Potash mining effluents and ion imbalances cause transient stress in adult common roach, *Rutilus rutilus*

Nora Baberschke, Laura Schulzik, Torsten Preuer, Klaus Knopf <https://orcid.org/0000-0001-9401-8145>, Thomas Meinelt, Werner Kloas

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Potash mining effluents and ion imbalances cause transient stress in adult common roach, *Rutilus rutilus*

Nora Baberschke^{a, *}
baberschke@igb-berlin.de

Laura Schulzik^a
Torsten Preuer^a

Klaus Knopf^{a, b}

Thomas Meinelt^a

Werner Kloas^{a, b, c}

^aDepartment of Ecophysiology and Aquaculture, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587, Berlin, Germany

^bAlbrecht Daniel Thaer-Institute, Faculty of Life Sciences, Humboldt-University of Berlin, Invalidenstraße 42, 10115, Berlin, Germany

^cDepartment of Endocrinology, Institute of Biology, Faculty of Life Sciences, Humboldt-University of Berlin, Unter den Linden 6, 10099, Berlin, Germany

*Corresponding author.

Abstract

A present ecological issue causing secondary salinization in different countries is the discharge of effluents by the potash mining industry. In Germany, the River Werra is used as a sink for potash mining discharges containing high concentrations of ions, predominantly Cl⁻, K⁺, Na⁺, and Mg²⁺ resulting in a strong decline of the biodiversity and abundance of local species. However, hardly anything is known about the acute and chronic physiological effects of high concentrations and imbalances of ions being prevalent in potash mining effluents in fish. Therefore, the stress response and selected immune and growth parameters were investigated in standardized laboratory experiments. A native freshwater fish species, *Rutilus rutilus*, was exposed to concentrations of the high currently allowed (HT) and lowered future thresholds (LT) and three different ion solutions (containing high Mg²⁺ (Mg), high K⁺ (K) and high Mg²⁺ + K⁺ (Mg + K) concentrations) for four different exposure times (24 h, 7 d, 21 d, 8 wk). Tank water (additionally after 9 and 12 h) and plasma cortisol, glucose and protein, hematocrit and hemoglobin were determined after each exposure time. Furthermore, plasma lysozyme and head kidney leucocyte respiratory burst activity (only after 21 d) were evaluated as well as growth parameters. A transient stress response was induced in almost all groups. Tank water cortisol was elevated after 9 h in HT, LT and Mg + K and in HT after 12 h, whereas glucose concentrations increased after 24 h in all exposure groups except K. HT led to enhanced hematocrit and hemoglobin content after 24 h. Plasma protein, immune system and growth were not affected in any group. None of the ion solutions induced acute toxicity but most triggered typical acute stress reactions. Rather the sum of high ion concentrations than single ions challenged the fish. Even though the effects observed in adult roach were only transient and indicate acclimatization under laboratory conditions, adverse effects observed in the river are evident and further research on physiological endpoints including reproductive parameters and impacts on younger life stages seem to be needed to scientifically base protective thresholds.

Keywords: Ion imbalances; Freshwater fish; Stress; Cortisol; Innate immune response; Growth

1 Introduction

Anthropogenic salinization of rivers, referred to as secondary salinization, is becoming an increasing global ecological issue as it potentially harms the aquatic biota by long-lasting effects and might destabilize the ecological balance (Arle and Wagner, 2012; Cañedo-Argüelles et al., 2013).

A serious local problem causing secondary salinization is the discharge of effluents of the potash mining industry into the River Werra in Germany. A part of the accruing effluents, which develop during the mining and

processing of potash salts and stockpiling of its residues, is directly released into surface waters. Besides other ions, they are highly rich in Cl^- , Mg^{2+} , Na^+ and K^+ . The current discharge thresholds in Germany permit concentrations as high as 2500 mg/L Cl^- , 340 mg/L Mg^{2+} and 200 mg/L K^+ and a total hardness of 90 °dH (Regierungspräsidium Kassel 2012). These thresholds strongly exceed environmental concentrations of major ions commonly found in river sections unaffected by potash mining effluents ($\text{Cl}^- < 100 \text{ mg/L}$, $\text{Mg}^{2+} 10\text{--}15 \text{ mg/L}$, $\text{K}^+ < 5 \text{ mg/L}$, 11–14 °dH) (Hübner, 2007). Moreover, these thresholds are not based on scientifically validated data (Arle and Wagner, 2012). Thus, the River Werra is one of the most affected European surface waters and, under such conditions, will not obtain the good ecological status according to the EU Water Framework Directive.

Ecological consequences are a strong decline of the biodiversity and abundance of local species in the River Werra. Moreover, structural alterations of biocenoses were documented (Hübner, 2007; IGF, 2009; Arle and Wagner, 2012). Studies on fish populations revealed a negative correlation between species richness, abundance and biomass of fish and the degree of salinization (Matthes and Werner, 2009). Even ubiquitous and frequently occurring fish species, such as the common roach, are present only in low numbers or completely absent and exhibit higher disease rates compared to species in undisturbed river sections (Matthes and Werner, 2005; Schwevers et al., 2005; EcoRing, 2008).

