University of Stavanger

**Faculty of Science and Technology**

# Master's Thesis

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# Abstract

Inner Oslofjord is vulnerable to contamination due to its enclosed features and threshold separated pools. The area is one of the most densely populated in Norway, which entails to high release of environmental contaminants. The purpose of this study was to investigate the effects of these contaminants on the cod populations in the area. This was conducted by using the outer fjord as a reference site. Both physiological indices and biomarkers were used to assess the effects from planar organic compounds, genotoxic compounds and heavy metals.

In the middle of December 2017, 80 cod specimens were collected in total, 40 from each of the sites. The physiological indices showed little difference. Both condition index (CI) and liver somatic index (LSI) showed no difference, but there was found higher values for gonadosomatic index (GSI) in the males from inner fjord.

In cod bile, higher concentrations of 2,3-ring and 4-ring polyaromatic hydrocarbon (PAH) metabolites were found in inner fjord compared to outer fjord. This indicates a higher exposure of PAHs for cod in the inner fjord compared to the outer fjord. No difference was found in Metallothionein content in fish liver when comparing the monitored areas, which indicates a heavy metal exposure of similar degree. In blood, the δ-aminolevulinic acid dehydratase (ALA-D) activity did not show any significant difference regarding lead exposure in the cod sampled in inner fjord compared to the ones sampled in outer fjord. The EROD activity measured in the liver did also not show any significant difference between the cods from inner and outer fjord, indicating an exposure of planar organic contaminants like polychlorinated biphenyls (PCBs) of similar degree.

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# Abbreviations

| | |
|---|---|
| ALA | δ-aminolevulinic acid |
| ALA-D | δ-aminolevulinic acid dehydratase |
| ANOVA | Analysis of variance |
| B[a]P | Benzo[a]pyrene |
| BOD | Biological oxygen demand (5 days test) |
| BSTFA | N,O-Bis(trimethylsilyl)trifluoroacetamide |
| Cd | Cadmium |
| CI | Condition index |
| COD | Chemical oxygen demand |
| Cu | Copper |
| CYP | Cytochrome P450 |
| CYP1A | Cytochrome P450 1A |
| DMSO | Dimethyl sulfoxide |
| DTNB | 5,5-dithiobis-2-nitrobenzic acid |
| EDTA | Ethylenediamine tetraacetic acid |
| ERA | Environmental risk assessment |
| EROD | 7-ethoxy-resorufin-O-deethylase |
| FF | Fixed fluorescence |
| GSH | Glutathione |
| GSI | Gonadosomatic index |
| $H_2O_2$ | Hydrogenperoxide |
| HCl | Hydrochloric acid |
| Hg | Mercury |
| MT | Metallothionein |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NFR | Nordre Follo renseanlegg |
| OH-PAH | OH-polyaromatic hydrocarbon |
| PAH | Polyaromatic hydrocarbon |
| Pb | Lead |
| PBG | Porphobilinogen |
| PCA | Principal component analysis |
| PCB | Polychlorinated biphenyls |
| PCDD | Polychlorinated dibenzodioxins |
| PFE | Pyrene fluorescence equivalents |
| PMSF | Phenylmethylsulfonyl fluoride |

| | |
|---|---|
| RA | Treatment plant (renseanlegg) |
| RT | Room temperature |
| SFS | Synchronous fluorescence spectroscopy |
| TMS | Trimetylsilyl |
| UC | Ultra-centrifuge |
| UV | Ultra violet |
| VEAS | Central treatment plant west |
| Zn | Zinc |

# 1. Introduction

## 1.1 Aim of study

The aim of this thesis was to monitor the presence of pollutants in inner Oslofjord using Atlantic cod (*Gadus morhua*) collected in the fjord as a bioindicator. The set of biomarkers assessed included poly aromatic hydrocarbon (PAH) metabolites, 7-ethoxy-resorufin-O-deethylase (EROD) activity, metallothionein (MT) content, δ-aminolevulinic acid dehydratase (ALA-D) activity, condition index (CI), liver somatic index (LSI) and gonadosomatic Index (GSI).

Biomarker results were also compared to previous year's results to find a pattern or change in the ecological state of the fjord.

## 1.2 Pollution in fjords

Marine environments can often act as the ultimate sink for environmental pollutants. Toxic compounds released in the environment may end up in some sort of water system. In fjords, where the effluents are not dispersed in the open sea very rapidly, pollution is of great concern. Several reports have been published showing increased concentration of heavy metals as well as chemical contaminants in water from such areas (Haug *et al.*, 1974).

### 1.2.1 Municipal wastewater

Municipal wastewater discharges and water pollution are linked together. Ever since larger cities created water pipes and sewers, water pollution started to be a concern (Arnesen, 2001). Domestic wastewater was used as irrigation already 3200 BC and was later also used for disposal and fertilizer purposes in Athene and Rome. It wasn't before heavy industrialization and urbanization, in the mid-19$^{th}$ century modern wastewater systems were built as a reaction to aggravation of unsanitary conditions. Contaminated water lead to cholera outbreaks in London in 1832, 1849 and 1855 killing tens of thousands, and in 1858 untreated human waste lead to the Great Stink in the River Thames (Angelakis and Snyder, 2015).

In Oslo, water pollution was discussed as early as the late 19$^{th}$ century. The first wastewater-treatment plant was built in 1910, and in the following decades, several treatment plants and intercepting sewerage systems were built. However, due to political disagreement about the source of pollution, which treatments that were necessary and generally insufficient knowledge of the situation, a complete sewerage system which connected all the households in Oslo to a treatment plant was not in place before 1983 (Arnesen, 2001).

## 1.3 Oslofjord

Oslofjord is a Norwegian fjord on the Skagerak trait which "splits" the southern coast for 100 km from about Fredrikstad to Oslo. It is roughly 2000 km$^2$ in total and was formed by glacier depression. Along the shoreline it is possible to find several small towns and the area is generally forested. This is one of Norway's most densely populated areas and is in continuous growth. The fjord is seperated into inner and outer fjord by the Drøbak Sound.

### 1.3.1 Inner Oslofjord

The inner fjord is an enclosed fjord with a size of 190 km$^2$ and several threshold-separated pools. (*figure 1.1*). The two biggest pools are Vestfjorden and Bunnefjorden which are as deep as 150-160 m (Arnesen, 2001, Lundsør *et al.,* 2017). All the water exchange between the inner fjord and Skagerak happens through the Drøbak Sound, which at its most critical spot is only about 20 m deep and 1 km wide. This results in a reduced water effluent dispersion into open seawater, meaning that pollutants and nutrients released into the fjord is of great concern.

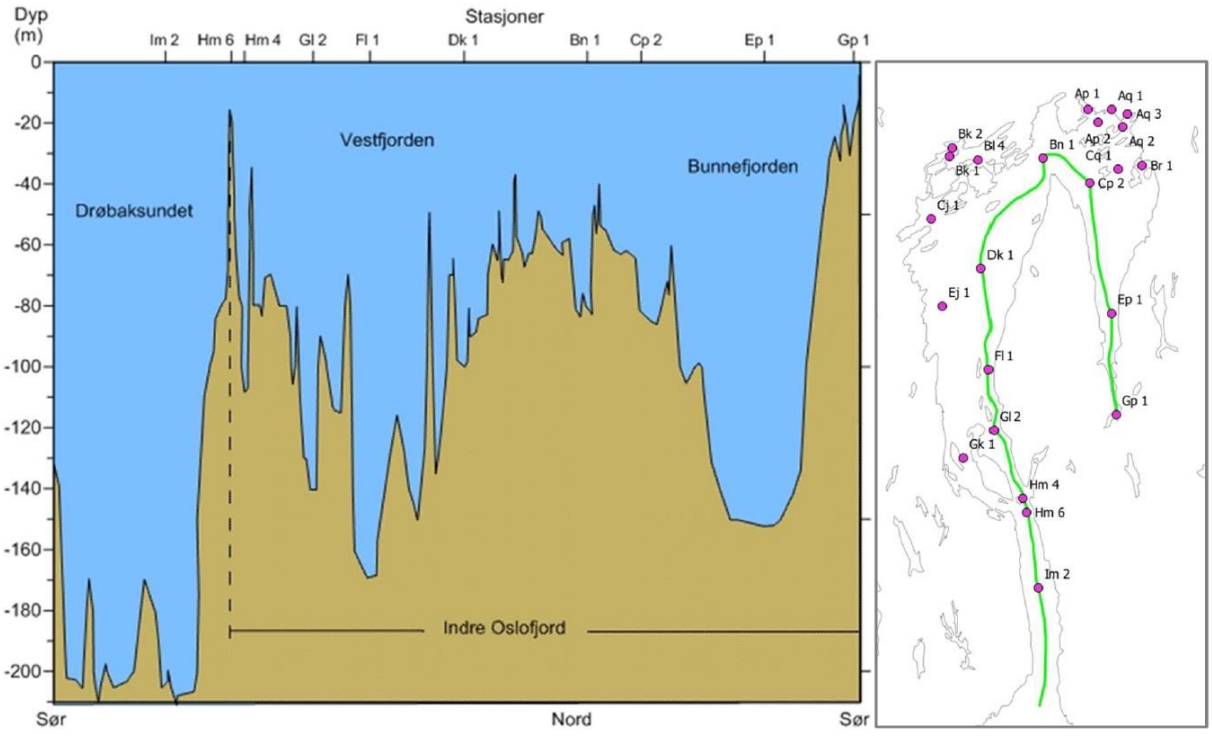


Figure 1.1 Topography of inner Oslofjord (left). The different thresholds result in lower effluent into the open sea, especially in the inner most part. Dyp; depth, stasjoner; stations, sør; south, nord; north. On the right is the route used to draw this profile (Lundsør *et al.,* 2017).

The main concern in inner Oslofjord has been massive release of organic materials and nutrients and also the low water exchange with fresh seawater from Skagerak. This is mainly due to the narrow inlet and shallow threshold in Drøbak. Water exchange of the bottom part of the fjord happens yearly in

Vestfjorden, and only every 3-4 years in Bunnefjorden. This can however be influenced by longer periods of north wind during the winter and higher density difference between the water streaming into the fjord and the water already there (Lundsør *et al.*, 2017). The high amount of available nutrition did result in increased eutrophication in the photic zone, which eventually lead to increased oxygen consumption. This is more crucial in the bottom part on the fjord, where algae residues and other organic material are broken down, resulting in a high oxygen debt.

Several decades of discussions, politics and disagreement on the reason behind the bad conditions in the fjord, postponed the improvement of the water condition. The failure of realizing the important of reducing nutrient release, not just organic matter, and a belief of much better self-purifying capacity of the fjord played a major part in this (Arnesen, 2001). The importance of nutrient removal was recognized in the 1970s, resulting in decreasing release until a minimum around 2002-2003 and has now slightly increased and stabilized (*Figure 1.2, Figure 1.3*) (Arnesen, 2001, Selvik and Høgåsen, 2016).

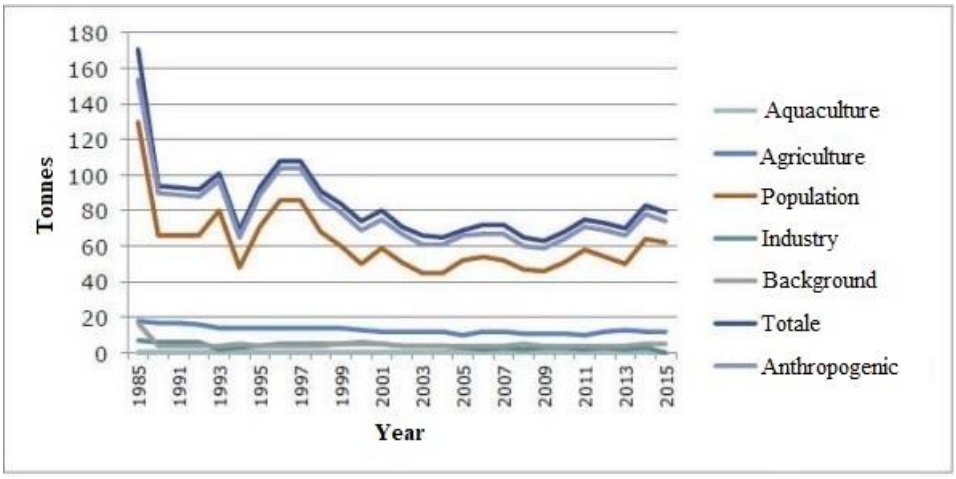


Figure 1.2 Anthropogenic release of phosphorous to inner Oslofjord (Selvik and Høgåsen, 2016)

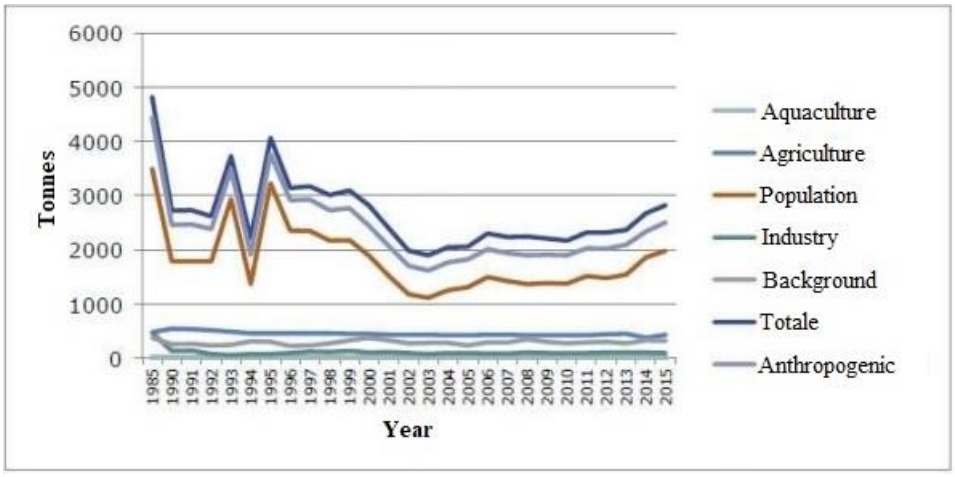


Figure 1.3 Anthropogenic release of nitrogen to inner Oslofjord (Selvik and Høgåsen, 2016)

Even though the continuous load of nutrients and organic materials have declined and stabilized at a much lower value, there is still a huge amount of oxygen debt in the fjord sediments. Consequently, the restitution of the bottom fauna is delayed. Good oxygen conditions are crucial to maintain both biodiversity and water quality (Lundsør *et al.*, 2017).

### 1.3.2 Effluents in inner Oslofjord

The wastewater effluents into inner Oslofjord come from several sources, and the main contributor of nitrogen, phosphorous and organic materials comes from householding wastewater via treatment plants. Even though the main concern has been nutrients and organic matter, the industrialization and urbanization of the area have led to releases of industrial wastes (e.g. PAHs, PCBs and heavy metals) which can cause severe effects on the biota and the ecosystem. These contaminants can be mutagenic, carcinogenic and supress immune-system functions and potentially cause biological damage to organisms (Abdel-Shafy and Mansour, 2015). Thus, they have gotten a lot of attention and restrictions to preserve the environment.

As mentioned before, the population growth in the area in later years has led to more pollutant releases into the fjord, even though it is still within given limits from the government. Regarding inner Oslofjord, which has been a major priority due to the population density, there are five main waste water facilities (*figure 1.4*). They are VEAS (central treatment plant west), Fagerstrand RA (RA stands for treatment plant), Buhrestua RA, Bekkelaget RA and NFR (Nordre Follo Treatment plant).

The biggest wastewater treatment plants in inner Oslofjord is the Central treatment plant west (VEAS) located at Bjerkåsholmen close to Vollen (*Figure 1.4)*. This facility is treating the water from 600 000 people which is about 11 % of the Norwegian population and has been running since 1982.

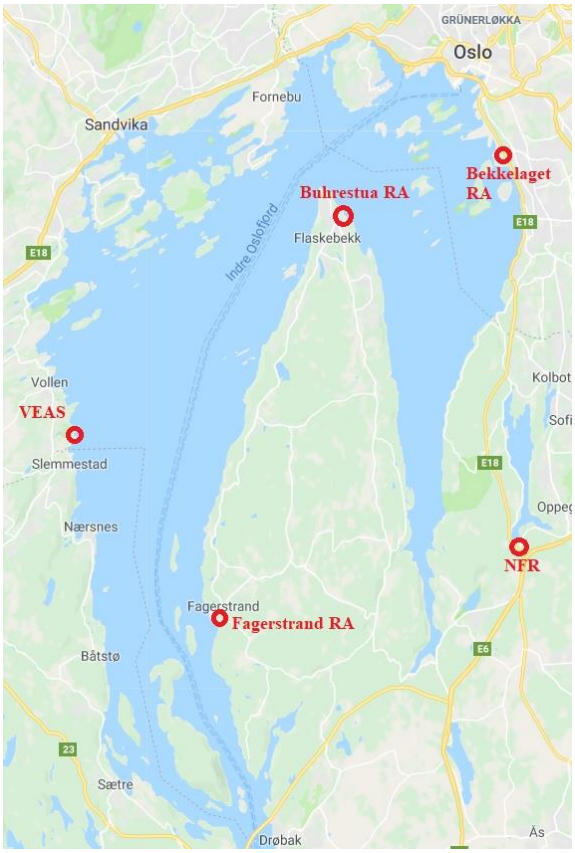

Figure 1.4 Overview over treatment plants in inner Oslofjord

The effluents from VEAS contains several environmental hazardous compounds, but the amount of the most severe ones are at a stable low level as a result of heavy restrictions and focus on environmental friendly conditions. Nitrogen, phosphorous, chemical oxygen demand (COD) and biological oxygen demand (5 days test) (BOD) are increasing, but still at a much lower rate than before. The annual releases of compounds from the waste water effluents from VEAS is shown in *Table 1.1*.

