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Published in:
Aquaculture

Link to article, DOI:
10.1016/j.aquaculture.2023.739405

Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

Aquaculture 570 (2023) 739405

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Acute hydrogen sulfide exposure in post-smolt Atlantic salmon (*Salmo salar*): Critical levels and recovery

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ARTICLE INFO

Keywords:
Respirometry
Sulfide toxicity
Excess oxygen uptake
Metabolic rate
Recirculating aquaculture system

ABSTRACT

Despite the importance of Atlantic salmon in marine aquaculture production systems, remarkably little is known about the effects of hydrogen sulfide (H_2S) on the physiology of the species. In recent years, mass mortalities of Atlantic salmon have been reported in recirculating aquaculture systems (RAS) due to acute H_2S exposure. This highlights the importance of obtaining a better understanding of tolerance thresholds and metabolic responses to this toxic gas. The toxicity of H_2S is exerted at the level of the mitochondria, where impairment of the enzyme cytochrome *c* oxidase inhibits cellular respiration. Because H_2S depresses oxygen uptake (MO_2), intermittent flow-through respirometry, a common method for assessing the metabolic response to various stressors in fishes, is a suitable method to determine concentration thresholds for when H_2S affects the metabolism of Atlantic salmon. During exposure trials, 3 size groups (range ~100-500 g) of fish were acclimated to control conditions to obtain baseline measurements, whereafter they were exposed to progressively increasing H_2S concentrations (0.53 ± 0.14 μM h^{-1}) until MO_2 decreased below the standard metabolic rate or loss of equilibrium occurred, which we considered to be the critical H_2S concentration (H_2S_{crit}). Fish were then allowed to recover in H_2S free water to determine the excess oxygen consumption (EOC) following H_2S exposure. The results show that Atlantic salmon have a lower tolerance to H_2S than previously estimated, with a mean H_2S_{crit} of 1.78 ± 0.39 μM H_2S, which was independent of size. During recovery, the estimated EOC greatly exceeded the accumulated oxygen deficit (DO_2) in all groups, and the small salmon had a significantly larger EOC. While the magnitude of the EOC was greater for small salmon, it did not differ in duration (recovery time) among the different sizes of fish. The larger EOC showed that H_2S exposure had a greater effect on the recovery phase of the small salmon, and exposure to H_2S may leave the fish more vulnerable to other stressors post-exposure. This study provides specific values that underline the sensitivity of Atlantic salmon to acute H_2S exposure and emphasizes the importance of the aquaculture industry to implement mitigating strategies for the occurrence of H_2S at production facilities.

1. Introduction

Atlantic salmon (*Salmo salar*) is a well-studied teleost species, due to its high commercial value as a species in aquaculture production, as well as a recreational target species. In aquaculture production, recent reports of mass mortalities in salmonids following exposure to hydrogen sulfide (H_2S) (Sommerset et al., 2020), emphasize the need for a broader understanding of the physiological effects of H_2S on fish, and to determine their tolerance thresholds and capacity to recover following exposure. Hydrogen sulfide is produced by microbes and is a result of the conversion of sulfate by sulfate-reducing bacteria (SRBs) (Muyzer and Stams, 2008). For the majority of animals, with the exception of a few species adapted to inhabiting H_2S-rich areas (Tobler et al., 2016), exposure to concentrations of H_2S in the micromolar range is considered lethal. Atlantic salmon in their natural habitat are unlikely to encounter toxic levels of H_2S, but under production in saltwater recirculating aquaculture systems (RAS), fish may experience acute and chronic exposure to H_2S, due to microbially produced H_2S. RAS consists of several environments that contain different microbial communities, each of which vary in their potential for H_2S production (Rojas-Tirado et al., 2021). Some of these communities have a high production potential for H_2S under anaerobic conditions, during which H_2S can reach lethal levels, and cause incidents of mass mortalities (Sommerset et al., 2020).

In an aqueous solution, sulfide can be present as one of three species: hydrogen sulfide (H_2S) gas, or in one of the two ionic forms as

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https://doi.org/10.1016/j.aquaculture.2023.739405
Received 26 October 2022; Received in revised form 25 January 2023; Accepted 22 February 2023
Available online 27 February 2023

hydrosulfide (HS^-) or sulfide (S^{2-}). Neither of the two latter exert the same toxic effect as H_2S (Smith et al., 1977). The chemical speciation, or fraction of each species present, is a function of pH, temperature, and ionic strength of the aqueous solution (Millero et al., 1988). The unionized gaseous form of H_2S easily diffuses from the ambient water across the gill epithelium (Hunn and Allen, 1974) and biological membranes. *In vitro* studies have demonstrated that H_2S present at low concentrations (5–20 μM) stimulates oxygen consumption in isolated mitochondria and donates electrons to the mitochondrial respiratory chain in several invertebrates and vertebrates (Cooper and Brown, 2008; Paul et al., 2021; Yong and Searcy, 2001). Furthermore, endogenously produced H_2S has been recognized as an important gasotransmitter and is implicated in various physiological processes, including cardiovascular control in the modulation of blood supply to tissues and organs (Olson and Whitfield, 2010). However, *in vitro* studies have shown that high H_2S concentrations (10–100 μM) inhibit cellular respiration in the mitochondria and aerobic ATP production (Szabo et al., 2014). Here, cytochrome *c* oxidase (COX) in the electron transport chain is inhibited, thereby preventing aerobic production of adenosine triphosphates (ATP) (Cooper and Brown, 2008). Consequently, H_2S either stimulates cellular respiration or impairs it through the reversible inhibition of COX (Giuffrè and Vicente, 2018).

