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The effect of aqueous aluminium on mortality and respiration in the amphipod *Gammarus lacustris*

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Abstract

The literature covering freshwater acidification and Al-toxicity in aquatic organisms has mainly focused on fish species sensitivity to acidic Al-rich water. A thorough review of the literature shows that knowledge is far more limited when it comes to various taxa of aquatic invertebrate species. This study looks at the effect of aqueous aluminium and acidic water on mortality and respiration in the amphipod *Gammarus lacustris*. I have performed three subsequent mortality experiments, lasting 18 days, exposing *G. lacustris* to; (1) acidic Al-rich medium (pH 5.8), (2) acidic Al-rich medium (pH 4.8), (3) acidic Al-poor medium (pH 4.8) and (4) untreated natural water (pH 7.2). This has been followed by three respirometry experiments, exposing *G. lacustris* to untreated natural water (pH 7.2), acidic Al-poor medium (pH 5.8), and acidic Al-rich medium (pH 5.8). *G. lacustris* was exposed to each medium for five days before transfer to respiratory chambers for oxygen consumption measurements.

There was a statistically significant difference in mortality between exposure mediums ($p < 0.001$ (log-rank)). Mortality in the untreated exposure medium varied between 0 and 8%. Mortality in the exposure medium acidic Al-rich water (pH 5.8) was 8% in experiment 1, 67% in experiment 2, and 63% in experiment 3.Animals exposed to acidic Al-rich water and acidic Al-poor water (pH 4.8) had a mortality of 100% in all three experiments. There was also a statistically significant difference in normoxic O_2-consumption in animals exposed to the three media ($F_{2, 98} = 17.78$, $p < 0.001$, (ANOVA)) and in the critical O_2-concentration in animals exposed to the three media ($F_{2, 105} = 29.537$, $p < 0.001$, generalized eta squared = 0.36).

The overall conclusion is that aqueous aluminium is toxic to G. lacustris. However, the species is far more sensitive to acidity, and aqueous aluminium is not the main cause of the previously reported high sensitivity to freshwater acidification in G. lacustris. Al-toxicity in G. lacustris is dependent on the degree of Al-polymerization, and the effect is more evident at pH 5.8 than at pH 4.8. The possible link between the degree of Al-polymerization and the respiration in G. lacustris was not evident. However, elevated concentrations of aqueous aluminium have a clear effect on respiration in terms of increased normoxic O2-consumption and higher critical O2-concentration.

Preface

When I am now debarking from the long journey of a master thesis, there is no doubt that the journey has played a big part in determining if I am to be a scientist. I am, therefore, happy to still be able to say that this is what I want. However, without all of you who, with or without meaning to, have helped me along the way, I think it is fear to say that the journey may have ended abruptly.

So a big thanks to you all!

Moreover, I will, first of all, like to thank all the *Gammarus lacustris* for giving their lives in the name of science. I will like to thank my supervisor, Antonio B. S. Poléo, for all the knowledge you are willing to share, motivation, discussions and help with the writing. Thank you, Ivar Optun Andersen and Else-Mari Slåttelid, for the collaboration under significant parts of the project. Thank you, Nessim Kleiche, for your motivation, help with parts of the respirometry experiments and data preparation. Thank you, Sergey Morozov, for taking the time to explain and make changes in the FishResp package just for me. Thank you, Nicholas Carey, for your help with the preparation of the data for critical O_2-consumption. Thank you, Kåre Sandklev and Olav Berge at the fish hatchery, for always helping out when needed.Thank you, Ruben A. Pettersen, for lending us equipment and Kjartan Østby and the rest and the rest of the E^4 group for critical questions and help. And thank you, Linda Kollandsrud, for just being you!

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Innholdsfortegnelse

Introduction

Already in the 1920's episodic fish deaths in rivers, increased mortality of roe and fry in hatcheries, and declining catches of Atlantic salmon (*Salmo salar*) and brown trout (*S. trutta*) in several rivers and lakes were reported from the southern parts of Norway (Dahl, 1921, 1926; Huitfeldt-Kaas, 1922). The events of fish deaths often appeared during spring flood periods, or during autumn storms with heavy rain episodes. Huitfeldt-Kaas (1922) suggested that periods with heavy rain could release toxic water from dried-up bogs that killed the fish. Later, Dahl (1926) measured decreased water pH during a heavy rain episode that killed fish in a hatchery and suggested that the water acidity could be the toxic agent.

The earliest mentioning of the phenomenon "acid rain", however, seems to be found in a book from 1872 by the Scottish chemist Robert Angus Smith (Smith, 1872). Despite this, it took more than 80 years before the acidity of the precipitation – the acid rain – was suggested to be the cause of freshwater acidity measured by Dahl (1926) and the subsequent declines in fish populations in affected areas of Scandinavia, Britain, and North America (Dannevig, 1959).

It was little doubt that acid rain was a central part of the explanation of the episodic fish deaths and fish population declines in the affected areas, but a study published already in 1937 pointed out that natural water with pH levels as low as 4.5, such as dystrophic lakes and ponds, or alkaline lakes and springs with a pH as high as 9.5 can support fish life (Ellis, 1937). This contradiction was not paid much attention. Interestingly, other studies in the same time period, and somewhat later, showed that aqueous aluminium found in polluted water was toxic to freshwater organisms (Anderson, 1944, 1950; Biesinger & Christensen, 1972; Bringmann & Kuhn, 1959; Jones, 1939, 1940; Pulley, 1950; Thomas, 1915). It was not until the late 1970s, however, that the link between acid precipitation and increased concentrations of aqueous aluminium in lakes and rivers were discovered (Dickson, 1978; Driscoll et al., 1980; Schofield, 1976).

Aluminium is the most abundant metal in the earth's crust, and the natural occurrence is generally limited to highly insoluble complex minerals, where aluminosilicates such as feldspars predominate, making the average concentration of aluminium in natural waters minute. The majority of soluble weathering products of Al-minerals are retained in the soil, bound to inorganic and organic particles and compounds. The acidification caused by acid rain leads to a chronic reduction of soil water pH, i.e. increased H^+-concentration. Ion exchange (by H^+) with the soil particles results in the mobilization of various cations from the

edaphic (soil) to the aquatic environment. First, the loosely bound base cations (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) and subsequently, when the acid neutralizing capacity (ANC) of the base cations have decreased, the more strongly bound metals such as aluminium (Al^{n+}) (Cronan & Schofield, 1979; Lawrence et al., 1999; Seip et al., 1989; Stoddard et al., 1999). This leads to extensively increased concentrations of aqueous aluminium in the watershed (Alexander et al., 2017; Kowalik & Ormerod, 2006; Pye et al., 2012; Wellington & Driscoll, 2004; Wigington Jr et al., 1992; 1996), creating toxic conditions for many freshwater organisms (Driscoll et al., 1980; Gensemer & Playle, 1999).

The literature covering freshwater acidification and Al-toxicity in aquatic organisms has focused mainly on fish (Gensemer & Playle, 1999), and our knowledge about various fish species sensitivity to acidic Al-rich water and the mechanism of Al-toxicity in fish is well documented (Gensemer & Playle, 1999; Neville, 1985; Poléo, 1995; Poléo & Bjerkely, 2000; Poléo et al., 1997). When it comes to aquatic invertebrate species, the knowledge is far more limited, especially in terms of the mechanisms of Al-toxicity in various taxa.

As indicated by Ellis (1937), fish are sensitive to low pH if the levels are low enough, for example 4.5 or lower. This also applies to other freshwater organisms, but many invertebrate species seem to be affected by smaller decreases in pH compared to fish (Fjellheim & Raddum, 1990). Several studies show an increase in mortality and a decrease in biomass with increasing concentration of H^{+}-ions in the water (Friberg et al., 1980; Meijering, 1984; Otto & Svensson, 1983; Sutcliff & Carrick, 1973; Økland & Økland, 1986). It has therefore been agreed that low pH has a negative effect on the invertebrate fauna in acidified freshwater ecosystems (Havas & Rosseland, 1995; Herrmann, 1993).

Crustaceans such as *Gammarus lacustris* are considered among the most sensitive freshwater organisms for low pH (Herrmann, 1993). For Scandinavia and parts of Europe, a decrease in the number of *G. lacustris* populations has been registered, and addressed as an effect of acid rain (Herrmann, 1993; Meijering, 1984; Økland, 1969; Økland & Økland, 1986). It is still unclear, however, whether the sensitivity to freshwater acidification in various invertebrates in general, or *G. lacustris* in particular, is directly related to decreasing pH or indirectly related to increased concentrations of aqueous aluminium, as is the case for fish. This question was raised as early as 1990 (Howells et al., 1990; Rosseland, 1990), but it has remained unanswered until today. More specifically, we need to find out if elevated concentrations of aqueous aluminium are the main cause of the toxicity of acidified water in various invertebrate taxa and species, and what is the mechanism of toxic action of aluminium

in these species, including G. lacustris. A thorough review of the current literature reveals that limited research has been conducted on invertebrates and aluminium in light of freshwater acidification, and the limited research that exist has not clarified the significance of aluminium compared to pH for the various species, or the mechanisms behind the toxic effect (Berrill et al., 1985; Burton & Allan, 1986; Gensemer & Playle, 1999; Havas & Likens, 1985; Havas & Rosseland, 1995; Herrmann & Andersson, 1986; Mackie, 1989; McCahon & Poulton, 1991; Ormerod et al., 1987; Storey et al., 1992).

Very often, studies of Al-toxicity in various freshwater organisms are based upon chemical equilibrium constants. The environment, however, is never in a steady-state, and chemical equilibrium does rarely exist (Andersen, 2006; Fakhraei & Driscoll, 2015; Hindar et al., 1994). Accordingly, some ecotoxicological studies have shown that non-steady-state aluminium chemistry predominates and dictates the Al-toxicity in fish (Lydersen et al., 1994; Poléo et al., 1994; Rosseland et al., 1992; Weatherley et al., 1991). Under such conditions, inorganic monomeric aluminium species transform into polymeric aluminium species (Hem & Roberson, 1967; Lydersen et al., 1990), and it has been proposed that the toxicity of aluminium in fish is most severe during ongoing Al-polymerization because under such conditions aluminium accumulates on the gill surfaces (Poléo, 1995; A. B. S. Poléo & F. Bjerkely, 2000; Rosseland et al., 1992). Aluminium exposure causes respiratory and ion regulatory disturbances in the fish (Gensemer & Playle, 1999; Neville, 1985). The respiratory disturbances seem to be the predominant effect at pH above 5.5 (Neville, 1985; Playle et al., 1989; Poléo & Bjerkely, 2000). Positively charged aluminium forms will bind to the negatively charged gill surface (Poléo, 1995; Wold & Selset, 1977), acting as polymerization nuclei causing clogging of interlamellar spaces by complexes of Al-polymers and mucus leading to hypoxia (Oughton et al., 1992; Poléo, 1995; Poléo & Bjerkely, 2000; Poléo et al., 2017). Ion regulatory disturbances, i.e. net loss of plasma ions such as Na^+ and Cl^-, seem to predominate at pH below 4.5 (Gensemer & Playle, 1999; Neville, 1985).

The acid deposition in the northern regions of Europa and America has declined substantially during the last four decades, causing considerable improvement of the water chemistry due to international agreements to reduce the emissions of sulphur and nitrogen compounds (Ø. A. Garmo et al., 2014; Skjelkvåle et al., 2007; Skjelkvåle et al., 1998; Wright, 2008). Fish and invertebrate populations has also started to recover from the acidification, but this recovery has been slower (Arseneau et al., 2011; Enge et al., 2016; Hesthagen et al., 2016; Hesthagen et al., 2011; Murphy et al., 2014). The explanation behind the mismatch between chemical

and biological recovery could be that the amounts of base cations in the soil, i.e. the ANC, is still low, and that aluminium leaches out from the catchments during heavy rain episodes. This has led to the suggestion that we might be witness to a shift from chronic acidification when acid rain was still abundant, towards an episodic acidification governed by more frequent heavy rain and storm events in the northern regions due to climate changes (Poléo et al., 2021; Wright, 2008). Thus, the catchments are not constantly leaching aluminium anymore, but seem to mobilize aluminium to the aquatic environment during heavy rain and storms. Consequently, freshwater organisms are no longer chronically exposed to toxic levels of aluminium, but face toxic episodes that might be more frequent than previously (Enge et al., 2016; Laudon & Bishop, 1999; Poléo et al., 2021; Serrano et al., 2008).

So, why is it important to learn more about the possible interaction between aqueous aluminium and invertebrates in acidified freshwater ecosystems? In my opinion, there are several answers to this. Firstly, we need to fill in the knowledge gaps concerning the importance of aqueous aluminium in acidified freshwater ecosystems. Secondly, we need to know how aluminium affects various invertebrate taxa and species in order to understand and predict the ecosystem effects of a shift from chronically to episodic acidification. This is also important concerning future climate changes. Thirdly, increasing knowledge over the last decades has shown the importance of biodiversity and interactions in ecosystems that calls for studies addressing a more comprehensive picture of the effect of acidification regarding mitigation and conservation. Therefore, it is crucial to unveil the effect of acidic and Al-rich water and possible mechanisms of Al-toxicity in organisms other than fish in the aquatic food chain.

In the present study, I investigate the effect of aqueous aluminium on the amphipod (Crustacea) *G. lacustris* in light of freshwater acidification, by exposing the species to various combination of pH and Al-concentration. The aim of the study is to find out if the sensitivity to acidification in this species is due to lowered water pH, to increased aqueous Al-concentrations, or to a combination of both these factors. Also, to find out if Al-polymerization is of importance for a possible effect of aluminium in this species, as it is in fish. Accordingly, my experiments aim to answer the following scientific research questions:

1. Is aqueous aluminium toxic to *G. lacustris*?
2. Is aqueous aluminium the main cause of the previously reported high sensitivity to freshwater acidification in *G. lacustris* or not?
3. Is a possible Al-toxicity in *G. lacustris* dependent on the degree of Al-polymerization, like what we know in fish?

I also wanted to find out if there is any similarity in the mechanism of toxic action of aqueous aluminium in *G. lacustris* compared to fish. This is an extensive task to undertake, but the first step towards a description of the mechanism of Al-toxicity in *G. lacustris* is to find out how the various combinations of pH and Al-concentration affect its respiration. Hence, my last scientific research question is:

4. Is there a possible link between the degree of Al-polymerization and the respiration in *G. lacustris*?

Material and methods

Experimental animals – capturing and husbandry

This study is part of a project called "Aluminium Toxicity in Aquatic Invertebrates" at the Inland Norway University of Applied Sciences, and was conducted as a series of experiments on the freshwater amphipod *G. lacustris* exposed to various combinations of water pH and Al-concentration. Being a crustacean of the taxonomic class Malacostraca (order Amphipoda), *G. lacustris* is not underlying any legislation regarding collection or ethical concerns when used for scientific purposes. Despite this, we have followed the general guidelines for animal husbandry and welfare as far as possible given by the Norwegian Animal Research Authority for fish. The number of animals collected in the field was in accordance with the need for specimens for the experiments.

