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Antioxidant Enzyme System in Fish from Contaminated Lakes in Chernobyl Exclusion Zone

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Abstract

Aquatic organisms in the Chernobyl exclusion zone have been chronically exposed to ionizing radiation since the Chernobyl nuclear power plant accident in 1986. Fish are considered the most radiosensitive aquatic organism, and in the highly contaminated lake Glubokoye in Chernobyl exclusion zone, the fish have been continuously exposed to ionizing radiation e.g., from ^{137}Cs and ^{90}Sr over many generations since the accident. Radionuclides in the environment can contribute to external radiation, internal radiation and chemical effects from uptake. Ionizing radiation can lead to biological effects on organisms, indirectly or directly. The radiation can interact with water in the organism and produce reactive oxygen compounds such as free radicals. These free radicals do in turn generate indirect effects by reacting with biomolecules and cause oxidative damage to DNA and other cell constituents.

In this study, the two fish species pike (Esox Lucius) and rudd (Scardinius erythrophthalmus) from the highly contaminated lake Glubokoye, were used to identify morphological changes and to determine activity of antioxidant enzymes. The results were compared with pike and rudd from less contaminated lake Kashovka to identify effects due to ionizing radiation. The enzyme activity of the detoxifying enzymes; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were determined in the liver and the kidney tissue samples.

Previous studies have shown high radioactivity in lake Glubokoye. The fish in Glubokoye experience both internal and external exposure of ionizing radiation from the radionuclides ^{137}Cs and ^{90}Sr. Measurements carried out in Kiev by laboratory personnel showed an activity in Glubokoye at 3.3 ± 0.7 Bq/L of ^{137}Cs and, 96 ± 17 Bq/L of ^{90}Sr, respectively. Based on the activity concentration of ^{137}Cs in the fish muscle, and ^{90}Sr activity in the bone, the total internal dose-rate in the pike from Glubokoye was calculated to 4.29 ± 3.06 µGy/h. The ^{137}Cs external dose rate from water in the pike from Glubokoye were $9.68 \times 10^{-4} \pm 3.48 \times 10^{-5}$ µGy/h. In the rudd from Glubokoye the total internal dose-rate of ^{137}Cs and ^{90}Sr was on $9.45 \pm 8.92 \times 10^{-1}$ µGy/h. The external dose-rate was $1.06 \times 10^{-3} \pm 6.52 \times 10^{-6}$ µGy/h from water.

The tissue samples from the liver and the spleen of the contaminated fish showed distinct morphological changes. From the histological analysis, it appears that the highly

contaminated Glubokoye fish have been affected probably by ionizing radiation, especially in the liver and the spleen.

Most of the measurements of the enzyme activity in the tissue samples showed no significant effect indicating that the contaminated fish have higher levels of free radicals than the control fish. However, SOD activity in the rudd kidney showed significant difference and indicated that the contaminated rudd have higher levels of free radicals than the control fish.

Thus, results indicate that the fish from Glubokoye have histological changes in the structure of cells and tissue probably due to ionizing radiation. However, the ionizing radiation has not led to a higher proportion of ROS in the contaminated fish.

Sammendrag

Akvatiske organismer i Tsjernobyl-eksklusjonssonen har blitt kronisk eksponert for ioniserende stråling siden atomkraftulykken i Tsjernobyl i 1986. Fisk anses som de mest radiosensitive akvatiske organismene, og i den høyt kontaminerte innsjøen Glubokoye har fisk blitt eksponert ioniserende stråling, f.eks. fra ^{137}Cs og ^{90}Sr over flere generasjoner etter ulykken. Radionuklider i miljøet kan bidra til ekstern stråling, intern stråling og kjemiske effekter fra opptak. Ioniserende stråling kan reagere med vann i organismen og produsere reaktive oksygenforbindelser som frie radikaler. Disse frie radikalene kan gi indirekte effekter ved å reagere med biomolekyler og forårsake oksidativ skade på DNA og andre cellebestanddeler.

I dette studiet ble gjedde (*Esox Lucius)* og sørv *(Scardinius erythrophthalmus*) fra den høyt kontaminerte innsjøen, Glubokoye, benyttet for å se på de morfologiske endringer og for å bestemme enzymaktiviteten. Enzymaktiviteten av de detoksifiserende enzymene; superoksid dismutase (SOD), katalase (CAT), glutation peroksidase (GPx) og glutation reduktase (GR) ble bestemt i vevsprøver fra lever og nyre.

Tidligere studier har vist høy radioaktivitet i både innsjø og fisk nært Tsjernobyl. Fisken i Glubokoye opplever både ekstern og innvendig eksponering fra ^{137}Cs og ^{90}Sr. Målinger utført i Kiev av laboratoriepersonell viste en aktivitet i vannet på henholdsvis 3,3 ± 0,7 Bq/L (^{137}Cs) og 96 ± 17 Bq/L (^{90}Sr). Basert på aktivitetskonsentrasjonen av ^{137}Cs i fiskemuskelen, og ^{90}Sr i fiskebein, ble den totale innvendige dose-raten i gjedde fra Glubokoye beregnet, 4,29 ± 3,06 µGy/t. Den ^{137}Cs eksterne dose raten fra vannet i gjedda fra Glubokoye var 9,68 $x10^{-4}$ ± 3,48 x 10^{-5} µGy/t. I sørven fra Glubokoye var den totale interne dose-rate fra ^{137}Cs og ^{90}Sr på 9,45 ± 8,92 x 10^{-1}µGy/t. Den eksterne dose raten av ^{137}Cs fra vannet på sørven i Glubokoye var 1,06 x 10^{-3} ± 6,52 x 10^{-6} µGy/t.

Vevsprøvene fra lever og milt av den kontaminerte fisken viste tydelige morfologiske forandringer. Fra den histologiske analysen ser det ut til at den sterkt forurensede Glubokoye-fisken sannsynligvis har blitt påvirket av ioniserende stråling, spesielt i leveren og milten.

De fleste av målingene av enzymaktiviteten i vevsprøvene viste ingen signifikant effekt som indikerte på at den forurensede fisken ikke hadde høyere nivåer av frie radikaler forhold til kontrollfisken. SOD-aktiviteten i nyren fra sørven viste derimot signifikant forskjell og indikerte på at den forurenset sørven har høyere nivåer av frie radikaler enn kontrollfisken. For å underbygge denne observasjonen er det viktig med videre arbeid.

Resultatene indikerer på at fisken fra Glubokoye har histologiske endringer i strukturen til celler og vev, sannsynligvis på grunn av ioniserende stråling. Den ioniserende strålingen har midlertidig ikke ført til høyere andel av ROS i den forurensede fisken.

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Abbreviations and definitions

CAT	Catalase
CEZ	Chernobyl exclusion zone
ChNPP	Chernobyl nuclear power plant
DCCs	Dose conversion coefficients
DNA	Deoxyribonucleic acid
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
NMBU	Norwegian university of life science
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UIAR	Ukrainian Institute of Agriculture Radiology

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1 Introduction

1.1 Background

To understand the risk of exposure to ionizing radiation, environmental studies on the long-term effects of radionuclide pollution are crucial. However, knowledge about the effect of long-term chronic exposure on the organism in the natural environment is still limited (Lerebours et al., 2018).

In contaminated lakes in the Chernobyl exclusion zone (CEZ) the fish have been chronically exposed to environmental radiation since the Chernobyl accident. After the Chernobyl accident, many nearby lakes were contaminated with radioactive fallout that included several short-lived and long-lived radionuclides such as ^{137}Cs and ^{90}Sr. Glubokoye is one of the most contaminated lakes after the accident (Alexakhin et al., 2006), and it is therefore interesting to see how affected the fish in Glubokoye is 34 years after the fallout. According to Sazykina, T., and Kryshev, A. (Cited byLerebours et al., 2018), fish are considered to be the most radiosensitive aquatic organism. The radioactive isotopes ^{137}Cs and ^{90}Sr are among some of the main dose contributors of the fission products (Kofstad & Pedersen, 2018). They have both long half-life (30 years and 29 years, respectively). ^{90}Sr emits ß-radiation, while ^{137}Cs emits both ß-radiation and γ-radiation that can affect the organism.

Ionizing radiation can lead to biological effects on organisms, indirectly or directly. Direct effects are when the energy from the ionizing radiation is deposited directly into the cell, and cause damage on the DNA. Indirect effects occur when ionizing radiation interacts with water in the organism and produces free radicals that can damage DNA and other cell constituents. Among wildlife, the dosimetry of fish is dependent upon external exposure from sediments and water in addition to internal dose if the radionuclides are accumulated in the fish. Right after the accident a dose of 400 µGy/h was assumed (Kryshev, 1998). In recent years, calculated total has varied between 50 µGy/h and 300 µGy/h (Gudkov et al., 2016). The dose is lower now than it was previously due to short-lived radionuclides have decayed out. From previous studies the morphological changes have been most frequently recorded in the reproductive system in fish from Glubokoye contaminated by the Chernobyl accident (Lerebours et al., 2018).

This study investigated the effects of ionizing radiation in the fish in the natural environment. It assesses whether there were any differences in oxidative stress enzyme activity in fish as well as histopathology in the liver and the kidney of pike (*Esox Lucius)* and rudd *(Scardinius erythrophthalmus*).

1.2 Objectives for this thesis

The main objective of this study was to determine radioactive isotope exposure levels, and to investigate whether the ionizing radiation cause adverse effects on the fish in Glubokoye. Due to chronic high exposure of ionizing radiation, it was hypotheses that:

H0: Exposure to radiation over three decades would negatively affect the general health of pike.
H1: The activity of antioxidant enzymes in the fish from Glubokoye is higher than in fish from the control lake.

2 Theory

2.1 Chernobyl nuclear power plant

On April 26, 1986, the most severe accident in the history of the world nuclear industry happened (Balonov et al., 2010). The accident at the Chernobyl nuclear power plant (ChNPP), then Sovjet – now Ukraine, resulted in release of large amounts of radioactive materials (Bennett et al., 2006). The reactor became unstable when a low power engineering test was conducted, and this caused thermal explosions and fires. The reactor burned for ten days (Choppin et al., 2013). From the burning reactor, radioactive substances like radioactive gases, condensed aerosols and large amount of fuel particles were ejected. The wind and the changing weather conditions caused contamination of local regions and trace contaminated throughout Eastern and Western Europe. The total release of radioactive substances includes many different nuclides, but the most significant of the radioactive substances are iodine-131 (^{131}I), cesium-137 (^{137}Cs), strontium-90 (^{90}Sr), and some plutonium radioisotopes because of their long half-life and effect right after the accident (Balonov et al., 2010).

Because of the direction of the wind over Chernobyl, radioactive gases and particles were carried in westerly and northerly directions. The material was deposited due to the rainfall. Radionuclides of short half-life were released in large amounts, and radionuclides with long half-life were released only in small amounts (figure 2.1 and 2.2). All countries of the northern hemisphere could measure trace concentrations of radionuclides (Nations, 2011).

The radioactive levels in water bodies nearby ChNPP were also affected. Right after the accident, the activity concentrations were high, but the levels of radionuclides decreased rapidly in rivers and lakes because of dilution, physical decay, and absorption of radionuclides by catchment soils (Balonov et al., 2010). Radionuclides, such as Cs and Sr bioaccumulate in the aquatic food chain, which led to significant activity concentrations in fish in the most affected areas. In some closed lakes, where there is no outflowing streams, both water and fish have been remained contaminated with ^{137}Cs and ^{90}Sr for more than 30 years (Alexakhin et al., 2006).

2.2 Glubokoye

The closed lakes in the Chernobyl affected areas are the most radioactive contaminated water bodies after the Chernobyl accident (Alexakhin et al., 2006). Glubokoye is located 10 km north of the ChNPP, and it is one of the most contaminated lakes from the accident (Murphy et al., 2011). The activity concentration in the water have been reported several times e.q., 7.57 Bq/L (^{137}Cs) and 77.7 Bq/L (^{90}Sr), and in the sediments were the activity on 40.706 Bq/kg (^{137}Cs) and 30.526 Bq/kg (^{90}Sr) (Fuller et al., 2017).

Several fish species exist in Glubokoye. Fish are a potential carrier of radionuclides from the aquatic environment to humans since fish are a major source of protein and nutrition (Arai, 2014). In previous studies, different fish species have been investigated several times, e.g., pike, perch, roach and rudd. Few years after the accident the highest specific activity of ^{137}Cs were recorded in predatory species, such as perch and pike. In the fish species that feed upon benthos and plankton organisms and vegetation such as silver bream, rudd, roach and bream were the lowest concentration of ^{137}Cs found (Ryabov, 2002).

Pike and rudd are from two different tropical levels. Rudd is an herbivore, and pike is a carnivorous fish-eating specie. The total dose of pike and rudd from Glubokoye have been determined, 0.4 Gy/year in pike and 0.47 Gy/year in rudd, this is similar to 45 µGy/h and 54 µGy/h (Kaglyan et al., 2019). The rudd from Glubokoye has a higher activity concentration than the pike from the same lake.

Earlier predicted dose rates in Glubokoye has been in a range between 13.0 to 294.0 µGy/h (Kaglyan et al., 2016). Negatively effect of ionizing radiation on the fish from the Chernobyl exclusion zone have earlier been reported. Dose-rate 1 mGy/d, equivalent to 41.66 µGy/h, is the threshold level for the appearance of first negative changes in the fish immune system. The organisms can adapt for radiation at lower dose-rates. Dose-rates 208.33 – 416.67 µGy/h are threshold levels for the development of adverse effects on the reproductive system (Kryshev et al., 2008; Kryshev & Sazykina, 2012; Sazykina, T. & Kryshev, A., 2003).

In figure 2.1 and 2.2 shows the deposition of ^{137}Cs and ^{90}Sr over the 30 km Chernobyl zone.

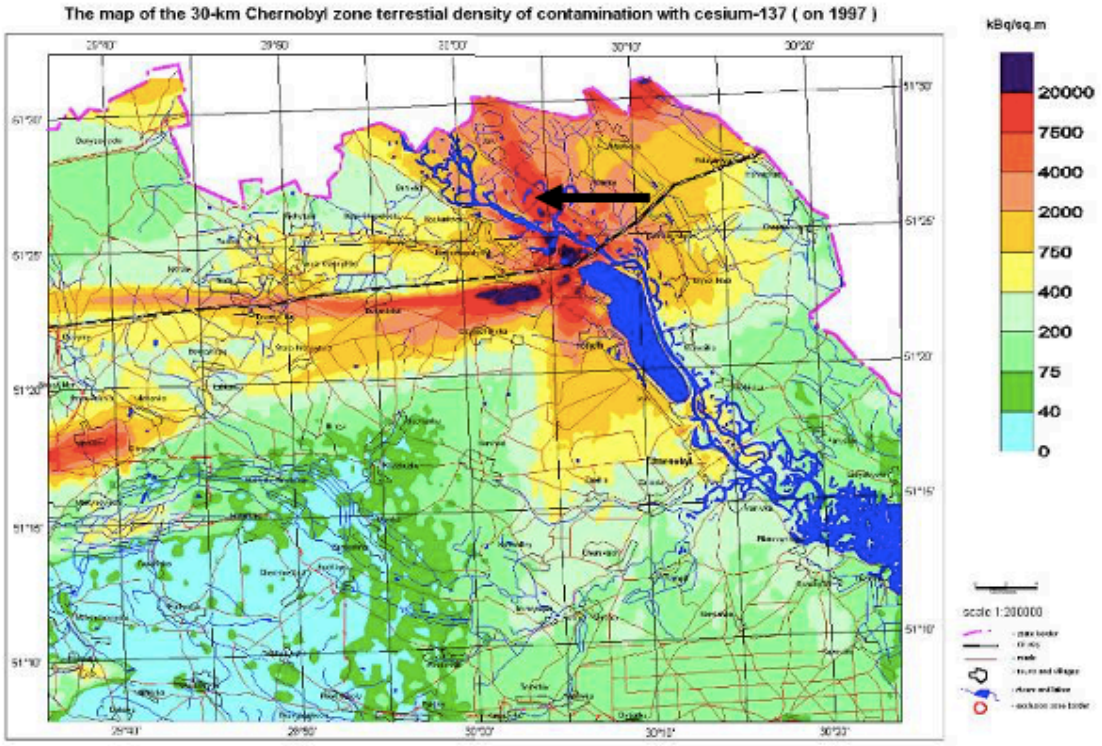


Figure 2.1 *Map of the contamination of ^{137}Cs over the 30 km Chernobyl zone. The lake, Glubokoye, is marked with an arrow (Kashparov V. et al., 2018).*

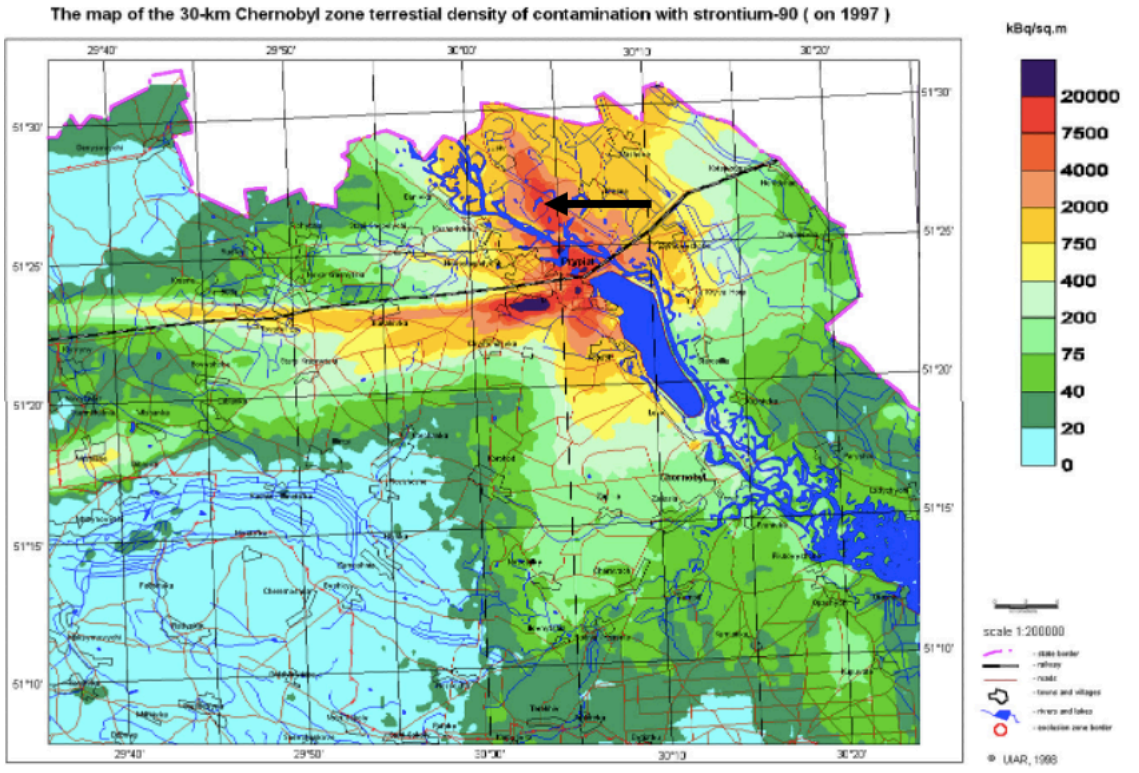


Figure 2.2 *Map of the contamination of ^{137}Cs over the 30 km Chernobyl zone. The lake, Glubokoye, is marked with an arrow (Kashparov V. et al., 2018).*

2.3 Radioactive isotopes

A radionuclide is an unstable form of a chemical element, and when the ratio between protons and neutrons changes, ionizing radiation is emitted. When the ionizing radiation interacts with an atom or matter the tightly bound electron is removed from the orbit, and the atom becomes charged or ionized (Publications, 2000). Ionizing radiation are either electromagnetic waves (gamma or X-rays) or particles like neutrons, beta, or alpha.

Radionuclides can be divided into natural and man-made sources. Natural sources of background radiation include cosmic rays and natural decay of radioactive isotopes such as uranium and daughters of uranium (Walker et al., 2016). Man-made sources include such as radionuclides released from nuclear explosions (weapon testing), reprocessing plants (Sellafield, Dounreay), nuclear accidents (Chernobyl, Fukushima), and non-nuclear sources like mines for the extraction of minerals, phosphorous and coal. Examples of man-made radionuclides are ^{137}Cs, ^{131}I, Pu, and ^{90}Sr.

