5. Chapter Five: Sediment Exposure Trials

5.1. Introduction

The decline in wild salmonid stocks of has been attributed to a variety of factors operating in the freshwater environment (Wood and Armitage, 1997; Greig *et al*., 2003; Lawson *et al*., 2004). The presence of contaminants in biological sensitive periods of the life cycle, spawning and embryonic development, could have a negative impact on future reproduction and recruitment. Much literature can be found on water-borne pesticides and other water-borne contaminants affecting salmonids (Petty *et al*., 1995; Waring and Moore, 1997; Moore and Waring, 2001). However, little research has been conducted on the contaminants which bind to the river bed sediments and the effect of these sediment-bound contaminants on salmonids. Sediments act as secondary contamination sources for organic pollutants, making it one of the principal reservoirs of environmental pollutants. Sediment represents a source from which residues can be gradually released into the groundwater and living organisms (Vagi *et al.*, 2007). Therefore sediment is an important factor when observing survival of early developmental stages of salmonids and when considering the contaminants present in salmonid spawning gravel.

5.1.1. Sedimentation in spawning gravels

Research has indicated that the sedimentation of salmonid spawning gravels may reduce the survival of embryos and alevins, and therefore the subsequent production of juvenile populations in rivers and streams (Hicks *et al*., 1991; Armstrong *et al*., 2003; Curry and MacNeill, 2004; Kemp *et al*., 2011). Reduced water flow and oxygen levels are believed to be the main reasons for this reduced survival within spawning gravels (Hicks *et al*., 1991; Wu, 2000; Ortlepp and Mürle, 2003). The delivery of fine sediment as a result of agriculture can hold chemicals such as pesticides and herbicides as well as sediment-bound nutrients (Hancock, 2002; Greig *et al*., 2005b; Kemp *et al*., 2011). Rivers within agricultural catchment areas are at high risk of pesticide inputs in both aqueous and sediment-bound forms. Field runoff has been established as one of the major routes of entry for pesticides into freshwater systems, especially following a storm event (Wheeldon,

2003). Some evidence has shown that pesticides derived from intensive agriculture may be adsorbed onto sediments and fine particulate matter which are deposited within the spawning gravels (Taylor *et al*., 2008). Sediment-bound pesticides may further reduce the survival of the intragravel stages of salmonids. Levels of other contaminants, such as hydrocarbons, have also been identified from samples of river sediments (Evans *et al*., 1990b; Liess *et al*., 1999).

Salmonid redds are built at the upstream limit of riffles with an area of a few square metres (Hobbs, 1937). They begin as a pocket, from which the female removes fines and small gravels (Chapman, 1988). The female fish removes a large proportion of fine particles (silt and sand) to increase both the gravel porosity and permeability (McNeil and Ahnell, 1964) and to favour the flow of oxygenated water within the redd (Guerrin *et al.*, 2001a). The 'dome' shape of redds also favours the intragravel water flow (Guerrin *et al*., 2000). Fine particles, which fill interstices of the spawning gravel reduce the intragravel flow velocity and consequently decrease the amount of dissolved oxygen supplied to the eggs and alevins (Guerrin *et al.*, 2001a). A study on Chum salmon showed that the female salmon did purge streambed gravels of fine sediment during redd preparation, but the egg pockets were returned to essentially pre-spawned conditions by subsequent sediment transport processes (Peterson and Quinn, 1996b). However, just prior to the deposition of the eggs, the egg pocket is always the cleanest part of the redd (White, 1942). Fine sediment is a natural component of streambeds, but land use practices can create an increase in fines (Collins *et al.*, 2009). In many catchments increasingly intensive use of land for agriculture and forestry practices has altered hydrological pathways, water quality characteristics and sediment transport (Malcom *et al.*, 2003a). Salmonid streams have been affected by channel modification for agricultural purposes. Low levels of intragravel dissolved oxygen and reduced gravel permeability have been implicated in reduced survival of eggs and alevins exposed to elevated levels of fine sediment (Malcom *et al.*, 2003b). Different authors have classified fines by various size classes. To account for all fine sediment, all classes below 0.85mm were included (Argent and Flabbe, 1999). Whereas Malcom *et al.* (2003b) and Soulsby *et al*. (2001) define fine sediments as less than 2mm. Peterson and Quinn (1996) classed fine sediment as less than 4mm for their research.

Sediment is defined as fragmental mineral material transported or deposited by water or air, and is believed to be the principle non-point pollutant from forestry and other land use activities (Argent and Flebbe, 1999). Sorption is a physicochemical process of pesticide-sediment-water interactions, correlated with the nature of the solids in the system and the characteristics of the pesticides and other contaminants. The ability of the pesticide to be adsorbed on sediments is dependent on polarity, a pesticide is adsorbed more when its polarity is low (Hutson and Roberts, 1990). Sediment with a high adsorptive capacity and a high strength of adsorption would release pesticides to the water at a slow rate, and in low amounts (Petit *et al*., 1995). This would present a smaller hazard to phytoplankton and zooplankton, but a greater threat to filter feeding organisms, which rely upon organic matter at the sediment water interface, and to those which rely upon the benthos as a food source (Petit *et al.*, 1995).

Modern streambed sediments are usually dominated by fine-grained minerals, especially clays and organic matter (Younger *et al.,* 1993). There are two hydrogeological consequences of this. Firstly, the fine grain size of the sediment gives it a low hydraulic conductivity and secondly, the high clay and organic matter content renders the sediment highly sorptive. According to Younger *et al.* (1993), several recent studies have investigated the role of the organic matter in streambed sediments in controlling sorption processes. It is suggested that the role of organic matter in streambed sediments is to control sorption processes (Younger *et al.,* 1993). Geffard *et al.* (2003) noted that sediments represent a vast sink for contaminants in aquatic systems and may pose a threat to pelagic and benthic organisms.

It is generally believed that the dissolved fraction of a pollutant, as opposed to the sediment-sorbed fraction, is much more available to micro-organisms and is therefore degraded rapidly. A study in the Netherlands by Vink and Van der Zee (1997) showed the behaviour of individual pesticides may indicate that the micro-organisms able to transform the individual pesticides differ in types. Following their findings, it is plausible that the presence and activity of micro-organisms tailored to specific pesticide, directly determine the pesticide's fate, but only when environmental conditions for the entire bacterial consortium are favourable.

Adsorption of chemicals onto sediment particles is an important process through which many contaminants are removed from the water column. Pollutants stored in sediments may be easily remobilised by sudden increases of river flow and consequently increase the toxicant concentrations (Bosch *et al.*, 2009). A study in Germany found that total pesticide loads are a better indicator of pesticide contamination than water concentrations especially if they are expressed as a fraction of the pesticide applied (Müller *et al.*, 2002).

The bioavailability of contaminants depends on several factors; physical (grain size of sediment and suspended particulate matter), chemical (solubility, reactivity or compounds, complexing agents), and biological (benthic or pelagic organisms, mode of exposure) (Geffard *et al.*, 2003). It has been suggested that PAHs associated with the fine sediment (clay and silt) are the most mobile and biodegradable and therefore are of the greatest concern (Talley *et al.*, 2002).

Polycyclic aromatic hydrocarbons (PAHs) and Organochlorine pesticides (OCPs), most of which are categorised as persistent organic pollutants (POPs), are of environment significance due to their widespread distribution and potential toxicity to organisms. PAHs mainly originate from anthropogenic sources and many of the PAHs with four or more rings are carcinogenic and mutagenic because of their metabolic transformation capability (Kuang and Xu, 2009). Concentrations of total PAHs (t-PAHs; sum of the 2-6 ring PAHs) in sediments of the northern South China Sea varied from 138 to 498ng/g with an average of 286ng/g (Chen, *et al*., 2003). Concentrations of total hexachlorocyclohexane (HCH)s (t-HCH; sum of α-, β-, γ-, δ-HCH isomers) ranged from 0.08ng/g to 1.38ng/g with a mean value of 0.36ng/g. The levels of total dichlorodiphenyltrichloroethanes (DDTs) (t-DDT; sum of *p,p'*-DDD, -DDE and –DDT) ranged from 0.04ng/g to 2.48ng/g with an average of 0.87ng/g (Chen *et al.*, 2006).

5.1.2. Organochlorine Pesticides

Increased sediment and chemical contaminant loads in run-off water as a consequence of intensive agricultural land-use are major areas of concern. Cavanagh *et al.* (1999) looked at

OCP residues in soils and sediments in rivers catchments of Australia. There was an absence of detectable concentrations of OCPs from historic agricultural activities in the catchments and no literature currently exists on the presence of OCPs from the River Avon. However, as further expansion of agricultural activities occurs it becomes more critical to understand the factors influencing both the transport of sediment and sediment-bound contaminants (Bhattacharya *et al*., 2003). This increase in sediment loading can potentially impact the level of OCPs entering freshwater systems.

A study looking at the contamination of bottom sediments in Swedish rural ponds found concentrations of an OCP (DDT) at all the test sites. DDT was withdrawn from the Swedish market in 1970 and totally banned in 1975 (Kreuger *et al.*, 1999). Frank *et al.* (1990) found higher concentrations of pesticide residues in sediment than those detected in water. However, most pesticides will leave the field in a dissolved form and only those with very low water solubilities or strong ion-exchange properties would adsorb to suspended solid particles (Larson *et al.*, 1997). Kreuger *et al.* (1999) demonstrated the importance of pesticide distribution between matrices as pesticides detected at the highest level in sediment samples were either not detected or only detected at low concentrations in water samples collected during the same period. However, a study in Germany looked at sediment-bound contaminants in small streams and suggested that sediment should not be seen as a pollutant accumulator in order to provide information about long-term contaminant trends (Honnen *et al.*, 2001). The study found that sampling during a low water period led to high pollutant concentrations in sediment and sampling the same site at high water conditions the concentrations in the sediment dropped significantly. Therefore it is important to consider the timing and conditions during sampling. A study on streambed sediments in New Jersey (Stackelberg, 1997) found that higher concentrations of chlorinated organic compounds were found widespread in drainage areas of various land use patterns. The most widely distributed compounds were DDT and its metabolites DDE and DDD, reflecting the historic distribution of these compounds.

Organic matter, which influences microbial activities and variation, play a most important role in accumulation, enrichment or degradation to OCPs. Xue *et al.* (2006) found that the concentration of OCPs in sediment was much higher than those in surface water in Guanting reservoir (Beijing, China). Among the analysed OCPs, *p,p'*-DDE, δ-HCH, aldrin,

γ-HCH and β–HCH were the most abundant compounds in water while *p,p'*-DDE, o,*p'*-DDT, β–HCH, δ-HCH, *p,p'*-DDT and aldrin accounted for 85% of total OCPs in sediment. A study looking at sediments in sewer system (Hanoi, Vietnam) found β–HCH was the only isomer (among three investigated HCH isomers) detected in quantifiable amounts in five of the 22 samples (Hoai *et al.*, 2010). The sediment concentrations of β–HCH determined in this study were <0.2-36ng g^{-1} (mean 3.8ng g^{-1}).

Some studies have shown the ability of contaminants to accumulate in aquatic organisms. Bioaccumulation studies on benthic animals (Knezovich and Harrison, 1988) have identified three potential pathways of chemical transfer; a) pore water, b) ingested sediment, c) direct contact with sediment particles. McHugh *et al.* (2010) looked at levels of OCPs in a diadromous species, glass eels (*Anguilla anguilla*). OCPs were measured in eel samples and overall levels were low. Extractable lipid levels were lower in the two yellow eel samples compared to those in the silver eels. POP levels, including some OCPs, observed in eel muscle from this study were relatively low.

5.1.3. Polycyclic Aromatic Hydrocarbons

PAHs are organic chemicals composed of fused benzene rings, whose environmental behaviour has been investigated for more than 20 years. The widespread occurrence of PAHs is largely due to their formation and release during the incomplete combustion of coal, oil, petrol and wood (Wild and Jones, 1995). Recent evidence suggests that petroleum hydrocarbons from oil spills can persist in sediment for decades longer than previously thought (Incardona *et al*., 2004). PAHs can be metabolically transformed by aquatic and terrestrial organisms into mutagenic, carcinogenic and teratogenic organisms such as dihydrodiol epoxides (Harrad, 2001). These metabolites bind to and disrupt DNA and RNA, which is the basis for tumour formation (Harrad, 2001). It is expected that PAHs rapidly transfer from the water column to the sediments. Most PAHs in water are associated with the particulate phase (Wild and Jones, 1995). It has been suggested that PAHs associated with the fines (silt/clay fraction) are much more mobile and biodegradable than other particle fractions and therefore are potentially the greatest concern for the environment (Talley *et al.*, 2002).

Urban storm runoff has been widely considered a large source of pollutants discharged into receiving waters. A study looking at spatial variation of runoff toxicity showed a consisitent magnitude of PAH toxicity at field site despite the difference in traffic volume. They concluded that dust particle which accumulated on the urban roads had attributed to this feature (Zhang *et al.,* 2011). Many studies have demonstrated the toxicity effects of free PAHs. Incardona *et al*. (2004) observed morphological malformations, yolk-sac oedemas and lordosis (spinal curvature) in zebrafish larvae which had been exposed to PAHs. Similar effects were noted by Carls *et al*. (1999) and Heintz *et al*. (1999) following the Exxon Valdez spillage, resulting in PAH exposure to aquatic organisms.

Since sediments are sinks and reservoirs of genotoxic PAHs, natural and anthropogenic perturbations may re-dissolve or re-suspend sediment-bound PAHs, and cause continual, long-term exposure to salmon and other aquatic organisms that inhabitat or migrate through these contaminated freshwater, estuarine and marine ecosystems. Results from a study conducted by Barbee *et al.* (2008) showed that juvenile salmon exposed to PAHs for relatively brief residence times (several weeks) increased induction of cytochrome P450 enzymes and associated damage to hepatic DNA, as evidenced by increased levels of xenobiotic-induced DNA adducts. The study suggested the potential exists for an incremental effect of increased exposure to anthropogenic chemicals on the physiological fitness of juvenile salmon as they migrate through contaminated environments. Although genotoxicity was correlated with PAH concentrations in the sediment, it was not correlated with PAH levels in the water column (Barbee *et al.*, 2008).

Research by Benedetti *et al.* (2007) investigating the Antarctic fish (*Trematomus bernacchii*) found significant interactions between metabolism of trace metals and aromatic xenobiotics (PAHs). They suggest that these interactions have implications for monitoring both bioaccumulation and biological effects in key sentinel species. In this study all metals influenced the metabolism of Benzo[*a*]pyrene (BaP). However, different metals were found to affect the rate of bioaccumulation and biotransformation rates of BaP (Benedetti *et al.*, 2007).

A study was carried out on concentrations of a range of PAHs in feral finfish (herring, sand lance and capelin; yellowtail flounder and American plaice) from the Northwest Atlantic

(Hellou *et al.*, 2006). It was noted that when concentrations of PAHs in the internal organs and carcasses of some finfish are lipid normalised then a certain level of food biomagnification is observed. In finfish species PAHs tend to be present at higher concentrations in smaller fish, although an exception is observed with herring (Hellou *et al.*, 2006). Many chemicals can reach higher concentrations in bottom sediment than in the upper water column. Therefore, all trophic levels within a food chain can be affected by the input of sediment-bound contaminants present in the substrate. This makes sediments a potential long term source of toxic substances which, when sorbed to sediment particles or in solution in pore water, can be the main source of threat to benthic organisms and, potentially, to the entire aquatic community (Vigano *et al.*, 1998).

When considering bioaccumulation, a study looking at phenanthrene in *Carassius auratus* concluded that bioaccumulation of this PAH was significantly affected by a fast metabolism clearance. The results from the research suggested that fish may be able to metabolise the phenanthrene (Sun *et al.*, 2006).

A study looking at the settlement behaviour of polychaetes found that no acute significant effects were observed in the PAH contaminated sediment compared to the controls. However despite the polychaete (*Streblospio benedicti*) tolerance of PAHs in terms of larval settlement and juvenile metamorphosis stage, longer term effects on subsequent growth and reproduction are still poorly known (Chandler *et al.*, 1997).

The condition factor, based on the length-weight relationship, is often used to express the overall wellbeing of a fish and some literature has suggested that the condition factor is a useful tool to assess the effect of pollution on fish when variation in other environmental factors is limited (Bervoets and Blust, 2003). For example, the PAH levels in the liver of brown trout (*Salmo trutta*) were examined in fish from high mountain lakes across Europe and Greenland (Vives *et al.*, 2004). It was observed that condition factor and liver concentration were inversely correlated. Therefore it is possible that contaminant exposure may reduce the condition factor in fish (Feist *et al.*, 2005).

5.1.4. Triazine Pesticides

The affinity of triazine herbicides to bind to sediments is still not clearly understood. Triazines are known to be more water soluble than some other contaminants, such as PAHs, however previous studies have recorded levels of triazines in sediment samples. A study on the Humber River detected agricultural herbicides, such as atrazine and propazine, within sediment (Long *et al*., 1998). Triazines are suggested to have high water solubility and to have only a moderate ability to adsorb onto soil, however Meakins *et al.* (1995) found triazine compounds in the sediments associated with saltmarshes. Moreover, Dagnac *et al.* (2005) did find atrazine in some dried agricultural soils in the Paris Basin. Other studies have found low concentrations of sediment-bound triazines in soil samples (Kulikova and Perminova, 2002) as well as sediment samples (Ahel *et al*., 1992; Mersie *et al*., 1998). So it is possible that the sediment-bound triazines will not persist in freshwater sediments due to high water solubilities, therefore concentrations may be expected to be low or not detected.

5.1.5. Fluctuating Asymmetry

Fluctuating asymmetry is a pattern of developmental instability caused by perturbations during development, giving rise to an imprecise expression of the developmental design (Waddington, 1942; Zakharov, 1992). Fluctuating Asymmetry (FA) is the random deviation from bilateral symmetry in meristic traits (Allenbach *et al.*, 1999), for which differences between the right and left sides are normally distributed around a mean of zero in a population (Mazzi & Bakker, 2001). Meristic traits are fixed at an early life stage and are therefore insensitive to environmental conditions later on in life (Kristoffersen and Magoulas, 2009). FA can be caused by environmental or genetic disturbances during early embryonic development (Østbye *et al.*, 1997). Environmental stressors that may induce FA usually include temperature extremes, oxygen and nutrition deficiency, larval density and chemical pollution. Insecticide exposure and temperature were observed to affect FA of damselflies (Chang *et al.*, 2007). If stress factors are present the regulation of normal ontogenetic developmental processes will be reduced, causing increased FA levels (Østbye *et al.*, 1997). A study demonstrated a change in normal developmental processes of meristic traits in wild *Salmo salar* as a result of environmental stress (Vøllestad and

Hiindar, 1997). After transportation from the wild to a common hatchery environment the pectoral and pelvic fin ray counts were found to show fluctuating asymmetry. Previous studies have indicated that FA is increased in organisms that inhabit stressful and/or marginal environments (Allenbach *et al*., 1999). More recent research suggests that this association could enable FA to be used as a bioindicator. Allenbach *et al*. (2009) studied two fish species (*Gambusia affinis* and *Notropis ludibundus*) and showed that individuals with smaller FA survived longer when exposed to two insecticides and an OCP (Lindane).

Previous research has reported correlations between FA and fitness such as survival, fecundity, growth and reproductive success in fish (Øxnevad *et al.*, 2002). FA has been suggested as a promising method for monitoring sub-lethal levels of pollutants (Zakharov, 1992; Clarke, 1994). More specifically, Allenbach *et al*. (2009) suggested that FA could be used as an indicator of susceptibility of fish to pesticides.

5.1.6. Genotoxicity

Exposure of fish to genotoxic contaminants can be the result of digestion, through ingestion of dietary chemicals, or by the respiratory route due to absorption of waterborne chemicals through the gills or body surface (De Flora *et al.,* 1993). A sensitive marker of genotoxic damage is DNA strand breakage. Kammann *et al.*, (2001) have shown that contaminants can cause single strand DNA breaks, even if other indicators are not observed (i.e. induction of metabolic enzymes cytochrome P450 or formation of DNA ducts). DNA strand breaks are potentially pre-mutagenic lesions. One of the most commonly used techniques for detecting the breaks in single stranded DNA is the comet assay (Singh *et al*., 1988). The comet or single-cell gel electrophoresis assay allows early detection of single strand DNA breaks which can be produced by a range of genotoxic agents (Lee and Steinert, 2003). Such a technique allows for the detection of subtle changes after exposures to potentially predict any possible future damaging effects on reproduction or other important endpoints in the longer term.