The tolerance to H_2S in fish differs between species, reportedly due to physiological adaptations to occupy specific habitats and life-stages, and concentration thresholds for acute H_2S exposure leading to fish mortality (LC50) range from 0.25 to 53 μM (Bagarinao and Vetter, 1989; Smith and Oseid, 1974). In general, the importance of H_2S tolerance for animals inhabiting extreme environments is reflected in the diversity of strategies evolved for coping with H_2S that are found in many bottom-dwelling organisms (Tobler et al., 2016). Some shallow-water environments have a high potential for H_2S production from the sediment, and in such areas adaptations to cope with increased H_2S can be found in the local species. On the contrary, pelagic species such as the speckled sanddab (*Citharichthys stigmaeus*), and the Northern anchovy (*Engraulis mordax)* show a lower tolerance to H_2S with mortalities occurring at 1 μM, which emphasizes that lifestyle and ecology are central factors in predicting the H_2S tolerance (Bagarinao and Vetter, 1989). A commonly used approach for evaluating the response of a fish species to changes in its environment, such as temperature and oxygen saturation, is respirometry (Schurmann and Steffensen, 1997). Measurements of the oxygen uptake (MO_2) provides information on the metabolic state of the animal. The energy required to support basic maintenance functions is commonly referred to as the standard metabolic rate (SMR) (Chabot et al., 2016). The difference between the active and resting metabolic rate was described as the scope for metabolic activity by Fry and Hart (1948), which is also referred to as the aerobic scope (AS). The AS represents the energy available for metabolic processes on top of the basic maintenance functions, including exercise, growth, recovery responses, and is influenced by a wide range of environmental factors. Because H_2S can impair cellular respiration and thus the ability of the mitochondria to utilize oxygen, the effect of H_2S can be addressed through oxygen uptake studies. In the present study, we assess the tolerance to acute H_2S exposure by determining the concentration of H_2S above which fish either are unable to maintain SMR or lose equilibrium. For this purpose, we have coined the term H_2S_{crit}, because in many ways it resembles P_{crit}, a commonly used concept to determine critical oxygen tensions (Reemeyer and Rees, 2019; Rogers et al., 2016) (a few refs). This measure is used as a proxy for assessing the metabolic response to increasing H_2S concentrations, and the specific concentration where the fish cannot maintain its MO_2 as a representation of the inhibition of COX. Thus, we hypothesized that H_2S exposure causes whole animal MO_2 to decline below SMR and that this point signifies the H_2S tolerance threshold. When the oxygen demand to maintain SMR cannot be satisfied, an oxygen deficit (DO_2) occurs. While some species may be able to cope with a DO_2 by metabolic depression, such as has been observed for carp (Nilsson and Renshaw, 2004) and Nile tilapia (Bergstedt et al., 2021), most species must rely on anaerobic energy production, and tolerate the accumulation of lactate and H^+ (Wang and Richards, 2011). When H_2S exposure ceases and the gas is removed from the water, the fish can begin a recovery phase which is reflected by an increase in MO_2 to repay DO_2. During the recovery from an exposure event, the major challenge is the clearance of the anaerobically produced metabolites and replenishment of depleted energy stores. These processes can be assessed and quantified through the excess oxygen consumption following H_2S exposure (EOC), which provides information on the severity of the exposure and serves as a measure of reliance on anaerobic energy production. Hence, the larger the accumulated DO_2, the more O_2 is needed for metabolic recovery and returning the fish to homeostasis, which is reflected in the increase in MO_2 (EOC). EOC is based on the concept of EPOC (excess post-exercise oxygen consumption) that was originally introduced to describe the effect of exercise on the subsequent oxygen uptake (Hill and Lupton, 1923), and the concept has frequently been used to examine the effect of both exercise and hypoxia in fish (Bergstedt et al., 2021; Mandic et al., 2008; Zhang et al., 2018). These concepts are applied in the present study, although here the factor limiting MO_2 is not the lack of environmental oxygen but a H_2S-induced impairment of cellular oxygen use. The interconnection between H_2S and O_2 has also been demonstrated by measuring the cardiovascular response to both hypoxia and H_2S exposure. The physiological response induced by exposure to exogenous H_2S is similar to that of exposure to hypoxia, across a wide range of vertebrates (Olson et al., 2006). This was demonstrated in rainbow trout (*Oncorhynchus mykiss*), where exposure to H_2S resulted in branchial vasoconstriction, a response observed in vertebrates that experience low oxygen levels in the tissues leading to a contraction of vascular smooth muscle to redirect blood flow to optimize ventilation and oxygen delivery (Skovgaard and Olson, 2012). This similarity in the responses has laid the basis for suggesting that oxidation of endogenously produced H_2S serves as an oxygen-sensing mechanism (Olson, 2021).

To our knowledge, only one other study (Kiemer et al., 1995) has examined the effect of acute H_2S exposure concentration in Atlantic salmon, but no critical H_2S concentration was determined, nor was the actual concentration of either H_2S or total sulfide that the fish were exposed to, measured. Thus, the objective of this study was to elucidate the effect of H_2S on the metabolism of Atlantic salmon to provide a better understanding of their sensitivity. Determining tolerance thresholds can further provide the aquaculture industry with an improved understanding of the critical concentrations and effects of H_2S, to maintain good welfare and avoid mortality events. To obtain values for the critical concentration of H_2S during acute exposure we set out to identify 1) the critical concentration and the onset of respiratory arrest (inhibited by H_2S) at which MO_2 decreases or the fish loose equilibrium (LOE) and 2) the recovery response quantified through excess oxygen consumption (EOC) post-exposure in Atlantic salmon. Three size groups were tested to assess whether fish size was a factor, and consequently if certain phases in the smolt to grow-out tanks are more vulnerable to acute H_2S exposure.

