SUBLETHAL EFFECTS OF EUCALYPTUS-BASED PULP MILL EFFLUENTS ON TASMANIAN NATIVE FISH

by

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ABSTRACT

The effects of eucalypt-based pulp mill effluent on organisms in marine receiving waters are relatively unknown. A previous study by Munday *et al.* (1991) assessed the acute toxicity of the effluent discharged from Australian Paper's Burnie pulp and paper mill. However, at that time the mill was discharging up to 20% pine-based effluent. To obtain an accurate assessment of the current situation at the mill, which is now processing 100% eucalypt pulp, the acute toxicity of the Burnie mill effluent to *Vibrio fisheri*, *Isochrysis galbana*, *Tetraselmis suecica*, *Nitzschia closterium* and *Artemia salina* was calculated. The acute toxicity of the effluent to microalgae was determined for two reasons; microalgae form the base of the food chain and has been shown to be sensitive to simulated eucalypt-based pulp mill effluent (Stauber *et al.* 1994), also, as the microalgae formed the base of the food chain in the experiments discussed in Chapter 3, the sensitivity of the microalgae and the *Artemia,* which fed on the microalgae, to the effluent needed to be assessed. The results obtained were related to the constituents of the effluents with the main toxic property of the effluents being the pH. After the pH had been modified to that of seawater the effluents were less toxic. The effluents were not acutely toxic to single cell algae at the concentrations discharged into Bass Strait.

When evaluating the effects of complex effluents such as eucalypt-based kraft pulp mill effluent the different routes of exposure must be considered. The contribution of dietary exposure as well as waterborne exposure needs to be taken into consideration as there has been much discussion in the literature about the extent of the influence of food chain bioaccumulation. To determine the significance of each route of exposure, and if any additive or synergistic effects of the routes occur, an experiment was designed to simulate effects of Burnie mill effluent in the environment. Common jollytails (*Galaxias maculatus*) were exposed to low levels (0.5%) of effluent via the food chain or via the water column or both food chain and water column. As biotransformation of toxicants occurs in the liver, this organ was analysed to detect any sublethal effects which may have been caused by exposure to the effluent. Significant increases in EROD (ethoxyresorufin-O-deethylase) activity in fish exposed to the effluent by the combined routes were detected. However, no significant differences in EROD activity occurred between control fish and fish exposed to the effluent

by only one route. The effects of the combined routes of exposure appeared to be synergistic rather than additive as a 3 - 4 fold increase in EROD activity was found in fish exposed to the effluent via both routes. This was supported by the increase in histological changes within the livers of fish exposed by the combined routes. Also, proliferation of rough endoplasmic reticulum within the hepatocytes of these fish as determined by transmission electron microscopy was an additional indicator of a sublethal response in fish receiving the effluent by the combined water column and dietary routes. An indirect ELISA to detect the presence of cytochrome P-4501A1 was performed but this method was not sensitive enough to detect effects of effluent exposure.

As exposure to pulp mill effluent can have adverse effects on the livers of fish it follows that the reproduction of the fish may be affected, as this organ is involved in hormone metabolism essential to the reproductive processes. To evaluate this aspect of the sublethal effects of pulp mill effluent, Tasmanian blennies were collected from locations in Emu Bay then transferred to the University and maintained in equivalent concentrations of pulp mill effluent to those found in Emu Bay. The blennies then spawned and the resulting embryos were also maintained in the same effluent concentrations until hatching. The exposed blenny larvae were compared with control larvae and showed a significant decrease in length. The number of larvae per spawn and the quality of eggs was also significantly less in Emu Bay fish than control fish. No significant differences between the Emu Bay sites were found so that the incidence of sublethal effects in blenny larvae was not related to distance of blenny populations from the mill outfall.

From the results of this series of experiments, simulating environmental exposure to the Burnie mill effluent, as discharged during 1994 - 96, it can be stated that levels of the effluent equivalent to those occurring in Emu Bay cause fish to exhibit minor changes in liver structure and ultrastructure, a slight increase in detoxification enzyme activity and a decrease in embryo / larval growth. These changes apparently do not severely affect the health of the fish. However, the effect of the effluent on reproduction is appreciable and would have a potential impact on the fish population dynamics.

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GLOSSARY

Acute bioassay	Short-term (relative to generation time) assay utilising relatively high concentrations of toxicants, usually used to calculate LC50
Additive effect	Refers to mixtures of toxicants in complex effluents; toxic effect in combination = sum of separate constituents
AHH	Aryl hydrocarbon hydroxylase
ANOVA	Analysis of variance
AOX	Adsorbable organic halogens
APPM	Australian Pulp and Paper Manufacturers now Australian Paper
Bioaccumulation	Sum of bioconcentration and biomagnification
Bioassay	Toxicity test using live organisms, may be acute (short term), chronic (long term) or subacute / subchronic (intermediate)
Bioconcentration	Concentration in organism in relation to the concentration in the water column.
Biomagnification	Food chain effect; substances at one trophic level are concentrated at higher trophic levels
Biomarkers	Biochemical and physiological changes that are taken as indicators of pollution or stress
Biotransformation	Metabolic alteration of a chemical or molecule
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
Chronic bioassay	Long-term (relative to generation time) and low concentrations of toxicants usually used to calculate EC50
CI	Condition index is a measure of mass per unit length
Cytochrome P4501A1	Highly conserved protein involved in the detoxification of both endogenous (e.g. endogenous steroids) and exogenous (xenobiotic) compounds

DARS	Direct alkali recovery system
EC50	Concentration that affects the designated criteria of 50% of the test population
ECF	Elemental chlorine-free
ECOD	Ethoxycoumarin-O-deethylase
ELISA	Enzyme linked immunosorbant assay
Emu Bay	Receiving water of eucalypt-based pulp mill effluent discharged from the Australian Paper Mill at Burnie
EROD	7-ethoxyresorufin-O-deethalyse
f2 Medium	Widely used medium for culturing marine algae
Genotoxic	Has an adverse effect on the genome
Hepatocyte	Individual liver cell
Histopathology	Technique for determining structure of cells at the light microscope level
Induction	'Switching on' of translation and transcription processes leading to synthesis and activation of a particular enzyme or protein
LC50	Concentration that kills 50% of the test population
Life-cycle	Series of stages, from a point in one generation to the same point in the next generation
Lipophilic	Fat soluble
LSI	Liver somatic index
MAb	Monoclonal antibody
MFO	Mixed function oxidases
Microalgae	Unicellular algae
Microtox	A toxicity test involving the luminous marine bacteria *Vibrio fisheri*. Changes in light output are taken as indicators of stress
n	Total number of organisms used in the experiment
NOEC	No observable effect concentration
OD	Optical density
PAS	Periodic acid Schiff reaction
PCB	Polychlorinated biphenyls
PBS	Phosphate buffered saline

PCDD	Polychlorinated dibenzidioxins
PCDF	Polychlorinated dibenzofurans
Population of a species	Group of organisms of same species living together in the same place at the same time
Reference toxicant	Standard chemical, with known toxicological properties that can be used to test a candidate bioassay
RER	Rough endoplasmic reticulum
RIA	Radioimmunoassay
SCE	Sister chromatid exchange
Semi-static	Exposure system in which the test solution is renewed at intervals during the study
Static	Exposure system in which the test solution is not renewed during the study
Sublethal effects	Effects from exposure to toxicants that are not directly lethal to the organism
Synergistic effects	Refers to mixtures of toxicants and complex effluents; toxic effect in combination is greater than the sum of the separate constituents
TCDD	Tetrachloro-dibenzodioxin
TCDF	Tetrachloro-dibenzofuran
TEF	Toxicity emission factor
TCF	Totally chlorine-free
TEM	Transmission electron microscopy
Toxicant	Chemical or mixtures of chemicals that have an adverse effect on exposed organisms
TTU	Total toxicity units
TU	Toxic unit = 100/EC50
v/v	Volume per volume
WAO	Wet air oxidation

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

The Australian Paper pulp and paper mill, located at Burnie on the north-west coast of Tasmania, Australia (Lat 41-04° S, Long 145-55° E) (Figures 2.1 and 2.2), discharges primary-treated effluent into a marine receiving water, Emu Bay. The effluent discharged is a combination of effluents from both the pulping and paper making processes of the mill. The pulping process uses anthraquinone, a promoter of delignification, and caustic soda with the bulk of the liquid produced during this process entering the mill's waste stream. The pulping process is one of the main contributors of colour, biological oxygen demand (BOD) and lignin derivatives to the final effluent. The Burnie mill uses a chemical bleaching sequence with four stages to bleach the dark coloured pulp :

1. Chlorination (C).
2. Caustic extraction (E) + hydrogen peroxide.
3. Chlorine dioxide (D).
4. Calcium hypochlorite (H).

Mill processes are discussed in more detail in Chapter 2.

The toxicity of pulp and paper mill effluents is considered to be mainly associated with the pulping and bleaching processes (Huuskonen and Lindstrom-Seppa 1995). The toxicity of the final effluent is determined by the type of wood stock and the bleaching process. The woodstock utilized by the Burnie mill for pulping is a eucalypt hardwood. Effluents from pulp mills utilizing hardwood stock for pulping have shown less acute toxicity than pine-based effluents (Davies *et al.* 1988, Lukatelich and Evans 1990, Munday *et al.* 1991). For example; LC50s for rainbow trout range from 10 to 41% for Australian pine-based effluents compared with >100% for eucalypt-based effluent (i.e. no mortalities in 100% effluent) (Davies *et al.* 1988, Lukatelich and Evans 1990, Munday *et al.* 1991). In general, the differences in toxicities can be attributed to the presence of chlorate, dioxins and furans, resin acids, chlorophenols and other lignin derivatives that are found in pine-based effluents (Sodergren *et al.* 1988, Ahtiainen *et al.* 1996, Verta *et al.* 1996). Chlorate, dioxins and furans have not been detected in the

Burnie mill effluent and resin acids are present only as background levels, this leaves the chlorophenols as the main identified toxic constituent (Appendix 1).

Field studies and laboratory-based studies have previously been performed to determine the toxic effects of the eucalypt-based effluent discharged from the Burnie mill. A field study was conducted in 1993 to determine the impact of the Burnie mill effluent on fish populations in Emu Bay (Deavin 1993). This study determined that the effluent adversely affected both the health and reproductive potential of Tasmanian blenny (*Parablennius tasmanianus*) populations living in the bay. Prior to this field study, the acute and chronic toxicity of the Burnie mill effluent to several marine species had been evaluated in laboratory studies by Munday *et al.* (1991). However, the woodstock that the Burnie mill was using in 1990/91 consisted of a percentage of pine (10 - 20%) combined with the eucalypt stock, and this may have influenced the toxicity of the effluent. Therefore, a comprehensive study of the toxicity of the 100% eucalypt-based effluent presently being discharged from the Burnie mill was required by Australian Paper. Australian Paper requested that this study be performed so that answers could be provided which would assist the Environment Manager with deciding if further field studies were required in the future. Thus, the aims of this study were:

1. To define the toxicity of the effluent that was discharged into Emu Bay;
2. To define the toxicity of the waste streams within the plant;
3. To define which biomarkers in fish were suitable for detecting exposure to eucalypt-based pulp mill effluent;
4. To determine the effect of dietary and water-borne exposure of the effluent alone and in combination on fish;
5. To determine the effects of the effluent on fish embryo development.

1.1 Review of Information Relevant to Aims

1.1.1 Definition of the Toxicity of Burnie Mill Effluent

The toxicity of the final discharge was monitored on a monthly basis to determine the variability in the acute toxicity of the Burnie mill effluent and the toxicity of the effluent at the dilution at which it enters the receiving water. Results obtained from the toxicity testing were used to assess the probable impact of the effluent on organisms living within the receiving

water. Also, any variations in toxicity which may impact on the long-term laboratory bioassays were detected.

1.1.2 Toxicity of Waste Water Streams

In conjunction with monitoring of the final discharge, the toxicity of the waste water from each mill processing stage was also assessed. These results were then used to calculate toxicity emission factors to trace the source of toxicity in the final discharge effluent.

1.1.3 Suitability of Fish Biomarkers

As mentioned previously, eucalypt-based pulp mill effluent is less toxic than pine-based pulp mill effluent and, therefore, exposure of fish to eucalypt-based effluent will result in less severe acute and chronic effects when compared to fish exposed to pine-based effluents (Munday *et al.* 1991). Due to the subtle effects of eucalypt-based effluent exposure in fish, a range of biochemical and physiological indicators of exposure needed to be assessed so that the appropriate biomarkers could be selected for detecting this type of exposure. Biomarkers which have been used extensively in the Northern Hemisphere to assess the impacts of pulp mill effluent include; ethoxyresorufin-O-deethylase (EROD) activity, induction of cytochrome P4501A1, haematology, immunology, histopathology of liver, skin, gills and gonads, liver ultrastructure, growth, carbohydrate metabolism, increased vitamin A metabolism, decreased levels of vitamin C, liver somatic index, condition factor, gonadosomatic index, reproductive status, changes in steroid profiles, parasitic infections and skeletal abnormalities (Lethinen 1990, Bucher *et al.* 1992, Goksoyr *et al.* 1994, Kloepper-Sams and Benton 1994, Biagianti-Risbourg and Bastide 1995, Collier *et al.* 1995, Forlin *et al.* 1995, Gagnon *et al.* 1995, Gilbert *et al.* 1995, Martel *et al.* 1995, Parrot *et al.* 1995, Soimasuo *et al.* 1995, van den Heuvel *et al.* 1995, Ahtiainen *et al.* 1996, Mosse *et al.* 1996, Tana and Lehtinen 1996, Struthridge *et al.* 1997). Not all of the biomarkers mentioned above are suitable for use in laboratory-based studies, however, several of these biomarkers have previously been utilized for detecting exposure to eucalypt-based effluents (Munday *et al.* 1991, Holdway *et al.* 1993, Brumley *et al.* 1996).

Elevated mixed function oxidase (MFO) activities have been reported in fish exposed to pine-based pulp mill effluent in many studies (Goksoyr *et al.* 1994, Kloepper-Sams and Benton

1994, Collier *et al.* 1995, Forlin *et al.* 1995, Parrot *et al.* 1995, Soimasuo *et al.* 1995, Ahtiainen *et al.* 1996, Tana and Lehtinen 1996). However, attempts to demonstrate induction

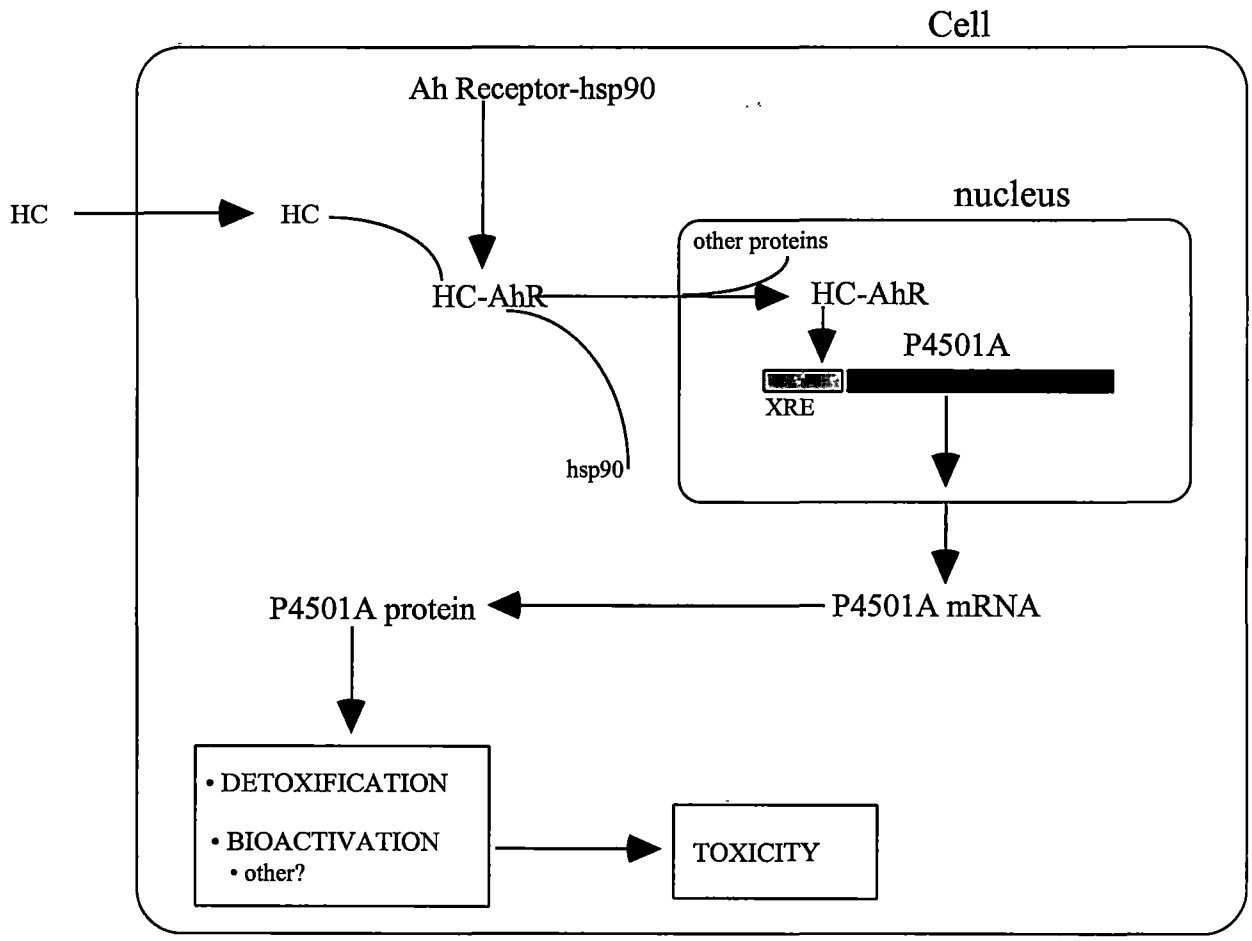


Figure 1.1
Model showing mechanism of cytochrome P4501A induction. Polycylic aromatic hydrocarbon inducer (HC) enters the cell, binds to the receptor (AhR) which is activated and then enters the nucleus. The receptor-inducer complex binds to the regulatory elements (XRE) in the DNA, stimulating transcription of P4501A mRNA. The message is translated to apoenzyme in the rough endoplasmic reticulum and insertion of heme results in an active enzyme. (Adapted from Stegeman *et al.* 1989)

of MFO activity in fish exposed to water-borne eucalypt-based pulp mill effluent have proven inconclusive in two laboratory-based studies (Munday *et al.* 1991, Brumley *et al.* 1996). Munday *et al.* (1991) detected a non-significant trend towards an increase in ethoxycoumarin-O-deethylase (ECOD) activity in common jollytails after 17 weeks of exposure to 0.5% and

5% v/v effluent. However, it has since been shown that EROD activity is a more sensitive method for measuring MFO induction (Holdway *et al.* 1993).

Following on from this work, Brumley *et al.* (1996) exposed sand flathead to concentrations of a simulated eucalypt-based effluent up to 8% v/v for 96 hours and did not detect EROD induction. Even though the use of ECOD and EROD assays have proven inconclusive in the cited studies for detection of exposure to eucalypt-based pulp mill effluent in the laboratory, EROD has proven to be a sensitive indicator of exposure to this type of effluent in field-based studies (Munday and Brand 1992), and, as such, it was selected as a biomarker to use in the assessment of the effects of eucalypt-based pulp mill effluent as described in this thesis.

Another method for detecting MFO induction is the presence of the protein cytochrome P4501A1 which has been shown to be induced by exposure to pine-based pulp mill effluents (Collier *et al.* 1992, Courtney *et al.* 1993, Goksoyr *et al.* 1994, Kloepper-Sams and Benton 1994). There is nothing in the literature to indicate that induction of P4501A1 has ever been utilized as a biomarker for detection of exposure to eucalypt-based pulp mill effluents. Therefore, cytochrome P4501A1 induction as detected using a monoclonal antibody against scup cytochrome P4501A1 (Stegeman *et al.* 1985) was selected for trial as a biomarker in the series of experiments described in this thesis.

Several constituents of pulp mill effluent are known to induce MFO activity (the mechanism of MFO induction is outlined in Figure 1.1). The most potent inducers are tetrachlorodibenzo-dioxin (TCDD) and tetrachlorodibenzo-furan (TCDF), however, most PCDDs and PCDFs will induce MFO activity to some extent (Parrott *et al.* 1995). As Burnie mill effluent has undetectable levels of PCDDs and PCDFs, the level of MFO induction was not expected to be high, however, other unidentified constituents of the effluent may be MFO inducers.

Even though there was no significant MFO induction in common jollytails exposed to eucalypt-based pulp mill effluent in the study by Munday *et al.* (1991) other sublethal effects were observed. The livers of the common jollytails showed histopathological changes and ultrastructural changes of hepatocytes. These changes were less severe than those observed in the livers of fish exposed to pine-based pulp mill effluent of pycnotic and displaced nuclei and vacuolated hepatocytes (Lehtinen and Oikari 1980, Heath 1987, Bucher *et al.* 1992, Khan *et al.*

1994, Kloepper-Sams *et al.* 1994). Histopathological changes are a sensitive biomarker and can provide more information on the health status of a fish than a single biochemical parameter (Teh *et al.* 1997). Therefore, histopathology of the fish livers was also selected as a biomarker for evaluation. Similarly, transmission electron microscopy of hepatocytes was selected for evaluation as a biomarker as this method can detect the subcellular changes which occur prior to cellular or organ damage. Transmission electron microscopy was used to detect ultrastructural changes in the livers of common jollytails in the study by Munday *et al.* (1991), where rough endoplasmic reticulum proliferation and an increase in myelinoid bodies were observed.

Blood plasma analysis for liver derived enzymes was not selected as a biomarker for evaluation, as major cellular damage that would result in the release of these enzymes has not been seen in the livers of fish exposed to eucalypt-based pulp mill effluent. Previously, Munday *et al.* (1991) did not detect any significant findings in the liver enzyme analine aminotransferase (ALAT) in *Aldrichetta forsteri* and *Onchorynchus mykiss* exposed to Burnie mill effluent.

Other biomarkers selected for evaluation were growth, condition index and liver somatic index. These indices reflect a response to chronic stress relating to changes in energy metabolism or feeding regimes (Andersson *et al.* 1987, Goede and Barton 1990, Munkittrick *et al.* 1991, Huuskonen and Lindstrom-Seppa 1995, Levine *et al.* 1995). These biomarkers have not previously been used to assess the impacts of exposure to eucalypt-based pulp mill effluent, however, they were adjudged to be appropriate for use with juvenile fish in long-term studies.

A decrease in steroid levels has been recorded in conjunction with an increase in MFO activity in fish exposed to primary treated pine-based pulp mill effluent (Adams *et al.* 1992, Munkittrick *et al.* 1992). A reduction in steroid levels can ultimately be related to perturbations of reproductive capabilities of fish within a population (Donaldson 1990, Adams *et al.* 1992, Holm *et al.* 1994, Tana and Lehtinen 1996). For instance, reduced steroid levels may have been related to an increased number of atretic oocytes found in Tasmanian blenny populations living in Emu Bay (Deavin 1993). The presence of atretic oocytes may also be directly or indirectly related to exposure to TCDD, as Holm *et al.* (1994) reported the presence of atretic oocytes in

fish after exposure to TCDD. Therefore, an experiment was designed to measure the steroid levels in blennies and assess this biomarker for its ability to detect exposure to eucalypt-based pulp mill effluent.

1.1.4 The Effect of Dietary and Water-Borne Exposure

As all previous studies exposed fish to the eucalypt-based pulp mill effluent via the water column only, the contribution of dietary exposure has been ignored (Munday *et al.* 1991, Brumley *et al.* 1996). As mentioned previously, one aim of this thesis is to assess the use of biomarkers for detecting exposure to eucalypt-based pulp mill effluent. In conjunction with this aim, another aim has been incorporated into this study, to quantify the contribution of both the water-borne and the dietary routes of exposure. Most studies measuring the effect of dietary and water column uptake of toxicants have used only a single toxicant (Macek *et al.* 1979, Cooper *et al.* 1992, Cleveland *et al.* 1993). In those experiments the body burden of the toxicant was measured, however, this is not an appropriate method for assessing the effects of a complex effluent. Instead, the use of biomarkers will enable the determination of the effects on the physiology of the fish and how these observed effects may relate to impacts on fish populations in the receiving environment.

1.1.5 Effects on Fish Embryo Development

Following on from the assessment of steroid levels as biomarkers, a study was designed to determine the possible effects of eucalypt-based pulp mill effluent on embryo development. Johnson *et al.* (1988) and Collier *et al.* (1993) have shown that parent fish from contaminated sites that were exposed to environmental toxicants have more severely impacted offspring than parents from control sites that were not exposed to environmental toxicants. Therefore, a study was designed to collect adult Tasmanian blennies from sites in Emu Bay which were exposed to pulp mill effluent and assess their offspring for abnormalities. The aim of this study was to determine if exposure of adults and embryos to eucalypt-based pulp mill effluent caused any abnormalities in the resulting larvae. Abnormalities may occur in offspring of exposed parents due to the accumulation of lipophilic compounds in the liver and the ovaries which will affect the developing embryo (Chen and Sonstegard 1984, Johnson *et al.* 1988, Collier *et al.* 1993, Holm *et al.* 1994). However, the mechanisms involved in reproductive toxicity are not completely understood but are thought to include modulation of hormone levels essential for

oocyte maturation and ovulation, toxicity to developing gametes or nutritive cells and generalized stress responses (Hose *et al.* 1989).

As very little information on the toxic effects of eucalypt-based pulp mill effluent exists in the literature, the aim of this series of studies was not to determine the mechanisms of toxicity caused by exposure to eucalypt-based pulp mill effluent or to identify toxic constituents, but to develop an understanding of the range of toxic effects caused by this effluent. As the majority of the experiments mirrored the receiving environment, as closely as possible in a laboratory situation, the results from the studies described in this thesis may be able to be related to impacts on fish populations living in Emu Bay.

CHAPTER 2

EUCALYPT-BASED PULP MILL EFFLUENT

CHAPTER 2

ACUTE TOXICITY OF EUCALYPT-BASED PULP MILL EFFLUENT

2.1 Introduction

As mentioned previously, the untreated eucalypt-based effluent discharged from the Australian Pulp and Paper Mill located at Burnie on the north-west coast of Tasmania, Australia enters Bass Strait, a marine receiving water (Figures 2.1, 2.2 and 2.3). The effect of this type of complex effluent on living organisms in marine receiving waters is due to both the physical and chemical properties of the effluent. These properties include:

- High biochemical oxygen demand which results in removal of oxygen from the water column (Adams and Bealing 1994);
- Suspended solids which can cause interference with respiration of fish and invertebrates by clogging the gills (Handlinger 1990);
- High or low pH will alter the solubility of metals in solution, resulting in precipitation of metals on contact with fish gills. Also, pH will affect the solubility of metals such as copper, cadmium and zinc making these toxic metals bioavailable. Further, acid or alkaline water will also interfere with respiration in aquatic organisms (Handlinger 1990a);
- An increase in colour will restrict light from entering the aquatic system and, therefore, decrease productivity of primary producers (Austin 1988);
- An increase of nutrients will cause increased growth of phytoplankton due to addition of nitrogen, phosphorus and carbon (Stauber *et al.* 1994).

As indicated above, effluents discharged from pulp and paper mills are complex mixtures of chemicals with various physical parameters which are determined by the operations performed by the mill. A number of mill processes influence the toxicity of the final discharge. To aid in understanding the causes of toxicity in the effluents from the Burnie pulp and paper mill which were used in this study, the processes and chemical compounds utilised during those processes are outlined below.

2.1.1 Process at Burnie Mill

The overall operation occurring at the Burnie mill (Figure 2.4) involves the processing of hardwood eucalypt wood chips into high quality paper. Annually the mill produces 70,000 tonnes of bleached chemical hardwood pulp and 130,000 tonnes of paper. Australian Paper also imports pulp to provide sufficient pulp for paper production. The entire process, which is documented in the mill's Environmental Management Plan of 1994, is discussed here while more detail appears in Appendix 1.

Mill processes begin with woodchips that are fed into two continuous pulping digesters that utilize a soda-anthraquinone (soda-AQ) process. The chips are placed in steaming vessels and are suspended in recirculating cooking liquor at 180°C, which flows countercurrently to the chips. Anthraquinone, a promoter of delignification, and caustic soda are added to the cooking process and the lignin dissolves in the circulating alkaline pulping liquor. The spent pulping liquor passes to the chemical recovery system, the wet air oxidation (WAO) plant and the direct alkali recovery system (DARS), where the dissolved organic matter is used as a fuel source. The cooked chips then undergo a washing process which removes soluble materials. Next, pressure inside the vessel causes the chips to disintegrate into pulp and the resulting brown pulp is diluted to 4.5% v/v by the addition of water, prior to being treated in screening lines to remove reject material. The brown pulp is washed again to remove residual black liquor in preparation for bleaching. Some of the water from both washings is returned to the screening system, but the bulk of the liquid enters the mill waste stream and is one of the main sources of colour and BOD in mill waste water. The pulps produced by this method are similar to kraft pulps, which utilise a mixture of sodium hydroxide and sodium sulphide as pulping chemicals, but the absence of sulphur in the soda-AQ method is an advantage as it eliminates the foul odour from sulphur-containing gases typical of kraft pulping (Nelson 1989).

Following the pulping process some spent black liquor is diluted and heated under pressure in a wet air oxidation (WAO) plant. This process recovers chemicals and generates steam for use in the pulping process. The oxidised liquor is then mixed with lime to form caustic soda, which is also used in pulping. Condensate, which may contain many soluble constituents of wood is formed when black liquor is heated. This product is subsequently discharged in the mill waste stream.

The remainder of the spent black liquor is concentrated into a heavy black liquor with 57% solids for use in the direct alkali recovery system (DARS) plant. The organic matter in the black liquor burns in a furnace to generate energy which is used throughout the plant. By incorporating the direct alkali recovery system into plant operations, black liquor effluent entering the mill waste water stream has been reduced.

The dark coloured pulp which has been produced is bleached to produce a white pulp. The lignin must be removed completely during this process to obtain the required whiteness of the pulp. After pulping, the washed brown pulp is diluted and mixed with chlorinated water for 45 minutes. This is the first stage of the bleaching sequence. The four stages are: (i) chlorination (C), (ii) caustic extraction (E), (iii) chlorine dioxide treatment (D) and (iv) calcium hypochlorite (H). After 45 minutes the chlorinated pulp is washed and fed to the extraction stage. The chlorine reacts with the residual lignin in the pulp to form a complex which is soluble in caustic soda and, therefore, the chlorinated pulp is mixed with a caustic soda solution and heated for one hour to dissolve the lignin. Hydrogen peroxide is also added during this stage as a pre-bleaching agent. After this second stage, the pulp is washed to remove the residual lignin/chlorine complex and then chlorine dioxide is added for one hour as a bleaching step. The final bleaching stage involves mixing the pulp with calcium hypochlorite for three hours at 50°C. After this stage is completed the bleached pulp is washed and sent for storage until required. Most of the water used for washing in this process is recycled, but 148 L/s are discharged into the mill waste water stream. This discharge represents 20% of the final discharge volume. Organochlorine compounds, that are formed during this procedure, also enter the waste water stream.

Numbers 4 and 10 paper machines process bleached pulp into copier paper. After dilution to 4% consistency the pulp is transferred from the storage tower to the paper machines. Other additives such as dyes, clay, alum and sizes including sulphuric acid (for pH adjustment) are mixed with the pulp. The pulp is further diluted with backwater from the paper machines and passed through a cleaning system to remove any residual dirt. The pulp is then distributed evenly over a forming wire and most of the water is drained and returned for use in pulp dilutions, while some enters the waste stream. The surface of the paper is treated through a size press and calender rolls to achieve the desired sheet density and requisite surface properties.

Finally, the paper is transferred to the finishing room where it is reeled or cut and wrapped as required by the customer.

2.1.2 Toxicity of Pulp and Paper Mill Effluents

The toxicity of pulp mill effluents is considered to be associated mainly with the pulping and bleaching processes (Huuskonen and Lindstrom-Seppa 1995). Therefore, as mentioned in Chapter 1, the toxicity of effluents is determined by the type of wood stock being pulped and the bleaching process that is used in the mill. In general, the toxicity of pulp mill effluents is attributed to chlorate, chlorophenols, resin acids and lignin derivatives (Sodergren *et al.* 1988, Ahtiainen *et al.* 1996, Verta *et al.* 1996). As chlorate has not been detected in the Burnie mill effluents and resin acids are present only as background levels, the main identified toxic constituents of this effluent consist of the chlorinated phenolics and total phenols (Table 2.1). It is pertinent that total phenol content has been shown to be associated with acute toxic effects in a wide range of aquatic organisms (Verta *et al.* 1996).

TABLE 2.1 Waste Water Data for the Burnie Pulp and Paper Mill
(Adapted from the Mill's Environmental Management Plan, 1994)

Quantity discharged	700 L / s
Suspended solids	160 mg / L
Biochemical oxygen demand	250 mg / L
Colour	1400 Pt-Co units
Dilution of waste water at outfall (1996)	12 : 1 (200 m from outfall) 50:1 (1.5 km from outfall)
AOX	1.9 kg / tonne pulp
Total Phenols	1059 µg / L
Chlorinated Phenolics	0.6 - 3.8 µg / L
Total Fatty Acids	< 3.0 mg / L
Resin Acids	0.416 µg / L
Chlorate	Not Detected
TCDD	Not Detected
TCDF	Not Detected

Chlorinated phenols are formed in the bleaching filtrates during delignification of the pulp as a result of chlorine and chlorine dioxide reacting with the lignin structure. This gives rise to products of electrophilic substitution of chlorine in the aromatic ring, electrophilic displacement of alkyl sidechains from the aromatic ring, demethylation of aromatic methoxyl

groups, dealkylation of other aromatic ethers, oxidative ring opening of aromatic rings and formation of quinones (Wallis *et al.* 1993). Oxidation reactions are more prevalent with chlorine dioxide than with chlorine. These reactions cause the formation of a variety of chlorinated phenols including: chlorophenols, chlorocatechols, chloroguaiacols, chlorovanillins, all four of which are also found in softwood filtrates, chlorosyringols and chlorosyringaldehydes which are found in hardwood filtrates only (Wallis *et al.* 1993). The levels of these compounds found in the Burnie mill effluent are shown in Appendix 1.

Some chlorinated phenolics are known to be moderately toxic, persistent and bioaccumulative in the environment (Volkman 1992, Kovacs *et al.* 1993, Stauber *et al.* 1994). Chlorinated phenols can exert both acute and chronic toxicity by the mechanism of uncoupling of oxidative phosphorylation and inhibition of mitochondrial respiration in the cells of fish (Argese *et al.* 1995). These are believed to be the most significant modes of toxic action for phenols with two or more chlorine substituents, whereas, mono-substituted chlorinated phenols act as "polar narcosis" poisons (Leach and Thakore 1975, Kovacs *et al.* 1993, Argese *et al.* 1995). Both Stauber *et al.* (1994) and Kovacs *et al.* (1993) demonstrated that more chlorine substituents present on the phenols the greater the toxicity of the compound. However, the toxicity of the highly substituted compounds can be reduced when the pH is changed to that of seawater (Leach and Thakore 1975, Kovacs *et al.* 1993). The reduction in toxicity at pH 8.2 is related to the greater proportion of ionised molecules to unionised molecules which indicates that unionised forms are more toxic than their corresponding ions. Also, under anaerobic conditions, chlorine atoms can be removed from organochlorines by microbially-mediated reductive halogenation, thereby reducing the toxicity of those compounds (Volkman 1992).

Most of the research on the environmental effects of chlorinated and non-chlorinated lignin derivatives, resin acids, fatty acids, chlorate and chlorophenolic compounds has been performed on pine-based effluent in the northern hemisphere (Leach and Thakore 1975, McKague 1981, Kovacs *et al.* 1993). That research has shown that most of the acute toxicity of the effluents is related to the compounds mentioned above, especially resin acids, fatty acids and chlorate. However, resin acids, fatty acids and chlorate have not been detected in the Burnie mill effluent (Table 2.1). The most toxic compounds commonly found in pine-based pulp mill effluents are polychlorinated dibenzodioxins and dibenzofurans, in particular 2,3,7,8-TCDD/F. Most dioxins and furans have not been detected in the Burnie mill effluent but have

been found in the sludge (see Appendix 1). This indicates that dioxins and furans may be present in the effluent, but at concentrations less than the detection limits of 6 pg/L. The only dioxin detected in the Burnie mill effluent was octochlorodibenzo-p-dioxin which is not considered to bioaccumulate as rapidly or to cause as severe toxic effects as other dioxins (Loonen *et al.* 1994, Grimwood and Dobbs, 1995, Haynes *et al.* 1995, van der Weiden *et al.* 1994).

The total amount of organochlorines in an effluent can be measured as AOX (adsorbable organic halogens), however, this figure gives no information about the types of compounds present or the toxicity of the effluent. Even so, the amount of AOX in the effluent is regulated in some countries including Australia. Environmental Guidelines for New Bleached Eucalypt Kraft Pulp Mills (Commonwealth of Australia, 1995) recommend a limit of 1 kg/air dried tonne of pulp, even though current research indicates that AOX has little environmental relevance and no relationship between AOX and environmental effects has been found (Volkman 1992, McCubbin and Folke 1995, Verta *et al.* 1996).

The acute toxicity of the Burnie mill effluent needed to be assessed as the effluent at the final discharge point was utilised for experiments to determine the sublethal effects on fish and fish larvae. Therefore, the concentration of effluent used in those experiments needed to be less than the concentration which was acutely toxic. Furthermore, a sub-project was incorporated to assess the source of the toxicity by sampling the waste water of all mill processes and the toxicity emission factor (TEF) for each mill process was calculated.

2.1.3 Toxicity Tests

The most common method for assessing acute toxicity of pulp mill effluents is by using single species bioassays (Stauber *et al.* 1994b, Ahtiainen *et al.* 1996, Leal *et al.* 1997). The acute toxicity of the Burnie mill effluent was assessed using the Microtox® bioassay which utilises the luminescent marine bacterium *Vibrio fisheri.* Toxicity is measured by the inhibition of light production by *V. fisheri.* Light inhibition indicates a disturbance of the bacterium's energy metabolism as the luminescence pathway is a direct branch of the electron transport chain. Therefore, the luminescence is a measurement of the metabolic status of the bacteria and can be used to assess the toxicity of a compound (Argese *et al.* 1995, Ahtiainen *et al.* 1996, Leal *et al.* 1997).

Another method for testing the acute toxicity of pulp mill effluents involves the use of a unicellular marine alga. Algal bioassays are based on measurements of inhibition of cell growth or photosynthetic activity (Argese *et al.* 1995, Ahtiainen *et al.* 1996). Unicellular algae are sensitive to a wide range of organic toxicants and are particularly suited to define the acute toxicity of pulp mill effluents (Stauber *et al.* 1994). The unicellular marine alga *Nitzschia closterium* was chosen as a standard bioassay organism by the National Pulp Mills Research Program as it had been extensively used in previous toxicity studies. It is widely distributed in Australian waters and is relevant to Australian ecosystems (Stauber *et al.* 1994). *N. closterium* was used in this study to determine the acute toxicity of the Burnie mill effluent. Also, the bioassay method was used with two other algal species *Isochrysis galbana* and *Tetraselmis suecica* to provide information on a range of algal species as well as ensuring that the effluent was not acutely toxic to those algae at the concentrations used in the dietary experiments reported later (Chapter 3).

The acute toxicity of the effluent to the brine shrimp, *Artemia salina*, which is a food source for fish in aquaculture situations, was also determined. Even though the use of *A. salina* has been criticised because these crustaceans are insensitive to many toxicants (Widdows 1993), the acute toxicity of the *A. salina* needed to be determined to ensure that it was not sensitive to the concentration of effluent used in the dietary exposure experiments as described in Chapter 3.

2.2 Materials And Methods

2.2.1 Sample Collection and Storage

Effluent samples were collected from the Australian Paper pulp and paper mill located at Burnie on the north west coast of Tasmania (Figures 2.1, 2.2 and 2.3). Samples were collected from several process areas which discharge into the main sewer, from a point on the main sewer and at the at the final discharge point (Table 2.2, Figure 2.5 and Appendix 1, Figure 1.2). Each site was sampled several times during 1995 (Table 2.3). Each sample was collected with the aid of a 200 mL stainless steel dipper which was rinsed thoroughly in each effluent prior to sampling. The sample was then transported, at 4°C, in acid washed 250 ml glass bottles, to the Aquaculture Centre at the University of Tasmania's Launceston campus. The samples were maintained at 4°C until testing which occurred within 24 hours. The colour and pH were recorded for each sample at the time of sampling.

The acute toxicity of effluent collected from the discharge point at the Burnie mill was tested approximately once a month when effluent was collected for use in experiments. The effluent was collected from the settlement pond (Figure 2.5) which flows directly to the discharge outflow located in Bass Strait. The effluent was collected using an eight litre plastic bucket and transported in 20 litre black polypropylene containers, with no air space, and was maintained at 0°C in a cold room until required or for a maximum of four weeks. Two litre aliquots of the effluent were removed from the container every four days, placed in 1 litre glass bottles and maintained at 4°C in the Toxicology Laboratory (Aquaculture Centre) for use in exposure experiments.

