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The development and application of a 28 day larval fish toxicity test

KL Cheng

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The development and application of a 28 day larval fish toxicity test

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Declaration

This thesis is submitted for the Degree of Bachelor of Science (Honours) and is my own work to the best of my knowledge and belief, except where acknowledged in the text. This material has not been submitted either in whole or in part of any other degree at this or any other University.

The work reported in this thesis was carried out at the Environmental Research Institute of the Supervising Scientist and the School of Science and Primary Industries, Charles Darwin University under the supervision of Professor DL Parry and Dr Richard Anthony van Dam.

Kim Leang Cheng, Darwin, November 2007

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And last, but certainly never the least, to my daughter Jade, Thank You for being so patient with me, and yes, Mum has finally finished her book.

Abstract

Chronic toxicity no-observed-effect concentration (NOEC) data for five local freshwater species have been used to derive a high reliability water quality 'Limit' of 6 μg/L for uranium in Magela Creek downstream of Ranger Uranium Mine. Two of the NOEC values, 400 and 810 μg/L, represent estimates for two fish species, the purple-spotted gudgeon, *Mogurnda mogurnda* and the chequered rainbowfish, *Melanotaenia splendida inornata*, respectively, based on mortality after only 7 days exposure (+ 7 days post-exposure for *M. mogurnda*). Although such endpoints satisfy a current national water quality guideline criterion for a 'chronic' endpoint, their appropriateness as indicators of longer-term, sub-lethal effects has been questioned.

The aims of this study were to (i) develop a 28 d chronic toxicity test for two species, *M. splendida inornata* and *M. mogurnda*, and (ii) use the resultant toxicity test protocol to determine the chronic toxicity of uranium to these species.

Toxicity test development experiments for *M. splendida inornata* focusing on diet and feeding regimes proved mostly unsuccessful, with a range of complex diets unable to result in greater than 40% larval survival over 28 d. Based on a minimum acceptable criterion for control larval survival of ≥80%, *M. splendida inornata* was considered inappropriate for a 28 d chronic toxicity test.

Test development experiments for *M. mogurnda* were successful in characterising the partitioning of uranium in the test environment and identifying a diet and feeding regime that resulted in >70% larval survival and strong larval growth over 28 d. Subsequent modifications to the diet/feeding regime resulted in larval survival rates of >90% in the toxicity tests, well above the control survival criterion. The resultant 28 d larval growth and survival test protocol for *M. mogurnda* was used to assess the chronic toxicity of uranium. Newly hatched larvae exposed to 3180 μg/L uranium resulted in 100% larval mortality within the first 24 hours of exposure. Larvae exposed to 1,400 μg/L uranium exhibited significant 13% and 30% reductions in length ($P < 0.001$) and dry weight ($P < 0.001$), respectively. Based on larval length and dry weight, the lowest-observed-effect concentration (LOEC) and the No-Observed-Effect-Concentration (NOEC) to larval *M. mogurnda* was 1400 and 770 μg/l U, respectively. These results are similar to the existing toxicity data based on shorter exposure durations, and suggest that the first two weeks post-hatch are critical in terms of the effects of uranium.

Contents

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Abbreviations

ANOVA – Analysis of variance

ANCOVA – Analysis of covariance

ARR – Alligator Rivers Region

ERA – Energy Resources Australia

ANZECC and ARMCANZ – Australian and New Zealand Environment and Conservation Council

USEPA – United States Environmental Protection Agency

OECD – Organisation for Economic Co-operation and Development

eriss – Environmental Research Institute of the Supervising Scientist

LC – Lethal concentration

LOEC – Lowest-Observed-Effect-Concentration

NOEC – No-Observed-Effect-Concentration

SD – Standard deviation

SE – Standard error

SL – Standard length

α – (alpha- level of significance)

°C – degrees Celsius

d – day

h – hour

l – litre

µg/l – micro-gram per litre

µm – micro-metre

µS/cm – micro-siemen per centimetre

$t_{0/24}$ – at time 0 and 24 h

rpm – revolutions per minute

U – uranium

> – greater than

≥ – greater than or equal to

< – less than

≤ – less than or equal to

± – plus or minus

NMCW – Natural Magela Creek water

FTDW – Filtered Darwin tap water

1 Introduction

1.1 Ecotoxicology

Ecotoxicology is the science of investigating and understanding the interactions between chemical contaminants (natural or anthropogenic) and the natural environment, including how such contaminants impact on ecological systems (Chapman 1995a). The analysis of the transfer processes of contaminants within a natural system and the subsequent occurrence of structural and functional biological effects is the main aim of ecotoxicological studies (Boudou & Ribeyre 1989). Historically, ecotoxicology has relied on the use of toxicity testing to anticipate how chemical contaminants are likely to impact on ecological systems. Ecotoxicology provides the basis for making decisions on the likely impact of a chemical or effluent on the environment. Ecotoxicity assessment addresses the effect of chemicals on a range of species and on inter-species interactions in the environment. It covers both laboratory tests and field assessments, as well as a variety of microcosm and mesocosm experiments (Chapman 1995a).

1.2 Ecotoxicological testing

Data from toxicity testing studies can be used for regulatory purposes (ie the development of water quality guidelines) and/or, combined with additional lines of evidence, to predict ecological risk (Chapman 1995a; LaPoint & Waller 2000). The major reason for conducting toxicity tests with fish and other aquatic organisms is to determine the concentrations of a substance which are harmful to the organisms and those which have no apparent effect (Sprague 1990). Toxicity tests with effluents or pure substances are used to determine the 'safe' or 'no effect' concentrations of these substances (US EPA 2002). The objective of measuring the toxicity of a chemical is to estimate as precisely as possible the range of chemical concentrations that produce readily observable and quantifiable responses in the same group of species tested under controlled laboratory conditions. The establishment of the link between the concentration of a pollutant in the environment and the adverse effects on the organism is known as the concentration-response model (Rand & Petrocelli 1985).

1.2.1 Acute and chronic toxicity testing

Acute toxicity tests provide information about the relative lethality of a toxicant and are designed to determine the highest concentration that is sufficient to affect some percentage of a limited number of test organisms over relatively short periods (one-to-four days). Mortality clearly indicates an adverse effect at the individual level, and most represents likely effects at the population level. Mortality is generally less sensitive than most sub-lethal endpoints as it does not provide information on the effects of a toxicant in terms of growth, development and reproduction whereas chronic toxicity test data can. Chronic toxicity tests usually runs over durations that exceed 10% of an organisms life span, and the associated endpoints are usually sub-lethal in nature. Typically, chronic toxicity tests have been used to estimate 'safe' concentrations also known as no-observed-effect concentrations (NOECs). Chronic toxicity tests methods are variable because they must be tailored to suit the life history of the individual test organisms and are substantially more expensive than acute tests in time, resources and personnel. However, the data produced are more useful in predicting a

concentration not likely to harm a population, ie a 'safe' concentration (Buikema Jr et al 1982). Because conducting a chronic toxicity test can be so intensive (ie time, expense and variability between organisms) short-term tests have been developed to estimate chronic toxicity of contaminants at a particular sensitive life stage of an organism. Short-term tests using sensitive life stages and sub-lethal endpoints have been well tested and applied, and are considered appropriate for estimating the toxicity of chemicals (ANZECC & ARMCANZ 2000: van Dam and Chapman 2001).

1.2.1.1 Acute toxicity tests

Mortality (ie a lethal effect) is the most common test end-point assessed in acute toxicity tests, and can be related to gross environmental effects and is often used as a simple, indirect indicator for sub-lethal effects (Chapman 1995a). Point estimation techniques are typically used to calculate toxicity estimates (see below). Such approaches utilise mathematical models that assume a continuous dose-response relationship. Point estimation techniques have the advantage of providing a point estimate of the toxicant concentration causing a given amount of adverse effect (eg 10%, 50%), the precision of which can be quantitatively assessed within tests by calculation of 95% confidence limits, or across tests by calculating a standard deviation and coefficient of variation (US EPA 1994).

For acute toxicity tests, the point estimate most often reported is the toxicant concentration causing a 50% effect, with the effect usually being death, over a relatively short period (eg one to four days) (US EPA 2002). This value is referred to as the median effect (EC_{50}) or lethal (LC_{50}) concentration. EC/ LC_{50} values are determined by point estimation techniques such as Probit analysis and the Spearman-Karber Method (US EPA 1994). The median (ie 50%) value is used as the variance is least at the median and therefore the concentration that causes some effect can be most accurately calculated (Warne 1998). Such acute toxicity results can provide meaningful comparisons of toxicant lethality between organisms. However, acute tests rarely measure sub-lethal effects, and therefore cannot readily be used to predict sub-lethal concentrations able to harm a population or ecosystem (Buikema Jr et al 1982). It has been traditional to use acute toxicity tests based on fish and invertebrate mortality, however, over the past decade there has been a major advance in the research towards the development of chronic bioassays.

1.2.1.2 Chronic toxicity tests

Chronic toxicity tests determine the response of a test species over a number of generations or at least a significant portion of the organism's life span. Short-term sub-chronic tests that measure effects at a sensitive life-stage can be used as estimates for chronic toxicity (ANZECC & ARMCANZ 2000). Chronic toxicity tests can provide information on the subtle effects of the toxicants on the survival, growth, development and reproduction of an organism (Buikema Jr et al 1982; Chapman 1995a). In contrast to acute toxicity data, chronic toxicity data are more resource-intensive to collect and may be more difficult to interpret in terms of ecological significance (Reily et al 2003). Results from chronic toxicity tests are usually expressed as the No-Observed-Effect-Concentration (NOEC) and the Lowest-Observed-Effect-Concentration (LOEC). NOECs and LOECs are determined by hypothesis testing (Dunnett's Test, a t test with a Bonferroni adjustment, Steel's Many-one Rank Test, or the Wilcoxon Rank Sum Test with Bonferroni adjustment) (US EPA 1994).

These estimates are dependent in large part on the design of the tests themselves. Factors that can affect the sensitivity of the test include the choice of statistical analysis, and the amount of variability between responses at a given concentration. Chronic data from this approach are generally easy to apply and provide statistical information on test variability. However, the tests

can have either poor or excessive statistical power, due to unconstrained type II errors and it does not derive a dose-response relationship, as the NOEC and LOEC values depend on the choice of test concentration, and *a priori* estimates of NOECs cannot be made (ANZECC & ARMCANZ 2000).

The NOEC is the highest concentration that does not cause a statistically significant effect while the LOEC is the lowest concentration that does cause a statistically significant effect (ie the test concentration directly above the NOEC) (ANZECC & ARMCANZ 2000). It is important to note, however, that the NOEC is not necessarily the concentration that produces no effect, but the concentration that produces an effect that is not statistically significantly different from that of the controls (Warne 2001). It is important to note that the NOEC and LOEC are limited to the concentrations selected for the test (US EPA 1994; Warne 2001). It is not possible to place confidence limits on the NOEC and LOEC derived from a given test, and it is difficult to quantify the precision of the NOEC-LOEC endpoints between tests. If the data from a series of tests performed under the same conditions (ie with the same toxicant, toxicant concentrations, and test species) were analysed with hypothesis testing techniques, precision could only be assessed by a qualitative comparison of the NOEC-LOEC intervals, with the understanding that the maximum precision would be attained if all tests yielded the same NOEC-LOEC interval (US EPA 1994). In practice, the precision of results of repeated chronic tests is considered acceptable if the NOECs vary by no more than one concentration interval above or below the central tendency (US EPA 1994).

More recently, point estimation has been employed to calculate toxicity estimates from chronic tests. It has been estimated that the current NOEC values correspond to IC_{25} (concentration that would cause a 25% inhibition in growth or reproduction) or less and values of 5 or 10% are considered preferable (van Dam and Chapman 2001; ANZECC & ARMCANZ 2000).

1.2.2 Test species

No single species can be defined as being the most sensitive to toxicant exposure. Therefore, the toxicity testing of organisms representing different taxonomic groups and/or trophic levels is needed to ensure a reasonable representation of a range of species is covered (Holdway 1992a). To achieve appropriate representation of species, it is generally considered that toxicity tests should be available for fish, invertebrates, microalgae and higher plants (Chapman 1995b).

1.2.2.1 Fish as toxicity test species

Fish are present in almost all aquatic environments and play an important role within their environment due to the various functions they have in the trophic networks, biomass and productivity, life spans and migration. The fundamental knowledge of the biology of fish is much more diverse and detailed than that of other zoological groups living in the aquatic environment. Freshwater species that can be bred in the laboratory form the basis of much research work in Ecotoxicology (Sprague 1973; Boudou & Ribeyre 1989).

1.2.2.2 Standard fish species

The development of a standard methodology of testing that will be usable for all fish species would be ideal. This cannot be the case however as it is extremely difficult, if not impossible to find one species of fish representing all the selection criteria where toxicity needs to be assessed. Fish species vary from one to the other with sensitivity, abundance, ecological importance, life-span, size and growth rate, susceptibility to stress, diseases and social

behaviour and culturing and rearing (under controlled laboratory conditions). Whilst it is impossible to standardise one fish species that have all these properties comparable to all other fish species, it is possible to standardise several species in the laboratory. Some standard fish species used in ecotoxicology are *Salmo gairdneri* (rainbow trout), *Salvenilus fontinalis* (brook trout), *Pimephales promelas* (fathead minnow), *Ictalurus punctatus* (channel catfish), *Lepomis macrochirus* (bluegill), *Brachydanio rerio* (zebra fish), *Carrassius auratus* (goldfish) and *Poecilia reticulata* (guppy). Precise research objectives are used to choose the test species. For example, to study the effects of contaminants on reproduction, a fish species that produces a large number of eggs should be used (Sprague 1973; Boudou & Ribeyre 1989).

The most sensitive life stage for fish are the embryo and fry (Woltering 1984; USEPA 2002) and partial life stage toxicity tests (eg 30 d post hatch) would be an appropriate approach to estimating chronic toxicity for fish species (ANZECC & ARMCANZ 2000; US EPA 2002; Holdway 1992a).

1.2.3 Fish species of Alligator Rivers Region

Kakadu National Park is of high conservational value (see Section 1.3 for details) and the introduction of exotic species is prohibited. Consequently, the biological test species need to be relevant to that ecosystem. Since the 1980s, 19 local species have been evaluated as potential toxicity testing species, including *Mogurnda mogurnda* (Holdway 1992a; Rippon & Hyne 1992) and two rainbowfish species, *Melanotaenia splendida inornata* (Holdway 1992b) and *Melanotaenia nigrans* (Williams et al 1998).

1.2.3.1 *Melanotaenia splendida inornata* (Peters, 1866)

Commonly known as the chequered rainbowfish, *M. splendida inornata* belongs to the family Melanotaeniidae and is found in the river systems of the Northern Territory and Queensland that flow into the Arafura Sea and Gulf of Carpentaria (Figure 1.1).

Figure 1.1 *Melanotaenia splendida inornata* (Pusey et al 2004)

Males are easily differentiated from females by their bright colours, longer first dorsal fin, and pointed profile of the posterior dorsal and anal fins. They commonly inhabit rivers, creeks, swamps, marshy lagoons, lakes and reservoirs and are often abundant where water

flow is minimal. Adult size can be up to about 14 cm but the more common size is about 8 cm or less (Allen et al 2002).

1.2.3.2 *Melanotaenia nigrans* (Richardson, 1843)

Commonly known as the Black-banded rainbowfish, *Melanotaenia nigrans* belongs to the family Melanotaeniidae. *M. nigrans* is smaller than the chequered rainbowfish growing to a maximum of 8.5 cm. Individuals are typically grey-brown dorsally and white on the lower sides with a prominent black mid-lateral stripe. Mature males develop reddish second dorsal and anal fins with black margins. They commonly inhabitat rainforest streams, lily lagoons and small creeks in swampy areas (Allen et al 2002).

1.2.3.3 *Mogurnda mogurnda* (Richardson, 1844)

Commonly known as the Northern trout gudgeon or Purple spotted gudgeon, *Mogurnda mogurnda* (Fig 1.2) belongs to the family Eleotridae. *M. mogurnda* inhabit rivers, creeks and billabongs, in quiet or slowly flowing sections among vegetation and rocks, and is widely distributed across northern Australia. They can be identified by a series of vertically elongated dark brown blotches or bars overlaid with numerous red spots on their sides. Males develop an intense bluish colouration during courtship (Allen et al 2002).

Figure 1.2 *Mogurnda mogurnda* (Pusey et al 2004)

1.3 Uranium

1.3.1 Speciation

Uranium (U) is a member of the lanthanide series of metals, has an atomic number of 92 (Sheppard et al 2005) and is one of the heaviest naturally occurring elements on Earth. It has 16 known isotopes, all of which are radioactive. In nature U consists of a mixture of three isotopes, ^{238}U (99.275%), ^{235}U(0.720%), and ^{234}U (0.005%) (Markich 2002). ^{238}U is the parent isotope of ^{234}U and exists in radioactive equilibrium. Uranium occurs in five valence states: +2, +3, +4, +5 and +6. The uranous (+4) and uranyl (+6) oxidation states are found in the environment (Hayes et al 2000).

All three isotopes are soluble in acid solutions as uranyl UO_2^+ ions, or in neutral and alkaline solutions as dicarbonate $UO_2(CO_3)_2^{2+}$ ions or tricarbonate $UO_2(CO_3)_3^{4-}$ ions (Sheppard et al 2005). Water having an excess of CO_3^{2-} results in the formation of the tri-carbonate complex which yields the dicarbonate complex upon dissolution (Hayes et al 2000).

The bioavailability of U is dependant on its physicochemical form and speciation. In anoxic waters (low Redox potential) U occurs as U^{4+} and/or UO_2^+. U^{4+} has a strong tendency to form complexes with inorganic ligands (uranyl fulvate or humate) and precipitate (eg Uranite, $UO_{2[s]}$) and remain immobile whereas UO_2^+ forms soluble complexes. In oxic waters, U occurs as UO_2^{2+} and forms stable readily soluble ionic and/or neutral complexes that are highly mobile and play the most important role in U transport during weathering. UO_2^{2+} is the most stable species and the most prevalent form in the environment (Sheppard et al 2005). The most important complexes formed by the uranyl ion are the carbonate, sulfate, fluoride, phosphate, and hydroxyl complexes (Hayes et al 2000). Uranium in water may be present in the three oxidation states, U^{4+}(U(IV)), UO_2^+ (U(V)) and UO_2^{2+} (U(VI) or uranyl ion) with UO_2^{2+} and UO_2OH^+ being the major bioavailable forms where UO_2^{2+} has approximately twice the toxicological effect of UO_2OH^+ (Markich 2002).

1.3.2 Bioavailability and toxicity

The toxic effect of a metal depends not only on its abundance but also on its bioavailability, defined as the ability of a metal to bind to or traverse the cell surface of an organism (Sunda & Huntsman 1998). The effect of a metal on the ecosystem depends on its exact chemical form, or speciation. Metal species that are typically available for uptake by organisms include their free metal ions (Bailey et al 2002). The characteristic of U in water (particulate, colloidal or dissolved) is governed by its solubility and its affinity for the adsorbing to particulate matter (driven by pH, Eh and ligand concentration) (Markich 2002).

The most basic operational definition of metal speciation is the fractionation of species on filtration through a 0.45μm filter membrane. The < 0.45 μm filtrate is operationally defined as the dissolved fraction and contains free metal ions, metals complexed to inorganic and organic ligands, and fine colloidal matter. The > 0.45 μm fraction that is excluded is classified as particulate matter and can contain a variety of material including micro-organisms, large organic molecules, clays, carbonates and metal oxides (Sondi & Pravdic 1998). The concentration of metals in the dissolved fraction is generally considered to be of greater importance as it contains the majority of bioavailable species. Metals in the particulate fraction are generally strongly associated with or adsorbed to large colloidal and suspended material, which substantially reduces their bioavailability (Fortin 2004; Nolan et al 2003). Not all metals in the dissolved fraction are necessarily an indication of toxicity as only the bioavailable fraction (free-metal ions or labile complexes) contribute to toxicity (Nolan et al 2003). Other constituents of the dissolved fraction, such as fine colloidal matter and stable complexes are not bioavailable and therefore, do not contribute to toxicity (Markich 2002; Sunda & Huntsman 1998).

Natural U is classified as both a radiological and toxicological agent and is the only radionuclide for which chemical toxicity is the limiting factor (Sheppard et al 2005). Uranium presents a very low radiological risk due to a low specific activity, and its biological action is considered to be primarily a stable element. Perhaps the most important factor influencing the potential health impact of natural U is its solubility. Solubility affects both availability and exposure determining health impacts (Hayes et al 2000).

1.4 Mining in the Alligator Rivers Region

1.4.1 General

There has been a culture of uranium mining in the Alligator Rivers Region since 1956. Uranium mines include Nabarlek, Jabiluka, Ranger and Rum Jungle with proposed mining activities at Koongarra, and Western Arnhem Land (Kay 1997).

Nabarlek is a decommissioned uranium mine and mill is situated in western Arnhem Land in the Cooper Creek catchment, a tributary on the East Alligator River. The orebody was discovered in 1970 and decommissioning began in 1994 and was completed in the dry season of 1995. Jabiluka uranium mine is situated within the Kakadu National Park and the Jabiluka lease occupies over 18 000 km^2 immediately adjacent the Ranger lease (Kay 1997) (see section 1.3.2).

1.4.2 Ranger

The Ranger Uranium Mine (Ranger) is located on the Ranger mineral lease within the Alligator Rivers Region (ARR), Northern Territory (Figure 1.3). The ARR comprises of an area of almost 33 000 km^2, which is roughly defined by the catchments of the East, South and West Alligator Rivers (Figure 1.3). Kakadu National Park, a World Heritage site occupies approximately two-thirds of the ARR (20 000 km^2) and encompasses the Ranger mining lease (Figure 1.3) (van Dam et al 2002).

