4. Chapter Four: Water Exposure Trials

4.1. Introduction

4.1.1. Pollution

Survival of the early life stages of salmonids and the consequent developmental success are paramount for the long-term persistence of salmonid stocks. It is therefore imperative that there is an understanding of the potential risks associated with degraded salmonid spawning habitats and the possible deleterious effects on the salmonid eggs and alevins as a result of water-borne pollutants.

In recent years salmonid numbers have been shown to be in decline across the UK. The number of anadromous sea trout (*Salmo trutta*) in the northern Atlantic have shown a dramatic decline in the last two decades (Jonsson and Jonsson, 2009). The numbers of Atlantic salmon (*Salmo salar*) in the UK have also experienced a recent marked decline (Hendry & Cragg-Hine, 2003), with an approximate 50% reduction in the number of adults returning to fresh water since the 1970s (Graham and Harrod, 2009). Fish are considered to be good indicators of water quality (Fausch *et al*., 1990; Soto-Galera *et al*., 1998; Uriarte and Borja, 2009) suggesting that the decline in salmonid numbers may be a result of a reduced water quality and an increase in pollution. The Water Framework Directive describes pollution as deliberate or accidental contamination from human activity that harms ecosystems, human health, material, property, amenities or other legitimate uses of the environment (EA, 2006b). Increasing industrialisation and anthropogenic activity produce many xenobiotics that threaten the environment (Wiegand *et al.*, 2001). Xenobiotics such as metals, chlorine, cyanides, ammonia, pesticides, polychlorinated biphenyls, herbicides and petroleum hydrocarbons are the major classes of toxic chemicals that enter the waterways and may affect non-target organisms such as fish eggs and larvae, resulting in decreasing recruitment to the adult population (Heath, 1995). There are two ways in which pollution enters the water course: point source pollution or non-point source pollution. Point-source pollution generally originates from wastewater discharged from industrial facilities and municipal sewage (Uriarte and Borja, 2009). A number of legislations on environmental welfare have been in place since the 1970s, such as the

Environment Protection Act 1990 (UK), which have helped to decrease the degree of point source pollution from industrial and agricultural emissions (Cave, 1998). However, non-point source pollution (diffuse pollution) is much harder to regulate and therefore a greater threat to water quality (Faulkner *et al*., 2000), with 87% of UK rivers thought to be at risk (EA, 2007). Diffuse pollution continues to persist as it is difficult to measure and control as a result of wide dispersal of sources and the tendency to fluctuate with seasons and weather (Carpenter, 1998).

A large amount of diffuse pollution arises from land use activities. Such pollutants can be persistent, resulting in long term damage to aquatic organisms (Ross *et al*., 1995). A major source of diffuse pollution is from agriculture. Diffuse pollution from agricultural sources includes herbicides, pesticides, organic and inorganic fertilisers (Wheeldon, 2003). Nutrients and chemicals contained in the fertilisers can leach from the soil ending up in rivers and ground water. Less soluble components can be washed overland to rivers, which are a particular problem during heavy rainfall. This leads to large concentrations of pesticides and chemicals being washed into surrounding waters (EA, 2007). In the UK, it is reported that 28,000 tonnes of pesticides are used each year and the majority of these chemicals end up in rivers and lakes (Huskes and Levsen, 1997).

4.1.2. Polycyclic Aromatic Hydrocarbons

As well as diffuse pollution from agricultural sources, urban runoff is another method of entry into the water course. Polycyclic Aromatic Hydrocarbons (PAHs) exist as components of crude oil, and arise from the chemical conversion of coal, asphalt, creosote and roofing tar (Lyons, 1997). PAHs are released into the environment directly from oil spills or from the incomplete combustion of carbon-containing fuels such as diesel, wood, coal and tobacco (Lyons, 1997). PAHs are capable of bioaccumulation in plant (Meudec *et al*., 2006) and animal (Harwell and Gentile, 2006) tissues and, due to their atmospheric presence, are able to travel long distances, resulting in worldwide occurrence (Ngabe and Poissant, 2003). Naphthalene tis a non-polar PAH which can be found in a wide range of products including petroleum products, mothballs, wood preservatives, solvents and dyes. However, the main source of naphthalene in the environment is believed to be vehicle

exhaust (Gavin *et al.,* 1996). Hydrocarbons deposited by vehicles on roadways can collect in roadside ditches and following sufficient rainfall can make their way into a stream or river (Incardona *et al.*, 2004). PAHs can also be manufactured into pesticides, which can also enter the waterways by agricultural and urban runoff. Naphthalene is the major raw material for Carbaryl, which is used as a general purpose insecticide, and phenanthrene is manufactured in the production of phenanthrenequinone, which is an intermediate for some pesticides (Nagpal 1993).

In the environment, lipophilic (hydrophobic) contaminants, such as halogenated aromatic hydrocarbons (HAHs), PCBs and PAHs readily bioaccumulate in fish. Bioaccumulation of these lipophilic chemicals by adult fish may have significant consequences on the development and survival of their offspring (Ostrander, 1996). However, uptake of hydrophobic chemicals into the developing embryo from water is not a significant route of exposure in the environment since concentrations of these chemicals freely dissolved in water are extremely low (Ostrander, 1996). As the development of the embryo progresses from fertilisation to hatching, and then larval maturation, the response to toxic substances also changes (Ostrander, 1996). Differences in sensitivity between early life stages and juvenile adult stages of fish may partly be caused by differences in accumulation (Petersen and Kristensen, 1998). The yolk absorption process is believed to be a major factor in the redistribution of chemicals that result in toxic effects in later developmental stages. The relative lipid content in early life stages is higher than in juvenile and adult stages, therefore it can be expected that there will be a higher accumulation of lipophilic chemicals during the embryonic stage (Petersen and Kristensen, 1998). Embryonic stages of salmonids depend on their yolk-sac prior to exogenous feeding. This lipid-rich sac can be up to 60-70% of the total body weight (Honkanen *et al.*, 2001). The relative proportion of lipid content in the early life stages is far greater than in the adult, indicating that they have a greater affinity for lipophilic chemicals per kilogram of total weight and thus accumulate more (Anderson *et al.*, 1997).

Both naphthalene and phenanthrene possess the capability to cross the egg chorion of sheepshead minnow (*Cyprinodon variegates*). By removing the chorion it was observed that it did not have a significant impact on hatching success when compared to eggs which had retained the chorion during exposure, thus illustrating chorion permeability (Anderson

et al., 1997). Another study has shown that lipophilic PAHs accumulate within the yolk sac of pink salmon reared in oiled gravel (Marty *et al.*, 1997). Embryonic stages of fish are the most vulnerable to PAH accumulation in rivers (Mäenpää *et al*., 2004) which could be a result of the critical differentiation of the nervous and circulatory system occurring over a short period of time (Peterson and Kristensen, 1998) or the undeveloped capability to metabolise xenobiotics which may have accumulated within the egg or alevin (Peterson and Kristensen, 1998). Vulnerability of early life stages was demonstrated by research following the 1989 Exxon Valdez oil spill in Alaska which contaminated near-shore and intertidal spawning grounds for Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*). Sub-lethal effects such as malformations, yolk sac oedema, premature hatching, jaw reductions and spine curvature were recorded (Carls *et al*., 1999; Heintz *et al*., 1999; Hose *et al.,* 1996) in the early life stages of the species. Additionally, Carls *et al.* (1999) and Heintz *et al*. (1999) noted an increase in the frequency rate of malformations with weathered crude oil exposure, which is believed to be a result of the composition shift from predominantly two-ring benzene structure (e.g. naphthalene) to a structure with a higher frequency of tri-cyclic PAHs (e.g. phenanthrene). It is this higher frequency composition which is believed to induce increased malformations in early life stages as Incardona *et al*. (2004) demonstrated with zebrafish. Phenanthrene exposed embryos exhibited morphological malformations (Figure 4.1.), however naphthalene exposed embryos appeared to undergo ordinary development (Figure 4.2). These findings support those of Carls *et al*. (1999) and Heintz *et al.* (1999) following the Exxon Valdez oil spillage.

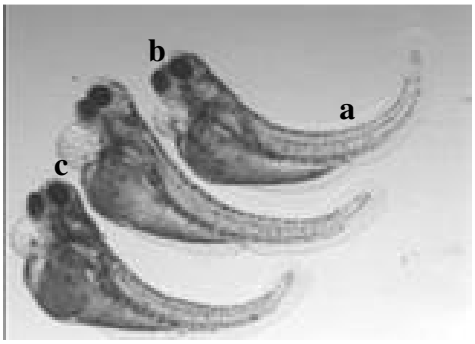

Figure 4.1. Zebrafish exposed to phenanthrene demonstrating spinal curvature (a), eye malformations (b) and yolk sac malformations (c) (adapted from Incardona *et al*., 2004).

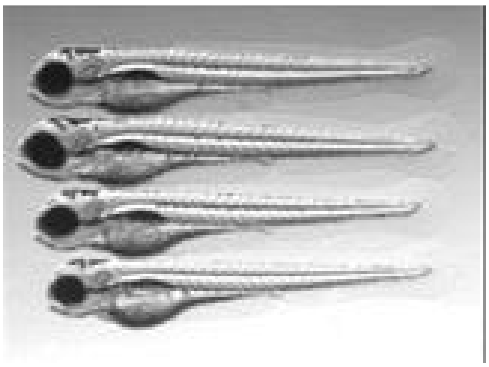

Figure 4.2. Zebrafish exposed to naphthalene showing normal development (Adapted from Incardona *et al*., 2004).

Additionally, a study by Solbakken *et al.* (1984) also showed that two PAHs; naphthalene and phenanthrene, demonstrated markedly different effects on the early life stages of cod (*Gadus morhua*). Following an exposure of 24 hours, phenanthrene had maximally accumulated whereas naphthalene had only slightly accumulated. Also upon return to uncontaminated seawater, naphthalene was readily eliminated whereas phenanthrene was slowly eliminated, suggesting that it was more lipophilic (Solbakken *et al.,* 1984). Such research suggests that PAHs can penetrate the chorion and accumulate within the developing egg for sustained periods of time. Kuhnhold and Busch (1977) examined the selective uptake and penetration of water dissolved hydrocarbons into eggs of Atlantic salmon (*Salmo salar*) during embryonic and larval development. The chorion, like other biological membranes, consists of lipids and proteins in varying proportions. Membrane lipids are amphilic, provided with polar and non-polar parts of the molecule chain, which is a key to their specific function allowing other compounds of similar or different polarities to pass, to be adsorbed or even absorbed in the chorion (Kuhnhold and Busch, 1977). The study showed that the majority of the accumulated hydrocarbons (naphthalene and 3,4-Benzopyrene) were concentrated in the yolk and the periviteline fluid.

Studies have demonstrated that the fresh water conditions during the salmonid early developmental stages can have an impact on the recruitment and survival of adults in the marine environment and can affect the success of spawning on return to their natal stream (Daye and Glebe, 1984; Moore *et al*., 2003; Regetz, 2003, Malcolm *et al*., 2004; Moore *et al*., 2011). Such research can indicate a potential decrease in populations as a result of contaminant exposure during the early life stages of development. Fairchild *et al.* (1999) displayed a decline in the number of returning Atlantic salmon adults to the spawning

grounds in Canada, believed to have been a result of nonylphenol exposure to the smolts. Additionally, Heintz *et al.* (2000) found a 15% reduction in marine survival and delayed effects on growth of pink salmon (*Oncorhynchus gorbuscha*) in oil exposed embryos compared to non-exposed embryos.

4.1.3. Pesticides

In Britain pesticides are mainly used in association with agriculture and horticulture and include herbicides, insecticides and fungicides. Pesticides are used to control weeds and pests and they are a major pollutant of river systems. Physical, chemical and biological processes transform most pesticides applied in agriculture into one or more transformation product (Belfroid *et al*., 1998). Due to their application, these compounds are introduced into the environment and may distribute into ground water, surface water and sediment (Belfroid *et al*., 1998). The triazine pesticide group includes a number of widely used herbicides. Triazines have a high solubility in water and are quite stable in soils (Lentza-Rizos, 1996). Atrazine and related triazine herbicides, such as simazine and cyanazine, have been widely applied to corn, soybean, barley and sorghum products for controlling broadleaf weed and grass (Weigand *et al*., 2001). Atrazine persists in the environment for relatively long periods. The half-life of atrazine in water and sediment have been quoted as being 244 days (Soloman *et al.*, 1996) and 578 and 330 days respectively (Brassard *et. al*., 2003). Atrazine is not expected to adsorb strongly to sediments and may partition only moderately from the water column (Hela *et al*., 2005). Once atrazine is in the water, the relatively small hydrolysis and aqueous photolysis rates can result in an extended presence in the stationary water (Soloman *et al.*, 1996). Due to its high mobility through soil, atrazine easily finds its way into the water systems. In 2004 the EA reported that atrazine and atrazine metabolites were some of the most commonly occurring pesticides found in ground water analysis across England and Wales (Figure 4.3). Research on the distribution of pesticides showed that following application, some atrazine is adsorbed by soil particles and organic matter, thus remaining in the surface soil. It is this part which may be washed out from the soil by rain and snow into surface water in dissolved and suspended form (Gao *et al.*, 1997). Another part may leach into the soil and may appear in preferential flow waters and may be transported into the aquatic environment by runoff as well as interflow

(Gao *et al.*, 1997). Levels of atrazine as high as 480µg/l have been observed following a storm event (Huber, 1993).

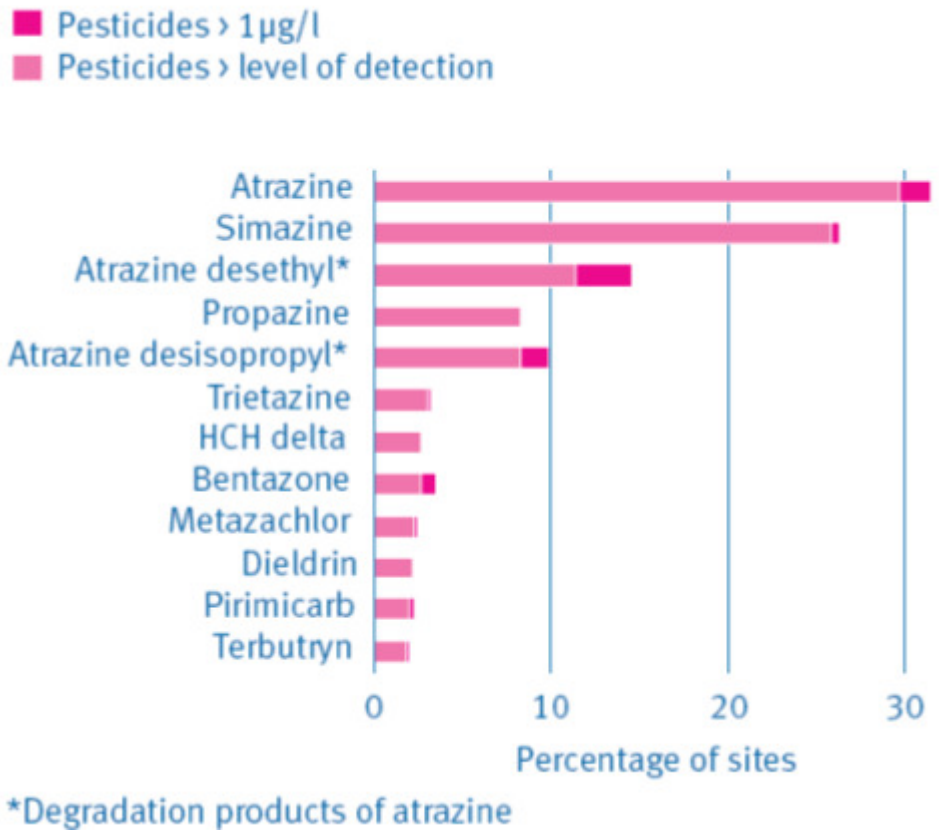

Figure 4.3. Pesticides detected in groundwater in England and Wales (sourced from EA, 2004).

Atrazine contamination in ground water is particularly significant since it has been well documented (Hansen, 1975) that spawning activity of salmonids tends to occur in the hyporheic zone, in areas with strong upwelling groundwater (Geist *et al.*, 2002). Non-agricultural uses of atrazine were banned in 1993 and it was eventually banned in the EU in October 2003, mainly due to its unpreventable water contamination, therefore detected concentrations have gradually declined in UK surface waters. However, due to its stability under hydrolysis, aqueous photolysis and its low potential to volatise from surface waters, atrazine is very persistent within the environment (Brassard *et al.*, 2003). The highest levels of atrazine are generally in the springtime following its use as a pre-emergence herbicide (Weigand *et al.*, 2001). Pesticides applied in agriculture may lead to the occurrence of transformation products in aquatic ecosystem. These transforms have the potential of actual similar or higher toxicity risk compared to the parent pesticide. Atrazine

is responsible for the formation of at least seven transformation products (Belfroid *et al.*, 1998), including desethylpropylatrazine (DEA) and desisopropylatrazine (DIA).

A study on the tiger salamander (*Ambystoma tigrinum*) demonstrated that in more highly atrazine-contaminated waters, larvae were likely to complete transformation (metamorphose) at a smaller size than they would in the absence of atrazine (Larson *et al.*, 1998). It is the smaller size which was associated with lower survival and fecundity. A study looking at the effects of atrazine on channel catfish (*Ictalurus punctatus*) embryos showed a relationship between the frequency of teratogenesis and the concentration of exposure (Birge *et al.*, 1983). Atrazine concentrations of 0.06, 4.8 and 46.7mg l^{-1}, exhibited teratogenesis in surviving larvae at frequencies of, 4, 69 and 100%, respectively. Additionally Wiegand *et al.* (2001) demonstrated that zebrafish (*Danio rerio*) embryos exposed to atrazine were affected in terms of organogenesis, movement, heart and circulatory systems as well as enzyme function. Atrazine in fresh water was found to cause a significant reduction in gill Na^+K^+ATPase activity in salmon smolts at concentrations of 2.0, 5.0 and 10.0 µg l^{-1} (Waring and Moore, 2004). Moore *et al*. (2003) examined the effects of atrazine on salmon smolts and found significant differences in the plasma Cl^- and Na^+ concentrations as well as increased mortalities when transferred to seawater.

