Potash mining effluents and ion imbalances cause transient osmoregulatory stress, affect gill integrity and elevate chronically plasma sulfate levels in adult common roach, *Rutilus rutilus*

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Potash mining effluents and ion imbalances cause transient osmoregulatory stress, affect gill integrity and elevate chronically plasma sulfate levels in adult common roach, *Rutilus rutilus*[☆]

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Abstract

Secondary salinization is a growing global ecological issue. One cause is the discharge of effluents by the potash mining industry into surface waters such as the River Werra in Germany. Increases of major ions require various physiological responses of freshwater organisms to maintain the hydromineral balance of body fluids. However, only little is known about the acute and chronic effects of high concentrations and imbalances of ions on osmoregulation in freshwater teleosts.

The present study aimed to elucidate the effects of potash mining effluents and different cation ratios on the osmoregulatory capacity and gill histopathology of a native fish species. Individuals of *Rutilus rutilus* were exposed to the currently allowed (HT) and intended future (LT) thresholds as well as to high concentrations of Mg^{2+} (Mg), K^+ (K), and Mg^{2+} and K^+ (Mg + K) for a period of 24 h, 7 d, 21 d and 8 wk. Plasma osmolality, $[Na^+]$, $[Mg^{2+}]$, $[K^+]$, $[Ca^{2+}]$, $[Cl^-]$ and $[SO_4^{2-}]$ and branchial Na^+/K^+ -ATPase activity were determined. Moreover, histological gill alterations after 21 d and muscle water content after 8 wk were examined. HT transiently (24 h) elevated plasma osmolality, plasma $[Na^+]$ and $[Ca^{2+}]$, whereas $[SO_4^{2-}]$ was chronically increased even after 8 wk. Exposure to LT, Mg and Mg + K led to increased $[SO_4^{2-}]$ levels for at least 21 d. It seems that $[SO_4^{2-}]$ is mainly disturbed by multiple ions at high concentrations and long-term effects are unknown. Hydromineral homeostasis was maintained as indicated by unchanged Na^+/K^+ -ATPase activity and muscle water content. However, mild structural alterations of the gills were observed in all exposure groups suggesting adaptational responses but with the potential to affect gas exchange capacity. Hence, the current thresholds for potash mining effluents affect osmineral regulation in roach and further investigations should address potential impacts on reproduction in native fish species and physiological effects of SO_4^{2-} .

Exposure of the native freshwater fish species, *Rutilus rutilus*, to high concentrations and imbalances of major ions induces transient changes of osmolality and electrolytes, affects gill structures as an adaptational response and elevates chronically plasma sulfate levels with unpredictable long-term effects.

Keywords: Osmoregulation; Ion imbalances; Electrolytes; Plasma sulfate; Gill histology

1 Introduction

Deviations of physiological ion concentrations and compositions in the freshwater environment require various physiological responses of teleost fish to maintain their hydromineral balance of body fluids. Increases in salinity

described as the total of occurring dissolved inorganic ions, can be of natural origin, for example in estuaries, or due to anthropogenic actions. Anthropogenic salinization of surface waters, referred to as secondary salinization, is of increasing global concern due to its ecotoxicological potential (Arle and Wagner, 2012; Cañedo-Argüelles et al., 2013). One of the main causes of secondary salinization is the potash mining industry, discharging ion-rich effluents, predominantly Cl^- , Na^+ , Mg^{2+} , K^+ and SO_4^{2-} , into rivers, for instance in Canada, Spain and into the River Werra in Central Germany. The effluents arise during the mining and processing of potash salts and German thresholds exist for Cl^- , Mg^{2+} and K^+ (2500 mg/L, 340 mg/L and 200 mg/L, respectively) and a total hardness of 90° dH (Regierungspräsidium Kassel, 2012). In comparison, ion concentrations in undisturbed river sections are commonly much lower ($\text{Cl}^- < 100 \text{ mg/L}$, $\text{Mg}^{2+} 10\text{--}15 \text{ mg/L}$, $\text{K}^+ < 5 \text{ mg/L}$, 11–14 °dH) (Hübner, 2007). Thus, the River Werra is considered to be one of the most disturbed rivers in Europe. A strong decline of the biodiversity and abundance of local species, along with structural alterations of biocenoses are seen as the result of excess ion concentrations in unphysiological ratios (Hübner, 2007; Arle and Wagner, 2012). Species richness, abundance and biomass of fish in the River Werra, including common roach, *Rutilus rutilus*, are negatively correlated with the degree of salinization and roach individuals show higher disease rates (Matthes and Werner, 2009).