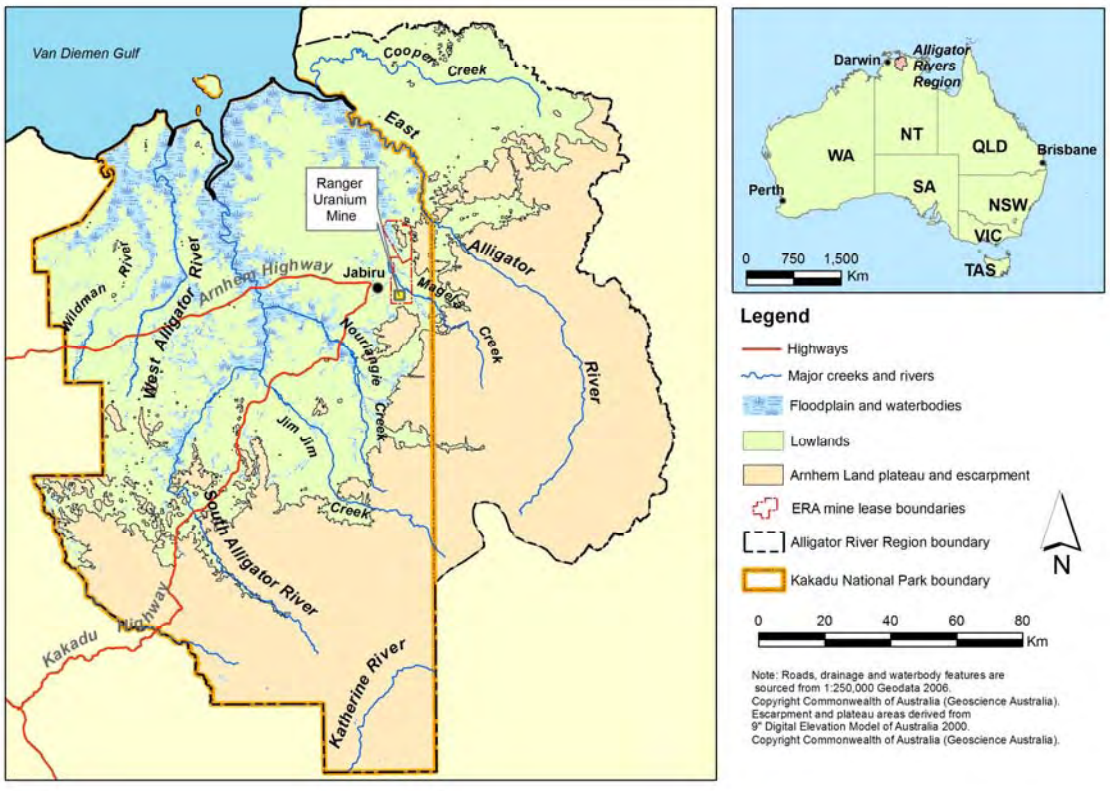

Figure 1.3 The Alligator Rivers Region and the location of the Ranger Uranium Mine

Ranger is operated by Energy Resources of Australia Ltd (ERA), and lies within the 78 km^2 Ranger Project Area. The mine is located less than 1 km from Magela Creek, a tributary of the

East Alligator River (Figure 1.3). Mining and commercial production of uranium concentrate has taken place at Ranger since 1981 (Johnston & Needham 1999; van Dam et al 2002).

The operation of Ranger uranium mine adjacent to the high conservation value aquatic ecosystems of the Magela creek catchment has necessitated ongoing site specific investigations to ensure that the aquatic ecosystems adjacent to and downstream of the mine are protected (Holdway 1992a; van Dam et al 2002). The main risk identified for ecosystems surrounding the mine site is from the dispersion of mine waste waters to streams and shallow wetlands during the intense wet seasons associated with the tropical monsoonal weather of Northern Australia (Supervising Scientist 2002). Each year Magela Creek receives mine-related effluent from a number of sources. The main source is Retention Pond 1 (RP1) from which low level contaminant waste water is released into Magela Creek via a nearby billabong toward the end of the wet season (Supervising Scientist 2002). Other waters that enter Magela Creek via surface water pathway originate from the Corridor Creek wetland filter and land application/irrigation areas within close proximity to Magela Creek (Supervising Scientist 2002; Energy Resources Australia 2003).

The main aquatic contaminants of concern from the Ranger site include uranium (U), magnesium (Mg) and sulphate (SO_4^{2-}) (Supervising Scientist 2002). Other metals, including copper (Cu) and lead (Pb) can be present in ore and waste rock with the potential of becoming mobilised in runoff water, although their concentrations in waters released to Magela Creek are very low and mining contaminants released into the environment may become available for uptake by biota from the air, water, and soil in addition to dietary uptake (Kay 1997).

1.5 Environmental monitoring of mining impact at Ranger

1.5.1 Overview

The Supervising Scientist's monitoring program to ensure the protection of the aquatic ecosystems downstream of Ranger involves a four tiered approach: i) deriving site specific water quality guideline trigger values (TVs); ii) determining 'safe' release dilution of waste water; iii) early warning monitoring following waste water release; and iv) longer-term monitoring to determine the ecological significance of any impacts. The first approach uses local aquatic species for assessing toxicant impacts at particular sites and was adopted as part of the environmental protection program for Ranger in the mid 1980s, with toxicity tests for at least 10 local species having been developed. Five local aquatic species are currently used for toxicity testing purposes, with the exception of the sac-fry survival test, all test endpoints represent chronic responses. These and other values have been used to determine water quality TVs and waste water release dilutions (van Dam et al 2002).

Water quality guidelines (WQG) are derived from ecotoxicological data from multiple species toxicity tests, and enables mine managers (and regulators) to manage (and regulate) mine water releases such that there are no deleterious effects to the downstream aquatic ecosystems (van Dam et al 2002).

Aquatic ecosystems are affected by physical and chemical variations that can occur naturally within the environment, or due to human intervention. The consequent risk posed to the health of the ecosystem can be assessed by monitoring physical, chemical and biological aspects of water and sediment, based on a number of indicators. These include biological (eg fish and macro invertebrates), physical and chemical (eg physico-chemical parameters) and toxicants (eg metal concentration). Metals are monitored based on comparison of measured

indicator values to guideline trigger values as outlined by the WQGs (ANZECC & ARMCANZ 2000).

Water quality criteria compile scientific data into a single figure that indicates the chance of magnitude of the effects of a contaminant on a particular aquatic organism under specific environmental conditions (Chapman 1995b). A WQG can be derived with the intention of providing with confidence, that there will be no significant impact in the environmental values if they are achieved and if in exceedance of the guidelines indicates, there is potential for an impact to occur (ANZECC & ARMCANZ 2000). Toxicity tests using local freshwater species have been employed to assess this issue (van Dam et al 2002).

Water quality guidelines (or trigger values) represent contaminant concentrations below which there will be no significant impact to the environmental values, and above which there is potential for an impact to occur (ANZECC & ARMCANZ 2000). ANZECC & ARMCANZ (2000) recommend deriving guideline values for physical and chemical stressors and toxicants using, in order of preference, (1) local biological effects data, (2) local reference site data, or (3) the default values available (ie regional reference data or global effects data). For Ranger, a key contaminant of concern, and therefore one that needs to be monitored, is uranium. The waterways within Kakadu National Park, which is a World Heritage listed site of very high conservation/ecological value, are monitored at the highest level of protection (for 99% of all species) as outlined in the WQGs (ANZECC & ARMCANZ 2000).

Over the past 20 years, the toxicity of uranium has been assessed for numerous freshwater species native to the ARR (van Dam et al 2002). Some of these toxicity data have been used to derive a high reliability site-specific water quality 'Limit' for Magela Creek downstream of Ranger (Hogan et al 2005), using the first approach recommended by the Australian and New Zealand WQGs (ANZECC & ARMCANZ 2000).

1.5.2 Site-specific uranium 'Limit' for Magela Creek

Toxicity tests using freshwater species local to the ARR have been employed since the late 1980s to assess the toxicity of uranium. Local toxicity data have been used to derive a high reliability site-specific water quality 'Limit' for Magela Creek downstream of Ranger, using the approach recommended by the Australian Water Quality Guidelines (WQG) (ANZECC & ARMCANZ 2000). Effective environmental monitoring is essential so that the impact of mining can be accurately measured and relevant mitigation procedures can be carried out.

The Limit of 6 μg/L was derived using chronic toxicity No-Observed-Effect Concentration (NOEC) data, ranging from 18–810 μg/L, for 5 species (Hogan et al 2005; van Dam et al 2002) (Table 1.1). However, two of the NOEC values, 400 and 810 μg/L, represent estimates for two fish species, the purple-spotted gudgeon, *Mogurnda mogurnda* and the chequered rainbowfish, *Melanotaenia splendida inornata*, respectively, based on mortality after only 7 days exposure (+ 7 days post-exposure for *M. mogurnda*) (Holdway 1992b). Although such endpoints satisfy the current WQGs criterion for a 'chronic' endpoint (ie >96 hour test duration), its appropriateness as an indicator of longer-term, sub-lethal effects has been questioned. Figure 1.4 shows the No-Observed-Effect-Concentrations from direct toxicity tests for each of the five species plotted on a log-logistic probability plot. The central line the toxicity data represents the line-of-best-fit. The curved dotted lines around the fitted line represent the 95% confidence limits.

Table 1.1 Summary of chronic toxicity of U to local species, in natural Magela Creek water (Hogan et al 2005)

Species	Test endpoint	NOEC (µg/L U)	Reference
Chlorella sp. (green alga)	Cell division rate (72 h)	117 [a]	Hogan et al (2005)
Moinodaphnia macleayi (water flea)	Reproduction (three brood	18 [a]	ERISS unpublished data Semaan et al (2001)
Hydra viridissima (green hydra)	Population growth (96 h)	150 [a]	Hyne et al (1992) [b]
Mogurnda mogurnda (fish)	Mortality (7 day exposure/7 days post-exposure)	400	Holdway (1992)
Melanotaenia splendida inornata (fish)	Mortality (7 days)	810	Holdway (1992)

[a] Toxicity values represent geometric means.

[b] Publication presented as nominal concentration, therefore, measured concentrations for Environmental Research Institute of the Supervising Scientist records were used for trigger value derivation.

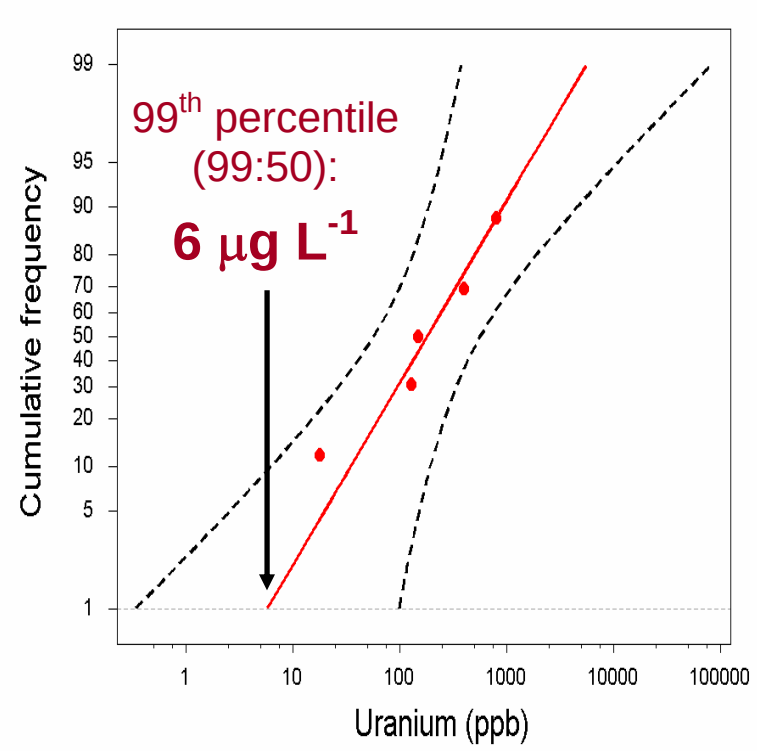

Figure 1.4 Log-logistic distribution fitted to U NOEC values from Table 1.1

1.5.2.1 Trigger value derivation using chronic toxicity data

Historically, toxicant trigger values have been derived by applying a safety factor to the LOEC, NOEC (chronic data) or EC/LC_{50} (acute data) of the most sensitive species tested (ANZECC & ARMCANZ 2000; Reily et al 2003). The magnitude of the safety factor is determined by the type of data and the nature of the toxicant; typically, a safety factor of 10 would be applied to chronic NOEC data compared to a safety factor of 100 for acute LC_{50} data (Warne 2001). Other factors, such as whether the toxicant is an essential ion or is known to biomagnify may also influence the choice of a safety factor. While the use of a safety

factor is simple to apply and is relatively easy to understand, the approach has been highly criticised because the number generated has no theoretical basis and is purely empirical (Warne 2001).

Over the past decade an alternative approach has been developed for deriving toxicant trigger values. The approach differs from the incorporation of a safety factor as it is statistically based and offers a different degree of protection as well as an uncertainty measure. In this approach, which has been adopted by ANZECC & ARMCANZ (2000), NOEC toxicity data from multiple species are used to construct a cumulative probability plot, to which the most appropriate distribution from the Burr Type III family of distributions is fitted. The resultant 'species sensitivity distribution' is used to derive an estimated concentration that should protect *x%* of the species in the environment. This approach, however, requires chronic NOEC toxicity data for at least 5 species from a minimum 4 taxonomic groups/trophic levels in order to derive a trigger value. If this cannot be met, the trigger value is derived using the safety factor approach (ANZECC & ARMCANZ 2000).

1.6 Aims

The aims of this study are to:

1 Develop an appropriate 28 day larval growth toxicity test protocol for the two fish species, *M. mogurnda* and *M. splendida inornata*; and

2 Determine the effects of uranium on growth of larval *M. mogurnda* and *M. splendida inornata*, over a 28 day exposure period

The current fish toxicity testing protocol at ***eriss*** is an acute test over four days. Therefore, an additional protocol needs to be established for longer-term chronic toxicity testing. The chronic toxicity testing protocol for *M. mogurnda* and *M. splendida inornata* will be generally based on the OECD Guideline 215, *Fish Juvenile Growth Test* (OECD 2000). However, a series of experiments will need to be undertaken to determine the final experimental design of the 28 day toxicity test protocol to be used to assess the chronic toxicity of uranium. Aspects that will be considered will include: feeding regimes and how they affect larval growth, water quality and uranium concentration; larval stocking/loading densities and how they affect larval growth, water quality and uranium fate; and measurement endpoints for larval growth.

2 Methods – general

2.1 Diluent water

Natural Magela Creek water (NMCW) was used as diluent/control water for all tests. NMCW from Bowerbird Billabong (latitude 12° 46’ 15”, longitude 133° 02’ 20”) or just upstream of the confluence of Georgetown Billabong (latitude 12° 40’ 28’’ longitude 132° 55’ 22’’) in the Magela Creek catchment was collected in acid washed high density polyethylene (HDPE) 20 L containers. Filtered NMCW typically was slightly acidic (pH 5.5–6.5) and had very low ionic strength (EC 5–20 µS/cm) and water hardness (<5 mg/L as $CaCO_3$). All waters were stored at 4˚C within 2 h of collection and filtered through Whatman #42 (2.5 µm) filter paper within 48 h. Each day of each test, conductivity, dissolved oxygen and pH were measured for each treatment (see 2.6.1).

2.2 General laboratory procedures

2.2.1 Cleaning

All equipment in contact with test organisms, control water or test solutions was made of chemically inert materials (eg Teflon, glass or polyethylene). All plastic and glassware were washed by soaking in 5% v/v nitric acid (HNO_3) for 24 h before undergoing a detergent (Gallay Clean A non-phosphate powder, Gallay Scientific, Melbourne, Australia) wash and two rinses in a laboratory dishwasher with high purity Elix (Millipore, Molsheim, France) water. All reagents used were analytical grade and stock solutions were made up in high purity Milli Q (Millipore) water.

2.3 Maintenance of brood fish stocks

2.3.1 Aquaria

Aquaria for holding fish broodstock were housed in a temperature controlled (28 ± 2°C) laboratory with a 12 h light 12 h dark photoperiod. Each aquarium was set up as a flow-through system, so that filtered Darwin tap water (FTDW) flowed continuously through to maintain optimal water quality and health of the fish (a full turnover of water in a 24 h period).

M. splendida inornata broodstock were collected from Nourlangie Creek (South Alligator Rivers Catchment, Kakadu National Park) and transported to the ERISS laboratory where they were maintained at 28 ± 1°C in 144-L, flow-through glass aquaria. Brood stock were selected and placed into breeding tanks with the ratio of 3 males to 7 females or 3 males to 10 females for optimal combination for spawning (Humphrey et al 2003b).

2.3.2 Fish feeding

2.3.2.1 *M. mogurnda*

M. mogurnda broodstock were fed daily on a diet consisting mainly of live worms and commercial fish pellet (Hikari sinking carnivore pellets).

2.3.2.2 *M. splendida inornata*

M. splendida inornata broodstock were fed a mixed diet 3 times daily consisting of commercial flake fish food (Sera san), newly hatched live *Artemia* nauplii (see 2.3.3) and frozen adult brine shrimp (thawed and rinsed in FDTW)

2.3.3 Culturing *Artemia* nauplii

Live nauplii (*Artemia* spp.) are used as food for many types of aquatic organisms, including hydra and larval fish. Cultures were maintained in 1 L conical separation funnels. A salt solution was made by dissolving approximately 30 g (about 1 tbsp) of un-iodised coarse rock salt or sea salt in about 1 L of FDTW. A funnel was attached to an air-line and the tap on the stopper opened to gently aerate the solution with oil-free compressed air. After the salt was dissolved, one teaspoon (~ 5 g) of commercially harvested, dried brine shrimp cysts were added. The solution was continuously aerated to prevent cysts from settling. The cysts hatched after 24-48 h at an incubation temperature of 27 °C, with a 12:12 h light:dark cycle. Nauplii were harvested by turning off the aeration and allowing for the nauplii to settle at the base of the funnel. The nauplii were then strained through a 250 µm mesh nylon net and rinsed thoroughly with the NMCW. Live nauplii were collected for feeding using a Pasteur pipette.

2.4 Preparation for testing

2.4.1 Test environment

Tests were conducted at 27 ± 1°C using a constant temperature incubator (Labec refrigerated incubator) with a 12 h light: 12 h dark photoperiod.

2.4.2 Data recording

Test animals were observed and data recorded at 24 h intervals after the commencement of the test. Water quality parameters (See Section 2.6.1) were measured and recorded at the beginning and end of each 24 h period, which corresponded with daily water renewals (See Section 2.5).

2.4.3 Commencement of a test

2.4.3.1 *M. mogurnda*

When a batch of eggs were produced, the eggs were left in the parent aquarium for 24–48 h allowing the male parent fish to maintain them. After this time, the developing embryos were carefully removed by placing the object on which they are laid into a 20-L flow-through hatching chamber of gently aerated FTDW at a temperature of (28 ± 2°C). The aeration was used to simulate the male ‘fanning’ water over the eggs to reduce the incidence of fungal spores settling. Hatching commenced between 4-5 days. The hatching chamber had an in-line pump (Quietone 1200, Aquasonics, Wauchope NSW) and a UV steriliser (Rainbow Lifegard QL-8, Aqauasonics, Wauchope NSW) to further reduce the number of fungal spores (and other micro-organisms).

2.4.3.2 *M. splendida inornata*

Spawning was induced by introducing a substrate (woollen mops) onto which the eggs could be spawned. The substrates were placed into the breeding tanks in the afternoon, with spawning taking place the following morning. When batches of eggs are produced, the

substrates were immediately relocated to prevent predation by the parent fish. The spawning substrates were placed into a separate tank containing FDTW with a temperature similar to which they were removed (28 ± 2°C) with gentle aeration until first signs of hatching (5–6 d). After hatching, larvae that were selected for testing were randomly placed in cylindrical 250 ml volume plastic containers (with lids) and rectangular 750 ml volume plastic containers with 200 and 500 ml NMCW water, respectively (see section 3.2 for full details). For all tests, larval fish were fed three times daily: morning, midday and late afternoon, with each treatment consisting of three replicates.

2.5 General test method for both species

The general test methods for both species were adopted or adapted from three existing formal protocols: (i) the 96 h larval survival test for *M. mogurnda* (Riethmuller et al 2003); (ii) a generic fish juvenile growth test (OECD 2000); and (iii) a generic fish early life stage test (OECD 1992). The methods described below were applicable to all test development experiments that involved fish and the uranium toxicity experiments. Additional specific details of the experiments are described in Sections 3.2 (for *M. splendida inornata*) and 4.2 (for *M. mogurnda*).

Once all the eggs had hatched (or at least sufficient numbers to enable a test to be started), they were carefully isolated into a Petri dish using a wide-mouthed Pasteur pipette. Neither embryos nor sac-fry were treated for fungus. Examination was made under the microscope to determine which sac-fry were free from overt disease or gross morphological deformity and were suitable test organisms. Damaged and excess sac-fry were euthanased in a 0.4 g/L solution of benzocaine.

Less than 10 h old sac-fry from the isolated stock were selected and placed into diluent water until there were ten sac-fry in each test container. Observations under the microscope were done to ensure that there were ten sac-fry in each test container, and any sac-fry that are damaged in any way (eg disrupted yolk sac, haemorrhaging etc) were replaced. Test containers were covered and randomly placed inside a temperature controlled incubator (27 ± 1°C).

