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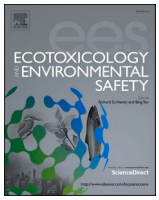

Ecotoxicological assessment of Cu-rich acid mine drainage of Sulitjelma mine using zebrafish larvae as an animal model

Shubham Varshney, Mikkel Lundås, Prabhugouda Siriyappagouder, Torstein Kristensen, Pål A. Olsvik *

Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

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ABSTRACT

Acid mine drainage (AMD) is widely acknowledged as a substantial threat to the biodiversity of aquatic ecosystems. The present study aimed to study the toxicological effects of Cu-rich AMD from the Sulitjelma mine in zebrafish larvae. The AMD from this mine was found to contain elevated levels of dissolved metals including Mg (46.7 mg/L), Al (20.2 mg/L), Cu (18.3 mg/L), Fe (19.8 mg/L) and Zn (10.6 mg/L). To investigate the toxicological effects, the study commenced by exposing zebrafish embryos to various concentrations of AMD (ranging from 0.75% to 9%) to determine the median lethal concentration (LC_{50}). Results showed that 96 h LC_{50} for zebrafish larvae following AMD exposure was 2.86% (95% CI: 2.32–3.52%). Based on acute toxicity results, zebrafish embryos (<2 hpf) were exposed to 0.1% AMD (Cu: 21.7 μg/L) and 0.45% AMD (Cu: 85.7 μg/L) for 96 h to assess development, swimming behaviour, heart rate, respiration and transcriptional responses at 116 hpf. Light microscopy results showed that both 0.1% and 0.45% AMD reduced the body length, eye size and swim bladder area of zebrafish larvae and caused phenotypic abnormalities. Swimming behaviour results showed that 0.45% AMD significantly decreased the locomotion of zebrafish larvae. Heart rate was not affected by AMD exposure. Furthermore, exposure caused a significant increase in oxygen consumption indicating vascular stress in developing larvae. Taken altogether, the study shows that even heavily diluted AMD with environmentally relevant levels of Cu caused toxicity in zebrafish larvae.

1. Introduction

Acid mine drainage (AMD) or acid rock drainage (ARD) is the runoff coming out from mines of sulphur-bearing minerals. AMD drainage usually contains elevated levels of metals like iron (Fe), aluminium (Al), copper (Cu), and zinc (Zn). (Jönsson et al., 2006). This runoff is formed due to oxidation reactions between surface water (rainwater, groundwater or snow-melt water) and sulphur-bearing mineral rocks (Akcil and Koldas, 2006; Simate and Ndlovu, 2014). These reactions lead to the formation of sulphuric acid resulting in extremely low water pH (Rezaie and Anderson, 2020). The phenomenon of AMD is associated with both abandoned and active mines (Acharya and Kharel, 2020), but the regular pumping out and better management of flow through water in active mines subsides this acidic runoff to some extent (Rezaie and Anderson, 2020). AMD poses a severe concern to the environment due to its low pH and high concentration of metals (Matlock et al., 2002; McCarthy, 2011). Only obligately acidophilic eukaryotes such as fungi, yeasts, algae and protozoa are known to survive the acrid conditions in undiluted AMD (Colmer et al., 1950; Druschel et al., 2003).

AMD is one of the most noxious forms of aquatic pollution. Runoff from a single mine tailing can spread and pollute large areas (Smuda et al., 2014). When AMD meets the waterbodies, low pH may disrupt the metabolic and reproductive efficiency of aquatic organisms (Hogsden and Harding, 2012). Some dissolved metals in AMD have the property to bioaccumulate in tissues and disrupt their normal functioning (Simate and Ndlovu, 2014). Calcium (Ca) and magnesium (Mg) are essential micronutrients for fish, and their levels in the water should be within a certain range for fish to thrive. However, in AMD, the levels of these metals become excessive, leading to metabolic disturbances in fish and reduced growth rates (Akcil and Koldas, 2006). Aluminium (Al) is a metal that is commonly found in AMD and it becomes toxic at low pH due to a high charge-to-volume ratio as Al^{3+} ions with subsequent binding to negatively charged biotic ligands (Neville and Campbell, 1988). Al toxicity can result in disruption of respiration across gills

* Correspondence to: Faculty of Biosciences and Aquaculture, Nord University, N-8049 Bodø, Norway.
E-mail address: pal.a.olsvik@nord.no (P.A. Olsvik).

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surfaces, loss of osmoregulatory capacity and specific enzyme inhibition (Na/K ATPase) occurring at relatively low concentrations in low pH waters (Thorstad et al., 2013). High concentrations of Al can cause physical deformities in fish, such as the bending of the spine (Capriello et al., 2021). Additionally, it can also cause neurological damage, leading to impaired swimming ability and reduced predator avoidance (Capriello et al., 2019; Senger et al., 2011). The presence of Fe in AMD can cause adverse effects, such as reduced oxygen levels in the water, which can lead to hypoxic or anoxic conditions, both of which can be fatal to fish (Faulkner and Skousen, 1994). Water contains Fe in various forms, including Fe^{2+} and transitory Fe^{3+}. While these forms of iron are not completely precipitated as $Fe(OH)_3$, they can still bind to o1ther substances in the water such as clay or sand, resulting in the formation of ochre (Casiot et al., 2009). When this ochre builds up in a body of water, it can cover fish gills and prevent them from obtaining oxygen from the water (Younger and Wolkersdorfer, 2004). Another toxic metal in AMD is Cu which can bioaccumulate in aquatic organisms and exert toxic effects on growth, reproduction and overall health (Czédli et al., 2014). Apart from getting bioaccumulated, it can also get biomagnified across specific marine food webs (Cardwell et al., 2013; Mirzaei VandKhanghah et al., 2022). In this way, organisms at higher levels have higher levels of Cu than organisms at lower levels. Cu even at low concentrations can cause oxidative stress, gill injury, and intestinal inflammation in fish (Craig et al., 2007; Pereira et al., 2016). Cu is also linked to increased NH_3^+ levels in plasma by inhibiting the gill Na^+/K^+ ATPase, which results in altered Na^+ ion levels in plasma and, ultimately, osmoregulatory dysfunction (Blanchard and Grosell, 2006; Grosell et al., 2007).

Apart from aquatic organisms, dissolved metals like mercury (Hg), cadmium (Cd), and arsenic (As) in AMD may also pose a serious threat to humans when they contaminate the drinking water. The negative effect of AMD is not only limited to aquatic life, AMD also degrades surrounding soil by heavy metal contamination (Rezaie and Anderson, 2020). Despite being a significant environmental issue, there is a lack of scientific studies showing the effect of AMD on aquatic life and one of the reasons could be that AMD often affects aquatic environments in remote or hard-to-reach locations.

The Sulitjelma mine, which is located in northern Norway, was started in 1884 to extract Cu, FeS_2 (pyrite) and Zn ore out of the Sulitjelma mountains and was closed in 1991. Metals were extracted from a total of 18 ore bodies. Upon closure, mine shafts gradually filled up with water, and AMD runoff from adjacent areas was also led into the mines as a temporary mitigating measure. During the following decades, the mine shafts overflowed, leading to increased runoff of AMD to the natural waterbodies downstream. Drainage from the Sulitjelma mine enters Langvatnet lake, which is still contaminated with heavy metals. Surveillance data showed that the water concentration of Cu, Zn and Cd near the outlet of Langvatnet in 2021 was 14.9, 17.7 and 0.03 μg/L, respectively (annual average) (Norconsult, 2022). Before closing the mine, a substantial effort was made to limit the acid mine runoff. Despite these efforts, continuous testing between 1993 and 2015 showed an increased volume of discharge having low pH and high metal concentrations (Norconsult, 2022). A direct effect of this can be seen at Grunnstollen, where AMD from one of the most polluting mine shafts enters Gikenelva, resulting in decreased pH and increasing levels of Cu (11.3 mg/L; 2021) over the years (Gjertsen and Risvoll, 2018; Norconsult, 2022). Lake Langvatnet is a 5.5 km^2 lake at 127 msl with a catchment area of 585 km^2. The naturally variable seasonal runoff due to snow cover in winter and its melting during summer is highly disrupted by upstream hydropower operation. This hydropower plant with high retainment capacity results in a much higher winter runoff and subsequent lower summer runoff that corresponds poorly with high AMD runoff during summer, thus lower AMD dilution. Regardless of this, the newly founded company "Nye Sulitjelma Gruver AS" plans to reopen the mine. It is essential to thoroughly evaluate the environmental risks, such as AMD, and take appropriate measures to minimise negative impacts on the environment before reopening the mine. Metal concentrations and water volumes from the main source of AMD, the water-filled mine shafts (Grunnstollen) have been monitored regularly, albeit with varying time resolution, since operations closed. Varying concentrations from 6 to 27 mg Cu/L, with peak concentrations during summers have been found (Norconsult, 2022). The resulting metal concentrations at the lake outlet has also been documented, along with infrequent surveys of fish population status in the lake. Overall, the highly contaminated AMD water is sufficiently diluted for the lake fish population to be sustained, but elevated well above assumed background level and sufficiently high to maintain ecosystem impact (Iversen et al., 2008). Seasonal variation from winter levels at around 10 μg Cu/L to summer peaks at 30–80 μg Cu/L have been documented in time series from 1990 to present time, with little to no signs of improved conditions (Norconsult, 2022).

