related with a decrease in the membrane viscosity and a higher ROS oxidation, thus increasing the rate and propagation of lipid radical chain reactions Cold-adapted organism may thus be more vulnerable to oxidative stress (Regoli et al., 2012). Aquatic organisms have a higher proportion of polyunsaturated fatty acids among their lipids, which are the substrate for oxidation (Regoli et al., 2012). As a result of the decomposition of PUFAs, malondialdehyde acid is formed, which is commonly used as a biomarker to evaluate the biological effects of pollutant in aquatic organisms (Buge and Aust, 1974; Milinkovitch et al., 2015). In sea-ice environments the oxygen concentration can be high due to photoautotrophic activity. If both DOM and irradiance are also high, it can lead to an elevated ROS production which results in oxidative stress on sea-ice organisms (Regoli et al., 2012). In the other hand low seawater temperatures will influence a higher O_2 solubility and pO_2. Abele (2004) reported a 40% increase between 15 - 0°C seawater temperature (Sidell, 1998). Higher O_2 solubility is related with higher ROS formation. In the cell cytosol the O_2 solubility is less influenced by the temperature due to the solutes concentration, but in tissues of polar ectotherms, higher O_2 steady-states concentrations might be consider. An increase of pO_2 will lead an increase of chemical and enzymatic ROS production, since they are pO_2 dependent processes, thus higher dissolved O_2 in tissues will may increase the potential lipid peroxidation (Abele, 2004). Thus

both homeoviscus adaptation to cold environment and higher O_2 dissolved in cytosol may yield polar organisms to be more sensitive to lipid peroxidation (Abele, 2004)

Benthic marine invertebrates from intertidal zones may experience low O_2 episodes due to environmental and physiological hypoxia, thus ROS production can increase during or after hypoxic episodes. Affected tissues such as gills also show an increased or higher level of antioxidant enzymes compared with other tissues. Such a high level can be considered a mechanism of damage protection when ROS increase during re-oxygation, unfreezing and reactivation episodes after hypoxia, freezing or hibernation periods. Euryoxic benthic species such as bivalves and cnidarians may have better antioxidant response due to their adaptation to changes in O_2 during air exposure (Livingstone, 2001). Also salinity variations have effects in intertidal or estuarine benthic organisms and the stress produced has been related with ROS production (González et al. 2015).

PAHs are one of the pollutant groups which increase pro-oxidant free radicals products in marine invertebrates by different processes such as enzyme induction (CYPs), auto-oxidation (cytochrome P450), disruption of membrane-bound electron transport or depletion of antioxidant defenses such as the reduction of glutathione (GSH) involved in phase II biotransformation. Different taxonomic groups experience different levels of oxidative stress, which depend on the physiological and environmental characteristics of each group (Livingstone, 2001). *Mytilus* spp have been proved to biotransform B(a)P into mutagenic metabolites, generating ROS species such as H_2O_2 (Marsh et al. 1993; Michel et al. 1992). Regoli et al. (2012) suggested that antioxidant defenses take place once ROS production reach a certain limit. Ansaldo et al. (2005) exposed *Nacella concinna* to 0,05% dose of marine diesel with no antioxidant response but considerable protein oxidation and lipid peroxidation.

1.7.3   DNA single strand break related with PAHs toxicity.

Oxidizing agents such as ROS may generate DNA adducts, base modification, single or double strand breaks and DNA protein cross links (Dexheimer, 2013). Single strand breaks (SSBs) are formed when one strand of the helix undergoes discontinuity by the breakage of the phosphodiester link (Shugart, 2000), and a nucleotide is lost by damage at the point of the break 5´- or 3´- termini. Since SSBs compromises genetic viability, cells have effective and fast repair mechanisms. DNA double strand break (DSBs) may be a potential dangerous type of DNA

damage, since the template strand for reparation is also damaged. SSBs is frequency much higher than DSBs by the effects of free radicals such as H_2O_2, and is a consequence of the breakup of the oxidized sugar or during the DNA base excision repair of oxidized bases (Caldecott, 2008). Uncorrected damages of DNA during replication will generate the deletion of one or more bases pairs or a base-pair substitution in the replicated DNA chain. Chromosomal aberrations and mutations may be the result of DNA strand breaks, which can lead to cell death. Mutations are further spread along the cell generations during DNA replication. (Alberts et al. 2014; Jha, 2008).

1.8 ARCTIC AMPHIPOD *GAMMARUS SETOSUS* - THE STUDIED ORGANISM

Gammarus setosus (Dementieva 1931) is an Arctic benthic gammarid amphipod with a circumpolar distribution and an Arctic restriction. It is found in sheltered or slightly exposed rocky beaches from the subtidal to the upper low intertidal range between 0-1m depth. It inhabits substrates composed of loose rocks with algae but can also be abundant in mud flats with detritus (Carrasco-Navarro et al. 2015; Wezlawski, 1994; Whiteley et al., 2011). Maximum population densities can reach about 3000 ind m^{-2}, mean value at a studied site about 396 ind m^{-2} (Weslawski et al. 1994)

Gammarus setosus is epibenthic and has been found to be sensitive to oil related compounds (Olsen et al., 2007). Gómez and Dauvin (2000) also remarked the sensitivity of the amphipods towards aromatic components of petroleum oil with high toxicity, showing a high initial mortality. Gulliksen and Taasen (1982) reported complete absence of *G. setosus* in the Van Mijenfjord, Svalbard from 2 monitoring sites two years after the oil spill from storage tanks. Densities in monitoring sites unaffected by the spill were reported to be 64-80 ind m^{-2}.

Scavenger species such as *G. setosus* feed on carrion, thus being a key recycling organisms of organic material which is transferred to higher trophic levels, thus becoming an important component in polar food webs (Nygård et al., 2012). *Gammarus setosus* feeding preferences are scavenging on planktonic organisms such as Dinophyceae, Tintinnina and protist cysts which can reach 50% of the intake volume in the gut (Legezynska et al., 2012). Previous studies, (Legezynska, 2001) of the gut content of 5 scavenging species of Lysianossoid amphipods from Kongsfjorden (*Onisimus caricus*, *Onisimus edwardsi* and *Orchomenella minuta, Anonyx nugax*

and *A. Sarsi*) showed that carrion and crustaceans remains were the larger part of the diet, followed by detritus. *Onisimus edwardsi* contained 50% proportion filamentous algae and diatoms in the gut. Under laboratory conditions three species were feed on dead or damaged zooplankton which were collected close to the bottom due to the lack of predation adaptations. Also sediment grains were found in three species, guts of *O. edwardsi* and *O. minuta* contained the coarser sediments compared with *O. caricus.*

G. setosus is a marine crustacean and as it counterparts of this taxonomic group the gas exchange takes place via gills, which are located in the ventral part of the animal beneath the thorax. Gills are simple, flat sacs through which hemolymph is pumped.

Rastrick and Whiteley (2011) reported the lower metabolic rate of some Gammarus inhabiting subarctic latitudes compared with Gammarus from temperated latitudes and Carrasco-Navarro (2015) remarked the lack of information about arctic vs subarctic species differences in metabolic rates, but suggested that similarities would be expected in Gammarus setosus compared with subarctic Gammarus species.

1.9 OBJECTIVES.

The aim of the study was to assess the bioaccumulation and genotoxic effects of PAHs and alkylated PAHs, from marine diesel fuel spiked and naturally coal polluted sediments in individual amphipods from two *Gammarus setosus* populations. The two G. setosus populations differed in their historical exposure to PAHs associated with coal.

The following hypothesis were tested:
1. Alkylated PAHs are accumulated to a larger extent that their parent homologues.
2. PAH bioaccumulation induces oxidative stress in G. setosus.
3. There is a difference in the toxic response between amphipod populations which have been historically exposed to coal contaminated sediments (Kongsfjorden) compared to populations from pristine environments (Krossfjoden)

2 MATERIALS AND METHODS.

2.1 KONGSFJORDEN – KROSSFJODEN SYSTEM: STUDY AREA

The study was carried out during June and July 2015 in the Kongsfjorden-Krossfjoden system, which is located between 78°40' and 77°33' N and 11°33' – 13°6' E on the north-west coast of Spitsbergen in Svalbard (Figure 2.1).

Figure 2.1. The Kongsfjorden-Krossfjoden system study area in Spitsbergen archipielago (https://www.norgeskart.no/)

The system is formed by two fjords that share a common mouth with no sill, instead a through that cross the wide shelf. Warm, salty Atlantic water meet the cold and fresh water from the shelf. These influences make the system sub-Arctic rather than Arctic (Hop, 2002). Krossfjodenen is south-east to north-west oriented, 30km long and its width is between 3 and 6 km. The fjord is divided by the King Haakon Peninsula at the inner part with a tidal glacier Liliehookenbreen. Kongsfjordenen is north-south oriented, 20 km long and from 4 to 10 km wide. The mouth is placed between Kvadehuken and Kapp Guissez (Svendsen et al. 2002). The inner fjord is shallow and divided in two basins separated by a sill and the archipielago Lovénøyane; the most inner basin is about 94m deep, reaching the outer basin a depth about 428m (Wlodarska-Kowalczuk, 2004, Elverhøi et al. 1980). Four tidal glaciers, Kongsbreen, Kongsvegen, Conwaybreen and Blomstrandbreen have a high impact on the hydrography (Hop, 2002), releasing fresh water and mineral sediments into the marine environment during run-

off, ice calving, and snowmelt. It is considered a glacial fjord with both Atlantic and Arctic influence on physical factors.

The outer part is influenced by the ocean and the inner part influenced by tidal glaciers, resulting in high amount of both primary production and particulate organic carbon (POC) from phytoplankton detritus, on the inner basin and lower amounts on the outer basin which is lower influenced by the tidal glaciers that limits the amount of light penetrating the water column and the dilution of the organic matter by the mineral fraction. Mineral sedimentation rates vary from the glacial bays over 800 g m^{-2} day^{-1} to 200 g m^{-2} day^{-1} at the distal part of the inner basin to reach rates about 25 g m^{-2} day^{-1} at the outer basin (Wlodarska-Kowalczuk, 2004).The biology is also influenced by these conditions, showing a gradient in biodiversity and abundance from Arctic to boreal species from the inner to the outer part (Hop, 2002; Wlodarsk-Kowaleczuk& Pearson, 2004).

2.1.1 Sampling sites

Bottom sediment and biological samples were collected at two sites. In Kongsforden the studied site was Thiisbukta (11°54.303'E, 78°55.802'N) (figure 2.2) and Ebeltofhamna (11° 55.863'E, 78° 55.645'N) in (figure 2.2).

Thiisbukta is the innermost part of Kolhamna and the closest sampling site to Ny-Ålesund. The bay is influenced by land and glacier runoff which is discharged during the warm season through a larger stream into the marine environment in the bay also bringing with it coal particles and pollutants. Spitsbergen is crossed by a large depression of Terciary deposits with a NNW trend axis, containing coal shale and coal rock in the central part. Paleogene strata with coal can be found in other basins in Spitsbergen but the Central Basin is the main one, being the uppermost unit of the Van Mijenfjorden Group. Kings Bay Coalfield in Ny-Alesund is the northwest strata of the Central Basin included in the Van Mijenfjorden Group (Cmiel et al. 2004). An Environmental Impact Risk Assessment performed in the area (NPI, 1994) detected high levels of PAH in marine sediments, which origin was identified as the landfill placed close to Thiisbukta instead of oil spill or leaks from ships coming into the fjord. Coal seams are exposed at the rocky shore in sedimentary rocks (limestone and dolostone) which are visible in the cliffs surrounding the bay. Coal from Svalbard is classified as bituminous, containing high quantity of carbon 75-90% (Dallmann, 2015). This coal bearing bedrock is also a source of coal

particles directly to the marine sediments by through weathering and erosion carried out during the melting season.

Ebeltofhama was selected as being considered a pristine area in comparison to Kongsfjordenen site. This natural port was the first whaling station stablished in 1611 and remains of these activities can still be found on the place as well as a graveyard at the lagoon´s mouth dated from that period. Although anthropogenic activities have been carried out during last 400 years, during the last century polluting activities have not been reported, thus anthropogenic polluted impact on the area can be disregarded. (NPI, 2015). Ebeltofhama is formed by a lagoon partially separated from the open to the fjord water by a long sandy barrier shoreline.
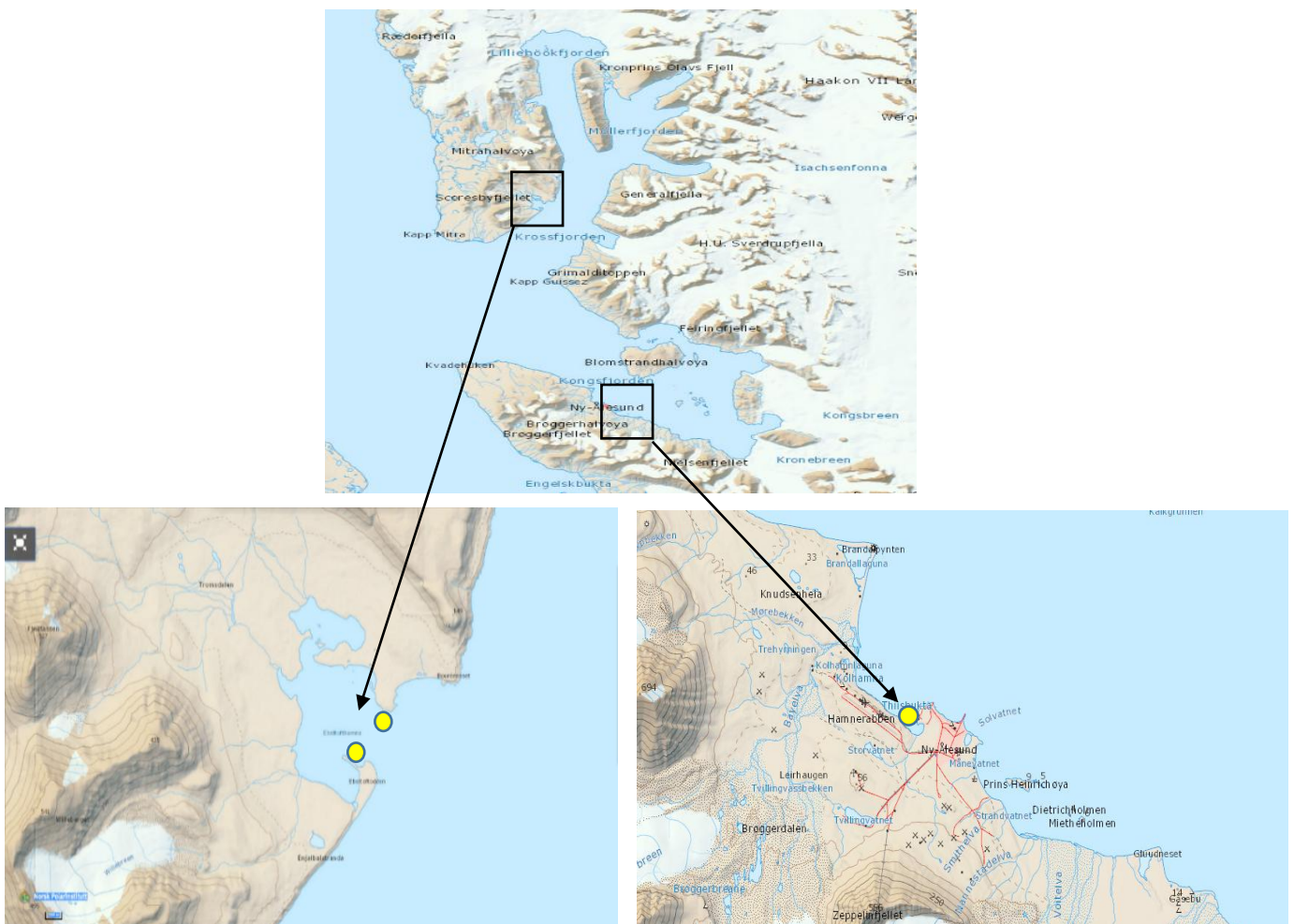