Due to their hyperosmolarity with respect to the surrounding environment, freshwater organisms including fish have to actively take up ions from the water. Osmoregulation is an energy demanding process and underlies hormonal control as for example sea water acclimation is promoted by the stress hormone cortisol and growth hormone (McCormick, 2001; Evans et al., 2005). Despite efficient mechanisms to maintain the hydromineral homeostasis, high concentrations as well as imbalances of ions can cause osmotic stress and eventually impair physiological functions of freshwater organisms (Mount et al., 1997; Goodfellow et al., 2000; Schwarz and Allen, 2014). In several studies, adverse effects on freshwater fish were attributed to high NaCl concentrations including elevated cortisol, effects on the immune system, reduced growth and even mortality (Dheer et al., 1986; Dominguez et al., 2005; Velasco-Santamaria and Cruz-Casallas, 2008; Kammerer et al., 2010). Although the toxic potential increases with increasing NaCl concentrations, there are indications that Na^+ and Cl^- are not the main cause for toxicity. In most cases, elevated NaCl concentrations allowed an adaptation of euryhaline fish to the stress condition, if K^+ and Mg^{2+} occur in normal ratios (Marshall et al., 1999; Kammerer et al., 2010; Malakpour Kolbadinezhad et al., 2012). In contrast to Na^+ and Cl^- , K^+ is potentially toxic as high concentrations can lead to cardiac arrest and paralysis (Ringer and Murreli, 1878; Mount et al., 1997). The physiologically effective K^+ concentrations for fish can be as low as 80 mg/L (Halsband, 1976) and high K^+ concentrations can cause a reduced tolerance of invertebrates to the total salinity (Schmitz and Kneissl, 1967). In contrast, Ca^{2+} has the potential to enhance the tolerance to the total salinity as it indirectly reduces the permeability of the gills for ions (Wendelaar Bonga, 1978). Mg^{2+} has similar but weaker osmoregulatory effects than Ca^{2+} (Wendelaar Bonga, 1978; Soucek et al., 2011) and studies on fish described a toxic potential for this ion (Mount et al., 1997; Van Dam et al., 2010).

A laboratory study addressed the toxic effects of potash mining effluents on the water flea, *Daphnia magna*, by exposure to different ion solutions. The authors suggested that the sum of ions as well as the unphysiological composition, mainly of Ca^{2+} and Mg^{2+} , are causing chronic adverse effects and reproductive impairments (EcoRing, 2008).

However, regarding potash mining effluents and ions apart from Na^+ and Cl^- , data and knowledge about the acute and chronic physiological effects on fish is scarce. In the recently published experimental study associated to the current study, impacts of ion imbalances concerning plasma ion disturbances and histopathological as well as biochemical changes in gills of the roach were determined (Baberschke et al., 2019). Despite the osmotic challenges, only transient effects have been found for plasma ions, except a significant increase of SO_4^{2-} over a period of 8 weeks, suggesting a great osmoregulatory capacity of the common roach. In addition, all ion solutions tested caused moderate but significant histopathological alterations in gills demonstrating that the osmotic challenges are causing a morphological response being potentially associated with stress. In a further recent study, Delompré et al. (2019) investigated the impacts of ion imbalances caused by hydraulic fracturing fluid on plasma ions and gill histology of rainbow trout. They revealed only significant elevations of strontium and bromide but no significant long term effects, neither on the main physiologically active plasma ions nor on gill morphology. Therefore, although it seems that some fish species might be able to adapt well to ion imbalances, it cannot be ruled out that such permanent osmotic challenges might elicit a stress response being indicative for hidden adverse effects impacting even population levels.

The evaluation of the stress response in fish is a useful tool to assess and identify physiologically adverse effects. The stress response is a mechanism by which an organism encounters any disturbance of homeostasis in order to maintain essential physiological body functions (Selye, 1956). First, teleost fish respond with the primary stress response by releasing catecholamines (CA) and cortisol. Especially CA further stimulate the secondary stress response consisting of immediate cardiovascular and hematological changes as well as mobilization of energy sources to counteract the increased oxygen and energy demand. An organism may withstand the stressor by adapting to the new condition or the stress response can become dysfunctional (maladaptive) when stress is chronic. During the tertiary stress response, stress may elicit debilitating effects e. g. on the immune system and growth by energy allocations or even leads to death. The stress hormone and major glucocorticoid cortisol plays a key role in mediating suppressive effects on immune functions and growth (Barton and Iwama, 1991; Wendelaar Bonga, 1997).

The common roach, *Rutilus rutilus* L., a cyprinid and moderately euryhaline freshwater teleost, is a suitable ecotoxicological study organism as it is highly abundant and ubiquitously occurring in Eurasia with a moderate sensitivity for stressors (Larmuseau et al., 2009).

The aim of the present study was to evaluate the stress response and selected parameters of the innate immune system and growth of a native freshwater species to detect possible physiological changes caused by potash mining effluents and ion imbalances. Acute and chronic stress parameters including tank water and plasma cortisol, plasma glucose and protein, blood hematocrit and hemoglobin as well as immune parameters, such as lysozyme and

respiratory burst activity of leucocytes, and different growth parameters were determined. The physiological effects of two different concentrations of potash salts, representing the currently permitted and lower future thresholds, were evaluated. In addition, three different ion solutions were employed to evaluate the effects induced by ion imbalances. In order to assess the time course of a potential stress response, the fish were exposed to test salt solutions for four different exposure times (24 h, 7 d, 21 d, 8 wk).