Table 2.2 Burnie Pulp and Paper Mill Sample Sites

Sample Sites	Volume Discharged L/s
1. Bleach Plant	148
2. Pulp Mill	110
3. N° 4 Paper Machine	80
4. N° 10 Paper Machine	79
5. DARS Plant (Direct Alkali Recovery System)	40
6. WAO Plant (Wet Air Oxidation)	80
7. Pulp / Paper	680
8. Discharge	**760**

Figure 2.1

Tasmania in Relation to Mainland Australia

Figure 2.2

Location of Burnie on the North-West Coast of Tasmania

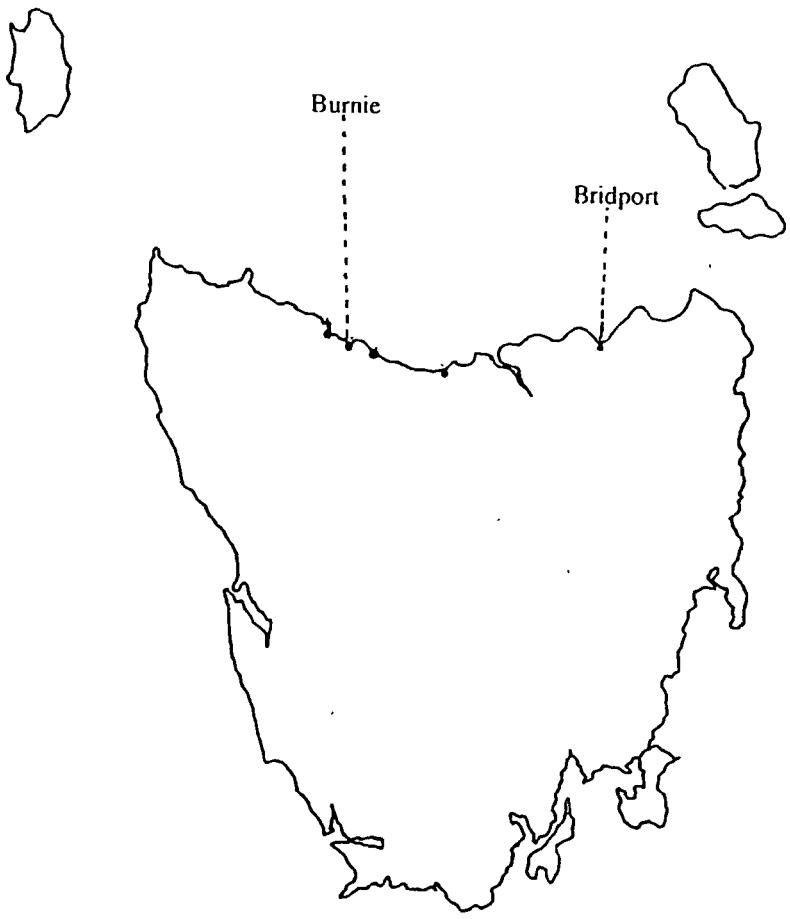


Figure 2.3

Map of Emu Bay showing the Effluent Outfall and Effluent Concentrations Within the Bay

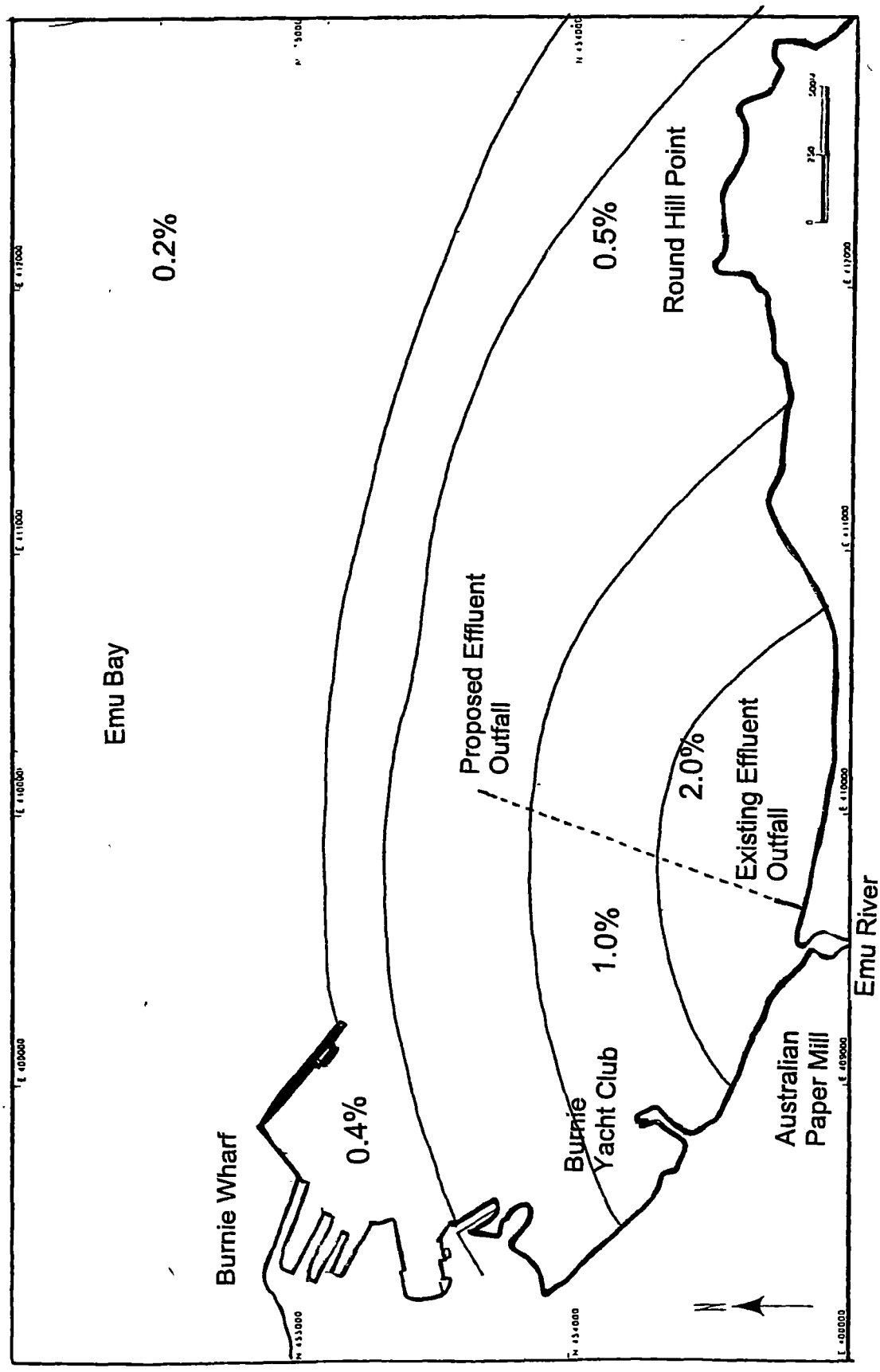


Figure 2.4

Australian Paper: Burnie Pulp And Paper Mill

Figure 2.5

Settlement Pond At The Burnie Mill

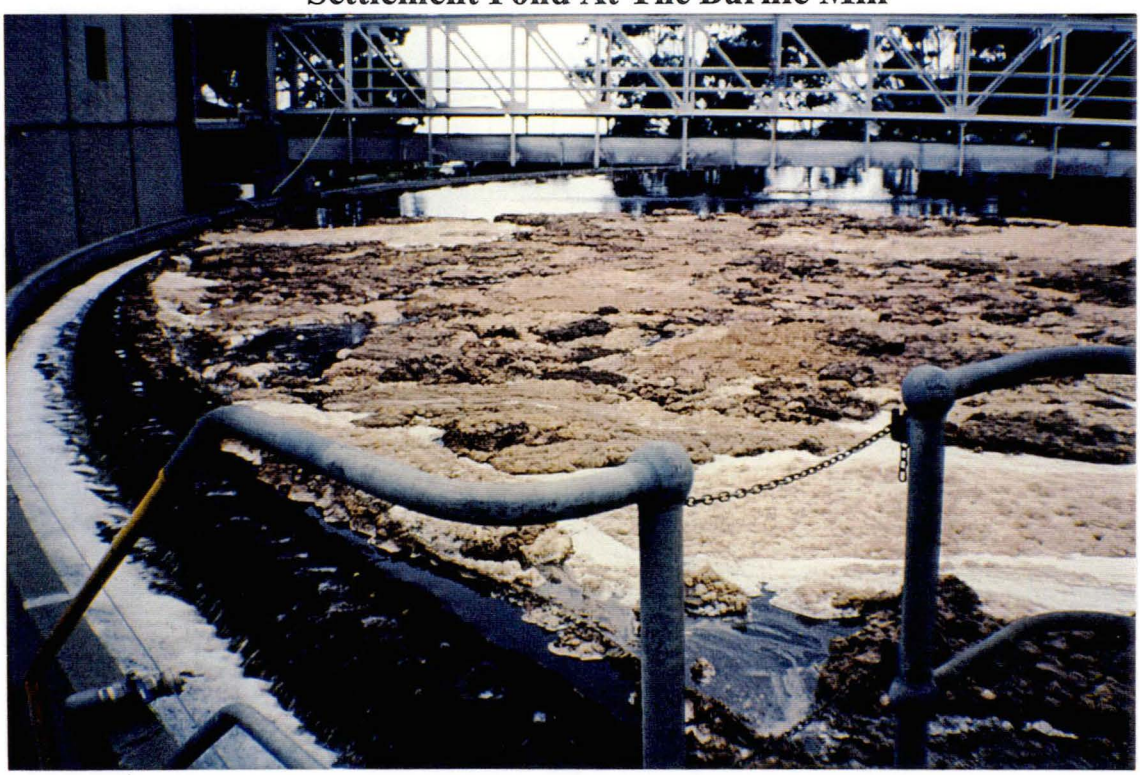


Table 2.3 Dates All Sites Sampled in 1995

Burnie Pulp and Paper Mill Sites 1 - 7

- 30 January
- 14 March
- 27 April
- 6 June
- 21 September

Burnie Pulp and Paper Mill Discharge - Site 8

- 30 January
- 9 March
- 14 March
- 27 April
- 5 June
- 25 July
- 28 August
- 21 September
- 19 October
- 27 November

2.2.2 Acute Toxicity of Effluents

The acute toxicity of all samples was measured with Microtox® Model 500 (Microbics Corporation, USA). All samples were tested less than 24 hours after sampling, following the test protocols described in the Microtox manual (Microbics 1992).

2.2.2a Microtox Protocol

A cuvette was placed in the reagent well of the Microtox analyser and 1000 µL of Reconstitution Solution were added to the cuvette. The Reconstitiution Solution was then added to a reagent vial and mixed. The Reagent Solution was then returned to the cuvette and mixed again.

Cuvettes were placed in each row of the Microtox analyser and 500 µL of Microtox diluent were added to each cuvette. 10 µL of the Reagent Solution were added to each cuvette and incubated for 15 minutes. The light emission was tested to obtain a time 0 emission.

250 µL of Microtox Osmotic Adjusting Solution were added to the end cuvette with 2.5 mL of sample (sample concentration 90%). 500 µL of this 90% sample were transfered to the next

cuvette containing 500 µL of diluent resulting in a 50% dilution. 500 µL of that dilution were then transfered to the next cuvette containing 500 µL of diluent until five dilutions had been achieved with a dilution factor of 50%. The light emissions were measured again at 5, 10 and 15 minutes. The light emissions were compared to time 0 readings and the 15 minute EC50s were calculated. A phenol standard reference toxicant was tested with each batch of reagent. Testing continued if the 15 minute EC50 was within the range of 13 – 26 ppm phenol.

The acute toxicity of each sample was measured prior to the pH being adjusted (with the exception of the sample taken on the 27/4/95, where, due to time constraints, only the toxicity of the pH adjusted samples was tested). The pH of each sample was then adjusted to between 8.0 and 8.3 with either 1 M NaOH or 1 N HCl to simulate the pH of seawater and the acute toxicity of the adjusted sample was remeasured.

Concentrations of effluent samples tested in the Microtox were 90%, 45%, 22.5%, 11.25% and 5.625%. The luminescence of the *Vibrio fisheri* bacteria in the effluent concentrations was measured at 5, 10 and 15 minutes, the decrease in luminescence was calculated, and the 15 minute value was used to determine the EC50 of the effluent. The EC50 was calculated using ToxCalc (Tidepool Scientific 1992) a U.S. EPA approved statistical analysis for toxicity data. The EC50 is expressed as the concentration of effluent that causes a 50% reduction in light output from *V. fisheri*.

Changes in acute toxicity of the effluent from site 8 (discharge) after storage were also measured by Microtox. Previous work had shown that Burnie mill effluent became less toxic over a period of time (N. Edyvane, pers. comm.). Therefore, acute toxicity for the June 1995 sample was measured after 48 and 72 hours. This tesing monitored the changes in toxicity over the period of a 96 hour bioassay. The acute toxicity of the August sample was measured after one week of storage at 4°C and five weeks at 0°C to determine changes in toxicity which occurred with storage.

2.2.3 Toxic Units and Toxicity Emission Factors

Toxicity emission factors (TEF) and toxic units (TU) were calculated using acute toxicity results from each discharge site sampled. Toxic units were calculated by dividing 100 by the mean EC50 for each sample site (Kim Oahn and Bengtsson 1995). The TEF was calculated

using the TU for each sample site following the formula from Kim Oahn and Bengtsson (1995):

TEF = TU x Flow (m^3t^{-1} product).

The flow rate was calculated from the volume discharged (Table 2.1) and the production of the Burnie mill (Australian Paper Environmental Management Plan, 1994).

2.2.4 Acute Toxicity of Effluent to Algae

Algae used for testing toxicity of effluent from the discharge point (site 8) were *Nitzschia closterium*, *Isochrysis galbana* and *Tetraselmis suecica*. *N. closterium* is the single cell alga recommended by the National Pulp Mill Research Program for use as a standard bioassay organism (Stauber *et al.* 1994). The golden brown alga *I. galbana* and the green alga *T. suecica* were used as feed for *Artemia salina* used in the subsequent experiments, described in the next chapter, to determine the effect of dietary exposure to pulp mill effluent. Stock cultures of the algae were obtained from CSIRO Marine Laboratories, Hobart, and maintained in 100 mL axenic cultures (f2 media) in 250 mL Erlenmyer flasks at 21°C within the algal stock culture room at the Aquaculture Centre.

The toxicity of K_2CrO_4, a reference toxicant recommended by the National Pulp Mill Research Program, to the three species of algae was determined as a quality control procedure and to ensure that the test method was suitable for *I. galbana* and *T. suecica*. The method used is given in detail in Stauber *et al.* (1994).

2.2.4a Algal EC50 Method

Algal cells were harvested from a five day old stock culture. From that culture a 15 mL volume was centrifuged at 20°C at 2500 rpm for 7 minutes. The nutrient medium was discarded and the algal pellet resuspended in 0.2 μm filtered sea water. The centrifugation and washing procedure was repeated three times to ensure that all the nutrient medium was removed. The algal cells were finally resuspended in 15 mL of filtered seawater.

2.2.4b Test Solutions

Duplicate controls were prepared by dispensing 50 mL of 0.2 μm filtered seawater into acid washed 200 mL Erlenmeyer flasks. Five dilutions of K_2CrO_4 and of effluent were prepared. K_2CrO_4 concentrations were 0, 0.25 ppm, 0.5 ppm, 1 ppm, 2 ppm and 4 ppm (based on EC50 from Stauber *et al.* 1994). Pulp mill effluent concentrations were 0, 2.5%, 5%, 10% and 20%

(based on Microtox EC50 results) for each of the three algal species. The salinity of the pulp mill effluent was adjusted to 32 ppt using Modified GP-2 artificial seawater (Stauber *et al.* 1994) and the pH adjusted to 8.2 with 1 N HCl. Fifty mL of each concentration were dispensed into 200 mL acid-washed Erlenmeyer flasks in duplicate. To each flask 0.5 mL of 26 mM sodium nitrate and 0.5 mL of 1.3 mM potassium dihydrogen phosphate were added.

Algae were added aseptically to each flask at a cell density between 3 - 5 x 10^4 cells per ml. Each flask was incubated at 21°C on a 12 hour light : 12 hour dark cycle at 14000 lux for 72 hours. Subsamples were taken aseptically each 24 hours to determine cell density in each flask. Cell density was calculated with the aid of an Improved Neubauer Haemocytometer and an Olympus BH2 microscope (100x magnification). Algal cells in each corner square and the middle square were counted in each chamber of the haemocytometer. The average of the two chambers was calculated then multiplied by 10^4 to obtain the cell density per mL. The EC50 for each experiment was calculated with a Trimmed Spearman-Karber analysis using growth data from all treatments (Tidepool Scientific, 1992).

Modified GP-2 Artificial Seawater

(Stauber *et al.* 1994)

COMPOUND	CONCENTRATION (g / L)
NaCl	21.03
Na_2SO_4	3.52
KCl	0.61
KBr	0.088
$Na_2B_4O_7.10H_2O$	0.034
$MgCl_2.6H_2O$	9.50
$CaCl_2.2H_2O$	1.32
$NaHCO_3$	0.17

2.2.5 Acute Toxicity of Pulp Mill Effluent to *Artemia salina*

The toxicity of the pulp mill effluent to *A. salina* was evaluated using the method described in detail by Widdows (1993). Instar II - III *A. salina* larvae (~24 hours old) (for detail on culturing see chapter 3, 3.2.8) were cultured and rinsed in the effluent concentration being tested prior to being placed in test wells (Nunc 12 well plate). The salinity and pH of the effluent were adjusted using methods previously mentioned. Ten larvae were placed in each well containing 1 ml of the test solution and five replicates of each concentration (0, 6.25%, 12.5%, 25%, 50% and 100%) were used. The multiwell plate was covered and placed in darkness at 25°C for 24 hours. After 24 hours the numbers of dead and living *A. salina* were counted using an Olympus stereomicroscope. Immobile *A. salina* were counted as mortalities and the LC50 was calculated using a Probit analysis (Davies *et al.* 1988). Quality control of the *A. salina* bioassay was assured by using the criteria of control mortalities. If the control mortality exceeds 10% the test is considered invalid. No reference toxicants were used for this bioassay as it has been shown that *A. salina* was not a sensitive test species (Persoone *et al.* 1989, Widdows 1993).

2.3 Results

2.3.1 Microtox EC50 Results For Burnie Mill

The effluents discharged from the bleach plant and the DARS plant were the most toxic of all the effluents at the Burnie mill, showing high toxicity with EC50s ranging from 5 - <11.25% v/v (Tables 2.4 and 2.8). The effluents from the pulp mill, WAO plant, paper machines, combined pulp and paper discharge and the final discharge all showed moderate to high toxicity (Tables 2.5, 2.6, 2.7, 2.9, 2.10 and 2.11). However, the toxicity of all discharges was reduced after adjusting the pH to that of sea water.

Table 2.4

Sample 1 - Bleach Plant

Date	Colour	pH	EC50 % v/v	95% CL
1/2/95	clear yellow	**2.81** 7.5	**8** 44	7.8 , 8.8 34.8, 56.5
27/4/95	pale yellow	8.20	7	5.2, 10.5
6/6/95	light brown	**2.78** 8.20	**5** 25	4.3, 5.9 21.0, 30.1

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.5

Sample 2 - Pulp Mill

Date	Colour	pH	EC50 % v/v	95% CL
27/4/95	dark brown	8.20	14	11.2, 18.0
6/6/95	dark brown	**7.30** 8.13	**36** 37	5.5, 243 32.5, 45.3
21/9/95	dark brown	**9.9** 8.3	**29** >90	25.7, 33.3

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.6

Sample 3 - N° 4 Paper Machine

Date	Colour	pH	EC50 % v/v	95% CL
1/2/95	cloudy, white ppt	**6.95**	**> 90**	
14/3/95	" "	**6.46** 8.19	**13** 12	11.0, 16.2 10.8, 14.2
27/4/95	" "	8.08	>90	
6/6/95	clear, white ppt	**7.22** 8.11	**34** 38	27.3, 42.1 30.7, 47.4
21/9/95	clear, white ppt	**6.4** 8.2	**19** 29	16.0, 22.4 24.9, 33.5

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.7

Sample 4 - N° 10 Paper Machine

Date	Colour	pH	EC50 % v/v	95% CL
1/2/95	cloudy, white ppt	**7.3**	**14**	11.9, 15.6
14/3/95	" "	**6.15** 8.22	**36** 46	23.4, 54.1 38.2, 54.5
27/4/95	" "	8.06	48	39.2, 58.2
6/6/95	clear, white ppt	**7.57** 8.14	**58** 59	45.8, 72.4 45.1, 76.8
21/9/95	clear white ppt	**6.67** 8.21	**>90** >90	

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.8

Sample 5 - DARS Plant

Date	Colour	pH	EC50 % v/v	95% CL
1/2/95	dark brown	**11.67** 7.5	**4** 20	3.5, 4.0 17.2, 24.6
14/3/95	light brown	**11.88** 8.20	**<11.25** 32	 24.2, 43.7
27/4/95	brown, brown ppt	8.35	>90	
6/6/95	light brown, ppt	**11.03** 8.15	**9** >90	7.9, 10.3
21/9/95	light brown	**11.83** 8.29	**6** >90	5.4, 6.5

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.9

Sample 6 - WAO Plant

Date	Colour	pH	EC50 % v/v	95% CL
1/2/95	brown, ppt	**7.75**	**38**	33.8, 44.1
14/3/95	dark brown	**7.98** 8.28	**18** 13	15.8, 19.5 12.3, 15.0
27/4/95	light brown	8.35	>90	
6/6/95	clear, white ppt	**7.78** 8.15	**26** 30	23.7, 32.0 26.0, 35.7
21/9/95	white, ppt	**6.54** 8.14	**14** 15	11.0, 17.8 10.6, 20.5

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.10

Sample 7 - Combined Pulp / Paper
(Does not include WAO)

Date	Colour	pH	EC50 % v/v	95% CL
1/2/95	dark brown	**7.70**	**83**	64.0, 107.2
14/3/95	dark brown	**11.15** 8.25	**8** 42	7.7, 8.5 36.0, 49.9
27/4/95	dark brown	8.20	65	54.1, 77.5
6/6/95	dark brown	**9.79** 8.17	**55** >90	48.9, 61.6
21/9/95	light brown, ppt	**9.32** 8.14	**>90** >90	

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.11

Sample 8 - Discharge

Date	Colour	pH	EC50 % v/v	95% CL
30/1/95	dark brown	**10.4** 7.5	**18** >90	16.6, 20.7
9/3/95	light brown	**11.75** 8.27	**5** >90	4.4, 5.3
14/3/95	light brown	**11.80** 8.26	**<11.25** >90	
27/4/95	light brown	8.23	>90	
6/6/95	dark brown	**10.43** 8.29	**17** 53	16.3, 18.5 47.0, 60.1
25/7/95	light brown	**9.94** 8.19	**14** 21	12.7, 16.7 16.1, 27.1
28/8/ 95	dark brown	**11.08** 8.34	**10** 64	9.4, 11.2 29.4, 137.6
21/9/95	light brown	**9.32** 8.19	**51** >90	43.9, 58.8
19/10/95	light brown	**11.83** 8.26	**16** 85	15.0, 18.1 72.1, 96.9
27/11/95	light brown	**10.76** 8.21	**25** >90	22.0, 28.1

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.12

Effects of Storage on Toxicity

Date	Colour	pH	EC50 % v/v	95% CL
30/1/95	dark brown	**10.4** 7.5	**18** >90	16.6, 20.7
30/1/95 6 wk at 4°C	dark brown	**10.50** 8.16	**23** >90	21.1, 25.3
6/6/95	dark brown	**10.43** 8.29	**17** 53	16.3, 18.5 47.0, 60.1
6/6/95 48 hr / 4°C	dark brown	**10.53** 8.09	**10** 38	8.5, 12.4 27.8, 51.3
6/6/95 72 hr / 4°C	dark brown	**10.45** 8.05	**10** 27	8.7, 12.8 17.6, 42.3
25/7/95	light brown	**9.94** 8.19	**14** 21	12.7, 16.7 16.1, 27.1
25/7/95 1 wk at 4°C	light brown	**9.94** 8.19	**16** 15	10.5, 25.4 9.6, 22.4
25/7/95 0°C 5 wks	light brown	**11.2** 8.25	**18** >90	15.0, 22.6

Note: Figures in bold type denote raw effluent. Figures in normal type denote effluent with modified pH.

Table 2.13
Toxicity Emission Factors (TEF) for Various Sites Within the Burnie Mill

Sample Site	Mean EC50 %v/v (from Tables 2.4 - 2.11)	Toxicity Unit* (TU)	Toxicity Emission Factor** (TEF)
Bleach Plant	6.7	14.9	347
Pulp Mill	9.7	10.3	178
N°4 Paper Machine	39	2.5	32
N°10 Paper Machine	49	2.0	25
DARS Plant	7.5	13.3	84
WAO Plant	24	4.2	53
Pulp/Paper	60	1.7	182
Discharge	18	5.5	660

*Toxicity Unit (TU) = 100/EC50
**Toxicity Emission Factor (TEF) = TU x Flow (m^3t^{-1} product)
Note: Flow calculated from discharge volume (Table 2.1) and annual production of pulp (70,000 t/a).

2.3.2 Toxicity Emission Factors and Toxic Units

Of all the imputs into the final discharge the bleach plant and the DARS plant showed the highest number of toxic units and the highest toxicity (Table 2.13). However, the bleach plant had a higher TEF than the DARS plant (347 and 84 respectively), indicating that the bleach plant contributed more toxicity to the final discharge than the DARS plant. Effluent from the pulp mill also showed a high TU value (10.3) and a high TEF value (178). The effluent sampled from the final discharge also showed a high TEF value of 660.

2.3.3 Algal and *Artemia salina* Acute Toxicity Results

Two algal species, *N. closterium* and *I. galbana*, were sensitive to the reference toxicant K_2CrO_4 at the concentrations tested (Table 2.14). *T. suecica* was not as sensitive as the other

species tested. However, the bioassay protocol proved suitable for use with all three species. Results obtained for the mill effluent again showed that *N. closterium* and *I. galbana* were more sensitive than *T. suecica* (Table 2.15) and that all algal species were more sensitive than either *A. salina* or Microtox (Table 2.15). All results in Table 2.14 show that the effluent was not toxic to the algae or *A. salina* at the concentrations that were used in the dietary experiments described in Chapter 3.

Table 2.14

EC50 Results For Reference Toxicant K_2CrO_4

Species	EC50 ppm	95% Confidence Limits
Nitzschia closterium	2.2	1.4, 2.6
Isochrysis galbana	3.1	1.9, 3.7
Tetraselmis suecica	>4.0	

Table 2.15

Acute Toxicity of Burnie Pulp and Paper Mill Effluent (sampled 25/7/95)

Species	EC50/LC50 % v/v	95% Confidence Limits
Nitzschia closterium	8	7.4, 8.1
Isochrysis galbana	8	7.7, 8.3
Tetraselmis suecica	16	14.8, 17.2
Artemia salina	>100	
Microtox (from Table 2.10)	21	16.1, 27.1

2.4 Discussion

2.4.1 Acute Toxicity of Burnie Pulp and Paper Mill Effluent

As mill processes alter from day to day, or even from hour to hour, variations in the acute toxicity of effluents from different manufacturing areas occur. The acute toxicity results for the Burnie mill operational areas, as tested with Microtox, show that these changes have a major influence on the acute toxicity of discharge effluents (Tables 2.4 - 2.11). Variations in toxicity between sampling dates occur, with differences being up to 10 fold at some sites. The Microtox EC50 results from each sample site were used to determine the source of the toxicity in the final discharge by calculating toxicity emission factors (TEF) for each sample point (Kim Oahn and Bengtsson, 1995).

The acute toxicity of waste water from the pulping process, as measured with Microtox, reveals that this effluent is less toxic than that from the bleach plant (Table 2.4 and Table 2.5). The toxicity of the waste water from the pulping process is generally attributed to the black liquor containing lignin derivatives from the wood stock (Sodergren *et al.* 1988, Kim Oahn and Bengtsson, 1995, Ahtiainen *et al.* 1996, Verta *et al.* 1996). The discharge from the pulp mill has the second highest TEF of the process samples (Table 2.13), indicating that the wood products in the waste water are major contributors to the acute toxicity of the final discharge.

The chemicals involved in the bleaching process create chlorinated compounds which enter the waste stream and, therefore, the bleach plant (Table 2.4) was the most acutely toxic of all the discharges tested. As the pH of the effluent from the bleach plant was low, some of the acute toxicity can be attributed to this factor, especially as the acute toxicity lessened when the pH was adjusted to that of seawater. However, as would be expected from this sample, adjusting the pH would not remove all the toxicity as chlorinated compounds in the sample would remain. As mentioned previously, the reduction in toxicity at pH 8.2 is related to the proportion of ionised molecules to unionised molecules (Leach and Thakore 1975, Kovacs *et al.* 1993) with ionised molecules showing less toxicity than unionised molecules. The effluent from the bleach plant is the main contributor to the toxicity of the final discharge as evidenced by the TEF of 347 (Table 2.13). The bleach plant contributed 52% of the final toxicity, but only 20% to the final volume of the discharge. The TEF from the Burnie mill bleaching sequence is

considerably less than the TEF obtained for the BAPACO mill in Vietnam, where a similar bleaching sequence is used on different raw products, leading to calculated TEFs of 500 - 750 for this mill (Kim Oahn and Bengtsson, 1995).

Volumes and constituents of the waste water created in various stages involved in paper-making will vary. Consequently, the most variable acute toxicities of effluents from the Burnie mill relate to the paper machines (Tables 2.6 and 2.7) with EC50s for the Microtox assays ranging from nontoxic at >90% to acutely toxic at about 13%, depending on the processing stage at the time of sampling. The toxicity of the discharge from these two sample sites is not related to pH, and thus it must be attributed to some other physical factor or chemicals. Waste water from both these sites contributed minimally to the acute toxicity of the final effluent discharge, as evidenced by the TEFs (Table 2.13).

Just as the black liquor has been shown to be the most toxic constituent of pulp mill effluents (Leach and Thakore 1975, McKague 1981, Kovacs *et al.* 1993, Huuskonen and Lindstrom-Seppa 1995) the waste from the DARS plant has also been shown to be acutely toxic (Table 2.8) as it may contain water-soluble compounds from the black liquor. However, some of the toxicity of this effluent is ameliorated when the pH is adjusted to that of seawater. Although the volume of this discharge is only 5% of the final discharge, the TEF is 13% of that of the discharge (Table 2.13). These results indicate that the DARS plant is contributing relatively more to the toxicity than to the final volume of discharge.

The toxicity of the effluent from the WAO plant may to be due to similar water-soluble wood derivitives found in the DARS plant effluent, as the pH has no influence on the acute toxicity (Table 2.9). However, the effluent from the WAO plant is less acutely toxic than that of the DARS plant. Consequently, the WAO plant does not have as much influence on the toxicity of the final discharge as the DARS plant, as evidenced by the TEF values (Table 2.13), even though the volume entering the waste stream is twice that of the DARS plant.

The sample site for the combined pulp and paper discharge combines all waste water streams for the entire mill operations with the exception of the WAO plant. The acute toxicity of the effluent sampled at this site varies by more than 10 fold between sample dates. This is due to varying operational procedures occurring at the mill at the time of sampling (Table 2.10). The

combined pulp and paper discharge contributes 90% volume to the final discharge but only 30% to the toxicity (Table 2.13). As this sample includes the two most toxic effluents, the bleach plant and the pulp mill, it would be expected that the effluent would be acutely toxic with a high TEF. However, the acute toxicity of these two waste water streams seem to be modified due to reactions occurring within the effluent during mixing.

The effluent sampled at the discharge site was moderately to highly toxic (Table 2.11) with the acute toxicity ranging from 5% to 51% (mean 17% ± 11%). Most of the acute toxicity was due to the pH of the effluent as, after the pH had been adjusted to that of sea water, the acute toxicity disappeared leaving the effluent, in most cases, nontoxic. This indicates that, once the effluent is diluted with seawater in Emu Bay, it is not acutely toxic.

The TEF for the final discharge of 660 (Table 2.13) is at the lower end of the range of TEFs (645 - 825) calculated for the BAPACO pulp and paper mill in Vietnam (Kim Oahn and Bengtsson, 1995). The TEF for the Burnie mill is considerably less than that for a Swedish mill (TEF = 985) (Kim Oahn and Bengtsson, 1995). The TEF for the Burnie mill discharge is lower than the combined TEF values for all the other waste water streams that were sampled. This indicates that the primary treatment of settlement and aeration has reduced the toxicity of the final discharge to a certain extent.

The acute toxicity of pulp mill effluent can change over a period of time due to additive, synergistic, antagonistic or independent action of the constituents of the effluent (Doi 1994). Acute toxicity can be reduced by particulate matter settling out of suspension and constituents of the effluent adsorbing onto that particulate matter. In 1990, acute toxicity tests performed on the Burnie pulp mill effluent by APPM, revealed that the acute toxicity decreased over time and that the general trend is for the effluent samples to detoxify spontaneously. Storage of the effluent at 4°C will delay that detoxification process (N. Edyvane, APPM, pers. comm.). However, the effluent used in that earlier series of experiments contained a proportion of softwood effluent, which would have altered the characteristics of the effluent. To assess the effects of storage on the 100% eucalypt effluent used in this study, and to confirm that the sampling strategy used was appropriate, a series of assays was performed on several effluent samples selected at random. Microtox results demonstrated that storage of the effluent, at 0°C and 4°C, did not change the acute toxicity characteristics of the effluent over time (Table 2.12).

2.4.2 Acute Toxicity to Algae and *Artemia salina*

Use of a reference toxicant is recommended to assess the reproducibility of the acute toxicity test procedure on algae (J. Stauber, pers. comm.). Chromium (VI) is a recommended reference toxicant (Stauber *et al.* 1994) and, as such, it was used to determine if the *N. closterium* acute toxicity test procedure were suitable for use with the other two algal species investigated: *I. galbana* and *T. suecica*. The 72 hr EC50 result for this acute toxicity test on *N. closterium* (Table 2.14), using K_2CrO_4 as the toxicant, was very close to the EC50 of 2.43 ± 0.18 ppm calculated by Stauber *et al.* (1994). *N. closterium* proved to be more sensitive to the reference toxicant than the other two algal species (Table 2.14).

The *N. closterium* bioassay has been proven to be the most sensitive, reproducible and fully-validated toxicity test in a suite of bioassays developed by the National Pulp Mill Research Program (Stauber *et al.* 1994b). In the study represented here the *N. closterium* and *I. galbana* bioassays were shown to be more sensitive to the pulp mill effluent than the *T. suecica* bioassay with an EC50 of 8% (Table 2.15). *T. suecica* was the least sensitive of the algae species tested, but all the of those tested were more sensitive to the effluent than the *V. fisheri* used in Microtox. This is in agreement with work by Blaise *et al.* (1987) which revealed that an algal bioassay was more sensitive to pine-based pulp mill effluent than both Microtox and a 96 hr LC50 using rainbow trout. Also, Eklund *et al.* (1996) found that the algal bioassay using reproduction of the red macroalga, *Ceramium strictum,* was also more sensitive than Microtox to pine-based pulp mill effluents. Several other researchers have also used a freshwater microalga, *Selenastrum capricornutum*, for assessing the acute toxicity of pulp mill effluents and all have found that algal assays are more sensitive than many assays using other organisms (Argese *et al.* 1995, Kim Oahn and Bengtsson, 1995, Ahtiainen *et al.* 1996, Bailey and Young, 1997).

For the duration of this project the effluent discharging into Emu Bay from the Burnie mill was diluted approximately 1:50 (2%) at the outfall point (Neilson 1993), but this changed in late 1996 to 1:150 (0.67%) with the addition of a diffuser to the outfall pipe. This latter level is less than the concentration of effluent required to cause acute toxicity in all the three species of marine algae tested and the marine bacteria used with the Microtox assay, but may still cause sublethal effects.

The algal EC50 results can be extrapolated to assess effects of the effluent in the receiving water. The safety level is generally assessed as < 1/10 of the EC50 (Kim Oahn and Bengtsson, 1995). Also, the average concentration should be <1/20 of the EC50 to prevent sublethal effects from occurring. Results from the *N. closterium* bioassay show that the maximum concentration of Burnie mill effluent should be 0.8% with an average of 0.4% to avoid sublethal effects on unicellular algae. Consequently, the levels of effluent found in the bay (0.4 - 2%) (Figure 2.3) would not cause acute mortalities in the phytoplankton, but the sublethal effects of the effluent at the concentration in Emu Bay may interfere with photosynthesis and reproduction of those primary producers. These effects must be taken into consideration when assessing the impacts of the effluent on the receiving water. Even with the deployment of the new diffuser, which will result in a rapid mixing of the effluent with sea water in the bay, chronic toxicity of the effluent to primary producers is still a possibility which needs to be considered.

As mentioned previously, the impact of pulp mill effluents in marine receiving waters is due, in part, to high BOD, suspended solids, dissolved substances, pH, colour and toxic chemicals. The main characteristics of the effluent which affect marine algae are; increased growth due to nutrients such as nitrogen, decreased photosynthesis/productivity due to the colour of the effluent, decreased growth due to toxic constituents of the effluent and changes to species composition (Stauber *et al.* 1994, Lowell *et al.* 1995, Dube and Culp, 1997). Some of the toxic constituents of pulp mill effluent have been shown to be chlorate, chlorophenolics, resin acids and lignin derivitives (Leach and Thakore 1975, McKague 1981, Kovacs *et al.* 1993), phytoplankton and macroalgae have both been shown to be sensitive to these constituents (Lehtinen, 1991). However, Burnie mill effluent has non-detectable levels of resin acids and chlorates so these are unlikely to be involved. Possibly some toxicity of the effluent to algae may be attributed to a wide range of chlorinated phenolics which are present in the effluent (see Appendix 1).

Stauber *et al.* (1994) have tested the acute toxicity of several types of chlorophenols to *N. closterium* (Table 2.16). The concentrations of chlorophenols that caused acute toxicity to *N. closterium* were orders of magnitude higher than the concentrations of those chlorophenols detected in Burnie mill effluent, with the exception of 3,4,5-trichlorocatechol and

tetrachloroguaiacol. This indicates that toxicity of this type of complex effluent may be due to additive or synergistic effects of the constituents rather than to any individual chlorophenol component as the total of the toxic units (TTU) calculated from the EC50 values was 0.63. This does not explain the effects observed at this concentration. As the TTU is < 1; either unmeasured toxic components are present in the effluent or there are synergystic effects occurring. As in other complex effluents, such as sewage, the potential for synergism and antagonism between constituents is difficult to quantify and the contribution to toxicity by individual components is unclear (Burridge *et al.* 1996).

Untreated whole effluents typically contain 20 - 200 mg chlorate (ClO_3^-) / L (Stauber *et al.* 1994). Chlorate, which is produced by incomplete reduction of chlorine dioxide to chloride during pulp bleaching, is toxic to brown algae including the unicellular alga *N. closterium.* However, chlorate has not been detected in the effluent from the Burnie mill (Table 2.1). TCDD and TCDF were also undetectable in the Burnie mill effluent, using a method with a sensitivity of 6 pg/L. Further, this detection limit is is below the NOEC of 0.05 ng/L determined for algae (Grimwood and Dobbs, 1995). Therefore, the acute toxicity of the Burnie mill effluents cannot be attributed to chlorate or dioxins and furans.

I. galbana, T. suecica and *A. salina* were exposed to the pulp and paper mill discharge to determine the EC50 value for each species. The EC50 values were then used to ensure that the concentrations of effluent used in the dietary exposure experiments (0.5 - 2.5%), described in the next Chapter, were not acutely toxic to these test species (Table 2.14). The effluent that was used in the EC50 experiments was one of the most toxic of all the discharge effluents sampled, consequently, none of the effluents used in the duration of the experiments would have caused acute toxicity to either the algae or *A. salina.* Further, the effluent was not acutely toxic to the *A. salina,* as no mortalities occurred in the assay at 100% effluent concentration (Table 2.14).