Figure 2.2. Sampling sites Ebeltofhamna in Krossfjoden (left) and Thiisbukta bay in Ny-Alesund (right)

2.2 FIELD SAMPLING

2.2.1   Sediment and amphipod collection.

Sediment was collected from Ebeltofhamna (Krossfjoden) and Thiisbukta (Kongsfjorden) using a Van Veen grab. Only the uppermost oxic layer (5 cm) was retrieved and transported to the laboratory in closed buckets. The sediment was sieved (1 mm mesh size) to remove macrofauna and larger particles. Buckets with the prepared sediments were kept cool (4^oC) and dark with a continuous water supply ($4\ ^oC$) until set-up and start of the bioaccumulation experiment. These sediment samples were stored frozen (-20°C) in Rilsan (Tub-ex Aps.) bags until further processing. Sediment from Thiisbukta contained darker and well sorted silt size particles while Ebeltofhamna sediment contained mixed sediment.

Coal samples were collected in Ny-Ålesund from the abandoned mining piles which are exposed to weathering and the runoff in Thiisbukta bay. The samples were collected in Rislan bags and frozen immediately to -20°C at the laboratory until analysis.

Amphipods *Gammarus setosus* for the bioaccumulation experiment were collected by hand from both Kongsfjordenen and Krossfjodenen in the intertidal zone during low tide when animals were sheltered under boulders at the shoreline. The amphipods were placed into coolers filled to the brim with seawater from the collection site, and transported to the laboratory where they were kept in aerated aquaria at 4 °C in darkness for acclimation until the start of the experiment. Animals were not feed at any moment.

2.3   EXPERIMENTAL DESIGN.

2.3.1   Bioaccumulation experiment

Bioaccumulation experiments were run 28 days according to the US-EPA standard for bedded sediment accumulation tests (EPA, 1993). Three different treatments were studied, control (Krossfjoden sediment), MDF spiked Krossfjoden sediment, naturally coal polluted Thiisbukta sediment. Individuals collected from the *G. setosus* populations at Kongsfjorden and Krossfjoden sampling sites were exposed to the three sediment treatments in parallel experiments. Six time sampling times ($ST_0 – ST_5$) were established from the start of the exposure with a geometrical progression. In order to establish the base line for the analysis, the ST_0 group of *G. setosus* samples were immediately frozen at -20^oC after acclimation, without

being exposed to any treatment. About 500 g of sediment from each treatment were frozen for further characterization (grain size, moisture and TOC) and PAHs, alkylated PHAs concentration analysis.

2.3.2   Sediment preparation and sampling

Krossfjoden sediment was divided into two buckets, 6.5 ml of marine diesel (density 0.84 g cm^{-3}) was spiked in 6 kg wet weight sediment (910 mg diesel kg^{-1} WW sediment). The diesel oil was added to a slurry of 500 g WW of sediment and seawater 1:4 and left to mix for 5 hours on a magnetic stirrer in cool and darkness. The slurry was mixed with the remaining sediment using a hand mixer, the released overlying water was collected as the MDF accommodated fraction (WFS). The remaining Krossfjoden sediment was kept as control treatment. From each treatment, 150 g of sediment was transferred into glass flasks about 250 ml and filled up with sea water and left overnight to settle. Three replicates of each experimental unit were made for all treatments. Each glass flask was supplied with seawater from the fjord at 80 m depth, thus natural conditions, physicochemical parameters and temperature at 4 ^{o}C where kept. Water from a main pipe was independently supplied to each glass flask maintaining continuous inflow and outflow. Water flow from the tank was filtered with a mesh 1mm size in order to avoid detritus from algae or organisms enter the distribution tubes system. The glass flasks were maintained in a continuous water flow system to maintain low and constant temperature. Flows and temperature were monitored daily. The outflow water from the flasks was filtered with active carbon before released to the sewage. Twelve individuals of *G. setosus* were transferred into each flask and kept until the time sampling established for that specific glass flask was finished. Glass flask of control and test sediment where placed randomly in a rack and kept in dark.

During destructive sampling, animals for PAH and alkylated PAH analysis were carefully collected from each jar and kept in replicate separated glass jars with clean seawater during 3 h for gut purging and then frozen (-20 ^{o}C) in brown prewashed and muffled glass vials. Animals for lipid peroxidation were collected at sampling times (ST) ST_1, ST_2 and ST_4, frozen in liquid nitrogen and kept at -80 ^{o}C. Animals for comet assay analysis were collected at ST_2 and ST_4 where hemolymph was extracted according to the method described below. Sediment from the glass flasks were transferred into individual Rilsan (Tub-ex Aps) bags and frozen (-20 ^{o}C) for further PAH and alkylated PAH analysis.

2.4   CHEMICAL ANALYSES

2.4.1   Extraction and quantification of PAHs and alkylated PAHs in sediment

Sediment and amphipods extraction of PAHs/alkylated PAHs was carried out by pressurized liquid extraction (PLE) in an ASE200 system. Amphipods from the same treatment and glass flask were pulled. The extraction cells for the animals were filled with 4 gram 2% deactivated silica to retain lipids, a bottom layer, 5-10 g of pulled samples and filled up with Ottawa sand. PLE extraction cells for the sediment were filled with 4 g of Silica gel 60 (0,063-0,0200mm, Merck) previously activated overnight at 180 ^{o}C, 4 g of copper powder (to remove elemental sulfur in sediments), 5 g of sediment grounded and homogenated with 5 g of Hydromatrix and Sodium sulfate, 200 μl of internal standard (Appendix 1) and filled up with Ottawa sand as inert matrix. Two pressurized liquid extractions from each cell was carried out with a solvent mixture of n-pentane:dichloromethane (9:1) at 1500 psi and 100 ^{o}C temperature into separated collection vials. Both extractions were mixed and concentrated down to 20 ml at 40 ^{o}C temperature, 200μL recovery standard was added to the vials and filled up to20ml with recostitued n-pentane:dichloromethane (90:10).

For the Extracts were analyzed for PAHs on an Agilent 5975C inert XL MSD with electron ionization with a selection ion monitoring. About 1 μl sample was injected in splitless mode at 300 °C, to a 60 m HP-5 capillary column with 0.25 mm inner diameter, 0.25 μm film thickness and a flow rate about 1.1ml/min. The initial temperature (40°C) was maintained for 2 minutes, and increased 25 °C min^{-1} to 100°C, followed by 5°C min^{-1} to 315 °C and maintained for 14 minutes with a total run time of 61 minutes. Peaks were quantified using Chemstation V2.0 (Agilent technologies).

2.4.2   Thiobarbituric acid assay (TBARs) – oxidative stress

The assay was based on the measurement of thiobarbituric acid reactive substances (TBARS) such as malondialdehyde (MDA) by the thiobarbituric acid TBARs assay. The test measures the amount of MDA present in the sample, which is generated as a degradation product from peroxided lipids as a side product of enzymatic metabolism of prostaglandins. MDA reacts with thiobarbituric acid (TBA) at low pH and high temperature (90-100°C) to form a colored complex, the MDA-TBA complex, with an absorption maximum at 532-600 nm can be measured by visible absorptions spectrophotometry. The amount of MDA formed depends on the lipid

content of the sample, metal ion contamination in the reagents and antioxidants in the sample (Södergren, 2000).

The method was carried out according to Ohkawa et al., 1979. Individually whole animals were added to 300-500 µL phosphate-buffer pH 7,4 with 0,1% Triton X-100 and 4% butylated hydroxytoluene in 1mL methanol and homogetanted in Precellys ®24 filled with five metal beads, at 3x6000 rpm for 3x5 seconds. Samples were kept in ice during the experiment. The standard solution containing 24 µL of MDA tetrabutylammonium salt solution and 226 µL of distilled H_2O was diluted in series of 48, 24, 12, 6, 3, 1,15 µM. From the homogenate, 100 µL was added to Eppendorf tubes containing 0,4 mL of 60mM Tris buffer (7,93 g/L Trizma HCL, 1,16 Trizma Base, pH 7,4), 0,1 mM Diethylenetriaminepentaacetic (DTPA), 0,5 mL of trichloroacetic acid (TCA) (12%) and 0,5 mL of thiobarbituric acid (TBA) (0,73%). Samples and standards were incubated at $95^{o}C$ for 60 minutes in Termarks T1056 incubator, cooled on ice and centrifuged at 20.000 rpm for 10 minutes. Form the supernatant, 0,1 mL was pipetted to a 96-well microplate in quadriplicates. Absorbance was measured at 532 nm and 600 nm and the results were expressed in nmol/g wet weight tissue.

2.4.3 Comet assay – DNA single strand breaks

Single cell gel electrophoresis or alkaline comet assay is a method that measures cellular DNA damage by quantifying breaks in single stranded DNA in individual cells. Damaged cells contain more fragmented DNA than undamaged cells do. Thus when running a gel electrophoresis the amount of DNA fragments that migrate is proportional to the frequency of DNA strand breaks.

When cells are lysed in detergent (Triton X-100) cell and nuclear membranes are removed and histones are also removed by adding NaCl. Thus the remaining structure are nucleoids which structure has been disrupted by the extraction of the histones but maintaining the supercoiling of DNA. DNA strand breaks may relax one loop of the supercoiling structure and since these loops are structural units when an electrophoretic field is applied to the loops, they migrate towards the anode. The number of breaks present in the gel are correlated with the number of the relaxed loops, and the results from the comet assay will be related with the DNA that appears in the comet tail. What is measured while scoring the cells is the relative intensity of the DNA tail fluorescence as index of DNA break frequency. Comet assay can be used to asses both DNA damage and repair as well as the antioxidant status of the cells measuring its resistance to oxidative H_2O_2 damage (Azqueta, 2013).

Four *Gammarus setosus* individuals were randomly selected from each flask during the specific sampling times. Hemolymph from the abdominal part of the telson was extracted with hand modified glass microcapillary tubes after gently drying the individuals with tissue paper. A drop of hemolymph was transferred to an Eppendorf tube containing 10 µL of phosphate buffered saline (PBS) (Ca/Mg free) for further analysis of DNA strands breaks as well as DNA repair. Gel electrophoresis was performed according to Hylland (2015). For the evaluation of the DNA damage by the tail intensity, films were stained with SYBR gold 1 µM for 20 minutes at room temperature and from each sample 50 cells were manually scored using fluorescence microscopy (ex/em 520/610) at 200x with Comet Assay IV software.

2.5   DATA TREATMENT AND STATISTICAL ANALYSIS

Data from the analysis was checked for outliers and normal distribution. Data outside the first and third quartiles of the distribution were considered outliers and removed from the data set. The hypothesis of normal distribution was tested by the Shapiro-Wilk test and the data set was logarithmic transformed when the assumption was violated. Multiple comparison of the treatments for each sampling time was carried out. Homogeneity of variances were tested by Brown-Forsythe test. The individuals from the same replicate were tested by nested analysis of variances (ANOVA), and comparisons of groups from the same factors and treatment were tested by One-Way ANOVA, followed by All Pairwise Multiple Comparison or Turkey tests. Groups which violated the assumption of equal variances were tested by the non-parametric Kruskal-Wallis One Way ANOVA test. Null hypotheses of equal variances was established at 95% significance level ($p < 0.05$).

3 RESULTS

3.1 Bioaccumulation experiment

The experiment was successfully performed during the five sampling times (ST) along 28 days. During the experiment, the behavior of the animals was reported without wandering swimming abilities or loss of mobility, vertical swimming movements were noticeable when the flasks were manipulated. Sediment samples were transferred from the experimental laboratory to the different analytical laboratories without breaking the cold chain, and storage temperatures were maintained at -20 ^{0}C in sediment and -80 ^{0}C biological samples.

Dead amphipods were reported in ST3, ST4 and ST5, all of them were individuals from the Kongsfjorden population (table 3.1). In order to avoid decreasing number of replicates ST3 and ST5 were repeated with amphipods from the Kongsfjorden, which were previously acclimated following the same procedure as previous individuals. After 18 days, two more Kongsfjorden animals from the control treatment were reported dead. These repeated replicates were finally no analyzed since ST3 and ST5 were not included in the biological analyses.

Table 3.1 Glass flask containing all 12 dead animals during the exposure experiment.

Sampling time (ST)	Glass flask	Treatment	Population
3	30	Control	Kongsfjorden
3	35	Control	Kongsfjorden
3	40	Thiisbukta	Kongsfjorden
3	51	Thiisbukta	Kongsfjorden
4	44	Control	Kongsfjorden
4	64	Thiisbukta	Kongsfjorden
5	68	Control	Kongsfjorden
5	61	Control	Kongsfjorden
5	88	Diesel	Kongsfjorden

Additionally, dead animals were reported in some of the glass flasks while the rest of animals from the same flask showed active movements and correct swimming (appendix 2). In some cases, the dead animal´s carcasses seemed to be partially eaten or just the exoskeleton was remaining, thus cannibalism and molting could infer the decreased number of individuals.

