The use of zebrafish embryos as an alternative approach for ecotoxicity testing

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ABSTRACT

The use of fish embryos for acute ecotoxicity tests has been widely documented and over recent years there has been significant progress in the use of zebrafish embryos as an alternative approach to juvenile fish. However, there are still some questions preventing the unilateral adoption of this surrogate life stage as an alternative to the use of juvenile fish for regulatory testing purposes. Many of the concerns that have been raised include the absolute sensitivity of fish embryos. For example, published work has shown that fish embryos are not as sensitive as other more developed life-stages (such as the eleutheroembryos) to certain classes of compounds including, but not exclusively, cationic polymers. However, there is limited understanding of why fish embryos do not behave similarly and exhibit a toxic response to these classes of chemicals. One possible reason is that fish embryos have a protective envelope called a chorion, which could prevent these certain chemicals from passing into the embryo. The research described within this thesis has focussed on understanding the need for alternative approaches in ecotoxicity testing, particularly regarding the use of fish embryos. In addition, the developmental ontogeny of zebrafish embryos has also been studied. Finally, the research investigated whether the chorion is indeed a barrier to entry to certain chemicals, and a technique for quantifying the proportion of bio-available chemical within the different components of the embryo was developed.

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CHAPTER 1: INTRODUCTION

The use of fish embryos for predicting environmental toxicity has, over the recent years, become increasingly recognised as an important tool particularly in terms of the development of alternative approaches to animal testing. Furthermore, fish embryos are not only being used to predict acute ecotoxicity but are increasingly being linked with predicting chronic ecotoxicity. In addition, their use has also been heralded as a surrogate approach in the early stages of drug discovery in place of more common mammalian endpoints.

This introductory chapter gives some background into the history surrounding the development of alternative approaches, and how fish embryos fit into this harmonised approach in terms of the three “Rs” of animal testing (namely Replacement, Reduction and Refinement). Importantly, this chapter focuses on the use of fish embryos in ecotoxicity testing, how fish embryos relate to alternative approaches and the potential research needs associated with fish embryos. A brief outline of the structure and direction of this thesis is also included within this chapter.

The need for alternative test methods

The scientific relevance, reliability and ethical acceptability of acute toxicity testing with mammals and other vertebrates have been under intense scrutiny for several years. Over twenty years ago, a United Kingdom (UK) Home Office advisory committee reported that some animals suffer severe pain during acute toxicity tests, and that results were not always reliable (Langley, 1997). According to this report, a survey in 1964 showed that acute toxicity studies performed on mammals were not

reproducible, and that variability could be as great as 100-fold, depending upon the species and strain used. This variability was attributed to factors such as differences in caging, temperature, lighting, bedding, handling, age and gender. Similarly, in fish, seasonal variations in environmental factors, manipulated by the holding and breeding conditions, can also result in pathological changes to the liver, which subsequently might affect the toxicity of a test substance (Rusche and Kohlpoth, 1993). Furthermore, seasonality causes a change in temperature and dissolved oxygen content, which can dramatically influence the concentration that causes a toxic response (Baer *et al*., 2002).

The suitability of acute toxicity testing to determine lethality should also be questioned. For example, in mammalian and avian testing it is generally accepted that the determination of a precise LD_{50}[1] value is not necessary (Yam *et al*., 1991) since safety factors (of at least one order of magnitude) are subsequently applied to the data at the risk assessment stage (Fentem and Balls, 1993). In addition, it is considered that LC_{50}[2] values determined for fish and amphibians are not sufficiently conclusive to justify the amount of animals used (Schulte and Nagel, 1994). These principles were reiterated by Fentem and Balls (1993) who concluded that “fish are used as an ecologically ‘representative’ species and, as such, the toxicity data obtained only provides an approximate guidance for environmental risk assessment purposes”. Such findings have signified the need for alternative approaches to chemical testing to be developed.

[1] The lethal dose which affects 50% of the population is used in human health assessments.
[2] The lethal concentration which affects 50% of the population is used in environmental acute toxicity assessments.

The 3Rs

A significant step towards such alternative approaches was first developed by Russell and Burch in their seminal book *The Principles of Humane Experimental Techniques*. In this book, the authors indicated that all experiments using animals should be designed to diminish or remove inhumane practices and should following the principles of the so-called 3Rs (Reduction, Refinement and Replacement). The principles of the 3Rs are described as follows:

- *Reduction* means reduction in the numbers of animals used to obtain information of given amount and precision;
- *Replacement* means the substitution for conscious living higher animals of insentient material;
- *Refinement* means any decrease in the incidence or severity of inhumane procedures applied to those animals, which still have to be used.

More recently, the 3Rs have been extended to include a further three Rs known as the Solna principles (Gimeno, personal communication). These further 3Rs require that any potential alternative approach should be Reliable/Reproducible, Relevant and gain Regulatory acceptance (i.e. from regulatory authorities such as the Organisation for Economic Cooperation and Development [OECD] or the European Centre for Validation of Alternative Methods [ECVAM]).

The principles of the traditional 3Rs have been reinforced by the European Union (EU) strategy ‘white paper’ for a future chemicals policy (EC, 2001) which has more recently become part of the Registration, Evaluation, Authorisation and restriction of Chemicals guidance document. Part of this document addresses the promotion of

non-animal testing, stating that there should be a maximisation of non-vertebrate animal test methods, that the development of new non-animal test methods should be encouraged, and that test programmes should be minimised (COM, 2001). According to Anon (2001), these principles are in stark contrast to current procedures concerning aquatic toxicity testing in Europe, particularly where fish are used. In the year 2006 for example, according to the UK Home Office annual report on the Statistics of Scientific Procedures on Living Animals, the overall number of scientific procedures went up from 2.89 million to just over 3.01 million (an increase of 4%) and the increases were attributed to the increase in the use of mice and fish. Specifically the fish increases were due to studies on the protection of man, animals and the environment and constituted 9% of all scientific procedures performed on protected organisms
(see http://www.homeoffice.gov.uk/rds/pdfs07/spanimals06.pdf) .

The use of fish in environmental toxicity testing

Fish represent the oldest and most diverse class of vertebrates. They encompass approximately 48% of the known member species in the subphylum Vertebrata. Fish live in a wide range of aquatic habitats: from fresh to salt water; from the cold polar seas to warm tropical reefs and from shallow surface waters to the ocean depths. The geographical and environmental diversity of fish thus makes them important experimental models in environmental toxicology.

Certain fish species also possess many specific characteristics that support their use in environmental toxicology. Examples of these favourable attributes in fish include the

fact that they are in intimate contact with the aquatic environment; they possess specialised functions of higher vertebrates; some species of fish have a relatively short life-cycle and can produce eggs in large quantities (favouring studies of chemical effects on reproduction); eggs can be externally fertilised and are transparent, thus embryonic development can be monitored visually and finally, many fish species are also suitable for both field and laboratory experiments (e.g. cage monitoring studies), facilitating extrapolations between lab-based studies and field observations. In addition, fish are fundamentally important to the function of most aquatic ecosystems and have a high economic value and thus there is a need to protect fish populations against the potential adverse effects of chemical discharges.

Historically, the advantages of fish for use in chemical testing and the economic importance of some fish, both for food and recreation, have made them favoured models in ecotoxicity studies. Consequently, fish have been used in ecotoxicity tests to assess the potential environmental impact of individual chemicals and their mixtures, (e.g. effluents), and establish criteria for monitoring water quality in rivers and estuaries. Fish are used for evaluating acute and chronic toxicity and the scientific community, regulatory bodies and the chemical industry as a whole have, in the past, considered fish to be an important (arguably the most important vertebrate) representative for studies on the aquatic environment.

Potential alternatives to the use of fish - a 3Rs approach

A number of alternative approaches have been suggested in order to implement the 3Rs in acute aquatic toxicity assessments and in 2005 a European Centre for Ecotoxicity and Toxicity of Chemicals (ECETOC) taskforce was set up in an attempt

to collate and coordinate the different methods that were available. To date, the findings from the taskforce are probably the most comprehensive source of potential alternatives, and a brief synopsis is presented herein. The alternative approaches were presented in a report from the taskforce that focused on whether the potential alternative was a Reduction, a Replacement or a Refinement of an existing technique (ECETOC, 2006).

Reduction

In terms of *reducing* the number of organisms, it was suggested that using fewer concentrations of the chemical tested would lower the numbers of organisms used with only a minimal loss of precision (Douglas *et al*., 1986). However, this is based on limited data and does not have regulatory acceptability. Another potential approach to reduce the number of fish used was discussed, called the acute threshold (step-down) test, as proposed by Hutchinson *et al*. (2003). The objective of this approach is to reduce the number of fish used (from ≥42 to ≥10 fish per substance) and to determine an LC_{50} by applying comparative threshold data obtained from the most conservative data from algae and/or daphnid acute tests. To date, this principle has been partially substantiated for 91 active pharmaceutical ingredients (API). Approximately 80% of the APIs had an LC_{50} value equal to or higher than the most sensitive algae or daphnid test. Hutchinson *et al*. (2003) proposed that, for the further 20% of the APIs, it would be possible to extrapolate a comparable LC_{50} value for the fish by employing a step-down factor of 3.2 to the EC_{50}[3] value derived from the most sensitive species. More recently, Jeram *et al*. (2005) demonstrated that, for more than

[3] The concentration which causes an effect in 50% of the population.

1,400 chemicals from the New Chemicals Database, a reduction between 53 and 71% of the number of fish used to establish the LC_{50} value could have been achieved by using the step-down approach.

Replacement

In terms of approaches to *replace* the use of fish, the taskforce looked at *in vitro* test methods using established fish cell lines. Of the different cell lines that are commercially available, the review included PLHC-1 (a hepatocellular carcinoma cell line from the fish top minnow), the RTG-2 (a rainbow trout gonad cell line) and the R-1 (a fibroblast-like cell line derived from liver tissue of rainbow trout) along with suspension-cultured fish cells. Considering each cell line independently, in terms of a suitable alternative to acute testing *in vivo*, it was evident that no cell line is without its problems for use: The PLHC-1 cell line, which is derived from a hepatocellular carcinoma, retains most of its metabolic functions and contains an aryl hydrocarbon (AH) receptor and stable, inducible cytochrome P450 enzymes. This cell line is also relatively simple to culture. However, these cells are more susceptible to DNA damage through excessive mitotic divisions (Vrba *et al*., 2002). For this reason, the use of primary cell lines in the determination of genotoxicity would be more appropriate. Hepatocyte cell lines require specialised procedures (e.g. liver perfusion) and cannot be passaged[4] (Babich and Borenfreund, 1991).

The RTG-2 cell line suffers through the fact that its absolute sensitivity appears to be less than that of cultured primary fish hepatocytes, juvenile fish and fish embryos.

However, this cell line (RTG-2) has been used to develop an estrogen-responsive reporter gene system that has excellent sensitivity (Fent, 2001).

The R-1 cell line has been reported to have a similar sensitivity when compared to acute toxicity of waste water samples in experiments performed on the same substance *in vivo*. However, the waste water samples which were tested may have had a specific mode of action that affected cells in culture and therefore, such a comparison is not a definitive answer to the sensitivity between *in vivo* and *in vitro* assays.

With respect to the use of suspension-cultured fish cells, it was noted that when compared to *in vivo* assays performed for 6 different anti-fouling compounds, the *in vitro* assays were always less sensitive (Voytic-Harbin *et al*., 1998). However, these experiments were not conducted over the same time period (i.e. a minimum of 7 days *in vivo* versus 24 hours *in vitro*) and this may have affected the outcome. Additionally, the suspension-cultured fish cells were reported to have no significant differences in their response to a series of chemicals when compared with primary cell cultures and epithelial cell lines even when the cells for all three culture systems were sourced from the same tissue. The suspension-cultured fish cell lines were also reported to be more rapid and simpler to use than monolayered cultured fish cells.

With regards to the use of the non-hepatic cell lines, the main problem is the fact that they do not have the xenobiotic metabolising capacity of hepatocyte cells. It is also evident that comparisons made between the *in vivo* and *in vitro* test methods are

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[4] The process of maintaining a continual cell line in culture.

relatively limited. Additionally, where these comparisons have been made, the class or types of substances used seem to be relatively selective and do not have a wide range of applicability domain. For example, Castaña *et al*. (1996) obtained excellent correlation coefficients between the *in vivo* and *in vitro* data that they produced (mean of r=0.97) for 16 chemicals. However, the chemicals tested involved four heavy metals and the remaining chemicals were all phenolic derivatives. The correlations were also slightly misleading due to the fact that, in some circumstances, the results from the assays performed *in vitro* were up to 200 times less sensitive than the *in vivo* data. Babich and Boreunfreund (1997) also investigated the *in vitro* cytotoxicity of 13 different phenolic derivatives. Their results gave similar correlation coefficients (r=0.98) between the Neutral Red Uptake (NRU) assay, using BF-2 cell lines, and the LC_{50} assays *in vivo* suggesting that the *in vitro* assay is as predictive as the *in vivo* assay. The phenolic derivatives did not include 2,4-dinitrophenol in the correlation comparisons due to the fact that BF-2 cells are not reactive to uncouplers of oxidative phosphorylation. Another important consideration is the fact that, in certain circumstances, a correlation coefficient was not reported even though the comparisons were indicated as being similar.

It is clear from the published literature that each cell type and assay offers advantages and disadvantages but currently, based on the previous discussion, *in vitro* tests with a single cell line type do not appear to represent a suitable alternative to acute testing *in vivo*. Primary cell cultures are generally reported to be more representative of situations *in vivo* than permanent cell lines (Behrens *et al*., 1998). This should not diminish the potential use of fish primary cell cultures and fish cell lines as part of a battery of techniques for establishing possible chemical potency and effects *in vivo* in

fish. A comprehensive review of the use of fish cells in ecotoxicology has been presented by Castaño *et al* (2003).

Other potential replacements of fish for toxicity assessments (that have been discussed at length) include *in silico* techniques such as Quantitative Structure Activity Relationships ([Q]SARs). (Q)SARs relate biological activity to the physico-chemical and structural properties of chemical compounds (Walker, 1998). Limited predictions from certain classes of chemicals may also be possible. For example, Verhaar *et al*. (1992) developed a classification scheme that separated small to intermediate sized organic chemicals into four distinct classes, which were: inert chemicals, less inert chemicals, reactive chemicals and specifically acting chemicals. Validation of this classification scheme showed that prediction of toxicity was only possible for inert organic chemicals, however, a range of potential toxicity values could be predicted for the other classes of chemicals (Verhaar *et al*., 2000). A major limitation of this SAR-based classification system is that toxicity of metals, inorganics and ionisable organic chemicals cannot be predicted by the Verhaar scheme. A detailed review of this scheme, along with other (Q)SARs, is provided by ECETOC (2003a).

Refinement

The third key area considered by the ECETOC taskforce (for assessing potential alternatives to the use of animals in chemical testing) focused on *refinement* of ecotoxicity testing. This was mainly based on non-lethal endpoints, such as

behavioural responses, and more importantly, in the context of this thesis, the use of alternative life stages of fish and will be discussed in more detail in Chapter 2.

The use of fish embryos in ecotoxicology

There are many potential benefits to the use of fish embryos in acute aquatic toxicity assessments, and these are detailed below. It is important, to first describe the definition of a fish embryo, as this can vary. According to the dictionary of ichthyology (Coad and McAllister, 2003), the embryonic stage of development in fish refers to the autotrophic stages of development. These include the stages of development from the fertilized oocyte to the sac fry stage where fish are still autotrophic, i.e. feeding on their own yolk reserves. This latter post hatch/sac fry stage of development is more commonly referred to as the eleutheroembryo (Ballon 1975; Woodland and Maly, 1997; Carvalho *et al*., 2004). However, for the context of this thesis, the term fish embryo will refer to the fertilised egg prior to hatching.

Benefits of using fish embryos

According to the UK Animals (Scientific Procedures) Act (1986), fish are classed as protected from the point when they are capable of independent feeding. Fish embryos, therefore, are excluded from this protection since they are autotrophic. Fish embryos are also less sentient than more advanced life-cycle forms, as they have less developed neuroendocrine and olfactory systems. Hence, the use of fish embryos is generally considered an ethical alternative to the use of whole fish.

The potential benefits of using fish embryos for toxicity testing compared with approaches using post yolk sac fish include:

- Single fish embryos can be maintained in small volumes of test solution, which reduces the need for large amounts of potentially expensive test substance. For example, zebrafish embryos can be maintained in volumes as small as 100 µl for up to six days (Phylonix, personal communication) or even as small as 50 µl for up to 48 hours.
- Fish embryos can be cultured in microtitre wells and processed on a standard microtitre plate reader, which can be used as a high-throughput screening tool.
- Most fish embryos are inexpensive to maintain and are relatively easy to produce in large numbers (e.g. single mating pairs of zebrafish [*Danio rerio*] normally can produce between 100 and 200 eggs in one spawning).
- The majority of fish embryos are completely transparent and, therefore, development can be assessed visually. This enables the possibility of determining teratogenicity, in addition to specific organ toxicity (e.g. liver and kidneys) through the use of immunochemical techniques.
- Fish embryos can be used as a tool for determining the genetic effects of substance exposure, since approximately 90% of the genome is active during embryogenesis, whilst only approximately 10% is functional during adult life.

The use of fish embryos in ecotoxicology

A considerable volume of work has already been undertaken in the field of fish embryo ecotoxicology, predominantly using zebrafish (*D. rerio*) embryos, although

other OECD recommended species have also been investigated, including rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*) (Cairns *et al*., 1965; Laale, 1977; Birge *et al*., 1979; Ensenbach *et al*., 1989; Schulte and Nagel, 1994; Lange *et al*., 1995; Canaria *et al*., 1999).

The effect of chemicals on fish embryos can be evaluated, not only in terms of overall lethality, typically characterised by coagulation of the egg, but also a series of other end-points relating to diverse physiological functions which may be indicators of chronic endpoints. These include gastrulation, number of somites, movement, development of organs, pigmentation, heartbeat and circulation (Schulte and Nagel, 1994). Effects on many of these endpoints allow specific assessment relevant to the mode of action of certain chemicals. These parameters of embryo toxicity have been further characterised into two groups of EC_{50} (the concentration effecting 50% of the population) classifications, EC_{50} I and EC_{50} II:

EC_{50} I endpoints (time taken for observation of each parameter shown in parentheses);

- Non-completion of gastrulation (12 hours),
- No somites (16 hours),
- No heartbeat (48 hours),
- No movement (48 hours),
- coagulated egg.

Evidence of deficiencies in any of these parameters will result in an inability to hatch or impact on hatching success, or incur survival problems.

EC_{50} II endpoints are summarised as:

- Arrested development.
- Developmental deficiencies.
- Reduced motility.
- Growth and fitness.

EC_{50} II endpoints are characterised as indicators of the mechanism of toxic action of a chemical.

Schulte and Nagel (1994) used this approach to evaluate six chemicals[5] with different Fish Acute Toxic Syndromes (FATS)[6] including organic herbicides, pesticides and phenolic compounds. Using zebrafish embryos, the EC_{50} values and the Lowest Observed Effect Concentrations (LOEC) for the compounds tested were found to be comparable to acute LC_{50} data (i.e. the results fitted into the same EU classification tier of very toxic, toxic, moderately toxic and low toxicity). However, in some instances the EC_{50} values differed by two to three orders of magnitude.

There are however, some conflicting studies, that have highlighted differences in the sensitivity of fish embryos compared with whole fish systems. For example, Ensenbach *et al*. (1989) considered that fish embryos were not as sensitive as juvenile/adult fish (but these authors did not adopt the same system of evaluation as Schulte and Nagel, making direct comparisons difficult across the studies). Cairns *et al*. (1965), found that adult fish were more sensitive to zinc chloride, potassium cyanide and potassium dichromate, but embryos were more sensitive to naphthenic

[5] Malathion, carbaryl, 4,6-dinitro-*o*-cresol, 2,4-dinitrophenol, phenol and 4-nitrophenol.
[6] Fish acute toxic syndromes are the different physiological responses that fish can exhibit following exposure, for example, swimming behaviour.

acid. Birge *et al*. (1979) commented on the fact that embryos and larvae were more sensitive to phenol, aniline and chlorobenzene and suggested that embryos are acceptable as a replacement to acute toxicity tests with adult fish when toxicity data is required on organic chemicals. Consistent with this, Kovrižnych and Urbančíková (2001) found that embryos exposed to methanol proved to be 77 times more sensitive than adult fish. Additionally, these authors found that LC_{50} values for zebrafish embryos and adult fish, for the eight chemicals tested, were comparable even though the actual sensitivity of the different assays varied with each substance.