A study in Southern Spain found the highest level of PAH contaminated sediments in Bay of Algeciras, which presented the highest relationship with the biological effects of the exposed organism (*Arenicola marina*). The highest area of PAH contamination correlated

with the elevation of single strand DNA breaks detected in the animals exposed to Bay of Algeciras sediments (Morales-Caselles *et al.*, 2009). Such results demonstrate that the comet assay is a sensitive tool for monitoring the genotoxic effects of PAH in marine organisms. Research by Billiard *et al*. (1999) exposed rainbow trout (*Oncorhynchus mykiss*) eggs to the PAH retene (32–320 μg l^{-1}) from the eyed egg stage to hatch (42 d) and from hatch to the onset of swim up. This caused exposure-related increases in blue sac disease posthatch. Other symptoms included yolk sac edema, subcutaneous hemorrhaging, reduced growth, and craniofacial malformations (Billiard *et al*., 1999). Couch *et al*. (1983) examined rainbow trout that were fed diets containing benzo(a)pyrene (1.006 mg kg^{-1}) for 12 months. The research showed that exposed trout developed liver tumors. Other research on rainbow trout fed on diets containing benzo(a)pyrene (1 mg kg^{-1}) for 18 months showed that 25% of the exposed fish had liver neoplasms, compared to 15% of exposed fish after 12 months with no evidence of neoplasia in controls (Hendricks *et al.,* 1985).

This chapter will look at the possible effects of environmentally relevant concentrations of sediment-bound contaminants and the effects of these pollutants on the early life stages of *Salmo trutta*. The concentrations chosen for the exposure trial reflect the average background concentrations detected in sediment samples taken from the River Avon and its tributaries (Chap. 3). The exposure trials aimed to identify potential risks of sediment-bound contaminants to salmonid eggs and alevin whilst they remain in the gravel. Biochemical parameters of the eggs will determine any metabolic differences and physiological effects were analysed. It can be expected that any effects noted during the egg stage of development may also affect the larval stage. Alevins were examined for any morphological deformities as well as physiological parameters. Genotoxicity and morphological analyses of emergenced fry was performed to distinguish whether any exposure during the very early life stages have the potential to effect the juvenile stages. Observations from this exposure trial may help to determine the potential effects of sediment-bound contaminants on the survival and development of early life stages of salmonids.

5.2. Material and Methods

5.2.1. Organism

Green brown trout diploid eggs and milt were sourced from Allenbrook Trout Farm, Dorset. The eggs and milt were transported to the CEFAS laboratory in Lowestoft, Suffolk in cool boxes on ice, where they were fertilised (as described in Chap.2) and placed in incubators. Some of the newly fertilised eggs were first placed in Netlon® egg baskets (as described in Chap.3), so that embryos and alevins could be sampled, and some were buried in gravel for emergence studies (Figure 5.1).

Figure 5.1. CEFAS, Lowestoft aquarium facilities; Incubators and Emergence Tanks.

5.2.2. Experimental Design

The incubators were dosed periodically with spiked sediment. The artificial sediment used for the experiment was purchased from Newplast Modelling Clay Ltd, Devon. The clay was incubated at 450ºC for 5 hours before use in order to rid of any organic matter. The toxicology data supplied by the company showed that there were no metal contaminants present in the clay. It was then necessary to mechanically macerate the clay with a pestle and mortar, so that it was representative of the fine fraction (<63um). The clay was used as a surrogate for fine sediments and it would only in part represent the fine sediments found

in a natural habitat. However the particle size (<63um) is representative of the size analysed for bound contaminants.

The aquarium facilities at the CEFAS Laboratories allowed for duplicate replications for the experiment. For Year 1 and Year 2 of this aspect of the research, there were six incubators, each with corresponding emergence tanks available. In Year 3 an additional four incubators and emergence tanks were installed to allow for a greater range of contaminant exposures, although still with duplicate replications.

The black incubators (Figure 5.2) in the aquarium facilities (height, 48.5cm; diameter, 66cm) were on flow-through with dechlorinated tap water that upwelled (300ml/min) from the bottom of the tank. The outflow was situated at the top of the tank and drained out into a fibreglass tanks (height=177cm; width=46cm; depth=24cm). In the bottom of the tanks a wire mesh square basket (height=10cm; width=18cm; depth=18cm) was positioned directly under the outflow of the incubators. Photoperiod was ambient and controlled by a mechanical time switch that was changed weekly to reflect natural (52˚) conditions.

Figure 5.2. Emergence tanks showing mesh box to catch the swim up fry.

River gravel (previously collected from the field sites) was pre-rinsed in freshwater several times following collection. The gravel was then thoroughly rinsed again in freshwater (from the same source as the experimental set up) to remove any residual fines before use. A mass of 60kg of the cleaned gravel was added to each incubator a week prior to the start of the sediment exposure trials. The mass of clay (representing the less than 63µm fraction) added to the incubators was equivalent to the average sediment loading data from the field work (Chap. 3). The calculated mass of artificial sediment equivalent to a monthly loading for Year 1 and Year 2 experiment and for a fortnightly loading for Year 3 was 18.62g and 9.31g of the less than 63µm sediment, respectively. This sediment was then spiked with the relevant treatment before being added to the incubators. When the sediment was added to the incubators, the outflow of each incubator was blocked using a gloved hand or bag for 10 minutes, so that the sediment had time to settle with no flow.

Different contaminants were used for each year. Tables 5.1, 5.2 and 5.3 indicate all of the compounds used for the sediment exposure trials and the individual nominal concentrations for each.

Table 5.1. Concentration of PAHs for Sediment Exposure Trials.

Compound	Concentration (ng g^{-1})	µg in 18.62g
Naphthalene	34.982	0.651
Acenaphthylene	117.806	2.193
Acenaphthene	61.484	1.144
Phenanthrene	1630.332	30.356
Anthracene	487.742	9.081
Pyrene	6955.222	129.506
Benz[a]anthracene	4366.476	81.303
Chrysene	4945.334	92.082
Benzo[b]fluoranthene	6576.088	122.446
Benzo[k]fluoranthene	2395.650	44.607
Benzo[a]pyrene	4691.132	87.349
Indeno[1,2,3-cd]pyrene	6042.632	112.514
Dibenz[a,h]anthracene	1526.226	28.418
Benzo[g,h,i]perylene	4767.324	88.768
Fluorene	105.44	1.963

Table 5.2. Concentration of OCPs for Sediment Exposure Trials.

Compound	Concentration (ng g^{-1})	µg in 18.62g
Beta-Lindane	57.47	1.070
Pentachloronitrobenzene	21.22	0.395
Gamma-Lindane	33.20	0.618
Heptochlor	85.30	1.588
Dieldrin	109.88	2.046

Table 5.3. Concentration of Triazines for Sediment Exposure Trials.

Compound	Concentration (ng g^{-1})	µg in 18.62g
Simazine	12.64	0.235
Atrazine	302.34	5.630
Propazine	14.36	0.267
Cyanazine	28.95	0.539
Prometryn	114.91	2.140

5.2.3. Contaminant Preparation

All pure compounds were purchased in their powder form from QMX Laboratories, UK. Stock solutions of the individual compounds were made up at the concentrations and volumes shown in Tables 5.4 – 5.9.

Table 5.4. Stock solution calculations and concentrations of the PAHs.

Compound	Mass/Volume of Stock	Concentration
Naphthalene	6.5 mg $10ml^{-1}$	650 µg ml^{-1}
Acenaphthylene	21.9 mg $10ml^{-1}$	2190 µg ml^{-1}
Acenaphthene	11.4 mg $10ml^{-1}$	1140 µg ml^{-1}
Phenanthrene	3.04 mg $10ml^{-1}$	303.6 µg ml^{-1}
Anthracene	9.08 mg $10ml^{-1}$	908 µg ml^{-1}
Pyrene	12.91 mg $10ml^{-1}$	1291 µg ml^{-1}
Benz[a]anthracene	8.13 mg $10ml^{-1}$	813 µg ml^{-1}
Chrysene	9.21 mg $10ml^{-1}$	921 µg ml^{-1}
Benzo[b]fluoranthene	2.44 mg $10ml^{-1}$	244 µg ml^{-1}
Benzo[k]fluoranthene	4.46 mg $10ml^{-1}$	446 µg ml^{-1}
Benzo[a]pyrene	8.73 mg $10ml^{-1}$	874 µg ml^{-1}
Indeno[1,2,3-cd]pyrene	2.25 mg $10ml^{-1}$	225 µg ml^{-1}
Dibenz[a,h]anthracene	2.84 mg $10ml^{-1}$	284 µg ml^{-1}
Benzo[g,h,i]perylene	8.88 mg $10ml^{-1}$	887 µg ml^{-1}
Fluorene	19.63 mg $10ml^{-1}$	1963 µg ml^{-1}

Table 5.5. Working solution concentrations of the PAHs.

Compound	Concentration	Volume of Stock
Naphthalene	13 µg $100ml^{-1}$	2ml
Acenaphthylene	43.8 µg $100ml^{-1}$	2ml
Acenaphthene	22.8 µg $100ml^{-1}$	2ml
Phenanthrene	607.2 µg $100ml^{-1}$	2ml
Anthracene	181.6 µg $100ml^{-1}$	2ml
Pyrene	2590.2 µg $100ml^{-1}$	2ml
Benz[a]anthracene	1626 µg $100ml^{-1}$	2ml
Chrysene	1841.6 µg $100ml^{-1}$	2ml
Benzo[b]fluoranthene	2449 µg $100ml^{-1}$	10ml
Benzo[k]fluoranthene	892.2 µg $100ml^{-1}$	2ml
Benzo[a]pyrene	1747 µg $100ml^{-1}$	2ml
Indeno[1,2,3-cd]pyrene	2250.2 µg $100ml^{-1}$	10ml
Dibenz[a,h]anthracene	568.4 µg $100ml^{-1}$	2ml
Benzo[g,h,i]perylene	1775.4 µg $100ml^{-1}$	2ml
Fluorene	3926 µg $100ml^{-1}$	2ml

Table 5.6. Stock solution calculations and concentrations of the OCPs.

Compound	Mass/Volume of Stock	Concentration
β-Lindane	10.700 mg $10ml^{-1}$	1070 µg ml-1
Pentachloronitrobenzene	3.960 mg $10ml^{-1}$	396 µg ml-1
γ-Lindane	6.180 mg $10ml^{-1}$	618 µg ml-1
Heptochlor	15.880 mg $10ml^{-1}$	1588 µg ml-1
Dieldrin	20.460 mg $10ml^{-1}$	2046 µg ml-1

Table 5.7. Working solution concentrations of the OCPs.

Compound	Concentration	Volume of Stock
β-Lindane	535 µg $100ml^{-1}$	500µl
Pentachloronitrobenzene	198 µg $100ml^{-1}$	500µl
γ-Lindane	309 µg $100ml^{-1}$	500µl
Heptochlor	794 µg $100ml^{-1}$	500µl
Dieldrin	1023 µg $100ml^{-1}$	500µl

Table 5.8. Stock solution calculations and concentrations of the Triazines (TRIs).

Compound	Mass/Volume of Stock	Concentration
Simazine	2.35 mg $10ml^{-1}$	235 µg ml^{-1}
Atrazine	58.30 mg $10ml^{-1}$	5830 µg ml^{-1}
Propazine	28.72 mg $10ml^{-1}$	2872 µg ml^{-1}
Cyanazine	5.39 mg $10ml^{-1}$	539 µg ml^{-1}
Prometryn	21.396 mg $10ml^{-1}$	2140 µg ml^{-1}

Table 5.9. Working solution concentrations of the Triazines (TRIs).

Compound	Concentration	Volume of Stock
Simazine	117.5 µg $10ml^{-1}$	500µl
Atrazine	2915 µg $10ml^{-1}$	500µl
Propazine	1436 µg $10ml^{-1}$	500µl
Cyanazine	269.5 µg $10ml^{-1}$	500µl
Prometryn	1069.8 µg $10ml^{-1}$	500µl

The volume of the stock solutions stated in Tables 5.4, 5.6, 5.8 were added to a 100ml volumetric flask and made up to the total volume with acetone. Volumes from all standard stock solutions were added together to make the mix working solution. These working solutions were then used to spike the sediment. For Year 1 and and Year 2 trials a volume of 5ml of the working solution was used to spike 18.62g of the artificial sediment before adding to the relevant incubators containing the fertilised eggs. For Year 1 the solvent control group was spiked with 5ml of acetone and added to the incubators at the same time. The control group received no treatment and 18.62g of sediment were added to the incubators simultaneously. For Year 3, 2.5ml of the working solutions were added to 9.31g of artificial sediment to spike the corresponding incubators. The control group received no treatment and 9.31g of sediment were added to the control incubators simultaneously.

5.2.4. Year 1 Experiment

For the first year of the sediment exposure experiment three treatments were used; the control; just the sediment was added to the incubators, the solvent control; acetone of the same volume used as the volume applied as a carrier solvent for the contaminant spike was added to the sediment prior to dosage, PAHs; a mix solution dissolved in acetone was added to the sediment prior to dosing (Figure 5.3). The incubators were dosed monthly.

Figure 5.3. Incubators used for sediment trials i) showing emergence tank; ii) incubator.

For this first sediment exposure trial, there were six egg boxes buried in the gravel, each containing 50 eggs. There were 150 eggs buried in the gravel for the emergence part of the study. Eggs were sampled on day 35 post-fertilisation and day 49 post-fertilisation. Two egg boxes were removed from each replicate incubation tank on each sampling day. The weight and diameter for each viable egg was measured and recorded. Volume, surface area and surface area to volume ratio were calculated using the egg weight (Chap. 2). Some eggs were fixed in FBS and some frozen (-4°C) for further analyses. Frozen eggs were used to determine the dry weight, percentage water content, protein concentration and NPS concentration, as well as chloride concentration (Chap. 2).

Alevins were sampled on day 70 post-fertilisation. Two egg boxes from each replicate incubation tank were removed for sampling. The lengths and weights were taken and recorded for all which survived. The presence of any yolk sac oedemas were also noted (Chap. 2). Alevins were then fixed in NBF or frozen (-4C) for further analyses. Percentage yolk sac and body weight were calculated, as well as dry weight and protein concentration.

Temperature, emergence and mortality were monitored daily. The swim-up fry were contained within the metal baskets, which were positioned at the end of the outflow in the emergence tank. Baskets were checked twice daily and numbers were counted and recorded. However, no fish were found to emerge during the day (between 8am and 5pm) so all data can be considered as night emergence (Crisp, 1991; Crisp and Hurley, 1991; Johnstone, 1997).

Once counted the fry were released from the mesh box into the emergence tank until sampling. The fry were terminated on day 98 post-fertilisation using a Schedule One method (using 2-phenoxyethanol and destruction of the brain) and individual fish were weighed and lengths measured. From the results the Condition Factor (*K*) was calculated to assess the overall condition of the fry from each treatment group. Fulton's Condition factor;

$$K = \frac{W}{L^3}$$

Where; W is weight (g) and L is length (mm).

Adult trout change in 'fatness' during the year, which can be measured by changes in the condition factor (K) (Frost and Brown, 1967). The condition factor represents whether the brown trout fry sample is too fat or too thin in relation to the body proportions meaning whether the fish is heavier or lighter than the average fish of that length. The average trout has K that is equal to 1, if K is less than 1 the fish is considered to be in poor condition and so weighs less than expected i.e. long and thin, and if K is more than 1 then it weighs more than is expected (fat).

5.2.5. Year 2 Experiment

For the second year of the sediment exposure experiment three treatments were used; the control; just the sediment was added to the incubators; PAHs, a mix solution dissolved in acetone was added to the sediment prior to dosing; Pesticides plus PAHs, a mix solution dissolved in acetone consisting of OCPs and PAHs. No solvent control was used for this trial as few differences were observed between the control and the solvent control in the previous trial. The incubators were dosed monthly.

For the second trial, four egg boxes were buried into the gravel, each containing 50 egg. For the emergence study, 300 eggs were buried directly into the gravel. Eggs were sampled on day 47 post-fertilisation. Two egg boxes were removed from each replicate incubation tank on the sampling day. The weight and diameter for each viable egg was measured and

recorded. Volume, surface area and surface area to volume ratio were calculated using the egg weight. Some eggs were fixed in NBF and some frozen (-4°C) for further analyses. Frozen eggs were used to determine the dry weight, percentage water content, protein concentration and NPS concentration, as well as chloride concentration (Chap. 2).

Alevins were sampled on day 68 post-fertilisation. Two egg boxes from each replicate incubation tank were removed for sampling. The lengths and weights were taken and recorded for all which survived. The condition factor (*K*) was calculated from the information. Alevins were then either fixed in NBF or frozen (-4°C) for further analysis. Percentage body and yolk sac measurements, protein concentration and dry weight were analysed at a later stage. Alevins fixed in NBF were analysed for fluctuating asymmetry (refer to section 5.2.5.1).

The swim-up fry were counted and recorded as soon as they reached the mesh box in the emergence tank. Once counted the fry were released from the mesh box into the emergence tank until sampling. The fry were terminated on day 134 post-fertilisation using a Schedule One method and individual fish were weighed and lengths measured. From the results the Condition Factor was calculated to assess the overall condition of the fry from each treatment group.

Fry body depth was measured, with the standard point of measurement just in front of the dorsal fin (Figure 5.4).

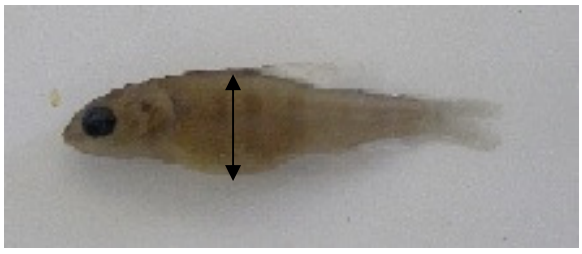

Figure 5.4. Photograph of a brown trout sample depicting the point of measurement for body depth.

The yolk sac of each sample was scored on a scale of 0-5, 0 being no yolk sac and 5 being the largest amount of yolk sac present (Figure 5.5).

Yolk Sac Score: 0

No yolk sac is present, emergence occurred after depletion of the reserves.

Yolk Sac Score: 1

Slight remnants of yolk sac visible around the abdominal area.

Yolk Sac Score: 2

Yolk sac swelling more pronounced than previously.

Yolk Sac Score: 3

Definitive yolk sac remnants present.

Yolk Sac Score: 4

Large yolk sac visible with clear orange colouration around the intestinal region.

Yolk Sac Score: 5

Very large yolk sac reserves present representing early emergence from the gravel.

Figure 5.5. Brown trout photographs representing each yolk sac score

5.2.5.1. Fluctuating Asymmetry

Fry which were fixed in 5% Neutrally Buffered Formalin (NBF) were analysed for fluctuating asymmetry. In order to prevent bias when measuring the samples, a blind study was conducted with the relabeling of the jars by an independent person. Prior to measuring, all the samples were rinsed thoroughly in tap water to get rid of any excess NBF. For both eye height and eye diameter, the left and right sides of every fish were measured using digital calipers. Finally each pectoral and pelvic fins, both left and right, were removed with dissecting scissors and placed on individual slides (pectoral separate from pelvic). The fins were covered in emersion oil prior to cover slips, and then the fin rays were counted, using a dissecting microscope.

5.2.5.2. Comet Assay

The comet assay method (Singh *et al.*, 1988) was adapted by Goodsir, F. (Pers.Com., CEFAS). Alkaline Solution, Electrophoresis Solution and Lysis Solution (Appendix 5) were made up and kept in the dark at 16ºC. Low Melting Point (LMP) agarose, 0.5% in Phosphate Buffered Saline (PBS), was put into a microwave for 2.30 minutes on Medium (Power Level 5). After 1 minute it was removed and stirred and after the full time it was removed and placed in a water bath and incubated at 37ºC. 10ml of Dimethyl Sulphate (DMSO) and 1000µl of Triton X was added to a beaker containing 90ml of Lysis Solution and left to incubate in a fridge (5ºC) for 10 minutes.