To date, research on AMD is tilted more towards metal characterisation and fewer studies focused on the ecotoxicological effects. It is reported that thousand-fold diluted AMD causes toxicity in phytoplankton (Oberholster et al., 2010), daphnia (Martins et al., 2009; Soucek et al., 2000) and adult zebrafish (Pereira et al., 2020). The zebrafish larvae is an animal model used considerably to evaluate the toxicity of various environmental pollutants. Due to the small size and transparent body of zebrafish larvae, it is possible to observe real-time developmental changes using microscopy. The transparent body also provides a non-invasive method to measure the heart rate. To date, there is a notable gap in the understanding of how AMD alters the behaviour of fish larvae and, to our knowledge, no scientific reports on this topic are available. Therefore, the present study aimed to evaluate whether a thousand-fold diluted AMD produces toxic effects in zebrafish larvae. For this study, we decided to go with a multiple endpoint approach as AMD can have multifaceted effects on organisms. These effects are often visible at different levels of the biological organization, from molecules (qPCR) to phenotype (development, behaviour, heart rate). The information generated from multiple molecular and phenotypic endpoints will provide toxicological information which can aid the conservation and remediation efforts in AMD-affected areas.

2. Materials and methods

2.1. Sample collection

Three AMD samples of two litres each were collected on 15th October 2021 from the tailing of the Sulitjelma mine at Grunnstollen (67°07'58.9"N 16°05'06.1"E; Fig. 1) in Sulitjelma, Norway. Water temperature, pH, dissolved oxygen and specific conductivity of the samples were determined on-site with portable instruments (Orion 4-star, Thermo Scientific, USA). The physicochemical properties of the AMD samples were determined at the Fish physiology laboratory at Nord University. The AMD samples were stored in dark at 4 °C before metal analysis and exposure studies.

2.2. Chemical analysis

To analyse the heavy metal content of the raw AMD, we sent one sample from one of the bottles having AMD samples to an accredited laboratory (ALS Laboratory Group, Luleå, Sweden). The concentration of 19 metals in AMD samples was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES), inductively coupled plasma-sector field mass spectrometry (ICP-SFMS) and inductively coupled plasma-atomic fluorescence spectrometry (ICP-AFS). The metal analysis was performed by an accredited laboratory (ALS Laboratory Group, Luleå, Sweden).

2.3. Zebrafish husbandry

Adult zebrafish (AB strain) were reared in a recirculatory system

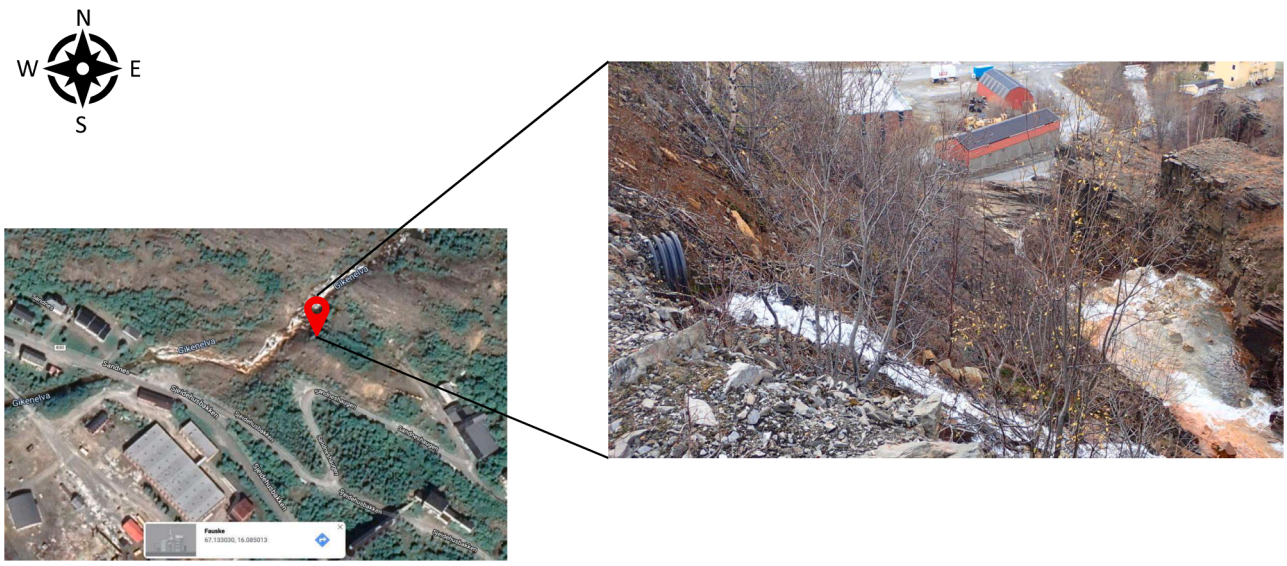

Fig. 1. Location of the sampling site for acid mine drainage (AMD) in the Sulitjelma Cu mines.

(Aquatic Habitats Z-Hab System) under optimal conditions (water temperature = 27 ± 1 °C, pH = 7.3–7.5, conductivity = 500–800 μS and 12 L:12D photoperiod) at the zebrafish facility of Nord University. Adult zebrafish were fed ad libitum with Zebrafeed® micro diet (Sparos Lda, Olhão, Portugal) twice a day. Zebrafish eggs were obtained by mass breeding of adult males and females in the breeding tanks (Lawrence, 2007). Fertilised eggs were reared in the ISO standard fish media (ISO water: ISO 7346–3; 79.99 mM $CaCl_2$.2 H_2O, 20.00 mM $MgSO_4$.7 H_2O, 30.83 mM $NaHCO_3$, 3.09 mM KCl, pH = 7.4 ± 0.1) (ISO, 2007). Since zebrafish embryos and larvae below 120 hr old are not protected animal stages, no animal test authorisation is required according to European legislation Directive 2010/63/EU (Commission, 2010). All exposure experiments were terminated before 120 hpf (hours post fertilisation) and fish were euthanised at 118 hpf by immersing in water containing 200 mg/L of MS222 along with 200 mg/L of sodium bicarbonate (Matthews and Varga, 2012).

2.4. Zebrafish embryo acute toxicity (FET) test

The zebrafish embryo acute toxicity test was conducted according to the OECD guidelines (OECD, 2013) with minor adaptations. The test was commenced within two hpf (<16 cell stage) and terminated at 96 hpf. For validation of the test 4 mg/L 3,4-Dichloroaniline (DCA) was used as a positive control and ISO standard fish media as a negative control (OECD, 2013). Viable eggs were selected based on their regularities under the binocular microscope (Leica Z00M 2000) (OECD, 2013). The selected eggs were placed in pre-treated 24-well plates (Cat. number: GREI662000–06_28, VWR, Norway) with 2 ml of test solution per embryo in each well (OECD, 2013). The test was performed under the semi-static system with a 90% renewal of the test solution every 24 h (OECD, 2013). Twenty eggs per well were exposed to twelve concentrations ranging from 0.75% to 9% AMD (~137.25 μg/L to 1647 μg/L Cu), spaced at intervals of 0.75%. These experiments were conducted in triplicates to ensure robust and reliable results. Soon after the exposure, the well plates were kept in a climate chamber (Sanyo MIR-254, Sanyo Scientific, Bensenville, Illinois, United States of America) at 27 ± 1 °C with a 12 L:12D photoperiod cycle. The pH of test solutions was not neutralised due to the lack of knowledge on the effect of pH alteration on metal speciation (Lourenço et al., 2017). The mortality (endpoint of FET) was counted every 24 h to calculate the lethal concentration (LC) values.

2.5. Experimental set-up

Doses for the developmental, behavioural, cardio-vascular and qPCR studies were selected based on the results obtained from the FET tests and metal concentration analysis. After the acute toxicity tests, we selected two concentrations as low (0.1% AMD; 4% of 96 h LC_{50}) and high (0.45% AMD; 16% of 96 h LC_{50}) to study their effect on behaviour, development, respiration and gene expression. The low dose (Cu; 21.73 μg/L, Zn; 4.79 μg/L) was also selected to match the level of pollutants in the nearby Langvatnet lake (Cu; 14.9 μg/L, Zn; 17.7 μg/L) whereas the high dose was selected as an environmentally relevant worst-case scenario given the high variability in dilution of AMD and higher concentrations close to discharge points. Another reason to select a high dose was to examine the sub-lethal effects of AMD in zebrafish larvae. The above said test solutions were prepared by diluting raw AMD in ISO standard fish media. The raw AMD was diluted using the same bottle whose sample was sent for heavy metal analysis. Additionally, the heavy metal analysis was conducted on three samples from the 0.1% and 0.45% groups. Fertilised eggs were exposed to 0% of AMD (Control), 0.1% of AMD (Low dose) and 0.45% AMD (High dose). Then about 100 fertilised eggs (<16 cell stage) were exposed to 200 ml of test solution in a 500 ml pre-treated glass beaker for 96 h. The glass beakers were covered with parafilm (with several holes) and were kept in a climate chamber (Sanyo MIR-154, Sanyo Scientific, Bensenville, Illinois, United States of America) at 27 ± 1 °C with a 12 L:12D photoperiod cycle. Around 90% of the test solution was renewed every 24 h to maintain an adequate concentration of contaminants (Busquet et al., 2014). At the end of 96 h, the test solution was replaced with ISO standard fish media for all the treatments until the last sampling point (118 hpf). We conducted three independent main exposure experiments (E1: Behavioural toxicity assay + Developmental toxicity studies; E2: Respiration toxicity assay and Heart rate assay; E3: Samples for qPCR). All experiments were performed in triplicates.