Chlorophenoxy Acid Herbicides, or more commonly known as Acid Herbicides (AH), are of significant importance because of their worldwide distribution and their extensive use as plant growth regulators (Jankowska *et al*., 2004). Acid herbicides are chemical compounds with physiological properties similar to the natural plant hormones and they have been used as common weed killers (Ozcan Oruc *et al*., 2004) for the control of broad leaf weeds in a variety of crops including wheat, oats and barley (Fletcher *et al.,* 1995). Gomez *et al.* (1998) found that following acute exposure to the acid herbicide, 2,4-Dichlorophenoxyacetic acid (2,4-D), the fresh and brackish water fish (*Tinca tinca*) expressed marked alterations of haematopoietic tissue, characterised by progressive swelling, cell necrosis and activation of the phagocyte system. Studies on the catfish (*Clarias batrachus*) showed that exposure to acid herbicides caused considerable DNA damage (Ateeq *et al*., 2006). Additionally a study on the mussel, *Mytilus galloprovincialis*, also found exposure to acid herbicides (specifically 2,4-D) caused damage to DNA replication and repair systems (Micic *et al.*, 2004).

Organochloride Pesticides (OCPs) are man-made chemicals (Scorecard, 2005). OCPs have been found to accumulate in the fatty tissues of fish (Laws, 1993). Yamaguchi *et al*. (2003) studied fish in the upper Thames and found that eels accumulated more OCPs than other species, possibly due to the fat content of that species. OCP concentrations were also found to increase as a function of fish size (Gutenmann *et al*., 1992). Other research noted that OCP accumulated at higher concentrations in the gill, liver and brain than other tissues. The highest levels were found in the gills, possibly due to the larger surface areas per gram of tissue and the fact that it is exposed to the external environment (Yang *et al*., 2007). The concentrations of OCP compounds in the muscle tissue of gravid salmonids were found to be significantly correlated with the concentration of these compounds in the eggs (Miller, 1993). Quintozene, also known as Pentachloronitrobenzene (PCNB), exposure to Japanese Medaka embryos have been shown to cause deformities of the eyes and also retarded development of the brain, somites, notochord and heart (Metcalfe *et al*., 2008). However another study has shown that organochlorine pesticides cause damage to the liver, premature cell death and a reduction in the muscle lipid content when exposed to a tropical fresh water fish (Miranda *et al*., 2008).

Ammonia is a main component of a number of fertilisers and is also leached from sewage and degradation of organic matter (Finn, 2007; Rice and bailey, 1980). It is found present in water in two different forms, NH_3 and NH_4^+. The unionised form is more toxic to aquatic organisms and is thought to interfere with a number of biological pathways (Luckenbach *et al.*, 2003). There have been several studies looking at the effects of ammonia on various fish species. Tolerance to ammonia exposure is species dependant (Portz *et al*., 2006). Short-term exposure to elevated ammonia levels can cause a range of symptoms in fish, including increased gill ventilation, erratic movement, loss of equilibrium and convulsions (Russo and Thorston, 1991). Exposure to concentrations in excess of 0.3 mg/l can cause nerve damage and corrosion of the skin and gills (Portz *et al*., 2006). When fish are exposed to chronically elevated levels of ammonia they have been found to express decreased growth rates, fecundity and resistance to disease (Timmons *et al*., 2002).

This Chapter will look at the possible effects of water-borne contaminants, both as mixes, as well as individual effects of each pollutant. The concentrations used were chosen to

mimic the minimum and maximum mean background concentrations observed from analyses of water samples taken from the river Avon and its tributaries (Chapter 3), where a local natural population of brown trout reside and also to test any effects these mixes may have upon the development of the *Salmo trutta* eggs. The first year water exposure trials used contaminant solutions of concentrations which were monitored in the rivers. As the preliminary experiment, these concentrations of contaminants were used for individual compounds and were intended to mimic levels of environmental relevance. The consequent trials exposed brown trout eggs to environmental levels of groups of contaminants. The physiological effects on egg weight and changes in shape were monitored along with any possible changes in metabolism, as indicated by protein and Ninhydrin Positive Substances (NPS) measurements. It could be expected that any effects of the eggs will also demonstrate affect on the hatchlings. Alevins were examined for physical deformities such as lordosis, yolk sac oedema and haemorrhaging as well as morphometric variations in weight and length. Such analyses will suggest whether any of the contaminants have an effect of the early life stages of salmonids at environmentally relevant concentrations.

4.2. Material and Methods

4.2.1. Organism

Eye-pigmented diploid brown trout embryos (Allenbrook Trout Farm, Dorset) were used for three water exposure trials. Eye-pigmented embryos were used for these water trials as to prevent large mortalities as a result of transportation and movement at the stages of development most sensitive to mechanical shock. Salmonid eggs are much more sensitive to mechanical shock before the eye pigmentation stage than afterwards (Crisp, 1988). On arrival at the Institute of Marine Sciences laboratory (IMS), the eggs were immediately separated into appropriate 1L beakers of Artificial Fresh Water (AFW) (Chap. 2 section **2.4**). However, additionally for the third year of water trials, green brown trout eggs and milt were collected from Allenbrook Trout Farm, Dorset and fertilised upon return to IMS (Chap.2 section **2.2**). Following fertilisation the perivitelline fluid forms very quickly and soon holds 22% of the egg weight (Potts and Rudy, 1969). This process makes the egg

impermeable, which is why it is given the name 'water hardening'. This final year of trials was to see if the contaminants had an effect during the water hardening process of fertilisation, therefore replicating a more representative environmental situation. Therefore during the fertilisation process the water used to fertilise each batch of eggs contained contaminants. Despite research indicating the sensitivity of eggs prior to the eye pigmentation stage (Crisp, 1988; Turnpenny and Williams, 1980), Jenson and Alderice (1983) did find that salmonid eggs may be handled or gently stirred and poured, without significant mortality during the first few minutes following activation.

4.2.2. Experimental Design

The AFW in the beakers was aerated using a Pasteur pipette attached to an inert plastic air line and the air flow (compressed gas) was kept low so as to not disturb the egg position in the beaker and to prevent too much movement. The eggs were then left in the beakers for 24hrs to acclimatise before dosing of any contaminants (Day 0). The temperature was maintained at 10±1 ºC as to replicate the temperature of the borehole-fed water system of the hatchery. The photoperiod represented the conditions of that of redds so the experiment was ran in permanent darkness. Studies have shown that salmonid embryos demonstrate photo sensitivity (Luckenbach *et al.*, 2001), so light exposure was kept to a minimum to prevent light-induced anomalies. Very low level natural light was used in order to perform the daily water changes for the experiment. Each beaker was filled with 800ml of AFW and the water was changed daily as a flow-through system was not available. A volume of 700ml of waste water from each beaker was removed and replaced with 700ml (2x350ml) of AFW. It was therefore necessary to alter the dosing volume accordingly.

Beakers were labelled and randomly positioned on trays situated on the shelves within the incubator room (Figure 4.4). Both eggs and alevins were sampled at specific time intervals. It is thought that alevins are more at risk from exposure to pesticides than eggs, as the larval stage does not possess the protective chorion barrier (Rice and Bailey, 1980; Carls *et al.*, 2008).

Figure 4.4. Positioning of beakers within the incubator room.

For the third year of water exposure trials, it was necessary to siphon the 700ml waste water using plastic aquarium tubing. This technique was developed to minimise the movement of the eggs during water changes as the sensitivity of these eggs was much greater prior to the eyeing stage (Crisp, 1988). For the trial using newly fertilised eggs, Astroturf discs were placed in the bottom of the beakers (Figure 4.5) so that the eggs would settle and the motility would again be reduced during the water changes. For reasons of sensitivity water changes were only conducted every other day so to minimise disturbance of the eggs.

Figure 4.5. Astro-Turf discs in beakers to reduce motility of newly fertilised eggs. (Year 3 Experiments)

4.2.2.1. Year 1 Experiment

Eye-pigmented (14 days after fertilisation) brown trout, *Salmo trutta,* eggs (Figure 4 .6) incubated at 10˚C were used as test organisms for this experiment. 500 embryos were collected from Allenbrook trout hatchery in Wimborne, Dorset on the 28th of November 2006. Eggs were transported without water, in ice-filled coolers to the Institute of Marine Sciences. Immediately upon arrival eggs were separated into 1 litre glass beakers containing 800 ml of aerated AFW, and were allowed to acclimatize in the dark for 24 hours (Day 0) before exposure, any abnormal or dead eggs, apparent due to an opaque white appearance, were removed and replaced from the stock beaker. The temperature of the water was maintained at 11 ± 1 ˚C. The embryos were fertilised and incubated at Allenbrook farm at 11˚C, and in order to place the least amount of stress on the embryos as possible this temperature was maintained throughout the duration of the experiment by control of a thermostatically controlled cool room.

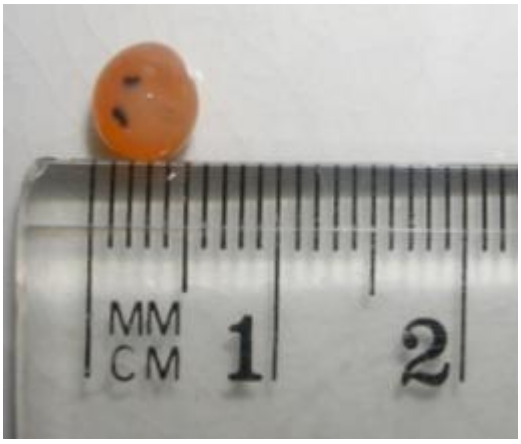

Figure 4.6. An eye-pigmented brown trout egg with scale.

There were two control groups used in this experiment; no solvent control and a solvent control (0.0025% acetone). There were six replicates of each control group; each one consisting of 25 eggs per replicate. The contaminant groups for this experiment were PAHs, and a Triazine; naphthalene (NAPH) and phenanthrene (PHEN), and atrazine (ATR), respectively, were used as individual compound contaminants. A mix contaminant was made up from all the individual compounds. All the contaminant exposures had six replicates; each replicate consisting of 25 eggs. This preliminary experiment aimed to test

the egg incubation method and to ensure that the design was optimised for consequential trials.

All pure compounds were dissolved in acetone which aided more rapid dispersion and solution of test substances (Hutchinson *et al.,* 2006). 1mg of pure compound of each contaminant was dissolved in 10ml of acetone to create stock solutions each with a resultant concentration of 100µg ml^{-1}. Solutions were stored in 10ml light-shielded volumetric flasks, and were kept cool in a refrigerator in between usage. Working solutions of each compound were prepared by diluting 280µl of NAPH stock solution, 200µl of ATR stock solution and 200µl of PHEN stock solution, respectively, in individual 10ml volumetric flasks in acetone. A MIX solution (680 µl mix ml^{-1} Acetone) of all three compounds was prepared using the working solutions of ATR, NAPH and PHEN at concentrations of 50ng l^{-1}, 70ng l^{-1} and 50ng l^{-1} respectively.

These concentrations were chosen to mimic the maximum mean background concentrations observed from previous analyses of water samples taken from the River Avon field sites (Chap. 3) where a local natural population of brown trout reside and also to test any effects this mix may have upon the development of the *Salmo trutta* embryos. After dosing, the nominal concentrations in the beakers were as follows: 50ng l^{-1} for ATR and PHEN- exposed eggs and 70ng l^{-1} for NAPH-exposed eggs. The equivalent nominal concentrations of each individual compound were also in the MIX beakers.

After 24 hours recovery from transportation, the eggs were transferred to a static exposure system consisting of 18 1L volume glass beakers (3 for each treatment), each containing 800ml of AFW and aerated using glass Pasteur pipettes. On Day 1 of the experiment, 20 µl of each exposure solution, including the acetone control, was added to each corresponding beaker using a Transferpettor. Piston tubes were changed during each dosing, and the piston tip was cleaned with methanol. This procedure avoided cross contamination of the chemicals. It was assumed that the aeration of the water would distribute each chemical uniformly within the Artificial Fresh Water (AFW).

Fresh AFW was prepared (see Chap.2) and aerated and was used to renew 87.5% volume (700ml) of the test solutions every 24 hours. Following renewal of the test water, each

beaker was subsequently dosed with 17.5 µl (87.5% of the initial 20ul dose) of each corresponding stock solution. Renewal of only 700ml of the test solution each day kept disturbance of the eggs to a minimum.

Each day any dead eggs were removed and recorded. Eggs (n=24) were sampled at day six post-exposure (d6PE) and day 13 post-exposure (d13PE) for weight, diameter, water content and biochemical analysis. The eggs were then exposed to their subsequent chemical until hatching (last egg hatched on day 16). At hatch each alevin was screened for anomalies and then anaesthetised with a lethal dose of 2-Phenoxyethanol and the brain destroyed (**2.3**). Alevins were weighed and lengths measured. Further analysis included % yolk sac and % of the total body weight.

4.2.2.2. Year 2 Experiment

Eye-pigmented diploid brown trout, *Salmo trutta,* eggs (14 days post fertilisation incubated at 10˚C) were also used as test organisms for this experiment. Eggs were collected from Allenbrook trout hatchery in Wimborne, Dorset on the 26th of November 2007. Eggs were transported without water, in ice-filled coolers to the Institute of Marine Sciences. Upon arrival eggs were immediately transferred into 56 1l beakers containing AFW only, and allowed to acclimatise in the dark for 24 hours maintained at 10 ± 1^{o}C (Day 0). The remaining embryos were transferred to a stock beaker. As before, any identified damaged or dead eggs were removed and replaced with eggs from the stock beaker.

Again, there were two control groups used in this experiment; no solvent control and a solvent control (0.0025% acetone). There were six replicates of each of the control groups; each of the six beakers containing 25 embryos. There were also six replicates of the contaminant mix exposure group, six beakers of the low concentration and six beakers of the high concentration. For the PAH, OCP, TRI and AH exposures there were four replicates for each low and high concentration; each of the four beakers containing 25 embryos.

For the Year 2 experiment, the levels of pesticides were also representative of those found in the field trials (Chap. 3). The contaminant groups PAHs, OCPs, Triazines, and AHs

were all used in the water exposure trials. Each contaminant group consisted of both a low concentration and a high concentration working solution. A mix incorporating all the classes of contaminants was also prepared, also incorporating a low and high concentration.

The PAHs mix was prepared using the compounds naphthalene, phenanthrene, Acenapthene, benzo [b] fluoranthene, benzo [k] fluoranthene, benzo [a] pyrene, indeno [1,2,3-cd] pyrene, dibenz [a, h] anthracene and benzo [g, h, i] perylene all at 50ng l^{-1} for the low concentration mix and 100ng l^{-1} for the high concentration mix (Table 4.1). The stock solution was prepared to a concentration of 100µg ml^{-1} by adding 1mg of each pure compound in 10ml amber volumetric of acetone. The low concentration working solution (LCWS) was prepared to a concentration of 2µg ml^{-1} by adding 200µl of the stock solution to 10ml amber volumetric of acetone. The high concentration working solution (HCWS) was prepared to a concentration of 4µg ml^{-1} by adding 400µl of stock solution to 10ml amber volumetric of acetone.

Table 4.1 Concentrations of PAH Solutions.

Compound	Low Conc (ng l^{-1})	High Conc (ng l^{-1})
Napththalene	50	100
Phenanthrene	50	100
Acenapthene	50	100
Benzo [b] fluoranthene	50	100
Benzo [k] fluoranthene	50	100
Benzo [a] pyrene	50	100
Indeno [1,2,3-cd] pyrene	50	100
Dibenz [a, h] anthracene	50	100
Benzo [g, h, i] perylene	50	100

These concentrations were chosen so that nominal concentrations of 50ng l^{-1} of each compound (Low mix) and 100ng l^{-1} of each compound (High mix) were prepared.

The triazine mix was prepared using the compounds desisopropylatrazine (DIA), desethylatrazine (DEA) and atrazine, at concentrations 2000ng l^{-1} and 40ng l^{-1}, respectively, for the low concentration and 4000ng l^{-1} and 80ng l^{-1}, respectively, for the high concentration (Table 4.2). The DIA stock solution was made up to a concentration of 800µg ml^{-1} by adding 8mg of pure compound in 10ml amber volumetric of acetone. The DEA stock solution was made up to a concentration of 100µg ml^{-1} by adding 1mg of pure

compound to 10ml amber volumetric of acetone. The LCWS was prepared from 1000µl of DIA stock solution and 160µl of DEA stock solution in 10ml amber volumetric of acetone. The HCWS was prepared from 2000µl of DIA stock solution and 320µl DEA stock solution in 10ml amber volumetric of acetone.

Table 4.2. Concentrations of Triazine Solutions.

Compound	Low Conc (ng l^{-1})	High Conc (ng l^{-1})
Desisopropylatrazine	2000	4000
Desethylatrazine	40	80
Atrazine	40	80

These concentrations were chosen so that nominal concentrations of 2000ng l^{-1} and 40ng l^{-1} of each compound (Low) and 4000ng l^{-1} and 80ng l^{-1} of each compound (High) were prepared.

The OCP mix was prepared using the compounds o,p-DDT, pentachloronitrobenzene (PCNB) and β-lindane at concentrations of 40ng l^{-1}, 40ng l^{-1} and 70ng l^{-1}, respectively, for the low concentration and 80ng l^{-1}, 80ng l^{-1} and 140 l^{-1}, respectively, for the high concentration (Table 4.3). The stock solution for o,p-DDT and PCNB was prepared by dissolving 1mg of each pure compound in a 10ml volumetric of acetone to achieve a concentration of 100ug ml^{-1}. The stock solution for β-lindane was prepared to the same concentration of 100ug ml^{-1}. The LCWS was prepared with 160µl of the o,p-DDT and PCNB stock solution and 320µl of the beta-lindane stock solution in 10ml amber volumetric of acetone. The HCWS was prepared with 320µl of the o,p-DDT and PCNB stock solution and 640µl of the β-lindane stock solution in 10ml amber volumetric of acetone.

Table 4.3. Concentrations of OCP Solutions.

Compound	Low Conc (ng l^{-1})	High Conc (ng l^{-1})
O,p-DDT	40	80
Pentachloronitrobenzene	40	80
Beta- Lindane	70	140

These concentrations were chosen so that nominal concentrations of 40ng l^{-1} and 70ng l^{-1} of each compound (Low) and 40ng l^{-1} and 140ng l^{-1} of each compound (High) were prepared.

The acid herbicide mix was prepared using the compounds mecoprop and 2-methyl-4-chlorophenoxy butyric acid (MCPB) both at concentrations of 35ng l^{-1} for the low concentration and 70ng ml^{-1} for the high concentration (Table 4.4). The stock solution of concentration 100µg ml^{-1} was prepared by dissolving 1mg of each pure compound in 10ml amber volumetric of acetone. The LCWS was prepared by adding 140µl of stock solution to acetone in 10ml amber volumetric and the HCWS was prepared by adding 280µl of sock solution to acetone in 10ml amber volumetric.

Table 4.4. Concentrations of Acid Herbicide Solutions.