G. lacustris were caught with a sieve attached to a pole in Lake Nedgardssjøen in Stor-Elvdal municipality, Innlandet County (GPS coordinates: 61.5235826, 10.8807032), and brought in to the experimental unit of the fish hatching facility at the University (Evenstad, Stor-Elvdal municipality) where the experiments were conducted. The water of Lake Nedgardssjøen has a higher pH (approximately 7.5) compared to the hatchery water (in average 7.2). In addition, the water temperature in Lake Nedgardssjøen was always a few degrees higher than in the hatchery water each time animals were collected. Therefore, the animals were always acclimated to the hatchery water for at least 14 days before the experiments. We observed some mortality of weak or injured animals during the first days of the acclimation period. The 14-day acclimation period was implemented because preliminary tests showed 98 % survival of the remaining animals after 14 days.

At the hatchery, the animals were placed in several 10-litre flow-through storage tanks, approximately 20 individuals in each. The storage tanks contained small rocks collected in a nearby stream, and vegetation and bottom substrate collected in Lake Nedgardssjøen to provide some natural food supply. Animals not used immediately in experiments were also given supplementary food (Nutra Olympic, Skretting AS) a few times during the storage period. Supplementary feeding was always terminated 14 days before experiments. The storage tanks were equipped with a water intake at the bottom and an overflow for outlet water on the top of the tanks, providing good circulation and oxygenation of the water.

The operating water in the experimental unit of the hatchery is taken from an artificial pond in the stream Tronka and defined as untreated natural water from the surrounding catchment area above the hatchery. Table 1 gives an indication of the water quality of the operating water. Throughout the experimental periods, the operating water was very stable and maintained an average pH of 7.2, conductivity around 23 µS/cm and temperature close to 2 ℃.

Test conditions

My study consisted of three separate mortality experiments followed by three respirometry experiments. In the mortality experiments, *G. lacustris* was exposed to four different water qualities: acidic Al-rich medium at pH 5.8 (labelled red), acidic Al-rich medium at pH 4.8 (labelled yellow), acidic Al-poor medium at pH 4.8 (labelled blue) and untreated natural water at pH 7.2 (labelled green) as the control (Table 2). In the respirometry experiments, *G. lacustris* was exposed to three different water qualities: acidic Al-rich medium at pH 5.8 (labelled red), acidic Al-poor medium at pH 5.8 (labelled black) and untreated natural water pH 7.2 (labelled green) as the control (Table 2).

The acidic Al-rich medium (pH 5.8, red) was prepared by the addition of an Al-stock solution ($Al(NO_3)_3 \cdot 9H_2O$ dissolved in distilled water (11.2 g/l) and 7.6 ml/l nitric acid (HNO_3)) to the operating water. Similarly, the acidic Al-rich medium (pH 4.8, yellow) was prepared by the addition of an Al-stock solution ($Al(NO_3)_3 \cdot 9H_2O$ dissolved in distilled water (11.2 g/l) and 15.2 ml/l nitric acid (HNO_3)) to the operating water. The nominal Al-concentration in the acidic Al-rich media was calculated to be 1000 µg/l. In order to minimize the effect of temperature on the aluminium chemistry, the water temperature was kept between 0.5 and 4.0 °C during the experimental period.

Table 1. Water quality variables of the operating water in the fish hatching facility at Evenstad, Stor-Elvdal municipality, used in the experiments. The analyses were performed by the Norwegian Institute of Water Research (NIVA). Samples were collected in February 2004, and the values are mean ± S.D. (n=3). Only the minimum and maximum values are given for pH.

Water quality variables	
pH	7.33 – 7.38
Conductivity (µS/cm)	45.6 ± 0.1
Alcalinity (mmol/l)	0.352 ± 0.002
N-tot (µg/l N)	207 ± 10
NO_3^- (µg/l N)	133 ± 5
TOC (mg/l C)	2 ± 0
Cl^- (mg/l)	0.47 ± 0.01
SO_4^{2-} (mg/l)	3.96 ± 0.02
Ca^{2+} (mg/l)	7.40 ± 0.01
K^+ (mg/l)	0.36 ± 0
Mg^{2+} (mg/l)	0.56 ± 0.01
Na^+ (mg/l)	1.16 ± 0
$Fe^{2+/3+}$ (µg/l)	53 ± 9
Al-reactive (µg/l)	12 ± 1
Al-non labile (µg/l)	7 ± 1
Al-tot (µg/l)	21 ± 1

The two acidic Al-poor media were made by adding an HNO_3-solution to the operating water, lowering the pH from 7.2 to either 4.8 (blue) or 5.8 (black). The various chemical solutions were added to the operating water by means of peristaltic pumps Watson Marlow 205S (Figure 1), and the rpm was adjusted to give the selected pH values. To the untreated natural control water, no additions of chemicals were made. In the respirometry experiments, the stock solution of acidic Al-rich (ph 5.8) and acidic Al-poor (pH 5.8) water was increased to contain the double dose of aluminium $Al(NO_3)_3 \cdot 9H_2O$ (22.4 g/l) and nitric acid (HNO_3) 15.2 ml/l per 25 litres of distilled water (Table 2). However, the changing of stock solution was compensated for by turning down the speed of the pumps (rpm), keeping the pH and Al concentration at the same level as in the mortality experiments.

Due to the low pH in the Al-stock solution (pH approximately 2.5), the total amount of aluminium was present as Al^{3+}. When the pH rapidly increased to either 4.8 or 5.8 as the Al-stock solution was mixed with the operating water, Al^{3+} may start to polymerize into larger molecules depending on the amount of increase in pH (Hem & Roberson, 1967; Poléo, 1995; Poléo & Bjerkely, 2000). The degree of Al-polymerisation was expected to be much higher at pH 5.8 compared to pH 4.8 (Lydersen, 1990; Poléo, 1995). Previous studies have shown that at pH as low as 4.8, most of the aluminium in the water will be present as Al^{3+}, with a low ability to polymerize. At pH 5.8, on the other hand, most of the aluminium in the water will be present as Al-hydroxides, which have the ability to polymerize (Lydersen, 1990; Poléo, 1995; Poléo & Bjerkely, 2000). Thus, in this way, we aimed to create an unstable Al-rich water with ongoing Al-polymerization at pH 5.8 and a more stable Al-rich water with minimal Al-polymerization taking place at pH 4.8.

Table 2. The test media made and used in the mortality and respirometry experiments.

Channel codes	Test medium	pH	Nominal Al-concentration	Chemicals added to 25 liter distilled water
Mortality experiments				
Red channel	Acidic Al-rich medium (unstable)	5.8	1000 µg/l	280g $Al(NO_3)_3$ and 190 ml HNO_3
Yellow channel	Acidic Al-rich medium (stable)	4.8	1000 µg/l	280g $Al(NO_3)_3$ and 380 ml HNO_3
Blue channel	Acidic Al-poor medium	4.8	0 µg/l	380 ml HNO_3
Green channel	untreated natural (operating) water	7.2	0 µg/l	None
Respirometry experiments				
Red channel	Acidic Al-rich medium (unstable)	5.8	1000 µg/l	560g $Al(NO_3)_3$ and 280 ml HNO_3
Black channel	Acidic and Al-poor	5.8	0 µg/l	280 ml HNO_3
Green channel	untreated natural (operating) water	7.3	0 µg/l	None

Experimental setup

The experimental setup (Figure 2) consisted of a 120-litre level tank receiving the operating water of the hatchery. The tank was made of plastic with the size 52 x 30 x 78 cm (with x depth x length). The water was distributed to four flow-through exposure channels from the level tank. The purpose of the level tank was to provide a steady water supply into the exposure channels. This was done by letting the surplus water into the level tank exit in an overflow (Figure 3). To make this system function, it was necessary to provide more water into the level tank than the total amount of water running to the four exposure channels.

Figure 1. Picture showing one of the peristaltic pumps (Watson Marlow 205S) delivering chemicals to the operating water through a silicon tube inserted into an extension pipe on top of a mixing pipe leading the test medium into the experimental channel.

The exposure channels were made of fibreglass (47 x 17 x 220 cm). The operating water was led from the level tank to each exposure channel through a “mixing pipe” made of PVC (Figures 1 and 3). The water flow into the level tank, as well as the four exposure channels, were controlled by PVC ball valves (seen as blue levers in Figure 2 and 3). The inlet of the mixing pipes was covered with a fine plastic mesh to prevent larger particles from entering the exposure channels from the level tank. Particles on the fine mesh were regularly removed to keep the water flow into the exposure channels stable throughout the experiments. The water flow through the mixing pipe into each exposure channel was kept at approximately 2

l/min. It was frequently checked, at least once a day, during experiments to secure a stable water flow through the exposure channels. Each mixing pipe had a built-in extension pipe on top of it to add chemical solutions to the experimental water (Figure 1) through a silicon tube via the peristaltic pumps. At the point where the water flowed out of the mixing pipe and into each exposure channel, there was “a mixing cup” at the bottom of the channel (Figure 4) to ensure that the chemical solution added was well mixed with the operating water before reaching the exposed animals.

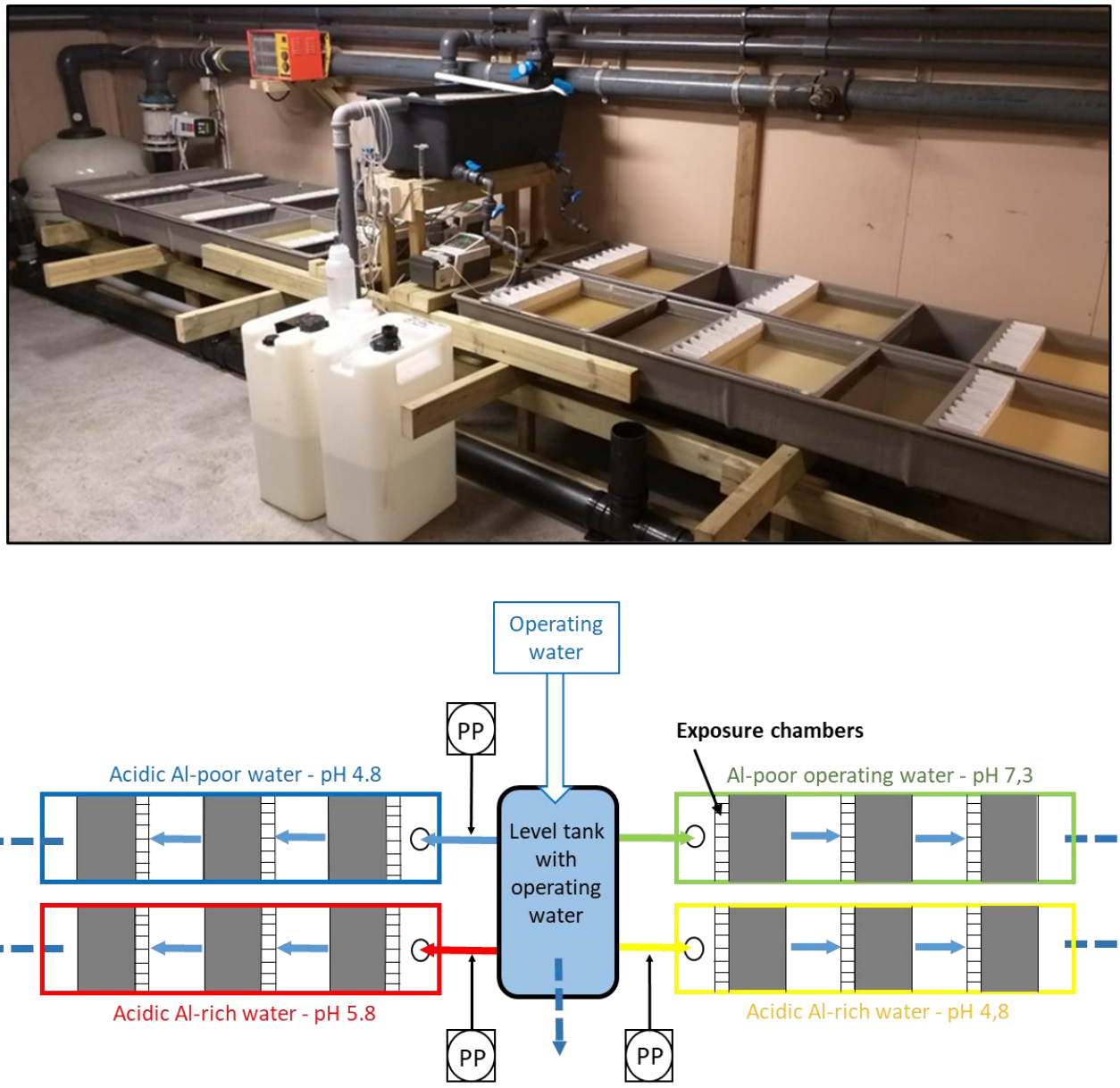

Figure 2. Top: picture of the experimental setup. The white tanks in front of the setup contained the stock solutions added to the operating water. Bottom: schematic representation of the experimental

setup showing the four exposure channels (coloured). The coloured arrows are the mixing pipes leading the operating water added chemicals by peristaltic pumps (PP) from the level tank to the channels. Blue arrows are showing the water flows through the setup, and dashed lines indicate exit water by overflow. The media indicated by coloured writing are those used in the mortality experiments.

Figure 3. Picture showing the level tank (black tank). The large pipe in front of the tank is the overflow, draining the excess water ensuring a stable water level in the tank. The four smaller pipes on the tank sides are the mixing pipes leading water from the tank to the experimental channels. Over the tank, in the background, is the inlet to the level tank supplying it with the operating water (large blue lever).

Each exposure channel was divided into three sections by three rows of exposure chambers in a way that forced the test medium to pass through the chambers on its way along the channel (Figure 2 and 5). Each row consisted of eight equal-sized exposure chambers (6 x 10 x 14 cm) made of plastic, PVC and fibreglass. Within each channel, the first row of exposure chambers was placed 7 cm from the inlet of water from the level tank, the second row 80 cm from the inlet, and the third row 150 cm from the inlet. The residence time of the test medium in

question, from the inlet in the exposure channel to the first row of exposure chambers, was 45 sec, 10 min to the second row, and 20 min to the third row. The total water residence time for a channel was approximately 45 min, from the inlet to the outlet at the end. The setup with the exposure chambers fixed at different water residence time was especially important for the acidic Al-rich medium (pH 5.8) to enable the exposure of the animals to different degrees of Al-polymerization. The theory behind this is that aluminium binds to, and polymerizes onto negatively charged surfaces such as gills, more extensively in the initial phase of the polymerization process when positively charge Al-hydroxides predominate in the solution (Poléo, 1995; Poléo & Bjerkely, 2000). As the polymers grow their net positive charge approach zero, and their ability to bind to surfaces like gills decreases.

