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

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Abstract

Alum (aluminium sulphate, $Al_2(SO_4)_3$) dosing is used to reduce eutrophication of lakes by sequestering phosphorus and preventing its uptake by phytoplankton for growth. When applied to water at circum-neutral pH, alum forms aluminium hydroxide ($Al(OH)_3$), a precipitate that is benign at environmentally relevant concentrations and moderate concentrations. However, at low and high pH, dissolved ionic aluminium species evolve which are known to be toxic to a range of aquatic organisms. In the Rotorua lakes, alum dosing occurs under circum-neutral pH (6–8) conditions, however, transiently elevated pH (10) conditions may occur due to algal uptake of carbon dioxide (CO_2) for photosynthesis. Under elevated pH, particulate $Al(OH)_3$ solubilises to form aluminate ($Al(OH)_4^-$), which can interfere with osmoregulation and respiration. Similarly, at circumneutral pH, solid $Al(OH)_3$ predominates and is generally benign, but in high concentrations can irritate and damage gill tissue causing inflammation and excess mucus production which impairs oxygen uptake, CO_2 elimination, and ultimately causing death. The effects of aluminium under intermittent elevated pH on fish gill function, respiration rates, and metabolism have not yet been investigated.

This study investigated the impacts of aluminium on rainbow trout (*Oncorhynchus mykiss),* common bully (*Gobiomorphus cotidianus*), and kōura (*Paranephrops planifrons*) osmoregulation and respiration under diurnal pH cycles, through two experiments. The first being a dose exposure study whereby rainbow trout and kōura were exposed to alum derived aluminium at 2 mg L^{-1} under diel pH cycling (pH 7–10) over 10 days. Measured parameters included haematocrit, haemoglobin, mean cell haemoglobin concentration (MCHC), and blood plasma osmolarity in rainbow trout and haemolymph osmolarity in kōura. A significant increase (Student's *t*-test, $P < 0.05$) in the mean cell haemoglobin concentration (MCHC) in the rainbow trout control group indicated erythrocyte swelling, suggesting a generalised stress induced response. However, other haematological variables such as plasma and haemolymph osmolarity, haematocrit and haemoglobin concentrations were not significantly different (Student's *t*-test, $P > 0.05$) between control and treatment groups. Histological examination of kōura and rainbow trout gill tissue was also conducted, with abnormalities observed within both the control and treatment groups for each species. However no significant differences (Kolomogorov-Smirnov, $P > 0.05$) were observed between groups. It was concluded that

organisms can physiologically compensate for aluminium induced stress and disturbance at the gills.

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 would inhibit the respiratory gas exchange resulting in increased gill ventilation from hypoxic and hypercapnic conditions. There was a significant negative correlation (Pearson's, $r^2 = 0.34$, $P < 0.0001$) between pH and MO_2 possibly indicating kōura are 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, mean MO_2 was within expected ranges for rainbow trout and kōura, indicating that significant respiratory impacts were not occurring within the period of exposure. 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 aluminium and diel pH cycling are unlikely to cause acute osmoregulatory or respiratory impairment or cause death in kōura, common bully, or rainbow trout.

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Chapter 1
Introduction

Impacts of Lake Eutrophication

Anthropogenic land-use changes such as deforestation, urbanisation and agricultural intensification have increased catchment loading of nitrogen and phosphorus to aquatic ecosystems, resulting in eutrophication and increased formation of algal blooms (Smith *et al.* 2016; Abell *et al.* 2020). Cyanobacterial algal blooms are of particular concern has they may produce a range of toxins with negative impacts ranging from a musty taste in drinking water to neurotoxicological effects and even death following consumption (Smith *et al.* 2016). Microcystin hepatotoxins can inhibit protein phosphatases, osmoregulation, and oxidative processes in freshwater fishes (Monserrat *et al.* 2003). Toxins from species such as *Microcystis aeruginosa* have been found to accumulate within the native New Zealand freshwater crayfish or "kōura" *(Paranephrops planifrons)* and the introduced rainbow trout (*Oncorhynchus mykiss*) to levels above safe limits for human consumption set by the World Health Organisation (Wood *et al.* 2006; Hickey & Gibbs 2009; Clearwater *et al.* 2014). Reports of microcystin impaired growth in common carp (*Cyprinus carpio*), osmoregulation in rainbow trout, and cardiac function in brown trout (*Salmo trutta*) alevins support the need for reductions in nutrient loading to reduce toxic algal bloom formation (Best *et al.* 2001; 2003; El Ghazali *et al.* 2010). Even on toxin producing blooms may have negative ecological impacts by reducing water clarity, which limits light availability to submerged macrophytes and reduces amenity values to the public. In poorly buffered lakes, such as Lake Rotorua, intensive algal blooms may induce diel pH fluctuations of up to 3–4 pH units (i.e., pH 6 –10).

Algal bloom driven pH cycles

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. At low pH, carbonic acid is most prevalent while carbonate is predominantly formed at high pH (Figure 1.1) (Dodds 2002), depicted by the bicarbonate equilibrium equation below (Dodds 2002):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^-$$

In this equation, moving from left to right, CO_2 reacts with water to form carbonic acid, which then dissociates into hydrogen ions and bicarbonate in solution, and then further dissociates into carbonate. Carbonate buffers water 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, while those with little available carbonate and bicarbonate are more likely to experience fluctuations in pH (Dodds 2002).

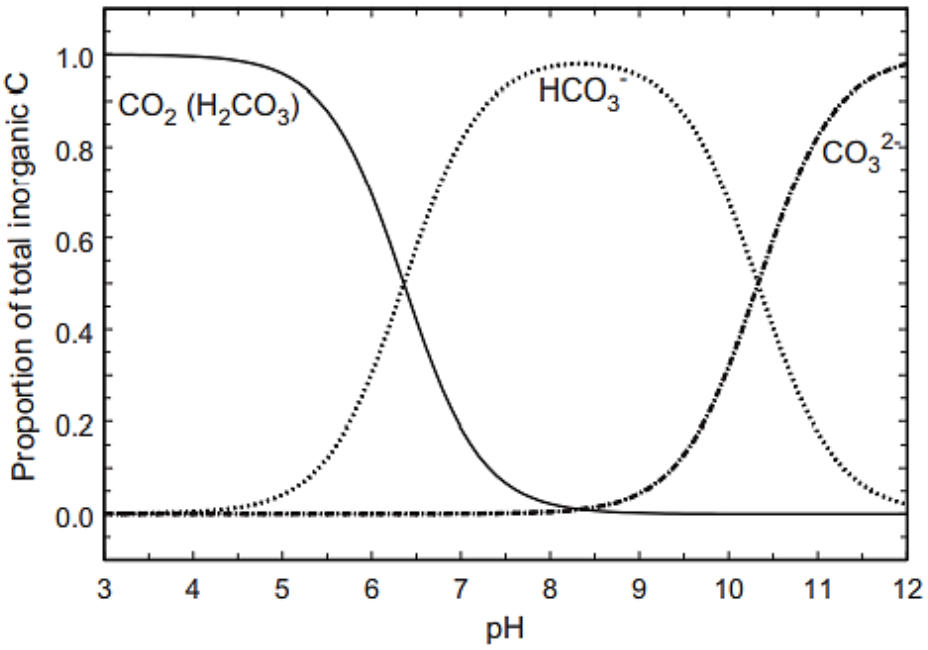


Figure 1.1: Changing concentrations of inorganic carbon compounds in the bicarbonate equilibrium as a function of pH (Dodds 2002, p. 232)

Photosynthesis involves the uptake of CO_2 which can lead to increases in pH as the bicarbonate equilibrium equation is driven to the right (Ping 2006; Tank *et al.* 2009; Acuña-Alonso *et al.* 2020). Respiratory release of CO_2 at night has the opposite effect, creating free protons and decreasing pH (Heini *et al.* 2014). The biomass of the algal bloom therefore dictates the magnitude of the flux of bicarbonate in the system and the magnitude of pH change over a 24-hour period. For example, Lake Rotorua in the Bay of Plenty Region 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 1.2) (Tempero *et al.* 2015).

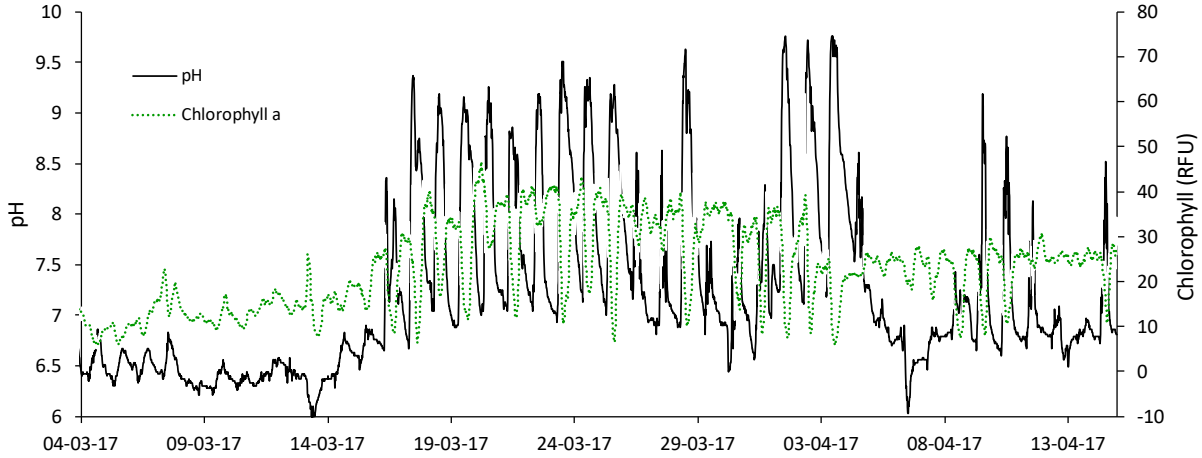


Figure 1.2: changes in pH between pH 6.5 and pH 10 correlating with fluctuations in Chlorophyll *a* in Lake Rotorua over a 3-week period in March 2017.

Alum dosing for control of phosphorus loading

Alum (aluminium sulphate) is used as a restoration tool by lake managers to reduce primary productivity by sequestering dissolved reactive phosphate (DRP). Reducing phosphorus availability limits phytoplankton growth and inhibits algal bloom formation (Hickey & Gibbs 2009). For example, two successive whole-lake alum applications of 1.5 mg Al L^{-1} to Lake Courtille (France) reduced DPR by approximately 30% at the surface and 50% at the sediment/water interface (Hullebusch *et al.* 2002). Similarly, two successive alum doses of Lake Ketchum (USA) decreased summer mean epilimnetic total phosphorus by 95% (Brattebo *et al.* 2017). A reduction of internal phosphorus loading of 81% was observed in Lake Harriet post alum treatment (Huser *et al.* 2011). The effects of reduced available phosphorus due to alum dosing saw large reductions in chlorophyll *a* (> 70%) and the reduced the frequency and density of nuisance algal blooms in these lakes (Huser *et al.* 2011; Brattebo *et al.* 2017).

Once applied to the waterbody, aluminium sulphate undergoes a hydrolysis reaction producing aluminium hydroxide ($Al(OH)_3$), with DRP subsequently adsorbing to the aluminium ions (Cooke *et al.* 1993). When applied as a bulk dose, alum may also form a surface barrier (sediment cap) capturing the release of phosphorus from sediment and reducing internal

loading (Rydin *et al.* 2000). Unlike iron, aluminium is unresponsive to redox changes, keeping phosphorus bound during periods of lake stratification and resulting hypoxia, thereby interrupting phosphorus cycling within the lake (Hickey & Gibbs 2009). This is advantageous as aluminium hydroxide mineralises over a period of 1–2 years, permanently removing the adsorbed phosphorus from uptake by phytoplankton (Cooke 1993; Hickey & Gibbs 2009). However, there are drawbacks to the use of alum, ideally dosing is conducted under circum-neutral conditions (pH 6.0–8.0), resulting in the formation of a amorphous aluminium hydroxide precipitate on contact with water which binds dissolved reactive phosphorus (DRP) and forming a low-density floc (Lewandowski *et al.* 2003). 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 decline, increasingly toxic soluble species of aluminium form, beginning with $Al(OH)^+$, then $Al(OH)^{2+}$, and finally free Al^{3+} ions. The release of H^+ ions occur during aluminium hydrolysis reactions:

$$Al(OH)_2^+ + H_2O \leftrightarrow Al(OH)_3\,(s) + H^+$$
$$Al(OH)^{2+} + H_2O \leftrightarrow Al(OH)_2^+ + H^+$$
$$Al^{3+} + H_2O \leftrightarrow Al(OH)^{2+} + H^+$$

Decreasing pH

Where (s) = solid precipitate (Cooke *et al.* 2005)

Concerns regarding the toxicity of aluminium within freshwater ecosystems began soon after the implementation of alum dosing for lake restoration in the 1970s and an increased awareness of aluminium toxicity mobilised by acid rain in some northern hemisphere catchments (Sparling & Lowe 1996). The mineralised form of $Al(OH)_3$ (Gibbsite) is relatively stable but the amorphous form can be solubilised under low and high pH conditions (Lewandowski *et al.* 2003), forming $Al(OH)_4^-$ under alkaline conditions and, $Al(OH)_2^+$, $Al(OH)^{2+}$, and Al^{3+} under increasingly acidic conditions (Figure 1.3) (Gensemer & Playle 1999; Winter *et al.* 2005).

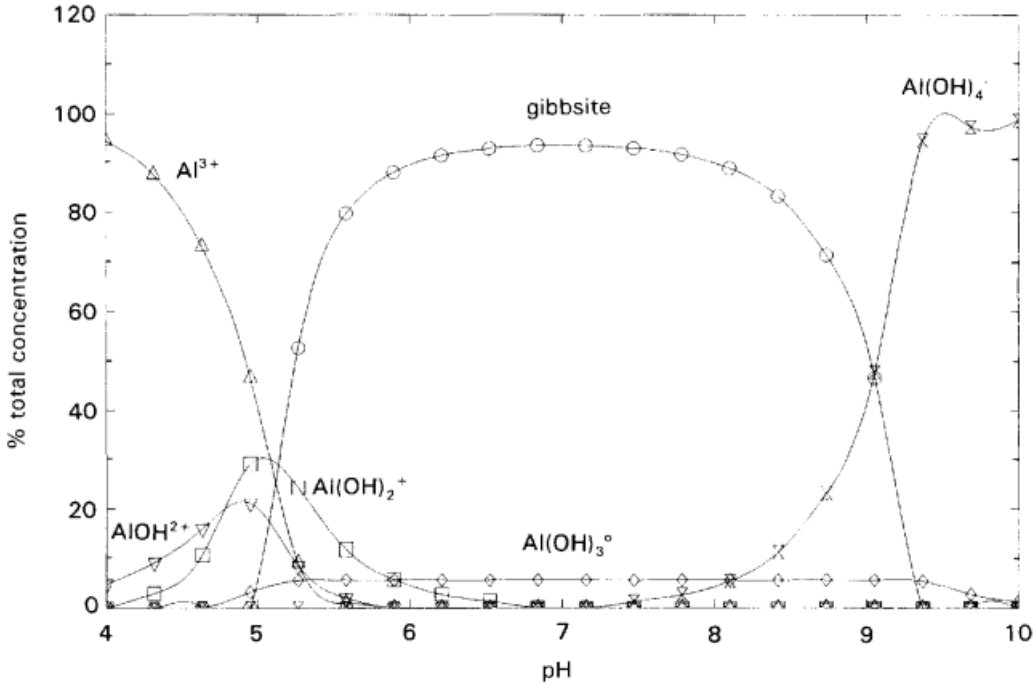


Figure 1.3: Aluminium speciation across a range of pH conditions from 4 to 10 (Gensemer & Playle 1999).

