Mercury accumulation in European perch (*Perca fluviatilis*) in Lake Mjøsa

Effect on the activity of Superoxide dismutase and Glutathione peroxidase enzymes.

Jerlane Njamba Mukonda

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Acknowledgment

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Dedication

The school going process is very long and sometimes boring. At one point, I did not see the point of going to school, so I dropped out of school in the 5^{th} grade and started fishing with friends. It was only after 3 moths when my mother realized, and she pursuaded me to go back to school. I dedicate this to her. May her soul rest in peace. The second person I dedicate this project to is my brother in-law, Mulele Mainga, who took charge of me immediately when I became parentless in 1998. Our friendship grew when we spend a lot of time fishing, hunting birds and herding cattle together. I can't forget those moments we enjoyed together in 1998. You are a true lover of nature.

Table of contents

Abbreviations

CAT	Catalase
CFC	Chlorofluorocarbons
ETC	Electron transport chain
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GS-SG	Glutathione (oxidized)
GST	Glutathione S-transferase
Hg	Hydragyrum/ mercury
LC_{50}	Lethal Concentration
NADH	Nicotinamide adenine dihydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NIVA	Norsk Institutt for Vannforskning
PMS	Phenazine methosulphate
PMSF	Phenylmethanesulfonyl fluoride
ppb	Parts per billion
ppm	Parts per million
ROS	Reactive oxygen species
Rpm	Rotations per minutes
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E	Standard error
SOD	Superoxide dismutase
WTO	World health organization
XO	Xanthine oxidase

1 Abstract

Mercury pollution in freshwater is widespread and a concern to human health. Administering mercury chloride to organisms under study to determine the acute toxicity of mercury has been a common approach in studying pathology and response of antioxidant enzymes. Little is documented on antioxidant response in freshwater fish living in their natural habitat in a lake that is moderately polluted with mercury. To address this question, levels of Mercury in white muscle and livers of European Perch (*Perca fluviatilis*) from Lake Mjøsa were studied. Age and length of fish were used to study the correlation of mercury levels against antioxidant response (enzyme activity). The fish samples used in the experiment were caught in June 2012 and May 2013. Enzyme activity analysis was determined on two antioxidant enzymes, Superoxide dismutase (SOD) and Glutathione peroxidase (GPx). The activity analysis of SOD was carried out using a non-enzyme superoxide generator. The results revealed mercury presence in studied samples in a range between 0.15 ppm and 1.69 ppm in the white muscle, and between 0.12 ppm and 1.81 ppm in the liver. Regression analysis using linear model, revealed age and length of fish correlated positively against mercury concentration in both white and liver tissues. Superoxide dismutase activity correlated negatively against $[Hg]_{muscle}$, $[Hg]_{liver}$ and fish length, while total protein concentration was positively correlated against $[Hg]_{muscle}$ and $[Hg]_{liver}$. No correlation was observed between SOD enzyme activity and [protein]. The negative enzyme activity observed in this study is a remarkable observation and opposite of what acute toxicity studies previously reported. The results can be concluded that chronic mercury exposure in the natural habitat may reduce enzyme activity, but the lost activity is compensated for by more protein production. In vitro addition of mercury chloride during enzyme activity analysis only reduced signal intensity instead of enzyme activity, an observation that may mean that mercury ions reacted with the superoxide ions and escape as vapour, without binding to the enzyme molecules.

2 Introduction

This project is a master's thesis carrying a weight of 60credits (ECT) in a two year study program of Master's in applied and commercial biotechnology at Hedmark University College. It was a stand-alone project with the aim of studying if there is any observable and explainable correlation between antioxidant enzyme levels, protein levels and mercury levels in freshwater fish in a lake that was once affected by mercury pollution. In other words, the study focuses on the effects of mercury pollution on antioxidant enzymes of freshwater fish in their natural habitat.

Much of the previously conducted mercury studies in fish relied mainly on administering mercury to live organisms to observe its effects. In this project, an idea was developed to study mercury levels in fish already living in mercury-contaminated waters, and to observe the difference in liver SOD enzyme response at different perch fish sizes (length and age). After extraction of SOD enzyme from fish liver, the liver homogenates that contained SOD enzyme were administered with mercury chloride ($HgCl_2$) to observe the SOD response in-vitro. Another hypothesis was therefore that an addition of mercury chloride to the liver homogenates during activity analysis will inhibit the SOD enzyme activity.

Mercury (Hydragyrum, Hg) is the only metallic element that is liquid at room temperature. It has an atomic weight of 200.59 grams per mole, boiling point of -38.83 °C and melting point of 356.73 °C. Its silver in colour, hence it is sometimes known as quicksilver. It is a heavy metal with a density of 13.534 g/cm³. Mercury is a naturally occurring element found in air, water and soil (WHO, 2013). There are several anthropogenic sources associated with the release of mercury into the environment, with only few natural events that cause mercury pollution, such as volcanic eruption, crustal degassing, forest, lakes and oceans (Lindqvist & Rodhe, 1985; Mason et al., 1994) (MERC, 2007).

2.1 Mercury pollution in Lake Mjøsa

Lake Mjøsa is the biggest lake in Norway and there are vast agricultural and industrial activities in town situated alongside it. The lake is also used as a recreational site for many of the inhabitants living in Hedmark, Akershus and Oppland counties, in the form of fishing, swimming and boating. Lake Mjøsa is an important source of raw water to the municipalities, private companies and as well farmers around it (Løvik, Jarl Eivind et al., 2009). The easiest fish species to catch with a fishing rod by the banks of Lake Mjøsa is European Perch (*Perca fluviatilis*) (Personal experience). Lake Mjøsa experienced a period of eutrophication in the 1970s as a result of agricultural, industrial and municipal sewage run-off (Holtan et al., 1979). It was not only algae blooms as a result of eutrophication that were the only problem, but experiments on the water quality from Lake Mjøsa revealed a lot of contaminants ranging from easily controlled salts like phosphates to persistent organic and heavy metal pollutants such as polychlorinated biphenyls (PCBs) and mercury, respectively . The Norwegian government embarked on a huge task to prevent pollution in Lake Mjøsa. A project called "Aksjon Mjøsa" (1976 – 1981) was launched in order to restore the water quality in Lake Mjøsa to acceptable or near level. The government efforts has hugely paid off in drastically reducing the nutrient levels from Lake Mjøsa, where there was a significant reduction in nutrients and heavy metals from 1976 onwards when the project "Aksjon Mjøsa" was launched (Løvik, Jarl Eivind et al., 2013).

Heavy metals such as mercury and lead are still persistent in the lake sediments as these are difficult to entirely get rid of. The total amount of mercury in the sediments in Lake Mjøsa is estimated to be 1.8 tons, with half of the mercury in the sediments is coming from a Lillehammer and the rest from Gjøvik and Hamar region. Much of the mercury in the sediments where deposited into the lake at around 1945, with the peak deposit taking place in the middle of the 1960s, with the main source being from a carton and paper factory "Mesna kartongfabrikk" in Lillehammer area (Rognerud, 1985). Today mercury pollution into Lake mjøsa is through aerial transport with an annual mercury deposit of about 2.9 kg, of which the major sources being combustion of fossil fuel for power and heating, Metal production, waste incineration and chlor-alkali industry all around Europe (Schlabach et al., 2009). As it is seen in figure 1, atmospheric sources into Scandinavia are mainly from mainland Europe (central,

south and Eastern Europe), and atmospheric mercury concentration is highest in autumn (fall) months.

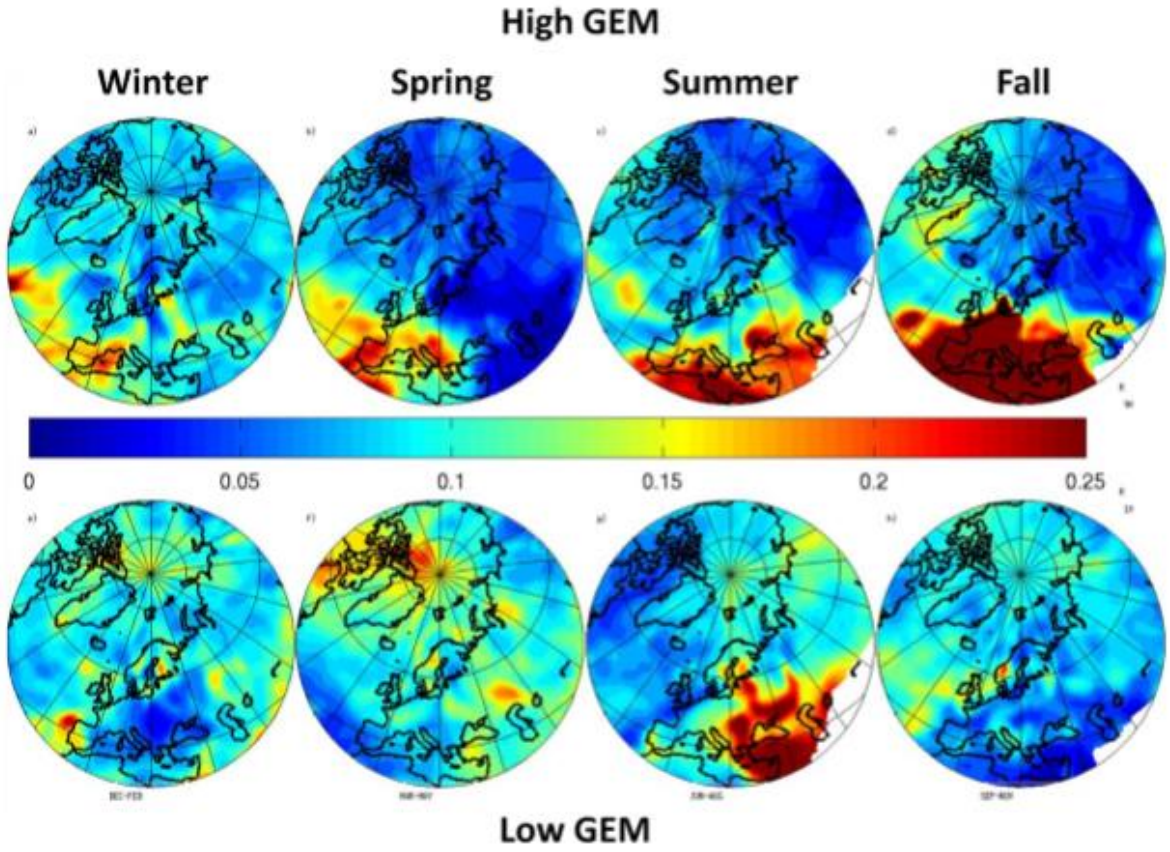

Figure 1: Source (upper row) and sink (botton row) region plots for gaseous elemental mercury (GEM) at Kise (location at which data was collected) during the year 2007 – 2009. The plots are splits in seasons; winter (left), spring (middle left), summer (middle right) and fall (right. The colour scale ranges to 0.25 for all panels. Values exceeding 0.20 indicates frequent transport from these areas. Source: (Schlabach et al., 2009)

2.2 Previous reports about Mercury pollution in fish from Lake Mjøsa and other nearby lakes.

Early attempts to measure the amount of mercury in perch fish from Lake Mjøsa revealed average mercury amount of 0.46 mg Hg/kg (0.46 ppm) wet weight, with average length of 26.8 cm (Fjeld et al., 1999). Table 1 below shows the results from the study in mercury levels in perch from Lake Mjøsa, conducted in 1998 by the Norwegian institute of water research (NIVA).

Table 1 Average mercury concentration (mg Hg/kg wet weight), length, weight and age in the analysed material of Perch fish from Lake Mjøsa, caught in 1998. Maximum and minimum values are also given. Source: (Fjeld et al., 1999).

	Mg Hg/kg (PPM)	Length (cm)	Weight (g)	Age (years)
Average	0.46	26.8	283	6.9
Standard deviation	± 0.46	± 5.6	± 220	± 2.6
Maximum	2.00	43.5	1054	17
Minimun	0.10	18.0	57	4

Some Norwegian lakes in Hedmark County towards the border with Sweden were reported to have pike and perch fish with mercury levels in their white muscles up to 4 mg Hg/kg (4 ppm) (Rognerud & Fjeld, 2002). This is eight times higher than the accepted consumption limit of 0.5 ppm set both by the Norwegian food safety authority (Mattilsynet) and the world health organization (WTO). “Mattilsynet” advise pregnant women not to consume Pike (*Esox Lucius*) and perch that are over 25 cm long, and to avoid eating trout (*Salmo trutta*) with mass over one kilogram. The rest of the population is advised not to eat these fish more than once in a month (Mattilsynet, 2011).

2.3 Unaccounted for mercury sources to the environment

Even though much of the primary mercury production activities have dropped remarkably, a lot of mercury stockpiles are still available. In World War 2, there have been a lot of weapons and ammunitions that were manufactured and used, contributing to mercury pollution in the water bodies. Example of world war remains emitting mercury into the Norwegian ecosystem is a submerged submarine wreckage (U-864) that was discovered in 2003 on the west coast of Norway , with a huge threat to the marine life due to the presence of over 65 tons mercury in it. Another submarine wreckage (U-486) suspected to contain fuel and unexploded torpedoes was discovered in 2012 about two kilometres from the site where U-864 was discovered (NRK, 2013). These discoveries where random, which leads to people to think that more unpleasant surprises are still yet to come with regard to anthropogenic mercury emissions into the ecosystem world-wide.

2.4 European Perch (*Perca fluviatilis*)

European perch fish is an entirely freshwater living fish, and is regarded as opportunistic feeders (Griffiths, 1976). It is a facultative ambush predator, hunts during the day, eating mostly invertebrates and young fish. When it is a juvenile perch feeds mainly on zooplanktons and changes its diet to macro-invertebrates when it grows to intermediate size. When fully grown perch fish feeds mainly on small fish, including their own kind (Svanbäck & Eklöv, 2002). About 40% of the annual ration is eaten during spring, 30% during summer, 15% during autumn, and up to 20% during the winter (Popova & Sytina, 1977). Common size of perch from Lake Mjøsa is between 10 and 40 cm ("Species in Lake Mjøsa," s.a.), but some perch individuals can grow to a size over 700 grams (Personal experience). Distinct intra-species morphologies and diet differences have been observed between perch caught in pelagic waters and those caught in littoral waters. It was found that perch caught in the littoral habitat, independently of size, had a deeper body, larger head and mouth and longer fins, with macro-invertebrates and fish being dominant in their diet than perch caught in the pelagic zone. The diet of the pelagic perch consisted mainly of zooplankton, and to some extent fish and small proportion of macro-invertebrates. The relationship between the length of perch and the size of the mouth and fins also found to be different between perch from the two

habitats, as it was observed that the increase in the length of the pelvic fin and the area of the mouth increased faster with size in the littoral perch (Svanbäck & Eklöv, 2002). The spawning season for perch in May and spawning is induced when water temperatures rose above 14º C (Gillet & Dubois, 1995). Perch fish tend to accumulate more methyl-mercury because of their predatory nature when they grow bigger (Mattilsynet, 2011).

Figure 2: An image of perch fish (perca fluviatilis) showing its typical identity of black vertical stripes on its sides, with red pelvic, anal and caudal fins. Source: (Fiskefoto.dk, s.a.)

2.5 Mercury types in the environment and associated human health risks.

Mercury exists in several forms in the environment, and these forms are elemental or metallic, inorganic, and organic mercury compounds, and the transformation process from one form to another is complex and involves many factors such as pH, temperature, physical and chemical influences (Biester et al., 2002; Lin, C.-J. & Pehkonen, 1999; Schroeder & Munthe, 1998; Schroeder et al., 1991). Mercury is regarded as a global problem because of its ability to be transported and deposited long distances from its point of origin (Boening, 2000; Cheng & Schroeder, 2000).

Of all forms of mercury, organic mercury (methyl-mercury) is the most common form of mercury in the tissues of aquatic organisms (Kainz et al., 2006) and its effect on antioxidant response and lipid peroxidation is more severe compared to inorganic mercury (mercury chloride) (Berntssen et al., 2003). Methyl-mercury is formed by microorganisms found in the water through methylation process (Jensen & Jernelov, 1969; Ribeiro et al., 1996; Trevors, 1986). Due to bioaccumulation, the predatory big fish that eat small fish are the ones with the highest amount of mercury in their flesh (Monteiro, L. R. et al., 1996; Shastri & Diwekar, 2008) . Both marine and freshwater fish are equally susceptible to mercury (Monteiro, L. R. et al., 1996; Power et al., 2002; Watras et al., 1998; Žižek et al., 2007). Figure 3 shows a general overview of how mercury is converted from one form to the other, and how methyl-mercury ends up in the food chain.