To assess whether radioactive isotopes are harmful to organisms, three factors are considered; (1) intensity of the radioactive decay based on the mass and energy of the particles produced, (2) half-life to the isotope, and (3) biochemistry of the radioactive isotope. Radioactive isotopes of essential elements will follow the same biochemical pathways as their stable forms (Walker et al., 2016). Radionuclides in the environment can contribute to external radiation, internal radiation and chemical effects during uptake.

2.3.1 Radioactive decay

Radioactive decay occurs when an unstable radioactive nuclide spontaneously loses energy by emitting nuclear radiation. This spontaneous nuclear transformation is unaffected by pressure, temperature, chemical form. Half-life ($t_{1/2}$) is the time required for half of the radioactive atom decays. Some nuclides have a half-life of seconds, and some have half-life for millions of years (Choppin et al., 2013). The energy difference between the mother and daughter nuclide corresponds to the decay energy. This energy appears in the form of emission of helium nuclei, an electron or a positron, or electromagnetic radiation (Choppin et al., 2013).

Radioactive decay can be characterized by α-, β- and γ–radiation. Alpha-decay is the emission of helium nuclei, ß-decay is the creation and emission of an electron or a positron or the process of electron capture. Gamma-decay is the emission of electromagnetic radiation (photons) where the transition occurs between energy levels of the same nucleus (Choppin et al., 2013).

2.3.2 ^{137}Cs

Cs is the last stable element of the alkali metals in the periodic table. Cs are in the same period in the periodic table as potassium (K). This is why Cs will follow the same pathways as K when Cs are taken up by indiscriminatory from K, both from food and from the water. Similar to potassium ions, cesium is concentrated in the cytoplasm of the cells and thus accumulated in edible muscle tissue (Phillips & Russo, 1978).

In the environment, both stable Cs and radioactive Cs occurs. Stable Cs occurs mainly from erosion and weathering of rocks and minerals. It is also released into air, water, and soil through mining and milling of ores. Radioactive Cs is released into the air by nuclear power plants, nuclear accidents, and nuclear weapons testing. Stable Cs and radioactive Cs are chemically alike and will, therefore, behave similarly (Health, 2004).

^{137}Cs is among the most common heavy fission products (Wessells, 2012). With a half-life on 30 years, and a combination of high-energy radioactivity and chemical reactivity makes ^{137}Cs a particularly dangerous fission product (Wessells, 2012). When the radionuclide decays, it emits ß-particles, and both ^{137}Cs and its metastable nuclear isomer, barium-137m, emits γ-radiation of moderate energy (NCIthesaurus, 2007) (Figure 2.3).

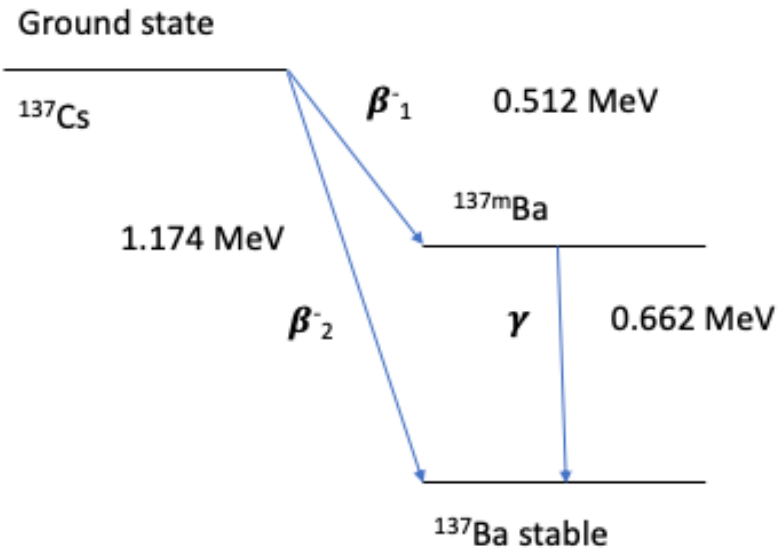


Figure 2.3 *^{137}Cs decay scheme*

The accumulation of ^{137}Cs in fish is very complex, and it leads to a wide difference in contamination levels according to water chemistry, fish types, size, and feeding patterns. The most important pathway of ^{137}Cs into fish is via food consumption (Smith et al., 2002).

2.3.3 ^{90}Sr

Sr is alkaline earth metal, and are in the same group as calcium, Ca. This is why the element exhibits biochemical behavior similar to calcium. Therefore is Sr a "bone seeker", it tends to accumulate in the bones and can replace calcium (Stigbrand et al., 2008).

Sr occurs both naturally and man-made. Stable Sr is found in rocks and soils and has four stable, non-toxic and non-radioactive isotopes (Pathak & Gupta, 2020). Anthropogenic Sr is produced from coal burning, processing of strontium compounds, and using phosphate fertilizer. There are different radioisotopes of Sr, but ^{90}Sr is the most dangerous Sr isotopes after a fallout. ^{90}Sr is a hazardous radioisotope due to its long half-life (29 years), and its ability to absorb in biological material (Pathak & Gupta, 2020). ^{90}Sr emits β-particles, and a decay scheme for the isotope is shown in figure 2.4.

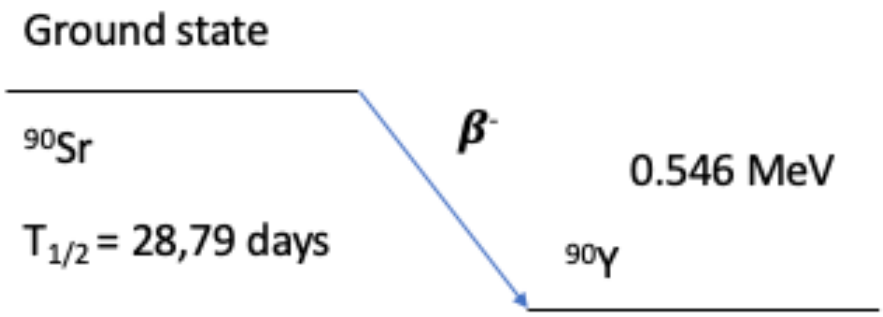


Figure 2.4 *^{90}Sr decay scheme*

According to Schiffman (Cited by Agnedal, 1966) the uptake of Ca and Sr in the fish occurs directly from the water. The main uptake occurs via the gills and the mucous membranes in the mouth. Only one-tenth is taken up through the food. The concentration of ^{137}Cs in the water is often lower than the concentration of ^{90}Sr, because ^{137}Cs binds easily to particles and organic matter such as sediments, while ^{90}Sr is more mobile and thus more accessible in the water. ^{90}Sr is the main dose-forming radionuclide for fish in the majority of water bodies within CEZ (Kaglyan et al., 2016).

2.4 Effects of ionizing radiation on fish

Fish is considered to be the most radiosensitive aquatic organism and have been continuously exposed to ionizing radiation in freshwater systems at CEZ since the accident (Sazykina, T. & Kryshev, A. I., 2003). The accumulation of radionuclides in fish are more intensive in the early periods of life (Kaglyan et al., 2016). In the common rudd it is previous observed increase in the specific activity of ^{90}Sr and ^{137}Cs, with preferred ^{90}Sr accumulation. There are observed higher ^{90}Sr activity than ^{137}Cs in the first years of the predatory fish, e.g., pike. But, the following years the specific activity of ^{137}Cs begins to exceed, probably with gradual prevalence of small fish in its feeding (Kaglyan et al., 2016).

Fish can be exposed to radiation in several ways; via the gills, exposure by ingestion, or exposure from water and sediments. The effect of the radionuclides on fish depends on three main factors; the external and internal dose and the chemical effects. Since water screens for ionizing radiation, the external dose is dependent on the distance from the radionuclide that emits ionizing radiation to the aquatic organism. The harmful effects of radiation depend on

the composition of matter and the amount of energy deposited by the radiation (Choppin et al., 2013). Ionizing radiation induces a variety of damages in the DNA, both direct and indirect (O'Neill & Fielden, 1993).

Indirect effects of ionization radiation arise when the penetrating radiation interact with water molecules, the major constituent of the cell, and other organic molecules in the cell (Desouky et al., 2015). Free radicals are then produced. Free radicals are molecules with an unpaired electron in the structure and are, therefore, very reactive (Desouky et al., 2015). They play an essential role in the origin of life and biological evolution, implicating their beneficial effects on the organisms (McCord, 2000). However, free radicals can interact with DNA and cause molecular structure damage. Inflammation is a biological response of the immune system which can be triggered by a variety of factors, like damaged cells, toxic compounds, or irradiation (Chen et al., 2018). It is the primary immune response to eliminate pathogens or to repair tissue damage (Dinh et al., 2014). Superoxide and hydrogen peroxide are reactive oxygen species (ROS), and they are produced by immune cells in order to kill pathogens (Crowley, 2014).

Direct effects arise when the energy from the ionizing radiation is deposited directly into the cell and cause damage on the DNA, as a single- or double-strand break. Figure 2.5 shows an overview of the outcome when the energy from ionizing radiation is deposited directly into DNA. If an organism is exposed to radiation, living cells can be damaged, causing death in some of them and modifying others. Most of the organs and tissue are not affected by the loss of a considerable number of cells. However, if the number of lost cells are high enough, there will be observable harm to organs that may lead to death (Nations, 2011). The damage can be repaired successfully, repaired unsuccessfully, or lead to cell death. This is called deterministic effect and are distinguished from the stochastic effect that is cancer or genetic effects (Commission, 2014).

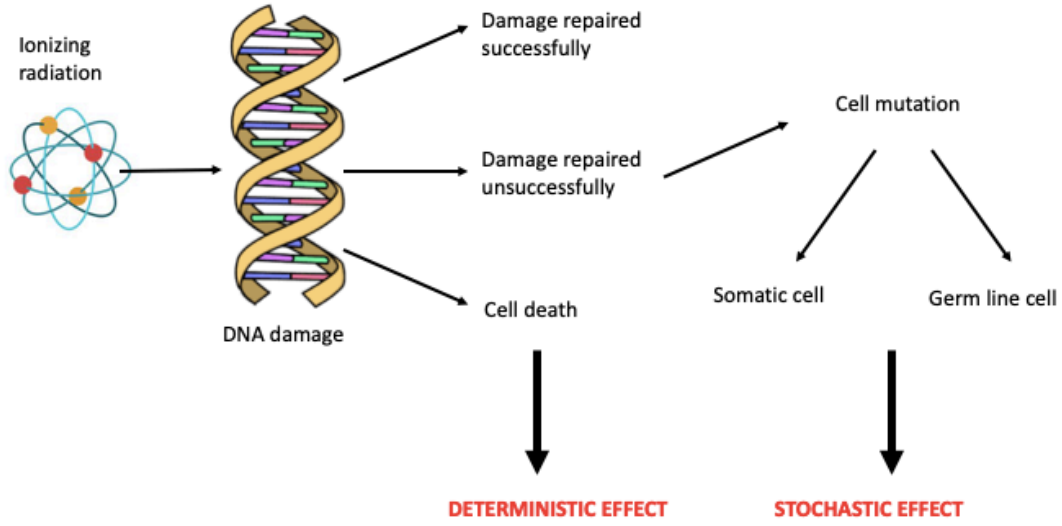


Figure 2.5 *Principle outline describing the outcome when the energy from ionizing radiation is deposited directly into DNA.*

2.5 Histopathology

Histopathology is the study of tissue changes by disease and injury. According to Adams, (cited by Kaur et al., 2018), histopathological changes are used as biomarkers to evaluate the overall health of fish exposed to contaminants. Specific vital organs which are responsible for fundamental functions such as accumulation and biotransformation of xenobiotics, excretion, and respiration in fish can be examined, by analyzing kidney and liver (Gernhöfer et al., 2001).

The liver is the organ that is most susceptible to damage caused by various toxins, and it is associated with detoxification and biotransformation due to its position, function, and blood supply (Camargo & Martinez, 2007). A healthy fish liver has a structure that consists of a continuous mass of cells called hepatocyte. The liver cells have a polygonal form with homogenous cytoplasm and either eccentric or centrally located distinct nuclei (Lakshmaiah, 2016). The kidney is a vital organ of the body, and proper kidney function is to maintain the homeostasis. It is also involved in the removal of waste from the blood (Tayel et al., 2014). The splenic tissue in the fish exerts an essential role for the proper functioning of several host defense system mechanism (Seker et al., 2011).

The spleen of fish consists of two types of tissue that have different functions: white pulp and red pulp (Fänge & Nilsson, 1985). Pathogens and cellular debris, as well as aging erythrocytes, are effectively removed from the blood by red macrophages. There are plenty of macrophages in the red pulp (Bronte & Pittet, 2013). The white pulp consists of lymphocytes (lymphatic tissue), and white blood cells are produced here. It consists of B lymphocytes and T lymphocytes (Mebius & Kraal, 2005).

In previous studies, abnormalities in fish exposed to ionizing radiation have been observed. In liver cells of the freshwater fish, *Oreochromis mossambicu* exposed to 3 – 300 mGy, congestion of blood vessels, structural alteration, cellular swelling, vacuolation and necrotic liver cells have been observed (Bukhari et al., 2012). Abnormalities in the spleen tissue has been observed in previous studies. Xu et al. (2008) observed that the white and red pulp areas became difficult to identify, and that the amount of red blood cells are reduced in zebrafish which had a virus infection.

2.6 Oxidative stress and antioxidant defense

Many organisms have an excess of water in the body, this is why water (H_2O) usually absorb the ionizing radiation that penetrates the material. The water is reduced to harmful reactive oxygen species (ROS) e.g., superoxide anion radial (O_2^-). Reactive oxygen species are free radicals, and a high proportion of ROS can cause oxidative stress in the organism. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defense (Halliwell, 1994). To prevent oxidative stress the organism has evolved antioxidant defense mechanisms. The antioxidant enzymes catalyze reactions to neutralize free radicals and reactive oxygen species. The major antioxidant enzymes in an organism are the superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase enzymes (Finosh & Jayabalan, 2013)..

A reduction of water in the cell may lead to formation of harmful superoxide anion radical (O_2^-). When superoxide anion radical is reduced, hydrogen peroxide (H_2O_2) is produced. This is not a free radical, but it is chemically more active than molecular oxygen (Lushchak, 2015). By accepting one electron, the hydrogen peroxide is split into hydroxyl radical (·OH), and hydroxyl anion (OH). A water molecule is produced if the hydroxyl anion interacts with a proton and an electron (Lushchak, 2015).

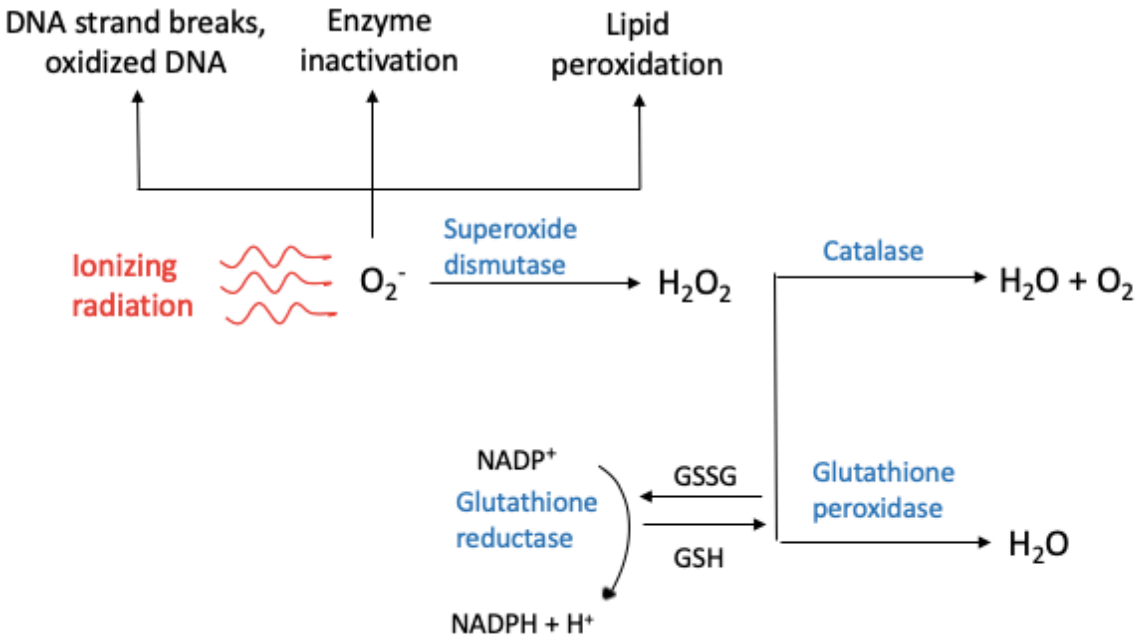


Figure 2.6 *Illustrates the relationship between superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as the potential damage of ROS (Carvan & Di Giulio, 2015).*

Measures of oxidative stress are biomarkers of exposure to ionizing radiation, while histopathological lesions are biomarkers of effects (Kelly & Janz, 2009).

2.7 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is a group of metalloenzymes (Kangralkar et al., 2010). It is the first detoxification enzyme and most powerful antioxidant in the cell, and acts as a component of the first-line defense system against reactive oxygen species (ROS) (Ighodaro & Akinloye, 2018). SOD catalyzes the dismutation of the harmful superoxide anion (O_2^-) into less hazardous hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Ighodaro & Akinloye, 2018).

Increased SOD activity indicates high level of superoxide anion, and the fish are exposed to higher levels of free radicals. Previous study, (Liu et al., 2013), indicates that the SOD activity is stable for several months at 0°C. Equation 1.1 shows how SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2.

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \text{Equation 1.1}$$

Catalase (CAT) or glutathione peroxidase (GPx) degrade hydrogen peroxide after SOD has dismutated superoxide into hydrogen peroxide (Nandi et al., 2019).

2.8 Catalase (CAT)

Catalase (CAT) catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen (O_2) and two molecules of water (H_2O). Catalase is a ubiquitous antioxidant enzyme present in most aerobic cells (Ighodaro & Akinloye, 2018), and catalyzes the reaction of hydrogen peroxide into water and oxygen. Overheating can inactivate catalase (Johansson & Borg, 1988). It is therefore essential to keep the enzyme cold during sample preparation and assaying. The enzyme is also very unstable at high dilution, thus should the samples be diluted immediately before the analysis (Herbert, 1955). In equation 1.2 is the catalytic activity of CAT shown.

Catalytic activity:

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \qquad \text{Equation 1.2}$$

Increased CAT activity indicates that there are higher levels of hydrogen peroxide (H_2O_2) in the organism, and the organism is exposed to higher levels of free radicals, which can lead to oxidative stress. The CAT activity is stable for several month at 0°C (Liu et al., 2013).

2.9 Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) is an enzyme that breakdown hydrogen peroxides (H_2O_2) to water (H_2O) (Ighodaro & Akinloye, 2018). These antioxidant enzymes have the capacity to capture free radicals to prevent lipid peroxidation and maintain intracellular homeostasis as well as redox balance (Mulgund et al., 2015). GPx reduces hydrogen peroxide by using GSH as an electron donor. The electron is transferred via the catalytic residue selenocysteine (Ismail et al., 2010).

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \textit{R-O-H} + \textit{GSSG} + \textit{H}_2\textit{O} \qquad \text{Equation 1.3}$$

$$\textit{GSSG} + \textit{NADPH} + \textit{H}^+ \xrightarrow{\text{GR}} \textit{2GSH} + \textit{NADP}^+ \qquad \text{Equation 1.4}$$

Higher levels of GPx in the organism indicates higher levels of free radicals, like hydrogen peroxide (H_2O_2). Previous studies has shown that GPx activity in tissue samples is stable over a couple of weeks when stored at -80 °C (Jung et al., 1993).

2.10 Glutathione reductase (GR)

Glutathione reductase (GR) is a flavoprotein and catalyzes the reduction of glutathione disulfide (GSSG) to glutathione (GSH). Higher amounts of hydrogen peroxide indicate higher amount of GR, since GSSG is produced when GPx breaks down hydrogen peroxide (H_2O_2) to water (H_2O). The reaction is essential for the maintenance of glutathione level (Carlberg & Mannervik, 1985). In this reaction, NADPH is a coenzyme which increases the rate of the chemical reaction. Glutathione (GSH) is a tripeptide which is widely distributed in plants, animals, and microorganisms (Ahmad, 2012). The antioxidant has a major role as a reductant in the oxidation-reduction process and serves in detoxification and several other cellular functions of great importance (Carlberg & Mannervik, 1985). In equation 1.5 are the reaction shown.

$$\textit{GSSG} + \textit{NADPH} + \textit{H}^+ \xrightarrow{\text{GR}} \textit{2GSH} + \textit{NADP}^+ \qquad \text{Equation 1.5}$$

An increased proportion of GR in the cell indicates an increased ratio of GSSG/GSH. High levels of GSSG indicates oxidative stress. The GR activity in the tissue is quite stable over a few weeks when the tissue are stored at -80 °C (Jung et al., 1993).