The major ions, prevalent in freshwater, are essential for diverse physiological functions as well as the osmomineral regulation in teleost fish. Since they retain their osmolarity and ionic composition at a constant level, teleosts are hypertonic in freshwater and production of copiously diluted urine and active absorption of ions from the external water is inevitable (Evans et al., 2005). The gills, as the primary site for osmoregulation, represent a multifunctional organ with a large surface and direct contact to the external environment. Chloride cells, located in the gill epithelium, allow the uptake of ions from the ambient water (Foskett and Scheffey, 1982). Numerous ion channels, pumps and ion exchangers are localized in the membrane of chloride cells, through which ions can be passively or actively transported (Perry et al., 2003). One of the major enzymes accountable for ion fluxes is the energy driven gill $\text{Na}^+/\text{K}^+ - \text{ATPase}$ (NKA). The regulation of plasma ions in teleosts is energy demanding and the neuroendocrine system plays a major role (McCormick, 2001). Besides the involvement of ion transporters in gills and other organs as a physiological response to changes in salinity, morphological alterations of the gill structures are another mechanism of teleosts to face environmental changes (Mallatt, 1985; Wendelaar Bonga, 1997).

However, despite the existing strategies to maintain the hydromineral homeostasis, high concentrations and imbalances of ions, as emanating from potash mining effluents, may lead to osmotic stress (Mount et al., 1997; Goodfellow et al., 2000; Schwarz and Allen, 2014). Various studies coping with osmoregulation addressed the effects of changing NaCl concentrations on euryhaline freshwater or anadromous fish (Handeland et al., 1998; Marshall et al., 1999). Alterations of plasma cortisol concentrations, plasma osmolarity and branchial NKA activity of euryhaline fish with increasing salinity were observed (Velasco-Santamaria and Cruz-Casallas, 2008; Malakpour Kolbadinezhad et al., 2012). Research considering physiological responses to ions other than Na^+ and Cl^- remains strongly underrepresented. Studies focusing on ions are mostly of toxicological nature, employing fairly high concentrations, or dedicated to mammals and invertebrates (Mount et al., 1997; Chapman et al., 2000; Semplicio et al., 2016). For example, toxic effects of excess K^+ include cardiac arrest in mammals and mortality in fish and invertebrates (Ringer and Murreli, 1878; Mount et al., 1997). Ca^{2+} and to a lesser extent Mg^{2+} are commonly associated with ameliorative effects in combination with other ions, reducing their toxicity to fish (Wendelaar Bonga, 1978; Elphick et al., 2011).

Khangarot and Ray (1989) assessed the 48 h EC_{50} (concentration with 50% immobilized individuals) of the water flea, *Daphnia magna*, for K^+ , Mg^{2+} and Na^+ (141.46 mg/L, 343.56 mg/L, 402.6 mg/L, respectively). Although considered the least toxic metal ions, concentrations are comparable to those found in the River Werra emphasizing critical effects on this invertebrate species. Acute toxicity testing of potash salts on juvenile zebra fish, *Danio rerio*, revealed increased mortality and developmental impairments even at concentrations as low as 2‰ (Meinelt and Stüber, 2008). Ion solutions comparable to this study induced moderate stress but chronic morphological alterations of gills and gonads in zebra fish (Irob et al. submitted). Recent laboratory results associated to this study (Baberschke et al. submitted) elucidated an induction of a typical but transient stress response in the common roach caused by potash mining effluents and ion imbalances suggesting an adaptive capacity. Due to the paucity of information of physiological effects of potash effluents on native species, the discharge thresholds in Germany are not based on scientific data despite the evident ecological impacts.

Therefore, the aim of the present study was to evaluate the osmoregulatory capacity and possible structural alterations of the gill epithelium of a native and moderately euryhaline freshwater teleost, *R. rutilus*, in order to assess the osmotic stress induced by high concentrations and imbalances of ions as prevalent in potash mining effluents. Plasma osmolarity, plasma electrolyte concentrations ($[\text{Na}^+]$, $[\text{Mg}^{2+}]$, $[\text{K}^+]$, $[\text{Ca}^{2+}]$, $[\text{Cl}^-]$, and $[\text{SO}_4^{2-}]$) and the enzymatic activity of branchial NKA were determined after 24 h, 7 d, 21 d and 8 wk. Furthermore, chronic histopathological alterations of the gills after 21 d as well as a possible chronic change of muscle water content after 8 wk were examined.

