EXAMINATION OF PULP AND PAPER EFFLUENT-HYPOXIA INTERACTIONS IN FISH

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ABSTRACT

Recent concerns have focused on the adverse effects of low dissolved oxygen (DO) combined with the potential chronic effects of pulp and paper mill effluent exposure on fishes in the Tarawera River, Bay of Plenty, New Zealand. A degassing system for the removal and control of dissolved oxygen (DO) capable of producing water with ~0.5 mg L^{-1} DO to saturation and two series of five separate DO concentrations each was first constructed. Because the Tarawera River receives effluent discharges from a chemithermomechanical (CTMP) tissue mill and an integrated thermomechanical (TMP)/bleached kraft pulp and paper mill (BK), these effluents were studied. Using the DO control system, the effects of simultaneous effluent and hypoxia exposure on the 48-h DO LC50 (median lethal concentration) were examined in fry and juvenile rainbow trout (*Oncorhynchus mykiss*) and common bully (*Gobiomorphus cotidianus*). TMP/BK mill effluent (ME) was diluted to 15 % v/v to reflect the upper concentration in the receiving environment, while CMTP effluent was extracted directly from the river. The presence of either effluent did not significantly increase the lethality of low DO for either species and life stage differences were not detected. Pre-exposure of juvenile trout to 15 and 70 % TMP/BKME also showed no significant effect on the DO LC50. No significant difference in the oxygen consumption of juvenile trout tested in 15 % v/v effluent were evident, but significant increases in oxygen consumption were observed for fish pre-exposed to 15 and 70 % effluent when tested in reference water. Four-week exposures were performed with juvenile trout to determine the chronic effects of TMP/BKME (15 % v/v) and CTMP effluent combined with non-lethal DO concentrations (2.5 mg L^{-1} to fully saturated), revealing excellent survival of fish in the combined effluent/hypoxia exposures, but marred by poor survival of reference water-exposed fish. Relationships for growth were observed for fish exposed to DO and effluent in the CTMP experiment. Several DO dose-response effects were also observed on general hematology in both effluent experiments, but were generally considered minor and typical of hypoxia exposure in fish. Four-week exposures using juvenile trout in 0, 10, 30 and 70 % TMP/BKME and 0, 35, 110 and 250 µg L^{-1} dehydroabietic acid (DHAA) were performed, where swimming performance, oxygen consumption and hematology were investigated. The chief finding was that of reduced swimming performance, indicated by lower critical swimming

(Ucrit) speeds, for fish exposed to 70 % TMP/BKME. Other moderate effects on various hematological parameters were also observed. However, all observed effects are considered relatively small in magnitude and at effluent concentrations well above those found in the receiving environment. Forty eight-hour DO LC50s were determined for juvenile inanga (*Galaxias maculatus*), common smelt (*Retropinna retropinna*), shortfin eel elvers (*Anguilla australis*), rainbow trout, common bully and the freshwater shrimp (*Paratya curvirostris*). Juvenile inanga were the most sensitive fish species, while eel elvers were the most tolerant fish species tested.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 PROJECT BACKGROUND

Concerns put forward by the Department of Conservation (DOC) during the appeal to the Fletcher Challenge Paper Ltd., Tasman Mill (now the Norske-Skog/Carter Holt Harvey joint venture) and the Carter Holt Harvey Tissue (CHHT, now CHH Consumer Brands) effluent discharge resource consents, under the Resource Management Act (1991), were focused on the effects of low dissolved oxygen (DO) combined with the potential chronic effects of pulp and paper mill effluent exposure on fishes in the Tarawera River, Bay of Plenty, New Zealand. This chapter outlines the overall problem, gives a general overview of relevant literature and sets out the project objectives.

1.2 THE TARAWERA RIVER

1.2.1 River Description

The Tarawera River is an important water resource located in the central North Island of New Zealand [1]. The river flows to the east coast at Matata from Lake Tarawera, which is situated approximately 50 – 60 km inland (Fig. 1.1). The river has a mean water flow of approximately 26 $m^3 s^{-1}$ and a bed of moving sediment dunes and pumice [2]. The Tarawera River was once connected to the Rangataiki River [3]. In the early 1900's, much of the lower Tarawera River was straightened and the Rangataiki was diverted. The straightening of the Tarawera resulted in considerable scouring, causing a decline of fish habitat in the lower section of the river [3].

Around the township of Kawerau, the Tarawera River is influenced by numerous natural, industrial and domestic inputs. The lower river is a receiving environment for geothermal, pulp and paper and municipal sewage discharges; the two pulp and paper mills have the greatest discharge volumes. The first pulp and paper mill (CHH Consumer Brands) is a tissue mill that currently discharges treated effluent directly to the river and to river-adjacent rapid infiltration basins. The second pulp and paper mill (Tasman Mill) currently discharges secondary-treated effluent directly to the river. As the Tarawera River is not especially large (Fig. 1.2), it receives a considerable volume of pulp and paper wastewater for its size. Mean

daily river flow down-stream of the mills may be composed of anywhere between 5 and 12 % v/v effluent [1, 4] (Fig. 1.3).

Figure 1.1. The Tarawera catchment originating inland at Lake Tarawera, flowing approximately 50 km east to the coast at Matata.

Dissolved oxygen fluctuations are a common problem in many river systems that are subject to industrial wastewater emissions [5], and deoxygenation in the Tarawera River has been an ongoing issue from the 1950's to the present day [3, 6]. The nature of the Tarawera River bed provides ideal habitat for oxygen consuming microbes that account for up to 90 % of the deoxygenation that occurs in the river [2, 7], and it has been suggested that the river may have a reduced ability to assimilate effluents with high BOD as a result [8]. Due to effluent loading and deoxygenation in the river, colour and DO are now regarded as key areas of concern in the management of the Tarawera River [8].

Figure 1.2. Segment of the Tarawera River above the township of Kawerau.

1.2.2 Tarawera Fish Studies

A number of fish studies have been conducted on the Tarawera River, particularly over the last decade. Recent analysis of fish health in the Tarawera River has shown that total fish densities are similar to those found in the nearby Rangataiki River [3]. Yet surveys have revealed the absence of previously recorded fish in the Tarawera, such as koaro (*Galaxias brevipinnis*), inanga (*Galaxias maculatus*), giant kokopu (*Galaxias argenteus*) and lamprey (*Geotria australis*) [9]. However, two new species previously not recorded in the Tarawera River, the shortjaw kokopu (*Galaxias postvectis*) and Cran's bully (*Gobiomorphus basalis*), were discovered [9]. The lack of certain fish species, especially koaro, has caused some concern. It has been suggested that young migratory fish might be actively avoiding the river due to effluent loading and/or reduced DO [10]. Accordingly, caging studies with juvenile inanga and koaro examined the *in situ* effects of downstream effluent exposure and DO on fish survival in the Tarawera River [10]. This study did not show any significant differences in survival between inanga exposed in the Tarawera River compared to caged reference fish in the Rangataiki River. However, in the koaro study, Young [10] did note that DO

levels at exposed sites in the Tarawera regularly fell below 5 mg L^{-1} and a relationship between mortality and reduced DO was found. Young [10] concluded that DO must remain above 5 mg L^{-1}, a point above the absolute minimum regulation of 4 mg L^{-1} in the Tarawera River, to ensure protection of fishes at these sites.

Figure 1.3. River colour in the Tarawera River downstream of Kawerau.

Controlled field and river-side laboratory studies assessing the effects of Tasman effluent have revealed some chronic effects in fish, in particular, on reproductive physiology [4, 11, 12]. Reproductively maturing rainbow trout (*Oncorhynchus mykiss*) exposed to 10 % Tasman effluent for two months revealed no effect of effluent exposure on growth, gonad development or gonadal steroid levels compared to river water-exposed control subjects [4]. In fact, the only observed effluent effect was a modest induction (x 2.5) of hepatic 7-ethoxyresorufin-*O*-deethylase (EROD). In a follow-up study [11], timing of exposure to effluent was seen to have reproductive effects in male and female rainbow trout. Adult trout exposed to 12 % Tasman effluent for eight months, beginning roughly three months before gonadal development, showed some effects on growth and

reproductive parameters [11]. Here it was observed that fish of both sexes had reduced growth, females had smaller ovaries, reduced plasma levels of testosterone and estradiol, and males showed induction of vitellogenin (the egg-yolk precursor protein) and lowered 11-ketotestosterone levels. Similar effects were not observed for fish exposed to 12 % effluent for two months, starting approximately half-way through gonadal development.

Laboratory studies on 21-day exposed mosquitofish (*Gambusia affinis*) to both untreated and secondary-treated Tasman effluents showed some effluent androgenicity (having androgen-like effects), expressed as pseudo-gonopodial development and male-like mating behaviour in females [12]. When Ellis et al. [12] repeated this experiment with filtered secondary-treated effluent, they discovered that filtration eliminated the masculinisation effects, indicating that the active components causing the observed effects were associated with suspended solids. However, these effects have subsequently disappeared [13].

1.3 PULP AND PAPER MILL DESCRIPTIONS

Information and data presented in this section were obtained from the Carter Holt Harvey Tasman (CHH Tasman) annual report 2002 [14], the Norske-Skog Tasman (NS Tasman) community report 2002 [15] and personal communication with the respective mill Environmental Managers; Helen Jenkins (CHH Tasman), Robert Donald (NS Tasman) and Kirstine Hulse (CHH Consumer Brands).

1.3.1 Carter Holt Harvey (Consumer Brands) Tissue Mill

The CHHCB mill is a medium-sized pulp and paper mill with a staff of around 300. The mill produces approximately 110 T d^{-1} of unbleached and peroxide-bleached chemithermomechanical pulp (CTMP) and 160 T d^{-1} of tissue paper. Mechanical pulp (CTMP) produced on site is made from purchased woodchips, making up roughly 60 % of the mills' total pulp requirements, with the remainder as purchased unbleached and bleached kraft pulp. Furnish may be 100 % *Pinus radiata*, 100 % *Eucalyptus spp.* or a 50/50 blend, dependent on the desired final paper product. Effluent from the CTMP process undergoes anaerobic treatment in

combination with Kawerau municipal sewage prior to discharge. Final treated CTMP effluent is currently discharged both directly to the Tarawera River and to adjacent river-side rapid infiltration basins. Grey water from the paper making process is sent to the Tasman facility where it undergoes primary and secondary treatment before discharge to the Tarawera River.

Figure 1.4. Aerial photo of the Tasman Mill site in Kawerau. The Tarawera River can been seen in the lower left corner.

1.3.2 Norske-Skog/Carter Holt Harvey Tasman Mill

The Tasman Mill (Fig. 1.4) is now two separate pulp (CHH Tasman) and paper (NS Tasman) business entities, employing roughly 1,000 combined staff. The Tasman facility is an integrated stone groundwood thermomechanical (TMP)/bleached kraft pulp and paper mill (BK), producing 950 and 700 T d^{-1} of

newsprint and pulp, respectively. The Norske-Skog mill uses up to 98 % mechanical fibre with a furnish of 100 % *Pinus radiata*, the remainder being kraft. The CHH mill produces kraft pulp that has a furnish of around 25 % *Eucalyptus spp.* and 75 % *Pinus radiata* fibre. Pulp undergoes chemical treatment with sodium hypochlorite or chlorine dioxide (DEopDnD or DeopPD), and has been elemental chlorine free (ECF) since 1998. TMP effluent (7 % total flow) is pre-treated in an activated sludge bioreactor system before combination with the remaining BK effluent. Combined effluents are primary treated by passage through a coarse screen and gravity clarifier to remove solid material prior to secondary treatment. Secondary treatment of effluent takes place in a 45-hectare aerated pond system (4 ponds) with a retention time of 4 – 6 days before final discharge to the Tarawera River (Fig. 1.5). On average, 133,000 $m^3 d^{-1}$ of water is taken from the Tarawera, while total wastewater discharge is approximately 175,000 $m^3 d^{-1}$.

Figure 1.5. Aerated effluent treatment pond at the Tasman site.

1.4 HYPOXIA IN FISH

In aquatic environments, hypoxia is typically regarded as a condition of very low DO (typically DO concentrations < 3 mg L^{-1}, but often ≤ 5 mg L^{-1}). To understand the problems associated with hypoxia, it is important to appreciate the challenges

facing fish when simply attempting to extract oxygen from water. The solubility of oxygen in fresh water is a function of temperature and partial pressure. As temperature rises, the solubility of oxygen decreases; as partial pressure decreases, the solubility of oxygen decreases. Thus, dissolved oxygen only comprises around 10 – 20 parts per million of water, compared to around 20 % of air. Additionally, water is up to 800 times denser than air and diffusion is slow. These factors essentially mean that aquatic organisms must not only be more efficient at taking up oxygen but must also expend more energy to do so, compared to terrestrial organisms.

The basic effects of hypoxia in fish are well known. A complete lack of spawning in fathead minnows (*Pimephales promelas*) experiencing hypoxia has been shown at very low DO concentrations of around 1 mg L^{-1} [16]. Brungs [16] also demonstrated reduced fecundity (number of eggs) at 2 mg L^{-1}, increased time to hatch and deformities in developing hatchlings below 5 mg L^{-1}, and reduced growth and survival at all DO concentrations below the saturated controls. It was suggested that growth and survival were the most sensitive measures of hypoxia exposure in fathead minnows. Similar evidence has been reported on the development of embryos and larvae of lake trout (*Salvelinus namaycush*) and largemouth bass (*Micropterus salmoides*) [17]. Carlson and Siefert [17] found that at 7 and 10 °C, DO levels of approximately 50 % saturation (~ 5.5 – 6 mg L^{-1} DO) and lower can cause delayed hatching, increased time to first feeding, reduced growth and reduced survival in lake trout. Reduced DO exposures in largemouth bass at 20 and 23 °C revealed that 35 % saturation (~ 3 mg L^{-1} DO) yielded comparative results to lake trout at 50 % saturation, although moderately reduced growth was still observed at higher DO levels of around 70 % saturation (~ 6 mg L^{-1}). While this study demonstrated similar results for both fish species examined, it also shows considerable differences in the sensitivies of fishes.

Brett and Blackburn [18] examined the effects of lowered DO in young coho (*Oncorhynchus kisutch*) and sockeye (*Oncorhynchus nerka*) salmon. This study also reviewed and reanalyzed several previously published studies for largemouth bass, carp (*Cyprinus carpio*) and coho salmon. From their own study they found strong dependence of growth on DO concentrations below 5 mg L^{-1}. They

concluded that for all species investigated, above critical DO concentrations ranging from 4 – 4.5 mg L^{-1} growth was not limited when exposed for periods of 4 – 8 weeks. More recent studies have demonstrated that hypoxia can cause reduced growth in turbot (*Scophthalmus maximus*) [19, 20] and sea bass (*Dicentrarchus labrax*) [20] at DO concentrations of 5 mg L^{-1} and below. In the first study [19], it was suggested that reduced growth could have been due to reduced food intake. The subsequent study [20] then confirmed the link between hypoxia and reduced feeding resulting in diminished growth. Pichavant et al. [20] suggest that by minimizing feeding, fish may be able to reduce energy usage, in turn, reducing overall oxygen demand.

Apart from influencing growth and survival, hypoxia can have a variety of other effects in fish. Fish may respond to hypoxia in a number of ways in an attempt to alleviate or reduce negative impacts, through enhancing respiratory performance or through behavioural modifications [21-23]. Elevated plasma catecholamines (adrenalin and nor-adrenalin) have been observed in rainbow trout exposed to varying degrees of hypoxia [24]. Increases in heart and ventilation rates have been demonstrated in rainbow trout [25, 26], bluegill (*Lepomis macrochirus*) and brown bullhead catfish (*Ictalurus nebulosus*) [25], and behavioural modifications often observed in various fish species include reduced activity, surfacing, leaving the water or avoidance [21, 27-29].

1.5 PULP AND PAPER EXPOSURE IN FISH

The effect of pulp and paper effluents in fish has been thoroughly examined and the mass of literature available on the subject is overwhelming. In the last 10 – 15 years there has been a concerted effort on the part of the pulp and paper industry to improve the overall quality of environmental emissions, particularly those to the aquatic environment [30]. Since the introduction of modified pulping, bleaching and treatment processes, much of the once observed effects in fish, such as acute toxicity, have disappeared altogether [30, 31]. Therefore, this section will only briefly touch on some of the more recent observations in fish.

A host of effects resulting from pulp and paper effluent exposure are consistently observed in both field and laboratory studies with numerous fish species. One of

the most widely reported responses, linked with detoxification processes in the liver, is increased mixed function oxygenase (MFO) activity, as indicated by EROD induction [32-36]. Other common effects of effluent exposure include, but are not limited to, reduced stress responsiveness [37], increased growth [34], increased liver size [33], decreased gonad size [33, 38], reduced sex steroid levels (testosterone and estradiol) [35, 38], reduced fecundity [39], immunosuppression [33, 34, 40-42] and DNA damage [36, 38, 43].

1.6 CUMULATIVE EFFECTS

Assessments of cumulative, or multiple, stressor effects are beginning to receive more attention as researchers attempt to understand population-level effects [44]. Through examination of brook trout (*Salvelinus fontinalis*) population modeling experiments, Power [44] has suggested that predictions based on the summing of individual stressors may result in considerable under-estimation of stressor effects.