2 Materials and methods

2.1 Ion solutions

The German discharge thresholds currently permitted until 2020 (high thresholds, HT) and future lowered thresholds (LT), intended as thresholds from 2021 onwards, were tested (Table 1). Furthermore, ion solutions with elevated Mg²⁺ (Mg), K⁺ (K), and Mg²⁺ and K⁺ (Mg+K) concentrations were evaluated. A control group (Ctrl) containing reconstituted tap water (see 2.3) served as a reference. For the ion solutions, NaCl, NaSO₄, MgCl₂·6H₂O, MgSO₄·7H₂O, KCl, KSO₄ and CaCl₂·2H₂O (S3 Chemicals, Germany) were adequately added to the tap water and ion concentrations of filtered (0.22 µm) water samples were verified once per week. Anion (Cl⁻, SO₄⁻²) concentrations were measured by ion chromatography (IC, conductivity detector CDD-10A, Shimadzu, Japan) with an anion analysis column (Shodex IC Si-90 4E) and an eluent containing 1.8 mM Na₂CO₃, 1.7 mM Na₂HCO₃ and 1% acetonitril. Cations (Na⁺, Mg²⁺, K⁺, Ca²⁺) were determined by inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 6000 Duo, Thermo Scientific, USA). A blank (ultrapure water) and standards (Mg²⁺, K⁺, Ca²⁺; 1 and 2 mg/L; Na⁺: 25 and 50 mg/L), acidified with HCl, were used. Deviations from nominal concentrations were kept within 10% (for measured ion concentrations refer to supplements Table 1).

Table 1 Nominal ion concentrations in mg/L, measured conductivity in µS/mL and osmolality in osmol/kg ± SD of the different test waters. Values in bold represent the characteristic ion concentrations for one exposure group according to the current and future thresholds.

alt-text: Table 1

Exposure group	Ion concentrations [mg/L]					Conductivity		Osmolarity
	Na ⁺	Cl ⁻	Mg ²⁺	K ⁺	Ca ²⁺	SO ₄ ⁻²	[µS/cm]	
Ctrl	90	60	9	4	80	190	877.6 ± 13.3	9.3 ± 2.5
HT	1160	2500	340	200	140	780	8673.8 ± 313.2	124.5 ± 6.4
LT	790	1700	230	150	95	490	6187.9 ± 349.0	99.5 ± 2.1
Mg	90	260	230	4	80	780	2270.8 ± 72.8	28.0 ± 3.1
K	90	190	9	150	80	190	1380.2 ± 33.2	20.7 ± 4.0
Mg+K	90	150	230	150	80	1150	2650.8 ± 72.2	36.7 ± 13.3

2.2 Preconditioning of the fish

Mature individuals of *Rutilus rutilus* L. were obtained from Lake Grosser Mueggelsee (Berlin, Germany) by electrofishing. The fish were transported to the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) Berlin and kept in aerated 800 L tanks with a constant flow-through of water at 15.5 ± 0.5 °C (mean ± standard deviation, SD) under a natural photoperiod. They were fed daily *ad libitum* with commercial 1.3 mm trout pellets (Aller Performa, Aller Aqua, Germany).

2.3 Experimental conditions

After a minimum of 2 months, the fish were exposed to six different ion solutions for a period of either 24 h, 7 d, 21 d and 8 wk in a high-standard continuous flow-through system using reconstituted tap water (Lutz et al., 2008). The tap water was priorly passed through several filters (5 - 0.45 µm) and sterilized by UV light. Each exposure group consisted of two 26 L tanks (flow rate 7.6 L/h), stocked with 12 fish each with a mean mass of 23.47 ± 7.11 g (total of n=24 fish per exposure group). The fish were allowed to acclimatize to the laboratory conditions for 14 days. As during preconditioning, the fish were fed twice daily at a feeding rate of 0.2% of body mass. Prior to the final sampling at the end of each exposure time, the fish were starved for 24 h. The artificial photoperiod was 14 h L/10 h D (light/dark) during summer (24h, 7 d, 21 d) and 12 h L/12 h D during autumn exposure (8 wk). Temperature (20.1 ± 0.2 °C), pH (7.7 ± 0.1), dissolved oxygen (80 ± 8.5%, 7.3 ± 0.8 mg/L) and conductivity (Table 1) were measured weekly (WTW, Germany). Photometric examinations of ammonium and nitrite concentrations (Hach Lange, Germany, NH₄⁺: 0.16 ± 0.09, NO₂⁻: 0.024 ± 0.04 mg/L) were also performed once per week. A multichannel peristaltic pump (Ismatec, Cole Parmer GmbH, Germany), continuously adding concentrated ion solutions, was used with inert tubing (MHILL Tygon, Cole

Parmer GmbH, Germany) and calibrated daily. The correct operation of the flow-through system and the condition of the fish was monitored twice a day. All tests were performed in compliance with the institutional guidelines for the care and use of animals and were approved by the Berlin State Office of Health and Social Affairs; LAGeSo (reference number G 0277/14).

