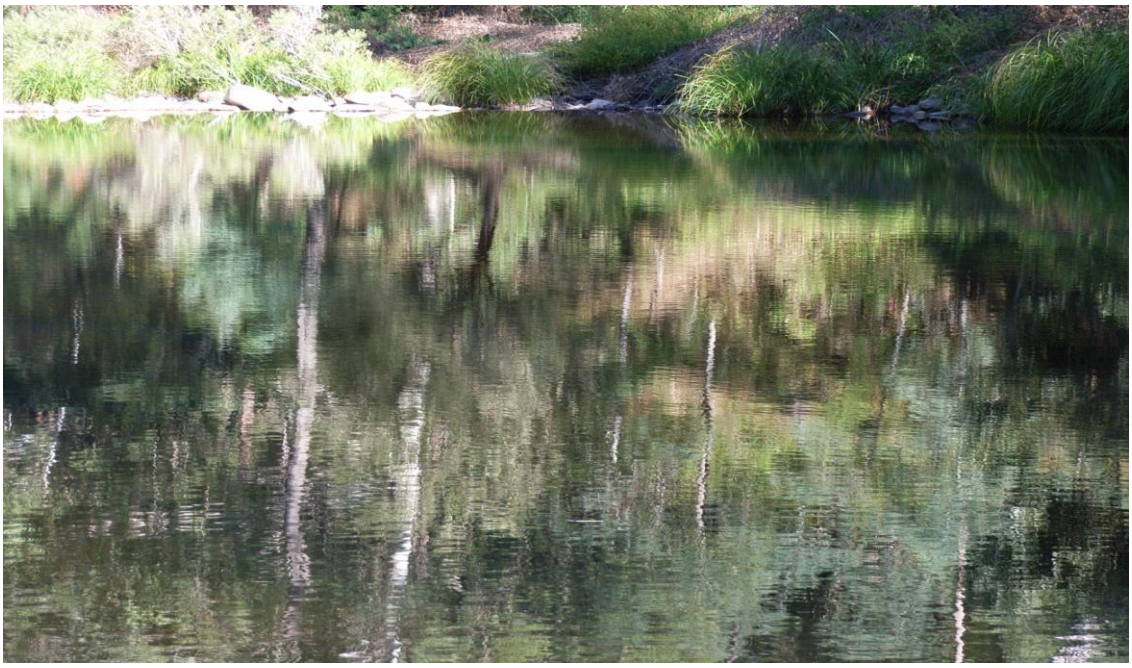
The Effects of Pulse Exposure of Six Agricultural Chemicals (including four herbicides used by G-MW) on the Early Life Stages of Selected Native Fish from the Goulburn-Murray River Region



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Glossary

48 hour LC_{50}: The concentration of the test chemical that results in 50% mortality of the test organisms in 48 hours.

96 hour LC_{50}: The concentration of the test chemical that results in 50% mortality of the test organisms in 96 hours.

Acute toxicity: Adverse effects occurring within a short time of administration of a single dose of a chemical, or immediately following short or continuous exposure, or multiple doses over 24 hours or less

ANZECC : Australia and New Zealand Environment Conservation Council

ARMCANZ: Agriculture and Resource Management Council of Australia and New Zealand

ASTM: American Society for the Testing of Materials

Chronic toxicity: Adverse effects occurring as a result of repeated dosing with a chemical on a daily basis, or exposure to the chemical, for a large part of an organism's lifespan (usually more than 50%).

Comatose: In a state of lethargy, with no movement.

Control: A test medium that does not contain the test chemical but is in all other respects identical to the test solutions.

Early life stage: A stage in the development of an organism which is within the first 10% of its total life span

Ecotoxicology: Ecotoxicology is the study of the harmful effects of chemical compounds on species, population and the natural environment. Or "the study of the harmful effects of chemicals upon ecosystems" (Walker *et al,* 1996)

Endpoints: Time or data points selected for assessment

Half life: the period of time that must elapse for a pesticide to lose half of its original toxicity or effectiveness.

Hydrolysis: breakdown of a chemical in the presence of water?

LC_{50}: Lethal $Concentration_{50}$ A concentration of a pollutant or effluent at which 50% of the test organisms die; a common measure of acute toxicity

LD_{50}: Lethal $Dose_{50}$ A dose of a toxicant at which 50% of the test organisms die; a common measure of acute toxicity when the toxicant is ingested or injected.

Lethal: causing death

MSDS: Material Safety Data Sheet

Macrocosm: A tests system/container that replicates a complete functioning ecosystem in roughly the appropriate proportions on a macroscopic or large scale.

Microcosm: A tests system/container that replicates a complete functioning ecosystem in roughly the appropriate proportions on a small scale.

NOEL: No observed effect level

NOEC: No observed effect concentration

Photolysis: breakdown of a chemical by light

Pulse exposed: exposed to the test chemical for a finite short duration and then transferred into control media.

Sympatric: of biological species or speciation occurring in the same or overlapping geographical areas

Toxicants: substances that is harmful to living organisms

Twitching: making small uncoordinated jerking movements

Sub-lethal: below the level which directly results in death.

WLW: Wet Lab Water. This is tap water that has been carbon filtered and aerated.

Executive summary

A series of ecotoxicological experiments were carried out to assess the lethal and sub-lethal effects of six agricultural chemicals including four aquatic herbicides and two other pesticides (used in the G-MW region) on two species of selected Australian native fish. These herbicides were glyphosate, amitrole, 2,4-D amine and acrolein which are commonly used by Goulburn -Murray Rural Water Authority to control aquatic plants in channels and drains. Two other pesticides that were regularly detected in G-MW irrigation channels (2004-2006) i.e. endosulfan and copper were also tested. There were no previous data on the toxicity of herbicides used by G-MW on native fish and an auditor while reviewing G-MW herbicides usage data recommended the comparison of published toxicity data on alien species with that of Australian native fish.

Early life stage (< 2 days old larvae) of two native fish including the iconic Australian species Murray Cod (*Maccullochella peellii peelii*) and the sensitive native fish Murray River Rainbowfish (*Melanotaenia fluviatilis*) were selected for a series of ecotoxicological experiments. All experiments were conducted at the RMIT University purpose built ecotoxicological laboratory following the American Society of Testing Materials (ASTM) protocols for independent treatments.

Larval fish were exposed to a variety of concentrations (1, 10, 100, 1000 and 10,000 µg/L) of six agricultural chemicals (referred to as 'toxicants' in this report) for 24 hours and then placed in a toxicant free medium (i.e. pulse-exposed). The main objectives of this study were to observe and record the effects of these toxicants on the early life stages of Murray cod and Murray River rainbowfish in relation to some selected end points including survival and mortality, growth and development (total length, yolk sac length, heart rate) and other behavioural responses at 24, 48, 72 and 96 hours.

The study found that acrolein can significantly affect the survival of Murray cod larvae, as 100% mortality was recorded when fish were exposed to acrolein at 10,000 µg/L (12h). However the study also recorded insignificant numbers of cod deaths with acrolein at other concentrations. The other effects due to acrolein were reduction in fish length, yolk sac length, and heart rate. On the other hand, there was virtually no effect on Murray cod larvae from exposure to other herbicides such as glyphosate, 2,4-D amicide and amitrole at the concentrations tested. All Murray cod larvae died from exposure to copper at 10,000 µg/L but no other behavioural or morphological variations were observed following exposure to other concentrations. Endosulfan did not result in any deaths in Murray cod larvae, but caused a significant reduction in cod growth due to spinal curvature.

In contrast, Murray River rainbowfish larvae were found to be very sensitive to pulse-exposed endosulfan and acrolein at very low concentrations with mortality recorded as low as 1 µg/L. Rainbowfish exposed to endosulfan and acrolein at the tested concentrations were also lethargic, motionless, comatose, or twitching.

Based on these preliminary experiments, the 96 hour LC_{50} on fish larvae could be estimated, With acrolein following a 24 hour pulse exposure for Murray cod, the 96 hour LC_{50} was >1000 and <10,000µg/L and for Murray River rainbowfish it was <10

µg/L while for endosulfan 96 hour LC_{50} was >10,000µg/L for Murray cod and for Murray River rainbowfish it was also <10µg/L. The current experimental results therefore indicate that the Murray River rainbowfish is very sensitive to pesticides, while Murray cod is a moderately hardy fish (in larval stage) and less sensitive to pesticides. The 96 hour LC_{50} values for Northern hemisphere fish, such as rainbow trout in continuous exposure were reported to be 150 µg/L for acrolein and 2 µg/L for endosulfan respectively.

The larger yolk sac and thicker yolk membrane in Murray cod larvae may play a key role in minimizing the toxic effects of herbicides and pesticides on this species whereas Murray River rainbow fish have a smaller yolk sac and much thinner yolk membrane which may have provided less protection from the toxicants.

This study generated for the first time ecotoxicological data for the iconic Australian native fish Murray cod i.e. lethal and sub-lethal effects of a range of agricultural chemicals on Murray cod. The data shows that larval Murray cod are less sensitive to the tested chemicals (in particular to acrolein, endosulfan and copper) than alien fish species

This preliminary study demonstrates the effects of individual herbicides and pesticides on Murray cod and Murray River rainbowfish. However, in natural waterways they are generally exposed to a mixture of chemicals simultaneously. Therefore, it is recommended that further research should be conducted using mixtures of G-MW herbicides and pesticides identified in G-MW irrigation areas to evaluate their effects on sensitive native fish such as rainbowfish.

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Introduction

Goulburn-Murray Rural Water Authority (G-MW) uses a number of herbicides to control aquatic weeds in channels, drains and natural waters within the Goulburn-Murray River region in northern Victoria. The lack of toxicity data on the effects of these herbicides on native Australian fish has resulted in the need for an ecotoxicological investigation into their possible effects. The four herbicides investigated include: acrolein (Magnacide H), 2,4,D amine (Amicide LO500A), amitrole (Amitrole T), and glyphosate (Roundup active). In addition, two insecticides (endosulfan and copper sulfate) are included in the investigation as a result of G-MW's concern for their toxic effects on the early life stages of native fish in the region. Both endosulfan and copper were regularly detected within G-MW irrigation channels during 2004-05 and 2005-06 irrigation season (see Rose and Kibria 2006).

(1) **Literature Review**: This consists of background information compiled on the toxicity of the selected toxicants (i.e. four herbicides and two pesticides) on native and alien fish species.

(2) **Laboratory Testing**: This contains a comprehensive report on the tests conducted using the four herbicides and two pesticides on Murray cod larvae and the two most toxic pesticides (acrolein and endosulfan) on Murray River rainbow fish; with details of the methods used, results obtained and a discussion to interpret the experimental results.

(1) Literature review

1.1 Toxicants

The physico-chemical properties and toxicity information of the six agricultural chemical are summarized in Appendix 1 . A brief literature review of each chemical is given below.

1.1.1 (Magnacide H; Acrolein 950 g/kg)

Acrolein is very toxic to both humans and aquatic organisms. Approximately 5445 kg of acrolein in the form of Magnacide H was applied to waterways within the G-MW catchment in 2001 predominantly for the control of submersed weeds such as ribbonweed, pondweeds and Elodea (G-MW, 2005). Chronic exposure of humans to acrolein is reported to result in the development of permanent lung damage in the form of decreased pulmonary function, and delayed pulmonary oedema that can lead to chronic pulmonary disease (ref??) . In addition, acrolein has been reported to induce a variety of genetic changes and at least three studies have reported it to be mutagenic in the Ames test.

Aquatic toxicity data indicate that it is highly toxic to fish with 96 h LC_{50} s of 24 µg/L in the bluegill (*Lepomis macrochirus*) and rainbow trout (*Onchorynchus mykiss*) and 570 µg/L in the sheepshead minnow (Table 1.1). This high toxicity has also been recorded in aquatic invertebrates, with a 48 h LC_{50} of 22 µg/L in *Daphnia magna* (Baker Hughes, MSDS, 2005). "The product 'directions for use' label states that it is toxic to fish and wildlife and must be kept out of rivers, lakes, ponds and streams and that fish, shrimps, and other aquatic species will be killed at recommended application rates". In the case of irrigation, water from treated channels must remain in the irrigation system for a total of 72 hours from the time an injection has ceased. The significant acute toxicity of environmentally realistic concentrations

of Magnacide H to northern hemisphere fish has emphasizes the need to determine if native fish species are similarly affected.

1.1.2. (**Amicide 2,4-D 625 g/L)**

Approximately 10,100 L of the herbicide 2,4-D amine (Amicide LO500A) was applied in the G-MW region in 2001 to control emergent weeds including arrowhead and some milfoils (G-MW, 2005). According to the MSDS of amicide supplied by Nufarm, 2,4-D amicide (625 g/L-low selective herbicide) does not appear to pose any threat to fish other than in very high concentrations, with an LC_{50} approximating 100 mg/L in rainbow trout. Boyle (1980) reported that 2,4-D (amicide) applied to ponds at rates of 5 and 10 kg/ha increased the survival and growth rates of bluegills (*L. macrochirus*) possibly via the stimulation of phytoplankton productivity. George and Hingorani (1982) reported that 2,4-D was not known to accumulate through the food web and was provisionally approved for use against freshwater weeds in India. They reported the 38 and 21 h LC_{50} s for the rotifer (*Brachionus calyciflorus*) and the daphnid (*Daphnia lumholtzi*) exposed to 2,4-D at 5 and 20 ppm, respectively (Table 1.1). Investigations into the toxicity of 2,4-D in the freshwater fish *Channa punctatus* found that concentrations as high as 75 ppm did not significantly increase mortality (Abul Farah *et al*., 2003). It is the aim of this investigation to determine if the high tolerance northern hemisphere species to 2,4-D is similar to Australian native fish species.

1.1.3 Amitrole T

Amitrole T (3-amino-1,2,4-triazole) is a herbicide with a very wide spectrum of activity against annual and perennial broad leaf and grass type weeds acting via inhibition of the

caretenoid biosynthesis and has been recorded to cause possible ecotoxicological side effects (Oesterreich *et al*., 1999), with slight toxicity to fish and freshwater invertebrates (Dow Agrosciences, MSDS, 2003). The reported 'slight toxicity to fish' failed to mention concentration or species.

Amitrole is widely used on fallow land prior to sowing, along roadsides and railways and for weed control in ponds (WHO, 1994). In the G-MW region the 28,666 L of Amitrole used in 2001 was for the control of water couch, barnyard grass, umbrella sedge and some broadleaf plants predominantly in drains and dry land situations (G-MW, 2005). In water amitrole does not breakdown by hydrolysis or photolysis, nor does it volatilize bioaccumulate in aquatic organisms. The biodegradation half-life is about 40 days. Degradation in open waters may occur through oxidation by other chemicals. In vegetation amitrole is readily absorbed and rapidly translocated in the roots and leaves of higher plants. Special care should be taken with the use of amitrole as it is considered by some authorities to be a suspected carcinogen (Dow Agrosciences, MSDS 2003). This investigation on the toxic effects of amitrole to Australian native fish species is of great interest to G-MW in light of the paucity of available information in the literature with respect to fish toxicity.

1.1.4 Glyphosate (weedmaster duo 360 g/L)

Glyphosate is a toxicant predominantly used for the control of terrestrial, emergent and floating vegetation. Glyphosate is characterized by a very specific action that blocks the synthesis of certain amino acids used in the construction of proteins. Glyphosate decomposes rapidly and is reported to be environmentally safe at concentrations used in the field (Anton *et al*., 1994; Franz *et. al*., 1997).

Glyphosate is used in the G-MW region to control grasses and some broadleaf plants such as cumbungi, water couch, and for the temporary control of arrowhead. Approximately 38,560 L of glyphosate, as Roundup Active ™, was applied to drains and surrounding areas in the G-MW region in 2001 (G-MW, 2001). The reported LC_{50} of 109 mg/L in rainbow trout (Anton *et al*,.1994)suggests that it does not pose an acute threat to fish. Rendon-von Osten *et al*., (2005) reported the 96 h LC_{50} for mosquito fish exposed to glyphosate to be 18 mg/L while Szarek *et al*., (2000) found that all carp, *Cyprinus carpio*, exposed to 205 and 410 mg/L glyphosate as formulated Roundup ™, died within an hour following exposure (Table 1.). Wan *et al.* (1989) found that the toxicity of glyphosate and its formulations depends on the type of dilution water type, with water hardness and pH to be the most contributing factors used. The observed variation of the 96 h LC_{50}s for glyphosate and its formulations were within an order of magnitude irrespective of water types. ANZECC& ARMCANZ (2000) has set the maximum recommended level of glyphosate contamination in the aquatic environment at 370 mg/L. They argue that at this level, 99% of Australian aquatic freshwater life will be protected from chronic toxicity.

1.1.5 Copper

Copper is a trace metal that finds its way into aquatic environments as a result of discharged industrial effluents, vehicle emissions, and from crops where it is used as an insecticide. In the G-MW region of eastern Australia approximately 55,000 kg of copper hydroxides were released to control a range of pests on terrestrial plants including various forms of spot, blight, rot, canker and downy mildew (G-MW, 2005). The regular detection of copper (range detected <0.9-4.2 µg/L) in waterways within the G-MW region (see Rose and Kibria, 2006) indicates that it has been transported from their original application sites into the aquatic environment.

While copper is an essential trace-element for a variety of metabolic processes, it can be extremely toxic to aquatic organisms including fish (Yang and Chen, 1996; Matsuo *et al*., 2005). The main route of copper toxicity in fish is reported to be the binding of copper to target sites at the gills and in the intestine (Di Toro *et al*., 2003; Grosell *et al*., 2002). Copper exposure clearly results in rapid copper accumulation by the gill followed by disturbance of especially Na^+ and Cl^- homeostasis, leading to osmoregulatory failure (Lauren & McDonald, 1987; Wood, 2001). Matsuo *et al*., (2005) and Grosell *et al*., (2002) reported that freshwater tambaqui (*Colossoma macropmum*) and the marine gulf toadfish (*Opsanus beta*) respectively were both very tolerant to the effects of copper exposure (Table 1.1). Kamunde and Wood (2003) reported a 96 h LC_{50} ranging from 0.17 to 0.21 µmol/L (10.52 – 13.2 µg/L) for rainbow trout exposed to waterborne copper and suggested that growth was not a sensitive endpoint for detection of copper exposure at these concentrations. The 24 to 96 h LC_{50} s on copper toxicity to silver bream, *Sparus sarba*, ranged from 1 to 2 mg/L (Wong *et al*., 1999) indicating that marine fish may be more tolerant to copper exposure compared with freshwater teleosts.

1.1.6 Endosulfan (Thiodan 350 g/L)

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9 methano-2,4,3-benzodiexathiepin-3-oxide) is a broad spectrum, photostable chlorinated hydrocarbon insecticide of the cyclodiene group, developed by Farbwerke Hoechst in 1952. Approximately 10,000 tonnes of technical grade endosulfan were applied globally in 1992 (Naqvi and Vaishnavi, 1993) with approximately 700 tonnes applied to cotton alone in New South Wales during the 2000/2001cotton season (Broomhall, 2002). The World Health Organization (WHO, 1986) classified endosulfan as moderately hazardous based on an oral LD_{50} in the rat, while the United States Environmental Protection Agency (US EPA) classified endosulfan as highly hazardous (US EPA, 1991).

In Australia, the insecticide endosulfan is a potentially serious contaminant as large quantities are used and it is very toxic to fish, its toxicity being one of the highest of all the organochlorine insecticides (Schoettger, 1970). Endosulfan was used mainly by the cotton industry in northern New South Wales and southern Queensland at an application rate of 3 to 3.5 kg/ha per year (Colton and Cutting, 1985) and was responsible for approximately 70% of fish kills caused by pesticides (Mann, 1989; Napier *et al.*, 1998). The Department of Land and Water Contamination's (DLWC) Central and North West Regions Water Quality Program – Pesticide Monitoring 1998, determined that 65% of samples containing detectable endosulfan concentrations were above national water quality guidelines for protecting aquatic ecosystems. The report also showed that there had been no reduction in endosulfan concentrations over the years 1996-1998. It is apparent that 'unsafe' levels of endosulfan were entering the waterways of New South Wales (NSW) and Queensland during this period.

Endosulfan is ranked as the pesticide having the greatest impact on the Australian riverine ecosystem according to Leonard *et al.* (1999). Gormley and Teather (2003) reported that eggs of the Japanese medaka (*Oryzias latipes*) took longer to hatch and the resulting fry were smaller at 1 week of age and had decreased mobility at 2 weeks following exposure to endosulfan for 24 h (Table 1.1.). Additionally, they observed that fry exposed to endosulfan shortly following hatch did not differ from control mobility. Endosulfan was regularly detected in passive samplers (range 0.0015-0.029 µg/L) in G-MW water supply channels during 2004-06 (see Rose and Kibria, 2006).

Although the precise molecular mechanisms responsible for cyclodiene toxicity are still poorly understood, previous data suggest that they act by altering the electrophysiological and associated enzymatic properties of nerve cell membranes (Videira *et al.*, 2002), causing a change in the kinetics of Na^+ and K^+ ion flow through the membrane. This neurotoxic effect probably underlies the extreme sensitivity of fish and other aquatic species to endosulfan.

Table 1.1: Selected references on the toxicity of selected herbicides and insecticides to aquatic organisms.