In previous studies, antioxidant enzymes like SOD, GPx and GR were significantly induced by 2.5 and 5 Gy gamma radiation at various embryonic stages of *K. marmoratus* (Rhee et al., 2012). Hardmeier et al. (1997) suggested that antioxidant enzymes have protective roles against radiation damage. If oxidative stress is induced in an organism, a subsequent antioxidant defense system will also be induced to detoxify oxidative stress-triggered radicals (Rhee et al., 2012).

3 Materials and methods

In two lakes of different radionuclide activity concentration within the CEZ, two types of fish were studied (pike and rudd), to identify effects upon exposure to ionizing radiation. This study is based on fish from two different trophic levels – herbivorous fish (rudd) and carnivorous fish (pike). The fish of the same species from both the control lake, Kashovka, and the contaminated lake, Glubokoye, were sampled at the same time, but at different times for each specie. In March 2019, nine pike from the control lake, Kashovka, and nine pike from the contaminated lake, Glubokoye were caught. In May 2018, ten rudd from Kashovka, and ten rudd from Glubokoye were caught. After measuring the length and weight, the fish were dissected in field by the team from Ukrainian Institute of Agriculture Radiology (UIAR) and (NMBU).

To obtain information on activity concentration of radionuclides in fish, samples of muscle and bone were collected and stored cold before freezing at -20°C. To obtain information of enzyme activity, samples of liver and kidney were snap-frozen in liquid nitrogen and stored in dry shipper with liquid nitrogen during transport. In addition, samples of liver, kidney and spleen of fish were fix on formalin to study histology.

Water samples were collected to obtain information of general water quality. Temperature and pH were measured in field by the NMBU team. Large water samples were filtered by 0.45 µm membrane filter before collecting 1 liter from lake Glubokoye and 20 liters from lake Kashovka, in addition 50 mL small water samples were collected for general ion composition and metals.

The 50 mL water samples, the fixed tissue samples and the dry shipper with liver and kidney samples were transported to NMBU, Ås. At the isotope laboratory, NMBU, the liver and kidney samples were stored at -80°C in Eppendorf vials until analysis. Analysis of 50 mL water samples were done by personnel at NMBU isotope laboratory, while analysis of enzyme activity and the histology were done as a part of this MSc study. Both water samples and fish samples were collected to determine the activity concentration of radionuclides. The samples were transported to UIAR in Kiev, and the activity concentration of both ^{90}Sr and ^{137}Cs in the samples were measured there.

3.1 Exposure characterization

To ensure that the water quality in the lakes was relative similar and not effecting the fish differently, the general water ion composition was analyzed at NMBU using ICP-MS (Agilent 8900) after acidification with 5% ultrapure HNO_3.

To determine the difference in exposure to radionuclides between lakes, the gamma activity of ^{137}Cs in water and the fish muscle were determined with a multi-channel analyzer ASEPEC-927 and GEM-30185 detector at UIAR. The ^{90}Sr activity in fish bone were determined in bone ash directly using SEB-01-07 beta spectrometer (AKP, Ukraine), while the activity of ^{90}Sr in water samples were determined by standard radiochemical analysis. The activity of radionuclides is presented as activity per liter water or per kg wet fish tissue. The results were used to calculate internal and external doses.

3.2 Dose calculation

Calculation of dose-rates were performed using ERICA by Justin Brown at the Norwegian Radiation Protection Authority (DSA). The "add organism" module in ERICA was used to generate the internal and external dose conversion coefficients (DCCs). In appendix A the mass and dimensions used in the "Add organism" function in ERICA are listed.

The fish used in the calculation had a large weight distribution, representative masses were therefore chosen to cover the range of the fish masses. The geometry which was closest to the actual measured mass was then selected by applying a DCC. For example, a pike mass of 0.4 kg is given, and DCCs corresponding to 0.5 kg are applied. In ERICA an equivalent ellipsoid shape is used to represent a given organism (ratio of length to breadth to height). For pike, a ratio of length:height:width of 1:0.14:0.14 was assumed. For rudd, which have a different shape, ratio of 1:0.333:0.167 was assumed.

The measured activity concentration of ^{137}Cs and ^{90}Sr, for muscle and bone respectively (wet weight), was converted to equivalent whole-body activity concentrations. The bones of the fish are a small proportion of the total weight of the fish relative to the muscle. To estimate the average activity concentration for the whole fish based on ^{90}Sr, a conversion factor of 0.140 was used. To estimate an average activity concentration for the whole fish based on

^{137}Cs in the muscle, a conversion factor of 1.10 was used. These conservation factors are given by Yankovich et al. (2010).

The equation used for calculation of the whole-body dose-rates are listed in appendix A. The total dose-rate is calculated by summing the internal and the external dose-rates.

Since there were only samples of water and fish collected in the project, the external dose-rate is just calculated based on the contribution from the water, and not the dose-rate from sediments. To evaluate the contribution from sediments, previous published dose rates in Glubokoye have been included in the discussion.

3.3 Equipment, reagents and solutions

An overview of the equipment used is found in table B.1, and an overview of the solutions and reagents used is found in table B.2. All tables are found in appendix B.

The 20 mM phosphate buffer pH 7.4 used to homogenize the fish samples were prepared by dissolving 2.8 g of $NaH_2PO_4 \cdot H_2O$ in 100 ml ddH_20 (solution A) and dissolving 3.5 g $Na_2HPO_4 \cdot 2H_2O$ in 100 ml ddH_20 (solution B). The next step was to dissolve 4.5 g of NaCl in 500 ml ddH_20 then add 1.0 ml of 0.5 mM EDTA, 0.5 ml of Triton, 20.25 ml of solution A and 4.75 ml of solution B. The 20 mM phosphate buffer were autoclaved.

3.4 Histology

Histology samples of pike liver, kidney and spleen from Glubokoye were examined to assess if there were any possible effect on the fish physiology caused by ionizing radiation. Proper sample preparation for histology specimens is essential for making useful histological sections of the tissue samples.

Due to staining the cytoplasm and the nuclei can be separated. The Hematoxylin staining will show blue colored nuclei and pink to red-colored cytoplasm. Light microscope with bright field optics (Leica DM6, Germany) was used to identify the nuclei and the cytoplasm, and images were taken. By identifying morphological changes in the contaminated tissue, it is possible to see if the ionizing radiation has affected the cell. Five samples of liver, kidney, and spleen from Glubokoye and six samples of liver, kidney, and spleen from Kashovka were

examined. The tissue samples were fixed in 10% buffered formalin for 12 hours immediately after dissection. Subsequently, samples were dehydrated in graded alcohol baths as described in subchapter 3.4.1.

3.4.1 Preparation of formalin

The PFA powder and fumes used for the fixation protocol are very toxic and was therefore handed carefully in the field. The solution was made in the laboratory by adding 16 g PFA powder (4 % PFA) to 200 ml dH_2O DEPC, then adding 2 ml of 1 M NaOH. This solution was dissolved at 60 °C. After the solution had cooled down 40 ml of 10 x PBS was added, and the pH was adjusted to 7.4 with concentrated HCl. The volume was adjusted to 400 ml. The samples were aliquoted in 50 ml and placed in the freezer (-20 °C) until used. The solution was diluted twice with the DW.

Tissue samples were transferred to tubes with 2 % PFA and incubated lying over the night at 4 °C. The samples were thoroughly washed twice in 25 ml 1 x PBS with 0.1 % Tween-20 (PBST). Subsequently, the solution was poured off and 30% EtOH was filled up. The tissue samples were then incubated lying for one hour at room temperature. Afterwards, 50% EtOH was added, and the samples were incubated lying for two hours at room temperature. Finally, 70% EtOH was added and the samples were incubated lying for two hours. The samples were then stored in -20°C.

After storage, fixed samples were infiltrated in paraplast (Sakura, Japan) at 60 °C, using a Tissue-Tek VIP Jr. automatic embedding machine (Sakura, Japan). Using a rotary microtome (Leica LM2255, Germany), 10 µm-thickness histological sections were produced and placed on superfrost glass slides (VWR, USA). All the histological sections were subsequently placed on 42 °C warming plates for 12 hours to adhere firmly on the glass slides. The sections were stained following “Meyer’s method” using hematoxylin and eosin.

To deparaffinize, the sections were submerged in Histoclear (Cell path, England) twice for 10 minutes. For rehydration, the sections were soaked in 300 mL absolute alcohol twice, and then transferred to 95% alcohol for 2 minutes. At the end of the rehydration process the sections were soaked in 70% alcohol for 2 minutes, before they were rinsed briefly in distilled water.

For staining, samples were placed in Mayer hematoxylin solution for 8 minutes and then washed in warm running tap water (30 °C) for 10 minutes. To remove superfluous Mayer hematoxylin solution, the histological sections were briefly rinsed in distilled water, and then dipped ten times in 95% alcohol. The sections were counterstained in eosin-phloxine B solution for 1 minute.

Furthermore, the sections were dehydrated in 95% alcohol for 5 minutes and twice in absolute alcohol for 5 minutes. Finally, sections were cleared in Histoclear twice for 5 minutes and mounted in DePeX (Sigma-Aldrich, USA) with xylene based mounting medium.

3.5 Method enzyme activity

The enzyme activity in the kidney and the liver was determined to assess whether the ionizing radiation affects ROS and oxidative stress in the fish. The essential antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were measured, and the total activity was calculated.

3.5.1 Sample preparation for determining enzyme activity

The following procedure was performed with samples on ice, to prevent temperature related loss of enzyme activity. The protocol was identical for all the different organ samples in the both fish species. A small proportion of the sample material was taken from the freezer (-80 °C) and weighted. The aliquots of pike samples were weighted accurately to about 58.2 ± 4.15 mg (liver) and 64.0 ± 13.49 mg (kidney), and the aliquots of rudd kidney samples were weighted accurately to about 40.1 ± 15.07 mg. After thawing the fish samples on ice, the fish samples were added to a vial containing six 1/8” (3.175 mm) stainless-steel grinding beads (Lysis Beads-Matrix S), with 1 ml of ice-cold 20 mM phosphate buffer 7.4 pH. Subsequently samples were homogenized in a FastPrep-24^{TM} 5G homogenizer (Illkirch, France) (figure 3.1) at the speed of 10 m/s for 10 seconds without cooling, and immediately transferred back on ice.

Figure 3.1 *Shows the FastPrep-24™ 5G homogenizer used to homogenize the fish samples.*

To reduce the handling of samples before analysis of CAT, GPx, and GR, the homogenates for all the samples were aliquoted into two different centrifuge tubes - approximately 500 µl to each tube. One aliquot used for pretreatment for SOD and one used for pretreatment for the other enzymes. CAT, GPx, and GR are mainly present in cytosol. The homogenates for these enzymes were centrifuged with an Allegra 64R Centrifuge (Indianapolis, USA) at 10.000xg for 15 minutes at 4°C, and the supernatant was carefully collected (Figure 3.2). Aliquot samples were prepared for each enzyme and stored at -80°C. All samples were analyzed within one month after homogenization.

Since SOD is present in both cytosol and the mitochondria, the homogenates for SOD was sonicated to release the mitochondrial SOD. Homogenates for SOD were sonicated with Ultrasound sonication (Danbury, USA) on ice. Sonication was done twice with a 20 % amplitude for 10 seconds, separated by 60 seconds interval for cooling on ice. The homogenates for SOD were then centrifuged with an Allegra 64R Centrifuge (Indianapolis, USA) at 10.000xg for 15 minutes at 4°C. The resulting supernatant was carefully collected (Figure 3.2). Aliquots were made for every sample. The supernatant is assumed to be stable for at least one month when stored in -80 °C.

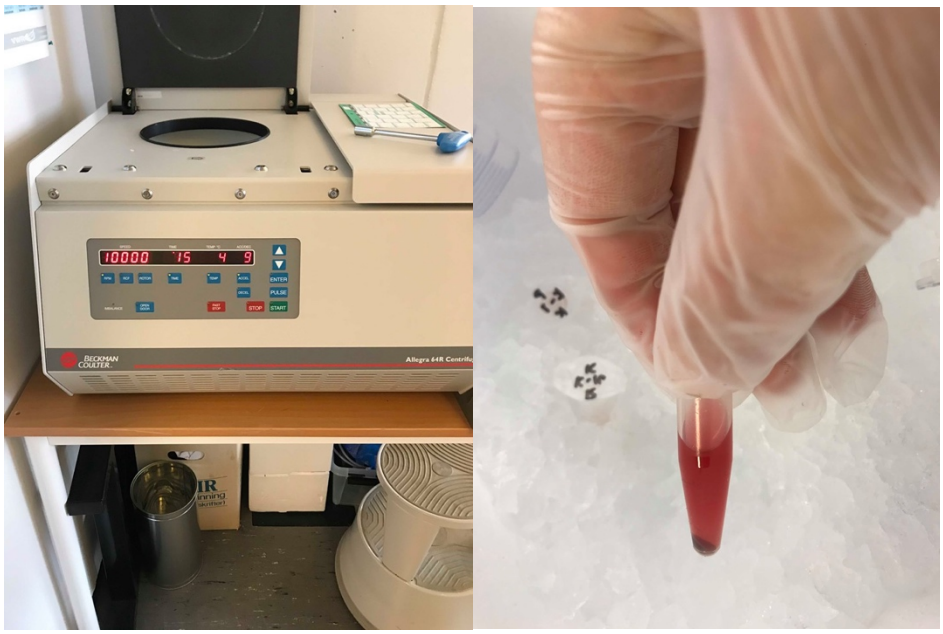


Figure 3.2 The *picture to the left shows the centrifuge that was used, Allegra 64R Centrifuge. The picture to the right is a picture of a kidney sample of pike after the centrifugation. The supernatant was carefully collected for measurements of the enzyme activity.*

The protein concentration in the supernatant for each enzyme was determined by using NanoDrop 2000, A280 (Wilmington, USA) (Figure 3.3) before the enzyme activity was analyzed.

In the first analysis of the enzyme activity, all the samples were diluted ten times before the protein concentration was measured. The analyses indicated that the enzyme activity of SOD, CAT, GPx, and GR in the pike liver correlated with the protein concentration. A correlation factor, R^2, higher than 0.4, indicates correlation. To minimize the influence of protein concentration of the results, all the samples were diluted to obtain a protein concentration between 0.5 to 0.8 mg/ml before determination of enzyme activity. Thus, the protein concentration was between approximately 0.5 and 0.8 mg/ml during the final analyzes of SOD in the pike liver. The same range in the protein concentration was used when measuring CAT and SOD activity in the kidney samples of pike and the kidney samples of rudd. As a blank measurement, the 20 mM phosphate buffer pH 7.4 was used.

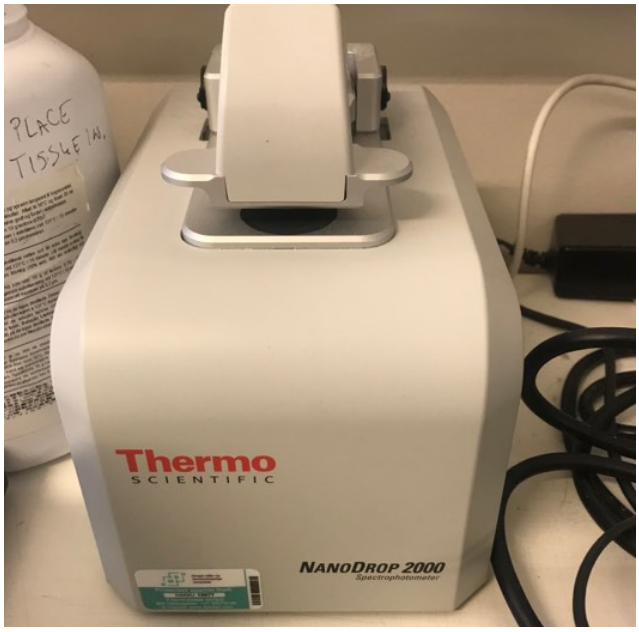


Figure 3.3 *The NanoDrop 2000 instrument used to measure the protein concentration in the fish samples after centrifugation*

3.5.2 Determination of enzyme activity

The enzyme activity in the fish samples was performed in precisely the same way for both fish types and the liver and kidney samples. All the samples were placed on ice during assaying. To measure the enzyme activity, assay kit for each specific enzyme was used. The enzyme kit used for each specific enzyme has a limited number of samples that can be analyzed.

3.5.2.1 Superoxide dismutase (SOD)

For determination of the superoxide dismutase (SOD) activity in the liver and the kidney samples, an assay kit purchased from Sigma-Aldrich was used. An indirect, colorimetric method is utilized in the SOD assay kit-WST. The method is based on the xanthine oxidase (XO) producing O_2^-, which in turn reacts quantitatively with and converts WST-1 to WST-1 formazan. This means that the absorbance at 440 nm is proportional to the amount of superoxide anion. The reaction rate is inhibited by SOD activity which consumes O_2^-. The inhibition by SOD activity can thus be quantified by measuring the decrease in the color development at 440 nm (Sigma-Aldrich, 2018). The principle of the SOD assay kit is shown in figure 3.4.

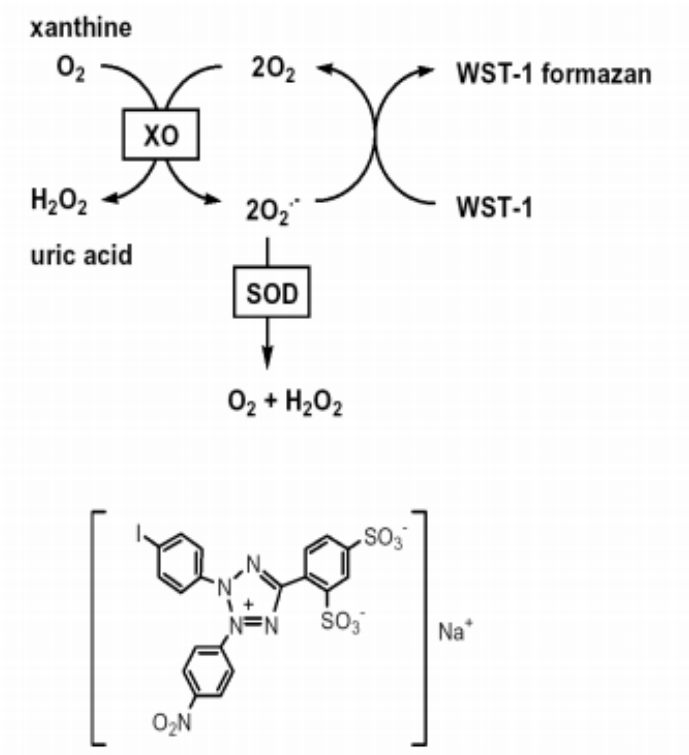


Figure 3.4 *The principle of the SOD assay Kit* (Sigma-Aldrich, 2018).

The exact dilution depends on each sample and suggests maintaining the inhibition rate between 20% -90% (Sigma-Aldrich, 2018). In figure 3.5 an example of the inhibition curve is shown. The absorbance at 25 minutes was chosen for further calculations. In the first analysis of SOD activity, the liver samples were diluted 10 times with the sample buffer before the protein concentration was determined. For assaying the samples, the samples were diluted five times after the protein concentration was determined. Based on the developed method in this study, a protein concentration within the interval (0.5 and 0.8 mg/ml) was desired. All the liver and kidney samples for SOD activity were therefore diluted differently. The dilution depends on how high enzyme activity there is in the samples. For assaying, the liver samples were diluted five times, and the kidney samples were diluted two times.