TABLE 2.16
Concentrations Of Chlorophenols In Burnie Mill Eucalypt-Based Pulp Mill Effluent Compared To 72 Hr EC50 Concentrations Of Individual Chlorophenols

Chlorophenol	Concentration in Burnie Mill Effluent (Neilson 1993) µg/L	Toxic Units in 8% Burnie Mill Effluent (EC50 for *N. closterium*)	72 hr EC50 *Nitzschia closterium* (Stauber *et al.* 1994) µg / L
phenol	40	6.4×10^{-5}	49878
4-chlorophenol	460	4.5×10^{-3}	8036
2,4-dichlorophenol	1400	1.3×10^{-2}	8916
2,4,6-trichlorophenol	1500	1.2×10^{-2}	10231
4,5-dichlorocatechol	600	3.9×10^{-3}	12284
3,4,5-trichlorocatechol	3000	0.30	793
tetrachlorocatechol	2700	7.1×10^{-2}	3024
4,5-dichloroguaiacol	NDR		4680
4,5,6-trichloroguaiacol	690	1.3×10^{-2}	4158
tetrachloroguaiacol	2700	0.12	1862
5,6-dichlorovanillin	190	8.0×10^{-4}	19000
2,6-dichloro-syringaldehyde	3800	1.9×10^{-2}	15939
3,4,5-trichlorosyringol	3600	7.1×10^{-2}	4032
Total Toxic Units		0.63	

NDR = Peak detected but did not meet quantification criteria (Appendix 1)

The use of *A. salina* for acute toxicity assays has several advantages such as: stock culture maintenance is not required, short exposure times, wide salinity tolerance, low cost and simple procedures (Widdows 1993). However, the disadvantages outweigh the advantages, with the main disadvantages being the low sensitivity of *A. salina* to toxicants, the lack of relevance to Australian ecological systems and the difficulty in distinguishing immobility from death.

The results from the toxicity tests described in this chapter indicate that the effluent from the Burnie mill is of lower toxicity than that of mills discharging untreated pine-based effluent (Ahtiainen *et al.* 1996). Although the Burnie mill is, in some areas, using 40 - 50 year old technology, the toxicity of untreated effluent from ECF (elemental chlorine free) and TCF (totally chlorine free) mills using pine and birch stock is higher than that of the untreated Burnie mill effluent (Ahtiainen *et al.* 1996). The untreated ECF and TCF effluents typically gave Microtox EC50 results of <10% v/v, which is approximately 50% more toxic than the Burnie mill effluent (Table 2.13). These results indicate that the natural constituents of the woodstock, resin acids and lignin derivitives, probably contribute the majority of the toxicity (Ahtiainen *et al.* 1996). Therefore, part of the toxicity of the Burnie mill effluents may be attributed to lignin derivitives and the rest to compounds which are biologically activated by mill processes.

2.5 Conclusions

- The acute toxicity of effluents from different manufacturing processes within the Burnie mill varies greatly with time.

- The bleach plant and the DARS plant effluent samples were the most acutely toxic.

- The bleach plant exhibited the highest TEF, but this decreased upon mixing with other waste water streams.

- The acute toxicity of all the discharge effluents decreased after the pH and salinity were adjusted to that of seawater.

- After the effluent discharge from the Burnie mill has been diluted in Bass Strait it would not be acutely toxic to *V. fisheri.*

- The Burnie mill effluent is discharged into Bass Strait at a lower concentration than that which causes acute toxicity to the marine algae *N. closterium, I. galbana* and *T. suecica.* However, the concentration may still cause sublethal effects on single-celled algae.

- The concentrations of chlorinated phenolics in the diluted effluent are orders of magnitude lower that those which cause acute toxicity to *N. closterium.*

- The concentrations of effluent used in the dietary exposure bioassays were not in the range likely to cause acute toxicity to *I. galbana, T. suecica* and *A. salina.*

CHAPTER 3

EFFECTS OF PULP MILL EFFLUENT ON TASMANIAN NATIVE FISH

CHAPTER 3

EFFECTS OF PULP MILL EFFLUENT ON TASMANIAN NATIVE FISH

3.1 Introduction

Biochemical and physiological indicators of environmental exposure to pollutants (biomarkers) are used to detect sublethal impacts on fish and can provide an early warning of adverse effects before the onset of serious pathological damage (Mosse *et al.* 1996). As mentioned in Chapter 1, biomarkers have been used extensively to assess the impacts of pulp mill effluent on fish species in the Northern Hemisphere (Forlin *et al.* 1995, Gagnon *et al.* 1995, van den Heuvel *et al.* 1995, Borton *et al.* 1996, Martel *et al.* 1996, McMaster *et al.* 1996, Sandstrom 1996, Tana and Lehtinen 1996, Williams *et al.* 1996). Biomarkers routinely used in those Northern Hemisphere studies include; ethoxyresorufin-O-deethylase (EROD) activity, induction of cytochrome P4501A1, haematology, immunology, histopathology of liver, skin, gills and gonads, liver ultrastructure, growth, carbohydrate metabolism, vitamin A metabolism, vitamin C status, liver somatic index, condition factor, gonadosomatic index, reproductive status, changes in steroid profiles, parasitic infections and skeletal abnormalities (Lethinen 1990, Bucher *et al.* 1992, Goksoyr *et al.* 1994, Kloepper-Sams and Benton 1994, Biagianti-Risbourg and Bastide 1995, Collier *et al.* 1995, Forlin *et al.* 1995, Gagnon *et al.* 1995, Gilbert *et al.* 1995, Martel *et al.* 1995, Parrot *et al.* 1995, Soimasuo *et al.* 1995, van den Heuvel *et al.* 1995, Ahtiainen *et al.* 1996, Mosse *et al.* 1996, Tana and Lehtinen 1996, Struthridge *et al.* 1997). The majority of the biomarkers mentioned above were determined from fish populations sampled in field studies and not all of these biomarkers are suitable for use in laboratory studies. Biomarkers such as parasitic infections, skeletal deformities, reproductive status and age to maturity, which indicate long-term exposure, are particularly useful in field studies. However, as laboratory studies are short-term, other, more sensitive biomarkers are used to assess changes. These biomarkers usually include; EROD activity, cytochrome P4501A1 induction, other metabolic processes, histopathology and haematology (Hektoen *et al.* 1994, Holm *et al.* 1994, Lindstrom-Seppa *et al.* 1994, van der Weiden *et al.* 1994, Levine *et al.* 1995, Williams *et al.* 1996).

The biomarkers mentioned are useful for detecting exposure to pine-based pulp mill effluent and laboratory studies have shown that several constituents of the effluent will induce similar responses (Hektoen *et al.* 1994, Holm *et al.* 1994, Lindstrom-Seppa *et al.* 1994, van der Weiden *et al.* 1994, Levine *et al.* 1995, Williams *et al.* 1996). The constituents in pine-based pulp mill effluent which show the most toxicity; TCDD and TCDF, are not present in detectable levels in Burnie mill eucalypt-based effluent and resin acids are present in low levels (Appendix 1). Therefore, the acute and chronic effects on fish exposed to eucalypt-based pulp mill effluent are not as severe as the effects resulting from exposure to pine-based pulp mill effluent (Munday *et al.* 1991). This is evidenced by the differences in acute toxicity of Australian pine-based pulp mill effluent when compared with the acute toxicity of eucalypt-based pulp mill effluent, with rainbow trout 96 hour LC50s ranging from 10 - 41% for pine-based effluent and no toxicity observed in undiluted eucalypt-based effluent (Davies *et al.* 1988, Lukatelich and Evans 1990, Munday *et al.* 1991). Acute toxicity of eucalypt-based pulp mill effluent has been determined (Munday *et al.* 1991, Stauber *et al.* 1993, Stauber *et al.* 1994), but very little research has been performed to evaluate the use of biomarkers to assess the chronic effects of eucalypt-based pulp mill effluent (Munday *et al.* 1991, Holdway *et al.* 1993, Brumley *et al.* 1996). Therefore, this series of experiments has two aims (1) to evaluate the use of biomarkers for detecting exposure to eucalypt-based pulp mill effluent and (2) the use of biomarkers to determine the significance of the route of exposure.

Little is known about the bioavailability of the constituents of eucalypt-based pulp mill effluent or the kinetics of accumulation, disposition and possible biological effects on fish living in the receiving waters. In the case of such a complex effluent there may be constituents which are accumulated only by uptake from the water and others that are accumulated only via dietary exposure, determined both by the chemisty of the constituent and the physiology of the fish. (Thomann 1981, Heath 1987, Connell 1995, Pastor *et al.* 1996). Uptake of constituents from the water (bioconcentration) can be up to 10^6 times that of the water concentration and via the diet (biomagnification) can be increased between 30 – 100 times from the prey to the predator (Nowak 1997). Consequently, when testing a complex effluent such as this, a test method is required that will take into account the different modes of accumulation. Usually, emphasis has been on a single or several individual constituents known to be in the effluent (Hektoen *et al.*

1994, Stauber *et al* 1994, van der Weiden *et al.* 1994, Abbott *et al.* 1995, Parrott *et al.* 1995, Struthridge *et al.* 1997). These types of tests do not take into account the reactions between the constituents, and, of course, the toxic action of many effluent compounds can be additive or synergistic (Lloyd 1991, Ribeyre *et al.* 1995).

Munday *et al.* (1991) performed a series of bioassays to assess the acute and chronic effects of eucalypt-based effluent. These bioassays evaluated the effects of effluent exposure through uptake from the water column only, as this route was assumed to have a greater effect on toxicity than dietary exposure (Schell *et al.* 1993, Levine *et al.* 1994, Connell 1995, Ribeyre *et al.* 1995, Post *et al.* 1996). The contribution of dietary exposure needed to be determined to obtain an improved representation of effects occurring in the receiving water, as the extent of the influence of food chain bioaccumulation of pulp mill effluent is unknown (Macek *et al.* 1979, Thomann 1981, Whittle *et al.* 1992, Barry *et al.* 1995, Post *et al.* 1996). The sublethal effects of exposure routes (dietary, water-borne and dietary + water-borne) of fish to eucalypt-based pulp mill effluent were determined using selected biomarkers. The effects were quantified to establish the significance of each route of exposure and the biomarkers were then evaluated for use in detecting sublethal responses to eucalypt-based pulp mill effluent.

A food chain was simulated using algae and *Artemia salina* nauplii or juvenile clams which were cultured in concentrations of effluent that are found in Emu Bay (Consulting Environmental Engineers 1991). The exposed *A. salina* and clams (*Katelysia scalarina*) were fed to the common jollytails (*Galaxias maculatus*) and Tasmanian blennies (*Parablennius tasmanianus*) respectively. *A. salina* and clams were cultured without effluent for the control diet. The common jollytails and blennies were divided into four groups to determine the effects of each route of exposure:

- Group 1 - control, no effluent exposure,
- Group 2 - dietary exposure , control water,
- Group 3 - water-borne exposure , control diet,
- Group 4 - dietary exposure and water-borne exposure.

To simulate environmental conditions found in Emu Bay, the fish were exposed to low levels of Burnie mill effluent in each of the treatments for up to 3 months in the chronic assays and for 96 hours in the short term live feed assay. The short term assays were included to detect if any

sublethal effects occurred within the 96 hours normally used for an LC50 bioassay. At the completion of each experiment selected biomarkers were assessed. Biomarkers selected were based on previous experience with acute and chronic bioassays (Munday *et al.* 1991). As the bioassays performed by Munday *et al.* (1991) showed that exposure of common jollytails to low concentrations of eucalypt-based pulp mill effluent had minimal effects on white blood cell differential counts and pathological cell damage, biomarkers for detecting these changes were not included in this study. Further, the use of histopathology instead of serum enzymes can be used to detect cellular changes before the onset of the pathological cell damage in organs such as liver, muscle and kidney, whereas, some serum enzymes, such as total proteins and aspartate aminotransferase, lack organ specificity (Cameron 1989). Therefore, the biomarkers selected for assessment in this study were:

- growth,
- condition index,
- liver index,
- histology,
- electron microscopy,
- EROD (ethoxyresorufin-O-deethylase) activity and
- cytochrome P4501A1 induction.

Each biomarker was assessed for its ability to detect exposure to low levels of eucalypt-based pulp mill effluent and was analysed to quantify the effects of each route of exposure.

Condition index, which reflects general nutritional status of the fish, was selected as a biomarker for assessment in this series of experiments as a decrease in condition index is often observed in response to pulp mill effluent exposure (Andersson *et al.* 1987, Huuskonen and Lindstrom-Seppa 1995). A decrease in condition index is usually due to depletion of energy reserves reflecting a change in feeding patterns or behaviour in response to stressors (Goede and Barton 1990, Levine *et al.* 1995). Other changes in condition index can be due to sexual maturation and this must be taken into account when analysing this parameter (Goede and Barton 1990).

Growth of fish was selected as a biomarker for use with juvenile fish and is usually assessed by measuring absolute weight gains of the fish over a period of time. Growth of fish is commonly used as endpoint in laboratory bioassays (U.S. EPA 1988). Growth, as a biomarker, is used to compare exposed and unexposed fish and decreased growth reflects secondary effects of chronic

stress (Goede and Barton 1990), as well as direct effects of chemicals in the effluent on metabolism and protein synthesis. Decreased growth has been observed in fish exposed to pine-based pulp mill effluent indicating a decreased ability to efficiently convert energy to somatic or reproductive tissue (Munkittrick *et al.* 1991, Huuskonen and Lindstrom-Seppa 1995).

The liver of the fish plays an important role in many vital functions. It is involved in basic food conversion, storage of glycogen and lipids, production of yolk proteins during oocyte development as well as the biotransformation and detoxification of xenobiotics (Heath 1987, Kohler and Pluta 1995). Therefore, any alterations to liver function may have an adverse effect on the health of the fish as well as impair reproductive capabilities. Therefore, several biomarkers targetting sublethal effects on the liver were selected.

Liver somatic index (LSI) was selected as a biomarker for detecting exposure to eucalypt-based pulp mill effluent as the LSI is a useful ratio for assessing the general condition and metabolic-energy demands of the fish (Forlin *et al.* 1995). The LSI usually reflects the nutritional status of the fish and therefore, can be sensitive to environmental contaminants, as changes are most likely due to variations in the storage of lipids and glycogen (Heath 1987, Goede and Barton 1990, Everaarts *et al.* 1993). Generally, liver size increases in response to exposure to toxicants as a result of impairment to biochemical pathways which can result in the inability to access storage compounds (Andersson *et al.* 1987, Heath 1987, Forlin *et al.* 1995, Huuskonen and Lindstrom-Seppa 1995). Also, proliferation of rough endoplasmic reticulum due to induction of cytochrome P4501A1 can cause hepatocyte hypertrophy and result in changes to liver size (Andersson *et al.* 1987, Hinton and Lauren 1990)

Biotransformation of toxicants occurs within the liver, where oxidation and reduction reactions are carried out by the mixed function oxidase (MFO) system (Heath 1987). The induction of the MFO system is readily quantified by measuring the amount of cytochrome P4501A1 produced and the EROD activity in the liver. As EROD and other MFO enzymes are located on/in the rough endoplasmic reticulum (RER) within the hepatocyte, proliferation of the RER often occurs in conjunction with induction of EROD activity and cytochrome P-450A1 production (Heath 1987, Moore 1992). Also, increased MFO activity can result in damage to cell membranes due to the increased production of oxyradicals produced by the biotransformation of toxicants (Collier *et al.* 1992b, Kohler and Pluta 1995). Therefore, histology and transmission electron microscopy

were used to identify and quantify any cellular and subcellular alterations occurring. These changes are more sensitive than growth or reproductive changes and provide a better evaluation of organism health than a single biochemical parameter (Teh *et al.* 1997)

Cytochrome P450 monooxygenases are a superfamily of proteins that oxidise a wide variety of environmental contaminants and can be induced in the liver of fish by chemicals such as polychlorodibenzo-p-dioxins (PCDD), polychloro-dibenzofurans (PCDF), and other polycyclic aromatic hydrocarbons commonly found in pulp mill effluents (Courtenay *et al.* 1993, Lindstrom-Seppa *et al.* 1994, Parrott *et al.* 1995). Due to their lipophilic and persistent properties these MFO inducers are bioconcentrated in fish mainly via the food chain (van der Weiden *et al.* 1994, Fisk *et al.* 1997) and the metabolism of these types of compounds by fish is an important factor in the toxicity of these compounds. The toxic mechanism of these compounds is mediated through the cytosolic Ah-receptor (Figure 1.1). The polycyclic aromatic hydrocarbons bind to the Ah receptor creating an activated receptor-ligand complex to instigate production of the P4501A1 protein (Celander *et al.* 1994). This induction is sometimes associated with changes in the metabolism of endogenous compounds such as steroids and can disturb energy metabolism which results in weakened immune response, decreased gonadal size and increased age to maturation (Soimasuo *et al.* 1995). Research by Klopper-Sams and Swanson (1992) and van der Weiden *et al.* (1994) indicates that induction of cytochrome P4501A is one of the most sensitive biomarkers of exposure to pine-based pulp mill effluent, but the use of this biomarker has not been reported in relation to exposure to eucalypt-based effluent. Therefore, this biomarker was selected to assess its ability to detect the effects of exposure to low levels of eucalypt-based pulp mill effluent.

The inductive response of cytochrome P450 is often reflected in the hepatic EROD activity because EROD is a dependent activity of cytochrome P4501A1 in fish (Holm *et al.* 1994, Lindstrom-Seppa *et al.* 1994). Pertinent research by Woodworth and Helders (unpublished) supports this, as common jollytails exposed to the inducer benzo-*a*-pyrene showed a positive correlation between cytochrome P4501A1 induction and EROD activity. Also, a positive correlation between cytochrome P4501A1 and EROD induction in blenny and carp occurred after exposure to a single toxicant (Celander *et al.* 1994, van der Weiden *et al.* 1994b). However, Lindstrom-Seppa *et al.* (1994) observed EROD activity in fathead minnows, exposed to various concentrations of tetrachlorobiphenyl, in a different pattern to the induction of cytochrome

P4501A1 protein. Further, in situations where there is a complex mixture of environmental toxicants present, variations have occurred in the correlation between the amount of cytochrome P4501A1 present and the EROD activity (Collier *et al.* 1992, Celander *et al.* 1994, Goksoyr *et al.* 1994). These differences in correlations between cytochrome P4501A1 induction and EROD activity indicate that individual compounds produce their own distinctive response and may even react differently when part of a complex effluent, and it is recommended that both measurements be utilised when assessing a complex effluent (Goksoyr *et al.* 1994, Peters *et al.* 1994). Therefore, as pulp mill effluent is such a complex mixture, and elevated EROD and ECOD (ethoxycoumarin-O-deethylase) activities have been detected previously in relation to exposure to Burnie mill effluent (Munday *et al.* 1991, Munday and Brand 1992), both the cytochrome P4501A1 protein content and the EROD activity were measured in this series of experiments.

Pine-based pulp mill effluents have been reported to impact on the production of reproductive hormones in fish (Munkittrick *et al.* 1992). For instance, in conjunction with increased mixed function oxygenase activity, a reduction in circulating gonadal steroids was recorded in fish exposed to primary treated pine-based pulp mill effluent in Jackfish Bay (Munkittrick *et al.* 1992). Also, female redbreasted sunfish showed reduced plasma estradiol levels after exposure to pine-based pulp mill effluent (Adams *et al.* 1992). However, no direct links between MFO induction and reduced steroid levels have been established (Munkittrick *et al.* 1992). Reduced levels of steroids can be related to an impairment in reproductive processes which ultimately influence the reproductive capabilities of the population leading to a gradual decrease in population numbers (Donaldson 1990, Holm *et al.* 1994). A decrease in estrogens affects the yolk precursor vitellogenin resulting in smaller oocytes, therefore, a smaller yolk sac would be available for use by the resulting larvae adversely affecting their viability (Pankhurst and Conroy 1987, Donaldson 1990). Results from a field study, which was performed to determine the effects of Burnie mill effluent on Tasmanian blenny populations (Deavin 1993), indicated that there was an impact on the reproductive capabilities of the blennies living in Emu Bay. Consequently, an assay using the Tasmanian blenny was performed in the laboratory to determine the effects of the Burnie mill effluent on estradiol and testosterone levels.

These experiments were designed to determine the sublethal effects of exposure routes (dietary, water-borne and dietary + water-borne) of fish to eucalypt-based pulp mill effluent. The effects were quantified to establish the significance of each route of exposure and the biomarkers were

then evaluated for use in detecting sublethal responses to eucalypt-based pulp mill effluent. These experiments were not designed to identify the biological mechanisms by which eucalypt-based pulp mill effluent affects the selected biomarkers. Common jollytails (*Galaxias maculatus*) were selected as the test species for this series of bioassays as they fulfil all of the selection criteria for bioassay species as listed in Rand and Peterocelli (1985). The common jollytail is a small diadromous fish found in Australia, New Zealand and South America (Berra *et al.* 1996). The fish adapt readily to laboratory conditions and its small size allows for large numbers in treatment aquaria.

3.2 Materials and Methods

3.2.1 Facilities

All experiments were run in a temperature controlled laboratory (17 ± 0.5°C with a light regime of 16 hours light / 8 hours dark) at the National Key Centre for Teaching and Research in Aquaculture at the University of Tasmania, Launceston campus, Tasmania (hereafter called the Aquaculture Centre).

3.2.2 Fish

Juvenile common jollytail (*Galaxias maculatus*) (Figure 3.1) were collected from the wild using a dip net in October /November 1993, 1994 and 1995. At that time of year the juveniles are moving from the sea into freshwater streams where they will mature. The fish were six months old and weighed approximately 0.5g at capture. These fish were transported to the Aquaculture Centre and acclimatised to 25 ppt seawater over one week. The fish were maintained until required in a 1500L recirculating system at 25 ppt salinity and fed daily with commercial trout pellets (Gibsons, 1/8") at a rate of 2% body weight / day.

Adult Tasmanian blennies (*Parablennius tasmanianus*) (Figure 3.2), 4.33 ± 2.4g were collected using fish traps from an unpolluted site in north-east Tasmania and transported to the Aquaculture Centre and placed in eight 20 litre aquaria. Six fish were placed in each aquaria, four females and two males, each with individual nests (Munday and Woodworth, unpublished). The fish were acclimatised to the aquaria for one week in sea water, 33 ± 1 ppt salinity and pH 8.2 ± 0.1. The fish were fed with meat from juvenile clams (*Katelysia scalarina*) at 2% body weight / day. Fifty percent water changes were undertaken daily.

3.2.3 Effluent

Primary treated effluent was collected at approximately four weekly intervals from the Australian Paper bleached eucalypt-based pulp and paper mill at Burnie, Tasmania, in 20 litre containers and transported directly to the Aquaculture Centre where it was stored at 0°C. The effluent was collected just prior to the discharge point. Toxicity, colour and pH of each batch of effluent were recorded. Processes of the paper mill were soda-AQ pulping to Kappa N° 16, conventional CE(D/H) bleaching and primary treatment of effluent prior to discharge. (C - chlorination, E - caustic extraction , D - chlorine dioxide, H - calcium hypochlorite) : See Chapter 2

Figure 3.1

Juvenile Common Jollytail (*Galaxias maculatus*)

(Actual size)

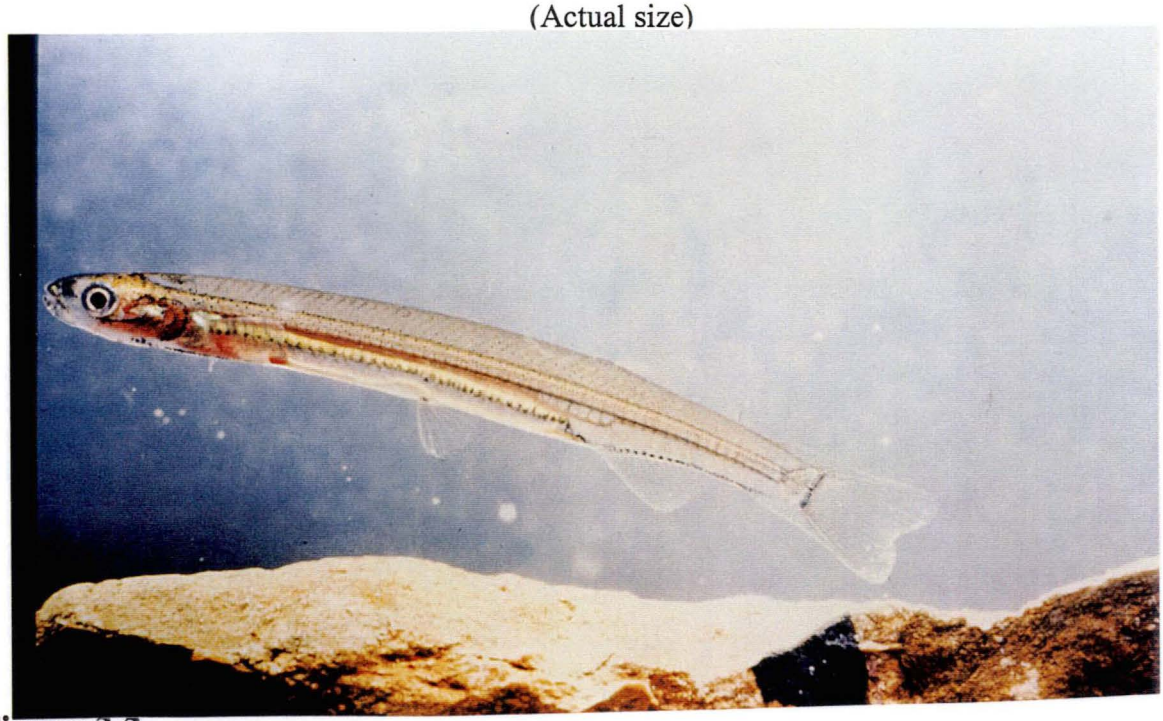


Figure 3.2

Adult Male Tasmanian Blenny (*Parablennius tasmanianus*)

(2/3 Actual size)

3.2.4 Diluent Water

Diluent water consisted of sea water, salinity 33 ppt and pH 8.2 ± 0.1, which was collected from an relatively unpolluted site in northern Tasmania and transported to the laboratory in a stainless steel road tanker. At the laboratory it was stored in a 22,000 litre concrete tank. Launceston town water supply was used to reduce the salinity of the sea water to 25 ppt for use with the common jollytails.

3.2.5 Microtox

The toxicity of the whole effluent was tested within 24 hours of being collected using a Microtox M500 toxicity analyser. The toxicity of the effluent was also tested after the pH and salinity were adjusted to that of seawater. See Chapter 2

3.2.6 Algae

Single celled marine algae were used as feed for the *Artemia salina* and clams which were cultured in semi-continuous batch culture at the Aquaculture Centre. Algal starter cultures were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Fisheries, Hobart, and maintained as stock cultures at the Aquaculture Centre. These stock cultures were maintained at 20°C, in low light with no aeration and they were subcultured regularly to maintain high quality cultures. Twenty to fifty mL of the algal stock culture were used to inoculate a 250 mL working culture, which was then grown to a phase of exponential growth and used to inoculate a 3 L working culture. When the algae again reached exponential growth, the flask was used to inoculate a 200 L semi-continuous culture. The algal cultures grown in the 200 L bags were exposed to a light regime of 18 hours light to 6 hours dark at a temperature of 24°C and supplied with air containing 0.5 - 3% carbon dioxide.

The algae used to feed the *A. salina* and clams in these dietary experiments were harvested from the 200 L semi-continuous batch culture by collecting the appropriate amount in a bucket and transporting the algae to the *A. salina* growing unit or the toxicology laboratory (for the clams). Two species of algae, *Isochrysis galbana* (Figure 3.3) and *Tetraselmis suecica,* (Figure 3.4) were used to feed the *A. salina* (Figure 3.5) and the juvenile clams.

The algae were grown in f2 media which is a balanced mixture of nutrients that has been found to support the growth of most marine algal species. The formulation is as follows:

f2 MEDIA

Stock Solutions

1.	$NaNO_3$	150 g / L
2.	$NaH_2PO_4.2H_2O$	13 g / L
3.	$Na_2SiO_3.5H_2O$	22.65 g / L
4.	Ferric Citrate	9.0 g / L
	+ Citric Acid	9.0 g / L

5.	Trace Metals	mg / 100 mL
	$CuSO_4.5H_2O$	1.96
	$ZnSO_4.7H_2O$	4.40
	$CoCl_2.6H_2O$	2.20
	$MnCl_2.4H_2O$	36.0
	$Na_2MoO_4.2H_2O$	1.26

6.	Vitamins	mg / L
	Thiamine	200
	Biotin	1.0
	Vitamin B12	1.0

After autoclaving (20 minutes at 121°C) store all stock solutions at 4°C. Add 0.5 mL of each stock solution per litre of 0.2µm filtered seawater. The silicate solution was only added to the *N. closterium* cultures.

Figure 3.3

***Isochrysis galbana* Mag. x 800**

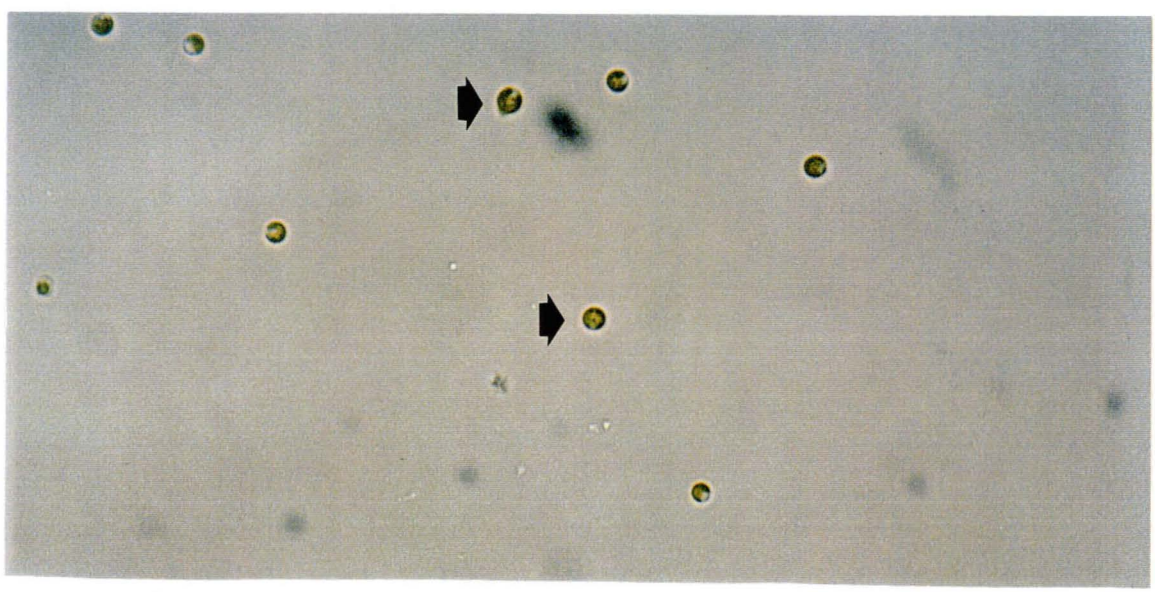


Figure 3.4

***Tetraselmis suecica* Mag. x 800**

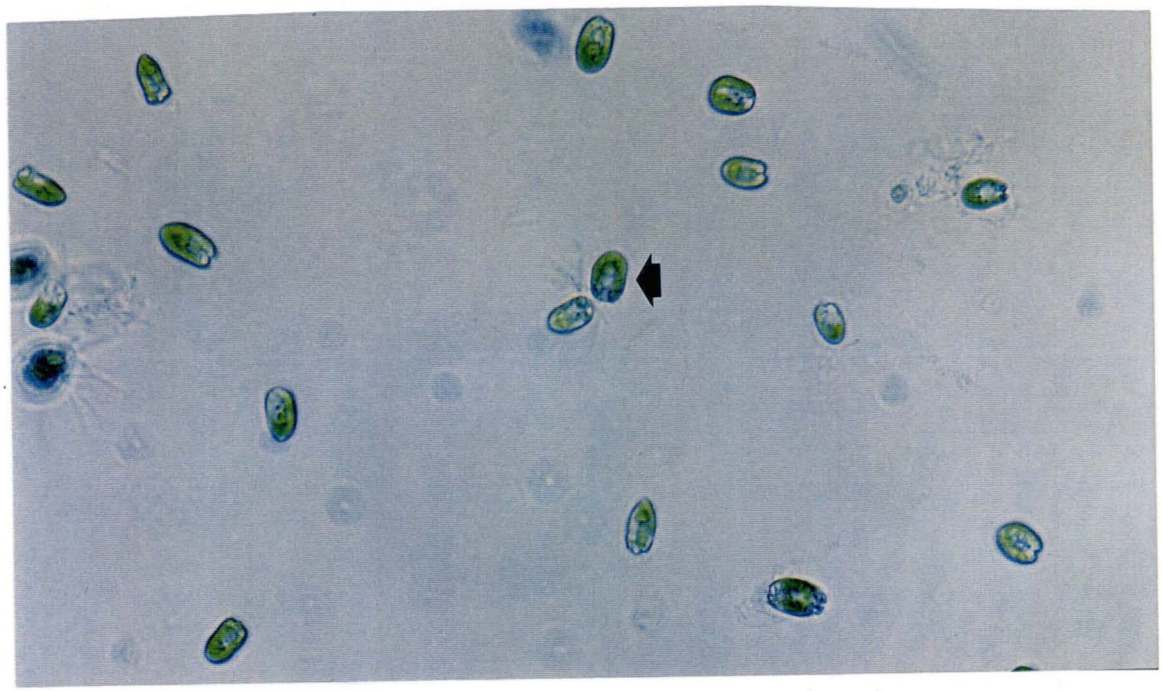
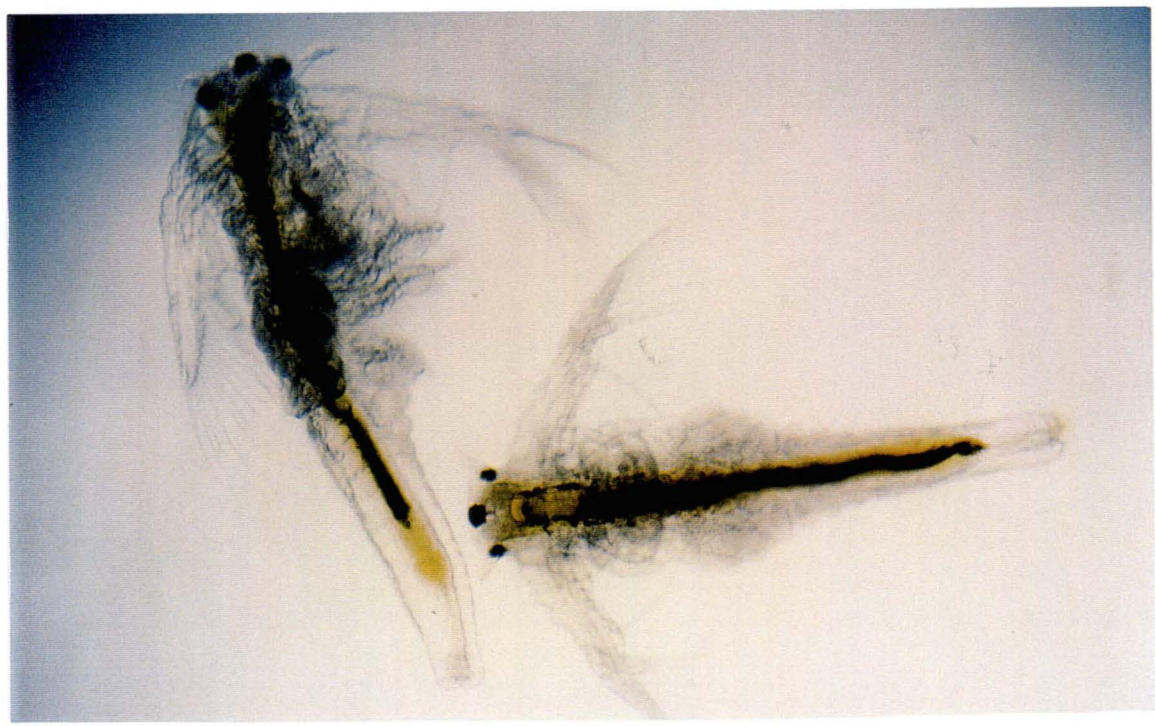


Figure 3.5

***Artemia salina* Mag. x 100**



3.2.7 *Artemia salina*

Four days prior to the start of the dietary experiments, six 80 L cultures of *Artemi salina* were set up in the temperature controlled *A. salina* growing unit, 28 ± 1°C. For each 80 L culture, 15 grams of *A. salina* cysts (Olympic) were weighed into 1 L beakers and 800 mL of seawater was added. An airstone was placed in each beaker and the cysts were then hydrated for 1 - 2 hours at room temperature. The cysts were next decapsulated by the addition of sodium hypochlorate to a concentration of 22g active chlorine / L for approximately 10 minutes, until the cysts turned orange. The cysts were immediately poured onto a 100 µm mesh screen and rinsed thoroughly with running freshwater. The rinsed cysts were then immersed in a 0.1 M sodium thiosulphate solution for 1 minute and rinsed again with running water. The cysts were then placed in 100 L cylinders with conical bases with 40 L of seawater at 28 ± 1°C, 24 hour light and vigorously aerated.

After hatching (day one of culture) 5 L of *I. galbana* and 5 L of *T. suecica* were added to each culture and seawater was added to reach a culture volume of 80 L. A volume of effluent was added to the exposed cultures to maintain the effluent concentrations at 2.5% or 0.5%. On day three of culture a water change was performed whereby 40 L of culture medium were removed and replaced with 30 L of seawater, 5 L of *I. galbana* and 5 L of *T. suecica*. Effluent was added to the exposed *A. salina* to maintain effluent concentration. On days four and five of culture, one quarter of the *A. salina,* 3 - 4 mm in size and having undergone up to 10 moults, were harvested and fed to the common jollytails. Each culture was then topped up to 80 L with seawater and 10 L of algae on day 5. On day 6 of culture half was harvested for feed and on day 7 the remaining *A. salina* were fed to the common jollytails. The *A. salina* were harvested by opening a tap at the base of the conical tank and catching the *A. salina* on a 200 µm mesh screen. The *A. salina* were rinsed prior to feeding to the fish and added to the aquaria daily at approximately 1000 per fish, this allowed for feeding *ad libitum.*

3.2.8 Clams

Juvenile clams (*Katelysia scalarina*) were collected from an unpolluted site in north-west Tasmania and transported to the Aquaculture Centre where they were placed in two 40 L aquaria in a flow through system (80 L per day). The clams were acclimatised for 1 week in sea water and were fed with *I. galbana* and *T. suecica* (10 L of each per day). Effluent was then added to

one of the aquaria at a concentration of 1%. Clam meat was fed to the blennies at a rate of 2% fish body weight / day.

3.2.9 Artificial Feed

The artificial feed was prepared by mixing 1kg of trout crumble with 5.5 mL of effluent and 5g of gelatine mixed with 100mL of hot water. The gelatin mix was cooled prior to being added to the effluent/pellet mix to make a trout crumble cake containing 0.5% effluent v/w. Control cake was prepared by deleting the effluent. Both the food cakes were stored at -18°C with the cake being fed to the fish at 2% fish body weight / day.

3.2.10 Chronic Experiments

3.2.10a Test Solutions

Twelve 40 L aquaria were filled with 25ppt seawater (3 replicates x 4 treatments). Two hundred mL of effluent were added to six of the aquaria to obtain an effluent concentration of 0.5%. The effluent used in these experiments was collected monthly and maintained at 0°C. See Chapter 2.

All water parameters were measured daily for the duration of the assays. Salinity of the test solutions was monitored with an Atago model S10 salinity refractometer and maintained at 25ppt. Aeration was provided in all aquaria and the dissolved oxygen for the assays was maintained at >9.0 ppm. Dissolved oxygen was measured with a WTW OXI 96 dissolved oxygen meter. The pH and temperature were also monitored during all assays with a HANA HI 8424 pH / temperature meter. The pH of all the aquaria was within the range of 8.2 ± 0.1. The laboratory temperature was maintained at 17.0 ± 0.5°C with two Philips reverse cycle air conditioners. Lighting consisted of fluorescent lights with a photoperiod of 16 hours light and 8 hours dark.

3.2.10b Assay 1

Ten juvenile common jollytail (~1g and 10 months old) were placed in each of twelve 40 L glass aquaria with 25 ppt seawater. Fish were exposed to the effluent as per Table 3.1.

Each treatment consisted of three aquaria. The fish were fed *ad libitum* with 4 - 6 day old *A. salina* and any uneaten food was removed from the aquaria. Daily maintenance of the aquaria involved a 50% water exchange with the addition of 100 mL of effluent to maintain the concentration in the aquaria at 0.5%. The loading rates were 1 g biomass / 2 L replacement water / day. The experiment was terminated after 3 months exposure.

At the end of the exposure the fish were anaesthetised, weighed, measured and the sex determined. The livers were then removed and weighed and processed either for histology, electron microscopy or EROD determination. The remains of the fish were placed in 10% buffered formalin.

Table 3.1 Exposure Method for Common Jollytails

	Dilution Water	Pulp Mill Effluent 0.5%	Unexposed Food	Exposed Food 0.5%
Group 1 Control	✓		✓	
Group 2 Food	✓			✓
Group 3 Water		✓	✓	
Group 4 Food/Water		✓		✓

3.2.10c Assay 2

As in Assay 1, with the differences being that fifteen mature common jollytail (~2g and 18 months old) were placed in each aquarium with the loading rate being 1g biomass / 1 L replacement water / day. The experiment was terminated after six weeks exposure due to the presence of moribund egg-bound females.

3.2.10d Assay 3

As for Assay 2 with the difference being that the live food was replaced by artificial food. The experiment was conducted for 3 months.