Toxicant	**Species**	**Effect measured**	Parameter		**Reference**
			Test	Result	
Glyphosate 360	Rainbow trout *Oncorynchus mykiss*	Survival	96 h LC_{50}	109 mg/kg	Dow Agrosciences, MSDS 2003
Glyphosate	Mosquitofish *Gambusia yucatana*	Survival	96 h LC_{50}	17.79 mg/L	Rendon-von Osten *et al*., 2005
Glyphosate (formulated Roundup ™)	Carp *Cyprinus carpio*	Mortality	LC100	< 205 mg/L	Szarek *et al*., 2000
Glyphosate 360	Daphnia *Daphnia magna*	Survival	48 h LC_{50}	105 mg/L	Dow Agrosciences, MSDS 2003
Glyphosate commercial formulation 54.9% ai	Rainbow trout *Oncorhyncus mykiss*	Survival	96 hr NOEC LC_{50}	823.5 4290.8	Anton *et al, 1994*
Glyphosate (technical grade)	Coho *Oncorhynchus kisutch*	Survival (range in different types of dilution water)	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	44-210 27-205 27-182 27-174	Wan *et al., 1989*
Glyphosate (technical grade)	Chum *Oncorhynchus keta*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	16-202 13-178 10-157 10-148	Wan *et al., 1989*

Glyphosate (technical grade)	Chinook *Oncorhyncus tshawytsha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	24-220 22-220 22-211 19-211	Wan *et al., 1989*
Glyphosate (technical grade)	Pink salmon *Oncorhyncus gorbuscha*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	26-380 14-245 14-190 14-190	Wan *et al., 1989*
Glyphosate (technical grade)	Rainbow trout *Salmo gairdneri*	Survival	24 hr LC_{50} 48 hr LC_{50} 72 hr LC_{50} 96 hr LC_{50}	21-220 11-220 11-220 10-197	Wan *et al., 1989*
Copper	Silver sea bream *Sparus sarba*	Survival (fingerlings) Growth	24 h LC_{50} 48 h LC_{50} 72 h LC_{50} 96 h LC_{50} 30 days	2.01 mg/L 1.28 mg/L 1.17 mg/L 1.03 mg/L significant reduction	Wong *et al*., 1999

Copper	Rainbow trout *Onchorynchus mykiss*	Survival Growth (30 days)	96 h LC_{50} No effect	10.52 – 13.2 µg/L 0.24 µmol/g/fish/day	Kamunde and Wood, 2003
Copper hydroxides (Kocide)	Rainbow trout *Onchorynchus mykiss*	Survival	96 h LC_{50}	80	G-MW, 2005
Copper	Japanese eel, *Anguilla japonica*	Survival	96 h LC_{50}	0.06-0.34 mg/L	Yang and Chen, 1996 (in Wong *et al*., 1999)
Copper	Gulf toadfish *Opsanus beta*	Survival	96 h LC_{50}	> 340µM	Grosell *et al*., 2004
Copper	*Tambaqui Colossoma macropomum*	Survival	96 h LC_{50}	>400 µg/L	Matsuo *et al*., 2005
2,4-D	Rotifer (*Brachionus calyciflorus*) Daphnia (*Daphnia lumholtzi*)	Survival (Exposure to 5 ppm) Survival (Exposure to 10 ppm)	LT_{50} LT100 LT_{50} LT100	24 h 31 h 38 h 71 h	George and Hingorani, 1982.
2,4-D	Freshwater fish *Channa punctatus*	Survival	96 h LC_{50}	> 75 ppm	Abul Farah *et al*., 2003.
Endosulfan	Freshwater fish *Macrognathus aculeatum*	Survival	96 h LC_{50}	3.5 ppb	Rao *et al*., 1981.

Endosulfan	Eel *Anguilla anguilla*	Survival	96 h LC_{50}	0.041 mg/L	Gimeno *et al*., 1995
Endosulfan	Rainbow trout *Oncorynchus mykiss*	Survival	96 h LC_{50}	8 µg/L	G-MW, 2005
Endosulfan	Japanese medaka *Oryzias latipes*	Time to hatch Growth (1 week) Mobility (2 weeks)	N/A	Significantly longer Smaller fry Reduced mobility	Gormley and Teather, 2003
Endosulfan (Thiodan EC 35)	*Colisa* (trichogaster) *fasciatus*	Reproduction (adult females)	Altered	1 mg/L	Pandy, 1988
Magnacide H (Acrolein 950 g/kg)	Water flea *Daphnia magna* Mysid shrimp *Mysidopsis bahia* Mysid shrimp *Holmesimysis costrata* Marine copepod *Acartia tonsa* Bluegill sunfish *Lepomis macrochirus* Rainbow trout *Oncorynchus mykiss* Sheepshead minnow *Cyprinodon variegates*	Survival	96 h LC_{50}	22 µg/L 500 µg/L 790 µg/L 55 µg/L 24 µg/L 22 µg/L 570 µg/L	Baker Hughes, 2005

1.2. Fish in Ecotoxicology

Australian freshwater environments are unique as are the organisms that inhabit them (Williams and Wan, 1972; Sunderam *et al.*, 1992). In freshwater ecosystems, fish are often the main predators of vertebrate (e.g.: larvae of other fish) and invertebrate (e.g.: rotifers, daphnids) organisms and consequently play a key role in the maintenance and structure of communities and ecosystems (Sunderam et al, 1992). In general, fish increase the species diversity of the ecosystem, which may lead to increased community stability (Moulton, 1980). Consequently, the removal or depletion of fish populations from freshwater environments may have broad deleterious effects on sympatric aquatic organisms (Paine, 1969; Steen, 1969; Giesy, 1980; LaPoint and Fairchild, 1992).

The use of fish as indicators of toxicity in freshwater ecosystems has a long history (Hart *et al.* 1945; Doudoroff and Katz, 1950, 1953; Tarzwell, 1957; Sprague, 1992; Chapman, 1999 in Rand). However, the majority of data on freshwater fish is based on organisms from the northern hemisphere and is consequently not representative of the Australian lotic environment. The paucity of ecotoxicological data based on Australian freshwater fish has highlighted the need to establish endemic species in the assessment of xenobiotics in Australian waterways. To set more appropriate guidelines for the use and impact of pesticides in Australia, we have chosen to focus on native fish species. The ecological importance, broad geographical distribution, and reproductive characteristics of Murray cod and rainbowfish; and their occurrence in the Murray-Darling river system make them suitable models for assessing the impacts of anthropogenic substances in Australian waterways, especially in the G-MW region.

1.2.1 Fish species

The fish species chosen for this ecotoxicological investigation include the iconic Murray cod, *Maccullochella peelii peelii*, and the Murray River rainbowfish, *Melanotaenia fluviatilis.* Murray cod are endemic to the freshwater Murray Darling River system of eastern Australia and are currently listed as vulnerable by the Victorian Flora and Fauna Guarantee Act (1984??) and threatened (species of national significance) under the Commonwealth Environmental Protection and Biodiversity (EPBC, 1999) Act. Estimated that wild Murray cod stock are now less than 10 % of the population present at the time of European settlement and are continued decline (http://www.nativefish.asn.au/cod.html, 2005). The reduction in abundance has been attributed to habitat alterations while it is possible that pollution of waterways by anthropogenic substances such as herbicides and insecticides could add to this decline.

Cod may be exposed to a variety of herbicides and insecticides that find their way into the aquatic environment as a result of runoff, leaching, spray-drift, and/or by direct application. As a top order carnivore, Murray cod play an integral role in the structure and maintenance of aquatic ecosystems. Their predominant lie-in-wait predatory life-style makes them particularly vulnerable to toxicant exposure for extended periods of time. The suspected influence of anthropogenic substances on Murray cod survival does not have a strong scientific base. This lack of published ecotoxicological data on the effects of herbicides and insecticides on Murray cod emphasized the need for the current investigation.

The Murray River rainbowfish, *Melanotaenia fluviatilis* is native to eastern Australian waterways and have become an important laboratory test organisms for assessing the toxicity of chemicals to Australian freshwater fish (Holdway *et al.*, 1994; Barry *et al.*, 1995; Reid *et al.*, 1995; Reid and Holdway, 1995; Pollino 2000). Murray River rainbowfish are predominantly surface feeders and play an integral role in the transfer of energy through the aquatic food chain. Cod and rainbowfish are potentially exposed to formulated herbicides such as Magnacide H (Acrolein), Amitrole T (250 g/L), Weedmaster duo (Glyphosate 360 g/L), Amicide 625, and the insecticides copper sulfate and Thiodan (350 g/L endosulfan) as both species inhabit waters in the immediate vicinity to land where these chemicals are used. The transitory nature of toxicants in flowing water, application events, breeding times, and rainfall events suggest that the early life stages of cod and rainbowfish are likely to be exposed to these toxicants in pulses.

1.2.2 Exposure duration

Historically, ecotoxicological investigations on the effects of xenobiotics on freshwater organisms have focused on the concentration of continuously exposed toxicant required to elicit a 50% acute response (LC_{50}) over a period of 24-96 hours. While providing for fundamental comparative research and the possibility of providing relevant intermittent lethality estimates (Handy, 1993) the continuous exposure of organisms to insecticides is in many cases environmentally unrealistic. A number of authors (Sprague, 1971; Environment Canada, 1994; Pollack 1997) argue that aquatic organisms are more likely to

be exposed to toxicants episodically than continuously. This is especially true for those organisms that inhabit streams and rivers.

Exposure of aquatic organisms to insecticides is dependent on the chemical and physical characteristics of the toxicant and receiving waters. The relatively short half-lives of the pesticides, duration of rain events, rates of binding to organic material and aquatic flow regimes indicate that aquatic organisms are unlikely to be exposed to pesticides at the same concentration for more than 24 hours. Consequently, this study will focus on the exposure of aquatic organisms to a single 'pulse' of each pesticide for a 24 h period in an attempt to replicate environmentally realistic conditions.

1.2.3 Age of fish

Fish less than 24 hours old were chosen for ecotoxicological investigation, as very young organisms are often the most sensitive to the effects of chemicals (Sprague, 1971; McKim, 1977; Spehar *et al.*, 1983; Barry *et al.*, 1995). The confounding effects of feeding older fish are removed when investigating effects of toxicants on organisms less than 24-hour-old. Fish less than 24 hours old focus on an age group in cod and rainbowfish that is likely to be present at the time of pesticide application (G-MW, pers. comm. Golam Kibria, 2006) The birth of many riverine organisms, particularly fish, occurs within the allocated pesticide-spraying regime currently used in Australia for cotton (Merrick and Schmida, 1984; Shaw *et al.*, 1986) and the growth of submerged aquatic vegetation. Cod have been recorded to breed from mid-October until at least mid-December in the southern Murray-Darling

(Koehn & O'Connor, 1990; Humphries *et al*., 2002; Humphries, 2005) and may extend this breeding until mid-January in dams (pers. comm.,Neil Hyatt of Snobs creek fish hatchery) while rainbowfish may breed throughout the year (Holdway *et al*., 1994). The regimes of pesticide application coincide with the 'wet' or rainy season, which may facilitate the transportation of anthropogenic substances into waterways. The influx of runoff may allow pesticides bound to sediment to become bioavailable to species inhabiting the water column.

1.2.4 Acute and chronic endpoints

Acute lethal toxicity is considered that which causes severe and rapid damage to the organism by the fastest acting mechanism of poisoning, fatal unless the organism escapes the toxic environment at an early stage (Sprague, 1973). A lethal concentration of toxicant that is capable of killing half of a population of organisms is recognized by the ASTM and the WHO as being sufficiently toxic to cause significant problems for the continued survival of a species. Acute toxicity may result in an alteration in the population genetics of a species, thus reducing their ability to adapt to future environmental perturbations.

Acute toxicity testing is more cost and labor efficient than most chronic toxicity endpoints, and is clearly less variable. A number of authors (Woltering, 1984; Crossland, 1985; Mayer *et al.*, 1986) argue that many chronic endpoints such as growth are often equally or less sensitive than survival. Gorge and Nagel (1990) and Woltering (1984) conducted over 170 toxicity tests, concluding that fry mortality was the clearest and most sensitive response to xenobiotic exposure. A comparison of endpoints for 28 chemicals and seven fish species in

34 studies found that survival was equal to or more sensitive than all other endpoints 56 to 69% of the time (Mayer *et al.*, 1986). Acute toxicity tests provide a clear and consistent means of comparing the toxicity of different compounds (Bloomquist, 2002).

While acute toxic responses to pesticides are of paramount importance in comparative fish toxicity testing, a number of sub-lethal responses have been shown to be equally valid. Consequently, we have chosen to investigate a number of sub-lethal toxic responses in cod following exposure of eggs and larvae to selected pesticides. The sub-lethal responses chosen for investigation included; time to hatch, percent hatch, swimming behaviour, fish length (growth), yolk-sac length (energy consumption), the ratio of fish length to yolk-sac length, heartbeat, and mean rates of morphological malformations (Klumpp & Westernhagen, 1995).

(2) Laboratory Testing

2. Materials and Methods

The procedures employed in this investigation consist of the exposure of a random sample of organisms to a concentration of pulsed toxicant in experimental chambers with periodic observations of organism mortality and behaviour. Organisms were observed to die at different times, thus indicating that there is a distribution of sensitivity, within the organisms population, to the toxicant at the concentration being tested. In this context the % mortality can be viewed as a measure of population sensitivity. For any distribution in the sensitivity of the organism population, organisms at a given sensitivity level in each of the pulsed concentration experiments can be assumed to have common or similar characteristics with respect to the effects of the chemical being tested.

2.1 Fish tested

2.1.1 Murray cod (*Maccullochella peeli peelii*)

Snobs Creek Fish Hatchery, Eildon, Victoria supplied Murray cod eggs and larvae for the experiments conducted in this report. Adult cod used for breeding were maintained in dams within the hatchery. As breeding commenced, a number of mesh-lined breeding substrates were placed within the dam to act as laying sites (Figure 1.1). The artificial substrates were

removed from the dams two to three times a week depending on the number of eggs laid. The mesh was removed from the breeding substrate and placed in a flow through hatching tank (Figure 1.2) where the eggs were treated with ammonium sulfate (1500 mg/L) for one hour to prevent bacterial and fungal infection. Cod eggs used for percent hatch and time to hatch were treated daily with 500 μL hydrogen peroxide as recommended by Yamamoto *et al*., (2001). This procedure was followed until hatching was complete. The use of cod eggs for experimental purposes was restricted to an ecotoxicological investigation into the effects of endosulfan poisoning. Endosulfan was chosen as it was previously shown to be the most toxic of the selected toxicants to fish as well as being involved in over 70% of fish kills within Australia. The limited supply of cod egg from Snobs Creek Fish Hatchery led the current investigation to focus on larval fish for the study on the effects of selected agricultural chemicals.

Larvae and eggs were transferred from the hatchery to RMIT University in temperature controlled polyethylene containers within an esky. At the university the eggs and/or larvae were maintained in aerated and temperature controlled static 50 L glass aquaria. Individual tanks were supplied with thermostat regulated heaters and temperature gauges to assure constant temperature. Tank temperature was recorded daily. Plastic air-driven filters containing nylon wool provided a substrate for nitrogenous bacteria. Photoperiod was set on a daily cycle of 16h light: 8h dark with a dusk/dawn transitional period of 15 minutes. Cool white fluorescent tubes (approximately 600 lux at water surface) provided light. Fish/eggs were removed from the aquaria for the purpose of toxicity testing.

Figure 2.1 Artificial mesh-lined breeding substrate used for the collection of Murray cod eggs from earthen dams at Snobs Creek Fish Hatchery, Eildon, Victoria.

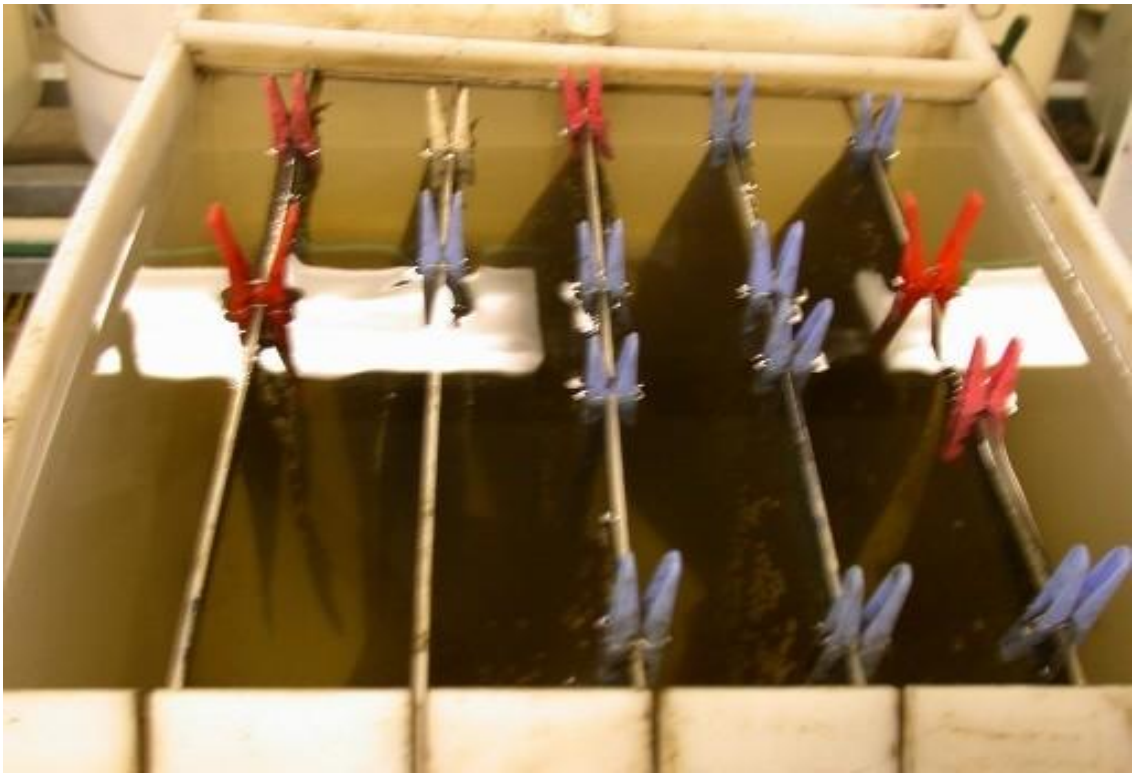

Figure 2.2. Flow through hatching tanks at Snobs Creek Fish Hatchery, Eildon, Victoria. Eggs were treated daily with 1500 μL/L ammonium sulphate in an effort to prevent fungal and bacterial buildup.

http://www.amonline.net.au

Figure 2.3 Adult Murray cod *Maccullochella peelii peelii*

2.1.2 Murray River Rainbowfish (*Melanotaenia fluviatilis*)

The original stock of *M. fluviatilis* was collected from the Murray River, Victoria in 1994 and housed at RMIT City campus, Melbourne, for use in ecotoxicological investigations. In April 2001 the laboratory population was transported and housed at RMIT, Bundoora, where they currently remain. Fish were acclimated to laboratory conditions for 6 months prior to testing.

Figure 2.4 Adult Murray river rainbowfish *Melanotaenia fluviatilis*

Rainbowfish used for experimental purposes were housed and bred in 50L flow-through glass aquaria (Figure 3). The water volume in each chamber was maintained at 45L, and flow rates through the test chambers were greater than six water volume additions in 24h, allowing for 99% replacement as recommended by Sprague (1969). Incoming wet-lab

water (WLW) was sand and carbon filtered and pre-heated to 25^0C prior to contact with fish and aquaria. Room temperature was maintained at 25^0C. Mean (SE) characteristics of the test water for static and flow-through test chambers are recorded in Tables 3.1 and 3.3 Water characteristics when measured at the start of the experiments, daily, and just before renewal (for static chambers) and, did not vary significantly, either from tank to tank within experiments or between experiments for both static and flow-through test chambers. All measurements were within acceptable limits (ASTM, 1989).

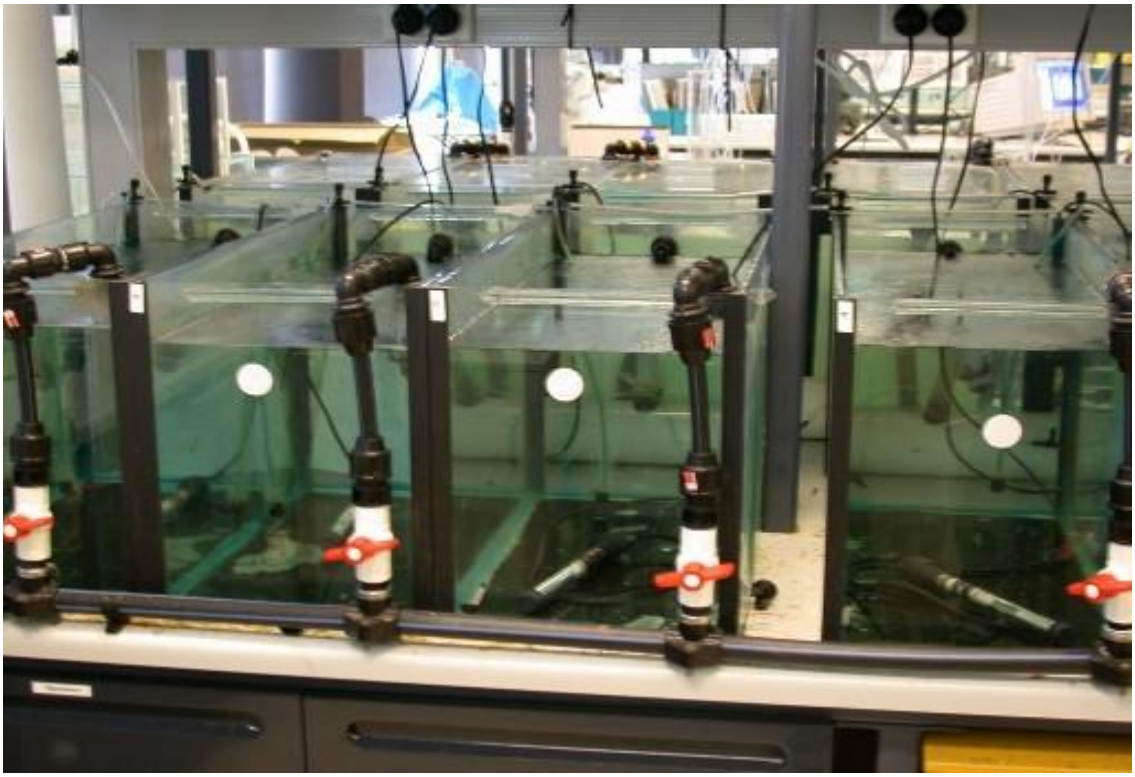

Figure 2.5. Flow through glass aquaria at RMIT University, Melbourne, Victoria.