Fry were fed 1–3 times daily, depending on the type and objectives of the experiment. Survival and health were observed, followed by the transfer of all live fry into a replenished container with the corresponding fresh diluent water each day for the duration of the test.

All fry surviving to day 28 were euthanased in a 0.4 g/L solution of benzocaine. Length was measured using a stage micrometer to 2 decimal places. Replicates were placed onto pre-weighed foil trays lined with paper and dried in an oven at 60°C for 72 h (OECD 2000). Dry weight was measured on a balance to five decimal places.

2.6 Quality control

2.6.1 Measurement of water quality

Temperature, dissolved oxygen (DO), pH, electrical conductivity (EC) and ammonia (NH_4) of test solutions were monitored regularly throughout the duration of each experiment. EC and pH measurements were taken with WTW Multiline P4 Meter using a TetraCon EC electrode and a Sentix 41-3 pH electrode.

DO was measured using a WTW inoLab Multi Level 1 with a CellOx 325 DO probe.

NH_4 was measured using Palentest and Merck test kits.

2.6.2 Chemical analysis

Sub-samples of newly collected natural Magela Creek water were analysed for dissolved organic carbon (DOC) and alkalinity (see Appendix 1) by the National Measurement Institute. A comprehensive suite of chemical analyses was undertaken by the Northern Territory Environmental Laboratories (Berrimah, NT) for Al, Ca, Cd, Cu, Fe, Mg, Na, Pb, SO_4, U and Zn, using inductively coupled plasma mass spectroscopy (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES) for all procedural blanks, Milli Q blanks and control treatments and all other test solutions were sub-sampled for uranium analysis only. All sub-samples were collected in 60mL plastic bottles and acidified to 1% and 5% HNO_3 for filtered (Suprapur, 69%; 0.45 µm syringe filter (mixed cellulose)) and totals, respectively.

2.7 Statistical analyses

Hypothesis testing statistical analysis was used to determine whether there were statistically significant differences in larval responses between experimental treatments. Where possible, the endpoints measured were survival and growth (dry weight and length).

2.7.1 Test development experiments

The software package Minitab® Version 15.1.1.0 was used for statistical analysis during the test development (Chapters 3 and 4). Prior to analysis, data were tested for the key assumptions of homogeneity of variances (Bartlett's Test; α=0.05) and normality (Shapiro-Wilks Test; α=0.05), and where necessary, appropriately transformed. Percent or proportion data form a binomial distribution rather than a normal distribution (Zar 1984), and therefore all such data were arcsine transformed using the following equation:

$$p' = \sqrt{\left(n + \frac{1}{2}\right)} \arcsin \sqrt{\frac{X + \frac{3}{8}}{n + \frac{3}{4}}}$$

where n is the proportion data being transformed (Zar 1984).

Survivorship data (ie survival over time) were analysed using 1-way analysis of covariance (ANCOVA; α=0.05). Other data, such as length, dry weight and uranium concentrations in uranium fate experiments, were analysed using 1-way analysis of variance (ANOVA; α=0.05). Where significant effects were detected, Tukey's test (α=0.05) was used to determine which experimental treatments were significantly different from each other.

Power analysis was used to determine the minimum number of replicates (ie sample size) needed for the toxicity tests given a specified effect size and statistical power. Data from the *M. mogurnda* feeding test (Test 2, Section 4), specifically the length and dry weight data, were used to estimate this. Full details of the power analyses, which were performed in Minitab® Version 15.1.1.0, are provided in Section 4.

2.7.2 Uranium toxicity tests

Toxicity test data were analysed using ToxCalc™ Software. Hypothesis testing was used to establish the NOECs and LOECs for uranium using the US EPA flow chart method. Where assumptions for homogeneity and normal distributions were met (using Bartlett's Test and

Kolmogorov D Tests or Shapiro-Wilks Test, respectively), Dunnett's Test (α=0.05) was performed if there were equal number of replicates. Where replicate number varied (ie length data) then the Bonferrroni t Test (α=0.05) was performed. Non-parametric tests were performed when assumptions for homogeneity and normal distributions were not met, using Steel's Many-One Rank Test and Wilcoxon Rank Sum Test for equal and varied replicates, respectively.

3 Toxicity test development – *Melanotaenia splendida inornata*

3.1 Introduction

Melanotaenia splendida inornata belongs to a group of small colourful freshwater fish of the family Melanotaenidiidae. There are 6 genera and 53 species of rainbowfishes, found in a variety of ecological conditions in Australia and New Guinea. They are classed as a forage species and play a valuable role in the food chain of the river environments. Rainbowfish are perhaps the most ubiquitous of all freshwater fishes in tropical Australia, occurring in large numbers throughout their range (Allen et al 2002).

Relatively little is known about the early life history of the majority of rainbowfish species in their natural habitat. There are only a few brief notes published on the reproduction of some species. There are few published descriptions of the development of rainbowfish embryo and larvae (Humphrey et al 2003a).

Rainbowfish spawning habits do not differ markedly from each other. Rainbowfish spawning involves simultaneous shedding of eggs and milt. Wild populations of rainbowfish normally reproduce in spring and summer and under controlled laboratory conditions, spawning can occur daily throughout the year. Newly hatched larvae are strong swimmers and remain predominantly in the upper 1cm of water. Larvae hatch 7–9d at 25°C, with hatching success generally in the range of 40–70%. Rainbowfish larvae have low lipid reserves and commence feeding within hours after birth (Holdway et al 1994).

The objective of the initial phase for this test development was to establish a suitable diet and feeding regime that would result in an optimal survival rate in *M. splendida. inornata*. Other main considerations were: stocking density/volume of diluent water and water quality.

3.2 Methods

Series of feeding trials involving different food types and feeding regimes was conducted to determine the most suitable feeding regime that would provide optimal survival in *M. splendida inornata* larvae. The individual food types tested, in various combinations were: Ocean Star International (OSI) (Micro-Food Special Diet. Red Jungle Brand. Ocean Star International Inc.), *Chlorella* (unicellular algae cultured at ***eriss***), Sera powder (Sera Micron) *Artemia* (brine shrimp) nauplii (homogenised and live), fermented food with vitamins (FFV), Aquasonic 'Freshwater' Fry starter and *Paramecium.* (See Appendix 4 for more details on food types). The details of the diets and feeding regimes for each experiment are provided below.

Where possible, the design of feeding Tests 2-6 were based on the results of the preceding test(s). Discussion of this is provided in Section 3.3-5.

3.2.1 Test 1: OSI, *Chlorella*, Sera powder and homogenised *Artemia nauplii*

Survival of newly-hatched *M. splendida inornata* larvae was assessed under eight different diets. The diets consisted of various food types, either alone or in combination, at one of two densities, as shown in Table 3.1.

Table 3.1 Larval *M. splendida inornata* diets assessed in Test 1

Diet	Food type and quantity			
	OSI Micro-food	***Chlorella***	**Sera powder**	**Homogenised *Artemia***
Density 1				
A	2ml	-	-	-
B	2ml	5 x 10^6 cells	-	-
C	2ml	5 x 10^6 cells	√	-
D	-	-	-	100/ml
Density 2				
E	4ml	-	-	-
F	4ml	1 x 10^7 cells	-	-
G	4ml	1 x 10^7 cells	√	-
H	-	-	-	200/ml

An OSI stock solution was prepared according to manufacturers instructions. Sera powder was administered at an approximate quantity that provided each larvae with a small amount. *Chlorella* sp., a single celled alga that was cultured at eriss for use in *Moinodaphnia macleayi* culturing and bioassays (Riethmuller et al 2003), was administered at a density of 5×10^6 cells for the lower density and 1×10^7 cells at the higher density.

The concentration of homogenised *Artemia* nauplii was quantified by first making a 1:4 dilution of newly harvested nauplii with NMCW. The solution of *Artemia* nauplii was swirled to uniformly resuspend the nauplii. Once the nauplii were resuspended, 1.01 ml was drawn up and immediately dispensed onto a Sedgwick rafter cell. Nauplii were counted in the cell as well as the inside of the pipette tip from which the sub sample was collected. The number of nauplii in 1 ml of the original live stock solution was calculated. The live stock solution was then homogenised using a Potter-Elvehejm tissue homogeniser and diluted accordingly.

Refer to Section 2 for all other testing details.

3.2.2 Test 2: OSI, *Chlorella*, Sera powder Fermented food with vitamins (FFV) and Aquasonic

Survival of newly-hatched *M. splendida inornata* larvae was assessed under five different diets. The diet from Test 1 consisting of OSI, *Chlorella* and Sera powder at the higher density was used as the base diet. Because unacceptably high mortality was observed in Test 1 (See Section 3.3), the diet was supplemented with one of two additional food types, FFV or Aquasonic. The final diets tested are shown in Table 3.2.

All other test methods were the same as described for Test 1 as in Section 3.2.1. FFV was prepared at ***eriss*** for use in *Moinodaphnia macleayi* culturing and bioassays (Riethmuller et al 2003). The test was terminated at day 9 (See Section 3.3).

Table 3.2 Larval *M. splendida inornata* diets assessed in Test 2

Diet	Food type and quantity				
	OSI Micro-food	Chlorella	Sera powder	FFV	Aquasonic
A	4 ml	1 x 10^7 cells	√	-	-
B	4 ml	1 x 10^7 cells	√	50 µl	-
C	4 ml	1 x 10^7 cells	√	150 µl	-
D	4 ml	1 x 10^7 cells	√	-	50 µl
E	4 ml	1 x 10^7 cells	√	-	150 µl

3.2.3 Test 3: Base Feed (OSI, *Chlorella*, Sera powder, FFV) and *Paramecium*

Survival of newly-hatched *M. splendida inornata* larvae was assessed under four different diets (Table 3.3). Following the results of Test 2 (See Section 3.3), the diet consisting of OSI, *Chlorella* and Sera powder + 50 µL FFV was adopted as the Base Feed (BF) for Test 3 and all subsequent tests using *M. splendida inornata*. Because larval mortality in Test 2 was unacceptably high (see Section 3.3) Test 3 assessed BF supplemented with live *Paramecium*.

Paramecium starter cultures were purchased from Southern Biological. Two cultures were started by preparing a 1:20 and 1:10 dilution of starter culture:filtered Darwin tap water (FDTW) containing several grains of rice and a small amount of lettuce. Cultures were replenished weekly with fresh FDTW and food. *Paramecium* were observed regularly under the microscope for health and density. In addition to the four diets, newly hatched *Artemia* nauplii were introduced at day 7 for all groups at an approximate rate of 5 per larval fish. *M. splendida inornata* mouthgapes were larger by day 7 and therefore commenced eating larger food sources after day 7.

Table 3.3 Larval *M. splendida inornata* diets assessed in Test 3

Diet	Details
A	OSI + *Chlorella* + Sera powder
B	BF*
C	OSI + *Chlorella* + Sera powder + Paramecium
D	BF+ Paramecium

* BF: Base Feed – consists of OSI, *Chlorella* and Sera powder + 50 µL FFV.

3.2.4 Test 4: Base Feed (OSI, *Chlorella*, Sera powder, FFV) and *Paramecium*

Survival of newly-hatched *M. splendida inornata* larvae was assessed under four different diets (Table 3.4). *Paramecium* showed to improve survival in Test 3 (see Section 3.3), Test 4 assessed BF supplemented with live *Paramecium* at varying densities. Variations to the base feed and *Paramecium* was used to determine the density of each diet was needed to promote optimal larval survival and maintain water quality. *Paramecium* in 100 and 300 µl in conjunction with the base feed (Table 3.2, diet *B*).

In addition to the four diets, newly hatched *Artemia* nauplii were introduced at day 7 at an approximate rate of 5 per larval fish for all groups.

Table 3.4 Larval *M. splendida inornata* diets assessed in Test 4

Diet	Details
A	BF* + Paramecium (100 µl)
B	BF + Paramecium (300 µl)
C	(BF + Paramecium (100 µl))/2
D	(BF)/2 + Paramecium (300 µl)

* BF: Base Feed – consists of OSI, *Chlorella* and Sera powder + 50 µL FFV.

3.2.5 Test 5: Base Feed (OSI, *Chlorella*, Sera powder, FFV) and *Paramecium* (at higher densities)

Survival of newly-hatched *M. splendida inornata* larvae was assessed under four different diets (Table 3.5). *Paramecium* at the highest density in Test 4 (Table 3.4) had the greatest survival (see Section 3.3), therefore, Test 5 assessed BF supplemented with live *Paramecium* at higher concentrations.

Table 3.5 Larval *M. splendida inornata* diets assessed in Test 4

Diet	Details
A	BF + *Paramecium* (150 µl)
B	BF + *Paramecium* (450 µl)
C	BF + *Paramecium* (900 µl)
D	BF + *Paramecium* (1800 µl)

3.2.6 Test 6: Base Feed (OSI, *Chlorella*, Sera powder, FFV) and *Paramecium* (using an aseptic method)

Survival of newly-hatched *M. splendida inornata* larvae was assessed under four different diets (Table 3.5). *Paramecium* at higher densities may have reduced water quality in Test 5 (see Section 3.3) and therefore, Test 6 assessed BF supplemented with live *Paramecium* at higher concentrations using septic culturing methods.

Paramecium cultures were centrifuged at 1000 revolutions per minute (rpm) for 10 minutes. The concentrated *Paramecium* suspension was then rinsed with FTDW and centrifuged a second time before being dispensed as food. Methods for aseptic techniques involved all media, flasks and pipettes being sterilised. Flasks and pipettes were alcohol wiped and all media UV sterilised. Media to which *Paramecium* were cultured were autoclaved and transfers were undertaken using aseptic techniques in a laminar flow cupboard.

3.3 Results

3.3.1 Test 1

Water quality during Test 1 is shown in Table 3.6, while the survival of larval *M. splendida inornata* fed the various diets is shown in Figure 3.1. Water quality was within acceptable limits with pH remaining 0.5 units from fresh water parameters (See Appendix 1) and DO

remaining above 60% saturated oxygen. None of the diets resulted in acceptably high (ie >80%) larval survival. By day 11 most larvae had died (Figure 3.1), and the test was terminated. There were, however, significant differences in the survivorship of larvae fed the different diets. Results of the ANCOVA and subsequent Tukey's Test showed that there was significant effects between the eight diets assessed. Diet *H* was statistically different from all the others. Diet *G* resulted in the highest proportion surviving and was statistically different from *E* and *H* (See Table 3.1).

OSI, *Chlorella* and Sera micron did not provide the larval fish enough nutrients to take them safely through their first 11 days of life. Survival was low with one larval fish surviving at day 11. Larvae fed OSI alone or a combination of OSI and *Chlorella* resulted in lower survival compared to those fed OSI, *Chlorella* and Sera. The combination of OSI, *Chlorella* and Sera at the higher density showed the strongest survival and this feeding regime was used as a basis for the test two. Homogenised nauplii did not provide sufficient nutrition and resulted in 100% mortality by days 4 and 5 for 100 and 200 nauplii/ml, respectively, and therefore was discontinued.

Table 3.6 Water quality taken during test 1 (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH_4)
A	6.55–6.92	77.6–87.8	19–22	0
B	6.52–6.97	79.1–92.6	19–22	0
C	5.45–6.93	65.8–101.5	20–24	0
D	6.67–6.75	82.4–91.9	22–23	0
E	6.22–6.66	71–88	19–22	0
F	6.26–7.04	65.9–93.3	19–21	0
G	6.53–6.66	49–78.2	21–26	0
H	6.53–6.66	74.9–80.7	20–24	0

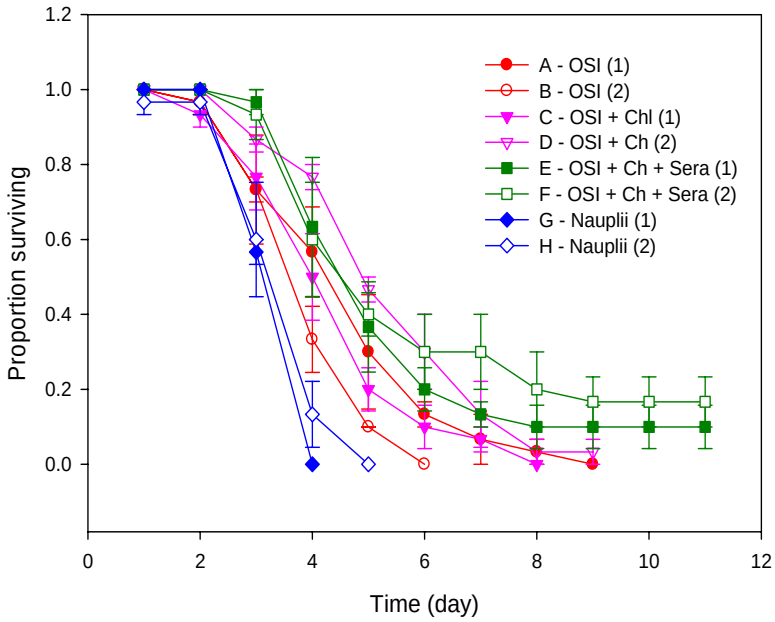

Figure 3.1 Test 1 – Survival of newly-hatched larval *M. splendida inornata* fed on eight different diets based around four food types, either in combination or alone (Table 3.1)

3.3.2 Test 2

Water quality during Test 2 is shown in Table 3.7, while the survival of larval *M. splendida inornata* fed the various diets is shown in Figure 3.2. Water quality was not within acceptable limits with pH shifting by more than 0.5 units from fresh water parameters and DO dropping below 60% saturated oxygen (See Appendix 1). None of the diets resulted in acceptably high (ie >80%) larval survival (Figure 3.2). By day 9 most larvae had died (Figure 3.2), and the test was terminated. Results of the ANCOVA and subsequent Tukey's Test showed that there was significant effects between the 5 diets assessed. Diets consisting of Aquasonic were significantly poorer from all other diets. Diet *B* consisting of OSI, *Chlorella*, Sera powder and FFV (50) was significantly better than from all other treatments and showed the strongest survival > 50% at day 8.

FFV appeared to complement OSI, *Chlorella* and Sera micron and survival improved slightly (50% at day 5). The combination of these four food types were given the identity as the 'base feed' (BF).

Aquasonic did not improve the survival of rainbowfish. In fact, water quality was reduced with dissolved oxygen dropping down to as low <1% saturated oxygen and conductivity rose up to > 1mS/cm (Diets *D* and *E*, Table 3.2). Larval fish remained in the upper stratum of the water column where oxygen level was highest. Aquasonic was therefore discontinued.

Table 3.7 Water quality taken during Test 2 (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH_4)
A	6.21–6.87	66.1–93.7	20-23	0
B	6.23–6.93	65.2–94.9	21-25	0
C	6.22–6.89	64.990.6	21-28	0
D	6.06–6.19	2.3–10	375-654	0
E	5.8–6.16	0.8–5	700-2450	0

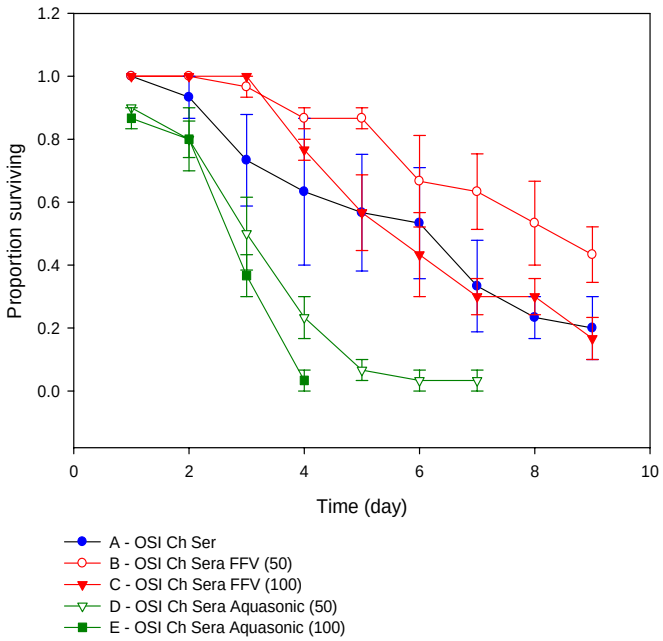

Figure 3.2 Test 2 – Proportion of *M. s. inornata* surviving with the feeding regime outlined in Table 3.2

3.3.3 Test 3

Water quality during Test 3 is shown in Table 3.8, while the survival of larval *M. splendida inornata* fed the various diets is shown in Figure 3.3. Water quality was within acceptable limits with pH remaining 0.5 units from fresh water parameters and DO remaining above 60% saturated oxygen (See Appendix 1). None of the diets resulted in acceptably high (ie >80%) larval survival. By day 21 most larvae had died (Figure 3.3), and the test was terminated. There were, however, significant differences in the survivorship of larvae fed the different diets. Results of the ANCOVA and subsequent Tukey's Test showed that there was significant effects between the four diets assessed. Diet *D* was significantly better than *A*, *B* and *C* (Table 3.3), indicating that larval survival was strongest when fed OSI, *Chlorella*, Sera, FFV and *Paramecium*.

To try to further improve survival, the live microscopic food source *Paramecium* was introduced into the diet as well as changes to stocking density 10 larvae/500 ml and light intensity inside the incubator was reduced. Original 250 ml cylindrical containers were replaced with rectangular 750 ml containers that provided a larger surface area for oxygen transfer into the water column. Some improvement to the percentage surviving was observed. *Paramecium* provided larval fish enough live food small enough to ingest until their mouthgape was large enough to feed off live nauplii which was introduced at day 7 with the first successful ingestion at day 11.