Emergence fry were killed using a schedule one method (i.e. fatal dose of anaesthetic, 2-Phenoxyethanol, Fisher, UK and destruction of the brain) and then 2µl of blood from each fish was diluted in 498µl of PBS (or 1µl in 249µl PBS). In total, 28 fry from each treatment were used for the comet assay. 10µl of the blood/PBS solution was pipetted into 160µl of LMP agarose and shaken vigorously to ensure a thorough mix. 50µl of this solution was pipetted into each replicate circle on a Comet slide (Trevigen, UK). Each blood sample was prepared and pipetted one at a time. A positive control was also used as an indication of the DNA damage, a blood/PBS sample from one of the treatment fish was exposed to UV light for 1 minute in order to damage the DNA. The control is a positive UV control which was duplicated on the slide. The slide was kept in the dark between samples and left to incubate at 5ºC for 10 minutes to allow the agarose to set. The Lysis solution at 5°C was gently poured over the prepared slide in a Petri dish and left to incubate at 5ºC for 60 minutes. The slide was then removed from the Petri dish and excess Lysis Solution gently tapped off. The Petri dish was washed in UltraPure (UP) water and dried with lint free tissue. The Alkaline Solution was then poured into the Petri dish with the slide and left to incubate at 5ºC for 30 minutes. The slides were removed, excess solution gently tapped off and placed in an electrophoresis tank (BioRad Horizontal tank) with the electrophoresis solution at 30V, 300mA for 30 minutes using a power supply (BioRad PowerPac). The slide were removed from electrophoresis tank and rinsed three times in RO water, taking care not to let the agarose slip off. The slides were then placed in a Petri dish with 70% Ethanol for five minutes, to fix the samples, and were left to dry overnight (in the dark). Once ready for analysis, 50µl of the SYBR green (Stratech Scientific Ltd, UK) in solution (Appendix 5) was added to each circle of the slide to stain the DNA. Slides were then ready to be visualised under a microscope.

A fluorescent microscope (Nikon, Eclipse E800) with a digital camera (Nikon, DN 100) was used to visualise the slides. The strand breaks (DNA damage) allows the migration of DNA strands and loops under electrophoresis and these appear as comet-like tails emerging from the nuclear DNA mass when viewed under fluorescence. The size of the comet tail is dependent on the amount of DNA damage.

Figure 5.6 demonstrates the components of a comet. The head region represents the DNA that doesn't migrate outside of the nucleus and the tail region represents the DNA migrating out of the nucleus as a result of fragmentation (damage). The amount of DNA is proportional to the fluorescent intensity at that location (Nelms, 1997).

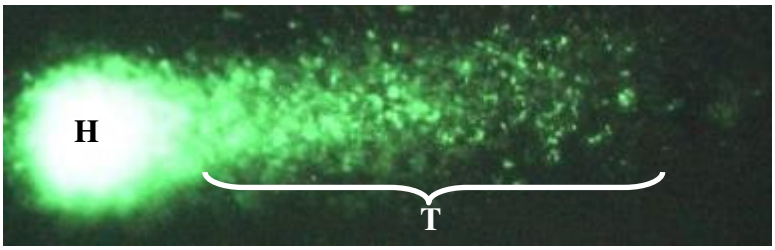

Figure 5.6. Image generated by the comet assay; H – head region; T – tail region.

The images captured were analysed using Comet Assay IV (Perceptive Instruments) software. The software gave values for Tail Length and Tail Intensity (percentage DNA in the tail), as well as calculating the Tail Moment (percentage DNA in the tail multiplied by the tail length).

5.2.6. Year 3 Experiment

For the third year of the sediment exposure experiment five treatments were used as four extra incubator and emergence tanks were installed ready for the season. The treatments included the control; just the sediment was added to the incubators; PAHs, OCPs, TRIs and MIX (a solution consisting of all the contaminant groups). Incubators were dosed evry two weeks.

For the third trial, four egg boxes were buried in each incubating, all containing 50 eggs. For the emergence study, 150 eggs were buried directly into the gravel. Eggs were sampled on day 51 post-fertilisation. Two egg boxes were removed from each replicate incubation tank on the sampling day. The weight and diameter for each viable egg was measured and recorded. Volume, surface area and surface area:volume ratio were calculated using the egg weight (Chap. 2). Some eggs were fixed in FBS and some frozen for further analyses. Frozen eggs were used to determine the dry weight, percentage water content, protein concentration and NPS concentration, as well as chloride concentration (Chap. 2).

Alevins were sampled on day 100 post-fertilisation. Two egg boxes from each replicate incubation tank were removed for sampling. The lengths and weights were taken and recorded for all which survived, from which the condition factor could be calculated (as described in section **5.2.4**). Some alevins were frozen and some fixed for further analysis. Alevins were analysed for yolk sac oedema, percentage weight of body and yolk sac, dry weight and protein content (Chap. 2).

The swim-up fry were counted and recorded as soon as they reached the mesh box in the emergence tank. Once counted the fry were released from the mesh box into the emergence tank until sampling. The fry were terminated on day 125 post-fertilisation using a Schedule One method and individual fish were weighed and lengths measured. From the results the Condition Factor was calculated to assess the overall condition of the fry from each treatment group. As with the second trial (**5.2.5**), blood collected from day 125 post fertilisation fry were used for the comet assay (please refer to section **5.2.5.2**).

5.2.7. Statistical Analysis

Where possible the data was analysed using a nested General Linear Model (GLM) to test for variations within the different replicates. Egg morphometrics, egg biochemical analysis, alevin and fry analysis all used a nested design where appropriate. Random factors for the analysis were egg box and tank, so that all stages of replication were accounted for. However when the appropriate data for a nested design was not available, a one way ANOVA were used to determine statistically significant differences in the results. Statistical software programmes SigmaStat® and Minitab 15® were used for the analysis. SigmaStat® tested data sets for normality and homogenous variances prior to analysis. If a significant difference ($P<0.05$) was observed a Holm-Sidak or Tukey 95% simultaneous confidence intervals, pair wise comparisons identified which treatments on which days were significant. Data represented in percentage were Arcsine transformed prior to statistical analysis.

Kruskal Wallis nonparametric test was used to establish whether the yolk sac score was affected by the treatment groups, by using the rank of the data values rather than the actual

values. If the p-value was less than 0.05 then the yolk sac score was statistically significant.

To statistically analyse the symmetry of the fry the values of the paired features measured were used in the formula L-R (left – right), to produce either a positive or negative value, or a value of 0. The orientation of the value indicates the type/preference of symmetry present, positive values indicate left asymmetry, negative values indicate right asymmetry and a value of 0 shows bilateral symmetry. The number which results from the formula L-R, referred to as signed asymmetry by Østbye *et al.*, (1997), enables deviations from ideal fluctuating asymmetry distributions (normal distributions with a mean of zero) to be identified. Deviations from normality were examined using Ryan- Joiner test (similar to Shapiro-Wilk *W* test). To detect directional distributions (normal distributions with a mean different from zero) the character values between the left and right sides were compared using a paired t test. The presence of a broad peaked distribution is indicative of antisymmetry (non-normal distributions with a mean of zero or different from zero) (Østbye *et al.*, 1997).

5.3. Results

5.3.1. Year 1

The daily measured temperature readings are shown in Figure 5.7. The temperature ranged between about 6-12°C throughout the trial. The temperature was at the highest at the start of the trial and the coldest temperature was generally just before the time of emergence.

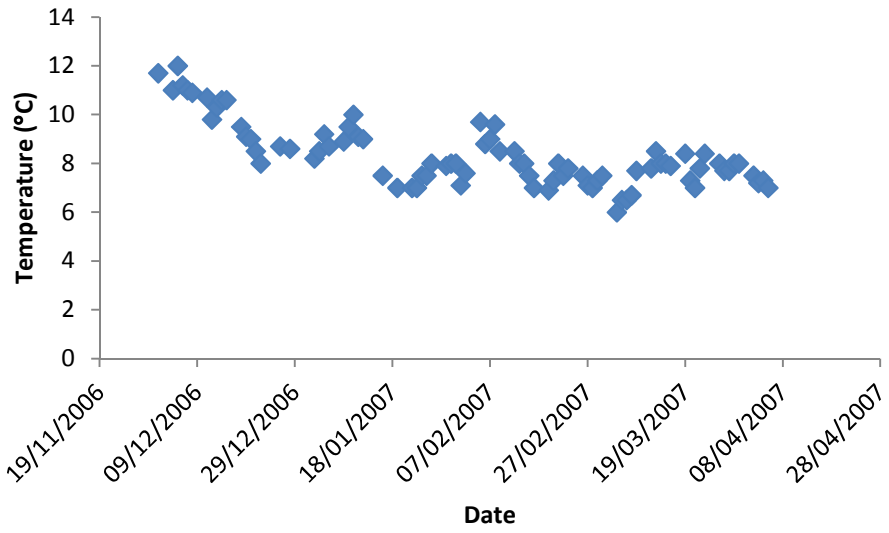

Figure 5.7. Daily temperature measurements of the incubator water.

The results for the brown trout mortality at different stages of development can be seen in Figure 5.8. The graph represents a total mean percentage of mortality for the combined replicates. It can be seen that mortality was greatest in the first developmental stage (day 35 post fertilisation) in the control treatment. Generally, mortality rates increased for all treatments for the consequent developmental stages, egg, alevin and fry.

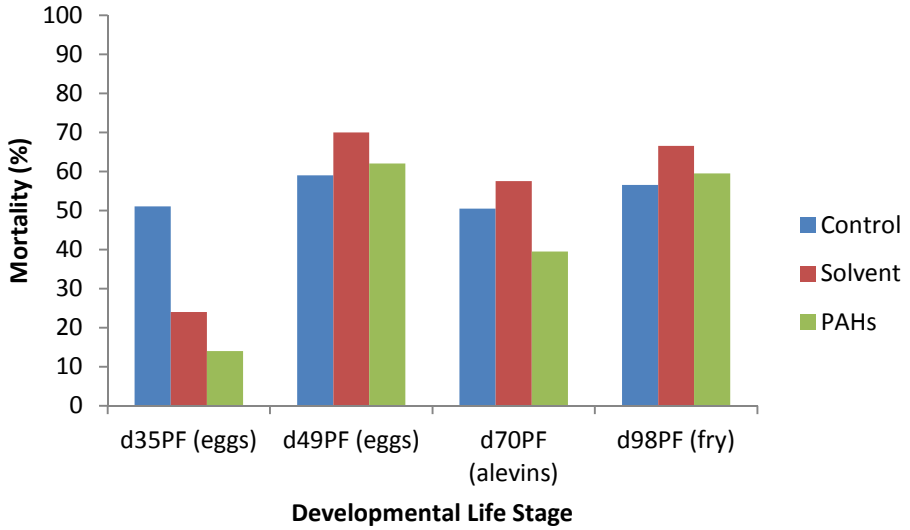

Figure 5.8. Mortality rate at different developmental stages.

5.3.1.1. Egg Results (d35PF and d49PF)

Table 5.10 shows the morphometric analysis, weight, diameter, volume, surface area and surface area to volume ratio, for the day 35 and day 49 post-fertilisation eggs. A nested GLM indicated that at day 35 post-fertilisation treatment had no significant effect on the weight (GLM (nested), F=0.24; d.f. =2; *P*=0.794). However the tank used also had no effect (GLM (nested), F=0.84; d.f.=3; *P*=0.506) and neither did the indivivual egg boxes selected for sampling (GLM (nested), F=0.72; d.f.=6; *P*=0.637). At day 49 post-fertilisation statistical analysis showed that treatment had no significant effect on egg weight (GLM (nested), F=0.54; d.f. =2; *P*=0.589). However the tank also had no effect (GLM (nested), F=0.30; d.f. =3; *P*=0.827) and neither did the individual egg boxes selected for sampling (GLM (nested), F=0.07; d.f. =6; *P*=0.999).

Statistical analysis showed that treatment at day 35 post-fertilisation did not have a significant effect on the diameter of the eggs (GLM (nested), F=1.18; d.f. =2; *P*=0.405), neither did the factor of tank (GLM (nested), F=0.66; d.f. =3; *P*=0.604) nor the individual egg box selected (GLM (nested), F=1.81; d.f. =6; *P*=0.095). At day 49 post-fertilisation statistical analysis showed that treatment had no significant effect on the diameter of the egg (GLM (nested), F=0.24; d.f. =2; *P*=0.791), neither did the tank selected (GLM (nested), F=0.51; d.f. =3; *P*=0.682) nor the individual egg box (GLM (nested), F=1.42; d.f. =6; *P*=0.209).

Statistical analysis for day 35 post-fertilisation showed that treatment did not have an effect on egg volume (GLM (nested), F=0.24; d.f. =2; *P*=0.794). The factor of tank also had no significant effect on egg volume (GLM (nested), F=0.84; d.f. =3; *P*=0.506) nor did the egg box selected for sampling (GLM (nested), F=0.72; d.f. =6; *P*=0.637). Eggs sampled on day 49 post fertilisation were not significantly different in volume as a result of treatment (GLM (nested), F=0.47; d.f. =2; *P*=0.646), tank (GLM (nested), F=1.04; d.f. =3; *P*=0.400) and egg box (GLM (nested), F=0.61; d.f. =6; *P*=0.722).

Statistical analysis for day 35 post-fertilisation showed that treatment did not have an effect on egg surface area (GLM (nested), F=0.26; d.f. =2; *P*=0.785). There was also no

significant effect of tank (GLM (nested), F=0.84; d.f. =3; *P*=0.504) or egg box (GLM (nested), F=0.68; d.f. =6; *P*=0.670) on the egg surface area. Eggs sampled on day 49 post fertilisation showed no significant differents as a result of treatment (GLM (nested), F=0.56; d.f. =2; *P*=0.595), tank (GLM (nested), F=1.01; d.f. =3; *P*=0.415) or selected egg box (GLM (nested), F=0.68; d.f. =6; *P*=0.670).

The results for ratio of egg volume to surface area at day 35 post fertilisation showed that treatment did not have an effect on egg surface area to volume ratio (GLM (nested), F=0.30; d.f. =2; *P*=0.756). The factor of tank also had no significant effect on the surface area to volume ratio of the brown trout eggs (GLM (nested), F=0.86; d.f. =3; *P*=0.498) neither did the selected egg box (GLM (nested), F=0.72; d.f. =6; *P*=0.631). Eggs sampled on day 49 post fertilisation did not show a significant difference as a result of treatment (GLM (nested), F=0.87; d.f. =2; *P*=0.463), tank (GLM (nested), F=0.90; d.f. =3; *P*=0.469) nor selected egg box (GLM (nested), F=0.87; d.f. =6; *P*=0.515).

Table 5.10. Morphometric analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPF	Weight (mg)	Diameter (mm)	Volume (mm^3)	Surface Area (mm^2)	SA:Vol	N value
CNT	**35**	102.57±0.49	5.26±0.02	93.73±0.49	99.76±0.35	1.07±0.00	97
CNT	**49**	112.90±10.00	5.69±0.03	95.60±0.49	101.08±0.34	1.06±0.00	151
SOL	**35**	102.62±0.34	5.23±0.02	93.78±0.41	99.80±0.24	1.07±0.00	123
SOL	**49**	104.08±0.87	5.78±0.04	94.74±0.74	100.47±0.53	1.06±0.00	77
PAH	**35**	102.37±0.41	5.18±0.02	93.53±0.41	99.62±0.29	1.07±0.00	46
PAH	**49**	102.53±1.47	5.78±0.04	95.36±1.34	100.82±0.87	1.06±0.00	66

Table 5.11 shows the the results for the biochemical analysis, dry weight, water content, NPS levels, protein concentration and chloride levels, for both day 35 and 49 post-fertilisation. Statistical analysis showed that for day 35 post-fertilisation, treatment did not have a significant effect on the egg dry weight (GLM (nested), F=0.43; d.f. =2; *P*=0.684). There was also no significant differences as a result of tank (GLM (nested), F=3.32; d.f. =3; *P*=0.094), however the selected egg box did have a significant effect (GLM (nested), F=5.83; d.f. =6; P<0.001). One of the egg boxes selected from a PAH tank had significantly larger dry weights than other treatments, tanks and egg boxes. For day 49 post-fertilisation, treatment did have a significant effect on the egg dry weight (GLM (nested), F=14.32; d.f. =2; *P*=0.026), however there was no significanty effect of the tank (GLM (nested), F=0.23; d.f. =3; *P*=0.870) nor the selected egg box (GLM (nested),

F=1.77; d.f. =6; *P*=0.140). Therefore the true significant difference was in the actual treatment rather than variation amongst the replicates.

Statistical analysis for day 35 post-fertilisation showed that treatment (GLM (nested), F=0.77; d.f. =2; *P*=0.536) and tank (GLM (nested), F=3.30; d.f. =3; *P*=0.0.96) did not have a significant effect on the egg water content. However the selected egg box did have an effect on the egg water content (GLM (nested), F=5.69; d.f. =6; *P*<0.001). One egg box from PAH and one from solvent control treatment group had significantly less egg water content than the control group. For day 49 post fertilisation, there was no significant effect as a result of treatment (GLM (nested), F=8.08; d.f. =2; *P*=0.055), tank (GLM (nested), F=0.61; d.f. =3; *P*=0.648) nor selected egg box (GLM (nested), F=1.27; d.f. =5; *P*=0.295).

Statistical analysis for day 35 post-fertilisation showed that the factor of treatment did not have a significant difference on NPS concentration of the eggs (GLM (nested), F=3.13; d.f. =2; *P*=0.183). The factor of tank (GLM (nested), F=1.21; d.f. =3; *P*=0.379) also had no significant effect for day 35 post fertilisation, however the selected egg box did have a significant effect (GLM (nested), F=3.59; d.f. =6; *P*=0.005). One of the PAH treatment group egg boxes had significantly lower NPS concentration than other egg boxes from all treatments. For day 49 post-fertilisation neither treatment (GLM (nested), F=3.30; d.f. =2; *P*=0.195) nor tank (GLM (nested), F=0.23; d.f. =3; *P*=0.870) had a significant effect on the egg NPS concentration. However the factor of egg box did have a significant effect (GLM (nested), F=5.55; d.f. =5; *P*=0.001). One PAH egg box had significantly lower NPS concentration than other boxes within all other treatments.

Statistical analysis showed that for day 35 post-fertilisation there was no significant difference for treatment (GLM (nested), F=3.50; d.f. =2; *P*=0.163) nor tank (GLM (nested), F=1.21; d.f. =3; *P*=0.378) on egg protein concentration. However the selected egg box did have a significant effect (GLM (nested), F=2.84; d.f. =6; *P*=0.019). One PAH and one solvent control egg box had higher protein concentrations than the other boxes. For day 49 post-fertilisation, treatment (GLM (nested), F=0.32; d.f. =2; *P*=0.749) and tank (GLM (nested), F=0.53; d.f. =3; *P*=0.681) had no significant effect on egg protein content, however egg box did have a significant effect (GLM (nested), F=2.85; d.f. =5; *P*=0.027).

As with day 35 post-fertilisation one PAH and one solvent control egg box had higher protein concentrations than the other boxes.

The data demonstrated that the factor of treatment for day 35 post-fertilisation did not have a significant difference on the egg chloride concentration (GLM (nested), F=0.42; d.f. =2; *P*=0.689). The factor of tank also had no significant effect on egg chloride concentration (GLM (nested), F=1.86; d.f. =3; *P*=0.242). However egg box did have a significant effect (GLM (nested), F=2.87; d.f. =5; *P*=0.031). PAH and solvent control treatment group had an egg box with a greater egg chloride concentration than other boxes. For day 49 post-fertilisation treatment (GLM (nested), F=3.95; d.f. =2; *P*=0.167) and tank (GLM (nested), F=0.18; d.f. =3; *P*=0.903) had no significant effect on egg chloride concentration. However the egg box selected did have a significant difference on the egg chloride concentration (GLM (nested), F=4.11; d.f. =3; *P*=0.023). PAH and solvent control treatment group had an egg box with a greater egg chloride concentration than other boxes.