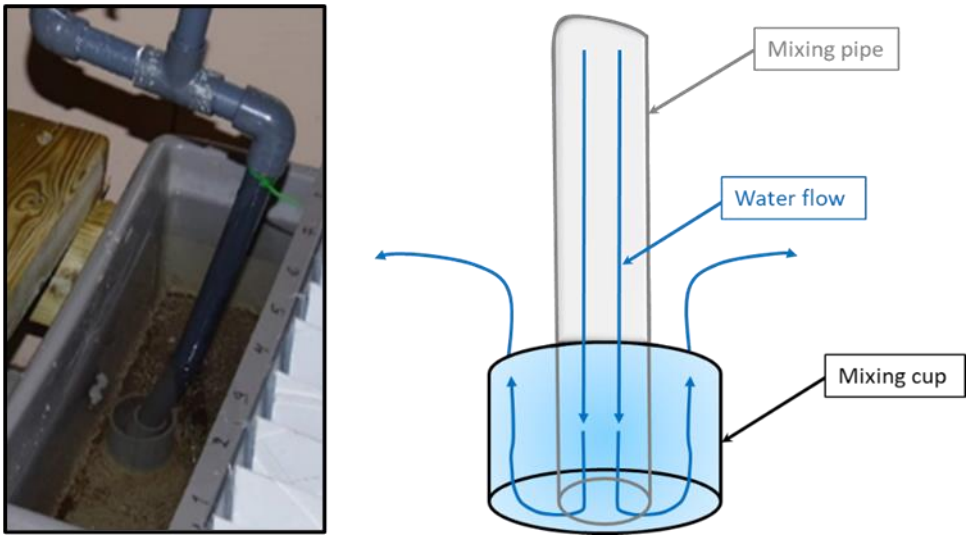

Figure 4. Left: Picture of a mixing pipe leading down to a mixing cup at the bottom of the experimental channel. Right: schematic presentation of the water flow (arrows) down the mixing pipe into the mixing cup and into the experimental channel.

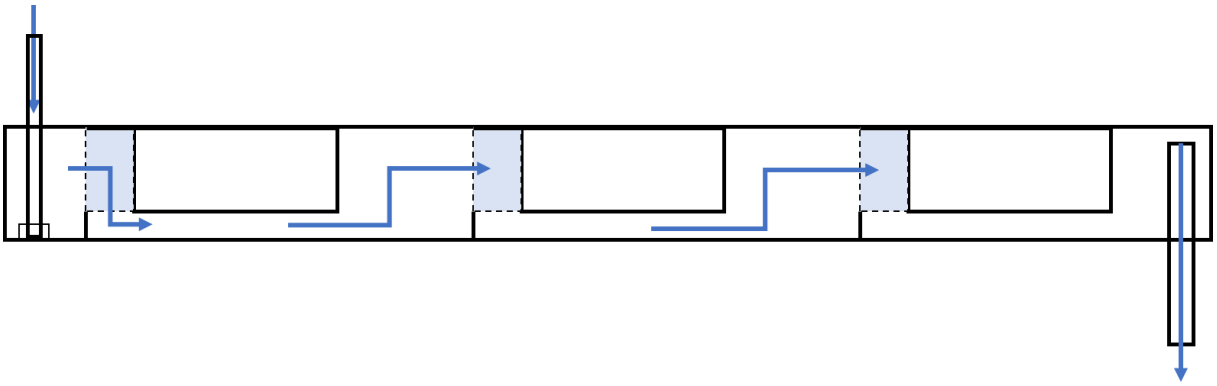

Figure 5. Schematic presentation of an experimental channel showing how it was constructed to force the water to flow (blue arrows) through the exposure chambers (light blue) on its way through the channel. The exposure chambers consisted of perforated PVC sheets in the front and at the bottom, providing an easy flow through the chambers (1 x 1 mm perforation). To the far left is the inlet via the mixing pipe into the mixing cup at the bottom of the channel, and to the far right is the overflow outlet from the channel.

Experimental protocol – mortality experiments

Each of the three subsequent mortality experiments consisted of four exposures: (1) acidic Al-rich medium (pH 5.8, red), (2) acidic Al-rich medium (pH 4.8, yellow), (3) acidic Al-poor medium (pH 4.8, blue) and (4) untreated natural water (pH 7.2, green). One channel was used for each medium.

At least one hour before the experiments were started, the addition of chemicals to the operating water entering the channels was started, to make sure that the desired pH in each channel was obtained by adjusting the peristaltic pumps delivering the chemical solutions. The experiments were started in the evening by placing one individual *G. lacustris* into each exposure chamber within the channels, 96 in total. In the first mortality experiment (mortality experiment 1), monitoring was performed three times every day throughout the experiment: morning, afternoon and evening. In mortality experiments 2 and 3, monitoring was performed twice a day: each morning and evening. All three experiments lasted for 18 days and were terminated by turning off the chemical dosage and removing the surviving animals from the exposure chambers. If all animals within a channel died before 18 days of exposure, this particular exposure was terminated by turning off the dosage.

The daily monitoring consisted of checking mortality in each exposure chamber, collecting a water sample for conductivity and pH measurements from each channel, checking the water

flow and dosage into each channel, and finally measuring the water temperature directly in each channel.

Water samples for Al-fractionation were collected in each channel from the central exposure chamber of each row of chambers, between one and three times during each experiment, depending on how fast the animals died in the particular exposures. In mortality experiment 1, a total of 12 water samples for Al-fractionation were collected just before the exposure started, another 12 samples after five days of exposure, and 6 samples on the last day of the experiment since the exposures to acidic Al-rich medium (pH 4.8, yellow) and acidic Al-poor medium (pH 4.8, blue) were already terminated. In the mortality experiment 2, water samples for Al-fractionation were collected twice from all four channels at exposure day 5 (a total of 24), then 3 samples were collected from the channel with acidic Al-rich medium (pH 4.8, yellow) at day 11, and 6 samples at day 18, from the channels with acidic Al-rich medium (pH 5.8, red) and untreated natural water (pH 7.2, green). In mortality experiment 3, 3 water sample for Al-fractionation were collected only once from each channel: at day 6 from the channels with acidic Al-rich medium (pH 4.8, yellow) and acidic Al-poor medium (pH 4.8, blue), and at the last day of the experiment from the channels with acidic Al-rich medium (pH 5.8, red) and untreated natural water (pH 7.2, green).

Experimental protocol – respirometry experiments

In the respirometry experiments, only one exposure channel was used for each experiment. In respirometry experiment 1, the *G. lacustris* was exposed to the untreated natural water (pH 7.2, green), in experiment 2 to the acidic Al-poor medium (pH 5.8, black), and in experiment 3 to the acidic Al-rich medium (pH 5.8, red). In these experiments, *G. lacustris* was exposed to the medium in question for five days before transfer to respiratory chambers for oxygen consumption measurements (Figure 6).

At least one day before the experiments were started, the addition of chemicals to the operating water entering the channel was started. When the desired pH in the channel was obtained by adjusting the peristaltic pump delivering the chemical solutions, the experiment was started by placing the first specimens of *G. lacustris* into the exposure chambers. I only had four respiratory chambers available, so the exposures had to be performed successively by placing new animals into the exposure chambers daily.

In the first respirometry experiment, two and two animals were placed into the same exposure chamber in the first row of chambers, four animals in total. This procedure was followed for the next two days until 12 animals were distributed two and two in the chambers. The day after, four animals were placed in the second row of chambers and so on, until 12 animals were distributed in each of the next chamber rows. After five days, I started to perform the oxygen consumption measurements on the first four animals placed into exposure chambers and continued with daily measurements until all 36 animals were tested. During this experiment, I became aware that I needed to monitor the background respiration in the water, due to the presence of microorganisms. Therefore, 12 consecutive oxygen consumption measurements were performed on pure water samples collected from the exposure channel at the various chamber rows (three from each) before the experiment was terminated.

In the respirometry experiments 2 and 3, the same protocol as for experiment 1 was followed. However, in these experiments, one of the four respiratory chambers was used for background oxygen consumption measurements each day. Hence, only three animals were placed into the chambers daily.

Daily monitoring of the channel, after the same protocol as in the mortality experiments, were also performed in the respirometry experiments. Water samples for Al-fractionation were collected three times during each respirometry experiments, after the same protocol as described for the mortality experiments

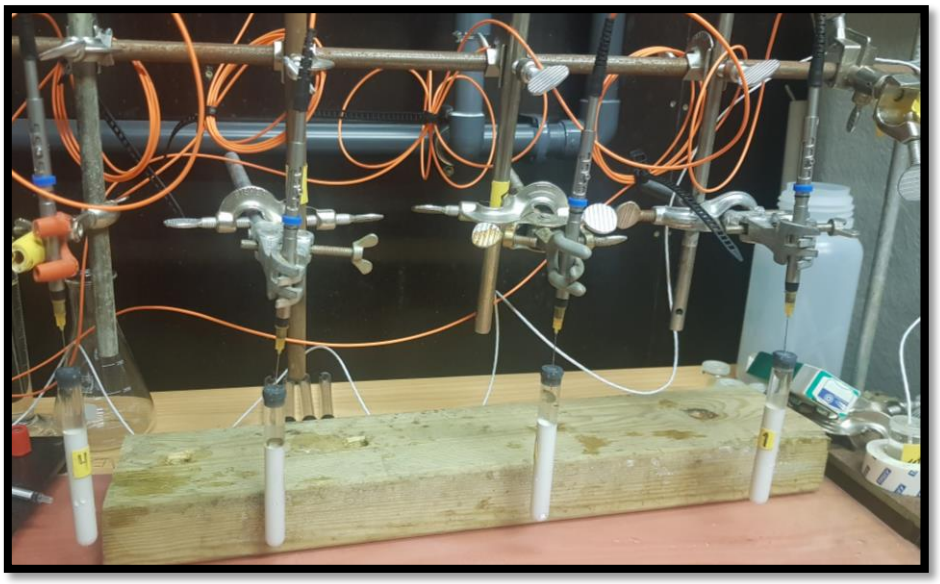

Figure 6. *The four respirometry chambers attached to the retractable fibre oxygen microsensors OXR50. In the right side corner, the temperature probe Pt100.*

Sampling and water chemical analyses

Colour labelled 1 litre plastic bottles were used to collect water samples for conductivity and pH measurements. One bottle for each channel. The bottles were rinsed in the water from the channel each day before sampling. Water samples for Al-fractionation were collected in pre-rinsed (nitric acid solution) 1 liter plastic bottles. All bottles used for water sampling were rinsed twice in the same water from which samples were to be taken, before the actual water samples were collected. Water samples for Al-fractionation were collected using a silicon tube siphon.

Water temperature was measured with a Testo 830-T4 thermometer. The electrolytic conductivity of the water was measured with a Radiometer Copenhagen CDM 80 Conductivity meter. The values were read to the nearest tenth, and the temperature corrected to 25 °C. The conductivity was measured repeatedly and determined when three consecutive measurements were identical within one-tenth of a unit (µS/cm). Water pH was measured using a WTW 3110 pH meter with a Hamilton Polilyte Plus H S8 120 glass electrode. pH buffers of 4.01 and 7.00 were used to calibrate the electrode daily before measurement. The pH meter has an indicator that flashes during the measurement. When the indicator stopped flashing, the pH was read.

Aqueous aluminium was fractionated by the so-called Barnes/Driscoll method, where 8-hydroxyquinoline (HQ) and methyl isobutyl ketone (MIBK) extraction (Barnes, 1975) is combined with a cation-exchange procedure (Driscoll, 1984), according to a protocol described by Poléo *et al.* (1997). Upon extraction of a water sample, aluminium is complexed with HQ (C_9H_7NO), and the Al-HQ complex is extracted into an organic phase of MIBK. The extraction time is 20 sec as Barnes (1975) recommends. The extracts were stored at 4°C for at least 24 hours before the amount of aluminium was analyzed spectrophotometrically on a Shimadzu UV-1201 spectrophotometer at 395 nm (Bloom et al., 1979; Tikhonov, 1973). Absorbance was also measured at 600 nm to correct for iron interference (Sullivan et al., 1986). The standard deviation of the method is estimated to be approximately 1% of the mean (Sullivan et al., 1986), and the detection limit is 13 µg Al/l according to Vogt *et al.* (1994).

Based on the extraction time of 20 sec, the total amount of monomeric aluminium (Ala) was determined by direct extraction of an untreated water sample (Driscoll, 1984). Water from the sample was also run through a cation exchange column with Amberlite IR-120 ion exchange resin (10 ml) before extraction was performed. Positively charged aluminium compounds are

retained in the ion exchange column while negatively charged, and uncharged compounds escape. Aluminium extracted (20 sec) from an ion-exchanged sample is defined as monomeric organic aluminium (Alo) (Driscoll, 1984). In some literature, this fraction is often named non-labile aluminium. Since monomeric inorganic aluminium compounds (Ali) are positively charges, and retained in the ion exchange resin, this fraction is calculated as the difference between Ala and Alo. Large aluminium compounds, such as colloidal aluminium, stable organic and hydroxyl organic aluminium compounds, are not extractable in 20 sec. Therefore, the total concentration of aluminium (Alr) in the water sample was determined by extraction of a sub-sample acidified with HNO_3 to pH 1.0 and stored for minimum 24 hours. Table 3 gives an overview of the fractions that were analyzed or calculated.

Table 3. Description of the various Al-fractions obtained according to the Barnes/Driscoll method.

Alr	Total aluminium, determined by extraction of an acidified water sample (pH 1.0) after 24 hours storage.
Ala	Total monomeric aluminium, determined by extraction of an untreated water sample.
Alo	Organic monomeric aluminium, determined by extraction of the eluate of a cation exchanged water sample.
Ali	Inorganic monomeric aluminium, calculated as the difference Ala – Alo.

The cation exchange column resin Amberlite IR-120 was on the Na^+-form. In order to avoid changes in the Al-fractions as water samples are run through the ion exchange column caused by changes in pH, the pH of the ion exchange resin was adjusted to the nearest 0.5 pH unit of the water samples in question (Driscoll, 1984). The fluid velocity through the column was 3.8 ml/min per ml of ion exchange resin. The ion exchange resin was prepared with 60 ml of 10^{-4} M NaCl solution between each run of a water sample, and 60 ml of water sample was always run through the ion exchanger before it was collected for extraction.

Each time aluminium was fractionated, a standard curve (0, 40, 100, 200, 400 and 600 µg Al/l) was produced for the calculating of Al-concentration from the optical absorbance measurements on the spectrophotometer by means of the formula:

$$[Al] = ((OD_{395} - (OD_{600} \times 1.12)) / slope) \pm intersection$$

Where OD is optical density (absorbance), slope is the slope of the regression line and intersection is where the lane crosses the x-axis.