Compound	Low Conc (ng l^{-1})	High Conc (ng l^{-1})
Mecoprop	35	70
MCPB	35	70

These concentrations were chosen so that nominal concentrations of 35ng l^{-1} of each compound (Low) and 70ng l^{-1} of each compound (High) were prepared. The contaminant mix solutions included a cocktail of all the individual stock solutions at volumes equivalent to that of each compound used to prepare the working solutions in 10ml amber volumetric of acetone (Table 4.5).

Table 4.5. Concentrations of MIX Solutions.

Compound	Low Conc (ng l^{-1})	High Conc (ng l^{-1})
Napththalene	50	100
Phenanthrene	50	100
Acenapthene	50	100
Benzo [b] fluoranthene	50	100
Benzo [k] fluoranthene	50	100
Benzo [a] pyrene	50	100
Indeno [1,2,3-cd] pyrene	50	100
Dibenz [a, h] anthracene	50	100
Benzo [g, h, i] perylene	50	100
Desisopropylatrazine	2000	4000
Desethylatrazine	40	80
Atrazine	40	80
O,p-DDT	40	80
Pentachloronitrobenzene	40	80
Beta- Lindane	70	140
Mecoprop	35	70
MCPB	35	70

On day 8 post-exposure (d8PE) and day 15 post-exposure (d15PE), six embryos were removed from each of the test beakers for measurement of their weight (after being blotted

dry) and their diameter (at their widest point using digital callipers). Three embryos from each replicate were frozen at -20˚C and three fixed in NBF. For both the control and solvent exposures and the contaminant mix exposures; 36 embryos were sampled for analysis (six from each of the six replicates) for each sampling day. For all the other contaminant exposures; 24 embryos were sampled for analysis (six from each of the four replicates) for each sampling day. Half of the embryos sampled were frozen and the other half were fixed in NBF. Embryos were also analysed for NPS (including water content) and Protein concentrations. Chloride analysis (see later) was also performed. Survival was recorded, as well as number of deaths and number hatched.

4.2.2.3. Year 3 Experiment

For the final year of water exposure trials, two main experiments were undertaken. The first exposed green (newly fertilised) eggs to selective contaminants and the second exposed eye-pigmented eggs to the same contaminants. Both experiments continued to hatching.

4.2.2.3.1. Experiment One: Exposure from Fertilisation

Brown trout eggs and milt were collected 11th November 2008 from Allenbrook Trout farm, Dorset and transported in polystyrene containers over ice to the Institute of Marine Sciences. One hundred eggs were fertilised at a time using a tea strainer and Pasteur pipette to minimise handling. Eggs were fertilised in batches of 100 according to the standard procedure (Chap. 2). However, the AFW added to the eggs and milt for fertilisation was first dosed with the relevant contamination treatment, using the concentrations described above (section). Once fertilised, the eggs were very carefully placed in beakers (20 eggs per beaker). Each beaker had been prepared with a disk of Astroturf to minimise movement of the newly fertilised eggs (Figure 4.7). To prevent the build up of stagnant water below the eggs, holes were created in the disks using a heated needle. These holes allowed for the underlying water to be aerated sufficiently by the aerating pipettes. This upwelling action was representation of the water movement seen in the natural environment of brown trout eggs (Raleigh, 1982).

Figure 4.7. Astroturf in beaker to reduce motility of eggs.

Each contaminant had five replicate beakers. These beakers were randomly positioned within the temperature controlled room. The eggs were incubated at 10±1°C in AFW for 24hrs prior to the first dosage of contaminant. However, for this exposure trial no damaged or dead eggs were replaced.

In addition to the main exposure experiment, a 48hr exposure trial was run. Eggs were fertilised, as described above, and incubated in a 10±1°C growth room. For each treatment, 25 eggs were exposed for the 48hr duration. This trial was to identify fertilisation success of eggs exposed to contaminants during the water hardening phase of their development. The presence of the chorion and blastodisc were used as indicators of successful fertilisation.

As with the previous two years, there were two control groups used; no solvent control, and a solvent control (0.0025% acetone). The solvent control consisted of an equivalent volume of acetone (HPLC Grade 99.8+%, Fisher, UK) which was required as the carrier solvent for the preparation of the contaminants. Therefore the same volume of acetone was added to the solvent control beakers as the volume of contaminant. There were five replicates of each of the control groups; each of the five beakers containing 20 eggs. There were also five replicates of each exposure group; PAHs, OCPs, Triazines and Acid Herbicides, each containing 20 eggs. For this trial, five replicates per treatment were exposed to ammonia, ammonia plus

contaminant mix low concentration and ammonia plus contaminant mix high concentration.

The exposure concentrations for the trials in Year 3 were the same as in Year 2. However, this trial included an exposure of ammonia. The ammonia solution was prepared using ammonium chloride (NH_4Cl) with a molecular weight (MW) of 53.49. The concentration of ammonia used for the trial represented the mean level found in the field sites (300µg l^{-1}). The solution was prepared so that a 50µl volume could be used to dose 800ml of AFW at a concentration of 300µg l^{-1}. Eggs were exposed to ammonia as well as, MIX (Low) plus ammonia and MIX (High) plus ammonia.

Eggs were sampled on day 30 post-fertilisation (d30PF) and alevins on day 93 post-fertilisation (d93PF). A sub-sample of alevins was also sampled on hatching. For the fertilisation success exposure trial, eggs were sampled on day 2 post-fertilisation (d2PF).

4.2.2.3.2. Experiment Two: Exposure from Hatching

Eye-pigmented eggs were collected from Allenbrook Trout Farm and transported to the Institute of Marine Sciences. Immediately on arrival the eyed eggs were placed in 1l beakers and left to acclimatise for 24hr. Any damaged or dead eggs were removed and replaced before the dosage of contaminants.

The eggs were not exposed to any contaminants until hatching. Four alevins from each contaminant replicate were sampled on hatch (T0) and then alevins were sampled on day 18 post-exposure (d18PE) and day 38 post-exposure (d38PE). The contaminants for this experiment were of the same concentrations as the green egg experiment. With the exception of the Amm plus Mix (Low), only the high contaminant concentrations were used for this exposure trial.

4.2.3. Dosing Methodology

The initial dosing (Day 1) required 20µl of each individual working solution, the acetone and the ammonia to be added to the corresponding beaker containing a volume of 800ml of AFW so that the desired contaminant concentration could be achieved. For days thereafter (Day 2, Day 3, Day n+1….), 700ml of the water from each beaker was removed (Figure 4.8) and initially only 350ml of AFW replaced. A volume of 17.5µl was required to dose each beaker, now containing 450ml AFW, with the relevant contaminant mix. Once each beaker was dosed then the remaining 350ml of AFW was added to each beaker so that the full 700ml was replaced to make up 800ml again. This same procedure was repeated for each consecutive day. Once all the beakers were dosed and all the water replaced, a sheet of parafilm was laid on the top of the beaker to prevent contamination from other beakers and possible air contaminants from the incubating room. Parafilm was also placed on top of the beaker whenever it was left between water removal, replacement or dosing (Figure 4.9). Daily water changes were carried out and each beaker was constantly aerated, therefore it was not deemed necessary to check the physicochemical parameters of each beaker. The conditions of the experiment were to minimise and potentially erradicate the accumulation of ammonia, or other physicochemical parameters.

Figure 4.8. Removal of water still left enough for the eggs to continue to be exposed.

Figure 4.9. Parafilm placed on top of each beaker to prevent contamination.

4.2.4. Egg Analysis

Sampled eggs were weighed (mg) and the diameters measured (mm) using digital calipers, as described in Chapter 2 (**2.8.1**). The volume and surface area of each egg were obtained from the weight measurements (Fleming & Ng 1987; Einum *et al.,* 2002) as described in Chapter 2. As only one diameter measuremnt was taken, it was considered that the variable of weight was a more accurate method to calculate the volume and surface area of eggs.
For the dry weight analysis, frozen eggs were thawed and blotted dry then placed in a pre-weighed 1.5 ml micro centrifuge tube. The tube was then reweighed with the egg and the total weights recorded. These tubes were then heated in an oven at 60°C for 72 hours, and then allowed to cool in a desiccator. From reweighing the micro-centrifuge tubes the egg dry weights and percentage water contents were calculated and recorded.

Ninhydrin Positive Substances (NPS) analysis and protein analysis was determined by the technique described in Chapter 2 (**2.8.5** and **2.8.6** respectively). Chloride concentration of the eggs was also measured using the procedure stated in Chapter 2 (**2.8.7**).

For the 48hr exposure trial (2dPF), eggs were fixed in Stockard's Solution (Appendix 2) and later examined under the microscope to check for fertilisation success. The presence of

the chorion and blastodisc were used as indicators that fertilisation occurred as it demonstrates development (Figure 4.10). The fertilisation rate was calculated as follows;

$$\% \text{ Fertilisation Rate} = \frac{No.fertilised}{Total\ no.of\ eggs} \times 100$$

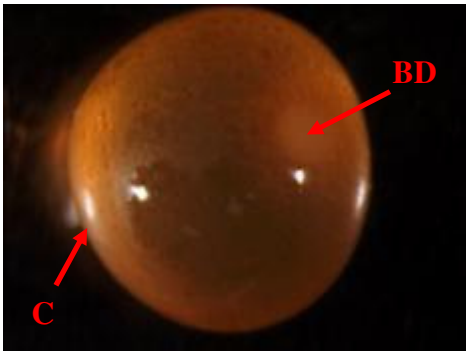

Figure 4.10. A fertilised egg (Control Treatment) Blastodisc (BD) and Chorion (C) identified by red arrows.

4.2.5. Alevin Analysis

Some alevins were sampled on the day of hatching. The number hatched from each beaker was recorded as well as the number of deaths. Hatched alevins were removed from the exposure beaker and killed using a fatal dose of anaesthetic (**2.3).** Lengths and weights were measured as described in Chapter 2 (**2.8.1**) and then the alevins were either frozen or fixed in NBF. Some alevins were sampled at a later stage prior to exogenous feeding at a known interval post-exposure of post-fertilisation and post-hatching. In order to achieve the percentage body weight compared to the percentage yolk sac weight; the alevin was firstly weighed as a whole. Using a scalpel, the yolk sac was carefully removed (Figure 4.11) and weighed separately.

Figure 4.11. YS removal for % body and yolk sac weights.

From the total weight (T), yolk sac weight (YS) and body weight (BW) of the alevin, the percentages were calculated as follows;

Yolk Sac Weight (%) $= \frac{YS}{T} \times 100$

Body Weight (%) $= \frac{BW}{T} \times 100$

4.2.6. Statistical Analysis

One way ANOVA were used to determine statistically significant differences in the results. However, a two ANOVA was used when using day as a factor. Statistical software programmes SigmaStat® and Minitab 15® were used for the analysis. SigmaStat® tested data sets for normality and homogenous variances prior to analysis. A method of One Way ANOVA was used to determine any significant differences between treatment and time (days) of exposure using the morphometrics measurements and calculations. If a significant difference ($P<0.05$) was observed then a Tukey 95% simultaneous confidence intervals, pair wise comparisons would identify which treatments on which days were significant. Data represented in percentage were Arcsine transformed prior to statistical analysis.

4.3. Results

4.3.1. Year 1

There were no mortalities in any of the treatments for the duration of the trial. Therefore the individual contaminants did not have an effect on the survival of the salmonid eggs.

The results for the brown trout egg morphometrics can be seen in Table 4.6. The table shows the mean weights, diameter, volume and surface area for day 6 post-exposure and day 13 post-exposure eggs. The data shows that the treatments had no significant effect on the weight of the eggs (ANOVA, F=0.04; d.f.=5; *P*=1.000). Statistal analysis also shows that the day post-exposure did not have a significant effect on the weight (ANOVA, F=0.05; d.f.=1; *P*=0.956). Treatment also had no significant affect on the diameter of the eggs for either d6PE or d13PE (ANOVA, F=0.45; d.f. =5; *P*=0.845). The data also shows that the day post-exposure did not have a significant effect on the size of the eggs (ANOVA, F=0.68; d.f. =1; *P*=0.505).

The Statistical analysis for egg surface area for d6PE and d13PE shows that treatment did not have an effect on the surface area of the eggs (ANOVA, F=0.92; d.f. =5; *P*=0.468). The age of the egg also had no significant difference on the egg surface area (ANOVA, F=0.18; d.f. =1; *P*=0.669). Statistical analysis of the surface area to volume ratio data, as calculated by egg weight, for day 6 and day 13 post exposure eggs shows that treatment did not have an effect on the surface area to volume ratio (ANOVA, F=0.07; d.f. =5; *P*=0.999). The age of the egg also had no significant difference on the surface area to volume ratio (ANOVA, F=0.12; d.f. =1; *P*=0.888).

Table 4.6. Morphometric analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPE	Weight (mg)	Diameter (mm)	Volume (mm^3)	Surface Area (mm^2)	SA:Vol	N value
CNT	**6**	109.10±2.52	5.36±0.12	100.26±2.52	104.23±1.76	1.04±0.01	24
CNT	**13**	105.65±2.64	5.39±0.12	96.81±2.64	101.81±1.83	1.06±0.01	24
SOL	**6**	109.20±3.21	5.35±0.10	100.36±3.21	104.21±2.23	1.05±0.01	24
SOL	**13**	113.70±3.55	5.38±0.10	104.86±3.55	107.26±2.47	1.03±0.01	24
ATR	**6**	106.78±3.05	5.35±0.11	97.94±3.05	102.57±2.11	1.05±0.01	24
ATR	**13**	107.60±3.17	5.25±0.11	98.76±3.17	103.10±2.22	1.05±0.01	24
NAPH	**6**	106.85±2.72	5.29±0.13	98.01±2.72	102.64±1.89	1.05±0.01	24
NAPH	**13**	111.00±2.52	5.30±0.10	102.16±2.52	105.55±1.71	1.04±0.01	24
PHEN	**6**	107.95±2.93	5.32±0.12	99.11±2.93	103.38±2.02	1.05±0.01	24
PHEN	**13**	106.10±2.34	5.26±0.11	97.26±2.34	102.15±1.63	1.05±0.01	24
MIX	**6**	105.75±2.22	5.29±0.12	96.91±2.22	101.92±1.56	1.06±0.01	24
MIX	**13**	106.00±3.08	5.28±0.12	97.16±3.08	101.99±2.14	1.06±0.01	24

Table 4.7 shows the results for the egg biochemical analysis of the brown trout eggs. The table shows the mean dry weights, percentage water content, NPS concentration, protein concentration and chloride concentration for day 6 post-exposure and day 13 post-exposure eggs. Statistical analysis showed that there was no significant difference in the egg dry weight for either the treatment (ANOVA, F=1.47; d.f. =5; *P*=0.216) or the day post exposure (ANOVA, F=0.05; d.f. =1; *P*=0.817).

Data for percentage water content showed that day post exposure had no significant effect on the water content of the eggs (ANOVA, F=0.00; d.f. =1; *P*=0.960). However, statistical analysis did show that treatment had a significant effect on the water content of the eggs (ANOVA, F=4.45; d.f. =5; P=0.001). A Tukey's pair wise comparison identified a significant increase in water content in NAPH exposed eggs compared with both PHEN and MIX exposed eggs but not for the control or solvent exposed eggs.

Statistical analysis for the egg ninhydrin positive substance concentrations for Day 6 and Day 13 post exposure showed that treatment did have a significant effect on the egg concentration (ANOVA, F=3.10; d.f. =1; *P*=0.016). Tukey's pair wise comparison identified that SOL treatment eggs had a lower ninhydrin positive substance concentration than PHEN exposed eggs. Also, day post exposure had a significant effect on the egg concentration (ANOVA, F=8.87; d.f. =1; *P*=0.004). Tukey's pair wise comparison confirmed that Day 13 eggs had a significantly higher ninhydrin positive substance concentration than Day 6 eggs.

Statistical analysis for the egg protein concentration for both d6PE and d13PE showed that day post exposure had no significant effect on the protein concentration (ANOVA, F=2.77; d.f. =1; *P*=0.102). The treatment also had no significant effect on the protein concentration (ANOVA, F=1.62; d.f. =5; *P*=0.171).

Statistical analysis for the egg chloride concentration showed that chloride concentration in d6PE eggs was not significantly greater than the chloride concentration in d13PE eggs (ANOVA, F=4.03; d.f. =1; P=0.05). Treatment also had no significant effect on the egg chloride concentration (ANOVA, F=0.40; d.f. =5; P=0.850).

Table 4.7. Biochemical analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPE	Dry Weight (mg)	Water Content (%)	NPS (NPS nM/mg Egg)	Protein (mg/mg Egg)	Chloride (nEq Cl/mg Egg)	N value
CNT	**6**	36.20±1.93	62.79±1.17	0.11±0.01	0.18±0.02	22.89±1.64	10
CNT	**13**	36.20±2.84	64.69±0.99	0.19±0.01	0.15±0.03	18.57±3.15	10
SOL	**6**	33.4±2.29	62.41±0.78	0.14±0.02	0.20±0.03	20.69±3.90	10
SOL	**13**	38.00±1.41	63.83±0.89	0.13±0.02	0.15±0.02	19.44±3.59	10
ATR	**6**	40.20±3.60	63.93±1.01	0.18±0.03	0.16±0.04	21.95±3.65	10
ATR	**13**	38.2±1.12	62.53±0.88	0.22±0.02	0.15±0.02	20.94±3.69	10
NAPH	**6**	40.00±3.10	65.98±0.39	0.13±0.09	0.19±0.03	23.20±1.50	10
NAPH	**13**	36.60±2.52	63.72±1.57	0.16±0.00	0.10±0.02	21.18±1.10	10
PHEN	**6**	36.60±2.06	60.43±0.90	0.19±0.03	0.15±0.02	23.79±3.76	10
PHEN	**13**	35.40±0.25	61.58±1.57	0.22±0.03	0.12±0.05	18.19±1.76	10
MIX	**6**	32.40±0.88	61.64±0.75	0.13±0.03	0.09±0.01	25.25±2.89	10
MIX	**13**	36.00±1.73	60.99±1.37	0.21±0.00	0.13±0.02	21.91±1.43	10

Figure 4.12 shows the mean hatch day of the alevins for each treatment. The non-solvent control alevins hatched later than the other treatment-exposed groups, with the phenanthrene-exposed alevins hatching the earliest.