2.4 Tank water and animal sampling

Water samples of 1 L were taken from each tank after 9 h, 12 h, 24 h, 7 d and 21 d for tank water cortisol determination. At the end of every exposure (24 h, 7 d, 21 d, 8 wk), all animals were sacrificed. The fish were anesthetized by a blow on the head, blood samples were immediately obtained by caudal vein puncture with heparinized syringes (2000 U ammonium heparin, Sigma-Aldrich, Germany) and they were killed by decapitation. 40 µL of blood were used for hematocrit and hemoglobin determination. To obtain the plasma, blood samples were centrifuged (5 min, 16100 g) and plasma immediately snap frozen in liquid nitrogen and stored at −80 °C until further processing. Liver, heart and spleen were excised for mass determination. After 21 d exposure, head kidneys were also taken to assess respiratory burst activity of leucocytes.

2.5 Cortisol, glucose and protein determination

Cortisol concentrations from tank water (non-invasive) and blood plasma (invasive) were measured by enzyme linked immunosorbent assay (ELISA). Prior to ELISAs, tank water and plasma underwent extraction processes. Plasma samples (5 µL) were added to 500 µL 99.8% ethanol and centrifuged (5 min, 3000 g).^{The supernatant was collected and evaporated overnight. Cortisol was dissolved in 500 µL phosphate buffered saline (PBS, with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, Germany), 5% ethanol, pH 7.2) for 1 h on a shaker (200 rpm). Water samples were collected and processed similarly as described by [Brüning et al. \(2015\)](#) with some modifications. 1 L tank water was collected from all tanks per exposure group after 9 h, 12 h, 24 h during the 24 h, 7 d and 21 d exposures to obtain a sample size of n=6 and after 21 d of the third and fourth exposure (21 d, 8 wk) to achieve a sample size of n=4. Tank water was immediately filtrated (1.6 µm glass fiber filters, Sigma-Aldrich, Germany) by using a vacuum pump. Cortisol was extracted using SolEx C18 cartridges (Thermo Scientific, USA) activated by ethyl acetate in a Dionex AutoTrace 280 device (Thermo Scientific, USA) and eluted with 6 mL ethyl acetate (Sigma-Aldrich, Germany) being evaporated overnight. Cortisol was redissolved in 600 µL PBS in an analogous manner to plasma cortisol. The extracted samples (tank water and plasma) were further processed according to the manufacturer's protocol using commercial 96-well Saliva Cortisol ELISA-Kits (IBL, Germany) and absorption was photometrically measured in a plate reader (Tecan infinite M200, Tecan, Switzerland).}

Plasma glucose concentration was photometrically determined in 96-well plates in duplicate by the aid of a glucose kit (Glucose GOD FS 10⁺, Diasys Diagnostic Systems, Germany).

Total protein concentration of 2 µL blood plasma (1:100 diluted in ultrapure water) was analyzed in duplicate with a Roti-Quant Universal Kit (Carl Roth, Germany) in 96-well plates according to the manufacturer's instruction.

2.6 Hematocrit (Hct) and hemoglobin (Hb) measurements

For Hct, 30 µL blood were absorbed in duplicate with heparinized microhematocrit capillary tubes (Th. Geyer, Germany) and centrifuged (5 min, 13000 g) in a hematocrit centrifuge (Hettich, Germany). Hb concentration of 10 µL blood diluted in ultrapure water (1:100) was photometrically determined by the Triton/OH method. A Hemoglobin Assay Kit (Sigma-Aldrich, Germany) was employed and all steps performed according to the manufacturer's instructions.

2.7 Lysozyme assay

Plasma lysozyme activity was determined by a turbidimetric assay according to [Ellis \(1990\)](#) with some modifications. Plasma samples were tested in duplicate in 96-well plates by using 5 µL plasma (diluted 1:5 in 0.025 M sodium phosphate buffer, pH 6.2) that was mixed with 175 µL of a suspension of 0.2 mg/mL lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich) in 0.025 M sodium phosphate buffer (pH 6.2). After shaking for 5 min, the absorption was measured at 530 nm and 25°C every min for a total of 17 min. The decrease in absorbance of 0.001/min was determined representing one unit of lysozyme activity.

2.8 Respiratory burst assay

The assay was performed following the methods of [Secombes \(1990\)](#) and [Liu et al. \(2017\)](#) with some adjustments. Suspensions of head kidney leucocytes were prepared by pressing the tissue through a 70 µm gauze cap (EASYstrainer; Greiner Bio-One, Austria) whilst rinsing with ice-cold RPMI 1640 medium (Biowest, France, with phenol red, supplemented with 100 U/mL penicillin-streptomycin 100 X, 2 mM L-glutamine, 20 U/mL sodium heparin) and then centrifuged (60 min, 600 g, 4 °C). Leucocytes were transferred to new tubes and washed 3 times in 2 mL ice-cold RPMI and centrifuged in between for 10 min. Cells were cultured in RPMI cell culture medium (Biowest, France, without phenol red, supplemented with 100 U/mL penicillin-streptomycin 100 X, 2 mM L-glutamine, 25 mM HEPES buffer). Cell numbers were determined with Neubauer improved counting chambers (chamber depth 0.1 mm, 0.0025 mm²) and adjusted to a density of 1x10⁷ cells/mL by dilution. 100 µL of cells were seeded into adhesive 96-well plates (Nunclon, Thermo Scientific, USA) and allowed to adhere for 90 min at 22 °C. Non-adhered cells were removed by rinsing with 100 µL cell culture medium.