Table 1.1 Components from municipal wastewater effluent from the Central treatment plant west (VEAS) 2012-2017 (norskeutslipp.no)

| Component | Unit | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 |
|---|---|---|---|---|---|---|---|
| Arsenic (As) | kg/year | 62,4 | 42,1 | 62,4 | 48,5 | 45,0 | 54,6 |
| Cadmium (Cd) | kg/year | 5,33 | 5,23 | 6,10 | 5,80 | 5,00 | 5,18 |
| Chromium (Cr) | kg/year | 58,5 | 56,0 | 62,6 | 78,0 | 52,0 | 53,6 |
| Copper (Cu) | kg/year | 2143 | 612 | 804 | 785 | 552 | 516 |
| Mercury (Hg) | kg/year | 0,31 | 0,29 | 0,41 | 0,37 | 0,33 | 0,34 |
| Nitrogen, total | tonnes/year | 851 | 903 | 1208 | 1401 | 1382 | 1424 |
| Nonylphenol and nonylphenol ethoxylates | g/year | 540 | 470 | 630 | 580 | 650 | 591 |
| Nickel (Ni) | kg/year | 196 | 231 | 264 | 306 | 236 | 298 |
| Phosphorus, total | tonnes/year | 29,2 | 29,7 | 34,2 | 32,5 | 26,1 | 32,7 |
| Lead (Pb) | kg/year | 45,7 | 37,1 | 78,9 | 82,0 | 49,0 | 42,9 |
| Zinc (Zn) | kg/year | 2046 | 1777 | 2016 | 2324 | 1933 | 2113 |
| Hexabromocyclododecane (HBCD / HBCDD) | kg/year | 0,54 | 0,47 | 0,63 | 0,43 | 0,39 | 0,50 |
| Polychlorinated biphenyls (PCB7) | g/year | 540 | 470 | 630 | 580 | 520 | 1642 |
| 2,2',6,6'-tetrabromobisphenol A (TBBPA) | g/year | 540 | 470 | 630 | 429 | 387 | 495 |
| Di-(2-ethylhexyl) phthalate (DEHP) | kg/year | 48,8 | 42,6 | 73,4 | 45,6 | 40,0 | 54,0 |
| 2,2',4,4'-tetrabromodiphenyl ether (BDE47) | g/year | 270 | 230 | 320 | 215 | 193 | 169 |
| 2,2',4,4',5-pentabromodiphenyl ether (BDE99) | g/year | 270 | 230 | 320 | 215 | 193 | 169 |
| 2,2',4,4',6-pentabromodiphenyl ether (BDE100) | g/year | 270 | 230 | 320 | 215 | 193 | 168 |
| 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183) | g/year | 270 | 230 | 320 | 215 | 193 | 178 |
| Bis(pentabromophenyl)ether(deca-BDE) | g/year | 540 | 900 | 790 | 429 | 387 | 652 |
| PAH-16 (NS9815) | kg/year | 0,66 | 0,47 | 0,63 | 0,58 | 0,52 | 1,31 |
| PAH Total | kg/year | 0,66 | 0,47 | 0,63 | N/A | N/A | N/A |
| Chemical oxygen demand (COD) | tonnes/year | 3559 | 4334 | 6295 | 6791 | 5339 | 5393 |
| Suspended substance (SS) | tonnes/year | 881 | 1045 | 2099 | 2084 | 1479 | 1532 |
| Biochemical oxygen demand (BOD), 5 days | tonnes/year | 811 | 1081 | 1948 | 2443 | 1774 | 1642 |

Previously, environmental toxicants like PAHs, PCBs and heavy metals has mainly been released into the fjord from treatment plants. Due to heavy restrictions and newer treatment procedures at the plants, these releases have been reduced. As a result, the main source of these contaminants are no longer effluents from the treatment plants. As the new sources were not recognised in previous decades, not much info about the amount of releases from these sources are documented. Hence, previous total effluents to the fjord is incomplete, but still a good estimate. *Table 1.2* reports a comprehensive list of releases of PAHs, PCBs and heavy metals to inner Oslofjord comparing 1995 to 2013 (Johnsen and Samdal, 1995; Berge *et al.*, 2013).

Table 1.2 Estimated releases of metals, polyaromatic hydrocarbons and polychlorinated biphenyls into inner Oslofjord in 1995 and 2013. TP = treatment plant (Johansen and Samdal, 1995; Berge *et al.*, 2013).

| **1995** | hg | Cd | Pb | Cr | Cu | Zn | Ni | PAH | PCB |
|---|---|---|---|---|---|---|---|---|---|
| TP effluents | 31,8 | 60,8 | 138 | 809 | 9397 | 13296 | N/A | 32,9 | 1 |
| TP overflow | 6 | 12,1 | 22 | 123 | 1617 | 3474 | N/A | 7,48 | 0,2 |
| River effluents | 1,72 | 42,7 | 680 | 484 | 1170 | 7945 | N/A | 29,2 | $188,55\times10^{-3}$ |
| Total | 39,5 | 116 | 840 | 1416 | 12184 | 24715 | N/A | 69,6 | 1,3 |
| **2013** | | | | | | | | | |
| River effluents | 2,2 | 14 | 429 | 398 | 2538 | 5397 | 684 | 35,5 | 0,1 |
| Atmospheric | 1,6 | 7 | 168 | 24 | 100 | 792 | 37 | 13,6 | 0,01 |
| Drainage overflow | 2,1 | 19 | 544 | 706 | 1081 | 5534 | 276 | 20,1 | 2,1 |
| TP effluents | 0,9 | 7 | 79 | 152 | 2528 | 4033 | 466 | 5,8 | 0,8 |
| TP overflow | 0,5 | 3 | 60 | 50 | 229 | 502 | 40 | 2,5 | 0,3 |
| Total | 7,3 | 50 | 1280 | 1330 | 6476 | 16258 | 1503 | 77,5 | 3,31 |

One might expect higher numbers from 1995 if atmospheric releases and drainage overflow also were included here, even though these most likely are much higher in 2013 due to more traffic and similar effects. Regarding the drainage overflow, the most consequential source of release is from the total area of roads. Typical sources of heavy metal and PAH pollution in drainage overflow is deterioration of wheels, pavement and road, exhaust release, oil spills, releases of brake linings, wear and tear of engines and so on. It is estimated that the sand traps used in drainage systems along roads can hold up to 50 % of heavy metals, potentially the same percentage of PAHs as well, meaning the releases from overflow can be drastically reduced. This does however rely on the sand traps being emptied often enough so no overflow will occur (Lindholm, 2015).

## 1.4 Environmental monitoring

Chemical pollutants released from human sources, such as industrial activity, agriculture or sewage into environmental compartments pose a risk to the ecosystem. The anthropogenic load and the ecological risk related to the pollutant stress are commonly evaluated by environmental assessments to determine the consequences this applies to the biological life (Walker *et al.,* 2012).

Assessment of pollutants in aquatic environments is determining the potential to affect biological elements and changing the ecological status of the water body. Historically these assessments have been centred around the determination of physical and chemical variables, such as the concentration of the pollutants in the biota and the water column. However, today there is a general agreement that these procedures have limited ability to determine the biological effect caused by the pollutants (Galloway *et al.,* 2004).

To fully understand the environmental effects of pollutants, it is important to address more than just the concentration in biota and in the water column. Several interacting environmental, ecological and biological factors will affect the behaviour, bioavailability, bioaccumulation potential and the toxic potential in different environmental compartments. Pollutants will almost always occur in mixtures (Howard, 1997), which may give rise to additive, synergistic and/or antagonistic effects in the uptake process of the biota (Walker *et al.,* 2012). Another key element to take into consideration is each individual organisms' response to different pollutants and mixture of pollutants.

To obtain a more accurate assessment of the overall state of an ecosystem, one must assess the effects of the physio-chemical environment on the different species in the given environment. The speciation uptake of pollutants and inherent inter-individual and inter-species differences in vulnerability to pollutants, and the toxicity of mixtures of pollutants are very important factors. With a better understanding of the species and environmental factors in play, one can more effectively link the effects from pollutants up through the hierarchical system from a biological organization to ecosystem and human health (Moore *et al.,* 2004).

Environmental assessments which take biomonitoring into account has gained momentum over the last decades. Instead of attempting to standardise different condition of laboratory experiments, where factors as interaction with other pollutants, soil and sediment type, rainfall, pH and salinity will affect the bioavailability, an easier approach is to monitor a natural population. The biomonitoring approach usually involves the traditional monitoring tools based on chemical analyses, but also the modern tools based on biological responses known as biomarkers (Walker *et al.,* 2012).

When exposed to pollutants, organisms may start exhibiting symptoms or reactions that are indicative of exposure and/or biological damages. The responses can be rated in a hierarchical sequence of where

they first are seen, and the importance of where effects are. *Figure 1.5* illustrates this and shows which of these responses that are useable as biomarkers in red.

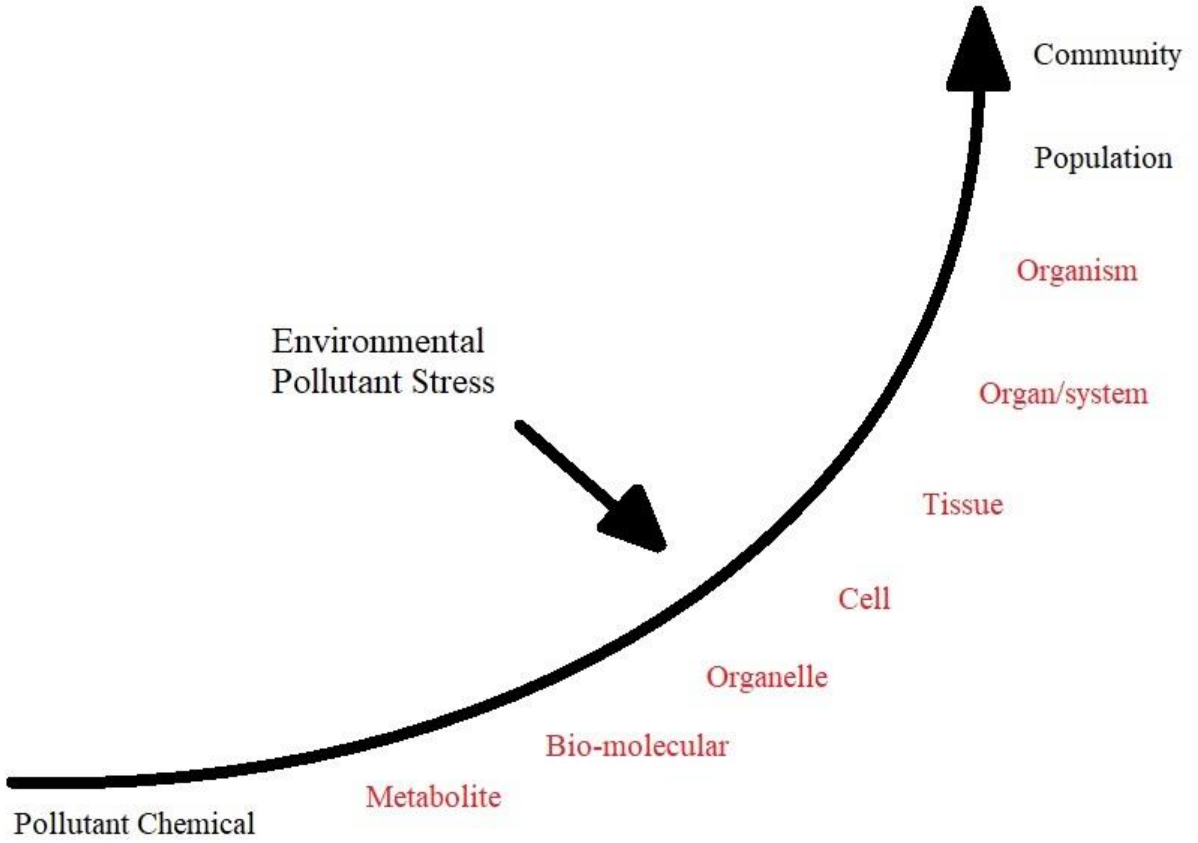


Figure 1.5 Hierarchical sequence of biological responses and which categories that are usable as biomarkers (red text).

Biological responses can be categorized into protective and non-protective. A protective response can be an induction of a protective measure to reduce or prevent the pollutants from causing any toxic effects. When exposed to heavy metal, the protective response is represented by the induction of MTs, metal-binding proteins. MTs bind heavy metals to it as a protective measure which are reducing the bioavailability. Non-protective biological response to pollutants can be an indication of toxic effects or harm that already have been done in cells, such as formation of DNA adducts from exposure to genotoxic

In any case, a sufficient exposure may lead molecular and cellular damage to develop into more adverse biological effects up through higher levels of biological organisations (*figure 1.5)*, and eventually lead to pathology with reduced physiological performance and reproductive success (Moore *et al.,* 2004).

With an effective integrated environmental management system, one can use the information biomarkers to prevent them from developing into more adverse effects on higher organisational levels concerning long-term consequences (Moore *et al.,* 2004).

### 1.4.1 Biomarkers

The term biomarker, which has been given acceptance in recent years, do still have several definitions. Walker *et al.* (2012) defines a biomarker as; "any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status". Thus biochemical, physiological, histological, morphological, and behavioural measurements are considered biomarkers, and can provide information related to pollution in biomonitoring assessments.

The most used classification of biomarkers divides them into two categories; biomarker of exposure and biomarker of effects. The biomarker of exposure indicates exposure to chemicals or a specific class of pollutants, but not the degree of adverse effect. Biomarker of effect demonstrates adverse effects in organisms (Walker *et al.*, 2012).

As biomarkers have a huge range, from subcellular effects to whole organism damage (*Figure 1.5*), they can reflect potential contamination in different organization levels in an ecosystem. Selection of which biomarkers to analyse and integrating them into a multivariant analysis can therefore be a powerful tool for evaluating the contamination effects. As biomarker results can be very specific and vary in relevance, a combination of them is preferable and needed for a sufficient evaluation of exposed organisms (Broeg *et al.*, 2005).

Environmental risk assessment (ERA) and monitoring programs have increasingly adapted the integration of biomarkers mostly due to their rapid responses and highly specific effects to different environmental contaminants (McCarthy and Munkittrick, 1996). Depending on the pollution, environment and the chosen specie, a given set of biomarkers are chosen and evaluated in combination with one another for a thorough assessment of the biological effects the contaminants introduce. Exposure to for example PAHs can be seen in the bile of fish, where the PAH metabolites will concentrate, and should also be seen in EROD activity, which is a highly sensitive early response biomarker that has proven to be one of the most effective at detecting aromatic contaminants. The combination of biomarkers is also very important to obtain a better picture of the biological threat the pollution is posing.

#### 1.4.1.1 advantages and limitations

Implementing biomarkers, often to complement the more traditional chemical methods in ERA can be quite considerable. A good example of this is the MT analysis, which measures the metal binding protein content in the given organisms' tissue (e.g. liver tissue in fish) rather than the trace metal content in water, sediment and/or biota. The following are some of the advantages (Handy *et al.*, 2003):

i Biomarker responses may indicate the presence and bioavailability of a pollutant, rather than just a biologically inert form of pollutant.

ii Using a suite of biomarkers may reveal the presence of contaminants not initially suspected.

iii Biomarker responses can be persistent and last long after transient exposure to pollutants that are degraded and not detectable anymore, which may result in detection of intermittent pollutant events that the traditional routine chemical monitoring may miss.

iv Biomarker analysis are quite often both easier to perform and less expensive than a wide range of chemical analysis.

Biomarkers do however come with some limitations, which if not fully understood, may result in inadequate results. Variability in biomarker responses is one of the most common error sources. Variability may be observed through the seasons and from change in environmental (e.g. temperature, dissolved oxygen, daylight) or biological (e.g. hormones, genotype, body size, sex, age) factors. Thus, natural variation in biomarker responses due to these changes needs to be known. Seasonal variations in the baseline levels of biomarkers are accepted in monitoring programs if one understands and can account for the causes (reproduction, temperature etc.) and timing (Nahrgang *et al.,* 2013).

Any environmental monitoring program which includes biomarkers should use methods to account for and minimalize the variability biomarker responses may give. When choosing a sentinel specie, one should carefully consider all the areas (polluted and reference) and choose a specie which is abundant and has a relevant status in the ecosystem. A good monitoring program is designed and timed to account for seasonal variations the populations may experience to achieve a better overall assessment of the status and effect it has on the population. Or in the case of yearly sampling, they should always be in the same period each year to achieve the most comparable results (Handy *et al.,* 2003, Nahrgang *et al.,* 2013).

### 1.4.2 Previous environmental assessment in oslofjord

The mixture of releases from municipal sewage, industrial activity and agriculture from around the fjord is exposing the marine ecosystem to a variety of pollutants and stress factors. The monitoring program for inner Oslofjord have been going on since the 1970s and is providing information regarding the status of the fjord, and mapping how the environmental conditions are changing over time.

Restrictions and regulations have reduced the release of contaminants into the fjord, and the results can be seen from the environmental monitoring. The monitoring programme includes a battery of assessments and analyses. The whole programme includes analyses of hydrography, hydrochemistry, phytoplankton content, the widespread of pliers, lower growth limit of algae and vertical widespread of sea urchins, biogeography: mapping and modelling of the marine nature, the occurrence of hyper benthos in response to oxygen levels, toxic blue-green algae in nearby rivers due to over fertilizing and species composition of fish from trawl and from the shore (Lundsør *et al.,* 2017, Berge *et al.,* 2014). The last decade has shown a small but persistent bettering of the conditions in the inner fjord, but they

are still worse than in the outer fjord. In addition to this, research on lowering the wastewater effluent released into the lake have shown great promise regarding the oxygen levels at bottom of the fjord and might get implemented. When the submerged outlet is further submerged, it will result in more forced vertical mixing in the bottom layers (Staalstrøm, 2017).

IRIS have contributed with biomonitoring of the fjord, sampling Atlantic cod and analysing a set of biomarkers. The biomonitoring programme is comparing the general health status of fish populations from the fjord, with reference populations from a clean site in the outer part of the fjord.

## 1.5 Atlantic cod (*Gadus morhua*)

The Atlantic cod was found suitable for evaluating the effects of pollutants in inner Oslofjord and was thus chosen as indicator species. The Atlantic cod is a very common North Atlantic fish species with a natural habitat from the north-east coast of USA to the Barents Sea and Baltic Sea (Heard, 2004). It is widespread in Norway and has a commercially and recreationally importance. It prefers temperate to boreal waters, which is reflected in its distribution. They live at the ocean floor at a depth ranging from 10-150 meters and do prefer a sea floor with coarse sediments rather than mud. Cod populations are relatively stationary (Godø, 1995), which is an important quality for an indicator species to relate toxicological effects to a small area. As their livers are quite fatty (40-80 %), they are viable in monitoring of accumulation of lipophilic contaminants (Goksøyr *et al.*, 1996). As an apex predator, they have a high relevance in their environment. Their diverse diet, which consist of both pelagic and benthic organisms (e.g. shrimps, crabs and fish) (Hop *et al.*, 1992) makes them highly likely to accumulate environmental contaminants from diet, but also from water through gills (Grung *et al.*, 2009).

