Faculty of Applied Ecology, Agricultural Sciences and Biotechnology

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Master Thesis

The effect of aqueous aluminium on mortality and respiration of the isopoda *Asellus aquaticus*

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// Abstract

The present study investigates the effect of aqueous aluminium (Al) and acidic water on mortality and respiration in the isopoda *Asellus aquaticus*. I conducted one mortality experiment lasting 22 days exposing *A. aquaticus* to an acidic Al-rich medium (pH 5.8), an acidic Al-poor medium (pH 5.8) and untreated natural water (control) (pH 7.0). Followed by three respirometry experiments exposing *A. aquaticus* to an acidic Al-rich medium, an acidic Al-poor and untreated natural water each for five days and then transferring the animals into closed-respirometry chambers for 48 hours.

In the mortality experiment *A. aquaticus* was more sensitive to the acidic Al-rich medium than the acidic Al-poor- and the control media. In the respirometry experiment Al-exposed *A. aquaticus* displayed a lower normoxic O_2 consumption than in the acidic Al-poor- and control media. Similarly, the critical O_2 tension was lower in Al-exposed *A. aquaticus* than acidic Al-poor- and control media. The critical O_2 tension was inconsistent with the mortality *A. aquaticus* experienced in the mortality experiment.

I draw the conclusion that *A. aquaticus* is more sensitive to aqueous aluminium than to an acidic Al-poor medium.

Acknowledgements

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Contents

Introduction 1
Aluminium 2
Aluminium and Aquatic Organisms 3
Material and Methods 6
Experimental Animals 6
Test Conditions 7
Experimental Setup 8
Experimental Protocol 12
Sampling and Water Chemical Analysis 13
Respirometry 16
Statistical Analyses 19
Survival Analysis 19
Oxygen Consumption Analysis 22
Results 24
Locomotor Activity 24
Water Chemistry 24
Mortality 28
Respirometry 30
Discussion 39
Al chemistry 39
Is aqueous aluminium toxic to *A. aquaticus* and is a possible Al-toxicity in dependent on the degree of aluminium polymerisation similar to fish? 40
Is there a link between the degree of aluminium polymerisation and respiration in *A. aquaticus*? 42
Conclusion 43
Literature 45
Appendix A: Figures 58

Introduction

Robert Angus Smith first described acid deposition as surface damage on buildings near industrial centres in the United Kingdom (Smith, 1872). His research was, however, forgotten until unknown consequences of acid deposition emerged in Norway a century later. Scientists recorded a decline in fish populations together with episodic fish deaths but struggled to explain what caused these events (Dahl, 1921; Huitfeldt-Kaas, 1922, 1923; Schofield, 1976). Only after Mackereth (1957), Gorham (1958) and Dannevig (1959) published their observations did scientists become aware of acid depositions' adverse effects on pH in lakes and streams. Later, Schofield discovered aluminium (Al) mobilised from the soil to be an essential factor in understanding why fish populations declined. Schofield's theory was that increased H^+-ion concentrations in freshwater were not the sole cause for the observed fish deaths, but rather increased amounts of mobilised aluminium in freshwater (Burrows & Hem, 1977; Schofield, 1977; Dickson, 1978). Building on Schofield's work Poléo (1995) presented a novel theory – where polymerisation of aluminium is the primary mechanism causing fish deaths and not aluminium *per se.*

The consequences of freshwater acidification in fish physiology (Neville, 1985; Howells et al., 1990; Poléo, 1995; Poléo et al., 1997; Poléo & Bjerkely, 2000) and population declines are well documented and understood (Schofield, 1976; Driscoll et al., 1980; Muniz & Leivestad, 1980; Schindler et al., 1985; Havas & Rosseland, 1995; Sparling & Lowe, 1996; Gensemer & Playle, 1999). However, research on the consequences of freshwater acidification on aquatic invertebrates is scarce. Most studies are field experiments, making it difficult to separate the effect of low pH from aqueous aluminium. The importance of water quality, especially pH, Ca, Al, and organic acids, are known but not very well understood in invertebrates (Schofield, 1977; Dickson, 1978; Schindler et al., 1985; Økland & Økland, 1986; Muniz, 1990; Havas & Rosseland, 1995; Lien et al., 1996; Gensemer & Playle, 1999). Lakes and streams in Europe and North America are still recovering from acidification. Although, recent research indicates that the water chemistry is recovering, acid-sensitive invertebrates are still sparse (Garmo et al., 2014; Berger et al., 2016; Driscoll et al., 2016; Beneš et al., 2017; Lawrence et al., 2021).

Aluminium

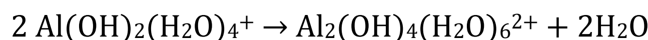
Despite being the most common metallic element in the Earth's crust, there is no known biological function for aluminium in our ecosystems and concentrations in freshwater are relatively low. In its most common form, aluminium is found as aluminosilicate in rocks, mainly as feldspars (Havas, 1986a; Howells et al., 1994; Michel & Ludwig, 2005). Because acid rain is a consequence of burning fossil fuels, it emits sulphur dioxide (SO_2) and nitrogen oxides (NO_X) into the atmosphere. In the atmosphere, these oxides react with water molecules (H_2O) and form sulfuric acid (H_2SO_4) and nitric acid (HNO_3). As a result, acidic compounds precipitate with rainwater, snow, or dry deposits (SO_4^{2-} and NO_3^-), making them more acidic due to increased levels of H^+ (Cronan & Schofield, 1979; Seip et al., 1989; Lawrence et al., 1999; Stoddard et al., 1999).

When rainwater precipitates to the ground, weathering of rocks and minerals occur naturally. Therefore, the leaching of base cations from the soil is part of the natural soil development process. Without this process, water quality in streams and lakes would be too poor to sustain fish and other freshwater organisms. When rainwater gets enriched in H^+, SO_4^{2-} and NO_x, these natural processes are accelerated. Weathering of parent minerals like aluminosilicates, especially feldspars, is the primary source of aluminium in the soil. Continued weathering forms secondary aluminium sources, which dissolve into their ionic constituents with time. These low molecular inorganic aluminium ions are the tertiary source of aluminium in the soil. Under normal circumstances, the soil withholds low molecular inorganic aluminium ions due to ion exchange and complex ion formation. With acid depositions, however, aluminium is replacing base cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, NH_4^+) loosely bound on the surface of inorganic and organic molecules. Low molecular inorganic aluminium will precipitate and form complexes with inorganic (F^-, OH^-, SO_4^{2-}, PO_4^{3-} and SiO_4^{4-}) and organic (COO^-) ligands. Continued ion exchange, leeching of base cations, and later H^+-ions, will deplete the soil of its buffer capacity and make Al^{3+}-ions start leaching into freshwater ecosystems (Havas, 1986a; Lydersen, 1990; Poléo, 1995).

Consequently, an increased supply of H^+ and Al^{3+}-ions in freshwater ecosystems can lower the pH to 4 or less (Cronan & Schofield, 1979; Seip et al., 1989; Lawrence et al., 1999; Stoddard et al., 1999). Aluminium now exists as $Al(H_2O)_6^{3+}$ (octahedral hexahydrate). If pH or temperature rises, water molecules surrounding the aluminium ion will deprotonate, and aluminium hydroxides will form (Figure 1) (Hem & Roberson, 1967; Lydersen, 1990). This

increases the amount of different hydroxy complexes (Appendix A1). Then, when deprotonated octahedra's are present in freshwater, as $Al(OH)(H_2O)_5^{2+}$ or $Al(OH)_2(H_2O)_4^{+}$ aluminium starts to polymerise (Hem & Roberson, 1967).

Deprotonated octahedra's then coalesce to form a dimer (Figure 1), during the release of two water molecules (dehydration):



$$2\ Al(OH)_2(H_2O)_4^{+} \rightarrow Al_2(OH)_4(H_2O)_6^{2+} + 2H_2O$$

With ongoing Al-polymerization of deprotonated dimers, chain structures will form (Figure 1). Further particle growth and reduction in the properties of cationic Al-polymers will interfere with its ability to bind onto biological surfaces (Lydersen, 1991; Lydersen et al., 1994; Poléo, 1995).

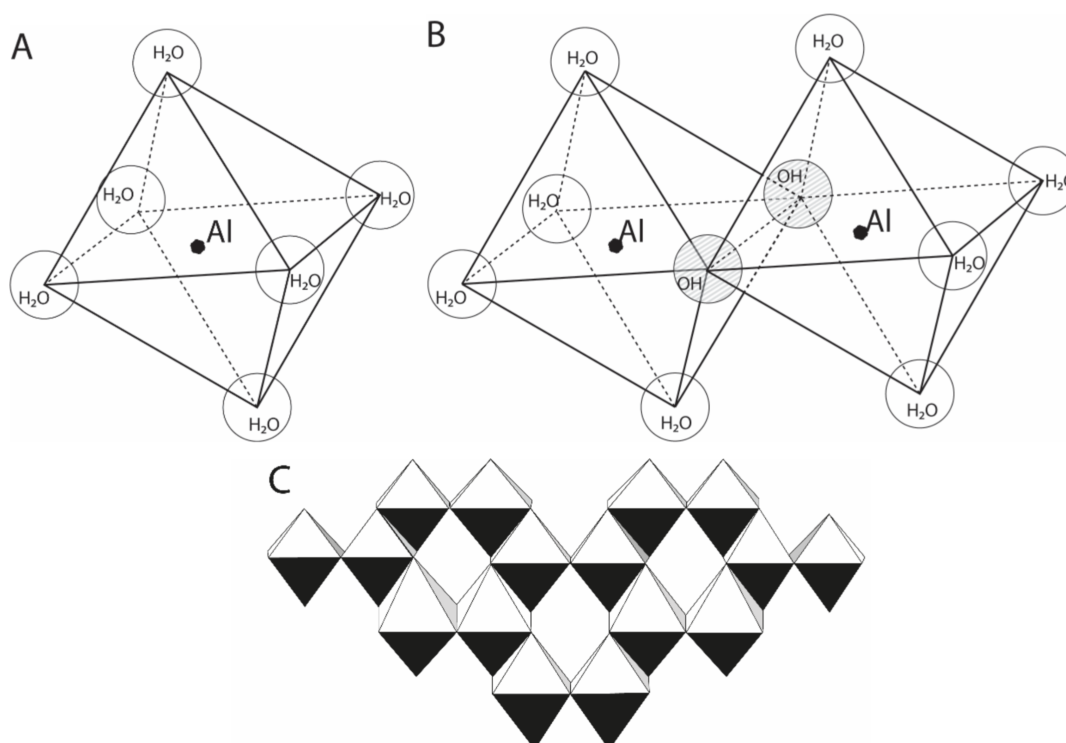


Figure 1. Schematic representation of A. hydrolysed aluminium ion $Al(OH_2)_6^{3+}$, B. Dimeric cation $Al_2(OH)_2(OH_2)_8^{4+}$ with a double OH^- bridge and C. Al-hydroxide octahedral chain structure after illustrations from Hem & Roberson (1967).

Aluminium and Aquatic Organisms

When the ambient water surrounding the fish or the gill microenvironment is favourable for continuous aluminium polymerisation, i.e., pH 5-7 (Hem & Roberson, 1967), Poléo (1995)

proposes that this is when aluminium is most toxic in fish. Positively charged low molecular Al species then accumulates on the fish's negatively charged gill surface (Appendix A2) (Wold & Selset, 1977; Muniz & Leivestad, 1980; Poléo, 1995; Poléo & Bjerkely, 2000). This decreases the surface available for gas exchange and increases the diffusion distance between water and blood, which causes respiratory and ion regulatory disturbances in fish (Hughes, 1981; Wood & McDonald, 1982; Neville, 1985; Poléo & Bjerkely, 2000). The respiratory disturbances predominate between pH 5-7, depending on water temperature (Lydersen et al., 1991; Exley et al., 1996). Ion regulatory disturbances, however, predominate at pH below 4.5, i.e., the net loss of plasma ions, e.g., Na^+ and Cl^- (Staurnes et al., 1984; Neville, 1985).

Aquatic invertebrates are a diverse and essential group for healthy ecosystems. They are an integral part of the food web and vital in breaking down material from primary production (Pechenik, 2014). Aquatic invertebrates have different respiratory strategies; some respire through diffusion over the skin, and others with gills. The gill-breathing invertebrates mainly respire by gills where oxygen diffuses from water into haemolymph fluid or gills with tracheal tubes (Moyes & Schulte, 2014). *Asellus aquaticus* is a freshwater *crustacea:isopoda* common in ponds and lakes with pH ranging from 4.7 to 8.8. It thrives in shallow water between detritus and rocks and is believed to be pollution tolerant (Økland, 1980). *A. aquaticus* adults' range between 8-25 mm in body size. Their body is dorsoventrally flattened with a segmented exoskeleton, i.e., the cuticula. Connected to the *cephalothorax* are two pairs of antennae, compound eyes and *maxillipeds*. The *pereon* is segmented into seven parts with seven pairs of *uniramous thoracic* walking legs (*pereopods*). The walking legs gradually increase in length from the 1st *thoracic* segment to the 7th. The *pleotelson* bears the *biramous uropods*, with the 3rd *pleopod* modified to work as an *operculum* attached ventrally. Ventrally on the *pleotelson* is the branchial cavity, a concave space holding the 4th and 5th *pleopods* functioning as gills, protected by the operculum (Minelli et al., 2013; Kemp et al., 2020). The gill function in *A. aquaticus* is very similar to freshwater fish. It is the primary organ for gas exchange, ion regulation, osmoregulation, and excretion of nitrogenous waste (NH_3/NH_4^+) (Wright, 1995; Freire et al., 2008; Henry et al., 2012).

Earlier ecotoxicological studies with Al-exposed aquatic invertebrates have indicated that aqueous aluminium only, to a limited extent, increases toxicity compared with lowered water pH (Havas, 1985; Havas & Likens, 1985a; France & Stokes, 1987; Tabakk & Gibbs, 1991; Storey et al., 1992). Despite this, there are indications of aqueous aluminium toxicity in certain aquatic invertebrates. Havens (1993) reported elevated mortality in *Skistodiaptomus*

oregonensis exposed to aqueous aluminium (200 µg Al/L) at pH 6, compared to a control group. Similar results have been reported for *Orconectes virilis, D. galeata mendotae* at pH 5.5-6 (Weatherley et al., 1989; Havens, 1992). Further indicated by histochemical staining showing aluminium to accumulate over the whole body, particularly on ionregulatory- (chloride cells) and respiratory-surfaces (Havas, 1986c; McCahon et al., 1987; Havens, 1990; Guerold et al., 1995; Vuori, 1996). Similar to all these studies, a net loss in Na^{+} and Cl^{-} is causing mortality, different from acute hypoxia in fish. These earlier studies were conducted on aquatic insects or with steady state Al-chemistry. This is important because aluminium must polymerise for Al-exposure to be toxic and cause acute hypoxia in fish (Poléo, 1995; Poléo & Bjerkely, 2000). Also, how aquatic organisms excrete waste products is essential. Aquatic insects excrete carbon dioxide (CO_2) over the gills (Eriksen & Mæur, 1990), similarly to *A. aquaticus* and fish (Minelli et al., 2013). As a result, the water constituting the gill microenvironment turns acidic. Ammonia (NH_3), however, is excreted as rectal fluids in aquatic insects (Staddon, 1964; Gensemer & Playle, 1999) and diffuses over the gill epithelium in *A. aquaticus* and fish (Dresel & Moyle, 1950; Minelli et al., 2013; Moyes & Schulte, 2014). Because the pH most likely will increase in the gill microenvironment for *A. aquaticus* similar to fish, conditions should be more favourable for aluminium polymerisation compared with aquatic insects. There are many known similarities between *A. aquaticus* and fish, enough to raise the question if aqueous Al and ongoing Al polymerisation will be toxic to *A. aquaticus*?

Because precipitation is increasing and becoming more intense with climate change (Benestad et al., 2022), questions on Al toxicity are once again relevant. With increased precipitation aluminium will now leach episodically into freshwater, causing toxic pulses (Laudon & Bishop, 1999; Serrano et al., 2008; Enge et al., 2016).