2. Materials and methods

2.1. Fish husbandry

Atlantic salmon (*Salmo salar*) all-female smolt were acquired from a commercial producer in Denmark (Danish Salmon, Hirtshals). The fish were kept at facilities at DTU Aqua in Hirtshals in seawater flow-through tanks of 2800 L (200 × 200 × 70 cm), where the temperature ranged from 11.6 to 16.5 °C throughout the experimental period. Fish were acclimated to their new facilities for 3 weeks before starting the experiments. Oxygen saturation was kept above 90% by aeration, and a diurnal rhythm of 15 h light and 9 h dark was maintained by an automatic system. The fish were reared on a commercial diet (EFICO Enviro 940, Biomar A/S, Denmark) at a daily ration of 1.2% body weight per

day. Fish were not fed for 24 h before experiments started. Experimental protocols and procedures used in this study involving animals were conducted in accordance with Danish and EU Legislation and approved by Danish Veterinary and Food Administration (permit:2022-15-0201-01138).

2.2. Experimental design

The experimental setup was comprised of two tanks, one larger main tank (137 L, 76 × 60 × 30 cm), and a smaller mixing tank (12 L). All experiments were conducted in seawater (33‰) pumped from the North Sea. The mixing tank was used to ensure that the dosed sulfide stock solution was well mixed before entering the main tank, and the main tank was used for the respirometry measurements (Fig. 1). Exchange of water between the two tanks was maintained by two aquarium pumps with a flow rate of 750 L/h. Oxygen saturation (% of air saturation) was maintained above 85% by aeration. Oxygen, temperature, and pH were monitored and recorded with an optical multi-analyte meter and compatible software (Pyroscience, Aachen, Germany). H_2S concentrations in the tanks were measured every minute using two H_2S microsensors (Unisense, Denmark) connected to an amperometric device and monitored ireal-timeme on a PC. A sulfide stock solution (0.1 M, details in Section 2.4) was dosed into the mixing tank by a peristaltic pump (Cole-Parmer, Wertheim, Germany) controlled by a feedback system. Using a digital acquisition device (U6 DAQ, LabJack, Inc., CO, USA) the continuous signal from H_2S microsensors was transmitted to a second PC running DAQfactory (AzeoTech, Inc., OR, USA). DAQfactory controlled the H_2S concentrations by an analog output signal to the peristaltic pump to turn on/off when concentrations deviated from the predefined levels. Continuous dosing of sulfide *via* the feedback system was chosen to minimize the decrease in sulfide concentrations caused by the oxidation of sulfide by O_2 (Bergstedt et al., 2022). The experiments were conducted in a temperature-controlled room, which kept the experimental setup at 14.0 ± 0.9 °C. To avoid visual disturbance of fish the main tank was partially shielded by non-transparent plastic.

2.3. Respirometry

Oxygen uptake (MO_2) of the fish was measured as a proxy for the metabolic response to H_2S exposure, using computerized intermittent flow-through respirometry. All experiments were conducted with one individual at a time. The volume of the respirometer, including tubing, was: 1.4, 2.5, and 10.6 L, and was used for the small (103.3 ± 21.0 g), medium (275.3 ± 26.3 g), and large (550.2 ± 39.3 g) size groups, respectively. Each oxygen consumption measurement loop lasted 6 min, with a 150 s flush, 30 s wait, and a 180 s measurement period. Oxygen saturation in the respirometer was measured with a fiber-optic oxygen meter (PreSens, Regensburg, Germany) inserted in the recirculation loop. The connected PC with AutoResp software (Loligo, Viborg, Denmark) managed the flush, wait, and measure phases, and processed the oxygen measurement data. After weighing, the fish were chased in a bucket with low water levels by hand for 3 min, after which fish were immediately transferred to the respirometer and the highest MO_2 value was used as MO_{2max} (Raby et al., 2020). The fish were left to acclimatize in the respirometer for 24 h to obtain values to be used subsequently for the determination of the individual SMR (Steffensen, 1989) before the H_2S exposure phase was initiated.

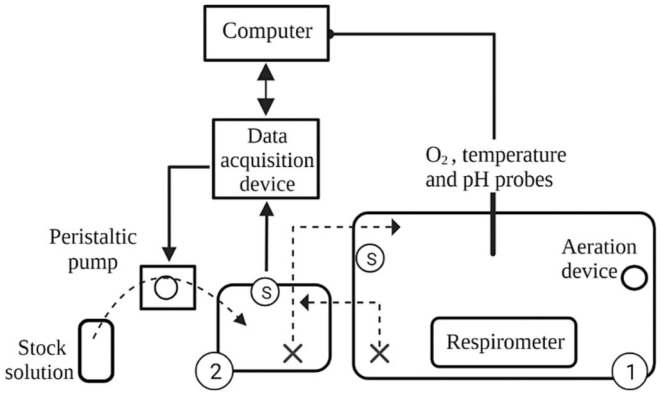

Fig. 1. Schematic representation of the experimental setup. Pumps are indicated with an X, and punctuated lines indicate tubes and flow direction (arrows). Hydrogen sulfide (H_2S) concentrations were continuously measured by H_2S- sensors marked with (S), in the main tank (1) and the mixing tank (2). An automatic feedback system controlled the H_2S concentrations, based on sensor information obtained in the mixing tank. Based on the measured H_2S concentration the peristaltic pump (connected to a 0.1 M Na_2S stock solution) was turned on/off.

2.4. H_2S exposure

H_2S concentrations were measured with the H_2S-sensors and recorded on the connected PC using the compatible software (SensorTrace Logger, Unisense, Denmark). The sensors were calibrated in seawater adjusted to ~ pH 2 with 5 M HCl, using a 9-point calibration, ranging from 0 to 20.5 μM ($R^2 < 0.99$). A sulfide stock solution of 0.1 M was prepared by adding $Na_2S{\cdot}9H_2O$ (Sigma-Aldrich, St. Louis, MO, USA) to ultrapure water that had been thoroughly bubbled with nitrogen gas for 20 min. The exposure experiments were divided into three phases: acclimatization, exposure, and recovery (Fig. 2). Throughout the acclimatization period measurement of MO_2 for SMR estimation was obtained, as well as the maximum oxygen uptake (MO_{2max}) as described in Section 2.5. During the exposure phase, H_2S concentrations in the main tank increased linearly at a rate of 0.53 ± 0.14 μM h^{-1}. The critical H_2S concentration (H_2S_{crit}), was defined as the point where two consecutive MO_2 values decreased below the SMR determined during the acclimatization phase or as the sudden occurrence of loss of equilibrium (LOE). At this point, the experimental water in the tank was exchanged as fast as possible with H_2S-free water of the same temperature, and the fish were allowed to recover overnight. The following morning the fish was euthanized in an overdose of benzocaine (0.1 g L^{-1} ethyl-p-aminobenzoate). During experiments, a daily water exchange of ~20% every morning was performed.