3.2.10e Assay 4

This experiment utilised a marine fish, the Tasmanian blenny (*Parablennius tasmanianus*), as the test fish. Duplicate 20 L aquaria containing six adult fish were used in this experiment which was

conducted using the same method as that in the previous experiments. The differences in this experiment were that the effluent concentration was 1% and the food source were juvenile clams fed on the same types of algae as the *A. salina*; also the salinity of the sea water was 33 ppt. This experiment was terminated after 6 weeks to preclude mortalities due to spawning stress.

3.2.11 96 Hour Assays

3.2.11a Test Solutions

Twelve 40 L aquaria were filled with 25ppt seawater (3 replicates x 4 treatments). Effluent was added to six of the aquaria to obtain an effluent concentration of 2.5%. A fresh batch of effluent was used for each 96 hour assay. See Chapter 2, Table 2.10 for effluent data. All water parameters were measured daily for the duration of the assay, as mentioned previously.

3.2.11b 96 Hour Live Feed Assay

Five common jollytails, approximately two years old and weighing 4.57 ± 2.37 g were placed in each of twelve 40 L glass aquaria one week prior to the commencement of the assay. Treatments were randomly allocated among aquaria.

On day one of the assay, effluent was added to the aquaria for water exposure and food/water exposure at a concentration of 2.5%. The experimental method was as per Table 3.1 with loading rates of 1 g biomass / 1 L replacement solution / day.

Water changes were performed daily where 20 L of the test solution was removed. This allowed for removal of uneaten *A. salina* and faecal material on the bottom of the aquaria. The test solutions were replaced with 20 L of 25ppt seawater and 500 mL of effluent was added to Group 3 and Group 4 aquaria.

After the fish had been exposed to the treatments for 96 hours, they were killed with an overdose of benzocaine (100 ppm) and processed for analysis.

3.2.11c 96 Hour Assay 2 and 3

Five fish, approximately 3 - 4 years old and weighing 23.57 ± 10.55 g for Assay 2 and 10 fish approximately 2 - 3 years old and weighing 7.70 ± 2.45g for Assay 3 were placed in each of

twelve 40 litre glass aquaria one week prior to the commencement of the assay. Treatments were randomly allocated among aquaria.

On day 1 of the assay, effluent which had been adjusted to a salinity of 25ppt and a pH of 8.2, was added to the allotted aquaria in concentrations of 10%, 20% and 30%. The fish were not fed for the duration of the assay but daily water changes were performed where 50% of the test solution was removed and replaced with 25ppt seawater and the appropriate concentration of effluent. Loading rates were 5g biomass / 1 L replacement solution / day and 3.5 g biomass / 1 L replacement solution / day, respectively.

After the fish had been exposed to the treatments for 96 hours, they were killed with an overdose of benzocaine and processed for analysis.

3.2.12 Parameters Measured

Each fish was weighed on a Sartorius balance to the nearest mg. Each fish was then measured to the nearest 0.1 mm. Fish were opened ventrally and the sex determined. The liver was removed and weighed to the nearest mg. A small portion of the common jollytail liver was placed in 10% phosphate buffered formalin for processing for histology and the remaining piece of liver was frozen at -80°C for EROD analysis (Holdway *et al.* 1993) the next day. Cytochrome P4501A1 analysis using an indirect ELISA technique (Goksoyr 1991) was performed the following week. The livers of the anaesthetised blennies were removed and processed for EROD analysis only. The blenny gonads were staged as per Table 3.2, then removed and frozen at -18°C for estradiol and testosterone analysis (Pankhurst and Conroy 1987).

Table 3.2
Criteria for Allocation to Sexual Maturity Stages of the Tasmanian Blenny

Stage	Gonad Condition
Females	
I Immature Virgins	Ovaries small, ribbon-like and transparent, occupying <1/3 of the length of the body cavity. Oocytes microscopic, <0.1mm.
II Maturing Virgins or Recovered Spents	Ovaries slightly swollen, occupying 1/2 the length of the body cavity, small oocytes just visible to the naked eye, up to to 0.1mm.
III Ripening	Ovaries enlarged, occupying 3/4 of the body cavity, several groups of oocytes visible to the naked eye, up to 0.5mm, yellow/white in colour.
IV Ripe	Ovaries distended ans occupying most of the body cavity, ripe ova 1mm, green in colour.
V Spent	Ovaries flacid and bloodshot.
Males	
I Immature Virgins	Testes transparent ans minute, occupying 1/6 length of body cavity.
II Maturing Virgins or Recovered Spents	Testes compact and opaque white, occupying 1/2 the length of the body cavity.
III Ripening	Testes larger, occupying 3/4 of the length of the body cavity, white in colour.
IV Ripe	Testes slightly larger than III, milky white in appearance.
V Spent	Testes shrunken, brownish in colour.

3.2.13 Growth, Condition Index and Liver Index

The growth of common jollytails was calculated for chronic Assays 1 to 3 and was expressed as a percentage of the control weight.

Growth was calculated as percentage growth of control fish, as weights of individual fish at the beginning and end of the experiment could not be correlated because the fish were not individually identified due to their small size.

% Growth = Weight of treated fish (g)/ weight of control fish (g) x 100.

Condition index for fish in all of the experiments was calculated using the formula;

Condition Index = 100 x body weight (g) / [length (cm)]^3

(Andersson *et al.* 1987).

The liver somatic index was calculated for all common jollytail using the formula;

Liver Somatic Index = 100 x liver weight (g) / body weight (g)

(Andersson *et al.* 1987).

3.2.14 Histopathology

A small piece of liver was removed from the anaesthetised fish and placed immediately into 10% phosphate buffered formalin where they were maintained until processing for histopathology. The livers were dehydrated through a series of alcohols, cleared in xylene and infiltrated with paraffin wax (Gordon 1991) prior to cutting 4 micron sections (See Appendix 2 for details). The sections were stained using haematoxylin and eosin (H & E) (Stevens 1991), (See Appendix 2). A periodic acid - Schiff (PAS) (Cook 1991) stain was used to demonstrate the presence of glycogen (See Appendix 2). Sections were examined using an Olympus BH2 microscope.

The types of histopathological changes observed in all livers were itemised and the number of changes per 100 cells in each liver were determined by counting using the battlement method (Munday and Nowak 1997). The results were then analysed using ANOVA.

3.2.15 Electron Microscopy

Small pieces of liver were fixed for 2 hours at room temperature in 5% glutaraldehyde / 0.2M sodium cacodylate buffer (pH 7.2) for examination by transmission electron microscopy. The fixative was removed and the samples washed three times, with a 10 minute soaking step, in 0.2M sodium cacodylate buffer. The samples were then post-fixed in 1% aqueous solution of osmium tetroxide for 2 hours at room temperature. The samples were then washed in distilled water for 10 minutes followed by dehydration through a series of ethanol dilutions to 70% ethanol for storage prior to embedding (See Appendix 2).

Immediately prior to embedding, the samples were dehydrated in 100% ethanol and then were infiltrated with a 100% ethanol : Spurrs resin (1:1) mixture overnight at room temperature. That solution was removed and replaced with 100% Spurrs resin which was left overnight at room temperature (see Appendix 2). The sample was next transferred to fresh resin and polymerised at 65°C overnight (Anon 1991).

Ultra thin sections were cut using a diamond knife, after which they were placed on a copper grid and double stained with uranyl acetate and lead citrate (see Appendix 2), 15 minutes in each solution. The sections were examined on a Hitachi H-300 transmission electron microscope.

3.2.16 EROD Activity

EROD activity was assayed using a modification of the method described by Holdway *et al.* (1993) and is detailed in Appendix 2. Livers were removed from anaesthetised fish and placed in Eppendorf tubes and frozen at -80°C for 24 hours prior to preparation of the microsomes.

Microsomes were prepared by homogenising the livers followed by centrifuging the homogenate at 13,000 rpm at 4°C for 20 minutes. The post mitochondrial supernatant (PMS) was then transferred to a precooled Eppendorf tube. Assays were performed in duplicate with a blank to record background fluorescence for each sample.

Each tube contained 450 µL 0.1M TRIS buffer, 100 µL BSA (12 mg mL^{-1}), 250 µL Cofactor (10mM MgCl2, 200mM KCl, 6mM glucose-6-phosphate, 1.25mM NADP, 100U G-6-dehydrogenase) and 100 µL PMS. Duplicate standards were prepared with concentrations of 9,

18, 27, 36 and 45 pM resorufin. To prevent breakdown of resorufin, 10 µL of dicoumarol was added to each tube. Methanol (2.5 mL) was added to all the blank tubes.

All tubes were incubated at 30°C for 2 minutes prior to the initiation of the reaction by the addition of 100 µL ethoxyresorufin (1.25 mg in 10 mL 1:1 methanol:DMSO). The samples were incubated for 10 minutes after which they were placed on ice and 2.5 mL of methanol was added to the samples and the standards. The tubes were centrifuged at 2000g for 5 minutes and the fluorescence of the supernatant was measured at an excitation wavelength of 530 nm and emission wavelength of 585 nm and compared to the standard curve. Protein analysis was determined by the method outlined by Lowry *et al.* (1951). EROD activity was calculated as pmols/min/mg protein.

3.2.17 ELISA Measurements

The PMS used in the EROD analysis were also used for an indirect ELISA technique to detect cytochrome P4501A1 isoenzyme (Goksoyr 1991). This method is detailed in Appendix 2.

Microsome preparations were diluted to a protein concentration of 10µg / mL in coating buffer and allowed to adsorb to microtitre plates overnight. The plates were then washed three times and blocked with 2% BSA for 1 hour. The plates were again washed three times prior to the addition of 100 µL of 1-12-3p6 mouse monoclonal antibody (MAb) against scup cytochrome P4501A1 (Stegeman *et al.* 1985) diluted 1:40 in 1% BSA/PBS.

The plates were left overnight, washed again, and 100µL of the secondary rabbit-anti-mouse IgG antibody (diluted 1:300 in 1% BSA/PBS) was added to each well and incubated in the dark for 1 hour. After washing, the colour reaction was developed for 30 minutes with 100 µL of developing solution (O-phenylenediamine in 37.5 mL sodium citrate/phosphate buffer and 15µL H_2O_2). Positive samples made of liver preparations from common jollytails exposed to benzo-a-pyrene (40mg/kg body wt) were included in each plate and processed in the same way as the samples from the assays.

The optical density (OD) was determined using an ELISA plate reader which read the absorbance at 492 nm. The optical densities were corrected by subtracting the OD for blank samples and a plate correction factor of the ratio of positive OD to negative OD for each plate was applied.

3.2.18 Estradiol and Testosterone Analysis

Testosterone and 17ß-estradiol were measured by radioimmunoassays (RIA) in gonads following ether extraction. The blenny gonads were weighed and approximately 0.125g of gonadal tissue was homogenised in 1 mL of ethyl acetate, vortexed and centrifuged at 3000 rpm. 100µL aliquots were transferred to assay tubes for evaporation, after 200µL of buffer (0.05M phosphate buffer containing 0.1% gelatine) was added. 200µL of gonad extract was incubated at room temperature for 24 hours with 200µL of tracer [tritiated testosterone and estradiol respectively (Amersham International), 3000cmp] and 200µL of antibody. After incubation, samples were cooled on ice for 10 minutes before addition of 200µL of dextran-coated charcoal suspension then left to stand for 10 minutes before centrifugation at 4°C (3600 rpm for 10 minutes). The supernatant was then combined with 4 mL of scintillation fluid for counting as ng steroid / g of gonadal tissue. All samples were assayed in duplicate and all values were corrected for tissue weight.

3.2.19 Statistical Analysis

LC50s were calculated using a probit program by Davies *et al.* (1988). EC50 values were calculated with a trimmed Spearman-Karber method using ToxCalc (Tidepool, 1992). EROD and ELISA data were analysed with the statistics package JMP2 to determine if the variances within the data were equal with Bartlett's test. If the data failed this test the nonparametric analysis of Kruskal-Wallis was used. If the data showed homogeneity of variance the data was then analysed with an analysis of variance (ANOVA), taking into account each fish, sex, replicate and treatment for the EROD and ELISA results and fish, stage of maturity, replicate and treatment for the steroid assays. The results were tested for significance at the 95% level using Tukey-Kramer HSD. All percentage data were arcsine transformed prior to analysis. All data were analysed within assays as physiological and species differences precluded comparisons between assays.

3.3 Results

3.3.1 Effluent

The colour of the effluent varied between ten batches from a dark brown containing flocculent matter to a clear light brown. The pH of the untreated effluent also varied between batches ranging from pH 11.83 to 9.32. The acute toxicity of the effluent, as measured by Microtox, ranged from an EC50 of 5% to 51% with 95% confidence limits (95% c.l.) of 4.4, 5.3 and 43.9, 58.8 respectively (Table 2.11, Chapter 2). A sample of each batch of effluent had its pH and salinity adjusted to that of sea water using HCl and artificial sea salts. The acute toxicity of the adjusted samples was also determined using Microtox with the EC50 results ranging from 21% (95% c.l. 16.1, 27.1) to >90% (Table 2.10).

3.3.2 Algal EC50 / *Artemia salina* LC50

See Chapter 2

The acute toxicity of the Burnie mill effluent to the algae *Nitzschia closterium* was assessed. *N. closterium* is the species being promoted as a standard bioassay species by the National Pulp Mills Research Program (Stauber *et al.* 1994), as discussed in Chapter 2. This species was more sensitive to the effluent, with an EC50 of 8%, than one of the two species that were used in the food chain experiments (Table 2.13). *I. galbana* had an EC50 of 8%, equal to that of *N. closterium*, and *T. suecica* was shown to be less sensitive with an EC50 of 16% (Table 2.13). The Burnie mill effluent was non-toxic to *Artemia salina* with an EC50 of >100% (Table 2.13).

3.3.3 Chronic Assays

3.3.3a Common Jollytail Growth

These studies were perturbed by a number of practical problems, especially sexual maturation of larger fish. Growth rates were calculated for 30 fish from each treatment instead of the 45 that were initially in each treatment in Assays 2 and 3 due to mortalities of the larger fish in all aquaria caused by the inability to spawn (this complication is discussed in detail later). The final weight of the control fish was set at 100% and all other treatments were compared to the controls. The differences in growth in Assay 1 were attributed to exposure via food (Table 3.3). Differences in the growth occurring in Assays 2 and 3 were noticable in the fish that were exposed to effluent in the water column. This is in contrast to the fish exposed by both routes in Assay 2 which showed greater growth than the control fish.

3.3.3b Condition Index

Condition indices for control fish in all assays were higher than for treated fish (Table 3.4). However, in Assay 3 these results were not significantly different. Assays 1 and 2 showed significant differences in condition indicies between treatments.

Table 3.3

Growth of Common Jollytails

(weight as percent of control weight)

Treatment	Assay 1 (n = 30)	Assay 2 (n = 30)	Assay 3 (n = 30)
Control (Group 1)	100%[a]	100%[a b]	100%[a b]
Food (Group 2)	85%[b]	100%[a b]	109%[b]
Water (Group 3)	98%[a]	94%[b]	81%[a]
Food/Water (Group 4)	79%[b]	110%[a]	90%[a b]

Note: Different superscript letters indicate that results are significantly different at the 95% level (P<0.05) within assays.ie. results with the same superscript are not significantly different.

Table 3.4

Condition Indices for Common Jollytails

Treatment	Assay 1 (n = 30)	Assay 2 (n = 30)	Assay 3 (n = 30)
Control (Group 1)	0.56 ± 0.04[a]	0.69 ± 0.02[a]	0.68 ± 0.02[a]
Food (Group 2)	0.50 ± 0.04[c]	0.58 ± 0.02[b]	0.66 ± 0.01[a]
Water (Group 3)	0.53 ± 0.04[b]	0.62 ± 0.02[cb]	0.63 ± 0.02[a]
Food / Water (Group 4)	0.47 ± 0.04[d]	0.64 ± 0.02[c]	0.64 ± 0.01[a]

Note: Different superscript letters indicate that results are significantly different at the 95% level (P<0.05) within assays.ie. results with the same superscript are not significantly different.

3.3.3c Liver Somatic Index

Liver somatic indices (LSI) for all common jollytails were calculated with control fish in all assays showing significantly higher LSIs when compared with group 4 fish, with the exception of Assay 3 (Table 3.5).

Table 3.5

Liver Somatic Indices for Common Jollytails

Treatment	Assay 1 n = 15	Assay 2 n = 15	Assay 3 n = 15
Control (Group 1)	2.00 ± 0.61^{a}	1.61 ± 0.63^{a}	1.59 ± 0.57^{a}
Food (Group 2)	1.22 ± 0.54^{b}	1.62 ± 0.69^{a}	1.54 ± 0.49^{a}
Water (Group 3)	1.73 ± 0.84^{a}	1.28 ± 0.46^{ab}	1.26 ± 0.44^{a}
Food/Water (Group 4)	1.34 ± 0.46^{b}	1.03 ± 0.44^{b}	1.48 ± 0.53^{a}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

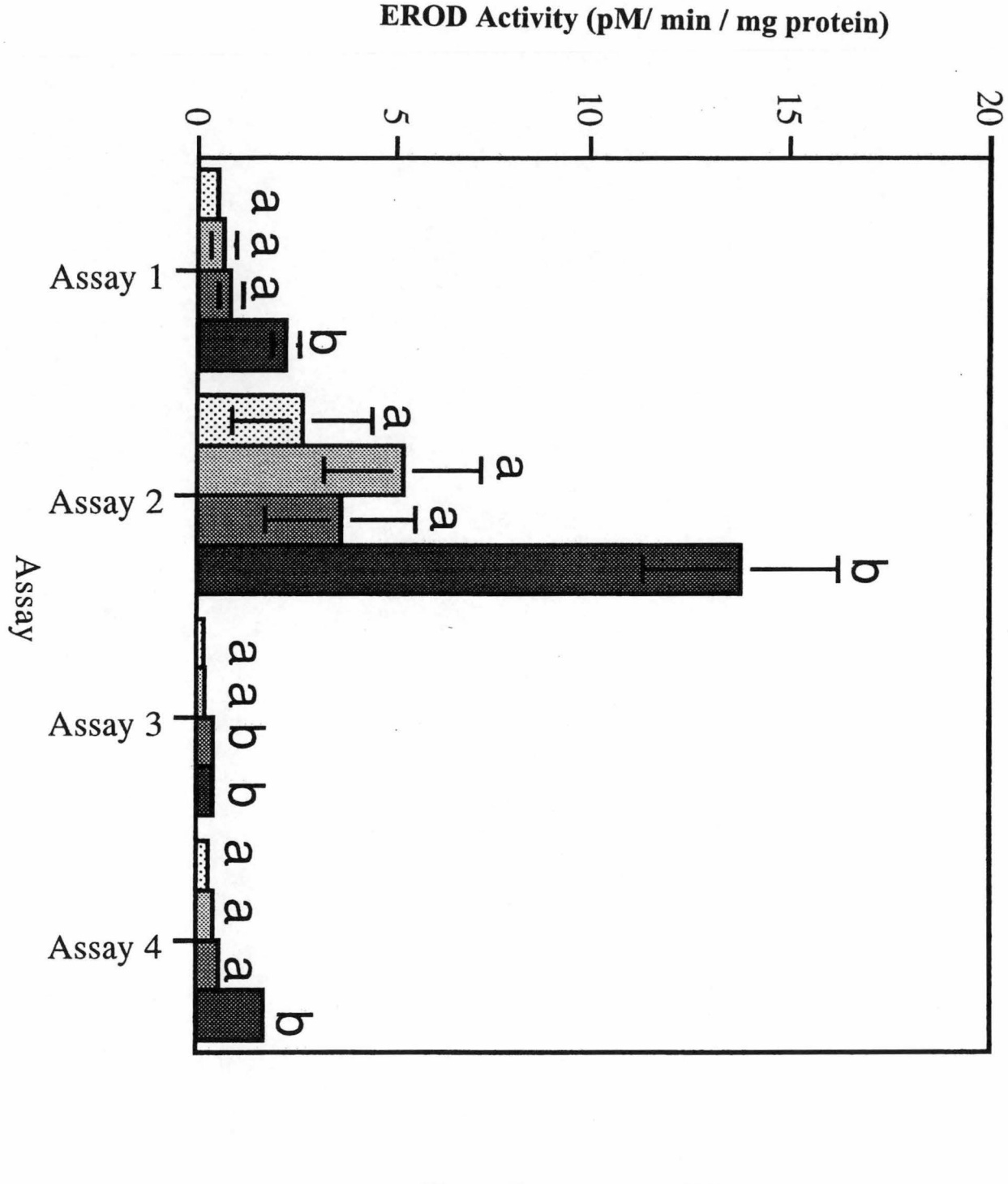
3.3.3d EROD Activity

EROD activity for each assay was analysed using a nested ANOVA with the variables being sex, aquaria and fish for each treatment. The nested ANOVA showed that there were no significant differences in EROD activity that were related to sex or replicate, therefore, results for each replicate were pooled. Blennies and common jollytails from the live feed assays 1, 2 and 4 showed a significant increase in EROD activity in group 4 fish which had been exposed to effluent by the combined food/water route (Figure 3.6). Fish in groups 1, 2 and 3 from these experiments did not show significant differences in EROD activity. The EROD induction in group 4 blennies and common jollytails was approximately 3 to 4 fold that of the control and other treatment groups.

Fish fed artificial food in Assay 3 from groups 1 and 2 were not significantly different from each other but were significantly different from the fish exposed to effluent from the water column in groups 3 and 4 (Figure 3.6).

Figure 3.6

EROD Activity (pM/ min / mg protein) in Livers of Experimental Fish



3.3.3e ELISA Measurements

The ELISA technique did not detect any significant differences between the optical densities of the samples and the blank wells. See Table 3.6 for example of Assay 1. However, the positive controls gave a high optical density.

Table 3.6

Optical Density Readings From Cytochrome P4501A1 ELISA

Treatment	Assay 1 Optical Density
Control (Group 1)	0.332 ± 0.017^{a}
Food (Group 2)	0.299 ± 0.015^{a}
Water (Group 3)	0.226 ± 0.015^{a}
Food/Water (Group 4)	0.225 ± 0.021^{a}
Blank Wells	0.207 ± 0.025^{a}
Positive Controls	0.885 ± 0.025^{b}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

3.3.3f Liver Histopathology

Several types of histopathological changes were observed in the livers of both the common jollytails and blennys that were exposed to the effluent. Livers from common jollytails in group 1 from all assays showed normal structural features. Livers from groups 2 and 3 showed low numbers of hepatocytes with different sized nuclei. However, large numbers of hepatocytes with different sized nuclei and smaller numbers of binucleate hepatocytes (Figure 3.8) were observed in group 4 fish. Vacuoles were observed in hepatocytes of fish (Figure 3.9) from all treatments but only the vacuoles from the control fish stained PAS positive indicating glycogen storage.

The types of changes observed in exposed fish consisted of binucleate cells, nuclear polymorphism, melanomacrophage aggregations, enlarged sinusoids, granulomas, foci of

necrosis, basophilic foci and eosinophilic foci. In some cases exposed fish showed more than one type of histopathological change in the liver. Consequently, the number of pathological changes were calculated by examining one hundred hepatocytes by the battlement method for each liver. These results were analysed, with Group 4 fish showing significantly more changes when compared with the other Groups (Figure 3.7).

3.3.3g Electron Microscopy of Common Jollytail Livers

Livers of control common jollytails from Assays 1 and 2 showed normal hepatocyte ultrastructure. Livers from groups 2 and 3, fish that were exposed to the effluent via one route only, showed a small increase in binucleated hepatocytes. However, livers from group 4 fish, which had been exposed to the effluent by the combination of routes, showed an increase in altered hepatocyte ultrastructure. These changes included an increase in the numbers of binucleate hepatocytes, hepatocytes with bizarre-shaped nuclei or enlarged nuclei, an increase in the number of lysosomes within the hepatocytes and a proliferation of rough endoplasmic reticulum (RER). The most common effect was that the cytoplasm of the hepatocytes was filled with dilated RER.

3.3.3h Estradiol and Testosterone Analysis

Testosterone and estradiol were analysed for both male and female blennies. The stage of sexual maturity of the fish and the replicate were included in the nested ANOVA statistical analysis but neither parameter was significant. No significant differences were detected for estradiol or testosterone in male or female fish in any treatment (Tables 3.7 and 3.8). Group 4 males showed an increase in testosterone levels but this increase was not statistically significant as individual variation in all assays was large.

Figure 3.7
Histopathological Changes per 100 Hepatocytes in Common Jollytails and Blennies Due to PME Exposure

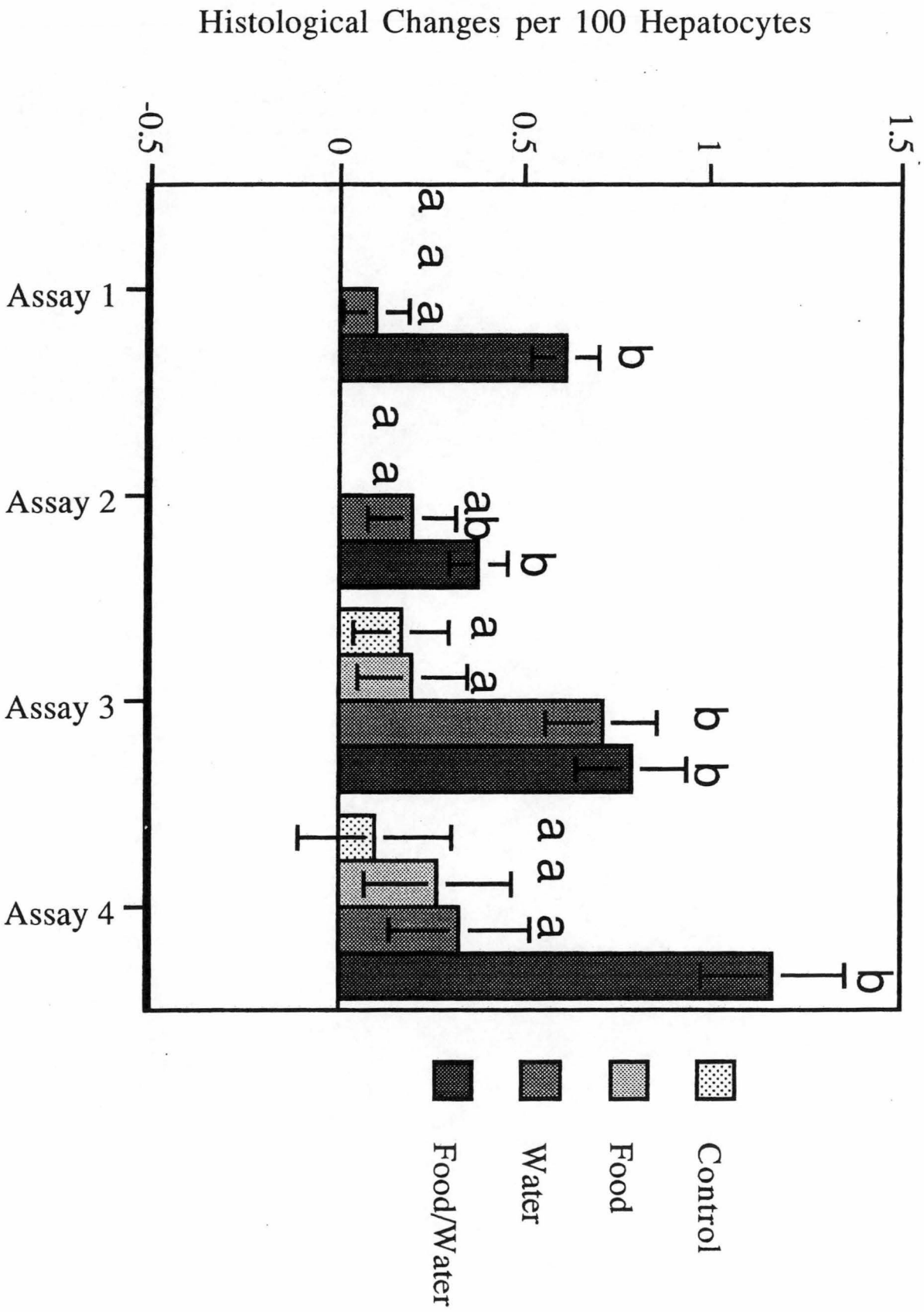


Figure 3.8

***Galaxias maculatus* Liver Showing Different Sized Nuclei and Binucleate Cells.**
H & E stain. Mag. x 400

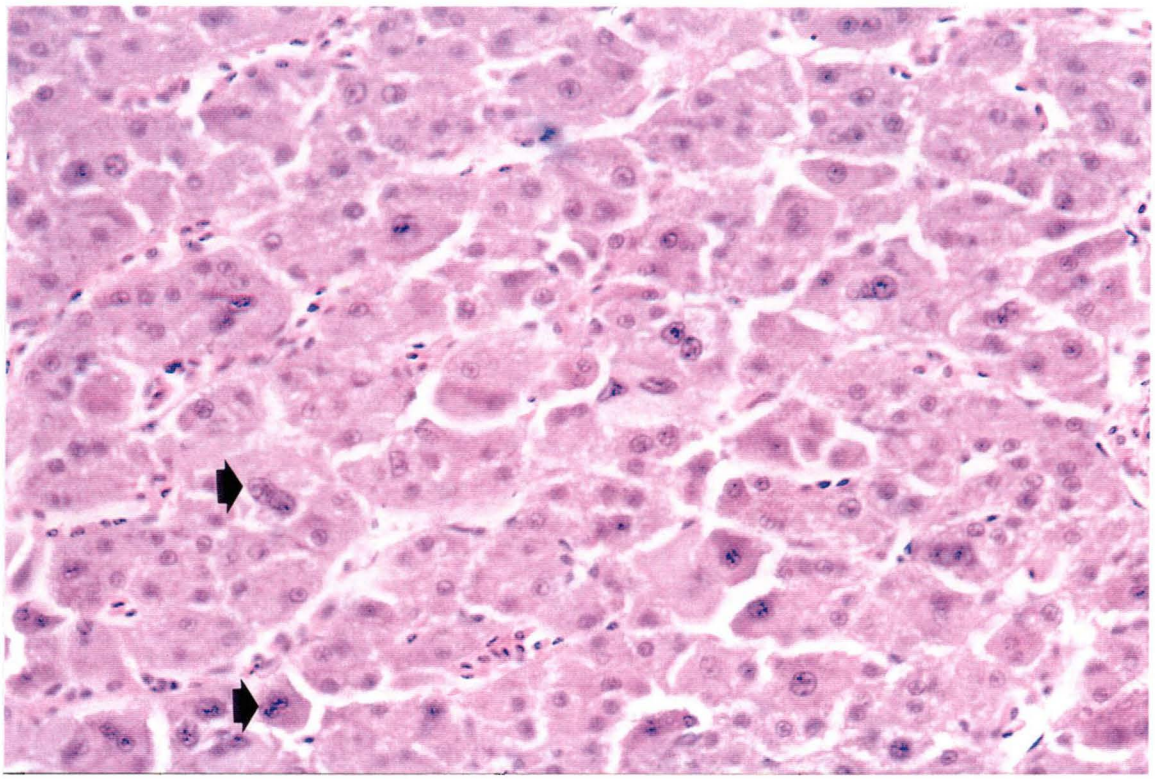


Figure 3.9

***Galaxias maculatus* Liver Showing Vacuoles**
H & E Stain. Mag. x 400

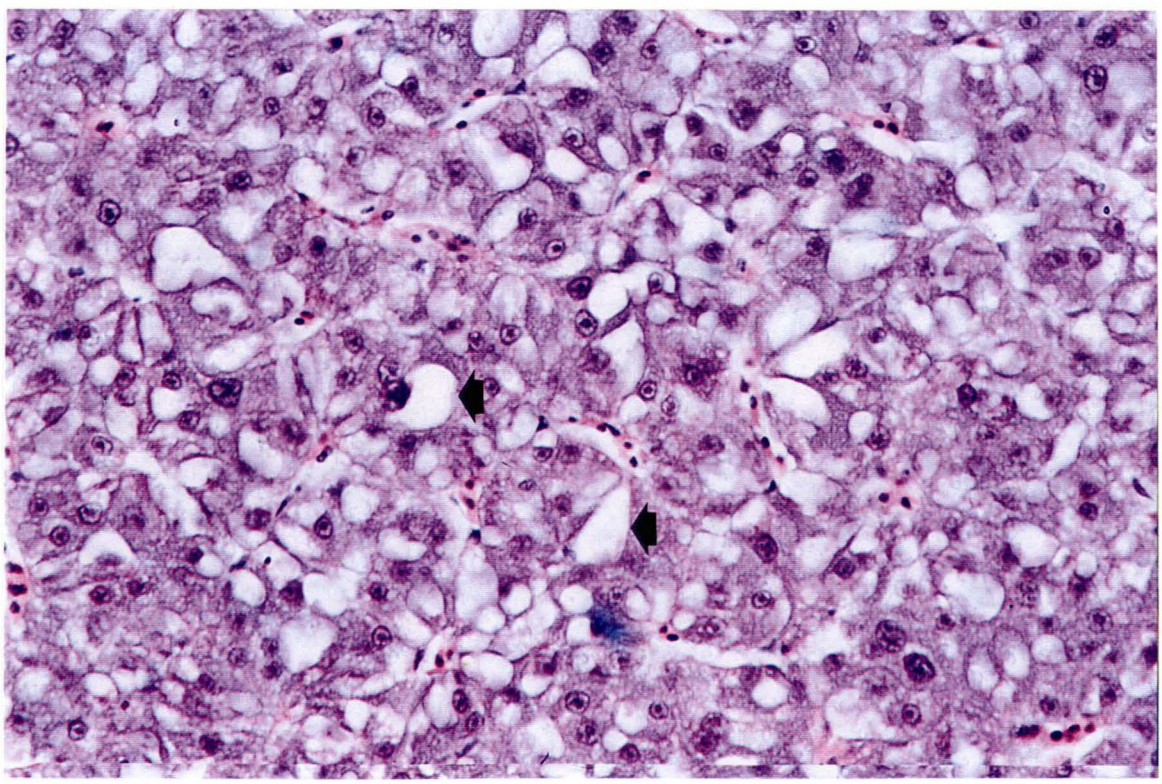


Table 3.7
Steroid levels of Female Tasmanian Blennies Exposed to Pulp Mill Effluent

Treatment	Estradiol ng/g gonad	Testosterone ng/g gonad	Stage (Range)
Control (Group 1) n = 8	1.74 ± 0.51^a	0.67 ± 0.32^a	III - IV
Food (Group 2) n = 6	1.92 ± 0.59^a	1.07 ± 0.37^a	II - IV
Water (Group 3) n = 5	2.97 ± 0.65^a	1.48 ± 0.41^a	IV
Food/Water (Group 4) n = 3	1.51 ± 0.84^a	1.13 ± 0.53^a	IV

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

Table 3.8
Steroid levels of Male Tasmanian Blennies Exposed to Pulp Mill Effluent

Treatment	Estradiol ng / g gonad	Testosterone ng / g gonad	Stage (Range)
Control (Group 1) n = 2	0.84 ± 0.42^a	0.74 ± 0.59^a	II - III
Food (Group 2) n = 4	1.23 ± 0.32^a	1.09 ± 0.39^a	II
Water (Group 3) n = 3	0.93 ± 0.37^a	1.19 ± 0.46^a	III
Food/Water (Group 4) n = 4	0.66 ± 0.33^a	2.18 ± 0.40^a	II - III

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

3.3.4 96 Hour Assays

3.3.4a EROD Activity

There were no significant differences in EROD activity among treatments in the 96 hour live feed assay (Table 3.9) and Assay 2 treatments (Table 3.10). However, EROD activity in fish from Assay 3 exposed to 10 and 20% effluents showed a significant decrease in EROD activity when compared with the controls and the fish exposed to 30% effluent (Table 3.10).

3.3.4b Histopathology

No significant histopathological changes were observed in the livers of the common jollytails from the 96 hour live feed assay. Small vacuoles were observed in the hepatocytes of livers from fish from all treatments. The vacuoles were PAS positive, indicating that the vacuoles were storing glycogen.

Histopathological changes were observed in the livers of fish from 96 hour Assays 2 and 3 (no feed). These changes included hepatocytes with nuclei of differing sizes, small granulomas and melanomacrophage aggregates. Most of the changes occurred in the fish from the 30% effluent treatment (Table 3.11). Vacuoles were also observed in the livers from the fish in all treatments. Only the vacuoles in the control fish and the fish exposed to 10% effluent stained PAS positive for glycogen, which indicated that the fish in the higher concentrations were depleting their glycogen stores.

Table 3.9

EROD Results for 96 Hour Live Feed Assay (2.5% pulp Mill Effluent)

Treatment	EROD Activity pM/ min / mg protein n = 15
Control	0.618 ± 0.104^{a}
Food	0.705 ± 0.105^{a}
Water	0.417 ± 0.110^{a}
Food/Water	0.478 ± 0.107^{a}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

Table 3.10

EROD Results for 96 Hour Assays 2 and 3

Treatment % Effluent	Assay 2 EROD Activity pM/ min / mg protein	Assay 3 EROD Activity pM/ min / mg protein n = 15
0	0.997 ± 0.241^{a}	0.676 ± 0.065^{a}
10	1.114 ± 0.231^{a}	0.226 ± 0.064^{b}
20	0.578 ± 0.252^{a}	0.377 ± 0.069^{b}
30	0.601 ± 0.232^{a}	0.764 ± 0.067^{a}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

Table 3.11

Histopathological Changes in Common Jollytail Livers

Treatment %Pulp Mill Effluent	96 Hour Assay 2 n = 5	96 Hour Assay 3 n = 5
0	0.167 ± 0.109^{a}	0.0000 ± 0.099^{a}
10	0.538 ± 0.105^{b}	0.333 ± 0.100^{b}
20	0.857 ± 0.143^{bc}	0.400 ± 0.121^{b}
30	1.000 ± 0.110^{c}	0.889 ± 0.128^{c}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$) within assays.ie. results with the same superscript are not significantly different.

3.4 Discussion

3.4.1 Effluent and EC/LC50

The toxic properties of the Burnie mill effluent were determined to ensure that the concentrations used in this series of experiments were not acutely toxic to any of the organisms. The main toxic property of the effluent, as measured by Microtox, was the pH. A reduction in toxicity was observed after the pH and salinity of the effluent had been adjusted to that of sea water and, in most cases, the effluent was not acutely toxic to *V. fisheri*. However, the effluent was acutely toxic to microalgae in low concentrations (as discussed in Chapter 2). The components of the Burnie mill effluent which caused the toxic effects are unknown.

The chemicals found in pine-based effluent that cause toxic effects, such as resin acids, TCDD and TCDF, have not been detected in the Burnie mill effluent. However, it has been suggested that chlorinated compounds, including phenols, guaiacols, catechols and syringols, are a major source of toxicity in pulp mill effluents (Stauber *et al.* 1994, Fisher *et al.* 1996). Chlorinated phenolics are the main source of chlorinated compounds in the Burnie mill effluent (see Chapter 2 and Appendix 1). The major effect of chlorophenolics on fish is the uncoupling of oxidative phosphorylation, in which the weak acid serves as a proton shuttle, collapsing the mitochondrial pH gradient necessary for ATP synthesis (Oikari *et al.* 1988). However, the assays performed on fish showed that up to 95% of chlorophenolic compounds were rapidly taken up from the water and were just as rapidly excreted with minimal effects, though some of these compounds and their metabolites can bioaccumulate (Oikari *et al.* 1988).

3.4.2 Growth

Growth of fish is a biomarker that is commonly used when assessing the impacts of toxicants, as decreased growth represents a chronic response to stress (Heath 1987, Mehrle *et al.* 1988, Holdway *et al.* 1995). This chronic response is evident in the decreased growth of fish in feral populations exposed to pine-based pulp mill effluents when compared to that of fish from reference sites (Anderson *et al.* 1987, Oikari *et al.* 1988, Munkittrick *et al.* 1991, Soimasuo *et al.* 1995). Growth of fish is also used as an endpoint in standard bioassay methods which were developed for use by regulatory agencies (U.S. EPA 1988). These U.S. EPA standard methods use only larval fish as test organisms, as this life-stage has been shown to be one of the most

sensitive to toxicant exposure (Sprague 1971, McKim 1977, Longwell 1988, von Westernhagen 1988, Holdway *et al.* 1995). As it was not possible to obtain larval common jollytails for use in this series of experiments juvenile fish were used in Assay 1 and adult fish were used in Assays 2 and 3.

As mentioned previously, Burnie mill effluent contains few of the chemicals found in pine-based pulp mill effluent which induce responses in exposed fish. The eucalypt-based effluent used for these studies has undetectable levels of TCDF and TCDD, which have been shown to reduce growth in rainbow trout (Mehrle *et al.* 1988, van der Weiden *et al.* 1994). Reduced growth can indicate responses to toxicants such as effects on assimilation efficiency, metabolic processing, metabolic pathways or maintenance costs (Webb and Brett 1972, Heath 1987, Lehtinen 1989). Elevated maintenance metabolic rates, which reduce the proportion of energy available for growth, have been associated with decreased growth of fish exposed to pine-based pulp mill effluent (Webb and Brett 1972).