Atlantic cod has been widely used as a bioindicator (Beyer *et al.*, 1996; Goksøyr *et al.*, 1994; Hylland *et al.*, 2009). They have a quite low seasonal variability baseline levels of biomarkers, which can be considered as a strong advantage for environmental monitoring as any deviations can be interpreted as anthropogenic impact (Nahrgang *et al.*, 2013). Due to health concerns, the Norwegian Food Safety Authority advised against consumption of cod liver from inner Oslofjord because of its high content of Hg and PCBs (Økland, 2005).

## 1.6 Assessment of Pollution in Biomonitoring

### 1.6.1 The Condition Index, Liver Somatic Index and Gonadosomatic Index

#### 1.6.1.1 General health status – The Condition Index

The CI is a measurement of the overall health of each individual and is commonly used in biomonitoring. It refers to the relative fatness of the individual which represents stored energy. The fat reserves of an organism are an important source of energy regarding biological activities, such as reproduction and migration, when many organisms do not feed or is not able keep up with the high energy demand it requires (Eliassen and Vahl, 1982). It is also important for survival in case of longer periods of scarcity.

CIs are usually calculated based on some ratio between body length and weight, or the pure weight ratio between dry/wet weight values in tissue or whole organism and tissue weight. A higher value indicates more energy reserves or fat.

In *G. morhua*, the CI is generally measured as some ratio between total body weight and fork length.

#### 1.6.1.2 Liver somatic index

The LSI is defined as the ratio between liver weight to body weight. It provides an indication of stored energy in liver which gives a status of energy reserves in an animal. In an environment with reduced food availability, the liver of fish is usually smaller (i.e. less energy reserved in the liver). LSI can also be affected by e.g. pulp and paper mill effluents, landfills and wastewater treatment plants (Hanson *et al.*, 2013). One can expect to see effects on LSI before the health of the individual is significantly affected, thus it is recommended as a supplemental biomarker in monitoring programs. Throughout the year, LSI can change markedly, and this variation must be considered for comparative reasons (ICES, 2012).

#### 1.6.1.3 Gonadosomatic index

The GSI is defined as the ratio between gonad weight to body weight. GSI supplies information about the health and gonadal maturation status. It can be used to assess potential risk for reduced reproductive potential of an organism. As the gonad size varies throughout the year, one must carefully assess the GSI. The use of GSI is not only for the maturation of the organism, it is commonly used to assess responses to exogenous stress. There is evidence that several environmental contaminants can lead to alterations in the gonads, like reduced GSI, morphological changes or both (Sakamoto *et al.*, 2003).

### 1.6.2. Genotoxic compounds

Genotoxicity can be caused by chemical compounds (e.g. PAH metabolites, PCBs or heavy metal ions) or physical agents (e.g. UV or X-ray). The compounds are known to cause chromosomal damage by DNA sequence altering. Even though cells have enzymatic mechanisms which repairs damage to DNA strands, they are not always able to repair the damage. When the DNA structure is altered it can lead to severe effects for the individual and future generations. The most usual damages are DNA adducts, strand break, modified bases or DNA crosslinks (Walker *et al.*, 2012).

PAHs are a group of ubiquitous hydrophobic organic compounds derived from pyrogenic or sources (Vuorinen *et al.*, 2006). As most PAHs have a high hydrophobicity and can be strongly sorbed by either organic or inorganic waterborne particles, they may eventually end up in the sediment compartment in an aquatic system. Here they may persist for a very long time as they are resistant to bacterial degradation and will thus act as a threat to the aquatic system being bioaccumulated in food chains (Dong *et al.*, 2012). Many PAHs are known as genotoxic pollutants which have highly reactive metabolites. During phase I metabolism through oxidation by cytochrome $P_{450}$, highly reactive by-products are formed with

high affinity for nucleophilic sites on cellular macromolecules, like DNA (UNEP/RAMOGE, 1999). The interaction between these compounds bound to DNA result in DNA adducts which can lead to formation of a variety of DNA lesions, which again pose a treat for later DNA replication (Walker *et al.*, 2012; UNEP/RAMOGE, 1999).

#### 1.6.2.1 Fixed fluorescence

Fluorescence can be summed up as absorption with a delayed emission. It is the result of a three-stage process which generally happens in PAHs. It starts off with excitation of an electron from a photon of an external source, creating an excited electron singlet state. The excited electron will stay excited for a fixed time (typically 1-10 nanoseconds). The electron will go back to its original relaxed state releasing the fluorescence emission. Not all initially excited electron returns to its original state by fluorescence emission which must be taken into account. The fluorescence emission is lower in energy due to energy dissipation during the excited lifetime. This energy difference is called the Stokes shift and is fundamental to the sensitivity of fluorescence detection, because it allows the emission to be detected against a low background (Fluorescence Fundamentals, 2018).

PAH metabolites in fish bile can be measured semi-quantitatively by fluorescens detection at certain fixed wavelength pairs. It works on the principle that the optimal excitation wavelength increases with the size of the PAH metabolites. Thus, the different sizes of PAH metabolites can be measured and distinguished from one another (Aas *et al*., 2000).

### 1.6.3. Metallothionein

MT was discovered by Margoshes and Vallee (1957) when they isolated a Cd-binding protein from the renal cortex (kidney) of horses. MT is a family of heat stable, cysteine-rich proteins with low molecular weight (6,000 – 7,000 Da) that belong to a superfamily of intracellular metal-binding proteins. MT proteins have a unique amino acid composition with up to 30% cysteine, no aromatic amino acids or histadine and a high metal content (6 to 7 metal atoms per mole of protein) (Hamilton and Mehrle, 1986). The amino-acid sequence is highly conserved, even when isolated from different animal species (Dziegiel *et al.*, 2016).

From protein sequencing it has been revealed that MT proteins are a single polypeptide chain, in which the cysteines are organised in the following sequences; Cys-X-Cys, Cys-X-X-Cys, and Cys-Cys, where X denotes an amino acid other than cysteine. The high content of cysteine means a high content of bind-able thiol groups (-SH) which is an important characteristic of MTs. The clustered binding sites which involves both terminal and bridging thiolate groups, can bind a variety of metals (e.g. Ag(I), Au(I), Bi(III), Cd(II), Co(II), Cu(I), Fe(II), Hg(II), Pb(II), Pt(II), Tc(IV) and Zn(II)) due to the electrophilic properties of the sulphur (Stillman, 1995).

The behaviour of MT is dominated by the chemistry of the thiol group (Templeton and Cherian, 1991), thus heavy metal sharing similar stoichiometric characteristics as the divalent essential metals Cu and Zn will be able to bind to the proteins thiol groups. MTs are usually not saturated by only one kind of metal, but by several different metals simultaneously, depending on its amino acid characteristics and affinity for metal ions. The *in vitro* affinity of the protein generally decreases in the hierarchical sequence $Hg^{2+} > Cu^{+}$, $Ag^{+}$, $Bi^{3+} >> Cd^{2+} > Pb^{2+} > Zn^{2+} > Co^{2+}$ (Amiard *et al.*, 2006), showing the essential metals are likely to be displaced, even by those considered to be most toxic.

MTs in *G. Morhua* are about average size compared to other species, with its predicted molecular mass of 6,1 kDa. In total, the chain consists of 60 residues, in which 20 are Cysteine (Cys) residues. (UniProtKB - P51902 (MT_GADMO), 2018). The N-terminal of *G. Morhua* MT is lacking the asparagine in position 4 which is present in mammalian MTs, as is the case for other piscines. Additionally, the *G. Morhua* N-terminal methionine is not acylated, which makes it differ from all other described vertebrate MT (Hylland *et al.*, 1994). The protein contains 2 metal-binding domains (α and β) and is regarded a class I MT. Within cluster A (α domain) the protein is capable of binding 4 divalent heavy metals which are coordinated via cysteinyl thiolate bridges to the 11 Cys residues located here. The β domain can bind three divalent heavy metals to 9 Cys.

MTs in fish are naturally present in different tissues, primarily including gills, liver, kidney and digestive tract (Kovarova *et al.*, 2009). A number of physiological and toxicologic factors are known to readily induce MTs. The induction has been proven to be influenced by exposure to inducing agents such as heavy metals, hormones, pharmaceuticals, thermal stress, steroids, organic solvents, alcohols, cytokines, alkylating agents, radiation, infection, and ROS (Ruttkay-Nedeckt *et al.*, 2013, Mao *et al.*, 2012, Viarengo *et al.*, 2000). MTs show varying sensitivity to different inducing agents. As the amino acid composition of MT influences the behaviour of the protein, the different isoforms will also show varying sensitivity to the different inducing agents. The most effective inducer is the essential metal Zn, which also shares several physiologically relevant interactions and is the reason for several functions of MT.

Regarding the high number of stimulating factors of MT induction, it is difficult to identify its biological functions. The protein is not yet fully understood and is still a subject of controversy. However, most authors do agree about its role as a multifunctional protein in metal regulating and detoxifying processes. While its definite function is unknown, its main function is widely accepted as homeostatic metabolism of the essential metals copper and zinc. It is also believed to provide a protective measure against excessive amounts of these metals by bioaccumulation and detoxification (Hamilton and Mehrle, 1986).

### 1.6.4. EROD

Through extensive research on the cytochrome P450 (CYP) enzyme system on mammals dating back to mid-1960s, came the suggestion to use the P450 1A subfamily of monooxygenase (CYP1A) as a biomarker. The cytochrome P450s are a diverse family of hemoproteins found in all species thus far examined with an extensive ability to metabolise xenobiotics and endogenous chemicals (Whyte *et al.*, 2000). In combination with several other enzymes, it acts as an electron transport system that catalyses a vast number of monooxygenase reactions (Olivia *et al.*, 2014).

In fish, these enzymes are primarily concentrated in the liver, but are also present in the kidney, gastrointestinal tracts, gill and other tissues (Varanasi *et al.*, 1989; Whyte *et al.*, 2000). The CYP system is responsible for the metabolism of a vast number of xenobiotics and endogenous compounds. When these compounds are biotransformed into more hydrophilic and excretable forms, by non-specific phase I and phase II transformation reactions, the CYP1A is involved in phase I. By either exposing a polar group or add one to the toxicant, it will enhance the water solubility for elimination from the organism (Andersson and Förlin, 1992). In that way, cytochrome P450s like CYP1A generally detoxicate xenobiotic compounds, even though in some cases the metabolite from phase I is more toxic than the parent compound (Olivia *et al.*, 2014).

In fish species, CYP1A seems to be a very sensitive biomarker of exposure to pollutants, both organic and inorganic. The enzyme induction can occur from stimuli to detoxify toxicants or transform them for easier excretion (Whyte *et al.*, 2000). It is dependent on mixed-function oxygenase (MFO) or monooxygenases. Assays which include MFO enzyme generally utilizes EROD by correlating it to e.g. phenanthrene-type metabolites in liver. MFOs are a family of inducible enzymes, which by single oxygen addition oxidizes chemicals, both anthropogenic and natural. The metabolism helps in excreting nonpolar compounds

### 1.6.5 ALA-D

Through industrial activities and other sources, Pb is released and enters aquatic environments where it can cause sublethal damage and change in reproduction, growth and behaviour. Pb poisoning is ubiquitous in fish and resulting toxic effects include muscular atrophy, lordoscoliosis, paralysis, black tails, degeneration of caudal fin, hyperactivity, erratic swimming, loss of equilibrium and mortality (Burden *et al.*, 1998). Black tails is a symptom of neurotoxicity as a result of Pb exposure, and is a precursor to deformities in the spine which eventually will lead to atrophy in the tail region, reducing swimming ability, interference with reproduction and death (Hodson *et al.*, 1978). Instead of measuring the bioavailability of Pb in water, the activity of ALA-D, an erythrocyte enzyme involved in heme synthesis is used as a biomarker (Burden *et al.*, 1998).

ALA-D is an enzyme that catalyses the condensation of porphobilinogen (PBG), which is a heme precursor, from aminolevulinic acid. Heme is a very essential component of hemoproteins, such as hemoglobin, which is the protein that transfers oxygen in the blood. The enzyme requires Zn as a cofactor in this condensation, but Zn can be replaced by Pb which changes the quaternary structure and effectively inhibits the ALA-D activity (Schmitt *et al.,* 2005; Moraes *et al.,* 2003; Warren *et al.,* 1998).

Because of this it is a very well-known biomarker for investigation of Pb exposure. Measuring ALA-D activity in an organism may give a more accurately prediction of Pb exposure than more traditionally analysis of Pb concentration in water. ALA-D activity has been used for, and believed to be Pb specific, but there are studies which suggests there are metals other than Pb which also is able to inhibit the activity of the enzyme (Rodriguez *et al.,* 1989; Hylland *et al.,* 2009).

# 2 Materials and methods

## 2.1 Sampling sites

The sampling was carried out from the 11th to the 13th of December in 2017 with the use of the University of Oslo sampling boat. Eighty specimens of Atlantic cod were collected in total, 40 from each of the sites; outer fjord (the clean reference site) and inner fjord (the potentially polluted site) (*Figure 2.1*).

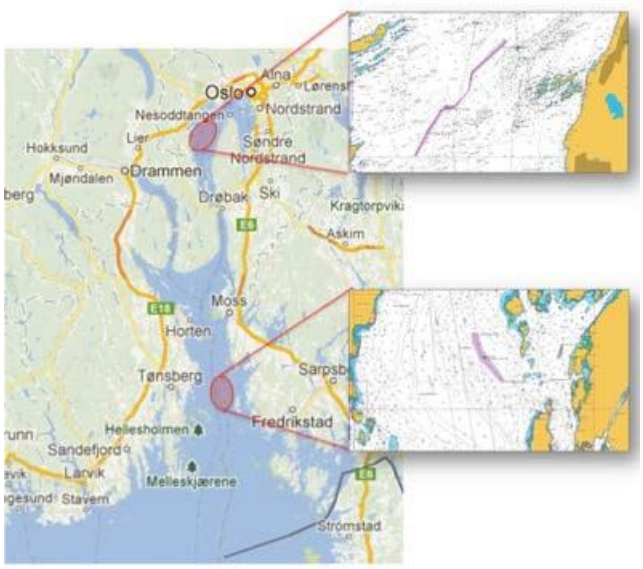


Figure 2.1 Overview of sampling areas in Oslofjord (Berge J. A. *et al.*, 2014)

The population of the nearby municipalities are close to 990 000 in Oslo (2017), 116 000 in Drammen (2017), 111 000 in Fredrikstad/sarpsborg (2017), 51 000 in Tønsberg (2017) and 32 000 in Moss (2017). The inner fjord is surrounded by urban districts, agricultural activities and forest where the main pollution releases are the effluents from wastewater treatment plants, river effluents into the fjord and drainage overflow, depending on the type of pollutant. These sampling sites have been investigated for several years for comparison (Berge *et al.*, 2014).

## 2.2 Sampling and pre-treatment

Cod were sampled by trawling. All fish were measured and weighed onboard the vessel, including the liver and gonad. Liver, bile and blood were put in sample tubes and stored in dry-ice on the boat before being sent back to the laboratory, where they were cryo-stored until further analyses were conducted.

## 2.3 Biological assays

### 2.3.1 Condition Index, Liver Somatic Index and Gonad Somatic Index

The length and total weight of each fish was measured with a measuring board and digital fish scale (Berkley® model BTDFS50-1). The fish were sexed by visually examining their gonads. A motion compensated balance (Marel M2000 series) was used to measure total liver and gonad weight onboard the vessel. The condition Index was determined as the ratio between total fish weight and the cube of the fork length of the fish.

$$Condition\ Index\ (CI) = \left[\frac{fish\ weight\ (g)}{fork\ lenght\ (cm^3)}\right] x100$$

The Liver Somatic Index (LSI) reflects the animal nourishment status. The LSI was calculated as:

$$Liver\ Somatic\ Index\ (LSI) = \left[\frac{Liver\ weight\ (g)}{fish\ weight\ (g)}\right] x100$$

The Gonadosomatic Index (GSI) is a measure of the sexual maturity of animals in correlation to ovary and testis development. The GSI was calculated as:

$$Gonad\ Somatic\ Index\ (GSI) = \left[\frac{Gonad\ weight\ (g)}{fish\ weight\ (g)}\right] x100$$

### 2.3.2 Fixed fluorescence method

Bile samples were thawed on ice before being diluted 1:1600 in methanol mixed 1:1 with distilled water (50% MeOH). The FF analyses were performed on a Thermo Scientific Lumina Fluorescence Spectrometer. Slit widths were set at 2,5 nm for both excitation and emission wavelengths, and all analyses were performed using quartz cuvettes. All bile samples were analysed by the following wavelength pairs: 290/335, 341/383 and 380/430 nm, optimised to detect 2-3 ring, 4-ring and 5-ring PAH metabolites, respectively. Synchronous fluorescence spectroscopy (SFS) was used to detect naphthalene, pyrene and benzo[a]pyrene metabolites. A constant difference of 42 nm ($\Delta\lambda$) between excitation and emission wavelength was used. This $\Delta\lambda$ was found to be optimal for the detection of pyrene metabolites and also suitable for detection of naphthalene and benzo[a]pyrene metabolites (Aas *et al*., 2000). The detected fluorescence signal was transformed into pyrene fluorescence equivalents (PFE) through a standard curve made by pyrene (Sigma St Louis, USA). The concentration of PAH in the bile samples were expressed as µg PFE/mL bile.

### 2.3.3 Metallothionein

The MT assay was performed using some minor modification compared to the method introduced by Viarengo *et al.* (1997). In this assay, the MT concentration was determined by utilizing the ethanol/chloroform fractionation of the tissue homogenate to obtain a partially purified metallothionein fraction. The concentration of MT is quantified spectrophotometrically by evaluating the SH residue content utilizing the Ellman's reagent. Precautions are taken to avoid oxidation and formation of intramolecular disulphide bonds, eliminate contamination by soluble low molecular weight thiols, both endogenous and exogenous, and to ensure complete MT precipitation. The spectrophotometric method is a very simple, repeatable and low-cost method for the detection of MT in tissue.

Metallothionein sample preparation

For each MT sample, dissected liver was homogenized in three volumes of 0.5 M sucrose, 20 mM Tris buffer with pH 8.6, with added 0.006 mM leupeptin, 0.5 mM PMSF and 0.01 % β-mercaptoethanol. Leupeptin and PMSF acts as antiproteolytic agents, while the β-mercaptoethanol acts as a reducing agent. The homogenate was centrifuged at 20.000 x G for 20 min at 4°C in 15 mL Falcon tubes to obtain a supernatant containing MTs. One mL of the supernatant was extracted by pipette and added to 1.05 mL cold (-20 °C) ethanol and 80 µL of chloroform in a new 15 mL Falcon tube and vortexed for a few seconds. The sample was then centrifuged at 6.000 x G for 10 min at 4 °C. The resulting supernatant was extracted and added to 3 volumes of cold (-20 °C) ethanol and 40 µL 37% HCl in a new 15 mL Falcon tube. The sample were stored at -20 °C for 1 hour and then split into 3 eppendorf tubes before being re-centrifuged at 6000 x G for 10 min at 4°C. The supernatant was removed, and the pellets were dried in a speed vacuum at 30 °C for 10 min.