2.2 Glassware.

All glassware was washed following a very strict cleaning regime. Purchased and pre-used glassware was acetone rinsed then washed in WLW three times. Following this, glassware was left to sit in 10g/L of pyrogenetically negative cleaner (Pyroneg ™) dissolved in WLW for 24 hours and then scrubbed thoroughly. Pyroneg residue was rinsed off with WLW three times and glassware was given a final rinse with Milli-Q prior to sitting in 5% nitric acid (with WLW) for 24 hours. Following removal from nitric acid, all glassware was rinsed three times with Milli-Q water and left to dry upside down on clean laboratory towel. All glassware was stored in containers lined with laboratory towel.

2.3 Treatment solutions.

The weighing and dispensing of all stock and treatment solutions was conducted within a fume hood. Individual stock solutions at each treatment concentration were prepared in acetone using a maximum dilution factor of 1:10. Fish were transferred to the treatment solution in polyethylene beakers. Fish remained in contact with water at all times by collecting in the edge of the tilted beaker during transfer. All of the pesticides investigated were supplied by GM-W with the exception of Magnacide H, which was supplied by Baker Hughes, Pty. Ltd.

2.4 Toxicity Testing

Tests were performed using three replicates and a minimum of five exposure concentrations, and controls. Results of previously conducted range-finding experiments were used to determine appropriate test solution concentrations. Less than two-day-old fish were removed from a 100 L rearing tank and randomly placed into mesh-lined 100 mL polyethylene beakers using a 20 mL plastic ladle. Each polyethylene beaker had 4; 25 mL holes drilled through the sides and were covered with 0.2 mm^2 mesh to facilitate water flow. A circular hollow plastic tube was fitted under the lip of each beaker to act as a floatation device.

Ten juvenile fish of mixed sex were randomly placed into each floating polyethylene beaker. The floating beakers were then placed in 600 mL short glass beakers each containing 500 mL of treatment solution. Fish were not fed for the duration of the experiment. Following the treatment pulse (24h), all polyethylene beakers were dipped in WLW for a period of 5 minutes to remove excess toxicant bound to beakers (Barry *et al.*, 1995). Beakers containing fish were then transferred to 50 L aerated static test-tanks for the remainder of the 96h test. Replicate post-pulse treatment beakers were maintained in the same tank in an attempt to reduce tank effects. Since exposures had taken place in isolated compartments it was concluded that ASTM (American Society of Testing and Materials, 1991) protocols for independent treatments had been satisfied. Numbers of live fish and behavioural observations were recorded at 24, 48, 72 and 96 hours. Total fish length and yolk-sac length were determined at the completion of the 96 h test following exposure to 5

mg/L of the registered fish anesthetic, MS222. A binocular microscope was fitted with a graticule for the purpose of measuring morphological features.

Fish death was determined by lack of heartbeat using a binocular microscope (WILD M32) fitted with an Intralux ™ light source. To assess death it was necessary to remove the motionless fish with a smooth-ended autoclaved glass pipette to a 1mL microscope dish for examination. All live fish were immediately returned to their respective treatment beakers. The impact/stress induced in live fish involved in this procedure is noted but unknown. Heartbeat was recorded using a stopwatch and microscope. As it was difficult to see the heart contractions of the Murray cod due to their upright posture (Figure 1.6) the movement of blood from the heart was used as a surrogate for heartbeat data.

Figure 1.6. A one-day-old Murray cod, M*accullochella peelii peelii*, larvae.

Based on the results of the toxicity of the experimental chemicals to Murray cod, the two most toxic chemicals (namely acrolein and endosulfan) were tested on Murray River rainbowfish.

All concentrations of test chemicals quoted throughout this report are nominal concentrations. The 24 hour pulse was a static non-renewal exposure in all tests.

2.5 Statistical analysis

Data analysis included the use of one-way Analysis of Variance (ANOVA) of treatment and control means. If the assumptions of ANOVA were not upheld, a non-parametric Kruskal-Wallis test was performed. Tukey's pairwise comparison and Mann-Whitney post-hoc tests were used to determine significant ($P<0.05$) differences between treatments and control means. Student T-tests were employed to determine the significance of differences between two treatment concentrations.

In all Figures and Tables throughout this report any depicted data that was significantly different to the control data in that respective test is denoted by an asterisk.

3. Results

3.1 Water quality

The water quality was maintained similar to control WLW and there was no significant change in water quality with the addition of toxicants in experiments with both the Murray cod and the Murray River rainbowfish (Tables 2 and 3)

Table 3.1. Water characteristics for larval Murray cod exposures in static test chambers. Mean (±SE) temperature (°C), dissolved oxygen (% saturation), pH, and conductivity (μS/cm) (n =7).

Chemical tested[a]	Temperature	Dissolved oxygen	pH	Conductivity
Magnacide H (Acrolein 950g/kg)	22.4 (0.2)	90.2 (0.11)	7.46 (0.02)	98.8 (0.8)
2,4,D amine (Amicide 625 g/L)	21.6 (0.1)	91.5 (0.38)	7.34 (0.04)	101 (0.4)
Amitrole (Amitrole T)	22.3 (0.1)	92.9 (0.18)	7.55 (0.02)	101 (1.0)
Weedmaster duo (Glyphosate 360 g/L)	21.5 (0.1)	93.7 (0.72)	7.43 (0.02)	139 (0.9)
Copper	22.5 (0.1)	91.2 (0.22)	7.46 (0.02)	144 (0.2)
Endosulfan (Thiodan 350 g/L)	23.2 (0.1)	90.9 (0.20)	7.20 (0.04)	143 (0.9)

[a] Water characteristics recorded at 0, 24, 48, 72 and 96 h were pooled.

Table 3.2 Water characteristics for larval rainbowfish exposures in static and flow-through test chambers. Mean (±SE) temperature (°C), dissolved oxygen ((% saturation), pH, and conductivity (μS) (n = 7).

Chemical tested[a]	Temperature	Dissolved oxygen	pH	Conductivity
Magnacide H (Acrolein 950g/kg)	20.1 (0.1)	90.2 (0.2)	7.37 (0.06)	121 (0.31)
Endosulfan (Thiodan 350 g/L)	20.4 (0.1)	90.9 (0.2)	7.24 (0.05)	133 (0.84)

[a] Water characteristics recorded at 0, 24, 48, 72 and 96 h were pooled.

3.2 Effects of pesticides on Murray cod larvae

The 96 h survival of Murray cod larvae following a 24h pulse-exposure to six agricultural chemicals used within the Goulburn-Murray Water catchment showed that copper and acrolein concentrations of 10,000 μg/L were responsible for the death of all exposed fish (Table 3.3). The remaining pesticides did not significantly affect the survival of larval cod at the concentrations tested. Pulse endosulfan concentrations of 10,000 μg/L were shown to significantly reduce the total length of exposed cod at 96 h while pulse-exposure of larvae to 100 μg/L endosulfan and above, significantly affected fish swimming behaviour. Normal swimming behaviour was also significantly altered in fish exposed to copper and acrolein

concentrations resulting in erratic movements and finally in death. No morphological or behavioural aberrations were noted in cod pulse-exposed to glyphosate, Amitrole T, or 2,4D Amicide in the current investigation.

Table 3.3. The percentage survival of larval Murray cod at 24 and 96 h following a 24 h pulse-exposure to six formulated pesticides. Technical grade products and/or concentrations of active ingredients are reported in parentheses.

Toxicant	**Concentration (µg/L)**	**Survival (%)**	
		24 h	**96 h**
Magnacide H (Acrolein, 950g/kg)	10,000 1,000	0 100	0 97
2,4-D (Amicide, 625 g/L)	100,000	100	100
Amitrole T (250g/L).	10,000	100	94
Weedmaster duo (Glyphosate, 360 g/L)	100,000	97	87
Copper (copper sulphate)	10,000 1,000	0 100	0 100
Thiodan (endosulfan, 350 g/L)	10,000	97	97

Table 3.4 The 96 h effects on morphological and behavioural characteristics of Murray cod larvae following a 24 h pulse-exposure to selected toxicants. Significant differences (SD) were determined by comparing controls with the highest treatment concentrations. Students T-Tests were used to compare treatment means. Significant differences (**SD**) in morphological and behavioural characteristics are highlighted while insignificant effects (n.e.) are noted.

Toxicant	Heart beat (n = 10)	Fish length (n = 10)	Yolk sac length (n = 10)	Fish length/ yolk sac length (n = 10)	Behaviour (n = 10)	Internal eye colour (n = 10)
Magnacide H (Acrolein, 950g/kg)	n.e.	n.e.	n.e.	n.e.	**S.D.**	n.e.
2,4-D (Amicide, 625 g/L)	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Amitrole T (250g/L).	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Weedmaster duo (Glyphosate, 360 g/L)	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Copper	n.e.	n.e.	n.e.	n.e.	**S.D.**	n.e.
Thiodan (endosulfan, 350 g/L)	n.e.	**S.D.**	n.e.	**S.D.**	**S.D.**	n.e.

3.2.1 Effect of Magnacide H (Acrolein 950g/kg)

All cod exposed to acrolein concentrations of 10,000 µg/L were dead at 12 h while insignificant fish death occurred at acrolein concentrations of 1, 10, 100, and 1,000 µg/L at 96 h. Cod exposed to non-lethal acrolein concentrations did not exhibit significant behavioural or morphological differences between treatment and control fish (Figs 3.1-3.4). An insignificant trend for decrease in cod length with increasing concentration, reduction in the ratio of mean fish length to mean yolk sac length, and a slightly reduced heart rate in fish pulse-exposed to acrolein concentrations was observed.

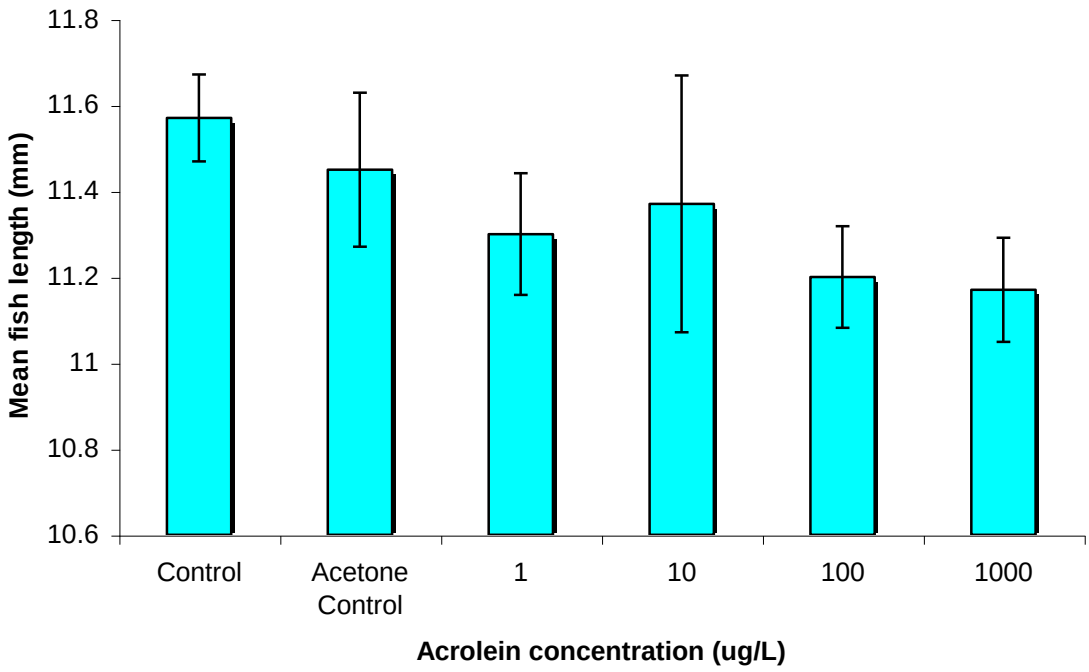

Figure 3.1. The mean length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Magnacide H (acrolein 950g/kg) (n = 10).

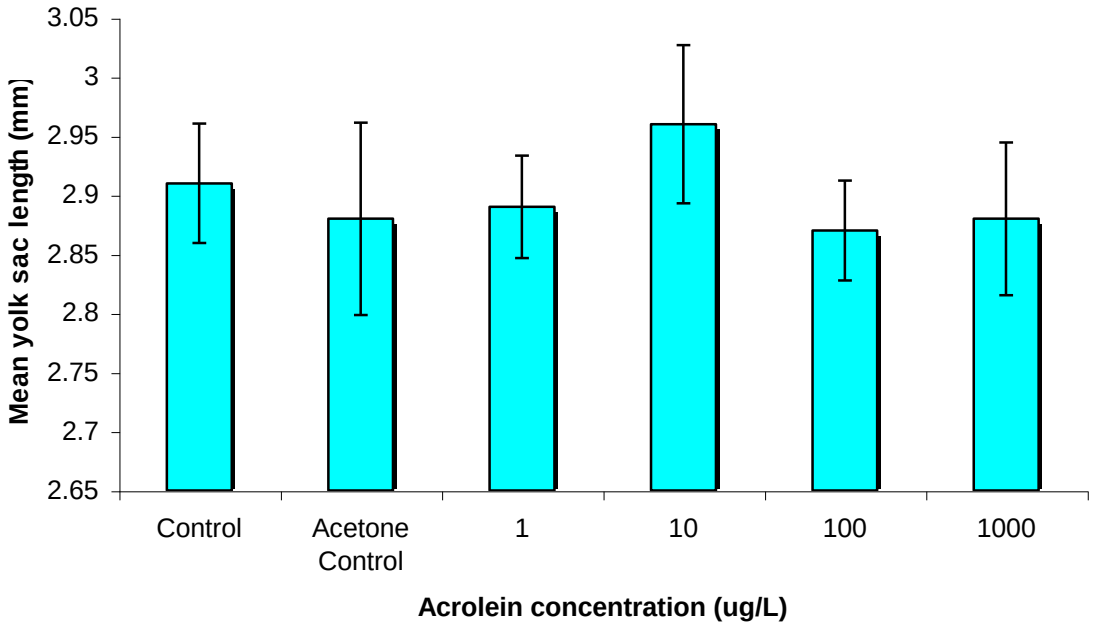

Figure 3.2. The mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Magnacide H (acrolein 950g/kg) (n = 10).

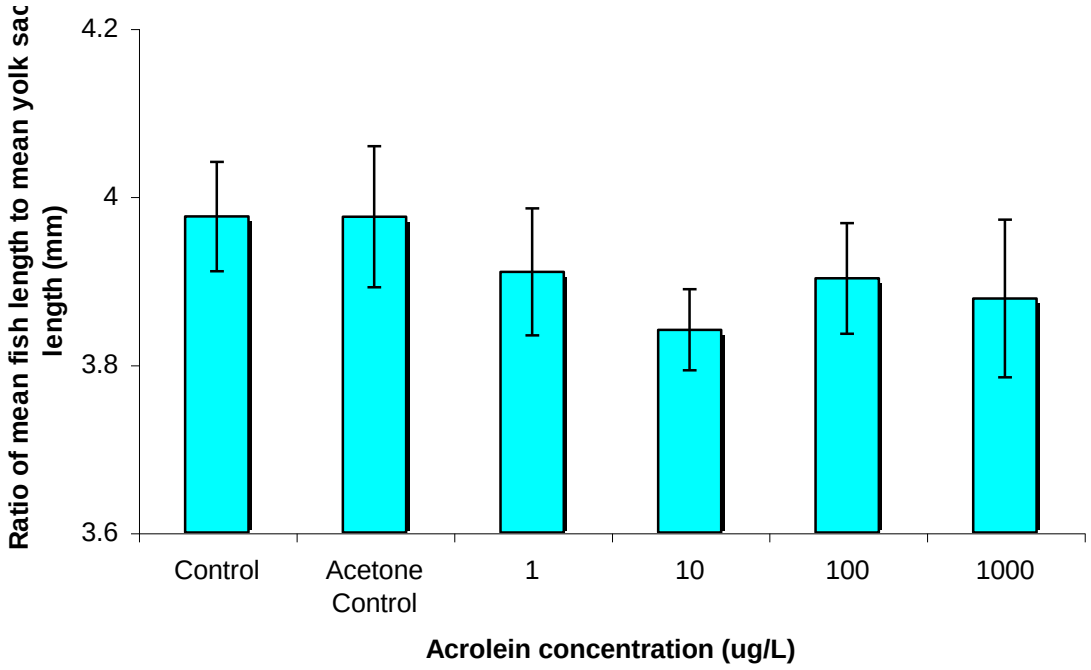

Figure 3.3. The ratio of mean fish length to mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Magnacide H (acrolein 950g/kg) (n = 10).

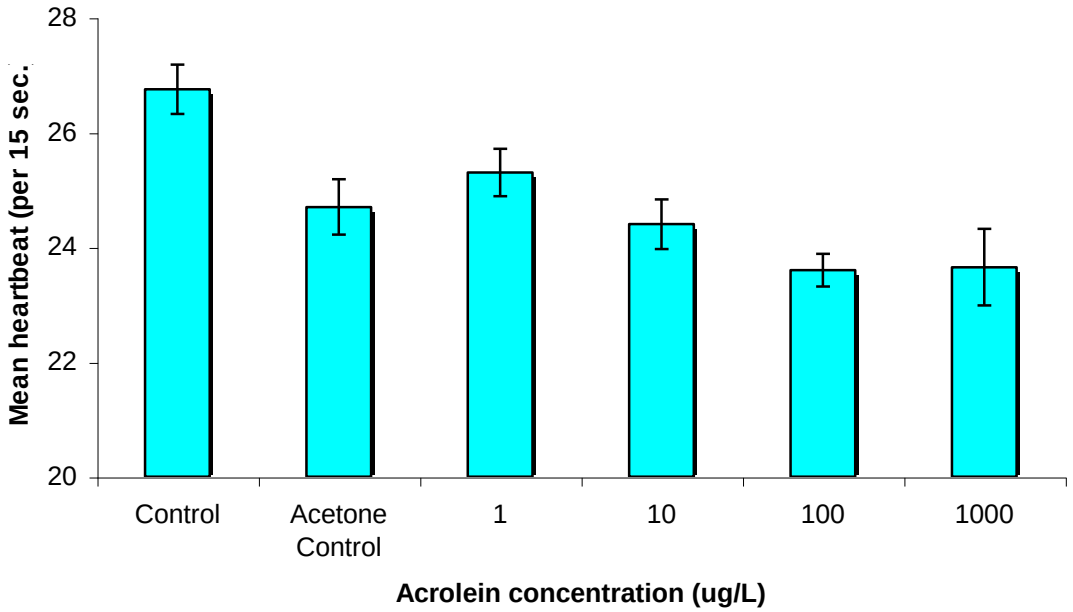

Figure 3.4. The mean heartbeat (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Magnacide H (acrolein 950g/kg) (n = 20).

3.2.2 Effect of 2,4-D Amicide (625 g/L)

All cod survived the 24 h pulse-exposure to 2,4-D Amicide (625 g/L) concentrations from 1 to 100,000 µg/L at 96 h. Measured behavioural and morphological attributes of the cod larvae did not vary significantly between treatment and control groups at any stage throughout the investigation (Figs 3.5-3.8). Pulse-exposure of <2 day-old cod larvae to 2,4-D Amicide did not significantly affect the parameters investigated in this ecotoxicological study. No clear trend was observed in mean fish length, mean yolk sac length, the ratio of mean fish length to mean yolk sac length, or mean heartbeat with 2,4-D Amicide concentration.