Table 5.11. Biochemical analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPF	Dry Weight (mg)	Water Content (%)	NPS (NPS nM/mg Egg)	Protein (mg/mg Egg)	Chloride (nEq Cl/mg Egg)	N value
CNT	**35**	46.20±1.84	55.05±1.11	40.97±1.61	0.56±0.02	23.25±1.75	20
CNT	**49**	14.70±1.37	27.35±1.82	114.96±6.51	0.93±0.04	29.90±4.82	20
SOL	**35**	30.10±3.58	41.54±3.16	69.40±4.93	0.80±0.04	29.95±2.26	20
SOL	**49**	22.89±1.95	36.61±2.04	109.29±6.98	1.04±0.09	38.66±4.57	20
PAH	**35**	31.50±2.27	44.27±2.06	77.62±6.98	0.79±0.05	31.39±2.34	14
PAH	**49**	19.79±2.41	33.95±2.49	90.75±4.93	1.04±0.05	39.01±5.88	18

5.3.1.2. Alevin Results (d70PF)

Alevin lengths were not measured due to the time constraints at sampling . The results for the alevin wet weights are shown in Table 5.12. Treatment showed to have a significant difference on the day 70 post fertilisation wet weight (GLM (nested), F=30.17; d.f. =2; *P*=0.010). A Tukey's pairwise comparison identified that PAH exposed alevins were significantly heavier than the control and solvent control-exposed alevins. Additionally tank had no significant effect on alevin weight (GLM (nested), F=0.16; d.f. =3; *P*=0.917)

but the egg box did show a significant difference (GLM (nested), F=2.90; d.f. =6; *P*=0.012).

Table 5.12 shows the results for the alevin dry weight for each treatment group. Statistical analysis shows that treatment did not have a significant effect on the alevin dry weight (ANOVA, f=0.26; d.f. =2; P=0.771).

The results for the alevin protein concentration can be seen in Table 5.12. Statistical analysis demonstrated that treatment did not have a significant effect on the protein concentration of the day 70 post fertilisation alevins (ANOVA, F=0.57; d.f. =2; P=0.573).

Table 5.12. Morphometric and biochemical analysis of *Salmo trutta* alevins (Data represents Mean±SEM)

Treatment	dPF	Wet Weight (mg)	Dry Weight (mg)	Protein (mg/mg body)	N value
CNT	**70**	118.71±1.24	40.40±3.77	0.31±0.04	42
SOL	**70**	121.19±0.96	42.00±3.32	0.25±0.02	37
PAH	**70**	126.85±1.23	44.70±5.35	0.32±0.06	39

The results for the weight of the body weight and yolk sac weight, represented as a percentage of total weight, can be seen in Figure 5.9. Statistical analysis demonstrated that trestment had no effect on the percentage weight of the yolk sac or body weight for treatment (GLM (nested), F=0.32; d.f. =2; *P*=0.746), tank (GLM (nested), F=0.10; d.f. =3; *P*=0.955) nor egg box (GLM (nested), F=1.68; d.f. =6; *P*=0.184).

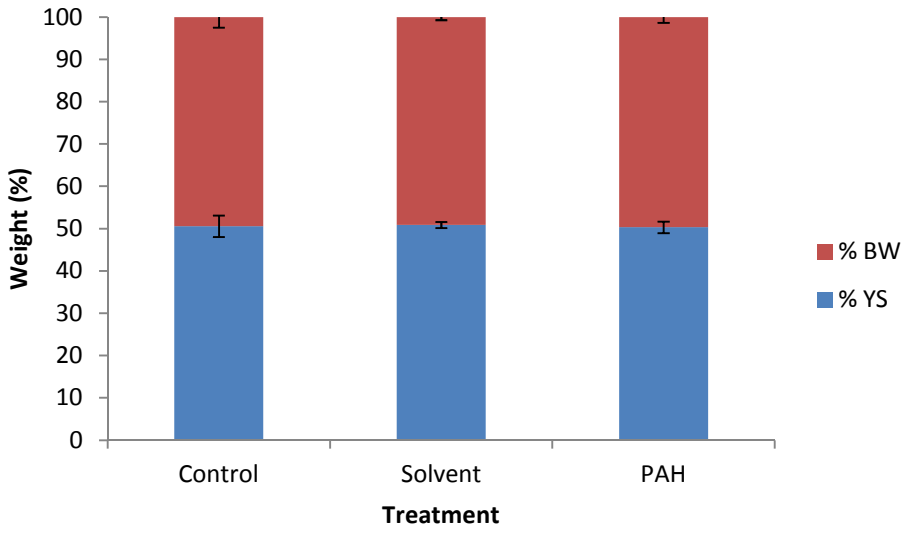

Figure 5.9. Percentage yolk sac (YS) and percentage body weight (BW) of alevins d70PF. (Data represents Mean±SEM). n=10.

Figure 5.10 shows some of the yolk sac oedemas which were observed in alevins day 70 post fertilisation. The accumulation of fluid withing the yolk sac were observed more in the PAH exposed alevins.

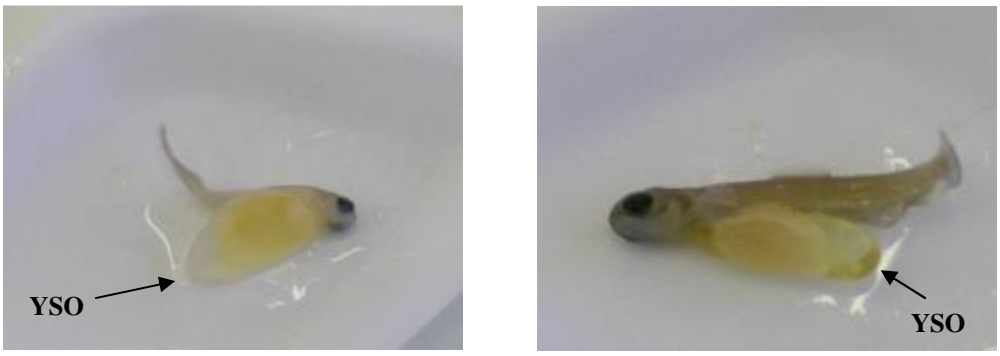

Figure 5.10. YS oedemas from PAH exposed alevins. YSO–yolk sac oedema.

Figure 5.11 shows the results for the scored yolk sac oedemas of the day 70 post fertilisation alevins as a percentage of the total number of alevins. Overall there were more frequent observations of yolk sac oedemas in the PAH treatment group compared to both the control groups (control and solvent control).

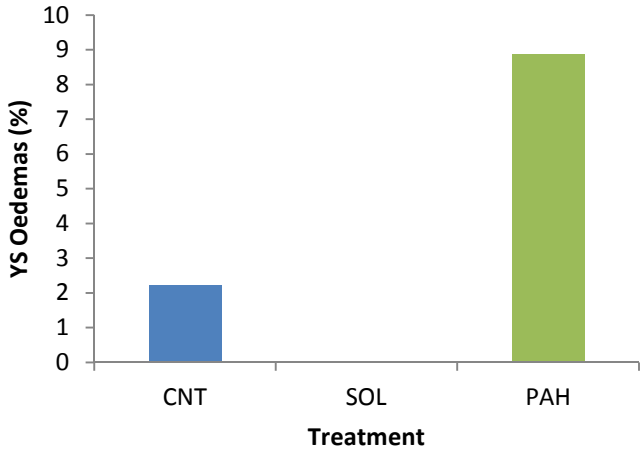

Figure 5.11. Total observed yolk sac oedemas as a percentage of total alevins.

5.3.1.3. Fry Results (d98PF)

Table 5.13 shows the morphometric analysis of the day 98 post-fertilisation fry. Statistical analysis of the fry demonstrated that treatment did not have a significant effect on the length of the fry (GLM (nested), F=0.07; d.f. =2; *P*=0.937). However, the factor of tank did have a significant effect on fry length (GLM (nested), F=3.49; d.f. =3; *P*=0.028). Emerged fry from one tank which had been exposed to PAHs were significantly longer than fry from any of the other tanks. Treatment had no significant effect on the weight of the emerged fry (GLM (nested), F=0.27; d.f. =2; *P*=0.782), but tank did have a significant effect on fry weight (GLM (nested), F=3.36; d.f. =3; *P*=0.032). Emerged fry from one tank which had been exposed to PAHs were significantly heavier than fry from any of the other tanks. Treatment (GLM (nested), F=1.35; d.f. =2; *P*=0.377) and tank (GLM (nested), F=0.80; d.f. =3; *P*=0.506) showed no significant effect on fry condition factor.

Table 5.13. Morpometric analysis of *Salmo trutta* fry (Data represents Mean±SEM)

Treatment	dPF	Weight (mg)	Length (mm)	Condition Factor (*K*)	N value
CNT	98	165.00±3.59	27.58±0.23	0.79±0.01	12
SOL	98	155.00±4.35	27.58±0.23	0.74±0.02	12
PAH	98	167.50±4.63	27.83±0.30	0.78±0.03	12

5.3.2. Year 2

The temperature readings taken for the duration of the trial are shown in Figure 5.12. The temperature ranged from about 5-10°C during the trial. The highest temperature was at the start of the trial and the lowest just before the start of emergence.

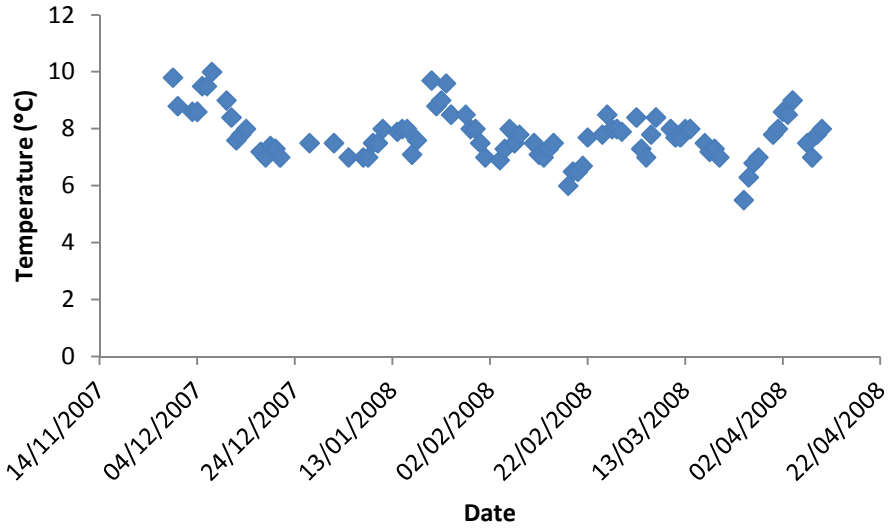

Figure 5.12. Regular temperature readings of the sediment exposure trial water.

The mortality rates for the brown trout at each developmental stage can be seen in Figure 5.13. The data is represented as a mean percentage and generally shows that the control group had slightly better survival compared to the exposed groups. Overall the egg survival rates were better than both the alevin and fry stage of development. The fry stage had the lowest survival compared to the earlier stages of development.

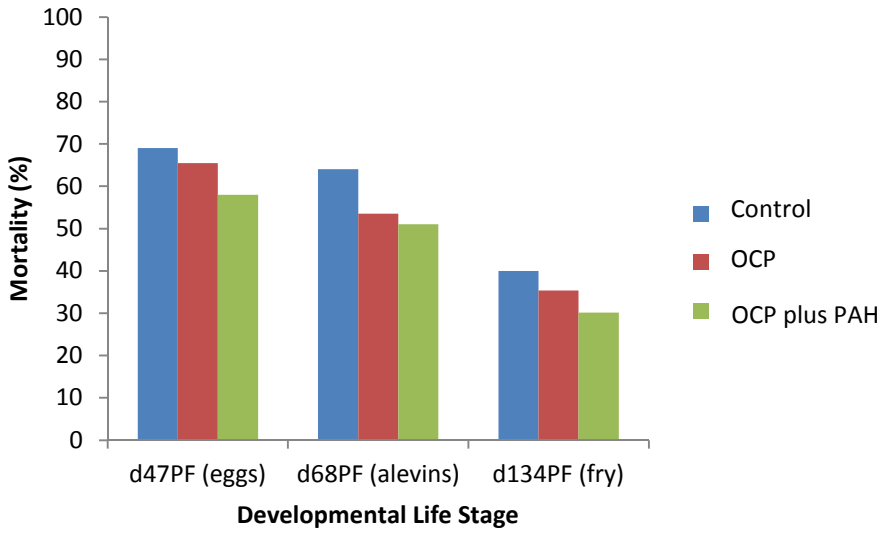

Figure 5.13. Mortality rate at different developmental stages.

5.3.2.1. Egg Results (d47PF)

Table 5.14 shows the morphometric analysis of day 47 post-fertilisation brown trout eggs. Treatment was not shown to have a significant effect on the weight of the eggs (GLM (nested), F=1.58; d.f. =2; *P*=0.341). The factor of tank was also shown not to have a significant effect on egg weight (GLM (nested), F=0.86; d.f. =3; *P*=0.525). However egg box did indicate a significant difference of egg weight (GLM (nested), F=2.37; d.f. =5; *P*=0.041). A Tukey's pairwise comparison identified that one egg box from OCP and one from OCP plus PAH-exposed eggs were significantly lighter than other egg boxes.

At day 47 post fertilisation, treatment did not have a significant effect on the diameter of the eggs (GLM (nested), F=1.09; d.f. =2; *P*=0.440). The factor of tank also had no significant effect on egg diameter (GLM (nested), F=1.76; d.f. =3; *P*=0.250). But egg box selection did have a significant effect on egg diameter (GLM (nested), F=2.75; d.f. =6; *P*=0.0.014). A Tukey's pairwise comparison demonstrated that one egg box from OCP plus PAH treatment tanks were significantly larger in diameter than other egg boxes.

Statistical analysis for day 47 post-fertilisation showed that treatment did not have an effect on egg volume (GLM (nested), F=0.49; d.f. =2; *P*=0.653) neither did tank (GLM (nested), F=2.90; d.f. =3; *P*=0.120). However egg box selection did have a significant

effect on egg volume (GLM (nested), F=3.35; d.f. =6; *P*=0.004). A pairwise comparison identified that one egg box from an OCP treatment tank and one from an OCP plus PAH-treatment tank had significantly smaller volumes than other boxes.

Results for egg surface area at day 47 post fertilisation showed that treatment did not have an effect on egg surface area (GLM (nested), F=0.38; d.f. =2; *P*=0.714). Tank also had no effect on egg surface area (GLM (nested), F=2.19; d.f. =3; *P*=0.186). However, egg box did have a significant effect on egg surface area (GLM (nested), F=3.05; d.f. =6; *P*=0.007). A pairwise comparison identified that one egg box in an OCP tank and one in an OCP plus PAH tank had significantly smaller surface areas than other boxes.

Statistical analysis for day 47 post-fertilisation eggs showed that treatment did not have an effect on egg surface area to volume ratio (GLM (nested), F=1.37; d.f. =2; *P*=0.377). Tank also had no significant effect (GLM (nested), F=4.46; d.f. =3; *P*=0.054). Although egg box selection did have a significant effect on egg surface area to volume ratio (GLM (nested), F=2.98; d.f. =6; *P*=0.008). A pairwise comparison identified that one egg box in an OCP tank and one in an OCP plus PAH tank had significantly smaller surface area to volume ratios than other boxes.

Table 5.14. Morphometric analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPF	Weight (mg)	Diameter (mm)	Volume (mm^3)	Surface Area (mm^2)	SA:Vol	N value
CNT	**47**	105.70±1.20	5.28±0.04	92.88±0.82	102.18±0.82	1.10±0.01	57
OCP	**47**	101.66±0.75	5.39±0.05	92.26±0.66	99.09±0.54	1.08±0.01	63
OCP+PAH	**47**	102.40±0.71	5.44±0.03	93.97±0.81	99.62±0.52	1.07±0.01	77

Table 5.15 shows the data from the biochemical analysis, dry weight, water content, NPS levels, protein concentration and chloride concentration. Statistical analysis showed that treatment had no significant effect on the dry weight of the eggs (GLM (nested), F=0.34; d.f. =2; *P*=0.734). Tank (GLM (nested), F=0.72; d.f. =3; *P*=0.579) and egg box (GLM (nested), F=1.50; d.f. =5; *P*=0.216) also had no significant effect on dry weight. Statistical analysis demonstrated that treatment (GLM (nested), F=0.72; d.f. =2; *P*=0.551), tank (GLM (nested), F=0.87; d.f. =3; *P*=0.508) and egg box (GLM (nested), F=1.19; d.f. =5; *P*=0.336) had no effect on the percentage water content of the eggs.

The results for the egg NPS concentration show the factor of treatment did not have a significant effect on the NPS concentration of the brown trout eggs (ANOVA, F=0.36; d.f. =2; *P*=0.700). Also the results for the protein concentration of the eggs from statistical analysis demonstrated that treatment had no effect on the protein concentration of the eggs (ANOVA, F=1.87; d.f. =2; *P*=0.166). Statistical analysis for the egg chloride concentration showed that there was a significant difference between the treatment groups (ANOVA, F=3.71; d.f. =2; *P*=0.038). A Tukey's pairwise comparison identified that the OCP plus PAH-exposed eggs had a significantly higher chloride concentration than either the control group or the OCP exposed eggs.

Table 5.15. Biochemical analysis of *Salmo trutta* eggs (Data represents Mean±SEM)

Treatment	dPF	Dry Weight (mg)	Water Content (%)	NPS (NPS nM/mg Egg)	Protein (mg/mg Egg)	Chloride (nEq Cl/mg Egg)	N value
CNT	**47**	35.73±0.54	63.84±0.41	60.47±2.14	0.30±0.00	30.35±2.04	11
OCP	**47**	36.69±0.73	62.74±0.42	57.79±2.30	0.30±0.00	27.87±2.98	16
OCP+PAH	**47**	36.16±0.65	62.97±0.29	59.34±1.85	0.31±0.01	37.85±3.21	19

5.3.2.2. Alevin Results (d68PF).

Table 5.16 shows the morphometric and biochemical analysis data for brown trout alevins. For day 68 post fertilisation, alevin length was not effected by the treatment (GLM (nested), F=1.51; d.f. =2; *P*=0.349) or tank (GLM (nested), F=1.46; d.f. =3; *P*=0.270). However the factor of egg box did significantly effect the alevin length (GLM (nested), F=8.88; d.f. =13; *P*<0.001). One egg box from OCP plus PAH treatment tank had alevins which were significantly shorter than the egg boxes.

Statistical analysis for day 68 post-fertilisation alevins showed that treatment did not have a significant effect on the weight of the alevins (GLM (nested), F=1.33; d.f. =2; *P*=0.384). However the factor of tank did have a significant effect on alevin weight (GLM (nested), F=4.04; d.f. =3; *P*=0.016). A comparison analysis showed that alevins from one OCP plus PAH treatment tank were lighter than those from other tanks. Egg box had no significant effect on alevin weight (GLM (nested), F=0.54; d.f. =14; *P*=0.895).

Statistical analysis demonstrated that treatment generated no significant difference on the dry weight of alevins (ANOVA, F=2.05; d.f. =2; P=0.149). There was also no significant difference between treatments (ANOVA, F=0.76; d.f. =2; P=0.479) for alevin protein content.

Table 5.16. Morphometric and biochemical analysis of *Salmo trutta* alevins (Data represents Mean±SEM)

Treatment	dPF	Length (mm)	Wet Weight (mg)	Dry Weight (mg)	Protein (mg/mg body)	N Value
CNT	**68**	18.92±0.19	118.93±1.12	47.80±4.54	0.18±0.02	72
SOL	**68**	18.96±0.14	116.52±1.49	36.70±4.04	0.24±0.05	93
PAH	**68**	17.95±0.36	113.82±1.88	44.10±3.14	0.21±0.03	98

Figure 5.14 shows the results for the yolk sac weight and body weight represented as a percentage of the total alevin weight. Treatment did not have a significant effect on the percentage yolk sac or body weight of the alevins weight (GLM (nested), F=1.37; d.f. =2; *P*=0.373). Tank (GLM (nested), F=1.40; d.f. =3; *P*=0.268) and egg box (GLM (nested), F=0.91; d.f. =14; *P*=0.552) also had no signifcant effect on the percentage yolk sac or body weight of the alevins.