2.6. Developmental toxicity studies

Developmental studies were performed separately at 72, 96 and 116 hpf. For this, we randomly selected ten larvae (n = 10) among the replicates of each exposure group. Then larvae were individually examined and photographed under the stereomicroscope Olympus SZX12 (Melville, USA). Images were taken using an Olympus SC50 video camera (Olympus soft imaging solutions, Münster, Germany). The open-source ImageJ software (http://imagej.net) measured body length, eye

size, yolk sac area and bent angle (head-to-trunk angle) (Schindelin et al., 2012). Images were also assessed for larval deformities across the treatment.

2.7. Heart rate assay

At 116 hpf, ten larvae per exposure group (n = 10) were randomly selected among the replicates. Selected larvae were fixed on cavity slides and were immobilised using 3.5% methylcellulose (Muntean et al., 2010; Varshney et al., 2023). Videos were taken using Olympus SZX12 (Melville, USA) equipped with an Olympus SC50 video camera (Olympus soft imaging solutions, Münster, Germany) (Zakaria et al., 2018). Heart rate videos of around 25–30 s at 25 frames per second were recorded with zebrafish larvae placed laterally (García-Cambero et al., 2019). The recorded videos were analysed for heart rate using the DanioScope *v*1.1 software (Noldus Information Technology, Netherlands).

2.8. Respiration toxicity assay

At 112 hpf, ten larvae per exposure group (n = 10) were randomly selected among the replicates to measure the oxygen consumption rate. Following the manufacturer's guidelines, the system was calibrated using oxygen-depleted (0.159 M sodium sulphite) and oxygen-saturated ISO standard fish media to reduce the error due to in-system oxygen. Zebrafish larvae were distributed into a 24-well plate sensor dish (PreSens, Germany) at a density of two larvae per well, except for one well that served as a blank control (Pan et al., 2019). Then, the sensor dish was submerged in a glass tank containing ISO-standard fish media (Wang, 2019). The temperature (27 ± 1 °C) was maintained by running the system in the dark inside a climate chamber (Sanyo MIR-154) (Vliet, 2019). The oxygen consumption rate was recorded for six hours using the program MicroResp® version 1.0.4 (Loligo Systems, Viborg, Denmark).

2.9. Behavioural toxicity assay

At 116 hpf, 24 larvae per exposure group were randomly selected among the replicates for measuring the locomotor behaviour using the DanioVision system (Noldus Information Technology, Wageningen, Netherlands). For the locomotor recordings, zebrafish larvae were placed in 24-well plates. Before starting the recording, the larvae were acclimatised in the dark for 5 min (Padilla et al., 2011). The 20 min recording incorporates two repeated cycles of 5 min light (60% intensity ~ 2640 lux) and 5 min dark. The 24-well plates were placed into the DanioVision™ observation chamber (Noldus Information Technology, Wageningen, Netherlands) to track larval locomotion at 27 ± 1 °C. All locomotor responses were recorded between 13:00 and 16:00 to reduce variability due to metabolic activity (Chiffre et al., 2016). The data obtained were analysed using the EthoVision® XT 16 software (Noldus Information Technology, Wageningen, Netherlands). To reduce the background noise such as instrument's own noise 0.2 mm MDM (minimum distance moved) smoothing was applied (Nüßer et al., 2016).

2.10. RNA extraction, cDNA synthesis and RT-qPCR

At 116 hpf, ten larvae belonging to the same exposure group were pooled as one replicate for the qPCR studies. The larvae were snap-frozen by immersing them in liquid nitrogen and were stored at − 80 °C before further RNA isolation. The pooled larvae were suspended in the QIAzol reagent (Cat. number: 79306, Qiagen, Hilden, Germany) followed by homogenisation. The total RNA was isolated using the Direct-zol™ RNA MiniPrep (Cat. number: R2052, Zymoresearch, CA, USA) following the manufacturer's instructions. The isolated RNA was suspended in 30 μL ultra-pure DNAse/RNAse-free water. The concentration of total RNA was measured using the NanoDrop OneC Microvolume UV-Vis Spectrophotometer (Cat. number: ND-ONEC-W, NanoDrop Technologies, Wilmington, DE, USA) and Qubit™ 4 Fluorometer (Cat. number: Q33238, Thermo Fisher Scientific, Waltham MA, USA). The quality of the RNA was checked using agarose gel electrophoresis. A total of 750 ng RNA was used to prepare cDNA using the QuantiTect reverse transcripton kit (Cat. number: 205311, Qiagen, Germany) following the manufacturer's protocol. Primers for qPCR studies were chosen that were associated with oxidative stress, apoptosis, Cu-transport, protein folding, neurological effect, Cd toxicity, endocrine disruption and swim bladder functioning. Some of the selected primers were taken from published literature while others were designed using the Primer Express software (version 2.0). The qPCR reactions were run with a sample size of six per group (n = 6). A total of 11 genes were selected as target genes and their details are provided in Table 1. All PCR reactions were carried out in triplicate with a reaction volume of 10 μL. The reaction mixture contained 5 μL of FastStart universal SYBR green master mix (Cat. number: 04707516001, Roche, Holding AG, Basel, Switzerland), 2 μL of primer pair and 3 μL of 10X diluted cDNA sample. The real-time PCR amplification was performed on a LightCycler® 96 real-time PCR System (Cat. number: 05815916001, Roche, Holding). The cycling conditions were initial holding at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 60 °C for 20 s. The specificity of the amplification of each reaction was verified by analysing a melting curve. The standard curve was plotted by running a series of dilutions (1:10, 1:100, 1:1000, 1:10000 and 1:100000) of pooled cDNA. A preliminary experiment was conducted on the stability of three reference genes (*uba52*, *tuba1* and *ef1a*) across all the samples. Based on the stability and uniform expression across all the samples, ubiquitin 52 and elongation factor 1 A were selected as reference genes and used for normalisation. The quantification results of the qPCR were analysed using the $2^{-\triangle\triangle Cq}$ method with PCR efficiency correction (Pfaffl, 2001).

2.11. Statistical analysis

The calculation of the FET was performed using the 'ecotox' package in R studio. The LC_{50} values were determined along with the 95% confidence interval (OECD, 2013). For all other exposure studies, the significant outliers were detected and omitted by Grubbs' method. Before proceeding towards the statistical tests, the normality of the data was evaluated using Kolmogorov-Smirnov and Shapiro-Wilk tests. The normally distributed data (development, heart rate, non-angular behaviour) was analysed using one-way ANOVA followed by Dunnett's multiple comparison tests deployed to test the effect of the treatment. Non-normal data (respiration) was analysed using Friedman's test followed by the Wilcoxon Signed-Rank test. The circular data (angular behaviour) was analysed using the Rayleigh test (test of uniformity) followed by Stephens Modified Watson's test. All statistical tests were performed in R studio, and plots were prepared using the same. A significance level of $p < 0.05$ was considered to be statistically significant (*) and $p < 0.005$ was considered to be very significant (**). Representative heatmaps and trajectory maps of the distance travelled by larvae were prepared using the EthoVision® XT 16.0 software (Noldus Information Technology, Wageningen, Netherlands).

3. Results

3.1. Physico-chemical characterisation of raw and diluted AMD sample

The raw AMD sample of the Sulitjelma Cu mine had a pH of 3.5, specific conductivity of 1.69 mS/cm and acid capacity of 66.5. The metal analysis of the raw AMD sample (Table 2) showed a very high concentration of metals, specifically Ca (193 mg/L), Mg (46.7 mg/L), Al (20.2 mg/L), Fe (19.8 mg/L), Cu (18.3 mg/L) and Zn (10.6 mg/L). Fish survival in raw AMD is impossible due to low pH and high metal toxicity.

Table 1
List of the primers used in the gene expression studies.