While embryo studies and comparisons with acute LC_{50} data conducted on warm-water species are relatively widely documented, studies carried out on cold-water species are sparse. Of the few studies documented, Canaria *et al*. (1999), proposed a method for a seven-day embryo toxicity test using rainbow trout. During the study, the fertilisation success, embryo mortality and morphogenesis of rainbow trout embryos exposed to effluents[7] and a reference toxicant (sodium dodecyl sulphate) were investigated. Morphological development was observed on the embryos after 7 days, when they had reached stage 11, as described by Ballard (1973). At this stage of development, the embryos were clearly visible after treatment with a clearing solution (acetic acid). Any embryos that had not reached this stage (stages 1 to 10 as described by Ballard, 1973) were recorded as non- viable. The results showed that the 7-day rainbow trout embryo toxicity test was more sensitive to sodium dodecyl sulphate than the 7-day juvenile fathead minnow survival and growth test. Furthermore, the 7-day embryo toxicity test was more sensitive, by almost an order of magnitude, compared with the 96 hour acute toxicity test with juvenile rainbow trout.

The 7-day rainbow trout embryo test has several advantages over some traditional fish early life-stage (ELS) protocols. Rainbow trout ELS studies are relatively long in duration (30 day embryo phase followed by a 90 day grow-out phase) and are consequently relatively expensive. They are also carried out under flow-through conditions whilst the 7-day embryo test is carried out under semi-static conditions. Furthermore, water hardening[8] can take place in rainbow trout embryos for up to 30 minutes post fertilisation whilst being exposed to the test chemical. Hence, during this 30-minute period the micropyle is open and the embryonic chorion will still be relatively permeable to the exposure chemical/mixture. The only ethical issue involved with such a study is the collection of milt from the gonads, usually from altered sex females that do not have sperm ducts and consequently, this is a terminal procedure. If the milt is obtained from a fish farm, this is not classed as a regulated procedure, and hence is not covered by the UK Home Office licence. Furthermore, milt collected from one fish can be cryopreserved and stored for use in many further embryo experiments.

In addition to the potential use of embryos for acute toxicity assessments, embryos can also be used for developmental toxicity testing. Morphological development of embryos can be affected by toxicants and can be quantified to determine sensitivity to test compounds (Nguyen and Janssen, 2002). Morphological differences such as reduction in pigmentation, yolk sac oedema and deformation of the notochord have been shown to be affected by chemicals such as cadmium and copper; sodium pentachlorophenol and malathion; and chromium and malathion (Nguyen and Janssen, 2002). The African catfish (*Clarias gariepinus*) was used in these

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[7] No comparative data was published for the 96-hour acute toxicity test or for the 7-day fathead minnow survival and growth test exposed to the effluents.
[8] The process of water hardening is the closure of the micropyle following fertilisation. The micropyle is the opening in the protective chorion created by the penetrating sperm.

investigations because of its well-documented biology, ease of culturing, short developmental time, transparent eggs and year-round reproduction. Following quantification, sensitivity was summarised as growth>abnormality>larval survival>embryo survival>hatchability. Results from five-day ELS studies with African catfish were similar to the sub-chronic results obtained from a 12 day ELS using zebrafish, and were also comparable with data from ELS studies conducted on other OECD species (Nguyen and Janssen, 2002).

The importance of the developmental stages of fish in determining their sensitivity to different chemicals has recently been highlighted. In 2003, a taskforce was commissioned by ECETOC to evaluate the ECETOC Aquatic Toxicity (EAT III) database (ECETOC, 2003). The findings from this report, based on the limited data that was analysed, concluded that there were no differences between the sensitivity of embryos and larvae for the chemicals that had been tested. In addition, there was no trend to suggest that larvae were more or less sensitive than juvenile fish. This is in contrast, however, to previous findings by Hutchinson *et al*. (1998). In their evaluations, they compared results using a previous EAT database (ECETOC, 1993) for fish at different levels of development. They concluded that fry were more sensitive than embryos for 68% of substances and, compared to juvenile fish, were more sensitive for 83% of the substances tested. Consistent with the current EAT report they found that juvenile fish were more sensitive than adult fish for 92% of the substances examined. The increased sensitivity of juvenile and larval fish was attributed to the surface area:volume ratio. In addition, young fish have accumulated less fat than adult fish and, therefore, it is thought that they have less capacity to accumulate lipophilic substances.

As discussed, there are many benefits to the use of embryos and a significant amount of research has been conducted on their use as an alternative approach. However, there appears to be conflicting evidence affecting the unilateral adoption of fish embryos in place of juvenile/adult fish, particularly pertaining to their absolute sensitivity. There are several possible reasons why fish embryos may be considered less sensitive. For example, Lange *et al*. (1995) suggested that the metabolic capability of fish embryos is not well developed, making them less sensitive to toxins that require metabolic activation (e.g. Benzo[a]pyrene). In relation to this, an additional complication with fish embryos is that not all metabolites are synthesised *in ovo*. Lange *et al*. (1995) reported that after 24 hours, the only metabolite of phenol in zebrafish embryos was phenyl sulphate, and only in 5 day-old embryos was the complete spectrum of phenolic metabolites present, that are found in adult zebrafish. This has implications for toxic metabolites that may not be synthesised within the exposure period of the fish embryo assay giving false negative results.

Another possible reason for reduced sensitivity in fish embryos is the protective envelope (chorion) surrounding a fish embryo, which could be acting as a barrier to certain chemicals, particularly once the micropyle has closed following fertilisation. Embryos used for acute toxicity tests, therefore, will always be less sensitive than embryos used in chronic toxicity tests, for example, in life-cycle studies where eggs and embryos are exposed continuously. According to Westerfield (1995), however, it is possible to remove the chorion through pre-treatment with a dilute solution of pronase or through mechanical disruption.

Chapter 1

Research needs and objectives

As detailed above, there are some questions surrounding the sensitivity of fish embryos when compared with whole fish. One of the potential reasons is that the protective chorion may be acting as a barrier to entry of certain chemicals. Therefore, the research described herein focuses on understanding whether the chorion is the reason for the reduced sensitivity that has been observed for certain chemicals in fish embryos when compared to whole fish *in vivo* studies. In addition, the use of fish embryos has been examined in order to better understand any other potential limitations of such an alternative approach.

In order to determine whether the chorion acts as a barrier to entry into zebrafish embryos, this thesis first investigates the different properties of the chorion during embryogenesis in addition to understanding and characterising the fundamental ontogeny of developing zebrafish embryos. Following this, the permeability of zebrafish embryos was investigated by dissecting embryos pre-exposed to chemicals with different physicochemical properties. This lead to identifying where these chemicals partitioned within the embryo (e.g. perivitiline fluid, embryo or chorion) and what quantities of the chemicals could be determined within each of these different partitions, thus enabling an understanding of whether certain chemicals are taken up into the embryos, or if the chorion is preventing/limiting the uptake. The next stage of this research focused on understanding if the permeability of zebrafish embryos can be manipulated to increase the uptake of chemicals and whether uptake of is influenced by different molecular sizes.

CHAPTER 2: DEVELOPMENTAL ONTOGENY OF ZEBRAFISH EMBRYOS

Introduction

An understanding of the ontogeny of zebrafish embryos is of particular importance in ecotoxicity testing and has been widely studied and documented (Hisaoka and Battle, 1958 and more recently Kimmell *et al*., 1995). Most fish embryos are completely transparent and, therefore, the developmental ontogeny can be easily assessed. This enables the possibility of determining affects such as teratogenicity, in addition to specific organ toxicity (e.g. liver and kidneys). Fish embryos can also be used as a tool for determining the genetic effects of substance exposure, since approximately 90% of the genome is active during embryogenesis, whilst only approximately 10% is functional during adult life. Furthermore, a thorough understanding of the transition from embryo to eleutheroembryo stage and further to the heterotrophic stage of development is necessary in order to avoid testing on potentially protected organisms as discussed in the previous chapter.

However, the process of embryogenesis may be potentially sensitive to variations in temperature and requires defining more precisely in order to understand these subtle differences. Therefore, this chapter first describes the process of embryogenesis in fish embryos and subsequently in zebrafish embryos. Following this, the normal developmental rates of zebrafish embryos have been investigated and the differences between the developmental rates of zebrafish embryos cultured at different temperatures documented. Importantly, this chapter details the development of zebrafish embryos with comparisons to the seminal and more traditional methodology that was originally

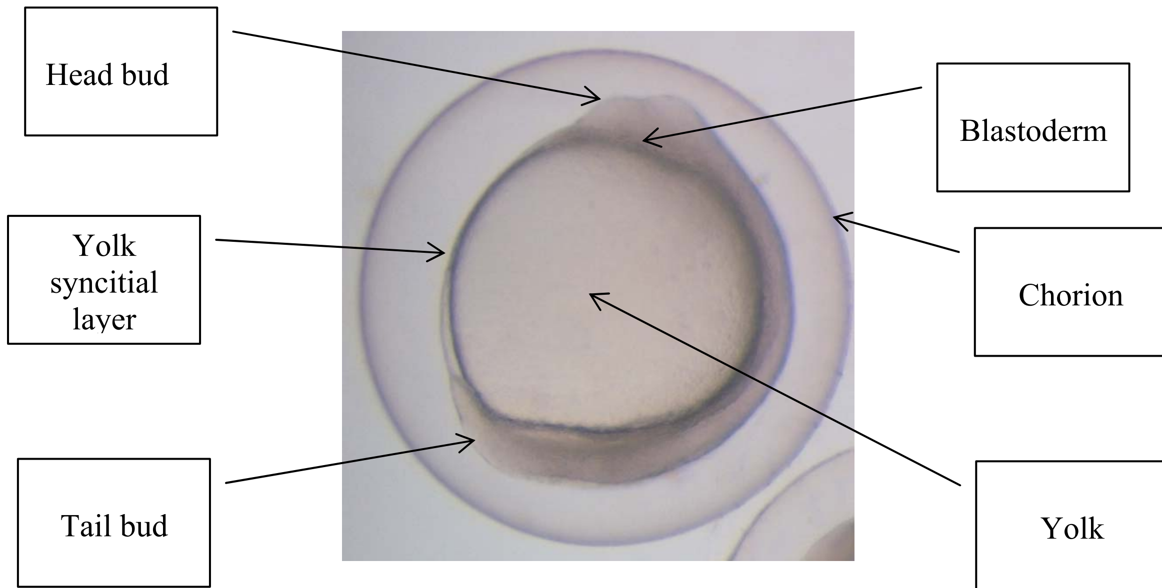
described by Hisaoka and Battle (1958) along with references to the findings of Kimmell *et al*. (1995).

Fish embryology

Embryogenesis is initiated by activation or by fertilisation. Fertilisation is a coordinated process with site-specific entry of the sperm into the egg dictated by the structural organisation of the chorion (Hart and Donovan, 1983). The site of entry through the chorion is known as the micropyle, which is a cone shaped vestibule approximately 30 µm in diameter with a tapered canal that traverses the chorion (Hart and Donovan, 1983). The micropyle is the only site of sperm entry and it is designed to prevent multiple sperm entry. Fertilisation requires the fusion of the male and female gametes following sperm entry. Fertilised fish embryos are composed of two major compartments, a large yolk[9] and a blastoderm surrounded by a multinucleated yolk syncitial layer (YSL) (see Figure 2.1) (Hagedorn *et al*., 1998).

[9] The yolk is constituted mainly from the phospholipid vitellogenin.

Figure 2.1. Zebrafish embryo at 100% epiboly



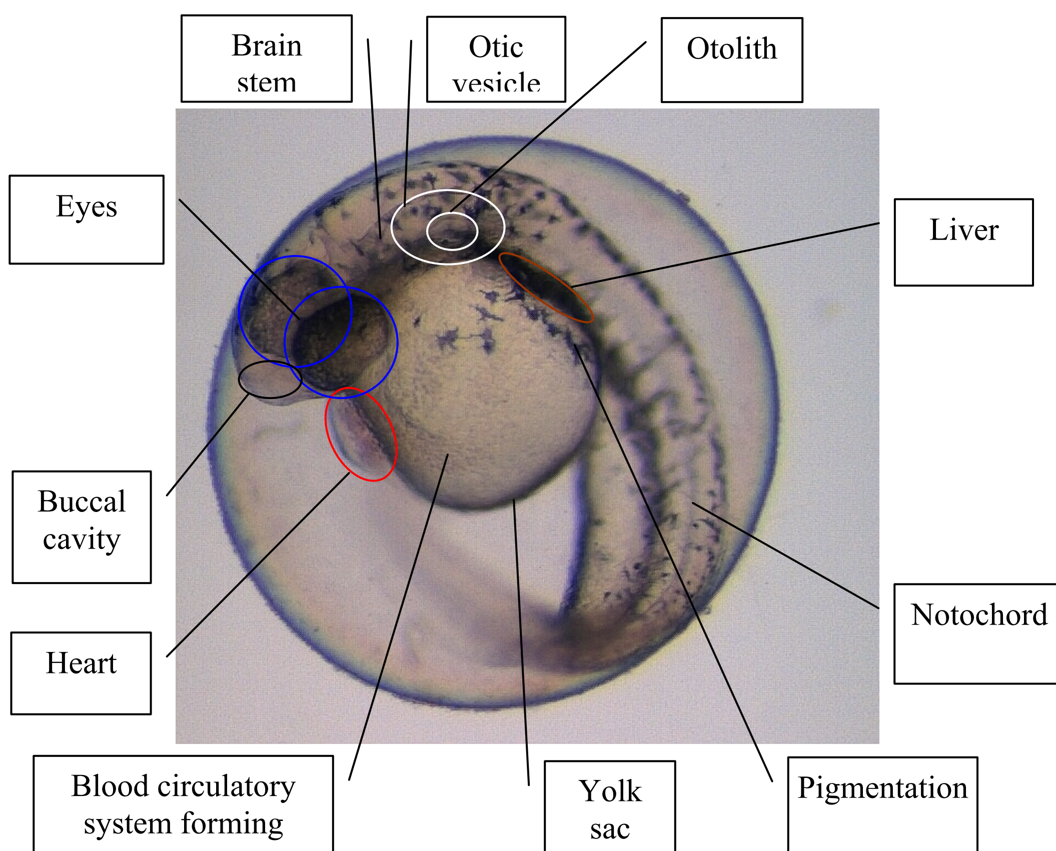
Embryogenesis in zebrafish

Hagedorn *et al*. (1998) described the early development of fish embryos beginning with the division of the blastoderm. The cells in the blastoderm continue to divide, and at the 10th division, nucleation of the YSL occurs at the margins of the blastoderm (Rawson *et al*., 2000). This nucleated YSL replaces the non-nucleated yolk cytoplasmic layer. The YSL, along with the overlying plasma membrane, are reported to be the main permeability barriers to water and cryoprotectants (Rawson *et al*., 2000). Once the cells of the blastoderm have divided to reach the 128 cell stage, epiboly[10] begins. This continues until the blastoderm completely surrounds the yolk. Below the blastoderm, the YSL develops and envelops the yolk by approximately 50-75% epiboly. Following the completion of epiboly, muscle somites begin to develop in the tail region of the embryo at a rate of three somites per hour (Westerfield, 1993) and the final stages of

[10] The expansion of a cell layer over other cells, which occurs during gastrulation.

development are characterised as the 26-somite stage (Hagedorn *et al*., 1998). During these final stages of development, organogenesis occurs, and Figure 2.2 shows a 48 hour old zebrafish embryo with all the major organs and vascularisation present.

Figure 2.2. Zebrafish embryo at 48 hours post (28 ± 1°c) fertilisation showing the major organs



Investigations of the developmental rates of zebrafish embryos

As we have seen from the background information on fish embryology in the preceding sections, the embryo begins as a basic structure consisting of a small number of rapidly

dividing cells, which develop into a highly structured and functioning embryo within a relatively short period of time (e.g. ca. 48 hours for zebrafish). The rapid development of a zebrafish embryo makes it especially attractive as a model for ecotoxicological assessment. A caveat however, is that relatively small changes in temperature can affect embryo development significantly. For example, zebrafish embryos cultured at 26°C have been shown to hatch only after 96 hours of development, whilst embryos cultured at 30°C have been shown to hatch within 48 hours of development. In such situations, there may be implications regarding the UK Home Office regulations for embryo tests since hatched fry may be considered as protected organisms as discussed in the previous chapter. Consequently, the first set of experiments described herein, investigated the developmental stages of zebrafish embryos. In addition, the time taken to reach the different stages of development, was investigated by recording a series of images of a zebrafish embryo at different time points throughout embryogenesis. This has defined the timing for progression to the different stages in addition to supporting information on the structural features of zebrafish embryos at the different stages. Subsequently, a second set of experiments was performed to further define the developmental rates of zebrafish embryos, cultured at three different temperatures. The different developmental rates of the zebrafish embryos were captured in a series of images recorded at time intervals of 3, 6, 24, 30 and 48 hpf.

Materials and methods

The first experiment was carried out in Plastic Petri dishes (90 mm diameter) (Bibby sterilin). The second set of experiments was performed in plastic 96 well plates (Linbro®/Titertek®) and plastic 24 well plates (Linbro®/Titertek®) and within a

temperature and photoperiod controlled laboratory. All images were taken using a Leica DC500 digital camera in conjunction with a Leica MZ8 stereomicroscope. The software used was the Leica QWin pro version 3.1.0.

Dilution water

The dilution water was dechlorinated tap water, which was passed through activated carbon, coarsely filtered to remove particulate material and dechlorinated with sodium thiosulphate. Salts were added, as required, to maintain minimum hardness levels, and the treated water was then passed through an ultraviolet steriliser to a second set of 20 and 10 µm filters. The dilution water supply was then delivered to a temperature controlled header tank in the test laboratory set to a nominal test temperature of 25 ± 1°C and was further filtered to 5 µm prior to delivery to the test vessels.

Test temperature and photoperiod

The first experiment test was conducted at a nominal temperature of 25 ± 1°C, and a photoperiod of 16 hours light: 8 hours of dark with a 20 minute dawn/dusk transition period. The second experiment test was conducted in two temperature controlled rooms set to a nominal test temperatures of 25 and 28 ± 1°C, and additionally in a Gallenkamp incubator set to a nominal test temperature of 30 ± 1°C. All tests were carried out with a photoperiod of 16 hours light: 8 hours of dark with a 20 minute dawn/dusk transition period.

Chapter 2

Test species and justification

Zebrafish (*Danio rerio*) embryos (wik strain, Brixham reference no 07/04), obtained from the University of Exeter, were used for this study. The zebrafish was the species of choice because it is readily available, easily bred in the laboratory, representative of a widely distributed warm water fish and has been extensively used in ecotoxicological assessments (Cairns *et al*., 1965; Laale, 1977; Ensenbach *et al*., 1989; Schulte and Nagel, 1994; Lange *et al*., 1995; Kovrižnych and Urbančíková, 2001).

Procedure

Zebrafish embryos were obtained from the husbandry unit of Brixham Environmental Laboratory (BEL) within two hours of the initiation of spawning. The embryos were cleaned using fresh dilution water, by rinsing the embryos over a nylon meshed container (mesh gauge of 0.1 mm diameter) to remove any uneaten food and faeces. The embryos were then carefully transferred to clean dilution water in a plastic Petri dish (90 mm diameter). Embryos were then visually assessed against a black background to identify any dead eggs, which were subsequently removed. Dead eggs appear white against a black background which is characterised by coagulation of the egg. Following the collection and cleaning procedure, the embryos were transported to the test facility and all observations were carried out using a Leica MZ8 stereomicroscope.