Table 3.8 Water quality taken during Test 3 (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH_4)
A	6.41–6.69	62.8–92.3	19–20	0
B	6.43–7.11	62.3–93.9	19–24	0
C	7.37–7.14	60.9–93	18–21	0
D	6.45–7.05	62.2–91.1	21–25	0

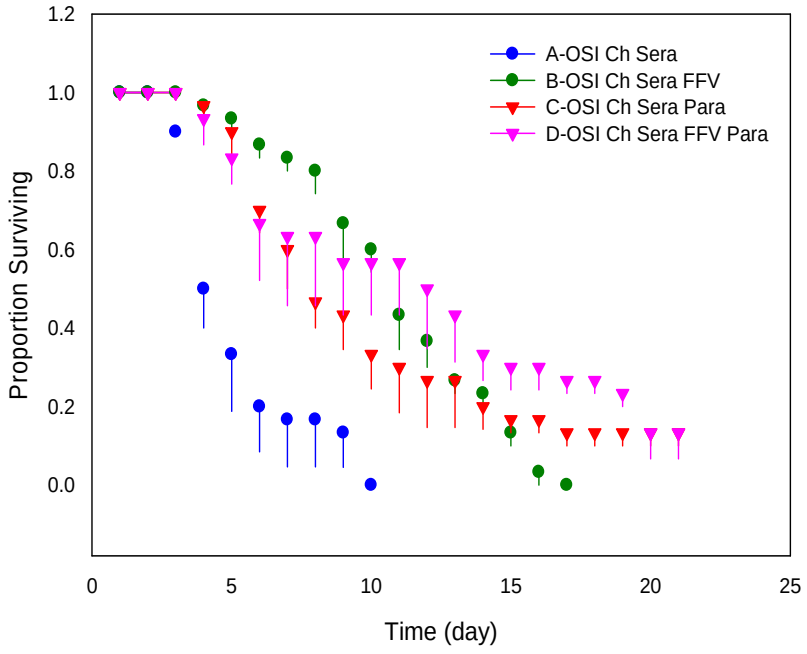

Figure 3.3 Test – 3 Proportion of *M. s. inornata* surviving when fed diets outlined in Table 3.3

3.3.4 Test 4

Water quality during Test 4 is shown in Table 3.9, while the survival of larval *M. splendida inornata* fed the various diets is shown in Figure 3.4. Water quality was within acceptable limits with pH remaining 0.5 units from fresh water parameters and DO remaining above 60% saturated oxygen (See Appendix 1). None of the diets resulted in acceptably high (ie >80%) larval survival at the end of the 28 d test. There were, however, significant differences in the survivorship of larvae fed the different diets. Results of the ANCOVA and subsequent Tukey's Test showed that there was a significant effect between the four diets assessed. Diets *A* and *C* were statistically different to *B* and *D* (See Table 3.4), indicating that *Paramecium* given at higher densities resulted in stronger survival.

Varying densities of *Paramecium* were assessed to determine whether more or less *Paramecium* given would have an effect on the survival of rainbowfish larvae. It was found that larval fish fed higher concentrations of *Paramecium* were more active and appeared healthier than those fed at the lower concentration. This observed result was consistent with the statistically significant difference in survivorship between the two concentrations of *Paramecium* diets. The first signs of live nauplii intake were seen in the groups being fed higher amounts of *Paramecium*. Once the larval fish were able to eat live nauplii growth increased markedly and percentage of survival reached a plateau once all larval fish could eat *Artemia* nauplii (at ~day 15). Based on trial and error and close observations, larval fish appeared 'safe' once they could eat live nauplii. Most fish could eat nauplii by day 15, therefore aspects to the test design up until day 15 needed to be improved. Fish that ate live nauplii before any of the others had a head start in terms of development. Other fish were beginning to show interest by this stage, but did not have mouth gapes large enough. They slowly began to eat brine shrimp but by this stage, those first commenced feeding off nauplii were already larger in size. The average length of 1 day old fry was 3.5 ± 0.023mm (mean ± s.e., n = 30). Average growth (in length) of larvae eating nauplii was 28% compared to the 18% for those that did not eat nauplii at all.

Table 3.9 Water quality taken during test 4 (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH_4)
A	6.53–6.92	77.8–99.3	17–23	0
B	6.56–6.93	83.2–97.5	17–23	0
C	6.63–6.96	87.3–95.9	17–21	0
D	6.59–6.93	86.8–95.4	17–22	0

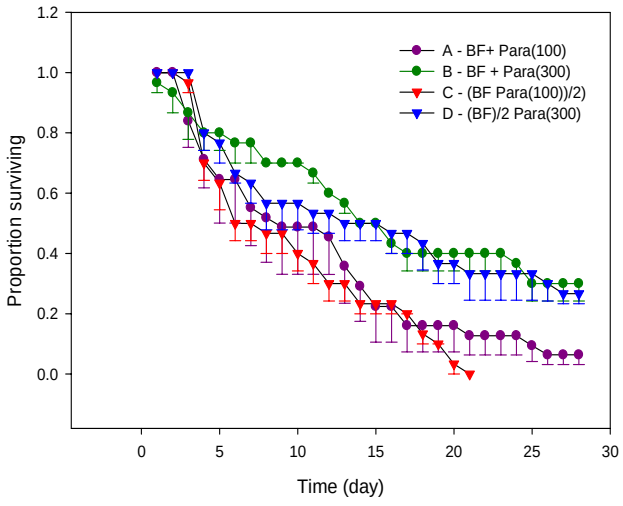

Figure 3.4 Test – 4 Proportion of *M. splendida inornata* surviving when fed the diet outlined in Table 3.4

3.3.5 Tests 5 and 6

Water quality during Tests 5 and 6 are shown in Tables 3.10 and 3.11, respectively, while the survival of larval *M. splendida inornata* fed the various diets from both in Tests 5 and 6 are shown in Table 3.5. Water quality was within acceptable limits for both of Tests 5 and 6 with pH remaining 0.5 units from fresh water parameters and DO remaining above 60% saturated oxygen (See Appendix 1). None of the diets resulted in acceptably high (ie >80%) larval survival, with or without aseptic techniques and the tests were terminated at days 3 and 4, respectively (Figure 3.5).

It was found from test 3 that *Paramecium* introduced at a higher concentration improved survival in the first 14 days and provided larval fish with a source of live food until their mouthgape was big enough to eat live nauplii. *Paramecium* was given at a higher concentration to try and improve survival in the days leading up to this. However, by increasing the *Paramecium* concentration, unknown live micro-organisms were also introduced. Introducing a higher volume of *Paramecium* culture to the diluent water affected water quality and thus larval health. As survival steadily improved with the introduction of new food sources, problems arising that involve the health of the larval fish became apparent. Fungal infections were common among all treatments (Figure 3.6 a and b). There was large variability in survival, however, what appeared to be a severe case of fungal infections was consistent across all groups. Larval fish carried mass amounts of filamentous debris. This debris was apparent throughout the diluent water and the larvae swam into it and became caught. The debris was most likely to have been introduced by the *Paramecium* culture, as this was the only change in the feeding regime and was not observed after the introduction of FFV. Although survival was still reasonable, a majority of larvae were heavily infected with fungus and the test was terminated at day 3. A further test using the same method as the previous with but with an axenic paramecium culture was trialled. This axenic culture did not eliminate fungal infection and debris within the diluent water.

Table 3.10 Water quality taken during test 5 (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH_4)
A	6.61–6.74	89.9–94.2	17–18	0
B	6.62–6.76	91.8–93.4	17–18	0
C	6.69–6.76	93.0–94.6	17–18	0
D	6.70–6.74	91.1–93.8	17–18	0

Table 3.11 Water quality taken during test 6 (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH4)
A	6.46–6.69	92.2–117.6	17–19	0
B	6.60–6.76	90.3–117.3	17–20	0
C	6.63–6.72	89.3–116-3	18–22	0
D	6.63–6.87	88.9–116-3	18–26	0

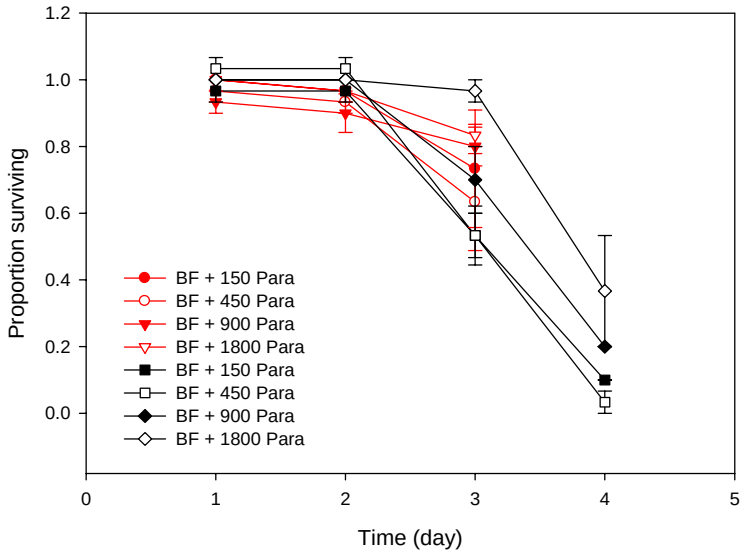

Figure 3.5 Comparison of *M. splendida inornata* survival using aseptic (black) and non aseptic (red) *Paramecium* cultures

a) b)

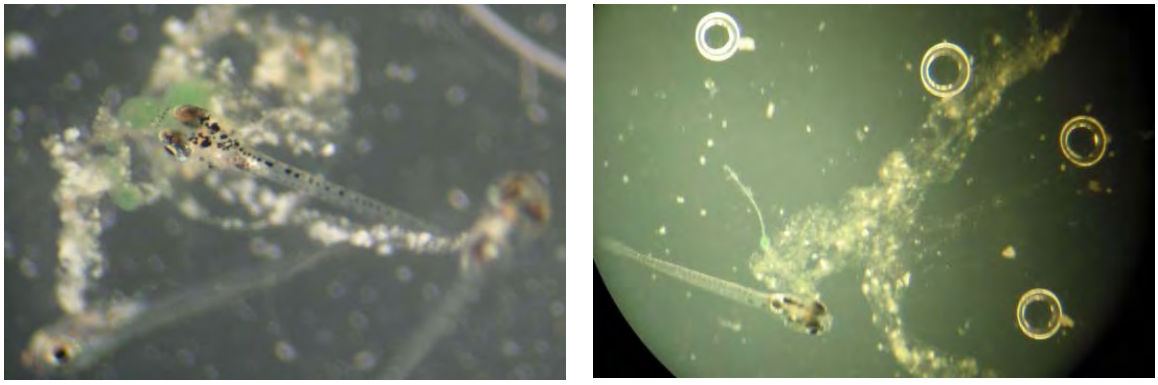

Figure 3.6 a and b: Larval fish with unidentifiable infection, possibly fungal or bacterial contamination introduced from the *Paramecium* food source (larval fish presented here are < 3 days old ~3.5 mm in length).

3.4 Discussion

Despite numerous attempts, no diet was found that would result in acceptable survival (≥80%) (US EPA 1994) of larval *M. splendida inornata* over a 28-d period. The best survival that was attained was approximately 38% after 28 days, when larvae were fed the base feed and *Paramecium* (300 µl) (Figure 3.4). In contrast, whilst assessing the early development and growth of the Eastern rainbowfish (*M. splendida splendida*), Humphrey et al (2003a) found that larval fish fed a mixed diet of Tetra MinE baby fish food, ground up *Artemia* nauplii and a "green water" culture containing *Paramecium*, rotifers and other various invertebrates generally resulted in greater than 90% survival. However, it is understood that numerous toxicity experiments needed to be undertaken before this percentage survival of control (ie unexposed) larvae was obtained (C. Humphrey, AIMS, pers comm).

Experiments conducted by Williams and Holdway (2000) to determine larval survival in Crimson-spotted rainbowfish (*M. fluviatilis*), involved feeding larval fish a diet consisting of Sera micron, newly hatched nauplii, vinegar eels nematodes and *Paramecium* four times a day. They found that larval rainbowfish diet must match their mouth development and food introduced over time can be changed as the larvae grow.

In the experiments in this study (Tests 3 and 4, Figures 3.3 and 3.4, respectively), larvae were fed live nauplii from day seven but did not successfully ingest it until about day 12, suggesting that there mouths were not large enough until day 12.

Williams et al (1998) assessed the suitability of using larval *M. nigrans* (Black-banded rainbowfish) as a laboratory toxicity test organism for 96 h acute tests. An initial test resulted in low mean survival (< 60%) where the larval fish were not fed during the 96 h test duration. These results prompted the investigators to assess the effects of survival with various feeding regimes. They found that survival improved (>80% in the controls) with the introduction of small doses of Aquasonic fry food (15 µl per 30 ml diluent water), although the tests were only 4 days in duration. *M. splendida inornata* in the present feeding study (Test 2, Section 3.2) were fed an even lower ratio of Aquasonic:diluent water (50 and 100 µl per 200 ml diluent water) and no improvement to larval survival was observed. In fact, Aquasonic reduced water quality with dissolved oxygen dropping to a low ~1% and electrical conductivity (EC) increasing up to 2450 µS/cm (Table 3.7). For a test to be considered valid, the dissolved oxygen concentration must be ≥60%, test water should remain constant during the testing period (OECD 2000) and survival being ≥ 80% (US EPA 1994). The dissolved oxygen levels in other feeding regimes that did not include Aquasonic (Table 3.2) achieved this criterion (Table 3.7). EC increased by an order of magnitude from the original value of 20 µS/cm before Aquasonic had been added (see appendix for new water quality). Williams et al (1998) also found that the health of the broodstock at the time of spawning had an effect on larval survival. Spawning was poor when fed a diet of only flake food, whereas when the diet was changed to crushed fish pellets and newly hatched nauplii, egg production increased and larval survival improved.

Rainbowfish broodstock used in this trial were fed twice daily on Sera Flake food and newly hatched *Artemia* nauplii. Initially, spawning produced a high number of eggs and after several spawns, the number of eggs declined. Frozen adult *Artemia* nauplii (thawed and rinsed in FDTW) was introduced into their diet and broodstock spawned opportunistically with larval hatch rate always being sufficient to commence a test. Whilst a good mixed diet led to a high number of eggs, it did not show improvement toward larval survival used in the feeding trials. Therefore, poor larval rainbowfish survival was most likely due to the test conditions itself not the maintenance of broodstock.

Barry et al (1995) found that the effects of esfenvalerate on 7 d old crimson spotted rainbowfish larvae could not be determined because of high control mortality. Larvae at this stage undergo transition from endogenous to exogenous feeding and may be more sensitive to handling and stress. Larval mortality in the control at day 28 was approximately 34%. Even though the test conditions were different to these feeding trials, with larger volumes of flow through diluent water for 26 days after the exposure period and a feeding regime that consisted of 6 feeds per day, unexposed larval survival was still relatively low (65% over 28 d).

According to growth development studies conducted by Humphrey et al (2003a), variation in length between individuals increased as they aged. The coefficient of variation increasing from 3% to 6%, up to 12 days after hatching, to 15% at 87 days after hatching. Growth rates for *M. splendida splendida* were considerably higher than those reported for other members

of the genus. Humphrey et al (2003a) also found that 28 day old *M. splendida splendida* had a standard length (SL) of 15mm (ie 55% growth) where the average length of *M. splendida splendida* on hatching was 3.7 ± 0.03 mm SL (mean ± s.e., *n*=30). The maximum length measured in this study (Test 4) was 9.0 mm at day 28 (ie 35% growth).

Comparing larval growth from different studies is difficult because growth rates vary with changes to test conditions (eg temperature, feeding rate and densities) (Reid & Holdway 1995).

There are many papers that have assessed the growth and development or the toxicity of chemicals to rainbowfish, although only a few have used test durations as long as 28 days (Holdway 1992b; Humphrey et al 2003a). Feeding regimes varied between different studies and these results were relied on for information and guidance on how to best approach this feeding trial. However, *M. splendida inornata* larval survival in the feeding trials in this study were poor even when fed feeding regimes similar to those used by previous researchers.

3.5 Summary

Diet and feeding regime was a significant modifying factor to larval survival in the present *M. splendida inornata* feeding trials. Of the six feeding trials conducted, it was found that a diet consisting of the base feed supplemented by *Paramecium* resulted in the strongest survival rate. This test (Test 4) was the only test that successfully reached day 28, although the maximum survival rate was 38%.

For a chronic toxicity test to be valid, there needs to be at least 80–90% survival in the controls (unexposed treatment) (OECD 1992; 2000). This was not achieved in any of the six feeding trails conducted and therefore a 28-d U toxicity test for *M. splendida inornata* could not proceed.

4 Toxicity test development – *Mogurnda mogurnda*

4.1 Introduction

A study conducted by Rippon & Hyne (1992) found *M. mogurnda* to be a suitable standard test species. The study showed that while *M. splendida inornata* and *M. mogurnda* had similar sensitivities to copper, *M. mogurnda* could produce larger amounts of eggs per breeding pair about every 5 days. *M. splendida inornata* egg production was daily, however, egg numbers were lower. *M. splendida inornata* have low lipid reserves and commence eating within hours after hatching (Holdway et al 1994) whereas *M. mogurnda* have developed yolk sacs and do not require additional nutrients in the first four days. *M. mogurnda* are capable of consuming live brine shrimp as early as day 3 and therefore no additional food source was needed. The current fish toxicity testing protocol at ***eriss*** is an acute test over four days using newly hatched and unfed *M. mogurnda*. Consequently, in order to be able to perform a chronic larval growth test, testing considerations similar to those described in Section 3 for *M. splendida inornata* need to be addressed for *M. mogurnda*. Series of experiments was undertaken to determine the final experimental design of the 28 day toxicity test protocol to be used to assess the chronic toxicity of uranium.

The objective of the second phase of this test development was to establish a suitable feeding regime that would result in an optimal survival rate in *M. mogurnda*. The following aspects were investigated with reference to OECD guidelines (OECD 2000) for test development: feeding regimes and how they affect larval growth, water quality and uranium concentration; uranium fate; and measurement endpoints for larval growth.

4.2 Methods

4.2.1 Test 1: Effect of feeding duration on the partitioning of uranium

As fish larvae need to feed during a chronic toxicity test, it is important to understand the implications of the presence of food on the partitioning of the toxicant being studied. This test assessed the partitioning of uranium in a test environment where the food type, live *Artemia* nauplii, was present for varying durations, namely 2, 4 and 6 h. The test was run in the absence of *M. mogurnda* larvae, hence providing a worst case scenario of the maximum amount of food being potentially available to interact with uranium. The test was run for 24 h, with the food being introduced during the last 6 hours, as it was anticipated that the most appropriate time to feed the larvae would be as soon as practicable prior to each 24 h period test solution renewal.

Test uranium solutions of 50 µg/L and 500 µg/L were prepared by diluting a stock solution of 5 g/L uranyl sulphate with NMCW. 200 ml of test solution was dispensed into 250 ml plastic vials. A total of eight treatments (four treatments per concentration of uranium) was assessed, as shown in Table 4.1 (with three replicates per treatment). Section 1.2.2 emphasises that the < 0.45 µm fraction of uranium contains the majority of the bioavailable species, therefore total and filtered (Table 4.3) uranium concentrations for each treatment were measured at the commencement of the test (ie at 0 h), just before food was introduced (ie at 18, 20 and 22 h) and at the end of the 24 h test period. In addition, a control treatment with no food was

included to determine the potential loss of uranium to the test container walls. Physico-chemical analysis of new and 24 h old water parameters were measured for electrical conductivity (EC), dissolved oxygen (DO) and pH (WTW, Germany). Filtered and unfiltered sub-samples of test solutions were collected for dissolved organic carbon analysis.

Table 4.1 Treatments used in Test 1 to assess the effect of the presence of food (*Artemia* nauplii) on the partitioning of uranium

Group	Uranium Concentration (µg/L) & associated treatment ID	
	50	500
Control (no food)	A	E
Food present for 2 h (22 h post-commencement)	B	F
Food present for 4 h (20 h post-commencement)	C	G
Food present for 6 h (18 h post-commencement)	D	H

Eighteen (18) h after the commencement of the test (6 h before end of test) approximately 10 ml of test solution from each replicate in group *D* and *H* was sampled and preserve using 13µL concentrated nitric acid and stored at 3-4 °C for uranium analysis. Approximately 200 *Artemia* nauplii were placed into the remaining solution. At 20 and 22 hours the procedure was repeated for groups *C*/*G* and *B*/*F*, respectively.

At 24 h, all test containers (including controls) were shaken vigorously and immediately sampled using a syringe for total and dissolved uranium analysis (dissolved fraction sample filtered through <0.45 µm mixed cellulose syringe filter).

ICP-MS analysis of uranium

Uranium analysis was performed using ICP-MS on a Perkin Elmer ELAN 6000 instrument with an AS90 auto-sampler (Perkin Elmer, USA). The instrument was operated according to manufacturers specifications (Table 4.2) and prior to use the nebuliser gas flow and ion lens voltage were optimised to give maximum sensitivity.

Table 4.2 Elan 6000 ICP-MS instrument conditions

Instrument parameter	**Setting**
Power (W)	1050
Argon plasma gas flow (L/min)	17
Argon auxiliary gas flow (L/min)	1.2
Argon nebuliser gas flow (L/min)	0.7
Sample rinse (s at 48 rpm)	20
Sample uptake (s at 48 rpm)	20
Scan mode (s at 16 rpm)	20
Sweeps/reading	6
Replicates	3
Dwell time (ms)	100

The accuracy of ICP-MS measurements was checked using custom made 'Calcheck', a standard solution of known metal concentrations.

