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Reinardy, Helena C.

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RELATING GENOTOXICITY TO DNA REPAIR AND REPRODUCTIVE SUCCESS IN ZEBRAFISH (*Danio rerio*) EXPOSED TO ENVIRONMENTAL TOXICANTS

By

Helena C. Reinardy

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Faculty of Science & Technology

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HELENA C. REINARDY: Relating genotoxicity to DNA repair and reproductive success in zebrafish (*Danio rerio*) exposed to environmental toxicants

ABSTRACT

The potential for environmental toxicants to cause genetic damage (genotoxicity) in organisms is a prominent concern because effects on DNA can compromise reproductive success and survival in organisms. Genotoxicity in male germ cells is of particular concern because damage to DNA in sperm may not be repaired and the consequences of damaged genetic material may be transgenerational (from parent to offspring). An integrated approach across multiple levels of biological organization is necessary to establish linkages between exposure to genotoxicants and subsequent effects at molecular and higher levels of biological organization. This thesis addresses the relation between toxicant-induced genotoxicity and reproductive success in zebrafish, and focuses on a model genotoxicant (hydrogen peroxide) and dissolved metals (radionuclide or non-radioactive forms) under controlled laboratory conditions. Uptake and depuration kinetics of a mixture of radionuclides (^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{109}Cd, ^{110m}Ag, ^{134}Cs, and ^{241}Am) were investigated, and radiation dose estimations were computed to link exposure and bioaccumulation with radiation dose. Cobalt (Co, non-radioactive) was selected as an environmentally relevant toxicant for investigation of genotoxicity and effects on reproductive success with a focus on male fish. Chronic exposure (12-d) to $0 - 25$ mg l^{-1} Co resulted in reduced numbers of spawned eggs, lower fertilization success, and reduced survival of larvae to hatching. In male fish, DNA damage was detected in sperm and genes involved in DNA repair (*xrcc5*, *xrcc6*, and *rad51*) were induced in testes from some Co treatments, generally consistent with reduced reproductive success. No change in expression of repair genes in larvae spawned from parents exposed to Co was observed. Overall, results indicate that DNA damage and induction of DNA repair genes can occur rapidly after exposure to genotoxicants and that, if exposure levels are elevated, negative effects on reproduction can occur. Results are considered with particular focus on implications of male genotoxicity on reproductive success and the potential for transgenerational effects of toxicants.

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Glossary

Ag	silver		HBSS	Hank's buffered saline solution
Am	americium		hpf	hours post fertilisation
BCF	bioconcentration factor		HR	Homologous recombination
BER	Base excision repair		IAEA-MEL	Internaltional Atomic Energy Agency Marine Environment Laboratories
BMF	biomagnification factor			
bp	base pairs			
Bq	Becquerels		manSv	man Sieverts
Cd	cadmium		Mg	magnesium
cDNA	copy DNA		Mn	manganese
Ci	Curies		NCBI	National Center for Biotechnology Information
Co	cobalt			
CR	concentration ratio			
Cs	caesium		NHEJ	Non-homologous end joining
DO	dissolved oxygen			
DCC	dose conversion coefficient		PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid		PU	Plymouth University
DPBS	Dalbecco's phosphate buffered saline		qRT-PCR	Quantitative reverse transcriptase PCR
dpf	days post fertilisation		R or r	Röentgen
dph	days post hatching		Rad	Radiation absorbed dose
DSB	Double strand break		RAPD	Randomly amplified polymorphic DNA
ERICA	Environmental Risk from Ionising Contaminants: Assessment and Management		*rad* genes	radiation genes
			RNA	Ribonucleic acid
eV	Electron Volts		Se	selenium
gadd genes	growth arrest and DNA damage genes		SSB	Single strand break
			Sv	Sieverts
GLM	General Linear Model		xrcc genes	X-ray cross-complementing genes
Gy	Grays			
H_2O_2	Hydrogen peroxide		Zn	zinc
			95 % C.I.	95%confidence interval

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Reinardy, H.C., Teyssie, J-L., Jeffree, R.A., Copplestone, D., Henry, T.B., Jha, A.N. (**2011**). *Uptake, depuration, and radiation dose estimation in zebrafish exposed to radionuclides via aqueous or dietary routes.* Science of the Total Environment. 409: 3771-3779. (Chapter 3).

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*infested with sea lice (*Lepeophtheirus salmonis*). Canadian Journal of Fisheries and Aquatic Sciences. 64 (10): 1360-1369.

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<u>PLATFORM PRESENTATIONS</u>

Reinardy, H.C. Nov 2011. *Stressed fish: from populations to genes*. Internal seminar, Akvaplan-Niva, Tromsø, Norway.

Reinardy, H.C., Henry T.B., and Jha, A.N. 2010. *Effects of Radionuclides in Zebrafish*. Co-ordinating Group for Environmental Radioactivity, annual meeting, Lancaster University. Received presentation prize. Awarded travel grant (PMSP Education Fund).

Reinardy, H.C., Henry, T.B., Jha, A.N. 2009. *Effects of Ionizing Radiation on Zebrafish*. Internal Seminar, Ecotoxicology Research and Innovation Centre, Plymouth University.

Reinardy, H.C., Henry, T.B., Jha, A.N. 2009. *Effects of Radionuclides on Zebrafish.* Internal Seminar, Marine Biology and Ecology Research Group, Plymouth University.

POSTER PRESENTATIONS

Reinardy, H.C., Syrett, J., Jeffree, R.A., Henry, T.B., Jha, A.N. 2011. *Linking DNA damage in sperm with reproductive success in adult zebrafish* Danio rerio *exposed to cobalt (Co^{2+}).* 21st SETAC Europe Annual Meeting, Milan. Awarded student travel grant (UK-SETAC).

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Author's declaration

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Introduction and

Literature Review

1.1 Introduction

An extensive evaluation of potential effects of toxicants on organisms requires investigation of impacts at different levels of biological organization (Mothersill and Seymour, 2012). A need for reproductive and genotoxic endpoints has been highlighted for radionuclides, in particular (Woodhead, 2003; Real *et al.*, 2004), but there is an overall need for increased linkages between genotoxicity and reproductive success. Implications of genotoxicity in germ cells are of concern because of the potential for transfer of damaged genetic material from parents to offspring, affecting future generations through increased genetic alterations (Dubrova, 2003a; Barber and Dubrova, 2006). Investigations into biological effects of radionuclides are focused on 4 endpoints: mortality, morbidity, reproduction, and mutation (Larsson, 2008) and more experimental data on these endpoints are required in most groups of organisms, including fish (Woodhead, 2003; Real *et al.*, 2004; Copplestone *et al.*, 2008; Dallas *et al.*, 2012). By way of focused studies of uptake, bioaccumulation, genotoxicity, DNA repair, and reproductive success in zebrafish, this thesis aims to link these divergent perspectives in order to evaluate the potential for toxicants to induce effects.

Investigation of links among different biological endpoints in response to toxicants involves multiple areas of specialist research, including ecotoxicology, genotoxicology, and reproduction of fishes. Some reviews have considered effects of ionizing radiation and other toxicants on fish and other aquatic organisms (Egami, 1980; Jha, 2004; Handy *et al.*, 2011; Dallas *et al.*, 2012), but there is no review which focuses on genotoxicity and implications on reproduction and potential for long-term transgenerational effects. The objective

of this literature review is to provide a broad and critical evaluation of available information on effects of environmental radionuclides on organisms, with emphasis on genotoxicity and reproduction in fish. The focus is on radionuclides of environmental concern, both from a radiological and non-radioactive (metal) perspective.

1.2 Environmental radionuclides and metals

Radionuclides and metals are ubiquitous in the environment and human activities have led to increased concentrations in numerous areas. Long-lived radionuclides, mainly part of the decay series of ^{238}U or ^{232}Th, were produced when the earth was formed and slow decay rates (half-lives of ^{238}U and ^{232}Th are 4.5 x 10^9 and 1.4 x 10^{10} years, respectively) ensure that they will be present for the considerable future (Valković, 2000a). Sources of natural radionuclides and metals are present in mineral deposits buried deep underground but can come into contact with surface organisms through events such as volcanic eruptions, earthquakes, or mine activities. Historical and current mine works are a major source of metal and radionuclide release into the environment by excavated deposits exposed to weathering and erosion (Valković, 2000b). For example, effluents from an abandoned uranium mine in Portugal led to riverbed sediment loads of up to 950 Bq kg^{-1} of ^{238}U, and elevated levels of ^{230}Th and ^{226}Ra (Carvalho *et al.*, 2007). Another source of radionuclides and metals to the environment is combustion of coal and other fossil fuels, which releases and redistributes trapped radionuclides such as radon, uranium, thorium, and respective decay isotopes (Valković, 2000b). In contrast with radionuclides, non-radioactive metals are more widely released and redistributed by

biogeochemical cycling and biological processes (Garrett, 2000). New sources of metals to the earth are limited to occurrence of meteorites and upwelling from the earth's core (Wood, 2012), and the majority of re-distribution and movement of metals is driven by anthropogenic activities, primarily mining (Rauch and Pacyna, 2009). Local geological differences in mineralogy determines environmental concentrations of metals (Garrett, 2000), and 'natural' concentrations of cobalt, for example, can vary from ng – µg l^{-1} in freshwater (Collins and Kinsela, 2010).

Development of technologies to harness energy from controlled nuclear reactions and generation of 'anthropogenic' radionuclides have led to a global expansion of nuclear power industries (IAEA, 2007). Generation of radionuclides from stable atoms initially began in laboratory experiments in the 20th century and has subsequently grown to an industrial scale. In 2007, there were 30 countries with a nuclear power industry, and Belgium, France, Lithuania, and Slovakia obtain over 50 % of their energy from nuclear reactors (IAEA, 2007). Reliance on nuclear power is projected to increase globally (IAEA, 2006), particularly as energy production from fossil fuels has been linked to global climate change and nuclear power has been proposed as a 'cleaner' alternative source of energy as it produces less carbon emissions (Bickerstaff *et al.*, 2008).

Principal sources of anthropogenic radionuclides into the environment are global fallout, authorised and accidental releases from industry (e.g. nuclear power plants), and military applications. A concern about expansion of nuclear power in the UK, and elsewhere, is the disposal of radioactive waste produced by power plants with least possible environmental impact or risk to humans

(Linsley, 1990; IAEA, 2007). High profile incidents in nuclear facilities such as the nuclear reactor explosion in Chernobyl and the break-down of the cooling system in Fukushima have demonstrated that released radionuclides can be transported across the globe (Parache *et al.*, 2011; Shozugawa *et al.*, 2012). Examples of radionuclides discharged from nuclear power plants are ^{60}Co, ^{90}Sr, ^{99}Tc, ^{134}Cs, ^{241}Pu, and ^{241}Am, and different radionuclides are released as liquid or gases (Copplestone *et al.*, 2001). In addition to radioactive particles, the nuclear power industry is a major source of environmental metals such as cobalt (Blust, 2012), strontium (Chowdhury and Blust, 2012) and uranium (Goulet, 2012).

Nuclear technologies developed from tests (underground, underwater, and atmospheric) that have led to localized and widespread radionuclide contamination (Templeton, 1980; Noshkin *et al.*, 1997). Elevated levels of radionuclides and metals have been reported in organisms in close proximity to fuel processing plants and discharge sites. Increased levels of plutonium in sediment and invertebrates, and above-background levels of ^{239}Pu, ^{241}Am and ^{242}Cm were reported in seaweeds and seagrasses (Noshkin, 1972).

Metals and radionuclides released into the environmental by human activity ultimately arrive in aquatic ecosystems where they are mainly sequestered in sediments. Many radionuclides have low water solubility and mostly associate with particles or sediments (Valković, 2000c; Salbu, 2009; Holmes *et al.*, 2012), remain bound for long periods of time, and are not generally bioavailable to organisms (Zhdanova 1991, from Dobrovolsky 1995). Americium is an example of an environmentally relevant radionuclide that is absorbed in sediments due to its high solid-liquid ratio distribution coefficient, although physical characteristics

are also dependent on environmental conditions such as soil type, temperature, and pH (Beresford *et al.*, 2008a; Das *et al.*, 2009; Gil-Garcia *et al.*, 2009). Mechanical disturbance can release bound radionuclides from sediments and re-suspend them in water, which increases their potential transport and bioavailability (Templeton, 1980; Bird and Evenden, 1996).

Metals and radionuclides discharged into water are affected by physico-chemical environmental factors such as pH (Kligerman, 1980; Koyanagi, 1980; Dobrovolsky and Lyalko, 1995). In addition, distribution and bioavailability will differ if co-discharged as a complex mixture (Schindler *et al.*, 1980; Ebbs *et al.*, 1998), or as they undergo decay (Noshkin, 1972). A general drop of 1 pH unit in soil (from an acid rain-like simulation) has been estimated to increase mobility of radionuclides such as ^{226}Ra and ^{137}Cs by factors of 2 or more (Sheppard and Sheppard, 1988). Acidification can also increase breakdown of larger radioactive aggregates, release individual components, and change or increase their solubility (Dobrovolsky and Lyalko, 1995). Currently, there is scientific debate on the possibility of a reduction in ocean pH due to increased concentrations of atmospheric carbon dioxide (Orr *et al.*, 2005). There is a possibility that a reduction in ocean pH will increase release of radionuclides and metals from a sediment-bound state into the water, or new sources will remain in solution and not bind to sediment (Lacoue-Labarthe *et al.*, 2009). However, predicted changes are relatively modest [approximate reduction in ocean pH from 8.2 to 7.7, dependent on modelled future scenarios, (Orr *et al.*, 2005; Jeffree, 2009)], which may not be within the range required to change the solubility of all metals (Breitbarth *et al.*, 2010). With recent nuclear accidents, an increase in global nuclear industries, issues of nuclear waste, and unknown

future climate conditions (e.g. ocean acidification), potential effects of environmental contaminants, particularly metals and radionuclides, are of immediate concern (Polikarpov, 2001; Mothersill and Seymour, 2012).

1.3   Uptake of radionuclides and metals by fish

Aquatic organisms such as fish can be exposed to metals and radionuclides in water. A controlled release of radionuclides (^{60}Co and ^{134}Cs) into a deep lake to simulate radionuclides leaked from ground water into sediments resulted in high levels in lake whitefish (*Coregonus clupeaformis*) even after levels in the water and sediment were no longer detectable (Bird *et al.*, 1998). This suggests long-term persistence and potential for biological effects beyond initial exposure, due to uptake and bioaccumulation in fish. Potential routes of uptake in fish are through skin (Kleinow *et al.*, 2008) and diet (Boyle *et al.*, 2011; Mustafa *et al.*, 2012). Uptake can also be influenced by environmental effects such as temperature and salinity (Hama-Furukawa and Egami, 1980; Hansen, 1980).

Rate and extent of uptake and bioaccumulation of toxicants in fish are dependent on many factors such as size, dimensions, physiology, and species differences (Jeffree and Teyssie, 2006; Jeffree *et al.*, 2006a). For instance, chondrichthyans were reported to have greater bioaccumulation, especially in skin, compared with actinopterygians (Jeffree and Teyssie, 2006), and bioaccumulation was greater in smaller compared with larger fish (Malek, 1999). Ionic composition (e.g. potassium and calcium) in water and fish can influence epithelial transport of metals and radionuclides that are taken up through the same uptake sites (Srivastava *et al.*, 1990; Srivastava *et al.*, 1994). Bioaccumulation can be measured by calculation of a bio-concentration factor

(BCF, ratio of concentration in fish compared with water, equivalent to concentration ratio, CR), or bio-magnification factor (BMF, ratio of concentration in fish compared with diet), and reported values of BCF and BMF vary among and within studies. BCF values for caesium (either ^{137}Cs or ^{134}Cs) have been reported to be greater than 600 in zebrafish (Srivastava *et al.*, 1994), 3.5 in turbot (*Scophthalmus maximus*) (Jeffree *et al.*, 2006a), 3 in perch (*Perca fluviatilis*) (Malek, 1999), and as low as 0.7 in dogfish (*Scyliorhinus canicula*) (Jeffree *et al.*, 2006a).

Metals and radionuclides can bioaccumulate to different extents within internal organs in fish. Muscle is the main component of fish consumed by humans and therefore bioaccumulation in muscle tissue is of particular interest from a human health perspective. Cs concentrates in soft tissues, including muscle, to a greater extent than in bone or liver (Saxén and Koskelainen, 2002). High muscle affinity of Cs is due in part to its isotopic similarity to potassium that enables it to be taken up via potassium ion channels (Peles *et al.*, 2000), in a similar manner to cobalt (Co^{2+}, or ^{60}Co) can be taken up via calcium channels (Kim *et al.*, 2006). Competition between toxicant and essential ion at uptake channels can inhibit normal ion uptake, as demonstrated by ^{90}Sr and calcium (Smith *et al.*, 2009). Zn-65 has an affinity to melanin-rich tissues such as those in the eye (Buhler, 1968), and ^{210}Po has been reported to bind to liver proteins (Durand *et al.*, 1999), which can result in localised regions of bioaccumulation. In contrast, other radionuclides (e.g. Ra, Th, Sr, Pb, Pu, and Po) show great affinity to bone tissue over skeletal muscle in fish (Noshkin, 1972; Clulow *et al.*, 1998; Pyle and Clulow, 1998). Tritium (^{3}H), however, is uniformly distributed throughout fish tissues (within the tissue water fraction as tritiated water)

(Kirchmann and Dupont, 1980), although differences among tissues have been reported in bivalves (Jha *et al.*, 2005; Jaeschke *et al.*, 2011).

1.4   Effects of ionizing toxicants on fish

1.4.1   Radiation dose estimation

A unique aspect of the study of effects of radionuclides is estimation of dose because, unlike stable metals, dose cannot be inferred directly from concentration. Estimation of radiation dose is a complex process that encompasses the physico-chemical properties of the nuclide in addition to the biology of the organism it comes into contact with (Beresford *et al.*, 2008c). Unstable radioactive particles (radionuclides) emit radiation energy (radio-activity) as they undergo decay by spontaneous transformation, expressed as Becquerels (1 Bq equals 1 transformation per second). Radiation absorbed dose (rad; SI unit Gy = 100 rads) is amount of energy deposited by radioactivity within a tissue, specific to radiation type (e.g. alpha-particles, beta- or gamma-rays) as well as tissue type (IAEA, 2004). Therefore, absorbance of radiation dose is tissue and radionuclide specific, and alpha particles deliver a greater absorbed dose compared with beta radiation or gamma rays. A radiation weighting factor (typically 10 for alpha and 1 for beta emitters) is applied to compare dose among different emitters, termed 'equivalent dose'. Effective dose is the sum of weighted (by application of tissue weighting factors) equivalent doses (because radionuclides can emit multiple types of radiation), calculated in Sieverts (Sv) for human populations. Collective effective dose represents dose received by a human population, termed man-Sieverts (manSv). Estimates of human dose (Sv) are relatively well defined, and different

weighting factors for human internal organs allow for tissue-specific assessment of radiation dose. However, estimation of equivalent dose for non-human organisms (effective dose unit gray, 1 Gy equal to 1 joule kg^{-1}) is less defined and there is a need for greater investigation of environmentally-relevant dose rates in non-human organisms within the environment (IAEA, 1992; Batlle *et al.*, 2011).

There is a large difference among radionuclides in their potential to cause biological effects. Beta particles and gamma rays can pass through tissue, but alpha-particles are larger and are blocked by barriers such as skin or scales. Long-lived radionuclides will continually deliver a radiation dose, but dose will change with formation of daughter (decay) isotopes (Noshkin, 1972). In contrast, low levels of short-lived radionuclides can be used as biomedical tracers without major health implications. All these factors combine to complicate estimations of dose in organisms (Blaylock and Frank, 1980).

1.4.2 Genetic damage and repair

Ionization of molecules caused by transfer of electrons from metals or radionuclides is a principal mechanism of effect of toxicants (Spitz *et al.*, 2004). Cells are approximately 80 % water and ionization of water molecules can result in production of free radicals including reactive oxygen species (e.g. ˙OH) (Ziech *et al.*, 2011). Free radicals are highly reactive and DNA, proteins, lipids, and other molecules can become oxidised. Oxidised DNA can result in mutation, strand fragmentation, and formation of micronuclei from exposure to, for instance, ionizing contamination (Jha, 2004; Dallas *et al.*, 2012). The most severe DNA lesions caused by ionizing toxicants are single- and double-strand

breaks (SSB and DSB, respectively) (Gu *et al.*, 1997; Jha, 2008; Collins, 2009). A dose-dependent increase in single and double strand breaks were reported in zebrafish larvae (5 – 6 dpf) after gamma-irradiation (7200 µGy hr^{-1}, ^{137}Cs) and exposure to alpha-particles (740 µGy hr^{-1}, ^{210}Po) (Knowles, 2002; Jarvis and Knowles, 2003).

DSBs and strand break repair can be induced by both endogenous and exogenous mechanisms. Oxidisation of sugars or bases in the DNA molecule can lead to a SSB in DNA (Cadet *et al.*, 2010). DSBs can be a result of an error in base excision repair (BER) of SSB, in addition to direct radiation or free radicals (Ohnishi *et al.*, 2009; Vandersickel *et al.*, 2010a). DSBs in DNA can lead to cell death and carcinogenesis (Gu *et al.*, 1997; Kobayashi *et al.*, 2008), therefore many repair mechanisms have evolved to repair and reduce strand breaks in damaged cells (Hagmann *et al.*, 1998; Bladen *et al.*, 2007a). Repair processes are initiated after damage is detected and the cell cycle is arrested (Bladen *et al.*, 2005). Strand breaks are primarily repaired via non-homologous end joining (NHEJ) or homologous recombination (HR) pathways (Kobayashi *et al.*, 2008). When homologous ends of single strand breaks are in close proximity, HR repair can utilise the intact sister template (Sonoda *et al.*, 2006) to bind broken ends and repair by DNA synthesis and final ligation (Takata *et al.*, 1998; Kobayashi *et al.*, 2008). NHEJ is more error-prone and involves formation of a complex of proteins to bind free ends of broken DNA and recruit ligation enzymes to re-join the strands (Gu *et al.*, 1997; Jones *et al.*, 2001; Kobayashi *et al.*, 2008).

1.4.3 Transgenerational genetic effects

Transgenerational effects can occur by transfer of damaged genetic material from toxicant-exposed parents to unexposed offspring via gametes (sperm and eggs) (Dubrova, 2003a; Shimada and Shima, 2004). Genetic mutations and chromosomal aberrations can be transmitted from parent to offspring and lesions can accumulate, with more severe effects in non-irradiated offspring than were evident in the original exposed parents (Natarajan, 2006; Ryabokon and Goncharova, 2006). Embryonic malformations in medaka (*Oryzias latipes*) exposed to X-rays were reported to be a result of inherited mutations transmitted through sperm (Ishikawa and Hyodo-Taguchi, 1997). DNA damage in sperm can be transferred to the embryo (McGregor and Newcombe, 1972) due, in part, to reduced capacity for repair in sperm, especially in later stages of spermatogenesis (Egami and Hama-Furukawa, 1980; Shima and Shimada, 1991). A study in a wild population of mosquito fish (*Gambusia affinis*) estimated that individual fish had received over 10 Gy from sediments contaminated with radionuclides (^{106}Ru, ^{137}Cs, ^{60}Co, and ^{90}Sr) since the establishment of Oak Ridge National Laboratory in 1945 (Blaylock and Frank, 1980). Genetic effects linked to chronic radionuclide exposure included increased levels of deleterious and recessive lethal genes (determined by presence of RAPD bands) and DNA strand breaks (single cell gel electrophoresis, 'comet', assay), with overall population-level shifts in genotype frequencies (Theodorakis *et al.*, 1999).

Transfer of genetically impaired material from exposed parent to unexposed offspring (or from exposed cells to later cell generations) can lead to 'genome instability', broadly defined as the phenomenon of genetic effects that are not a

consequence of direct exposure (Little, 2003; Barber *et al.*, 2006; Geigl *et al.*, 2008). Alterations in the genome of un-irradiated cells include chromosome aberrations (chromatid aberrations and structural rearrangements), ploidy changes, micronucleus formation, gene mutations, amplification disruption, cellular transformation, clonal heterogeneity, delayed reproductive cell death, and tandem repeat instability (reviewed in Morgan, 2003; Barber and Dubrova, 2006). In mice (*Mus musculus*), increased DNA strand breaks were reported in the first generation (F1) after parental (F0) irradiation with X-rays (Barber *et al.*, 2006), and in utero irradiation (1 Gy) resulted in elevated rates of mutation and genetic instability (Barber *et al.*, 2009). Chromosome aberrations in irradiated trout sperm (up to 4 Gy of gamma-radiation from a ^{60}Co source) can result in malformations in embryo eyes (McGregor and Newcombe, 1972), and increased mutation frequency was reported in zebrafish sperm following gamma-radiation (^{137}Cs, 10 Gy) resulting in fertilized embryos with elevated levels of mutation (Chakrabarti *et al.*, 1983; Walker and Streisinger, 1983). Genome instability can occur by mutations in regions involved in damage sensing and signal transduction (Niwa, 2006), or DNA damage that is incorrectly restored by repair mechanisms (Little, 2003), and is a critical mechanism of cancer initiation and promotion in mammalian cells (Little, 2003; Loeb *et al.*, 2003; Barber *et al.*, 2006).

1.4.4 Effects of ionizing toxicants on germ cells and embryos

Higher level effects of ionizing toxicants depend on which cells are affected. Germ cells (eggs and sperm) in gonads are of particular importance because effects can directly influence reproductive success and may lead to transgenerational effects (Jha, 2004). In addition, germ cells can be highly

37

sensitive to radiation, with greater levels of damage compared with other cells (Hamaguchi, 1980; Konno, 1980). Accelerated spermatogenesis can result in an increase in malformed sperm in medaka following irradiation (Kuwahara *et al.*, 2003), and stage of sperm development can determine level of effects. Mature stages of spermatogenesis (spermatids and spermatozoa) had higher total estimated mutations compared with earlier stages (spermatogonia) in medaka exposed to 9.5 Gy of ^{137}Cs (Hyodo-Taguchi, 1980; Shima and Shimada, 1991).This could be a result of high levels of cell death in early stages of spermatogensis, or reduced repair mechanisms during spermiogenesis (development of spermatids to spermatozoa (Shima and Shimada, 1991; Hales *et al.*, 2005; Leal *et al.*, 2009).

Gonads, particularly testes, are affected by exposure to radiation. Chronic irradiation of medaka to both X- and gamma-rays resulted in reduction of testicular weight (reduced gonadal somatic index) and reduced numbers of spermatogonia. At lower dose rates, removal from the radiation field resulted in regeneration of spermatogonia and indications of a return to control numbers (Hyodo-Taguchi and Egami, 1969; 1976; Hyodo-Taguchi, 1980). Reduced gonad development and complete sterility were reported in adult medaka reared from fertilised eggs exposed to chronic gamma-irradiation (Egami and Hama-Furukawa, 1980). Reduced spermatogenic tissue and oocyte-like cells were observed in histological sections of zebrafish testes after gamma-irradiation (up to 7200 µGy hr^{-1}) (Knowles, 2002). Also testes degeneration and presence of oocyte-like cells occured in male Chinook salmon (*Oncorhynchus tshawytscha*) exposed (as eggs) to 0.1 Gy day^{-1} (^{60}Co source) (Bonham and Donaldson, 1972).

Effects of ionizing toxicants on female reproduction are less clear. In zebrafish, effects in ovaries were less severe compared with levels of degradation in testes (Knowles, 2002), but ovarian tissue damage was reported in salmon (Bonham and Donaldson, 1972). Fecundity (total number of eggs) can be a measure of effects on females because the number of eggs spawned is dependent on female condition. A mixed exposure to gamma-rays (7400 µGy hr^{-1}) and alpha-particles (740 µGy hr^{-1}) resulted in reduced egg output and reduced egg viability in zebrafish (Knowles, 2002). In a wild population of mosquito fish (*Gambusia affinis*) exposed to environmental levels of radiation up to 109 mGy day^{-1} (radionuclide mix including ^{137}Cs, ^{106}Ru, ^{60}Co, ^{90}Sr, and ^{65}Zn), brood size was higher in irradiated populations, although offspring had higher levels of mortality and developmental abnormalities (Blaylock, 1969). Increased levels of mutation from chronic exposure to environmental radiation may select for individuals with high fecundity and result in large brood sizes (Blaylock, 1969), although the results were not reproducible under laboratory conditions (Trabalka and Allen, 1977).

The sensitivity of germ cells and gonads to ionizing damage can be exploited in fish aquaculture. Sublethal doses of radiation can reduce gonad development, induce sterility, and increase somatic growth, which are highly desirable characteristics for aquaculture and an alternative to other methods of sterility induction (e.g. hormone-induced sterility) (Woodhead and Setlow, 1980). Sperm exposed to low doses of radiation can result in greater effects (e.g. embryonic mortality) compared with sperm exposed to higher doses (termed 'Hertwig effect') (Ijiri, 1980). A dose-dependent reduction in sperm viability can be a result of low-dose exposure, and increased radiation dose can induce

gynogenesis (no male contribution to embryonic DNA resulting in all-female offspring) at higher doses (Ijiri and Egami, 1980), as seen in turbot (Piferrer *et al.*, 2004), catfish (*Rhamdia sapo*) (Valcarcel *et al.*, 1994), and tilapia (*Oreochromis niloticus*) (Don and Avtalion, 1993) exposed to UV irradiation. Exposure of fertilised zebrafish eggs to gamma-rays (^{137}Cs) at the cleavage stage of embryogenesis induced maleness, with a peak in males (95 %) at doses of 4.25 Gy, exposed at 180 min post fertilization (Walker and Streisinger, 1983). Gynogenesis can also be beneficial in aquaculture as it can increase somatic growth rates (Valcarcel *et al.*, 1994; Piferrer *et al.*, 2004).

Embryos and larvae have been the target for studies of ionizing toxicants due to their sensitivity and their often visible developmental abnormalities. Effects can depend on developmental stage and, in general, early embryonic stages are more vulnerable to ionizing damage (Walker and Streisinger, 1983; Hagger *et al.*, 2005; McAleer *et al.*, 2005), although radiosensitivity increased as cellular differentiation advanced in medaka embryos (Hamaguchi, 1980). This is because early developmental stages are particularly vulnerable to induced mutations, apoptosis, and oxidative stress responses (Yabu *et al.*, 2001). Embryonic abnormalities such as twinning and vertebral malformations have been reported in mosquito fish from a lake contaminated with radionuclide-rich effluents (Blaylock and Frank, 1980). Also, in a laboratory study, zebrafish eggs exposed to a radiation dose (from ^{137}Cs and ^{241}Am) had abnormalities that included spinal curvature, shortened body length, inhibition of yolk resorbance, eye abnormalities (micro-opthalmia), and reduced brain volume (Geiger *et al.*, 2006; Yum *et al.*, 2009).

1.5   Conclusions and hypotheses

Genotoxicants including metals and radionuclides can induce genetic damage through oxidative injury to DNA, and effects can result in reduced reproductive success and affected offspring. Because of the importance of successful reproduction to maintain stable populations, an understanding of how reproduction is impacted by toxicants is critical to link molecular mechanisms of toxicology with potential transgenerational effects. There is a lack of studies linking effects of toxicants on different levels of biological organisation (i.e. molecular to individual and population levels). In particular, there is a need to link defined exposures (e.g. concentration-dependent dose delivery for environmental radionuclides) to genetic damage, repair mechanisms, and reproductive success. Establishing linkages among effects at different biological levels is critical to fully evaluate potential effects of toxicants on organisms.

The principal objectives and hypotheses addressed in this thesis are:

1.   To assess the kinetics of uptake, bioaccumulation, and depuration of a mixture of radioactive metals (radionuclides) in adult zebrafish exposed via water or diet, and to estimate radiation dose rates (**Chapter Two**).

> **Hypothesis 1**:   Bioaccumulation of radionuclides in whole body and gonads of adult zebrafish differs depending on whether exposure is aqueous or via the diet.

> **Hypothesis 2**:   Dose estimations of radionuclides differ according to route of exposure (i.e. dietary or aqueous).

2. To establish protocols for non-invasive sub-lethal sperm sampling for assessment of DNA damage in sperm of adult zebrafish (**Chapter Three**).

> **Hypothesis 1**:   Hydrogen peroxide will induce concentration-related damage to DNA (DNA strand breaks) in zebrafish sperm exposed *in vitro*, detectable by single-cell gel electrophoresis (comet) assay.
>
> **Hypothesis 2**:   The frequency of non-invasive sperm sampling from individual zebrafish will affect cell density (sperm count) and DNA damage (DNA strand breaks) in the sperm sample.

3. To assess the expression profiles of key DNA repair genes after *in vivo* exposure of larval zebrafish to hydrogen peroxide, and link assessment of DNA repair to DNA damage (**Chapter Four**).

> **Hypothesis 1**:   Hydrogen peroxide will induce concentration-related DNA damage (DNA strand breaks), detectable by comet assay, in zebrafish larvae exposed both *in vitro* and *in vivo*.
>
> **Hypothesis 2**:   Expression of DNA repair genes will be induced after DNA damage (DNA strand breaks) occurs, and result in reduction in levels of DNA damage over time.
>
> **Hypothesis 3**:   Expression profiles of DNA repair genes over time can be modelled and models can enable comparisons in timing and extent of induction of expression among genes.

4.   To assess the effects of cobalt (as a model metal toxicant) on adult zebrafish, and investigate links between genotoxicity, DNA repair, and reproductive success (**Chapter Five**).

   Hypothesis 1: After detection of concentration-related damage in sperm (DNA strand breaks, comet assay), DNA repair genes will be induced in zebrafish testes exposed to cobalt.

   Hypothesis 2: Reproductive success (egg output, fertilisation success, and larval development) will be affected by chronic exposure to cobalt.

   Hypothesis 3: Expression of DNA repair genes will be affected in larvae spawned from adult zebrafish chronically exposed to cobalt.

2:

General methodological approach

2.1 Test Organism

Fish are ideal model organisms for environmental toxicology because they are sensitive to many toxicants, they have a vital position in aquatic systems and food chains, and their biological responses are comparable with other vertebrates including humans (Di Giulio and Hinton, 2008). Zebrafish have well-characterised molecular, genetic, and developmental mechanisms that make them a suitable and useful model organism to assess environmental effects on aquatic vertebrates (Ankley and Johnson, 2004; Hill *et al.*, 2005; Carvan *et al.*, 2007). However, interpretation of ecotoxicological responses in a model species such as zebrafish must be done with care as laboratory-reared animal populations are generally genetically less diverse than wild, or non-model, organisms, which might affect their responses (Coe *et al.*, 2009; Brown *et al.*, 2012). Laboratory studies with environmentally relevant, non-model, species are not without technical challenges (Hogstrand *et al.*, 2002), and the advantages of organisms that are easy to maintain in the laboratory (Lawrence, 2007) with well characterised genetic and developmental mechanisms and available techniques (Parng, 2005) can outweigh considerations of potential genetic inbreeding or environmental relevance (Brown *et al.*, 2011). Fish, including zebrafish, continue to be a useful model for effects studies of toxicants

such as metals and radionuclides (Hinton and Whicker, 1997; Garnier-Laplace *et al.*, 2000).

2.2 Zebrafish water

The Zebrafish Research and Teaching Facility in Plymouth University is supplied by South West Water Limited, Exeter. Prior to use for fish husbandry or exposures (Chapters 4, 5, and 6), water was left to stand for >24 hr. Standing was constantly aerated, with addition of coral to buffer. Concentrations of key ions were stable (Table 2.1), as was temperature (26 ± 1 s.d. °C), pH (6.7 ± 0.3 s.d.), and dissolved oxygen (92 % ± 3 s.d.). Temperature, pH, and dissolved oxygen were measured daily and ammonium, nitrate, and nitrite were analysed weekly (< 0.02, < 20, and < 0.1 mg l^{-1}, respectively).

Table 2-1: Concentrations (mg l^{-1}) of sodium, calcium, potassium, and magnesium in water used for fish husbandry and exposures in Plymouth University.

	Mean	Min.	Max.
Sodium	7.7	6.8	8.5
Calcium	13.8	12.2	15.6
Potassium	0.7	0.5	1.3
Magnesium	0.9	0.8	1.4

2.3 Use of hydrogen peroxide as model genotoxicant

Hydrogen peroxide is a genotoxicant able to produce DNA strand breaks in exposed cells, primarily though the production of oxygen radical species such as ·OH molecules (Horváthová *et al.*, 1998; Collis *et al.*, 2005). It has been

widely used as a model genotoxicant and positive control in studies detecting DNA damage by the single cell gel electrophoresis (comet) assay (Reeves *et al.*, 2008; Oggier *et al.*, 2011). Despite some concerns of the heterogeneity of cell response to challenges from H_2O_2 (Kruszewski *et al.*, 1994; Fairbairn *et al.*, 1995), it is particularly suited for time-course experiments (Chuang *et al.*, 2002) as it is highly reactive with no residual exposure from precipitation or depuration once replaced with clean water. Therefore, hydrogen peroxide was selected to validate the comet assay and the responses in DNA repair genes after initiation of DNA strand breaks induced by exposure to H_2O_2.

2.4   Selection of exposure toxicants

To investigate uptake and bioaccumulation characteristics in adult zebrafish, a mixture of radionuclides was selected (^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{109}Cd, ^{110m}Ag, ^{134}Cs and ^{241}Am) to reflect varying types of radiation (alpha, beta, and gamma emitting radionuclides) and to compare with similar studies in other fish species (e.g. Brown *et al.*, 1996; Jeffree *et al.*, 2006a,b; Jeffree *et al.*, 2007; Matthews *et al.*, 2008). For investigation of toxicological effects on reproduction and genotoxicity, cobalt was selected to reflect an important but understudied environmental toxicant and to complement the initial study with information on uptake and bioaccumulation characteristics of cobalt in adult zebrafish.

2.5   General statistical approach

Specific statistical methods are covered in each Chapter. In general, differences in endpoints (e.g. reproductive effects, DNA damage effects, and gene expression responses) were tested parametrically (directly or on transformed data) against factors of tank, individual, or treatment. Where means of

unequally balances subsamples (comparison between unequal n) are illustrated to reflect whole population (within tanks) distribution (mean and error), standard error of the mean was used; where all samples are compared (e.g. water tests of equal n) standard deviation was used to compare means (Keppel, 1991; Dytham, 2006).

3:

Uptake, depuration, and radiation dose estimation in zebrafish exposed to radionuclides via aqueous or dietary routes

Hypothesis 1: Bioaccumulation of radionuclides in whole body and gonads of adult zebrafish differs depending on whether exposure is aqueous or via the diet.

Hypothesis 2: Dose estimations of radionuclides differ according to route of exposure (i.e. dietary or aqueous).

Abstract

Understanding uptake and depuration of radionuclides in organisms is necessary to relate exposure to radiation dose and ultimately to biological effects. We investigated uptake and depuration of a mixture of radionuclides to link bioaccumulation with radiation dose in zebrafish, *Danio rerio*. Adult zebrafish were exposed to radionuclides (^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{109}Cd, ^{110m}Ag, ^{134}Cs and ^{241}Am) at tracer levels (< 200 Bq g^{-1}) for 14 d, either via water or diet. Radioactivity concentrations were measured in whole body and excised gonads of exposed fish during uptake (14d) and depuration phases (47d and 42d for aqueous and dietary exposures respectively), and dose rates were modelled from activity concentrations in whole body and exposure medium (water or diet). After 14-d aqueous exposure, radionuclides were detected in decreasing activity concentrations: ^{75}Se > ^{65}Zn > ^{109}Cd > ^{110m}Ag > ^{54}Mn > ^{60}Co > ^{241}Am > ^{134}Cs (range: 175 – 8 Bq g^{-1}). All radionuclides (except ^{54}Mn) reached equilibrium during the uptake phase. Equilibrium also occurred for radionuclides during dietary exposure (except for ^{65}Zn and ^{109}Cd), but the order of radionuclide activity concentration in tissues (Bq g^{-1}) was: ^{65}Zn > ^{60}Co > ^{75}Se > ^{109}Cd > ^{110m}Ag > ^{241}Am > ^{54}Mn > ^{134}Cs (range: 91 – 1 Bq g^{-1}). Aqueous exposure resulted in higher whole body activity concentrations for all radionuclides except ^{60}Co. Route of exposure did not appear to influence activity concentrations in gonads, except for ^{54}Mn, ^{65}Zn, and ^{75}Se, which had higher activity concentrations in gonads following aqueous exposure. Highest gonads activity concentrations (Bq g^{-1}) were for ^{75}Se (211), ^{109}Cd (142), and ^{65}Zn (117), and highest dose rates (µGy h^{-1}) were from ^{241}Am (aqueous, 1050; diet 242). This study links radionuclide bioaccumulation data obtained in laboratory experiments with radiation dose determined by application of a dosimetry modeling tool, an approach that will enable better linkages to be made between exposure, dose, and effects of radionuclides in organisms.

3.1 Introduction

Anthropogenic activities have generated radionuclides for the last 70-80 years (Valković, 2000a), and production of radionuclides is likely to increase with greater demand for energy, and expansion of nuclear industries (IAEA, 2007). Most releases of radionuclides arrive, either directly or indirectly, in aquatic environments, and water bodies and sediments become the ultimate sinks for these materials (Egami, 1980, Jha, 2004). In addition to potentially higher releases, radionuclides from previous releases that have accumulated in sediments can become more bioavailable as a consequence of changes in water chemistry (e.g. acidification) (Koyanagi, 1980; Sheppard and Sheppard, 1988; Dobrovolsky and Lyalko, 1995; Macdonald *et al.*, 2005). Increases in amount and bioavailability of radionuclides in the environment could lead to greater risk to organisms and to humans via the food chain.

Radionuclides can bioaccumulate in the aquatic environment, and studies have been conducted to model the uptake and depuration dynamics of specific radionuclides in various species of fish (Dobrovolsky and Lyalko, 1995; Brown *et al.*, 1996; Jeffree *et al.*, 2007). Dissolved radionuclides are primarily taken up across gill membranes or epithelia of the gastrointestinal tract depending on exposure (aqueous or dietary); and, if exposure is of sufficient duration, equilibria will be established between radionuclides in tissues and in the abiotic environment (Jeffree *et al.*, 2006b; Mathews *et al.*, 2008). Information on uptake/depuration kinetics of some radionuclides is available for some fish and these results can be applied to help understand bioaccumulation of radionuclides in wild fish that inhabit contaminated environments. A particular aspect that is missing from previous investigations of radionuclide

bioaccumulation in fish is the link between exposure, bioaccumulation, and delivered dose. New techniques in dosimetry modeling and dose estimations (Brown *et al.*, 2008; Wood *et al.*, 2009) can now be applied to extend results of bioaccumulation studies to provide estimates of radiation dose received by fish and other organisms exposed to radionuclides either alone or as mixtures.