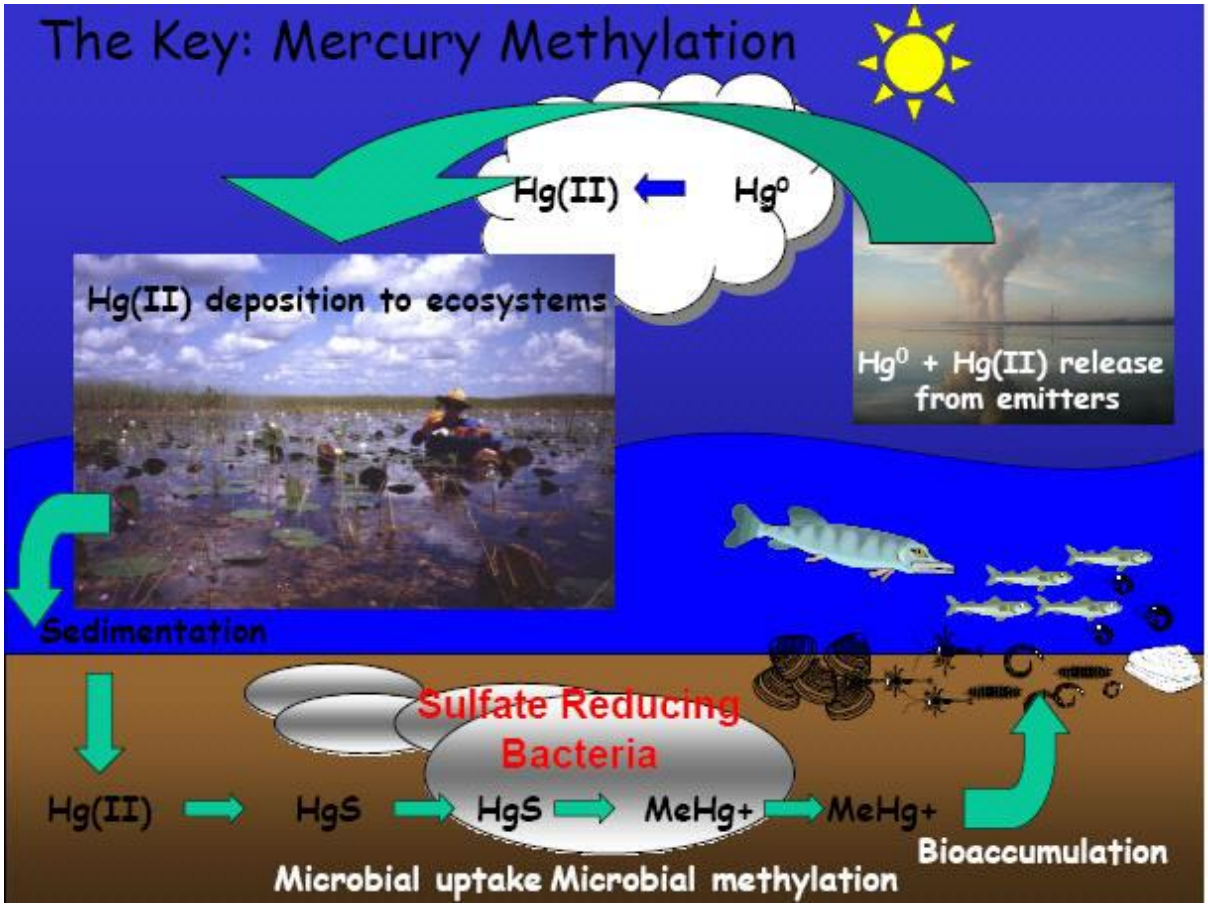

Figure 3: A detailed description of mercury cycle, including the methylation stage carried in the water sediments by Sulphate reducing bacteria. Source: ("Mercury methylation process," s.a.).

Apart from the ability of mercury to persist in the body of organisms on top of the food chain through bioaccumulation, serious pathological risks to human beings can occur. The best example of the dangers of mercury to human beings is well documented after the outbreak that happened in Minamata in Japan. The observations done in Minamata led to the coining of "Minamata disease". Cats could be seen spinning around and dying everywhere. Affected people had sensory disturbances and their hair was detected to contain high levels of mercury. Later it was discovered that the cause of the disease was the ingestion of fish and shellfish contaminated with methylmercury from the Minamata, and the source of the mercury was from the chemical plant that discharged its wastes directly into the Minamata bay (Harada, 1995).

2.6 Antioxidant enzymes affected by mercury

After the Minamata disease, more research was done on mercury to discover its negative effects. A variety of effects were reported when mercury was tested in fish (Berntssen et al., 2003; Bleau et al., 1996; GÜL et al., 2004; Jagoe et al., 1996; Verma et al., 1983) . The occurrence of the Minamata disease was a very special case and an eye-opener to the world. Because of exposure to high levels of mercury, it was easy to connect the cases in affected people to mercury as the cause and the chemical factory as the source. Studies to determine the lethal dosages and the biochemical effects of mercury in living organisms were carried out. Monteiro et al (2010) observed an LC_{50} of 0.71 mg L^{-1} when live fish species were exposed to inorganic mercury for 96 hours. The same study also observed an increase in antioxidant enzymes such as Superoxide dismutase (SOD), Glutathione reductase (GR), Catalase (CAT), and Glutathione S-transferase (GST) in liver, gills, white muscles and heart in the mercury treated samples compared to the control groups.

The mercury induced antioxidant enzymes' role in the organism's body is to prevent lipid peroxidation by fighting against reactive oxygen species (ROS). But LC_{50} values may vary depending on fish species, size, water hardness and pH. (Alam & Maughan, 1995; Bleau et al., 1996; GÜL et al., 2004). Methyl mercury is more toxic than inorganic mercury, as it was observed that only a small amount of exposure to methyl mercury triggered high protein, biochemical response and oxidative damage by ROS than inorganic mercury (Berntssen et al., 2003; Kim et al., 2013).

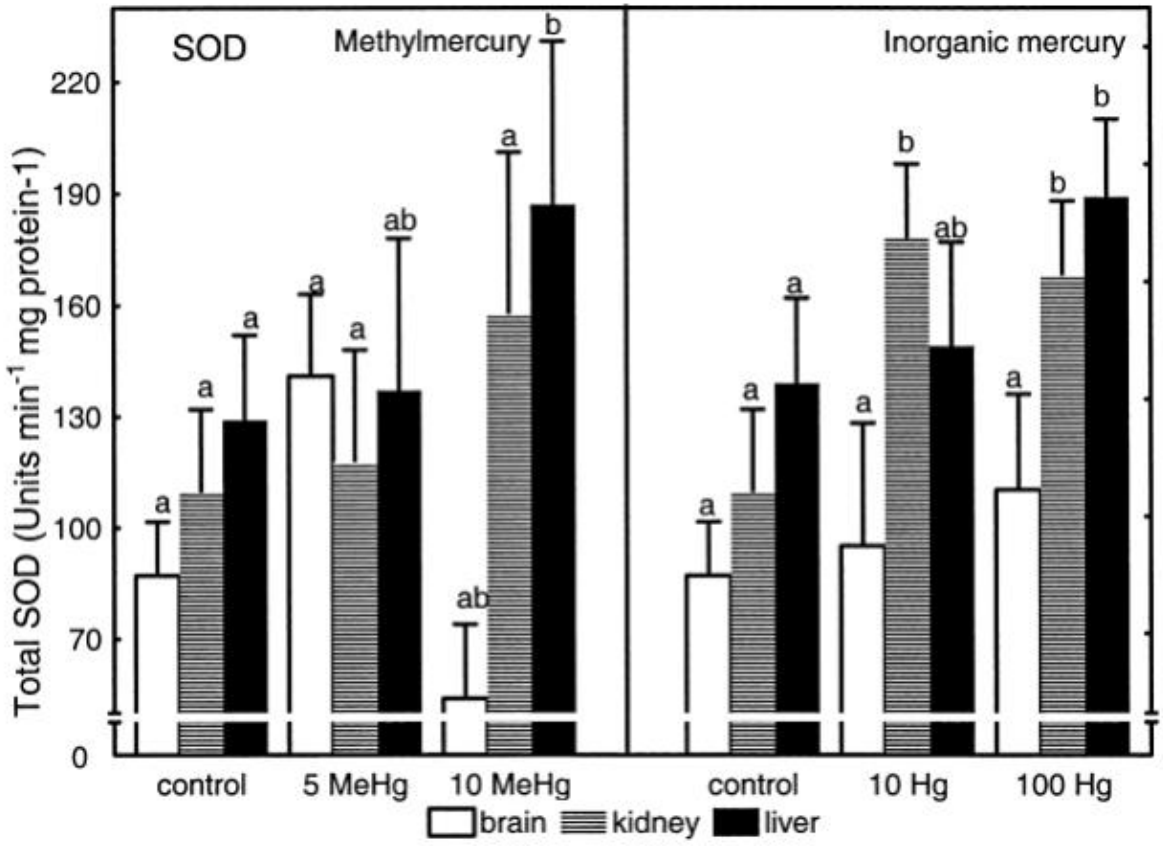

Figure 4: SOD response in the brain, kidney and liver in salmon fish exposed to methyl mercury and mercury chloride. The figure shows that SOD response is highest in the liver, and that methyl-mercury is more effective than mercury chloride. Source (Berntssen et al., 2003).

Mercury was found to interact with membrane proteins mostly in the mitochondria, where and electron transport chain is disrupted causing electrons to leak out and form superoxide that causes lipid peroxidation and DNA damage (Berntssen et al., 2003; Verlecar et al., 2008; Yee & Choi, 1996), as well as unregulated flow of potassium ions (K^+) which causes a loss of membrane potential (SONE et al., 1977). Glutathione (GSH), a three peptide molecule, is widely produced in living organisms and it is employed by antioxidant enzymes in the redox reactions to fight ROS, and the ratio between reduced and oxidized glutathione in the tissues of organisms is also used as an indicator of oxidative stress (Asensi et al., 1999; Jerca et al., 1993). Low concentrations of ROS are beneficial to the cells because they are involved in cell signalling (del Río et al., 2006; Droge, 2002; Sharma et al., 2012).

It is not only external substances such as heavy metal pollution that trigger the production of ROS in living organisms. Xanthine oxidase (XO) is an enzyme that is involved in purine catabolism and the reduction of nitric oxide under hypoxic conditions, and in the process producing super oxide and hydrogen peroxide endogenously (Harrison, 2002; Millar et al., 1998). Metallothionein is a cysteine-rich low molecular weight protein employed in the anti-oxidative responses in cells of living organisms by chelating heavy metals and it is synthesized predominantly in the liver and kidneys (Coyle et al., 2002). Studies have shown that organisms exposed to metal pollutants such as copper, zinc, and cadmium have elevated levels of metallothionein than in control organisms (Coyle et al., 2002; Klaassen et al., 1999). Some studies did not find correlation between mercury and metallothionein concentration, thereby questioning the use of metallothionein as a biomarker for mercury exposure (Mieiro et al., 2011).

2.7 Description of enzymes heavily employed as indicators of stress caused by Mercury, and their analytical protocols

SOD (Superoxide dismutase) enzyme (EC 1. 15. 1. 1) is a homodimer with a molecular weight of approximately 30 kDa (Lin, C.-W. et al., 1997). The activity of SOD in vivo is the conversion of superoxide ions into hydrogen peroxide and oxygen (O_2) (McCord & Fridovich, 1969). The most common and old method for the activity analysis of SOD is based on the reduction of cytochrome c by superoxide ions, and these superoxide ions are produced by another enzyme, xanthine oxidase. The activity is monitored at 550 nm (Beauchamp & Fridovich, 1971). Another newer SOD protocol uses PMS and NADH to generate superoxide, hence it is referred as a non-enzyme superoxide generator, and the activity is monitored at 560 nm (Ewing & Janero, 1995). Various forms of SOD enzymes exist, depending on the metal cofactor that is attached to them. Copper and Zinc SOD (Cu-Zn-SOD) are SOD forms that are predominant in eukaryotic cells, while Manganese SOD (Mn-SOD), Iron SOD (Fe-SOD) and Nickel SOD (Ni-SOD) are predominant in prokaryotic cells (Fridovich, 1989).

Glutathione peroxidase (GPx) (EC 1. 11. 1. 9) is made up of four subunits, with a combined weight of ca. 95 kDa (Broderick et al., 1987). There eight various forms of GPx enzymes (GPx1 – GPx8) currently identified (Brigelius-Flohé & Maiorino, 2013), and the main function of GPx works by converting lipid and hydrogen peroxides into water with the help of glutathione as an electron donor (thereby oxidizing GSH to GS-SG). GPx activity is an indirect reaction coupled with GR (Glutathione reductase), and it is monitored at 340 nm following the oxidation of NADPH (Nakamura et al., 1974). Instead of using common hydrogen peroxide as a substrate for GPx, cumene hydrogen peroxide or tert-butyl hydroperoxide is prefereby used in order to measure the total activity of GPx and avoid other enzymes' side reaction (Liu et al., 2006). Sodium azide is also recommended to be added if the GPx activity is measured from crude samples, in order to block the side reaction of other enzymes such as catalase

Catalase (CAT) (EC 1.11.1.6) is also a molecule made up of four subunits, with a total molecular weight of about 240 kDa (Esaka & Asahi, 1982). The activity of CAT is to convert hydrogen peroxide into water and molecular oxygen and the activity is monitored at 240 nm (Shangari & O'Brien, 2001). Glutathione reductase (GR) (EC 1.8.1.7) enzyme is a homodimer with a total weight of ca. 114 kDa (McCallum & Barrett, 1995), and is involved in reducing glutathione that is oxidized (GS-SG) in the body, and the activity analysis uses glutathione and monitor the activity at 340 nm. Glutathione S-transferase (GST) (EC 2.5.1.18) is also a homodimer with a combined molecular weight of ca. 50 kDa (Shichi & O'Meara, 1986). SOD, GPx, GR, GST and CAT work together to fight against ROS, as shown in figure 5.

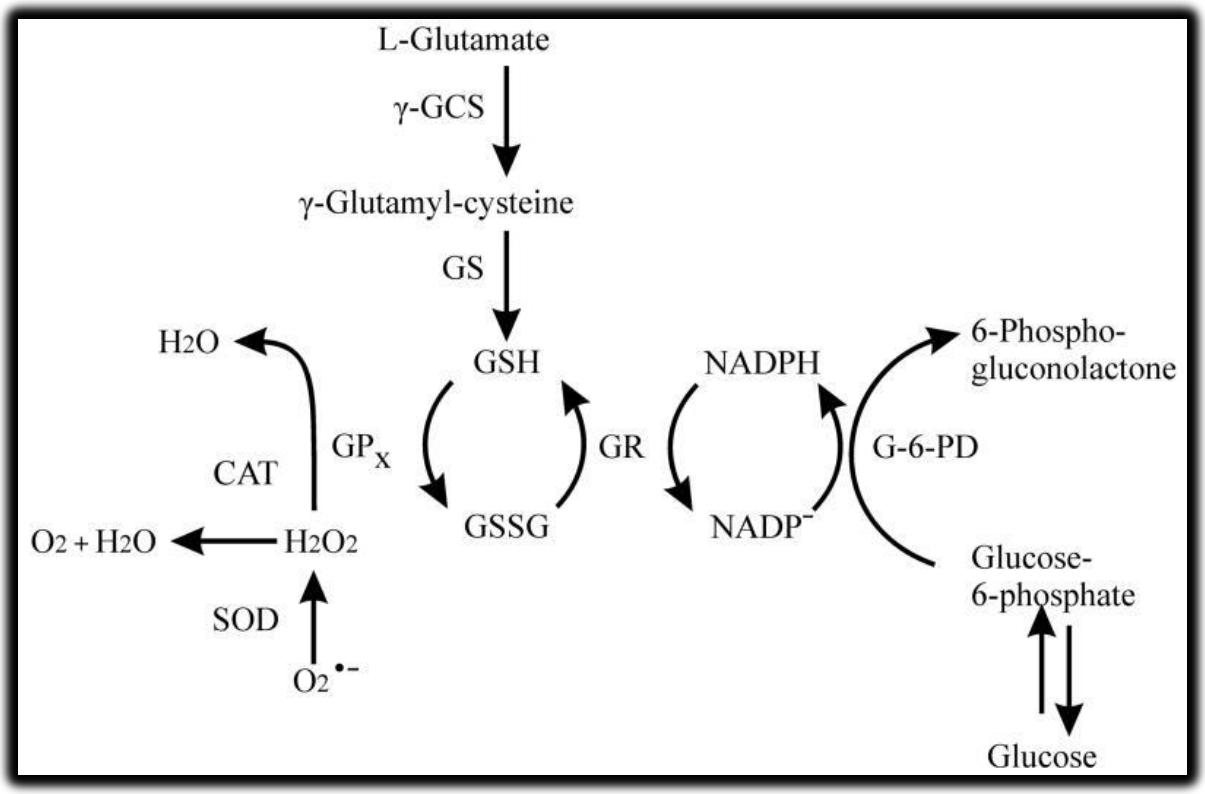

Figure 5: A schematic diagram showing the activity of antioxidant enzymes. Source: (Weydert & Cullen, 2010)

2.8 Background (project origin)

. A lot of information in the study of mercury pollution to living organisms is widely published. The majority of these previous studies report on acute mercury toxicity tests, where mercury chloride, methyl-mercury or any other derivative of mercury is administered to organisms under study to observe and record the lethal dosage and document the change in physiology, pathology and antioxidant response against mercury. It is not only reports about acute mercury toxicity being that are observed to be abundantly published, but also it is found that inorganic mercury is predominantly used in these early studies. Also due to the emphasis of research in heavy metal pollution in developed countries, the author of this paper got motivated to acquire knowledge about mercury pollution in order to get hands-on skills to work with developing countries. In Lake Mjøsa early studies confirmed the presence of mercury, both in the lake's sediments and in the tissues of fish species caught from the mentioned lake.