The respiratory burst activity was determined in triplicate using nitroblue tetrazolium (NBT). Cells were incubated in 150 μ L cell culture medium containing 1 mg/mL NBT for 90 min at 22 °C in the dark. Production of reactive oxygen species (ROS) was induced by 1 μ g/mL 12-phorbol 13-myristate acetate (PMA, Sigma-Aldrich). Wells without PMA were used to determine spontaneous ROS production without stimulation. Finally, the supernatant was removed. Cells were rinsed with cell culture medium, fixed by adding 100 μ L of 100% methanol, washed twice with 100 μ L 70% methanol and air dried. Formazan was dissolved in 100 μ L 2 M KOH and 100 μ L dimethyl sulfoxide (DMSO) per well (all chemicals from Carl Roth, Germany) and optical densities were measured at 620 nm. The stimulatory capacity of the cells to ROS production was calculated as the quotient of the mean OD values with and without PMA stimulation.

2.9 Growth parameters and organo-somatic indices

The total and standard length and body mass of all fish were determined. The condition factor ($CF = 100 \times (\text{body mass})/(\text{standard length})^3$) as well as organo-somatic indices of liver, heart and spleen ($OSI = (\text{mass}_{\text{tissue}}/\text{mass}_{\text{body}}) \times 100$) were calculated.

2.10 Statistical analyses

For statistical analysis, IBM SPSS Statistics 24 (IBM, USA) was used. In the case of normal distribution and homogeneity of variances, one-way ANOVA with subsequent Tukey-HSD post-hoc test was performed. If necessary, a log-transformation ($\log_{10}x$) was conducted to normalize data. If homogeneity of variances was not given, Tamhane post-hoc test was employed. Kruskal-Wallis test including a Bonferroni correction was chosen as non-parametric test. In order to examine a successful stimulation of leucocytes of the respiratory burst assay, Wilcoxon rank-sum test was used. The significance level was set as significant at $p \leq 0.05$. Data are presented either in box and whisker plots (box with 25, 50 and 75 percentiles, whiskers stretching from minimum to maximum value and outliers indicated as stars or circles) or as the mean $\pm$ SD.

3 Results

3.1 Cortisol, glucose and protein

No mortality occurred in any group and after any exposure time. After 9 h, tank water cortisol concentration was significantly elevated in all groups, except the exposure groups Mg and K, relative to the control ([Fig. 1 A](#)). Thus, HT ($p=0.001$) responded with the highest increase, followed by LT ($p=0.007$) and Mg+K ($p=0.034$). For group Mg ($p=0.09$), a trend (defined as $p \leq 0.1$) of an increase was observed. Furthermore, HT ($p=0.005$) and LT ($p=0.044$) had significantly higher cortisol levels than group K. After 12 h ([Fig. 1 B](#)), cortisol levels were only significantly increased in HT ($p=0.021$) compared to control, and LT ($p=0.074$) showed a trend of an increase. Again, HT showed a higher cortisol concentration than K ($p=0.014$). The means and medians of all groups, except those of K and Mg, were still higher than control after 24 h ([Fig. 1 C](#)), although not significantly. For the following exposure times, no significant differences between groups were found (for p-values and pooled means of data not graphically presented refer to [Supplementary Material Table 2](#)). Although HT exhibited higher plasma cortisol levels after 24 h ([Fig. 1, D](#)) as observed in tank water, data of plasma cortisol did not reveal any significant effect.

Fig. 1 Concentrations of tank water cortisol in ng/g biomass after 9 h (A), 12 h (B), 24 h (C) (n=6, Tukey), plasma cortisol in ng/mL after 24 h (D, n=9-12, Kruskal-Wallis) and plasma glucose in mg/mL after 24 h (E, n=11-15, Tamhane) for each exposure group. Unequal lower cases indicate significant differences at a significance level of $p \leq 0.05$.

alt-text: Fig. 1

The concentration of plasma glucose was significantly increased in all groups, except in K, relative to the control after 24 h (Fig. 1E). Alike cortisol, group HT ($p=0.07$) induced the highest rise in plasma glucose levels, followed by LT ($p=0.023$), Mg+K ($p=0.018$) and then Mg ($p=0.04$), HT ($p=0.04$), LT ($p=0.01$), LT ($p=0.023$) and Mg ($p=0.01$) as well as Mg+K ($p=0.011$) were also different to K. No significant differences were detected after 7 d, 21 d and 8 wk.

The total protein concentration of the blood plasma of all groups did not reveal any significant effect at all exposure times.