Therefore, knowing the alkalinity and buffering capacity of the receiving environment is critical to avoid the release of H^+ beyond the buffering capacity of the system (Tempero 2018). Klapper (2002) stated that alum dosing should not exceed 3 mg Al L^{-1} for every 10 mg L^{-1} of $CaCO_3$ to stay within the water's buffering capacity, while Cooke *et al.* (2005) stated that no more than 5 mg Al L^{-1} should be dosed to low alkalinity (<50 mg L^{-1} $CaCO_3$) lakes to avoid toxic effects. Because of the low buffering capacity in Lake Rotorua (alkalinity ~8.3 mg L^{-1} $CaCO_3$ and hardness ~14.8 mg L^{-1} $CaCO_3$) , the recommended limit for total aluminium concentrations is 0.2 mg L^{-1} between pH 6.0 and pH 9.0, and 0.1 mg L^{-1} above pH 10 (Tempero *et al.* 2015).

Alum dosing of Lake Rotorua

Whole lake bulk dosing and continuous inflow dosing of alum have been used to mitigate phosphorus loading in New Zealand (Pilgrim & Brezonik 2005; Paul *et al.* 2008; Landman & Ling 2011). Whole-lake dosing primarily targets the internal loading of phosphorus and is most effective in lakes suffering from internally driven algal blooms (Welch & Cooke 2005). Inflow dosing focuses on mitigating external phosphorus loading by removing phosphorus from within the water column of tributary streams and is most effectively used in catchments with

high external phosphorus loading (Pilgrim *et al.* 2007; Smith *et al.* 2016). Both techniques have been applied within the Te Arawa lakes district with inflow dosing of the Utuhina and Puarenga Streams of Lake Rotorua and the Waitangi Stream of Lake Rotoehu. Bulk alum dosing successfully reduced total phosphorus (TP) in Lake Okaro surface waters by ~32% between 2003 and 2004 (Özkundakci *et al.* 2010).

Lake Rotorua is a shallow (mean depth 8.2 m), moderately sized (79.8 km^2) polymictic lake (Tempero *et al.* 2015). The lake is one of twelve volcanic lakes located in the Te Arawa Lakes district in the Central Volcanic Plateau of the North Island, (Smith *et al.* 2016). Lake Rotorua is naturally mesotrophic to eutrophic due to geothermal inputs of dissolved nitrogen and phosphorus (Scholes & McIntosh 2010). Urbanisation (e.g., wastewater discharge), forestry, and farming activity beginning in the 1880's caused a gradual decline of lake water quality, shifting the Trophic Level Index (TLI) from approximately 4.2 prior to the 1960s to above 5.0 in the mid-1980s (Smith *et al.* 2016; McBride *et al.* 2018; Tempero 2018). In response, the Bay of Plenty Regional Council implemented continuous alum dosing into the Utuhina (2006) and Puarenga (2010) tributaries to reduce phosphorous loading to the lake (Tempero *et al.* 2015). Dosing rates fluctuate daily however, the combined average aluminium dose for both streams was c. 202 kg Al day^{-1} between July 2010 – June 2014 (Figure 1.4) with a total dose of 722.4 tonnes of aluminium added into Lake Rotorua by 2016 (Tempero *et al.* 2015; Tempero & Hamilton 2016).

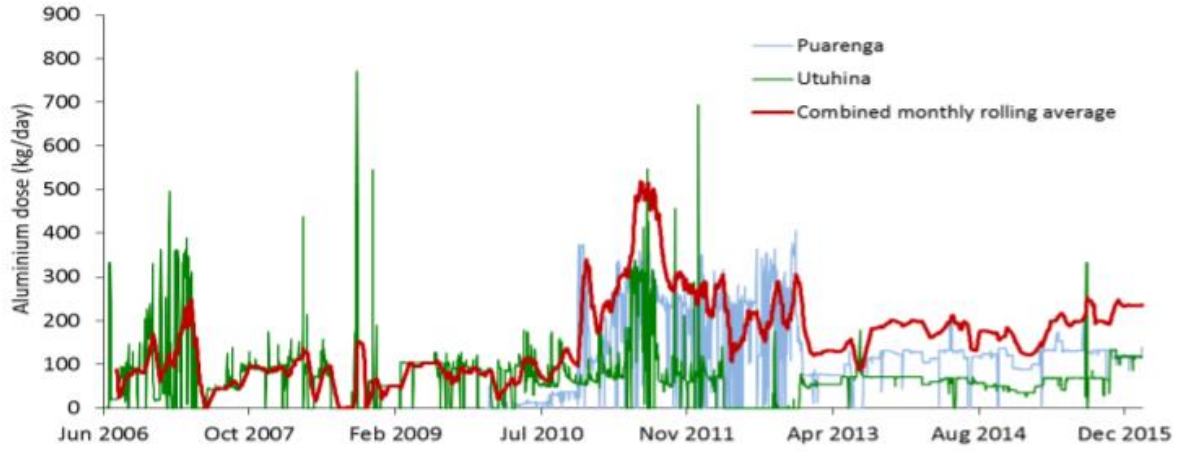


Figure 1.4: Daily aluminium dose rates for Lake Rotorua inflow streams - Puarenga and Utuhina streams until 1 March 2016. Reproduced from Tempero and Hamilton (2016).

The consented aluminium application limits are 1 mg Al L^{-1} in the Utuhina stream and 2 mg Al L^{-1} in the Puarenga stream (McIntosh 2012). Continuous dosing is used to remove phosphorus within the streams, minimising phosphorus loading into the lake, however, the accumulation of alum-derived aluminium in lake sediments may also provide the additional benefit of sequestering in-lake phosphorus (Tempero *et al.* 2015). Due to dilution effects, the estimated water column aluminium concentration within Lake Rotorua is approximately 0.02 mg Al L^{-1} (Tempero *et al.* 2015). Lake Rotorua aluminium concentrations are notably lower than international uses of alum which can range from >2 mg L^{-1} to 20 mg L^{-1}, due to differences in mode of application (Berkowitz *et al.* 2005). Typically, one-off bulk doses are applied to small, deep lakes with high natural buffering capacity or applications that contain buffering agents to counter the acidifying hydrolysis reaction of aluminium sulphate. Bulk dosing of Lake Rotorua is not feasible due to its large surface area and poor buffering capacity, increasing the risk of alum induced acidification (Tempero *et al.* 2015).

Aluminium toxicity

Aluminium is the most common metal in the Earth's crust and is therefore abundant in the natural environment (Sparling & Lowe 1996). In fish and crustaceans, aluminium toxicity is primarily due to dissolved ionic forms of aluminium (e.g., Al^{3+}) which occur at high and low pH (Gensemer & Playle 1999). Toxic effects primarily occur at the gills disrupting osmoregulation and respiration (Alexopoulos *et al.* 2003; Gensemer *et al.* 2018).

pH changes in the gill micro-environment

In fish and crustaceans, gills are the primary site of gas exchange, ion transport, and waste excretion (Playle & Wood 1989a). Metabolic waste products such as ammonia (NH_3) and CO_2 are excreted from the gills, which can make the gill micro-environment either acidic or basic depending upon the pH of the inspired water and the buffering capacity of the environment (Playle & Wood 1989a). 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 & Jensen, 1993; Wilkie, 1996). Playle and Wood (1989b) reported that pH changes at the surface of rainbow trout gills were sufficient to change the solubility of aluminium, resulting in 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 low pH

Under low pH conditions aluminium hydroxides can polymerise onto the negatively charged gill surfaces causing physiological disruption including loss of electrolytes (Na^+ and Cl^-), hypercapnia, plasma acidosis, increased blood haematocrit, and decreased plasma osmolarity (Poléo 1995). Aluminium disruption of ionoregulation occurs at low pH due to the displacement of Ca^{2+} by aluminium, allowing Na^+ and Cl^- to passively diffuse out through the gills and H^+ to diffuse in, thereby disrupting homeostasis (Hwang *et al.* 2011). Studies by Baker and Schofield (1981), Poléo *et al.* (1997), and Winter *et al.* (2005), provide evidence for toxic action of aluminium on a range of teleost fish species including perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) in acidic waters. However, acidic pH conditions are uncommon in Lake Rotorua apart from the geothermal Sulphur Bay where pH levels differ substantially from the rest of the lake (pH 7.0), reaching as low as pH 3.5 near the head of the bay (Ling 2016a). This area is uninhabitable for biota other than a highly tolerant *Chironomus* species, however, dilution effects increase the pH as the geothermal waters mix with the main lake, and pH is near 7 at the mouth of Sulphur Bay (Ling 2016a).

Toxicity at circumneutral and high pH

Toxicological studies of aluminium in high pH waters are limited; however, Poléo and Hytterød (2003) and Winter *et al.* (2005) do provide insights into high pH (>8.5) mortality rates. Osmoregulatory disruption is the predominant mode of toxic action at high pH. Poléo and Hytterød (2003) exposed Atlantic salmon (*Salmo salar*) to aluminium (350 µg Al L^{-1}) undergoing transitional states from low pH (pH 1.5 to 5.8) and from high pH conditions (pH 9.5 to 6.3). While no acute effects were found, physiological indicators of stress, including a 30% increase in blood haematocrit and 300% blood glucose increase were observed after 3 weeks exposure. However, the pH conditions represented highly artificial environmental states and are unlikely to be encountered in natural environments. Winter *et al.* (2005) found moderate aluminium accumulation on trout gills at pH 8.8. The accumulation was attributed to the differences in water chemistry (pH ~6) in the gill micro-environment allowing aluminium

in the inspired water to polymerise and precipitate in the gill environment and onto the gill surfaces (Winter *et al.* 2005). Findings also revealed that aluminium accumulation did not occur at pH 10 and toxic effects, including mortality, were caused by high pH. These authors conclude that at high pH, aluminium will not cause significant impacts on fish health so long as exposures are short (1–4 days), and that high pH toxicity will eventually outweigh any adverse effects of aluminium.

Aluminium toxicity in the Te Arawa Lakes

Ling (2016a; 2016b) carried out toxicological assessments of alum dosing at Lake Rotorua in the Puarenga Stream and at Lake Rotoehu in the Waitangi Soda Springs Stream. Baseline monitoring commenced in the Puarenga Stream in 2009 prior to alum dosing, to provide a baseline on aluminium accumulation in organism tissues for comparison in subsequent sampling studies. Ling (2016a) measured aluminium bioavailability within the digestive glands of freshwater mussels (*Echyridella menziesii*), whole common bully (*Gobiomorphus cotidianus*), plant roots (*Eleocharis acuta*) and chironomids (*Chironomus zealandicus*) found within the vicinity of the Puarenga Stream outlet (a total of 4 sites) in Sulphur Bay. Aluminium concentrations were highest in chironomid larvae across all sites, while low to moderate accumulation was found in the macrophyte *Eleocharis acuta,* and the lowest accumulation occurred in mussels and common bully. Results of this study were similar to pre-dosing aluminium concentrations, although Sulphur Bay is naturally high (~1 mg L^{-1}) in geothermally sourced aluminium (Ling 2016a). A similar study was conducted comparing aluminium accumulation in the gills, flesh and hepatopancreas/liver in kōura (*Paranephrops planifrons*) and goldfish (*Carassius auratus*) sourced from Lake Rotoehu to species sampled from Lake Rotorua and Rotoiti (due to the lack of pre-dosing data in Lake Rotoehu) (Ling (2015; 2016b). Aluminium levels were higher in the gills of Lake Rotoehu goldfish sampled in 2015 (~350 mg kg^{-1} dry weight) compared to samples taken from Rotoiti in 2013 (250 mg kg^{-1} dry weight) while aluminium concentrations were slightly lower (by ~ 100 mg kg^{-1} dry weight) in kōura gill samples. Additionally, gill aluminium for both kōura and goldfish from Lake Rotoehu were greater by ~60–270 mg kg^{-1} dry weight than samples collected from Lake Rotorua, indicating an increased proportion of water-derived aluminium and bioavailability within Lake Rotoehu (Ling 2016b).

Tempero *et al.* (2015) reported that mean total aluminium concentration within Lake Rotorua was 0.02 mg L^{-1}, which was lower than the United States Environmental Protection Agency

(USEPA) acute exposure guidelines of 0.75 mg Al L^{-1} at pH 6.5–9.0 for no more than 1 hour and the total aluminium 5% hazardous concentration (HC5) estimates of 0.45 mg L^{-1} at pH 6.5 to 0.644 mg L^{-1} at pH 8. These studies suggest that current alum dosing regimens within the Te Arawa Lakes do not cause significant adverse effects on fish and kōura health. However, osmoregulatory and respiratory tolerances of New Zealand native species under an increased aluminium dose of 1−2 mg Al L^{-1} are unknown and further consideration of aluminium toxicity during photosynthetically driven diel pH shifts is required. Further research is required as it is possible for the concentration of aluminium dosed into the tributary streams (~1 mg Al L^{-1}) to be found in Rotorua Lake, near stream inflows. However, it is unlikely that average aluminium levels in the main body of the lake would ever reach these concentrations due to dilution.

Determination of toxicological impacts

The study of toxicology is broad and aims to determine toxic effects of chemicals on organisms. More specifically, aquatic toxicology focuses on effects of chemical agents on living organisms in their natural aquatic environment (freshwater, estuarine, or marine) by determining organism responses (either lethal or sublethal), over a range of time scales (acute and chronic), and doses under various environmental conditions (Dodds 2002). Acute studies are used to identify rapid effects of a toxicant (usually <96 h), while chronic exposures occur over weeks to months depending on the life stage and lifespan of the test organism (Wang *et al.* 2016). Historically, toxicology has largely focused on species sensitivities by determining the lethal dose and effective concentration of potentially toxic chemicals such as heavy metals, pesticides, and industrial effluents (Dodds 2002). More recently, toxicological studies have shifted to focus on chronic effects such as impaired growth and reproduction and sublethal acute physiological effects, including emerging toxicants such as pharmaceuticals and nanoparticles which are unlikely to enter systems at high concentrations (Chowdhary & Raj 2020).