Spectrophotometric assay (Ellman's reaction)

The three pellets were resuspended in 50 µL 0.25 M NaCl and 50 µL 1 M HCl containing 4 mM EDTA each and subsequently gathered together in a 15 mL Falcon tube. A volume of 4.2 mL 0.2 M phosphate buffer at 8 pH containing 2M NaCl and 0.43 mM DTNB (5,5-dithiobis-2-nitrobenzic acid) was then added to the sample. One mL of the sample was evaluated in the spectrophotometer at 412 nm utilizing reduced glutathione (GSH) as a reference standard.

GSH reference standard preparation

The reference standard was plotted utilizing 4 different GSH reference concentrations; 15 µM, 30 µM, 60 µM and 90 µM and evaluating the absorbance at 412 nm. One mole of GSH yields 1 mole of thiol groups (-SH).

Metallothionein concentration calculation

Metallothionein were analysed at 412 nm and the absorbance were interpolated using the GSH reference curve. The corresponding X-axis values obtained represented the total molar concentration of SH groups present in the MT sample. Considering the size and residue content of the MT protein in Atlantic cod, the dilution factor of the homogenizing of the tissue, the concentration of MT in the sample can be obtained from the following formula:

$$[MT]\left[\frac{ng}{g}\right] = \frac{Interpolatd\ value\ \left(\frac{nmol}{ml}\right)}{20\ cys\ residues} * 6106\ Da * 4{,}5 * 4$$

## 2.3.4 EROD

EROD sample preparation

For each sample, dissected liver was homogenized in four volumes of ice-cold 0.1 M $NaH_2PO_4xH_2O$, 0.15 M KCl and 1 mM ethylenediamine tetraacetic acid (EDTA) in distilled water with pH 7.4 (adjusted with NaOH). The homogenate was centrifuged at 12.000 G for 20 min at 4°C in 5 mL Eppendorf tubes. The supernatant was carefully collected to ensure a clean supernatant. One mL was collected and stored in -80°C for further analysis, while 2 mL were transferred into ultra-centrifugation (UC) tubes for further processing. The UC tubes were balanced carefully in pairs to within 0.01 g and placed opposite of each other in the ultra-centrifuge rotor (70.1 Ti). The samples were then centrifuged at 100.000 G for 1 hour at 4°C. The supernatant was carefully transferred into storage vials, without affecting the microsome layer. Then, 0.5 mL per gram of initial tissue of resuspension buffer containing 0.1 M $NaH_2PO_4xH_2O$, 0.15 M KCl, 1 mM EDTA and 87 % glycerol (230 mL/litre solution) with 7.4 pH (adjusted with NaOH) was added into the UC tube to resuspend the microsome layer and the pellet before being put into storage at -80°C.

Cuvette method

Microsome samples were thawed on ice prior to the analysis. For each sample, 1.96 ml EROD buffer (0.1 M Na phosphate buffer adjusted to 7.8 pH (optimum for cod)), 10 µl 7-ethoxyresorufin substrate solution (1 mg 7-ethoxyresorufin per 10 ml DMSO) and 20 µl microsome fraction was added to a cuvette and mixed well by inverting 3-4 times. The sample was placed into the spectrofluorometer and started to record the baseline signal. The cuvette was removed and added 10 µl 9 mM nicotinamide adenine dinucleotide phosphate (NADPH) solution, mixed well by inverting the cuvette 3-4 times and placed back into the spectrophotometer. The change in fluorescence was recorded as a continuous linear line (i.e. 1 min recording per sample). The cuvette was then taken out once more and added 10 µl resorufin

internal standard (10-20 µM resorufin in DMSO), mixed well by inverting 3-4 times and placed back in to record the rise in fluorescence level.

The fluorescence change per amount of resorufin added (pmol) was calculated. Then the specific enzymatic activity (pmol/min/mg protein) of each measured sample was calculated using the following formula:

$$pmol\ resorufin/min/mg\ protein\ = \frac{F_S}{min} * \frac{R}{F_R} * \frac{1}{V_S} * \frac{1}{C_S}$$

| | |
|---|---|
| $F_S$ / min | Increase in sample fluorescence per minute |
| R | pmol resorufin added as internal standard |
| $F_R$ | Increase in fluorescence due to the addition of the resorufin standard |
| $V_S$ | Volume of sample (0.02 ml) |
| $C_S$ | Protein concentration in analytical mix (mg/ml) |

The protein concentration was determined using the Bradford assay (Bradford, 1976), see section 2.3.6.

### 2.3.5 ALA-D

The ALA-D assay was performed using some minor modification from those of Hylland (2004) and Alves *et al.* (2006). Blood samples were thawed on ice and diluted 1:1 with a dilution buffer (0.1 M potassium phosphate buffer at pH 7.0 with 0.2 % Triton x-100) in centrifugation tubes. The mixture was homogenized by hand with a Teflon pestle. Fifteen µl of homogenate was then transferred into 5 different tubes. Two of these were for blanks, two for ALA-D analysis and one for protein measurement. 75 µl each of dilution buffer and ALA reagent (3.35 mg Amino-levulinic acid in 5 ml dilution buffer) was added to the blanks and ALA-D analysis tubes, respectively. All tubes were vortex for a couple of seconds before 2 hours incubation in RT. Four porphobilinogen standards were prepared with a concentration of 2, 4, 8 and 16 µg/ml from a stock solution of 40 µg/ml and diluted with dilution buffer,

After 2 hours of incubating, a volume of 600 µl precipitation solution (0.24 M Trichloro-acid and 0.22 M n-ethylmaleimide in distilled water) was added to all tubes. All tubes were mixed for a couple of seconds and left to stand for 5 minutes, before being centrifuged at 1000 G for 5 minutes. 100 µl each of supernatant from the tubes and Ehrlich's solution (750 µl double distilled water, 2.5 ml of 70 % perchloric acid and 10.5 ml glacial acetic acid, with 0.25 g 4-dimethyl-amino-benzaldehyde dissolved into the finale volume) were added to each plate and the plates were shaken for 30 seconds. All samples were then incubated for 15 minutes at RT before the absorbance were measured at 540 nm on a plate (insert name of the plate reader) reader.

The activity was calculated as the quantity of porphobilinogen (ng) produced per hour per mg protein from the homogenized blood samples. The protein content in each sample was analysed utilizing the Bradford assay (see section 2.3.6).

### 2.3.6 Bradford Assay

The Bradford assay is a procedure to determine the concentration of solubilized protein (Bradford, 1976). With the addition of an acidic solution blue dye Coomassie Brilliant Blue G-250 to the protein solution, one can measure the optical density at 595 nm using a spectrophotometer or microplate reader. A higher concentration of protein will result in a bluer mixture whereas lower concentration will be weaker in colour as a response to the protein. This response is linear within a certain concentration range and provides a relative measurement of protein concentration when comparing to a standard curve.

One part of the Bio-Rad dye Concentrate was diluted with 4 parts distilled water and filtered to remove particulates. Appropriate aliquots of Sigma BSA 5% standard curve solution (50 mg/ml, A-4268) (BSA) were obtained and one aliquot of BSA house control reference sample. The house control reference sample was verified to be within the acceptance range (+/- 2 standard deviation). All thawed aliquots were mixed by vortexing. The appropriate dilution factor for the unknown samples were decided (usually 0-5 µg protein). Ten µl of sample or standard was added to each well, meaning a sample with 10 mg/ml should be diluted 1:50 resulting in a sample with 2 µg protein in the applied well. For each standard, there were three parallels, whereas there were four parallels for each unknow sample. To each well there was added 200 µl of diluted dye reagent and the content in the well was mixed well. The plate was incubated for at least 5 minutes. As the absorbance will increase over time, the incubation should last no more than one hour. In case of air bobbles, these were popped with a clean pipette. The absorbance was measured at 595 nm and the parallels were examined to exclude clear outliers.

The protein concentration measurement is necessary for both the EROD and ALA-D analysis.

## 2.4 Statistical analysis

All results were analysed using the statistical programs IBM SPSS Statistic 25 and Minitab 18. All collected cod data were analysed for comparison between the two sampling sites and between the years using one-way ANOVA if the variance was homogenous, or by the Scheffé F-test using SPSS.

The correlation between the physiological indices and the biomarkers were assessed by the Spearman's rank order correlation test using SPSS.

The Principal Component Analysis (PCA), a multivariate analysis, were performed in Minitab to assess the variance and determine if the two areas could be distinguished based on the test data.

# 3 Results

In this thesis sampled cod collected in December 2017 were used for biomarker analyses, and the data was compared to previous samples in 2015.

All raw physiological data are reported in Appendix A, and all raw biological data are reported in Appendix B.

## 3.1 Condition Index

CI results are summarized in *figure 3.1*. Mean CI values in *G. morhua* from the reference site varied from 0.94 (2015) to 0.97 (2017), whereas in the exposed site the mean values varied from 0.87 (2015) to 0.91 (2017). There was no significant statistical difference between the recorded values in cod from the inner fjord when compared to the ones in the outer fjord, in any of the years. There was also no significant difference between the years.

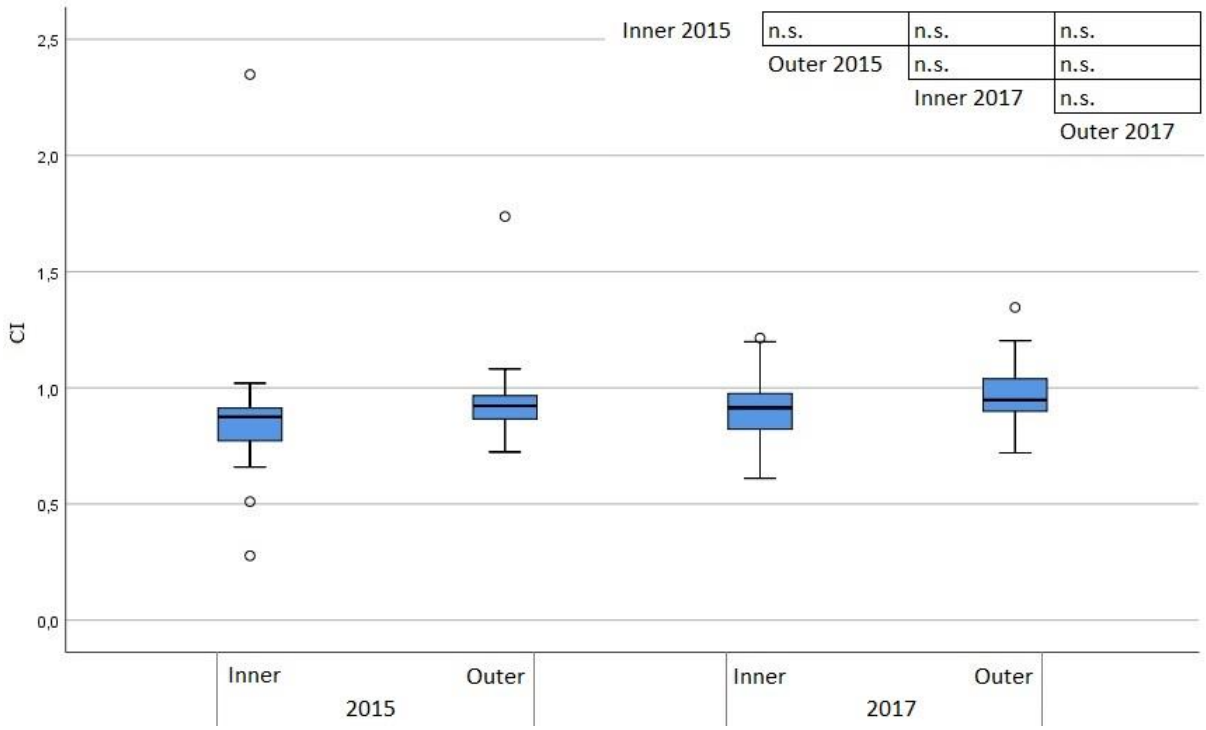


Figure 3.1 Box and whisker diagram of CI values. The bottom part of the boxes indicates the 25$^{th}$ percentile, while the upper part of boxes indicates the 75$^{th}$ percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. Statistical comparisons were performed using the post hoc Scheffé test and results are reported in the top-right corner of the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.2 Liver somatic index

LSI results are summarised in *figure 3.2*. Mean LSI values in *G. morhua* collected in the reference are range from 2.10 (2015) to 2.40 (2017), whereas in the exposed area it ranged from 1.88 (2015) to 3.10 (2017). There was significant difference between the results from inner fjord in 2017 and the 2015 results, as well as outer 2017 and inner 2015 which indicates an increase in LSI between the years. However, no significant difference between the two sampling sites were observed within the same year.

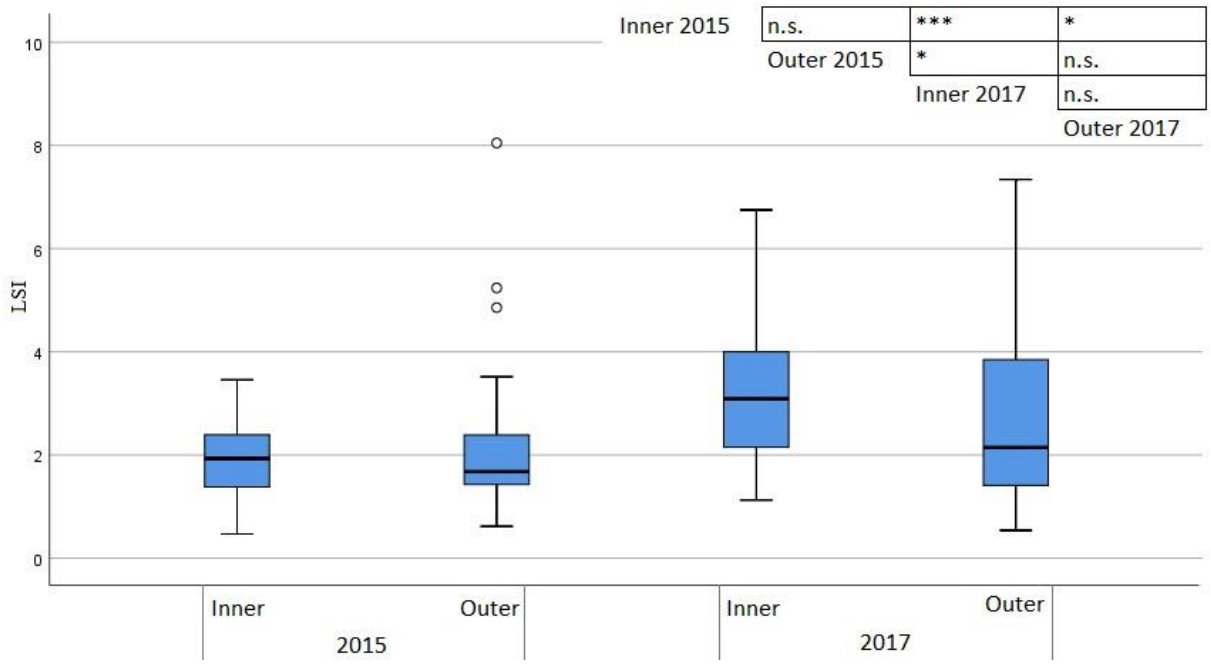


Figure 3.2 Box and whisker diagram of LSI values. The bottom part of the boxes indicates the 25$^{th}$ percentile, while the upper part of boxes indicates the 75$^{th}$ percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. Statistical comparisons were performed using the post hoc Scheffé test and results are reported in the top-right corner of the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.3 Gonadosomatic index

GSI results are summarised in *Figure 3.3*. The GSI analysis was only conducted in 2017, not 2015. Mean values ranged from 2.38 (outer fjord) to 2.64 (inner fjord) for females, and from 2.5 (outer fjord) to 5.37 (inner fjord) for males. The only statistically difference was found between males from inner fjord. There was a significant difference between males and females in inner fjord, and also between males in inner fjord and both genders in outer fjord. By two-way ANOVA there was also found a significant difference between the areas, between the sex and area x sex ($p<0.05$).

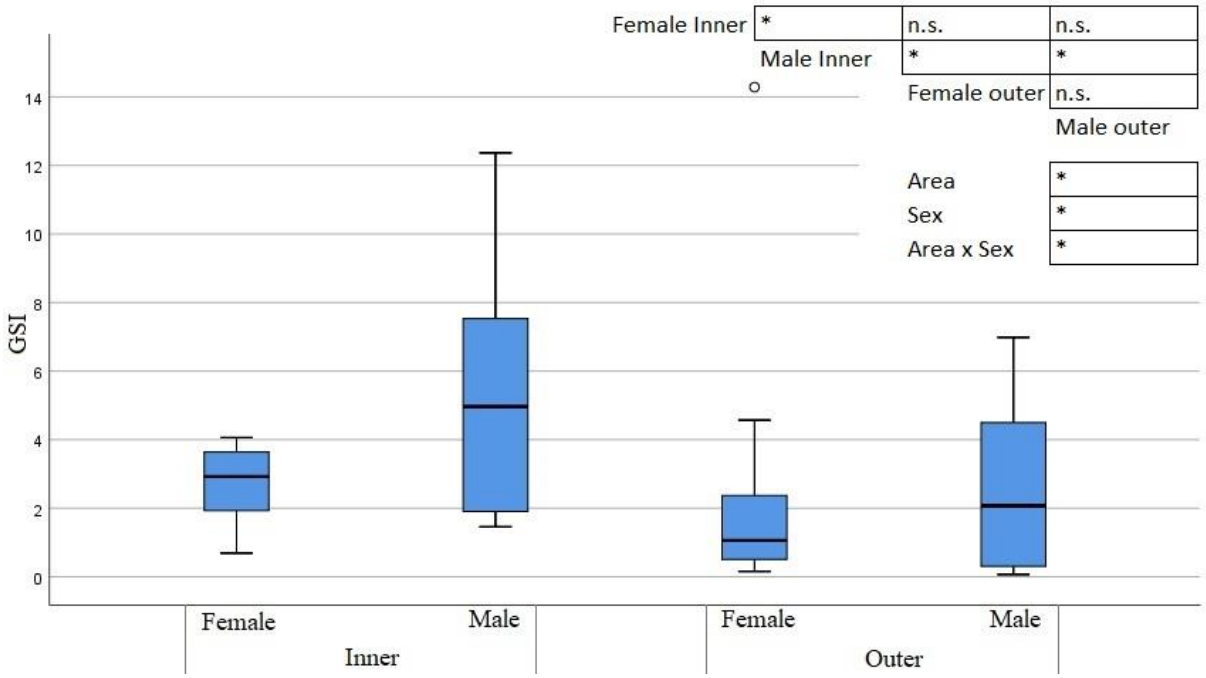


Figure 3.3 Box and whisker diagram of GSI values. The bottom part of the boxes indicates the 25$^{th}$ percentile, while the upper part of boxes indicates the 75$^{th}$ percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. Statistical comparisons were performed using the post hoc Scheffé test and 2-way ANOVA (sex and area). Statistical results are reported in the top-right corner of the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.4 PAH metabolites in bile

FF assay results are summarised in *Figure 3.4.* Median values in the reference area (outer fjord) varied from 271 (2017) to 431 (2015) for 2,3-ring PAH metabolites, 126 (2017) to 144 (2015) for 4-ring PAH metabolites, and from 36 (2017) to 53 (2015) for 5-ring PAH metabolites. There was a statistically significant difference ($p<0.001$) between the areas in both 2015 and 2017 regarding 4-ring PAH metabolites. There was also recorded a significant difference ($p<0.05$) between the areas for 2,3-ring PAH metabolites in 2017, but not in 2015. For 5-ring metabolites there were no significant difference between areas in any of the years, only between the inner fjord in 2015 and outer fjord in 2017.