Experiment 1: Investigations of the normal ontogeny of the zebrafish embryo

In the first experiment, approximately 20 embryos, which were newly fertilised and at the first stage of development as described by Hisaoka and Battle (1958), were selected and transferred to a separate Petri dish. Individual embryos, were selected and used for the duration of the observations. For the purposes of these investigations, the images of a single embryo were used as an example of the development of the population of embryos. Photographic images were taken at the different stages of embryo development described by Hisaoka and Battle (1958) and the time taken to reach the different stages was recorded.

Experiment 2: Development of zebrafish embryos cultured at different temperatures

For the second experiment, the embryos were again transported to the test facility following the collection and cleaning procedure, and observed using a Leica MZ8 stereomicroscope. Embryos, which were newly fertilised and at the first stage of development as described by Hisaoka and Battle (1958), were selected and transferred to a separate Petri dish. From these embryos, three single embryos were selected and added individually to single wells in each of three 96 well plates. Each embryo was cultured in approximately 200 µl of dilution water and each plate was sealed with a loose fitting lid to prevent evaporation. Each plate was then transferred to each of the different culture facilities set up to each respective temperature (i.e. nominal test temperature of 25, 28 and 30 °C). One plate was then placed into each of the different culture facilities and observed for the different rates of development at different time points (3, 6, 24, 30 and 48 hours post fertilisation).

To supplement these detailed observations, additional 12 and 24 well plates were also set up, with individual embryos per well, to determine the variability in the developmental rates of zebrafish embryos cultured at different temperatures. The stages of development of these embryos were based on the description of the periods of early development described by Kimmel *et al*. (1995). In addition to the investigations of the developmental rates of the embryos at different temperatures, the time to hatch was also documented to enable an understanding of when fish embryos could potentially be considered as a protected organism. The periods of early development as described by Kimmel *et al*. (1995) are shown in Table 2.1. However, for these investigations, embryos were selected within approximately 2 hours of fertilisation, therefore, the first two stages (zygote and cleavage) were not included. Furthermore, the plates were incubated at 2 different temperatures of 25 and 28 ± 1 °C in temperature controlled rooms and with the same conditions as detailed previously. Embryos, cultured in the 12 well plates, were cultured in approximately 5ml of dilution water, and those cultured in the 24 well plates were cultured in approximately 2ml of dilution water. Each plate was covered with a loose fitting lid to prevent excessive evaporation.

Table 2.1. Periods of early development of zebrafish embryos at 28.5°C as described by Kimmel *et al*. (1995)

Period	Time (h)	Description
Zygote	0	Newly fertilised egg through the completion of the first zygotic cell cycle
Cleavage	0.75	Cell cycles 2-7
Blastula	2.25	Rapid cell cycles (8,9) leading to more longer asynchronous cell cycles at the midblastula transition leading on to the beginning of epiboly
Gastrula	5.25	Formation of the epiblast, hypoblast and embryonic axis- end of epiboly
Segmentation	10	Development of somites, pharyngeal arch primordial, and neurones, organogenesis begins, the tail starts to form and early movements occur.
Pharyngula	24	Phylotypic-stage of embryogenesis; straightening of the body axis, circulation, pigmentation and fins begin to develop
Hatching	48	Completion of primary organogenesis, cartilage development in the head and pectoral fins- hatching begins asynchronously
Early larva*	72	Swim bladder inflates, predator prey behaviours occur

*This is the eleutheroembryo stage which is more commonly referred to in embryology

Results and discussion

Investigations of the normal ontogeny of the zebrafish embryo (experiment 1)

The pH, conductivity, hardness (as $CaCO_3$), alkalinity and free and residual chlorine of the laboratory dechlorinated water supply were measured and determined to be: pH 7.86, conductivity 230 $\mu S\ cm^{-1}$, alkalinity 21.2 mg l^{-1}, hardness 42.0 mg l^{-1}, and free and residual chlorine <2 $\mu g\ l^{-1}$.

The photographic images of the embryos at the different stages of development are shown in Figure 2.3. From these images it can be seen that the early development of the embryo begins shortly after fertilisation with the first division of the blastoderm (Figures 2.3a & 2.3b). The cells in the blastoderm continue to divide (Figures 2.3c to 2.3h), and

at the 10th division (Figure 2.3i), according to Rawson *et al*. (2000), nucleation of the Yolk Syncitial Layer (YSL) occurs at the margins of the blastoderm. This nucleated YSL replaces the non-nucleated yolk cytoplasmic layer. The YSL, along with the overlying plasma membrane, are reported to be the main permeability barriers to water and cryoprotectants (Rawson *et al*., 2000). Once the blastoderm reaches the 128 cell stage, epiboly begins until the blastoderm completely surrounds the yolk (Figures 2.3j to 2.3m). Below the blastoderm, the YSL develops and envelops the yolk by approximately 50-75% epiboly. Following the completion of epiboly (Figure 2.3n), muscle somites begin to develop in the tail region of the embryo at a rate of three somites per hour (Westerfield, 1993). Images of stages 9-11 and 18-20, as described by Hisaoka and Battle (1958), were not obtained in this experiment. After 23 hours post fertilisation (Figure 2.3o), the otoliths had formed and the notochord was clearly visible. The final stages of development are characterised as the 26-somite stage (Hagedorn *et al*., 1998), which was clearly evident by 48 hours post fertilisation (Figure 2.3p). At this time, the embryo is fully developed and pigmentation has started to develop in the skin and eyes.

Figure 2.3. Developmental stages of a zebrafish embryo cultured at 25 ± 1°C

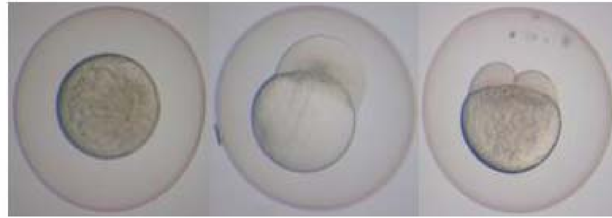


Fig a- Stage 1 Fertilised egg 0 h	Fig b- Stage 2 1 cell blastodisc 0.1 hpf[11]	Fig c- Stage 3 2 cell early cleavage 0.3 hpf

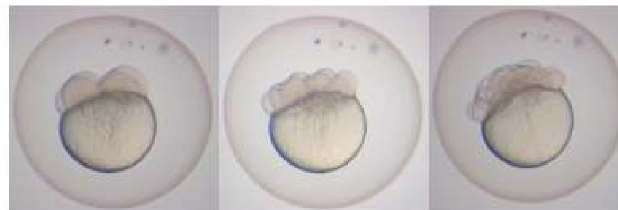


Fig d- Stage 4 4 cell early cleavage 0.4 hpf	Fig e- Stage 5 8 cell early cleavage 0.9 hpf	Fig f- Stage 6 16 cell early cleavage 1.2 hpf

Fig g- Stage 7 32 cell early cleavage 1.5 hpf	Fig h- Stage 8 64 cell late cleavage 1.8 hpf

[11] hpf hours post fertilisation

Figure 2.3 (continued). Developmental stages of a zebrafish embryo cultured at 25 ± 1°C

Fig i- Stage 12 Very late blastula 2.4 hpf	Fig j- Stage 13 Early gastrula 3.1 hpf	Fig k- Stage 14 Blastoderm envelopes ⅓of the yolk 4.4 hpf

Fig l- Stage 15 Blastoderm envelopes ½ of the yolk 4.9 hpf	Fig m- Stage 16 Blastoderm envelopes ¾ of the yolk 7.4 hpf	Fig n- Stage 17 Closure of the Blastoderm 8.4 hpf

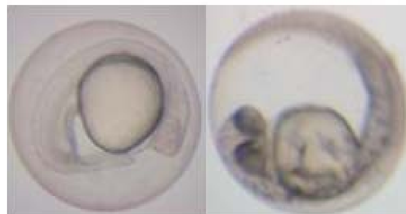


Fig o- Stage 21 Otolith formation 23 hpf	Fig p- Stage 23 Pigment formation (melanophore) 48 hpf

Development of zebrafish embryos cultured at different temperatures (experiment 2)

The pH, conductivity, hardness (as $CaCO_3$), alkalinity and free and residual chlorine of the laboratory dechlorinated water supply was measured and determined as: pH 7.63, conductivity 206 µS cm^{-1}, alkalinity 26.4 mg l^{-1}, hardness 52.0 mg l^{-1}, and free and residual chlorine <2 µg l^{-1}.

The time taken to reach the different stages of development at a nominal temperature of 26°C, as described by Hisaoka and Battle (1958), are shown in Table 2.2. To facilitate a comparison between the work of these authors and the current study, the development stages of the embryos at different temperatures and time intervals is also included in the table. Photographic images of the different stages of embryo development that were obtained at the different time points are shown in Figure 2.4.

From the photographic images presented in Figure 2.4, it can be seen that there are clear differences in the developmental rates of zebrafish embryos cultured at the different temperatures. According to the descriptions of the developmental stages by Hisaoka and Battle (1958), after 3 hours the embryo cultured at 25 ± 1°C had reached stage 12 representing very late blastula, while the embryos cultured at the higher temperature were both at stage 13, classed as early gastrula.

After 6 hours, the embryo cultured at 25 ± 1°C had reached stage 14 where the blastoderm had enveloped a third of the yolk sphere. The embryo cultured at 28 ± 1°C had reached stage 15 where the blastoderm had enveloped half of the yolk sphere, and

the embryo cultured at 30 ± 1°C had reached stage 16 where the blastoderm had enveloped three quarters of the yolk sphere.

After 24 hours, the embryos had reached stages 20, 21 and 22 at temperatures of 25, 28 and 30°C respectively. Stage 20 is characteristic of the optic cup formation, stage 21 is where the otolith begins to form and stage 22 is the point when retinal pigmentation starts to form. The embryo cultured at 25 ± 1°C took 30 hours to reach stage 21 whilst the other 2 embryos had reached stage 23 between 24 and 30 hours. Stage 23 is characteristic of body pigmentation developing. After 48 hours, the embryo cultured at 25 ± 1°C had only reached stage 23, the embryo cultured at 28 ± 1°C had reached stage 24, characteristic of xanthophore development, and the embryo cultured at 30 ± 1°C had already hatched.

The time taken to reach the different stages of development at 25, 28 and 30°C were compared with the findings of Hisaoka and Battle (1958) at 26°C. Comparisons revealed that the embryos cultured at a nominal temperature of 25°C and 26°C (Hisaoka and Battle, 1958) developed at a similar rate. Conversely, the embryos cultured at the higher temperatures (28 and 30°C) were always at an advanced stage of development when compared with those cultured at the lower temperatures other embryos. In addition, the embryo cultured at 30°C had hatched within 48 hours post fertilisation while Hisaoka and Battle (1958) did not observe hatching until 96 hours post fertilisation at a temperature of 26°C.

Table 2.2. Different stages of development at specific time intervals and different temperatures

Time post fertilization (h)	Stage according to the different temperature conditions			Stage according to Hisaoka and Battle (1958) 26°C
	25°C	28°C	30°C	
3	Stage 12 Very late blastula	Stage 13 Early gastrula	Stage 13 Early gastrula	Stage 10-11 Late high-flat blastula
6	Stage 14 Blastoderm enveloping $^{1}/_{3}$ yolk sphere	Stage 15 Blastoderm enveloping $^{1}/_{2}$ yolk sphere	Stage 16 Blastoderm enveloping $^{3}/_{4}$ yolk sphere	Stage 14 Blastoderm enveloping $^{1}/_{3}$ yolk sphere
24	Stage 20 Optic cup	Stage 21 Otolith formation	Stage 22 Retinal pigmentation	Stage 20 Optic cup
30	Stage 21 Otolith formation	Stage 23 Body pigmentation (melanophore)	Stage 23 Body pigmentation (melanophore)	Stage 21 Otolith formation
48	Stage 23 Body pigmentation (melanophore)	Stage 24 Xanthophore development	Stage 25 Hatch	Stage 23 Body pigmentation (melanophore)

Figure 2.4. Stages of development of zebrafish embryos cultured at different temperatures

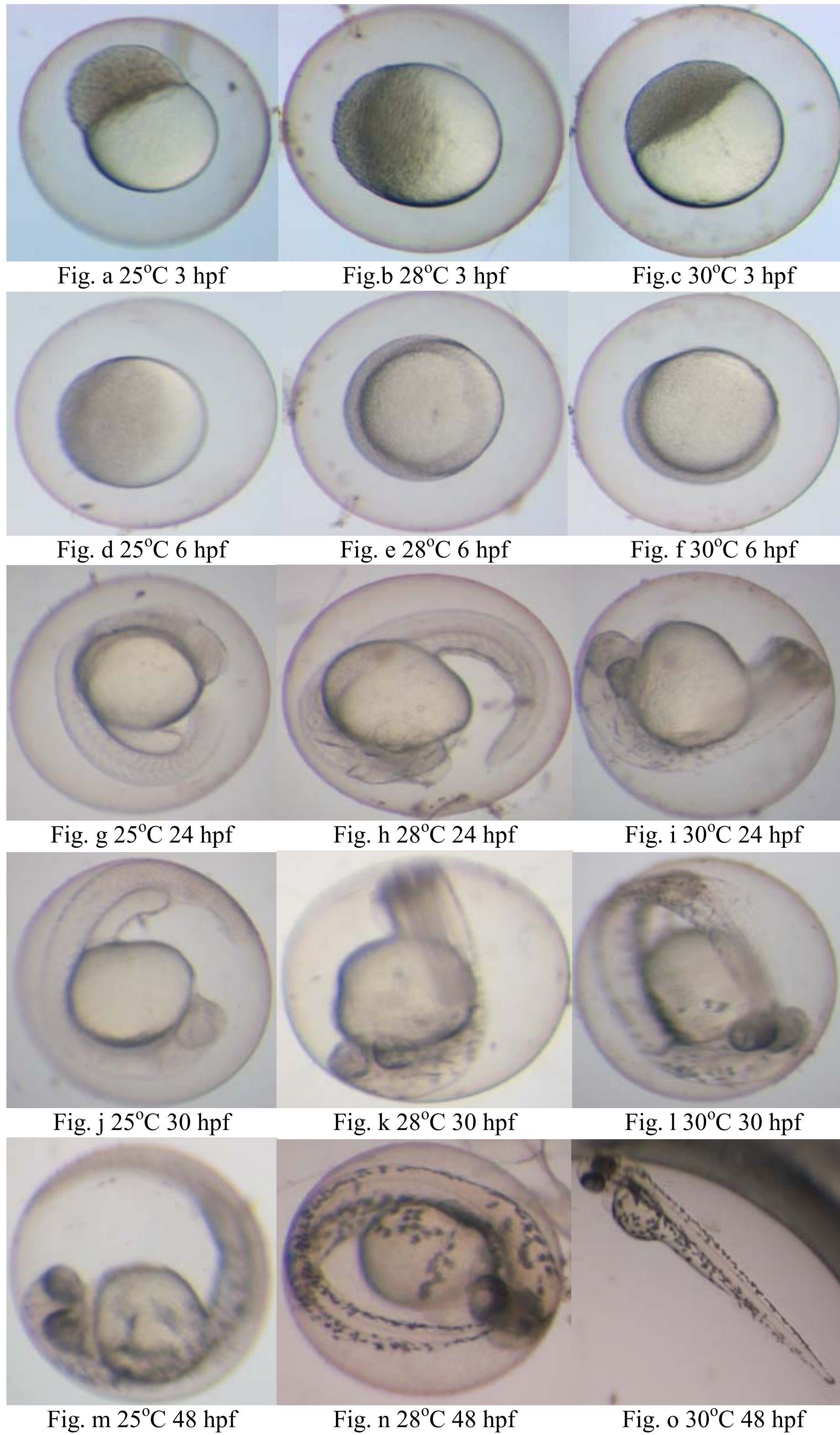


Fig. a 25°C 3 hpf
Fig.b 28°C 3 hpf
Fig.c 30°C 3 hpf
Fig. d 25°C 6 hpf
Fig. e 28°C 6 hpf
Fig. f 30°C 6 hpf
Fig. g 25°C 24 hpf
Fig. h 28°C 24 hpf
Fig. i 30°C 24 hpf
Fig. j 25°C 30 hpf
Fig. k 28°C 30 hpf
Fig. l 30°C 30 hpf
Fig. m 25°C 48 hpf
Fig. n 28°C 48 hpf
Fig. o 30°C 48 hpf

The results from the investigations carried out to supplement the work detailed above, focussing on the variability of the developmental rates of zebrafish embryos cultured at different temperatures, is shown in Table 2.3. The pH, conductivity, hardness (as $CaCO_3$), alkalinity and free and residual chlorine of the laboratory dechlorinated water supply was measured and determined as: pH 7.56, conductivity 211 µS cm^{-1}, alkalinity 22.2 mg l^{-1}, hardness 45.3 mg l^{-1}, and free and residual chlorine <2 µg l^{-1}.

Table 2.3. Periods of early development of zebrafish embryos cultured at 25 and 28°C using the descriptions of Kimmel *et al*. (1995)

Culture type	Stage of development				
	2h	24h	48h	72h	96h
12 well 28°C	All Blastula	11 embryos at pharyngula stage, 1 dead	11 embryos in hatching period, 1 dead	All remaining hatched	All remaining hatched
24 well 28°C	All Blastula	22 embryos at pharyngula stage, 2 dead	22 embryos in hatching period, 2 dead	88% hatched, 1 remaining at hatching stage	All remaining hatched
12 well 25°C	All Blastula	4 dead, 25% of the embryos between segmentation and pharyngula, remaining 75% at pharyngula	4 dead, 1 embryo remaining between segmentation and pharyngula, remainder entering hatching period	5 dead (including less developed embryo from 48h), 1 hatched, remaining 86% at hatching stage	All remaining hatched
24 well 25°C	All Blastula	23 embryos at pharyngula stage, 1 dead	2 dead, 1 at pharyngula stage, remainder entering hatching period	2 dead, 1 still at pharyngula stage, remainder all in hatching period	3 dead (including the less developed embryo from 72h), all remaining hatched

These data indicated that zebrafish embryos cultured in individual wells of 12 or 24 well plates, developed relatively synchronously at 28±1 °C, however, they developed relatively asynchronous at the lower temperature and at a slower rate compared to

those cultured at 28±1 °C. In addition, the point of hatch differed by up to 24 hours. This is in contrast to embryos cultured in groups in Petri dishes, which tend to hatch very synchronously. However, hatching synchrony of embryos cultured in Petri dishes may be due to the presence of hatching enzymes being released into the medium as the chorion of the hatching embryo ruptures.

Interestingly, in these investigations, the presence of under developed embryos was observed in the cooler temperature conditions. This indicates that the development of zebrafish embryos cultured at lower temperatures may be adversely affected and even retarded to the point where survivability could be compromised. There also appeared to be no relation to the position of the slower developing individual embryos within the plates.

Summary

The ontogeny of developing zebrafish embryos, discussed in this chapter, has shown that even a subtle difference in temperature can have a pronounced effect on the rates of development in zebrafish embryos. This could have significant implications regarding the use of zebrafish embryos in ecotoxicology as an alternative lifestage, particularly at high temperatures when embryos have hatched within 48 hours. Particularly as they have become technically classed as a protected organism according to the definitions applied in the Home Office Animals (scientific procedures) Act (1986). This will also mean that work carried out on hatched embryo/fry which are capable of independent feeding will need to be covered by a

Home Office license and may be subject to scrutiny by the Home Office inspectors. Furthermore, post hatched embryos should be covered by a Home Office license if it can be demonstrated that they have the capacity to feel pain and distress. This is a current area of research which is considered quite contentious within fish physiologists. In addition, there may also be issues regarding zebrafish embryos cultured at lower temperatures (e.g. <25 °C) for use in ecotoxicity assessments and particularly in developmental toxicity, as there may be issues regarding retardation, which could be recorded as false positives.

