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Hepatic responses to chronic exposure to hydrogen sulfide in post-smolt Atlantic salmon

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Abstract

A major threat in marine land-based recirculating aquaculture systems (RAS) is the production of hydrogen sulfide (H_2S). In many physical and biological systems, hydrogen sulfide (H_2S) is a naturally occurring chemical that can be produced either endogenously or exogenously. In recent years, mass mortality events associated with H_2S has become an issue in RAS-based farming of Atlantic salmon. We have limited understanding however, on how H_2S affects Atlantic salmon physiology. In this study, the effects of chronic sub-lethal level of H_2S in post smolts Atlantic salmon in RAS was investigated. Three different concentrations were 0 µg (control), 1 µg (low), and 5 µg H_2S/L (high), and continuous exposure was carried out for 4 weeks. Thereafter, fish were allowed to recover for 2 weeks Due to its importance in xenobiotic metabolism, understanding the effects of H_2S exposure was focused on the liver using gene expression analysis of key biomarker genes and histology. In addition, biochemical markers for liver health were analysed in plasma. The expression of most gene markers was significantly upregulated in the low H_2S concentration group at week 2 followed by downregulation at week 4. With the increase in the concentration of H_2S, the recovery rate was decreased except in *catalase (cat)* and *creba* where the recovery rate was found higher in low concentrations as compared to control and high H_2S concentrations. Further biochemical marker test shows very prominent recovery in high doses of H_2S after the removal of H_2S. These results show that though H_2S influences the liver it can recover from that even though the recovery is minimal and provide information on how Atlantic salmon react to H_2S.

List of Abbreviations

ALAT	Alanine transaminase
ASAT	Aspartate aminotransferase
aldob	*aldolase, fructose-biphosphate B*
cat	*catalase*
CK	Creatine Kinase
cDNA	Complementary DNA
creba	*cyclic-AMP response element binding protein A*
cu/znsod	*cu/zn superoxide dismutase*
cyp1a	*cytochrome P450 1AS*
fbp1	*fructose-biphosphate*
FAO	Food and Agriculture Organization
gpx	*glutathione peroxidase*
hsp70	*heat shock protein 70*
hsp90	*heat shock protein 90*
H_2S	Hydrogen sulfide
LDH	Lactate dehydrogenase
MBBR	Moving bed biofilter reactor
PCR	Polymerase chain reaction
pkm	*pyruvate kinase M 1/2*
pgk	*phosphoglycerine kinase*
RAS	Recirculating Aquaculture System
RNA	Ribonucleic acid
ROS	Reactive oxygen species
soux	*sulfite oxidase*
sqor1	*sulfide: quinone oxidoreductase 1*
taldo	*transaldolase*
UV	Ultraviolet

Table of Contents

1. Introduction 1

2. Background 2

2.1 Atlantic salmon life cycle 2

2.2 Atlantic salmon aquaculture in Norway 3

2.3 Challenges in salmon farming 3

2.4 Recirculating aquaculture system (RAS) 5

2.5 H_2S in RAS 6

2.6 H_2S in liver 8

3. Materials and methods 10

3.1 Experimental approach 10

3.2 Ethical use of animals in research 10

3.3 Experimental fish 10

3.4 Husbandry conditions 10

3.5 Experimental setup 11

3.5.1 Dosing of H_2S 11

3.6 Sampling procedure 11

3.7 Transport of sample 12

3.8 Gene expression analysis 13

3.8.1. Primers 13

3.8.2 RNA Isolation and Quantification 14

3.8.3 Preparation for complementary DNA (cDNA) 15

3.8.4 Real-time Quantitative PCR and gene analysis expression 15

3.9 Histological assessments 16

3.11 Data Analysis 17

4. Results 18

4.1 Gene expression profiles in the liver 18

4.1.1 Sulfide detoxification genes 18

4.1.2 Stress response genes 19

4.2 Histology 21

5. Discussion 23

5.3 Metabolic genes in liver 25

5.4 Histology 26

5.5 Biochemical marker test 26

6. Conclusion 28

7. References 29

8. Appendix 40

1. Introduction

Aquatic food production is anticipated to grow by 14% in 2030, with aquaculture accounting for a significant portion of this expected expansion. Aquaculture significantly contributes to the security of the world's food supply by providing 89% (157 million tonnes) of produce from the aquatic environment used for direct human consumption and the remaining for non-food uses like fish meal and fish oil (FAO, 2022) Because of the industry's rapid growth, new intensive farming techniques have been developed, thereby improving production, environmental control, biosecurity, and environmental sustainability. Land-based recirculating aquaculture systems (RAS) are one of the promising farming technologies.

Atlantic salmon (*Salmo salar*) aquaculture is rapidly expanding, with around 2.7 million tonnes production in 2020 (FAO, 2022). The production cycle has two phases: a land based smolt production and grow out phase in sea cages until harvest (Bergheim et al., 2009). According to FAO fisheries and aquaculture estimates, output, consumption, and trade will all expand through 2030, albeit at slower growth rates. The increase in global trade, decrease in wild fish availability, competitive product pricing, rising incomes, and urbanization have all fuelled the growth of aquaculture, which has increased the global per capita consumption of seafood. The advantages of a controlled production environment, such as the reduction of adverse environmental impacts, a flexible location, and high biosecurity, have contributed to the increased adoption of RASs to produce smolts and post-smolts (Martins et al., 2010; Lazado & Good, 2021).

Recirculating aquaculture systems (RAS) enable high-yield fish production with little water consumption (0.1–1 m^3/kg feed) (Martins et al.,2010). However, intensive production and water reuse are not without their obstacles. In recent years, hydrogen sulfide (H_2S) has been recognized as an issue in RAS due to the potential for H_2S formation, especially under saline conditions. H_2S is a hazardous gas linked to salmonid mass mortalities, which has negative economic ramifications for aquaculture producers (Sommerset et al., 2020). H_2S production has been a problem in wastewater treatment facilities and different industrial processes for decades, partly because of its corrosive qualities, high toxicity, and odor nuisance. The physiological impacts of H_2S on salmon is limited, hence can be a challenge in knowing how to solve the issues related mortality, fish health and welfare.

2. Background

2.1 Atlantic salmon life cycle

Atlantic salmon (*Salmo salar*), a member of the Salmonidae family, is an anadromous fish species which means they are born in freshwater environments (lakes, rivers, and streams), migrate to the sea as juveniles and develop into adults, and then return to the freshwater environment to spawn and deposit their eggs (Webb et al., 2007). More than 400 waterways in Norway contain Atlantic salmon, and the country is home to 25% of the world's healthy populations (Forseth et.al, 2017). The life cycle of an Atlantic salmon has several stages, i.e., egg, alevins, fry, parr, smolt, and adult salmon (**Figure 1**).

The eggs, which are laid in the gravel of freshwater rivers and streams, mark the beginning of the life cycle of the Atlantic salmon. The temperature significantly impacts egg and alevin development, making it the most significant indicator of egg hatching. It takes around 70 to 160 days depending on temperature to transform from egg to alevins, and it stays in the gravel for an additional time while feeding on the yolk sac connected to their bodies (Webb et al., 2007). The alevins emerge from the rock and become free-swimming fry once the yolk sac is completely consumed. The young spend their first year in freshwater, eating insects and other tiny aquatic organisms for food. Fry transforms into parr with spots and vertical lines for camouflage.

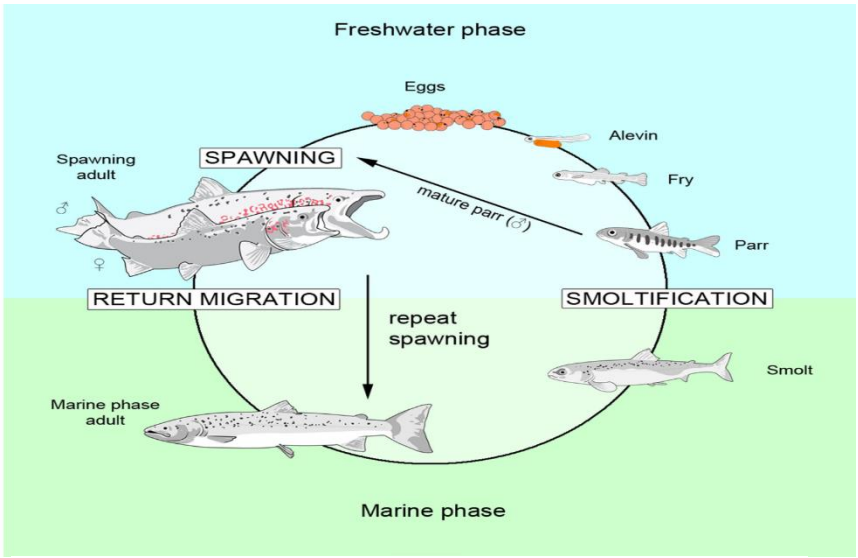


Figure 1. The life cycle of Atlantic salmon (Mobley et al., 2021)

Atlantic salmon undergo a physiological transformation known as smoltification in the second stage of their life cycle, which helps them adjust to a saltwater habitat. It returns to its original freshwater habitats to reproduce to complete the process. Migrating adults go through colour and morphological changes, especially when they enter freshwater rivers. Spawners have brownish-red skin, but males grow larger fins and a unique hooked jaw than females.

2.2 Atlantic salmon aquaculture in Norway

Norway is the world's top producer of farm-raised Atlantic salmon. Norway's total harvest of Atlantic salmon increased by 12% from 2020 to 2021, totalling 1,532,100 tonnes (Stormer, 2022). The majority of Norwegian aquaculture is industrial, contemporary, and fiercely competitive. In the 1970s, businesses in Norway started breeding and growing Atlantic salmon in cages in the nation's coastal waters (Thodesen & Gjedrem, 2006). The Norwegian fish farming sector is anticipated to grow five times faster than the global average by the year 2050 due to the expanding markets for seafood and bio marine products (Olafsen et al., 2012). Most of these farms are found in the northern and western parts of the nation, where the salmon can develop most effectively in the cold, clear waters. Although salmon farming has significantly boosted Norway's economy, there are concerns about how it affects the environment. Organic wastes like feces and uneaten feed, as well as inorganic nutrients like ammonium (NH_4^+) and phosphate (PO_4^{-3}) are discharged into the environment (Skogen et al., 2009). These nutrients may contribute to hazardous algal blooms, which can be highly destructive to fish, seabirds, marine mammals, and other marine life. The Norwegian government has taken action to control the salmon farming sector and lessen its adverse environmental effects. To lessen the pollution salmon farms cause, the government unveiled new restrictions in 2017 (FAO, 2021). These rules mandate that farmers limit waste production and monitor and report on the amounts of nitrogen and phosphorus in the water surrounding their fields.