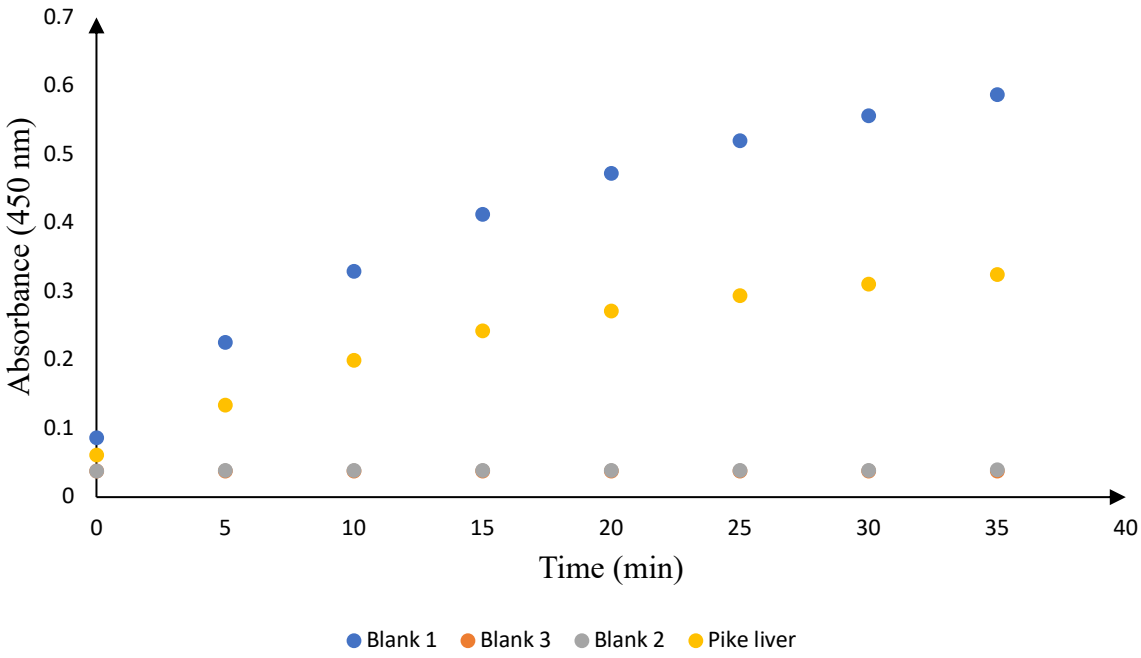


Figure 3.5 *Inhibition curve for a pike liver sample from Glubokoye. The absorbance when the incubation time was at 25 minutes was chosen for further calculations.*

The main modification of the protocol, was using 25°C as an incubation temperature for the fish samples instead of 37°C. In the sample wells, 20 µL of sample and 220 µL of reagents from the assay kit were added. In the blank samples 240 µL of reagents from the assay kit were added. The 96-well plate reader was then mixed thoroughly, and the absorbance was read at 450 nm every five minutes after the enzyme working solutions were added. A Multiskan Ascent 96 well plate reader (USA) was used.

The SOD activity inhibition rate (%) were calculated using equation 1.6. Blank 1 consist of superoxide anion, which is the reagent with the highest absorbance. Blank 3 measures the background absorbance of the reagents and is subtracted from blank 1. The corrected absorbance in blank 1 is used in equation 1.6. Blank 2 measures the background absorbance in the samples and is subtracted from the absorbance in the samples. This is the corrected absorbance in the sample and used to calculate the inhibition rate. To calculate the inhibition rate (%), the corrected absorbance in the sample is subtracted from the corrected absorbance in blank 1. The result is then divided on the corrected absorbance in blank 1 and multiplied with 100.

SOD activity (inhibition rate %) =

$$\left\{\frac{corrected\ absorbance\ blank\ 1 - corrected\ absorbance\ sample}{corrected\ absorbance\ blank\ 1}\right\} * 100 \qquad \text{Equation 1.6}$$

To normalize the SOD activity to the protein concentration, the SOD inhibition rate is divided on the measured protein concentration. Finally, the dilution factor is multiplied to calculate the SOD activity in the undiluted sample (equation 1.7). The normalized SOD activity is thus given as U/mg protein. On unit (U) is the amount of SOD that inhibits the rate color formation by 50 %.

$$\text{Normalized SOD activity} = \frac{SOD\ activity}{protein\ concentration} \text{ x sample dilution} \qquad \text{Equation 1.7}$$

Increased SOD activity indicates high level of superoxide anion, and the fish are exposed to higher levels of free radicals. For more details and information, see the handbook to the SOD assay kit-WST (Sigma-Aldrich, 2018).

3.5.2.2 Catalase (CAT)

For determination of the catalase (CAT) activity in the fish, an assay kit purchased from Cayman Chemical was used. The peroxidic function of CAT is used to determine the enzyme activity in this kit. The hydrogen peroxide (H_2O_2) binds to the CAT enzyme and produce water (equation 1.8). Methanol (CH_3OH) reacts with the complex, CAT-H_2O, and produce water and formaldehyde (CH_2O) (equation 1.9) (Wheeler et al., 1990). Is a colorimetric method where the formaldehyde produced is measured with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) (Cayman Chemical, 2017). The enzyme samples were kept on ice during sample preparation and assaying, according to manufactures recommendations.

$$CAT + H_2O_2 \rightarrow CAT\text{-}H_2O \qquad \text{Equation 1.8}$$

$$CAT\text{-}H_2O + CH_3OH \rightarrow CAT + 2H_2O + CH_2O \qquad \text{Equation 1.9}$$

In the first analysis of CAT activity, the liver samples were diluted ten times before the protein concentration was determined, and before assaying the samples the liver samples

were diluted 7.5 times further using the catalase sample buffer. The analysis indicated that the enzyme activity of CAT in the pike correlated to the sample protein concentration. Since the enzymatic kit only can analyze a certain number of samples, only the kidney samples for pike and rudd were analyzed with a protein concentration within the interval, 0.5 to 0.8 mg/ml. Before assaying the samples, the kidney samples were diluted five times with the catalase sample buffer after the protein concentration was determined.

In the sample wells, 20 µL of sample and 130 µL of reagents from the assay kit were added. In the blank and standard wells, 150 µL of reagents from the assay kit were added. The reaction was initiated by adding 20 µL of diluted Hydrogen peroxide and incubated on a shaker for 20 minutes at room temperature. To terminate the reaction, 30 µL Potassium Hydroxide was added to the wells. Subsequently, 30 µL of Catalase Purpald was added to the wells. The 96-well plate was then incubated on a shaker for 10 minutes at room temperature. Subsequently, 10 µL of Catalase Potassium Periodate was added to the wells and incubated for five minutes in room temperature. The absorbance was then read at 540 nm using a Multiskan Ascent 96 well plate reader (USA). To obtain reproducible results, the amount of CAT added to the well should result in activity between 2-35 nmol/min/ml.

For determination of the reaction rate, a formaldehyde standard curve was made by plotting the corrected absorbance of standards as a function of final formaldehyde concentration (Figure 3.6). A standard curve shows the linearity of the assay. The correct absorbance of the standards is calculated by subtracting the average absorbance of standard A from itself and all the other standards. Standard A was also subtracted from the samples.

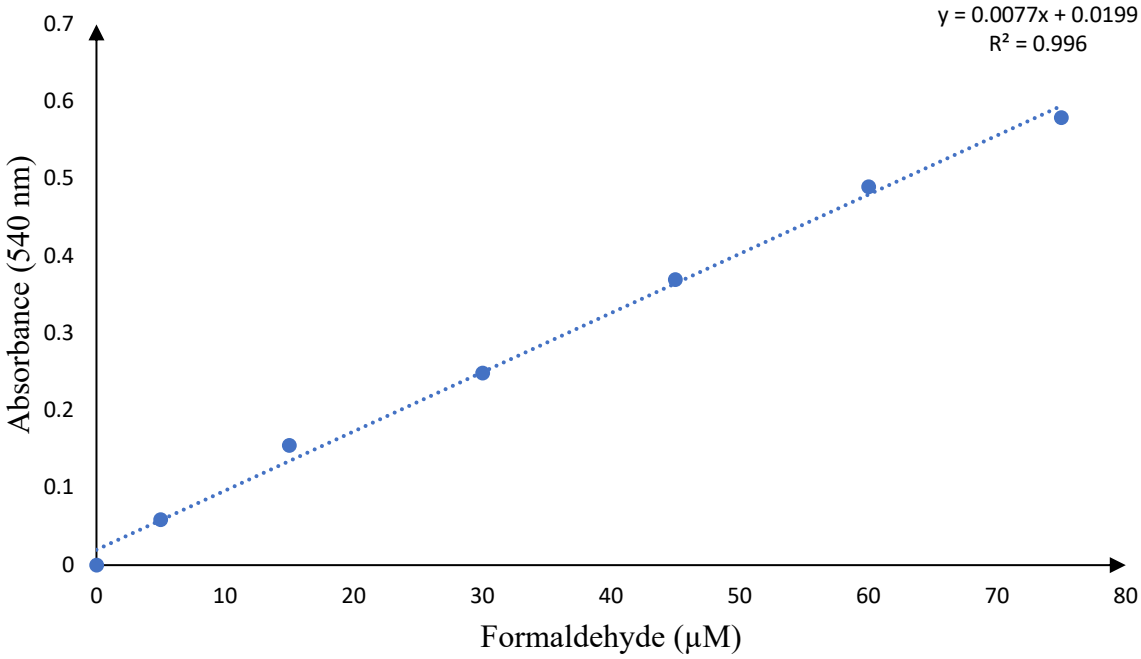


Figure *3.6* *Formaldehyde standard curve. To calculate the CAT activity in the sample the equation y = 0.0077x + 0.0199 was used. The curve had a regression, R^2 of 0,996.*

The CAT activity was calculated by using equation 1.10. The CAT concentration in the samples was calculated by the linear equation from the formaldehyde curve, y = 0.0077x + 0.0199. The concentration in the samples was then divided on the incubation time, 20 minutes. Finally, dilution factor is multiplied to calculate the CAT activity in the undiluted sample. CAT activity is presented as nmol/min/ml unit.

CAT activity =

$$\frac{\mu M) of\ sample}{20\ min}\ x\ sample\ dilution \qquad \text{Equation 1.10}$$

For more details and information about reagents preparation and the execution of the assay, see the handbook (Cayman Chemical, 2017).

3.5.2.3 Glutathione Peroxidase (GPx)

For determination of the glutathione peroxidase (GPx) activity in the liver samples, the GPx assay kit (Cayman Chemical) was used. This technique measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced

upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH (Cayman Chemical, 2019). The equation for this reaction is shown in equation 1.11 and 1.12.

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-O-H} + \text{GSSG} + H_2O \qquad \text{Equation 1.11}$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + NADP^+ \qquad \text{Equation 1.12}$$

For both GPx and GR, the pike liver samples were diluted ten times before protein concentration was determined. Before adding the samples in the wells, the liver samples were diluted two more times. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the absorbance is directly proportional to the GPx activity in the sample, since GPx is rate limiting (Paglia & Valentine, 1967).

In the sample wells, 20 µL of sample and 150 µL of reagents from the assay kit were added. In the control and the background wells, 170 µL of reagents from the assay kit were added. The reaction was initiated by adding 20 µL of Cumene Hydroperoxide to the wells. The 96-well plate was carefully shaken before reading the absorbance. A Multiskan Ascent 96 well plate reader (USA) was used to read the absorbance once every minute at 340 nm, to obtain at least five time points.

To obtain reproducible results, the amount of GPx in the samples added to the well should have a decrease in the absorbance between 0.02 and 0.135 per min. The change in absorbance per minute was decided by plotting the absorbance as a function of time (Figure 3.7). A linear portion of the curve was obtained.

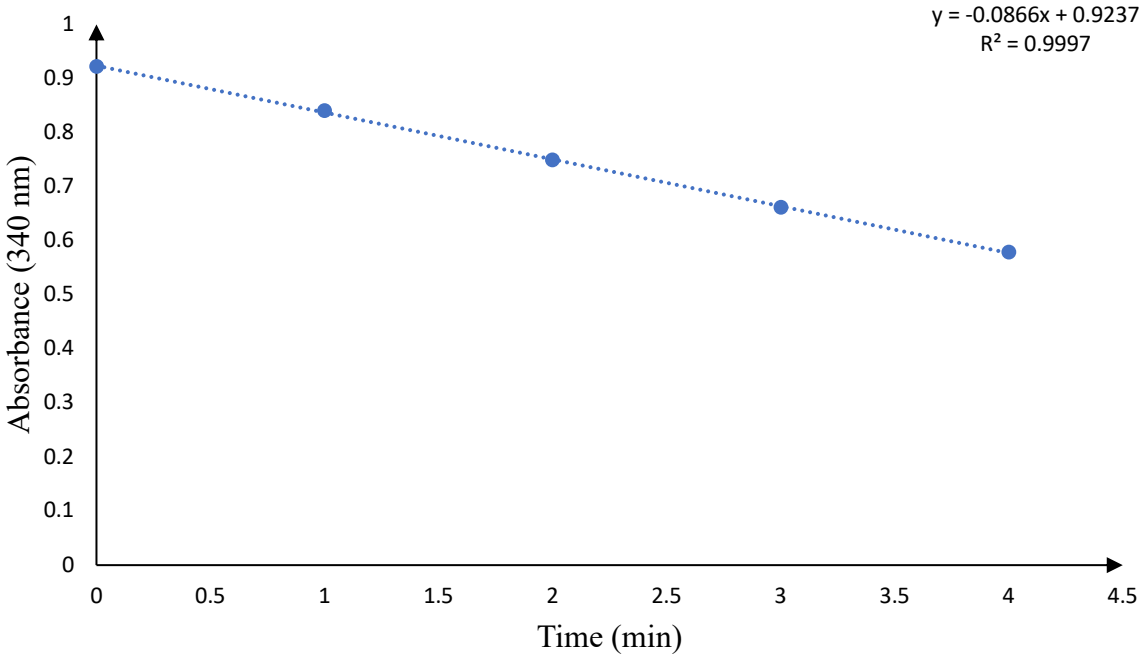


Figure 3.7 *The decrease in absorbance per minute (*ΔA340/min*) in liver sample. The decrease in the sample absorbance is 0.0866. Five time points were used, from 0 to 4 minutes. The curve had a regression, R^2, of 0.9997.*

The rate of absorbance per minute (ΔA340/min) for the background of non-enzymatic wells was also determined (figure 3.8).

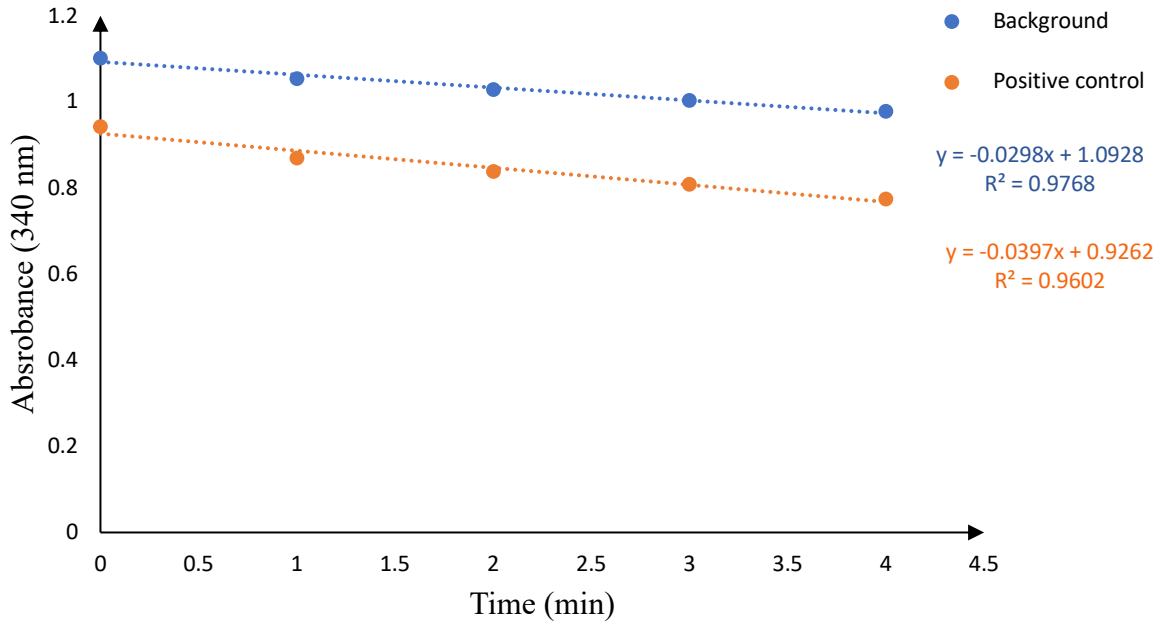


Figure 3.8 *Glutathione peroxidase positive control activity. The blue graph is the background absorbance. The orange graph shows the positive control with added GPx. Five*

time points were obtained, from 0 minutes to 4 minutes. The decrease in the background absorbance is 0.0298.

To calculate the GPx activity in the sample, equation 1.13 was used. The background corrected decrease in ΔA340/min was calculated by subtracting the decrease in the background absorbance from the decrease in the sample absorbance. NADPH consumption per minute was calculated using the NADPH extinction coefficient, 0.00373 μM^{-1}. This ratio is multiplied with the ratio of amount solution added in the wells (0.19 ml) and the amount of sample added in the wells (0.02 ml). Eventually, dilution factor is multiplied to calculate the GR activity in the undiluted sample. GR activity is presented as nmol/min/ml unit.

GPx activity =

$$\frac{\Delta A_{340}/min}{0.00373\ \mu M^{-1}}\ x\ \frac{0.19\ ml}{0.02\ ml}\ x\ sample\ dilution \qquad \text{Equation 1.13}$$

Higher levels of GPx in the organism indicates higher levels of free radicals like hydrogen peroxide, (H_2O_2). For more information about reagents preparation and execution of the assay, see the handbook to the given kit (Cayman Chemical, 2019).

3.5.2.4 Glutathione Reductase (GR)

For determination of the glutathione reductase (GR) activity in the liver, an assay kit purchased from Cayman Chemical was used. The principle is that the GR activity oxidize the added amount of NADPH. GR activity is determined by measuring the rate of NADPH oxidation. The reaction (equation 1.14) is essential for the maintenance of glutathione levels (Carlberg & Mannervik, 1985).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \qquad \text{Equation 1.14}$$

Before adding the samples to the wells, the GR liver samples were diluted two times. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. In the sample wells, 20 µL of sample and 120 µL of reagents from the assay kit were added. In the control and the background wells, 140 µL of reagents from the assay kit were added. The reaction was initiated by adding 50 µL of NADPH to the wells. The 96-well plate was

carefully shaken before reading the absorbance. A Multiskan Ascent 96 well plate reader (USA) was used to read the absorbance once every minute at 340 nm to obtain at least five time points. To obtain reproducible results, the amount of GR added to the well should cause an absorbance decrease (ΔA340/min) between 0.008 and 0.1 per minute (Cayman Chemical, 2016). By plotting the absorbance as a function of time the change in absorbance per minute was determined (Figure 3.9). A linear portion of the curve was obtained.

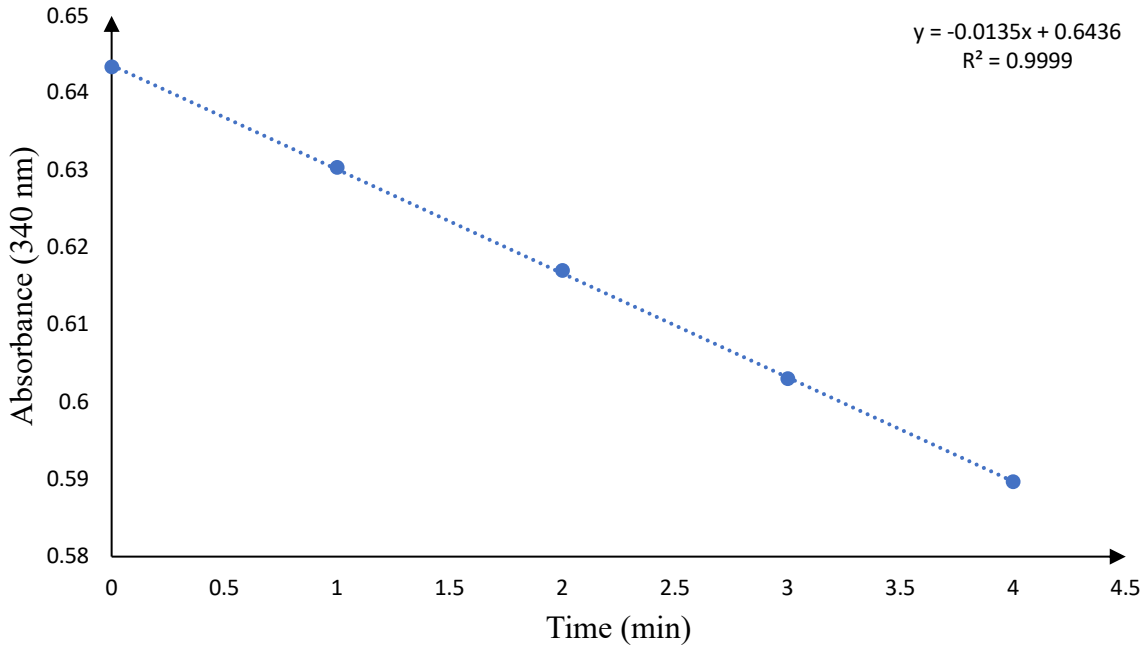


Figure 3.9 *The decrease in absorbance per minute (*ΔA340/min*) in liver sample. The decrease in the sample absorbance is 0.0135. Five time points were used, from 0 to 4 minutes. The curve had a regression, R^2, of 0.9999.*

The rate of absorbance per minute (ΔA340/min) for the background of non-enzymatic wells, and the positive control were also determined (Figure 3.10).

