An Assessment of Endocrine Disrupting Potential of a New Zealand Pulp and Paper Mill Effluent using Rainbow Trout *Oncorhynchus mykiss* and Mosquitofish *Gambusia affinis*.

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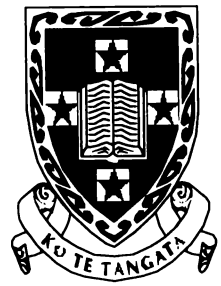

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Abstract

I examined the effects of a modern mixed thermo-mechanical pulp/bleached kraft (TMP/BK) mill effluent on potential reproductive-endocrine impacts on two fish species, rainbow trout and mosquitofish using a combined approach of laboratory and on-site mesocosm exposures. Rainbow trout *Oncorhynchus mykiss* were utilised in a combination of early life stage and juvenile (+1 year) long- and short-term exposures. Exposure of rainbow trout eggs to 15% (v/v) effluent until 10.5 months of age had no effect on fertilisation, hatching success, time to hatch, time to swim-up, juvenile mortality rate, growth and development. Juvenile (+1 year) rainbow trout were exposed to secondary treated TMP/BK mill effluent at a range of concentrations from environmentally relevant (10%) to 70% (v/v) in two exposure studies. During both 21- and 56-day exposures to 10% and 30% (v/v) effluent, no statistically significant impacts on spleen weight, liver weight, condition factor, and circulating testosterone and pregnenolone levels were observed. Consequently, vitellogenin induction as well as the expression of the estrogen receptor in juvenile males was not observed in either experiment. Statistically significant differences in spleen and liver size as well as high experimental mortality were observed in the 70% (v/v) effluent 21-day treatment and was linked to an atypically high suspended solids load. Two mosquitofish *Gambusia affinis* experiments were conducted, both consisted of a 21-day exposure using adult females. The first experiment was a multiple concentration exposure to primary and secondary treated effluents. A statistically significant masculinisation response (gonopodial development) was noted in all effluent

treatments. However, secondary treatment of the effluent resulted in a significant decrease in gonopodial development. The second experiment focused on filtered and unfiltered secondary treated effluent at an environmentally relevant concentration (15%). Filtration of the treated effluent resulted in the almost complete elimination of the female masculinisation response. In all experiments, male mating behaviour was observed in the masculinised female mosquitofish. I concluded that a significant species difference regarding sensitivity to reproductive-endocrine modulating compounds occurred following exposure to a mixed TMP/BKM effluent. In addition, the data suggest that the compounds of concern are bound to solid matter within the effluent and these effluents have the potential to exert effects through an androgen-like mode of action.

Acknowledgements

Similar to the statistical analyses, I have saved the most difficult section to write until the end (literally the last few hours). It really is not possible to thank all the people who have encouraged and helped me throughout the past three and a half years. If I have left anybody out it is not because I have not appreciated your efforts but mostly because I am severely sleep-deprived and will most likely kick myself when I realise the mistake I've made (Expect a bottle of wine in repent).

First and foremost, a very special and sincere thank you to Dr Michael van den Heuvel and Prof. Dr. Daniel Dietrich. Mike was definitely worth the wait in the beginning stages down in Rotorua, and well, Dan justified braving a bitterly cold European winter to work with him and his colleagues at the University of Konstanz, Germany. Thank you both for your continual support and guidance. A thank you also to Dr Trevor Stuthridge, whom I could always rely on to provide me with sarcastic remarks and signatures on those dreaded requisition forms. As for Dr Lynda McCarthy, where do I start with my always positive, ever cheerful infusion of estrogen! Thank you for looking at things from the same side of the fence. I would also like to thank my university supervisors Drs Nick Ling and Ian Hogg, for their support and advise particularly during the final months.

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I would also like to take this opportunity in thanking all the team in the 'Waste Management Systems and Technologies' group, particularly Nicola, Suzanne Murray, Sheree and Peter. Here's to eventually finding a more user-friendly project name! Lee and Gavin, thanks for helping me on those treacherous mosquitofish collection trips. Finally I would like to thank the team from 'AG Dietrich' in Konstanz, especially Biggi, Alex, Anke, Vlasta, Bettina and Evelyn.

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So on that note, as I promised my parents, it's time to get out there and get a "REAL" job.

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Chapter One: Introduction

1.1. Thesis introduction

Traditionally, risk assessment of environmental contaminants has focused on acute (severe) lethality, with mortality used as a common endpoint in conventional toxicity testing. However, over the past 20 years there has been a shift in focus towards chronic (less-severe) effects at environmentally relevant concentrations, including the assessment of complex whole effluents rather than single compound toxicity.

Endocrine disruption was first brought to public attention in the late 1970s-1980s with the connection between chlorinated insecticides (including DDT and its' metabolites) and adverse impacts on sexual development and reproductive capacity of bird populations (Cheek et al. 1998). More recently, research in the United Kingdom has demonstrated feminisation of male fishes and linked this to the degradation products of alkyl polyethoxylate detergents, nonylphenol and octylphenol (White et al. 1994). Estrogenic activity has also been linked to exposure to sewage treated wastewaters (Jobling and Sumpter 1993). Other studies have shown gender biases towards males and the masculinisation of female fishes exposed to pulp and paper mill effluents (Cody and Bortone 1997; Larrson et al. 2000). Public concern has now been raised about feminisation effects in males exposed to estrogen-like compounds, including both naturally-occurring (plant sterols) and xenobiotic estrogen-mimics (Colborn and Clement 1992; Jobling and Sumpter 1993; Carmichael 1998).

The impacts of greatest concern are changes to endocrine system function or "functional deficits" of the reproductive, behavioural, central nervous (neuro-endocrine) and immune systems. These effects can result from exposure to compounds capable of endocrine disruption at concentrations far below levels where any acute response may be apparent.

Endocrine disruption can be manifested in many ways, not always simultaneously e.g. multi-generational. Endocrine disrupting compounds may impair or alter reproductive function by acting at the hypothalamus, pituitary, gonad or liver, a key site of estrogen receptor induction and the synthesis of vitellogenin (McTavish et al. 1998; Kime and Nash 1999). Disruption at any of these sites may result in changes in the rate of gonadal development or in the viability of gametes, (including sperm viability and morphology) and hormonal disruption. Reproductive dysfunction at low, non-lethal exposure concentrations can be caused either by direct mutageneic action on the gametes, or indirectly by modulation of the endocrine system so that gamete development takes place during a significant disturbance to the 'normal' hormonal environment. Reproductive impairment can occur at any stage including gonadal development, fertilisation and subsequent larval development (Kime and Nash 1999). The development and production of viable sperm and eggs is essential for successful fertilisation, hatching and embryonic development and survival.

Regardless of the reproductive-endocrine disruption action site, the net result is a change in gamete quantity or quality, and may include trans-generational impacts i.e. genetic, phenotypic mutations or physiological abnormalities, or a subsequent

decrease in reproductive success in both offspring of, and exposed parents. Ultimately, these are the most significant endpoints since production of sufficient numbers of viable gametes (enough to ensure population survival and maintenance) is the primary goal of the reproductive system. Therefore, the study of reproductive impairment following exposure to compounds (effluents) with reproductive-endocrine disrupting potential, at non-lethal and "environmentally relevant" concentrations in the receiving environment has much significance with regard to assessing impacts on fish populations.

Accordingly, the main objectives of this thesis were to 1) determine if pulp and paper mill effluents from a modern facility have the potential to alter the reproductive capacity of fishes; 2) determine if reproductive impairment occurred at environmentally relevant concentrations; and 3) isolate the compound(s) responsible for any observed endocrine disruption.

This study addressed these key aims by employing a combination of long- and short-term exposures utilising two fish species, rainbow trout, *Oncorhynchus mykiss* and mosquitofish, *Gambusia affinis*. Experiments were conducted using both laboratory and "on-site" mesocosm facilities, which afforded the opportunity to investigate potential impacts from a point source effluent without confounding factors such as additional discharges from other facilities (e.g. the Caxton mill, sewage effluent), as well as allowing control of variables like energy intake, and the severity of exposure. The thesis is arranged as a series of chapters each consisting of journal articles which have been submitted for peer review with the exception of chapter two. A reference section accompanies each chapter. This thesis consists of six chapters; chapter one contains an introduction to the topic

and background information on the study site. Chapter two follows with a preliminary toxicity assessment of primary and secondary treated effluents. Chapters three and four present results from long-term exposure of early life stage and juvenile rainbow trout to TMP/BK effluent at ecologically relevant concentrations to determine if endocrine disrupting effects occur. Chapter five presents evidence of an androgenic response in adult female mosquitofish (*Gambusia affinis*) exposed to both primary and secondary treated effluent at multiple effluent concentrations. The thesis concludes with a summary of results and the overall thesis conclusions in chapter six.

1.1.2 Endocrine disruption and other impacts associated with the pulp and paper industry

The effects of bleached kraft mill effluent (BKME) on aquatic organisms have been extensively investigated since the 1960s. A variety of toxic effects have been observed from biochemical disturbances to changes in population dynamics, including reproductive impairment (Owens 1991; McMaster et al. 1995; Kovacs et al. 1997; Munkittrick et al. 1997).

Some of the reproductive impacts observed include decreased egg and gonad size, reduced fertilisation and hatching success and delayed maturity (McMaster et al. 1991, 1996; Munkittrick et al. 1992a, 1994); decreases in serum sex steroid levels and overall gonadal steroid production (Van Der Kraak et al. 1992; Munkittrick et al. 1992b; McMaster et al. 1994, 1995; McCarthy et al. 1997); disrupted fecundity-weight relationships (Gagnon et al. 1994a). Bioaccumulation of chlorophenolic compounds and increased growth rates have been observed in fish

in effluent-receiving populations (Gagnon et al. 1994a, 1994b). Increased induction of cytochrome P450 monooxygenases (Andersson et al. 1987; Lindstrom-Seppa and Oikari 1990a, 1990b) including induction of the hepatic mixed function oxidase (MFO) system usually measured as (EROD) activity have been associated with exposure to pulp and paper wastewaters (McMaster et al. 1991; Munkittrick et al. 1994; Gagnon et al. 1994a; van den Heuvel et al. 1995; Donald 1997; Jones et al. 1997).

The reduction of male secondary sex characteristics and modification of female secondary sex characteristics have been observed in some BKME exposed fish species (McMaster et al. 1991; Howell et al. 1980; Drysdale and Bortone 1989). For instance female mosquitofish (*Gambusia affinis*) with partially or fully developed gonopodia, a male reproductive organ which enables sperm transfer during mating. Along with this morphological alteration, changes in behaviour (Howell et al. 1980) and reproductive potential (Rosa-Molinar and Williams 1984) have also been reported following *Gambusia* exposure to kraft mill effluents.

1.1.3. Estrogenic and androgenic potential of BKMEs

Estrogenic activity in pulp and paper effluent and black liquor has been reported by Zacharewski et al. (1995) using *in vitro* recombinant receptor/reporter gene assays. More recently, the synthesis of plasma vitellogenin (Vtg), an egg yolk precursor protein and Vtg gene expression in the liver of male fish has been reported following BKM effluent exposure, in both laboratory and caging studies (Soimasuo et al. 1998; Mellanen et al. 1999; Tremblay and Van Der Kraak, 1999). Hepatic synthesis of vitellogenin is generally considered to be dependent on

stimulation of the liver by estrogens or estrogen-like compounds, following synthesis vitellogenin is secreted into the blood and sequestered by the oocytes and stored as yolk. Vtg is normally detected in males at background levels only. (Pelissero et al. 1993). To date, there have been few studies which have demonstrated androgenic activity in pulp and paper effluent, and a paucity of research investigating androgenic-potentials of these wastewaters. However, Hewitt et al. (2000) have recently demonstrated that some constituents of BKME have the ability to interact with the androgen receptor in hepatic tissues of white sucker.

1.1.4. Reduction in effects following modernisation

In recent years the international pulp and paper industry has committed considerable resources to environmental improvement through process modifications and the installation or upgrade of secondary treatment facilities (Kovacs et al. 1997).

The benefits of these investments, in terms of improved effluent quality include virtual elimination of chlorinated dioxins/furans, significant decreases in other chlorine- and non-chlorine-containing organics, the elimination of acute lethal toxicity and a reduction of the chronic/sublethal toxicity of effluents and therefore the decreased impact on physiology of receiving water fish populations have been well documented (Kovacs et al. 1997), with a number of studies have reporting no significant deleterious effects on fish reproduction and/or health (Swanson et al. 1993, 1994; Gagnon et al. 1994a, 1994b; Kloepper-Sams et al. 1994a, 1994b). However, significant reproductive impacts have still been recorded at some sites where process and treatment modifications have been implemented (Munkittrick

et al. 1992b, 1992c, 1994; MacLatchy et al. 1997) and neither elemental chlorine free (ECF) bleaching nor secondary treatment facilities were adequate to eliminate all chronic toxic responses (Munkittrick et al. 1997). For example, a field study reinvestigating gonopodial development in female mosquitofish in BKME receiving waters demonstrated that mill process modifications were unable to eliminate the masculinisation response observed in pre-modification situations, although gonopodial development was less pronounced than the initial study conducted in 1980 (Cody and Bortone 1997).

Studies of bleached kraft mills discharging secondary treated effluent have been conflicting, with the pattern and extent of many observed effects appearing to be site specific and not able to be easily extrapolated to all situations. Many of the observed impacts have been indiscriminant of mill 'type', in that similar physiological changes have been observed at mills with and without ECF bleaching, secondary effluent treatment and other treatment/technology upgrades in place (Munkittrick et al. 1997).

The question still remains as to whether the improvements made to process, production and wastewater treatment during the production of pulp and paper are sufficient to ensure that effluents are not detrimental to the health of the aquatic environment and whether further efforts are required (Kovacs et al. 1997).

1.2 Study Site

1.2.1 Tarawera River system

The Tarawera River originates from Lake Tarawera (NZMS260 V16 168-297) and flows a distance of 55km (Figure 1.1.) to the coast at Matata (NZMS260 V15

1.2.2 Map of study area

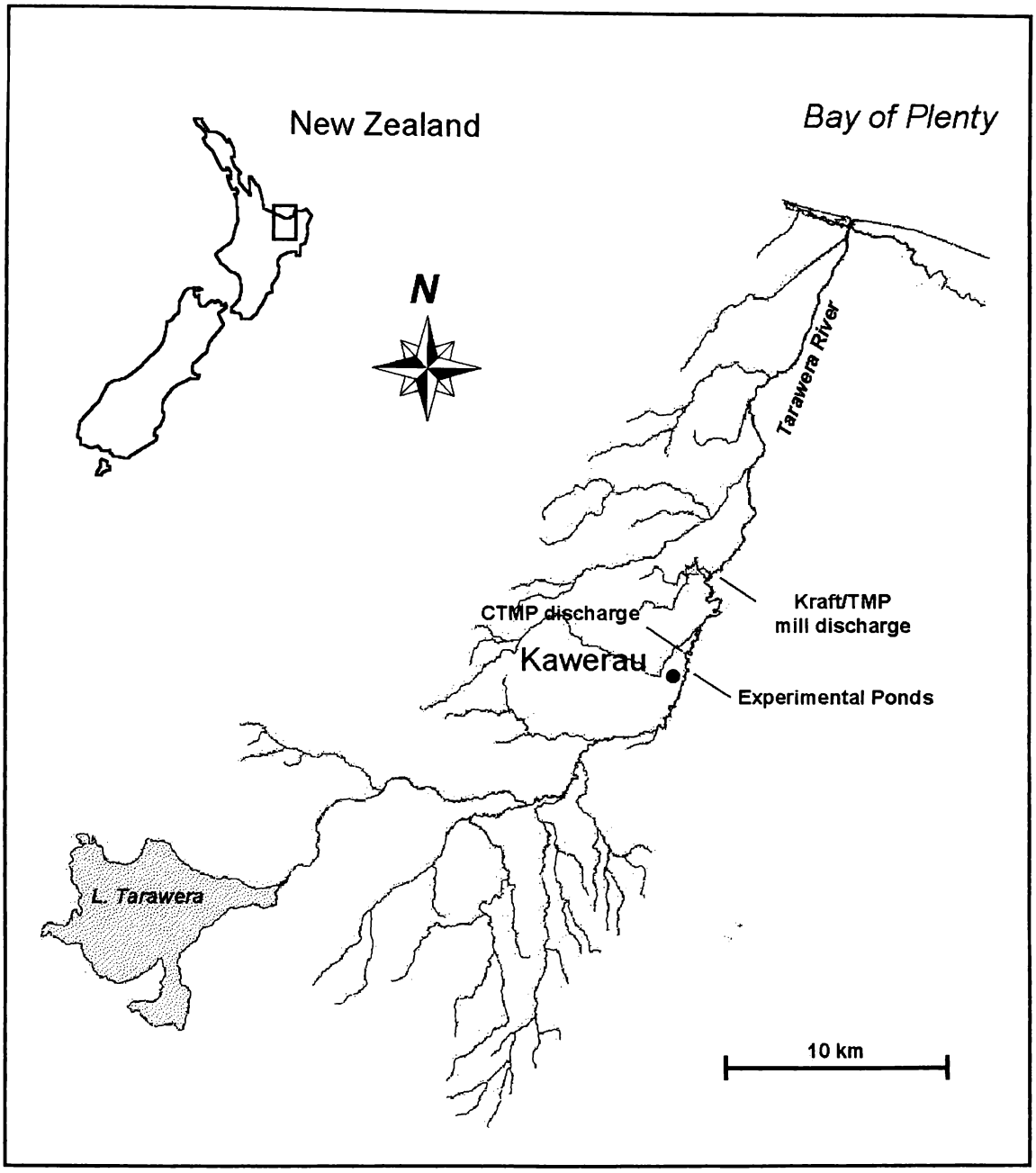

Figure 1.1. The Tarawera River catchment

396-619). The Tarawera River has a low variability of flow with a mean flow rate of 26 $m^3 \cdot s^{-1}$ with minimum and maximum flow of 14.6 $m^3 \cdot s^{-1}$ and 54.8 $m^3 \cdot s^{-1}$ respectively, the flow regime is stable due to the lake reservoir and the sponge-like nature of the pumice soils (Dell et al. 1996). Lake Tarawera is situated in an area of high volcanic and geothermal activity in the central North Island. The upper catchment area is forested with relatively little human habitation (Rutherford 1997). Long-term changes in vegetation cover, increases in production forestry and a decline in regional rainfall have all contributed to a reduction in flow over the last 30 years (Dell et al. 1996).

The Tarawera River is a major water resource in the Bay of Plenty Region. In the lower catchment the township of Kawerau (NZMS260 V16 355-395) is situated approximately 30km from the sea and has a population of approximately 7,830. Two pulp and paper mills at Kawerau discharge effluent into the river as well as sewage from the town. Carter Holt Harvey Caxton paper mills operate a bleached sulfonated-chemithermomechanical pulp mill with a rated capacity of 50,000 $t \cdot yr^{-1}$ paper and 75,000 $t \cdot yr^{-1}$ pulp. Effluent from this pulp mill and sewage from Kawerau township is treated in an anaerobic system with discharge (ca. 0.25 $m^3 \cdot s^{-1}$) to the river and/or rapid infiltration basins (Donald 1997). The combined effluent is discharged approximately 1 km upstream of the river water intake site of the second mill, Norske Skog-Tasman Ltd.

In addition to the discharge from these two pulp and paper mills, the Tarawera River also receives discharges from a geothermal bore field. Works Geothermal operates a bore field supplying steam to Norske Skog-Tasman Ltd. The discharge

of waste geothermal fluid occurs at two points, giving a combined river discharge of 0.24 $m^3 \cdot s^{-1}$ (Dell et al. 1996).

Water chemistry in the Tarawera catchment is greatly influenced by the extensive geothermal activity in the area, resulting in higher than average dissolved salt concentrations compared with other NZ rivers. In addition, geothermal discharges elevate concentrations of several metals including mercury, lithium, arsenic and boron. Although many of these compounds are known to have toxic effects, concentrations in the river do not exceed guidelines for the protection of aquatic life (Dell et al. 1996).

The main issue affecting aquatic life in the Tarawera River is dissolved oxygen (DO). Deoxygenation has been a problem since the 1950s and river DO commonly drops by up to 5 $g \cdot m^{-3}$ in eight hours travel time (Rutherford 1997). Pulp mill effluent discharged into the river significantly reduces DO concentrations, in some instances to below current classification standards (6.5 $g \cdot m^{-3}$). The high deoxygenation rate is a result of high microbial activity in a highly mobile sand river bed, where there is a high rate of mass transfer of DO and solutes between the bed and the river (Rutherford 1997).

1.3 Mill Description

This thesis focused solely on assessing the effects of effluent discharged into the Tarawera River from the Norske Skog-Tasman pulp and paper mill.

The Norske Skog-Tasman Ltd mill (formally Fletcher Challenge Paper) is an integrated bleached kraft mill and thermo-mechanical (BK/TMP) pulp and paper mill i.e. uses both kraft and thermo-mechanical pulping processes. The main differences in these treatments are that kraft pulping uses alkaline chemicals, pressure and heat for separation of wood fibres from lignin, whereas thermo-mechanical pulp production utilizes heat and mechanical refiners to breakdown wood chips. The fibre is then used to make newsprint. Approximately 757 tonnes of kraft pulp and 1010 tonnes of newsprint are produced each day (270,000 and 310,000 $t \cdot yr^{-1}$ respectively). Norske Skog-Tasman has been 100% elemental chlorine free (ECF) since April 1997. Mill production is based primarily on softwood (*Pinus radiata*) with occasional use of eucalypts. The mill draws 147 million litres of water from the river per day.

Norske Skog-Tasman implements biological secondary wastewater treatment. The wastewater treatment system consists of a thermomechanical pulping pre-treatment bioreactor facility within the TMP mill. Kraft mill effluent is then combined with this TMP effluent into a single drain and passed through two bar screens and a gravity clarifier and solids de-watering plant for partial solids removal, followed by secondary treatment in a four pond aerated stabilisation basin system 5km from the mill. The ponds have an area of 45 hectares, with a retention time of 5-6 days. The main function of the pond system is to reduce biological oxygen demand (BOD), absorbable organic halides (AOX) and

toxicity, by utilising high bacterial and microorganism activity within the treatment ponds. The system removes 80-90% of the BOD and final effluent is non-toxic at environmentally relevant concentrations (chapter two). Following treatment in the aerated lagoon system, effluent is discharged into the Tarawera River at a total mean volume of 180,000 $m^3 \cdot d^{-1}$ (186 ML·d). River effluent dilution ranges between 5 and 12 percent and enters the river system with an average temperature of 26-28°C.

During the last decade, the Norske Skog-Tasman mill has undergone process and effluent treatment modifications including the introduction of oxygen delignification and the substitution of elemental chlorine with chlorine dioxide, primary solids dewatering, improvements to treatment pond performance, foul condensate stripping, and isothermal cooking. In addition to these, Norske Skog-Tasman Ltd has also improved in-mill and treatment pond monitoring equipment and reduced the risk of liquor spills within the mill with the installation of a recovery boiler spill system. These modifications have resulted in reductions in the quantities of contaminants discharged and demonstrated a marked reduction in chlorinated compounds (54-97%), with a 92% decrease in chlorinated resin acids (Dell et al. 1996). Dioxin and furan levels have also decreased considerably due to ECF bleaching and AOX has decreased by 84%. Over the past ten years, colour discharge has decreased by more than 75% due to changes in pulping and bleaching processes (oxygen delignification) and improved operational control (Norske Skog-Tasman Ltd, Environmental Report, 1999).

1.4 On-site 'mesocosm' exposure facility

The experimental ponds constituting the 'mesocosm facility' (Plate 1.1.) are situated on site of the Norske Skog-Tasman Ltd water intake and clarifier. Located along the west wall of the water reservoir, the exposure facility is exposed to consistent sunlight and wind conditions across the wall of the reservoir. Additionally, the reservoir wall provides a concrete support for the experimental plumbing as well as providing shading from the east. A lean-to shelter has been constructed along the concrete reservoir wall, providing shelter to the ponds and over-head electrical lighting. A metal-wire fence also follows the perimeter of the experimental facility (Plate 1.1). All experiments are conducted under ambient conditions with additional lighting used for sampling periods only.

The mesocosm ponds consist of three 10,000 L epoxy-coated fibreglass tanks divided into 3 sections for juvenile trout exposures (Plate 1.2), and six non-divided 12,000 L epoxy-coated fibreglass tanks used for adult trout (large fish) exposures. Reference water is pumped directly from the Tarawera River at a point 900 m upstream of the mesocosm facility and upstream of the points of discharge of mill effluent and municipal sewage. River water is pumped continuously into a concrete reservoir (170,000 L) adjacent to the exposure ponds, which is kept at a constant level by pumping an excess of water into the tank then draining the excess to waste (Plate 1.4). There is constant head pressure (approx 3 meters) down to the reference and effluent mixing tanks of the exposure facility. Reference and dilution flows are controlled using stainless steel globe valves. Reference water temperature is ambient, reflecting that of the Tarawera River which can vary between 12 and 20°C throughout the year.

Alongside the reference water reservoir is another large reservoir (80,000 L) used to hold secondary treated effluent (Plate 1.3). Effluent is transported by road tanker on a weekly basis and sourced immediately prior to discharge from treatment pond number four into the Tarawera River. The effluent storage reservoir is continually re-circulated using submersible pumps in order to prevent solids settling and the effluent becoming anaerobic. From here, 100% effluent is gravity fed to mixing tanks alongside the mesocosm ponds. Both the river water and effluent holding reservoirs are covered with plastic tarpaulins for extra shading.