If the use of fish embryos is proposed as a replacement/refinement to the use of whole fish for ecotoxicological experiments then this should be approached with some caution. A potential solution to this problem would be to maintain the embryos at lower temperature (e.g. 26-27°C) for the duration of the experiment (48 hours), or to reduce the exposure time at the higher temperature (>28°C) so that the experiments are concluded before the embryos hatch. Furthermore, the potentially protective chorion could be a major barrier to entry of certain classes of chemical and the possibility of permeating the chorion could be essential should the use of fish embryos be accepted as an alternative approach to the use of fish for ecotoxicity testing. The chorion of zebrafish embryos will be investigated in greater depth in the following chapter.

CHAPTER 3 INVESTIGATIONS INTO THE STRUCTURE AND FUNCTION OF ZEBRAFISH CHORIONS

Introduction

To further supplement the understanding of developmental ontogeny of zebrafish embryos, the focus of this chapter is to investigate the structure and function of the protective envelope (chorion) of fish embryos *per se*, and then more specifically in zebrafish. An understanding of the structure and function of the chorion would enable a better understanding of the protective properties of this potential barrier to entry of certain chemicals. To investigate the structure and function of the chorion of zebrafish embryos, a series of scanning electron microscopic (SEM) images were obtained. The aim of these SEM images was to study the external surface of the chorion in order to develop a better understanding of how chemicals might pass through this membrane. These investigations included identifying and investigating the structure of the micropylar apparatus on the outside of the chorion. In addition, the pores on the inside surface of the chorion were identified and characterised. Complementary to these images, a series of supplementary images of fathead minnow embryos were also obtained to enable the chorions of fathead minnow embryos to be compared with that of zebrafish embryos.

Basic structural composition of fish chorions

The protective envelope of fish oocytes is commonly referred to as the chorion, although it is also known as the *zona radiata*. In Amphibians, it has been referred to as the vitelline envelope, in reptiles and birds as the perivitelline envelope and in

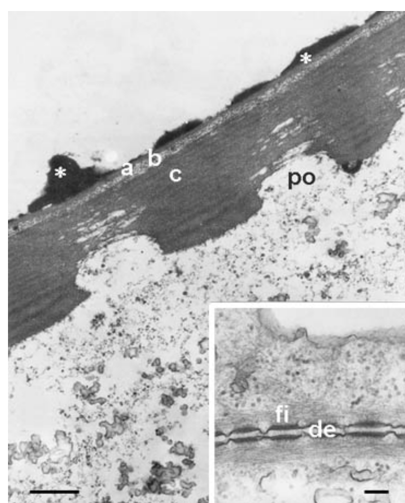
mammals as the *zona pellucida* although these definitions are interchangeable (Spargo and Hope, 2003). In terms of fish, the chorion is the outermost non-gelatinous envelope (Alderice, 1988), which encloses the developing oocyte forming a protective barrier to the external environment (Mizell and Romig, 1997). The structure of the chorion is usually multi-layered with varying thickness and numbers of layers depending on the species of fish (Bonsignorio *et al*., 1996). According to Guraya (1986), the ultra-structure of the chorion consists of mucopolysaccharides, glycoproteins, carbohydrate-protein matrices and protein-polysaccharide combinations. However, the exact biochemical combination depends upon the stage of development of the oocyte or the species of fish (Guraya, 1986; Bonsignorio *et al*., 1996). For example, in the early development of flounder oocytes the chorion is made up mainly from polysaccharides, and predominantly of proteins during the latter stages of oogenesis (Guraya, 1986). Furthermore, in most teleosts the inner layer is made up from proteins whilst the outer layer also contains polysaccharides (Guraya, 1986). There are also major differences between the structure of the chorion of freshwater and marine teleosts and also between pelagic and demersal marine teleosts (Hart and Donovan, 1983). Choriogenesis is thought to be related directly to the development of the egg and may be linked to follicular or hepatic activity (Bonsignorio *et al*., 1996). The embryo is separated from the chorion by the perivitiline space, which is filled with perivitiline fluid.

Ultra-structural analysis of the zebrafish chorion

The chorion of zebrafish embryos is an acellular envelope with three layers and is between 1.5 and 3.5 µm in thickness (Bonsignorio *et al*., 1996; Rawson *et al*., 2000). The three different layers of the envelope that are associated with the zebrafish

chorion are highlighted in Figure 3.1. In this image it can be seen that the inner layer (Z3) in the zebrafish constitutes approximately 75-80% of the chorion and is structurally similar to that of goldfish (*Carassius auratus*) and the monolayered chorion of rainbow trout (*Oncorhynchus mykiss*). Bonsignorio *et al.* (1996), described the inner layer as a fibrillar wave shaped component embedded in an amorphous matrix. The macromolecular structure of the zebrafish chorion, following analysis by Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE), has shown that it is constructed from four major polypeptides (molecular weights 116, 97, 50 and 43 kDa) and several minor components. The 116 and 50 kDa polypeptides are suggested to be N-linked glycoproteins and the 97 and 43 kDa polypeptides are not glycosylated (Bonsignorio *et al.*, 1996). The two internal layers of the envelope have been reported to have well defined pores traversing both layers, which contain microvilli (Hart and Donovan, 1983; Rawson *et al.*, 2000). However, the outer layer is an electron-dense lamina with osmiophilic material blocking the ends of the pores (Hart and Donovan, 1983).

Figure 3.1. Ultrastructural analysis of the zebrafish chorion showing the three layers (a,b and c) plus the pores (po)



http://www.harrisinternational.com/images/zebrafish.jpg accessed 26.04.2007

Permeability of the chorion

Most of the research that has been performed on investigating the permeability of fish embryos has been associated with the cryopreservation of fish embryos. The most common cryoprotectants that have been used are glycerol, dimethyl sulphoxide (DMSO), methanol and propane-1,2-diol (Harvey *et al*., 1983; Zhang and Rawson, 1996a; Zhang and Rawson, 1996b; Robles *et al*., 2003; Cabrita *et al*., 2003). Robles *et al*. (2003) indicated that the chorion is a potential barrier to cryoprotectants since it prevents water and solute movement in and out of the embryo.

Consistent with this, Harvey *et al*. (1983) reported that DMSO, glycerol and methanol permeate to relatively low levels in zebrafish embryos but of these, methanol permeates the most effectively. In addition, it was shown that glycerol entered the embryo relatively easily but only reached about 8% of the equilibrium level after two hours (equilibrium relates to the external concentration of the glycerol solution). DMSO reached about 2.5% equilibrium in the same period of time. Following dechorionation of the zebrafish embryos, the amount of DMSO permeating in to the embryo was shown to increase. Harvey *et al*. (1983) suggested that these findings indicated that the chorion retards the free exchange of solute. They also reported that the permeability of zebrafish embryos increases between the early cleavage stage of development and closure of the blastopore, and that permeability is maximal during epiboly.

The findings of Harvey *et al*. (1983), suggesting the relatively low permeation of cryoprotectants across the chorion, are in contrast to the results of Zhang and Rawson (1996 a and b). These authors reported that the chorion is effectively freely permeable to water and cryoprotectants and the actual barrier to cryoprotectants is the

inner vitelline membrane. Furthermore, these authors suggest that the pores, which transverse the chorion, are the major pathways for cryoprotectants entering into the embryo. However, it is unclear whether the cryoprotectants are responsible for dissolving the osmiophilic material that blocks the ends of the pores thus enabling the transfer of the cryoprotectants and water across the chorion. In addition, the authors hypothesised that the embryo, and the combined effect of the chorion and the perivitiline fluid, control the osmotic influx of chemicals in fish embryos and that permeability is not just influenced by the chorion.

Understanding the permeability of the chorion may also relate to comparative sensitivities between fish embryos and hatched eleutheroembryos. For example, Leonard *et al*. (2006), presented data to suggest that zebrafish embryos were not sensitive to certain classes of chemicals, but that newly hatched eleutheroembryos exhibited a similar toxic response to that of juvenile fish. These chemicals were from the quaternary ammonium group and included cationic and amphoteric polymers such as Merquat 100 and Luviquat HM552. Interestingly, the molecular weight of these chemicals is very high which is characteristic of surfactant-like chemicals. For example, Merquat 100 has a molecular weight of 150,000 and Luviquat HM552 has a molecular weight of 400,000. On initial discussions, Leonard (pers com) suggested that these chemicals were affecting respiration via the gills in the eleutheroembryos, which was not evident in the unhatched embryos, as at that stage of development they do not have fully functioning gills. However, more recent discussions with Leonard (pers com) indicated that dechorionated embryos were as sensitive to these compounds when compared to the hatched eleutheroembryos. This suggests that the chorion is preventing these chemicals from entering in to the embryos by acting as a barrier to entry from this particular group of chemicals.

Chapter 3

Possible routes for chemicals to enter into zebrafish embryos across the chorion

The chorions of fish embryos are multi-layered coatings designed to protect the developing embryo and on initial consideration it would seem unlikely that the envelope itself is permeable or porous to chemicals. For example, after the initiation of embryogenesis (post fertilisation), fish embryos take on water via the micropyle in a process called water hardening. When water hardening is complete, the embryo is characterised as being turgid, which is a function of positive pressure from within the embryo. To maintain this positive pressure, it is necessary for the chorion to prevent water escaping through the envelope of the embryo. Therefore, it is likely that if the envelope prevents water escaping through the chorion then it is equally likely that material cannot also enter into the embryo through the envelope. However, fish embryos are not completely resistant to desiccation if they are permanently removed from the aqueous environment. Hence, fluid inside the embryo must be able to escape from the embryo but it has yet to be ascertained how or from where this fluid is able to escape.

A possible entry route for fluid and other materials in to the embryo is via the micropyle. After sperm enters the embryo through the micropyle, this pore remains open and permeable to the external environment for a period of time as water is taken up and until the process of water hardening is completed. The process of sperm entry into the embryo via the micropyle has been comprehensively documented (Hart and Donovan, 1983; Wolenski and Hart, 1987 and Amanze and Iyengar, 1990), however, the exact process governing water hardening is less well understood. During the process of water hardening the micropylar apparatus changes to prevent polyspermic fertilisation (Haley and Wessel, 2004).

The micropylar apparatus is described as a funnel shaped vestibule, tapering from approximately 8 µm in diameter at the opening, to approximately 2.3 µm in diameter at the inner part of the canal (Hart and Donovan, 1983). At its narrowest point, the micropylar aperture is only just larger than that of the diameter of the head of a zebrafish sperm. Following sperm entry, the head of the sperm comes in to contact with the egg plasma membrane beneath the inner layer of the chorion, which triggers a cortical reaction (Haley and Wessel, 2004). This reaction causes the intracellular free calcium concentration to increase, which in turn causes cortical granules to fuse and release its contents into the surrounding space within the embryo. The contents of these granules are not fully characterised. However, the contents of these granules cause the osmotic pressure to decrease, which consequently causes water to enter the embryo via the open micropyle.

As the embryo inflates with water, during water hardening, it is likely that the miropylar apparatus constricts in diameter, and it is this constriction, which prevents polyspermic fertilisation. One thing, which is not fully understood, is whether the micropyle closes completely during the water hardening process and if so if the micropyle sealed permanently. For example, it is possible that although the opening of the micropylar apparatus contracts during water hardening, it could still remain open slightly even after the embryo has inflated fully and is turgid. The contents of the embryo could then remain within the boundary of the chorion via osmotic pressure and/or simply through water tension. Furthermore, if the micropyle closes over completely following water hardening, it may not be closed permanently and could perhaps be opened if the pressure inside the embryo changed. For example, it is possible that the osmotic pressure within the embryo fluctuates due to the contents of

the perivitiline fluid changing. These fluctuations could occur spontaneously or as part of a homeostatic response within the embryo.

In both examples suggested above, it is possible that if the internal pressure within the embryo increases significantly, that some of the contents of the perivitiline fluid would need to be released via a different route other than the micropyle. One potential route for the release of this aqueous material could be via the pores, which traverse the envelope. For example, if the micropyle is open and the osmotic pressure of the perivitiline fluid is low, then aqueous material will enter into the embryo. As the internal pressure increases within the chorion, it could be possible (if the osmiophilic material, blocking the external section of the pores, is semi permeable) that fluid could be released via the pores if the internal pressure reaches a critical level. Under such circumstances, as fluid is released via the pores, the internal pressure on the chorion would decrease which would cause the micropylar apparatus to dilate, thus enabling fluid to enter the embryo through the micropyle when the osmotic pressure decreases. Furthermore, it could be possible that the osmotic pressure could be modified if the contents of cortical granules are released into the perivitiline fluid. This influx and efflux of fluid may explain why zebrafish embryos appear to pulsate during embryogenesis if observed using time-lapse photography.

It should be noted that the osmiophilic material blocking the external section of the pores, have yet to be fully characterised and the assumptions of fluid transfer via the pores assumes that the osmiophilic material is semi permeable. However, if the osmiophilic material is impermeable, it is also possible that these pore plugs could act as a valve allowing material to exit via the pores when the internal pressure increases past a critical level. In addition to being a route for perivitiline fluid to be released

from the embryo, the pores may also be the route for fluid to enter into zebrafish embryos, assuming that the osmiophilic material allows fluid to pass through the pores. If the pores are partially permeable and allow fluid to enter in and out of the embryo, depending on the osmotic potential across the membrane, then the chorion should not be looked as being a barrier to entry for chemicals. In comparison to the level of understanding regarding the structure and function of the osmiophilic material, the structure of the pores have been well documented. For example, the diameter of the pore at its narrowest point is reported to be approximately 0.2 µm wide, which increases to 0.3-0.4 µm diameter at the inner opening of the canal (Hart and Donovan, 1983). The total number of pores, traversing a zebrafish embryo, has been calculated to be in the region of approximately 7 million (Hart and Donovan, 1983).

In addition to considering which route chemicals enter into the embryo, it is also important to consider other factors, which may influence uptake such as molecular size of the compound and physicochemical properties such as those of positively charge substances. In terms of the assumptions discussed herein, it is understood that chemicals, which have a large molecular weight generally, also have a large molecular size.

The work of Leonard and Braunbeck (pers com) has indicated that chemicals which are not toxic to embryos but that are toxic to eleutheroembryos are chemicals with large molecular weights, and include positively charged surfactant-like chemicals. The chemicals studied were all quaternary ammoniums including, amphoteric and cationic polymers (e.g. Merquat 100 and Luviquat HM552) (Leonard *et al*., 2006). However, when the same chemicals were tested using dechorionated embryos, the

embryos were found to be as sensitive as the eleutheroembryos. This suggests that chemicals with a large molecular weight and hence molecular size and that are positively charged, are in fact not able to enter the embryo. Therefore, it could be assumed that these large molecular substances are either too big, or form complexes which are too big, to pass through the envelope, the micropylar apparatus or the pores in the envelope.

In order to fully investigate the different possible routes for chemicals to enter through the chorion, a series of electron microscopic images were obtained. The following section focuses on these images with a particular focus on identifying the possible routes for chemicals to enter the embryo through the chorion.

Electron microscopic investigations into the structure of the chorion of zebrafish embryos

To investigate the structure and function of the chorion of zebrafish embryos, a series of scanning electron microscopic (SEM) images were obtained. These investigations included characterising the external surface of the chorion plus, identifying and investigating the structure of the micropylar apparatus on the outside of the chorion. In addition, the pores on the inside surface of the chorion were identified and characterised. Complementary to these images, a series of supplementary images of fathead minnow embryos were also obtained to enable the chorions of fathead minnow embryos to be compared with that of zebrafish embryos.

Materials and methods

Zebrafish embryos were obtained from the husbandry unit at BEL, as described in the previous chapters. Twenty embryos were fixed in a 2% ($^{v}/_{v}$) solution of glutaraldehyde and stored in a centrifuge tube until they were processed for analysis with a scanning electron microscope (SEM). In addition to the zebrafish embryos, fathead minnow (*Pimephales promelas*) embryos were also obtained from the husbandry department at BEL and fixed using 2% ($^{v}/_{v}$) glutaraldehyde.

The embryos were transported to the Bioimaging centre of the University of Exeter, where they were prepared for imaging by critical point drying using a series of alcohol solutions. Once prepared, the embryos were added to 12 mm diameter carbon tabs, attached to Jeol mounting stubs and then coated with osmium using a Fisons SEM coating system. The images were then observed using a Jeol JSM-6390LV scanning electron microscope.

Results

A series of images of the zebrafish embryos were obtained. The first image (Figure 3.1) is a zebrafish embryo 4 hours post fertilisation and the subsequent images (Figures 3.2-3.4) are more detailed images of the chorion. To investigate the internal structure of the chorion, several zebrafish embryos were broken open post dehydration but prior to coating with osmium. An example of one of these embryos is shown in Figure 3.5, and Figures 3.6 and 3.7 are from the internal surface of this chorion particularly focussing on identifying the pores. The final images (Figures 3.8-3.11) are taken from the chorion of a fathead minnow embryo.

Chapter 3

Investigations into the structure of the external surface of the chorion

Figure 3.1, shows the image of a zebrafish embryo 4 hours post fertilisation taken at a magnification of X 85. The embryo shown has lost its spherical shape and has started to collapse possibly due to the process of critical point drying. This can be a problem with preparing fish embryos for SEM as they are comprised mainly of water and easily loose their shape and structure when the water is removed. However, this does not prevent the structure of the chorion from being observed and at a magnification of X 8000 (Figure 3.2) the small extrusions appear to have fibrilar extensions spreading out from the body of the extrusions and penetrating into, or from, the surface of the chorion. These extrusions could be specific structures associated with the chorion or could be fungal bodies attached to the chorion by mycelium spreading out through the junctions between the polypeptides on the surface of the chorion. In addition to these unidentified extrusions on the surface of the chorion, other micro organisms such as bacteria are visible. Interestingly, on the external surface of the zebrafish chorion individual polypeptides can be observed fitting together very closely. How tight these junctions are and the potential permeability between these molecules is unknown. Therefore, chemicals may be able to enter through the chorion via the gaps in the polypeptides. Furthermore, it appears that the surface of the chorion is undulated with the polypeptides recessing into holes. The areas where these recesses occur are likely to be due to the pores on the inside surface of the chorion as they appear at a similar frequency to that of the pores detailed by Hart and Donovan (1983).