Additive and synergistic toxicant effects have been known for some time. Early studies have demonstrated increased mortality in fish exposed simultaneously to various toxicants with hypoxia. Survival times of rainbow trout exposed to a range of potassium cyanide concentrations (0.105 – 0.155 ppm) all increased with increasing DO (10 – 100 % saturation) [45]. The same pattern in rainbow trout has also been demonstrated with exposure to ammonium salts, monohydric phenols, zinc, lead and copper salts [46]. Thurston et al. [47] also found a strong positive correlation between DO and the 96-h median lethal concentration (LC50) of aqueous ammonia. Here it was shown that as DO was increased (2.6 – 8.6 mg L^{-1}) the 96-h LC50 for NH_3 increased (0.32 – 0.81 mg L^{-1}).

Similar results have been obtained for fish exposed to pulp and paper mill effluents. Hicks and DeWitt [48] examined median survival times of juvenile coho salmon exposed to 22.5 and 33 % v/v kraft mill effluent (KME), over a range of DO concentrations from 3.4 – 13.9 mg L^{-1}. They discovered that at DO concentrations of 9 mg L^{-1} and below, survival times were drastically reduced at both KME concentrations. Graves et al. [49] observed differences in acute toxicity of bleached kraft mill effluent (BKME) with DO in sheapshead minnows

(*Cyprinodon variegatus*) at different stages of development. Fish (embryos to adults) were exposed to BKME concentrations of 0, 50 and 100 % v/v at DO concentrations of 1, 3 and 5 mg L^{-1}. They found that embryo survival was dependent on effluent concentration, fry survival was dependent on DO concentration, juveniles were more sensitive to BKME at 1 mg L^{-1} DO, and adult survival was not influenced by combinations of either. However, such findings no longer hold as much relevance. These initial pulp mill effluent studies were performed with relatively untreated and toxic effluents that can no longer be extrapolated to present effluents.

1.7 PROJECT AIM

The lower Tarawera River is subject to intervals of reduced dissolved oxygen concentration, which are more pronounced in the warmer summer months. Despite the large body of work on hypoxia in fish, there is a paucity of recent research on the combination of low DO and pulp and paper effluent exposure. Since an adequate database on the effects of low DO in fish already exists, the objective of this DO-effect study on fishes was to focus on possible interactions between effluent exposure and hypoxia. If effluent exposure does not add significantly to the stress caused by low DO, then the already existing criteria for DO should be adequate to safeguard fish health in the Tarawera River.

Therefore, the aim of this project was to determine what, if any, are the combined effects of pulp and paper mill effluents and hypoxia in fish. To do this, acute (lethality) and chronic (growth, survival, hematology, respiration and energetic fitness) endpoints were examined.

1.8 ETHICS STATEMENT

All animal manipulations were approved by Animal Ethics Committees prior to experiments commencing. Forest Research ethics protocol numbers are 2001-01 (acute study), 2002-01 (chronic study), 2003-01 (swimming study) and 2003-02 (native fish study).

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CHAPTER TWO

AN IMPROVED SYSTEM FOR THE CONTROL OF DISSOLVED OXYGEN IN FRESHWATER AQUARIA

As published in the Journal of Water Reseach
Landman and van den Heuvel (2003) 37: 4337-4342

2.1 ABSTRACT

A system for the removal and control of dissolved oxygen (DO) from freshwater was designed and constructed with aquarium-type fish studies in mind. Degassed water was obtained using a partial vacuum of -14 psi, and DO regulated at an aquarium scale using electronically controlled aeration with timed partial water renewal. The degassing system was capable of producing water with ~1.7 $mg \cdot L^{-1}$ DO within 10 minutes of operation, and 0.55 $mg \cdot L^{-1}$ after 2 hours. The control system was capable of maintaining DO levels of *ca.* 0.8 $mg \cdot L^{-1}$ over 48 hours in the absence of aeration and further capable of precisely controlling DO levels as low as 1.16 ± 0.002 $mg \cdot L^{-1}$ (mean ± SEM) with aeration over a 48 hour period.

2.2 INTRODUCTION

Dissolved oxygen (DO) is critical for the survival of aquatic organisms and low DO is known to be a modifying factor of toxicant impacts [1-5]. However, in laboratory experiments, DO is difficult to reliably control, and there is a need for methods that would enable accurate, simple and preferably automated assessments of effects of hypoxic conditions to be made. The removal of oxygen from water can be achieved through either physical or chemical methods [6]. The chemical methods of oxygen removal are not often used for biological tests due to undesirable effects such as direct toxicity or increased solid content from the chemical compounds added to the water. Physical methods include thermal degassing, vacuum degassing and nitrogen stripping. With the exception of thermal degassing, the physical methods for oxygen removal have been extensively employed in fish studies over the last 50 years [1, 2, 4, 7-10]. Although vacuum degassing and nitrogen stripping have their own intrinsic drawbacks, these methods are still relatively fast and simple. Nitrogen stripping is probably more cost effective in the short-term, because vacuum degassing requires a greater capital cost. However, in the long-term, vacuum degassing will be more economical as there is a reduced consumable cost.

Vacuum degassing presents some unique engineering challenges, but once a suitable system is developed, it can produce an almost inexhaustible supply of degassed water. A system for vacuum degassing was developed by Mount [11,

12] that has been successfully used in numerous studies since (e.g. [4, 13-15]). This method is based on passing a continuous flow of water through a variable partial vacuum dependent on the desired DO level. The solubility of oxygen is dependent on the partial pressure of oxygen (and temperature). The vacuum causes a drop in partial pressure, thereby resulting in reduced solubility, which forces oxygen to move out of solution until equilibrium is reached. However, the intrinsic drawback with Mount's system is that it is only capable of producing one concentration of DO at any given time, dependent on the degree of vacuum applied.

The current paper describes an application of the vacuum degassing apparatus designed by Mount [11, 12] that enables the production of multiple DO concentrations. The key innovation of this system is the use of electronic control of water reaeration to obtain precision control of DO in the aquarium.

2.3 MATERIAL AND METHODS

2.3.1 Design of the degassing system

Two identical 200 L total capacity drums were constructed, from 5 mm stainless steel, as degassing vessels (Fig. 2.1a). Two 5 mm-thick steel straps were welded around the circumference and another two across the top of each vessel for reinforcement. Eight mm-thick stainless steel covers were welded to the top of each vessel with a 20 cm hole cut in the centre to allow for a clear plastic lid making internal viewing possible and also allowing access to the inner cavity of the vessel. The plastic lid was bolted down securely to form an airtight seal. The pressure gauge, vacuum and water circulation lines were then attached to the plastic lid.

The vacuum was obtained with an oil sealed, rotary vane, high vacuum pump (Woosung - series TRP-12, Incheon-City, Korea) set with a vacuum regulator. Water entered the vacuum vessel through a model Extraflo-25 armless float valve (Apex Valve Ltd, Auckland, New Zealand) and a 25 mm PVC pipe directed into the headspace. Water was continuously circulated from the bottom of each vessel

to the top using a model 413 centrifugal water pump (Onga Ltd, Melbourne, Australia). The diffuser was created by making a "T" out of PVC pressure piping with numerous holes drilled on the underside. This ensured maximum water agitation, thereby increasing the rates of degassing of incoming and circulating water.

Continuous circulation of water was desirable to 1) provide thorough mixing, thereby preventing oxygen stratification, 2) increase agitation, thereby optimising the rate and degree of degassing, and 3) to enable water removal. Two stainless steel globe valves were placed in the circulation line at the top of each vessel; the first to regulate water flow back into the vessel and the second to control water removal. A pressure-vacuum gauge was placed in-line just before the first globe valve so that flow rates and line pressure could be set and monitored.

2.3.2 Design of the dissolved oxygen control system

The DO control system consisted of the two degassing vessels and two paired sets of five exposure chambers (aquaria) constructed from PVC (Fig. 2.1b). The basic concept of this system involved controlled aeration of degassed effluent water at individual aquaria (Fig. 2.2). Aquaria were 15 L capacity and each set of five had an external water jacket to aid in temperature control. Each aquarium was equipped with a COS 4 DO sensor and Liquisys M COM 223 meter (Endress and Hauser, Weil am Rhein, Germany). Small 12 V DC air compressors (Thomas Air Compressors, Hanover, Germany) and model E5CK digital controllers (Omron, Osaka, Japan) were used to provide the controlled aeration as required (Fig. 2.2). The voltage output from the DO metering system was then transferred to the parallel port of a desktop PC via an ADC-11 adapter (Pico Technology, St. Neots, UK). Data was recorded using PicoLog 5.06.3 for Windows (Pico Technology, St. Neots, UK).

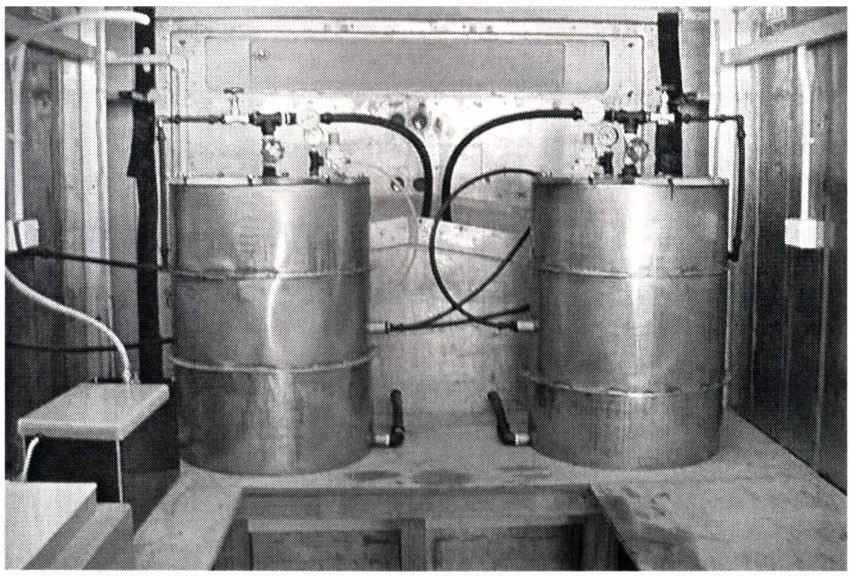

Figure 2.1a. Degassing vessels for removal of DO from water using a vacuum.

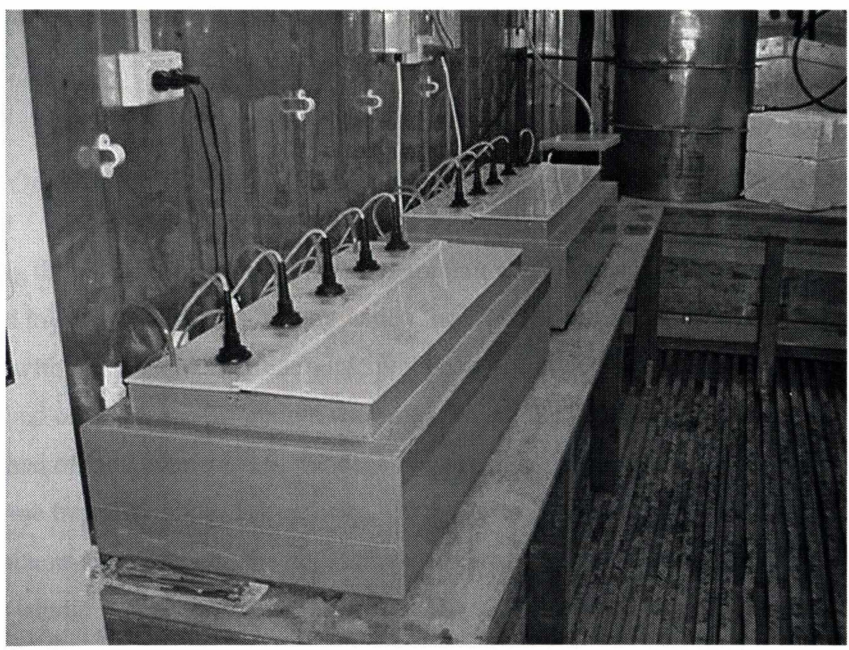

Figure 2.1b. Chambers used for the control of DO and exposure studies.

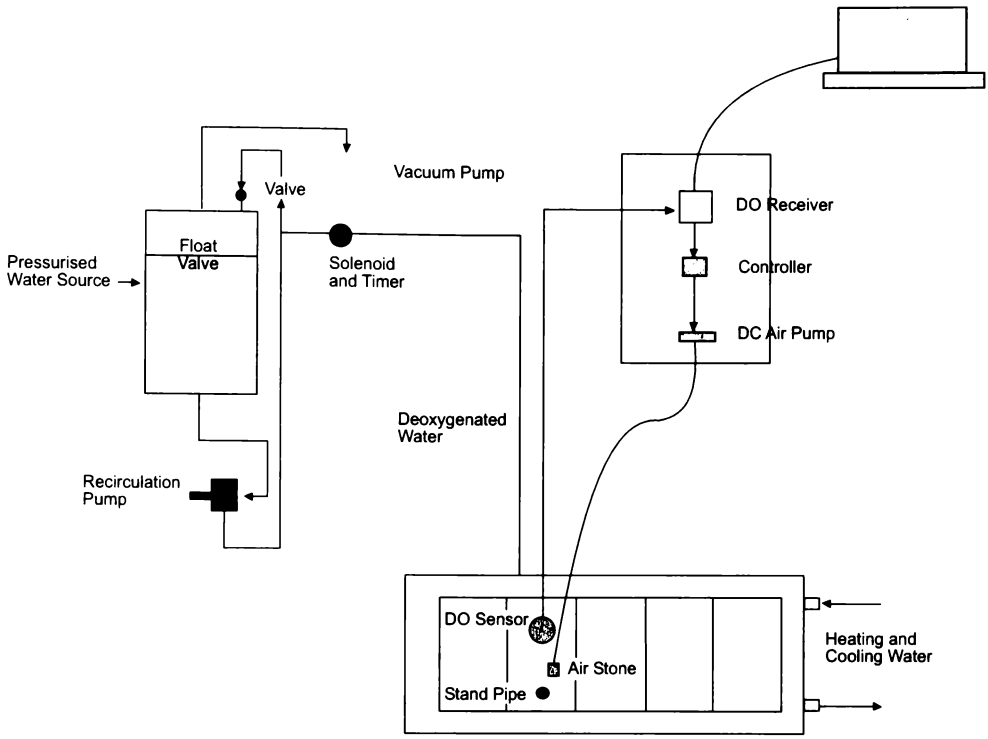

Figure 2.2. Schematic of the DO control system.

2.3.3 Operation of the dissolved oxygen control system

During the initial set-up phase, the vacuum pump was allowed to warm up with a closed inlet for 30 minutes, during which time the two degassing vessels were filled with fresh dechlorinated municipal tap water and the circulation pumps switched on. After the 30-minute warm-up period, the vacuum pump was switched off and connected to the degassing vessels. A model ZFD 145 (02) moisture trap (Rietschle, Schepfheim, Germany) was packed in ice and placed in the vacuum line, between the degassing vessels and vacuum pump, to condense water vapour before it reached the pump. The vacuum pump was then switched on again and the vessels were vacuumed down to -14 psi. During initial degassing the first globe valve in the water circulation line was completely opened to provide maximal flow and accelerated deoxygenation. To test the efficiency of the degassing chambers, 600 mL water samples were taken every 10 minutes for DO

measurement with a hand held model 55 DO meter (Yellow Springs Instrument Co. Ltd, Ohio, USA).

To test the DO control system, both degassing vessels and both exposure chambers were used in the absence of test organisms. Following initial set-up and degassing, the exposure chambers were slowly filled to ensure that any re-oxygenation in the chambers would be minimal. Each aquarium was filled with approximately 10 L of water and volume was set with standpipes. Because a large volume of water was removed from each degassing vessel, another 30 – 40 minutes of degassing was allowed to ensure complete deoxygenation of the replacement water. Prior to initiation of the experiment, sensors were calibrated and controllers tuned under test conditions.

One aquarium from each chamber was designated as a fully air saturated control (continuous aeration) and another was designated as essentially devoid of oxygen (no aeration). The remaining aquaria were set to 1.1, 1.5 and 2.3 $mg \cdot L^{-1}$ DO, respectively. Clear plastic barriers were constructed to place just under the water surface in each aquarium. Holes were cut in the barriers for the DO sensors, stand pipes and hoses. To allow for movement of water and air under the barriers, a 1.5 mm clearance was provided on all sides between the barrier and aquarium walls. The purpose of the barrier was primarily to prevent fish from being able to agitate the water surface during experimentation, thus minimising the amount of oxygen re-dissolving into the water. Degassed water was supplied to the exposure chambers on a timed basis. Timers were built to control small irrigation solenoid valves for the delivery of water to each chamber. Water was supplied in 30-second bursts at 4-minute intervals providing approximately 3.5 L per aquarium per hour. This corresponds to a 95 % replacement time of 10 hours. Data readings for DO were taken at 5-minute intervals. The system was allowed to operate over a 48-hour period without alteration.