Experimental approaches

A range of *in situ* and laboratory-based approaches ranging in scale and duration have been employed to ascertain acute and chronic physiological endpoints for a diversity of aquatic organisms. Large-scale experiments typically utilise generalised field surveys or more rigorous before-after control-impact (BACI) experimental approaches. These approaches reveal the impact of the toxicant within the natural environment and are often used to estimate cumulative

effects of multiple stressors within the ecosystem such as excess nutrients, fertilizers, and pesticides on biota health (Barmentlo *et al.* 2018). Large-scale experiments often assess contaminant consequences at the population, community, and whole ecosystem level, however, they are difficult to replicate and often lack suitable controls (Gessner & Tlili 2016).

In comparison, laboratory-based experimental designs, such as mesocosms and tank studies, explore the direct effects of toxicants under controlled environments (Barmentlo *et al.* 2018). Mesocosm experimental designs are larger laboratory-based or field enclosure experiments that simulate actual environmental conditions in order to investigate complex interactions between biotic and abiotic factors (Sharma *et al.* 2021). Laboratory tank studies are small-scale and primarily conducted on a single species of a limited size/age range. They can be used to determine a range of sublethal endpoints such as alterations in physiology and behaviour (i.e., altered haematological parameters or changes in swimming activity or feeding) (Graney *et al.* 2020). These types of experiments are easier to replicate and environmental conditions such as temperature and light can be tightly controlled. However, they generally only examine acute effects on a subset of the population and do not account for chronic effects from cumulative stressors.

Toxicological endpoints

The focus of aquatic toxicology has shifted focus from lethal to sublethal effects due to a greater appreciation of toxicity occurring at low and continuous doses within complex receiving environments (Heath 1996). Lethal exposure studies focus on the dose/concentration at which death for a percentage of the population occurs, usually over short timescales (i.e., 96 h LD_{50}). Measures such as LD_{50} curves represent the lethal dose resulting death in 50% of the population within a given time period. Alternatively, LT_{50} values give the time taken for 50% of individuals to die during exposure to a given toxicant concentration. For example, Poléo *et al.* (1997), found that Atlantic salmon were particularly sensitive to aluminium-rich (~402 µg Al L^{-1}) acidic (~ 5.0 pH) waters reflected by their low LT_{50} of 85 h. While this information is useful for providing a maximum concentration limit for toxicants, these studies do not consider sublethal impacts that may result at lower concentrations or under prolonged exposure.

Chronic, sublethal endpoints include effects on reproduction, growth and development, and behaviour. Acute endpoints may examine physiological and behavioural responses through

haematological, biochemical, histological, and observation of behavioural changes over short time-periods (Sloman & McNeil 2012). For example, Saravanan *et al.* (2011) reported haematological parameters such as haemoglobin concentration (Hb), haematocrit (Hct), erythrocyte counts (RBC), and biochemical parameters such as plasma glucose to determine the toxicity of synthetic organochlorine pesticide, lindane, to common carp fingerlings in acute (24 – 96 h) and sub-chronic (25 d) exposures. An observed increase in Hb and Hct was indicative of respiratory impairment and the increase in plasma glucose levels was attributed to the stress induced metabolic demands imposed by the chemical (Saravanan *et al.* 2011). Assessment of complex behavioural endpoints such as predator avoidance, food capture, and spontaneous activity provide insight into potential effects on survival (Sloman & McNeil 2012). Niyogi *et al.* (2006) observed feeding behaviours of juvenile rainbow trout chronically exposed to waterborne copper (Cu) at a concentration of 55 µg L^{-1} revealing appetite and growth impairment, which could reduce overall physiological function and survival over time.

Sublethal physiological effects of aluminium

The gills are responsible for osmoregulation, respiration, and acid-base balance (Gensemer & Playle 1999). Chloride and pavement cells within the gill epithelium are responsible for transporting ions to and from the environment via ion channels, ion pumps, and ion exchanges. Polymerisation and precipitation of environmental aluminium to the gill surface can disrupt the transfer of ions in and out of the body as well as impairing respiratory exchange of gases (Gensemer & Playle 1999). Ionic imbalances from disruption of osmoregulation can have direct effects on the functioning of the digestive and renal tissues, muscles, impairment of energy balances and hormone regulation (Baldisserotto *et al.* 2018). Gill damage by aluminium precipitation can cause excessive mucus secretion, impairing gas exchange by increasing the diffusional distance for oxygen (Gensemer *et al.* 2018). A reduction in oxygen delivery to the cells initiates an increase in gill ventilation, however, a sustained lack of oxygen will limit metabolism. Additionally, acid-base balances may be impaired due to the coupled exchange of ions to the exchange of protons and bicarbonate, resulting in the acidosis (accumulation of H^+) or alkalisation (accumulation of HCO_3^-) of intra- and extracellular fluids, leading to cellular damage (Gilmour 2012).

Osmolarity regulation

Freshwater provides a significant challenge to osmoregulators such as fish and kōura as they are hypertonic to the environment, resulting in continuous diffusion of water into the body and essential loss of essential ions such as Ca^{2+}, K^+, Na^+ and Cl^- to the environment as they diffuse down the ionic gradient towards the lower solute concentration (Griffith 2017). Osmoregulation maintains the concentration of salt ions within intercellular and extracellular fluids (Moyes & Schulte 2013). In order to maintain homeostasis, ions are transported from the environment across the apical cell plasma membrane of specialised mitochondria-rich chloride cells (ionocytes) and associated pavement cells located within the gill filament epithelium (Figure 1.5) (Moyes & Schulte 2013). Ion exchange is achieved through a number of mechanisms including passive ion channels which allow the diffusion of selected ions such Cl^-, co-transporter and exchangers which are carrier proteins that transport two different species from one side of the membrane to the other at the same time (i.e., Cl^- and HCO_3^- or Na^+ and H^+). This type of transport is known as secondary active transport and is powered by the energy derived from the concentration gradient of the ions across the membrane the cotransporter. In contrast, active transport ATPase pumps such as Na^+, K^+-ATPase, which actively move ions against concentration gradients using ATP (Moyes & Schulte 2013; Griffith 2017). Carbonic anhydrase provides H^+ and HCO_3^- from CO_2 for branchial ion transport and is primarily involved in acid-base regulation (Gilmour 2012). These processes are hormonally controlled and may be upregulated or downregulated depending on metabolic demands. Furthermore, under prolonged exposure to osmotic stressors, proliferation of chloride cells in the gill epithelium may occur to help compensate for the osmotic challenge.

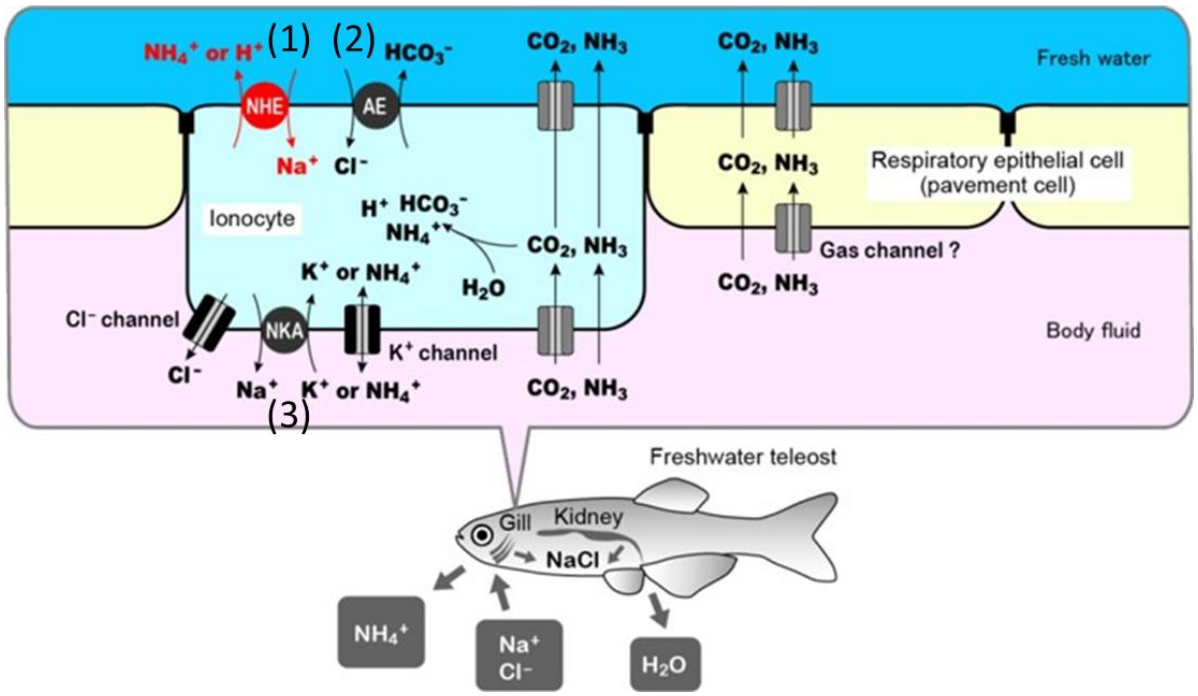


Figure 1.5: Schematic diagram of sodium and chloride uptake in the gills of a freshwater teleost. In the chloride cells, (1) a H+-ATPase pump in the apical membrane generates an electrochemical gradient for the passive diffusion (uptake) of Na^+ through an epithelial Na^+ channel and acidifies the gill boundary layer of the gill. (2) In the apical membrane a Cl^-–HCO_3^- cotransporter, exchanges Cl^- into the cell and Cl^- exits into the body fluid via a passive ion channel. (3) Na^+ uptake is completed at the basolateral membrane via Na^+, K^+-ATPase. K^+ pumped into the cell by Na^+,K^+-ATPase recycles via channels in the basolateral membrane. Between cells are well-developed tight junctions to minimize diffusive ion loss by the paracellular pathway. Repurposed from Ito *et al.* (2014).

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 & Playle 1999). Brown trout (*Salmo trutta*), exposed to aluminium (12.5 ug Al L^{-1}) at pH 5.0 experienced a decline in Na^+ (~25%) and Cl^- (~15%) plasma concentrations after 6 hours of exposure, although some recovery was evident after 120 h (Waring & 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).

Haemolymph parameters reflect crustacean immune responses. Haemocytes (hyaline, semi-granular, granular cells) carry out a range of immune functions (phagocytosis, cellular communication, and cytotoxicity) and disperse through the open-circulatory system allowing for a non-specific, effective immune response (Ward *et al.* 2006). Total haemocyte counts are commonly used to determine changes in crayfish haemolymph. Studies show conflicting responses of crustacean haemocyte abundance under different stressors, including toxicant exposure and fluctuations in dissolved oxygen (Taylor *et al.* 2009). Lorenzon *et al.* (2001) found that depression in total haemocyte counts (THC) in shrimp (*Palaemon elegans*) resulted after heavy metal exposure. Poor recovery, handling and confinement stress caused prolonged haemocyte depression in captured New Zealand kōura (*Paranephrops planifrons)* (Taylor *et al.* 2009). Ward *et al.* (2006) and Alexopoulos *et al.* (2003) reported haemocyte proliferation in crayfish (*Pacifasticus leniusculus*) exposed to aluminium which was attributed to gill damage and mucus secretion causing hypoxic stress.

Fish respiration and metabolism

Metabolism is defined as the totality of energy consumption, manipulation, and storage by organisms and can be measured as oxygen consumption rate as, under normoxic resting conditions, all metabolism is fuelled aerobically (Chabot *et al.* 2016; Nelson 2016; Rosewarne *et al.* 2016). Catabolism, involving the breakdown of metabolites and anabolism, responsible for maintenance, growth, and reproduction, require energy. Energy in the form of ATP is produced via cellular respiration which is most effective in the presence of oxygen (Reece *et al.* 2015).

In fish, feedback from the body's metabolism controls gill ventilation rates, which moves water over the gill surfaces allowing uptake O_2 and elimination of CO_2 (Reece *et al.* 2015). Some fish species ventilate via buccal cavity pressure while others carry out ram- ventilation or are able to undertake both (Reece *et al.* 2015). Crayfish use paddle-like appendages creating a current over their gills (Reece *et al.* 2015). Exchange of O_2 and CO_2 occurs by diffusion across the gill pavement cells (PVCs) within the gill lamellar epithelium. Rates of gas exchange are dependent

on the partial pressures of each gas, the area and thickness of the diffusional surface, and the metabolic requirements of the individual (Reece *et al.* 2015).

Metabolic requirements are influenced by body mass and temperature (Fernandes 2007). Smaller animals have a higher metabolic rate per gram of body weight than larger animals, therefore, require a greater oxygen delivery rate to the cells (Reece *et al.* 2015). Variations in environmental conditions such as temperature cause fluctuations in metabolic demand (Reece *et al.* 2015). For ectothermic poikilotherms, such as fish and crayfish, increased environmental temperatures accelerate the rate of metabolic processes which require increased delivery of oxygen to the cells to maintain standard metabolic rates (SMR) (Chabot *et al.* 2016). Additionally, temperature increases reduce the amount of oxygen dissolved within water providing further challenge for metabolic maintenance (Harper 1992).

Intermittent flow respirometry

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 & 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 is comprised of the chamber, a recirculation loop, a flush pump, and a recirculating pump, and is typically submerged in a tank of 100% O_2 saturated water. There are two distinct cycles – the recirculation period where the chamber is closed off from the external environment allowing the organism to consume oxygen, decreasing the % O_2 saturation over time, and the flush period where the chamber is flushed with fully saturated water, increasing the dissolved oxygen back to normoxic conditions (Figure 1.6) (Claireaux & 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.

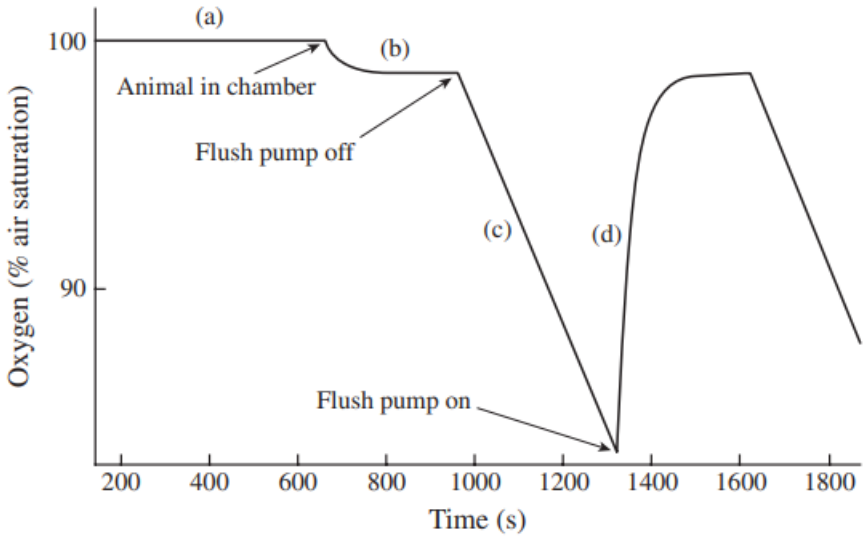


Figure 1.6: A graphical depiction of a typical intermittent flow respirometry chart, modelling the rate of fish oxygen consumption from 100% air saturation over time (s). Oxygen levels should be similar to air saturation before animal is placed into the chamber (a) and will reach a new equilibrium after the animal is introduced (b). The period where the flush pump is turned off will see a linear decline in oxygen (c) and the slope of this line is used to calculate mass specific oxygen consumption (mg O_2 g^{-1} h^{-1}). Before the onset of hypoxia (<80%) the flush pump is switched on and clean water increases oxygen levels back up to equilibrium in a linear fashion (d) (Claireaux & Chabot, 2016).