Figure 3.1. Zebrafish embryo approximately 4 hours post fertilisation (X85 magnification)

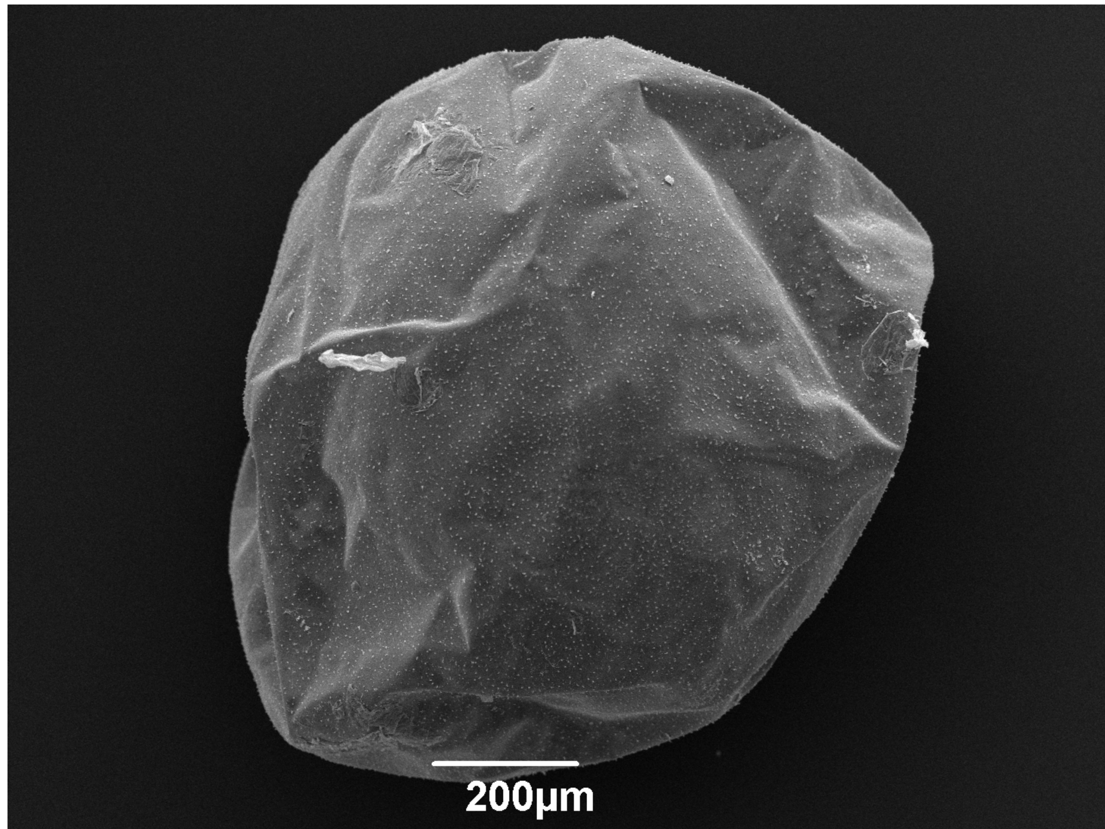


Figure 3.2. Chorion of the zebrafish embryo (X8000 magnification)

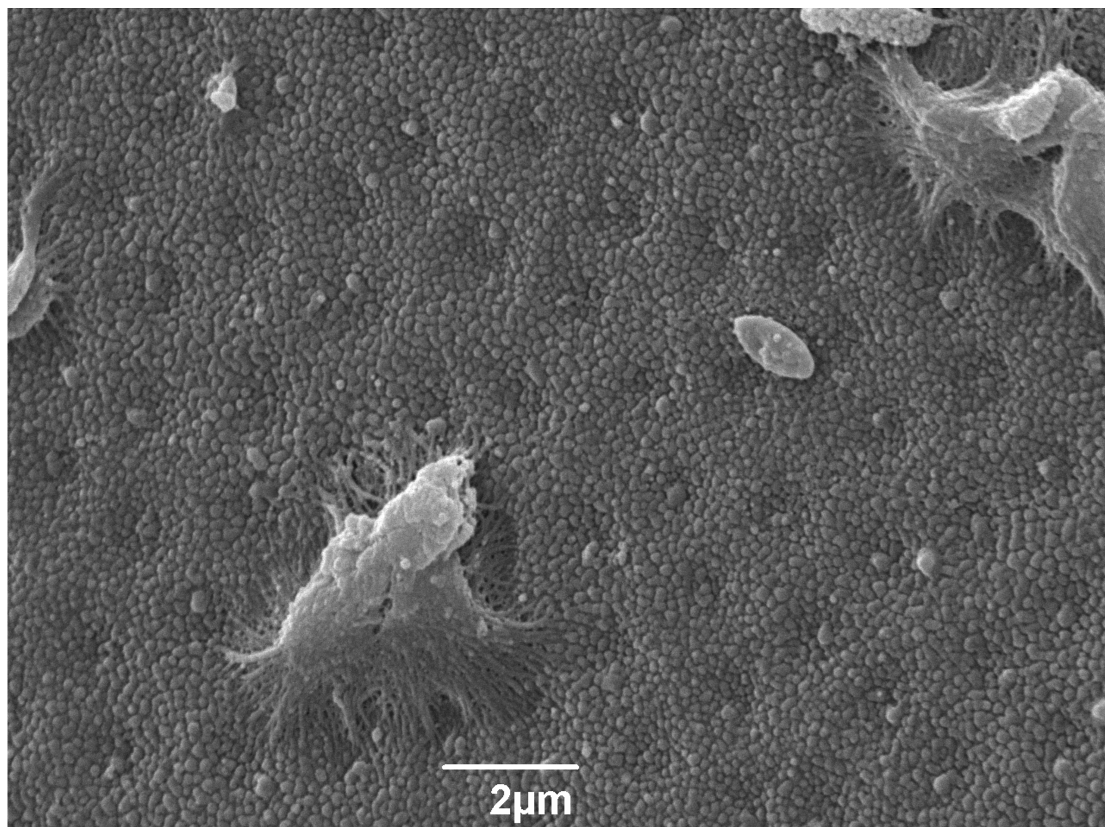


Figure 3.3 shows the external surface of the chorion with the micropyle identified (d). Figure 3.4 is of the micropyle at a higher magnification of X 3700 and this image clearly shows the spiral structure of the micropyle tapering to a diameter of approximately 2.5 µm. This is consistent with the descriptions of Hart and Donovan (1983) who detailed that the micropyle tapered from a diameter of approximately 8 µm on the outside to approximately 2.3 µm at its narrowest point. The concentric appearance of the micropyle would perhaps indicate that chorion is constructed from many layers, however, as discussed previously, characterisation of the zebrafish chorion has shown that it is formed from only 3 distinct layers (Bonsignorio *et al*., 1996; Rawson *et al*., 2000). It is possible that the site of the micropyle is the final attachment point of the chorion to the wall of the follicle prior to release of the oocyte into the ovary. Furthermore, the concentric appearance of the micropyle could be a result of the process of choriogenesis during the development of the oocyte.

Considering the micropyle as a route of entry for water and dissolved substances into the embryo, it is clear from the images presented herein that the micropyle is a hole in the chorion which is not completely sealed. This may be due to the dehydration process, whilst preparing the embryos for imaging, or because the embryo is no longer turgid. Nevertheless, it is a distinct possibility that this is the portal for transfer of aqueous material between the inside and the outside environment.

Figure 3.3. External surface of the chorion with micropyle (a) (X 370 magnification)

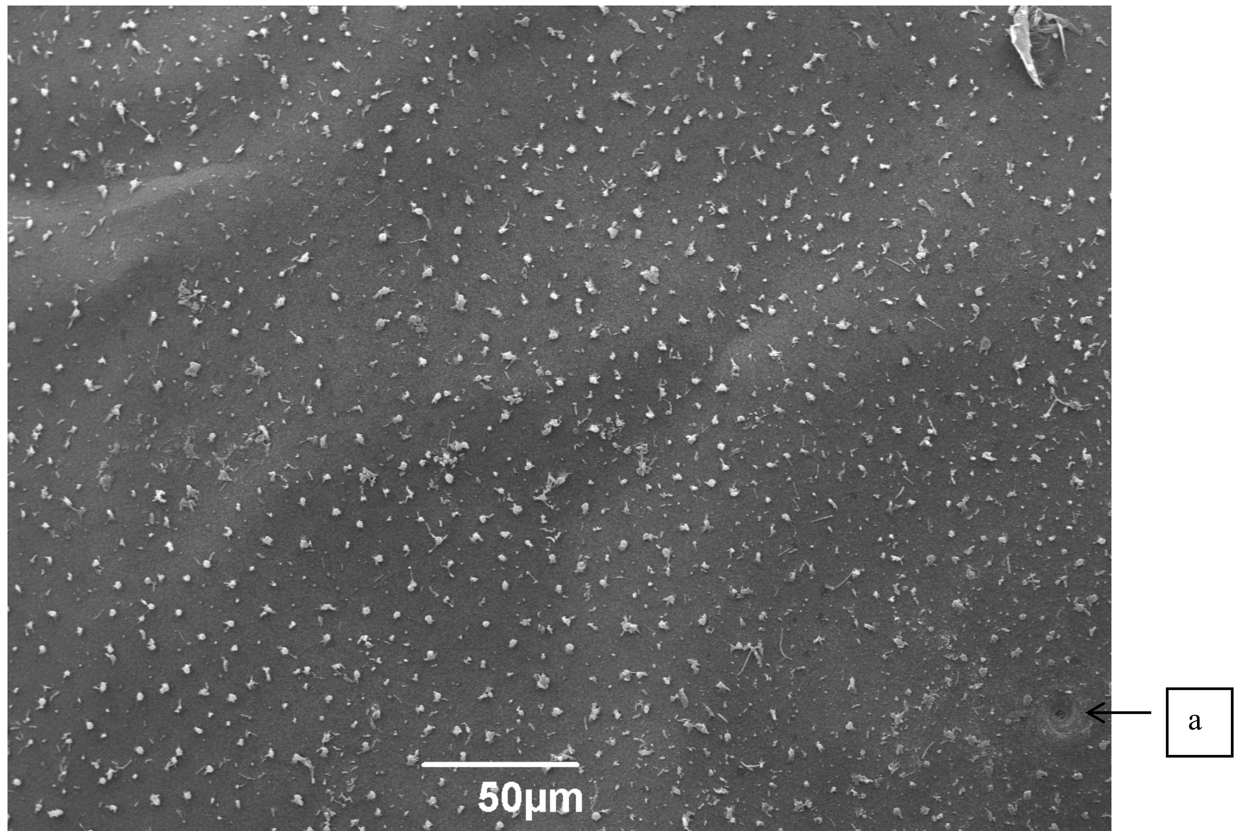
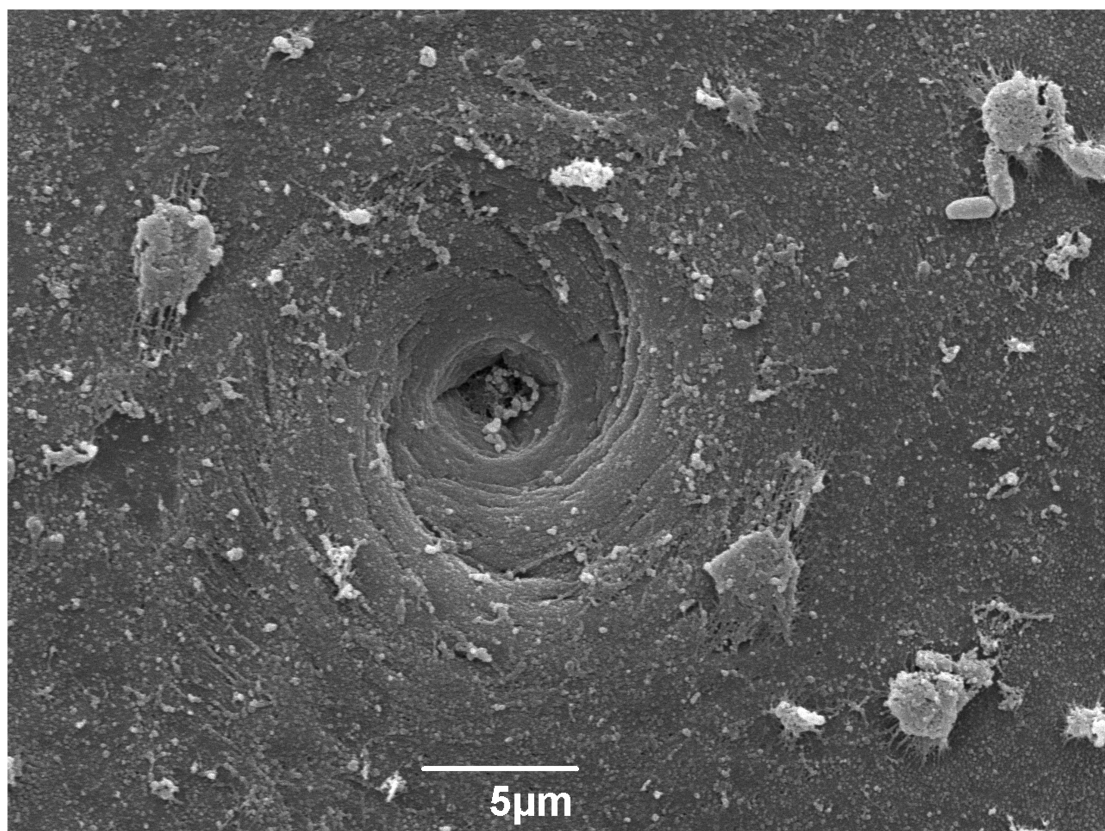


Figure 3.4. External surface of the chorion with micropyle (X 3700 magnification)



Investigations into the structure of the internal surface of the chorion

Figure 3.5 shows the image of a zebrafish embryo broken open prior to coating with osmium at a magnification of X 55. The subsequent images (Figures 3.6 and 3.7) are also from the inner surface of the chorion at a higher power of magnification (X 2700 and X 10000 respectively). From these images (Figures 3.6 and 3.7), it is possible to see pores on the inner surface of the chorion. Each pore is approximately 0.5 µm in diameter and the frequency of pores appears to be distributed relatively uniformly across this inner layer. The pores are spaced approximately 2 µm apart and appear at a frequency consistent with that detailed by Hart and Donovan (1983). At the higher magnification of X 10000 (Figure 3.7), the surface of the inner layer of the chorion appears to be very different from the outer structure of the chorion as shown in Figure 3.2. The inner layer does not appear to be constructed of the individual polypeptides but looks more like an amorphous layer. From looking at this inner layer it appears relatively rigid in comparison to the glycoprotein outer layer, which may indicate that the inner layer has a structural function supporting the chorion of the zebrafish embryo. If this inner layer is more rigid than the outer surface it is possible that the pores function as an expansion gap when the embryo expands during water hardening.

Figure 3.5. Zebrafish embryo broken open prior to coating (X 55 magnification)

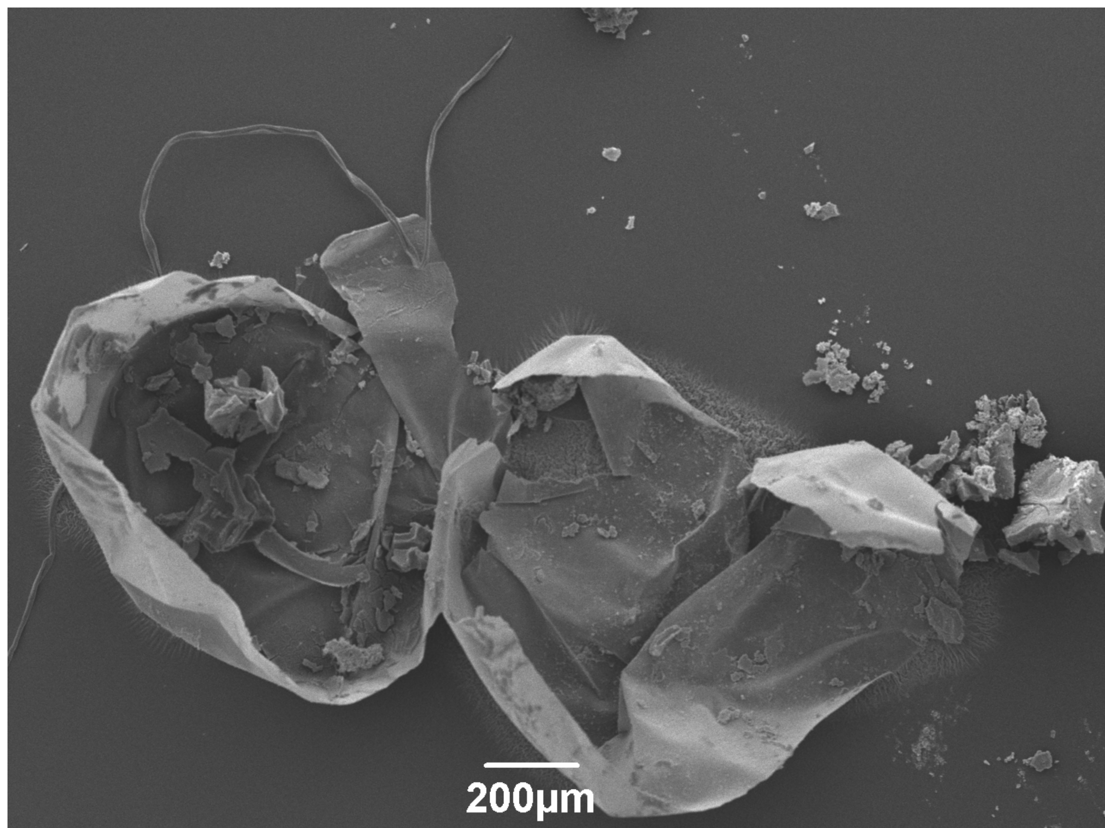


Figure 3.6. Internal surface of the chorion (X 2700 magnification)

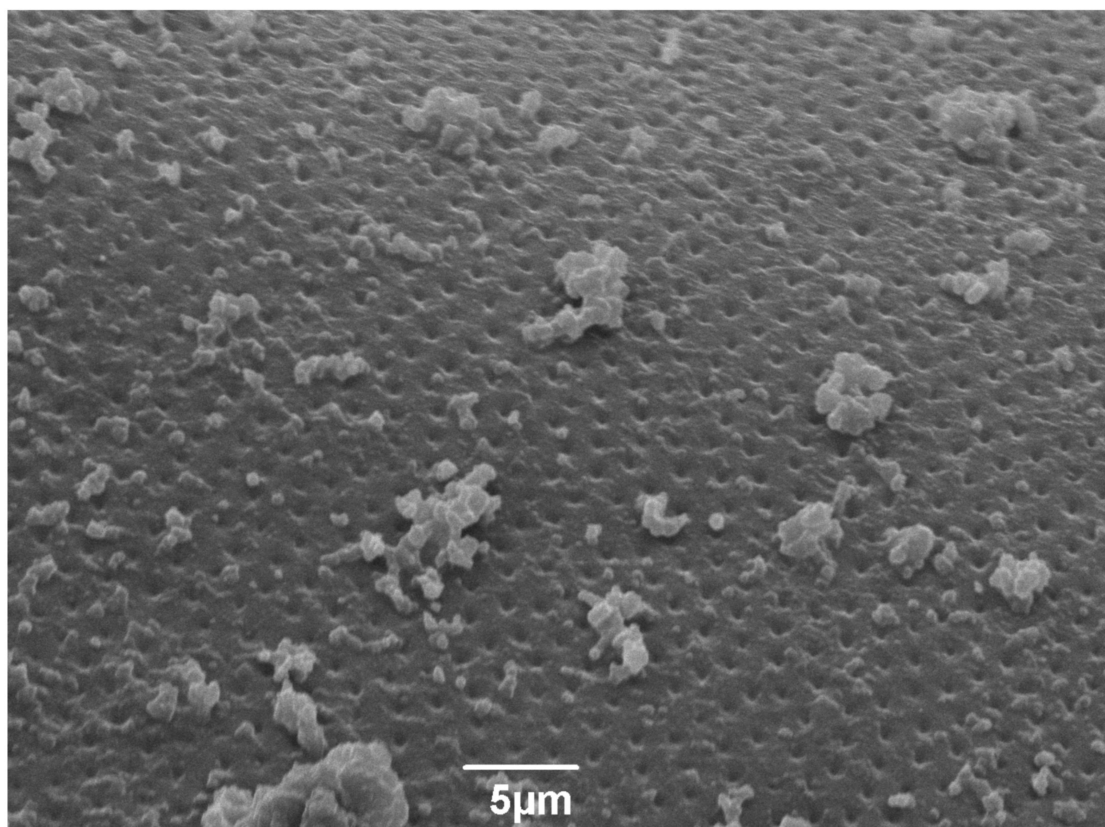
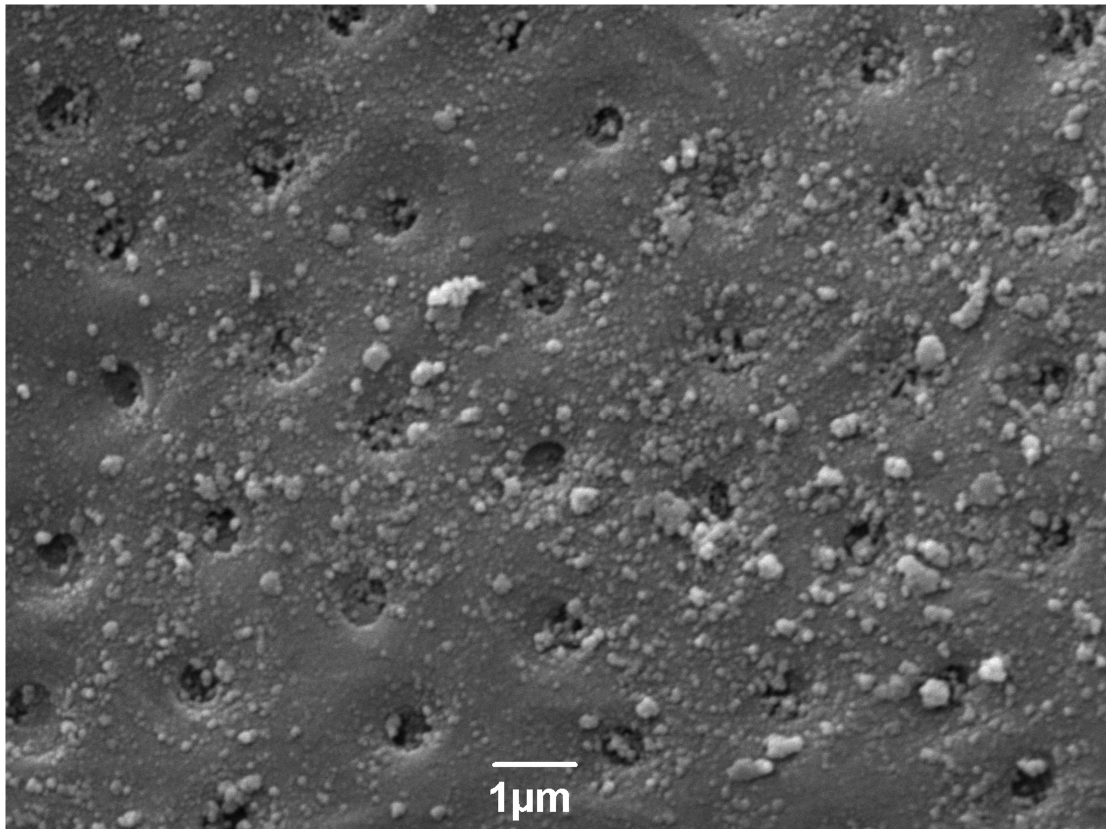


Figure 3.7. Internal surface of the chorion (X 10000 magnification)



Investigations into the structure of the chorion of fathead minnows

Fathead minnow embryos were obtained and observed for comparative purposes with the zebrafish images. The following images (Figures 3.8-3.9) are of a fathead minnow embryo approximately 4 hours post fertilisation. As with the untreated zebrafish embryos, the fathead minnow embryos had collapsed during the drying process. Interestingly, the fathead minnow embryos showed some striking differences to the zebrafish embryos. The main visible difference in the appearance of the fathead minnow chorion is that it has a much smoother and lustrous appearance, whereas the zebrafish chorion is matt and textured in comparison. Furthermore, in comparison to the zebrafish embryos, there were considerably less particulates and micro organisms stuck to the outside of the chorion.