Sample preparation was carried out in a class 100 clean room using trace metal clean procedures. To each sample, 1 mL of an internal standard solution (400 μg/L Rhodium, Indium and Rhenium) was added per 10 mL of water sample.

Calibration standards for ICP-MS analysis were made using mixed metal stock solutions prepared from 1000 mg/L metal standards (BDH, Merck) in MQ water.

4.2.2 Test 2: Effect of feeding on larval survival and growth

Three feeding regimes were assessed to determine a suitable feeding regime that will result in optimal larval survival and growth over 28 d.

Less than 1-day old larvae were placed in clean water and provided with a standardised quantity of food (*Artemia* nauplii per larva, Table 4.3). Nauplii are a standard food for test animals in aquatic toxicology because of the availability, ease of use, and presumed good nutritional quality (ASTM 1988). Nauplii were placed as close to their mouths as possible at the early stages until they were able to swim and source food on their own. All larvae were fed 3 h prior to water being replenished and groups that were fed twice were provided with a second feed after diluent water change (Table 4.3).

Table 4.3 Feeding regime assessed in Test 2

	Day 1–6 (*Artemia* nauplii per larva)	Day 7–23 (*Artemia* nauplii per larva)	Day 24–28 (*Artemia* nauplii per larva)
A	10-20 daily	20-30 daily	30-40 daily
B	5-10 twice daily	10-20 twice daily	20-30 twice daily
C	10-20 twice daily	20-30 twice daily	30-40 twice daily

4.3 Results

4.3.1 Test 1

The effect of the presence of food on uranium concentration in NMCW is shown in Figure 4.1. Table 4.4 shows the total and filtered uranium concentrations in the treatments over the course of the experiment, while Figure 4.1 presents the data in terms of the filtered uranium concentration at 24 h as a proportion of the total uranium concentration at 24 h. The data showed that the loss of uranium over 24 h was less than 10% at both concentrations and all food treatments. Results of the ANOVA showed that there was no significant difference between dissolved (filtered) and total (unfiltered) fractions of uranium after 24 hours. In addition, there was no significant difference in filtered and total uranium concentrations between the different food durations (ie no food, food present for 2, 4 or 6 h). Finally, in the 'no food' control treatment, filtered and total uranium concentrations at 24 h were not significantly different to those at test commencement.

Table 4.4 Measured uranium concentrations for t_0 – t_{24} in Test 1

Food presence	Uranium concentration (µg/L) during experiment (± SEM)									
	0 h		18 h		20 h		22 h		24 h	
	total	*filtered*	*total*	*filtered*	*total*	*filtered*	*total*	*filtered*	*total*	*filtered*
Nominal U – 50 µg/L										
No food	48 (0.9)	44 (0.3)	49 (0.8)	44 (0.06)	49 (0.5)	43 (1.1)	51 (0.4)	47 (0.3)	52 (0.6)	46 (0.3)
2 h food	-	-	-	-	-	-	49 (0.9)	44 (0.2)	47 (0.7)	43 (0.6)
4 h food	-	-	-	-	49 (0.6)	45 (0.3)	-	-	50 (1.0)	44 (1.3)
6 h food	-	-	47 (0.4)	43 (0.8)	-	-	-	-	48 (1.3)	41 (0.6)
Nominal U – 500 µg/L										
No food	467 (4.2)	483 (6.5)	484 (8.8)	466 (3.0)	494 (13.4)	472 (4.6)	512 (15.8)	462 (8.8)	442 (17.8)	407 (5.06)
2 h food	-	-	-	-	-	-	484 (3.9)	457 (8.7)	432 (3.9)	420 (9.5)
4 h food	-	-	-	-	508 (9.9)	474 (14.5)	-	-	505 (8.9)	372 (12.8)
6 h food	-	-	481 (4.7)	452 (9.2)	-	-	-	-	428 (2.6)	410 (4.0)

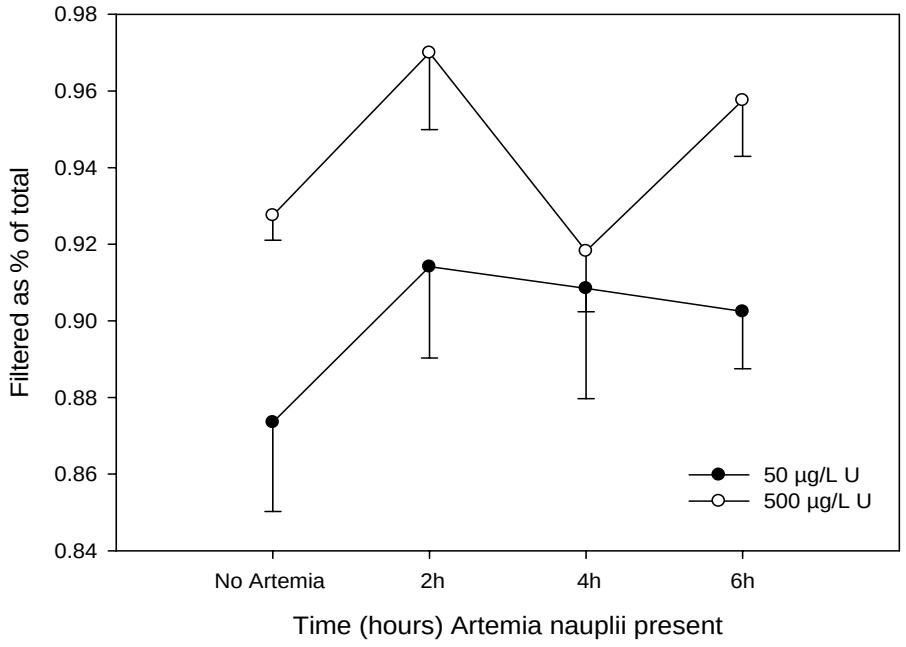

Figure 4.1 Mean (±SEM) uranium loss to *Artemia* nauplii present for 2, 4 and 6 h expressed as a proportion of total uranium

4.3.2 Test 2

Water quality during Test 2 is shown Table 4.5, while the survival of larval *M. mogurnda* is shown in Figure 4.2. Water quality was within acceptable limits with pH remaining 0.5 units from fresh water parameters and DO remaining above 60% saturated oxygen (See Appendix 1).

Results of the ANOVA and subsequent Tukey's Test showed that there was significant difference of survival and growth in feeding regime *A* from feeding regimes *B* and *C*.

Table 4.5 Water quality taken during *M. mogurnda* (min-max)

Diet	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH)
A	5.4–6.31	87.8–102.3	13–16	0
B	5.64–6.19	83.5–99.3	13–16	0
C	5.69–6.21	85.1–97	13–16	0

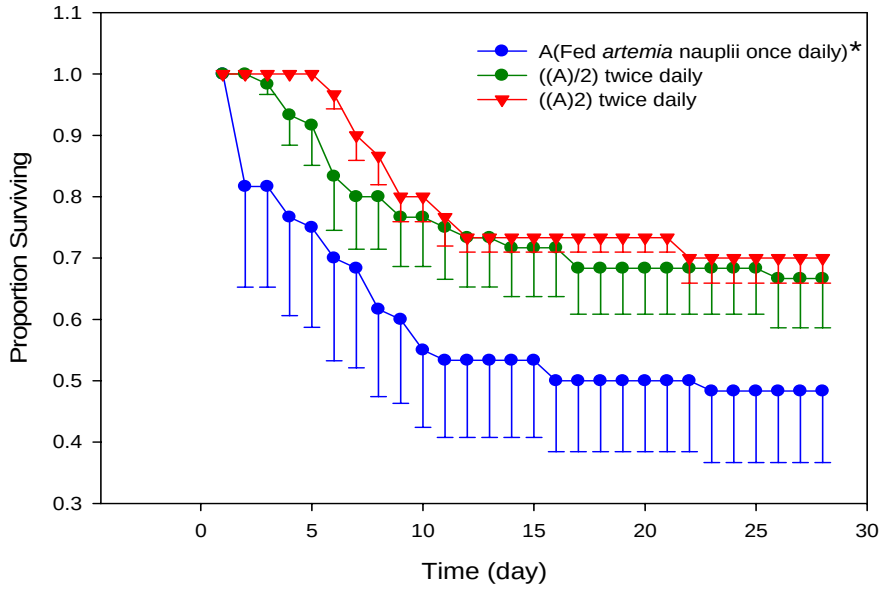

Figure 4.2 Effect of feeding regime (Table 4.2) on mean (±SEM) survival of larval purple-spotted gudgeon (*Mogurnda mogurnda*) over 28 days. Data in blue, green and red represent results for diets A, B and C, respectively. *Denotes a significant difference from the other two feeding regimes ($P \leq 0.05$).

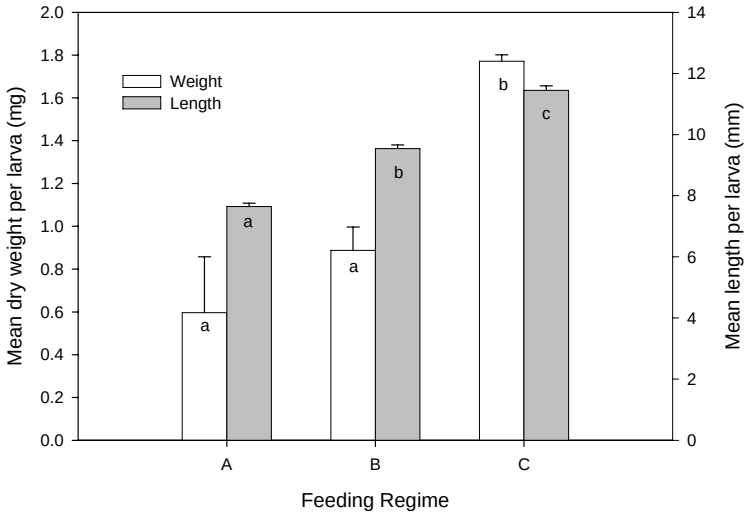

Figure 4.3 Effect of feeding regime (Table 4.2) on mean (±SEM) dry weight and length over 28 days. Bars for dry weight and length that do not have a letter in common are significantly different from each other ($P \leq 0.05$).

The minimum number of replicates (ie sample size) needed for the toxicity tests given a specified effect size and statistical power was determined using Power analysis (See section 2.7.1). Power, denoted as $1 - \beta$ (Type II Error), is the probability of having detected an effect in the event it actually existed (Zar 1984). Power analysis can be used for a range of reasons, including determining sample sizes (as applied here), minimum detectable differences and power itself. Data from the *M. mogurnda* feeding test (Section 4.3.3), specifically the length and dry weight data, were used to estimate this. The parameters entered into the power analysis were:

No. treatments: 7

α: 0.05

Standard deviation: the SD of the feeding regime *C* treatment (Table 4.3) x 1.5 was used, where 1.5 is a slight overestimation to account for the event that the SD measured during the feeding trial was unusually low. For the mean length SD of 0.134, this was 0.2. For the mean dry weight SD of 0.074, this was 0.11.

Target Power: 0.8

Maximum difference between means: a value corresponding to 20% of the control value was used. For the mean length of 11.44 mm, this was 2.3. For the mean dry weight of 1.77 mg, this was 0.35.

For length, the calculated sample size was 2, and the actual power was 0.999 (Figure 4.4) and for dry weight, the calculated sample size was 4, and the actual power was 0.823 (Figure 4.5).

Hence, a sample size of 4 (per treatment) was the minimum required to ensure a power of at least 0.8 for both length and dry weight endpoints.

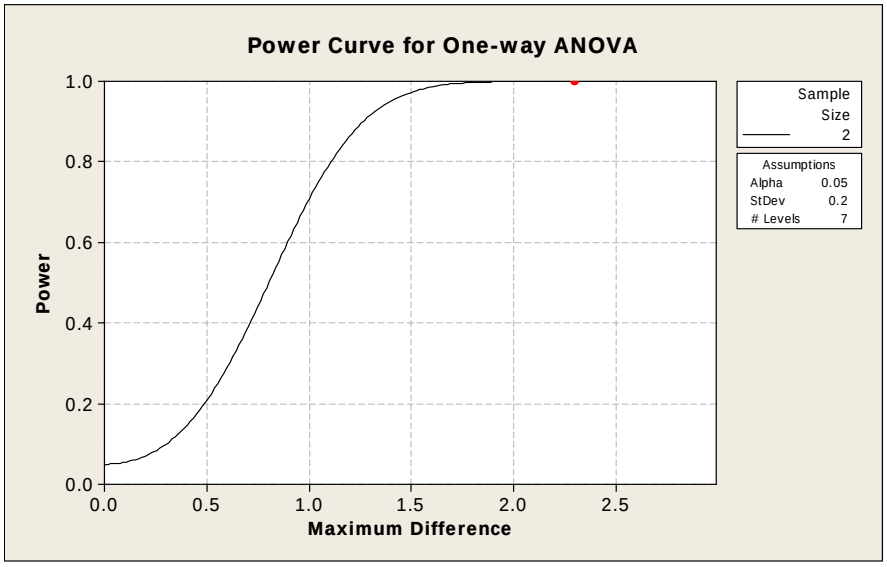

Figure 4.4 Length data Power curve. A power of 0.999 with a sample size of two (ie two replicates per treatment) calculated based on length data

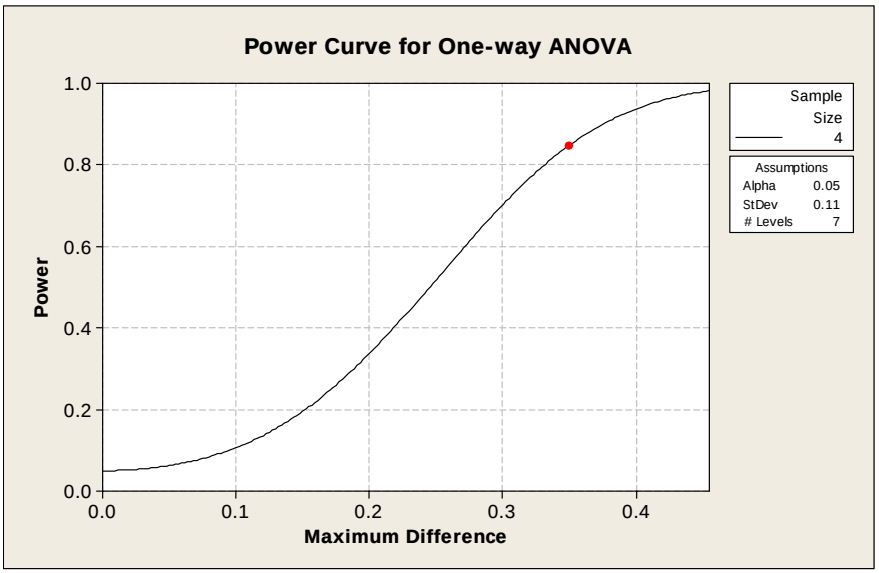

Figure 4.5 Dry weight data Power curve. A power of 0.823 with a sample size of four (ie four replicates per treatment) calculated based on dry weight data

4.4 Discussion

Results from Test 1 (4.3.1) showed that the loss of uranium to the test environment was not significant, and therefore would not affect the level of uranium exposure to the test fish.

The feeding trial experiment (Test 2; 4.3.2) found that feeding regime *C* (10–20 (days 1–6), 20–30 (days 7–23) and 30–40 (days 24–28) nauplii, twice a day) resulted in strong larval growth at the end of the 28 day test period (Figure 4.3). In comparison, larval fish fed regime *A* had a reduction in growth of 33 and 66% for length and dry weight, respectively, while larval fish fed feeding regime *B* had a reduction in growth of 17 and 50% for length and dry weight, respectively.

Survival rate was also the strongest in feeding regime *C* (Figure 4.2), although the minimum acceptable survival rate for toxicity tests, of ≥80% was not achieved (see below for further discussion). This was due to a notable decline in survival between days 5 to 10. During this period, larval fish were eating more, and by day 10, all the food being provided was being consumed. This critical period was assessed closely to determine the reason for the mortality. It was evident that larval fish were not receiving sufficient food and, as a result, the amount of nauplii provided per fish was increased after day 7. Larval survival remained constant from day 10 and close observations for left-over food (if any) were made for the remainder of the test. Little or no food was used as an indication for when larval fish needed more food as they grew larger. The groups fed the least amount of nauplii (*A* and *B*) were always the first to show signs of food depletion. When this was observed, all groups were fed more accordingly.

The toxicity development for *M. mogurnda* was primarily based on OECD (2000). Validity of the test was achieved with pH and DO remaining within an range acceptable range over the 24 h before the diluent water was replenished. According to OECD (2000), the appropriate loading rates and stocking densities for the rainbow trout are ~ 2 g/l to avoid overcrowding. Larval stocking density rate should be low enough to maintain a DO concentration of at least 60% without the need for aeration.

Larval stocking density used in this toxicity development test was 17 mg/l at day 28 with survival being > 70%. While this percent survival does not meet the 80% requirement (US

EPA 1994) nor the 90% requirement of the OECD juvenile growth test guideline (2000), feeding was initially based on trial and error, and the > 70% survival rate was considered high considering that larval fish did not get enough food between days 5–10 (Figure 4.2). The feeding regime recommended in OECD (2000) is that larval fish are to be fed two equal portions per day, with the ration being based on the weight for each fish. As weight could not be taken throughout the 28 d testing period, observations of larval health, and the monitoring of remaining food after the 2–3 h feeding duration were used to determine if larval fish were getting a sufficient amount to eat. It is important to note that the 90% survival criterion (OECD 2000) is based on juvenile survival, not newly hatched larval survival. The OECD fish early life stage test (OECD 1992), which exposes embryos then the hatched larvae for a substantial period of time, specifies survival criteria of 70–80% depending on the species. Given this, the survival rate of larvae fed feeding regime *C* could be considered acceptable. Moreover, the results of the test, and the observations on feeding made during and thereafter, enabled the development of a suitable diet and feeding regime that would further improve survival, as evidenced by the high control survival in both toxicity tests (see Chapter 5).

In this study, *M. mogurnda* larval survival was higher than *M. splendida inornata*. There are a number of likely reasons for this. It is well known that *M. splendida inornata* are opportunistic spawners (Bishop et al 2001). However, problems arising from low numbers of eggs per spawning (*M. nigrans*), difficulties in feeding in early life stages (*M. nigrans* and *M. splendida inornata*) and a inability to survive in water quality associated with peak flow conditions (*M splendida inornata*), limit the suitability of these fishes for laboratory bioassays. Rainbowfish larvae need to be reared under stringent conditions and require various diets in order to survive. Many toxicity tests have been undertaken (Holdway 1992b; Reid and Holdway 1995; Barry et al 1995; Williams and Holdway 2000; Humphrey et al 2003a; Humphrey et al 2003b) with test conditions and diets varying to a certain extent, but almost always involving complex diets being fed up to six times a day (Bywater et al 1991). Bywater et al (1991) identified *M. mogurnda* to be the most suitable species for toxicity testing as they can reproduce in numbers sufficient for bioassays and in early larval stages, can be easily fed. Perhaps the biggest advantage for *M. mogurnda* larvae is that they have larger mouthgapes than rainbowfish larvae, and as such can be fed more easily on just *Artemia* nauplii rather than the complex diets required by rainbowfish, which must be tailored to suit this until they are able to eat larger food sources such as live nauplii (Williams and Holdway 2000).

To further refine the design of the toxicity test protocol, the growth data from the feeding experiment were used to determine the sample size required in order to be able to detect a 20% change in growth with a power of 0.8 (ie an 80% probability of successfully detecting a 20% effect). The power analysis indicated that a sample size of 4 was the minimum required to achieve the above criterion for both larval length and dry weight.

4.5 Summary

The *M. mogurnda* feeding trial was completed with a >70% survival rate at day 28. Although this did not meet the accepted >90% criterion for survival specified in OECD (2000) for juvenile fish, it was within the general criterion of >70% (species-dependent) specified in OECD (1992) for larval fish. The feeding regime that resulted in the highest rate of survival was a standardised amount of *Artemia* nauplii two times a day, once in the morning and again in the early evening, at a feeding rate of approximately 10–20, 20–30 and 30–40 nauplii per larva, through days 1–7, 8–25 and 26–28, respectively. This feeding regime was adopted for the toxicity tests described in the following chapter.

Loss of uranium to the test environment was found to be less than 10% over 24 h indicating that uranium concentration will remain significantly constant and therefore will not affect uranium exposure to larvae over the 24 h period before test waters are replenished.

5 28-d uranium test application

5.1 Introduction

Section 4 detailed the development of a chronic toxicity testing protocol to assess the effects of chemicals on the growth and survival of newly hatched larval *M. mogurnda* over a 28 d exposure period. Sufficient developmental work was done to result in a test protocol that was appropriately optimised and would enable the detection of relatively small (ie 10–20%) changes in larval growth as a result of toxicant exposure. The 28 d exposure duration of the chronic test represents at least twice the exposure duration of any other toxicity test previously undertaken using *M. mogurnda*. The chronic toxicity protocol provided the opportunity to assess the relevance of the historical fish uranium toxicity estimates (Holdway 1992b) in the dataset used to derive the current Limit for uranium in Magela Creek (See Section 1.4.2).

The objective of the final phase of this study was to determine the effects of uranium on *M. mogurnda* larval growth over a 28 day exposure period, and subsequently to determine the Lowest-Observed-Effect-Concentration and No-Observed-Effect-Concentration values.

5.2 Methods

Uranium stock solutions were prepared from uranyl sulphate dissolved in Milli Q water at a concentration of 5g/L. On the day before test commencement, test solutions were prepared using the stock uranyl sulphate solution with filtered NMCW (See Section 2). Test solutions were made in 2 X 22 L and 1 X 20 L batches to provide a sufficient amount for 28 days of testing.