Benefits to using the intermittent respirometry approach include omission of prolonged hypoxia to the animal, progressive hypercapnia, and waste accumulation associated with closed and flow-through respirometry (Svendsen *et al.* 2016). However, there are limitations with intermittent respirometry such as leaks, gas bubbles, and noise in oxygen meter readings which need to be considered during the experimental set up (Svendsen *et al.* 2016). Mixing of water within the chamber via the recirculation loop and the measurement of background (microbial) respiration are essential for the accuracy of oxygen consumption data produced (Rodgers *et al.* 2016). Additionally, oxygen consumption may not directly relate to metabolic rate due to the potential for the organisms to switch between aerobic and anaerobic metabolism (Nelson 2016). Aerobic metabolism is the most efficient way to produce ATP, however, under reduced oxygen conditions, most species are able to compensate for reduced oxygen levels and ATP production by initiating anaerobic metabolism in a limited capacity (Nelson 2016). Maintaining dissolved oxygen concentrations within the respirometers above 80% ambient saturation will minimise the chance of oxygen consumption being influenced by hypoxia-induced metabolic changes (Clark *et al.* 2013).

Respiration impairment by aluminium

At circumneutral pH, aluminium can precipitate onto the gills causing irritation and excessive mucus secretion which increases the gas diffusion distance across the gill surface (Gensemer *et al.* 2018). Reduced oxygen uptake leads to reduction of oxygen within the blood and delivery to cells. This effect was demonstrated by Waring and Brown (1995) where brown trout (*Salmo trutta*) were exposed to various pH levels and aluminium concentrations (pH 7.0, 5.0, and pH 5.0 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 h exposure (Waring & Brown 1995). Reduced oxygen delivery due to inhibited oxygen uptake slows cellular respiration and the rate of energy production. Depressed ATP production 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 necrotic 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 in attempts to maintain oxygen delivery to the cells in order to meet biochemical oxygen demands (Gensemer & Playle 1999).

Summary

Algal blooms pose a toxic risk to biota by altering environmental pH, reducing light availability, production of toxins, disruption of food webs and loss of amenity values. Alum dosing is a widely used mitigation measure for controlling harmful algal blooms in freshwater systems and has been employed in the Te Arawa Lakes. However, there is limited knowledge of the potential toxic effects of continuous alum dosing on fish and kōura during diel alkaline pH changes from algal blooms. Alum to control eutrophication can only be warranted if dosing does not cause adverse effects on the environment and the ecological community. Internationally, alum toxicology studies have documented aluminium toxicity at a range of environmental pH, either through precipitating onto the gills causing respiratory impairment or binding to the surfaces of the gill inhibiting osmoregulation.

Previous monitoring by Landman and Ling (2011) and Ling (2016a; 2016b) have reported that alum dosing of inflows to Lake Rotorua and direct dosing of Lake Okaro did not result in

significant negative impacts on aquatic biota, which was attributed to the low dosing rates. The total aluminium continuous inflow dose rate of 1–2 mg Al L^{-1} within Lake Rotorua is conservative compared to international uses. Specific toxicity testing of alum to New Zealand native freshwater fish and crayfish has yet to be conducted, and investigation of aluminium toxicity in relation to photosynthetically driven diel changes in pH has not been previously investigated. Examining the physiological responses of aquatic organisms may reveal potential adverse impacts on fish and crustaceans, assist in setting dosing limits, and guide future lake management plans.

Study species

Rainbow trout (*Oncorhynchus mykiss*) are an introduced pelagic species inhabiting riverine and lacustrine environments throughout New Zealand (Rowe & Chisnall 1995). They primarily prey upon macroinvertebrates and smaller native fish and are a significant recreational sport fish maintained by stocking by Fish and Game New Zealand (Blair *et al.* 2013). Rainbow trout have an upper thermal limit of ~21˚C and oxygen threshold of ~2.5 g m^{-3}, which has become an increasing concern due to the effects of climate change creating a habitat squeeze during summer months endangering the success of recreational stocking efforts (Rowe & Chisnall 1995). Rainbow trout are also one of the most commonly used species for toxicological studies involving freshwater fish as they are relatively easy to keep and breed in captivity.

Common bully (*Gobiomorphus cotidianus*) are an endemic amphidromous species that inhabit rivers, streams, and lakes throughout New Zealand (Figure 1.7) (Rowe 1999). They are benthic dwelling, pH- and hypoxia-tolerant and are commonly found within the littoral zone (Rowe 1999; Landman & Ling 2011). They are often prevalent in lakes with greater nutrient status and higher turbidity which aid in predator avoidance (Rowe 1999). Breeding, occurs during the summer months, producing planktonic larvae which feed on zooplankton until reaching ~18 mm in length (Rowe 1999). Adults prey on chironomid larvae and aquatic invertebrates and are an important part of the lake food webs, supporting piscivorous species such as trout and eels (Rowe & Chisnall 1995; Rowe 1999).

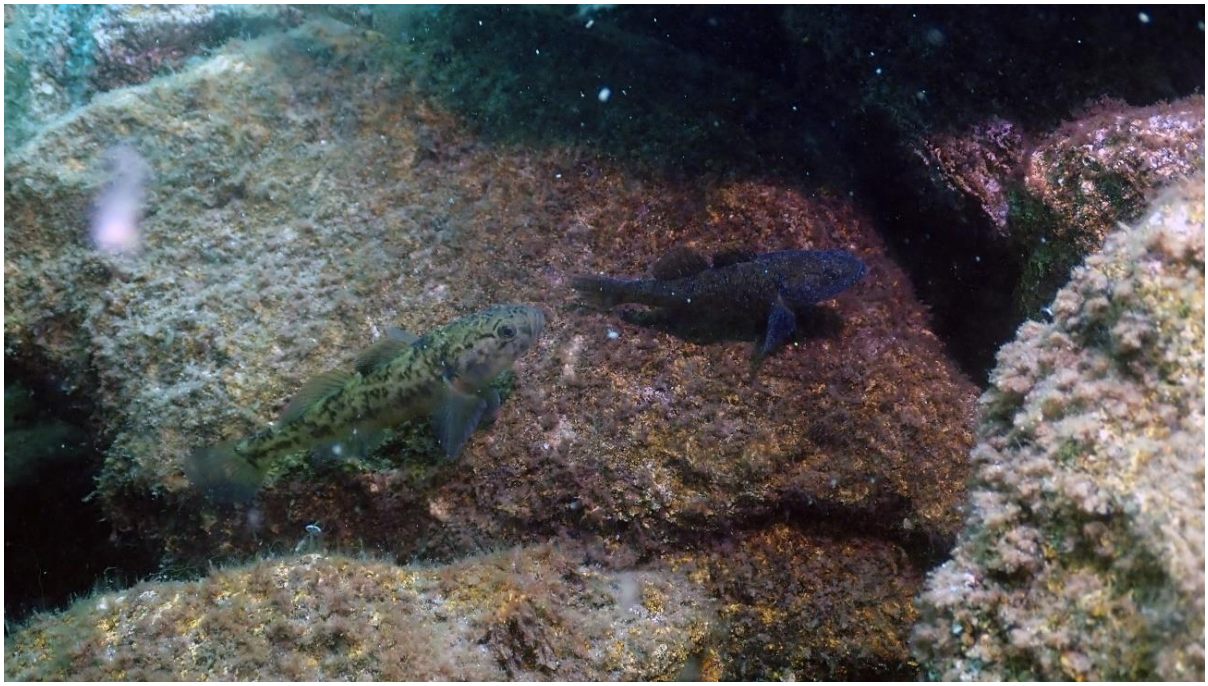


Figure 1.7: Two common bully (*Gobiomorphus cotidianus*) in Lake Rotoma. The specimen on the right showing the darker colouring of breeding males. Photo credit: Warrick Powrie.

The endemic freshwater crayfish species *Paranephrops planifrons* is predominantly found in freshwater streams and lakes across the North Island of New Zealand as well as in northern and western parts of the South Island (Figure 1.8) (Kusabs *et al.* 2018). Traditionally known as kōura, this species and its South Island relative *Paranephrops zealandicus* are considered 'taonga'. They are an important food source in Māori culture and are also commercially farmed (Kusabs *et al.* 2015a; Kusabs *et al.* 2018). As a keystone species, they play an important ecological role in streams and lakes as scavengers and shredders and provide food for native and recreational fish species (Kusabs *et al.* 2015b; Kusabs *et al.* 2018). In lakes, kōura tend to move to deeper parts of the lake during daylight hours and migrate into the more productive littoral zone to feed at night (Kusabs *et al.* 2018). The introduction of invasive fish species and the effects of cultural eutrophication have impacted kōura populations and declines within the Te Arawa lakes district have been observed (Kusabs & Quinn 2009).

Figure 1.8: Adult kōura (*Paranephrops planifrons*) in Lake Rotoma, Bay of Plenty. Photo credit: Warrick Powrie.

Thesis objectives

The potential toxicological effects of alum dosing poorly buffered lakes during algal bloom driven pH cycling have not been investigated. This research will 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 will be examined by exposure of rainbow trout and kōura to 2 mg Al L^{-1} with diel pH cycling for 10 days. Physiological sampling with include 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 will be examined using intermittent flow respirometry. Oxygen uptake rates in individual test subjects will be measured over 48 hours and compared to determine whether changes in mass specific metabolism occur in response to pH cycling at 2 mg Al L^{-1}. Information from these experiments will then be synthesised to provide recommendations as to the toxicological risk of alum dosing during algal blooms. All experiments within this study (Protocol #1110 and Protocol #1128) were approved by the University of Waikato Animal Ethics Committee prior to testing and sampling

Chapter 2

Effects of aluminium on rainbow trout and kōura osmolarity regulation under diel pH cycling

Introduction

Toxicological studies of aluminium on aquatic organisms have primarily focused on impacts under acidic conditions (Baker & Schofield 1981; Waring & Brown 1995; Poléo *et al.* 1997). However, there has been a recent shift to investigations of aluminium toxicity at high pH as naturally alkaline waters or highly eutrophic systems may produce toxic soluble aluminium species (i.e., $Al(OH)_4^-$) causing osmoregulatory disruption (Winter *et al.* 2005; Cardwell *et al.* 2018; Gensemer *et al.* 2018).

Precipitation of $Al(OH)_3$ onto gill surfaces occurs due to differences in the pH between the environment and the microclimate near the gill surface. This can result in irritation, cellular erosional damage, excess mucus secretion, and chloride cell proliferation leading to haematological and histological changes from respiratory and osmoregulatory impairment (Alexopoulos *et al.* 2003; Parkyn *et al.* 2011). Aluminium precipitation onto gill surfaces at circumneutral pH can be identified by lamellar fusion and clubbing, lifting of the epithelia, lesions, vascular congestion, and the proliferation of ionocytes (Wood 2001). In contrast, polymerisation between the gill surface and dissolved aluminium species occurs at high and low pH (pH < 5.5 and >8.5), resulting in decreasing plasma osmolarity and erythrocyte swelling, which is reflected in increased haematocrit (Burgos-Aceves *et al.* 2019; Garcia Parra & Baldisserotto 2019).

In order to sequester phosphorus and reduce the formation of harmful cyanobacterial blooms within Lake Rotorua alum dosing (~1-2 mg Al L^{-1}) of the Utuhina Stream (since 2006) and the Puarenga Stream (since 2010) has been conducted by the Bay of Plenty Regional Council (Tempero *et al.* 2015; Ling 2016a; 2016b). However, due to the low buffering capacity of Lake Rotorua (alkalinity ~8.3 mg L^{-1} $CaCO_3$ and hardness ~14.8 mg L^{-1} $CaCO_3$), algal bloom induced pH changes may increase the prevalence of toxic aluminium species (i.e., $Al(OH)_4^-$). The photosynthetic uptake of carbon dioxide (CO_2) during daylight hours reduces the amount of free H^+ ions in solution, increasing the pH, while respiration overnight releases CO_2,

decreasing environmental pH (Heini *et al.* 2014; Acuña-Alonso *et al.* 2020). Shifts from circumneutral pH, where insoluble $Al(OH)_3$ is most abundant, to alkaline conditions increases the proportions of toxic soluble aluminate ions ($Al(OH)_4^-$) 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).

Research objectives

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) over a 10-day period. This was design was intended to emulate diel pH changes observed in Lake Rotorua during a phytoplankton bloom. Plasma (rainbow trout) and haemolymph (kōura) osmolarity, as well as haematocrit and haemoglobin concentration in rainbow trout will be measured to determine if osmotic disruption occurred in response to aluminium and cyclic pH exposure. Additionally, gill histological analysis was conducted for abnormalities caused by aluminium and pH.

Methods

Collection and Maintenance of Study Animals

All procedures followed those set out in the University of Waikato Animal Ethics Committee Standard Operating Procedure (SOP number: 7) for capture, handling, and captive maintenance of fish. 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. Rainbow trout were held in a 5000 L fiberglass tank supplied with continuous dechlorinated municipal water at a rate of 1.5 L $minute^{-1}$ and constant aeration was provided to maintain oxygenation within the tank. Kōura were housed in two 100 L glass tanks (containing no more than seven individuals) and two 60 L glass tanks (containing no more than four individuals), each with a recirculating filtration

system (Figure 2.1). To mitigate aggression within the 100 L holding tanks, crayfish were separated by a Perspex plastic divider, and provided with a half terracotta pot for refuge.

Figure 2.1: Kōura housed in 100 L glass tank with dividers and half pots to limit aggression.

Rainbow trout were fed a commercial pellet feed (grain size 0.8 mm) every 2 days and the kōura were fed frozen bloodworms (chironomid larvae) every 3 days. Feeding was monitored and excess food was removed after 30 minutes. All animals were monitored at least once daily to manage signs of stress and general health.

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. A pH 12 sodium hydroxide (Merck, Germany) stock solution was prepared from 25 g of solid NaOH dissolved into 50 L of dechlorinated water, pH was adjusted to 12 if necessary.