Mortality monitoring and respirometry

The mortality of *G. lacustris* was checked by visual inspection. When it was difficult to decide if an animal was dead or not, observation of reaction to direct illumination with a flashlight and water pumped towards the animal using a pipette was applied inside the exposure chamber. If the animal showed no reaction, it was classified as dead. Dead animals were removed from their chambers. The length of the animals was measured before they were conserved in 70% ethanol and stored for possible later examination.

Respirometry, i.e. oxygen consumption measurements, were performed using an optical oxygen and temperature meter FireStingO2-FSO2-4 powering four retractable fibre oxygen microsensors OXR50 placed into self-made respiratory chambers. Automatic temperature compensation of the O_2-measurements was achieved by a temperature probe Pt100 connected to the temperature port of the meter. The FireSting O_2-meter was connected to a laptop running the computer program *Pyro Oxygen Logger*, version 3316. The O_2-meter, the O_2 and temperature sensors, and the computer program were provided by the company PyroScience GmbH, Germany.

The respiratory chambers used were self-made from blood sample test tubes (6 ml Vacutainer®) with airtight lids. The tip of oxygen microsensors was inserted through a rubber stopper in the test tube lids. The test tubes had to be modified to reduce their volumes. This was done by filling the tubes with epoxy to the desired volume, appropriate for the size and movement of individual *G. lacustris*, and the space needed for the microsensors. The specimen inside the respiratory chamber could move freely below and around the microsensor, stirring the water inside the chamber, ensuring as smooth as possible curves of O_2-consumption. The volume of each respiratory chamber was determined by weighing the chamber with and without water at 4°C.

Each O_2-consumption measurement was started by placing an animal together with water from its exposure chamber into a respiratory chamber. Measurement of the background respiration was performed the same way, but only with water and no animal inside the respiratory chamber. The respiratory chamber was closed, making sure that no air bubbles were present inside the chamber. The O_2-concentration (mg O_2/l) of the water inside the

respiratory chamber was continuously logged (10 min intervals) by the FireStingO2-meter and plotted as curves on the computer by the *Pyro Oxygen Logger* program, until all oxygen within the chamber was consumed by the animal. When the measurement was terminated, the animal inside the chamber was collected and stored in a freezer at –25 °C for later determining of body weight, important for calculating the specific O_2-consumption rate (ml O_2/kg x hour).

To be able to compare the O_2-consumption rate between individuals, it was important that the O_2-consumption rate was as close as possible to the animal standard metabolic rate (SMR). To ensure this, animals were not fed 14 days before the exposures, during the exposures or during the respirometry. The light was switched off at all-time in the room where the respirometry was performed, except for the short periods when new animals were placed into the exposure chambers or the respiratory chambers.

The calculated specific O_2-consumption rate for each individual was plotted against the measured O_2-concentration in the respiratory chamber. The critical O_2-concentration for each individual was determined as the crossing point between two trend lines fitted to the plots, by the packages *caRey* and *respR* in the computer program *R* version 4.0.5. The *pcrit* function in *respR* was used to calculate the rolling rate internally from the time intervals and O_2-concentrations (ml/l).

Statistical analyses

The computer program *R* version 4.0.5 and *Rstudio* version 1.4.1106 were used for all data analysis (R Core Team 2021). For the mortality data obtained in the three mortality experiments, the *packages* survival and *servminer* were used for Kapmlin-Meier estimates and log-rank tests. The mortality data for each experiment were individually fitted for Kapmlin-Meier estimates of the effect of the test media and the water residence time, i.e. row of exposure chambers as the grouping factors. LT_{50}-values were calculated using the function *surv.median.line* in *servminer*. LT_{50} = Lethal Time 50, which means exposure time until 50 % of the exposed group of individuals are dead. Pairwise log-rank tests were then run between the exposure groups (test media) and between the water residence times. Comparisons were made between the four test media (exposure channels), between the water residence time (rows of exposure chambers) within each experimental channel, and between all the water residence times of an experiment (12 rows of exposure channels). Due to no other mixing of

the water within the respiratory chambers in the respirometry experiments, a 10 min interval between measurements was applied. This time interval was needed to detect a steady decrease of O_2-concentration. The data measurements still had some noise (not entirely linear) and were smoothed with *R* package *caRey* before analysis.

The differences between the breakpoints for the critical O_2-concentration between the exposures (test media) and subsequently between the residence times within each exposure (exposure chamber rows) were checked for assumptions for one-way ANOVAs. Normality assumptions were met for the Shapiro-Wilk test for the total dataset of the respirometry experiments ($p = 0.14$) and for the between residence times within each exposure (exposure chamber rows) for acidic Al-rich medium (pH 5.8) and the acidic Al-poor medium (pH 5.8), but not for the experiment with the untreated natural water (pH 7.2). However, boxplots and Q-Q plots did not show substantial violations of normality. The homogeneity of variance assumption was not fully met for the standard one-way ANOVAs and was followed by Welch's ANOVAs. This was to see if there was a significant difference in the results of the two tests, as Welch's ANOVA is better fitted for reduced homogeneity of variance.

The specific O_2-consumption rate was calculated in *R* using the package *FishResp*. In *FishResp*, the background respiration was adjusted for, and the effect of water temperature variation and the individual animal body mass (weight) was integrated into the O_2-consumption rate. Six individuals were removed from the data (five from the untreated natural water, and one from the acidic Al-rich (pH 5.8) medium) as the R-squared for the fitted regression line of O_2-consumption was under r^2 95. The difference in O_2-consumption rate between the exposures (test media) and subsequently between the residence times within each exposure (exposure chamber rows) was checked for assumptions for one-way ANOVA's. One individual (outlier) was removed from the acidic Al-poor water (pH 5.8) exposure data as the body weight value was wrong and could not be corrected. Normality assumptions were met for the Shapiro-Wilk test for the total dataset ($p = 0.16$). This was also the case for the data representing the residence times within the exposures (exposure chamber rows) for the acidic Al-rich medium (pH 5.8) and the acidic Al-poor medium (pH 5.8), but not for the untreated natural water (pH 7.2). Nevertheless, ANOVAs are quite robust in terms of moderate violation of the normality assumption if the sample size is not too small (Agresti & Franklin, 2018; McKillup, 2011). The homogeneity of variance assumption for one-way ANOVAs was not fully met for the O_2-consumption and was therefore followed by Welch's ANOVA.

Results

Water chemistry

The water temperatures during all the experiments were very stable. In the toxicology experiments, water temperature varied between 0.4 and 3.0 ℃, and in the respirometry experiments, between 0.5 and 4 ℃ (Table 4).

Table 4. Water temperature (°C) during the experiments (mean ± SD).

Mortality experiments (all four channels)		Respirometry Experiments (one single channel)	
Experiment 1	1.77 ± 0.79 (n = 49)	**Untreated nat. (pH 7.2)**	1.90 ± 1.18 (n = 50)
Experiment 2	0.97 ± 0.43 (n = 36)	**Acidic Al-rich (pH-5.8)**	1.17 ± 0.38 (n = 18)
Experiment 3	1.26 ± 0.30 (n = 36)	**Acidic Al-poor (pH-5.8)**	3.60 ± 0.95 (n = 19)

The water electrical conductivity was also very stable during the experiments but varied somewhat between the different media due to the chemical additions made to obtain them (Table 5).

Table 5. Water electrical conductivity (µS/cm) in the various exposure media used in the experiments (mean ± SD).

	Experiment 1	**Experiment 2**	**Experiment 3**
Mortality experiments			
Acidic Al-rich (pH 5.8)	28.7 ± 1.6 (n = 49)	29.0 ± 1.9 (n = 36)	30.6 ± 1.2 (n = 36)
Acidic Al-rich (pH 4.8)	34.7 ± 4.4 (n = 25)	37.6 ± 9.4 (n = 20)	34.5 ± 2.2 (n = 13)
Acidic Al-poor (pH 4.8)	32.0 ± 5.2 (n = 37)	32.4 ± 2.5 (n = 9)	35.2 ± 6.3 (n = 7)
Untreated nat. (pH 7.2)	23.4 ± 1.3 (n = 49)	22.7 ± 1.1 (n = 36)	24.4 ± 0.5 (n = 36)
Respirometry experiments			
Untreated nat. (pH 7.2)	24.1 ± 1.1 (n = 50)		
Acidic Al-poor (pH 5.8)		28.3 ± 0.7 (n = 18)	
Acidic Al-rich (pH 5.8)			30.2 ± 1.5 (n = 19)

pH in the untreated natural water (green) was somewhat unstable in the mortality experiments, causing a similar or even higher variation in pH in the various test media made

for the experiments (Figure 7). pH in the untreated natural water (green) varied between 6.8 and 7.9 (n = 121), with the median for each experiment between 7.2 and 7.3. In the acidic Al-rich medium (pH 5.8, red), pH varied between 5.4 and 6.6 (n = 121) during the three mortality experiments, with a median between 5.7 and 6.0 for each experiment. This was fairly close to the nominal pH aimed for in this treatment. In the acidic Al-rich medium (pH 4.8, yellow), pH varied between 4.3 and 5.8 (n = 58) during the mortality experiments, except for the last measurement in experiment 2, where the pH was as low as 3.8. In this medium, however, the nominal pH aimed for was more difficult to obtain, with the median pH for each experiment between 5.1 and 5.3 (Figure 7). In the acidic Al-poor medium (pH 4.8, blue), pH varied between 4.4 and 5.6, (n = 53) with a median between 4.6 and 5.1 for each experiment. This was quite close to the nominal pH aimed for the medium. In experiment 1, there were a couple of outliers we cannot explain, pH 4.1 at day 12 and pH 6.2 at day 13. These values were measured the last two days of the exposure when 96 % of the animals exposed to the acidic Al-poor medium had already died. Therefore, they can be ruled out as being very influential (see mortality results below).

In respirometry experiment 1, water pH in the untreated natural water (green) varied between 6.9 and 7.3 (n = 50, median 7.1, Figure 8). In experiment 2, pH in the acidic Al-poor medium (pH 5.8, black) varied between 5.9 and 6.3 (n = 18) with a median of 6.1. In experiment 3, pH in the acidic Al-rich medium (pH 5.8, red) varied between 6.0 and 6.3 (n = 19) with the median 6.2. In both these acidic media, water pH was somewhat higher than the nominal pH aimed for (5.8) but comparably quite similar (Figure 8).

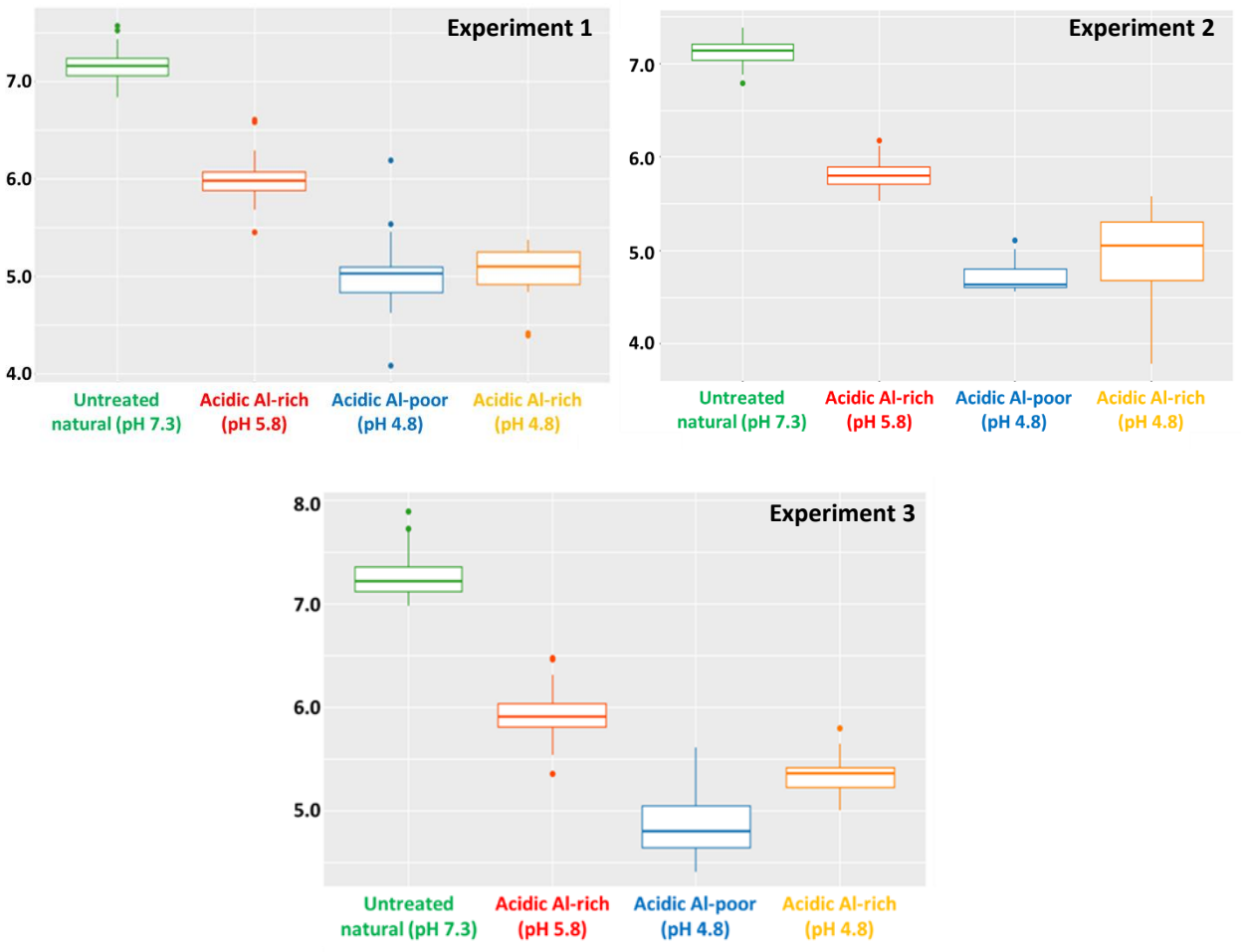

Figure 7. Boxplots of water pH (y-axis) in the four different media used in the mortality experiments, bold line inside the box represent the median, the box represents 50 % of the values, and the vertical lines are the whiskers representing minimum and maximum. Dots are outliers.