Decreased growth was observed in juvenile fish from Assay 1 which were exposed to eucalypt-based pulp mill effluent via the dietary source and via the combined dietary and water-borne route (Table 3.3). These results may be an indication that the palatability or chemical composition of the feed may be affected. The appetite of the fish or alterations in metabolic processes influenced by organic chemicals from wood extracts that are present in the effluent may result in reduced food intake or assimilation (Soimasuo *et al.* 1995, Dube and Culp 1996). Growth results from Assays 2 and 3 are inconclusive as mortalities due to failure to spawn occurred in the exposed adult fish. These mortalities caused Assay 2 to be discontinued after seven weeks. Growth seems to be a useful biomarker for detecting exposure in laboratory studies providing the duration of the study is sufficient to record changes in growth of control fish and the fish are at either larval or juvenile life-stages. Further, increased mortalities of adult fish highlight the need to use juvenile fish for long term assays to monitor the sublethal effects of complex effluents.

In contrast to the results obtained in Assay 1, increased growth has been observed in fish exposed to low concentrations of pine-based pulp mill effluent in both field and laboratory studies (McLeay and Brown 1974, Soimasuo *et al.* 1995). In some instances this increase in growth has been attributed to an increase in food availability caused by enhanced algal productivity from

elevated nutrients (McLeay and Brown 1974, Haley *et al.* 1995, Soimasuo *et al.* 1995, Dube and Culp 1996). Other factors that may produce a stimulatory effect on growth such as; the presence of sitosterol, abietic resin acids or hormone analogs from pine used for pulping, have been suggested (McLeay and Brown 1974, Soimasuo *et al.* 1995, Dube and Culp 1996). Conversely, reduced growth has also occurred in fish exposed to pine-based pulp mill effluent in some studies (Oikari *et al.* 1988, Lehtinen 1989, Munkittrick *et al.* 1991, Soimasuo *et al.* 1995). Andersson *et al.* (1987) has shown that chlorophenolic constituents of pine-based effluent can reduce the growth rate of fish as well as affecting the food conversion efficiency. Chlorinated phenolics are known to cause uncoupling of oxidative phosphorylation, thus, causing an increase in metabolic rate which may adversely impact on growth (Webb and Brett 1972, Davis 1976). However, this effect is related to the concentration of chlorinated phenols as Oikari *et al.* (1984) showed that the addition of low levels had no effect on the energy metabolism of fish after three months of exposure.

3.4.3 Liver Index and Condition Index

Fish exposed to toxicants often show reduced food consumption, therefore, changes in liver and condition indicies can be associated with variations in glycogen and fat content related to the nutritional status of the fish (Heath 1987, Oikari *et al.* 1988). In this series of experiments consumption of exposed *Artemia salina* may account for the significant differences observed in LSI and condition indice (CI) in Assay 1 (Tables 3.4 and 3.5) as nutritional content or palatability of exposed *A. salina* may vary from the control *A. salina*. The condition indices for Assay 1 follow the same trends as the growth results with Group 4 fish showing significantly reduced growth and condition index. Condition index results for Assays 2 and 3 were inconclusive, most likely due to the effects of sexual maturation, and could not be related to the route of exposure. LSI results from fish in Assay 1 showed the same trends as growth and condition indicies, with lower values for Group 2 and Group 4 fish (Table 3.5). LSI was smaller in Group 4 fish in Assay 2 which were exposed by the combined routes. A reduction in liver size is in accord with results from Oikari *et al.* (1984) who showed that rainbow trout exposed to low levels of pine-based pulp mill effluent also had a decreased LSI. This is in contrast to results obtained by Andersson *et al.* (1987) which showed an increase in LSI of fish exposed to high concentrations of pine-based pulp mill effluent. The absence of glycogen in the livers of the exposed fish suggests that the fish had utilised their readily-available energy

stores as a result of reduced intake of food. However, toxic effects may also have been involved.

Liver somatic indices (LSI) in all assays indicated a trend towards smaller livers in exposed common jollytails, which is in accord with Oikari *et al.* (1984) who showed that rainbow trout exposed to low levels of pine-based pulp mill effluent also had decreased LSIs. Reduced liver size was also recorded in perch living in the receiving water of Iggesund (Sweden) pulp mill (Forlin *et al.* 1995). The reduction in LSI observed may be due to changes in nutritional status of the fish, as discussed previously, rather than direct toxic effects on the liver causing atrophy or severe malfunction of the liver (Forlin *et al.* 1995). This is in contrast to LSI of fish exposed to pine-based pulp mill effluent in other receiving waters which showed enlarged livers due to hepatocyte hypertrophy (Andersson *et al.* 1987, Forlin *et al.* 1995). An increase in LSI is usually associated with toxicant-induced metabolic disturbances; including increased fat storage or stimulated protein synthesis (Forlin *et al.* 1995). However, no changes in liver indices were reported by Hall *et al.* (1992) in rainbow trout exposed to various levels of biologically treated pine-based effluent for 42 months. Also, mountain whitefish showed no differences in liver or condition indices after exposure to pine-based pulp mill effluent (Kloepper-Sams *et al.* 1994). The apparently contradictory results obtained for LSI and CI indicate that these are probably inappropriate for use when assessing the effects of low levels of either pine-based or eucalypt-based pulp mill effluent.

3.4.4 EROD Activity

Biochemical biomarkers have previously been used to detect exposure to eucalypt-based pulp mill effluent in a series of experiments by Munday *et al.* (1991). Common jollytails exposed to Burnie mill effluent at concentrations of 0, 0.5 and 5% for three months showed a non-significant trend towards an increase in ECOD (ethoxycoumarin O-deethylase) activity (Munday *et al.* 1991). However, EROD activity has since been shown to be a more sensitive method for detecting MFO activity (Ahokas *et al.* 1994) and consequently was used in this series of experiments.

The EROD results for the live feed experiments, Assays 1, 2 and 4 show that there were no significant differences between control fish and fish exposed by only one route (Figure 3.6). However, EROD results from those experiments showed that activity of Group 4 fish was

significantly different from all other groups. A three to four fold increase in EROD activity in Group 4 fish was observed, indicating that the effects of the combined routes of exposure appear to be synergistic rather than additive. These results contrast to those of Assay 3, in which the common jollytails were fed an artificial diet. The Assay 3 results show that the two fold EROD induction in Groups 3 and 4 fish was entirely due to water-borne exposure. This is the first instance where a statistically significant increase in EROD induction has occurred in response to exposure to eucalypt-based effluent via the water column only (Munday *et al.* 1991, Brumley *et al.* 1996). Further, this study has shown that exposure to 30% eucalypt-based effluent via the water column will not induce EROD activity within 96 hours. Therefore, time, concentration of effluent and method of exposure all need to be considered when assessing EROD induction due to eucalypt-based pulp mill effluent.

The EROD results obtained in all of the experiments were very low when compared with induction in common jollytails by benzo-a-pyrene (BaP) (Woodworth and Helders, unpublished). However, BaP is known to be a potent EROD inducer (Levine *et al.* 1994), whereas previous work has shown that eucalypt-based pulp mill effluent is not a good EROD inducer when fish are exposed by only one route (Munday *et al.* 1991, Brumley *et al.* 1996). Differences in levels of EROD induction between the BaP study and this study can also be attributed to the methodology used. One major difference in the preparation of the livers for the EROD assay between the BaP study and this study was that, due to time constraints, the livers were frozen whole overnight, at -80°C, prior to being homogenised and assayed the next day. According to Monod and Vindimian (1991) freezing the livers prior to homogenising results in approximately a 50% loss of EROD activity. Also, in this study the S9 fraction of the homogenate was used for the EROD enzyme assay. It has been shown that by using ultracentrifugation to purify the microsomes the sensitivity of the assay is increased by three times (O'Hare *et al.* 1995). Even so, it has been shown that the use of the S9 fraction is an acceptable method for measuring EROD activity (Monod and Vindimian 1991). Therefore, theoretically, the method used in this study was six times less sensitive than that used by Woodworth and Helders (unpublished).

The results obtained in this series of experiments have implications in relation to the methodologies used for assessing the effects of any complex effluent. By exposing the fish to the effluent by only one route, the impacts of the effluent may be under-estimated. One

example of this effect is that, as mentioned previously, Brumley *et al.* (1996) obtained no EROD induction in sand flathead exposed to 0.5, 2 and 8% simulated eucalypt-based pulp mill effluent by only one route for 96 hours. Consequently, Brumley *et al.* (1996) concluded that EROD was not an appropriate biomarker for exposure of eucalypt-based pulp mill effluent. Work presented in this thesis supports that conclusion for a 96 hour exposure. As mentioned previously, EROD activity was not induced in common jollytails in 96 hours by water-borne exposure to 30% eucalypt-based pulp mill effluent. Therefore, results obtained in laboratory studies which expose fish to complex effluents via the water column only may not give a true indication of effects that may be occurring in the environment. This is evidenced by an increase in EROD activity detected in red cod living in the vicinity of a Tasmanian pulp mill and a busy port (Munday and Brand 1992) and also in sand flathead collected from industrialised areas of Port Phillip Bay, Victoria (Holdway *et al.* 1994).

The stage of maturation and the sex of the fish have been reported to influence EROD activity in several fish species (George *et al.* 1990, Levine *et al.* 1995, Lindstrom-Seppa and Stegeman 1995, O'Hare *et al.* 1995, Fisk *et al.* 1997). The presence of estradiol in maturing female fish suppresses EROD induction, possibly due to a mechanism to ensure sufficient estrogen levels to initiate and maintain vitellogenesis (George *et al.* 1990, O'Hare *et al.* 1995). Also, George *et al.* (1990) reported two to four fold higher EROD activity in male plaice than in immature females, indicating that there may also be a seasonal increase in male EROD activity. To determine if the EROD activity in common jollytails was sex dependant, the sex of each fish was statistically analysed in combination with the EROD activity. The analysis did not show significant differences between induction in male and female fish in any assay (Appendix 3). Further, due to the influence that sexual maturity has on EROD activity inter-assay comparisons cannot be performed as the fish in each assay were at different stages of maturity. Also, interspecies differences occur (George *et al.* 1990, Stein *et al.* 1993, Kloepper-Sams and Benton 1994). This means that the results from the common jollytail and the blenny assays cannot be directly compared with each other. Even so, results obtained from this series of experiments indicate that EROD activity is a potential biomarker for the detection of exposure to eucalypt-based pulp mill effluent under appropriate conditions. Also, the results indicate that both the dietary route and the water-borne route are of equal importance in the induction of EROD activity.

EROD induction in fish exposed to pulp mill effluent has been mainly attributed to the presence of TCDDs and TCDFs (Holdway *et al.* 1993, Parrott *et al.* 1995, Soimasuo *et al.* 1995b). If these compounds were responsible for the responses to the eucalypt-based pulp mill effluent they must have been acting at concentrations below the level detectable by analytical methods in use in 1996 (see Appendix 1). The low levels of EROD inducers present in the effluent would explain the lack of induction in fish exposed via only one route. These results indicate that further research needs to be undertaken to determine if the length of exposure or the concentration of the effluent has the most influence on EROD induction in response to eucalypt-based pulp mill effluent. Also, chemical analysis of the fish's liver to detect the presence of these types of EROD inducing compounds would be helpful. For instance, Fisk *et al.* (1997) has found that liver tissue concentrations of TCDD correlate closely with EROD induction.

The specific compounds in pulp mill effluent which induce EROD activity in exposed fish have not yet been fully identified (Williams *et al.* 1996, Parrot and Tillitt 1997). However, Williams *et al.* (1996) speculated that EROD inducers were mainly generated from lignin liberated during the bleaching or pulping processes. Certain chlorinated compounds that are produced during these processes at the Burnie mill, the chlorinated phenolics including guaiacols and catechols, are not recognised as EROD inducers. While these chemicals are readily taken up by the fish, the majority are also rapidly excreted after conjugation with glucuronic acid (Kloepper-Sams and Benton 1994, Tana and Lehtinen 1996, Williams *et al.* 1996). Further, the intraperitoneal injection of several types of chlorinated phenolics including 4,5,6-trichloroguaiacol and tetrachloroguaiacol (<100 mg/kg body weight) did not induce EROD activity (Forlin *et al.* 1989). However, exposure to high levels of these compounds can induce low EROD activity and skeletal deformities in fish (Bengtsson *et al.* 1989) but chronic exposure to low levels does not alter physiological parameters (Tana and Lehtinen 1996). Therefore, the chlorinated phenolic compounds found in the Burnie mill effluent are unlikely to have had an effect on EROD activity and induction was most probably caused by the presence of undetectable levels of PCDDs and PCDFs or other unidentified constituents of the effluent.

3.4.5 Cytochrome P4501A

Induction of cytochrome P4501A1 in fish has proved to be one of the most sensitive indicators for evaluating the effects of pine-based pulp mill effluent (Huuskonen and Lindstrom-Seppa

1995). The indirect ELISA method for detecting P4501A1 induction, using either a polyclonal antibody against cod P-4501A1 IgG (Goksoyr 1991) or a monoclonal antibody against scup P-4501A1 Ig MAb 1-12-3 (Stegeman *et al.* 1985), has been used to show increases in fish exposed to toxicants in the laboratory and fish from contaminated communities (Courtney *et al.* 1993, Goksoyr *et al.* 1994, Lindstrom-Seppa *et al.* 1994). These fish also showed high levels of EROD activity. Woodworth and Helders (unpublished) also demonstrated a positive correlation between EROD activity and cytochrome P4501A1 induction in common jollytail exposed to benzo-a-pyrene (40mg/kg body wt). However, an indirect ELISA method using the scup monoclonal antibody did not detect induction of cytochrome P4501A1 in any of the common jollyails or blennies exposed to the eucalypt-based pulp mill effluent. However, the positive controls, collected from common jollytails injected with benzo-a-pyrene (40 mg/kg body wt) gave high levels of P4501A1 induction, as measured by optical density at 492 nm, (OD 0.885) and high EROD activity (352 pM/min/mg protein). Apparently, the ELISA technique is not sufficiently sensitive to detect differences in low levels of cytochrome P4501A1 induction (Stegeman pers. comm.). This lack of sensitivity of the ELISA technique is supported by Collier *et al.* (1995) who obtained P4501A1 ELISA values very much lower than the values obtained for either of the EROD or AHH enzyme assays. Also, Collier *et al.* (1993b) did not detect any increase of cytochrome P4501A1 in toadfish exposed to environmental contaminants, but detected small increases in EROD activity. Lack of cytochrome P4501A1 activity has also been reported when severe hepatocyte damage, such as increase in hepatic neoplasia, has occurred (Collier *et al.* 1992, Soimasuo *et al.* 1995b). However, histological changes of that type were not observed in the common jollytails or blennies and would rule out hepatocyte damage as an influencing factor.

On the basis of this series of experiments, an indirect ELISA technique using a monoclonal antibody against scup P4501A1 Ig MAb 1-12-3 does not seem to have sufficient sensitivity to detect P4501A1 induction in common jollytails or Tasmanian blennies exposed to low levels of eucalypt-based pulp mill effluent. The results indicate that the ELISA technique is not sensitive enough to detect small increases in P4501A1 induction and its use as a biomarker for detection of low level exposure to eucalypt-based pulp mill effluent is limited. However, these antibodies may be effective in detecting the presence of P4501A1 in the tissues of exposed fish using immunohistochemistry techniques (Collier pers. comm.) or Western blots (Goksoyr *et al.* 1991).

3.4.6 Histopathology and Electron Microscopy

Histopathology and transmission electron microscopy (TEM) of livers from exposed fish showed a significant increase in the number of hepatocytes with large nuclei or twin nuclei in fish exposed to the effluent via the combined dietary and water-borne routes. Common jollytails previously exposed to 0.5% and 5% effluent for the same period of time also exhibited binucleate hepatocytes (Munday *et al.* 1991). These types of histopathological changes have also been observed in caged fish exposed to blue-green algal (cyanobacterial) toxins (Kent *et al.* 1988) and in English sole from Puget Sound (Myers *et al.* 1990). Kent *et al.* (1988) has suggested that these cells are sublethally injured but are able to survive for months.

The histopathological liver changes observed in this study are less severe than those usually described for fish exposed to pine-based pulp mill effluents. Fish exposed to pine-based pulp mill effluents commonly show pycnotic and displaced nuclei and vacuoles within the hepatocytes which indicate changes in liver function (Lehtinen and Oikari 1980, Heath 1987, Bucher *et al.* 1992, Khan *et al.* 1994, Teh *et al.* 1997). However, Kloepper-Sams *et al.* (1994) reported that the only histopathological changes observed in fish exposed to pine-based pulp mill effluent in receiving waters was the presence of vacuoles within the hepatocytes. These types of vacuoles have also been observed in the Tasmanian blennies exposed to the eucalypt-based effluent in this study and that of Deavin (1993). Fisk *et al.* (1997) have found vacuolisation within hepatocytes and loss of glycogen content in fish exposed to TCDD. This is additional evidence to suggest that low levels of dioxins may be present in Burnie mill effluent and TCDD may be accumulating in fish livers, causing similar effects in common jollytails and blennies.

The proliferation of rough endoplasmic reticulum (RER) within the cytoplasm of exposed fish has been associated the detoxification process as measured by EROD activity (Kohler 1989, Moore 1992, Engwall *et al.* 1994). Proliferation of RER has also been linked to enlarged hepatocytes and increased liver somatic index (Hinton and Lauren 1990). However, even though proliferation of RER was observed in the common jollytails, the liver indices for each treatment were not significantly different and any changes were mainly due to the nutritional status of the fish. The absence of RER proliferation in control common jollytails and in fish exposed to the effluent by only one route indicated that a synergistic effect of effluent constituents may have occurred due to the combination of dietary and waterborne exposure. The histopathology and

transmission electron microscopy results from this study indicate that both methods are suitable and sensitive for use as biomarkers to detect exposure of fish to low concentrations of eucalypt-based pulp mill effluent.

3.4.7 Steroid Analysis

Histopathology results from a previous study (Deavin 1993) showed that the Burnie mill effluent had an adverse effect on the reproductive abilities of a blenny population living in Emu Bay. Females from that population possessed gonads with an increased number of atretic oocytes and smaller oocyte size, indicating reduced steroid levels (Donaldson 1990, Adams *et al.* 1992, Holm *et al.* 1994, McMaster *et al.* 1994). Therefore, it was decided to determine the steroid levels in blenny gonads from blennies used in Assay 4. The methodology used for determining testosterone and estradiol levels in Tasmanian blennies has not been validated, however, methodology has been validated for other fish species. Also, small gonad sizes made accurate determination of steroid levels difficult (C. Barnett pers. comm.), therefore, the results obtained from the testosterone and estradiol analysis may not be precise. However, the results were analysed using ANOVA which showed that male and female blennies from Assay 4 exhibited no significant differences in estradiol or testosterone levels. This result was most likely due to the variability of the data. However, there was a trend in the male fish exposed to the effluent via both routes to show increased testosterone, but, due to the sample size, which was too small to show significant results when dealing with such variable data, this trend was non-significant.

As the blennies are multiple spawners, spawning every 3 - 5 days, the levels of gonadal steroids will vary with the stage of sexual maturity (Donaldson 1990). Therefore, the stage of the gonads was also included in the ANOVA when analysing the steroid results. However, statistical analysis showed that steroid levels were not significantly related to stage of gonadal development. The results from the steroid analysis are inconclusive due to the small sample sizes and the variability in the data (which may, in part, be due to lack of material for analysis). Therefore, in order to obtain a better understanding of eucalypt-based pulp mill effluent on steroid levels in fish the use of larger sample sizes, a different fish species and a longer exposure period are recommended for any further laboratory studies.

Because female blennies from a previous study showed adverse affects on reproductive abilities, as mentioned above (Deavin 1993), it would be appropriate to test the steroid levels of blennies

living in Emu Bay. This would give a better indication of the effects of Burnie mill effluent on steroid levels, as McMaster *et al.* (1992) showed that confinement and handling stress can change steroid levels. Also, there was no correlation between steroid levels and EROD activity in the blennies, which is in agreement with the findings of Munkittrick *et al.* (1992).

The results from this experiment indicate that measurement of steroid levels is not an appropriate biomarker for use with blennies in laboratory studies of exposure to low levels of eucalypt-based pulp mill effluent. Several limitations of laboratory based studies, which may have affected these results, are evident when assessing steroid levels in fish. The results indicate that the duration of exposure to low levels of the effluent (seven weeks) may have been insufficient to induce changes or the concentration of effluent was too low for changes to be induced, the sample size, especially the number of male fish tested, was too small and confinement and handling stress may have confounded results. Also, as shown, the multiple spawning biology of blennies make correlation of steroid results difficult, a species of fish which spawns only yearly may be more suited to laboratory-based steroid experiments. However, the use of steroid analysis as a biomarker of exposure to pine-based pulp mill effluent of feral populations gives meaningful results and, consequently, may be suitable for testing feral fish populations exposed to eucalypt-based pulp mill effluents. Certainly, further studies on this aspect are warranted.

3.4.8 96 Hour Assays

Both EROD activities and histopathology results from the 96 hour live feed assay showed that there were no significant differences between the control fish and the fish exposed to 2.5% effluent via the food, water column or the combined routes. A further two 96 hour assays were performed to determine the level of effluent that was required to induce EROD activity within that time frame and without causing mortalities. The maximum concentration of effluent used was 30% which caused slight histopathological changes in the livers of the exposed fish in both assays. As the fish were not fed for the duration of these assays the fish exposed to the effluent metabolised all their glycogen stores while the control fish still exhibited some glycogen storage after 96 hours. This result indicates that the exposed fish required more energy reserves to cope with the pulp mill effluent as has been described by Hinton and Lauren (1990).

Results from all of the 96 hour assays showed no relationship between route of exposure or effluent concentration and EROD induction. However, Assay 3 did show a 50% reduction in

EROD activity in the 10% and 20% treatments (Table 3.10). This is difficult to explain as a reduction in EROD activity is usually related to hepatocyte injury or death, thereby rendering the cell unable to produce the enzyme (Hinton and Lauren 1990, Kloepper-Sams and Benton 1992). The reduction in EROD activity in Assay 3 cannot be attributed to hepatocyte damage as histopatholgy results (Table 3.11) show that no significant liver damage was caused by eucalypt-based pulp mill effluent exposure during the assay.

As EROD was induced in one of the chronic assays by the waterborne route and not significantly induced in the short term assays, it would seem that 96 hours is insufficient exposure time to induce EROD activity in response to eucalypt-based pulp mill effluent. Also, as mentioned previously, Brumley *et al.* (1996) did not induce EROD activity in that time period with waterborne exposure to simulated eucalypt-based pulp mill effluent. However, 96 hours is sufficient time to produce changes to hepatocytes as detected by histopathology. Therefore, histopathology is a more sensitive biomarker of short-term exposure to eucalypt-based pulp mill effluent than EROD activity.

3.5 Conclusions

Results from this series of experiments show that growth, condition index and liver somatic index are appropriate parameters for detecting impacts of eucalypt-based pulp mill effluent in laboratory studies using juvenile fish. However, these parameters may be appropriate for use with adult fish if exposure duration is prolonged and the experiments are not perturbed by physiological changes such as sexual maturation. The growth, condition and liver indice results for juvenile common jollytails from Assay 1 indicate that the dietary route has more influence on these parameters than the water-borne route.

Results obtained indicate that eucalypt-based pulp mill effluent has a minor effect on fish at concentrations that are found in the receiving water. Fish exposed via the combined routes (dietary and water-borne) showed minimal changes in liver structure and ultrastructure, a three to four fold increase in ethoxyresorufin-O-deethylase (EROD) activity and no detectable elevation of cytochrome P4501A1. EROD induction, histopathology and ultrastructure of liver were proven to be useful biomarkers for the detection of exposure to low levels of eucalypt-based pulp mill effluent in laboratory studies. These biomarker results show that water-borne or dietary exposure alone is not statistically distinguishable from that of unexposed fish and

that the combined routes may cause significant changes in these biomarkers. Further, and most importantly, the results obtained from this series of bioassays have implications in relation to the methodologies used for assessing the effects of any complex effluent. By exposing the fish to the effluent via only one route the impacts of the effluent may be under-estimated in many instances.

In addition to the assessment of the use of biomarkers for determining the effects of exposure to eucalypt-based pulp mill effluent, the results obtained in this series of bioassays indicate that there are potential impacts on fish populations in the receiving waters. Fish populations that are exposed to the Burnie mill effluent in concentrations that are found in Emu Bay will show sublethal effects such as; liver damage and increased EROD activity, and that these impacts will increase with exposure time. Deavin (1993) has shown that resident blenny populations in Emu Bay do exhibit adverse sublethal effects after long term exposure to the effluent and further studies are required on these and other fish populations. These further studies should involve EROD, testosterone and estradiol analyses and a chemical analysis of fish livers to detect and identify effluent constituents that may induce EROD activity.

CHAPTER 4

EXPOSURE OF TASMANIAN BLENNY (*Parablennius tasmanianus*) PARENTS AND EMBRYOS TO PULP MILL EFFLUENT

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4.1. Introduction

The early life history stages of fish are known to be more vulnerable to environmental changes than either adults or juveniles (Sprague 1971, McKim 1977, Longwell 1988, von Westernhagen 1988). Therefore, it follows that embryonic and larval development of fish are good indicators of the quality of the aquatic environment. The development of aquatic organisms in the vulnerable early life stages may be impacted by an increase of toxicants in the environment caused by spills, agricultural runoff and deliberate discharges into the aquatic environment (Klumpp and von Westernhagen 1995). The effects of these toxicants may cause high mortalities or be responsible for sublethal effects in the exposed embryos and larvae (Walker *et al.* 1991, Hall *et al.* 1993). These sublethal effects may be cellular, morphological or physiological and may result in a reduced survival potential (Rosenthal and Alderdice 1976, von Westernhagen 1988). Consequently, as larval mortality is naturally high, any increases in mortality due to environmental changes or presence of toxicants is potentially detrimental to the population (von Westernhagen 1988).

Larval viability has been shown to be adversely affected by exposure to eucalypt-based pulp mill effluent in a study by Deavin (1993). Results from that study showed that Tasmanian blenny embryos exposed to pulp mill effluent concentrations of greater than 2.5% had higher mortality at hatch, decreased growth and increased morphological abnormalities which would preclude swimming and feeding. Some of the abnormalities observed in that study included spinal curvature, jaw deformities and craniofacial deformities and the severity of these effects increased with higher pulp mill effluent concentrations. These types of abnormalities are common effects of toxicant exposure but can not be specifically related to a single compound (Hose *et al.* 1981, Smith *et al.* 1991). These effects are indicative of changes to the metabolism of the embryo, possibly on specific hormonal or enzyme systems, and can reduce the survival potential of the larvae (McKim 1985, Lye *et al.* 1997). Environmental stressors such as salinity, dissolved oxygen, pH and temperature will also

have an effect on larval viability (Rosenthal and Alderdice 1976, von Westernhagen 1988). However, these environmental stressors will usually not impact on a laboratory-based study because of controlled environmental conditions but they must be taken into account when performing field-based assessments of larval development.

Larval viability and egg quality have also been shown to be impaired by the exposure of female fish to organic contaminants in several studies, as lipohpilic organic compounds can bioconcentrate within the liver and then be transfered to the reproductive tissues (Bresch 1982, Johnson *et al.* 1988, Collier *et al.* 1993, Lye *et al.* 1997). Adams *et al.* (1992) suggests that female reproductive impairment is the main mechanism through which pulp mill effluents affect reproduction in fish populations. This may either be due to mutagenic or teratogenic effects caused by components of the effluent or impairment of the parent's reproductive system due to the bioconcentration of lipophillic organochlorines (Adams *et al.* 1992, Lye *et al.* 1997). However, a combination of these is the most likely method affecting the reproduction of fish.

The reproduction of female Tasmanian blennies in Emu Bay populations has been shown to be adversely affected by exposure to eucalypt-based pulp mill effluent (Deavin 1993). The reproductive potential of Emu Bay populations was shown to be affected as the female blennies showed an increase in the number of atretic oocytes within the gonad and a shorter spawning season when compared to control sites. The results obtained from that study prompted a study to assess the viability of larvae from parent fish from Emu Bay which had been continuously exposed to the pulp mill effluent. To assess this, a laboratory-based study took place at the University of Tasmania in 1993/1994 (Munday and Deavin 1994). This study involved exposing a pair of Tasmanian blenny adults, captured in Emu Bay, and their resulting embryos to the same concentration of pulp mill effluent as that found at the capture site. The study indicated that parental exposure to eucalypt-based pulp mill effluent increased the number of resulting larvae exhibiting sub-lethal effects when compared to larvae exposed as embryos from unexposed parents (Deavin 1993). The type and number of sublethal effects occurring in larvae from exposed parents would adversely affect the survival potential of the larvae and, possibly, the population to which they belonged. As this initial study exposed only one pair of blenny adults, the results obtained were inconclusive. A new study was initiated to compare

viability of resulting larvae from Emu Bay blennies from several sites, with varying pulp mill effluent concentrations, and blennies from Bridport as the control fish.

The Tasmanian blenny (*Parablennius tasmanianus*) was selected as the species for both field studies (Deavin 1993) and laboratory studies (Munday and Deavin 1994) as the blenny fulfils all of the requirements for use as a bioassay organism as outlined by Rand and Peterocelli (1985). It is a common cryptobenthic inhabitant of rocky shorelines and is widely distributed around the coastline of Australia (Cook 1986, Gaughan *et al.* 1990). The blenny lives in the littoral and sublittoral zones in coastal areas, the habitat most at risk from environmental contaminants. The blenny is an appropriate representitive of the ecosystem in Emu Bay as the fish are sedentary bottom dwellers and the males are territorial during the breeding season, so that the movements of the fish are restricted to a relatively small area (Cook 1986, West 1988).

The reproductive biology of the blenniids has been well studied in terms of breeding cycle, induced spawning and egg and larval development (Jillet 1968, Patzner 1983, von Westernhagen 1983). The blennies are multiple spawners with the females spawning every 3 - 5 days, laying the eggs in a single layer on the inside surface of the male's nest during the spring / summer spawning period. The Tasmanian blenny has proven to be amenable to laboratory handling and is relatively easy to induce to spawn under laboratory conditions (Cook 1986, West 1988, Munday and Woodworth unpublished). Previous work by Munday and Woodworth (unpublished) has shown that the blennies adapt quickly to laboratory conditions and spawning has been induced, in winter, within four weeks of collection.

Several studies have exposed fish embryos to pine-based pulp mill effluent (Burton *et al.* 1983, Vuorinen and Vuorinen 1987, Kovacs *et al.* 1995a, Kovacs *et al.* 1995b) and effluent constituents (Prince and Cooper 1995, Zabel *et al.* 1995). The results of these studies indicate that the time at which the embryos are first exposed to the effluent is important. The embryo has been shown to be more tolerant of toxicant exposure than other early life-stages as the chorion and perivitelline fluid protect the fish embryo, to a certain extent, from water borne toxicants (Rosenthal and Alderdice 1976). The chorion is a protective membrane which acts as a physical barrier to the influx of some chemicals while it acts as a semipermeable membrane for exchange of respiratory gases thereby facilitating osmoregulation (Heath 1987, Weis and Weis 1989). However, if embryos are exposed to water-borne contaminants prior to the formation of the

perivitelline space, the contaminants may be able to enter with the water through the pores of the chorion. This happens due to water uptake occurring prior to the hardening of the chorion and the formation of the perivitelline space (von Westernhagen 1988). This effect is evidenced by work by Burton *et al.* (1983) where striped bass embryos, which were exposed to pulp-mill effluent 6 hours after fertilisation, showed a hatch rate of 58% and the same species first exposed to the effluent at 24 hours after fertilisation showed a 96% hatch rate. Further evidence of uptake of pulp mill constituents through the chorion is demonstrated by Vuorinen and Vuorinen (1987), who exposed adult brown trout to 0.2% pine-based pulp mill effluent and incubated the resulting embryos in the same concentration of effluent. Upon hatch the larvae showed reduced length and a slower heart rate than control larvae. However, larvae from the exposed parents which were incubated in clean water showed no differences from control larvae.

To determine the impacts of eucalpyt-based pulp mill effluent on the reproduction and embryonic development of Tasmanian blenny populations living in several areas of Emu Bay an experiment was designed which utilised fish from selected populations within the bay. The experiment was designed to simulate conditions occurring in Emu Bay in areas where the blenny populations are located. Adult male and female blennies from sites along the effluent discharge gradient were collected and transported to the laboratory where they were placed in aquaria containing the concentration of effluent at the collection site (Environmental Consulting Engineers 1991). The fish were allowed to spawn naturally and the embryos were placed into effluent concentrations the same as the parent fish, to simulate conditions at each sample site. At hatch several parameters were measured and the larvae were assessed for any morphological abnormalities. The aim of this experiment was to simulate conditions occurring in Emu Bay to determine if any effects on larvae of Emu Bay parents can be attributed to effluent exposure.

4.2. Materials and Methods

4.2.1 Facilities

All experiments were conducted in the temperature controlled toxicology laboratory (17 ± 0.5°C), with a light regime of 16 hours light / 8 hours dark, at the National Key Centre for Teaching and Research in Aquaculture at the University of Tasmania (hereafter called the Aquaculture Centre).

4.2.2 Pulp Mill Effluent

Pulp mill effluent was collected from the discharge sewer of the Australian Paper mill at Burnie prior to entering Emu Bay. Twenty litre polypropylene containers of effluent were transported to the Aquaculture Centre approximately every three weeks. The effluent was stored at 4°C. The pH (10.3 - 11.5) and salinity (0 ppt) of the effluent were not adjusted. The colour of each batch of effluent was dark brown. The acute toxicity of three batches of effluent were assessed with Microtox.

4.2.3 Dilution Water

Sea water was collected from an unpopulated site in north-eastern Tasmania and transported to the laboratory in a stainless-steel road tanker. At the laboratory it was stored in a 22,000 litre concrete tank. The sea water was passed through a sand filter prior to use and the salinity was 33 ppt and pH 8.0 - 8.2. This water was utilised as both the control and dilution water.

4.2.4 Collection Of Tasmanian Blenny Adults

Tasmanian blenny adults were collected from several sites in Emu Bay, Burnie, Tasmania and a control site in north-eastern Tasmania in April, May, June and November 1994. The Emu Bay sites are locations of blenny populations living in the vicinity of the pulp mill outfall. The effluent concentrations used in this experiment are representative of concentrations found at those sites as measured by Consulting Environmental Engineers (1991) (Table 4.1 and 4.2 and Figure 4.1).

Table 4.1 Blenny Collection Sites

Site	Location
Emu Bay	Wharf 1 km west of the mill outfall
Emu Bay	Yacht Club 500 m west of the mill outfall
Emu Bay	Round Hill Point 1.5 km east of the mill outfall
Bridport (Control)	Brid River 100 m from the river mouth

Sample sites are shown in Figures 2.2 and 4.1.

Blennies were caught using fish traps and some were netted in rock pools. Three pairs of adult blennies (4.33 ± 2.4g) were taken from each site and transported live to the Aquaculture Centre.

The fish were transported to the Aquaculture Centre in 60 L plastic-lined bins with no aeration. Once at the Aquaculture Centre the fish were placed in 20 L glass aquaria which contained two 10 - 15 cm lengths of PVC pipes (50 - 80 mm diameter) cut in half longitudinally for use as nests. The aquaria were set up as static systems consisting of under gravel filters with airlifts which helped to circulate water through the aquaria. Washed gravel approximately 3-4 cm deep covered the under-gravel filters and sand-filtered sea water was used to fill the aquaria. Water changes of approximately 50% were performed daily. The fish were fed daily with diced squid and prawn meat at 2% fish body weight / day. Any uneaten food was siphoned from the aquaria during water exchanges. All fish from the second and third collections from each site were placed in a 40 L aquarium, ie. three pairs of adult fish were placed in the same aquarium. The 40 L aquaria were set up in the same way as the 20 L aquaria.

Dissolved oxygen concentrations were maintained at >7.0 ppm using aeration and was measured daily using a WTW OXI 96 dissolved oxygen meter. The laboratory temperature was controlled at 17 ± 0.5°C by two reverse-cycle air conditioners. Water pH (pH 8.0 - 8.2) and temperature were measured daily using a Hana HI 8424 pH / temperature meter. Salinity, 33 ± 1 ppt, was measured with an Atago model S-10 refractometer to the nearest

1.0 ppt. Lighting consisted of fluorescent lights with a photoperiod of 16 hours light and 8 hours dark.

4.2.5 Exposure Method

All effluent exposure concentrations were based on the plume distance from the effluent outlet as measured by Consulting Environmental Engineers (1991). Pulp mill effluent concentrations to which the adult and embryonic blennies were exposed are shown in Table 4.2. Effluent collected from the mill was added to the treatment aquaria to obtain the concentrations listed in Table 4.2. Effluent was added to dilution water in each aquaria after water exchanges to maintain the appropriate concentration for each site.

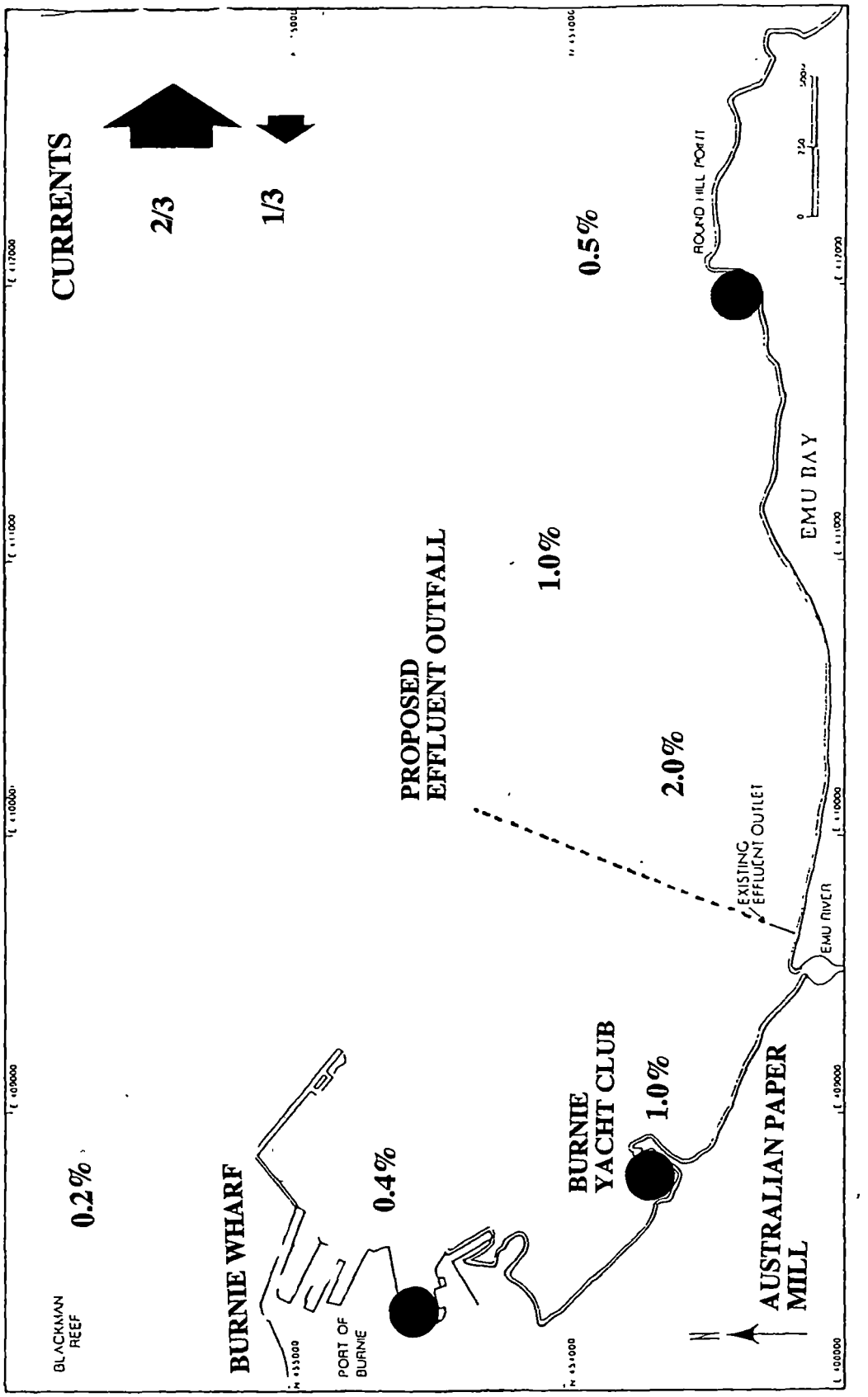
To induce spawning the blennies collected in April/May were placed in aquaria with pulp mill effluent of the appropriate concentration for that site and maintained at 10 ± 1°C for two weeks prior to the temperature being increased to 17 ± 0.5°C for the remainder of the study. The blennies trapped in June and November were allowed to spawn without being subjected to temperature changes. Any fertilised eggs were removed from the aquaria and placed in an embryo exposure chamber (Deavin 1993) in the same concentration of pulp-mill effluent as the parents.