Target genes	Gene function	Primer sequences	Length (bp)	GenBank ID	Efficiency (%)	Reference
uba52 - ubiquitin	Reference gene	F: CGAGCCTTCTCTCCGTCAGT R: TTGTTGGTGTGTCCGCACTT	126	NM_001037113	94.12	
ef1a - elongation factor 1A	Reference gene	F: AGACAACCCCAAGGCTCTCA R: CTCATGTCACGCACAGCAAA	126	AY422992	94.12	
tuba1 - tubulin A1	Reference gene	F: GGTGCCCTCAATGTGGATCT R: GCCACAGAGAGCTGCTCATG	131	AF029250	95.50	
cat - catalase	Oxidative stress	F: TCTCCTGATGTGGCCCGATA R: GGTTTTGCACCATGCGTTTC	169	NM_130912	94.45	(Santos et al., 2020)
gpx1 - glutathione peroxidase 1	Oxidative stress	F: TACGTCCGTCCTGGAAATGG R: GTCACTGGGCTGAGGAAGCT	123	BC083461	93.27	
casp3 - caspase 3	Apoptosis	F: CCCAGATGGTCGTGAAAGGAT R: TGAACCATGAGCCGGTCATT	107	NM_131877	93.28	
bclx - bcl2l1 – anti- or pro-apoptotic regulator	Apoptosis	F: GGGCTTGTTTGCTTGGTTGA R: AGAACACAGTGCACACCCTT	128	NM_131807	89.41	
slc31a1 - high-affinity copper transporter 1	Cu transporter	F: GGCCACGGAGATCACATGAT R: CTCCACATTTTTGTAGCCGAAGT	63	NM_001320405	93.68	(Kwok & Chan, 2020)
hsp70 - heat shock protein 70	Protein folding	F: GCACCACCTACTCCTGTGTGGG R: CTGTGAAGGCAACATAGCTGGG	102	AF210640	104.59	(Tiedke et al., 2013)
tfap2a - transcription factor AP-2 alpha	Neurological effect	F: AAGAGTTCACGGACCTGCTG R: AAACAGCTCTGAATCCCGGG	91	NM_176859	100.29	
cyp1a1 - cytochrome P450 1A1	Cd toxicity	F: GGTGTTGGTTTTCGGTTTGG R: GGCATCCCGGTGAACTTTAA	114	AF210727	90.88	
esr1 - estrogen receptor 1	Endocrine disruption	F: TGCCCTCATTTACTGCATCA R: CACCTTCCCTGAAGACTGGA	214	NM_152959	98.40	
pbx1a - pre-B-cell leukemia homeobox 1a	Swim bladder function	F: GAGGGAAGAAAACAGGACATTG R: TTTCTTTTATTTCACACAGGACGTT	151	AJ245962	89.41	(Wu et al., 2020)
atp1a1 - ATPase Na/K Transporting Subunit α1	Homeostasis	F: TCTGACTCTCACCGCCAAAC R: GCCAAGAGTCTCCACAGCTT	76	NM_131686	99.97	

Table 2
Metal analysis of the raw and diluted AMD samples along with the Norwegian water quality index for the reference.

Metal	Norwegian freshwater quality index			Raw AMD	High group (0.45% AMD)		Low group (0.1% AMD)		Control group	
	Good	Moderate	Bad	Mean	Mean	SD	Mean	SD	Mean	SD
Aluminum (μg/L)				20200	51.63	39.26	27.6	1.15	2.06	-
Arsenic (μg/L)	0.15–0.5	0.5–8.5	8.5–85	< 0.5	< 0.5	-	< 0.5	-	< 0.5	-
Barium (μg/L)				10.7	90.71	61.93	109.36	76.34	3.21	0.004
Cadmium (μg/L)	< 0.08	< 0.45	< 4.5	32.3	0.18	0.01	< 0.05	-	< 0.05	
Calcium (mg/L)				193	14.83	0.88	14.13	0.95	13.6	0.77
Chromium (μg/L)	0.1–3.4	0.1–3.4	0.1–3.4	27.7	< 0.5	-	< 0.5	-	< 0.5	-
Cobalt (μg/L)				249	1.47	0.04	0.32	0.004	< 0.05	-
Copper (μg/L)	0.3–7.8	0.3–7.8	7.8–15.6	18300	85.70	1.28	21.73	2.22	< 1	-
Iron (mg/L)				19.8	0.03	0.01	0.0045	-	< 0.004	-
Lead (μg/L)	0.02–1.2	1.2–14	14–57	20.2	< 0.2	-	< 0.2	-	< 0.2	-
Magnesium (mg/L)				46.7	9.42	0.01	9.22	0.012	9.16	0.032
Manganese (μg/L)				2500	13.67	0.63	3.46	0.21	0.3645	0.1
Mercury (μg/L)	0.001–0.047	0.047–0.07	0.07–0.14	< 0.02	< 0.02	-	< 0.02	-	< 0.02	-
Molybdenum (μg/L)				0.7	< 0.5	-	< 0.5	-	< 0.5	-
Nickel (μg/L)	0.5–4	4–34	34–67	81.2	0.61	-	< 0.5	-	< 0.5	-
Potassium (mg/L)				11.6	7.17	0.03	7.11	-	7.11	0.03
Sodium (mg/L)				8.14	112.33	0.47	113	0.81	112.33	1.69
Vanadium (μg/L)				< 0.05	< 0.05	-	< 0.05	-	< 0.05	-
Zinc (μg/L)	1.5–11	1.5–11	11–60	10600	68.73	4.79	22.43	4.19	9.27	1.53

According to the Norwegian classification of environmental quality of water (shown in Table 2), both dilutions used in this study are considered bad (Miljødirektoratet, 2016). The only possible way to test the sub-lethal effects of AMD on fish is by dilution (Chamorro et al., 2018). The pH of test solutions (0.75%, 1.5%, 2.25%, 3.0%, 3.75%, 4.5%, 5.25%, 6.0%, 6.75%, 7.5%, 8.25% and 9%) used the acute toxicity tests were found to be 7.18 ± 0.02 (n = 3), 7.20 ± 0.03 (n = 3), 6.95 ± 0.01 (n = 3), 6.91 ± 0.02 (n = 3), 6.73 ± 0.06 (n = 3), 6.67 ± 0.02 (n = 3), 6.22 ± 0.04 (n = 3), 6.29 ± 0.03 (n = 3), 5.98 ± 0.003 (n = 3), 5.76 ± 0.05 (n = 3), 5.21 ± 0.021 (n = 3) and 5.09 ± 0.03 (n = 3) respectively. Even after thousand-fold dilution, low test concentration (0.1% raw AMD) used in the present study contained Ca (14.13 mg/L), Mg (9.22 mg/L), Al (0.027 mg/L), Cu (0.021 mg/L), Zn (0.022 mg/L) and Fe (0.0045 mg/L). The pH of the 0.1% and 0.45% AMD solution was 7.18 ± 0.02 (n = 12) and 6.95 ± 0.04 (n = 12). The concentration of heavy metals in Grunnstollen and Lake Langvatnet has shown a notable

similarity over the years (Supplementary Table. S1).

3.2. LC_{50} values for AMD to zebrafish larvae

We observed a survival rate of 95% in the negative control group and a mortality rate of 70% in the positive control group, demonstrating the successful validation of the FET as per the OECD guidelines. The LC_{50} values were found to be 6.97%, 4.76%, 3.62% and 2.86% AMD when exposed to 24 h, 48 h, 72 h and 96 h, respectively (Table 3). Finney's probit method was used to get LC_{50} values and regression line for all the time points, as shown in Fig. 2. The results indicate that AMD-induced toxicity is both time and concentration-dependent.

3.3. Growth and development

At 48 and 72 hpf, the incidence of unhatched larvae was checked. The percentage of unhatched larvae was < 5% across all the treatments. To check the quantitative alterations in the development, we measured body length, head-to-trunk angle (HTA), swim bladder area and eye size at 72, 96 and 116 hpf. At 72 hpf, there was no significant difference in the body length of the zebrafish larvae across all the treatments (Fig. 3A). At 96 hpf, there was a significant reduction in the body length of the larvae after exposure to 0.1% AMD ($p < 0.05$) and 0.45% AMD ($p < 0.005$) when compared to control (Fig. 3A). A similar reduction in body length was seen in the 0.45% AMD exposure group at 116 hpf with a p-value < 0.005 (Fig. 3A).

Exposure to 0.45% AMD resulted in a significant reduction ($p < 0.005$) in the eye size measured at 116 hpf (Fig. 3B). However, exposure to 0.1% AMD did not produce a significant difference in eye size compared to the control. No significant differences in eye size were observed in larvae exposed to 0.1% or 0.45% AMD at 72 and 96 hpf (Fig. 3B).

Exposure to 0.45% AMD resulted in a significant reduction ($p < 0.005$) in the swim bladder measured at 116 hpf (Fig. 3C). However, exposure to 0.1% AMD did not produce a significant difference in swim bladder size compared to the control (Fig. 3C). No significant differences in swim bladder size were observed in larvae exposed to 0.1% or 0.45% AMD at 72 and 96 hpf.

Exposure to 0.45% AMD resulted in a significant reduction ($p < 0.005$) in the head-to-trunk angle (Fig. 3D). Exposure to 0.1% AMD did not have any significant difference in the head-to-trunk angle compared with the control (Fig. 3D).

We found that 96 h exposure to 0.45% AMD caused various developmental abnormalities in zebrafish larvae when observed at 116 hpf. These abnormalities include uninflated swim bladder, kyphosis, reduced yolk sac resorption, pericardial edema and yolk sac edema (Supplementary Fig. S1).