Figure 3.8. Fathead minnow embryo approximately 4 hours post fertilisation (X 80)

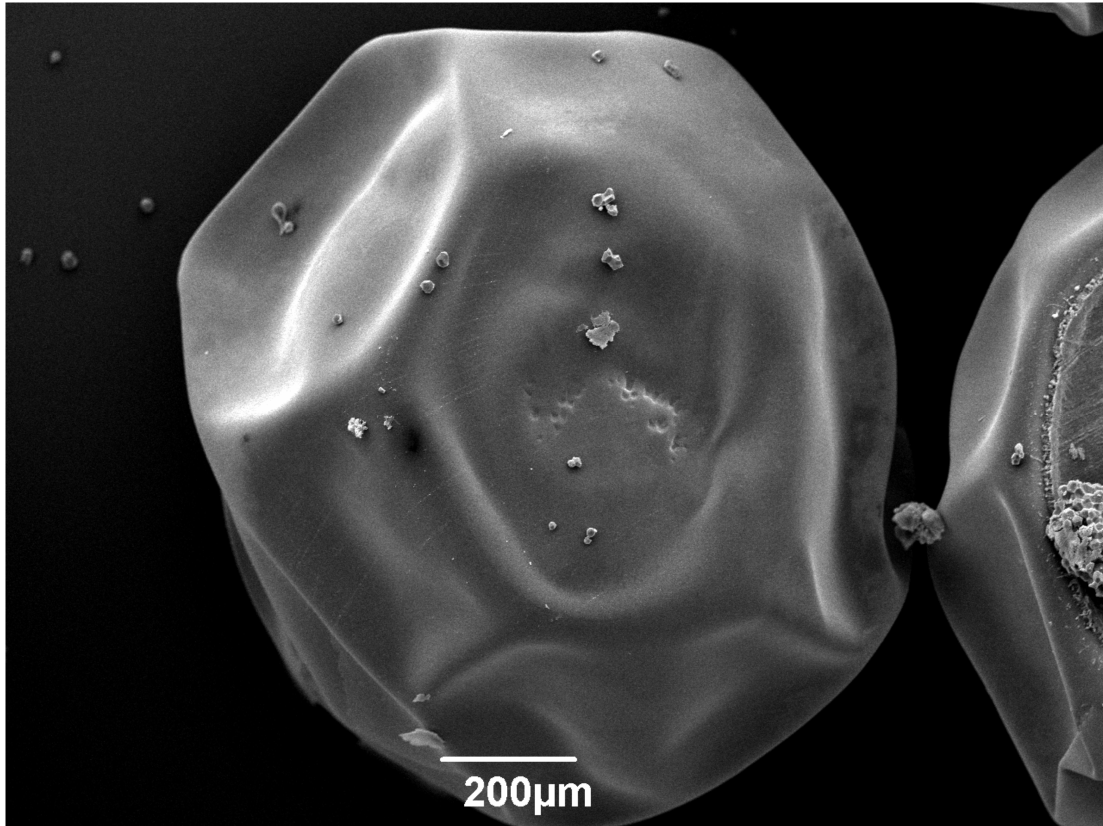
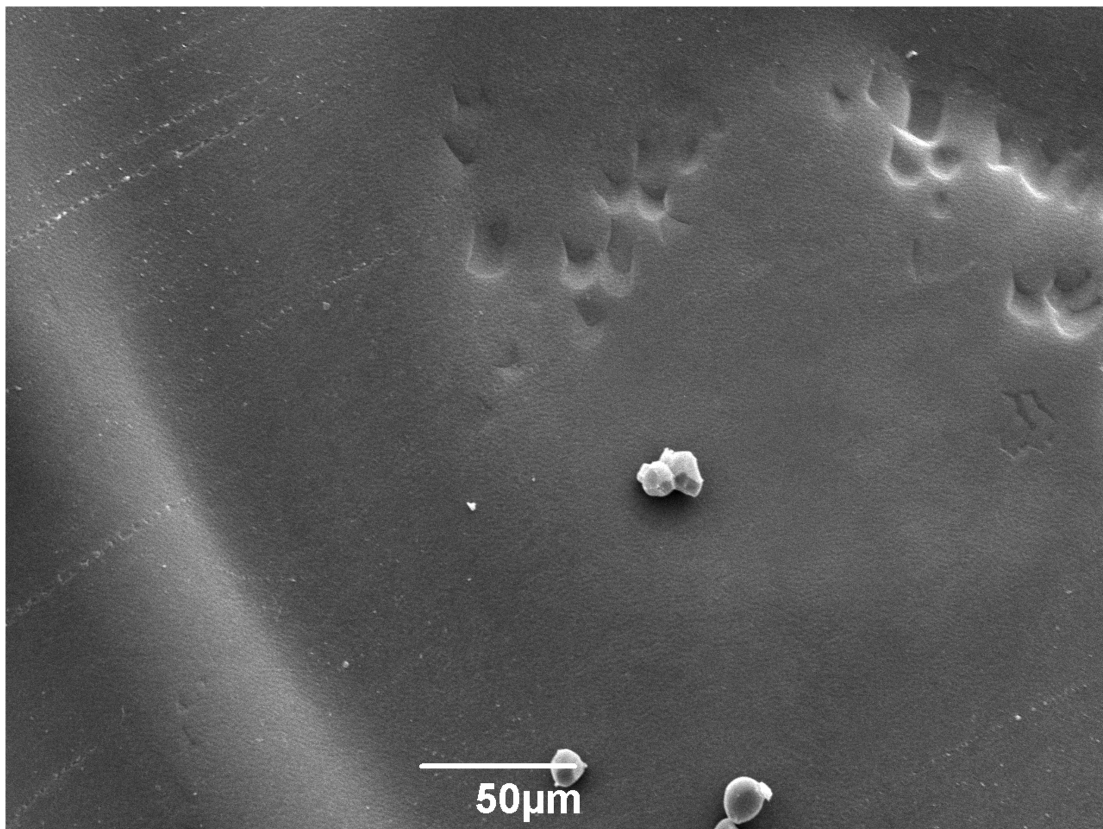
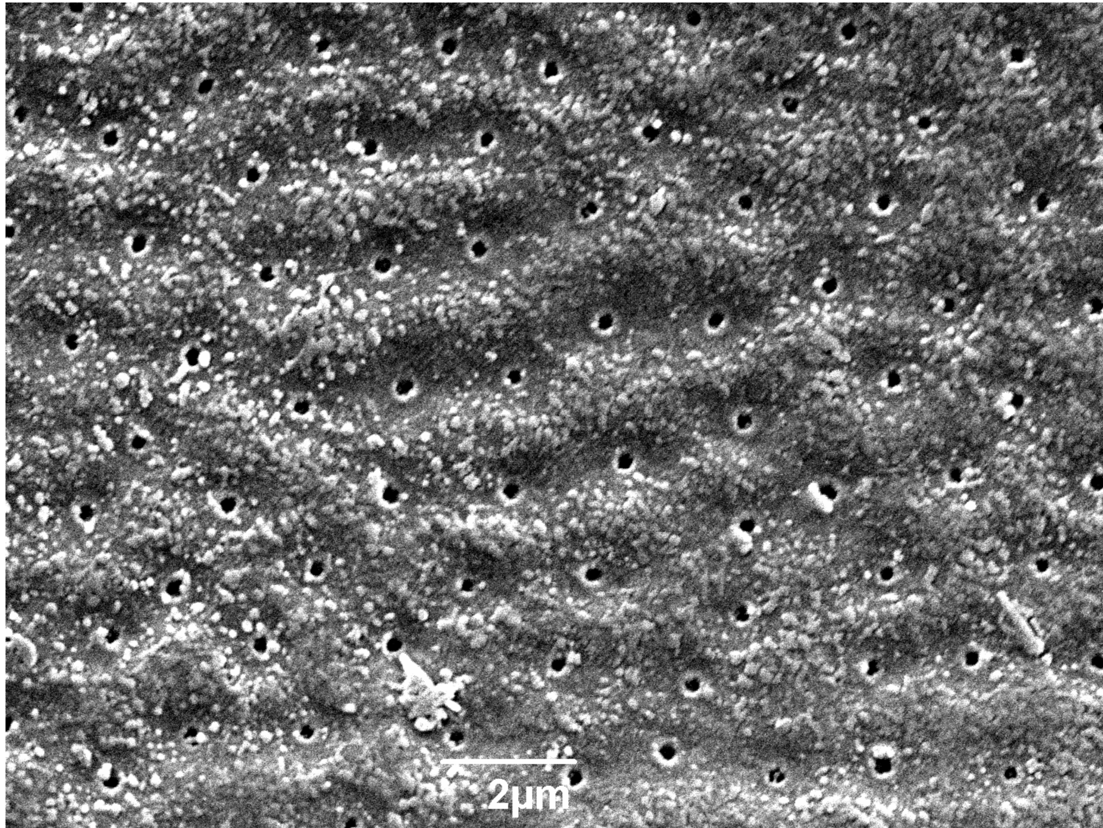


Figure 3.9. Fathead minnow embryo approximately 4 hours post fertilisation (X 370)



The most interesting discovery from the images of the fathead minnow chorion, observed at the higher power magnification of X 8000 (Figure 3.10), was that pores are clearly evident on the outside surface of the chorion. These pores are distributed across the chorion at a similar frequency to the pores on the internal surface of the zebrafish chorion. The size of the pores on the external surface of the chorion are approximately 0.2 µm in diameter and are relatively consistent in size across the surface (±0.05 µm). Unfortunately, there were no images obtained from the internal surface of the fathead minnow chorion, hence it was not possible to determine if these pores traversed all the way through the chorion or only through the outer layers of the chorion. The ultrastructure of the fathead minnow chorion also differs considerably with the zebrafish chorion. For example, individual glycoproteins are evident on the external surface of the zebrafish chorion but the chorion of the fathead minnow embryos appears to be relatively amorphous and more similar to the internal surface of the zebrafish chorion.

Figure 3.10. Fathead minnow embryo approximately 4 hrs post fertilisation (X 8000)



Conclusions

The chorion of zebrafish embryos has been studied from the SEM images presented within this chapter. The glycoproteins, which constitute the outer layer of the chorion are clearly visible using SEM techniques and it is also evident that this external surface is a flexible membrane to which particles and micro organisms adhere. On inspection of the internal surface of the chorion, it is clear that it is permeated with numerous pores, which could be the route of aqueous transfer into and out of the embryo across the chorion. However, the glycoprotein outer layer of the chorion may possibly prevent the transfer of aqueous material into and out of the embryo. The other possibility for chemical entry, as discussed in the previous chapters, is the micropyle. The images that were taken of the micropyle could be considered to

substantiate this hypothesis as it appears to be a significant hole through the membrane. However, it is not known if any of the drying processes during the sample preparation, affected the degree of opening of the micropyle. Furthermore, it was not possible to obtain any images of the micropyle from the internal surface of the chorion so it was not possible to confirm whether the micropyle was open on the inside or whether the hole becomes plugged post fertilisation. Further investigations of the inner surface of the chorion would be required to visualise the micropyle on the inside of the chorion. However, there may be questions regarding the efficacy of images of membranes that have been treated with such an invasive technique. Therefore, further work would be beneficial to develop a non-destructive technique to obtain some supplementary images.

In comparison, the chorion of the fathead minnow embryos has revealed some interesting differences between the membranes of the two different species of fish. The chorion of the fathead minnow appears to be much smoother and lustrous in appearance and there are pores evident on the external surface of the chorion. However, it is unclear whether the pores transverse the entire membrane and therefore, this would warrant further investigations to understand if the pores are present on the internal surface. The clear difference between the chorions may have some link with the method of spawning for each species of fish. For example, zebrafish are a broadcast spawner, which means that they disperse their embryos during the mating process. During this process these embryos simply fall to the bottom and then develop within the substrate at the bottom of the habitat. Whilst on the bottom, detritus and micro organisms, which come in to contact with the embryos, become attached to the surface of the chorion. In comparison, the fathead minnows

use an egg depositing technique. This involves the male fathead minnow encouraging the female to deposit her eggs on the underside of a substrate such as an over hanging rock. During egg depositing, the male fathead minnow releases his milt to fertilise the eggs. Subsequently, he will then tend and clean the embryos until they hatch. This cleaning process is evident as the surface of the chorion of the fathead minnows is very clean compared to the zebrafish chorion. In addition, the very smooth and lustrous appearance is likely to be a physical adaptation, enabling the embryos to stick to the substrate on which the eggs are deposited. In addition, the pores on the outside of the chorion may have a physical role in causing the embryos to adhere to substrates as the embryo inflates during the water hardening process post fertilisation. Nevertheless, these pores on the outside edge of the fathead minnow embryos warrant further investigation. In addition, it would be important to understand if chemicals such as the cationic polymers (e.g. quaternary ammoniums), which do not have a toxic effect on chorionated embryos, are able to pass through the chorion of fathead minnow embryos. Such information would be important from the regulatory aspect of the use of fish embryos as a surrogate for juvenile fish in ecotoxicity assessments. For example, the proposed OECD Fish Embryo Toxicity (FET) draft test guideline is focussed on the use of zebrafish as the test species. However, in light of the fact that fathead minnow embryos appear to have pores on the outside of the chorion could suggest that the chorion of these embryos may be more permeable to substances such as the cationic polymers.

CHAPTER 4: INVESTIGATIONS INTO THE UPTAKE OF RADIOLABELLED COMPOUNDS INTO ZEBRAFISH EMBRYOS

For the purpose of this thesis, in order to better understand the permeability of zebrafish chorions and uptake of chemicals into the embryo, a procedure was needed to dissect and then measure compounds within the different partitions of the embryo. At the outset of these investigations, it was not known if it would even be possible to measure radiolabelled material within a single zebrafish embryo. Therefore, a preliminary set of investigations were performed (phase 1) to develop an appropriate method for dissecting the embryos and then to determine if either radiolabelled Estradiol or Fluoranthene could be measured within the internal contents of the embryo. These preliminary investigations were based on a small number of comparative data, which meant that it was difficult to draw any firm conclusions about the results apart from some general findings. However, from these preliminary investigations, the data showed that it was possible to measure [4-^{14}C]Estradiol or [3-^{14}C]Fluoranthene within individual embryos which had been exposed for 24 hours at a concentration of 0.1 and 1.0 mg l^{-1} to a solution of and a preferred method for dissecting the embryos was determined.

Following these preliminary investigations, a further set of optimisation studies (Phase 2) were performed to enable the uptake of [4-^{14}C]Estradiol or [3-^{14}C]Fluoranthene into zebrafish embryos to be quantified. To optimise the methods a series of experiments were performed, each quantifying the amount of radio labelled compound taken up into the different partitions. These investigations are presented as individual experiments following the reporting of the Phase 1 preliminary investigations.

Materials and methods

Test substance

The test substances used in these investigations were all standard reagent based chemicals and are specified in Table 4.1. The chemicals were chosen on the basis of availability within the laboratory and of those that had different octanol water partition coefficients ($\log_{kow}$), i.e. different characteristics of lipophilicity. The exposure concentration of each chemical was selected based on published acute toxicity data for fish along with an appropriate safety factor to prevent lethal effects from occurring. An additional consideration in the choice of the test concentrations was that the test solutions should contain sufficient amounts of radioactivity to enable detection in relatively small volumes without causing an adverse effect to the embryos. Therefore, in Phase 1 the concentration of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol was 1.0 and 0.1 mg l^{-1} respectively, and for Phase 2 the concentrations used were 0.5 and 0.1 mg l^{-1}respectively.

Table 4.1. Test chemicals and concentrations tested

Chemical name	**$\log_{kow}$**	**Supplier**	**96 h LC_{50} (fish species)**	**Reference**
[4-^{14}C]Estradiol	4.01	Perkin Elmer	0.46 mg l^{-1} (Medaka embryo)	Shosaku *et al* (2002)
[3-^{14}C]Fluoranthene	5.22	Sigma-Aldrich	4.0 mg l^{-1} (Bluegill sunfish)	Buccafusco *et al* (1991)

Test conditions

The dilution water, and methods for obtaining the embryos were as described in the previous chapter. The preliminary investigations were conducted in plastic 96 well microtitration plates (Linbro/Tiertek), with a well capacity of approximately 0.35 ml. And the further optimisation studies were carried out in plastic and glass[12] Petri dishes (90 mm diameter). All work was carried out in a temperature-controlled laboratory (27 ± 2°C) with a photoperiod of 16 hours light: 8 hours of dark and with a 20 minute dawn/dusk transition period.

Combustion method used prior to liquid scintillation counting

To determine if the radiolabelled material had been taken up into the embryo, the samples first needed to be combusted using a biological oxidizer to release the carbon (as CO_2) from the biological material. The samples were oxidized in a Packard 307 sample Oxidizer. Samples were placed in cellulose combustion cones (Perkin-Elmer Life & Analytical Sciences, USA), covered with cellulose powder (Perkin-Elmer Life & Analytical Sciences, USA) and then combusted for a burn time of approximately 30 seconds. The time for complete combustion could be adjusted and any samples, which had not fully combusted within 30 seconds, were further oxidized prior to the addition of Carbosorb (Perkin-Elmer Life & Analytical Sciences, USA [≈10 ml]) and Permafluor (Perkin-Elmer Life & Analytical Sciences, USA [≈10 ml]). Carbosorb was added to trap $^{14}CO_2$ produced in the combustion cycle followed by Permaflour,

[12] The initial investigations with [3-^{14}C]Fluoranthene were in plastic Petri dishes, however, all subsequent investigations in Phase 2 were performed in glass Petri dishes to avoid substance adhering to the plate.

which was then added as a scintillator. Lids were then screwed onto the LSC combustion vials and the samples placed in a LSC counter for analysis. The quantity of each chemical within the different partitions was calculated from the amount of becquerels measured in each sample, using the formula:

Quantity of compound (ng) = amount of becquerels/specific activity*1000

Where specific activity of [3-^{14}C]Fluoranthene was 8230 and the specific activity of [4-^{14}C]Estradiol was 7063.