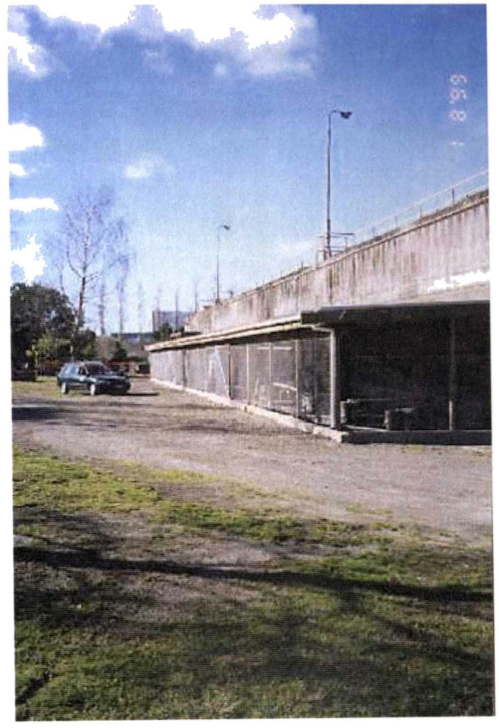

Plate 1. External view of on-site exposure 'mesocosm' facility.

Plate 2. Divided tanks used for juvenile rainbow trout exposures (chapter four).

Plate 3. 100% effluent reservoir at the on-site mesocosm facility.

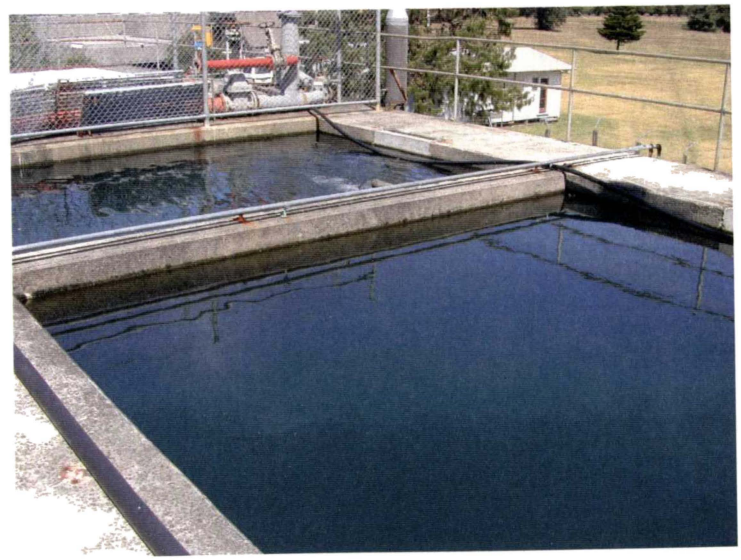

Plate 4. Tarawera River (reference) water reservoir at the on-site mesocosm facility.

1.5 Test Species

The species utilised in the present study were rainbow trout (*Oncorhynchus mykiss*), mosquitofish (*Gambusia affinis*), and the waterflea (*Daphnia carinata*). Rainbow trout were selected as the primary test species because they form the basis for valuable recreational fisheries and tourism in the lakes of the central North Island (McDowall 1990), are specifically mentioned in the Resource Management Act and have protection within the act, are an ideal reference species with widespread use in aquatic toxicological testing and a wide body of literature is available on their physiology and finally, they are easily accessible and amenable to laboratory conditions. Furthermore, because *O.mykiss* have an extensive global distribution, results from this study will have widespread applicability.

For similar reasons, mosquitofish were selected as a secondary test species because of their ease of maintenance and breeding under laboratory conditions, aided by their small size. *Gambusia* are also particularly easy to source and capture in the wild. A major advantage of working with *Gambusia* is that they display strong external sexual dimorphism which is obvious within three months of age. Given their small size and shorter life cycle, generational studies are relatively short-term. Additionally, they also offer an alternative reproductive strategy to rainbow trout in that *Gambusia* are a live-bearing species and thus make for an excellent species to compare with salmonids for the examination of differences in species' responses.

Finally, *Daphnia carinata* were used additionally for initial effluent toxicity assessments. The species has become a recognised and standard test organism internationally, and standard chronic toxicity tests are a specific requirement of

Norske Skog-Tasman's discharge consent to water permit under the Resource Management Act.

1.6 New Zealand Perspective

1.6.1. Previous research conducted on the Tarawera River.

A number of studies have been undertaken in an attempt to assess the biotic impacts of pulp and paper, municipal and geothermal discharges into the Tarawera River. However, very little research has been conducted investigating reproductive impacts on fishes in the Tarawera River catchment.

Between 1990 and 1994, Environment Bay of Plenty carried out detailed investigations of the water quality and flow in the Tarawera River catchment. The study incorporated toxicity, ecology, chemistry, and hydrology of the area. Results indicated that although the concentrations of pulp mill contaminants are relatively low, the chronic effects observed in receiving biota are consistent with bleached kraft mill effluent exposure (Dell et al. 1996). The ecological study demonstrated significant impacts on macroinvertebrate and plant communities. The status of fish communities in the river and tributaries was also assessed. The diversity of indigenous fish species tended to be lower in the down-river catchment area compared with the upper catchment. It is thought possible that poor water quality in the lower river is influencing juvenile fish migration and ultimately distribution (Dell et al. 1996).

Other studies have identified bioaccumulation of pulp and paper contaminants in freshwater and marine mussels (Hickey et al. 1993), as well as the induction of detoxifying mixed function oxygenase (MFO) enzyme activity in the livers of exposed fish. Significant increases in MFO activity measured as hepatic 7-

ethoxyresorufin-O-deethylase (EROD) have been recorded in both caged and feral eels at sites downstream of the Norkse Skog-Tasman effluent discharge point. Studies were undertaken in 1996 in an effort to determine the kinetics of EROD induction, the rate of loss of EROD activity following removal from the source, and to characterise the compounds causing the induction of EROD activity (in eels) in the Tarawera River (Jones et al. 1997).

Relatively few studies have focused on rainbow trout in the Tarawera River. However, one study by Donald (1997) investigated the health status of rainbow trout exposed to bleached kraft and chemithermomechanical pulp mill effluent in the river. Differences in health, measured as health condition profiles, were observed in downstream trout compared to a reference population. Some of the parameters identified in the profile included lower hematocrit, plasma protein and fat, as well as a reduction in liver size of exposed trout, suggesting a nutritional deficiency in these fish (Donald 1997). Trout in the lower river tended to congregate around stream mouths and consumed less food than reference populations. Organic contaminants associated with pulping and bleaching processes were detected in bile and tissue samples. In addition, relatively low induction of EROD activity was measured in exposed fish. A number of fish in the lower river had resorbed eggs, however gonad weights were not measured. It cannot be certain if this is due to reproductive impairment by compounds within pulp mill effluent or merely a reallocation of energy in response to an environmental stressor (Donald 1997).

Despite these observations, it has been suggested that the combination of effects observed were more likely the result of low DO levels, high river temperatures (which tend to exacerbate the effects of low DO concentrations) and decreased

water clarity, rather than sub-lethal effects of organic contaminants (Donald 1997).

1.6.2. Legislative requirements

In the early 1990's, community concerns regarding the effects of the mill discharges led to the development of the Regional Plan for the Tarawera River Catchment. An important aim of the plan was the development of water quality conditions adequate to support trout in the lower river (Donald 1997). Around the same time, environmental monitoring and regulation began to rely more upon the measurement of biological effects in exposed organisms rather than chemical concentrations in those organisms or their environment. The introduction of the Resource Management Act (RMA) in 1991 added further incentive towards 'effects-based' environmental monitoring and management (Jones et al. 1997). The primary purpose of the RMA was/is to promote the 'sustainable management' of natural and physical resources and the need to avoid, remedy or mitigate adverse effects (Mitchell 1997).

The Tarawera River Regional Plan stemmed from the RMA. Under the act, regional councils are responsible for the control of discharges of contaminants into air and water, as well as setting thresholds for managing effects of activities on the environment. Obviously, regional councils have a key role in the resource consent process and are required/responsible on receipt of a resource consent application to determine if further information is required to enable assessment of the application and whether or not the application should go before the public. Under the RMA, if regional councils believe an application should be publicly

notified (i.e. usually industry discharges) they are then required to serve notice of the application on every person or organisation considered to be an affected party, and place a public notice in the newspaper and affixed to the site (Mitchell 1997). Following public notice, any person/organisation wishing to make a submission against or supporting the proposal may do so within a limited period of time.

Within the Bay of Plenty region, much public concern and focus has been on the issue of colour in the Tarawera River. Public perception of the lower Tarawera River is mainly influenced by the poor visual appearance of the water. Perception studies of NZ waters have clearly defined the colour and clarity thresholds for bathing and aesthetic purposes (Dell et al. 1996). The discharge from pulp and paper effluents degrade the appearance and visual appeal of the river. The achievement of an "acceptable" green hue in the lower Tarawera River is dependent on a 95% reduction in the colour of the Tasman effluent, and at present discharges of large amounts of colour are a non-complying activity under current waste water discharge permits (Dell et al. 1996).

The Norske Skog-Tasman mill holds permits under the Resource Management Act (1991) for water abstraction, waste water and air discharges and solid waste disposal. These permits are issued by the Bay of Plenty Regional Council (Environment BOP) and have conditions relating to the volume and nature of discharges, monitoring and reporting requirements and staged improvements.

Norske Skog-Tasman currently holds permits to:

- Discharge contaminants to air
- Divert water from Urupa Lagoon and discharge to Tarawera River

- Discharge primary and secondary solids, asbestos, septic tank waste, solid and special wastes to land
- Discharge wastewater to the Tarawera River from ecology research tanks
- Discharge lime mud to land

Permits which have expired and applications to renew these are still being processed:

- Discharge treated wastewater to the Tarawera River
- Discharge stormwater, car-wash water, and filter backwash water to the Tarawera River
- Discharge oxygen into the Tarawera River to enhance oxygen levels

Norske Skog-Tasman Ltd is allowed under the RMA to continue operating under the old permits while the new applications are being processed.

Regarding waste water discharge consents, examples of some of Norske Skog-Tasman's consent orders include: waste water discharge less than 260 $ML \cdot day^{-1}$, suspended solids discharged less than 20 $t \cdot day^{-1}$ and the monthly average must be less than 14 $t \cdot day^{-1}$, effluent temperature less than 35°C, pH must be between 6 and 9, pentachlorophenol less than 1.87 kg·day and triclorophenol below 6.01 kg·day. There are restrictions on BOD_5 which varies with the river temperature and flow, but should not be higher than 3.5 ppm, as well as restrictions on allowable DO, colour, organic discharges and mixing zone limits into the river. There is also an added requirement to have regular consultation with groups in the area that are affected by mill operations and have regional concerns regarding mill impacts including local tangata whenua. This is specifically mentioned in the

RMA as well as issues such as water colour, clarity, and any significant adverse effects on aquatic life.

Under current legislation, the mill has DO load limits of greater than 6.5 $g \cdot m^{-3}$ 30-day mean, and a 7-day minimum of more than 5.0 $g \cdot m^{-3}$.

Toxicity limits are also included in the waste water discharge permit. Treated effluent must be non-toxic at environmentally relevant concentrations and the mill is required to perform standard acute toxicity tests using early life stage rainbow trout. In addition, the mill is required to conduct standard chronic (*Daphnia*) and Microtox™ (bacteria) toxicity tests (Environment Canada, 1990).

The Norske Skog Tasman waste water discharge permit currently in the application process will expire in 2012, once approved, with permit reviews every five years.

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Chapter Two: Preliminary Toxicity Assessment of Primary and Secondary Treated Effluent

2.1 Introduction

The assessment of the toxicity (acute and chronic) of pulp and paper and geothermal effluents discharging into the Tarawera River has been an issue of concern and investigated using several standard test protocols for some time now. In 1994, a study was undertaken to assess the toxicity of the Tarawera River by testing effluent discharges, the receiving waters and geothermal discharges. Responses of three different test organisms were investigated, with the assumption that a range of test species provides a greater level of ecosystem protection. The tests consisted of a 14-day chronic reproduction test using the cladoceran species *Daphnia magna* and *Daphnia carinata,* a 96-hour acute toxicity test assessing growth with a freshwater alga, *Selenastrum capricornutum* and a Microtox^{TM} acute toxicity test using the bioluminescent bacterium *Photobacterium phosphoreum* (Hickey 1994). Results suggested that effluent concentrations capable of affecting 10% and 20% of the test organisms (EC_{10} and EC_{20}) were 46% to 100% for Norske Skog-Tasman mill effluent, 19% to 43% for Caxton mill effluent and 4% to 19% for the geothermal effluents respectively. High concentrations of arsenic and hydrogen sulfide were thought to contribute significantly to the toxicity of the geothermal effluent (Dell et al. 1996). However, no significant acute or chronic toxicity was demonstrated in river water samples collected below the points of effluent discharge. Contrasting results utilising vertebrate species have shown some toxic effects. Beresford (1993) exposed early

life stage zebrafish (*Brachydanio rerio*) to various concentrations of Norske Skog-Tasman and Caxton effluents and found 20% (v/v) Caxton effluent had a significant impact on hatching and mortality of eggs and larvae. Norkse Skog-Tasman effluent at concentrations between 20-100% had no impact on mortality although hatching was delayed slightly. The effluent concentration range for these studies was set well above the actual dilution range of Norske Skog-Tasman effluent into the Tarawera River which is between 5 to 12%. Therefore it cannot be assumed that such effects would be repeated in the receiving environment.

As part of the Norske Skog-Tasman wastewater discharge to the Tarawera River resource consent, treated effluent must be non-toxic at environmentally relevant concentrations and the mill is required to perform standard acute toxicity tests using early life stage rainbow trout. In addition, the mill is required to conduct standard chronic D*aphnia* and Microtox™ (bacteria) toxicity tests.

Given the number of standard toxicity tests that have been and are regularly undertaken, the purpose of this chapter was not solely to reassess acute and chronic toxicity of Norske Skog-Tasman treated effluent at environmentally relevant concentrations but also to look at the toxicity of effluent prior to treatment in an aerated stabilisation pond system (primary effluent) in comparison to secondary treated effluent over a wide range of concentrations. A secondary aim was to then identify a range of effluent concentrations which would be suitable for use in multi-concentration exposures for subsequent mosquitofish experiments (chapter five). Two test species were utilised in the acute and chronic toxicity tests, *Daphnia carinata* and the mosquitofish. *Daphnia* because

they are a standard test species and mosquitofish to establish non-lethal effluent concentrations suitable for future experimentation with this species.

Daphnia carinata are a native freshwater cladoceran species, often referred to as "water fleas". *Daphnia* are a widely used standard test species for environmental impact assessment and the monitoring of discharges. They are used for routine acute and chronic toxicity assessment (ASTM, 1997). However, *Daphnia carinata* are not normally found in the fast flowing Tarawera River and more typically inhabit lakes, ponds and backwaters (Hickey 1994). *Gambusia affinis* (mosquitofish) are a poeciliid species and were introduced to New Zealand in the 1930s. They are a subtropical freshwater fish found throughout the North Island (McDowall 2000). Mosquitofish are found along the length of the Tarawera Catchment particularly around the warmer areas of geothermal input as well as at the Matata estuary on the coast (Donald pers comm. 1999). See chapter five for a detailed description of *G.affinis*.

2.2 Methods and Materials

2.2.1. *Daphnia*

The protocol for *Daphnia* acute and chronic toxicity testing was conducted according to guidelines recommended by ASTM Standard Guide (1997) for conducting *Daphnia magna* Life-Cycle Toxicity Tests and the Environment Canada Reference method EPS 1/RM/14 (1990). Biological test method: Reference method for determining acute lethality of effluents to *Daphnia magna*. *Daphnia carinata* were used instead of *D.magna* because Forest Research did not have the appropriate MAF permits to house and conduct tests on this particular

species. *Daphnia carinata* were kindly donated by Dr Chris Hickey and identified by Dr Ann Chapman.

2.2.1a. 48-hour acute toxicity test

Test organism and culturing

Individuals selected for the acute toxicity tests were neonates less than 24 hours old, all originating from a laboratory culture. The brood stock had been cultured for three generations and remained healthy and stable without production of ephippia over this period. The ephippium is an egg case which develops inside an adult female daphnid in response to adverse conditions and the eggs have usually been fertilised through sexual reproduction. The culturing protocol followed Environment Canada EPS methods (1990). The brood culture was maintained in Ngongotaha Spring water with temperature and lighting conditions identical to those used in the acute test. Brood chambers consisted of 4 L glass aquaria, which had gentle aeration and weekly water replacement. The brood stock was fed a combination natural and synthetic diet consisting of YCT and freshwater algae (*Selenastrum capricornutum*). YCT is a combination of yeast, chlorophyl and digested trout pellets blended together and allowed to ferment for 2-3 days. *Selenastrum* was cultured following the protocol recommended by the ASTM (1997).

Test conditions and procedure

A 48-hour static test was conducted inside incubators which had a photoperiod set at 16:8 (L:D) and light intensity of 600 lux. Temperature was maintained at 20 ±2

°C. Each test treatment had a pH between 6-9, and dissolved oxygen was never below the critical experimental level of 5.5 $mg \cdot L^{-1}$. During the test, test chambers were not aerated, nor was pH adjusted. Test chambers consisted of 60 mL polyethylene cups which were covered during the experiment and prior to use were soaked in distilled water for 48 hours. All chambers and plastic covers were identical. Neonate daphnids were randomly assigned to each replicate test chamber and were not fed during the course of the test. The test consisted of nine treatments with five replicates per treatment each containing five daphnids. The test volume was 50 ml. Daphnids were exposed to either primary or secondary treated effluent, both of which were at concentrations of 15%, 25%, 50% and 100% (v/v). Reference (control) and diluent water was sourced from the Ngongotaha Spring supply. Primary effluent was sourced from pond one of the Norske Skog-Tasman four-pond aerated stabilisation basin system, while secondary or final treated effluent were sourced immediately prior to discharge from pond four into the Tarawera River. Effluent samples were transported to the laboratory in 20 L polyethylene carboys and stored in the dark, refrigerated at 4 °C, until use. At experimental termination the number of surviving daphnids was recorded. The test began two days following effluent collection.

Physicochemical conditions

Basic physicochemical parameters were measured in each of the treatment groups prior to random assignment of daphnids and placement in the incubator.

Table 2.1. Physicochemical test conditions. P1=pond one, primary effluent. P4=pond four, secondary effluent

Physicochemical parameters			
Treatment	**Temp ^{o}C**	**pH**	**DO % saturation**
Control	18.3	6.84	119
P1 15%	19.4	7.55	94.4
P1 25%	19.1	7.63	96.2
P1 50%	19	7.4	87.4
P1 100%	19.3	6.75	98
P4 15%	19.6	7.53	95.1
P4 25%	18.9	7.69	96.9
P4 50%	19.3	7.87	94.1
P4 100%	19.1	6.9	136

Statistical analyses

Experimental mortality/survival was analysed using analysis of variance (ANOVA), followed with Tukey's post-hoc test for multiple comparisons. The critical level of statistical differences for analyses was assessed at α=0.05. All statistical testing was completed using the GraphPad Prism 3® software package.

2.2.1b. 21day chronic toxicity test

Test organism and culture

Individuals selected for the chronic test were all neonates less than 24 hours old, originating from a laboratory culture. The brood stock had been cultured for three generations using the same food, temperature, photoperiod and light intensity as that in the chronic test. The culturing protocol followed Canadian EPS methods (1990). The brood culture remained healthy and stable without ephippia production over this period and was maintained in 4L glass aquaria containing

Ngongotaha Spring water which was replaced weekly. The culture was fed a combination natural and synthetic diet consisting of YCT and algae as described above.

Test conditions and procedure

One neonate daphnid was randomly assigned to each replicate. There were five replicates per treatment and a total of nine treatments consisting of primary and secondary treated effluents, both at concentrations of 15%, 25%, 50% and 100% (v/v). The test volume was 50 mL. Control and diluent water was sourced from the Ngongotaha Fish and Game Trout Hatchery spring water supply. Primary and secondary effluents were sourced and stored in the same manner as those described in the *Daphnia* acute toxicity test. Exposure began within two days of effluent collection. Individual daphnids were exposed for a period of 21 days. The number of offspring they produced was recorded and offspring removed every second day, which coincided with effluent and water changes. Daphnids were fed approximately 0.3 mL of algae (1.0×10^8 algae cells$\cdot L^{-1}$) to 0.15 ml YCT once a day. Test chambers were 60 mL polyethylene cups which had been soaked in distilled water for 48 hours prior to use. Chambers were covered with clear plastic sheets throughout the course of the test. The exposure was undertaken in an incubator with a photoperiod of 16:8h (L:D), temperature of $20 \pm 2^{\circ}C$ and the light intensity was 600 lux. During the 21-day exposure, test chambers were not aerated nor was pH adjusted, however, pH remained between 7 and 8 and dissolved oxygen was at saturation levels.

Physicochemical conditions

Basic physicochemical parameters were measured in each of the treatment groups prior to random assignment of daphnids and placement in the incubator.

Table 2.2. Physiochemical test conditions. P1=pond one, primary effluent. P4=pond four, secondary effluent

Physicochemical parameters			
Treatment	**Temp °C**	**pH**	**DO % saturation**
Control	18.9	7.2	112
P1 15%	18.6	7.52	107.1
P1 25%	18.8	7.57	102.4
P1 50%	18.5	7.6	101.6
P1 100%	18.7	7.39	102
P4 15%	18.8	7.46	108.3
P4 25%	18.7	7.55	104.7
P4 50%	18.7	7.68	102.5
P4 100%	18.7	7.59	102.6

Statistical analyses

The total number of offspring produced by each daphnid over the 21-day period was analysed using analysis of variance (ANOVA), followed with Tukey's post-hoc test. The critical level of statistical differences for analyses was assessed at α=0.05. All statistical testing was completed using the GraphPad Prism 3® software package.

2.2.2 Mosquitofish

Fish and housing facility

Mosquitofish (*Gambusia affinis*) were captured from a population found in the University of Waikato campus lakes, Hamilton using hand nets. Fish were transported to the laboratory where the sexes were separated. Only adult male mosquitofish (20-30 mm long) were used for the initial toxicity test due to low numbers of females. Mosquitofish had been held under laboratory conditions for four weeks prior to testing and showed no signs of disease or stress. Fish were held and acclimated in dechlorinated city water and were fed standard tropical fish flake feed daily until satiation. Holding aquaria were aerated gently, had activated charcoal filters and 90% water replacement weekly.

Experimental design and exposure

The test was conducted under ambient laboratory temperature conditions, with a 12:12h (L:D) photoperiod. Due to *Gambusia's* high tolerance of low DO levels, the test treatments were not aerated. The test volume was 3L with no replacement over the course of the test. Five individuals were randomly placed into each treatment. Fish were exposed to either primary or secondary treated effluent, both of which were tested at concentrations of 5%, 15%, 25%, 50%, 75% and 100% (v/v). Due to low mosquitofish numbers there was no experimental replication. Exposure was for a 48-hour period, during which the fish were monitored hourly for the first eight hours. After 48-hours, survival was recorded. Primary effluent was sourced from pond one of the Norske Skog-Tasman four-pond aerated stabilisation basin system, while secondary or final treated effluent was sourced immediately prior to discharge from pond four into the Tarawera River. Effluent

was transported to the laboratory in 20 L polyethylene carboys and stored refrigerated at 4°C until use. Dechlorinated Rotorua City tap water was used for control and diluent waters.

Physicochemical parameters

Basic physicochemical parameters were measured prior to random placement of fish into each treatment in order to ensure that dissolved oxygen, conductivity and pH were within the tolerance range for *Gambusia*.

Table 2.3. Physicochemical conditions. P1=pond one representing primary effluent. P4 = pond four representing secondary effluent.

Physicochemical parameters				
Treatment	**Temp ^{0}C**	**DO % saturation**	**conductivity $\mu S \cdot cm^{-1}$**	**pH**
control	18.5	101.4	70	7
P1 5%	18.6	99.8	118	7.59
P1 15%	18.4	85.1	212	7.74
P1 25%	18.5	85	290	7.81
P1 50%	18.3	73.2	503	8.02
P1 75%	18.5	59	679	8.05
P1 100%	18.8	49	876	8.09
P4 5%	18.3	92.1	103	7.2
P4 15%	18.7	90.4	158	7.46
P4 25%	18.8	79.1	218	7.8
P4 50%	19.4	71.8	347	7.97
P4 75%	19.4	60.7	470	8.01
P4 100%	19.3	59.7	587	8.02

Statistical analyses

Due to the small sample size and no replication, statistical analyses could not be conducted.

2.3 Results

2.3.1. *Daphnia*

48-hour acute toxicity test

During a 48-hour exposure of neonate daphnids, secondary treated effluent at concentrations of 15%, 25%, 50%, 100% had no effect upon daphnid mortality (Figure 2.1.) Survival was also unaffected in primary effluent at concentrations of 15%, 25% and 50%. However, 100% primary effluent had a significant impact with only 68% survival ($P < 0.05$). None of the individuals in the dilution-water control groups produced ephippia and both groups had 100% survival. Following ASTM guidelines, control or reference treatments must not produce ephippia to be considered acceptable.

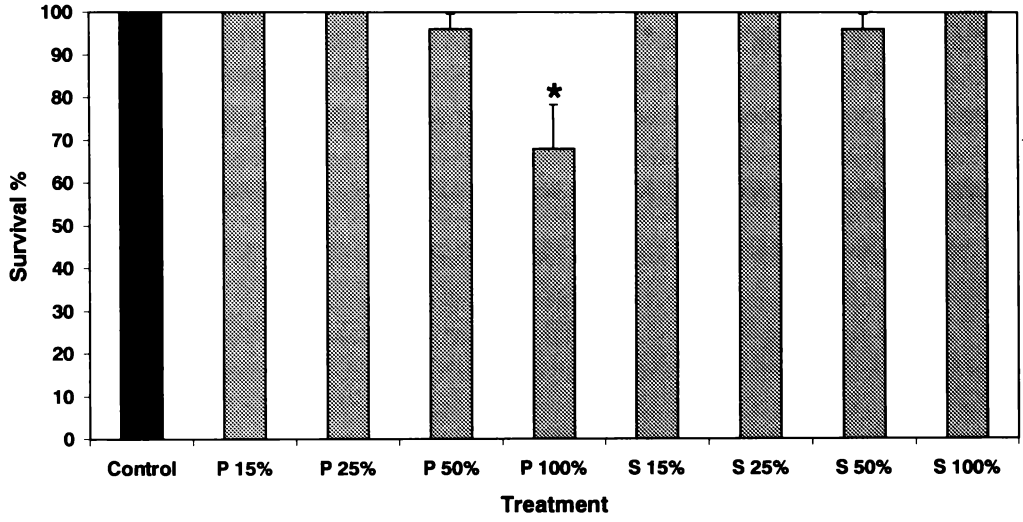

Figure 2.1. Total percentage survival of *Daphnia carinata* exposed to primary and secondary treated effluent at concentrations of 15, 25, 50 and 100% (v/v) and Ngongotaha spring water, for a period of 48-hours. Each bar represents the total percent for 5 replicates with a sample size of 5 each. Asterisks indicate significant differences from the control (ANOVA, $\alpha=0.05$). 'P' represents primary effluent and 'S' represents secondary treated effluent.