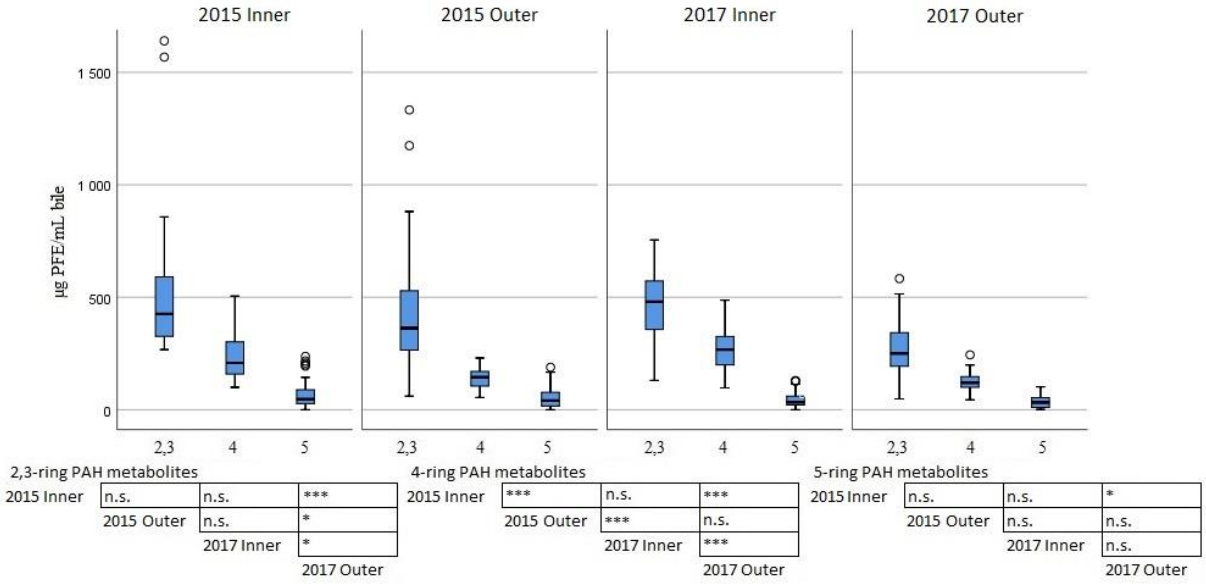


Figure 3.4 Box and whisker diagram of PAH metabolites results. The bottom part of the boxes indicates the 25$^{th}$ percentile, while the upper part of boxes indicates the 75$^{th}$ percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. 2,3: 2,3-ring PAH metabolites, 4: 4-ring PAH metabolites, 5: 5-ring PAH metabolites. Statistical comparison done using the post hoc Scheffé test and results are reported under the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.5 The metallothionein assay

MT assay results are summarized in *figure 3.5*. The mean values in the cod collected at the reference station (outer fjord) was 2.89 mg/g both years, even though the results from 2017 had a significant bigger variance. The mean values from the exposed area (inner fjord) ranged from 2.96 mg/g (2017) to 2.98 mg/g (2015). There was no significant statistical difference between the recorded values in collected cod from the stations within the same year, or between them.

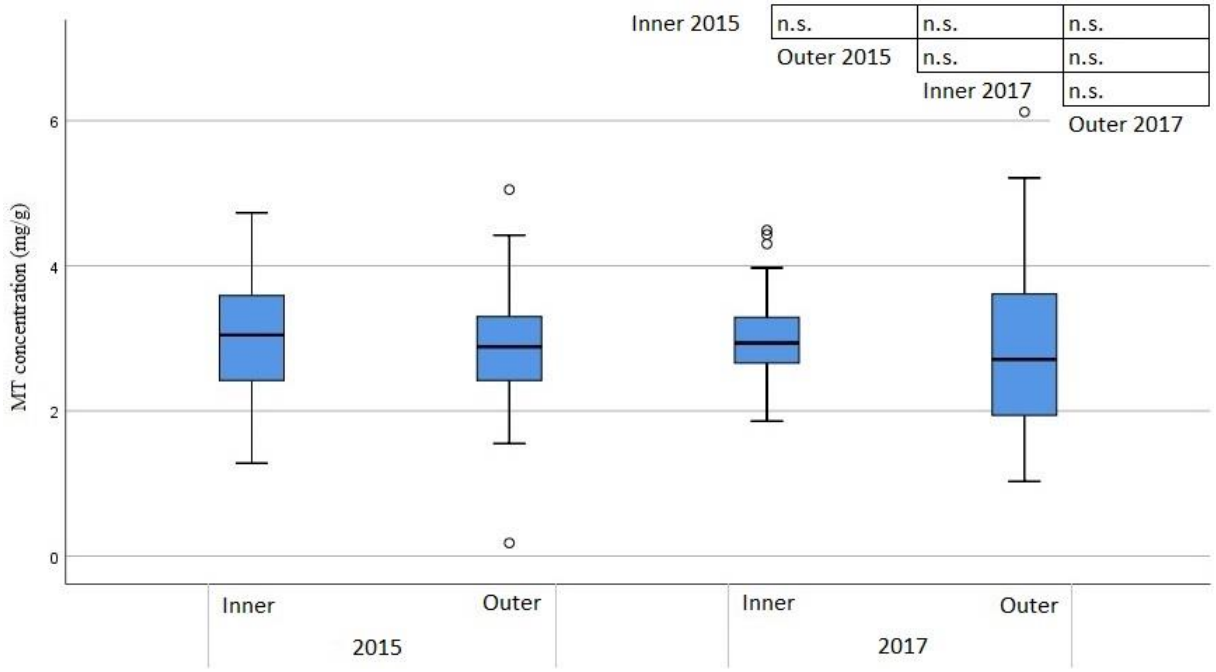


Figure 3.5 Box and whisker diagram of MT results. The bottom part of the boxes indicates the 25th percentile, while the upper part of boxes indicates the 75th percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. Statistical comparisons were performed using the post hoc Scheffé test and results are reported in the top-right corner of the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.6 EROD

The EROD results are summarized in *Figure 3.6.* The mean value in the cod collected in the reference area (outer fjord) was 46.9 in 2015 and 21.8 in 2017. The mean values from the exposed area (inner fjord) was 68.2 in 2015 and 18.7 in 2017. There was no significant difference between the two stations in any of the years. There was however a significant difference ($p<0.001$) between the values from inner fjord 2015 and both areas in 2017, as well as a significant difference ($p<0.05$) between the values from outer fjord 2015 and inner fjord 2017.

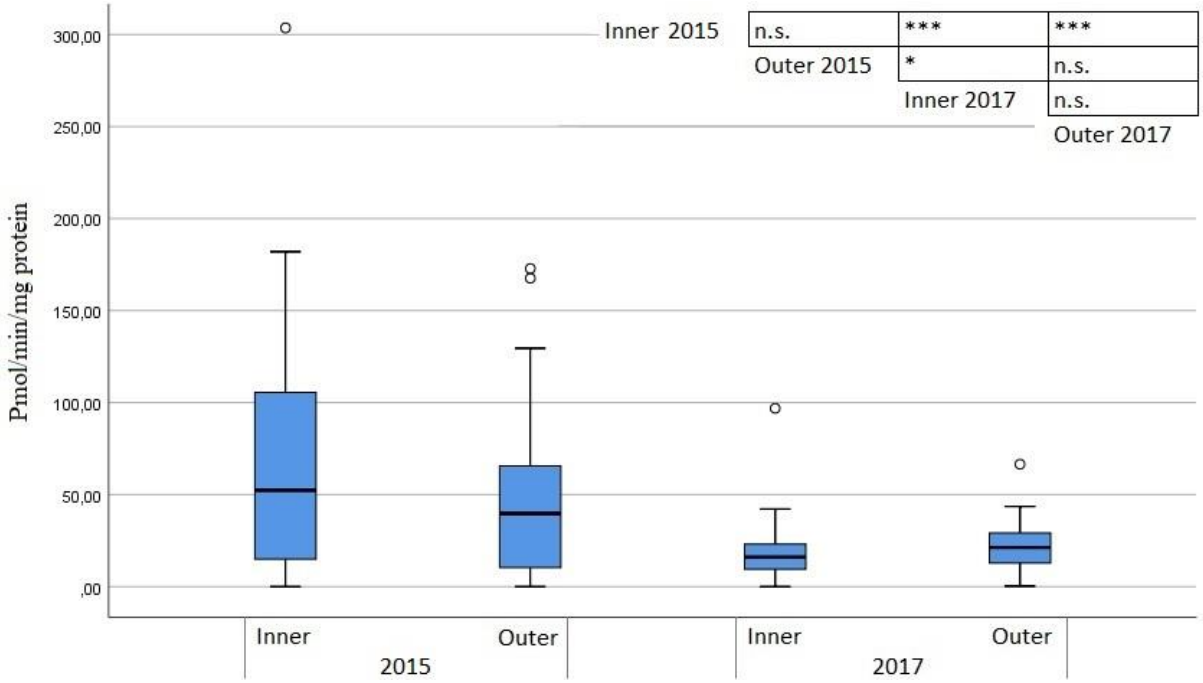


Figure 3.6 Box and whisker diagram of EROD activity results. The bottom part of the boxes indicates the 25th percentile, while the upper part of boxes indicates the 75th percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. Statistical comparisons were performed using the post hoc Scheffé test and results are reported in the top-right corner of the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.7 ALA-D

ALA-D results are summarised in *Figure 3.7*. The mean values in the cod collected at the reference area (outer fjord) were 80.0 in 2015 and 39.5 in 2017. The mean values in the reference site (outer fjord) were 49.2 in 2015 and 30.6 in 2017. There was no statistical difference between the stations in 2017, but there was a significant difference ($p<0.001$) between the values from inner fjord 2015 and the rest of the values. There was also a significant difference ($p<0.01$) between the values from the outer fjord when 2015 and 2017.

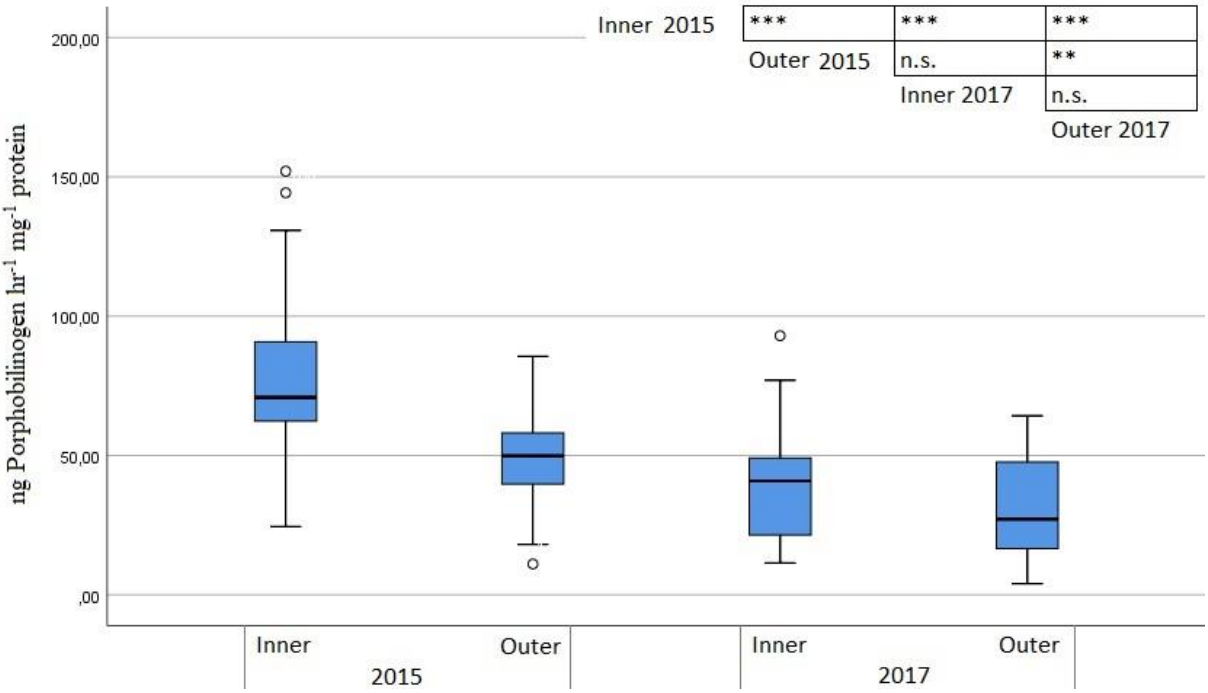


Figure 3.7 Box and whisker diagram of ALA-D values. The bottom part of the boxes indicates the 25th percentile, while the upper part of boxes indicates the 75th percentile; Horizontal lines in boxes indicate median values; whiskers are maximum and minimum value, not taking outliers into consideration. The dots are outliers. Statistical comparisons were performed using the post hoc Scheffé test and results are reported in the top-right corner of the boxplot, ***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$, n.s.: not significant.

## 3.8 Correlations

Correlation coefficients between the measured values from 2017 are shown in *Figure 3.8*. The 4-ring PAHs showed a significant negative correlation ($p<0.05$) with EROD. It also showed a positive correlation with 2,3-ring PAH metabolites ($p<0.01$), GSI ($p<0.01$) and 5-ring PAH metabolites ($p<0.05$). GSI values showed a significant positive correlation ($p<0.01$) with LSI, and a significant negative correlation ($p<0.01$) with EROD activity.

| | | ALA-D | EROD | MT | GSI | LSI | CI | 5-ring PAHs | 4-ring PAHs | 2,3-ring PAHs |
|---|---|---|---|---|---|---|---|---|---|---|
| 2,3-ring PAHs | Corr. Coef. | 0,244 | -0,183 | 0,089 | 0,310* | 0,220 | -0,077 | 0,197 | 0,666** | |
| | sig. | 0,052 | 0,152 | 0,486 | 0,014 | 0,083 | 0,549 | 0,120 | 0,000 | |
| | n | 64 | 63 | 63 | 62 | 63 | 63 | 64 | 64 | |
| 4-ring PAHs | Corr. Coef. | 0,212 | -0,319* | 0,171 | 0,501** | 0,202 | -0,232 | 0,256* | | |
| | sig. | 0,093 | 0,011 | 0,179 | 0,000 | 0,113 | 0,068 | 0,041 | | |
| | n | 64 | 63 | 63 | 62 | 63 | 63 | 64 | | |
| 5-ring PAHs | Corr. Coef. | 0,080 | 0,111 | 0,116 | 0,109 | 0,063 | -0,122 | | | |
| | sig. | 0,527 | 0,385 | 0,364 | 0,401 | 0,621 | 0,339 | | | |
| | n | 64 | 63 | 63 | 62 | 63 | 63 | | | |
| CI | Corr. Coef. | -0,140 | 0,164 | -0,023 | 0,044 | -0,098 | | | | |
| | sig. | 0,219 | 0,150 | 0,844 | 0,704 | 0,394 | | | | |
| | n | 79 | 78 | 77 | 78 | 78 | | | | |
| LSI | Corr. Coef. | 0,116 | -0,042 | 0,164 | 0,545** | | | | | |
| | sig. | 0,310 | 0,716 | 0,156 | 0,000 | | | | | |
| | n | 78 | 77 | 76 | 77 | | | | | |
| GSI | Corr. Coef. | 0,096 | -0,312** | 0,055 | | | | | | |
| | sig. | 0,401 | 0,006 | 0,638 | | | | | | |
| | n | 78 | 77 | 76 | | | | | | |
| MT | Corr. Coef. | 0,202 | -0,164 | | | | | | | |
| | sig. | 0,076 | 0,154 | | | | | | | |
| | n | 78 | 77 | | | | | | | |
| EROD | Corr. Coef. | -0,103 | | | | | | | | |
| | sig. | 0,366 | | | | | | | | |
| | n | 79 | | | | | | | | |
| ALA-D | Corr. Coef. | | | | | | | | | |
| | sig. | | | | | | | | | |
| | n | | | | | | | | | |

Figure 3.8 Spearman's rank order correlation between the nine biological markers. Corr. Coef.: Correlation coefficient. Sig.: p-value. n: number of samples. ***: Correlation is significant at the 0.001 level; **: Correlation is significant at the 0.01 level; *: Correlation is significant at the 0.05 level.

## 3.9 Multivariate analysis – Principal Component Analysis

A PCA analysis was conducted and is presented graphically with the two components which contributed the most in explaining the total variation in the dataset. The model explained 40.1 % of the total variation, where the first component (x-axis) explained 25.3 % and the second (y-axis) explained 14.8 % of the total variation (*Figure 3.9*). The recorded values are clearly separated by area in the plot, with only minor mixing between them.