Radiation dose is a function of the energy level of the radionuclide, the nature of the exposure, and the size and characteristics of the target organism. The dose delivered by a particular radionuclide within an organism is the combination of all its radiation emissions and radiation type(s) (e.g. alpha, beta, gamma), which makes estimation of dose rates and accumulated doses complex. The Environmental Risk from Ionizing Contaminants: Assessment and Management (ERICA) Tool [www.ceh.ac.uk/PROTECT/ERICAdeliverables.html, (Brown *et al.*, 2008)] is a dosimetry model that can estimate internal, external, and total dose rates of selected radionuclides (individual or radionuclide mixtures) within a selected organism, based on organism size and the environmental compartment in which the organism resides. The ERICA Tool was made publicly accessible in 2008 (Larsson, 2008) and has been applied to a variety of environmental contamination situations with several types of organisms (e.g. Beresford *et al.*, 2008b; Stark and Pettersson, 2008; Wood *et al.*, 2008; Wood *et al.*, 2009). However, the application of the ERICA Tool (or any other dosimetry modelling package) to estimate dose rates over time for fish exposed to complex mixtures of radionuclides through multiple routes of exposure has not been reported.

Arguably, the most useful fish model for investigating biological responses to stressors is zebrafish, *Danio rerio,* a common model in ecotoxicological studies

(Hill *et al.*, 2005; Carvan *et al.*, 2007). Clarifying how radionuclide exposure relates to dose in zebrafish will enable interrogation of the effects of radionuclides to be conducted in the context of the radiation dose. Effects of radiation have been investigated in zebrafish and include DNA damage and developmental abnormalities (Jarvis and Knowles, 2003), gene expression changes (Yum *et al.*, 2009), and apoptosis (Yabu *et al.*, 2001). Only two studies have investigated bioaccumulation of radionuclides in zebrafish (Srivastava *et al.*, 1990; Srivastava *et al.*, 1994). In the study of Srivastava *et al.*, (1990), adult zebrafish had high bioaccumulation of ^{137}Cs (bio-concentration factor, BCF, of 600) following an aqueous exposure. There is presently no information on dosimetry or the kinetics of other radionuclides in zebrafish.

The objective was to investigate the uptake and depuration of a mixture of radionuclides (^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{109}Cd, ^{110m}Ag, ^{134}Cs and ^{241}Am) in adult zebrafish exposed via aqueous or dietary routes. In addition to modelling uptake and depuration kinetics of these radionuclides, we used the ERICA Tool to estimate dose rates and accumulated doses received by zebrafish to link radionuclide exposure with dose.

3.2 Methods

3.2.1 Fish

Adult zebrafish (*Danio rerio*) were obtained from Poisson d'Or suppliers (Belgium) and were maintained in a 700-L tank with aerated and charcoal-filtered water within a recirculation system in the Radioecology Laboratory of the Marine Environmental Laboratories (IAEA-MEL), Monaco. For the experimental exposures, fish were transferred into 70-L glass aquaria for each exposure (100

fish tank^{-1}) (Figure 3-1). Water chemistry was checked daily during acclimation and exposure periods and was found within acceptable ranges (temperature 21° ± 0.5, pH 7.9, dissolved oxygen at saturation, ammonia, nitrate and nitrite below detection limit). Fish were fed with live adult brine shrimp (*Artemia* sp.) once a day. Fish exposed through the diet were fed brine shrimp with elevated levels of accumulated radionuclides from previous exposure (Table 3-1). The dietary exposure tank contained six control fish, placed within the same tank water but separated from the rest of the fish by mesh, and fed unlabelled brine shrimp. This was to test for dissolution of the radionuclides from the brine shrimp, and to ensure there was no additional aqueous exposure.

Figure 3-1: Experimental set-up for radionuclide exposure to adult zebrafish.

3.2.2 Selection of radionuclides and exposure scenario

The radionuclide mixture contained the following eight radionuclides: ^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{110m}Ag, ^{109}Cd, ^{134}Cs, and ^{241}Am. The radionuclides were obtained from Isotopes Products (Germany, ^{54}Mn, ^{60}Co, ^{109}Cd, and ^{241}Am), Polatom (Poland, ^{75}Se, ^{110m}Ag and ^{134}Cs), and Cyclotron (Russia, ^{65}Zn). The carrier eluant was 0.1 M HCl for ^{54}Mn, ^{60}Co, ^{65}Zn, ^{110m}Ag and ^{109}Cd, 0.1 M HNO$_3$ for ^{110m}Ag, 1 M HCl for ^{241}Am and H$_2$O for ^{134}Cs and ^{75}Se. For the aqueous exposure the water was spiked with the radionuclide mixture every 1-3 days over the 14-day exposure period. For the dietary exposure, commercially available adult brine shrimp (*Artemia* sp.) were exposed to the radionuclide mixture for 9-days after which they were frozen in batches. Fish were fed the frozen exposure-diet daily. Mean activity concentrations in the water and the brine shrimp are listed in Table 3-1.

Table 3-1: Mean (S.E.M., n = 8 d) activity concentrations in the water and the brine shrimp diet for the aqueous and dietary exposure respectively.

Exposure	Isotope activity concentration (mean ± s.d.)							
	^{54}Mn	^{60}Co	^{54}Zn	^{75}Se	^{110m}Ag	^{109}Cd	^{134}Cs	^{241}Am
Aqueous	0.47	0.51	0.48	0.50	0.49	0.45	0.52	0.19
(Bq ml^{-1})	(0.01)	(0.01)	(0.02)	(0.02)	(0.02)	(0.01)	(0.01)	(0.01)
Dietary	20.8	159.4	188.8	38.0	35.7	61.4	1.9	43.3
(Bq g^{-1})	(0.8)	(6.1)	(7.3)	(1.5)	(1.1)	(2.8)	(0.1)	(1.7)

3.2.3 Determination of tissue activity concentrations

Radionuclide activity concentration in water and fish were determined by a high-resolution gamma-spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors (EDNC 33-195-R, Intertechnique; 40-70% efficiency) that were connected to a multi-channel analyzer and a personal computer employing spectral analysis software (Interwinner 6, Intertechnique), as previously reported (Jeffree *et al.*, 2010). The radioactivity levels were determined by comparison with known standards of appropriate geometry and were corrected for background and isotope physical decay, as previously reported (Jeffree *et al.*, 2010). Fish were randomly sampled from the exposure tanks for live individual radioactivity measurements (9 fish sampled at the start of aqueous exposure, 24 fish sampled at the start of depuration of dietary exposure, and 12 fish sampled at all other time points). Each individual fish was placed in a vial containing 50 ml of clean water with a plastic 'tunnel' in place to restrict movement (Figure 3-2). The vial was placed in a gamma spectrometry system and activity counts were collected after 17 min (range of counting period 15-20 min), after which the fish was returned to the exposure tank. Samples were taken every 1-2 d during the 14-d uptake and beginning of depuration phases for each exposure, then every 7-10 d for the remaining period of depuration. Subsamples of six fish were removed and frozen for later dissection and measurement of gonad activity concentrations.

Bioaccumulation was calculated as bio-concentration factor (BCF) for aqueous exposure, and as bio-magnification factor (BMF) for dietary exposure. BCF is radionuclide activity concentration in tissue (Bq g^{-1}) divided by radionuclide

activity concentration in water (Bq kg^{-1}), at equilibrium. BMF is activity concentration in tissue
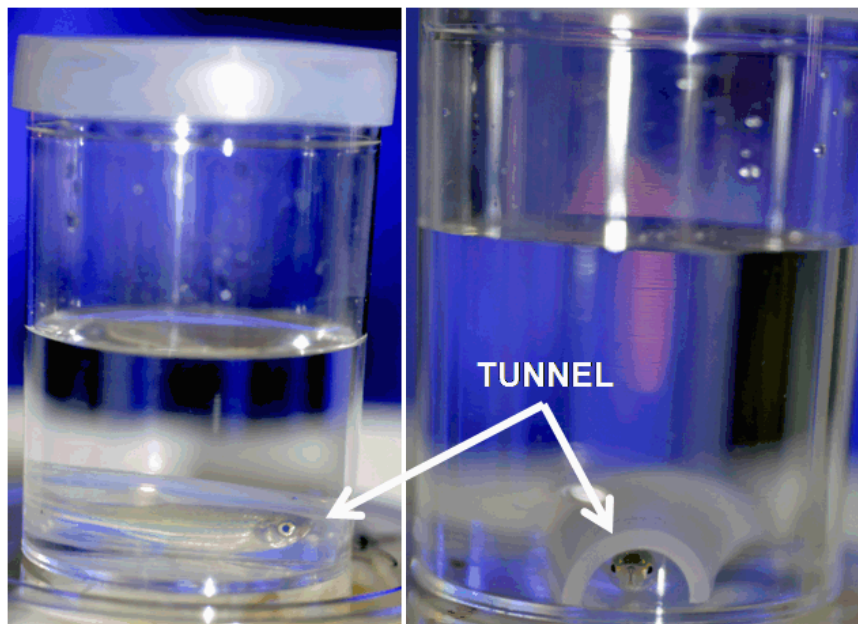
Figure 3-2: Vial containing tunnel for live fish activity measurements.
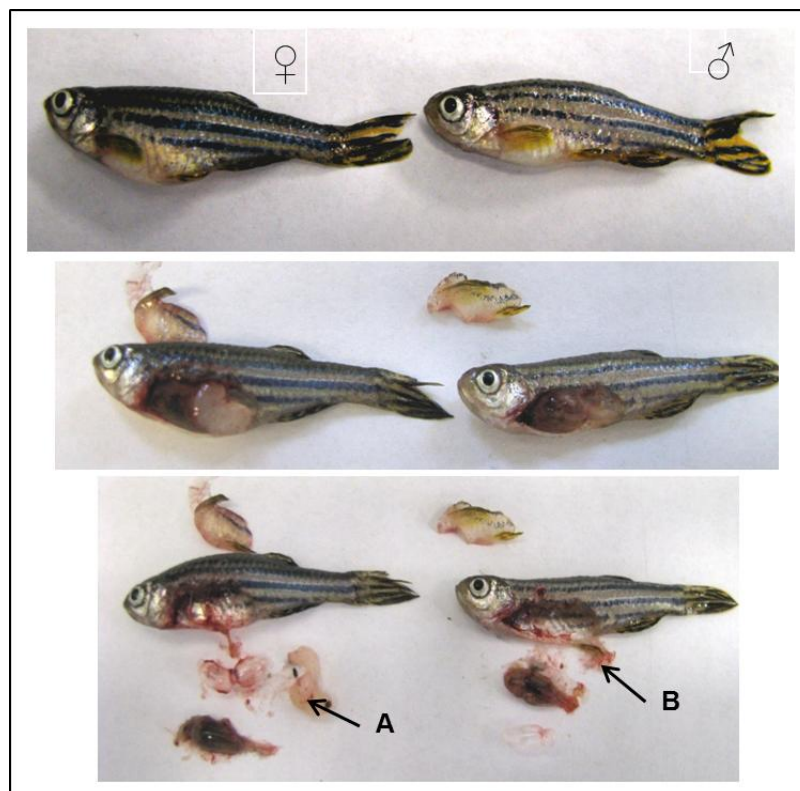
Figure 3-3: Dissection of ovaries (**A**) and testes (**B**) from adult zebrafish.

(Bq g^{-1}) divided by activity concentration in diet (Bq g^{-1}). The amount (%) of radioactivity in the gonads was calculated by dividing total Bq counts in the gonads by the total Bq counts in the whole body.

3.2.4 Dosimetry calculations

The ERICA Tool (www.ceh.ac.uk/PROTECT/ERICAdeliverables.html, Brown *et al.*, 2008) was used to calculate internal, external, and total dose rates, and accumulated doses received by zebrafish after aqueous and dietary exposure (see Appendix 1 for ERICA Tool method development). The ERICA Tool (tier 2) uses dimensions for organisms of interest to calculate dose rate in the whole body. To obtain the dimensions, a 'model' zebrafish was constructed based on measured fish weights applied to regression equations of fish weights and other dimensions (length, height and width). The regressions were calculated from a database of 88 individual adult fish. Model zebrafish dimensions were: weight 0.294 g, length 33.6 mm, width 4.0 mm, and height 6.8 mm. The model zebrafish was set to a freshwater environment with a water occupancy factor of 1 to best match the tank environment of the exposures.

Dose rates for eight isotopes (^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{110m}Ag, ^{109}Cd, ^{134}Cs and ^{241}Am) were calculated by the ERICA Tool (Brown *et al.*, 2008), Tier 2. Zn-65 was added to the default list of isotopes with the 'add isotope' function. The ERICA Tool is designed to model exposure of organisms, including aquatic ecosystems and, as such, calculates the external dose in organisms living in the water column from both water and sediment. However, there was no sediment compartment in the experiment and therefore the distribution coefficient (K_d) was set to zero, to prevent the ERICA Tool from estimating any sediment-derived external dose. By setting the water activity concentration to 1 (sediment

and whole body activity concentration set to zero) the tool calculates weighted dose conversion coefficient (DCC) for each radionuclide for external dose only (giving a DCC value as µGy h^{-1} Bq^{-1} ml^{-1}, Table 3-2). By setting the whole body activity concentration to 1 (sediment and water activity concentrations set to zero) the tool calculates weighted DCC for internal dose (giving a DCC value as µGy h^{-1} Bq^{-1} g^{-1} of body tissue, Table 3-2). The ERICA Tool default radiation weighting factors of 10 for alpha, 3 for low energy beta, and 1 for beta/gamma were used and these are included in the dose estimation to reflect the differing biological effect of the respective types of radiation in tissue.

For the aqueous exposure, external and internal DCCs were applied to water activity concentrations and whole body activity concentrations respectively to give the external and internal dose rates at each time point (µGy h^{-1}). The total dose rate was calculated by adding external dose rate to internal dose rate at each time point. Total accumulated dose (µGy) was calculated by taking different dose rates at different time points, multiplying them by the corresponding hours at that rate, and adding the cumulative total over each time point. The same was done for dietary exposure removing the external dose component to give a single estimate of internal dose rate (there being no external dose rate when exposed through the diet).

Table 3-2: Weighted dose conversion coefficients (DCCs) for internal and external dose rate estimations for selected radionuclides in adult zebrafish (model zebrafish dimensions: 0.294 g, length 33.6 mm, width 4.0 mm, and height 6.8 mm), as calculated by the ERICA Tool.

Isotope	Internal DCC ($\mu Gy\ h^{-1}\ Bq^{-1}\ g^{-1}$)	External DCC ($\mu Gy\ h^{-1}\ Bq^{-1}\ ml^{-1}$)
^{54}Mn	0.01	0.48
^{60}Co	0.06	1.44
^{65}Zn	0.01	0.33
^{75}Se	0.02	0.22
^{110m}Ag	0.05	1.59
^{109}Cd	0.06	0.01
^{134}Cs	0.09	0.90
^{241}Am	31.65	0.02

3.2.5 Kinetics

The radionuclide activity concentration was measured in fish exposed either via the water or the diet, and recorded as Bq g^{-1}. The uptake kinetics were modelled with a single-component first order kinetic model (1):

$$C_t = C_{ss}\,(1 - e^{-k_e t})$$

where C_t and C_{ss} represent activity concentration at time t (d) and at steady state, respectively, and k_e represents biological uptake rate constant (d^{-1}) (Whicker and Schultz, 1982; Jeffree *et al.*, 2006b). If there was no indication of reaching a steady state during the time of exposure (non-significant fit to model 1), a simple linear regression model was applied (2):

$$C_t = k_u t$$

where k_u is the slope of regression (rate of increase in Bq g^{-1} d^{-1}). Depuration after return to clean water or return to normal diet was modelled using either a single-component exponential model (3):

$$A_t = A_0 \, e^{-k_e t}$$

where A_t and A_0 are activity concentrations (Bq g^{-1}) at time t (d) and 0 respectively, and $k_e t$ is biological depuration rate constant, or a double-component exponential model (4):

$$A_t = A_{0s} \, e^{-k_{es} t} + A_{0l} \, e^{-k_{el} t}$$

where A_{0s} and A_{0l} are activity concentrations (Bq g^{-1}) at time t(d) and 0, respectively, for short-lived (s) and long-lived (l) component, and k_e is the biological depuration rate constant (d^{-1}) (Whicker and Schultz, 1982; Jeffree *et al.*, 2006b). To account for the delay in uptake into internal organs, the gonad uptake was modelled from the second sampling point (d 11). Uptake and depuration into the gonads were modelled with the same single-component first order kinetic model for uptake and the single-component exponential model for depuration, fitted to the data means. Parameters and statistics of uptake and depuration models were estimated by iterative adjustments by non-linear exponential rise to maximum and exponential decay functions in SigmaPlot 11.0 (Systat Software, Inc., USA), respectively. Where the significance of the model was not satisfied (p<0.05), the model was not applied to the data. Other statistical analyses were conducted with StatGraphics 5.1 (Statistical Graphics Corp., USA).

3.3   Results

3.3.1   Fish Growth

No fish died during the experiment and no significant differences in fish mass (0.294 ± 0.003 S.E.M., n = 384) or gonad size were observed between exposures over the experimental uptake and depuration period. Ovaries were significantly heavier than testes (mean female gonad weight 0.021 g ± 0.003 S.E.M., n = 48, mean male gonad weight 0.0023 g ± 0.0002, n = 78, Mann-Whitney $p < 0.05$).

3.3.2   Bio-concentration/Bio-magnification

Activity concentrations of radionuclides increased in the whole body following both aqueous and dietary exposure, and whole body activity concentrations were higher in fish exposed to aqueous radionuclides compared with dietary exposed fish for all radionuclides except ^{60}Co (Figure 3-4). Uptake of radionuclides by zebrafish appeared to reach equilibrium during aqueous exposure for all radionuclides except ^{54}Mn, and the highest radioactivity concentrations were for ^{65}Zn and ^{75}Se (120.3 and 175.0 Bq g^{-1} whole body, respectively) at the end of uptake phase (Figure 3-4).

Depuration of radionuclides was defined to begin when fish were transferred to water that was not spiked with radionuclides (aqueous exposure) or when fish were returned to a diet not amended with radionuclides (dietary exposure). For fish exposed to aqueous radionuclides, whole body levels decreased immediately upon initiation of depuration for ^{54}Mn, ^{60}Co and ^{241}Am, while other radionuclides (particularly ^{65}Zn, ^{75}Se, ^{109}Cd and ^{134}Cs) showed slower depuration. Zn-65 decreased slowly during depuration phase, and 40 % of

activity concentration at equilibrium was still present after 60 d. Se-75 also showed slow depuration, and activity levels were 50 Bq g^{-1} (23 % of equilibrium levels) at the end of experiment (d 60).

The order of decreasing activity concentrations in whole body for aqueous exposure was: ^{75}Se > ^{65}Zn > ^{109}Cd > ^{110m}Ag > ^{54}Mn > ^{60}Co > ^{241}Am > ^{134}Cs. In comparison, the order for dietary exposure was quite different: ^{65}Zn > ^{60}Co > ^{75}Se > ^{109}Cd > ^{110m}Ag > ^{241}Am > ^{54}Mn > ^{134}Cs. In gonads the aqueous and the dietary exposures resulted in most radionuclides having equally high activity concentrations, except for ^{75}Se which had up to 200 times greater accumulation in the gonads after aqueous exposure compared with dietary exposure. Maximum level of uptake in gonads was earlier through dietary than aqueous exposure for all radionuclides. The order of decreasing counts in gonads for aqueous exposure was: ^{75}Se > ^{65}Zn > ^{109}Cd > ^{110m}Ag > ^{54}Mn > ^{241}Am > ^{60}Co > ^{134}Cs. In comparison, the pattern for the dietary exposure was different: ^{109}Cd > ^{65}Zn > ^{110m}Ag > ^{75}Se > ^{241}Am > ^{54}Mn > ^{60}Co > ^{134}Cs.
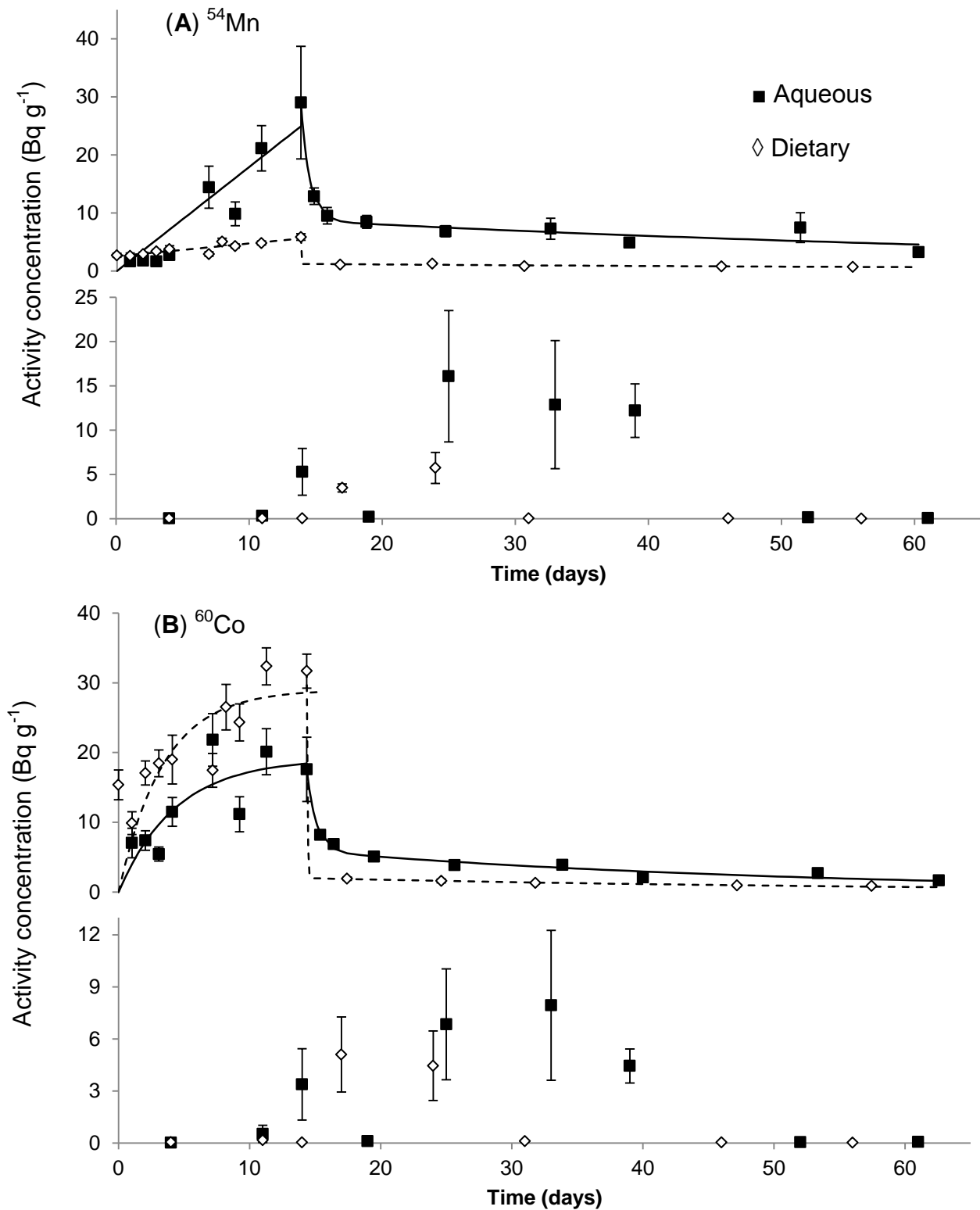
Figure 3-4: Activity concentrations (Bq g⁻¹) in the whole body (upper panels) and in the gonads (lower panels) after aqueous (filled squares) and dietary (open diamonds) exposures. (**A**) ^{54}Mn, (**B**) ^{60}Co, (**C**) ^{65}Zn, (**D**) ^{75}Se, (**E**) ^{109}Cd, (**F**) ^{110m}Ag, (**G**) ^{134}Cs, and (**H**) ^{241}Am.

Figure 3-4 cont.

64
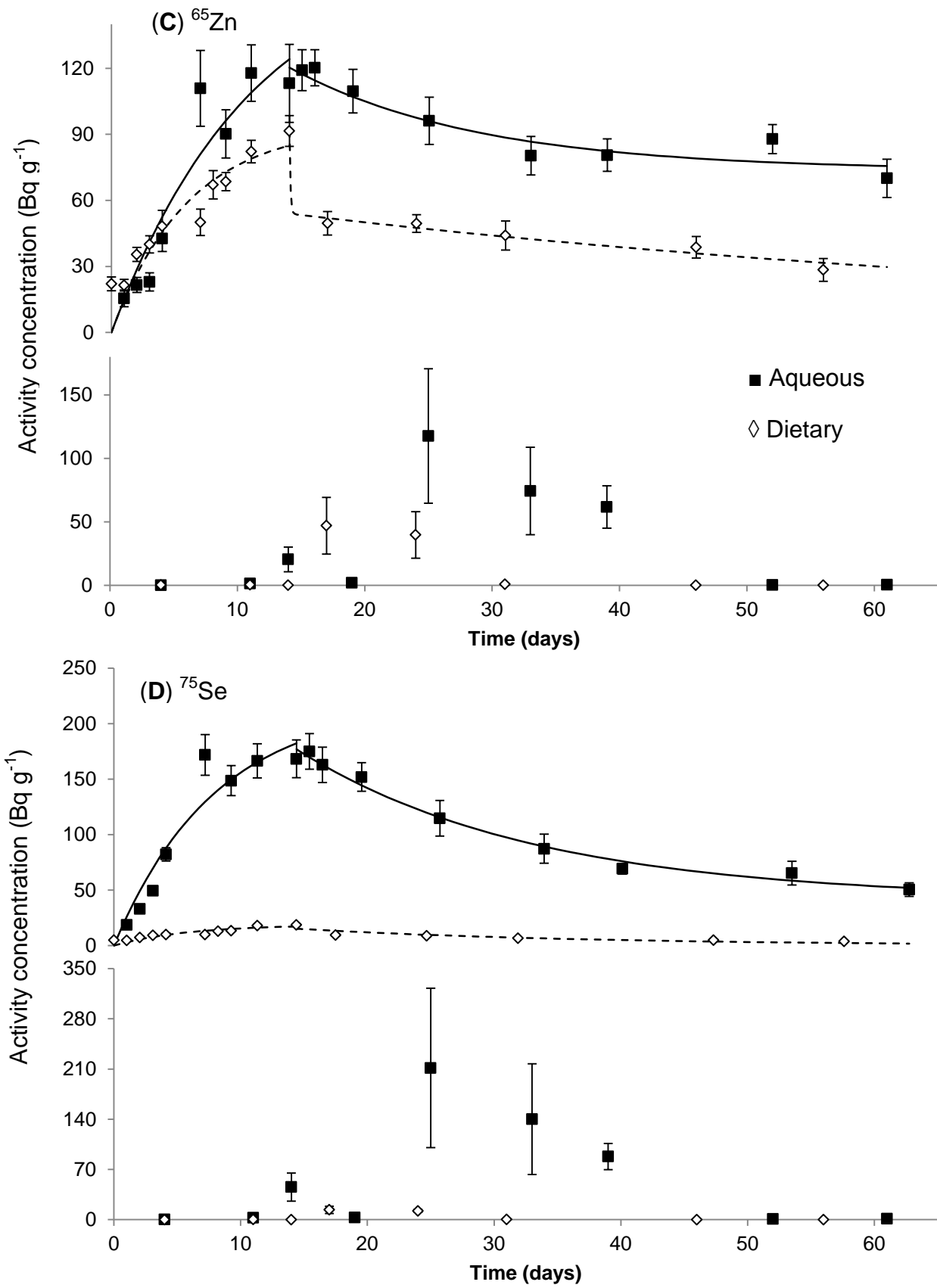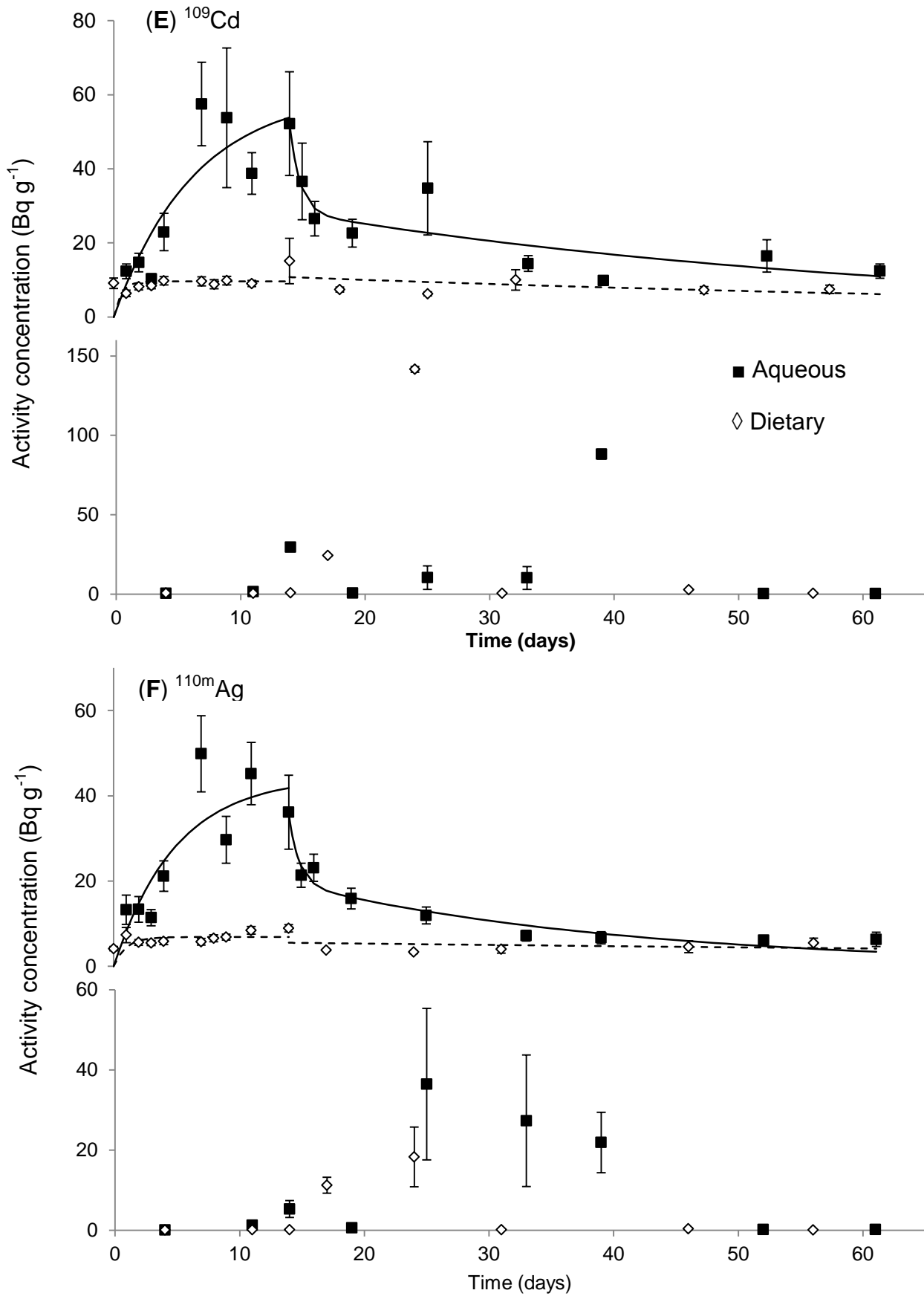
Figure 3-4 cont.
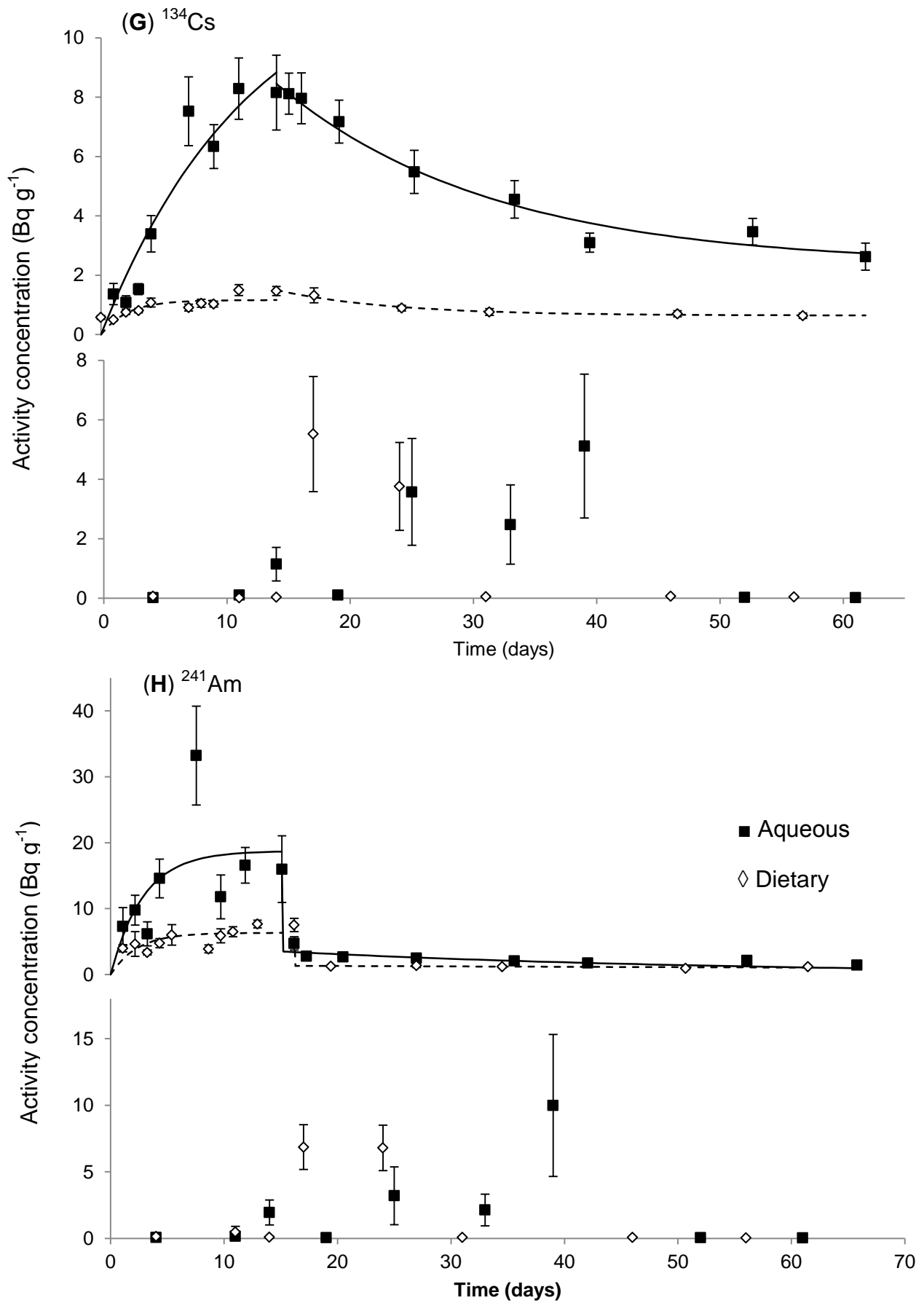
Figure 3-4 cont.

Amount of radioactivity in gonads relative to amount in whole body varied among radionuclides and between exposures (Table 3-4). Most radionuclides did not show much difference in amount of uptake into gonads between the two routes of exposure, with only slight indications of higher concentrations from dietary exposure in ^{109}Cd, ^{134}Cs and ^{241}Am. Although tissue activity concentrations and BCF of ^{54}Mn, ^{109}Cd, and ^{110m}Ag were not the highest among the radionuclides tested, they did have the highest values in gonads (% of whole body activity concentrations). All radionuclides had low initial concentrations that subsequently increased between d 25 and 39. The order of radionuclide bioaccumulation (percentage of whole body activity concentration) in gonads was: ^{109}Cd > ^{110m}Ag > ^{54}Mn > ^{75}Se > ^{241}Am > ^{60}Co > ^{134}Cs > ^{65}Zn. The pattern was again different in the dietary exposure: ^{109}Cd > ^{241}Am > ^{54}Mn > ^{60}Co > ^{134}Cs > ^{110m}Ag > ^{75}Se > ^{65}Zn. The main difference between the two routes of uptake was the timing of uptake into gonads, with dietary route showing much earlier uptake into gonads compared with the aqueous route.

Based on model-derived radionuclide activity concentrations at the end of uptake phase (d 14, C_{ss} or C_t for single component model, a, or linear, b model, respectively), BCF and BMF were similar for most of the radionuclides (Figure 3-5). An exception was ^{134}Cs, which had the lowest BCF but highest BMF of all the radionuclides. BCF and BMF values varied among radionuclides with ^{65}Zn and ^{75}Se having highest BCFs (359 and 433 respectively), compared with the other radionuclides (≤ 100) (Figure 3-5).

Table 3-3: Parameters of single component (1) or linear (2) uptake models, and single component (3) or double component (4) depuration models, for radionuclides in whole body of zebrafish after aqueous or dietary exposure.

Isotope	Exposure	Uptake			Depuration		
		$C_t{}^a$	URC[b]	Model fit[c]	C[d]	DRC[e]	Model fit[f]
[54]Mn	Aqueous	24.93	1.78	87.3[2]	20.31, 8.70	1.54, 0.01	0.0001[4]
	Dietary	4.75	0.35	0.0004[1]	4.64, 1.21	130959.1, 0.01	0.0046[4]
[60]Co	Aqueous	37.33	0.24	0.0028[1]	11.69, 5.87	1.43, 0.03	<0.0001[4]
	Dietary	29.83	0.23	0.0003[1]	19.12	2.01	<0.0001[3]
[65]Zn	Aqueous	172.88	0.09	<0.0001[1]	46.72, 73.62	0.07, 4.3E-12	0.0063[4]
	Dietary	108.48	0.11	<0.0001[1]	37.63, 53.94	12.63, 0.013	0.0177[4]
[75]Se	Aqueous	218.04	0.129	<0.0001[1]	135.08, 41.87	0.06, 2.4E-11	<0.0001[4]
	Dietary	21.38	0.12	<0.0001[1]	15.22	0.05	0.0201[3]
[109]Cd	Aqueous	60.69	0.16	0.0049[1]	24.08, 28.37	1.23, 0.02	0.021[4]
	Dietary	9.64	1.70	0.0029[1]	10.76	0.01	0.3314[3]
[110m]Ag	Aqueous	44.55	0.20	0.0111[1]	16.39, 19.43	1.24, 0.04	0.0008[4]
	Dietary	6.88	1.02	0.0004[1]	5.53	0.006	0.6829[3]
[134]Cs	Aqueous	12.78	0.08	<0.0001[1]	6.11, 2.34	0.06, 1.2E-11	0.0001[4]
	Dietary	1.33	0.25	<0.0001[1]	0.86, 0.64	0.12, 2.5E-11	0.0192[4]
[241]Am	Aqueous	18.76	0.38	0.0327[1]	12.49, 3.50	766.24, 0.02	<0.0001[4]
	Dietary	6.33	0.46	0.0014[1]	6.20, 1.33	334940.1, 0.01	0.0025[4]

[a]Activity concentration (d 14) C_{ss} or C_t for single component ([1]) or linear ([2]) uptake models respectively.

[b]Uptake rate constants k_e or k_u for single component or linear uptake models respectively.

[c]Significance of model as p-value or R^2 (%) for single component or linear uptake models respectively.

[d]Activity concentrations A_0, or A_{0s} and A_{0l} for single ([3]) or double ([4]) component depuration models respectively.

[e]Depuration rate constants k_e, or k_{es} and k_{el} for single or double component depuration models respectively.

[f]Significance of model as a p-value.
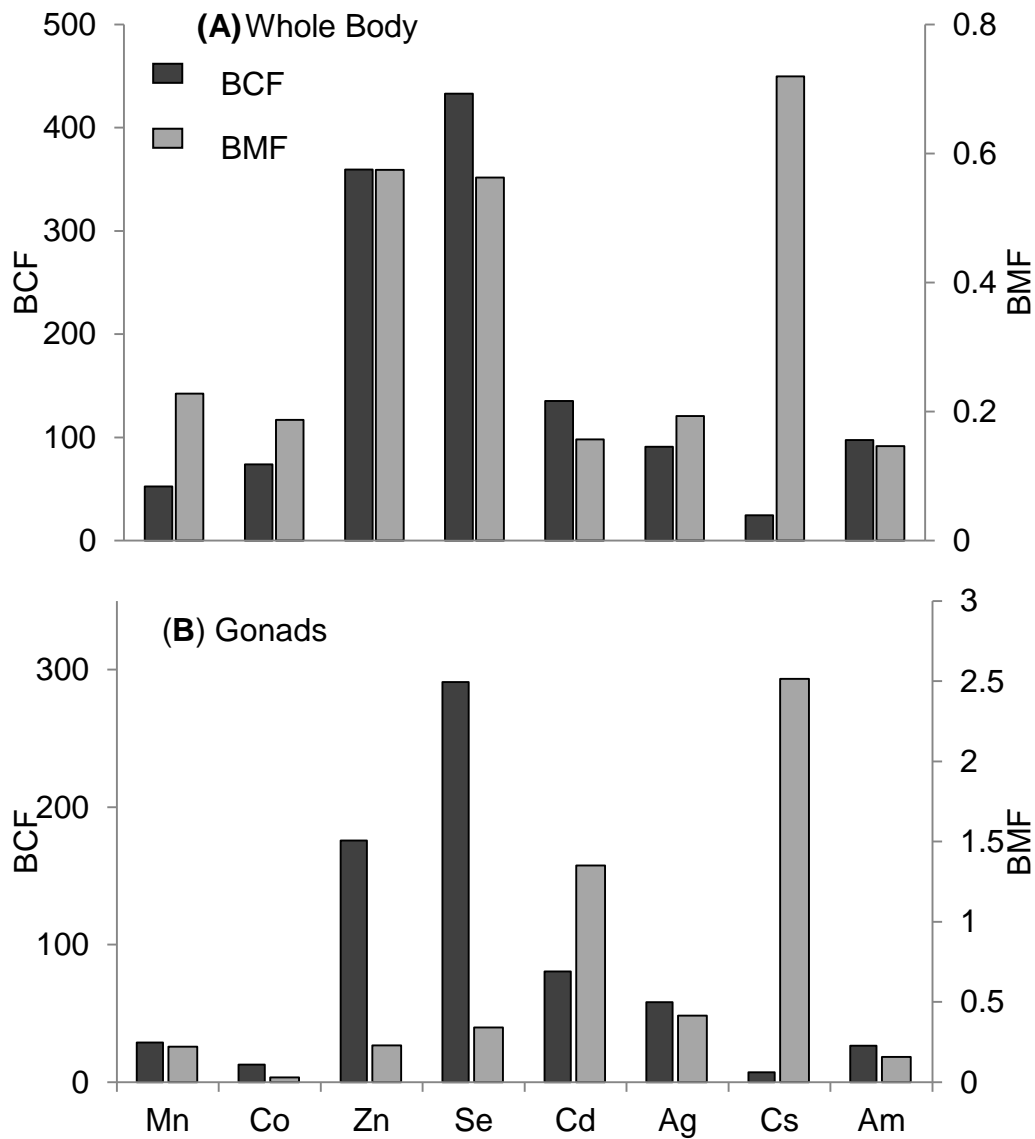
Figure 3-5: Bioaccumulation after aqueous (BCF, dark bars) and dietary (BMF, light bars) exposures for whole body (**A**) and gonads (**B**) of zebrafish. Note the secondary y-axis for BMF.