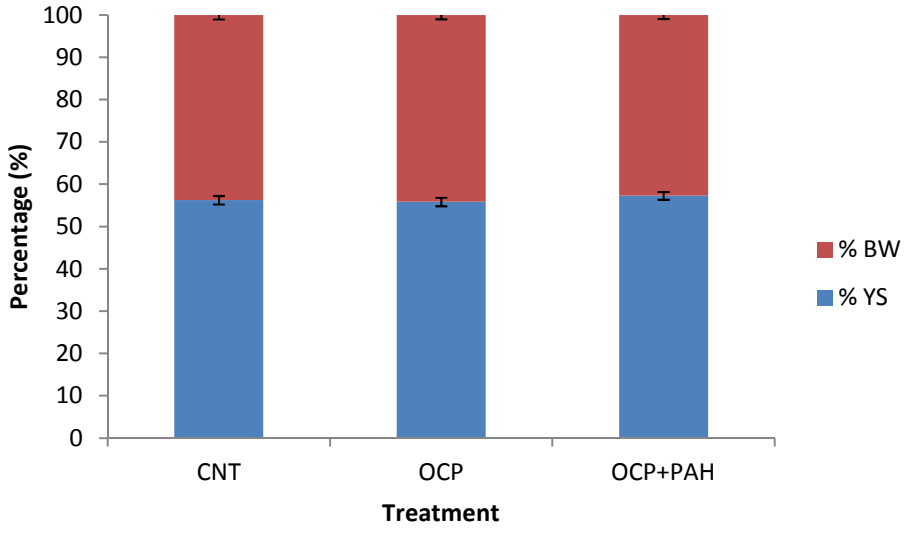

Figure 5.14. Percentage yolk sac (YS) and percentage body weight (BW) of alevins d68PF (Data represents Mean±SEM). n= 20, 24, 25 respectively.

Figure 5.15 shows the percentage yolk sac oedemas scored for the total number of alevins observed. It clearly shows that a greater porportion of yolk sac oedema were seen in the contaminant exposed fry compared to the control group.

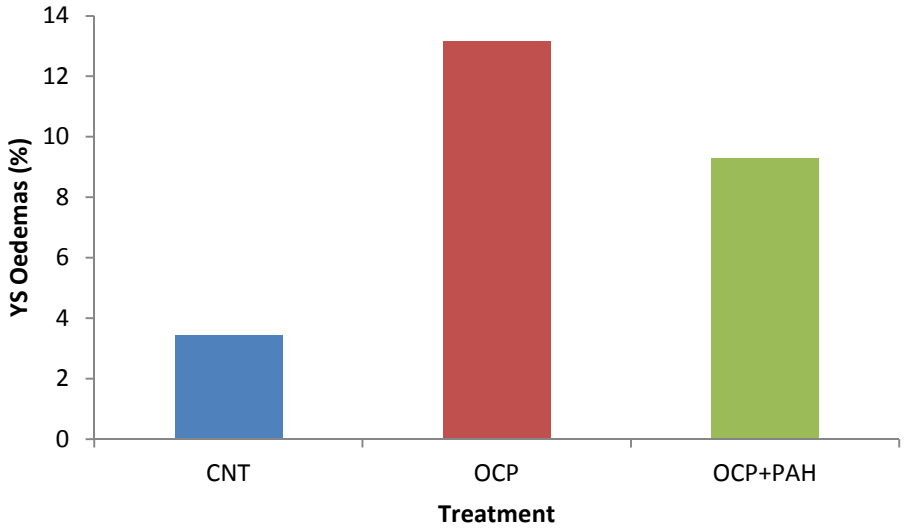

Figure 5.15. Total observed yolk sac oedemas as a percentage of total alevins.

5.3.2.3. Fry Results (d134PF)

Table 5.17 shows the morphometric analysis data for the day 134 post-fertilisation fry. Statistical analysis showed that treatment was not found to be a significant factor in effecting fry weight (GLM (nested), F=0.19; d.f. =2; P=0.834). However, the factor of tank did have a significant effect on the fry weight (GLM (nested), F=5.46; d.f. =3; *P*=0.001). Fry from one OCP plus PAH treatment tank were lighter than those from the other tanks.

For day 134 post fertilisation, treatment did not have a significant effect on the length of the fry (GLM (nested), F=0.09; d.f. =2; *P*=0.915). However, the factor of tank did have a significant effect on the fry length (GLM (nested), F=6.22; d.f. =3; *P*<0.001). Fry from one OCP plus PAH treatment tank were smaller in length than those from the other tanks.

Statistical analysis showed that treatment did not have a significant effect on the condition factor of the fish at day 134 post-fertilisation (GLM (nested), F=0.41; d.f. =2; *P*=0.696).

Tank also had no significant effect on the condition factor of the fry (GLM (nested), F=2.04; d.f. =3; *P*=0.108).

Table 5.17. Morphometric analysis of *Salmo salar* fry (Data represents Mean±SEM)

Treatment	dPF	Weight (mg)	Length (mm)	Condition Factor (*K*)	N value
CNT	134	350.90±8.83	32.86±0.24	0.97±0.01	147
OCP	134	345.98±9.58	32.45±0.22	0.96±0.01	152
O+P	134	332.96±7.57	32.64±0.26	0.95±0.01	135

Figure 5.16 shows the results for body depth for brown trout fry. Statistical analysis showed that treatment did not have an effect on fry body depth (ANOVA, F=0.85; d.f. =2; *P*=0.430).

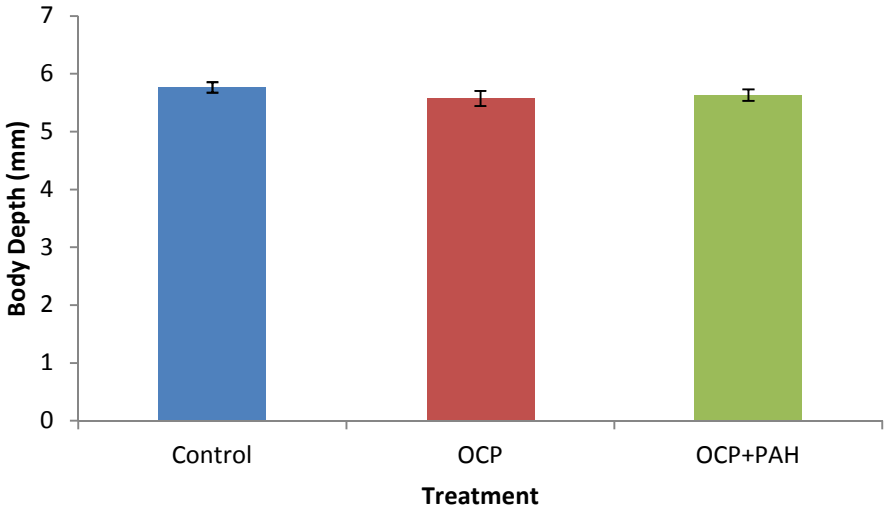

Figure 5.16. Body depth of brown trout fry for each treatment. (Data represents Mean±SEM). n=120, 97, 120 respectively.

The results for the yolk sac score for the brown trout fry are shown in Figure 5.17. Statistical analysis showed that treatment did not have an effect on the assigned yolk sac score (0-5) of the fry (Kruskal-Wallis Test, H=0.71; d.f. =2; *P*=0.702). Therefore treatment did not significantly affect the absorption of the yolk sac during the gravel stages of development. This indicates that premature emergence was not observed during this trial as a result of treatment.

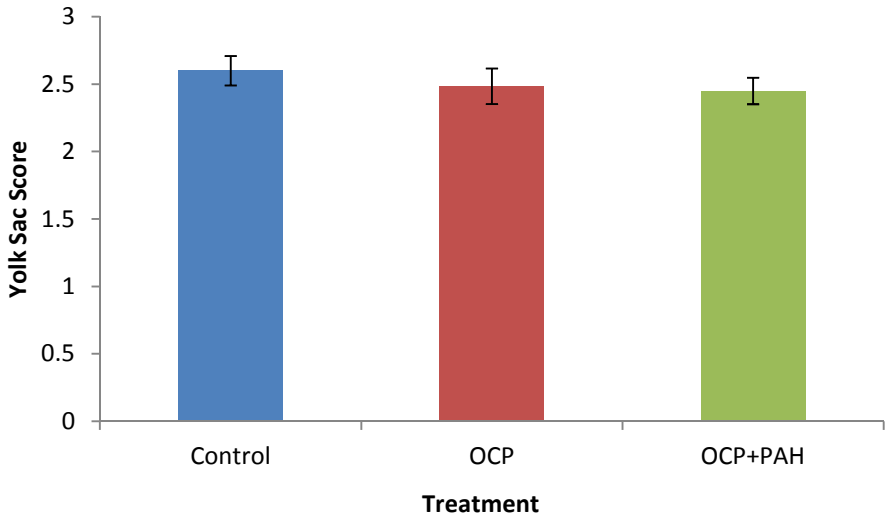

Figure 5.17. Yolk sac score of brown trout fry (0-5) for each treatment. (Data represents Mean±SEM). n=120, 97, 120 respectively.

5.3.2.3.1. Fluctuating Asymmetry

Swim-up fry were used for the fluctuating asymmetry analysis in the second sediment exposure trial. Figure 5.18 shows the results for the formula L-R and statistical analysis was found to not be statistically significant (Kruskal Wallis, H=3.10; d.f. =2; *P*= 0.212). The results for the eye height of brown trout fry showed that treatment did not have a significant effect on the eye height on the left side of the fry (Kruskal Wallis, H=0.16; d.f. =2; *P*=0.925) nor the right side of the fry (Kruskal Wallis, H=3.08; d.f. =2; *P*=0.215).

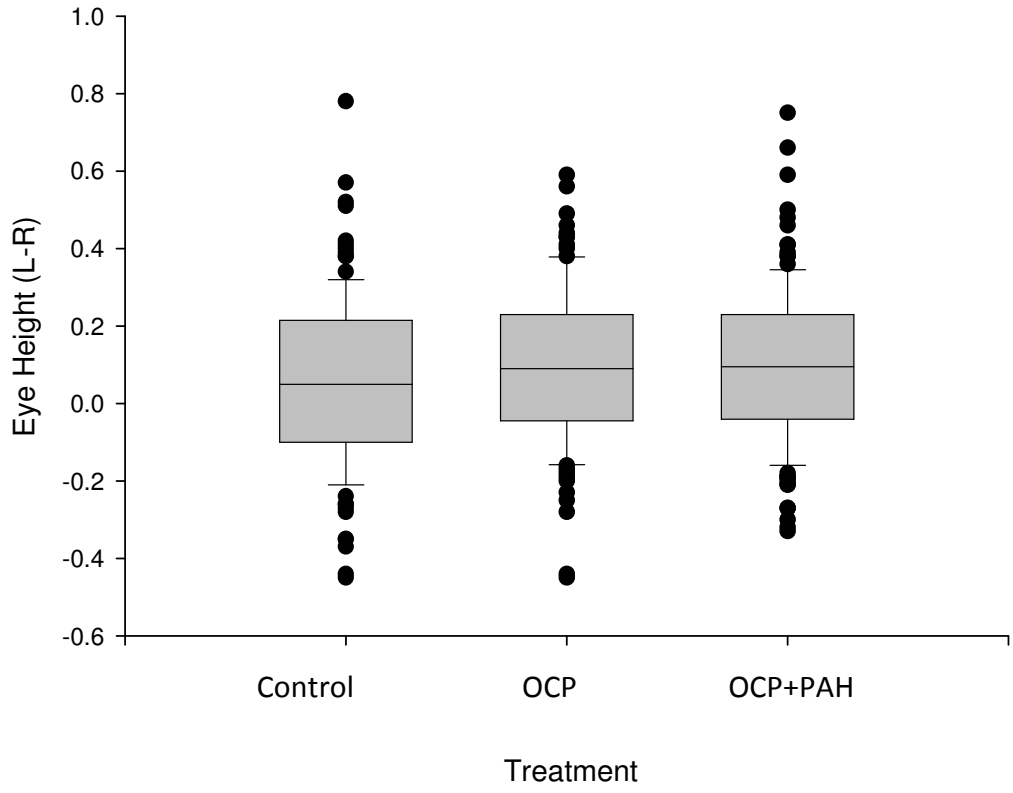

Figure 5.18. Eye height (L-R) of brown trout for each treatment. (Data represents Mean±SEM). n=120, 97, 120 respectively.

The Ryan-Joiner test was carried out to detect deviations from normality for eye height. The analysis showed the data to not have a mean of 0 (Control = 0.058, OCP = 0.098, OCP+PAH= 0.101). A paired t test was carried out to establish whether the symmetry was directional. The control group showed the left eye height to be significantly larger from the right (T-test; t=2.88; *P*= 0.005). Both the OCP (T-test, t=4.67; *P* <0.001) and OCP plus PAH (T-test, t=5.50; *P* <0.001) treatments showed significant differences between the left and right eye heights, with the left side eye displaying a greater height for both treatments.

The percentage of symmetry was calculated using the L-R formula to discover whether eye height showed a tendency to deviate in one direction, whether the variation was random or if a deviation from bilateral symmetry was shown at all. The percentages showed a distinct pattern in all the treatments, with left symmetry being the most pronounced characteristic and bilateral symmetry decreasing with the increase in organic pollutants (Figure 5.19).

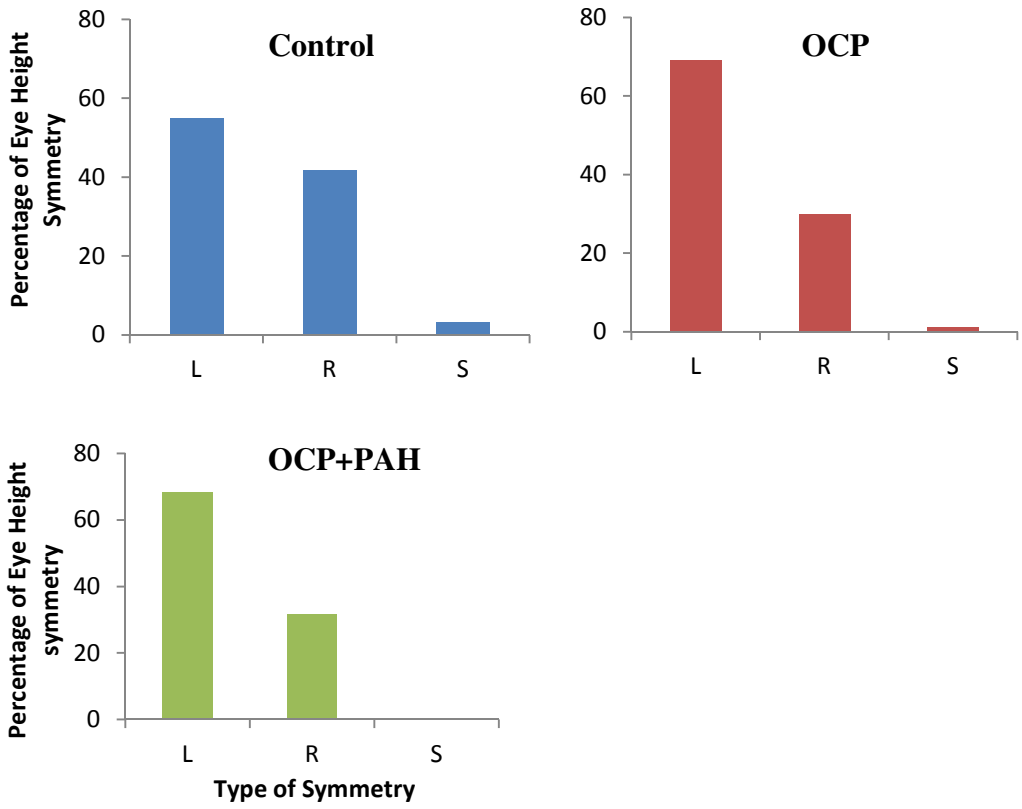

Figure 5.19. The type of symmetry (left (L), right (R) and symmetrical (S)) and the percentage of symmetry found for each treatment for eye height. n=120, 97, 120 respectively.

The results for the eye width of brown trout fry are shown in Figure 5.20. The figure shows the results for the eye width formula L-R and statistical analysis showed that treatment had no significant difference (K, H=0.07; d.f. =2; *P*=0.965). The mean width measurements for both the left and the right eye of each brown trout fry. Statistical analysis showed that treatment did not have a significant effect on the eye width of the emerged fry in neither the left side (Kruskal Wallis, H=0.91; d.f. =2; *P*=0.635) nor the right (Kruskal Wallis, H=0.51; d.f. =2; *P*=0.776).

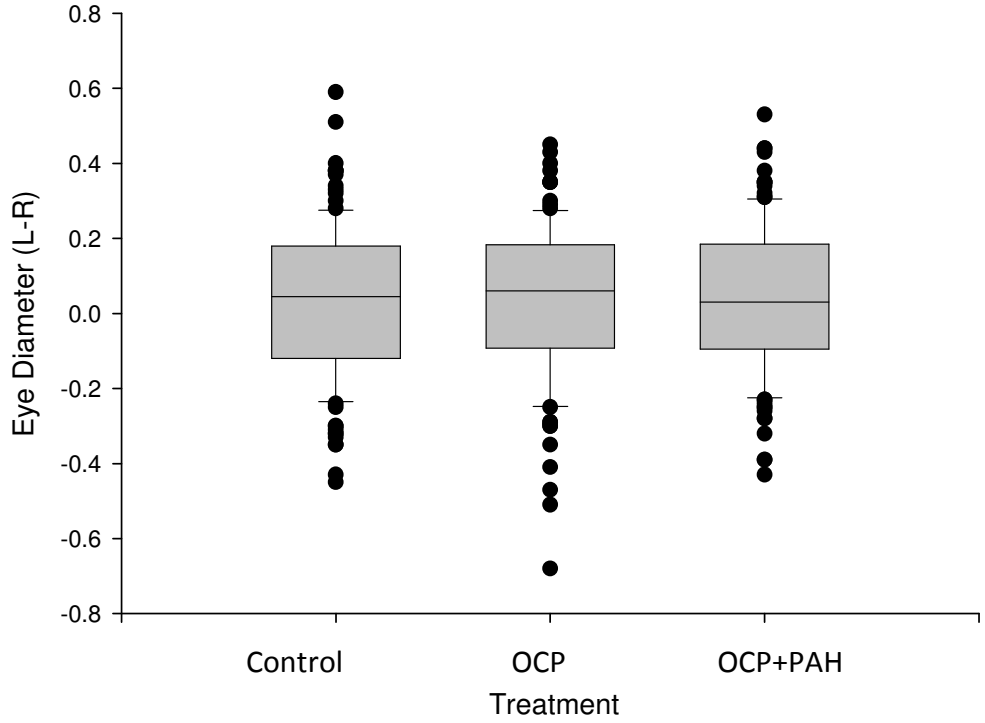

Figure 5.20. Eye width (L-R) of brown trout for each treatment. (Data represents Mean±SEM). n=120, 97, 120 respectively.

A normality test was conducted and the analysis showed the data did not have a mean of 0 in any of the treatments (control = 0.032, OCP = 0.029, OCP plus PAH = 0.042). A paired T test was carried out, comparing the left and right eye widths within each treatment. The control treatment indicated no significant difference between the left and right sides (T-test, t=1.723; *P*= 0.087). OCP treatment also showed no significant difference (T-test, t=1.334; *P*= 0.185). OCP plus PAH showed a significant difference between left and right eye diameters (T-test, t=2.334; *P*= 0.021), with left asymmetry more expressed.

The percentages of symmetry/asymmetry show high deviation towards the left in all three of the treatments and similar percentages of right asymmetry (Figure 5.21).