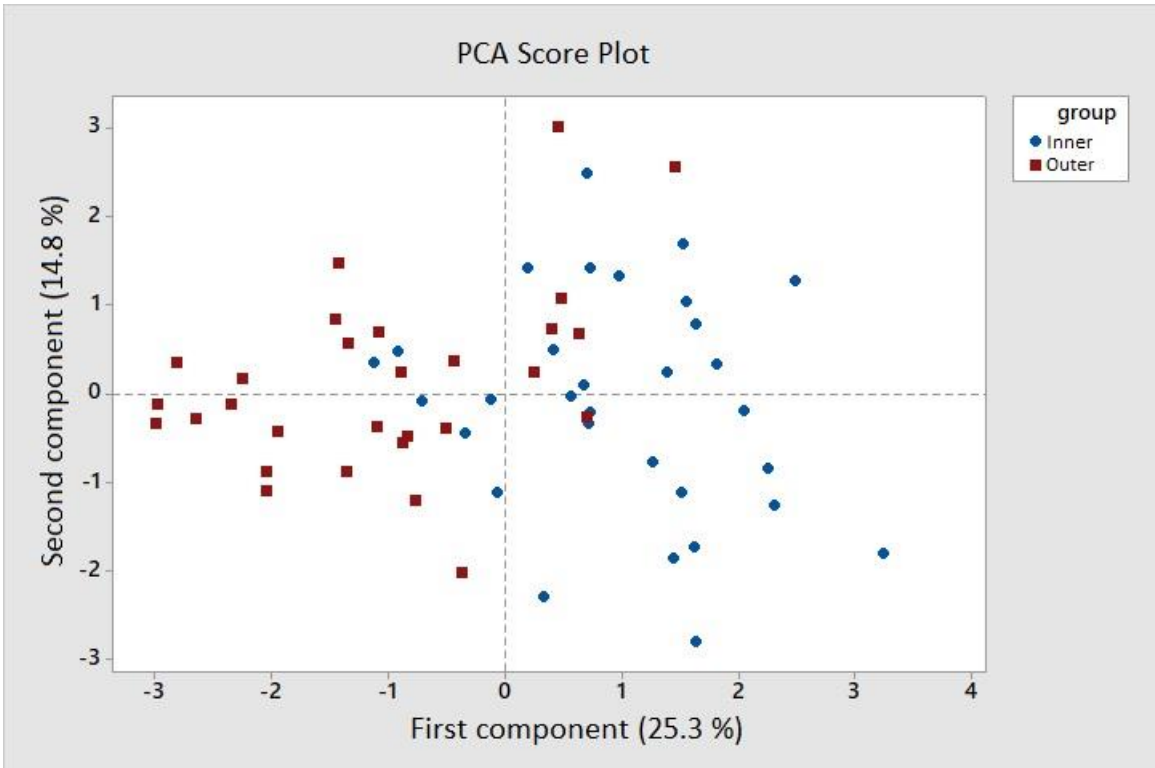


Figure 3.9 PCA score plot of the sampling. Total variance explained in the plot: 40.1 %

# 4. Discussion

## 4.1 Physiological indices

In this study we choose to analyse all collected cod as they are representatives of the populations in the fjord. The size of the sampled cod ranged from 33.5 cm to 81.5 cm, and the weight ranged from 300 g to 4640 g. The distribution of both weight and length was of similar character which provides a comparable basis. The age of the collected cod was not taken into consideration. Fish with different age may also have a difference in maturation stage. This might influence measured values and responses in the biomarkers but is assumed to not have a significant impact.

There was no significant difference in the CI when comparing the inner fjord to the outer fjord. The LSI did not show any significant difference when comparing the inner fjord to the outer fjord within the same year but indicated a significant increase from 2015 to 2017. The GSI showed significant higher values for males in the inner fjord when comparing to females in inner fjord and both genders in the outer fjord. The difference in GSI may have been caused by age difference, where the males in inner fjord potentially were more mature than the rest. This is hard to confirm as no age of any of the caught fish were considered. This could potentially influence the LSI as well, as the gonad development is energy demanding and has shown to reduce the LSI. Reduced LSI values might be regarded as an indication of exposure to environmental pollution.

Both CI and LSI will vary throughout the year as the energy loss regarding spawning can be quite huge and give pronounced manifestations (Mello and Rose, 2005). Variation can also occur between the seasons as food availability, especially the availability of fat-rich food, and the temperature is assumed to be factors of influence (Krohn *et al.,* 1997). Organic pollutants have been reported to influence LSI in fish (van der Oost *et al.,* 2003), whereas Aas *et al.* (2001) reported reduction in both LSI and CI from conducted field studies on cod exposed to PAHs.

The CI is a measurement of somatic weight to length and reflects the overall health and condition of fishes. A value of 0.85 or above is regarded as normal (Lambert and Dutil, 1997), whereas under will be considered low. Values below 0.7 indicates scarcity of food. Only one fish was measure below 0.7, which indicates that there is no scarcity of food in any of the areas. The median value of CI was 0.92 in fish collected in the inner fjord and 0.95 in the ones from the outer fjord, without any significant difference between them. This may indicate a food availability of similar degree, and that environmental pollution does not have a negative effect on the condition in the cod population in inner fjord. This has proven to be a normal situation between the areas since the project started in 2002 (Berge *et al.,* 2014). Food availability has generally been found the same in both areas. It also indicates that the environmental pollution in inner fjord does not influence the CI of the populations here.

The median value of LSI was 3.05 in fish collected in the inner fjord and 2.21 in the ones collected in the outer fjord. LSI values between 2 and 6 percent is considered normal regarding wild Atlantic cod (Jobling, 1988). The index may be influenced by food availability and diet. A diet consisting mostly of pelagic organisms have proven to give an increased LSI compared to a benthic diet (Sherwood *et al.,* 2007). Increased LSI has also been observed in cods from exposed sites and when experimentally exposed to PAHs, PCBs and Polychlorinated dibenzodioxins (PCDDs) (van der Oost *et al.,* 2003). Even though the median LSI in inner fjord is larger than in the outer fjord, there was not a significant statistical difference when comparing the areas to each other. This has been the normal situation from previous studies comparing the inner fjord to the outer fjord (except 2011) (Berge *et al.,* 2014). This is an indication that cods from the inner fjord do not have reduced LSI values as a result of environmental pollution. Nevertheless, variations might occur, like in 2011, which might be explained by variation in diet of e.g. fat-rich prey.

The mean GSI value was significantly higher in male cods sampled in the inner fjord compared to females sampled in inner fjord and both genders sampled in the outer fjord. This might be an indication of more mature males in inner fjord, but as mentioned, age was not measured and can thus not be confirmed. The cods were collected outside of the spawning season to minimize differences due to maturation stages and gender (Brander, 1994). As mentioned before, more mature fish could affect the LSI as well. By analysing the mean GSI and LSI values of both male and female cod sampled in the inner fjord, the observed values may indicate an age difference. The average LSI value for female cods in the inner fjord was 3.65 and the average LSI value for males were 2.69. This makes it a reasonable assumption that both the LSI and GSI difference in inner fjord may be caused by age difference. There might also be effects which could have an impact on the spawning times between the areas or expedite the maturation in one area compared to the other, but this is less likely. It appears reasonably to assume that the difference in mean GSI value is due to age difference, even though this could not be confirmed. From previous analyses it has seemed that no significant difference in mean GSI value is a normal situation when comparing the inner fjord to the outer fjord (Berge *et al.,* 2014).

The PCA analysis showed a significant positive correlation between LSI and GSI. This would most likely have been a negative correlation during the spawning period, as the gonad development is demanding a high amount of energy. But as the cods were sampled outside of the spawning period, this is considered an indication of high food availability. There was also found a negative correlation between GSI and EROD activity. Exposure to several environmental contaminants have been linked to reduced GSI values in fish (Sakamoto *et al.,* 2003), and increased EROD activity is also observed (Whyte *et al.,* 2000). Meaning the higher GSI values are found in the cods with lower EROD activity, which may indicate that the environmental pollution resulting in higher EROD activity can also influence the gonad development of the cod. But as the only correlation between GSI and PAH

metabolites is positive, this is most likely not the case. These results are hard to interpret, and the correlation cannot be explained.

Summarized, there was no significant difference in the CI or LSI values in sampled cod from the inner fjord compared to the outer fjord. The increased GSI value in sampled male cod from inner fjord is most likely due to age difference, even though this was not confirmed.

## 4.2 PAH exposure

### 4.2.1 PAH metabolites in bile

There was a significant difference in 2,3-ring and 4-ring PAH metabolites in the bile of cod from the inner fjord compared to the outer fjord, but no significant difference in 5-ring PAH metabolites in the bile. There was also found a correlation between 4-ring PAH metabolites with both 2,3-ring and 5-ring PAH metabolites, but not between 2,3-ring and 5-ring PAH metabolites. PAH metabolites measured in bile are an indication of recent exposure (van der Oost *et al.,* 2003) either by uptake through the gills or from diet (Grung *et al.,* 2009). As cods sampled in inner fjord had a significant higher concentration of both 2,3-ring and 4-ring metabolites, this indicates a higher recent exposure to PAHs in inner fjord compared to outer fjord. Thus, the population of cod in the inner fjord may experience more negative effects caused by PAHs compared to cods in the outer fjord. The observed correlation between the metabolites may also indicate similar ratios and/or sources of release.

PAHs in the marine environment will most often appear in complex mixtures, with up to several hundred different individual components (Neff *et al.,* 2005). In this thesis, groups of 2,3-, 4-, and 5-ring PAH metabolites in cod bile was measured by FF. The biggest difference between inner and outer fjord in PAH metabolite content was found in the 4-ring group, or pyrene type of PAHs. A significant higher content was found in the inner fjord compared to the outer fjord. The 2,3-ring PAH metabolites did also show a higher content in the inner fjord compared to the outer fjord. The smaller PAHs have a higher solubility in water compared to bigger PAHs that are more hydrophobic. Hence the smaller PAHs are more available for uptake through the gills, while the bigger ones tend to bind to the sediment and be less bioavailable. They are however accumulated up through food chains, and some prey, like polychaetes, have a much higher bioaccumulation of PAHs as they have a less effective metabolism of them. A potential source of pyrene metabolites in cod, is by consuming *Hediste diversicolor* which bioaccumulates PAHs from contaminated sediments, especially pyrene (Ruus *et al.,* 2005). As sampled sediments have shown concentrations of pyrene over 600 µg/kg sediment, this is most likely one of the bigger contribution paths of pyrene regarding cod in inner fjord (Berge *et al.,* 2013).

PAHs in the environment are derived from pyrogenic or petrogenic sources. The pyrogenic PAHs originate from incomplete combustion of organic material and consist mostly of 3 or more aromatic rings. The petrogenic PAHs originate from oil and petroleum products and consist mainly of 2 to 3

aromatic rings (Neff *et al.,* 2005). Pyrene type of metabolites, which showed the most significant difference between the areas is associated with pyrogenic sources (Aas *et al.,* 2001). Thus, it would be conceivable to assume the increased exposure to 4-ring PAHs in inner fjord is originated from combustion processes like engines in cars and boats, but also industrial combustion activities. This is further supported by the estimated sources of PAH to inner fjord (see section 1.3.2), where atmospheric, drainage overflow and river effluents are major sources. The river effluents may very well consist of PAHs from industrial combustion and combustion processes from cars. Combustion of wood is also recognized as an important source of PAH exposure and considering the higher population density around inner fjord compared to outer fjord, it will result in an increased exposure here (Sørensen, 2012). Naphthalene and phenanthrene type of PAHs are mostly associated with petrogenic sources, and higher values in inner fjord can be caused by oil spills from harbours, boats and different activities up-stream in rivers which leads to the inner fjord. The boat traffic in the fjord is a known source of pollutant regarding PAHs (Koehler and Hardy, 1999).

Another plausible reason for a higher PAH exposure in inner fjord compared to outer fjord is the enclosed feature of the fjord. This leads to reduced water exchange with the open ocean. PAHs are persistent in sediments, especially 4- and 5-ring PAHs which have a half time over 100 days (Wilcock *et al.,* 1996). As the water exchange regarding the bottom most part only happens once a year for Vestfjorden and every 3-4 years for Bunnefjorden (Lundsør *et al.,* 2017), PAHs are more likely to up-concentrate in both the water and the sediments.

The majority of PAHs will be metabolised to more water-soluble and excretable forms and sent to the bile. From here, the PAHs leave the organism as a part of digestion (Andersson and Förlin, 1992). The liver is metabolizing the PAHs quite effectively, which is why PAHs are usually concentrated in the bile. As the bile is being emptied reasonably often as a part of the digestion, measured PAH concentration will indicate PAH exposure from recent days (van der Oost *et al.,* 2003).

Previously conducted studies have shown persistent higher values of PAH metabolites in the bile of cod from inner fjord compared to outer fjord (Berge *et al.,* 2014). From the Spreaman's rank order correlation analysis there were found a strong correlation between 4-ring PAH metabolites and 2,3-ring PAH metabolites. This may indicate that they can originate from the same sources. The results from this study indicates the cod from inner fjord is exposed to PAHs in a higher degree than cod from the outer fjord.

### 4.2.2 EROD activity

There was no observed significant difference in EROD activity when comparing the inner fjord with the outer fjord in 2017 or in 2015. There was however significant difference between the years.

The induction of CYP1A can be caused by both endogenous and exogenous chemicals that bind to the aryl hydrocarbon receptor and thus upregulate the CYP1A gene (Hahn, 1998). Several planar aromatic hydrocarbons are known to induce the CYP1A. This includes dioxins, PAHs, PCBs, polybrominated biphenyls (PBBs) and polychlorinated tert-phenyls (PCTs) (Whyte *et al.,* 2000).

The only correlation between PAH metabolites in the bile and EROD activity was with 4-ring PAH metabolites. This might indicate that only the 4-ring PAH metabolites are present in a high enough concentration to influence the EROD activity. The correlation was however negative, meaning increased 4-ring metabolites seems to lead to decreased EROD activity. A previously conducted experiment with cod and flounder showed a positive correlation. Cod and flounder were placed in cages and exposed to contaminated sediments, where phenanthrene, pyrene and benzo[a]pyrene showed a significant correlation with CYP1A in liver tissue in both species (Beyer *et al.,* 1996). Why the correlation in this study was observed as negative remains unknown.

Regarding PCB as a CYP1A inducing agent, concentrations in cod liver was reported to exceed the new concept denoted high reference concentration (PROREF) by a factor between 2 to 5 in 2016 (Green *et al.,* 2016). The PCB content in blue mussel decreased from exceeding the PROREF by factor between 5 to 10 in 2015 to a factor between 2 to 5 in 2016. This might be a reason for the higher EROD activity observed in 2015 compared to 2017.

Factors as temperature has been proven to influence the CYP1A induction from previously conducted experiments (Lyons *et al.,* 2011; Sleiderink *et al.,* 1995). As the cod were collected within a timespan of two days it is assumed there was no temperature difference between the days. The two areas are also geographically close, assuming no temperature difference between the two stations. Therefore, temperature was not assumed to influence the CYP1A induction in this experiment.

During starvation it has been reported reduction in EROD activity in rainbow trout (*Oncorhynchus mykiss*) (Andersson *et al.,* 1985). However, both CI and LSI values from the two areas indicates the cod populations do not suffer from scarcity. It was assumed that scarcity did not affect the obtained EROD activity.

Another factor which might influence the CYP1A induction is that continuous exposure to CYP1A inducing agents may affect the fish to become more resistant towards these contaminants (Wirgin and Waldman, 2004).

Hylland *et al.* (2012) defined the background level for EROD activity as 145 pmol/min/mg protein. This means all recorded data in 2017 was within the background level. Previous studies have shown a higher activity in the inner Oslofjord compared to the outer fjord (Berge *et al.,* 2014). This might indicate the exposure to CYP1A inducing agents have been reduced from previous years.

## 4.3 metallothionein

There were no significant differences regarding MT content in liver of the cods sampled in the inner fjord compared to the ones in outer fjord. MT is used as a biomarker for heavy metal exposure. Conducted experiments have shown induction of the protein from the non-essential metals like cadmium and the essential metals zinc and copper (Goksøyr *et al.,* 1996; Hylland *et al.,* 1994). One could have expected higher MT induction in cod sampled in inner fjord, as Green *et al.* (2016) reported high content of Hg in cod fillet (exceeded PROREF by a factor of 5-10), and elevated values of mercury, lead and zinc in the sediments. Significantly high Hg concentrations have been measured several places in the sediments in inner fjord (Helland *et al.,* 2003).

Other environmental contaminants can influence the induction of MT. Conducted experiments on flounder have shown that both PCB-156 and benzo[a]pyrene (B[a]P) influenced and reduced the induction effect of Cd, and that the order of the exposure mattered (Sandvik *et al.,* 1997). Both contaminants are present in inner Oslofjord. B[a]P concentration is on a downward trend, and ΣPCB-7 is exceeding the PROREF by a factor between 5 to 10. The ΣPCB-7 has been within the PROREF limit in outer fjord but was found to be exceeding by a factor of 2 in 2016 (Green *et al.,* 2016). This might have influenced more reduction of MT induction in inner fjord compared to outer fjord. The B[a]P content in bile was not assessed in this thesis, but as there was not any significant difference in 5-ring PAH metabolites in the bile when comparing cod from inner fjord to cod from outer fjord, it is assumed PAHs did not influence the MT concentration.

Both Zn and Cu concentrations are increased in female cod during vitellogenesis and in males during gonad development. This results in higher MT concentrations in the liver (Hylland *et al.,* 1992). As the males in inner fjord had significant higher GSI value, this might have influenced the measured MT concentration. However, no significant correlation was found between MT and GSI, so it is assumed it had no influence.

## 4.4 ALA-D activity

There was not found any significant difference in ALA-D activity when comparing the sampled cods from inner fjord to the ones from outer fjord in 2017. There was found a significant difference between the years, and between the sampled cod in inner fjord compared to the outer fjord in 2015. All results were above the normal baseline activity (10-20 ng/min/mg protein) (OSPAR, 2007). This indicates that cod in the areas do not have inhibited ALA-D activity from Pb exposure.

ALA-D activity in red blood cells is a Pb specific biomarker (Walker *et al.,* 2012). A good correlation between the amount of Pb in blood and ALA-D activity have been reported (Schmitt *et al.,* 2007). There are however other metals which has been reported to also inhibit the ALA-D activity to some extent. (Rodriguez *et al.,* 1989; Hylland *et al.,* 2009). The enzyme in fish do demand a zinc ion as a cofactor to function (Schmitt *et al.,* 2005; Moraes *et al.,* 2003; Warren *et al.,* 1998), and studies have proven that

zinc has the ability to reactivate inhibited ALA-D (Lombardi *et al.,* 2010). The Zn level in inner Oslofjord is however considered to be low (Green *et al.,* 2016) and is therefore assumed to not affect the obtained results.

Pb exposure, on a national basis, mainly comes from products containing Pb. This includes ammunition, fishing gear, landfill seeps, sediments, sludge, drains and overflow from e.g. storm water. Pb is also transported over long distances via water and air flows from Europe and is an important source of Pb in Norway. Since 1995, several restrictions and actions to reduce Pb discharge has resulted in an approximately 80 % reduction in 2010 (Sørensen, 2012).