Therefore, I have set out to investigate the following scientific questions:

1. Is aqueous aluminium toxic to *A. aquaticus*?
2. Is a possible Al-toxicity in *A. aquaticus* dependent on the degree of aluminium polymerization, similarly to fish?
3. Is there a link between the degree of aluminium polymerization and respiration in *A. aquaticus?*

Material and Methods

I conducted this study as a series of experiments on the freshwater crustacean *A. aquaticus* (Isopoda). It is a part of the project Aluminium Toxicity in Aquatic Invertebrates at Inland University College of Applied Sciences. I conducted all experiments at the Evenstad fish hatchery facility in Stor-Elvdal municipality. *A. aquaticus* were exposed to various combinations of water pH and Al-concentrations. I performed both mortality trials and respiratory experiments.

Since *A. aquaticus* is an invertebrate species, Norwegian legislation on research animals does not cover it. The legislation states that vertebrates, decapods and squids are research animals that require permission for use in experiments (Ministry of Agriculture and Food, 2015).

Experimental Animals

I collected *A. aquaticus* for use in the experiments in the Frognertjernet pond (Global Positioning System (GPS) coordinates: 60.799951, 11.163539), Hamar municipality, Inland County, Norway. I used a rod sieve to collect the animals while wading in shallow water, leading the sieve back and forth over the bottom of the pond. *A. aquaticus* were identified and stored in plastic containers with fresh water from the pond and transported to the experimental unit of the fish hatchery at Evenstad University College. Also, sediments, water vegetation and rocks were brought back to the hatchery to help the animals thrive better in captivity (Graça et al., 1993). I collected animals on seven different occasions during November 2020. Since water temperature and pH differed between pond Frognertjernet and the research facility at Evenstad (Table 1). I placed the animals in 20-litre flow-through storage tanks for acclimation. Here 30 animals were kept together in each tank and the acclimation period was four weeks before experiments started.

A. aquaticus is very sensitive to currents in the water. Therefore, I turned off the normal water flow through the storage tanks and exchanged the water in the tanks every 14 days instead. The water of the research facility comes from the creek "Grøfta" (hereafter referred to as operating water). Before entering the experimental setup, the operating water was passed through a sand filter to remove the humus. During the experiments, the operating water temperature fluctuated between 0.6 and 14.3 °C, water pH between 5.9 and 7.5 and water electrical conductivity between 10.4 and 36.4 µS.

Table 1. Temperature and pH for capture- and research location at given dates for collection of animals from the field.

Date	Location	pH	Temperature °C
26.10.20	Frognertjernet (Ridabu)	8.0	3
26.10.20	Research facility (Evenstad)	7.3	4
6.11.20	Frognertjernet (Ridabu)	8.0	3
6.11.20	Research facility (Evenstad)	7.3	4
7.11.20	Frognertjernet (Ridabu)	8.0	3
7.11.20	Research facility (Evenstad)	7.3	4
8.11.20	Frognertjernet (Ridabu)	8.0	3
8.11.20	Research facility (Evenstad)	7.3	4
13.11.20	Frognertjernet (Ridabu)	8.0	2
13.11.20	Research facility (Evenstad)	7.3	3
14.11.20	Frognertjernet (Ridabu)	8.0	2
14.11.20	Research facility (Evenstad)	7.3	2
15.11.20	Frognertjernet (Ridabu)	8.0	2
15.11.20	Research facility (Evenstad)	7.3	2

Test Conditions

I used three different test media in the experiments: an acidic Al-rich medium, an acidic Al-poor medium and a control medium (Table 2). The control medium I used was untreated operating water. For the preparation of the acidic Al-rich medium, an acidic $Al(NO_3)_3$ stock solution was added to the operating water by means of a Watson Marlow 205S peristaltic pump. The stock solution was prepared by dissolving 560 g (11.2 g/L) of Al-nitrate ($Al(NO_3)_3 \cdot 9H2O$) and adding 380 ml (7.6 ml/L) nitric acid (HNO_3) in a 50L tank with deionised water. The $Al(NO_3)_3$ stock solution had a pH of 2.0. The low pH in the stock solution was to ensure that the aluminium present was on the Al^{3+}-form before adding it to the operating water. I prepared the Al-poor medium similar to the Al-rich medium by making an HNO_3 stock solution where I only added nitric acid (HNO_3) to deionised water in a 50L tank. (Table 2, Table 3). I calculated the nominal Al-concentration in the Al-rich medium to be 1000 µg/L and close to zero in the two other media (Table 2).

Table 2. Test mediums used in toxicity- and respirometry experiments.

Medium	Nominal pH	Nominal Al-concentrations	Additions
Acidic Al-rich (labelled red)	~5.8	1000 µg/l	$Al(NO_3)_3 + HNO_3$
Acidic Al-poor (labelled purple)	~5.8	0 µg/l	HNO_3
Control (labelled green)	~7	0 µg/l	None

Table 3. Chemical composition of department water at Evenstad fish hatchery facility in Stor-Elvdal, Inland, Norway.
Water samples were analysed at the Norwegian Institute for Water Research (NIVA) in 2004.

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

Experimental Setup

The experimental setup consisted of one 80L level tank (785 x 300 x 485 mm: length x width x height). It received and distributed operating water to three different flow-through channels. From here on, I refer to the flow-through channels as exposure channels. Each exposure channel contained three rows, each with eight exposure chambers. From here on, I refer to the three rows as Level 1-3 (Figure 2, Figure 3).

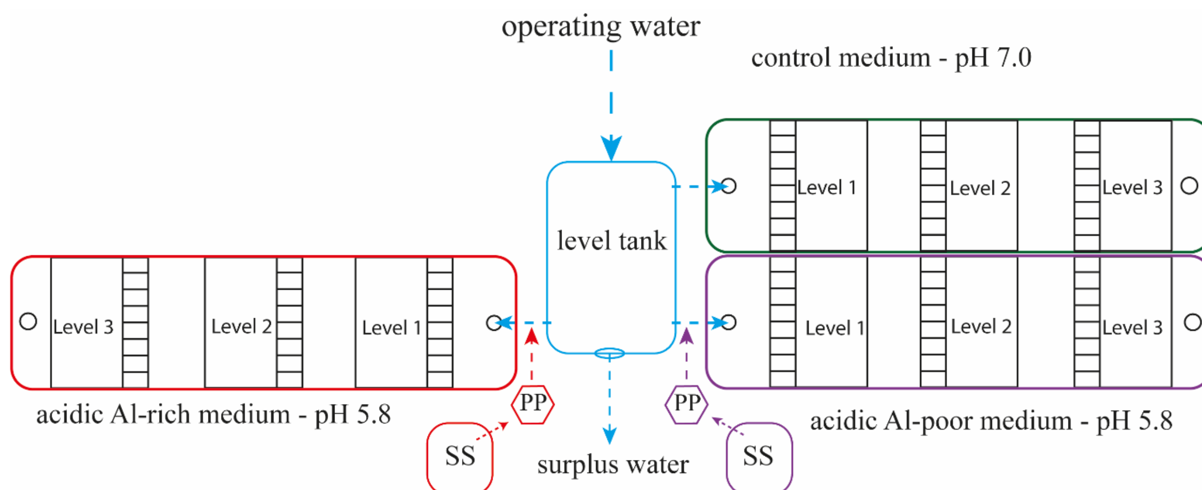


Figure 2. Schematic overview of the experimental setup. Operating water is distributed from the level tank to all three exposure channels. Each exposure channel had three Levels, with a row containing eight exposure chambers. Stock solutions (SS) were pumped with peristaltic pumps (PP) into a supply pipe and mixed with operating water as it entered the channel. The water exit was through an overflow pipe at the end of each exposure channel. Acidic Al-rich (red), acidic Al-poor (purple), control (green).

Figure 3. Overview of the experimental setup. Three Levels with one row of exposure chambers (white) were placed inside each exposure channel. White tanks contain Al(NO3)3 (left) and HNO3 (right) stock solution used in the experiments. In the middle of the photo, above the channels, is the level tank with overflow (grey pipe)- and supply pipes (on the side).

The purpose of the level tank was to ensure a stable water flow and to act as a backup reservoir in case of lowered water flow into the facility. Ball valves on the pipes leading water from the level tank were used to control the water flow into each channel (Figure 3). The water flow into each channel was approximately two l/min. Respective stock solutions were added to the operating water through a small pipe on top of the supply pipe, leading

water from the level tank into the exposure channel (Figure 3, Figure 4, Figure 5). To ensure the stock solutions were mixed properly with the operating water as they entered the channels, I made a mixing device at the bottom of the exposure channel where the pipe entered (Figure 4).

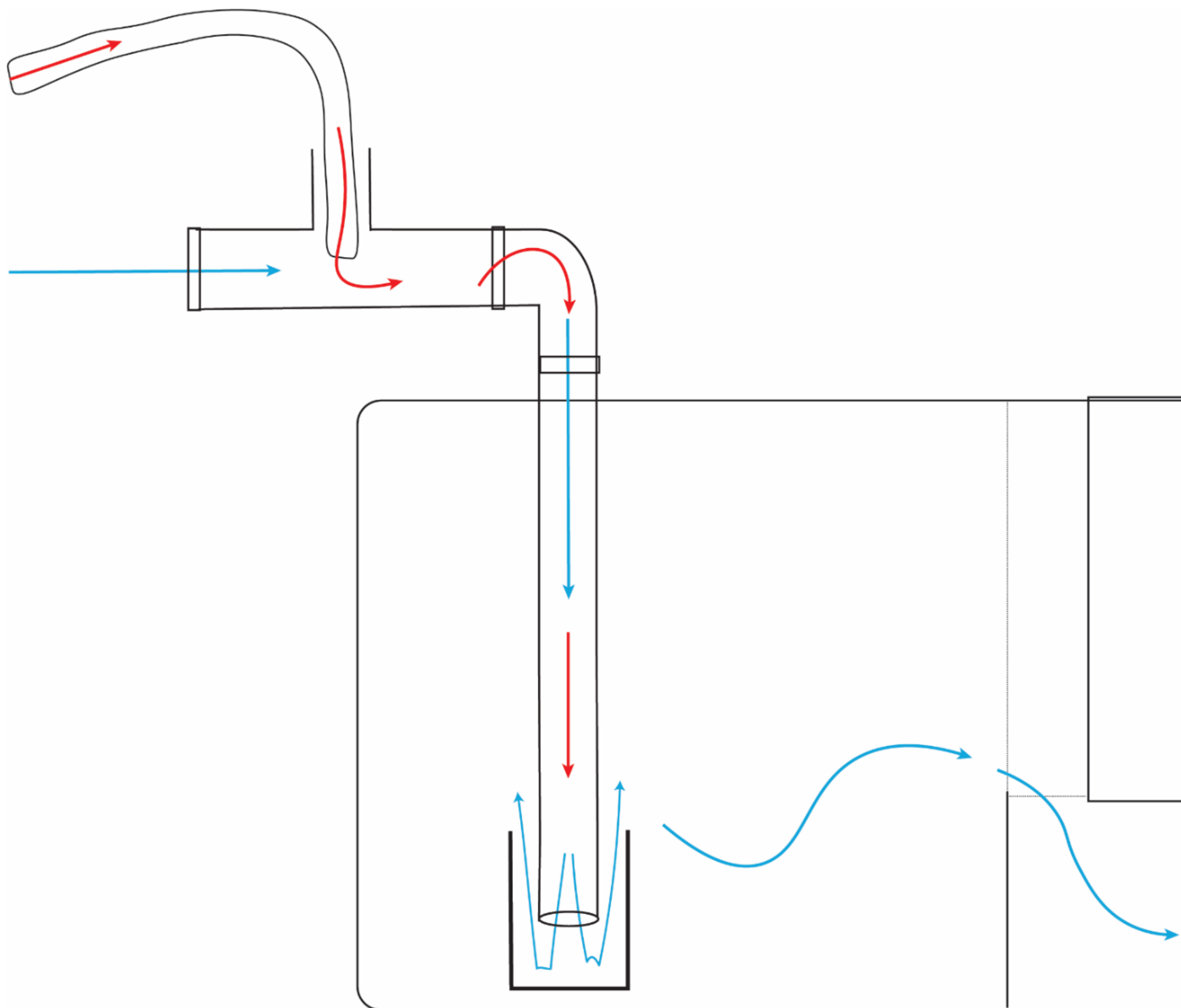


Figure 4. Schematic representation of the supply pipe, the addition of chemical the solution, mixing cup and parts of the exposure channel. Stock solution flow (red arrows) through a silicone tube into the supply pipe. Water flow (blue arrows), mixed with a stock solution, through the supply pipe into the mixing cup inside the entrance of the exposure channel, securing thoroughly mixing the operating water and stock solution. Before it flowed through the perforated polyvinyl chloride (PVC) sheets into the exposure chamber.

Figure 5. The stock solution is pumped with a peristaltic pump through the riser on top of the supply pipe in a silicone tube. Water and stock solution are then mixed thoroughly in the mixing cup at the bottom of the exposure channel. Further, the different media flow through the exposure chambers (white sections inside insert trays). Ball valves are visible as blue knobs on the supply pipe.

To force the water to flow through the exposure chambers on its way through the exposure channels (Figure 6), I used non-toxic silicone to seal the gaps between the channel walls and the insert tray. As mentioned before, in the exposure channel, there are three different levels (Figure 2). These levels act as toxicity gradients for *A. aquaticus* in the exposure channel with an acidic Al-rich medium. In Level 1, the water residence time is 1 minute; in Level 2: 12 minutes; in Level 3: 25 minutes.

Residence time for the acidic Al-rich medium is essential because polymerising aluminium will lose its toxicity with time (ageing). The distribution of aqueous Al-species is closely linked with pH, but temperature and organic ligands are also important. The pH of the stock solution I used in this experiment was ~2. Therefore, in the stock solution with Al, the only significant Al species present was Al^{3+} (Hem & Roberson, 1967; Lydersen et al., 1991). As mentioned before, Al hydrolysis depends on pH and temperature (Appendix A1). Thus, when

the stock solution (pH ~2) is added to the operating water (pH 7) and mixed in the mixing cup pH in the medium will rapidly rise to ~5.8. As a result, the Al^{3+}-ion will deprotonate and change its form to $Al(OH)_2(H_2O)_4^+$, and Al will start polymerising in front of Level 1. Under these conditions, the degree of Al polymerisation is important and what decides how toxic Al can be to *A. aquaticus*. Since Al polymers will bind to organic ligands as they flow through the exposure channel, the medium will become less toxic when it passes through levels 2 and 3. Eventually, the Al polymers net charge will approach zero. When the net charge of Al polymers is close to zero, it is difficult for them to bind to negatively charged organic surfaces (Poléo et al., 1994; Poléo, 1995). Therefore, it is vital that *A. aquaticus* is exposed to an acidic Al-rich medium with increasing residence time.

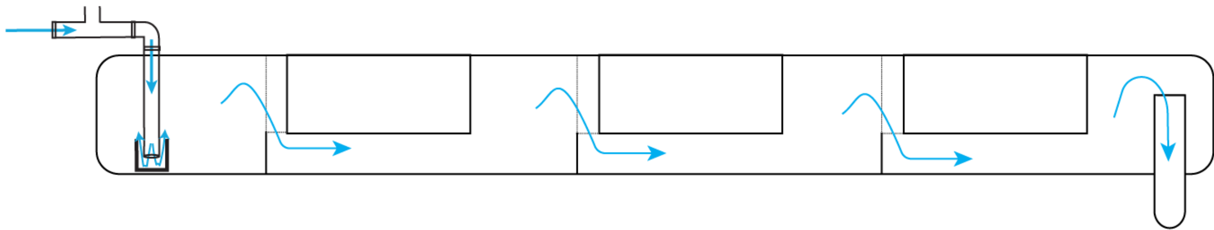


Figure 6. Water (blue arrows) enters through a supply pipe in the front of the exposure channel and is mixed with the stock solution in the mixing cup. Next, it flows through the exposure chambers with perforated PVC sheets (1 mm x 1 mm) in the front and bottom. The water exits through an overflow pipe at the end of the exposure channel.

I made all pipes used in the experiment from polyvinyl chloride (PVC), fibreglass exposure channels and PVC exposure chambers (Figure 5).

Experimental Protocol

This study consisted of one mortality experiment followed by a series of three respirometry experiments. In the toxicity experiment, I exposed *A. aquaticus* to three different test media in parallel: acidic Al-rich medium (pH 5.8), acidic Al-poor medium (pH 5.8), and control medium (pH 7.0). The experiment started when three animals were placed in each exposure chamber, making it 24 at each level. At least 12 hours before the experiment started, the addition of stock solutions was initiated by turning on the peristaltic pumps. For the next 21 days, until the experiment's termination, I monitored mortality daily, as well as water pH, temperature, conductivity, stock solution dosage flow and water flow in each channel. Samples for aqueous aluminium fractionation were collected on day 1, day 11 and day 21

from the centre of each level of exposure chambers. I adjusted if there were significant deviations from the nominal pH level, chemical solution flow or water flow.