21-day chronic toxicity test

A significant decrease in offspring production was observed over a 21-day exposure to primary effluent at concentrations of 50% and 100% (v/v) ($P<0.05$). The mean number of offspring produced were 92 and 10 for 50% and 100% respectively (Figure 2.2.). By comparison, the mean number of offspring produced in the control treatment was 177. No individuals in the control treatment produced ephippia. Secondary treated effluent at a concentration of 100% also resulted in a significant decrease in offspring with a mean of 101 individuals produced from five replicates. Effluent concentrations of 15% and 25% in both effluents and 50% in secondary treated effluent, had no significant impact on offspring production in comparison with the control treatment. The first broods produced were on day eight in all treatments except the 100% concentrations. At the end of the 21-day exposure two individuals were dead in 100% primary effluent and one in 25% secondary treated effluent.

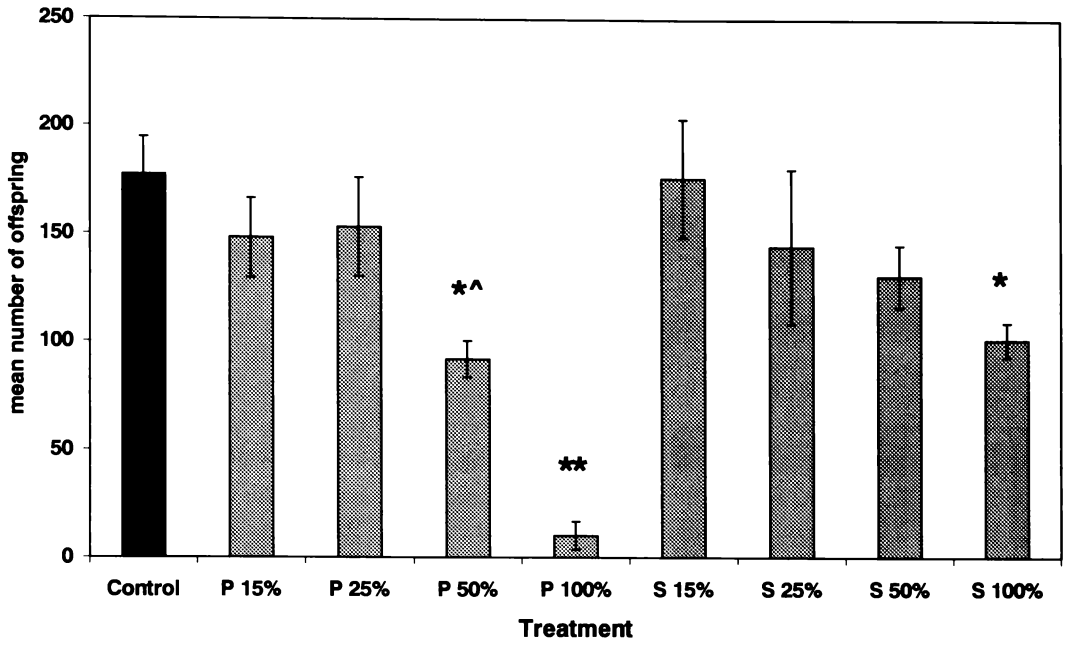

Figure 2.2. Mean (±95% C.I.) offspring production of *Daphnia carinata* exposed to primary and secondary treated effluent at concentrations of 15, 25, 50 and 100% (v/v) and Ngongotaha spring water over a 21-day period. Each bar represents five individuals. Single asterisks indicate a significant difference from the control, a double asterisk indicates a significant difference from all other treatments, and '^' indicates a significant difference from all treatments except S50 and S100% (ANOVA, α=0.05). 'P' represents primary effluent and 'S' represents secondary treated effluent.

2.3.2 Mosquitofish

Exposure to secondary treated effluent for a period of 48-hours had no effect on adult mosquitofish survival at concentrations of 5%, 15%, 25%, 50%, 75%, or 100% (v/v) (Figure 2.3.). In addition, no individuals in any of these treatments showed signs of disease or stress such as discolouration or unusual behaviour. Both of the controls had 100% survival with no signs of stress in any individuals. In contrast, mortality was extremely rapid in primary effluent concentrations of 75% and 100% (v/v). Within three hours of exposure 100% mortality was observed. Primary effluent concentrations at 50% also proved to be lethal with

100% mortality within six hours. Twenty percent mortality was noted in 25% primary treated effluent, however the surviving individuals showed no obvious signs of stress. Concentrations of 5% and 15% primary effluent did not affect mosquitofish survival.

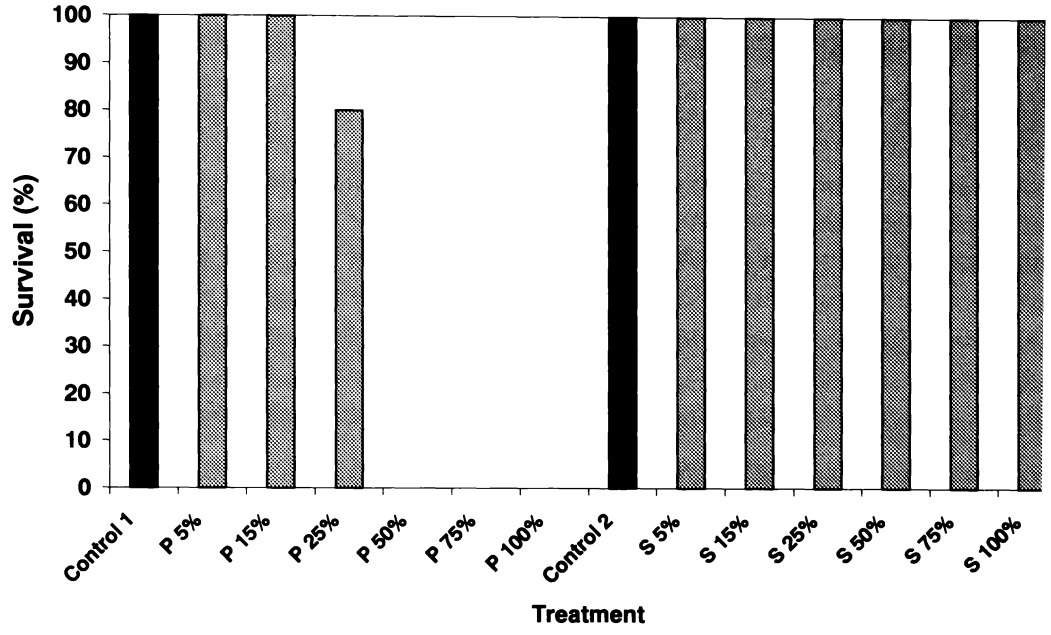

Figure 2.3. Percentage survival of *Gambusia affinis* exposed to primary and secondary treated effluent at concentrations of 5, 15, 25, 50, 75 and 100% (v/v) and dechlorinated tap water for a period of 48-hours. Each bar represents a sample size of five. 'P' represents primary effluent and 'S' represents secondary treated effluent.

2.4 Conclusions

One of the most notable observations from this preliminary study were the differences in species response. Mosquitofish are far more susceptible to effluent exposure than *Daphnia*. Mosquitofish had much higher mortality in primary effluent at concentrations of 50%, 70% and 100% compared with *Daphnia*. Given

that effluent is secondary treated in a stabilisation pond system prior to discharge, toxicity from primary effluent exposure has no environmental relevance, however, it enabled the determination of non-lethal concentrations for future mosquitofish exposures (chapter five). Secondary treated effluent had no significant impact on acute lethality in mosquitofish or *Daphnia* at any of the test concentrations, demonstrating that most of the effluent acute toxicity is lost following secondary treatment. One hundred percent secondary effluent did have a significant impact on daphnid offspring production. A significant decrease in the number of juveniles produced by *Daphnia* was also observed in primary effluent at concentrations of 50% and 100%. Again, primary effluent has no environmental relevance and Norske Skog-Tasman's treated effluent dilution in the Tarawera River is between 5 and 12%.

Ngongotaha Spring water was a successful control and diluent water in the present study, however, an early report by NIWA tested Ngongotaha Spring water in a 14-day daphnid chronic toxicity test and concluded that exposure to Ngongotaha Spring water resulted in a 21% decrease in offspring production compared to control water supplied from a local spring (NIWA report FRI170201/2237, 1997). According to ASTM (1997) guidelines the Ngongotaha spring water used in the present tests was appropriate since the average number of offspring produced in the control group was 177 neonates over a 21-day period. Guidelines recommend control total offspring production must be greater than 60 individuals per parent over 21 days for *Daphnia* species (ASTM,1997).

It was concluded that treated effluent at any concentration and primary effluent at concentrations less than or equal to 15% would not have a lethal toxic effect and would be suitable for future mosquitofish experiments.

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Chapter Three: Long-term Early Life Stage Exposure of Rainbow Trout to Treated New Zealand Pulp and Paper Mill Effluent

3.1 Abstract

Internationally, studies have noted effects of pulp and paper effluents on the reproductive physiology of fishes including reduced fertilisation and hatching success, smaller gonads, increased age to maturation, alterations in secondary sex characteristics and reduced plasma sex steroid levels. The chronic effects of secondary-treated effluent from a modern New Zealand pulp and paper mill were assessed using a long-term, early life stage laboratory exposure of rainbow trout, *Oncorhynchus mykiss*. Rainbow trout were exposed to a mixed thermomechanical pulp and bleached kraft mill (TMP/BK) effluent at an environmentally relevant concentration (15 % whole secondary treated effluent by volume) during a 320-day exposure. Results indicate that from egg fertilisation to ten months of age, no statistically significant difference between exposed and reference groups occurred with regard to fertilisation and hatching success, time to hatch, length at hatch, or time to swim up. Growth and development throughout the course of the experiment was not affected by exposure to effluent. At experimental termination, effluent exposed fish had significantly (statistically) smaller livers and reduced condition compared to the reference group. Estrogen receptor expression and vitellogenin induction in the juvenile rainbow trout was not observed.

3.2 Introduction

The impact of pulp and paper mill effluents on aquatic organisms has been extensively investigated since the 1960s, and linked to a variety of toxic effects including reproductive impairment (Owens 1991, Zacharewski 1997). Some of the observed reproductive impacts include decreased egg and gonad size, reduced fertilisation and hatching success, delayed maturity (McMaster et al. 1991, 1996; Munkittrick et al. 1992a, 1994), as well as decreases in serum sex steroid levels and overall gonadal steroid production (Van Der Kraak et al. 1992; Munkittrick et al. 1992a; McMaster et al. 1994, 1995; McCarthy et al. 1997). In addition, decreases in egg and sperm viability and embryo and fry quality have also been noted (Tana and Nikunen 1986). Recently, the synthesis of plasma vitellogenin (an egg yolk precursor protein) and vitellogenin gene expression in the liver of male fish has been reported following BKM effluent exposure. (Soimasuo et al. 1998; Mellanen et al. 1999; Tremblay and Van Der Kraak 1999).

Egg hatchability and fry development are considered to be the sensitive stages of fish exposed to chemical toxicants and effluents. Many laboratory studies have been conducted to investigate the effects of chemical pulp mill effluents on reproduction and early life stages (Johnsen et al. 2000). These studies have focused on direct exposure of early life stages as well as generational impacts, exposing sexually mature adults prior to spawning and laboratory exposures of eggs and milt taken from fish in pulp and paper mill effluent receiving water populations (Tana and Nikunen 1986; Kovacs et al. 1995b). One such study found eggs from a pike population previously living in BKME receiving waters had higher survival/viability when exposed to effluent compared with eggs from a

reference population (Tana and Nikunen 1986). Kovacs et al. (1995a) have also investigated survival and hatching success of eggs from laboratory pre-exposed sexually mature fathead minnows and found no effluent-related effects on hatching of the second generation. Other laboratory based studies have focused on exposing early life stages to compounds with endocrine disrupting potential which naturally occur within pulp and paper mill effluents. For example, Lehtinen et al. (1999) found a causal link between effects on eggs and brood through dose-dependent increases in phytosterols in the roe, indicating that wood-derived compounds in pulp and paper effluents may be responsible for previously observed reproductive impacts.

In recent years the international pulp and paper industry has committed considerable resources to environmental improvement through process modifications and the installation or upgrade of secondary treatment facilities (Kovacs et al. 1997). The benefits of these investments in terms of improved effluent quality and the decreased impact on physiology of receiving water fish populations have been well documented (Swanson et al. 1993, 1994; Gagnon et al. 1994a, 1994b; Kloepper-Sams et al. 1994a, 1994b). However, significant impacts on reproductive parameters continue to be recorded at sites where process and treatment modifications have been implemented (Munkittrick et al. 1992b, 1992c; MacLatchy et al. 1997). Results have been conflicting, in that, physiological changes have been observed at mills with and without ECF bleaching, secondary effluent treatment and other treatment/technology upgrades in place and it has been suggested that reproductive endocrine impacts are not

solely due to the bleaching process (MacLatchy et al. 1997) or chemical additives of pulping, but also naturally occurring plant compounds.

Long-term, early life stage (ELS) and life cycle studies which expose organisms to effluents from birth to sexual maturity and reproduction, are very comprehensive means of evaluating effluent impact in the laboratory (Kovacs et al. 1995b). To date no long-term ELS studies have been undertaken in New Zealand to assess potential impacts of BKME. However, a study assessing fish health in the Tarawera River was conducted by Beresford (1993) and investigated hatching and mortality of zebrafish eggs and larvae exposed to Caxton and Norske Skog-Tasman effluents. She concluded that exposure to 10 to 100% Norske Skog-Tasman effluent had no effect on mortality and resulted in a slight delay in hatching. Mortality was high in eggs and larvae exposed to concentrations of 40 to 100% Caxton effluent and hatching occurred earlier than normal.

The aim of the present study was to enable a long-term assessment of the effects of a modern New Zealand pulp and paper mill effluent at an ecologically relevant concentration, on the early life stages of rainbow trout (*Oncorhynchus mykiss*) in a laboratory exposure. Two experiments were conducted, the first focused on water-hardening, fertilisation and subsequent hatching success. The second experiment was a long-term assessment of gamete viability, growth and development, as well as other physiological and reproductive and physiological parameters (e.g. vitellogenin induction and condition factor).

3.3 Methods and materials

Mill description

The Norske Skog-Tasman Mill (formally Fletcher Challenge Paper) is an integrated bleached kraft mill and thermomechanical (BK/TM) pulp and paper mill, 760 and 1010 a.d.t.d^{-1} (air dried tonne per day) respectively. Mill production is primarily softwood (*Pinus radiata*) with occasional eucalypt production and has been elemental-chlorine free (ECF) since April 1998. Norske Skog-Tasman implements biological secondary wastewater treatment and elemental-chlorine-free (ECF) bleaching. The wastewater treatment system consists of a thermomechanical pulping pre-treatment bioreactor facility within the TM pulp mill. Kraft mill effluent is then collected into a single drain and passed through two bar screens and a clarifier for partial solids removal and final treatment in a four pond aerated stabilisation basin system. The ponds have an area of 45 hectares with a retention time of 5-6 days. Following treatment in the aerated lagoon system effluent is discharged into the Tarawera River at a total mean volume of 180,000 $m^3 \cdot d^{-1}$. River effluent dilution ranges between 5 and 12% and enters the river system with an average temperature of 26-28°C.

Fish

Rainbow trout eggs for experiment one were obtained from the Fish and Game Rotorua trap situated on Lake Tarawera, Rotorua, New Zealand. Eggs and sperm were stripped and pooled from three females and three males respectively, at the trap.

Rainbow trout eggs for experiment two were obtained from the Department of Conservation National Trout Hatchery trap situated on the Tongariro River, located near Turangi, New Zealand. Eggs were stripped and pooled from three ripe females and fertilized on-site with sperm pooled from two males. Eggs were left undisturbed for 15 minutes while water-hardening and fertilisation occurred, then transported on ice back to the laboratory and immediately allocated to treatments and replicates. Water-hardening generally occurs during fertilisation and is the process by which the egg absorbs all water needed for embryonic growth and development, once this occurs the egg hardens (Cudby pers comm. 2001).

Experiments began in late-July towards the final stages of spawning. The main spawning in New Zealand takes place in the late autumn and through the winter, with peak migration occurring in June and July. However spawning continues well into August and some areas have late spawning into October.

Experimental design and exposure

The first experiment was a preliminary study assessing the effects of effluent exposure on fertilisation and water-hardening of rainbow trout eggs. Approximately 1000 eggs were mixed with sperm pooled from 3 males and immediately halved into two groups and placed in 15% effluent or Lake Tarawera water and left undisturbed for 15 minutes. Eggs were transported back to the laboratory (travel time of 20 minutes) and divided between four treatments. No transport mortalities were observed. Eggs water-hardened in 15% effluent were divided into one group of 100 held in 15% effluent (EE) and another group of 200

held in reference water (ER). Those eggs water-hardened in reference water were divided into a group of 200 (RR) remaining in reference water and another group of 100 (RE) placed in 15% effluent for the remainder of the experiment. Effluent was obtained from the Norske Skog-Tasman mill in Kawerau, immediately before discharge into the Tarawera River, and was transported to the laboratory in Rotorua. Approximately 90,000 L of 100% treated effluent was transported on a weekly basis and stored in a cool, dry site outside the laboratory. Dechlorinated Rotorua City tap water was used as reference and diluent water for all laboratory exposures. Sodium thiosulphate (NaS_2O_3) at a concentration of $1mg \cdot L^{-1}$ was used to remove chlorine from the tap water.

It was the intention to continue the exposure for 28 days or until hatch. The experimental set-up consisted of four PVC pots (15 cm diameter) with a total volume of 2 L. Eggs were housed in fine mesh baskets (5 cm deep), held at the top of each pot. Water was set-up in a gravity flow through each pot via plastic flexible tubing (15 mm diameter) at a rate of 250 $mL \cdot min^{-1}$. Mixing and cooling header tanks were orientated at one end of each treatment. Final treatment effluent was continuously pumped via a peristaltic pump into the effluent exposure mixing tank and set for a desired concentration of 15% (v/v). The experimental pots were covered with black plastic sheets for the first 20 days because days 5 to 20 are considered the most sensitive stages of embryonic development. Temperature was maintained between 11.8 and 14 °C, measurements of pH, DO and temperature were recorded daily. Dead eggs were also removed daily. Twenty-four hours following fertilisation twenty eggs were removed from treatments EE and RE and 75 eggs from ER and RR and preserved in Stockard's Solution to harden and clear the eggs for determination of fertilisation success (by light microscopy). Those

eggs that had a collapsed blastodisc were considered unfertilised or unable to continue development during the cleavage stage (Knight 1963). Stockard's solution consists of 5% formaldehyde, 4% glacial acetic acid, 6% glycerol in distilled water.

A further sample of 10 eggs per treatment was taken at the early-eyed stage (14 days) to access embryonic development. At this stage high mortality was observed and the experiment was terminated after 25 days of exposure when some hatching was occurring. Experimental termination coincided with the discovery of chlorine toxicity due to peristaltic pump failure.

The second experiment was a 320-day laboratory exposure to investigate embryonic development, hatching success and timing, swim-up and growth and development of juvenile trout exposed to treated TMP/BK effluent at 15% (v/v) and a reference water. Effluent was collected, transported and stored as described in experiment one. Dechlorinated city water was used as reference and diluent. Sodium thiosulphate (NaS_2O_3) at a concentration of 1 $mg \cdot L^{-1}$ was used to remove chlorine from the tap water, which was added to the reference water holding tank with a peristaltic pump. The sodium thiosulphate flow into the reference tank was measured daily.

Fertilised and water-hardened eggs were transported back to the laboratory and placed into one of two treatment groups. No transport related mortalities were observed. Approximately 1500 eggs were divided among five replicates per treatment (300 eggs per pot). The initial experimental set-up consisted of five PVC pots (15 cm diameter) with a total volume of 2 L. Eggs were housed in fine

mesh baskets (5 cm deep), held at the top of each pot. Water was set-up in a gravity flow through each pot via plastic flexible tubing (15 mm diameter) at a rate of 400 $mL \cdot min^{-1}$ to allow for the greater number of eggs used in experiment two. Mixing and cooling head tanks were orientated at one end of each treatment. Final treatment effluent was continuously pumped using a peristaltic pump into the effluent exposure mixing tank and set for a desired concentration of 15% (v/v). Effluent flows were monitored daily. The experimental pots were covered with black plastic sheets during the first 20 days of exposure.

When trout reached swim-up (had absorbed their yolk-sac and were feeding at the surface) they were transferred into larger hatchery rearing troughs with divided baskets (70 cm x 45 cm x 20 cm). Baskets were divided into two sections with mesh screens and were suspended in the rearing troughs (3 m x 0.5 m x 0.25 m) which had a drain of 75 mm diameter at one end to allow continuation of flow-through exposure. The flow rate was increased to 600 $mL \cdot minute^{-1}$. In addition, small pumps circulated water about the troughs in order to maintain consistent temperature within the system. Transparent plastic covers were placed over the troughs to prevent fish from jumping out. Once the fingerlings were six months old the baskets were removed and the five replicates per treatment were collapsed into one large group per treatment. This was essential due to the physical limitations of space, experimental apparatus, water and effluent supply in the laboratory.

Temperature was maintained between 11.8 and 14°C and was measured hourly with temperature probes (data loggers). The average temperature over the experimental period of 12.9 °C and 12.8°C for reference and effluent treatments

respectively. Temperature, dissolved oxygen, conductivity and flow rates were monitored daily and a constant photoperiod was set at 12h light:12h dark.

Trout were fed commercial salmon food (Pacific start, 10% lipid, 53% crude protein, NRM Feed Mills, Hope-Nelson, NZ). The size of pellet was adjusted as the fish grew, ranging from well ground (crumbles) fry-size to 2.7 mm diameter pellets. Trout were fed ad libidum daily until satiation.

Experimental endpoints were fertilisation and hatching success, sex ratio, growth and development including embryo development, length at hatch and swim-up, as well as occurrence of visible morphological abnormalities and mortality. The occurrence of any developmental abnormalities, most commonly cephalic twinning, symmetric conjoined twinning, kryptosis and other spinal malformations, as well as mortality were recorded daily and dead individuals removed. Sub-sampling periods for chemistry, growth and development were taken at one, two and three week intervals following egg fertilisation, samples were stored at -20°C. A further sample was taken at swim-up. During the course of the experiment the rainbow trout passed through various development stages, the most critical stages being; 1. 24 hour eggs, 2. early eyed (18 days), 3. late eyed stage, 4. hatching and 5. swim-up and feeding. These stages were monitored with dissecting scope observations made on individuals sacrificed during sub-sampling intervals.

Following 320 days of exposure, fish were sacrificed with a blow to the head and measurements of length, weight and liver size were taken for calculations of somatic indices and condition factor. If possible, sex was determined. Blood samples were collected (via caudal puncture) and pooled, then placed on ice in

heparinized collection tubes until centrifugation and storage at -80°C. Liver and pooled bile samples were removed and snap frozen in liquid nitrogen and stored at -80°C prior to vitellogenin, estrogen receptor and chemical analyses of organic chemicals typically found as constituents of treated effluent. Muscle samples for chemical analyses were individually wrapped in pre-fired tin foil and stored at -20°C.

Analyses

Vitellogenin.

Vitellogenin (vtg) induction at the protein level was determined using western blotting techniques for detection of the protein and an enzyme linked immunosorbent assay (ELISA) for quantification of plasma protein concentration. Western blotting was conducted following an in-house protocol adapted from the methods of Gershoni (1983); Copeland et al. (1986); Denis et al. (1988) and Pelissero et al. (1993). Plasma samples were prepared for protein determination by the method of Bradford (1976). Plasma proteins were separated by gel electrophoresis and blotted to nitrocellulose membranes for staining and antibody binding. An ELISA to determine levels of vtg in trout plasma samples was performed using a Biosense Laboratories Quantitative Rainbow Trout vitellogenin ELISA kit (Vtg-102). The kits are based on a competitive binding assay and a monoclonal vtg antibody developed towards salmonid species. The assay has a working range of 150-6000 $ng \cdot mL^{-1}$ of vtg.

Vitellogenin induction at the mRNA level was determined by reverse transcriptase- polymerase chain reaction (RT-PCR) using a Primus 25/96 Thermocycler, according to 'in-house' methods adapted from LeGuellec et al.

(1988); Pakdel et al. (1989); Ren et al. (1996); Vanden Heuvel (1998). In brief, RNA was isolated from liver samples by phenol extraction with TRIzol Reagent and collected via isopropanol precipitation and ethanol washes. RNA was then denaturated at 58°C (15 minutes) and the nucleic acid concentration determined. RNA was translated to cDNA by a reverse transcriptase (RT). Using specific primers, the sample cDNA-species was amplified by polymerase chain reaction (PCR), followed by cDNA separation on agarose gel for detection with ethidium bromide and UV light photography. Expression of the estrogen receptor was determined at the mRNA level using RT-PCR techniques described above.

Water extractable trace organics

Weekly effluent samples were collected over a three month period towards the end of the experiment and filtered using 15 cm Whatman GFC filters. Filtrate and filter papers were stored at 4°C and -20°C respectively. Aqueous effluent samples for determination of organic extractives (i.e. resin acids, fatty acids, phytosterols, phenolics or monoterpenes) were extracted by continuous liquid-liquid extraction at pH 9. The solvent volume was reduced, samples were dried with anhydrous sodium sulphate and then derivatised (sitylation) for analysis by gas chromatography/mass spectrometery (GC-MS). The filter papers were ground with sodium sulfate and placed in a soxhlet extractor overnight with methylene chloride. All organics were corrected for extraction blanks and adjusted for the recovery of the appropriate surrogate standard.