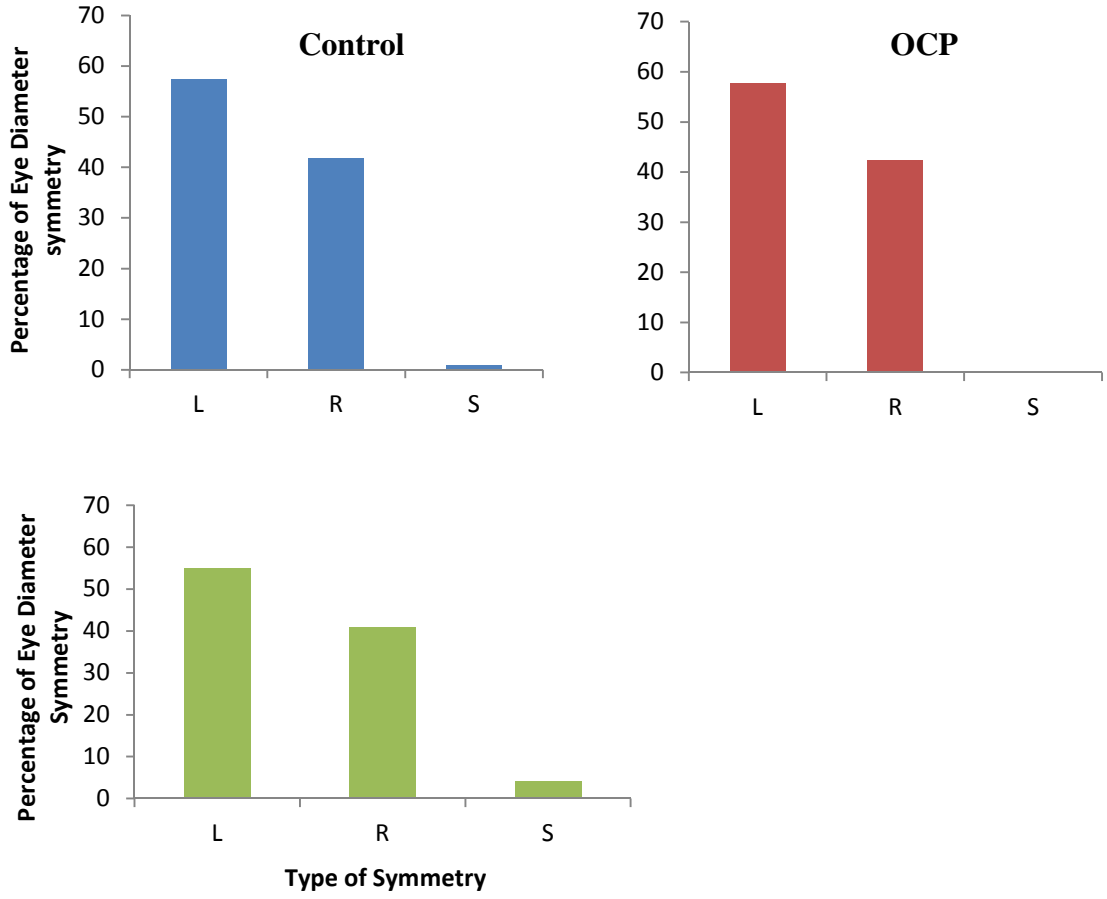

Figure 5.21. The type of symmetry (left (L), right (R) and symmetrical (S)) and the percentage of symmetry found for each treatment for eye width. n=120, 97, 120 respectively.

The results for the number of pectoral fins of the emerged fry are shown in Figure 5.22. No statistical significance was found between the pectoral fin ray counts using the formula L-R of the treatment groups (K, H=0.77; d.f. =2; *P*=0.681). Statistical analysis showed that treatment did not have a significant effect on the number of pectoral fin rays of the emergenced fry for neither the left side (Kruskal Wallis, H=0.15; d.f. =2; *P*=0.928) nor the right side (Kruskal Wallis, H=2.05; d.f. =2; *P*=0.359).

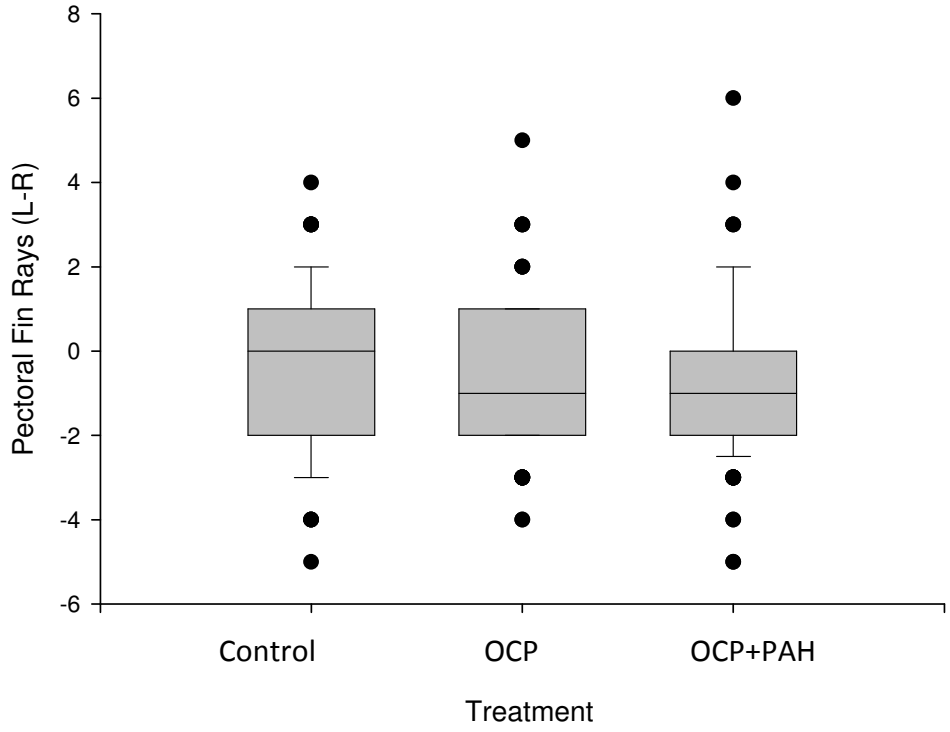

Figure 5.22. Pectoral fin ray counts (L-R) of brown trout for each treatment. (Data represents Mean±SEM). n=120, 97, 120 respectively.

A normality test indicated that none of the treatments have a normality distribution mean equal to 0 (control = -0.4833, OCP = -0.5258, OCP+PAH = -0.6333). However there were variations within the individual treatments. There were significantly more pectoral fin rays on the right side of the brown trout fry for the control group (T-test, t=-2.99; *P* =0.003), the OCP exposed group (T-test, t =-3.11; *P* =0.002) and the OCP plus PAH exposed group (T-test, t=-3.89; *P* <0.001). This can be observed in Figure 5.25 by the negative number of fin ray counts.

The percentage of symmetry was calculated using the L-R pectoral fin ray count data and can be seen in Figure 5.23. A similar pattern can be observed in all the treatment groups, identifying a greater tendency for right asymmetry. Higher percentages of bilateral symmetry were displayed for the pectoral fin rays compared to the eye measurements.

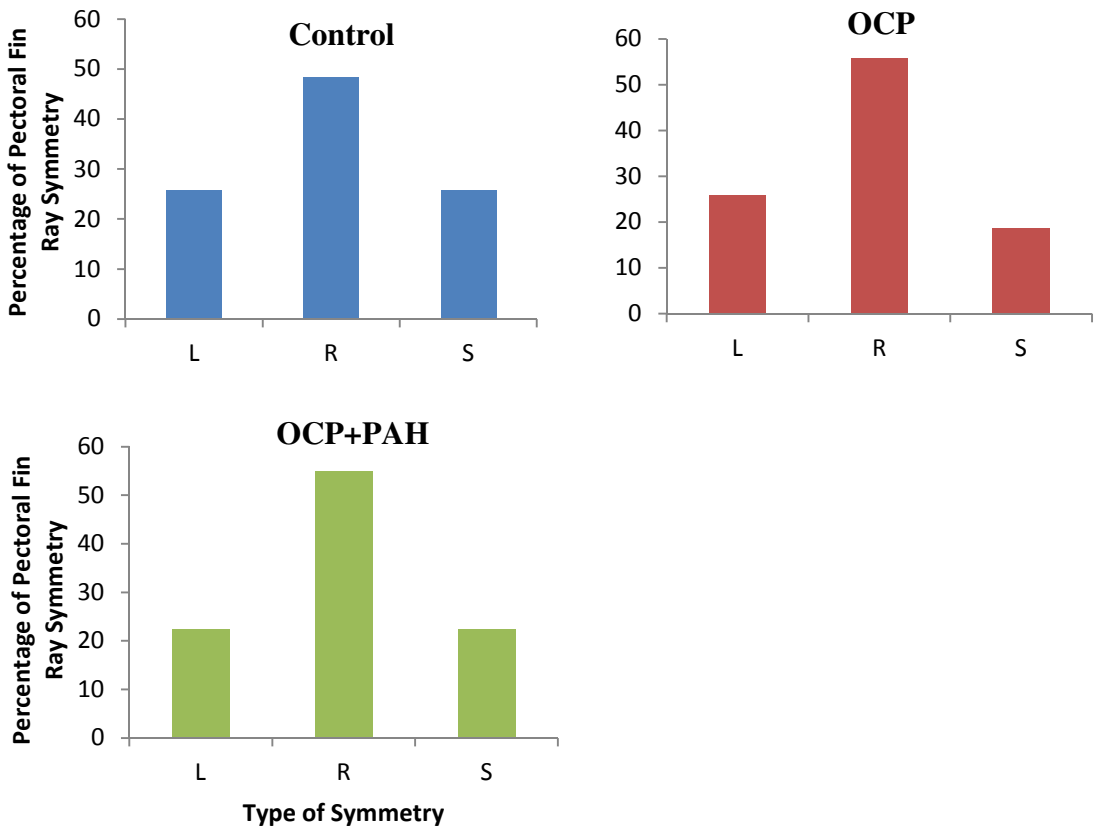

Figure 5.23. The type of symmetry (left (L), right (R) and symmetrical (S)) and the percentage of symmetry found for each treatment for pectoral fin ray counts. n=120, 97, 120 respectively.

The results for the number of pelvic fins of the emergence fry are shown in Figure 5.24. No statistical significance was found between the pelvic fin ray counts using the formula L-R of the treatment groups (K, H=0.60; d.f. =2; *P*=0.743). Statistical analysis showed that treatment did not have a significant effect on the number of pelvic fin rays of the emerged fry for neither the left side (K, H=2.99; d.f. =2; *P*=0.225) nor the right side (K, H=1.59; d.f. =2; *P*=0.452).

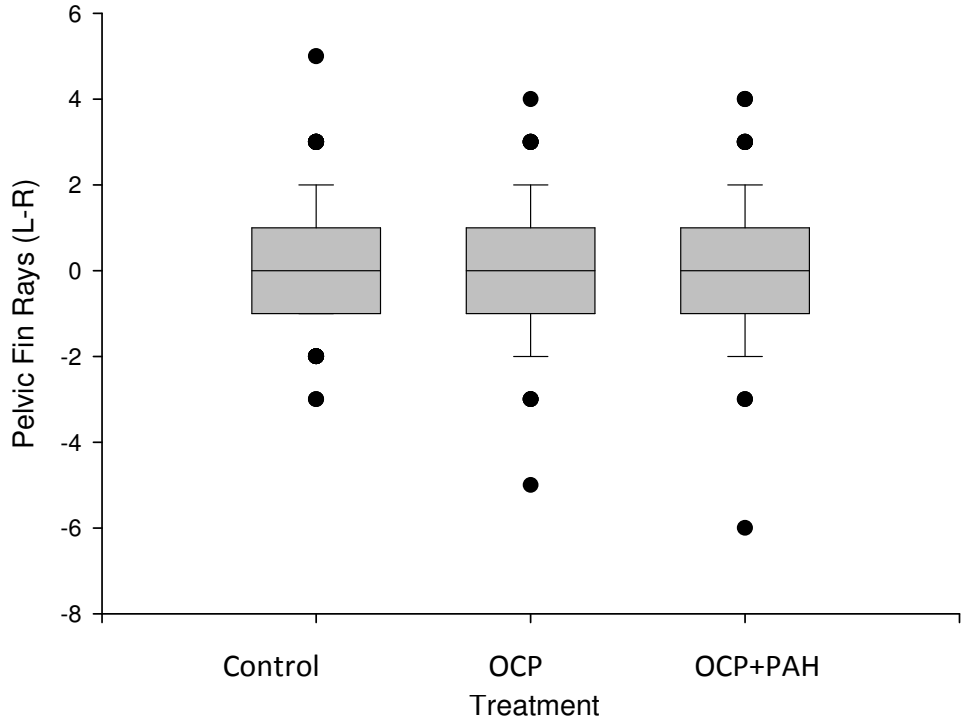

Figure 5.24. Pelvic fin ray counts (L-R) of brown trout for each treatment. (Data represents Mean±SEM). n=120, 97, 120 respectively.

A Ryan-Joiner test indicated whether a normal distribution was present and revealed the treatments to have mean values not equal to 0 (Control = 0.2417, OCP = 0.2268, OCP+PAH = 0.125). There were no significant differences between the left and right side for the pelvic fin ray counts within the control group (T-test, t=1.76; *P* =0.080), the OCP group (T-test, t= 1.41; *P* =0.162) or the OCP plus PAH group (T-test, t =0.88; *P* =0.378).

The percentage of symmetry for pelvic fin rays shows a gradual decrease between the three types of symmetry/asymmetry across all the treatments, with left asymmetry proving to be the most prominent (Figure 5.25).

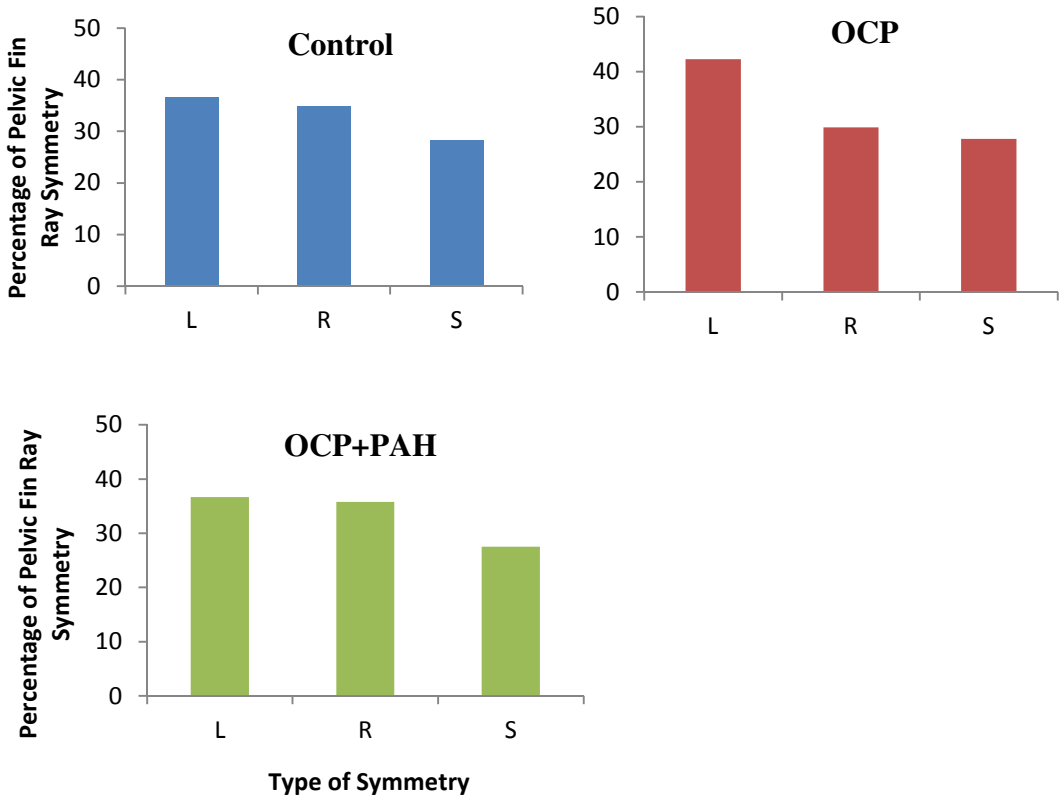

Figure 5.25. The type of symmetry (left (L), right (R) and symmetrical (S)) and the percentage of symmetry found for each treatment for pelvic fin ray counts. n=120, 97, 120 respectively.

5.3.2.3.2. Genotoxicity

Example images for the control and UV positive control comets can be seen in Figures 5.26 and 5.27 respectively. It is clearly visible that the comet tail for the positive control (Figure 5.27) is much greater than the control exposed group (Figure 5.26), which represents a greater amount of DNA damage.

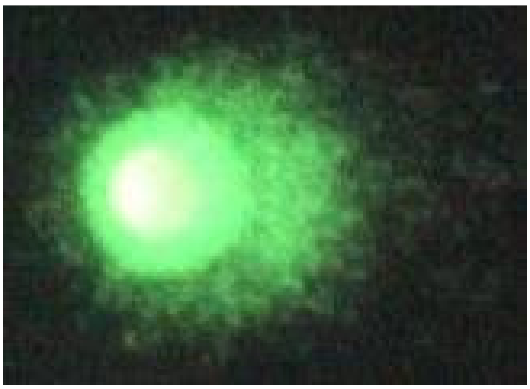

Figure 5.26. Comet from a blood cell of a control group fry.

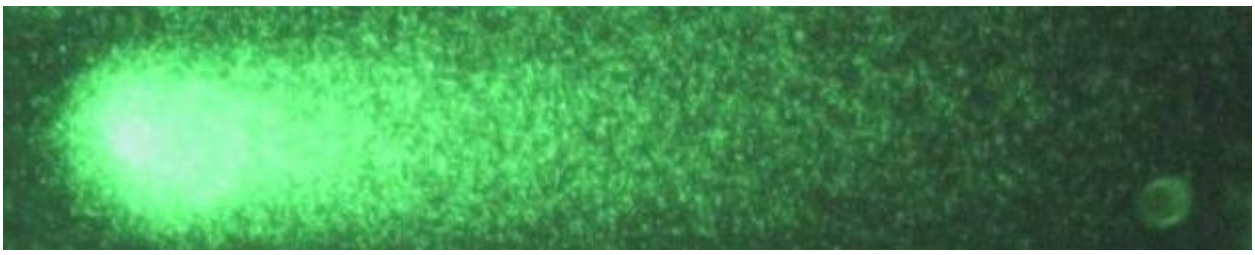

Figure 5.27. UV+ve Control (UV exposed blood cell to validate the assay).

The tail length measurements of the comets can be seen in Figure 5.28. Statistical analysis showed that there was a significant difference between the treatments. (ANOVA, F=64.85; d.f. =2; P<0.001). A pairwise comparison identified that the fry exposed to OCP and OCP+PAH treatments had significantly greater tail lengths than those fry in the control group.

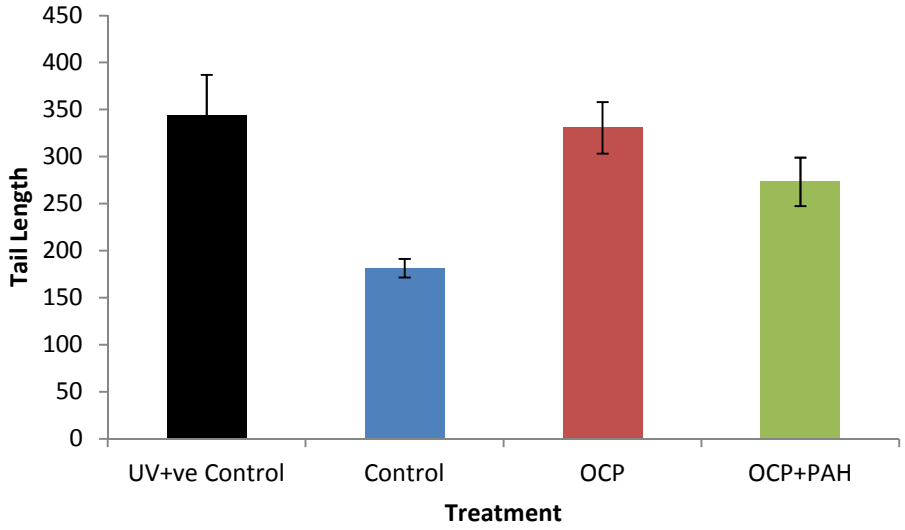

Figure 5.28. Comet Assay analysis showing Tail Length (Data represents Mean±SEM) n= 28 for each exposure treatment.

Images for the comets representing the OCP exposed fry can be seen in Figure 5.29, and OCP plus PAH exposed fry in Figure 5.30. These images are representative of the exposure groups and clearly show an extended comet tail which is indicative of DNA damage (Singh *et al.,* 1988).

Figure 5.29. Comet from the blood cell of an OCP exposed fry.

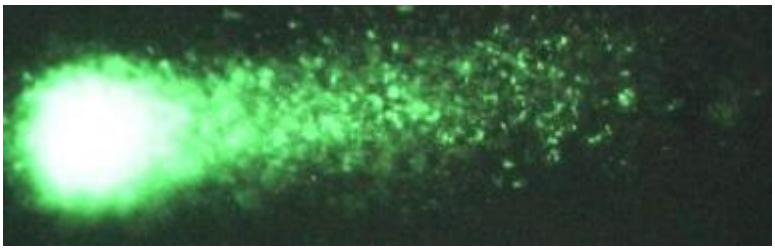

Figure 5.30. Comet from the blood cell of an OCP plus PAH exposed fry.

The tail intensity measurements of the comets can be seen in Figure 5.31. Statistical analysis showed that there was a significant difference between the treatments. (ANOVA, $F=49.25$; d.f. $=2$; $P<0.001$). A pairwise comparison identified that the fry exposed to OCP and OCP plus PAH treatments had significantly greater tail intensity than those fry in the control group.