I conducted the respirometry experiments as three separate experiments, with one of the test media at the time: the acidic Al-poor medium, the control medium, and finally, the acidic Al-rich medium. Each experiment consisted of an initial exposure of *A. aquaticus* to the test medium in question, followed by oxygen consumption measurements on the exposed animals in a respirometer (see below). At least 24 hours before starting the two experiments with the acidic test media, the addition of stock solution was initiated. Each experiment started by placing five animals into separate exposure chambers within one of the exposure chamber levels. I exposed the animals for five days before transferring them to the respirometer. The five-days exposure time was chosen based on when mortality first occurred in the mortality experiment. Due to limited respirometry capacity, exposures were performed successively, and new animals were placed into the exposure chambers every second day. Three animals were exposed in each exposure chamber, nine in each level, making it 27 in each experiment. Thus, each experiment lasted for 24 days. I followed the same daily monitoring protocol in the respirometry experiment as in the mortality experiment. On day 1, day 12 and day 24, I collected samples for aqueous aluminium fractionation.

Sampling and Water Chemical Analysis

I visually examined each animal to see if it was dead. Sometimes it was difficult to decide if an animal was dead or not. Illuminating the animal with a flashlight was helpful. If the animal did not react to the light, I used a pipette to pump water towards the animal, looking for a reaction. If the animal did not react to both methods, it was considered dead and removed from the exposure chamber. To collect water samples for pH and conductivity measurements, I used plastic bottles marked with colour labelling according to the media (Table 2). A separate set of bottles labelled the same way was used to collect water samples for aluminium fractionations (Table 2). I rinsed all bottles used for water sampling according to a protocol described by Gill (2021). I collected water samples behind each channel's first level for pH and conductivity measurements. For collecting water samples for aluminium fractionation, I used a siphon from the central exposure chamber within each row – three samples from each channel. The siphon was cleansed in acidified water and rinsed in deionised water before use. I measured water temperature with a Testo TC type K, class 1 temperature probe attached to a Testo 830-T4 thermometer in the level tank. Water conductivity was measured using a

Radiometer Copenhagen CDM 80 Conductivity meter. Conductivity readings were temperature corrected to 24°C. Conductivity was measured repeatedly and determined when three consecutive measurements gave the same reading. I used a WTW portable ProfiLine pH 3110 pH meter connected to a Hamilton Polilyte Plus H S8 120 glass electrode to measure the pH. To calibrate the pH electrode daily before measurements, I used pH buffers of 4.01 and 7.00. The pH meter is equipped with a flashing indicator, I determined the pH after the indicator stopped flashing.

For aluminium fractionation, I used the Barnes/Driscoll method to fractionate aqueous aluminium (Barnes, 1975; Driscoll, 1984) after a protocol described by Poléo et al. (1997). The method combines 8-hydroxyquinoline (HQ) and methyl isobutyl ketone (MIBK) extraction (Barnes, 1975) with a cation-exchange procedure (Driscoll, 1984). This method is well suited for aqueous aluminium fractionation in field surveys and laboratory experiments (Barnes, 1975; Driscoll, 1984; Sullivan et al., 1986; Lydersen et al., 1994). Dissolved aluminium is complexed with HQ (C_9H_7NO) when extracting a water sample to an Al-HQ complex. The Al-HQ complex, which is not water-soluble, moves from the water into an organic phase of MIBK. I used an extraction time of 20 seconds, as recommended by Barnes (1975). The fractionation of aluminium must be done immediately after the water sample has been taken. To prevent the distribution of different aluminium species from changing while the samples are stored (Lydersen et al., 1990; 1994). To determine the amount of total monomeric aluminium species (Ala) in the water, I extracted an untreated sub-sample right after sampling. To determine the amounts of organic monomeric aluminium species (Alo) and inorganic monomeric aluminium compounds (Ali). I ran another sub-sample through the cation exchange column containing Amberlite IR-120 ion exchange resin (10 ml) and extracted it in the same way as the Ala sample. Positively charged Ali species are retained by the cation exchange resin, while negatively- and uncharged Alo species pass through the resin (Driscoll, 1984). I calculated Ali as the difference between Ala and Alo. To avoid any changes in the distribution of aluminium species due to changes in water pH during the ion exchange process, I adjusted the pH in the ion exchange column, so it did not deviate with more than 0.5 pH units from the pH in the water sample. The speed of the water running through the ion exchange column was 3.8 ml/min per ml ion exchange resin. To prepare the ion exchange resin between each water sample, I ran 60 ml of $10^{÷4}$ M NaCl solution and 60 ml of the water sample through the ion exchange column. Before, I collected another 60 ml of the water sample for extraction. Colloidal aluminium, stable organic and hydroxy-organic

aluminium compounds are not extractable within 20 seconds. I, therefore, acidified the remaining water sample with HNO_3 to pH 1.0 and stored it at 4°C in a refrigerator for at least 24 hours before I extracted it to determine the total concentration of aluminium (Alr) in the water.

All aluminium MIBK extracts were stored in a refrigerator at 4°C for a minimum of 24 hours. Before, the amount of aluminium was analysed spectrophotometrically on a Shimadzu UV-1201 spectrophotometer at 395 Nm (Tikhonov, 1973; Bloom et al., 1979). To correct for iron interference, I measured the absorbance at 600 Nm (Sullivan et al., 1986). The standard deviation for this method is calculated to be 1 % of the average (Sullivan et al., 1986), and the limit of detection is 13 µg Al/l according to Vogt et al. (1994)

Each time I performed aluminium fractionation, I also made a standard curve by extracting aqueous aluminium standards (0, 40, 100, 200, 400 and 600 µg Al/L) to calculate the aluminium concentrations from the absorbance measurements. I did the calculations with R version 4.2.1 (R Core Team, 2022) in RStudio version 2022.07.1+554 (RStudio Team, 2022) by calculating the squared r-value (Rsquared), the slope and the intersection on the y-axis for each standard curve:

$$[Al] = ((OD395 - (OD600 \times 1.12)) / slope) \pm intersection$$

$$OD = \text{Optical Density (Absorbance)}$$

Table 4 gives an overview of the different fractions that were analysed or calculated, and Figure 7 shows the schematic overview of how the fractionation was done.

Table 4. Description of the different Al-fractions being analysed or calculated following the Barnes/Driscoll protocol.

Alr:	Total aluminium is determined by extracting a water sample acidified to pH 1.0 with HNO_3 and storing it for a minimum of 24 hours.
Ala:	Total monomeric aluminium is determined by the extraction of an untreated water sample.
Alo:	Organic monomeric aluminium is determined by the extraction of the eluate from a cation-exchanged water sample.
Ali:	Inorganic monomeric is defined as the difference between Ala - Alo

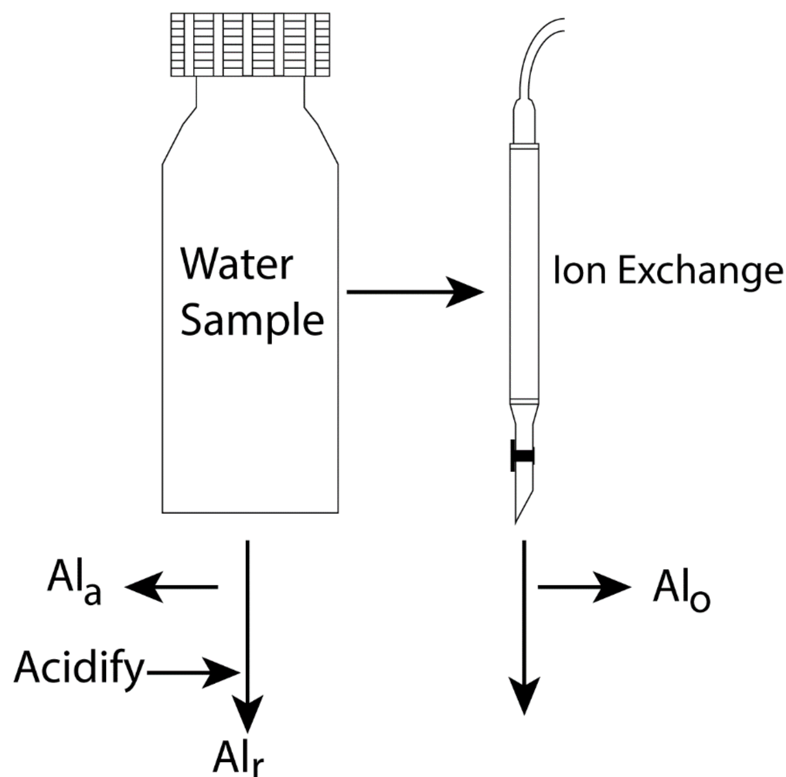


Figure 7. Schematic overview of the fractionation process by ion exchange and extraction after the Barnes/Driscoll method. The Ali-fraction is missing since it is calculated as the difference between Ala and Alo.

Respirometry

Oxygen consumption rates were measured using closed-system respirometry (Figure 8). To compare the physiological response of animals in different treatments and different levels, I used customised 6 ml $^{-1}$ blood collection tubes (BD Vacutainer) as respiratory chambers (Figure 9).

Because of *A. aquatics*' small size (0.5-20 mm) compared to the much larger volume in each respiratory chamber (Figure 9). I lowered the volume of all four respiratory chambers with epoxy resin ($C_{21}H_{25}ClO_5$). This made it easier for the animals to stir the water inside the chamber and produced more reliable measurements of normoxic O_2 consumption (Mo_2). I equipped each respiratory chamber with a standard rubber cap making them hermetically sealed. Through the rubber cap, I inserted the fibre-optic respirometry microsensors (FireSting OXR50, PyroScience GmbH, Aachen, Germany). I filled every respiratory chamber with media from the exposure channel where I collected the animal. Before I sealed

the respiratory chamber, I inserted a cannula (BD Microlance 3, 30G 13mm) through the cap to let excess water exit from the respiratory chamber through the cannula. This prevented unwanted air from getting trapped inside the respiratory chamber. Every replicate had three chambers with animals and one chamber only with medium acting as control (to measure background oxygen consumption from bacteria in the water).

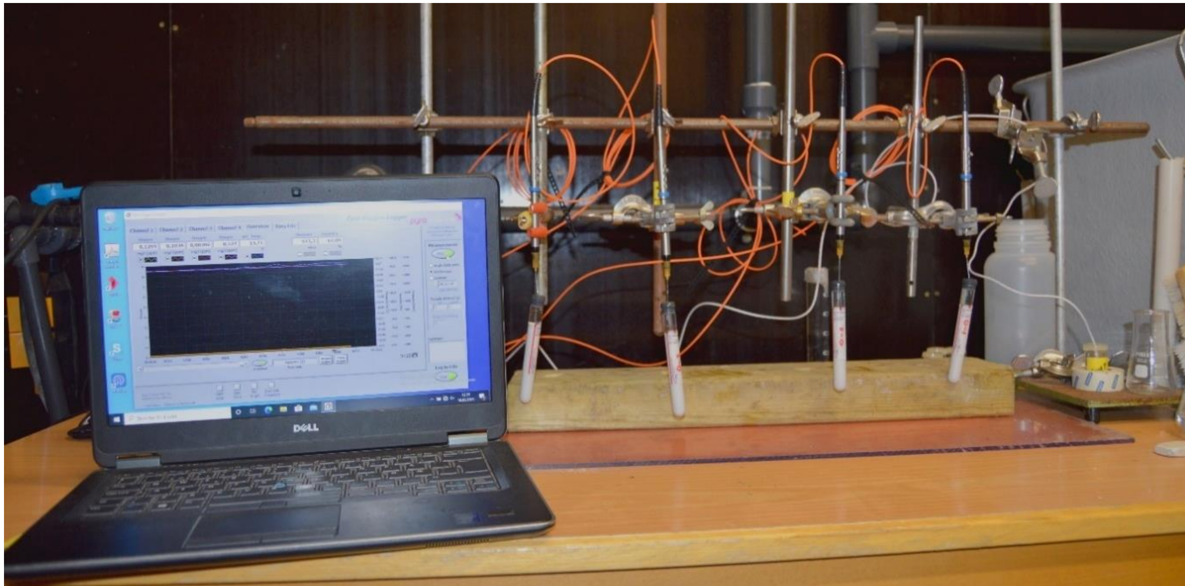


Figure 8. Overview of the respirometry setup. The personal computer (PC) powers four fibre-optic respirometry microsensors and the temperature logger. The fibre-optic microsensors are inserted into the respirator chamber with a needle and the temperature logger sensor is submerged in a water bottle on the right.

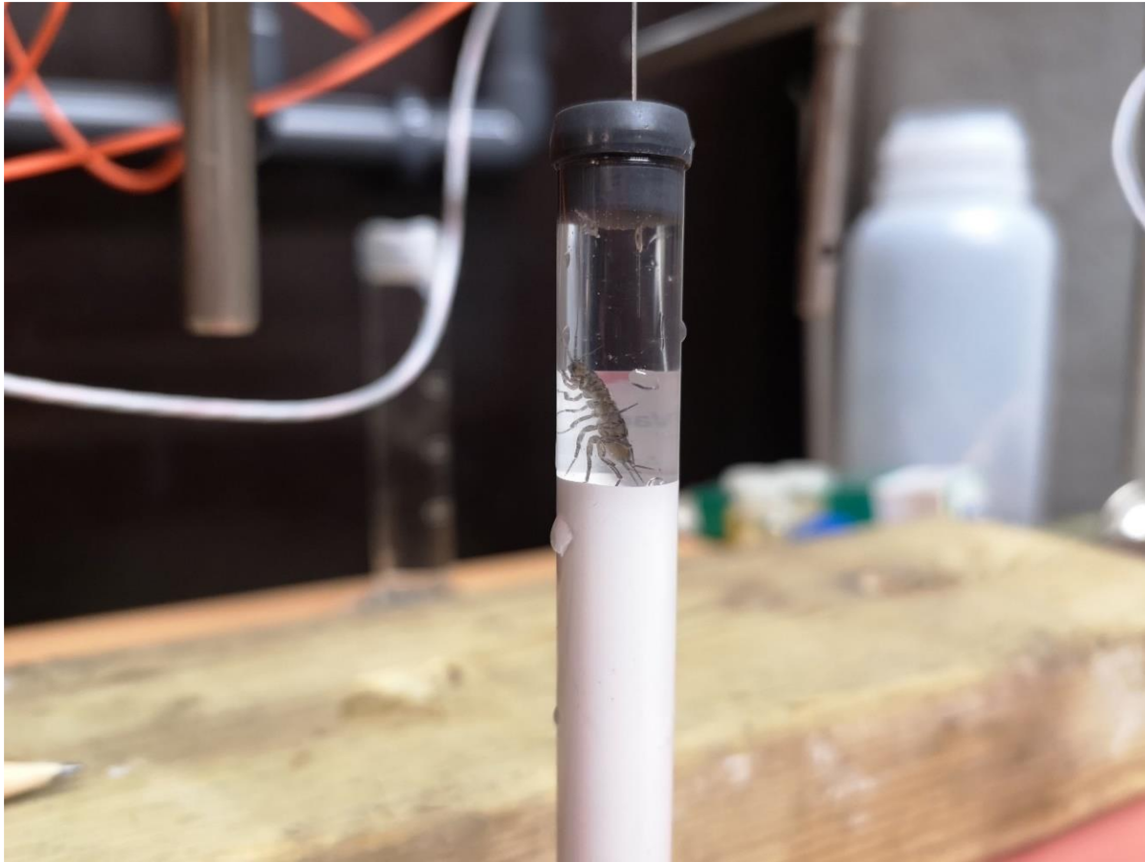


Figure 9. A. aquaticus inside one of the respirometry chambers filled with acidic Al-rich medium. Below the rubber cap is the needle which protects and holds the respirometry microsensor visible.

I performed three replicates per level – nine in total per treatment. Dissolved oxygen measurements (DO) were recorded simultaneously in all four respiratory chambers at 1-second intervals. I recorded compensation temperature measurements parallelly in a separate 20 ml water container with a Pt 100 TSUB21 temperature sensor (PyroScience GmbH, Aachen, Germany) (Figure 8). I gave all animals enough time to consume all the oxygen within their chamber. After 48 hours, the animals were collected from the respirometry chambers and transferred to separate (serial numbered) laboratory test tubes (virgin polypropylene (PP)) 0.2 ml and kept in a freezer (-24°C) for later weighing. I logged all O2 measurements with a Pyro Oxygen Logger software version 3316 (PyroScience GmbH, Aachen, Germany) running on a personal computer (PC) with a Microsoft Windows 8 operating system. I also used the PC to power the PyroScience FireSting O2-FSO2-4 Oxygen and Temperature meter (PyroScience GmbH, Aachen, Germany) through a Universal Serial Bus (USB). The four respirometry microsensors were connected to and powered by the Oxygen and Temperature meter (Figure 8).