Trace organics in bile

Total organics in bile were determined after hydrolysis of the bile sample with ethanolic potassium hydroxide. Hydrolysed bile was acidified and extracted with methyl tertiary-butyl ether and the extract was dried through sodium sulphate, transferred into hexane and applied to a florisil cartridge. The compounds retained on the cartridge were eluted with 1:9 ethanol:hexane, derivitised, and analysed by GC-MS. Samples were corrected for surrogate recovery and blank determinations.

Statistical analyses

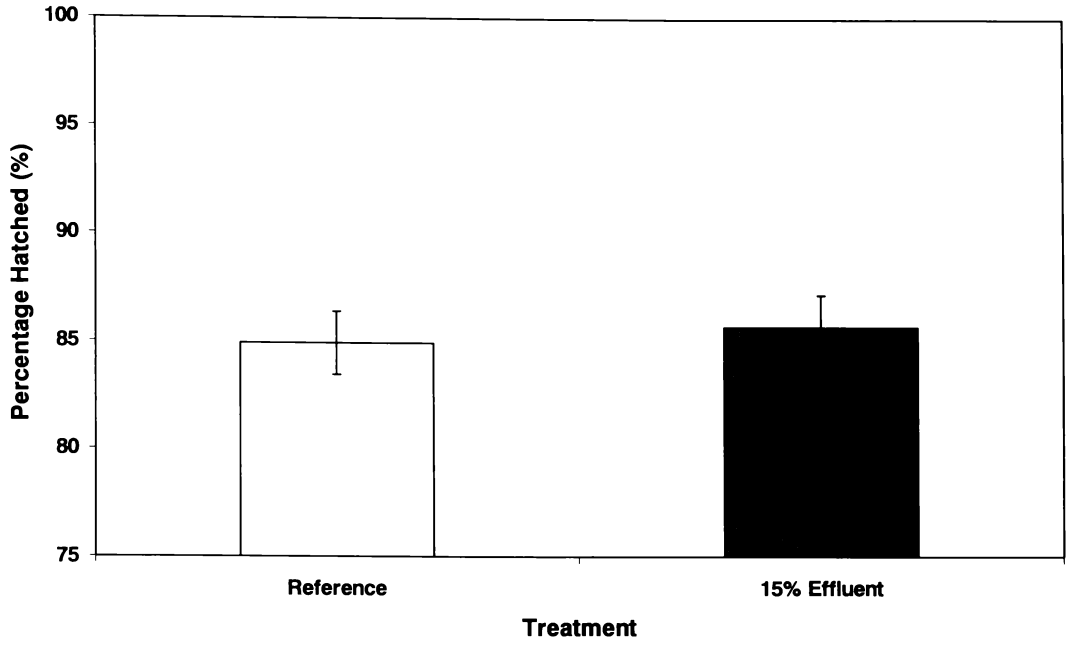
Condition factor, liver size, and body weight data were analysed using analysis of covariance (ANCOVA). Data were log-transformed prior to using ANCOVA. It should be noted that although statistical comparisons using ANCOVA were completed on body and liver weight, data are presented as somatic indices for ease of comparison. Fulton's condition factor was calculated as body weight ÷length^3·100, liver-somatic index (HSI) as liver weight÷ body weight·100. Length, sex ratio, hatching success and length at hatch data were compared using unpaired t-tests. All statistical testing was completed using SYSTAT® and GraphPad Prism 3 software packages. The critical level of statistical differences for all analyses in this paper was assessed at α=0.05.

3.4 Results

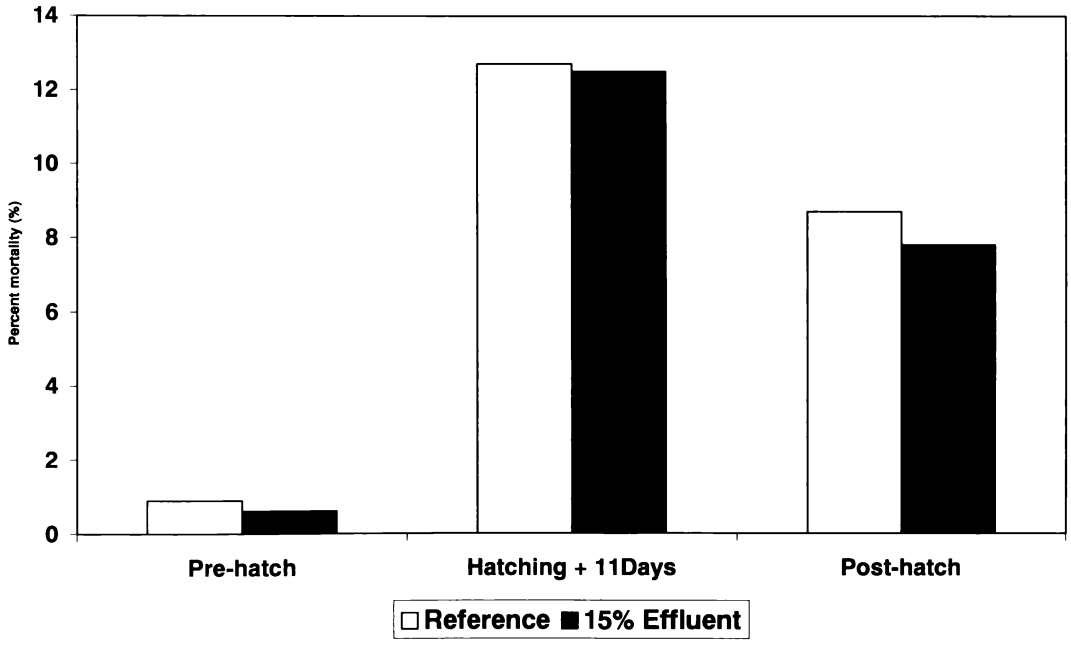
Experiment one demonstrated that the fertilisation and water-hardening of eggs in 15% effluent had no impact on fertilisation success. Due to technical problems with the sodium thiosulphate perstaltic pump and subsequent chlorine toxicity

approximately five days prior to expected hatch, this experiment was terminated after 25 days exposure, therefore no data was available on hatching success.

The second experiment, utilising the same source of effluent also at 15% (v/v) demonstrated that effluent exposure did not significantly affect hatching of the eggs, length at hatch, sex ratio or mortality (Figures 3.1, 3.2, 3.3). Some fibre and particulate matter appeared to attach to the eggs, with fungal growth beginning around these areas in the effluent-exposed treatment only. However, this did not have any effect upon hatching and survival of effluent exposed embryos nor did any noticeable fungal infection occur in either treatment. Growth and the timing of consequential developmental stages were not affected by effluent exposure. In both treatments, within the first eight days, embryonic movement was observed. By days 10-14 the eyes were fully pigmented and visible through the chorion. The heart beat, circulatory system and gills were also prominent. Within 21 days the skin had much greater pigmentation and pectoral fin movement was clear. By day 25 the embryos were very active, responding to stimulation and displaying typical 'swimming' movements. Hatching was completed in both treatments within days 26 to 28. Sac-fry began feeding around day 39 even though the yolk-sacs had not been fully resorbed. However, actual 'swim-up' was estimated to occur between days 42 to 48.



A)



B)

Figure 3.1. Experiment two. A) Mean (±95% CI) percentage hatched in rainbow trout eggs exposed to 15% (v/v) secondary treated effluent and reference water. N=5 replicates per treatment. Total of 1487 and 1491 eggs in reference and effluent treatments respectively. B) Percent mortality recorded throughout the exposure period. Each bar represents the total % value for number dead per treatment, for each critical period over the course of the experiment.

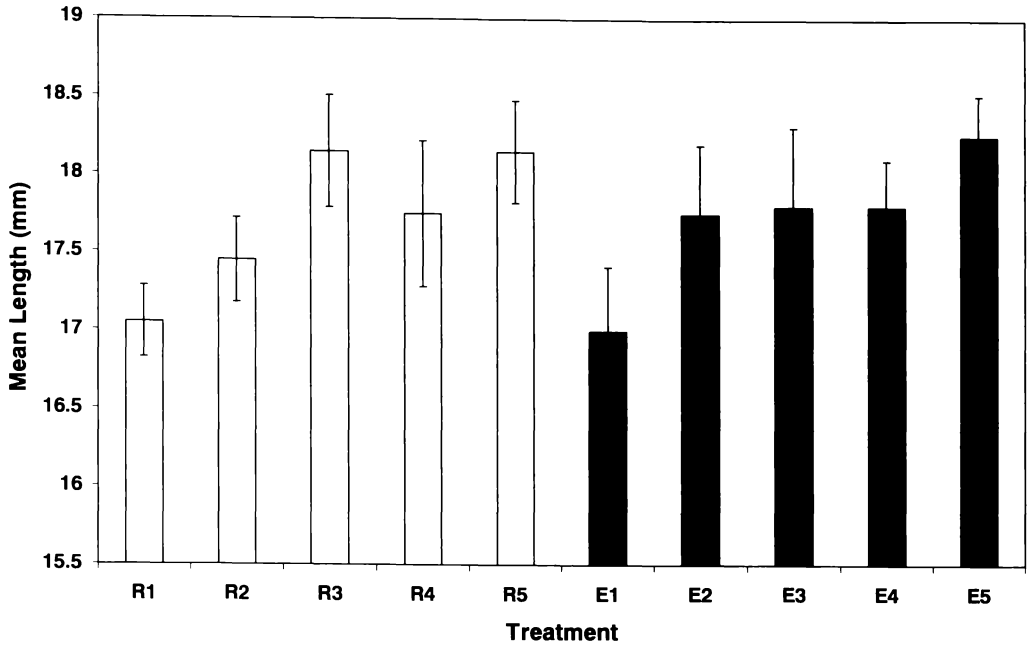


Figure 3.2. Experiment two. Mean (±95%C.I.) length at hatch of rainbow trout eggs exposed to 15% v/v secondary treated effluent (E) and reference water (R). Each bar represents the mean value of 10 individuals sampled per treatment replicate.

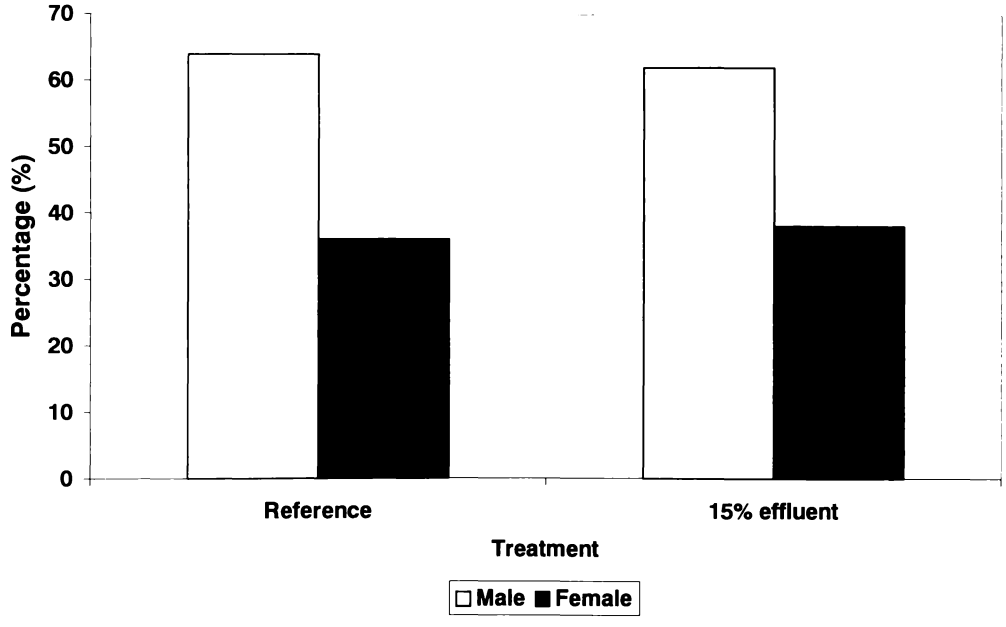


Figure 3.3. Experiment two. Percentage sex ratio as determined at experiment termination. Each bar represents the total percentage per treatment, n= 50. Note: as the individuals were only ten months old, visual gender determination by dissection under a dissecting-microscope may not have been consistently accurate.

Visible morphological abnormalities (Figure 3.4) were observed, although there was no indication that deformities were linked to effluent exposure as similar percentages of deformities were observed between treatments. Seven individuals were identified as having three eyes. Total deformities were 2.5% and 3% for reference and effluent treatments respectively. It is estimated that 50% of all deformed sac-fry had died by day 50.

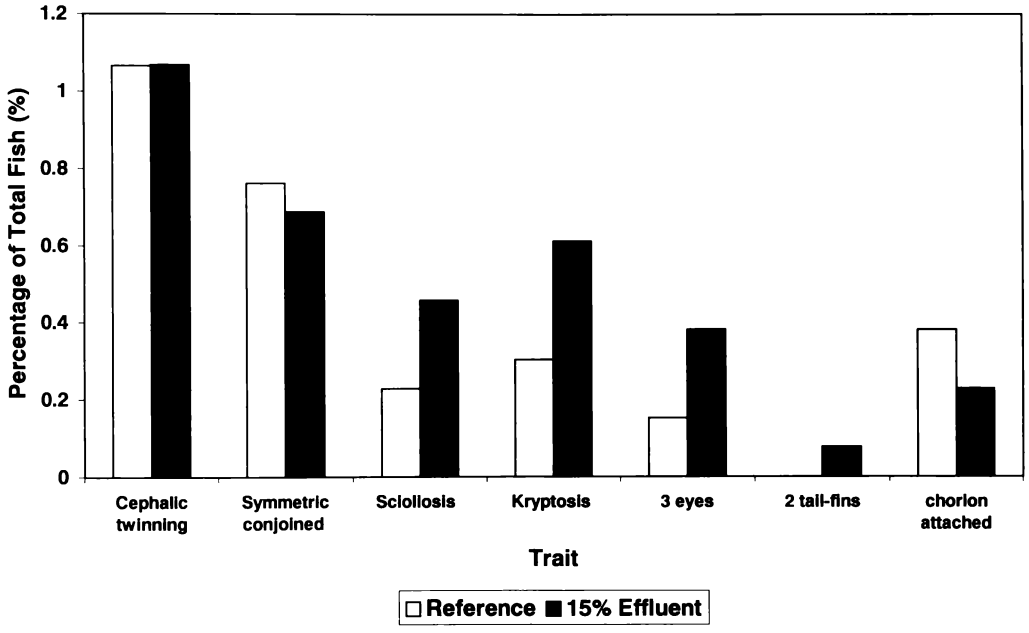


Figure 3.4. Experiment two. Percentage of deformities observed in rainbow trout exposed to reference water and secondary treated effluent (15%v/v).

At the end of the experiment, length and weight did not differ between treatments. However, both liver size (hepato-somatic index) and condition factor were significantly (statistically) reduced in the effluent exposed fish (Figures 3.5 and 3.6). Vitellogenin production (Figure 3.7) and estrogen receptor expression were not induced in fish exposed to 15% effluent, with all experimental concentrations similar to negative control levels.

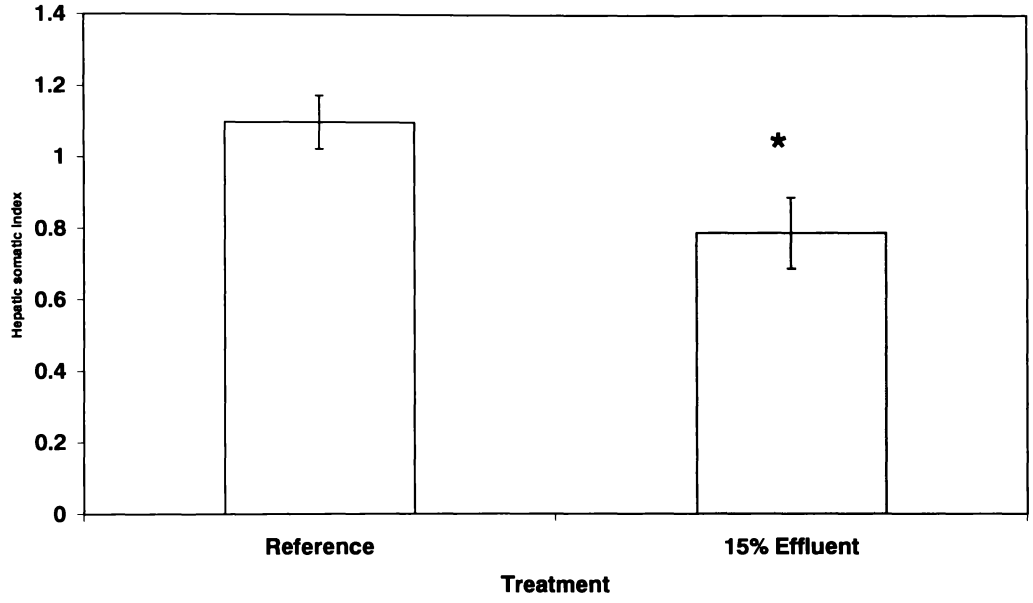


Figure 3.5. Experiment two. Mean (±95%C.I.) hepatic-somatic index in rainbow trout exposed to reference water and 15% (v/v) secondary treated effluent. Each bar represents the mean value of 50 fish sampled. Asterisk represents a significant difference from the reference treatment (ANCOVA, $p<0.05$).

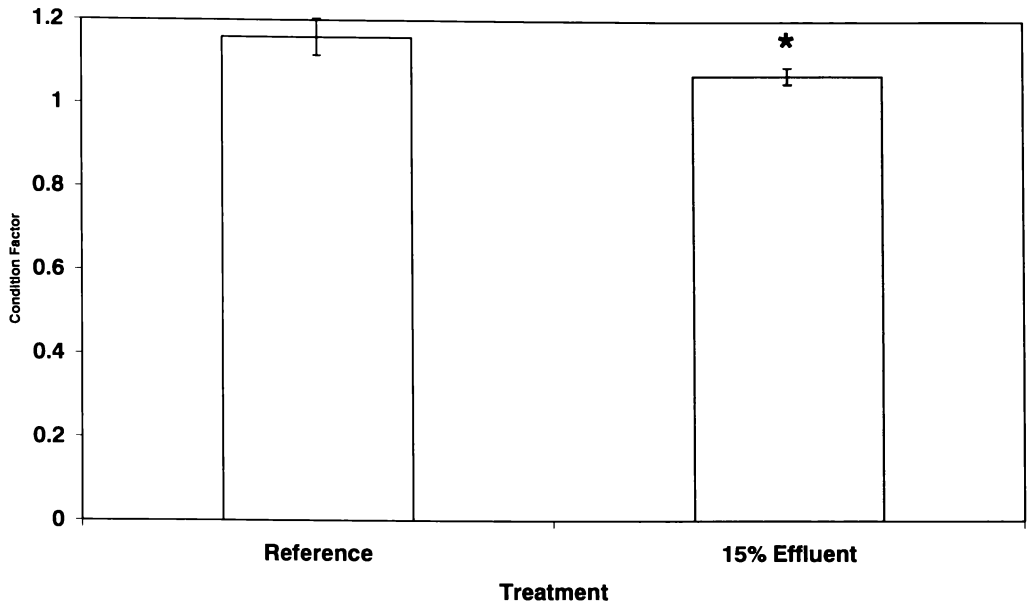


Figure 3.6. Experiment two. Mean (±95%C.I.) condition factor value for juvenile rainbow trout exposed to reference water and 15% (v/v) secondary treated effluent. Each bar represents the mean value of 50 individuals. Asterisk represents a significant difference from the reference treatment (ANCOVA, $p<0.05$).

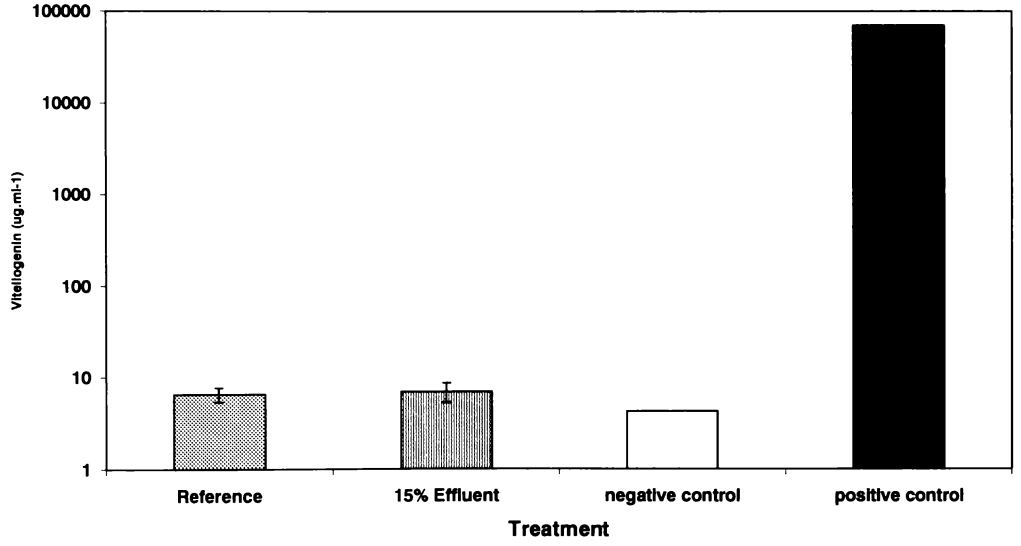


Figure 3.7. Experiment two. Mean (±95%C.I.) concentrations of plasma vitellogenin in juvenile rainbow trout exposed to reference water and 15% (v/v) secondary treated effluent. N= 3 for the negative and positive controls. Negative control represents hatchery reared adult male rainbow trout and the positive control represents estradiol injected hatchery reared adult male trout. Reference and 15% effluent bars represent the mean value of 50 gender-pooled samples.

Total organics removed following filtration of effluent samples showed substantial total phytosterol (87.9%) extraction (Table 3.1), likewise a substantial proportion of total resin acid neutrals and resin acids were removed through filtration with 78% and 75.6% extraction respectively.

Results from bile chemical analyses indicated that individuals exposed to 15% effluent treatment had substantial concentrations of resin acids compared with that found in reference fish (Table 3.2). Phytosterols and resin acids were measured in bile extractives of effluent exposed fish, however the resin acid neutrals were not found.

Table 3.1. Mean (SEM) percentage particulate and dissolved ($\mu g \cdot L^{-1}$) organics concentrations in 100% effluent sampled over April to July 1999. N=7 for all means. GFC= glass fibre filter.

Compound	Mean Total Concentration	SEM	Mean percent in GFC filtrate
Resin acid neutrals			
Fichtelite	11	3	94.2
Dehydroabietin	1.5	0.4	87.7
Tetrahydroretene	20.8	5.9	86.4
Retene	16.2	5.6	84.9
Methyldehydroabietin	1.8	0.5	73.6
Resin acids			
Pimaric acid	60.7	10	67.7
Sandaracopimaric acid	15	2.2	74.8
Isopimaric acid	30.1	4.1	72.2
Palustric acid	23.3	5.1	89.4
Dehydroabietic acid	83.7	9.2	68
Abietic acid	151.9	27.2	78
Neoabietic acid	9.3	2.1	97.1
Pimarenic acid	25.2	3.8	71.1
Sandaracopimarenic acid	54.2	14.2	56.9
Isopimarenic acid	75.3	13.7	70.2
13-Abietenic acid	176.6	30.8	69.9
Dihydroisopimaric acid	18.4	3.5	90.3
Pimaranic acid	21.7	3.6	75
Isopimaranic acid	16.1	2.6	75.9
Abietanic acid	216.9	34.7	73.9
Seco-1-dehydroabietic acid	111.5	28.4	40.1
Seco-2-dehydroabietic acid	62.1	15.7	34.7
Phytosterols			
Cholesterol	32.2	7.2	85.1
Campesterol	7.6	1.5	93.7
Stigmasterol	21.2	6.2	88.1
Sitosterol	165.4	29.3	87.8
Sitostanol	61.2	10.2	84.8

Table 3.2. Total organics concentrations ($\mu g \cdot g^{-1}$ –dry weight) in bile extracts. Each value represents bile pooled from 30 fish. (n.d.= not detected)

Compound	15% Effluent	Reference
Resin Acid Neutrals		
Fichtelite	n.d.	n.d.
Dehydroabietin	n.d.	n.d.
Tetrahydroretene	n.d.	n.d.
Retene	n.d.	n.d.
Methyldehydroabietin	n.d.	n.d.
Total Resin Acid Neutrals	**0.00**	**0.00**
Resin Acids		
Pimaric acid	24.8	n.d.
Sandaracopimaric acid	n.d.	n.d.
Isopimaric acid	11.7	n.d.
Palustric acid	17.3	25.21
Levopimaric Acid	n.d.	n.d.
Dehydroabietic acid	78.4	n.d.
Abietic acid	121.4	n.d.
Neoabietic acid	n.d.	n.d.
Pimarenic acid	31.7	n.d.
Sandaracopimarenic acid	199.4	n.d.
Isopimarenic acid	50.5	n.d.
13-Abietenic acid	38.2	n.d.
Pimaranic acid	31.9	n.d.
Isopimaranic acid	15.2	n.d.
Abietanic acid	160.2	n.d.
Seco-1-dehydroabietic acid	3399	n.d.
Seco-2-dehydroabietic acid	2354	n.d.
12-Chlorodehydroabietic acid	n.d.	n.d.
14-Chlorodehydroabietic acid	13.5	n.d.
12,14-Dichlorodehydroabietic	n.d.	n.d.
7-Oxodehydroabietic acid	n.d.	n.d.
Total Resin Acids	**6548**	**25.2**
Phytosterols		
Cholesterol	19668	23870
Campesterol	13.4	5.1
Stigmasterol	n.d.	n.d.
Sitosterol	14.1	n.d.
Sitostanol	n.d.	n.d.
Total Phytosterols	**19696**	**23875**

3.5 Discussion

In this study a modern secondary-treated mixed TMP/BKM effluent, at an environmentally relevant concentration, was not observed to have any significant impact on early life stage (ELS) rainbow trout during a 320-day laboratory exposure. Growth and the timing of critical developmental stages in both treatment groups followed typical stages in salmonid physiological development. Embryonic development, time to hatch and swim-up were consistent with the known timeframe predictions and observations (Knight 1963, Frost 1967, Crisp 1988) and no treatment differences were observed in the present study. An earlier assessment of fish health in the Tarawera River noted a delay in the time to hatch of zebrafish exposed to the same effluent used in the present study (Beresford 1993). Results similar to that of the present long-term exposure have previously been observed in a 202-day life cycle exposure of fathead minnows to a TMP effluent. Effluent exposure from egg stage to sexual maturity had no effect on hatching, mortality, growth and development and the subsequent hatchability of first generation eggs (Kovacs et al. 1995a). Previous studies have observed gender biases towards male offspring in BKME exposed early life stages (Kovacs et al. 1995b; Larsson et al. 2000), however, no substantial sex ratio differences were noted in the present study.