2.3 Challenges in salmon farming

Salmon farming has expanded dramatically in recent years to accommodate the rising demand for fish as a protein source. Salmon production is economical because the cycle is well understood and under control (Southgate & Lucas, 2012), and efficient farming technology is available (Lekang, 2013). Two stages are usually used in the production process: a freshwater phase in tanks on land from egg to smolt, ready to survive in sea water (typically 100 g), and a grow-out stage until harvest (about 4-6 kg) (Lekang et al., 2016). Open sea cages are used for salmon farming during the grow-out period when the fish get more extensive and require more

room and water exchange. These offer an efficient and cost-effective system when managed well and at an appropriate scale.

As any other industries, salmon aquaculture faces several challenges which include high mortality events. In marine farms, losses of 60 million fish were reported in 2021. The exact causes of such high mortality levels remain unclear. However, many fish died after rigorous handling techniques, such as delousing methods (Dyrevernalliansen, 2022). Also, sea lice feeding behavior may result in mechanical harm, such as skin and fin erosion on fish (Bjørn & Finstad, 1997; Dawson et al. 1998) and can result in osmotic stress and mortality (Grimnes and Jakobsen 1996; Nolan et al. 1999; Finstad et al. 2000). The environmental impact of salmon aquaculture is one of the main problems. Large, open-net pens are frequently used in the sea for salmon farming, which may hurt wild fish populations and the local ecosystem. Salmon from farms may get out of their confines and breed with salmon from the wild, which may have genetic effects on the wild population. The presence of disease in salmon farms is another challenge. The industry may suffer considerable financial losses because of disease that can spread quickly and affect salmon farmed in close quarters. Diseases have historically been the biggest issue in the Atlantic salmon farming industry in which the first diseases to be identified as being responsible for fish deaths were cold-water vibriosis (caused by *Vibrio salmonicida*), furunculosis (caused by *Aeromonas salmonicida* subsp. *salmonicida*), and infectious pancreatic necrosis (caused by IPN virus) (Tilseth, 1991). On the other hand, increasing organic loads and nutrients in the marine environment because of salmon aquaculture intensification were primarily caused by surplus feeds, fish excrement, and other metabolic waste products (Strain & Hargrave 2005). The build-up of this organic material causes eutrophication and sedimentation, which may significantly alter the hydrochemical and biogeochemical processes in the nearby waters (Strain & Hargrave, 2005; Valdemarsen et al., 2012).

These significant difficulties in raising Atlantic salmon in open sea cages have made it easier for farmers to embrace cutting-edge farming techniques, like recirculating aquaculture systems (RAS). These land-based salmon farming techniques shorten the time spent maturing at sea, where they are more exposed to challenging environmental circumstances. 90% of European farms raising Atlantic salmon, mainly in Scotland and Norway, use land-based flow-through systems to produce salmon smolts; however, due to seasonal water shortages and low water temperatures, conversion to RAS technology became an alternative (Glover et al., 2019). In recent years, RAS-produced smolts make up around 70% of those used in marine cage farming in Norway (Meriac, 2019) and to prevent disease from spreading and, if it does, to limit the

spread of pathogens, disinfection is first and foremost a crucial component of biosecurity measures (Lazado & Good, 2021). The aquaculture sector can boost productivity, lower biosecurity hazards, and enhance ocean quality by implementing cutting-edge digital technology like blockchain and AI feeding (Global Salmon Initiative, 2021).

2.4 Recirculating aquaculture system (RAS)

One of the key drivers of the expansion of salmon farming has been the creation and enhancement of new farming technologies that are expected to address a variety of challenges that hamper development. Recirculating aquaculture system (RAS) raises fish, crustaceans, and other aquatic organisms by simulating a natural marine habitat. The method reduces the amount of water needed for aquaculture production and lessens the environmental impact of fish farming by constantly filtering and reusing the water up to 90-99% (Badiola et al., 2012). RAS technology has gained favor recently as it responds to challenges in sustainable food production.

RAS is a closed system with fish tanks, a filtration system, and water treatment incorporated into one production line (**Figure 2**). It aims to ensure a rearing environment that supports fish's health and welfare. RAS consists of (1) growing tank, (2) sump of particulate removal device, (3) biofilter, (4) oxygen injection with U-tube aeration and, (5) water circulation pump. The most crucial RAS process is solids removal, which, when carried out well, improves the performance of other system elements. As soon as they are produced, solids like as uneaten feeds and excreta must be eliminated since their presence can cause biofouling, NH_3 generation, oxygen depletion, a high microbial load, and finally the onset of disease in the system (Neethu et al., 2020). A vital element of the filtration section of a RAS is the biological filter, or biofilter. The biofilter is the main location where biological nitrification takes place, and it houses the nitrifying bacteria. The dissolved nitrogenous waste products emitted by the aquaculture aquatic species are processed by nitrifying bacteria. This biofilter has a medium (corrugated plastic sheets, beads, or sand grains) where bacteria can grow. Beneficial bacteria eliminate (detoxify) fish excretory products, primarily ammonia, in the biofilter (Holan et al., 2020). Thus, making RAS the most environmentally friendly at a commercially viable level.

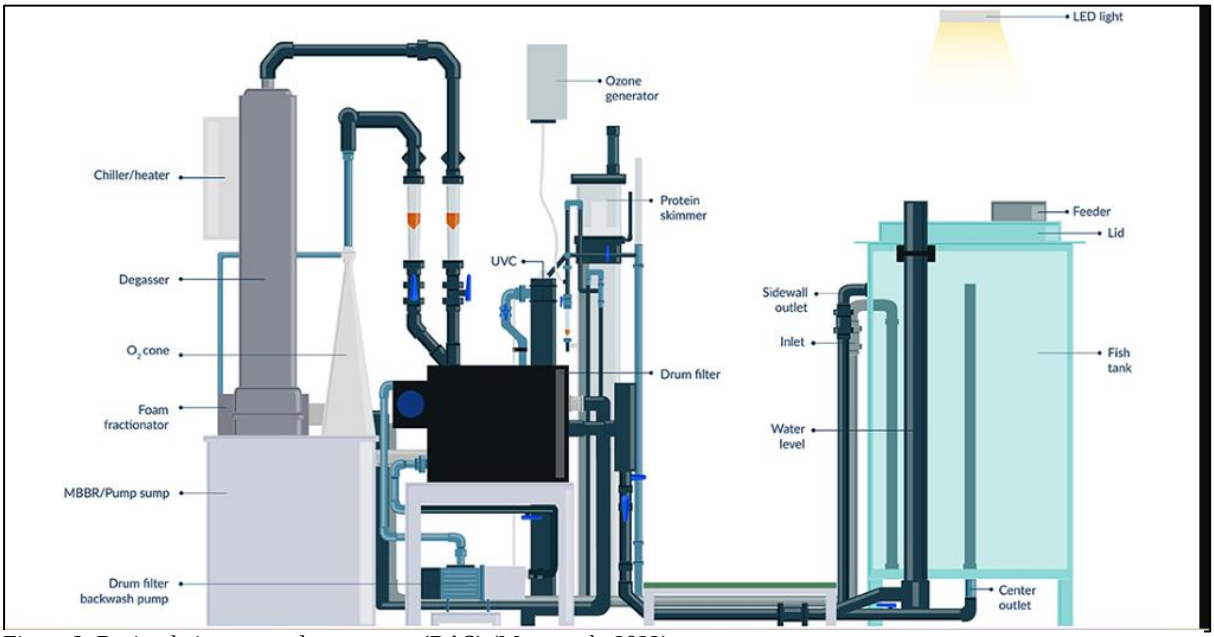


Figure 2. Recirculating aquaculture system (RAS) (Mota et.al., 2022)

The moving bed biofilter reactor (MBBR) and trickling filters are the two most often utilized forms of biofilter in Norwegian RAS farms. Water from the biofilter reactor is sent to the degassing unit to remove primarily unfavorable gases for fish, such as carbon dioxide (CO_2) and free nitrogen (N_2). The water's oxygen saturation decreases after going through the biofilter and degasser; therefore, an aeration unit raises it to 90%. An oxygen enrichment device is used to add pure oxygen to the water to ensure that there is enough oxygen present. Microorganisms in the water, including bacteria, viruses, fungi, and tiny parasites, are then killed and eliminated by either an ultraviolet (UV) or ozone treatment unit before returning to the fish tanks. To make sure the water is suitable for the growth of both biofilms and fish in the system, critical water quality parameters, including pH, temperature, and dissolved oxygen, are measured, and monitored (Ebeling and Timmons, 2010). Although the principle sounds straightforward, the system's management and functioning are complicated. RAS's productivity is influenced by the culture species, stocking densities, feeding rates, length of the production cycle, and other management factors, and RAS have better control over the production outcomes (Badiola et al., 2012).

2.5 H_2S in RAS

Because of reuse of water in RAS there is a higher chance of accumulation of toxic compounds like particulate matters, ammonia, nitrite and nitrate which will have a negative impact on the system's operation and output while putting fish safety at risk (Liu et al., 2013). Sulphate is

abundant in seawater, with a more complex chemistry and ion concentrations 10-1000 times higher than freshwater. This is the primary reason why the risk of H_2S production is higher in saline than in fresh and marine water (Nazaroff & Alvarez-Cohen, 2001). Other factors include stagnant water and sludge, dead zones or corners, thick biofilm, too little water flow, which can cause dirt to collect inside pipes, and infrequent pipe.

H_2S is poisonous at higher concentrations to fish and other aquatic species and has a foul odour of "rotten eggs" (Harbison & Bourgeois, 2015; Cabillon & Lazado, 2022). Production of H_2S, a potent chemical asphyxiant, poses an even greater hazard to the welfare and health of fish in closed aquaculture systems (Letelier-Gordo et al., 2020). H_2S is produced as a by-product of microbial activity, especially in regions with low oxygen levels, such as sediment or biofilters. When sulphate-reducing bacteria (SRB) break down organic materials, they use sulphate (SO_4^{2-}) as a terminal electron acceptor, which produces H_2S (Dalsgaard, 2019; Muyzer & Stams, 2008). The sulfide production rates of SRB are in cleaning and flushing, and other technical issues that could cause water to backlash around the sediments and release all the H_2S that the sediments have formed (Yu & Bishop, 1998). H_2S has detrimental effects on fish, blocking oxygen release, causing cellular anoxia, and ultimately preventing the generation of ATP (Kiemer et al., 1995).