Table 4.2 Effluent Concentrations

Site	Pulp Mill Effluent Concentration %
Bridport	0
Burnie Wharf	0.4
Round Hill Point	0.5
Burnie Yacht Club	1.0

Figure 4.1

Map of Emu Bay at Burnie Showing Location of Sample Sites and Effluent Concentrations in the Bay



4.2.5a Embryo Exposure Chamber

Fertilised eggs were maintained in embryo exposure chambers consisting of PETE plastic containers, holding 1 L of test solution. Aeration was provided for the embryos and they were maintained in the exposure chambers until hatching. Each test solution was exchanged at a rate of 100% daily.

4.2.6 Parameters Monitored

Upon hatching the larvae were anaesthetised in 20 ppm benzocaine, then measured using an Olympus dissecting microscope with an eyepiece graticule to measure to the nearest 0.1mm. The larvae were next observed under a Leitz dissecting microscope for morphological abnormalities and photographed. The larvae were then fixed in Davidson's fixative for future reference (Handlinger 1990b).

The number of days to hatch for each batch of embryos was recorded. Also, 20 larvae from each days hatch in each exposure chamber were anaesthetised and a number of parameters of the newly hatched larvae were monitored. The parameters monitored were:

- Length of larvae at hatch, mm,
- % larvae with large yolk sacs,
- % larvae exhibiting a snub nose effect,
- % larvae displaying spinal curvature,
- % larvae manifesting jaw deformities
- % larvae with eye deformities.

4.2.7 Statistical Analysis

All percentage data was arcsine transformed prior to analysis. All data were analysed using Bartlett's test to determine that variances were equal. If the data failed the Bartlett's test the non-parametric analysis of Kruskal-Wallis was used. If the data showed homogeneity of variance the data were then analysed using a one way analysis of variance (ANOVA) and tested for significance at the 95% level using Tukey-Kramer HSD.

4.3 Results

4.3.1 Acute Toxicity

The batches of pulp mill effluent used in the experiment were all of similar toxicity and the toxicity did not decrease with storage (Table 4.3). Further information on acute toxicity of pulp mill effluent can be found in Chapter 2.

Table 4.3

Microtox Results Of Pulp Mill Effluent

Date	Storage	pH	EC50 % Effluent v/v
July 1994	3 weeks @ 4°C	11.4	18
August 1994	24 hours @ 4°C	10.9	18
January 1995	24 hours @ 4°C	10.4	15

4.3.2 Spawning of Tasmanian Blennies

Fish from all sites spawned successfully, with approximately 25% of all the 6500 resultant larvae analysed (Table 4.4). There was a significant difference in the number of larvae per spawn between Burnie Wharf blennies and the blennies from the control site (Table 4.5). Also, Burnie Wharf blennies had a smaller number of larvae per spawn than the other Emu Bay sites, but the differences were not significant due to the large variations between spawns. The quality of the embryos from the Burnie fish was inferior to that of control fish as fertilisation was reduced; see Figures 4.2 and 4.3 in which green eggs indicate fertilization and yellow or clear eggs were unfertilized. In Figure 4.3, Round Hill Point (0.5% effluent), approximately 20% of the eggs were unfertilized, whereas the control fish showed 100% fertilization.

Figure 4.2

Developing Embryos from Control Parents
(Approx. 0.75 actual size)

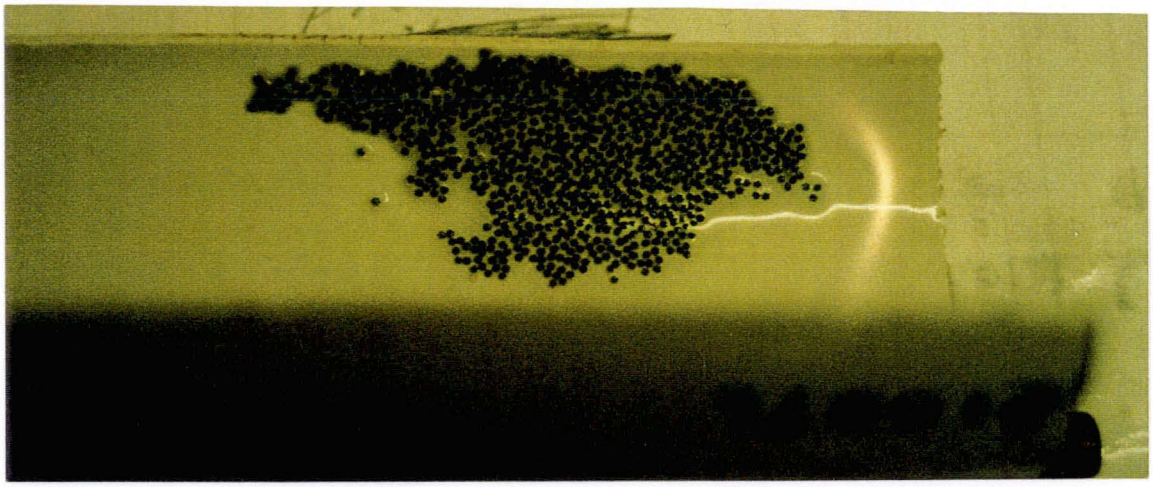


Figure 4.3
Developing Embryos from Round Hill Point Parents (0.5% effluent)
(Approx. 0.75 actual size)

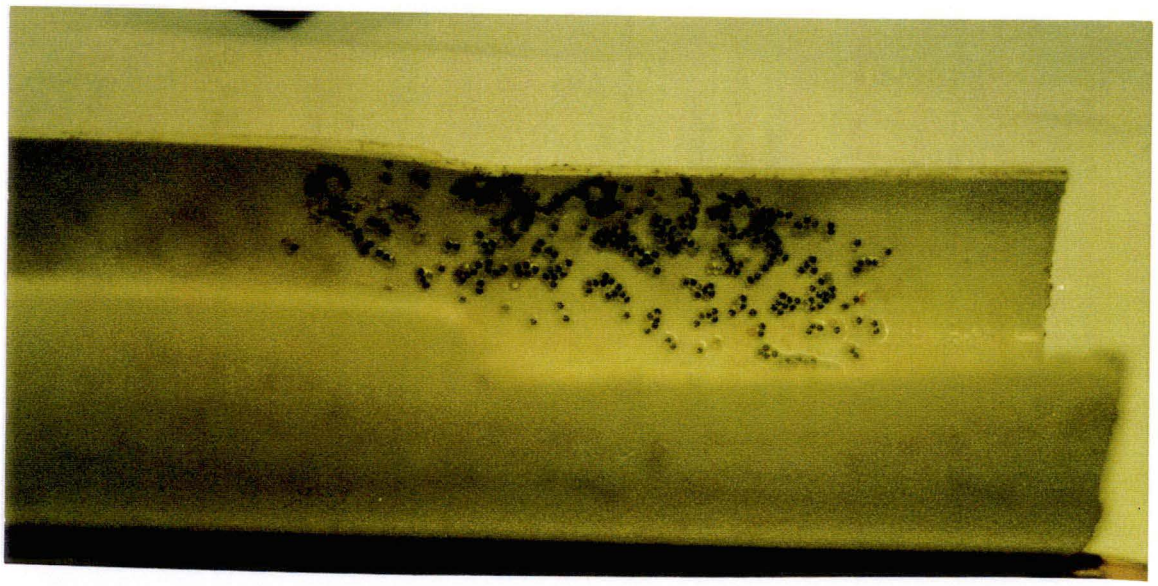


Table 4.4

Spawning Data For Tasmanian Blennies

Site	% Pulp Mill Effluent	N° Spawns	N° Pairs	Total N° Larvae
Control	0	3	3	1794
Round Hill Point	0.5	4	3	1045
Burnie Wharf	0.4	5	3	445
Burnie Yacht Club	1.0	9	6	3053

Table 4.5

Average Number Of Larvae Per Spawn For Tasmanian Blennies

Site (% Effluent)	Average N° Larvae / Spawn
Control (0%) (n = 3)	598.0 ± 218.3^{a}
Round Hill Point (0.5%) (n = 4)	433.7 ± 198.1^{ab}
Burnie Wharf (0.4%) (n = 5)	148.3 ± 30.7^{b}
Burnie Yacht Club (1.0%) (n = 9)	496.0 ± 285.5^{ab}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$).ie. results with the same superscript are not significantly different.

4.3.3 Length

Blenny larvae from the Burnie Yacht Club parents (1% effluent) were significantly smaller than those from the control parents and Round Hill Point parents (0.5% effluent) (Table 4.6). Larvae from all Emu Bay sites were significantly smaller than those from control parents.

4.3.4 Mean Number of Days to Hatch

The larvae from the Burnie fish also showed a delay in hatching when compared with the control larvae (Table 4.6) with Round Hill Point and Burnie Wharf larvae showing a slightly longer delay compared to the Yacht Club larvae.

Table 4.6

Length And Number Of Days To Hatch Of Tasmanian Blennies

Site	Length mm	N° Days To Hatch
Control (0%)	4.64 ± 0.02^{a} (n = 229)	17.60 ± 1.26^{a}
Round Hill Point (0.5%)	4.19 ± 0.06^{b} (n = 320)	22.91 ± 2.96^{b}
Burnie Wharf (0.4%)	4.08 ± 0.08^{bc} (n = 170)	22.44 ± 1.27^{b}
Burnie Yacht Club (1.0%)	3.92 ± 0.06^{c} (n = 602)	20.16 ± 4.04^{ab}

Note: Different superscript letters indicate that results are significantly different at the 95% level ($P<0.05$).ie. results with the same superscript are not significantly different.

4.2.5 Morphological Effects

Abnormalities were observed in all treatments with significant differences between the prevalence of abnormalities exhibited by control larvae and larvae from the Burnie sites (Figure 4.4). Abnormalities observed included; snub nose, eye deformity, spinal curvature, large yolk sacs with slight oedema and jaw deformities in larvae from all sites and cephalic deformities in Burnie yacht club larvae. There was also a significant difference in the number of large yolk sacs exhibited by larvae from the Burnie Yacht Club site when compared to other Emu Bay sites. There was also a significant difference in percentage of jaw deformities of larvae from Round Hill Point when compared to control larvae and larvae from the other sites. Examples of morphological abnormalities are shown in Figures 4.5 to 4.12.

Figure 4.4

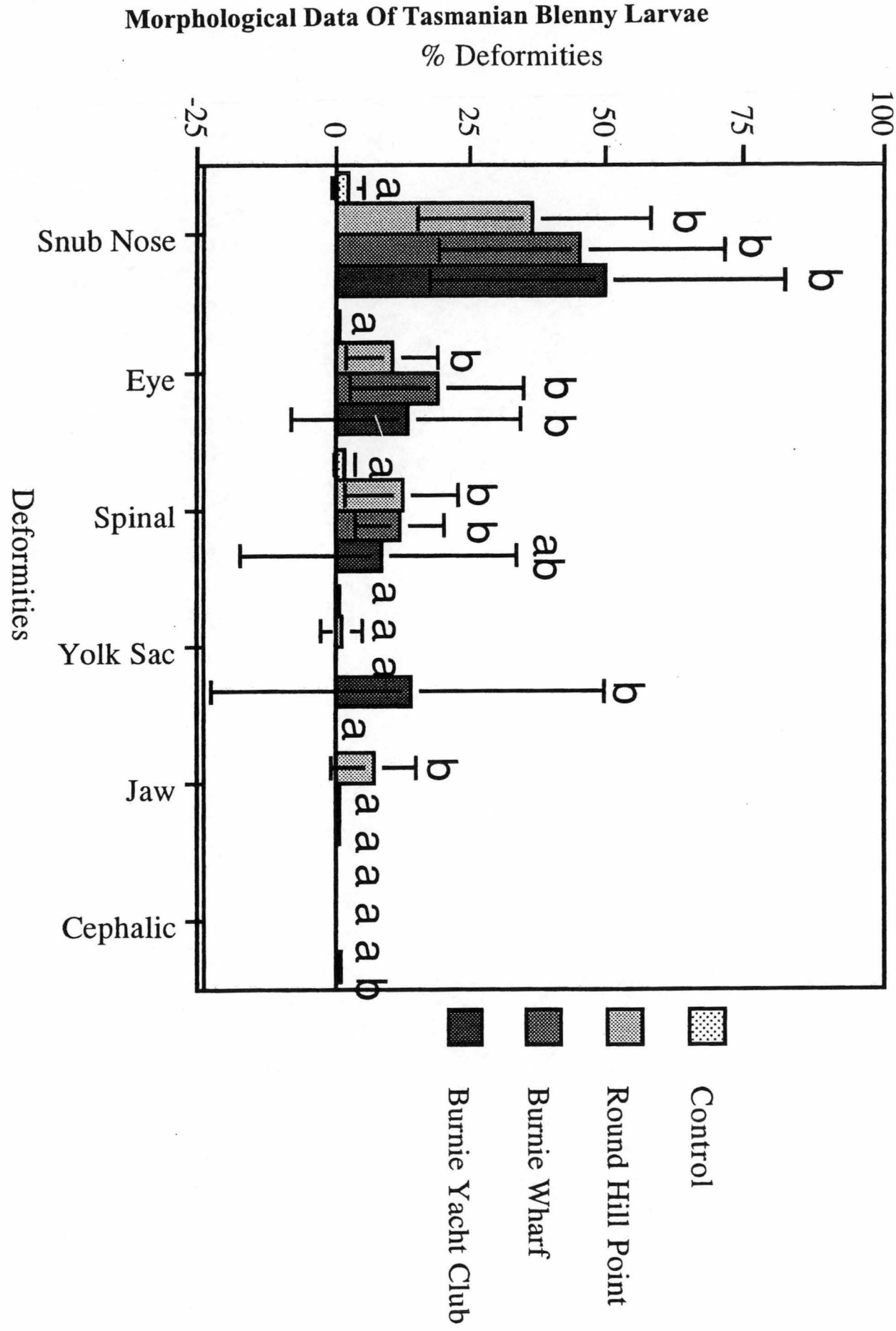

Morphological Data Of Tasmanian Blenny Larvae
% Deformities
-25
0
25
50
75
100
Snub Nose
Eye
Spinal
Yolk Sac
Jaw
Cephalic
Deformities
Control
Round Hill Point
Burnie Wharf
Burnie Yacht Club

Figure 4.5

Normal Tasmanian Blenny Larva

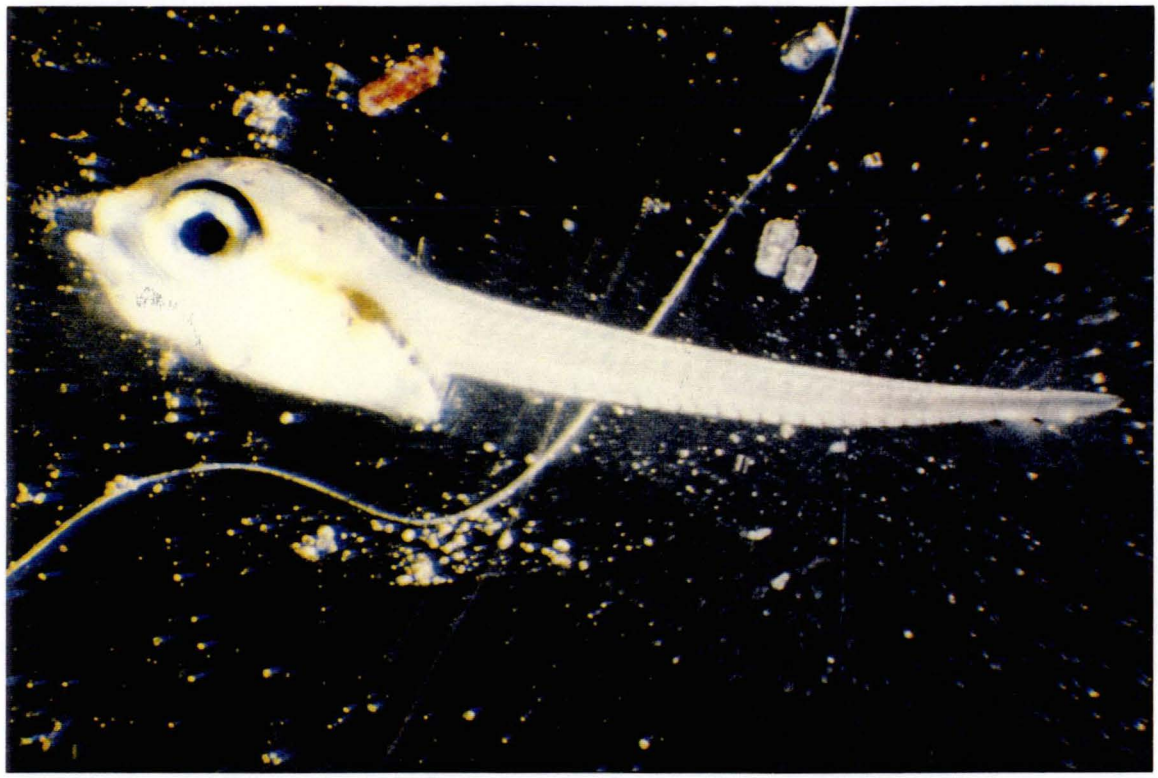


Figure 4.6

Tasmanian Blenny Larva Showing Spinal Curvature

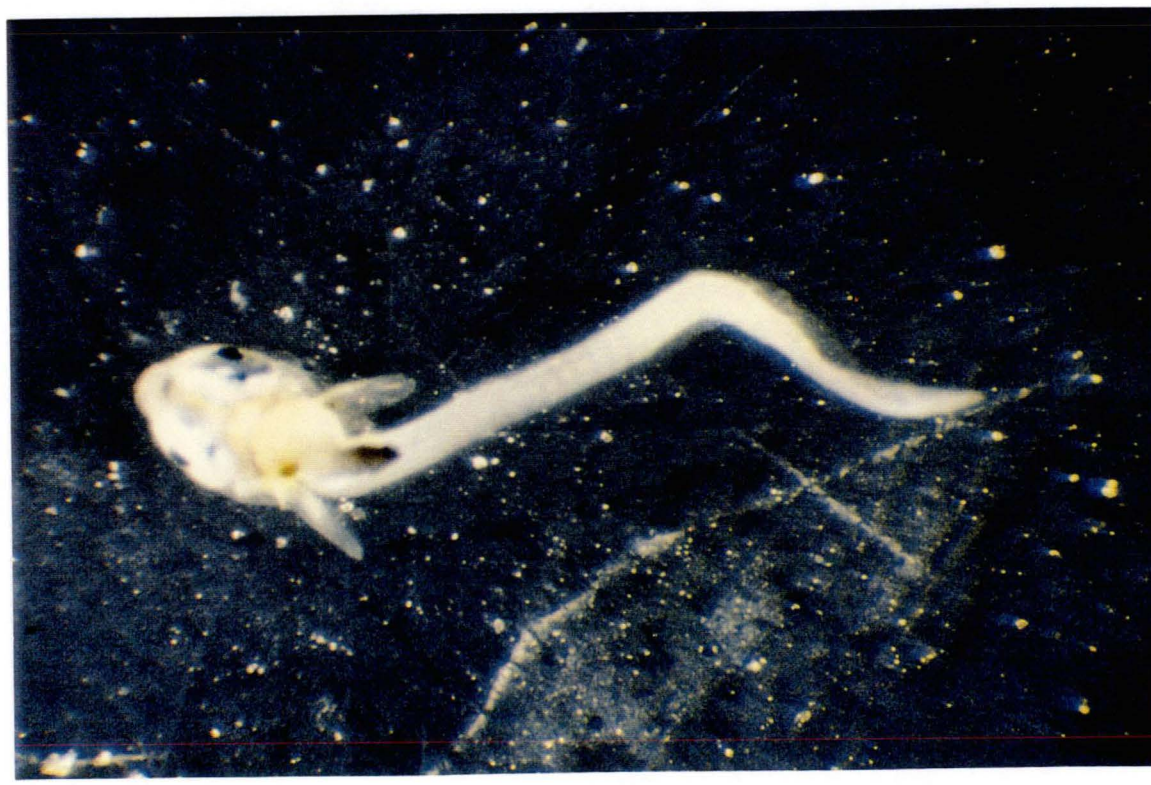


Figure 4.7

Tasmanian Blenny Larva Showing Jaw Deformity

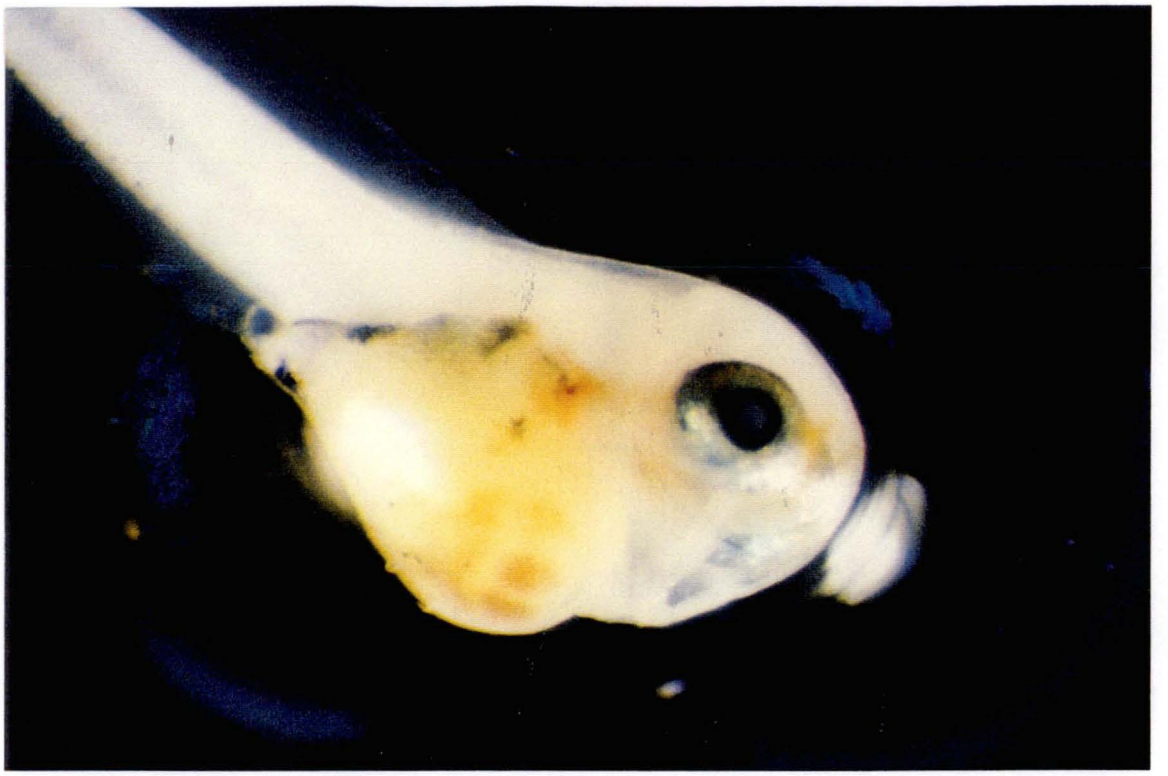


Figure 4.8

Tasmanian Blenny Larva Showing Jaw Deformity

Figure 4.9

Tasmanian Blenny Larva Showing Eye Deformity

Figure 4.10

Tasmanian Blenny Larva Showing Snub Nose

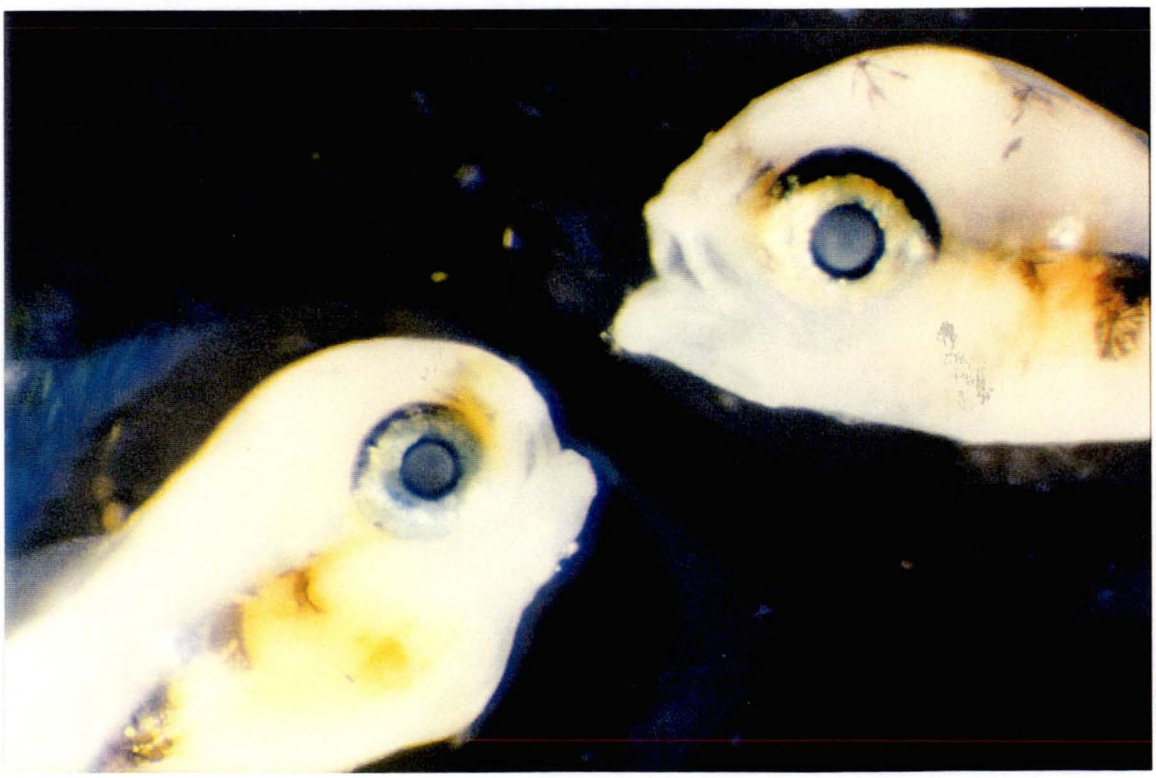


Figure 4.11

Tasmanian Blenny Larva Showing Large Yolk Sac

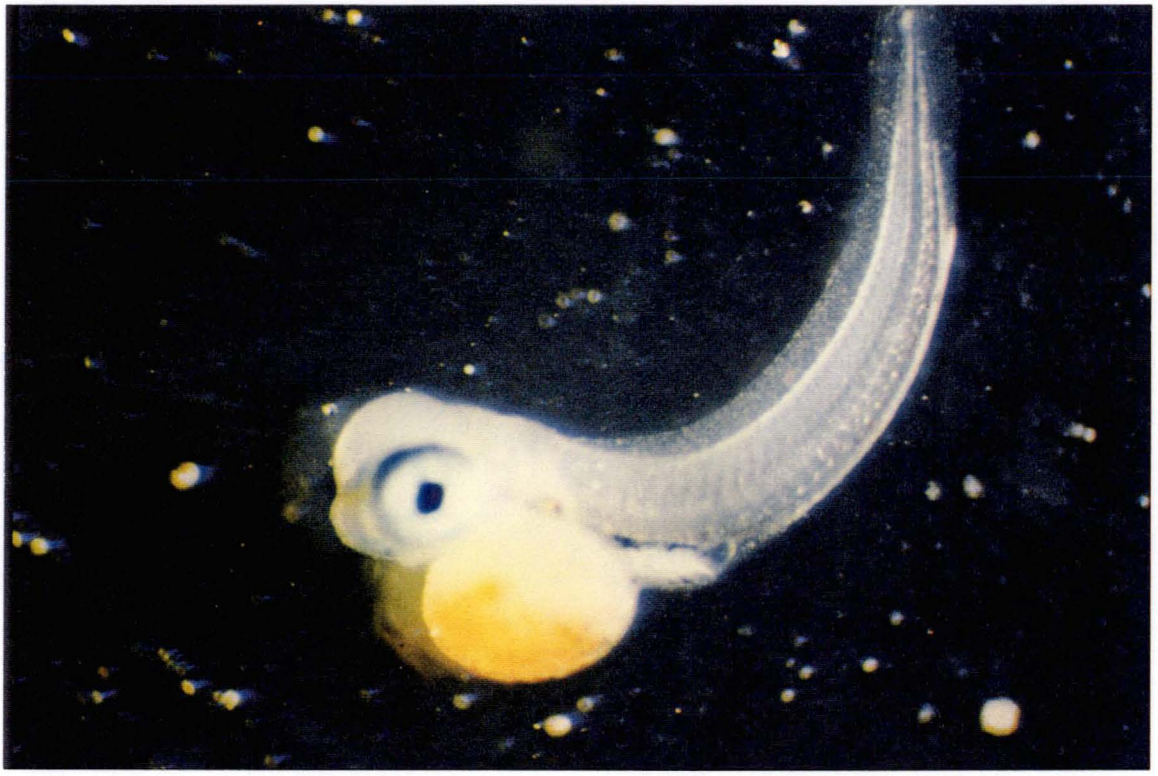
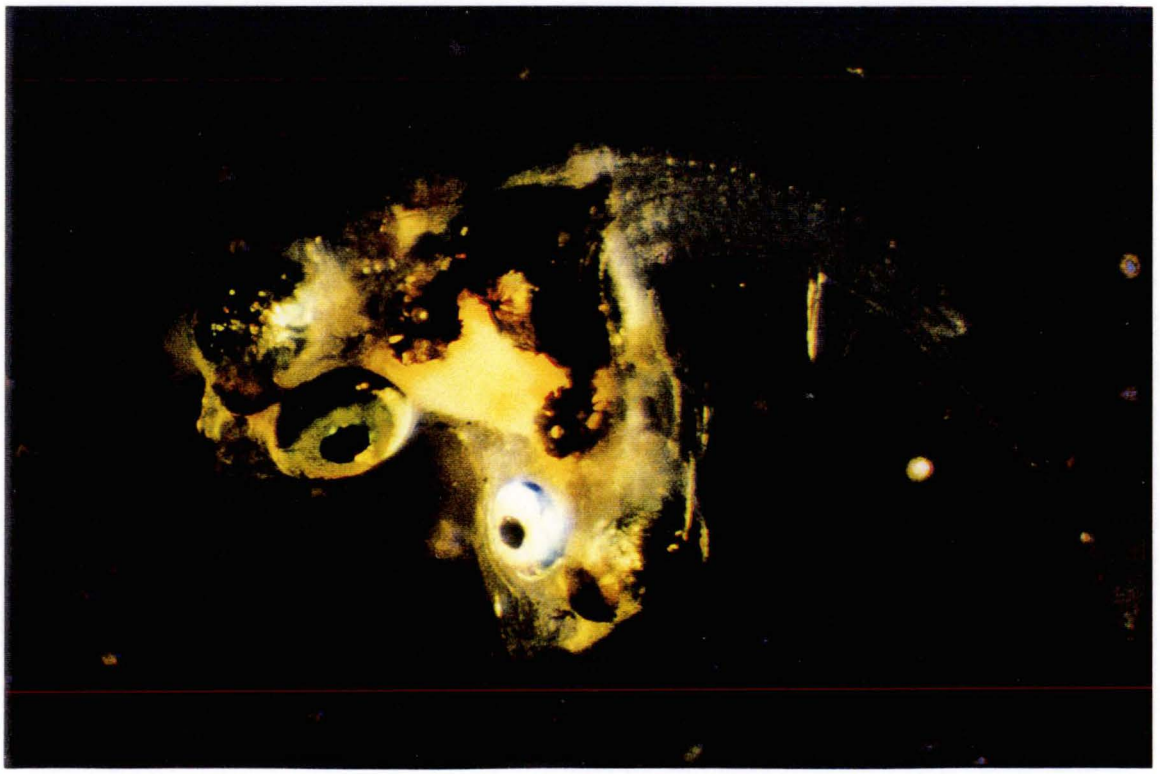


Figure 4.12

Tasmanian Blenny Larva Showing Cephalic Deformity



4.4 Discussion

4.4.1 Microtox

The pulp mill effluent was tested in July, August 1994 and January 1995. The EC50 results obtained ranged from 15% - 18% v/v, indicating that the batches of effluent were of similar toxicity. Subsequent Microtox assays have shown that by adjusting the pH to that of seawater the EC50, as tested with Microtox, is greatly reduced (see Chapter 2). However, as shown in Chapter 3, the effluent still produces sublethal effects in exposed fish.

4.4.2 Embryo Exposure

As mentioned previously, the fertilised egg is sensitive to those compounds that are able to penetrate the chorion and that have the potential to interfere with metabolism and energy production (Rosenthal and Alderdice 1976). The embryos and parent fish used in this experiment were continuously exposed to pulp-mill effluent for the duration of the experiment. Consequently, there were opportunities for components of the effluent to be incorporated into the embryo. Pulp-mill effluent contains a large number of high- and low-molecular weight particles such as chlorophenols and guaiacols (Neilson 1993) which may be able to enter through the chorion during the water-hardening process.

4.4.3 Number Of Larvae Per Spawn

The spawns from control blennies were larger with an even distribution over the spawning substrate (Figure 4.2) when compared with the Emu Bay sites. The spawns from the Emu Bay fish were significantly smaller with patchy distribution and lower fertilisation rates and an example is given of Round Hill Point embryos in Figure 4.3. The causes of these effects are not known at this stage and further work is required to determine if it is a direct cytological effect or mediated via effects on reproductive hormones.

A significant difference in the number of larvae per spawn was observed between the control and Burnie Wharf fish. A difference was also observed between Emu Bay fish, with blennies from the Burnie Wharf site showing fewer resulting larvae per spawn than either the Round Hill Point or Burnie Yacht Club blennies, this may indicate that factors other than pulp mill effluent are influencing the adult blennies. This is quite possible as Emu Bay is a busy port and the wharf area in particular would be subject to contamination by minor oil spills and other possible pollutants. The yacht club area, where the blennies were captured, incorporates a

launching ramp for motor craft and small petroleum spills would occur from time to time. However, Round Hill Point is relatively remote from both these influences

4.4.4 Number Of Days To Hatch

Hatch rate was not monitored in this experiment as hatching success has not proven to be a sensitive indicator of exposure to pulp-mill effluent (Burton *et al.* 1983, Burton *et al.* 1984, Kovacs *et al.* 1995). However, changes in duration of the hatching period, either an increase or decrease, will have an effect on larval viability as it may desynchronise food availability at the time of first feed and, if an external source of food is unavailable after the yolk sac has been utilised, survival will be affected (Hall *et al.* 1993). Therefore, the number of days to hatch was recorded for all larvae as the time to hatch has been shown to be affected by exposure to toxicants (Rosenthal and Alderdice 1976, Weis and Weis 1989). The larvae from all three sites in Emu Bay had a similar average number of days to hatch. However, larvae from the control site had significantly fewer days to hatch than the Burnie Wharf site. This may indicate that the larvae exposed to pulp mill effluent have a decreased metabolic rate which would result in the longer incubation times observed. These results are in contrast to Burton *et al.* (1983) and Kovacs *et al.* (1995) who found no delayed hatching or other anomaly associated with hatching success in fish embryos exposed to treated pine-based pulp-mill effluent.

4.4.5 Length

The length of fish larvae has been reduced by exposing developing embryos to many types of toxicants and is one of the most sensitive measurements of exposure (Rosenthal and Alderdice 1976, Weis and Weis 1989). Heart rate and reduced yolk conversion are also sensitive parameters of toxicant exposure which will lead to a reduction in growth. These parameters can therefore be utilised as an early indicator of harmful concentrations of toxicants. In this experiment the shorter length and large yolk sac size of Emu Bay larvae compared to control larvae indicates that they had converted less yolk to tissues and that the development had been retarded. Retarded development in embryos exposed to toxicants can occur due to alterations in biochemical processes that result in changes to adenosine triphosphate (ATP) levels that may lead to reduced energy availability (von Westernhagen 1988, Hall 1991). As mentioned previously, chlorinated phenolics can exert acute and chronic toxicity by promoting uncoupling of oxidative phosphorylation which inhibits ATP production by the mitochondria. Therefore,

the reduced growth and delayed hatch detected in the blenny larvae exposed to the Burnie mill effluent may be related to the presence of chlorinated phenolics.

Control larvae were significantly larger than the larvae of all the Emu Bay parents (Table 4.5). Within the Emu Bay treatments, larvae of Burnie Yacht Club parents were significantly smaller than larvae of Round Hill Point parents. However, the length of larvae of Burnie Wharf parents was not significantly different from either larvae of the Round Hill Point parents or the Burnie Yacht Club parents. This result indicates that a concentration effect on growth may be occurring. Larvae of Burnie Yacht Club parents which were exposed to the highest concentration of effluent (1%), showed the least growth when compared to larvae of parents from the other Emu Bay sites. Further, there was no significant difference between the growth of larvae from Burnie Wharf parents (0.4% effluent) and Round Hill Point parents (0.5% effluent). However, larvae of Burnie Wharf parents were slightly smaller than the larvae of Round Hill Point parents indicating that the Burnie Wharf parents may have been exposed to another source of toxicant such as petroleum hydrocarbons.

4.4.6 Morphological Abnormalities

The larvae from the Burnie Yacht Club parents which were exposed to the highest concentration of effluent (1%) exhibited a significantly higher percentage of larvae with a large yolk sac when compared to larvae from other treatments. The presence of a large yolk sac together with a slight degree of oedema is similar to the blue-sac disease found in salmonid larvae after exposure to TCDD in the laboratory, which was demonstrated by Walker *et al.* (1991, 1992, 1994). The oedema and large yolk-sac exhibited by the Burnie Yacht Club larvae may indicate the presence of TCDD in the effluent. As mentioned previously, TCDD is a lipophillic compound and can bioaccumulate in lipid rich tissues such as liver and gonads. Walker *et al.* (1994) has shown that TCDD can be translocated from parent fish to oocytes resulting in non-viable oocytes and craniofacial abnormalities and symptoms resembling those of blue-sac disease in resulting fry. Yolk sac and pericardial oedema, haemorrhages and fore-shortened maxilla have also been exhibited in several species of fish larve in response to TCDD exposure (Helder 1980, Wisk and Cooper 1989, Spitsbergen *et al.* 1991, Walker *et al.* 1991, 1992). Therefore, the abnormalities exhibited by the larvae from Emu Bay parents may possibly be due, in part, to exposure to TCDD.

Other morphological abnormalities have also been observed in larval fish in response to toxicant exposure, with spinal curvature being commonly detected (Rosenthal and Alderdice 1976). Spinal curvature will impair the swimming ability of the larvae, thereby, resulting in an inability to feed and an increased susceptibility to predation (Rosenthal and Alderdice 1976, Heath 1987). Exposure to pulp mill effluent has been implicated in the increase of spinal curvatures in fish larvae (Burton *et al.* 1984, Karas *et al.* 1991). This study showed that exposure to eucalypt-based pulp mill effluent also correlates with spinal curvature in larval fish (Figure 4.5). There is a significant difference between the larvae of control parents exhibiting spinal curvature and larvae of Emu Bay parents, indicating that exposure to Burnie mill effluent apparently causes an increase in spinal curvatures. However, there are no significant differences in the percentage of larvae exhibiting spinal curvature between the Emu Bay sites, indicating that there are no concentration dependent effects. Spinal curvature can be related to a lack of vitamin C as Vitamin C is required for the formation of collagen which is essential for skeletal development (Foster 1990). Vitamin C is also utilised in the MFO detoxification pathway (Mehrle and Meyer 1975, von Westernhagen 1988, Goksoyr *et al.* 1991). Therefore, if a larval fish was exposed to a MFO inducer, vitamin C would be used for the detoxification of the toxicant, thus, limiting the amount available for collagen production and resulting in scoliosis or lordosis in the larvae (Foster 1990).

Spinal curvatures in blenny larvae have been observed in a previous study (Munday and Deavin 1994). In that study, Tasmanian blenny larvae exposed to pulp-mill effluent exhibited significantly higher percentage of spinal curvature than control larvae. Spinal curvature was exhibited by 13% of larvae exposed to 1% effluent, whereas less than 1% of control larvae exhibited spinal curvature. However, Burton *et al.* (1983) exposed striped bass embryos to 2% treated pine-based pulp-mill effluent with no spinal curvatures being observed in the resulting larvae. Spinal curvatures, which may be related to effluent exposure, have been observed in up to 10% of fish larvae trapped in the wild living near pulp-mill effluent outflows (Karas *et al.* 1991). However, spinal deformities can result from embryo exposure to other types of toxicants and environmental stressors including low dissolved oxygen, low or high temperatures, mercury, copper, cadmium and pesticides and herbicides (McCormick *et al.* 1977, Crawford and Guarino 1985, Weis and Weis 1989, Anderson *et al.* 1991, Devlin and Mottet 1992). Therefore, care must be taken when assessing these types of field studies. However, none of the above mentioned stressors would have influenced embronic development in this study as all

conditions in the blenny embryo exposure experiments were strictly controlled and cadmium, the most toxic stressor, is not found in Burnie mill effluent (Nielson 1993).