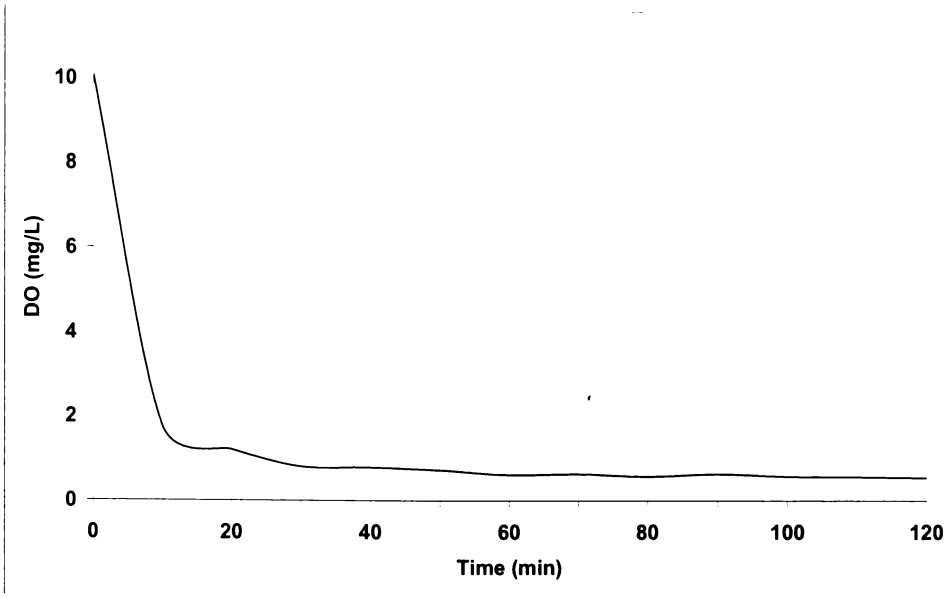

Figure 2.3a. Performance of the degassing vessel for DO removal over time.

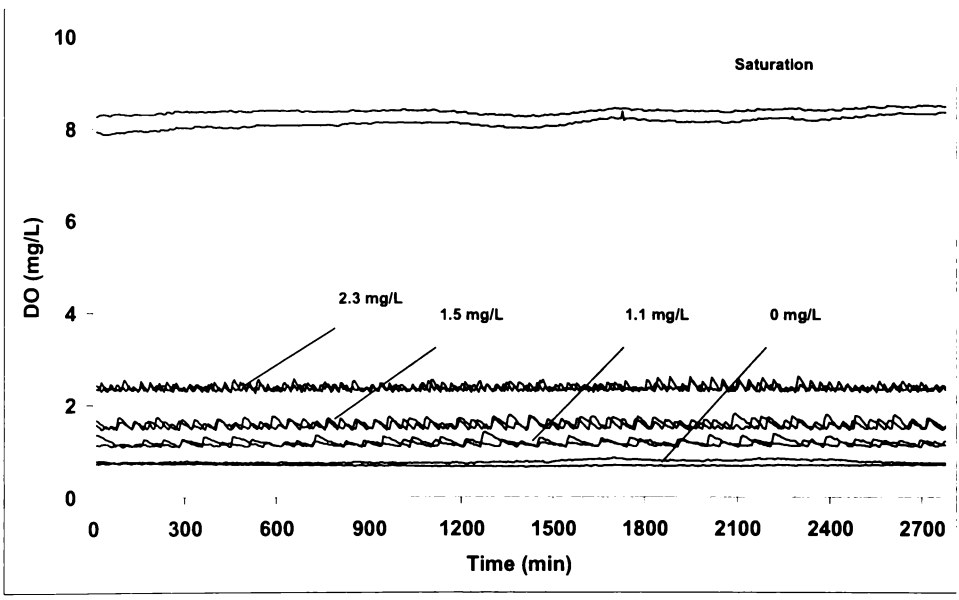

Figure 2.3b. Comparison of DO control with the two vessel and chamber sets over a 48 hour period. Nominal DO concentrations are indicated for each set of measured concentrations.

2.4 RESULTS AND DISCUSSION

2.4.1 Degassing efficiency

The performance of a single degassing vessel can be seen in Fig. 2.3a. Target vacuum pressure was achieved within the first 10 minutes of operation, during which time the majority (80 %) of DO removal had occurred. Dissolved oxygen was 10.07 $mg \cdot L^{-1}$ in the circulating water prior to application of the vacuum, and within 10 minutes of degassing, was reduced to 1.77 $mg \cdot L^{-1}$. After one hour of operation, DO had fallen to 0.62 $mg \cdot L^{-1}$ and finally to 0.55 $mg \cdot L^{-1}$ after 2 hours. These results formed the basis of operation and initial set-up times for later use of the degassing system.

Table 2.1. Comparisons of DO concentrations (mean ± SEM $mg \cdot L^{-1}$, n = 554) for the two water degassing vessels and oxygen control/exposure chamber sets at 17 °C and 300 m altitude.

Nominal Oxygen Concentration ($mg \cdot L^{-1}$)	Measured Oxygen Concentration ($mg \cdot L^{-1}$)	
	Control Set 1	Control Set 2
0	0.77 (0.001)	0.69 (0.001)
1.1	1.19 (0.003)	1.16 (0.002)
1.5	1.59 (0.004)	1.58 (0.003)
2.3	2.38 (0.002)	2.38 (0.003)
Saturation	8.15 (0.005)	8.40 (0.002)

2.4.2 Forty eight-hour dissolved oxygen control trial

The control system was tested without organisms using both degassing vessels and exposure chambers. Table 2.1 compares mean recorded DO concentrations over the 48-hour trial. Dissolved oxygen remained relatively constant at each set point and similar between the two test sets (Fig. 2.3b). Some variation is evident within the pairs of non-aerated and air-saturated chambers. This variation is not

considered to be a problem with regard to experimentation, as the lowest and highest DO groups will be used effectively as positive (expected effect) and negative (no expected effect) controls, respectively. At nominal concentrations of 1.1 $mg \cdot L^{-1}$ or greater, the control system operated with a precision range of around $\pm$ 0.01 - 0.1 $mg \cdot L^{-1}$, corresponding to a standard error no greater than 0.004 mg·
L^{-1}.

2.4.3 Proof of concept

The data presented in this paper demonstrates the precision control of DO at an aquarium scale. The system has been successfully tested with fish to determine its suitability for acute lethality testing. Since these initial tests, the system has been operational for a further nine months and continuously run for periods of up to six weeks in duration. While the system is essentially automated, daily maintenance is required to empty the moisture trap and replace the packed ice. Total daily maintenance does not exceed 30 minutes.

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CHAPTER THREE

ACUTE PULP AND PAPER EFFLUENT-HYPOXIA INTERACTIONS IN FISH

As accepted for publication.
Landman, van den Heuvel and Ling (2003), in the proceedings of the 5th International Conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents, Seattle, USA, June 2003, DEStech Publications

3.1 ABSTRACT

The Tarawera River receives effluent discharges from a chemithermomechanical (CTMP) tissue mill and an integrated thermomechanical (TMP)/bleached kraft pulp and paper mill (BK). The Tarawera River is also known to experience fluctuating oxygen concentrations, particularly over the warmer summer months. To investigate possible effluent-hypoxia toxicity interactions, effluents from both mills were tested separately. TMP/BK effluent was diluted to 15 % v/v to reflect the upper concentration in the receiving environment, while CMTP effluent was extracted directly from the river and not diluted further. Using an oxygen concentration series, parallel experiments were conducted with and without effluent to determine 48-h median lethal concentrations (LC50s) for dissolved oxygen (DO) in fry and juvenile rainbow trout (*Oncorhynchus mykiss*) and common bully (*Gobiomorphus cotidianus*). The presence of either pulp mill effluent did not significantly increase the toxicity of low DO for either species. Mean LC50 values ranged from 1.43 to 1.83 mg L^{-1} for trout and 0.69 to 0.99 mg L^{-1} for bullies. While life stage differences were not evident, differences between species are obvious with rainbow trout clearly more sensitive to hypoxic conditions. Stand-alone experiments with TMP/BKME showed no significant difference in the oxygen consumption of juvenile trout tested in 15 % v/v effluent, but significant increases in oxygen consumption were observed for fish pre-exposed to 15 and 70 % effluent when tested in reference water.

3.2 INTRODUCTION

The Tarawera River is considered a major water resource in the Bay of Plenty Region of New Zealand [1]. Two pulp and paper mills located at the township of Kawerau discharge effluents into the river approximately 20 – 30 km from the coast [1-3]. The Tarawera River bed is composed of pumice and moving sediment dunes, providing ideal habitat for oxygen consuming microbes that account for at least 90 % of the river deoxygenation that occurs [2-4]. It has been suggested by Dell et al. [3] that because of the high oxygen depletion, the river may have a reduced ability to assimilate effluents with a high biochemical oxygen demand (BOD). The predominant pulp and paper mill in Kawerau currently discharges approximately 175 ML d^{-1} of secondary-treated effluent directly to the river, with

a BOD of just over 5 T d^{-1} [5, 6]. Dissolved oxygen (DO) levels were reported below the minimum standard of 5 mg L^{-1} frequently during the 1990s [3, 4, 7], and river DO levels may also drop by a total of up to 5 mg L^{-1} in the 20 – 30 km stretch of river below the effluent discharge points [2]. More recent data continues to show depressed river DO, with monthly averages ranging between 4.2 and 6 mg L^{-1} for the year 2002 [5].

Increased toxicity in fish exposed to a variety of toxicants under hypoxic conditions has long been observed [8-10]. Increased pulp mill effluent toxicity under hypoxic conditions has also been demonstrated in fish [11-13]. Despite recent concerns regarding hypoxia downstream of pulp and paper mills internationally [14, 15] and in New Zealand [2, 3, 16], consideration for effluent-hypoxia interactions in fish have not received significant scientific scrutiny. Previous North American studies were performed with relatively untreated and toxic effluents and can no longer be extrapolated to present day effluents. Recently, evidence with mayflies [15] has suggested that increased cumulative effects (e.g. increased toxicity) of some pulp mill effluents under hypoxic conditions may no longer be biologically or statistically significant.

The primary aim of this study was to determine if environmentally relevant concentrations of two New Zealand pulp and paper mill effluents influenced acute DO lethality in two species of fish. This study also examines the effects of pre-exposure of fish to a thermomechanical/bleached kraft mill effluent (TMP/BKME) on acute DO lethality and routine oxygen consumption in trout.

3.3 MATERIALS AND METHODS

3.3.1 Mill descriptions

Effluents were collected from two mills; an integrated thermomechanical (TMP)/bleached kraft pulp and paper mill (BK) and a chemithermomechanical (CTMP) tissue mill. The furnish of both mills is softwood (*Pinus radiata*), with some pulping of eucalypt hardwood in the TMP/BK mill. Effluent from the TMP waste stream of the TMP/BK mill is pre-treated in a moving bed bioreactor prior

to being combined with the remaining effluent streams within the mill. Combined effluent is settled in a primary treatment pond, followed by secondary treatment in an aerated oxidation lagoon system for 4 – 5 days prior to discharge into the Tarawera River. CTMP effluent, along with Kawerau municipal wastewater, is treated in an anaerobic system and discharged into rapid infiltration basins (RIBs) adjacent to the river.

3.3.2 Animals

Rainbow trout (*Oncorhychus mykiss*) in this study were hatched in the laboratory using fertilised eggs obtained from the Department of Conservation Turangi Trout Centre (Turangi, New Zealand). Trout were housed under standard laboratory conditions, supplied with activated-carbon-dechlorinated Rotorua City tap water at 14 °C with a 12:12 light:dark photoperiod, and fed daily with a commercially available salmon feed (Reliance StockFoods, Dunedin, New Zealand). Common bullies (*Gobiomorphus cotidianus*) were captured from Lake Tarawera by seine net and transported back to the laboratory. Bullies were housed in 80 L glass aquaria (approximately 100 fish per aquarium) at 16 °C with 50 % water replacement daily, and fed freshly hatched *Artemia* (San Francisco Bay Brand, Newark, USA) every second day. To minimise the risk and occurrence of disease, untreated sea salt was added to all bully aquaria (0.25 % w/v NaCl; Dominion Salt, Mt. Maunganui, New Zealand). Approximate age of bullies was determined from weight and length measurements [17].

3.3.3 Experiments

3.3.3.1 Dissolved oxygen lethality

Acute lethality bioassays were performed with 4 – 6 week-old trout fry (0.14 - 0.58 g; 2.39 - 3.65 cm) and 2 – 3 month-old parr (0.95 - 1.60 g; 4.68 - 5.35 cm), and with 1 – 2 month-old bully fry (0.08 - 0.14 g; 2.14 - 2.48 cm) and 3 – 4 month-old juveniles (0.21 - 0.31 g; 2.58 - 2.96 cm). Using a vacuum degassing and DO control system [18] (Chapter 2) fish were exposed in flow-through plastic aquaria to a series of five DO concentrations. Nominal DO concentrations used

were <0.8, 1.2, 1.7, 2.3 mg L^{-1} and saturation for trout and <0.8, 0.8, 1.3, 1.6 mg L^{-1} and saturation for bullies. Nominal oxygen concentrations were selected based on preliminary testing to establish reasonable sensitivity ranges for DO. Ten fish per oxygen concentration were exposed at 15 °C for 48 h, and mortalities recorded periodically (0, 0.75, 1.5, 3, 6, 12, 24, 48 h) to determine the 48-h median lethal concentration (LC50) for DO.

Effluent and reference water experiments were conducted simultaneously: five DO concentrations with effluent and five without effluent. All tests were replicated three times. TMP/BK mill effluent (ME) was collected at the mill outflow and transported back to the laboratory where it was diluted to 15 % v/v with dechlorinated tap water to reflect the upper concentration in the receiving environment. Reference river water was also collected and diluted to 15 % v/v with tap water for reference water exposures. Diluted CTMP effluent and reference river water were taken directly from the river and transported back to the laboratory in 10,000 L tanker trucks. CTMP effluent and reference river water were not diluted. For the purpose of this study, water presumed to contain CTMP effluent leachate from the RIBs is considered diluted CTMP effluent. The CTMP effluent collection point on the river was chosen in consultation with mill staff at a site below the RIBs but above the discharge area of the TMP/BK mill. Reference river water was collected upstream from both mills.

Acute lethality bioassays were repeated with juvenile trout (3.03 - 3.50 g; 5.76 - 6.18 cm) that had been pre-exposed to 15 and 70 % v/v TMP/BKME for two weeks. Fish were exposed in 80 L glass aquaria (150 fish per aquarium) at 15 °C on a 12:12 light:dark photoperiod and supplied with constant aeration. Water replacement was conducted daily (60 %) and a maintenance feed ration of approximately 0.5 – 1 % of wet body weight per day was provided. Lethality tests were conducted in fresh dechlorinated tap water. Median lethal concentrations for DO were calculated using the Spearman-Karber method for estimation of LC50 values [19].

3.3.3.2 Respiromtery

Static respirometers (2.25 L) were constructed for the purpose of determining resting oxygen consumption in juvenile trout. The respirometers were based on a previous design [20] and allowed for operation in open and closed modes. The respirometer was composed of a main cylindrical chamber constructed from PVC, connected by PVC tubing to a flow cell with a COS 4 DO sensor connected to a Liquisys M COM 223 meter (Endress Hauser, Weil am Rhein, Germany). The output from the DO meter was transferred to the parallel port of a desktop PC via an ADC-11 adapter (Pico Technology, St. Neots, UK). Data were recorded using PicoLog 5.06.3 for Windows (Pico Technology, St. Neots, UK). A small dual-use Seltz L20i submersible/external aquarium pump (Hydor, Bassano, Italy) was used to circulate water through the respirometer during closed operation and to flush the respirometer when in open mode. Each respirometer was completely submersed in an 80 L glass aquarium filled with fresh dechlorinated tap water supplied with constant aeration. Water temperature was controlled by maintaining a constant ambient air temperature of 15 °C.

Juvenile trout (8.5 - 21.6 g; 9.5 - 13.9 cm) were pre-exposed to fresh/reference water (control) and 15 % TMP/BKME in 80 L glass aquaria (20 fish per aquarium) for two weeks at 15 °C with a 12:12 light:dark photoperiod, supplied with constant aeration, 60 % daily water replacement, and a maintenance feed ration of approximately 1 % of wet body weight per day. Test conditions corresponded to pre-exposure conditions, so that fish pre-exposed in fresh water were tested in fresh water, and fish pre-exposed to 15 % effluent were tested in 15 % effluent. Each test was initiated by transferring a single fish (10 per treatment) to the respirometer the day before actual testing; allowing it to recover for 18 h overnight under ambient test conditions with the respirometer flushing aerated "test-water" through the apparatus. The following day, four repeat 1 h oxygen consumption readings were taken by closing the respirometer and measuring the decrease in DO. The respirometer was opened and flushed for 20 min between each 1-h reading period.

An additional experiment was performed using a separate batch of juvenile trout (10.2 - 15.6 g; 9.5 - 10.9 cm). Oxygen consumption was determined for fish tested

in fresh/reference water only (5 fish per treatment group), following the two-week pre-exposure to reference water (control), 15 and 70 % TMP/BKME.

Oxygen consumption was calculated from DO concentration, respirometer volume and time: $VO2 = [\Delta CO2 \times V] \div [T \times Wt]$, where VO2 = oxygen consumption (mg O2 g^{-1} h^{-1}), $\Delta CO2$ = change in oxygen concentration of water (mg L^{-1}), V = volume of the respirometer (L), T = duration of measurement (h) and Wt = mass of fish tested (g).

3.3.4 Water chemistry

Samples were taken at the time of effluent collection for the determination of organic components. A sample of 100 % TMP/BKME was filtered through 15-cm GF/C filters. The filtrate and filter paper were subsequently stored at -20 °C prior to analysis. Analysis was performed as per previous methods [16]. River water from the CTMP experiment (10 L) was extracted using solid phase extraction on SPEC 47 mm C18AR extraction disks (Varian Inc., Lake Forest, USA) due to the lower level of extractives expected.

3.3.5 Statistics

Acute lethality data were subjected to a paired T-test to determine significant differences between LC50 values for acute lethality treatments. Oxygen consumption data were subjected to an analysis of variance with Dunnett's post-hoc tests (ANOVA; $\alpha < 0.05$). All statistical analyses were performed using SYSTAT 10 (SPSS, Chicago, IL, USA) [21] for Windows. Values are presented as means ± SEM.