2.5. Calculations, data processing, and statistics

The standard metabolic rate was determined for each individual by continuously measuring MO_2 for 24 h before the H_2S exposure experiments and using the mean of the lowest normal distribution (MLND) (Chabot et al., 2016). MO_2 values with an $R^2 < 0.90$ were excluded in the determination of SMR. During exposure, MO_2 was reduced, which caused a decrease in the calculated slope and consequently, the R^2 values were lower, thus $R^2 > 0.70$ were included in the analysis during H_2S exposure. The aerobic scope (AS) was calculated as the difference between MO_{2max} and SMR (Svendsen et al., 2012). Background respiration was measured after each fish was removed from the respirometer but was found to be negligible and was not corrected for.

For graphical presentation and analysis, an average of every 10 H_2S measurements was taken, and a regression line was fitted. As H_2S concentrations were measured every minute, each open circle represents an average of 10 min of measurements in the main tank (Fig. 3). H_2S_{crit} for each fish was defined as the value (μM H_2S) obtained from the intercept between the regression line of the H_2S concentration and the vertical line extended from the MO_2 measurement prior to the occurrence of one of the predetermined events. The predetermined events used to indicate the inhibition of respiration were the occurrence of LOE and/or 2 consecutive MO_2 values below SMR. The indicator used (LOE or two consecutive MO_2 values) was determined by whichever occurred first.

Based on the individual MO_2 measurements, the oxygen deficit

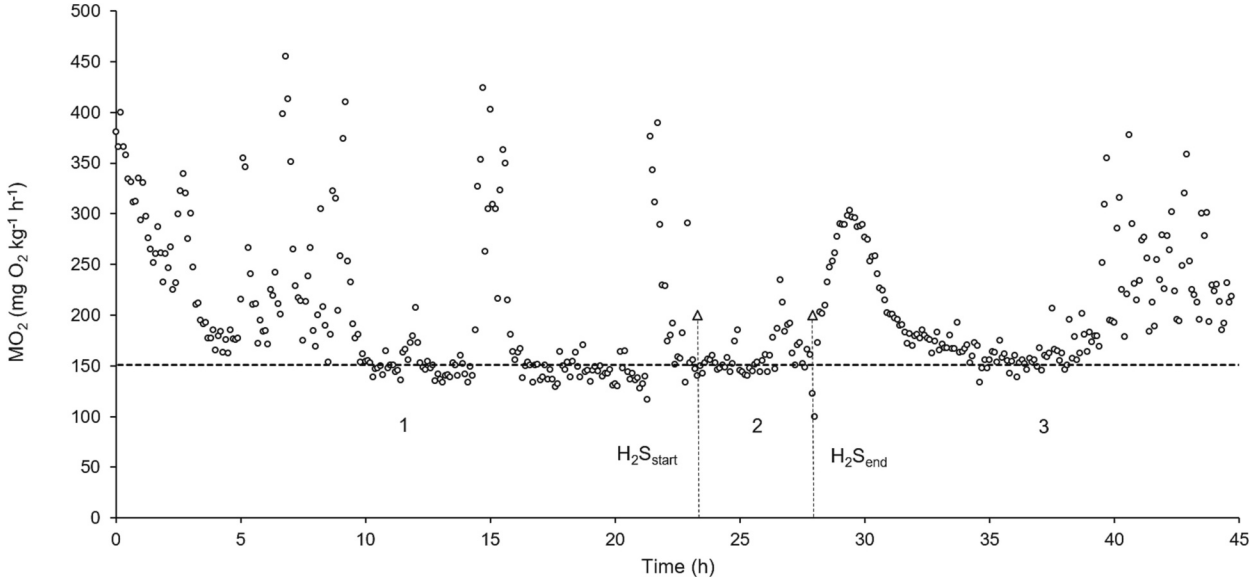

Fig. 2. Representative graph for the oxygen uptake (MO_2; mg O_2 kg^{-1} h^{-1}) during the whole experiment. The experiment consists of three phases: (1) acclimatization and MO_2 measurements for estimation of standard metabolic rate (SMR), (2) exposure to increasing concentrations of H_2S, and (3) post-exposure recovery. Example shown for an 84 g Atlantic salmon in seawater (33‰), with an estimated SMR of 150.8 mg O_2 kg^{-1} h^{-1}. The dashed line indicates the SMR and dotted vertical lines the initiation and termination of the H_2S exposure.