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 each tank with the stock 2 mg L^{-1} aluminium solution 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 treatments tanks with the pH 12 2 mg L^{-1} aluminium solution at a rate of 6 mL h^{-1}. All tanks were initially filled with the stock aluminium solution and the test subjects introduced and 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) (Figure 2.2). Over the 10-day testing period, tank flow-through was switched between pump 1 and pump 2 every 12 hours. The two control tanks remained at pH 7, while the two treatment tanks were subjected to increases in pH from pH 7 to pH 10 over a 12-hour period. The pH subsequently declined back to pH 7 within 12 hours of pump 1 starting.

Figure 2.2: Experimental setup showing the two control tanks, two treatment tanks (left), the peristaltic pumps (centre), and the stock NaOH and 2 mg Al L^{-1} stock bottles (right).

A YSI Prosolo 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 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 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 (Figure 2.3). 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.

Figure 2.3: Kōura haemolymph sampling from the pericardial sinus between the carapace and the first abdominal segment.

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 calculated using the following equation.

$$\text{Haemoglobin concentration (g L}^{-1}) = \frac{(Hb\ ABS \times 64458 \times df)}{44000}$$ (Dacie & 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 Hb 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 haemoglobin concentration inside each red blood cell.

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 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 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 the addition of 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 (Sartorius, Germany), 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

Students' *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.

Results

Total aluminium was maintained around 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. At high pH (pH >8.5) it was assumed soluble forms of aluminium would become more prevalent as described by Gensemer & Playle (1999).

Table 2.1: Total aluminium and particulate aluminium (mean ± SEM) in treatment and control tanks for rainbow trout and kōura.

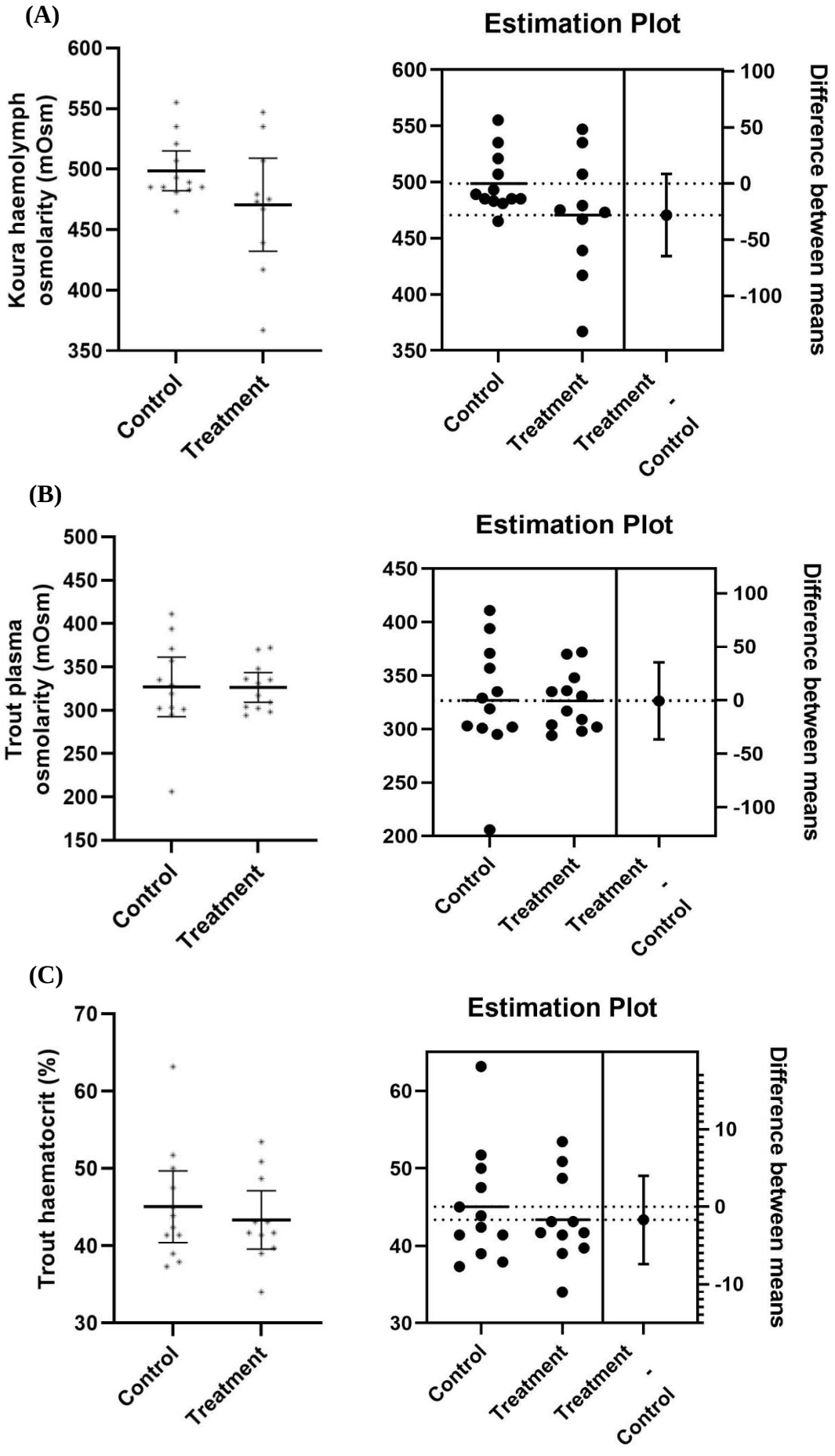
Species	Tank	Total Aluminium (mg L^{-1})	Soluble aluminium (mg L^{-1})
Rainbow trout	Control	2.28 ± 0.12	0.03 ± 0.010
	Treatment	2.08 ± 0.37	0.09 ± 0.045
Kōura	Control	2.94 ± 0.15	0.02 ± 0.003
	Treatment	1.93 ± 0.62	0.10 ± 0.034

No statistically significant differences in the osmolarity, haematocrit or haemoglobin observed between treatment and control groups of either species were at the end of the 10-day experiment (Student's *t*-test, $P > 0.05$, Table 2.2). 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 (mOsm), rainbow trout osmolarity (mOsm), haematocrit (Hct), whole blood (Hb), and mean cell haemoglobin concentration (MCHC) show the differences between the means at the 95% confidence interval (Figure 2.4).

Table 2.2: Mean ± SEM are presented for rainbow trout and kōura haemolymph osmolarity (mOsm) and rainbow trout haemoglobin (Hb) and haematocrit (Hct), mean cell haemoglobin concentration (MCHC) for control and treatment groups. The *P*- values and *F*- test values represent the significant of differences and variances between control and treatment means.

Species	**Tank**	**n**	**Mean ± SEM**	***P*- value**	***F*- test**
Rainbow trout (mOsm)	Control	12	326.9 ± 15.58		
	Treatment	12	326.3 ± 7.77	0.97	0.0297*
Kōura (mOsm)	Control	12	498.7 ± 7.50		
	Treatment	10	470.6 ± 17.00	0.12	0.0269*
Rainbow trout (Hct)	Control	12	45.0 ± 2.10		
	Treatment	12	43.3 ± 1.69	0.54	0.4208
Rainbow trout (Hb)	Control	12	67.8 ± 2.98 (g/L)		
	Treatment	11	72.8 ± 5.11 (g/L)	0.41	0.0876
Rainbow trout MCHC	Control	6	151.2 ± 8.87 (g/L) 2.3 ± 0.13 (mM)		
	Treatment	6	211.7 ± 11.69 (g/L) 3.3 ± 0.24 (mM)	<0.01*	0.231

Asterisks (*) indicate significant difference ($P < 0.05$) in the control and treatment data.


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

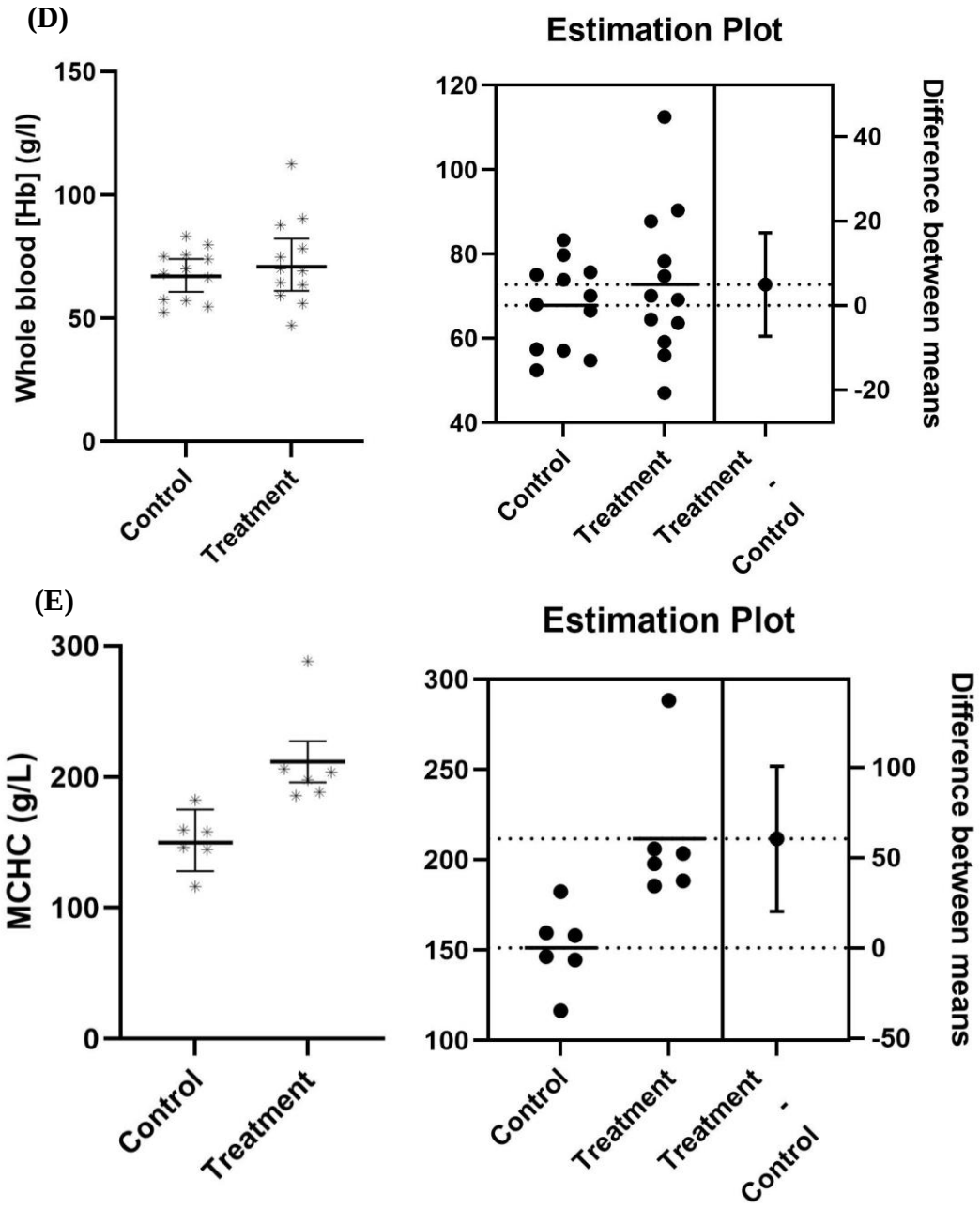


Figure 2.4: Scatter plots of (A) kōura haemolymph osmolarity (mOsm), (B) rainbow trout plasma osmolarity (mOsm), (C) rainbow trout haematocrit (%), (D) rainbow trout whole blood [Hb] (g L^{-1}), and rainbow trout (E) rainbow trout mean cell haemoglobin concentration (MCHC) (g L^{-1}) for individuals in control and treatment groups and associated estimation plots. Data is presented in means with 95% confidence intervals.

Abnormalities within both the control and treatment groups for rainbow trout and kōura were observed (Figure 2.5). 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.

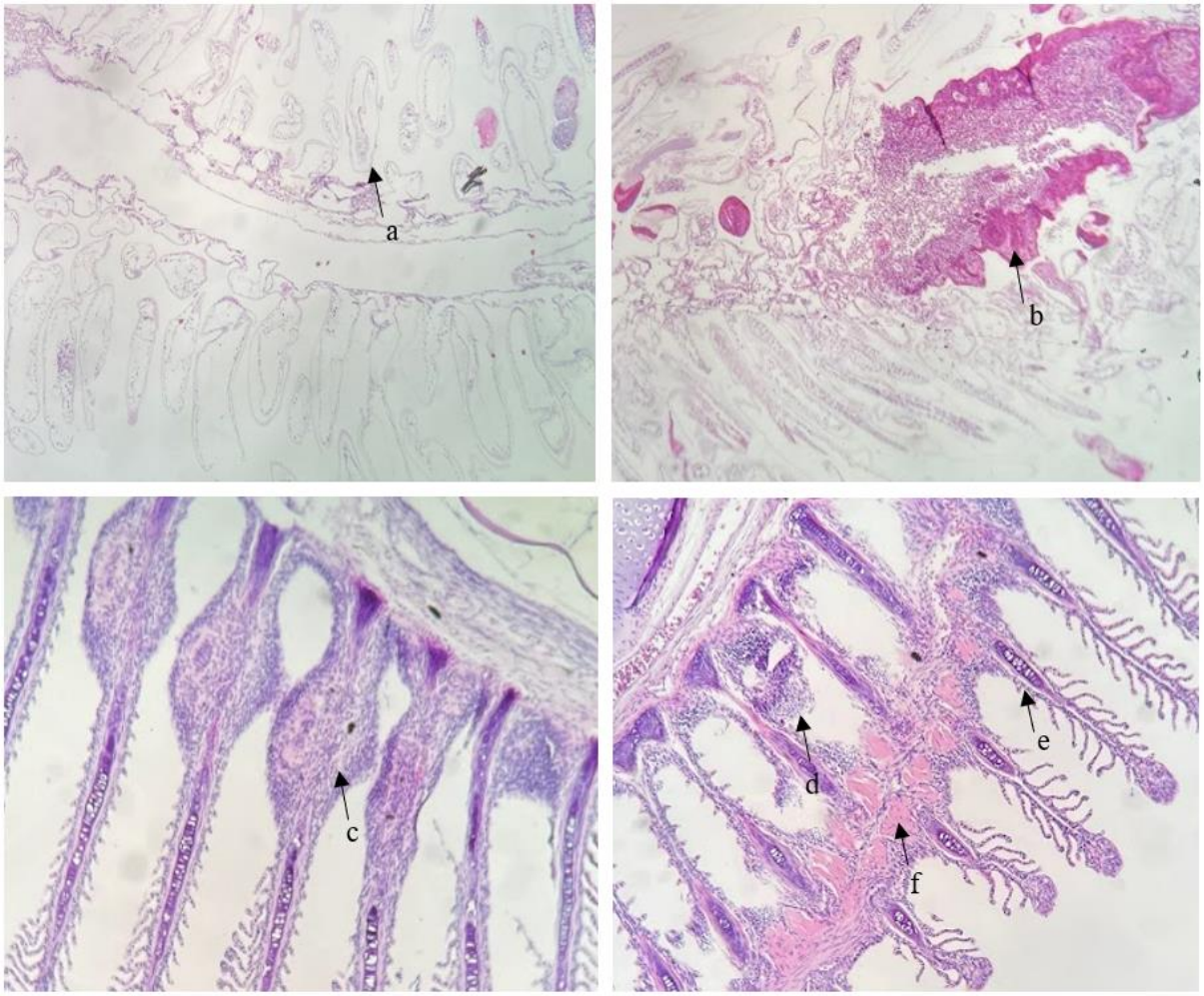


Figure 2.5: Histological gill sections of treatment kōura (top) and rainbow trout (bottom) individuals showing epithelial lifting from the cuticle, lamellae swelling, lamellae disorganisation (a), and a cellular anomaly (b) in kōura gills and hyperplasia (c), RBC proliferation (d), epithelial lifting (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 2.6) and observed abnormalities were more prolific in the treatment gill sections. The most notable effect occurring in the kōura treatment gills was the cellular anomaly, which could potentially be an increase in haemocytes which may be evidence of an immune response. While there was evidence of damage found in rainbow trout controls and treatments, there was no statistical difference (P = 0.8475, Figure 2.6).