Jaw deformities and snub nose have not commonly been described in fish larvae exposed to pulp mill effluent (Burton *et al.* 1983, Vuorinen and Vuorinen 1987, Shenker and Cherr 1990). However, these types of deformities have been described in fish larvae exposed to TCDD (Wisk and Cooper 1990, Spitsbergen *et al.* 1991, Walker *et al.* 1991, 1992). As TCDD is a MFO inducer, deformities involving the skeletal system such a snub nose and jaw deformities may also be related to a lack of vitamin C. Snub nose was a commonly observed abnormality in larvae of exposed Emu Bay parents and jaw deformities were observed to a lesser extent (Figures 4.6, 4.7 and 4.9). The occurrence of snub nose in exposed larvae was significantly different to those in the larvae of control fish. However, the prevalence of jaw deformities was not significantly different between control and exposed larvae. As a high incidence of snub nose was observed in larvae of Emu Bay parents, this seems to be a more sensitive indicator of eucalypt-based effluent exposure than the other abnormalities examined. However, as with all of the other abnormalities, with the exception of large yolk sac, there was no concentration dependent response.

Eye malformations, such as anopthalmia, micropthalmia, monoopthalmia and haemorrhages, can occur in larvae due to toxicant exposure and can result in reduced visual perception which will affect phototaxis, reduce the ability to hunt prey and avoid predation (Rombough and Garside 1982, Klein-MacPhee *et al.* 1984, Anderson *et al.* 1991, Devlin and Mottet 1992). However, eye abnormalities have not previously been described for larval fish exposed to pulp-mill effluent, but up to 18% of exposed larvae in this bioassay exhibited eye deformities. A common eye deformity observed in the Emu Bay larvae was the presence of an hour-glass shaped eye (Figure 4.8).

Cephalic deformities were observed in larvae from the Burnie yacht club site only. The deformity observed is termed twinning (Figure 4.11), where the larvae has two heads joined onto one body. In some cases the larvae had only a rudimentary second head and a joined yolk sac. It is unlikely that larvae with this type of deformity would survive. This type of deformity has not been described in the literature as occurring in response to pulp mill effluent exposure. However, this abnormality has been shown in larvae exposed to metals such as cadmium (Weis

and Weis 1989) and this aetiology would need to be excluded before accepting involvement of pulp mill effluent.

4.4.7 Parental Exposure

When the results obtained from this study are compared with results obtained from a study which exposed blennies at the embryo stage only (Deavin 1993) several differences in the frequency of morphological abnormalities are observed. For example; 50% of larvae from Burnie Yacht Club parents exposed to 1% effluent showed evidence of snub nose, whereas, <1% of larvae exposed to 10% effluent as embryos showed signs of snub nose. Further, no morphological abnormalities were observed in larvae exposed to 1.25% effluent as embryos. This contrasts to the present study, where larvae resulting from parental and embryo exposure to a 1% concentration exhibited 8% spinal curvature, 13% large yolk sac and oedema and <1% jaw deformities. These differences in the frequency of abnormalities indicate that parental exposure to pulp mill effluent makes a significant contribution to the abnormalities occurring in the resulting larvae. Further, these results have implications for experimental method, as embryo exposure alone will underestimate the impacts of the toxicant, ignoring the contribution of the parent fish.

Some of the differences between larvae of Emu Bay parents and larvae that were exposed to Burnie mill effluent as embryos only, could be attributed to the physiology of the parent fish. As mentioned previously, bioaccumulation of lipophillic compounds such as TCDD can be transfered to the gonads and resulting larvae (Walker *et al.* 1991, 1992, 1994). Even though PCDDs and PCDFs were not detected in the Burnie mill effluents they were detected in Burnie mill sludge (Appendix 1). This indicates that while these compounds are present in the effluent in undetectable concentrations they are potentially available via the food chain, and thus, liable to be bioaccummulated by the parent fish. If these MFO inducing compounds are bioaccumulated by the parent fish, the MFO activity would increase as a result and this has, indeed, been evidenced by an increase in EROD activity in blennies as discussed in the previous chapter. As EROD induction is occurring in the adult fish it is reasonable to assume that elevated EROD activity occurs in the embryos in response to exposure to Burnie mill effluent. Such an increase on MFO could interfere with vitamin C metabolism in both the parent and embryos. This interference with vitamin C metabolism would impair collagen production, resulting in the skeletal deformities such as those observed in the larvae. However, EROD

induction and vitamin C levels in adult or embryo blennies were not assessed in this study and any relationship between EROD induction and vitamin C levels is speculation under the circumstances. Future studies should include the monitoring of MFO inducers, EROD and vitamin C levels in fish and larvae from Emu Bay to determine the long-term impacts of the Burnie mill effluent on reproduction and productivity of fish in the receiving waters.

While it is difficult to attribute the other abnormalities occurring in the exposed embryos to mutagenic activity of the effluent, mutagenicity of pulp mill effluents has been determined in several studies (Langi and Priha 1988, Wong *et al.* 1994). Langi and Priha (1988) showed that untreated pine-based pulp mill effluents were mutagenic according to the Ames and SCE (Sister Chromatid Exchange) tests. However, the effluents were not mutagenic at the concentrations found in the receiving waters. These findings are supported by Wrisberg and van der Gaag (1992), Rao *et al.* (1994) and Rannug *et al.* (1981), who also stated that the mutagenic components were of low molecular weight, some of which were water soluble. Wrisberg and van der Gaag (1992) attribute the production of chemicals causing mutagenicity to the pulping process and the chlorine dioxide bleaching process. This finding agrees with Langi and Priha (1988) who showed that non-chlorinated compounds were capable of causing mutagenic effects. Wrisberg and van der Gaag (1992) also showed that genotoxic effects occurred in mussels collected from the receiving waters. Further studies using SCE, micronuclei or chromosome aberrations need to be undertaken to determine if the abnormalities exhibited by the exposed blenny larvae are, in fact, due to genotoxic effects of the effluent or are the result of direct cytotoxicity.

The results indicate that exposure of fish to pulp mill effluent in concentrations that are found in Emu Bay does have an adverse effect on larvae of parents living in the bay. As mentioned previously, the effluent may affect biochemical processes occurring within the embryos and adult fish. In addition, the effect of the effluent on the unfertilised eggs and sperm, after they leave the body of the adult, must be taken into account. A bioassay using eggs and sperm of the macroalgae *Hormosira banksii* exposes the sperm to a toxicant for 30 minutes prior to fertilising the eggs (Stauber *et al.* 1993). The end point of this bioassay is inhibition of fertilisation, therefore, exposure of the sperm to the toxicant for 30 minutes can have an adverse effect on the viability of the sperm. However, it is unlikely that the blenny sperm would be exposed to the pulp mill effluent for such a period, as the male closely follows the

female as she is laying the eggs and the eggs are fertilised immediately (Cook 1986). However, McMaster *et al.* (1992) has shown reduced sperm motility in feral white sucker exposed to pine-based pulp mill effluent. This may also be a factor in these assays and could account, in part at least, for the lower fertilisation rates of the eggs produced by the exposed blennies. The effect of pulp mill effluent on fish sperm requires further investigation. However, Von Westernhagen *et al.* (1987) showed that sperm from PCB contaminated male fish did not exert a substantial influence on viable hatch and concluded that sperm contamination has only a minor influence on viable hatch.

Several studies of reproduction in feral flatfish (Johnson *et al.* 1988, Casillas *et al.* 1991) have shown that female fish living in contaminated sites have significantly reduced reproductive capabilities and results from a field study of Emu Bay agree with these findings (Deavin 1993). This reduction in reproductive capabilities can, most likely, be partly attributed to an accumulation of lipophilic compounds in the maturing gonad which affects maturation of the gametes and subsequent development of the resulting embryos (Longwell 1988, Monosson *et al.* 1994). As many pulp mill effluent constituents are hydrophobic, these chemicals may accumulate in lipid-rich organs such as the liver and the gonad, especially the ovary (Johnson *et al.* 1988). To show that lipophilic compounds do, in fact, accumulate in the gonad, Walker *et al.* (1994) exposed lake trout to TCDD and measured the amount in the gonads and also monitored effects in the resulting larvae. That experiment showed that TCDD was incorporated into the eggs of exposed fish and that mortalities increased in the resulting larvae. Further studies on the body burdens of pulp mill effluent constituents in blennies are needed so that these can be related to reproductive effects.

4.5 Conclusions

From this series of bioassays it is apparent that parental and embryo exposure to eucalypt-based pulp mill effluent does have an effect on the incidence of sublethal effects in blenny larvae. However, these effects were not related to concentrations of effluent used and, therefore, there is probably not a strict correlation between the distance of blenny populations from the mill outfall and the incidence of sublethal effects. It is important to note that all of the sublethal effects observed in the Emu Bay larvae can result in an adverse effect on the survival potential of the individual larvae and, therefore, the population to which they belong.

The skeletal abnormalities observed in the larvae of Emu Bay fish may, in part, be attributed to exposure to MFO-inducing compounds with the resulting elevated MFOs impacting on vitamin C which is required for collagen production.

The results obtained from this study indicate that parental exposure to eucalypt-based pulp mill effluent will increase the number of resulting larvae exhibiting sublethal effects when compared to larvae exposed as embryos, but from unexposed parents, (Deavin 1993). Furthermore, and most importantly, the results obtained from this bioassay have implications in relation to the methodologies used for assessing the effects of pulp mill effluent on embryos. By exposing only the embryos to the effluent the impacts of the effluent may be under-estimated.

CHAPTER 5

GENERAL DISCUSSION

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5.1 Acute Toxicity

The Australian pulp and paper mill located at Burnie uses a chemical pulping process with chlorine-based bleaching compounds. The acute toxicity of the final discharge varies according to the processes occurring within the different manufacturing areas at the time of sampling. Variations in acute toxicity, as measured by Microtox, have demonstrated differences of up to 10 fold between sampling dates, at some sites. The bleach plant and the direct alkali recovery system (DARS) plant are the main contributors to the toxicity of the final discharge. Waste water from the bleach plant contains chlorinated lignin derivatives, while the waste water from the DARS plant contains water-soluble compounds from the black liquor from the pulping process. Black liquor has been demonstrated to contain the most acutely toxic constituents of pulp mill effluents (Leach and Thakore 1975, McKague 1981, Kovacs *et al.* 1993, Huuskonen and Lindstrom-Seppa 1995). The effluent sampled at the discharge site was moderately to highly acutely toxic. However, most of the toxicity of that effluent, and of other waste waters which were sampled throughout the mill, was related to the pH. After the pH of the effluents had been adjusted to that of sea water the acute toxicity was reduced, leaving the effluent, in most cases, relatively non-toxic. Therefore, after the effluent mixes with sea water in Emu Bay, the effluent is unlikely to be acutely toxic to organisms living in the receiving water.

Although the effluent did not demonstrate acute toxicity at the concentrations that are found in the receiving water, the effluent did demonstrate sublethal toxicity at those concentrations. Results from the *Nitzschia closterium* bioassays show that the maximum concentration of the Burnie mill effluent in Emu Bay should be 0.8% with an average of 0.4% to avoid sublethal effects on unicellular algal species. Consequently, the levels of effluent known to occur in the bay (0.4 - 2%) would not cause acute mortalities in the phytoplankton, but, long-term exposure to the effluent at these levels may interfere with photosynthesis and reproduction of those primary producers. These effects must be taken into consideration when assessing the impacts

of the effluent on the receiving water. Even with the deployment of the new diffuser, which will result in a rapid mixing of the effluent with sea water in the bay, chronic toxicity of the effluent to primary producers is still a possibility which needs to be assessed.

5.2 Sublethal Effects

Although the Burnie mill effluent did not demonstrate acute toxicity to Microtox and unicellular algal species at concentrations found in Emu Bay, several sub-lethal effects were exhibited by exposed fish. By exposing juvenile common jollytails to the effluent via both the dietary route and the water column, growth was significantly affected when compared to control fish. However, results of growth trials with maturing fish exposed by the dietary and water-borne routes were inconclusive. Liver somatic index (LSI) and condition index (CI) of juvenile common jollytails decreased in response to exposure to Burnie mill effluent. LSI and CI results for maturing fish were, again, inconclusive. Therefore, the use of these biomarkers are of limited use in laboratory-based studies using adult fish. However, these biomarkers may be appropriate for use with juvenile fish in laboratory-based experiments.

Ethoxyresorufin-O-deethylase (EROD) activity, liver histopathology and electron microscopy were the only biomarkers tested that were sufficiently sensitive to detect exposure to low levels of eucalypt-based pulp mill effluent in both juvenile and adult fish. The results from the live feed experiments showed that there were no differences between control fish and those which were exposed to Burnie mill effluent by only one route. However, EROD and histopathology results demonstrated significantly different effects on fish exposed by the combined dietary and water-borne route when compared to fish exposed by only one route. The results obtained in this series of experiments have implications in relation to the methodologies used for assessing the impacts of complex effluents. By exposing the fish to the effluent by only one route, the impacts may be under-estimated; this is particularly relevant to the use of water borne exposure alone which is frequently recommended as being adequate and is even specified by some regulatory bodies (U.S. EPA 1988).

5.3 Sublethal Effects on Larvae

The concentrations of Burnie mill effluent that were found in Emu Bay were not acutely toxic to the blenny larvae. However, Burnie mill effluent produced sub-lethal effects in larvae of parent fish collected from Emu Bay. These results indicate that blenny populations in Emu Bay are probably adversely affected by exposure to Burnie mill effluent, as the morphological

abnormalities exhibited by the larvae would have an effect on the survival potential of each individual. The results reported in this thesis have demonstrated sub-lethal effects on the resulting larvae were not concentration-dependent within the limits of this work. Therefore, the incidence of sub-lethal effects cannot be strictly related the to the distance of blenny populations from the outfall. The results obtained from this study also have implications in relation to the methodologies for assessing the effects of a complex effluent on fish embryos. By exposing only the embryos to the effluent, the contribution of the parent fish is ignored, potentially underestimating the toxicity of the effluent.

As discussed in Chapter 2, it is difficult to attribute the toxicity of the Burnie mill effluent to a specific constituent, as many of the chemicals identified as toxic were not present in detectable levels in the Burnie mill effluent. The toxicity of the pulping process waste water is usually attributed to lignin derivatives, such as the resin acids and chlorinated phenolics (Sodergren *et al.* 1988, Kim Oahn and Bengtsson 1995, Ahtiainen *et al.* 1996). As resin acids are present only at background levels in Burnie mill effluent, the acute toxicity may be due to levels of chlorinated phenolics in the effluent. However, in addition to the chlorinated phenolics, unidentified toxic components may be present in the effluent. In addition, additive or synergistic effects between constituents may be occurring.

Results obtained from both the common jollytail experiments and blenny embryo exposures indicate that PCDDs and PCDFs may be present in the effluent as well as the sludge (Appendix 1). Results obtained in the experiments described in this thesis are similar to results obtained in other studies which exposed fish to TCDD (Mehrle *et al.* 1988, van der Wieden *et al.* 1994). As discussed in Chapter 3, the increased EROD activity exhibited by fish exposed to the effluent by the combined dietary and water-column route can be attributed to an increase in exposure to lipophillic aromatic hydrocarbons, such as PCDDs and PCDFs. These EROD inducers will bioaccumulate through the food chain. However, this study has shown that the water-borne route has the same significance as the dietary route. The results from the embryo exposure study also indicate that EROD inducing compounds are present in the effluent. Blenny larvae exhibited similar morphological abnormalities as lake trout embryos exposed to TCDD (Walker *et al.* 1991, 1992, 1994). The skeletal abnormalities, in particular, may be attributed to a relative lack of vitamin C, which is consumed in the MFO pathway. This lack of vitamin C will result in the inhibition of collagen production essential for skeletal formation.

5.4 Conclusions

The results obtained in the bioassays described in this thesis indicate that:

- Growth, condition index and liver somatic index will detect exposure to eucalypt-based pulp mill effluent in juvenile fish in laboratory-based studies.

- Compounds that cause EROD induction are present in the Burnie mill effluent.

- EROD activity, liver histopathology and electron microscopy are appropriate biomarkers for detecting exposure to eucalypt-based pulp mill effluent such as that discharged by the Burnie mill.

- Exposure of experimental animals to eucalypt-based pulp mill effluent by one route will potentially under-estimate the impacts of the effluent in the environment.

- Exposure of embryo fish to eucalypt-based pulp mill effluent will potentially under-estimate the toxicity of the effluent by ignoring the contribution of the parent(s).

- Burnie mill effluent probably adversely impacts on fish populations in Emu Bay as the survival potential of individual larvae is potentially affected by exposure to the effluent at concentrations found in the bay.

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APPENDIX 1

BURNIE AND WESLEY VALE PULP AND PAPER MILLS DATA

Table 1.1

Comprehensive Specific Compound Analysis of Burnie and Wesley Vale Mill Effluents

ESR
ENVIRONMENTAL

HE4/3
Client File: CLAU9B
Lab ref: K0871

19 April 1996

Australian Paper
P O Box 201
Burnie
TAS 7320
Australia

Attention: Mr Dave Campin

Dear Dave

Please find enclosed a copy of the analytical certificates for the effluent and sludge samples analysed for polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).

The results for the effluent samples are reported as picograms per litre (pg L^{-1}) on an as received basis. The results for the sludge samples are reported as picograms per gram (pg g^{-1}) on a dry weight basis. The total toxic equivalents (TE) was calculated using NATO toxic equivalents factors.

The only quantifiable PCDD or PCDF congener in the effluent samples was octachlorodibenzo-p-dioxin. The total TEs determined for these samples were in the range 5.2 - 10.0 pg L^{-1}. Greater than 95% of these values was contributed from limits of detection.

There were a range of PCDD and PCDF congeners determined in the Burnie sludges, while in the Wesley Vale sludges only the higher hepta- and octa- chlorinated dibenzo-p-dioxins were determined. The congener pattern shown by the tetrachlorinated dibenzofurans in the Burnie sludges was consistent with a chlorine bleach pulp source.

The moisture contents of your sludge samples as determined by oven drying to constant weight are listed below.

Burnie Sludge 18-3-96	62.7 %
Burnie Sludge 20-3-96	60.4 %
Wesley Vale Sludge 18-3-96	78.0 %
Wesley Vale Sludge 20-3-96	79.5 %

If you have any queries please do not hesitate to contact me.

Regards

Scott V Leathem
Environmental Chemistry

Institute of Environmental Science & Research Limited
Wellington Science Centre
Gracefield Road, PO Box 30-547, Lower Hutt, New Zealand
Telephone: (64-4) 570-1555 Facsimile: (64-4) 569 4500

A Crown Research Institute

19-Apr-96

Certificate of Analysis

Laboratory Reference: K0872

Client: Australian Paper
P O Box 201
Burnie
TAS 7320
Australia

Attention: Dave Campin

Sample Type: Sludge

Date Received: 26 March 1996

Analysis: Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans

Method: Isotope Dilution

The samples were dried, spiked with $^{13}C_{12}$ labelled internal standards then extracted with organic solvents. The extracts were then purified with chemical treatment and solid phase chromatographic techniques. Measurement was by high resolution gas chromatography and high resolution mass spectrometry. Full details are available on request.

TELARC endorsement applies only to the application of an approved analytical method for the determination of PCDDs and PCDFs in these samples.

CDD	= chlorodibenzo-p-dioxin	**Hp**	= hepta
CDF	= chlorodibenzofuran	**O**	= octa
T	= tetra	**TEF**	= toxic equivalents factor (NATO basis)
Pe	= penta	**TE**	= toxic equivalent
Hx	= hexa	**pg g^{-1}**	= picogram-per-gram

All tests reported herein have been performed in accordance with the laboratory's terms of registration

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19-Apr-96

RESULTS

Laboratory Reference: K0872/1

Sample: Burnie Sludge 18-3-96

Congeners		Level# pg g^{-1}	TEF	TE level pg g^{-1}
2378 TCDF		5.12	0.1	0.512
Non 2378 TCDF		12.3	0	0
2378 TCDD	<	0.5	1	0.25
Non 2378 TCDD	<	0.9	0	0
12378 PeCDF	<	0.4	0.05	0.01
23478 PeCDF	<	0.3	0.5	0.075
Non 2378 PeCDF		5.82	0	0
12378 PeCDD	<	0.4	0.5	0.1
Non 2378 PeCDD		3.54	0	0
123478 HxCDF	<	0.4	0.1	0.02
123678 HxCDF	<	0.2	0.1	0.01
234678 HxCDF	<	0.3	0.1	0.015
123789 HxCDF	<	0.4	0.1	0.02
Non 2378 HxCDF		2.08	0	0
123478 HxCDD	<	0.4	0.1	0.02
123678 HxCDD	<	0.5	0.1	0.025
123789 HxCDD	<	0.3	0.1	0.015
Non 2378 HxCDD		2.28	0	0
1234678 HpCDF	<	0.5	0.01	0.0025
1234789 HpCDF	<	0.3	0.01	0.0015
Non 2378 HpCDF	<	0.4	0	0
1234678 HpCDD		3.32	0.01	0.0332
Non 2378 HpCDD		3.07	0	0
OCDF	<	0.9	0.001	0.00045
OCDD		410	0.001	0.410

< = Less than limit of detection (0.5 of value used in calculating TE)

Results calculated on a dry weight basis.

Total toxic equivalents: 1.5 pg g^{-1} (NATO basis)

19-Apr-96

RESULTS

Laboratory Reference: K0872/2

Sample: Burnie Sludge 20-3-96

Congeners		Level# pg g^{-1}	TEF	TE level pg g^{-1}
2378 TCDF		4.04	0.1	0.404
Non 2378 TCDF		12.1	0	0
2378 TCDD	<	0.3	1	0.15
Non 2378 TCDD	<	1	0	0
12378 PeCDF	<	0.5	0.05	0.0013
23478 PeCDF	<	0.3	0.5	0.075
Non 2378 PeCDF		5.11	0	0
12378 PeCDD	<	0.3	0.5	0.075
Non 2378 PeCDD		3.21	0	0
123478 HxCDF	<	0.6	0.1	0.03
123678 HxCDF	<	0.3	0.1	0.015
234678 HxCDF	<	0.1	0.1	0.005
123789 HxCDF	<	0.3	0.1	0.015
Non 2378 HxCDF		2.17	0	0
123478 HxCDD	<	0.2	0.1	0.01
123678 HxCDD	<	0.2	0.1	0.01
123789 HxCDD	<	0.2	0.1	0.01
Non 2378 HxCDD		2.06	0	0
1234678 HpCDF	<	0.9	0.01	0.0045
1234789 HpCDF	<	0.7	0.01	0.0035
Non 2378 HpCDF		1.57	0	0
1234678 HpCDD		3.06	0.01	0.0306
Non 2378 HpCDD		3.01	0	0
OCDF		1.92	0.001	0.00192
OCDD		345	0.001	0.345

< = Less than limit of detection (0.5 of value used in calculating TE)

Results calculated on a dry weight basis.

Total toxic equivalents: 1.2 pg g^{-1} (NATO basis)

PAPRO New Zealand.

Mill Effluents Sample name Date Papro No.	Wesley Vale Effluent 18/3/96 E96164	 19/3/96 E96165	Burnie Mill Effluent 18/3/96 E96166	 20/3/96 E96167
Target compounds (ng/L)				
4-chlorophenol	0	0	0	0
2,4-dichlorophenol	0	0	573	847
2,4,6-trichlorophenol	38	49	2852	4657
2,3,4,6-tetrachlorophenol	0	0	504	0
pentachlorophenol	271	93	267	120
4-chloroanisole	0	0	0	0
2,4-dichloroanisole	0	0	0	0
2,4,6-trichloroanisole	0	0	0	0
2,3,4,6-tetrachloroanisole	0	0	0	0
pentachloroanisole	0	0	0	0
4,5-dichloroveratrole	0	0	0	0
3,4,5-trichloroveratrole	0	0	0	0
tetrachloroveratrole	0	0	0	0
2-chlorovanillin	0	0	49217	160446
5-chlorovanillin	0	0	0	0
6-chlorovanillin	0	0	1249	2181
2,5-dichlorovanillin	0	0	0	0
5,6-dichlorovanillin	0	0	2685	5640
3-chlorocatechol	0	44	0	0
4-chlorocatechol	0	23	0	0
3,5-dichlorocatechol	0	0	0	0
3,6-dichlorocatechol	0	0	0	0
3,4-dichlorocatechol	0	0	0	0
4,5-dichlorocatechol	0	0	0	0
3,4,6-trichlorocatechol	0	0	0	0
3,4,5-trichlorocatechol	0	0	0	0
tetrachlorocatechol	0	0	0	0
3-chlorosyringol	0	0	348	92
4-chlorosyringol	0	0	0	0
3,5-dichlorosyringol	0	0	0	0
3,4-dichlorosyringol	0	0	0	0
trichlorosyringol	0	0	0	0
4-chloromethoxycatechol	0	0	0	0
6-chloromethoxycatechol	0	0	0	0
5-chloromethoxycatechol	0	0	0	0
4,6-dichloromethoxycatechol	0	0	0	0
5,6-dichloromethoxycatechol	0	0	0	0
4,5,6-trichloromethoxycatechol	0	0	0	0
4-chloropyrogallol	0	0	0	0
5-chloropyrogallol	0	0	0	0
4,6-dichloropyrogallol	0	0	0	0
4,5-dichloropyrogallol	0	0	0	0
4,5,6-trichloropyrogallol	0	0	0	0
5-chloroguaiacol	0	0	1615	81
4-chloroguaiacol	0	0	409	51
6-chloroguaiacol	0	0	0	18
3,4-dichloroguaiacol	0	0	6669	17115
4,6-dichloroguaiacol	0	0	111	0
4,5-dichloroguaiacol	0	0	626	256
3,5,6-trichloroguaiacol	0	0	0	0
3,4,6-trichloroguaiacol	0	0	298	456
3,4,5-trichloroguaiacol	0	0	1124	437
4,5,6-trichloroguaiacol	0	0	823	1401
tetrachloroguaiacol	0	0	1767	2805
2-chlorotrimethoxybenzaldehyde	0	0	637	586
4,5,6-trichlorotrimethoxybenzene	0	0	72	255
2,6-dichlorotrimethoxybenzaldehyde	0	0	543	496
2-chlorosyringaldehyde	0	0	9923	19858
2,6-dichlorosyringaldehyde	0	0	4505	6797
2-chloroacetosyringol	0	0	425	260
2,6-dichloroacetosyringol	0	0	415	0
Total	309	209	87657	224853

19-Apr-96

Certificate of Analysis

Laboratory Reference: K0871

Client: Australian Paper
P O Box 201
Burnie
TAS 7320
Australia

Attention: Dave Campin

Sample Type: Effluent

Date Received: 26 March 1996

Analysis: Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans

Method: Isotope Dilution

The samples were spiked with $^{13}C_{12}$ labelled internal standards then extracted with organic solvents. The extracts were then purified with chemical treatment and solid phase chromatographic techniques. Measurement was by high resolution gas chromatography and high resolution mass spectrometry. Full details are available on request.

TELARC endorsement applies only to the application of an approved analytical method for the determination of PCDDs and PCDFs in these samples.

CDD	= chlorodibenzo-p-dioxin	**Hp**	= hepta
CDF	= chlorodibenzofuran	**O**	= octa
T	= tetra	**TEF**	= toxic equivalents factor (NATO basis)
Pe	= penta	**TE**	= toxic equivalent
Hx	= hexa	**pg L^{-1}**	= picogram-per-litre

All tests reported herein have been performed in accordance with the laboratory's terms of registration

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19-Apr-96

RESULTS

Laboratory Reference: K0871/3

Sample: Burnie Mill Effluent 18-3-96

Congeners		Level# pg L^{-1}	TEF	TE level pg L^{-1}
2378 TCDF	<	6	0.1	0.3
Non 2378 TCDF	<	6	0	0
2378 TCDD	<	6	1	3
Non 2378 TCDD	<	6	0	0
12378 PeCDF	<	3	0.05	0.075
23478 PeCDF	<	2	0.5	0.5
Non 2378 PeCDF	<	3	0	0
12378 PeCDD	<	6	0.5	1.5
Non 2378 PeCDD	<	6	0	0
123478 HxCDF	<	3	0.1	0.15
123678 HxCDF	<	3	0.1	0.15
234678 HxCDF	<	2	0.1	0.1
123789 HxCDF	<	5	0.1	0.25
Non 2378 HxCDF	<	3	0	0
123478 HxCDD	<	4	0.1	0.2
123678 HxCDD	<	5	0.1	0.25
123789 HxCDD	<	4	0.1	0.2
Non 2378 HxCDD	<	4	0	0
1234678 HpCDF	<	4	0.01	0.02
1234789 HpCDF	<	4	0.01	0.02
Non 2378 HpCDF	<	4	0	0
1234678 HpCDD	<	5	0.01	0.025
Non 2378 HpCDD	<	5	0	0
OCDF	<	6	0.001	0.003
OCDD		104	0.001	0.104

< = Less than limit of detection (0.5 of value used in calculating TE)

Results calculated on an as received basis.

Total toxic equivalents: **6.8** **pg L^{-1} (NATO basis)**

19-Apr-96

RESULTS

Laboratory Reference: K0871/4

Sample: Burnie Mill Effluent 19-3-96

Congeners		Level# pg L^{-1}	TEF	TE level pg L^{-1}
2378 TCDF	<	4	0.1	0.2
Non 2378 TCDF	<	4	0	0
2378 TCDD	<	4	1	2
Non 2378 TCDD	<	4	0	0
12378 PeCDF	<	2	0.05	0.05
23478 PeCDF	<	2	0.5	0.5
Non 2378 PeCDF	<	2	0	0
12378 PeCDD	<	4	0.5	1
Non 2378 PeCDD	<	4	0	0
123478 HxCDF	<	2	0.1	0.1
123678 HxCDF	<	3	0.1	0.15
234678 HxCDF	<	2	0.1	0.1
123789 HxCDF	<	4	0.1	0.2
Non 2378 HxCDF	<	2	0	0
123478 HxCDD	<	4	0.1	0.2
123678 HxCDD	<	4	0.1	0.2
123789 HxCDD	<	3	0.1	0.15
Non 2378 HxCDD	<	4	0	0
1234678 HpCDF	<	3	0.01	0.015
1234789 HpCDF	<	3	0.01	0.015
Non 2378 HpCDF	<	3	0	0
1234678 HpCDD	<	9	0.01	0.045
Non 2378 HpCDD	<	9	0	0
OCDF	<	6	0.001	0.003
OCDD		268	0.001	0.268

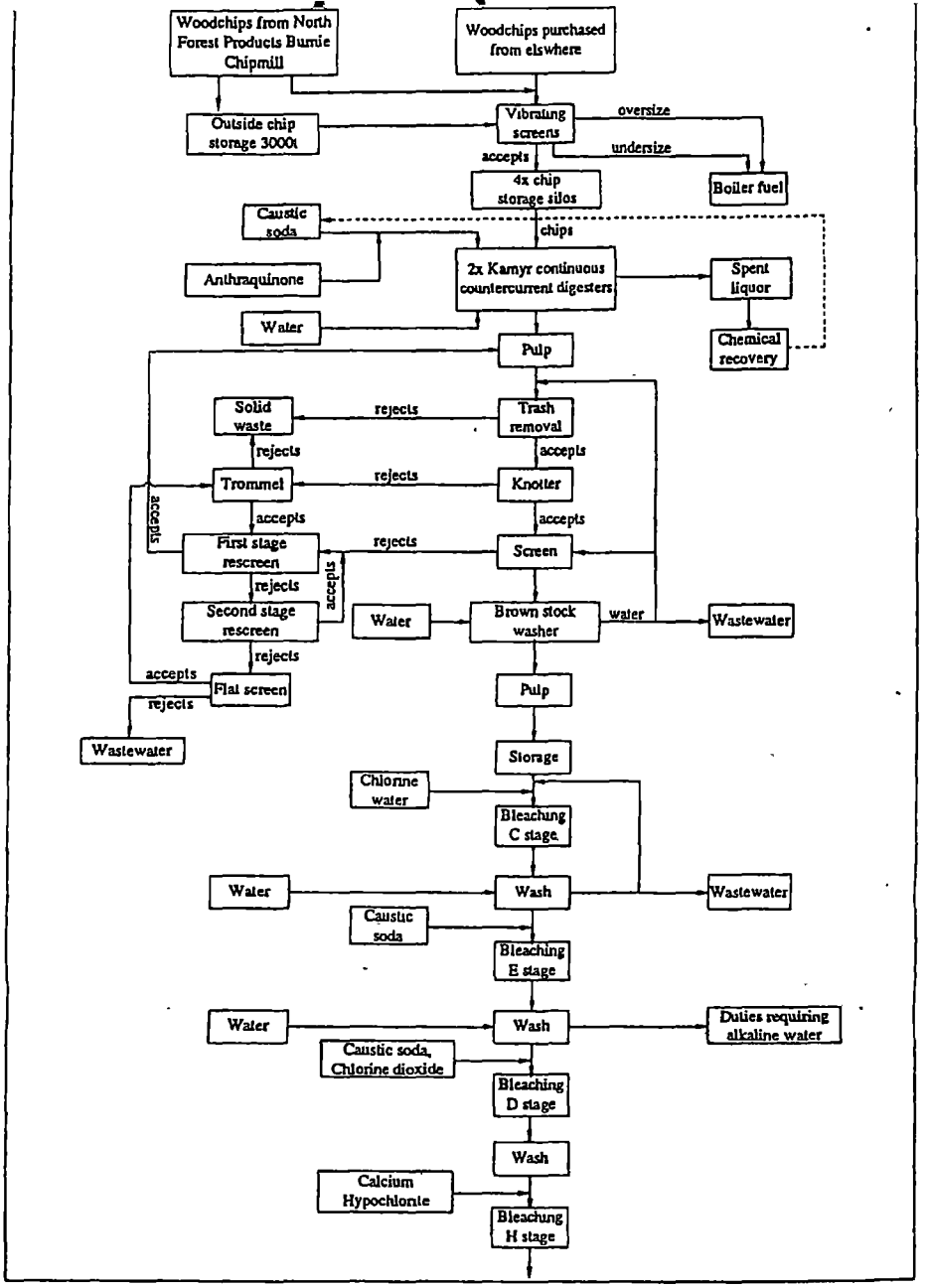
< = Less than limit of detection (0.5 of value used in calculating TE)

Results calculated on an as received basis.

Total toxic equivalents: 5.2 pg L^{-1} (NATO basis)

k0871dx

Figure 1.1
BURNIE MILL PROCESSES


Woodchips from North Forest Products Burnie Chipmill
Woodchips purchased from elswhere
Outside chip storage 3000t
Vibrating screens
oversize
undersize
accepts
4x chip storage silos
Boiler fuel
Caustic soda
chips
Anthraquinone
2x Kamyr continuous countercurrent digesters
Spent liquor
Water
Chemical recovery
Pulp
Solid waste
rejects
Trash removal
accepts
Trommel
Knotter
First stage rescreen
Screen
Second stage rescreen
Water
Brown stock washer
water
Wastewater
Flat screen
Pulp
Storage
Chlorine water
Bleaching C stage
Wash
Caustic soda
Bleaching E stage
Dulies requiring alkaline water
Caustic soda, Chlorine dioxide
Bleaching D stage
Calcium Hypochlorite
Bleaching H stage

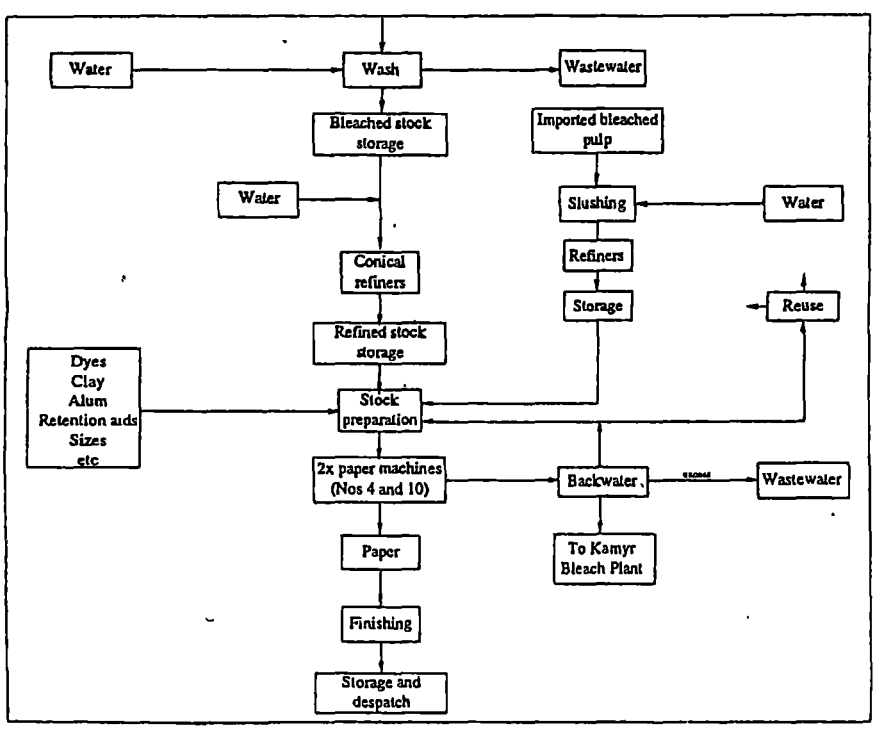

Water
Wash
Wastewater
Bleached stock storage
Imported bleached pulp
Slushing
Refiners
Conical refiners
Storage
Reuse
Refined stock storage
Dyes
Clay
Alum
Retention aids
Sizes
etc
Stock preparation
2x paper machines (Nos 4 and 10)
Backwater
Paper
To Kamyr Bleach Plant
Finishing
Storage and despatch

Figure 1.2

BURNIE MILL SAMPLE SITES

1. Bleach Plant

2. Pulp Mill

3. N° 4 Paper Machine

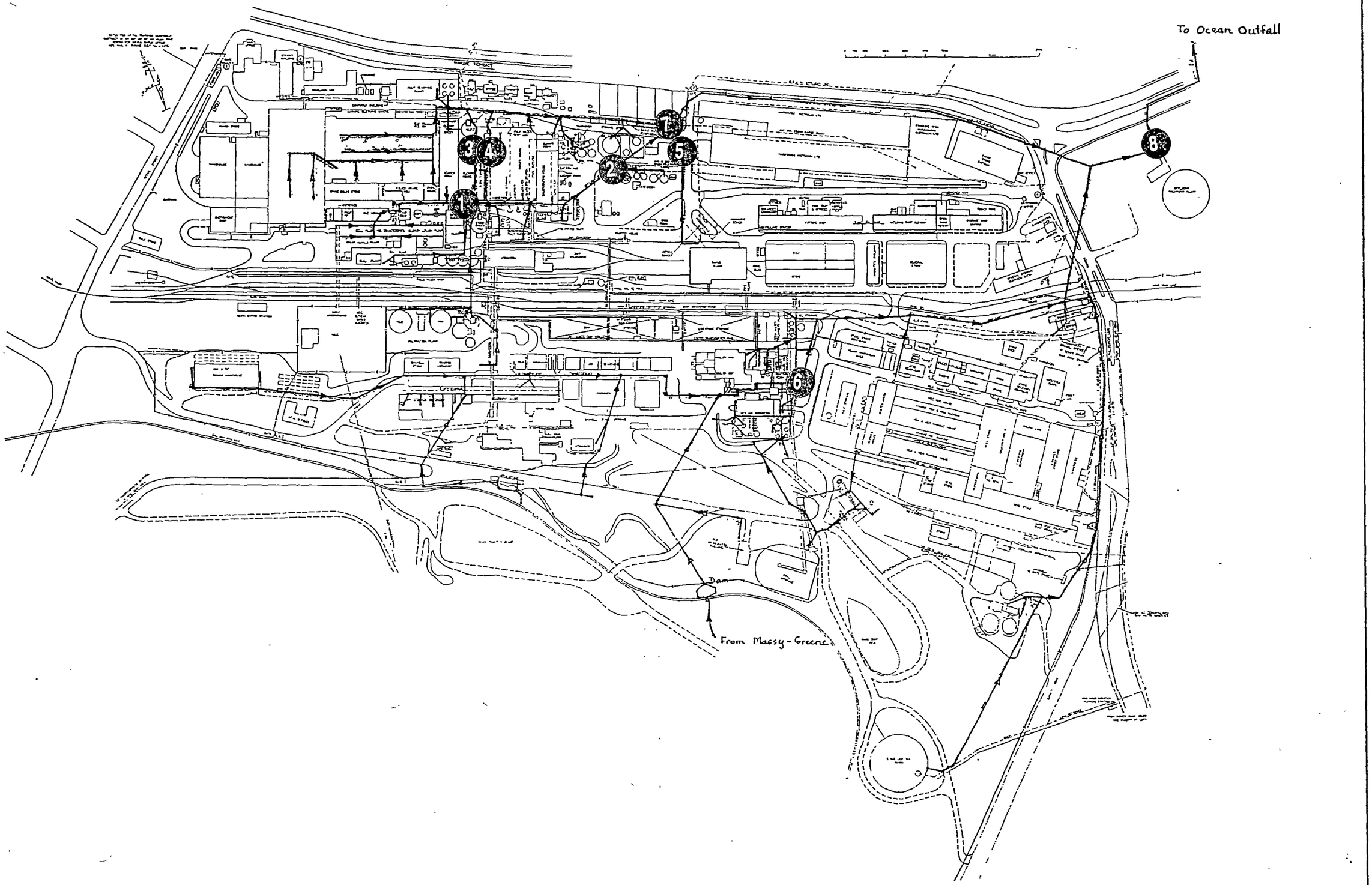
4. N° 10 Paper Machine

5. DARS Plant

6. WAO Plant

7. Pulp / Paper

8. Discharge



A0-8152 001 B

Figure 8.2 Process water

APPENDIX 2

DETAILED METHODS FOR PROCEDURES OUTLINED IN CHAPTER 3 MATERIALS AND METHODS

2.1 Procedure For MFO Assay (EROD)

(Holdway *et al.* 1993)

2.1.1 Preparation Of Microsomes

1/ Weigh livers (~0.5 g)

2/ Place in cooled homogeniser barrel and add 4 volumes of cold homogenising buffer.