Contrary to radionuclide accumulation in whole body, accumulation in gonads continued to increase after initiation of depuration for all radionuclides in both aqueous and dietary exposures (Figure 3-4). Variations in radionuclide activity concentrations in gonads prevented statistical fitting of uptake depuration kinetic models. Maximum activity concentrations in gonads were observed earlier for all radionuclides in the dietary exposure (17-24 d) compared to the aqueous exposure (25-39 d). Radionuclide activity concentrations in gonads were near control concentrations after 39 d (aqueous) and 24 d (dietary). For most radionuclides (except ^{109}Cd and ^{134}Cs), gonads had higher bioaccumulation of radionuclides after aqueous exposure.

3.3.3 Dose

The ERICA Tool estimated dose rates from average whole body activity concentrations and, despite lower water radionuclide activity concentrations compared with diet amended with radionuclides, the aqueous exposure resulted in higher accumulated doses for all radionuclides except ^{60}Co (Figure 3-6). Aqueous radionuclide exposure produced both an internal and an external dose to the fish, and the external accumulated doses of ^{65}Zn, ^{75}Se, ^{109}Cd, and ^{241}Am were considerably greater than the internal accumulated doses (five orders of magnitude greater in ^{241}Am, three orders of magnitude greater in ^{109}Cd, and approximately one order of magnitude greater in ^{65}Zn and ^{75}Se). Co-60 and ^{134}Cs had equal internal and external contribution to accumulated dose, and ^{110m}Ag had a slightly greater internal accumulated dose at the end of the uptake phase. Mn-54 was the only radionuclide with a higher external accumulated dose in the aqueous exposure.

The doses received by the fish from most radionuclides were low; accumulated doses, except for [241]Am (due to the x10 weighting factor applied to alpha emitters) were < 2.2 Gy for aqueous or dietary exposures (Table 3-4).
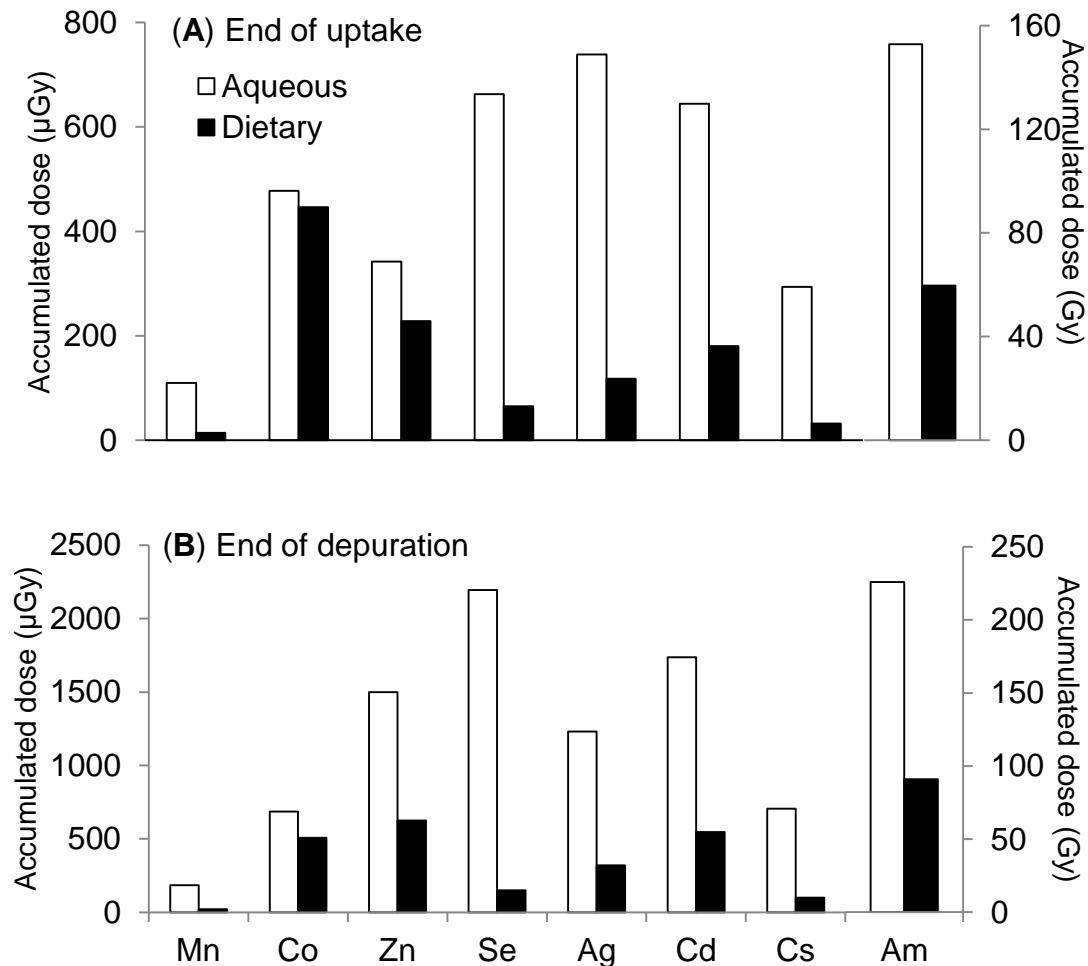
Figure 3-6: Accumulated doses (µGy) to adult zebrafish at the end of uptake (**A**) and depuration (**B**) periods following aqueous or dietary exposure to a mixture of radionuclides. Note secondary y-axis for americium in Gy, not µGy as for the other radionuclides.

The aqueous exposure resulted in over double the total accumulated dose compared with the dietary exposure. Maximum dose rates observed during exposure periods varied among the radionuclides and between exposure regimes, with aqueous exposure producing consistently higher dose rates than the dietary exposure (Table 3-5). Aqueous exposure resulted in maximum dose rates between days 7 and 14, depending on the radionuclide, but dietary exposure resulted in maximum dose rates between days 11 and 14.

Table 3-4: Activity concentrations (Bq g^{-1}) in gonads and maximum % of whole body activityconcentrations found in the gonads. Aqueous activity concentrations are means between d 25 and d 39 (± S.E.M.) and dietary activity concentrations are means between d 17 and d 24 (± S.E.M.). Maximum percentage of whole body activity concentrations found in the gonads was reached between d 25 and d 39 in the aqueous exposure and between d 17 and d 24 in dietary exposure.

Isotope	Aqueous		Dietary	
	Activity concentration (Bq g^{-1})	Max. transfer (%)	Activity concentration (Bq g^{-1})	Max. transfer (%)
^{54}Mn	13.7 ± 1.2	15.0 ± 5.1	4.6 ± 1.1	14.8 ± 4.9
^{60}Co	6.4 ± 1.0	10.0 ± 3.0	4.8 ± 0.3	14.6 ± 10.1
^{65}Zn	84.6 ± 16.9	6.5 ± 3.3	43.3 ± 3.6	7.0 ± 5.6
^{75}Se	146.6 ± 35.8	14.5 ± 9.0	13.0 ± 0.7	10.1 ± 7.3
^{109}Cd	36.2 ± 26.0	19.7 ± 6.2	82.9 ± 58.6	34.3 ± 15.5
^{110m}Ag	28.6 ± 4.2	19.3 ± 6.1	14.8 ± 3.5	11.7 ± 2.8
^{134}Cs	3.7 ± 0.8	7.8 ± 4.8	4.6 ± 0.9	12.7 ± 4.7
^{241}Am	5.1 ± 2.5	12.5 ± 1.3	6.8 ± 0.02	23.8 ± 6.7

Table 3-5: Accumulated doses and maximum dose rates in the whole body of adult zebrafish following aqueous or dietary exposure to radionuclides. Accumulated doses at end of depuration (d 61 for aqueous exposure and d 56 for dietary exposure), and maximum dose rates were during the uptake phase.

Isotope	Aqueous		Dietary	
	Accumulated dose (d61, Gy)	Mx. Dose rate ($\mu Gy\ h^{-1}$)	Accumulated dose (d61, Gy)	Mx. Dose rate ($\mu Gy\ h^{-1}$)
^{54}Mn	0.2	0.5	0.02	0.1
^{60}Co	0.7	2.1	0.5	2.0
^{65}Zn	1.5	1.6	0.6	1.1
^{75}Se	2.2	3.0	0.2	0.3
^{110m}Ag	1.2	3.4	0.3	0.5
^{109}Cd	1.7	3.2	0.6	0.8
^{134}Cs	0.7	1.2	0.1	0.1
^{241}Am	226.0	1051.4	91.0	242.4

3.4   Discussion

Whole body activity concentrations of radionuclides differed between exposure routes tested (water or diet), and activity concentrations were higher after aqueous exposure for ^{54}Mn, ^{65}Zn, ^{75}Se, ^{109}Cd, ^{110m}Ag, ^{134}Cs, and ^{241}Am (not ^{60}Co). It is possible that aqueous radionuclides partitioned onto skin surfaces and led to elevated whole body activity concentrations, a pattern that has been reported for some radionuclides in dogfish, *Scyliorhinus canicula* (Jeffree *et al.*, 2006b). Whole body activity measurements included gut contents, and

accumulation of radionuclides in the gut lumen (but not absorbed across epithelial membranes) could result in over estimation of whole body activity concentrations for the dietary exposure. Despite the potential for over estimation (in both dietary and aqueous exposure routes) the dietary exposure resulted in lower tissue activity concentrations.

Uptake kinetics of radionuclides into zebrafish were similar among radionuclides during aqueous exposure with all (except [54]Mn) approaching steady state. These results were consistent with a previous study of radiotracer uptake in marine fish which found similar kinetics for [109]Cd, [134]Cs and [51]Cr (but not for [241]Am, [57]Co, [54]Mn or [65]Zn) after 24 days aqueous exposure in juvenile bream, *Sparus auratus* (Mathews *et al.*, 2008). A linear uptake pattern, however, with no equilibration between water activity concentrations and body accumulation, was found in the essential metals [65]Zn, [57]Co and [54]Mn, a pattern only matching [54]Mn from this study. Differences in extent of uptake are not wholly accounted for by slight differences in exposure activity concentration among the radionuclides but could also be a result of radionuclide-specific differences and use of different ion uptake channels by different metals.

Different c and rates of radionuclide bioaccumulation have been reported in fish species (Jeffree and Teyssie, 2006) and the present results indicate zebrafish are also different. A comparison in uptake and depuration rates between *S. auratus*, *S. canicula*, and turbot, *Psetta maxima,* highlighted species differences (Mathews *et al.*, 2008), and zebrafish k_e and k_u are consistently higher than any of the marine species for [54]Mn, [65]Zn, [109]Cd, [134]Cs, and [241]Am. Species differences in radionuclide bioaccumulation in fish could be particularly influenced by whether they live in freshwater or seawater. Higher radionuclide

accumulation, observed in zebrafish, could be because freshwater fish actively take up ions from the environment to maintain internal ion concentrations (Sloman *et al.*, 2006). In zebrafish some of the radionuclides, namely ^{241}Am and ^{65}Zn, do appear to be even higher than *S. auratus¸* and their small body size supports the hypothesis that size differences, as well as species-specific differences, play a role in determining radionuclide uptake. The importance of body size in determining body burdens of aqueous radionuclides has been reported in some studies (Jeffree *et al.*, 2007; Mathews *et al.*, 2008). The results of aqueous ^{65}Zn and ^{75}Se bioaccumulation in this study suggest that radionuclides with stable elements essential for fish metabolism use the same mechanisms for active uptake or depuration. The slow and low rates of depuration in both ^{65}Zn and ^{75}Se in the aqueous exposure indicate incorporation of the radioactive form of zinc and selenium into biological processes of these essential metals (Jaramillo *et al.*, 2009; Ye *et al.*, 2009). Similar high levels of retention of ^{54}Mn during the depuration phase were found for the benthic seawater teleost, *P. maxima* (Jeffree *et al.*, 2006b), and rainbow trout, *Oncorhynchus mykiss*, where > 50 % of initial body burden of ^{54}Mn was retained after 42 days of depuration (Adam *et al.*, 1997). The variation in the extent of depuration in the other radionuclides could be indicative of different levels of interaction with metabolically active stable elements, possibly resulting in the effective depuration rates of ^{241}Am, ^{134}Cs and ^{110m}Ag that have no stable counterparts essential to fish metabolism. In comparison to *P. maxima*, zebrafish had lower activity concentrations at the end of uptake and depuration phases for ^{54}Mn and ^{109}Cd only, and Co, ^{65}Zn,^{134}Cs and ^{241}Am had higher concentrations in the zebrafish. After the end of the depuration phase only ^{109}Cd

was higher in the zebrafish but the depuration phase was also four days shorter in the zebrafish exposure compared with the *P. maxima* study. Cs-134 can move easily across biological barriers with a reported BCF below 10 in *S. canicula*, (Jeffree *et al.*, 2006a; Jeffree *et al.*, 2007) and *P. maxima*, (Jeffree *et al.*, 2006b; Mathews *et al.*, 2008), and below 20 in zebrafish (present study). In our study ^{134}Cs had the highest BMF but the lowest BCF, and this result was consistent with previous results that have shown rapid uptake and rapid depuration for ^{134}Cs (Jeffree *et al.*, 2007). An high BCF (600) was reported previously for ^{137}Cs in zebrafish (Srivastava *et al.*, 1994), as the fish compensated for the lack of potassium by taking up caesium. The interaction with other key metabolic ions highlights the complexity of the interactions between environmental contaminants, such as radionuclides, and their stable metal counterparts having functional roles in the organism.

Route of exposure (water or diet) led to similar amounts of radionuclides (^{60}Co, ^{65}Zn, ^{109}Cd, ^{110m}Ag, ^{134}Cs and ^{241}Am) in gonads for the exposure activity concentrations tested. However, the maximum amounts of radionuclides detected in gonads were observed at different time points between the aqueous and dietary exposures. All of the radionuclides in the aqueous exposure reached maximum activity concentrations (Bq g^{-1}) by day 39 compared with day 24 in the dietary exposure. Contaminated prey items in the environment may have a more immediate impact on dose delivery to gonads when compared with dissolved radionuclides.

This study used tracer concentrations of a mixture of radionuclides to enable investigation of uptake depuration kinetics and dosimetry, and was not conducted to evaluate potential biological impact of accumulated radionuclides

to establish dose-response relationships. However, based on available information and estimations of accumulated doses and dose rates, it could be assumed that ^{241}Am, the only alpha-emitter included in the exposure, delivered a dose that could induce detrimental effects (accumulated dose from aqueous exposure 226 Gy, max dose rate 1 Gy h^{-1}). An accumulated dose of over 200 Gy (over 500 µGy h^{-1}) has been shown to have adverse biological effects such as DNA damage and impaired reproduction in zebrafish (Knowles, 2002; Jarvis and Knowles, 2003), but these endpoints were not considered in the present study. The high dose rate in americium is largely a consequence of the weighting factor of 10 applied to alpha emitters by the ERICA Tool, and there is some debate over whether this weighting factor is appropriate for non-human organisms (Chambers *et al.*, 2006). Without the weighting factor applied, the accumulated dose and maximum dose rates drop by an order of magnitude, below any expected dose effects concentrations. The ERICA Tool does not address differential doses to internal organs therefore estimating doses in zebrafish gonads was not possible.

Previous studies have investigated bioaccumulation of radionuclides in fish (e.g. Garnier-Laplace *et al.*, 1997; Mathews *et al.*, 2008; Jeffree *et al.*, 2010) or effects of doses of radiation from radiation sources (Yabu *et al.*, 2001; Yum *et al.*, 2009), but the linkages between bioaccumulation and radiation dose has not been established previously for any fish species. Specific calculations for individual radionuclides (e.g., tritium) have been used to estimate dose from bioaccumulation data in other organisms including invertebrates (Jha *et al.*, 2005; Jaeschke *et al.*, 2011), however the complexity of species-specific dose modelling for other radionuclides, and radionuclide mixtures, requires

sophisticated modelling packages such as ERICA Tool. The combination of experimental activity concentration measurements with estimations of accumulated doses and dose rates described in the present study allows for direct linking of radionuclide dose to biological effects. This approach will enhance our ability to assess potential risks of radionuclides in the environment by linking experimental data for species-specific uptake with direct estimations of dose. In addition, the novel application of the ERICA Tool (primarily designed for environmental risk assessments) to laboratory exposures of radionuclides can initiate investigations of dosimetry in organisms through different exposure scenarios (e.g. routes and time). It is a useful tool to allow linkage between physicochemical kinetic investigations and biological effects to assist in evaluations of the effects of radionuclides in the environment. Gonads are a relevant target organ for radionuclide studies because germ cells are particularly susceptible to radiation damage, and radiation-induced changes in the testes or the ovaries can impede reproductive success of the organisms (Hamaguchi, 1980; Konno, 1980). Damage to DNA can result from radiation exposures and transgenerational effects are possible that could affect long-term survival of populations (Dubrova, 2003a; Jha, 2004, 2008; Pentreath, 2004).

3.5   Conclusion

This is the first study to link radionuclide exposure and bioaccumulation to estimation of radiation dose in zebrafish exposed to a mixture of environmentally relevant radionuclides at tracer level concentrations via dietary and aqueous routes. The study suggests that all radionuclides (except ^{54}Mn) reached equilibrium during 14 days uptake phase for aqueous or dietary exposures, although the magnitude of activity in tissues varied significantly for

different radionuclides either for whole body or for gonads. The highest whole body dose received as estimated using the ERICA modelling tool was for ^{241}Am (the only alpha-emitter included in the exposure scenario) from either of the exposure routes. The usefulness of ERICA tool suggests that despite certain limitations it will continue to contribute to dose estimation in organisms including different fish species. Furthermore, studies from zebrafish can provide insight into bioaccumulation and dosimetry of radionuclides in other species, and they can aid in environmental and human risk assessments of the biological impacts of radionuclides.

3.6 Acknowledgements

4:

Development and validation of non-invasive methodology for repeated collection and assessment of DNA damage in sperm of zebrafish, *Danio rerio*

Hypothesis 1:   Hydrogen peroxide will induce concentration-related damage to DNA (DNA strand breaks) in zebrafish sperm exposed *in vitro*, detectable by single-cell gel electrophoresis (comet) assay.

Hypothesis 2:   The frequency of non-invasive sperm sampling from individual zebrafish will affect cell density (sperm count) and DNA damage (DNA strand breaks) in the sperm sample.

In review *Journal of Fish Biology*, July 2012

Abstract

Environmental stress including exposure to toxicants can negatively affect sperm, fertilization success, and survival of offspring. To develop methods for incorporation of sperm assessment into investigations with zebrafish *Danio rerio*, the effect of repeated sub-lethal sampling was evaluated on sperm quantity and induction of DNA damage [single cell gel electrophoresis (comet assay)]. Mean number of sperm decreased after 12 d by 57 % in fish sampled every 2 days (p < 0.05). There was a non-significant trend in reduced sperm counts (22 % after 12 d, p > 0.05) in fish sampled every 4 d, but counts did not decrease in fish sampled every 7 d (p > 0.05). *In vitro* DNA damage in sperm increased significantly with concentration of H_2O_2 (0 - 200 µM), and 200 µM H_2O_2 produced 88.7 ± 3.9 % tail DNA compared to unexposed controls [12 ± 0.7 % tail DNA (mean ± S.E.M., n = 3)]. There was no difference in DNA damage in sperm after 14 d of repeated (2-d, 4-d, or 7-d) sample collection (p > 0.05, n = 7 - 9 males). Results indicate that repeated sampling of sperm decreased sperm counts if sampling is too frequent (every 2 d), but DNA damage is not induced. Assessment of sperm quantity and presence of DNA damage can be included in numerous research investigations with zebrafish.

4.1 Introduction

The effects of environmental stressors such as toxicants can be transgenerational (i.e. effects on parents transmitted to offspring), and lesions in gametes can compromise survival of offspring. Evaluations of transgenerational effects are important and investigations of sperm quantity and quality provide a unique sub-lethal approach for determination of some of these effects. Spermatozoa have been reported to be more important than ova regarding the potential to transmit DNA damage to the next generation (Anderson and Wild, 1994; Dubrova, 2003a; Lewis and Aitken, 2005), due in part to the presence of damage repair systems in oocytes (Ashwood-Smith and Edwards, 1996) that are assumed to be largely absent in sperm (Hales *et al.*, 2005; Leal *et al.*, 2009). It is therefore important to be able to evaluate the quality of sperm in organisms, and a key parameter to assess is damage in DNA, which has consequences for the fertility and fecundity of organisms including for humans (Lewis and Aitken, 2005). Zebrafish *Danio rerio* (Hamilton, 1822) are a useful model organism applied to numerous research areas (Steenbergen *et al.*, 2011) including genotoxicological studies (Teraoka *et al.*, 2003; Diekmann *et al.*, 2004). Sperm can be sampled from adult male zebrafish (Morris *et al.*, 2003; Jing *et al.*, 2009; Wilson-Leedy *et al.*, 2009). However, the influence of repeated non-invasive sperm sampling on sperm counts and induction of DNA damage in sperm has not been previously investigated. Only one study has used the comet (single cell gel electrophoresis) assay to detect DNA damage in zebrafish sperm, but that study evaluated excised testes after euthanasia (Uren-Webster *et al.*, 2010). The objective of this study was to develop and validate methods for repeated non-invasive sampling of sperm with minimal impact on adult zebrafish and to

evaluate DNA damage in sperm cells using the comet assay (Collins, 2004; Jha, 2008; Kosmehl *et al.*, 2008), a technique used widely in assisted reproduction technology to determine quality of sperm (Lewis and Agbaje, 2008).

4.2   Materials and Methods

4.2.1 Fish husbandry

Adult zebrafish (*Danio rerio*, age ~120 d) were obtained from the Zebrafish Research Facility at the Plymouth University, UK, which is maintained under routine approved animal welfare protocols (Figure 4-1). Water quality characteristics were measured daily (mean ± S.D.) for temperature (26 ± 1 °C), pH (6.7 ± 0.3), and dissolved oxygen (92 % ± 3), and ammonium, nitrate, and nitrite were analysed weekly (< 0.02, < 20, and < 0.1 mg l^{-1}, respectively). Photoperiod was 12L:12D h, and fish were fed twice daily with live brine shrimp nauplii, *Artemia* sp. or dry fish flake mix (equal proportions ZM Systems flake, brine shrimp, spirulina, and TetraMin® stable flake). Stock fish were maintained in 20-L glass aquaria in a recirculating system, and males were used for the *in vitro* comet assay validation tests.

4.2.2 Method for repeated sperm sampling

The most active spawning time for zebrafish is at the beginning of the light photoperiod (Lawrence, 2007) so sperm from all adult male fish were sampled within 1 h of the start of light period, after anesthetization (250 mg l^{-1} tricaine methansulfonate, MS-222, PHARMAQ Ltd, Hampshire, UK). Fish were held still by placing within a 1 cm deep groove cut in a sponge, and the ventral surface

was exposed for observation by stereomicroscope to facilitate sperm collection.

Figure 4-1: Zebrafish Research Facility, Plymouth University.

Slight abdominal finger pressure was applied (Wilson-Leedy and Ingermann, 2007; Jing *et al.*, 2009) and expelled sperm was collected in a hand-pulled glass capillary tube (Na-hep micro-haematocrit tubes, diameter 1.55 ± 0.05 mm, Fisher Scientific, UK), following the method from Wilson-Leedy and Ingermann (Wilson-Leedy and Ingermann, 2007) (Figure 4-2). Sperm collected in the tube was expelled onto a glass microscope slide that was kept on ice (to reduce evaporation from sperm sample drop). The average volume of sperm obtained per fish per sampling time was 2 µl (range 1-5 µl), and was consistent with other studies (Harvey *et al.*, 1982; Xu *et al.*, 2008). A 0.5 or 1 µL (depending on volume obtained from fish) subsample was pipetted from the glass slide into a tube, and stored on ice for analysis within 6 h. Fish were allowed to recover in freshwater before they were returned to the system aquaria. The whole sampling process from anaesthesia to recovery took < 5 min, and fish were held out of water for < 1.5 min.
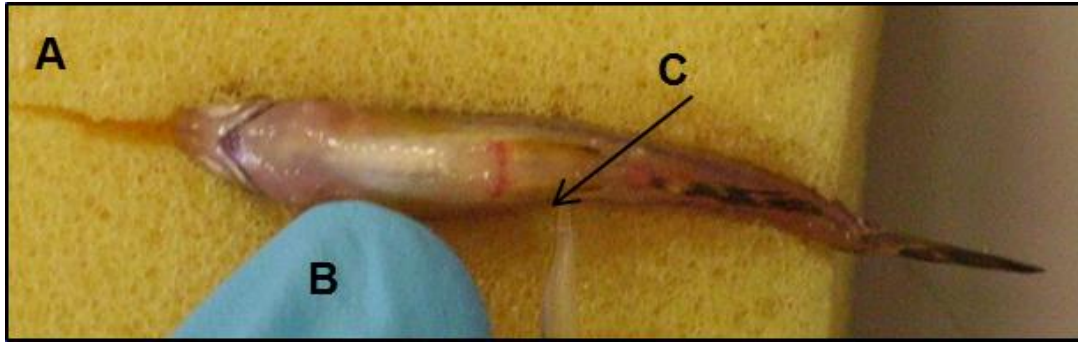
Figure 4-2: Non-invasive extraction of sperm zebrafish. (A) Fish held inverted in sponge groove; (B) slight abdominal pressure; (C) fine glass capillary tube.

4.2.3 Effect of sampling frequency on sperm

Experimental fish were stocked into nine 10-L glass aquaria supplied with partial (10% exchange per day) recirculating water within the same system and allowed to acclimate for 7 d prior to initiation of the experiment. Each aquarium was divided in half with a mesh and six reproductively active females (demonstrated by previous pair spawning with > 50 embryos produced) were placed on one side of the mesh. Three reproductively active male fish (mean total length = 33.8 mm ± 0.3 S.E.M., n = 27 fish) were placed into each tank on the opposite side of the mesh from females. Male fish were individually identifiable by their size within each aquarium and were allowed to acclimate to the aquarium for 7 d. The frequency of sperm sampling was the test variable with three tanks per treatment: fish were sampled every 2, 4, or 7 d (n = 3 tanks, n = 9 males per treatment). Fish in the 2-d treatment were sampled (as described above) every other day for 14 d, the 4-d treatment group were sampled on days 0, 4, 8, and 12, and the 7-d treatment group were sampled on days 0, 7, and 14.

For sperm cell counts, a haemocytometer (Neubauer) was pre-coated with two applications of polyvinyl alcohol (Rain Repellent, Halfords PLC, Worcestershire, UK) (Skinner and Watt, 2007; Wilson-Leedy and Ingermann, 2007), to prevent cells from sticking to glass surface and allow free movement of sperm. Sperm was initially diluted 1:25 (Hank's Buffered Saline Solution, HBSS 300), followed by a second dilution (1:50) in trypan blue to stain viable cells and initiate activation of sperm (nominal osmolality at activation, 150 mOsmol (Jing *et al.*, 2009). Cells were viewed under a compound light microscope (40x objective, 400x magnification, Leitz Laborlus S), and the number of sperm cells in each sample was determined as the mean of five independent counts of a 1 µl volume (grid dimensions 1 x 1 x 0.1 mm, Neubauer haemocytometer). On the final sperm sampling event for each group of fish (d 14 for 2-d and 7-d treatment fish, and d 12 for 4-d treatment group), a subsample of sperm from each individual male (approximately 1 µl) was diluted (1:100) with HBSS and used for assessment of DNA damage (comet assay) as outlined below (section 4.2.4).

4.2.4 Comet (Single cell gel electrophoresis) assay

Validation of the comet assay in zebrafish sperm was conducted in three separate experiments. Sperm samples were exposed *in vitro* to hydrogen peroxide (H_2O_2); unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). Sperm samples (total volume collected) from three male fish were pooled, diluted in 100 µl HBSS, and triplicate aliquots of the sperm dilution were incubated with the following final H_2O_2 concentrations: 0, 10, 25, 50, 100, and 200 µM [range based on sensitivity of aquatic organisms (Cheung *et al.*, 2006)]. After 10 min incubation, cells were

centrifuged (13,000 rpm, 4 min) into a pellet, H_2O_2 was removed, and the cells were re-suspended in 10 µl HBSS.

The comet assay protocol used previously for human sperm was adapted from Simon *et al.* (2010). In brief, cells were mixed with 180 µl low melting point agarose (0.5 % agarose), pipetted onto a slide pre-coated with 1.5 % normal melting point agarose (dried overnight), and covered with a glass coverslip. Gels were allowed to set for 1 h in a cold room (5 ºC) before cover slips were removed and then slides placed in lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h. The procedure differed from Simon *et al.* (2010) in that decondensation steps were not used because it caused increased damage in control cells. After lysis, slides were placed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min (to unwind DNA), followed by electrophoresis (25 V 280-350 mA) for 20 min. Slides were washed in neutralising buffer (0.4 M Tris HCl, pH 7.5) and distilled water, allowed to dry overnight, and scored for % tail DNA (100 cells per slide, Leica DMR fluorescent microscope, Komet 5, Kinetic Imaging, Nottingham) following routine procedures (Collins, 2004, 2009; Kumaravel and Jha, 2006).

4.2.5 Statistics

Statistical analysis of results was conducted with STATGRAPHICS 5.1 (Statistical Graphics Corp., USA). The effect of sampling frequency on sperm cell counts was tested by general linear model (GLM) with sampling day and sampling frequency as model factors (effects of individual fish or fish length were not significant and removed from the model). Concentration response in sperm DNA damage following *in vitro* exposure to H_2O_2 was tested by logistic regression. DNA damage following sub-lethal sperm extraction was tested

(GLM) on arcsine transformed % tail DNA data, and the independent variables were sampling frequency and individual fish.

4.3   Results

Two of the total of 27 males died during the experiment (both from the 4 d treatment group and apparently due to aggressive interactions between fish), but all other fish appeared normal and had no gross external lesions, abnormal behaviour, or reduction in feeding rate. Of 140 total attempts to collect sperm, 18 (13 %) were unsuccessful, and, of these unsuccessful attempts, 8 (5.7 %) were attributed to two males that were consistently poor at producing sperm for sample collection. At time 0, before repeated sampling of sperm began, sperm counts were variable among aquaria in the treatment groups (mean 2.8 x 10^6 μl^{-1}, min 0.5 x 10^6 μl^{-1}, max. 6.0 x 10^6 μl^{-1}), and time 0 sperm counts were excluded from analysis of the effect of repeated sampling over time. The number of sperm in fish sampled every 2 d decreased 57 % after 14 d sampling (mean down from 1.7 ± 0.3 S.E.M. day 2 to 0.7, ± 0.1 S.E.M. cells x10^6, day 14, GLM $p < 0.01$). Fish sampled every 4 d had a tendency for lower sperm counts (mean down from 1.8 ± 0.3 S.E.M. day 4 to 1.4 ± 0.1 S.E.M. day 12, cells x10^6), but this decrease was not significant (GLM $p > 0.1$). No change in the number of sperm was observed in fish sampled every 7 d (2 % decline, mean down from 1.31 ± 0.1 S.E.M., day 7 to 1.28 ± 0.1 S.E.M. day 14, cells x10^6, GLM $p > 0.1$) (Figure 4-3).
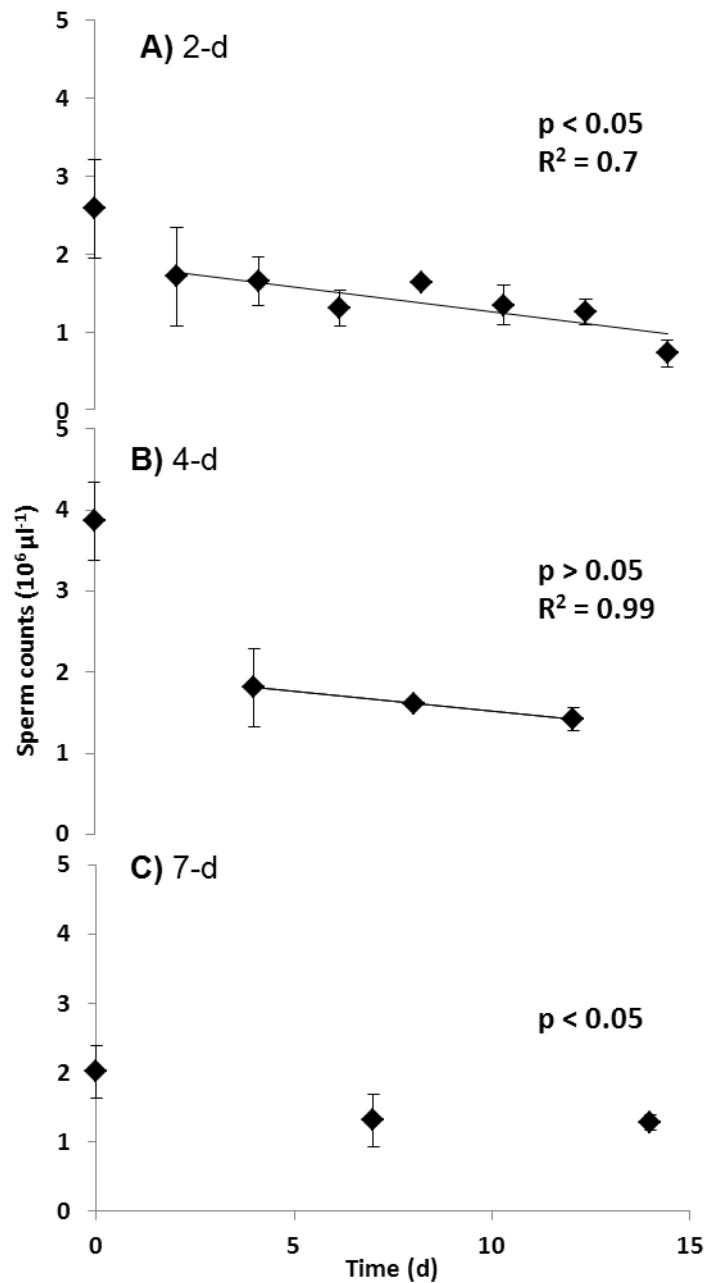
Figure 4-3: The effect of sperm sampling frequency on sperm counts (mean ±

S.E.M., n = 6 – 9 fish) in adult zebrafish sampled every 2, 4, and 7 days.

Levels of DNA damage in sperm increased with concentration of H_2O_2 (*in vitro* incubation for 10 min) (Figure 4-4, A), and levels of DNA damage in unexposed sperm was 9 – 12 % tail DNA, consistent with levels of DNA damage in control sperm samples (Bony *et al.*, 2010). Evaluation of DNA damage in sperm from fish that were sampled repeatedly during the 14-d experiment indicated no significant differences in fish sampled every 2, 4, or 7 days (Figure 4-4, B), and low variance among treatments was observed (2-d fish mean 18.5 ± 0.1 S.E.M., 4-d fish mean 16.7 ± 0.2 S.E.M., and 7-d fish mean 15.8 ± 0.2 S.E.M., % tail DNA, n = 7 - 9 fish per treatment).
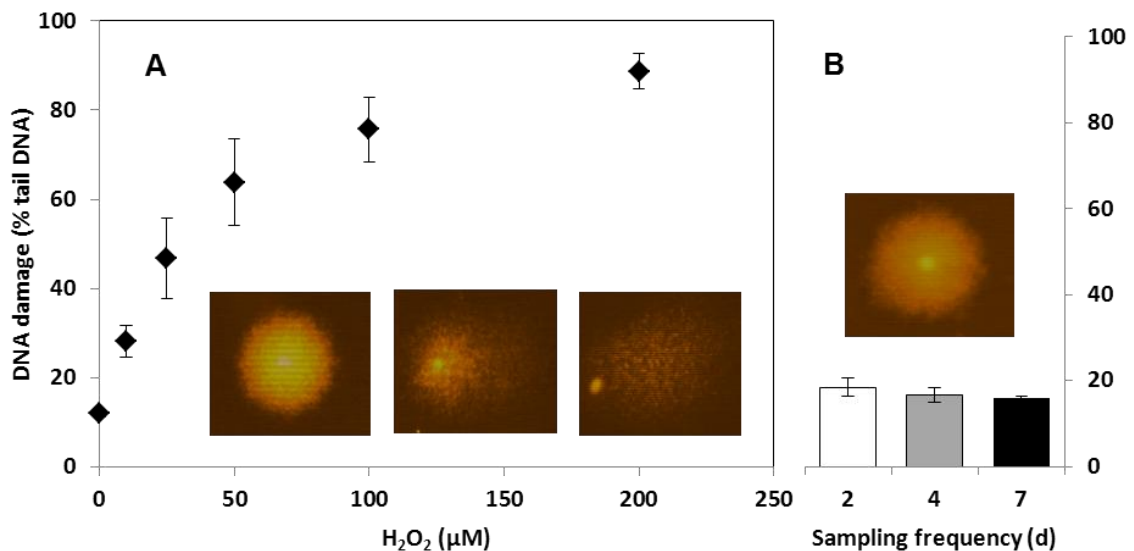
Figure 4-4: Mean (± S.E.M., n = 3 independent experiments) DNA damage assessed by comet (single cell gel electrophoresis) assay in zebrafish sperm exposed (10 min) *in vitro* to different concentrations of H_2O_2 (**A**), and the mean (± S.E.M., n = 7 – 9 fish) DNA damage in zebrafish sperm obtained from males that were sampled every 2, 4, or 7 d over a 14-d experiment (**B**).

4.4   Discussion

The present results indicate that repeated sampling of sperm can be conducted in zebrafish and measurements of sperm quantity and quality can be considered among the endpoints that are tested in diverse investigations that apply to the zebrafish model. The repeated sperm sampling procedure did not appear to negatively affect fish (i.e., no lesions, abnormal behaviour, or alteration in feeding, were observed) indicating that this non-invasive approach can be integrated into experiments with adult fish with minimal impact. The frequency of sperm sampling affected sperm concentration indicating that use of a sampling frequency that does not impact sperm concentration is important. It is possible that the decrease in sperm counts in fish sampled every 2-d, and the non-significant trend of reduced sperm counts in the 4-d sampled fish would become more pronounced over longer sampling periods. Repeated sampling of sperm has been reported to reduce or eliminate sperm present in samples obtained from birds (Lombardo *et al.*, 2004). However, little information is available on the impact of sperm sampling frequency on sperm characteristics in other organisms including important laboratory models.

Sperm production in zebrafish follows a pattern of cystic spermatogenesis over a 6-d cycle from early spermatid formation to production of mature spermatozoa (Leal *et al.*, 2009; Uren-Webster *et al.*, 2010). The method of sampling in the present study is expected to enable assessment of mature spermatozoa only, and the observed decrease in sperm counts in fish sampled every 2 or 4 d is consistent with limitations in capabilities of fish to produce sperm. The effect of repeated sampling on numbers of sperm produced has not been investigated previously in zebrafish. Previous studies with zebrafish have reported large

differences in sperm counts ranging from 250 cells μl^{-1} (Kemadjou Njiwa *et al.*, 2004; Njiwa *et al.*, 2004), 8.8 x 10^4 cells μl^{-1} (Tan *et al.*, 2010), and 8 x 10^6 (Xu *et al.*, 2008) cells μl^{-1}, but accurate sperm concentration is often not the focus of studies directed at cryopreservation of sperm and preserving sperm quality characteristics (Jing *et al.*, 2009; Wilson-Leedy *et al.*, 2009; Tan *et al.*, 2010). Lower proportions of spermatozoa and higher proportions of spermatocytes have been reported after histological examination of adult male zebrafish following toxicant exposure (Uren-Webster *et al.*, 2010); however, assessment of sperm concentration has not been included in any other study that investigated the effects of experimental treatments in zebrafish.

An objective of this study was assessment of sperm production and the influence of repeated sampling over time. The endpoint directly links with other measures of sperm quality (e.g. motility) that are important in fertilization success. Motility of sperm is highly dependent on sperm concentration (Jing *et al.*, 2009), and motility characteristics including sperm velocity has been reported to be related to reproductive success in zebrafish (Wilson-Leedy and Ingermann, 2007; Paull *et al.*, 2008). In addition to sperm quantity and motility, the integrity of DNA within sperm is important. DNA damage in sperm can affect subsequent reproductive success (Zhou *et al.*, 2006; Lewis and Galloway, 2009) but studies of DNA damage in zebrafish sperm are limited to one study (Uren-Webster *et al.*, 2010). In the present investigation, repeated sampling of sperm from zebrafish did not lead to increased incidence of DNA damage as detected by the comet assay.

Evaluating DNA damage following *in vitro* exposure to H_2O_2 demonstrated that zebrafish sperm is similar to H_2O_2-induced damage in sperm of other organisms.

In mammals, sperm exposed *in vitro* to 100 mM H_2O_2 had similar levels of DNA damage as reported in zebrafish (present study), with up to 60 – 70 % tail DNA observed in humans, bulls, and mice (Villani *et al.*, 2010). Repeated sampling of sperm from zebrafish did not induce DNA damage or DNA damage in sperm regardless of the frequency of sampling, indicating that the sampling procedure did not induce adverse effects in sperm. The level of *in vivo* DNA damage in sperm in the present study was similar to levels of damage in zebrafish sperm reported in the study of Uren-Webster *et al.* (2010), in which adult zebrafish sperm had up to 12 % tail DNA by comet assay in sperm obtained by crushing testes after euthanasia. The repeated method for non-invasive sperm sampling is preferable to obtaining sperm via crushing testes because the comet assay can be conducted on a more homogeneous cell type (i.e., mature spermatozoa) with similar sensitivities rather than a mixture of gonad cells of differing sensitivities. Furthermore, the fish do not need to be euthanized.

4.5   Conclusion

The present study indicated that repeated sampling of sperm at intervals of 2, 4, or 7 d can be done with minimal impact on adult zebrafish and produce samples that are useful for assessment of sperm quantity and quality measurements. If sperm is sampled too frequently, the number of sperm that are obtained decreases over time, which is likely related to the time required for spermatogenesis to generate mature spermatozoa. The comet assay can be effectively applied to assess DNA damage in zebrafish sperm, as has been done in sperm from other organisms, and repeated sperm sampling from zebrafish does not lead to elevated DNA damage in sperm. Integration of assessments to determine the quantity and quality of sperm into experiments

with zebrafish could be applied readily to add information on the effects of different types of experimental treatments on an important component of male reproductive success.