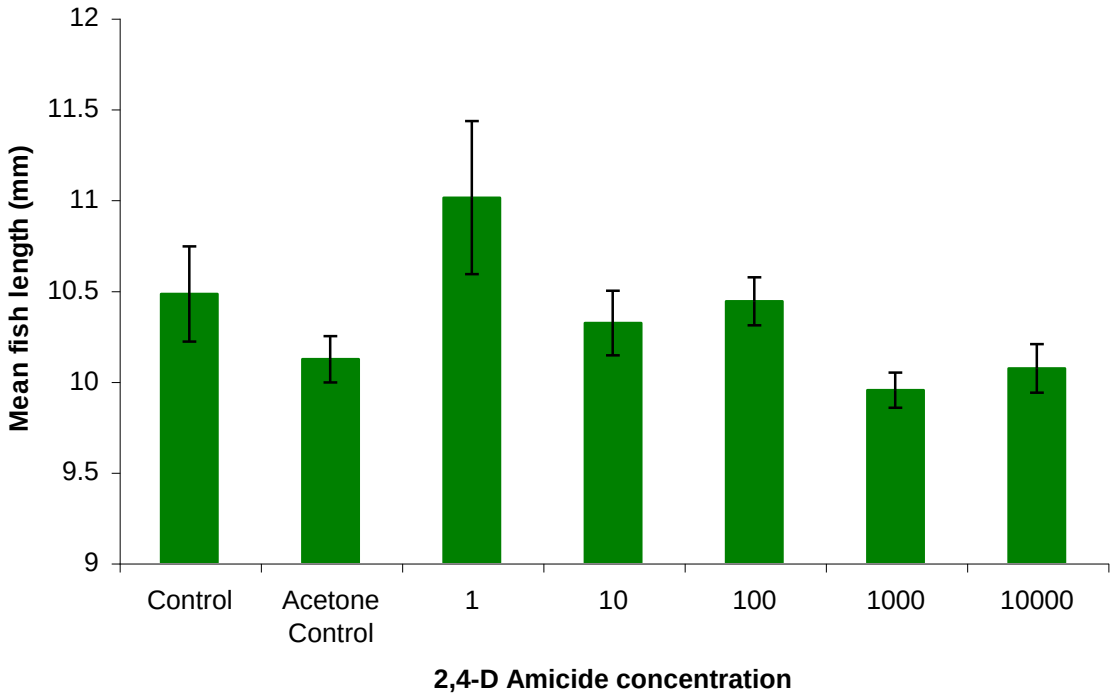

Figure 3.5. The mean length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to 2,4-D Amicide (625 g/L) (n = 10).

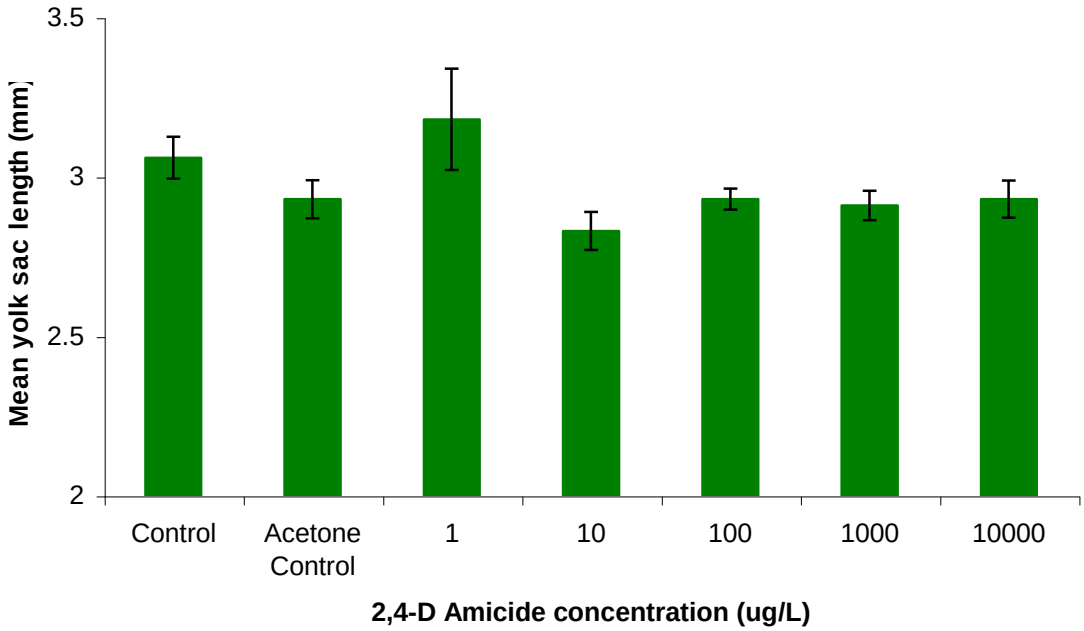

Figure 3.6. The mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to 2,4-D Amicide (625 g/L) (n = 10).

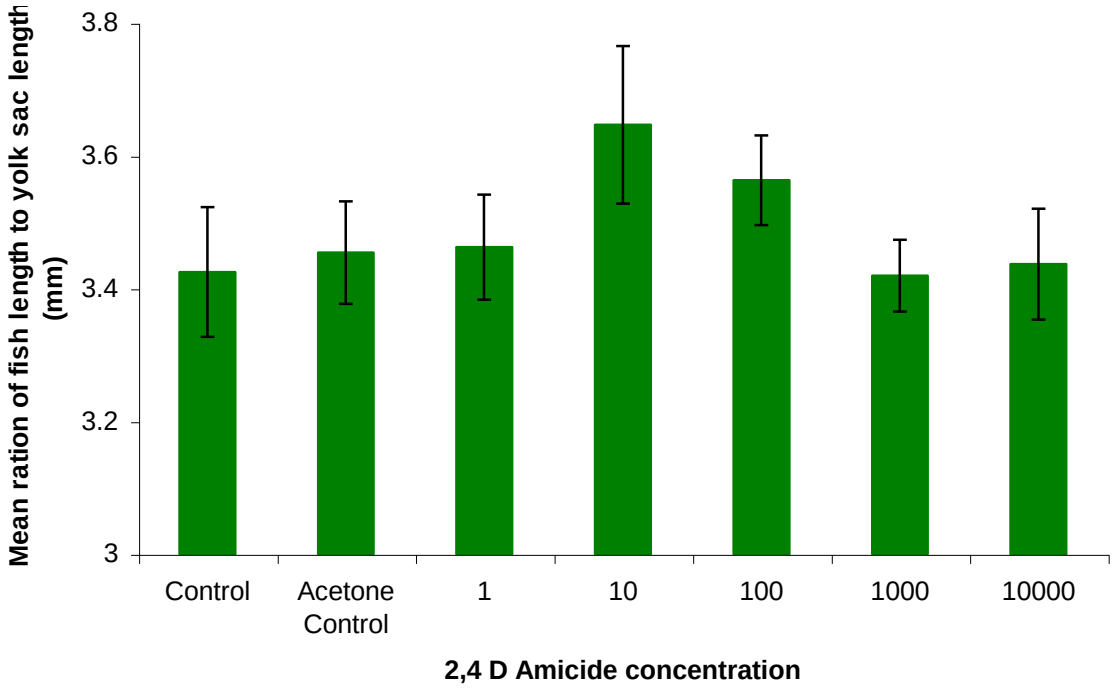

Figure 3.7 The ratio of mean fish length to mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to 2,4-D Amicide (625 g/L) (n = 10).

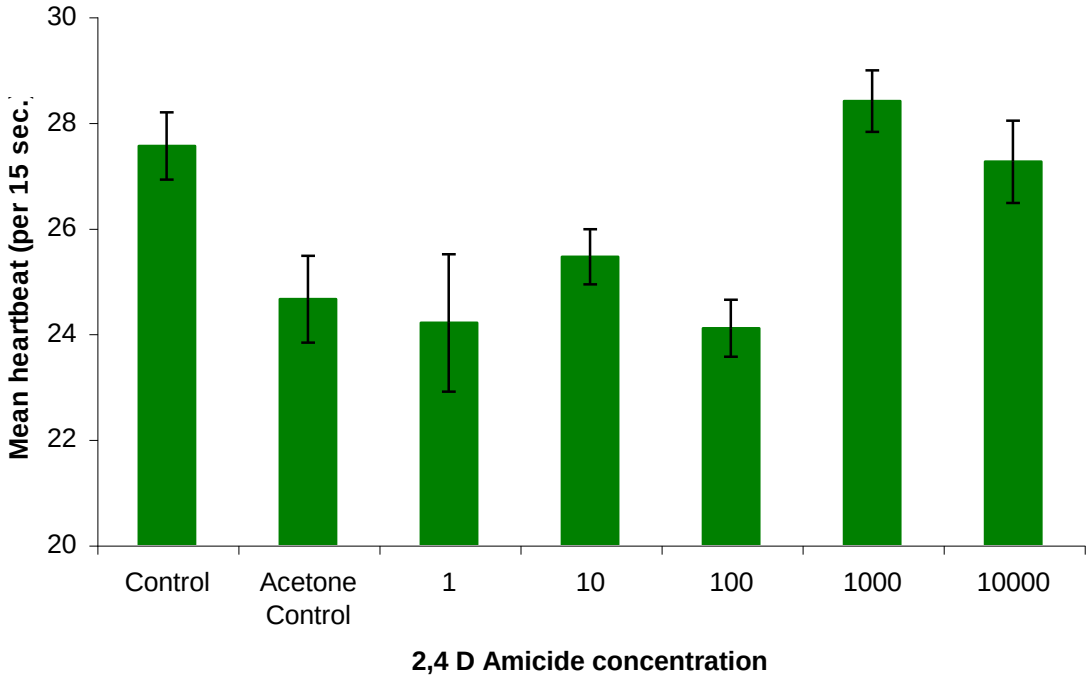

Figure 3.8. The mean heartbeat (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to 2,4-D Amicide (625 g/L) (n = 10).

3.2.3 Effect of Amitrole T (250g/L).

No significant difference in 96 h survival of Murray cod larvae was observed following a 24 h exposure to Amitrole T concentrations up to 10,000 µg/L compared with control fish. Larval survival in controls and AmitroleT treatments of 1, 10, and 100 µg/L was 100% while treatments of 1,000 and 10,000 µg/L resulted in 97 and 94% survival, respectively, at 96 h (Table 3.3). The death of a single cod exposed to 1,000 µg/L Amitrole T occurred at 96 h while one cod died at 48 and 96 h following exposure to 10,000 µg/L Amitrole T.

An analysis of measured and observed 96 h sub-lethal responses similarly indicated a lack of significant effect on larval cod following a 24 h pulse-exposure to Amitrole T (Table 3.4). Average fish length, yolk sac length, ratio of fish length to yolk sac length, and larval heartbeat did not vary significantly between treatment and control fish (Figures 2.9 to 2.12). While insignificant, a general increase in fish length with concentration (Figure 1) and an elevated heart rate of fish pulse-exposed to Amitrole T treatments (Figure 3.12) compared with control fish was evident. The swimming behaviour of larval Murray cod was not observed to vary within or between treatment and control fish for the duration of the investigation.

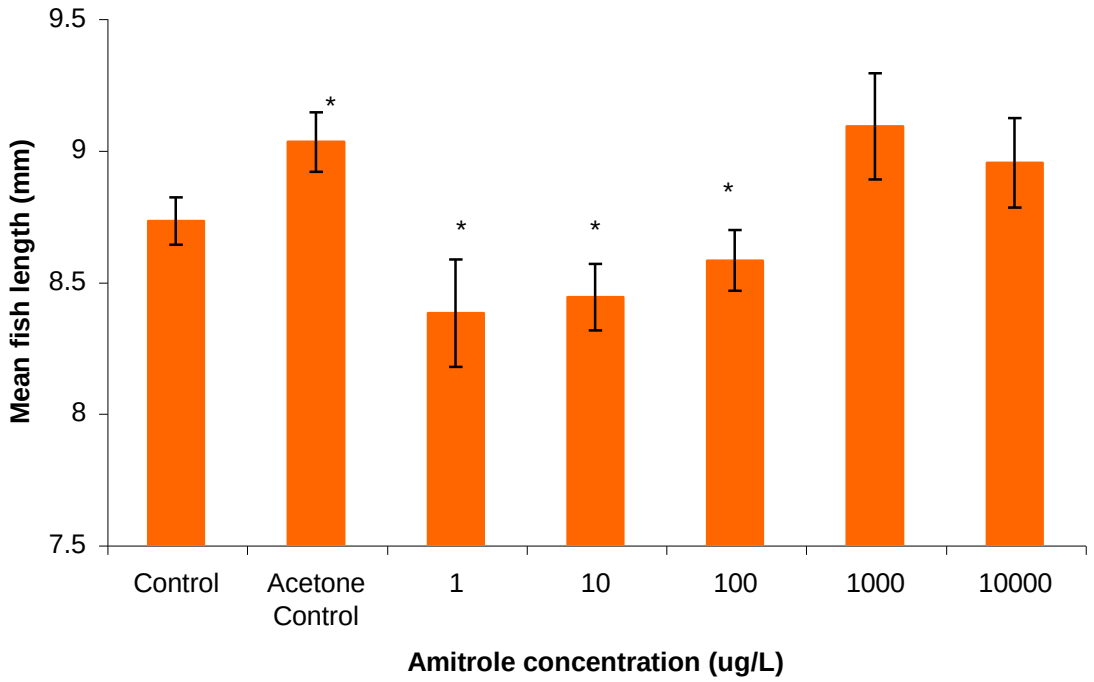

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Figure 3.9. The mean length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Amitrole T (250g/L) (n = 10).

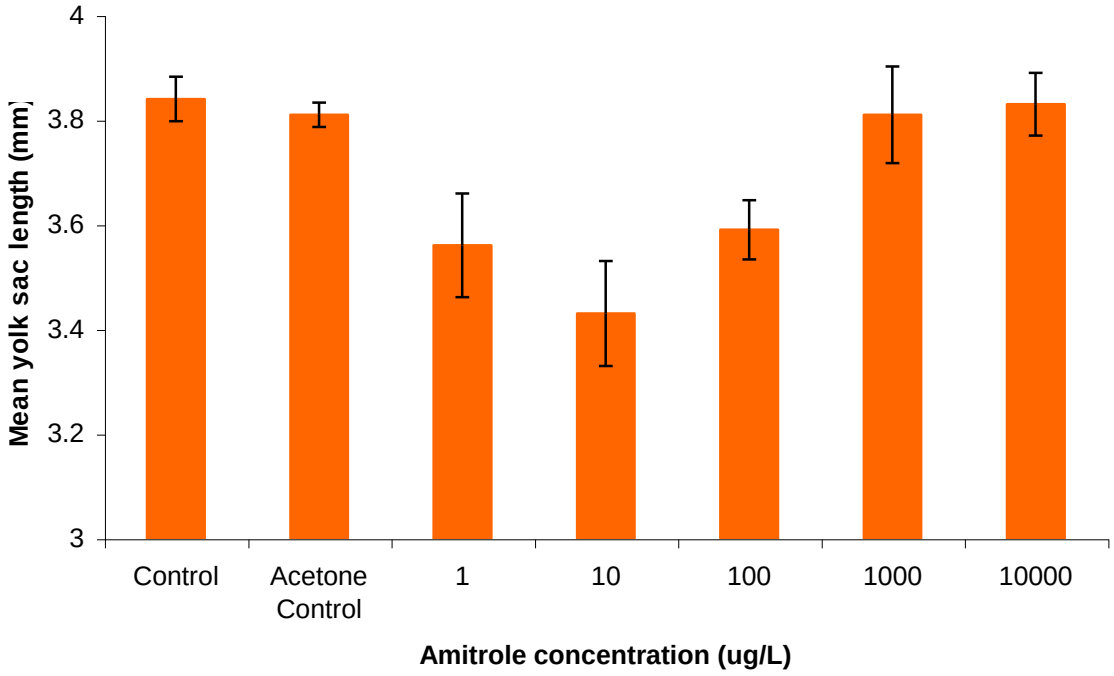


Figure 3.10. The mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Amitrole T (250g/L) (n = 10).

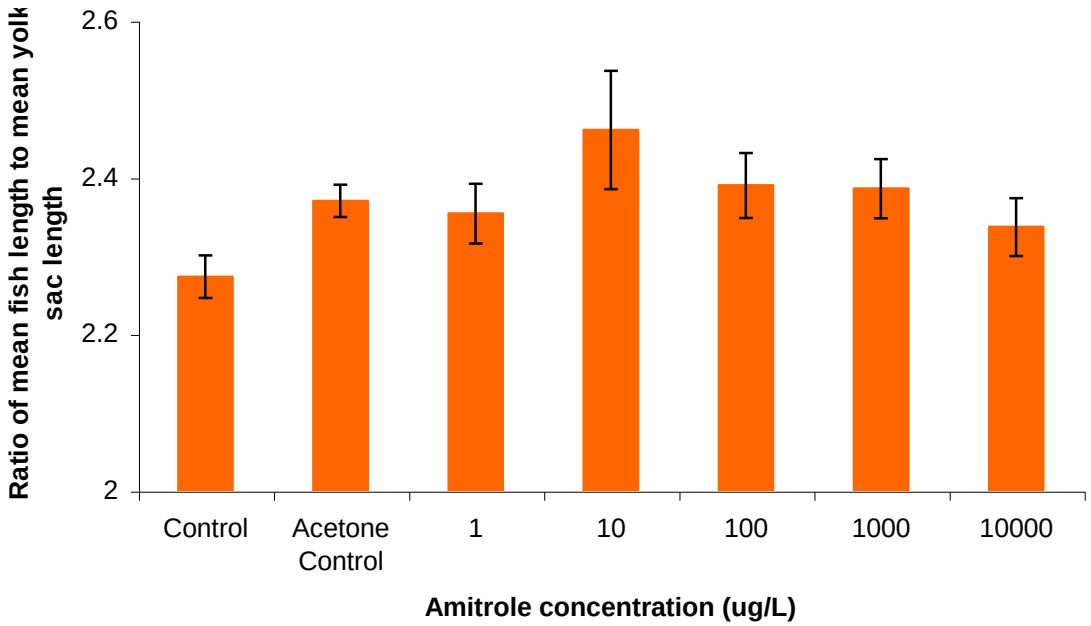


Figure 3.11. The ratio of mean fish length to mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to AmitroleT (250g/L) (n = 10).

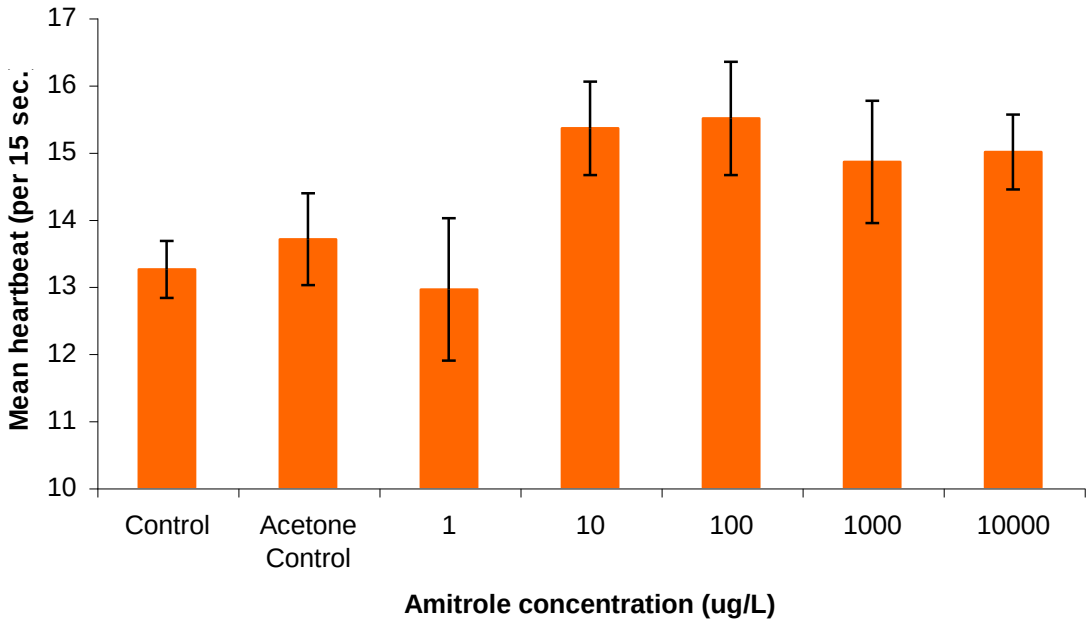


Figure 3.12 The mean heartbeat (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Amitrole T (250g/L) (n = 10).

3.2.4 Effect of Weedmaster duo (Glyphosate 360 g/L)

The pulse-exposure of < 2-day-old Murray cod to 100,000 µg/L formulated glyphosate resulted in the death of approximately 3% and 13% of fish at 24 and 96 h, respectively (see Table 3.3). Prior to their death, the fish pulse-exposed to 100,000 µg/L glyphosate did not exhibit any signs of twitching normally associated with poisoning. No significant variation in measured morphological or behavioural observations was noted over the duration of the experiment in the remaining treatments including those fish surviving in the highest test concentration (Figures 13 to 16). An insignificant concentration dependent decrease in mean fish length was recorded in larval cod exposed to Weedmaster duo (Figure 13).