2 Materials and methods

2.1 Preconditioning and exposure conditions of the fish

The experimental setup corresponds to the protocol as previously described by Baberschke et al. (submitted). Briefly, mature individuals of *R. rutilus* were caught from Lake Grosser Müggelsee (Berlin, Germany) and kept for a minimum of two months in aerated 800 L tanks. After transfer to a continuous flow-through system and a 14 d acclimatization period, the fish ($23.47 \pm 7.11 \text{ g}$, mean mass $\pm$ SD) were exposed to the ion test solutions (Table 1) for a period of either 24 h, 7 d, 21 d and 8 wk. Multichannel peristaltic pumps continuously added ion solutions to 26 L glass aquaria (flow rate 7.6 L/h, stocking density 12 fish, two replicates per group). The fish were fed twice daily (0.2% of body mass) with commercial dry food. A weekly measurement of water parameters was performed (temperature: $20.1 \pm 0.2 \text{ }^\circ\text{C}$, pH: 7.7 ± 0.1 , dissolved oxygen: $80 \pm 8.5\%$, $7.3 \pm 0.8 \text{ mg/L}$, NH_4^+ : 0.16 ± 0.09 , NO_2^- : $0.024 \pm 0.04 \text{ mg/L}$).

Table 1 Nominal ion concentrations in mg/L, measured conductivity in $\mu\text{S/cm}$ and osmolality in $\text{osmol/kg} \pm \text{SD}$ of the different test waters. Values in bold represent the characteristic ion concentrations for one exposure group according to the current and future threshold values.

Exposure		Ion concentrations [mg/L]					Conductivity	Osmolarity
group	Na ⁺	Cl ⁻	Mg ²⁺	K ⁺	Ca ²⁺	SO ₄ ²⁻	[μS/cm]	[m ^{os} mol/kg]
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

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 Affair, LaGeSo (reference number G 0277/14).

2.2 Ion test solutions

Currently allowed discharge thresholds (until 2020, high thresholds, HT) and reduced future thresholds (from 2021, lower thresholds, LT) were tested ([Table 1](#), for measured mean ion concentrations refer to supplements [Table 1](#)). In addition, ion solutions with high Mg²⁺ (Mg), high K⁺ (K) and high Mg²⁺ and K⁺ concentrations (Mg + K) were investigated. Tap water was spiked with NaCl, NaSO₄, MgCl₂·6H₂O, MgSO₄·7H₂O, KCl, KSO₄ and CaCl₂·2H₂O (S3 Chemicals, Germany) to obtain the intended ion concentrations. Fish of the control group (Ctrl) were held in tap water and ion concentrations were examined weekly.

2.3 Blood plasma and tissue sampling

Fish were anesthetized by a blow on the head before blood samples were obtained by caudal vein puncture with heparinized syringes and then killed by decapitation. Blood plasma and dissected gill filaments (after 24 h, 7 d, 21 d, 8 wk) and muscle tissue (after 8 wk) were immediately snap frozen in liquid nitrogen and stored at −80 °C. Gill arches were excised after 21 d for histological examination.

2.4 Plasma osmolality and ion concentrations

The osmolality of 15 μL plasma was examined by means of a freezing-point osmometer (Osmomat, Gonotec, Germany). Plasma [Na⁺], [Mg²⁺], [K⁺] and [Ca²⁺] were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Fisher, USA). Prior to measurement, plasma samples were diluted 1:201-fold in ultrapure water. Plasma [Cl⁻] and [SO₄²⁻] were measured by ion chromatography (IC, conductivity detector CDD-10A, Shimadzu, Japan). In order to remove interfering lipids, 10 μL plasma (diluted 1:10 in ultrapure water) was mixed with 1 mL *n*-hexan and placed on a shaker for 30 min. After centrifugation (1425 g, 2 min), samples were deep-frozen and quickly separated from the liquid *n*-hexan phase. Proteins of the diluted plasma samples were precipitated in 450 μL 99.8% ethanol and 450 μL ultrapure water was added. Samples were centrifuged (1425 g, 15 min) and the supernatant collected. For measurement, an anion analysis column was used (Shodex IC St-90 4 E) with an eluent containing 1.8 mM Na₂CO₃, 1.7 mM Na₂HCO₃ and 1 % acetonitril.

2.5 Na⁺/K⁺-ATPase activity

The activity of NKA was determined according to the method of [McCormick \(1993\)](#) with slight modifications. Small pieces of frozen gill filaments were excised and 150 μL ice-cold 0.1% SEID buffer (250 mM sucrose, 10 mM Na₂EDTA, 50 mM imidazole, 0.1% sodium deoxycholate) was immediately added to each sample. After homogenization with a hand-operated Teflon pestle for 15 s, samples were processed as described by [McCormick \(1993\)](#) and enzyme activity measured in a 96 well plate reader (Tecan infinite M200, Tecan, Switzerland) for 12 min at 340 nm and 25 °C. Inhibition of NKA was induced by 3 mM ouabain (Sigma-Aldrich, Germany). Total protein concentration of 5 μL gill homogenate (1:10 diluted in ultrapure water) was determined in duplicate with a Roti-Quant Universal Kit (Carl Roth, Germany) according to the manufacturer’s instruction. A standard series was prepared from bovine serum albumin (BSA, Sigma-Aldrich, Germany) and activity of NKA is expressed as $\mu\text{moles ADP/mg protein/hour}$.