Abnormal morphological alterations were observed, however, there were no treatment differences in the number or incidences recorded. It is suspected these abnormalities were due to a brief (~12h) temperature increase of approximately 4-6°C (maximum temperature reached was 18°C) due to water-cooling equipment

failure during the first ten days of embryonic development. A time when developing embryos are considered to be 'most' sensitive to any perturbations in their immediate environment. Symptoms were typical of those described by Weis and Weis (1989), see paper for a more detailed descriptions of deformations.

Initial studies investigating aspects of early life stages in fishes had primarily focused on the determination of LC50 values for known environmental toxicants over a narrow period within the "more sensitive" stages of early development (Van Leeuwen et al. 1985). However, little information had been available concerning the long-term effects of low concentrations of BKM effluents on fish. Although a few early studies have examined growth rates in juvenile and ELS salmonids following exposure to kraft mill effluents (Ellis, 1967; Webb and Brett 1972), more recently life cycle studies have been conducted using fish species which have shorter reproductive cycles (Kovacs et al. 1995a, 1995b, 1996). A similar study to the present exposed juvenile coho salmon to BKME for a 200-day period, although exposure did not begin until alevins were 100 days old and the primary focus was directed towards growth rates, blood cell counts, lactate and glucose levels (McLeay and Brown 1974). Results from the McLeay and Brown study did not concur with those observed in the present study regarding growth and there is now much debate as to the reliability of the use of growth response in chronic fish and ELS toxicity tests (Woltering 1984).

No statistically significant differences in length or weight were observed in the present study, however, effluent exposed fish had smaller livers and a significant reduction in condition factor, which may have been attributable to high effluent colour and therefore a reduced visual distance for detecting food.

Although the present study was unable to demonstrate significant impacts from mixed TMP/BK mill effluent exposure, concurrent studies exposing sexually mature rainbow trout eight months prior to spawning, to the same TMP/BK mill treated-effluent (at 10% v/v) and subsequent fertilisation of eggs from exposed parents, resulted in reduced egg size and smaller fry at hatch (van den Heuvel et al. unpublished). Other studies have also demonstrated reproductive and ELS impacts following the exposure of sexually mature brown trout four months prior to spawning, to pulp and paper mill effluents and phytosterols (Lehtinen et al. 1999; Johnsen et al. 2000). The subsequent impacts on offspring would suggest the crucial period may be final maturation (vitellogenesis) in the female parent fish, whereby impacts could more likely be manifested in the offspring (Lehtinen et al. 1999). Results from the present and concurrent studies by van den Heuvel et al. (unpublished) support the idea that pre-exposure of parents prior to spawning may have a significant impact on offspring in contrast to exposure of early life stages alone. However, further studies are required and results from a whole life cycle assessment would prove to be extremely valuable.

The TMP/BK mill effluent in this experiment failed to induce expression of the estrogen receptor in hepatic tissues or vitellogenin production at gene and protein levels in juvenile rainbow trout. Evidence of estrogenic activity in bleached kraft mill effluent (BKME) and black liquor was first noted by Zacharewski et al. (1995) using *in vitro* recombinant receptor-reporter gene assays. More recently, caging studies by Soimasuo et al. (1998) and Mellanen et al. (1999) observed vitellogenin gene expression in male white sucker exposed to a Finnish BKME.

Laboratory exposures of juvenile rainbow trout by Tremblay and Van Der Kraak (1999) demonstrated a Canadian BK mill effluent was also capable of inducing the synthesis of plasma vitellogenin. In spite of these findings, induction of vitellogenin in wild male fishes from known BKME receiving waters has yet to be observed (Van Der Kraak et al. 1998).

It is well known that some constituents of pulp and paper mill effluents contain wood extractives, processing additives and other compounds that are potentially capable of causing endocrine disruption. Suspected compounds include resin acids, polycyclic aromatic hydrocarbons (PAHs), surfactants, chlorinated compounds (PCDD and PCDFs) and phytosterols (Owens 1991; Zacharewski 1997). There has been widespread suggestion that a potential source of estrogen agonists (although not identified), are likely to come from the wood-derived phytosterols (Koistinen et al. 1998; Mellanen et al. 1996, 1999) and compounds not removed by secondary treatment (Munkittrick et al. 1994). A number of studies have demonstrated reproductive impacts in fish exposed to various phytosterols, including impacts on early life stages which have tentatively linked estrogenic as well as androgenic responses to phytosterol exposure (Denton et al. 1985; Lehtinen et al. 1999; Tremblay and Van Der Kraak 1999). To date, the mechanisms of action of phytosterols are unknown. Break-down or by-products from these compounds have also demonstrated androgenic effects (Denton et al. 1985).

Androgenic effects have been seen in fishes exposed to BK mill effluents including morphogenesis in female mosquitofish (Cody and Bortone 1997; Ellis et al. 2000) and tubercle development in female sucker (Munkittrick et al. 1997).

Recently, Hewitt et al. (2000) demonstrated that constituents of BKME have the ability to interact with the androgen receptor in white sucker hepatic tissue. At present the authors' unpublished data reveal extracts from the TMP/BK mill effluent used in the present study, have the potential to bind to the androgen receptor in goldfish gonadal tissue. It is the intention of the authors to further investigate the androgenic potential of the TMP/BK mill effluent and to address any differences in species related response observed to date.

3.6 Conclusions

The present study demonstrates that this particular TMP/BK mill effluent at 15% (v/v) has no marked impact on fertilisation and hatching success, survival and development in ELS rainbow trout and would appear to have no significant reproductive or estrogenic impacts on juvenile rainbow trout. However, effluent exposed fish had smaller livers and reduced condition factor. This paper does not discount the possibility that the effluent of concern may have androgenic potential, as suggested by previous findings (Ellis et al. 2001) contrasting two fish species.

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Chapter Four: Lack of Estrogenic Effects in Juvenile Rainbow Trout Exposed to a Modern NZ Pulp and Paper Mill Effluent

4.1 Abstract

A programme to determine the potential impacts of a modern New Zealand pulp and paper mill effluent on fish populations, used a combination of long- and short-term exposures of the rainbow trout, *Oncorhynchus mykiss* to the effluent. Juvenile (1+ aged) rainbow trout were exposed to a mixed thermomechanical pulp/bleached kraft (TMP/BK) mill effluent at a range of concentrations from environmentally relevant (10%) to 70% (effluent by volume) in two exposure studies. During both 21- and 56-day exposures to 10% and 30% (v/v) effluent, no significant impacts on spleen and liver size, condition factor, and circulating testosterone and pregnenolone levels were observed. Vitellogenin induction or expression of the estrogen receptor in juvenile males was not observed in either experiment. Significant differences in spleen and liver size as well as high experimental mortality were observed in fish exposed to 70% (v/v) secondary treated effluent compared to a reference treatment during the 21-day exposure and was linked to an atypically high suspended solids load. Thus, the combined data from these experiments demonstrated a lack of estrogenicity or impacts on steroidogenesis following exposure to TMP/BK mill effluent. Results from parallel studies have instead linked androgenic activity to the effluent of investigation.

4.2 Introduction

The impacts of pulp and paper mill effluents on aquatic organisms have been extensively investigated since the 1960s, and linked to a variety of toxic effects, including reproductive impairment (Owens 1991, Zacharewski 1997). Some of the observed reproductive impacts include decreased egg and gonad size, delayed maturity (McMaster et al. 1991, 1996; Munkittrick et al. 1992a, 1994), reductions in serum sex steroid levels and overall gonadal steroid production (Van Der Kraak et al. 1992; Munkittrick et al. 1992a; McMaster et al. 1994, 1995; McCarthy et al. 1997). Reductions in male secondary sex characteristics and modification of female secondary sex characteristics have been observed in some species (Howell et al. 1980; Drysdale and Bortone 1989; McMaster et al. 1991; Ellis et al. 2000). Increased induction of cytochrome p450 monooxygenases (Andersson et al. 1987; Lindstrom-Seppa and Oikari 1990a, 1990b) and induction of hepatic mixed function oxidase (MFO) activity (McMaster et al. 1991; Munkittrick et al. 1994; Donald 1997) have also been noted in fishes exposed to pulp and paper wastewaters.

Estrogenic activity in pulp and paper effluent and black liquor has been reported by Zacharewski et al. (1995) using in vitro recombinant receptor/reporter gene assays. More recently, the synthesis of plasma vitellogenin (vtg) and vtg gene expression in the liver of male fish has been reported following BK mill effluent exposure (Soimasuo et al. 1998: Mellanen et al. 1999; Tremblay and Van Der Kraak 1999). Many studies have linked a number of the observed estrogenic effects to wood-derived phytosterols (including β-sitosterol), of which there are

considerably high quantities in pulp mill effluents (Zacharewski et al. 1995; McLatchy et al. 1995, 1997; Mellanen et al. 1996, 1999). In spite of the androgenic responses observed in *Gambusia* species there is still a paucity of research investigating androgenic-potentials of pulp and paper wastewaters (and endocrine disrupting compounds per se). However, Hewitt et al. (2000) have demonstrated that some constituents of bleached kraft mill effluent (BKME) have the ability to interact with the androgen receptor in white sucker hepatic tissues.

In recent years, the international pulp and paper industry has committed considerable resources to environmental improvement through process modifications and the installation or upgrade of secondary treatment facilities (Kovacs et al. 1997). The benefits of these investments in terms of improved effluent quality and decreased physiological impacts on receiving water fish populations have been well documented, with a number of studies reporting no obvious deleterious effects on fish reproduction and/or health at some sites (Swanson et al. 1993,1994; Gagnon et al. 1994a, 1994b; Kloepper-Sams et al. 1994a, 1994b) which could conclusively be linked to pulp and paper effluent exposure. Nonetheless, significant reproductive impacts have still been recorded at sites where process and treatment modifications have been implemented (Munkittrick et al. 1992b, 1992c, 1994; MacLatchy et al. 1997). Another confounding factor is that the pattern and extent of many observed effects, appear to be site specific and may not be easily extrapolated to all situations (Bussieres et al. 1998). Results from bleached kraft mills discharging secondary treated effluent have been conflicting; for example, physiological changes have been observed at

mills with and without ECF bleaching, secondary effluent treatment and other treatment/technology upgrades in place (Kovacs et al. 1997).

Previous findings suggest that reproductive impacts are not due to the bleaching process or chemical additives (of pulping) alone (MacLatchy et al. 1997). Some studies have mentioned the growing evidence that secondary treatment of effluent may actually enhance concentrations of potential endocrine disrupting compounds, following microbiological activity (degradation) within the treatment ponds (MacLatchy et al. 2000). Nonetheless, compounds with such potential have not yet been identified.

This study investigates the impacts of a modern TMP/BK mill with secondary treatment facilities and 100% ECF bleaching on juvenile rainbow trout (*Oncorhynchus mykiss*) at an on-site exposure facility. To date no studies of this kind have been undertaken in New Zealand in order to ascertain reproductive endocrine impairments in fishes exposed to TMP/BK mill wastewaters. The present studies focus on chronic impacts in juvenile rainbow trout investigating reproductive endpoints such as changes in gonadal and liver size, circulating sex steroids, and induction of vitellogenesis in males as an indicator of estrogenic acitivity. Two experiments were conducted, the first a longer-term exposure to 10% (v/v) secondary treated effluent with multiple sampling intervals and a depuration period. The second experiment was a short-term exposure to multiple concentrations of secondary treated effluent.

4.3 Methods and materials

Mill description

The Norske Skog-Tasman Mill (formally Fletcher Challenge Paper) is an integrated thermomechanical pulp/bleached kraft (TMP/BK) pulp and paper mill i.e. uses both kraft and thermomechanical pulping processes producing 760 and 1010 air dried tonne per day ($a.d.t.d^{-1}$) respectively. Mill production is primarily softwood (*Pinus radiata*) with occasional eucalypt production and has been elemental-chlorine free (ECF) since April 1998. Norske Skog-Tasman implements secondary wastewater treatment and ECF bleaching. The wastewater treatment system consists of a thermomechanical pulping pre-treatment bioreactor facility within the TMP mill. Kraft mill effluent is then collected into a single drain and passed through two bar screens and a clarifier for partial solids removal and then treated in a four pond aerated stabilisation basin system. Pond one serves as a primary treatment sludge/dredging pond. The ponds have an area of 45 hectares, with a retention time of 5-6 days. Following treatment in the aerated lagoon system, effluent is then discharged into the Tarawera River at a total mean volume of 180,000 $m^3 \cdot d^{-1}$. River effluent dilution ranges between 5 and 12 percent and enters the river system with an average temperature of 26-28°C.

Exposure system

Juvenile rainbow trout experiments were conducted at an on-site exposure/mesocosm facility at the Norske Skog-Tasman Mill in Kawerau, New Zealand. Fish exposure tanks were 7,500 L epoxy-coated fibreglass tanks divided

into three 2,500 L sections. The facility was roofed and fenced to provide shade during the summer months.

Reference water was pumped directly from the Tarawera River upstream of any points of discharge of two pulp and paper mills further downstream. Water was pumped continuously via pipeline into a 170,000 L concrete reservoir adjacent to the exposure ponds, which was kept at a constant level by pumping an excess of water into the tank then draining the excess with an overflow. Reference and dilution flows were controlled using stainless steel globe valves.

Secondary treated effluent was transported via road tanker on a weekly basis and sourced immediately prior to discharge into the Tarawera River. Effluent was held in a 85,000 L concrete reservoir and continually re-circulated using submersible pumps in order to prevent solids settling and effluent becoming anaerobic. 100% effluent was gravity fed to mixing tanks alongside the exposure tanks, where effluent dilution was controlled for a desired concentration prior to flow into the exposure tanks. Effluent and dilution water flow in the exposure treatment of experiment one were measured using digital in-line flowmeters (Great Plains Industries Inc., Wichita, Kansas, USA) and flow was adjusted daily. Effluent flows for experiment two were measured and adjusted manually on a daily basis.

Fish and Experimental design

Juvenile rainbow trout were obtained from the Eastern Fish and Game Ngongotaha Hatchery, Rotorua, New Zealand. Trout were age 1+ averaging 25-30 cm and 200-300 g. Trout were transported to Kawerau (approximately 80km) and acclimated for 2 weeks in Tarawera River (reference) water prior to the start of

both experiments. Trout were held under ambient light conditions at the on-site exposure facility and the exposure tanks were aerated continuously. Fish were fed commercial salmon food (Pacific Lite, 14% lipid, 43% crude protein, NRM Feed Mills, Hope-Nelson,NZ). The daily ration was maintained at 1% of estimated wet body weight.

Two separate experiments were conducted at the on-site facility. The first (experiment one), undertaken in April 1999, was a 56-day experiment whereby fish were exposed to either reference water or 10% secondary treated effluent for a period of 28 days. During this period, twenty fish per treatment were sampled at days 7,14 and 28. Following day 28, effluent exposure ceased and was replaced with reference water. During the depuration period, 20 fish per treatment were again sampled on days 7,14 and 28 from the cessation of effluent exposure. A pre-exposure (time zero) sample of 24 fish was taken two days prior to initiation of the experiment during the acclimation period. Each treatment was divided into triplicated tanks, with 40 fish per tank. Flow rates into each 2,500 L replicate were 4 $L \cdot min^{-1}$.

Experiment Two commenced in February 2000, and was a 21-day exposure to 10%, 30% or 70% final treated effluent. Tarawera River water used as reference and diluent water. There were two replicates per treatment containing 20 fish per replicate. An initial acclimation sample of 24 fish was taken prior to the commencement of the experiment. Flow rates were maintained at 3 $L \cdot min^{-1}$ per 2,500 L tank.

After both exposures, fish were sacrificed by a blow to the head, measured, weighed and gender noted. Gonad, liver and spleen weights were also recorded. Blood samples were collected immediately after the blow to the head via caudal puncture and kept on ice in heparinized collection tubes until transported to the laboratory for 15 minutes centrifugation (500 g) and storage of plasma at -80°C. Liver tissue samples were divided, half each stored at -80°C for mixed-function-oxygenase (MFO) and mRNA analyses. Whole gall bladders were removed and quick frozen in liquid nitrogen and stored at -80°C for chemical analysis.

Vitellogenin and estrogen receptor analyses

Vitellogenin (vtg) induction at the protein level was determined using western blotting techniques for detection of the protein and an enzyme linked immunosorbent assay (ELISA) for quantification of plasma protein concentration. Western blotting was conducted following protocol adapted from the methods of Copeland et al. (1986) Denis et al. (1988) and Pelissero et al. (1993). Plasma samples were measured for total protein by the method of Bradford (1976). Proteins were separated by gel electrophoresis and blotted to nitrocellulose membranes for staining and antibody binding (Gershoni and Palade 1983).

ELISA's were performed using a Biosense Laboratories Quantitative Rainbow Trout vitellogenin ELISA kit (Vtg-102). The kits are based on a competitive binding assay and a monoclonal Vtg antibody has been developed towards salmonid species. The assay has a working range of 150-6000 $ng \cdot ml^{-1}$.

Vitellogenin induction at the mRNA level was determined by reverse transcriptase- polymerase chain reaction (RT-PCR) using a Primus 25/96

Thermocycler, according to methods adapted from Ren et al. (1996), Pakdel et al. (1989) and Vanden Heuvel (1998). In brief, RNA was isolated from liver samples by phenol extraction with TRIzol Reagent and collected via isopropanol precipitation. RNA was then denaturated at 58°C (15 minutes) and the nucleic acid concentration determined. RNA was translated to cDNA by reverse transcriptase (RT). Using specific primers, the sample cDNA-species was amplified by a polymerase chain reaction (PCR), followed by cDNA separation on agarose gel for detection with ethidium bromide and UV light photography.

Expression of the estrogen receptor was also determined at the mRNA level using RT-PCR techniques described above.

Steroid hormone analysis

Circulating sex steroid hormones were measured according to McMaster et al. (1992). Plasma samples were thawed and steroid hormones were extracted with diethyl ether. The plasma extract from males and females was analysed for testosterone and pregnenolone using standard radioimmunoassay (RIA) procedures.

Water extractable trace organics

Weekly effluent samples were collected from April to June 1999 and during March 2000 and filtered using 15cm Whatman GFC filters. Filtrate and filter papers were stored at 4°C and -20°C respectively. Aqueous effluent samples for determination of organic extractives (i.e. resin acids, fatty acids, phytosterols, phenolics or monoterpenes) were extracted by continuous liquid-liquid extraction

at pH 9. The solvent volume was concentrated with nitrogen using a Zymark Turbovap and the sample dried with anhydrous sodium sulphate and then derivatised (sitylation) for analysis by gas chromatography/mass spectrometry (GC-MS). The filter papers were ground with sodium sulfate and placed in a soxhlet extractor overnight with methylene chloride. All organics were corrected for extraction blanks and adjusted for the recovery of the appropriate surrogate standard.

Trace organics in bile

Total organics in bile were determined after hydrolysis of the bile sample with ethanolic potassium hydroxide. The hydrolysed bile was acidified and extracted with methyl tertiary-butyl ether and the extract was dried through sodium sulphate, transferred into hexane and passed through a florisil cartridge. The sample was eluted with 1:9 ethanol:hexane, derivitised, and analysed by GC-MS. Samples were corrected for surrogate recovery and blank determinations.

7-ethoxyresorufin-O-deethyase analysis

Hepatic mixed function oxygenase (MFO) enzyme activity was estimated as 7-ethoxyresorufin-O-deethylase (EROD) activity using a modification of the fluorescence plate-reader technique outlined by van den Heuvel et al., (1995). Liver extracts were homogenised in a cryopreservative buffer (0.1 M phosphate, 1mM EDTA, 1mM dithiothreitol , and 20% glycerol, pH 7.4) and spun at 9000 g to obtain the post-mitochondrial supernatant (PMS). The EROD reaction mixture contained 0.1M HEPES buffer, pH 7.8 (Sigma, St Louis, MO, USA), 5.0 mM Mg^{++}, 0.5mM NADPH (Applichem, Darmstadt, Germany), 1.5 μM 7-

ethoxyresorufin (Sigma), and approximately 0.5 $mg \cdot ml^{-1}$ of PMS protein. EROD activity was determined kinetically in 96-well plates using one reading every 10 minutes on a BMG POLARstar Galaxy microplate fluorometer (BMG Labtechnologies, Offenburg, Germany). Resorufin was determined using 544nm excitation and 590nm emission filters. Protein content was estimated from fluorescamine fluorescence (390 nm excitation, 460nm emission filters) against bovine serum albumin (Sigma).

Statistical analyses

Condition factor, liver size, and body weight data were analysed using analysis of covariance (ANCOVA). Data were log-transformed prior to using ANCOVA. It should be noted that although statistical comparisons using ANCOVA were completed on body and liver weight, data are presented as somatic indices for ease of comparison. Fulton's condition factor was calculated as body weight $\div length^3 \cdot 100$, liver-somatic index (HSI) as liver weight $\div$ body weight$\cdot 100$. Steroid, vitellogenin, EROD, and length data, were compared using analysis of variance (ANOVA) with a Bartlett test for homogeneity of group variances, and a Tukey post-hoc test. ANCOVA and ANOVA statistical testing were completed using the SYSTAT® and GraphPad Prism 3 software packages. The critical level of statistical differences for all analyses in this paper was assessed at $\alpha=0.05$.

4.4 Results

Routine effluent chemistry measurements demonstrated that parameters such as dissolved oxygen, pH, and absorbable organic halogens (AOX) remained fairly

consistent over the period of both experiments one and two. However, total suspended solids in experiment two were substantially higher than experiment one, as was conductivity. Conductivity of 100% effluent averaged 916 $\mu S \cdot cm^{-1}$ and 1186 $\mu S \cdot cm^{-1}$ for experiments one and two respectively. Experiment two had a considerably higher solids loading than usual; total suspended solids averaged 55 $mg \cdot L^{-1}$ compared to a typical mill average of 38.3 ±4.4 $mg \cdot L^{-1}$. Effluent in experiment one averaged 33.8 $mg \cdot L^{-1}$ solids. There were no treatment differences in pH or dissolved oxygen levels for either experiment. Reference water temperature was ambient, reflecting that of the Tarawera River and over the course of both experiments averaged between 14.2°C and 18.0°C for experiments one and two respectively. Some effluent exposures were elevated by up to 0.7°C above these values.

Differences between the two effluents used for each experiment were obvious regarding resin acid and phytosterol concentrations, although in both, resin acids remained the most abundant extractive found, typical of softwood pulping. Secondary treatment of the effluent removes approximately 90% of the total resin acids. Resin acid and resin acid neutral concentrations for 100% effluent, during experiment one were almost double that of experiment two (Table 4.1).Total phytosterol concentrations showed similar trends to resin acids and neutrals, whereby significantly higher concentrations (287.6 $\mu g \cdot L^{-1}$) were found in experiment one effluent compared with experiment two, 87.3 $\mu g \cdot L^{-1}$. Approximately 60% of β–sitosterol is removed (Taverndale and Stuthridge, unpublished data) following secondary treatment. In the present study, ß-sitosterol

accounted for more than 50% of the total phytosterols in final effluent of both experiments.

Results from bile chemical analyses indicated that individuals in 10 and 30% effluent treatments were exposed to effluent-related compounds, revealing significant uptake of resin acids from the effluent (Table 4.2). Phytosterols and resin acids were measured in bile extractives of effluent exposed fish, however the resin acid neutrals were not detected in 10% effluent or reference treatments. Phytosterols were also measured in reference fish at concentrations similar to those of effluent exposed fish. The source of these phytosterols is due to dietary intake. Results from chemical analyses of food show substantial concentrations of β-sitosterol and cholesterol at levels substantially higher than those found in 100% secondary treated effluent (author's unpublished data 2001).

Table 4.1. Mean (SEM) total concentration of organics ($\mu g \cdot L^{-1}$) in 100% secondary treated effluent sampled during experiments one (April-July 1999) and two (February-March 2000). N=7 for all means in experiment one. N=5 for all means in experiment two.