3.2 Hct and Hb

21 d	SSI	0.25	±	0.06	0.26	±	0.06	0.23	±	0.06	0.24	±	0.06	0.22	±	0.05	0.25	±	0.06
	CSI	0.18	±	0.06	0.19	±	0.07	0.17	±	0.05	0.17	±	0.04	0.17	±	0.03	0.15	±	0.02
	CF	1.59	±	0.17	1.54	±	0.12	1.55	±	0.14	1.54	±	0.12	1.60	±	0.13	1.58	±	0.11
	Standard length [cm]	11.7	±	0.9	11.6	±	0.8	11.8	±	0.9	11.7	±	0.9	11.7	±	1.0	11.6	±	1.1
	Body mass [g]	24.66	±	5.22	24.92	±	5.73	25.35	±	6.26	24.81	±	5.56	24.94	±	6.39	25.00	±	7.31
	HSI	0.90	±	0.18	1.00	±	0.20	0.90	±	0.18	0.88	±	0.21	0.84	±	0.23	0.71	±	0.27
8 wk	SSI	0.24	±	0.06	0.31	±	0.09	0.31	±	0.07	0.26	±	0.08	0.31	±	0.09	0.22	±	0.04
	CSI	0.18	±	0.03	0.20	±	0.12	0.17	±	0.02	0.19	±	0.04	0.18	±	0.04	0.16	±	0.06
	CF	1.51	±	0.13	1.56	±	0.16	1.50	±	0.17	1.52	±	0.13	1.51	±	0.16	1.59	±	0.11
	Standard length [cm]	11.4	±	1.1	11.3	±	1.1	11.4	±	1.0	11.3	±	0.9	11.4	±	0.9	11.3	±	0.9
	Body mass [g]	23.79	±	6.77	23.50	±	6.57	23.05	±	5.85	23.31	±	6.06	23.38	±	5.44	23.46	±	5.25
	HSI	0.85	±	0.19	0.89	±	0.20	0.85	±	0.24	0.91	±	0.16	0.92	±	0.21	0.85	±	0.24
	SSI	0.26	±	0.07	0.33	±	0.19	0.25	±	0.08	0.25	±	0.06	0.24	±	0.07	0.27	±	0.10
	CSI	0.16	±	0.03	0.16	±	0.02	0.16	±	0.02	0.16	±	0.03	0.18	±	0.03	0.19	±	0.07
	CF	1.56	±	0.08	1.61	±	0.09	1.56	±	0.12	1.59	±	0.10	1.57	±	0.09	1.59	±	0.13

4 Discussion

Since ecological implications of potash mining effluents in the River Werra are undeniable but experimental determinations of osmotic impacts on plasma ions and gill histology revealed only moderate effects (Baberschke et al., 2019), effects of ion solutions according to the current and future thresholds as well as ion imbalances on the stress response and selected immune and growth parameters of the common roach were investigated. In the recent study, no mortality occurred and a transient stress response was induced in the fish of most exposure groups, whereas innate immune system and somatic growth were not affected.

The rise in cortisol as a response to a variety of stressors has been described in many studies (Pickering and Pottinger, 1989; Kammerer et al., 2010). The results of the present study revealed noticeable elevated cortisol levels in the tank water after 9 h in all groups except group K, with no significant change, and Mg, showing a trend of an increase (Fig. 1 A). HT was significantly increased and LT responded with a trend even after 12 h (Fig. 1 B). Although the significant effect of HT diminished after 24 h (Fig. 1 C, D), concentrations of almost all groups were still higher than control and it could be speculated that there may be an effect that was masked by the high variance. Considering the elimination of potential handling effects by tank water collection, it is suggested that the non-invasive sampling method of tank water is more suitable for the assessment of cortisol levels. Furthermore, the method allows the collection of samples at the same time and at additional time points independent from the final animal sampling.

Besides CA, cortisol mobilizes energy sources to counteract the increased energy demand. Furthermore, it plays a crucial role during adaptation processes to higher salinities and affects growth, immune system and reproduction (Pickering, 1990, Wendelaar Bonga, 1997; McCormick, 2001). Since concentrations rose in most groups only for a relatively short time (9-12 h), the fish seem to suffer from acute stress but to be capable of regaining homeostasis. In many studies, stressors of different nature induced a transient elevation of cortisol with subsequent adaptation (Jeney et al., 1996; Marshall et al., 1999). Roach stressed by acute handling responded with rapidly elevated cortisol levels returning to basal levels after 4 h (Pottinger et al., 1999). Kammerer et al. (2010) observed an increase in plasma cortisol with concomitant transient osmoregulatory distress in euryhaline tilapia, *Oreochromis mossambicus*, transferred to 25‰ seawater. Since the effects disappeared after 3 days, the authors concluded subsequent acclimation of the fish.