After the first three replicates with the acidic Al-rich medium, one of the microsensors was damaged and stopped working. Therefore, I used the three remaining respirometry chambers to record oxygen consumption and performed separate measurements of background respiration at the end of the experiment. I did not feed the animals during experiments; therefore, I consider my measurements to represent standard metabolic rate (SMR) (Beamish & Mookherjii, 1964; Schurmann & Steffensen, 1997; Svendsen et al., 2016).

Before weighing the animals, I air-dried them at 60°C for 24 hours in a Binder ED 720 Avantgarde air cabinet. I weighed on a Mettler AE 260 DeltaRange scale to 0.001 g precision. To get precise estimates of the O_2 consumption for *A. aquaticus*, I had to know the effective volume of each respirometry chamber. The effective volume is the volume inside the respirometry chamber subtracted by each animal's mass. In the statistical analyses chapter, I explain how I estimated the effective volume in more detail. I calculated the volume of each respirometry chamber by weighing them separately with and without water and subtracting the weight without- from the weight with water. I weighed all chambers on a Mettler AE 260 DeltaRange scale (Table 5).

Table 5. Weight (g) of respiratory chambers with and without water, the water temperature was 4°C.

Respirometry Chamber	With water	Without water	n
#1	17.90 g	16.23 g	2
#2	17.87 g	16.25 g	2
#3	17.88 g	16.24 g	2
#4	17.91 g	16.25 g	2

Statistical Analyses

I performed all data analyses in R version 4.2.1 (R Core Team, 2022) in RStudio version 2022.7.1.554 (RStudio Team, 2022). I conducted Survival analyses with the Kaplan-Meier (Kaplan & Meier, 1958) graphical representation of the survival curve and Cox’s proportional hazard regression model (Cox, 1972). I created aluminium extraction- and respirometry figures with ggplot2 (Wickham 2016).

Survival Analysis

The Kaplan-Meier analysis and Cox's proportional hazard regression model are *time-to-event* analyses. In this study, *event* is when one animal dies and is removed from the experiment.

The Cox proportional hazard model is semi-parametric and expressed by the hazard function:

$$\lambda(t) = \lambda_0 \lambda(t)\ x\ \exp(b_{1x1} +\ b_{2x2} +_{...} + b_p x_p)$$

The hazard function, denoted $\lambda(t)$, is determined by the p-covariates $(x_1, x_2, \dots, x_p)$. Next, the coefficients $(b_1, b_2, \dots, b_p)$ are a measurement of the impact of covariates. The baseline hazard is denoted by λ_0 and is equivalent to the hazard value when all the x_i is equal to zero. The hazard may vary over time, denoted $\lambda(t)$, where t is time. Hazard ratios (HR) are expressed by $exp(b_i)$. If the value is greater than zero, this indicates an increasing value of the ith covariate. And the event hazard also increases, and survival times decrease.

In the Cox proportional hazard model, the fundamental assumption is a proportional hazard, i.e., that the effect of each predictor variable on survival remains constant with time. Another essential part of this analysis is the hazard ratio. The Cox proportional hazard model compares survival times between the different treatments and describes the risk for each animal in that treatment. One of the treatments is set as a *reference treatment*, and the other treatments are compared with that treatment. Subjects in the reference treatment are assigned a hazard ratio of *1*, also referred to as the baseline hazard. Subjects in the other treatments have hazard ratios relative to the reference level. If the hazard ratio is lower than *1*, the treatment has a reduction in hazard compared with the reference treatment, and the opposite is true for a hazard ratio higher than *1*. If the hazard ratio is *1*, the treatment had no effect compared with the reference treatment (Vittinghoff et al., 2012; Harell Jr, 2015; Moore, 2016).

The Kaplan-Meier estimator is nonparametric and given by the function:

$$\grave{S}(t) = \prod_{t_i \leq t} \left(1 - \frac{d_i}{n_i}\right)$$

The survival function denoted $\grave{S}(t)$, is the animal's probability of being event-free at time t, i.e., the survival time is greater than time t. Next, d_i denotes the number of animals failing at time t_i, and n_i is the number of animals dying at time $\geq t_i$. Kaplan-Meier curves are visualised as non-increasing step functions, where one step or multiple steps indicate the deaths of one or multiple animals (Vittinghoff et al., 2012; Harell Jr, 2015; Moore, 2016).

To create the Kaplan-Meier plots, calculate p-values and inspect the Schoenfeld residuals, I used the package *survminer* (v0.4.9; Kassambara et al, 2021). To compute the Cox's proportional hazard ratio, test the assumption of proportional hazards and test for differences between survival times of Al-exposed *A. aquaticus* between Levels 1-3 (alpha-level 0.05) I

used the *survival* package (v3.3-1; Therneau, 2022). Next, I created forest plots with the 'survivalAnalysis' package (v0.3.0; Wieswag, 2022). The Cox proportional hazards model makes two assumptions: survival curves have a proportional hazard across covariates over time, and there is a linear relationship between the log hazard and quantitative covariates. For categorical covariates, the assumption of linearity does not apply. I fitted two univariate Cox proportional hazard models where *time* and *event* variables represented the survival object and *treatment* the explanatory variable. The *time* variable is survival time in days. The event variable is the animal's status, 1 is dead, and 0 is censored. Censored animals can be, e.g., animals lost from the experiment. The *time* variable is numerical, and the *event* variable is binary. The explanatory variable is categorical with three levels. The *treatment* variable corresponds to the medium in question: *Green* is the control medium, *Purple* is the acidic Al-poor medium, and *Red* is the acidic Al-rich medium (Figure 2). In my models, *Green* is the reference treatment.

To test the assumption of proportional hazards, I used the *cox.zph* function in the *survival* package. The function tests the Schoenfeld residuals computed for each covariate in Cox's proportional hazard analysis. They are tested to be independent with time, and the function returns a p-value for each covariate. The H_0 hypothesis for the proportional hazard assumption cannot be rejected if the survival curves have a proportional hazard over time (alpha-level 0.05). I also inspected the Schoenfeld residuals visually to look for a non-random pattern with the *ggcoxzph* function from the *survminer* package. Test scores from the *cox.zph* test was not statistically significant, *Media* (log-rank test; X^2 (2) = 4.86, p = <0.088, n = 3, Table 6), which indicates that the assumption of proportional hazard was respected (Table 6). Accordingly, the Schoenfeld residuals showed a random pattern.

Table 6. Test scores from the proportional hazard assumption test with *cox.zph* function

Variable	**Chisq**	**df**	**p-value**
Media	4.86	2	0.088

Oxygen Consumption Analysis

To prepare for the oxygen consumption analysis, I used the package respfun (v0.4.4; Carey, 2020). I converted water mass to water volume with the function *wm_to_vol*, and I calculated the effective volume of each respiratory chamber for all animals with the function *eff_vol*.

I used the respR package (v2.0.2; Harianto et al., 2019) to analyse oxygen consumption and critical O_2-concentration ($[O_2]_{crit}$). I inspected the oxygen consumption visually with the function *inspect*. To reduce the influence of stress after handling the animals, I excluded the first 30 minutes (Edwards, 1960; Adcock, 1975; Hervant et al., 1998; Wilhelm et al., 2006; Ros et al., 2021). To calculate the rate of oxygen uptake, I used the function *calc_rate*. I visually inspected the data and excluded obviously unstable regions. Unstable regions are spikes to the up- or downside in the O_2-consumption curves. The reason for these spikes is the lack of a stirring mechanism inside the respirometry chambers. Therefore, when the animal moved inside the chamber, the water would be stirred and cause irregularities in the O_2-consumption curves. Next, I extracted O_2 consumption values from stable regions above 80% saturated water concentration with a minimum duration of 30 minutes. All selected regions had a minimum R-squared (r2) value of 0.95 and the same window width of 30 minutes (Svendsen et al., 2016; Prinzing et al., 2021; Ros et al., 2021). To correct for possible oxygen consumption of microorganisms in the water or fungi growth on the internal surface of the respirometry chamber. I calculated the background respiration rate for each replicate with the function *calc_rate.bg*. From a blank respiration sample, I calculated background respiration and extracted data from similar stages in the experiment as to *A. aquaticus*. Next, I adjusted the oxygen consumption to account for potential background respiration with the function *adjust_rate*. Since respR uses unitless rates, I converted the rates into specified output units with the function *convert_rate*. I used the effective volume variables to calculate the correct oxygen consumption rates. The standard output unit in respiration studies with invertebrates is µg O_2 dry weight (DW), hour (h). Therefore, I converted all the output units to this unit (Sandeman & Lasenby, 1980; Kedwards et al., 1996; Wilhelm et al., 2006). I calculated the critical O_2 concentration with the function *oxy_crit*. The function uses the broken-stick regression as the standard approach. The method iteratively fits two segments of the data and reports the intersection with the smallest sum of the residual sum of squares between the linear models as the estimated critical point. It reports breakpoints as intercept values (Yeager & Ultsch, 1989).

To detect differences between the treatments and the levels in the acidic Al-rich media on *A. aquaticus*, I conducted an analysis of variance (ANOVA) (Fisher, 1925). ANOVA uses the F-test to test for statistical significance (alpha-level 0.05). It compares the variance across means of two or more groups. I fitted two different models, both with interaction terms. I fitted the first model with normoxic oxygen consumption *(MO_2)* as the dependent variable and *treatment* and *level* as independent variables with an interaction effect between them. For the second model, I fitted the critical O_2 concentration *($[O_2]_{crit}$)* as the dependent variable and *treatment* and *level* as independent variables with an interaction effect between them. I carried out diagnostic tests to ensure that the assumptions of the ANOVA were respected. The ANOVA test makes four assumptions about the data used in the analysis. 1. Independence of observations – each subject should only belong to one group (i.e., one should not affect the other). Independence of observations can only be ensured with good experimental design for categorical variables. I separated animals in exposure chambers into smaller groups inside each tray and measured only one animal per respirometry chamber. 2. None of the subjects can be classified as significant outliers. I tested for outliers with the *identify_outliers* function in the *rstatix* package version 0.7.0 (Kassambara, 2021). It identifies values above Quartile (Q) 3 + 1.5xInterQuartileRange (IQR) or below Q1 – 1.5IQR and classifies them as significant outliers. 3. ANOVA assumes data to have a Gaussian distribution. I visualised the residuals from each model with Quantile-Quantile (QQ) plots and histograms. For QQ plots, residuals should fall along the reference line, and histograms should form a Bell curve. Further, I tested for normality with a Shapiro-Wilks test which returns a p-value. If the p-value is larger than 0.05, H_0 can be kept. There was no evidence of non-normality for the MO_2 variable ($W = 0.98$, $p = 0.59$) and the $[O_2]_{crit}$ variable ($W = 0.98$, $p = 0.26$). Last, 4. the ANOVA test assumes homogeneity of variance. I used the base R function 'plot' to make a residuals vs fit plot and found no evident relationship between residuals and fitted values. Therefore, I assumed homogeneity of variance and continued with the models I had fitted without any changes. ANOVA models only give results for each variable used in the analysis and not sub-levels in variables. Therefore, I used the *emmeans* package (v1.8.2; Lenth et al., 2022) to conduct post hoc comparisons among factors in my models. *Emmeans* calculates estimated marginal means. In the background, *emmeans* use Tukey's post hoc test for each factor in an ANOVA analysis. It returns p-values for all factor levels in each model. P-values indicate if there is a statistical difference between *treatments* and *levels* (alpha-level 0.05). To make figures 1, 2, 4, 6, 7, I used Adobe Illustrator version 26.3.1 (Adobe Inc., 2022).

Results

Locomotor Activity

In the mortality and respirometry experiments, I observed locomotor activity changes in the different exposure channels. I could observe that Al-exposed *A. aquaticus* reduced its locomotor activity and became stationary compared with animals exposed to the acidic Al-poor- or the control media. To provoke a reaction from an Al-exposed animal, I had to pump water towards it with a pipette, while in animals exposed to the acidic Al-poor- or control media, it was sufficient with a beam of light. I did not quantify these observations, but they are important observations, and therefore I mention them in my result section.

Water Chemistry

Water temperatures in the mortality experiment were stable throughout the experiment and were 1.56 ± 0.53 °C on average (n = 30, Table 7). Water temperatures in the respirometry experiments differed between the experiments. It was 5.55 ± 2.03 °C (n = 30) on average in the experiment with the acidic Al-poor medium, 13.42 ± 0.93 °C (n = 30) in the acidic Al-rich medium, and 9.88 ±1.89 °C (n = 30) in the control medium (Table **7**). The main reason for the water temperature differences was due to differences in the ambient temperature from where the operating water originated because I performed experiments from December 2020 until July 2021.

Table 7. Water temperature for various water chemical regimes (mean ±SD)

Experiment	**Media**		**Temperature (°C)**	**n**
Mortality Experiment	**control**	(pH 7)	1.56 ±0.53	30
	acidic Al-poor	(pH 5.8)	1.56 ±0.53	30
	acidic Al-rich	(pH 5.8)	1.56 ±0.53	30
Respirometry Experiment	**control**	(pH 7)	9.88 ±1.89	30
	acidic Al-poor	(pH 5.8)	5.55 ±2.03	30
	acidic Al-rich	(pH 5.8)	13.42 ±0.93	30

The average water electrical conductivity was relatively stable throughout the experimental period. It varied between 19.24 ± 6.42 µS/cm (n = 30) in the acidic Al-poor medium during the respirometry experiment and 39.04 ± 4.36 µS/cm (n = 30) in the acidic Al-rich medium in the same experiment (Table 8). Conductivity differences among experiments and media were

partly due to differences in the amount of chemicals added to the water and partly due to seasonal variation in the ambient run-off to the source of the operating water.

Table 8. Water electrical conductivity (µS/cm) for various water chemical regimes (mean ±SD)

Experiment	Media		µS/cm ±SD	n
Mortality Experiment	control	(pH 7)	20.60 ±1.28	30
	acidic Al-poor	(pH 5.8)	25.29 ±2.02	30
	acidic Al-rich	(pH 5.8)	26.72 ±2.89	30
Respirometry Experiment	control	(pH 7)	23.05 ±5.14	30
	acidic Al-poor	(pH 5.8)	19.24 ±6.42	30
	acidic Al-rich	(pH 5.8)	39.04 ±4.36	30

Water pH was very stable in all the experimental media in the mortality and respirometry experiments, and it corresponded well with the nominal pH values I aimed to create (Table 9). In the mortality experiment, the pH of the natural water (control medium) was 7.03 ± 0.11 on average. In the acidic Al-poor and acidic Al-rich media, the average pH was 5.73 ± 0.48 and 5.72 ± 0.38, respectively. In the respirometry experiment, the average water pH was 7.04 ± 0.18, 5.94 ± 0.59 and 5.95 ± 0.17 in the control-, acidic Al-poor- and acidic Al-rich media, respectively.

Table 9. Water pH for various water chemical regimes (mean ±SD)

Experiment	Media		pH ±SD	n
Mortality Experiment	control	(pH 7)	7.03 ±0.11	30
	acidic Al-poor	(pH 5.8)	5.73 ±0.48	30
	acidic Al-rich	(pH 5.8)	5.72 ±0.38	30
Respirometry Experiment	control	(pH 7)	7.04 ±0.18	30
	acidic Al-poor	(pH 5.8)	5.94 ±0.59	30
	acidic Al-rich	(pH 5.8)	5.95 ±0.17	30

During the mortality experiment, the Alr concentration in the acidic Al-rich medium was Level 1: 954 ±38 µg/L (n = 3), Level 2: 1007 ±55 µg/L (n = 3), Level 3: 1002 ±68 µg/L (n = 3) (Figure 10), and relatively stable through the experiment. Throughout the acidic Al-rich exposure channel, the Al-fractions changed as anticipated. In Level 1: 833 ±20 µg/L (n = 3) was present as monomeric Al-species (Ala), Level 2: 797 ±24 µg/L (n = 3), and Level 3: 741 ±29 µg/L (n = 3) (Figure 10). The concentration of organic monomeric Al-fraction (Alo) in Level 1: 528 ±113 µg/L, Level 2: 554 ±111 µg/L and Level 3: 605 ±68 µg/L (Figure 10) and

increased with water residence time. As a result, the inorganic monomeric Al-fraction (Ali) decreased with water residence time from 305 ±98 µg/L (n = 3) in Level 1 to 242 ±98 µg/L (n = 3) in Level 2 and 136 ±77 µg/L (n = 3) in Level 3 (Figure 10).