Newly hatched (<10 hours old) *M. mogurnda* larvae were exposed to one of six concentrations of uranium, determined using information from Holdway (1992), plus a control. Each treatment consisted of 4 replicates with 10 larvae per replicate in a volume of 500 ml. The fish were provided with a standard amount of feed (*Artemia* nauplii) two times a day, once in the morning and again in the early evening, at a feeding rate of approximately 10–20, 20–30 and 30–40 nauplii per larva, through days 1–7, 8–25 and 26–28, respectively, for a set period prior to test solution renewals. Various endpoints were measured based on data recorded throughout the 28 day exposure period as well as at the end of testing. Larval length were measured at various times during the test (ie days 1, 7, 14, 21 and 28) using a Canon 7 mega pixel digital camera and analysed for length using Leica QWin Standard version 2.4, while survival, final length, wet weight and dry weight being measured at day 28.

Survival was recorded daily, just prior to test solution renewal. Larval length, as measured and recorded on days 0 (test commencement), 7, 14, 21 and 28. At the termination of the test (ie at day 28), fish were collected and prepared for dry weight analysis (See Section 2.5)

Two uranium toxicity tests were undertaken, the nominal and measured uranium concentrations for which are presented in Table 5.1.

Table 5.1 Nominal and measured (filtered) uranium concentrations in tests assessing the chronic toxicity of uranium to *M. mogurnda*

Uranium concentration (μg/L)			
Test 1		**Test 2**	
Nominal	*Measured (±SEM)*	*Nominal*	*Measured (±SEM)*
0	0.08 (0.05)	0	0.02 (0.03)
27.5	27 (2.3)	110	90 (2.2)
55	46 (1.1)	220	184 (3.2)
110	90 (3.3)	440	381 (2.54)
220	166 (1.4)	880	768 (11.7)
440	329 (1.84)	1760	1397 (160)
880	685 (6.8)	3520	3180*

* One measurement taken

5.3 Results

5.3.1 Test One

The effects of uranium on *M. mogurnda* larvae are shown in Figures 5.1 and 5.2. Water quality is presented in Table 5.2. Water quality was within acceptable limits with pH remaining 0.5 units from fresh water parameters and DO remaining above 60% saturated oxygen (See Appendix 1).

Larval fish exposed to 166 and 329 μg/L U experienced some sort of illness (Figures 5.3 and 5.4) that could not be defined. The ailment became prominent by day 14 and affected their gills and head. Their gills flared hyperactively and at day 16 their gills carried stringy green debris. It could not be determined whether the debris became caught from an outside source or whether the larval fish were discharging the infection out through their gills (Figure 5.4b). The diluent water was analysed to determine if the water was the cause of this problem. There was no evidence to suggest that the infection came from the diluent water as no unusual particulate matter was observed in test solutions at 166 and 329 μg/L U. Water quality was within acceptable ranges for pH, EC and DO. The effect of uranium was eliminated for these treatments as there was no growth or survival effects in treatment 685 μg/L U. The source of infection could be due to possibly a bad (however unlikely) selection of larval fish at the commencement of the test for those particular treatments. It could be possible that the infection was 'contagious' and spread across the two treatments. When the larval fish were transferred each day, the same pipette was used moving up the concentration gradient. The infection may have spread through this way. However, if this was the case, the disease would have spread up to the larval fish exposed to 685 μg/L U. The two diseases observed in 166 and 329 μg/L U may not even have any relation to each other. Although the symptoms were similar there were also subtle differences in the effect it had on them. Growth at 166 μg/L U was significantly higher than at 329 μg/L U but survival was significantly lower (Figures 5.1 and 5.2). The disease 166 μg/L U did not effect the entire treatment as there was only one replicate that had the disease. There was high mortality in this replicate with only 40% surviving by day 28 while the three other replicates appeared to be healthy.

Table 5.2 Water quality taken during Toxicity Test 1 (min-max)

Exposure concentration (µg/l U)	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH)
0	6.07–6.87	84.5–97.2	13–16	0
27	6.19–6.69	85.8–94.8	13–15	0
46	6.2–6.62	86–96.8	13–15	0
90	6.2–6.61	85.3–97.3	13–15	0
166	6.24–6.46	88.3–101.3	12–14	0
329	6.24–6.62	87.6–97.3	13–15	0
880	6.24–6.46	89.2–96.4	13–15	0

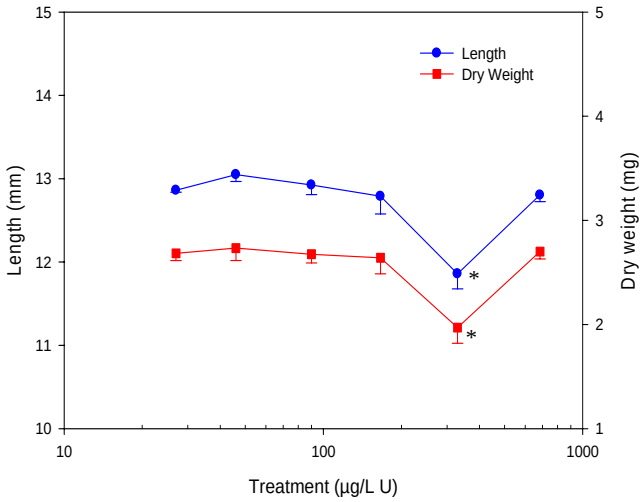

Figure 5.1 Effect of 28 days' exposure to uranium on mean (±SEM) dry weight and length of larval purple-spotted gudgeon (*Mogurnda mogurnda*). Mean control (±SEM) dry weight and length were as follows: dry weight – 2.96 (±0.04) mg; and length – 13.16 (±0.12) mm. *Denotes a significant difference in growth from the control larvae (unexposed) ($P \leq 0.05$).

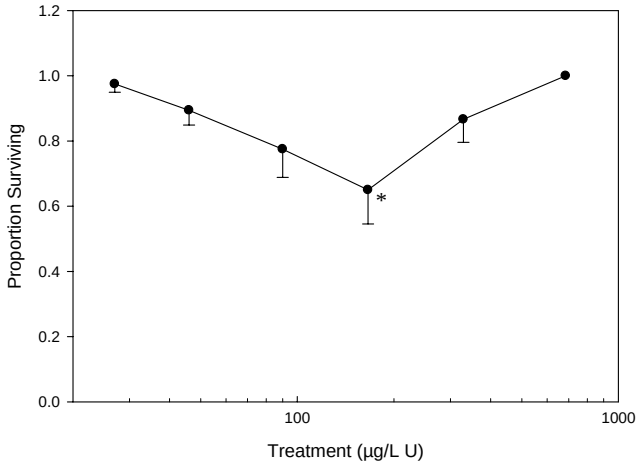

Figure 5.2 Effect of 28 days exposure to uranium on mean (±SEM) survival of larval purple-spotted gudgeon (*Mogurnda mogurnda*) * Denotes a significant difference in survival from control larvae (unexposed) ($P \leq 0.05$).

a) b)

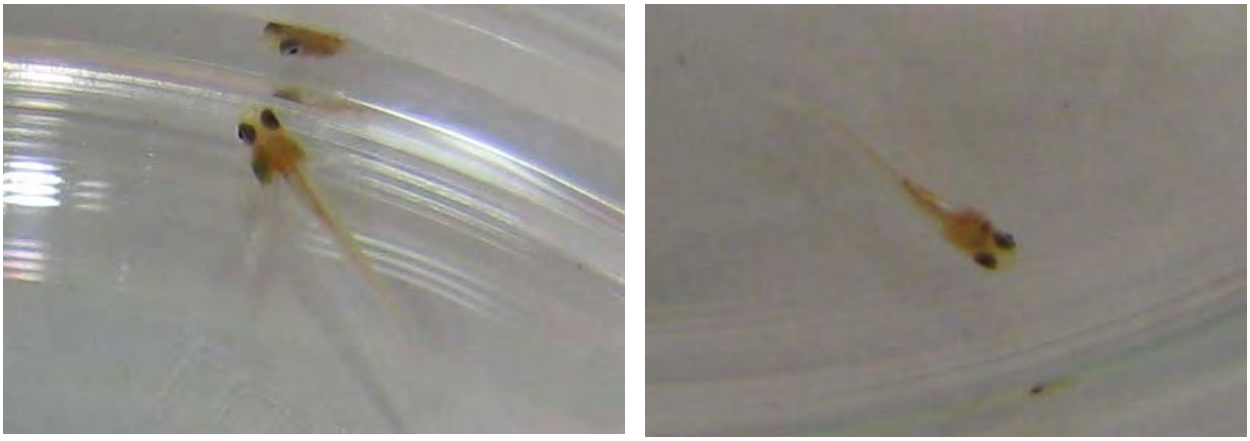

Figure 5.3 a) Larval fish exposed to 166 µg/l U at day 21. b) Larval fish exposed to 329 µg/l U at day 21

a) b)

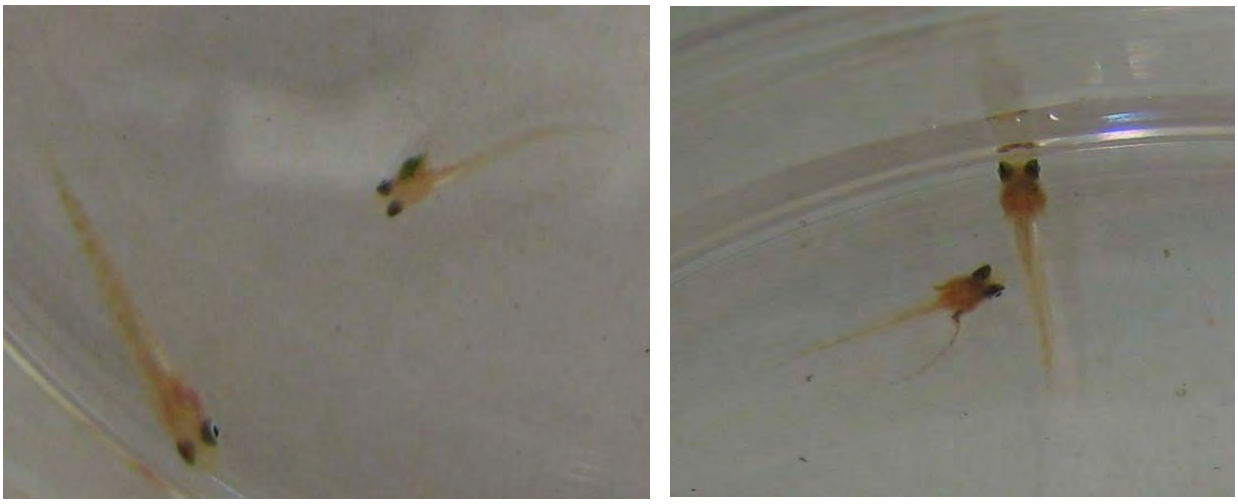

Figure 5.4 a) Larval fish exposed to 166 µg/l U at day 28 b) Larval fish exposed to 386 µg/l U at day 28

5.3.2 Test two

Larval growth is presented in Figure 5.5, while survival is presented in Figure 5.6. Water parameters are presented in Table 5.3. Water quality was within acceptable limits with pH remaining 0.5 units from fresh water parameters and DO remaining above 60% saturated oxygen (See Appendix 1).

M. mogurnda larvae exposed 3,180 µg/L uranium resulted in 100% larval mortality, with all fish dying within the first 24 hours of exposure (Figure 5.6). There was no significant mortality compared to contral larvae (unexposed) observed in any of the other uranium concentrations. Survival for all treatments (excluding 3,180 µg/L U) did not fall below 90%. Observations from the feeding test (Section 4) showed that larval fish needed more food from an early age. Hence, throughout toxicity testing, close observations on eating habits were in

place to ensure that larval fish always received an excess of food to avoid mortality related to starvation.

Larvae exposed to 1400 µg/L U exhibited significant reductions in length (13%) and dry weight (30%) compared to control larvae after 28 d exposure. Length data taken over the duration of the test at days 1, 7, 14, 21, and 28 showed that uranium at this concentration had a significant effect on length by day 14, with the reduction in length being 10%. Larval length of fish exposed to 1,400 µg/L was also significantly lower at days 21 and 28, with reductions being 15 and 13%, respectively (Figure 5.7). Larvae exposed to uranium concentrations below 1,400 µg/L showed no adverse effects to exposure over the test period relative to the control (unexposed) larvae. Based on larval length and dry weight, the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) were 1,400 and 880 µg/L, respectively (Figure 5.5).

Table 5.3 Water quality taken during Toxicity Test 2 (min-max)

Exposure concentration (µg/l U)	pH	Dissolved Oxygen (DO) %	Electrical Conductivity (EC) µS/cm	Ammonia (NH)
0	4.76–6.97	88.5–99	18–27	0
90	5.82–6.94	88.7–97.1	18–21	0
185	6.12–6.94	88.2–96.4	18–21	0
381	6.1–6.93	83.7–99.2	18–21	0
770	6.3–6.93	82.9–97.6	18–21	0
1400	6.26–7.01	87.6–96	18–20	0
3182	6.83	95.4	19	0

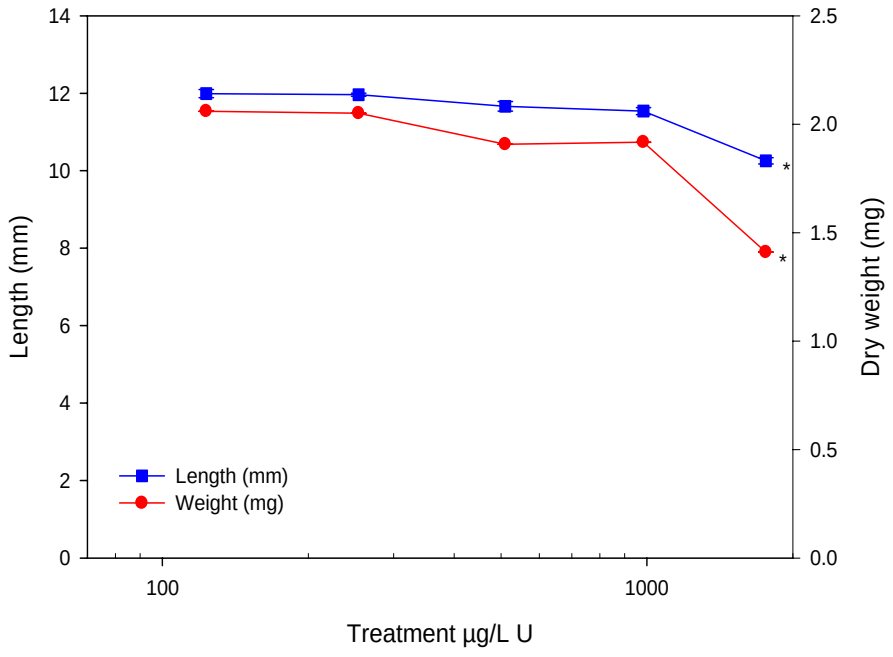

Figure 5.5 Effect of 28 days exposure to uranium on mean (±SEM) dry weight and length of larval purple-spotted gudgeon (*Mogurnda mogurnda*). Mean control (±SEM) dry weight and length were as follows: dry weight – 2.02 (±0.13) mg; and length – 11.83 (±0.16) mm. * Denotes a significant difference in growth from control larvae (unexposed) ($P \leq 0.05$).

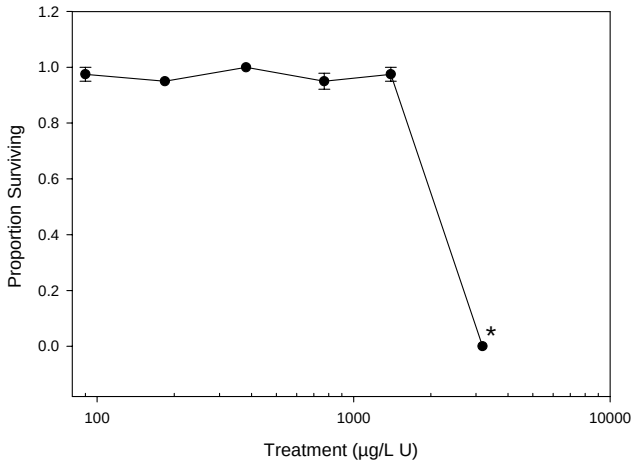

Figure 5.6 Effect of 28 days' exposure to uranium on mean (±SEM) survival of larval purple-spotted gudgeon (*Mogurnda mogurnda*). Mean control response (±SEM) in survival was 98 (±3)% * Denotes a significant difference in survival from control larvae (unexposed) ($P \leq 0.05$).

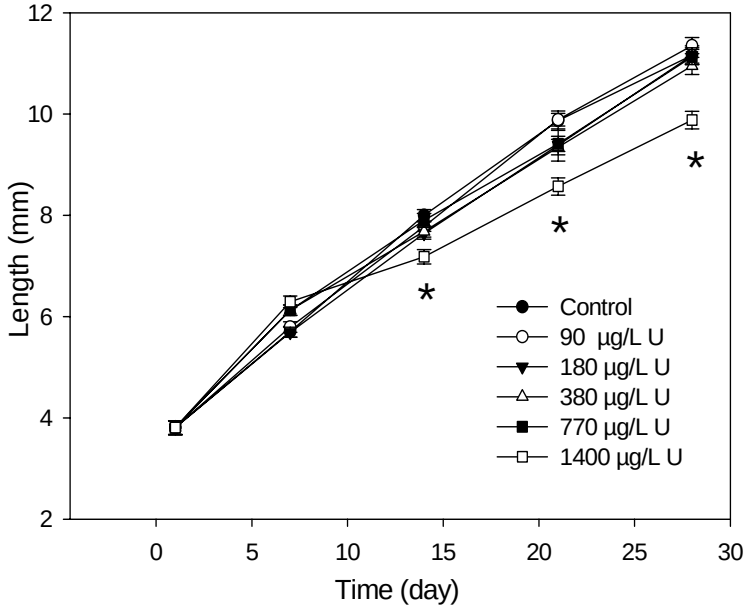

Figure 5.7 Growth data (mean length (±SEM)) over 28 days taken on days 1, 7, 14, 21 and 28. *Denotes a significant difference in length from the control larvae (unexposed) and other treatment concentrations for that time period ($P \leq 0.05$).

Figure 5.8 Larval fish exposed to 1400 µg/L exhibited curvature to the spine at day 14

5.4 Discussion

The results of the toxicity tests completed in this study found that the growth and survival of newly hatched larval *M. mogurnda* were significantly reduced at 1400 μg/L and 3180 μg/L, respectively. Holdway (1992b) exposed 1 d old larval *M. mogurnda* to uranium for 14 d followed by a 15 d post exposure period, reporting a LOEC and NOEC of 1790 μg/L and 880 μg/L, respectively for the 14 d exposure period. He found that exposure at 1790 μg/L resulted in a significant reduction in growth in larval wet weight and length relative to the controls and that during the post exposure period, no significant reduction in size was apparent. Mortality was also significantly higher for larvae exposed to 1400 μg/L over 14 days. The 15 day post exposure period showed delayed mortality with larvae exposed to 880 μg/L and 1400 μg/L. Factors often constraining fish chronic toxicity tests, such as exposure duration, have resulted in the use of early life stage tests (Petocelli 1985). Woltering (1984) reviewed 25 years of fish life cycle toxicity data (chronic, partial and early-life stage) for a variety of hazardous chemicals and found that fry survival was consistently the most sensitive response, with a significant reduction in survival for 57% of the 173 test reviewed. Thirty six percent (36%) of the tests had significant reductions in larval growth, whereas the significant effects on adult survival and growth represented 13 and 5% of the tests reviewed, respectively. While none of the 173 tests reviewed involved uranium as a toxicant, it can be a clear and succinct indication that for a majority of cases, the early life stage of fish are the most sensitive. Exposure to a toxicant during the critical developmental period can induce stress and subsequently change the timing of these developmental events. Hence, the early development period is a key factor in the usefulness of early life stage tests when seeking a short and sensitive toxicity test (McKim 1985).

Sprague (1969) noted that there is a general consensus that acute toxicity to fish occurs in the first 4 days of exposure but this time limit must not be taken arbitrarily, as the acute effects of some toxicants can continue beyond day 4. Sprague (1973) also mentioned that the duration of a toxicity test should be dependant on the toxicity curve, where mortality ceases and a threshold is reached or if this is not the case, that the test should continue until the shape of the toxicity curve is clearly established. In some cases, tolerance increases in larger fish, which have a lower weight-specific metabolic rate that smaller fish, with a two- or three-fold increase in tolerance as fish grow from 1 g to 10 g (Sprague 1990). Larger fish may be more tolerant to a toxicant, which seems to fit a fairly standard explanation that the weight-specific metabolic rate decreases in larger fish and that the smaller fish, with higher metabolic rates, would take up more toxicant. It is also important to note that hydrolytic breakdown of a toxicant in the liver is more effective in larger and mature fish as their physiological development is more advanced than smaller and younger fish (Sprague 1973).

The similarities in sensitivity between life cycle tests and partial life cycle tests on Brook trout and Fathead minnows have been established for copper, methyl mercury, cadmium and lead. Partial life cycle tests were considered to be equivalent to life cycle tests in the development of water quality criteria for aquatic animals (McKim 1985). In the case of *M. mogurnda*, older gudgeons are significantly more tolerant of uranium exposure than younger gudgeons. Significant mortality was observed with 6 d old larvae exposed to 1090 μg/L but not with 40 and 70-day old gudgeons, nor was there any significant effect on growth after the 7-d exposure, 7-d day post exposure period (Holdway 1992b).