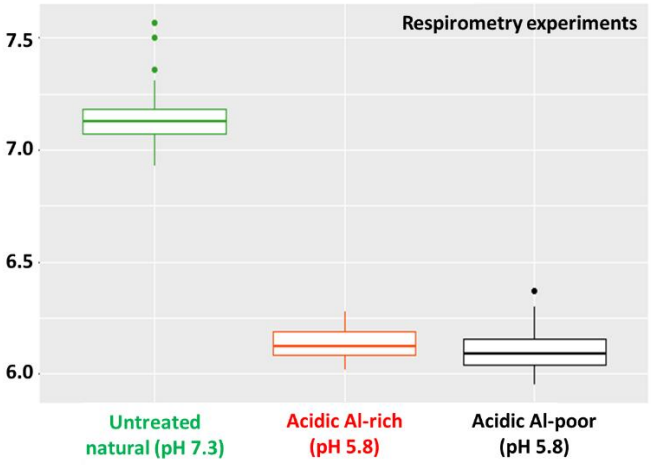

Figure 8. Boxplots of water pH (y-axis) in the three respirometry experiments.

In all three mortality experiments, the concentrations of the various aqueous aluminium fractions were, as expected, much higher in the two Al-rich media compared to the two media where no aluminium was added. In the acidic Al-rich medium (pH 5.8, red), the mean total concentration of aluminium (Alr) varied between 990 and 1346 µg/l during the experimental period (Table 6–8). In the acidic Al-rich medium (pH 4.8, yellow), the mean Alr-concentration varied between 914 and 1098 µg/l. Thus, both media were quite close to the nominal Al-concentration of 1000 µg/l. In the two media where no aluminium was added (acid Al-poor medium and untreated natural water), the Alr-concentration was between 35 and 106 µg/l during the mortality experiments. These values represent the background values, or the natural amount of aluminium present in the operating water.

Table 6. Aluminium fractions measured (µg/l) in the four exposure media used in mortality experiment 1. Se table 3 for an explanation of the Al-fractions. Values are mean ± SD.

		Mortality experiment 1		
		Chamber row 1	Chamber row 2	Chamber row 3
Acidic Al-rich (pH 5.8)	Alr	1346 ± 256 (n = 3)	1133 ± 86 (n = 3)	1229 ± 233 (n = 3)
	Ala	907 ± 136 (n = 3)	767 ± 70 (n = 3)	806 ± 89 (n = 3)
	Alo	547 ± 144 (n = 3)	601 ± 228 (n = 3)	572 ± 208 (n = 3)
	Ali	360 ± 276 (n = 3)	173 ± 184 (n = 3)	243 ± 255 (n = 3)
Acidic Al-rich (pH 4.8)	Alr	1075 ± 178 (n = 2)	1088 ± 212 (n = 2)	1098 ± 199 (n = 2)
	Ala	977 ± 192 (n = 2)	920 ± 199 (n = 2)	875 ± 144 (n = 2)
	Alo	212 ± 109 (n = 2)	222 ± 90 (n = 2)	347 ± 70 (n = 2)
	Ali	765 ± 301 (n = 2)	698 ± 289 (n = 2)	528 ± 74 (n = 2)
Acidic Al-poor (pH 4.8)	Alr	54 ± 42 (n = 2)	47 ± 31 (n = 2)	50 ± 32 (n = 2)
	Ala	2 ± 3 (n = 2)	2 ± 2 (n = 2)	2 ± 3 (n = 2)
	Alo	2 ± 3 (n = 2)	0 ± 1 (n = 2)	1 ± 1 (n = 2)
	Ali	2 ± 3 (n = 2)	2 ± 2 (n = 2)	1 ± 2 (n = 2)
Untreated nat. (pH 7.2)	Alr	70 ± 13 (n = 3)	61 ± 16 (n = 3)	60 ± 15 (n = 3)
	Ala	28 ± 27 (n = 3)	22 ± 22 (n = 3)	23 ± 23 (n = 3)
	Alo	42 ± 53 (n = 3)	32 ± 39 (n = 3)	34 ± 42 (n = 3)
	Ali	1 ± 1 (n = 3)	1 ± 1 (n = 3)	1 ± 2 (n = 3)

The mean Ala-concentration in the acidic Al-rich medium at pH 5.8 (red) during the mortality experiments was somewhat lower than the Alr-concentration, and varied between 767 and

1009 µg/l (Table 6–8). In the acidic Al-rich medium at pH 4.8 (yellow), however, the mean Ala-concentration was almost as high as the Alr-concentration, between 872 and 1000 µg/l. In the two media without Al-addition, the mean Ala-concentration was also lower than the Alr-concentration, between 2 and 59 µg/l. The mean Alo-concentration in acidic Al-rich medium at pH 5.8 (red), varied between 420 and 614 µg/l during the mortality experiments (Table 6–8), and was substantially higher than in the acidic Al-rich medium at pH 4.8 (yellow) where it varied between 123 and 347 µg/l. Consequently, the Ali-concentration was substantially lower in the Al-rich medium at pH 5.8 compared to the Al-rich medium at pH 4.8, between 173 and 439 µg/l and 528 and 877 µg/l, respectively (Table 6–8). Consistent with the fact that the Ala-concentration was low in two media without Al-addition (blue and green), the concentrations of both Alo and Ali were low (Table 6–8).

Table 7. Aluminium fractions measured (µg/l) in the four exposure media used in mortality experiment 2. Se table 3 for an explanation of the Al-fractions. Values are mean ± SD.

		Mortality experiment 2		
		Chamber row 1	Chamber row 2	Chamber row 3
Acidic Al-rich (pH 5.8)	Alr	990 ± 300 (n = 2)	1092 ± 52 (n = 2)	1049 ± 40 (n = 2)
	Ala	820 ± 50 (n = 2)	820 ± 212 (n = 2)	782 ± 98 (n = 2)
	Alo	420 ± 72 (n = 2)	440 ± 9 (n = 2)	465 ± 73 (n = 2)
	Ali	399 ± 122 (n = 2)	381 ± 221 (n = 2)	317 ± 171 (n = 2)
Acidic Al-rich (pH 4.8)	Alr	1069 ± 165 (n = 3)	1085 ± 112 (n = 3)	1059 ± 53 (n = 3)
	Ala	1000 ± 88 (n = 3)	999 ± 100 (n = 3)	971 ± 77 (n = 3)
	Alo	123 ± 46 (n = 3)	125 ± 45 (n = 3)	126 ± 48 (n = 3)
	Ali	877 ± 119 (n = 3)	874 ± 124 (n = 3)	845 ± 144 (n = 3)
Acidic Al-poor (pH 4.8)	Alr	58 ± 9 (n = 2)	44 ± 6 (n = 2)	39 ± 10 (n = 2)
	Ala	18 ± 2 (n = 2)	14 ± 3 (n = 2)	16 ± 4 (n = 2)
	Alo	17 ± 5 (n = 2)	15 ± 3 (n = 2)	15 ± 0 (n = 2)
	Ali	2 ± 2 (n = 2)	2 ± 2 (n = 2)	2 ± 3 (n = 2)
Untreated nat. (pH 7.2)	Alr	66 ± 22 (n = 3)	53 ± 19 (n = 3)	56 ± 15 (n = 3)
	Ala	32 ± 27 (n = 3)	28 ± 25 (n = 3)	26 ± 24 (n = 3)
	Alo	23 ± 2 (n = 2)	17 ± 1 (n = 2)	15 ± 1 (n = 2)
	Ali	0 ± 0 (n = 2)	0 ± 0 (n = 2)	0 ± 0 (n = 2)

There was some variation in the concentration of the different Al-fractions through the channels (between the exposure chamber rows). This is most probably explained by the relatively few numbers of Al-fractionations performed and the fact that the Al-fractionations were performed by me and my co-students having limited experience with the Al-fractionation methodology. The Al-fractionation results did not reveal any gradual decrease in the Ala-fraction, or increase in the Alo-fraction, with the water residence time through the channels with the Al-rich media (red and yellow). Especially in the acidic Al-rich medium at pH 5.8 (red), this could be expected since the Al-chemistry was unstable, favouring Al-polymerization. This can also be explained, at least partly, by our inexperience and low number of Al-fractionations performed.

Table 8. Aluminium fractions measured (µg/l) in the four exposure media used in mortality experiment 3. Se table 3 for an explanation of the Al-fractions. Values are mean ± SD

		Mortality experiment 3		
		Chamber row 1	Chamber row 2	Chamber row 3
Acidic Al-rich (pH 5.8)	Alr	1055 (n = 1)	1174 (n = 1)	1089 (n = 1)
	Ala	951 (n = 1)	1009 (n = 1)	944 (n = 1)
	Alo	614 (n = 1)	573 (n = 1)	505 (n = 1)
	Ali	337 (n = 1)	436 (n = 1)	439 (n = 1)
Acidic Al-rich (pH 4.8)	Alr	997 ± 241 (n = 2)	914 ± 104 (n = 2)	921 ± 81 (n = 2)
	Ala	894 ± 188 (n = 2)	872 ± 102 (n = 2)	881 ± 72 (n = 2)
	Alo	276 ± 77 (n = 2)	284 ± 80 (n = 2)	276 ± 80 (n = 2)
	Ali	617 ± 111 (n = 2)	588 ± 22 (n = 2)	605 ± 7 (n = 2)
Acidic Al-poor (pH 4.8)	Alr	42 ± 11 (n = 2)	35 ± 0 (n = 2)	35 ± 2 (n = 2)
	Ala	37 ± 18 (n = 2)	29 ± 13 (n = 2)	59 ± 54 (n = 2)
	Alo	14 (n = 1)	13 (n = 1)	12 (n = 1)
	Ali	10 (n = 1)	7 (n = 1)	9 (n = 1)
Untreated nat. (pH 7.2)	Alr	58 ± 38 (n = 2)	106 ± 131 (n = 2)	72 ± 16 (n = 2)
	Ala	30 ± 8 (n = 2)	40 ± 33 (n = 2)	24 ± 4 (n = 2)
	Alo	46 ± 17 (n = 2)	34 ± 5 (n = 2)	32 ± 10 (n = 2)
	Ali	0 ± 0 (n = 2)	13 ± 18 (n = 2)	1 ± 2 (n = 2)

During the exposures in the respirometry experiment 3, to the acidic Al-rich medium (pH 5.8, red), the mean Alr-concentration decreased gradually from 2025 to 1314 µg/l with the water

residence time through the channel (Table 9). Similarly, both the mean Ala-concentration and Alo-concentration decreased gradually with the water residence time, from 1137 to 893 µg/l and from 582 to 462 µg/l respectively. The mean Ali-concentration did not show a gradual change with water residence time as the other fractions and varied between 405 and 555 µg/l (Table 9). In this experiment, the acidic Al-rich medium (pH 5.8, red) was not so close to the nominal Al-concentration of 1000 µg/l as it was in the mortality experiments, especially in the initial part of the experimental channel.

In the respirometry experiments 1 and 2, with the acid Al-poor medium (pH 5.8, black) and untreated natural water (pH 7.2, green), the Alr-concentrations, and all other fractions, were low, between 0 and 29 µg/l during the mortality experiments (Table 9). As these values represent the background values of the operating water, they confirm that it was a substantial decrease in dissolved aqueous aluminium with residence time through the channel in respirometry experiment 1.

Table 9. Aluminium fractions measured (µg/l) in the three respirometry experiments. Se table 3 for an explanation of the Al-fractions. Values are mean ± SD.

Respirometry experiments 1-3				
		Chamber row 1	Chamber row 2	Chamber row 3
Untreated nat. (pH 7.2)	Alr	29 ± 18 (n = 3)	17 ± 8 (n = 3)	20 ± 3 (n = 3)
	Ala	9 ± 4 (n = 3)	5 ± 4 (n = 3)	4 ± 2 (n = 3)
	Alo	0 ± 0 (n = 3)	0 ± 0 (n = 3)	0 ± 1 (n = 3)
	Ali	9 ± 4 (n = 3)	5 ± 4 (n = 3)	4 ± 3 (n = 3)
Acidic Al-poor (pH 5.8)	Alr	21 ± 1 (n = 3)	20 ± 1 (n = 3)	21 ± 1 (n = 3)
	Ala	4 ± 3 (n = 3)	4 ± 2 (n = 3)	6 ± 2 (n = 3)
	Alo	1 ± 1 (n = 3)	1 ± 1 (n = 3)	3 ± 1 (n = 3)
	Ali	3 ± 4 (n = 3)	3 ± 3 (n = 3)	3 ± 3 (n = 3)
Acidic Al-rich (pH 5.8)	Alr	2025 ± 656 (n = 3)	1466 ± 591 (n = 3)	1314 ± 197 (n = 3)
	Ala	1137 ± 277 (n = 3)	899 ± 173 (n = 3)	893 ± 180 (n = 3)
	Alo	582 ± 165 (n = 3)	495 ± 168 (n = 3)	462 ± 169 (n = 3)
	Ali	555 ± 113 (n = 3)	405 ± 51 (n = 3)	431 ± 174 (n = 3)

Mortality

In mortality experiment 1, mortality of *G. lacustris* exposed to all four media was observed (Figure 9), and there was a statistically significant difference in the mortality between the different media (Table 10). It was only in the two pH 4.8 media (yellow and blue) that all animals died (100 % mortality) during the exposure time, and the mortality in these two media was not statistically different (p = 0.099). In the acidic Al-rich medium at pH 5.8 (red) and in the untreated natural water at pH 7.2 (green), only two animals died (8 % mortality) during the experiment, and they showed no statistical difference between them (p = 0.999).

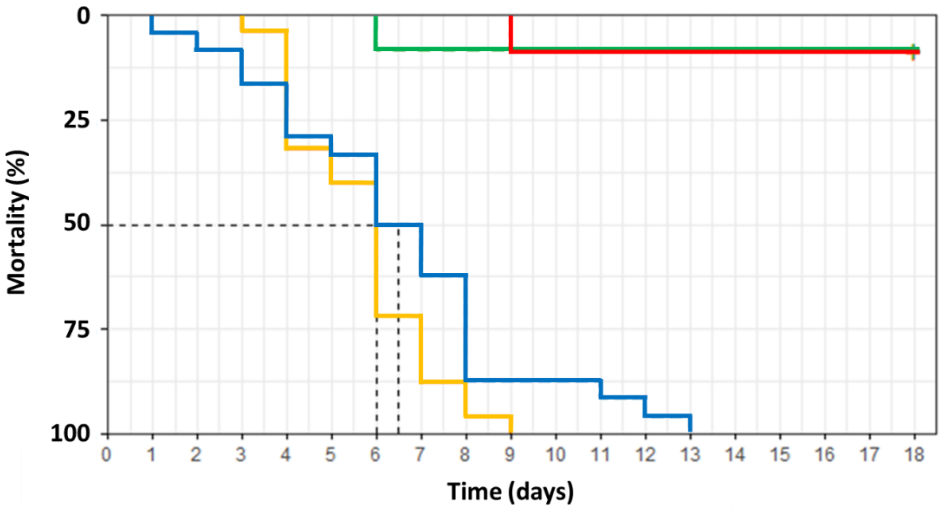

Figure 9. Accumulated mortality of *Gammarus lacustris* in mortality experiment 1. Acidic Al-rich medium at pH 5.8 (red), acidic A-rich medium at pH 4.8 (yellow), acidic Al-poor medium at pH 4.8 (blue) and untreated natural water at pH 7.2 (green). Dotted lines indicate LT_{50}-values.