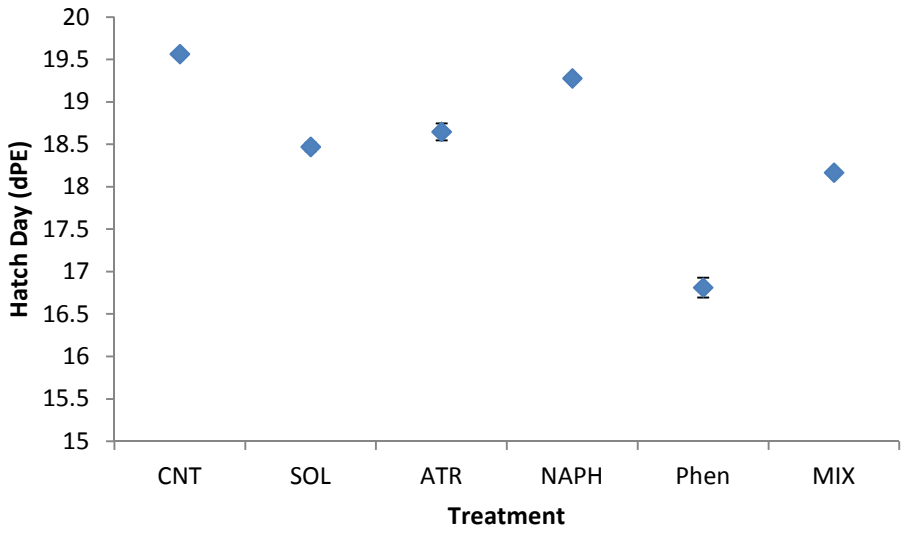

Figure 4.12. Mean hatch day (dPE) for alevins for each treatment. Data represents Mean±SEM (n= 85,17,17,18,16, and 18 respectively).

Alevin weights measured on hatching are represented as mean values with standard errors in Figure 4.13. Statistical analysis showed that treatment did have a significant effect on the weight of the hatchlings (ANOVA, F=3.33; d.f. =5; *P*=0.007), however a Tukey's pair wise did not identify a significant difference between specific treatments.

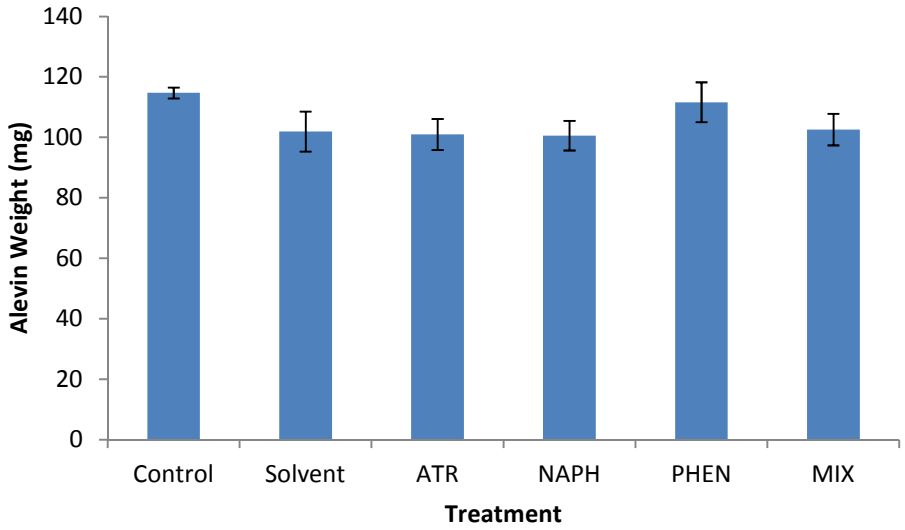

Figure 4.13. Alevin total weight on hatch for each treatment. Data represents Mean±SEM (n= 85,17,17,18,16, and 18 respectively).

The results for alevin body weight and yolk sac weight, as a percentage of total weight, is displayed in Figure 4.14. Treatment did not have a significant effect on either the alevin body weight (ANOVA, F=0.88; d.f. =5; *P*=0.504) or the alevin yolk sac weight (ANOVA, F=0.88; d.f. =5; *P*=0.504).

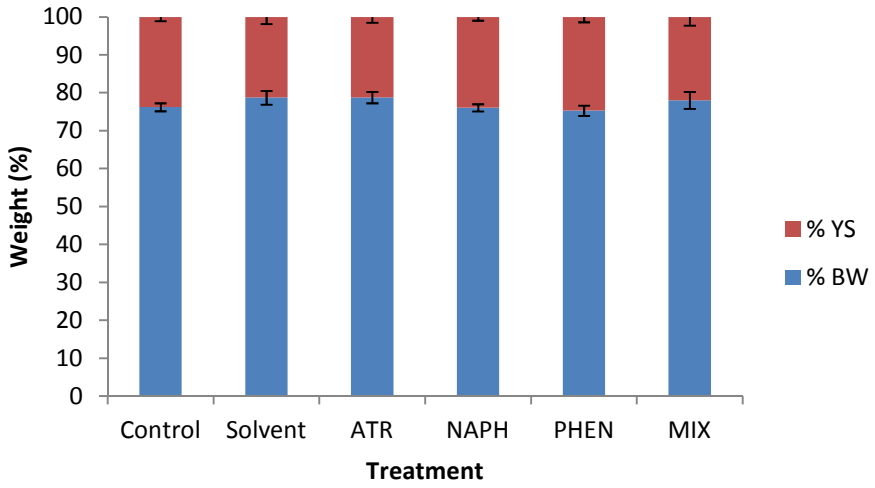

Figure 4.14. Alevin body weight and yolk sac weight on hatching, shown as a percentage of total weight. Data represents Mean±SEM (n=5; Control, n=15).

Visual observations were also noted for the hatchlings. Only one alevin from MIX treatment demonstrated any abnormalies. This alevin had a twisted spinal cord and there was only one eye.

4.3.2. Year 2 Results

The results for the egg survival is represented as a percentage in Figure 4.15. Statistical analysis showed that treatment had no significant effect on either the egg survival (ANOVA, F=0.75; d.f. =11; *P*=0.682).

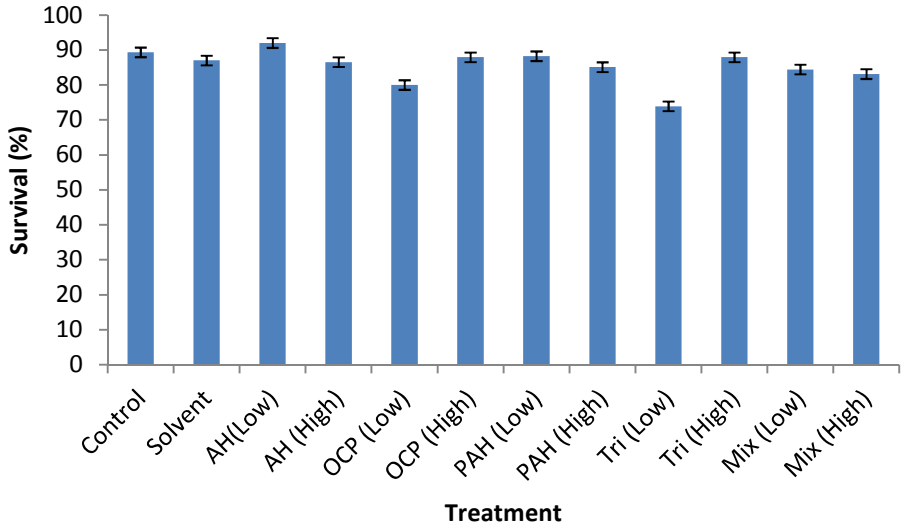

Figure 4.15. Egg Survival of brown trout (Data represented as percentage of total number).

Figure 4.16 shows the data for the percentage number of alevins found dead on hatching and the percentage number of eggs with arrested development. Arrested development was defined as eggs which failed to hatch but did not display the opaque colour of dead eggs. These eggs failed to hatch for at least 10 days after the other alevins from the same beaker had hatched. Statistical analysis showed that treatment had no significant effect on either the number of alevins dead on hatching (ANOVA, F=0.26; d.f. =11; *P*=0.989) or the number of eggs with arrested development (ANOVA, F=0.71; d.f. =11; *P*=0.724).

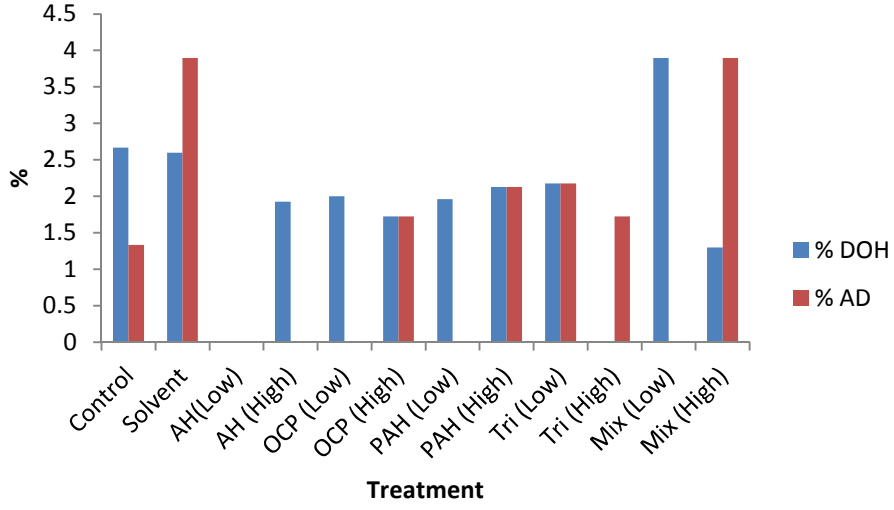

Figure 4.16. Brown trout eggs dead on hatch and eggs with arrested development (Data represented as percentage).

The results for the brown trout egg morphometrics can be seen in Table 4.8. The table shows the mean weights, diameter, volume and surface area for day 8 post-exposure and day 15 post-exposure eggs. Statistical analysis showed that day post exposure did not have a significant effect on the egg weight (ANOVA, F=1.47; d.f. =1; P=0.226). Additionally treatment did not have a significant effect on egg weight (ANOVA, F=1.60; d.f. =11; *P*=0.166).

Statistical analysis for day 8 post-exposure and day 15 post-exposure showed that day had no significant effect on the diameter of the eggs (ANOVA, F=0.38; d.f. = 1; *P*=0.539). However, statistical analysis showed that treatment did have a significant effect on the egg diameter (ANOVA, F=25.22; d.f. =11; *P*<0.001). A Tukey's pair wise comparison identified that all contaminant exposed eggs were significantly heavier than control eggs with the exception of the OCP exposed eggs at both concentration levels and solvent control eggs.

Statistical analysis for egg volume on day 8 and day 15 post-exposure showed that treatment did not have a significant effect on the volume of the exposed eggs (ANOVA, F=1.41; d.f =11; *P*=0.165). Day post-exposure also had no effect on the volume of the exposed eggs (ANOVA, F=1.48; d.f. =1; P=0.224). Statistical analysis for the egg surface area showed that treatment did not have a significant effect on the surface area of the exposed eggs (ANOVA, F=1.38; d.f =11; *P*=0.180). Day post-exposure also had no effect on the surface area of the exposed eggs (ANOVA, F=1.49; d.f. =1; P=0.222).

Statistical analysis for day 8 and day 15 post-exposure showed that day post exposure had no significant effect on the surface area to volume ratio (ANOVA, F=1.41; d.f. =11; *P*=0.235). Additionally, treatment also had no significant effect on surface area to volume ratio (ANOVA, F=1.25; d.f. =1; *P*=0.252).

Table 4.8. Morphometric analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPE	Weight (mg)	Diameter (mm)	Volume (mm^3)	Surface Area (mm^2)	SA:Vol	N value
Control	**8**	100.22±2.39	5.10±0.05	91.38±2.39	97.86±1.70	1.08±0.01	36
Control	**15**	104.86±2.71	4.88±0.08	96.02±2.71	101.09±1.92	1.06±0.01	36
Solvent	**8**	104.19±2.49	5.72±0.06	95.35±2.49	100.67±1.75	1.06±0.01	36
Solvent	**15**	99.19±2.47	5.61±0.06	90.35±2.47	97.05±1.86	1.09±0.01	36
AH (Low)	**8**	97.04±2.32	5.53±0.07	88.20±2.32	95.65±1.68	1.09±0.01	24
AH (Low)	**15**	100.67±2.92	5.62±0.06	91.83±2.92	98.18±2.08	1.08±0.01	24
AH (High)	**8**	101.87±2.63	5.48±0.06	93.04±2.63	99.10±1.85	1.07±0.01	24
AH (High)	**15**	97.17±2.02	5.67±0.05	88.33±2.02	95.78±1.47	1.09±0.01	24
OCP (Low)	**8**	103.46±3.08	5.31±0.10	94.62±3.08	100.14±2.20	1.07±0.01	24
OCP (Low)	**15**	106.71±3.80	2.61±0.36	97.87±3.80	102.28±2.72	1.06±0.02	24
OCP (High)	**8**	101.08±3.53	4.85±0.08	92.24±3.53	98.37±2.49	1.08±0.01	24
OCP (High)	**15**	101.21±2.64	2.57±0.36	92.37±2.64	98.61±1.89	1.07±0.01	24
PAH (Low)	**8**	99.71±3.25	5.49±0.09	90.87±3.25	97.42±2.35	1.08±0.01	24
PAH (Low)	**15**	99.29±3.33	5.61±0.08	90.45±3.33	97.12±2.35	1.08±0.01	24
PAH (High)	**8**	98.17±3.35	5.60±0.07	89.33±3.35	96.30±2.37	1.09±0.01	24
PAH (High)	**15**	101.38±2.80	5.68±0.07	92.53±2.80	98.71±1.98	1.07±0.01	24
TRI (Low)	**8**	97.96±3.45	5.42±0.07	89.12±3.45	96.12±2.47	1.09±0.01	24
TRI (Low)	**15**	102.71±3.94	5.53±0.10	93.87±3.94	99.41±2.86	1.07±0.02	24
TRI (High)	**8**	100.33±3.37	5.61±0.08	91.49±3.37	97.85±2.41	1.08±0.01	24
TRI (High)	**15**	97.42±2.81	5.51±0.08	88.58±2.81	95.85±2.03	1.09±0.02	24
MIX (Low)	**8**	93.33±2.65	5.42±0.06	84.49±2.65	92.77±1.94	1.11±0.01	36
MIX (Low)	**15**	99.81±2.16	5.62±0.06	90.97±2.16	97.60±1.54	1.08±0.01	36
MIX (High)	**8**	95.94±2.35	5.45±0.07	87.10±2.35	94.77±1.69	1.10±0.01	36
MIX (High)	**15**	98.47±2.64	5.62±0.06	89.63±2.64	96.53±1.89	1.09±0.01	36

Table 4.9 shows the results for the egg biochemical analysis of the brown trout eggs. The table shows the mean dry weights, percentage water content, NPS concentration, protein concentration and chloride concentration for day 8 post-exposure and day 15 post-exposure eggs. Statistical analysis for the egg dry weight on day 8 and day 15 post-exposure showed that day had no significant effect on the egg dry weight (ANOVA, F=1.29; d.f. = 1; *P*=0.279). Additionally, treatment also had no significant effect on the egg dry weight (ANOVA, F=1.52; d.f. =11; *P*=0.121).

Statistical analysis for the brown trout egg NPS concentration showed that day post-exposure had a significant effect on the egg NPS concentration(ANOVA, F=6.85; d.f. =1; *P*=0.001). Egg NPS at d15PE was significantly greater than egg NPS at d8PE. Additionally treatment also had a significant effect on the egg NPS concentration (ANOVA, F=31.80; d.f. =11; *P*<0.001). Tukey's pair wise comparison analysis showed the PAH exposed eggs had significantly higher NPS concentrations than control eggs, however the solvent control; and mix, OCP and triazine exposed eggs also had significantly higher

NPS concentrations than control eggs but no significant difference with acid herbicide exposed eggs.

Statistical analysis for the egg protein concentration showed that day post exposure had no significant effect on the egg protein concentration (ANOVA, F=0.47; d.f. =1; P=0.627). However, treatment did have a significant effect on the egg protein concentration (ANOVA, F=3.45; d.f. =11; P<0.001). Tukey's pair wise comparison identified that the mix exposed eggs at both concentrations had significantly higher concentrations of egg protein than the control treatment eggs.

Statistical analysis for egg chloride concentration for day 8 and day 15 post exposure showed that there was no significant effect on egg chloride by days post exposure (ANOVA, F=1.29; d.f. =1; P=0.259) nor treatment (ANOVA, F=1.37; d.f. =11; P=0.194). However, the interaction of day and treatment did display a significant effect (ANOVA, F=2.00; d.f. =11; P=0.034) but only indicated that for the treatment triazine (Low), day 15 post exposure eggs had a significantly greater chloride concentration than day 8 post exposure eggs.

Table 4.9. Biochemical analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPE	Dry Weight (mg)	NPS (NPS nM/mg Egg)	Protein (mg/mg Egg)	Chloride (nEq Cl/mg Egg)	N value
Control	**8**	51.33±2.07	0.50±0.01	0.24±0.01	24.94±2.64	12
Control	**15**	51.78±3.12	0.52±0.01	0.23±0.01	19.77±1.36	12
Solvent	**8**	57.11±2.55	0.37±0.01	0.26±0.01	30.11±2.75	12
Solvent	**15**	51.22±4.40	0.46±0.01	0.25±0.02	25.99±2.45	12
AH (Low)	**8**	52.83±2.70	0.53±0.03	0.26±0.02	31.71±3.56	12
AH (Low)	**15**	57.83±3.51	0.55±0.01	0.29±0.01	23.82±4.32	12
AH (High)	**8**	61.17±4.14	0.52±0.02	0.28±0.02	23.84±2.09	12
AH (High)	**15**	58.67±2.16	0.47±0.01	0.27±0.02	26.96±3.03	12
OCP (Low)	**8**	61.50±2.70	0.37±0.03	0.28±0.01	27.92±3.47	12
OCP (Low)	**15**	55.17±2.12	0.35±0.03	0.27±0.01	19.85±2.00	12
OCP (High)	**8**	54.33±4.74	0.42±0.03	0.29±0.01	26.63±3.83	12
OCP (High)	**15**	57.50±3.00	0.34±0.03	0.26±0.01	18.91±1.14	12
PAH (Low)	**8**	56.67±2.70	0.58±0.01	0.30±0.02	33.68±2.41	12
PAH (Low)	**15**	52.50±3.86	0.69±0.04	0.30±0.01	28.89±3.01	12
PAH (High)	**8**	51.17±3.84	0.61±0.02	0.29±0.00	24.23±2.70	12
PAH (High)	**15**	53.77±4.74	0.61±0.03	0.28±0.01	28.40±3.57	12
TRI (Low)	**8**	50.00±4.11	0.34±0.02	0.28±0.02	22.08±3.55	12
TRI (Low)	**15**	54.00±4.62	0.36±0.04	0.27±0.01	32.46±5.71	12
TRI (High)	**8**	52.00±2.93	0.36±0.03	0.26±0.01	25.85±4.35	12
TRI (High)	**15**	51.50±4.24	0.39±0.03	0.28±0.02	22.98±2.39	12
MIX (Low)	**8**	57.11±3.12	0.39±0.02	0.31±0.02	20.81±2.49	12
MIX (Low)	**15**	60.44±3.06	0.42±0.01	0.28±0.01	27.36±2.07	12
MIX (High)	**8**	53.67±2.10	0.38±0.02	0.28±0.01	25.61±2.29	12
MIX (High)	**15**	59.89±4.16	0.39±0.02	0.32±0.04	24.97±2.62	12

Table 4.10 shows the alevin morphometric analysis on hatching . Statistical analysis showed that treatment had a significant effect on the length of hatched alevins (ANOVA, F=3.78; d.f. =11; P<0.001). Tukey's pair wise comparison identifried that mix (Low) exposed alevins were significantly longer than solvent control treatment alevins and OCP (Low) exposed alevins. Statistical analysis for the alevin weight measured on hatching showed that treatment did have a significant effect on the alevin weight (ANOVA, F=2.33; d.f. =11; *P*=0.010). A Tukey's pair wise comparison identified that acid herbicide exposed alevins (for both concentration levels) had significantly higher weights than control treatment alevins.