Compound	Mean Total Concentration		Mean Total Concentration	
	Exp One	SEM	Exp Two	SEM
Resin Acid Neutrals				
Fichtelite	11	3	8.1	2.7
Dehydroabietin	1.5	0.4	0.2	0.2
Tetrahydroretene	20.8	5.9	5.2	5.3
Retene	16.2	5.6	7.5	7.5
Methyldehydroabietin	1.8	0.5	0.2	0.2
Resin Acids				
Pimaric acid	60.7	10	39.7	24.5
Sandaracopimaric acid	15	2.2	3.1	2.7
Isopimaric acid	30.1	4.1	18.2	10.4
Palustric acid	23.3	5.1	2.2	2.5
Dehydroabietic acid	83.7	9.2	66.3	32.1
Abietic acid	151.9	27.2	43.9	25.1
Neoabietic acid	9.3	2.1	3.0	4.1
Pimarenic acid	25.2	3.8	10.3	6.0
Sandaracopimarenic acid	54.2	14.2	18.8	14.5
Isopimarenic acid	75.3	13.7	21.8	13.8
13-Abietenic acid	176.6	30.8	109.3	37.1
Dihydroisopimaric acid	18.4	3.5	8.6	5.9
Pimaranic acid	21.7	3.6	9.0	7.2
Isopimaranic acid	16.1	2.6	4.5	3.9
Abietanic acid	216.9	34.7	77.8	39.9
Seco-1-dehydroabietic acid	111.5	28.4	54.9	50.8
Seco-2-dehydroabietic acid	62.1	15.7	33.4	33.7
12-Chlorodehydroabietic acid	2.4	0.4	1.2	1.1
14-Chlorodehydroabietic acid	8.8	1.4	5.8	5.4
12,14-Dichlorodehydroabietic acid	0.7	0.1	0.1	0.2
7-Oxodehydroabietic acid	1.3	0.2	2.7	4.4
Phytosterols				
Cholesterol	32.2	7.2	4.6	3.9
Campesterol	7.6	1.5	2.2	1.5
Stigmasterol	21.2	6.2	5.6	3.2
Sitosterol	165.4	29.3	57.2	27.9
Sitostanol	61.2	10.2	17.7	12.7

Table 4.2. Total organics concentrations ($\mu g \cdot g^{-1}$ –dry weight) in bile extracts. Each value represents bile pooled from 40 fish. (n.d.= not detected)

Compound	30% Effluent	10% Effluent	Reference
Resin Acid Neutrals			
Fichtelite	n.d.	n.d.	n.d.
Dehydroabietin	n.d.	n.d.	n.d.
Tetrahydroretene	n.d.	n.d.	n.d.
Retene	7.81	n.d.	n.d.
Methyldehydroabietin	12.79	n.d.	n.d.
Total Resin Acid Neutrals	**20.60**	**0.00**	**0.00**
Resin Acids			
Pimaric acid	28.12	15.50	n.d.
Sandaracopimaric acid	16.81	n.d.	n.d.
Isopimaric acid	16.41	8.34	n.d.
Palustric acid	9.93	12.17	11.04
Levopimaric Acid	n.d.	n.d.	n.d.
Dehydroabietic acid	32.75	18.33	6.14
Abietic acid	89.88	52.17	13.88
Neoabietic acid	n.d.	n.d.	n.d.
Pimarenic acid	4.98	5.89	n.d.
Sandaracopimarenic acid	10.22	11.70	n.d.
Isopimarenic acid	11.85	11.14	n.d.
13-Abietenic acid	46.56	34.70	n.d.
Pimaranic acid	6.71	8.38	n.d.
Isopimaranic acid	1.54	3.42	n.d.
Abietanic acid	37.05	31.31	n.d.
Seco-1-dehydroabietic acid	42.67	30.48	n.d.
Seco-2-dehydroabietic acid	22.05	14.09	n.d.
12-Chlorodehydroabietic acid	6.45	n.d.	n.d.
14-Chlorodehydroabietic acid	5.86	2.51	n.d.
12,14-Dichlorodehydroabietic	n.d.	n.d.	n.d.
7-Oxodehydroabietic acid	6.46	n.d.	n.d.
Total Resin Acids	**396.32**	**260.13**	**31.06**
Phytosterols			
Cholesterol	17677.76	21060.82	17396.05
Campesterol	46.50	38.23	33.20
Stigmasterol	n.d.	n.d.	n.d.
Sitosterol	40.06	35.66	29.86
Sitostanol	n.d.	n.d.	n.d.
Total Phytosterols	**17764.32**	**21134.71**	**17459.12**

Trout survival was 98% in reference water and 98.7% in 10% effluent throughout the 56 day experiment. Experiment two had 95% survival in both reference and 10% effluent treatment groups. No mortality was observed in 30% treated effluent, however 70% treated effluent produced 88% mortality within the first four days of exposure. The remaining five fish still alive on day 5 of the experiment as well as thirteen recently dead fish from this treatment were sampled, where possible, for blood and tissue.

During the 56-day experiment with depuration period, condition factor (Figure 4.1) was not affected by exposure to final treated effluent at a concentration of 10%. Within treatment differences in weight were observed over time, as well as between the time zero (acclimation) sample and all other treatments, however, none could be linked to effluent exposure. Length was also significantly different between the time zero (acclimation) sample and all other treatments and sampling periods. Both length and weight were reduced in the time zero sample as expected however, over the course of the experiment, no treatment related differences were observed. Treated effluent at 10% (v/v) had no impact upon hepato-somatic or splenic-somatic indices (Figure 4.1), nor on circulating testosterone and pregnenolone levels in both males and females (Figure 4.2 and 4.3). There were, gender differences in circulating testosterone levels, expectedly males had higher circulating testosterone than females. Measurements of circulating vitellogenin (Figure 4.4) and vitellogenin gene expression in male fish, demonstrated that at a concentration of 10%, this effluent was not able to induce induction at the protein or mRNA level. Nor was exposure to effluent able to induce expression of the estrogen receptor measured at the mRNA level.

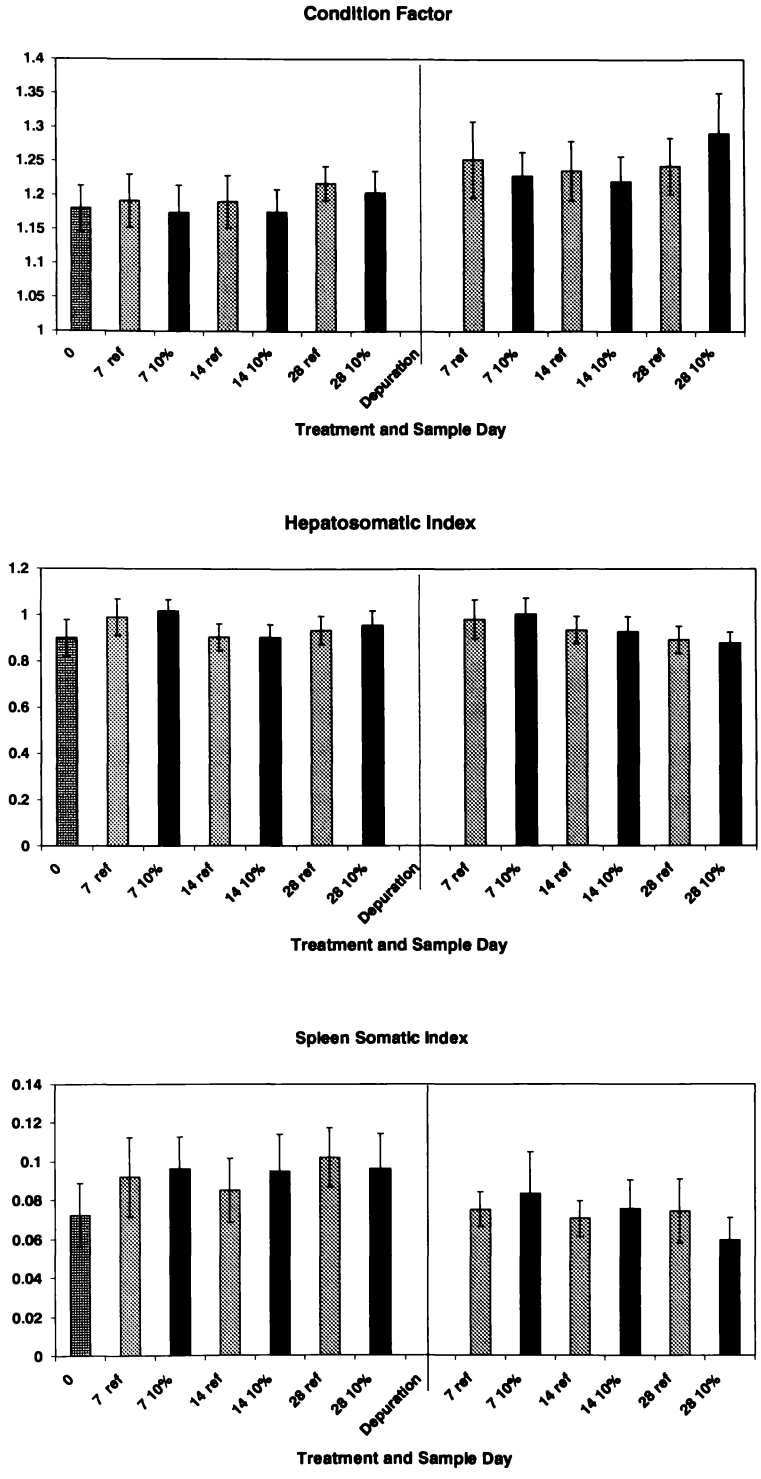
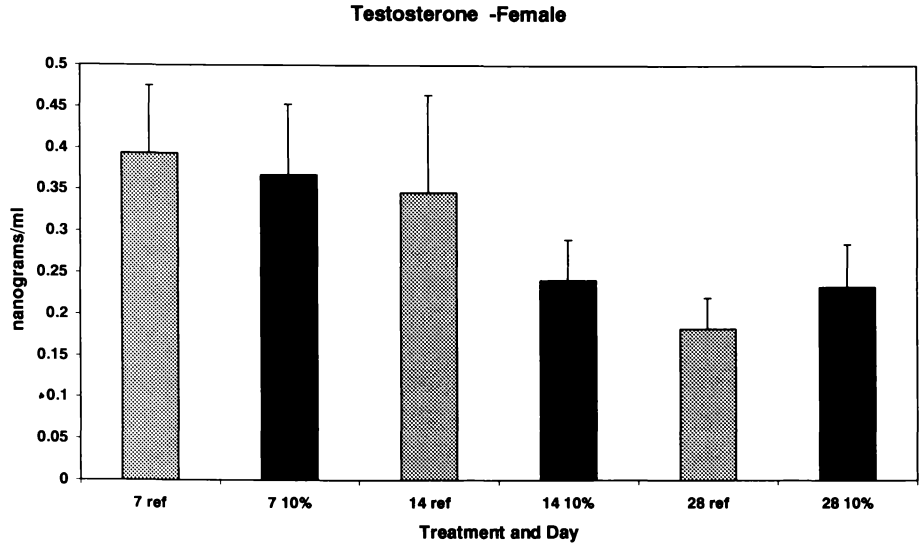
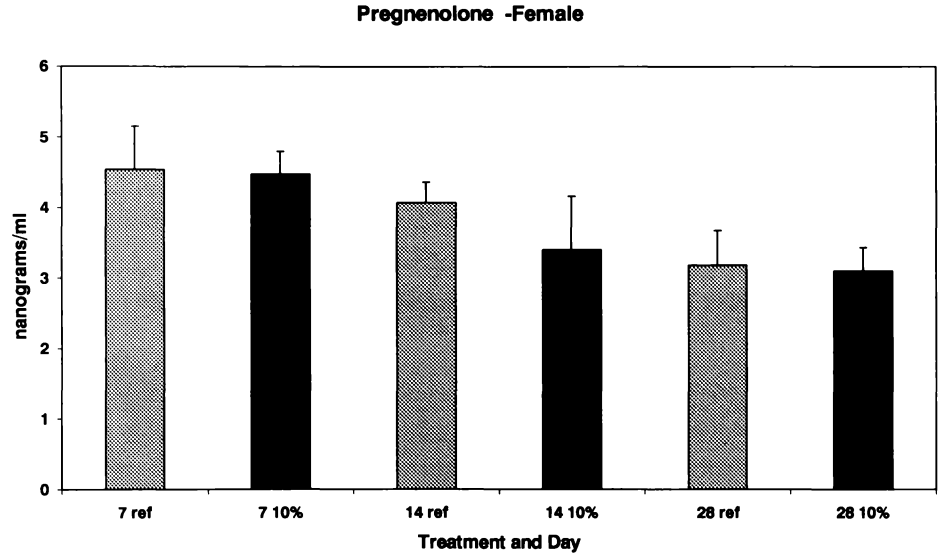


Figure 4.1. Mean (± 95% C.I.) condition factor, hepatic and spleen somatic indices in juvenile rainbow trout exposed to reference water and 10% treated effluent for 28 days and the following 28-day depuration period. N=20 for each data point.

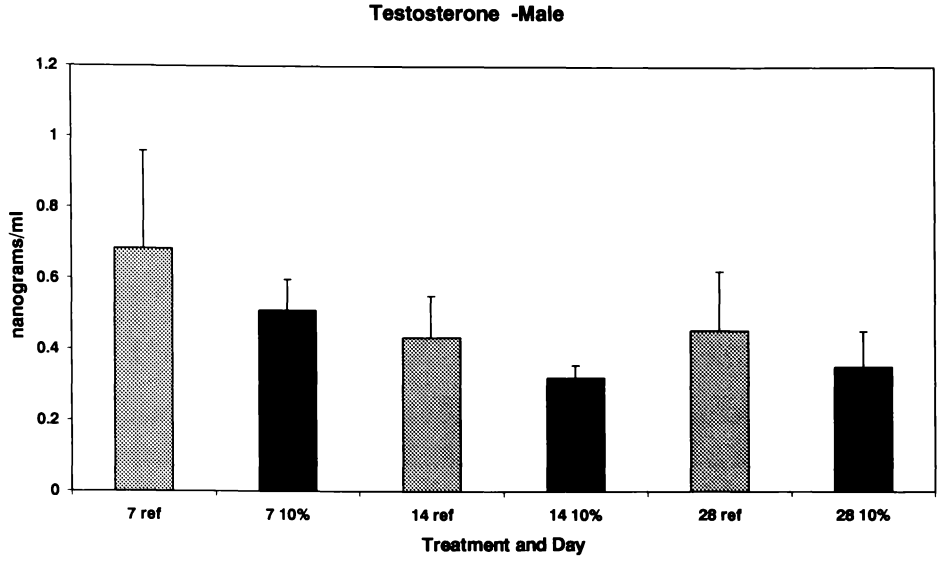


A)

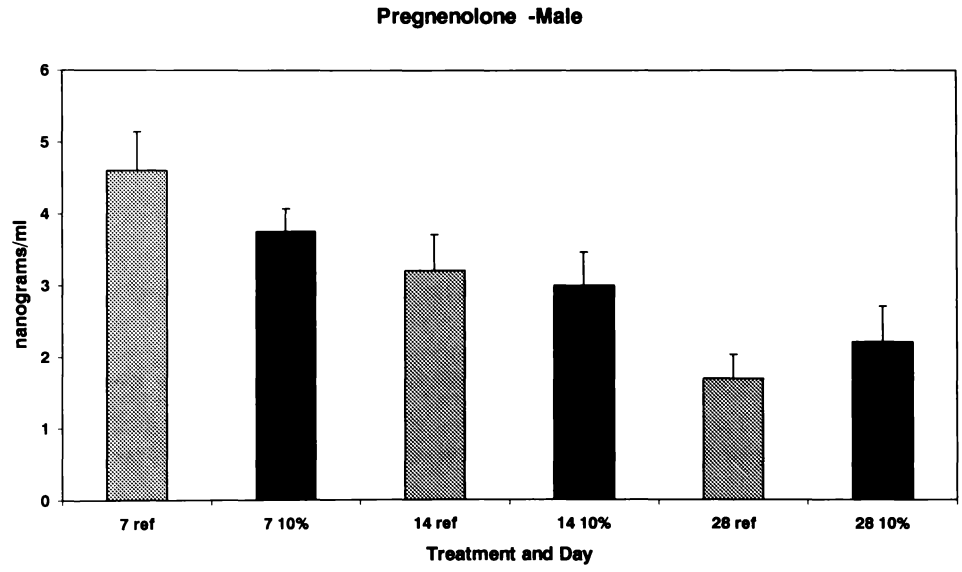


B)

Figure 4.2. Mean (± 95% C.I.) concentrations of plasma steroid sex hormones a) testosterone and b) pregnenolone in juvenile female exposed to Tarawera (reference) river water and 10% effluent in river water for 28-days. Sub-samples were taken on days 7,14, and 28. Sample size per bar = 11,10,9,12,8 and 11 left to right respectively.



A)



B)

Figure 4.3. Mean (± 95% C.I.) concentrations of plasma steroid sex hormones a) testosterone and b) pregnenolone in juvenile males exposed to Tarawera (reference) river water and 10% effluent in river water for 28-days. Sub-samples were taken on days 7,14, and 28. Sample size per bar = 8,10,9,8,11 and 8 left to right respectively.

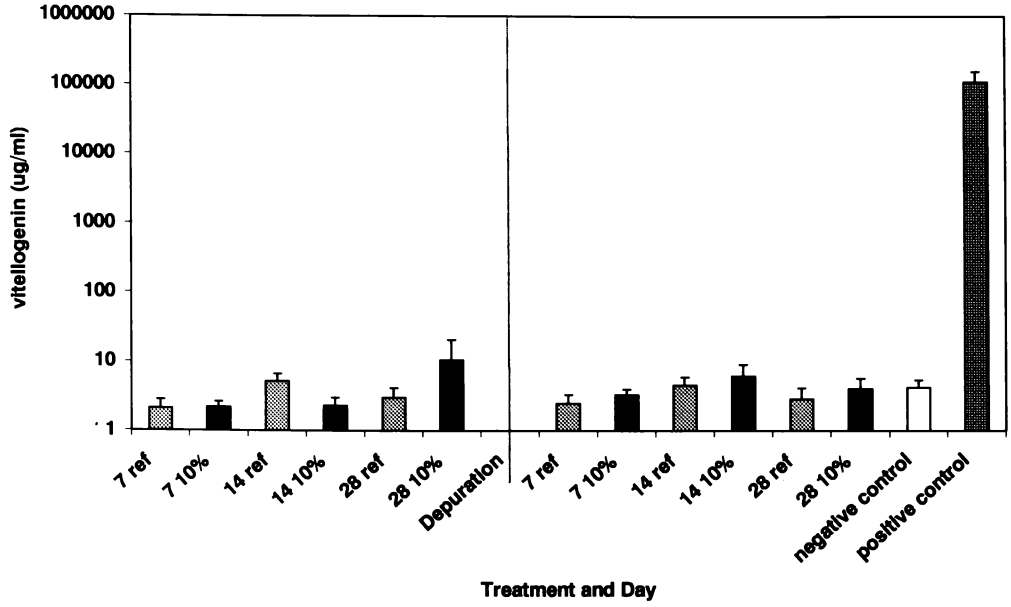
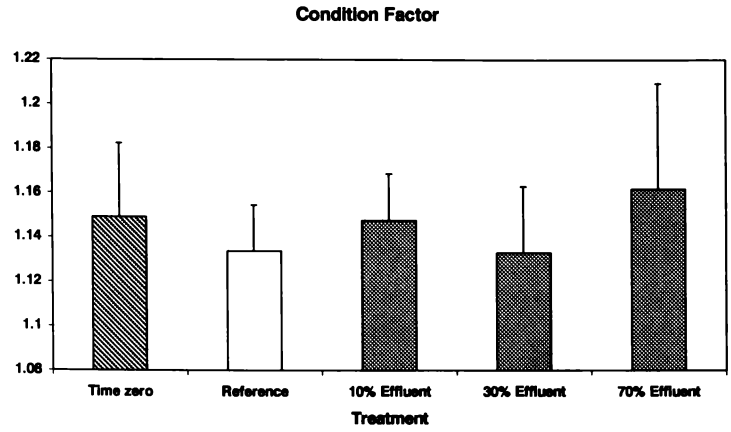


Figure 4.4. Mean (± 95% C.I.) plasma vitellogenin concentrations in juvenile male rainbow trout exposed to reference water and 10% effluent in reference water for 28-days. Samples were taken on days 7,14 and 28 and then following depuration sampled on day 7,14 and 28. N= between 7 and 11 for all bars except the negative and positive controls which have a sample size of 3. Negative control represents hatchery reared adult male rainbow trout, and the positive control represents estradiol injected hatchery reared male rainbow trout.

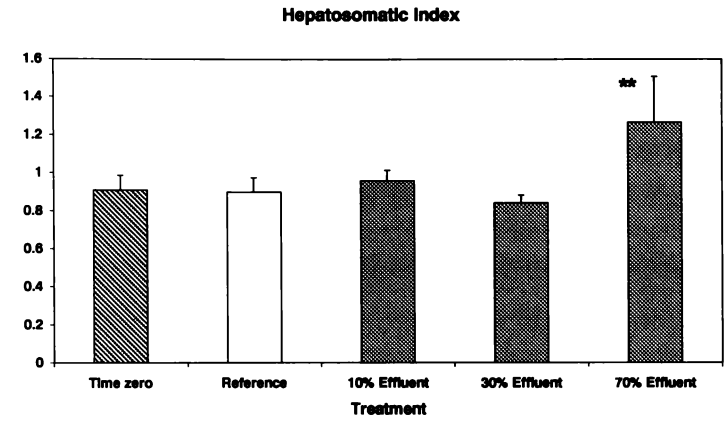
The second experiment, a 21-day exposure to multiple effluent concentrations, demonstrated that length, weight were not affected by exposure to final treated effluent, nor was condition factor (Figure 4.5a). However, significant treatment differences in liver and spleen size, shown as somatic indices, were observed (Figure 4.5b&c). Results from Tukey's pairwise comparisons indicate that at concentrations of 10% and 30% (v/v), treated effluent had no effect on liver or spleen size in exposed fish. However, treatment differences were observed between 70% effluent and all other treatments. Spleens were also significantly enlarged compared to time zero in the 30% effluent-treated fish. Exposure to 70% effluent was very brief (four days) due to high mortality. Circulating sex steroid

levels of testosterone and pregnenolone were not affected due to 10%, 30% or 70% effluent exposure (Figure 4.6). There were obvious sex-related differences in testosterone levels. Again, effluent at concentrations of 10%, 30% and 70% were not capable of stimulating vitellogenin production or gene expression in exposed males (Figure 4.7). PCR analyses for detection of estrogen receptor gene expression also failed to show induction following effluent exposure at any of the exposure concentrations. Analyses of MFO enzyme activity, measured as EROD induction indicated that activity was in all treatments, although the level of activity was very low (Figure 4.8). EROD induction was used as a method of indicating that fish were exposed/affected by effluent. No gender differences were noted in EROD activity nor were there any significant treatment differences.

A)



B)



C)

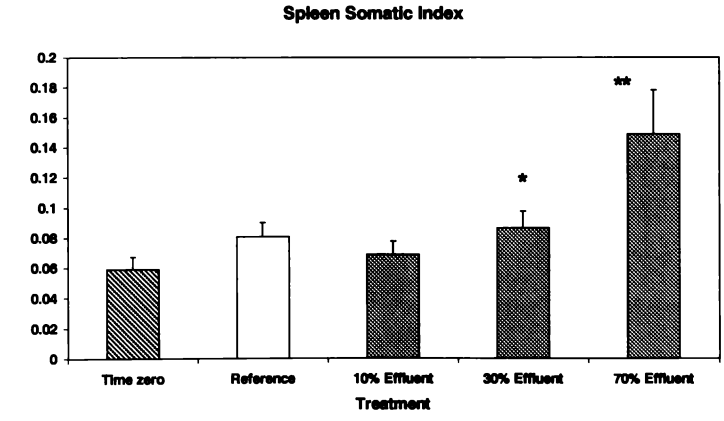
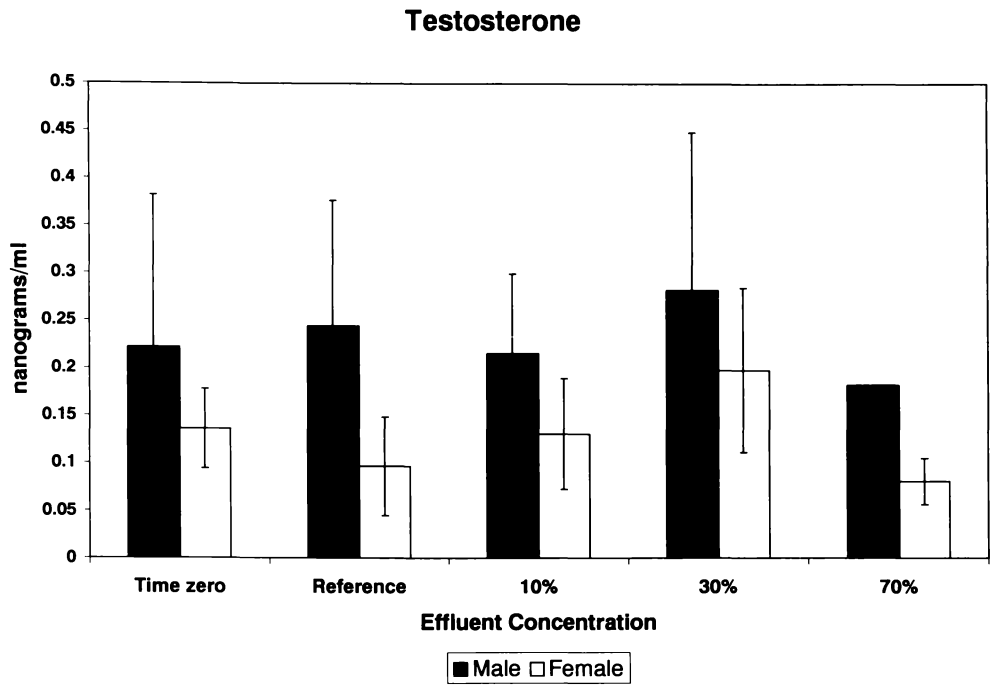
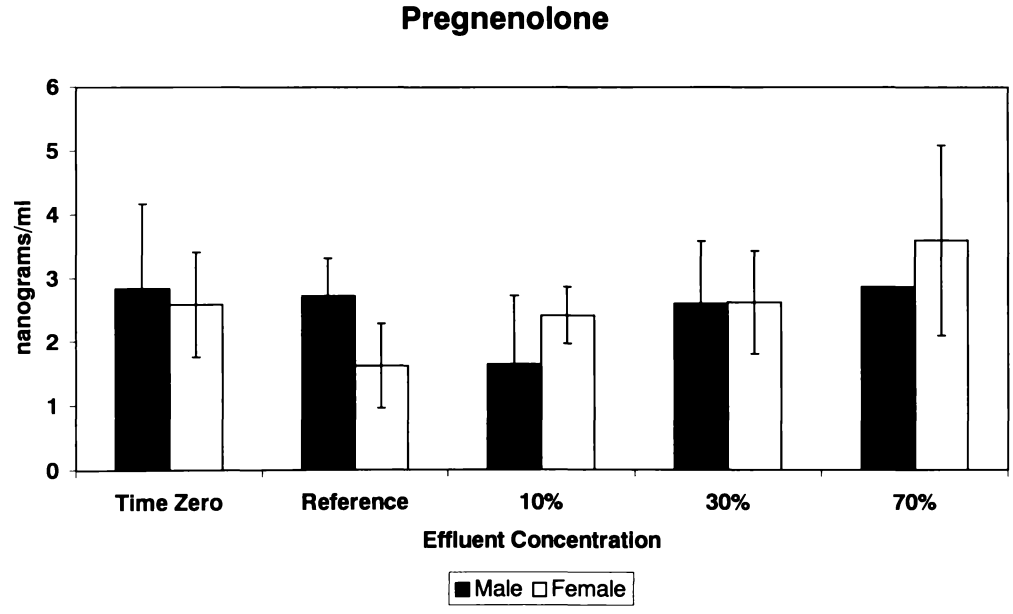


Figure 4.5. Mean (± 95% C.I.) condition factor (A), hepatic (B) and spleen somatic (C) indices in juvenile rainbow trout exposed to reference water and 10, 30 and 70% treated effluent for a 21-day period. Each bar represents a sample size of 40. A single asterisk indicates a significant difference from time zero, and a double asterisk represents a significant difference from all other treatments.(ANCOVA, $P<0.05$)



A)



B)

Figure 4.6. Mean (± 95% C.I.) concentrations of plasma steroid sex hormones a) testosterone and b) pregnenolone in juvenile rainbow trout exposed to Tarawera (reference) river water and 10, 30 and 70% effluent in river water for 21-days. Sample size for all bars representing females = 12,9,9,10 and 4 and males n= 6,10,8,9, and 1 for time zero, reference, 10, 30 and 70% treatments respectively.