Table 3.1. Mean (± SEM; μg L^{-1}) organic extractives in 15 % v/v TMP/BKME (measured in 100 % samples and multiplied by 0.15). Due to small sample sizes (n = 2), CTMP effluent extracted from the Tarawera River and reference river water are presented as value ranges (μg L^{-1}).

Compound	River Water	TMP/BKME	CTMP
Fichtelite	0.13 – 0.39	1.17 (0.42)	n.d. – 1.33
Dehydroabietin	n.d.	0.02 (0.01)	n.d.
Tetrahydroretene	n.d.	0.12 (0.07)	n.d.
Retene	n.d.	0.19 (0.07)	n.d.
Methyldehydroabietin	n.d.	0.01 (0.01)	n.d.
Total Resin Acid Neutrals	**0.13 – 0.39**	**1.52 (0.48)**	**n.d. – 1.33**
Pimaric acid	2.34 – 4.40	13.62 (3.41)	24.01 – 29.19
Sandaracopimaric acid	0.14 – 0.24	1.49 (0.37)	1.41 – 1.44
Isopimaric acid	0.74 – 1.47	7.09 (1.96)	6.41 – 6.47
Palustric acid	n.d.	1.23 (0.88)	n.d.
Levopimaric Acid	n.d.	n.d.	n.d.
Dehydroabietic acid	1.25 – 1.92	16.31 (4.66)	5.10 – 6.77
Abietic acid	0.90 – 1.52	53.74 (16.36)	2.71 – 5.76
Neoabietic acid	n.d.	0.65 (0.51)	0.21
Pimarenic acid	n.d.	1.13 (0.36)	n.d.
Sandaracopimarenic acid	n.d.	2.17 (0.65)	n.d.
Isopimarenic acid	n.d.	2.00 (0.92)	n.d.
13-Abietenic acid	n.d. – 0.06	9.11 (2.86)	0.09 – 0.13
Pimaranic acid	n.d.	1.58 (0.49)	n.d.
Isopimaranic acid	n.d.	0.39 (0.13)	n.d.
Abietanic acid	n.d.	5.51 (1.74)	n.d.
Seco-1-dehydroabietic acid	1.25 – 1.45	3.94 (1.13)	3.71 – 10.61
Seco-2-dehydroabietic acid	0.95 – 0.99	2.10 (0.51)	2.36 – 6.19
12-Chlorodehydroabietic acid	n.d. 0.04	0.22 (0.07)	0.04 – 0.93
14-Chlorodehydroabietic acid	0.05 – 0.18	0.79 (0.22)	0.12 – 5.84
12,14-Dichlorodehydroabietic	n.d.	0.08 (0.04)	n.d.
7-Oxodehydroabietic acid	0.13 – 0.29	0.19 (0.07)	0.37 – 1.00
Total Resin Acids	**8.84 – 11.45**	**123.33 (33.22)**	**46.40 – 74.47**
Cholesterol	0.66 – 0.85	2.76 (0.51)	0.73 – 0.95
Campesterol	0.05 – 0.20	0.27 (0.07)	n.d. – 0.08
Stigmasterol	0.42 – 0.56	0.31 (0.12)	n.d. – 1.65
Sitosterol	0.58 – 1.13	5.63 (1.32)	0.47 – 1.22
Sitostanol	n.d. – 0.15	1.80 (0.44)	n.d. – 0.2
Total Phytosterols	**2.19 – 2.42**	**10.76 (2.04)**	**1.21 – 4.09**

Note. n = 7 for TMP/BKME samples and n = 2 for CTMP effluent and river water samples. n.d., not detected, method detection limit is 0.01μg L^{-1}.

3.4 RESULTS

3.4.1 Water chemistry

Extractives measurement from both effluents is shown in Table 1. Chemistry for TMP/BKME effluent was determined for 100 % effluent samples and shown as 15 % in Table 1 to reflect the relevant exposure concentrations. Analysis of the TMP/BKME appears to be characteristic of a secondary-treated pulp mill effluent, shown by the predominating resin acids. At present, little is known about neither the relative proportion of sewage to CTMP effluent, nor the environmental fate of the combined effluents in question following their discharge into the river-side rapid infiltration basins (RIBs). This study is the first known attempt to extract this particular effluent directly from the river and to use it in biological testing. Analysis of the CTMP effluent shows increased concentrations of resin acids compared to reference river water collected further upstream. The presence of these resin acids makes it likely that at least some pulp and paper effluent is leaching to the river below the where effluent is discharged into the RIBs. However, the full extent of leaching process is unknown.

3.4.2 Dissolved oxygen lethality

Effluent did not influence the median lethal concentration for dissolved oxygen (DO LC50) of rainbow trout and common bullies (Figs. 3.1 and 3.2). Dissolved oxygen LC50s ranged from approximately 1.3 to 1.8 mg L^{-1} in trout and 0.7 to 1.0 mg L^{-1} in bullies. Life stage (size) did not influence oxygen sensitivity, but trout were significantly more sensitive ($p < 0.001$) to hypoxia than bullies. The only other point of significant difference was for the case of juvenile trout exposed to CTMP effluent. CTMP effluent exposure significantly reduced the sensitivity ($p = 0.026$) of juvenile trout to hypoxia. Pre-exposure of juvenile trout to 15 and 70 % TMP/BKME had no significant effect on the DO LC50; with mean DO LC50 values of 1.62 and 1.56 mg L^{-1}, respectively (Fig. 3.3).

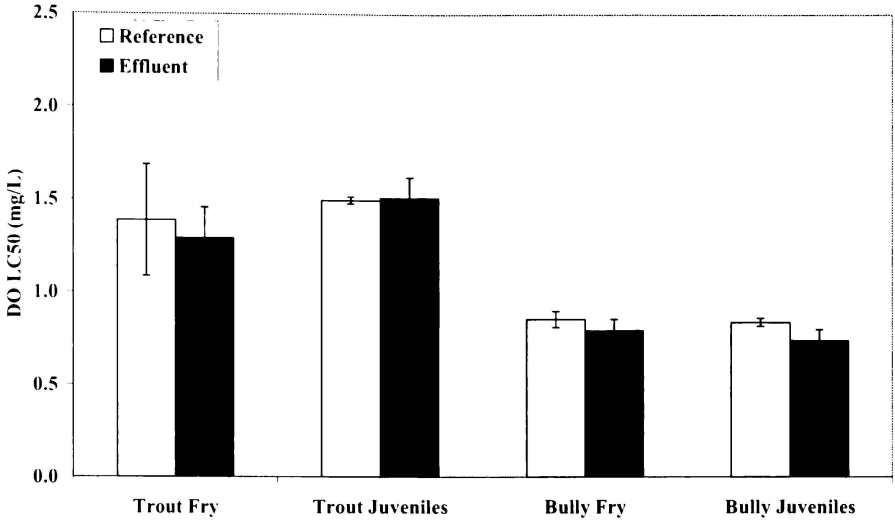

Figure 3.1. Comparison of DO LC50 values (mean ± SEM mg L^{-1}) for TMP/BK effluent.

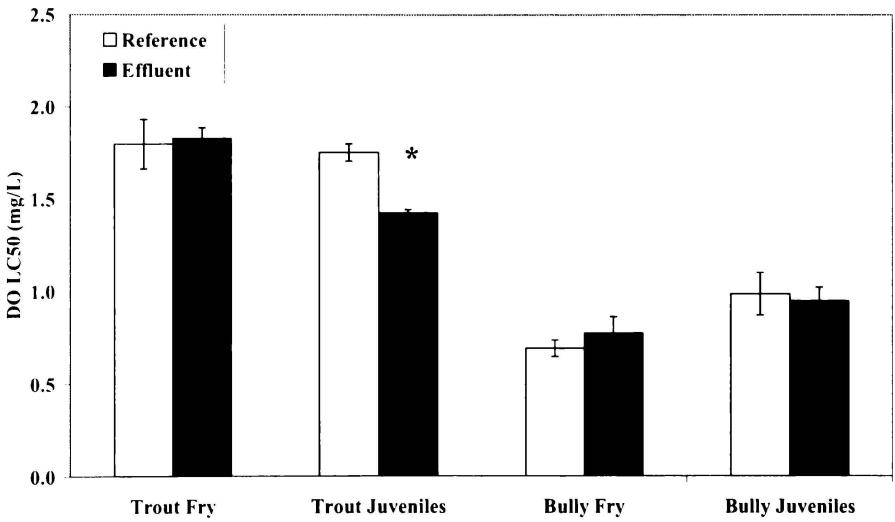

Figure 3.2. Comparison of DO LC50 values (mean ± SEM mg L^{-1}) for CTMP effluent. * = $P< 0.05$

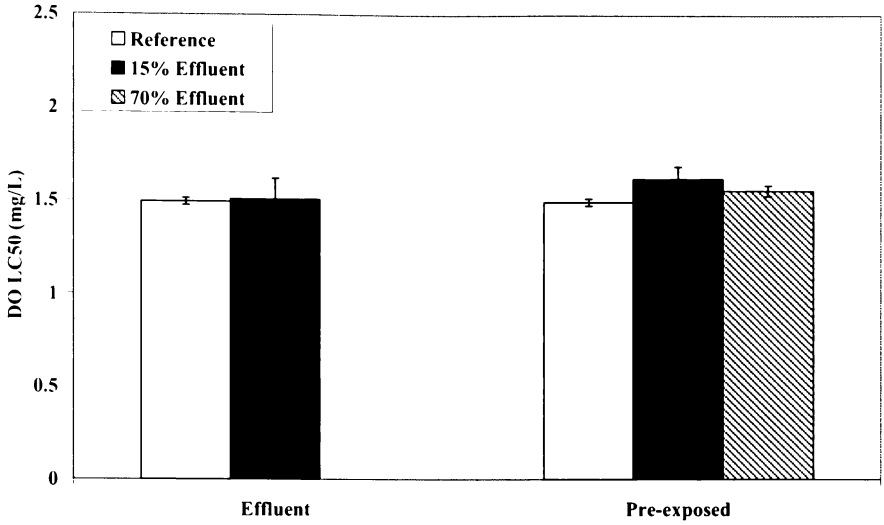

Figure 3.3. Effect of TMP/BKME on DO LC50s (mean ± SEM mg L^{-1}) in juvenile rainbow trout. Bars marked 'effluent' were taken directly from the initial DO lethality tests. Bars marked 'pre-exposed' compare reference fish with those pre-exposed to 15 and 70 % TMP/BKME for 2 weeks before LC50 tests were performed with fresh water.

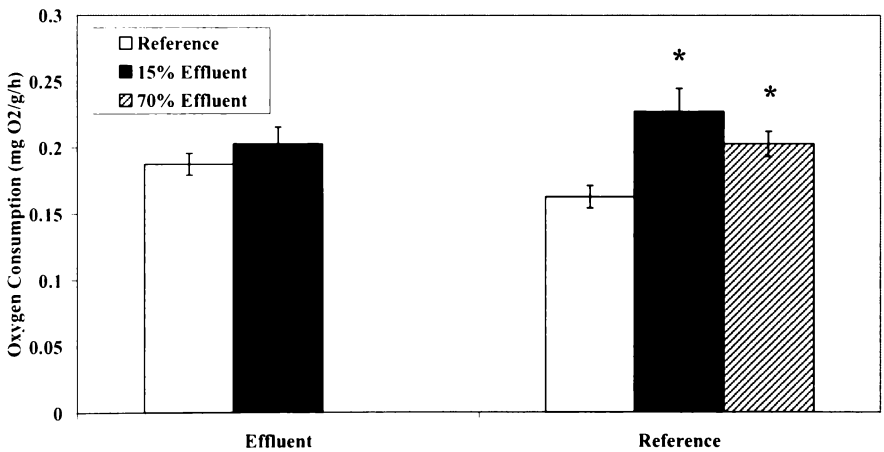

Figure 3.4. Mean (± SEM) routine oxygen consumption (mg O_2 g^{-1} h^{-1}) of juvenile rainbow trout. Bars marked 'effluent' compare reference fish to those pre-exposed and tested in 15 % TMP/BKME. Bars marked 'reference' compare reference fish to those pre-exposed in 15 and 70 % TMP/BKME but tested in reference water. * = $P < 0.05$

3.4.3 Respirometry

The oxygen consumption of juvenile trout pre-exposed to and tested in 15 % TMP/BKME was not different from controls (Fig. 3.4). However, fish tested in fresh water only following pre-exposure to both 15 and 70 % TMP/BKME for two weeks consumed significantly more ($p < 0.001$ and $p = 0.004$, respectively) oxygen than the controls (Fig. 3.4).

3.5 DISCUSSION

The results from the DO lethality experiments showed that the pulp and paper effluents present in the Tarawera River do not enhance the acute sensitivity of trout or bullies to hypoxia at environmentally relevant concentrations. This is consistent with a recent mayfly study [15] demonstrating a lack of effluent-hypoxia effects with another modern pulp mill effluent.

It is not surprising that trout were the more sensitive of the two species. Salmonids are generally regarded to be one of the most sensitive groups of fish to hypoxic conditions and bullies are known to be quite tolerant of hypoxia [22]. It should be noted that trout in these experiments appeared relatively tolerant (48-h LC50s < 2 mg L^{-1} DO) considering that they are regarded as a "sensitive" species. However, while DO concentrations below 4 – 4.5 mg L^{-1} may be limiting to growth, juvenile coho (*Oncorhynchus kisutch*) and sockeye (*Oncorhynchus nerka*) salmon have been shown to tolerate levels as low as 2 and 3 mg L^{-1} for at least two weeks [20]. The United States Environmental Protection Agency [23] guidelines also suggest that mortalities should not be observed in salmonids at DO concentrations above 3 mg L^{-1}. In the case of the present study, where trout were resting, mortalities were not observed at concentrations above 2.2 mg L^{-1} in trout and 1.3 mg L^{-1} in bullies.

Oxygen consumption rates of juvenile trout in this study are consistent with previously reported values for rainbow trout under routine conditions [24, 25] and during exposure to BKMEs [26]. Some limited effects of BKME exposure on routine oxygen consumption in rainbow trout have been reported, such as increased cough frequency [27], increased ventilation rate [28] and marginal

increases in oxygen consumption [26]. Recent findings have demonstrated numerous effects of dehydroabietic (DHAA) and isopimaric (IPA) resin acids on rainbow trout hepatocytes [29-31]. Rissanen et al. [31] suggested that DHAA, in particular, results in cellular ATP depletion and activation of glycolysis in hepatocytes, thereby increasing the oxygen requirement for ATP replenishment. Our results appear somewhat contradictory in the sense that trout exposed to TMP/BKME did not appear to consume more oxygen when exposed to and tested in effluent, only when tested in reference water following effluent exposure. However, it has been speculated that while increased ventilation rates may increase the uptake of DHAA, it may likewise increase the active clearance of DHAA at the gills [32]. The fact that oxygen consumption increased in effluent pre-exposed trout only once they were transferred to fresh water in the present study supports the notion of a toxicant clearance or recovery process. The potential for effects such as cellular ATP depletion; resulting in a greater oxygen demand, cannot be ruled out here.

3.6 SUMMARY AND CONCLUSIONS

Results from this study showed that if DO levels remain above the current regulatory limit of 5 mg L^{-1}, the presence of pulp mill effluent does not likely constitute a serious problem for fishes in the Tarawera River with regard to cumulative effluent-hypoxia effects based on the acute and chronic endpoints assessed herein. However, the observed changes in trout oxygen consumption cautions that while acute or severe effects are not observed, sub-lethal effects of modern TMP/BKMEs on fish energetics may still be of some interest and concern.

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CHAPTER FOUR

CHRONIC PULP AND PAPER EFFLUENT-HYPOXIA INTERACTIONS IN FISH

4.1 ABSTRACT

The effects of chronic exposure to simultaneous pulp and paper effluent and hypoxia were examined with rainbow trout (*Oncorhynchus mykiss*). Effluent from a thermomechanical (TMP)/bleached kraft (BK) pulp and paper mill and a chemithermomechanical (CTMP) tissue mill were studied. Fish were exposed to a series of dissolved oxygen (DO) concentrations (2.5, 3, 4, 5 mg L^{-1} and saturation) in side-by-side effluent and reference water experiments for four weeks. At the conclusion of the experiments, fish growth and survival, critical swimming speed (Ucrit) and a suite of hematological parameters were measured. This study showed poor survival of fish exposed to reference water conditions during both effluent experiments. However, survival was excellent for effluent-exposed fish (100 % survival at all DO concentrations in TMP/BK mill effluent (ME); 67 % survival at 4 mg L^{-1} DO and 100 % for the remainder in CTMP effluent). Relationships for growth were observed for fish exposed to DO and effluent in the CTMP experiment. Several DO dose-response effects were also observed on general hematology in both effluent experiments, but were generally considered minor and typical of hypoxia exposure in fish. There was also some evidence suggesting the presence of these effluents was having effects on the swimming performance of fish, as was shown by very low Ucrit values for fish exposed to TMP/BKME. DO and effluent effects on Ucrit of CTMP effluent and reference water-exposed fish were also seen. However, all observed effects were deemed to be minor and the main finding of this study was that rainbow trout were capable of surviving combined effluent-hypoxia exposure for four weeks, for both effluents examined.

4.2 INTRODUCTION

Large bodies of literature exist on both the effects of hypoxia and pulp and paper effluent exposure in fish. The effects of chronic hypoxia exposure have been well described for numerous fish species. Common effects include lack of spawning, reduced fecundity, reduced growth and survival, increased stress hormone levels and modified behaviour [1-6]. Wide-ranging effects of pulp and paper effluent have been reported in fish, such as reduced steroid levels, decreased gonad size,

reduced/increased growth, reduced stress responsiveness, and mixed function oxygenase induction [7-11] to mention just a few.

