Toxicological effects of aluminium in relation to diel pH changes on fish and kōura

ERI Report Number 160

Client report prepared for Bay of Plenty Regional Council
By Emily Fensham, Nick Ling and Grant Tempero
2022

Environmental Research Institute
Division of Health, Engineering, Computing & Science,
University of Waikato, Private Bag 3105
Hamilton 3240, New Zealand

Te Tumu Whakaora Taiao
Environmental
Research Institute
THE UNIVERSITY OF WAIKATO

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Cite report as:
Fensham E., Ling N., and Tempero G.W. 2022. Toxicological effects of aluminium in relation to diel pH changes on fish and kōura. ERI Report No. 160, a client report prepared for Bay of Plenty Regional Council. Environmental Research Institute – Te Tumu Whakaora Taiao, Division of Health, Engineering, Computing & Science, University of Waikato, Hamilton, New Zealand. 43 pp. ISSN 2463-6029 (Print) & ISSN 2350-3432 (Online)

Cover photo: Kōura (*Paranephrops planifrons*) in Lake Rotoma, Bay of Plenty. Photo: Warrick Powrie.

Reviewed by:

Associate Professor Deniz Özkundakci

Toihuarewa Waimāori - Chair in Lake and Freshwater Science

Environmental Research Institute

Approved for release by:

Dr Charles Lee

Academic Co-Director

Environmental Research Institute

Executive Summary

Since 2006, alum (aluminium sulphate; $Al_2(SO_4)_3$) has been applied to the Utuhina and Puarenga Streams at a targeted dose rate of 1 mg Al L^{-1} to control phosphorus loading to Lake Rotorua. Alum dosing is widely used for water quality restoration and, under circumneutral pH (6-8), is considered to have no significant toxicological impacts at low to moderate (0.1–2 mg Al L^{-1}) concentrations. At circumneutral pH, alum forms aluminium hydroxide ($AlOH_3$), a white insoluble precipitate which adsorbs phosphorus, reducing its availability for phytoplankton growth. However, at high or low pH, monomeric and hydroxy aluminium species occur in varying proportions with respect to acidic (i.e., Al^{3+}, $AlOH^{2+}$, $Al(OH)_2^{+}$) and alkaline (i.e., $Al(OH)_4^{-}$) conditions with increasing toxicity to aquatic organisms.

Under typical conditions, the pH of Lake Rotorua is near pH 7, but due to its limited buffering capacity may reach pH 10 during intensive algal blooms. These diel increases in pH occur due to the photosynthetic uptake of CO_2 during the day, thereby increasing the environmental hydroxide concentration and raising the pH of the lake. At night, respiration releases CO_2, driving down the pH due to the formation of carbonic acid. Diel pH cycling has the potential to solubilise alum-derived aluminium, resulting in toxicological impacts on aquatic biota. Previous research has primarily focused on the toxicological impacts of aluminium under acidic or, more recently, alkaline conditions, however, potential impacts during transient exposure to alkaline pH have not been reported.

The University of Waikato was contracted to investigate the effects of aluminium at 2 mg L^{-1} in association with diel pH cycling on rainbow trout (*Oncorhynchus mykiss*), common bully (*Gobiomorphus cotidianus*) and kōura (*Paranephrops planifrons*) osmoregulation and respiration. Potential osmoregulatory effects were investigated by exposure of rainbow trout and kōura to aluminium at 2 mg L^{-1} under diel pH cycling (pH 7–10) over 10 days. No significant differences between control and treatment groups were observed in either plasma and haemolymph osmolarity, haematocrit or haemoglobin concentration (Student's *t*-test, $P > 0.05$). A significant difference (Student's *t*-test, $P < 0.05$) in the mean cell haemoglobin concentration (MCHC) in the rainbow trout control group was attributed to erythrocyte swelling, suggesting a generalised stress-induced response rather than an impact from aluminium. Histological examination of kōura and rainbow trout gill tissue was also conducted, with abnormalities observed within both control and treatment groups for each species. A significant difference (Kolomogorov-Smirnov, $P < 0.05$) was observed between kōura control and treatment groups, indicating that kōura gills may be more susceptible to erosional damage from precipitated aluminium hydroxide. It was concluded that diel pH cycling and exposure to 2 mg Al L^{-1} were unlikely to significantly impact osmoregulatory function in rainbow trout and kōura in Lake Rotorua.

A second experiment utilised intermittent flow respirometry to determine mass-specific metabolic oxygen consumption rates (MO_2) of rainbow trout, common bully, and kōura exposed to 2 mg Al L^{-1} and diel pH fluctuations (pH 7–10) over 60 hours. It was expected that precipitation of dissolved aluminium onto the gill surface during pH transitions and/or binding of dissolved aluminium species could inhibit the respiratory gas exchange resulting in increased gill ventilation from hypoxic and hypercapnic conditions. There was a significant negative correlation (Pearson's, $r^2 = 0.34$, $P < 0.001$) between pH and MO_2, possibly indicating kōura were sensitive to the experimental conditions. However, there were no consistent trends in differences between control and treatment groups for any of the tested species. Data interpretation was hindered by the necessity to exclude samples from the rainbow trout and common bully control groups due to aberrations in the oxygen sensor data. However, the mean MO_2 was within expected ranges for rainbow trout and kōura, indicating that significant respiratory impacts were not occurring during the exposure period. Although definitive conclusions could not be made regarding changes to MO_2 in response to aluminium and diel pH cycling, it is unlikely that acute impacts would result from these conditions in the natural environment.

From this research, it is concluded that the combined impacts of diel pH cycling and aluminium exposure at twice the current dosing rate (i.e., 1 mg Al L^{-1}) are unlikely to significantly impact respiratory and osmoregulatory function in fish and kōura in Lake Rotorua. There was some indication that at circumneutral pH and moderate aluminium concentrations (2 mg Al L^{-1}), particulate aluminium may cause erosional damage to kōura gill tissue. However, such conditions are highly unlikely to be encountered within the lake where the mean total aluminium concentrations are approximately one hundred times lower (0.02 mg Al L^{-1}). These experimental results are consistent with observations from the Al-dosed Utuhina stream, which show no evidence of effects on macroinvertebrate communities or kōura downstream of the Al input. Further investigation of aluminium exposure on more susceptible and less mobile life stages (e.g., larval rainbow trout) would help to define potential impacts and species tolerances. Further testing of potential impacts on gill tissues of kōura from particulate aluminium is also recommended.

Acknowledgements

We would like to thank Deniz Özkundakci and John Tyrrell for reviewing this document. The collection of kōura was done with the assistance of Warrick Powrie, and Te Arawa iwi are thanked for their permission to collect specimens from lakes Tarawera and Rotoma. ICP-MS analysis was conducted by Danielle Blackwell. Additional support and funding were provided by the Bay of Plenty Regional Council Chair in Lakes and Freshwater Science.

Table of Contents

Executive Summary 3
Acknowledgements 5
Table of Contents 6
Background.....9
Alum dosing and aluminium toxicity.....9
Aluminium toxicity at alkaline pH 10
Osmoregulation impairment by aluminium.....11
Respiration impairment by aluminium 11
Lake Rotorua: Aluminium dosing and ecological monitoring 12
Phytoplankton bloom driven pH changes.....13
Methods 15
Osmolarity impacts on rainbow trout and kōura.....15
Respiratory impacts on rainbow trout, common bully and kōura.....19
Results 23
Osmolarity impacts on rainbow trout and kōura.....23
Respiratory impacts on rainbow trout, common bully and kōura.....28
Discussion.....33
Osmolarity impacts on rainbow trout and kōura.....33
Gill histology.....35
Respiratory impacts on rainbow trout, common bully and kōura.....35
Conclusions 37
References 38
Appendix 1 42

LIST OF FIGURES

Figure 1. Comparative total aluminium 5% hazardous concentrations (HC5) for Lake Rotorua under differing pH values based on mean dissolved organic matter concentration of 2 mg L^{-1} and mean water hardness of 14 mg L^{-1} (as $CaCO_3$). Values were calculated using DeFrost *et al*. (2018) and updated by Defrost *et al*. (2020) multiple linear regression models. 10

Figure 2. Daily aluminium dose rates to the Utuhina and Puarenga Streams. Combined 180-day average daily dose rate to Lake Rotorua is also presented. 12

Figure 3. Changing concentrations of inorganic carbon compounds in the bicarbonate equilibrium as a function of pH (Dodds 2002, p. 232).....14

Figure 4. Changes in surface water pH correlating with fluctuations in chlorophyll-*a* in Lake Rotorua over a 6-week period in March 2017.....15

Figure 5. Stylised experimental design for determining potential osmotic and haematological disruption from alum derived aluminium at 2 mg Al L^{-1} under diel pH cycling.....17

Figure 6. Common bully within respirometry chamber. 21

Figure 7. Scatter plots and associated estimation plots of individual (A) kōura haemolymph osmolarity (mOsm), (B) rainbow trout plasma osmolarity (mOsm), (C) rainbow trout haematocrit (%), (D) rainbow trout whole blood [Hb] (g L^{-1}), and (E) rainbow trout mean cell haemoglobin concentration (MCHC) (g L^{-1}) for individuals in control and treatment groups and. Data is presented with group means with 95% confidence intervals. 26

Figure 8. Histological gill sections of treatment kōura (top) and rainbow trout (bottom) individuals showing epithelial lifting from the cuticle, lamellae swelling, lamellae disorganisation (a), and a cellular anomaly (b) in kōura gills and hyperplasia (c), RBC proliferation (d), epithelial lifting (e), and a cellular anomaly (f) in rainbow trout gills.....27

Figure 9. Box and whisker plots displaying cumulative abnormality scores for kōura and rainbow trout control and treatment groups with whiskers representing 10% and 90% bounds. A Kolmogorov-Smirnov test revealed a significant difference (P <0.05) between the kōura treatment and control samples. 28

Figure 10. Mean (± SEM) metabolic consumption rate (MO_2) for rainbow trout, common bully and kōura exposed to either 2 mg Al L^{-1} pH 7 (control) or 2 mg Al L^{-1} pH 7–10 cycling (treatment). 29

Figure 11. Effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) for kōura control (n = 6) and treatment groups (n = 5) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SEM. Shaded areas represent the dark photoperiod (12 h). 30

Figure 12. Effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) for rainbow trout control (n = 2) and treatment groups (n = 5) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SEM. Shaded areas represent the dark photoperiod (12 h). 31

Figure 13. Effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) for common bully control (n = 3) and treatment groups (n = 4) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SEM. Shaded areas represent the dark photoperiod (12 h). 31

Figure 14. Plot of t-statistic values for the differences between the kōura control and treatment mean MO_2 for each hour, with diel pH cycling. If the t-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level. 32

Figure 15. Plot of t -statistic values for the differences between the rainbow trout control and treatment mean MO_2 for each hour, with diel pH cycling. If the t-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.....32

Figure 16. Plot of t -statistic values for the differences between the common bully control and treatment mean MO_2 for each hour, with diel pH cycling. If the t-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.....33

Background

Alum dosing and aluminium toxicity

Alum (aluminium sulphate; $Al_2(SO_4)_3$) dosing is widely used for lake restoration due to its property of sequestering dissolved reactive phosphate (DRP), thereby limiting phytoplankton growth and inhibiting algal bloom formation. Once applied to the waterbody, aluminium sulphate undergoes a hydrolysis reaction producing insoluble aluminium hydroxide ($Al(OH)_3$), with DRP subsequently adsorbing to the aluminium ions (Cooke *et al*. 2005). When applied as a bulk dose, alum floc may also form a surface barrier (sediment cap), capturing phosphorus released from the sediment and reducing internal loading (Rydin *et al*. 2000). Aluminium is unresponsive to redox changes, keeping phosphorus bound during periods of lake stratification and resulting anoxia, thereby interrupting phosphorus cycling within the lake (Hickey and Gibbs 2009). Aluminium hydroxide is relatively insoluble at pH 6–8, but solubility increases under alkaline or acidic conditions (Gensemer and Playle 1999). Various monomeric and hydroxy aluminium species (i.e., Al^{3+}, $AlOH^{2+}$, $Al(OH)_2^+$, $Al(OH)_3$ and $Al(OH)_4^-$) occur in varying proportions with regard to pH (Gensemer and Playle 1999). In addition, the hydrolysis of $Al_2(SO_4)_3$ to $Al(OH)_3$ results in the release of H^+ ions, lowering the pH of the receiving waters. If the environmental pH continues to decrease, soluble aluminium species form, beginning with $Al(OH)^+$, then $Al(OH)_2^+$, and finally free Al^{3+} ions. These inorganic monomeric aluminium species are considered the most toxic forms of aqueous aluminium (Sparling and Lowe 1996).

Aluminium is the third most abundant crustal element but has little, if any, known biological function and it is generally agreed that biological systems do not require aluminium to function (Gensemer and Playle 1999; Poléo and Hytterød 2003). In aquatic animals, aluminium toxicity is primarily due to disruption of osmoregulation and respiration by dissolved monomeric aluminium species at the gills (Alexopoulos *et al*. 2003; Gensemer *et al*. 2018). In fish and crustaceans, gills are the primary site of gas exchange, ion transport, and waste excretion (Playle and Wood 1989). Metabolic waste products such as ammonia (NH_3) and CO_2 are excreted from the gills, making the gill micro-environment either acidic or basic depending upon the pH of the inspired water and the buffering capacity of the environment (Playle and Wood 1989). This may result in acidic water from the environment becoming more alkaline as it passes over the gills, while neutral and alkaline waters may become acidic due to proton excretion at the gill epithelium (Rankin and Jensen 1993; Wilkie 1996). Playle and Wood (1989) reported that pH changes at the surface of rainbow trout (*Oncorhynchus mykiss*) gills were sufficient to change the solubility of aluminium, resulting in polymerisation and precipitation of dissolved aluminium onto the gill surface causing disruption of Na^+/K^+-ATPase ion pumps in the chloride cell membranes and impairing osmoregulation (Wilkie *et al*. 1999; Cardwell *et al*. 2018).