Previous reports of acute mortalities in salmon smolt production linked to H_2S toxicity were from Norwegian and Danish RAS plants (Hjeltnes et al., 2017; Sommerset et al., 2021; Bergstedt et al., 2022; Alipio et al., 2023; Bergstedt & Skov, 2023) influenced by factors like pH, temperature, sulphate concentrations, and the bioavailability of organic materials (Laanbroek & Pfennig, 1981; Muyzer & Stams, 2008; Plugge et al., 2011). A single acute dose of H_2S between 0.75 and 0.99 mg/L caused a tremendous deal of stress and gill tissue destruction (necrosis), which was thought to eventually cause liver damage, stunted growth, and increased susceptibility to illnesses (Letelier-Gordo et al., 2020). Higher (millimolar) H_2S exposure tends to be cytotoxic to cells because it produces free radicals and oxidants, mobilizes calcium, depletes glutathione, releases intracellular iron, and activates pathways that lead to mitochondrial cell death. Additionally, H_2S can cause rats to go into reversible hypothermia and a state like that of suspended animation (Szabó, 2007). After initial exposure to sulfide, general neurological problems in people frequently persist for longer than a month (National Research Council, 1979).

2.6 H_2S in liver

The liver is the primary detoxifying organ, with the capacity to process and eliminate toxic substances, medications, toxins from the environment, and endotoxins (Gao et al., 2008; Chen et al., 2019). It is also a primary site of H_2S synthesis and removal (Dilek et al., 2020; Wang, 2012). Exposure to H_2S causes the liver's enzyme activity to diminish, impacting the liver's capacity to detoxify hazardous compounds. Oxidative stress, brought on by H_2S exposure, harms liver cells and compromises liver function.

As gasotransmitter, H_2S is known to have a significant role in a variety of conditions including inflammation, septic shock, ischaemia-reperfusion injuries, cardiovascular disease, and more recently, liver physiology and chronic liver illnesses such non-alcoholic fatty liver disease (NAFLD) (Mateus & Prip-Buus, 2022). H_2S is produced either endogenously (host) or exogenously (environmental microbe). H_2S plays a variety of physiological functions in the body at low quantities, including vasodilation and neurotransmitter modulation. Higher effect at high concentrations, H_2S can damage the body excessively, leading to neurological, heart, and lung issues. Organisms have evolved several detoxification processes for H_2S, where liver plays an important role. The liver plays a crucial function in H_2S detoxification by converting H_2S to non-toxic compounds that can be excreted from the body. With the help of thiosulphate sulfurtransferase, the liver detoxifies H_2S by converting it to thiosulfate, and with the help of sulfite oxidase, the liver detoxifies H_2S by converting it to sulfate. According to the study (Shatalin et al., 2011), thiosulfate sulfurtransferase is essential for the liver's ability to detoxify H_2S. The researchers also discovered that elevated H_2S levels result in an upregulation of thiosulfate sulfurtransferase expression. The effects of various toxins on the livers of several fish species have been detailed by Malins, 1988, Myers et al. (1992), and Hinton et al. (1988). They demonstrated how the liver lesions evolved progressively throughout exposure and how each individual's development differs. They also demonstrated the possibility of liver regeneration and recovery from those injuries and neoplasm development.

Aim of the study

The goal of the present study was to explore the hepatic responses of Atlantic salmon post smolts to chronic sub-lethal exposure H_2S. The specific objectives are:

- to study the effect of sulfide, stress, and metabolism on gene expression of Atlantic salmon

- to study how the treatment groups are working by biochemical marker test
- to study about the increase in the calibre of hepatic sinusoids which would correspond to hepatic vascularization.

The main hypothesis was chronic sub-lethal level of H_2S affected the metabolic state and stress response in the liver in a dose-dependent manner, but recovery was from quick and not influenced by exposure concentration.

3. Materials and methods

3.1 Experimental approach

The fish trial was conducted at the research facility of Nofima (Norwegian Institute of Food, Fisheries, and Aquaculture Research) in Sunndalsøra, Norway. The laboratory analyses were performed in its biotechnology lab in Ås.

3.2 Ethical use of animals in research

All fish-handling techniques followed EU guidelines (Directive 2010/63/EU). The Norwegian Food Safety Authority approved the fish trial under FOTS ID 28927.

3.3 Experimental fish

The post-smolts Atlantic salmon (*Salmo salar*) of Bolaks strain ER 4-21 (ER =Erfjord) was produced and smoltified at Sunndalsøra Research Station. The fish were raised in a freshwater flow-through system before the experiment. Smoltification was verified by visual examination, a seawater test, and measurements of plasma chloride levels, which showed the ability to regulate plasma chloride levels below 150 mmol $L^{-}1$.

3.4 Husbandry conditions

Nine 1150-liter RAS units (Landing Aquaculture, Boxtel, Netherlands) received 450 mixed-sex post-smolts. Processes for removing sediments, ammonia, and CO_2, as well as for oxygenation and temperature control, were installed in the units. The units had a moving bed bioreactor (MBBR) and a drum filter upstream of a 500 L Cornell dual drain tank that ended in a pump sump. Water was returned to the tank through an oxygen cone, a chiller, and a CO_2 degasser with a side stream loop. The hydraulic retention time (HRT) was 16.7 minutes, and the water flow was 30 L/min. The average weight of the 50 fish in each tank was 162±2.5 grams.

The water was maintained at the optimum temperature (12.4 ± 0.3 °C), salinity (12.4 ± 0.2 ppt), pH (7.8 ± 0.1, adjusted daily with bicarbonate [$NaHCO_3$]), and dissolved oxygen saturation (>90%). Fish were fed a commercial meal (Micro Boost 80, Ewos, Bergen, Norway) four times per hour via a belt feeder, introduced to experimental tanks, and allowed to acclimatize for 3 weeks. The fish had not eaten for twenty-four hours before the tissue collection.

3.5 Experimental setup

The long-term sub-lethal exposure study involved continuous H_2S treatment over four weeks. Each of the three treatment groups had three RAS units in duplicate. Control (0 µg), low dose (1 µg), and high dose (5 µg H_2S/L) made up the treatment groups. The background H_2S concentrations in the control groups were discovered to be less than 0.3 µg/L. Dosing was stopped after four weeks, and the surviving fish were raised for another two weeks in the same systems and with the same husbandry practices. Each day, mortality was noted.

3.5.1 Dosing of H_2S

A 100-liter stock solution of sodium hydrosulfide hydrate ($NaSH*xH_2O$, Sigma-Aldrich, MO, USA) was made in a removable lid dosing tank filled with brackish water. Before being put in the dosing tank, the sodium hydrosulfide hydrate flakes were dissolved in water. A peristaltic pump (Watson-Marlow Fluid Technology Solutions, U530 series) and dosing tubes were used to deliver the solution to the treatment tanks after a stirring pump was initiated to ensure homogeneity. The stock solution dosage was the same for replica tanks.

With one sensor for each treatment group, three SeaRAS AquaSenseTM cabinets were used to monitor applied H_2O concentrations continuously. The sensors were positioned in the water exits of the fish tanks. The tanks were switched once each week to maintain accuracy, and one tank from each treatment group was observed for a week. It was anticipated that the H_2S concentrations in the tanks receiving the same dosing flow would be the same. H_2S levels were routinely maintained by adjusting the dose, automatically reported, and plotted online (Grafana) in real-time.

3.6 Sampling procedure

50 post-smolts salmon were reared in one tank. There were three tanks per treatment groups which were control (0 µg H_2S/l), low H_2S (1 µg H_2S/l) and high H_2S (5 µg H_2S/l). Sampling was performed before the exposure of H_2S i.e., 0 weeks and with exposure it was executed at 2weeks and 4 weeks, and after the removal of H_2S week 6. 18 samples were taken from each treatment group and dipped in Finquel for 5 minutes.

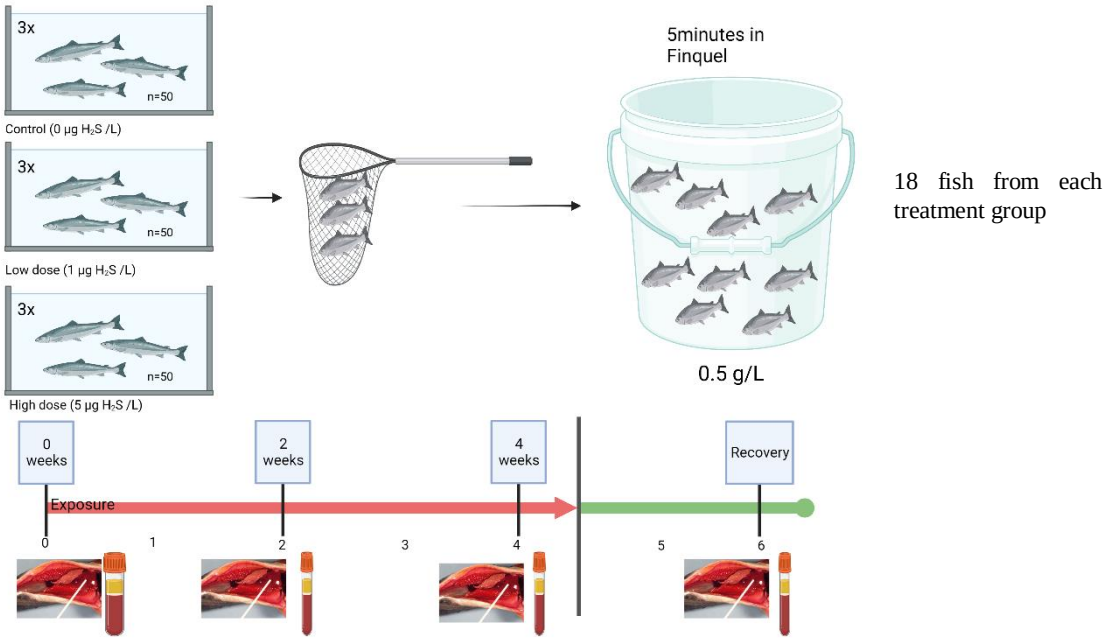


Figure 3. Sampling procedure. Created with BioRender.com

Liver samples: The fish was opened ventrally, and the liver was cut out.