Table 4.10. Morphometric analysis of *Salmo trutta* alevins (Data represents Mean±SEM)

Treatment	Length (mm)	Weight (mg)	N value
Control	15.66±0.23	98.93±2.83	30
Solvent	15.49±0.19	102.21±2.61	29
AH (Low)	16.05±0.17	114.00±3.29	18
AH (High)	15.25±0.26	113.50±3.70	18
OCP (Low)	14.93±0.30	104.56±3.07	18
OCP (High)	15.49±0.16	101.95±3.43	20
PAH (Low)	16.10±0.25	101.85±3.64	20
PAH (High)	15.61±0.24	103.85±2.86	20
TRI (Low)	16.20±0.27	109.70±3.89	18
TRI (High)	15.57±0.33	100.70±3.42	20
MIX (Low)	16.56±0.25	108.78±3.28	18
MIX (High)	16.40±0.15	105.00±2.92	18

Figure 4.17 shows the body weight and yolk sac weight as a percentage of total weight. Statistical analysis showed that treatment had a significant effect on both the body weight (ANOVA, F=3.68; d.f. =11; P<0.001) and yolk sac weight ANOVA, F=3.69; d.f. =11; P<0.001). Tukey's pair wise comparison showed that acid herbicide (Low) exposed eggs had significantly greater body weights than acid herbicide (High) and PAH (High) exposed eggs. Also acid herbicide (High) exposed eggs had significantly smaller body weights than acid herbicide (Low) and OCP (High) exposed eggs, but not to controls.

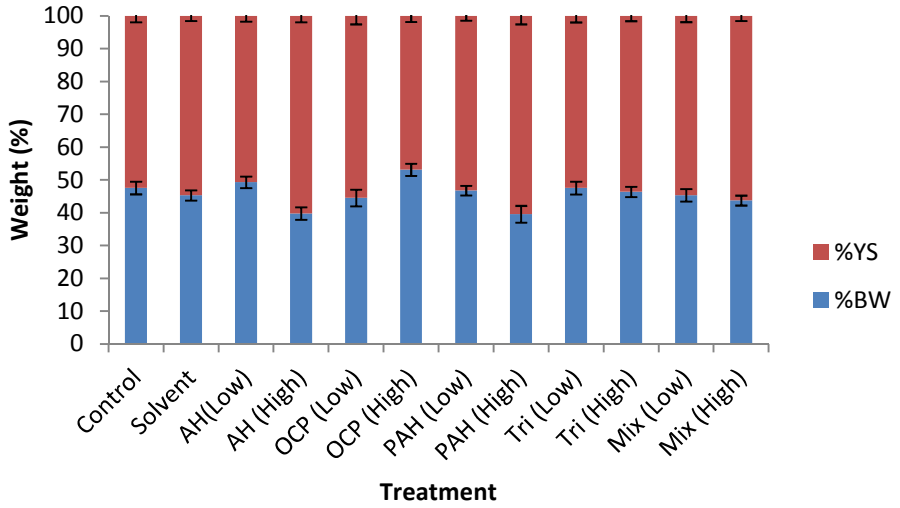

Figure 4.17. Body weight and yolk sac weight, as a percentage of total weight. Data represents Mean±SEM (n= 30,29,18,18,18,18,18,20,20,20,18,20 respectively).

Visual observations of the brown trout hatchlings were noted during the experiment. Several abnormalies were observed in specific treatments; four Control treatment alevins were described as being concave in appearance; two OCP exposed alevins were observed to have twisted spines; ten PAH exposed alevins were described as being concave and two PAH exposed alevins had eyes which were not spherical; one triazine exposed alevin had no eyes and seven triazine exposed alevins were described as having a bent spine. No yolk sac oedemas were observed during this trial.

4.3.3. Year 3 Results

4.3.3.1. 48hr Fertilisation Experiment

Table 4.11 shows the results for egg morphometric analysis for the 48hr exposure trial. Statistical analysis showed that treatment had a significant effect on egg weight (ANOVA, F=1.78, d.f. =13; P=0.046). Fisher Method (pair wise comparison) indicated various significant differences between the treatments. The OCP (Low)-, triazine (Low)-, Ammonia-, mix (Low)- and acid herbicide (High)-exposed eggs were significantly lighter compared to the control eggs.

Egg diameter results for the 48hr exposure trial showed that treatment had a significant effect on the size of the eggs (ANOVA, F=15.62; d.f. =13; P<0.001). Tukey's pair wise comparison identified that both the acid herbicide and triazine exposed eggs, for both concentration levels, had a significantly smaller diameter than the control treatment eggs.

Statistical analysis showed that volume for 48 hour exposure eggs are showed that treatment did not have a significant effect on the volume of the brown trout eggs (ANOVA, F=1.78; d.f. =13; P=0.056). The results for egg surface area for 48 hour exposure eggs also showed that treatment did not have a significant effect on the surface area of the eggs (ANOVA, F=1.79; d.f. =13; P=0.054). However, statistical analysis showed that treatment did have a significant difference on the egg surface area to volume ratio (ANOVA, F=1.80; d.f. =13; P=0.042). A pairwise comparison identified that acid

herbicide and triazine exposed eggs had a greater surface area to volume ratio compared to the control group.

Table 4.11. Morphometric analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	Weight (mg)	Diameter (mm)	Volume (mm^3)	Surface Area (mm^2)	SA:Vol	N value
Control	97.63±1.96	5.55±0.04	88.78±1.96	96.11±1.46	1.09±0.01	20
Ammonia	92.08±1.85	5.53±0.04	83.24±1.79	92.08±1.30	1.11±0.01	20
AH (Low)	99.08±1.79	5.26±0.06	90.24±2.02	97.20±1.50	1.08±0.01	20
AH (High)	91.79±2.02	5.09±0.04	82.95±2.17	91.84±1.59	1.11±0.01	20
OCP (Low)	93.21±1.89	5.59±0.05	84.37±2.11	92.90±1.56	1.11±0.01	20
OCP (High)	95.79±1.68	5.57±0.05	86.95±1.89	94.82±1.40	1.09±0.01	20
PAH (Low)	95.38±2.00	5.53±0.06	86.54±1.68	94.48±1.22	1.10±0.01	20
PAH (High)	96.29±2.03	5.63±0.05	87.45±2.00	95.14±1.46	1.09±0.01	20
TRI (Low)	92.58±2.60	5.00±0.06	83.74±2.03	92.32±1.49	1.11±0.01	20
TRI (High)	98.33±2.17	5.24±0.05	89.49±2.60	96.67±1.97	1.08±0.01	20
MIX (Low)	91.83±2.17	5.52±0.05	82.99±1.64	91.85±1.19	1.11±0.01	20
MIX (High)	93.96±2.11	5.50±0.04	85.12±1.85	93.42±1.36	1.10±0.01	20
MIX (Low)+Amm	98.42±1.64	5.64±0.05	89.58±1.64	96.73±1.19	1.08±0.01	20
MIX (High)+Amm	95.79±1.73	5.51±0.06	86.95±1.73	94.82±1.26	1.09±0.01	20

Eggs scored positive for fertilisation by the presence of the chorion and blastodisc. Only two eggs scored negative for fertilisation following the 48 hour exposure; one exposed to mix (High) plus ammonia and one exposed to triazine (Low) (Figure 4.18).

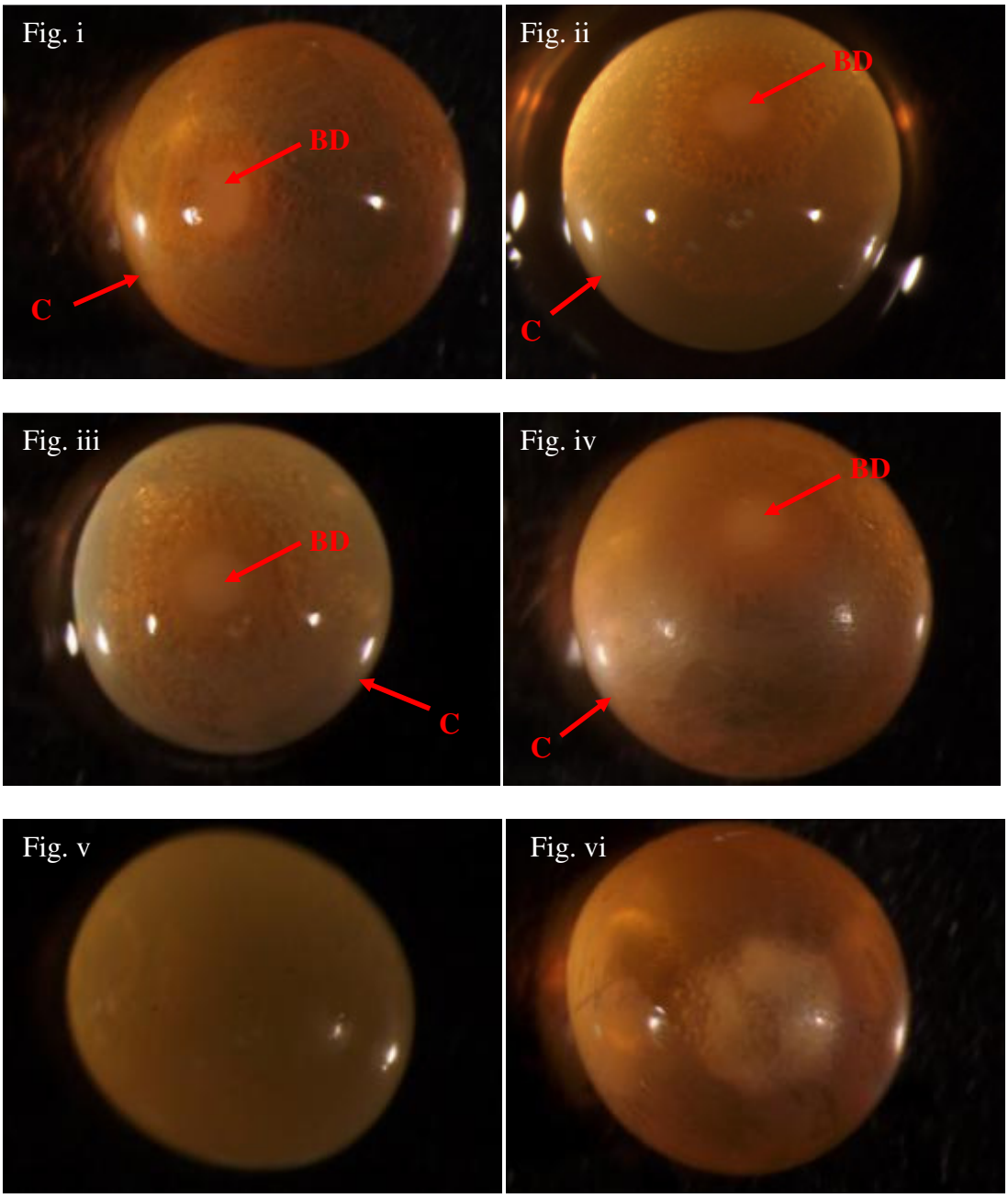

Figure 4.18. 48hr exposure trial images. Blastodisc (BD) and Chorion (C) are identified; Fig. i) Control egg, Fig. ii) TRI (High) exposed egg, Fig. iii) OCP (Low) exposed egg, Fig. iv) PAH (Low) exposed egg, Fig. v) Unfertilised TRI (Low) exposed egg, Fig. vi) Unfertilised MIX (High) plus ammonia exposed egg.

Fertilisation success was calculated as 100% for all treatments, except for triazine (Low) and mix (High) plus ammonia where fertilisation success was 95.83% for both those specific treatments.

4.3.3.2. Green Egg Experiment

Figure 4.19 shows the survival data for the eggs to the eyeing stage of development. Survival varied between the treatments with the highest survival observed for the high concentration of triazine exposure.

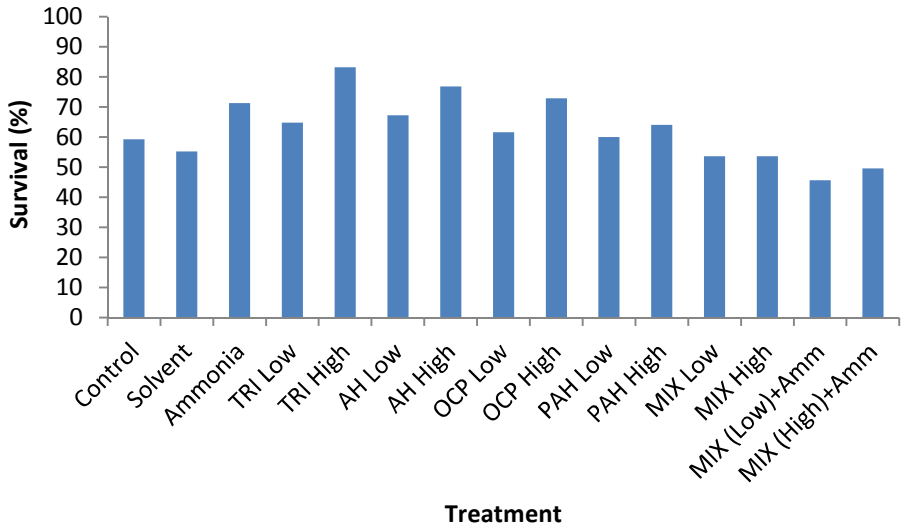

Figure 4.19. Survival of the fertilisation to eyeing stage.

Figure 4.20 shows the survival of the eyed eggs to hatching. The survival of this stage of development was much greater than the initial survival from fertilisation to eyeing stage. There was some inter-treatment variation, however generally the survival was high.

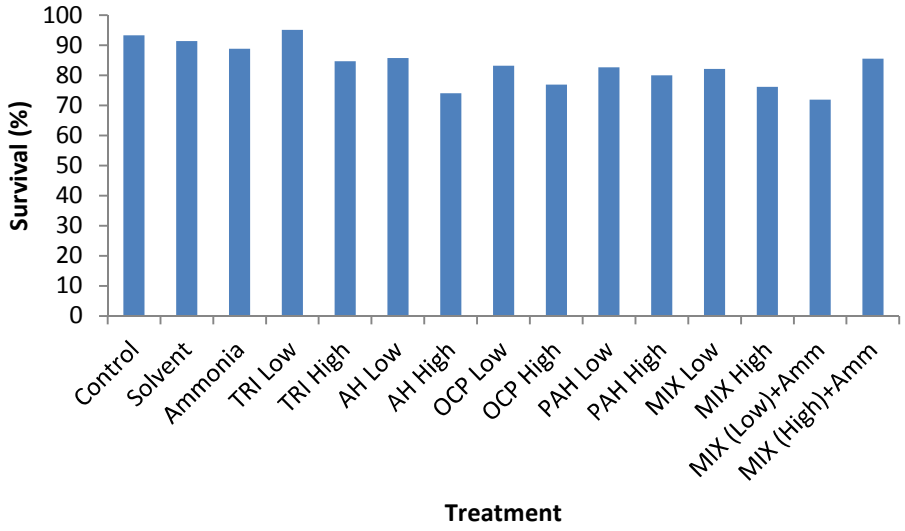

Figure 4.20. Survival from eyed to hatch (to end).

Table 4.12 shows the egg morphometric analysis for day 30 post fertilisation. Statistical analysis showed that treatment had a significant effect on the weight of the eggs (ANOVA, F=19.53; d.f. =15; P<0.001). Tukey's pair wise comparison identified that the acid herbicides and triazine exposed eggs, for both levels of concentration had a significantly smaller weight than that of control treatment eggs. Additionally, treatment also had a significant effect on egg diameter (ANOVA, F=8.57; d.f. =15; P<0.001). Tukey's pair wise comparison identified that acid herbicides and triazines (both concentration levels) had a smaller diameter than those eggs exposed to control treatment eggs.

Statistical analysis for egg volume at day 30 post-fertilisation showed that treatment did have a significant effect on the volume of the eggs (ANOVA, F=2.49; d.f. =15; P=0.002). A pairwise comparison indicated that no treatment group differed from the non-solvent control. However acid herbicide (High) and mix (High) plus ammonia had significantly greater volumes than OCP, mix, triazines and ammonia exposure groups. Results also showed that treatment had a significant effect on the surface area of the eggs (ANOVA, F=2.41; d.f. =15; P=0.003). A pairwise comparison indicated that no treatment group differed from the non-solvent control, however AH (High) had significantly greater volumes than OCP, Mix, Triazines and Ammonia exposure groups.

Statistical analysis for egg surface area to volume ratio showed that treatment did have a significant effect on the surface area to volume ratio of the eggs (ANOVA, F=2.08; d.f.

=15; *P*=0.011). Tukey's pair wise comparison identified that triazine (High) exposed eggs had a larger ratio than acid herbicide (High) exposed eggs, but not compared to the controls.