Therefore this master project was aimed at determining the mercury levels in Perch fish (*P. fluviatilis*) caught from Lake Mjøsa, as well as investigating correlation of mercury levels against selected antioxidant enzymes from the sampled perch livers. With reference to results in early studies about the positive response in the activity of antioxidant enzymes, namely Superoxide dismutase (SOD) and Glutathione peroxidase (GPx), the hypothesis in this study is that high mercury levels in the liver of European perch (*Perca fluviatilis*) affects the activity of these two mentioned enzymes (SOD and GPx), and that correlation between mercury levels, age of fish and protein concentration is expected to be positive. With positive correlation, it is meant that the total protein concentration and enzyme activities of SOD and GPx are expected to be high in bigger and older fish with high mercury levels in their tissue. With high confidence, all the fish samples obtained from Lake Mjøsa were expected to test positive against mercury because previous studies carried out by the Norwegian institute of water research (NIVA) reported the presence of mercury in the sediments and in the fish.

In developing countries, environmental pollution by heavy metals is an on-going concern because strict measures to curb the release of mercury into the environment are ignored. Countries in Africa such as Zambia and Namibia generate a lot of income from mining activities, but it is questionable how the mining factories dispose their wastes. There are reports of heavy metal discharge from mining wastes in copper mines of Zambia and Zimbabwe that led to the contamination of the water systems near the mining sites (Berg et al, 1995; Nakayama et al, 2010). Strangely enough is that the majority of the mining industry in Africa is controlled by companies from the industrialized countries (Europe and North America, including China), who should be aware and abide to rules and regulations for environmental safety and protection. Yet there is little these companies do to take care of the environment. This causes a lot of suffering and health hazards to the local residents who rely mainly on fishing and farming as their source of livelihood.

The pursuit to high living standard led to industrial revolution, but the industrialization of the western world led to mass environmental pollution. Why can't we regard this as a lesson to learn from so that another path could be followed in the modernization of the third-world, instead of following the exact steps that lead to destruction?

The specific objectives are:

1. Determine the levels of mercury (in ppm) in the white muscles and livers of Perch fish.
2. Conduct enzyme activity analysis to determine the correlation between antioxidant response of SOD and GPx enzymes in the liver and mercury levels in the white muscle of perch.
    a. For control purposes during enzyme activity analysis, commercial SOD enzyme was used.
3. Perform a SOD activity analysis method with the use of a non-enzyme superoxide generator.
4. Determine total protein concentration in the perch liver homogenates.
5. Perform statistical analysis using linear model to determine the correlation between SOD and GPx activities against fish length, mercury content (ppm) and protein content (mg/g).

3 Materials and Methods

3.1 Frame of the project

The project was conducted using 36 freshwater fish samples from European perch species (*Perca fluviatilis*). Fish samples were caught on two occasions, the first batch consisted on 16 perch caught in spring 2012 (batch 1), while the second batch consisted of 20 perch fish caught in spring 2013 (batch 2). Mercury level determination was done from both liver and white muscle tissue, while total protein and enzyme activity were done only from liver homogenates. Below is a flow diagram of study showing the frame of the project.

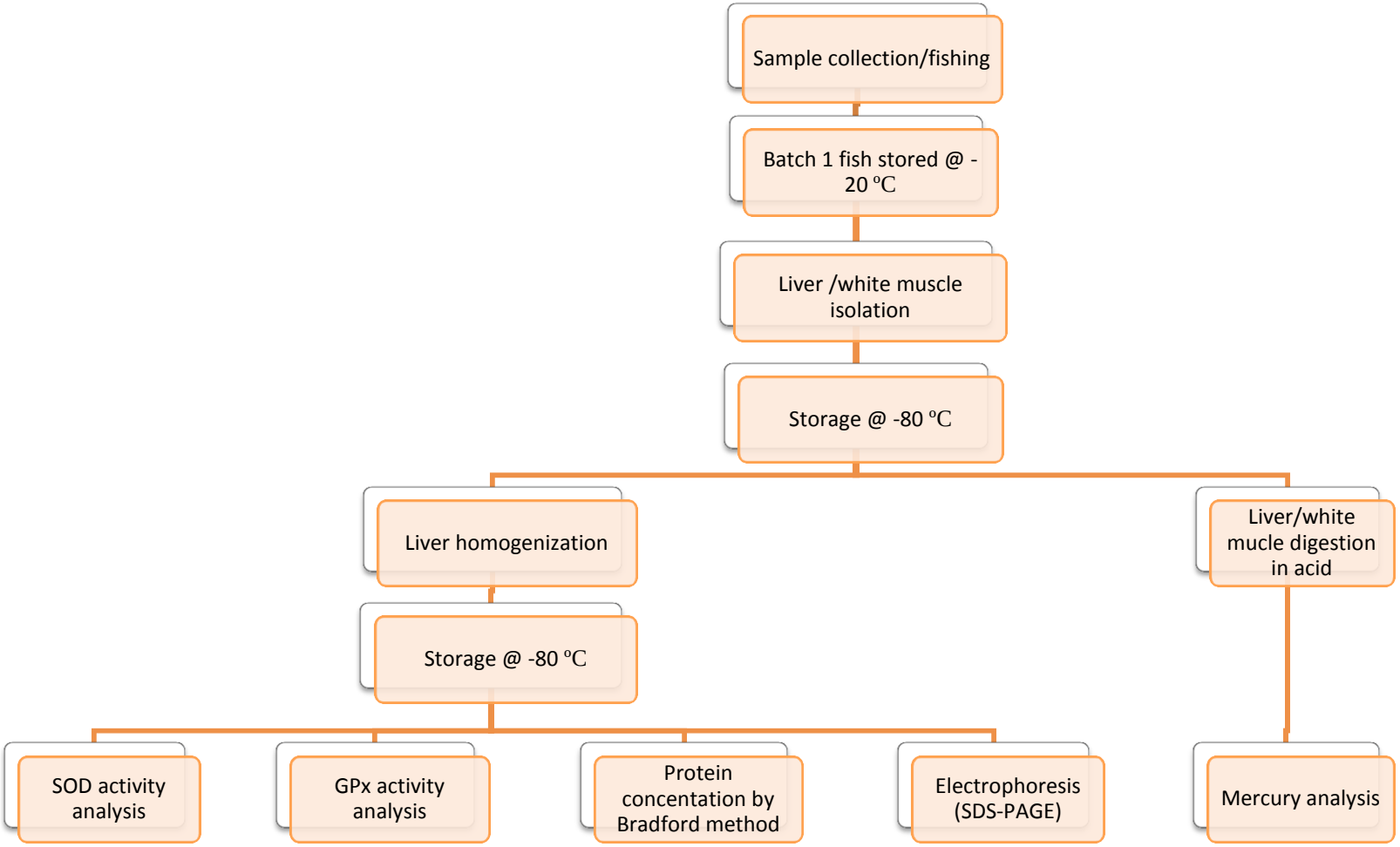

Figure 6: Flow diagram showing activities carried out during the master's thesis execution.

3.2 Chemicals and equipment needed

Nitro blue tetrazolium (NBT) and 50 ml teflon tubes (PTFE) with pressure-tight screw caps obtained from VWR-International LTD (Radnor, PA, USA). Nicotinamide adenine dihydrogen (NADH) from AMRESCO LLC (Solon, OH, USA). Phenazine methosulphate (PMS) and Nicotinamide adenine dinucleotide phosphate (NADPH) were from AppliChem GmbH (Darmstadt, GERMANY). Mercury stock solution (1000 mg Hg/L) was from PerkinElmer (Waltham, Massachusetts, USA). Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione reductase (GR), and Glutathione (GSH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). FLUOster optima spectrophotometer obtained from BMG LABTECH GmbH (Ortenberg, Germany). Flat botton 96-wells assay plates were from Corning (Corning, NY, USA). Hydrogen peroxide (H_2O_2) was obtained from Merck KGaA (Darmstadt, Germany).

3.3 Fish sample collection

European perch (*Perca fluviatilis*) used in the project were caught in benthic gill nets (1.5 x 25 m; 24, 29, 35, 39, 45) in Lake Mjøsa, southeast Norway in July and September 2012 (batch 1), and in May 2013 (batch 2. Place of sample collection (fishing) for both batches were at marinesenteret Hamar (N 60° 47.448', E 11° 4.543'). Figure 1 below show the exact location of the place where the fish samples were collected. The gill nets were set in the evening and picked in the morning, at the water depth between 2 and 6 meters. The perch that were caught were taken to the lab right after being removed from the nets. Batch 1 was stored at -20^{o} C immediately after arriving at the laboratory. The livers were removed and homogenized four months later (in January 2013). In batch 1, livers from 16 perch fish were removed and included in the enzyme activity analysis project, together with the 20 liver samples from batch 2. Even though the livers from batch 1 were prepared on a different date as those ones from batch 2, and that they were prepared after freezing the fish, the way in

which they were handled during the homogenization process was similar to the 20 liver samples from batch 2 fish. In batch 2, twenty (20) European perch fish were caught in the spring season of 2013 (May). All the fish that were caught here were immediately put on ice after removal from the nets and transported to the laboratory. In the laboratory on the same day, the livers were removed and stored at -80^{o} C to be used later in enzyme activity analysis.

In both batches (1 and 2), the mass, sex and length of each fish was recorded. For the determination of age of the fish, the otoliths and gill-covers (operculum) were removed and used for this purpose. Table A1 (Appendix) gives a summary of age, sex and length of the sampled perch fish. A piece of white muscle (approximately 1 gram) was removed from the left shoulder of each fish and stored at – 80 ºC and later used for the determination of mercury levels.

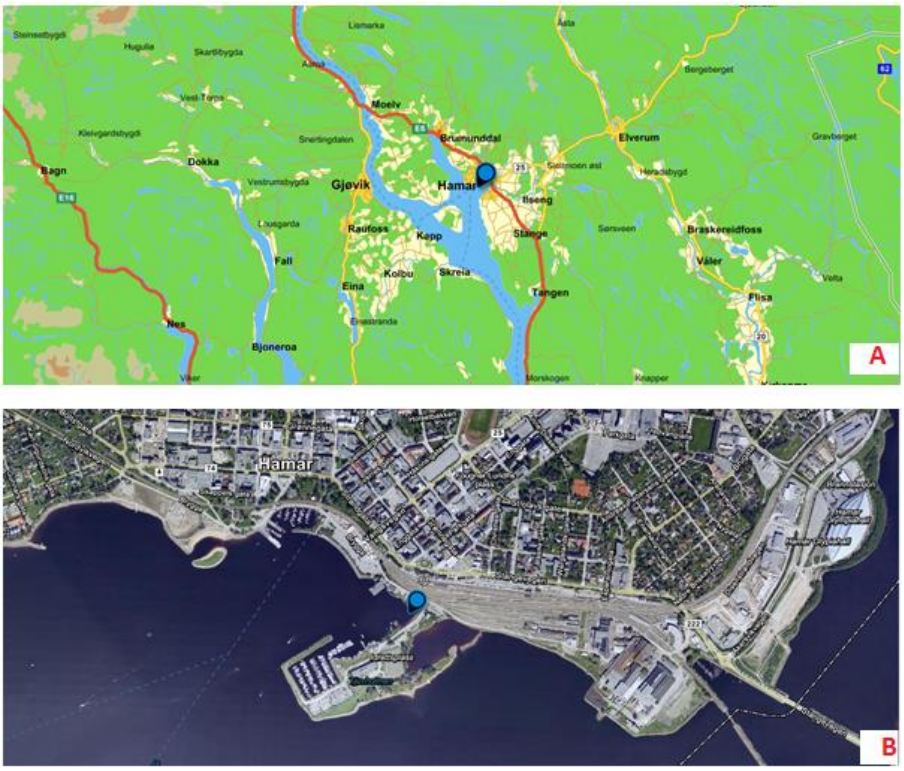

Figure 7: Marinesenteret Hamar was the site from where the perch fish used in the project were collected. Portion A on the figure shows a wider satellite view of the city of Hamar, while portion B shows the exact fishing location in Hamar. The exact coordinates for the fishing site are N 60° 47.448', E 11° 4.543'.

3.4 Mercury level determination

The mercury level measurements in both muscle and liver from each perch fish were carried out using the procedure for analysing fish for total mercury (Nriagu, 1996), using the PSA 10.025 Millennium Merlin spectrophotometer (Orpington, Kent, UK). About 1.0 g of muscle tissue from each perch fish were weighed and transferred into 50 ml Teflon tubes (PTFE) (VWR-International LTD, Radnor, PA, USA) with pressure tight screw-caps, where digestion was carried out in 5 ml concentrated nitric acid (HNO_3) and 1 ml H_2O_2 for 2 hours at 95-98^o C. An amount of 1 ml 10% Sulfamic acid (H_3NSO_3) was added to remove the yellow colour (nitrous gases that could affect the analysis). There was no need to centrifuge or filter, since the solution was clear after digestion. Then 0.50 or 1.0 ml (depending on Hg concentration) of the solution from each Teflon tube was diluted to 50 ml in distilled water and analysed in the Merlin spectrophotometer, to nearest 0.01 ppb. A standard curve for references was created, and this was made using commercial mercury (mercury chloride, $HgCl_2$) from the stock solution concentration of 1000 mg Hg/L, or 1000 µg/mL, which corresponds to 1000 parts per million, or 1000 ppm. Unknown samples were analysed in parallels. The standard curve was used as reference, and was made by diluting the stock solution of mercury chloride (1000 µg Hg/mL) to 5 concentrations, namely 0 parts per billion (ppb), 0.20 ppb, 050 ppb, 1.0 ppb, 2.0 ppb and 3.0 ppb. The dilution of the samples (0.50 or 1.00 ml to 50 ml) was chosen to fit the mercury measurements between 0.50 and 3.00 ppb.

3.5 Preparation of extracts for enzyme assays

The livers obtained from the perch fish were subjected to homogenisation. The whole homogenisation process was carried out on ice. Tris-HCl buffer containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.1% triton-100 was used, with pH 7.8. The homogenisation ratio was 1:9, which means 9 ml buffer for every 1 gram of perch liver. Homogenisation was done using an IKA T18 basic ultra-turax homogeniser (Staufen, Germany). The speed on the ultra-turax was set at one, and the homogenisation time for each liver was three (3) minutes. After homogenisation with ultra-turax, sonication using an ultra-

sonic Branson 250 sonicator was carried out. The timer on the ultra-sonic sonicator was set at hold, duty cycle set at 20 percent (20%), output control set at 2. Each liver homogenate received three cycles of 30 pulses (30 pulses X 3 cycles). This process was carried out on ice. After sonication, the homogenates were subjected to centrifugation for 30 minutes at 15 000 rotations per minutes (rpm). Centrifugation was repeated twice and the centrifuge temperature was set between 1^{o} C and 4^{o} C. The pellets were discarded and the supernatants were saved and distributed in cryopreserving tubes, 1 ml in each tube. The tubes were then stored at -80^{o} C for later analyses.

3.6 Protein determination

Protein measurement in the liver homogenates was done according to the Bradford method (Bradford, 1976). Bovine serum albumin (BSA) (Sigma-Aldrich, USA), was used to create the standard curve. BSA powder was diluted to a final concentration of 10 mg/ml in distilled water, with 1% glycerol added. Coomassie brilliant blue solution was prepared separately. The Coomassie brilliant solution was made by diluting 100 mg Brilliant Blue G powder in 50 ml denatured ethanol (96%) and 100 ml phosphoric acid (85%). Distilled was added to the brilliant blue solution to a total volume of 1000 ml. The solution was ready for use after it was subjected to filtration. In order to make the standard curve, 5 dilutions of BSA solution were made, and these were 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1.0 mg/ml. An amount of 50 µl from each of the five BSA dilutions was mixed into 2.5 ml Coomassie brilliant blue solution, and subjected to absorbance measurement in a spectrophotometer at 595 nm. The spectrophotometer measurements from the five BSA dilutions were done in parallels. A solution containing only Coomassie blue solution was also made and it was used as a Blanc.

3.7 SOD activity analysis

Commercial SOD was used for references during the analysis of enzyme activity. FLUOster optima spectrophotometer (BMG LABTECH GmbH, Germany) was used in the measurement of SOD activity. The wavelength used to measure SOD activity was 560 nm and at 25ºC. Enzyme activity measurement was carried out in 96-well flat bottom assay plates (Corning, NY, USA).

The SOD protocol followed had a non-enzyme superoxide generator (Ewing & Janero, 1995). During the activity analysis for SOD enzyme, NBT, NADH and PMS were the main chemicals needed. Also it was required to test the effect of mercury on the activity of SOD. Therefore mercury chloride was used.

50mM Potassium buffer, with 0.1mM EDTA, at pH 7.4 was used. This was called buffer A. Buffer B was made by adding 98 µM NADH and 62 µM NBT to buffer A. Mercury (11 ppm) and 33µM PMS solutions were prepared using buffer A.

According to the report by the producer of the SOD protocol used in this project (Erwing & Janero, 1995), the mixture of NADH and PMS generate $O_2^{\cdot -}$ under nonacidic conditions. The produced super oxide ions can then react with NBT to form a stable formazan product that is blue in colour and the color intensity is detected in the spectrophotometer at 560 nm. The SOD enzyme in the reaction will consume super oxide ions, making them unavailable to react with NBT, therefore preventing the formation of the blue color that is detectable at 560 nm. The more the SOD enzymes in the reaction, the less the blue color formation. The equations below show the type of reactions taking place in the SOD activity analysis protocol.

1. NADH + PMS → $O_2^{\cdot -}$
2. $O_2^{\cdot -}$ + NBT → Phenazan product (blue color)

The blue color is detectable in the spectrophotometer at 560 nm. The presence of SOD in the reaction mixture prevents equation 2.