4.6   Acknowledgements

5:

Changes in expression profile of genes associated with DNA repair following induction of DNA damage larval in zebrafish *Danio rerio*

Hypothesis 1:   Hydrogen peroxide will induce concentration-related DNA damage (DNA strand breaks), detectable by comet assay, in zebrafish larvae exposed both *in vitro* and *in vivo*.

Hypothesis 2:   Expression of DNA repair genes will be induced after DNA damage (DNA strand breaks) occurs, and result in reduction in levels of DNA damage over time.

Hypothesis 3:   Expression profiles of DNA repair genes over time can be modelled and models can enable comparisons in timing and extent of induction of expression among genes.

Abstract

Repair of damaged DNA is a continuous process in organisms and is initiated by transcription of genes that are induced in response to specific types of DNA damage. Strand breaks (single and double) are a common type of DNA damage that is repaired predominantly through non-homologous end-joining or homologous recombination. Progression of DNA strand break repair and the changes in expression profiles of genes involved are unknown. In this experiment, DNA damage was induced in zebrafish larvae by brief exposure (10 min) to hydrogen peroxide (H_2O_2, 100 mM), and the presence of DNA strand breaks was assessed by single cell gel electrophoresis (comet) assay over 24 h. H_2O_2 was selected because it is eliminated rapidly after induction of DNA damage. DNA damage was detected immediately after 10-min H_2O_2 exposure (mean exposed 35.4 ± 3.8 S.E.M. % tail DNA, mean control 17.2 ± 2.0 S.E.M. % tail DNA, n = 3), and damage was reduced to within control levels within 24 hours (mean exposed 9.2 ± 0.4 S.E.M. % tail DNA, mean control 9.9 ± 0.9 S.E.M. % tail DNA, n = 3). At 0, 6, 12, and 24 h post-exposure, expression of specific genes involved in DNA repair were analysed by quantitative reverse transcriptase PCR (qRT-PCR), including *xrcc5*, *xrcc6* (non-homologous end-joining), *rad51* (homologous recombination), and *gadd45a* (DNA damage recognition). The expression of each gene increased within 6 h after exposure with max. 2.1 fold change for *rad51*, followed by *gadd45* (1.71 fold change), *xrcc6* (1.48 fold change), and *xrcc5* (1.46 fold change). Modelling of gene expression profiles (critical exponential model) indicated that highest expression occurred for each gene within 5 h of exposure to H_2O_2, and expression profiles were similar among all genes. Results indicate that repair mechanisms were initiated rapidly after DNA damage and that evaluation of gene expression profiles throughout the repair process is essential to establish the time course of these processes.

5.1   Introduction

Endogenous and exogenous agents cause DNA strand breaks that must be repaired to preserve cellular function and organism survival. A strand break can occur in one or in both strands, and, depending on the type of breakage, different repair mechanisms will be induced to resolve the DNA damage and reduce breaks within damaged cells (Dhawan *et al.*, 2009). Once damage such as strand breaks are detected, the cell cycle is arrested and repair is initiated (Bladen *et al.*, 2005). A series of proteins mediate damage recognition, signalling, end processing, and end re-joining, via either non-homologous end-joining (NHEJ) or homologous recombination (HR) pathways (Kobayashi *et al.*, 2008). When the homologous sister ends of the broken strands are in close proximity, repair using the intact sister template can be straightforward and accurate through the HR pathway (Sonoda *et al.*, 2006). Rad proteins bind to broken ends, locate the appropriate homologous sequence, and recruit intermediates for DNA synthesis and final ligation (Takata *et al.*, 2000; Kobayashi *et al.*, 2008). However, in tightly packed chromatin, close proximity of homologous template is not always possible, and NHEJ is suggested as the predominant strand break repair pathway in vertebrate cells (Takata *et al.*, 1998). NHEJ involves formation of Ku80/Ku70 heterodimer that binds the free ends of broken DNA strands, spanning the gap and recruiting ligating enzymes to re-join the strands (Gu *et al.*, 1997; Jones *et al.*, 2001; Kobayashi *et al.*, 2008).

Repair of DNA damage is initiated by induction of genes that code for products that drive the repair process. Key genes involved in NHEJ are *xrcc5*, *xrcc6* (encoding for Ku80 and Ku70 protein, respectively), and *rad51* is critical for HR

repair (Thacker and Zdzienicka, 2004). These genes involved in NHEJ and HR repair are induced after radiation (Bladen *et al.*, 2007; Sandrini *et al.*, 2009b) and metal (Gonzalez *et al.*, 2006; Sandrini *et al.*, 2009a) exposure, in response to induction of strand breaks. *In vitro* studies in zebrafish reported induction of *xrcc5* 12 h after exposure to UV-B (Sandrini *et al.*, 2009b) and 24 h after exposure to copper (Sandrini *et al.*, 2009a). In adult zebrafish, a 10-fold induction of *rad51* expression was reported in liver after 63-d dietary exposure to methylmercury (Gonzalez *et al.*, 2005), a 32-fold induction in expression after 7-d exposure to cadmium (Gonzalez *et al.*, 2006), and a 5-fold peak in expression in gills after 8-d exposure to copper (Lerebours *et al.*, 2009). It has been suggested that single strand breaks occur rapidly and are rapidly repaired compared with more complex double-strand breaks (Brendler-Schwaab *et al.*, 2005), but the timing of induction of damage and expression of these repair genes has not been established.

Understanding the timing of gene expression is important for interpreting the dynamics of gene regulation (Chechik and Koller, 2009). Frequently, gene expression studies have not collected samples at enough timepoints to permit effective assessment of the change in gene expression over time (e.g. Gonzalez *et al.*, 2006; Oggier *et al.*, 2011), and this can lead to comparisons in expression of particular genes at timepoints that do not necessarily reflect important moments in the expression profile of the genes. Advances have been made in modelling large microarray datasets of gene-expression changes among treatments and over time, but a focused comparison of the timings of expression in single genes are uncommon (Eastwood *et al.*, 2008), and this approach has not been applied to DNA repair genes. Evaluation of time-related

expression profiles of individual genes has been approached by a critical exponential curve model, which was reported to be an improvement over other models including ANOVA, clustering, or network models (Eastwood *et al.*, 2008). The critical exponential curve model enables time and level of expression to be compared for individual genes across experimental treatments, and this approach could be useful for investigating changes in expression of DNA repair genes.

The objectives of this study were to induce DNA strand breaks in larval zebrafish and to evaluate changes in expression of key genes involved in DNA repair during the period of DNA repair (24 h). H_2O_2 was selected as the agent to induce DNA damage because it is highly reactive and does not persist after fish are transferred to clean water (Chuang *et al.*, 2002; Reeves *et al.*, 2008). Expression profiles of *xrcc5*, *xrcc6*, *rad51*, and *gadd45a* (a gene involved in detection of DNA damage) were evaluated, and the time course of expression was modelled by a critical exponential model (Eastwood *et al.*, 2008).

5.2 Methods

5.2.1 Zebrafish larvae

Adult zebrafish (*Danio rerio*, age 4-5 months) were reared in re-circulating aquaria in the Zebrafish Research Facility at Plymouth University, maintained under routine approved animal welfare protocols. Water quality parameters were measured daily (mean ± s.d.) for temperature (26 ± 1 °C), pH (6.7 ± 0.3), and dissolved oxygen (92 % ± 3), and ammonium, nitrite and nitrate were analysed weekly (< 0.02, < 20, and < 0.1 mg l^{-1}, respectively). Photoperiod was 12L:12D h, and fish were fed three times daily with live brine shrimp nauplii,

Artemia sp. or dry fish flake mix (equal proportions ZM Systems flake, brine shrimp, spirulina, and TetraMin® stable flake). Stock fish were routinely bulk spawned, and eggs were collected and reared in 50 ml petri dishes with daily water changes. For all larval exposures, 72 hours post fertilisation (hpf) hatched larvae were selected (Figure 5-1).

Figure 5-1: Hatched zebrafish larvae (centre) approx. 72 hpf surrounded by younger unhatched embryos at various stages of development.

5.2.2 Validation of single cell gel electrophoresis (comet) assay

Unless otherwise specified, all chemicals were sourced from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). Zebrafish larvae (30 newly hatched, 72 hpf) were mechanically homogenised (pellet pestle, Sigma) in 100 µl Dalbecco's phosphate buffered saline (DPBS, Gibco), and 5 µl aliquots of cell suspension were incubated for 10 min with hydrogen peroxide (0, 1, 10, 25, 50, 100, 200, and 500 µM), before centrifugation (8000 g) to pellet the cells. The supernatant was removed, and the cell pellet washed and re-suspended in 10 µl

DBPS. The re-suspended cells (10 µl) were mixed with 200 µl low melting point agarose, and DNA damage was assessed by alkaline single cell gel electrophoresis (comet) assay. Comet assay was carried out following the routine procedures (Reinardy *et al.*, 2012), with some modifications for somatic cells. In brief, 10 µl of re-suspended cells was mixed with 180 µl low melting point agarose (0.5 %), dropped onto a slide pre-coated with 1.5 % normal melting point agarose (dried overnight), and flattened with coverslips. Gels were left to set for 1 h at 5 ^{0}C before removal of cover slips, and then slides were placed in lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10 % DMSO, 34 mM N-Lauroylsarcosine, pH 10) for 1 h. After lysis, slides were placed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min unwinding, followed by electrophoresis (25 V 280-350 mA) for 20 min. Slides were washed in neutralising buffer (0.4 M Tris HCl, pH 7.5) and distilled water, allowed to dry overnight, and scored for % tail DNA (Leica DMR fluorescent microscope, 100 cells per slide, Komet 5, Kinetic Imaging, Nottingham) following routine procedures (Collins, 2004; Kumaravel and Jha, 2006).

5.2.3 *In vivo* exposures of larvae to hydrogen peroxide

Due to the highly reactive nature of H_2O_2, exposures were carried out in sets of 3 to ensure triplicate treatments and triplicate controls were closely time-matched, and each individual experiment used the same H_2O_2 stock for each treatment. Newly hatched larvae (72 hpf) were counted into 50-ml plastic dishes (9 cm diameter, 40 larvae per dish, 3 dishes per treatment) in clean 'fish water' (aquarium system water). For each concentration, H_2O_2 was added to the dishes (total volume 40 ml) and the larvae were exposed for 10 minutes.

Control dishes contained 40 ml of 'fish water'. After 10 min exposure to H_2O_2, larvae were poured through a sieve and returned to clean 'fish water' to start the recovery period. Control dishes were also washed through sieve and larvae returned to clean 'fish water'.

An initial experiment was conducted to establish the dose-response of larvae exposed to 50, 100, and 200 mM H_2O_2 (controls 0 mM in triplicate), followed by 0 and 24 h recovery in clean water. Larvae were collected and sampled for comet assay as outlined above (with the exception of mechanical homogenisation in RLT buffer), three slides analysed per sample. Toxicity was assessed by mortality after 24 h recovery.

A recovery time-course experiment exposed larvae for 10 min to 100 mM H_2O_2, followed by a recovery period of 0, 6, 12, and 24 hours in clean water. All samples (triplicate exposed and triplicate controls at each timepoint) were mechanically homogenised in RLT buffer (RNeasy MiniKit, Qiagen). Subsamples (5 µl) from the first and last timepoints (0 and 24 hours recovery) were removed and immediately analysed for DNA damage by comet assay. Remaining sample homogenates were frozen at -80 ^{0}C for gene expression analysis.

5.2.4 RNA extraction and cDNA synthesis

Total RNA was extracted (RNeasy MiniKit for animal tissue, Qiagen) from samples of 30 larvae, mechanically homogenised in RLT buffer, and frozen at -80 ^{0}C, following manufacturer's protocol with initial sonication (3 - 5 secs). Additional steps included further tissue break-up (QiaShredder column, Qiagen) and a 15 min DNase treatment. RNA was eluted into 30 µl and concentration

and quality of total RNA was determined by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer). All samples were diluted to 100 ng µl^{-1} total RNA, and 800 ng was used to synthesise cDNA following the manufacturer's protocol (ImProm-II^{TM} Reverse Transcription System, Promega), with hexanucleotide primers and deoxynucleotide mix (Sigma-Aldrich). cDNA was synthesized in the following conditions: annealing at 25 ^{0}C, extending at 42 ^{0}C, and heat-inactivating transcriptase at 70 ^{0}C (GeneAmp® PCR System, 9700, Applied Biosystems). cDNA was stored at -80 ^{0}C until qRT-PCR gene expression analysis.

5.2.5 Selection of primers for DNA repair genes

Primers were selected by Primer Blast (National Center for Biotechnology Information, NCBI). Primers were designed to give amplicons spanning 1 intron junction, and were checked to avoid secondary structure, self-annealing, complementarity, and potential hairpins by DNA calculator (Sigma-Aldrich) and OligoCalc (Northwestern University, USA). Amplicon size was verified on a 2 % agarose gel after PCR amplification. Primer details are listed in Table 5-1.

5.2.6 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Lyophilised primers (Eurofins MWG Operon, Ebersberg, Germany) were reconstituted to 100 µmol with RNase-free water and mixed with SYBR Green JumpStart *Taq* ReadyMix to give a final reaction concentration of 375 nmol in 20 µl total volume. Fluorescence was detected (StepOne Real-Time PCR System, Applied Biosystems) over 40 cycles, cycling conditions of 94 ^{0}C for denaturing, primer-specific annealing 55-60 ^{0}C (see Table 5-1), and extension

at 72 ^{0}C. For analysis, the cycle threshold was set to 25,000 for all qRT-PCR runs. A standard curve of cDNA template (pooled template from each sample within experiment) was run on each plate to allow for within-experiment plate normalisation.

5.2.7   Data analyses

Statistical analyses were conducted with STATGRAPHICS 5.1 (Statistical Graphics Corp., USA). Comet assay data (% tail DNA) was arcsine transformed before simple linear regression was applied to test for significant dose response following both *in vitro* and *in vivo* exposures.

For gene expression analysis, the efficiency of qRT-PCR was calculated ($e = 10^{(-\frac{1}{slope})} - 1$) from the standard curve for each plate. Samples from the same experiment run over multiple plates were adjusted to the plate with the efficiency closest to 1 by resolving for slope and intercept of the standard curves. Only efficiencies between 0.9 – 1.1 were accepted for further analysis, and comparative quantification ($2^{-\Delta\Delta C_t}$) was used for calculating fold-changes in the gene of interest normalised to *β-actin*, (ΔC_t calculated by mean *β-actin* C_t for 0, 6, and 12 hour timepoints, and mean C_t for 24 hour timepoint) and to time-point controls ($\Delta\Delta C_t$) (Henry *et al.*, 2009). The kinetic response in gene fold-change over time was modelled by a critical exponential curve y(x) = A + (B+Cx)R^x, where A, B, C, and R and parameters, *y* is the fold-change gene expression response, and *x* is time, as proposed for time-course analysis of gene expression (Eastwood *et al.*, 2008).

Table 5-1: Zebrafish (*Danio rerio*) gene specific primers for DNA repair genes (*xrcc5, xrcc6,* and *rad51*), growth arrest gene

(*gadd45a*), and housekeeping gene (*β-actin*). Reference sequence numbers from NCBI, and product length in base pairs (bp).

Gene	Ref seq no.	Forward (5'-3')	Reverse (5'-3')	Product (bp)	Annealing temp (^{0}C)
xrcc5	NM_001017360.1	AGAAGTTTGTCCAGCGGCAGGTG	GAGCATCGAGCCAGTCTGCCTG	216	59
xrcc6	NM_199904.1	TCGGAGAGGCTCTGTGGTGCT	CTCCGGGCTTTGAGAGGTGCATC	201	55
rad51	NM_213206	ACTAGCCGTCACCTGCCAGC	ACTGCCCACCAGACCATACCGTT	133	60
gadd45a	NM_001002216.1	AGAGAACCGTGCGCGACACAT	ACAGAGTCCATTCTTTCCGTTGCGT	105	60
β-actin	NM_131031.1	ACACAGCCATGGATGAGGAAATCG	TCACTCCCTGATGTCTGGGTCGT	138	55

5.3   Results

No changes in behaviour of larvae were observed upon addition of H_2O_2 to the fish water. After 10-min exposure to H_2O_2 and rinsing in clean fish water, larvae initially maintained their position motionless on the bottom of the container before resuming normal swimming behaviour. No larvae died 24 h after the 10-min exposure to concentrations of 50 mM H_2O_2 and below; mortality was 27.5 % and 23.1 % at concentrations of 100 and 200 mM, respectively.

DNA strand breaks were induced by exposure to H_2O_2 in both larval cell homogenates (*in vitro*) and in exposed larvae (*in vivo*). Control levels of DNA damage in larvae cell homogenates (*in vitro*, no exposure to H_2O_2) were 26.6 ± 2.1 S.E.M. % tail DNA. Damage increased significantly ($p < 0.001$) to 70.8 ± 2.1 S.E.M. % tail DNA after *in vitro* exposure to 500 μM H_2O_2 (Figure 5-2). In comparison, levels of *in vivo* DNA damage in control larvae was 8.3 ± 3.4 S.E.M. % tail DNA, and larvae exposed to 200 mM H_2O_2 had levels of DNA damage up to 36 ± 1.2 S.E.M. % tail DNA (Figure 5-3). Larvae exposed to H_2O_2 *in vivo* had significantly lower levels of DNA damage 24 h after return to clean water, and damage did not differ significantly from controls (% of tail DNA 8.6 ± 0.8 S.E.M., 200 mM H_2O_2; unexposed control larvae, 11.1 ± 1.6 S.E.M., % tail DNA) (Figure 5-3). An independent *in vivo* exposure to 100 mM H_2O_2 produced levels of DNA damage of 35.4 ± 3.8 % tail DNA (unexposed controls, 17.2 ± 2.0 S.E.M. % tail DNA), and 24-h recovery in clean water confirmed that levels of DNA damage returned to control levels (exposed 9.2 ± 0.4 S.E.M., unexposed controls 9.9 ± 0.9 S.E.M., % tail DNA) (Figure 5-4).
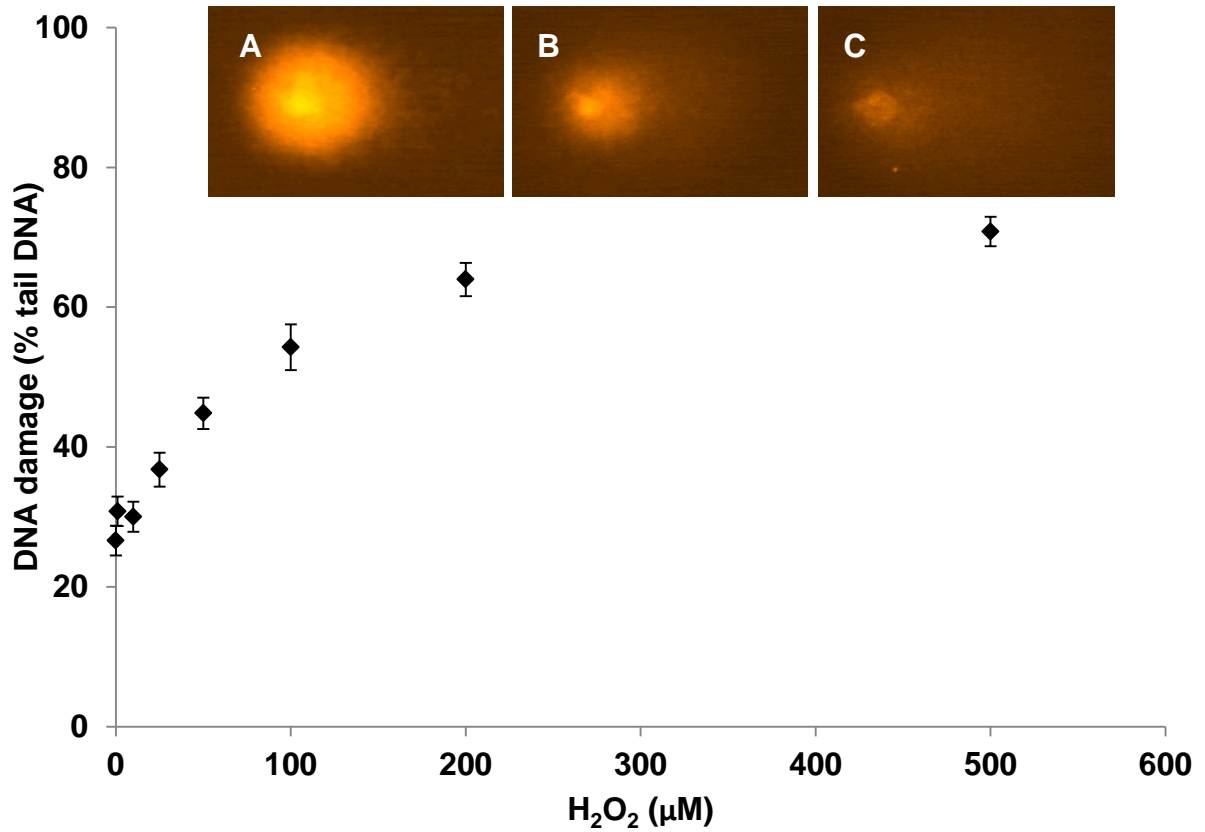
Figure 5-2: Concentration-dependent increase in DNA damage (% tail DNA, comet assay) with exposure to H_2O_2 in mechanically homogenised larvae (72 hpf) exposed *in vitro* for 10 min. Inserts A - C, indicate representative comet assay images with increasing H_2O_2 concentration.
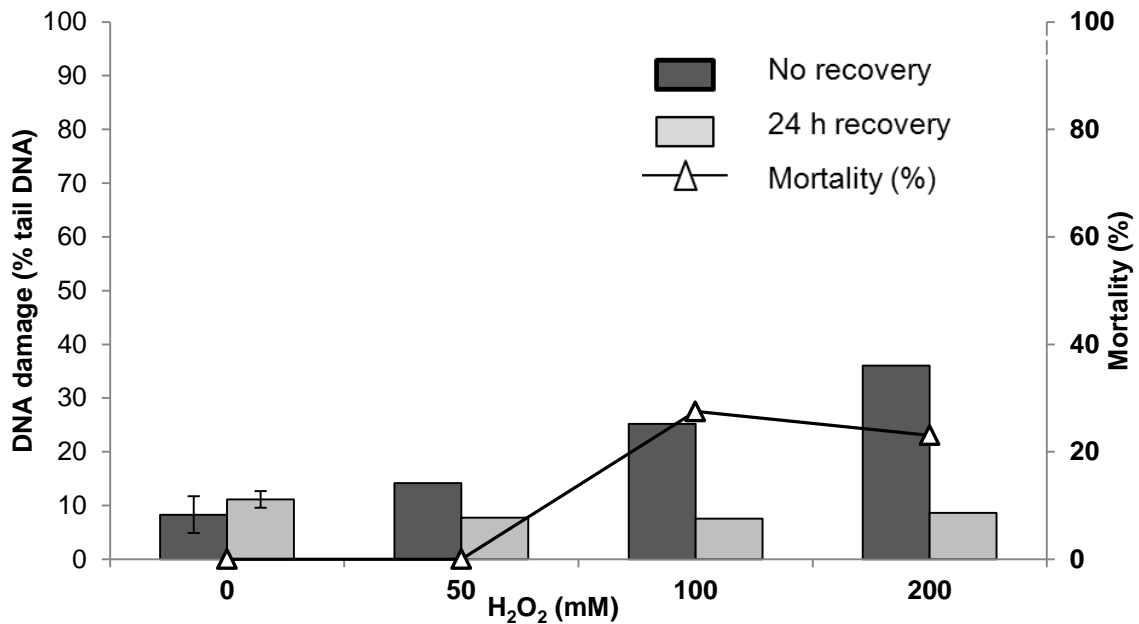
Figure 5-3: DNA damage (% tail DNA, comet assay) in larvae exposed *in vivo* to increasing concentrations of H_2O_2. Dark grey bars indicate larvae sampled immediately after 10 min incubation with H_2O_2, light grey bars indicate larvae exposed for 10 min and allowed to recover in clean water for 24 h. Line graph shows increasing mortality with increasing concentration after 24 h.

There was no statistically significant difference in mean C_t values for *β-actin* between exposed and control larvae at each time point evaluated, therefore use of *β-actin* as a housekeeping gene for normalisation was justified. There was a difference in expression of *β-actin* over time, and larvae sampled between 0 - 12 h post-exposure had significantly lower mean C_t (17.2 ± 0.1 S.E.M., n = 18) compared with larvae sampled at 24 h post-exposure (17.9 ± 0.2 S.E.M., n = 6).
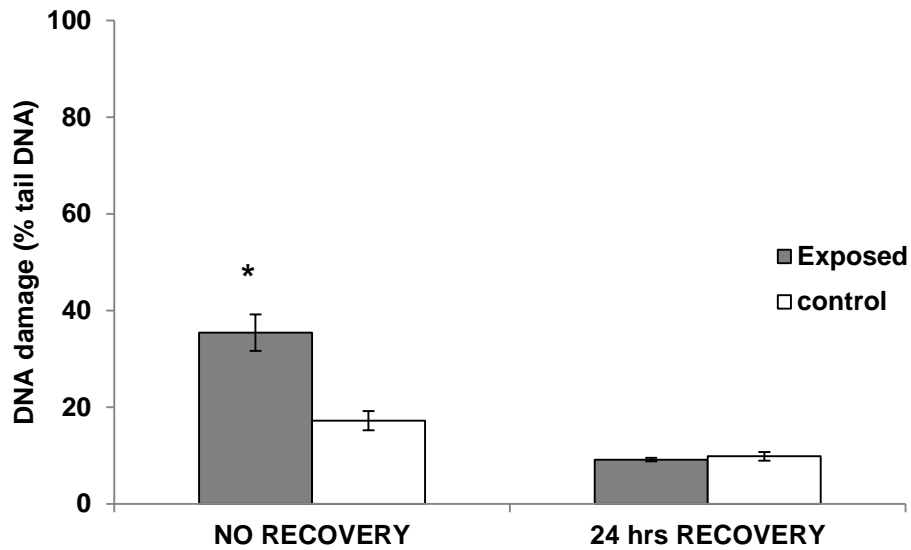
Figure 5-4: DNA damage (% tail DNA) in zebrafish larvae, as analysed by alkaline comet assay. *Significantly higher DNA damage in larvae immediately after 10 min exposure to 100 mM H_2O_2 (arcsine transformed, one-way ANOVA $p < 0.05$); no significant difference in exposed relative to control at 24-h post exposure.

Exposure to H_2O_2 induced expression of *xrcc5*, *xrcc6*, *rad51*, and *gadd45a*. Expression of *rad51* was up-regulated (max. fold change 1.80 ± 0.28, mean ± S.E.M., n = 3) after initial 10-min exposure followed by 6-h recovery in clean water. Comparable levels of induction for *xrcc5*, *xrcc6*, and *gadd45a* were 1.46 ± 0.26, 1.48 ± 0.10, and 1.71 ± 0.31 (mean ± S.E.M., n = 3), respectively (Figure 5-5). The level of *rad51*, *xrcc5*, and *xrcc6* expression returned to control levels after 12-h recovery in clean water, but expression of *gadd45a* remained marginally elevated (mean 1.4 ± 0.1 S.E.M., n = 3) at the same time point (not statistically higher than controls). After 24-h recovery in clean water, expression of *rad51*, *xrcc5*, and *xrcc6* appeared lower than controls (0.83 ± 0.11, 0.76 ±

0.13, and 0.76 ± 0.12, respectively, mean ± S.E.M., n = 3), but this difference was not significant.
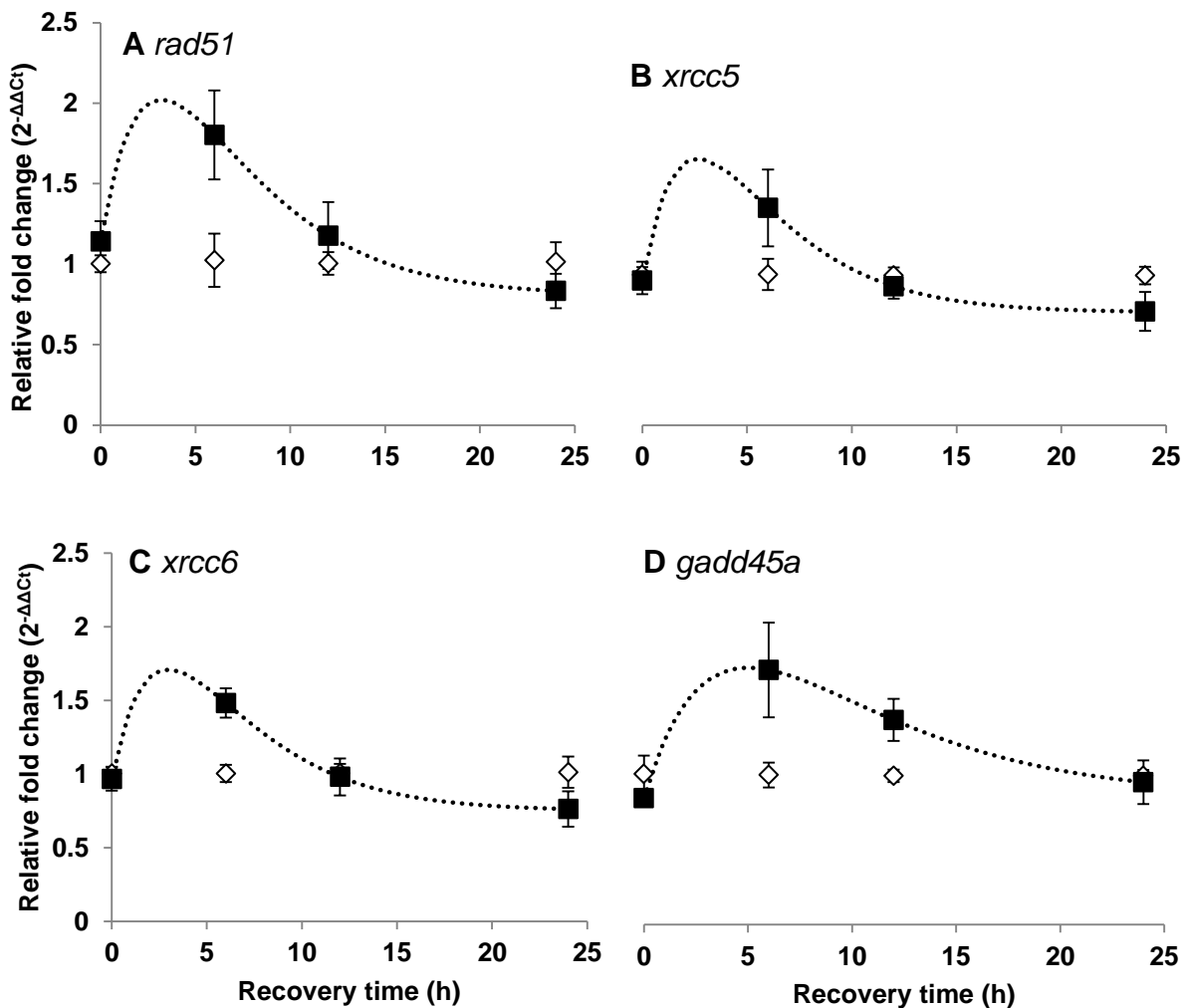
Figure 5-5: Relative fold change ($2^{-\Delta\Delta Ct}$) of DNA repair genes (A – *rad51*, B – *xrcc5*, C – *xrcc6*, and D – *gadd45a*) in zebrafish larvae exposed to 100 mM H_2O_2 for 10min. n = 40 larvae per dish, n = 3 dishes per treatment, n = 6 dishes per timepoint (3 exposed, 3 control), black squares exposed dishes, and white diamonds control dishes. Dotted line indicates fitting of critical exponential curve.

The critical exponential model was fitted to describe the time-course changes in expression profile of the four genes, and the modelled peak expression values were 2.67, 2.98, 3.22, and 5.02 hours for *xrcc5*, *xrcc6*, *rad51*, and *gadd45a*, respectively (Figure 5-6, Table 5-2). The model suggested that expression of *gadd45a* did not decrease as rapidly as the other three genes. A comparison between the model parameters (Table 5-2) suggested that *xrcc5* and *xrcc6* had similar and closely-matched peak expression profiles.
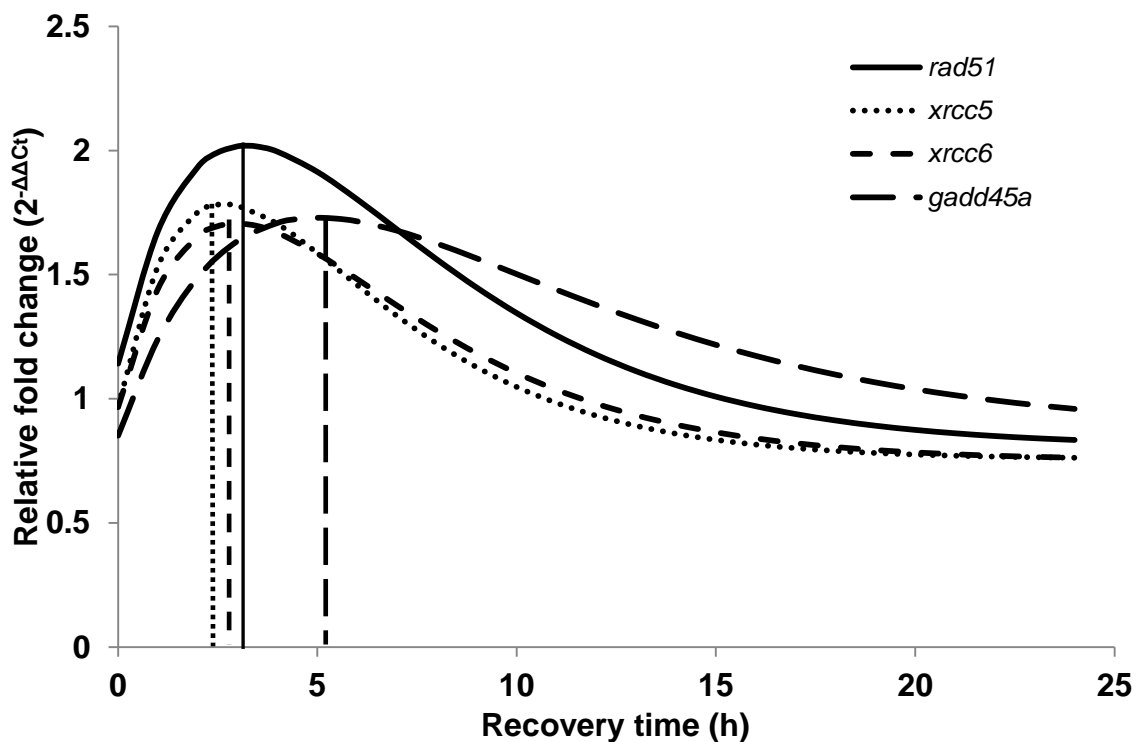
Figure 5-6: Comparison of modelled time-course profiles of relative fold change in gene expression of DNA repair genes, *rad51* (solid line), *xrcc5* (dotted line), *xrcc6* (short dashed line), and *gadd45a* (long dashed line), expressed in zebrafish larvae exposed to 100 mM H_2O_2 for 10 min, followed by increasing recovery time in clean water. Model: $y(x) = A + (B+Cx)R^x$, where A, B, C, and R are parameters, *y* is the fold-change gene expression response, and *x* is time (adapted from Eastwood *et al.*, 2008). Time of peak expression is indicated by dotted lines.

Table 5-2: Parameters of fitting a critical exponential curve, $y(x) = A + (B + Cx) R^x$, where A, B, C, and R are parameters, y is the fold-change ($2^{-\Delta\Delta Ct}$) gene expression response, and x is time. Fitted model used to determine estimated timing of peak in gene expression response, and magnitude of estimated response.

Gene	Fitted parameters[a]				Estimated response	
	A	**B**	**C**	**R**	**Time of peak response (h)**	**Peak response ($2^{-\Delta\Delta Ct}$)**
rad51	0.8073	0.3341	0.509	0.7595	3.22	2.02
xrcc5	0.7569	0.2121	0.8836	0.7091	2.67	1.78
xrcc6	0.751	0.2153	0.735	0.7371	2.98	1.71
gadd45a	0.8666	-0.0153	0.4726	0.8185	5.02	1.73

[a](after Eastwood *et al.*, 2008)

5.4 Discussion

Exposure to H_2O_2 generated DNA strand breaks in larval zebrafish that were detected by the comet assay. H_2O_2 was selected as a model genotoxicant as it induces strand breaks through production of reactive oxygen species (Horváthová *et al.*, 1998), but is rapidly neutralized and does not persist in the organism (i.e., no residual effects) (Chuang *et al.*, 2002; Reeves *et al.*, 2008). Although there are concerns regarding heterogeneity of cell response after H_2O_2 exposure (Kruszewski *et al.*, 1994; Fairbairn *et al.*, 1995), H_2O_2 is commonly used as a reference toxicant for genotoxicity studies (including zebrafish) (Oggier *et al.*, 2011) and is particularly suited for time-course experiments (Chuang *et al.*, 2002). The high reactivity of H_2O_2 complicates inter-experiment comparisons of concentration responses (e.g. between the *in*

vitro and *in vivo* exposures) because it is difficult to establish that H_2O_2 is of similar potency among different experiments. Exposure of larvae to 100 mM H_2O_2 is likely to have primarily induced single-strand breaks in DNA (Vandersickel *et al.*, 2010b), however, double-strand breaks can form when high levels of accumulated single strand breaks occur in close proximity (Jin *et al.*, 2011). The alkaline version of the comet assay assesses predominantly single- but also double-strand breaks and is an indicator of overall DNA strand fragmentation (Collins, 2004). *In vivo* exposure in the present study resulted in levels of DNA strand breaks consistent with levels detected in zebrafish larvae exposed to 1 % H_2O_2 (presumed equivalence of 8.8 mM, 7 – 8 % tail DNA) (Oggier *et al.*, 2011). Detection of reduced DNA damage after the 24-h recovery period indicates that repair of fragmented DNA occurred.

Following DNA damage in zebrafish larvae, genes involved in DNA repair processes were induced. The three DNA repair genes investigated (*xrcc5*, *xrcc6*, and *rad51*) were induced up to 2-fold, and this induction was consistent with the relatively low levels of induction observed in other studies [e.g., *in vitro* exposure to copper resulting in approx. 1.2- and 1.4- fold induction of Ku80/*xrcc5* (Sandrini *et al.*, 2009a,b)]. However, *rad51* can be highly induced in adult zebrafish, and a 32-fold change in expression has been reported in liver after 7-d exposure to cadmium (Gonzalez *et al.*, 2006), and 5-fold change in gills after 8-d exposure to uranium (Lerebours *et al.*, 2009). The similarity in extent of induction of genes involved in NHEJ and HR suggests that both repair pathways were activated as a consequence of the H_2O_2 exposure, which agrees with suggestions that DNA repair pathways are not mutually exclusive (Takata *et al.*, 1998; Rapp and Greulich, 2004). DNA repair genes can be

constitutively expressed in cells (Kim *et al.*, 2001; Bladen *et al.*, 2005) due to the importance of DNA repair on cell survival, and have been used as housekeeping genes (Iwanaga *et al.*, 2004). Therefore, the relative population of DNA repair gene mRNA within actively dividing cells may be constantly high (Lu and King, 2009), and subtle relative induction in expression appears sufficient to address increased levels of DNA damage.

The H_2O_2 exposure (10 min) induced up-regulation of *gadd45a* in larvae that was observed after 6- and 12-h recovery in clean water. Growth arrest and DNA damage-inducible (GADD) proteins are involved in cell cycle regulation (Kawahara *et al.*, 2005), and induction of *gadd45* has been reported after exposure to ionizing radiation (Hollander *et al.*, 1993) and metals (Gonzalez *et al.*, 2005; Gonzalez *et al.*, 2006). Expression of *gadd45* was induced 55-fold after 7-d exposure to cadmium (Gonzalez *et al.*, 2006), and this high induction indicated potential use of *gadd45* expression as a positive control to detect DNA damage. In this study, exposure to H_2O_2 did not result in high induction of *gadd45a* (max. fold change 1.7 ± 0.3 S.E.M.), suggesting either a role in long-term cell-cycle regulation beyond the 24-h study period, or involvement of other gene variants encoding for the diverse GADD45 family of proteins (Moskalev *et al.*, 2012).

The profile of the gene expression response varied over time for all genes evaluated. Timing of induction may be of particular importance for DNA repair-related genes, as the timing of repair genes has been suggested to be critical (*in vitro*) in triggering alternative pathways to repair, such as apoptosis (Kim and Hyun, 2006). If a single timepoint is selected for analysis of gene expression without knowledge of the expression profile over time, conclusions made about

treatment effects on gene expression may not be valid (Chechik and Koller, 2009). As expected, the expression and time profile of *xrcc5* and *xrcc6* were similar perhaps because they are involved in the same NHEJ repair mechanism; however, *gadd45a* appeared to reach highest expression level later than *xrcc5* and *xrcc6* suggesting its involvement in subsequent cellular processes. While further evidence is required to understand the relations between gene expression profiles among genes and how these relate to cellular physiology, models of the gene expression profiles can give some indications from which hypotheses can be drawn for subsequent testing.

The critical exponential model suggested that time-related expression profiles of *xrcc5*, *xrcc6*, *rad51*, and *gadd45a* were similar. The model indicated that the maximum expression for each gene occurred after the initial measured gene expression timepoint (0 h), but before the measured 6-h post-exposure samples, and thus the interpretation of the modelled time profile is somewhat speculative. The model was selected because the shapes of the time profiles of the genes selected in this study were similar to other transcription profiles (for genes involved in fungal morphogenesis) where the model has been applied (Eastwood *et al.*, 2008). The model can be applied to microarray data as an improved method for clustering gene responses from large datasets, but applying this model to individual gene expression profiles allows for comparisons in model parameters (Table 5-2) of key genes of interest, reflecting underlying cell processes beyond simple comparisons of significant differences at certain times (Eastwood *et al.*, 2008). The *xrcc* genes modelled in this study had similar values for parameter A, which dictates the magnitude of response, and parameter B, which dictates the initiation of response. Modelled

expression of *rad51* differed from the *xrcc* genes in parameters A and B, suggesting that it is functionally distinct, despite overall similarity in the shape of the curve (parameter C). A comparison between fitted model parameters for *gadd45a* and the three DNA repair genes indicate reduced initial (0 h) amount of *gadd45a* mRNA (negative parameter B), suggesting a delayed induction of response and functional dissimilarity from the action of DNA repair. A limitation in modelling the response of the selected genes in this study is lack of early timepoint samples to verify early induction of gene response. More resolution in characterising the early induction period (0 – 6 h post-exposure), in addition to extending the baseline expression before damage was induced, would allow for alternative modelling approaches (e.g. the 'split-line' model proposed to evaluate time of gene induction) (Eastwood *et al.*, 2008).