2.6 Histological analysis of gills

Dissected gill arches were sampled after 21 d and processed according to Gröner et al. (2017). Briefly, gills were preserved in Bouin's fixative (Sigma-Aldrich, Germany). Samples were dehydrated by flushing them twice in 70 % ethanol. After different dehydration steps with 70-99.8 % ethanol and xylol in the Excelsior ES Tissue Processor (Thermo Fisher, USA), gills were embedded in paraffin wax. The embedded tissues were cut (4 µm) with a rotary microtome (HM 340 E, Thermo Fisher, USA), transferred to a water bath (ultrapure water, 45°C) and mounted on microscope slides. For staining, paraffin was removed and tissue was stained with hematoxylin and eosin. The microscopic analysis was performed at 400-fold magnification (Olympus BX50, Japan). Of each gill sample, four primary filaments with 20 lamellae (total of 80 lamellae) were blindly analyzed for pathological alterations that were categorized into the severity levels 'minimal', 'moderate' and 'severe'. The mean of the four primary filaments was taken representing 20 lamellae.

Muscle water content

Fresh weight of small pieces of dorsal epaxial muscle tissue (approx. 0.5 g) was determined in clean sealed pre-weighted glass vials at room temperature after 8 wk. Samples were dried for 48 h at 105 °C and remaining moisture removed in a desiccator. After 48 h, the dry weight was determined. Muscle water content (moisture in % = (wet weight-dry weight/wet weight) x 100) was calculated.

2.7 Statistical analyses

The software SPSS Statistics 24 (IBM, USA) was used for all statistical analyses and graphs. If the assumptions for parametric testing were met (normal distribution and homogeneity of variances), one-way ANOVA with Tukey-HSD post-hoc test was performed. Tamhane post-hoc test was chosen if homogeneity of variances was not given. In the case of non-parametric testing, the Kruskal-Wallis test followed by a Bonferroni correction was employed. A significance level of $p \leq 0.05$ was considered significant. Values are expressed as box (25, 50 and 75 percentiles) and whisker (minimum to maximum value and outliers indicated by stars and circles) plots or as mean $\pm$ SD.

3 Results

3.1 Plasma osmolarity and electrolytes

Plasma osmolarity of HT ($p = 0.03$) was significantly enhanced after 24 h compared to control (Fig. 1 A). There were no differences between the exposure groups and after any further exposure time (for all p-values and pooled means $\pm$ SD of data not shown refer to supplements Table 2). Corresponding to osmolarity, plasma $[Na^+]$ ($p = 0.048$, Fig. 1 B) and $[Ca^{2+}]$ ($p = 0.01$, Fig. 1 C) were significantly elevated in group HT after 24 h. Moreover, HT responded with significantly higher $[Mg^{2+}]$ compared to group K ($p = 0.018$, Fig. 1 D). No further changes of plasma ions were detected between groups after the exposure of 7 d, 21 d and 8 wk. Plasma $[Cl^-]$ as well as $[K^+]$ were not significantly altered by the exposure groups after any exposure time.

Fig. 1 Plasma osmolarity in mosmol/kg (n = 12-14, Tukey), [Na⁺] (n = 13-14, Tukey), [Ca²⁺] (n = 13-14, Kruskal-Wallis) and [Mg²⁺] (n = 13-14, Kruskal-Wallis) in mg/L after 24 h exposure. Unequal lower cases indicate significant differences at a significance level of p ≤ 0.05.

alt-text: Fig. 1

The measurement of [SO₄²⁻] revealed no significant effects after 24 h (Fig. 2 A). However, after 7 d, HT (p = 0.001), LT (0.007) and Mg + K (0.003) responded with a significant increase relative to control (Fig. 2 B). HT was also significantly higher than K (p = 0.034). After the 21 d exposure, HT (p = 0.00), Mg (p = 0.00) and Mg + K (p = 0.00) had noticeable higher [SO₄²⁻], HT (p = 0.00), LT (p = 0.002), Mg (p = 0.027) and Mg + K (p = 0.00) were also higher than K (Fig. 2 C). Furthermore, HT (p = 0.044) was different to Mg. After 8 wk (Fig. 2 D), only HT showed significantly higher [SO₄²⁻] than control (p = 0.039) and K (p = 0.041).

Fig. 2 Plasma [SO₄²⁻] in mg/L after 24 h, 7 d, 21 d and 8 wk (n = 9–10, Tukey). Unequal lower cases indicate significant differences at a significance level of p ≤ 0.05.

alt-text: Fig. 2

3.2 Na⁺/K⁺-ATPase activity

There were no significant changes of NKA activity in any group and after all exposure times.