3.8 Procedure for SOD activity analysis

An amount of 200µL buffer B was added into the assay plate wells using an eight channelled pipette. Then 25 µL of enzyme SOD/sample were added into the assay plate wells. An amount of 25 µL mercury solution was added into some of the wells designated for mercury effect analysis. The mercury solution holds 11 ppm, and this will have a final concentration of 1ppm when mixed to the final volume of 275 µL into the assay plate well. In order to have a total volume of 275 µL in each well, 25 µL of buffer A added to micro-plate wells without mercury effect analysis. Also those wells that were used to measure the blank were filled with 275 µL buffer A. The micro-plate was incubated for 60 minutes at room temperature. The reaction was started when an amount of 25 µL freshly prepared 33 µM PMS was added into the assay plate wells. Control wells were free from PMS, and Buffer A was applied in them to bring the total volume to 275 µL in each well. The absorbance was monitored at 560 nm for about 5 minutes. Three amount of commercial SOD were used: 1000 ng, 10 ng and 0.1 ng, and these were used as references.

3.9 GPx activity analysis

GPx protocol followed was according to Flohè L and Günzler W.A (1982). The only modification done to this procedure was to use 96-well assay plate in the spectrophotometer, than to use cuvettes. Main chemicals needed for this assay were GSH, NADPH and H_2O_2. During the activity analysis for GPx, buffer A was used. This buffer contained 100 mM Potassium phosphate (K_2PO_4) and 1 mM EDTA. The pH of the buffer was set at 7.0. Buffer A was made by adding 13.61 grams of K_2PO_4 and 0.372 grams of EDTA into 1000 mL dH_2O. Buffer B was also prepared and it contained 10 mM GSH, 1.5 mM NADPH. Buffer B was made by adding 153.66 mg GSH, 62.5 mg and NADPH into buffer A. No pH adjustments were made this time, as it was assumed that the pH of 7.0 was not changed. In order to measure the effect of mercury on the enzyme, 11 ppm of mercury was used. This was prepared by taking 550 µl Hg from the stock solution (1000mg/L) to the final volume of 50 ml in buffer A. The GPx activity analysis is a coupled reaction in which the GPx-oxidised GSH (GS-SG) has to be reduced by GR enzymes with the help of NADPH as a source of

electron. Therefore, 2.4U/mL GR solution was required to be made. This 2.4 U/mL was made by taking out 960 µL GR stock solution (10U/mL) to the final volume of 1ml in buffer A. H_2O_2 was one of the chemicals needed in the activity analysis of GPx, as this is the main chemical were the GPx enzyme works to convert it into H_2O (water). A solution of 12 mM H_2O_2 was prepared for this purpose.

3.10 Procedure for GPx analysis

An eight channelled pipette was used to add 125 µL of buffer B added into the wells. An amount of 25 µL GR added into the wells, and then followed by the addition of 25 µL GPx and 25 µL Mercury. The mixture was incubated at room temperature for one hour to allow mercury to bind to the enzyme. The overall reaction was started by the addition of 25 µL H_2O_2 into the wells. The decrease in absorption was monitored at 340 nm for the first five minutes of reaction time. The non-enzymatic and non-hydrogen peroxide reactions were assessed by replacing the enzyme and the H_2O_2 by buffer A. This was done on the same assay plate, but in separate wells. The total volume of the solution mixture in each of the wells was 225 µL. The wells designated for blanks were filled with 225 µL buffer A.

3.11 Statistical analysis

Data were handled and charts were made in Microsoft Excel worksheet. The analysis of variance and linear models were run in the R-program (Crawley, 2007; R-program, 2013) in an attempt to explain variation of mercury content in white muscle and liver of perch from Lake Mjøsa, and to explain variation in enzyme activity (expressed as SOD slope). For mercury content, fish length and fish age were used as predictors. For enzyme activity, fish length, fish age, mercury content and protein concentration were used as predictors.

4 Results

This section will present the results observed after measuring mercury concentration in both white muscle and liver homogenates, protein concentration from liver homogenates, SOD and GPx activities from liver homogenates, as well as the correlation of these above mentioned observations against age and length of the analysed fish samples.

4.1 Mercury content in muscle and liver of perch

Like early mercury pollution studies conducted by the Norwegian institute of water research (NIVA) in Lake Mjøsa, this study confirmed the presence of mercury in all the 36 perch fish samples that were analysed. The detected level in perch white muscles ranged between 0.15 and 1.69 ppm (mg Hg/kg), while the detected mercury levels in the liver was between 0.12 and 1.81 ppm. With all 36 fish samples taken into account, the average amount of mercury in white muscle was found to be 0.69 ppm, and the average length of the sampled fish was 344 mm. It is important to mention that the fish samples used in the study were caught in two different years (2012 and 2013), but at the same place. The fish samples in batch 1 (fish caught in 2012) had an average white muscle mercury content of 0.34 ppm and an average length of 304 mm, while the fish samples in batch 2 (fish caught in 2013) had an average white muscle mercury content of 0.97 ppm and an average length of 376 mm. Figure 8 graphically shows the differences in range, average and standard errors of length, muscle and liver mercury observed during the measurement of mercury content in both batch 1 and batch 2 perch fish samples. The results clearly show that the fish samples in batch 2 were bigger and had higher mercury content than the fish samples in batch 1. The analysis of variance only revealed the length to be significant different between batch 1 and batch 2. Muscle and liver mercury concentration did not give a significant difference when ANOVA was carried out.

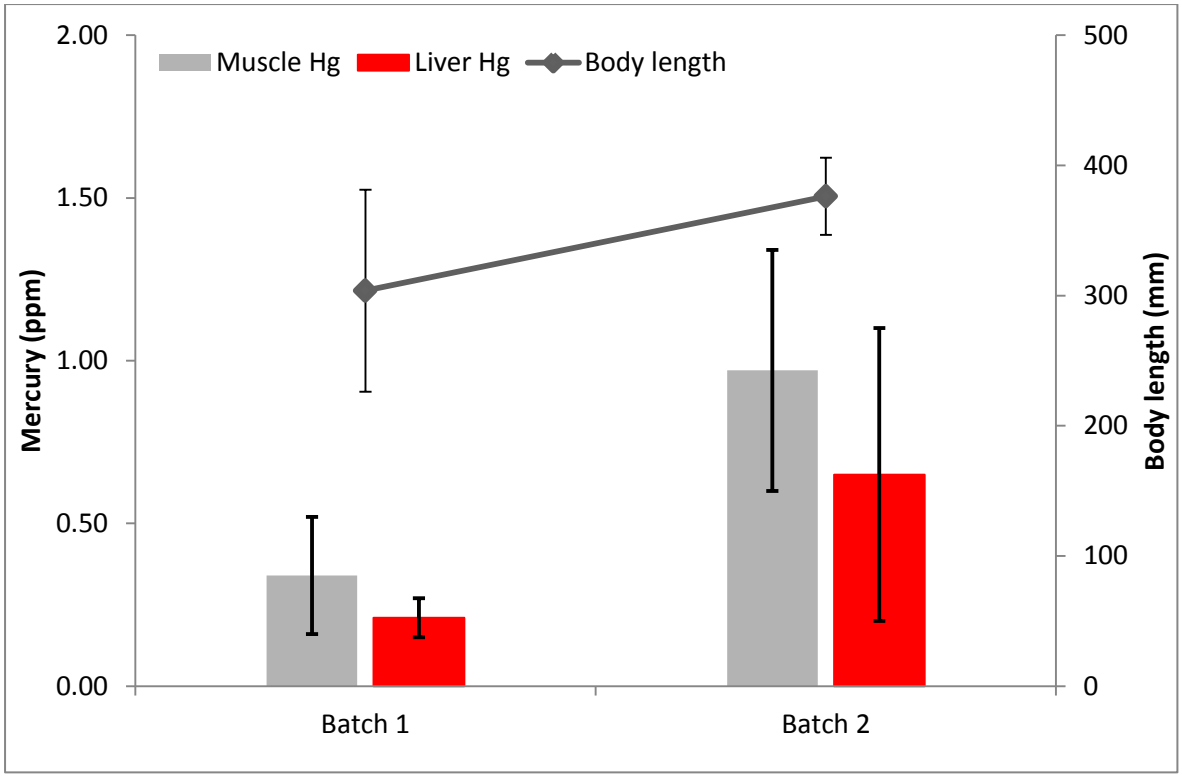

Figure 8: The differences in range, average and standard errors of length, muscle and liver mercury observed between batch 1 and batch 2 perch fish samples, observed during the measurement of mercury content.

4.2 Mercury correlation against age, length, protein concentration and enzyme activity

The correlation of Mercury concentration against the fish length, fish age, superoxide dismutase slope (SOD slope), Glutathione peroxide slope (GPx slope) and protein concentration in the liver homogenates in perch fish was determined using linear regression analysis. Mercury concentration in white muscles and liver was positively correlated with age and length. Mercury concentrations were higher in batch 2 than in batch 1 for all measurements (Fig 8). Age and year as categorical as predictors gave the highest coefficient of explanation for both muscle and liver mercury (R^2 = 0.662 and 0.384, respectively). Age explained 37.9% ($F_{1, 33}$ =36.97, $P < 0.0001$), and difference between years explained 28.2% of variation ($F_{1, 33}$ =27.51, $P < 0.0001$) in muscle mercury. Similarly for liver mercury concentration was 22.0% ($F_{1,32}$ =11.45, $P < 0.01$) of variation explained by age and 16.4% ($F_{1,32}$ =8.53, $P < 0.01$) by difference between years. When linear regression analysis was

carried out between muscle and liver mercury, a coefficient of explanation of 81.5% of variation was explained by mercury concentration ($F_{1,33} = 146.3$), $P < 0.0001$.

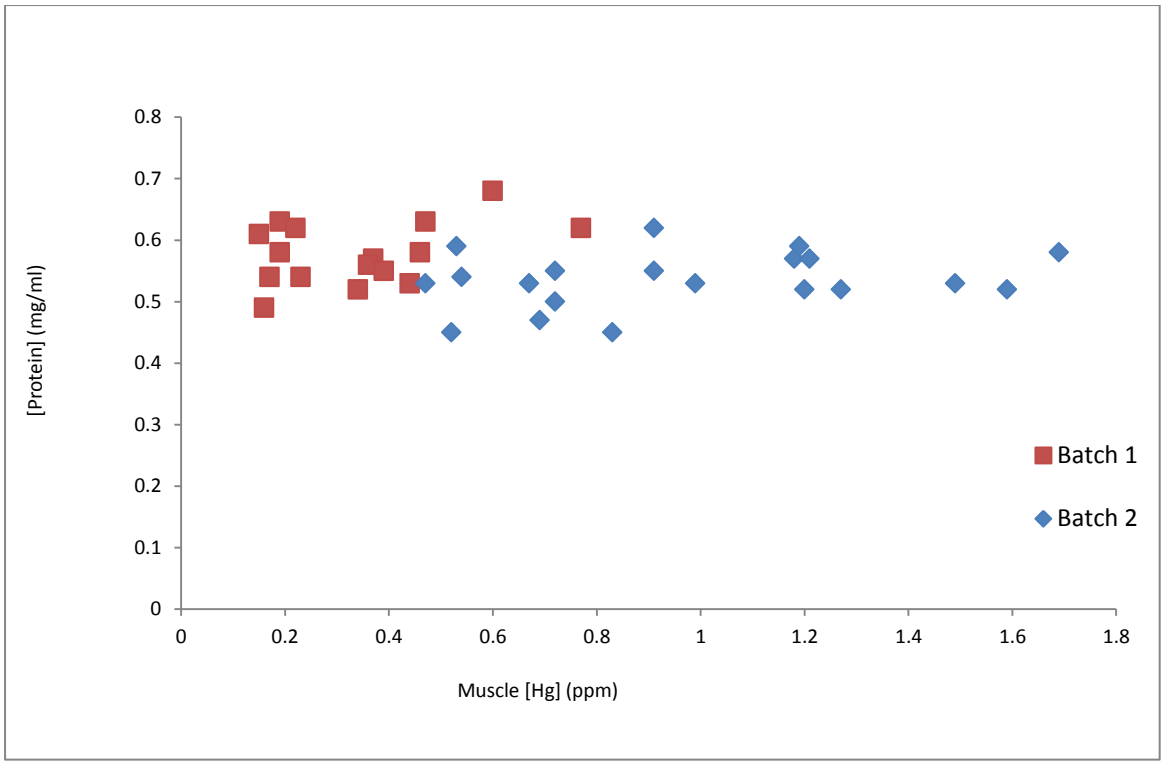

Figure 9: Scatter diagram of white muscle mercury content (ppm) plotted against protein concentration (mg/g). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

Table 2 Linear models of mercury concentration (ppm, ±S.E) in white muscle $[Hg]_{muscle}$ and liver $[Hg]_{liver}$ of perch caught in Lake Mjøsa in 2012 and 2013 with fish length, age and year as explanatory variables. Also $[Hg]_{muscle}$ was used as an explanatory variable to determine $[Hg]_{liver}$ response.

Models	R^2	d.f.	P
$[Hg]_{muscle}$ = 0.622 (±0.101)factor[2013] + 0.344 (±0.075)	0.527	34	< 0.0001
$[Hg]_{muscle}$ = 0.002 (±0.001)Length+ 0.472 (±0.472) factor[2013] – 0.282 (±0.271)	0.597	33	< 0.0001
$[Hg]_{muscle}$ = 0.064 (±0.018)Age + 0.472 (±0.492) factor[2013]– 0.504 (±0.243)	0.662	33	< 0.0001
$[Hg]_{liver}$ = 0.444 (±0.118)factor[2013] + 0.210 (±0.089)	0.300	33	< 0.001
$[Hg]_{liver}$ = 0.001 (±0.001)Length+ 0.387 (±0.140) factor[2013] - 0.052 (±0.349)	0.313	32	< 0.01
$[Hg]_{liver}$ = 0.047 (±0.023)Age + 0.352(±0.121) factor[2013] - 0.419 (±0.313)	0.384	32	< 0.001
$[Hg]_{liver}$ = 0.993 (±0.097)$[Hg]_{muscle}$ - 0.144 (±0.056) factor[2013] - 0.162 (±0.083)	0.835	32	< 0.0001

4.3 Protein concentration in liver homogenate

Figure 10 below shows a graphical comparison on average length, average muscle mercury and average protein concentration between batch 1 and batch 2. Total protein concentration in the liver homogenates ranged from 0.43 mg/ml to 0.65 mg/ml protein per gram of liver. Linear regression analysis revealed that protein concentration correlated positively in logarithmically (ln) transformed mercury concentration in both white muscle and liver in the pooled samples ($p<0.05$) (Table 3). This significant correlation shows that more proteins are produced in fish that contains more mercury. But the opposite is observed on the graph, as it is seen in figure 10 that the average protein concentration in Batch 1 is slightly higher than the average protein concentration of batch 2.

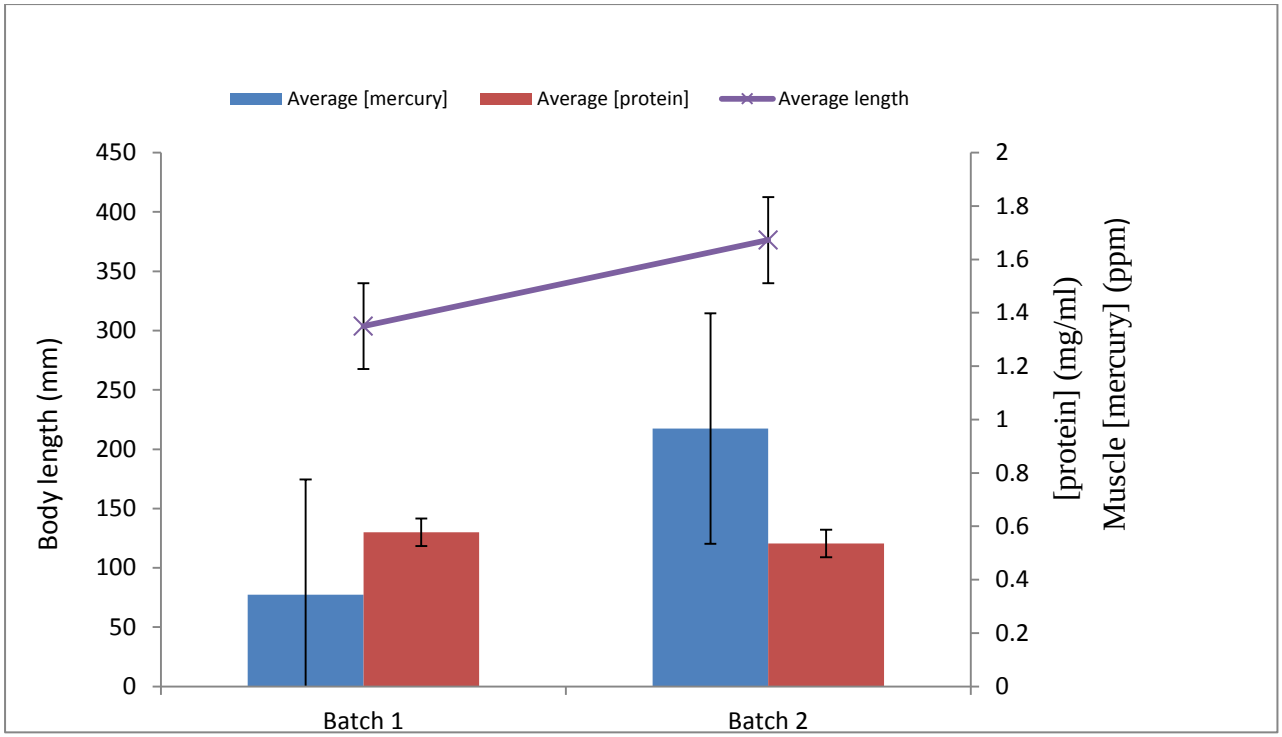

Figure 10: graphic representation comparing average body length, white muscle mercury and protein concentration between batch 1 and batch 2 samples.