In the previous chapter [12] (Chapter 3), acute effects of simultaneous effluent and hypoxia exposure were examined where it was demonstrated that the presence of two pulp and paper mill effluents did not influence the sensitivity of rainbow trout (*Oncorhynchus mykiss*) and the common bully (*Gobiomorphus cotidianus*) to hypoxia. However, increased oxygen consumption of trout was demonstrated following chronic effluent exposure. The purpose of the current study was to examine the chronic effects of combined effluent-hypoxia exposure using the same two pulp and paper effluents studied previously.

The aim of this study was to examine the chronic effects of simultaneous effluent and hypoxia exposure primarily on the growth and survival of juvenile rainbow trout. This study also provided an opportunity to examine more general hematological effects and make the first attempt to assess the swimming performance of fish exposed to these specific effluents.

4.3 MATERIALS AND METHODS

4.3.1 Fish

Laboratory-hatched and reared juvenile rainbow trout (*Oncorhynchus mykiss*; 6.89 ± 0.04 cm, 3.49 ± 0.06 g) were housed in well-aerated, 400 L indoor tanks in dechlorinated Rotorua city tap water at 11 – 13 °C under a 12:12 photoperiod and fed daily with a commercial salmon feed (Reliance Stock Foods, Dunedin, New Zealand).

4.3.2 Mills and effluents

Two pulp and paper mill effluents were used in this study. The first from an integrated thermomechanical (TMP)/bleached kraft pulp and paper mill (BK) and the second from a chemithermomechanical (CTMP) tissue mill. These mills are

discussed in detail in Chapters 1 and 3. Effluents were collected in the same manner as described in Chapter 3.

4.3.3 Exposures

Using a vacuum degassing and DO control system [13] (Chapter 2), fish were tested in 15 L flow-through plastic aquaria to two series of five nominal DO concentrations (2.5, 3, 4, 5 mg L^{-1} and fully saturated), one DO series with effluent and the other without (reference). The first experiment investigated the effects of TMP/BK mill effluent (ME) and hypoxia, where 20 fish were exposed to each oxygen concentration at 20 °C for four weeks. Fish were exposed to TMP/BKME diluted to 15 % v/v with dechlorinated tap water, reflecting the upper concentration in the receiving environment, and reference river water diluted to 15 % v/v with tap water. The second experiment examined the effects of CTMP effluent and hypoxia, where 12 fish were exposed to each oxygen concentration at 18 °C for four weeks. The lower temperature of 18 °C was used in attempt to mitigate problems encountered during the first study at 20 °C. Diluted CTMP effluent and reference river water were taken directly from the river (as per Chapter 3) and collected on a weekly basis. CTMP effluent and river water were not diluted.

All fish were fed an approximate feed ration of 1 % wet body weight per day. Where appropriate, mortalities were recorded and bodies removed.

4.3.4 Swimming performance

Critical swimming speed (Ucrit) was determined using a custom-built, 230 L, recirculating flume (University of Waikato, Hamilton, New Zealand). Ucrit determinations were based on the standardised protocol described by Brett [14] involving an incremental swimming test. All Ucrit determinations were made by testing fish in reference water. Approximately 10 % water replacements (20 – 30 L) were made daily. For each exposure group, five fish were netted from their tanks and fork length was measured before transfer to the flume. Flume speeds were calculated using the average length of the five fish. Fish were allowed to acclimate in the flume for 2 h at a routine swimming speed of 0.5 BL s^{-1} (body

lengths per second) at 18 °C. Following acclimation, water velocity was increased in increments of 0.5 BL s^{-1} every 15 min until exhaustion. Exhaustion was assumed when all five fish were no longer able to maintain their position in the water flow and were confined against the rear grill of the flume. Fish were then removed, weighed and measured.

Ucrit was calculated using the formula: Ucrit = Ui + [Uii x Ti/Tii], where Ucrit = critical swimming speed (BL s^{-1}), Ui = highest swimming velocity reached by fish that was maintained for the full increment duration (BL s^{-1}), Uii = incremental velocity increase (0.5 BL), Ti = duration swum by fish at the highest speed reached (min) and Tii = increment time (15 min).

4.3.5 Blood sampling and analysis

After the four-week exposure period, blood samples were taken from five fish in each exposure group. Fish were individually netted from tanks and stunned by a blow to the head. Blood samples were subsequently taken before sacrifice by a further blow to the head. Approximately 50 µL of blood was taken via caudal venepuncture into pre-heparinized syringes (400 i.u. mL^{-1}) and placed on ice. Samples were immediately analysed for haematocrit (Hct), haemoglobin (Hb), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV) and differential white blood cell counts according to standard methods [15]. Plasma samples were stored at -20 °C for later analysis of glucose, lactate and triglycerides using Sigma kits (Sigma-Aldrich Pty. Ltd., Sydney, Australia).

4.3.6 Water chemistry

Water and effluent sub-samples were taken at the time of collection for the determination of organic components. A sample of 100 % TMP/BKME was filtered through 15-cm GF/C filters. The filtrate and filter paper were subsequently stored at -20 °C prior to analysis. Analysis was performed as per previous methods [16, 17]. River water from the CTMP experiment (10 L) was

extracted using solid phase extraction on SPEC 47 mm C18AR extraction disks (Varian Inc., Lake Forest, USA) due to the lower level of extractives expected.

4.3.7 Statistics

All statistical analyses were performed using SYSTAT 10 (SPSS, Chicago, IL, USA) for Windows. Because white cell counts were measured as proportions of various cell types, data were arcsine transformed [18] prior to ANOVA testing. Initial statistical analyses were performed for whole data sets from the combined DO/effluent exposures (both effluent studies). All data were subjected to one-way analyses of variance (ANOVA; $\alpha < 0.05$) with Dunnett's post-hoc tests to determine the effect of DO on measured parameters.

All data (DO/effluent and DO/reference exposures) for both effluent studies were then subjected to two-way ANOVA tests (general linear model; $\alpha < 0.05$) using DO, treatment (reference/effluent) and DO x treatment as independent factors. All values are presented as means ± SEM.

Due to their small size, fish could not be tagged and individually identified. Growth was determined as an absolute value by subtracting the average initial weight of all fish in each treatment group, from final weights of each individual fish. Growth data was then subjected to the same statistical analyses as for all other data.

Table 4.1. Mean (± SEM) organic extractives in 15 % v/v TMP/BKME (measured in 100 % samples and multiplied by 0.15) and CTMP effluent (μg L^{-1}).

Compound	TMP/BKME	CTMP
Fichtelite	1.23 (0.38)	n.d.
Dehydroabietin	0.05 (0.02)	n.d.
Tetrahydroretene	0.07 (0.04)	n.d.
Retene	0.27 (0.11)	0.01 (0.00)
Methyldehydroabietin	n.d.	n.d.
Total Resin Acid Neutrals	**1.63 (0.49)**	**0.00 (0.00)**
Pimaric acid	13.84 (1.76)	0.03 (0.00)
Sandaracopimaric acid	1.62 (0.19)	n.d.
Isopimaric acid	6.50 (0.78)	0.01 (0.00)
Palustric acid	0.13 (0.10)	n.d.
Levopimaric Acid	n.d.	n.d.
Dehydroabietic acid	17.64 (3.17)	0.07 (0.01)
Abietic acid	66.49 (7.46)	n.d.
Neoabietic acid	0.08 (0.03)	n.d.
Pimarenic acid	1.42 (0.17)	n.d.
Sandaracopimarenic acid	2.82 (0.26)	n.d.
Isopimarenic acid	1.93 (0.88)	n.d.
13-Abietenic acid	10.58 (2.11)	0.05 (0.00)
Pimaranic acid	2.05 (0.26)	n.d.
Isopimaranic acid	0.50 (0.08)	n.d.
Abietanic acid	6.41 (0.80)	0.03 (0.00)
Seco-1-dehydroabietic acid	6.09 (0.71)	0.08 (0.03)
Seco-2-dehydroabietic acid	2.40 (0.48)	0.07 (0.02)
12-Chlorodehydroabietic acid	0.24 (0.05)	n.d.
14-Chlorodehydroabietic acid	0.99 (0.12)	n.d.
12,14-Dichlorodehydroabietic	0.22 (0.03)	n.d.
7-Oxodehydroabietic acid	0.30 (0.06)	n.d.
Total Resin Acids	**142.25 (18.09)**	**0.24 (0.06)**
Cholesterol	3.09 (0.77)	1.53 (0.30)
Campesterol	0.42 (0.07)	0.32 (0.13)
Stigmasterol	0.15 (0.05)	0.09 (0.04)
Sitosterol	7.76 (1.31)	0.72 (0.07)
Sitostanol	2.12 (0.27)	0.05 (0.01)
Total Phytosterols	**13.53 (2.35)**	**2.63 (0.46)**

Note. $n = 5$ for TMP/BKME samples and $n = 4$ for CTMP effluent and river water samples. n.d., not detected, method detection limit is 0.01 μg L^{-1}.

4.4 RESULTS

4.4.1 Water chemistry

The TMP/BKME organic components measured in this study (Table 4.1) are characteristic of a modern secondary-treated pulp mill effluent, and are consistent with previous findings (Chapter 3; Table 3.1). However, sample analysis of river water collected downstream of the CTMP mill (CTMP effluent) was not typical, as shown by the lack of resin acid neutrals and resin acids. This contrasts with previous findings, where river water presumed to contain CTMP leachate, possessed elevated resin acid concentrations (Chapter 3; Table 3.1). However, CTMP effluent in this study was shown to have relatively high concentrations of fatty acids and a comparison with historical TMP/BKME samples (2001 and 2002 samples) obtained from Forest Research (Rotorua, New Zealand) is shown in Table 4.2.

Table 4.2. Mean (± SEM) fatty acid extractives in 15 % v/v TMP/BKME (measured in 100 % samples and multiplied by 0.15) and CTMP effluent (μg L^{-1}).

Compound	TMP/BKME	CTMP
Decanoic acid	n.d.	0.04 (0.01)
Dodecanoic acid	0.12 (0.2)	0.06 (0.01)
Tetradecanoic acid	0.35 (0.08)	0.12 (0.01)
Palmitoleic acid	8.95 (2.22)	0.32 (0.07)
Palmitic acid	3.61 (1.01)	2.04 (1.09)
Margaric acid	0.17 (0.03)	0.05 (0.01)
Linoleic acid	1.31 (0.47)	0.97 (0.63)
Oleic acid	2.17 (0.71)	1.26 (0.59)
Linolenic acid	0.93 (0.35)	21.31 (13.42)
Elaidic acid	3.15 (1.35)	0.20 (0.04)
Stearic acid	0.61 (0.11)	0.34 (0.07)
Eicosanoic acid	2.78 (0.68)	0.08 (0.00)
Docosanoic acid	1.06 (0.29)	0.22 (0.07)
Tetracosanoic acid	n.d.	0.30 (0.03)
Total Fatty Acids	**16.88 (4.47)**	**26.84 (15.82)**

Note. $n = 24$ for TMP/BKME samples and $n = 4$ for CTMP effluent and river water samples. n.d., not detected, method detection limit is 0.01μg L^{-1}.

Table 4.3. All data (mean ± SEM) from fish exposed to 15 % v/v TMP/BKME (effluent exposure) and various DO concentrations at 20.29 ± 0.02 °C for four weeks. *N = 20* for growth and survival and *n* = 5 for all other data. * = P < 0.05 (post-hoc).

Nominal DO concentration	Saturation	5 mg/L	4 mg/L	3 mg/L	2.5 mg/L
Growth (g)	-0.13 ± 0.27	-0.06 ± 0.26	-0.11 ± 0.25	-0.19 ± 0.25	-0.15 ± 0.23
Survival (%)	100	100	100	100	100
Ucrit (BL/s)	2.89 ± 0.47	3.02 ± 0.17	3.19 ± 0.15	2.91 ± 0.20	2.62 ± 0.19
Hct (%)	26.9 ± 2.1	32.9 ± 0.7	30.2 ± 2.3	29.8 ± 2.9	25.6 ± 2.8
Hb (g/L)	63.1 ± 3.7	75.2 ± 2.8	73.5 ± 2.3	73.3 ± 6.1	73.7 ± 5.0
RBCC (x10^{11} cells/L)	8.7 ± 1.1	10.1 ± 0.4	10.0 ± 1.2	9.9 ± 1.6	10.2 ± 0.8
MCHC (g/L)	236.9 ± 9.0	229.5 ± 13.1	247.3 ± 14.9	247.8 ± 12.6	241.5 ± 9.3
MCH (pg)	77.4 ± 9.7	70.2 ± 5.0	77.1 ± 10.4	81.0 ± 11.1	60.5 ± 6.7
MCV (fl)	324.1 ± 33.3	305.6 ± 10.0	307.5 ± 25.4	321.6 ± 30.8	252.4 ± 31.1
Glucose (mmol/L)	1.50 ± 0.28	2.18 ± 0.19	2.96 ± 0.38*	1.93 ± 0.05	2.46 ± 0.17*
Lactate (mmol/L)	1.13 ± 0.17	1.05 ± 0.30	2.92 ± 0.87*	1.05 ± 0.10	2.19 ± 0.38
Triglycerides (mmol/L)	1.31 ± 0.07	1.18 ± 0.10	1.16 ± 0.06	1.26 ± 0.07	1.00 ± 0.07
Thrombocytes (per 100 WBCs)	2.0 ± 0.6	3.2 ± 0.5	2.4 ± 0.7	5.6 ± 1.0*	1.4 ± 0.2
Granulocytes (per 100 WBCs)	10.9 ± 3.0	14.0 ± 1.6	16.2 ± 1.6	11.2 ± 0.8	11.6 ± 2.4
Lymphocytes (per 100 WBCs)	84.2 ± 2.4	82.8 ± 1.6	81.4 ± 1.9	84.8 ± 1.7	87.0 ± 2.3

Table 4.4. All data (mean ± SEM) from fish exposed to 15 % v/v river water (reference water exposure) and various DO concentrations at 20.29 ± 0.02 °C for four weeks. *N = 20* for growth and survival and *n* = 5 for all other data unless otherwise indicated. n/a., not available.

Nominal DO concentration	Saturation	5 mg/L	4 mg/L	3 mg/L	2.5 mg/L
Growth (g)	n/a	-0.06 ± 0.26 *n* = 17	-0.06 ± 0.21	0.08 ± 0.34 *n* = 19	-0.26 ± 0.30
Survival (%)	0	85	0	35	100
Ucrit (BL/s)	n/a	3.42 ± 0.28	n/a	n/a	3.58 ± 0.09
Hct (%)	n/a	23.7 ± 2.1	n/a	30.7 ± 5.7	30.7 ± 1.8
Hb (g/L)	n/a	76.8 ± 2.0	n/a	63.6 ± 8.9	84.5 ± 3.5
RBCC (x10^{11} cells/L)	n/a	8.9 ± 1.5	n/a	8.9 ± 1.7	10.0 ± .07
MCHC (g/L)	n/a	255.4 ± 14.0	n/a	219.6 ± 16.8	229.4 ± 11.1
MCH (pg)	n/a	98.0 ± 17.8	n/a	77.0 ± 7.7	82.5 ± 18.6
MCV (fl)	n/a	381.0 ± 63.7	n/a	349.6 ± 16.8	359.5 ± 18.6
Glucose (mmol/L)	n/a	2.01 ± 0.17	n/a	2.24 ± 0.39	2.21 ± 0.20
Lactate (mmol/L)	n/a	2.26 ± 0.21	n/a	3.11 ± 0.61	2.20 ± 0.43
Triglycerides (mmol/L)	n/a	1.13 ± 0.07	n/a	0.96 ± 0.04	1.03 ± 0.04
Thrombocytes (per 100 WBCs)	n/a	3.0 ± 0.3	n/a	2.8 ± 0.9	2.2 ± 0.5
Granulocytes (per 100 WBCs)	n/a	16.6 ± 3.5	n/a	27.8 ± 3.0	10.2 ± 3.0
Lymphocytes (per 100 WBCs)	n/a	80.4 ± 3.6	n/a	69.4 ± 2.8	87.6 ± 2.8

Table 4.5. All data (mean ± SEM) from fish exposed to CTMP effluent (effluent exposure) and various DO concentrations at 16.08 ± 0.02 °C for four weeks. N = 12 for growth and survival and n = 5 for all other data unless otherwise indicated. * = $P < 0.05$, † = $P < 0.1$ (post-hoc).