3.3 Histological alterations of gill epithelia after 21 d

Considering all gill histological alterations at a time, all exposure groups responded with a significantly higher mean number of affected lamellae compared to control after 21 d (Fig. 3 A, p-values for HT: 0.00, LT: 0.00, Mg: 0.00, K: 0.007, Mg + K: 0.00). The number of epithelial cells, located in the interlamellar region and on lamellae, affected by hyperplasia of moderate degree was significantly increased in HT (p = 0.034) (Fig. 4). Considering either hyperplasia in the interlamellar region or on lamellae, no significant changes were detected for any severity level. Every exposure group responded with an increased mean number of lifted epithelial cells of minimal degree compared to control. Mg + K exhibited the highest increase (p = 0.001), followed by HT (p = 0.002), LT (p = 0.009), Mg (p = 0.005) and K (p = 0.047). No significant alterations of moderate and severe degree were identified. There was no significant change of the number of fused lamellae at each severity level. HT (p = 0.00) and Mg + K (p = 0.001) contained more hypertrophic chloride cells of minimal degree than control and Mg (p = 0.082) showed a trend (defined as p ≤ 0.1) of an increase. Group Mg + K had significantly more shortened lamellae (p = 0.001) and exhibited a higher number of infiltrated leucocytes (p = 0.003) whereas HT responded with a trend (p = 0.086) (Fig. 5).

Fig. 3 Mean number of altered lamellae considering all histological alterations after 21 d (A). Mean number of altered lamellae for each alteration presented as stacked bars (B). Unequal lower cases indicate significant differences at a significance level of $p \leq 0.05$ (n = 6-9, Kruskal-Wallis).

Fig. 4 Number of altered lamellae for hyperplasia, epithelial lifting, lamellar fusion and chloride cell hypertrophy after 21 d (n = 6-9, Kruskal-Wallis). Alterations were categorized into the severity levels minimal, moderate and severe. Unequal lower cases indicate significant differences at a significance level of $p \leq 0.05$.

Fig. 5 Number of altered lamellae for lamellae shortening and leucocytes after 21 d (n = 6–9; Kruskal-Wallis). Unequal lower cases indicate significant differences at a significance level of $p \leq 0.05$.

alt-text: Fig. 5

3.4 Muscle water content after 8 wk

Muscle water content was determined after 8 wk and remained homogeneous within all exposure groups.

4 Discussion

Investigations of physiological effects of stressors are essential to elaborate solution approaches of anthropogenic interferences with the environment. In a prior study, it has been demonstrated that only a transient stress response is induced in roach by potash mining effluents and ion imbalances under laboratory conditions (Baberschke et al. submitted). In order to evaluate the observed adverse impacts of those effluents in the River Werre, the effects on the osmoregulatory capacity of the common roach were evaluated. Only the currently allowed thresholds (HT) caused transient changes of plasma osmolality and $[\text{Na}^+]$ and $[\text{Ca}^{2+}]$. However, in most groups plasma $[\text{SO}_4^{2-}]$ was elevated over a period up to 21 d and only in HT, $[\text{SO}_4^{2-}]$ remained chronically increased. All exposure groups induced chronic morphological alterations of the gill structure.

Fish gills represent a large surface area with close proximity to the environment for diffusion and ion fluxes via ion channels and transporters. Thus, teleosts are prone to disturbances of the internal hydromineral balance. As a consequence of osmotic stress and impaired osmoregulation, a change of plasma osmolality and plasma electrolytes may occur (Marshall et al., 1999; Kammerer et al., 2010). A transient increase in plasma osmolality was detected following exposure to HT for 24 h (Fig. 1 A). Accordingly, plasma $[\text{Na}^+]$ and also $[\text{Ca}^{2+}]$ were increased (Fig. 1 B, C). Due to the substantially higher number of ions, $[\text{Na}^+]$ and $[\text{Cl}^-]$ are the major electrolytes accountable for the osmotic pressure. Thus, the slight increase in $[\text{Na}^+]$ was most likely the reason for the change of osmolality in HT. It seems that ion absorption and excretion mechanisms, including ion pumps and morphological changes of gills, did not yet adapt to the greater availability of Na^+ and Ca^{2+} concentrations of the surrounding water (Malakpour Kolbadinezhad et al., 2012). Shifts of electrolytes and osmolality also occur as a general response to stress due to alterations of fluids between cellular and extracellular compartments caused by the rapid secretion of catecholamines (Lock and Wendelaar Bonga, 1991). Thus, $[\text{Na}^+]$ and $[\text{Ca}^{2+}]$ increases could also be attributed to catecholamine action. As demonstrated by Lin et al. (2011), Ca^{2+} uptake is induced by elevated cortisol resulting in a upregulation of epithelial Ca^{2+} channels. Therefore, the observed increases in $[\text{Ca}^{2+}]$ could be explained by the hypercalcemic action of elevated cortisol levels as previously determined for roach exposed to HT (Baberschke et al. submitted). According to Mount et al. (1997), high Na^+ and Ca^{2+} concentrations in the external media evoke only minor toxicity and the corresponding anions are accountable for the toxic potential.