Two tests of 173 chronic fish life cycle tests that Woltering (1984) reviewed found that growth was delayed in the early life stages of the Fathead minnow when exposed cadmium, zinc, copper and chromium, although larval fish attained equal size to the controls by the end of the

test. In the second toxicity test, *M. mogurnda* length was significantly reduced by about 10% after 14 d exposure to 1400 µg/L, although the extent of this growth effect did not increase dramatically by day 28, where the reduction in length was 13%. Growth may eventually reach a stage where it is no longer statistically different at a later stage of their life, although this was not apparent over the 28 d test duration. Larval fish exposed to 1400 µg/L (see 5.3.2) exhibited curvatures (Figure 5.8) to their spines and they remained on their sides during the first week whereas the control group were all upright and swimming by days 2-3. However, the effects of uranium appeared to lessen over the final 14 days of exposure, with some of their spines straightening slightly. Early development stages are the most sensitive and many toxicologists agree that the toxicity endpoints assessed during these life stages are acceptable indications to the chronic toxicity effects from full life cycle exposures (Woltering 1984).

Bywater at al. (1991) compared the relative acute sensitivities of uranium to five species of fish from Magela Creek at various life stages, and found that *M. mogurnda* was the second most sensitive species with Blue eye (*Pseudomugil tenellus*) being the most sensitive. Those that were found to be progressively less tolerant to uranium were the Mariana's Hardyhead (*Craterocephalus marianae*), Black-striped rainbowfish (*M. nigrans*) and Chequered rainbowfish (*M. splendida. inornata*).

Liber et al (2004) assessed the chronic toxicity of uranium to the White Sucker Fry (approximately 40 day old). The white sucker fry was found to be relatively tolerant of chronic uranium exposure, with no significant mortality in the highest test concentration (27.86 mg/L) over the 30 d exposure. There were significant sub-lethal effects observed at this concentration but not at the concentration below it (7.33 mg/L). It is important to note however, that the fry were ~40 days old at first exposure and this may have been the reason for the higher tolerance to uranium exposure when compared to *M. mogurnda* exposed to uranium in this study.

The sensitivity of uranium toxicity for five local species from four taxonomic groups are *M. splendida. inornata* > *M. mogurnda* > *Hydra viridissima* > *Chlorella* sp. > *Moinodaphnia macleayi*. A site specific guideline trigger value for uranium in Magela Creek was derived using new and historic toxicity data for uranium from these five local species. Currently the trigger value for uranium is 6 µg/L. This value was derived using a log-logistic distribution of the available NOEC dataset with most recent NOEC for *Chlorella* sp. established by Hogan et al. (2005) and is predicted to protect 99% of species. This value replaces all other interim uranium trigger values derived for Magela Creek and will be reviewed again when more site-specific toxicity data become available.

Table 5.4 Comparison of uranium toxicity for *M. mogurnda* from this present study to Holdway (1992)

	This Study	Holdway (1992)
Exposure Period	28-d	14-d
LOEC µg/l U	1400	1790
NOEC µg/l U	770	880

The comparison of the LOECs and NOECs between this study (Test 2, Section 5) to Holdway (1992) shows that the results from a 28 d exposure period is not more sensitive to shorter term toxicity tests.

The NOEC of 770 ug/L for *M. mogurnda*, obtained from Test 2, is three to four orders of magnitude higher than measured concentrations of uranium in Magela Creek at the

compliance point (GS009) downstream of Ranger (median – 0.05 ug/L, 99th percentile – 0.4 ug/L; data from 2001–2007). Thus, the risk of direct uranium toxicity to fish in Magela Creek is extremely low.

5.5 Summary

A 28 d toxicity test protocol was established based on the results attained from the test development studies (Section 4), and which was based on the OECD Test Guidelines 210 and 215 (OECD 1992; 2000). *M. mogurnda* larval fish were exposed to various uranium concentrations over a period of 28 days. Toxicity test 2 (Section 5. 3. 2) resulted in a LOEC and NOEC of 1400 and 770 µg/L, respectively, based on length and dry weight data. Growth data taken over the duration of the test showed that a significant difference in growth became apparent at day 14 and remained significantly different for the rest of the test period.

6 Summary and conclusions

Although there have been extensive studies on the toxicity of uranium to freshwater fish of the Alligator Rivers Region, the majority has assessed acute toxicity and are not necessarily applicable to use for the derivation of water quality trigger values (van Dam 2004). Furthermore, although the fish toxicity data in the dataset currently used to derive the water quality Limit for uranium downstream of Ranger (ie 7 day mortality for *M. splendida inornata* and 7 day exposure/7 day post-exposure mortality for *M. mogurnda*) meet WQGs criteria as chronic toxicity values, realistically, they cannot be considered as reliable indicators of chronic toxicity. In general, greater confidence in water quality guidelines is gained by incorporating species data derived from longer-term, sub-lethal experiments as opposed to short term experiments measuring lethality.

Such information is important when considering the need to ensure the protection of a World Heritage Area such as Kakadu National Park, particularly while mining is carried out in the Park's catchments. The data produced from these tests (and subsequent follow-up testing as identified below) will provide a more reliable uranium trigger value with respect to the fish component of the aquatic ecosystems, and will also contribute to the wider toxicological database. The final point is important when one considers the quantities of chemical contaminants entering water bodies throughout Australia and internationally, and the need for additional Australian species data to contribute to regional and national water quality guidelines for key toxicants in freshwaters.

When conducting a fish chronic toxicity test, survival of at least 80% in control treatments needs to be attained for the test to be considered valid. All rainbowfish feeding trials did not meet this criterion. It was found that diet and feeding regime was an important factor in rainbowfish survival as larval rainbowfish requires an extensive mixed diet of processed and live food fed several times daily. Six feeding trials were conducted with varied diets. Larval rainbowfish fed a base feed diet of OSI, *Chlorella*, Sera powder and FFV had a survival rate of > 40% at day 7. Survival continued to improve as the extent or complexity of their diet increased. When the base feed was supplemented with live *Paramecium*, survival increased to 38% at day 28. A toxicity test protocol for *M. splendida inornata* could not be developed due to the inability to establish a suitable feeding regime and test conditions that would result in an optimal survival rate (ie ≥80%).

The feeding trial involving *M. mogurnda* resulted in >70% survival at day 28 when fed twice daily at a feeding rate of approximately 10–20, 20–30 and 30–40 nauplii per larva, through days 1–7, 8–25 and 26–28, respectively. Observations made during and subsequent to the test resulted in minor modifications to the feeding regime that resulted in >90% survival of control larvae in toxicity tests.

A 28 d uranium toxicity test protocol was established and the effect of uranium toxicity to *M. mogurnda* was assessed. Larval fish exposed to 3180 μg/L resulted in 100% mortality within the first 24 h. There was no significant mortality at uranium concentrations 1400, 770, 380, 180 and 90 μg/L relative to the controls. However, growth, both in terms of length and dry weight, was significantly lower at 1400 μg/L U compared to the controls and all other uranium treatments with surviving larvae. Consequently, the LOEC and NOEC based on length and weight were found to be 1400 and 770 μg/L, respectively. The significant growth effect at 1400 μg/L was apparent in terms of length at day 14, and did not increase substantially in terms of its extent throughout the remainder of the 28 d exposure period.

The toxicity of uranium to *M. mogurnda* over a 28 days exposure period did not appear to be higher than that previously reported following shorter (ie 7 and 14 day) exposure periods, indicating that the most critical life-stage for the toxic effects of uranium on *M. mogurnda* larvae is within the first two weeks post-hatch.

Further research into the 28 d uranium chronic toxicity to *M. mogurnda* still needs to be assessed as historical fish toxicity data currently incorporated in the dataset used to derive the uranium Limit appear to be reasonably representative of uranium concentrations that will result in longer-term chronic effects. Further data analysis and interpretation will enable a closer examination of this issue. In order to complete this study, an additional 28 day larval growth toxicity test should be undertaken to measure effects on *M. mogurnda* growth between the uranium concentrations of 770 and 3180 μg/L.

Ideally, more research focusing on the development of a toxicity test for *M. splendida inornata* would further increase the confidence in the uranium Limit as fish toxicity data representing two chronic endpoints would be more ideal than one.

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Appendix 1

Water Collections and Test Parameters

Table A1.1. NMCW and water parameters.

Test	Conductivity (μS/cm)		Dissolved Oxygen (%)		pH		Dissolved organic carbon (mg/l)		Alkalinity (mg/l $CaCO_3$)	
	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
Test 1 Ch.4	13	15	106.9	99.9	5.73	5.67	4.5	4.4	9	9
Test 1 Ch.5	14	13	95.7	87.5	6.30	6.31	2.6	3.1	6	6
Test 2 Ch.5	NR	NR	NR	NR	NR	NR	NR	3.4	NR	7

Table A1.2. Test development water parameters

Test	Treatment	Conductivity (μS/cm)	Dissolved Oxygen (%)	pH
Test 1 (Ch. 3)	New water	19-24	79.0-122.5	6.53-7.15
Lower Density	OSI	19-22	77.6-87.8	6.55-6.92
	OSI + Ch	19-22	79.1-92.6	6.52-6.97
	OSI + Ch + Sera	20-24	65.8-101.5	5.45-6.93
	nauplii	22-23	82.4-91.9	6.67-6.75
Higher Density	OSI	19-22	71-88	6.22-6.66
	OSI + Ch	19-21	65.9-93.3	6.26-7.04
	OSI + Ch + Sera	21-26	49-78.2	6.53-6.66
	nauplii	20-24	74.9-80.7	6.53-6.66
Test 2 (Ch. 3)	New water	19-24	88.3-113.2	6.63-7.15
	OSI + Ch +sera	20-23	66.1-93.7	6.21-6.87
	OSI + Ch + sera + FFV 50	21-25	65.2-94.9	6.23-6.93
	OSI + Ch + sera +FFV 100	21-28	64.9-90.6	6.22-6.89
	OSI + Ch + sera + Aqua 100	375-654	2.3-10	6.06-6.19
	OSI + Ch + sera + Aqua 200	700-2450	0.8-5	5.8-6.16
	FFV 50	20-22	79.9-86.7	6.2-6.87
	FFV 100	21-26	74.9-86.7	6.75-6.95
	H: Aqua 100	406-2650	2.1-15.2	5.96-6.16
	I: Aqua 200	453-1001	1.4-3.8	5.94-6.13

Test 3 (Ch. 3)	New water	18-21	94.5-110.3	6.63-6.89
	OSI Ch Sera	19-20	62.8-92.3	6.41-6.69
	OSI Ch Sera FFV (BF)	19-24	62.3-93.9	6.43-7.11
	OSI Ch Sera Para	18-21	60.9-93	7.37-7.14
	OSI Ch Sera FFV Para	21-25	62.2-91.1	6.45-7.05
Test 4 (Ch. 3)	New water	18-20	93.2-118.1	6.42-6.8
	BF + Para(100)	17-23	77.8-99.3	6.53-6.92
	BF + Para(300)	17-23	83.2-97.5	6.56-6.93
	(BF + Para)/2	17-21	87.3-95.9	6.63-6.96
	(OSI Ch Ser FFV)/2 Para(300)	17-22	86.8-95.4	6.59-6.93
Test 5 (Ch.3)	New water	17	100.3-101.3	6.69-6.56
	BF + Para (150)	17-18	89.9-94.2	6.61-6.74
	BF + Para (450)	17-18	91.8-93.4	6.62-6.76
	OSI Ch Sera FFV Para (900)	17-18	93.0-94.6	6.69-6.76
	BF + Para(1800)	17-18	91.1-93.8	6.70-6.74
Test 6 (Ch. 3)	New water	16-17	100.2-123.0	6.3-6.59
	BF + Para (150)	17-19	92.2-117.6	6.46-6.69
	BF + Para (450)	17-20	90.3-117.3	6.60-6.76
	BF + Para (900)	18-22	89.3-116-3	6.63-6.72
	BF + Para(1800)	18-26	88.9-116-3	6.63-6.87
Test 1 (Ch. 4)	New water	12-13	91.9-110.9	5.34-6.5
	(A) nauplii x 1	13-16	87-8-102.3	5.4-6.31
	((A)/2)2	13-16	83.5-99.3	5.64-6.19
	((A)(2)2)	13-16	85.1-97	5.69-6.21

Table A1.3. Test water parameters

Test	Nominal U concentration (μg/l U)	Conductivity (μS/cm)	Dissolved Oxygen (%)	pH
Test 1 (Ch. 5)	0	13-16	84.5-97.2	6.07-6.87
	27.5	13-15	85.8-94.8	6.19-6.69
	55	13-15	86-96.8	6.2-6.62
	110	13-15	85.3-97.3	6.2-6.61
	220	12-14	88.3-101.3	6.24-6.46
	440	13-15	87.6-97.3	6.24-6.62
	880	13-15	89.2-96.4	6.24-6.46
Test 2 (Ch. 5)	0	18-27	88.5-99	4.76-6.97
	110	18-21	88.7-97.1	5.82-6.94
	220	18-21	88.2-96.4	6.12-6.94
	440	18-21	83.7-99.2	6.1-6.93
	880	18-21	82.9-97.6	6.3-6.93
	1760	18-20	87.6-96	6.26-7.01
	3500	19	95.4	6.83

Appendix 2

Nominal and Measured Uranium Concentrations

Table A2.1. Nominal and measured uranium concentrations for U exposure tests.

Test	Time	Nominal Uranium Concentration (μg/l)	Measured Uranium Concentration (μg/l) Totals	Measured Uranium Concentration (μg/l) Dissolved
Test 1 (Ch. 4)	t0	50	48.3	44.3
		500	466.9	483.1
	t2			
	t4			
	t6			
Test 1 (Ch. 5)	Test solution batch 1	0	0.03885	
		27.5	30.03	
		55	61.32	
		110	121.8	
		220	254.1	
		440	483	
		880	936.6	N/R
	Test solution batch 2	0	0.06615	0.03045
		27.5	32.655	24.675
		55	66.15	47.145
		110	134.4	86.94
		220	257.25	164.85
		440	514.5	327.6
		880	1010.1	678.3
	Test solution batch 3	0	0.0798	0.13736
		27.5	37.905	29.29
		55	61.215	44.945
		110	127.05	93.627
		220	243.6	167.66
		440	484.05	331.28
		880	1010.1	691.85
Test 2 Ch. 5	Test solution batch 1	0	0.0168	0.01414
		110	122.85	85.345
		220	253.05	183.82
		440	501.9	378.75

		880	934.5	745.38
		1760	1890	1515
		3520	3832.5	3181.5
	Test solution batch 2	0	0.0168	0.01616
		110	120.75	92.92
		220	258.3	189.88
		440	513.45	385.82
		880	1016.4	783.76
		1760	1354.5	1080.7
	Test solution batch 3	0	0.021	0.02424
		110	126	90.496
		220	250.95	178.77
		440	514.5	377.74
		880	1000.65	775.68
		1760	2037	1595.8
Test.. (adsorption to test container) (Chapter…)	t0	220	175.35	119.18
		3520	3591	3141.1
	t24	220	163.8	112.11
		3520	3622.5	3242.1

Appendix 3

Statistical analyses

Table A3.1. Test 1 (Section 5) One-way ANOVA survival summary

Larval Fish Growth and Survival Test-Percent survival				
Start Date:	Test ID: 803M		Sample ID:	Survival
End Date:	Lab ID:		Sample Type:	
Sample Date:	Protocol: 28D CHRONI		Test Species:	MMO-Mogurnda mogurnda
Comments:				

Conc-%	1	2	3	4
MCW	1.1851	1.1851	1.1851	1.1851
27.5	1.1393	1.1851	1.1851	1.1851
55	1.1393	1.0811	1.1851	1.1393
110	1.0918	1.0426	1.0918	1.0918
220	0.8809	0.9913	1.0426	1.1393
440	1.1393	1.1851	1.0257	1.1393
880	1.1851	1.1851	1.1851	1.1851

			Transform: Untransformed					Rank	1-Tailed
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical
MCW	1.1851	1.0000	1.1851	1.1851	1.1851	0.000	4		
27.5	1.1737	0.9903	1.1737	1.1393	1.1851	1.953	4	16.00	10.00
55	1.1362	0.9587	1.1362	1.0811	1.1851	3.753	4	12.00	10.00
*110	1.0795	0.9109	1.0795	1.0426	1.0918	2.281	4	10.00	10.00
*220	1.0135	0.8552	1.0135	0.8809	1.1393	10.620	4	10.00	10.00
440	1.1224	0.9470	1.1224	1.0257	1.1851	6.054	4	12.00	10.00
880	1.1851	1.0000	1.1851	1.1851	1.1851	0.000	4	18.00	10.00

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.85884	0.896	-0.4495	3.60388
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	55	110	77.7817	1.81818
Treatments vs MCW				

Table A3.2. Test 1 (Section 5) One-way ANOVA growth data (length d 28) summary

Larval Fish Growth and Survival Test-Length

Start Date:	Test ID: 803M	Sample ID:	Final day length (mm)
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4	5	6	7	8	9	10
MCW	13.100	13.200	13.200	13.200	13.300	14.600	13.600	12.700	12.300	13.000
MCW	11.900	11.700	13.100	13.000	13.300	12.400	14.300	13.300	13.100	13.500
MCW	13.200	13.500	12.100	12.800	12.500	12.400	13.300	13.800	13.800	13.500
MCW	12.800	14.900	13.300	11.100	13.600	13.000	13.000	13.600	13.400	15.100
27	14.200	13.300	12.600	12.500	13.500	13.900	11.800	12.600	10.800	14.400
27	12.800	13.900	11.800	11.000	11.400	13.400	14.000	13.000	11.200	13.000
27	12.800	14.000	13.500	11.900	12.400	13.900	13.900	12.400	13.500	12.800
27	13.100	11.400	12.000	12.600	13.200	14.200	13.000	13.100		
55	12.400	12.700	12.600	13.400	14.000	13.800	13.100	12.200	14.300	12.300
55	13.500	13.300	12.800	12.300	13.300	12.800	13.400	13.100	12.500	12.300
55	12.800	13.200	14.100	12.500	13.200	13.000	11.900	13.700	13.900	13.300
55	13.400	11.200	13.400	13.500	13.300					
110	13.800	13.600	13.600	13.000	11.800	12.900	14.100	11.200	11.700	13.100
110	13.000	12.500	13.800	12.700	12.600	14.000	12.900	12.400	13.000	12.600
110	12.400	12.700	11.700	13.700	13.900	13.300	13.400	11.200	13.400	13.500
110	13.300									
220	9.900	13.300	11.900	13.800	10.400	11.900	15.500	13.300	12.500	13.000
220	12.500	13.800	13.500	13.400	13.000	14.000	12.500	11.900	13.400	14.200
220	14.000	13.000	12.600	13.100	11.400	12.700				
440	12.100	12.100	11.100	11.800	9.300	10.200	11.000	13.000	12.000	9.600
440	11.500	11.000	12.900	12.100	13.500	13.200	12.000	10.600	13.600	13.000
440	12.400	13.200	11.500	10.200	9.700	12.500	13.700	13.700	13.300	10.900
440	10.100	13.100	12.100	11.100						
880	13.400	12.700	12.700	11.600	11.300	11.500	11.900	12.800	14.500	13.400
880	12.000	13.000	12.100	13.900	11.500	10.700	13.500	14.200	13.400	14.100
880	14.300	12.000	12.100	12.200	11.600	14.000	14.200	13.000	13.500	11.500
880	12.700	11.800	11.300	11.900	13.700	13.500	13.300	14.400	13.000	14.000

			Transform: Untransformed					Rank	1-Tailed		
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical		
MCW	13.163	1.0000	13.163	11.100	15.100	5.969	40				
27	12.863	0.9773	12.863	10.800	14.400	7.484	38	1384.50	1262.00		
55	13.043	0.9909	13.043	11.200	14.300	5.121	35	1285.00	1105.00		
110	12.929	0.9823	12.929	11.200	14.100	6.140	31	1043.50	910.00		
220	12.865	0.9774	12.865	9.900	15.500	9.173	26	805.50	689.00		
*440	11.856	0.9007	11.856	9.300	13.700	10.858	34	881.50	1054.00		
880	12.805	0.9728	12.805	10.700	14.500	8.225	40	1481.50	1371.00		

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Kolmogorov D Test indicates normal distribution (p > 0.01)	1.0027	1.035	-0.3367	0.01025
Bartlett's Test indicates unequal variances (p = 1.44E-03)	21.5848	16.8119		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Wilcoxon Rank Sum Test	220	440	311.127	0.45455
Treatments vs MCW				

Table A3.3 Test 1 (Section 5) One-way ANOVA growth data (dry weight d 28) summary

Larval Fish Growth and Survival Test-Dry Weight

Start Date:	Test ID: 803M	Sample ID:	Dry Weight (mg)
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4
MCW	3.0050	2.8640	2.9480	3.0540
27	2.5733	2.8711	2.7110	2.5820
55	2.7833	2.5100	2.5930	3.0478
110	2.7300	2.4929	2.5975	2.8800
220	2.2050	2.7083	2.9286	2.7222
440	1.6067	2.1370	1.8517	2.2867
880	2.4940	2.7540	2.7360	2.8200

			Transform: Untransformed					1-Tailed		
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD
MCW	2.9678	1.0000	2.9678	2.8640	3.0540	2.750	4			
27	2.6844	0.9045	2.6844	2.5733	2.8711	5.197	4	1.882	2.451	0.3692
55	2.7335	0.9211	2.7335	2.5100	3.0478	8.733	4	1.555	2.451	0.3692
110	2.6751	0.9014	2.6751	2.4929	2.8800	6.264	4	1.943	2.451	0.3692
220	2.6410	0.8899	2.6410	2.2050	2.9286	11.648	4	2.169	2.451	0.3692
*440	1.9705	0.6640	1.9705	1.6067	2.2867	15.342	4	6.621	2.451	0.3692
880	2.7010	0.9101	2.7010	2.4940	2.8200	5.281	4	1.771	2.451	0.3692