5.5   Conclusion

Damage in DNA is induced in zebrafish larvae exposed to H_2O_2 and this damage is repaired rapidly following induction of various genes involved in DNA repair. Up-regulation of *xrcc5*, *xrcc6*, and *rad51* is consistent with induction of both NHEJ and HR repair pathways within 6 h of recovery. Induction of gene expression was subtle (below 2-fold change), in agreement with other studies, and within the expected context of genes that are constitutively expressed in cells. Establishing time-related changes in expression of the genes evaluated in this study is important because, if single timepoints are selected inappropriately, incorrect conclusions can be drawn. For example, if samples were collected only at 24 h post-exposure in the present study, no change in expression of DNA repair genes would have been detected. Modelling profiles of gene expression changes can enable comparisons in the timing of induction among

genes and permit hypotheses to be drawn regarding the cellular processes that are influenced by the gene products. In the context of the present study, expression profiles of xrcc genes were similar (initiation of induction, time of peak induction, and magnitude of induction), and shared an overall response curve shape with *rad51*, supporting the involvement of both repair pathways (NHEJ and HR) in the overall repair response to damage by H_2O_2. The gene expression profile of the functionally distinct *gadd45a* pathway was illustrated by differences in model parameters (compared with the DNA repair genes) that indicated differences in timing of the cellular processes that these gene products support.

6:

Genotoxicity in male zebrafish (*Danio rerio*) exposed to cobalt, with implications on reproduction and expression of DNA repair genes

Hypothesis 1: After detection of concentration-related damage in sperm (DNA strand breaks, comet assay), DNA repair genes will be induced in zebrafish testes exposed to cobalt.

Hypothesis 2: Reproductive success (egg output, fertilisation success, and larval development) will be affected by chronic exposure to cobalt.

Hypothesis 3: Expression of DNA repair genes will be affected in larvae spawned from adult zebrafish chronically exposed to cobalt.

Abstract

Although cobalt (Co) is an environmental contaminant of surface waters in both radioactive (e.g. ^{60}Co) and non-radioactive forms, there is relatively little information about Co toxicity in fishes. The objective of this study was to investigate acute and chronic toxicity of Co in zebrafish, with emphasis on male genotoxicity and implications for reproductive success. The lethal concentration for 50 % mortality (LC$_{50}$) in larval zebrafish exposed (96 h) to 0 – 50 mg l^{-1} Co was 35.3 ± 1.1 (95 % C.I.) mg l^{-1} Co. Adult zebrafish were exposed (13 d) to sub-lethal (0 - 25 mg l^{-1}) Co and allowed to spawn every 4 d and embryos were collected. After 12-d exposure, fertilization rate was reduced (6 % total eggs fertilized, 25 mg l^{-1}) and embryo survival to hatching decreased (60 % fertilized eggs survived, 25 mg l^{-1}). A concentration-dependent increase in DNA strand breaks was detected in sperm from males exposed (13 d) to Co, and DNA damage in sperm returned to control levels after males recovered for 6 d in clean water. Induction of DNA repair genes (*rad51*, *xrcc5*, and *xrcc6)* in testes was complex and not directly related to Co concentration, although there was significant induction in fish exposed to 15 and 25 mg l^{-1} Co relative to controls. Induction of 4.0 ± 0.9, 2.5 ± 0.7, and 3.1 ± 0.7 fold change (mean ± S.E.M. for *rad51*, *xrcc5*, and *xrcc6*, respectively) was observed in testes at the highest Co concentration (25 mg l^{-1}). Expression of these genes was not altered in offspring (larvae) spawned after 12-d exposure. Chronic exposure to Co resulted in DNA damage in sperm, induction of DNA repair genes in testes, and indications of reduced reproductive success.

6.1 Introduction

Cobalt (Co) can become an environmental contaminant of concern when released in high concentrations from industries including mining and mineral processing (Lauwerys and Lison, 1994). The most soluble and stable form of dissolved Co in freshwater of neutral pH is Co(II) oxidation state (Co^{2+}) (Collins and Kinsela, 2010, Blust, 2012), and environmental concentrations vary from very low (range from ng – ug l^{-1} in freshwater) to contaminated sites with water concentrations > 3 mg l^{-1} (Collins and Kinsela, 2010; Payne, 1977). Ground and surface water concentrations from contaminated mining sites have been measured up to 625 mg l^{-1}, which are well above the recommended limit of 8 μg l^{-1} indicated for protection of freshwater organisms (Kim *et al.*, 2006). The radionuclides ^{60}Co is released into the environment through nuclear weapons testing and radioactive waste (Valković, 2000d) and 37 GBq were reported to be released into the air at one site over three decades (1944 – 1972, Hu *et al.*, 2010). Both stable and radioactive Co are potential toxicants to organisms in surface waters.

Aqueous cobalt (Co, predominantly Co^{2+}) can be absorbed by fish and can accumulate within tissues. In whole adult zebrafish, the bioconcentration factor (BCF) was 74 after 14-d exposure to aqueous ^{60}Co (Reinardy *et al.*, 2011), and was consistent with Co BCF values reported for other freshwater fish species (Blust, 2012). Co-60 bioaccumulated in gonads (BCF 13) and, after return to clean water, rapid depuration reduced whole body concentrations by over 50 % within 24 h (Reinardy *et al.*, 2011). Bioaccumulation of Co in fish is similar to other divalent metals, and other metals (including Cu, Cr, Zn, and Pb) are reported to bioaccumulate in gonads (Celechovska *et al.*, 2007; Terra *et al.*,

2008). Compared with other divalent metals (e.g. Cu and Zn) there is considerably less information on uptake, bioaccumulation, and toxic effects of Co.

Information to date indicates that the biological effects of Co are dependent on the exposure concentration and extent of bioaccumulation. Co (as the main component of cobalamin) is an essential nutritional component and is involved in many cellular processes including formation of vitamin B12 (Banerjee and Ragsdale, 2003). Without sufficient dietary Co supplementation, carp (*Cyprinus carpio*) had reduced weight gain and specific growth rates (Mukherjee and Kaviraj, 2009). Conversely, aqueous concentrations above 10.8 mg l^{-1} resulted in reduced time to hatching and lower survival of zebrafish larvae (Dave and Xiu, 1991), and oxidative injury at concentrations of over 100 mg l^{-1} in goldfish, *Carassius auratus* (Kubrak *et al.*, 2011). Exposure to some metals can negatively affect fish reproduction (Boyle *et al.*, 2008; Simon *et al.*, 2011), but consequences of exposure to Co on fish reproduction are unknown.

Cobalt is a genotoxicant that can cause DNA damage and chromosomal fragmentation (e.g., Figgitt et al., 2010) and, if genotoxicity occurs in germ cells, negative effects on reproductive success are possible (Anderson and Wild, 1994). DNA damage in sperm can lead to transfer of damaged DNA to offspring (Dubrova, 2003) and the level of DNA damage in sperm can indicate paternal exposure to genotoxicants (Sipinen *et al.*, 2010). Genetic damage from sperm is important in overall embryo success (Anderson and Wild, 1994), and paternal DNA damage (in sperm) has been linked to disease in offspring (Aitken *et al.*, 2004). Sperm motility characteristics of fish can be altered by exposure to zinc and cadmium (Kime *et al.*, 1996), and reproductive success has been reported

to be adversely affected by exposure to uranium (Simon *et al.*, 2011) and arsenic (Boyle *et al.*, 2008), but there are no studies on genotoxicity and reproductive impacts of Co exposure in fish. In marine invertebrates, paternal exposure to genotoxicants resulted in significantly elevated levels of DNA damage in sperm and an increase in developmental abnormalities in their offspring (Lewis and Galloway, 2009). Although metals can bioaccumulate in gonads, induce genotoxicity (based on evidence from *in vitro* exposures), and affect reproduction, the extent that negative effects on reproduction are mediated by metal-induced genotoxicity in sperm is unknown.

Repair of damaged DNA in is critical to prevent transfer of impaired genetic material to offspring via germ cells. DNA repair mechanisms in eukaryotic cells are constitutively active to repair DNA damage caused by both endogenous agents and exogenous genotoxicants (Shin *et al.*, 2004). However, little is known about mechanisms of repair of metal-induced DNA damage in fish; although in some tissues (including brain, liver, gill, and skeletal muscle) genes involved in repair of single- and double- strand breaks have been shown to be up-regulated after exposures to uranium (Lerebours *et al.*, 2009) and cadmium (Gonzalez *et al.*, 2006). Repair of DNA is limited in spermatozoa due to tight packaging of genetic material and the potential longevity of the spermatozoa (Aitken *et al.*, 2004; Lewis and Galloway, 2009), therefore repair of damaged DNA could take place in testes or in the fertilised embryo. Induction of DNA repair genes in fish testes has not been investigated, and the tissue-specific induction of repair following genotoxicity of metals is not known.

The objectives of this study were first to establish the acute toxicity of aqueous Co in zebrafish larvae; and second, to investigate the chronic toxicity of

aqueous Co in adult zebrafish. The investigation focused on male genotoxicity (DNA damage in sperm), the capacity for DNA repair (expression of specific genes involved in DNA strand break repair in testes), and overall reproductive success (spawning success and survival of larvae).

6.2 Methods

6.2.1 Fish

Zebrafish were obtained from the Zebrafish Research Facility, Plymouth University, and maintained under routine approved animal welfare protocols. Photoperiod was 12 h, and stock fish were fed three times daily with live brine shrimp nauplii, *Artemia* sp. or dry fish flake mix (equal proportions ZM Systems flake, brine shrimp, spirulina, and TetraMin® stable flake). Larvae were routinely bred from bulk spawning of stock fish. Developing larvae were kept in plastic dishes (90 mm diameter, 50 ml) with daily water changes to remove unfertilized or un-developing eggs, and debris. Hatched embryos, 72 hours post fertilisation (hpf), were used for acute larval exposures. Sex of stock adults was determined through visual identification, and individuals were selected after successful pair spawning resulting in fertile eggs. For the chronic adult exposure, fish were placed in 10-L experimental static tanks, with 50 % water changes daily. Water quality parameters were measured daily (mean pH: 7.0; dissolved oxygen: 6.8 mg l^{-1}; temperature: 26.6 $°C$; total ammonia: 1.2 mg l^{-1}).

6.2.2 Acute exposure of larvae

Unless specified, all chemicals were sourced from Sigma Aldrich (Sigma-Aldrich Company Ltd., Dorset, UK). To confirm appropriate sub-lethal concentrations for adult zebrafish exposure, two trials with zebrafish larvae were conducted to

establish acute toxicity of Co. For each trial, larvae were exposed (96 h), in four separate experiments with different batches of fish (n = 20 per beaker, three beakers per concentration), to Co (0, 5, 10, 20, 30, 40, and 50 mg l^{-1}, Co). The first trial exposed larvae to Co, added as $CoCl_2$, and the second trial exposed larvae to Co, added as $CoSO_4$, to elucidate any toxic effects of ion carrier. Mortality after 96 h exposure was recorded and concentration of Co resulting in 50 % mortality (LC_{50}) was computed and compared between $CoCl_2$ and $CoSO_4$.

6.2.3   Chronic exposure of adult zebrafish

6.2.3.1   Experimental design

Eight experimental tanks were randomly assigned a treatment of control (triplicate tanks, zero added Co, nominally 0 mg l^{-1} Co, measured water concentrations range 0.07 – 0.4 mg l^{-1}, mean 0.2 mg l^{-1}, n = 11 control water samples), 5, 10, 15, 20, or 25 mg l^{-1} Co. A stock solution of 100 g Co l^{-1} was made up from $CoCl_2 \cdot 6H_2O$ (Sigma Aldrich). After the daily water change, tanks were spiked with 0 - 2.5 ml stock solution to reach the nominal concentrations. Exposure was confirmed by analysis of Co by inductively coupled plasma optical emissions spectroscopy (ICP-OES, Varian 725 ES, Mulgrave, Australia) in exposure water sampled over 4 days at start of exposure (n = 4 water samples per tank). Water samples (5 ml) were acidified with nitric acid and analysed for Co and Pt. Analytical grade reference material was used to generate a standard curve and 1 % Pt was added to all samples, standards, and blanks as an internal instrument control. Co was detected at two wavelengths (230.786 and 238.892 nm) with an instrument detection limit of 0.05 µg l^{-1}, and average emissions (counts sec^{-1}) were converted to concentrations according to the input standard curve.

Selected fish were placed in eight 10-L tanks and males and females (n = 6 pairs) were separated by a screen divider that allowed free movement of water and prevented spawning. A preliminary bulk spawning event (before addition of Co) initialised a 4-d spawning routine, which continued during exposure (3 spawning events in total over 12-d exposure). For spawning, fish were placed into a separate 10-L spawning tank containing a glass grid raised 10 cm from base of tank to allow eggs to fall through (Figure 6 1). Fish were allowed to spawn for 3 - 4 h before being returned to their original exposure tank. With the fish removed from the spawning tank the glass grid was lifted, eggs were siphoned into beakers, and pipetted into plastic dishes for counting.

Figure 6-1: Bulk spawning tank with glass grid to allow eggs to fall through for collection after spawning.

At the end of the exposure (12-d) the fish were spawned for a final time, and 3 males were sampled for testes. For assessment of DNA damage, sperm from the remaining 3 males were sampled the day after the final spawning event (d 13) and 100 % water change ensured all Co was removed and exposure ended. After sperm sampling [following non-invasive procedure (Chapter 4)], males were returned to clean water for 6 d, after which sperm was sampled from the same males for assessment of DNA damage after recovery period.

All non-fertile eggs (no distinct chorion, small, discoloured) and all fertile un-developing eggs (distinct chorion, normal sized, discoloured, no distinct yoke or blastodisc) were discarded. Fertile and normally developing embryos (distinct blastomeres, normal shape and size, no discolouration) (Kimmel *et al.*, 1995) were transferred to clean water in dishes and kept at 26 ˚C for 4 d. Any embryos that ceased developing were counted and discarded during the daily water changes. At 96 hpf hatched embryos were counted and any abnormal malformed larvae were noted.

6.2.3.2 *DNA damage in sperm*

Following non-invasive sampling, sperm samples were kept on ice for same-day analysis of DNA damage by alkaline single-cell gel electrophoresis (comet) assay. Comet assay assessment of DNA damage in sperm was performed following validated protocols for zebrafish (Chapter 4). Individual sperm samples (total volume approximately 1-2 µl) were diluted in 100 µl Hank's buffered saline solution (HBSS), and 5 µl of diluted sperm was mixed with 180 µl low melting point agarose to give a dilution of approx. 60,000 cells. The cell/agarose mixture was dropped onto glass slides pre-coated with normal melting point agarose, covered with a glass cover slip and left to set for 1 hr at 4

°C. Cells were lysed for 1 hr by immersing slides in buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10), and DNA was unwound in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min before being run on electrophoresis for 20 min (25V, 280-350 mA). Slides were neutralised (0.4 M Tris HCl, pH 7.5) for 10 min and washed in distilled water for a further 10 min before being left to dry overnight (4 ^{0}C). Dried slides were stained with ethidium bromide (10 mg ml^{-1}) and 100 nucleoids were scored per sample (Leica DMR fluorescent microscope, Komet 5, Kinetic Imaging, Nottingham) following routine procedures (Collins, 2004; Kumaravel and Jha, 2006).

6.2.3.3   RNA extraction and cDNA synthesis

Gene expression analysis was carried out in larvae and excised testes following chronic exposure to Co. For RNA extraction, 30 larvae per sample (72 hpf, hatched, n = 1 - 3 samples per tank) were mechanically homogenised in RLT buffer before storage at -80 ^{0}C, and testes were dissected from individual males and frozen at -80 ^{0}C. Total RNA was extracted following manufacturers protocol (RNeasy MiniKit for animal tissue, Qiagen) with initial sonication (3 - 5 secs), additional tissue break-up with QiaShredder column, and a 15 minute DNase treatment. RNA was eluted into 30 µl and the concentration and quality of total RNA was determined by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer). All samples were diluted to 100 ng ul^{-1} total RNA, and 800 ng were used to synthesise cDNA following the manufacturer's protocol for ImProm-II^{TM} Reverse Transcription System (Promega), with hexanucleotide primers and deoxynucleotide mix (Sigma-Aldrich). cDNA was synthesized under the following conditions: annealing at 25 ^{0}C, extending at 42 ^{0}C, and heat-

inactivating transcriptase at 70 ^{0}C (GeneAmp® PCR System, 9700, Applied Biosystems). cDNA was stored at -80 ^{0}C until q-RT-PCR gene expression analysis.

6.2.3.4   Selection of primers for DNA repair genes

Primers were selected by Primer Blast (NCBI). The amplicons were designed to span 1 intron junction, and were checked to avoid secondary structure, self-annealing, complementarity, and potential hairpins by DNA calculator (Sigma-Aldrich) and OligoCalc (Northwestern University, USA). Amplicon size was verified on a 2 % agarose gel after PCR amplification. Primer details are listed in Table 6-1.

6.2.3.5   Quantitative reverse transcriptase PCR (qRT-PCR)

Lyophilised primers (Eurofins MWG Operon, Ebersberg, Germany) were reconstituted to 100 µmol with RNase-free water and mixed with SYBR Green JumpStart *Taq* ReadyMix to give a final reaction concentration of 375 nmol in 20 µl total volume. Fluorescence was detected (StepOne Real-Time PCR System, Applied Biosystems) over 40 cycles, cycling conditions of 94 ^{0}C for denaturing, primer-specific annealing 55-60 ^{0}C (Table 6-1), and extension at 72 ^{0}C. For analysis, the cycle threshold was set to 25,000 for all qPCR runs. A standard curve of cDNA template (pooled template from each sample within experiment), was run on each plate to allow for within-experiment plate normalisation.

Table 6-1: Zebrafish (*Danio rerio*) gene specific primers for DNA repair genes (*xrcc5, xrcc6,* and *rad51*), growth arrest gene (*gadd45a*), and housekeeping gene (*β-actin*). Reference sequence numbers from NCBI, and product length in base pairs (bp).

Gene	Ref seq no.	Forward (5'-3')	Reverse (5'-3')	Product (bp)	Annealing temp (^{0}C)
xrcc5	NM_001017360.1	AGAAGTTTGTCCAGCGGCAGGTG	GAGCATCGAGCCAGTCTGCCTG	216	59
xrcc6	NM_199904.1	TCGGAGAGGCTCTGTGGTGCT	CTCCGGGCTTTGAGAGGTGCATC	201	55
rad51	NM_213206	ACTAGCCGTCACCTGCCAGC	ACTGCCCACCAGACCATACCGTT	133	60
gadd45a[a]	NM_001002216.1	AGAGAACCGTGCGCGACACAT	ACAGAGTCCATTCTTTCCGTTGCGT	105	60
β-actin	NM_131031.1	ACACAGCCATGGATGAGGAAATCG	TCACTCCCTGATGTCTGGGTCGT	138	55

6.2.4 Statistical analyses

Statistical analyses were conducted with STATGRAPHICS 5.1 (Statistical Graphics Corp., USA). LC_{50} of Co was established by logistic regression ($p = e^{logit(p)}/1 + e^{logit(p)}$) where $logit(p) = a + bx$, a and b are constants and x is Co (mg l^{-1}). Comet assay data (% tail DNA) was arcsine transformed before analysis. Estimation of bioaccumulation of Co in testes was calculated from the reported BCF for ^{60}Co (Reinardy *et al.*, 2011) applied to the analysed water concentrations of Co.

For gene expression analysis, the efficiency of qRT-PCR was calculated ($e = 10^{(-1/slope)} -1$) from the standard curve for each plate. Samples from the same experiment run over multiple plates were adjusted to the plate with the efficiency closest to 1 by resolving for slope and intercept of the standard curves. Only efficiencies between 0.9 – 1.1 were accepted for further analysis, and comparative quantification ($2^{-\Delta\Delta Ct}$) was used for calculating fold-changes in the gene of interest normalised to *β-actin*, and to control tanks (0 Co, $\Delta\Delta Ct$) (Henry *et al.*, 2009).

6.3   Results

No changes in behaviour of larvae were observed after addition of Co and there was no mortality in control larvae (0 mg l^{-1} Co). Mortality increased with Co concentration (logistic regression, p < 0.05, Figure 6-2), and the 96-h Co concentration that resulted in 50 % mortality (96-h LC_{50}) was 35.3 ± 1.1 mg l^{-1} (mean ± 95 % C.I., n = 20 larvae per sample, n = 8 independent exposures). There was no significant difference in acute toxicity between $CoCl_2$ and $CoSO_4$, and respective LC_{50} values were 34.7 ± 1.5 and 36.0 ± 1.6 mg l^{-1} (mean ± 95 %

C.I., n = 4 independent exposures). Concentrations of ≤ 25 mg l^{-1} $CoCl_2$ were selected for the chronic Co exposures in adult zebrafish.

Addition of $CoCl_2$ for chronic exposure in adult fish resulted in some initial behavioural changes (reduced swimming), but normal behaviour resumed within 2-3 h. Two female fish died during the exposure and both appeared to have external lesions associated with aggressive interactions among fish. Water Co concentrations were within 81 ± 13.3 % (mean ± s.d., n = 28 water tests) of nominal. Accumulation of Co into whole body and testes, computed from measured aqueous Co concentrations (present study) and reported BCFs of 74 (whole body) and 13 (gonads) (Reinardy $et~al.$, 2011), was estimated to be up to 1.37 and 0.24 mg g^{-1} respectively (Figure 6-3).
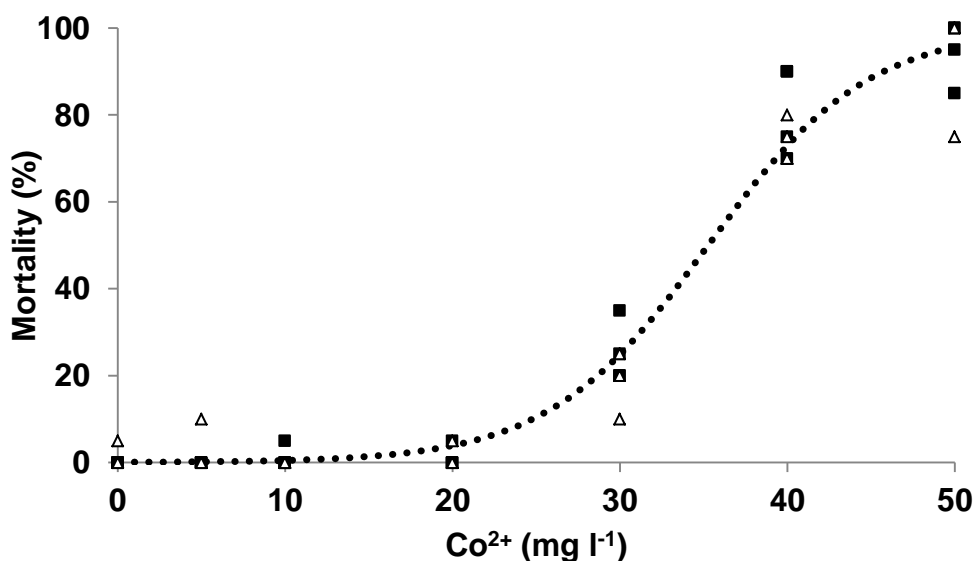
Figure 6-2: Concentration response in 72 hpf zebrafish larvae exposed for 96 h to Co, added as $CoCl_2$ (black squares) or $CoSO_4$ (open triangles). No significant difference between $CoCl_2$ and $CoSo_4$ (Logistic regression, p > 0.05), LC_{50} was 34.7 ± 1.5 and 36.0 ± 1.6 mg l^{-1} (mean ± 95 % C.I., n = 20 larvae per sample, n = 4 independent exposures) for $CoCl_2$ and $CoSO_4$, respectively. Combined LC_{50} is 35.3 ± 1.1 mg l^{-1} (mean ± 95 % C.I., Logistic regression, p < 0.05), n = 20 larvae per sample, n = 8 independent exposures.

Reproduction was adversely affected by chronic exposure to aqueous Co. The total number of fertilised eggs was reduced over time with increasing Co concentration (% of total eggs fertilised, d 12 control mean ± S.E.M. 82 ± 9.6 %, d 12, 25 mg l^{-1} Co 5.6 %, GLM, p < 0.05) (Figure 6 - 3). At higher Co concentrations (10, 20, and 25 mg l^{-1} Co) there was a trend of lower rates of cumulative egg production and fertilization success over time (Table 6 - 2; Figure 6 - 3). Embryo mortality appeared higher at higher Co concentrations, although this trend was not significant (% mortality, d 12 control mean ± S.E.M. 22 ± 4.7 %, d 12, 25 mg l^{-1} Co 40 %, GLM, p = 0.052) (Figure 6 - 2).

Chronic exposure to Co did not affect sperm sampling success; sampling was unsuccessful from 3 males (12.5 %), out of a total of 24 males sampled sub-lethally for sperm (3 per tank, 8 tanks). DNA damage in sperm increased with Co concentration (GLM, p < 0.05, n = 1 - 3 males per tank) (Figure 5-4). After 6-d recovery in clean water there was no difference among concentrations on levels of DNA damage in sperm (GLM, p > 0.05). The highest level of sperm DNA damage (70 % tail DNA) occurred in a single male (unable to sample from other two males) exposed to 20 mg l^{-1} Co; however, even with removal of this high value from the analysis, DNA damage in sperm increased with Co concentration (GLM, p < 0.05, n = 2 – 3 males per tank). In the highest Co concentrations (15 - 25 mg l^{-1} Co), the mean level of DNA damage in sperm was 52 % tail DNA, compared with < 30 % tail DNA for concentrations below 10 mg l^{-1} Co.
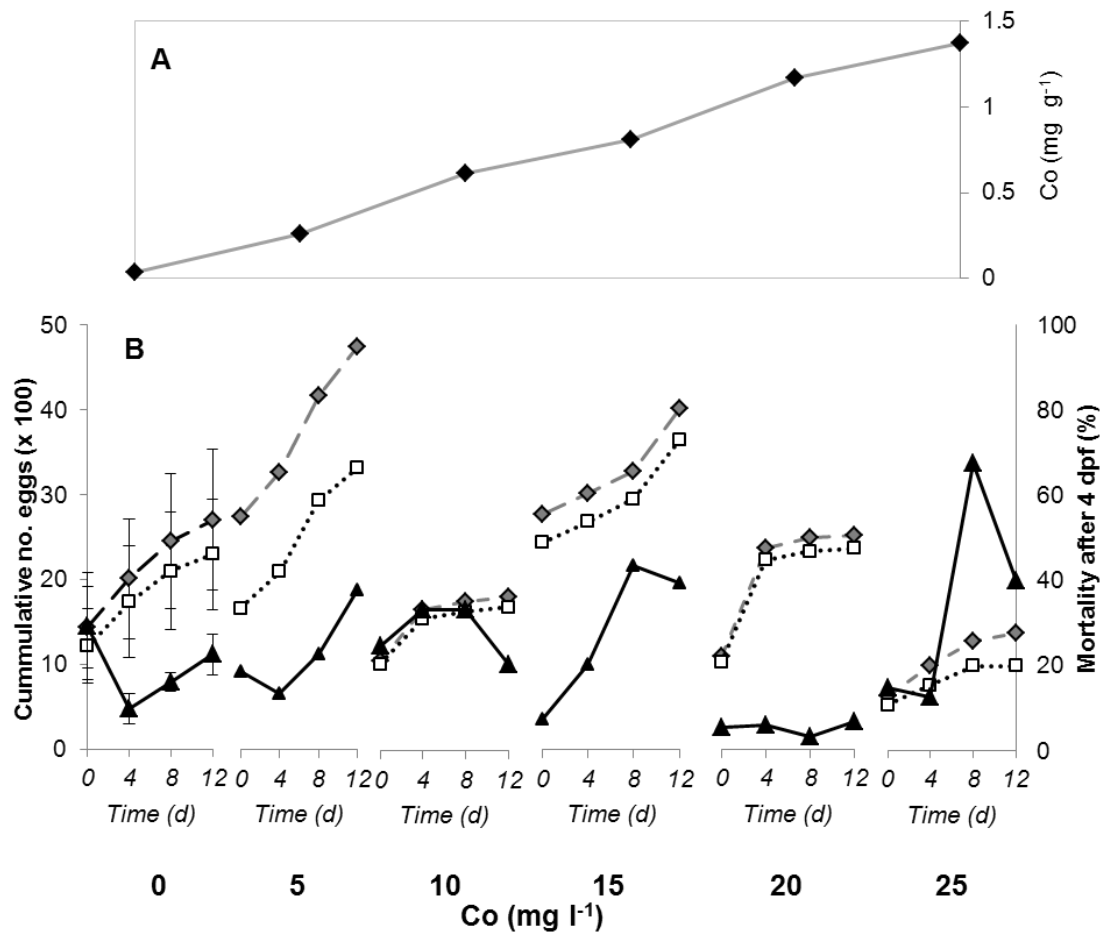
Figure 6-3: Reproductive success and larval mortality in zebrafish exposed to aqueous cobalt for 14 d and bulk spawned every 4-d. (**A**) Estimated bioaccumulation of Co in whole adults, based on whole zebrafish BCF of 74 (Reinardy *et al.*, 2011) and measured water Co concentrations in each tank. (**B**) Grey diamonds indicate total number of cumulative eggs, white squares indicate number of cumulative fertilised eggs, and black triangles indicate mortality (% of fertilised eggs) after 96 hpf. Control (0 mg l^{-1} cobalt) data is mean ± S.E.M., n = 3 tanks; Co treatment tanks, n = 1 tank per concentration.

Table 6-2: Linear regression parameters (slope and R^2) of number of cumulative eggs (total and fertilised, Table 5-3) from adult zebrafish exposed to aqueous Co for 4, 8, and 12 days. Values in bold indicate significant interaction term between regression slopes from treated tanks compared with control tanks (GLM, $p < 0.05$). [a]Interaction term $p = 0.0524$ by slope comparison with control (GLM).

	Co (mg l^{-1})					
	0	**5**	**10**	**15**	**20**	**25**
Cumulative total no. eggs [slope (R^2)]	0.8738 (0.9733)	1.7875 (0.9826)	**0.1813** (0.9868)	1.2125 (0.9336)	**0.1888** (0.9085)	0.4788 (0.9128)
Cumulative total no. eggs fertilised [slope (R^2)]	0.7063 (0.9719)	1.4688 (0.9563)	**0.1613** (0.9856)	1.16 (0.94)	0.1625[a] (0.9164)	0.2838 (0.7665)

The expression of *β-actin* was not affected by experimental treatments in either testes or larvae (GLM, $p > 0.05$), and therefore use of *β-actin* as a housekeeping gene was justified. The selected DNA repair genes analysed (*rad51*, *xrcc5*, and *xrcc6*) were induced at 15 mg l^{-1} Co, and, in addition, *rad51* and *xrcc6* were induced at 25 mg l^{-1} Co (Figure 6-5, A). There was no change in expression of *gadd45a* in testes with increasing Co concentration. There was no effect of Co concentration on induction of genes (*rad51*, *xrcc5*, *xrcc6*, and *gadd45a*) in larvae spawned after 12-d adult exposure, and no differences were detected among different Co concentrations (Figure 6-5, B).
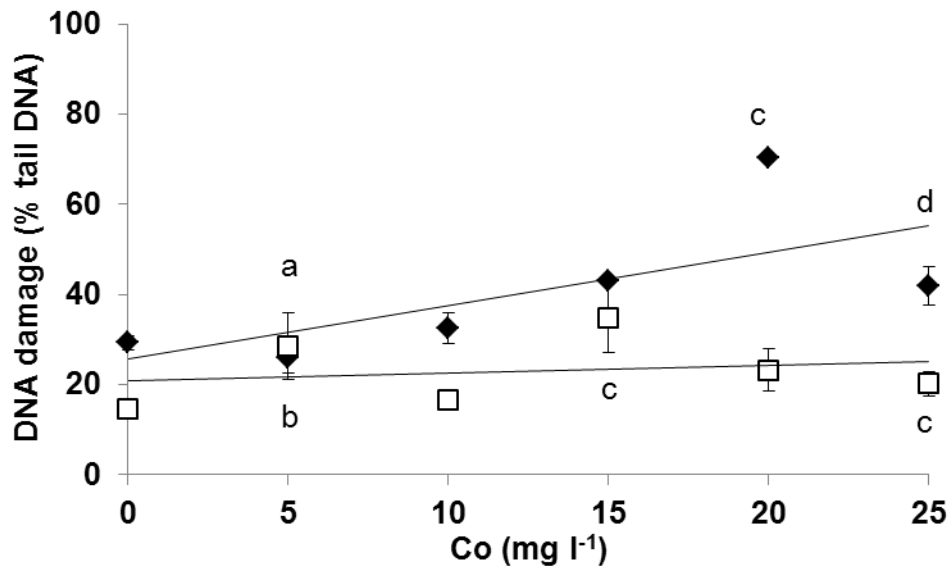
Figure 6-4: DNA damage (% tail DNA, comet assay) in zebrafish sperm sampled sub-lethally after a 13-d adult exposure to aqueous Co (mg l^{-1}, added as $CoCl_2$, black diamonds) followed by a 6-d recovery period (return to clean water, white squares). Data are mean ± S.E.M., n = 3 males except a (n = 9), b (n = 7), and c (n = 2), d (n = 1). DNA damage increased significantly with Co concentration immediately after exposure (linear regression, GLM, $p < 0.05$), but damage did not differ from controls after 6-d recovery period (linear regression, GLM, $p > 0.05$). The interaction term (concentration X sampling time) was significant (GLM, $p < 0.05$, interaction term $p < 0.05$).
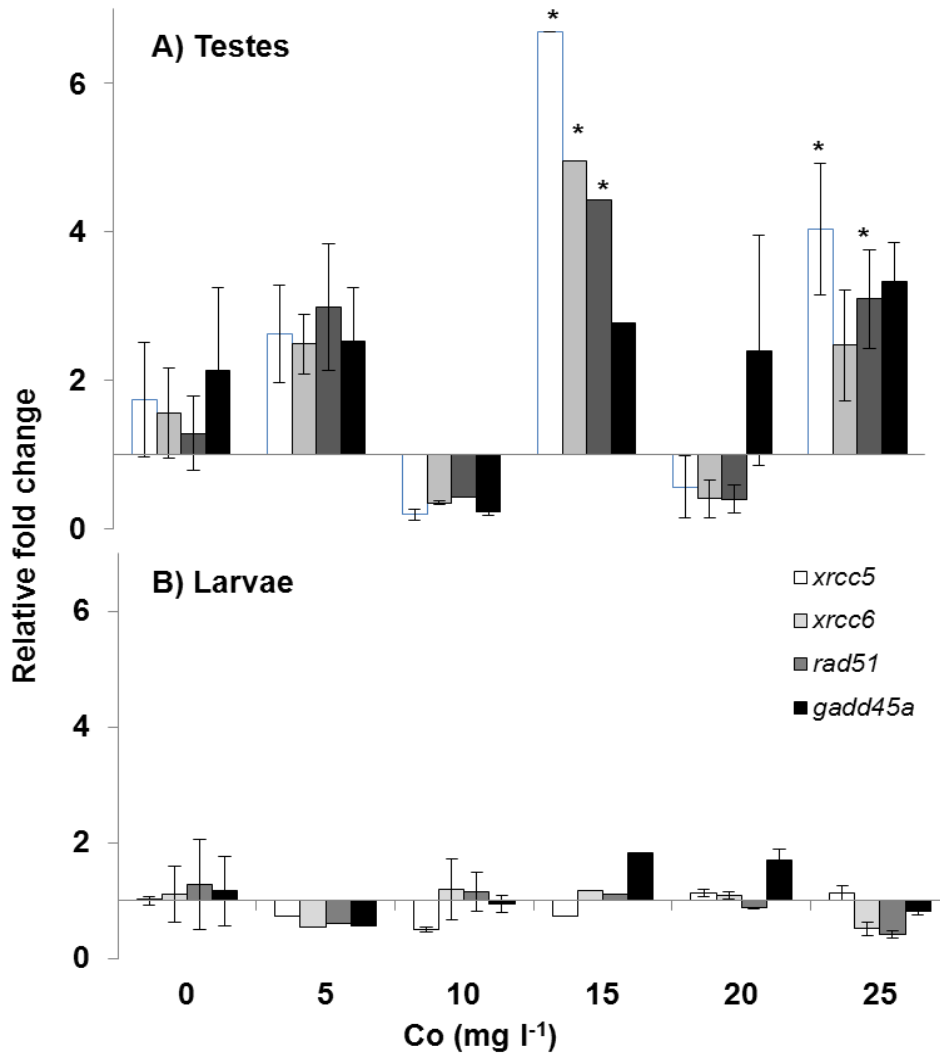
Figure 6-5: Gene expression (relative fold change, $2^{-\Delta\Delta Ct}$) in testes (**A**) of adult zebrafish exposed to aqueous Co for 12 d. No significant concentration-dependent response in induction (GLM, $p > 0.05$). *Significant induction of DNA repair genes compared with control (0 mg l^{-1} Co) (One-way ANOVA, Fisher's LSD $p < 0.05$). DNA repair gene expression in larvae (96 hpf, **B**) spawned from 12-d exposed adults.

6.4 Discussion

The 96-h acute toxicity (LC_{50}) of Co was 35 mg l^{-1} for zebrafish larvae in the present study. This LC_{50} value is approximately 1 order of magnitude greater (indicating lower toxicity) than that reported for adult fathead minnow (*Pimephales promelas* 4-d LC_{50} 3.45 mg l^{-1}), but similar to that reported in other adult freshwater fishes (4-d LC_{50} 66.8 mg l^{-1} for gold fish, *Carassius auratus*, and 4-d LC_{50} 82.7 mg l^{-1} for common carp, *Cyprinus carpio*) (Blust, 2012). Co^{2+} can compete with Ca^{2+} ions at Ca^{2+} uptake sites (Janssen, 2000; Kim *et al.*, 2006) therefore uptake of Co^{2+} can be dependent on water hardness (Diamond *et al.*, 1992); and the increased sensitivity of fathead minnows to Co could be due to unreported differences in water Ca^{2+} concentration, although species-specific differences cannot be discounted. The present study resulted in an average mortality of 94 % at the highest concentration of 50 mg l^{-1} Co, indicating greater sensitivity in larval zebrafish compared with adult goldfish (*Carassius auratus*) [concentrations up to 150 mg l^{-1} induced oxidative stress but no mortality, 96-h exposure (Kubrak *et al.*, 2011)] and adult blind Mexican cave fish *Astyanax fasciatus* [100 % mortality after 17 h exposure to 118 mg l^{-1} Co (Janssen, 2000)]. Chronic adult exposure to environmentally-relevant concentrations of Co (0 – 50 mg l^{-1}) in the present study did not affect adult zebrafish survival, and the results for acute larval toxicity and chronic adult exposure are consistent within the range of limited published toxicity data of Co in fish.

Assessment of reproductive endpoints can be complex in fish, and there was no direct concentration-dependent relationship in reproductive endpoints in the present study. Fish exposed to 15 mg l^{-1} Co had consistently high reproductive

output and fish exposed to 10 mg l^{-1} Co had consistently low reproductive output, in addition to variation among control tanks (0 mg l^{-1} Co) contributing, in part, to absence of strong concentration-related response. It is possible that reproductive endpoints do not follow a simple concentration-dependent response, and high variability in reproductive output has also been reported for zebrafish exposed to uranium (Simon *et al.*, 2011) and arsenic (Boyle *et al.*, 2008). It is also possible that the differences between concentrations selected in the present study were not great enough to allow discrimination in effects between concentrations. The approach to analysis of reproductive endpoints adopted in this study highlighted differences before and after exposure within each tank and took into account tank differences in spawning endpoints. For example, fish exposed to 20 mg l^{-1} Co had low rates of mortality of larvae (6 % after 12-d exposure), but this was due to low numbers of fertilised eggs (n = 31 fertilised eggs). Conversely, fish exposed to 5 mg l^{-1} Co had high mortality of larvae (36 % after 12-d exposure) but much higher numbers of fertilised eggs (n = 550 fertilised eggs). Exposure of adult zebrafish to arsenic resulted in reduced rates of cumulative total number of eggs (Boyle *et al.*, 2008) and an inverse concentration-dependent regression response in fertilisation (%) was reported in adult zebrafish exposed to phthalates (Uren-Webster *et al.*, 2010). The present study adopted a combination of these approaches to analyse complex reproduction data, and emphasises within-tank changes in reproductive outcomes after addition of Co. Consideration of individual tank performance at the start and end of exposure to Co indicates reduced rates of cumulative total eggs spawned, cumulative fertilised eggs, and increased mortality of larvae at

the highest concentrations, and therefore exposure to Co reduced reproductive success in zebrafish.

DNA damage in sperm increased with Co exposure and estimated bioaccumulation of Co in testes. In comparison, *in vitro* exposure to 100 mM H_2O_2 produced levels of DNA damage over 60 % in humans, bulls, and mice (Villani *et al.*, 2010), as well as in zebrafish (Reinardy *et al.*, 2012) with an approximate 50 % increase in damage from control levels. An *in vivo* adult exposure to phthalates, in contrast, resulted in low DNA fragmentation of sperm in zebrafish testes (12 % tail DNA), despite reduced reproductive success and induced expression of selected genes involved in hormone regulation (Uren-Webster *et al.*, 2010). Repair of DNA damage in germ cells is critical to prevent transfer of damage to offspring, but no studies have linked germ cell genotoxicity with reproductive success. Genotoxicity in sperm can reduce fertility (Lewis and Agbaje, 2008), and transgenerational impacts (e.g. increased mutations and genome instability) can result from transmission of genetic damage from exposed parents to non-exposed offspring (Aghajanyan and Suskov, 2009; Dubrova, 2003).

Expression of DNA repair genes did not show a clear concentration-dependent induction response in testes. DNA repair genes were induced up to 4-fold in testes exposed to the highest Co concentration (25 mg l^{-1}), in addition to a reduction in DNA damage in sperm following 6-d recovery in clean water. If repair is initiated in testes, it is possible that spermatogonia with damaged DNA could be repaired before they develop into spermatozoa when they lose their capacity for repair (Hales *et al.*, 2005; Leal *et al.*, 2009). Fish exposed to lower concentrations of Co did not have increased levels of expression of DNA repair

genes and it is possible that repair mechanisms were induced earlier (before 12 d exposure) and peak expression was missed by single timepoint sampling (Chechik and Koller, 2009). Exposure of adult zebrafish to phthalates detected changes in gene expression (e.g. *acox1*) in testes that also did not follow a concentration-dependent pattern of induction (Uren-Webster *et al.*, 2010), indicating that consideration of both concentration and sampling time may play a part in regulation of gene expression in testes. No significant induction of DNA repair genes was detected in larvae, suggesting either there was no DNA damage in larvae or that damage was too low to induce DNA repair mechanisms. Either way, it suggests that larval DNA was not greatly damaged (damaged DNA in sperm not transferred), and it is possible that repair of DNA damage occurred in the testes following exposure to Co.