Plasma levels of $[\text{Cl}^-]$ were not altered in any exposure group although HT contained an uneven ratio of Na^+ and Cl^- , with a Cl^- surplus. Excess Cl^- is able to exit the cell via anion channels as a possible reason for the unchanged concentrations (Marshall, 2002; Evans et al., 2005). Another reason could be that high levels of Ca^{2+} promote the tightening of cellular junctions, thus lowering passive Cl^- diffusion (Soucek et al., 2011).

Similar to Cl^- , excess Mg^{2+} did not lead to changes of plasma $[\text{Mg}^{2+}]$, as levels remained balanced in all groups at all times. Van Dam et al. (2010) evaluated the influence of Ca^{2+} on Mg^{2+} toxicity on the Northern trout gudgeon, *Mogurnda mogurnda*, with respect to soft freshwater and determined the medium lethal concentration (LC_{50}) of 40 mg/L for Mg^{2+} . Toxic effects exerted by Mg^{2+} were diminished by high Ca^{2+} levels probably by competition for binding sites of both cations and thus reducing Mg^{2+} uptake. Adverse effects were also greater in invertebrates than in the fish tested. In the present study, the normal to relatively high Ca^{2+} levels (Table 1) may prevent from more serious impacts as the ion has also ameliorative effects by reducing the permeability of membranes to ions (Wendelaar Bonga, 1978).

Unfortunately, it is hardly possible to tell whether there is an alteration of $[\text{K}^+]$ due to highly variable values within the exposure groups, although no significant differences were detected. The measurement of $[\text{K}^+]$ is prone to

strong deviations from its actual concentration by either the sampling method (Oikari and Soivio, 1975) or hemolysis resulting in a release of K^+ by damaged blood cells (Marino et al., 2001). Hence, the lack of effects on plasma $[K^+]$ may be interpreted with caution.

In the study conducted by Malakpour Kolbadinezhad et al. (2012), the moderately euryhaline Caspian roach, *Rutilus rutilus*, transferred stepwise to increasing salinity levels did not respond with any changes of plasma osmolality, $[Na^+]$, $[Cl^-]$ and $[K^+]$ and hematocrit at 5‰ salinity. Changes of a few of these parameters occurred at a salinity of 10 and others at 15‰ indicating a strong ion and osmoregulatory capacity. Many euryhaline teleost species are capable of keeping their plasma osmolality at constant levels with rising salinity containing ions in a physiological ratio (Behrens et al., 2017). The rapid but transient osmoregulatory imbalance, expressed by shifts of osmolality and $[Na^+]$ and $[Ca^{2+}]$ in HT, and the unchanged parameters in the other exposure groups suggest that roach is capable of efficient ion and osmoregulation. This ability is reflected by the fact that roach as a secondary freshwater fish, naturally inhabits brackish waters (Rückert et al., 2007). To our knowledge, other studies on the osmoregulatory capacity of fish, focusing on ions other than Na^+ and Cl^- and considering ion imbalances do not exist. Since toxicity strongly depends on ionic composition (Mount et al., 1997; Chapman et al., 2000), fluctuations of such in potash effluents may lead to other effects than provided by this study with constant ion concentrations.

Unexpectedly, $[SO_4^{2-}]$ was continuously elevated in group HT, LT and Mg + K after 7 d and in all exposure groups, except K, after 21 d (Fig. 2 B, C). In group HT, the effect was persistent even after 8 wk (Fig. 2 D). Group K contained an almost equal water SO_4^{2-} concentration to the control explaining the absence of an effect. Even though group Mg + K was exposed to SO_4^{2-} concentrations approximately a third higher than HT (Table 1), the effect was diminished after 8 wk. Furthermore, water SO_4^{2-} concentrations in group HT were about the same as in Mg. Hence, it is conceivable that the simultaneous but effective regulation of several ions or in combination with high Cl^- and Na^+ concentration either reduces the ability of SO_4^{2-} excretion or increases the uptake. In freshwater and marine teleosts, excess SO_4^{2-} is excreted by ion exchangers and co-transporters in the kidneys and plasma levels are maintained within a narrow range (Kato et al., 2009). In contrast, no mechanism for branchial SO_4^{2-} uptake has been identified for freshwater fish and passive diffusion via ion channels seems likely (Elphick et al., 2011). Elphick et al. (2011) evaluated effective concentrations of SO_4^{2-} in association with varying water hardness on different plant, invertebrate and fish species in order to establish water quality guidelines. They recommend a threshold of 675 mg/L in hard water when other ions are in a physiological range. Also Mount et al. (1997) considered SO_4^{2-} to be one of the least toxic ions. However, knowledge about SO_4^{2-} toxicity is scarce and the focus mostly lies on the associated cations. Here, for the first time persistently elevated plasma $[SO_4^{2-}]$ in freshwater fish was demonstrated and the acute and chronic effects remain unclear. This lack of knowledge urgently calls for further research with emphasis on physiological effects including reproduction. Moreover, due to the unpredictable long-term effects, SO_4^{2-} is recommended to be included as a threshold for protective effluent management.