3/ Homogenise on ice with 6 strokes of the homogenising plunger.

4/ Centrifuge at 10,000g for 20 minutes at 2-4°C.

5/ Decant the post-mitochondrial supernatant (PMS) into pre-cooled test tubes.

6/ Take an aliquot of PMS for protein analysis - diluted 100:1 with 0.5M NaOH ie. 10 µL of PMS in 990 µL 0.5 M NaOH (can be stored at room temperature for several days).

Steps 7 - 10 Optional

7/ Centrifuge PMS at 100,000g for 60 minutes at 2-4°C.

8/ Discard the supernatant.

9/ Microsomes (pellet) are resuspended in suspension buffer - 1 mL per gram of wet weight liver.

10/ This can be frozen at -70°C.

11/ For each assay combination set up 3 tubes, test in duplicate with a blank and 5 tubes for the standards.

	TEST	**BLANK**
TRIS BUFFER	450 µL	450 µL
COFACTOR	250 µL	250 µL
MICROSOMES	100 µL	100 µL
ALBUMIN	100 µL	100 µL

STANDARDS

	9 pM	**18 pM**	**27 pM**	**36 pM**	**45 pM**
TRIS Buffer	640 µL	630 µL	620 µL	610 µL	600 µL
Cofactor	250 µL	250 µL	250 µL	250 µL	250 µL
Albumin	100 µL	100 µL	100 µL	100 µL	100 µL
Standard	10 µL	20 µL	30 µL	40 µL	50 µL

12/ Add 10µL dicoumarol to all tubes and 2.5mL methanol to blanks.

13/ Pre-incubate all tubes at 30°C for 2 minutes.

14/ To start reaction add 100µL of ethoxyresorufin (substrate) to tests and blank. Incubate all tubes at 30°C for 10 minutes.

15/ Remove tubes from water bath to ice and stop reaction with the addition of 2.5 mL of methanol to tests and standards.

16/ Vortex and centrifuge tubes at 2000g for 5 minutes.

17/ Remove 1 mL of supernatant from each tube and record fluorescence with fluorescence spectrophotometer at EX wavelength 530nm and EM wavelength 585nm.

2.1.2 Reagents For Microsome Preparation

1/ Homogenising Buffer

0.1M K_2HPO_4 + 0.1M KH_2PO_4
ADD

Dithiothreitol	0.1542 g/L
EDTA	0.2923 g/L
KCl	7.456 g/L
Phenanthroline	0.01982g/L

pH 7.4

2/ Suspension Buffer

0.1M K_2HPO_4 + 0.1M KH_2PO_4
20% Glycerol pH 7.4

2.1.3 Reagents For EROD Assay

1/ Substrate
Stock Solution
Dissolve 1.25 mg ethoxyresorufin in 10 mL 1:1 methanol:DMSO.
Store in freezer.
Dilute stock solution 1:50 in 0.1M Tris buffer (light sensitive).

2/ Resorufin Standard
5.3mg resorufin
5 mL 0.01N NaOH
Dilute 100µL to 500 mL distilled water for working solution .

3/ Cofactor Solution
10 mL is enough for 40 assays.

To prepare	10mL:	20 mL	30 mL
Part A			
$MgCl_2$	20.33mg	40.66mg	60.99mg
KCl	149.12mg	298.24mg	447.36mg
Glucose-6-phosphate	18.25mg	36.5mg	54.75mg
0.1M Phosphate Buffer pH 7.4	4 mL	8 mL	12 mL
Part B			
NADP	9.84mg	19.68mg	29.52mg
G-6-dehydrogenase	100U	200U	300U
Distilled water	6 mL	12 mL	18 mL

4/ Dicoumarol Solution

Dicoumarol	20.23 mg
DMSO	100mL

5/ Albumin
12 mg Albumin per mL dist water

6/ 0.1M TRIS Buffer pH 7.8

Trizama HCL	10.64 g
Trizma Base	3.94 g
Distilled water	1 litre

2.2 Procedure For Protein Assay

(Lowry *et al.*1951)

1/ Add 200µL of the diluted PMS (see 6/ of EROD procedure) to test tube with 800µL of 0.5 NaOH.

2/ Prepare a blank containing 1.0 mL of 0.5 NaOH.

3/ Add 5mL freshly prepared copper reagent to all samples, mix thoroughly with a vortex mixer and allow to stand for 10 minutes

4/ Add 0.5mL of 1N Folin reagent, mix immediately with a vortex mixer and stand for 30 minutes.

5/ Read the absorbance at 750 nm after zeroing with the blank.

6/ Prepare standard curve and read absorbances as mg/mL.

$$\text{Protein Conc. mg/mL} = \text{Slope of standard curve} \times \frac{500}{1000} \times \text{Absorbance}$$

2.2.1 Reagents For Protein Assay

1/ 0.5M NaOH: dissolve 20.0g NaOH in 1 L distilled water.

2/ 2% (w/v) Na_2CO_3: dissolve 40g of anhydrous Na_2CO_3 in 2 L distilled water.

3/ 1% (w/v) copper sulphate: dissolve 2.5g $CuSO_4.5H_2O$ in 250 mL distilled water.

4/ 2% (w/v) potassium sodium tartrate: dissolve 5.0g of $KNaC_4H_4O_6.4H_2O$ in 250mL distilled water.

5/ Folin Reagent: Stock commercial reagent is diluted 1:1 with distilled water immediately before use.

6/ 100µg/mL bovine serum albumin standard: dissolve 10mg of bovine serum albumin in 100mL 0.5 M NaOH.

7/ Copper Reagent: mix 1 volume each of 1% copper sulphate and 2% potassium sodium tartrate with 100 volumes of the sodium carbonate solution. Prepare the reagent immediately before use.

2.2.2 To Prepare Standards For Protein Assay

1/ Make a stock solution of bovine serum albumin 100µg/mL (see 6/ protein reagents).

2/ Take 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL of the above stock solution and make up to a final volume of 1.0 mL with 0.5M NaOH (equiv. to 0, 20, 40, 60, 80 and 100 µg protein/mL)

3/ Process the standard curve as for protein assay.

2.3 ELISA Method

(Goksoyr *et al.* 1991)

1/ Dilute protein samples (PMS) to 10 µg / mL in coating buffer.
Calculation:
10 µg / Conc. in sample µg/mL = Y mL of PMS
1 - Y = mL coating buffer

2/ Coat wells of a 96 well microtitre plate with 100 µL of diluted sample, 3 wells per sample and leave overnight at 4°C. Place carbonate buffer in all wells not containing samples. A blank control and a positive control should be used with each plate.

3/ Wash 3 times with TTBS and leave the last washing solution in the wells for 3 - 5 minutes as a soaking step.

4/ Block wells with 200 µL 2% BSA or 2% Tween 20 (dilute in PBS/TBS) for 1 hour at room temperature.

5/ Wash 3 times with TTBS and leave the last washing solution in the wells for 3 - 5 minutes as a soaking step.

6/ Add 100 µL of monoclonal antibody to scup P450 IgG (diluted 1:40 in 1% BSA/PBS) to all wells. Wrap the microtitre plates in aluminium foil and incubate at 4°C overnight.

7/ Wash 3 times with TTBS and leave the last washing solution in the wells for 3 - 5 minutes as a soaking step.

8/ Add 100µL of secondary antibody (rabbit-anti-mouse IgG) diluted 1:300 in 1% BSA/PBS. Wrap plates in aluminium foil and leave at room temperature for 1 hour.

9/ Wash 5 times with TTBS and leave the last washing solution in the wells for 3 - 5 minutes as a soaking step.

10/ Develop colour with 1 tablet OPD (O-phenylenediamine) in 37.5 mL sodium citrate/phosphate buffer + 15µL H_2O_2 until reaction looks stable ~10 - 15 minutes.

11/ Stop reaction by adding 50µL 4N H_2O_2 to each well.

12/ Read the absorbance at 492nm on a Titretek Multiscan Plus Mk 2 ELISA plate reader. To calculate optical density of the samples subtract the absorbance of the blank wells.

2.3.1 Solutions For ELISA

PBS - Phosphate Buffered Saline

$Na_2HPO_4.2H_2O$	1.7g	
KH_2PO_4	0.5g	
NaCl	17.0g	
Distilled water	2 L	pH 7.2

Coating Buffer

$NaHCO_3$	9.5g	
Distilled water	1 L	pH 9.5

TBS - TRIS-Buffered Saline

Trisma base	4.84g	
NaCl	58.48g	
Distilled water	2 L	pH 7.5

TTBS

Tween 20	1 mL
TBS buffer	2 L

TMB Buffer - sodium citrate/phosphate buffer

0.2M aqueous Na_2HPO_4	25.7 mL	
0.1M Citric Acid	24.3 mL	
Distilled water	50 mL	pH 5.0

2.4 Solutions and Methods for Histology

2.4.1 Gill's Haematoxylin

(Stevens 1991)

Distilled water	730 mL
Ethylene glycol	250 mL
Haematoxylin (anhydrous)	2.0g
$Al_2(SO_4)_3.18H_2O$	17.6g
Glacial acetic acid	20 mL
Sodium iodate	0.2g

Method

Dissolve Haematoxylin in Ethylene Glycol and water using heat and a magnetic stirrer. When dissolved add Sodium Iodate then add other ingredients.

2.4.2 Eosin

(Stevens 1991)

1% Aqueous eosin Y	100mL
1% Aqueous phloxine	10mL
Ethanol 95%	750mL
Glacial acetic acid	4mL

2.4.3 Haematoxylin And Eosin Staining Method

(Stevens 1991)

Sections to water	Xylene	2 mins
	Xylene	2 mins
	Absolute Alcohol	30 sec
	Absolute Alcohol	30 sec
	70% Alcohol	30 sec
	Water	
Gill's haematoxylin	3 min	
Wash in running water		
Wash in Scott's tap water	1 min	
Wash in running water	1 min	
70% Alcohol	30 sec	
1% Eosin	3 min	
Differentiate in 70% alcohol	30 sec	
Dehydrate in 70% alcohol	15 sec	
Absolute Alcohol	30 sec	

Absolute Alcohol	30 sec
Clear in xylene	30 sec
Xylene	30 sec
Mount in DPX	

2.4.4 Periodic Acid- Schiff (PAS) Method

(Cook 1991)

Sections to water	As Above
1% aqueous Periodic acid	10 min
Wash in running water	5 min
Schiff reagent	30 min
Sulphite rinse 1	2 min
Sulphite rinse 2	3 min
Sulphite rinse 3	2 min
Wash in running water	10 min
Gill's Haematoxylin	30 sec
Wash in running water	30 sec
Scotts tap water	30 sec
Wash in running water	3 min
Dehydrate, clear and mount	As Above

Results:

PAS positive substances	Bright red
Nuclei	Blue
Other tissue constituents	Pale blue

Sulphite Rinse

Distilled Water	92.5 mL
20% Potassium metabisulphite	2.5 mL
1N HCl	5.0 mL

2.5 Solutions and Methods for Transmission Electron Microscopy

2.5.1 Spurrs Resin

Embedding Kit - Probing and Structure

ERL 4206	10.0g
(Vinyl cyclohexene dioxide)	
DER 732	6.0g
(Diglycidal ether of polypropylene glycol)	
NSA	26.0g
(Nonenyl succinic anhydride)	
DMAE	0.36g
(Dimethylaminoethanol)	

2.5.2 Uranyl Acetate

0.5% $UO_2(C_2H_3O_2)_2.2H_2O$ in 30% ethanol

2.5.3 Reynolds Lead Citrate

Lead nitrate $Pb(NO_3)_2$	1.33g
Sodium citrate $Na_3(C_6H_5)_7.2H_2O$	1.76g
Distilled water	30 mL

Mix in 50 mL volumetric flask for 30 min.
Add 8.0 mL 1N NaOH
Distilled water to 50 mL mix gently.

APPENDIX 3

EROD RAW DATA

Table 3.1
3 Month Assay 1 0.5% Pulp Mill Effluent

Start: 7th February 1995 End:3rd May 1995

Fish N°	Tank N°	Treatment	Sex	EROD Activity pM/min/mg protein
1	1	C	?	1.216
2	1	C	F	0.237
3	1	C	F	0.248
4	1	C	F	0.048
5	1	C	F	0.118
6	3	C	M	0.487
7	3	C	F	0.120
8	3	C	F	0.241
9	3	C	F	0.908
10	3	C	M	1.465
11	10	C	F	0.363
12	10	C	M	0.604
13	10	C	F	0.667
14	10	C	M	0.312
15	10	C	F	0.413
16	5	F	M	0.821
17	5	F	M	0.798
18	5	F	M	0.789
19	5	F	M	0.433
20	5	F	M	0.169
21	7	F	F	0.263
22	7	F	F	0.238
23	7	F	F	0.191
24	7	F	F	0.555
25	7	F	F	1.225
26	11	F	M	0.127
27	11	F	M	0.818
28	11	F	M	1.269
29	11	F	F	0.472
30	11	F	M	1.491
31	4	W	M	0.633
32	4	W	M	0.548
33	4	W	F	0.220
34	4	W	M	0.629
35	4	W	M	0.508

Fish N°	Tank N°	Treatment	Sex	EROD Activity pM/min/mg protein
36	6	W	F	0.523
37	6	W	M	3.464
38	6	W	M	0.762
39	6	W	M	0.215
40	6	W	F	0.781
41	9	W	F	0.857
42	9	W	M	1.646
43	9	W	M	0.203
44	9	W	F	0.685
45	9	W	F	0.623
46	2	FW	M	0.888
47	2	FW	F	8.321
48	2	FW	F	0.782
49	2	FW	F	0.956
50	2	FW	F	0.827
51	8	FW	F	4.139
52	8	FW	F	0.843
53	8	FW	F	0.419
54	8	FW	M	1.065
55	8	FW	?	1.838
56	12	FW	M	1.420
57	12	FW	F	5.985
58	12	FW	M	1.639
59	12	FW	M	2.283
60	12	FW	F	1.751

Statistical Analysis of 3 Month Assay 1

Response: EROD

Summary of Fit

Rsquare	0.285337
Root Mean Square Error	1.30826
Mean of Response	1.04265
Observations (or Sum Wgts)	60

Lack of Fit

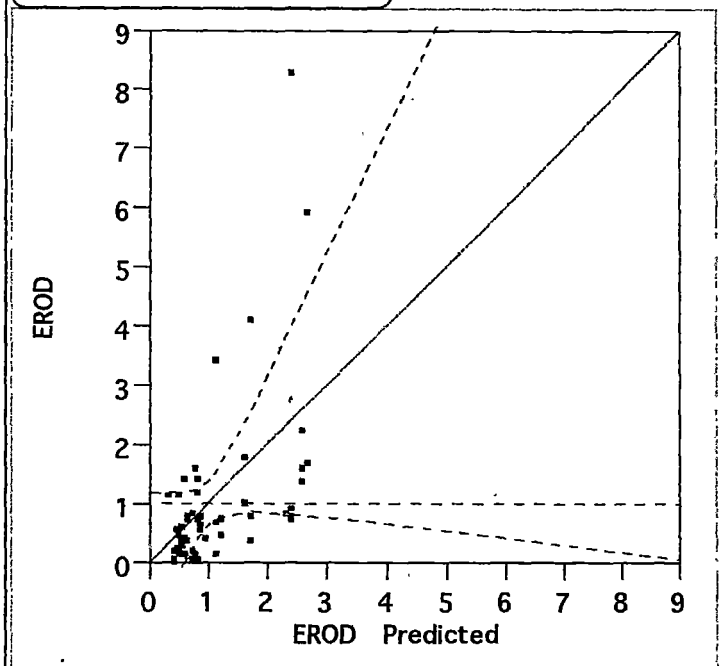
Parameter Estimates

Singularity Details

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F	
TREATMENT	3	0	0.0000000	•	•	LostDFs
TANK No	11	8	4.0484703	0.2957	0.9639	LostDFs
SEX	1	1	0.1006671	0.0588	0.8094	

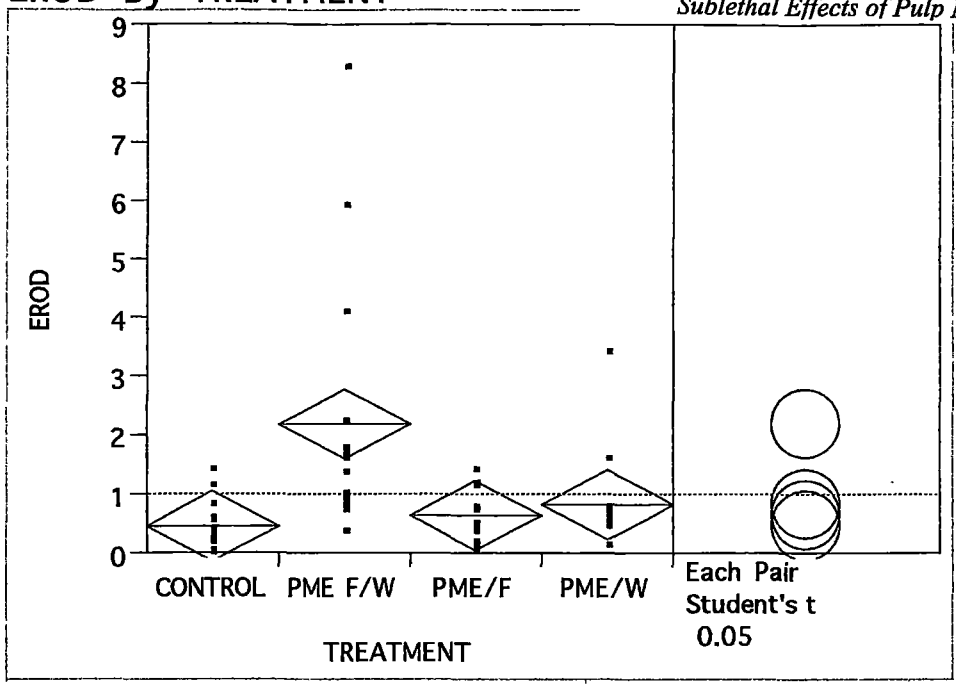
Whole-Model Test



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	12	32.11750	2.67646	1.5638
Error	47	80.44257	1.71154	Prob>F
C Total	59	112.56007		0.1357

EROD By TREATMENT



Means

Summary of Fit

Rsquare	0.249279
Root Mean Square Error	1.228393
Mean of Response	1.04265
Observations (or Sum Wgts)	60

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	28.05890	9.35297	6.1983
Error	56	84.50117	1.50895	Prob>F
C Total	59	112.56007		0.0010

Mean Estimates

Level	number	Mean	Std Error
CONTROL	15	0.49647	0.31717
PME F/W	15	2.21040	0.31717
PME/F	15	0.64393	0.31717
PME/W	15	0.81980	0.31717

Means Comparisons

Dif=Mean[i]-Mean[j]	PME F/W	PME/W	PME/F	CONTROL
PME F/W	0.00000	1.39060	1.56647	1.71393
PME/W	-1.39060	0.00000	0.17587	0.32333
PME/F	-1.56647	-0.17587	0.00000	0.14747
CONTROL	-1.71393	-0.32333	-0.14747	0.00000

Alpha= 0.05

Comparisons for each pair using Student's t

t
2.00324

Abs(Dif)-LSD	PME F/W	PME/W	PME/F	CONTROL
PME F/W	-0.89854	0.492055	0.667922	0.815389
PME/W	0.492055	-0.89854	-0.72268	-0.57521
PME/F	0.667922	-0.72268	-0.89854	-0.75108
CONTROL	0.815389	-0.57521	-0.75108	-0.89854

Positive values show pairs of means that are significantly different.

Table 3.2
7 Week Assay 2 Common Jollytails 0.5% Pulp Mill Effluent

Start: 22nd May 1995 End: 29th June 1995

Fish N°	Tank N°	Treatment	Sex	EROD Activity pM/min/ mg protein
C1	3	Control	F	0.798
C2	3	Control	F	1.38
C3	3	Control	M	2.76
C4	3	Control	M	1.118
C5	5	Control	F	0.57
C6	5	Control	F	1.078
C7	5	Control	M	0.53
C8	5	Control	F	1.56
C9	5	Control	F	0.71
C10	3	Control	M	0.747
C11	7	Control	F	0.58
C12	7	Control	M	0.51
C13	7	Control	M	1.75
C14	7	Control	F	0.383
C15	7	Control	F	2.88
F1	6	Food	?	2.001
F2	6	Food	M	2.29
F3	6	Food	F	3.92
F4	6	Food	F	2.07
F5	10	Food	F	4.48
F6	10	Food	F	0.66
F7	10	Food	F	0.43
F8	10	Food	M	1.32
F9	10	Food	F	1.96
F10	10	Food	F	5.12
F11	11	Food	F	2.58
F12	11	Food	M	1.04
F13	11	Food	M	1.11
F14	11	Food	F	3.47
F15	11	Food	M	5.98
W1	4	Water	F	0.52
W2	4	Water	M	1.38
W3	4	Water	M	0.62
W4	4	Water	F	0.09
W5	4	Water	M	1.62
W6	9	Water	F	2.03
W7	9	Water	F	0.38

Fish N°	Tank N°	Treatment	Sex	EROD Activity pM/min/ mg protein
W8	9	Water	M	0.13
W9	9	Water	M	6.55
W10	9	Water	F	1.46
W11	12	Water	M	7.03
W12	12	Water	M	14.42
W13	12	Water	M	0.80
W14	12	Water	F	0.37
W15	12	Water	F	2.15
FW1	1	Food/Water	M	3.41
FW2	1	Food/Water	M	2.7
FW3	1	Food/Water	M	12.37
FW4	1	Food/Water	M	12.58
FW5	1	Food/Water	F	9.47
FW6	2	Food/Water	M	20.3
FW7	2	Food/Water	F	25.83
FW8	2	Food/Water	M	3.71
FW9	2	Food/Water	F	2.78
FW10	2	Food/Water	M	2.09
FW11	8	Food/Water	F	11.03
FW12	8	Food/Water	F	24.76
FW13	8	Food/Water	F	53.72
FW14	8	Food/Water	F	2.47
FW15	8	Food/Water	M	19.58

Statistical Analysis of 7 Week Assay 2

Response: EROD

Summary of Fit

Rsquare	0.396023
Root Mean Square Error	7.820332
Mean of Response	6.30175
Observations (or Sum Wgts)	60

Lack of Fit

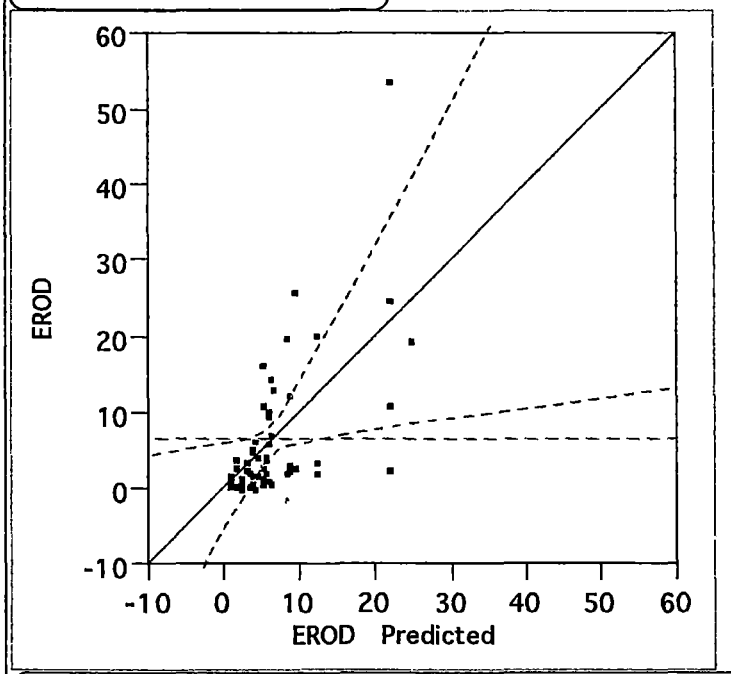
Parameter Estimates

Singularity Details

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F	
TREATMENT	3	0	0.00000	•	•	LostDFs
TANK	11	8	693.84907	1.4182	0.2139	LostDFs
SEX	1	1	107.75347	1.7619	0.1908	

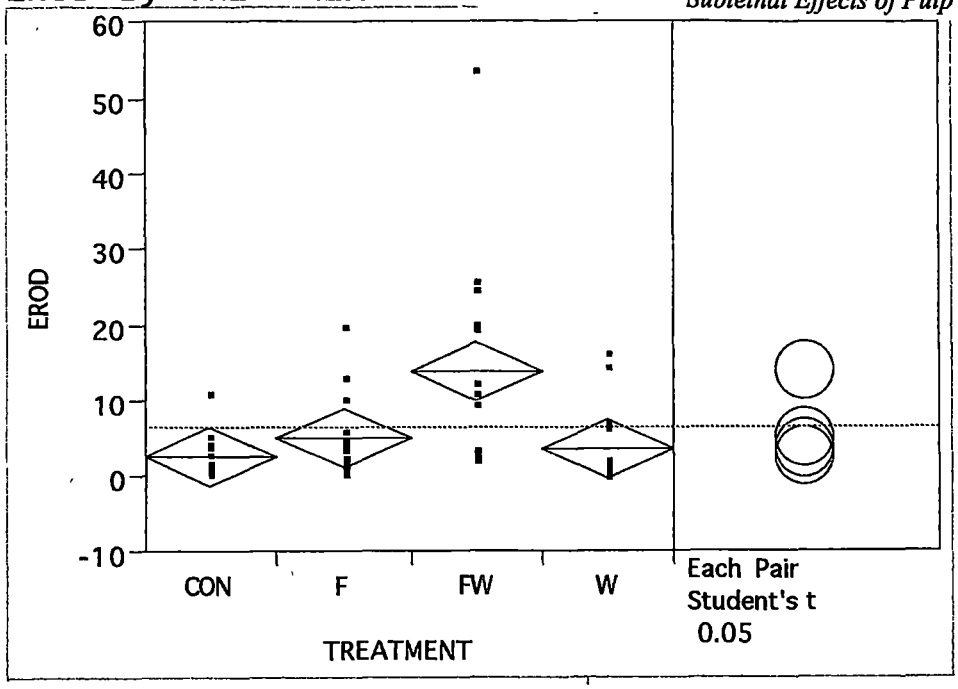
Whole-Model Test



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	12	1884.7287	157.061	2.5681
Error	47	2874.4071	61.158	Prob>F
C Total	59	4759.1358		0.0105

EROD By TREATMENT



Means

Summary of Fit

Rsquare	0.246133
Root Mean Square Error	8.004192
Mean of Response	6.30175
Observations (or Sum Wgts)	60

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	1171.3787	390.460	6.0945
Error	56	3587.7571	64.067	Prob>F
C Total	59	4759.1358		0.0012

Mean Estimates

Level	number	Mean	Std Error
CON	15	2.6261	2.0667
F	15	5.1823	2.0667
FW	15	13.7901	2.0667
W	15	3.6085	2.0667

Means Comparisons

Dif=Mean[i]-Mean[j]	FW	F	W	CON
FW	0.0000	8.6077	10.1816	11.1639
F	-8.6077	0.0000	1.5739	2.5562
W	-10.1816	-1.5739	0.0000	0.9823
CON	-11.1639	-2.5562	-0.9823	0.0000

Alpha= 0.05

Comparisons for each pair using Student's t

t
2.00324

Abs(Dif)-LSD	FW	F	W	CON
FW	-5.85490	2.75283	4.32670	5.30903
F	2.75283	-5.85490	-4.28104	-3.29870
W	4.32670	-4.28104	-5.85490	-4.87257
CON	5.30903	-3.29870	-4.87257	-5.85490

Positive values show pairs of means that are significantly different.

Table 3.3
3 Month Assay 3 Pulp Mill Effluent 0.5%

Live feed replaced by trout pellets + 0.5% effluent
Start: 28th August 1995 End: 4th December 1995

Fish N°	Tank N°	Treatment	Sex	EROD Activity pM/min/mg protein
C1	3	C	F	0.242
C2	3	C	F	0.067
C3	3	C	F	0.115
C4	3	C	F	0.071
C5	3	C	F	0.186
C6	4	C	F	0.091
C7	4	C	F	0.109
C8	4	C	M	0.356
C9	4	C	F	0.254
C10	4	C	M	0.247
C11	10	C	F	0.152
C12	10	C	F	0.309
C13	10	C	F	0.155
C14	10	C	F	0.163
C15	10	C	M	0.530
F1	1	F	M	0.275
F2	1	F	M	0.339
F3	1	F	F	0.171
F4	1	F	F	0.151
F5	1	F	M	0.254
F6	2	F	F	0.138
F7	2	F	F	0.071
F8	2	F	F	0.163
F9	2	F	F	0.128
F10	2	F	F	0.226
F11	5	F	M	0.306
F12	5	F	F	0.213
F13	5	F	F	0.639
F14	5	F	F	0.173
F15	5	F	M	0.123
W1	6	W	M	0.375
W2	6	W	F	0.151
W3	6	W	F	0.739
W4	6	W	F	0.139
W5	6	W	M	0.405
W6	8	W	F	0.198

Fish N°	Tank N°	Treatment	Sex	EROD Activity pM/min/mg protein
W7	8	W	M	0.432
W8	8	W	F	0.503
W9	8	W	F	0.275
W10	8	W	F	0.144
W11	12	W	F	0.122
W12	12	W	M	0.130
W13	12	W	M	0.724
W14	12	W	M	0.318
W15	12	W	M	1.773
FW1	7	FW	F	0.747
FW2	7	FW	F	0.128
FW3	7	FW	M	0.234
FW4	7	FW	M	0.294
FW5	7	FW	F	0.142
FW6	9	FW	F	0.500
FW7	9	FW	F	0.167
FW8	9	FW	F	0.177
FW9	9	FW	M	0.539
FW10	9	FW	F	0.345
FW11	11	FW	F	0.872
FW12	11	FW	M	1.060
FW13	11	FW	M	0.281
FW14	11	FW	F	0.593
FW15	11	FW	F	0.374

Statistical Analysis of 3 Month Assay 3

Response: EROD

Summary of Fit

Rsquare	0.21403
Root Mean Square Error	0.262636
Mean of Response	0.32165
Observations (or Sum Wgts)	60

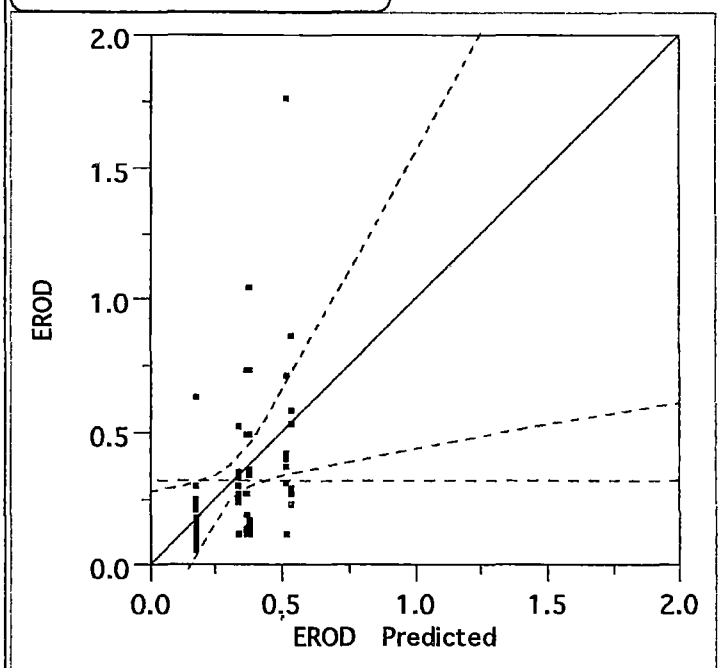
Lack of Fit

Parameter Estimates

Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
TREATMENT	3	3	0.52043268	2.5150	0.0678
SEX	1	1	0.33299371	4.8276	0.0322

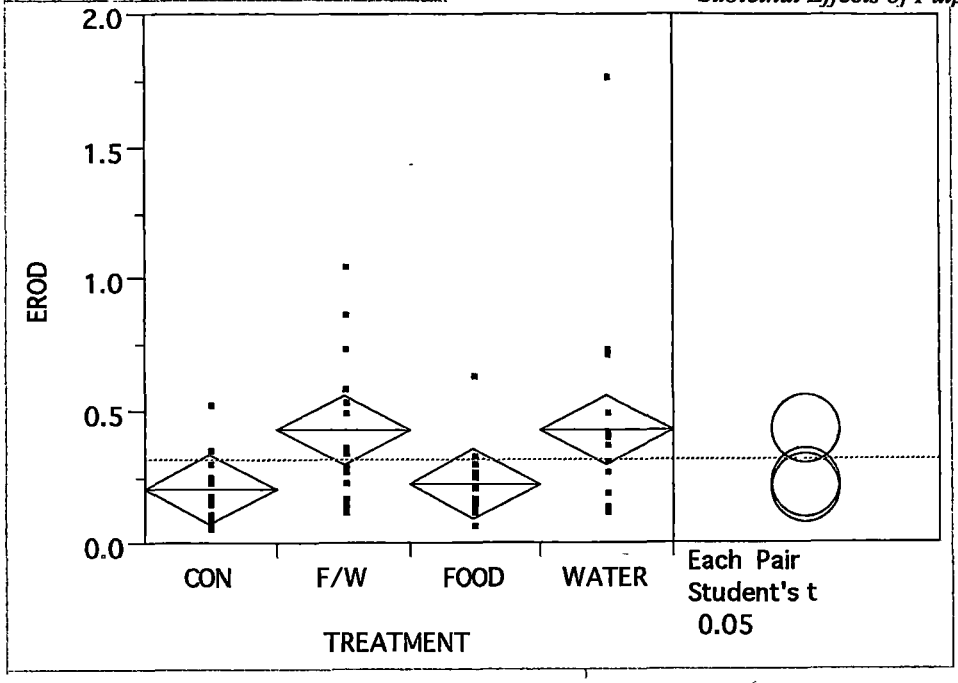
Whole-Model Test



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	1.0330976	0.258274	3.7443
Error	55	3.7937760	0.068978	Prob>F
C Total	59	4.8268736		0.0092

EROD By TREATMENT



Means

Summary of Fit

Rsquare	0.145043
Root Mean Square Error	0.271463
Mean of Response	0.32165
Observations (or Sum Wgts)	60

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.7001039	0.233368	3.1668
Error	56	4.1267697	0.073692	Prob>F
C Total	59	4.8268736		0.0313

Mean Estimates

Level	number	Mean	Std Error
CON	15	0.203133	0.07009
F/W	15	0.430267	0.07009
FOOD	15	0.224667	0.07009
WATER	15	0.428533	0.07009

Means Comparisons

Dif=Mean[i]-Mean[j]	F/W	WATER	FOOD	CON
F/W	0.000000	0.001733	0.205600	0.227133
WATER	-0.00173	0.000000	0.203867	0.225400
FOOD	-0.2056	-0.20387	0.000000	0.021533
CON	-0.22713	-0.2254	-0.02153	0.000000

Alpha= 0.05

Comparisons for each pair using Student's t

t
2.00324

Abs(Dif)-LSD	F/W	WATER	FOOD	CON
F/W	-0.19857	-0.19684	0.007030	0.028563
WATER	-0.19684	-0.19857	0.005297	0.026830
FOOD	0.007030	0.005297	-0.19857	-0.17704
CON	0.028563	0.026830	-0.17704	-0.19857

Positive values show pairs of means that are significantly different.

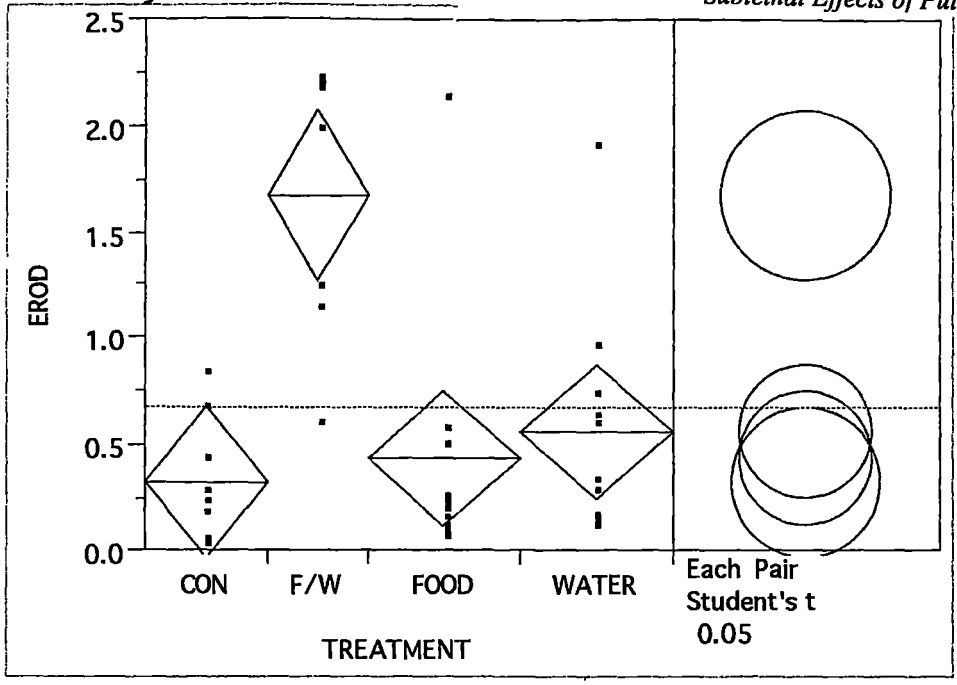
Table 3.4
6 Week Blenny Assay 1% Pulp Mill Effluent

Start: 23rd October 1995 End: 30th November 1995

Fish N°	Treatment /Tank	Sex	Stage	EROD Activity pM/min/mg protein
1	C1	M	III	0.685
2	C1	F	IV	0.060
3	C1	F	IV	0.251
4	C1	F	IV	0.048
5	C1	F	I	0.070
6	C3	F	IV	0.853
7	C3	F	IV	0.299
8	C3	F	IV	0.199
9	C3	F	II	0.451
10	C3	M	II	Not Tested
11	F2	F	IV	0.203
12	F2	M	II	0.238
13	F2	F	II	2.159
14	F2	F	II	0.276
15	F2	M	II	0.517
16	F6	F	IV	0.585
17	F6	F	II	0.245
18	F6	M	II	0.114
19	F6	F	IV	0.128
20	F6	F?	II	0.087
21	F6	M	II	0.169
22	W4	F	III	0.973
23	W4	F	IV	0.131
24	W4	M	III	0.747
25	W4	M?	III	1.927
26	W4	F	II	0.653
27	W4	F	IV	0.184
28	W8	F	IV	0.155
29	W8	M	III	0.354
30	W8	F	IV	0.189
31	W8	F	IV	0.293
32	W8	F	II	0.614
33	FW5	F	IV	2.008
34	FW5	M	III	0.615
35	FW5	M	III	2.186
36	FW7	M	II	2.246
37	FW7	M	III	2.213
38	FW7	F	IV	1.162

Fish N°	Treatment /Tank	Sex	Stage	EROD Activity pM/min/mg protein
39	FW7	F	IV	1.254
40	FW7	F	IV	Not Tested

EROD By TREATMENT



Means

Summary of Fit

Rsquare	0.479406
Root Mean Square Error	0.530725
Mean of Response	0.672368
Observations (or Sum Wgts)	38

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	8.819063	2.93969	10.4367
Error	34	9.576746	0.28167	Prob>F
C Total	37	18.395809		0.0001

Mean Estimates

Level	number	Mean	Std Error
CON	9	0.32400	0.17691
F/W	7	1.66914	0.20060
FOOD	11	0.43000	0.16002
WATER	11	0.56545	0.16002

Means Comparisons

Dif=Mean[i]-Mean[j]	F/W	WATER	FOOD	CON
F/W	0.00000	1.10369	1.23914	1.34514
WATER	-1.10369	0.00000	0.13545	0.24145
FOOD	-1.23914	-0.13545	0.00000	0.10600
CON	-1.34514	-0.24145	-0.10600	0.00000

Alpha= 0.05

Comparisons for each pair using Student's t

t
2.03223

Abs(Dif)-LSD	F/W	WATER	FOOD	CON
F/W	-0.57651	0.582213	0.717668	0.801602
WATER	0.582213	-0.4599	-0.32444	-0.24332
FOOD	0.717668	-0.32444	-0.4599	-0.37877
CON	0.801602	-0.24332	-0.37877	-0.50844

Positive values show pairs of means that are significantly different.