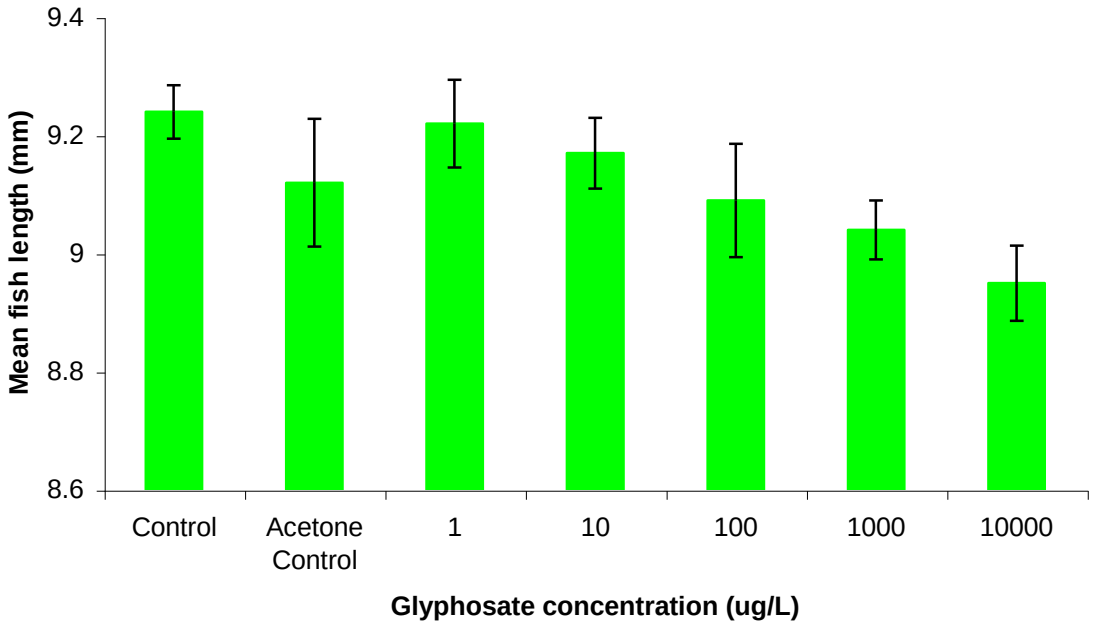


Figure 3.13. The mean length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Weedmaster duo (glyphosate 360 g/L) (n = 10).

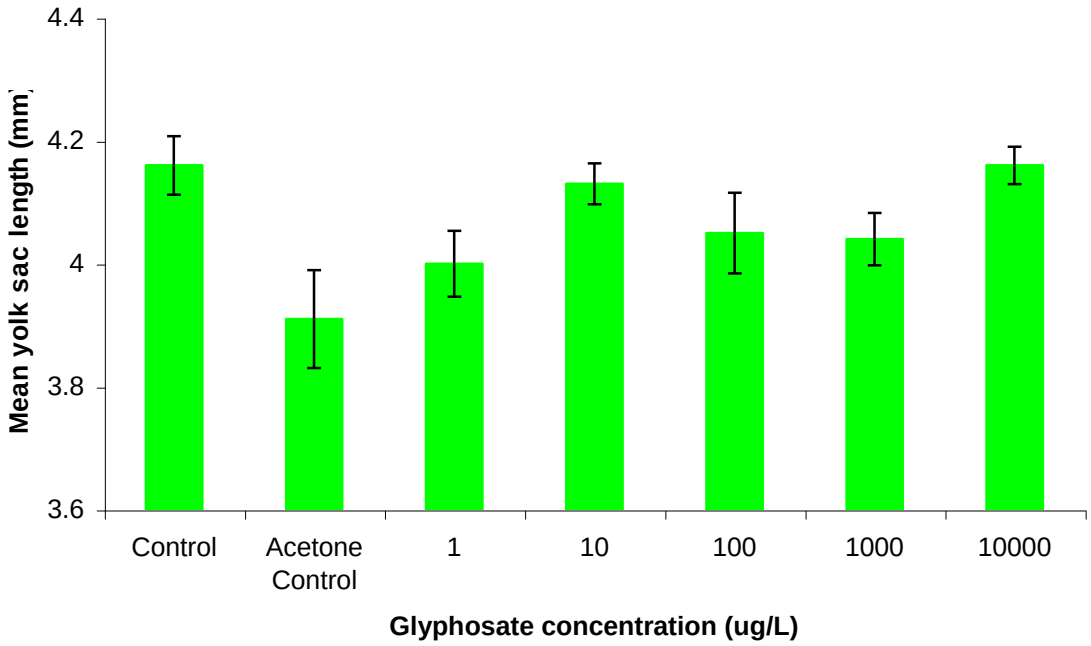


Figure 3.14. The mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Weedmaster duo (glyphosate 360 g/L) (n = 10).

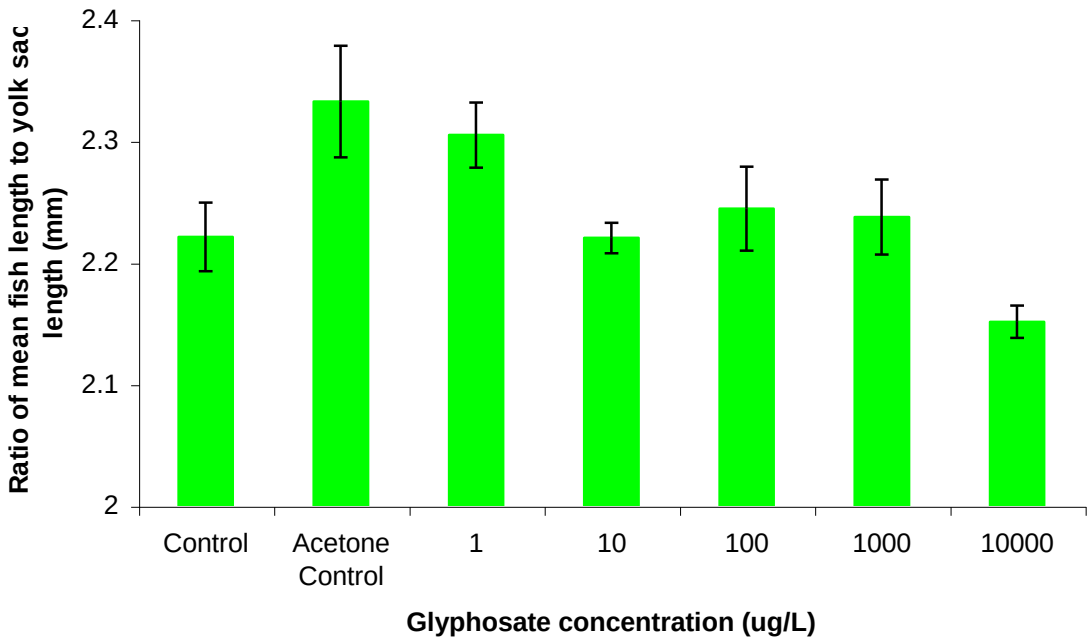


Figure 3.15. The ratio of mean fish length to mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Weedmaster duo (glyphosate 360 g/L) (n = 10).

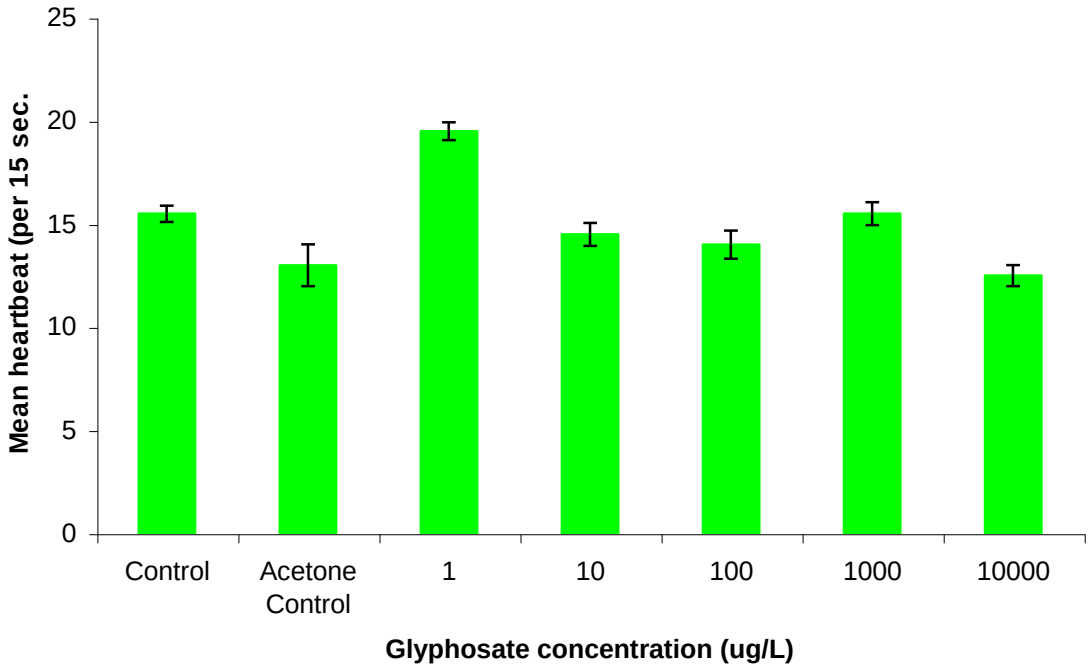


Figure 3.16. The mean heartbeat (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Weedmaster duo (glyphosate 360 g/L) (n = 20).

3.2.5 Effect of Copper

All cod exposed to copper concentrations of 10,000 µg/L were dead at 24 h while fish exposed to 1, 10, 100 and 1,000 µg/L copper survived to 96 h (Table 3.3). No significant variation in measured morphological or behavioural observations was noted over the duration of the experiments in the remaining treatments (Figure 3.17-3.20). There was no clear trend in mean fish length, yolk sac length, the ratio of mean fish length to mean yolk sac length or heartbeat with copper concentration.

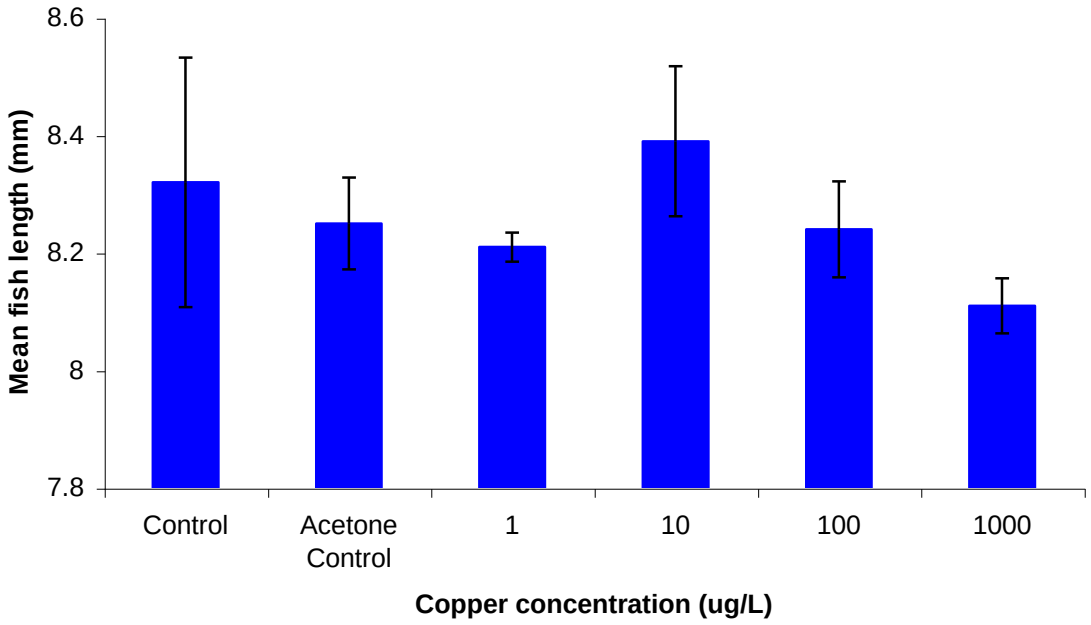


Figure 3.17. The mean length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to copper (n = 10).

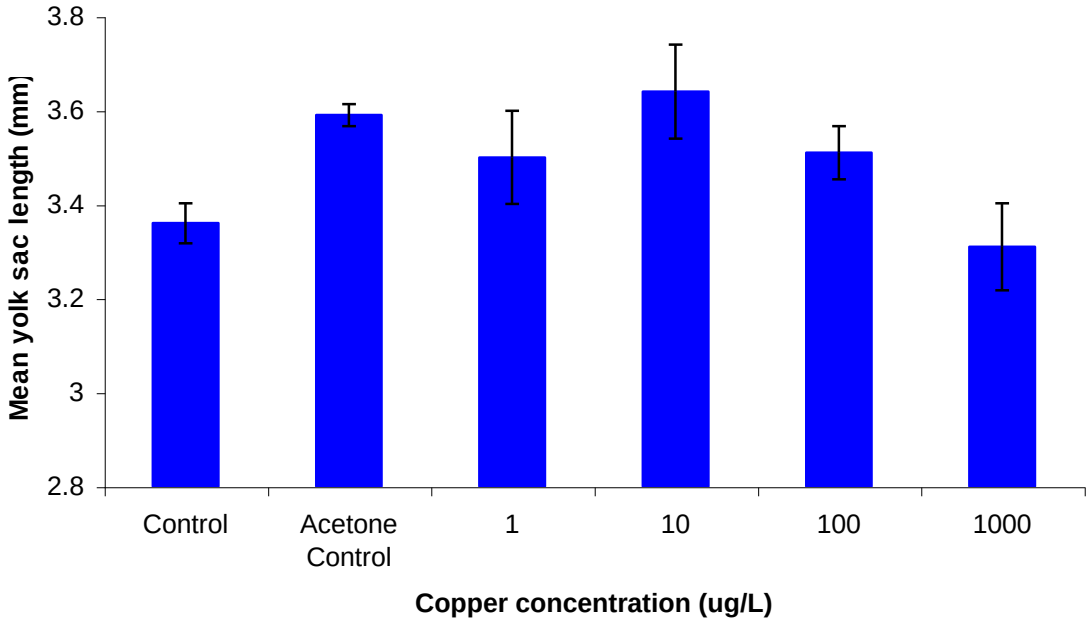


Figure 3.18. The mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to copper (n = 10).

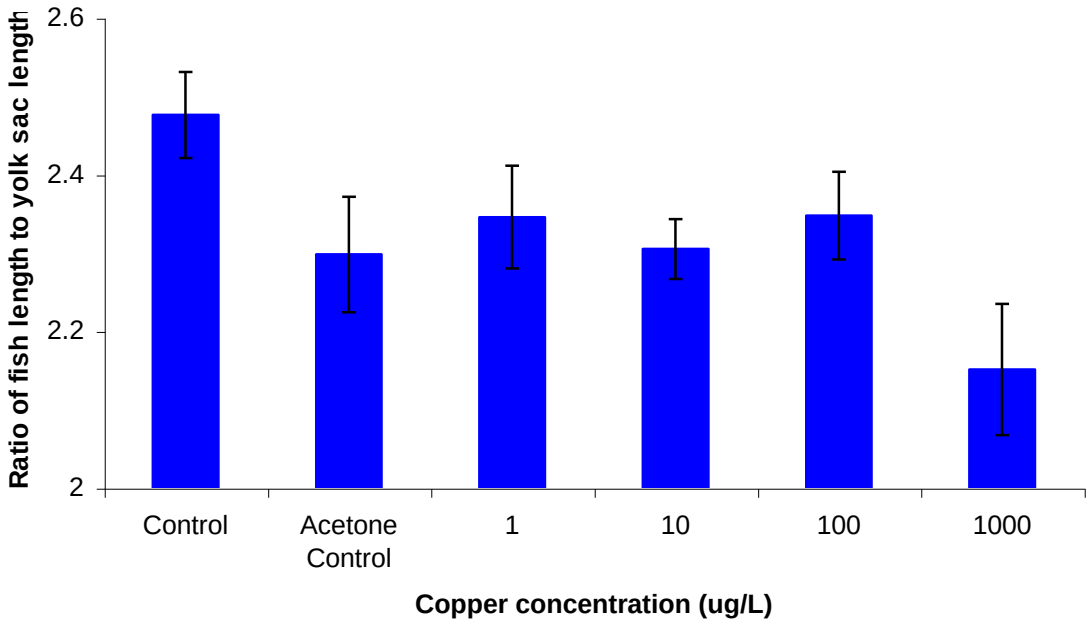


Figure 3.19. The ratio of mean fish length to mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to copper (n = 10).

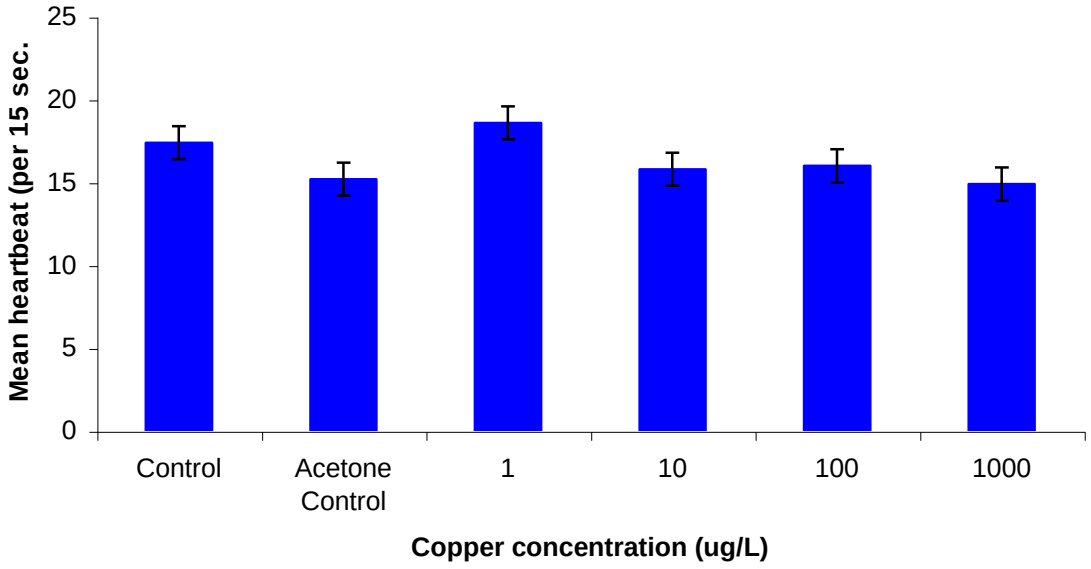


Figure 3.20. The mean heartbeat (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to copper (n = 20).

3.2.6 Effect of Endosulfan (Thiodan 350 g/L)

A number of serious behavioural and morphological modifications were observed in Murray cod exposed to the highest endosulfan treatment concentration (10,000 µg/L) (Figures 3.21 to 3.24). At the completion of the pulse-exposure (24 h), approximately 90% of the cod exposed to 10,000 µg/L endosulfan were comatose with the remaining 10% in a highly agitated 'twitching' state. Recovery from this 'twitching' state was not observed in cod pulse-exposed to endosulfan throughout the investigation. The inability of rainbowfish to recover from endosulfan induced 'twitching' has been previously recorded (Raymond, unpublished RMIT University.)

Approximately three quarters (73%) of the cod exposed to 10,000 µg/L endosulfan showed serious morphological differences compared with fish from all remaining treatments at 96 h. The main difference was observed to be a curvature in the body of the cod. This 'spinal curvature' was responsible for the significant reduction in the total body length of larval cod pulse-exposed to 10,000 µg/L endosulfan compared with fish in the remaining treatments at 96 h. Spinal curvature was not evident in cod pulse-exposed to lower endosulfan concentrations at 96 h, however, an insignificant reduction in total mean cod length at 96 h with increasing endosulfan concentrations from 1 to 320 µg/L was noted, but this trend was not continue at 1,000 µg/L (Figure 3.21). There was no clear effect of endosulfan concentration on mean yolk sac length or the ratio of mean fish length to mean yolk sac length in larval Murray cod. The mean heart rate of cod exposed to 100 and 1,000

µg/L endosulfan was slightly elevated compared with control fish but not at 320 µg/L (Figure 3.24).

While spinal curvature was not evident in cod exposed to 1,000 µg/L endosulfan, a number of behavioural aberrations were recorded. Modifications to fish behaviour were significant, with 50% of the cod exposed to 1,000 µg/L observed to be comatose/lethargic while the remaining larvae were observed to be in a state of vigorous 'twitching'. No fish pulse-exposed to endosulfan concentrations of 100 µg/L and below showed signs of twitching and/or were comatose. The transition from 'normal' to 'abnormal' (twitching, sporadic jerking) swimming and from 'twitching' to comatose in the current investigation was observed to be unidirectional; no larvae were observed to recover from an 'abnormal' state of swimming or from the comatose state. The behavioural and/or morphological modifications noted in cod larvae following pulse-exposure to the highest endosulfan treatments did not result in significantly different survival rates at 96 h when compared with the remaining treatments including controls.