Nominal DO concentration	Saturation	5 mg/L	4 mg/L	3 mg/L	2.5 mg/L
Growth (g)	0.43 ± 0.30	0.64 ± 0.31	0.16 ± 0.22	-0.44 ± 0.19*	-0.30 ± 0.22*
Survival (%)	100	100	67	100	100
Ucrit (BL/s)	4.81 ± 0.68	3.19 ± 0.16	3.64 ± 0.34	3.96 ± 0.17	3.75 ± 0.33
Hct (%)	24.6 ± 2.6	24.3 ± 0.8	24.0 ± 2.3 n = 3	21.7 ± 1.5	31.9 ± 2.8†
Hb (g/L)	64.2 ± 6.8	64.4 ± 1.6	68.5 ± 2.5 n = 3	71.4 ± 3.5	82.0 ± 3.9*
RBCC (x10^{11} cells/L)	10.7 ± 1.5	11.9 ± 1.0	9.4 ± 0.7 n = 3	7.9 ± 0.7	10.1 ± 1.1
MCHC (g/L)	261.8 ± 6.2	265.3 ± 5.4	289.4 ± 19.1 n = 3	331.2 ± 12.9*	261.3 ± 16.2
MCH (pg)	61.2 ± 20.2	65.5 ± 7.7	73.2 ± 5.2 n = 3	92.4 ± 6.7*	76.4 ± 9.0
MCV (fl)	234.4 ± 20.2	247.7 ± 31.1	254.6 ± 22.8 n = 3	278.2 ±13.8	295.3 ± 37.8
Glucose (mmol/L)	4.69 ± 0.56	3.79 ± 0.40	2.09 ± 0.30* n = 3	2.77 ± 0.28*	3.97 ± 0.19
Lactate (mmol/L)	1.78 ± 0.05	1.79 ± 0.14	2.09 ± 0.83 n = 3	1.44 ± 0.24	2.64 ± 0.70
Triglycerides (mmol/L)	1.20 ± 0.05	1.15 ± 0.11	0.87 ± 0.12 n = 3	1.08 ± 0.06	0.82 ± 0.19
Thrombocytes (per 100 WBCs)	4.0 ± 0.9	2.0 ± 0.7	3.0 ± 1.1 n = 4	4.0 ± 0.4 n = 4	5.6 ± 3.4
Granulocytes (per 100 WBCs)	19.8 ± 5.2	9.6 ± 3.3	20.0 ± 3.7 n = 4	9.0 ± 3.2 n = 4	6.8 ± 1.9
Lymphocytes (per 100 WBCs)	76.2 ± 5.7	88.4 ± 3.6	77.0 ± 3.6 n = 4	87.0 ± 3.2 n = 4	87.6 ± 3.4

Table 4.6. All data (mean ± SEM) from fish exposed to river water (reference water exposure) and various DO concentrations at 16.08 ± 0.02 °C for four weeks. *N = 12* for growth and survival and *n* = 5 for all other data unless otherwise indicated. * = P < 0.05 (post-hoc). n/a., not available.

Nominal DO concentration	Saturation	5 mg/L	4 mg/L	3 mg/L	2.5 mg/L
Growth (g)	0.61 ± 0.23	0.48 ± 0.19	0.64 ± 0.25	-0.10 ± 0.29	0.02 ± 0.30
Survival (%)	25	42	17	100	100
Ucrit (BL/s)	3.40 ± 0.74 *n* = 3	3.83 ± 0.34	n/a	3.64 ± 0.33	5.88 ± 0.26*
Hct (%)	n/a	n/a	n/a	28.3 ± 1.5	35.1 ± 1.5
Hb (g/L)	n/a	n/a	n/a	66.1 ± 2.7	79.0 ± 3.3
RBCC (x10^{11} cells/L)	n/a	n/a	n/a	11.9 ± 1.1	12.2 ± 0.8
MCHC (g/L)	n/a	n/a	n/a	234.4 ± 7.6	225.4 ± 2.8
MCH (pg)	n/a	n/a	n/a	57.0 ± 4.3	65.7 ± 3.5
MCV (fl)	n/a	n/a	n/a	242.2 ± 12.9	291.6 ± 15.7
Glucose (mmol/L)	n/a	n/a	n/a	4.13 ± 0.41	4.20 ± 0.38
Lactate (mmol/L)	n/a	n/a	n/a	2.45 ± 0.62	2.50 ± 0.49
Triglycerides (mmol/L)	n/a	n/a	n/a	1.18 ± 0.08	1.23 ± 0.07
Thrombocytes (per 100 WBCs)	n/a	n/a	n/a	3.2 ± 0.9	8.2 ± 4.8
Granulocytes (per 100 WBCs)	n/a	n/a	n/a	8.6 ± 1.6	7.2 ± 1.1
Lymphocytes (per 100 WBCs)	n/a	n/a	n/a	88.2 ± 1.0	84.6 ± 4.5

4.4.2 TMP/BKME exposures

Fish exposed to TMP/BK effluent revealed several DO dose-response effects (one-way ANOVA), as shown by significant increases in glucose ($p = 0.005$), lactate ($p = 0.026$) and thrombocyte count ($p = 0.002$) (Table 4.3). All other measured parameters were not affected. There was 100 % survival at all concentrations, and while there was a mean loss in weight for all groups, growth was not affected (Table 4.3).

Two-way ANOVA revealed no significant differences between effluent and reference water-exposed fish. However, as significant mortalities were observed in the reference water exposures (Table 4.4), statistical comparisons between effluent and reference water-exposed fish is reduced in authority due to the lack of reference data. Observations of fish during the study determined that reference fish were suffering from severe infestations of the white spot *Ichthyophthirius* parasite, with the exception of those fish exposed to the very lowest DO concentration of 2.5 mg L^{-1}. Fish exposed to effluent also did not show signs of parasite sensitivity.

4.4.3 CTMP mill effluent exposures

Fish exposed to CTMP effluent revealed DO dose-response effects (one-way ANOVA), shown as a decrease in growth ($p = 0.001$), increased Hct ($p = 0.029$), Hb ($p = 0.041$), MCHC ($p = 0.002$) and MCH ($p = 0.048$), and decreased glucose ($p = 0.022$) (Table 4.5). A significant DO dose-response ($p = 0.001$) for Ucrit of reference-water exposed fish was also observed, where it was seen that Ucrit was greater at low DO (Table 4.6). It should be noted that general behavioural observations throughout the experiment, it was seen that at the lower oxygen concentrations fish became less active and did not feed as aggressively. After feeding, larger amounts of uneaten food were also removed from these exposure groups.

Two-way ANOVA showed significant effects only on Ucrit for DO ($p = 0.022$), treatment ($p = 0.081$) and DO x treatment ($p = 0.004$). A near significant

treatment ($p = 0.081$) effect on growth was observed as overall greater mean growth in reference water-exposed fish. Again, survival was an issue for the reference water exposures (Fig. 4.6) and apart from Ucrit and growth, the statistical comparisons are questionable due to insufficient reference data. Fish mortalities were also observed coinciding with incidences of white spot infestation.

4.5 DISCUSSION

Interpreting the findings from this study is complicated. For both experiments, survival was a significant problem for fish exposed to the reference water conditions as a result of parasite infestation. However, it appears that effluents and very low DO (2.5 and 3 mg L^{-1}) in reference water exposures confer some level of protection to fish from the white spot parasite, shown as greater survival (Tables 4.3 – 4.6). As full data was primarily only available for the effluent exposures in each experiment, some limited analysis was still possible to determine dose-response effects of DO.

Directly comparing the two effluent experiments is not entirely appropriate as each was conducted under different conditions. Stemming from the problems encountered with the first effluent experiment (TMP/BKME), the CTMP effluent experiment was conducted at a slightly lower temperature (set to 18 °C, measured as 16.08 ± 0.02 °C) and with fewer fish in each treatment group ($n = 12$), in an attempt to prevent or reduce whatever factors contributed to the poor survival in the TMP/BKME experiment. The differences in experimental protocol may go a long way to explaining some of the observed differences between the separate effluent studies, particularly for growth and survival.

An exponential increase in oxygen consumption has been observed in resting rainbow trout when temperature was increased from 12 to 27 °C [19]. In the present study, fish subjected to the higher temperature of 20 °C (measured as 20.29 ± 0.02 °C) for TMP/BKME exposures most likely experienced greater energy demands through the associated increased metabolic activity, compared to the CTMP effluent experiment. It has also been demonstrated that under hypoxic conditions, seabass (*Dicentrarchus labrax*) and turbot (*Scophthalamus maximus*)

may reduce food intake in order to reduce the energetic demands of feeding, thereby resulting in diminished growth [6, 20]. The combination of temperature and hypoxia in the present study may have resulted in reduced growth for the TMP/BKME exposure. However, it is expected that these two factors would probably be additive, so the results cannot be fully explained by this theory alone. It is possible that fish exposed to the lower DO concentrations also employed behavioural strategies, such as reduced activity, to further offset the effect of reduced oxygen availability [4, 21]. While not measured, casual observations of fish indicated that those at the lower DO concentrations were less active and did not feed aggressively. The lower temperature of ~16 °C for CTMP effluent exposed fish may have been more optimal for normal growth across DO concentrations. In lowering the temperature for this experiment, temperature-effects on feeding, metabolism and growth may have been reduced or mitigated, resulting in more clearly defined specific DO growth-effects.

It has been suggested that growth and survival are the most sensitive measures of hypoxia exposure in fathead minnows (*Pimephales promelas*) [1], but growth is not expected to be affected at DO concentrations of around 4 – 5 mg L^{-1} in salmonids [22], although the data presented in this study may suggest otherwise. For growth, at least, this appears somewhat similar in the present study for CTMP effluent exposure. Separate analyses of CTMP effluent and reference water exposures (one-way ANOVA) indicated reduced growth at 3 mg L^{-1} DO during effluent exposure only (Table 4.5). However, analysis of all growth data from this study (two-way ANOVA) showed less over-all growth in the effluent-exposed fish compared to reference water-exposed fish (Tables 4.5 and 4.6). Previous studies have also shown reduced growth in sockeye salmon (*Oncorhynchus nerka*) and pinfish (*Lagodon rhomboides*) exposed to pulp and paper mill effluents [9, 23]. The similar finding here is confusing given that extractives analysis of CTMP effluent showed little in the way of typical pulp and paper constituents. However, some fatty acids were seen, particularly linolenic acid, and overall fatty acid profiles differed considerably for the two effluents (Table 4.2). Therefore, it is possible that differences in growth between CTMP effluent and reference water-exposed fish may be attributable to effects of fatty acid exposure.

For the TMP/BKME experiment, increases in blood glucose were observed for fish exposed to 2.5 and 4 mg L^{-1} DO, and blood lactate at 4 mg L^{-1} DO (Table 4.3). The hyperglycemic response in fish is typically regarded as a secondary stress response resulting from elevation of the stress hormone cortisol [24]. As cortisol was not measured in this study (because of small fish size), the cause of glucose elevation can not be established. However, all observed glucose values fall within those that have been previously reported for rainbow trout at rest [25]. The significant elevation of lactate in the 4 mg L^{-1} DO group is likely to be a result of anaerobic metabolism from the mildly hypoxic conditions and elevated temperature. Again, all lactate levels measured here (approximately 1.5 – 3 mmol L^{-1}), while slightly elevated for rainbow trout at rest, are not indicative of severe aerobic impairment. For example, lactate concentrations as high 15 – 20 mmol L^{-1} are not uncommon in rainbow trout subjected to exhaustive exercise [26]. For CTMP effluent-exposed fish, the opposite effect on blood glucose levels occurred. Here it was seen that with decreasing DO, there was also a reduction in glucose. As it is assumed that the reduced growth in this experiment is a result of diminished food intake, the lower circulating glucose levels are expected.

In order to alleviate or reduce the effects of hypoxia, fish may attempt to enhance their respiratory capabilities [21]. This appears to be the case for CTMP effluent exposed fish here, where DO-dependent increases in Hct, Hb, MCHC and MCH values were observed. Such changes serve to increase the oxygen transport capacity of blood, and are likely a response to direct hypoxia exposure or stress associated with the acute sampling procedure [3, 27-29].

An interesting effect on swimming performance, measured as Ucrit, was seen in the CTMP effluent experiment. The data presented suggest that for fish exposed to the effluent, there is a decrease in swimming performance with decreasing DO. However, the opposite pattern is observed for reference water-exposed fish, at least for the very lowest DO exposure (2.5 mg L^{-1}) where Ucrit is considerably greater (5.88 ± 0.26 BL s^{-1}) than for oxygen saturated controls (3.40 ± 0.74 BL s^{-1}). While significant effects were not observed on swimming performance in the TMP/BKME experiment, Ucrit appears to be lower for all DO-effluent exposure groups. Comparing effluent exposure values to the two reference water values

obtained, it can be seen that mean Ucrit is roughly 1 BL s^{-1} lower, and around 2 BL s^{-1} lower when compared to effluent and reference water exposures from the CTMP effluent experiment.

4.6 SUMMARY AND CONCLUSIONS

Despite problems associated with the poor survival of reference water-exposed fish, it was clearly demonstrated that juvenile rainbow trout were capable of surviving simultaneous effluent and hypoxia exposure at approximately 16 and 20 °C with two pulp and paper mill effluents for up to four weeks. The presence of these effluents significantly improved the chance of survival, presumably by offering some degree of protection against the white spot parasite. While some DO dose-response effects were observed for fish exposed to the two effluents, the responses are considered typical of hypoxia exposure, relatively small in magnitude, and clearly not a significant threat to fish survival in the medium to long-term. However, there is some evidence suggesting the presence of these effluents is having effects on the swimming performance of fish, as was shown by very low Ucrit values for fish exposed to TMP/BK effluent, and the measurable effects on Ucrit of CTMP effluent and reference water. Further studies examining the effects of pulp mill effluents on swimming performance would be valuable to shed further light on this subject.

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CHAPTER FIVE

SWIMMING PERFORMANCE OF JUVENILE TROUT EXPOSED TO A PULP AND PAPER MILL EFFLUENT AND A RESIN ACID

5.1 ABSTRACT

The effects of a thermomechanical (TMP)/bleached kraft pulp and paper mill effluent (BKME) and a major pulp and paper effluent constituent, dehydroabietic acid (DHAA), on juvenile rainbow trout was examined. Trout were exposed to 0, 10, 30 and 70 % TMP/BKME, and also to 0, 35, 110 and 250 μg L^{-1} DHAA for four weeks to investigate the chronic dose-response effects of these toxicants on fish swimming performance, oxygen consumption and hematology. Reduced swimming performance, as indicated by lower critical swimming (Ucrit) speeds, was found for fish exposed to 70 % TMP/BKME. Moderate increases in mean cell haemoglobin concentration (MCHC) at 70 % TMP/BKME, and blood glucose at 30 and 70 % TMP/BKME were seen. The opposite trend for glucose was found for DHAA-exposed fish, where a slight decrease in glucose was seen at 110 and 250 μg L^{-1} DHAA. No effects of TMP/BKME and DHAA on other measured parameters were noted, with the exception of marginal changes in blood glucose. This study demonstrates the ability of a modern pulp and paper effluent to have effects on the energetic fitness of rainbow trout. However, all observed effects are considered relatively small in magnitude and at effluent concentrations well above those found in the receiving environment for this particular effluent. Soft water used in the resin acid experiment, compared to harder river water used in the effluent study, is also offered as another possible explanation for the differences in swimming performance between these experiments.

5.2 INTRODUCTION

Pulp and paper industry process and treatment changes have resulted in effluents with reduced whole-organism responses, virtually no acute toxicity [1, 2], and considerable improvements in receiving environments [3]. Early studies reported numerous effects of pulp mill effluents on basic fish physiology, including reduced growth and food conversion in sockeye salmon (*Oncorhynchus nerka*) and pinfish (*Lagodon rhomboides*) [4, 5], altered respiration and ventilation in coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) and pinfish [5, 6], and modest increases in oxygen consumption in rainbow trout [7]. Other studies have revealed effects on swimming performance, such as reduced maximal swimming ability in juvenile sockeye and coho salmon [8, 9] and

impaired ability of perch (*Perca fluviatilis*) to maintain position in a rotary-flow apparatus [10].

Resin acids have become increasingly studied since their abundance in effluents and identification as significant contributors to fish toxicity was realised [11]. Peng and Roberts [12] recently described an inversely proportional relationship between toxicity and solubility of the seven most abundant resin acids, identifying dehydroabietic acid (DHAA) as the most soluble but the least toxic to *Daphnia magna* and rainbow trout. However, this relates to the toxokinetic properties of DHAA and not to potency, *per se*. Regardless of overall toxicity, the effects of DHAA in fish are thoroughly documented. The most notable effects of DHAA have been observed on the liver and blood [13-16], but more recent research is starting to shed light on the effects and mechanisms relating to disruption of cellular energetics [17]. There is also some evidence that acute exposures to components of some effluents, such as tetrachloroguaiacol, can result in reduced swimming performance in rainbow trout [18]. However others have failed to demonstrate an effect of acute chlorinated DHAA exposure (24-h exposure to 80 % of the 96-h LC50 for 14-monochloroDHAA and 12,14-dichloroDHAA) on swimming performance in trout [19].

Recent studies have examined a New Zealand pulp and paper effluent with the potential to have effects on reproductive physiology [20] and oxygen consumption [21] (Chapter 3) in rainbow trout, and secondary sexual characteristics in mosquitofish (*Gambusia affinis*) [22]. However, no attempts have been made to determine the effect of this effluent on swimming performance, other than that described in the previous chapter (Chapter 4). Therefore, this study aims to determine if chronic exposure to a modern pulp and paper mill effluent, and the major effluent constituent dehydroabietic acid, has the potential to affect swimming performance, respiration and hematology in rainbow trout.

5.3 MATERIALS AND METHODS

5.3.1 Fish

Laboratory-hatched and reared rainbow trout (*Oncorhynchus mykiss*; 12 ± 0.3 cm, 18 ± 0.8 g) were housed in well aerated, 12,000 L outdoor tanks in dechlorinated Rotorua city tap water at 11 – 13 °C under a natural photoperiod and fed daily with a commercial salmon feed (Reliance Stock Foods, Dunedin, New Zealand). Two groups of 80 fish were taken two weeks apart to be used in the two separate exposure studies.