For qPCR: Before being moved to a freezer at -80°C until RNA isolation, a small portion of the liver was stored in RNAlater (ThermoFisher Scientific Inc., MA, USA) for 24 hours at 4°C.

For histology: A transverse piece of the liver measuring 1 centimeter in length was fixed for 24 hours at room temperature in 10% buffered formalin before being stored at 4°C for analysis (CellPath, Newtown, UK).

For Biochemical marker test: A vacuum blood collection needle measuring 0.08 x 38 mm was used to draw blood samples from the caudal vein. The blood was centrifuged for ten minutes at 3700 rpm using an Avanti J-15R Centrifuge from Beckman Coulter in Switzerland. Plasma was pipetted, transferred to a new 1,5 ml Eppendorf tube, and kept in a freezer at -80°C.

3.7 Transport of sample

While tissues preserved in 70% ethanol were chilled in another styrofoam box and flown from Sunndalsøra to Ås, frozen tissues in RNAlater® were sealed in a styrofoam box with dry ice. The histology samples were preserved at 4 °C while the RNAlater® samples were kept in a -70 °C freezer upon arrival at Nofima in Ås.

3.8 Gene expression analysis

3.8.1. Primers

Molecular changes in the liver following H_2S exposure were determined by analyzing the expression of some essential marker genes including sulfide genes (*sulfite oxidase* (*soux*) and *sulfide: quinone oxidoreductase 1* (*sqor1*)), stress genes (*heat shock protein 70* (*hsp70*), *heat shock protein 90* (*hsp90*), *catalase*(*cat*), *cu/zn superoxide dismutase* (*cu/znsod*), *glutathione peroxidase* (*gpx*), *cytochrome P450 1A* (*cyp1a*)) and metabolic genes (*pyruvate kinase M1/2 (pkm*), *transaldolase* (*taldo*), *phosphoglycerine kinase* (*pgk*), *fructose-bisphosphatase 1* (*fbp1*), *aldolase, fructose-bisphosphate B* (*aldob*), *cyclic-AMP response element binding protein A* (*creba*)) (**Table 1**).

Table 1: Primers used in the study.

Gene name	**Abbreviation**	**Sequences (5' → 3')**	**Reference**
18S ribosomal RNA	*18s*	F: TGTGCCGCTAGAGGTGAAATT R: GCAAATGCTTTCGCTTTCG	(Jorgensen et al., 2006)
Elongation factor 1-	*elf1a*	F: GAATCGGCTATGCCTGGTGAC R: GGATGATGACCTGAGCGGTG	(Garcia & Johnston, 2013)
Sulfide: quinone oxidoreductase 1	*sqor1*	F: GGATAGGAAGTATGATGGCTACAC R: GGTCAATAGGGAATGTCTCCA	(Alipio et al., 2022)
Sulfite oxidase	*soux*	F: TGTCTGAGTATAAGGTGGGTGAG R: GGTGATGTAGTTGTCGGAGAG	(Alipio et al., 2022)
Heat shock protein 70	*hsp70*	F: CCCCTGTCCCTGGGTATTG R: CACCAGGCTGGTTGTCTGAGT	(Solberg, et al., 2012)
Heat shock protein 90	*hsp90*	F: CCACCATGGGCTACATGATG R: CCTTCACCGCCTTGTCATTC	(Olsvik, et al., 2013)
Catalase	*cat*	F: GGGCAACTGGGACCTTACTG R: GCATGGCGTCCCTGATAAA	(Olsvik, et al., 2013)
Cu/Zn superoxide dismutase	*cu/znsod*	F: CCACGTCCATGCCTTTGG R: TCAGCTGCTGCAGTCACGTT	(Solberg, et al., 2012)
Glutathione peroxidase	*gpx*	F: GATTCGTTCCAAACTTCCTGCTA R: GCTCCCAGAACAGCCTGTTG	(Solberg, et al., 2012)

Cytochrome P450 1A	*cyp1a*	F: AGGTGGGAATGACTCGTACTC R: GATGTATCCTTGACTGTGCAGT	(Beemelmanns et al., 2021)
Pyruvate kinase M1/2	*pkm*	F: GTGACCATGATGCACTCGATC R: GGACAGCGTGGGCGATAC	(Bower et al., 2009)
Transaldolase	*taldo*	F: AGGTAGACGCCAGGCTTTC R: CCATGTTGAGGAGAGCTTGA	(Bower et al., 2009)
Phosphoglycerine Kinase	*pgk*	F: CTCGGTGATGGGGCTTAGG R: TCATTGGTGGAGGCGACA	(Bower et al., 2009)
Fructose-Bisphosphatase 1	*fbp1*	F: TGGGATTGCCAACCTCTATG R: GCCCTCTCGTTCTCCTCTG	(Bower et al., 2009)
Aldolase, Fructose-Bisphosphate B	*aldob*	F: TCCGTGACCTCCTGTTCTCT R: CTGTGCCTTTGTCCACCTTA	(Bower et al., 2009)
Cyclic-AMP response element binding protein A	*creba*	F: GGAGTCTGTTTCGCTAAGTCG R: CGTAGGACCGCTGGATGT	(Bower et al., 2009)

3.8.2 RNA Isolation and Quantification

Two beads and 400 µL of lysis buffer per tube were used in the Qiagen collection tube plate. 54 liver samples were taken, i.e., 18 representatives from each tank and placed in each tube. Since the liver is rich in RNA, smaller pieces were used. Scalpel blade was used to cut the samples and was discarded between samples. The forceps used to put the samples inside the tubes were kept in 70% ethanol for a few minutes before moving to distilled water and were cleaned between every sample. The samples in the lysis buffer were added with 20 µL proteinase k/sample and were homogenised for 1-minute homogenization. Thereafter the samples were centrifuged for 1-minute at 1600 rpm and incubated at 37.1 °C for 25 minutes. The lysed samples were then transferred to a deep well plate. Biomek 4000 automated workbench station (Beckman Coulter, Inc., CA, United States) was used for the RNA isolation.

After everything is set up, the process takes around 2.5 to 3.25 hours. The extracted RNA quantity was determined using a NanoDrop 8000 Spectrophotometer (ThermoFisher Scientific, United States). The nucleic acid-protein ratio of RNA samples was considered satisfactory if it was between 1.9 and 2.1 (Manchester, 1996).

3.8.3 Preparation for complementary DNA (cDNA)

The RNA from the robot was normalized to obtain 200 ng of RNA in a 15 µL solution. Using nuclease-free water, we dilute the RNA before normalizing it if RNA is less than 3 µL. For cDNA, the master mix was prepared using a High-Capacity RNA-to-cDNA Kit (Beckman Coulter, Inc., CA, United States) in which 10 µL of buffer and 1 µL of the enzyme was mixed for 60 samples making a total of 660 µL of master mix. From that master mix, 11 µL was transferred to 96 deep well plates, and 9 µL of normalized RNA (200 ng) was added. On that plate, -RT control (10 µL buffer, 1 µL nuclease-free water, and 9 µL normalized RNA from 20 random samples), an 11 µL mixture of buffer and enzymes, and 9 µL nuclease-free water were also placed. Then the plate was sealed and centrifuged for 1 minute. The thermocycling procedure was completed in a VeritiTM 96-Well Thermal Cycler 7 (Applied Biosystems, California, United States) under a "high-capacity cDNA ABIO" program under the following conditions: 60 min at 37°C, 5 min at 95°C, and at 4°C for infinite time.

3.8.4 Real-time Quantitative PCR and gene analysis expression

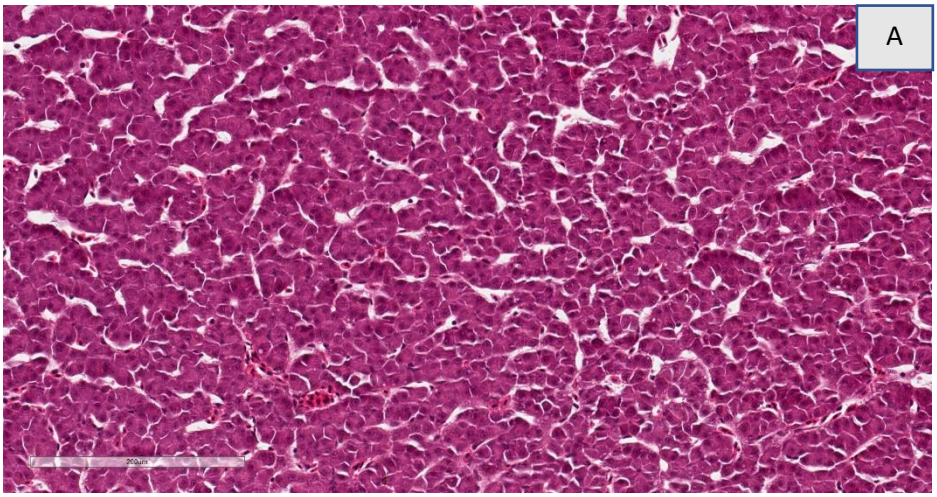
Applied Biosystems QuantStudioTM 5 Real-Time PCR instrument was used to quantify gene expression using Power SYBR Green PCR Master Mix (Applied Biosystems). In a nutshell, a 10 µL reaction mixture of 4 µL of 30x dilution cDNA (i.e., 3 µL cDNA and 145 µL nuclease-free water) and 6 µL master mix was used to get 10 µL of total volume per well. In the 6 µL master mix, there were 5 µL SYBR green and 0.5 µL of each 5 µM forward and reverse primers of the target genes indicated in Table 2 were used. 0.5 µL of forward and reverse primers were obtained by mixing 95 µL nuclease-free water and 5 µL gene primers. RT control and a pool of nuclease-free water with enzyme and buffer were also included. The following thermocycling conditions were used on all samples, which were conducted in duplicates: Pre-incubation at 95 °C for 2 min, 40 cycles of amplification lasting 1s at 95 °C and 30 °C, a melt curve stage lasting 15s at 95°C, 1 min at 60 °C, and 15s at 95 °C. Using the geometric mean of two housekeeping genes: *18s*, and *elongation factor 1-a (elf1a)*, the relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ technique. The reference gene *elf1a* is involved in protein synthesis and has undergone extensive validation as a trustworthy reference gene in quantitative real-time PCR examination of gene expressions in Atlantic salmon (Olsvik et al., 2005; Moore et al., 2005) and Atlantic cod (Aursnes et al., 2011) as well as in a wide range of other organisms, including plants (Mallona et al., 2010), copepods (Frost & Nilsen, 2003), and human (Hamalainen et al., 2001).