Table 3: Linear model for SOD slope (NBT reduction/min) with fish length (mm), mercury content (ppm) and protein content (mg/g). Values in parentheses are ± standard errors, while r^2 is simple regression and R^2 is multiple regressions. Significant positive correlation is detected between SOD slope and fish length in fish samples obtained in 2013.

Models	R^2	d.f.	*P*
SOD slope = -6.433 (±1.559)factor[2013] + 38.352 (±1.162)	0.333	34	< 0.0001
SOD slope = 0.0360 (±0.013)Length – 9.048 (±1.707) factor[2013] + 27.40(±4.083)	0.460	33	< 0.0001
SOD slope = 5.089 (±2.537)$[[Hg]_{muscle}$ - 9.596 (±2.172) factor[2013] + 36.600 (±1.416)	0.406	33	< 0.001
SOD slope = 1.505 (±2.337)$[[Hg]_{liver}$ - 7.499 (±1.893) factor[2013] + 38.435 (±1.294)	0.373	32	< 0.0001
[Protein] = 3.031 (±1.192)•ln$[Hg]_{liver}$ + 56.476 (±1.555)	0.164	33	< 0.05
[Protein] = 3.037 (±1.269)•ln$[Hg]_{muscle}$ + 55.086 (±1.148)	0.144	34	< 0.05

4.4 Enzyme activity of commercial superoxide dismutase (SOD) and liver homogenates

The SOD analysis measured in the fish samples detected SOD activity. From the SOD activity analysis, the reduction rate of nitro-blue tetrazolium (NBT) was monitored. A very high SOD activity was observed when undiluted liver homogenates were used, which produced very fast reaction of which only flat curves were observed after spectrophotometric analysis. The liver homogenates were subjected to a range of dilution series, and the 10^{-7} dilution was observed to have SOD activity with reaction rate that could be measured easily.

Fig 12 shows the signal curves obtained during the SOD enzyme activity analysis. The first 3 minutes of the reaction were used for the calculation of the SOD slope values in the liver homogenates (appendix). The first three minutes of observation were chosen because it was necessary to record the outcome in the initial state when enzymes were still in fresh state, as well as when reactants were still fresh and unaffected by changes in pH, temperature or too much light exposure. The first 4 cycles in the first 3 minutes were also preferred

because they gave curves with slopes that were ascending linearly. The curves in figure 12 represent average curves from three replicates from each fish sample measured.

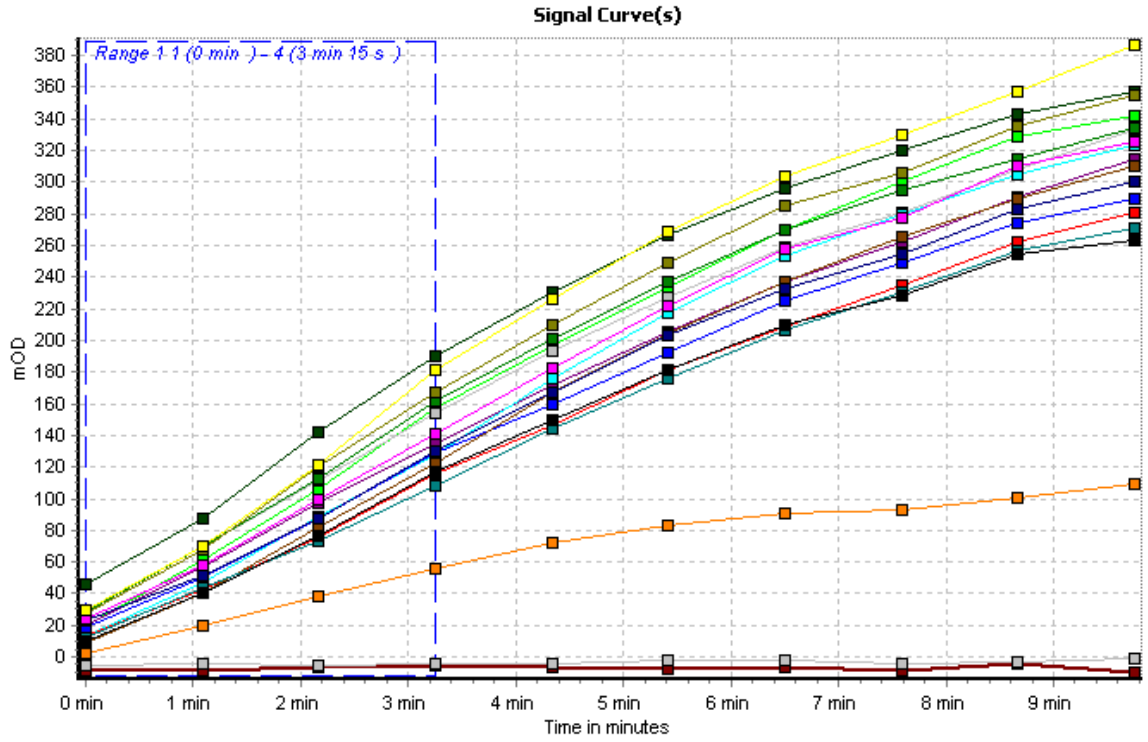

Figure 11: Spectrophotometer generated signal curves during the measurement of SOD enzyme activity in liver homogenates. The first 3 minutes (shown with blue broken line) of the reaction were used in the slope calculation (rate of NBT reduction). Each is an average of three replicate from each fish sample.

4.5 SDS-PAGE

SDS-PAGE was carried out to map both SOD and GPx bands. Due to many fish samples, only one fish sample had its liver homogenates used. During SOD band determination, one liver homogenate vial from batch 1 samples was chosen randomly. Figure 13 shows the commercial SOD band, in a red circle in lane one. Lane two had liver homogenates applied in it and shows in the same region (about 15 kDa) that there are proteins with molecular weight similar to that of SOD subunits. Lane 3 and 5 were applied with ladder (protein standard). Lane 4 was applied with commercial CAT enzyme, showing a band at ca. 55 kDa.

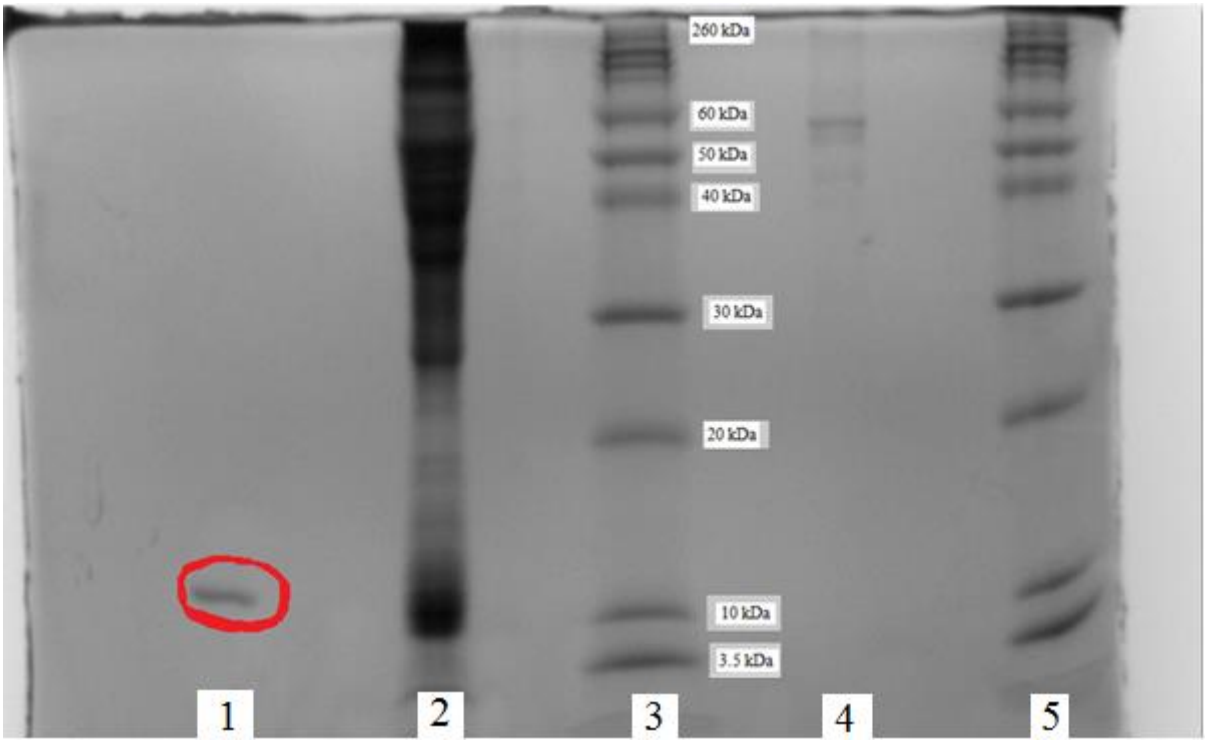

Figure 12: SDS-PAGE picture. Lane one shows the commercial SOD band, in a red circle. Lane two had liver homogenates applied in it and shows in the same region (about 15 kDa) that there are proteins with molecular weight similar to that of SOD subunits. Lane 3 and 5 were applied with ladder (protein standard). Lane four was applied with commercial CAT enzyme, showing bands at ca. 55 kDa.

The same was done for GPx, in which a random sample from batch 2 was used in the SDS-PAGE. Figure 14 shows the results. The band with a red circle in lane 4 depicts the commercial GPx, with molecular weight for subunits at ca. 25 kDa. Liver homogenates were applied in lane two, and in the similar region (about 25 kDa) we see proteins with similar molecular weight as the GPx subunits.

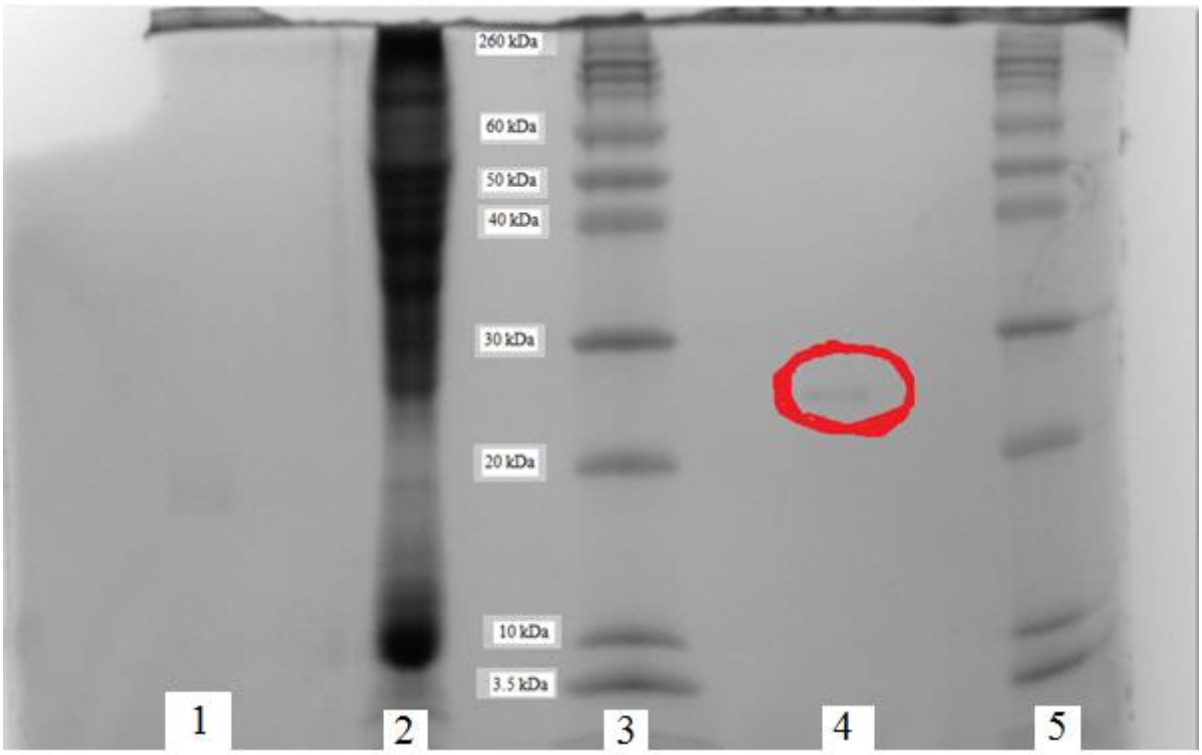

Figure 13: SDS-PAGE picture depicting the protein band of commercial GPx enzyme in the red circle. Fish sample is shown in well 2, while the ladder (protein standard) is shown in both well 3 and 5.

The statistical linear model was used to determine the correlation between SOD slope, fish length (mm), mercury content (ppm) and protein content (mg/g). The linear model revealed that there is a significant positive correlation between SOD slope, muscle mercury, liver mercury and fish length Table 4. No correlation was observed to exist between SOD slop and protein concentration (Table A2 in appendix).

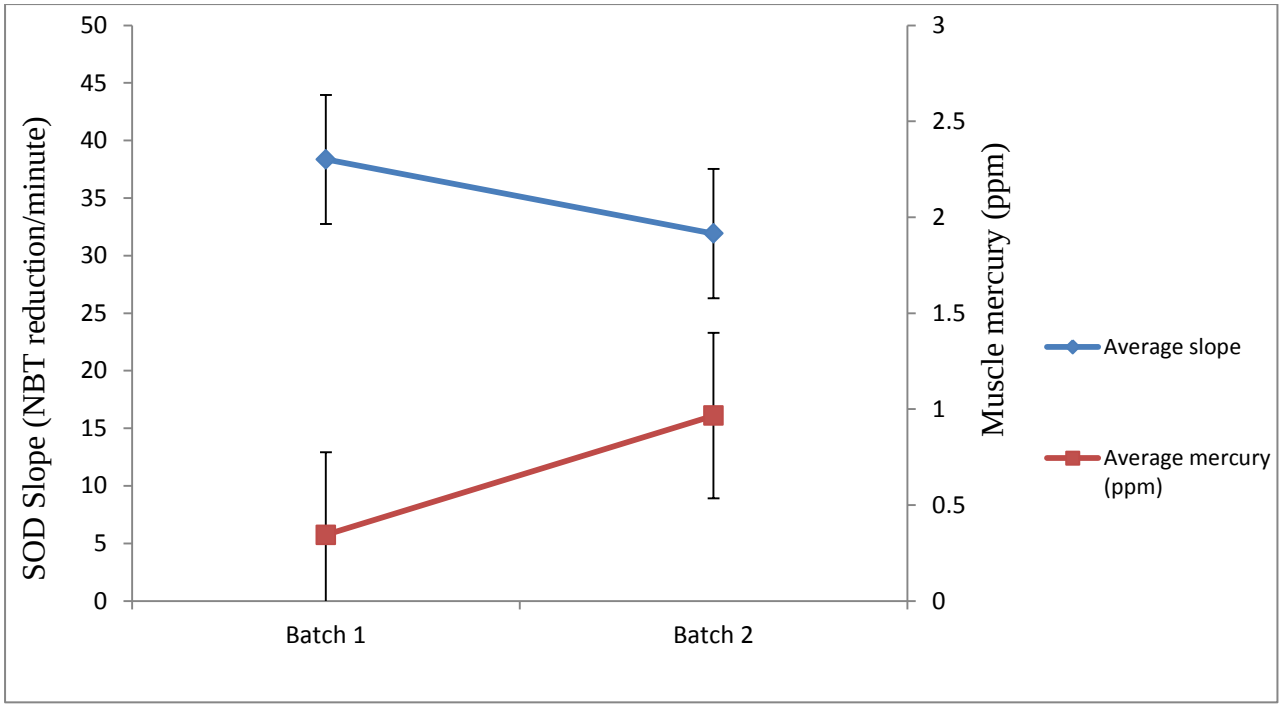

Figure 14: Graphical representation of result showing average mercury concentration and SOD slope values between batch 1 and batch 2 samples.

Fig 16 shows the slope values obtained from the signal curves (appendix) during the enzyme activity analysis of commercial SOD enzymes. Series1 represents slope values of SOD enzymes that was not treated with mercury, while series2 are slope values from mercury treated commercial SOD. From the SOD activity analysis, the rate of NBT reduction (SOD slope) by commercial SOD with mercury treatment (1ppm) produced faster reactions than the untreated commercial SOD. In series1, the highest concentration of commercial SOD (1000 ng) produced faster reaction rate (low slope value), while the lowest concentration of commercial SOD (0.1 ng) produced the lowest reaction rate (high slope value). There was a little difference observed in slope values between 10 ng SOD (28.8) and 0.1 ng SOD (30.9). Also it was observed that the slope values between 0.1 ng SOD and the positive control (no SOD added) gave slope values that are identical (both slope values were 30.9). The series2 slope values (mercury treated SOD enzymes) did not differ much, with the only significant difference being the slope value from 1000 ng SOD (3.5), while the rest of the mercury treated samples gave similar slope values of 6.8. The mercury treated positive control a slope

value of 6.3, slightly lower that mercury treated 10 ng and 0.1 ng commercial SOD. The negative control lacked both SOD and PMS, and did not produce any slope.