Secondary stress effects triggered by CA include immediate cardiovascular changes and a fast mobilization of glucose via glycogenolysis. In contrast to CA, cortisol leads to a slower release of glucose using gluconeogenetic pathways (Mommensen et al., 1999). In one of few studies dealing with roach, cortisol and glucose were elevated after confinement stress indicating a typical stress response (Pottinger, 2010). In the present study, glucose displayed a similar pattern to cortisol but with a time delay. All groups except K responded with hyperglycemia after 24 h with HT showing the strongest increase (Fig. 1 D). The findings of cortisol are thus confirmed by the results of glucose although the rapid hyperglycemia is most likely a response to CA. After 7 d, the effects on glucose levels were abolished providing further indication that the fish experienced stress but they were able to acclimatize to the changed condition.

In addition to glucose, protein is used as an energy source to compensate an increased energy demand during stress conditions. For example, Sancho et al. (1998) recorded decreased protein levels after exposing European eel to pesticides. Furthermore, changes of plasma protein levels may be due to ionoregulatory disfunctions, hepatotoxic or other pathological effects (Pfeifer and Weber, 1979; Gluth and Hanke, 1985). In this study, the total protein content of the blood plasma was homogenous throughout all groups and exposures. This suggests that protein metabolism was not affected by the different ion solutions and no acute toxicity occurred.

Alterations of Hct have been observed in many fish species exposed to physical, chemical or biological stressors (Dheer et al., 1987; Barton et al., 1986; Barton et al., 1987; Biron and Benfey, 1994). Hct, the percentage proportion of blood cells of the blood volume, predominantly erythrocytes, was increased in HT after 24 h (Fig. 2). Triggered by CA just as cortisol, an increase can either be caused by a swelling of the cells or by hemoconcentration. Erythrocytes can be quickly released from the spleen or head kidneys to antagonize oxygen deficiency during stress (Barton et al., 1987; Witeska, 2013). A cortisol-mediated reduction of extracellular fluid leading to increased Hct has also been assumed for juvenile rainbow trout fed with cortisol (Barton et al., 1987). Also dehydration leading to plasma reduction in hypersaline solutions was frequently described (Kammerer et al., 2010), but is rather unlikely since the fish remained hypotonic relative to the ion solutions used in this study. As Hb concentrations are simultaneously elevated in group HT after 24 h (Fig. 2), it might be assumed that hemoconcentration by splenic contraction, as a response to acute stress and increased metabolic activity, or fluid movements, due to hormone action, is applicable, as described for several fish species (Barton et al., 1987; Eslamloo et al., 2014).

It is widely accepted that stress, independent of its nature, is linked to immune functions. Whereas acute or mild stress can enhance immune responses, chronic or severe stress with prolonged elevated cortisol levels is often correlated to immunosuppression (Elsaesser and Clem, 1987; Wendelaar Bonga, 1997; Weyts et al., 1999).

In fish, the innate immune system is a major non-specific mechanism to defeat pathogens (Secombes and Fletcher, 1992; Saurabh and Sahoo, 2008). Two mechanisms, the respiratory burst activity of leucocytes and plasma lysozyme activity, were tested in this study. During phagocytosis, leucocytes produce and release antibacterial reactive oxygen species (ROS) resulting in a respiratory burst (Klebanoff, 1980). However, no effect was detected in any exposure group after 21 d. Since the respiratory burst was only measured after 21 d, possible alterations after other exposure times, for instance during the transient stress phase when cortisol was elevated, cannot be excluded. For example, *R. rutilius* exposed to UVB-light and handling stress responded with elevated cortisol levels and reduced lymphocyte numbers for up to 7 days but increased respiratory burst activity only after one day (Salo et al., 2000). Pietsch et al. (2014) revealed pro- and anti-inflammatory immune responses including a reduced respiratory burst activity of head kidney cells, while cortisol, Hct and Hb were not affected in carp exposed to the toxin deoxynivalenol for 4 weeks. Lysozyme is a hydrolytic enzyme that breaks cell wall structures of bacteria for their elimination (Saurabh and Sahoo, 2008) but plasma lysozyme activity was not altered in any group after the different exposure times. There was no changed lysozyme activity following the cortisol increase as described in different studies. For example in goldfish, lysozyme activity was increased after acute stress and suppressed after repeated stress with transient increases in cortisol and glucose (Eslamloo et al., 2014). In this study, the activity of both parameters was neither induced nor reduced in any exposure group. There are no indications for stimulatory or suppressive effects by cortisol action. Hence, the involvement of cortisol and immune responses after stress is variable. Considering the results of this study, it seems that the induced stress was too weak to affect the immune system. However, fish populations in the disturbed Werre, including roach, exhibited significantly higher disease rates according to externally visible damages (Matthes and Werner, 2009). With the two immune parameters tested in the lab experiments, the obvious increased susceptibility to pathogens observed in the field cannot be explained. However, laboratory rearing provides optimum water parameters without pathogenic load and thus a challenge experiment exposing fish to pathogens during exposure might reveal impacts on the immune system as seen in the wild.