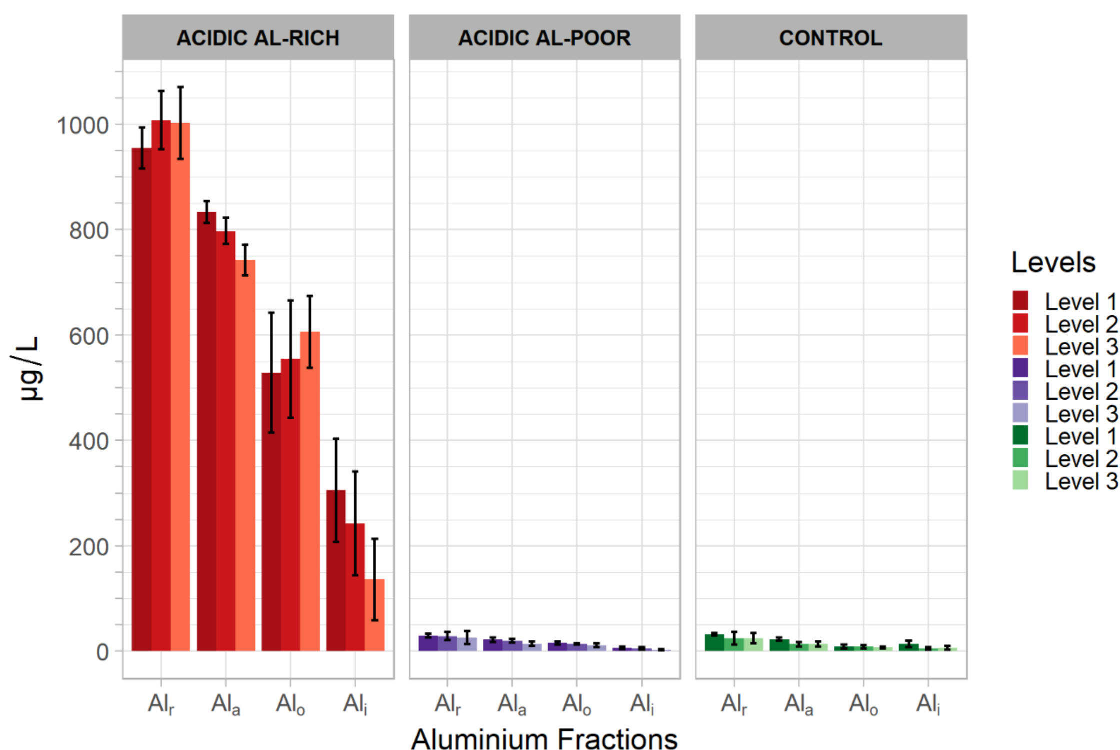


Figure 10. Aluminium fractions in the acidic Al-rich-, acidic Al-poor- and control media, respectively. The X-axis represent the different Al-species in the water samples analysed with the Barnes/Driscoll method per level. The Y-axis is the amount of each Al-species in µg/L for each level inside the exposure channel. Each level represents increasing water residence times, starting at Level 1 (1 min), Level 2 (12 min), and Level 3 (25 min). Values are displayed as mean µg/L ± SD acidic Al-rich, acidic Al-poor, control. Level 1: Alr: 954 ±38, 21 ±3, 32 ±3 µg/L, Ala: 833±20, 22 ±4, 22 ±3 µg/L Alo: 528 ±113, 15 ±3, 8 ±3 µg/L, Ali: 305 ±98, 6 ±2, 13 ±6 µg/L, Level 2: Alr: 1007 ±55, 28 ±7, 24 ±12, Ala 797 ±24, 19 ±3, 22 ±3 µg/L, Alo: 554 ±111, 13 ±1, 8 ±2 µg/L, Ali: 242 ±98, 5 ±2, 5 ±1 µg/L, Level 3: Alr: 1002 ±68, 25 ±12, 24 ±9 µg/L, Ala: 741 ±29, 14 ±4, 13 ±3 µg/L, Alo: 605 ±68, 11 ±3, 7 ±1 µg/L, Ali: 136 ±77, 2 ±1, 6 ±3 µg/L.

During the respirometry experiment, the Alr concentration in the acidic Al-rich medium was 900 ±193 µg/L (n = 3) in Level 1, 936 ±178 µg/L (n = 3) in Level 2 and 889 ±143 µg/L (n = 3) in Level 3 (Figure 11) and relatively stable throughout the experiment (Figure 11). The concentration of monomeric Al-species in the acidic Al-rich channel; Level 1: 724 ±146 µg/L (n = 3), Level 2: 755 ±94 µg/L (n = 3) and Level 3, 707 ±130 µg/L (n = 3) (Figure 11). The concentration of organic monomeric Al-fraction (Alo) in Level 1: 604 ±148 µg/L (n = 3), in Level 2: 600 ±107 µg/L (n = 3) and in Level 3: 608 ±130 µg/L (n = 3) (Figure 11) and was

stable with water residence time. As a result of this, the inorganic monomeric Al-fraction (Ali) did not change with water residence time Level 1: 119 ±10 µg/L (n = 3), Level 2: 153 ±13 µg/L (n = 3), Level 3: 99 ±21 µg/L (n = 3) (Figure 11). In the acidic Al-poor- and the control media, the Alr-concentration in Level 1 was 60 ±37 and 66 ±10 µg/L (n = 3), in Level 2 53 ±11 (n = 3) and 31 ±38 µg/L (n = 3) and Level 3, 52 ±7 (n = 3) and 31 ±7 µg/L (n = 3) respectively (Figure 11). The reportedly toxic Ali-concentration was in Level 1: 14 ±5 (n = 3) and 5 ±7 µg/L (n = 3), Level 2: 12 ±4 (n = 3) and 11 ±3 µg/L (n = 3), and Level 3: 6 ±1 (n = 3) and 7 ±3 µg/L (n = 3) (Figure 11).

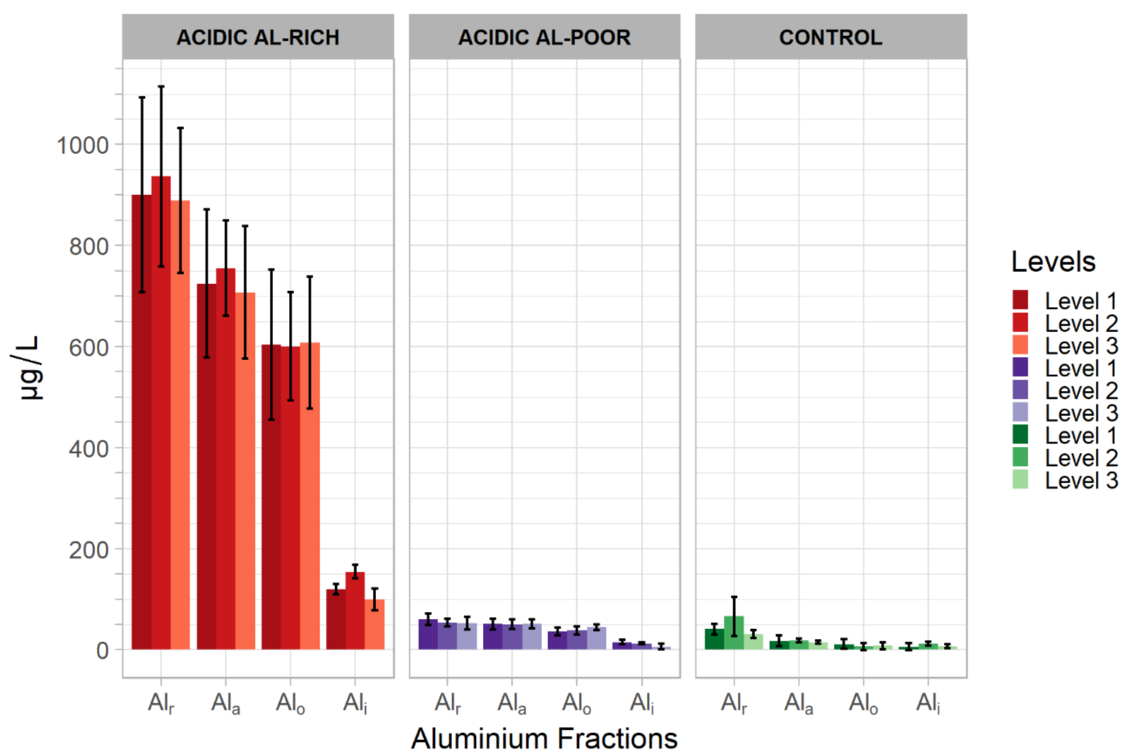


Figure 11. Aluminium fractions in the acidic Al-rich-, acidic Al-poor- and control media, respectively. The X-axis represent the different Al-species in the water samples analysed with the Barnes/Driscoll method per level. The Y-axis is the amount of each Al-species in µg/L for each level inside the exposure channel. Each level represents increasing water residence times, starting at Level 1 (1 min), Level 2 (12 min), and Level 3 (25 min). Values are displayed as mean µg/L ± SD, acidic Al-rich, acidic Al-poor, control. Level 1: Alr: 900 ±193, 60 ±37, 40 ±5 µg/L, Ala: 724 ±146, 50 ±12, 17 ±7 µg/L, Alo: 604 ±148, 35 ±8, 11±9 µg/L, Ali: 119 ±10, 14 ±5, 5 ±7 µg/L, Level 2: Alr: 936 ±178, 53 ±11, 66 ±10 µg/L, Ala: 755 ±94, 50 ±11, 17 ±10 µg/L, Alo: 600 ±107, 38 ±7, 6 ±7 µg/L, Ali: 153 ±13, 12 ±4, 11 ±3 µg/L, Level 3: Alr: 889 ±143, 52 ±7, 31 ±38 µg/L, Ala: 707 ±130, 50 ±10, 14 ±3 µg/L, Alo: 608 ±130, 44 ±8, 7 ±7 µg/L, Ali: 99 ±21, 6 ±1, 7 ±3 µg/L.

Mortality

Cumulative mortality for *A. aquaticus* compared between the different media in the mortality experiment was statistically significant (log-rank test; X^2 (2) = 17.13, p = <0.001, n = 75, Figure 12). Observed mortality in the acidic Al-rich medium was 22.67% (n = 75) and 4% (n = 75) in the control medium (Figure 12). As a result of this, the risk of dying was six times greater for *A. aquaticus* when exposed to the acidic Al-rich medium (HR 6.07, 95% CI 1.78-20.73, n = 75, Figure 13) compared with the control medium (n = 75, Figure 13). In contrast, 4% of the animals died in the acidic Al-poor medium, similar to the control medium, where 4% died (Figure 13). Therefore, no elevated risk was associated with the acidic Al-poor medium (HR 1, 95% CI 0.20-4.95, n = 75, Figure 13) compared with the control medium (n = 75, Figure 13).

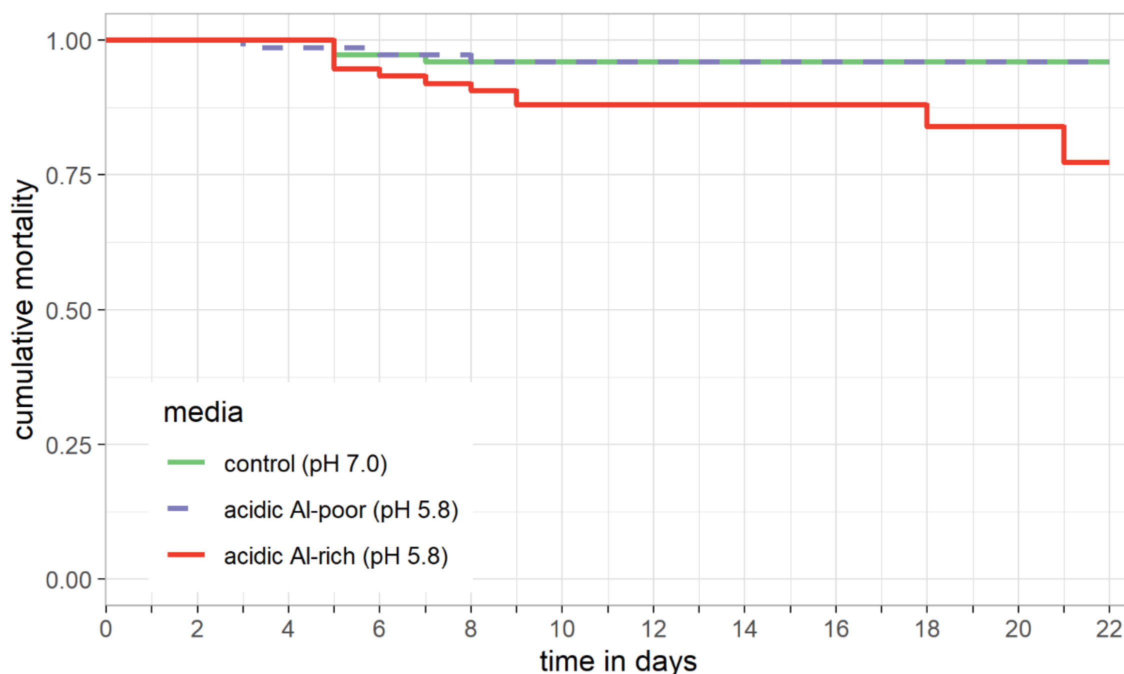


Figure 12. Cumulative mortality for A. aquaticus exposed for 22 days to control medium (pH 7.0, n = 75), acidic Al-poor medium (pH 5.8, n = 75) and acidic Al-rich medium (pH 5.8, n = 75). In the control medium: 4% of the animals died; in the acidic Al-poor medium: 4% died; and in the acidic Al-rich medium: 22.67% of the animals died.

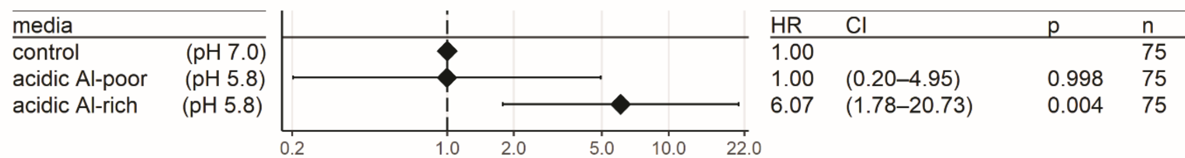


Figure 13. Univariate Analysis (Cox Proportional Hazard Regression Model) of overall mortality between the control medium (n = 75), compared with acidic Al-poor (n = 75)- and acidic Al-rich media (n = 75). The control medium is the reference level. Therefore, the acidic Al-poor and acidic Al-rich mortality are compared with the control medium. Values below 1 represent less toxic- and values above 1 represent more toxic than the reference level. Mortality comparisons are displayed with a hazard ratio (HR), 95% confidence intervals (CI), p-values and the number of animals exposed in each treatment (n). In the acidic Al-rich- the hazard ratio was 6.07 (C. I. = 1.78-20.73) and in the acidic Al-poor media 1 (C. I. = 0.20-4.95) compared with the control medium.

Comparing cumulative mortality between levels in the acidic Al-rich channel shows no statistically significant difference (log-rank test; $X^2(2) = 1.1$, $p = 0.6$, $n = 75$, Figure 14, Table 10). Therefore, there was no visible toxicity gradient between the levels in the acidic Al-rich exposure channel, where Level 1 was assumed to be most toxic, Level 2 less toxic and Level 3 slightly toxic.