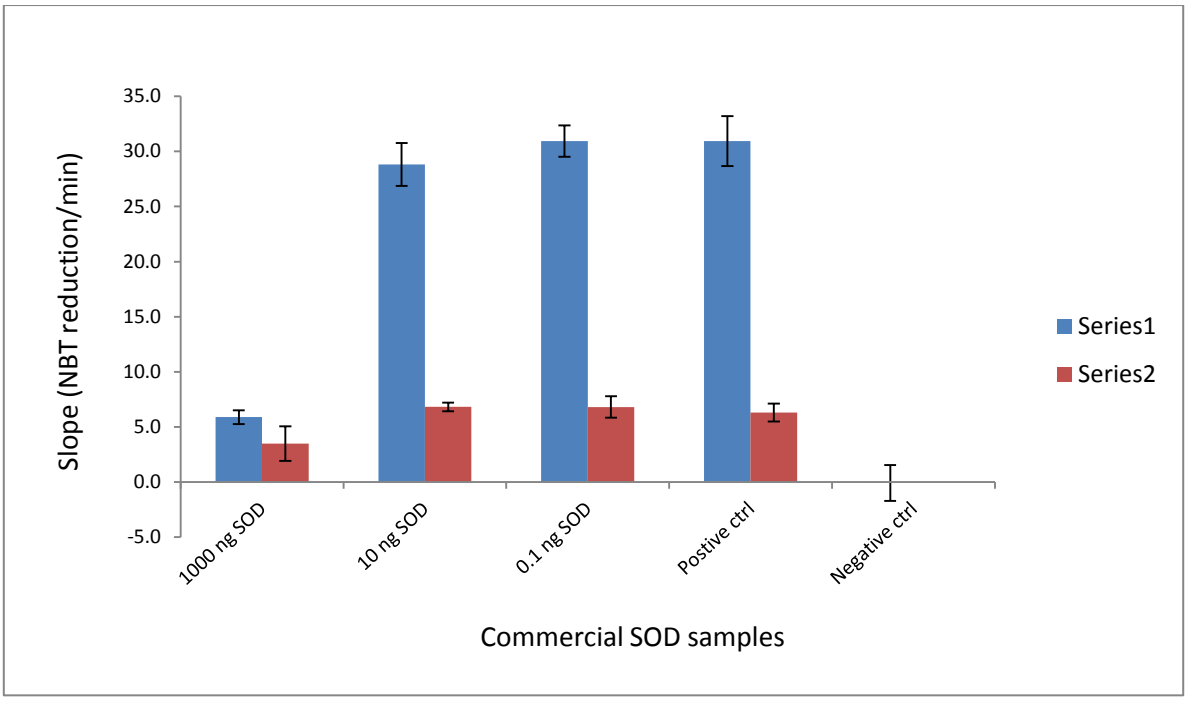

Figure 15: The slope values obtained during the enzyme activity analysis of commercial SOD enzymes. Series1 represents slope values of SOD enzymes untreated with mercury. Series2 are slope values from mercury treated commercial SOD enzyme. Vertical bars show standard deviation. Positive control samples did not contain SOD enzyme, while negative control sample did not contain both SOD and PMS.

4.6 GPx enzyme activity analysis

GPx activity analysis was carried out. Just like SOD activity analysis, commercial GPx was analysed first to observe the response. The amount of commercial GPx enzyme observed to give ideal reaction rates where those not exceeding 1 nanogram (1 ng). Figure 16 shows the bar graphs of slopes obtained from the commercial GPx activity analysis. The graph shows that there is a downward trend in slope values when commercial GPx is diluted. Activity analysis of GPx enzyme from liver homogenates was done on samples from the first 12 fish samples from 2012 (batch). Figure 17 shows the results of GPx slope values from fish samples presented as a scatter diagram. Linear regression analysis on the GPx results showed that there is a negative correlation between GPx activity and mercury contents, but this negative correlation was not reveal to be significant by linear model.

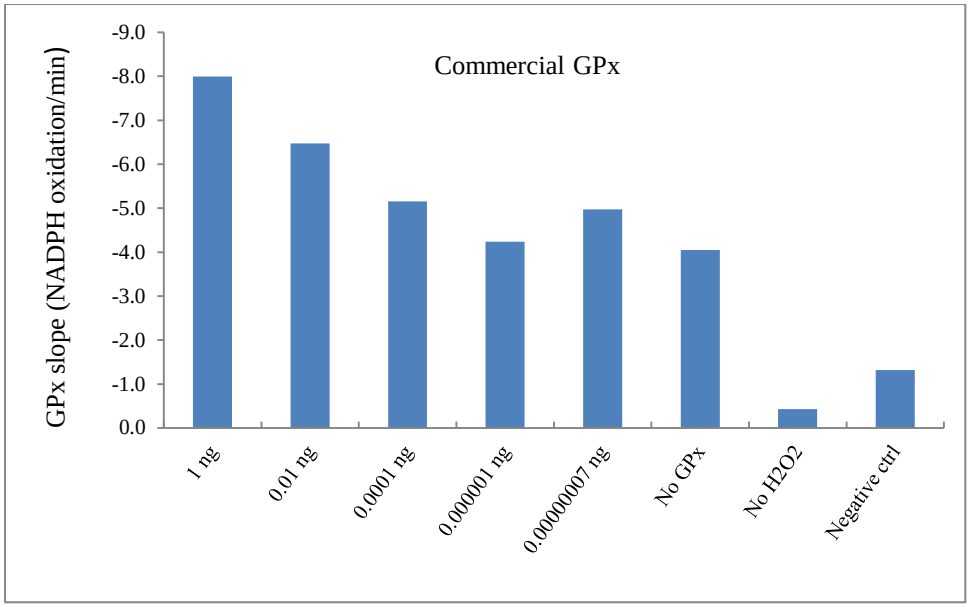

Figure 16: Figure 16: The slope values obtained during the enzyme activity analysis of commercial GPx enzyme at different concentrations. No GPx is a GPx independent reaction.No H_2O_2 is a hydrogen peroxide independent reaction. Negative control is a reaction without both H_2O_2 and GPx added into the reaction.

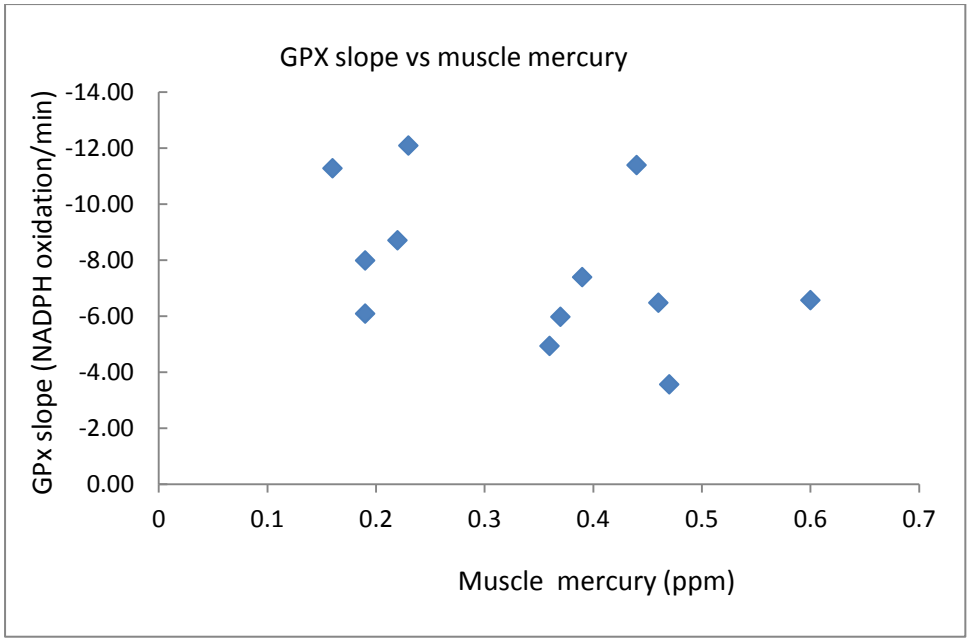

Figure 17: scatter diagram showing slope values of GPx activity from fish samples plotted against muscle mercury content.

5 Discussion

5.1 Levels of mercury in perch from Lake Mjøsa

Lake Mjøsa is the biggest lake in Norway and there are vast agricultural and industrial activities in town situated alongside it. The lake is also used as a recreational site for many of the inhabitants living in Hedmark, Akershus and Oppland counties, in the form of fishing, swimming and boat tours. Lake Mjøsa is an important source of raw water to the municipalities, private companies and as well farmers around it (Løvik, Jarl Eivind et al., 2009). The easiest fish species to catch with a fishing rod is perch (*P. fluviatilis*) (Personal experience). The total amount of mercury in the sediments in Lake Mjøsa is estimated to be 1.8 tons (Rognerud, 1985).

This study confirmed the presence of mercury in all the 36 perch fish samples in analysis, with detected levels between 0.15 and 1.69 mg/kg in white muscles. This clearly shows that some big perch with high mercury concentration in their flesh still exists in Lake Mjøsa, with levels over three times higher than the limit (0.5 mg Hg/kg) set by the Norwegian food safety authority (mattilsynet) and the world health organization. Statistical linear model revealed that there is a significant positive correlation between mercury content in fish and both age and length of fish. This confirms the bio accumulative nature of mercury (Monteiro, D. A. et al., 2010; Monteiro, L. R. et al., 1996). Of these two predictors, length is easier to determine than age, since it only requires a tape measure to determine the length of the fish than ortoliths or operculum for age determination. But this should not rule out the important of age, because due to different metabolical capacities of different fish, some fish can grow faster than the others because they have access to more food. Taking all 36 samples into account, the average mercury concentration level was observed to be 0.69 ppm (0.69 mg Hg/kg wet weight). But when the results were observed closely, it was noticed that the sampled fish under 30 cm long had mercury levels lower than the set limit of 0.5 mg/kg (table A1 in appendix). Compared to studies done in 1998 where it was found average mercury levels of 0.46 mg/kg in perch fish with average length of 268 mm (Fjeld et al., 1999), mercury levels reported in this study in perch fish with approximately the same size are lower (0.34 ppm and 303.75 mm). This is a down-hill trend, and very good news to consumers of perch from Lake Mjøsa. But it is still highly recommended to still follow the 25 cm

benchmark set by the Norwegian food safety authority if an individual would consume perch, so that the lowest possible mercury is consumed.

5.2 Commercial and sample SOD activity measurements

A none-enzymatic superoxide generator assay for SOD activity analysis was used (Ewing & Janero, 1995). During the response analysis of commercial SOD, A maximum amount of 1 mg was added into the wells of the assay plate. This amount was chosen because the rate of reaction was ideal to work with and observe in the spectrophotometer. Amounts above 1 mg SOD gave faster reactions that started and finished before the assay plate could be placed in the spectrophotometer for measurement. Compared with other concentrations (10 ng and 0.1 ng), it was indeed confirmed that, when other reactants' concentrations remained unchanged, different commercial SOD amounts gives different slope values, with the highest SOD content giving the lowest slope value (fastest reaction rate). The wells in which no SOD added (but having NBT, NADH and PMS) was designed as the positive control, and this in theory was suppose the give the highest slope values because the detectable blue color (formazan product) could be formed undisturbed. This was not always the case in all the repeated experiments, as it was observed that sometimes the positive control had a slope lower than the slope of 0.1 ng. Because superoxide ions are substrates for both SOD and NBT (to form formazan product), it is therefore assumed that the positive control reactions tends to reach equilibrium faster because there is no competition for the superoxide anions, while the reaction mixture with SOD present could hold longer reaction. A zero slope value was observed in the negative control, a reaction mixture that contained NADH, NBT, but without mercury, SOD and PMS. The observed behaviour of the negative control reaction mixture not giving any slope is what was expected, because there was no PMS that could react with NADH to produce superoxide ions.

5.3 What happens to slopes when mercury chloride is added?

In both commercial and sample SOD activity analysis, it was observed that when1 ppm mercury chloride was added the reaction went faster and produced lower slopes than in mercury untreated reactions. This is opposite from the hypothesis, in which it was expected that the mercury would inhibit the activity of SOD enzymes, so that the reaction rate could produce high slope values that are similar to that of the positive control. On first thought, this observed trend could be assumed to be because the added mercury activated the SOD enzymes to react faster with the superoxide ions, but then it was also observed that the positive control treated with mercury also had reduced slope values, almost as low as the slope values from mercury treated SOD reactions. The assumption for this observation is that mercury cations reacted with superoxide anions (instead of activating SOD enzymes), thereby forming metallic mercury that escaped into the atmosphere. This assumption is supported by the observation from a study which revealed that mercuric ions can be reduced by superoxide ions (Aikoh, 1991). The reaction between mercury cations and superoxide anions seems to be fast enough to out-compete the NBT/superoxide reaction. Because of this fast NBT/superoxide reaction, the colour formation (blue formazan product) that is registered in the spectrophotometer at 560 nm is reduced. Even though direct addition of mercury chloride to the fish samples did not give results as hypothesis (deactivation enzyme activity to create high slopes), the observed results were satisfactory in a sense that they could act as a guideline on what to expect in future experiments.

There are no available reports that say mercury chloride has a direct effect on the SOD enzymes. Yee and Choi (1996) assessed the effect of mercury in the electron transport chain of the mitochondria. Their findings showed that mercury disrupted the electron transport chain, giving a significant increase in the reactive oxygen species (ROS) and a reduction in glutathione. Indeed many studies showed that mercury binds to the membrane proteins; the most affected is the ubiquinol (cytochrome c oxidoreductase region) (Yee & Choi, 1996). When the electron transport chain (ETC) is affected, electrons are not channelled properly, and they randomly combine with oxygen atoms to form superoxide ions, free radicals which in turn can cause oxidative damage. The produced free radicals induce the release of antioxidant enzymes such as SOD, GPx, CAT and GR.

5.4 Correlation of enzyme activity and protein concentrations against mercury content.

Protein concentration revealed positive correlation against mercury content, an observation that that is supported by literature (Monteiro, D. A. et al., 2010). Negative correlation was observed when enzyme activity SOD slope) was plotted against mercury content and fish length. This observation is new and opposes early studies. An assumption for this observation could be that, the longer the fish gest exposed to mercury in its natural habitat, the weaker its antioxidant defence becomes. This weakness in antioxidant response therefore necessitates the fish to produce more proteins to compensate for lost enzyme activity, as we observed that there is positive correlation between protein concentrations against mercury concentration. With only 36 samples used in the experiments, it would difficult to conclude if this observed non-correlation is indeed the way it is. Probably the sample experiment should be done with more fish samples covering the whole spectrum, so that surety could be attained.

5.5 GPx activity analysis

GPx analysis was briefly done. The response activity analysis from the commercial GPx enzyme yielded the expected slope patterns; with the highest enzyme content (1 ng) giving the highest (sharpest) downhill slope values (Figure 17). During the execution of the GPx activity analysis, common hydrogen peroxide (H_2O_2) was used during both commercial GPx analysis and the analysis of samples from 2012 (batch 1), following the protocol by Flohé et al (1971) which the continuous formation of GS-SG ((Floh et al., 1971). When added with mercury chloride, it was also observed that the slope values were reduced just like in SO. Reason for this observation was later discovered that new and improved protocols for GPx activity assays stopped using common hydrogen peroxide, and the use of cumene hydrogen peroxide or tert-butyl hydrogen peroxide as substrate has become preferred. This substrate discriminates against other enzymes (such as CAT) that can react with common hydrogen peroxide, and the spontaneous reaction with GSH is low, measuring the total GPx activity (Thomson, 1985). Also new GPx protocol recommends the addition of sodium azide

to block side reaction caused CAT enzyme in crude samples (homogenates) (Esworthy et al., 2001).

The measured GPx activity analysis gave slope values with a downward pattern, when plotted again white muscle mercury concentration, but the linear model revealed that this pattern is of no significant correlation. Therefore no conclusion could be made if this was expected.

5.6 Further studies

No correlation was observed between SOD slope and protein concentration in the samples. In order to validate this observation, it is required to test at lot of fish with approximately the same size. It was only 36 fish used in this project, and the fish caught in 2012 were significantly smaller that the fish from 2013. During the testing of the effect of mercury chloride on enzyme activity, the observed results were opposite to what was hypothesized. But the mercury form that is most poisonous and bio-accumulates in living organisms is methyl mercury. Therefore methyl mercury should be added directly to the reaction mixture in future to observe its effect on enzyme activity.

There is needed more experiments in future needs to be done to confirm if 1 ng GPx is the ideal amount to work with. The first twelve samples from 2012 were tested against GPx activity, with slope values that did not show any specific pattern, with no correlation to mercury content. Also it was later discovered that in order to measure GPx values in the fish samples, sodium azide needed to be added to supress the Catalase activity to stop competing with GPx enzymes, as well as the use of cumene hydrogen peroxide to discriminate against GST enzyme that would otherwise compete with GPx for common hydrogen peroxide. A lot of time was invested in the design of experiments during the execution of this project, since it was the first time when this project was carried out. Therefore it is worth to note that not all angles were explored. For example enzyme activity and protein concentration were measured only after the samples were stored at – 80 ºC, and that only 10% glycerol was added to the samples. In follow-up studies it could be recommended to test all parameters in order to obtain a solid conclusion.