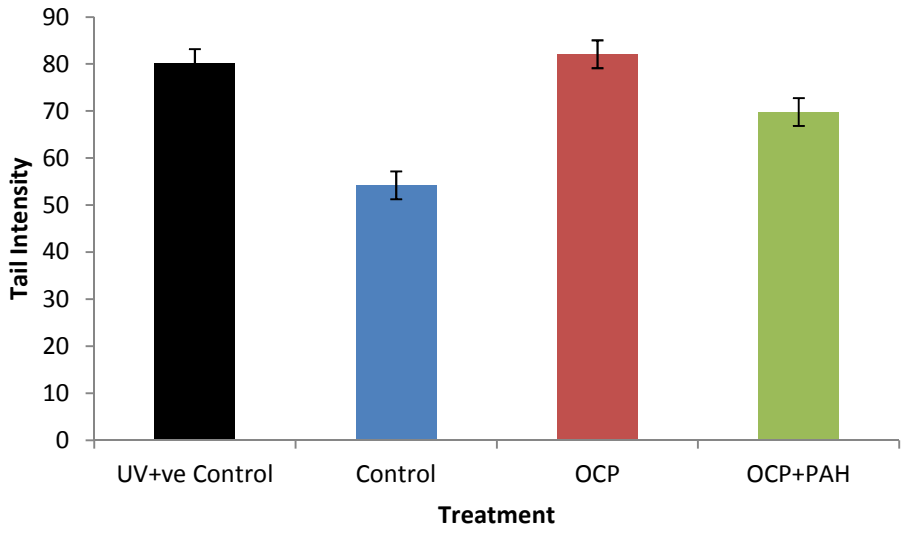

Figure 5.31. Comet Assay analysis showing Tail Intensity (Data represents Mean±SEM) n= 28 for each exposure treatment.

The tail moment calculations of the comets can be seen in Figure 5.32. Statistical analysis showed that there was a significant difference between the treatments. (ANOVA, F=64.81; d.f. =2; P<0.001). A pairwise comparison identified that the fry exposed to OCP and OCP plus PAH treatments had significantly greater tail moments than those fry in the control group.

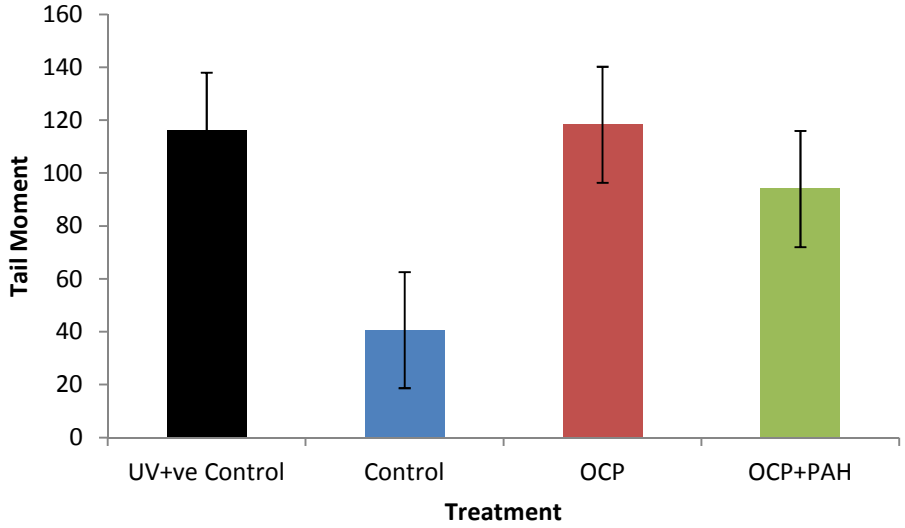

Figure 5.32. Comet Assay analysis showing Tail Moment (Data represents Mean±SEM) n= 28 for each exposure treatment.

5.3.3. Year 3

The daily temperature readings of the incubators are shown in Figure 5.33. The temperature ranged from 5-10°C.

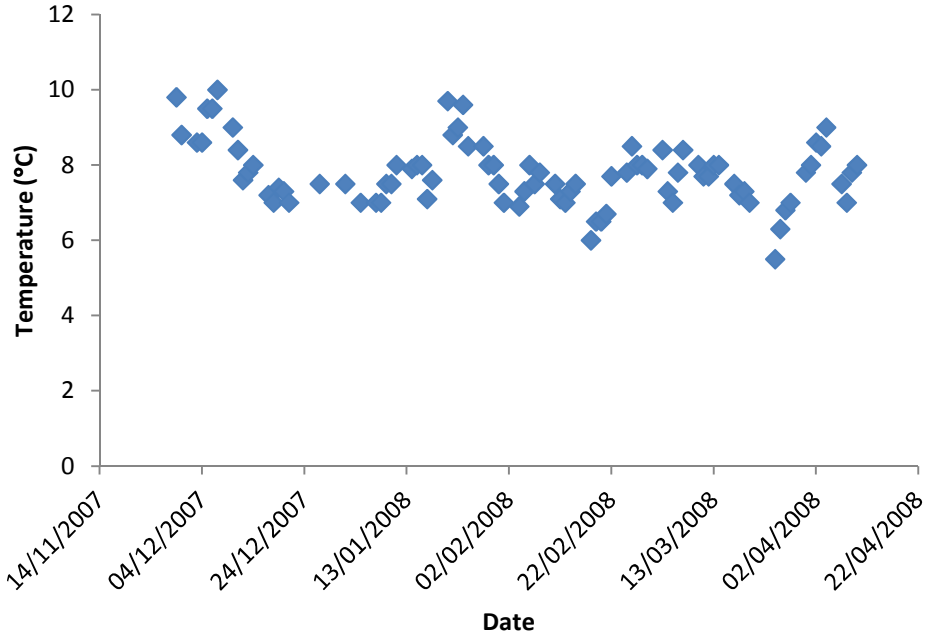

Figure 5.33. Daily temperature readings throughout the sediment exposure trial.

Figure 5.34 shows the mortality of the brown trout for the trial. Mortality was extremely high across the treatments.

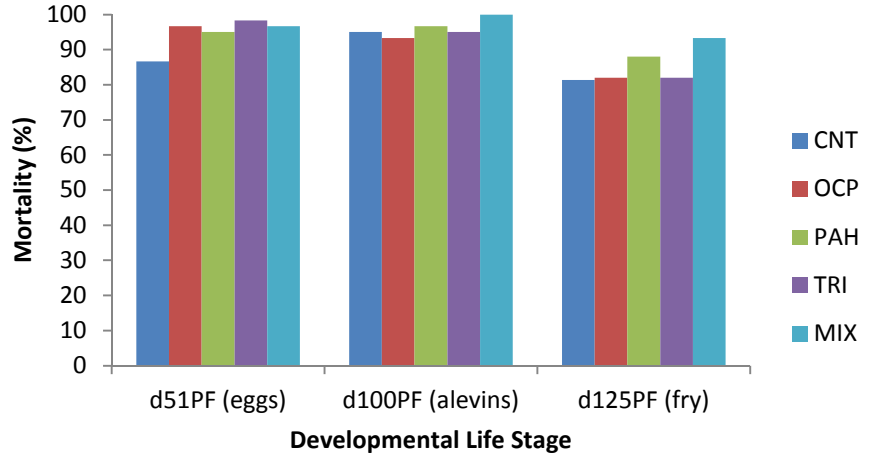

Figure 5.34. Mortality rates of brown trout at each stage of development. Data represents mean average percentage of mortality.

5.3.3.1. Egg Results (d51PF)

Table 5.18 shows the morphometric analysis data of the brown trout eggs. Statistical analysis showed that treatment had no effect on the egg weight (ANOVA, F=0.30; d.f. =4; *P*=0.874). Theer was also no significant effect of the factors tank (GLM (nested), F=0.05; d.f. =3; *P*=0.983) or egg box (GLM (nested), F=2.17; d.f. =3; *P*=0.121) on the egg weight.

Statistical analysis showed that treatment had no effect on the egg diameter (GLM (nested), F=8.06; d.f. =4; *P*=0.312). Tank (GLM (nested), F=0.26; d.f. =3; *P*=0.855) and egg box (GLM (nested), F=2.14; d.f. =3; *P*=0.125) selected also had no significant effect on egg diameter.

Statistical analysis showed that treatment had no effect on the egg volume (ANOVA, F=0.30; d.f. =4; *P*=0.874). Statistical analysis also showed that treatment had no effect on the egg surface area (ANOVA, F=0.29; d.f. =4; *P*=0.882) nor egg surface area to volume ratios (ANOVA, F=0.26; d.f. =4; *P*=0.903).

Table 5.18. 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
CNT	115.44±1.87	5.70±0.06	106.60±1.87	108.67±1.27	1.02±0.01	16
OCP	116.00±5.12	5.71±0.14	107.16±5.12	109.02±3.51	1.02±0.02	4
PAH	119.50±3.99	5.90±0.09	110.66±3.99	111.38±2.72	1.01±0.01	6
TRI	117.00±11.00	5.43±0.29	108.20±11.00	109.65±7.45	1.02±0.04	2
MIX	114.25±3.71	5.67±0.06	105.41±3.71	107.87±2.55	1.03±0.01	4

Table 5.19 shows the dry weight and water content of day 51 post-fertilisation eggs. Statistical analysis demonstrated that treatment did not have a significant effect on the dry weight of the d51PF eggs (GLM (nested), F=3.61; d.f. =4; *P*=0.519). Tank (GLM (nested), F=0.32; d.f. =3; *P*=0.809) and egg box (GLM (nested), F=0.95; d.f. =3; *P*=0.441) also had no significant effect on egg dry weight.

Statistical analysis demonstrated that treatment did not have a significant effect on the water content of d51PF eggs (GLM (nested), F=2.78; d.f. =4; *P*=0.263). Tank (GLM

(nested), F=1.46; d.f. =3; *P*=0.346) and egg box (GLM (nested), F=1.90; d.f. =3; *P*=0.170) also had no significant effect on egg water content.

Table 5.19. Egg dry weight and water content analysis (Data represents Mean±SEM)

Treatment	Dry Weight (mg)	Water Content (%)	N value
CNT	41.36±0.64	62.42±0.48	11
OCP	39.75±2.25	63.40±0.81	4
PAH	42.67±1.02	61.91±0.60	4
TRI	39.50±2.50	63.47±1.23	6
MIX	42.25±1.49	58.47±0.73	2

5.3.3.2. Alevin Results (d100PF)

Figure 5.35 shows the alevin lengths at day 100 post-fertilisation. Statistical analysis showed that treatment had no effect on the alevin length (GLM (nested), F=0.54; d.f. =3; *P*=0.697). No alevins survived in the incubators dosed with mix-exposed sediment, therefore no results for this group are shown. Tank (GLM (nested), F=0.34; d.f. =2; *P*=0.780) and egg box (GLM (nested), F=4.05; d.f. =1; *P*=0.100) also had no effect on the alevin length.

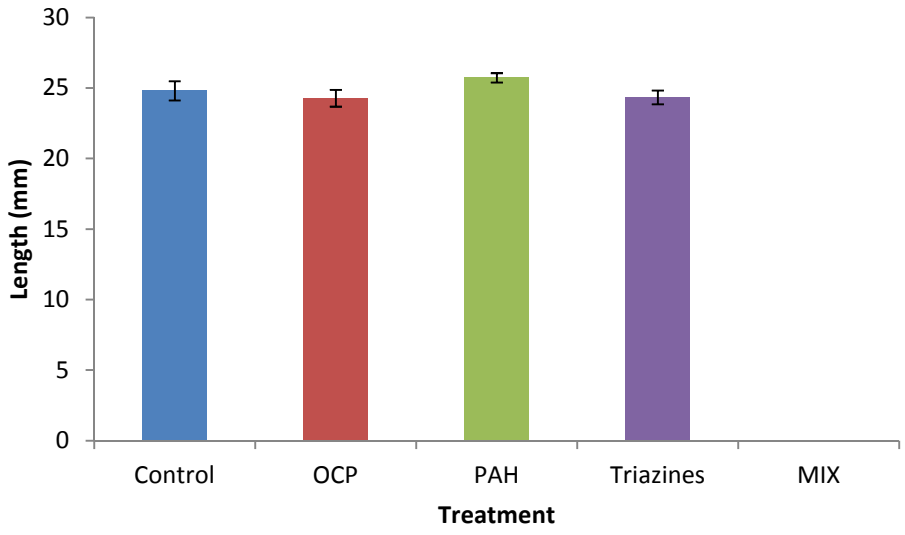

Figure 5.35. Lengths of juveniles at day 100 post fertilisation (Data represents Mean±SEM). n=3, 4, 2, 3, 0 respectively.

Figure 5.36 shows the alevin weight at day 100 post-fertilisation. Treatment had no effect on the alevin weight (GLM (nested), F=0.29; d.f. =3; *P*=0.832) nor did tank (GLM (nested), F=0.20; d.f. =2; *P*=0.846). However, egg box did have a significant effect on the

alevin weight (GLM (nested), F=15.57; d.f. =1; *P*=0.011). One egg box in the PAH treatment tank had a significantly greater weight than those in boxes of other treatments.

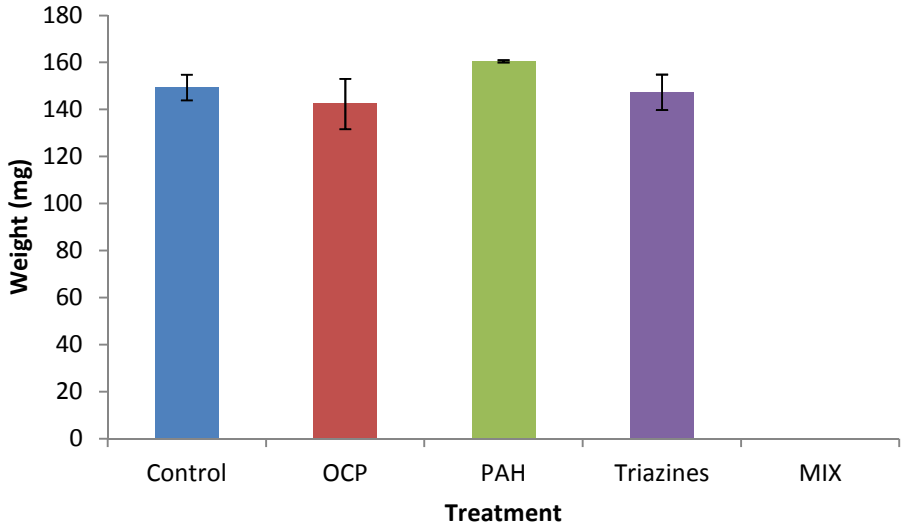

Figure 5.36. Weights of juveniles at day 100 post fertilisation (Data represents Mean±SEM). n=3, 4, 2, 3, 0 respectively.

5.3.3.3. Fry Results (d125PF)

Table 5.20 shows the morphometric analysis of the day 125 post-fertilisation fry. Treatment was found to significantly effect the weight of the fry (ANOVA, F=2.95; d.f. =4; *P*=0.024). A pairwise comparison indicated that the mix exposed fry were significantly lighter than the control group. Treatment also significantly effect the length of the fry (ANOVA, F=2.74; d.f. =4; *P*=0.032). A pairwise comparison indicated that the mix exposed fry were significantly shorter than the control group. However, the condition factor of the fry demonstrated that treatment did not have a significant effect on the condition factor of the emerged fry (ANOVA, F=2.21; d.f. =4; *P*=0.072).

Table 5.20. Morphometric analysis of *Salmo salar* fry (Data represents Mean±SEM)

Treatment	dPF	Weight (mg)	Length (mm)	Condition Factor (*K*)	N value
CNT	125	265.89±5.97	30.90±0.21	0.90±0.01	28
OCP	125	243.50±11.20	29.93±0.28	0.89±0.02	27
PAH	125	246.80±14.00	30.52±0.43	0.88±0.03	18
TRI	125	256.89±8.99	30.46±0.30	0.90±0.02	27
MIX	125	204.40±21.20	29.16±0.66	0.80±0.04	10

Table 5.21 shows the dry weight and water content of the day 125 post-fertilisation fry. Statistical analysis demonstrated that treatment did have a significant effect on the fry dry weight (ANOVA, F=2.80; d.f. =4; *P*=0.042). A pairwise comparison identified that mix and OCP exposed fry had a lower dry weight than the control group, as well as PAH and triazine exposed fry. Statistical analysis also demonstrated that treatment did have a significant effect on the fry water content (ANOVA, F=2.80; d.f. =4; *P*=0.042). A pairwise comparison identified that triazine and PAH exposed fry had a lower water content compared to the control group.

Table 5.21. Dry weight and water content of *Salmo salar* fry (Data represents Mean ±SEM)

Treatment	dPF	Dry Weight (mg)	Water Content (%)	N value
CNT	125	41.36±0.64	62.42±0.48	11
OCP	125	39.75±2.25	63.40±0.81	4
PAH	125	42.67±1.02	61.91±0.60	4
TRI	125	39.50±2.50	63.47±1.23	6
MIX	125	42.25±1.49	58.47±0.73	2

5.3.4 Summary

Table 5.22 is a summary matrix of the significant results observed in the treatments across all three years of experiments. Any significant effects as a result of the tank or the egg box are not listed in the summary table.

Table 5.22. Summary matrix of significant results for sediment exposure trials

	Mortality	Egg/Alevin/Fry Morpometrics	Egg/Alevin/Fry Biochemistry
Year 1	•Lowest egg mortality 14% (PAH)-51%(CNT) at d35PF. • Highest mortality 59%(CNT)-70%(SOL) at d49PF. • Lowest mortality (39.5%) at d70PF in PAH. • Lowest mortality (56.5%) at d98PF in CNT.	•PAH greater alevin weight than SOL and CNT at d70PF.	•PAH greater egg dry weight than CNT at d49PF.
Year 2	• Highest mortality at egg stage 58%(OCP+PAH)-69% (CNT). • Lowest mortality (51%) at d68PF in OCP+PAH. •Lowest mortality at fry stage 30.17% (OCP+PAH) 40%(CNT).	•No significant effects	•OCP+PAH eggs greater chloride than CNT and OCP.
Year 3	• High mortality at egg stage 86.66%(CNT) – 98.33%(TRI). • Highest mortality at alevin stage 93.33%(OCP) - 100%(MIX). •High mortality at fry stage 81.33%(CNT) – 93.33% (MIX).	•MIX smaller length and weight than other treatments.	•TRI and PAH smaller dry weight and water content than CNT.

5.4. Discussion

Very few studies have examined the effects of sediment-bound contaminants on aquatic species. The research carried out for this chapter measured the effects of a selection of contaminant groups; groups of compounds, and mixes of compound groups. The exposure trials were conducted at environmentally relevant levels so any significant effects of these sediment-bound contaminants can be directly related to natural conditions and could be used to predict possible impacts of pollution on wild salmonid populations in the Avon catchment. This chapter looked at acute and sublethal effects of the sediment-bound contaminants on the early life stages of brown trout.

The laboratory environment eliminated other variables that could effect the survival of brown trout which could not be done *in situ* in rivers. Dumas *et al.* (2007) found that redds positioned upstream from fish farms appeared of better quality than those downstream of the effluents. The interstitial water quality of redds was influenced by that of surface water and nitrogen contents were of the same magnitude. Therefore other contaminants present

in the natural environment, such as those associated with fish farm effluent, would not have influenced the results of a controlled laboratory environment.

The sediment-bound contaminant trials was not however able to remove the possibility that sedimentation itself could be playing a part in the brown trout survival. For all of the exposure trials, the control group consisted of sediment which had not been spiked with contaminants. Optimal survival to emergence of brown trout alevins was found in gravel with a size of 18mm. Finer gravel resulted in a lower survival rate although the gross water flow was the same through all the redds independent of gravel size (Olsson and Persson, 1986). Therefore, it is possible that the fine sediment alone had a contributing factor in survival of the early life stages of the *Salmo trutta*. Survival of the early life stages is paramount for subsequent survival but during this period there are high mortalities in terms of sedimentation. This is mainly due to clogging of the gravel interstices reducing intragravel oxygen supply to eggs and alevins (McNeil, 1962; Guerrin and Dumas, 2001a).

The survival data showed that the control treatment groups had a greater percentage of mortality compared to the contaminant exposed groups for all three years of trials. This was particularly evident for the d35PF eggs in the first trial. Reasons for this observation are unclear, as once the trout eggs were fertilised, they were randomly distributed among the incubators. However there is already a naturally high mortality rate of salmonid embryos without the added effects of pollutants (Weis & Weis 1989). The final trial possibly demonstrated this naturally high mortality rate, as few eggs, alevins and even fry survived the experiment. Therefore it is unfortunate that the n-values for this final year trial were very low.