3.9 Histological assessments

The formalin-preserved liver was transferred in 70% ethanol at room temperature. The samples underwent a series of dehydration, clearing and paraffin embedding using an automated tissue processor (TP1020, Leica Biosystems, Nussloch, Germany) following an 11-hour long process.Paraffin blocks were prepared using a modular tissue-embedding system (Leica EG1150H, Leica Biosystems, Nussloch, Germany). Thereafter, the tissue blocks were cut into 5 µm sections using a rotary microtome (Leica RM2165, Leica Biosystems, Nussloch, Germany). The slice was then put carefully onto a glass slide. The tissue section was then kept in the oven 60◦C overnight (TS 9026, Termacks, Sweden). The slide was stained with Haematoxylin and Eosin (H&E) (Appendix I) using an automated stainer (ST5010, Leica Biosystems, Nussloch, Germany), which took 40 minutes to complete. After staining, cover slip was mounted, and the stained sections were allowed to dry overnight. The stained tissue sections were scanned with a digital slide scanner (Aperio CS2, Leica Biosystems, Illinois, USA).

3.9.1 Histological evaluation

The scanned tissue was snapshot into five more pictures covering most of the tissue on 20x resolution from ImageScope×64. The snapped picture was then put in ImageJ for further analysis. In the ImageJ the picture's known distance was kept 200 µm and area and mean of whole picture was calculated. After that the saturation of picture was increased until the picture looks like in **Figure 4B**. The mean and area of the slide images of each liver's interlobular space were calculated.



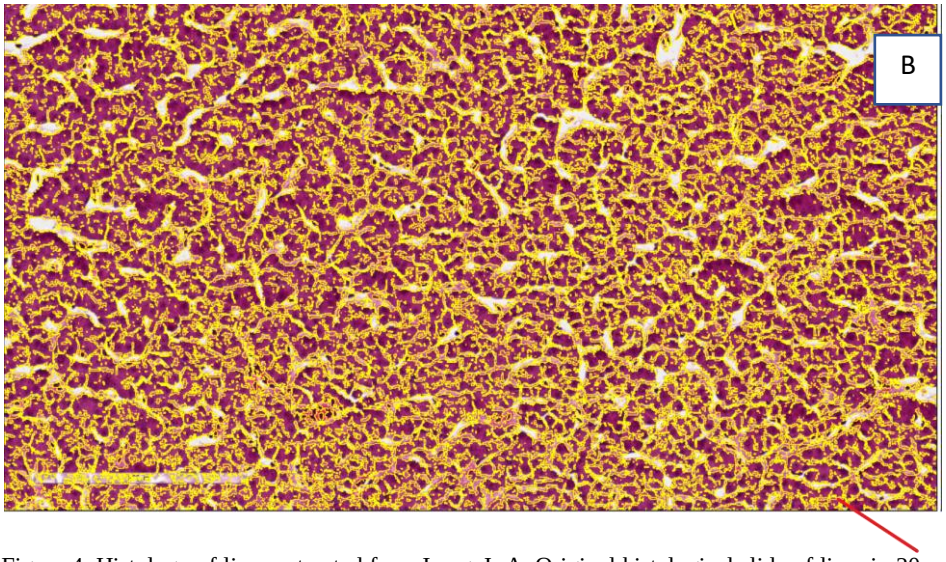


Figure 4. Histology of liver extracted from ImageJ. A: Original histological slide of liver in 20x, B: Image after measuring liver sinusoids i.e., white area

3.10 Biochemical marker test

Blood plasma samples were sent to Nofima's AKVA Laboratory in Sinndalsøra to analyze Lactate dehydrofenase (LDH), asparate aminotransferase (ASAT), alanine transaminase (ALAT) and creatine kinase (CK) in Horiba Pentra C400 Clinical Chemistry Analyzer (HORIBA FRANCE SAS – Lyon, France).

3.11 Data Analysis

A significant difference in gene expression, biochemical marker, and histology was analyzed using R-studio 2022.07.02. The normality of data sets was tested using the Shapiro-Wilk and Levene's tests for homogeneity of variance. Some outliers were removed from data set and were log-transformed except in creatine kinase as assumptions of ANOVA was not met. Two-way ANOVA was used, and Tukey tests was applied when significancy was found in ANOVA. *catalase*, *glutathione peroxidase*, *sqor1*, lactate dehydrogenase, aspartate aminotransferase, alanine transaminase, creatine kinase was used Friedman test as it didn't meet the ANOVA assumptions. Each number is expressed as its mean and associated standard deviation (SD). The significance threshold was set at $p<0.05$.

4. Results

4.1 Gene expression profiles in the liver

4.1.1 Sulfide detoxification genes

The relative expression profiles of the sulfide detoxification genes *soux* and *sqor1* are shown in **Figure 5**. There was a significant upregulation of *soux* in low H_2S group compared to control and high concentrations at week 4 of exposure (**Figure 5A**). Significant temporal variation was observed in *soux* gene expression in the control group displaying significantly higher levels at 2 weeks following recovery than 2 and 4 weeks of exposure. The expression of *sqor1* showed significant temporal variations for both the control and high H_2S groups. For the control group, *sqor1* expression was significantly higher at 2 weeks following recovery compared to the levels during H_2S exposure. On the other hand, the expression of *sqor1* in high H_2S group was significantly higher at week 4 of exposure and 2 weeks of recovery compared with the level at week 2 of exposure. Moreover, there were significant inter-treatment differences at week 4 of exposure, where the expression of *sqor1* was significantly higher in both H_2S exposed groups than the control.

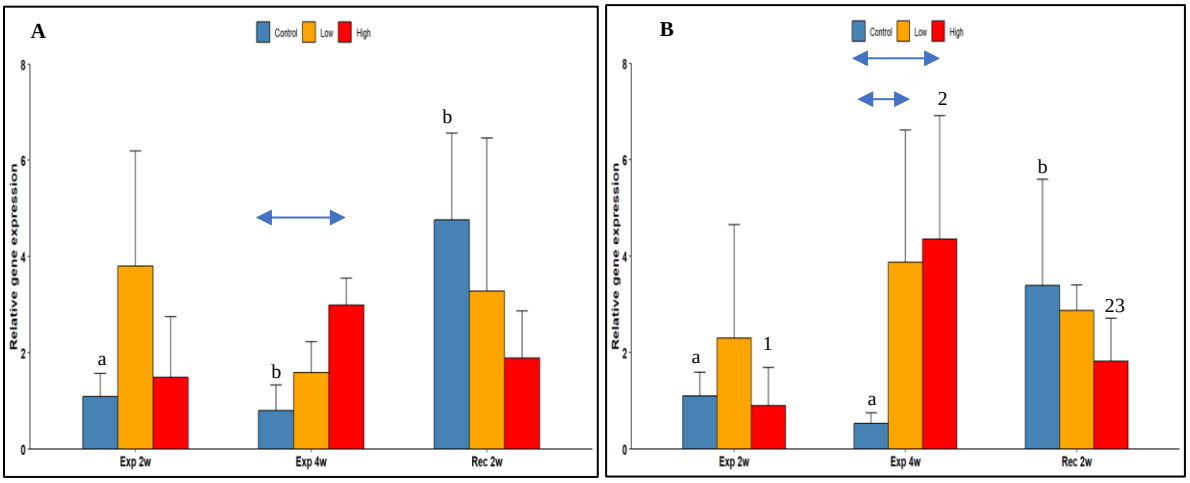


Figure 5. Relative expression of sulfide detoxification genes; A: *sulfite oxidase* (*soux*), B: *sulfide: quinone oxidoreductase 1* (*sqor1*) of Atlantic salmon with the exposure of hydrogen sulphide in 2 weeks, 4 weeks and 2 weeks after removal of H_2S with control, low and high H_2S concentrations. The bars indicate the mean and standard deviation. Statistically significant differences (p-value<0.05) within samples are denoted by an arrow while numeric marks denote the differences within the high group and small alphabet denote the differences within the control group. The data were log-transformed before analysing with two-way ANOVA in A and friedman test was used for B since it didn't meet the assumptions of ANOVA.

4.1.2 Stress response genes

There was no significant difference between treatment group or in any time-point in the expression levels of *hsp70*, *hsp90*, *cat* and *gpx* (**Figure 6A, B, C, E**). However, tendencies showed elevated gene expression after 2 weeks exposure to low H_2S concentration compared to control and high H_2S concentrations. There was a significant difference in *cu/znsod* mRNA levels between control and high, and control and low in exposure 2 week and significant difference was found between exposure 4 week and recovery 2 week of control H_2S concentrations (**Figure 6D)**. In *cyp1A* there was a significant difference between low and high H_2S in exposure 2 week and between control of exposure 4 week and recovery 4 week (**Figure 6F**).