Sampling times ST4 and ST5 seemed to be especially critical due to the increasing number of dead animals.

3.2 Polycyclic aromatic hydrocarbons in coal and sediment samples.

Figure 3.1 shows the total ΣPAH, total alkylated PAHs and total 16 US EPA PAHs concentrations in coal samples from the mining area and Thiisbukta, in the MDF and in the sediment samples from Thiisbukta and Krossfjoden. Total ΣPAHs include the US EPA 16 PAH, alkylated PAHs plus dibenzothiophene, benzo(e)pyrene and perylene (appendix 3).
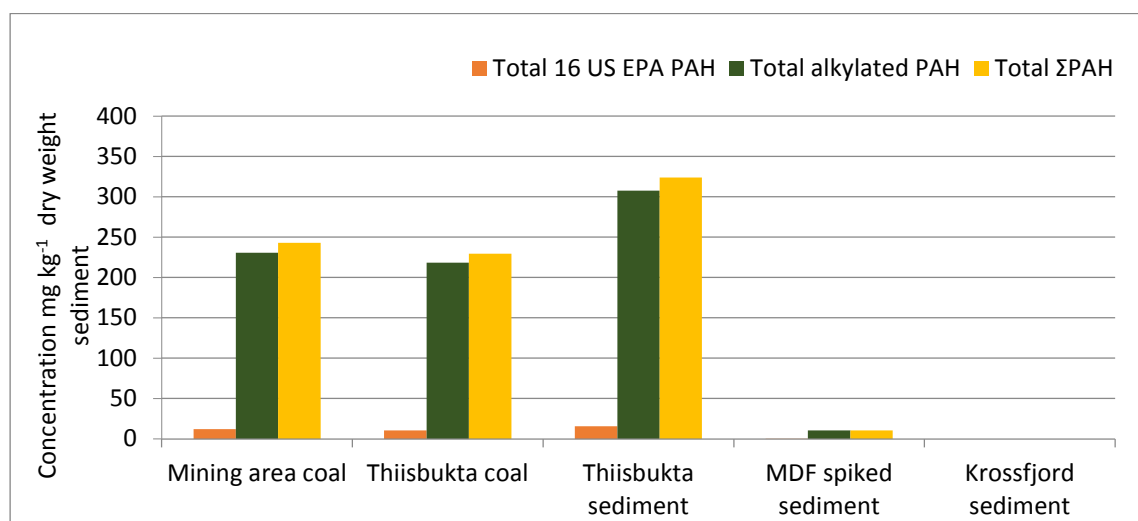
Figure 3.1. Total 16 US EPA priority PAH, total alkylated PAHs and total ΣPAHs concentrations in coal, and sediment samples from the bioaccumulation experiment at the start of the exposure. Values are expressed in mg kg^{-1} dry weight sediment

All the sampling sites except Krossfjoden showed a higher proportion of alkylated PAH in relation to their parent compounds, as well as similar proportions between alkylated PAHs and US EPA 16 PAH among the sampling sites. Sediment sample from Thiisbukta contained the higher concentrations of PAHs, reaching 324 mg kg^{-1} dry weight (DW) of total ΣPAH, 308 mg kg^{-1} DW of total alkylated PAHs and 15 mg kg^{-1} DW of total US EPA 16 PAHs. The coal sample from Thiisbukta contained 229 mg kg^{-1} DW of total ΣPAH, 219 mg kg^{-1} DW of alkylated PAH and 10 mg kg^{-1} DW of US EPA 16 PAHs. The coal sample from the mining area contained 243 mg kg^{-1} DW of total PAH, 231 mg kg^{-1} DW of total alkylated PAH and 12 mg kg^{-1} DW of total US EPA 16 PAHs. The MDF contained 277 mg kg^{-1} DW of total ΣPAH, 263 mg kg^{-1} DW of total ΣPAH and 13 mg kg^{-1} DW of US EPA 16 PAHs. Krossfjoden PAHs concentrations were under the detection limits for all the compounds (appendix 4).

Alkylated PAHs accounted for the 94.95 % of total ΣPAHs in the mining pile sample, 95.27% in the coal from Thiisbukta and 95.04 % in the Thiisbukta sediment from the exposure experiment, in the MDF 97 % of the total ΣPAHs were alkylated homologues.

Appendix 5 shows the PAH fingerprint of coal and sediment samples from the four sampling sites. Similar patterns were found for both coal and sediment samples except for Krossfjoden sediment samples. Napthalenes, phenanthrenes, as well as alkylated chrysenes, pyrenes, fluorenes and dibenzothiophenes were the compounds showing the highest concentrations. The alkylated forms were consistently higher in concentrations than the respective parent compounds. The coal sample from the mining area contained a higher proportion of naphthalene compared with the Thiisbukta coal and sediment samples from Kongsfjorden, reaching 7100 ng g^{-1} DW (± 0.0 SD) of naphthalene and 41808 ng g^{-1} DW (± 0.0 SD) of C3-naphthalene in the coal, versus the 9700 ng g^{-1} DW (± 958 SD) of naphthalene and 28580 ng g^{-1} DW (± 4268 SD) of C3-naphthalene in the Thiisbukta sediment. The mining pile contains a higher concentration of C2 and C3-naphthalene, the coal sample from Thiisbukta contained a higher proportion of C3 and C4-naphthalene and the Thiisbukta sediment contained the highest concentration of naphthalene and a similar proportion of C1 to C4-naphthalene. Both Thiisbukta coal and Thiisbukta sediment samples showed a higher proportion of phenantrene compound compared with the mining coal, C4-phenanthrene being extremely high with a concentration of 92492 ng g^{-1} DW (± 39571 SD) in Thiisbukta sediment samples.

Figure 3.2 and 3.3 show the total PAHs concentrations in sediments from the three different treatments during the experiment exposure time for the Kongsfjorden and Krossfjoden populations. In both cases the Thiisbukta treatment showed the highest concentrations compared with control and diesel treatment. The relation between parent compounds and its alkylated forms followed the same pattern as at the start of the experiment, higher concentrations were detected in the alkylated forms compared to the parent compounds.

For the Kongsfjorden population (figure 3.2) among the experimental treatments, Thiisbukta showed an irregular pattern along the exposure time since the highest values of PAH concentrations were found in ST3 and ST5. For ST3 values of total PAH raised 736 mg kg^{-1} DW, being the alkylated forms the 96,19 % of the total PAH with 708 mg kg^{-1} DW, and for ST4, the lowest concentrations, reached 215 mg kg^{-1} DW of total PAH and 198 mg kg^{-1} DW the alkylated forms.

Figure 3.2 PAH concentrations along the exposure treatment times for the Kongsfjorden population. Values are expressed in mg kg^{-1} DW sediment.

For the Krossfjoden population (figure 3.2) the Thiisbukta treatment also showed the highest values but with a different pattern compared with Kongsfjorden populations exposure sediment, since the highest values were obtained at ST5 with 645 mg kg^{-1} DW total ΣPAH and 614 mg kg^{-1} DW of total alkylated PAH and ST3 remains similar to the other sampling times with 311 mg kg^{-1} DW of total ΣPAH and 295 mg kg^{-1} DW of total alkylated PAHs. Values are presented in appendix 7.
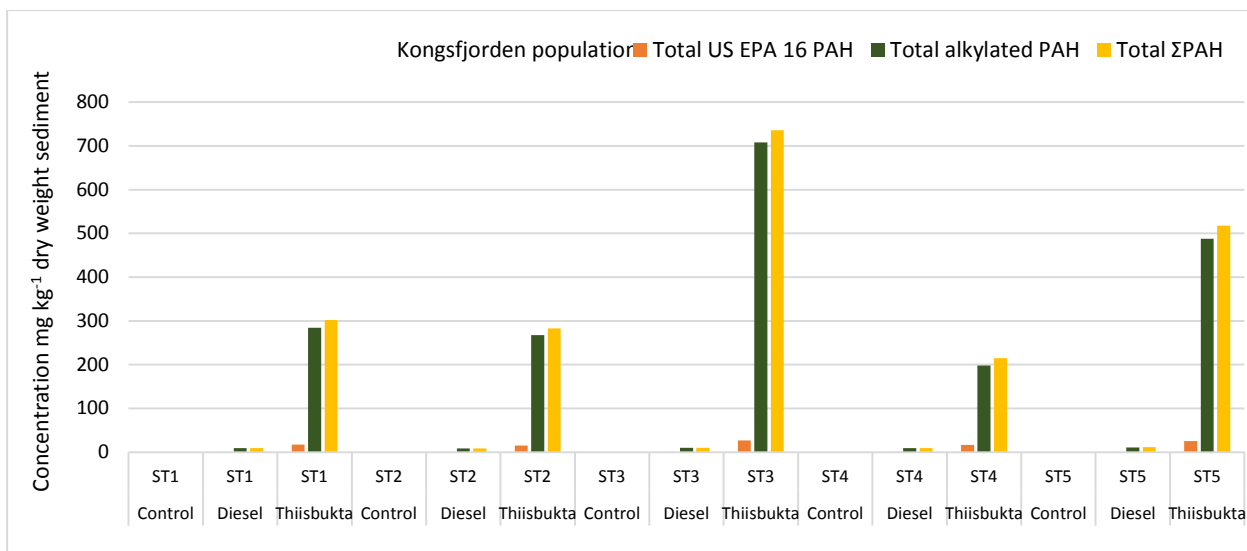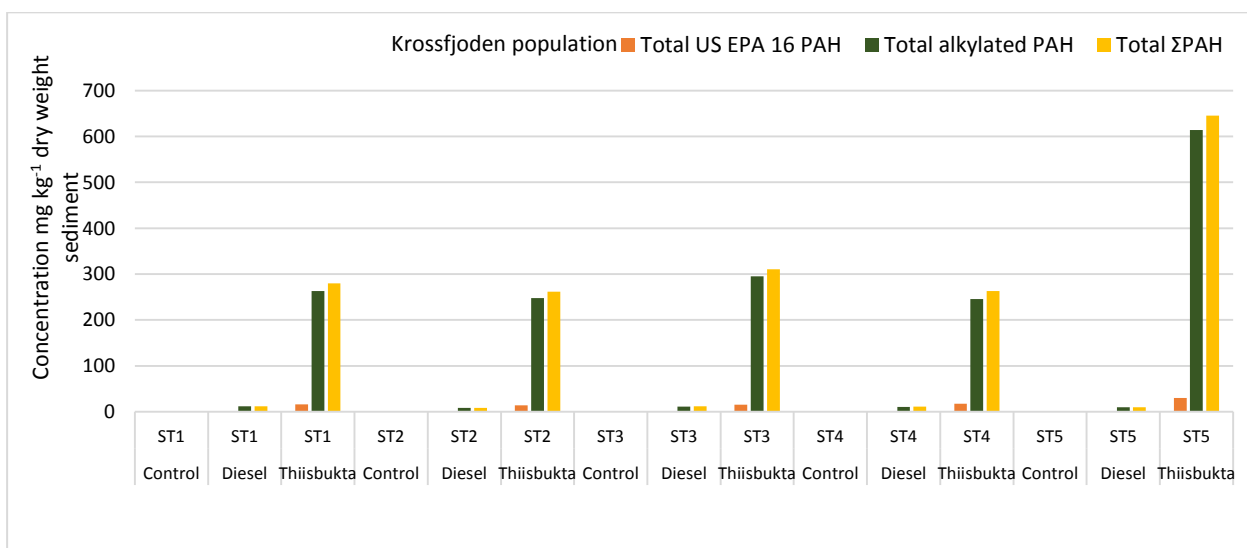

Figure 3.3 PAHs concentrations along the exposure treatment times for the Krossfjoden population. Values are expressed in mg kg^{-1} DW sediment.

Appendix 8 and 9 shows the PAH fingerprint in sediment samples along the exposure time for each treatment and Kongsfjorden, Krossfjoden populations respectively. Control treatment showed values under the detection limit. The compounds detected in the MDF treatment were naphthalene, fluorene, phenanthrene and their alkylated homologs as well as the alkylated forms C1 and C2 - benzo(a)anthracene. Detected compounds from the Thiisbukta treatment sediments were more broad, with highest values in naphtalenes, phenanthrene and chrysene compounds and their alkylated forms, and the lower values of fluorene, dibenzothiophene, pyrene and benzo(a)anthracene. A common pattern among treatments is the bell-shape of the compound groups, from the parent to its alkylated forms.

3.3 Polycyclic aromatic hydrocarbons concentration in amphipods.

Polycyclic aromatic hydrocarbons compounds in amphipods where only detected in amphipods from ST2 in diesel treatment and ST4 in both diesel and Thiisbukta treatments. Results from sampling times ST0 and ST1 were under the detection limit and are thus not presented.

Figure 3.4 and 3.5 shows the PAH fingerprint detected at the ST2 and ST4 in both Kongsfjorden and Krossfjoden amphipods exposed to the diesel treatment. Only the compounds over the detection limit are included in the plots. Krossfjoden amphipods contained higher amounts of the lightest alkylated C1-naphthalene, 1242 ng g^{-1} wet weight (WW) (±679 SD) in ST2 compared with Kongsfjorden 317 ng g^{-1} WW (±549 SD) in ST2 that contained lower concentration of heavy alkylated naphthalene. The variability of the results was also higher in Krossfjoden compared to Kongsfjorden (appendix 10). Body burdens of alkylated naphthalenes decreased markedly about one order of magnitude, from the ST2 to ST4 and a shift from less alkylated naphtalenes to more alkylated naphtalenes were detected in both amphipods populations. In both sampling times ST2 and ST4 the alkylated C2-naphthalene was the compound with higher concentrations in ng g^{-1} wet weight (WW) from 2.702 ng g^{-1} WW (±1369 SD) ST2 to 368 ng g^{-1} WW (±673 SD) ST4 in Krossfjoden animals from the MDF treatment, and from 1579 ng g^{-1} WW (±486 SD) ST2 to 274 ng g^{-1} (±474 SD) ST4 in Kongsfjorden animals from the MDF treatment.