5.3.2 Effluent and Resin Acid

Whole effluent was collected from the Tasman Mill, Kawerau, New Zealand. The mill is an integrated thermomechanical (TMP)/bleached kraft (BK) pulp and paper mill. Mill furnish is primarily softwood (*Pinus radiata*) with some *Eucalyptus spp.* pulping. Effluent from the TMP waste stream of the TMP/BK mill is pre-treated in a moving bed bioreactor prior to being combined with the remainder of effluent streams within the mill. Combined effluent is settled in a primary treatment pond, followed by secondary treatment in an aerated oxidation lagoon system for 4 – 6 days prior to discharge into the Tarawera River (175,000 m^3 d^{-1}; 5 – 12 % total river flow). Secondary treated final discharge effluent was collected from the mill in 1,000 L batches, with 1 L sub-samples taken for the determination of organic components. Effluent sub-samples were filtered through 15-cm GF/C filters, with filtrate and filter paper stored at -20 °C prior to analysis. Organic component analysis was performed as per previous methods [22, 23]. Pure (> 99 %) DHAA was obtained from Forest Research (Rotorua, New Zealand). A 5 g L^{-1} stock solution of DHAA was made by dissolving 1 g DHAA in 200 mL of 0.1 M NaOH. The stock solution was diluted with NaOH as needed (1 mL DHAA solution per 20 L of tank water) for the exposure series. Two tank-water samples were collected from the 250 µg L^{-1} treatment group; the first 30 minutes after water replacement and the second 24 h later. Samples were collected in 200 mL plastic bottles and stored at -20 °C for 4 weeks prior to analysis.

5.3.3 Exposures

In Experiment 1, fish were exposed to 0 (control), 10, 30 and 70 % v/v effluent (20 fish per tank) in 80 L glass tanks at 14.1 ± 0.1 °C. Up-stream reference Tarawera River water (total hardness ~ 35 g m^{-3} CaCO3) was collected at the same time as effluent, for diluent and control study uses. For Experiment 2, fish were exposed to 0 (control), 35, 110 and 250 µg L^{-1} DHAA (20 fish per tank) in 80 L glass tanks at 14.5 ± 0.1 °C. Four mL of the appropriate concentration of DHAA solution was added to each tank (4 mL 0.1 M NaOH in the control) at the beginning of the experiment. Dechlorinated tap water (total hardness < 10 g m^{-3} CaCO3) was used as diluent. Water pH was not adjusted after NaOH additions.

Fish were exposed in the laboratory for 4 weeks with a 12:12 light:dark photoperiod. Fifty percent water replacements (40 L) were made daily, fish fed an approximately 1 – 2 % wet body weight ration every second day, and tanks treated with 100 g of sea salt (Dominion Salt, Mt. Maunganui, New Zealand) every second day following water replacement. Tanks were re-dosed with DHAA after each water replacement.

5.3.4 Blood sampling and analysis

After the 4-week exposure period, blood samples were taken from 5 fish at each effluent and DHAA concentration. Fish were individually netted from tanks and stunned by a blow to the head. Blood samples were subsequently taken before sacrifice by a further blow to the head. Approximately 120 µL of blood was taken via caudal venepuncture into pre-heparinized syringes (400 i.u. mL^{-1}) and placed on ice. Samples were immediately analysed for haematocrit (Hct), haemoglobin (Hb), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH) and mean cell volume (MCV) according to standard methods [24]. Plasma samples were stored at -20 °C for later analysis of glucose, lactate, triglycerides and cortisol. Glucose and lactate were analysed using Sigma kits (Sigma-Aldrich Pty. Ltd., Sydney, Australia). Cortisol was measured from plasma samples with a commercial

radioimmunoassay kit (Coat-a-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA, USA).

5.3.5 Respirometry

Routine oxygen consumption was determined using static respirometers based on a previous design [25] as described by Landman et al. [21] (Chapter 3). A small submersible pump was used to circulate water through the respirometer during closed operation and to flush the respirometer with fresh oxygenated water when in open mode. Each respirometer was completely submersed in an 80 L, air saturated, constant temperature water bath (15 °C). A single fish (5 fish per effluent and DHAA concentration) was transferred to each respirometer and allowed to acclimate for 18 h overnight with the respirometer in open mode. Following acclimation, four repeated 1-h oxygen consumption readings were taken by closing the respirometer and measuring the decrease in dissolved oxygen (DO). Total DO did not fall below 65 – 70 % saturation during testing. The respirometer was opened and flushed for at least 10 min between each determination.

Oxygen consumption was calculated from DO concentration, respirometer volume and time: $VO2 = [\Delta CO2 \times V] \div [T \times Wt]$, where VO2 = oxygen consumption (mg O2 g^{-1} h^{-1}), $\Delta CO2$ = change in oxygen concentration of water (mg L^{-1}), V = volume of the respirometer (L), T = duration of measurement (h) and Wt = mass of fish tested (g).

Oxygen consumption was determined for fish under reference water conditions. Approximately 10 % water replacements (8 – 10 L) were made daily. For statistical analysis, the first oxygen consumption measure was ignored for all fish tested.

5.3.6 Swimming performance

Critical swimming speed (Ucrit) was determined using a custom-built, 230 L, recirculating flume (University of Waikato, Hamilton, New Zealand). Ucrit determinations were based on the standardised protocol described by Brett [8] involving an incremental swimming test. Ucrit determinations were made using the same water used for control fish and test diluent. Approximately 10 % water replacements (20 – 30 L) were made daily. Pairs of fish were netted from their tanks and measured for fork length before transfer to the flume. Flume speeds were calculated using the average length of both fish. Fish were allowed to acclimate in the flume for 2 h at a routine swimming speed of 0.5 BL s^{-1} (body lengths per second) at 15 °C. Following acclimation, water velocity was increased in increments of 0.5 BL s^{-1} every 15 min until exhaustion. Exhaustion was assumed when fish were no longer able to maintain their position in the water flow and were confined against the rear grill of the flume. Fish were then removed and weight and length measurements taken.

Ucrit was calculated using the formula: Ucrit = Ui + [Uii x Ti/Tii], where Ucrit = critical swimming speed (BL s^{-1}), Ui = highest swimming velocity reached by fish that was maintained for the full increment duration (BL s^{-1}), Uii = incremental velocity increase (0.5 BL), Ti = duration swum by fish at the highest speed reached (min) and Tii = increment time (15 min).

5.3.7 Statistics

To determine the effect of whole effluent and DHAA on measured parameters, data were subjected to analyses of variance with Dunnet's post-hoc tests (ANOVA; $\alpha < 0.05$). All statistical analyses were performed using SYSTAT 10 (SPSS, Chicago, IL, USA) for Windows. Values are presented as means ± SEM.

Table 5.1. Organic extractive concentrations ($\mu g\ L^{-1}$) in 100 % (v/v) whole effluent (n = 3) used in Experiment 1, and measured DHAA concentration ($\mu g\ L^{-1}$) after 24 h and 30 min after water replacement in Experiment 2 (n = 1).

	Whole Effluent		DHAA	
Compound	**Mean**	**SE**	**24 h**	**30 min**
Fichtelite	n.d.	n.d.	n.d.	n.d.
Dehydroabietin	n.d.	n.d.	n.d.	n.d.
Tetrahydroretene	n.d.	n.d.	n.d.	n.d.
Retene	n.d.	n.d.	n.d.	n.d.
Methyldehydroabietin	n.d.	n.d.	n.d.	n.d.
Total RA Neutrals	**0.0**	**0.0**	**0.0**	**0.0**
Pimaric acid	59.9	30.9	n.d.	n.d.
Sandaracopimaric acid	1.9	1.9	n.d.	n.d.
Isopimaric acid	30.3	16.9	n.d.	n.d.
Palustric acid	10.9	10.9	n.d.	n.d.
Levopimaric Acid	n.d.	n.d.	n.d.	n.d.
Dehydroabietic acid	52.7	22.8	n.d.	32.6
Abietic acid	90.5	47.4	n.d.	n.d.
Neoabietic acid	5.0	5.0	n.d.	n.d.
Pimarenic acid	n.d.	n.d.	n.d.	n.d.
Sandaracopimarenic acid	n.d.	n.d.	n.d.	n.d.
Isopimarenic acid	n.d.	n.d.	n.d.	n.d.
13-Abietenic acid	31.0	14.5	n.d.	n.d.
Pimaranic acid	n.d.	n.d.	n.d.	n.d.
Isopimaranic acid	n.d.	n.d.	n.d.	n.d.
Abietanic acid	5.0	3.2	n.d.	n.d.
Seco-1-dehydroabietic acid	2.5	1.4	n.d.	n.d.
Seco-2-dehydroabietic acid	1.1	1.1	n.d.	n.d.
12-Chlorodehydroabietic acid	n.d.	n.d.	n.d.	n.d.
14-Chlorodehydroabietic acid	n.d.	n.d.	n.d.	n.d.
12,14-Dichlorodehydroabietic	n.d.	n.d.	n.d.	n.d.
7-Oxodehydroabietic acid	n.d.	n.d.	n.d.	n.d.
Total Resin Acids	**280.5**	**154.2**	**0.0**	**32.6**
Cholesterol	n.d.	n.d.	n.d.	10.9
Campesterol	n.d.	n.d.	n.d.	n.d.
Stigmasterol	12.2	12.2	n.d.	n.d.
Sitosterol	8.5	8.5	n.d.	n.d.
Sitostanol	n.d.	n.d.	n.d.	n.d.
Total Phytosterols	**20.7**	**20.7**	**0.0**	**10.9**

Note. n.d. not detected.

Table 5.2. Summary of all measured parameters from Experiments 1 and 2 shown as mean ± SEM values (n = 5). * = $P < 0.05$ (post-hoc).

	Whole Effluent Concentration (%)				DHAA Concentration (µg L^{-1})			
Measure	**0**	**10**	**30**	**70**	**0**	**35**	**110**	**250**
Ucrit (BL/s)	5.38 ± 0.13	5.32 ± 0.18	5.39 ± 0.09	4.88 ± 0.07*	4.83 ± 0.23	4.80 ± 0.30	4.06 ± 0.31	4.79 ± 0.20
O_2 Con. (mg O_2/g/h)	0.17 ± 0.01	0.18 ± 0.01	0.21 ± 0.02	0.19 ± 0.01	0.16 ± 0.01	0.18 ± 0.03	0.17 ± 0.01	0.16 ± 0.01
Hct (%)	36.3 ± 2.78	33.1 ± 1.92	33.8 ± 1.93	30.3 ± 2.24	31.3 ± 1.5	31.6 ± 1.0	31.4 ± 2.2	30.6 ± 30.6
Hb (g/L)	70.1 ± 2.75	72.2 ± 3.88	72.4 ± 2.79	68.8 ± 4.24	73.4 ± 4.6	66.4 ± 1.5	69.5 ± 5.7	67.5 ± 3.3
RBCC ($x10^{12}$cells/L)	1.05 ± 0.07	1.09 ± 0.08	1.09 ± 0.08	1.07 ± 0.11	1.47 ± 0.07	1.18 ± 0.09	1.23 ± 0.16	1.16 ± 0.12
MCHC (g/L)	195.6 ± 9.0	219.1 ± 6.4	215.0 ± 5.9	228.2 ± 7.9*	234.7 ± 9.9	210.8 ± 6.4	228.6 ± 9.2	222.7 ± 14.6
MCH (pg)	67.5 ± 3.52	66.9 ± 2.34	67.4 ± 3.46	65.6 ± 3.8	50.5 ± 3.8	57.3 ± 3.7	59.0 ± 4.9	60.0 ± 5.3
MCV (fl)	348 ± 24.4	306 ± 15.1	314 ± 11.9	287 ± 12.1	214.4 ± 10.8	274.5 ± 24.4	259.2 ± 23.2	271.0 ± 24.3
Glucose (mmol/L)	4.37 ± 0.27	4.96 ± 0.24	5.38 ± 0.30*	5.37 ± 0.12*	6.87 ± 0.52	5.63 ± 0.28	5.32 ± 0.21*	4.97 ± 0.31*
Lactate (mmol/L)	0.94 ± 0.08	0.99 ± 0.20	1.18 ± 0.26	0.84 ± 0.18	0.82 ± 0.28	1.09 ± 0.40	0.62 ± 0.19	0.38 ± 0.10
Cortisol (ng/mL)	12.2 ± 5.8	20.1 ± 8.4	55.7 ± 41.3	8.4 ± 4.7	9.6 ± 3.6	5.9 ± 3.0	29.4 ± 25.5	6.7 ± 0.7

5.4 RESULTS

Measured organic extractives from whole effluent of Experiment 1 (Table 5.1) indicate a somewhat atypical Tasman effluent as shown by the complete lack of resin acid neutrals and the absence of numerous common resin acids and phytosterols. The relative abundance of the major resin acids in this effluent (pimaric, isopimaric, dehydroabietic, abietic and 13-abietenic acids), while marginally lower than expected, are still in normal relative proportions for this effluent. Dehydroabietic acid from Experiment 2 after 24 h exposure and 30 min after water replacement in the 250 $\mu g\ L^{-1}$ DHAA treatment group was measured once, and found to be 0 and 32.6 $\mu g\ L^{-1}$, demonstrating an obvious partitioning effect. It is unclear whether DHAA was coming out of solution, was taken up by fish or bound to the plastic bottles that the water samples were stored in.

After 4 weeks of exposure to whole effluent, dose-dependent responses were observed for Ucrit and MCHC. A significant reduction ($p = 0.033$) in swimming performance (Ucrit) was seen in fish exposed to 70 % effluent (Table 5.2). A significant increase ($p = 0.041$) in MCHC for fish also exposed to 70 % effluent, and a corresponding near-significant MCV decrease ($p = 0.110$) were also observed (Table 5.2). Significant increases ($p = 0.030$) in blood glucose for fish exposed to 30 and 70 % effluent were also found. Effluent dose-response effects were not observed for other measured parameters.

Significant decreases ($p = 0.008$) in blood glucose for fish exposed to 110 and 250 $\mu g\ L^{-1}$ DHAA were the only observed effects in the DHAA experiment (Table 5.2).

5.5 DISCUSSION

Bleached kraft mill effluent (BKME) effects have been seen in juvenile coho salmon (*Oncorhynchus kisutch*) where it was shown that even after relatively short effluent exposures (0 – 168 h) to concentrations below lethal levels (as low as 20 % of the 96-h LC50), a reduction in swimming performance was possible [9]. Howard [9] suggested that impaired oxygen uptake was the most likely factor influencing swimming performance. In supporting evidence, BKMEs have also

been shown to increase cough response, ventilation and oxygen consumption in fish [5-7]. Some increases in routine oxygen consumption have previously been demonstrated in juvenile trout exposed to 15 and 70 % TMP/BK mill effluent, which was presumed to be the result of either active toxicant clearance by the fish or altered cellular energetics [21] (Chapter 3). Since resting oxygen consumption did not appear to be affected in the present study, it is not clear if the reduced swimming ability following 70 % effluent exposure was due to reduced oxygen uptake. The lower values measured for MCHC coupled with high MCV in the control fish is indicative of adrenalin-mediated cell swelling [26, 27], most likely as a result of handling and sampling stress. Because similar responses were not seen in the higher effluent treatment groups, it is suggested that this could be the result of depressed catecholamine levels or desensitization of the red blood cell receptors due to prolonged stress in these fish [28, 29].

Changes in blood glucose were seen for fish of both the effluent and DHAA experiments. Increases in glucose were measured for fish exposed to 30 and 70 % whole effluent, while the opposite effect of glucose was seen for fish exposed to 110 and 250 $\mu g\ L^{-1}$ DHAA. Overall, blood glucose levels were moderately higher than expected. Elevated glucose levels are most frequently associated with the presence of the stress hormone cortisol [30]. However, changes in glucose were not mirrored by plasma cortisol concentrations in this experiment. Therefore, it implied that overall elevation of glucose, and changes according to effluent and resin acid dose, were metabolic in origin and not a function of stress.

Kennedy et al. [19] have demonstrated numerous effects of chlorinated DHAAs in rainbow trout, such as increased Hct, plasma lactate, plasma cortisol, liver protein and reduced disease resistance, but failed to show any effect of short-term exposure (24 h) of approximately 80 % of the LC50 (LC50 0.9 – 1.0 $mg\ L^{-1}$ DHAA) on swimming performance. Additionally, Johansen et al. [18] have also shown that while acute exposures of rainbow trout to tetrachloroguaiacol have numerous effects, including reduced swimming performance, measured parameters in chronically exposed fish were similar to controls. However, the lack of any obvious DHAA effects in this study is surprising because it has been

suggested that as little as 20 μg L^{-1} may be close to the "minimum effective concentration" of DHAA in rainbow trout [13].

The observed differences in overall swimming performance of whole effluent and DHAA exposed fish is potentially due to differences in water hardness. The effects of soft-water exposure are well known [31], the most striking effect being branchial chloride cell proliferation [32-34]. Greater chloride cells numbers are believed to increase the blood-to-water barrier, thereby reducing the efficiency of the gills and their ability to extract oxygen from water [33-35]. Assuming this was the case in this study, it would be no surprise that a reduced swimming performance was seen in soft tap-water (total hardness < 10 g m^{-3} $CaCO_3$) exposed fish, compared to river water (total hardness ~ 35 g m^{-3} $CaCO_3$) exposed fish. The fact that similar oxygen consumption values were seen between experiments could be explained by increased ventilation rates in tap-water exposed fish, compensating for a reduced ability to extract oxygen from water, as has been seen in rainbow trout with increased chloride cell numbers [33, 35].

5.6 SUMMARY AND CONCLUSIONS

The current study has demonstrated physiological effects in rainbow trout exposed to high doses (30 – 70 % v/v) of a pulp mill effluent and no significant effects of dehydroabietic acid exposure. These findings show that subtle sublethal effects in fish may still occur in a modern, well-treated, pulp mill effluent. The observation that swimming performance is hindered by effluent exposure is a significant one, as swimming ability in fish is often critical to survival. Impaired swimming ability may compromise migration, predator avoidance and foraging ability.