Previous results show that there has been no significant difference in ALA-D activity in the sampled cod collected in the inner fjord and the ones collected in the outer fjord, except 2011 (Berge *et al.*, 2014). This seems to correlate with the reduction of Pb discharge reported by Sørensen (2012). The results from this analysis does indicate that inner fjord is not exposed to a higher content of Pb than outer fjord.

# 5. Conclusions

The collected cod did not have any significant difference in CI or GSI. Males from inner fjord did have a significant higher GSI than females, and both genders in outer fjord, but is assumed to not influence the results of the biomarkers used in this study. This may indicate that there is no difference in food availability between the two monitored area, as regarding cod.

There were found higher concentrations of 2,3-ring and 4-ring PAH metabolites in cod bile in the inner fjord compared to the outer fjord. It was not found a higher concentration of CYP1A in liver, measured as EROD activity in inner fjord compared to outer fjord. No difference was recorded in MT concentration in cod liver as a response to heavy metal pollution. The measured ALA-D values did also not show any difference between the two monitored areas.

The PCA analysis showed that the individuals from the two areas was separated, which indicates the two areas are different. This difference may be an indication of the inner fjord being exposed to environmental contaminants in a higher degree than the outer fjord is.

# 6. Future Prospects

The EROD did not show any difference between the areas, even though there was a difference in PAH metabolites in the bile. As cod from areas with a continuous exposure to CYP1A inducing agents have shown a reduced EROD response, this could be further investigated. As fish from the inner fjord has been exposed to CYP1A inducing agents over a long period of time, it is plausible that they might have developed a resistance to these toxicants. This can be checked by capturing and replacing cod from the inner and outer fjord in clean waters for a longer period of time, before exposing them to CYP1A inducing agents.

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# Appendix A – Raw physiological data

Attachment 1 – Raw physiological data for collected cod in inner fjord 2017

| Fish | Station | Sex | Weight (g) | Lenght (cm) | Liver weight (g) | Gonad weight (g) | CI | LSI | GSI |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Inner | f | 1040 | 48 | 46 | 30,2 | 0,94 | 4,42 | 2,90 |
| 2 | Inner | m | 850 | 48,5 | 9,7 | 34,2 | 0,75 | 1,14 | 4,02 |
| 3 | Inner | m | 1300 | 52 | 32,2 | 19 | 0,92 | 2,48 | 1,46 |
| 4 | Inner | f | 3210 | 70 | 99,9 | 82,1 | 0,94 | 3,11 | 2,56 |
| 5 | Inner | m | 630 | 41 | 25,8 | 12 | 0,91 | 4,10 | 1,90 |
| 6 | Inner | f | 4640 | 81,5 | 263,2 | 188,6 | 0,86 | 5,67 | 4,06 |
| 7 | Inner | f | 520 | 40 | 9,1 | 3,8 | 0,81 | 1,75 | 0,73 |
| 8 | Inner | f | 1270 | 51,5 | 54,6 | 46,2 | 0,93 | 4,30 | 3,64 |
| 9 | Inner | m | 730 | 45 | 13,5 | 13,4 | 0,80 | 1,85 | 1,84 |
| 10 | Inner | f | 510 | 38,5 | 11,3 | 3,5 | 0,89 | 2,22 | 0,69 |
| 11 | Inner | m | 750 | 43,5 | 22,3 | 56,5 | 0,91 | 2,97 | 7,53 |
| 12 | Inner | f | 700 | 41 | 27,3 | 24,2 | 1,02 | 3,90 | 3,46 |
| 13 | Inner | m | 770 | 42 | 21,2 | 56,8 | 1,04 | 2,75 | 7,38 |
| 14 | Inner | m | 870 | 48 | 22,7 | 16,5 | 0,79 | 2,61 | 1,90 |
| 15 | Inner | m | 450 | 39,5 | 13,8 | 40,1 | 0,73 | 3,07 | 8,91 |
| 16 | Inner | f | 950 | 48 | 28,8 | 19,4 | 0,86 | 3,03 | 2,04 |
| 17 | Inner | f | 590 | 43 | 8,5 | 11,4 | 0,74 | 1,44 | 1,93 |
| 18 | Inner | f | 520 | 44 | 35,1 | 15,3 | 0,61 | 6,75 | 2,94 |
| 19 | Inner | m | 460 | 39,5 | 14,7 | 27,8 | 0,75 | 3,20 | 6,04 |
| 20 | Inner | m | 660 | 42 | 10,2 | 23,2 | 0,89 | 1,55 | 3,52 |
| 21 | Inner | f | 350 | 35,5 | 5,6 | 2,8 | 0,78 | 1,60 | 0,80 |
| 22 | Inner | m | 1460 | 53,5 | 41,8 | 180,5 | 0,95 | 2,86 | 12,36 |
| 23 | Inner | f | 1240 | 52,5 | 49,6 | 22,3 | 0,86 | 4,00 | 1,80 |
| 24 | Inner | m | 670 | 42 | 14,4 | 10,4 | 0,90 | 2,15 | 1,55 |
| 25 | Inner | m | 600 | 40 | 21,9 | 50,3 | 0,94 | 3,65 | 8,38 |
| 26 | Inner | m | 1030 | 47,5 | 36,9 | 77,5 | 0,96 | 3,58 | 7,52 |
| 27 | Inner | m | 920 | 45 | 26,7 | 54,3 | 1,01 | 2,90 | 5,90 |
| 28 | Inner | m | 700 | 41,5 | 14,7 | 16,6 | 0,98 | 2,10 | 2,37 |
| 29 | Inner | f | 700 | 42,5 | 22,6 | 26,6 | 0,91 | 3,23 | 3,80 |
| 30 | Inner | f | 1180 | 53 | 63,3 | 45,1 | 0,79 | 5,36 | 3,82 |
| 31 | Inner | f | 450 | 34 | 20,3 | 16,9 | 1,14 | 4,51 | 3,76 |
| 32 | Inner | f | 810 | 46 | 29,7 | 24,9 | 0,83 | 3,67 | 3,07 |
| 33 | Inner | f | 1110 | 47 | 35,7 | 23,5 | 1,07 | 3,22 | 2,12 |
| 34 | Inner | f | 1070 | 44,5 | 37,9 | 36,9 | 1,21 | 3,54 | 3,45 |
| 35 | Inner | m | 720 | 42 | 33,7 | 70,5 | 0,97 | 4,68 | 9,79 |
| 36 | Inner | m | 890 | 44 | 10 | 29,1 | 1,04 | 1,12 | 3,27 |
| 37 | Inner | m | 790 | 43 | 19,3 | 50,2 | 0,99 | 2,44 | 6,35 |
| 38 | Inner | f | 670 | 41,5 | 9,9 | 3,8 | 0,94 | 1,48 | 0,57 |
| 39 | Inner | f | 1590 | 51 | 66,1 | 49,9 | 1,20 | 4,16 | 3,14 |
| 40 | Inner | f | 820 | 43 | 10,5 | 13,5 | 1,03 | 1,28 | 1,65 |

Attachment 2 – Raw physiological data for collected cod in outer fjord 2017

| Fish | Station | Sex | Weight (g) | Lenght (cm) | Liver weight (g) | Gonad weight (g) | CI | LSI | GSI |
|---|---|---|---|---|---|---|---|---|---|
| 41 | Outer | f | 600 | 36 | 0,0107 | 0,0034 | 0,97 | 1,78 | 0,57 |
| 42 | Outer | f | 1490 | 50,5 | 0,0306 | 0,0353 | 0,95 | 2,05 | 2,37 |
| 43 | Outer | f | 390 | 31 | 0,0179 | 0,0053 | 1,04 | 4,59 | 1,36 |
| 44 | Outer | f | 1050 | 45,5 | 0,021 | 0,0044 | 0,87 | 2,00 | 0,42 |
| 45 | Outer | | 300 | 31 | 0,0115 | | 0,76 | 3,83 | |
| 46 | Outer | f | 830 | 40,5 | 0,019 | 0,0117 | 0,97 | 2,29 | 1,41 |
| 47 | Outer | f | 350 | 34 | 0,006 | 0,0051 | 0,72 | 1,71 | 1,46 |
| 48 | Outer | f | 960 | 42 | 0,0258 | 0,0242 | 0,99 | 2,69 | 2,52 |
| 49 | Outer | | 770 | 37 | 0,0117 | 0,0022 | 1,20 | 1,52 | 0,29 |
| 50 | Outer | m | 1840 | 54 | | 0,0361 | 0,87 | | 1,96 |
| 51 | Outer | m | 830 | 39 | 0,022 | 0,0037 | 1,12 | 2,65 | 0,45 |
| 52 | Outer | m | 1260 | 47 | 0,0315 | 0,0495 | 0,92 | 2,50 | 3,93 |
| 53 | Outer | m | | | 0,0208 | 0,0033 | | | |
| 54 | Outer | f | 2520 | 57,5 | 0,0617 | 0,0108 | 1,06 | 2,45 | 0,43 |
| 55 | Outer | m | 850 | 42 | 0,012 | 0,0026 | 0,87 | 1,41 | 0,31 |
| 56 | Outer | m | 850 | 42 | 0,0205 | 0,0286 | 0,90 | 2,41 | 3,36 |
| 57 | Outer | m | 1310 | 45 | 0,0221 | 0,0454 | 1,35 | 1,69 | 3,47 |
| 58 | Outer | f | 1160 | 44 | 0,0355 | 0,0425 | 1,05 | 3,06 | 3,66 |
| 59 | Outer | f | 930 | 43 | 0,0156 | 0,0047 | 0,90 | 1,68 | 0,51 |
| 60 | Outer | m | 930 | 41 | 0,0262 | 0,0129 | 1,06 | 2,82 | 1,39 |
| 61 | Outer | m | 520 | 34,5 | 0,0108 | 0,0041 | 0,95 | 2,08 | 0,79 |
| 62 | Outer | m | 900 | 43,5 | 0,02 | 0,0363 | 0,87 | 2,22 | 4,03 |
| 63 | Outer | f | 520 | 34 | 0,0094 | 0,0031 | 0,99 | 1,81 | 0,60 |
| 64 | Outer | f | 420 | 32,5 | 0,0071 | 0,0029 | 0,94 | 1,69 | 0,69 |
| 65 | Outer | f | 740 | 39,5 | 0,0247 | 0,0039 | 0,93 | 3,34 | 0,53 |
| 66 | Outer | f | 460 | 34,5 | 0,0069 | 0,002 | 0,91 | 1,50 | 0,43 |
| 67 | Outer | f | 1280 | 47,5 | 0,0174 | 0,0087 | 0,91 | 1,36 | 0,68 |
| 68 | Outer | m | 1700 | 49,5 | 0,0342 | 0,0425 | 1,08 | 2,01 | 2,50 |
| 69 | Outer | f | 2630 | 56 | 0,0789 | 0,1814 | 1,10 | 3,00 | 6,90 |
| 70 | Outer | m | 1270 | 46,5 | 0,019 | 0,0009 | 0,99 | 1,50 | 0,07 |
| 71 | Outer | m | 1380 | 47,5 | 0,0599 | 0,0806 | 0,98 | 4,34 | 5,84 |
| 72 | Outer | m | 1380 | 44,5 | 0,028 | 0,0471 | 1,17 | 2,03 | 3,41 |
| 73 | Outer | f | 1000 | 42,5 | 0,022 | 0,0129 | 1,03 | 2,20 | 1,29 |
| 74 | Outer | m | 540 | 35 | 0,0224 | 0,0171 | 0,95 | 4,15 | 3,17 |
| 75 | Outer | f | 380 | 31,5 | 0,0057 | 0,0015 | 0,93 | 1,50 | 0,39 |
| 76 | Outer | m | 380 | 32,5 | 0,0123 | 0,0011 | 0,81 | 3,24 | 0,29 |
| 77 | Outer | m | 1310 | 46 | 0,0377 | 0,0031 | 0,99 | 2,88 | 0,24 |
| 78 | Outer | m | 1300 | 48 | 0,0155 | 0,0666 | 0,85 | 1,19 | 5,12 |
| 79 | Outer | f | 1300 | 46 | 0,0477 | 0,0703 | 1,04 | 3,67 | 5,41 |
| 80 | Outer | m | 650 | 39 | 0,0152 | 0,0008 | 0,85 | 2,34 | 0,12 |

Attachment 3 – Raw physiological data for collected cod in inner fjord 2015

| Fish | Station | Sex | Weight (g) | Lenght (cm) | Liver weight (g) | Gonad weight (g) | CI | LSI | GSI |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Inner | F | 520 | 38,5 | 5,3 | | 0,91 | 1,02 | |
| 2 | Inner | F | 600 | 49 | 11,2 | | 0,51 | 1,87 | |
| 3 | Inner | F | 980 | 47,5 | 21 | | 0,91 | 2,14 | |
| 4 | Inner | F | 1820 | 60 | 57 | | 0,84 | 3,13 | |
| 5 | Inner | F | 460 | 38 | 10 | | 0,84 | 2,17 | |
| 6 | Inner | M | 460 | 37 | 15,7 | | 0,91 | 3,41 | |
| 7 | Inner | F | 480 | 40 | 12 | | 0,75 | 2,50 | |
| 8 | Inner | F | 660 | 42 | 16 | | 0,89 | 2,42 | |
| 9 | Inner | F | 760 | 44,5 | 10,8 | | 0,86 | 1,42 | |
| 10 | Inner | F | 480 | 38 | 5,4 | | 0,87 | 1,13 | |
| 11 | Inner | M | 1200 | 49 | 28,7 | | 1,02 | 2,39 | |
| 52 | Inner | M | 1420 | 80 | 19,6 | | 0,28 | 1,38 | |
| 53 | Inner | F | 1620 | 56 | 32,9 | | 0,92 | 2,03 | |
| 54 | Inner | F | 540 | 40 | 6,1 | | 0,84 | 1,13 | |
| 55 | Inner | M | 520 | 38,5 | 5,6 | | 0,91 | 1,08 | |
| 56 | Inner | F | 700 | 44,5 | 24,2 | | 0,79 | 3,46 | |
| 57 | Inner | F | 500 | 40 | 6,6 | | 0,78 | 1,32 | |
| 58 | Inner | M | 800 | 45 | 14,9 | | 0,88 | 1,86 | |
| 59 | Inner | F | 680 | 43 | 13,8 | | 0,86 | 2,03 | |
| 60 | Inner | F | 640 | 41,5 | 13,2 | | 0,90 | 2,06 | |
| 61 | Inner | F | 840 | 45 | 17,3 | | 0,92 | 2,06 | |
| 62 | Inner | F | 400 | 38 | 4,7 | | 0,73 | 1,18 | |
| 63 | Inner | M | 440 | 39,5 | 8 | | 0,71 | 1,82 | |
| 64 | Inner | M | 520 | 39 | 9,4 | | 0,88 | 1,81 | |
| 65 | Inner | M | 660 | 41,5 | 12,2 | | 0,92 | 1,85 | |
| 66 | Inner | F | 1220 | 57 | 16,6 | | 0,66 | 1,36 | |
| 67 | Inner | M | 300 | 34 | 8,5 | | 0,76 | 2,83 | |
| 68 | Inner | F | 880 | 46 | 18,2 | | 0,90 | 2,07 | |
| 69 | Inner | M | 710 | 43,5 | 18,9 | | 0,86 | 2,66 | |
| 70 | Inner | M | 1360 | 53 | 11,1 | | 0,91 | 0,82 | |
| 71 | Inner | F | 720 | 44 | 12 | | 0,85 | 1,67 | |
| 72 | Inner | F | 700 | 41,5 | 17,6 | | 0,98 | 2,51 | |
| 73 | Inner | F | 720 | 43 | 11,9 | | 0,91 | 1,65 | |
| 74 | Inner | F | 600 | 43 | 6,7 | | 0,75 | 1,12 | |
| 75 | Inner | F | 860 | 44,5 | 15,6 | | 0,98 | 1,81 | |
| 76 | Inner | F | 400 | 38 | 8 | | 0,73 | 2,00 | |
| 77 | Inner | M | 520 | 41 | 12,5 | | 0,75 | 2,40 | |
| 78 | Inner | M | 1560 | 40,5 | 7,3 | | 2,35 | 0,47 | |
| 79 | Inner | M | 900 | 46 | 18,8 | | 0,92 | 2,09 | |
| 80 | Inner | F | 680 | 42 | 7,8 | | 0,92 | 1,15 | |

Attachment 4 – Raw physiological data for collected cod in outer fjord 2015

| Fish | Station | Sex | Weight (g) | Lenght (cm) | Liver weight (g) | Gonad weight (g) | CI | LSI | GSI |
|---|---|---|---|---|---|---|---|---|---|
| 12 | Outer | M | 1200 | 50 | 19 | | 0,96 | 1,58 | |
| 13 | Outer | F | 790 | 44 | 10,4 | | 0,93 | 1,32 | |
| 14 | Outer | M | 860 | 45 | 13,9 | | 0,94 | 1,62 | |
| 15 | Outer | F | 240 | 30,5 | 3,8 | | 0,85 | 1,58 | |
| 16 | Outer | M | 1300 | 52,5 | 30,9 | | 0,90 | 2,38 | |
| 17 | Outer | F | 1020 | 49 | 6,3 | | 0,87 | 0,62 | |
| 18 | Outer | M | 200 | 30 | 3,1 | | 0,74 | 1,55 | |
| 19 | Outer | F | 880 | 37 | 16,2 | | 1,74 | 1,84 | |
| 20 | Outer | F | 3400 | 68 | 273,6 | | 1,08 | 8,05 | |
| 21 | Outer | F | 900 | 45,5 | 8,6 | | 0,96 | 0,96 | |
| 22 | Outer | F | 1180 | 49,5 | 17,8 | | 0,97 | 1,51 | |
| 23 | Outer | F | 760 | 43 | 18,1 | | 0,96 | 2,38 | |
| 24 | Outer | F | 2170 | 65 | 105,4 | | 0,79 | 4,86 | |
| 25 | Outer | M | 900 | 47,5 | 8,6 | | 0,84 | 0,96 | |
| 26 | Outer | F | 560 | 40 | 7,5 | | 0,88 | 1,34 | |
| 27 | Outer | F | 3600 | 70 | 84,2 | | 1,05 | 2,34 | |
| 28 | Outer | F | 1350 | 50 | 42,9 | | 1,08 | 3,18 | |
| 29 | Outer | F | 2860 | 65 | 149,8 | | 1,04 | 5,24 | |
| 30 | Outer | F | 1240 | 49,5 | 20,7 | | 1,02 | 1,67 | |
| 31 | Outer | F | 640 | 42 | 11,4 | | 0,86 | 1,78 | |
| 32 | Outer | F | 640 | 41 | 12,8 | | 0,93 | 2,00 | |
| 33 | Outer | F | 860 | 46 | 11,6 | | 0,88 | 1,35 | |
| 34 | Outer | F | 900 | 46 | 17,9 | | 0,92 | 1,99 | |
| 35 | Outer | F | 980 | 47 | 14,8 | | 0,94 | 1,51 | |
| 36 | Outer | F | 420 | 36 | 6 | | 0,90 | 1,43 | |
| 37 | Outer | F | 460 | 37 | 5 | | 0,91 | 1,09 | |
| 38 | Outer | F | 340 | 34 | 4,5 | | 0,87 | 1,32 | |
| 39 | Outer | M | 1020 | 46,5 | 17,8 | | 1,01 | 1,75 | |
| 40 | Outer | M | 1640 | 57 | 50 | | 0,89 | 3,05 | |
| 41 | Outer | F | 380 | 37 | 6,4 | | 0,75 | 1,68 | |
| 42 | Outer | M | 660 | 41 | 15,9 | | 0,96 | 2,41 | |
| 43 | Outer | F | 1800 | 58 | 43 | | 0,92 | 2,39 | |
| 44 | Outer | M | 700 | 44,5 | 9,5 | | 0,79 | 1,36 | |
| 45 | Outer | M | 1520 | 52 | 36,4 | | 1,08 | 2,39 | |
| 46 | Outer | M | 280 | 32 | 4,6 | | 0,85 | 1,64 | |
| 47 | Outer | F | 580 | 39 | 6,1 | | 0,98 | 1,05 | |
| 48 | Outer | F | 1200 | 51,5 | 17,3 | | 0,88 | 1,44 | |
| 49 | Outer | F | 1780 | 59 | 62,6 | | 0,87 | 3,52 | |
| 50 | Outer | M | 260 | 33 | 6,8 | | 0,72 | 2,62 | |
| 51 | Outer | M | 860 | 45,5 | 12,4 | | 0,91 | 1,44 | |