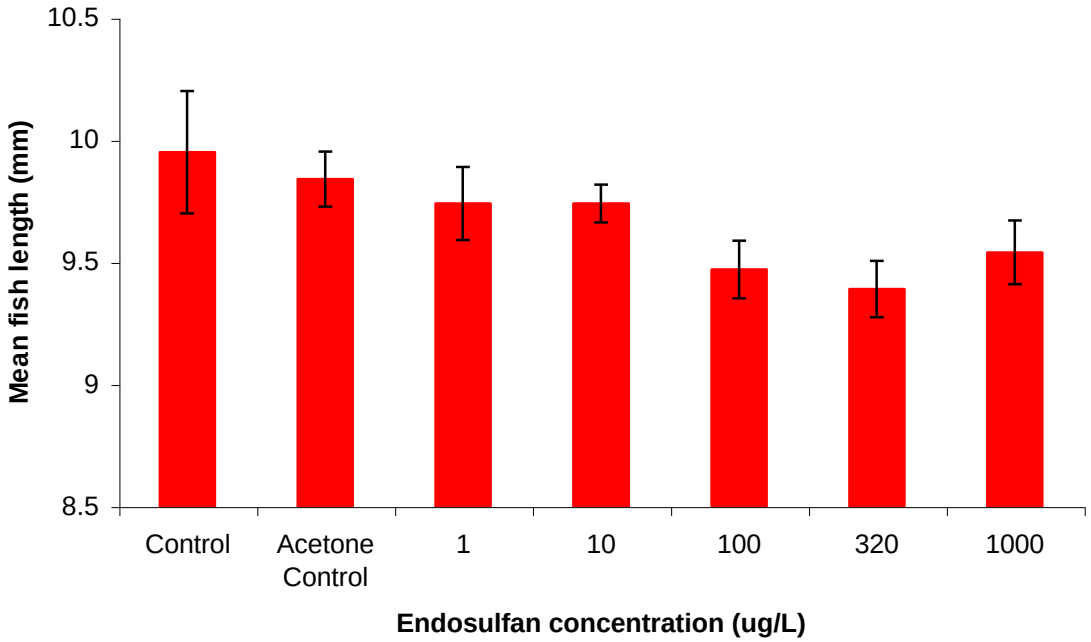


Figure 3.21. The mean length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Thiodan (350 g/L endosulfan) (n = 10).

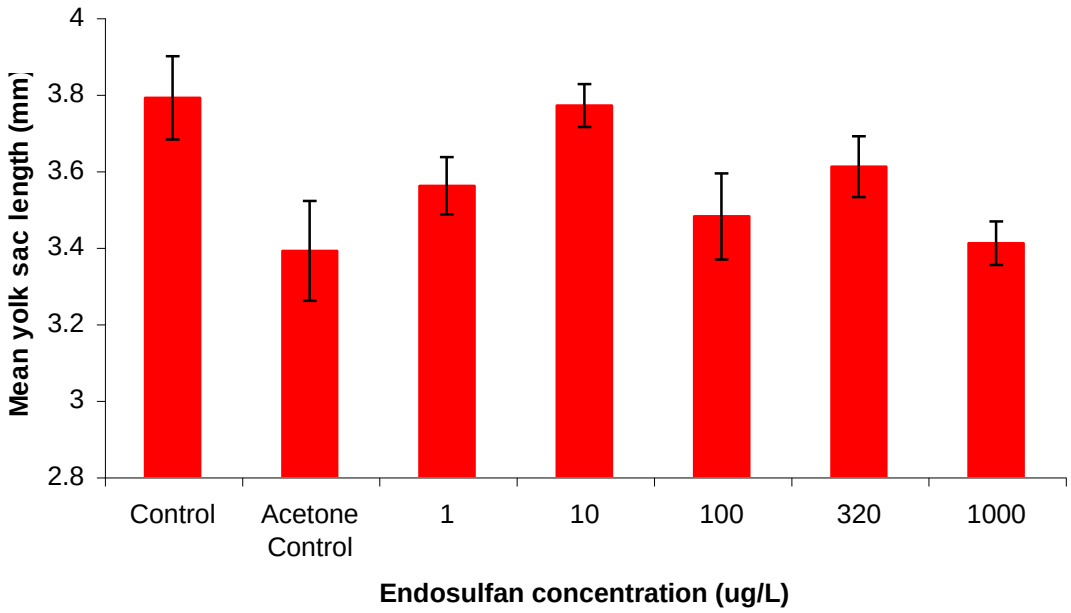


Figure 3.22. The mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Thiodan (350 g/L endosulfan) (n = 10).

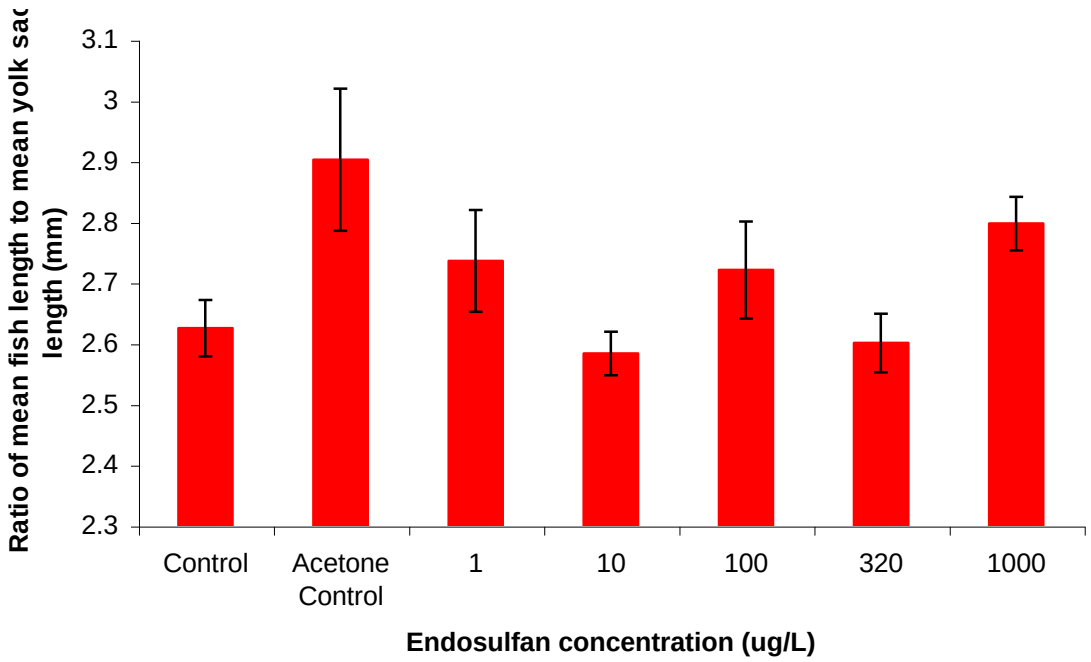


Figure 3.23. The ratio of mean fish length to mean yolk sac length (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Thiodan (350 g/L endosulfan) (n = 10).

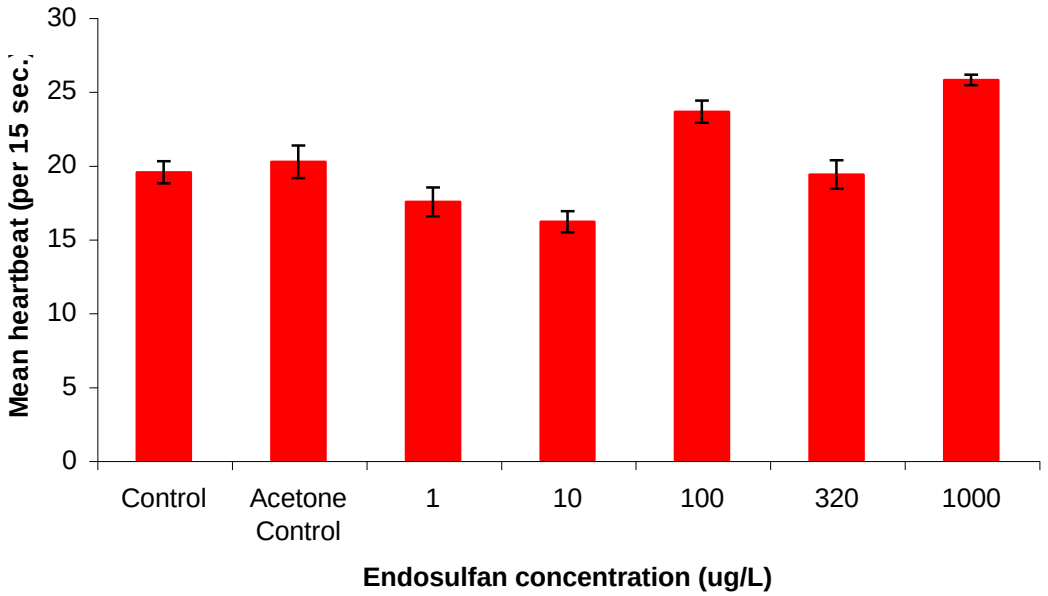


Figure 3.24. The mean heartbeat (±SE) of Murray cod larvae at 96 h following a 24 h pulse-exposure to Thiodan (350 g/L endosulfan) (n = 10).

There was no clear relationship between endosulfan concentration and the percentage of larval hatching (Figure 3.25). In excess of 90% of treated fish successfully hatched with the exception of the control fish where the hatch rate was approximately 85%. Murray cod eggs began to hatch on day 4 following pulse-exposure to endosulfan (Figure 3.26). The highest percent hatch on day 4 occurred in fish pulse-exposed to 1,000 µg/L endosulfan. Approximately 90% of fish hatched on day 5 of the experiment with insignificant (only 5%) hatching noted in the following days from day 6 to 9. The experiment was terminated on day nine as > 95% of the fish had hatched.

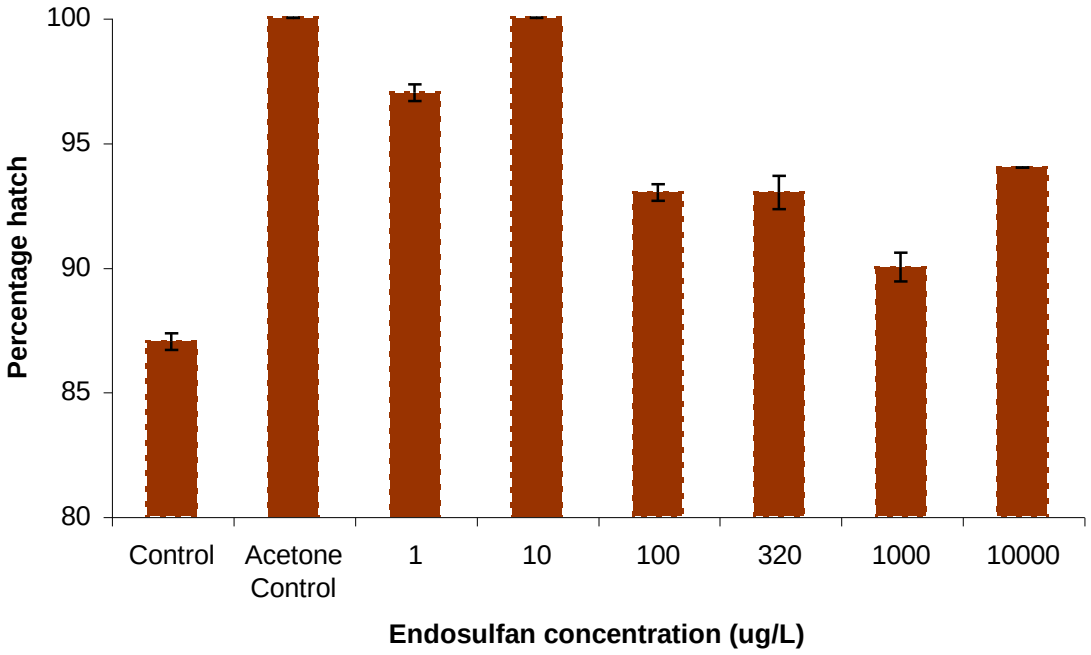


Figure 3.25. The percentage hatch of larval Murray cod eggs following a 24 h pulse-exposure to endosulfan as Thiodan (350g/Kg). Final hatch data was recorded nine days following exposure as this coincided with > 85% hatch in both controls.

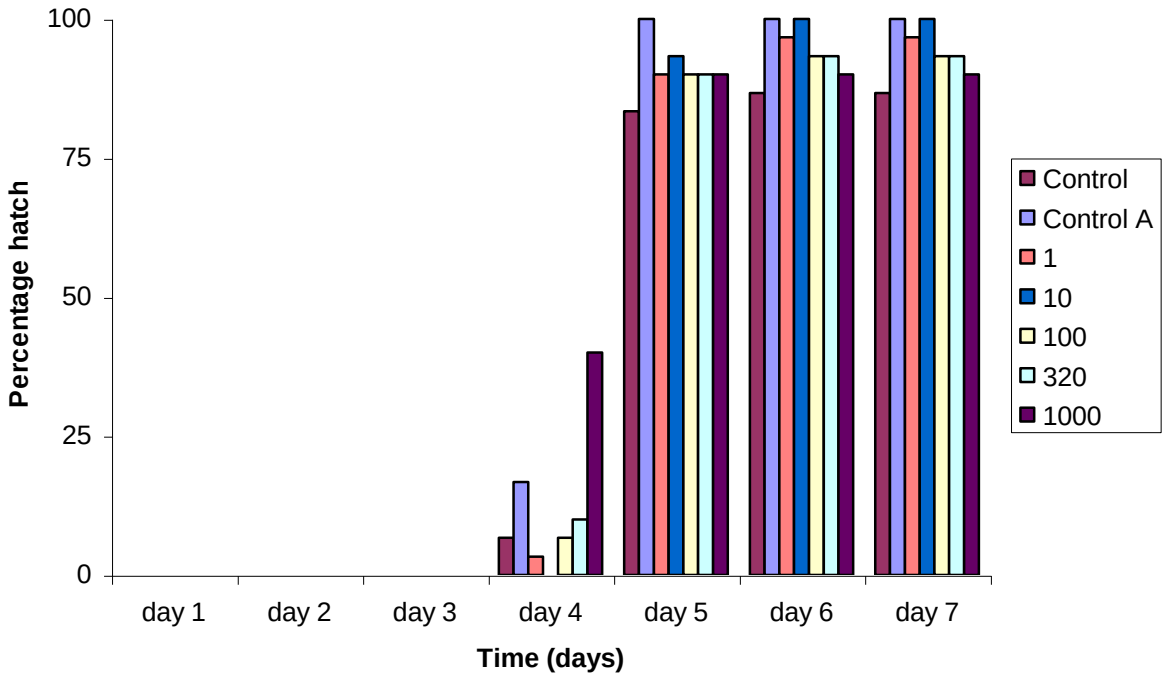


Figure 3.26. The time taken for larval Murray cod eggs to hatch following a 24 h pulse-exposure to endosulfan as Thiodan (350g/Kg).

3.3 Effects of pesticides on Rainbowfish larvae

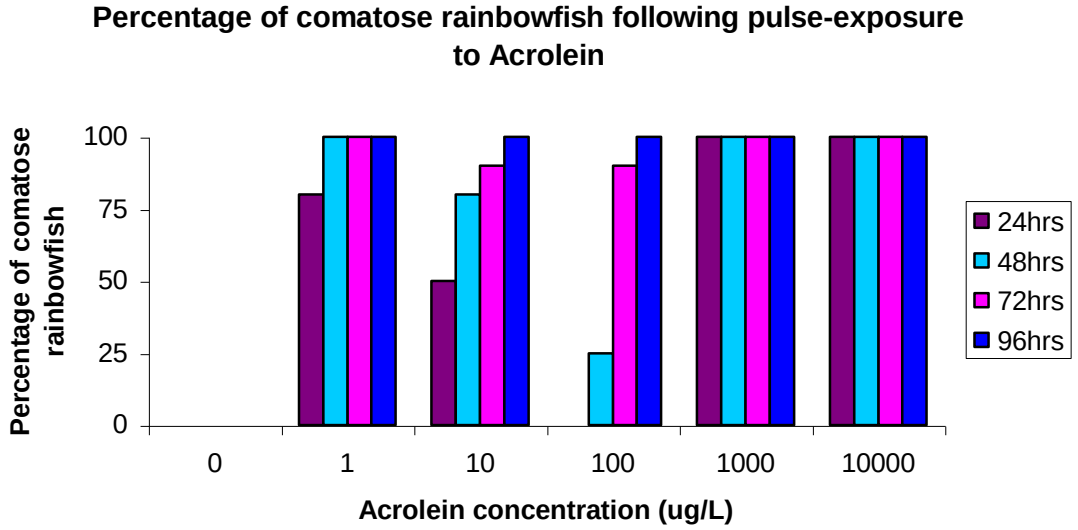
3.3.1 Effect of Acrolein (Magnacide H -950g/Kg).

Rainbowfish were very sensitive to the effects of pulse-exposed Acrolein with an LC_{50} of 0.5-1.0 μg/L (Figure 3.27). The effects of pulse-exposed acrolein were first recorded at 24 h where 80% of fish exposed to 1 μg/L acrolein were in a comatose state (Figure 3.27). By 48 h all of the fish pulse-exposed to 1 μg/L acrolein were comatose and unable to recover from this state for the duration of the experiment. Fig 3.27 shows the percentage of comatose fish was calculated as a percentage of fish with a heart-beat at 24, 48, 72 and 96 hours after exposure to acrolein. The insignificant rainbowfish mortality at 24 and 48 h rose dramatically to approximately 70 and 90% of fish exposed to 1 μg/L acrolein at 72 and 96 h, respectively.

In contrast with the 80 and 100% of fish that were comatose following exposure to 1 μg/L acrolein, 50 and 80% of fish exposed to 10 μg/L acrolein were comatose at 24 h and 48 h, respectively (Fig 3.27). By 72 h approximately 90% of fish exposed to 10 μg/L acrolein were comatose. This temporal reduction in comatose fish with an increase in acrolein concentration continued when rainbowfish were pulse-exposed to 100 μg/L acrolein. At this concentration no fish entered into the comatose state at 24 h, while approximately 25 and 90% of fish were comatose at 48 and 72 h. While less fish entered into a comatose state at 48 h following exposure to 10 μg/L (compared with 1 μg/L) significant fish mortality approximating 37% occurred within the same temporal scale. The rate of mortality increased to 75 and 95% at 72 and 96 h, respectively.

Rainbowfish pulse-exposed to 100 μg/L of acrolein showed signs of periodic twitching at 24 h. By 48 h 6% of the fish had died with 25% of fish entering into the comatose state,

comparatively fewer than that recorded from fish exposed to lower acrolein concentrations. While the number of fish entering a comatose state at 72 h (pulse-exposed to 100 µg/L acrolein) was similar to that observed in fish exposed to 1 and 10 µg/L acrolein, only 50% of fish died compared with 70 and 75%, respectively. All rainbowfish pulse-exposed to 1,000 and 10,000 µg/L acrolein died within 24 h. Fish exposed to 1,000 µg/L acrolein began to twitch 15 minutes into the exposure while all fish exposed to 10,000 µg/L acrolein were comatose at the same temporal scale.



- All rainbowfish pulse-exposed to 1,000 µg/L and above of Magnacide H died prior to the 24 h observation period.

Figure 3.27. The percentage of < 1-day-old rainbowfish that were comatose after 24, 48, 72, and 96 h following a 24 h pulse-exposure to Magnacide H (950g/Kg Acrolein) (n = 10).

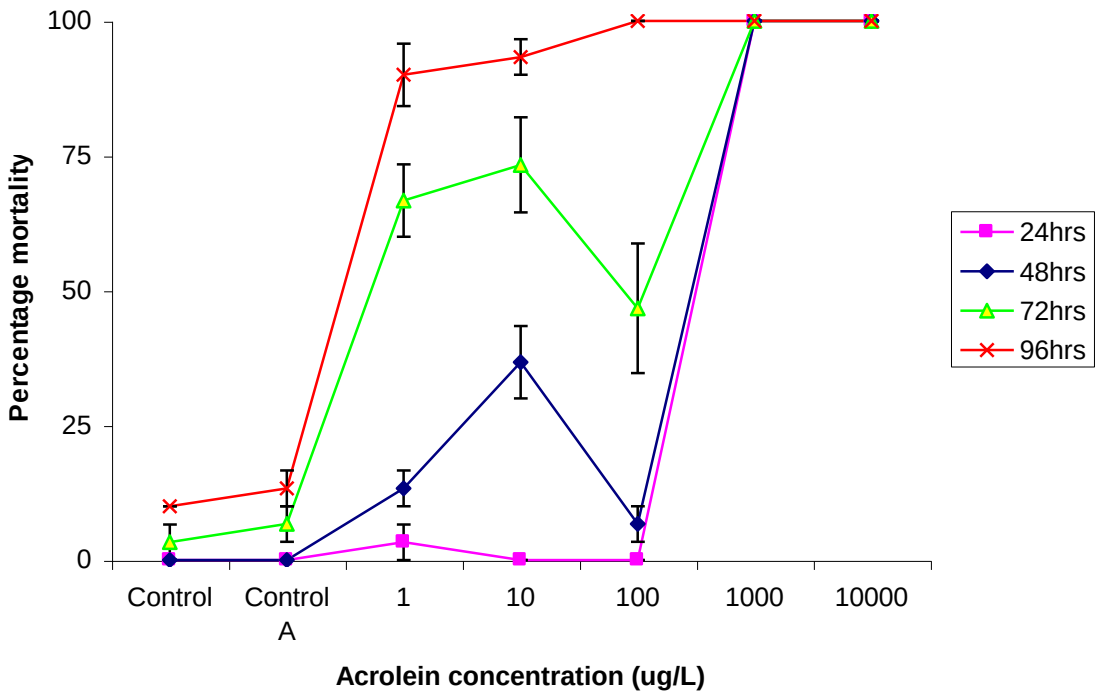


Figure 3.28. The percentage mortality (±SE) of < 1-day-old rainbowfish at 24, 48, 72, and 96 h following a 24 h pulse-exposure to Magnacide H (950g/Kg Acrolein) (n = 10).