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CHAPTER SIX

LETHALITY OF HYPOXIA IN NEW ZEALAND FRESHWATER FISH

6.1 ABSTRACT

Acute lethality of low dissolved oxygen (48-h DO LC50) was examined in several New Zealand freshwater fish species and one freshwater invertebrate over 48 h at 15 °C. Contrary to previous reports, juvenile inanga (*Galaxias maculatus*) was the most sensitive fish species tested. Common smelt (*Retropinna retropinna*) and rainbow trout (*Oncorhynchus mykiss*) were similar in sensitivity, while shortfinned eel elvers (*Anguilla australis*) were the most tolerant fish species tested. Fish LC50s varied from 0.54 to 2.65 mg L^{-1}. The shrimp (*Paratya curvirostris*) was also tolerant of low DO with a 48-h DO LC50 of 0.82 mg L^{-1}.

6.2 INTRODUCTION

A recent study examined the responses of several New Zealand freshwater fish species to hypoxic conditions (low dissolved oxygen) [1]. The key finding of this study revealed that rainbow trout (*Oncorhynchus mykiss*) was the most sensitive of seven fish and one shrimp (*Paratya curvirostris*) species tested. Based on these findings, Dean and Richardson [1] have suggested that by adopting USEPA guidelines for dissolved oxygen (DO) in salmonid waters, adequate protection should be conferred upon native New Zealand fish species.

Impetus for this research was based on two main factors: (1) that little is known about the sensitivities of New Zealand fish to hypoxia and (2) a study on lowland streams in the Waikato Region (North Island, New Zealand) showed daily summer DO minimums in the vicinity of 3 – 4 mg L^{-1} [2]. Dissolved oxygen fluctuations are a common problem in many aquatic systems, especially those that are subject to industrial wastewater discharges [3]. Similar findings have been recorded in a pulp and paper mill-impacted system, the lower Tarawera River (Bay of Plenty Region, New Zealand), where episodes of depressed DO have been recorded below the regulatory absolute minimum of 4.5 mg L^{-1} during summer [4]. It has also been reported that in the downstream Tarawera River, DO may drop by a total of up to 5 mg L^{-1} over the 20 – 25 km stretch of river from the pulp and paper mill discharges at Kawerau to the coast [5].

Such findings may be of significance as a number of New Zealand's freshwater fishes are known to be diadromous (having a marine life stage) [6] and may be required to migrate back through rivers and streams with reduced DO concentrations at critical times. Another recent study examined the survival of caged inanga (*Galaxias maculatus*) and koaro (*Galaxias brevipinnis*) in the Tarawera River, where a relationship between DO and koaro mortality was found when river DO concentrations dropped below 5 mg L^{-1} for a considerable portion of the exposure duration [7]. Therefore, it is perhaps no surprise that a survey of fishes present in the Tarawera River found that several galaxiid species, inanga, koaro and giant kokopu (*Galaxias argenteus*) were absent altogether [8]. Young [7] has suggested that young migratory fishes might be actively avoiding the river due to the industrial inputs, reduced DO or a combination of the two.

Since the initial Dean and Richardson [1] study, a more functional system for the removal and control of DO has been developed [9] (Chapter 2). Using this new system, the intention of this study was to complement the work of Dean and Richardson by examining the sensitivity of several New Zealand freshwater fish and invertebrate species over a more defined range of DO concentrations, allowing accurate median lethal concentration (LC50) predictions to be made. This could not be achieved by Dean and Richardson as fish were only exposed to 1, 3 or 5 mg L^{-1} DO for 48 h in their study.

Therefore, the current study was also conducted to broaden the limited understanding of important New Zealand freshwater organisms. New data is presented for wild captured fish, one invertebrate and compared with some recent findings for rainbow trout and common bully (*Gobiomorphus cotidianus*) [10] (Chapter 3).

6.3 MATERIALS AND METHODS

6.3.1 Animals

Wild captured animals used in this study were shortfinned eel elvers (*Anguilla australis*), inanga whitebait (*Galaxias maculatus*), common smelt (*Retropinna*

retropinna), common bully (*Gobiomorphus cotidianus*) and freshwater shrimp (*Paratya curvirostris*). Laboratory-hatched rainbow trout (*Oncorhynchus mykiss*) were also used. All captured animals were kept in the laboratory for at least five days prior to experimentation, housed in well aerated tanks supplied with fresh dechlorinated Rotorua tap water, under a 12:12 light:dark photoperiod.

Eel elvers were captured in the Rangitaiki River by a commercial trapping operation (Bill Kerrison) at the base of the Matahina hydro dam (Matahina village, New Zealand). Inanga whitebait and smelt were collected from the Waikato River (Hamilton, New Zealand) by seine net. Common bullies were collected from Lake Tarawera (Rotorua, New Zealand) by seine net. Shrimp were captured by backpack electrofishing in the Kaituna River (Te Puke, New Zealand). Rainbow trout were hatched in the laboratory using fertilized eggs obtained from the Department of Conservation (DOC) Turangi Trout Centre (Turangi/Taupo, New Zealand).

6.3.2 Experimental Protocol

Using a previously described vacuum degassing and oxygen control system [9] (Chapter 2) fish were exposed to a range of DO concentrations (e.g. < 1, 1.0, 1.4, 1.8, 2.4 mg L^{-1} and fully saturated) to determine median lethal concentrations for DO (DO LC50). Oxygen concentration series were modified to suit the differing sensitivities of each fish species. Ten fish were exposed to each oxygen concentration in a set of five 15 L plastic aquaria at a constant temperature of 15°C for 48 h. Mortalities were recorded at 0.75, 1.5, 3, 6, 12, 24 and 48 h exposure. Clear plastic barriers were placed just under the water surface in each aquarium to minimise re-aeration of water and also to prevent fish surfacing. Bioassays were replicated three to four times, depending on the number of available fish, for DO LC50 calculations and statistical comparisons.

6.3.3 Calculations and Statistics

The 48-h DO LC50s were calculated from mortality data using the Spearman-Karber method [11]. Comparisons between pooled trout and bully life stage data from [10] (Chapter 3) were made with paired T-tests using SYSTAT 10 (SPSS, Chicago, IL, USA).

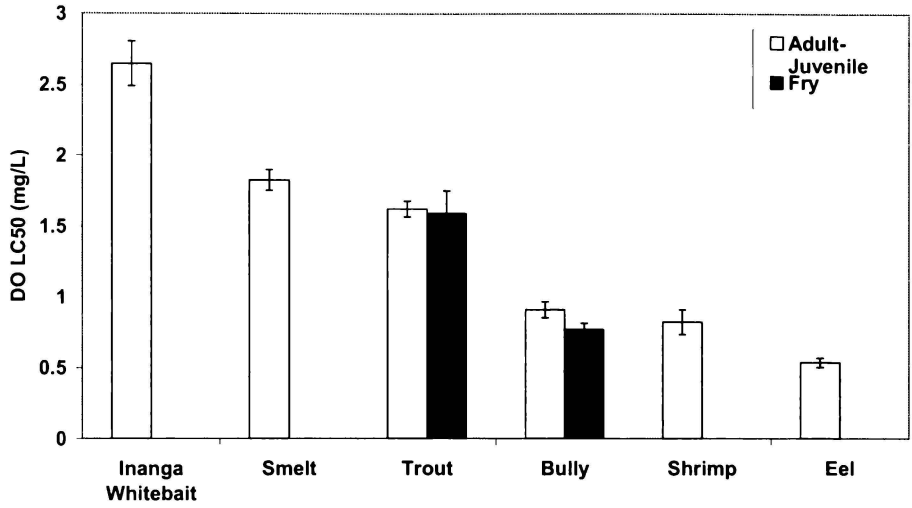

Figure 6.1. Mean (± SEM) 48-h DO LC50 values for inanga (n = 3), smelt (n = 4), trout (n = 6), bullies (n = 6), shrimp (n = 4) and eel (n = 3).

6.4 RESULTS

Relative sensitivities and tolerances to hypoxia varied considerably among the tested fish species (Fig. 6.1). Inanga whitebait was the most sensitive organism with a mean DO LC50 of 2.65 ± 0.16 mg L^{-1}. Trout and smelt had similar mean LC50s of 1.59 ± 0.16 (trout fry), 1.62 ± 0.06 (trout parr) and 1.83 ± 0.06 mg L^{-1} (adult smelt). Common bully were relatively tolerant of hypoxic conditions with DO LC50 values of 0.77 ± 0.04 (fry) and 0.91 ± 0.06 mg L^{-1} (juveniles), while shortfinned eel elvers were the most tolerant species tested with a DO LC50 of 0.54 ± 0.03 mg L^{-1}. Freshwater shrimp were also tolerant of hypoxia, with a mean DO LC50 of 0.82 ± 0.09 mg L^{-1}. For both eel and shrimp, 100 % mortalities were

not observed at the lowest measured test oxygen concentrations (0.673 ± 0.002 mg L^{-1} for shrimp and 0.501 ± 0.002 mg L^{-1} for eel exposures).

6.5 DISCUSSION

Previous studies have shown that chronic hypoxia exposure can result in reduced fish growth and survival [12-15], as well as numerous other effects including reproductive disruption [16, 17]. New Zealand study [1] has shown that acute exposures (48-h) of New Zealand freshwater species to low DO concentrations (1 mg L^{-1}) can cause at least some mortality in all species tested with the exception of elvers (*Anguilla* spp.), and partial mortality (14 %) at higher DO concentrations (3 mg L^{-1}) in rainbow trout. Additional behavioural observations revealed that at low DO concentrations (1 and 3 mg L^{-1}) most fish demonstrated at least some surfacing behaviour, while some fish (banded kokopu and shrimp) left the water completely at 1 mg L^{-1} DO.

The current study has demonstrated some differences in species sensitivity. In particular, it was shown that inanga whitebait were more sensitive to hypoxia, reflected by a mean 48-h DO LC50 of 2.65 ± 0.16 mg L^{-1}, which was considerably higher than for the other species tested. Dean and Richardson [1] found inanga whitebait and adults to be one of the more tolerant species they examined, with 61 and 38 % mortality at 1 mg L^{-1} DO, respectively. For most species tested, similar results were found between the two studies, with the only other notable exception being rainbow trout. In the current study, trout were found to be more tolerant as shown by mean 48-h DO LC50s of 1.58 ± 0.16 and 1.62 ± 0.06 mg L^{-1} with no mortalities at DO concentrations above 2.2 mg L^{-1}, compared to the 13 % mortality reported by Dean and Richardson after 48 h exposure to 3 mg L^{-1}.

The overall implication for this research may be that inanga are more sensitive to hypoxia than previously thought. Migratory inanga whitebait lack red blood cells and therefore might be expected to be less tolerant of hypoxia than other species. Recent research has demonstrated that hypoxia has a greater effect on the aerobic swimming ability of inanga whitebait than for juvenile rainbow trout (H.J. Bannon pers. comm.). However, this observation was confounded by the

additional finding that the swimming ability of post-migratory inanga juveniles is more sensitive to hypoxia than for whitebait. This may imply that the sensitivity of inanga whitebait, compared to other species, is not actually related to their lack of red blood cells.

While it can be concluded that current recommended regulatory limits might still protect native species at the 5 mg L^{-1} limit, the adoption of salmonid water guidelines [12, 18] may be premature. As inanga are a diadromous species [6], their migration back into fresh water may also carry with it additional demands, potentially exacerbating the effects of reduced DO. As toxicants are also known to increase the sensitivity of fish to low DO [19-23], the problems facing fish migrating through impacted systems is only confounded further. While cumulative effects of acute hypoxia exposure combined with two separate pulp and paper mill effluents were not previously demonstrated in rainbow trout and common bully [10] (Chapter 3), this is not to say that other species, such as inanga, may not respond differently under the same conditions.

6.6 CONCLUSIONS

New Zealand freshwater fishes vary in their tolerance to hypoxia. This study has highlighted differences between studies with respect to observed sensitivities of fish to hypoxic conditions. Caution is recommended when interpreting current laboratory data before extrapolating it to the natural environment. Clearly, more research is desirable in this area before absolute environmental DO recommendations are to be made.

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CHAPTER SEVEN

GENERAL DISCUSSION

This thesis examined combined effluent-hypoxia exposure, as well as stand-alone effluent and hypoxia exposures on fish. The initial experiments were designed to establish if the presence of two New Zealand pulp mill effluents had an effect on the median lethal concentration of dissolved oxygen 48-h DO LC50 in fish. It was decided that if an effluent-effect was observed on the 48-h DO LC50, then a severe toxicity problem would have already been established and further experiments would not be necessary. However, if there was no effluent-effect, then the focus of the project was to move toward chronic endpoints.

Particular emphasis was placed on the construction of an accurate and reliable DO control and exposure system (Chapter 2), taking around a year to build and perfect before experiments began. The first set of experiments (Chapter 3) examined the effect of 15 % (v/v) TMP/BK and river extracted CTMP mill effluents on the 48-h DO LC50 of fry and juvenile rainbow trout (*Oncorhynchus mykiss*) and common bully (*Gobiomorphus cotidianus*). It was determined from these experiments that the presence of either effluent did not increase the sensitivity of either fish species to hypoxia. These results are consistent with a recent mayfly study [1] demonstrating a lack of effluent-hypoxia effects with a North-American pulp mill effluent. Subsequent respirometry experiments (Chapter 3) demonstrated that pre-exposure of trout to 15 and 70 % (v/v) TMP/BKME for two weeks resulted in increased routine oxygen consumption for fish when tested in fresh water, but no change in oxygen consumption of those fish tested directly in 15 % (v/v) effluent. Therefore, it was considered prudent to repeat the oxygen lethality experiments with trout that had also been pre-exposed to 15 and 70 % (v/v) TMP/BKME. Here it was observed that pre-exposure had no effect on the 48-h DO LC50 of trout.

As part of one later experiment (Chapter 5), oxygen consumption was measured again with trout that had been exposed to 10, 30 and 70 % (v/v) TMP/BKME and 35, 110 and 250 $\mu g\ L^{-1}$ DHAA for four weeks. Interestingly, changes in oxygen consumption were not seen at all here. However, extractives analysis of the effluents revealed resin acid concentrations of around 4-fold greater in the experiment where an effect was observed (Chapter3; Table 3.1), compared to that where there was no effect (Chapter 5; Table 5.1). These differences are significant

as recent research has shown the ability of predominant resin acids to disrupt cellular energetics [2].

Nonetheless, the findings in Chapter 3 that environmentally relevant concentrations of a pulp and paper effluent can interfere with respiration may be a significant one. If the requirements for oxygen are greater when exposed to effluents, the additional demands placed on fish during hypoxia may still be additive. Since it was clear that the two effluents examined did not enhance the effects of hypoxia on survival in the short-term, other parameters such as long-term growth and survival might presumably have been more biologically relevant.

Unfortunately, this hypothesis could not be fully tested in the subsequent chronic effluent-hypoxia experiments (Chapter 4) due to poor survival of fish exposed to reference water for the two chronic pulp mill effluent (CTMP and TMP/BKMEs) experiments conducted. However, survival of effluent-exposed fish was excellent. Therefore, it was concluded that juvenile rainbow trout were capable of surviving simultaneous effluent and hypoxia exposure for four weeks at 16 – 20 °C. There were also some interesting effects noted on the swimming performance of fish. For fish exposed to CTMP effluent, the data presented suggested a decrease in swimming performance with decreasing DO, with the opposite pattern occurring in reference water-exposed fish. Although significant effects were not observed on swimming performance in the TMP/BKME experiment, overall swimming performance appeared to be diminished for all combined DO-effluent exposures.

To further investigate swimming performance (Chapter 5), trout were exposed to 0, 10, 30 and 70 % (v/v) TMP/BKME. In an attempt to establish whether resin acids, as major pulp mill effluent constituents, were the cause of any potential changes in swimming performance, trout were also exposed to 0, 35, 110 and 250 $\mu g\ L^{-1}$ DHAA. This experiment demonstrated that at 70 % (v/v) TMP/BKME, swimming performance was reduced in trout, although no effect of DHAA exposure was seen. As changes in oxygen consumption were seen in fish exposed to 70 % (v/v) TMP/BKME (Chapter 3), the finding that TMP/BKME may also reduce swimming ability continues to suggest that some subtle, sublethal effects on fish are still occurring in this modern, well-treated, pulp mill effluent. Both the

effects on oxygen consumption and swimming ability are important observations, as any reduction in the aerobic scope and adaptive ability of fish may be critical to survival. Not only might increased demand for oxygen reduce the ability of fish to tolerate episodes of hypoxia, but may also further diminish swimming capacity in the hypoxic environment.

The final study performed during this project aimed to utilize the DO control system to re-examine previously established [3] hypoxia sensitivities of several important New Zealand freshwater fish species by providing accurate DO LC50 determinations (Chapter 6). Stark contrasts between the relative sensitivity of trout and the tolerance of inanga whitebait (*Galaxias maculatus*) were found between this study, and that of Dean and Richardson [3]. The particular implication for these differing observations may be that inanga are more sensitive to hypoxia than previously thought. Relating these findings back to the Tarawera River problem, Young's [4] assertion that certain migratory fish species such as inanga, koaro and kokopu might be avoiding the river due to effluent loading and hypoxia may be justified. Additionally, although it can be concluded that current recommended regulatory limits for DO in the Tarawera River might still protect native species, the adoption of salmonid water guidelines [5, 6] may be premature, given that trout do not appear to be the most sensitive fish species examined to date.

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