In the Thiisbukta treatment (figure 3.6), only Kongsfjorden amphipods during ST4 showed PAH concentrations over the detection limit. Also C2-napththalene showed the highest PAHs concentrations in amphipods, reaching 920 ng g^{-1} WW (±1593 SD) and C2-phenantrene concentration was 262 ng g^{-1} WW (±545 SD). These concentrations correspond to dead animals

from the glass flask number 64, which contained all dead animals. Other glass flask contained all dead animals as well, but in these cases, concentrations were under the detection limit.
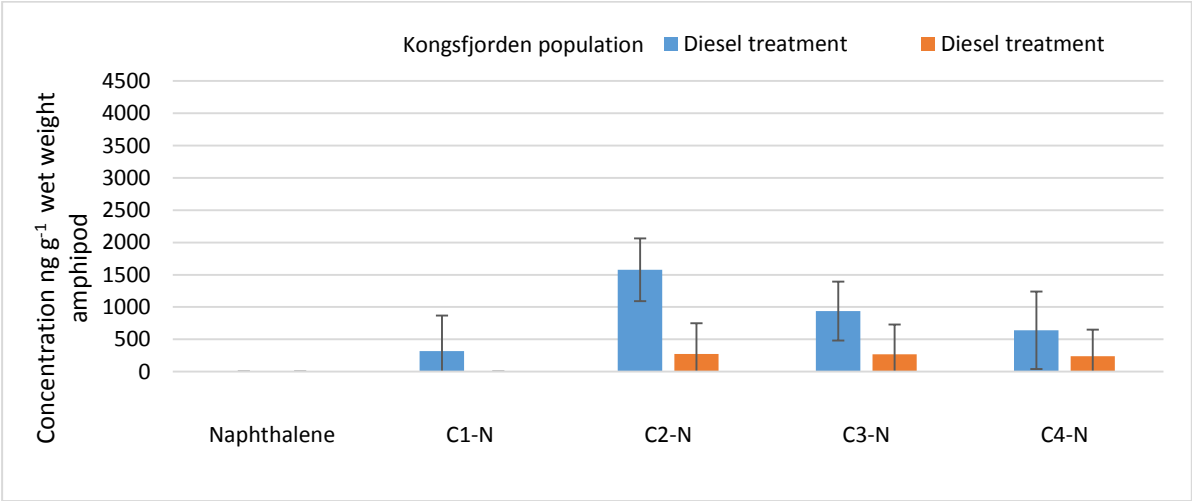
Figure 3.4 PAH detected compounds in Kongsfjorden population for diesel treatment during ST2 and ST4. Only compounds with values over the detection limit are presented in the plot. Concentrations are expressed in ng g^{-1}.
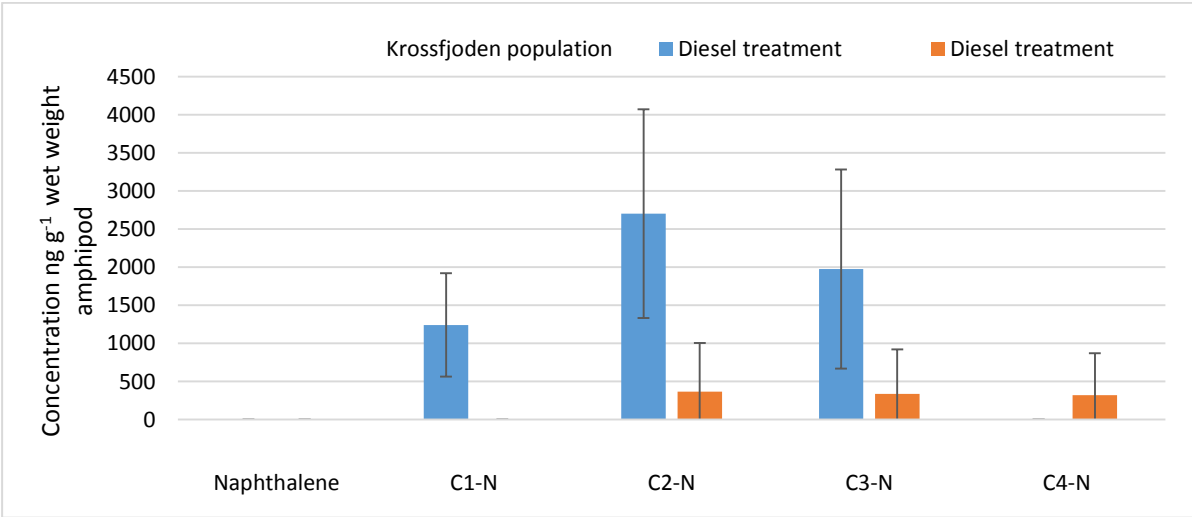
Figure 3.5 PAH detected compounds in Krossfjoden population with diesel treatment during ST2 and ST4. Only compounds with values over the detection limit are presented in the plot.
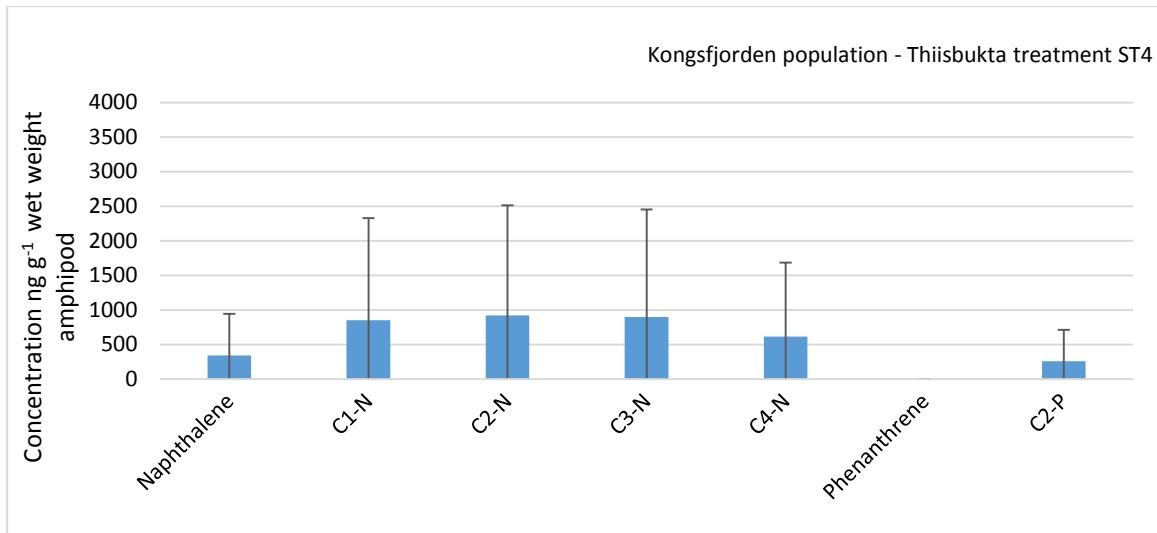
Figure 3.6 PAH detected compounds in Kongsfjorden population for Thiisbukta treatment during ST4. Only compounds with values over the detection limit are presented in the plot.

3.4 TBARs – Lipid peroxidation

Amphipods from both the Kongsfjorden and Krossfjoden populations had similar TBARs results at the start of the exposure experiment, t-test resulted in no significant difference (p=0,552). The concentration in Kongsfjorden animals reached 30.31 nmol g^{-1} WW (± 11,57SD) and 27.89 nmol g^{-1} WW (± 10,37SD) in Krossfjoden animals. Amphipods from both places had equal oxidative stress levels after the acclimation period in the laboratory (figure 3.7). Values are displayed in the appendix 11.
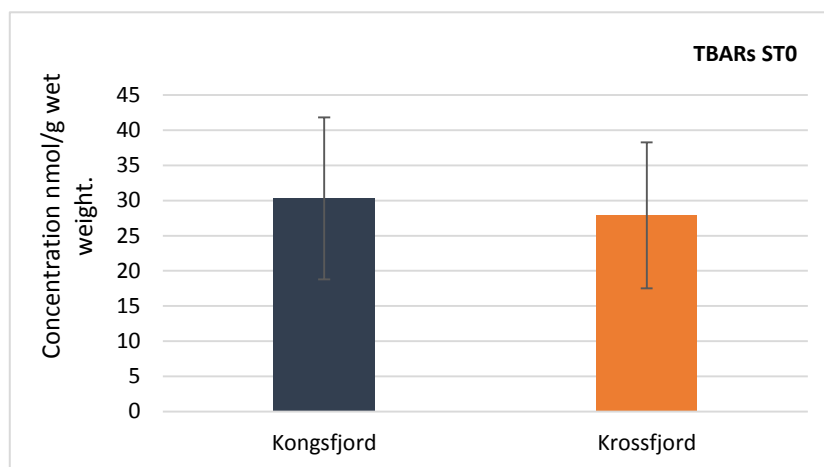
Figure 3.7 Amphipods TBARs concentration in nmol g^{-1} wet weight from the two sampling sites at the start of the exposure experiment.
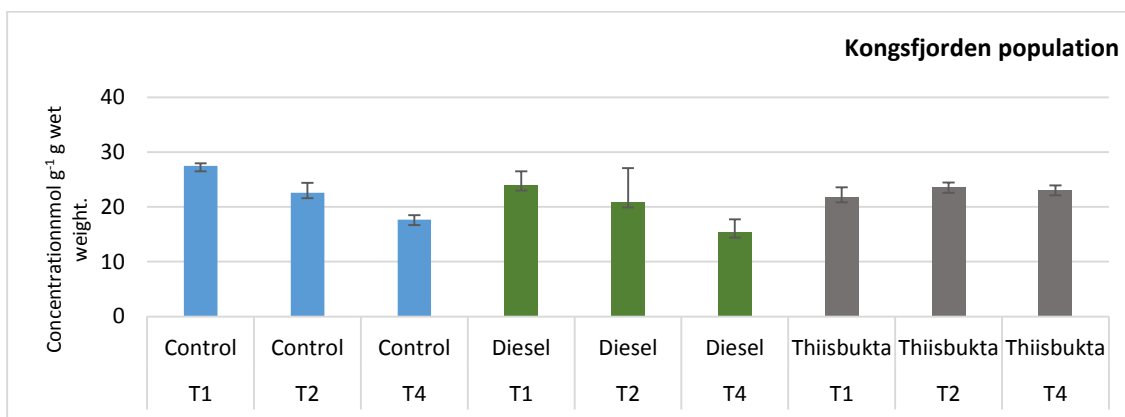
Figure 3.8 Amphipod TBARs concentration in nmol/g wet weight in ST1, ST2 and ST4 for amphipods from Kongsfjorden under all three treatments.
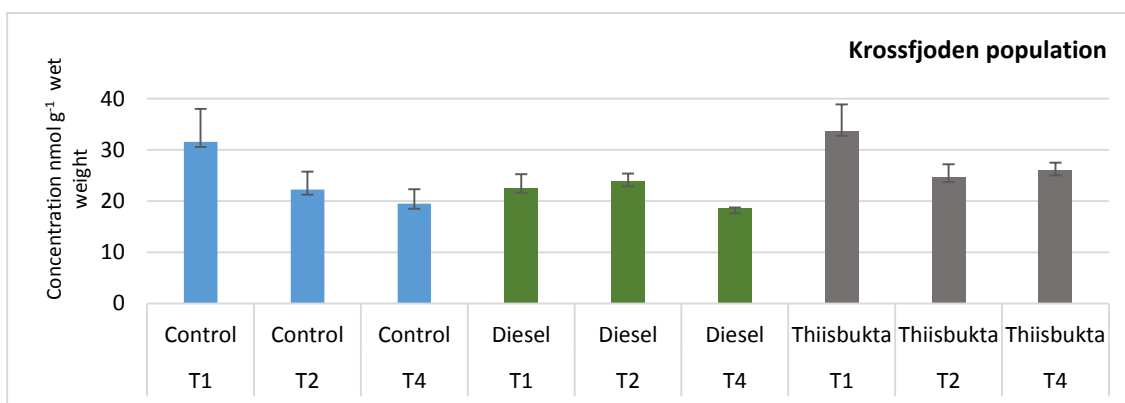
Figure 3.9 Amphipod TBARs concentration in nmol g^{-1} wet weight in ST1, ST2 and ST4 for amphipods from Krossfjoden for all three treatments.

One-way ANOVAs were performed for each population and sampling time separately. No statistical difference was obtained except for ST4 Kongsfjorden (p= 0,003) and Krossfjoden (p=0,007) populations under Thiisbukta treatment. Thus, amphipods from both fjords showed a significant difference in oxidative stress after 16 days of exposure to Thiisbukta treatment.

Krossfjoden amphipods (figure 3.9) showed an initial increase of oxidative stress values from the the ST0 to ST1 except for the MDF treatment. After that increase, TBARs levels were progressively reduced in ST2 and ST4. Kongsfjorden (figure 3.8) amphipods did not show this initial increase of lipid peroxidation levels at ST1 but a decrease in levels from ST0 to ST4 in both control and MDF treatments, ranging from 30.32 ng g^{-1} WW (ST0) to 17.67 ng g^{-1} WW

(ST4) in control treatment and to 15.44 ng g^{-1} WW (ST2) in MDF treatment. Animals from Thiisbukta treatment values were more irregular along the sampling times.