Aluminium toxicity at alkaline pH

Toxicological studies of aluminium under basic conditions are limited compared to those conducted at acidic pH. Also, differentiation of physiological effects originating from aluminium as opposed to pH is problematic, as high or low environmental pH can disrupt acid-base regulation, impacting osmoregulatory processes. However, it is generally postulated that osmoregulatory disruption by aluminate ($Al(OH)_4^-$) is the predominant mode of toxic action at high pH. DeForest *et al*. (2018) developed a multiple linear regression (MLR) model to predict chronic aluminium toxicity to algae, zooplankton and fish under varying conditions of dissolved organic matter (DOM), pH and water hardness. Hazardous concentrations to 5% (HC5) of species or genera were then derived for these parameters. The HC5 is a statistically derived value from sensitivity data of multiple species to a single toxicant and is analogous to the Predicted No-Effect Concentration (PNEC) for a single species. The MLR model was subsequently updated by DeForest *et al*. (2020) to include a greater range of pH (pH 6–8.7), DOM and water hardness conditions. When the modified MLR model is applied using DOM (2 mg L^{-1}) and water hardness values reported (14 mg L^{-1} as $CaCO_3$) for Lake Rotorua, the model notably deviates from the previous version above pH 8 (Figure 1). This indicates that, at a general community level, aluminium may be less toxic under alkaline conditions than previously indicated. However, it should be recognised that the DeForest *et al*. (2020) MLR model is still based on a relatively limited pH range where insoluble $Al(OH)_3$ is the dominant form. Above pH 8, $Al(OH)_4^-$ becomes the increasingly abundant form, and toxicological effects are likely to increase.

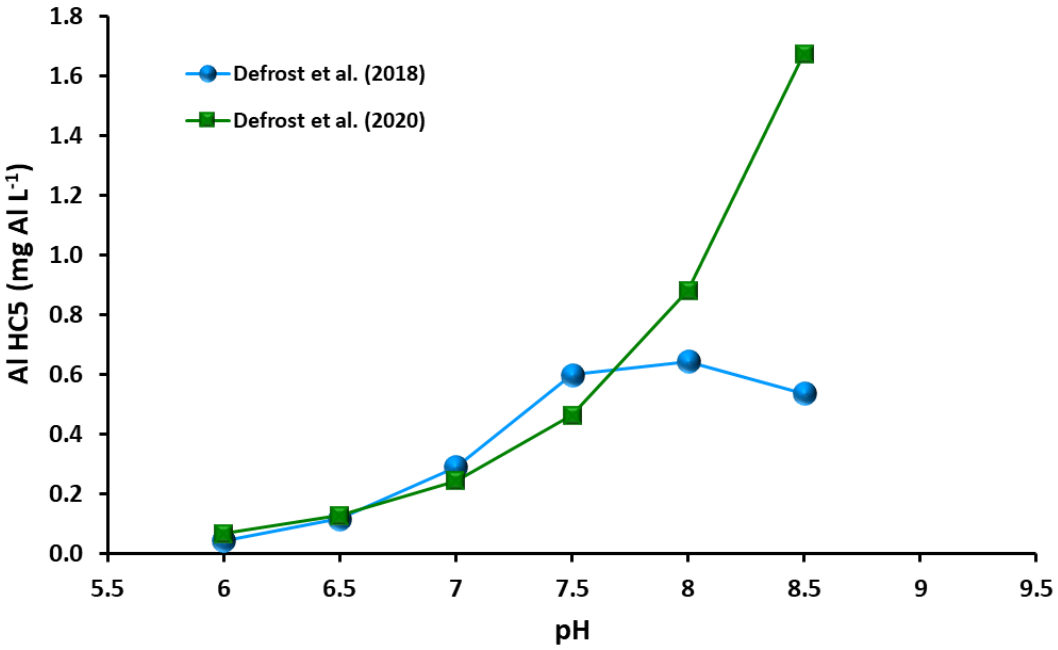


Figure 1. Comparative total aluminium 5% hazardous concentrations (HC5) for Lake Rotorua under differing pH values based on mean dissolved organic matter concentration of 2 mg L^{-1} and mean water hardness of 14 mg L^{-1} (as $CaCO_3$). Values were calculated using DeFrost *et al.* (2018) and updated by Defrost *et al.* (2020) multiple linear regression models.

Osmoregulation impairment by aluminium

During alkaline conditions and in the presence of aluminium, osmoregulatory disturbances can occur inhibiting the maintenance of ion concentrations within the body (Ali *et al*. 2017). Studies by Wilkie and Wood (1991, 1996) and Wilkie *et al*. (1999) demonstrated the adverse effects of high pH (>8) on gill ion transportation capacity, ionoregulation, and acid-base balance. Ionoregulatory effects from aluminium exposure arose due to the binding of soluble aluminium species (Al^{3+} and $Al(OH)_4^-$) to gill ion channels (Gensemer and Playle 1999). Brown trout (*Salmo trutta*) exposed to aluminium (12.5 ug Al L^{-1}) at pH 5 experienced a decline in sodium (~25%) and chloride (~15%) plasma ion concentrations after 6 hours of exposure, although some recovery was evident after 120 hours (Waring and Brown 1995). This effect is attributed to inhibition of ion influx by Al^{3+} and increased ion efflux resulting from opening or weakening of the tight junctions between the epithelial cells (McWilliams 1983; Marshall 1985; Freda *et al*. 1991). Reduction in blood osmolarity can lead to osmotic swelling of the red blood cells, resulting in increased haematocrit and decreased blood cell haemoglobin concentration. Increased haematocrit may lead to elevated blood viscosity which can impair circulatory function. Elevated haematocrit may be exacerbated by release of red blood cells from the spleen in response to reduced oxygen delivery resulting from decreased haemoglobin concentration (Witters *et al*. 1990).

Respiration impairment by aluminium

At circumneutral pH, aluminium may precipitate onto the gills causing irritation and excessive mucus secretion, increasing the gas diffusion distance across the gill surface (Gensemer *et al*. 2018). Reduced oxygen uptake leads to a reduction in oxygen delivery to tissues within the body. This effect was demonstrated by Waring and Brown (1995) where brown trout (*Salmo trutta*) were exposed to various pH levels and aluminium concentrations (pH 7, pH 5, and pH 5 with Al at 50, 25, and 12.5 ug L^{-1}) for up to 5 days. All experimental groups exposed to aluminium suffered decreases in arterial oxygen, with 100% death in the 50 ug L^{-1} and 67% death in the 25 ug L^{-1} group after 120 hours of exposure (Waring and Brown 1995). Reduced aerobic function and the prioritisation of gas exchange reduces the capacity for ion regulation, allowing cellular Na^+ influx, K^+ efflux, and Ca^{2+} accumulation, ultimately resulting in cell death (Brauner *et al*. 2009). Compensatory responses to reduced oxygen uptake can include increased ventilation rates, gill permeability, and perfusion rates. These responses are initiated to maintain oxygen delivery to meet the cells' biochemical oxygen demands (Gensemer and Playle 1999). There has also been some suggestion that at circumneutral pH and high aluminium concentrations (>2 mg Al L^{-1}), colloidal aluminium may cause erosional damage to the gill surface resulting in inflammation and excess mucus production, inhibiting oxygen uptake (Burrows 1977). However, this area has not been investigated as most aluminium applications at this concentration are episodic and limited in duration or continuous aluminium dosing is not applied at this level.

Lake Rotorua: Aluminium dosing and ecological monitoring

Alum dose rates to the Utuhina and Puarenga inflows have varied considerably since the initiation of alum dosing by the Bay of Plenty Regional Council in 2006 and 2010, respectively (Figure 2). Dosing to the Puarenga Stream was halted from 30 September 2018 to 22 November 2020, although alum dosing of the Utuhina Stream was increased during this period to partially compensate for the shutdown. As of 1 April 2022, 844 tonnes of aluminium had been dosed to Lake Rotorua. The mean aluminium dose rate (excluding shutdowns) to the Puarenga Stream has been greater (134.7 kg Al day^{-1}) compared to the Utuhina Stream (76.3 kg Al day^{-1}).

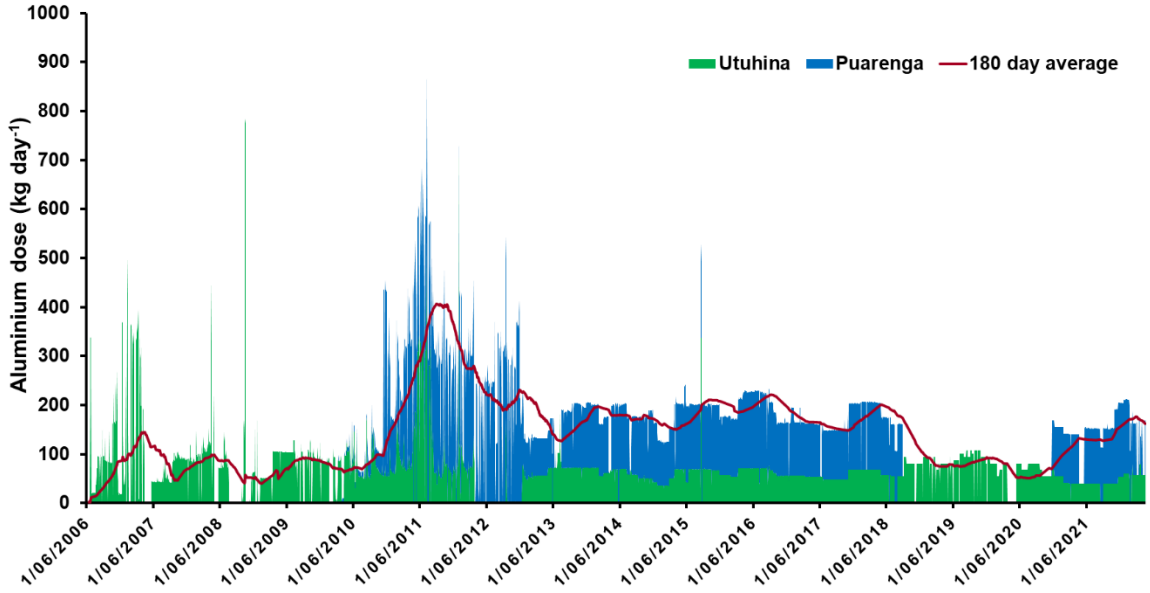


Figure 2. Daily aluminium dose rates to the Utuhina and Puarenga Streams. Combined 180-day average daily dose rate to Lake Rotorua is also presented.

Ongoing annual monitoring of the fish and aquatic macroinvertebrate communities in the Utuhina and Puarenga streams has been conducted by the University of Waikato (Ling and Brijs 2009, Ling 2014, 2016, 2017, 2021). The alum dosing site to the Utuhina Stream is located approximately 1,500 m upstream of the discharge point to Lake Rotorua. One upstream and two downstream sites of the dosing point are monitored annually. While differences in species abundance have been observed between years, this has been attributed to flood-related disturbances to stream bank morphology and in-stream vegetative cover or physical displacement of fish. Analysis of the stream macroinvertebrate community also showed no consistent differences in abundance between the upstream control site and the downstream sites. In some years, aluminium bioaccumulation was seen in the gills and liver tissues of common bully. However, there was no evidence of bioaccumulation of aluminium in the tissues of kōura (*Paranephrops planifrons*). Alum exposure in these species does not appear to affect their health or abundance in the stream (Ling 2021).

The Puarenga Stream alum dosing station is located approximately 500 m upstream of the discharge point to Sulphur Bay, a continuously active geothermal area. The geothermal activity at Sulphur Bay restricts fish passage upstream from Lake Rotorua and only three fish species have been recorded in the Puarenga Stream (Landman and Ling 2008). The very low pH at the head of the bay (~pH 2.5 – 3.0) results in high levels of bioavailable aluminium, with natural aluminium concentrations of approximately 1 mg Al L^{-1} within Sulphur Bay (Landman and Ling 2009). However, water column total aluminium concentrations quickly drop to near background levels (0.02 mg Al L^{-1}) near the mouth of the bay due to dilution (Ling 2016, Hamill 2021). The geothermally influenced environmental conditions within Sulphur Bay have excluded all but *Chironomus* spp., although the area is widely recognised as an important area for water birds (Landman and Ling 2009). A plume of geothermal water containing colloidal sulphur and silica exits Sulphur Bay. It is carried eastwards along the southern shore, driven by primarily north-westerly wind, where it finally mixes with the main body of lake water, disperses, and reaches near-neutral pH (Ling 2017). Although the plume does appear to influence the presence of biota along the south-eastern near-shore zone of the lake, the affected area is highly dependent on prevailing wind and hydrodynamic circulation patterns (Ling 2016). In addition, analyses of aluminium bioaccumulation in the tissues of macrobiota (Chronomids, *Chironomus zealandicus;* kakahi, *Echyridella menziesii*; common bully and *Eleocharis acuta*) found no significant effects of alum dosing could be distinguished from samples taken prior to alum dosing of the Puarenga Stream (Ling 2016).

Phytoplankton bloom driven pH changes

Diel pH cycling in lakes is driven by the photosynthetic biomass within the lake (Acuña-Alonso *et al*. 2020). In aquatic systems, inorganic carbon can be present as carbon dioxide (CO_2), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) depending upon environmental pH. Carbonic acid is most prevalent at low pH, while carbonate is predominantly formed at high pH (Figure 3). Carbonate buffers environmental waters from pH changes in neutral and slightly alkaline conditions as it reacts with free protons (H^+) to form bicarbonate, decreasing the concentration of H^+ and maintaining the pH of the solution. Therefore, environments with high buffering capacity (acid-neutralizing capacity) have large amounts of carbonate, resisting large changes in pH. In contrast, those with little available carbonate and bicarbonate are more likely to experience fluctuations in pH (Dodds 2002).