The balance of the major electrolytes $[Na^+]$ and $[Cl^-]$ is crucial in order to sustain normal physiological concentrations in body fluids. Branchial NKA activity has often been observed to increase during seawater acclimation and many studies addressed the endocrine mechanisms in euryhaline freshwater and anadromous fish (Hwang et al., 1989; Handeland et al., 1998; Marshall et al., 1999). Cortisol is involved in seawater and freshwater acclimatization and stimulates the proliferation of chloride cells and NKA activity (McCormick, 2001). Linking these results with formerly published cortisol measurements (Baberschke et al. submitted), transiently elevated cortisol levels during 24 h appear not to modulate NKA response. This may be explained by the fact that changes of ambient salinity and ion composition were too weak, as the fish remained hyperosmotic, and that *R. rutilus* might possess sufficient NKA containing chloride cells for rapid adaptation. The latter was suggested by Marshall et al. (1999) for the strongly euryhaline killifish, which responded with elevated cortisol but no NKA changes after seawater transfer. As outlined above, Malakpour Kolbadinezhad et al. (2012) investigated the effects of gradual salinity increase on osmoregulation of the Caspian roach. There was no change of NKA activity after transfer to 5‰ but a transient decrease at 10 and 15‰ before activity returned to control levels. The results confirm the observed lack of change since group HT exhibits a salinity about 5‰ and in the other groups, salinity is even lower. Only *in vitro* studies with euryhaline teleosts revealed that NKA activity increases strongly with external K^+ starting at concentrations comparable to those used in this study (Lin and Lee, 2005). Besides the fairly variable relationship between NKA activity and salinity found in literature, there are no similar *in vivo* studies investigating NKA activity with respect to ion imbalances and excess single ions.

Stressors of different nature, including salinity changes in the external medium, are able to induce unspecific morphological changes of the gills as adaptational responses or as a consequence of tissue damage (Mallatt, 1985; Wood, 2001). For example, the severity of alterations of branchial structures and functions in monea fish, *Melnyinis orinocensis*, were positively correlated with increasing NaCl concentrations (Velasco-Santamaria and Cruz-Casallas, 2008). The present study revealed mild to moderate histological alterations such as epithelial lifting of minimal degree in all groups (Figs. 3-5). Furthermore, hyperplasia (of interlamellar cells and on lamellae) of moderate degree was found in group HT. Hyperplasia, the proliferation of epithelial cells, and epithelial lifting, due to fluid infiltration, increase the epithelial area for diffusion between the blood and external medium as a protecting defense mechanism (Mallatt, 1985). Thus, the observed changes can be interpreted as an adaptation mechanism since this barrier function reduces the absorption of excess ions from the surrounding water. This is supported by the fact that plasma osmolality and electrolyte levels, except SO_4^{2-} , were unaffected after 21 d. Furthermore, filament shortening occurred significantly more frequently in Mg + K. All of the observed alterations diminish ion uptake but in turn potentially reduce the surface area for effective gas exchange (Wood, 2001). There was also an increased number of hypertrophic chloride cells of minimal degree in HT and Mg + K and a trend for Mg. The size increases may be explained by osmoregulatory distress (Mazon et al., 2002), although transient plasma electrolyte shifts give no indication. Hypertrophy is also assumed to increase the blood to water distance. A higher number of infiltrated leucocytes was found in Mg + K and a trend of an increase in HT. Infiltrated leucocytes are often described as an indicator for inflammatory events indicating possible cell damage or intruding pathogens (Höger et al., 2005).

Since no irreversible or severe lesions were detected, the physiological modifications are considered rather unspecific and adaptive to the ion stress by reducing ion uptake. However, since gill integrity is impaired in all

groups, albeit to different degrees, it cannot be ruled out that in the long-term, gas exchange capability would be negatively affected if compensation mechanisms fail.