3.5 Comet assay – DNA strandbreaks

During the scoring of the comets (figure 3.10) 50 cells of each individual were quantified for DNA damage. From each individual, the median of the 50 scores was calculated and the mean of all the individuals from the same glass flask was calculated as a replicate of the treatment.
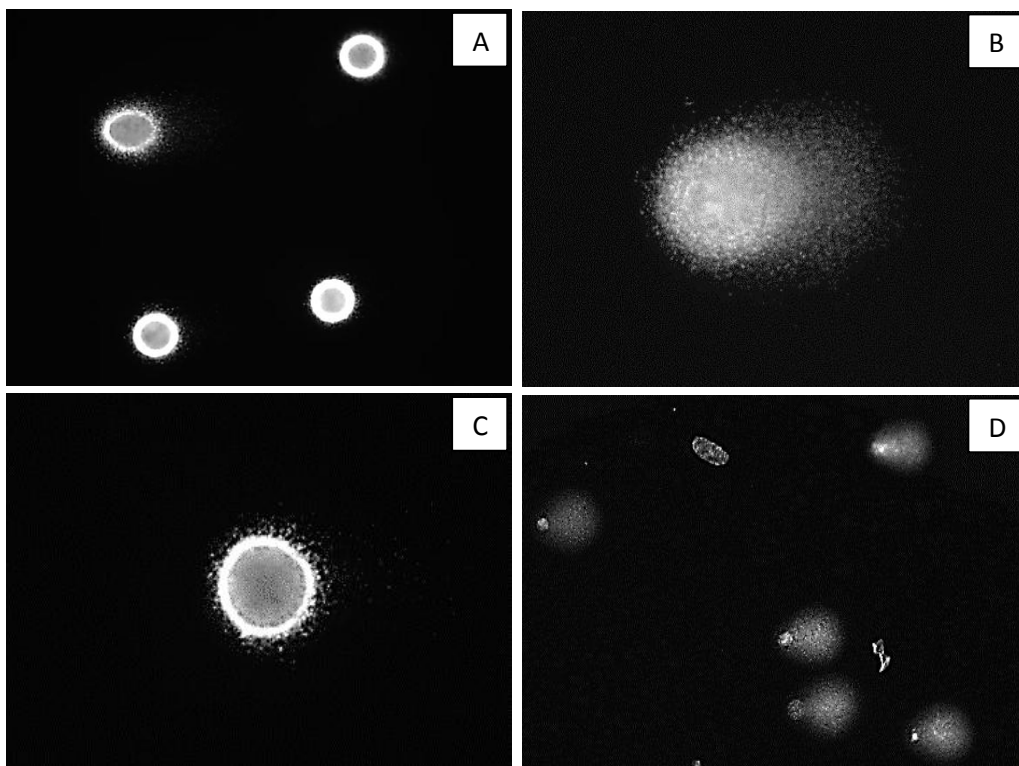
Figure 3.10 Photomicrographs of haemocytes from Gammarus setosus hemolymph extraction for Comet assay showing different DNA damage level. A, B and C: : Krossfjorden individual from diesel treatment. D: Krossfjorden individual from control treatment.

The DNA damage in amphipods haemocytes was expressed as the tail % intensity of the comet, which is the difference of the fluorescence intensity in the head and the tail, ranging from zero to 100 % damage. Haemocytes are considered appropriate cells for genotoxic studies because they are involved in physiological processes such as immune defense, transport, excretion and detoxification (Iwanaga and Lee, 2005). During the scoring process, lack of individuals from the same replicate and low cell density were the two main issues. Non-damaged cells were scored as a nucleoid core, characterized by no DNA migration or comet. Individuals showed different

levels of damage, from almost 100% of non DNA damage to a high number of nucleoids with a large comet. Also both types of nucleoids, damage and non-damage were present in the same individual.

Kongsfjorden amphipods showed an initial 6,83 % (± 6,66 % SD) tail intensity while in amphipods from the Krossfjoden was 1,16 % (± 0,11 % SD) (figure 3.11) but there was no statistically significant difference (p=0,091) between the two groups. Only cells from few Krossfjoden individuals were possible to score due to the lack or the low density of cells in the rest of individuals.
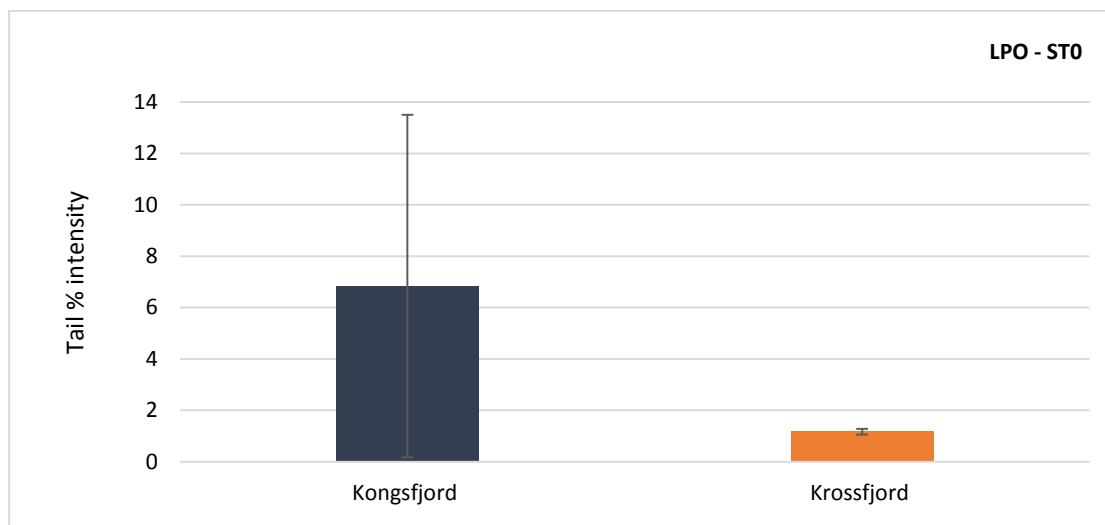
Figure 3.11 DNA damage in amphipods from the two sampling sites at the start of the exposure experiment and after acclimation.

Figures 3.12 and 3.13 shows the DNA damage resulting from the treatments at ST2 and ST4 after 4 and 16 days of exposure respectively in Kongsfjorden and Krossfjoden respectively.

In Kongsfjorden population (figure 3.12), the control treatment tail intensity values decreased during ST2 0.98% (± 082% SD) and increased to 6.41% (± 0.764% SD) during ST4. As contrary, MDF and Thiisbukta treatments showed an initial fast increase during ST2 reaching 9.89% (±8.44% SD) in MDF treatment and 10.16% (± 6.95% SD) in Thiisbukta treatment and a substantial decrease to 0.10% (±0.026% SD) in MDF and 3.95% (±0.39% SD) in Thiisbukta treatment at ST4. In Krossfjoden population (figure 3.13), the DNA damage level at ST2 remained as ST0 in all three treatments. Tail intensity values were 0,00% in control treatment,

0.68% (±0.47% SD) in MDF treatment and 0.59% (±0.54% SD) in Thiisbukta treatment. But in ST4 amphipods showed an increase in DNA damage under all three treatments, with % tail intensity 3.9% (±2.32% SD) in control treatment, 3.98% (± 4.70% SD) in MDF and 3.08% (±0,19 % SD) in Thiisbukta treatment. Tail intensity values from the Comet assay are presented in appendix 12.
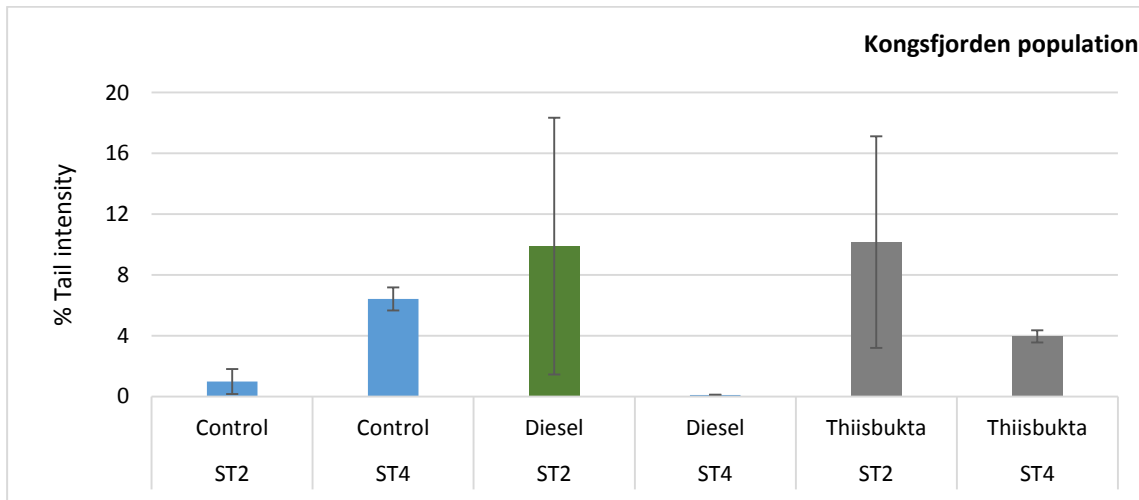
Figure 3.12 DNA damage in amphipods from Kongsfjorden for the three treatments during the sampling times ST2 and ST4
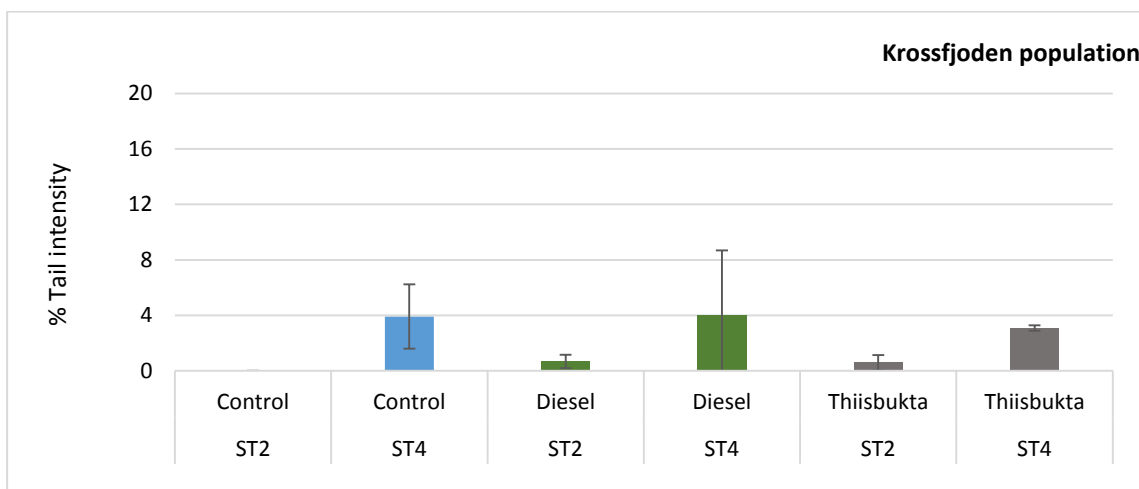
Figure 3.13 DNA damage in amphipods from Krossfjoden for the three treatments during the sampling times ST2 and ST4.

4. DISCUSSION

4.1 Polycyclic aromatic hydrocarbons concentrations in sediment

Fingerprint methods are tools to determine the pollutants source, type of contaminant, transport and hazardous concentrations in the environment, by the compound composition. The distribution pattern of the PAHs compounds has been used to identify the source of oil spills released in the environment due to the PAHs physico-chemical properties, more stable than other hydrocarbon compounds such as n-alkanes or isoprenoids (Alimi et al., 2003).

The total ΣPAH concentration in Thiisbukta sediments was higher compared with the mining pile sample and the sampling core from Thiisbukta, which may indicate the source of the PAHs from Thiisbukta is originated on the surrounding areas such as the abandoned mining area and coal seams. PAHs undergo degradation processes during weathering and the physicochemical properties of the PAHs, as well as the relative abundances of the compounds become modified by these processes (Page et al. 1996; Wang et al. 2004). In weathered crude oils, for example, higher relative concentrations of PAHs are found when compared with the unweathered oils (Alimi et al., 2003).

Naphthalene and phenanthrene compounds from the coal and sediment samples taken close to Ny-Ålesund and Thiisbukta display different concentrations among the sites which may be related to the weathering processes such as evaporation, dissolution, photooxidation and biodegradation thus reducing the total PAH concentration in sediments and the relative composition (Alimi et al. 2003). Naphthalenes (2 rings) predominate in the coal sample from the mining area while, while phenantrenes (3 ring) predominate in the Thiisbukta sediment sample. Compared to pyrogenic sources, petrogenic PAHs become more easily biodegraded due to their higher bioavailability and relatively lower association with carbon particles (Gogou et al. 2000). The 2 and 3 ringed compounds are more easily degraded while the 4 rings such as chrysenes are the more persistent (Sauer and Boehm, 1991). The microbial degradation process in both aerobic and anaerobic conditions is the factor most effective of the total degradation (Alimi et al. 2003) but also the slower one (Stogiannidis and Laane, 2015). Lighter alkylated naphthalenes are predominant in the coal sample from the mining area compared with the coal from Thiisbukta, it seems that weathering processes has been degraded the lighter C1 and C2 – naphthalenes in the coal from Thiisbukta but the C3 and C4 – naphthalenes are similar than those ones from the mining area coal. Parent PAHs and lighter alkylates are the first

compounds to be oxidized by microbes but photodegradation have effects on the higher alkylated homologs which become degraded faster than the lighter alkylated compounds (Sauer and Boehm, 1991)

The 16 US EPA priority pollutants are the main components of petrogenic fossil fuels as well as their specific alkylated homologs the "five target" naphthalene, phenantrene, dibenzothiophene, fluorene and chrysene alkylated PAHs. The C1 to C4 alkylated series are specific to fossil fuels and their relative abundance may identify the petrogenic sources since they are source specific (Boll et al. 2008; Wang et al. 2001). Fingerprints from both Konsfjord and MDF samples showed a 95 % content of alkylated PAH from the total ΣPAH which is characteristic of petrogenic sources.

In the MDF fingerprint, the main compounds were the lighter ones, mainly 2 to 3-ring which is characteristic of petroleum derivates, as well as some 4-ring compounds from the heaviest ones (Jiang et al. 2009). The 5-6 ring compounds and both anthracene and fluoranthene may result under detection in refined fuel oils while benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluroranthene and chrysene may be detected in low concentrations (Jiang et al. 2009; Sheadah et al. 2011). In contradiction fingerprints from Thiisbukta sediment sample showed compounds over the detection limit up to 6 rings such as benzo[ghi]perylene. Perylene which is also in a very low concentration in the Thiisbukta sediment, is a PAH found in coal and petroleum from diagenetic processes, with a terrigenous or marine source. In marine sediments, diatoms have been suggested to be the specific source (Venkatesan, 1988). Fingerprints from MDF and Kongsfjorden sediment samples showed the common petrogenic PAHs "bell-shape" pattern in the homologs series while the C2 and C3 alkylated PAH predominate over the parent compound as well as over the C1 and C4 alkylated homologs (Douglas et al. 2007) (appendix 11). Finally, the presence of chrysene and dibenzothiophenes in coal and Thiisbukta samples, but its absence in MDF samples is due to the chrysene alkylates are removed from diesel during the refining processes (Benze et al. 1996) and the abundances of dibenzothiophenes compounds containing sulfur, may be changed during the hydrodesulfurization process in crude oil (Page et al. 2006)

The study carried out by Dahle et al. (2006) on PAHs concentration in sediment samples from the Barents Sea showed the highest levels of PAHs in the southeast Spitsbergen shelf as well as in Svalbard inshore sediments. Total ΣPAH from the Svalbard inshore sediments concentrations

reached 8697 (± 5167 SD) ng g^{-1} DW, while in the Northwest Barents Sea reached 2109 (± 1640 SD) ngg^{-1} DW. The analyzed compounds were the same as we have studied except for alkylated pyrene, fluorene, benzo(a)anthracene and chrysene. The predominant alkylated forms were naphthalenes. In the study by Dale et al. (2006) the highest concentrations of petrogenic PAHs were found in the same areas, which were related with the Svalbard coal deposits. The identified sources in Svalbard were coal, crude oil and atmospheric dust with a petrogenic origin concluding that the hydrocarbon pollution in Svalbard was not related with human activities.