3.3.2 Effect of Endosulfan (Thiodan 350g/L)

Rainbowfish were very sensitive to the effects of pulse-exposed Thiodan (endosulfan, 350 g/L) with an LC_{50} of 3-10 µg/L (Figure 3.30). The behavioural effects of endosulfan exposure on larval rainbowfish were evident throughout the investigation in all treatment concentrations. The initial observation period (24 h post-pulse) showed that the majority (80 to 90%) of fish exposed to 1 µg/L endosulfan had become lethargic compared with control fish. This lethargic state was replaced by mild twitching at 48 h. By 72 h approximately 10% of fish pulse-exposed to 1 µg/L endosulfan were motionless on the bottom of the exposure beakers with the remaining fish swimming normally. At the completion of the experimental period (96 h) approximately half the fish pulse-exposed to 1

µg/L endosulfan were in a comatose state, with 23% of the fish dead. Fig 3.29 shows the percentage of comatose fish calculated as a percentage of fish with a heart-beat at 24, 48, 72 and 96 hours after exposure to endosulfan.

Rainbowfish pulse-exposed to 10 µg/L endosulfan were significantly more impaired than fish exposed to 1 µg/L endosulfan. At 24 h, 80-90% of rainbowfish were comatose while 6% of fish pulse-exposed to 10 µg/L had died. By 48 h all fish were comatose with mortality increasing to 23%. Rainbowfish mortality escalated to 93 and 100% at 72 and 96 h, respectively, following exposure to 10 µg/L endosulfan.

A dramatic change was noted in rainbowfish pulse-exposed to 100 and 1,000 µg/L endosulfan compared with fish exposed to 10 µg/L endosulfan. In contrast with the high comatose rate observed in fish exposed to 10 µg/L endosulfan at 24 h, fish exposed to 100 and 1,000 µg/L endosulfan were observed to be lethargic and/or twitchy at 24 h with only 3 fish evidently comatose following exposure to 1,000 µg/L endosulfan. The mortality rate at 48 h was 10 and 0% following exposure to 100 and 1,000 µg/L endosulfan, respectively. The large increase in mortality observed in fish exposed to 10 µg/L endosulfan at 72 h, was not observed in fish exposed to 100 and 1,000 µg/L where 5-10% of fish had died. While 80% of fish exposed to 100 µg/L endosulfan were swimming at 72 h, half of the fish exposed to 1,000 µg/L endosulfan were comatose. Approximately 50 and 36 % of fish pulse-exposed to 100 and 1,000 µg/L endosulfan died at 96 h, respectively.

Pulse-exposure of rainbowfish to 10,000 µg/L endosulfan resulted in the death of all fish at 24 h. Rainbowfish began titching within 1 minute of exposure to 10,000 µg/L endosulfan

while all fish were comatose 15 minutes following exposure. Control mortality was insignificant.

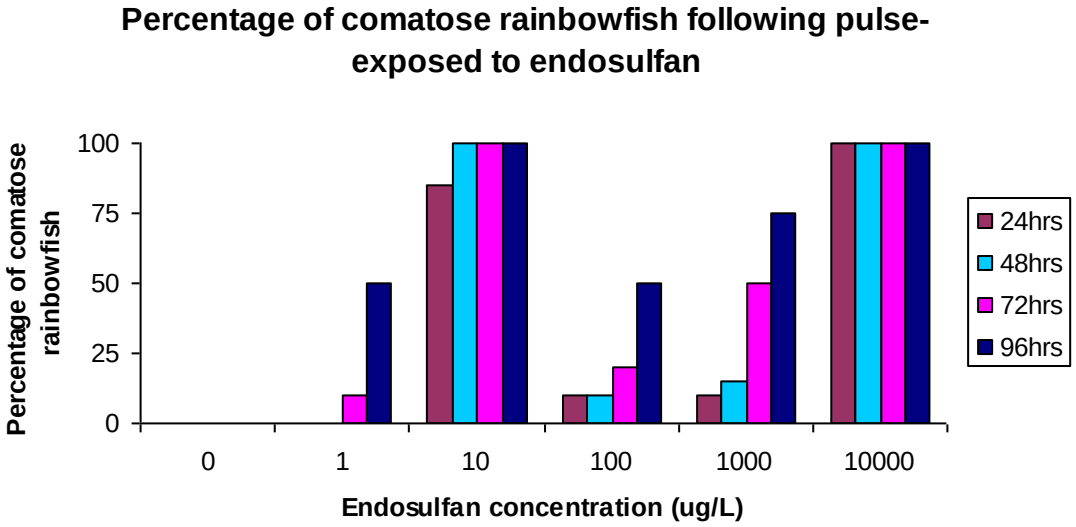


Figure 3.29. The percentage of < 1-day-old rainbowfish that were comatose after 24, 48, 72, and 96 h following a 24 h pulse-exposure to Thiodan (350g/L endosulfan) (n = 10)

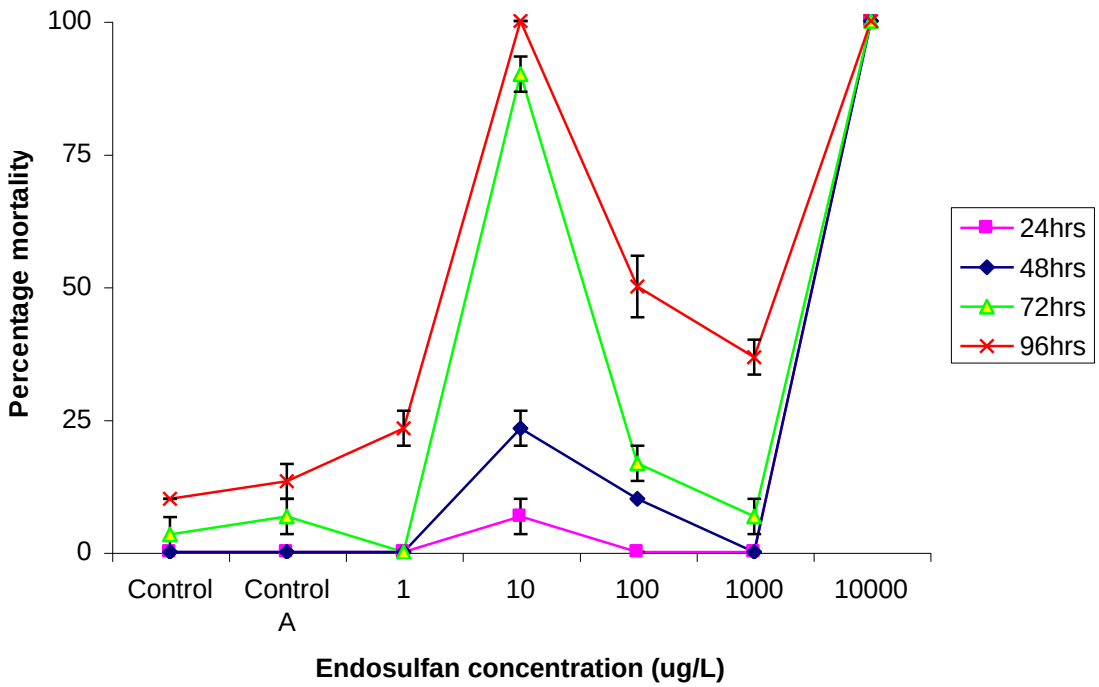


Figure 3.30. The percentage mortality (±SE) of < 1-day-old rainbowfish at 24, 48, 72, and 96 h following a 24 h pulse-exposure to Thiodan (350g/L endosulfan) (n = 10).

4. **Discussion.**

The current investigation found that Murray cod, *Maccullochella peelii peelii,* were most sensitive to the acute effects of acrolein and copper and relatively insensitive to the acute effects of pulse-exposed amitrole, 2,4-D, glyphosate and endosulfan at the concentrations investigated. In contrast, cod were observed to be very sensitive to sub-lethal concentrations of endosulfan.

Murray River rainbowfish, *Melanotaenia fluviatilis,* were acutely sensitive to the effects of pulse-exposed acrolein and endosulfan with LC_{50} s in the low parts per billion. In addition, rainbowfish were found to be very sensitive to sub-lethal concentrations of acrolein and endosulfan.

A number of behavioural and morphological characteristics of both species were affected by exposure to pulses of insecticides. Total body length, heartbeat, and swimming behaviour were the most sensitive characteristics investigated while yolk-sac length and the ratio of total body length to yolk-sac length were found to be the least sensitive parameters to the six pesticides investigated. Variations in acute and chronic toxicity of pulse-exposures of the six pesticides to cod and rainbowfish may be attributed to differences in fish biology and behaviour.

4.1 Toxicity of chemicals tested

4.1.1 Acrolein (Magnacide H -950g/L).

Rainbowfish were more acutely sensitive to the effects of acrolein poisoning compared with cod. The differences in behaviour of the two fish may have contributed to this disparity of toxic effect induced by exposure to acrolein. While cod are known to be lie-in-wait predators, rainbowfish actively search for their food with resulting comparatively higher respiration rates. As swimming requires greater amounts of energy compared with resting, it may be that the rainbowfish increased their uptake of the toxicant due to higher movement and consequently higher respiration rates. An increase in the rate of swimming may have contributed to greater damage to the gills of rainbowfish, as it is these organs that are involved in respiration (Wood, 2001). While differences in behaviour may have contributed to the greater uptake of acrolein by rainbowfish, the proportionally greater yolk sac size of cod may also be responsible, in part, for its relative insensitivity to acrolein. It may be that the lipids present in the yolk sac act as a binding and storage site for pesticides, thus preventing them from attaching to receptor sites on nerves and associated cells.

Larval Murray cod possess a significantly larger yolk sac compared with larval rainbowfish. The yolk sac of larval cod is large enough to prevent them from maintaining swimming for more than a few seconds at a time. Consequently, larval cod spent the majority of the exposure and post-exposure period on the bottom of their beakers. In contrast, rainbowfish were observed to spend their time swimming within the water column of their treatment enclosures. The comparatively smaller yolk sac of rainbowfish may be

depleted 3 to 4 days post hatch (Reid and Holdway, 1995) while the yolk sac of Murray cod may last from 8 to 10 days post-hatch (Humphries, 2005). It may be that the amount of energy required to combat the toxic effects of acrolein, while maintaining homeostasis, was greater than that provided by the yolk sac of larval rainbowfish. If so, then the larval rainbowfish may have succumbed to the toxic effects of the poison on tissues and organs along with the effects of starvation due to the depletion of energy reserves. This notion is supported by the conclusion put forward by Reid *et al*., (1995) that fish death after 24 h may be due to indirect effects of starvation rather than the direct effects of physiological damage. The death of large numbers of rainbowfish over the second half of the investigation is in agreement with this proposition. It may be that both direct and indirect effects induced by the toxic action of acrolein were responsible for the high sensitivity of rainbowfish to this toxicant.

While differences in yolk sac size between larval cod and rainbowfish may have resulted in greater rainbowfish mortality, a causal link between yolk sac size, energy consumption, and higher mortality in fish has not been established. The physiological and biochemical differences between the two fish species may have significantly contributed to the observed variation in sensitivity to acrolein. The possession or absence of enzymatic systems involved in the transportation, degradation, and excretion of toxic metabolites may vary significantly between the two fish species, resulting in vastly different effects. While the exact mechanisms of acrolein toxicity are unknown in aquatic vertebrates, it is evident that both cod and rainbowfish populations may be significantly reduced following exposure to this toxicant.

The sensitivity of the larval Australian native fish tested was shown to vary considerably following pulse-exposure to the herbicide, acrolein . The very high sensitivity of rainbowfish to acrolein supports the current recommendation that this product should not be used in waterways known to hold rainbowfish. While cod were comparatively less sensitive to acrolein, the results showed that all larval cod pulse-exposed to acrolein concentrations of 10,000 µg/L died within 12 h. The observation that acrolein concentrations as low as 1 µg/L resulted in cod death and the admission that acrolein is toxic to fish and wildlife and must be kept out of rivers, lakes, ponds and streams and that fish, shrimps, and other aquatic species will be killed at recommended application rates' (Baker Hughes, MSDS, 2005) suggests that more research into the effects of acrolein is required before Australian waterways containing cod and/or rainbowfish may be treated with this toxicant.

A comparison of the results from the current investigation using native fish with those in the literature indicate that rainbowfish are more sensitive to the toxic effects of pulse-exposed acrolein with a 96 h LC_{50} < 10 µg/L, compared with northern hemisphere fish and crustaceans with continuously exposed 96 h LC50 s ranging from 22 to 790 µg/L (Baker Hughes, MSDS 2005). Murray cod were less sensitive (96 h LC_{50} between 1,000 and <10,000 µg/L) to acrolein poisoning compared with reported toxicity values for bluegill sunfish *(L. macrochirus*) (96 h LC_{50}, 24 µg/L), rainbow trout (96 h LC_{50}, 22 µg/L) and Sheepshead minnows (96 h LC_{50}, 570 µg/L) as noted in Table 1.1. The shorter exposure period and differences between fish species may account for much of the variability in their

sensitivity to acrolein exposure. It is also possible that Murray cod larvae are more robust and therefore less sensitive to this pesticide.

4.1.2 2,4-D Amicide (625 g/L)

The lack of fish death following pulse-exposure to 100,000 μg/L 2,4-D indicates that this formulation of 2,4-D amine may not result in the death of cod under field conditions.

Therefore the results of this study indicated that Amicide is unlikely to pose a threat to Murray cod larvae at the reported spray concentrations in the G-MW region especially since it has a very short half life in the aquatic environment (Tomlin, 2000).

4.1.3 Amitrole T

Murray cod were found to be relatively insensitive to a pulse of Amitrole T. The specific use of this chemical as a herbicides does not appear to pose an acute threat to the survival of Australian Murray cod populations at current application rates. The death of two fish pulse-exposed to 10,000 μg/L amitrole T in the current investigation was consistent with the limited literature reports that Amitrole T is slightly toxic to fish and freshwater invertebrates (Oesterreich *et al*., 1999). There is some evidence for effect in that the heartbeat of larval cod following exposure to amitrole T concentrations of 10 μg/L or more was elevated. Such an increase may lead to the premature exhaustion of energy reserves used for homeostasis.

Both 2,4-D Amicide and Amitrole T seem to be of very low toxicity to Murray cod, similar to that reported in the literature for their toxicity to exotic species. It is possible that these pesticides are unavailable for uptake by larval gills and do not penetrate the yolk sac or if they do, are quickly detoxified by binding to the yolk.

Results indicated that Amitrole is unlikely to pose a threat to Murray cod larvae exposed to a pulse of the herbicide in the aquatic environment. However the half life in the environment can be up to 4 weeks (Tomlin, 2000) and it is possible that continuous exposure for this duration could result in different effects in the field.

4.1.4 Weedmaster duo (Glyphosate 360 g/L)

Pulse-exposure of Murray cod to 100,000 μg/L Glyphosate (Weedmaster duo) resulted in the death of 13% of test cod, indicating that short-term exposure to high concentrations of glyphosate may be lethal to native Australian fish. The highly specific nature of glyphosate toxicity suggests that glyphosate-induced mortality in Murray cod was the result of impaired construction of proteins from amino acids. The observation that glyphosate concentrations below 100,000 μg/L did not result in fish death is consistent with those in the literature that indicate that fish are relatively insensitive to the effects of glyphosate poisoning at field concentrations. The insignificant decrease in total cod length with increasing glyphosate concentrations suggests that sub-lethal effects of glyphosate poisoning may impact on the individual and population ecology of cod exposed to high concentrations of glyphosate. The maximum recommended level of glyphosate in Australia

has been set at 370 g/L (99% protection for aquatic ecosystem protection) (see Appendix II). Our findings indicate that formulated glyphosate (Weedmaster duo) may not pose an acute threat to the long-term survival of Murray cod populations at current application levels.

4.1.5 Copper

Murray cod were up to two orders of magnitude less sensitive to pulse-exposed copper compared with continuously exposed (96 h) northern hemisphere fish reported in the literature (Yang and Chen, 1996; Wong *et al*., 1999; Kamunde and Wood, 2003; Grosell *et al*., 2004; G-MW, 2005; Matsuo *et al*., 2005). While 24 h pulse-exposure to 10,000 μg/L of copper resulted in 100% larval cod mortality, 100% survived exposure to 1,000 μg/L for 96 h. Greater energy reserves combined with lower respiration rates and shorter exposure times may be responsible for much of the recorded relative insensitivity of Murray cod to copper toxicity. The existence of a 96 h acute toxicity threshold for Murray cod pulse-exposed to copper for a period of 24 h was established to be between 1,000 and 10,000 μg/L. The application of copper to crops in close proximity to waterways may need to be regulated in an effort to prevent copper concentrations above 1,000 μg/L from entering aquatic environments.

The large Murray cod yolk sac may provide the fish with a proportionally greater amount of energy required for detoxication and excretion of copper while providing the cod with

reserve energy necessary for homeostatic and osmoregulatory functioning. As behavioural observations were not reported in many of the northern hemisphere studies, it is possible that the low respiration rate noted for the 'lie-in-wait' predatory cod may have contributed to their lower sensitivity to copper exposure. Additionally, the reduction in exposure time of cod to copper may have contributed to their lower sensitivity to the toxicant by limiting the damage to internal and external organs such as the liver and gills, which are argued to be the main route for copper toxicity in fish. While higher energy reserves, reduced exposure time and behavioural variations may explain some of the variability in fish sensitivity to copper, other factors, such as fish size, temporal liver functioning, presence/absence of detoxifying enzymes, gill surface area and a number of physiological differences between fish species, may be equally plausible.

While larval Murray cod were less sensitive to copper toxicity compared with fish from the northern hemisphere, this relative insensitivity does not infer protection from toxicity. It is reasonable to suggest that a risk assessment be conducted to ensure the survival of cod in waters known to be susceptible to copper exposure within the Goulburn-Murray River regions in addition to other areas where copper is used as an insecticide. Such an assessment would necessarily include data on application rates, environmentally realistic copper concentrations and the lethal and sub-lethal effects on a range of Australian freshwater organisms.

4.1.6 Endosulfan (Thiodan (350g/L)

Endosulfan was the only pulse-exposed toxicant recorded as having a significant impact on the morphological and behavioural responses tested on less-than 2-day-old Murray cod larvae. The total length of larval Murray cod was significantly reduced following pulse-exposure to 10,000 μg/L endosulfan while cod pulse-exposed to endosulfan concentrations of 1,000 μg/L exhibited significant behavioural modifications compared with control fish. The No Observed Effect Concentration (NOEC) for behavioural modification in larval Murray cod was 100 μg/L while the NOEC for larval rainbowfish pulse-exposed to endosulfan was < 1 μg/L for acute and behavioural modifications. Cod length was the only morphological characteristic significantly affected by exposure to endosulfan.

The reduction in total cod length following exposure to 10,000 μg/L may be attributed to the spinal curvature of larval cod at 96 h. As total fish length was measured from tip to tail any curvature of the spine may have influenced this measurement. As endosulfan acts by altering the electrophysiological and associated enzymatic properties of nerve cell membranes causing changes in the kinetics of Na^+ and K^+ ion flow (Videira *et al.*, 2002), it is likely that the neurotoxicity of endosulfan may be responsible for the damage caused to the normal development of the spine in larval Murray cod and hence the comparatively lower total body length.. It is likely that a trade off between growth and energy requirements dedicated to the metabolism and removal of endosulfan from fish cells occurred. More energy may have been allocated to combating the toxic effects of endosulfan poisoning, resulting in a general decrease in fish length with an increase in

endosulfan concentration. Other energy-dependent factors such as homeostasis and osmoregulation may have contributed to the concentration dependent reduction in fish growth.

The reported action of endosulfan on Na^+, K^+ -ATPase, which is intimately coupled to osmoregulation, may be the irreparable affect on the osmoregulatory capacity of fish (Dalela *et al.*, 1978). The ability of fish to osmoregulate may be seriously compromised by changes to the internal distribution of sodium and potassium. Inhibition of these enzyme systems and their ability to alter nerve transmission may, in part, explain the high toxicity of endosulfan to fish. Such a change may influence the normal development of nerves, tissues, organs and other morphological structures.

The neurotoxic effect of endosulfan poisoning may be the cause of the sporadic 'twitching' and eventual slides into the comatose state of Murray cod and rainbowfish larvae pulse-exposed to 1,000 and 1 µg/L endosulfan, respectively. A number of authors have reported similar observations of sporadic and comatose symptomology following exposure of fish to endosulfan (Schoettger, 1970; Gopal *et al.*, 1981; Singh and Srivastava, 1981). The symptomatic twitching and comatose states have been associated with the calcium-induced release of neurotransmitters and the consequent disruption to nerve impulse transmission in fish (Ecobichon, 1996). As Na^+ and K^+ -ATPase enzymes are essential for the generation of membrane potentials and the maintenance of tissue osmolarity, inhibition of these enzyme systems and their ability to alter nerve transmission may, in part, explain the toxicity of endosulfan to fish (Dalela *et al.*, 1978; Naqvi and Vaishnavi, 1993). Murray cod eggs were

tolerant to endosulfan concentrations as high as 10,000 µg/L with a 24 hour pulse exposure with no significant effect on % hatch and only a one day delay in hatching. These results are unlike those observed for Northern Hemisphere species of fish (Table 1.1). It is possible that the egg membrane in Murray cod provides protection from this pesticide and results in the low toxicity, even though it is an organochlorine pesticide classified as highly hazardous (US EPA, 1991).