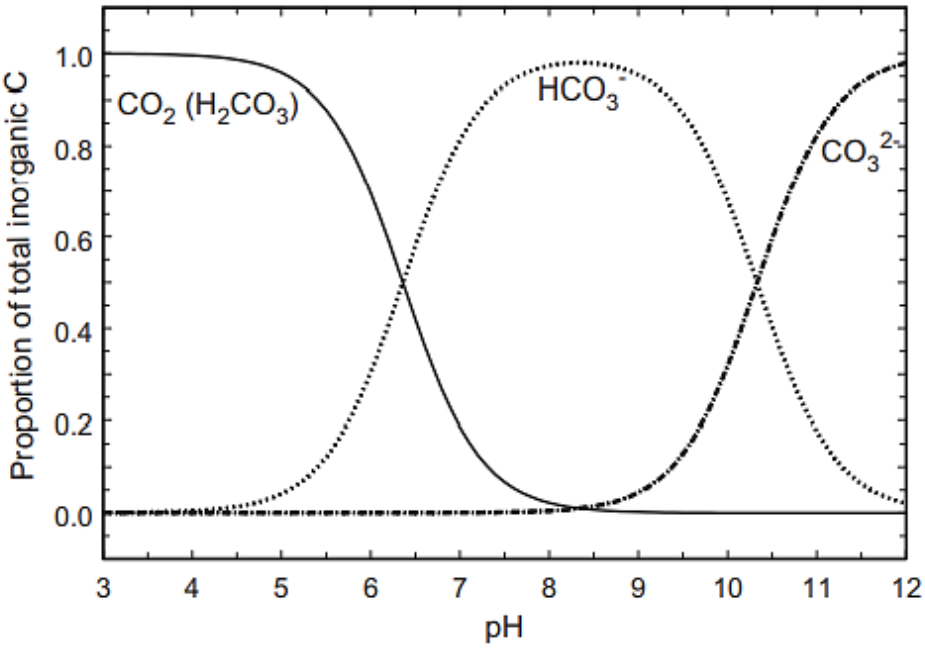


Figure 3. Changing concentrations of inorganic carbon compounds in the bicarbonate equilibrium as a function of pH (Dodds 2002, p. 232)

The process of photosynthesis involves the uptake of CO_2 which can lead to increases in pH as the bicarbonate equilibrium is driven to the right (Ping 2006, Tank *et al*. 2009, Acuña-Alonso *et al*. 2020). Conversely, the respiratory release of CO_2 at night produces the opposite effect, creating H^+ and decreasing pH (Heini *et al*. 2014). The biomass of the algal bloom therefore dictates the magnitude of bicarbonate flux in the system and the range of pH change over 24 hours. For example, Lake Rotorua has poor buffering capacity, and the presence of algal blooms can rapidly produce large shifts in pH from 6.5 to 10 over a 24-hour cycle (Figure 4) (Tempero *et al*. 2015). Shifts from circumneutral pH, where insoluble $Al(OH)_3$ is most abundant, to alkaline conditions increases the abundance of soluble aluminate ($Al(OH)_4^-$) ions within the environment. While numerous studies have investigated the osmoregulatory effects of prolonged continuous exposure to aluminium at high and low pH, there is no published information regarding aluminium toxicity in fish and crustaceans under cyclical (diel) pH conditions (pH ~6.5–10).

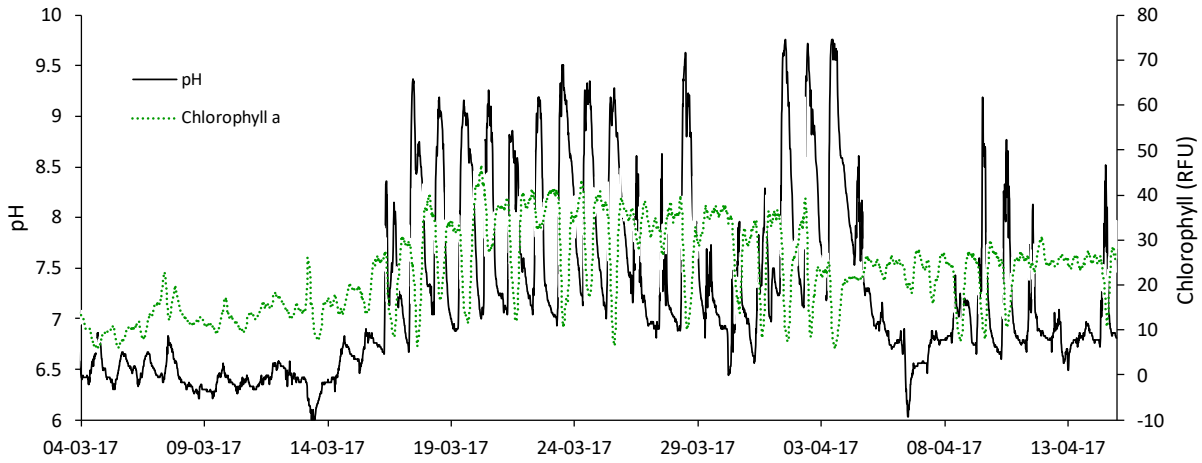


Figure 4. Changes in surface water pH correlating with fluctuations in chlorophyll-*a* in Lake Rotorua over a 6-week period in March 2017.

The University of Waikato was contracted by the Bay of Plenty Regional Council to examine the cumulative impacts of transient alkaline pH (7–10) and aluminium (2 mg L^{-1}) on rainbow trout, common bully, and kōura. Potential disruption of osmoregulation were examined by exposure of rainbow trout and kōura to 2 mg Al L^{-1} with diel pH cycling for 10 days. Physiological sampling included haemoglobin concentration, haematocrit, osmolarity, and gill histology of rainbow trout and kōura. The cumulative effects of 2 mg Al L^{-1} and diel pH cycling on the metabolic rate (MO_2) of rainbow trout, common bully, and kōura were examined using intermittent flow respirometry. Oxygen uptake rates in individual test subjects were measured over 48 hours to determine whether changes in mass-specific metabolism occur in response to pH cycling at 2 mg Al L^{-1}. Information from these experiments was then synthesised to provide recommendations on the toxicological risk of alum dosing during algal blooms. Before testing and sampling, all experiments within this study (Protocol #1110 and Protocol #1128) were approved by the University of Waikato Animal Ethics Committee.

Methods

Osmolarity impacts on rainbow trout and kōura

The purpose of this study was to determine whether osmotic and haematological disruption occurred in rainbow trout (*Oncorhynchus mykiss*) and kōura (*Paranephrops planifrons*) in response to exposure to 2 mg Al L^{-1} aluminium and cyclic pH (7-10-7) under laboratory conditions over 10 days. This was intended to emulate diel pH changes observed in Lake Rotorua during a phytoplankton bloom (Appendix 1). Plasma (rainbow trout) and haemolymph (kōura) osmolarity and haematocrit and haemoglobin concentration in rainbow trout were measured to determine whether osmotic disruption occurred in response to

aluminium and cyclic pH exposure. Additionally, gill histological analysis was conducted for abnormalities caused by precipitated particulate aluminium.

Collection and maintenance of study animals

Juvenile rainbow trout (mean weight 24.7 ±1.0 g SEM; mean fork length: 130.2 ±1.8 mm SEM) were sourced from the Fish and Game Ngongotaha hatchery in Rotorua. Mature kōura (mean weight 34.8 ±2.1 g SEM; ocular carapace length: 37.2 ±0.9 mm SEM), excluding breeding females, were collected by SCUBA from Lake Tarawera, Bay of Plenty. Animals were kept at the Waikato University Aquatic Research Centre, with a constant room temperature of 18°C and a photoperiod of 12L:12D

Experimental design

Alum and sodium hydroxide (NaOH) stock solutions were prepared at least 24-hours prior to use. A 1750 L tank was filled with dechlorinated water (water hardness ~40.9 g m^{-3} as $CaCO_3$, pH 7.3, dissolved organic carbon (DOC) 1.2 g m^{-3}), and 28% w/w liquid aluminium sulphate (Ixom, New Zealand) was added to achieve a concentration of 2 mg Al L^{-1}, and a submersible pump was used to keep the solution mixed. Fifty litres of pH 12 sodium hydroxide (Merck, Germany) stock solution was also prepared.

Toxicity tests were conducted in four 60 L flow-through glass tanks, divided into two control and two treatment tanks, each containing three test subjects. Twenty-four individuals (i.e., 12 control and 12 treatment) from both species were used in the toxicity testing, with two experimental runs conducted for each species. The tanks were fed by two peristaltic pumps, each with four independent pump heads. Pump 1 supplied all four tanks with the stock 2 mg L^{-1} aluminium solution (pH 7) at a rate of 0.2 L h^{-1}. Pump 2 supplied the two control tanks with 2 mg L^{-1} aluminium solution and the two treatment tanks with a pH 12 2 mg L^{-1} aluminium solution at a rate of 6 mL h^{-1} (Figure 5). All tanks were initially filled with the stock aluminium solution and the test subjects allowed to acclimate for 3 days prior to initiation of pH cycling. During the acclimation period, the tanks were flushed daily for 12-hours by pump 1 with the stock aluminium solution (pH 7). Over the 10-day testing period, tank flow-through was switched between pump 1 and 2 every 12 hours. The two control tanks remained at pH 7, while the two treatment tanks were subjected to increased pH from pH 7 to pH 10 over 12 hours. The pH subsequently declined back to pH 7 within 12 hours of pump 1 starting (Appendix 1).

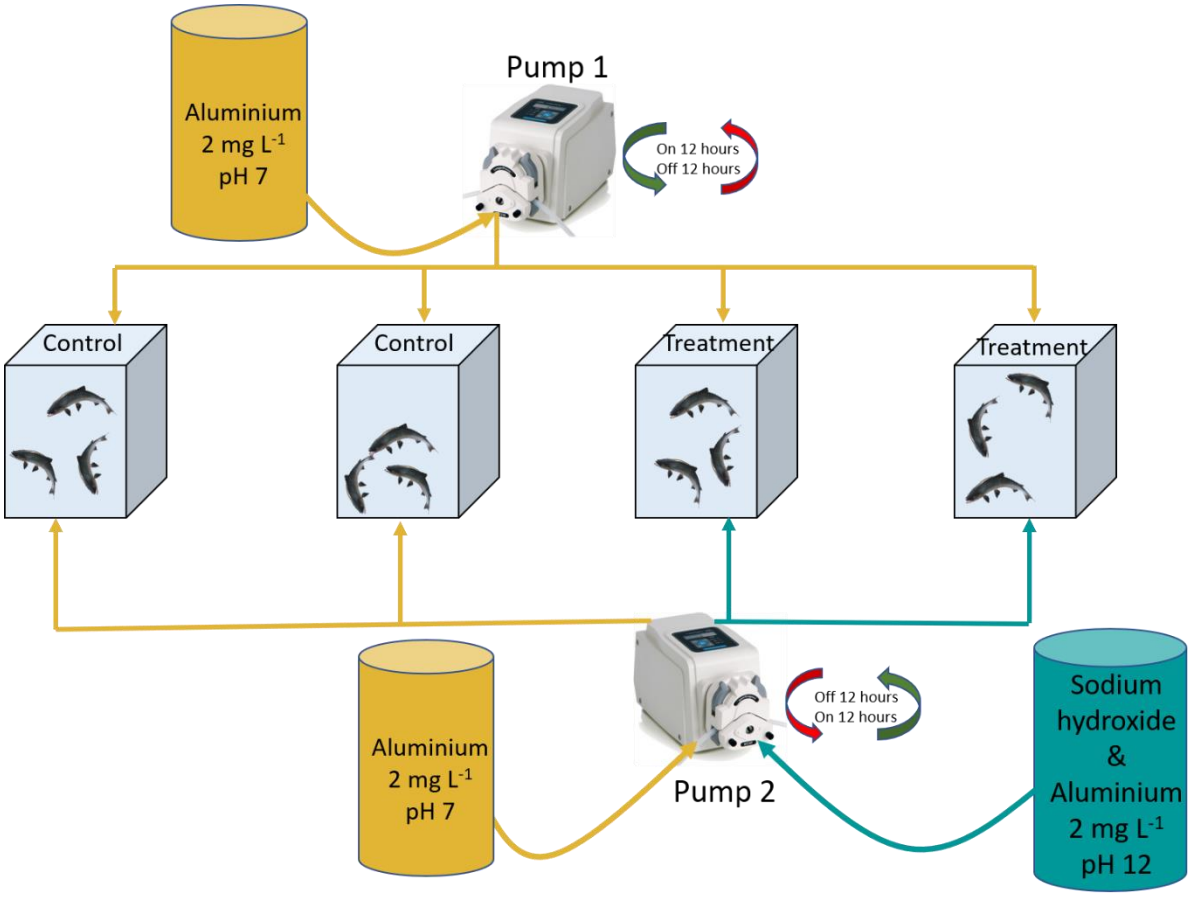


Figure 5. Stylised experimental design for determining potential osmotic and haematological disruption from alum-derived aluminium at 2 mg Al L^{-1} under diel pH cycling.

A YSI Prosolo multi-parameter meter was used to measure pH, temperature (°C), conductivity (µS/cm), and dissolved oxygen (%) at the start of each day. The pH of the treatment tanks was continuously recorded using a Radiometer MeterLab PHM20 pH meter connected to a Sekonic SS 250F chart recorder. Visual observations of the experimental subjects for signs of stress, such as surface breathing and the loss of equilibrium, were conducted for the first 10 minutes of the experiment, followed by 5-minute observations twice daily for the duration of the experiment. Any fish showing signs of distress or ill health were promptly removed from the experiment and euthanised.

Haematology

After 10 days of exposure, the test subjects were euthanized either by administering a lethal dose of 0.1 g L^{-1} benzocaine (rainbow trout) or Aqui-S (kōura). Individual weights (±0.1 g), trout fork length (±1 mm), and kōura ocular carapace length (±1 mm) were measured, and the subjects were examined for external injuries. Rainbow trout blood samples (200 µL) were taken by caudal venepuncture into a heparinised (lithium heparin, Sigma, New Zealand) 0.5

mL syringe and then transferred to a micro-centrifuge tube. Kōura haemolymph samples (100 µL) were drawn from the pericardial sinus between the carapace and the first abdominal segment. A 4% buffered formalin fixative (1:1) was used to preserve cell integrity and prevent cell clotting (Taylor *et al*. 2009).

Rainbow trout haematocrit (Packed Cell Volume) was determined by drawing blood into a 75 mm ammonium heparin capillary tube (Drummond Scientific Company). The tube was then sealed and centrifuged at 4,000 rpm for 5 minutes. Haematocrit was calculated as the percentage of the packed cell volume to total blood volume.