DNA damage can be repaired by induction of DNA repair pathways. The main two pathways for repair of strand breaks are non-homologous end-joining (NHEJ), mediated by the Ku70-Ku80 protein complex (encoded by the *xrcc6* and *xrcc5* genes respectively), (Schulte-Uentrop *et al.*, 2008; Thacker and Zdzienicka, 2004) and homologous recombination (HR), encoded by the *rad* genes, including *rad51* (Thacker and Zdzienicka, 2004). Initiation of different DNA repair pathways can be dependent on the cell cycle stage (Wu *et al.*, 2008), but NHEJ and HR are also not mutually exclusive and can work simultaneously to repair strand breaks (Rapp and Greulich, 2004; Takata *et al.*, 1998). A comparison among the *rad51* and *xrcc* genes suggests both NHEJ and HR are induced in testes after exposure to Co, and this observation is consistent with suggestions that both strand-break repair pathways can work

simultaneously, although not ruling out the possibility of induction of additional pathways of repair not included in this study.

The affected reproductive success in the present study indicates that either male or female factors are being impacted by exposure to Co. Genotoxicity in sperm and induction of DNA repair genes in testes were detected and additional effects in females are possible although not investigated in the present study. Extensive repair mechanisms (e.g. recombination) are reported in oocytes with repair activity up to three orders of magnitude greater than in somatic cells (Ashwood-Smith and Edwards, 1996). Whereas reduced total egg output in the present study is likely a result of an effect in females exposed to aqueous Co, it is possible that reduced fertilisation and reduced larval survival are due to affected sperm, because any genotoxic damage in oocytes could be repaired before fertilisation. A possible explanation for the lack of induction of DNA repair genes in larvae is because genotoxic damage is repaired prior to 96 hpf, (i.e. during earlier stages of embryogenesis) and/or in testes, or damaged sperm were unable to fertilise eggs resulting in no transfer of damage. It is possible that repair mechanisms in testes were effective in reducing DNA damage in sperm, and no transfer of effects from exposed adults to unexposed offspring was detected in the present study.

6.5   Conclusion

The present study demonstrated that chronic sub-lethal exposure to Co can reduce reproductive success, cause genotoxicity in sperm, and induce expression of DNA repair genes in testes. Despite effects on reduced fertility

(possible effect of male exposure) and fecundity (possible effect of female exposure), no induction of DNA repair genes was detected in larvae spawned from exposed adults. Little is known of the effects of Co toxicity on reproduction or genetic integrity in fish, and links between molecular mechanisms of DNA repair are complex. Many questions still remain, including whether Co-induced genotoxicity in sperm leads directly to induction of DNA repair mechanisms in testes, and whether DNA repair in testes can prevent transfer of genetic damage from exposed males to unexposed larvae. However, impaired reproductive success and genotoxicity in germ cells is of wider environmental and human health concern (DeMarini, 2012) and warrants further investigation of potential biological impacts of Co in the environment.

7:

Discussion

The focus of this thesis was to examine bioaccumulation, genotoxicity, expression of DNA repair genes, and reproductive success with the aim of adopting an integrated approach to investigate links among germ cell genotoxicity and reproductive outcome in a model fish species. It is possible that statistically significant effects might be detectable at lower levels of biological organisation (e.g. gene expression or other genetic endpoints) at low concentrations without impacts at the individual level (Jha, 2004; Jha, 2008). Equally, reproductive effects are possible without a toxic link to the individual; and only a combination of both can provide a complete view of potential toxic effects of contaminants. This is important to understand key biological processes, in addition to implementation of environmental policies and risk assessment (Moore *et al.*, 2004).

A unique aspect of the toxicology of radionuclides is the potential for radioactive as well as metal toxicity. The results discussed in Chapter 3 are the first to link radionuclide exposure and bioaccumulation for estimation of radiation dose. The problem of conversion of activity concentrations to dose has been circumvented in many instances by use of radiation sources that provide dose-delivery systems set to required levels (e.g. Hyodo-Taguchi, 1980; Barquinero *et al.*, 2004). This is not possible for studies of environmental toxicology because radionuclide contamination consists of deposits (as concentrations) in e.g. sediments, and the dose delivered is based on multiple factors such as combined radiological properties, route(s) of exposure, and biological characteristics of exposed organisms. The majority of radiation-effects studies are based on a specific radiation dose and a link between dose and activity

concentrations is required. Development of the publically-available ERICA Tool has newly provided an opportunity for estimation of internal and external dose in radio-ecological studies. The study described in Chapter 3 is the first to illustrate successful application of this tool to a classic laboratory uptake experiment. It also provides an example of how to produce estimates of dose rates from a mixture of radionuclides under different experimental conditions. The study emphasises that time and magnitude of accumulation is highly dependent on the organism, route of exposure, and chemical and radiological characteristics of the toxicant, and only once these have been established can the potential biological impacts be assessed. With available dosimetry modelling tools (e.g. ERICA Tool), radio-ecological investigations can link with radiation dose-effect relationships to provide a broader assessment of the environmental risk of radioactive contamination. This approach is vital in light of increasing importance of historical and novel contaminants in the environment. The issue of environmental radioactivity is particularly topical with recent high profile nuclear accidents, and in changing environments potentially releasing previously-sequestered historical deposits of radionuclides. It is essential that novel approaches such as environmental dose estimations are applied to evaluate toxicants and to anticipate environmental effects in advance of environmental disasters. An additional advantage of the ERICA Tool is the provision of dose data for in detail comparison between different radionuclides, and an assessment of the risk between different mixtures of radionuclides.

It is critical to assess the bioavailability of toxicants in organisms through uptake, depuration, and bioaccumulation kinetics to confirm exposure. Uptake and depuration characteristics of a suite of environmentally relevant radionuclides

were established in adult zebrafish exposed both through diet and water (Chapter 3). The kinetics of uptake and magnitude of bioaccumulation can link the environmental presence of the toxicant and the potential for biological effect in the organism (Kleinow *et al.*, 2008). Whereas a whole-body bioaccumulation approach can inform on overall biological effects on an organism, accumulation in internal organs allows for targeted studies of tissue-specific effects. Modelling uptake and depuration of radionuclides into organisms can be used for whole body accumulation (Whicker and Schultz, 1982; Jeffree *et al.*, 2006a). However, modelling uptake into internal organs is not as easy because movement of substances between different tissue types and interaction with cellular and metabolic processes are complex (Allen and McVeigh, 2004). The ability to model uptake into internal organs will provide an interesting and useful platform to investigate differential toxico-kinetic data for different environmental contaminants. In addition, tissue-specific bioaccumulation can inform on targeted studies of tissue-specific effects, which can link questions of mechanisms of toxicity to overall assessment of potential toxic risk.

Toxicants can bioaccumulate in gonads, a critical organ for reproductive effects and potential for transmission of effects to offspring. Germ cells within gonads are key targets of ecotoxicological investigations because it is through germ cells that impacts such as genetic damage are transmitted from exposed individuals to offspring. Sperm can be repeatedly sampled non-invasively from zebrafish, and the study discussed in Chapter 4 is the first to assess DNA damage, a key indicator for overall genotoxicity, in zebrafish sperm. Male germ cells have been suggested as the key route by which genotoxic effects are transferred to offspring (Dubrova, 2003b; Lewis and Aitken, 2005) due to lack of

DNA repair systems in tightly packed sperm DNA (Baumgartner *et al.*, 2009; Leal *et al.*, 2009). Therefore, evaluation of male genotoxicity following exposure to toxicants is critical for long-term assessment of transgenerational risks. This field is currently of much interest for assessment of human fertility (Aitken and De Iuliis, 2007; Lewis and Agbaje, 2008) but there are many questions about long-term impacts on other organisms and implications for environmental toxicity assessments. Some insights have come from studies of endocrine disrupting chemicals affecting males (e.g. increased 'feminisation', hormonal disruption, and intersexuality) with major environmental implications including effects on population dynamics, sex ratios, and potential local extinctions (Sumpter, 2005; Shenoy and Crowley, 2011). It is possible that the effects of male genotoxic agents may have similar consequences on wild populations. An evaluation of the potential for male genotoxic impacts is critical to determine long-term impacts of exposure, in particular for transgenerational impacts.

The overall genotoxic effect of a toxicant is a result of impairment of DNA repair processes rather than purely DNA damage. Repair of DNA damage is initiated by induction of genes encoding for products that drive the repair process. It is estimated that 175 genes are involved in DNA repair processes in humans (Wood *et al.*, 2005). NHEJ and HR are predominant repair pathways for DNA strand break repair, and the study in Chapter 5 focused on DNA strand breaks induced by exposure to H_2O_2 and subsequent repair via expression of selected genes involved in both NHEJ and HR. Both repair pathways involve many additional genes and gene products (Thacker and Zdzienicka, 2004), and the targeted approach of selecting key genes may miss unexpected changes in other genes. In addition, induction of DNA repair pathways is dependent on cell

cycle stage (Wu *et al.*, 2008). The results from Chapter 5 indicated that both DNA repair pathways were initiated, but clarifying the differential induction between the two main pathways, and possible induction of other DNA repair process (e.g. base excision repair or global genome repair) would be of interest in the DNA damage/repair system of zebrafish larvae exposed to H_2O_2. DNA strand break repair is of great interest to human cancer researchers (DeMarini, 2012) and the zebrafish model can be a useful bridge between such divergent fields of research such as biomedical and environmental toxicology.

Interpretation of gene expression information can be complex and investigation of the profile of gene expression over time can be critical, as illustrated in the study in Chapter 5. In simple terms, induction of expression, through e.g. presence of toxicant, results in increased gene product (RNA) which in turn translates into increased levels of proteins which can ultimately reduce levels of original toxicant. The reality, however, is a lot more complex, but it is clear that levels of RNA will show a kinetic response over the time of exposure, and low levels of gene product can be indicative of both un-induced gene expression and highly induced gene expression (with rapid translation into protein). There is still much to be done to integrate new techniques focused on toxicogenomics and other molecular aspects with environmental toxicology (Fedorenkova *et al.*, 2010; Fent and Sumpter, 2011). However, integrating underlying molecular changes with effects on individuals and populations will allow for a more comprehensive assessment of potential toxicity, and aid in further linkages between different levels of biological organisation (Villeneuve *et al.*, 2012).

An integrated assessment of toxicity of cobalt (Co) in adult fish exposed via the water resulted in genotoxic damage in sperm, induction of DNA repair genes in

testes, and reduced reproductive success (Chapter 6). Reduced reproductive success and impaired larval development could be due to multiple factors including direct toxicity on spawning behaviour, physiological effects in the female or male, or a result of the genotoxic damage detected in sperm. It is likely that all effects in combination resulted in the observed reproductive impairment. Lack of induction of DNA repair genes in larvae indicated lack of damage in larvae or successful repair, and, if the former is considered more likely, then transfer of genetic damage from sperm to offspring did not occur. Damaged sperm may not have fertilised any eggs, or those eggs fertilized by the damaged DNA may not have developed successfully to hatching. Repair mechanisms were induced in testes following induction of genotoxic damage in sperm. However, despite this active repair, sperm still showed elevated levels of DNA damage, indicating incomplete repair, or re-damage in the day between sampling gonads for RNA extraction (d 12) and evaluation of damage in sperm (d 13). It is probable that without the induced repair, DNA damage would be considerably higher in sperm.

A multi-level approach (genotoxicity, gene expression, and reproductive success) can assess the impacts of a toxicant on the individual level and, in addition, inform on the potential for further transgenerational impacts involving transfer of un- or mis-repaired genetic damage. Transfer of genotoxic damage to offspring via maternal DNA (eggs) is less likely than via paternal genetic contribution (sperm) due to active repair processes in oocytes (Ashwood-Smith and Edwards, 1996), but further work is required to tease apart the maternal and paternal contribution to offspring viability after adult exposure. Additional studies could include bulk spawning of exposed males with unexposed females,

and *vice versa*, and the zebrafish model, including genotoxic assessment and induction of DNA repair genes, is an ideal system to address these questions. Whether greater levels of genotoxic damage in sperm may result in detectable short-term DNA damage in larvae, whether larvae are able to initiate repair processes to reduce levels of DNA damage, and whether DNA damage can be transferred from oocytes to offspring are open questions for further investigation.

8:

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Appendix 1:

Radiation dosimetry: Use of the ERICA Tool

for dose rate assessment in zebrafish

(*Danio rerio*)

A1.1 Introduction

Over 60 European scientists collaborated to produce the 'ERICA (Environmental Risk from Ionising Contaminants: Assessment and Management) Integrated Approach to the Assessment and Management of Environmental Risks from Ionising Radiation (www.ceh.ac.uk/PROTECT/ERICAdeliverables.html). A part of this project involved development of an easy-to-use software package, ERICA Tool, which is a model for dose calculations for many types of organisms in different environments. The ERICA Tool combines several databases together. These databases provide parameters for transfer and dosimetry of radionuclides in reference organisms, and link these to data on biological effects of ionising radiation (using the FREDERICA database, www.frederica-online.org). The ERICA Tool was made accessible in 2008 (Brown *et al.*, 2008; Larsson, 2008; Wood *et al.*, 2008) and has been applied to a wide variety of environmental contamination situations involving several types of organisms (e.g. Beresford *et al.*, 2008b; Stark and Pettersson, 2008; Wood *et al.*, 2009).

The objective of adapting the ERICA Tool for zebrafish was to optimise, validate, and set input parameters to apply to data from the uptake and depurations experiment carried out in the International Atomic Energy Agency, Marine Environment Laboratories (IAEA-MEL), Monaco (Chapter 2). The dose received by an organism depends on several factors: type and amount of radiation, source of radiation (sediment, air, or water) and body size and shape of organism of interest. The type and amount of radiation and the source is straightforward to input, but more care has to be taken with inputted organisms dimensions. The ERICA tool dose calculations are based on inputted

dimensions of the organisms of interest, as well as raw data on radiation activity concentrations in the organism (internal dose) and the substrate (external dose); fish of different sizes will receive different doses, all other aspects being equal. Internal dose rates are calculated separately from external dose rates. Internal dose rates are calculated from concentration levels in the whole body (Bq kg^{-1}) and the external dose rates are calculated from concentration levels in the water (for pelagic fish, Bq l^{-1}).

The ERICA Tool was primarily intended for an overall impact assessment on wildlife in contaminated environments, therefore the reference organisms in the ERICA Tool are broad and non-specific, encompassing whole habitat dose estimations. The closest relevant reference organism for data on activity levels in zebrafish is the 'freshwater pelagic fish' setting, which is a model with average dimensions taken from rainbow trout. As dose calculations are sensitive to specific body size and shape, to calculate specific doses in zebrafish it was necessary to optimise the ERICA Tool and create model zebrafish dimensions to make it fully applicable to data gathered from the uptake and depuration in zebrafish (Chapter 2).

Validation of the ERICA Tool for zebrafish involved the following steps:

- Create a database of Plymouth University (PU) stock zebrafish dimension measurements including weight, total length, width, and height.
- Calculate regression equations for weight versus the other three parameters (length, height and width) based on the database of PU fish.
- Apply the regression equations to weight measurements from fish used in IAEA-MEL to estimate length, width, and height of the IAEA-MEL fish.

- Calculate mean dimensions for male, female, and all fish based on IAEA-MEL fish for inputting into ERICA Tool.

- Compare the outputted dose rates for different IAEA-MEL fish dimensions (female, male, and all fish).

- Compare the difference in output dose rates for individual fish compared to mean fish dimensions.

- Decide on particular input settings for final dose calculations from experimental data (Chapter 3).

-

A1.2 Methods

A1.2.1   Measurement of Fish

All PU fish were maintained and handled according to routine approved animal welfare protocols. 88 stock fish from PU (males n = 35, females n = 53) were measured for weight, length (total), width, and height using electronic digital callipers (Precision Gold) (Figure A1.1). Fish were lightly anaesthetised until loss of orientation in water, and placed on a tissue for total length and height measurements. Fish were inverted for width measurement before being weighed.

Figure A1-1: Dimensions used for the fish measurements including total body length and height (a and b, respectively, scale bar 35 mm), and width (c, scale bar 8 mm).

A linear regression was applied to fish dimension data. Based on these equations the length, width, and height of IAEA-MEL fish were estimated and average dimensions for female, male, and all fish were calculated.

A1.2.2 Parameter Setting for ERICA Tool

The Tier 2 start point was selected for all assessments. The isotopes selected for dosimetry calculations were the same as were used in the uptake/depurations experiment (Chapter 3): ^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{110m}Ag, ^{109}Cd, ^{134}Cs and ^{241}Am. Measurements from three model fish were added, having the dimensions of an average female, an average male, and an overall zebrafish, based on estimated dimensions of IAEA-MEL fish. Except for different dimensions, the three model fish were the same for all other settings: ecosystem was freshwater and water occupancy factor was set to 1 (water surface, benthos, and sediment occupancy factors all remaining 0).

For internal and external dose rate calculations, values of distribution coefficients (Kd) and concentration ratios (CR) were not used, but were set to 1 in order to progress through the analysis. For initial tests, analysis was limited to internal dose rates only, which required input of organisms' activity concentration (whole body counts, Bq kg^{-1} f.w.) with activity concentrations in water and sediment remaining at 0. For later analysis of external dose rates, input data was activity concentrations in water (Bq L^{-1}) with activity concentrations in sediment and fish remaining 0. Dose calculations involved different weighting settings for alpha, beta, or gamma emitters, with alpha emitters having a weighting factor of 10 compared with 1 for beta and gamma and 3 for low energy beta emitters.

A1.2.3   Effects of fish size

To investigate the sensitivity of the ERICA Tool to different sizes of fish, the whole body counts from the end of the uptake period (day 14, mean counts for all fish) was used as input data for internal dose rates. The same input was run for the different model zebrafish: female, male and all fish.

A1.2.4   Dose estimation using individual fish

A comparison was made between calculating a dose rate for an average fish with calculating individual dose rates followed by taking an average dose rate. This test used the individual fish data (activity counts in whole body) at day 14 to calculate individual dose rates for individual fish using the female model for females and male model for males to make the test as realistic as possible. The individual dose rates were averaged to give a mean and standard error value for all fish at the specific time (day 14). For this test, fish of unknown sex were not used, therefore the third model zebrafish (all fish) was not used either.

A1.3 Results

A1.3.1 Fish measurements

Average dimensions were calculated from the dataset of PU fish dimensions (Table A1.1). Regression equations were calculated based on a linear model defining the relationship between weight and the other three measurements (Figure A1.2), and the regression equations are listed in Table A1.2.

Table A1-1: Average dimensions of stock PU fish. Data are mean ± S.E.M.

Sex	Weight (g)	Length (mm)	Width (mm)	Height (mm)
F (n=53)	0.475 ± 0.01	34.91 ± 0.20	5.49 ± 0.10	8.54 ± 0.10
M (n=35)	0.374 ± 0.02	35.69 ± 0.44	4.11 ± 0.08	7.08 ± 0.11
All (n=88)	0.435 ± 0.01	35.22 ± 0.22	4.94 ± 0.10	7.96 ± 0.11

Table A1-2: Regression equations for PU fish dimension data. Weight (kg); length (m); width (m); height (m).

Sex	x = weight, y = length	x = weight, y = width	x = weight, y = height
F (n=53)	y=12.04x+0.0292	y=5.9866x+0.0026	y=7.5843x+0.0049
M (n=35)	y=22.77x+0.0272	y=2.9351x+0.003	y=5.6203x+0.005
All (n=88)	y=11.3x+0.0303	y=6.6814x+0.002	y=8.4634x+0.0043

The regression equations obtained from PU fish were applied to the weight data from IAEA-MEL fish to provide estimates of the average lengths, widths, and heights to be input into the ERICA Tool (Table A1.3).
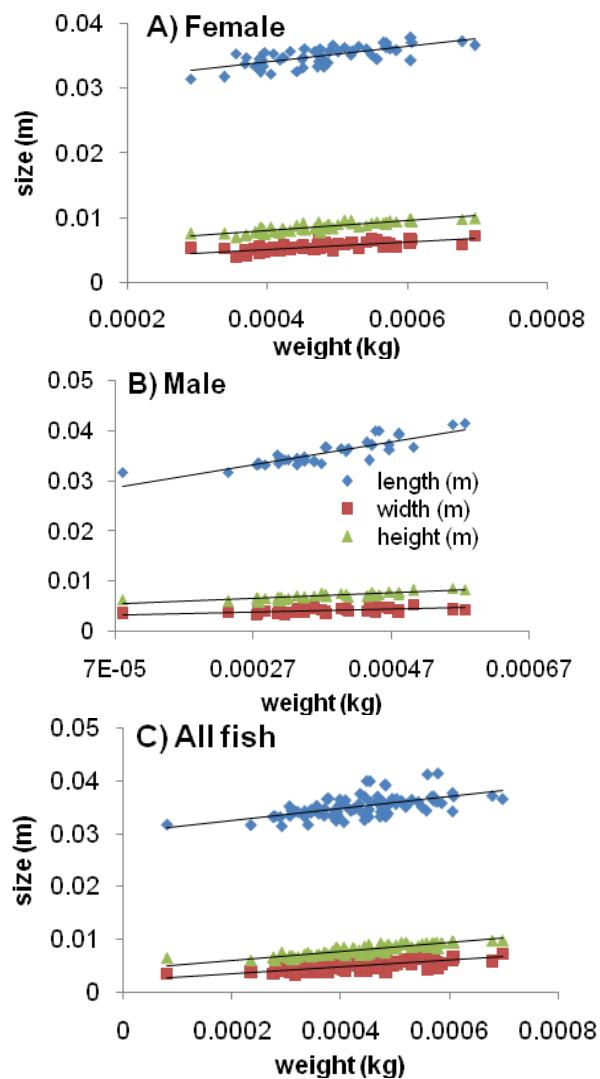
Figure A1-2: Linear regression relationships between fish weight and other dimensions for PU fish. (A) female fish n = 53, (B) male fish n = 35, (C) all fish combined n = 88. Units reflect the units required to input into the ERICA Tool.

Table A1-3: Mean (± SEM) dimension measurements of IAEA-MEL fish calculated from the regression equations based on measurements from PU fish.

Sex	Weight (g) mean ± SEM	Length (mm) mean ± SEM	Width (mm) mean ± SEM	Height (mm) mean ± SEM
F (n=25)	0.34 ± 0.01	33.33 ± 0.16	4.66 ± 0.08	7.50 ± 0.10
M (n=41)	0.29 ± 0.01	33.74 ± 0.24	3.85 ± 0.03	6.62 ± 0.06
All (n=192)	0.30 ± 0.004	33.64 ± 0.05	4.01 ± 0.03	6.84 ± 0.04

A1.3.2 Effect of fish dimensions on dose rate calculations

Using the input of whole body counts (average Bq kg^{-1}, male and female fish combined) at day 14 of the aqueous exposure of the uptake/depurations experiment (Chapter 3) the ERICA Tool was tested for the different model zebrafish: model female, model male, and model overall zebrafish. The output values are listed in Table A1.4. The high dose rate values for ^{241}Am reflects the high weighting factor for alpha emitters.

A1.3.3 Effect of individual activity concentrations on dose rate calculations

Individual dose rates for 18 fish (4 females and 14 males, complete data from day 14 of aqueous exposure) were calculated and the mean and SEM of these individual analyses are shown in Figure A1.3.

Table A1-4: Comparison of analysis output (internal dose rate, µGy hr^{-1}) for selected radionuclides (^{54}Mn, ^{60}Co, ^{65}Zn, ^{75}Se, ^{110m}Ag, ^{109}Cd, ^{134}Cs and ^{241}Am) for different model zebrafish: female, male and all fish.

Isotope	Input Whole body activity Concentration (kBq kg^{-1})	Output Female µGy hr^{-1}	Male µGy hr^{-1}	All µGy hr^{-1}
^{54}Mn	29.0	0.32	0.31	0.31
^{60}Co	17.6	1.08	1.06	1.09
^{65}Zn	113.2	1.40	1.37	1.38
^{75}Se	168.3	2.88	2.83	2.85
^{110m}Ag	36.1	1.97	1.91	1.93
^{109}Cd	52.2	2.90	2.89	2.89
^{134}Cs	8.2	0.76	0.74	0.74
^{241}Am	16.0	506.0	506.0	506.0
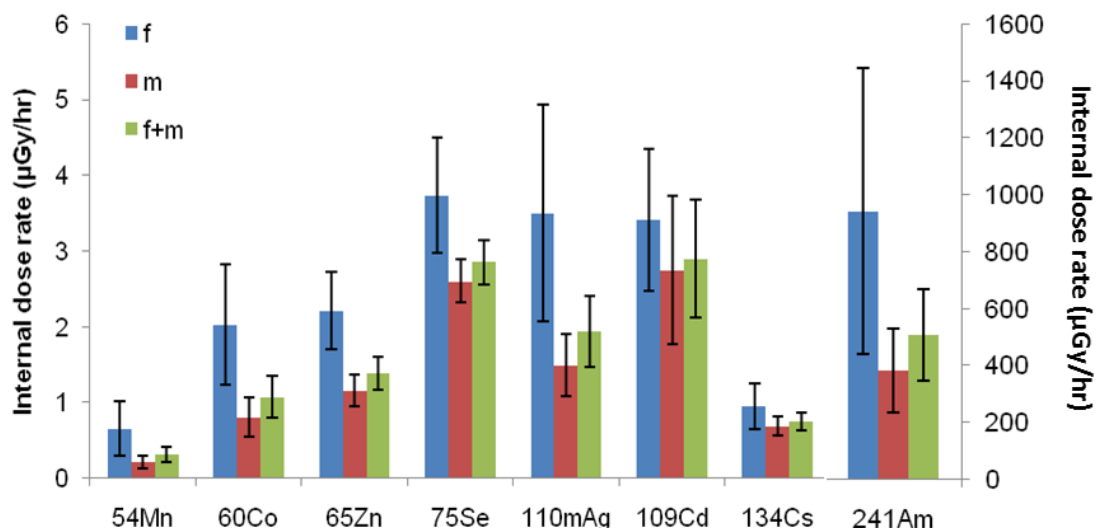
Figure A1-3: The internal dose rates calculated for individual fish (female n = 4, male n = 14, combined female and male n = 18) for eight radionuclides at day 14. Note the secondary axis for [241]Am which has an internal dose rate several orders of magnitude higher than other radionuclides, units remain the same.

A1.4 Discussion

The ERICA Tool is one of several models which seek to make dose calculations easy and accessible, but a comparison between the available models has highlighted the need for more work to increase the comparability between specific models (Beresford *et al.*, 2008c). In addition to inter-model differences highlighted in Beresford *et al.*, there are other sources of possible error, primarily through use of model organisms, which can be unrepresentative of individual dose rates, in addition to use of whole body activity levels that do not take into account different internal dose rates in different internal organs (Gomez-Ros *et al.*, 2008). Dimensions alone cannot account for differences in tissue densities and cell types, which may also affect overall species-specific differences in internal dose rate estimation.

The tests carried out on the model zebrafish are based on a model freshwater environment, which may not truly reflect the environment within a standard laboratory tank. The tank set-up of the uptake and depuration experiments included a high density of other fish within the tank and this may have an effect on the external dose rate, an effect which cannot be determined by a model that calculates an external dose rate based on activity levels within water alone.

A1.4.1   Fish Dimensions

There is a difference in overall body size between males and females of stock PU fish, and female fish are heavier, wider, and higher than males. Male fish are longer than female fish. This same pattern is reflected in estimated dimensions of IAEA-MEL fish, giving more weight to the method of estimating dimensions based on regression equations of a different stock of fish (PU fish).

The dataset of fish dimensions was based on a group of fish bred from PU stock zebrafish. These fish should be representative of other stocks of zebrafish, including IAEA-MEL fish. It can be seen from Figure A1-2 that an individual male was considerably smaller (lighter) than the other fish, yet it still lies well within the regression line. It is possible that the regression equations may become more representative with a greater dataset but it is likely that they will not change greatly. The regression equations provide a basis for estimating the other dimensions of the IAEA-MEL fish and, as such, slight variations in equations may not have a marked effect on final estimated dimensions.

A1.4.2.   Effect of fish dimensions on dose rate estimations

Female fish had a higher dose rate compared with males, most likely due to their greater size. The only radionuclide that was unaffected by differences in

tested fish dimensions was [241]Am. For all radionuclides (except [241]Am), dose rates for males were max. 3.5 % lower than dose rates for females, which represents the maximum error using a mis-matching model fish for dose rate calculations. When comparing model zebrafish (all fish) to model female and model male fish, the error drops to a maximum of 1 % and 2.5 % for females and males respectively. If these errors are considered acceptable then the model combined male and female zebrafish (with dimensions based on averages of all fish measured, n=53 females and n= 35 males) can be used for all dose rate calculations, irrespective of sex of measured fish. For a specific investigation into sex differences in dose rates it may be important to use sex-specific model zebrafish. However, for investigations into overall changing dose rates over time and between exposure scenarios (e.g. aqueous versus dietary exposure) activity counts averaged over a specific time-point can be run using an overall model zebrafish, and differences between sexes are likely to be overshadowed by time and treatment (aqueous versus dietary) effects.

A1.4.3   Effect of individual activity concentrations on dose rate calculations

The benefit of calculating dose rates for individual fish is that it allows a better estimate of individual variation in dose rate (because of individual variation in activity counts). However, the drawback could be in amplification of error because individual lengths, widths, and heights are estimated from regression equations of individual weights (the only raw data available for fish from the uptake and depuration experiment, Chapter 3). Each data point (n = 129 for aqueous exposure and n = 192 for dietary exposure) would need its own model zebrafish to reflect its individual (estimated) dimensions, and this is laborious

and unnecessary. A compromise for this approach would be to input individual activity counts but to use the same model zebrafish (model female for female fish, model male for male fish, and model zebrafish for undefined sexes).

For the tests the overall model zebrafish (females + males) was based on estimated dimensions averaged for the one timepoint (day 14); a further decision is whether an overall model zebrafish reflecting the average dimensions from all data points (129 for the aqueous experiment and 192 for the dietary experiment) can be used, or whether timepoint-specific model zebrafish should be used. An additional drawback is the added time required to run the model for each individual fish activity levels, but this additional time is not as great as the time required to create individual model zebrafish. The decision as to whether to run the model on individual fish models, timepoint-specific fish models, one overall fish model, individual activity levels, or average activity levels for each timepoint, can be made in light of the tests carried out so far.

Once decisions are reached further tests are necessary. A comparison between independent internal and external dose rates can be made. Independent internal and external dose rates can be compared with a combined analysis of input of both water activity counts and body activity counts.

The data on individual gonad counts measured in the uptake and depuration experiment (Chapter 3) is available to be used for organ-specific dose rate calculations. However, the available models (e.g. ERICA Tool) have so far not been able to achieve such complexity (involving shape within a shape dosimetry) but the data is available for use when the technology becomes available.

A1.5 Conclusions

For analysis of dose rates in adult zebrafish, model zebrafish dimensions are calculated based on regression equations applied to mean weights of IAEA-MEL fish. This decision is based on differences between sex-specific models being below the overall sensitivity of the model (personal communication with D. Copplestone). The tests here were for fish exposed via water (n=192 weight measurements). A final model zebrafish was calculated using the same regression equations and applied to all weight data from both aqueous and the dietary (n=192) exposures (total weight measurements n = 384). The dimensions of the model IAEA-MEL zebrafish were:

Weight = 0.294 g, length = 33.6 mm, width = 4.0 mm, and height = 6.8 mm.

This model IAEA-MEL fish was used for analysis of internal and external dose rates from the IAEA data (Chapter 3).

Appendix 2:

Toxicity of aqueous americium (^{241}Am) to zebrafish (*Danio rerio*) larvae

A2.1 Introduction

Larval zebrafish are commonly used to investigate dose response relationships and lethal toxicity of toxicants. They are particularly suited to investigating suitable experimental concentrations for more targeted toxicity effects experiments. In addition, embryos and larvae have been the target for radiation effects studies due both to their radiosensitivity and their often visible developmental abnormalities. Larval zebrafish (5-6 days post fertilisation, dpf) showed a dose dependent increase in DNA damage, measured through the alkaline comet assay, following both γ-irradiation (7200 µGy hr^{-1}, ^{137}Cs) and α-particle exposure (740 µGy hr^{-1}, ^{210}Po) (Knowles, 2002; Jarvis and Knowles, 2003). A threshold of 1000 µGy hr^{-1} was suggested for a wide range of organisms to show effects (Real *et al.*, 2004). The majority of effects studies have used sources of radiation emitting controlled doses, but linking environmental or experiment activity concentrations of radionuclides to doses within organisms, and biological effects caused by those doses is uncommon (Reinardy *et al.*, 2011). The Environmental Risk from Ionizing Contaminants Assessment and Management (ERICA) Tool (www.ceh.ac.uk/PROTECT/ERICAdeliverables.html) is a dosimetry software package developed to aid environmental risk assessment through linking activity concentrations to dose in organisms (Brown *et al.*, 2008) and can be applied to estimate doses following experimental radionuclide exposures (see Appendix 1 for method development).

The objective of this study was to establish a dose response in larvae exposed to a range of americium concentrations in order to inform on suitable aqueous americium concentrations for further effects studies in adult zebrafish.

A2.2 Rationale

An aqueous exposure of 0.2 Bq ml^{-1} in adult zebrafish reached an equilibrium of 18.8 Bq g^{-1} (whole body activity concentration) after 7 d, resulting in a maximum dose rate of 1000 µGy h^{-1}, and accumulated dose of 83000 Gy (Reinardy *et al.*, 2011). Dose rates of 8, 25, 185, and 740 µGy hr^{-1} (alpha-emitter ^{210}Po) produced some reduced egg output and viability but no distinct reproductive disruption in adult zebrafish (Knowles, 2002; Jarvis and Knowles, 2003). Other fish studies suggest induction of biological effects at doses over 1 Gy hr^{-1} (ERICA Tool database). Therefore a target dose of 10 Gy h^{-1} is hypothesised to result in detectable sub-lethal reproductive and genotoxic effects in adults, through an aqueous exposure of 2 Bq ml^{-1} ^{241}Am following dose estimations calculations using the ERICA Tool.

To verify the concentration of americium required for an adult aqueous exposure, trials were carried out with larvae, to establish an initial dose response from larvae exposed up to 100 Bq ml^{-1}, followed by a second acute dose response experiment exposing larvae up to 1000 Bq ml^{-1}.

A2.3 Methods and Results

A2.3.1 Exposure and sampling set-up

All radionuclide exposures were carried out in the Consolidated Radio-isotope Facility (CORiF, Plymouth University). Plastic sealable containers holding 200 ml exposure water were floated in a large water bath containing water pumps, heater, and data logger to ensure uniform constant temperature (27.6 ± 0.03 ^{0}C, mean ± S.E.M., TinyTag Datalogger, TinyTag Explorer 4.5). A lamp on a timer ensured 12L:12D photoperiod (Figure A2.1).
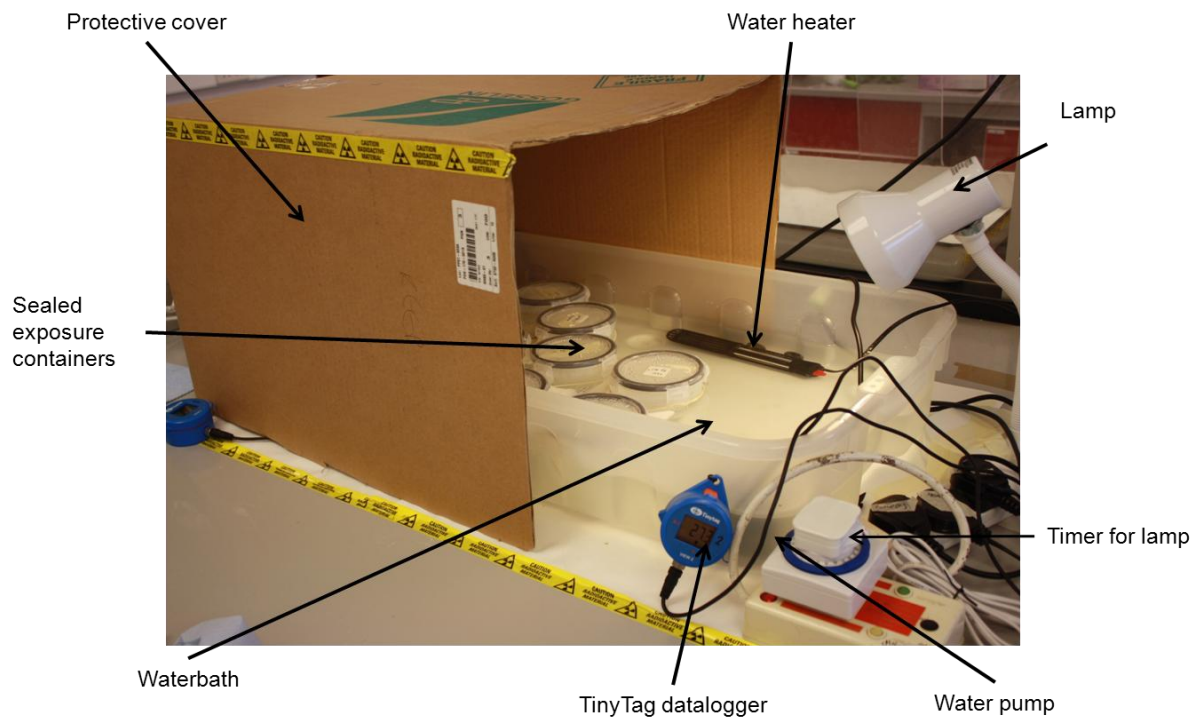
Figure A2-1: Experimental set-up for exposure of zebrafish larvae to radionuclides. Sealed plastic containers with larvae in 200 ml spiked water, floated in water bath to maintain temperature (27.6 ± 0.03 ^{0}C, mean ± S.E.M.), with a lamp on a timer to maintain 12L:12D photoperiod.

At the start of the exposure, 30 newly hatched (3 dpf) larvae (obtained by routine bulk spawning of stock fish from the Zebrafish Research Facility, Plymouth University) were placed into each sealable plastic container. Before spiking, the containers held 298 ml (by weight) 'fish water' (system water from aerated re-circulating aquaria in the Zebrafish Research Facility). The containers were moved to a fume hood for spiking.

All handling of radionuclides and experimental protocols involving exposure water were carried out according to the health and safety requirements of the CORiF facility, in agreement with the Radiation Protection Supervisor and trained technicians. Spiking was carried out by pipetting appropriate volume, and checking for neutral pH with narrow range indicator paper (for ease of discarding once contaminated with water containing radionuclide). After spiking, containers were sealed and moved to a water bath for the duration of exposure. At the end of the exposure period, containers were removed from water bath and placed in fume hood. Container lids were carefully removed and larvae were visually inspected for mortality or abnormalities. For sampling, larvae and exposure water were carefully poured and washed through a filter, and the larvae were pipetted into tubes for later analysis.

A2.3.2 Radionuclide

Americium was selected based on its potential for high radiation dose (Reinardy *et al.*, 2011), environmental relevance (Noshkin, 1972; Copplestone *et al.*, 2001), and lack of stable metal counterpart ensuring toxicity from americium is a combination of metal and radiological exposure. Americium (^{241}Am, Eckert & Ziegler Isotope Products, California, USA, supplied by High Technology Sources Ltd, Oxfordshire, UK) was received as 5 ml of $AmCl_3$ in 1M HCl with a specific activity of 3.43 Ci g^{-1}, total activity 81.08 µCi, purity >99 %. The activity concentration of americium in larvae was analysed for 30 min (ORTEC planar type gamma spectrometry).

A2.3.3 Toxicity of HCl carrier and NaCl controls

A non-radioactive trial exposure was carried out to match the exposure scenario of exposing to 100 Bq ml^{-1} ^{241}Am, added as either a 1 or 0.1 M HCl solution. A

1ml spike of 1 M HCl into 200 ml of exposure water ('fish water', see A2.3.1) resulted in a drop from neutral to pH 2.2, which is below the tolerance of zebrafish larvae. NaOH was added to buffer the HCl, resulting in a nominal addition of 5 mM NaCl. NaCl-matched controls were included, and each treatment was run in triplicate. One larva in three containers died during the exposure (3 out of 360, 0.83%) and there was no effect of treatment on mortality after 96 h (ANOVA, $p > 0.05$).

The acid carrier was sufficiently buffered by addition of NaOH, and the resulting increase in salt in the water did not affect larvae mortality, therefore a 1M HCL stock solution of americium was used for subsequent exposure, including suitable salt controls.

A2.3.4 Low-range dose response

For the low-range dose response test 30 larvae were counted into eight containers with 200 ml fish water and spiked with americium in 1 M HCl to produce the following respective activity concentrations: control 0 in triplicate, 0.1, 1, 10, 50, and 100 Bq ml^{-1}. After 96 hours exposure there was a single mortality out of a total of 240 larvae (0.4%) from the highest dose treatment.