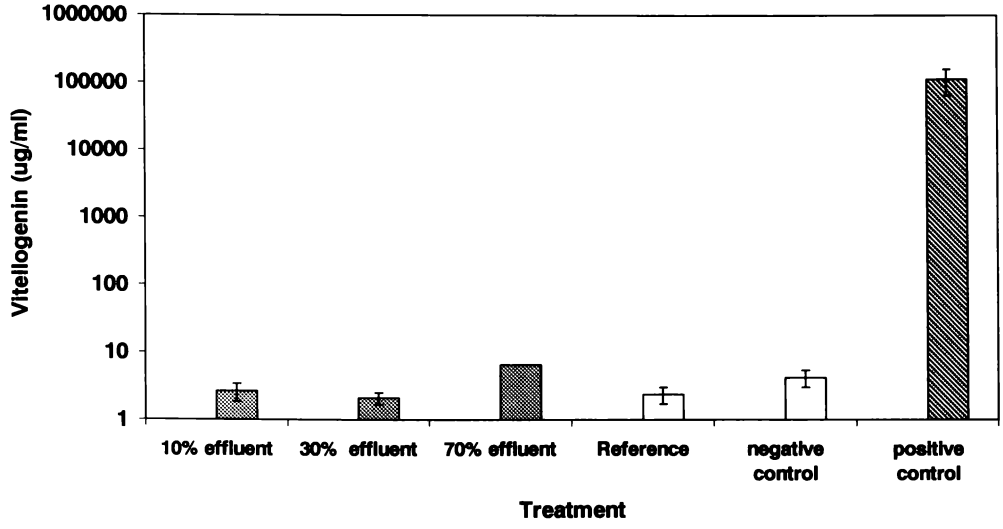


Figure 4.7. Mean (± 95% C.I.) plasma vitellogenin concentrations in juvenile male rainbow trout exposed to reference water and 10, 30 and 70% effluent, n= 23,30,1,20 for 10%, 30%, 70% and reference bars respectively. N=3 for the negative and positive controls. Negative control represents hatchery reared male rainbow trout, and the positive control represent estradiol injected hatchery reared male rainbow trout.

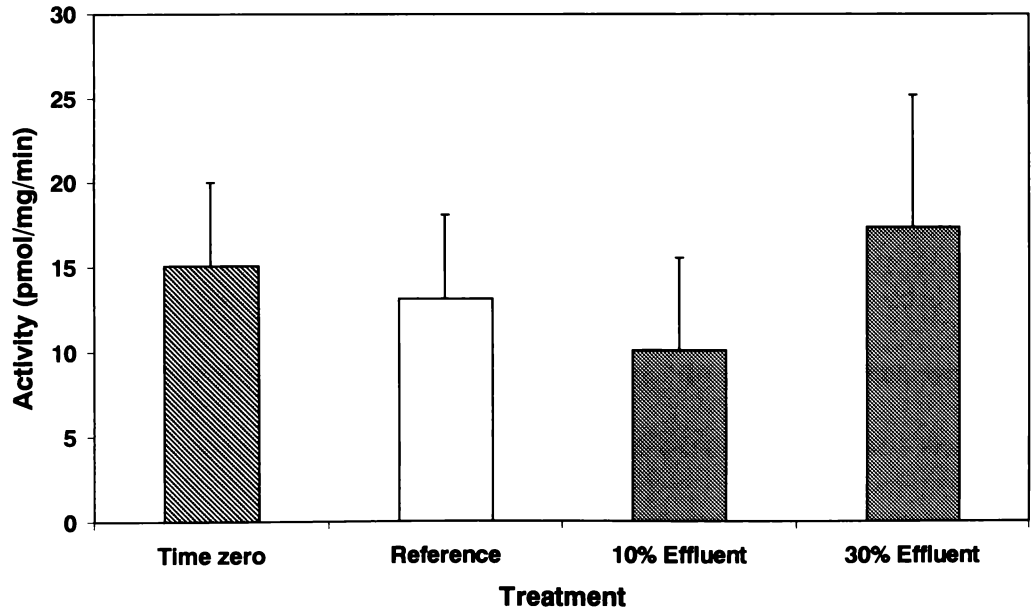


Figure 4.8. Mean (± 95% C.I.) hepatic 7-ethoxyresorufin deethylase (EROD) activity in male and female rainbow trout exposed to reference water and 10 and 30% effluent. Each bar represents mixed sexes. N= 24,18,9, and 13 for time zero, reference, 10 and 30% effluent treatments.

4.5 Discussion

During both long and short-term mesocosm exposures of juvenile rainbow trout to secondary treated effluent, concentrations ranging from 10% to 30% (v/v) failed to impact upon physiological or reproductive parameters including length, weight, condition factor, liver, spleen and gonad size. However, exposure to 70% effluent resulted in high mortality, as well as significantly larger spleen and livers compared to the other treatments. Most significant was the lack of estrogenicity or alteration of steroidogenesis that has been observed in past studies (McMaster et al. 1994; McCarthy et al. 1997; Tremblay and Van Der Kraak 1999). High mortality observed in the 70% effluent concentration was unexpected as initial toxicity tests demonstrated that 100% treated effluent was not acutely toxic to fish (*Gambusia*) or *Daphnia* (chapter two). It is possible that toxicity was due to atypically high suspended solids loading during the experimental period (55 $mg \cdot L^{-1}$ *cf* annual mean of 38.3 ± 4.4$mg \cdot L^{-1}$) which may have resulted in difficulties detecting food, increased levels of ammonia associated with high suspended solids. Differences in species sensitivity may also account for acute toxicity at differing effluent concentrations. Toxicity due to resin acids could potentially be another reason for high mortality. Resin acid concentrations recorded in the present experiment were approximately 0.535 $mg \cdot L^{-1}$ in 100% effluent, while the LC50 value is 1$mg \cdot L^{-1}$, although the highest test concentration used was 70% (Leach and Thakore 1976). It seems unlikely that sampling stress or lower oxygen levels were responsible for mortality as dissolved oxygen measurements were similar between all treatments. Despite such high mortality, it is important to consider the fact that environmentally relevant effluent concentrations in the Tarawera River range between 5-12%.

Depressions in circulating sex steroid hormones have been found in fish downstream of pulp and paper mills that implement a variety of treatment and process/production procedures (i.e. with/without chemical bleaching and with/without secondary treatment) (Munkittrick et al.1994; McMaster et al. 1995, 1996; McCarthy et al. 1997). The present studies failed to produce any evidence supporting decreases in circulating sex steroids or impairment of steroidogenesis following exposure to TMP/BK mill secondary treated effluent. Testosterone levels from the 21-day exposure showed no between-treatment effects, apart from obvious sex-related differences within treatments. Pregnenolone was more stable within treatments but still demonstrated no differences between treatment groups. During the 56-day experiment, no statistically significant differences in testosterone or pregnenolone production between treatments were noted. In contrast to the present findings, a similar 21-day exposure of juvenile rainbow trout to bleached kraft mill effluent (BKME) conducted by Tremblay and Van Der Kraak (1999) resulted in reductions in plasma testosterone and pregnenolone levels of exposed fish. Although some research has been able to link disturbances in hormonal levels to reproductive impairment, others have demonstrated that the use of biochemical and physiological parameters similar to those used in the present study did not clearly relate to impaired reproduction as measured by gonad weight and fecundity (Gagnon et al. 1994a).

EROD activity, as in other studies, was used as an indicator of exposure to BKME –related chemicals (Gagnon et al. 1994a). However, in the present study, EROD activity was relatively low compared to international studies with activity between 8-60 $pmol \cdot mg^{-1} \cdot min^{-1}$, although control values were high. A previous study of the same site saw a 2.7-fold induction in EROD activity in male trout (van den

Heuvel et al. unpublished data). In the present study the induction levels are consistent with a previous study in which rainbow trout sampled downstream of pulp and paper discharge sites in the Tarawera River catchment were tested (Donald 1997).

There is some evidence to suggest that compounds present in pulp and paper mill effluents have weak estrogenic potential (Van Der Kraak et al. 1998). Estrogenic activity of whole bleached kraft mill effluent as well as black liquor has been reported by Zacharewski et al. (1995) using *in vitro* recombinant receptor-reporter gene assays. Koistinen et al. (1998) used similar cell line methods for the detection of estrogenicity in pulp and paper effluent, sludge and sediment extracts. Caging studies by Soimasuo et al. (1998) and Mellanen et al. (1999) observed vitellogenin gene expression in male whitefish exposed to a Finnish BKME, and laboratory exposures of juvenile rainbow trout by Tremblay and Van Der Kraak (1999) demonstrated a Canadian BK mill effluent was capable of inducing synthesis of plasma vitellogenin. TMP/BK mill effluents at various concentrations from 10% to 70% (v/v) in the present study failed to induce expression of the estrogen receptor in hepatic tissues and the synthesis of vitellogenin at gene and protein levels in juvenile rainbow trout. It must be noted that vitellogenin induction has yet to be observed in 'wild' male fishes from known BKME receiving waters. In the three mills studied by Mellanen et al. (1999) only one was capable of inducing vitellogenin production. It is also possible that effects are masked due to a high energy intake (ration) as compared to field situations. The timing of exposure and reproductive condition of the individual may also affect responses. Other studies have shown more severe responses when exposure was initiated prior to the initiation of reproductive

development in fathead minnows (Robinson 1994). Experiments run concurrently with the present studies exposed adult female rainbow trout to identical secondary treated effluent demonstrated subtle effects in fish exposed 8 months prior to spawning during the pre-vitellogenesis stage. In contrast, females exposed during vitellogenesis were not affected by effluent exposure (van den Heuvel et al., unpublished 2000).

Highlighted was the fact that this particular mill discharged wood-derived compounds, such as sterols and resin acids, into the receiving environment in amounts vastly exceeding those of the other two mills. An increasing number of studies have linked hormonal changes and reproductive effects to estrogenic contaminants within such effluent suggesting that these compounds are predominantly wood-derived phytosterols (Denton et al. 1985; Servos et al. 1994; Mellanen et al.1999). In particular, ß-sitosterol has received much attention and is found in high quantities in pulp mill effluents (Zacharewski et al. 1995; McLatchy et al. 1995, 1997; Mellanen et al. 1996, 1999). β-sitosterol has been shown to increase plasma vitellogenin and decrease plasma testosterone, pregnenolone and cholesterol levels in juvenile rainbow trout (Tremblay et al. 1995). Similar effects have been seen in goldfish following intraperitoneal injection or waterborne exposure (MacLatchy and Van Der Kraak 1995; MacLatchy et al. 1997). A survey of 22 US mills found total sterol concentrations ranged from 71 to 535 $ug \cdot L^{-1}$ (Cook et al. 1996). Total sterol concentrations in the present study averaged 87 $ug \cdot L^{-1}$ to 287 $ug \cdot L^{-1}$, with ß-sitosterol contributing approximately 50% of the total phytosterol concentration with 165.4 $ug \cdot L^{-1}$ and 57.24 $ug \cdot L^{-1}$ for experiments one and two respectively. In comparison to previous studies which have observed estrogenic activity, phytosterol concentrations in the

present study were considerably greater. Our findings would suggest that phytosterols (ß-sitosterol) are not the primary agent or cause of estrogenic activity in BKME. Regardless of the 'tendancy' to hold phytosterols as a primary cause of estrogenic activity, a range of compounds present in BKME are known to be weakly estrogenic including lignans, stilbenes and resin acids (Van Der Kraak et al. 1998). Additives used in pulp and paper production have also been suspected as having potential to cause reproductive impairment including chlorinated compounds and surfactants. The pulp and paper industry are major users of alkylphenol ethoxylates, also known as nonionic surfactants and are commonly used as detergents, emulsifiers, wetting and defoaming agents (Lee and Peart 1999). Nonylphenol, a metabolite of alkylphenol ethoxylates is widespread and persistant in the environment and is well-known for its endocrine disrupting potential, including the ability to act as an estradiol agonist.

Although much focus has been on estrogenic activity, evidence suggests that pulp and paper effluents are capable of androgenic effects. Plant sterols such as ß-sitosterol, stigmasterol, and stigmastanol may be broken down by microorganisms to produce androgenic steroids or androstane-like compounds (Denton et al. 1985). Androgenic effects in fishes exposed to bleached kraft mill effluents have also been linked to phytosterols. Effects include morphogenesis in female mosquitofish and tubercle development in female sucker (Munkittrick et al. 1997; Cody and Bortone 1997; Ellis et al. 2000). Additionally, Hewitt et al. (2000) have demonstrated that some constituents of BKME have the ability to interact with the androgen receptor in white sucker hepatic tissue. Although TMP/BK mill effluent used in the present study did not impact on the reproductive physiology, growth or

mortality of juvenile rainbow trout, previous experiments conducted by the authors (Ellis et al. 2000; van den Heuvel et al. unpublished data) utilising the same effluent source, would suggest androgen-like potential/effects in the effluent. Further research is currently being undertaken by the authors to investigate the androgenic potential of this effluent and unpublished data have revealed that the TMP/BK mill effluent extracts used in the present study have the potential to bind to the androgen receptor in goldfish gonadal tissue.

In summary, the degree and extent of responses to BKME have varied considerably and the pattern and extent of responses of fish in receiving environments appear to be site specific and not easily extrapolated to all situations. Variations in characteristics of the receiving environments and exposed species (Kloepper-Sams et al. 1994a) and mode of action of endocrine modulating compounds must also be considered. Given that research is closer to isolating compounds responsible for the observed reproductive effects, at present the exact causative agents have not been identified and the assumption cannot be made that the effects observed are due to phytosterols, the absence of secondary treatment, or chlorine bleaching alone.

4.6 Conclusions

During both 21-day and 56-day experiments, exposure of juvenile (1+) rainbow trout to a mixed TMP/BK mill effluent failed to demonstrate estrogenic activity or effects including the induction of vitellogenin and estrogen receptor expression in male fish, or a reduction in steroidogenesis. Growth, condition factor, liver and spleen size were also not altered due to effluent exposure at concentrations of 10%

and 30% (v/v). However, an effluent concentration of 70% (v/v) resulted in unexpectedly high mortality, as well as enlarged liver and spleen size in exposed individuals. This is believed to be due to atypically high solids loading in the effluent during the period of experiment two.

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Chapter Five: Evidence of an Androgenic Response in Adult Female Mosquitofish Exposed to a Modern Pulp and Paper Mill Effluent

5.1 Abstract

Internationally, studies have noted effects of pulp and paper effluents on the reproductive physiology of fishes. The present study is part of an ongoing programme to determine the potential impacts of a New Zealand pulp and paper mill's effluent on fish populations. Adult female mosquitofish, *Gambusia affinis*, were exposed to pulp mill effluent prior to and post secondary treatment for 21 days. Occurrence of gonopodial development (masculinisation) was noted. The use of glass fibre (GF/C) filtered and unfiltered secondary treated effluent initiated examination of the route of exposure. Gonopodial development was noted in all the unfiltered samples at environmentally relevant concentrations (15% by volume), but was significantly less in secondary treated effluent. Filtered samples produced considerably less gonopodial development than unfiltered samples. Male mating behaviour was observed in masculinised adult female mosquitofish. The above data suggest significant endocrine modulating and potential population-level effects of combined thermo-mechanical pulp/bleached kraft (TMP/BK) mill effluent in this species.

5.2 Introduction

The impacts of pulp and paper mill effluents on receiving biota have included reproductive impairment in fishes. Observed effects include reduced plasma sex steroid levels (Van Der Kraak et al. 1992; McCarthy et al. 1997), decreased egg and gonad size (Andersson et al. 1988; Munkittrick et al. 1992a, 1992b, 1992c),

reduced fertilization and hatching success, decreased occurrence of secondary sex characteristics and delayed maturity (McMaster et al. 1991). Changes in sexual differentiation have been observed in pulp effluent-exposed eelpout, resulting in an increase and bias towards male offspring (Larsson et al. 2000).

Modification of secondary sexual characteristics has also been linked to pulp and paper effluent exposure. In mosquitofish (*Gambusia affinis*), a viviparous poeciliid, females have been observed to develop a male sexual organ, or gonopodium. Along with this morphological alteration, changes in behaviour (Howell et al. 1980) as well as reproductive potential (Rosa-Molinar and Williams 1984) have also been reported upon exposure to kraft mill effluents (KME). A study by Drysdale and Bortone (1989) exposed newly born *Gambusia* to whole BKME in the laboratory and noted precocious male development resulting in reduced adult size.

Gonopodial development has been studied in great detail in Poeciliidae species (Clemmens et al. 1966) including *Gambusia* (Turner 1941b, 1942a, 1942b). The treatment of female *Gambusia* with androgenic steroids, including methyl- and ethyltestosterone, readily induces gonapodial development although the effect is dramatically reduced in older individuals. The gonopodium of a masculinized female is identical in structure to that of a male. Further studies by Turner (1947) have linked castration of males to the cessation of gonopodial development. Following castration, hormone replacement using ethynyl testosterone results in the normal development of gonopodia in these individuals.

Although the specific chemicals or factors responsible for masculinization in BKME exposed *Gambusia* have not yet been identified, it is known that pulp and paper mill wastewaters contain wood extractives, processing additives and other compounds that are potentially capable of endocrine disruption (Owens 1991; Zacharewski 1997). Some of these compounds include phytosterols, resin acids, polycyclic aromatic hydrocarbons (PAHs), surfactants and organochlorines. It has been suggested that a wide variety of potential compounds occur as by-products from the processing of wood pulp (Bortone et al. 1989). Plant sterols have been suspected as the chief source of androstane steroids that can alter sex characteristics (Rosa-Molinar and Williams 1984). Previous research has demonstrated that plant sterols such as β–sitosterol, stigmasterol and stigmastanol may be broken down by microorganisms to produce androgenic steroids or androstane-like compounds. Denton et al. (1985) have shown that by-products of microbially degraded stigmastanol and β-sitosterol (using the micobacterium *Mycobacterium smegmatis)* can exert morphogenic effects in female *Gambusia* similar to those of Turner (1942a) and Howell et al. (1980).

The primary goal of this study was to ascertain if a modern, secondary-treated, elemental chlorine free (ECF), New Zealand TMP/BK mill effluent would induce abnormal morphogenic responses and altered behavioural traits in adult female mosquitofish. Secondly, the study sought to establish whether secondary treatment of effluent and subsequent filtration of this final treated effluent had a significant effect on the aforementioned responses. Finally, the need to isolate and identify the compounds within the TMP/BK mill effluent that could potentially be responsible for the observed effects was addressed.

5.3 Methods and materials

Mill description

The Norske Skog-Tasman Mill (formerly Fletcher Challenge Paper) is an integrated thermomechanical and bleached kraft (TMP/BK) pulp and paper mill producing 760 and 1010 air dried tonne per day (a.d.t.·d^{-1}) respectively. Mill production is primarily softwood (*Pinus radiata*) with the occasional use of eucalypt. Norske Skog-Tasman implements secondary wastewater treatment and elemental-chlorine-free (ECF) bleaching. The wastewater treatment system has a TMP pre-treatment bioreactor facility located adjacent to the TMP mill. Thermo-mechanical pulp and Kraft mill effluent is then collected into a single drain and passed through two bar screens and a clarifier for partial solids removal. Secondary treatment occurs in a four-pond aerated stabilisation basin. The ponds have an area of 45 hectares, with a retention time of 5-6 days. Following treatment in the aerated lagoon system, effluent is then discharged into the Tarawera River at a total mean volume of 180,000 $m^3 \cdot d^{-1}$. River effluent dilution ranges between 5 and 12 percent and enters the river system with an average temperature of 26-28°C.

Fish

This study focuses on changes in the reproductive morphology of adult female mosquitofish, *Gambusia affinis*. The mosquitofish is native to southern USA around the Gulf of Mexico and including the southern states from Texas to Alabama (McDowall 1990). They have been introduced into many tropical and

subtropical regions, chiefly as a means to control mosquito populations (McDowall 1990). Mosquitofish were introduced into New Zealand in the 1930s and are found throughout the North Island, but predominantly in Northland, the Waikato and the Bay of Plenty.

Typical to the Poeciliidae family, mosquitofish display strong sexual dimorphism with females averaging 60 mm and males 35 mm. Apart from size differences, males possess an anal fin with a modified gonopodium. The gonopodium is a complex structure derived from the elongation and modification/fusion of fin-rays 3,4 and 5, which functions as an intromittant organ during copulation (Howell and Denton 1989). The tip of the gonopodium has hooks and spines that act as a grasping device during sperm transfer.

The anal fin of females continues to grow proportionately to body size throughout the life of the female. However in males, the modification of the anal fin is hormone dependent (under androgenic control) and at sexual maturity, body size and gonopodia discontinue further growth (Turner 1941b). While gonopodia do not normally occur in female *Gambusia* they have been experimentally induced by treatment with androgenic hormones (Turner 1941a, 1941b, 1942a) and degradation products of phytosterols (Denton et al. 1985).

Mosquitofish, *Gambusia affinis,* were captured from a population in the University of Waikato campus lakes, Hamilton. Fish were caught using hand nets and transported alive back to the laboratory where the sexes were separated and allowed a two week acclimation period. If gender was unclear, those individuals

in doubt were removed from female only tanks. Adult female mosquitofish were selected for experimental exposures.

Experimental design and exposure

Mosquitofish exposures were performed under laboratory conditions with a 12:12h photo-period. Test chambers were gently aerated and arranged in a water bath maintained at 26-28°C. The total number of fish per treatment was twenty-four. Each treatment consisted of four replicates of six fish. The test volume per replicate was 4 liters with a 50% daily static renewal within all treatments.

Effluent for both experiments was sourced either directly from the first in-flow drain into pond one of the four pond aerated stabilization basin system or the final outflow drain from pond four, immediately prior to discharge into the Tarawera River. Effluent was transported to the laboratory in 20 L polyethylene carboys. Upon arrival at in the laboratory (within one hour of collection), effluents were stored refrigerated at 4°C in multiple 20 L containers, and were shaken vigorously prior to use in daily effluent replacement.

Two experiments were conducted during this study, both 21-day exposures to pulp and paper effluent. Exposures were either to effluent prior to treatment through the stabilisation pond system or effluent post-treatment and directly before immediate discharge into the Tarawera River. In both experiments, dechlorinated Rotorua City tap water was used for reference and diluent waters. Experiment one was initiated mid-December 1998 and experiment two was conducted in July 1999.

The first experiment contrasted untreated effluent at 15% v/v dilution (taken from the inlet of pond one of the treatment system) with secondary treated effluent (taken from the outlet of pond four) at 15% and 70% dilution. The second experiment compared filtered secondary treated effluent with unfiltered effluent (both at 15% v/v dilution). Filtration was conducted immediately after effluent collection using 15cm Whatman GFC filters. Filtrate and filter papers were stored at 4°C and -20°C respectively. Effluent dilutions needed for 50% daily renewals were aliquoted once filtration was completed, for all 21 days of the experiment.

Extractable organics analysis

Routine effluent chemistry measurements and samples for organic chemistry analyses were taken over the period when the experiments took place. Aqueous effluent samples for determination of organics (and removal due to filtration) were extracted by continuous liquid-liquid extraction at pH 9. The solvent volume was reduced, samples were dried with anhydrous sodium sulphate and then derivatised (silylation) for analysis by GC-MS. All organics were corrected for extraction blanks and adjusted for the recovery of appropriate surrogate standards.

Morphological and behavioural analyses

Morphological changes in the female anal fin were monitored daily by visual observation and classified into 5 categories (Howell and Denton 1989) in order to quantify and interpret the degree of anal fin modification. Morphological development was recorded as one of five gross developmental stages; 1). no obvious change; 2). tip of fin fused i.e. thickening of the 3rd ray; 3). addition of

new segments to 3rd,4th&5th rays, giving appearance of elongation; 4). 3,4,5-ray fusion and elongation, obvious to naked eye; 5). full gonopodial development, the length as long as that of a normal male. At termination of the experiment, all individuals that were in stages 3 to 5 were considered masculinized and were recorded as having undergone some degree of morphogenesis. This gave a degree of conservatism to the final results.