Murray cod larvae were very tolerant to the acute effects of endosulfan exposure with an LC_{50} in excess of 10,000 µg/L. In contrast, rainbowfish were highly sensitive to the acute effects of endosulfan poisoning with an $LC_{50} < 10$ µg/L. This value is in agreement with the findings of Raymond (RMIT University, unpublished) who reported a 96 h LC_{50} of 5.1 µg/L for crimson-spotted rainbowfish pulse-exposed to technical grade endosulfan for 4 h. In comparison, the majority of northern hemisphere fish also appear to be very intolerant to endosulfan exposure, with LC_{50}s in the vicinity of 1 to 50 µg/L. Field concentrations of endosulfan regularly exceeded the former Australian and New Zealand Environment and Conservation Council (ANZECC) water quality guideline of 0.03 µg/L (see appendix II and Edge *et al.*, 1999) and the highest recorded field level of total endosulfan in south-eastern Australia was 4.58 µg/L (Peterson & Batley, 1991) and in G-MW irrigation area was 0.0007-0.029 µg/L (Rose and Kibria, 2006). The data suggest that recorded field concentrations of endosulfan may not adversely affect the survival or behaviour of larval Murray cod in the Goulburn-Murray Water catchments. In contrast, pulse-exposure of rainbowfish to endosulfan concentrations below 1 µg/L led to significant acute, morphological and behavioural modifications. In light of these findings we recommend that

the use of endosulfan on crops surrounding waterways containing rainbowfish be managed in an effort to prevent this insecticide from entering aquatic ecosystems. Research into the biochemical and reproductive effects of endosulfan poisoning in rainbowfish and other sympatric species should be conducted to ensure the long-term survival of these populations and communities. Appropriate risk assessment modelling and preventative management should be undertaken to facilitate adequate protection of aquatic organisms found in waterways where endosulfan poisoning is likely.

4.1.6.1. Effects of endosulfan on hatching of Murray cod eggs

The percentage hatch of Murray cod eggs was not significantly affected by endosulfan concentrations from 1 to 1,000 μg/L indicating that cod eggs may be impermeable to pulse-exposed endosulfan. In contrast, we found that approximately 40% of cod eggs pulse-exposed to 1,000 μg/L endosulfan began to hatch a day earlier than significant numbers of eggs pulse-exposed to lower endosulfan concentrations. The cause and repercussions of early development may have a significant impact on the long-term survival of a species. A reduction in hatch time may confer a competitive advantage in light of the ability of these fish to procure food and territories. However, early hatch may result in exposure to predators and/or unfavorable environmental conditions.

The relationship between early hatch and high concentrations of endosulfan are not easily established. It may be that higher endosulfan concentrations may have altered the structure or shape of the eggs resulting in early destruction of the outer egg membranes. Alternately,

the developing fish exposed to higher concentrations of endosulfan may have been stimulated to accelerate growth and shortened their developmental time, albeit with spinal curvature and deformation of the body. This presumption assumes that the egg membrane is at least semi-permeable to endosulfan. It is also possible that higher concentrations of endosulfan adsorbed to the eggs prevented the normal exchange of ions through the membrane, resulting in the intra-chorion build-up of these ions leading to the early hatch of cod. While the exact mechanisms responsible for the early hatch of cod pulse-exposed to endosulfan remain unclear, the results of hatching prematurely may be advantageous. The absence of endosulfan concentrations above 5 μg/L in the field suggests that the majority of cod eggs from the same clutch would hatch within a day of one another.

4.3 Future research

Results of the current investigation into the effects of pulse-exposed toxicants to Murray cod and Murray River rainbowfish indicate that these toxicants may affect the survival, morphology, and behavioural characteristics of both species. What we do not know is how these changes are influenced by the action of internal systems within individual fish and the repercussions of these changes on fish populations. On a broader scale, we need to determine how the depletion or change in fish populations may influence the structure and functioning of the ecosystems within which they inhabit. Investigations into the long-term genetic effects of toxicant exposure may provide the scientific community with an avenue for detecting population changes.

More research into the biochemical effects of toxicant exposure would increase our understanding of the factors responsible for fish mortality and reductions in fish length. Establishing cause and effect relationships between individual toxicants and biochemical changes would increase our understanding of the factors responsible for acute and chronic changes in fish and other aquatic organisms. These relationships may provide us with indicators of toxic response such as the inhibition of cholinesterases, cytochrome P450s, carboxylesterase and other enzymes used to metabolise anthropogenic substances. Such bio-indicators may be used to manage the use of toxicants as well as assess the potential risks associated with their use.

Research into the effects of these selected toxicants on a range of sympatric macro and micro-invertebrate species need to be completed if we are to gain more insight into how these toxicants may impact on population and community structure and functioning. Future research into the toxicity of surfactants and other formulated additives may help to manage the use and effect of poisons in the aquatic environment. Research into the combined effects of toxicant mixtures would be most beneficial as many aquatic organisms may be exposed to a plethora of toxicants at the same time. Current research has shown that exposure to two or more chemicals may dramatically alter the toxicity of individual toxicants.

The use of lethal body burdens (LBB) rather than just toxicant concentrations within the water would help resolve problems associated with behavioural uptake of toxicants and the

factors responsible for symptomatic poisoning. The use of endogenous toxicant concentrations may provide more robust information on the establishment of cause and effect relationships for individual aquatic species.

The addition of microcosom and macrocosm studies may provide more useful information on the effects of toxicant exposure to community structure. These pond or stream experiments may be used to provide additional information on relationships between toxicant exposure and ecological effects, such as predator prey relationships, biomass investigations, biodiversity studies, the effects of vegetation clearing on feeding and breeding, as well as the bioavailability of toxicants to aquatic organisms inhabiting environments containing vegetation and other possible sinks for toxicants. Future research into the effects of herbicides on Australian fish should include detailed investigation into the effects of vegetation removal. While the direct effects of herbicides on native fish are of great interest in ecotoxicology it may be the indirect effects of vegetation removal that significantly impact on the long-term survival of aquatic organisms.

The current drought across the state of Victoria could result in pesticides that are sprayed becoming more concentrated in drying waterways and it is imperative that further similar studies be conducted on their possible effects on native fish and other fauna to understand and help prevent the detrimental effects of agricultural chemicals on ecosystem health.

4.4 Conclusions and Recommendations

The findings from the current investigation have led the authors to develop a number of recommendations for the use of selected toxicants in and around waterways containing Murray cod and Murray River rainbowfish.

Findings

- The results from the current investigation indicate that Murray cod larvae were most sensitive to the acute effects of pulse exposed copper and acrolein exposure and least sensitive to pulse-exposed 2,4-D, glyphosate, endosulfan, and Amitrole T (see Table 3.3 and Appendix II). It is important to note that these results are confined to the concentrations of individual toxicants tested and the lethal or sub-lethal characteristics investigated, which need to be related to application rates, and maximum allowable limits imposed by statutory legislation.
- The lack of significant acute and sub-lethal effects induced in Murray cod larva (see Table 3.4) following exposure to Amitrole T, 2,4-D, acrolein, glyphosate, and copper indicate that pulse-exposure to these toxicants at the concentrations investigated may not pose a real threat to the long-term survival of Murray cod populations. Future research into biochemical effects of these toxicants to larval cod is required to substantiate this claim.
- The acute and sub-lethal effects of endosulfan toxicity on Murray cod behaviour and growth were restricted to concentrations of 100 μg/L and above indicating that

the ANZECC and ARMCANZ (2000) water quality guideline of 0.03 µg/L (99% species protection) is sufficient to protect the long-term survival of Murray cod.

- Murray River rainbowfish were found to be very sensitive to the acute effects of pulse-exposed acrolein and endosulfan with 96 h LC_{50}'s is 1 - 10 µg/L, respectively.(See Figure 3.27 and 3.38 and appendix II) Consequently, we suggest for a more comprehensive review into the acute and sub-lethal effects of pulse exposure of these two chemicals (acrolein and endosulfan) to Murray River rainbowfish.

Recommendations

1. In light of the high sensitivity of Murray River rainbowfish to endosulfan and acrolein we recommend that a more comprehensive investigation into the sub-lethal effects of endosulfan and acrolein poisoning be conducted on rainbowfish larvae.
2. We also recommended that a range of pesticides used in the G-MW region should be tested on the sensitive Murray River rainbowfish since it is a useful indicator species.
3. This preliminary study demonstrates the effects of individual herbicides and pesticides on Murray cod and Murray river rainbow fish, however, in natural waterways they are generally exposed to a mixture of chemicals simultaneously. We therefore, recommend that further research should be conducted using mixtures of G-MW herbicides and pesticides identified in G-MW irrigation areas to evaluate their effects on sensitive native species such as Murray River Rainbow fish.

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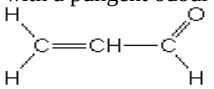
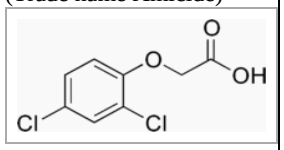
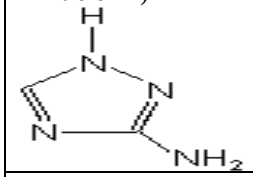
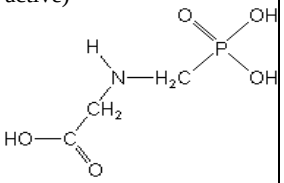
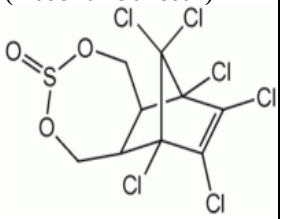
Appendix 1: Physico-chemical properties and toxicity of agriculture chemicals tested in the current study

Chemicals	**Mammalian Toxicology**	**Ecotoxicology**	**Partition coefficient or Log K_{ow}**	**Half life or DT_{50} (time for 50% loss)**	**Water solubility and Stability**	**Comments**	**References**
Acrolein – herbicide (Trade name Magnicide H) colourless mobile liquid with a pungent odour 	LD50 for rats-29 mg/kg NOEL (90 d) for rats 5mg/kg b.w daily	Highly toxic to fish, ***Fish*** : LC_{50} (24h) for rainbow trout 0.15 mg/L ***Daphnia*** : LC_{50} (48h) 20 ppb ***Algae*** : EC_{50} (5 d) *Selenastrum capricornutum* 0.050 mg/L; *Anabaena flos-aquae* 0.042; *Navicula pelliculos*a 0.07mg/L	1.08	Half life in water as follows : 3.5 d (pH 5); 1.5 d (pH 7) and 5-4 h (pH 10)	Water solubility : 208 g/kg (20^o C) Stability : stable ≤80 C; highly reactive chemical; may polymerise if exposed to light	It does not accumulate, Acrolein is not persistent in aquatic environment The rate of reaction of acrolein increased with increasing pH. In flowing water, the rate of loss is faster reflecting the influence of turbulence in increasing volatilization loss	Tomlin (2000); Nasim et. al. 2007
-2-4-D amine Herbicide (Trade name Amicide) 	LD50 for rats-639-764 mg/kg NOEL (2 yr) for rats 5mg/kg b.w daily	Some formulations of 2-4,D are highly toxic and others are less toxic ***Fish*** : LC_{50} (96 h) for rainbow trout >100 mg/L ***Daphnia*** : LC_{50} (21 d) 235 mg/L ***Algae*** : EC_{50} (5 d) *Selenastrum capricornutum* 33.2 mg/L	2.58-2.83 (pH 1); 0.04-0.33 (pH 5);	short half life in soil and aquatic environments; Half life is <7 days	Water solubility : 311 (pH 1); 20031 (pH 5); 23180 (pH 7); 34196 (pH 9) mg/L at 25 C) Stability :2,4-D is a strong acid and forms water soluble salts with alkali metals and amines	Low persistence Detected in groundwater supplies in the USA, and Canada	Tomlin (2000); Extoxnet (1996)
Amitrole-herbicide (Trade name Amitrole T or Amitrole TL) 	LD50 for rats->1100-24600 mg/kg NOEL (24 months) for rats 10 ppm	Slightly toxic to various freshwater fish and invertebrates ***Fish*** : LC_{50} (96 h) for rainbow trout >1000 mg/L ***Daphnia*** : LC_{50} (48h) > 10mg/L ***Algae*** : NOEC for *Scenedesmus subspictus* 8.96mg/*L*	-0.97	Half life is 2-4 weeks	Water solubility :280 g/L (23^oC); 530 g/L ((53^o C); Stability : stable in neutral, acidic and alkaline media powerful chelating agent	Low soil persistence Readily soluble in water Moderate potential for groundwater contamination as it does not adsorb strongly in soil particles (Tomlin (	Tomlin (2000); Extoxnet (1996)
Glyphosate-herbicide (Trade name Round up active) 	LD50 for rats-5600 mg/kg NOEL (2 years feeding trails- no ill effect) for rats 410 mg/kg	Known as non-toxic to fish and slightly toxic to invertebrates ***Fish*** : LC_{50} (96 h) for rainbow trout 86 mg/L ***Daphnia*** : LC_{50} (48h) 780 mg/L ***Algae*** : EC_{50} (72 h) *Selenastrum capricornutum* 485 mg/L (7 d) 13.8 mg/L; *Anabaena flos-aquae* 15mg/L	<-3.2(pH 2-5, 20^o C)	Average half life in soil is 47days (range 3-174 days) Half life in water is few to 91 days	Water solubility :11.6 g/L(25^oC); Stability : non-volatile donot photochemically degrade	In water, glyphosate is strongly adsorbed to suspended organic and mineral matter and is broken down primarily by microorganisms	Tomlin (2000); Extoxnet (1996)
Copper hydroxide – fungicide (Trade name kocide)	LD50 for rats-489 mg/kg	Highly toxic to aquatic organism (fish) Bioaccumulate into fish tissues	-	Persistent naturally occurring element. No chemical half-life. Biological half-	Water solubility :2.9 mg/L(pH 7, 25^oC); Stability : dehydrated > 50 ^oC for extended	Persist indefinitely, copper is bound or adsorbed to organic materials and to clay and mineral surfaces	Tomlin (2000); Extoxnet (1996); Eisler, (2000)

$Cu(OH)_2$		***Fish*** : LC_{50} (24 h) for rainbow trout 0.08 mg/L ***Daphnia*** : LC_{50} 6.5 ppb		life is species dependent.	periods, decomposes at 140^{o} C	Fish and invertebrates have mechanisms to detoxify (in Cu-rich granules and metallothioneins) and excrete Cu at low environmental concentrations.	Phillips and Rainbow, (1993)
Endosulfan-insecticide (Trade name thiodan) 	LD50 for rats-70 mg/kg (aqueous suspension), 110 mg/kg (in oil), 76 mg/kg (alpha-isomer), 240 g/kg (beta-isomer) NOEL (2 years for rats 15 ppm diet	Very highly toxic to fish Bioaccumulate ***Fish*** : LC_{50} (96 h) for golden orfe 0.002 mg/L ***Daphnia*** : LC_{50} (48h) 75-750 µg//L ***Algae*** : EC_{50} (72 h) for green algae >0.56 mg/L		For the alpha & beta endosulfan the half life is 30-70 days The main metabolite is endosulfan sulfate, which is degraded most slowly. DT_{50}. for total endosulfan (alpha- and beta-endosulfan and endosulfan sulfate) in the filed is 5-8 months	Water solubility :alpha –endosulfan 0.32 mg/L, beta-endosulfan 0.33 mg/L (22^{o}C); Stability : stable to sunlight, slowly hydrolysed in aqueous acids and alkalis	Moderately persistent in soil Endosulfan does not easily dissolve in water and has a low solubility Is broken down by fungi and	Tomlin (2000); Extoxnet (1996)

Appendix II. A comparison of LC_{50} (96 h) values of Murray cod, Murray River Rainbow fish (based on current experiments) and alien fish species (Rainbow trout) and guideline trigger value for the protection of aquatic species for each tested chemical

Toxicant	LC_{50} (96 h) for Murray cod Current study)	LC_{50} (96 h) for Murray River Rainbow Fish (current study)	LC_{50} (96 h) for Alien fish (Tomlin, 2000; ANZECC, 2000)	Guideline trigger value for the protection of freshwater species (95% or 99%) (ANZECC, 2000)	Comments
Acrolein (950g/L)	>1000 <10,000 µg/L	<10 µg/L	150 µg/L (rainbow trout) 14-125 µg/L (4 spp.)	0.01 µg/L Interim working levels	Murray cod are less sensitive than native rainbow fish and alien fish to acrolein Murray Rainbow fish are very sensitive to acrolein
				▪	▪
2,4-D amicide (625g/L)	>10,000 µg/L	not done	100,000 mg/L (rainbow trout) 1400-4800,000 µg/L (23 spp)	280 µg/L (95% protection) 140 µg/L (99% protection)	2,4-D affects murray cod larvae at very high concentration ▪
				▪	▪
Amitrole T (250g/L)	>10,000 µg/L	not done	>1000,000 mg/L (rainbow trout) 65,000-410,000 µg/L (4 spp.)`	22 µg/L (not specified)	Amitrole affects murray cod larvae at very high concentration ▪
				▪	▪
Glyphosate (360 g/L)	>100,000 µg/L	not done	86,000 µg/L (rainbow trout) 11,000-4290,000 µg/L (10 spp)	1200 µg/L (95% protection) 370 µg/L (99% protection)	Glyphosate may affect murray cod larvae at very high concentration
				▪	▪
Copper	>100 µg/L	not done	40-80 µg/L (salmonid including rainbow trout)	1.4 µg/L (95% protection) 1.0 µg/L (99% protection)	Copper is highly toxic to murray cod larvae at low concentrations
				▪	▪
Endosulfan, (350 g/L)	>10,000 µg/L	10 µg/L	2 µg/L (golden orfe) 0.1-63µg/L (42 spp)	0.2 µg/L (95% protetction) 0.03 µg/L (99% protection)	Murray cod are less sensitive than murray rainbow fish and alien fish to endosulfan Rainbow fish are very sensitive to endosulfan
				▪	▪

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Appendix IV: Research findings confirm responsible pesticide management (Media Release)
Friday 24 November, 2006

Two important research projects into Goulburn-Murray Water's involvement with pesticides have produced positive results for water users and the environment.

The research, showcased at a recent workshop in Tatura, confirmed that current management practices are minimising pesticide residue levels in channels and protecting our native fish.

The Department of Primary Industries, CSIRO, the Cooperative Research Centre for Ecotoxicology, and Goulburn-Murray Water presented the final report on new passive sampling techniques to detect residues of pesticides used by its customers in Goulburn-Murray Water irrigation channels.

G-MW Executive Manager Planning and Environment Alex Marshall said traces of five pesticides were found, but not at levels that would adversely affect channel water users or the environment.

"The results of this exhaustive study are a tribute to our customers' care for the environment," Mr Marshall said.

"Irrigators, town water users, domestic and stock water users and those who care for the environment should have their confidence in channel water quality bolstered."

RMIT University also presented the results of a study into the effects that copper, endosulfan and the herbicides used by Goulburn-Murray Water have on Australian native fish.

"This is a ground-breaking study, as chemicals are usually only tested on overseas species, like rainbow trout, before they are passed as safe to use," Mr Marshall said.

"RMIT found that native fish can safely tolerate higher levels of the herbicides we use compared to the foreign test species.

"This confirms that we are unlikely to harm native fish, as we operate in accordance with the regulations covering these herbicides."

Mr. Marshall said Goulburn-Murray Water had commissioned the two research projects as part of its continual improvement process to fill information gaps on pesticide and herbicide residues.

"There are a couple of other similar pesticide and herbicide research projects in the pipeline, the results of which G-MW will share with government regulators, customers and the public as they become available," he said.

"As each project is completed, we review the results to see if our chemical management can be improved."

Last updated: 22 Dec 2010

Source: http://www.g-mwater.com.au/news/media-releases/media-releases-2006/pesticidemanagement.html

Appendix V: R&D breakthrough – Murray Cod are more tolerant to herbicides than Alien Fish (G-MW Outlet artcle)

Research conducted by G-MW in collaboration with the RMIT University has found that Australia's iconic fish, the Murray cod is more tolerant to herbicides (glyphosate, amitrole, 2,4-D amine and acrolein) and other pesticides (endosulfan, copper) than alien fish rainbow trout.

The breakthrough ecotoxicological data, generated for the first time in Australia, provides new information about our native fish (tolerance to toxicants) and will assist in protecting the native Murray cod. Australia and New Zealand (ANZECC) guidelines to protect the aquatic ecosystems including native fish are based on the toxicity data of North American fish or alien fish such as rainbow trout.

The study was conducted as part of G-MW's environmental management program - R&D, and involved Dr Scott Richmond and Professor Dayanthi Nugegoda (RMIT) and Dr Golam Kibria (G-MW).
The experiments were conducted at the RMIT University's purpose built ecotoxicological laboratory. The study observed and recorded the effects of herbicides and pesticides on the early life stages of Murray cod in relation to survival and mortality, growth and development and other behavioural responses.

For more information about the study and its findings contact Golam x722 or email golamk@gmwater.

Source: G-MW Outlet: August 2007 ISSUE 210