Larvae from the 10, 50, and 100 Bq ml^{-1} treatments were sampled and analysed for ^{241}Am activity concentrations after 96 h exposure (Figure A2.2). The maximum activity in larvae exposed to 100 Bq ml^{-1} was 242.7 Bq. The bio-concentration factor at equilibrium [wet weight tissue concentration / water concentration, (Reinardy *et al.*, 2011)], based on an estimated weight of 100 μg per larvae, was between 1 and 2.1 (Figure A2.3).
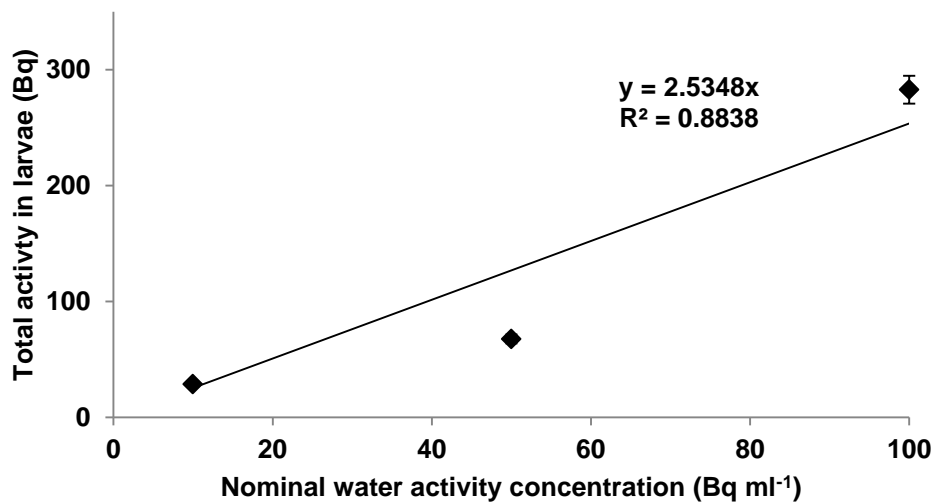
Figure A2-2: Total activity (Bq) in larvae exposed to a range (10, 50, and 100 Bq ml^{-1}) of ^{241}Am over 96 h. n = 30 larvae per concentration. Data are mean ± S.E.M. over a 30 min counting period (ORTEC gamma spectrometry).
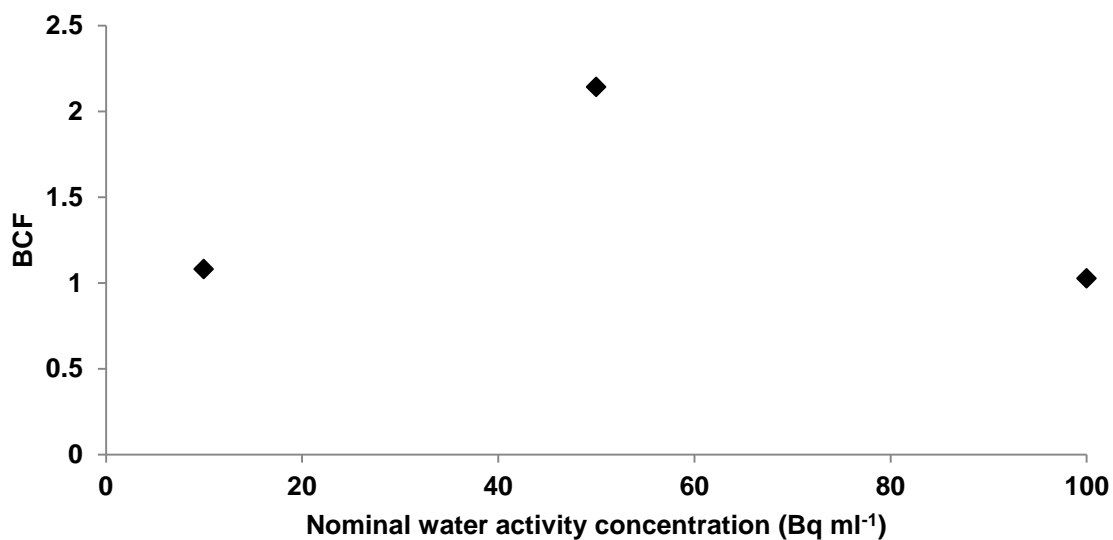
Figure A2-3: Bio-concentration (BCF) in zebrafish larvae (estimated to have an individual weight of 100 μg) exposed to aqueous ^{241}Am for 96 h.

A2.3.5   High range dose response

For the high-range dose response test 30 larvae were counted into eight containers with 200 ml fish water and spiked to with americium in 1 M HCl to produce the following respective activity concentrations: control 0 (quadruple), 100, 500, 750, and 1000 Bq ml^{-1}. After 96 hours exposure there was a single mortality out of a total of 244 larvae (0.4%) from a control container.

A2.4 Discussion

Estimation of radiation dose from activity concentrations involves complex calculations based on all emitted radiation from radionuclide (alpha-particles, beta- and gamma-irradiation) as well as size and shape of organism. The ERICA Tool is a useful method of dose estimation based on activity concentrations, and takes into account the differential biological effects of alpha, beta, or gamma radiation. The ERICA Tool default radiation weighting factors of 10 for alpha, 3 for low energy beta, and 1 for beta/gamma are designed to reflect the differing biological effect of the respective types of radiation in tissue. This high weighting factor for alpha radiation results in the high estimations of dose rates and accumulated doses from americium, and it is possible that in the case of an aqueous exposure of larval zebrafish the weighting factor is unsuitable, overestimating dose rates from water activity concentrations. Despite biological effects not being the focus of the study, the high estimated dose rates from [241]Am in adult zebrafish (Reinardy *et al.*, 2011) were within the range of doses that would have been expected to result in some biological effects (Knowles, 2002; Jarvis and Knowles, 2003), and no mortality or abnormalities were observed. This also supports an overestimation of dose

rates, in addition to the lack of lethal effects observed in larvae at considerably higher activity concentrations.

It was not possible to safely increase the activity concentrations beyond 1000 Bq ml^{-1} in the available facilities, and it was unexpected that such high exposures would not result in any mortality in larvae. Therefore an adult exposure to aqueous americium at an activity concentration level that would be expected to induce effects in reproduction and genotoxicity was not possible. This study suggests that validation of the weighting factor applied in estimating dose rates from alpha emitters may aid in furthering studies involving linking of environmental activity concentrations to biological effects.

A2.5 Acknowledgements

Appendix 3:

Standard Operating Procedures (SOPs)

LIST OF SOPs

SOP 1: Standard Operating Procedures for the alkaline comet assay on Rainbow trout blood

Principal Investigator: Ted Henry and Awadhesh Jha

Student Researcher: Helena Reinardy

Objective: Establish a protocol for performing the alkaline comet assay on adult rainbow trout (*Oncorrhynchus mykiss*), blood following specific experimental treatments (e.g., exposure to toxicants)

Reference: This SOP is a modification of the protocol by Seb Rider for general fish blood (salmonids).

Procedure:

This procedure is designed for using the comet assay on adult zebrafish blood, and is based on the protocol for salmonid blood (Seb Rider), with additions derived from the protocol for common carp and tilapia (Sana'a Mustafa).

1) Slide Preparation

a) Initial preparation
   i) Normal melting point agarose (A0169, Sigma) for initial coat to slides. 1.5 g into 100 ml dH_2O (in 200 ml bottle to leave space for bubbles when it boils). Melt in microwave set at highest power setting. Watch continuously as it will boil quickly. Remove from microwave when it starts to boil and check it is all dissolved. Store in cupboard.
b) Day Before Assay
   i) Re-melt in microwave. Pour carefully (it's hot!) into coplin jar. Before it sets dip slides (plain glass slides, or superfrost slides) into agarose to coat, wipe underside with tissue and place coated-side up on towel on tray. When all slides are coated, pour agarose back into bottle and return to cupboard. Place slides on tray into oven set at 37 ˚C and leave overnight.

2) Low Melting Point (LMP) Agarose

a) Make 0.75 % low melting point agarose for making cell gels.
b) Add 0.375 g LMP (Sigma 9414) agarose to 50 ml dH_20. Melt in microwave and aliquote 10 ml into 50 ml bottles (leaving space for boiling when needed).
c) Store in fridge until required.

3) Lysing Solution

a) Initial preparation
Using large (1L or 2L) Schott bottle, fill with 700 ml dH_2O. Place on a stirrer with a flea and dissolve the following, one at a time in order (NaOH helps the others to dissolve):

NaOH	7 g	(Fisher, S/4920/53)
NaCl	146.4 g	(Fisher, S/3160/53)
Na$_2$ EDTA	37.2 g	(Sigma, E5134-500g)
Trizma® Base	1.2 g	(Sigma, T1503-500g)
N-Lauryol-sarcosine	10 g	(Sigma, L5125-100g)

Mix all and leave overnight mixing until all has dissolved. Then adjust with NaOH (4M) to pH 10. Make up to 890 ml with dH$_2$O. This solution can be left at room temperature but refrigerate before use. If left refrigerated crystals may form. Note only 200 ml lysing solution is required for each assay filling 4 coplin jars.

b) IMMEDIATELY BEFORE USE ADD:
(Depends on how many slides are used, 10 slides into one slide bath, 100 ml volume)

Triton X 100 (cell lysis)   0.5 ml per 50 ml (2 ml for 200 ml)
(Sigma T-8787)

DMSO (prevents oxidative damage)   5 ml per 50 ml (20 ml for 200 ml)
(Fisher D/4121/PB08)

The final solution contains:

NaOH	to pH 10
NaCl	2.5 M
Na$_2$ EDTA	100 mM
Trizma® Base	10 mM
N-Lauryol-sarcosine	10g (1 %)
Triton X 100	1 %
DMSO	10 %

4) Electrophoresis Buffer

a) Make fresh for each set of runs (can be made whilst slides are setting in fridge or during lysis).
b) Make stock solution of 0.5M EDTA (Sigma E-7889).
   i) (0.465 mg EDTA into 250 ml dH$_2$O = 0.5M EDTA).
c) For 2 litres (needs to fill electrophoresis bath) add:
   i) EDTA stock (0.5M)   4 ml

    ii) NaOH (Fisher, S/4920/53)   10 g

    iii) pH 12.7 – 12.8

         (a) Check pH with new cell samples to see if it needs changing. Carp erythrocytes around 12.1/12.2.May have to reduce amount of NaOH if pH is closer to 13. 20g = 13.02 pH; 18.01g = 12.77 pH; 15.03g = 12.89!!

    iv) Refrigerate before use.

5) Neutralisation Buffer

a) To 800 ml dH_2O add 48.44 Trizma® Base.

b) Adjust pH to 7.5 using concentrated (4M) HCl. A lot is needed, take note of volumes added.

c) Make up to 1 litre.

    d) Can be stored at room temperature but refrigerate before use. This solution contains 0.4M Tris Base.

6) Ethidium Bromide Staining Solution

a) Stock solution 10 mg/ml

    i) Dilute to 0.02 mg/ml (1:500). Add 10µl to 4990µl distilled water to give total of 5ml of working solution.

7) Sample preparation

a) A cell dilution of 1 in 5000 is optimum. For fresh samples keep fresh blood on ice and perform all dilution on ice.

b) For all dilution steps make sure the solution is thoroughly mixed by pipetting and inverting tube. Try and avoid vortexing as it is too violent and may damage the cells.

c) DPBS (Invitrogen GIBCO 14190).

    i) 1:5000 dilution: serially dilute fresh blood 1:10, 1:10 and 1:50 (cumulatively 1:10, 1:100, 1:5000).

	Blood (µl)	DPBS (µl)	total volume (µl)
(1) First dilution	5	45	50
(1:10)	4	36	40
	3	27	30
	2	18	20
	1	9	10
(2) Second dilution	50	450	500
(1:100)	40	360	400
	30	270	300
	20	180	200
	10	90	100
(3) Third dilution			
(1:5000)	20	980	1000
(1:6666)	15	985	1000
(1:10,000)	10	990	1000

8) Assay Procedure

a) Slide preparation/resuspension in LMP agarose
i) Melt previously made LMP agarose in the microwave and store in water bath set at 40 °C (melted at 60 °C and stabilized at 37 °C).
ii) Spin final cell dilution solution for 3 min at 2000g. Use centrifuge in cold room to ensure samples are kept cold throughout.
iii) A small pellet may be visible but this is not always the case. Keep samples on ice.
iv) Discard the supernatant and resuspend in 180 µl warm LMP agarose from water bath.
v) For each slide drop 2x 75 µl onto slide (to make 2 gels side by side) and quickly place a cover slip on top and leave to set.
vi) Leave all the slides on tray at 4 °C for 1 hour.
(rest of assay is performed in the cold (4 °C) room).

b) Lysing
i) Whilst gels are setting at 4 °C prepare the lysing solution by adding the Triton-X and DMSO to the lysing solution, as outlined above.
ii) When the gels are set remove the cover slips gently and place slides into lysing solution in coplin jars at 4 °C.
iii) Leave for at least 1 hour.

c) Prepare electrophoresis buffer

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i) This can be made up before the start of the assay, whilst the gels are setting before lysis or during lysis. To ensure the buffer is cold either make it up early and leave to cool in cold room, or use previously-cooled distilled water.

d) **Unwinding**
   i) Pour cold electrophoresis buffer into chamber but make sure the slide holder is no yet covered. (Check pH 12.1/12.2 and temperature 4 ˚C).
   ii) Remove slides from lysing solution and place on slide holder in electrophoresis chamber.
   iii) Make sure all slides are orientated towards the current flow the same (labeled end towards the anode and randomly distribute slides), and fill any empty gaps with blank slides to prevent them from sliding (8 slides fit onto 1 row in chamber).
   iv) Add more electrophoresis buffer to a level that is just enough to cover the slides (otherwise the electrical current will run over the top of the gels instead of through them).
   v) When all the slides are covered immediately start the timer for unwinding.
   vi) Unwinding time depends on cell type. 5 min optimum for rainbow trout erythrocytes, 20 min optimum for carp erythrocytes.

e) **Electrophoresis**
   i) Immediately after unwinding place lid on electrophoresis chamber and turn the power supply on. Aim for 25 volts, 300 mA, but may need to reduce the voltage to reach 300 mA. Make sure to note the voltage and amps when its on – its not always the same as what was set. Voltage should be kept between 20 and 25 volts to ensure electrophoresis is working correctly.
   ii) Leave electrophoresis current to run for 20 min.

f) **Neutralization**
   i) After electrophoresis current is switched off, remove slides and carefully place into neutralization buffer in coplin jar. For rainbow trout erythrocytes leave for 10 min then transfer to dH_2O for a further 10 min. For carp erythrocytes do 3 x 5 min washes in neutralization buffer then 3 x 5 min washes in dH_2O.
   ii) When slides are washed, remove and leave to dry in cold room overnight. Slides are scored dry rather than wet because as the gel dries and shrinks all the cells end up on the same plane making it easier to score them.

g) **Scoring**
   i) Wear gloves at all times when handling ethidium bromide or the microscope used for scoring the comet assay.
   ii) Place 20 µl of ethidium bromide working solution onto each gel and cover with a cover slip.
   iii) Place onto microscope and score using the Komet 5 software.

SOP 6: Standard operating procedures for the alkaline comet assay on zebrafish larvae

Principal Investigator: Ted Henry

Student Researcher: Helena Reinardy

Objective: Establish a protocol for performing the alkaline comet assay on zebrafish, *Danio rerio*, larvae following specific experimental treatments (e.g., exposure to toxicants)

Reference: This SOP is a modification of the protocols for zebrafish sperm (SOP 13) and zebrafish blood (SOP 14), with some reference to the protocol for larvae (Jarvis and Knowles, 2003).

Procedure:

This procedure is designed for using the comet assay on zebrafish blastomeres, 5 hpf, but may be applied with modifications to younger and older blastomeres as well as larvae homogenates.

1) **Solution Preparation**

   a) **Agarose**
      i) 1.5% normal melting point agarose (A0169, Sigma-Aldrich)
      ii) 0.5% low melting point agarose (A9414, Sigma-Aldrich) was prepared in DPBS (invitrogen), 50 ml of each.

   b) Make stock of 10 M **NaOH**. Can be kept at room temperature for a few months.

   c) **EDTA** solution for lysis
      i) Make stock (200 ml) of 0.5 M EDTA
      ii) 37.224 g in 200 ml dH_2O
      iii) Add 10 M NaOH to dissolve.
      iv) Can be kept at room temperature for a few months.

   d) **EDTA** solution for electrophoresis
      i) Make stock of 200 mM EDTA
      ii) (FW 372.25. 372.25 in 1L = 1M. 372.25/5/5 = 200 mM in 200 ml)
      iii) Add 14.8896 g to 200 ml = 200 mM
      iv) Add 10 M NaOH to increase pH to 13, and help EDTA to dissolve.
      v) Can be kept at room temperature for a few months.

   e) Make stock of 1 M **Tris HCl** (can use Tris base instead).
      i) (FW 121.14, 60.57 g in 500ml = 1M)
      ii) Used for lysis buffer.

   f) Make stock of 0.4 M **Tris HCl** (can use Tris base instead).

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g) Dilute 1M stock by 2.5.
  i) 80 ml (1M Tris stock) + 120 ml dH$_2$O = 200 ml of 0.4 M Tris
  ii) pH 7.5
  iii) Use for Neutralisation Buffer.

h) **Lysis Buffer** stock (made up weekly)
  i) Make stock of 200 ml (for approx 24 slides).
    (1) 29.2 g NaCl
    (2) 40 ml EDTA (0.5M)
    (3) 2 ml Tris (1M)
    (4) 100 ml milliQ water
  ii) Mix
  iii) Adjust the pH to 10 using stock (10 M) NaOH
  iv) Make up the volume to 200 ml.
  v) Store at 4 ⁰C.

2) Sample preparation
a) 3-7 dpf larvae used
b) 30 larvae usually sampled per treatment. 30 larvae pipetted into 1.5 ml eppendorf tube, centrifuged to pellet larvae and water removed.

3) H$_2$O$_2$ preparation
a) Stock hydrogen peroxide (Sigma H1009) 8.8 M
  i) First dilution: 11.5 µl stock (8.8 M) + 988.5 µl distilled water → 0.1 M
  ii) Second dilution: 10 µl (0.1 M) + 990 µl DPBS → 1 mM
    (1) Final dilutions:

	1 mM stock (µl)	DPBS (µl)	Molarity
Total volume 1 ml	0	1000	control
	1	999	1 µM
	10	990	10 µM
	25	975	25 µM
	50	950	50 µM
	100	900	100 µM
	200	800	200 µM
	500	500	500 µM

4) *In vitro* exposure
a) Exposure done in triplicate with pooled sperm from 3 fish.
b) Use all 20 µl of blastomere cell solution for the H$_2$O$_2$ concentrations.
c) Add 200 µl of respective stock concentrations as quickly as possible and set time for 10 minutes.
d) Mix by flicking gently.
e) Set in centrifuge and spin after 6 minutes to pellet cells.
f) Remove 220 µl supernatant after total of 10 minutes exposure.
g) Add 10 µl L-15 medium to wash cells.

5) Slide preparation
    a) Melt NMP in microwave and pour into coplin jar.
    b) Dip superfrost slides into molten agarose and wipe underside. Place on tray and leave in oven or incubator at 30 ˚C overnight or until dry.
    c) Melt previously made up LMP and NMP in microwave at same time. Place LMP in waterbath set to 37-40 ºC.

6) Lysis Buffer
    a) Measure 25 ml of lysis buffer stock to 2 x 25 ml plastic screw-capped coplin jars (fits 10 slides, back to back).
    b) Add 250 µl Triton X-100 (cut end of tip off to help pipetting).
    c) If need 30 ml, add 300 µl Triton X-100
    d) Mix tip well and gently invert jar.
    e) Leave in fridge until use.

7) LMP
    a) 5 µl diluted semen (approx. 60,000 cells) was mixed with 180 µl LMP (using warmed tip) and place in 2x 75 µl drops onto the NMP gel.
    b) Place cover slips on drops as soon as possible.
    c) Slides left in cold room (4 ˚C) for an hour for gels to set.

8) Cell Lysis
    a) Gently remove the cover slips from the slides and place them back to back into the lysis buffer.
    b) Leave in cold room for 1 hour.

9) Electrophoresis Buffer
    a) For 2.4 L (new electrophoresis tank)
    b) 12 ml EDTA (200 mM stock, pH 13, take care with correct volume)
    c) 72 ml NaOH (10 M)
    d) 2316 ml distilled water
    e) Check pH – should be 13.

    f) If only 2 L needed
    g) 10 ml EDTA (200 mM stock, pH 13)
    h) 60 ml NaOH
    i) 1930 ml distilled water
    j) Check pH – should be 13
    k) This should result in a buffer with 1mM EDTA and 300 mM NaOH.

10) Unwinding
    a) Place slides in electrophoresis chamber.
    b) Leave to unwind for 20 min.

11) Electrophoresis
   a) It is very important to standardize the voltage and amplitude of the electric current.
   b) Set the power supply to 25 V and switch on.
   c) Check for bubble at either side of the bath to confirm current is working.
   d) Check the mA and adjust to 300 mA. Amps will increase by adding more buffer to raise the volume. Amps will decrease by removing buffer. Adjust volume until it reaches 300 mA.
   e) Run for 10 min.

12) Neutralisation and wash
   a) Place slides in jar and fill with neutralisation buffer (0.4 M Tris HCl, pH 7.5).
   b) Leave for 10 minutes.
   c) Replace buffer with distilled water
   d) Leave for 10 minutes.
   e) Remove slides and place on towel to dry.

13) Staining
   a) 10 mg/ml stock ethidium bromide. Keep in foil to avoid degradation in the light.
   b) Dilute 1:1000 by adding 0.5 µl stock to 500 µl milliQ water to give a working solution of 10 µg/ml ethidium bromide.
   c) Place 50 µl of working solution onto wet gel and replace cover slips (can use original cover slips).

14) Scoring
   a) Imaging software was Komet 5 (Kinetic Imaging, Nottingham).
   b) 50 cells scored per gel, 100 cells per slide.
   c) The means of % Tail DNA were calculated as well as the SE.

SOP 8   Alkaline comet assay on adult zebrafish blood after *in vitro* hydrogen peroxide exposure

Principal Investigator: Ted Henry and Awadhesh Jha

Student Researcher: Helena Reinardy

Objective: Establish a protocol for performing a positive control test of *in vitro* hydrogen peroxide exposure in zebrafish erythrocytes coupled with an alkaline comet assay as a measure of DNA damage.

Reference: This SOP is a modification of SOP 1 (comet assay on zebrafish erythrocytes) and the experimental protocol by Sana'a Mustafa for an *in vitro* hydrogen peroxide exposure in carp.

Procedure:

This procedure is designed for using the comet assay on adult zebrafish blood in a positive control *in vitro* exposure to H_2O_2.

1) **Slide Preparation**
   a) Initial preparation
      i) Normal melting point agarose (A0169, Sigma) for initial coat to slides. 1.5 g into 100 ml dH_2O (in 200 ml bottle to leave space for bubbles when it boils). Melt in microwave set at highest power setting. Watch continuously as it will boil quickly. Remove from microwave when it starts to boil and check it is all dissolved. Store in cupboard.
   b) Day Before Assay
      i) Re-melt in microwave. Pour carefully (it's hot!) into coplin jar. Before it sets dip slides (plain glass slides, or superfrost slides) into agarose to coat, wipe underside with tissue and place coated-side up on towel on tray. When all slides are coated, pour agarose back into bottle and return to cupboard. Place slides on tray into oven set at 37 ˚C and leave overnight.

2) **Low Melting Point (LMP) Agarose**
   a) Make 0.75 % low melting point agarose for making cell gels.
   b) Add 0.375 g LMP (Sigma 9414) agarose to 50 ml dH_2O. Melt in microwave and aliquote 10 ml into 50 ml bottles (leaving space for boiling when needed).
   c) Store in fridge until required.

3) **Lysing Solution**
   a) Initial preparation
   Using large (1L or 2L) Schott bottle, fill with 700 ml dH_2O. Place on a stirrer with a flea and dissolve the following, one at a time in order (NaOH helps the others to dissolve):

NaOH	7 g	(Fisher, S/4920/53)
NaCl	146.4 g	(Fisher, S/3160/53)
Na$_2$ EDTA	37.2 g	(Sigma, E5134-500g)
Trizma® Base	1.2 g	(Sigma, T1503-500g)
N-Lauryol-sarcosine	10 g	(Sigma, L5125-100g)

Mix all and leave overnight mixing until all has dissolved. Then adjust with NaOH (4M) to pH 10. Make up to 890 ml with dH$_2$O. This solution can be left at room temperature but refrigerate before use. If left refrigerated crystals may form. Note only 200 ml lysing solution is required for each assay filling 4 coplin jars.

b) IMMEDIATELY BEFORE USE ADD:
(Depends on how many slides are used, 10 slides into one slide bath, 100 ml volume)

Triton X 100 (cell lysis)   10 ml per 50 ml (2 ml for 200 ml)
        (Sigma T-8787)

DMSO (prevents oxidative damage)   5 ml per 50 ml (20 ml for 200 ml)(Fisher D/4121/PB08)

The final solution contains:

NaOH	to pH 10
NaCl	2.5 mM
Na$_2$ EDTA	100 mM
Trizma® Base	10 mM
N-Lauryol-sarcosine	10g (1 %)
Triton X 100	1 %
DMSO	10 %

4) Electrophoresis Buffer
   a) Make fresh for each set of runs (can be made whilst slides are setting in fridge or during lysis).
   b) Make stock solution of 0.5M EDTA (Sigma E-7889)**.**
      i) (0.465 mg EDTA into 250 ml dH$_2$O = 0.5M EDTA).
   c) For 2 litres (needs to fill electrophoresis bath) add:
      i) EDTA stock (0.5M)   4 ml
      ii) NaOH (Fisher, S/4920/53)   10 g

iii) pH 12.7 – 12.8
   (a) Check pH with new cell samples to see if it needs changing.
       Carp erythrocytes around 12.1/12.2.May have to reduce
       amount of NaOH if pH is closer to 13. 20g = 13.02 pH; 18.01g
       = 12.77 pH; 15.03g = 12.89!!
iv) Refrigerate before use.

5) **Neutralisation Buffer**
   a) To 800 ml dH_2O add 48.44 Trizma® Base.
   b) Adjust pH to 7.5 using concentrated (4M) HCl. A lot is needed, take note
      of volumes added.
   c) Make up to 1 litre.
   d) Can be stored at room temperature but refrigerate before use. This
      solution contains 0.4M Tris Base.

6) **Ethidium Bromide Staining Solution**
   a) Stock solution 10 mg/ml
      i) Dilute to 0.02 mg/ml (1:500). Add 10µl to 4990µl distilled water to give
         total of 5ml of working solution.

7) **Sample preparation**
   a) A cell dilution of 1 in 50 is optimum. For fresh samples keep fresh blood
      on ice and perform all dilution on ice.
   b) For all dilution steps make sure the solution is thoroughly mixed by
      pipetting and inverting tube. Try and avoid vortexing as it is too violent
      and may damage the cells.
   c) DPBS (Invitrogen GIBCO 14190).

8) **H_2O_2 preparation**
   a) Stock hydrogen peroxide 8.8 M
      i) First dilution: 11.5 µl stock (8.8 M) + 988.5 µl distilled water → 0.1 M
      ii) Second dilution: 10 µl (0.1 M) + 990 µl DPBS → 1 mM

(1) Final dilutions:	1 mM stock (µl)	DPBS (µl)	Molarity
Total volume 1 ml	0	1000	control
	1	999	1 µM
	10	990	10 µM
	25	975	25 µM
	50	950	50 µM
	100	900	100 µM
	200	800	200 µM
	500	500	500 µM

9) *In vitro* **exposure**
   a) Exposure done in triplicate with blood from 3 fish.

b) Final blood dilution placed into 8 tubes per fish (for 8 exposure concentrations, total number of tubes and slides 24).

c) Sample spun at 2000 rpm for 3 minutes to pellet cells.

d) Remove supernatant from all samples.

e) Add 200 µl of respective stock concentrations as quickly as possible and set time for 10 minutes.

f) Mix pellet by flicking gently.

g) Set in centrifuge and spin after 6 minutes to re-pellet cells.

h) Remove supernatant after total of 10 minutes exposure.

i) Add 200 µl DPBS to wash cells.

j) Re-spin and pellet.

10) Assay Procedure

a) Slide preparation/resuspension in LMP agarose

i) Melt previously made LMP agarose in the microwave and store in water bath set at 40 ˚C (melted at 60 ˚C and stabilized at 37 ˚C).

ii) A small pellet may be visible but this is not always the case. Keep samples on ice.

iii) Discard the supernatant and resuspend in 180 µl warm LMP agarose from water bath.

iv) For each slide drop 2x 75 µl onto slide (to make 2 gels side by side) and quickly place a cover slip on top and leave to set.

v) Leave all the slides on tray at 4 ˚C for 1 hour.
(rest of assay is performed in the cold (4 ˚C) room).

b) Lysing

i) Whilst gels are setting at 4 ˚C prepare the lysing solution by adding the Triton-X and DMSO to the lysing solution, as outlined above.

ii) When the gels are set remove the cover slips gently and place slides into lysing solution in coplin jars at 4 ˚C.

iii) Leave for at least 1 hour

c) Prepare electrophoresis buffer

i) This can be made up before the start of the assay, whilst the gels are setting before lysis or during lysis. To ensure the buffer is cold either make it up early and leave to cool in cold room, or use previously-cooled distilled water.

d) Unwinding

i) Pour cold electrophoresis buffer into chamber but make sure the slide holder is no yet covered. (Check pH 12.1/12.2 and temperature 4 ˚C).

ii) Remove slides from lysing solution and place on slide holder in electrophoresis chamber.

iii) Make sure all slides are orientated towards the current flow the same (labeled end towards the anode and randomly distribute slides), and fill any empty gaps with blank slides to prevent them from sliding (8 slides fit onto 1 row in chamber).

iv) Add more electrophoresis buffer to a level that is just enough to cover the slides (otherwise the electrical current will run over the top of the gels instead of through them).

v) When all the slides are covered immediately start the timer for unwinding.

vi) Unwinding time depends on cell type. 5 min optimum for rainbow trout erythrocytes, 20 min optimum for carp erythrocytes.

e) **Electrophoresis**
i) Immediately after unwinding place lid on electrophoresis chamber and turn the power supply on. Aim for 25 volts, 300 mA, but may need to reduce the voltage to reach 300 mA. Make sure to note the voltage and amps when its on – its not always the same as what was set. Voltage should be kept between 20 and 25 volts to ensure electrophoresis is working correctly.

ii) Leave electrophoresis current to run for 20 min.

f) **Neutralization**
i) After electrophoresis current is switched off, remove slides and carefully place into neutralization buffer in coplin jar. For rainbow trout erythrocytes leave for 10 min then transfer to dH_2O for a further 10 min. For carp erythrocytes do 3 x 5 min washes in neutralization buffer then 3 x 5 min washes in dH_2O.

ii) When slides are washed, remove and leave to dry in cold room overnight. Slides are scored dry rather than wet because as the gel dries and shrinks all the cells end up on the same plane making it easier to score them.

g) **Scoring**
i) Wear gloves at all times when handling ethidium bromide or the microscope used for scoring the comet assay.

ii) Place 20 µl of ethidium bromide working solution onto each gel and cover with a cover slip.

iii) Place onto microscope and score using the Komet 5 software.

SOP 9 Standard operating procedures for non-lethal collection of sperm from adult male zebrafish

Principal Investigator: Ted Henry and Awadhesh Jha

Student Researcher: Helena Reinardy

Objective: Establish a protocol for obtaining a sample of sperm sub-lethally from mature adult male zebrafish.

Reference: This SOP is a modification of a technique from (Jing *et al.*, 2009) . This technique involves sub-lethal anaesthesia and recovery and therefore is a Home Office licensed procedure. The procedure is only carried out by a trained person with a Personal License, under a Project Licence.

Equipment

- Wide forceps
- Spoon
- Sponge cut approximately the same depth as the fish to wedge and restrain fish.
- Capillary tubes (pulled in Bunsen burner to give smaller diameter tube).
- 2x 200ml beakers
- Dissecting microscope

Procedure:

1) Fish Selection
   a) For maximum volume of sperm, select males which are reliable and regular spawners.
   b) Pairs can be placed in pair spawning chambers overnight (see SOP 5). Remove the separator when the lights come on and the fish are ready to spawn. Observe the male and female come into contact and remove the male before spawning.
   c) If not using pair spawning chambers the volume of sperm sample may be less and the proportion of males which fail to give a sample may be greater.

2) Anaesthesia
   a) Prepare MS222 in about 2 cm of water (preferably tank water or water matching tank water).
   b) Place single male in MS222.
   c) Prepare a second recovery beaker filled with tank water to place the fish in after the procedure.
   d) Observe fish constantly. When the fish can no longer maintain position and shows no sign of swimming movement (opercular movement still rapid), remove fish gently with a teaspoon (forceps may induce tail damage).

3) Sample Extraction
   a) Place fish on tissue to gently remove excess water.

b) Wedge the fish belly up in the sponge and place under the dissection microscope.

c) Gently spread the pelvic fins to expose the anal and gonadal openings (very hard to see and not possible to distinguish between the holes).

d) Place the capillary tube at the opening with one hand and with the other, gently press down on the abdomen.

e) If successful a drop of milky liquid should exude from the opening and be drawn up into the capillary tube (< 1 µl!). If unsuccessful at first attempt return fish to the recovery water and try again with a new fish to avoid terminal injury.

f) Expel sperm from the capillary tube into a sample tube (for further processing) or onto a slide (for observation).

g) Once sample has been collected, place the fish in the recovery water. Observe the fish. It should be able to right itself and swim within a few minutes. If it shows no sign of recovery then terminate as a schedule 1 termination.

SOP 11   Standard operating procedures for the alkaline comet assay on human sperm

Principal Investigator: Ted Henry and Awadhesh Jha

Student Researcher: Helena Reinardy

Objective: Establish a protocol for performing the alkaline comet assay on human sperm.

Reference: This protocol is performed routinely by Luke Simon in the Reproductive Medicine Research Group, School of Medicine Dentistry and Biomedical Sciences, Queens University, Belfast.

Procedure: The assay room is lit by yellow light to avoid DNA damage from white light and daylight. Assay can be carried out at room temperature (18 °C) as sperm cell do not contain digestive enzymes and will not break down. During lysis cells should be placed in the fridge, and they can remain there for decondensation if convenient, although not necessary.

1) **Solution Preparation**

   a) **Agarose**
      i) 1 tablet of PBS dissolved in 200 ml of sterile water (as per instructions).
      ii) 1.5% normal melting point agarose (A0169, Sigma-Aldrich) and 0.5% low melting point agarose (A4018, Sigma-Aldrich) was prepared in PBS, 50 ml of each.

   b) Make stock of 10 M **NaOH**. Can be kept at room temperature for a few months.

   c) **EDTA** solution for lysis
      i)   Make stock (200 ml) of 0.5 M EDTA
      ii) Add 10 M NaOH to dissolve.
      iii) Can be kept at room temperature for a few months.

   d) **EDTA** solution for electrophoresis
      i)   Make stock of 200 mM EDTA
      ii) Add 10 M NaOH to increase pH to 13, and help EDTA to dissolve.
      iii) Can be kept at room temperature for a few months.

   e) Make stock of 1 M **Tris HCI** (can use Tris base instead).
      i)   Used for lysis buffer.

   f) Make stock of 0.4 M **Tris HCI** (can use Tris base instead).
      i)   pH 7.5
      ii) Use for Neutralisation Buffer.

   g) **Lysis Buffer** stock (made up weekly)

    i) Make stock of 200 ml (for approx 24 slides).
       (1) 29.2 g NaCl
       (2) 40 ml EDTA (0.5M)
       (3) 2 ml Tris (1M)
       (4) 100 ml milliQ water
    ii) Mix
    iii) Adjust the pH to 10 using stock (10 M) NaOH
    iv) Make up the volume to 200 ml.
    v) Store in fridge, 4 ºC.

h) **Decondensation**
    i) **DDT** (DL-Dithiothereitol, Sigma D9779-25G).
       (1) 1.58 g dissolved in 100 ml milliQ water
    ii) **LIS** (Lithium 3,5=diiodo-salicyate, Sigma D3635-25G)
       (1) 1.54 g dissolved in 100 ml milliQ water.
    iii) Aliquot both into 2.5 or 1.25 ml and store in freezer at -20 ºC.

2) Slide preparation

a) Melt previously made up LMP and NMP in microwave at same time. Place LMP in waterbath set to 37-40 ºC.
b) NMP
    i) Lay out fully frosted microscope slides (Surgipath) on towel.
    ii) 150 µl NMP pipetted into warmed tip. Place large (22 x 50 mm) cover slip resting at an angle on the slide and expel LMP underneath by drawing along slide, lowing cover slip at same time.
    iii) Slides left at room temperature to set for 15 min.

3) Lysis Buffer

a) Measure 25 ml of lysis buffer stock to 2 x 25 ml plastic screw-capped coplin jars (fits 10 slides, back to back).
b) Add 250 µl Triton X-100 (cut end of tip off to help pipetting).
c) Mix tip well and gently invert jar.
d) Leave in fridge until use.

4) LMP

    i) Cover slips were gently removed from the set NMP.
    ii) 10 µl diluted semen (approx. 60,000 cells) was mixed with 75 µl LMP (using warmed tip) and placed in drops into the NMP gel, replacing the cover slips imediately.
    iii) Slides left for further 15 min.

5) Cell Lysis

a) Gently remove the cover slips from the slides and place them back to back into the lysis buffer.
b) Place in fridge for 1 hour.

6) Electrophoresis Buffer

a) 1254.5 ml milliQ water
b) 39 ml NaOH (10M stock)
c) 6.5 ml EDTA (200 mM stock, pH 13, take care with correct volume)
d) This gives 1.3 liters buffer, sufficient to fill electrophoresis chamber.
e) Fill electrophoresis chamber (E-C Maxicell EC360 M Electrophoretic Gel System, with a BioRad Power Pac 300 power supply).

7) Decondensation
a) Remove required aliquots from freezer and leave to thaw.
b) Remove slides from jar. Add 2.5 ml of DDT. Mix and replace slides. Leave for 30 min.
c) Remove slides from jar. Add 2.5 ml of LIS. Mix and replace slides. Leave for 90 min.

8) Unwinding
a) Place slides in electrophoresis chamber.
b) Leave to unwind for 20 min.

9) Electrophoresis
a) It is very important to standardize the voltage and amplitude of the electric current.
b) Set the power supply to 25 V and switch on.
c) Check for bubble at either side of the bath to confirm current is working.
d) Check the mA and adjust to 300 mA. Amps will increase by adding more buffer to raise the volume. Amps will decrease by removing buffer. Adjust volume until it reaches 300 mA.
e) Run for 10 min.

10) Neutralisation
a) Remove slides from electrophoresis chamber and place on tissue in tray, gel side up.
b) Flood with neutralization buffer (0.4 M Tris HCl, pH 7.5).
c) Leave for 5 min before drain and reflood.
d) Repeat for 3 washes.

Stain and score immediately. If slides are left to dry the DNA will continue to spread and comet tails will be more diffuse. It is possible to fix gels in alcohol and leave to dry for later scoring but DNA may still spread and for best results score on same day.

11) Staining
a) 10 mg/ml stock ethidium bromide. Keep in foil to avoid degradation in the light.
b) Dilute 1:1000 by adding 0.5 µl stock to 500 µl milliQ water to give a working solution of 10 µg/ml ethidium bromide.
c) Place 50 µl of working solution onto wet gel and replace cover slips (can use original cover slips).

12) Scoring

    a) Slides were viewed on a Nikon E600 epifluorescence microscope (Nikon, Tokyo) equipped with an excitation filter of 515-560 nm from a 100 W mercury lamp and a barrier filter of 590 nm.

    b) Imaging software was Komet 5.5 (Kinetic Imaging, Nottingham).

    c) 50 sperm were scored per slide, two slides prepared per sample.

    d) The means of % Tail DNA were calculated as well as the SE.

SOP 13   Standard operating procedures for an *in vitro* exposure of zebrafish sperm to hydrogen peroxide and processed through the alkaline comet assay

Principal Investigator: Ted Henry and Awadhesh Jha

Student Researcher: Helena Reinardy

Objective: Establish a protocol for performing the alkaline comet assay on zebrafish sperm.

Reference: This protocol is adapted from the protocol (SOP 12) for zebrafish sperm.

Procedure: Assay can be carried out at room temperature (18 °C) as sperm cell do not contain digestive enzymes and will not break down.

1) Solution Preparation

a) **Agarose**
   i) 1.5% normal melting point agarose (A0169, Sigma-Aldrich)
   ii) 0.5% low melting point agarose (A9414, Sigma-Aldrich) was prepared in DPBS (Invitrogen), 50 ml of each.

b) Make stock of 10 M **NaOH**. Can be kept at room temperature for a few months.

c) **EDTA** solution for lysis
   i) Make stock (200 ml) of 0.5 M EDTA
   ii) 37.224 g in 200 ml dH$_2$O
   iii) Add 10 M NaOH to dissolve.
   iv) Can be kept at room temperature for a few months.

d) **EDTA** solution for electrophoresis
   i) Make stock of 200 mM EDTA
   ii) (FW 372.25. 372.25 in 1L = 1M. 372.25/5/5 = 200 mM in 200 ml)
   iii) Add 14.8896 g to 200 ml = 200 mM
   iv) Add 10 M NaOH to increase pH to 13, and help EDTA to dissolve.
   v) Can be kept at room temperature for a few months.

e) Make stock of 1 M **Tris HCl** (can use Tris base instead).
   i) Used for lysis buffer.

f) Make stock of 0.4 M **Tris HCl** (can use Tris base instead).

g) Dilute 1M stock by 2.5.
   i) 80 ml (1M Tris stock) + 120 ml dH$_2$O = 200 ml of 0.4 M Tris
   ii) pH 7.5
   iii) Use for Neutralisation Buffer.

h) **Lysis Buffer** stock (made up weekly)
  i) Make stock of 200 ml (for approx 24 slides).
    (1) 29.2 g NaCl
    (2) 40 ml EDTA (0.5M)
    (3) 2 ml Tris (1M)
    (4) 100 ml milliQ water
  ii) Mix
  iii) Adjust the pH to 10 using stock (10 M) NaOH
  iv) Make up the volume to 200 ml.
  v) Store at 4 ºC.

2) Sample preparation
a) Sperm extracted as per SOP 9.
b) Diluted (if < 1 µl) with 100 µl L-15 medium. Check a subsample under the microscope to ensure adequately diluted.
c) Aliquot 5 µl into eppendorf tube for assay.

3) H_2O_2 preparation
a) Stock hydrogen peroxide (Sigma H1009) 8.8 M
  i) First dilution: 11.5 µl stock (8.8 M) + 988.5 µl distilled water → 0.1 M
  ii) Second dilution: 10 µl (0.1 M) + 990 µl DPBS → 1 mM
    (1) Final dilutions:

	1 mM stock (µl)	DPBS (µl)	Molarity
Total volume 1 ml	0	1000	control
	1	999	1 µM
	10	990	10 µM
	25	975	25 µM
	50	950	50 µM
	100	900	100 µM
	200	800	200 µM
	500	500	500 µM

4) *In vitro* exposure
a) Exposure done in triplicate with pooled sperm from 3 fish.
b) 5 µl aliquots of sperm placed into 3 x 8 tubes for 8 different H_2O_2 concentrations.
c) Add 200 µl of respective stock concentrations as quickly as possible and set time for 10 minutes.
d) Mix by flicking gently.
e) Set in centrifuge and spin after 6 minutes to pellet cells.
f) Remove 200 µl supernatant after total of 10 minutes exposure.
g) Add 10 µl L-15 medium to wash cells.

5) Slide preparation
a) Melt NMP in microwave and pour into coplin jar.

b) Dip superfrost slides into molten agarose and wipe underside. Place on tray and leave in oven or incubator at 30 ˚C overnight or until dry.

c) Melt previously made up LMP in microwave. Place in waterbath set to 37-40 ºC.

d) Mix 180 µl LMP with the sperm sample (15 µl) and place 2x 75 µl as two drops on the NMP-coated slides.

   i)   Place cover slips on drops as soon as possible.

   ii) Slides left in cold room (4 ˚C) for an hour for gels to set.