Method used for liquid scintillation counting

Samples for LSC analysis were added to glass scintillation vials and Optiphase 'Hisafe' 3 scintillator (Perkin-Elmer Life & Analytical Sciences, USA) added to fill the vial. Each sample was counted in a Canberra-Packard (now Perkin-Elmer) spectrometer. All samples were preceded by one background determination, and all test solution samples were counted in triplicate. All samples were corrected for quenching by comparison to known standards using the Packard tSIE external quench correction technique. Each sample was counted for at least 10 minutes, or until a 2 sigma value of less than 2.0 was obtained, whichever was the sooner.

LSC Method

Radionuclide:

Radionuclide:	14^{C}
Count Mode:	Normal
Quench Set:	14C SLD 2006
Quench Indicator:	tSIE
External Std Terminator:	0.5 2s%

Count Parameters:

Precount Delay (min):	0.00		
Count time (min):	20.00		
Assay Cycles:	1		
Repeat Sample Count:	1		
Calculate Reference:	No		
#Vials/sample:	1		
Regions:	Lower limit		Upper limit
	A	0.0	156.0
	B	4.0	156.0
	C	0.0	0.0
Background subtract:	1st Vial		
Low CPM Threshold:	A	0.0	
	B	0.0	
	C	0.0	
2 Sigma % Terminator:	Any Region		
	A	1.0	
	B	1.0	
	C	1.0	
Count Corrections			
Static Controller:	On		
Coincidence time (nSec):	18		
Delay before burst (nSec):	75		
Apply Half Life Correction:	No		

Phase 1: Preliminary investigations

The preliminary investigations to determine an effective method for dissecting the embryos involved exposing 20 embryos to a single concentration of either [4-^{14}C]Estradiol (0.1 mg l^{-1}) or [3-^{14}C]Fluoranthene (1.0 mg l^{-1}). Embryos were exposed individually in single cell wells of a 96 well plate (n=20 embryos pre treatment) each well containing 200 µl of test substance. Embryos were selected for dissection and subsequently analysed after 2 or 24 hours exposure, however, due to the time taken to dissect the individual embryos only a limited number of embryos were successfully dissected for both substances (n=3).

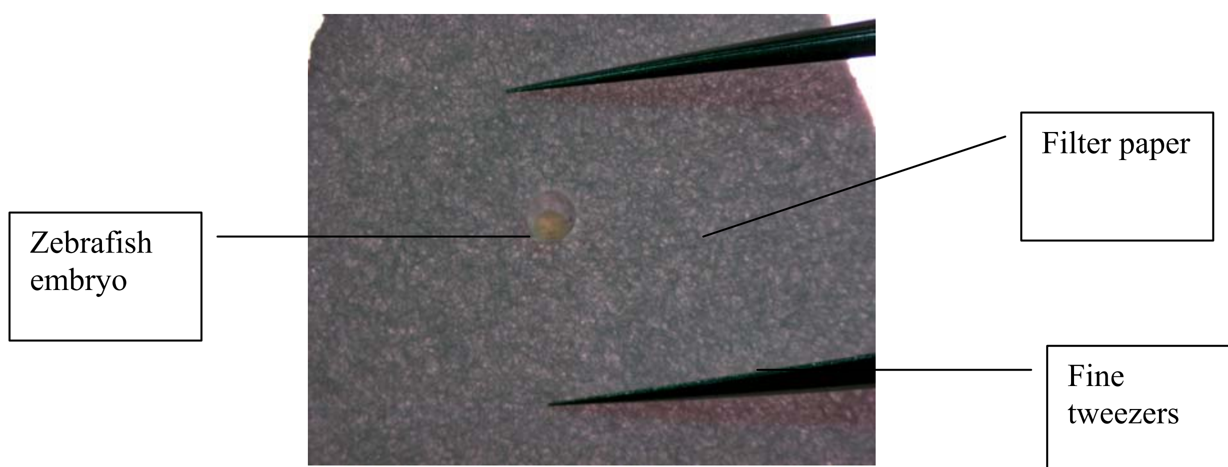
Dissection of embryos

The first dissection method involved dissecting the embryos in clean dechlorinated water in a Petri dish. Prior to dissection, the embryos were rinsed to remove any test substance transferred with the embryos. Individual embryos were then replaced into individual cell wells containing clean dechlorinated water, and the chorion of the embryo carefully teased apart using a pair of fine tipped tweezers. The embryos and chorions were then removed from the cell well with great care taken to minimise transfer of contaminants. The samples were then combusted and analysed as detailed above. The perivitiline fluid was analysed in the clean dechlorinated water of the cell well directly via LSC.

The second dissection method involved dissecting the embryos onto clean filter paper of approximately 50 mm diameter (see Figure 4.1). Prior to dissection, the embryos were blotted onto a piece of filter paper to remove any test substance from the outside

of the embryo. Following this, the chorion of the embryo was carefully teased apart and the perivitiline fluid released onto the filter paper. The chorion was then separated from the embryo and placed onto separate pieces of filter paper. All pieces of the filter paper were combusted separately prior to LSC as detailed above.

Figure 4.1. Zebrafish embryo dissected using the filter paper method



RESULTS

Determination of [3-^{14}C]Fluoranthene in zebrafish embryos

In these preliminary investigations, the embryos exposed to [3-^{14}C]Fluoranthene were dissected with some difficulty and only one embryo was successfully separated into all three different partitions. In addition, the numbers of embryos available to dissect were limited due to the number of non-viable eggs observed during the exposure. The results of the level of radioactivity measured in the samples and subsequent quantity of compound in the different partitions are shown in Table 4.2.

Table 4.2. Quantity of [3-^{14}C]Fluoranthene in different partitions of zebrafish embryos exposed for 2 hours and 24 hours

Dissection method	Exposure duration (hours)	Description of sample	Radioactivity (Bq)	Quantity of compound in sample (µg)*
Filter paper	2	embryo + chorion + perivitiline fluid	46.1	0.0056
Filter paper	24	chorion	27.9	0.0034
		embryo + perivitiline fluid	126	0.0153
Petri dish	24	chorion	2.0	0.0002
		embryo	63.7[a]	0.0077
		perivitiline fluid	49.6[a]	0.0060

* = radioactivity/specific activity where, specific activity of was 8230.

[a] chorion split during the first rinse therefore this sample could have been contaminated.

It is difficult to draw comparisons between the two dissection methods used on the embryos exposed to [3-^{14}C]Fluoranthene as there were some difficulties dissecting the different partitions using the filter paper method. However, [3-^{14}C]Fluoranthene could be detected within the different partitions of the embryo suggesting that the compound had passed through the chorion, into the perivitiline fluid and/or the embryo. When considering the Petri dish method, there appears to be some consistency between the level of [3-^{14}C]Fluoranthene in the perivitiline fluid and the embryo. However, during the rinsing process the chorion ruptured suggesting that the different partitions could have been contaminated with test substance from the cell well during transfer of the embryo.

Determination of [4-^{14}C]Estradiol in zebrafish embryos

Embryos exposed to [4-^{14}C]Estradiol were dissected relatively successfully at both 2 and 24 hours post exposure. It was not possible to separate the embryo and perivitiline fluid successfully from one embryo at 24 hours post exposure, consequently the embryo and perivitiline fluid were analysed together for this sample. The results of the level of radioactivity measured in the samples and subsequent quantity of compound in the different partitions are shown in Table 4.3.

Table 4.3. Levels of radioactivity and calculations of quantity of compound in different partitions of zebrafish embryos exposed to [4-^{14}C]Estradiol for 2 and 24 hours

Dissection method	Exposure duration (hours)	Description of sample	Radioactivity (Bq)	Quantity of compound in sample (µg)*
Filter paper	2	chorion	0.5	0.0001
		embryo	<0.5	<0.0001
		perivitiline fluid	2.8	0.0004
Filter paper	24	embryo + perivitiline fluid	9.4	0.0013
Petri dish	2	chorion	<0.5	<0.0001
		embryo	24.1	0.0034
		perivitiline fluid	6.9	0.0009
Petri dish	24	chorion	2.1	0.0003
		embryo	7.9	0.0011
		perivitiline fluid	6.8	0.0009

*= radioactivity/specific activity, where specific activity was 7063.

The embryo dissected using the filter paper method after 2 hours exposure, revealed that the level of [4-^{14}C]Estradiol in the perivitiline fluid was over 4 times higher than the quantity measured in the embryo and the chorion. This suggests that the [4-^{14}C]Estradiol passed through the chorion but was not taken up in to the embryo and had not bound to the chorion. Unfortunately, it is not possible to extrapolate from these results whether the level of [4-^{14}C]Estradiol in the embryo increased after 24 hours exposure as it was not possible to dissect the embryo from the perivitiline fluid. However, the level of [4-^{14}C]Estradiol in the combined partitions appeared to have increased after 24 hours exposure. This could mean that the concentration in the perivitiline fluid had not reached equilibrium within the 2 hours or that the embryo was taking up the [4-^{14}C]Estradiol. If the embryo had taken up the [4-^{14}C]Estradiol then this would be consistent with the embryos dissected using the Petri dish method, which at 24 hours had a similar quantity in the embryo as in the perivitiline fluid. However, these results should be viewed with some caution since the embryo at 2 hours post exposure had over 3 times more [4-^{14}C]Estradiol than the perivitiline fluid, which suggests that there may have been some issues with contamination.

Phase 2: Optimisation of experimental procedures for determining the uptake of chemicals into zebrafish embryos

As detailed above, preliminary investigations (Phase 1) were undertaken to provide a method for dissecting zebrafish embryos. The overall objective was to determine the uptake of radiolabelled chemicals into zebrafish embryos and identify whether such techniques could help identify potential barriers to entry of certain chemicals. Following Phase 1, further investigations were carried out in order to optimise and refine the techniques for dissecting zebrafish embryos, and importantly, to accurately

quantify the uptake of test compounds ([3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol) into the embryos. The preferred technique used for the dissection of the embryos, in the Phase 2 experiments, was the filter paper method. The materials and methods were as detailed prior to the Phase 1 investigations. Statistical analysis was performed and data sets which were normal and variances were homogeneous were assessed for significant differences using ANOVA. Any which failed the test for homogeneity of variances or which were non normal were assessed for significant differences using a non-parametric procedure (e.g. Kruskal-Wallice). The quantity of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol was measured in zebrafish embryos exposed for different periods of time and with different treatment regimes (e.g. pre sterilising). These investigations are presented as separate experiments below.

Experiment 1: Uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol into zebrafish embryos following 48 hours exposure

Experimental details

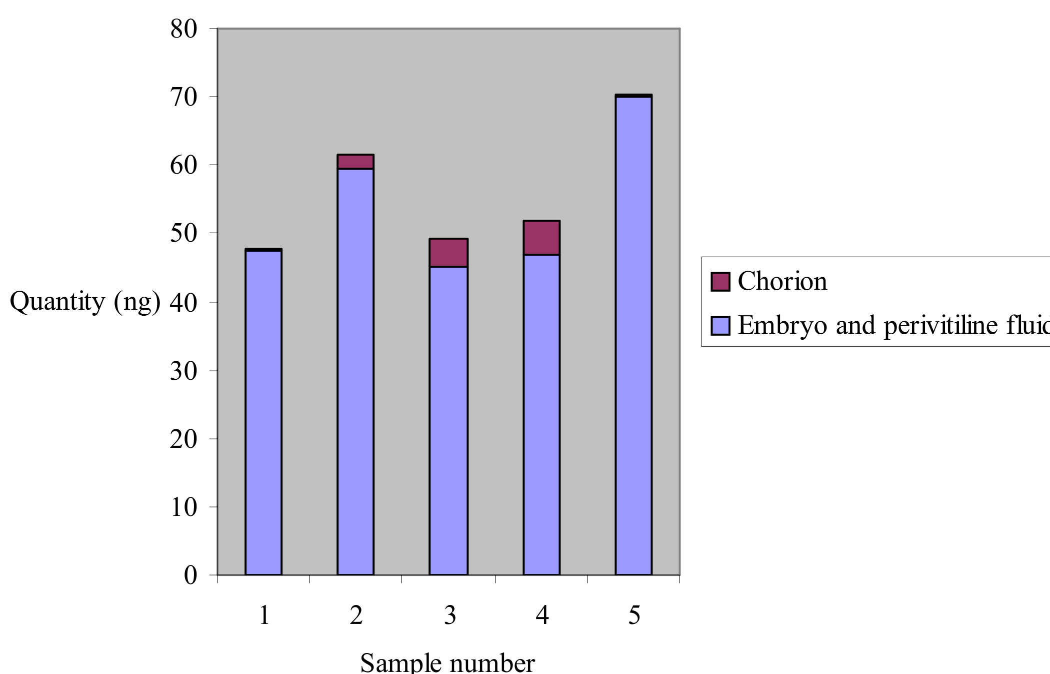
Experiment 1 investigated the quantity of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol that could be measured in zebrafish embryos exposed to the nominal concentrations of 0.5 and 0.1 mg l^{-1} respectively for 48 hours. Following exposure, embryos were removed and dissected into separate partitions- the chorion, and the remaining components inside the embryo (i.e. embryo plus perivitilline fluid) prior to analysis.

Experiment 1: Results

Uptake of [3-^{14}C]Fluoranthene

The uptake of [3-^{14}C]Fluoranthene (ng) taken up into the different partitions of the embryo individual zebrafish embryos (chorion and the embryo plus the perivitiline fluid) is shown in Figure 4.4. The data is also presented in the appendices. These data confirmed that [3-^{14}C]Fluoranthene can be measured inside the chorion of the embryo after 48 hour exposure, and that some level of [3-^{14}C]Fluoranthene can also be detected on or within the chorion. The quantity of [3-^{14}C]Fluoranthene measured within the embryo was also relatively consistent with an average (±SD) quantity of [3-^{14}C]Fluoranthene of 53.8 (±11) ng being detected from within the embryos.

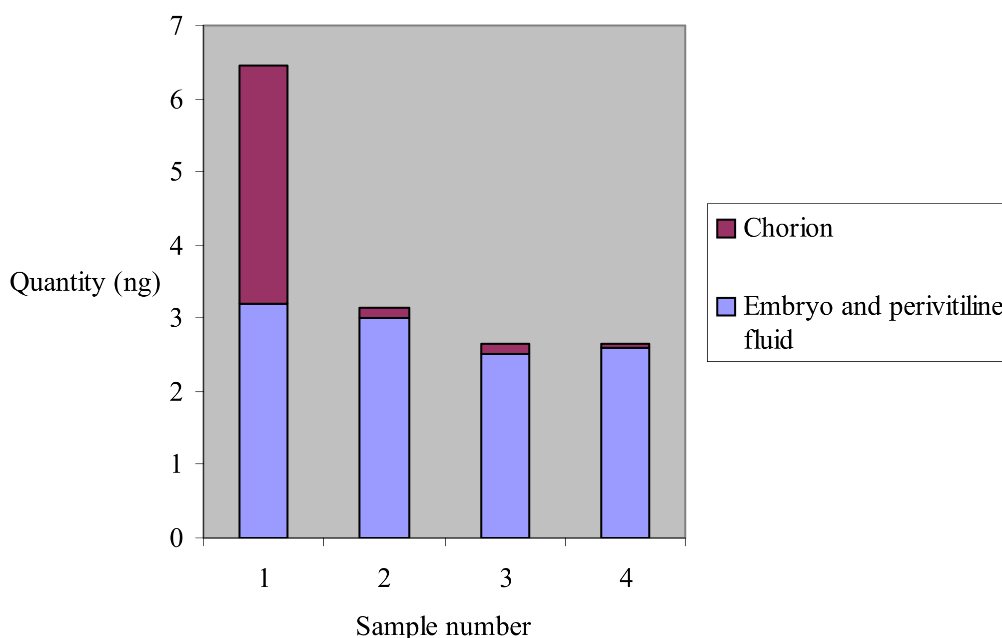
Figure 4.4. Uptake of [3-^{14}C]Fluoranthene into zebrafish embryos after 48 hour exposure



Uptake of [4-^{14}C]Estradiol

The results of the quantity of [4-^{14}C]Estradiol (ng) taken up into individual zebrafish embryos is shown in Figure 4.5. The data is also presented in the appendices. These data also confirm that [4-^{14}C]Estradiol can be measured within the embryo after 48 hour exposure, and again that some level of [4-^{14}C]Estradiol can also be detected on or within the chorion. However, it should be noted that the first dissection indicated that the chorion had 3.27 ng of radio labelled compound associated with the partition. This was possibly associated with contamination during the dissection process. The quantity of [4-^{14}C]Estradiol measured within the embryo was also relatively consistent with an average (±SD) quantity of [4-^{14}C]Estradiol of 2.83 (±0.326) ng being detected from within the embryos. Interestingly, the quantity of [4-^{14}C]Estradiol measured inside the embryo was significantly less than the quantity of [3-^{14}C]Fluoranthene measured inside the respective embryos.

Figure 4.5. Uptake of [4-^{14}C]Estradiol into zebrafish embryos after 48 hour exposure



Experiment 1: Discussion

In these initial investigations, the numbers of embryos available at the end of the exposure period were limited (n=5 and n=4) due to viability of the embryos after 48 hours exposure, and also due to other factors involved with the dissection process. For example, the process of dissecting embryos took longer than predicted due to the difficulty of manipulating such a small and delicate structure. The main issue, associated with the time taken to dissect the embryos, was that the embryos were transferred to clean water after the washing procedure. This could have affected the quantity of the test substances remaining inside the embryo, particularly if the passage of chemicals into the embryo occurs via a passive process. For this reason, embryos were maintained in clean water for as short a period of time as possible to prevent compromising the internal contents and hence the quantity of radio labelled material. Furthermore, on some occasions, it was not possible to separate the internal contents from the chorion so these samples were disregarded from the results.

The other reason for reduced sample size at the end of the process was the number of viable embryos, which were actually available post exposure. It was considered that this was not a function of the toxicity of the chemical because of the safety factors incorporated to the final exposure concentration that was chosen, plus the initial screening work, discussed earlier, did not highlight any toxicity at the concentration used. However, the viability issue could have been due to either a hereditary factor or possibly due to fungal contamination of the embryos.

Another important observation was that there appeared to be a relatively large variation associated with the quantity of material measured in the chorion which

suggests that the dissection process may not have been as reliable as that for the embryo/perivitiline fluid. For example, there may have been some issues with potential contamination of the chorion from the perivitiline fluid. However, the results did show that although there may have been some test material adhering to the outside of the chorion, the radiolabelled material was also getting in to the embryo and perivitiline fluid. This indicates that both [3-^{14}C]Fluoranthene and [3-^{14}C]Estradiol are able to pass through the chorion.

Experiment 2: Uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol into zebrafish embryos after 24 and 48 hours exposure

Experimental details

In order to better understand the dynamics of uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol into zebrafish embryos after different exposure periods, experiment 2 focussed on determining the uptake of the radio labelled compounds after 24 hours and 48 hours exposure. Following exposure, embryos were removed and dissected into separate partitions (i.e. chorion and remaining internal components [perivitiline fluid plus embryo]) prior to analysis to determine the quantity of radiolabelled material, which had passed through the chorion. The results of the uptake of the different compounds will be discussed separately with the uptake of [3-^{14}C]Fluoranthene preceding the [4-^{14}C]Estradiol investigations.