Table 10. p-values from log-rank test comparing the mortality of *Gammarus lacustris* exposed to the four test media in mortality experiment 1. Statistically significant differences are shown in bold.

	Acidic Al-rich (pH 4.8)	Acidic Al-rich (pH 5.8)	Acidic Al-poor (pH 4.8)
Acidic Al-rich (pH 5.8)	**0.001**		
Acidic Al-poor (pH 4.8)	0.099	**0.001**	
Untreated nat. (pH 7.2)	**0.001**	0.999	**0.001**

Acidic Al-rich (pH 5.8) n = 24, acidic Al-rich (pH 4.8) n = 25, acidic Al-poor (pH 4.8) n = 24, Untreated natural water (pH 7.2) n = 24.

The mortality was slightly faster in the acidic Al-rich medium at pH 4.8 (yellow), 100 % mortality after 9 days of exposure and LT_{50} = 6 days, compared to the acidic Al-poor medium at pH 4.8 (blue), 100 % after 12 days and LT_{50} = 6.5 days (Figure 9). No statistically significant difference in mortality was found between the three water residence times within each of the four experimental channels (Table 11).

Table 11. Comparison of the mortality of *Gammarus lacustris* at the three different water residence times (exposure chamber rows) within each experimental channel in mortality experiment 1. Statistically significant differences are shown in bold.

	P-value (log-rank test)
Acidic Al-rich (pH 5.8)	0.59
Acidic Al-rich (pH 4.8)	0.66
Acidic Al-poor (pH 4.8)	0.078
Untreated nat. (pH 7.2)	0.12

For all groups, n = 8, except for the third row of exposure chambers in the acidic Al-rich medium (pH 4.8) where n = 9.

In mortality experiment 2, mortality of *G. lacustris* exposed to the acidic Al-rich media at pH 5.8 and 4.8 (red and yellow) and to the acidic Al-poor medium at pH 4.8 (blue) was observed, but not in untreated natural water at pH 7.2 (green) (Figure 10). In this experiment, it was a statistically significant difference in mortality between all four media (Table 12). As in mortality experiment 1, it was only in the two pH 4.8 media (yellow and blue) that all animals died (100 % mortality) during the exposure time in experiment 2. In the acidic Al-rich medium at pH 5.8 (red), the mortality was 67 % after 18 days when the experiment was terminated, while there was no mortality in the untreated natural water at pH 7.2 (green).

The mortality was faster in the acidic Al-poor medium at pH 4.8 (blue), 100 % mortality after 5 days of exposure and LT_{50} = 2 days, compared to in acidic Al-rich medium at pH 4.8 (yellow), 100 % after 10 days and LT_{50} = 4 days (Figure 10). In the acidic Al-rich medium at pH 5.8 (red), LT_{50} was 17 days. No statistically significant difference in mortality was found between the three water residence times within each of the four experimental channels (Table 13).

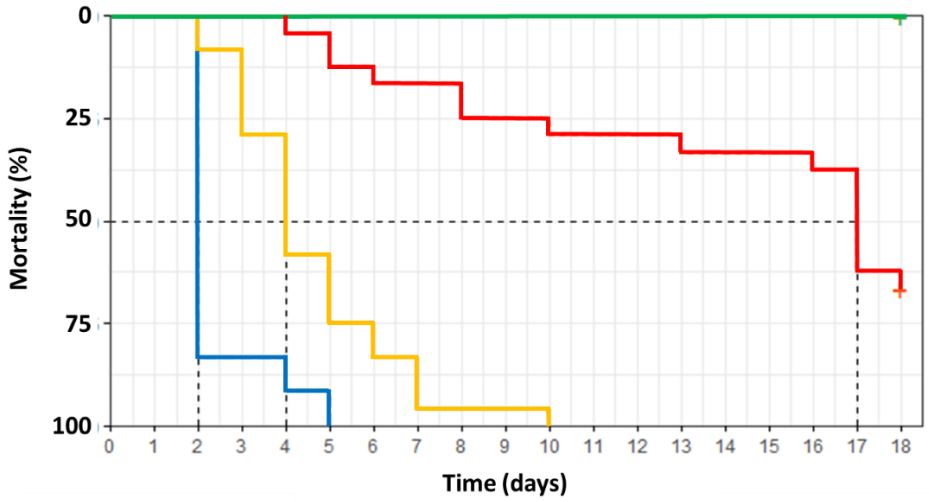

Figure 10. Accumulated mortality of *Gammarus lacustris* in mortality experiment 2. Acidic Al-rich medium at pH 5.8 (red), acidic A-rich medium at pH 4.8 (yellow), acidic Al-poor medium at pH 4.8 (blue) and untreated natural water at pH 7.2 (green). Dotted lines indicate LT_{50}-values.

Table 12. p-values from log-rank test comparing the mortality of *Gammarus lacustris* exposed to the four test media in mortality experiment 2. Statistically significant differences are shown in bold.

	Acidic Al-rich (pH 4.8)	Acidic Al-rich (pH 5.8)	Acidic Al-poor (pH 4.8)
Acidic Al-rich (pH 5.8)	**0.001**		
Acidic Al-poor (pH 4.8)	**0.001**	**0.001**	
Untreated nat. (pH 7.2)	**0.001**	**0.001**	**0.001**

For all four media n = 24.

Table 13. Comparison of the mortality of *Gammarus lacustris* at the three different water residence times (exposure chamber rows) within each experimental channel in mortality experiment 2. Statistically significant differences are shown in bold.

	P-value (log-rank test)
Acidic Al-rich (pH 5.8)	0.069
Acidic Al-rich (pH 4.8)	0.24
Acidic Al-poor (pH 4.8)	0.18
Untreated nat. (pH 7.2)	1

For all groups, n = 8.

In mortality experiment 3, mortality of *G. lacustris* was observed in all media (Figure 11), and there was a statistically significant difference in the mortality between the different media (Table 14). As in the previous two experiments, it was only in the two media at pH 4.8 that all animals died during mortality experiment 3. In the acidic Al-rich medium at pH 5.8 (red), the mortality was 63 % after 18 days when the experiment was terminated, while there was 8 % mortality in the untreated natural water at pH 7.2 (green).

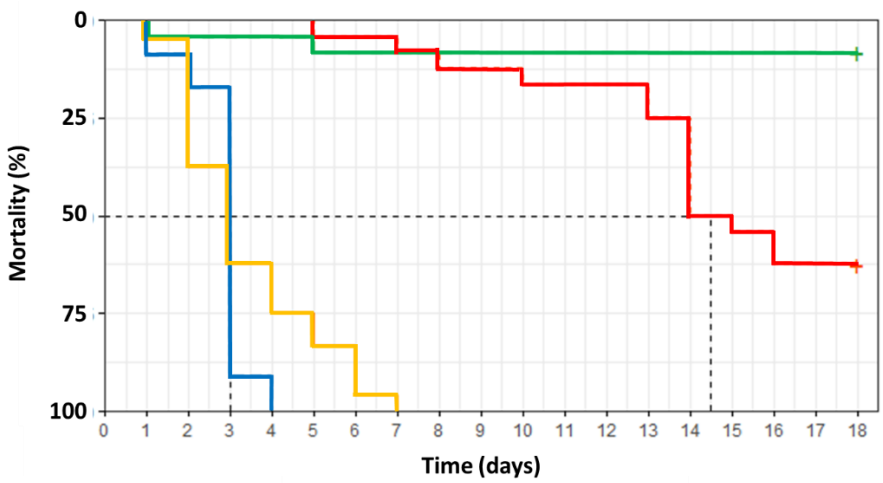

Figure 11. Accumulated mortality of *Gammarus lacustris* in mortality experiment 3. Acidic Al-rich medium at pH 5.8 (red), acidic A-rich medium at pH 4.8 (yellow), acidic Al-poor medium at pH 4.8 (blue) and untreated natural water at pH 7.2 (green). Dotted lines indicate LT_{50}-values.

Table 14. p-values from log-rank test comparing the mortality of *Gammarus lacustris* exposed to the four test media in mortality experiment 3. Statistically significant differences are shown in bold.

	Acidic Al-rich (pH 4.8)	Acidic Al-rich (pH 5.8)	Acidic Al-poor (pH 4.8)
Acidic Al-rich (pH 5.8)	**0.001**		
Acidic Al-poor (pH 4.8)	0.181	**0.001**	
Untreated nat. (pH 7.2)	**0.001**	**0.001**	**0.001**

Acidic Al-rich (pH 5.8) n = 24, acidic Al-rich (pH 4.8) n = 25, acidic Al-poor (pH 4.8) n = 23, Untreated natural water (pH 7.2) n = 24.

The mortality was faster in the acidic Al-poor medium at pH 4.8 (blue), 100 % mortality after 4 days of exposure and LT_{50} = 3 days, compared to in acidic Al-rich medium at pH 4.8 (yellow), 100 % after 7 days and LT_{50} = 3 days (Figure 11). This difference, however, was not

statistically significant (p = 0.181). In the acidic Al-rich medium at pH 5.8 (red), LT_{50} was 14 days, and the mortality was statistically significantly different from both the media at pH 4.8 (yellow and blue) (p = 0.001), and compared with the untreated natural water at pH 7.2 (green) (p = 0.001).

Table 15. Comparison of the mortality of *Gammarus lacustris* at the three different water residence times (exposure chamber rows) within each experimental channel in mortality experiment 3. Statistically significant differences are shown in bold.

	P-value (log-rank test)
Acidic Al-rich (pH 5.8)	**0.0029**
Acidic Al-rich (pH 4.8)	**0.03**
Acidic Al-poor (pH 4.8)	0.34
Untreated nat. (pH 7.2)	0.12

For all groups, n = 8, except for the third row of exposure chambers in the acidic Al-rich medium (pH 4.8) where n = 9, and the second row in the acidic Al-poor medium (pH 4.8) where n = 7.

In mortality experiment 3 there was a statistically significant difference in mortality between the three water residence times within the two experimental channels with Al-rich media (Table 15). In the acidic Al-rich medium at pH 5.8 (red), the highest mortality was observed in the first part of the channel, 100 % after 18 days and LT_{50} = 13.5 days. The mortality gradually decreased through the channel (Figure 12A) and was 63 % after 18 days and LT_{50} = 14.5 days in the middle of the channel, and 25 % after 18 days at the end of the channel. In the acidic Al-rich medium at pH 4.8 (yellow), there was 100 % mortality throughout the channel, but LT_{50} gradually increased through it; LT_{50} = 2 days in the initial part, LT_{50} = 3 days in the middle, and LT_{50} = 5 days at the end of the channel (Figure 12B).

In mortality experiment 2, the same gradual decrease mortality through the channel with the acidic Al-rich medium at pH 5.8 (red) was observed, but the difference between the water residence times within the channel was not statistically significant (p = 0.069, Table 13). The mortality was 88 % and LT_{50} = 8 days in the initial part, 63 % and LT_{50} = 17 in the middle, and 50 % and LT_{50} = 17.5 days at the end of the channel (Figure 12C).

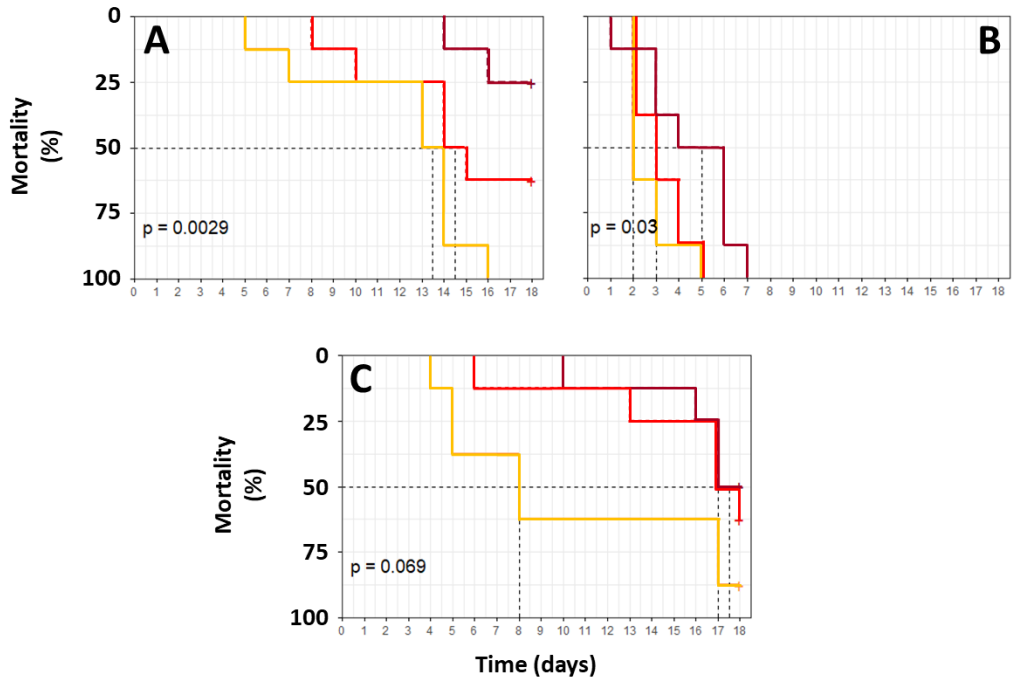

Figure 12. Accumulated mortality of *Gammarus lacustris* within experimental channels according to the water residence time of the water. **Yellow line:** 45 sec residence time (first row of exposure chambers), **red line:** 10 min residence time (second row of exposure chambers) and **deep red line**: 20 min residence time (third row of exposure chambers). **A:** acidic Al-rich medium at pH 5.8 in mortality experiment 3, **B:** acidic A-rich medium at pH 4.8 in mortality experiment 3, and **C:** Al-rich medium at pH 5.8 in mortality experiment 2. Dotted lines indicate LT_{50}-values.

Respirometry

G. lacustris exposed to the acidic Al-rich medium (pH 5.8) showed a mean normoxic O_2-consumption of 99.9 ± (SD) 41.0 mg O_2/kg x hour (n = 35). In comparison, the normoxic O_2-consumption in animals exposed to the acidic Al-poor medium (pH 5.8) was 79.6 ± (SD) 29.5 mg O_2/kg x hour (n = 35) and to the untreated natural water (pH 7.2) it was 52.8 ± (SD) 21.4 mg O_2/kg x hour (n = 31) (Figure 13). There were statistically significant differences in normoxic O_2-consumption in animals exposed to the three media ($F_{2, 98} = 17.78$, $p < 0.001$, generalized eta squared = 0.26). Tukey post-hoc analyses revealed that the difference in O_2-consumption between animals exposed to the two media at pH 5.8 (red and black) ($p = 0.017$), the acidic Al-rich medium and the untreated natural water (red and green) ($p < 0.001$), and the acidic Al-poor medium and the untreated natural water (black and green) ($p = 0.004$) were all statistically significant (Table 16). The Welch's ANOVA and Games-Howell post-hoc tests did not significantly differ from the normal ANOVA and Tukey post-hoc analyses.