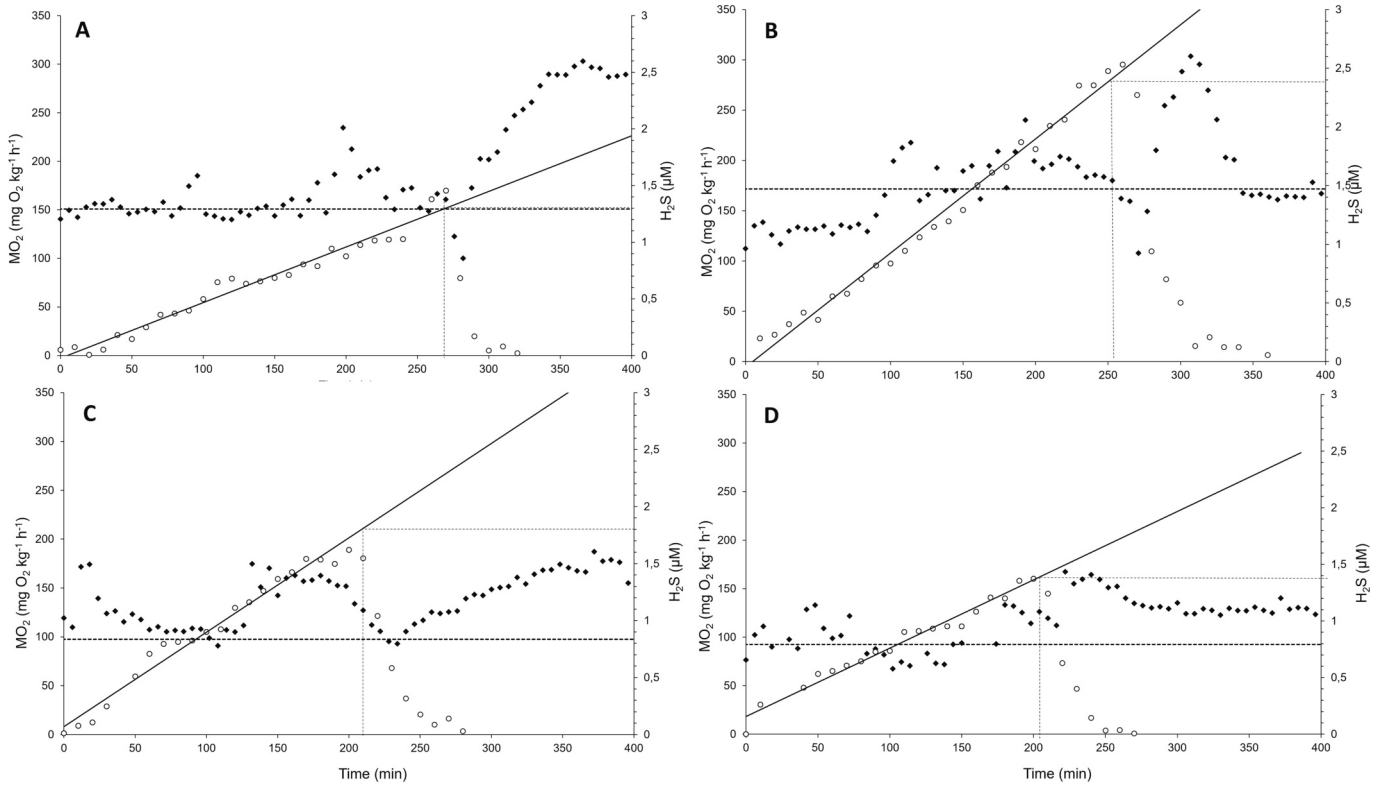

Fig. 3. A-D. Oxygen uptake (MO_2; mg O_2 kg^{-1} h^{-1}, black circles) in four Atlantic salmon (*Salmo salar*) during exposure to gradually increasing H_2S concentrations (µM, open circles). Exposure was terminated when A-B) two consecutive MO_2 values were reduced below the standard metabolic rate due to the increasing H_2S, C-D) and/or due to loss equilibrium (LOE) before a decrease in MO_2 could be observed. A regression line (solid) was fitted to the H_2S concentration. Every point represents the mean of 10 measured values. Square dotted line indicates the individual standard metabolic rate (mg O_2 kg^{-1} h^{-1}). Round dotted line shows the intercept between the predefined metabolic response indicator (reduction in MO_2/LOE) and the corresponding H_2S concentration, which is termed $H_2S_{crit.}$ Two distinct response types were observed in the fish leading up to H_2S_{crit}, an increase in MO_2 (B & C) or an unchanged MO_2.

(DO_2) generated during H_2S exposure was calculated as:

$$DO_2 = \sum_{i=0}^{n} (SMR - MO_2)t$$

where n is the number of MO_2 (mg O_2 L^{-1} h^{-1}) values below SMR as a result of the H_2S exposure, until the first MO_2 value that returned to or above the SMR, and t is the respirometry loop time (h). The excess

oxygen consumption (EOC) post-exposure was determined as:

$$EPOC = \sum_{i=0}^{n} (MO_2 - SMR)t$$

where n is the number of measurements obtained after the termination of H_2S exposure and the following increase in MO_2 until the fish returned to baseline metabolic rate, defined as two consecutive values being within 10% of the estimated SMR of the individual. MO_{2peak} values were identified as the highest MO_2 measurement upon return to H_2S-free water during the time of EOC.

The differences between the three size groups were tested with a one-way analysis of variance (ANOVA) and followed by pairwise multiple comparison procedures (Holm-Sidak). Prior to the ANOVA analysis, data were tested for normal distribution (Normality Test, Shapiro-Wilk) and homogeneity of variance (Equal Variance Test, Brown-Forsythe). All statistical analyses were performed using SigmaPlot (v. 14.0, Systat Software Inc.). The interquartile range (IQR) method was used to identify and discern outliers. Differences between size groups were considered statistically significant when $P < 0.05$. Values are presented as mean ± S·D.

3. Results

3.1. Oxygen consumption before exposure

The chasing procedure caused an elevated MO_2 which stabilized after 10–13 h in the respirometer, with occasional peaks in MO_2 due to spontaneous activity (Fig. 2). The estimated SMR was lower in the medium group compared to the large ($P = 0.026$) but did not differ from the small group. However, no differences were found between the MO_{2max} ($P = 0.261$) measured after chasing or the calculated AS ($P = 0.651$) between the three size groups. Values of the different metabolic parameters are listed in Table. 1.