6 Conclusion

- The project revealed that there is mercury in both the white muscles and livers of perch from Lake Mjøsa. Positive correlation of mercury against age and length of the fish reveals the bio-accumulation nature of mercury, which means that when a fish consumes the mercury contaminated food, the more the mercury accumulates in its body as it grow.
- It was observed that protein concentration was positively correlated with mercury content and fish length, but not with SOD activity, with showed neither negative correlation. The non-correlation of SOD activity against mercury and fish length can mean that fish exposure to mercury in a long run can reduce the antioxidant response of fish.
- The lack of correlation between SOD activity and protein concentration can lead to a conclusion proteins are produced at a constant and steady level to compensate for the lost enzyme activity, so that protein production gives stable enzyme balance in the body of the organism. This assumption takes into account that enzymes are also proteins, and this steady-state protein production take into account that both SOD and GPx are included into this steady-state protein production.
- It is useful to mention that the samples (liver homogenates) were stored for some time at -80 ºC before measuring protein concentration, SOD and GPx activity. Therefore it should be accepted that there is a possibility that the length of storage could have affect the stability and influence the enzyme activity, as it is reported that storage can affect the stability of proteins (Cryer & Bartley, 1974) .
- Also during liver homogenization, mechanical means were used, namely ultra-turax and ultra-sound sonication. The choice of homogenization method has its own effects on the activity of enzymes (Özbek & Ülgen, 2000).
- The addition of mercury chloride in the reaction mixture for SOD activity did not inhibit enzyme activity, but the signal intensity was reduced. This can be concluded that mercury chloride reacted with superoxide ions and escaped into the atmosphere as vapour. Both 10 ppm and 1 ppm mercury chloride

concentration were used for this test, and an incubation of 60 minutes was done before measuring the activity. The same results were observed. It is suggested that more samples and more tests using methyl mercury in future need to be done to confirm if this observation can be validated.

This project was done for the first time at Hedmark University College, so there were a lot of trial and errors involved. This took a lot of time to sort out. But because of a lot of trial and errors, a lot of knowledge was acquired during the project period.

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8 Appendix

Table A1: Summary of data obtained from the fish samples used in the experiment.

Species	Age (years)	Length (mm)	Lake name	Fish ID	Muscle Hg (ppm)	Liver Hg (ppm)	SOD slope (NBT (reduction/min)	GPx slope (NADPH oxidation/min)	[Protein] (mg/ml)
Perch	19	480	Mjøsa	2012_31	0.6	0.27	46.88	-6.57	0.68
Perch	12	415	Mjøsa	2012_32	0.47	0.23	41.57	-3.56	0.63
Perch	16	365	Mjøsa	2012_33	0.46	0.25	43.59	-6.48	0.58
Perch	13	330	Mjøsa	2012_34	0.37	0.16	48.27	-5.97	0.57
Perch	13	285	Mjøsa	2012_35	0.36	0.16	38.82	-4.94	0.56
Perch	13	285	Mjøsa	2012_36	0.39	0.14	34.46	-7.39	0.55
Perch	11	280	Mjøsa	2012_37	0.19	0.18	33.8	-7.98	0.63
Perch	12	260	Mjøsa	2012_38	0.19	0.16	39.92	-6.08	0.58
Perch	14	312	Mjøsa	2012_39	0.44	0.2	37.44	-11.39	0.53
Perch	10	260	Mjøsa	2012_40	0.23	0.2	34.76	-12.09	0.54
Perch	13	225	Mjøsa	2012_41	0.22	0.18	35.88	-8.71	0.62
Perch	11	205	Mjøsa	2012_42	0.16	0.16	42.03	-11.28	0.49
Perch	9	240	Mjøsa	2012_43	0.15	0.29	35.35		0.61
Perch	12	230	Mjøsa	2012_44	0.17	NA	32.38		0.54
Perch	12	278	Mjøsa	2012_45	0.34	0.21	31.15		0.52
Perch	21	410	Mjøsa	2012_46	0.77	0.36	37.34		0.62
Perch	18	410	Mjøsa	2013_01	1.69	1.26	40.81		0.58
Perch	16	440	Mjøsa	2013_02	0.83	0.38	31.94		0.45
Perch	16	420	Mjøsa	2013_03	0.67	0.26	34.39		0.53
Perch	16	390	Mjøsa	2013_04	1.49	1.1	41.7		0.53
Perch	16	410	Mjøsa	2013_05	0.99	0.68	33.7		0.53
Perch	16	360	Mjøsa	2013_06	1.18	0.96	27.07		0.57
Perch	16	370	Mjøsa	2013_07	1.21	1.08	28.36		0.57
Perch	15	380	Mjøsa	2013_08	1.27	1.04	33.11		0.52
Perch	13	380	Mjøsa	2013_09	0.69	0.48	32.03		0.47
Perch	15	375	Mjøsa	2013_10	1.2	0.87	27.26		0.52
Perch	15	360	Mjøsa	2013_11	0.53	0.51	26.52		0.59
Perch	11	330	Mjøsa	2013_12	0.91	0.45	33.29		0.55
Perch	17	390	Mjøsa	2013_13	0.47	0.14	31.87		0.53
Perch	13	370	Mjøsa	2013_14	0.52	0.12	26.75		0.45
Perch	18	390	Mjøsa	2013_15	1.59	1.81	28.7		0.52
Perch	13	340	Mjøsa	2013_16	0.54	0.23	36		0.54
Perch	17	360	Mjøsa	2013_17	0.72	0.28	32.1		0.5
Perch	14	370	Mjøsa	2013_18	0.72	0.24	27.46		0.55
Perch	13	320	Mjøsa	2013_19	0.91	0.33	30.76		0.62
Perch	16	360	Mjøsa	2013_20	1.19	0.86	34.58		0.59

Table A2: Linear model revealing no observed correlation between protein concentrations and length, age, muscle mercury, liver mercury and SOD slope. No correlation was also observed between SOD slope and Age.

Models	R^2	d.f.	*P*
[Protein] = 3.700 (±1.795) factor[2013] + 51.250 (±1.338)	0.111	34	< 0.05
[Protein] = 0.015 (±0.016)Length + 2.635 (±2.158) factor[2013] + 46.788 (±5.160)	0.132	33	0.10
[Protein] = 0.290 (±0.370)Age + 3.112 (±1.953) factor[2013] + 47.421 (±5.062)	0.127	33	0.11
[Protein] = 3.602 (±3.029)$[Hg]_{muscle}$ + 1.461 (±2.594) factor[2013] + 50.009 (±1.690)	0.148	33	0.07
[Protein] = 1.703 (±2.730)$[Hg]_{liver}$ + 3.260 (±2.211) factor[2013] + 50.576 (±1.511)	0.138	32	0.09
[Protein] = 0.146 (±0.199) SOD slope + 4.641 (±2.214) factor[2013] + 45.641 (±7.742)	0.125	33	0.11
SOD slope = 0.565 (±0.309)Age – 7.570 (±1.631) factor[2013] + 30.90(±4.23)	0.395	33	0.08

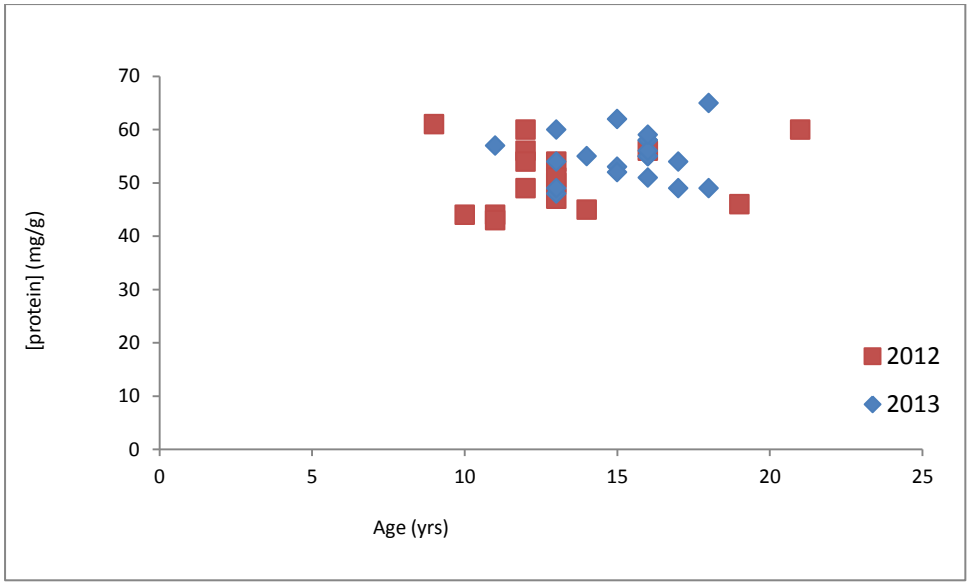

Figure A2: Scatter diagram of fish age (years) plotted against protein concentration (mg/g)). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

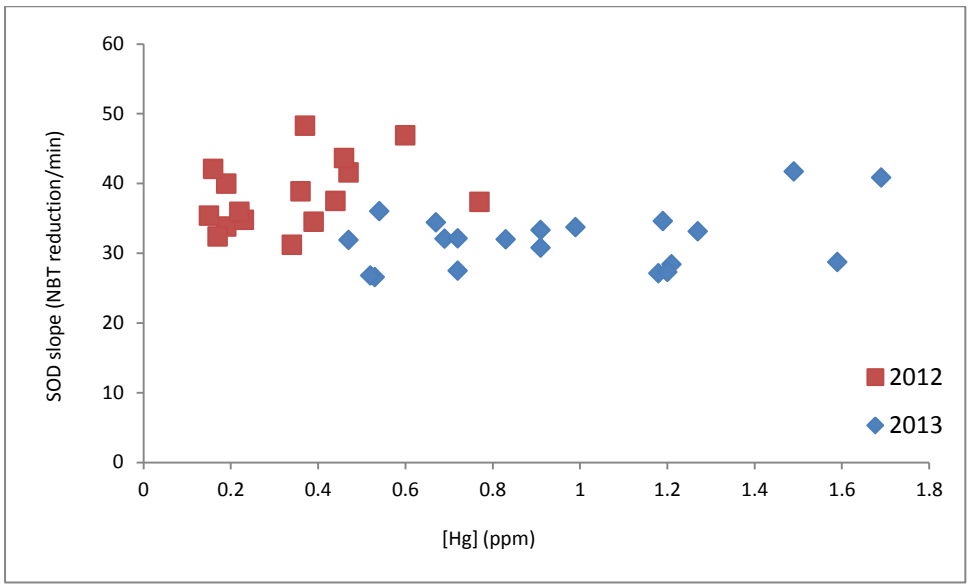

Figure A3: Scatter diagram of white muscle mercury content (ppm) plotted against SOD slope (NBT reduction/min). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

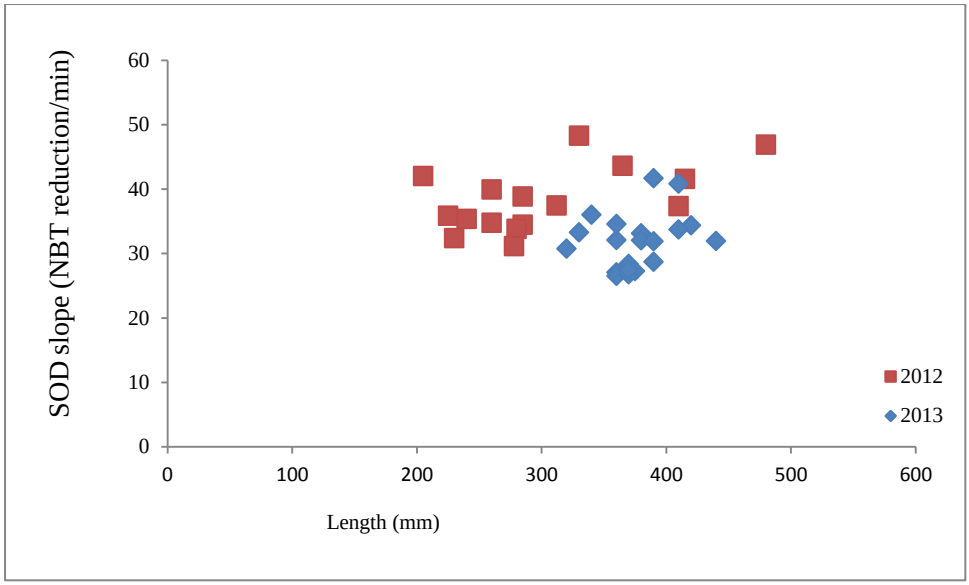

Figure A4: Scatter diagram of fish length (mm) plotted against SOD slope (NBT reduction/min). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

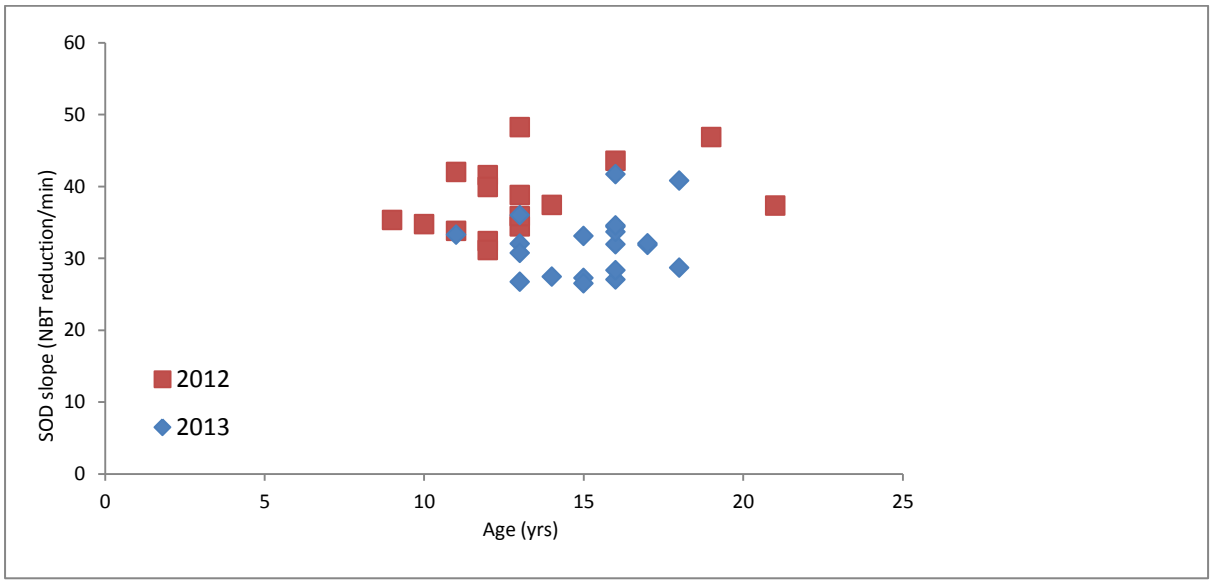

Figure A5: Scatter diagram of fish age (years) plotted against SOD slope (NBT reduction/min). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

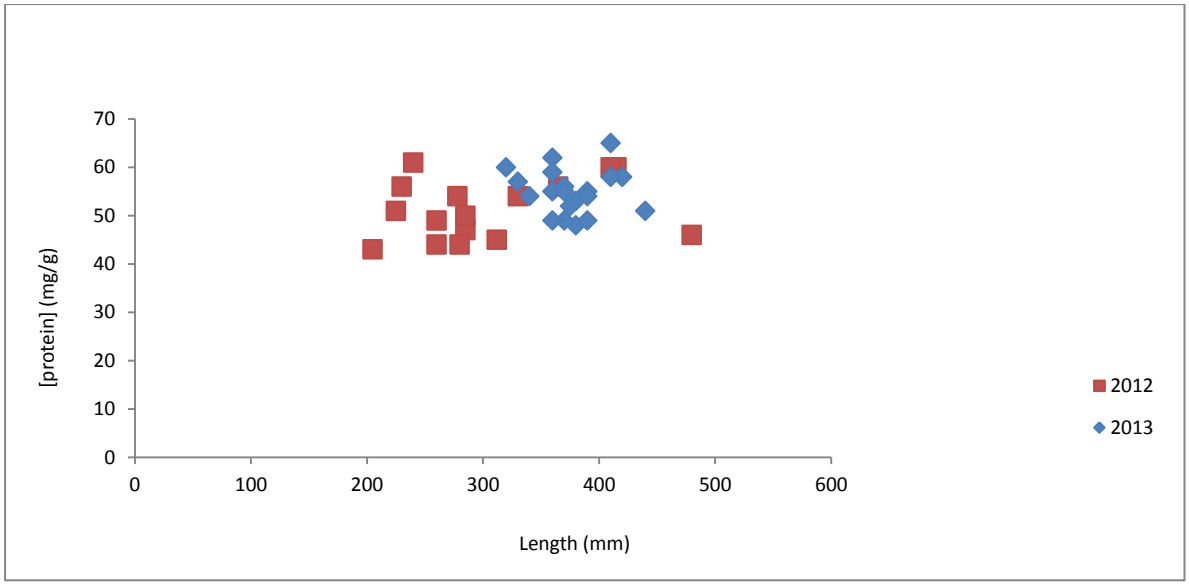

Figure A6: Scatter diagram of fish length (mm) plotted against protein concentration (mg/g). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

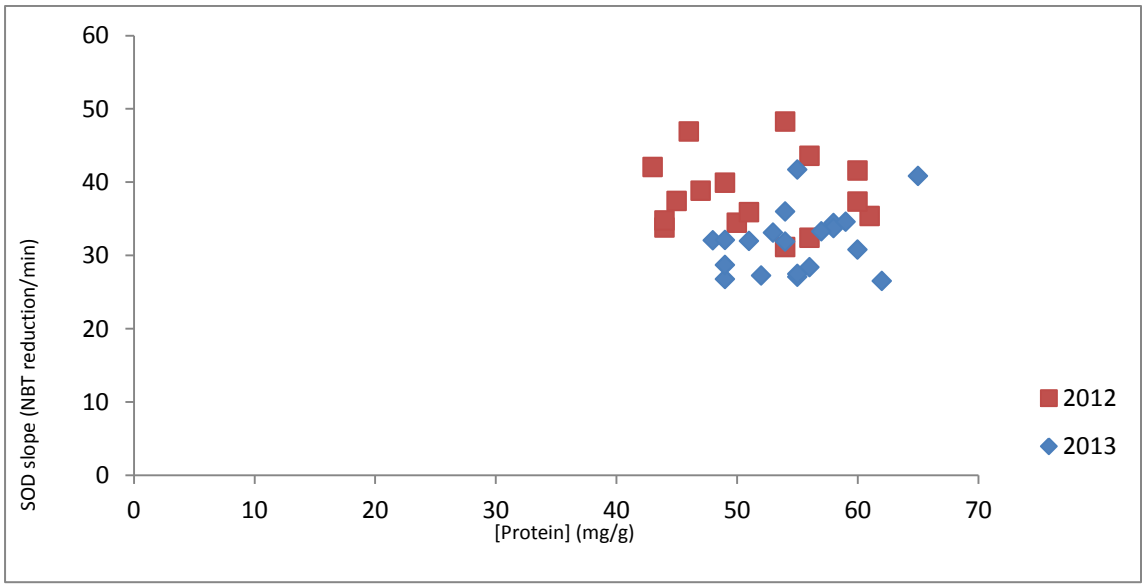

Figure A7: Scatter diagram of protein concentration (mg/g) plotted against SOD slope (NBT reduction/min). 2012 represents fish samples caught in 2012, while 2013 is fish samples caught in 2013.