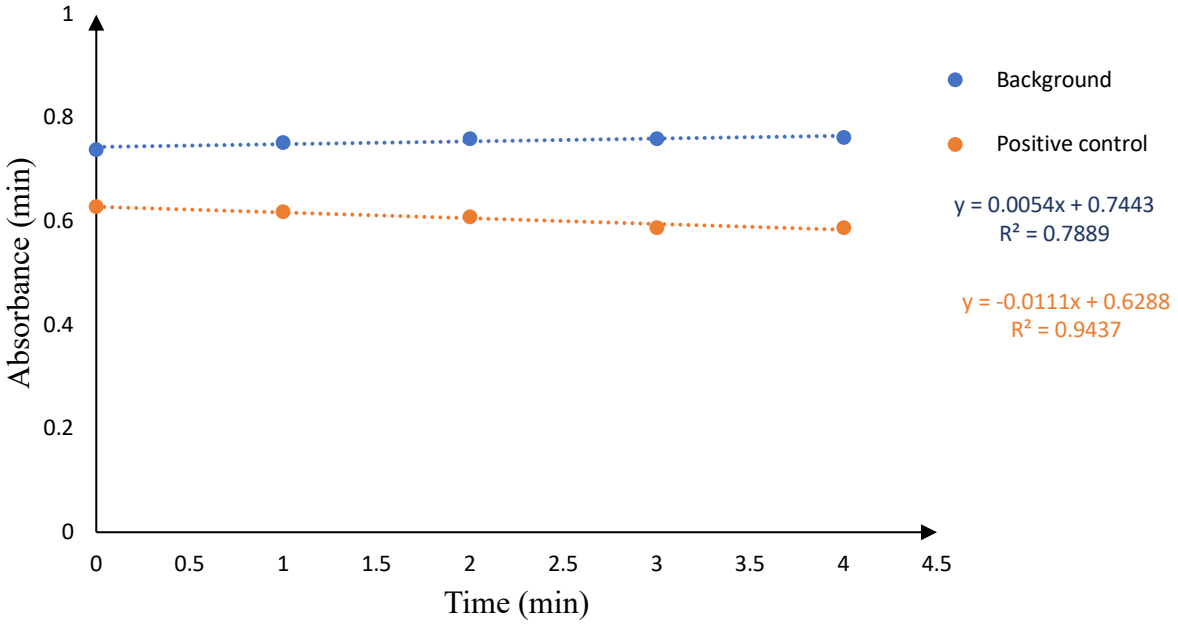


Figure 3.10 *Glutathione reductase positive control activity. The blue graph is the background absorbance. The orange graph shows the absorbance to the positive control with added GR. Five time points were obtained, from 0 minutes to 4 minutes. The decrease in background absorbance is 0.0054.*

To calculate the GR activity the same method as for GPx are used (see section 3.5.2.3). High levels of GSSG indicates oxidative stress. For further information about reagents preparation and execution of the assay, see the handbook to the given kit (Cayman Chemical, 2016).

3.6 Statistical analysis

For data handling, Excel 2016 and Minitab 17 were used. To identify significant differences between two groups, Minitab 17 t-test was used, with a p-value lower than 0.05 (α=0.05). The p-value of the weight, length and condition factor between the fish from the control lake Kashovka and the contaminated lake Glubokoye were calculated. The p-value of the enzyme activity (SOD, CAT, GPx, GR) between the fish from each lake were calculated as well. Excel was used to process all the data and calculate the enzyme activity in the fish. The equations used for calculation of the enzyme activity is stated in chapter 3.5.2.

The condition factor for fish was estimated using equation 1.15;

$$K = \frac{W*100}{L^3} = g/cm^3 \qquad \text{Equation 1.15}$$

Where;
W - is the weight of the fish in grams
L – the total length of the fish in centimeters

The condition factor can be used as an indicator of the water quality or the general health of fish populations (Ighwela et al., 2011). In this experiment the condition factor was used as an indicator of the fish population health in the two different lakes, Kashovka and Glubokoye.

Correlation is a statistical measure of how much two measurable sizes are related to each other (Ganti, 2020). In this study the correlation between the enzyme activity and the protein concentration was assessed. A correlation factor, R^2, larger than 0.4 was used as a threshold that the two measurable sizes are related to each other (Appendix C).

4 Results and Discussion

Previous measurements have shown that different lakes nearby the Chernobyl accident are highly contaminated with radionuclides, while other nearby lakes received very low amount (Lerebours et al., 2018). The fallout after the accident was very unevenly distributed. Although some lakes are heavily contaminated it is not clear to what extent the radionuclides have affected the aquatic organisms. Therefore, in this study fish from two similar lakes in the CEZ that have quite the similar water quality and resembling fish species diversity, but highly different in radionuclide contaminations, were investigated. Fish are considered among the most radiosensitive organisms in the freshwater ecosystem (Sazykina, T. & Kryshev, A. I., 2003). Thus, this study investigated effects in two species of different tropical levels. Rudd is an herbivore, and pike is a carnivorous fish-eating specie.

4.1 Water quality in Kashovka and Glubokoye

Glubokoye was one of the most contaminated lakes after the accident (Murphy et al., 2011). Kashovka, on the other hand, is considered as a noncontaminated lake in the CEZ, and is used as a control lake. The mean values for general water quality parameters (temperature, conductivity and ion composition) recorded in June 2018 and March 2019 are shown in table 4.1. The activity concentration of ^{137}Cs and ^{90}Sr is also given in the table.

Table 4.1 *The mean values for general water quality parameters and the activity concentration of* ^{137}Cs and ^{90}Sr *in lake Kashovka and Glubokoye. The data of water activity were carried out by laboratory personnel at NMBU, Ås and UIAR, Kiev.*

	Unit	Kashovka	Glubokoye
Temperature June 2018	°C	21.1	23.8
Temperature March 2019	°C	8.4	7.4
pH		7.2	7.7
Conductivity	µS/cm	235 ± 4	201.5 ± 10
^{137}Cs	Bq/L	0.023 ± 0.005	3.3 ± 0.7
Cs	ng/L	4.4 ± 1.5	4.9 ± 1.6
K	mg/L	3.4 ± 0.9	1.2 ± 0.1
^{90}Sr	Bq/L	0.118	96 ± 17
Sr	µg/L	141 ± 5	106 ± 2
Ca	mg/L	35 ± 3	30 ± 2

In the control lake the conductivity was relatively high, and the Ca concentration of 35 ± 3 mg/L was similar to moderate hard water qualities (Sengupta, 2013). The water in the control lake was neutral with a pH value on 7.2. The activity concentration of ^{137}Cs and ^{90}Sr were low in the control lake, 0.023 ± 0.005 Bq/L and 0.118 Bq/L (^{90}Sr), respectively. The temperature in Kashovka was higher in June 2018 compared to March 2019.

The concentrations of ions in the contaminated lake, Glubokoye, was moderate and the Ca concentration of 30 ± 2 mg/L was similar to moderate hard water quality (Sengupta, 2013). However, the Cs and Sr concentration were quite low, 4.9 ± 1.6 ng/L and 106 ± 2 µg/L. In the contaminated lake the pH value is 7.7. The activity concentration of ^{137}Cs and ^{90}Sr are 3.3 ± 0.7 Bq/L and 96 ± 17 Bq/L, respectively. The temperature in Glubokoye was higher in June 2018 compared to March 2019.

The result suggests that there were no major differences in the water quality (conductivity, pH and ion composition) between the control lake and the contaminated lake. Both Kashovka and Glubokoye are characterized as hard water quality. However, the conductivity was lower and less variable in Glubokoye. This is accordance with the determined concentration of major ions. The concentration of Ca and K were lower in Glubokoye compared to Kashovka. The activity concentration of ^{137}Cs and ^{90}Sr are significantly higher in Glubokoye, than in the control lake. Inwardly in the water of the lakes, the activity concentration of ^{90}Sr is significantly higher than the activity concentration of ^{137}Cs. ^{137}Cs binds easily to particles and organic matter that settles by time, while ^{90}Sr is more mobile and thus more accessible in the water. This difference in behavior of ^{137}Cs and ^{90}Sr could explain why the activity concentration of ^{137}Cs is lower than the activity concentration of ^{90}Sr in the waterbody. The concentration of ^{137}Cs is reported to be highest in the sediments (IAEA, 2019). In previous measurements, the activity concentration of ^{90}Sr and ^{137}Cs in the water of Glubokoye have been 99-120 Bq/L and 13-14 Bq/L, respectively (Gudkov & Nazarov, 2006). The activity concentration of the radionuclides is lower now than previous measurements due to decay.

4.2 Fish characteristics

Pike is a fish-eating specie that lives close to the land. Rudd is a smaller fish that often live on the shallows. This study examined the effects of ionizing radiation on pike and rudd. Due to regulations of sampling procedures the number of fish were restricted to ten of each

species per lake. Table 4.2 presents an overview of the sizes and fitness factors of sampled fish.

Table 4.2 *Overview of average length (cm), weight (kg), and the condition factor of fish collected from Kashovka and Glubokoye is given.*

Fish	Site	Length (cm)	Weight (kg)	Condition factor (g/cm^3)	Number of fish (N)
Pike	Kashovka	59.28 ± 18.38	1.56 ± 1.28	0.60 ± 0.09	9
Pike	Glubokoye	48.22 ± 14.70	0.98 ± 0.83	0.63 ± 0.27	9
Rudd	Kashovka	11.86 ± 2.22	0.022 ± 0.019	1.12 ± 0.11	10
Rudd	Glubokoye	12.60 ± 0.94	0.019 ± 0.006	0.93 ± 0.11	10

The pike from the control lake showed an average length of 59.28 ± 18.38 cm and weight of 1.56 ± 1.28 kg, with a condition factor of 0.60 ± 0.09 g/cm^3. The length and the weight of the rudd have an average of 11.86 ± 2.22 cm and 0.022 ± 0.019 kg, with a condition factor of 1.12 ± 0.11 g/cm^3. The pike from the contaminated lake, Glubokoye, showed an average length of 48.22 ± 14.70 cm and weight of 0.98 ± 0.83 kg, with a condition factor of 0.63 ± 0.27 g/cm^3. The rudd have an average length of 12.60 ± 0.94 cm and weights 0.019 ± 0.006 kg and have a condition factor of 0.93 ± 0.11 g/cm^3.

Although some of the pike collected in Kashovka was larger than pike collected in Glubokoye, there were no statistically significant differences (length $p=0.18$, weight $p=0.27$) between the pike from the two different lakes. The condition factor of the pike from the control lake was similar to the condition factor of the pike from the contaminated lake. The results demonstrate no difference in the general health of the fish (Ighwela et al., 2011).

The rudd from the two different lakes have quite similar length and weight. The condition factor is higher for the rudd from the contaminated lake, Glubokoye, compared to the rudd from the control lake, Kashovka ($p = 0.001$). The result indicates that there is a significant difference between the general health of the collected fish. More fish should be sampled to identify if the condition factor really is significant for the whole fish population.

4.3 Activity concentration of radionuclides in fish

To obtain information of the ionizing radiation, the activity concentration of radionuclides in the fish were analyzed. The activity concentration of the radionuclides accumulated in the fish is shown in figure 4.3.

Table 4.3 *^{137}Cs and ^{90}Sr activity concentration in the muscle and the bone ash of the pike and the rudd from Kashovka (K) and Glubokoye (G).* The *measurements in the fish were carried out by laboratory personnel at UIAR, Kiev.*

Site	Fish	N	Muscle ^{137}Cs (Bq/kg ww)	Bone ash ^{90}Sr (Bq/kg dw)	^{90}Sr in bone (Bq/kg ww)
Kashovka	Pike	9	193 ± 46	967 ± 631	202 ± 137
Glubokoye	Pike	9	8857 ± 6146	183889 ± 153051	30305 ± 23262
Kashovka	Rudd	10	118 ± 74	1500 ± 710	280 ± 103
Glubokoye	Rudd	10	8277 ± 2984	505700 ± 23500	101100 ± 4700

In the pike the activity concentration of ^{137}Cs is 193 ± 46 Bq/kg (ww) and the activity concentration of ^{90}Sr is 202 ± 137 Bq/kg (dw). In the rudd from the control lake the activity concentration of the radionuclides respectively is, 118 ± 74 Bq/kg (ww) and 280 ± 103 Bq/kg (dw).

In the pike from the contaminated lake, Glubokoye, the activity concentration of ^{137}Cs is 8857 ± 6146 Bq/kg (ww) and the activity concentration of ^{90}Sr is 30305 ± 23262 Bq/kg (dw). In the rudd from Glubokoye the activity concentration of ^{137}Cs and ^{90}Sr are 8277 ± 2984 Bq/kg (ww) and 101100 ± 4700 Bq/kg (dw).

The pike from the contaminated lake, Glubokoye, have higher activity concentration of ^{137}Cs in the muscle, but lower ^{90}Sr in the bone than the rudd from the same lake. The fish in Glubokoye have seven times higher activity concentration of ^{137}Cs and four hundred times higher activity concentration of ^{90}Sr than the fish from the Kashovka. In comparison, the limit for consumption of food and drinks contaminated with ^{137}Cs is in Norway is 65 Bq/kg

(Statens strålevern & Mattilsynet, 2013). Thus, the activity concentration of the radionuclides in the fish from Glubokoye is therefore quite high.

4.4 Internal and external dose-rates fish

Previous studies have reported high total doses from the contaminated lake, Glubokoye. Fish in contaminated lakes are exposed to radionuclides and ionizing radiation both internal and external. External dose is ionizing radiation from the sediments and the water, while the internal dose is radiation from accumulated radionuclides inside the fish. In this study, the sediments were not sampled, and the activity concentration not determined. Thus, external dose from sediments were not calculated. The internal and external dose-rates from the control lake Kashovka and the contaminated lake Glubokoye are shown in table 4.4.

Table 4.4 *Calculated dose-rates for ^{137}Cs and ^{90}Sr in the pike and rudd from the control lake, Kashovka and the contaminated lake, Glubokoye. The external dose consider only dose from water.*

Site	**Fish**	**N**	**Intern dose-rate ^{137}Cs**	**Intern dose-rate ^{90}Sr**	**Total intern dose-rate**	**External dose-rate ^{137}Cs**	**Total (int + ekst) dose-rate**
			µGy/h	µGy/h	µGy/h	µGy/h	µGy/h
Kashovka	Pike	9	$3.86 \times 10^{-2} \pm 7.84 \times 10^{-3}$	$1.76 \times 10^{-2} \pm 1.17 \times 10^{-2}$	$5.63 \times 10^{-2} \pm 1.75 \times 10^{-2}$	$6.63 \times 10^{-6} \pm 2.89 \times 10^{-7}$	$5.63 \times 10^{-2} \pm 1.75 \times 10^{-2}$
Glubokoye	Pike	9	1.66 ± 1.16	2.63 ± 2.02	4.29 ± 3.06	$9.68 \times 10^{-4} \pm 3.48 \times 10^{-5}$	4.29 ± 3.06
Kashovka	Rudd	10	$2.10 \times 10^{-2} \pm 8.79 \times 10^{-3}$	$2.24 \times 10^{-2} \pm 8.37 \times 10^{-3}$	$4.34 \times 10^{-2} \pm 1.70 \times 10^{-2}$	$7.35 \times 10^{-6} \pm 7.71 \times 10^{-8}$	$4.41 \times 10^{-2} \pm 8.78 \times 10^{-3}$
Glubokoye	Rudd	10	$1.36 \pm 5.04 \times 10^{-1}$	$8.09 \pm 3.88 \times 10^{-1}$	$9.45 \pm 8.92 \times 10^{-1}$	$1.06 \times 10^{-3} \pm 6.52 \times 10^{-6}$	$9.46 \pm 6.32 \times 10^{-1}$

There are low internal and external dose-rate values in the control lake. Each radionuclide, ^{137}Cs and ^{90}Sr constitutes almost equal internal dose-rate. The total internal and external dose-rates in the pike from the control lake are $5.63 \times 10^{-2} \pm 1.75 \times 10^{-2}$ µGy/h and $6.63 \times 10^{-6} \pm 2.89 \times 10^{-7}$ µGy/h, respectively. In the rudd from the control lake are the total internal and external dose-rates $4.34 \times 10^{-2} \pm 1.70 \times 10^{-2}$ µGy/h and $7.35 \times 10^{-6} \pm 7.71 \times 10^{-8}$ µGy/h,

respectively. The external dose-rate for rudd are a little higher than the external dose-rate for pike. However, the total dose in pike and rudd are very similar.

The fish from the contaminated lake, Glubokoye, are exposed to higher ionizing radiation than the fish from Kashovka. ^{90}Sr constitutes to a higher internal dose in both pike and rudd from Glubokoye than ^{137}Cs. In the pike from Glubokoye the total internal and external dose-rates are 4.29 ± 3.06 μGy/h and $9.68 \times 10^{-4} \pm 3.48 \times 10^{-5}$ μGy/h, respectively. The total internal and external dose-rates in the rudd from Glubokoye are $9.45 \pm 8.92 \times 10^{-1}$ μGy/h and $1.06 \times 10^{-3} \pm 6.52 \times 10^{-6}$ μGy/h, respectively. In the pike and rudd from both lakes, Kashovka and Glubokoye, contributes the total internal dose-rate almost 100% to the total dose-rate. The pike and rudd from Glubokoye are exposed to ionizing radiation which is almost hundred times higher than the ionizing radiation in the control lake. The external dose from water are lower compared to the internal dose. In both the control fish and the contaminated fish, the internal radiation contributes to a higher percentage of the total dose-rate relative to the external.

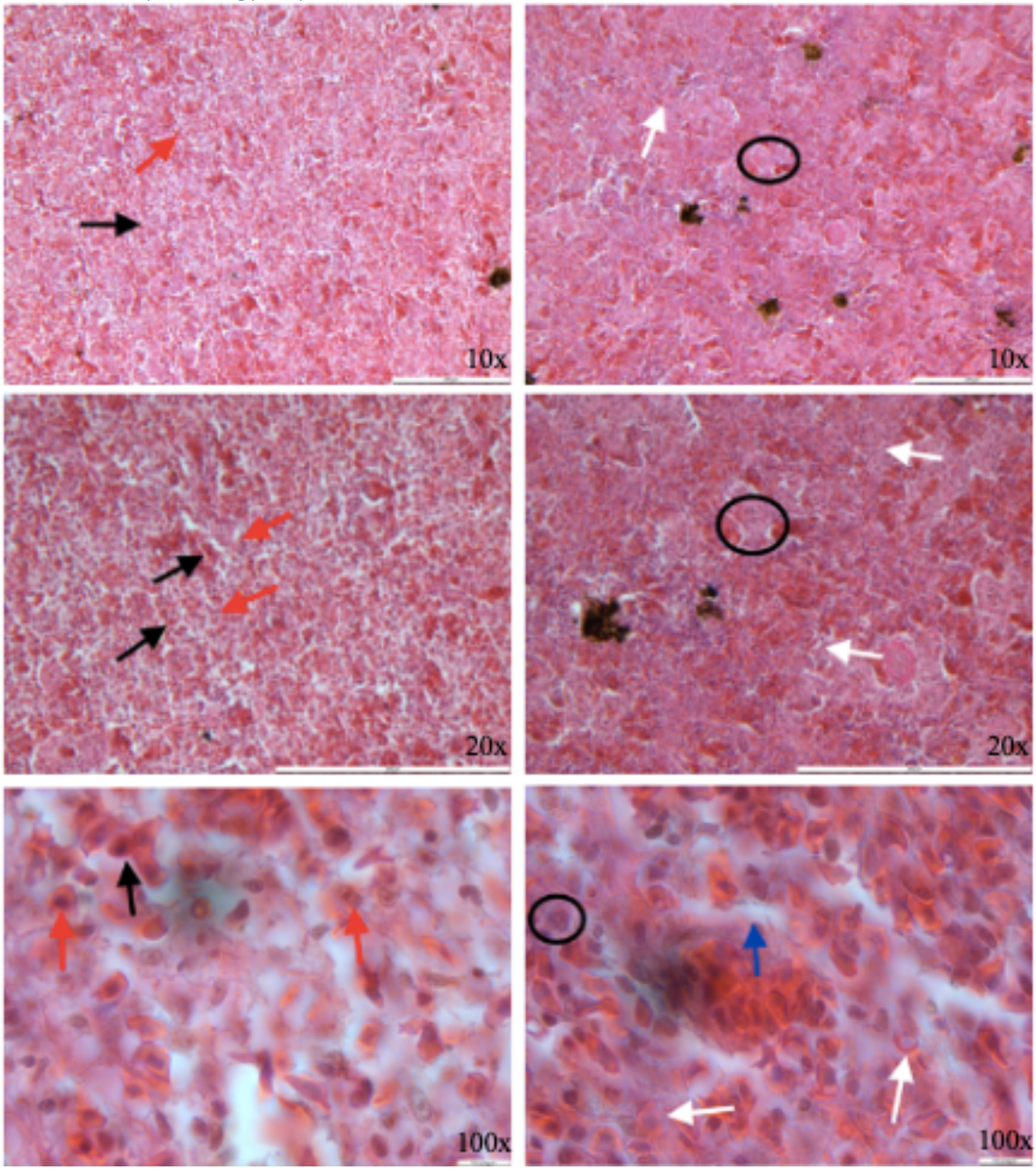
In previous studies, the total dose-rate of pike and rudd form Glubokoye have been determined, 45 μGy/h and 54 μGy/h (Kaglyan et al., 2019), were 80% of the total dose is predicted to contribute from the sediments. Radioactive isotopes can accumulate in sediments, and this may contribute to external ionizing radiation to fish. Previous studies, (Murphy et al., 2011), have shown high exposure from sediments. Estimated total external dose-rate for organisms on the sediment surface is 27.1 μGy/h (Fuller et al., 2017). The dose contribution depends on the behavior of the fish. Bottom dwelling (benthic) or mud foraging fish are more exposed than pelagic fish.