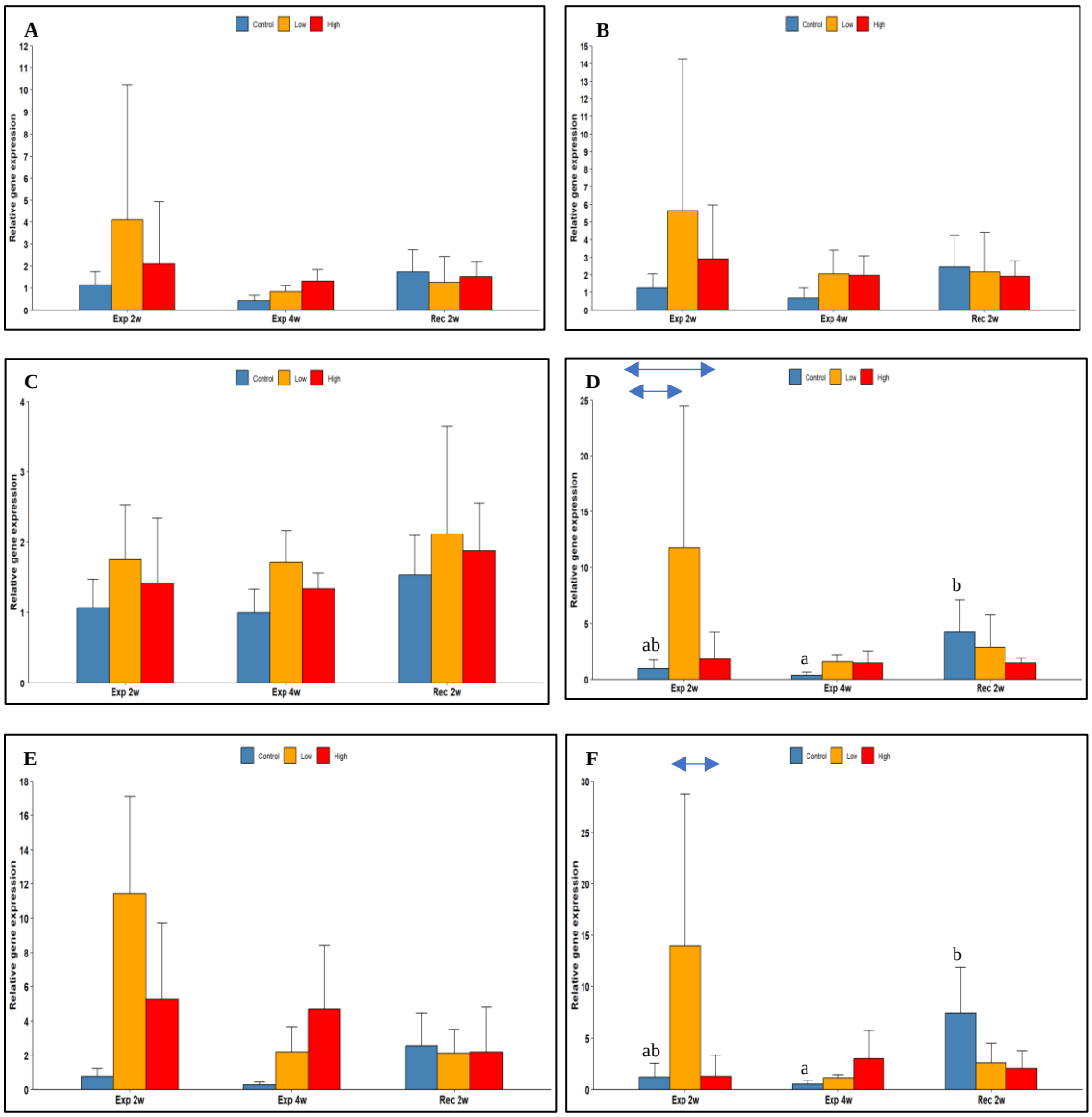


Figure 6. Relative gene expression of stress genes; A: *heat shock protein 70 (hsp70)*, B: *heat shock protein 90 (hsp90)*, C: *catalase (cat)*, D: *cu/Zn superoxide dismutase (cu/znsod*), E: *glutathione peroxidase (gpx)*, F: *cytochrome P450 1A* (*cyp1a).* Exp 2w means exposure 2 week, Exp 4w means exposure 4 week and Rec 2w means recovery 2 weeks. The bars indicate the mean and standard deviation of n = Statistically significant differences (p-value<0.05) within samples are denoted by arrow while small alphabet denote the differences within control group. The data were log transformed before analysing with two-way ANOVA and friedman test was used for C and E as it didn't meet the assumption of ANOVA.

4.1.3 Metabolic regulatory genes

The relative gene expression of metabolic genes; *pkm* (A), *t*aldo(B), *pgk* (C), *fbp1* (D), *aldob* (E) and *creba* (F) are shown in **Figure 7**. There was an upregulation of metabolic genes in low H_2S concentrations compared to control and high concentrations at week 2 of exposure (**Figure 7**). Significant temporal variation was found in *pyruvate kinase M1/2*, *phosphoglycerine kinase*, *fructose-bisphosphatase 1*, *aldolase*, *fructose-bisphosphate B* and *cyclic-AMP response element binding protein A* gene expression, but only in the control group where the level at 2 weeks following recovery was significantly higher than at 2 and 4 weeks of exposure. The expression of *fbp1* was upregulated after 2 weeks recovery in control and high H2S concentrations in exposure 2 week.

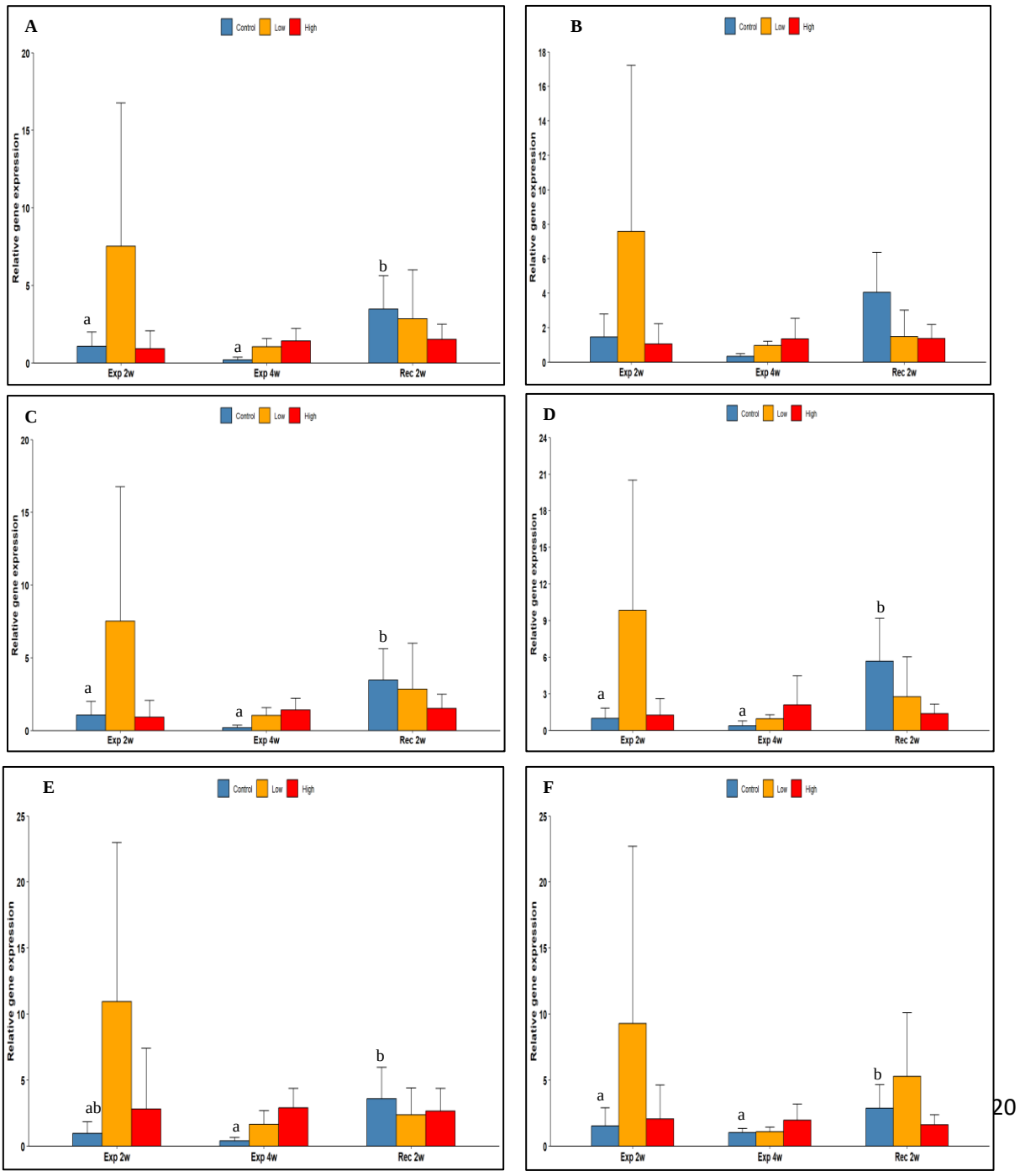


Figure 7. Relative gene expression of metabolic genes; A: *pyruvate kinase M1/2 (pkm)*, B: *transaldolase (taldo)*, C: *phosphoglycerine kinase (pgk)*, D: *fructose-bisphosphatase 1 (fbp1*), E: *aldolase, fructose-bisphosphate B (aldob)*, F: *cyclic-AMP response element binding protein A (creba)* of Atlantic salmon with the exposure of hydrogen sulphide in 2 weeks, 4 weeks and 2 weeks after removal of H_2S with control, low and high H_2S concentrations. The bars indicate the mean and standard deviation. Statistically significant differences (p-value<0.05) within control group are denoted by small alphabet. The data were log transformed before analysing with two-way ANOVA.

4.2 Histology

For the histology, area and mean of interlobular space was measured. There was a significant difference between low H_2S of 100% and white% and with high H_2S concentrations of 100% and white% in average of interlobular space (**Figure 8A**). No significant difference was found in average area of white % i.e., liver sinusoids (**Figure 8B**). White % of average of area shows the increased value in high H_2S concentration as compared to control and low H_2S concentrations (**Figure 8B**).