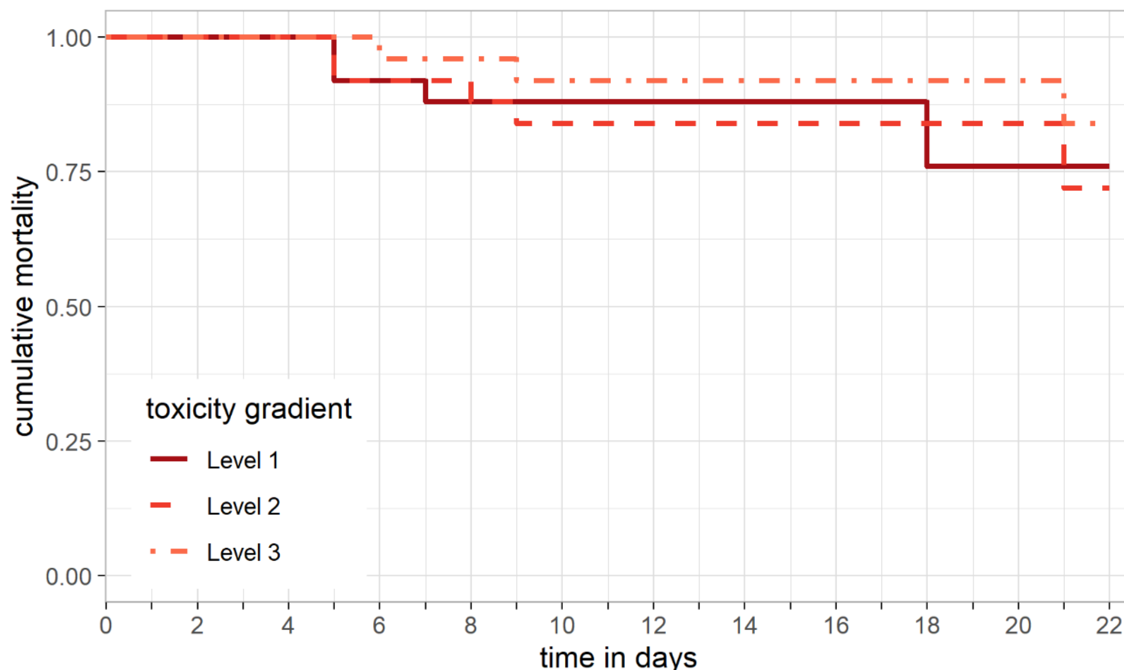


Figure 14. Cumulative mortality for *A. aquaticus* exposed to the acidic Al-rich (pH 5.8, N = 75) medium. Each level represented increasing water residence time and assumed decreasing toxicity, starting at Level 1 (1min., n = 25), dark red colour. Level 2 (12 min., n = 25), light red colour, and Level 3 (25min., n = 25), orange-reddish colour. One step down on the line represent one dead animal. Mortality in Level 1: 24%, Level 2: 32%, Level 3: 16%.

Table 10. Frequencies and Chi-Square results comparing overall survival between Level 1, Level 2, and Level 3 in the acidic Al-rich exposure channel (N = 75)

toxicity gradient	n	%	$X^2(2)$	p-value
Level 1	25	24	0.05	0.6
Level 2	25	32	0.35	0.6
Level 3	25	16	0.64	0.6

Respirometry

In the experiment with the acidic Al-rich medium (pH 5.8), *A. aquaticus* showed a median normoxic O2 consumption of 0.006, and the IQR was 0.003 µgO_2/mg DW h (n = 9, Figure 15). For the control- (pH 7.0) and acidic Al-poor media (pH 5.8), the median normoxic O_2 consumption was higher 0.023- and 0.028, and the IQR was 0.029- and 0.045 µgO_2 mg DW h, respectively (n = 9, n = 9, Figure 15).

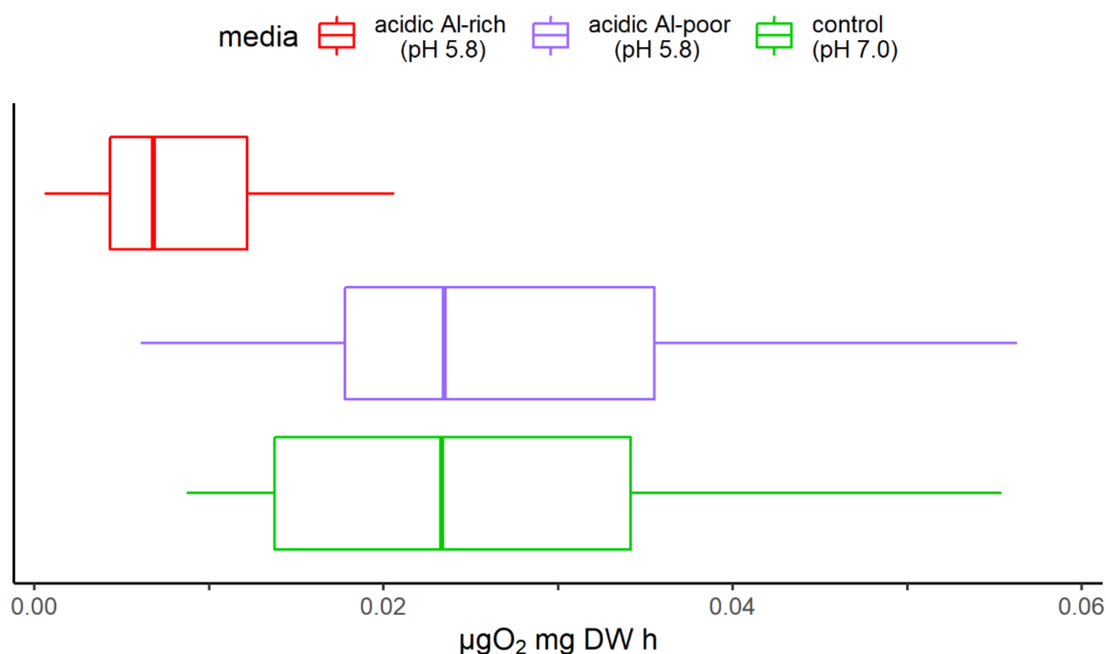


Figure 15. Comparative values of normoxic O_2-uptake (µgO_2/mg DW h) for *A. aquaticus* exposed to acidic Al-rich- (n = 9), acidic Al-poor- (n = 9) and control media (n = 9). The left side of the box represents the first quartile (1Q), the line inside the box represents the median, and the right side represents the third quartile (3Q). The lines outside the box represent the minimum and maximum values. The difference between the third and first quartile is the interquartile range (IQR). Normoxic O_2-uptake in the acidic Al-rich medium 1Q: 0.004, median: 0.006, 3Q: 0.012, IQR: 0.003, acidic Al-poor medium: 1Q: 0.017, median: 0.023, 3Q: 0.035, IQR: 0.029, control medium: 1Q: 0.013, median: 0.023, 3Q: 0.034, IQR: 0.045.

Tukey's post hoc test shows a statistically significant difference when comparing the MO_2-(p <0.001, 95% C.I = -0.024, -0.009, n = 81, Table 11) and $[O_2]_{crit}$- (p <0.001, C.I = -0.94, -0.51, n = 81, Table 12) values between the acidic Al-rich- and control media. Similarly, between the acidic Al-rich- and the acidic Al-poor media, the MO_2- (p = <0.001, C. I. = -0.025, -0.011, n = 81, Table 11) and the $[O_2]_{crit}$- (p = <0.001, C. I. = -0.076, -0.032, n = 81, Table 12) values were statistically significant. However, when comparing MO_2^- (p = 0.89, C. I. = -0.005, 0.008, n = 81, Table 11) and $[O_2]_{crit}$-values (p = 0.11, C. I. = -0.40, 0.03, n = 81, Table 12) between the acidic Al-poor- and control media, they were not statistically significantly different.

Table 11. Tukey's post hoc pairwise test results of the MO_2 rates in *A. aquaticus*.

Comparison	MO_2 (μgO_2/mg DW h)	DF	Std. Error	p-values	95% Confidence Interval	
					Lower Bound	Upper Bound
acidic Al-rich	0.006 ± 0.005	72	0.00271	<0.001	-0.025	-0.011
acidic Al-poor	0.017 ± 0.013					
acidic Al-rich	0.006 ± 0.005	72	0.00272	<0.001	-0.024	-0.009
control	0.023 ± 0.012					
acidic Al-poor	0.017 ± 0.013	72	0.00272	0.89	-0.005	0.008
control	0.023 ± 0.012					

Table 12. Tukey's post hoc pairwise test results of the $[O_2]_{crit}$ tension in *A. aquaticus*.

Comparison	$[O_2]_{crit}$ (mg O_2/L)	DF	Std. Error	p-values	95% Confidence Interval	
					Lower Bound	Upper Bound
acidic Al-rich	0.82 ± 0.303	72	0.0929	<0.001	-0.76	-0.32
acidic Al-poor	1.34 ± 0.344					
acidic Al-rich	0.82 ± 0.303	72	0.0931	<0.001	-0.94	-0.51
control	1.56 ± 0.357					
acidic Al-poor	1.34 ± 0.344	72	0.0929	0.11	-0.40	0.03
control	1.56 ± 0.357					

The median critical O_2-concentration ($[O_2]_{crit}$ (mg O_2/L) for *A. aquaticus* exposed to the acidic Al-rich medium (pH 5.8) was 0.82 mg O_2/L, and the IQR was 0.42 mg O_2/L (n = 27, Figure 16). In comparison, animals exposed to the acidic Al-poor- (n = 27) and control (n = 27) media had a higher median critical O_2-concentration ($[O_2]_{crit}$) 1.34 and 1.56 mg O_2/L, and the IQR was 0.52 and 0.5 mg O_2/L (Figure 16).

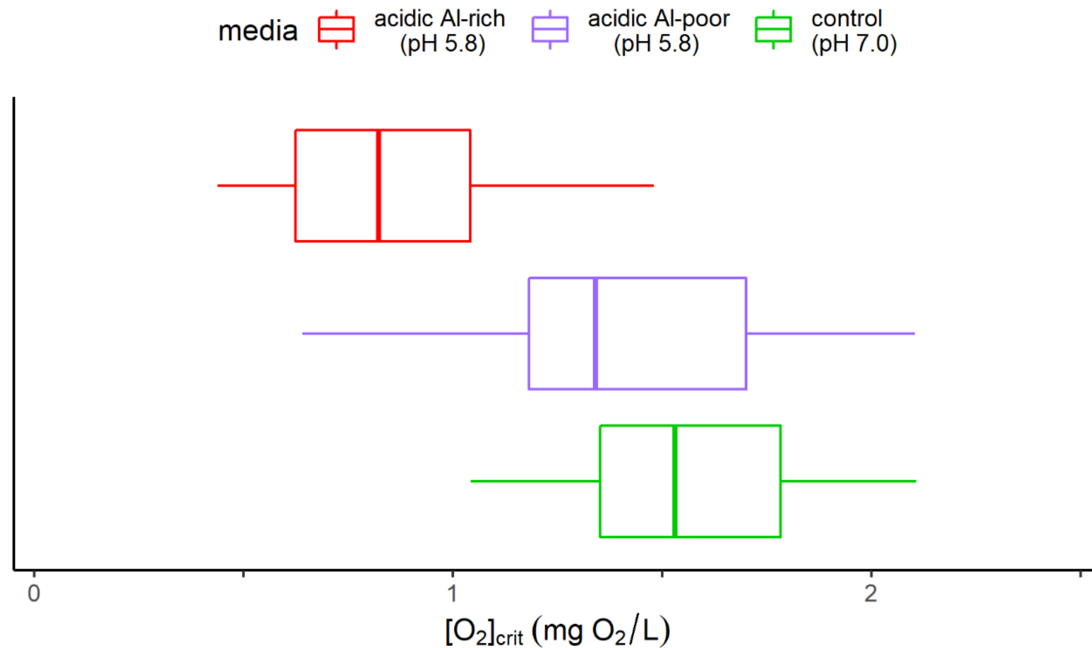


Figure 16. Comparative values of critical O_2-tension ($[O_2]_{crit}$ (mg O_2/L)) for *A. aquaticus* exposed to acidic Al-rich- (n = 27), acidic Al-poor- (n = 27) and control media (n = 27). The left side of the box represents the first quartile (1Q), the line inside the box represents the median, and the right side represents the third quartile (3Q). The lines outside the box represent the minimum and maximum values. The difference between the third and first quartile is the interquartile range (IQR). $[O_2]_{crit}$-tension in the acidic Al-rich medium 1Q: 0.62, median: 0.82, 3Q: 1.04, IQR: 0.42, acidic Al-poor medium 1Q: 1.18, median: 1.34, 3Q: 1.78, IQR: 0.6, and in the control medium 1Q: 0.01, median: 0.02, 3Q: 0.03, IQR 0.02.

The median normoxic O2 uptake overlapped between all three levels in the acidic Al-rich exposure channel (n = 27, Figure 17). Animals from Level 1 had the most consistent O2 consumption; the median MO_2 was 0.006 µgO_2/mg DW h (n = 9), and the IQR was relatively low at 0.002 µgO_2/mg DW h (Figure 17). In Level 2, the median MO_2 was 0.005 µgO_2/mg DW h (n = 9), and the IQR was 0.011 µgO_2/mg DW h, and animals displayed a larger variation in O2 consumption compared with Level 1 (Figure 17). However, animals from Level 3 had the highest median normoxic O2 consumption of 0.01 µgO_2/mg DW h (n = 9) and the highest IQR of 0.009 µgO_2/mg DW h (Figure 17).

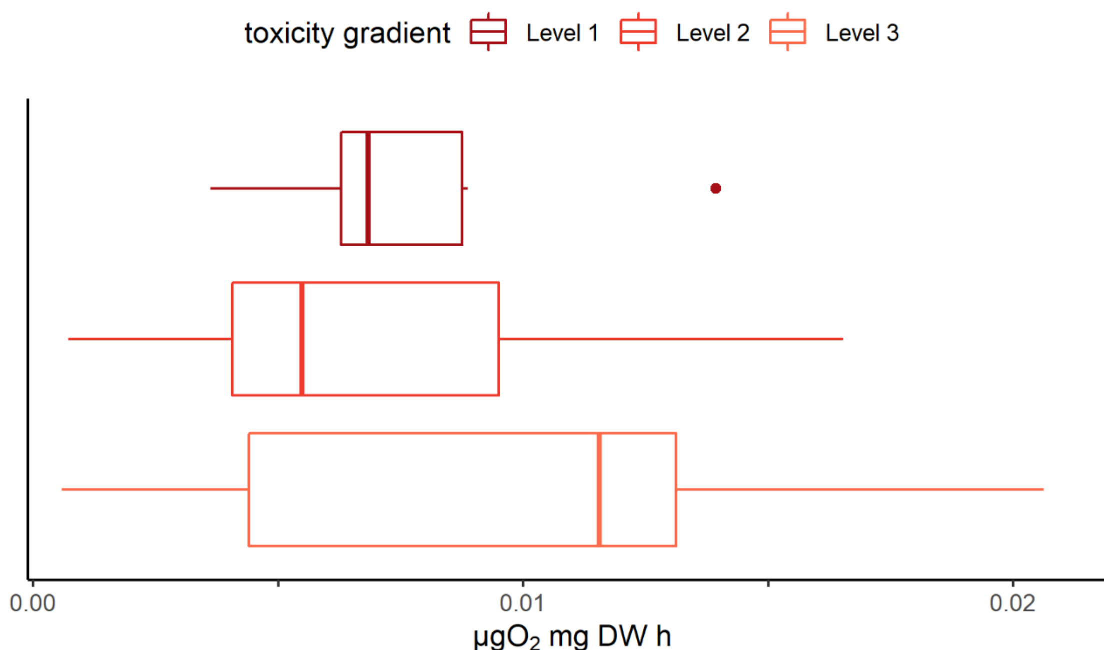


Figure 17. Comparative values of normoxic O_2-uptake (MO_2 (μgO_2/mg DW h)) in *A. aquaticus* exposed to acidic Al-rich medium between Level 1, Level 2, and Level 3. Each level represents an increase in water residence time and a decrease in assumed toxicity. The left side of the box represents the first quartile (1Q), the line inside the box represents the median, and the right side represents the third quartile (3Q). The lines outside the box represent the minimum and maximum values, and the dot is a potential outlier. The difference between the third and first quartile is the interquartile range (IQR). MO_2 rates in Level 1 1Q: 0.006, median: 0.006, 3Q: 0.008, IQR: 0.002, Level 2 1Q: 0.004, median: 0.005, 3Q: 0.009, IQR, 0.011 Level 3 1Q: 0.004, median: 0.011, 3Q: 0.013, IQR: 0.009

For *A. aquaticus* exposed to the acidic Al-rich medium, the median critical O_2-tension in Level 1: 0.82 mg O_2/L (n = 9), IQR: 0.42 mg O_2/L (Figure 18). In Level 2, the median critical O2 tension was higher: 0.94 mg O_2/L (n = 9) and the IQR: 0.39 mg O_2/L (Figure 18). Level 3 had the lowest median critical O2 tension: 0.77 mg O_2/L (n = 9), and the IQR was 0.4 mg O_2/L (Figure 18).