3.4. Heart rate

To evaluate the effect of AMD on heart rate, videos of larvae were recorded at 116 hpf. The results showed that AMD did not have a significant effect on the heart rate of zebrafish larvae even at the highest tested concentration, i.e., 0.45% AMD (Fig. 4A). Besides this, we found pericardial edema in some of the larvae belonging to the 0.45% AMD group (Supplementary Fig. S1).

Table 3
LC_{50} values in zebrafish larvae following 96 h exposure to raw AMD.

Sample	Time	LC_{50} (%)	95% Fiducial confidence interval (%)	Regression equation	R^2
Raw AMD	24 h	6.97%	5.83–8.33	y = 3.4362x + 2.1254	0.8696
Raw AMD	48 h	4.76%	4.14–5.47	y = 4.5391x + 1.9026	0.9194
Raw AMD	72 h	3.62%	2.96–4.42	y = 2.9635x + 3.34	0.9153
Raw AMD	96 h	2.86%	2.32–3.52	y = 2.9263x + 3.6503	0.9641

3.5. Rate of oxygen consumption

The effect of AMD on oxygen consumption was measured at 112 hpf and is shown in Fig. 4B. In general, the amount of available oxygen decreased with time and after six hours (or 6 h), almost 50% of the available oxygen was consumed in all the treatments. We observed a significant increase in the oxygen consumption in larvae after exposure of 0.1% and 0.45% AMD over time ($p < 0.005$), treatment ($p < 0.005$) and the interaction term ($p < 0.005$).

3.6. Swimming behaviour assessment

The effect of AMD on swimming behaviour was measured in a light-dark rhythm. Results showed a significant decrease in velocity ($p < 0.05$) and distance moved ($p < 0.05$) by larvae when they were exposed to 0.45% AMD (Fig. 5C & 5D). A significant increase ($p < 0.05$) in the movement and stasis phases was also found when larvae were exposed to 0.45% AMD (Fig. 6A & 6B). Exposure to 0.1% AMD did not result in any significant difference ($p > 0.05$) in the distance moved, velocity, movement and stasis when compared with the control. In a broader sense, behavioural results indicated larval hypoactivity following 0.45% AMD exposure as documented in heatmaps and trajectory maps (Fig. 5A & B). Exposure to 0.45% had a significant effect ($p < 0.05$) on the meandering and frequency of clockwise rotation. Exposure to AMD (0.1% & 0.45%) did not cause any significant changes in heading, CCW-rotation (counter-clockwise rotation), turn angle and angular velocity compared with control.

3.7. Gene expression

After observing the pronounced negative impact of AMD on larval growth, respiration, and behaviour, our goal was to further search for effects at the molecular level. Given that AMD has been linked to oxidative stress, apoptosis, protein folding, metal toxicity, endocrine disruption and neurological dysfunction, we chose to investigate the expression of key genes associated with these processes. The relative fold change of *cat*, *gpx1, casp3*, *bclx, slc31a1, hsp70*, *atp1a1, tfap2a*, *cyp1a1*, *esr1* and *pbx1a* was measured. After 96 h AMD exposure, *cat* ($p < 0.005$; 0.45% AMD), *slc31a1* ($p < 0.05$; 0.1% AMD, $p < 0.005$; 0.45% AMD), *tfap2a* ($p < 0.005$; 0.1% AMD, $p < 0.005$; 0.45% AMD), *cyp1a1* ($p < 0.005$; 0.1% AMD, $p < 0.005$; 0.45% AMD) and *atp1a1* ($p < 0.005$; 0.1% AMD, $p < 0.005$; 0.45% AMD) were significantly downregulated (Fig. 7). Furthermore, all significantly down-regulated genes showed a clear dose-response relationship (Fig. 7). No significant difference in relative fold change of *bclx*, *casp3*, *hsp70*, *esr1*, *pbx1a* and *gpx1* was found in exposed zebrafish larvae compared to the control group (Supplementary Fig. S2).

4. Discussion

This study shows that even thousand-fold diluted AMD can have detrimental effects on fish larvae. AMD is a major environmental issue resulting from mining activities that can have a detrimental impact on both aquatic and terrestrial ecosystems. Mine drainage causes degradation of water quality, damage to aquatic habitats and reduction of biodiversity. In 2017, the United Nations identified AMD as the second most significant global environmental issue, following global warming (Tuffnell, 2017). Estimates show that approximately 20,000–50,000 AMDs worldwide affect around 19,300 kilometres of rivers and 72,000 ha of lakes (Blowes et al., 2003). This problem requires significant financial resources to address, with mine waste remediation costs

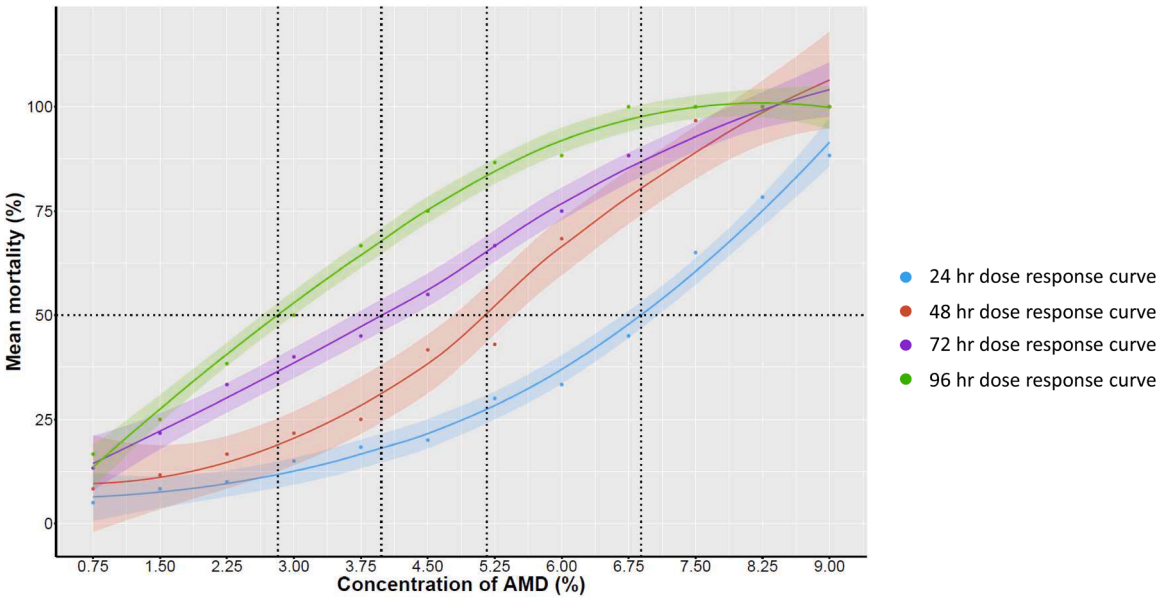

Fig. 2. Dose-response curves of zebrafish embryos exposed to acid mine drainage (AMD) for 96 h (n = 20).