Haemoglobin concentration was determined using the cyanomethaemoglobin method. Five µL of whole rainbow trout blood was added to 1 mL of Drabkin's Reagent (dilution factor 1:200) and refrigerated until quantification. Haemoglobin samples were allowed to stand for 10 minutes in order to come to room temperature, and then briefly agitated to mix. Absorbance at a wavelength of 540 nm was then determined by spectrophotometry (Shimadzu Model 1601, Shimadzu Corporation, Japan) and haemoglobin concentration was calculated using the following equation:

$$\text{Haemoglobin concentration (g L}^{-1}) = \frac{(Hb\ ABS \times 64458 \times df)}{44000}$$ (Dacie and Lewis 1991)

where *Hb ABS* is the sample absorbance at 540 nm and *df* is the dilution factor. The mean cell haemoglobin concentration was calculated by dividing the haemoglobin value by PCV and presented as a proportion. Mean cell haemoglobin concentration was then calculated by dividing haemoglobin concentration by the haematocrit proportion to give the mean red blood cell haemoglobin concentration.

Osmolarity of rainbow trout plasma and kōura haemolymph was determined using a Wescor VAPRO Vapour Pressure Osmometer. Rainbow trout blood samples were first centrifuged for 5 minutes at 4,000 rpm and the resulting plasma was collected into a micro centrifuge tube. Following osmometer calibration, 10 µL of blood plasma or haemolymph were used to determine osmolarity as mOsm. A control of 4% formalin sample was analysed to exclude any influences of the fixative on the haemolymph osmolarity results.

Gill histology

Gills removed from rainbow trout and kōura were preserved in 10% neutral buffered formalin. Gill tissue sections were mounted on slides, fixed with methanol, and stained with eosinophilic stain followed by basophilic stain according to general staining procedures. Gill sections were analysed at 40x and 100x magnification to examine signs of abnormality. Abnormalities, including epithelial lifting, primary and secondary lamellae fusion, hyperplasia, red blood cell proliferation, and cellular anomalies were identified according to Mallatt (1985)

and Abd El-Atti *et al*. (2019), and distributions of significant abnormalities were estimated. Images of abnormalities were taken at 40x and 100x magnification.

Tank aluminium content

Water quality samples were taken from selected control and treatment tanks at 10 am when tank pH was neutral. Particulate and dissolved aluminium content were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Total aluminium samples were acidified to pH 3 by adding 100 µL of trace metal grade 67% nitric acid to 10 mL of tank water, followed by filtering using 0.2 µm minisart filters (Sartorius, Germany). Dissolved aluminium samples were prepared by filtering 10 mL of tank water through a 0.45 µm minisart filter, followed by nitric acid acidification and further filtering through a 0.2 µm filter. Samples were then analysed for aluminium by ICP-MS (Agilent 8900 with a triple-quadrupole) at the University of Waikato Mass Spectrometry Facility.

Statistical Analysis

Student's *t*-tests were used to determine significant differences between the haematological variables of the control and treatment groups for rainbow trout and kōura. Cumulative scores for treatment and control groups were used in the Kolomogorov-Smirnov test to determine significant differences.

Respiratory impacts on rainbow trout, common bully and kōura

Respirometry measures the rate of oxygen uptake by an animal and is used to gain insight into the respiratory responses of animals to environmental challenges such as temperature and toxicants. Estimates of multiple metabolic states, such as standard metabolic rate (SMR), maximum metabolic rate (MMR), and aerobic metabolic scope (AMS) can be calculated over time (Claireaux and Chabot 2016). The most common respirometry protocol is intermittent flow respirometry, which measures the SMR of a fasted ectothermic individual in a rested state (Reece *et al*. 2015). The intermittent flow respirometry apparatus comprises the chamber, a recirculation loop, a flush pump, and a recirculating pump and is typically submerged in a tank at 100% oxygen saturation. There are two distinct cycles, (i) the recirculation period, where the chamber is closed off from the external environment allowing the organism to consume oxygen, decreasing the oxygen saturation over time, and (ii) the flush period where the chamber is flushed with fully saturated water, increasing the dissolved oxygen back to normoxic conditions (Claireaux and Chabot 2016). The mass-specific oxygen consumption (MO_2) is calculated from the change in oxygen saturation during closed chamber periods, normalised to the mass of the animal.

For this study, the combined effects of sublethal aluminium concentrations (2 mg L^{-1}) and diel pH cycling (7-10-7) on fish and crustacean respiratory exchange were examined. Oxygen consumption rates of rainbow trout (*Oncorhynchus mykiss*), common bully (*Gobiomorphus*

cotidianus), and kōura (*Paranephrops planifrons*) exposed to aluminium and diel pH cycling (pH 7–10) were measured, and the mass-specific data used to identify potential impacts in respiration rates over 60 hours of exposure.

Collection and maintenance of study animals

Juvenile rainbow trout (body mass: 37.2 g ±1.3 SEM; total length: 157 mm ±3.7 SEM) were sourced from the Fish and Game Ngongotaha hatchery in Rotorua. Adult common bully (body mass: 2.3 g ±0.1 SEM; total length: 57 mm ±0.75 SEM) were caught from Chapel Lake University of Waikato using minnow traps. Mature kōura (body mass: 53.9 g ±2.25 SE; ocular carapace length: 40.43 mm ±1.2 SE), excluding breeding females, were collected by SCUBA in Lake Rotoma, Bay of Plenty. Animals were kept at the Waikato University Aquatic Research Centre, with a constant room temperature of 18°C and a photoperiod of 12L:12D.

Respirometry procedure

A total of 12 individuals (i.e., 6 control and 6 treatment) of each species were used in the respirometry testing, with six paired experimental runs conducted for each species. Individual animals were placed in an appropriately sized respirometer (volumes; large = 1.8 L, medium = 314 mL, and small = 115 mL), so that they did not occupy >10% or <1% of the total respirometer volume (Figure 6). For each experimental run three respirometers were placed in separate 60 L glass tanks, two containing one test subject and the third respirometer remaining empty to measure background respiration. Tanks were continuously oxygenated with compressed air to maintain 100% oxygen saturation; water temperature was maintained at 18°C.

The aluminium and sodium hydroxide (pH 12) stock solutions were prepared as described above. Test subjects were introduced to tanks, and the aluminium concentration increased over a 12 hour period to allow for acclimation before initiation of pH cycling. During the test phase all tanks were initially flushed with the stock aluminium solution (pH 7) at a rate of 0.2 L h^{-1}. The pumps were automatically switched over and the second pump supplied the two control tanks with 2 mg L^{-1} aluminium solution (pH 7) and the one treatment tank with the pH 12 2 mg L^{-1} aluminium solution at a rate of 6 mL h^{-1}. Over the 48-hour testing period, tank flow-through was switched between pump 1 and pump 2 every 12 hours. The control tank and the background respiration tank remained at pH 7, while the treatment tank was subjected to increased pH from 7 to 10 over 12 hours. The pH subsequently declined back to pH 7 within 12 hours of pump 1 starting.

Test subjects were monitored for the first 30 minutes while pH changes commenced for signs of stress and to ensure the experiment was operating correctly. Open and closed periods in the respirometers were 30 minutes for rainbow trout and kōura, and 15 minutes open and 45 minutes closed for common bully. Prior testing has found these intervals were sufficient to prevent oxygen falling below 80% saturation and reduced the build-up of waste products

such as CO_2 within the respirometer. During open periods, oxygen was maintained as close to 100% saturation while during closed periods, oxygen levels decreased from 100% saturation as the animal used it up. Oxygen concentrations (mg L^{-1}) were measured continuously by fibre-optic oxygen sensors fitted within the recirculating loop of the respirometer and recorded using Presens Fibox 3 dataloggers (PreSens Precision Sensing, Germany). Experiments ran for 48 hours unless adverse effects were observed, in which case the experiment would be terminated early. At the end of the 48-hour experimental period, animals were euthanised (SOP#6 Euthanasia and anaesthesia of fish).

Figure 6. Common bully within respirometry chamber.

Data analysis

Individual oxygen consumption data for fish and kōura were plotted and a linear regression slope calculated for each oxygen consumption period when the respirometers were closed. The regression slopes were used to determine the rate of oxygen decrease in the respirometry chamber. To account for background respiration (i.e., microbial oxygen consumption), the rate of oxygen decline was subtracted from oxygen consumption rates for each fish and kōura. Mass-specific oxygen consumption (MO_2) was calculated as:

$$MO_2 = [(V_r - V_f) \times \Delta C_{wO2}] / (\Delta t \times M_f),$$

Where, V_r is the respirometry chamber volume (L), V_f is the volume of the fish (L), ΔC_{wO2} is the changes in dissolved oxygen concentration in the respirometer (mg L^{-1}), Δt is the time over which ΔC_{wO2} is measured (h), and M_f is the wet mass of the organism (g) as described in

Svendsen *et al*. (2016). For fish, volume was assumed to be equal to fish mass, while kōura density was determined as the mean volume displacement of three individuals covering the size range of the test subjects (i.e., 1.035 kg L^{-1}. Negative oxygen consumption rates were excluded as error in measurement was assumed and excessive fish activity in the first 12 hours of the organism in the respirometer were excluded and considered as acclimation period.

To account for background microbial respiration effects on oxygen consumption rates, the corrected oxygen consumption rate (MO_{2corr}) was determined as:

$$MO_{2corr} = MO_2 - MO_{2B}\, V_{RT}\, V_{RE}^{-1},$$

Where MO_2 is oxygen consumption (mg O_2 kg^{-1} h^{-1}), MO_{2B} is the background oxygen consumption (mg O_2 kg^{-1} h^{-1}), V_{RT} is the total volume of the empty respirometer including the recirculation loop, and V_{RE} is the effective respirometer volume as per Svendsen *et al*. (2016).

Statistical analysis

Statistical analyses were conducted using Microsoft Excel 2020 Data Analysis Toolpak and Prism 9 (Graphpad Software, LLC). Plots of mean mass-specific oxygen consumption (mg O_2 kg^{-1} h^{-1}) and standard error over time with diel pH cycling were produced to ascertain differences in mass-specific oxygen consumption between control and treatment groups. Multiple unpaired *t*-tests were conducted to determine differences between control and treatment groups, corrections were made for multiple comparisons. *t*-statistics were plotted over time, with values >1.96 being statistically different at the 95% confidence level. Pearson's *r* correlation was used to determine if linear correlations occurred between *t*-statistics and pH.

Results

Osmolarity impacts on rainbow trout and kōura

Total aluminium was maintained at approximately 2 mg L^{-1} in both control and treatment tanks for rainbow trout and kōura trials, with low levels of soluble aluminium present at pH 7 (Table 1). At high pH (pH >8.5), it was assumed that soluble forms of aluminium would become more prevalent as described by Gensemer and Playle (1999).

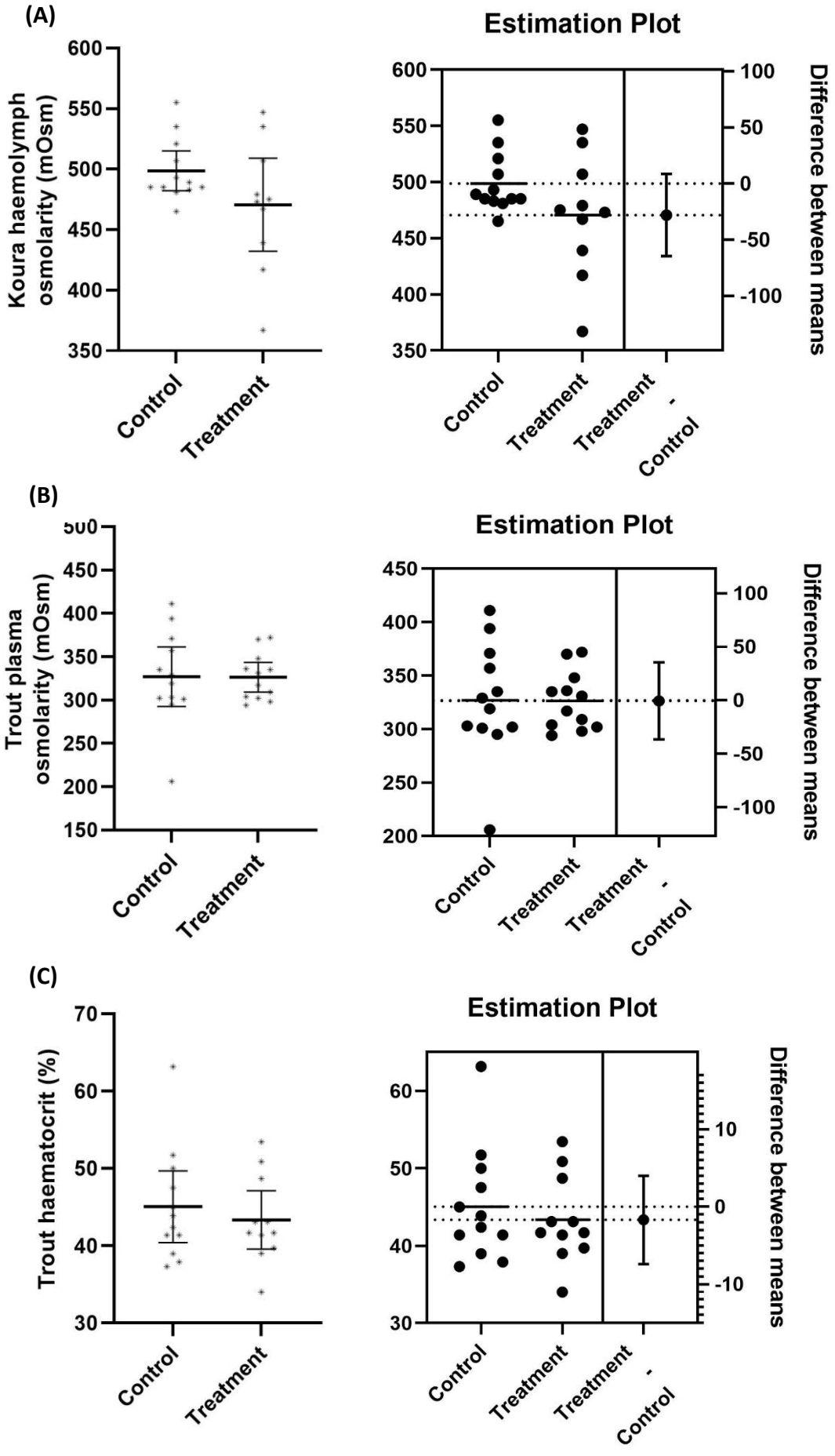
Table 1. Total aluminium and particulate aluminium (mean ± SEM) in rainbow trout and kōura treatment and control tanks at pH 7.