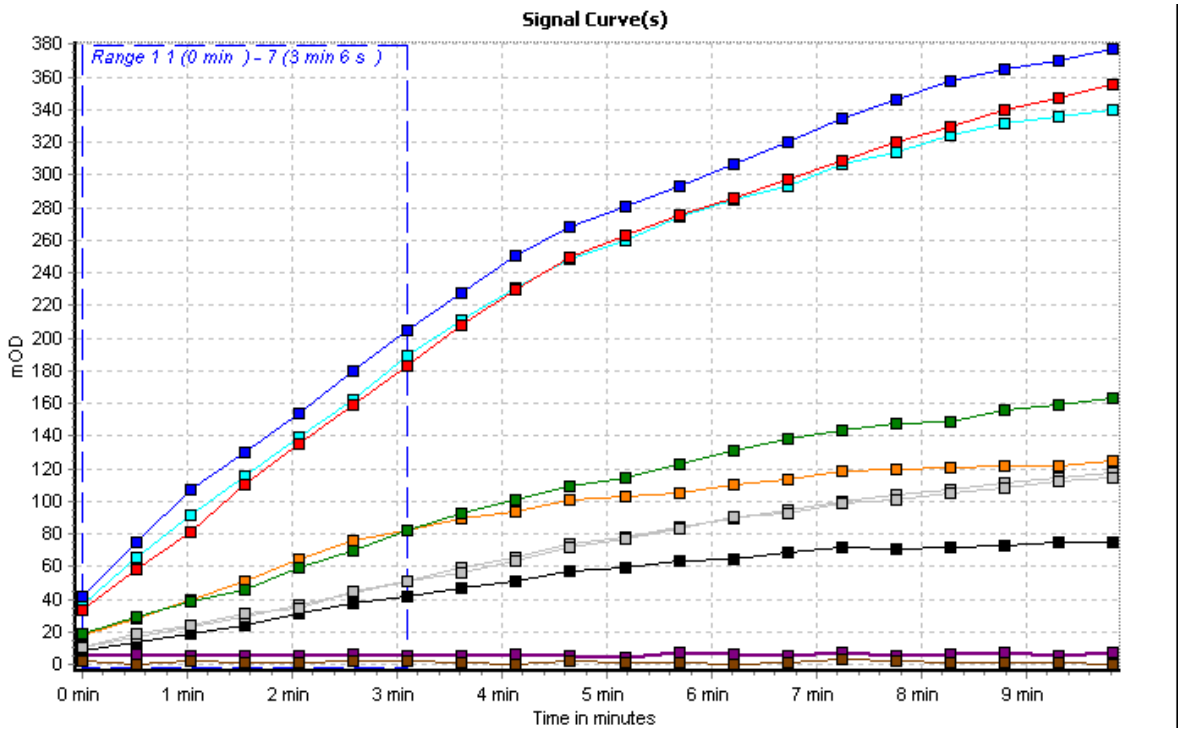

Figure A8: Signal curves generated during activity analysis of SOD commercial enzyme (both with and without mercury treatment). The amount of enzyme ranged from 1000 ng (highest amount) down to 0.01 ng (lowest amount).

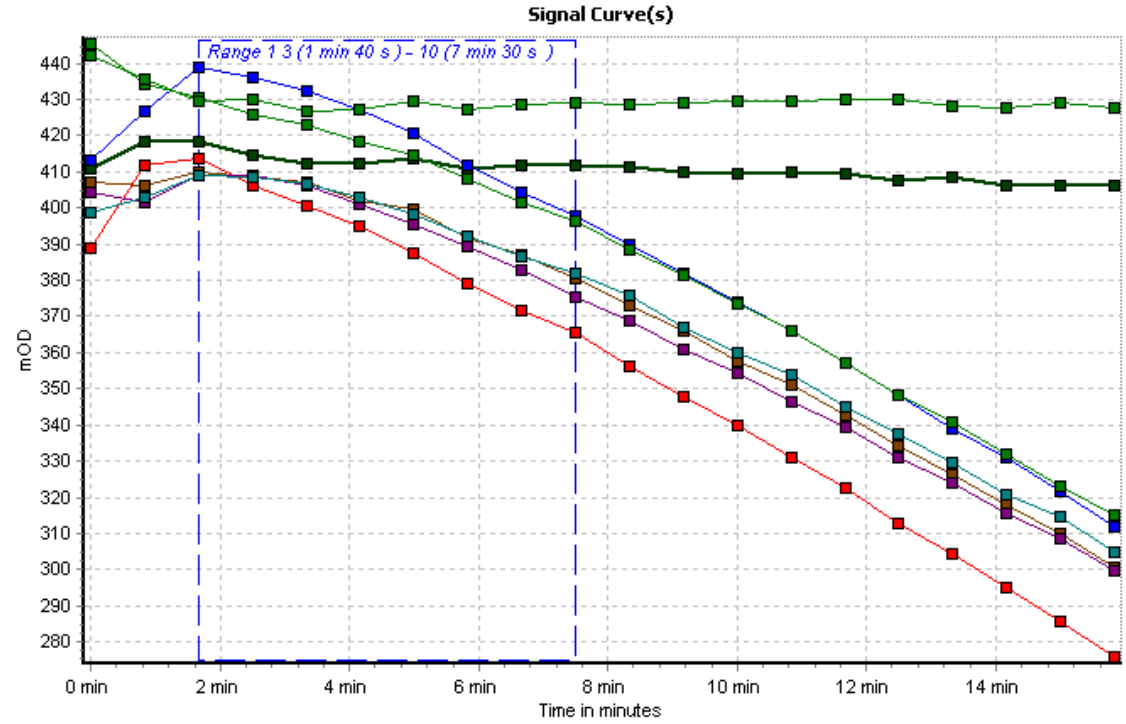

Figure A10: Signal curves generated during activity analysis of GPx commercial enzyme. The amount of enzyme ranged from 1ng (highest amount) down to 0.00000001 ng (lowest amount).

Protein concentration	Absorbance
0	0
0.2	0.218
0.4	0.442
0.6	0.583
0.8	0.744
1	0.912

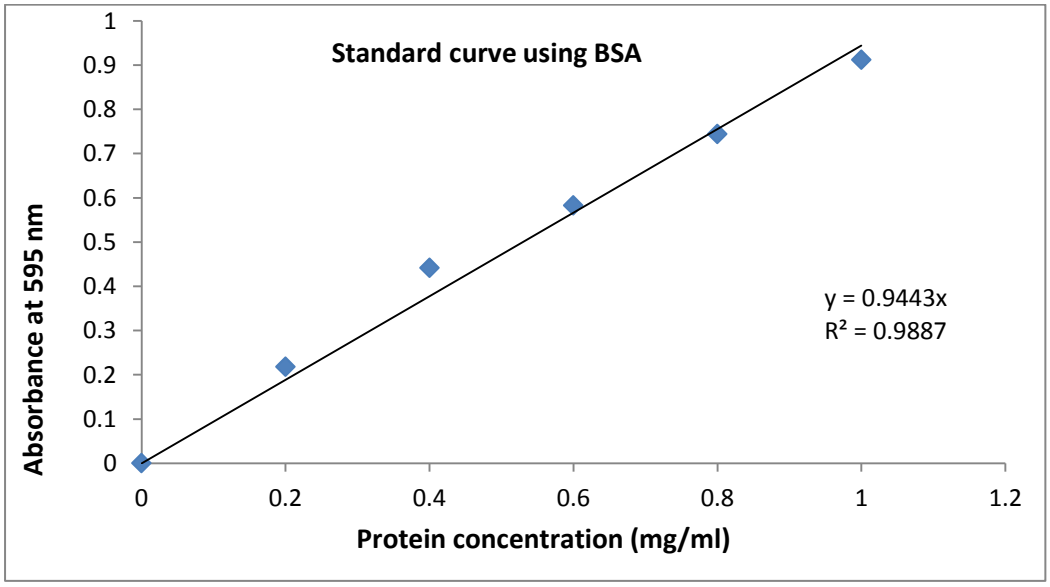

Figure A11: Standard curve constructed using data obtained by measuring BSA protein at 595 mn

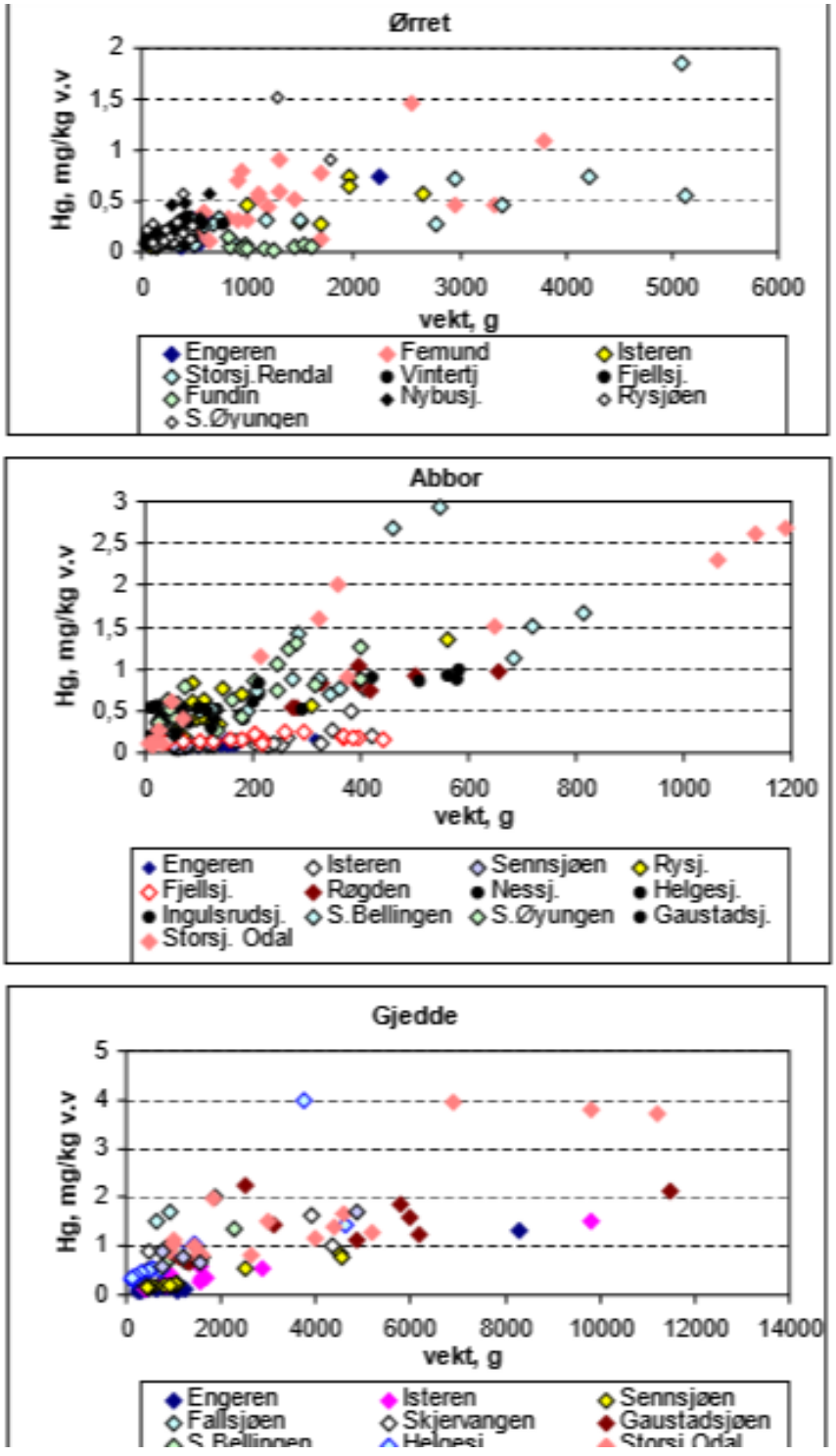

Figure A12: Comparison between mercury concentration and weight in brown trout (Salmo trutta), Perch (Perca fluviatilis) and Pike (Esox lucius) from lakes in Hedmark county, close to the Swedish border. Ørret = trout, Abbor = perch, Gjedde = Pike, v.v = wet weight, vekt = weight. Source: (Rognerud & Fjeld, 2002).

8.1 World industrial activities utilizing mercury

The usefulness of mercury in the early days before its harmful effect were known led to many countries producing it, with an annual global output of 1800 metric tons as of the year 2000 (UNEP, 2002). Brazil is one of the countries affected with mercury pollution because of artisanal gold mining activities in the sensitive amazon region (Pfeiffer et al., 1989; Pfeiffer et al., 1993). In Brazil's amazon region near the gold mining active sites, mercury levels in fish have been measured and were observed to reach up to 2.7 mg/kg in the fish muscles (Pfeiffer et al., 1989), with some studies in the same amazon region recording even higher mercury levels of up to 5.4 mg/kg in predatory fish species (Dorea et al., 2006). Amalgamation, a process in which Gold was separated from its ore using mercury, has been in use in gold extraction in the ancient world (Vaughn & Tripcevich, 2013), and it is still continuing to be used in artisanal gold mines in the developing countries (Donkor et al., 2006; Tschakert & Singha, 2007; Veiga, 1997).

Various other industries use mercury. Paper mill factories have also been found to have some negative effects on the aquatic organisms, as it was observed by Ahmad et al (2000) that the paper mill effluents induced antioxidant response in fish (Ahmad et al., 2000). Some chemicals used in the paper industry contained phenyl-mercury-acetate which was used as a preservative against fungi. The discharge from the paper industry polluted fish species (Johnels et al., 1967). The chlor-alkali industry produces caustic soda and chlorine using technologies such as the mercury cell, the diaphragm cell and the membrane cell process. In the mercury process, mercury is utilized as a cathode to trap sodium ions to form sodium-mercury amalgam, which is then reacted with water to form sodium hydroxide and mercury (Reis et al., 2009; Ullrich et al., 2007). The early chlor-alkali industry used mainly the mercury cell due to the lack of knowledge at that time about the harmful effects of mercury to the living organisms. The modern chlor-alkali factories understand the dangers of discharging mercury into the environment, and so the membrane cell is the most modern of all the three process. In the health and pharmaceutical sector, mercury has been used in dental fillings (Spencer, 2000; Yip et al., 2003), vaccines (Hessel, 2003), skin care products (al-Saleh & al-Doush, 1997; Weldon et al., 2000) and many other chemicals and instruments (EPA, 2013). Paints that contain mercury have also been reported (Agocs et al., 1990).

8.2 Status of mercury mining in the world

Almost all mercury mines in the world have closed down, including the biggest mercury mine from Spain that used to supply one third of the world's total mercury (Hernández et al., 1999), which shut down its operations in 2000 due to unprofitability and international pressure. As of 2009 there was reportedly only one mine involved in primary production of mercury intended for global supply. This mine is in Khaidarkan town in Kyrgyzstan. Other countries including China and are reported to produce mercury, but only for local use without supplying to the international market (Kirby, 2009). Apart from primary production of mercury, burning of coal and other fossil fuels emits mercury into the atmosphere (Joensuu, 1971).