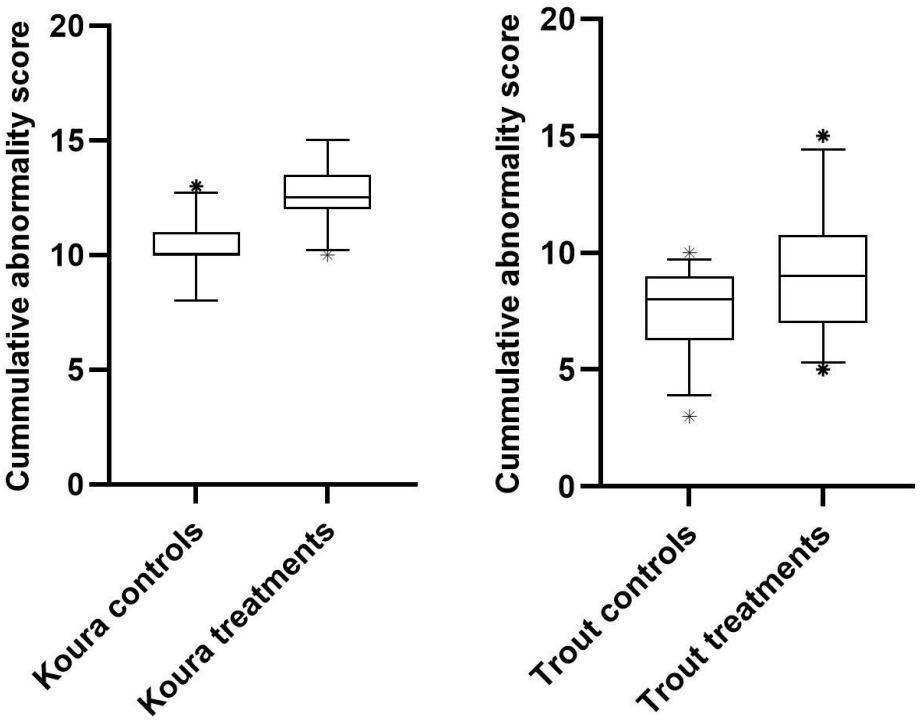


Figure 2.6: Box and whisker plots displaying cumulative abnormality scores for kōura and rainbow trout control and treatment groups with whiskers representing 10% and 90% bounds. The bounds of the box indicate the 25% and 75% quartiles and the median is represented by the line within the box. Outliers, points outside the lower and upper limits, are represented with asterisks (*).

Indicators of stress such as the loss of equilibrium and surface breathing were not observed in rainbow trout during daily monitoring in treatment and control tanks. However, two kōura were casualties of interspecific aggression.

Discussion

The toxicity of aluminium to aquatic organisms under continuous acidic or alkaline conditions has been widely explored, however, examination of toxicological effects under cyclic pH elevation has not been undertaken. The aim of this study was to determine the sublethal effects of alum derived aluminium on juvenile rainbow trout and kōura under diel pH cycling. A total of 24 rainbow trout and 24 kōura were exposed to 2 mg Al L^{-1} for 10 days with half the test group undergoing diel pH cycling from pH 7 to pH 10 and then back to pH 7, while the control group remained at pH 7.

Aluminium transitions from particulate $Al(OH)_3$ to soluble $Al(OH)_4^-$ as pH shifts from 7 to 10 and then back to 7 over a 24-hour period. Filtering and ICP−MS analysis confirmed that aluminium was predominantly present in particulate form at pH 7 and it was assumed that aluminium speciation changed from particulate to soluble forms as pH increased to 10, as reviewed by (Gensemer & Playle 1999). Previous studies have established that toxicological effects of dissolved aluminium occur at significantly lower concentrations (<100 µg L^{-1} to 800 µg L^{-1}) compared to particulate aluminium (Gensemer & Playle 1999; Poléo & 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 5.0 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 dependant on a number of 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 decrease (15%) in Cl^- 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 2.2, Figure 2.4). However, significant differences in the variances of osmolarity between the control and treatment groups for both rainbow trout and kōura were observed (*F*- value = 0.0297, 0.0269), 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 normal MCHC values for rainbow trout (~3.5 mM), while the control group MCHC was lower at 2.34 mM (Wells & Weber 1990), indicating erythrocyte swelling in the control group. Erythrocyte swelling can occur as a generalised adrenergic stress response, increasing 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, which consequently increases oxygen uptake at the gills. However, the increased intracellular Na^+ induces osmotic uptake of water causing the cell to swell. (Heming *et al.* 1987). This suggests that a more generalised stress response was occurring in the treatment group as metabolic resources were mobilised in order to maintain homeostasis.

Studies of aluminium toxicity have tended to focus on osmoregulatory effects as both acidic pH and dissolved aluminium result in synergistic impacts on osmoregulation in fish and crustaceans. Research into physiological toxicity of aluminium is complicated by the inter-relationship between aluminium toxicity and pH. At low and high pH, aluminium toxicity increases however, pH alone can also cause similar osmoregulatory disruptions (Wilkie & Wood 1991; Wilkie *et al.* 1999; Regish *et al.* 2018). Body pH is maintained through acid-base regulation whereby Na^+ and Cl^- is 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 & 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. Additionally, the loss of CO_2 increases in response to the increased CO_2 blood-water gradient (Garcia Parra & Baldisserotto 2019). 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 regulation makes determining specific physiological effects from aluminium challenging given the dependence of dissolved aluminium on pH.

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 & 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*), finding 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, histological examination of gill tissue revealed that damage occurred in both control and treatment groups of both rainbow trout and kōura, although statistically significant differences between control and treatment groups were only detected in kōura (Figure 2.6). This data suggests that kōura gills tissues may be more

susceptible to gill damage from elevated pH when high concentrations of aluminium are present. However, it is important to note that abnormalities were observed in all histological tissue samples for both species and treatment groups. This suggests that gill tissue damage was likely due to the high concentration of particulate aluminium present in both treatment and control groups.

Conclusions

No significant differences were found in rainbow trout plasma osmolarity or kōura haemolymph. Similarly, no significant differences were observed in rainbow trout haematocrit and whole blood haemoglobin samples. However, a significant decrease in MCHC of the rainbow trout control group was observed. This difference was likely due to a generalised stress response from exposure to high concentrations of particulate aluminium. There was also evidence of gill tissue damage in both species across both treatment and control groups suggesting that the high aluminium concentration may be causing some erosional damage of the gill tissue. However, it is unlikely that the co-stressors of cyclic pH increases and aluminium will result in acute toxicological effects in rainbow trout and kōura. The lack of effect in treatment groups is attributed to the transient nature of pH cycling, giving animals reprieve from continuous exposure from aluminium at either high or circumneutral pH. It is therefore concluded that organisms are able to metabolically compensate for any osmoregulatory disturbance which may be caused at high pH levels and aluminium at 2 mg Al L^{-1}. It should be noted that these exposure conditions are highly unlikely to occur in Lake Rotorua due to the dilutional effects of the lake on the dosed inflows. In addition, induced pH cycling from algal blooms is unlikely to persist for an extended period of time and the adaptive processes of the lake fauna are likely able to compensate for this stressor.

Chapter 3

Effects of aluminium on rainbow trout, common bully, and kōura respiration under diel pH cycling

Introduction

Aluminium sulphate ($Al_2(SO_4)_3$) is used to inhibit the formation of algal blooms due to its ability to sequester dissolved phosphorus from the water column. It is typically applied to water bodies with circumneutral pH (pH 6–8), but at concentrations >0.15 mg Al L^{-1} may cause gill irritation, excess mucus production and even damage gill tissue as it forms insoluble particulate aluminium hydroxide ($Al(OH)_3$) (Gensemer and Playle, 1999). Outside of circum-neutral pH aluminium forms soluble hydroxides which may interfere with osmoregulation, acid-base homeostasis, and respiration. The proposed mechanism for these effects is precipitation of dissolved aluminium onto the gill surface. This occurs due to differences in pH between the external environment and the microenvironment around the surface of the gill. The pH of the gill microenvironment is around pH 6 due to the release of waste products such as ammonium (NH_4^+), carbon dioxide (CO_2) and H^+ (Playle & Wood 1989b). More acidic or basic environmental waters containing dissolved aluminium passing over the gills encounter the more neutral pH environment causing $Al(OH_3)$ to precipitate onto the gill surface (Playle & Wood 1989a; Gensemer & Playle 1999). As well as causing gill irritation, aluminium may also bind to ion channels or Na^+/K+ – ATPase enzymes in the apical surface of the gill cells interfering with ion exchange, acid-base regulation and respiratory gas exchange (Svendsen *et al.* 2016).

The extent of aluminium effects on respiration rates and metabolism have previously been determined using experimental techniques such as respirometry. Respirometry, examines oxygen consumption rate (MO_2) by the animal, which is used as a proxy for metabolic rate (Rodgers *et al.* 2016). Reduced oxygen supply to cells hinders aerobic metabolic ATP production and physiological function (Reece *et al.* 2015). In the case of aluminium toxicity, reduced oxygen consumption is indicative of aluminium precipitation onto the gills causing interference with gas exchange and in severe cases increased gas diffusion distance due to excessive mucilage (Poléo 1995; Gensemer & Playle 1999; Winter *et al.* 2005). In response, compensatory mechanisms such as increased gill permeability, perfusion rates, and ventilation

rates are initiated in order to maintain sufficient gas exchange, however these come at a metabolic cost to the animal (Laitinen & Valtonen 1995; Onukwufor & Wood 2020). Compensatory mechanisms require high energy expenditure and increased gill permeability can cause rapid ion losses and circulatory failure and cannot be sustained indefinitely (Garcia Parra & Baldisserotto 2019).

Aluminium is currently dosed to the Utuhina Stream at approximately 1 mg L^{-1}, while this rate is not considered high in comparison to international dosing (~2–20 mg Al L^{-1}) (Berkowitz *et al.* 2005) and is further diluted upon entering Lake Rotorua where mean total aluminium concentration is 0.02 mg Al L^{-1} (Tempero *et al.* 2015). However, in terms of toxicological effects, osmoregulatory and respiratory disruptions have been reported at aluminium concentrations between 0.15–0.9 mg Al L^{-1} depending on environmental pH (Gensemer & Playle 1999). The additional effect of photosynthetically driven diel pH cycling has the potential to increase aluminium toxicity with shifts in pH from pH 7 to ~10 to pH 7 again, changing aluminium solubility over a 24-hour period. This has the potential to cause precipitation of $Al(OH_3)$ on the gills of fish and crustaceans inhabiting the lake, disrupting respiration and impacting the animals ability to meet metabolic oxygen demand. This is of particular importance as algal blooms often occur during the summer when water temperatures are higher. Increasing water temperature increases the metabolic rate of poikilothermic animals such as fish and crustaceans, while also reducing oxygen solubility in the water.

Study objectives

Examination of oxygen consumption will provide insight into the combined effects of sublethal aluminium concentrations and pH cycling on fish and crustacean respiratory exchange. Oxygen consumption rates of rainbow trout (*Oncorhynchus mykiss*), common bully (*Gobiomorphus cotidianus*), and kōura (*Paranephrops planifrons*) exposed to 2 mg L^{-1} aluminium and diel pH cycling (pH 7− pH 10) will be measured using intermittent-flow respirometry. The mass-specific data will be used to identify any trends in respiration rates over 60 h and significant differences between treatment and control groups will indicate that aluminium and pH have an effect on rainbow trout, common bully, and kōura respiration.

Methodology

Collection and Maintenance

All procedures followed those set out in the University of Waikato Animal Ethics Committee Standard Operating Procedure (SOP number: 7) for capture, handling, and captive maintenance of fish.

Juvenile rainbow trout (body mass: 37.2 g ±1.3 SE; total length: 157 mm ±3.7 SE) were sourced from the Fish and Game Ngongotaha hatchery in Rotorua. Adult common bully (body mass: 2.3 g ±0.1 SE; total length: 57 mm ±0.75 SE) 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 diving 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. The rainbow trout were held in a 5000 L fiberglass tank supplied with continuous dechlorinated municipal water at a rate of 1.5 L minute^{-1} and constant aeration was provided to maintain oxygenation within the tank. Kōura were housed in two 100 L tanks (containing no more than seven individuals) and two 60 L tanks (containing no more than four individuals), each with a recirculating filtration system. To mitigate aggression within the 100 L holding tanks, kōura were separated by a Perspex plastic divider and provided with a half terracotta pot for refuge. Common bullies were divided between two 60 L glass tanks filled with dechlorinated freshwater and the addition of artificial seawater (3 ppb – 4 ppb) to reduce osmotic stress and disease (Figure 3.1).

Study animals were not fed for the first week of being in the laboratory. The rainbow trout were fed two-three handfuls of small grain (0.8 mm) fish pellets every 2 days and the common bully and kōura were fed bloodworms every 3 days. Feeding was monitored to ensure that excess food was removed, and that fish were fed to satiation. All animals were monitored at least once daily to manage signs of stress.

Figure 3.1: Common bully (*Gobiomorphus cotidianus*) 60 L holding tank with half pots, plants, and aeration.

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}, a submersible pump was used to keep the solution agitated. A pH 12 sodium hydroxide (Merck, Germany) stock solution was prepared from 25 g of solid NaOH dissolved into 50 L of dechlorinated water, pH was adjusted to 12 if necessary.

Respirometry procedure

Intermittent respirometry was used to measure oxygen consumption of rainbow trout, common bully, and kōura (Svendsen *et al.* 2016). Twelve individuals (i.e., 6 control and 6 treatment) of each species were used in the toxicity testing, with six 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 the fish did not occupy more than 10% or less than 1% of the total respirometer volume (Figure 3.2). Three respirometers were

individually placed in separate 60 L glass tanks (two containing one test subject and the third remaining empty to measure background respiration). The tanks were continuously oxygenated with compressed air to maintain 100% oxygen saturation and water temperature was maintained at 18°C.