# Appendix B – Raw biomarker data

Attachment 1 – Raw biomarker data for collected cod in inner fjord 2017

| Fish | Station | PFE290/334 µg/ml | PFE341/383 µg/ml | PFE380/430 µg/ml | MT mg/g | EROD pmol/min /mg protein | ALA-D ng PBG/min /mg protein |
|---|---|---|---|---|---|---|---|
| 1 | Inner | 555 | 253 | 104 | 3,30 | 29,15313 | 47,6 |
| 2 | Inner | 476 | 264 | 50 | 1,86 | 17,3173 | 63,6 |
| 3 | Inner | | | | 2,27 | 96,86139 | 73,7 |
| 4 | Inner | 534 | 344 | 19 | 3,96 | 16,01178 | 93,0 |
| 5 | Inner | 127 | 94 | 57 | 2,53 | 21,36869 | 55,8 |
| 6 | Inner | | | | 4,30 | 7,97103 | 77,0 |
| 7 | Inner | | | | 2,76 | 14,50682 | 65,4 |
| 8 | Inner | 500 | 279 | 21 | 3,85 | 4,3628 | 61,1 |
| 9 | Inner | 702 | 474 | 56 | 3,07 | 7,8922 | 57,9 |
| 10 | Inner | 576 | 365 | 126 | 2,76 | 37,59639 | 47,2 |
| 11 | Inner | 358 | 233 | 1 | 2,18 | 11,81557 | 21,2 |
| 12 | Inner | 577 | 252 | 31 | 2,17 | 10,31422 | 19,9 |
| 13 | Inner | | | | | 15,03109 | 20,9 |
| 14 | Inner | | | | 2,28 | 19,65935 | 20,6 |
| 15 | Inner | 269 | 299 | 28 | 4,49 | 1,96267 | 21,7 |
| 16 | Inner | 425 | 296 | 49 | 2,73 | 8,0992 | 18,6 |
| 17 | Inner | 338 | 164 | 58 | 2,99 | 0,22969 | 13,8 |
| 18 | Inner | 356 | 183 | 9 | 2,97 | 9,49987 | 11,4 |
| 19 | Inner | 414 | 261 | 85 | 3,29 | 12,72803 | 19,3 |
| 20 | Inner | 414 | 190 | 49 | 4,43 | 0 | 40,8 |
| 21 | Inner | 624 | 358 | 58 | 2,35 | 22,21454 | 42,8 |
| 22 | Inner | 560 | 333 | 19 | 2,81 | 2,63546 | 42,0 |
| 23 | Inner | 563 | 377 | 14 | 3,27 | 10,56162 | 50,5 |
| 24 | Inner | 234 | 199 | 27 | 2,52 | 17,64919 | 39,6 |
| 25 | Inner | 478 | 260 | 21 | 3,40 | 9,39197 | 43,8 |
| 26 | Inner | 433 | 212 | 6 | 2,68 | 2,87391 | 46,1 |
| 27 | Inner | 498 | 382 | 110 | 3,97 | 14,15885 | 40,9 |
| 28 | Inner | 232 | 184 | 24 | 3,12 | | 57,5 |
| 29 | Inner | 297 | 117 | 96 | 3,02 | 32,78531 | 42,4 |
| 30 | Inner | 454 | 301 | 32 | 2,78 | 18,4758 | 44,1 |
| 31 | Inner | 579 | 223 | 32 | 3,03 | 17,75927 | 31,8 |
| 32 | Inner | 525 | 175 | 8 | 2,67 | 21,94936 | 39,8 |
| 33 | Inner | | | | 2,94 | 16,31329 | 22,8 |
| 34 | Inner | | | | 2,93 | 15,17081 | 26,3 |
| 35 | Inner | 253 | 272 | 34 | 3,20 | 33,24502 | 42,8 |
| 36 | Inner | 459 | 269 | 126 | 2,66 | 30,0824 | 27,7 |
| 37 | Inner | 325 | 158 | 30 | 2,41 | 42,19994 | 19,8 |
| 38 | Inner | | | | 2,37 | 31,52438 | 24,4 |
| 39 | Inner | 515 | 412 | 123 | 3,14 | 24,75354 | 18,3 |
| 40 | Inner | 735 | 209 | 0 | 2,10 | 23,89619 | 26,8 |

Attachment 2 – Raw biomarker data for collected cod in outer fjord 2017

| Fish | Station | PFE290/334 µg/ml | PFE341/383 µg/ml | PFE380/430 µg/ml | MT mg/g | EROD pmol/min /mg protein | ALA-D ng PBG/min /mg protein |
|---|---|---|---|---|---|---|---|
| 41 | Outer | 275 | 118 | 18 | 1,095416 | 27,9653 | 6,2 |
| 42 | Outer | 266 | 185 | 78 | 2,643287 | 5,48887 | 15,6 |
| 43 | Outer | 390 | 44 | 13 | 6,123707 | 21,09448 | 15,8 |
| 44 | Outer | 163 | 84 | 6 | 1,855613 | 38,05033 | 7,2 |
| 45 | Outer | 244 | 124 | 48 | 1,452617 | 30,88069 | 7,0 |
| 46 | Outer | 231 | 155 | 86 | 2,725718 | 31,76604 | 16,0 |
| 47 | Outer | | | | 1,031303 | 21,71991 | 16,5 |
| 48 | Outer | 246 | 143 | 15 | 3,476756 | 15,02613 | 11,2 |
| 49 | Outer | 240 | 91 | 58 | | 66,51605 | 11,6 |
| 50 | Outer | | | | 3,513392 | 16,79553 | 20,1 |
| 51 | Outer | 280 | 102 | 9 | 3,650777 | 36,83405 | 40,2 |
| 52 | Outer | 397 | 191 | 49 | 3,943865 | 12,92743 | 55,6 |
| 53 | Outer | 201 | 142 | 32 | 3,6233 | 19,51598 | 32,6 |
| 54 | Outer | 205 | 104 | 8 | 5,207807 | 43,57325 | 54,9 |
| 55 | Outer | 211 | 122 | 102 | 1,938044 | 17,05439 | 61,1 |
| 56 | Outer | 135 | 115 | 45 | 3,971342 | 8,09765 | 46,9 |
| 57 | Outer | 60 | 66 | 10 | 2,047952 | 17,01288 | 48,8 |
| 58 | Outer | 185 | 111 | 5 | 1,51673 | 1,86975 | 38,2 |
| 59 | Outer | 308 | 199 | 43 | 3,293576 | 7,97741 | 47,6 |
| 60 | Outer | 464 | 142 | 90 | 3,614141 | 28,93478 | 47,8 |
| 61 | Outer | 583 | 168 | 31 | 2,652446 | 29,4052 | 27,8 |
| 62 | Outer | 48 | 123 | 17 | 2,689082 | 19,65695 | 23,9 |
| 63 | Outer | | | | 2,515061 | 27,98522 | 16,7 |
| 64 | Outer | | | | 3,788162 | 24,86156 | 17,0 |
| 65 | Outer | 439 | 67 | 67 | 5,061263 | 13,84571 | 23,0 |
| 66 | Outer | 122 | 111 | 8 | 3,403484 | 21,42371 | 26,5 |
| 67 | Outer | 229 | 114 | 50 | 2,15786 | 25,25463 | 16,6 |
| 68 | Outer | 181 | 110 | 1 | 3,147032 | 26,09344 | 17,5 |
| 69 | Outer | 317 | 244 | 37 | 2,661605 | 0,22735 | 4,0 |
| 70 | Outer | 255 | 197 | 33 | 2,753195 | 34,37101 | 17,1 |
| 71 | Outer | 422 | 143 | 7 | 1,800659 | 6,31826 | 42,1 |
| 72 | Outer | 357 | 139 | 72 | 2,121224 | 17,82382 | 25,2 |
| 73 | Outer | | | | 1,800659 | 11,17049 | 49,6 |
| 74 | Outer | | | | 3,34853 | 11,13989 | 48,0 |
| 75 | Outer | 515 | 97 | 33 | 1,69991 | 23,86098 | 56,7 |
| 76 | Outer | | | | 3,742367 | 30,27566 | 64,2 |
| 77 | Outer | 128 | 70 | 10 | 1,901 | 35,1536 | 30,5 |
| 78 | Outer | 308 | 97 | 24 | 2,662 | 12,59634 | 37,7 |
| 79 | Outer | | | | 2,551697 | 3,67249 | 33,0 |
| 80 | Outer | 328 | 151 | 57 | 3,6233 | 27,28136 | 47,7 |

Attachment 3 – Raw biomarker data for collected cod in inner fjord 2015

| Fish | Station | PFE290/334 µg/ml | PFE341/383 µg/ml | PFE380/430 µg/ml | MT mg/g | EROD pmol/min /mg protein | ALA-D ng PBG/min /mg protein |
|---|---|---|---|---|---|---|---|
| 1 | Inner | 328 | 205 | 32 | 3,40 | 90,26 | 62,34 |
| 2 | Inner | 298 | 137 | 18 | 2,94 | 12,31 | 53,57 |
| 3 | Inner | 778 | 312 | 84 | 2,92 | 149,73 | 47,30 |
| 4 | Inner | 296 | 154 | 6 | 3,24 | 0,00 | 70,83 |
| 5 | Inner | 643 | 253 | 99 | 3,85 | 52,37 | 62,66 |
| 6 | Inner | 1568 | 146 | 144 | 4,08 | 25,17 | 65,79 |
| 7 | Inner | 271 | 141 | 88 | 2,41 | 13,47 | |
| 8 | Inner | 310 | 184 | 21 | 2,16 | 55,45 | 66,08 |
| 9 | Inner | 656 | 371 | 125 | 2,46 | 126,98 | |
| 10 | Inner | 451 | 103 | 20 | 2,15 | 29,60 | |
| 11 | Inner | 364 | 263 | 47 | 3,78 | | 63,22 |
| 52 | Inner | 412 | 184 | 3 | 3,06 | 71,22 | 90,77 |
| 53 | Inner | | | | 3,14 | 41,24 | 123,92 |
| 54 | Inner | 579 | 369 | 28 | 3,30 | 8,70 | 100,84 |
| 55 | Inner | 267 | 174 | 54 | 2,84 | 7,94 | 84,81 |
| 56 | Inner | 280 | 154 | 42 | 2,66 | 109,40 | 54,70 |
| 57 | Inner | 1640 | 133 | 0 | 2,42 | 2,62 | |
| 58 | Inner | 342 | 292 | 67 | 3,08 | 176,50 | |
| 59 | Inner | 438 | 260 | 79 | 1,74 | 303,64 | 54,82 |
| 60 | Inner | | | | 4,73 | 108,59 | 73,30 |
| 61 | Inner | 755 | 448 | 111 | 3,59 | 42,37 | 90,28 |
| 62 | Inner | 857 | 167 | 16 | 4,00 | 35,83 | 107,29 |
| 63 | Inner | 621 | 268 | 21 | 3,03 | 34,60 | 79,54 |
| 64 | Inner | 314 | 243 | 35 | 3,74 | 107,66 | 24,44 |
| 65 | Inner | 813 | 505 | 80 | 2,34 | 55,80 | 70,09 |
| 66 | Inner | 426 | 151 | 89 | 2,65 | 6,68 | 152,02 |
| 67 | Inner | 299 | 291 | 64 | 1,28 | 13,48 | 52,70 |
| 68 | Inner | 321 | 234 | 77 | 3,32 | 8,36 | 144,29 |
| 69 | Inner | 351 | 100 | 45 | 3,00 | 72,83 | 63,33 |
| 70 | Inner | 547 | 195 | 18 | 3,72 | 16,42 | 83,69 |
| 71 | Inner | 386 | 217 | 28 | 2,34 | 34,40 | 79,06 |
| 72 | Inner | 554 | 208 | 26 | 3,37 | 168,92 | |
| 73 | Inner | 488 | 341 | 208 | 3,68 | 40,86 | 62,79 |
| 74 | Inner | 508 | 348 | 217 | 3,40 | 0,00 | 130,84 |
| 75 | Inner | 602 | 401 | 199 | 1,86 | 107,21 | 100,39 |
| 76 | Inner | 357 | 172 | 30 | 2,91 | 63,30 | 89,09 |
| 77 | Inner | 324 | 163 | 33 | 1,91 | 78,78 | 59,07 |
| 78 | Inner | 516 | 339 | 194 | 3,32 | 103,95 | |
| 79 | Inner | 573 | 321 | 237 | 2,73 | 182,03 | 60,00 |
| 80 | Inner | 420 | 195 | 56 | 2,67 | 101,69 | 115,22 |

Attachment 4 – Raw biomarker data for collected cod in outer fjord 2015

| Fish | Station | PFE290/334 µg/ml | PFE341/383 µg/ml | PFE380/430 µg/ml | MT mg/g | EROD pmol/min /mg protein | ALA-D ng PBG/min /mg protein |
|---|---|---|---|---|---|---|---|
| 12 | Outer | 377 | 130 | 38 | 0,18 | 77,56 | 65,27 |
| 13 | Outer | 195 | 135 | 0 | 2,42 | 28,53 | 47,29 |
| 14 | Outer | 84 | 122 | 110 | 4,13 | 67,61 | 59,25 |
| 15 | Outer | 285 | 135 | 16 | 2,50 | 0,00 | 30,38 |
| 16 | Outer | 461 | 142 | 123 | 3,83 | 0,00 | 53,75 |
| 17 | Outer | 269 | 188 | 33 | 3,09 | 0,00 | 50,99 |
| 18 | Outer | 246 | 141 | 26 | 2,89 | 11,95 | 44,47 |
| 19 | Outer | 284 | 173 | 13 | | 76,99 | 49,78 |
| 20 | Outer | 529 | 122 | 11 | | 11,58 | 45,93 |
| 21 | Outer | 305 | 170 | 0 | 3,35 | 129,50 | 53,28 |
| 22 | Outer | 480 | 99 | 41 | 3,20 | 73,07 | |
| 23 | Outer | 287 | 157 | 0 | 2,24 | 0,00 | 64,39 |
| 24 | Outer | 684 | 84 | 21 | 2,94 | 63,58 | 51,55 |
| 25 | Outer | 487 | 79 | 107 | 2,89 | 36,66 | 11,03 |
| 26 | Outer | 307 | 230 | 52 | 3,12 | 55,71 | 32,83 |
| 27 | Outer | 646 | 96 | 43 | 2,22 | 47,77 | 34,49 |
| 28 | Outer | 1174 | 169 | 165 | 2,68 | 3,84 | 71,12 |
| 29 | Outer | 798 | 105 | 68 | 1,55 | 10,05 | |
| 30 | Outer | 378 | 204 | 91 | 2,92 | 92,73 | 85,56 |
| 31 | Outer | 475 | 199 | 16 | 2,65 | 53,04 | 63,49 |
| 32 | Outer | 1333 | 165 | 40 | 5,05 | 33,44 | |
| 33 | Outer | 278 | 125 | 100 | 3,18 | 53,34 | 49,86 |
| 34 | Outer | 251 | 120 | 47 | 2,95 | 2,82 | 36,32 |
| 35 | Outer | 191 | 54 | 26 | 2,64 | 112,15 | 59,17 |
| 36 | Outer | 367 | 163 | 72 | 2,97 | 13,82 | 26,77 |
| 37 | Outer | | | | 3,52 | 41,04 | 31,85 |
| 38 | Outer | 366 | 143 | 15 | 2,30 | 5,46 | 18,06 |
| 39 | Outer | 593 | 68 | 22 | 2,88 | 59,07 | 37,62 |
| 40 | Outer | 222 | 79 | 44 | 1,92 | 10,76 | 41,93 |
| 41 | Outer | 60 | 98 | 25 | 2,33 | 14,63 | 83,23 |
| 42 | Outer | 470 | 164 | 167 | | 3,71 | 61,36 |
| 43 | Outer | 198 | 102 | 0 | 2,40 | 167,55 | 49,78 |
| 44 | Outer | 141 | 162 | 37 | 2,81 | 38,42 | 55,69 |
| 45 | Outer | 373 | 150 | 11 | 2,78 | 51,86 | 53,32 |
| 46 | Outer | 332 | 186 | 43 | | 172,82 | |
| 47 | Outer | 360 | 215 | 189 | 3,30 | 55,48 | 42,45 |
| 48 | Outer | 265 | 202 | 80 | 4,42 | 33,75 | 57,07 |
| 49 | Outer | 615 | 215 | 77 | 3,99 | 8,79 | 54,65 |
| 50 | Outer | 780 | 146 | 67 | 2,55 | 100,28 | 49,61 |
| 51 | Outer | 881 | 162 | 15 | 3,38 | 58,60 | |