3.2. H_2S exposure

During H_2S exposure, fish showed distinct responses in their MO_2, which could be either categorized as reactive or passive. Three different response patterns were observed where fish 1) began increasing MO_2 shortly after dosage of H_2S had begun, 2) maintained a steady MO_2 until H_2S reached a higher concentration, 3) maintained MO_2 independent of H_2S until H_2S_{crit} was reached at which point MO_2 decreased or LOE occurred. During exposure trials, 6 of 23 tested fish (26%) lost equilibrium without showing a decline in MO_2 below SMR. The critical H_2S concentration (H_2S_{crit}) for individual fish was identified as the point where the increasing H_2S concentration caused MO_2 to decline (Fig. 3. A-B) or the loss of equilibrium (LOE) (Fig. 3. C—D). This point indicates the tolerance threshold towards H_2S, defined as the concentration at which MO_2 was impaired to such an extent that a recognizable response was elicited. Two general response types were observed; leading up to H_2S_{crit} fish responded with either an increased or unchanged MO_2. As such, four distinct response profiles could be described: an unaffected MO_2 with increasing H_2S concentrations until reaching H_2S_{crit}, followed by a decrease in MO_2 (Fig. 3A) or a LOE (Fig. 3D), or an elevated MO_2 in response to increasing H_2S until H_2S_{crit}, followed by a decline in MO_2 (Fig. 3B) or a LOE (Fig. 3C).The H_2S_{crit} did not differ between different size groups of Atlantic salmon tested ($P = 0.786$), and the overall mean obtained for all tested individuals was 1.78 ± 0.39 μM H_2S (60.7 ± 13.2 μg /L). The mean exposure time from the onset of H_2S dosing until the fish reached H_2S_{crit} was 206.6 ± 58.4 min. Due to the difference in the estimated SMR between the groups, SMR was plotted against H_2S_{crit} to examine whether the difference affected the values of H_2S. The identified H_2S_{crit} value was independent of SMR ($R^2 = 0.063$).

Table 1
General parameters calculated based on oxygen uptake (MO_2; mg O^2 kg^{-1} h^{-1}) measurements obtained from intermittent flow-through respirometry. Values are presented as mean ± S.D., for three size groups of Atlantic salmon (*Salmo salar*) in air-saturated seawater (33%), and the critical concentration of H_2S (H_2S_{crit}) during acute exposure. Superscript letters indicate significant differences between the three groups. Values presented as mean ± S.D.

	Small ($n = 9$)	Medium ($n = 7$)	Large ($n = 7$)
Weight (g)	103.3 ± 21.0	275.3 ± 26.3	550.2 ± 39.3
SMR (mg O_2 kg^{-1} h^{-1})	155.5 ± 25.8^{ac}	119.4 ± 30.7^{a}	161.1 ± 38.7^{c}
MO_{2max} (mg O_2 kg^{-1} h^{-1})	383.6 ± 46.2	330.8 ± 84.5	371.5 ± 58.2
AS (MO_{2max}-SMR)	228.1 ± 37.2	207.3 ± 67.9	210.5 ± 30.2
H_2S_{crit} (μM)	1.76 ± 0.38	1.81 ± 0.46	1.71 ± 0.39

3.3. Recovery from H_2S exposure

The increase in MO_2 during recovery from H_2S exposure lasted up to 5 h before returning to values approaching SMR. Elevations in MO_2 occurred shortly after H_2S concentrations decreased, and often before H_2S was completely removed from the system. During recovery, MO_2 became significantly elevated, with the highest increase measured in small salmon where the mean MO_{2peak} was 43.9 ± 12.2% above SMR (Fig. 4). Despite the elevated MO_{2peak} measured upon return to H_2S-free water, values were significantly ($P < 0.05$) lower in all groups compared to the MO_{2max} measured after the chasing procedure. Within the size groups, MO_{2peak} was lower in the medium group. All calculated recovery parameters are listed in Table 2. Altogether four individuals were excluded from the calculation due to recording errors and responses that prevented the calculation of EOC. The responses included maintaining an elevated MO_2 exposure and not returning to baseline values, and one maintained a reduced MO_2 during the recovery phase for 2.5 h after the termination of exposure. The accumulated DO_2 was similar between the groups ($P = 0.547$). The EOC was significantly larger in the small salmon ($P = 0.016$). EOC was considerably larger than the accumulated DO_2 for all three size groups, thus the oxygen needed to recover from exposure to critical H_2S concentrations greatly exceeded the deficit accumulated during exposure where MO_2 was impaired.

4. Discussion

4.1. Critical H_2S concentration

In the current experiments, the mean H_2S_{crit} for all three size groups of Atlantic salmon during acute exposure to H_2S was found to be 1.78 ±

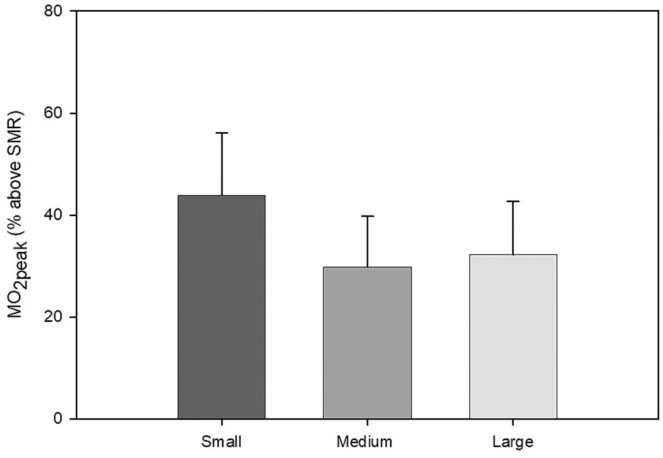

Fig. 4. Percentual increase in the maximum measured oxygen consumption (MO_{2peak}) from the estimated standard metabolic rate. Values are obtained during recovery from acute hydrogen sulfide exposure in three size ranges (Small = 103.3 ± 21.0 g, Medium = 275.3 ± 26.3 g, Large = 550.2 ± 39.3 g) of Atlantic salmon (*Salmo salar*).