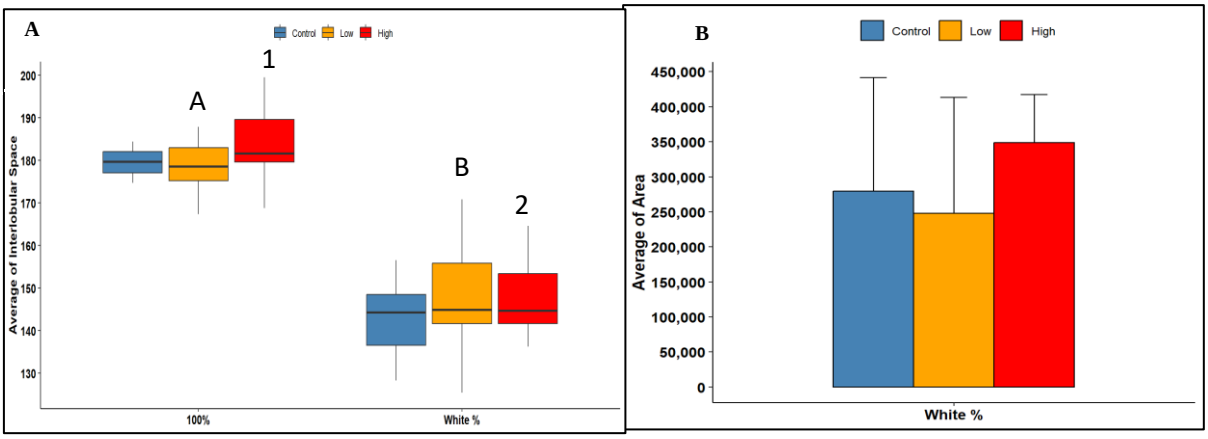


Figure 8. A: Mean of interlobular space, B: Average of Area of Atlantic salmon with the exposure of hydrogen sulphide in 2 weeks, 4 weeks and 2 weeks after removal of H_2S with control, low and high H_2S concentrations. Statistically significant differences (p-value<0.05) within low group are denoted by big alphabet and for high group by number.

4.3 Biochemical marker test

The graph shows the level of several key biochemical markers for liver health (**Figure 9**). Lactate dehydrogenase (**Figure 9A**) shows significant temporal difference between control group of exposure 2 week and exposure 4 week. LDH shows higher levels in first 2 week of exposure in comparison to exposure 4 week. There was no significant difference in aspartate aminotransferase (ASAT) and alanine transaminase (ALAT) (**Figure 9A and 9B**). Creatine kinase (CK) has shown significant downregulation between control and high H_2S concentrations in exposure 2 week and exposure 4 week and also with control and low H_2S concentration in exposure 4 week. Liver has shown great recovery in response to all biochemical markers i.e., LDH, ASAT, ALAT and CK especially with high H_2S concentration.

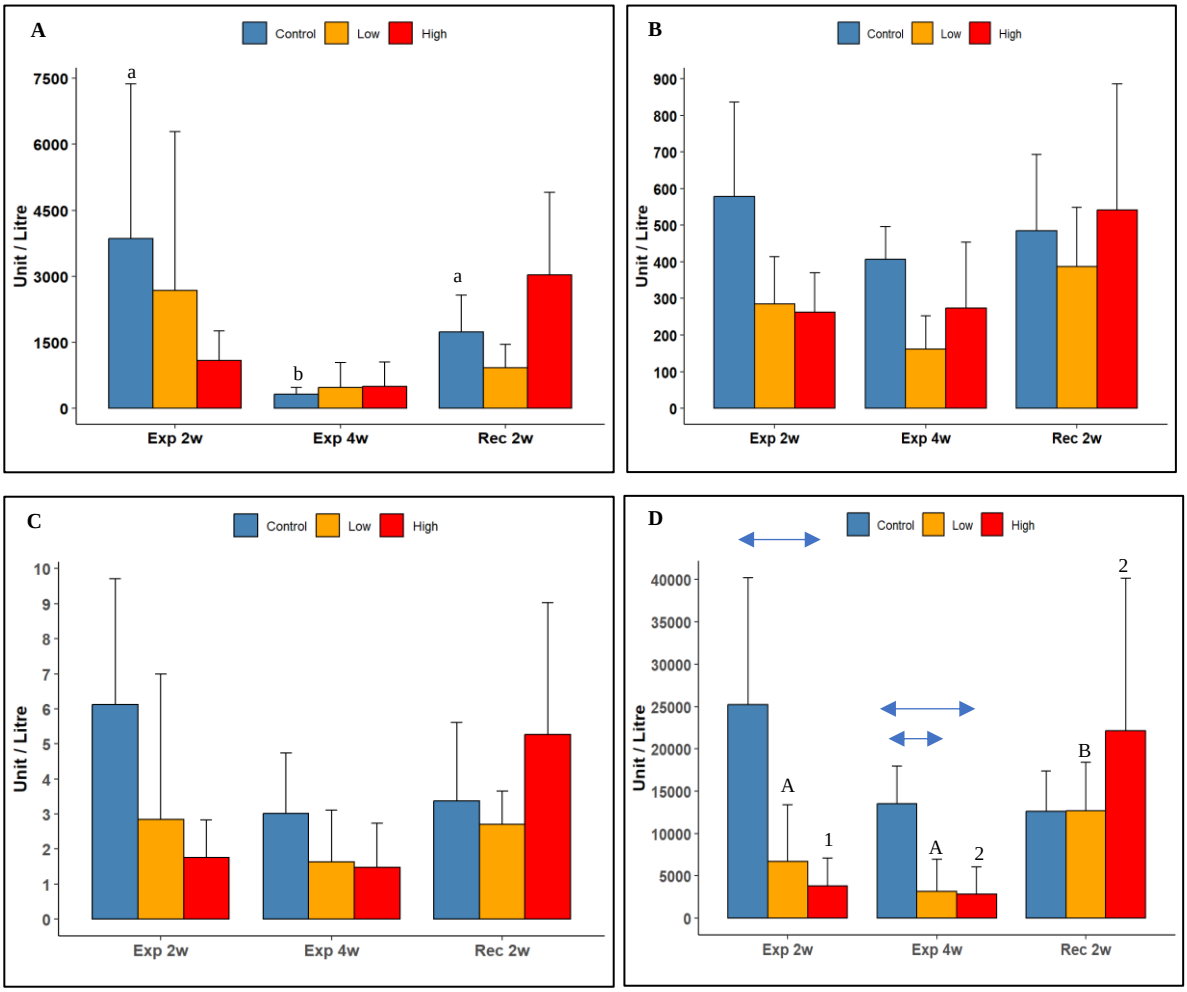


Figure 9. A: Lactate dehydrogenase (LDH), B: Aspartate aminotransferase (ASAT), C: Alanine transaminase (ALAT), D: creatine kinase (CK) of Atlantic salmon with the exposure of hydrogen sulphide in 2 weeks, 4 weeks and 2 weeks after removal of H_2S with control, low and high H_2S concentrations. The bars indicate the mean and standard deviation. Statistically significant differences (p-value<0.05) within samples are denoted by an arrow, while small alphabet, big alphabet, and numeric value denote the differences within the control group, low and high respectively. Friedman tests were used as it didn't meet the assumptions of ANOVA.

5. Discussion

Fish health is affected by different factors and causes physiological and morphological changes. The current study highlights how liver responses to H_2S.

5.1 Sulfide genes in liver

The effects of H_2S can vary depending on the dose; lower levels may be beneficial, whereas higher levels may be hazardous (Paul et al., 2021). The ability of marine fish to maintain the balance of aerobic respiration and to tolerate and survive in sulfide-rich environments is through mitochondrial detoxification of sulfide (Bagarinao & Vetter, 1990). Enzymes involved in the mitochondrial degradation of hazardous H_2S into its non-toxic forms are encoded by the genes *sqor1* and *suox* (Quinzii et al., 2017, Olson, 2012).

Research on the precise effects of hydrogen sulfide exposure on *sqor*1 is scarce. However, research has indicated that H_2S gas exposure may affect the expression and operation of several proteins involved in H_2S metabolism, including *sqor1*. When H_2S enters the liver, enzymes like Mercaptopyruvate sulfurtransferase (MPST) and *sulfide quinone oxidoreductase* (*sqor1*) convert it into less toxic forms like thiosulfate and sulfate. The capability of these enzymes can be overloaded by prolonged or excessive H_2S exposure, which causes hazardous quantities of H_2S to build up in the liver and other tissues. According to Horsman & Miller, 2016 to stop the integrated stress response from being activated when exposed to H_2S, *sqor1* activity is necessary. *sqor1* expression was rapidly upregulated with the exposure of H_2S and is essential for survival in H_2S. Internal H_2S levels will most likely drop to an acceptable range because of the decrease in H_2S generated by *sqor1* (Budde & Roth, 2011). The increase in expression of *sqor1* in this study in high dose of H_2S concentration may be the mechanism by organism to adapt hydrogen sulfide-rich environments (Zhang et al., 2021). Cysteine and methionine are two examples of sulphur-containing amino acids that are metabolized by the enzyme *soux*. A consequence of the metabolism of these amino acids is hydrogen sulfide (H_2S). This study shows the upregulation of *soux* gene in high dose of H_2S over 2 week of time and it may be due to the function of liver to act as a compensatory mechanism to aid the body in getting rid of extra H_2S or it may be due to the involvement of liver in endogenous and exogenous sulfide metabolism (Allore et al., 2021; Wu et al., 2019). Other reason for the rise in *soux* gene expression after H_2S exposure in high treatment is that *soux* is involved in converting sulfite, a hazardous intermediary in the metabolism of H_2S, to sulfate, a less hazardous compound that

may be eliminated from the body (Cohen et al., 1971). Lack of *sulfite oxidase* causes sulphite to build up, which has been known to be toxic to cells (Mellis et al., 2021).

5.2 Stress genes

Abiotic factors including toxins in water (Debes et al., 2012), dissolved oxygen (Huang et al., 2009; Fridovich, 1998; Waagbø et al., 2008), temperature (Aursnes et al., 2011), and diet type (Olsvik et al., 2007), as well as biotic factors like age and feeding behavior (Martínez-Álvarez at el., 2005), can cause oxidative stress in fish. In this study all the factors are maintained except for the toxins i.e H_2S. In fish, the heat shock response-related alterations in gene expression are often tissue-specific (Buckley et al., 2006; Currie et al., 2010; Madeira et al., 2014). We chose the liver for our investigation because of its high metabolic activity.