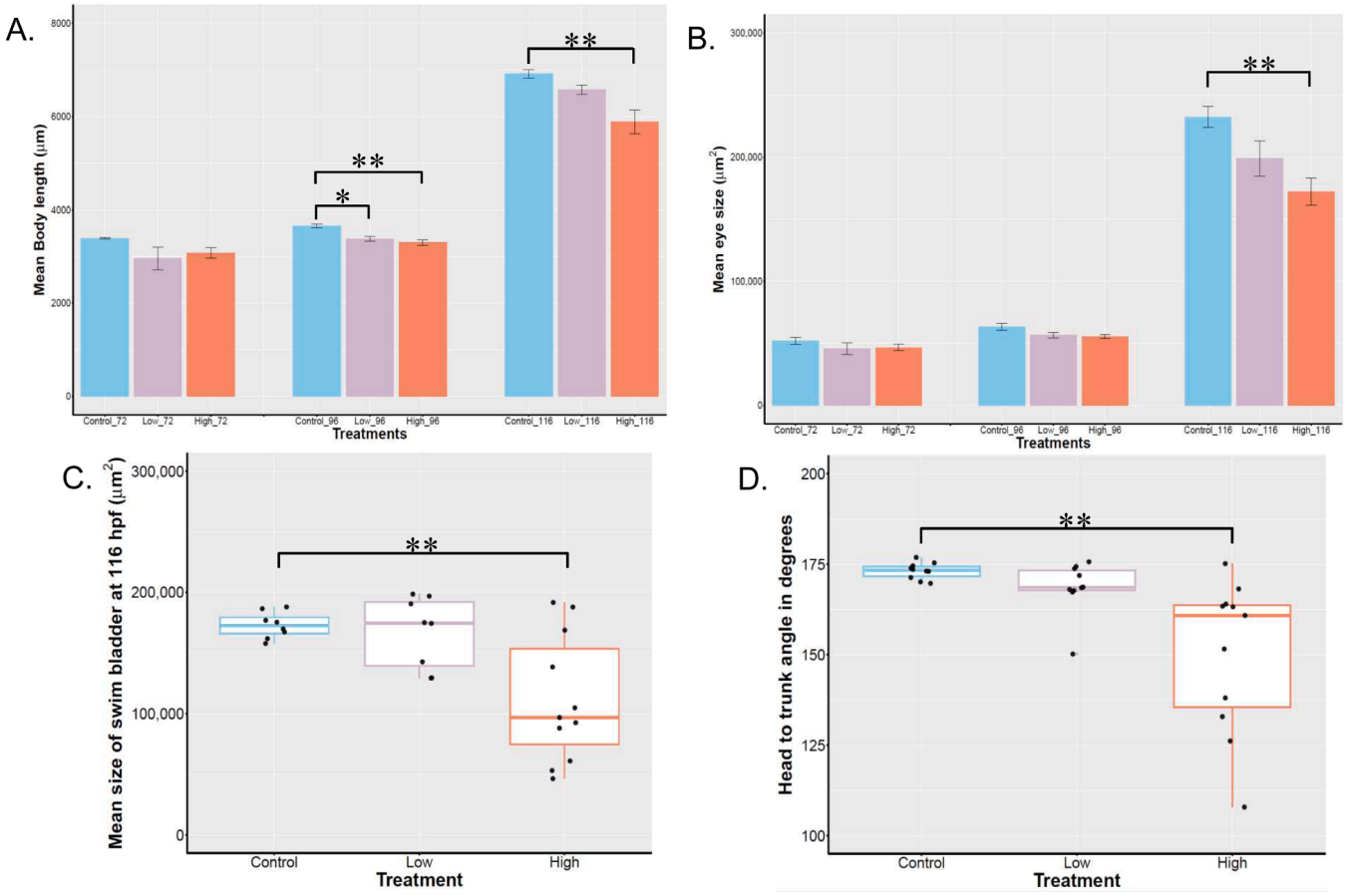

Fig. 3. Effects of acid mine drainage (AMD) on the larval (A) mean body length, (B) mean eye size, (C) mean swim bladder size, (D) head to trunk angle. Zebrafish embryos were exposed to 0% AMD (Control group), 0.1% AMD (Low group) and 0.45% AMD (High group) for 96 hr. Data represent the mean ± S.E. * *p < 0.005; compared with the control group (n = 10).

estimated to be in the tens of billions of dollars worldwide (Feasby, 1991). Given that the contaminants in AMD are primarily heavy metals, it is crucial to thoroughly investigate their harmful effects. The present study aims to improve our understanding of the impact of AMD on fish by using a multi-end point approach.

Exposure to AMD resulted in a significant increase in mortality rates among zebrafish larvae, with the LC_{50} values for 24, 48, 72, and 96 h determined to be 6.97%, 4.76%, 3.62% and 2.86% respectively. These

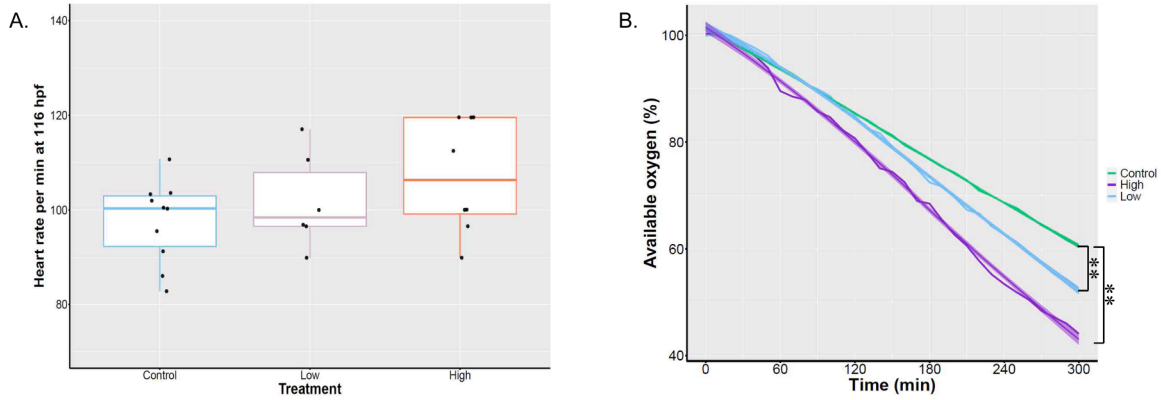

Fig. 4. Effects of acid mine drainage (AMD) on the larval (A.) heart rate and (B.) available oxygen. Zebrafish embryos were exposed to 0% AMD (Control group), 0.1% AMD (Low group) and 0.45% AMD (High group) for 96 hr. Data represent the mean ± S.E. * *p < 0.005; compared with the control group (n = 10).

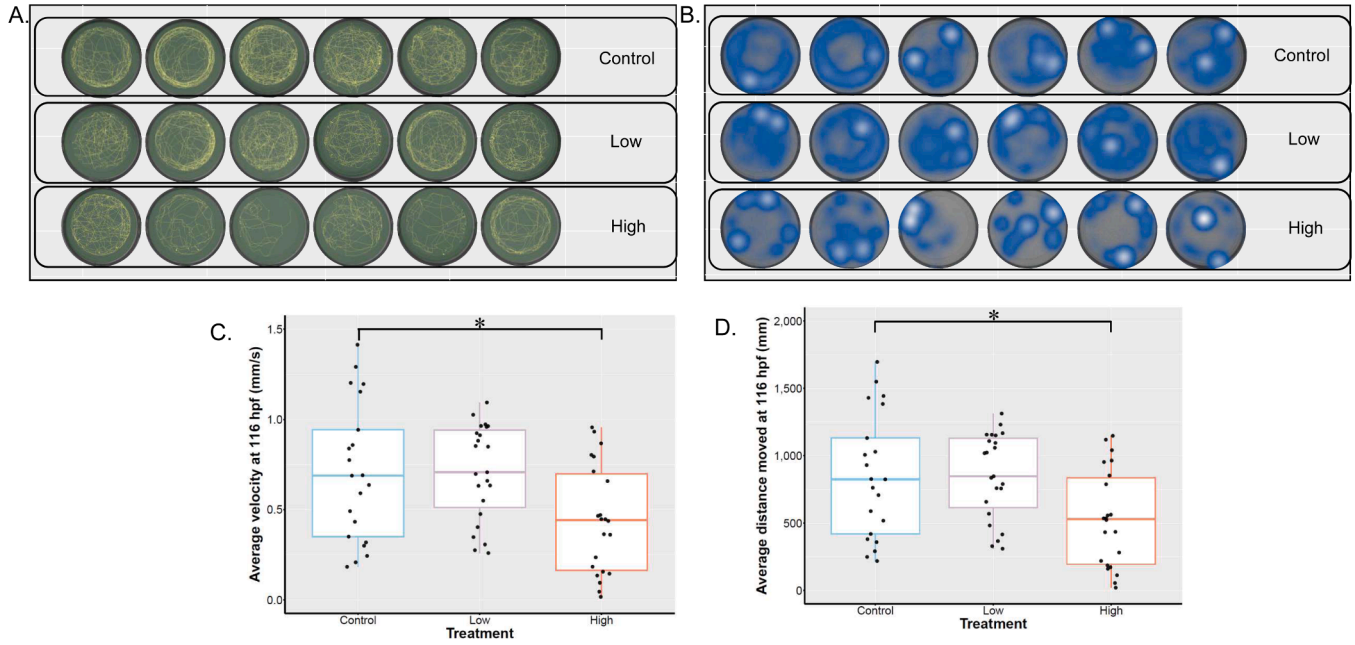

Fig. 5. Effects of acid mine drainage (AMD) on the swimming behaviour in zebrafish larvae. Zebrafish embryos were exposed to 0% AMD (Control group), 0.1% AMD (Low group) and 0.45% AMD (High group) for 96 hr, their locomotor activity was analysed using the DanioVision at 116 hpf. (A) representative trajectory chart, (B) representative locomotory heatmap of time spent, (C.) average velocity and (D.) average distance moved. Data represent the mean ± S.E. * *p < 0.005; compared with the control group (n = 18).

results are in line with a prior study by Chen et al. (2021), which reported LC_{50} values of 5.68%, 4.86%, 4.13% and 3.80% after exposing zebrafish larvae to raw mining effluent from a molybdenum mine in the Qinling Mountains of China. However, Chamorro et al. (2018) observed an LC_{50} of just < 1% in zebrafish larvae after 116 h of exposure to AMD from an active Cu mine in Chile. The variance in LC_{50} values can be attributed to differing concentrations and types of various heavy metals present in the AMD and water quality characteristics such as alkalinity. Other reasons could be differences in experimental methodology such as duration of exposure, temperature, pH, etc.