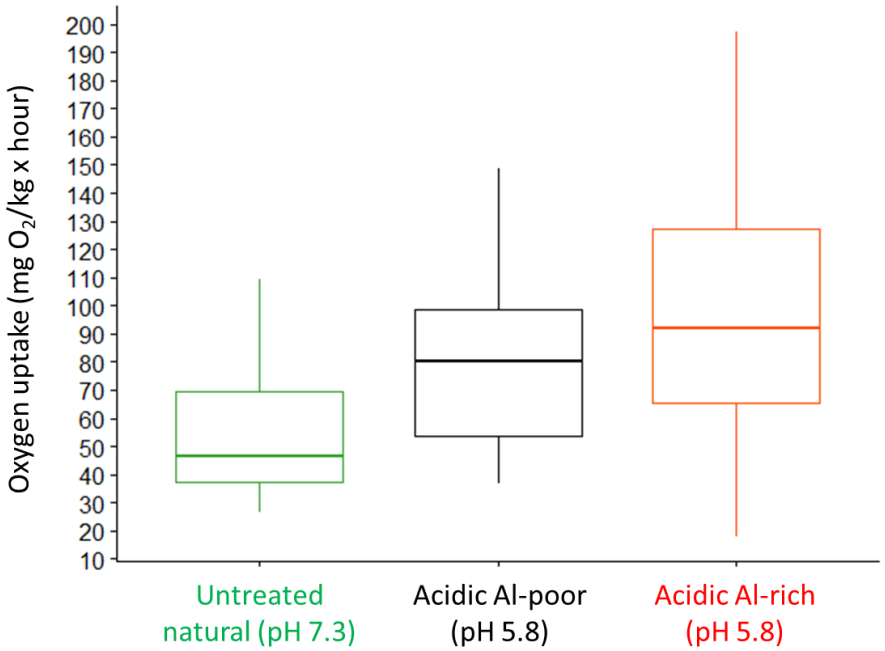

Figure 13. Boxplots of normoxic O_2-uptake *Gammarus lacustris* exposed to three different media (x-axis) in the respirometry experiments, bold line inside the box represents the median, the box represents 50 % of the values, and the vertical lines are the whiskers representing minimum and maximum.

Table 16. Results from the Tukey post-hoc analyses comparing the normoxic O_2-consumption (V_{O2}) and critical O_2-concentration ($[O_2]_{crit}$) in *Gammarus lacustris* exposed to the three different media in the respirometry experiment. Showen with the mean ± the confidence interval and statistically significant differences indicated by asterisks: $p < 0.001$***, $p < 0.01$** and $p < 0.05$*.

Comparison	**V_{O2} (mg O_2/kg x hour)**	**$[O_2]_{crit}$ (mg O_2/l)**
Acidic Al-rich (pH 5.8) Untreated nat. (pH 7.2)	99.9 ± 14.1 / 52.8 ± 7.9 ***	2.89 ± 0.32 / 1.71 ± 0.18 ***
Untreated nat. (pH 7.2) Acidic Al-poor (pH 5.8)	52.8 ± 7.9 / 78.6 ± 10.1 **	1.71 ± 0.18 / 1.86 ± 0.19
Acidic Al-rich (pH 5.8) Acidic Al-poor (pH 5.8)	99.9 ± 14.1 / 78.6 ± 10.1 *	2.89 ± 0.32 / 1.86 ± 0.19 ***

Animals exposed to the acidic Al-rich medium (pH 5.8) showed a mean critical O_2-concentration of 2.89 ± (SD) 0.96 mg O_2/l (n = 36). In comparison, the critical O_2-concentration in animals exposed to the acidic Al-poor medium (pH 5.8) was 1.86 ± (SD) 0.57 mg O_2/l (n = 36) and to the untreated natural water (pH 7.2) it was 1.71 ± (SD) 0.54 mg

O_2/l (n = 35) (Figure 14). There were statistically significant differences in the critical O_2-concentration in animals exposed to the three media ($F_{2, 105}$ = 29.537, p < 0.001, generalized eta squared = 0.36). Tukey post-hoc analyses revealed that the critical O_2-concentration in animals exposed to the acidic Al-rich medium (red) was statistically significantly different from both the untreated natural water (green) (p < 0.001), and the acidic Al-poor medium (black) (p < 0.001) (Table 16). There was no statistically significant difference in critical O_2-concentration between the animals exposed to the acidic Al-poor medium and the untreated natural water (p = 0.65). The Welch's ANOVA and Games-Howell post-hoc tests did not differ significantly from the normal ANOVA and Tukey post-hoc analyses.

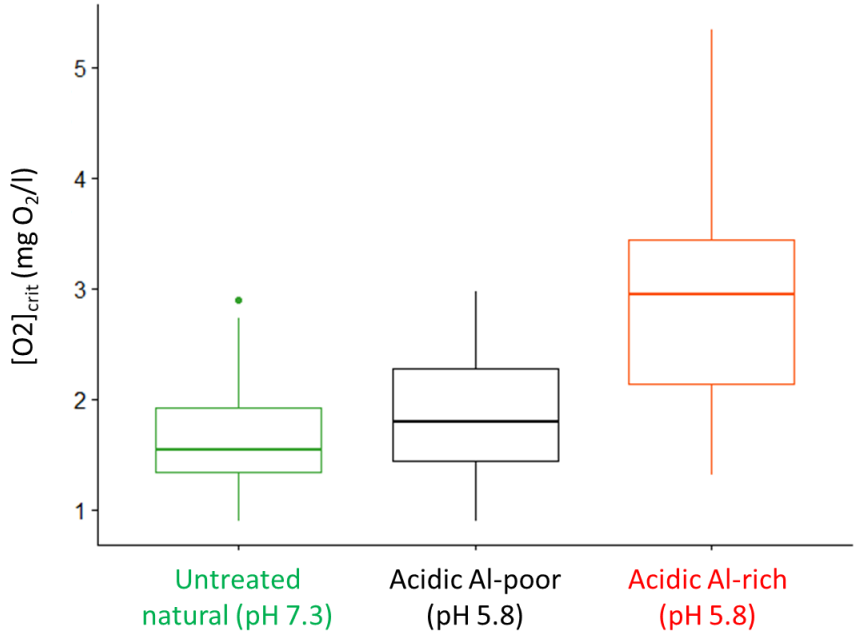

Figure 14. Boxplots of critical O_2-concentration for *Gammarus lacustris* exposed to three different media (x-axis) in the respirometry experiments, bold line inside the box represents the median, the box represents 50 % of the values, and the vertical lines are the whiskers representing minimum and maximum.

Comparing the normoxic O_2-consumption and critical O_2-concentration in *G. lacustris* exposed at different water residence times within the three experimental channels revealed that there was very little difference (Table 17). There were no statistically significant differences in normoxic O_2-consumption within each of the three exposure channels (one-way ANOVA and Welsh's ANOVA). The same was the case for the critical O_2-concentration in the two acidic media (pH 5.8). In the control channel with the untreated natural water (pH

7.2), however, there was a statistically significant difference ($F_{2, 33} = 6.75$, $p = 0.003$, generalized eta squared = 0.29). Tukey post-hoc analyses revealed that the difference in critical O_2-concentration was statistically significant between animals exposed in the initial part (45 sec residence time) and in the middle (10 min residence time) of the channel ($p = 0.002$) (Table 17). The Welch's ANOVA and Games-Howell post-hoc tests did not significantly differ from the normal ANOVA and Tukey post-hoc analyses.

Table 17. Results from the Tukey post-hoc analyses comparing the normoxic O_2-consumption (V_{O2}) and critical O_2-concentration ($[O_2]_{crit}$) in *Gammarus lacustris* exposed at different water residence time within each experimental channel in the respirometry experiment. Showen with the mean ± the confidence interval and statistically significant differences are indicated by asterisks: $p < 0.001$***, $p < 0.01$** and $p < 0.05$*.

Comparison	**V_{O2} (mg O_2/kg x hour)**	**$[O_2]_{crit}$ (mg O_2/l)**
Acidic Al-rich (pH 5.8)		
Row 1 / Row 2	90.1 ± 35.9 (n=11) / 101.0 ± 32.7 (n=12)	3.02 ± 1.18 (n=12) / 2.38 ± 0.72 (n=12)
Row 1 / Row 3	90.1 ± 35.9 (n=11) / 108.0 ± 52.9 (n=12)	3.02 ± 1.18 (n=12) / 3.28 ± 0.73 (n=12)
Row 2 / Row 3	101.0 ± 32.7 (n=12) / 108.0 ± 52.9 (n=12)	2.38 ± 0.72 (n=12) / 3.28 ± 0.73 (n=12)
Untreated natural water (pH 7.2)		
Row 1 / Row 2	61.1 ± 23.5 (n=12) / 42.5 ± 20.2 (n=10)	2.08 ± 0.70 (n=12) / 1.39 ± 0.25 (n=12) **
Row 1 / Row 3	61.1 ± 23.5 (n=12) / 53.4 ± 16.2 (n=9)	2.08 ± 0.70 (n=12) / 1.66 ± 0.31 (n=12)
Row 2 / Row 3	42.5 ± 20.2 (n=10) / 53.4 ± 16.2 (n=9)	1.39 ± 0.25 (n=12) / 1.66 ± 0.31 (n=12)
Acidic Al-poor (pH 5.8)		
Row 1 / Row 2	68.6 ± 22.1 (n=12) / 74.3 ± 27.2 (n=12)	1.78 ± 0.50 (n=12) / 1.84 ± 0.65 (n=12)
Row 1 / Row 3	68.6 ± 22.1 (n=12) / 104.0 ± 48.3 (n=12)	1.78 ± 0.50 (n=12) / 1.95 ± 0.58 (n=12)
Row 2 / Row 3	74.3 ± 27.2 (n=12) / 104.0 ± 48.3 (n=12)	1.84 ± 0.65 (n=12) / 1.95 ± 0.58 (n=12)

Discussion

Al-chemistry

The Ala-concentration in the Al-rich medium at pH 5.8, accounted for a smaller proportion of the total amount of aluminium in the water (Alr-concentration) compared to the Al-rich medium at pH 4.8, at least in mortality experiment 1 and 2 (Table 6–8). This indicates that a more significant proportion of aluminium was present as large polymeric forms at pH 5.8 than at pH 4.8 and can be explained by more unstable chemical conditions for aluminium at pH 5.8 than at pH 4.8, favouring Al-polymerization. Furthermore, the Alo-concentration in the Al-rich medium at pH 5.8 was much higher than in the Al-rich medium at 4.8. This can be explained by a higher degree of ongoing Al-polymerization at pH 5.8 compared to pH 4.8, and is supported by previous studies in which Al-rich water under unstable conditions has been artificially made (Poleo & Hytterød, 2003; Poléo & Bjerkely, 2000; Poléo et al., 1994). When the simple monomeric Al-forms present in solution at pH 5.8 start to polymerize in the initial part of the experimental channel, small polymeric Al-forms (dimers, trimers, etc.) will first be formed. These small polymers continue to grow into large Al-polymers as the water residence time increases (Lydersen et al., 1991). The small Al polymers are extractable within 20 seconds but pass through the cation exchanger because their net charge has begun to approach zero and are analyzed as Alo, even though they are inorganic (Hem & Roberson, 1967; Lydersen et al., 1994). I, therefore, have support for the assumption that *G. lacustris* was exposed to ongoing Al-polymerization when exposed to the Al-rich medium at pH 5.8.

However, it cannot be ruled out that there were some Al-polymerization going on in the Al-rich medium at pH 4.8 as well. The Alo-concentration in this medium was about four times higher than the total concentration of aluminium (Alr) in the Al-poor medium at pH 4.8 (Table 6–8). Because pH of the Al-stock solution added to the operating water was 2.0 or lower, all aluminium was exclusively present as Al^{3+} before it was added (Hem & Roberson, 1967; Lydersen, 1990). Therefore, the Alo-fraction in the Al-rich medium at pH 4.8 has to originate from the stock solution and not the background aluminium present in the untreated operating water. In the same way, as for the Al-rich medium at 5.8, the small Al-polymers in the Al-rich medium at 4.8 pass through the ion exchanger and are analyzed as Alo. Nevertheless, since the Ali-fraction in the Al-rich medium at pH 4.8 was very high, between 528 and 877 µg/l, compared to the Al-rich medium at pH 5.8, between 173 and 439 µg/l (Table 6–8), it is reasonable to believe that *G. lacustris* was exposed to high concentrations of

monomeric inorganic aluminium, primarily Al^{3+} when exposed to the Al-rich medium at pH 4.8.

The results also show quite clearly that *G. lacustris* exposed to the Al-poor media at pH 4.8 and 5.8, as well as the untreated natural water at pH 7.2, was exposed to low concentrations of aqueous aluminium (Table 6–8), and in particular low Ali-concentrations that were below the detection limit of 13 µg/l reported by Vogt et al. (1994). This is relevant because Ali is often considered the most toxic Al-fraction, see review by Gensemer & Playle (1999).

Toxicity of aqueous aluminium to *Gammarus lacustris*?

The results from the mortality experiments clearly show that water with pH 4.8 is highly toxic for *G. lacustris*, regardless of the presence of aqueous aluminium or not (Figure 9–11). When it comes to the mortality observed in *G. lacustris* exposed to aluminium at pH 5.8, it is not possible to say if this is caused by the reduced pH from 7.2 to 5.8, the elevated concentration of aqueous aluminium from 35–106 µg/l to 990–1346 µg/l (Table 6–8), or both, since no mortality experiment was performed with the Al-poor medium at pH 5.8. The respirometry experiments, on the other hand, revealed that *G. lacustris* exposed to aluminium at pH 5.8 showed significantly higher normoxic O_2-consumption and elevated critical O_2-concentration compared to those exposed to the Al-poor medium at the same pH (Table 16).

This indicates that aqueous aluminium is toxic to *G. lacustris*. Normoxic O_2-consumption and critical O_2-concentration, however, was also significantly higher at pH 5.8 than at pH 7.2 when only background levels of aluminium were present in the water (Table 16). This supports the results from the mortality experiments, clearly showing that reduced pH in the water is toxic for *G. lacustris* per se, but it adds to this that elevated concentrations of aqueous aluminium increases the toxicity of acidified water to *G. lacustris*, at least at pH around 5.8. This is important and of relevance, because freshwater acidification by acid rain most often cause drops in water pH down to between 5.5 and 5.8, and not as low as 4.8.