Species	Tank	Total aluminium (mg L^{-1})	Soluble aluminium (mg L^{-1})
Rainbow trout	Control	2.28 ±0.12	0.03 ±0.010
	Treatment	2.08 ±0.37	0.09 ±0.045
Kōura	Control	2.94 ±0.15	0.02 ±0.003
	Treatment	1.93 ±0.62	0.10 ±0.034

During daily monitoring in treatment and control tanks, indicators of stress, such as the loss of equilibrium and surface breathing, were not observed in rainbow trout. However, two kōura were casualties of intraspecific aggression. No statistically significant differences in the osmolarity, haematocrit or haemoglobin were observed between treatment and control groups of either species at the end of the 10-day experiment (Student's *t*-test, $P > 0.05$, Table 2). However, there were significant differences in the variance (F-test, $P < 0.05$) between the treatment and control groups for trout osmolarity and kōura haemolymph osmolarity. Additionally, there was a significant difference (Student's *t*-test, $P < 0.001$) between rainbow trout mean cell haemoglobin concentration between control and treatment groups. Scatter plots and corresponding estimation plots of kōura haemolymph osmolarity, rainbow trout osmolarity, haematocrit, whole blood haemoglobin concetration, and mean cell haemoglobin concentration (MCHC) show the differences between the means with 95% confidence intervals (Figure 7).

Table 2. Rainbow trout mean (± SEM) blood plasma and kōura haemolymph osmolarity (mOsm), rainbow trout and haematocrit (%), whole blood haemoglobin concentration (g L^{-1}) and mean cell haemoglobin concentration (MCHC; as both g L^{-1} and mM) for control and treatment groups. Asterisks (*) indicate significant differences between means (t-test; P <0.05) and variance (F-test) between the control and treatment groups.

Species		Tank	*n*	Mean ± SEM	ANOVA *P*- value	*F*- test *P*-value
Kōura	**Osmolarity**	Control	12	498.7 ± 7.5 (mOsm)		
		Treatment	10	470.6 ± 17.0 (mOsm)	0.12	0.0269*
Rainbow trout	**Osmolarity**	Control	12	326.9 ± 15.6 (mOsm)		
		Treatment	12	326.3 ± 7.8 (mOsm)	0.97	0.0297*
	Haematocrit	Control	12	45.0 ± 2.1 (%)		
		Treatment	12	43.3 ± 1.7 (%)	0.54	0.4208
	Haemoglobin	Control	12	67.8 ± 3.0 (g/L)		
		Treatment	11	72.8 ± 5.1 (g/L)	0.41	0.0876
	MCHC	Control	6	151.2 ± 8.9 (g/L) 2.3 ± 0.1 (mM)		
		Treatment	6	211.7 ± 11.7 (g/L) 3.3 ± 0.2 (mM)	<0.01*	0.231


(A)
Estimation Plot
Koura haemolymph osmolarity (mOsm)
600
550
500
450
400
350
Control
Treatment
Difference between means
100
50
0
-50
-100
Treatment - Control
(B)
Estimation Plot
Trout plasma osmolarity (mOsm)
500
450
400
350
300
250
200
150
Control
Treatment
Difference between means
100
50
0
-50
-100
Treatment - Control
(C)
Estimation Plot
Trout haematocrit (%)
70
60
50
40
30
Control
Treatment
Difference between means
10
0
-10
Treatment - Control

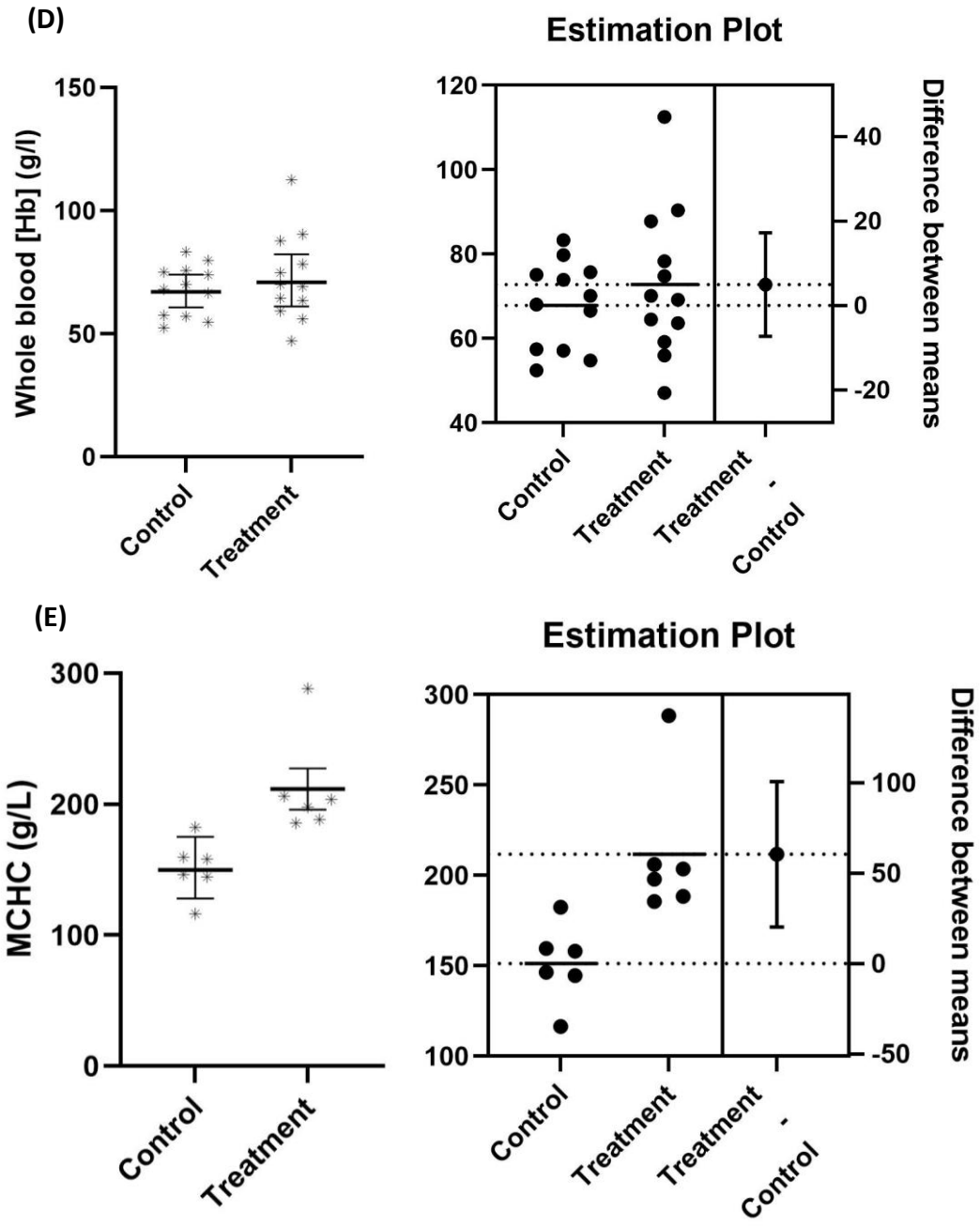


Figure 7. Scatter plots and associated estimation plots of individual (A) kōura haemolymph osmolarity (mOsm), (B) rainbow trout plasma osmolarity (mOsm), (C) rainbow trout haematocrit (%), (D) rainbow trout whole blood [Hb] (g L^{-1}), and (E) rainbow trout mean cell haemoglobin concentration (MCHC) (g L^{-1}) for individuals in control and treatment groups and. Data is presented with group means with 95% confidence intervals.

Abnormalities within the control and treatment groups for rainbow trout and kōura were observed (Figure 8). Epithelial lifting from the cuticle, lamellae swelling, lamellae disorganisation, and cellular anomaly were observed in kōura gills. Hyperplasia, red blood cell proliferation, epithelial lifting, cellular anomaly, and primary and secondary lamellae fusion were observed in rainbow trout gills.

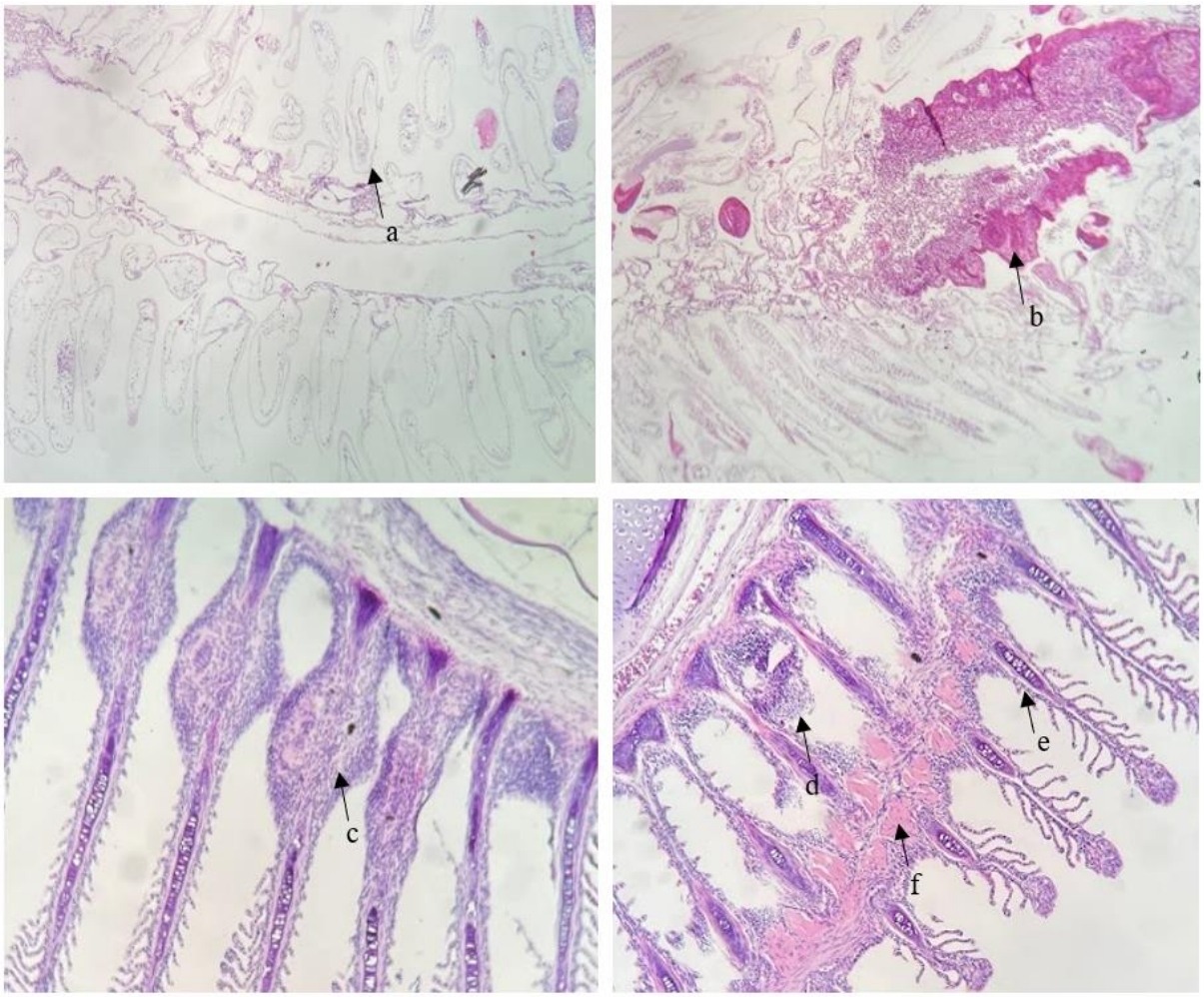


Figure 8. Histological gill sections of treatment kōura (top) and rainbow trout (bottom) individuals showing epithelial lifting from the cuticle, lamellae swelling, lamellae disorganisation (a), and a cellular anomaly (b) in kōura gills and hyperplasia (c), RBC proliferation (d), epithelial lifting (e), and a cellular anomaly (f) in rainbow trout gills.

A Kolmogorov-Smirnov test of cumulative scores for gill abnormalities revealed a significant difference (P <0.05) between the kōura treatment and control samples (P <0.01, Figure 9) and observed abnormalities were more prolific in the treatment gill sections. The most notable effect in the kōura treatment gills was the cellular anomaly, which could potentially be an increase in haemocytes that may be evidence of an immune response. While there was

evidence of damage in rainbow trout controls and treatments, there was no statistical difference between the two groups (P = 0.8475).

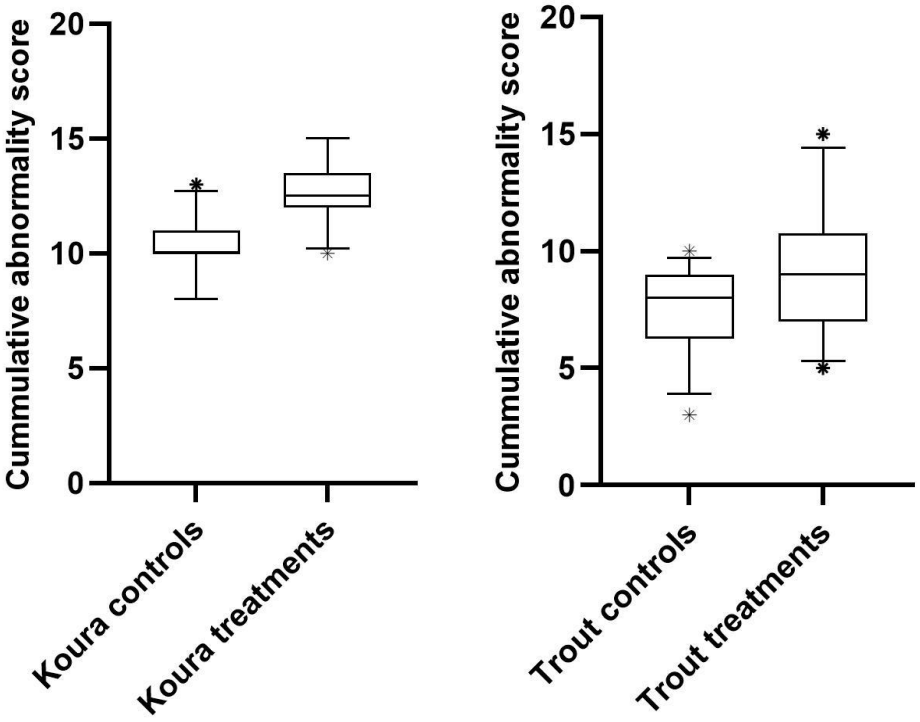


Figure 9. Box and whisker plots displaying cumulative abnormality scores for kōura and rainbow trout control and treatment groups with whiskers representing 10% and 90% bounds. A Kolmogorov-Smirnov test revealed a significant difference (P <0.05) between the kōura treatment and control samples.