In this study, pike and rudd were examined. Pike and rudd from the contaminated lake may have been exposed to ionizing radiation from the sediments. The external dose-rate from sediments might be in the range of 30-43 μGy/h as previous reported for pike and rudd. For that reason, it can be assumed that the fish from Glubokoye in this study are exposed to 40-53 μGy/h included dose from sediments. These values are much lower than 208.33 μGy/h which is predicted in previous studies, and have shown to have a chronic effect on the fish.

4.5 Histopathology of tissue from pike

The hypotheses for this study was that the exposure to radiation over three decades would negatively affect the general health of pike and rudd and disturb the antioxidant enzymes activity. By identifying cell morphological changes in the contaminated pike it is possible to see if the ionizing radiation has affected the cell. Since the kidney samples were not homogenous and porous, it was challenging to make proper histology preparations of the kidney samples. Thus, only liver and spleen were evaluated. In the subsequent subchapters two representative specimens of the pike liver and kidney tissue from the control pike and the contaminated pike are presented consecutively.

4.5.1 Histopathology of pike liver



Kashovka *Glubokoye*

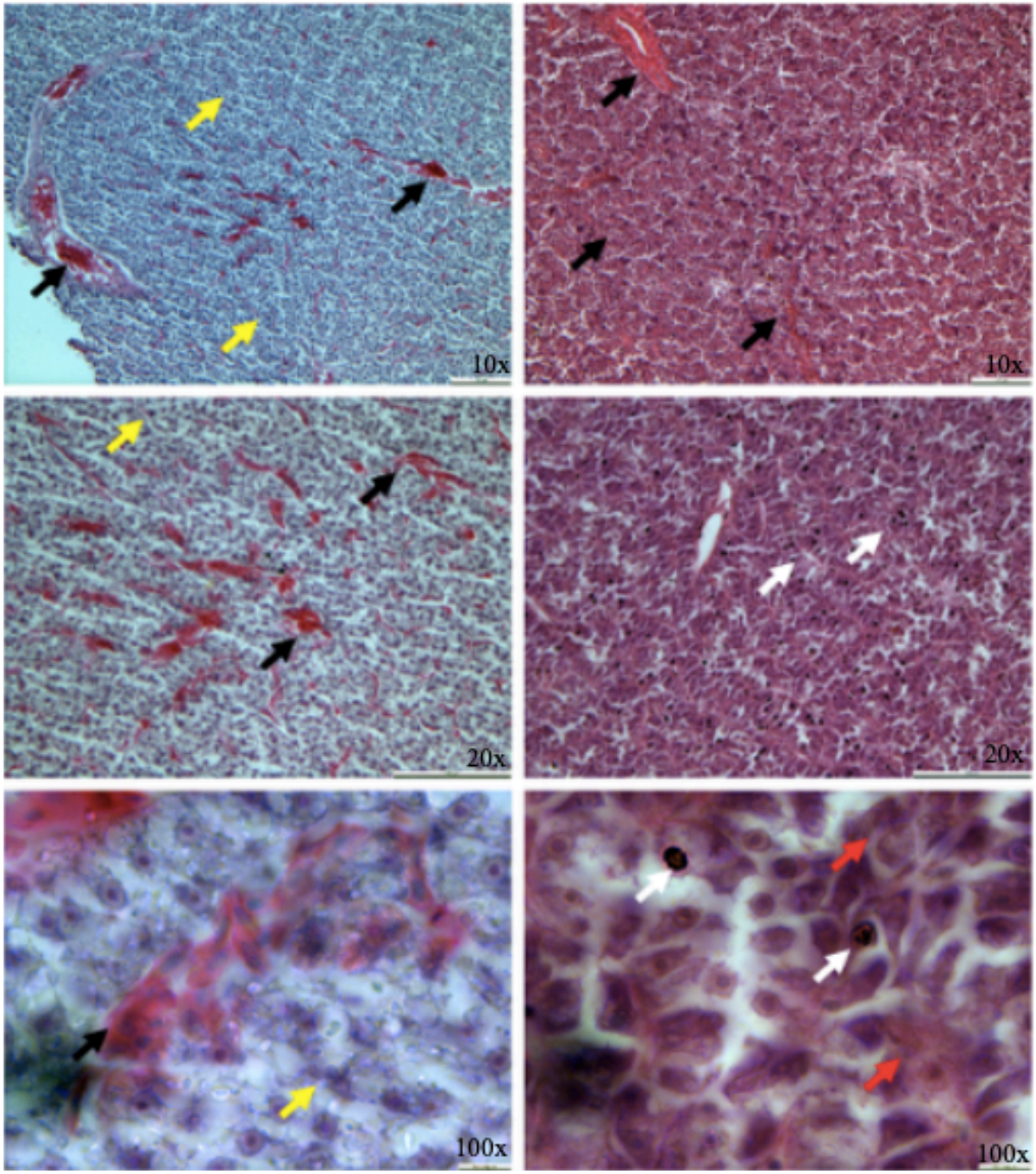
Figure 4.1 *Pictures of the pike liver from the control lake, Kashovka (left) and the contaminated lake, Glubokoye (right). The pictures are magnified 10x, 20x and 100x. It is observed hepatocytes (black arrow), nuclei (red arrow), degenerative changes (black circle), focal necrosis (blue arrow), and vacuolization (white arrow).*

The liver tissue from the control pike shows continuous mass of polygonal cells. Hepatocytes and nuclei are observed in the liver tissue of the control pike. The hepatocytes are marked with black arrows, and the nuclei is marked with white arrows in figure 4.1.

In the contaminated pike liver, it is observed degenerative changes (black circle), focal necrosis (blue arrow), and vacuolization (white arrow). By comparing the pictures in figure 4.1, it is possible to see pathological changes in the contaminated pike liver. The control pike tissue consists of a continuous mass of hepatocytes. The nuclei in the cells of the control liver tissue is normal in size and shape. The continuous mass of hepatocytes and nuclei is not observed in the contaminated liver tissue.

Abnormalities in liver of fish exposed to ionizing radiation have been observed in previous studies as mentioned previously in chapter, 2.5. Liver cells of freshwater fish were exposed to gamma radiation, 3-300 mGy, and congestion of blood vessels, structural alteration, cellular swelling, vacuolation and necrotic liver cells were observed (Bukhari et al., 2012). Although the fish in this study are exposed to a lower radiation, abnormalities in the liver cell like vacuolation is observed. The liver is an important organ in the fish, performing vital functions like detoxification (Dutta et al., 1993). In this study, no other factors than chronic exposure to radionuclides can explain the effects observed in the liver of the contaminated pike, thus it is likely that ionizing radiation have caused the damage to the liver tissue.

4.5.2 Histopathology of pike spleen



Kashovka Glubokoye

Figure 4.2 *Pictures of the pike spleen from the control lake, Kashovka (left) and the contaminated lake, Glubokoye (right). The pictures are magnified 10x, 20x and 100x. It is observed white pulp (yellow arrows), red pulp (black arrows), and cell degeneration (red arrows) are indicated.*

In the spleen tissue from the control pike it is possible to see areas with red and white pulp, the white pulp is marked with yellow arrows in figure 4.2, and the red pulp is marked with

black arrows. This indicates that the pike from the control lake not have abnormalities, since normal fish spleen has definite areas of white and red pulp.

In the spleen tissue from the contaminated pike, it is observed some areas with red pulp. The white pulp in the spleen tissue are more reddish, and it is therefore difficult to define areas of the white pulp. Cell degeneration (red arrows) and black dots (white arrows) are also observed in the spleen tissue from the contaminated pike

The spleen tissue from the control lake has more defined areas of white pulp and red pulp than the spleen from the contaminated pike. In the contaminated spleen tissue, it is possible to observe some abnormalities compared to the control pike. The spleen consists of two types of tissue which have different functions: white pulp and red pulp. In the contaminated spleen tissue the white- and red pulp areas were more reduced and difficult to identify. In addition, it appears like the amount of red blood cells in the contaminated fish is reduced. Cell degeneration was observed in the spleen tissue from the contaminated pike, which indicates that the cell structure is in bad conditions. In the contaminated spleen tissue, there are some black dots, which may be caused by accumulation of pollution. It can also be related to recycling of apoptotic, necrotic cells. Previous studies (Xu et al., 2008) have shown that white and red pulp areas are difficult to identify, and that the amount of red blood cells are often reduced. These abnormalities are shown in this study.

The histological changes in the structure of cells and tissue can be used to evaluate which health effects the radiation have on organisms. Based on histological analysis, it appears like the contaminated Glubokoye fish have been affected, probably by ionizing radiation, both in the liver and the spleen tissue. The chronic exposure of the radionuclides, ^{137}Cs and ^{90}Sr, seems to affect the tissue, and that the ionizing radiation causes damage to the fish tissue. This suggest that the contaminated fish is under stress.

4.6 Antioxidant enzymes in pike and rudd

To assess potential oxidative stress effects related to exposure to radionuclides and ionizing radiation, the activity of four central antioxidant defense enzymes were determined. The hypothesis of this study was that the activity of antioxidant enzymes in the fish from Glubokoye is higher than in the fish from the control lake since ionizing radiation can produce free radicals. In the subsequent subchapters the activity for SOD, CAT, and GPx in the pike and rudd are presented consecutively.

4.6.1 SOD activity in pike and rudd

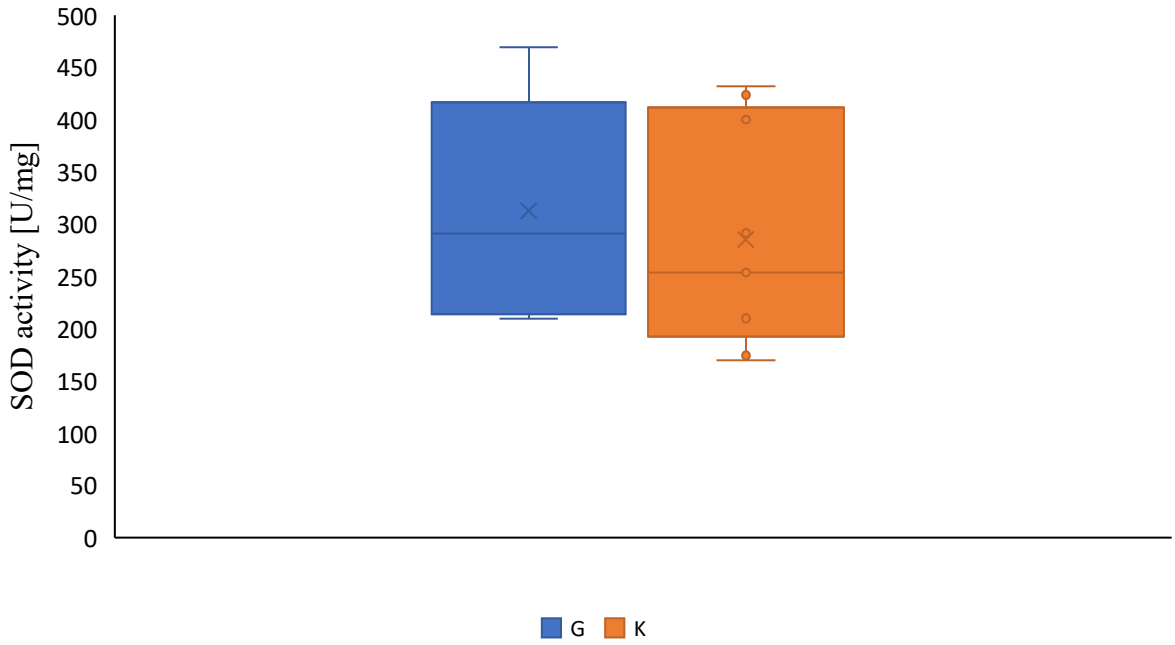


Figure 4.3 *Box plot of pike liver SOD activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of three technical replicates (sample size N=9). All measurements are presented in Appendix D.*

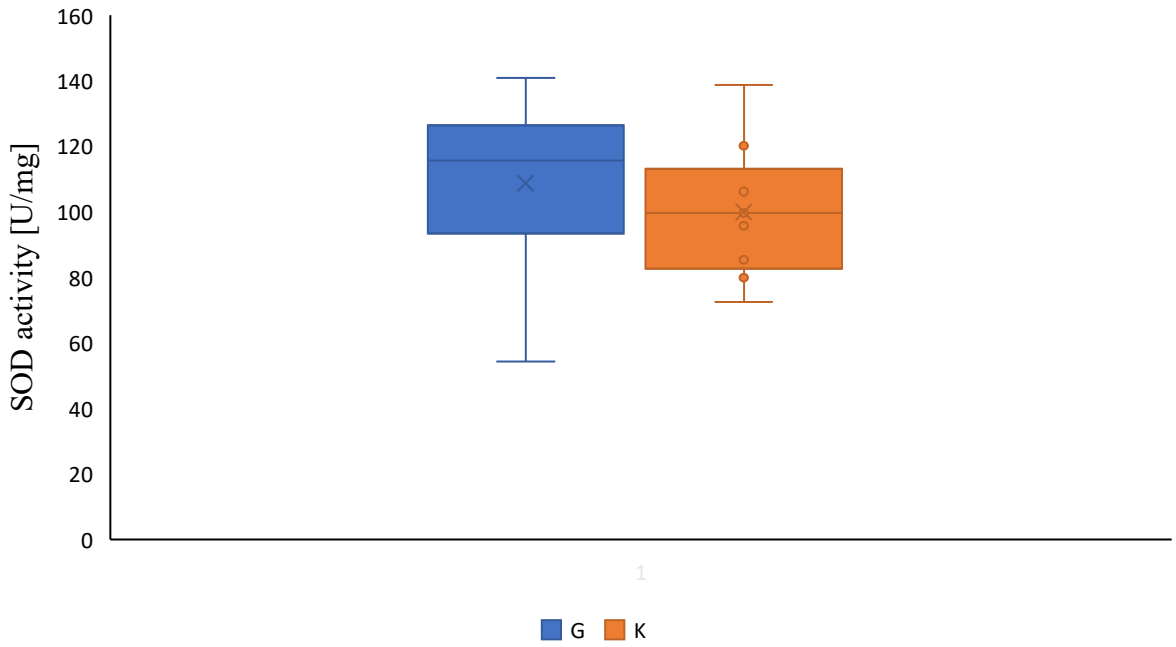


Figure 4.4 Box plot of pike kidney SOD activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of three technical replicates (sample size N=9). All measurements are presented in Appendix E.

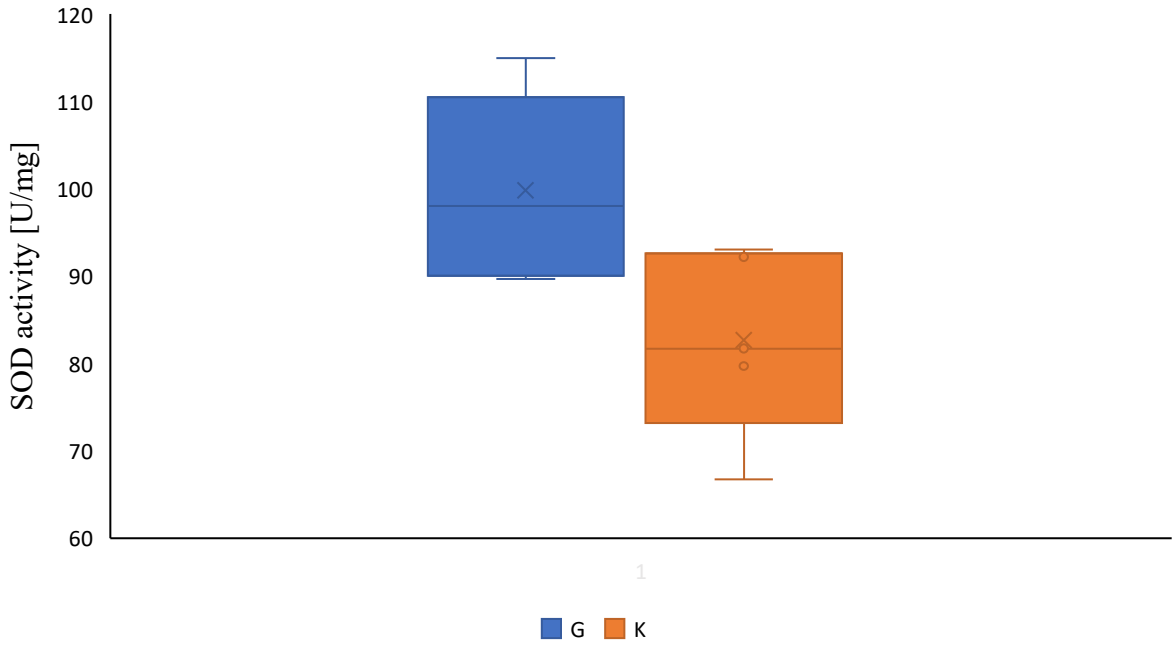


Figure 4.5 Box plot of rudd kidney SOD activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of two technical replicates (sample size N=10). All measurements are presented in Appendix F.

Based on the results of the SOD activity in the control pike liver (RSD = 37.5%), the kidney (RSD = 20.4 %) and the kidney of rudd (RSD = 13.0%), the measured values showed a quite high variability between the individual fish, resulting in a quite large standard deviation. The measured values showed a quite high variability in the contaminated pike liver (RSD = 32.2%), kidney (RSD = 24.1%) and in the rudd kidney (RSD = 10.8%) as well.

The SOD activity in the pike liver ($p = 0.58$), and in the pike kidney ($p = 0.45$) showed no significant difference between the control fish and in the highly contaminated fish. This indicates that the ionizing radiation does not affect the enzyme activity in the contaminated pike. However, the SOD activity in the rudd kidney ($p = 0.002$) showed significant differences between the control and the contaminated rudd. Higher SOD activity indicates higher proportion of the harmful oxygen specie, superoxide anion (O_2^-), which may be due to the increased dose of ionizing radiation in Glubokoye.

The SOD activity in the pike kidney is a little higher than the SOD activity in the rudd kidney. However, there are no significant differences between the SOD activity in the pike kidney and the SOD activity in the rudd kidney ($p = 0.34$ and $p = 0.10$). The SOD enzyme is rapidly excreted from the kidney since the enzyme does not bind to cellular membranes (Asakura & Kitahora, 2018). Therefore, is the SOD activity in the pike liver is higher than the SOD activity in the pike kidney.

The storage of the samples can have affected the enzyme activity. The rudd kidney have been stored in the freezer since June 2018, and the long storage time can have affected the enzyme activity.