As a tertiary response and strategy to cope with stress, energy is allocated from growth to maintain homeostasis. Moreover, growth depends on many external environmental factors such as salinity (Dheer et al., 1986; Bøuf and Payan, 2001). Euryhaline aligator gar, *Atractosteus spatula*, tolerated hyperosmotic salinities but responded with decreased growth (Schwarz and Allen, 2014). Many studies addressed the growth inhibitory role of cortisol, for example by daily cortisol administration to rainbow trout leading to reduced growth (Barton et al., 1987). At the same time, trout receiving normal feed but stressed daily did not show elevated cortisol levels and growth remained unchanged. In the present study, no significant differences of length, mass and CF were detected indicating that sufficient energy was available to sustain a normal growth rate and that transiently increased cortisol levels exerted no suppressive effects. The common roach is a fairly slow growing fish, hence, the 8 wk exposure might not be sufficient to detect long-term changes. Furthermore, the feeding rate was set to 0.2% of body mass per day which should be just enough to maintain the biomass rather than to induce growth. Thus, a prolonged stress period might have resulted even in a loss of weight. A reduced growth is also more likely under natural conditions when food is scarce and energy needs to be utilized for other essential activities and physiological processes.

Alterations of the hepatosomatic index (HSI), cardiosomatic index (CSI) and splenosomatic index (SSI) were recorded in association with different environmental stressors. For example, increased or decreased HSI may be indicative for poor food availability, liver damage and increased detoxification due to pollutants (Guimarães et al., 2009). Heart size changes can result from stress, with cortisol as a mediator, leading to cardiac diseases (Johansen et al., 2011). However, no significant alterations of HSI and CSI were found giving no indications for organ dysfunctions or adjustments. Only after 21 d there was a trend of a reduced liver size in Mg+K, but so far, the other results give no reason to assume toxic liver effects. After 21 d exposure, there was a trend of an increased spleen size in HT and LT. An enlargement of the spleen may be indicative of a response to infections or immune impairments (Dekić et al., 2016). Pietsch et al. (2011) recorded an increased SSI with simultaneously decreased immune parameters after cortisol administration. Since in this study, respiratory burst and lysozyme activity were not altered, immune failure and diseases are not likely the reason.

Under laboratory conditions, the common roach seems to be relatively tolerant to the different ion solutions. Exposure groups containing multiple highly concentrated ions (HT, LT and Mg+K) exhibited the most pronounced effects, whereas Mg triggered a rather mild stress response. Surprisingly, K⁺ as a single ion did not lead to any response at the concentrations used in this study, although it is considered to be the most toxic ion in general (Mount et al., 1997).

Several monitorings regularly evaluate abundance, biodiversity and health status of local fish species and documented a persistent negative correlation between these parameters and conductivity, in spite of some improvements compared to the last century (Matthes and Werner 2009, 2012). Although the common roach is usually one of the most dominant and robust species, only single individuals were recorded in the River Werra (Matthes and Werner 2005, 2012; Schwevers et al., 2005). In 2005, more than 50% of the common roach was classified as diseased (Matthes and Werner, 2005). However, as outlined above, no obvious immunosuppressive effects were detected, providing no explanation for the findings in wildlife populations. But, under the controlled laboratory conditions, water quality was always near optimum and the load of any bacteria at minimum and thus, any disease provoking factors being present in wildlife are missing or at low levels and might be no challenge for the immune system.

This experimental study focused on effects on the stress response to gain first insights about physiological effects caused by potash mining effluents. Other important parameters including the reproductive capacity of this species were not investigated yet. It can be assumed that in the natural environment, the presence of multiple stressors can lead to a loss of many even quite resistant species. Besides depending on duration and intensity of a stressor, toxicity is strongly species-specific and further depends on the developmental stage (Chapman et al., 2000). A laboratory study assessed the acute toxicity of different potash salts, similar in composition to those used this study, on juvenile zebra fish, *Danio rerio* (Meinelt and Stüber, 2008). Even a fairly low concentration of 2‰ led to increased mortality and deformations during embryonic and larval development. Moreover, other recent studies dealing with potash mining effluents on zebra fish give indications for strong adverse effects on the reproductive success (Wagler, pers. comm.) while physiological effects on adults were comparatively moderate (Irob et al., submitted). Schwevers et al. (2005) proposed that many of the occurring fish in the River Werra, including roach, have most likely migrated or drifted into the disturbed parts of the river, as proven for many macroinvertebrates by Arle and Wagner (2012), since a successful reproduction of fish is assumed to be unlikely.

5 Conclusion

Since knowledge about physiological effects of potash mining effluents is scarce, this experimental study was conducted to assess the stress response of a native fish species. Contrary to expectations, the study revealed an induction of only a transient stress response in adult *R. rutilus*, predominantly in groups with multiple highly concentrated ions. All ion solutions, except K, elicited rapid increases of cortisol and glucose, and in group HT, Hct and Hb was elevated. No activating or suppressive effects on innate immune parameters and growth were detected under optimum laboratory water conditions. However, the transient and rather moderate effects do not explain the devastating situation for *R. rutilus* in the River Werra. Since implications in nature are commonly greater due to multiple stressors, it cannot be excluded that the thresholds exceed the tolerable concentrations for normal physiological functioning. Therefore, the gain of further valuable scientific data including reproductive parameters and effects on younger life stages of *R. rutilus* is necessary to establish scientifically based thresholds.

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Appendix A. Supplementary data

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Appendix A. Supplementary data

The following is the Supplementary data to this article:

[Multimedia Component 1](#)

Multimedia component 1

alt-text: Multimedia component 1