The present study investigated the developmental effects of AMD exposure on zebrafish larvae. We found a significant reduction in body length in larvae exposed to 0.1% and 0.45% AMD at 96 hpf and in the 0.45% AMD exposure group at 116 hpf. We also found a significant reduction in eye size and swim bladder areas. These results are in line with a study where the authors observed significant effects on eye volume, body length and morphological aberrations in zebrafish larvae exposed to 2.5 μg/L of Pb (Curcio et al., 2022). Although the Pb levels in our samples were below the detection limit (<0.2 μg/L) in both the low and high exposure groups, the AMD diluted samples still contained detectable levels of other pollutants (such as Al, Cu, Zn and Fe), which contributed to larval deformities in our study. To explore the underlying mechanisms driving this decrease in swim bladder size, we examined the expression of *pbx1a*, a gene encoding a protein involved in swim bladder development and regulation (Teoh et al., 2010). However, we did not find any significant expression difference between the treatment groups and the control for this gene. Exposure to 0.45% AMD resulted in physical deformities such as yolk sac edema, pericardial edema, and curved spines. A study by Chamorro et al. (2018) also found similar deformities in zebrafish larvae at 72 hpf following AMD exposure. One of the primary mechanisms by which AMD causes developmental alterations in zebrafish larvae is through the release of heavy metals and metalloids (Sonnack et al., 2018). Cu, commonly found in AMD, can cause severe developmental abnormalities (Zhang et al., 2015). *Atp1a1* is a gene that encodes the alpha subunit of the sodium-potassium ATPase, which is a critical enzyme involved in the maintenance of ion

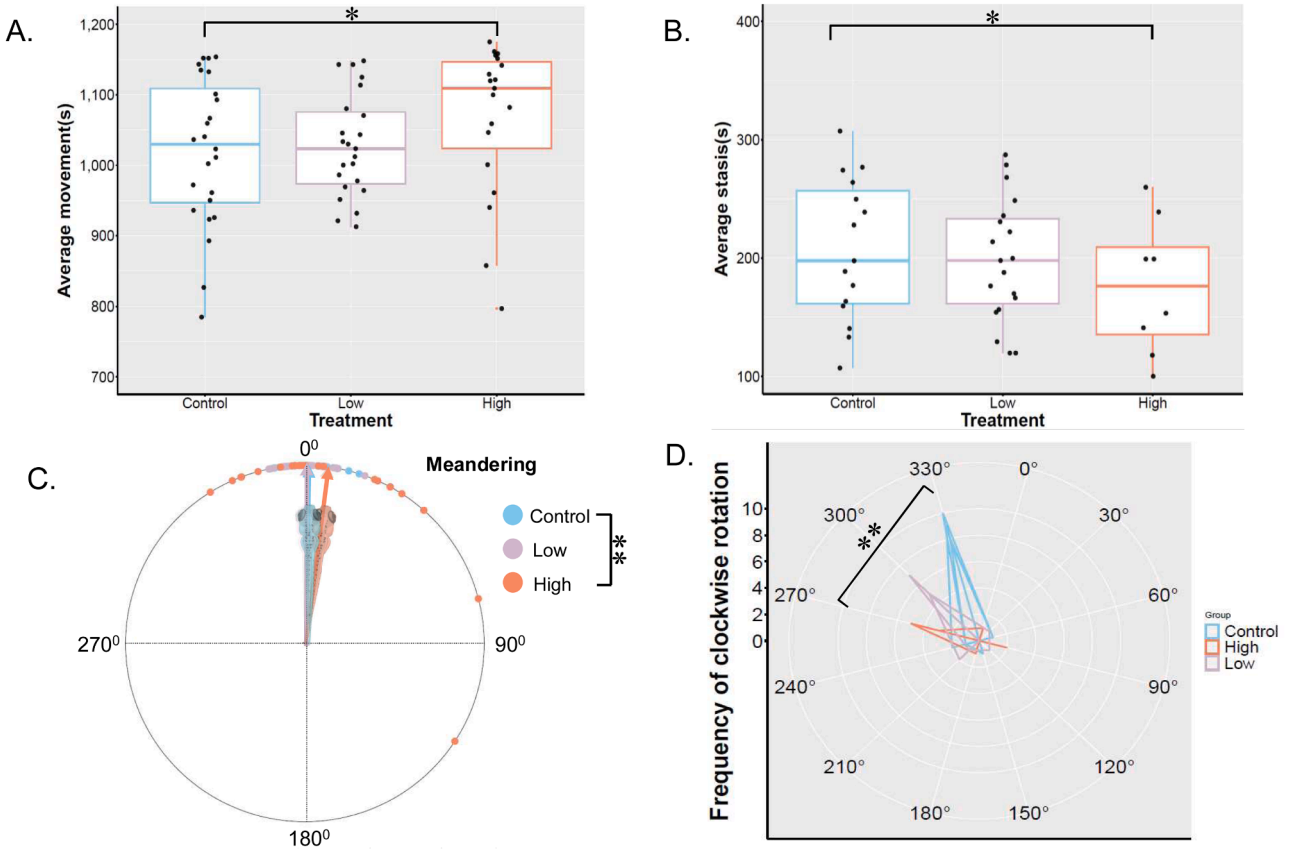

Fig. 6. Effects of acid mine drainage (AMD) on the swimming behaviour in zebrafish larvae. Zebrafish embryos were exposed to 0% AMD (Control group), 0.1% AMD (Low group) and 0.45% AMD (High group) for 96 hr, their locomotor activity was analysed using the DanioVision at 116 hpf. (A) average movement, (B) average stasis, (C.) meandering and (D.) clockwise rotation. Data represent the mean ± S.E. * *p < 0.005; compared with the control group (n = 18).

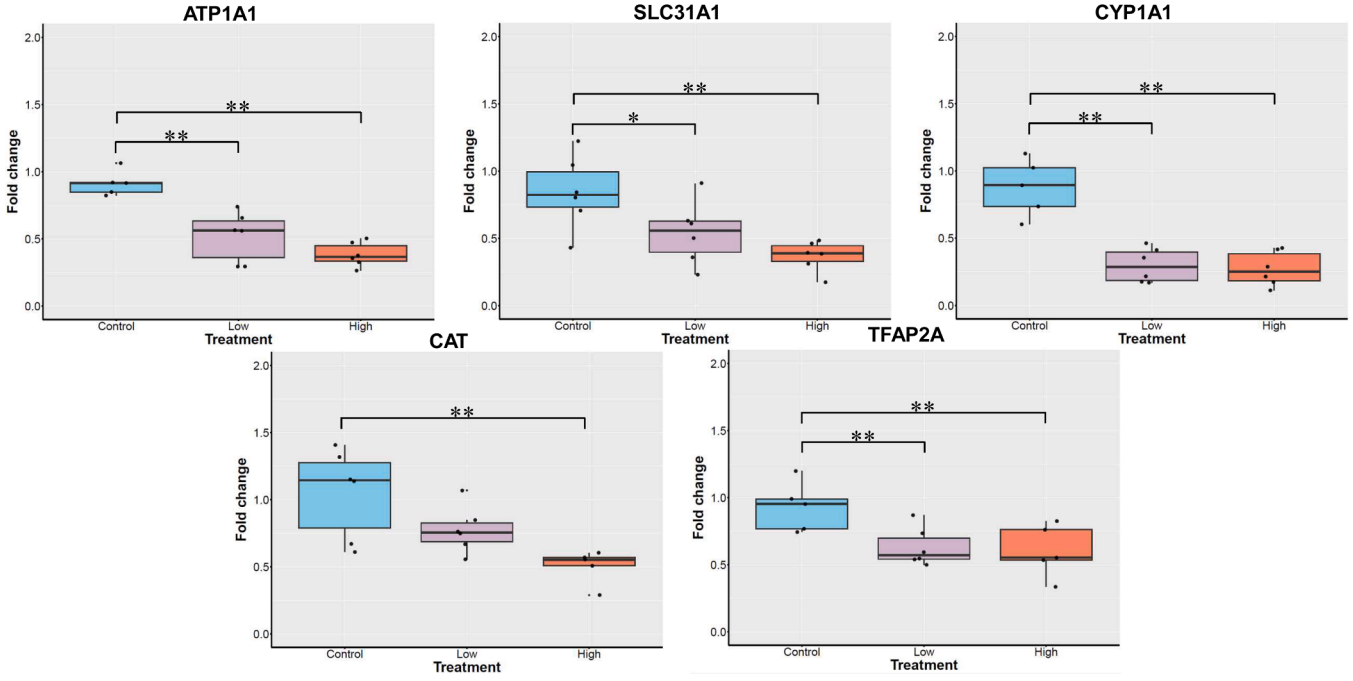

Fig. 7. The effect of acid mine drainage (AMD) on the larval gene expression. Zebrafish embryos were exposed to 0% AMD (Control group), 0.1% AMD (Low group) and 0.45% AMD (High group) for 96 hr. Data represent the mean ± S.E. Data represent the mean ± S.E. * *p < 0.005; compared with the control group (n = 6). ATP1A1 = ATPase Na/K Transporting Subunit α1, SLC31A1 = high-affinity copper transporter 1, CYP1A1 = cytochrome P450 1A1, CAT = catalase and TFAP2A = transcription factor AP-2 alpha.