4.6.2 CAT activity in pike and rudd

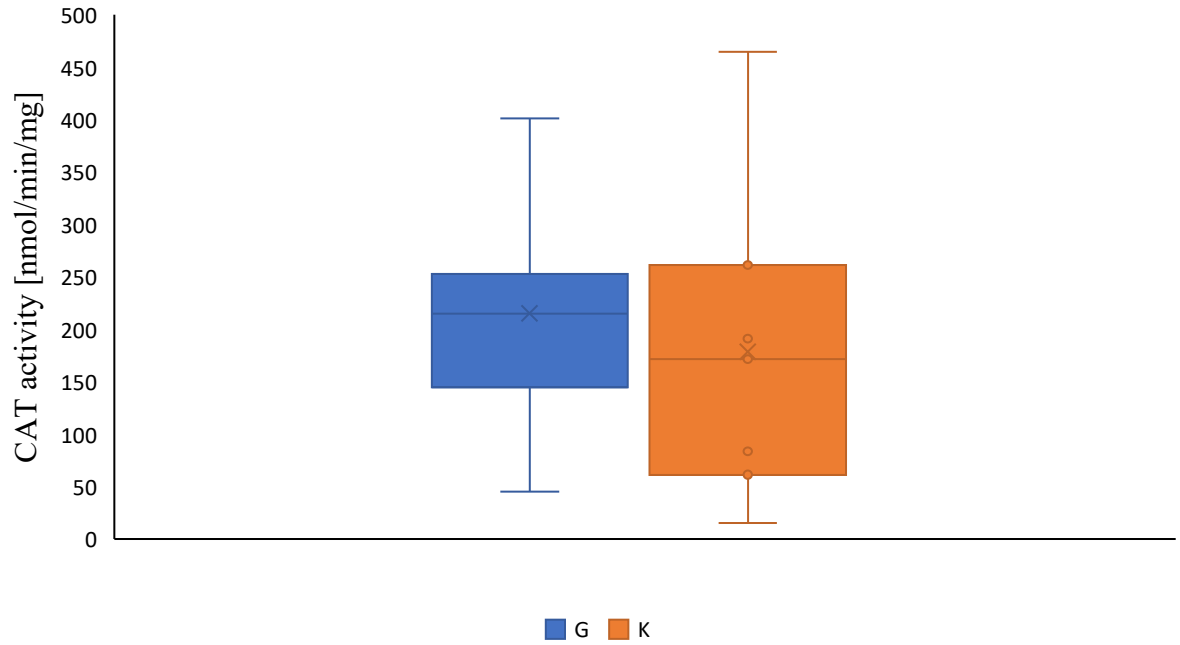


Figure 4.6 *Box plot of pike liver CAT activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of three technical replicates (sample size N=9). All measurements are presented in Appendix G.2.*

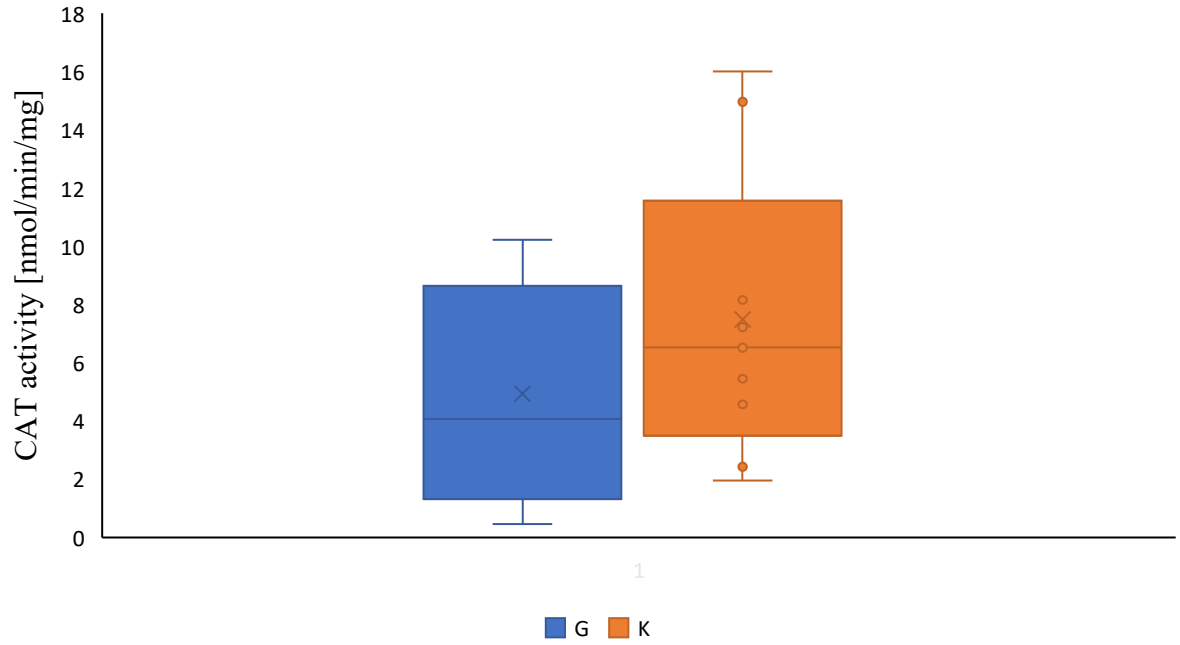


Figure 4.7 *Box plot of pike kidney CAT activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of two technical replicates (sample size N=9). All measurements are presented in Appendix H.2.*

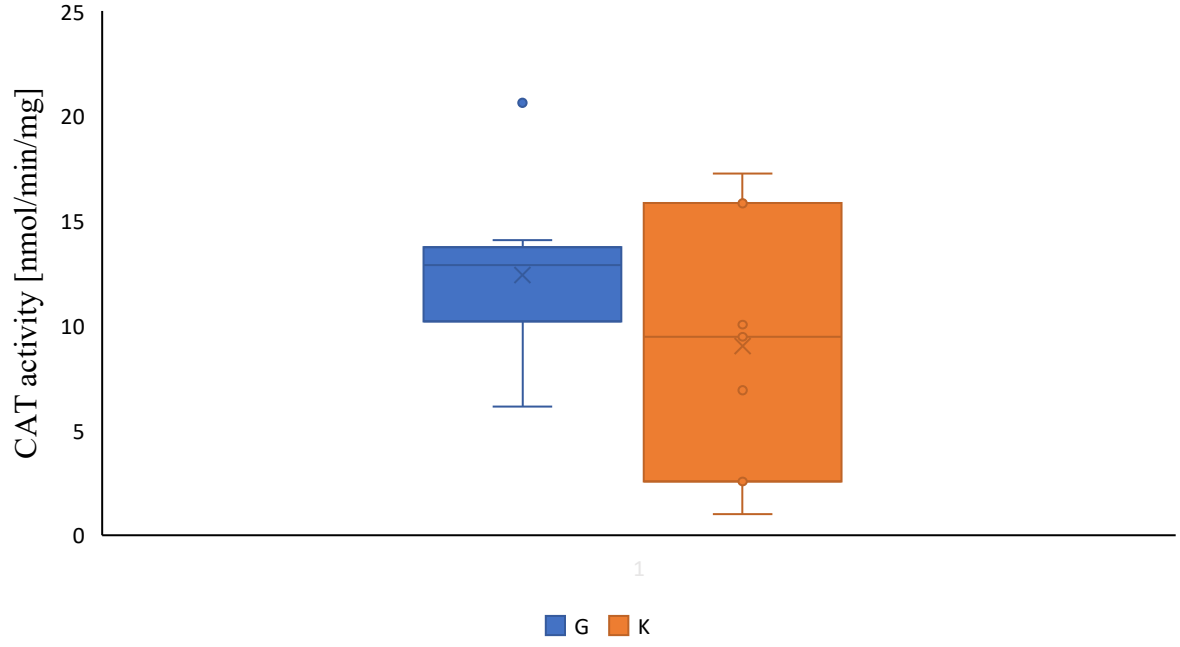


Figure 4.8 *Box plot of rudd kidney CAT activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of two technical replicates (sample size N=10). All measurements are presented in Appendix I.2.*

The results of the CAT activity in the control pike liver (RSD = 85.2%), the pike kidney (RSD = 69.1%), and the rudd kidney (RSD = 68.1%) showed quite high variability between the individual fish, resulting in a quite large standard deviation. In the contaminated pike liver (RSD = 50.7%), kidney (RSD = 81.8%), and in the rudd kidney (RSD = 31.8%) the measured values showed a quite high variability as well.

The CAT activity in the pike liver ($p = 0.56$), the kidney ($p = 0.39$), and the rudd kidney ($p=0.18$) showed no significant difference between the control fish and the contaminated fish. However, the analysis of the CAT activity in the pike and the rudd kidney showed too low CAT amount in the samples added to the wells (2-35 nmol/min/ml). The samples were therefore too low to be considered valid (Appendix H.1 and I.1). Even though the amount of CAT was too low, the samples were analyzed. The CAT activity in the pike kidney is higher than the CAT activity in the rudd kidney. Since the samples used are considered to be not valid in both of the analysis, the results are not representative.

The SOD activity in the contaminated rudd kidney were significantly higher than the SOD activity in the control rudd kidney, it was therefore expected higher CAT activity in the contaminated rudd kidney compared to the control rudd kidney. This were not the case in this study. However, since the CAT samples for the rudd were considered to be too low to be valid it is difficult to conclude anything about the CAT samples.

4.6.3 GPx activity in pike

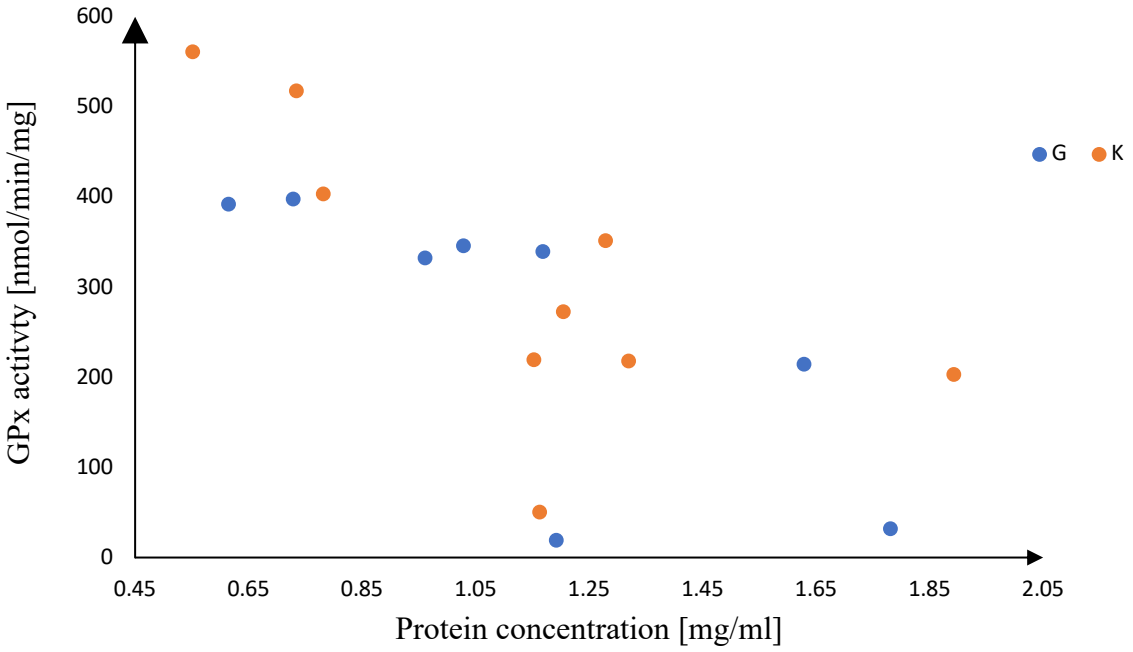


Figure 4.9 *GPx activity in the pike liver. The orange dots represent the fish from the control lake, Kashovka, and the blue dots represent the fish from the contaminated lake, Glubokoye. The values represent average of three technical replicates (sample size N=9), All measurements are presented in Appendix J.*

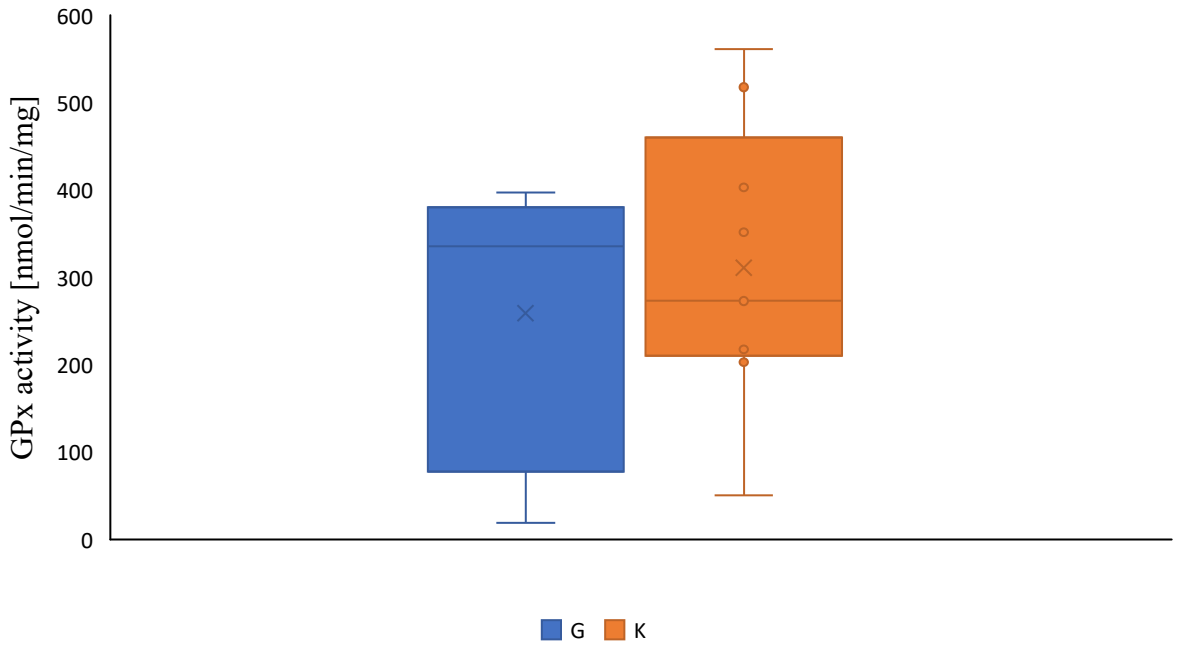


Figure 4.10 *Box plot of pike liver GPx activities. The orange box represents the control fish from Kashovka. The blue box represents the contaminated fish from Glubokoye. The values represent averages of three technical replicates (sample size N=9). All measurements are presented in Appendix J.*

The results in figure 4.10 shows the GPx activity in the control pike liver (RSD = 52.6 %) and the contaminated pike liver (RSD = 59.7%). The measured values show a quite high variability between the individual fish, resulting in a quite large standard deviation. The GPx activity in the pike liver showed no significant differences between the control pike and the contaminated pike ($p = 0.51$). In figure 4.9 is the GPx activity in the control fish relative to the contaminated pike. The correlation factor ($R^2 = 0.50$ and $R^2 = 0.56$) indicates that there is a correlation between the GPx activity and the protein concentration. The storage time of the samples can also have affected on the enzyme activity. The samples may have been stored for too long in the freezer so that the enzyme activity have been inactivated. Thus, the GPx samples were therefore not analyzed one more time.

4.6.4 Enzyme activity in the contaminated pike and rudd

The results of enzyme activity indicated that only the rudd kidney from the contaminated lake have significant higher SOD activity than the rudd from the control lake. In the pike there were no significant differences. Since SOD activity is proportional to superoxide anion (O_2^-),

the results indicate that the contaminated rudd have higher ROS proportion than the control rudd. However, the measured SOD activity in the rudd were analyzed with only five samples from each lake. To substantiate and get representative results, several rudd kidney samples should be analyzed. More fish samples will also reduce the %RSD, which was quite high for all the enzymes, and thus improve the possibility to identify significant differences, if any.

SOD catalyzes the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Because it was measured higher SOD activity in the contaminated rudd, higher CAT activity was expected. This was not the case, and the storage time may have affetcted the enzyme.

The condition factor is an indicator on the fish health. As stated previously in chapter 4.2, there were significant differences in the condition factor between the rudd from the control lake and the contaminated lake. This suggests that the differences in the fish health may affect the enzyme activity in the rudd.

4.6.5 Quality assessment of the analysis of the enzyme activity

It is different requirements in the various enzyme kit that have been used in this study. The different requirements are to obtain reproducible and representative results. Poor pipetting can cause erratic values, such as scattering of the triplicate values. This was solved by carefully pipetting the reagents and the samples in the wells. Bubbles can impact the analysis and were therefore removed by tapping gently on the side of the plate.

In all the analysis were either blank samples, backgrounds samples or positive controls used. By analyzing these samples, it is possible to get an indication of possible contamination or interferences. The backgrounds and the blank samples are also used to remove the background absorbance from the samples. Similar absorbance in the replicates of the blank samples, the background samples and the positive controls indicated that there were no contamination or interferences. Because of possible interferences, all the samples in this study were diluted. The diluted samples still contain various compounds and components that may interfere with the sample and the analysis, but the concentration of the compound and the components are lower.

Correlation is a statistical measure of how much two measurable sizes are related to each other (Ganti, 2020). In this study, correlation between the enzyme activity and the protein concentration was demonstrated (Appendix C). Since the R^2 values for the first measurements of the SOD, CAT, GPx and GR activity in the pike liver showed correlation between the protein concentration and the enzyme activity. In order to exclude the protein concentration as a possible source of error, new measurements of SOD and CAT were made with a protein concentration within a given interval (0.5 to 0.8 mg/ml). The new measurements showed no influence in variation of protein concentration for the measured enzyme activity. Since R^2 was less than 0.4 there is no correlation between the protein concentration and the enzyme activity.

Temperature can inactivate the enzyme activity. Therefore, if the samples are handled for too long in room temperature, during sampling or during handling at the laboratory, the enzyme activity can be inactivated. It is thus important to snap-freeze the tissue as soon as possible after the fish have been caught and dissected and keep the samples on ice during handling at the laboratory. In the subsequent chapter the requirements for each enzyme SOD, CAT, GPx, and GR are discussed.

4.6.6 Superoxide dismutase (SOD)

The SOD activity was measured by determining the inhibition of superoxide anion (O_2^-), which was quantified by measuring the decrease in the color development at 440 nm. Therefore, to obtain SOD activity measurements, it is suggested to maintain the inhibition rate between 20% and 90%. In this range the correlation between SOD activity and xanthine oxidase (XO) inhibition rate is linear. The inhibition curve for one pike sample from the contaminated lake is shown in figure 3.10.

All the SOD activity samples were within the suggested inhibition rate, which makes the measurements to be considered valid. In previous studies, the SOD activity in the *Siluris glanis* liver exposed to pollution from petroleum refinery industry have been measured, 218.6 – 253 U/mg protein (Avci et al., 2005). The previous measured SOD activity is similar to the measured SOD activity in the pike liver, 280 U/mg protein. The SOD activity in pike and rudd kidney measured in this study is also similar to previous measured SOD activity in *C.*

carpio and *O. niloticus* kidney, $0.150 \pm 6.31 \times 10^{-2}$ and 2.19 ± 0.07 U/mg protein (Oruc et al., 2004).

4.6.7 Catalase (CAT)

A requirement for reproducible results is that the amount of CAT activity added to the wells should result in an activity between 2 and 35 nmol/min/ml. This was solved by diluting all the samples differently. In the analysis of the pike liver, four samples had higher activity in the well than the requirement (Appendix G.1). They were therefore considered to have too low CAT amount to be considered valid. These four samples were not included further in the calculation of the CAT activity.

In the analysis of the pike and rudd kidney, there were only a few samples that had CAT amount within the given interval (Appendix H.1 & I.1). Thus, the results were too low to be considered valid. The enzymatic kit can only analyze a certain number of samples, the kidney samples of pike and rudd were therefore not analyzed one more time. All the samples of the pike and the rudd kidney were diluted too much. In order to obtain measurements within the criteria, the kidney samples from pike and rudd should have been diluted 2 times, not 5 times.

Catalase is a very sensitive enzyme and can quickly be inactivated when the samples are overheated (Johansson & Borg, 1988). In addition, the enzyme is very unstable at high dilution, hence the samples were diluted right before assaying. Therefore, the handling and storage of the CAT activity samples may have affected the analysis and the results.