Respiratory impacts on rainbow trout, common bully and kōura

Total aluminium was within the targeted exposure of 2 mg L^{-1} in exposure groups (Table 1). It was assumed that soluble forms of aluminium became more abundant with increasing pH. (Gensemer and Playle 1999).

Table 3. Mean (n =2) total and particulate aluminium in background respiration, control, and treatment tanks sampled at pH 7. Data presented in mean ±SEM.

Tank	Total Aluminium (mg L^{-1})	Soluble Aluminium (mg L^{-1})
Background	2.15 ±0.45	0.11 ±0.095
Control	2.40 ±0.40	0.11 ±0.095
Treatment	1.95 ±0.15	0.15 ±0.145

During daily monitoring, indicators of stress such as the loss of equilibrium and surface breathing were not observed in rainbow trout or kōura. However, one causality did occur when a common bully became trapped in the recirculation loop during the acclimation phase. Mean MO_2 for the entire exposure period was slightly greater in the treatment groups compared to control groups for each species, statistical tests were not performed on control and treatment group means due to the inherent variation in pH with the treatment groups (Figure 10).

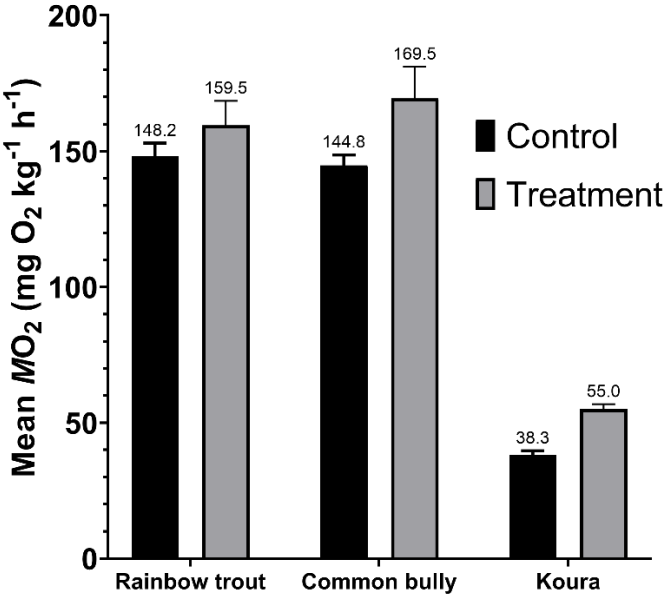


Figure 10. Mean (± SEM) metabolic consumption rate (MO_2) for rainbow trout, common bully and kōura exposed to either 2 mg Al L^{-1} pH 7 (control) or 2 mg Al L^{-1} pH 7–10 cycling (treatment).

Plots of the mass-specific oxygen consumption over time revealed a diurnal pattern in the kōura treatment and control groups, likely due to increased nocturnal activity influencing respiration rather than treatment effects (Figure 11). There was extensive variation in the rainbow trout (Figure 12) and common bully (Figure 13) data, likely related to the reduced sample numbers.

A Pearson's *r* correlation test was used to determine if a statistically significant correlation occurred when *t*-statistics between paired control and treatment groups were plotted against pH over time for each species. There was a significant correlation between the control and treatment *t*-statistics for kōura (r^2 = 0.34, P <0.0001, Figure 14), indicating a cyclic pattern in mass-specific oxygen consumption and aluminium-pH interactions may have been occurring. Pearson's *r* correlation tests for the common bully (r^2 = 0.02, P = 0.33, Figure 15) and rainbow trout (r^2 = 0.06, P = 0.054, Figure 16) were not statistically significant. However, the variability in the data and the reduced number of rainbow trout controls increased uncertainty in the

means. Trends in the *t*-statistics for rainbow trout and common bully reveal small differences between control and treatment respiration rates at circumneutral pH and larger differences at high pH. While the opposite is depicted for the *t*-statistic for kōura, indicating low pH and aluminium potentially have an effect on respiration rates for kōura. Large variances in the kōura treatment group towards the end of the experiment could be attributed to an active individual skewing the data. This data during could be considered statistical outliers, however, it removal would further decrease the sample size (n = 5).

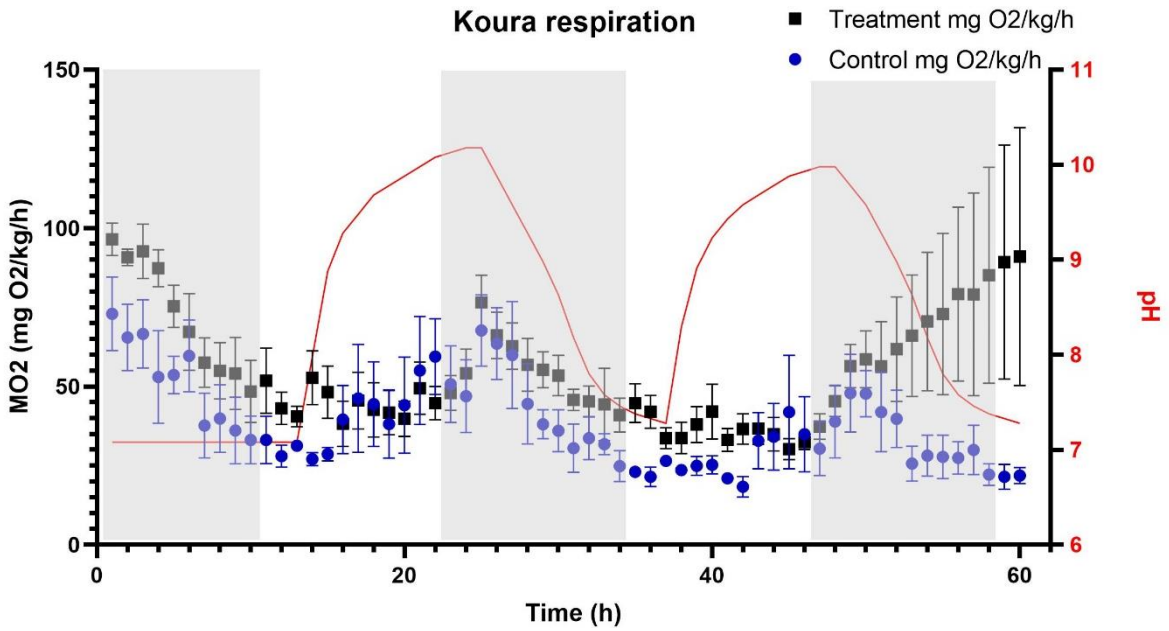


Figure 11. Effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) for kōura control (n = 6) and treatment groups (n = 5) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SEM. Shaded areas represent the dark photoperiod (12 h).

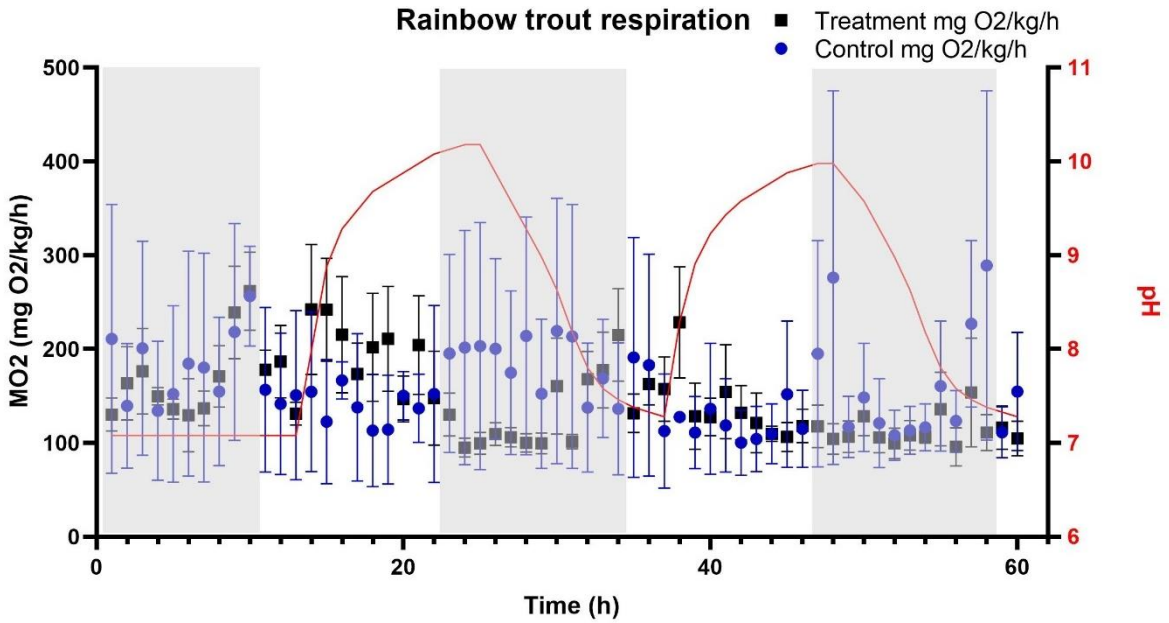


Figure 12. Effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) for rainbow trout control (n = 2) and treatment groups (n = 5) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SEM. Shaded areas represent the dark photoperiod (12 h).

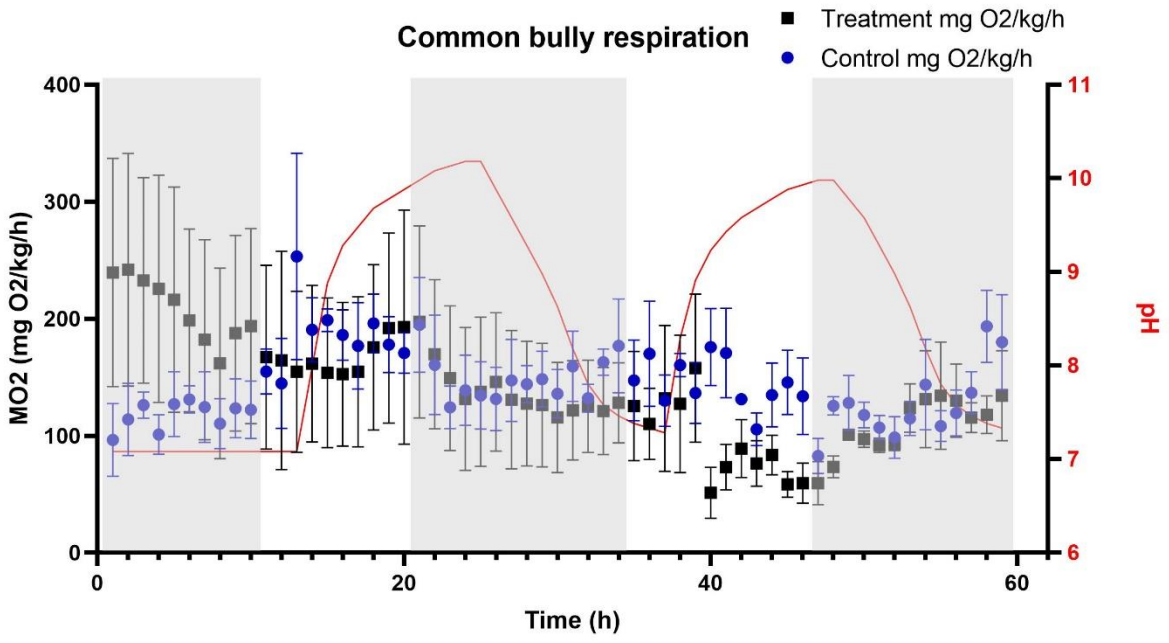


Figure 13. Effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) for common bully control (n = 3) and treatment groups (n = 4) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SEM. Shaded areas represent the dark photoperiod (12 h).

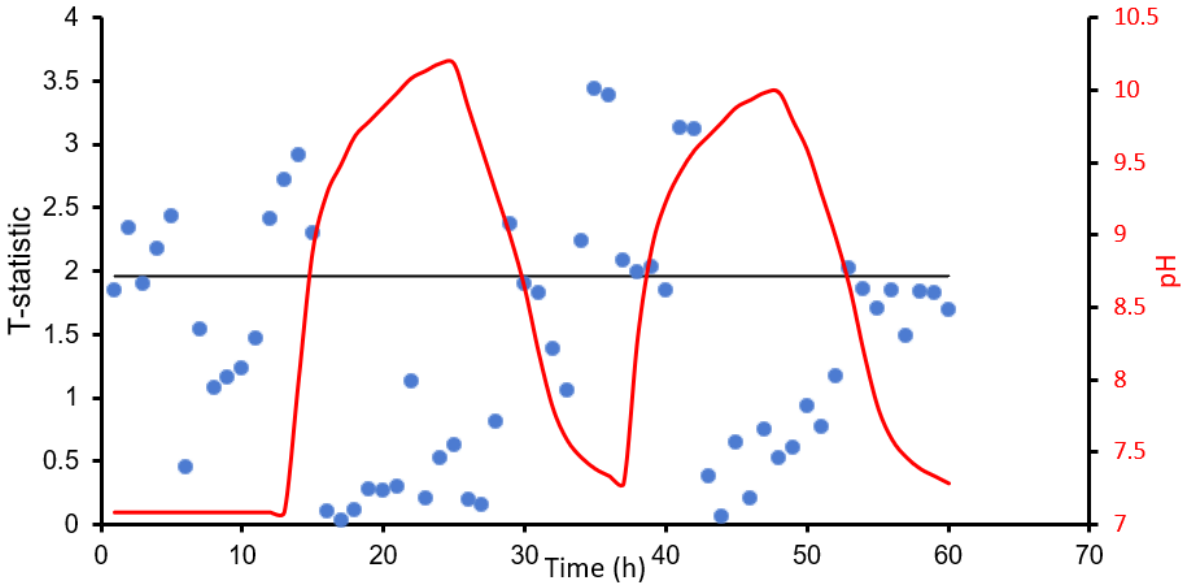


Figure 14. Plot of *t*-statistic values for the differences between the kōura control and treatment mean MO_2 for each hour, with diel pH cycling. If the *t*-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.