Since I have not been able to find any other studies investigating the effects of aluminium on *G. lacustris*, my results appear to be the first evidence that aqueous aluminium has little or no significance for the toxicity of acidic water at pH 4.8, but increases the toxicity of acidic water at pH 5.8. In mortality experiment 2 and 3, *G. lacustris* died faster in the Al-poor medium than in the Al-rich medium at pH 4.8, while it was the other way around in mortality experiment 1 (Figure 9–11). The differences in mortality, however, were minor, and only in

experiment 2 was its significantly faster mortality in the Al-poor medium compared to the Al-rich medium (Table 12). I, therefore, have no evidence that high concentrations of aqueous aluminium contribute to increased toxicity of the water at pH 4.8. On the other hand, it cannot be ruled out that aluminium have a weak counteracting effect on mortality. This effect, however, is so minor that my conclusion remains that aqueous aluminium is of no importance for the toxicity of acidified water at pH 4.8 in *G. lacustris*.

Previous studies with fish have shown that aluminium is more toxic at pH around 5.8 than at around pH 4.8 (Muniz & Leivestad, 1980; Poléo et al., 1994). The present study gives no evidence that this is the case with *G. lacustris*. On the contrary, as already mentioned, the water at pH 4.8, regardless of the Al-content, was more toxic to *G. lacustris* than water at pH 5.8. This indicates that the mechanism of Al-toxicity in *G. lacustris* is very different from what is found in fish.

A review of the literature reveals that there are many studies that deal with the effects of acidic Al-rich water on a number of other invertebrate species, for example: (Berrill et al., 1985; Burton & Allan, 1986; Gensemer & Playle, 1999; Havas & Likens, 1985; Havas & Rosseland, 1995; Herrmann & Andersson, 1986; Mackie, 1989; McCahon & Poulton, 1991; Ormerod et al., 1987; Storey et al., 1992). A problem with several of these studies is that they are performed on field data, and only to a limited extent distinguish between low pH and aqueous aluminium when addressing the cause of the effects reported. One exception, however, is a study by Burton & Allan (1986), who showed experimentally that a reduction in pH from 6.8–7.2 to 4.0 led to about 50 % mortality in four different species of invertebrates during 28 days of exposure. Similar to the present study, low pH led to mortality. The mortality, however, was substantially lower in the four species tested than in *G. lacustris* at pH 4.8 in my experiments (100 %), even though pH was lower and the exposure time 10 days longer in the study by Burton & Allen (1986). Furthermore, they found that mortality increased to around 80 % for three of the species tested when 500 µg Al/l was added to the water. One of these species was the crustacean isopod *Asellus intermedius*. Thus, their results do not correspond to mine, showing that the mortality in *G. lacustris* was similar at pH 4.8 if aluminium was added or not. Also, despite the fact that twice as much aluminium (1000 µg/l) was added in the present study compared to the study by Burton & Allan (1986). This strongly supports the conclusion that *G. lacustris* is much more sensitive to low pH compared to several other invertebrates, and that high concentrations of aluminium in the water have a minor effect on the toxicity at pH 4.8. This high sensitivity is further supported by another

experiment conducted by Burton & Allan (1986), in which they reduced the water pH to 5.0 and added 250 µg Al/l. This exposure had no effect on the four species tested. In my study, the pH was only slightly lower (4.8), but led to 100 % mortality of *G. lacustris* in all three mortality experiments.

The results from my study give evidence that *G. lacustris* not only is very sensitive to acidic water compared to other invertebrates, but also compared to other crustaceans in particular. Burton and Allan (1986) have, as already mentioned, shown that the isopod *A. intermedius* is substantially more tolerant to low water pH compared to *G. lacustris* in my study. Storey et al. (1992) studied the closely related amphipod *G. pulex* and show that this species is also substantially more tolerant of acidic water than *G. lacustris*. They found no mortality of *G. pulex* during 7 days of exposure to various combinations of pH (4.5–6.9) and Al-concentrations (0–1000 µg/l). In my experiments, *G. lacustris* exposed to the two media at pH 4.8 showed a mortality between 63 and 100 % after 7 days. Eventually, Storey et al. (1992) observed between 70 and 100 % mortality within 7 days of *G. pulex* when water pH was lowered to 4.0. They also found that adding aluminium in the acidic water increased mortality somewhat, which is also contrary to the results of the present study.

A study by McCahon & Poulton (1991) is more in line with the results from the present study. They reported between 70 and 90 % mortality in *G. pulex* after 6 days exposure to acidic Al-rich water in which pH was lowered from 7.2 to about 5.0 and added 570–940 µg Al/l. Furthermore, Ormerod et al. (1987) reported that *G. pulex* exposed to acidic Al-rich water at pH 5.0 and 400 µg Al/l, showed 20 % mortality after the 3 days of exposure. They also observed that Al-poor water at pH 4.5 had exactly the same effect. This is similar to the mortality in the acidic Al-poor medium (pH 4.8) observed in mortality experiment 1, but lower than in experiment 2 and 3, where the mortality of *G. lacustris* after 3 days was 83 and 87 %, respectively (Figure 9–11).

It has been suggested that aluminium may have an ameliorating effect on injuries in invertebrates exposed to acidic water (Havas & Likens, 1985; Havens, 1993). The present study gives somewhat room for speculating if this might be the case for *G. lacustris* as well, but the results are contradictory. In mortality experiment 1, there was a non-significant faster mortality in the Al-rich medium than in the Al-poor medium at pH 4.8 (Table 10). In mortality experiment 2, however, there was a significantly faster mortality in the Al-poor medium than in the Al-rich medium pH 4.8 (Table 12). This was also the case in mortality experiment 3, but the difference was not statistically significant (Table 14). Since these

differences in two out of three cases were not statistically significant, more data is needed to conclude that aluminium has a protective effect on *G. lacustris* at pH 4.8.

The overall conclusion to the first research question is that aqueous aluminium is toxic to *G. lacustris*. However, the species is far more sensitive to acidity, i.e. reduced pH, than to aluminium. Following this, the answer to the second research question is that aqueous aluminium is not the main cause of the previously reported high sensitivity to freshwater acidification in *G. lacustris.* The species also seems to be more sensitive to acidified water than other invertebrates, including other freshwater crustaceans. Accordingly, *G. lacustris* is not found in lakes with a pH lower than 6.0 regardless if influenced by acid rain or not (Økland & Økland, 1985). The extent of the toxicity of aqueous aluminium to *G. lacustris* at still unclear since no mortality experiment was performed with the acidic Al-poor medium at pH 5.8.

The importance of Al-polymerization for the Al-toxicity in *G. lacustris*

As already discussed, the Al-fractionation and analyses have confirmed that the acidic Al-rich medium at pH 5.8 represented unstable chemical conditions in which dissolved aluminium showed the tendency of polymerizing. On the other hand, it is not possible to say if the mortality observed in *G. lacustris* exposed to aluminium at pH 5.8 was caused by the reduced pH or the elevated concentration of aqueous aluminium since no mortality experiment was performed with the Al-poor medium at pH 5.8. This makes it difficult to discuss if Al-polymerization is important for the Al-toxicity or not. But, if I turn the question around and ask whether the degree of Al-polymerization can be linked to a possible toxicity of aluminium, it is easier to discuss and give some answers.

The *G. lacustris* was exposed to ongoing Al-polymerization in the Al-rich medium at pH 5.8, and the Al-fractionations and analyses also revealed that to a limited extent, some Al-polymerization also took place in the Al-rich medium at pH 4.8. In mortality experiment 1, almost no mortality of *G. lacustris* was observed in the Al-rich medium at pH 5.8, but in the two other mortality experiments, the mortality was highest in the first row of exposure chambers, and gradually decreased through the red channel (Figure 12, Table 13 and 15). These observations are in good agreement with previous studies where fish have been exposed to ongoing Al-polymerization (Poleo & Hytterød, 2003; Poléo & Bjerkely, 2000; Poléo et al., 1994). In these studies, mortality as well as anatomical and physiological damage

in surviving fish, decreased with the residence time of the water. This is explained by Al-polymers growing as water residence time increase, and that the Al-polymers approach a net zero charge as they grow larger (Hem & Roberson, 1967). Furthermore, that their ability to bind to biological surfaces decreases (Poléo, 1995).

In mortality experiment 3, it was also found a decreasing mortality rate in *G. lacustris* exposed to the Al-rich medium at 4.8 through the experimental channel (Figure 12, Table 15). This may be due to some Al-polymerization taking place even under more stable chemical conditions, indicated by an increased Alo-fraction in this medium (Table 6–8). Thus, it is possible that aluminium can have an effect on mortality also at pH 4.8, but that this is masked by the strong effect of the acidity, and therefore does not become detectable in my results.

The fact that the mortality of *G. lacustris* can be linked to the degree of ongoing Al-polymerization suggests that aluminium has an effect on the toxicity of acidified water, even if it seems to be of lesser or no importance at pH as low as 4.8. Fjeld et al. (1988) observed extensive accumulation of aluminium and damage to the gills of the noble crayfish (*Astacus astacus*) when acidic Al-rich water was neutralized by the addition of lime slurry, i.e. causing Al-polymerization to take place in the water. They had no good explanation, however, for why the toxicity increased when the pH increased, contrary to the concept of acid rain! The water originally maintained pH 5.2 and 280 µg Al/l (Alr), of which 130 µg/l was labile aluminium (corresponding to Ali) and 150 µg/l was non-labile aluminium (corresponding to Alo + Al-polymers). After lime addition, the pH had increased to 6.6, and the Alr-concentration dropped from 280 to 250 µg/l. The largest changes were in the amount of labile aluminium, which had fallen from 150 to 20 µg/l and non-labile aluminium, which had risen from 150 to 230 µg/l. This agrees well with my results showing that the same happens in the acidic Al-rich medium at pH 5.8, compared to the acidic Al-rich medium at pH 4.8. In the study of Fjeld et al. (1988), monomeric inorganic aluminium, mainly Al^{3+}, probably began to polymerize when the pH increased with the addition of lime, causing aluminium to accumulate on the crayfish gills. My results are therefore the first documentation after Fjeld et al. (1988) that the degree of Al-polymerization plays a role in the toxicity of acidic Al-rich water in organisms other than fish.

Based on what is known from fish, it could be expected that, at pH 5.8, where the Al-chemistry is significantly more unstable, and there are good conditions for Al-polymerization (Hem & Roberson, 1967; Lydersen, 1990), aluminium will bind and accumulate on the gill surfaces of *G. lacustris* as it does in crayfish and fish (Fjeld et al., 1988; Oughton et al., 1992;

Poléo, 1995; Poléo & Bjerkely, 2000; Rosseland et al., 1992). In the present study, accumulation of aluminium on the respiratory surfaces of *G. lacustris* was not measured, but in the respirometry experiments, normoxic O_2-consumption was higher in *G. lacustris* exposed to the Al-rich medium compared to the two Al-poor media tested (Figure 13). This is contrary to what has been observed in comparable experiments with fish, where respiration seems to be impaired by aluminium (Poléo & Bjerkely, 2000; Poléo et al., 2017; Poléo et al., 2021). It suggests that aluminium is not causing hypoxia in *G. lacustris*. On the other hand, high content of aluminium in the water caused a higher critical O_2-concentration in Al-exposed *G. lacustris* (Figure 14), suggesting that the animals were less able to extract oxygen from the water in the Al-rich medium compared to the two Al-poor media. There is no obvious explanation for the contradiction that *G. lacustris* increase it's O_2-consumption while the ability of extracting oxygen from the water was reduced and remains to be investigated further.

It is well known that ion regulatory disturbances are common symptoms of Al-toxicity in fish, typically evident by extensive loss of plasma ions (Gensemer & Playle, 1999; Neville, 1985; Poléo, 1995). Maintaining high body fluid levels of ions in freshwater organisms, against large concentration gradients between the body fluids and the surrounding freshwater low in ions is very energetically costly. It has been shown that crucian carp (*Carassius carassius*) might adjust the plasma ion content to a lower level than normal to reduce the diffusion gradient between the body fluids and the surrounding water, when exposed to copper, aluminium or anoxic water (Poléo et al., 2017; Schjolden et al., 2007; Sollid et al., 2003). It might be that the observed elevated normoxic O_2-consumption observed in *G. lacustris* exposed to the Al-rich medium at pH 5.8 is a compensatory oxygen demand caused by counteracting an Al-induced ion loss in this species. This also accounts for the elevated normoxic O_2-consumption in *G. lacustris* exposed to the Al-poor medium at pH 5.8 compared to those exposed to the untreated natural water at pH 7.2 (Table 16). This study therefore needs to be followed up by studies where the effect of acidic Al-rich water on ion balance in *G. lacustris* is addressed.

The overall conclusion to the third research question is that the Al-toxicity in *G. lacustris* is dependent on the degree of Al-polymerization, and that the effect is more evident at pH 5.8 than at pH 4.8. The answer to the fourth research question is that a possible link between the degree of Al-polymerization and the respiration in *G. lacustris* is not evident, but that elevated

concentrations of aqueous aluminium have a clear effect on respiration in terms of increased normoxic O_2-consumption and higher critical O_2-concentration.

Additional remarks

Since aqueous aluminium plays a role in the effect of acidified water on *G. lacustris*, it is reason to believe that this is also the case for many other invertebrate species or taxa. Future research within the project where this study was a part will most probably bring answers to many such questions. So far, this study has contributed with results suggesting that elevated concentrations of aqueous aluminium in acidified waters are of less importance in invertebrates compared to fish, but studying more species might change this. It should also be noticed that it is rare for acidified waters and watercourses in Norway to become as acidic as pH 4.8, and the present study indicates that aluminium is of importance for the toxicity of acidified water in *G. lacustris* at pH 5.8. It might therefore still be that aluminium is the main reason why some species of invertebrates disappear from acidified localities, especially in the initial phase of acidification and in moderately acidified localities.

It turns out that the biological recovery of the acidified freshwater ecosystems after the reduction in acid precipitation during the last 35 years, is slower than the recovery of the water quality (Enge et al., 2016; O. A. Garmo et al., 2014; Hesthagen et al., 2011; Skjelkvåle et al., 2007; 1998; Wright, 2008). The reason for this "mismatch" between chemical and biological recovery seems to be that the buffering capacity of the catchments is still low, and that there has been a shift between chronic acidification to episodic acidification (Wright, 2008). The catchments do not constantly leak aluminium to the surface waters as before, but increased Al-concentrations occur episodically in connection with heavy rain and storm events (Enge et al., 2016; Laudon & Bishop, 1999; Serrano et al., 2008). As the pH of the waters increases, the acidity will gradually become less important for fish and aquatic invertebrates such as *G. lacustris*. Increased surface water pH might favour Al-polymerization in the future as more unstable chemical conditions may be created when acidic Al-containing soil-water leaks into and mixes with the surface water during heavy rain episodes. Thus, aluminium could have a greater impact as the recovery continues. From a climate perspective, with the increasing frequency of storms and extreme weather due to global warming, this might be of significant importance.

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