The tanks were fed by two peristaltic pumps, each with four independent pump heads. Pump 1 supplied each tank with the stock 2 ppm aluminium solution at a rate of 0.2 L h^{-1}. Pump 2 supplied the two control tanks with 2 ppm aluminium solution and the two treatments tanks with the pH 12 2 ppm aluminium solution at a rate of 6 mL h^{-1}. All tanks were initially filled with the stock aluminium solution and the test subjects introduced and allowed to acclimate for 12-hours prior to initiation of pH cycling. During the acclimation period, the tanks were flushed by pump 1 with the stock aluminium solution (pH 7). 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 increases in pH from pH 7 to pH 10 over a 12-hour period. The pH subsequently declined back to pH 7 within 12 hours of pump 1 starting.

The 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 min open and 45 min closed for common bully. These intervals were sufficient to prevent oxygen falling below 80% of the ambient full 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 concentration (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 are observed. At the end of the 48-hour experimental period animals were euthanised (SOP#6 Euthanasia and anaesthesia of fish).

Figure 3.2: Individual common bully in the small sized (115 mL) respirometer.

Data analysis

Oxygen consumption data for individual fish and kōura were plotted and 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 individual 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).

Tank aluminium content

Particulate and dissolved aluminium content from selected background, control, and treatment tanks were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Total aluminium samples were acidified to pH 3 by the addition of 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 (Sartorius, Germany), 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

Statistical analyses were carried out 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 if there were 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. *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

Total aluminium was within the targeted exposure of 2 mg L^{-1} in exposure groups (Table 3.1). It was assumed that soluble forms of aluminium became more abundant with increasing pH. (Gensemer & Playle 1999).

Table 3.1: Total and particulate aluminium (*n* =2) 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

Indicators of stress such as the loss of equilibrium and surface breathing were not observed in rainbow trout or kōura during daily monitoring. 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 with the treatment groups (Figure 3.3).

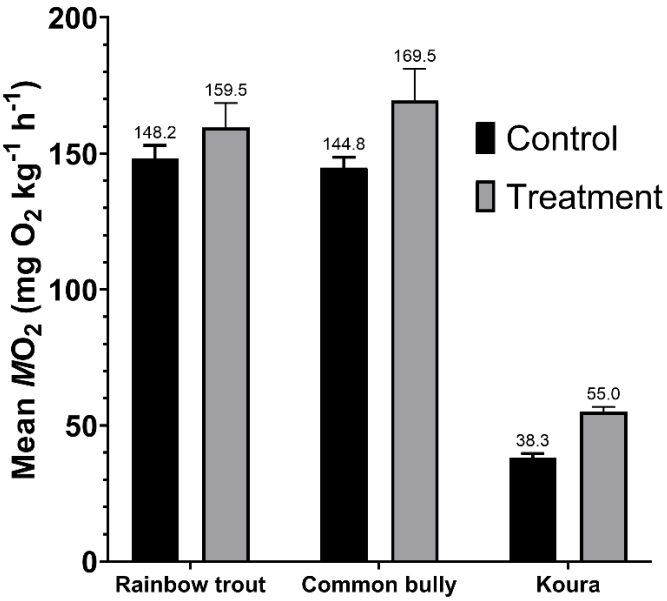


Figure 3.3. Mean (± SEM) metabolic consumption rate (MO_2) for rainbow trout, common bully and kōura exposed to either 2 mg Al L^{-1} pH 7 (control) or 2 mg Al L^{-1} pH 7-10 cycling (treatment).

Plots of the mass-specific oxygen consumption over time revealed a diurnal pattern in the kōura data (Figure 3.4). However, there is extensive variation for rainbow trout (Figure 3.5), and common bully (Figure 3.6) which can be related to the reduced sample numbers. However, from this data trends can still be seen. Mass-specific oxygen consumption data for all species in treatment groups decreased as pH lowered from pH 10 to 7 and is also observed in the kōura control group. This common change in both kōura treatment and control groups may be due to diurnal cycle in activity influencing kōura respiration and not related to treatment effects.

A Pearson's *r* correlation test determined that there was a statistically significant negative correlation when *t*-statistics were plotted against pH and time. There was a significant difference between the control and treatment *t*-statistic (r^2 = 0.34, P <0.0001, Figure 3.7), indicating that a cyclic pattern in kōura 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 3.8) and rainbow trout (r^2 = 0.06, P = 0.054, Figure 3.9) were not statistically different. However, the variability in the data and the low sample number of controls for rainbow trout increases the 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 that low pH and aluminium was having a significant (*t*-statistic >1.96) effect on respiration rates for kōura which is consistent with trends found in the kōura mass-specific oxygen consumption data.

Large variances in the kōura treatment group can be seen towards the end of the experiment which may be attributed to an active individual skewing the data. Increased activity could be due to disruptions (i.e., excessive noise or visual disturbance) within the laboratory environment. Data during this time could be considered as statistical outliers and it is expected that the removal of these points would result in the treatment data decreasing with the controls, continuing the diurnal pattern in oxygen consumption rate, however, would decrease the small sample size (n = 5) further.

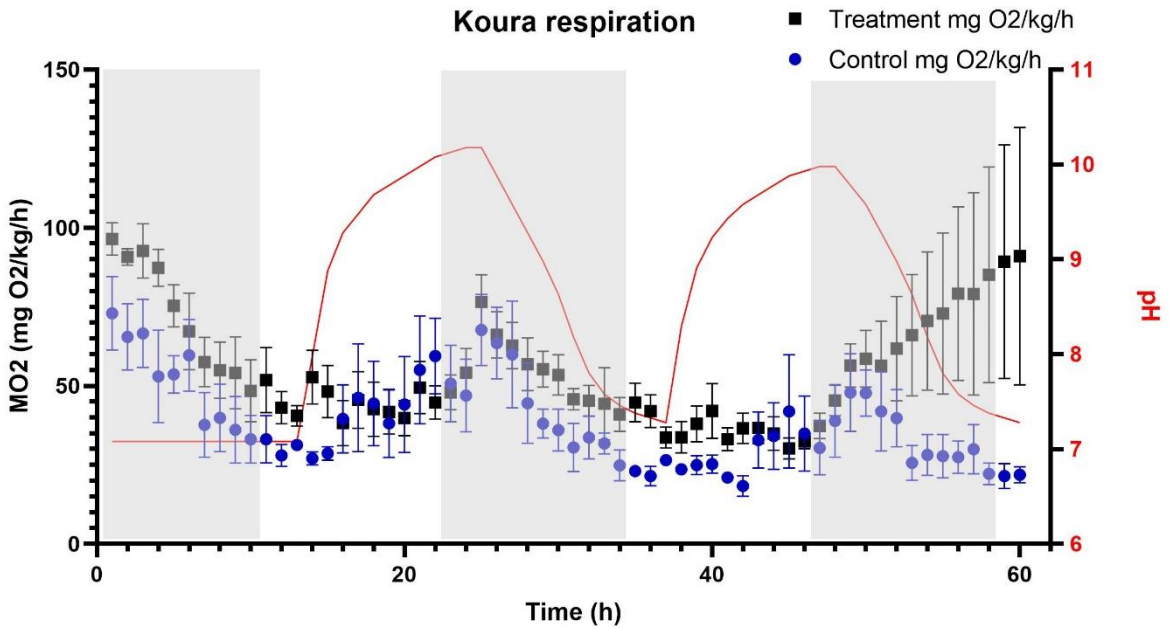


Figure 3.4: The combined effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) (mg O_2 kg^{-1} h^{-1}) for kōura control (n = 6) and treatment groups (n = 5) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SE. Shaded areas represent the dark photoperiod (12 h).

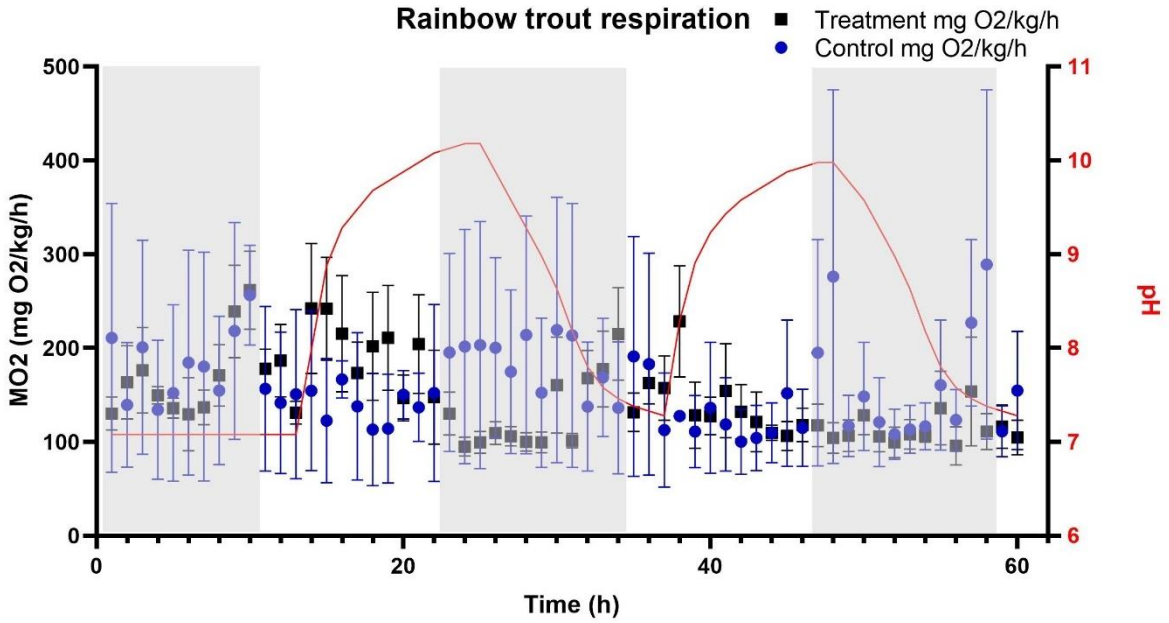


Figure 3.5: The combined effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) (mg O_2 kg^{-1} h^{-1}) for rainbow trout control (n = 2) and treatment groups (n = 5) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SE. Shaded areas represent the dark photoperiod (12 h).

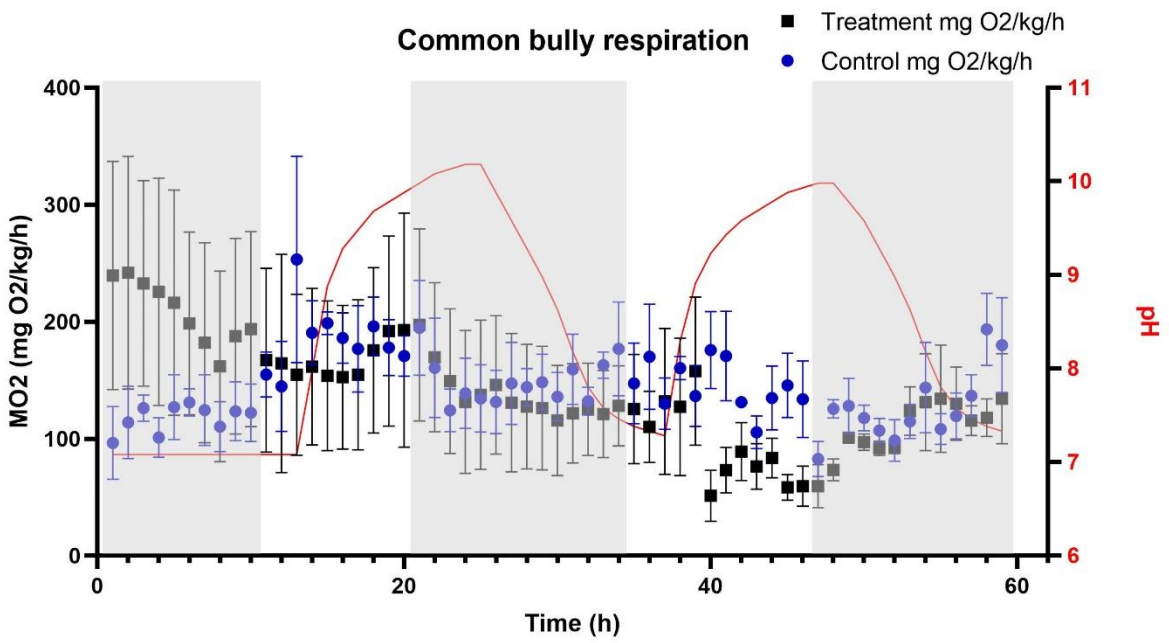


Figure 3.6: The combined effects of aluminium (2 mg L^{-1}) and pH (control = pH 7; treatment = pH 7–10) on mass-specific oxygen consumption (MO_2) (mg O_2 kg^{-1} h^{-1}) for common bully control (n = 3) and treatment groups (n = 4) exposed to 2 mg Al L^{-1} and diel pH cycles (pH 7–10) over time. Data are mean ± SE. Shaded areas represent the dark photoperiod (12 h).

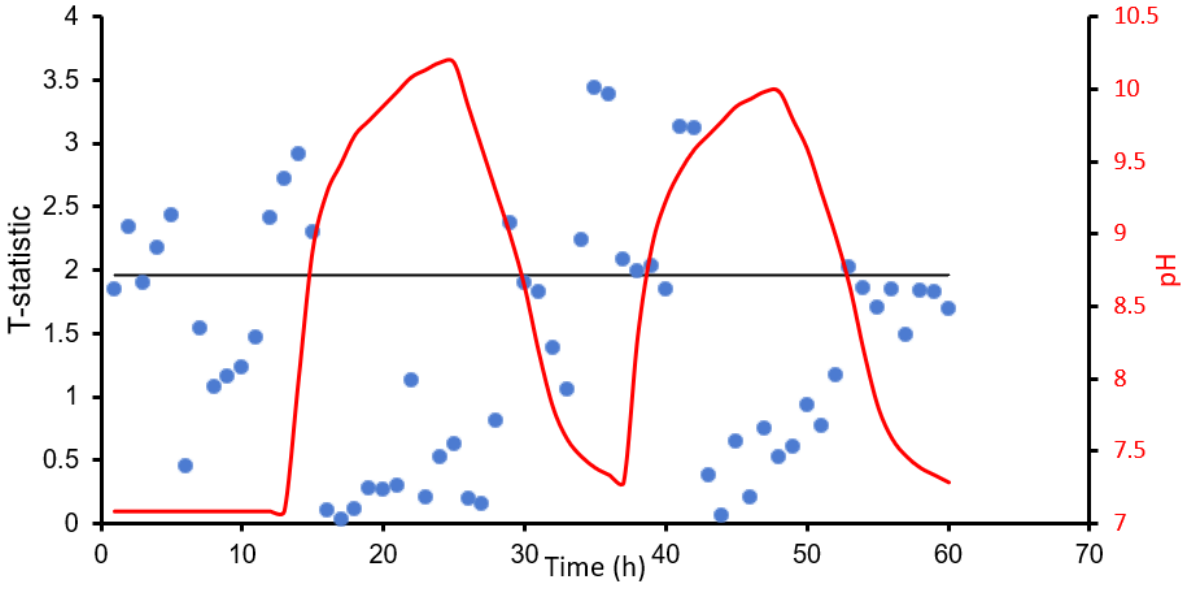


Figure 3.7: Plot of t-statistic values for the differences between the kōura control and treatment mean MO_2 for each hour, with the diel pH cycling. If the t-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.