Table 2
Metabolic parameters of three size groups of Atlantic salmon (*Salmo salar*) during recovery from acute hydrogen sulfide exposure. Superscript letters indicate significant differences between the three groups. Values presented as mean ± S.D.

	Small ($n = 8$)	Medium ($n = 6$)	Large ($n = 5$)
MO_{2peak} (mg O_2 kg^{-1} h^{-1})	321.4 ± 52.8[a]	217.4 ± 36.5[b]	316.3 ± 57.34[a]
DO_2 (mg O_2 kg^{-1} h^{-1})	10.1 ± 6.9	5.8 ± 7.3	10.8 ± 8.9
EOC (mg O_2 kg^{-1} h^{-1})	405.7 ± 176.3[a]	163.5 ± 120.6[b]	186.2 ± 119.1[b]
Time to SMR (min)	256.0 ± 75.5	214.8 ± 106.1	190.0 ± 153.1

0.39 μM H_2S (60.7 ± 13.2 μg/L). Upon reaching the threshold for H_2S, MO_2 drastically decreases, illustrating the limited time for removing H_2S before the consequences from exposure become irreversible. Literature investigating the tolerance and effects of H_2S exposure *in vivo* in teleosts is scarce, and the majority of studies have focused on species with a high tolerance to H_2S, such as the channel catfish (*Ictalurus punctatis*), goldfish (*Carassius auratus,* 96-h LC50 at 3.5 μM H_2S), tambaqui (*Colossoma macroponum,* 34 μM H_2S), and the California killifish (*Fundulus parvipinnis,* >20-h LC50 at 1.5 mM sulfide)(Adelman and Smith Jr., 1972; Affonso et al., 2002; Bagarinao and Vetter, 1989; Torrans and Clemens, 1982). These reported tolerance levels exceed by far the critical concentrations found for Atlantic salmon in this study. The high variation in reported values could be attributed to the differences in techniques for measuring H_2S, as well as the inconsistency between the estimated values of critical H_2S concentrations, which in many studies have been reported as "total sulfide", *i.e.*, the sum of H_2S, HS^- and S^{2-}. The actual H_2S concentration can be calculated from the measured total sulfide, but this is only possible if information on the chemical water parameters (pH, salinity, temperature) is reported. Similar complications occur when providing only the concentration of the sulfide donors such as Na_2S and NaHS. Due to the oxidation of sulfide by oxygen, the half-life of H_2S in a solution is <3 h (Bergstedt et al., 2022). Therefore, if a continuous dosage of H_2S in the system is not maintained, a mismatch between the actual experimental concentration and the theoretically intended concentration will occur. Ultimately this may lead to an overestimation of the H_2S concentration and the tolerance threshold. Another parameter that may affect the reported critical concentration of H_2S exposure in fish is the rate of induction. Rapid dosages of H_2S into the system will result in a lag phase between measured H_2S, uptake by the fish, and the exerted effect on the whole organismal level, which may also overestimate tolerance thresholds. In contrast, a slow dosage may underestimate the maximum tolerance and instead reflect the cumulative effect of H_2S exposure and the capacity for metabolizing sulfide by the fish. Thus, experiments that examine both the effect of rapidly increasing H_2S concentrations (acute exposure) as well as long-term effects of lower concentrations will help in advancing the understanding of H_2S exposure and the relevant *in vivo* concentrations.

Currently, knowledge gaps regarding the tolerance and response to H_2S still exist and much of the information available from the preceding literature is based on species that exhibit a high tolerance to H_2S. Though, apart from their capability to survive high levels of H_2S, these species do not appear to share a common ecology, being native to both marine and freshwater systems, temperate and tropical. However, species with high H_2S tolerance share the trait of being tolerant to hypoxia, despite employing different strategies to cope with low O_2 concentrations (Borowiec et al., 2015; Mandic et al., 2008; Scott and Rogers, 1981). In contrast, hypoxia-sensitive species, such as the bluegill (*Lepomis macrochirus*, 96-h LC50 of 1.3 μM H_2S*)* and rainbow trout*,* show a lower tolerance to H_2S (Marvin and Burton, 1973; L. L. Smith et al., 1976). The difference in the tolerance threshold of H_2S reported for the different species appears to reflect the general risk of encountering H_2S, and as a pelagic and highly active species, it is not surprising that Atlantic salmon have a lower tolerance to H_2S, compared to species found in environments where the H_2S production potential is greater (Bagarinao and Vetter, 1989). The reported values point to a link between the tolerance towards hypoxia and H_2S. This connection between H_2S and O_2 has also been demonstrated in fish, where H_2S has been suggested to function as an oxygen-sensing mechanism (Olson et al., 2008). In environments that are prone to hypoxic events, there is also a risk of H_2S production (Grieshaber and Völke, 1998), and it seems reasonable that an organism that can tolerate environmental hypoxia also tolerates tissue hypoxia induced by H_2S, and is likely related to DO_2 tolerance. Skovgaard and Olson (2012) further examined the response in perfused trout gills and found that exposure to hypoxia or H_2S caused vasoconstriction of the gills, which is a common response to hypoxia that leads to an increased functional surface area of the gills by lamellae recruitment (Pettersson and Johansen, 1982). The exact mechanism that defines the tolerance to H_2S has yet to be discovered, and specific adaptations, such as modified COX, are not present in all species that tolerate high H_2S levels (Tobler et al., 2016).

To advance the knowledge of the effects of H_2S on fish, the connection between tolerance towards H_2S and the capacity to metabolize H_2S needs to be examined. In addition, the development of a standardized protocol is needed, as such would facilitate comparison between studies. The standardized procedure should as a minimum include a description of *e.g.*, chemical water parameters for calculation of the fraction of H_2S present if it is total sulfide that is measured, the rate of induction of H_2S, and a common indicator for when the fish is encountering the critical H_2S concentration.