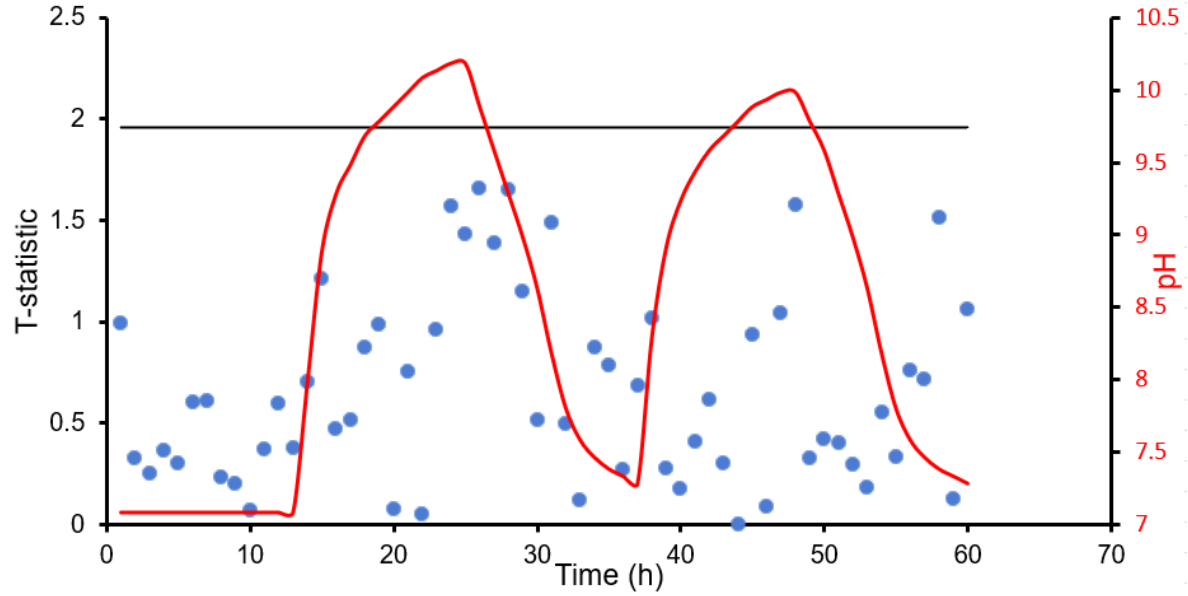


Figure 15. Plot of *t* -statistic values for the differences between the rainbow trout control and treatment mean MO_2 for each hour, with diel pH cycling. If the *t*-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.

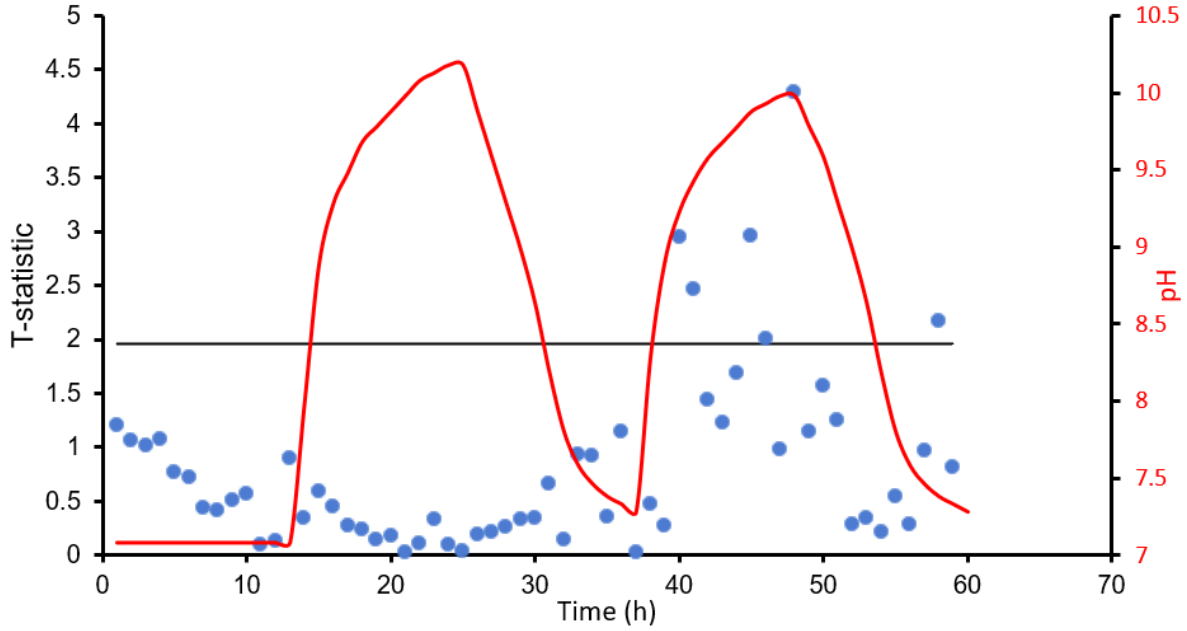


Figure 16. Plot of *t* -statistic values for the differences between the common bully control and treatment mean $\dot{M}O_2$ for each hour, with diel pH cycling. If the *t*-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.

Discussion

The objective of this study was to determine whether diel pH cycling (7-10-7) in association with aluminium (2 mg Al L^{-1}) resulted in disruption of osmoregulation and gill tissue damage in rainbow trout and kōura, and if changes in metabolic oxygen consumption occurred in kōura, rainbow trout and common bully in response to the same conditions. Significant changes were found in rainbow trout MCHC indicating cell swelling in the treatment group. Additionally, gill damage across all samples was observed, however, statistically significant differences were only found in kōura. Intermittent flow respirometry was used to determine mass-specific metabolic oxygen demand ($\dot{M}O_2$). There was some indication that kōura were impacted by the experimental conditions, although this was difficult to distinguish from possible increased nocturnal activity patterns. Ultimately, no consistent changes in $\dot{M}O_2$ were observed in response to diel pH changes and aluminium exposure.

Osmolarity impacts on rainbow trout and kōura

Previous studies have established that toxicological effects of dissolved aluminium occur at significantly lower concentrations (0.1–0.8 mg Al L^{-1}) compared to particulate aluminium (Gensemer and Playle 1999; Poléo and Hytterød 2003; Burgos-Aceves *et al.* 2019). For example, Witters *et al.* (1990) found a decrease in plasma volume by 60% in rainbow trout

exposed to aluminium (0.06 mg L^{-1}) at pH 5 for 3 days and elevated haematocrit (up to 55%) at 0.2 mg Al L^{-1}. However, physiological effects appear to be species-specific and depend on several factors, including pH, water hardness, humic content and length of exposure. For example, in contrast to acidic conditions, Poléo and Hytterød (2003) reported that Atlantic salmon (*Salmo salar*) exposed to 0.35 mg Al L^{-1} at pH 9.5 did not suffer any significant acute effects. However, after 3 weeks of continuous exposure, an increase in haematocrit (30%) and a decrease (15%) in chloride ion concentrations were observed.

In the current study, exposure of rainbow trout and kōura to 2 mg L^{-1} aluminium at varying pH for 10 days did not result in significant differences haematocrit and haemoglobin concentration compared to control groups exposed to 2 mg L^{-1} aluminium at constant pH 7 (Table 2, Figure 7Table 1). This finding partially supports the DeFrost *et al*. (2020) multiple linear regression (MLR) model, which indicated that within cirum-neutral pH range (6-8) toxicological effects are lower under alkaline pH compared to acidic pH, given the same water hardness and dissolved organic matter concentrations present in Lake Rotorua (Total aluminium and particulate aluminium (mean ± SEM) in rainbow trout and kōura treatment and control tanks at pH 7.). Although, effects at higher alkalinity >pH 8.5 may become more severe as $Al(OH)_4^-$ becomes more abundant. This suggests that H^+ concentration and the resulting prevalence of Al^{3+} are primarily responsible for osmotic disruption. In addition, H^+ concentration alone can cause similar osmoregulatory disruptions and may have a synergistic negative impact on aquatic species ability to osmoregulate at low pH (Wilkie and Wood 1991; Wilkie *et al.* 1999; Regish *et al.* 2018). Body pH is maintained through acid-base regulation whereby Na^+ and Cl^- are actively taken up from the environment in exchange for internal H^+ and HCO_3^- (Claiborne *et al.* 2002). At low environmental pH, there is an abundance of H^+ ions which inflict acid load onto the gills by inhibiting apical Na^+/H^+, NH_4^+ exchangers (Garcia Parra and Baldisserotto 2019). While in high pH waters, the lack of available H^+ ions ultimately cause a decrease in the ammonia (NH_3) blood-water gradient, slowing ammonia excretion rates. The alteration in osmoregulation disrupts acid-base regulation causing the imbalance of extracellular and intracellular fluid pH (Claiborne *et al.* 2002). The interdependence of the osmoregulatory and acid-base regulatory systems makes determining specific physiological effects from aluminium challenging, given the dependence of dissolved aluminium on pH.

Significant differences in the variances of osmolarity between the control and treatment groups for both rainbow trout and kōura were observed (*F*- test; *P* values= 0.0297 and 0.0269, respectively), reducing the sensitivity of the statistical test to detect differences between groups. Additionally, rainbow trout mean MCHC was greater ($P < 0.01$) in the treatment group compared to the control group. The treatment group MCHC (3.2 mM) was similar to typical MCHC values for rainbow trout (~3.5 mM). In comparison, the control group MCHC was lower at 2.34 mM (Wells and Weber 1990), indicating erythrocyte swelling in the control group. Erythrocyte swelling can occur as a generalised adrenergic stress response increasing the activity of the cellular membrane Na^+/H^+ exchanger pumping Na^+ into and H^+ out of the cell

(Heming *et al.* 1987). The resulting decrease in intraerythrocytic H^+ causes cell pH and haemoglobin-oxygen affinity to rise, consequently increasing oxygen uptake at the gills. However, the increased intracellular Na^+ induces osmotic water uptake, causing the cell to swell. (Heming *et al.* 1987). This suggests that a more generalised stress response occurred in the treatment group as metabolic resources were mobilised to maintain homeostasis.

Gill histology

The gill epithelium is comprised of various cell types such as pavement cells, mucous cells, and chloride cells which aid in gill function (Dymowska *et al.* 2012). Soluble aluminium, present at low and high pH, can polymerise and precipitate when moving through the gills and high concentrations of precipitated aluminium in circumneutral pH can abrade gill surfaces (Gensemer and Playle 1999). Commonly documented gill morphological changes due to aluminium include epithelial lifting, lamellae swelling, hyperplasia, primary and secondary lamellae fusion and excess mucus excretion (Evans *et al.* 1988; Alexopoulos *et al.* 2003). For example, hyperplasia, excess mucus secretion, and necrosis were documented in the gills of rainbow trout exposed to aluminium (0.13, 0.27, 0.54 mg L^{-1}) at pH 4.7 and 5.2 (Evans *et al.* 1988). Also, Alexopoulos *et al.* (2003) investigated the effects of freshly neutralised aluminium (0.5 mg L^{-1}) at circumneutral pH on freshwater crayfish (*Pacifastacus leniusculus*). They found that aluminium interacted predominantly at the surface of the gill, in the mucus layer and increased haemocytes in the treatment groups. In the present study, the histological examination of gill tissue revealed that damage occurred in both control and treatment groups of rainbow trout and kōura. However, statistically significant differences between control and treatment groups were only detected in kōura (Figure 9). This suggests that kōura gills tissues may be more susceptible to gill damage from elevated pH when high aluminum concentrations are present. However, it is important to note that abnormalities were observed in all histological tissue samples for both species and treatment groups which may indicate that gill tissue damage was due to the high concentration of particulate aluminium in both treatment and control groups.

Respiratory impacts on rainbow trout, common bully and kōura

The gills are responsible for respiratory gas exchange, ionoregulation, acid-base balances, and osmoregulation in fish and invertebrates. Aluminium can disrupt these processes through polymerisation and precipitation onto gill surfaces (Gensemer and Playle 1999). Prolonged decreases in the rate of oxygen influx may result in hypoxia, while restriction of CO_2 efflux can result in hypercapnia and respiratory acidosis (Svendsen *et al.* 2016). Slower oxygen uptake may initiate a compensatory response to increase the permeability of the branchial epithelial membranes. This improves the oxygen uptake efficiency of the gills, allowing cellular metabolic oxygen demands to be met (Onukwufor and Wood 2020). However, this mechanism cannot be sustained indefinitely due to elevated losses of plasma ions beyond the body's compensatory mechanisms for increased ionic uptake (Onukwufor and Wood 2020).

Alkaline waters (pH >8.5) impose similar challenges to acid environments, by inducing respiratory alkalosis (Wilkie and Wood 1996). The abundance of OH^- and HCO_3^- within the environment increases the electrochemical gradient favouring the influx of OH^- and loss of H^+ ions. This not only disrupts the acid-base balance of the animal but causes osmoregulatory issues as ionic uptake of Na^+ and Cl^- are inhibited (Wilkie and Wood 1996). Limited metabolic function and the reduced production of energy for activity disrupts homeostasis and increases the animal's susceptibility to disease and predation. This effect has been observed in freshwater crayfish (*Pacifasticus leniusculus*), where impairment of immune responses occurred under low oxygen tension due to aluminium precipitation on the gills, potentially lowering the immune response (Ward *et al.* 2006).

In the current study, it was expected that the MO_2 would increase when exposed to aluminium and periods of circumneutral pH or during periods of declining pH due to precipitation of particulate aluminium onto the gill surface causing inhibition of respiratory gas exchange (Playle and Wood 1989b; Gensemer and Playle 1999). Increased oxygen uptake rates were expected to compensate for possible respiratory acidosis and increased gill ventilation (Gensemer and Playle 1999). Kōura MO_2 values (25–100 mg O_2 kg^{-1} h^{-1}) were within a similar range to those observed for exposure to modified zeolite at circumneutral pH (Parkyn *et al.* 2011). The MO_2 vales for rainbow trout (100–250 mg O_2 kg^{-1} h^{-1}) were within the range observed for rainbow trout in a respirometry study by Svendsen *et al.* (2012) under normoxic conditions. However, common bully MO_2 were similar to rainbow trout respiration rates, when higher rates were anticipated due to their smaller size (Reece *et al.* 2015). Although there was a significant negative correlation between pH and kōura MO_2, there was no consistent trend in differences between control and treatment groups for any of the tested species. Apparent increases in the kōura treatment group MO_2 during periods of declining pH are mirrored in the control group, suggesting that the increased MO_2 was due to increased nocturnal activity.