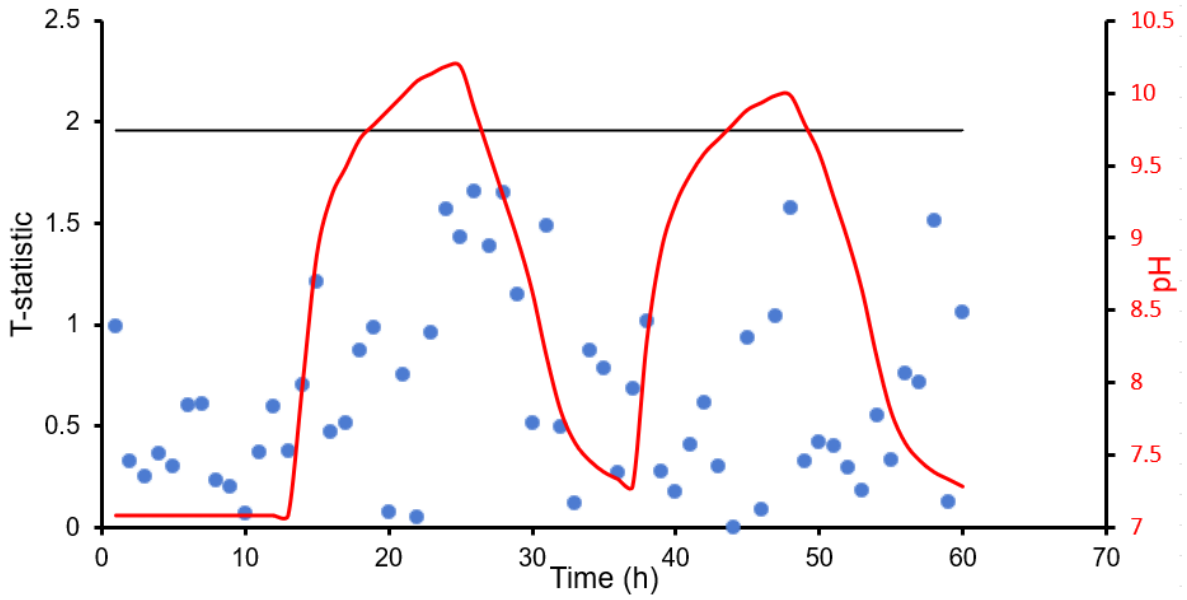


Figure 3.8: Plot of *t* -statistic values for the differences between the rainbow trout control and treatment mean MO_2 for each hour, with the diel pH cycling. If the *t*-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.

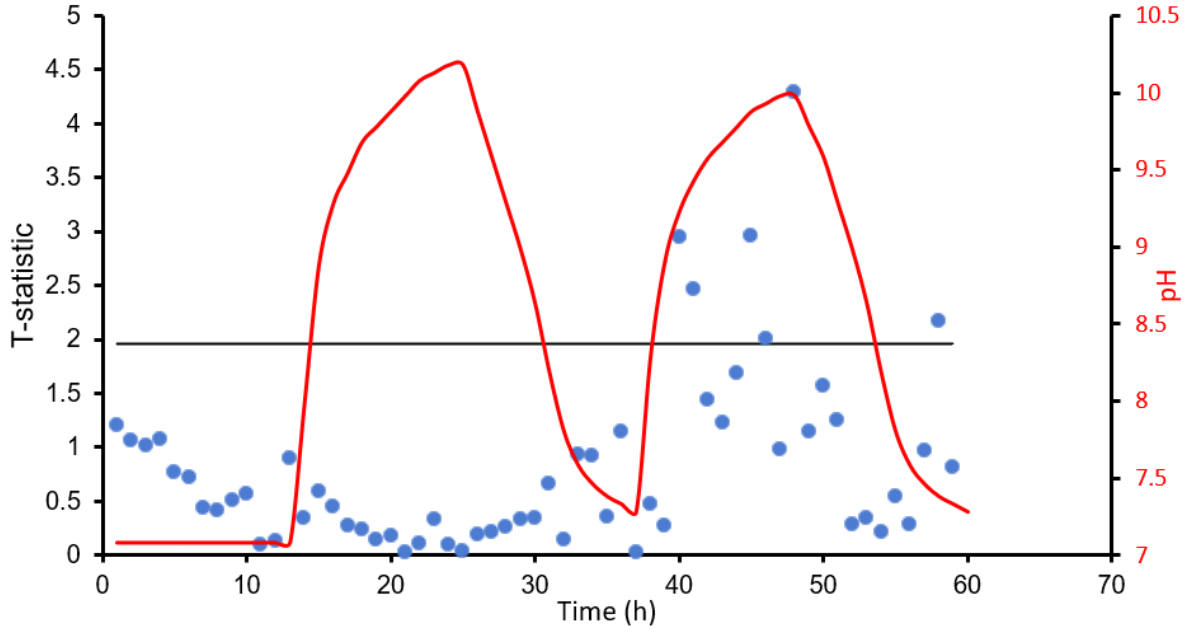


Figure 3.9: Plot of *t* -statistic values for the differences between the common bully control and treatment mean $\boldsymbol{MO_2}$ for each hour, with the diel pH cycling. If the *t*-statistic is >1.96 the control and treatment means were statistically different at the 95% confidence level.

Discussion

The gills are responsible for respiratory gas exchange, ionoregulation, acid-base balances, and osmoregulation in both fish and invertebrates. Aluminium has the potential to disrupt these processes through polymerisation and precipitation onto gill surfaces (Gensemer & 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 O_2 demands to be met (Onukwufor & 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 & Wood 2020). Alkaline waters (pH >8.5) impose similar challenges to acid environments, by inducing respiratory alkalosis (Wilkie & 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 & 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 previously 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 this study, the effects of alum derived aluminium on juvenile rainbow trout, common bully, and kōura respiration under diel pH cycling were examined. A total of 12 rainbow trout, 12 common bully, and 12 kōura were exposed to 2 mg Al L^{-1} for 60 hours in intermittent flow respirometry with half the test group undergoing diel pH cycling from pH 7 to pH 10 and then back to pH 7, while the other half remained at pH 7. Under these conditions precipitation of $Al(OH)_3$ is possible during periods of circumneutral pH or during pH decreases from 10 to 7. It was expected that the MO_2 would increase when exposed to aluminium and periods of circumneutral pH or during declining pH due to particulate aluminium and precipitation of aluminium onto the gill surface causing inhibition of respiratory gas exchange (Playle & Wood 1989b; Gensemer & Playle 1999). Increased rates of O_2 uptake were expected as a compensatory response for possible respiratory acidosis and increased gill ventilation

(Gensemer & Playle 1999). The kōura MO_2 values found in this study (25–100 mg O_2 kg^{-1} h^{-1}) were within a similar range to those observed for exposed to modified zeolite at circumneutral pH (Parkyn *et al.* 2011). Also, 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 carried out 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.

Oxygen concentration measurements taken within the recirculation loop of the respirometer were inaccurate for some individuals within the control groups resulting in 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 which reduced 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 results observed in Chapter 2 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 h may have been of insufficient duration to produce respiratory responses to the combination of cyclic pH and aluminium as impacts from both particulate and dissolve aluminium may be cumulative over time.

Conclusions

Findings of this study indicate that there was a negative correlation between oxygen consumption in kōura and the combined effects of aluminium (~2 mg Al L^{-1}) and diel pH cycling (pH 7−10). This suggests that kōura may be more susceptible aluminium and pH cycling, although significant population impacts are not likely to occur. Due to necessary exclusions in oxygen saturation measurements for rainbow trout and common bully control groups, no significant conclusions could be made as to the respiratory impacts of pH cycling and aluminium. However, mean MO_2 was within expected ranges for rainbow trout and kōura, indicating that significant respiratory impacts were not occurring within the period of exposure. It should also be noted that limitations in the number of respirometry chambers and available time meant that only the combined effects of pH and aluminium could be explored, rather than individual effects of aluminium and pH. Therefore, it is recommended that further study of individual pH and aluminium effects under both circumneutral and high pH be undertaken. Further exploration on the relative susceptibility of kōura is also highly recommended.

Chapter 4
Conclusions

Research summary and implications

The specific aims of this study were to investigate whether:

1) aluminium at 2 mg L^{-1} and cyclic pH changes impair osmoregulatory function in fish and kōura as measured by the following variables: haemoglobin, haematocrit, and plasma/ haemolymph osmolarity
2) aluminium (2 mg L^{-1}) at circumneutral pH will precipitate onto gill surfaces impairing oxygen uptake supressing metabolic function.

To determine these objectives, physiological and respiratory studies were utilised. To achieve the first aim, the physiological effects of aluminium at 2 mg L^{-1} under pH shift from pH ~7 to pH ~9.5 were assessed (Chapter 2). 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. The lack of effects found in other haematological variables suggests that this effect does not cause acute effects and is unlikely to result in death. While chronic conditions could potentially induce physiological disturbance and stress effects resulting in reduced growth and this is unlikely to occur in Lake Rotorua as algal blooms resulting in diel pH cycles do not persist beyond 3−4 weeks. Therefore, the lack of acute osmoregulatory disturbance in rainbow trout and kōura indicates that osmoregulation was not impaired to the point of acute physiological disruption. This can be attributed to the transient nature of the pH shifts allowing aluminium to remain predominantly in solid form, which is less bioavailable. The brief exposures (few hours) to soluble aluminium ($Al(OH)_4$) appears to be tolerable for juvenile rainbow trout and mature kōura.

Intermittent flow respirometry was used to achieve the second goal of this study, whereby oxygen consumption under normoxic conditions (≥ 80% O_2 saturation) were used as a proxy for aluminium effects on metabolism under diel pH shifts (Chapter 3). At circumneutral pH, aluminium particulates are known to clog gills, increasing the gas diffusion gradients and hindering metabolic processes (Chabot *et al.* 2016). There was some indication that kōura were

impacted by the experimental conditions, although this was difficult to distinguish from possible nocturnal activity patterns. Ultimately, no consistent changes in MO_2 were observed in response to diel pH changes and aluminium exposure. Due to experimental limitations and variation in data, it is recommended that further respirometry studies are carried out on juvenile rainbow trout, common bully, and kōura in a way that can allow for the differentiation between high pH and aluminium effects.

The goal of this study was to determine the toxic potential of 2 mg L^{-1} of aluminium to rainbow trout, common bully, and kōura under diel pH cycling between pH 7 and pH 10. By international standards, a dose rate of 2 mg Al L^{-1} is relatively conservative as alum is typically used in a single, bulk dose ($\leq$ 20 mg L^{-1}), and is often applied to small, deep lakes with a high buffering capacity (Welch & Cooke 1999; Berkowitz *et al.* 2005; Churchill *et al.* 2009; Huser *et al.* 2011). In addition, individual dose rates to the Utuhina and Puarenga Streams have rarely exceeded 1 mg Al L^{-1} and are substantially diluted upon reaching Lake Rotorua. In this study toxicological tests were conducted at 2 mg Al L^{-1}, as it was assumed that this was likely to be beyond the minimum effects threshold. However, it should be noted that this concentration is substantially higher than the mean aluminium concentration for Lake Rotorua (0.02 mg Al L^{-1}) and unlikely to be encountered by biota in Lake Rotorua.

In Lake Rotorua, algal bloom driven pH cycles only occur in the lake, therefore, the combined effect of pH and aluminium only occurs at low aluminium concentrations outside of the immediate areas discharged to by the Puarenga and Utuhina Streams. Testing at a higher aluminium concentration compared to what is found within the environment provides insight into the physiological tolerances of study animals, helping to set dosing limits. While aluminium concentrations are much higher in flows downstream from the dosing stations, the extent of the effect area is comparatively limited.

The results of this study reveal that the transient algal bloom driven shifts in pH (pH ~7–10) on aluminium toxicity at 2 mg L^{-1} in Lake Rotorua do not cause acute osmoregulatory or respiratory impairment and is unlikely to cause death in kōura, common bully, or rainbow trout. These results, along with the monitoring work undertaken in Lake Rotorua tributaries by Ling (2016a) and Ling (2016b), provide evidence that the combined effect of aluminium at 2 mg L^{-1} and pH cycling driven by algal blooms does not have a significant acute effect in Lake Rotorua. However, further investigation considering the cumulative effects of aluminium and algal

bloom driven pH shifts and other environmental challenges such as climate change and emerging contaminants is required.

Recommendations for future research

The benefits of conducting this research include gaining insight into the osmoregulatory and respiratory tolerances of rainbow trout, common bully, and kōura at 2 mg Al L^{-1} during cyclic shifts between circumneutral and high pH. Results from this study show no significant osmoregulatory and respiratory disturbance from combined 2 mg Al L^{-1} and diel pH cycling. From this, it can be concluded that the current alum dosing techniques applied in Rotorua Lake and its tributary streams are highly unlikely to be causing any acute physiological disturbances to rainbow trout, common bully, or kōura.

Due to the lack of physiological disruption in juvenile rainbow trout and mature common bully, and kōura, it may prove useful to determine the effects of aluminium exposure on more susceptible life stages (i.e., larval rainbow trout) which would help to define species tolerances. Exposing additional species dwelling in Lake Rotorua such as inanga (*Galaxias maculatus*), kakahi (*Echyridella menziesi*), or extending research of aluminium toxicity to chironomid communities would provide more information of aluminium toxicity in the lake. 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 (1 mg Al L^{-1}) of geothermally sourced aluminium 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 to circumneutral pH.

Furthermore, it may be valuable to repeat respirometry studies at higher temperatures (18 – 22 ˚C) to determine the cumulative effects of temperature, which decreases the environmental capacity for dissolved oxygen while increasing metabolic requirements, and aluminium exposure. Finally, additional experimentation could include a behavioural study at a range of aluminium concentrations to determine whether avoidance behaviours are employed. Gaining further knowledge of aluminium toxicity in the context of Lake Rotorua will become increasingly important as anthropogenically and geologically derived phosphorus

concentrations are expected to increase via aged groundwater inputs. Consequently, the frequency and longevity of algal blooms will increase, endangering ecological and local community health (Morgenstern *et al.* 2015). Alum could be required as a mitigation measure long-term therefore, gaining further knowledge of aluminium toxicity to local organisms under a range of realistic environmental conditions is highly recommended.

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