4.2. Response to increasing H_2S

Large individual variability in the response pattern of MO_2 to increasing H_2S concentrations was observed post-exposure to H_2S. Similar variability in the response of MO_2 to decreasing oxygen levels has been observed during hypoxia tolerance studies in Atlantic salmon (Barnes et al., 2011). Combined with the lack of differences in H_2S tolerance, this may suggest that this response variability is rooted in the behavioral phenotype of fish. The majority of the fish showed an increase in MO_2 as H_2S concentrations increased, while some individuals did not show an increase in MO_2 before reaching H_2S_{crit}. Individual variability in the response and coping mechanisms to various stressors has been demonstrated previously, and the sources of the individual variation have been ascribed to measurement errors, experimental design, genetic variation, and phenotypic plasticity (Nikinmaa and Anttila, 2019). To elucidate if the variability measured could be attributed to experimental parameters or the individual fish, potential connections between biological measurements and estimates (SMR, AS, MO_{2max}, DO_2, EOC, time to SMR, and duration of exposure) as well as ambient parameters (experimental temperature, thermal history) were examined, but did not reveal any interdependence. Thus the mechanism and causes behind the measured individual plasticity in the metabolic response to acute H_2S exposure, and the variability in recovery profile is not clear, and more experimental work on the pathways that are central for the removal of H_2S, such as sulfide quinone oxidoreductase that is oxidizing H_2S in the mitochondria (Paul et al., 2021), is needed to obtain further information on the underlying mechanisms that determine H_2S tolerance. The absence of a uniform response to increasing H_2S concentration may limit the feasibility of using computerized monitoring systems based on behavioral indicators. The technology for monitoring H_2S concentrations and H_2S mitigation protocols to prevent a build-up of H_2S should rely on sensor systems that provide quick feedback. The negative effects on fish exposed to high H_2S concentrations are rapid, and actions in order to prevent casualties, such as the addition of oxidative agents, need to be taken fast to remove H_2S from the system (Bergstedt et al., 2022).

4.3. Recovery from H_2S exposure

Individual variability was not limited to the response and coping

style to increasing H_2S concentrations, but also to the recovery response and measured recovery parameters such as DO_2, EOC, and time to SMR, following exposure to an acute stressor (Ferrari et al., 2020). The individual coping styles were not quantified in this study, but it cannot be excluded that a difference in physiological-behavioral traits was contributing to the difference in the sensitivity to H_2S and the extent of the recovery period across the three size groups. Despite differences in SMR, all three groups had the same AS and thus a similar capacity to repay EOC. Thus, the three groups had the same capacity for supporting oxygen-demanding processes, such as recovering from H_2S. The EOC measured was several-fold higher than the accumulated DO_2, illustrating that the oxygen needed to recover from exposure to critical H_2S concentrations far exceeded the deficit. The small salmon had a larger EOC than the two other size groups, which suggests that the effect of H_2S was greater in smaller individuals, and more O_2 was needed to clear the metabolic by-products and for re-synthesis of the depleted energy storage (Mandic et al., 2008). Especially during exposure to H_2S where aerobic ATP production is prevented, the highly metabolically active organs such as the heart, gills, and brain are supported by continuous delivery of glucose derived from the mobilization of glycogen reserves. Larger body size can therefore be an advantage as the lower mass-specific metabolic rate result in a reduced depletion of muscle and liver glycogen stores (Nilsson and Östlund-Nilsson, 2008). Quantification of the glucose and glycogen storages and utilization can elucidate the extent to which glycogen supports energy production, and if the quantity and substrate available in the tissues is a limitation for the tolerance to H_2S and the specific H_2S_{crit} of the fish.

5. Conclusion

In summary, this study demonstrates that the tolerance of Atlantic salmon to acute H_2S exposure is much lower than previously reported. While such a low tolerance has been suspected, due to the occasional mass mortalities observed in aquaculture systems, it has so far not been confirmed. This underlines the challenge that H_2S poses for the aquaculture industry, the necessity for short response times following an H_2S incident, and the importance of implementing systems for monitoring H_2S. If the behavioral response to H_2S shows a similar degree of variability as the metabolic response to gradually increasing H_2S levels, implementation of behavioral monitoring software could be impeded, if a uniform behavioral response to H_2S that can be used as a warning indicator is absent as well. Thus, the most fail-safe approach would be the implementation of H_2S-sensor systems for continuous measurement in order to alert production managers well in advance. Although the upper tolerance threshold to H_2S did not differ between the three groups of Atlantic post-smolt, exposure to H_2S caused a greater effect on the recovery phase of the small group. Here, the fish had a higher excess oxygen consumption than the two other groups, and production facilities need to consider the excess oxygen requirement for the fish if they are exposed to H_2S. Future efforts on H_2S toxicity and tolerance should focus on 1) examining and comparing the activity of enzymes that metabolize H_2S (*e.g.* sulfide quinone oxidoreductase and superoxide dismutase) and their role in relation to H_2S tolerance of various tissues and across species, 2) obtaining information on the metabolic recovery processes, detoxification of sulfide, and the metabolites accumulated from the onset of anaerobic metabolism during H_2S exposure, to gain knowledge on the mechanisms that facilitates removal of sulfide and whether they differ between species of either high or low H_2S tolerance. Finally, it is imperative that a standardized protocol for conducting H_2S-tolerance studies is determined, which will allow for a comparison of H_2S tolerance within species.

CRediT authorship contribution statement

Julie Hansen Bergstedt: Conceptualization, Methodology, Writing – original draft, Investigation, Formal analysis, Visualization, Data curation. **Peter Vilhelm Skov:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

This study was partially funded by the project H_2Salar, financed by the Research Council of Norway (Project number 300825).

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