Prior to the termination of experiment one, a 15 minute ethogram was compiled on one replicate per treatment. Five male-reproductive behavioural characteristics were observed and recorded over a 30 minute interval following the protocol and descriptions of Bortone et al. (1989). The five behaviours monitored were approach, chase, display, thrust and penetrate. Brief definitions of the behaviours include: Approach- fish slowly but deliberately, moved toward other; Chase- fish abruptly moves towards other; Display- fish's body remained rigid, quivering slightly with fins held erect; Thrust- fish's erect anal fin was moved toward the gonopore of the other fish; Penetrate- fish makes contact with gonopore of other fish, this usually follows the thrust.

Statistical analyses

Experimental mortality and gonopodial development were analysed using analysis of variance (ANOVA), followed with Dunnett's multiple comparison and Tukey's post-hoc tests. The critical level of statistical differences for analyses was assessed at α=0.05. All statistical testing was completed using the GraphPad Prism 3® software package.

5.4 Results

Mean chemistry measurements of 100% secondary treated effluent over the period of both experiments were demonstrated: conductivity 910 $\mu S \cdot cm^{-1}$, total suspended solids 33.8 $mg \cdot L^{-1}$, pH 7.4 and adsorbable organic halogens 1.2 $mg \cdot L^{-1}$. Resin acids, characteristic of softwood pulping, were the highest concentration extractive found in this effluent at 1.16 $mg \cdot L^{-1}$ total concentration (Table 5.1). Prior to primary and secondary treatment, resin acids average 12.2 $mg \cdot L^{-1}$. Therefore, the combined primary and secondary treatment systems achieved greater than 90% removal of resin acids. Phytosterols and resin acid neutral concentrations in treated effluent were much lower than resin acid concentrations with totals of 255 and 51.3 $\mu g \cdot L^{-1}$ respectively. Of the phytosterols, ß-sitosterol was the dominant compound accounting for more than half of the total phytosterols. Approximately 60% of ß-sitosterol was removed following secondary treatment of effluent (Taverndale and Stuthridge unpublished 1996). Total phenolics (> 90% guiacol) were almost totally removed by treatment showing a reduction from 277 $\mu g \cdot L^{-1}$ in untreated effluent to 1 $\mu g \cdot L^{-1}$ in treated effluent.

Due to the high particulate loading typical of pulp and paper effluent, and the hydrophobicity of the effluent extractives, a significant proportion of these compounds was removed from the effluent by GF/C filtration (Table 5.1). Phytosterols were most effectively removed by filtration showing an 88% removal. Total resin acid neutrals and resin acids removed through filtration were 78% and 76% respectively. Filtration also removed about 95% of the phenolics. The compounds remaining in the effluent after filtration should not be considered

as 'dissolved' since GF/C filtration may not remove small particulates or colloidal solids.

Mortality during experiment one was less than 15% throughout all treatment groups. Experiment two had higher variability in mortality across the treatment groups. This coincided with an atypically high effluent solids loading (TSS) during experiment two (TSS averaged 66 $mg \cdot L^{-1}$) compared with effluent sampled for experiment one (33.8 $mg \cdot L^{-1}$), which is more representative of the mean annual suspended solids loading. Mortality in the reference treatment of experiment two was 16%, whereas filtered and unfiltered treatments had mortalities of 20% and 25% respectively.

Table 5.1. Mean (SEM) total concentration of organics ($\mu g \cdot L^{-1}$) in 100% secondary treated effluent and percent of total concentration retained on filtrate, over the duration of the exposure. N= 7 for all means.

Compound	Mean Total Concentration	SEM	Mean percent retained on GFC filtrate
Resin acid neutrals			
Fichtelite	11	3	94.2
Dehydroabietin	1.5	0.4	87.7
Tetrahydroretene	20.8	5.9	86.4
Retene	16.2	5.6	84.9
Methyldehydroabietin	1.8	0.5	73.6
Resin acids			
Pimaric acid	60.7	10	67.7
Sandaracopimaric acid	15	2.2	74.8
Isopimaric acid	30.1	4.1	72.2
Palustric acid	23.3	5.1	89.4
Dehydroabietic acid	83.7	9.2	68
Abietic acid	151.9	27.2	78
Neoabietic acid	9.3	2.1	97.1
Pimarenic acid	25.2	3.8	71.1
Sandaracopimarenic acid	54.2	14.2	56.9
Isopimarenic acid	75.3	13.7	70.2
13-Abietenic acid	176.6	30.8	69.9
Dihydroisopimaric acid	18.4	3.5	90.3
Pimaranic acid	21.7	3.6	75
Isopimaranic acid	16.1	2.6	75.9
Abietanic acid	216.9	34.7	73.9
Seco-1-dehydroabietic acid	111.5	28.4	40.1
Seco-2-dehydroabietic acid	62.1	15.7	34.7
Phytosterols			
Cholesterol	32.2	7.2	85.1
Campesterol	7.6	1.5	93.7
Stigmasterol	21.2	6.2	88.1
Sitosterol	165.4	29.3	87.8
Sitostanol	61.2	10.2	84.8

Treated and untreated pulp and paper mill effluent caused gonapodium development (Figure 5.1). In experiment one, untreated effluent had a greater gonapodium inducing potency than secondary-treated effluent. Treatment through

the aerated stabilisation basin appeared to decrease the morphogenic response in adult female mosquitofish by approximately 25% (Figure 5.2). However, secondary treated (post-treatment) effluent was still capable of inducing gonopodial development in adult females at an environmentally relevant concentration of 15% (v/v). An obvious concentration response was observed in post-treatment effluent (Figure 5.2). Masculinisation was first observed after 7 to 8 days of exposure and occurred predominantly in the younger (smaller) females.

In the second experiment, filtration of post-treatment effluent resulted in a significant decrease in morphogenic response. In the post-treatment 15% (v/v) filtered treatment group, only one individual developed a gonopodium (Figure 5.3). Gross examination of ovaries in masculinised females showed no abnormal ovarian tissue development or intersex.

Masculinised females exhibited many of the behavioural patterns typical to 'normal' males, i.e., chasing non-masculinised females with gonopodial erection and thrusting. However, very rarely was penetration or contact with the non-masculinised female made. Approach and chase were the predominantly displayed behaviours, whilst display and thrust very rarely occurred. Penetration was observed in two incidents only. Occasionally, masculinised females were observed to attempt mating with other masculinised females.

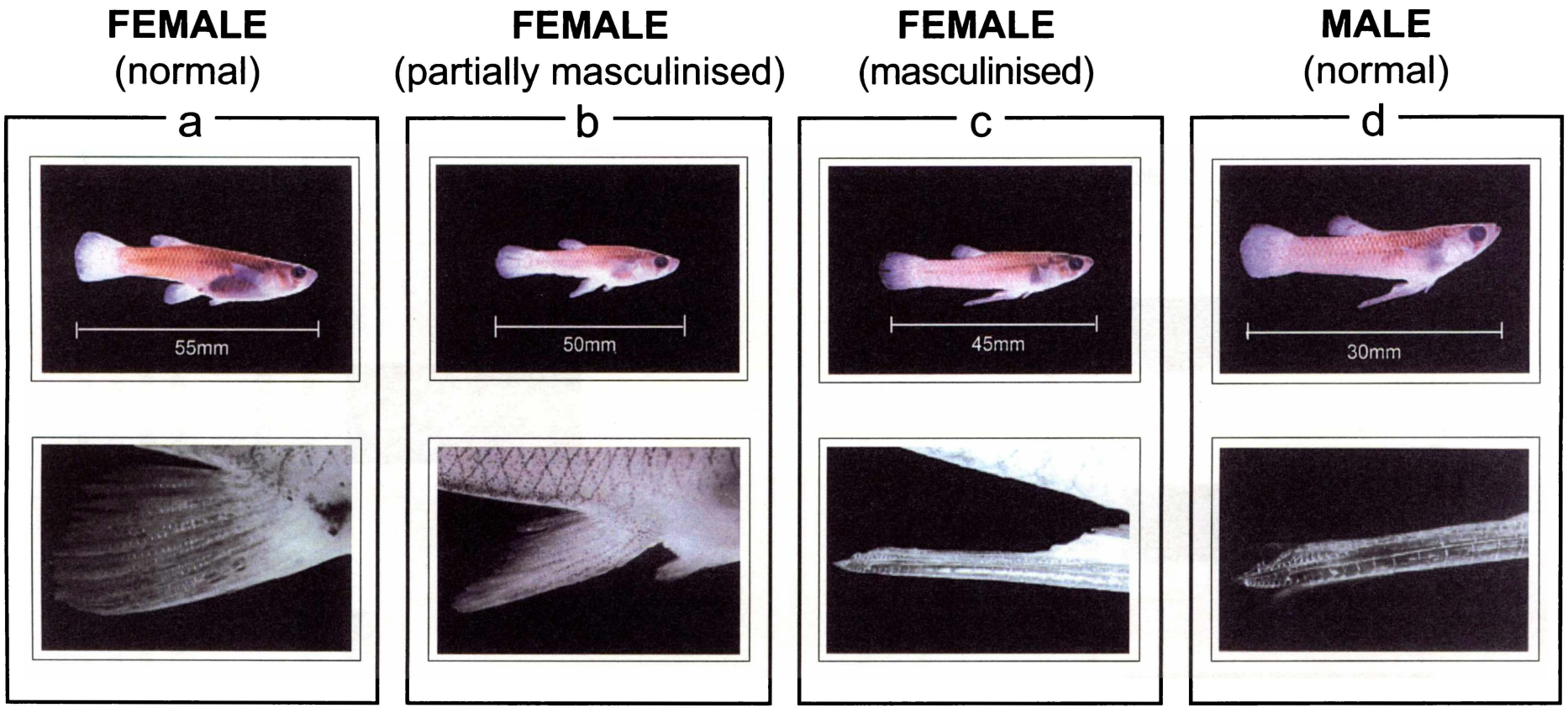

Figure 5.1. Gambusia affinis external and close-up anal fin morphology a) normal female, b) partially masculinised female, c) masculinised female, d) normal male.

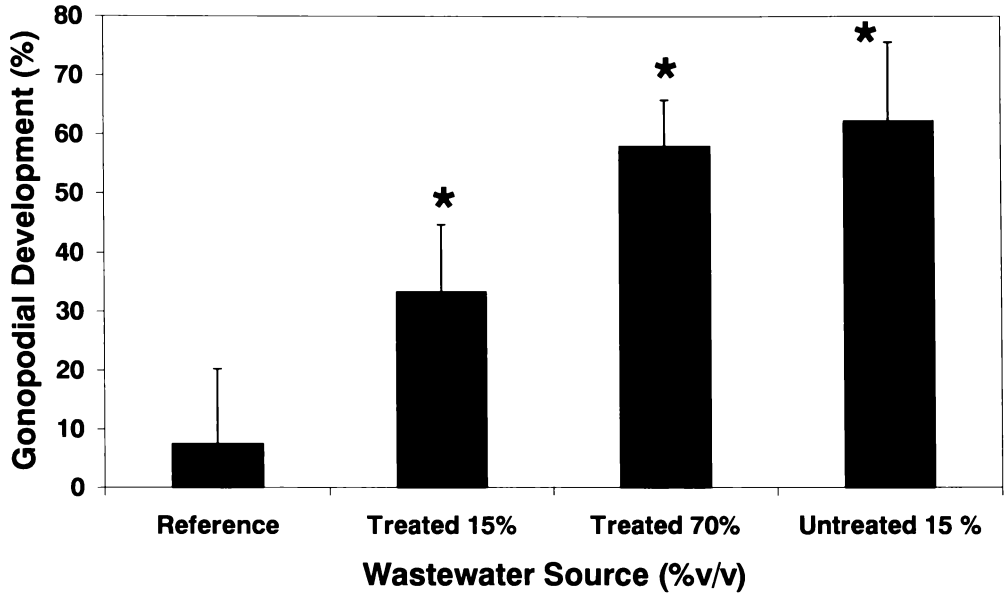

Figure 5.2. Mean (± 95% C.I.) gonapodial development in female mosquitofish exposed to various concentrations of treated and untreated effluent. Each bar represents the mean of four replicate tanks. Each tank contained 5 females. Asterisks indicate significant differences from the reference group (ANOVA, $P<0.05$).

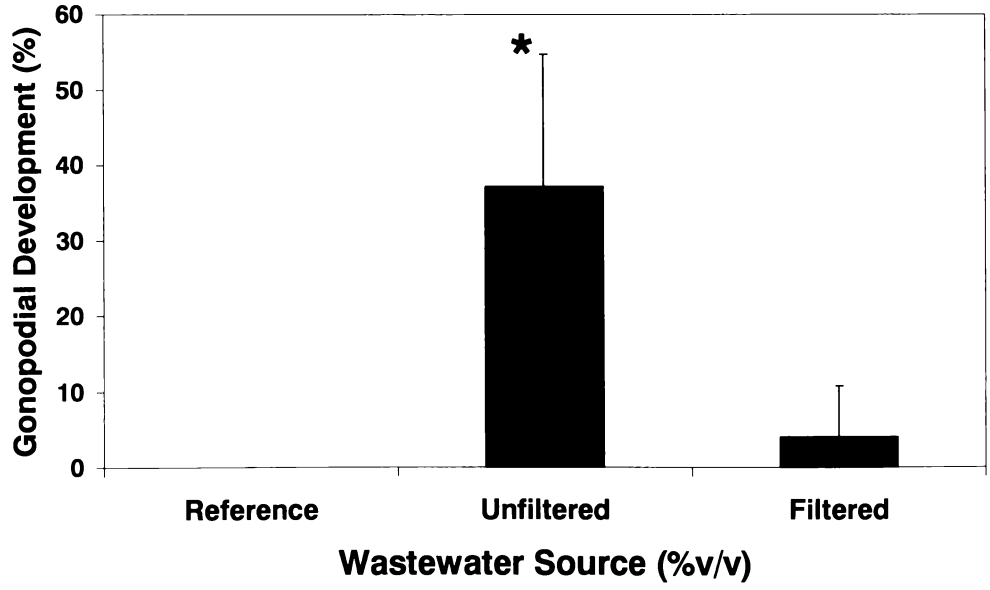

Figure 5.3. Mean (± 95% C.I.) gonapodial development in female mosquitofish exposed to 15% (v/v) GF/C filtered and unfiltered treated effluent. Each bar represents the mean of four replicate tanks. Each tank contained 5 females. Asterisks indicate significant differences from the reference group (ANOVA, $P<0.05$).

5.5 Discussion

In this study, a modern secondary-treated pulp and paper mill effluent was observed to induce androgenic effects in female mosquitofish. Specifically, effluent exposure induced the formation of a male sexual organ, or gonopodium. This morphological change was accompanied by the observation of male behavioural traits. Untreated and secondary-treated effluent were both capable of inducing these androgenic responses. Secondary treatment of the effluent partially reduced the frequency of masculinisation while filtration of effluent eliminated the response almost entirely.

Evidence of environmentally induced masculinisation was first noted by Howell et al. (1980) who observed a masculinised population of mosquitofish (*Gambusia affinis holbrooki*) in bleached kraft mill effluent (BKME) receiving waters in Florida, USA. These observations probably constitute the earliest documentation of 'endocrine effects' associated with pulp and paper effluent exposure. Prior to the Howell et al. field observations of 1980, extensive research had been conducted on the induction and development of gonopodia in female Poeciliids in response to androgenic hormones. Results of the present study concerning gonopodial development are consistent with the findings of Howell et al. (1980), and Drysdale and Bortone (1989).

In this study, morphogenesis began within 7-8 days of exposure with a greater number of younger females developing gonopodia than older individuals. Turner (1942b) observed similar responses and concluded that individuals 35-59 mm in length had anal fins "more" fixed in structural pattern. Both male and female

mosquitofish have an identical genetic pre-disposition for gonopodial development, however, it is the absence of endogenous androgenic hormones in the female that prevent development under normal circumstances (Turner 1942a). In the present study, masculinisation did not appear to interfere with ovarian development. Previous studies have shown that following histological examination, no abnormal ovarian tissue development or sex reversal occurred. In the previous studies, the ability to copulate and produce offspring also did not appear to be inhibited. Howell et al. (1980) observed masculinised females to be pregnant and produce offspring over summer months but the fecundity of these fishes was lower than for unexposed *Gambusia*. Thus it would appear that there is the potential for masculinised mosquitofish populations to suffer some degree of reproductive impairment, thereby impacting on population integrity.

Sterols are ubiquitous in pulp and paper mill effluents, ß-sitosterol usually being the most abundant, as was found in this study. The removal of these compounds during the pulping process presents great difficulty as they are hydrophobic and strongly bound to sediment. Data from the Norske Skog-Tasman mill show only 60% removal through effluent treatment. The sterols, or derivatives thereof, have been implicated as potentially causing some of the reproductive alterations observed when fish are exposed to effluents (Denton et al. 1985; Mellanen et al. 1996).

Microbiological transformations of these sterols into C_{19} steroids including testosterone has been recognised and is suspected as the chief source of androgenic steroids which can alter sex characteristics (Howell et al. 1980; Rosa-

Molinar and Williams 1984). A number of possible modes of action have been suggested including the possiblity that *in-vivo* biotransformation of a testosterone precursor to a physiologically-active androgen could also account for observed precocious and morphogenic effects. Alternatively, a testosterone sterol precursor in effluent might become transformed to an active androgen by microorganisms existing in the effluent, or stream sediment (Denton et al. 1985). Parrott et al. (2000) have observed reduced steroidogenesis in goldfish exposed to a treated pulp mill effluent but failed to find this effect in untreated effluent or any other in-mill waste stream supporting the hypothesis that the active compound(s) are formed during the treatment process. This theory was not supported in the present study, as biological degradation of effluent in an aerated stabilisation basin system (prior to discharge into the Tarawera River) resulted in a 30% reduction in gonopodial development. This coincided with the observed removal of large proportions of most of the measured extractives, with the exception of sterols. At the Norske Skog-Tasman mill, approximately 60% of sterols are removed during biological/anaerobic treatment in the stabilisation basin system. This mill differs from others in that 'untreated' effluent contains TMP effluent that has been aerobically treated in-mill before the various effluent streams are mixed. Thus it is possible that some of the bioactive compounds were formed there.

Although these data suggest a direct androgenic mechanism, other possible mechanisms exist. Stressors/factors other than the direct effect of androgenic hormones are capable of inducing morphogenesis in females including treatment with pregnant mare serum, chorionic gonadotropin, incomplete hypophysectomy, old age, parasites and ichthyophonus fungal infection (Howell et al. 1980). It is

possible that the above 'stressors' have an adverse effect either directly on the ovaries or indirectly through the pituitary gland. Increased levels of gonadotropin released from the pituitary could potentially increase ovarian androgen production if the conversion of testosterone to estradiol were limited. At the level of the ovaries, compounds in effluent may directly cause the inhibition of the conversion of testosterone to estradiol. Due to the complex nature of the hypothalamo-pituitary-gonadal axis, further study is required to resolve a mechanism.

Since the initial studies on mosquitofish masculinisation in the 1980's, the pulp and paper industry in general has undergone significant process and treatment improvements designed to reduce toxicity, BOD, solids and organochlorines in its effluent. The main changes are the nearly universal adoption of secondary treatment (predominantly aerobic) and the replacement of molecular chlorine with alternate bleaching compounds and methods. These changes coupled with other minor improvements in technology and a generally improved environmental awareness have produced dramatic improvements in effluent quality. Despite these changes, modern effluents can still be observed to produce subtle reproductive alterations in fishes including reduced gonad size, reductions in serum sex steroid levels and overall gonadal steroid production (Munkittrick et al. 1992b, 1992c, 1997). The results presented here demonstrate that a modern pulp mill effluent is also capable of inducing an androgenic response in mosquitofish. Future work will seek to determine the identity, mechanism of action and source of the morphogenetically active compounds in order to aid in the formulation of effluent treatment solutions.

5.6 Conclusions

Constituents associated with a modern pulp and paper mill wastewater were observed to exert a significant androgenic effect in *G.affinis* at environmentally relevant concentrations. The present study demonstrated a decrease in effects following secondary treatment of wastewater, which was coincident with the observed removal of potentially androgenic organic constituents (e.g. phytosterols) following secondary treatment in aerated stabilisation basins. Subsequent filtration of final treatment effluent further supported the conclusion that potential endocrine modulating compounds appear to be bound to solid matter within the effluent, and are not totally removed following secondary treatment. The compounds of concern removed following filtration included phytosterols (85-94%) and resin acids (74-94%).

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Chapter Six: Summary and Conclusions

This thesis assessed the reproductive-endocrine disrupting potential of a modern New Zealand pulp and paper mill effluent by examining reproductive endpoints in two fish species. Previous studies have shown that pulp and paper mill effluents have the potential to exert either estrogenic (Mellanen et al. 1999) or androgen-like (Cody and Bortone 1997) effects in fishes. My study addressed both of these concerns by examining estrogenic potential of TMP/BK mill effluent in rainbow trout and androgenic potential in mosquitofish. Exposures of early life stage (ELS) trout investigated hatching success, juvenile mortality rate, growth and development as well as reproductive-endocrine effects, namely the induction of vitellogenin synthesis and depression of steroidogenesis. Two further rainbow trout experiments consisted of long and short-term exposures of juvenile (1year +) trout. The shorter (21-day) experiment used a wide range of effluent concentrations, while the longer-term (56-day) experiment was exposure to a single environmentally relevant concentration. Both experiments focused on reproductive-endocrine effects including expression of the estrogen receptor and vitellogenesis in male fish, changes in organ size and condition factor and depressions of circulating sex steroid hormones. Mosquitofish were exposed to primary and secondary treated effluent to assess androgenic potential and to investigate the reduction and/or alteration of effects following treatment of the effluent, and also to undertake preliminary steps towards the isolation of compounds responsible for the observed effects. The mosquitofish experiments examined gonopodial development in adult females in two 21-day exposures. The first experiment compared primary effluent (15% v/v) with secondary effluent

(15% and 70% v/v), while the second was an exposure to either filtered or unfiltered secondary treated effluent (15% v/v).

Results from the long-term exposure of early life stage rainbow trout until ten months of age, at an environmentally relevant effluent concentration (15% v/v), had no effect on fertilisation, hatching success, mortality, growth or development. Two short-term exposures of juvenile (1 year +) rainbow trout to treated effluent clearly demonstrated that exposure to a wide range of effluent concentrations (10%, 30% and 70% v/v) produced no estrogenic (reproductive-endocrine) effects, including the induction of vitellogenin and estrogen receptor expression in male fish, or a reduction in steroidogenesis. As contrast, exposure of adult female mosquitofish to effluent revealed a significant androgenic response measured as gonopodial development (chapter five). Secondary treatment of the effluent resulted in a substantial decrease in this response, which coincided with the removal of potentially androgenic organic constituents (e.g. phytosterols). Filtration of treated effluent (15% v/v) identified the route of exposure and compounds within the effluent that may be responsible for the observed effects. The effluent constituents with endocrine disrupting potential are bound to particulate matter and the subsequent removal of these solids resulted in almost complete elimination of the androgenic response in female mosquitofish. This is one of the first studies providing evidence that further filtration of final secondary treated effluent removes the reproductive-endocrine disrupting potential of TMP/BK mill effluents.

An unexpected finding was the lack of reproductive-endocrine (estrogenic) response or indication of reproductive impairment in exposed rainbow trout as

previously measured in a number of international studies utilising several fish species (Munkittrick et al. 1992a, 1994, 1997; McMaster et al. 1995; Mellanen et al. 1999). In contrast, a significant androgenic response to TMP/BKME exposure was observed in mosquitofish.

There is a growing body of research highlighting androgenic (or anti-estrogenic) potential of BKM effluents (Drysdale and Bortone 1989; Kovacs et al. 1995a; Hewitt et al. 2000; Larsson et al. 2000; McCarthy et al. unpublished 2000). Although no reproductive impacts or "obvious" signs of androgenicity were observed in juvenile rainbow trout in my study, subtle effects have been observed in adult female rainbow trout exposed to the same effluent (van den Heuvel, unpublished 1999). However, no significant reproductive or estrogenic impacts were observed in exposed males.

My study has advanced towards the process of characterising suspected constituents of TMP/BK mill effluent responsible for the observed reproductive-endocrine effects. Results demonstrated that secondary treatment of effluent removed a large proportion of resin acids and phytosterols, while the majority of compounds further removed by filtration were also phytosterols and resin acids. Internationally, research has demonstrated that plant sterols such as β-sitosterol, stigmasterol and stigmastanol may be broken down by microorganisms to produce androgenic steroids or androstane-like compounds (Denton et al. 1985). In the case of the effluent used in the present study, microbial degradation of constituents associated with pulp and paper effluents (including phytosterols) through secondary treatment in oxidation ponds resulted in a decrease in effects rather than an increase as seen in previous studies (MacLatchy et al. 2000).

Research conducted in conjunction with this thesis has demonstrated that extracts of the same effluent utilised in the present studies, are capable of binding to the androgen receptor in goldfish. β-sitosterol was also tested and showed no binding in the bioassay (van den Heuvel et al. unpublished 2001). This is one of the earliest studies to give positive evidence of androgenic potential in TMP/BK mill effluents. To date, there is no conclusive evidence that the responses observed here are specifically androgenic i.e. mediated through the androgen receptor. The mode of action still remains unclear as do the exact compounds or combination of compounds that are responsible for the observed androgen-like effects. On-going studies is this area aim to develop/provide stronger evidence for androgenicity in TMP/BK mill effluents by incorporating a combination of whole organism (mosquitofish) exposures to effluents and extracts in combination with androgen-blocking compounds, with the development of further androgen-receptor bioassays.

Future directions in the assessment of endocrine disrupting potential of pulp and paper mill effluents must shift from the measurement and detection of effects and the development of more sophisticated assays to detect these effects, towards the identification of compounds within these effluents that are responsible for observed effects as well as the mechanisms (mode of action) by which these compounds act. Identification of the compounds causing effects may then enable prediction of the 'type' of pulp and paper mill that may be more likely to have significant endocrine disrupting impact on fish populations in the receiving environments and consequently the modification/upgrade steps necessary to reduce these impacts.

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