Table 4.12. Morphometric analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	Weight (mg)	Diameter (mm)	Volume (mm^3)	Surface Area (mm^2)	SA:Vol	N value
Control	96.15±1.75	5.56±0.04	90.45±2.16	97.33±1.56	1.08±0.01	20
Solvent	93.00±2.53	5.40±0.07	82.98±2.84	91.76±2.15	1.12±0.01	20
Ammonia	93.40±2.30	5.50±0.09	88.16±4.11	95.36±2.97	1.10±0.02	20
AH (Low)	93.65±1.62	5.09±0.06	69.36±2.47	81.43±1.91	1.18±0.01	20
AH (High)	101.30±1.60	5.06±0.07	68.36±2.53	80.59±2.08	1.19±0.02	20
OCP (Low)	94.55±1.85	5.36±0.04	81.07±1.94	90.47±1.46	1.12±0.01	20
OCP (High)	92.40±1.59	5.45±0.06	85.28±2.64	93.50±1.97	1.10±0.01	20
PAH (Low)	98.15±1.92	5.37±0.05	81.53±2.21	90.79±1.65	1.12±0.01	20
PAH (High)	99.65±2.36	5.38±0.06	82.02±2.91	91.05±2.17	1.12±0.01	20
TRI (Low)	93.85±2.41	4.96±0.09	65.04±3.28	77.77±2.68	1.22±0.02	20
TRI (High)	90.70±1.83	5.05±0.07	68.20±2.87	80.41±2.30	1.19±0.02	20
MIX (Low)	94.25±1.78	5.45±0.06	85.31±2.58	93.53±1.90	1.10±0.01	20
MIX (High)	94.55±2.03	5.49±0.06	87.11±2.55	94.85±1.87	1.10±0.01	20
MIX (Low)+Amm	97.50±1.99	5.44±0.07	85.12±3.20	93.28±2.40	1.11±0.02	20
MIX (High)+Amm	100.10±1.76	5.47±0.06	86.33±2.85	94.23±2.11	1.10±0.01	20

Table 4.13 shows the biochemical analysis of the day 30 post-fertilisation eggs. Statistical analysis for egg dry weight at day 30 post-fertilisation showed that treatment did have a significant effect on the egg dry weight (ANOVA, F=1.93; d.f. =15; *P*=0.036). However, Tukey's pair wise comparison did not indicate a significant difference between specific treatments. Although statistical analysis of egg water content data of the day 30 post-fertilisation eggs is showed treatment did not have a significant effect on the water content of the eggs (ANOVA, F=1.49; d.f. =15; *P*=0.135).

Statistical analysis for day 30 post-fertilisation eggs showed that treatment did have a significant effect on the egg ninhydrin positive substance concentration (ANOVA, F=10.71; d.f. =15; *P*<0.001). Tukey's pair wise comparison showed that mix, OCP, PAH, mix plus ammonia exposed eggs had significantly lower concentrations than both egg control treatments; control and solvent control. However results for the egg protein concentration at day 30 post-fertilisation showed that treatment did not have a significant effect on the egg protein concentration (ANOVA, F=0.86; d.f. 15; *P*=0.613). Statistical

analysis at day 30 post-fertilisation showed that treatment did not have a significant effect on the egg chloride concentration on d30PF (ANOVA, F=0.90; d.f. =15; *P*=0.567).

Table 4.13. Biochemical analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	Dry Weight (mg)	Water Content (%)	NPS (NPS nM/mg Egg)	Protein (mg/mg Egg)	Chloride (nEq Cl/mg Egg)	N value
Control	52.00±1.18	54.90±1.85	56.72±2.94	0.23±0.01	30.00±2.94	5
Solvent	54.60±1.81	58.12±2.90	56.53±3.05	0.22±0.01	32.79±2.82	5
Ammonia	53.20±2.27	58.03±2.80	46.58±1.48	0.23±0.01	27.48±0.52	5
AH (Low)	54.60±1.78	56.24±1.25	43.78±0.64	0.16±0.04	28.37±1.38	5
AH (High)	54.2±1.16	55.58±0.89	49.82±3.08	0.22±0.02	25.85±4.39	5
OCP (Low)	58.20±1.02	60.68±1.36	38.47±2.25	0.21±0.01	33.50±3.36	5
OCP (High)	50.40±3.03	57.54±3.18	40.74±1.78	0.22±0.01	31.45±3.09	5
PAH (Low)	59.80±2.31	60.48±1.74	28.42±3.18	0.23±0.01	32.43±2.47	5
PAH (High)	57.80±1.77	56.90±1.26	33.47±3.87	0.22±0.01	28.95±4.19	5
TRI (Low)	48.00±4.42	55.68±1.22	44.63±1.52	0.22±0.01	25.77±4.12	5
TRI (High)	52.40±3.09	58.81±1.98	50.05±2.61	0.22±0.01	22.91±2.43	5
MIX (Low)	47.80±2.85	54.04±1.42	33.05±3.81	0.19±0.04	28.89±2.95	5
MIX (High)	54.00±2.39	59.02±1.21	33.37±3.35	0.19±0.01	30.53±5.08	5
MIX (Low)+Amm	53.80±2.89	57.19±2.22	38.46±1.68	0.22±0.01	26.56±3.12	5
MIX (High)+Amm	50.00±2.39	53.36±2.27	28.53±4.78	0.20±0.04	27.08±1.84	5

Table 4.14 shows the results for the morphometric analysis of the Salmo trutta alevins on hatching. Statistical analysis showed that treatment had a significant effect on the alevin length on hatch (ANOVA, F=1.91; d.f. =15; *P*=0.029). A Tukey's pair wise comparison identified that OCP (High) and mix (High) were significant longer than both control groups. Statistical analysis did not show that treatment had a significant effect on the alevin weight on hatch (ANOVA, F=1.52; d.f. =15; *P*=0.110).

Table 4.14. Morphometric analysis of *Salmo trutta* alevins (Data represents Mean±SEM)

Treatment	Length (mm)	Weight (mg)	N value
Control	15.88±0.35	92.12±2.19	8
Solvent	16.50±0.19	89.75±2.94	8
Ammonia	16.03±0.37	84.50±2.48	8
AH (Low)	16.86±0.36	89.13±3.07	8
AH (High)	16.68±0.21	93.00±2.53	8
OCP (Low)	17.44±0.32	92.38±1.32	8
OCP (High)	16.33±0.37	91.62±4.11	8
PAH (Low)	16.26±0.54	84.25±1.60	8
PAH (High)	16.52±0.28	87.62±3.78	8
TRI (Low)	16.52±0.53	93.50±2.60	8
TRI (High)	16.87±0.17	81.75±3.30	8
MIX (Low)	16.10±0.37	87.37±3.92	8
MIX (High)	17.27±0.29	91.25±2.44	8
MIX (Low)+Amm	16.66±0.21	87.62±2.49	8
MIX (High)+Amm	17.10±0.14	89.62±1.40	8

Table 4.15 shows the results of morphometric and biochemical analysis of the day 93 post-fertilisation alevins. Statistical analysis showed that treatment did have a significant effect on the alevin weight (ANOVA, F=14.79; d.f. =14; $P<0.001$). Tukey's pair wise comparison showed that PAH (High) and triazine (High) exposed alevins were significantly heavier than control treatment alevins. Statistical analysis for day 93 post-fertilisation alevins showed that treatment did have a significant effect on the length of alevins (ANOVA, F=13.42; d.f. =14; $P<0.001$). Tukey's pair wise comparison identified that acid herbicide (Low) and triazine (High) exposed eggs were significantly shorter than control treatment eggs.

Statistical analysis for day 93 post-fertilisation alevins showed that treatment showed to have a significant effect on the alevin dry weight (ANOVA, F=2.97; d.f. = 15; $P=0.001$). Tukey's pair wise comparison identified that acid herbicide (Low), OCP (High), PAH (Low), TRI and ammonia exposed alevins had significantly lower dry weights than non-solvent control treatment alevins. However treatment did not have a significant effect on the percentage water content of the alevins at day 93 post-fertilisation (ANOVA, F=1.02; d.f. = 15; $P=0.448$). Statistical analysis showed that treatment had no effect on the day 93 post-fertilisation alevin protein concentration (ANOVA, F=1.43; d.f. =15; $P=0.58$).

Table 4.15. Morphometric and biochemical analysis of *Salmo trutta* alevins (Data represents Mean±SEM)

Treatment	Weight (mg)	Length (mm)	Dry weight (mg)	Water Content (%)	Protein (mg/mg alevin)	N value
Control	124.46±2.43	25.66±0.20	18.33±1.50	82.52±0.49	0.14±0.01	26
Solvent	118.14±4.02	25.86±0.32	14.33±0.80	83.52±0.57	0.15±0.01	14
Ammonia	108.28±1.81	24.39±0.18	13.33±0.62	82.38±0.46	0.17±0.01	20
AH (Low)	114.40±4.68	24.04±0.23	13.50±0.85	83.63±0.66	0.15±0.01	24
AH (High)	125.29±2.73	25.28±0.18	16.00±1.37	84.29±0.83	0.14±0.01	15
OCP (Low)	120.00±3.43	24.68±0.22	15.50±1.31	83.88±0.87	0.15±0.01	11
OCP (High)	123.23±3.05	25.87±0.25	14.17±2.12	83.90±1.07	0.14±0.00	23
PAH (Low)	120.20±4.15	24.80±0.54	13.17±1.85	85.07±0.75	0.15±0.01	22
PAH (High)	141.71±1.90	26.37±0.21	18.17±0.65	84.05±0.33	0.16±0.01	20
TRI (Low)	115.19±2.09	24.88±0.18	14.00±1.53	83.81±0.74	0.16±0.00	21
TRI (High)	99.94±2.19	23.51±0.22	12.67±1.33	84.18±0.70	0.15±0.01	37
MIX (Low)	130.40±3.49	25.80±0.23	16.00±1.821	84.06±0.69	0.15±0.01	34
MIX (High)	133.18±2.74	26.01±0.24	7.67±1.20	84.46±1.01	0.15±0.01	36
MIX (Low)+Amm	125.00±10.20	24.43±0.64	13.50±0.50	83.33±0.21	0.18±0.01	4
MIX (High)+Amm	123.13±3.55	25.23±0.37	16.75±1.18	83.23±0.32	0.15±0.01	8

Figure 4.21 shows the body weight and yolk sac weight of day 93 post fertilisation alevins. Data is represented as percentage weight of total weight. Treatment did have a significant effect on the percentage body weight of the alevins (ANOVA, F=6.58; d.f. =15; $P<0.001$). Tukey's pair wise comparison showed that ammonia and triazine (High) exposed alevins had significantly lower body weights compared to control treatment alevins. Additionally, treatment also had a significant effect on the percentage yolk sac weight of alevins (ANOVA, F=5.25; d.f. =15; P<0.001). Tukey's pair wise comparison showed that ammonia and triazine (High) exposed alevins had significantly heavier yolk sacs compared to control treatment alevins.

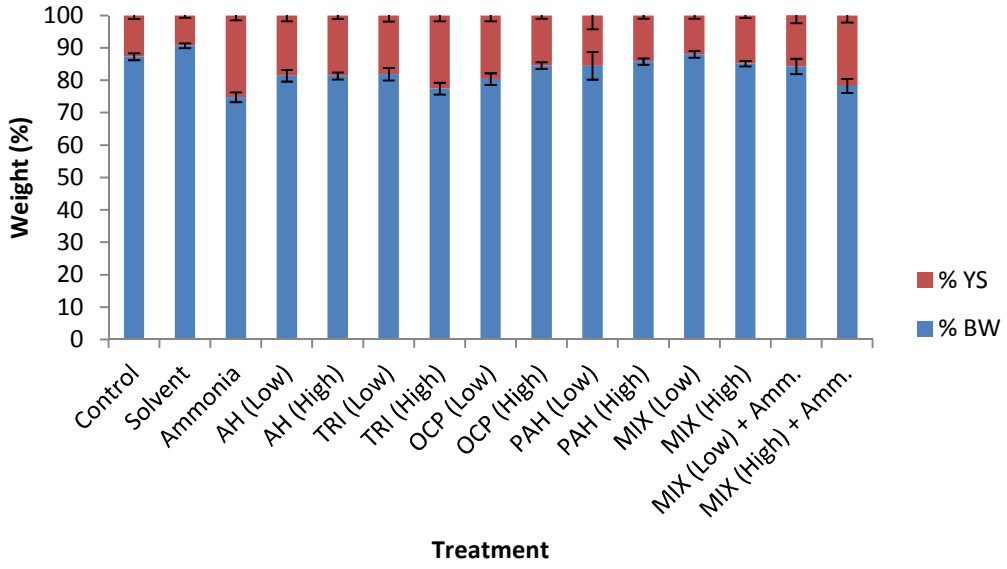

Figure 4.21. Alevin body weight and yolk sac weight, as a percentage of total weight, on day 93 post fertilisation, from eggs which had been fertilised in the relevant treatment prior to exposure. Data represents Mean±SEM (n= 10; MIX (High), n=8; A+M (Low), n=2; A+M (High), n=4).

Visual observations of the hatching alevins were noted throughout the experiment. One PAH (High) exposed alevin was described as displaying a yolk sac oedema (Figure 4.65); one PAH (Low) was also observed as having a yolk sac oedema (Figure 4.22); one PAH (Low) exposed alevin had a curved spine; one triazine (Low) exposed alevin and mix plus ammonia (Low) also had yolk sac oedemas (Figure 4.22). All observations were compared to control treatment alevins (Figure 4.22).

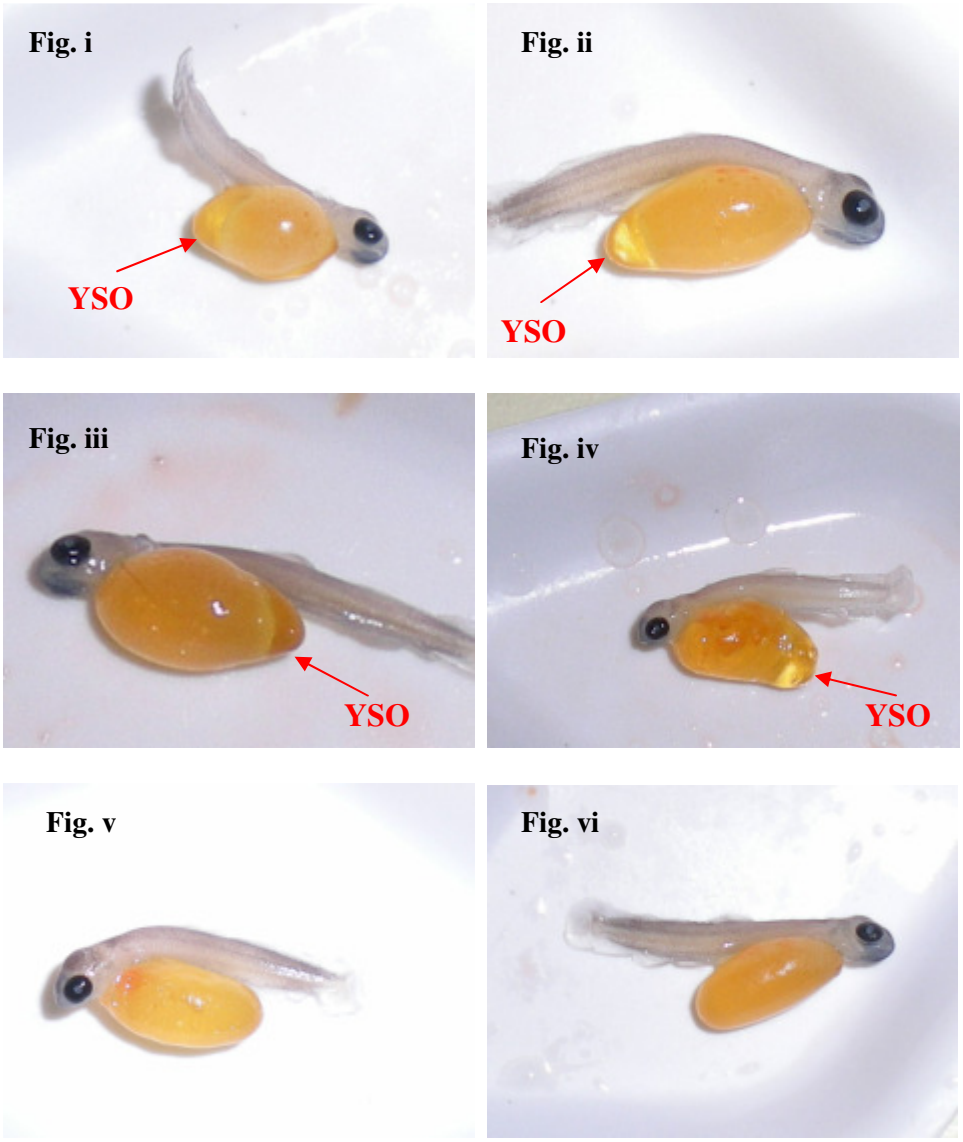

Figure 4.22. Alevins at day 93 post fertilisation. Yolk sac oedemas (YSO) are identified; Fig. i) PAH (High) exposed alevin Fig. ii) PAH (Low) exposed alevin, Fig. iii) MIX (Low) plus Amm. exposed alevin, Fig. iv) TRI (Low) exposed alevin, Fig. v) Control treatment alevin 1 showing normal yolk sac development, Fig. vi) control treatment alevin 2 showing normal yolk sac development.

4.3.3.3. Eyed Egg Experiment: Exposure from Hatching

Figure 4.23 shows the survival of the eyed eggs from hatching to the end of the experiment, during the actual exposure time to contaminants.

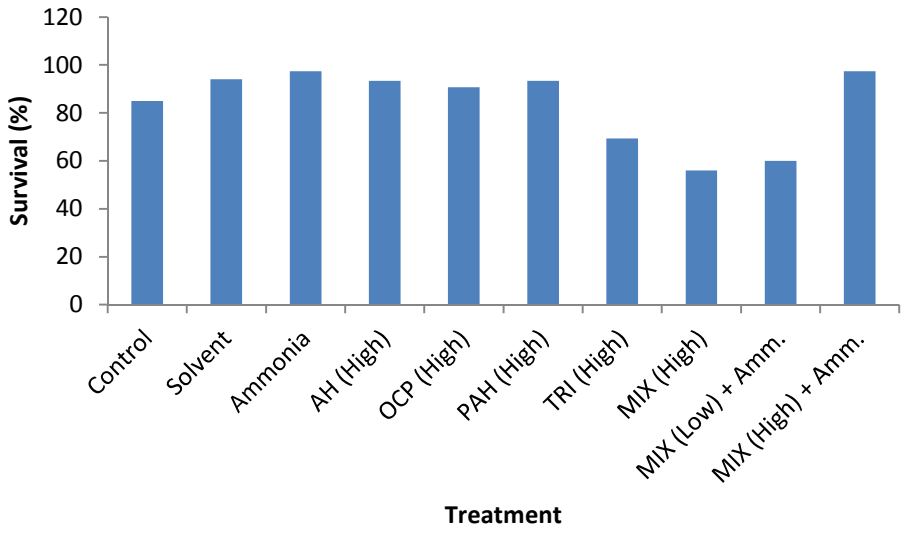

Figure 4.23. Survival of the eyed eggs from hatching to end of exposure

Table 4.16 shows the morphometric and biochemical analysis of day 18 and day 38 post-exposure alevins. Statistical analysis showed that treatment did have a significant effect on the alevin length at day 18 post-exposure (ANOVA, F=3.48; d.f. =9; P=0.001). Tukey's pair wise comparison identified that mix plus ammonia (Low) exposed alevins were significantly shorter than solvent control treatment alevins. Also OCP exposed alevins were significantly longer than PAH exposed alevins and mix plus ammonia (Low) exposed alevins when compared to the control group. Statistical analysis for day 38 post-exposure alevins showed that treatment had a significant effect on the alevin length (ANOVA, F=6.71; d.f. =9; P<0.001). Tukey's pair wise comparison showed that OCP exposed alevins were significantly longer than the control treatment alevins and that mix plus ammonia (Low) exposed alevins were significantly shorter than control treatment alevins. For further statistical analysis, Day 18 and Day 38 post exposure alevin data was analysed together. Day post exposure did have a significant difference on the alevin length (ANOVA, F=2453.81; d.f. =1; P<0.001). Tukey's pair wise comparison confirmed that Day 38 alevins were longer than Day 18 alevins. However, once the data for both exposure days was analysed together, treatment had no significant effect on the alevin length (ANOVA, F=1.59; d.f. =9; P=0.115).