Heat shock proteins (HSP) are produced by the cells in response to stressful condition like heat shock (Ritossa, 1962), cold (Matz et al., 1995), UV light (Cao et al., 1999) and wound healing/ tissue remodelling (Laplante et al., 1998). Their primary function is to defend cells from stressors by assisting in the proper protein folding of newly generated proteins, mending misfolded proteins, and transporting proteins to their sites of action (Lanneau et al., 2008). To prevent further physiological changes and maintain cellular homeostasis, they congregate in the cells to promote the correct folding of nascent and stress-accumulated misfolded proteins, proteolytic destruction of misfolded or denatured proteins, and prevent protein aggregation (Gupta et al., 2010). One of the first stress proteins to be activated under stressful circumstances is *hsp 70* and *hsp 90*, which build up when heat or other stresses cause the expression of their encoding genes to spike (Gupta et al., 2010; Morimoto & Tissières, 1994) which can be seen in this study that there is spike of expression in exposure 2 week of H_2S than in exposure 4 week. *hsp70* is a stress-inducible protein that functions to defend cells from a range of conditions, such as heat, oxidative stress, and toxin exposure. A prominent generator of reactive oxygen species (ROS) and oxidative stress in the liver is cytochrome P450 (Koop, 1992). ROS are pervasive, extremely reactive chemicals that are created when molecular oxygen is reduced. Oxidative stress is a result of an imbalance between the production of ROS and the cellular antioxidant defense system, which comprises molecules that scavenge and repair damage (Corsello et.al., 2018). *cu/znsod, glutathione peroxidase, catalase* are the main antioxidants providing a defense net during the process of oxidative stress (Dominko & Đikić, 2018). The activation of stress genes with low concentration H_2S exposure for 2 weeks in this study has shown that H_2S exposure has caused an oxidative stress. The antioxidant genes *gpx* and *cat*

encode the production of the intracellular enzymes' *glutathione peroxidase* and *catalase*, respectively, in accordance with oxidative stress. Additionally, these proteins have a role in oxidative stress (Allen & Tresini, 2000) which is clearly seen in this study. *gpx* and *cat* were more pronounced in liver of Atlantic salmon with the exposure for 2 and 4 weeks as compared to other stress genes indicating that mucosal oxidative stress is strongly induced by exogenous H_2S.

5.3 Metabolic genes in liver

The liver serves as the primary organ for the metabolism of lipids and glucose and regulates the amount of lipids in the blood through lipoprotein metabolism. The synthesis of lipoproteins, insulin sensitivity, glucose metabolism, and mitochondrial biogenesis and biogenetics are all impacted by hepatic H_2S metabolism (Mani et al., 2014). The metabolism of glucose and lipids, as well as the synthesis of energy, are just a few of the physiological activities that are inextricably linked to the liver's metabolic reactions. The final phase of glycolysis, which is crucial for producing adenosine triphosphate (ATP), is catalyzed by pyruvate kinase. Four main pyruvate kinase (pkm) isozymes are present in mammals: muscle (M1), liver (L), erythrocyte (R), and the ubiquitous M2 (Uyeda, 2013). According to studies, H_2S can enhance pkm activity in the liver, which enhances ATP synthesis and improves liver function. H_2S's capacity to improve mitochondrial performance and activate oxidative phosphorylation, which can boost the synthesis of ATP, may be the cause of this impact. On the other hand, some research contends that excessive H_2S concentrations can harm the liver and impede pkm action. The ability of H_2S to cause oxidative stress and inflammation, which can result in liver damage and reduced liver function, may be the cause of this impact but it's not seen in this study. The pentose phosphate pathway (PPP) enzyme transaldolase (taldo) produces NADPH to fend against oxidative damage. Mice lacking taldo are viable and suffer progressive liver illness that is ascribed to oxidative stress, in contrast to mice lacking other PPP enzymes, such as transketolase (TKT), which are incompatible with mammalian cell viability (Oaks et al., 2020). As shown in this study the expression of taldo is homozygous which has historically been linked to many types of liver illness, from minor elevations in liver enzymes (AI-Shamsi et al, 2015) to cirrhosis (Lipiński et al., 2018). Phosphoglycerol kinase (pgk) activity is highest in the liver in both humans and mice (MacLennan et al., 2006) but this study in Atlantic salmon shows little activity except for the low concentrations of H_2S when exposed for 2 weeks. It may be due to exposure to H_2S or maybe it's different in liver of Atlantic salmon. In teleosts, the fructose-1,6-bisphosphatase gene has not been fully characterized (Wang et al., 2010). A

study by Li et al., 2021 observed downregulation of *fbp1* in ovarian tumor tissues and vice-versa. There is a downregulation of expression of *fbp1* in this study except for low H_2S concentration while exposed for 2 weeks. It also reduces the glucose metabolism (Garrido et al., 2015, Soh et al., 2011). Fasting causes the liver to produce more of the cAMP response element binding protein (creb), which promotes the transcription of rate-limiting gluconeogenic genes to keep metabolic balance (Akhmedov et al., 2016). It also controls the expression of key genes in dopaminergic neurons (Wang et al., 2018).

5.4 Histology

We investigated the effect of H_2S on hepatic sinusoids in this study. All hepatic cell types, including hepatocytes, depend on communication and the proper functioning of the liver sinusoids, a special form of microvascular bed (Gibert-Ramos et al., 2021). Hepatic stellate cells (HSCs), which surround specific sinusoids, play a major role in controlling hepatic sinusoidal tone (Zhang et al., 1994). These contractile pericytes adjust sinusoidal tone in response to a regional equilibrium between vasoactive mediators (Kawada et al., 1993). Fiorucci et al., 2005 were the first to document the impact of H_2S on the liver's vascular system. In isolated livers from normal and cirrhotic rats, H_2S considerably reduced the rise in intrahepatic resistance following norepinephrine (NE) infusion. By boosting hepatic perfusion and lowering portal hypertension, the vasodilatory impact of H_2S is probably advantageous during cirrhosis. Based on this finding, one may hypothesize that H_2S, a vasodilator, would enhance hepatic tissue perfusion as found in this study and be advantageous in sepsis. But multiple investigations have demonstrated that endogenous H_2S has a role in the development of sepsis-related illness. By relaxing the smooth muscle cells that surround the liver sinusoids, H_2S has been shown to control blood flow through them. Because the liver's metabolic processes depend on proper blood flow through the sinusoids, this may have significant effects on liver function.

The impact of hydrogen sulfide (H_2S) on hepatic microcirculation has not been studied, even though it influences vascular resistance. Sepsis leads to dysregulated hepatic sinusoidal perfusion, which damages the liver.

5.5 Biochemical marker test

The assessment and treatment of patients with hepatic dysfunction benefit from a variety of biochemical testing. It is well recognized that the liver is important in the metabolism of most

chemicals. The level of the biochemical parameters present in the liver might therefore be impacted by any disturbance in the liver. Whenever liver cells and tissues are damaged, the activities of these enzymes are typically increased (Traynor et al., 2006).

Lactate dehydrogenase (LDH) is an enzyme that transforms lactate into pyruvate, which is a crucial step in the energy metabolism process. LDH is a typical indicator of tissue injury because it can be seen in many tissues, including the liver. The quantity of LDH in the serum that escapes from injured tissues can be measured using LDH tests which uses the catalytic feature of LDH that causes reversible oxidation of L-lactate to pyruvate, which is mediated by the hydrogen acceptor, NAD+ (Farhana & Lapping, 2021). Through the S-sulfhydration of lactate dehydrogenase, H_2S increases its activity, promoting mitochondrial electron transport (Borisov & Forte, 2021).

Alterations in permeability or the more visible breakdown of the cell membrane are reflected in variations in the amounts of the leakage enzymes ALAT and ASAT. ALAT is frequently incorporated into biochemical profiles to evaluate hepatic injury and is also thought to be a sign of liver toxicity (Smith et al., 2002). In this study ASAT and ALAT are increased when first exposed so that increase may be the body defence mechanism to H_2S and in 4 weeks it gradually decreases. Since, liver plays an important role in metabolism of most of the substances, the level of biochemical parameters present in the liver cells can therefore be impacted by any disturbance in the liver (Ukpo et al., 2012). The liver is evaluated and monitored using the enzymes ASAT and ALAT, which are concentrated within the cells of the liver (Wada & Snell, 1962). Therefore, an increase in their values may cause organ dysfunction (Wells et al., 1986) but this study found not so much increment in the values with the exposure of hydrogen sulfide over time and recovered well after the removal of hydrogen sulfide. CK is the biomarker of muscle damage (Kim & Wierzbicki, 2021). CK clearance in blood circulation was mostly carried out by the liver and blood cells. **Figure 9D** shows decreased or little CK levels which may be due to excessive CK clearance by non-muscle organs/tissues (Pan et al., 2023). The liver in this study shows progressive recovery in CK levels after the removal of hydrogen sulfide.

6. Conclusion

This study provides insights into the hepatic responses of post smolt Atlantic salmon to sub-lethal H_2S levels in RAS. The results demonstrated that H_2S exposure upregulated sulphide gene expression which facilitated the mobilization of anti-inflammatory and antioxidant response to H_2S as a stressor. Stress genes with low concentration H_2S exposure for 2 weeks in this study has shown that H_2S exposure has caused an oxidative stress. The metabolism of glucose and lipids, as well as the synthesis of energy, are just a few of the physiological activities that are inextricably linked to the liver's metabolic reactions to H_2S. H_2S may potentially affect nutrition digestion and overall liver function by regulating metabolic enzymes and signalling pathways involved in energy homeostasis. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) had increased with the increment of exposure time which indicate the sign of liver injury or malfunction while lactate dehydrogenase (LDH) and creatine kinase (CK) only shows prominent effect on 2 weeks of exposure of H_2S. With the larger liver sinusoids in high H_2S concentrations it signified the congestion of liver was prominent in that group.

To conclude, it seems that Atlantic salmon can withstand prolonged exposure to quite high amounts of H_2S, although they might eventually break down by progressive liver damage. But low amount of H_2S causes salmon to experience significant stress. It is believed that this study's findings may shed light on hydrogen sulfide toxicity, which has been linked to fish mortality or poor performance. The low concentration of H_2S needs to be further investigated as why it has more prominent effect than that of high concentrations.

7. References

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8. Appendix

Appendix I Protocol for Hematoxylin and eosin staining (H & E stain)

The liver section was H & E stained using an automated (ST5010, Leica Biosystems) with the following steps:

Appendix Table 1. Steps for Hematoxylin and eosin staining (H & E stain).

Plassering	Løsning	Tid
1	Xylen	5
2	Xylen	5
3	Abs	3
4	Abs	3
5	96%	2
6	96%	2
7	70%	2
Vask 1	Vann	3
8	Hematoxylin	4
Vask 2	Vann	2
9	Ammoniakk	1
Vask 3	Vann	0,3
10	Saltsyre	0,05
Vask 4	Vann	0,3
11	Erytrosin B	0,3
Vask 4	Vann	0,35
12	70%	0,15
13	96%	0,15
14	Abs	0,3
15	Abs	1
18	Xylen	1
Exit	Xylen	

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