Amphipods from the Kongsfjorden population exposed to the Thiisbukta treatment showed two sampling times ST3 and ST5 with extreme concentrations of alkylated naphtalenes and phenantrenes compared with the rest of sampling times. In this case death animals were reported for sampling time ST3 but not in ST5. In Krossfjoden animals from the same treatment only the sampling time ST5 showed extreme values, but no animals were reported dead for this case. The animal deaths during the experiment may not be related with extreme PAHs concentrations in some of the glass flasks. But the PAHs analysis of those glass flask were carried out during the same period, which may indicate a methodological difference during the extraction or quantification of the pollutants.

4.2 Polycyclic aromatic hydrocarbons body burden in amphipods

The marine diesel treatment was the only treatment showing body burden concentrations over the detection limit, and being accumulated by the amphipods during two sampling times with a different pattern after 4 and 16 days. The amphipods from the Krossfjoden population accumulated higher concentrations of the lightest alkylated PAHs compared with amphipods from the Kongsfjorden population.

Coal PAHs display a lower bioavailability to organisms due to their strong association with refractory and condensed organic carbon (Stogiannidis et al. 2015; Granberg, 2004), thus the MDF PAHs are the compounds resulting more bioavailable. Also the hydrolysis and absorbance of the lightest compounds of MDF by the amphipods may be faster and easier. However, information on the environmental impact and toxicity of unburnt coal associated PAHs is vastly unexplored (Achen, 2015). Marine diesel PAHs are mainly composed by LPAH with two – three rings and their alkylated forms (Douglas et al. 2007). From the five target alkylated PAH in MDF

the most abundant is Napthalene (>55%) and the least abundant is Chrysene (0,02%) (Wang et al. 1999)

A shift from the lightest PAHs from the ST2 to the heaviest C4-napththalene during ST4 shows the elimination of the lightest compound in a lapse of time about less than 12 days and the latest bioaccumulation of the heaviest compounds. Thus a slower absorbance of the C4 alkylated napththalenes may be delayed in time by its lower bioavailability compared with the lightest alkylated naphthalenes. Sagerup et al. (2016) exposed red king crab (*Paralithodes camtschaticus*) to dispersed marine diesel oil during one week, during the three weeks of recovery in nonpolluted water, the elimination rate of the pollutant was fast, naphthalene being the faster compound to be depurated.

Glutatione-S-transferase (GST) is involved in the phase II of enzymatic biotransformation of pollutants by the excretion of ROS. (Sheehan et al., 2001). It has been also related with detoxification of MDF compounds, and pointed as a suitable biomarker for diesel contamination in molluscs (Milinkovitch et al., 2015). Studies carried out by Milinkovitch et al., (2015) and Pan et al., (2009) showed an increase of GST activity in molluscs due to the exposure to MDF in the tested group, with no effects in the control group.

The fact that the bioaccumulation of MDF without a corresponding effect in lipid peroxidation may be due to the low concentration of spiked pollutant in the sediment (910mg diesel Kg^{-1} WW sediment). Effects of pollutants exposure on antioxidants defenses in mollusks are strongly dependent on the concentration of the pollutant (Solokova et al., 2012)

4.3 TBARs - Lipid peroxidation

No differences were found between the three treatments related with the oxidative stress in the amphipods after 16 days of exposure. The lipid peroxidation levels for the MDF treatment during the three sampling times cannot be related with the bioaccumulation levels in amphipods from the same treatment. No significant difference between the three treatments were detected. At the ST4 the Thiisbukta treatment showed a significant difference in oxidative stress for amphipods from both the Kongsfjorden and Krossfjoden populations compared with the other two treatments control and MDF. Other factors rather than the pollutants may have determined the obtained results, MDF concentration could be too low to have any toxic effect on the amphipods, which triggers the degradation of the lipids. Despite of, biomarkers of stress

such as lipid peroxidation may be difficult to interpret due to the high variability influenced by biological functions as well as environmental factors (Van der Oost et al., 2003; Shaw et al. 2004; Nahrgang et al. 2013). Milinkovitch et al., (2015) commented on the lack of significant differences of lipid peroxidation, between control and MDF contaminated groups after 7 days of exposure in the Iceland scallop (*Chlamys islandica*) but marine diesel did induce an increase of LPO in the tested group.

A decrease in the peroxidation levels from ST1 to ST4 in most of the treatments and in animals from both populations may be related with the antioxidant defenses of the amphipods, which could have been triggered during the sampling and transportation of the animals to the laboratory facilities or during the acclimation period by the habitat and environmental conditions changes. Since no significant differences resulted at the beginning of the experiment ST0 between the two factors or between the treatments during the sampling times, results of TBARs are not conclusive. Camus et al. (2005) remarked the fact that polar ectotherms have a better antioxidant system compared to temperate ones, despite lower metabolic rates and endogenous ROS production characteristic of polar marine organisms (Heise et al. 2003), thus suggesting that ROS production may be a function of external factors.

Studies conducted with molluscs exposed to marine diesel, water accommodated fractions of MDF or crude oil have concluded limited increases in lipid peroxidation levels (Lüchmann et al., 2011; Milinkovitch et al., 2015; Hannam et al., 2010). Milinkovitch et al. (2015) concluded that the lack of antioxidant activity (SOD) as well as lipid peroxidation after 7 days of exposure would be related by the effectivity of the detoxification system (GST) involved in the PAHs excretion, without ROS or antioxidant production. In contrast bivalves where exposed to benzo(K)fluoranthene or benzo(a)pyrene (4-rings) in two studies (Pan et al., 2005; 2009) showing in both of them an increase in LPO.

Three enzymatic systems, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the main antioxidant enzymes in the amphipod *Gammaurs locusta* as well as in other marine invertebrates (Correia et al., 2003). Catalase converts H_2O_2 into H_2O and O_2 with a fast induction compared with other antioxidant enzymes, and glutathione peroxidase is involved in the oxidation of glutathione with H_2O_2. (Lesser, 2006; Schvezov and Amin, 2011). SOD activity as antioxidant has been proved to be fast and sensitive to hydrocarbon compounds (Milinkovitch et al., 2013).

Lipid content in amphipods species have a high percentage of PUFA in the phospholipid membrane (Clarke et al., 1985). Correia et al. (2003) measured fatty acid composition in cultured *Gammarus locusta*, determining that unsaturated acids accounted for the 70% of total fatty acids and PUFA accounted for the 40% of the unsaturated fatty acids.

No significant differences resulted at the start of the experiment (ST_0) or between the treatments but on the other hand both populations show significant differences under Thiisbukta treatment. This fact implies that amphipods from the Kongsfjorden which were exposed to the sediment collected at the site which they inhabit do show oxidative stress. Thus results from the TBARs test may be inconclusive. Individual samples should perhaps have been frozen immediately in liquid nitrogen after collected in order to measure the initial oxidative stress level, as well as before and during the acclimation period in order to check for possible variations and asses the effects of transportation and manipulation of the animals in the stress level.

4.4 Comet assay - DNA strandbreaks

Amphipods from the Kongsfjorden show a higher initial DNA damage compared with amphipods from the Krossfjoden, which can be related to the polluted environment that they inhabit close to the mining area in Ny-Ålesund, which is reasonable and expected.

In Kongsfjorden animals from the control treatment there was an initial marked decrease in DNA damage after 4 days of exposure (ST2), which may be caused by the absence of polluted sediment, an initiated DNA repair systems or enzymatic antioxidant launch. However, in the other two treatments, MDF and Thiisbukta the Krossfjodenen animals showed the opposite pattern, after 4 days the DNA damage level increased slightly to markedly decrease after 16 days of exposure. Weber et al. (2013) evaluated the effects of the WSF of heavy oil with the Comet assay after having exposed the amphipod *Quadrivisio aff. Lutzi*, finding a DNA damage oscillation along the sampling times with and increase damage after 6h and 48 h and a decrease after 24h and 72 h. Weber concluded that this pattern was a response by the DNA repair system. Morley et al. (2006) also showed a similar oscillation pattern in human skin cells expose to UVA for 25h.

Both DNA strandbreaks and repair mechanism in organisms may occur as early responses during the first hours or days after the exposure of PAHs compounds, and the level of damage

may depend on the repair system of the organism, the exposure period and the dose of pollutants (Shugart, 2000). Single strand breaks (SSB) are more quickly repaired than double strand breaks (DSBs) and DNA repair processes are found in both vertebrates and invertebrates organisms (Moustacchi, 2000)

All three treatments showed the same pattern in Krossfjoden amphipods, a low DNA damage during ST2, similar to ST0 DNA damage, followed by an increase during ST4 after 16 days of exposure. This pattern is opposite to the one observed for the Kongsfjorden amphipods, except for the control treatment and may indicate a less efficient DNA repair system in the Krossfjoden population compared with Kongsfjorden population. The fact of the different responses between both populations may be related with an adaptation to the pollutants effects in the Kongsfjorden amphipods which is not visible in the Krossfjoden amphipods. The response to PAH initiated DNA damage in Krossfjoden amphipods would be slower compared with Kongsfjorden amphipods. Costa et al. 2016 studied the DNA SSBs in *Gammarus locusta* exposed to X-rays and cooper, suggesting two possibilities to the absence of DNA strandbreaks after 48 hours of exposure, the strong induction of the repair mechanism after 48h and its depletion after 96h or the effectiveness of the repair mechanism only after a recent attach (48 h).

The fluctuations of DNA strandbreak level along the time may be result from the dynamics of the DNA damage and the reparation mechanisms (Ching et al. 2001). The detection of DNA strand breaks may lead the identification of a genotoxic pollutant effect, becoming a useful biomarker in environmental toxicity, but it will not identify the specific pollutant (Shugart, 2000).

DNA strandbreaks may be produced directly by compounds such as H_2O_2 and indirectly after the metabolic activation of certain PAHs compounds, thus the level of DNA damage originated by the toxicity of PAHs will depend on the organism antioxidant level as well as the DNA repair capacity (Mitchelmore, 1998). Environmental factors such as hypoxic and thermal stress conditions may also affect the increase of SSBs in aquatic organisms (Mitchelmore, 1998). PAHs must be biotransformed and activated in order to exert DNA damage, being the metabolites by the ROS production the compounds damaging DNA (Lee and Steinert, 2003)

5. CONCLUSIONS

The bioaccumulation and genotoxic effects of PAHs and alkylated PAHs from MDF spiked and naturally coal polluted sediments were tested in two Gammarus setosus populations which differ in their historical exposure to PAHs associated with coal.

From the two sampling sites only Kongsfjordenen showed PAHs concentrations over the detection limit, thus no pollution related with petrogenic fossil fuels were found in Krossfjoden. Alkylated PAHs were predominant in coal and sediment samples and the petrogenic source of the PAHs found in the samples remains clear since the alkylated forms accounted for the 95% of the total ΣPAHS and the PAHs alkylated forms showed the characteristic "bell-shaped". Degradation processes such as weathering, bio and photodegradation may be reasons of the modified patterns between the lightest alkylated and the heavier PAHs compounds from the different coal and sediment samples as well as the high values of total ΣPAHs detected in Thiisbukta sediments.

PAHs were not detected in the organisms exposed to Thiisbukta sediments, indicating low bioavailability of coal associated PAHs. MDF spiked sediment was the only treatment showing body burden concentrations of PAHs in *G. setosus*, from which the alkylated forms were bioaccumulated rather than the parent compounds. With time, bioaccumulation shifted from the lightest alkylated forms to the heaviest alkylated PAHs.

The bioaccumulation of PAHs from the MDF didn´t show relevant effects in the lipid peroxidation from the organisms since no differences between the three treatments were obtained, but the decreasing levels in lipid peroxidation related with a possible increase of the antioxidant system may be related with an induction of the oxidative stress in a DNA level. Amphipods from Krossfjoden showed a higher bioaccumulation of alkylated PAHs compared with Kongsford amphipods and a later response at cellular damage during ST4 when the DNA strandbreak results increased. Alkylated forms of PAHs are considered more toxic than the parent compounds.

The opposite pattern response between the two amphipods populations related with the DNA strand breaks, denotes the different pollutant adaptation of the populations. The population inhabiting the historically coaly polluted area Kongsfjorden, undergoes a repair response when oxidative stress is triggered, but for the population inhabiting the pristine area a toxic response

appeared not to be so developed. Thus a biological adaptation to survival in such an environment might be an ability.

Previous studies in relation to PAHs contamination in the Barents Sea showed the influence that the natural coal has in the northeast area, where high concentrations of PAHs are found in the sediments in the in-shore Svalbard and the shelf. This fact may influence the studies conducted in organisms when assessing PAHs levels in biota, and estimations of the effects may be misinterpreted.

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APPENDIX

Appendix 1. List of standards used in the sediment and amphipods extraction of PAHs/alkylated PAHs which was carried out by pressurized liquid extraction (PLE).

Internal standard	PAHs	Recovery standard
Naphthalene-d8	Naphthalene, Acenaphthylene	Acenaphthylene-d8
Acenaphthene-d10	Acenaphthene	Anthracene-d10
Fluorene-d10	Fluorene	Anthracene-d10
Dibenzothiophene-d8	Dibenzothiophene	Anthracene-d10
Phenanthrene-d10	Anthracene, phenanthrene	Anthracene-d10
Pyrene-d10	Pyrene, fluoranthene	Fluoranthene-d10
Chrysene-d12	Chrysene, benzo(a)anthracene	Benzo(a)anthracene-d12
Benzo(k)fluoranthene-d12	Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(e)pyrene, Benzo(a)pyrene, perylene	Benzo(a)pyrene-d12
Benzo(g,h,i)perylene-d12	Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene, Indeno(1,2,3-c,d)pyrene	Indeno(1,2,3-c,d)pyrene-d12

Appendix 2. Number of dead animals reported dead during the bioaccumulation experiment, for each treatment and amphipod population.