It should also be noted that oxygen concentration measurements taken within the recirculation loop of the respirometer were inaccurate for some individuals within the control groups. This resulted in the exclusion of the data, reducing sample numbers in the rainbow trout and common bully control groups, and increasing uncertainty around the means. Inaccuracies can be attributed to the accumulation of air within the oxygen sensor probe space and within the respirometer, reducing the sensor's ability to measure the magnitude of cyclic oxygen saturation changes. Additionally, periods of spontaneous activity are typically removed from the respirometry data sets by only considering the lowest 10^{th} percentile of values. However, this was not feasible in the current study as it would have resulted in the exclusion of any increases in respiration attributed to the experimental treatment. Ultimately, the higher than anticipated uncertainty in the data precluded substantive conclusions as to the effect of diel pH cycling and aluminium on respiration in common bully and rainbow trout. Increasing the length of exposure may aid in ascertaining consistent responses to changes in

pH and resultant aluminium speciation over time. From the observed results, substantial physiological disturbances were not observed in the treatment groups in relation to the control groups, indicating that compensatory mechanisms were sufficient to cope with the metabolic challenges, even after 10 days exposure. Therefore, an exposure time of 60 hours may have been of insufficient duration to produce respiratory responses to the combination of cyclic pH and aluminium as impacts from both particulate and dissolved aluminium may be cumulative over time.

Algal bloom driven diel shifts in pH (pH 7–10) are unlikely to result in acute osmoregulatory or respiratory impairment in kōura, common bully, or rainbow trout. The tested aluminium concentration was approximately double the concentration dosed to the Utuhina and Puarenga Streams and would be substantially diluted upon entering Lake Rotorua. In addition, diel shifts in pH are restricted to periods of intensive algal bloom formation. Such conditions do not persist for longer than 2-3 weeks and are becoming increasingly infrequent as water quality improves (McBride *et al.* 2018). Water hardness has been demonstrated to have an ameliorating effect on aluminium toxicity in fathead minnow (*Pimephales promelas)* and the water flea *Ceriodaphnia dubia* (Gensemer *et al*. 2018). However, the difference in EC10 valves was minor within the water hardness range for Lake Rotorua (14 mg L^{-1} as $CaCO_3$) and Hamilton City tap water (40.9 mg L^{-1} as $CaCO_3$), and there was no difference above pH 8. These results, along with the monitoring work undertaken in the Utuhina and Puarenga Streams by Ling (2016a) and Ling (2016b), provide evidence that the current aluminium dosing rate is unlikely to have a significant effect on biota in Lake Rotorua. However, further investigation of aluminium exposure on more susceptible life stages (i.e., larval rainbow trout) would help to define species tolerances. Exposing additional species common to Lake Rotorua, such as inanga (*Galaxias maculatus*), kakahi (*Echyridella menziesi*), or extending the research of aluminium toxicity to chironomid communities could provide more information on the potential aluminium toxicity. Determining the combined effects of aluminium and pH on chironomid communities and kakahi would be particularly important in Sulphur Bay. This part of Lake Rotorua has naturally high inputs of geothermally sourced aluminium (1 mg Al L^{-1}) and highly acid waters (pH ~3.5) (Tempero *et al.* 2015). Acid-tolerant chironomid species reside in this part of the lake, while kakahi have been found within the circulation zone between the bay and the rest of the lake (Tempero *et al.* 2015). Therefore, these species could be adversely impacted by the effects of aluminium and low pH.

Conclusions

The main conclusions were:

1) Cyclic changes in pH driven by algal blooms in combination with current alum dosing levels are highly unlikely to result in acute osmoregulatory impacts on common fish and macroinvertebrate species in Lake Rotorua.
2) There were no significant impacts on metabolic oxygen consumption under similar exposure conditions.
3) There was some evidence that extended exposure to 2 mg Al L^{-1} may damage gill tissue from particulate aluminium, with kōura being more susceptible than rainbow trout.

Recommendations

1) Further investigation of aluminium exposure on more susceptible and less mobile life stages (i.e., larval rainbow trout) which would help to define potential impacts and species tolerances.
2) Further testing of potential impacts on gill tissues from particulate aluminium is recommended, particularly with regard to kōura.

References

Abd El-Atti M, Desouky MMA, Mohamadien A and Said RM (2019). Effects of titanium dioxide nanoparticles on red swamp crayfish, *Procambarus clarkii*: Bioaccumulation, oxidative stress and histopathological biomarkers. *Egyptian Journal of Aquatic Research* **45**: 11-18.

Acuña-Alonso C, Lorenzo O, Álvarez X, Cancela Á, Valero E and Sánchez Á (2020). Influence of *Microcystis* sp. and freshwater algae on pH: Changes in their growth associated with sediment. *Environmental Pollution (1987)* **263**: 114435-114435.

Alexopoulos E, McCrohan CR, Powell JJ, Jugdaohsingh R and White KN (2003). Bioavailability and toxicity of freshly neutralized aluminium to the freshwater crayfish *Pacifastacus leniusculus*. *Archives of Environmental Contamination and Toxicology* **45**: 509-514.

Ali MY, Pavasovic A, Mather PB and Prentis PJ (2017). Expression patterns of two carbonic anhydrase genes, Na+/K+-ATPase and V-type H+-ATPase, in the freshwater crayfish, *Cherax quadricarinatus*, exposed to low pH and high pH. *Australian Journal of Zoology* **65**: 50.

Brauner CJ, Farrell AP and Richards J (2009). Hypoxia. London, Academic Press.

Burrows WD and Hem JD (1977). Aquatic aluminum: Chemistry, toxicology, and environmental prevalence. *Critical Reviews in Environmental Science and Technology* **7**: 167–216.

Cardwell AS, Adams WJ, Gensemer RW, Nordheim E, Santore RC, Ryan AC and Stubblefield WA (2018). Chronic toxicity of aluminum, at a pH of 6, to freshwater organisms: Empirical data for the development of international regulatory standards/criteria. *Environmental Toxicology and Chemistry* **37**: 36-48.

Claireaux G and Chabot D (2016). Responses by fishes to environmental hypoxia: integration through Fry's concept of aerobic metabolic scope. *Journal of Fish Biology* **88**: 232-251.

Cooke GD, Welch EB, Peterson S and Nichols SA (2005). Restoration and Management of Lakes and Reservoirs. Boca Raton, CRC press. 601

Dacie JV and Lewis SM (1991). Practical Haematology. New York. Churchill Livingstone.

DeForest DK, Brix KV, Tear LM and Adams WJ (2018). Multiple linear regression models for predicting chronic aluminum toxicity to freshwater aquatic organisms and developing water quality guidelines. *Environmental Toxicology and Chemistry* **37**: 80-90.

DeForest DK, Brix KV, Tear LM, Cardwell AS, Stubblefield WA, Nordheim E and Adams WJ (2020). Updated multiple linear regression models for predicting chronic aluminum toxicity to freshwater aquatic organisms and developing water quality guidelines. *Environmental Toxicology and Chemistry* **39**: 1724-1736.

Dodds WK (2002). Freshwater ecology concepts and environmental applications. San Diego. Academic Press.

Freda J, Sanchez DA and Bergman HL (1991). Shortening of branchial tight junctions in acid-exposed rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Science* **48**: 2028-2033.

Gensemer RW, Gondek JC, Rodriquez PH, Arbildua JJ, Stubblefield WA, Cardwell AS, Santore RC, Ryan AC, Adams WJ and Nordheim E (2018). Evaluating the effects of pH, hardness, and dissolved organic carbon on the toxicity of aluminum to freshwater aquatic organisms under circumneutral conditions. *Environmental Toxicology and Chemistry* **37**: 49-60.

Gensemer RW and Playle RC (1999). The bioavailability and toxicity of aluminum in aquatic environments. *Critical Reviews in Environmental Science and Technology* **29**: 315-450.

Hamill KD (2021). Alum dosing to the Puarenga Stream: Compliance monitoring 2020. River Lake Ltd. Whakatane. 26.

Heini A, Puustinen I, Tikka M, Jokiniemi A, Lepparanta M and Arvola L (2014). Strong dependence between phytoplankton and water chemistry in a large temperate lake: spatial and temporal perspective. *Hydrobiologia* **731**: 139-150.

Hickey CW and Gibbs MM (2009). Lake sediment phosphorus release management-Decision support and risk assessment framework. *New Zealand Journal of Marine and Freshwater Research* **43**: 819-856.

Landman MJ and Ling N (2008). Potential environmental impacts of Puarenga Stream alum dosing: A review of aquatic toxicological data on aluminium, possible impacts on Sulphur Bay biota, and recommendations for impact monitoring. Scion. Rotorua. 18.

Landman MJ and Ling N (2009). Sulphur Bay baseline monitoring for the Puarenga Stream alum discharge. Scion. Rotorua. 10.

Ling N (2014). Utuhina Stream monitoring 2013: Effects of continuous alum dosing on fish and aquatic invertebrates. ERI Report No. 54. Client report prepared for Environment Bay of Plenty. Environmental Research Institute, Faculty of Science and Engineering, The University of Waikato, Hamilton, New Zealand. 20.

Ling N (2016). Puarenga Stream alum dosing – summary of effects on lake biota 2015. ERI Report No. 82. Client report prepared for Bay of Plenty Regional Council. Environmental Research Institute, Faculty of Science and Engineering, University of Waikato, Hamilton, New Zealand. 12 pp.

Ling N (2017). Puarenga Stream alum dosing – effects on Lake Rotorua/Sulphur Bay biota 2016. ERI Report No. 102. Client Report prepared for the Bay of Plenty Regional Council. Environmental Research Institute, Faculty of Science and Engineering, The University of Waikato, Hamilton, New Zealand. 12.

Ling N (2021). Utuhina Stream 2006 - 2020. In-stream alum dosing effects on fish and aquatic invertebrates ERI Report No. 153. Client report prepared for Bay of Plenty Regional Council. Environmental Research Institute, School of Science, The University of Waikato, Hamilton, New Zealand. 28.

Ling N and Brijs J (2009). Utuhina Stream Monitoring 2009:Fish and aquatic invertebrates. CBER Contract Report 106. University of Waikato. Hamilton, New Zealand. 25.

Mallatt J (1985). Fish gill structural changes induced by toxicants and other irritants: a statistical review. *Canadian Journal of Fisheries and Aquatic Sciences* **42**: 630 - 648.

Marshall WS (1985). Paracellular ion transport in trout opercular epithelium models osmoregulatory effects of acid precipitation.*Canadian Journal of Zoolology* **63**: 1816-1822.

McBride CG, Abell JM and Hamilton DP (2018). Long-term nutrient loads and water quality for Lake Rotorua: 1965 to 2017. ERI report 123. Environmental Research Institute, The University of Waikato, Hamilton, New Zealand. 70.

McWilliams PG (1983). An investigation of the loss of bound calcium from the gills of the brown trout, Salmo trutta, in acid media*. Comparative Biochemistry and Physiology* **74A**: 107-116.

Ping XIE (2006). Biological mechanisms driving the seasonal changes in the internal loading of phosphorus in shallow lakes. *Science China. Earth Sciences*: 14-27.

Playle RC and Wood CM (1989). Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **159**: 527-537.

Playle RC and Wood CM (1989). Water pH and aluminum chemistry in the gill micro-environment of rainbow trout during acid and aluminum exposures. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **159**: 539-550.

Poléo ABS and Hytterød S (2003). The effect of aluminium in Atlantic salmon (*Salmo salar*) with special emphasis on alkaline water. *Journal of Inorganic Biochemistry* **97**: 89-96.

Rydin E, Huser B and Welch EB (2000). Amount of phosphorus inactivated by alum treatments in Washington Lakes. *Limnology and Oceanography* **45**: 226-230.

Sparling DW and Lowe TP (1996). Environmental hazards of aluminum to plants, invertebrates, fish, and wildlife. Springer. 127.

Svendsen MBS, Bushnell PG and Steffensen JF (2016). Design and setup of intermittent-flow respirometry system for aquatic organisms. *Journal of Fish Biology* **88**: 26-50.

Tank SE, Lesack LFW and McQueen DJ (2009). Elevated pH regulates bacterial carbon Cycling in lakes with high photosynthetic activity. *Ecology (Durham)* **90**: 1910-1922.

Taylor S, Landman MJ and Ling N (2009). Flow cytometric characterization of freshwater crayfish hemocytes for the examination of physiological status in wild and captive animals. *Journal of Aquatic Animal Health* **21**: 195-203.

Tempero GW, McBride CG, Abell JM and Hamilton DP (2015). Anthropogenic phosphorus loads to Lake Rotorua. Client report prepared for Bay of Plenty Regional Council. ERI Report No. 66. Environmental Research Institute, Faculty of Science and Engineering, The University of Waikato, Hamilton, New Zealand. 31.

Waring CP and Brown JA (1995). Ionoregulatory and respiratory responses of brown trout, *Salmo trutta*, exposed to lethal and sublethal aluminium in acidic soft waters. *Fish Physiology and Biochemistry* **14**: 81-91.

Wilkie MP, Laurent P and Wood CM (1999). The physiological basis for altered Na and Cl^- movements across the gills of rainbow trout (*Oncorhynchus mykiss*) in alkaline (pH = 9.5) water. *Physiological and Biochemical Zoology* **72**: 360-368.

Wilkie MP and Wood CM (1991). Nitrogenous waste excretion, acid-base regulation, and lonoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. *Physiological Zoology* **64**: 1069-1086.

Wilkie MP and Wood CM (1996). The adaptations of fish to extremely alkaline environments. *Comparative Biochemistry and Physiology, Part B* **113**: 665-673.

Witters HE, Van Puymbroeck S, Van Den Sande I and Vanderborght OLJ (1990). Haematological disturbances and osmotic shifts in rainbow trout, *Oncorhynchus mykiss*

(Walbaum) under acid and aluminium exposure. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology* **160**: 563-571.

Appendix 1

Comparison between pH cycling in experimental tanks (red) and measured pH variation in Lake Rotorua (green) during an algal bloom in March 2017.