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.96978	0.896	-0.3356	0.00907
Bartlett's Test indicates equal variances (p = 0.39)	6.34289	16.8119		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs MCW	220	440	311.127	0.45455	0.36921	0.12441	0.37979	0.04537	1.0E-04	6, 21

Larval Fish Growth and Survival Test-Percent survival

Start Date:	Test ID: 834M	Sample ID:	Survival
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4
MCW	0.8207	0.6863	0.6088	0.6863
110	0.6088	0.6944	0.6088	0.6863
220	0.6088	0.6863	0.6088	0.6844
440	0.6108	0.6088	0.6088	0.6088
880	0.6088	0.6088	0.6088	0.6088
1760	0.6088	0.6088	0.6088	0.6088

			Transform: Untransformed					Rank	1-Tailed
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical
MCW	0.7005	1.0000	0.7005	0.6088	0.8207	12.573	4		
110	0.6495	0.9273	0.6495	0.6088	0.6944	7.264	4	16.00	10.00
220	0.6471	0.9237	0.6471	0.6088	0.6863	6.832	4	14.00	10.00
440	0.6093	0.8698	0.6093	0.6088	0.6108	0.168	4	12.50	10.00
880	0.6088	0.8691	0.6088	0.6088	0.6088	0.000	4	12.00	10.00
1760	0.6088	0.8691	0.6088	0.6088	0.6088	0.000	4	12.00	10.00

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.86694	0.884	0.74415	3.54327
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test Treatments vs MCW	1760	>1760		0.05682

Table A3.4. Test 2 (Section 5) One-way ANOVA survival data summary

Larval Fish Growth and Survival Test-Percent survival

Start Date:	Test ID: 834M	Sample ID:	Survival
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4
MCW	0.8207	0.6863	0.6088	0.6863
110	0.6088	0.6944	0.6088	0.6863
220	0.6088	0.6863	0.6088	0.6844
440	0.6108	0.6088	0.6088	0.6088
880	0.6088	0.6088	0.6088	0.6088
1760	0.6088	0.6088	0.6088	0.6088

			Transform: Untransformed					Rank	1-Tailed
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical
MCW	0.7005	1.0000	0.7005	0.6088	0.8207	12.573	4		
110	0.6495	0.9273	0.6495	0.6088	0.6944	7.264	4	16.00	10.00
220	0.6471	0.9237	0.6471	0.6088	0.6863	6.832	4	14.00	10.00
440	0.6093	0.8698	0.6093	0.6088	0.6108	0.168	4	12.50	10.00
880	0.6088	0.8691	0.6088	0.6088	0.6088	0.000	4	12.00	10.00
1760	0.6088	0.8691	0.6088	0.6088	0.6088	0.000	4	12.00	10.00

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.86694	0.884	0.74415	3.54327
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	1760	>1760		0.05682
Treatments vs MCW				

Table A3.5. Test 2 (Section 5) One-way ANOVA growth (length d 7) data summary

Larval Fish Growth and Survival Test-Length

Start Date:	Test ID: 834M	Sample ID:	Growth d 7(mm)
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4	5	6	7	8	9	10
MCW	6.0900	5.5100	5.6400	6.6500	6.5000	6.0600	6.2600	5.6100	5.3800	5.7200
MCW	5.6600	4.5600	6.3200	6.0300	5.9900	4.4800	5.5800	5.6300	6.0900	5.7200
MCW	5.9400	6.1300	4.9300	5.8800	7.0800	6.3000	5.7200	5.5900	5.7500	5.5500
MCW	4.5900	5.0400	5.6100	5.1400	5.5600	6.6600	7.0200	6.9400	5.2100	7.3100
110	6.6000	7.4700	6.1800	5.8200	5.0200	4.6800	6.0100	6.6800	5.1100	4.9700
110	5.8800	5.6600	6.7400	5.3800	5.2800	5.0400	4.9700	5.5200	5.3400	5.5800
110	7.4100	6.7900	6.2500	6.6400	6.0600	6.9300	5.3500	5.7200	5.9300	6.0200
110	5.4100	7.2200	5.4300	6.0800	5.7600	6.0900	5.5800	5.4800	5.4200	5.6300
220	5.4900	5.4900	5.4300	4.5200	5.7400	5.8200	5.1200	6.0900	6.1200	4.5800
220	6.3500	6.6200	6.1100	5.8200	5.8500	5.3000	4.8100	5.9100	5.2300	5.1700
220	7.1100	7.1800	7.3300	6.4300	4.8800	7.0500	6.5200	5.7300	6.5600	5.8900
220	5.8700	6.1600	5.5000	5.4000	4.4800	5.3400	5.9200	6.4900	5.8500	5.7400
440	6.9800	7.6700	7.1900	6.3100	7.2100	7.0500	5.8700	6.2300	8.1700	6.1300
440	6.9000	7.3200	6.7400	6.1200	5.0200	4.9600	6.1600	6.1900	5.1200	5.2200
440	6.5700	6.7300	6.1500	6.0100	6.1600	6.0600	6.0900	5.4200	7.0000	6.4000
440	6.5400	6.1500	7.0100	6.9000	5.5300	6.3900	5.7800	5.8700	5.8500	6.9100
880	6.8100	4.9700	5.5100	6.5700	6.5900	6.5900	5.9700	6.2300	5.7600	5.8200
880	5.9600	5.8300	6.1500	5.7000	6.1600	5.2500	5.4900	5.7900	5.7800	7.1300
880	7.5300	8.0100	6.8800	6.7400	6.0300	6.0300	5.4900	6.0700	4.4100	7.3200
880	6.0300	6.4000	6.4300	5.4700	6.0300	6.8200	6.2200	6.2000	6.3300	
1760	7.6600	7.7200	6.7800	6.5100	6.5900	6.7500	5.8500	6.6100	6.3300	5.8400
1760	6.2100	5.3400	6.1600	5.9300	5.9400	6.1000	4.6200	4.7700	6.3600	5.8600
1760	6.8700	6.9000	5.7100	6.1600	7.3100	6.4700	6.7900	6.1600	5.8800	5.5800
1760	6.9300	7.0300	6.7800	6.5600	5.5300	5.2600	6.1100	5.6300	5.6800	6.5900

			Transform: Untransformed						1-Tailed	
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD
MCW	5.8358	1.0000	5.8358	4.4800	7.3100	11.414	40			
110	5.8783	1.0073	5.8783	4.6800	7.4700	11.883	40	-0.273	2.342	0.3644
220	5.8250	0.9982	5.8250	4.4800	7.3300	12.242	40	0.069	2.342	0.3644
440	6.3520	1.0885	6.3520	4.9600	8.1700	11.306	40	-3.319	2.342	0.3644
880	6.1667	1.0567	6.1667	4.4100	8.0100	11.203	39	-2.114	2.342	0.3667
1760	6.2465	1.0704	6.2465	4.6200	7.7200	10.982	40	-2.640	2.342	0.3644

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Kolmogorov D Test indicates normal distribution (p > 0.01)	0.92405	1.035	0.19177	0.00398
Bartlett's Test indicates equal variances (p = 1.00)	0.28998	15.0863		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test Treatments vs MCW	1760	>1760		0.05682	0.3644	0.06244	2.1529	0.48399	6.9E-04	5, 233

Table A3.6. Test 2 (Section 5) One-way ANOVA growth (length d 14) data summary

Larval Fish Growth and Survival Test-Length

Start Date:	Test ID: 834M	Sample ID:	Growth d 14 (mm)
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4	5	6	7	8	9	10
MCW	7.9500	7.7000	7.5900	8.0700	6.7800	7.2600	6.5600	6.9000	6.2100	7.2400
MCW	8.3700	7.9200	7.7500	6.8100	6.7100	8.1800	8.0200	7.5800	8.2100	8.2800
MCW	7.5600	10.1100	8.9300	9.5100	9.2000	9.1000	9.0000	8.4200	9.1100	7.8000
MCW	9.7000	7.8300	7.9500	7.6500	7.6200	8.2800	8.3400	7.8800	7.2800	
110	9.3500	9.0900	8.2800	8.3500	7.6500	6.8300	7.1000	8.3300	7.0400	8.4500
110	7.5600	8.2300	7.3400	7.1500	8.0700	7.8200	7.8600	7.8500	8.2600	7.7000
110	7.8300	7.4100	7.2100	7.7600	8.6600	7.8500	7.3400	7.6500	7.6500	7.2800
220	7.1300	7.2300	7.6800	7.9300	7.1600	8.6700	7.2300	6.7700	7.1600	6.9900
220	7.2300	7.9700	7.6300	8.1400	7.4700	7.0000	7.7900	6.8300	7.6800	7.5300
220	7.8600	8.5600	7.2200	7.2100	7.7000	8.2000	8.9800	7.8100	7.1600	7.9800
220	8.4700	7.3200	7.7800	7.8900	7.7900	8.4400	7.3100	8.4000	7.2200	7.5500
440	6.2700	8.0700	8.1500	7.7600	7.8400	7.4100	7.3100	5.6900	6.6800	7.8500
440	7.8100	8.2300	8.8400	7.5200	8.1100	8.2900	7.2700	8.2400	8.1800	7.6000
440	8.9000	8.6100	7.8600	8.7600	8.9900	7.7300	9.3000	7.5300	9.4300	7.9300
440	8.0700	8.9100	8.6800	7.8100	7.2600	7.5600	8.3400	6.6900	7.1000	7.6700
880	9.4200	9.0500	9.1600	8.0100	7.8400	8.5300	7.5200	6.7200	8.2000	8.3200
880	8.5100	9.1800	9.3200	9.0300	7.4100	8.3800	8.4700	8.2300	8.3800	6.3700
880	8.8000	9.1400	9.1900	6.7100	8.9700	8.3000	7.6800	7.5600	7.7800	
1760	8.3700	8.4100	7.1900	6.8300	8.1100	7.7300	7.4300	7.6200	7.5500	7.4700
1760	7.8600	7.6400	7.2400	7.0100	6.9800	6.6300	5.8000	7.4700	9.3900	9.9100
1760	7.9400	7.2400	7.0100	6.9800	6.6300	5.8000	7.4700	7.3100	7.6300	8.1500
1760	7.4300	7.3500	6.3400	6.5800	6.4900	5.8500	6.1800	5.8000	6.4900	

Transform: Untransformed

Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N
MCW	7.9836	1.0000	7.9836	6.2100	10.1100	11.088	39
110	7.8317	0.9810	7.8317	6.8300	9.3500	7.592	30
220	7.6518	0.9584	7.6518	6.7700	8.9800	6.999	40
440	7.9063	0.9903	7.9063	5.6900	9.4300	9.963	40
880	8.2821	1.0374	8.2821	6.3700	9.4200	9.918	29
1760	7.2644	0.9099	7.2644	5.8000	9.9100	12.369	39

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Kolmogorov D Test indicates normal distribution (p > 0.01)	0.7726	1.035	0.14714	0.70743
Bartlett's Test indicates unequal variances (p = 9.45E-03)	15.2233	15.0863		

Table A3.7. Test 2 (Section 5) One-way ANOVA growth (length d 21) data summary

Larval Fish Growth and Survival Test-Length

Start Date:	Test ID: 834M	Sample ID:	Growth d 21
End Date:	Lab ID:	Sample Type:	
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda
Comments:			

Conc-%	1	2	3	4	5	6	7	8	9	10
MCW	11.360	11.700	12.680	10.820	11.260	9.280	9.250	11.220	12.450	9.470
MCW	8.950	9.450	10.860	8.170	9.580	11.100	10.320	9.300	10.300	10.640
MCW	9.460	10.800	10.840	10.690	10.380	7.980	11.950	10.110	7.960	11.310
MCW	10.270	9.140	11.710	10.570	10.030	10.150	11.430	7.170	9.770	
110	9.450	11.500	11.360	11.350	9.390	9.870	9.840	9.400	9.580	9.970
110	10.540	10.020	10.260	11.060	11.100	8.670	9.130	9.930	10.920	11.870
110	10.050	11.100	11.110	11.120	11.590	10.980	11.400	11.710	9.940	11.290
110	11.160	9.790	12.180	10.660	12.090	11.450	10.330			
220	11.180	9.570	11.170	10.000	11.200	13.580	11.960	12.150	10.680	9.050
220	9.580	10.590	11.090	10.860	10.820	10.890	8.240	8.530	9.770	12.460
220	10.470	9.660	9.280	10.590	9.870	10.340	10.830	10.820	8.980	11.600
220	10.540	8.490	10.970	8.830	9.390	8.900	9.820			
440	10.860	10.390	9.700	8.960	10.150	8.800	9.040	11.730	9.510	9.420
440	10.530	9.960	9.950	10.820	10.240	10.240	9.700	7.680	10.150	11.710
440	9.740	11.590	10.080	11.420	11.390	10.380	8.430	7.850	10.750	10.070
440	10.240	8.950	9.090	11.460	9.960	13.050	9.360			
880	10.190	10.890	10.220	9.920	10.860	11.020	10.170	10.710	9.970	11.360
880	11.250	11.640	10.650	9.530	9.860	10.670	10.260	10.880	9.920	12.080
880	8.230	8.370	9.250	8.950	9.550	8.460	9.400	8.730	9.980	11.030
880	10.710	11.320	9.400	10.460	10.660	9.730				
1760	8.730	9.810	9.440	9.450	8.530	8.240	8.060	9.880	6.590	9.460
1760	8.590	8.950	10.160	9.290	8.330	7.000	9.240	8.660	9.840	7.900
1760	11.060	11.150	8.910	9.260	10.170	10.750	9.370	8.530	8.130	10.060
1760	9.320	10.040	10.420	9.740	8.390	7.190	7.520	7.260	7.680	7.620

			Transform: Untransformed						1-Tailed	
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD
MCW	10.253	1.0000	10.2533	7.1700	12.6800	12.124	39			
110	10.626	1.0363	10.6259	8.6700	12.1800	8.467	37	-1.481	2.343	0.5896
220	10.345	1.0089	10.3446	8.2400	13.5800	11.473	37	-0.363	2.343	0.5896
440	10.091	0.9841	10.0905	7.6800	13.0500	11.128	37	0.647	2.343	0.5896
880	10.174	0.9923	10.1744	8.2300	12.0800	9.232	36	0.311	2.343	0.5938
*1760	8.968	0.8746	8.9680	6.5900	11.1500	12.571	40	5.210	2.343	0.5781

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Kolmogorov D Test indicates normal distribution (p > 0.01)	0.76585	1.035	-0.0579	-0.0375
Bartlett's Test indicates equal variances (p = 0.32)	5.81627	15.0863		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test Treatments vs MCW	880	1760	1244.51	0.11364	0.57814	0.05639	12.9017	1.2019	3.0E-09	5, 220

Table A3.8. Test 2 (Section 5) One-way ANOVA growth (length d 28) data summary

Larval Fish Growth and Survival Test-Length

Start Date:	Test ID: 834M	Sample ID:	Final day length (mm)	
End Date:	Lab ID:	Sample Type:		
Sample Date:	Protocol: 28D CHRONI	Test Species:	MMO-Mogurnda mogurnda	
Comments:				

Conc-%	1	2	3	4	5	6	7	8	9	10
MCW	11.400	10.900	9.400	11.300	10.100	11.600	11.800	10.600	12.800	12.300
MCW	11.600	11.700	13.300	12.300	13.600	11.500	10.300	11.400	11.600	12.600
MCW	10.700	10.700	12.700	12.100	12.700	12.100	12.700	12.400	13.600	11.800
MCW	11.500	12.300	11.800	10.000	11.700	12.700	12.900	13.800	11.100	
110	12.300	11.200	11.500	12.400	11.300	10.800	11.700	12.700	11.300	12.600
110	12.300	11.000	12.800	11.100	11.900	12.200	10.900	13.100	12.300	12.100
110	12.600	12.300	11.700	13.800	12.200	10.600	12.700	11.100	11.900	12.000
110	11.900	12.700	11.300	11.900	12.000	13.200	11.700	12.800		
220	12.900	11.600	11.200	12.700	11.500	11.200	10.700	12.500	11.100	13.400
220	12.800	12.300	12.000	12.200	12.900	10.700	10.900	11.700	11.700	11.900
220	11.900	11.800	12.200	12.100	12.600	12.600	12.700	10.300	12.300	12.000
220	12.500	12.200	12.500	11.900	10.800	11.600	12.400	12.300		
440	13.100	10.500	11.100	11.300	11.300	11.700	11.600	12.300	10.900	11.100
440	12.500	10.700	11.700	12.100	11.800	12.100	11.700	13.000	12.000	10.500
440	11.500	13.500	12.300	10.700	11.600	13.000	12.200	9.400	9.600	12.200
440	11.500	10.900	11.400	12.900	10.800	13.200	11.200	11.500	12.100	
880	10.900	11.000	11.300	11.700	12.200	11.100	11.900	12.600	12.600	11.000
880	10.900	11.800	10.700	11.500	12.000	11.700	12.600	10.000	11.700	10.600
880	11.100	10.800	11.500	14.400	11.100	12.200	11.000	11.200	9.800	11.600
880	12.400	10.200	11.700	12.000	12.200	12.300	12.400	10.500		
1760	9.800	10.100	10.800	11.100	10.300	10.100	11.000	9.600	9.500	9.900
1760	11.000	9.700	11.300	10.700	10.900	9.600	11.200	7.100	10.000	10.800
1760	10.300	10.300	11.300	9.300	9.700	11.500	11.800	10.500	9.400	10.200
1760	10.000	10.200	9.400	9.500	10.300	10.400	11.300	9.400	10.700	

			Transform: Untransformed						1-Tailed	
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD
MCW	11.831	1.0000	11.831	9.400	13.800	8.701	39			
110	11.997	1.0141	11.997	10.600	13.800	6.108	38	-0.846	2.343	0.462
220	11.963	1.0112	11.963	10.300	13.400	5.991	38	-0.672	2.343	0.462
440	11.654	0.9850	11.654	9.400	13.500	7.974	39	0.904	2.343	0.459
880	11.532	0.9747	11.532	9.800	14.400	7.611	38	1.519	2.343	0.462
*1760	10.256	0.8669	10.256	7.100	11.800	8.312	39	8.044	2.343	0.459

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Kolmogorov D Test indicates normal distribution (p > 0.01)	0.50927	1.035	-0.1879	0.78086
Bartlett's Test indicates equal variances (p = 0.21)	7.10289	15.0863		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test Treatments vs MCW	880	1760	1244.51	0.11364	0.45859	0.03876	16.562	0.74701	4.8E-18	5, 225

Table A3.9. Test 2 (Section 5) One-way ANOVA growth (Dry weight d 28) data summary

Larval Fish Growth and Survival Test-Dry Weight				
Start Date:	Test ID: 834M		Sample ID:	Dry weight data
End Date:	Lab ID:		Sample Type:	
Sample Date:	Protocol: 28D CHRONI		Test Species:	MMO-Mogurnda mogurnda
Comments:				

Conc-%	1	2	3	4
MCW	0.0163	0.0212	0.0199	0.0212
110	0.0178	0.0189	0.0206	0.0208
220	0.0209	0.0184	0.0200	0.0186
440	0.0182	0.0177	0.0193	0.0190
880	0.0179	0.0180	0.0185	0.0182
1760	0.0131	0.0127	0.0154	0.0139

			Transform: Untransformed						1-Tailed	
Conc-%	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD
MCW	0.0196	1.0000	0.0196	0.0163	0.0212	11.745	4			
110	0.0195	0.9935	0.0195	0.0178	0.0208	7.256	4	0.135	2.410	0.0023
220	0.0195	0.9925	0.0195	0.0184	0.0209	5.997	4	0.156	2.410	0.0023
440	0.0186	0.9466	0.0186	0.0177	0.0193	3.907	4	1.110	2.410	0.0023
880	0.0182	0.9255	0.0182	0.0179	0.0185	1.608	4	1.549	2.410	0.0023
*1760	0.0138	0.7005	0.0138	0.0127	0.0154	8.592	4	6.229	2.410	0.0023

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.94142	0.884	-0.8049	1.25868
Bartlett's Test indicates equal variances (p = 0.09)	9.44995	15.0863		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs MCW	880	1760	1244.51	0.11364	0.00227	0.11588	2E-05	1.8E-06	4.6E-05	5, 18

Power and Sample Size (Length)

```
One-way ANOVA
Alpha = 0.05  Assumed standard deviation = 0.2  Number of Levels = 7
          Sample  Target                    Maximum
SS Means    Size   Power  Actual Power  Difference
   2.645       2     0.8      0.999979         2.3
The sample size is for each level.
```

Power and Sample Size (Dry weight)

```
One-way ANOVA
Alpha = 0.05  Assumed standard deviation = 0.11  Number of Levels =
7
          Sample  Target                    Maximum
SS Means    Size   Power  Actual Power  Difference
  0.0578       4     0.8      0.823155        0.34
The sample size is for each level.
```

Appendix 4

Additional information-food types used (section 3)

Aquasonic Ingredients: Shrimp, fish, egg, sodium alginate, sorbic acid, sodium benzoate, vitamin A. (Quality analysis not available).

OSI Ingredients: Whole egg solids, fish mean, wheat flour, Torula dried yeast, fish oils, natural and artificial colours, sodium silico aluminates. (Quality analysis not available).

Quality analysis: Crude protein: 37%, Fat: 28%, Crude fibre: 5%, Phosphorous: 0.8%

Sera Micron (ingredients not available).

Quality analysis: Crude protein: 50.2%, Crude ash: 8.1%, Crude fat: 8.1% crude fibre: 4.2%.