6) Lysis Buffer

a) Measure 25 ml of lysis buffer stock to 2 x 25 ml plastic screw-capped coplin jars (fits 10 slides, back to back).

b) Add 250 µl Triton X-100 (cut end of tip off to help pipetting).

c) If need 30 ml, add 300 µl Triton X-100

d) Mix tip well and gently invert jar.

e) Leave in fridge until use.

7) Cell Lysis

a) Gently remove the cover slips from the slides and place them back to back into the lysis buffer.

b) Leave in cold room for 1 hour.

8) Electrophoresis Buffer

a) For 2.4 L (new electrophoresis tank)

b) 12 ml EDTA (200 mM stock, pH 13, take care with correct volume)

c) 72 ml NaOH (10 M)

d) 2316 ml distilled water

e) Check pH – should be 13.

f) If only 2 L needed

g) 10 ml EDTA (200 mM stock, pH 13)

h) 60 ml NaOH

i) 1930 ml distilled water

j) Check pH – should be 13

9) Unwinding

a) Place slides in electrophoresis chamber.

b) Leave to unwind for 20 min.

10) Electrophoresis

a) It is very important to standardize the voltage and amplitude of the electric current.

b) Set the power supply to 25 V and switch on.

c) Check for bubble at either side of the bath to confirm current is working.

d) Check the mA and adjust to 300 mA. Amps will increase by adding more buffer to raise the volume. Amps will decrease by removing buffer. Adjust volume until it reaches 300 mA.

e) Run for 20 min.

11) Neutralisation and wash

a) Place slides in jar and fill with neutralisation buffer (0.4 M Tris HCl, pH 7.5).
b) Leave for 10 minutes.
c) Replace buffer with distilled water
d) Leave for 10 minutes.
e) Remove slides and place on towel to dry.

12) Staining
a) 10 mg/ml stock ethidium bromide. Keep in foil to avoid degradation in the light.
b) Dilute 1:1000 by adding 0.5 µl stock to 500 µl milliQ water to give a working solution of 10 µg/ml ethidium bromide.
c) Place 50 µl of working solution onto wet gel and replace cover slips (can use original cover slips).

13) Scoring
a) Imaging software was Komet 5 (Kinetic Imaging, Nottingham).
b) 50 sperm were scored per gel, 100 sperm per slide.
c) The means of % Tail DNA were calculated as well as the SE.

SOP 18   <u>**Standard operating procedures for comet assay on zebrafish larvae – including *in vitro* validation with H$_2$O$_2$**</u>

Principal Investigator: Ted Henry and Awadhesh Jha

Student Researcher: Helena Reinardy

Objective: Establish a protocol for performing the alkaline comet assay on zebrafish larvae.

Reference: This protocol is adapted from the protocol (SOP 12) for zebrafish sperm.

<u>**Procedure:**</u>

Assay should be carried out on ice or in the cold room.

1) **Solution Preparation**

   a) **Agarose**
      i) 1.5% normal melting point agarose (A0169, Sigma-Aldrich)
      ii) 0.5% low melting point agarose (A9414, Sigma-Aldrich) was prepared in DPBS (Invitrogen), 50 ml of each.

   b) Make stock of 10 M **NaOH**. Can be kept at room temperature for a few months.

   c) **EDTA** solution for lysis
      i) Make stock (200 ml) of 0.5 M EDTA
      ii) 37.224 g in 200 ml dH$_2$O
      iii) Add 10 M NaOH to dissolve.
      iv) Can be kept at room temperature for a few months.

   d) **EDTA** solution for electrophoresis
      i) Make stock of 200 mM EDTA
      ii) (FW 372.25. 372.25 in 1L = 1M. 372.25/5/5 = 200 mM in 200 ml)
      iii) Add 14.8896 g to 200 ml = 200 mM
      iv) Add 10 M NaOH to increase pH to 13, and help EDTA to dissolve.
      v) Can be kept at room temperature for a few months.

   e) Make stock of 1 M **Tris HCl** (can use Tris base instead).
      i) 121.14 g in 1L = 1M; 60.57g in 500ml
      ii) Used for lysis buffer.

   f) Make stock of 0.4 M **Tris HCl** (can use Tris base instead).

   g) Dilute 1M stock by 2.5.
      i) 80 ml (1M Tris stock) + 120 ml dH$_2$O = 200 ml of 0.4 M Tris
      ii) pH 7.5
      iii) Use for Neutralisation Buffer.

    h) **Lysis Buffer** stock (made up weekly)
       i) Make stock of 200 ml (for approx 24 slides).
          (1) 29.2 g NaCl
          (2) 40 ml EDTA (0.5M)
          (3) 2 ml Tris (1M)
          (4) 2 g N-Lauroylsarcosine sodium salt
          (5) 100 ml milliQ water
       ii) Mix
       iii) Adjust the pH to 10 using stock (10 M) NaOH
       iv) Make up the volume to 200 ml.
       v) Store at 4 ºC.

2) Sample preparation

    a) Hatched larvae collected into microcentrifuge tube (approx. 30 used for gene expression).
    b) Remove as much water as possible (by placing tube on ice the larvae will fall to the bottom).
    c) Add 100 µl of cold DPBS and mechanically homogenise larvae (electronic pellet pestle, Sigma).Add required volume to make up suitable cell dilution (for gene expression samples, add a further 250 µl to match RLT volume from extraction SOP 107).

3) H_2O_2 preparation

    a) Stock hydrogen peroxide (Sigma H1009) 8.8 M
       i) First dilution: 11.5 µl stock (8.8 M) + 988.5 µl distilled water → 0.1 M
       ii) Second dilution: 10 µl (0.1 M) + 990 µl DPBS → 1 mM

(1) Final dilutions:	1 mM stock (µl)	DPBS (µl)	Molarity
Total volume 1 ml	0	1000	control
	1	999	1 µM
	10	990	10 µM
	25	975	25 µM
	50	950	50 µM
	100	900	100 µM
	200	800	200 µM
	500	500	500 µM

4) *In vitro* exposure

    a) Aliquot 5 µl into microcentrifuge tubes, in triplicate per H_2O_2 concentration.

b) 5 µl aliquots of homogenised larvae placed into 3 x 8 tubes for 8 different H_2O_2 concentrations.
c) Add 200 µl of respective stock concentrations as quickly as possible and set time for 10 minutes.
d) Mix by flicking gently.
e) Set in centrifuge and spin to pellet cells.
f) Remove 200 µl supernatant after total of 10 minutes exposure.
g) Add 10 DPBS to wash cells.

5) Slide preparation
a) Melt NMP in microwave and pour into coplin jar.
b) Dip superfrost slides into molten agarose and wipe underside. Place on tray and leave in oven or incubator at 30 ˚C overnight or until dry.
c) Melt previously made up LMP in microwave. Place in waterbath set to 37-40 ºC.
d) Mix 180 µl LMP with the sample (10 µl) and place 2x 75 µl as two drops on the NMP-coated slides.
   i) Place cover slips on drops as soon as possible.
   ii) Slides left in cold room (4 ˚C) for an hour for gels to set.

6) Lysis Buffer
a) Measure 25 ml of lysis buffer stock to 2 x 25 ml plastic screw-capped coplin jars (fits 10 slides, back to back).
b) Add 250 µl Triton X-100 (cut end of tip off to help pipetting).
c) If need 30 ml, add 300 µl Triton X-100
d) Pipette to mix.
e) Add 2.5 ml DMSO
f) Mix tip well and gently invert jar.
g) (If need total of 30 ml to cover slides, add 300 µl Triton-X and 3 ml DMSO).
h) Leave in fridge until use.

7) Cell Lysis
a) Gently remove the cover slips from the slides and place them back to back into the lysis buffer.
b) Leave in cold room for 1 hour.

8) Electrophoresis Buffer
a) For 2.4 L (new electrophoresis tank)
b) 12 ml EDTA (200 mM stock, pH 13, take care with correct volume)
c) 72 ml NaOH (10 M)
d) 2316 ml distilled water
e) Check pH – should be 13.

f) If only 2 L needed
g) 10 ml EDTA (200 mM stock, pH 13)

h) 60 ml NaOH
i) 1930 ml distilled water
j) Check pH – should be 13

9) Unwinding
a) Place slides in electrophoresis chamber.
b) Leave to unwind for 20 min.

10) Electrophoresis
a) It is very important to standardize the voltage and amplitude of the electric current.
b) Set the power supply to 25 V and switch on.
c) Check for bubble at either side of the bath to confirm current is working.
d) Check the mA and adjust to 300 mA. Amps will increase by adding more buffer to raise the volume. Amps will decrease by removing buffer. Adjust volume until it reaches 300 mA.
e) Run for 20 min.

11) Neutralisation and wash
a) Place slides in jar and fill with neutralisation buffer (0.4 M Tris HCl, pH 7.5).
b) Leave for 10 minutes.
c) Replace buffer with distilled water
d) Leave for 10 minutes.
e) Remove slides and place on towel to dry.

12) Staining
a) 10 mg/ml stock ethidium bromide. Keep in foil to avoid degradation in the light.
b) Dilute 1:1000 by adding 0.5 µl stock to 500 µl milliQ water to give a working solution of 10 µg/ml ethidium bromide.
c) Place 50 µl of working solution onto wet gel and replace cover slips (can use original cover slips).

13) Scoring
a) Imaging software was Komet 5 (Kinetic Imaging, Nottingham).
b) 50 sperm were scored per gel, 100 sperm per slide.

The means of % Tail DNA were calculated as well as the SE.

SOP 100   Standard operating procedure for designing primers for zebrafish

Principal Investigator: Dr. Theodore B. Henry

Objective: Establish steps for designing good primers

Reference:www.zfin.org; www.ncbi.nlm.nih.gov;
http://www.basic.northwestern.edu/biotools/oligocalc.html,
http://www.sigmaaldrich.com/life-science/custom-oligos.html

Primers already listed in paper:

- Check zfin website and do search for nucleotide or gene information.
- Check accession number or RefSeq from paper or zfin. Best to use RefSeq number as it has been verified to a greater extent, but accession number can be used if no RefSeq number available.
- Search for gene using RefSeq number in ncbi website.
- Copy and paste whole sequence into new primer document. Use find/replace function in word to remove numbers and spaces (enter ^# to search for numbers, enter 'space' to search for spaces).
- Find and highlight any published primers using the find/replace function.
  - [**NB for reverse primers, note that its written for backwards complimentary strand so you need to work out complimentary sequence, then reverse it to find it in the full sequence.**]
- Use the Primer-BLAST function on the ncbi website to check conditions of published primers, or search/design new ones.
-

Criteria for choosing primers:

  - Keep GC content in the 40-60% range
  - Avoid runs of an identical nucleotide, this is especially true for guanine, where runs of four or more Gs should be avoided
  - When using Primer Express software, the T_m should be 58-60 °C
  - Both primers should anneal at the same temperature
  - The five nucleotides at the 3' end should have no more than two G and/or C bases.
  - The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA
  - The primer pair has to be specific to the target gene and does not amplify pseudogenes or other related genes.
  - Test the amplicons and select ones that have the highest signal-to-noise ratio (i.e., low C_T with cDNA and no amplification with no template control or genomic DNA
  - Always Blast the primers

- Check primers for potential hairpins, complementarity, and self-annealing sites by copying primers into the OligoCalc website.
- Check for secondary structure of primers using the DNA calculator on the Sigma Aldrich website (should be weak or none, with no primer dimers).
- Complete primer-specific document, listing chosen primers, highlighting position in sequence, outlining the conditions of the primers (e.g. GC content, Tm, etc), outcome of the OligoCalc check, and any relevant references.

SOP 107   Standard operating procedures for extracting total RNA from zebrafish larvae for microarray or qPCR analysis

Principal Investigator: Dr. Theodore B. Henry

Objective: Establish a protocol for the extraction of total RNA from zebrafish *Danio rerio* following specific experimental treatments (e.g., exposure to toxicants)

Reference: This SOP was developed in CEB from the procedure used by Wan-Bin (Oct 2005) based on RNA easy mini kit (cat#74106, Qiagen) and was further modified to improve efficiency of handling of larvae.

Procedure:

This procedure is designed to be used for extracting RNA from zebrafish larvae following aqueous exposure of larvae to specific substances. In general, zebrafish embryos will be obtained from the stock colony, hatching will occur at 48-72 h post fertilization, and larvae will be placed in 400-ml glass beakers containing 200 ml exposure water.

Larvae Collection

1. Pour off as much exposure water as possible from experimental beaker into clean beaker. Pick up larvae that are swimming too near to the edge with pipet as you pour. Be careful not to pour off larvae. Pick up any larvae present in clean beaker and discard the water.
2. Pour the remaining water from experimental beaker into a small petri dish. Make sure ALL larvae are in the dish and not stuck to the wall of beaker.
3. Pipet out as much water as possible from petri dish without sucking up larvae.
4. When water amount is about 2ml, pipet water with larvae into 2ml microcentrifuge tube. (Tube pre-labeled with experiment number, sample number and date).
5. Place tube in ice-water to kill the larvae.
6. Pipet out excess water from microcentrifuge tube, leaving the pellet undisturbed.
7. If collecting many samples, store tubes in temporary liquid nitrogen tank before transferring to -80C freezer.

Storage

1. If extracting within 2 weeks after sample collection, storing in -80C is acceptable. If extraction is going to happen much later than 2 weeks, storing samples in liquid nitrogen is recommended. Samples can be stored this way for several months without compromising quality and quantity.

2. There should be little to no water in microcentrifuge tube after sample collection. If sample is immersed in RNAlater, discard RNAlater before adding RLT buffer (for RNA extraction). Note: Ratio of RNAlater to sample is 10:1. RNAlater can be used when there is no access to freezer or liquid nitrogen. Otherwise storing sample in RNAlater is not necessary.
3. After removing water or RNAlater, add 350 µl RLT (for RNA extraction from < 20 mg tissue).
4. After extraction, samples can be stored in -80C.

RNA Extraction

1. <u>Sonicate to break cells</u>: Place the 2 ml eppendorf tube containing larvae in 100-ml glass beaker filled with crushed ice (this will keep the sample cool during sonication). Clean the sonication probe with 70 % EtOH (or IMS) before and after each sample. Insert the sonication probe into the eppendorf tube such that the tip is approximately in the middle of the sample (do not want to be too near surface or bottom of tube). Set sonicator to "continuous" rather than "remote". Sonicate for an initial 5 second burst on level 4-5 and then check. If unbroken larvae still visible, sonicate for another 5 second burst on level 4-5. Store sample on ice until ready to proceed with other samples (i.e., until other beakers containing larvae have been sonicated).

   <u>NOTE: Too much sonication may cause nucleic acids, DNA and RNAto break.</u>

2. Pipette the lysate (350 µl) onto a QIAshredder spin column placed in 2 ml collection tube, centrifuge for 2 min at max speed, >8000xg (>10,000 rpm).
3. Transfer the flow-through to a new microcentrifuge tube, and centrifuge for 3 min at max speed.
4. Carefully transfer the supernatant into a new microcentrifuge tube. Add 1 vol (350 µl) of 70% ethanol to the cleared lysate, mix well by pipetting.
   a. [<20 mg tissue – 350 µl; 20-30 mg tissue – 600 µl; if tissue is stored in RNAlater, or difficult to lyse, then add 600 µl].
5. Add 700 µl of the sample (all of it) including any precipitation into an RNeasy minicolumn placed in a 2 ml collection tube. Centrifuge for 15 s at >8000x g (>10,000 rpm), discard the flow-through.
6. <u>DNase treatment</u>:
   a. Pippette 350 µl buffer RW1 into the RNeasy spin column, and centrifuge for 15 s at >8,000xg (>10,000 rpm) to wash. Discard the flow-through.
   b. In a separate tube prepare DNA solution by adding 10 µl DNase I stock solution to 70 µl RDD buffer **per sample**. Mix by gently inverting the tube, or by gently pipetting. DNase is found in -80 °C freezer in lab 422.

    i. [To make DNase I stock solution:
Add 550 µl molecular water to lyophilized vial by removing shield and lid carefully. Replace lid and mix by inverting vial, ensuring all powder is dissolved (there may be some in the lid).

Aliquote 50 µl into clean 0.5 ml tubes (10 µl per sample needed so good volume to store in).

Label 'DNase plus date'.

Store in -80 °C freezer in lab 422.

When thawing for dilution, do not re-freeze. Thawed DNase can be kept in fridge for 4-6 weeks.]

    c. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy silica-gel membrane, and place on the benchtop or in water bath (20-30°C) for 15 min (make sure to pipet the DNase I directly onto the membrane).
    d. Pipet 350 µl Buffer RW1 into the RNeasy mini column, and centrifuge for 15 s at >8,000 x g (>10,000 rpm). Discard the flow-through.

7. Transfer the column into a new 2 ml collection tube, pipette 500 µl buffer RPE onto the column, centrifuge for 15 s at > 8,000x g (>10,000 rpm) to wash the column. Discard the flow-through.
8. Add another 500 µl RPE buffer onto the column, centrifuge for 15 s at >8,000x g (>10,000 rpm) to wash the column again. Discard the flow-through, and centrifuge again at >8,000x g (>10,000 rpm) for 2 more min to dry the RNeasy silica-membrane.
9. Transfer the column into a new 1.5 ml collection eppendorf tube, pipette 30 µL of RNA storage solution (room temperature RNase-free water) directly onto the membrane. The RNase-free water can be kept at ~50°C by immersing in water from the hot water tap. Cut off lid of column and seal using 1.5 ml eppendorf lid. Let sit for 2 mins. (Can increase sitting time and reduce elution volume if concentrations of extracted RNA are low).
10. Centrifuge for 1 min at >8,000 x g (> 10,000 rpm) to elute the RNA out.
11. Discard the RNeasy minicolumn.
12. Store samples in -80°C freezer.

Using spectrophotometer NanoDrop (located in lab rm# 723) to measure RNA concentration

2. Switch on laptop.
3. Password: specmysample1
4. Clean NanoDrop with wipe before using machine. Open program ND-1000V3.5.2 on the computer. Add 2-3 µl molecular water before entering the program. Double click Nucleic Acid.
5. Initialize machine with 2µl of molecular water (or whatever is used for elution). Click OK. (Clean NanoDrop with wipe)

6. Change Sample Type to RNA-40. Then blank with 2µl of water. Click BLANK (Clean NanoDrop with wipe)
7. Measure all samples using 1-2µl. (Remember to clean NanoDrop with wipe after every measurement)
8. Put name of sample in Sample ID box. Then click MEASURE to read concentration.
9. To print report click show report. Give report a name click file then Print. (Papers print to printer in graduate office.)
10. Close program by clicking EXIT.

RNA easy mini kit (Cat #74106, Qiagen)

QIA shredder 250 (Cat #79656, Qiagen)

Notes: Optimum quality values for spectrophotometry of RNA: > 100 ng/µl,

260/280 - 1.9-2.2

260/230 – 1.9-2.2

Possible problem:

- 260/230 ratio very low: Sample likely to be contaminated with something which absorbs light at 230 nm.
  One source of contamination is guanidine thyocyanate salt which is in RLT and RW1 buffers. It may affect RT and/or qPCR so it is possible to run samples through a new column and repeat extraction to re-purify. If so, start by mixing RNA sample with estimated volume of RLT buffer, and equal volume of ethanol and then place in new column. Proceed with steps, excluding DNase treatment and RW1 step. RLT buffer and ethanol are needed for binding RNA to column. Also could increase washing steps with RPE buffer, increasing drying time by spinning for longer, and increasing elution time.

SOP 126   Standard operating procedures for reverse transcription (RNA – cDNA)

Principal Investigator: Dr. Theodore B. Henry

Objective: Establish a protocol for reverse transcription of RNA to generate cDNA in preparation for assessment of gene expression.

Reference: This SOP was developed in part from resources provided by Promega Complete manual on file: ImProm-II™ Reverse Transcription System, Instructions for use of product A3800. For specific citations visit: www.promega.com/citations/

Materials: Promega, ImProm-II™ Reverse Transcriptase (catalogue #A3802); RT (100 µL), 5X reaction buffer (600 µL), $MgCl_2$ (25 mM). Also need dNTPs (D7295-2ml) and Hexanucleotide primers (H0268-1UN).

Notes:

- Kit (ImProm-II™ Reverse Transcriptase) can be used to reverse transcribe RNA templates starting with either total RNA, poly(A)+ mRNA, or synthetic transcript RNA.

Procedure:

1. Preparation of the RNA template: Dilute extracted RNA to 100 ng/µl (if not already diluted at end of RNA extraction SOP).
    a. Remove RNA extraction from -80°C freezer and allow to defrost on ice.
    b. Label sterile nuclease-free tubes for each sample
    c. Concentration of extracted RNA measured on NanoDrop as part of extraction SOP 107. Optimum parameters:

        ≥100 ng/µL

        260/280 1.8-2.1

        260/230 > 1.5

    d. After RNA extraction and Nanodrop measurement there should be at least 20 µL for each extracted sample (30 µl minus 2µl for each nanodrop).
    e. Dilution: pipette 20 µL of the extracted RNA sample into sterile nuclease-free tube (this will allow you to be sure the volume is 20 µL; the few µL remaining can be discarded).
    f. Add the correct volume of nuclease-free water (keep tube on ice).
        Sample conc/100 = dilution factor

        Dilution factor × 20 = total volume

        Total volume – 20 = µL of water

i.e. (sample conc/100 x 20) - 20 = µl of water to add to 20 µl of RNA.

e.g. conc is 350 ng/µl: (350/100 × 20) – 20 = 50 µL; so add 50µl of water to 20 µl RNA to give 70 µl of 100 ng/µl RNA

 g. Keep tubes on ice and return to -80°C when finished with step 3 (below).

2. <u>RT Mix</u>:

 a. Remove all reagents from -20°C freezer and allow to defrost on ice. Vortex each one gently before use.
 b. Prepare RT-Mix in sterile, nuclease-free, 1.5 ml eppendorf tube (label 'RT-Mix).
 c. Prepare mix for all samples you plan to run at the same time (16 µL mix per sample, S), including no-template control.
 d. Combine reagents in order specified below. Keep tubes on ice.

Component	Final concentration	Volume to add
Nuclease-free water		6.6 µl (×S)
ImProm-II^{TM} 5X Reaction Buffer	0.83X	4 µl (×S)
$MgCl_2$ (25mM)	2.5 mM	2.4 µl (×S)
dNtp mix (10 mM each dNTP)	0.36 mM	1 µl (×S)
Hexanucleotide primers		1µl (xS)
ImProm-II^{TM} Reverse Transcriptase		1 µl (×S)
Final volume RT		16 µl (×S)

 e. Vortex RT-Mix gently to mix components and keep on ice.
 f. New Hexanucleotide primers will need preparing before use – defrost each tube and add 40 µl nuclease-free water and mix well (flick tube and spin down four or five times). Be sure to transfer over the last few µl of the previous tube's contents. This makes 0.5 µg/µl stock solution.

Notes:

 $MgCl_2$ may be optimized between 1.5-8.0 mM

3. <u>Preparation of RT reaction</u>

 a. Prepare a sterile, nuclease-free, thin-walled Bilatec 0.2ml PCR tube for each sample (including no-template control), and label appropriately for storage of cDNA. Place tubes on ice.

      b. Add 16 µL RT-Mix to eachRT reaction tube (PCR tube). Add 8 µL (i.e., 800 ng) of RNA sample [diluted to concentration of 100 ng/µL (i.e., step 1 above)] to RT reaction tube. This should provide enough cDNA after the RT reaction for triplicate samples for Q-PCR.

          i. Always keep tubes closed to prevent contamination and evaporation.

4. ReverseTranscription

      a. Be sure you have enough time to run the thermocycler as the machine cannot be left to run overnight. The whole procedure should take <1.5 hours.

      b. Use GeneAmp PCR System 970 thermocycler, Davy 428.

      c. Power on, front, bottom, left.

      d. Place tubes in heating block.

      e. User > Simon > Run > RT3 > Start > enter total volume (24 µl).

      f. Once plate has heated up it will display estimated running time.

          i. Settings should be as follows:

             ▪ Anneal at 25 °C for 5 minutes (may require optimization).

             ▪ Extend for 60 minutes at 42 °C (may require optimization 37-55 °C)

             ▪ Heat inactivate transcriptase by incubating at 70 °C for 15 minute

      g. Can analyze cDNA concentration and quality with NanoDrop, using ssDNA setting.

      h. See SOP107 RNA extraction for details on use of NanoDrop.

      i. Proceed with PCR or store cDNA in -80 freezer.

SOP 130   Standard operating procedures for qPCR using SYBR green method

Principal Investigator: Dr. Theodore B. Henry

Objective: Establish a protocol for the quantitative analysis of gene expression in zebrafish *Danio rerio* embryos and larvae exposed to stress-inducing chemicals using SYBR Green Q-PCR

Reference: This SOP was developed in UoP from the procedure outlined in the Sigma SYBR® Green JumpStart™ Taq ReadyMix™; CATALOGUE NUMBER S4438

Reagents:
- SYBR Green JumpStart Taq ReadyMix, Catalogue Number S9939, containing 20mM Tris-HCl, pH 8.3, 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), stabilizers, 0.05 unit/µL Taq polymerase, JumpStart Taq antibody, and SYBR Green I
- Internal Reference Dye, Catalogue Number R4526, 100× dye. Provided in a 0.3 ml vial.

Materials required but not supplied in kit:
- Water, PCR reagent, Catalogue Number W1754
- Primers
- DNA template
- Thermal cycler for quantitative PCR

Notes:
- SYBR Green JumpStart Taq ReadyMix can be stored at 2-8 °C for 3 months or for up to one year -20 °C for. There was no detectable loss of performance after 10 freeze-thaw cycles.
- Controls, a positive control is necessary to show the kit is working properly; a negative control is necessary to determine if contamination is present. A signal in the negative control indicates DNA contamination or presence of primer dimers
- After running a melting curve, any additional runs involving the same PCR product can be done with data collected in an additional detection step to eliminate primer-dimer and other mis-primed product signal. Melting Curve 50-95°C, read every 1°C, hold 5 sec.

A. Procedure for optimizing primer concentrations:
1. Objective is to determine the primer concentration that will give the most sensitive and reproducible product. Both the forward and reverse primers will be tested with dilutions of 1000, 500, 250, and 62.5 nM; and each combination of forward and reverse primer concentrations will be tested relative to each other.
   a. Obtain rack for holding tubes
   b. Prepare five new nuclease-free tubes for the forward primer and label 1-5; prepare five tubes for the reverse primer and label 1-5.
   c. Dissolve lyophilised primers into volume of RNase-free water, as directed on container, and freeze until needed.

d. Prepare 60 µL of 8 µM working solution for forward primer and place in forward primer tube 1; place 30 µL of water into tubes 2-5. Prepare 60 µL of 8 µM working solution for reverse primer and place in reverse primer tube 1; place 30 µL of water into tubes 2-5.

e. For the 8 µM forward primer working solution, dilute into forward primer tubes 2-5. First take 30 µL from tube 1 and place in tube 2, mix thoroughly by pipetting up and down at least 5 times. Repeat and mixing from tube 2 to 3, 3 to 4 , and 4 to 5.

f. For the 8 µM reverse primer working solution, dilute into forward primer tubes 2-5. First take 30 µL from tube 1 and place in tube 2, mix thoroughly by pipetting up and down at least 5 times. Repeat and mixing from tube 2 to 3, 3 to 4 , and 4 to 5.

g. Arrange 25 PCR tubes (5 X 5) for PCR reactions to test primer concentrations. Into each of the first column of tubes, aliquot 5 µL of forward primer tube 1, into second column use forward primer 2, etc. for each column. Into each of the first row of tubes, aliquot 5 µL of reverse primer tube 1, into second column use reverse primer 2, etc. for each column. (thus the tube located at position identified by column 1 row 1 will have the highest primer concentration, and tube located at column 5 row 5 will have the lowest concentration of primers).

h. Prepare qPCR Master Mix:
    i. Add reagents in appropriate-sized nuclease-free tube. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube. Reagents include:
        1. 520 µL 2×SYBR Green JumpStart *Taq* ReadyMix
        2. 156 µL water
        3. Final volume is 676 µL

i. Aliquot 26 µL of Master Mix to each of 25 PCR tubes (i.e., those tube containing primers). Mix thoroughly. Arrange 25 PCR tubes (new set of tubes from above) in the same order as described in 1f above. Transfer 18 µL from tube located in column 1 row 1 (above) to the same location in the second set of tubes. Repeat this for each of the tubes. The reason for this is to have one set of 25 tubes that have template and one set of 25 tubes that do not have template.

j. Add 2 µL of cDNA (10-50 ng) template to each of the first set of 25 tubes. Add 2 µL water to each of the second set of 25 tubes. Mix by gentle vortexing and briefly centrifuge to collect all components to the bottom of the tube.

k. Perform thermal cycling: (typical cycling parameters for 100 bp – 600 bp fragments)
    i. Initial denaturation 94 °C for 2 minutes
    ii. 40 cycles:
        1. Denaturation 94 °C for 15 seconds
        2. Annealing, extension, read fluorescence 60 °C (or 5 °C below the lowest primer T_M) 1 minute
    iii. Optional hold at 4 °C if products are to be run on a gel.

2. Evaluate fluorescent plots (ΔRn) for reactions containing target nucleic acid (first set of 25 tubes). Primer combinations with the lowest C_t and

the highest fluorescence will give the most sensitive and reproducible assays.

B. Procedure for Routine Analysis
1. Prepare Master Mix
    a. Add reagents in appropriate-sized nuclease-free tube, using ~10% more than is needed.
    b. Multiply the below volumes by S [where S = the number of samples + 1(no template control)]:
        i. 12.5 µl SYBR Green JumpStart *Taq* ReadyMix
        ii. 0.75 µl forward primer (optimal concentration determined in part A above)
        iii. 0.75 µl reverse primer (optimal concentration determined in part A above)
    c. Mix contents by gently vortexing and briefly centrifuge to collect components at the bottom of the tube
    d. If running a gene as positive control (e.g. ß-actin) prepare a second Master Mix containing ß-actin forward and reverse primers.
2. Prepare plate
    a. Dilute template cDNA (from SOP 128 RT reaction for RNA, 15 µl, 10-100 ng)
        i. 1:10 dilution (check for optimal dilution), volume based on number of samples, 6 µl of dilution per well
    b. Add 20 µl RNase-free water into 'water only' controls
    c. Add 6 µl RNase-free water into triplicate 'no template' controls
    d. Add 6 µl sample template into appropriate wells (each in triplicate)
    e. Add 14 µl Master Mix into each well, excluding water controls
    f. Cover with film securely.
    g. Tap to bring all volume to bottom of wells. Optional: place plate with suitable balances in plate centrifuge (Davy 422) and spin at 200 rpm for 30 sec.
    h. Plate can be stored in the fridge or in a covered ice box for up to 2 hours if the machine is not free.

3. Perform cycling using Applied Biosystems StepOne RT PCR System, and StepOne software v2.0
    a. Switch on q-RT-PCR machine at plug FIRST then at back of maching.
    b. Switch computer on (plug and laptop). Password for computer is simonwfox.
    c. Pull out drawer on the front of the machine, load plate and close the drawer.
    d. Click StepOne software v2.0 icon on desktop.
    e. Program Set Up
        i. Design wizard
        ii. Enter experimental name
        iii. Select 48-well setting – next
        iv. Select for standard curve
        v. Select SYBR®Green reagent
        vi. Select cDNA template – next

<div style="margin-left:2em">
<div style="margin-left:2em">
vii. Select 1 for no of targets - next
viii. Select 4 points for standard curve
ix. Select for 1 replicates - next
x. Select samples (e.g. if using whole plate select 37 and no replicates, or 12 samples x 3 replicates), selected wells indicated. – next
xi. Select cycling conditions (page 2D):
</div>
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1. Holding stage
   a. temp 94 ˚C, time 2 min
2. Cycling stage
   a. Step 1 temp 94 ˚C, 15 sec
   b. Step 2 temp 55˚C, unselect fluorescence, 1 min
   c. add step 3 after step 2
   d. Step 3 temp 72 ˚C, select fluorescence on, 1 min
3. Melt curve
   a. Step 1 temp 94 ˚C, 15 sec
   b. Step 2 temp 60 ˚C, 1 min
   c. Step 3 temp 94 ˚C, 15 secs
4. Optional hold at 4 °C if products to be run on a gel
5. Reaction volume per well :20 µl - next

xii. Move to page 3A without changing default settings

xiii. Page 3A select 'finish designing experiment'

f. Select Start Run
   i. Save in appropriate location (Teds file)
   ii. A box may pop up indicating standards have expired, click yes

g. When pre-cycling is initiated, return to Set Up page - Assign targets and samples – bottom left of screen select dye as passive reference to 'none'

h. Return to Analysis

Saving Results/Removing Plate

1. Once run has finished, go to file, click save as. You can then copy and paste your file from Ted's file to a memory stick.
2. Open the drawer on the front of the machine and remove your plate.
3. Close the StepOne software.
4. Power off the PCR machine by pressing the blue button on the bottom left of the touch screen.
5. Switch PCR machine off using button on the back of machine and switch plug off at the wall.
6. Shut down laptop.

Analyzing Results

1. Select standard wells and examine standard curve. Eliminate outliers if an entire group strongly deviates from expected value.
2. Select all wells. Adjust Ct to 25,000
3. Click "Quantify" tab at bottom of screen. Copy and paste data into Excel. Copy desired graphs as well.

SOP 130b   Standard operating procedures for performing agarose gel electrophoresis on RT-PCR products from zebrafish

Principal Investigator: James R. Syrett

Objective: To establish a protocol for identification of the sizes and relative quantities of the products of RT-PCR from zebrafish (*Danio rerio*) mRNA.

Reference: This SOP was developed from the procedure used by Michelle Kiernan (July 2006).

Notable Hazards: Take care when handling hot solutions. Risk of superheating solution, causing it to boil over without warning. Swirl regularly when heating and hold at arm's length. Ethidium bromide is toxic and carcinogenic. Wear gloves when handling and dispose of all contaminated materials in the ethidium bromide waste bin. Do not allow any contaminated gloves or tools outside the area marked with biohazard tape. Store ethidium bromide at 4°C in the dark, marked with a TOXIC label. UV radiation is carcinogenic. Avoid direct exposure to eyes and skin.

Procedure:

This technique was based on one used to identify bacterial plasmids, and has been modified for use on zebrafish eggs and larvae at the age of 0-7 days post-fertilisation, though should be applicable to any RT-PCR product.

Reagent Preparation

Prepare 10xTAE **or** 10xTBE buffer as per the manufacturer's instructions, or from the following recipes.

10xTAE buffer (1l)

- 1.86g EDTA (disodium salt)
- 48.4g Tris Base
- 1.1ml glacial acetic acid
- Add 900ml deionised water, adjust pH to 8.5 with NaOH, then make up to 1l with water.

10xTBE buffer (1l)

- 109g Tris base
- 55g Boric acid
- 4.8g EDTA (disodium salt)
- Dissolve in 900ml deionised water, adjust pH to 8.3 with NaOH, then make up to 1l with water.

Preparing Agarose Gel

1. For a 2% agarose minigel (8x10cm), add to a conical flask:
   - 1.0g agarose
   - 5ml 10xTAE buffer (or 10xTBE)
   - 45ml deionised water
2. Heat on full power in a microwave oven for 1 minute, swirling after 45s. Check that all the agarose is dissolved.
3. Leave to cool for 5min at room temperature.
4. Prepare the gel mould by taping up both ends. press along the edges to ensure a good seal. Set on a level surface.
5. Once the gel has cooled to about 60°C (enough to hold comfortably), add enough ethidium bromide to give a final concentration of 0.5μg/ml (say, 2.5μl of a 10mg/ml stock). Swirl to mix.
6. Slowly pour the molten gel into the mould. When finished, use a disposable pipette tip to sweep any air bubbles to the sides of the gel.
7. Carefully add the gel comb, making sure that no air bubbles form on the teeth.
8. Leave the gel to set for 30 minutes to 1 hour.

Preparing Electrophoresis Tank

1. Prepare 0.5xTAE (or TBE) buffer by diluting 1 part of the 10x stock with 19 parts deionised water. This is the running buffer. There should be enough to cover the gel in the tank.
2. Remove the tape from the ends of the gel mould and place on the white platform in the tank, ensuring that the comb is above the red lines at the **end** of the platform.
3. Pour on the running buffer, ensuring the gel is well covered.
4. Carefully remove the gel comb, making sure that the wells aren't torn, and do not contain air bubbles.
5. Check before loading any samples that the leads from the power pack are correctly connected and that the power pack is working properly.

Loading Samples

1. Prepare a clean 0.2ml eppendorf tube for each sample. Add 8μl of PCR product to each tube, then add 2μl of gel loading buffer to each tube.
2. Transfer the entire contents of the tube to one of the wells in the agarose gel. Take care not to pierce the gel with the pipette tip and **do not** depress the plunger completely, to prevent ejecting air into the gel.
3. When all the samples are loaded, load 10μl of DNA ladder solution another well (this should already contain loading buffer. If not, use 8μl DNA / 2μl loading buffer again).
4. Record each well's contents for reference after the gel has run.

Running the Gel

1. Place the lid on the gel tank and connect to the power pack, ensuring the negative (black) terminal is closer to the sample wells.
2. Switch on the power pack and adjust the voltage to 100V (5V/cm with a 20cm long tank). A stream of bubbles coming from the terminals indicates that a current is flowing.
3. Leave the gel to run for 40-45 minutes until the blue loading dye has moved between ⅔ and ¾ of the length of the gel (past the second set of red lines).

Removing the Gel

1. Turn off the power pack and disconnect the tank. Remove the lid and carefully extract the gel.
2. View or photograph the gel under UV illumination. Discard in the ethidium bromide waste bin.

Tips and Alternative Methods

More concentrated buffer stocks may be prepared by appropriately adjusting the masses of solutes. The dilution factor must be adjusted accordingly to give 0.5x buffer. For best results, the running buffer and gel buffer should be the same concentration (here 0.5x).

Check which size of gel comb is being used. This protocol uses 10µl samples, which completely fill the wells made by the smallest comb. Larger samples call for larger combs. If possible, avoid using the wells at the sides of the gels, as these can run unevenly.

It is a good idea to load the gel asymmetrically to avoid confusion should the gel be flipped over. You can do this by leaving one well empty, using only one well for the ladder, or avoiding using two ladders in the outside lanes (use one on outside lane, one nearer the centre).

As a test that the pipette tip is correctly in the well for loading, gently wiggle the pipette side to side. You should feel the walls of the well and the gel should move on the platform. You can keep the pipette steady whilst loading by placing one finger next to the tip.

Better resolution can be obtained by running the gel at 0.5V/cm for 10 minutes at the start, to allow the DNA to move into the gel evenly, before going up to 5V/cm. This is often not necessary.

Tools can be decontaminated of ethidium bromide by immersion in a dilute bleach bath.

2% gels give good resolution for 200bp - 1kbp DNA fragment. 0.7% is useful for 2kbp - 5kbp fragments.

SOP 131   Standard operating procedures for conducting PCR

Principal Investigator: Dr. Theodore B. Henry

Objective: Establish a protocol for routine PCR with samples obtained from fish tissue

Reference: This SOP was developed in part from a kit provided by Sigma: ReadyMix^{TM} Taq PCR Reaction Mix with MgCl$_2$ (Catalogue number P4600)

Materials: For kit see attached sheet from Sigma.

Notes:

- The PCR reaction on the thermocycler takes ~2 hours. Be sure you have enough time to run the reaction because the machine cannot be left to run overnight.
- Forward and reverse primers obtained from the manufacturer are ordered separately and based on specific design criteria. They are shipped as lyophilized vials and must be reconstituted with nuclease-free water. The amount of primer is given on the literature sent with the primer. Following the direction for reconstituting primers, make a 100 µmole/L (=100 pmol/µl) stock solution and also prepare at least four vials of primer working solution (this is to prevent too much freezing and thawing of primers). Working solution is prepared by taking 5 µL of stock solution and diluting to 500 µL with nuclease-free water. This results in a primer working solution concentration of 1µmole/L (1 µM).
- The concentration of primers in the final reaction volume is important for the PCR reaction. If PCR does not work well, primer concentrations may need to be optimized to improve the success.
- The procedure below is designed for a final primer concentration of 300 nM. Thus, 10 µL of primer working solution will be added for each 50 µL reaction (each sample requires 10 µL).
- The DNA template concentration is important. If the sample is cDNA obtained from reverse transcription of RNA see the procedure for the reverse transcription of RNA. The reverse transcription reaction of RNA is performed on 800 ng (obtained from total RNA extracted from sample and diluted to 100 ng/µL; and final volume of RT reaction of 24 µL) of total RNA and the final concentration of DNA is ~33.3 ng/µL.
- The procedure below is designed for a final DNA template concentration of ~165 ng in the 50 µL reaction volume. Thus, 5 µL of the cDNA template (concentration 33.3 ng/µL) will be added for each reaction.

Procedure:

1. Obtain a new 0.5 ml nuclease-free tube and label as PCR Master Mix. Keep tube on ice and add the following (where S = the number of samples + 1):
   a. S×(25 µL) ReadyMix (from kit)

       b. S×(10 µL) Forward primer

       c. S×(10 µL) Reverse primer

2. Obtain new 0.2 ml PCR tubes and label for each of your samples and for the no-template control

    a. Keep all tubes on ice.

    b. Add 45 µl of PCR Master Mix (prepared in step 1) to each tube

    c. Add 5 µl (~165 ng) of the sample cDNA (DNA template) to the correct tube.

    d. Add 5 µl of water to the tube labeled "no template control"

    e. Total final volume is 50 µl.

3. PCR cycling parameters (use GeneAmp PCR system 9700 machine in room 428):

    a. Select User > Sheren > Run > Exp001. Parameters should be as below

        i. Heat Lid at 94 °C for 5 minutes

        ii. Denature the template at 94 °C for 45 seconds

        iii. Anneal primers at 55 °C for 45 seconds

        iv. Extension at 72 °C for 45 seconds

        v. 30 cycles of amplification

        vi. Hold at 72 °C for 7 minutes

        vii. Hold at 4 °C

        viii. Total running time is ~ 2 hours

    b. Remove samples when PCR machine has cooled to 4 °C and store at -80 °C or proceed directly to evaluate product on gel.

    c. Do **NOT** leave the PCR machine to remain at 4 °C overnight.

Appendix 4:

Manuscript of PhD publications

Reinardy, H.C., Teyssie, J-L., Jeffree, R.A., Copplestone, D., Henry, T.B., Jha, A.N. (**2011**). *Uptake, depuration, and radiation dose estimation in zebrafish exposed to radionuclides via aqueous or dietary routes*. Science of the Total Environment. 409: 3771-3779.