gradients across cell membranes (Kasas et al., 2022). We found a significant downregulation of this gene in larvae exposed to 0.1% and 0.45% AMD. The downregulation of this gene in larvae exposed to Cu-rich AMD might hinder the proper functioning of this enzyme and ultimately have an adverse effect on ion regulation. One possible mechanism for this dysfunction is the binding of Cu ions to the enzyme, which can lead to changes in its conformation (Azizan et al., 2013; Hwang et al., 2011) and thereby impact transcriptional regulation. *Slc31a1* is a gene that encodes the Cu transporter 1, which is a transmembrane protein that plays a crucial role in the regulation of Cu uptake and homeostasis in mammals (Craig et al., 2009; Olsvik et al., 2016). *Slc31a1* was downregulated in larvae exposed to 0.1% and 0.45% AMD. The downregulation of this gene in response to Cu-rich AMD suggests that exposure to this pollutant can disrupt mechanisms associated with the influx-efflux of Cu ions. Dysregulation of the proteins encoded by *atp1a1* and *slc31a1* could seriously affect the health and survival of larvae as these enzymes play a crucial role in osmoregulation and maintaining ion gradients in the nervous system. Additionally, heavy metals like Cd can disrupt organogenesis, a study by Cheng et al. (2000) found craniofacial and skeletal deformities in zebrafish larvae exposed to molybdenum mining effluent containing 0.26 mg/L Cd in it. Similarly, a study by Avallone et al. (2015) found that chronic exposure of 0.3 mg/L $CdCl_2$ to zebrafish larvae affects sarcomeric pattern, glycoprotein composition and swimming performance. Cd is a potent inhibitor of cytochrome P450 (CYP) enzymes, which are essential for their ability to protect against toxic substances by facilitating detoxification reactions (Yang et al., 2016). Cd binds to the heme group of the CYP enzyme, which is essential for its catalytic activity (Benedetti et al., 2015). In this study, *cyp1a1* was significantly downregulated in larvae exposed to 0.1% and 0.45% AMD. This indicates that AMD exposure can interfere with mechanisms associated with the larvae's ability to metabolise certain toxic substances, potentially leading to their accumulation and increased toxicity (Benedetti et al., 2015). Beside to the toxic effects at the molecular level, Cd is also known to negatively affect body weight, food intake, metabolism and oxygen extraction efficiency in the freshwater fishes (Baudou et al., 2021; Ferro et al., 2021).

Oxidative stress induced by AMD exposure is another mechanism that can cause developmental abnormalities in zebrafish larvae and can also damage cellular macromolecules (Chen et al., 2021; Pereira et al., 2016). Cellular stress often affects the levels of the antioxidants such as glutathione and catalase in cells (Adeyemi et al., 2015; Contreras et al., 2005; Massarsky et al., 2017). Exposure to 0.45% AMD significantly downregulated the expression of the *cat* gene in this study. Several mechanisms could explain this observation. Downregulation of *cat* could be due to the suppression of transcription factors that normally activate the *cat* gene. For example, the nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that binds to specific regulatory regions of the *cat* gene and enhances its expression (Hou et al., 2012). However, AMD exposure can inhibit the activity of Nrf2, leading to a decrease in *cat* gene expression. Another potential mechanism of *cat* downregulation is the modulation of epigenetic marks such as DNA methylation and histone modifications (Min et al., 2010). AMD exposure can alter the methylation status of CpG sites in the promoter region of the *cat* gene, which will repress its transcriptional activity. Histone modifications like deacetylation and methylation can also reduce the *cat* gene's availability to transcription factors and RNA polymerase, causing the gene to be downregulated. (Min et al., 2010). AMD exposure had no significant effect on the expression of *gpx1*, the other antioxidant gene examined.

Heart rate can be considered a measure of stress (McGrath and Li, 2008). We found an increase in the heart rate following 0.1% and 0.45% AMD, but the results were not statistically significant (p-value; 0.11). However, we did observe pericardial edema in some of the larvae belonging to the 0.45% AMD-exposed group. This suggests that although AMD did not affect heart rate, it may have affected other aspects of cardiac function. An increase in oxygen consumption is an indication of increased metabolic demand, which could result from the larvae trying to compensate for the effects of the stressor (Barrionuevo and Burggren, 1999). We also investigated the effect of AMD on oxygen consumption in zebrafish larvae at 112 hpf. We observed a significant increase in oxygen consumption in larvae after exposure to 0.1% and 0.45% AMD. This finding suggests that AMD exposure affects the metabolic rate of the larvae.

The swimming behaviour of zebrafish larvae is closely linked to the development and functioning of neural circuits that control motor behaviour, making it an important measure of nervous system development. Our findings indicate a significant reduction in the distance moved and velocity of zebrafish larvae exposed to 0.45% AMD. This finding was also supported by the trajectory maps, which showed a clustering of the larvae in a smaller area. Additionally, the exposure to 0.45% AMD resulted in a significant increase in the stasis phase of the larvae. This can also be seen on the heatmaps, indicating a greater amount of time spent not moving or moving very slowly. Meandering of zebrafish larvae refers to the irregular and wandering movement pattern of their swimming behaviour, which is characterised by frequent changes in direction and speed (Kalueff et al., 2013). We found a significant difference in the meandering of larvae exposed to 0.45% AMD. Overall, the findings suggest that exposure to 0.45% AMD not only causes hypoactivity but also affects swimming direction. Our results are in line with a recent study that also found reduced swimming activity in zebrafish larvae exposed to AMD (Yoon and Yoon, 2022). Moreover, we also found downregulation of the *tfap2a* gene. This gene is encoding a protein essential for neurogenesis in the hindbrain (Holzschuh et al., 2003), and the downregulation of this key gene might be associated with abnormal behaviour in zebrafish.

Extrapolating controlled laboratory results to complex AMD-affected environments is challenging. Usually, lab experiments are designed with one to two specific variables, but in real-world scenarios, an intricate web of interactions or variables co-exists. Many real-world variables, such as changing pH levels, microbial communities, geological substrates, and flow dynamics, are absent from lab-based experiments. To address these issues, multifaceted approaches such as integrated research between laboratory and field, long-term monitoring and multiple-endpoint analysis should be adopted. In conclusion, using a multiple-endpoint approach is essential to understand the fundamental mechanisms and to build a broader conceptual understanding of hazards associated with AMD.

The Sulitjelma area has a long history of mining operations and has been identified as a significant source of AMD. The impact of AMD on Langvatnet Lake has been the subject of several former studies. Some studies have found that the lake's water quality has deteriorated as a result of the discharge of acidic mine water, with increased levels of Cu as one of the main environmental challenges (Dale et al., 2018; Gjertsen and Risvoll, 2018). Studies have suggested that AMD can also impact the lake's ecosystem to be more localised and limited to areas close to the mine discharge point (Gray, 1997; Rezaie and Anderson, 2020). Discharge of mine drainage into lake Langvatnet has raised concerns about the harmful impact on the lake's ecosystem. AMD discharge can lower the lake's pH and harm aquatic life. It can also reduce nutrient and oxygen availability, and lead to accumulation of toxic metals like Cu and Zn in fishes. Brown trout (Salmo trutta) caught near the outlet of Langvatnet in 2021 contained elevated levels of Cu and Cd in liver tissue. For Cd, the concentration in liver tissue was above the EU's legal limit, while for Cu the concentration was above the daily dietary recommendations (Norconsult, 2022). In aquatic ecosystems, AMD drops the water pH, disrupts nutrient balance, and releases heavy metals. These challenging situations can harm fish populations and lead to alteration or destruction of aquatic habitats. In terrestrial ecosystems, runoff from AMD-contaminated areas can harm plants, soils, and wildlife, reducing biodiversity and creating barren lands. In essence, we found that a thousand-fold diluted AMD can adversely affect the growth, development, behaviour and impact the expression of important genes

in zebrafish. These results also highlight the possible harm of diluted AMD on aquatic life. Overall, the effects of AMD from the Sulitjelma mine on Langvatnet lake highlight the need for careful management of mining activities and effective mitigation measures to protect the environment and aquatic ecosystems. Efforts need to be made to mitigate the impact of AMD from the Sulitjelma mine on Langvatnet lake, including the construction of passive or active treatment systems to neutralize the acidity and remove metals from the mine water before it enters the lake. The other possible mitigating method could be the development of artificial wetlands to reduce the harmful effects of AMD through bioremediation.

5. Conclusion

Mining operation, widely distributed around the world, plays a key role in extracting minerals and metals from the earth's crust. Mining has many negative environmental impacts and one of them is the release of acid mine drainage (AMD). The present study showcases the toxicological effects of Cu-rich AMD in zebrafish larvae. This study presents compelling evidence that even 100–1000-fold diluted AMD water collected from an abandoned mine has significant impacts on the biological functioning of zebrafish. 1000-fold diluted AMD reflects the concentration of metals in the 5.5 km^2 Langvatnet lake downstream of the discharge site. With some of the parameters being significantly affected by environmental relevant concentrations of metals, the study suggests that wild fish in this region might be negatively impacted by the metal-rich mine drainage water. This information can be used to develop strategies to mitigate the environmental impact of abandoned mines and to help in decision-making on whether to keep them closed or reopen them.

CRediT authorship contribution statement

Shubham Varshney: Conceptualization, Methodology, Data Curation, Writing - Original Draft. **Mikkel Lundås**: Methodology, Data Curation. **Prabhugouda Siriyappagouder**: Methodology. **Torstein Kristensen**: Supervision, Writing - Review & Editing. **Pål A. Olsvik**: Conceptualisation, Supervision, Funding acquisition, Writing - Review & Editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115796.

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