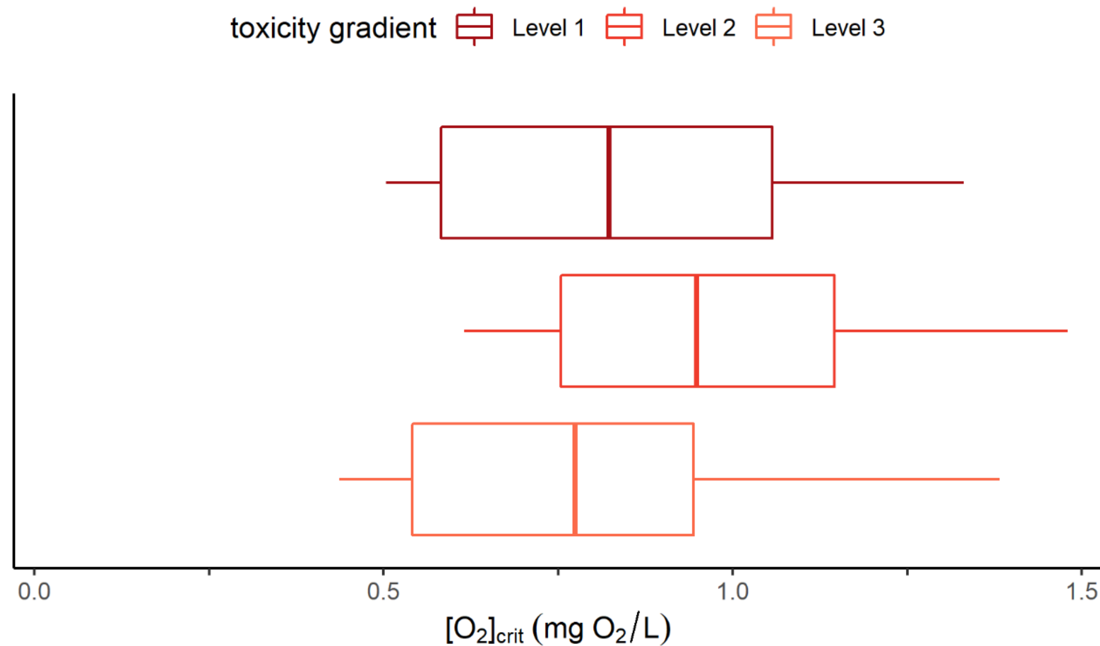


Figure 18. Comparative values of critical O_2-tension (mg O_2/L) in A. aquaticus exposed to acidic Al-rich medium between Level 1 (n = 9), Level 2 (n = 9) and Level 3 (n = 9). Each level represents an increase in water residence time and a decrease in assumed toxicity. The left side of the box represents the first quartile (1Q), the line inside the box represents the median, and the right side represents the third quartile (3Q). The lines outside the box represent the minimum and maximum values, and the dot is a potential outlier. The difference between the third and first quartile is the interquartile range (IQR). The $[O_2]_{crit}$-tension (mg O_2/L) in Level 1 1Q: 0.58, median: 0.82, 3Q: 1.05, IQR: 0.47, Level 2 1Q: 0.75, 0.94, 3Q: 1.14, IQR: 0.39, Level 3 1Q: 0.54, median: 0.77, 3Q: 0.94, IQR: 0.4.

Results from Tukey's post hoc test are reported in Table 13. When comparing the normoxic O2 consumption between the different media, Tukey's post hoc test results showed that the acidic Al-rich medium Level 1 (n = 9) was statistically significant from the acidic Al-poor medium Level 2 (n = 9) (p <0.001, C. I. = 0.01, 0.04). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 1 (n = 9) with the acidic Al-poor medium Level 3 (n = 9) shows a statistically significant difference (p <0.001, C. I. = 0.006, 0.036). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 1 (n = 9) with the control medium Level 1 (n = 9) shows a statistically significant difference (p <0.001, C. I. = -0.038, -0.008). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 1 (n = 9) with the control medium Level 2 (n = 9) shows a statistically significant difference (p = 0.002, C. I. = 0.004, 0.034). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 2 (n = 9) with the acidic Al-poor medium Level 2 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = -0.04, -0.01). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 2 (n = 9) with the control medium Level 2 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = -0.034, -0.003).

Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 3 (n = 9) with the acidic Al-poor medium Level 2 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = -0.04, -0.01). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 3 (n = 9) with the acidic Al-poor medium Level 3 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = -0.034, -0.005). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 3 (n = 9) with the control medium Level 1 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = -0.036, -0.007). Comparing the normoxic O2 consumption in the acidic Al-rich medium Level 3 (n = 9) with the control medium Level 2 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = -0.032, -0.003). Comparing the normoxic O2 consumption in the acidic Al-poor medium Level 1 (n = 9) with the acidic Al-poor Level 2 (n = 9) shows a statistically significant difference (p < 0.001, C. I. = 0.003, 0.033). Comparing the normoxic O2 consumption in the acidic Al-poor medium Level 1 (n = 9) with the control medium Level 1 (n = 9) shows a statistically significant difference (p = 0.04, C. I. = -0.030, 0.8^{5}). Comparing the normoxic O2 consumption in the acidic Al-poor medium Level 2 (n = 9) with the control medium Level 3 (n = 9) shows a statistically significant difference (p < 0.001, C.I. = -0.032, - 0.002).

Table 13. Normoxic O_2 consumption values from the different test media. Significant difference when the normoxic O_2 rate for *A. aquaticus* exposed to different media with different water residence time was compared using the Tukey's post hoc test are marked with asterixis. Values represent median μgO_2/mg DW h and ± SD for all animals in one level. The top number in the row represents the horizontal media, and the bottom number represents the vertical media. Asterisks indicate statistically significant differences: $p < 0.05$ = *, $p < 0.01$ = ** and $p < 0.001$ = ***

Media	**acidic Al-rich Level 1**	**acidic Al-rich Level 2**	**acidic Al-rich Level 3**	**acidic Al-poor Level 1**	**acidic Al-poor Level 2**	**acidic Al-poor Level 3**	**control level 1**	**control level 2**	**control level 3**
acidic Al-rich Level 1									
acidic Al-rich Level 2	0.006 ±0.003 0.005 ±0.005								
acidic Al-rich Level 3	0.006 ±0.003 0.009 ±0.006	0.005 ±0.005 0.009 ±0.006							
acidic Al-poor Level 1	0.006 ±0.003 0.014 ±0.009	0.005 ±0.005 0.014 ±0.009	0.009 ±0.006 0.014 ±0.009						
acidic Al-poor Level 2	0.006 ±0.003 *** 0.032 ±0.016	0.005 ±0.005 *** 0.032 ±0.016	0.009 ±0.006 *** 0.032 ±0.016	0.014 ±0.009 ** 0.032 ±0.016					
acidic Al-poor Level 3	0.006 ±0.003 *** 0.032 ±0.008	0.005 ±0.005 ** 0.032 ±0.008	0.009 ±0.006 ** 0.032 ±0.008	0.014 ±0.009 0.032 ±0.008	0.032 ±0.016 0.032 ±0.008				
control level 1	0.006 ±0.003 *** 0.029 ±0.015	0.005 ±0.005 *** 0.029 ±0.015	0.009 ±0.006 *** 0.029 ±0.015	0.014 ±0.009 0.029 ±0.015	0.032 ±0.016 0.029 ±0.015	0.032 ±0.008 0.029 ±0.015			
control level 2	0.006 ±0.003 ** 0.025 ±0.010	0.005 ±0.005 ** 0.025 ±0.010	0.009 ±0.006 ** 0.025 ±0.010	0.014 ±0.009 0.025 ±0.010	0.032 ±0.016 0.025 ±0.010	0.032 ±0.008 0.025 ±0.010	0.029 ±0.015 0.025 ±0.010		
control level 3	0.006 ±0.003 0.013 ±0.007	0.005 ±0.005 0.013 ±0.007	0.009 ±0.006 0.013 ±0.007	0.014 ±0.009 0.013 ±0.007	0.032 ±0.016 0.013 ±0.007	0.032 ±0.008 0.013 ±0.007	0.029 ±0.015 0.013 ±0.007	0.025 ±0.010 0.013 ±0.007	

Results from Tukey's post hoc test are reported in Table 14. Comparing the critical O_2 concentration between the acidic Al-rich medium Level 1 ($n = 9$) and the acidic Al-poor medium Level 1 ($n = 9$) ($p < 0.001$, -1.06, -0.03), Level 2 ($n = 9$) ($p < 0.001$, 0.07, 1.10) and Level 3 ($n = 9$) ($p < 0.001$, C. I. = 0.05, 1.08) there is a statistically significant difference. Also control medium Level 1 ($n = 9$) ($p < 0.001$, C. I. = -1.40, - 0.37), Level 2 ($n = 9$) ($p < 0.001$, C. I. = 0.25, 1.28) and Level 3 ($n = 9$) ($p < 0.001$, C. I. = 0.09, 1.12) was statistically significant compared with the acidic Al-rich medium Level 1 ($n = 9$). For Level 2 ($n = 9$) in the acidic Al-rich medium only the control medium Level 1 ($n = 9$) ($p < 0.001$, C. I = -1.33, -0.27), Level 2 ($p < 0.001$, C. I. = 0.25, 1.28) and Level 3 ($p < 0.001$, C. I. = -0.006, 1.054) was statistically significant compared with it. For Level 3, critical O2 concentrations in the acidic Al-rich medium compared with the acidic Al-poor medium Level 2 ($p < 0.001$, C. I. = -1.09, -0.09) and Level 3 ($p < 0.001$, C. I. = -1.07, -0.07) were statistically significantly different. However, for the control medium Level 1 ($p < 0.001$, C. I. = -1.39, -0.39), Level 2 ($p < 0.001$, C. I. = -1.27, -0.26) and Level 3 ($p < 0.001$, C. I. = -1.11, -0.10) was statistically significant different from Level 3 in the acidic Al-rich medium.

Table 14. Critical O_2-tension ($[O_2]_{crit}$) values from the different test media. Significant difference when the critical O_2-tension ($[O_2]_{crit}$) for *A. aquaticus* exposed to different media with different water residence time was compared with the Tukey's post hoc test are marked with asterixis. Values represent median µgO_2/mg DW h and ± SD for all animals in one level. The top number in the row represents the horizontal media, and the bottom number represents the vertical media. Asterisks indicate statistically significant differences: $p < 0.05$ = *, $p < 0.01$ = ** and $p < 0.001$ = ***

Media	**acidic Al-rich Level 1**	**acidic Al-rich Level 2**	**acidic Al-rich Level 3**	**acidic Al-poor Level 1**	**acidic Al-poor Level 2**	**acidic Al-poor Level 3**	**control level 1**	**control level 2**	**control level 3**
acidic Al-rich Level 1									
acidic Al-rich Level 2	0.82 ±0.311 0.94 ±0.293								
acidic Al-rich Level 3	0.82 ±0.311 0.74 ±0.299	0.94 ±0.293 0.74 ±0.299							
acidic Al-poor Level 1	0.82 ±0.311 * 1.34 ±0.224	0.94 ±0.293 1.34 ±0.224	0.74 ±0.299 * 1.34 ±0.224						
acidic Al-poor Level 2	0.82 ±0.311 * 1.30 ±0.313	0.94 ±0.293 1.30 ±0.313	0.74 ±0.299 ** 1.30 ±0.313	1.34 ±0.224 1.30 ±0.313					
acidic Al-poor Level 3	0.82 ±0.311 * 1.51 ±0.487	0.94 ±0.293 1.51 ±0.487	0.74 ±0.299 ** 1.51 ±0.487	1.34 ±0.224 1.51 ±0.487	1.30 ±0.313 1.51 ±0.487				
control level 1	0.82 ±0.311 *** 1.69 ±0.242	0.94 ±0.293 *** 1.69 ±0.242	0.74 ±0.299 *** 1.69 ±0.242	1.34 ±0.224 1.69 ±0.242	1.30 ±0.313 1.69 ±0.242	1.51 ±0.487 1.69 ±0.242			
control level 2	0.82 ±0.311 *** 1.49 ±0.520	0.94 ±0.293 ** 1.49 ±0.520	0.74 ±0.299 ** 1.49 ±0.520	1.34 ±0.224 1.49 ±0.520	1.30 ±0.313 1.49 ±0.520	1.51 ±0.487 1.49 ±0.520	1.69 ±0.311 1.49 ±0.520		
control level 3	0.82 ±0.311 ** 1.47 ±0.201	0.94 ±0.293 * 1.47 ±0.201	0.74 ±0.299 ** 1.47 ±0.201	1.34 ±0.224 1.47 ±0.201	1.30 ±0.313 1.47 ±0.201	1.51 ±0.487 1.47 ±0.201	1.69 ±0.311 1.47 ±0.201	1.49 ±0.520 1.47 ±0.201	

Discussion

In the present study, it is documented for the first time how *A. aquatics* responds to transient Al chemistry. In the first experiment, the mortality experiment, I found that *A. aquaticus* was less tolerant to the acidic Al-rich medium compared with acidic Al-poor- and control media (Figure 12, Figure 13). I did not find a decrease in mortality with the degree of ongoing Al polymerisation (Table 10). In the second experiment, the respirometry experiment, I found that *A. aquaticus* had lower normoxic O_2 consumption and lower critical O_2 tension in the acidic Al-rich medium compared with the acidic Al-poor- and control media (Figure 15, Figure 16, Table 11). Neither the MO_2 nor $[O_2]_{crit}$ decrease with water residence time in the acidic Al-rich exposure channel as was expected. Therefore, my results are inconclusive. They support that aqueous aluminium is more toxic to *A. aquaticus* compared with the acidic Al-poor- and control media. However, there is no support for that toxicity is dependent on the ongoing Al polymerisation, similar to fish. Neither is there a link between the degree of Al polymerisation and the respiration in *A. aquaticus*.

Al chemistry

My Al analyses show that *A. aquaticus* were exposed to ongoing Al polymerisation in the acidic Al-rich medium in the mortality experiment. In the respirometry experiment, however, most of the Al polymerisation was finished before it reached Level 2 and -3. Therefore, I did not measure increasing Alo- or decreasing Ali-concentrations in the acidic Al-rich exposure channel in the respirometry experiment. I will, therefore, describe the Al chemistry in the mortality experiment. During the experiment, I measured a change in Al chemistry through the acidic Al-rich exposure channel. With increased water residence time, the total monomeric Al fraction, Ala, decreased (Figure 10, Figure 11). This is because large Al polymers are not extractable after 20 sec. with the Barnes/Driscoll method (Barnes, 1975; Driscoll, 1984). For the organic monomeric Al fraction, Alo, I measured an increase due to ongoing Al polymerisation, where positively charged Al species will start to bind to other ligands. With time (ageing), their net charge will approach zero and pass through the ion-exchange column as organically bound aluminium. Following is a decrease in the concentration of inorganic monomeric Al, Ali, with increased water residence time because the inorganic aluminium binds to organic ligands in the water as part of the Al polymerisation process. The aluminium I added to the operating water was on $Al3^{+}$-form; with a rapid

increase in pH, it starts to polymerise. This changes the distribution of aqueous monomeric Al products from toxic inorganic species to less toxic organic species (Lydersen et al., 1990). Therefore, a decrease in Ali-, and an increase in Alo-concentration, show that Al polymerisation was taking place (Figure 10, Figure 11) (Hem & Roberson, 1967; Driscoll, 1984; Lydersen et al., 1994). Earlier studies looking at aluminium toxicity in freshwater organisms have made an Al-rich medium for Al hydrolysis equivalent to mine (Poléo et al., 1994; Poléo & Bjerkely, 2000; Poléo & Hytterød, 2003; Poléo et al., 2021). Their Al chemistry was similar to what I observed in my experiment.

Is aqueous aluminium toxic to *A. aquaticus* and is a possible Al-toxicity in dependent on the degree of aluminium polymerisation similar to fish?