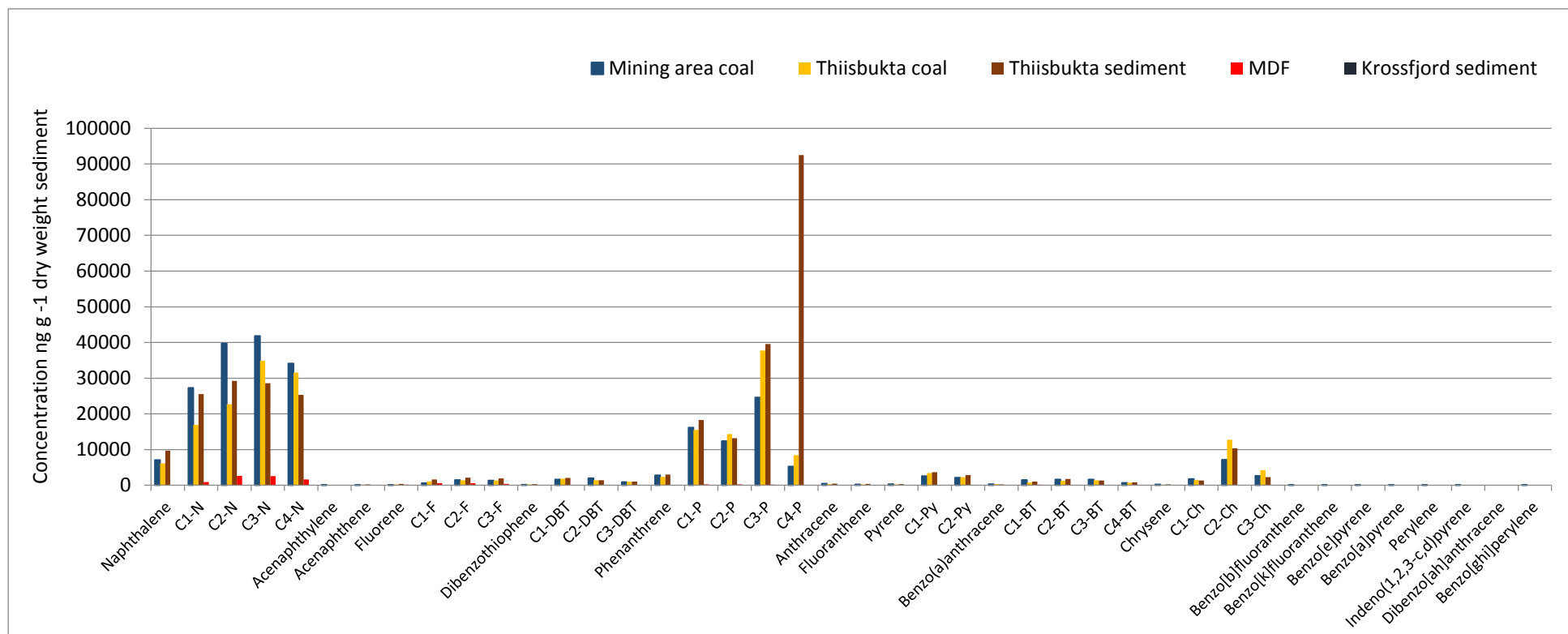| Dead animals from the bioaccumulation experiment.			
Sampling time (ST)	**Treatment**	**Population**	**Number of dead animals**
1	Control	Krossfjoden	3
1	Diesel	Krossfjoden	2
1	Thiisbukta	Krossfjoden	2
1	Control	Kongsfjorden	1
1	Diesel	Kongsfjorden	0
1	Thiisbukta	Kongsfjorden	2
2	Control	Krossfjoden	1
2	Diesel	Krossfjoden	2
2	Thiisbukta	Krossfjoden	2
2	Control	Kongsfjorden	0
2	Diesel	Kongsfjorden	3
2	Thiisbukta	Kongsfjorden	1
3	Control	Krossfjoden	2
3	Diesel	Krossfjoden	5
3	Thiisbukta	Krossfjoden	4
3	Control	Kongsfjorden	24
3	Diesel	Kongsfjorden	0
3	Thiisbukta	Kongsfjorden	25
4	Control	Krossfjoden	7
4	Diesel	Krossfjoden	7
4	Thiisbukta	Krossfjoden	8
4	Control	Kongsfjorden	12
4	Diesel	Kongsfjorden	8
4	Thiisbukta	Kongsfjorden	16
5	Control	Krossfjoden	16
5	Diesel	Krossfjoden	16
5	Thiisbukta	Krossfjoden	13
5	Control	Kongsfjorden	29
5	Diesel	Kongsfjorden	21
5	Thiisbukta	Kongsfjorden	12

Appendix 3. Detection limits for sediment and biological samples.

Code	PAH / alkylated PAH	Limit of Detection (LOD)	Limit of Quantification (LOQ)	Limit of Detection (LOD)	Limit of Quantification (LOQ)
		Sediment samples		Amphipod samples	
1	Naphthalene	0,068711	0,148059	0,26804	0,89348
2	C1-N	0,068711	0,148059	0,54673	1,82244
3	C2-N	0,068711	0,148059	0,54673	1,82244
4	C3-N	0,068711	0,148059	0,54673	1,82244
5	C4-N	0,068711	0,148059	0,54673	1,82244
6	Acenaphthylene	0,025125	0,078511	0,09534	0,31780
7	Acenaphthene	0,033639	0,105081	0,32159	1,07197
8	Fluorene	0,015580	0,051934	0,16765	0,55882
9	C1-F	0,068711	0,148059	0,54673	1,82244
10	C2-F	0,068711	0,148059	0,54673	1,82244
11	C3-F	0,068711	0,148059	0,54673	1,82244
12	Dibenzothiophene	0,034591	0,115304	0,47668	1,58893
13	C1-DBT	0,068711	0,148059	0,54673	1,82244
14	C2-DBT	0,068711	0,148059	0,54673	1,82244
15	C3-DBT	0,068711	0,148059	0,54673	1,82244
16	Phenanthrene	0,034324	0,090214	0,34910	1,16365
17	C1-P	0,068711	0,148059	0,54673	1,82244
18	C2-P	0,068711	0,148059	0,54673	1,82244
19	C3-P	0,068711	0,148059	0,54673	1,82244
20	C4-P	0,068711	0,148059	0,54673	1,82244
21	Anthracene	0,009781	0,032602	0,03500	0,11666
22	Fluoranthene	0,003265	0,007285	0,00744	0,02479
23	Pyrene	0,007320	0,016712	0,10843	0,36142
24	C1-Py	0,068711	0,148059	0,54673	1,82244
25	C2-Py	0,068711	0,148059	0,54673	1,82244
26	Benzo(a)anthracene	0,014708	0,049027	0,09899	0,32996
27	C1-BT	0,068711	0,148059	0,54673	1,82244
28	C2-BT	0,068711	0,148059	0,54673	1,82244
29	C3-BT	0,068711	0,148059	0,54673	1,82244
30	C4-BT	0,068711	0,148059	0,54673	1,82244
31	Chrysene	0,041786	0,139286	0,33063	1,10209
32	C1-Ch	0,068711	0,148059	0,54673	1,82244
33	C2-Ch	0,068711	0,148059	0,54673	1,82244
34	C3-Ch	0,068711	0,148059	0,54673	1,82244
35	Benzo[b]fluoranthene	0,042062	0,140207	0,02520	0,08401
36	Benzo[k]fluoranthene	0,038046	0,126818	0,08484	0,28279
37	Benzo[e]pyrene	0,023991	0,079969	0,08123	0,27075
38	Benzo[a]pyrene	0,031672	0,105574	0,12415	0,41383
39	Perylene	0,000076	0,000253	0,11027	0,36756
40	Indeno(1,2,3-c,d)pyrene	0,008784	0,029279	0,50523	1,68411
41	Dibenzo[ah]anthracene	0,014265	0,047549	0,36353	1,21177
42	Benzo[ghi]perylene	0,011664	0,038879	0,54673	1,82244

Appendix 4. Total PAH concentrations in coal and sediment samples from the mining area in Ny-Ålesund, Thiisbukta coal and both sampling sites from the exposure experiment, Thiisbukta and Krossfjoden. Values are expressed in mg kg $^{-1}$ dry weight.

	Mining area coal	Thiisbukta coal	Thiisbukta sediment	MDF spiked sediment	Krossfjoden sediment
Total PAH in coal and sediment samples					
Total 16 US EPA PAH	12	10	16	0	0
Total alkylated PAH	231	219	308	10	0
Total ΣPAH	243	229	324	11	0

Appendix 5. Fingerprints plot for the PAHs compounds from the two coal sampling sites and the exposure treatments Thiisbukta, MDF and Krossfojrd (control treatment). Concentrations are presented in ng g^{-1}

PAH concentration in ng g-1 dryweight sediment, Including LOD

Sample Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Mining area coal	7100	27281	39709	41808	34092	80	94	96	591	1459	1304	193	1609	2010	889	2790	16140	12355	24572	5273	475	229	314	2600	2161	325	1447	1645	1608	691	217	1789	7154	2631	44	53	32	87	120	12	0	18
Thiisbukta coal	6100	16875	22731	34836	31589	71	147	212	1086	1440	1357	258	1872	1389	949	2356	15502	14336	37760	8445	338	265	262	3433	2299	289	734	1270	1426	730	198	1508	12754	4264	79	63	53	66	64	16	0	24
Thiisbukta sediment	9700	25556	29224	28580	25321	91	271	429	1593	2163	1892	335	2036	1437	1067	2986	18252	13180	39577	92492	478	380	348	3681	2838	284	1029	1760	1353	795	253	1365	10359	2255	68	98	70	99	107	18	0	48
MDF	80	874	2651	2579	1623	0	0	147	598	645	417	0	0	0	0	67	259	281	173	0	0	3	9	0	0	0	151	105	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Krossfjord sediment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Standard deviation

Sample Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Mining coal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiisbukta coal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MDF Kongsfjord	52	173	411	342	180	0	0	23	287	60	49	0	0	0	0	9	35	34	20	0	0	1	1	0	0	0	19	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thiisbukta sediment	958	4291	6874	4268	5112	12	51	179	248	292	162	69	295	258	370	407	3569	3302	11198	39571	74	114	109	833	289	100	115	217	623	88	81	251	2274	1204	26	51	37	42	31	22	0	48
Krossfjord sediment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 7. Total PAHs concentrations in sediments from the three different treatments during the experiment exposure sampling times for both Kongsfjorden and Krossfjoden populations. Values are presented in mg kg $^{-1}$.

Kongsfjord population

TREATMENT	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta
Sampling time (ST)	ST1	ST1	ST1	ST2	ST2	ST2	ST3	ST3	ST3	ST4	ST4	ST4	ST5	ST5	ST5
Total US EPA 16 PAH	0	0	17	0	0	15	0	0	27	0	0	16	0	0	25
Total alkylated PAH	0	9	285	0	9	267	0	10	708	0	9	198	0	11	488
Total ΣPAH	0	10	302	0	9	283	0	10	736	0	10	215	0	11	518

Krossfjord population

Treatment	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta	Control	Diesel	Thiisbukta
Sampling time (ST)	ST1	ST1	ST1	ST2	ST2	ST2	ST3	ST3	ST3	ST4	ST4	ST4	ST5	ST5	ST5
Total US EPA 16 PAH	0	0	16	0	0	14	0	0	15	0	0	17	0	0	30
Total alkylated PAH	0	11	263	0	8	248	0	11	295	0	11	245	0	10	614
Total ΣPAH	0	12	280	0	8	262	0	12	311	0	11	263	0	10	645