Statistical analysis for day 18 post-exposure alevins showed that treatment had no significant effect on alevin weight (ANOVA, F=1.77; d.f. =9; P=0.079). However,

statistical analysis for day 38 post-exposure alevins showed that treatment did have a significant effect on the alevin weight (ANOVA, F=5.57; d.f. =9; *P*<0.001). Tukey's pair wise comparison identified that mix plus ammonia (Low) exposed alevins were significantly lighter than control treatment alevins. For further statistical analysis, data from Day 18 and Day 38 were analysed together. Day post exposure did have a significant effect on the alevin weight (ANOVA, F=305.06; d.f. =1; *P*=0.003). Tukey's pair wise comparison confirmed that day 38 alevins were significantly heavier than Day 18 alevins. Additionally, treatment also showed to have a significant effect on the alevin weight (ANOVA, F=2.80; d.f. =9; *P*=0.003). Tukey's pair wise comparison identified that solvent control treatment alevins, acid herbicide and OCP exposed alevins were all significantly heavier than mix plus ammonia (Low) exposed alevins.

Table 4.16. Morphometric and biochemical analysis of *Salmo trutta* alevins (Data represents Mean±SEM)

Treatment	dPE	Weight (mg)	Length (mm)	Protein (mg/mg alevin)	N value
Control	18	105.35±1.80	21.20±0.19	0.15±0.01	20
Control	38	125.30±1.45	25.77±0.13	0.14±0.00	40
Solvent	18	107.25±1.53	21.47±0.20	0.14±0.01	20
Solvent	38	127.65±1.30	25.77±0.12	0.14±0.00	40
Ammonia	18	103.73±1.69	21.07±0.10	0.14±0.00	15
Ammonia	38	127.17±1.12	25.30±0.15	0.15±0.00	30
AH (High)	18	114.13±5.66	21.23±0.20	0.13±0.00	15
AH (High)	38	126.57±2.32	25.38±0.19	0.14±0.00	30
OCP (High)	18	110.53±2.13	21.82±0.20	0.14±0.01	14
OCP (High)	38	131.70±1.63	26.14±0.14	0.14±0.00	30
PAH (High)	18	102.53±1.62	20.83±0.12	0.16±0.01	15
PAH (High)	38	126.43±1.64	25.44±0.16	0.15±0.01	30
TRI (High)	18	108.50±2.55	20.99±0.19	0.14±0.01	15
TRI (High)	38	121.23±2.06	24.92±0.20	0.15±0.01	30
MIX (High)	18	109.40±1.09	21.09±0.12	0.14±0.01	15
MIX (High)	38	123.77±2.02	25.57±0.15	0.15±0.00	30
MIX (Low)+Amm	18	107.29±2.51	20.61±0.16	0.15±0.01	14
MIX (Low)+Amm	38	114.00±3.31	24.56±0.21	0.15±0.00	22
MIX (High)+Amm	18	107.47±1.97	20.97±0.20	0.16±0.01	15
MIX (High)+Amm	38	127.03±1.54	25.62±0.15	0.13±0.01	30

Figure 4.24 shows the results for the day 18 post exposure alevin body tissue weight and alevin yolk sac weight, as a percentage of total weight, showed that treatment had a significant effect on the alevin body weight (ANOVA, F=4.27; d.f. =9; *P*<0.001). Tukey's pair wise comparison identified that triazine and mix plus ammonia (Low) exposed alevins had significantly lower body tissue weights than control treatment alevins. Also, OCP

exposed alevins had significantly greater body weights than triazine and mix plus ammonia (Low) exposed alevins. Additionally treatment had a significant effect on alevin yolk sac weights (ANOVA, F=4.23; d.f. =9; $P<0.001$). Tukey's pair wise comparison identified that triazine and mix plus ammonia (Low) exposed alevins had significantly larger yolk sacs compared to control treatment alevins. Also, OCP exposed alevins had significantly smaller yolk sac weights than triazine and mix plus ammonia (Low) exposed alevins. Treatment was also shown to have a significant effect on the body weight of the alevins at day 38 post-exposure (ANOVA, F=2.64; d.f. =9; $P=0.006$). Tukey's pair wise comparison identified that OCP exposed alevins had significantly greater body weights than triazine and mix plus ammonia (Low) exposed alevins. Treatment also had a significant effect on the alevin yolk sac weight (ANOVA, F=2.66; d.f. =9; $P=0.006$). Tukey's pair wise showed that OCP exposed alevins had significantly smaller yolk sac weights than triazine and mix plus ammonia (Low) exposed alevins. For further statistical analysis, Day 18 and Day 38 data sets were analysed together. Day post exposure did have a significant effect on the alevin body weight (ANOVA, F=4439.47; d.f. =1; $P<0.001$). Tukey's pair wise comparison confirmed that Day 38 post exposure alevins had significantly greater body weights than day 18 post exposure alevins. Additionally day post exposure also had a significant effect on the alevin yolk sac weight (ANOVA, F=5046.77; d.f. =1; $P<0.001$). Tukey's confirmed that Day 18 post exposure alevins had a greater yolk sac weight than Day 38 post exposure alevins. However, treatment did not have a significant effect on the either the alevin body weight (ANOVA, F=1.02; d.f. =9; $P=0.421$) or the alevin yolk sac weight (ANOVA, F=1.02; d.f. =9; $P=0.420$).

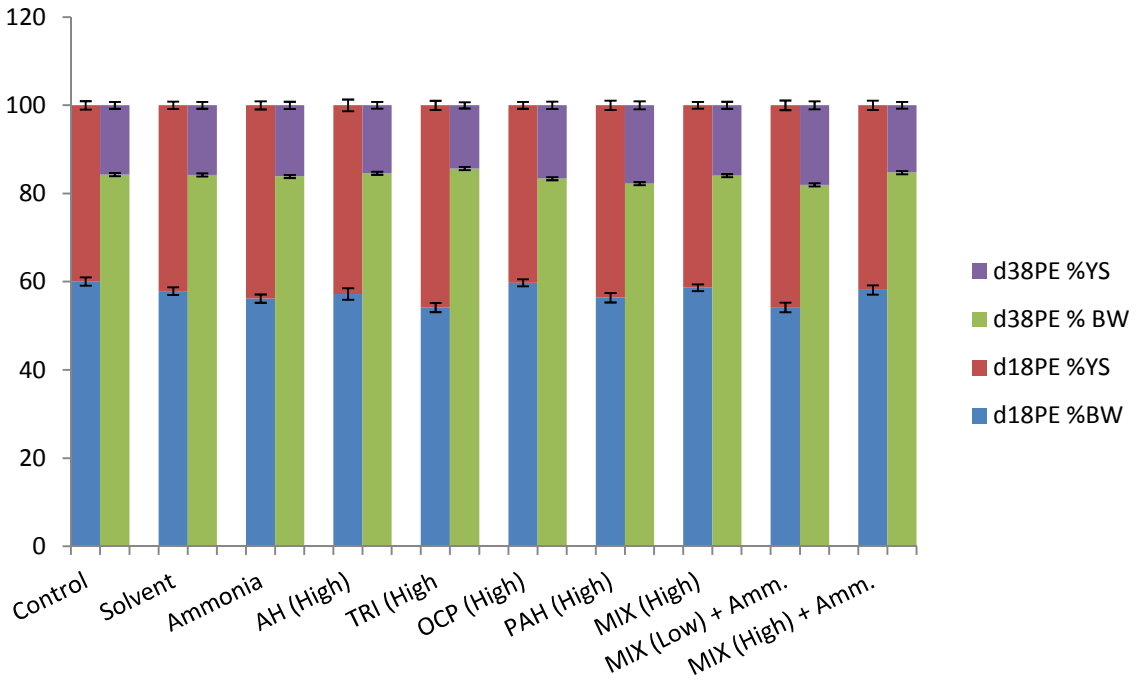

Figure 4.24. Alevin body weight and yolk sac weight, as a percentage of total weight, at day 18 post exposure (n=20, 20, 15,15,14,15,15,15,14,15 respectively) and at day 38 post exposure (n=37, 39, 31, 23, 29, 29, 21, 31, 18, 30 respectively). Data represents Mean±SEM.

Visual observations were noted for all sampled alevins. On Day 38 three PAH exposed alevins were described as having yolk oedemas (Figure 4.25); two triazine exposed alevins had yolk sac oedemas (Figure 4.25); one OCP exposed alevin had a yolk sac oedema (Figure 4.25).

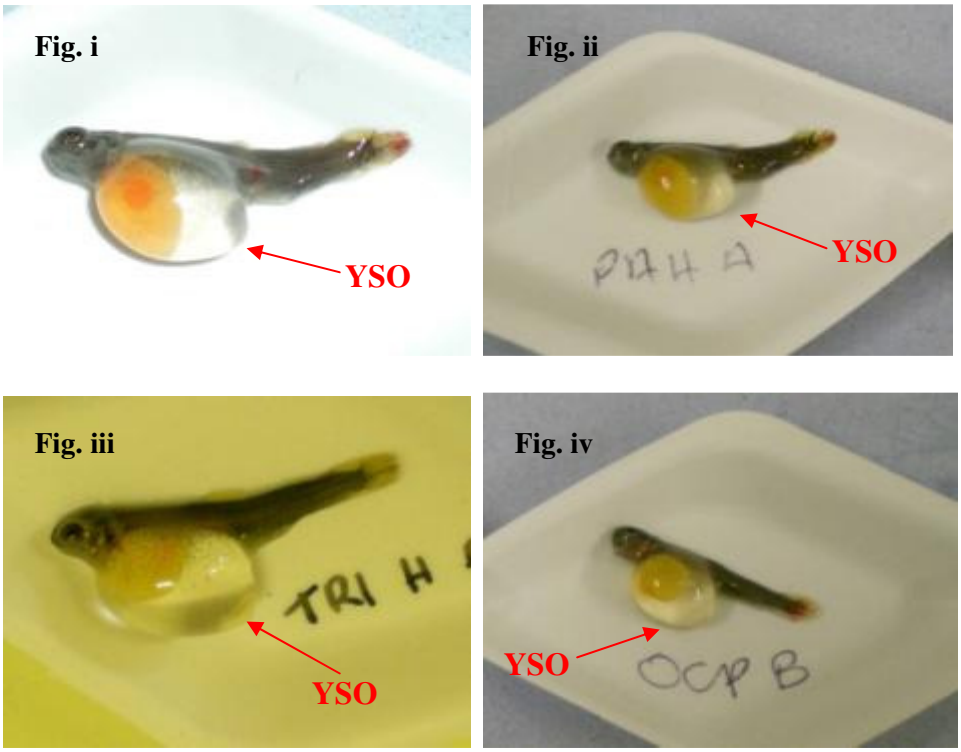

Figure 4.25. Alevins at day 38 post exposure. Yolk sac oedemas (YSO) are identified; Fig. i) PAH exposed alevin Fig. ii) PAH exposed alevin, Fig. iii) TRI exposed alevin, Fig. iv) OCP exposed alevin

4.3.4 Summary

Table 4.17 is a summary matrix of the significant results across all three years of experiments.

Table 4.17. Summary matrix of significant results for water exposure trials.

	Survival/Hatching	Egg/Alevin Morpometrics	Egg/Alevin Biochemistry
Year 1	•100% survival. •Treatment alevins hatched earlier than CNT group.	No significant differences	d13PE greater NPS than d6PE eggs.
Year 2	•73.91-92.00% survival (CNT – 89.33%).	•Treatment eggs greater diameter than CNT, except OCP. •AH (Low)-exposed alevins heavier than CNT.	•Treatment eggs greater NPS than CNT, except AH. •MIX greater protein than CNT.
Year 3 - (48hr exposure trial)	•100% survival. •100% fertilisation except 95.87% for TRI (Low) & MIX (High)+ Amm.	•OCP(Low), TRI(Low), MIX(Low) & AH(High) lighter than CNT. •AH & TRI smaller diameter than CNT. •AH & TRI greater SA:Vol than CNT.	n/a
Year 3 – (Green Egg Experiment)	•45.66 (MIX+Amm)-83.20% (TRI) to eyed stage survival (CNT - 59.20%) •71.93 (MIX+Amm)-95.06% (TRI) from eyed to hatch survival (CNT – 93.24%)	•AH & TRI smaller diameter & lighter than CNT. •OCP(High) & MIX(High) greater alevin length on hatch than CNT & SOL. •PAH(High) & TRI(High) greater d93PF weight & smaller length than CNT & SOL.	•MIX, OCP, PAH, MIX+Amm. eggs less NPS than CNT & SOL. •AH(Low), OCP(High), PAH(Low), TRI & Amm. smaller d93PF dry weight than CNT. •Amm. & TRI(High) smaller %BW and greater %YS than CNT.
Year 3 – (Eyed Egg Experiment)	•56.00 (MIX)-97.33% (Amm+) survival (CNT - 85%).	•MIX(Low)+Amm. d18PE shorter than SOL.	•TRI & MIX+Amm. smaller %BW and greater %YS than CNT.

4.4. Discussion

Although many studies have examined the impact of pollutants on aquatic organisms, few studies have examined effects in laboratory trials involving exposure to pollutants at concentrations measured at field sites. The research carried out for this study examined the effects of a selection of contaminant groups on the early life stages of the brown trout. The contaminants were investigated as individual compounds, groups of compounds, and as mixtures of different compound groups. Any significant effects of these water-borne contaminants could be directly related to natural conditions and could be used to predict possible impacts of pollution on wild populations of salmonids in the Avon catchment, and potentially other river systems supporting salmonid species.

4.4.1. Year 1 Trial

The preliminary laboratory trials for the water-borne contaminants focused on the use of individual compounds (atrazine, naphthalene, phenanthrene) to ascertain the effects each had on the developmental processes of brown trout embryos. The concentrations used for this trial were equivalent to environmental levels detected in the Avon catchment (Chapter 3). The data from this first trial suggested that individual contaminants can impact the early life stages of brown trout, although very few findings revealed significant effects on the exposed eggs. Similar data were recorded when these individual compounds were mixed, revealing that although there were some significant effects, they occurred infrequently.

Overall there were no mortalities observed in any of the treatments indicating that the individual pollutants had no impact on the survival of the salmonid eggs. However, certain contaminants did affect the eggs. Exposure of eggs to the contaminants revealed that NPS concentrations were significantly elevated when they were exposed to phenanthrene as compared to exposure to the solvent control. However, there was no significant difference in the NPS levels of the phenanthrene exposed eggs compared to the non-solvent exposed eggs. It is therefore possible that the effect could be due to solvent exposure and not an exposure to phenanthrene. The concentration of NPS is used as a proxy of free amino acids (FAA). In teleost fishes FAA have been found to regulate cell volume (Fyhn and Serigstad, 1987) and they are expected to increase during the normal development of salmonids (Srivastava *et al.*, 1994). As FAA can control the osmoregulation of fish eggs (Fyhn and Serigstad, 1987) the percentage water content of the eggs is closely linked to FAA levels. Despite the increase in NPS levels in the phenanthrene exposed eggs the water content was not shown to be significantly different for any of the contaminant exposed eggs compared to either of the control groups. However, eggs exposed to naphthalene were found to have a significantly greater water content compared to the phenanthrene and contaminant mix exposed eggs, but not compared to the control groups. The reason for these findings is not fully understood. It is possible that because PAHs have been reported to cause oedemas to both the yolk sac and pericardium in the embryo (Barron *et al.*, 2004) the increased water content in the naphthalene exposed group could potentially be explained by these malformations. However no yolk sac oedemas were observed in the hatched alevins of this

exposure group. Therefore it is unclear why there was an increase in water content in eggs exposed to naphthalene.

Treatment was not found to significantly affect the egg chloride concentration. However, eggs at day 6 post exposure were found to have a significantly greater chloride concentration than eggs at day 13 post exposure. Data for the hatching rate demonstrated that contaminants could induce premature hatching, as the contaminant exposed eggs hatched on average earlier than non-solvent control eggs. In particular, the phenanthrene exposed eggs hatched much earlier than either the solvent control or the non-solvent control eggs. Other studies have shown the acute toxicity of phenanthrene compared to other PAH compounds (Incardona, 2004). Premature hatching of embryos could be a concern as this would increase the time spent at the vulnerable yolk-sac stage compared to normal development, which may increase larval mortality (Rosenthal & Alderdice 1976). However, other studies have suggested that early hatching could be advantageous. Steele *et al.* (2001) noted that hatchlings have an increased surface area which is in direct contact with the surrounding waters and therefore alevins may be more at risk from increased transportation of contaminants into the body. Only one deformed alevin, which was exposed to the mix contaminant treatment, was observed throughout the experiment. The alevin was extremely deformed with a twisted spinal cord and one eye. With only one sample showing a deformity it is impossible to determine the contribution of the contaminants on the development of this individual. It is possible that this deformity could have occurred naturally, without the contaminant exposure. The results for the year 1 trial were shown to be relatively inconclusive, with very few effects being observed. Although there were few developmental impacts on the early life stages of brown eggs exposed to contaminants, it is feasible that the effects of egg exposure to pollutants could manifest itself at a later stage in development. Naphthalene and atrazine are both lipophillic compounds (Cho *et al*., 2004; Stoker and Cooper, 2007) and may readily accumulate in the embryo yolk sac (Korn & Rice 1981). In many teleost fish, such as brown trout and salmon, the lipid or oil globule is the last part of the yolk to be utilised by the developing larvae. This may result in the toxicological effects of contaminants to the embryo not being observed until the yolk is fully utilized at a later period in the fishes development (Pain *et al.,* 1992). It may also be possible that the *Salmo trutta* alevins are capable of detoxification processes. A study looking at the effects of PAHs on tilapia found that a

detoxification process was induced and CYP1A enzyme levels increased in order to transform PAHs to metabolites, with higher water solubility, so they are more readily excreted (Nongnutch, 2011). In freshwater catfish, the principle detoxification sites of the organochlorine pesticide, endosulfan, were found to be the liver and kidney (Rao and Murty, 1980). Evidence has shown that detoxification in fish is possible which may suggest that this could eliminate the possibility of observing any potential effects of the contaminants. It could suggest the reason why few significant acute effects were seen in this research. However this study focused on contaminant exposure of eggs up to the hatching stage and the full impacts of this on future development were not assessed. It is therefore possible that the potential toxic effects of environmental concentrations of individual pollutants were not accurately demonstrated as they may be seen in future life stages.