4.6.8 Glutathione peroxidase (GPx)

In the GPx enzyme kit it is a requirement that the added amount of GPx in the well should cause a decrease in absorbance at 340 nm per minute between 0.02 and 0.135. In the analysis of the GPx activity in the pike liver, all the samples had a decrease in absorbance within the given interval. All the samples were therefore considered valid. One pike sample from Glubokoye (G-3) had a decrease in absorbance of 0.027 per minute (Appendix J), which is close to the limit of the given range for valid results. However, the background ΔA_{340} absorbance was 0.0298 per minute, which is a higher value than the absorbance for liver sample G-3. Since the background absorbance is subtracted from absorbance to the sample, the final value for the one sample "G-3" would be negative. For that reason, the G-3 sample

was excluded from further analysis of the GPx activities. There were no significant differences in the GPx activity in the contaminated fish compared to the control fish. The storage time can have affected the GPx activity. It may thus be important to analyze the samples shortly after the fish are caught and dissected.

4.6.9 Glutathione reductase (GR)

In the GR kit it is a requirement that the amount of GR added to the well should cause a decrease in absorbance at 340 nm between 0.008 and 0.1 per minute. The results of the analysis showed that only one liver sample (K-2) from Kashovka and two liver (G-3 and G-11) samples from Glubokoye had a decrease within the given range (Appendix K). All the liver samples, except those three, had a reduction in A_{340} absorbance below 0.008 per minute. Therefore, the results are too low to be considered valid. The samples could have been concentrated using an Amicon centrifuge concentrator with 10,000 MW cut-off. However, the samples were not analyzed one more time since the first analysis indicated that the GR activity in the fish samples was inactivated. *Previous studies, (Jung et al., 1993),* have shown that the enzyme is stable for a few weeks when the tissue are stored in the freezer at -80 °C. The pike tested in this study have been stored since March 2019. The long storage time may have inactivated the GR enzyme in the sample.

5 Suggestion for further work

There is still a high concentration of long-lived radionuclides, such as ^{137}Cs and ^{90}Sr, in both Glubokoye and the fish. However, it is not proven that ionizing radiation affects the enzyme activity in the fish. The stability of the enzyme during storage is questionable (Jung et al., 1993; Liu et al., 2013). Superoxide dismutase is known to be a stable enzyme, while glutathione reductase is known to be less stable. To exclude the storage time as a source of error, the fish could have been analyzed shortly after it was caught.

In the contaminated rudd there was an indication of higher enzyme activity of SOD relative to the control rudd. Higher SOD activity was not observed in the contaminated pike. The total dose-rate were higher for rudd compared to the pike from Glubokoye. For further work, there should be more focus on the rudd, as well as study of pathological changes in the rudd tissue. With only five samples from each lake, the SOD activity in the rudd kidney was determined. To substantiate the observation that there is higher SOD activity in the contaminated fish, more fish should be analyzed. Multiple number of samples are recommended in all studies, since this makes the results more representative.

6 Conclusion

The exposure levels of ^{137}Cs and ^{90}Sr and the dose calculation of the radionuclides clearly showed that the fish from the contaminated lake, Glubokoye, was exposed to high ionizing radiation. Both internal and external radiation. In addition, there were signs of pathological changes in the fish tissue. Both the liver and the spleen tissue showed morphological changes in the structure of the cells and the tissue. However, the results have not identified if exposure to radiation over three decades have negatively affected the general heath in the pike from Glubokoye.

Even though the ionizing radiation have an effect on the tissue, measurements of the enzyme activity showed no significant differences in the proportion of reactive oxygen species. The activity of antioxidant enzymes in the fish from Glubokoye is not higher than in the fish from the control lake. These results indicate that the fish from the contaminated lake, Glubokoye, does not experience oxidative stress due to chronic exposure of ionizing radiation from ^{137}Cs and ^{90}Sr after the Chernobyl accident.

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Appendix A – Calculation of exposure doses

Table A.1 *Masses and dimensions used in "Add organism" function in ERICA.*

Species	Ratios	Mass
	L:H:W	**Kg**
Pike	1:0.14:0.14	0.1
	1:0.14:0.14	0.25
	1:0.14:0.14	0.5
	1:0.14:0.14	1
	1:0.14:0.14	2
	1:0.14:0.14	3
Rudd	1:0.333:0.167	0.01
	1:0.333:0.167	0.02
	1:0.333:0.167	0.03
	1:0.333:0.167	0.075

Equation used for calculation of the whole-body dose-rates:

Internal whole-body dose-rate, $\dot{D}^{j}_{int,r}$ (µGy/h):

$$\dot{D}^{j}_{int,r} = A^{j}_{tissue,r} \times k_{tissue \rightarrow wb,r} \times DCC^{j}_{int-wb,r}$$

Where:

$A^{j}_{tissue,r}$ - activity concentration of radionuclide 'r' in tissue (muscle or bone) of organism 'j', unit Bq/kg

$k_{tissue \rightarrow wb,r}$ - tissue to whole-body conversion factor for radionuclide 'r' for fish

$DCC^{j}_{int-wb,r}$ - Dose conversion factor (internal whole-body) for radionuclide 'r' and geometry corresponding to organism 'j', unit µGy/h per Bq/kg

External whole-body dose-rate, $\dot{D}_{ext,r}^{j}$ µGy/h:

$$\dot{D}_{ext,r}^{j} = A_{water,r} \times DCC_{ext-wb,r}^{j}$$

Where:

$A_{water,r}$ - Activity concentration of radionuclide ‘r’ in water, unit Bq/L

$DCC_{ext-wb,r}^{j}$ - Dose conversion factor (external whole-body) for radionuclide ‘r’ and geometry corresponding to organism ‘j’, unit µGy/h per Bq/KG

Appendix B – Overview: Equipment, reagents, solutions and plate set up

Table B.1 *shows which instruments and equipment used in the work with the study. All the plastic equipment was new.*

Instrument/equipment	Specification	Supplier
Homogenizer	FastPrep-24™ 5G	MP Biomedicals Europe, Illkirch, France
Ultrasound sonication	1/8" Microtip, Digital Sonifier 450	Branson Ultrasonics Corporation, Danbury, CT, USA
Allegra 64R Centrifuge	For Eppendorf tubes, Allegra 64R Centrifuge	Beckman coulter, Life Sciences Division Headquarters, Indianapolis, IN, USA
NanoDrop	A280, 2000 spechtrophotometer	Thermo Scientific, Wilmington, DE, USA
Multiskan Ascent	Spectrophotmeter, 96-well plate reader	Thermo Labsystems, USA
Multi-channel pipettes	20 µl and 200 µl pipettes	Thermo scientific, Finland
Automatic pipettes	20 µl and 100-1000	Thermo scientific, Finland
Pipette tips		
Eppendorf tubes		
96 well micro-plate		
Ultracut microtome	Leica EM UC6	Germany
Light microscope	Leica DM6B	Germany

Table B.2 *presents an overview of the reagents and solutions used in determination of SOD, CAT, GPx, and GR.*

Equipment/reagents	Specification	Supplier
Sample buffer	20mM phosphate buffer pH 7.4, 1 mM EDTA, 0,9% NaCl and 0,1 % Triton X-100)	NMBU
SOD Assay Kit-WST	Sigma-Aldrich item number 19160	Sigma-Aldrich, St. Louis, MO, USA
WST solution	5 ml	Sigma-Aldrich, St. Louis, MO, USA
Enzyme solution	100 µl	Sigma-Aldrich, St. Louis, MO, USA
Buffer solution	100 ml	Sigma-Aldrich, St. Louis, MO, USA
Dilution buffer	50 ml	Sigma-Aldrich, St. Louis, MO, USA
Manual	Item number 707015	Sigma-Aldrich, St. Louis, MO, USA
Catalase Assay Kit	Cayman Chemical kit, item number 707002	Cyman Chemical, Ann Arbor, MI, USA
Catalase assay buffer (10x)	Item number 707010	Cyman Chemical, Ann Arbor, MI, USA
Catalase sample buffer (10x)	Item number 707012	Cyman Chemical, Ann Arbor, MI, USA
Catalase formaldehyde standard	Item number 707014	Cyman Chemical, Ann Arbor, MI, USA
Catalase control	Item number 707013	Cyman Chemical, Ann Arbor, MI, USA
Catalase potassium Hydroxide	Item number 707015	Cyman Chemical, Ann Arbor, MI, USA
Catalase hydrogen peroxide	Item number 707011	Cyman Chemical, Ann Arbor, MI, USA

Catalase purpald (Chromogen)	Item number 707017	Cyman Chemical, Ann Arbor, MI, USA
Catalase potassium periodate	Item number 707018	Cyman Chemical, Ann Arbor, MI, USA
96-well solid plate	High-binding Item number 400010	Cyman Chemical, Ann Arbor, MI, USA
96-well cover sheet	Item number 400012	Cyman Chemical, Ann Arbor, MI, USA
Glutathione reductase assay kit	Cayman Chemical kit, item number 703202	Cyman Chemical, Ann Arbor, MI, USA
GR assay buffer (10x)	Item number 703210	Cyman Chemical, Ann Arbor, MI, USA
GR sample buffer (10x)	Item number 703212	Cyman Chemical, Ann Arbor, MI, USA
GR Glutathione reductase (control)	Item number 703214	Cyman Chemical, Ann Arbor, MI, USA
GR GSSG	Item number 703216	Cyman Chemical, Ann Arbor, MI, USA
GR NADPH	Item number 703218	Cyman Chemical, Ann Arbor, MI, USA
96-well solid plate	Colorimetric assay Item number 400014	Cyman Chemical, Ann Arbor, MI, USA
96-well cover sheet	Item number 400012	Cyman Chemical, Ann Arbor, MI, USA
GPx assay kit	Cayman Chemical kit, item number 703110	Cyman Chemical, Ann Arbor, MI, USA
GPx assay buffer (10x)	Item number 703110	Cyman Chemical, Ann Arbor, MI, USA
GPx sample buffer (10x)	Item number 703112	Cyman Chemical, Ann Arbor, MI, USA
Glutathione peroxidase (control)	Item number 703114	Cyman Chemical, Ann Arbor, MI, USA

GPx Co-substrate mixture	Item number 703111	Cyman Chemical, Ann Arbor, MI, USA
GPx cumene hydroperoxide	Item number 703118	Cyman Chemical, Ann Arbor, MI, USA
GPx NADPH	Item number 703119	
96-well solid plate	Colorimetric assay Item number 400014	Cyman Chemical, Ann Arbor, MI, USA
96-well cover sheet	Item number 400012	Cyman Chemical, Ann Arbor, MI, USA

Appendix C – Correlation factor

Table C.1 *Correlation factor (R^2) between the enzyme activity and the protein concentration. $R^2 < 0.4$ indicates no correlation. The fish tissue marked with * is analyzed with a great spread in the protein concentration (0.551 to 1.893 mg/ml). The other tissue is analyzed with a protein concentration within 0.5 to 0.8 mg/ml.*

Fish tissue	N	Site	R^2 SOD activity	R^2 CAT activity	R^2 GPx activity
Pike liver	9	Kashovka	0.2072	0.2061*	0.4989*
Pike liver	9	Glubokoye	0.1506	0.0718*	0.5594*
Pike kidney	9	Kashovka	0.0949	0.0439	-
Pike kidney	9	Glubokoye	0.075	0.0771	-
Rudd kidney		Kashovka	0.2122	0.0135	-
Rudd kidney	9	Glubokoye	0.0304	0.064	-

Appendix D – Superoxide dismutase in pike liver

Table D.1 *SOD activity for the liver sample of the pike from Kashovka (K) and Glubokoye (G). The protein concentration within the range 0.5 to 0.8 mg/ml. Three replicates for each pike have been used. N = 18.*

Fish	Protein concentration [mg/ml]	SOD activity [U/mg protein concentration]
K-1	0.621	291.54
K-2	0.732	169.45
K-3	0.653	423.55
K-4	0.726	400.06
K-5	0.732	253.65
K-6	0.825	174.06
K-7	0.603	432.10
K-8	0.629	213.99
K-9	0.770	209.86
G-1	0.724	430.96
G-2	0.614	291.10
G-3	0.726	251.50
G-6	0.578	469.18
G-7	0.504	402.02
G-8	0.706	333.77
G-9	0.618	209.40
G-10	0.693	212.71
G-11	0.700	215.03

Appendix E - Superoxide dismutase in pike kidney

Table E.1 *SOD activity in the kidney samples of the pike from and Kashovka (K) and Glubokoye (G). The protein concentration within the range 0.5 to 0.8 mg/ml. Three replicates for each pike have been used. N = 18*

Fish	Protein concentration [mg/ml]	SOD activity [U/mg protein concentration]
K-1	0.882	85.33
K-2	0.512	120.16
K-3	0.537	72.53
K-4	0.569	138.67
K-5	0.962	99.59
K-6	0.824	101.73
K-7	0.632	106.18
K-8	0.701	95.73
K-9	0.731	79.91
G-1	0.697	140.94
G-2	0.877	101.89
G-3	0.600	119.55
G-6	0.513	133.43
G-7	0.847	118.14
G-8	0.721	54.32
G-9	0.693	115.73
G-10	0.743	109.12
G-11	0.684	84.76

Appendix F – Superoxide dismutase in rudd kidney

Table F.1 *An overview over the kidney samples of the rudd from Kashovka (K) and Glubokoye (G). Protein concentration within the range 0.5 to 0.8 mg/ml. Three replicates for each pike have been used. N = 10.*

Fish	Protein concentration (mg/ml)	SOD activity [U/mg protein concentration]
K-11	0.604	92.21
K-12	0.689	81.71
K-13	0.622	66.73
K-14	0.602	79.68
K-15	0.799	93.05
G-1	0.552	98.00
G-2	0.587	89.66
G-3	0.829	105.92
G-4	0.853	90.44
G-5	0.576	114.97

Appendix G – Catalase in pike liver

Table G.1 *CAT activity of the pike kidney added in the cells. For reproducible results should the CAT amount added to the well be between 2-35 nmol/min/ml. K-1, K-6, G-8 and G-10 is not within the range (marked with red). These samples are considered non-reproducible and, are not included in further calculations of CAT activity. Three replicates have been used, N=18.*

Fish	CAT activity [nmol/min/ml]
K-1	51.24
K-2	2.32
K-3	25.58
K-4	17.87
K-5	9.83
K-6	64.40
K-7	34.12
K-8	32.55
K-9	21.07
G-1	24.40
G-2	31.50
G-3	5.49
G-6	32.90
G-7	34.64
G-8	68.13
G-9	34.31
G-10	45.80
G-11	33.45

Table G.2 *CAT activity for pike liver samples from Kashovka (K) and Glubokoye (G). CAT activity is shown and calculated using the equation from the formaldehyde standard curve (Figure 4.2). K-1, K-6, G-8 and G-10 (marked in red) had activity in the wells below the specific interval for reproduceable results. Three replicates have been used, N = 18.*

Fish	Protein concentration [mg/ml]	CAT activity [nmol/min/mg]
K-1	1.153	333,33
K-2	1.163	14,99
K-3	0.734	261,37
K-4	0.781	171,61
K-5	1.204	61,23
K-6	1.320	365,91
K-7	0.551	464,37
K-8	1.279	190,88
K-9	1.893	83,48
G-1	0.728	251,39
G-2	1.192	198,22
G-3	0.916	44,94
G-6	0.615	401,24
G-7	1.029	252,48
G-8	0.961	531,75
G-9	1.781	144,48
G-10	1.620	210,86
G-11	1.168	214,81

Appendix H - Catalase in pike kidney

Table H.1 *Amount CAT activity of the pike kidney added in the cells. For reproducible results should the CAT amount added to the well be between 2-35 nmol/min/ml. Only K-6 is within the given interval. N=18*

Fish	CAT activity [nmol/min/ml]
K-1	0.741
K-2	1.623
K-3	0.628
K-4	0.267
K-5	0.809
K-6	2.460
K-7	1.058
K-8	0.990
K-9	0.357
G-1	1.352
G-2	0.086
G-3	1.194
G-6	0.583
G-7	0.877
G-8	0.063
G-9	0.312
G-10	1.442
G-11	0.448

Table H.2 *CAT activity in the kidney samples of the pike from Kashovka (K) and Glubokoye (G). Protein concentration within the range 0.5 to 0.8 mg/ml. Two replicates for each pike have been used. N = 20.*

Fish	Protein concentration [mg/ml]	CAT activity [nmol/min/mg]
K-1	0.680	5.45
K-2	0.507	16.01
K-3	0.687	4.57
K-4	0.686	1.94
K-5	0.621	6.52
K-6	0.822	14.96
K-7	0.733	7.22
K-8	0.607	8.16
K-9	0.735	2.43
G-1	0.742	9.11
G-2	0.665	0.65
G-3	0.731	8.16
G-6	0.718	4.06
G-7	0.702	6.25
G-8	0.684	0.46
G-9	0.786	1.98
G-10	0.706	10.21
G-11	0.648	3.45

Appendix I - Catalase in rudd kidney

Table I.1 *Amount CAT activity of the pike kidney added in the cells. For reproducible results should the CAT amount added to the well be between 2-35 nmol/min/ml. K-13, K-15 and K-18 (marked in red) have negative CAT activity Only K-6 is within the given interval. N=20*

Fish	CAT activity [nmol/min/ml]
K-11	0.922
K-12	0.357
K-13	-0.005
K-14	0.154
K-15	-0.321
K-16	1.420
K-17	2.595
K-18	-0.027
K-19	2.347
K-20	1.329
G-1	1.465
G-2	1.781
G-3	0.809
G-4	1.691
G-5	1.103
G-6	2.686
G-7	1.488
G-8	1.420
G-9	2.007
G-10	1.374

Table I.2 *CAT activity of the kidney samples of the rudd from Kashovka (K) and Glubokoye (G). Protein concentration within the range 0.5 to 0.8 mg/ml. K-13, K-15 and K-18 (marked in red) have negative CAT activity. Two replicates for each pike have been used. N = 20.*

Fish	Protein concentration [mg/ml]	CAT activity [nmol/min/mg]
K-11	0.667	6.91
K-12	0.699	2.55
K-13	0.681	-0.03
K-14	0.763	1.01
K-15	0.589	-2.73
K-16	0.750	9.46
K-17	0.752	17.26
K-18	0.736	-0.18
K-19	0.741	15.83
K-20	0.662	10.04
G-1	0.537	13.64
G-2	0.769	11.58
G-3	0.659	6.14
G-4	0.625	13.53
G-5	0.735	7.50
G-6	0.651	20.63
G-7	0.548	13.57
G-8	0.639	11.11
G-9	0.714	14.06
G-10	0.562	12.23

Appendix J – Glutathione peroxidase (GPx) in pike liver

Table J.1 *GPx activity in the pike liver sample from Kashovka (K) and Glubokoye (G). The decrease in absorbance after the background absorbance been subtracted. G-3 (marked in red) has a negative decrease in absorbance and has therefore considered to be non-reproducible. Three replicates have been used for sample. N = 18.*

Fish	ΔA340/min	GPx activity [nmol/min/ml]	GPx activity normalized [nmol per min/mg]
K-1	0.0496	252.65	219.13
K-2	0.0115	58.58	50.37
K-3	0.0746	380.00	517.71
K-4	0.0618	314.80	403.07
K-5	0.0645	328.55	272.88
K-6	0.0564	287.29	217.65
K-7	0.0607	309.20	561.15
K-8	0.0883	449.79	351.67
K-9	0.0754	384.08	202.89
G-1	0.0568	289.33	397.43
G-2	0.0044	22.41	18.80
G-3	-0.0027		
G-6	0.0473	240.94	391.77
G-7	0.0698	355.55	345.53
G-8	0.0627	319.38	332.34
G-9	0.0112	57.05	32.03
G-10	0.0685	348.93	214.20
G-11	0.0778	396.30	339.30

Appendix K – Glutathione reductase (GR) in pike liver

Table K.1 *GR activity in the pike liver sample from Kashovka (K) and Glubokoye (G). The decrease in absorbance after the background absorbance has been subtracted. Background absorbance is 0.0011. The samples marked in red (K-2, G-3 and G-11) has an absorbance within the given range (0.008 and 0.1 absorbance per minute) for reproducible results. Three replicates have been used for each pike liver sample. N = 18.*

Fish	ΔA340/min	GR activity [nmol per min/mg]
K-1	0.0021	0.928
K-2	0.0166	7.27
K-3	0.0033	2.29
K-4	0.0034	2.22
K-5	0.002	0.846
K-6	0.0066	2.55
K-7	0.0023	2.13
K-8	0.0018	0.717
K-9	0.0019	0.511
G-1	0.0015	1.05
G-2	0.0017	0.73
G-3	0.0124	6.90
G-6	0.0012	0.99
G-7	0.0021	1.04
G-8	0.0034	1.80
G-9	0.0034	0.97
G-10	0	0.00
G-11	0.0081	3.53

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