Owing to changes of the osmotic pressure caused by alterations of the ambient salinity, teleost fish have to regulate their cellular volume to maintain hydromineral homeostasis (Assem and Hanke, 1979). Luz et al. (2008) reported that stenohaline goldfish, *Carassius auratus*, exposed to salinities up to 8‰ responded with unaltered muscle water content whereas muscle dehydration occurred at higher salinities. In contrast, an increase of muscle water content can be due to enhanced consumption of organic compounds to offset energetic demands during stress (Martem'yanov 2001). In this study, muscle water content was not affected after 8 wk supporting the assumption that *R. rutilus* did not suffer from chronic osmotic stress following exposure to the different ion solutions.

Together with previously published results (Baberschke et al. submitted), the present study does not give indications for acute toxicity caused by potash mining effluents and ion imbalances but certainly challenges the fish as evident by osmotic and ionic disturbances and gill impairments.

Exposure groups, containing multiple highly concentrated ions, exhibited the strongest effects in the following order $HT > Mg + K/LT$ compared to groups with single ions ($Mg > K$).

K^+ is considered to be the ion with the strongest toxic potential and studies recorded that high concentrations are the cause of a reduced tolerance of invertebrates to the total salinity (Schmitz and Kneissl, 1967; Mount et al., 1997). Hence, K^+ could rather manifest its toxic effects in combination with other ions than acting as a single ion. Water hardness (Ca^{2+} and Mg^{2+}) and the presence of other cations can ameliorate adverse effects of ions (Mount et al., 1997; Soucek et al., 2011). In this case, the relatively high Ca^{2+} concentrations could moderate potential toxic effects whereas this potential is unlikely for Mg^{2+} as indicated by the results of $Mg + K$. The results further demonstrate that effects are rather caused by the sum of ions and its deviation from physiological concentrations and ratios than by ion species. But it remains unclear why, regarding gill histology, $Mg + K$ induced more alterations than LT despite equal concentrations of Mg^{2+} and K^+ . It is feasible that effects in group LT may be attributed to combined action of Mg^{2+} and K^+ . Unfortunately, differences and effects of both groups are too weak and variable to speculate where they originate from and what exact role Na^+ , Cl^- and SO_4^{2-} might play.

R. rutilus seems to be tolerant to ion stress to some extent and obviously possesses mechanisms including morphological alterations of the gills to balance the resulting physiological disturbances. With the exception of $[SO_4^{2-}]$ that remained elevated, most likely passively, since no regulatory mechanism is known for freshwater fish. Most teleosts (about 95%) are stenohaline (Evans et al., 2005; McCormick, 2001), bearing only slight changes of the external salinity. This may also be applicable to less adaptive species and in terms of other ions than Na^+ and Cl^- . Combining the observed acute and chronic effects, especially elevated $[SO_4^{2-}]$, and the likely potentiation of effects by multiple stressors as well as the evident decline of *R. rutilus* and other species in the River Werra suggest that potash mining effluents pose a risk to the health of fish. Moreover, the effect on reproductive success, as the main driver for species preservation, is still questionable and there are strong indications for impairments of reproduction for example in zebra fish (Wagler et al., pers. comm.) whereas physiological and histopathological effects on adults remained comparatively moderate (Irob et al. submitted). Furthermore, potash mining effluents potentially act on immune functions as indicated by strongly increased disease rates of fish populations in the River Werra (Matthes and Werner, 2005; Schwevers et al., 2005).

5 Conclusion

In this experimental study, osmoregulatory stress was induced in *R. rutilus* by all exposure groups, albeit to different degrees. The effects were most pronounced in the group containing the concentrations corresponding to the currently allowed high thresholds (HT) expressed by transient shifts in osmolarity, $[Na^+]$ and $[Ca^{2+}]$ and chronically elevated plasma $[SO_4^{2-}]$. Exposure to the future suggested lower thresholds (LT) and the group containing high Mg^{2+} and K^+ concentrations ($Mg + K$) and to a lesser extent high Mg^{2+} (Mg) caused increased $[SO_4^{2-}]$ levels for at least 21 d suggesting that $[SO_4^{2-}]$ is mainly disturbed by multiple ions at high concentrations. All ion solutions affected branchial structures of minimal to moderate degree suggesting adaptational alterations but conceivably on the costs of gas exchange capacity. Moreover, the inability to maintain normal $[SO_4^{2-}]$ levels with unpredictable long-term effects urgently calls for further investigation. Thus, it can be assumed that the thresholds for potash mining effluents exceed the concentrations for normal physiological functioning in teleost fish and further investigations should address potential impacts on reproduction in native fish species.

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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](#)

Supplementary material_osmo_V2

alt-text: Supplementary material_osmo_V2

Graphical abstract