4.4.2. Year 2 Trial

The range of waterborne contaminants used for the trial in year two was increased in order to more closely represent the range of pollutants found in the environment. Previous research has shown that a mix of contaminants can be more acutely toxic to fish eggs than independently tested compounds at similar nominal concentrations (Rhodes *et al.,* 2005). Also compounds are rarely applied alone, especially in agriculture, rather they are used with other contaminants that may have their own effects and that may interact in various ways with each other. Hayes et al. (2006) found that mortality in *Rana pipiens* and *Xenopus laevis* was significantly greater when exposed to a mixture of contaminant compounds rather than a single compound.

In this study the morphological characteristics of the eggs were examined. The results revealed that both egg diameter and SA:Vol were affected by contaminant exposure. The egg diameters of the contaminant exposed eggs were significantly larger than all of the control treatment eggs, with the exception of the OCP contaminant group. A larger egg size has been suggested to be a weakness in the natural environment, as it thought that they are at a selective disadvantage under hypoxic conditions (Rombough, 2007). Larger eggs have also been shown to emerge later as fry than smaller eggs and at an earlier developmental stage (Rollinson and Hutchings, 2010). Mix treatment eggs had a higher

protein concentration compared to the control eggs. Protein is broken down in to free amino acids during development (Srivastava *et al.*, 1994), and this may suggest that the metabolism of the mix exposed eggs has been reduced. A reduced metabolism may effect the development of the egg, although the size (diameter) of the brown trout eggs in the contaminant exposed eggs were greater than both the controls. It is unclear why the eggs in the contaminant groups were larger than in the control group. Another explanation is that the contaminants could affect the ion regulation across the boundary of the egg, causing the egg to accumulate water and expand. However, this theory would need further investigation.

The SA:Vol was calculated using egg weight (Fleming and NG, 1987; Einum *et al*., 2002). Where only one measurement of diameter was recorded, egg weight was a more consistent and accurate way of calculating the SA:Vol than the alternative calculation method using egg radius (Bonislawka and Winnicki, 2000). Contaminant exposed eggs were found to have a significantly lower SA:Vol compared to control treatment eggs, with the exception of the OCP exposed eggs. However, the SA:Vol values recorded were similar to previous studies for *Salmo trutta* (Imanpoor *et al.*, 2009). A smaller SA:Vol is considered to generate a less effective exchange with substances in the water. This could be an advantage over eggs with a greater SA:Vol as a small SA:Vol is thought to protect the developing egg from contaminated water (Finn, 2007). It is possible that the eggs could react developmentally to the surroundings and that the contaminant exposed eggs displayed a smaller SA:Vol as a result of the presence of pollutants in the water in order to minimise the impact of the exposure.

When recording both egg diameter and SA:Vol it was noted that all contaminants affected egg morphology except OCPs. It is not clear why the OCPs had no effect on the developing eggs, when other contaminants did. Again, it is possible that the effects of OCP exposure could be more evident in the latter stages of development and may not affect the egg stage in the same way as the other contaminants examined. It is also possible that OCPs are far more toxic when mixed with other pollutants, as suggested by Rhodes *et al.* (2005).

Alevin weight on hatch was also examined. It was found that weight on hatch was significantly higher for the acid herbicide exposed alevins compared to the control treatment alevins. However, there was no significant difference found in the body weight or yolk sac weight, as a percentage of total weight. It is not possible, therefore, to conclude whether the higher weight in alevins exposed to acid herbicides was a result of reduced yolk sac absorption. No yolk sac oedemas were observed in any contaminant exposed alevins during the trial. It can be concluded from this that the increased weight on hatch was not a result of a malformation. As the alevin length on hatch also failed to show a significant difference it can be assumed that the overall size of the hatchlings did not vary as a result of exposure to contaminants.

Contaminants may also affect the actual fertilization success of egg and sperm during spawning in contaminated waters (Hall *et al*., 1984). It is possible that, as with the year 1 trial, the use of eye pigmented eggs for the exposure experiments prevented the contaminants fully affecting early development of brown trout eggs.

This research and the majority of other research on the early life stages of fish rely on monitoring the effects of contaminants on eggs which have already been fertilised and reached the eye pigmented stage of development (Heintz *et al.,* 1998; Petersen & Kristensen, 1998; Wiegand *et al*., 2000; Wiegand *et al.,* 2001). Despite the fertilisation process of salmonids taking a relatively short period of time it is this phase may still be the main route of contaminantion. Few studies have exposed salmonids to contaminants from the fertilisation stage. Moore and Waring (2001) looked at the effects of cypermethrin on the survival of *Salmo salar* and found that fertilisation success was reduced significantly in eggs which had been exposed to cypermethrin during the fertilisation process. Another study found that cypermethrin and diazinon exposure during fertilisation reduced both the total number of *Salmo salar* fry that emerged, as well as the timing of emergence (Lower and Moore, 2003). However, there are few other studies which have exposed freshwater fish to environmental levels of organic pollutants from the fertilisation stage. The permeability of fish eggs is relatively high before laying and during the process of 'water-hardening' (Potts & Rudy, 1968). Water hardening occurs during the first few minutes post-fertilisation and is quickly followed by the formation of a perivitelline fluid beneath the chorion by the uptake of water (Potts & Rudy, 1968). The egg is then hardened and the

permeability to water is reduced (Potts & Rudy, 1968). Therefore, it is possible that the permeability to waterborne contaminants also declines. Moreover, during the formation of the perivitelline fluid, Atlantic salmon eggs have been found to increase by about 16% of their initial weight in the first two hours (Potts & Rudy, 1969). If the 'water hardening' stage of development was to occur in contaminated water, then these contaminants could enter the egg along with the imbibed water (Rosenthal & Alderdice, 1976).

4.4.3. Year 3 Trial

The previous trials revealed few significant effects of environmentally relevant levels of contaminants on the early life stages of salmonids. It has been suggested that a potential reason for non-significant and inconclusive data from the previous experimental trials was the use of eye pigmented eggs, rather than from eggs that had been fertilisated in contaminated water. The final year trial used green eggs and milt and fertilisation of the gametes occurred in water containing the contaminants. Some research has found that when broadcast spawners, such as invertebrates, have a low sperm concentration in conjunction with toxicant exposure a large reduction in fertilisation is observed (Marshall, 2006). However salmonid milt contains an extremely large number of sperm per unit of milt, approximately 107 sperm cells per 1µl milt (Bouk and Jacobson, 1976) and high fertilisation is consistently achieved after only 5-10 seconds of exposure (Hoysak and liley, 2001). Although it is important that the sperm do not come in contact with water prior to fertilisation, as the sperm are motionless until this stage and will only remain motile for 30 seconds following the addition of water (Billard, 1992; Hoysak and Liley, 2001). Therefore, the standard salmonid egg fertilisation procedure used for this trial should not have affected the fertilisation success which is also highlighted by the resuts from this trial.

Although, as a comparison, the final year trial also exposured eyed eggs to contaminants, but prolonged the exposure time to the alevins once hatched.

4.4.3.1. 48hr Exposure Trial

This initial trial was conducted in order to observe the fertilisation success of eggs which had been fertilised in contaminated waters. Fertilisation was measured using a positive or

negative scoring system following microscopic evaluation of the blastodisc and chorion development. From this data a total fertilisation success percentage was calculated. Overall, all eggs fertilised in contaminated water and AFW (control), fertilised successfully (100%). However, eggs fertilised in triazine (Low) and mix (High) plus ammonia contaminated water both had a 95.83% success rate. Previous research supports the findings that contaminants have no effect on fertilisation success. A study by Holth *et al.* (2008) found that zebrafish displayed no differences in fertilisation success when exposed to PAH. It is difficult to conclude that the unfertilised eggs were a result of the contaminated water as there is a naturally high mortality rate of salmonid embryos without the added effects of pollutants (Weis & Weis 1989).

Morphometric measurements showed that the acid herbicides and triazine exposed eggs had significant effects on the diameter and SA:Vol of salmonid eggs. Eggs exposed to acid herbicides and triazines both had a significantly smaller diameter than the control treatment eggs. The smaller eggs may show decreased development as a result of the pollutant. It is possible that the metabolism is increased in order to excrete the contaminants. If this is the case then less energy would be used for growth.

Acid herbicide and triazine exposed eggs had a significantly larger SA:Vol compared to control treatment eggs. Larger SA:Vol could indicate a more effective exchange of substances in the environment, including any contaminants present in the water. Finn (2007) suggested that a reduced SA:Vol may be a mechanism to protect a developing egg from a high intake of contaminated water. The fact that the eggs in this study were found to be larger when exposed to contaminants could suggest that the eggs are not adapted to cope with the sub-optimal water and may be affected by exposure to certain contaminants. However the eggs exposed to the mix of contaminants did not demonstrate the same enlarged SA:Vol which could indicate that the different groups of compounds are reacting together which may influence the overall effect of the individual contaminant groups.

4.4.3.2. Green egg experiment

The second experiment for this trial exposed brown trout eggs to contaminants from fertilisation. This was to ascertain whether earlier exposure to pollutants would initiate a

greater toxicity compared to previous trials where exposure occurred following the eyed stage.

As with the 48hr exposure, the eggs exposed to acid herbicides and triazine had an impact on the diameter and SA:Vol of the eggs. Eggs exposed to acid herbicides and triazines were found to be significantly smaller in diameter compared to the control treatment eggs. It is possible that the presence of the contaminant may affect the growth of the exposed eggs, which may also affect metabolism. Two seperate experiments have shown that contaminants (acid herbicides and triazines) cause brown trout egg size to be reduced and this may be due to toxicity exposure. It is also possible that the smaller eggs of the contaminant exposed groups may be developing more slowly than the larger control eggs. However, there were no significant differences in the egg metabolism of acid herbicide and triazine exposed eggs compared to the control. The mix, OCP, PAH and mix plus ammonia exposed eggs had a significantly greater NPS concentration compared to both solvent and non-solvent control treatment eggs. As NPS concentrations are used as a proxy of free amino acids, the results provide an indication of egg metabolism (Srivastava *et al.*, 1994). As the protein is broken down in the developing egg, more free amino acids are released (Srivastava *et al.*, 1994). Therefore, it is possible that mix, OCP, PAH and mix plus ammonia contaminants are having a negative impact on the developing egg embryo metabolism.

Acid Herbicide and triazine exposed eggs had a significantly larger SA:Vol compared to the non-solvent control treatment. Finn (2007) suggested that a reduced SA:Vol protects developing eggs from external waterborne contaminants. The surface area to volume ratio was greater for acid herbicide and triazine exposed eggs for the 48hr exposure trial and at day 30 post fertilisation in this trial. Although the eggs exposed to these contaminants were smaller in size, the increased SA:Vol would potentially allow a more effective exchange of contaminants across the chorion as the egg develops. Other studies have found that atrazine can penetrate the chorion of zebrafish eggs (Wiegand *et al*., 2000; Mukhi, 2005). It is possible that acid herbicides and triazines affect some aspects of morphology of brown trout eggs during the early life stages. Other research on aquatic organisms has also shown triazines to cause a reduction in the growth, resulting in lower survival and fecundity (Larson *et al*., 1998). PAHs have been found to accumulate more rapidly in smaller eggs as

the absolute uptake rate of the contaminant has been shown to be directly proportional with the surface area to volume ratio (Heintz *et al.*, 1999).

Alevins measured on hatching showed no significant morphometric variations between the different treatments. However, results for alevins measured at Day 93 post fertilisation did display significant differences. PAH (High) and triazine (High) exposed alevins weighed significantly more than control treatment alevins. These findings could suggest that the contaminant exposed alevins may have experienced more water ingress during development, resulting in a greater weight than the control treatment alevins. Moreover, yolk sac oedemas were observed in some PAH and triazine exposed alevins. However, acid herbicides (Low) and triazine (High) exposed alevins were significantly shorter than control treatment alevins. This corroborates with the egg size data, as the acid herbicide and triazine exposed eggs were sinificantly smaller than the controls. It may be possible that the alevin stage of development may be more sensitive than the egg stage. Enzymes and receptors that allow contaminants to affect the alevins may not be present in the egg stage (Viant *et al*., 2006). The egg metabolic system may not be sufficiently developed to metabolise any contaminants, therefore, effects of any pollutant may not be observed until a later development stage (Oliva *et al.,* 2008). However, as the eggs and alevins are smaller in size compared to the controls, it could be suggested that both acid herbicides and triazines affect the developmental growth of brown trout during early life stages. Luckenbach (2003) found higher mortality, lower hatching success and lower growth values in brown trout exposed to a polluted stream compared to a non-polluted stream. Although the mortality and hatching was not significantly affected by contaminants in this trial, reduced growth rates were noted for some pollutants. It has also been suggested that difference sizes in hatching become more pronounced at a later stage of development (Debowski *et al.,* 2006) and could influence migration, and therefore recruitment of smaller fish. Results from the body weight and yolk sac weight data could support the earlier hypothesis that some contaminant exposed alevins may have allowed a greater permeability of water into the yolk sac. Triazine and ammonia exposed alevins did have a significantly lower body weight (and therefore lower yolk sac weight) than control treatment alevins. Visual observations of alevins exposed to PAH (Low and High), triazine (Low) and mix (Low) plus ammonia had yolk sac oedemas. These results could impact on the alevin weight, as a PAH exposed alevins were significantly heavier than the control

treatment alevins. Other research has shown the potential of exposure to PAHs to induce yolk sac oedemas (Incardona *et al.*, 2004).

4.4.3.3. Alevin Exposure Experiment

Alevins were exposed to environmentally relevant levels of pollutants and the effects observed from hatching to determine if exposure from a later stage of development showed different signs of toxicity. However, few significant differences were apparent in this experiment.

The lengths of the mix (Low) plus ammonia exposed alevins at day 18 were found to be significantly smaller than alevin lengths for the solvent control treatment. In common with the results from the green egg experiment, some of the contaminant exposed alevins (triazine and mix (Low) plus ammonia exposed alevins) had significantly lower body weights than the non-solvent control treatment alevins. However, OCP exposed alevins were significantly longer than non-solvent control treatment alevins at day 38. Mix (Low) plus ammonia exposed alevins were significantly shorter than non-solvent control treatment alevins. For both sample days mix (Low) plus ammonia exposed alevins were shorter than non-solvent control alevins. The mix (Low) plus ammonia exposed alevins were also significantly lighter than non-solvent control treatment alevins. It is therefore possible that from a later stage of early development, exposure to pollutants does not greatly affect morphology of the brown trout. However, some PAH, OCP and triazine exposed alevins did display very large yolk sac oedemas, whereas none were observed in either control group. The increased surface area of the alevins may have resulted in a greater ingress of water into the yolk sac (Steele *et al.*, 2001).

4.4.4. Conclusion

Various effects of different contaminants at both low and high environmentally relevant concentration levels on the very early life stages of brown trout have been observed. This study has investigated the effects of water-borne contaminants on the fertilisation, egg development, hatching and alevin development of the brown trout. However, few significant results were observed and it is therefore not possible to draw specific

conclusions. The results suggest that waterborne contaminants at environmental concentrations found in the River Avon catchment do not significantly effect the survival or development of salmonids when in isolation. There are several possible reasons why particular visual, morphometric and chemical reactions were not observed as a result of contaminant exposure. There are potentially synergistic and antagonistic effects of the contaminants which could affect the toxic ability of compounds (Rhodes *et al*., 2005). Ammonia has been found to cause both synergistic and antagonistic results when combined with PAH mixes (Luckenbach *et al*., 2003). The toxicology reaction of contaminants when combined may not yet be known. Other factors, such as sediment-bound contaminants, siltation and nutrient loading may all contribute to the high mortalities which were observed during the field trials (Chapter 3). It is also possible that there may be no acute effects of waterborne contaminants and it is necessary to research sub-lethal effects in order to understand potential risks.

There has been contradicting research on toxicity of freshwater organisms exposed to contaminants. Wan *et al.* (2006) found that triazines were not as acutely toxic to salmonids as previously suggested by Solomon *et al.* (1996). However, organisms may survive exposure to contaminants without displaying any visible effects. They may suffer in other ways, such as disadvantages in intraspecific competition (Wiegand *et al*., 2000) or other sub-lethal effects (Wiegand *et al*., 2001).

It is also suggested that sub-lethal effects may be more important than lethal effects (Paine and Leggett, 1992). Disruption of a few cells in the early life stages may remain undetected, so would be observed as undergoing normal development and may even hatch successfully. However, eventually, such cellular disruption could prevent normal functionality of the organism at a later stage of development, thereby affecting their ability to catch prey and avoid predators (Paine and Leggett, 1992; Moles *et al*., 1981). This may also lead to poor spawning and recruitment (Moles *et al*., 1981). A sub-lethal effect may cause disruption of the functional biochemistry of a cell or a group of cells at an embryonic level, which may be detrimental to the efficiency of a tissue or an organ at a later developmental stage (Rosenthal & Alderdice, 1976).

Further examination of the contaminant exposed eggs and alevins on a cellular level could be carried out. Analysis of DNA damage, enzyme activity and function, as well as exposure to known toxic levels of the contaminants, would improve the understanding of the effects of contaminants on brown trout and other salmonids. As many studies suggest, effects of contaminants may not be observed until much later stages of development. Therefore, conducting similar experiments, but allowing development to the juvenile stages, and possibly even adult stages, would allow a better analysis of the effects of contaminant exposure on the recruitment and population structure of brown trout. The next chapter will examine the effects of sediment-bound contaminants at environmentally relevant concentrations.