5.4.1. Morphometric measurements

For Year 1, there were no significant morphometric differences observed in either the day 35 or day 49 post fertilisation eggs. Although there were significant variations noted within the replication. Therefore the data suggests that there was a requirement for a greater number of true replication for the trial, rather than pseudo-replication. However, the experimental design for the sediment trial was limited to the facilities which were available at the CEFAS laboratory. The majority of the parameters measured to assess the toxicity showed no significant differences (morphometric and some biochemical) between the non-

solvent control and the solvent controls, however there were a few significant differences in biochemical analysis (protein, NPS and water content). Therefore at the time it was decided that the non-solvent control group would remain for further trials and the solvent control would be omitted. However, ideally both controls should have remained for subsequent sediment-bound contaminant experiments. For the day 70 post-fertilisation alevins it was shown that the PAH exposure increased the weight of the fish compared to the control alevins. Frost and Brown (1967) state that eggs of equal size produce equal size fry ready to feed at all temperatures between 7 and 12˚C. So as there were no significant differences in the egg weight or diameter, the larger PAH exposed alevins may indicate a slower adsorption of the yolk sac or premature hatching. It has been suggested that different sizes in hatching become more pronounced at a later stage of development (Debowski *et al.,* 2006) Although there is still a possibility that the few significant differences observed are an indication that the sediment-bound contaminants used in this trial did not effect the development of the early life stages of *Salmo trutta*.

For Year 2, no significant morphometric differences were observed at any of the developmental stages of the *Salmo trutta*. Although, as with the previous trial, significant variation was shown within the replications in term of the tanks and the egg boxes. Again this suggests a requirement of a greater number of tanks for each contaminant group. This trial only consisted of one control group, as the solvent control group was dropped in order to expose the brown trout to another group of contaminants. As there were very few significant differences observed between any of the treatments in the first year trial it was considered that, at the time, the omission of the solvent control was the most appropriate. However, it does suggest that the interpretation of any significant future results, with just the use of the non-solvent control, should be done so with caution. There were also no significant differences in the alevin or fry morphometrics. The results suggest that the contaminants used in this trial did not promote early emergence, as this would be represented by a greater yolk sac score. Frost and Brown (1967) found that outside this temperature range (7-12˚C) the alevins are smaller because the less favourable temperatures cause them to use more yolk for energy and less for growth purposes.

For Year 3, the only morphometric differences were observed in the day 125 post-fertilisation fry. The mix exposed fry had smaller lengths and weights compared to the

other treatments. Interestingly it is only the mix treatment which had a significant effect on the fry in this trial. This may be a result of an interaction between the compounds within the mix treatment. Hayes *et al.* (2006) noted that the mortality of Rana pipiens and Xenopus laevis increased with a nine compound mixture (35%) compared to a single pesticide exposure (4%). It has been suggested that some pesticides enhance to toxicity of other compounds when combined (Hayes *et al*., 2006).

Osterauer and Köhler (2008) also found hatching to occur early with increasing temperature. In the laboratory experiment the temperature was kept consistent and was regularly monitored which most likely explains the similarity in size. However in their natural environment the temperature is more prone to fluctuations due to a varying climate, so the outcome may result in varied egg and alevin sizes.

For Year 3, treatment had no significant effect on the egg or alevin morphometrics. However if a greater number of brown trout had survived this trial, a larger sample size may have generated a different result. However, the results for the fry emergence study showed that the mix exposed fry were lighter and smaller in length compared to the control group. It has been suggested that the difference in hatching size can become more pronounced at later stages of development (Debowski *et al*., 2006), so it is possible that the mix exposed *Salmo trutta* may have hatched later with smaller yolk reserves. Smaller fry could result in poor recruitment, and potentially population growth, if migration is influenced (Debowski *et al*., 2006).

Yolk sac oedemas were recorded for the contaminant and control groups. For Year 1, the PAH exposed alevins displayed a greater frequency of yolk sac oedemas than the control. The solvent control did not score positive for any yolk sac oedemas. PAH exposure has been found to cause a range of sub lethal effects including yolk sac oedema (Barron *et al.*, 2004; Incardona *et al*., 2004). Yolk sac oedemas were also found to be more frequent in the contaminant exposed alevins (OCP and OCP plus PAH). The presence of yolk sac oedemas could be a sign of 'blue sac syndrome'. In the early life stages of zebrafish, certain PAHs have been associated with 'blue sac syndrome' which is identified from various developmental defects such as reduced growth, crania-facial malformations, yolk sac oedema and subcutaneous haemorrhaging (Incardona *et al.*, 2006). An oedema is the

accumulation of fluid within the yolk sac so effectively increases the size of the yolk sac. A study on emergence of brown trout noted that premature alevins were both smaller and had larger yolk reserve than alevins at peak emergence. Since alevins with a large yolk reserve are poor swimmers, they can probably be easily captured by predators (Olsson and Persson, 1986). A yolk sac oedema, among other things, would impede the swimming ability of the alevin and therefore could inhibit successful recruitment. However, it must be remembered that overall fry survival was not significantly affected in these groups despite the presence of oedemas, so at a population level this had little or no obvious impact.

Hose *et al.* (1984) noted four major anomalies in rainbow trout embryos which were continuously exposed to the PAH, benzo[*a*]pyrene, from fertilisation through to hatching. These malformations included ocular defects, neural defects, skeletal defects and haematologic defects. Thus suggesting that such morphological abnormalities could significantly decrease feeding, reduced growth, increased predation and therefore reduced survival (Hose *et al.,* 1984; Rosenthal and Alderdice, 1976). Such research corroborates with the current study as brown trout exposed to PAH were shown to have a higher frequency of yolk sac oedemas as well as displaying reduced growth on hatching. Oliveira Ribeiro *et al.* (2005) looked at the effects of OCPs, PAHs and heavy metals on the European Eel (*Anguilla anguilla*) when taken in through the gills, skin and contaminated foods. The European eels live in both the sediment and the open water, within a wetland environment, for 9-15 years, prior to migration. They found high concentrations of OCPs in the muscles of the eel, which may have occurred through the skin (percutaneous route). The eels they sampled were all from a reserve in the catchment area of different agricultural activities, so the effects the eels have experienced result from long term exposure (Oliveira Ribeiro *et al.*, 2005). It is possible that the eels were exposed to contamination of the water, and thus the infiltration of food sources, or may be the result of the compounds binding to the sediment in which the eels inhabit. The chronic exposure may have entered the eel species at an early life stage, which may explain the extensive contamination within the organs of the eels (Oliveira Ribeiro *et al*., 2005).

The condition factor (*K*) represents how proportionate the salmonid's weight is to the length. It aids in deciphering whether the fish stocks are developing at an expected rate and indicate if any pollutants are causing abnormal effects, inhibiting even growth. The

condition factor values represented no treatment dependent significant differences for all trials. The values ranged from 0.74-0.79, 0.79-0.90 and 0.95-0.97 for Year 1,2 and 3 respectively. The *K* values were less than 1 which represents a poor conditioned fish. *K* is the measurement of the individual fish's well being, its fatness and the state of its gonads. Literature has indicated that the condition factor of fry of diploid Atlantic salmon ranges from 1.05-1.11 (O'Flynn *et al*., 1997). However, the lower values in this current study is potentially a result of the measurements of length and weight being taken from newly emerged fry with a recently absorbed yolk sac, as K is usually found to be higher in mature fish with ripe gonads (Frost and Brown, 1967). Therefore it seems that sediment-bound contaminants had no effect on the condition factor of the salmonids in this trial.

5.4.2. Biochemical analysis

Treatment had few effects on the biochemical analysis of the brown trout eggs for any of the trials. In Year 1, the PAH exposed eggs had a significantly larger dry weight than the control group eggs for day 35 post-fertilisation. In Year 2, the OCP plus PAH exposed day 47 post-fertilisation eggs had a greater chloride concentration than the control eggs. Chloride measurements give an indication of the ionic regulatory ability of the eggs, as the chloride inside the egg would be expected to be greater than the environmentso that there is effective excretion of metabolic waste. However as the contaminant exposed eggs had a greater concentration of chloride than the controls, this may indicate the regulation of ion concentrations in the egg may have been effected. An increase in ions within the egg may result in a greater movement of water into the egg. However there were no significant differences in the water content of the eggs. No significant differences were observed for the NPS levels, protein concentration and the body weight and yolk sac weight (as a percentage of total weight) for either Year 1 or Year 2. As a result of the high mortality there were insufficient numbers for protein analysis and body and yolk sac weight in Year 3 exposure trial. However, the day 125 post-fertilisation fry in the final trial did show a significantly reduced dry weight in the OCP tanks and a reduced water content in the triazine and PAH tanks compared to the control groups.

5.4.3. Fluctuating Asymmetry

The early life stages of various fish species are considered to be a useful early bioindicator of pollution in freshwater systems as during the incubation period, salmonids are in direct contact with sediment during their embryonic stage. Fish embryos are ideally suited to use as a monitoring and warning system, providing information on the effects of sediment-bound pollutants (Vigano *et al*., 1995). Fluctuating asymmetry is a population parameter that measures random deviations from perfect symmetry in bilaterally symmetric traits (Øxnevad *et al.*, 2002). Some authors dispute the use of FA stating current methods of analyses usually employ FA of single traits and appear to be weak and unreliable and it is suggested that composite measures of FA can provide vast advantages over single traits in terms of the probability of detecting FA differences between populations (Leung *et al*., 2000). However, FA is widely used as a measure of developmental stability, which is the ability to regulate development and produce the desired genetically determined phenotype despite environmental perturbations (Øxnevad *et al.*, 2002). FA has been proposed as a tool to monitor stressors in the environment (Allenbach *et al.*, 1999). As well as an indicator of environmental conditions FA has also been used in various studies as a potential indicator of genetic stresses such as inbreeding (Wagner, 1996), captivity stress (Almeida, 2008) and the overall condition of the larvae (Somarakis *et al*., 1997). In fish, both individual and population levels of bilateral asymmetry have been shown to positively relate to a wide range of biotic, abiotic and genetic stresses. Certain abiotic factors, including heavy metals, toxic chemicals and acidification, commonly cause elevated levels of FA (Almeida *et al*., 2008). Østbye *et al*. (1997) found the majority of meristic and morphometric characters they examined to exhibit high mean FA values in populations in aluminium rich acidic lakes than those in unaffected lakes. Aluminium can be stored within sediment and rocky substrate. This heavy metal is very toxic when in its free ion aqueous state (Al^{3+}) and unbinds from the substrate when the pH of the water drops below 6, becoming acidic. Perch spawn in the spring, which coincides with the time when acid and aluminium levels are at their highest due to snowmelt, leading to exposure at a very early life stage (Østbye *et al*., 1997).

FA indications were observed in some of the morphometric traits of the fry. Eye height was shown to be greater on the right hand side of the fry compared to the left, however

these findings did not represent a treatment dependent effect as all groups displayed the same significant differences. Although for the eye width, only the OCP plus PAH exposed fry displayed signs of FA. Eye width was significantly greater in the left eye than the right in the brown trout exposed to OCP plus PAH. Allenbach *et al*. (1999) exposed mosquitofish (*Gambusia affinis*) and sand shiner (*Notropis ludibundus*) to two insecticides, parathion (an organophosphate) and lindane (an organochlorine). They found eye diameter to exhibit a normal distribution and only a small proportion of the samples showed asymmetry in eye diameter.

Some of the meristic traits of the fry observed did indicate FA. Pectoral fin ray counts suggested FA as there were a greater number of rays on the right side of the fry compared to the left. This was indicative of all the treatment groups. The control group would be expected to possess close to perfect symmetry with the ideal fusiform shape. The fusiform design, of many aquatic species, reduces friction drag. This streamline shape is most efficient with smooth body surfaces, small scales and non bulging eyes. The perfect fusiform shape is expressed as the diameter of the width of the fish is equal to a quarter of their length (maximum width = 0.25 x length). The pelvic fin rays displayed no signs of FA. Such findings corroborate with that of Allenbach *et al.* (1999). Pelvic fin rays were shown to display low asymmetry when exposed to pesticides. Although Allenbach *et al*. (1999) also found pectoral fin rays were determined significant following exposure to pesticides. Results from this trial did not show a significant difference between the treatments which suggests that the sediment-bound contaminants did not encourage FA.

Chang *et al.* (2007) found that insecticides and temperature, and their interaction, had a significant effect on some traits of FA of larval damselflies. However, low FA was observed in those survivors from treatments with high mortality. Sediment-bound contaminants have shown to possibly cause some traits of FA in eye width. However, as other measured traits were also found in the control group there is no evidence to suggest that FA was a result of the exposure to the specific contaminants, or even that any effects are a result of just the presence of sediment alone.

Fluctuating asymmetry is a population parameter that measures random deviations from perfect symmetry in bilaterally symmetric traits (Øxnevad *et al*., 2002). FA is often used as

a measure of developmental stability, which is the ability to regulate development and produce the desired genetically determined phenotype despite environmental perturbations (Øxnevad *et al*., 2002). FA has also been proposed as a measure of developmental precision, which is the net outcome of the events from developmental noise and developmental stability (Allenbach *et al*., 1999). However exposure to sediment-bound contaminants did not induce FA during the early stages of development in salmonids. However, this does not indicate whether traits of FA could be observed at later stages of development in salmonid fish.

5.4.4. Genotoxicity

Enzymes and receptors required to allow the contaminants to act may not be present before the alevin 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). Sublethal effects of sediment-bound contaminants were investigated using the technique of the comet assay.

In order to accurately analyse the results for the comet assay, three parameters were measured; tail length, tail intensity and the preferred tail moment. The healthy DNA remains within the cell nucleus. One way to quantify the amount of DNA damage is by combining the length of the migrated DNA fragments, the tail, by the distribution of the DNA in the tail, tail intensity. These two important quantities define the tail moment. (Nelms, 1997). For all three suggested methods of analysis the same significant result were appararent. Both contaminant groups, OCP and OCP plus PAH exposed fry displayed greater DNA damage compared to the control group. So despite few observations of acute toxicity of brown trout to sediment-bound contaminants there is evidence to suggest sublethal effects of these compounds. Other studies have noted the possibility of unknown sublethal effects of contaminants on aquatic organisms. Research on polychaetes showed no significant acute effects on larval settlement, mean numbers of larvae settling in PAH contaminated sediments were consistently lower than PAH-free controls. Chandler *et al.* (1997) suggested that the absence of a PAH effect on *Streblospio benedicti* settlement may have been due to these compounds having no acute toxicity, but possibly more long-term

chronic effects. Recent research has shown that atrazine can induce significant DNA damage in the freshwater fish *Channa punctatus* and was found to be both concentration and time dependent (Nwani, 2011), which demonstrates the genotoxic potential of this triazine. The comet assay has been proven to be a sensitive tool in assessing genotoxic effects in fish (Nwani, 2001). Unfortunately the comet assay in the final sediment-bound contaminant trial did not work, so no molecular analysis of the triazine exposed fish was undertaken to see if this study found triazines to be genotoxic.

This trial has shown the comet assay to be a useful tool in assessing the toxicity of sediment-bound contaminants. It has shown the potential sublethal effects of xenobiotics which may not have been apparent as acute toxicity. Other studies have also shown the ability of the comet assay to show the genotoxicity potential of contaminants. A study looking at the effects of the PAH benzo[*a*]pyrene (BaP) and endosulfan sulphate on oyster embryos observed a positive correlation between oyster embryotoxicity and comet assay data, indicating the significance of genotoxicity in the molecular mechanisms of BaP embryotoxicity. The genotoxic effects of the environmental pollutant was measured by oxidative DNA damage, in terms of DNA strand breaks. The results suggested the oysters ability to biotransform the contaminants from early stages. Bioactivation is thought to result in the induction of an oxidative stress involved in the measured oxidative DNA damage (Wessel *et al.*, 2007).

Many tissues and cell populations, both in development and in adult stages, undergo apoptosis. Such a cellular event in the early life stage has many potential implications for future development. Analysing single cells for apoptosis helps in understanding the ability and the extent of the cells response to external stimuli, such as xenobiotics. The comet assay allows for the analysis of apoptosis of cells by measuring the quantity of DNA fragmentation and extent to which the fragments migrate away from the remaining cell body under an electric current (Nelms, 1997). Tail moment calculations for the contaminant exposed fry in the trial were shown to cause apoptosis. Dependent on the system used, a tail moment of greater than about 110 is indicative of apoptosis (Nelms, 1997). The tail moment in this trial for the OCP-exposed brown trout did indeed exceed the value of 110, thus indicating apoptosis of the cells. The PAH-exposed fish did also demonstrate levels close to those of apoptosis (~100). In a study by Cantrell *et al.* (1998) a

dose dependent survey of tissue specific apoptotic cell death and P450 expression revealed the embryonic vasculature in medaka was highly reponsive to the hydrocarbon TCDD. At sublethal concentrations the medaka embryos expressed apoptosis in the gill and digestive tissue suggesting the function of these tissues may be compromised. Although it must be noted that some DNA damage shown by the strand breaks in the comet assay technique could be reversible (Frenzilli *et al*., 2004). Therefore it is recommended that in further research the comet assay should be incorporated with another genotoxicity test, such as the micronucleus assay (Frenzilli *et al*., 2009; Vasquez, 2010). There are many advantages to the comet assay, the results are reproducible (Vasquez, 2010), it is very fast, sensitive (Vrzoc and Petraz, 1997) and can effectively detect the earliest stages of cell death (Vasquez, 2010) and only a small number of cells are required (Frenzilli *et al*., 2009) which could prevent destruction of test organisms. It the case of this study, comet assays could be run on the blood of smolts and adults without the need to destroy the fish.

Other studies have also shown sediment-bound contaminants to cause sublethal effects in early life stages of fish. Strmac *et al.* (2002) noted an increase in the mortality of zebrafish embryos following exposure to polluted freshwater sediments and Luckenbach *et al.* (2001) suggested that PAHs in sediment contribute to sublethal effects observed in brown trout embryos. This study also demonstrated the potential effects of sediment-bound contaminants on the early life stages of salmonids, in particular, at a molecular level.

5.4.5. Conclusions

There have been various effects of different sediment-bound contaminants at environmentally significant concentration levels on the very early life stages of brown trout. Trials have investigated the effects of sediment-bound contaminants on the fertilisation, egg development, hatching, alevin development and emergence of the brown trout. However, there were few significant results in terms of acute toxicity. There are several possible reasons why particular visual, morphometric and chemical reactions were not observed as a result of contaminant exposure. The sediment matrix is complicated, so it is possible that the contaminants used to spike the sediments were not bioavailable to the developing brown trout during the time scale of the trial. Other environmental factors, such

as temperature, may be an important variable in the release of adsorbed xenobiotics from the sediment particles. Also burrowing invertebrates play a key role in releasing contaminants from particulates in bioturbation in field situations (Reible *et al*., 1997; Qin *et al*., 2010). Under laboratory conditions such functions would not have existed.

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 by human eye or even microscopes, 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 *et al*., 1992 and Moles *et al*., 1981) and potential failing to be successful spawners and recruiters. 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 and Alderdice, 1976). However, with the technique of the comet assay, genotoxicity was investigated for sub-lethal effects.

The DNA damage analysis failed to work in the Year 3 trial. It is possible that data collected from such an assay may have given a better insight to the sub-lethal effects of other sediment-bound contaminants. The results from the comet assay suggested that sediment-bound contaminants do promote DNA damage in salmonids. As many studies suggest, effects of contaminants may not be observed until much later stages of development. So the effects of this DNA damage are still unknown. Therefore, conducting similar experiments, but allowing development to parr, smolt and even adult stages, would allow a better analysis of the effects of sediment-bound contaminant exposure on recruitment and populations of salmonids.

In fish, both individual and population levels of bilateral asymmetry have been shown to positively relate to a wide range of biotic, abiotic and genetic stress. Certain abiotic factors, including heavy metals, toxic chemicals and acidification, commonly cause elevated levels of FA (Almeida *et al.*, 2008). Meristic and morpological characteristics in the trial did not suggest that sediment-bound contaminants are promotors of FA. However, further investigation with a wider range of contaminant may generate interesting findings and a

greater overall knowledge of the effects of sediment-bound contaminants on the survival and success of salmonids.