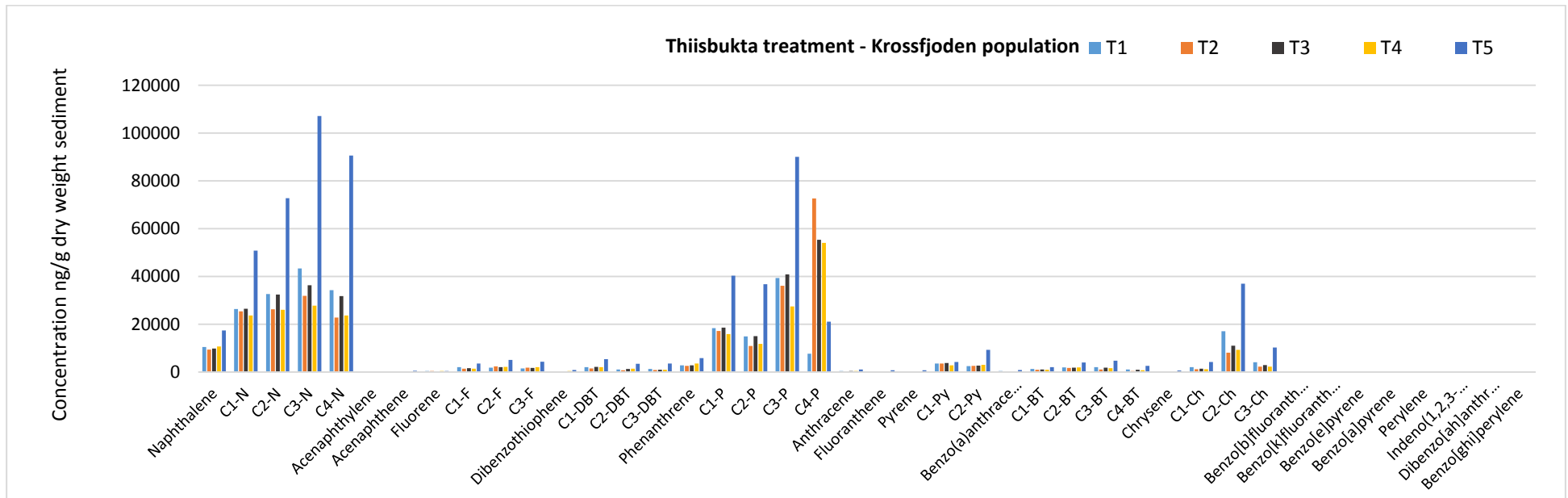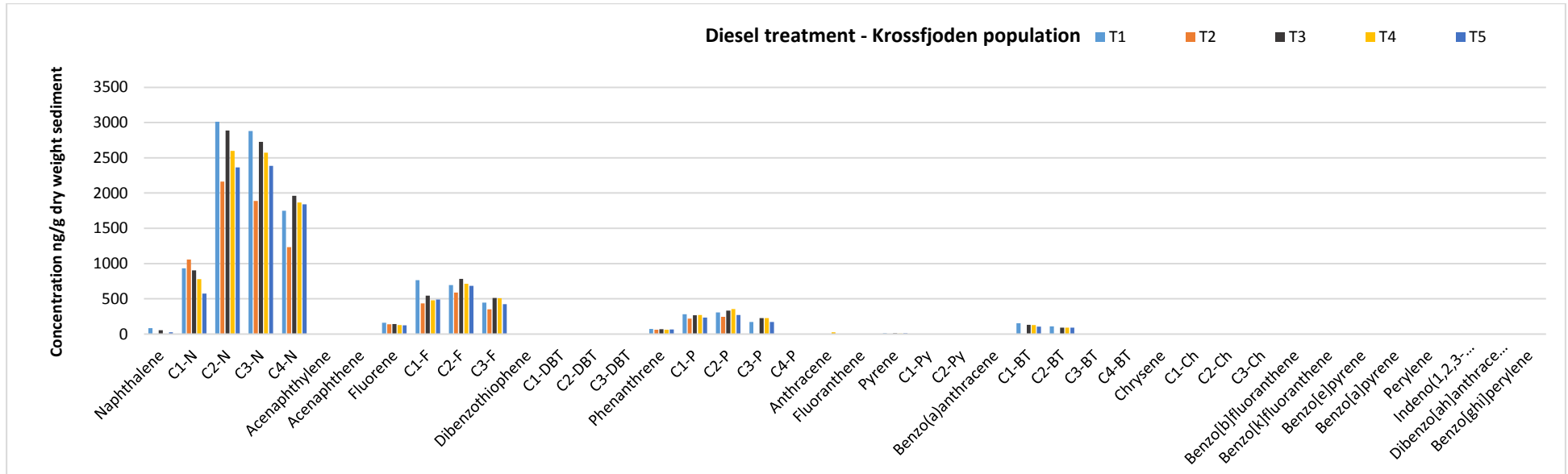**Appendix 8**. Fingerprints PAHs concentrations for Kongsfjorden population for each treatment during the five sampling times.
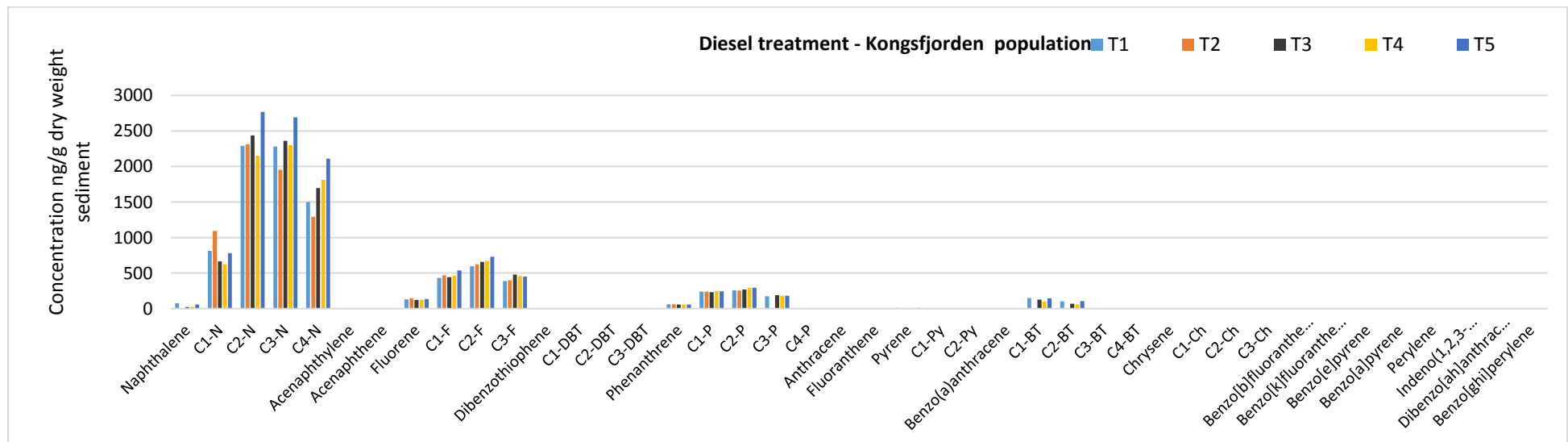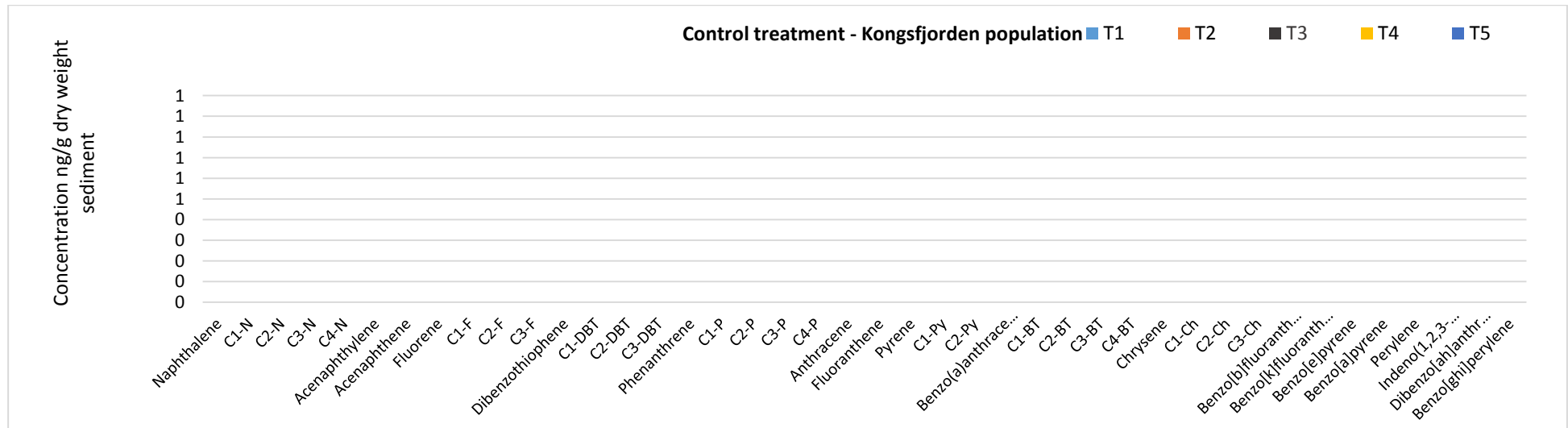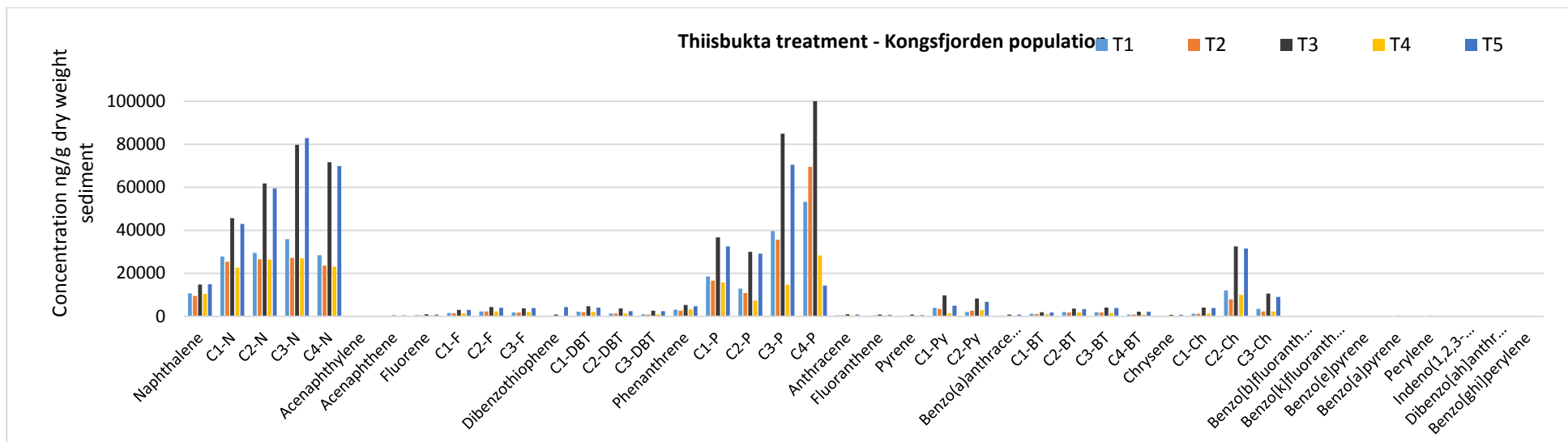
Appendix 9. Fingerprints PAHs concentrations for Krossfjoden population for each treatment during the five sampling times.

Diesel treatment - Krossfjoden population (T1, T2, T3, T4, T5)

Thiisbukta treatment - Krossfjoden population (T1, T2, T3, T4, T5)
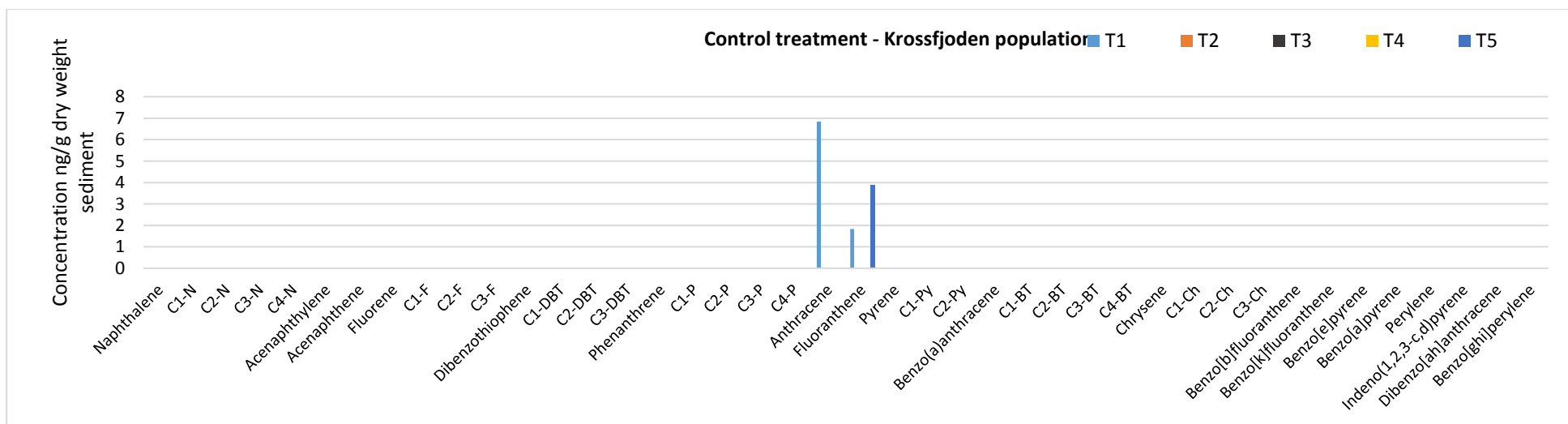
Appendix 10. PAHs body burden in amphipods. Only the detected values are presented and expressed in ng g^{-1} wet weight.

PAHs concentrations in amphipods									
Sampling time	Treatment	Amphipod	Naphthalene	C1-N	C2-N	C3-N	C4-N	Phenanthrene	C2-P
T2	Diesel	Krossfjoden	0	1242	2702	1975	0		
T2	Diesel	Kongsfjorden	0	317	1579	939	639		
T4	Diesel	Krossfjoden	0	0	368	337	319		
T4	Diesel	Kongsfjorden	0	0	274	266	237		
T4	Thiisbukta	Kongsfjorden	345	853	920	898	616	0	262

Standard deviation									
Sampling time	Treatment	Amphipod	Naphthalene	C1-N	C2-N	C3-N	C4-N	Phenanthrene	C2-P
T2	Diesel	Krossfjoden	0	679	1369	1305	0		
T2	Diesel	Kongsfjorden	0	549	486	456	599		
T4	Diesel	Krossfjoden	0	0	637	585	553		
T4	Diesel	Kongsfjorden	0	0	474	461	411		
T4	Thiisbukta	Kongsfjorden	597	1477	1593	1556	1067	0	454

Appendix 11. Lipid peroxidation results by the TBARs assay on amphipods collected at the start of the experiment and sampling times ST1, ST2 and ST4. Values expressed in nmol g^{-1} wet weight.

Lipid peroxidation					
		Kongsfjorden		Krossfjoden	
Sampling time	Treatment	TBARs nmol/g ww.	Stand dev.	TBARs nmol/g ww.	Stand dev.
T0		30,32	11,512	27,90	10,377
T1	Control	27,49	0,478	31,57	6,472
T2	Control	22,62	1,797	22,28	3,469
T4	Control	17,67	0,852	19,50	2,784
T1	Diesel	24,04	2,498	22,64	2,620
T2	Diesel	20,93	6,166	23,90	1,464
T4	Diesel	15,44	2,277	18,61	0,152
T1	Thiisbukta	21,84	1,726	33,78	5,129
T2	Thiisbukta	23,62	0,831	24,71	2,501
T4	Thiisbukta	23,11	0,856	26,02	1,486

Appendix 12. DNA damage quantification by the Comet assay on amphipods collected at sampling times 2 and 4, after 4 and 16 days of exposure to the treatments. Damage is expressed in terms of tail % intensity in relation to the head intensity of the comet.

		DNA damage % tail intensity			
		Kongsfjorden		Krossfjoden	
Sampling time	Treatment	Tail intensity	Standard dev.	Tail intensity	Standard dev.
T2	Control	0,98	0,826	0,00	0,000
T4	Control	6,41	0,764	3,92	2,323
T2	Diesel	9,89	8,449	0,68	0,479
T4	Diesel	0,10	0,026	3,98	4,703
T2	Thiisbukta	10,16	6,958	0,59	0,544
T4	Thiisbukta	3,95	0,396	3,08	0,199

Appendix 13. Distribution of parent PAHs and alkylated PAHs as function of the pollutant source. First histogram shows the "bell-shape" pattern characteristic of the petrogenic PAHs with mainly alkylated compounds. Second and third histograms show the skewed pattern with parent compound predominant over alkylated compounds (Stout et al. 2015)