I have not found other studies exposing *A. aquaticus* to ongoing Al polymerisation. Earlier studies were conducted with a steady-state Al chemistry or focused on the bioaccumulation of aluminium (Økland, 1980; Burton & Allan, 1986; Havas, 1986b; Martin & Holdich, 1986; Herrmann & Frick, 1995; Elangovan et al., 1999; O'Callaghan et al., 2019). The majority of studies with invertebrates did not find an added effect of Al to lowered water pH (Biesinger & Christensen, 1972; Lamb & Bailey, 1981; Havas & Hutchinson, 1982; Berrill et al., 1985; Hall et al., 1985; Havas & Likens, 1985b; Burton & Allan, 1986; Martin & Holdich, 1986; Hall et al., 1987; Ormerod et al., 1987; Weatherley et al., 1988; Mackie, 1989; McCahon & Poulton, 1991; Tabakk & Gibbs, 1991; Wren & Stephenson, 1991; Storey et al., 1992; Havens, 1993; Havas & Rosseland, 1995; Sparling & Lowe, 1996; Gensemer & Playle, 1999). For those that did, there is a weakness; many of the studies are field experiments or did not consider transient Al chemistry. Therefore, it is difficult to separate the effect of low pH from the effect of transient Al chemistry. One study, however, by Martin & Holdich (1986) indicated that aqueous Al was more toxic to A. aquaticus than *Crangonyx pseudogracilis* at pH 6.5, opposite of what they assumed. Burton & Allan (1986) compared Al sensitivity in *Asellus intermedius* at pH 4.0 with 500 µg Al/L added to the operating water and at pH 5.0 with 250 µg Al/L. They found no elevated sensitivity in Al exposed *A. intermedius* at pH 4.0 or 5.0. Because both studies added aluminium to water with pH either above or below what is considered favourable for aluminium polymerisation, I conclude that the animals were not exposed to a transient Al chemistry. Still, the study by Martin & Holdich (1986) indicates that A. aquaticus can be more sensitive to aqueous aluminium than other invertebrates. The studies by Martin & Holdich (1986) and Burton & Allan (1986)

seem to be the only examples of aluminium exposure in the *Asellus* genus. Because of this, I will discuss my results based on earlier studies where transient Al chemistry has been investigated in fish.

Mortality in acidic Al-poor exposed *A. aquaticus* was 4%, similar to the control medium, while in Al-exposed *A. aquaticus*, mortality was 22.67% (Figure 12, Figure 13). In the acidic Al-poor- and control exposure channels, the total concentration of Al, Alr, was much lower than in the acidic Al-rich (Figure 10). This showed that *A. aquaticus* is more sensitive to the acidic Al-rich medium than the acidic Al-poor- and control media (Figure 12, Figure 13) . However, mortality in the different levels in the acidic Al-rich exposure channel was relatively constant, Level 1: 24%, Level 2: 32%, and Level 3: 16 (Figure 14, Table 10). I observed a decrease in the anticipated toxic Ali-concentration with increased water residence time throughout the acidic Al-rich exposure channel. From what is reported in studies with Al-exposed fish, mortality in *A. aquaticus* is not as I expected. In studies on fish, toxicity is reported to decrease with the degree of ongoing Al-polymerisation (Rosseland et al., 1992; Poléo et al., 1994; Poléo, 1995; Poléo & Bjerkely, 2000; Poléo et al., 2021). When inorganic Al^{3+} at pH 2 is added to water with higher pH aluminium starts to polymerise. In the first stage of polymerisation Al is assumed to be most toxic to fish because it attaches and polymerises on its negatively charged operculum and gill epithelium surface and mucous, then the effect decreases with time. I expected similarities for *A. aquaticus,* too, because an operculum protects the gills, and waste excretion products (CO_2 and NH_3) diffuse similarly to fish, which should render the gill microenvironment favourably for Al polymerisation.

In the acidic Al-rich exposure channel, I observed behavioural changes in *A. aquaticus*. The locomotory activity was reduced compared with animals in the acidic Al-poor- and control channels. These behavioural changes seemed constant throughout the exposure channel and did not change with water residence time. One reason for lowering the locomotory activity could be to preserve energy. Because observations were not quantified, I cannot draw any conclusions. However, they are still interesting because they differ from behavioural changes in fish. Where the effect of transient Al-chemistry on behaviour is most severe in the initial phase and decreases with water residence time (Poléo & Bjerkely, 2000; Poléo et al., 2017).

Herrmann & Anderson (1986) proposed that Al-toxicity at pH 4.8 (2000 µg/L Al) in three species of lotic mayfly nymphs could be because Al-hydroxides precipitated on the gills and mucous. Therefore, preventing access to O_2, lowering respiration efficiency, and forcing the

animals to compensate. Under such conditions, adding 2000 µg/L Al is likely creating oversaturated conditions and physically blocking access to O_2.

The observations by Herrmann & Andersson (1986) together with my observation of locomotory activity changes in *A. aquaticus,* could help explain why toxicity is not dependent on the degree of Al polymerisation. On my part, this is only speculation since I did not quantify locomotory activity changes in *A. aquaticus*. Nevertheless, it is worth noting and should be investigated further in future studies.

Is there a link between the degree of aluminium polymerisation and respiration in *A. aquaticus*?

My results show that Al-exposed *A. aquaticus,* in most cases, displayed a significantly lower Mo_2 rate and $[O_2]_{crit}$ tension than in the acidic Al-poor- and control media, indicating less sensitivity to the acidic Al-rich medium (Figure 15, Table 11, Figure 16, Table 12)**.** In the acidic Al-rich exposure channel, the Alr concentration was much higher than in the acidic Al-poor- and control media (Figure 11). The Ali concentration was relatively stable in the acidic Al-rich exposure channel compared to what I aimed for. It did not decrease with increased water residence time, indicating that Al-polymerisation finished before it reached Levels 2 and 3 (Figure 11). The MO_2 rate and $[O2]_{crit}$ tension in Al-exposed *A. aquaticus* was relatively constant and did not vary significantly with increased water residence time (Figure 17, Figure 18, Table 13, Table 14)**.** Suppose the MO_2 rate and $[O_2]_{crit}$ tension changes in Level 1 came from Al-polymerisation. Then, MO_2 rate and a $[O_2]_{crit}$ tension for Levels 2 and 3 should have decreased with water residence time, as is known from studies on fish (Rosseland et al., 1992; Poléo et al., 1994; Poléo, 1995; Poléo & Bjerkely, 2000; Poléo et al., 2021). Similar to observations in the mortality experiment, Al-exposed *A. aquaticus* displayed reduced locomotor activity compared with the acidic Al-poor- and control media in the respirometry experiment. How *A. aquaticus* regulate its metabolic rate can help explain these changes. Under hypoxic conditions, it can regulate its metabolism by reducing locomotory activity. This reduction in locomotory activity will consequently lower the MO_2 rate, i.e., oxy-regulation. It can look like Al-exposed *A. aquaticus* reduces its energy expenditure and enters into metabolic depression in an attempt to survive (Edwards, 1960; Herreid, 1980; Hervant et al., 1996). This reduction in activity can help explain why the MO_2 rates are lower in Al-exposed animals. Childress (1971) showed a linear relationship between MO_2 rate and $[O_2]_{crit}$ tension for *Gnathophausia ingens* at hypoxic conditions. His results can help explain why $[O_2]_{crit}$ tension is lower for Al-exposed *A. aquaticus* than acidic Al-poor-

and control media. The low $[O_2]_{crit}$ tension in Al-exposed *A. aquaticus* indicates a higher tolerance than in the acidic AL-poor- and control media. This is contradictory to what I observed in the mortality experiment because mortality occurred in Al-exposed *A. aquaticus* after five days (Figure 12). It is worth mentioning the higher water temperature in the respirometry experiment than in the mortality experiment (Table 8). However, Rotvit & Jacobsen (2013) only found MO_2 to increase with water temperature, not $[O_2]_{crit}$.

Similarly to the mortality experiment, I speculate that the changes caused in MO_2 rate and $[O_2]_{crit}$ tension are caused by to oversaturated conditions. Therefore, Al-hydroxides precipitate on the operculum and gill surface of *A. aquaticus*, physically blocking access to O_2, lowering respiration efficiency, and forcing the animals to compensate by reducing their metabolic rate. The exposure time in the respiration experiment was much shorter than in the mortality experiment. Mortality first occurred after five days in the mortality experiment. Therefore, I moved animals from the exposure channels into the respirometry chambers after five days. When the animals were moved from the acidic Al-rich exposure channel into the respirometry chamber the supply of Al-hydroxides ceases. Because mortality occurred at day five and continue to increase, five days exposure time seems enough for Al-exposed *A. aquaticus* to have gill damages and ,therefore, stimulate to a continued energy saving strategy. Similar behaviour is observed in *Carassius carassius* as a strategy to survive anoxia (Nilsson et al., 1993; Poléo et al., 2017).

Previous studies analysing the standard metabolic rate in *A. aquaticus* obtained similar results to me under similar environmental conditions to the control medium (Hamburger & Dall, 1990; Hervant et al., 1997). Therefore, I am confident that my results in the respirometry experiment are valid even though they are inconsistent with the results from the mortality experiment.

Conclusion

Aqueous aluminium plays a significant role in the toxicity observed by *A. aquaticus* in acidified water. This study contributes with results suggesting that elevated concentrations of aqueous aluminium are essential in understanding the toxicity of *A. aquaticus* in acidified water. However, my results show that the ongoing Al-polymerisation process, as we know from fish, is not toxic to *A. aquaticus*.

Because the toxicity in *A. aquaticus* is probably not linked to a specific Al species similar to fish, it is unclear which mechanisms drive the toxicity and why Al-exposed *A. aquaticus* experiences higher mortality compared with the acidic Al-poor- and control media.

My results show that Al-exposed *A. aquaticus* had a lower MO_2 rate and $[O_2]_{crit}$ tension than in the acidic Al-poor- and control media. These results are inconclusive with the results from the mortality experiment, and future research is needed to gain more knowledge on Al-toxicity in *A. aquaticus*. In a future where precipitation is becoming more intense and occurs more often, waters can experience episodic acidification and leaching of aluminium, and it is essential to know what mechanisms drive the toxicity in invertebrates.

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Appendix A: Figures

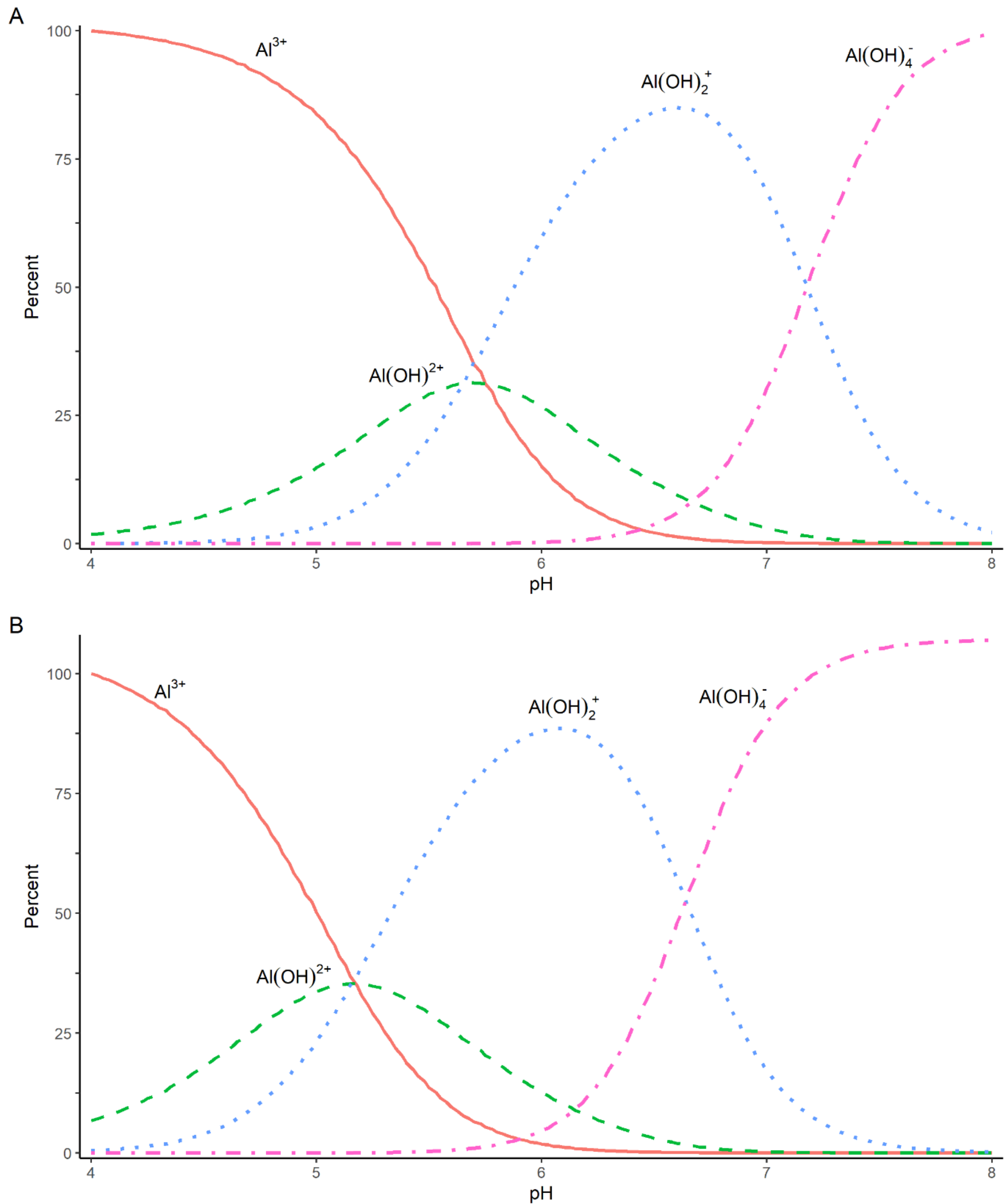

Figure A1. Distribution of aqueous monomeric hydrolysis products of aluminium. The equilibrium is shifted from left to right with increased pH and temperature. A is aluminium hydrolysis in 2°C and B is in 25°C water temperature. After Lydersen (1990).

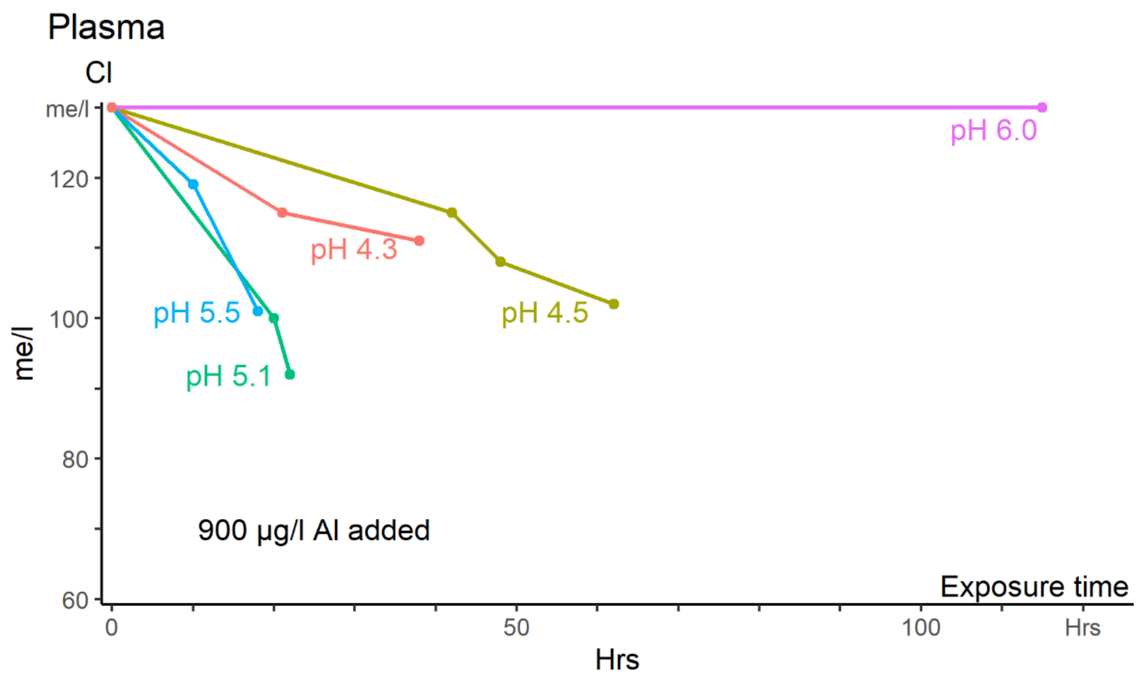

Figure A2. Loss of chloride (Cl) from blood plasma in *S. trutta* exposed to water added with 900 µg Al/L under different pH regimes. Recreated with data from Muniz & Leivestad (1980).