Experiment 2: Results

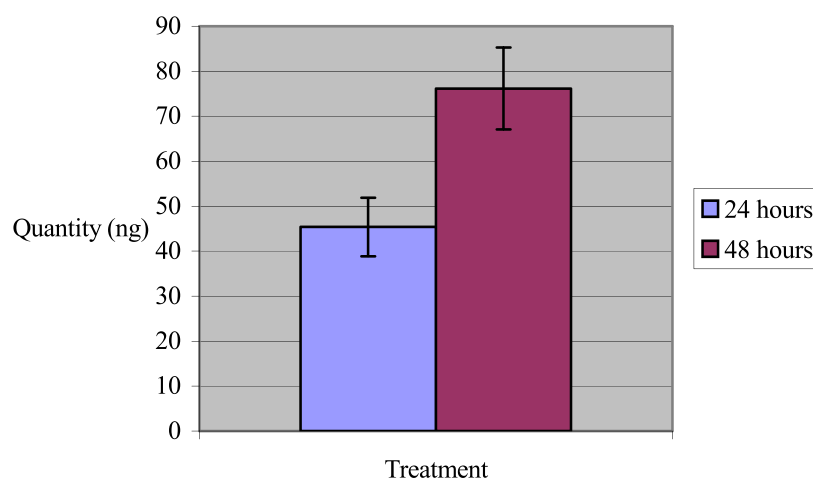
Uptake of [3-^{14}C]Fluoranthene into zebrafish embryos

The results of the initial uptake experiment using [3-^{14}C]Fluoranthene after 24 and 48 hour exposure are shown in Table 4.6 of the appendices. However, these data indicated that the level of [3-^{14}C]Fluoranthene measured within the internal contents of the embryo (embryo plus perivitiline fluid) was considerably lower than the values obtained in experiment 1 (48 hour mean quantity of 53.8 ng in experiment 1 compared with a mean of 2.11 ng in experiment 2). In addition, the measured concentration of the [3-^{14}C]Fluoranthene in the test solution had dropped from the initial measured concentration of 0.5 mg l^{-1} at the start of the exposure to 0.01 mg l^{-1} after 24 hours exposure and 0.008 mg l^{-1} after 48 hours exposure (2.0 and 1.6 % of nominal respectively). This decrease in concentration was considered to be due to the fact that the [3-^{14}C]Fluoranthene was possibly adhering to the plastic container that the solution had been prepared in and the plastic Petri dish. Therefore, all subsequent experiments using [3-^{14}C]Fluoranthene were carried using a fresh solution prepared in a glass amber bottle. In addition, to further prevent loss of test material onto the plastic Petri dishes, all the experiments using [3-^{14}C]Fluoranthene were carried out in glass Petri dishes.

Subsequently, experiment 2, using [3-^{14}C]Fluoranthene, was repeated with freshly prepared test solution to determine the uptake into zebrafish embryos after 24 and 48 hours exposure. The data are presented in Figure 4.7 and are also presented in the appendices. The concentration of [3-^{4}C]Fluoranthene was measured in the test solutions after 0, 24 and 48 hours. The mean concentration of [3-^{14}C]Fluoranthene at

0 hours was measured as 0.40 mg l^{-1}, at 24 hours the concentration had decreased to 0.19 mg l^{-1} (38% of the nominal concentration) and at 48 hours was measured as 0.14 mg l^{-1} (28% of the nominal concentration). The quantity of [3-^{14}C]Fluoranthene measured in the embryos after 24 and 48 hours exposure is shown in Figure 4.7 and the data is also presented in the appendices. The average quantity (expressed as mean [±SD, n=x]) of [3-^{14}C]Fluoranthene, after 24 hours exposure, was 45 (±6.5, n=10) ng, compared with an average of 76 (±8.9, n=13) ng after 48 hour exposure.

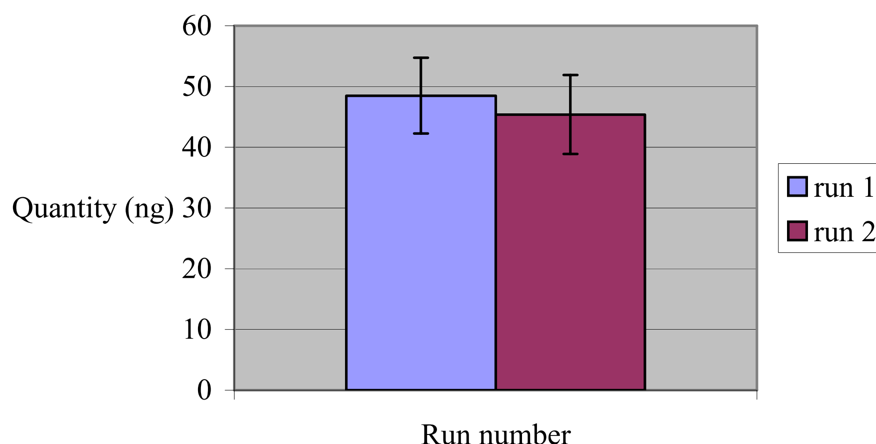
Figure 4.7 Mean uptake (± SD) of [3-^{14}C]Fluoranthene into zebrafish embryos after 24 and 48 hour



The number of individual embryos used for the 24 hour evaluations (n=4) was limited due to the limited numbers of embryos provided from broodstock in addition to unsuccessful dissections. However, despite the small numbers of individual embryos at 24 hours, the quantity of [3-^{14}C]Fluoranthene measured in these embryos was consistent with corresponding data obtained on an additional sampling occasion as shown in Figure 4.8. The data is also presented in the appendices. Statistical analysis of these data showed that there were no significant differences between the quantity

of [3-^{14}C]Fluoranthene taken up into the zebrafish embryos on the two separate occasions.

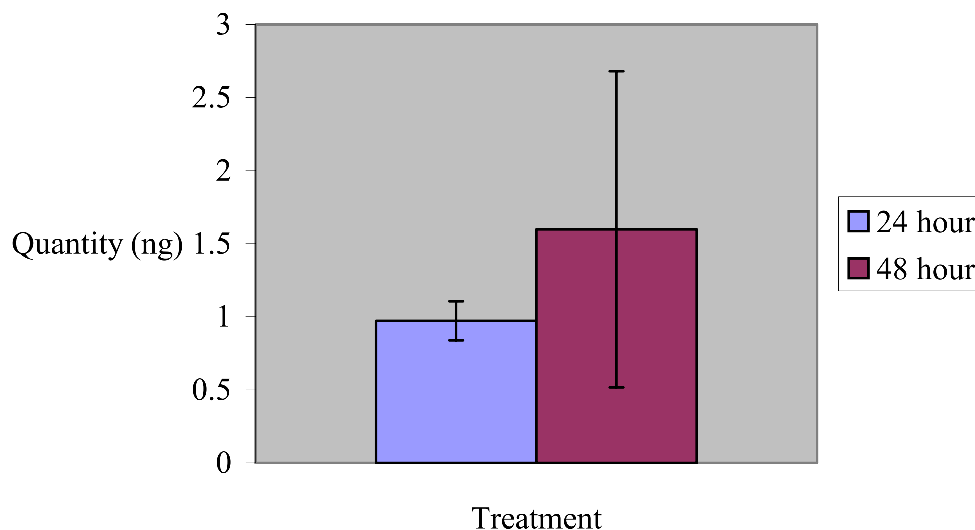
Figure 4.8. Mean uptake (±SD) of [3-^{14}C]Fluoranthene into zebrafish embryos after 24 hours



Uptake of [3-^{14}C]Estradiol into zebrafish embryos

Fortunately, the loss of concentration, identified in the [3-^{14}C]Fluoranthene investigations, was not a problem in the investigations involving the uptake of [3-^{14}C]Estradiol into zebrafish embryos after 24 and 48 hours exposure. In all the [3-^{14}C]Estradiol exposures, the test solutions remained stable throughout the exposure periods however the actual measured values were below nominal and in this investigation the measured concentration was 0.073 mg l^{-1} at 24 hours and 0.068 mg l^{-1} at 48 hours. The results of the uptake of [3-^{14}C]Estradiol after 24 and 48 hours are shown in Figure 4.9 and the data is also presented in the appendices.

Figure 4.9. Mean uptake (±SD) of [4-^{14}C]Estradiol into zebrafish embryos after 24 and 48 hours exposure



These data indicated that the quantity of [4-^{14}C]Estradiol had increased inside the embryos between 24 hours and 48 hours exposure. The quantity (mean (±SD)) of [4-^{14}C]Estradiol after 24 hours was 0.973 (± 0.478) ng and after 48 hours was 1.60 (± 1.08) ng.

Experiment 2: Discussion

Experiment 2 focussed on the uptake profile of both [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol after exposure for 24 and 48 hours. With regards to the exposure of [3-^{14}C]Fluoranthene, there were some initial problems regarding maintaining the concentration of [3-^{14}C]Fluoranthene within the test system. Therefore glass equipment was used for the repeat work, and fresh solutions were prepared. However, even though the concentration of [3-^{14}C]Fluoranthene at the beginning of the exposure was within 80% of the nominal concentration (0.4 mg l^{-1}), by the end of

the 48 hours it had declined to only 28% of the nominal concentration. This decrease in concentration may have been due to uptake and partitioning of the chemical into the embryo or there was also the possibility of the chemical adsorbing to the surfaces of the test vessels irrespective of the fact that the exposures were being carried out in glass Petri dishes. Nevertheless, the data showed that [3-^{14}C]Fluoranthene could be measured within the internal contents (i.e. embryo plus perivitiline fluid) of the embryo, which further substantiates the earlier findings that [3-^{14}C]Fluoranthene can pass through the chorion of zebrafish embryos. Furthermore, the quantity of [3-^{14}C]Fluoranthene had almost doubled in amount between 24 and 48 hours which indicates that [3-^{14}C]Fluoranthene was still being taken up into the zebrafish embryos through the chorion. In addition, the 24 hour data (all be it based on a small number of embryos, n=4) was consistent with the quantity measured on an additional investigation. This suggests that the process of dissecting embryos at 24 hours, for quantification of test substance within the internal contents of zebrafish embryos, maybe reproducible.

Furthermore, using these data (quantity of [3-^{14}C]Fluoranthene measured in runs 1 and 2) it is possible to make some extrapolation to the concentration of the radiolabelled substance within the embryo if the volume of the embryo is considered as a sphere. Using the formula $4/3\pi r^3$ to determine the volume of the embryo and assuming a diameter of the zebrafish embryo to be approximately 0.7mm, the volume can be calculated to be approximately 0.18 ml. Furthermore, the mean quantity of [3-^{14}C]Fluoranthene, measured in runs 1 and 2, was calculated to be 46.9 ng per embryo which would equate to 46.9 ng/0.18 ml. Therefore, this would mean that the concentration of [3-^{14}C]Fluoranthene in the embryos after 24 hours would be approximately 0.28 mg l^{-1}. It should however be noted that these values are

approximations based on a theoretical approach to determining the volume within a zebrafish embryo and do not take into account the different partitions within the embryo which may affect the real concentration within the whole embryo. For example, if the chemical is only within the perivitiline fluid and has not actually entered the embryo then this would not be representative of the concentration within the whole embryo. Interestingly, this does show that the concentration of [3-^{14}C]Fluoranthene is close to the concentration in the exposure solution which may indicate that the level of [3-^{14}C]Fluoranthene within the embryo is approaching equilibrium with the external medium.

In terms of the uptake of [4-^{14}C]Estradiol in experiment 2, the exposure regime appeared robust compared to the initial exposures using [3-^{14}C]Fluoranthene as the concentrations measured remained relatively consistent between 0 and 48 hours. In experiment 2, the mean quantity of [4-^{14}C]Estradiol measured in the internal components of the embryos (i.e. embryo plus perivitiline fluid) after 48 hours was consistent with the results from experiment 1. However, the quantity was slightly lower in experiment 2 (mean quantity 1.60 ng [±1.082] compared with 2.83 ng [±0.326]). However, the data from experiment 2 may have been skewed by unusually low results for 2 of the embryos that were analysed. This also explains the high standard deviation for the uptake data into the embryos after 48 hours. Furthermore, the quantity of radio labelled compound measured in the chorions of the embryos was, as expected, very low. On only one occasion it exceeded the background level of radioactivity, of approximately 1 becquerel. On this one occasion, the radioactivity was measured at 10.4 Bq (1.48 ng), which was an order of magnitude greater than that seen in the other dissections. This was attributed to possible contamination of the sample and therefore was not considered representative of the actual result.

Experiment 3: Uptake of [3 14C]Fluoranthene and [4-14C]Estradiol following pre treatment with halomid

Experimental details

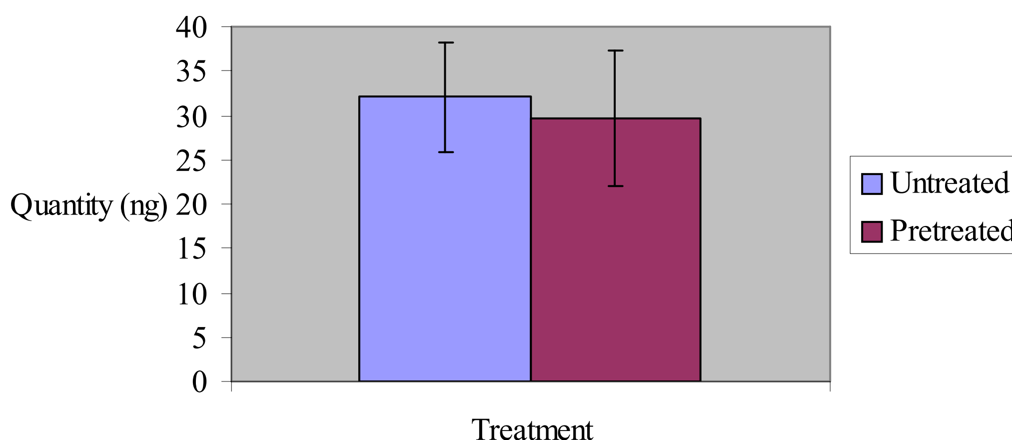
In the first 2 experiments, the numbers of embryos that were viable after 24 and 48 hours exposure limited the number of embryos that could be dissected. This could have been a consequence of the parent fish (P0) producing inferior egg quality, or it could have been due to microbial contamination of the embryos. In order to address the potential microbial contamination issue, it was suggested that subsequent experiments might require the use of embryos that had been previously sterilised in a bath of halomid (1% $^{v}/_{v}$) prior to exposure of the test compound. Zebrafish embryos are routinely sterilised in many breeding facilities prior to use in research and regulatory ecotoxicity assessments in order to prevent microbial infections. However, it is not known if any pre treatment would affect the uptake of chemicals. Therefore experiment 3 focussed on the uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol after 24 hours exposure, both with and without treatment of a dilute solution of halomid (1% $^{v}/_{v}$). The embryos were dissected into partitions of chorion and internal contents (embryo plus perivitiline fluid).

Experiment 3: Results

Uptake of [3-^{14}C]Fluoranthene into zebrafish embryos pre-treated with halomid

The results showing the uptake of [3-^{14}C]Fluoranthene into zebrafish embryos after 24 hours exposure with and without a sterilising agent (halomid) are shown in Figure 4.10. The data is also presented in the appendices.

Figure 4.10. Mean uptake (±SD) of [3-^{14}C]Fluoranthene into pre-treated and untreated zebrafish embryos (24 hours)



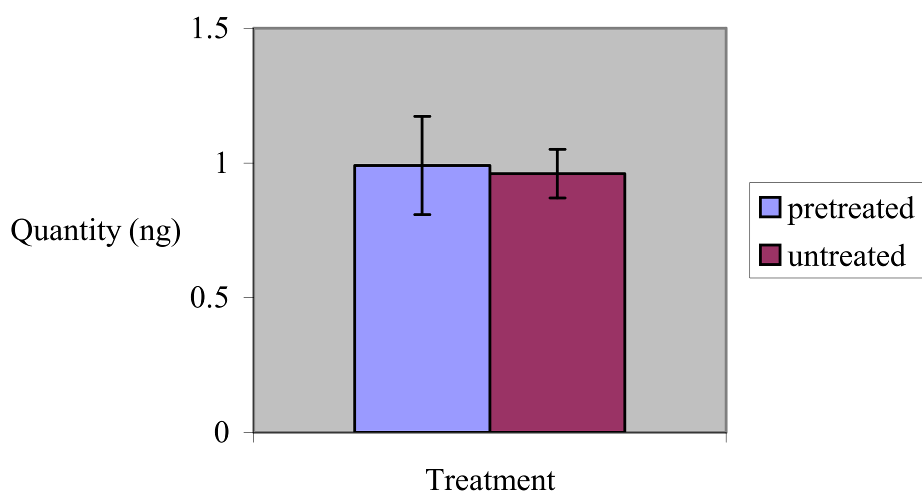
The mean (±SD) quantity of [3-^{14}C]Fluoranthene detected within the embryos, with no pre treatment of halomid, following 24 hours exposure to the test substance was 32.02 (±6.21) ng. The mean quantity of [3-^{14}C]Fluoranthene in pre treated embryos was 29.62 (±7.65) ng. Statistical analysis of these data indicated that the presence of the sterilising agent (at a concentration of 1% $^{v}/_{v}$) did not significantly affect (p=0.05) the uptake of [3-^{14}C]Fluoranthene into zebrafish embryos. Therefore, in order to prevent potential fungal contamination of embryos it was considered that future

assessments on zebrafish embryos, should proceed with pre-treatment using halomid (1% $^{v}/_{v}$), which would not influence the uptake of [3-^{14}C]Fluoranthene.

Uptake of [4-^{14}C]Estradiol into zebrafish embryos pre-treated with halomid

The results from the effect of pre-sterilising zebrafish embryos on the uptake of [4-^{14}C]Estradiol after 24 hours exposure, are shown in Figure 4.11. The data is also presented in the appendices. The mean (±SD) quantity of [4-^{14}C]Estradiol detected within the embryos, with no pre treatment of halomid, following 24 hours exposure to the test substance was 0.99 (±0.18) ng. The mean quantity of [4-^{14}C]Estradiol in the pre treated embryos was 0.96 (±0.09) ng.

Figure 4.11. Mean (±SD) Uptake of [4-^{14}C]Estradiol into pre and un treated zebrafish embryos (24 hours)



Consistent with the investigations into the uptake of [3-^{14}C]Fluoranthene, statistical analysis of the data for uptake of [4-^{14}C]Estradiol into zebrafish embryos indicated that the sterilising agent, halomid (1% $^{v}/_{v}$), did not have a significant affect (p=0.05) on the uptake. Therefore, it was considered that for future assessments on zebrafish

embryos, pre-treatment with halomid (1% $^{v}/_{v}$), would not influence the uptake of [4-^{14}C]Estradiol, and data obtained with such pre-treated embryos could be considered comparable with data obtained on embryos which had not been pre-treated.

The theoretical concentration of [4-^{14}C]Estradiol within a zebrafish embryo, using a similar approach to Experiment 2 (i.e. using the formula $4/3\pi r^3$, assuming a diameter of approximately 0.7mm and hence a calculated volume of approximately 0.18 ml) has also been calculated. For example, if the quantity of [4-^{14}C]Estradiol in zebrafish embryos after 24 hours exposure is assumed to be approximately 1.0 ng this would mean that the concentration in the embryos would be approximately 0.0056 mg l^{-1}. Consistent with before, it should be noted that these values are approximations based on a theoretical approach to determining the volume within a zebrafish embryo and do not take into account the different partitions within the embryo which may affect the real concentration within the whole embryo. Interestingly, if the concentration within the embryo is compared to the concentration in the test solution (0.1 mg l^{-1}), it is clear that the embryo concentration is significantly lower and is not approaching equilibrium with the external medium.

Experiment 4: Uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol into the different partitions of zebrafish embryos

Experimental details

The results from experiments 1 to 3 above indicate that both chemicals are taken up into zebrafish embryos, and that the chorion does not appear to prevent the entry of

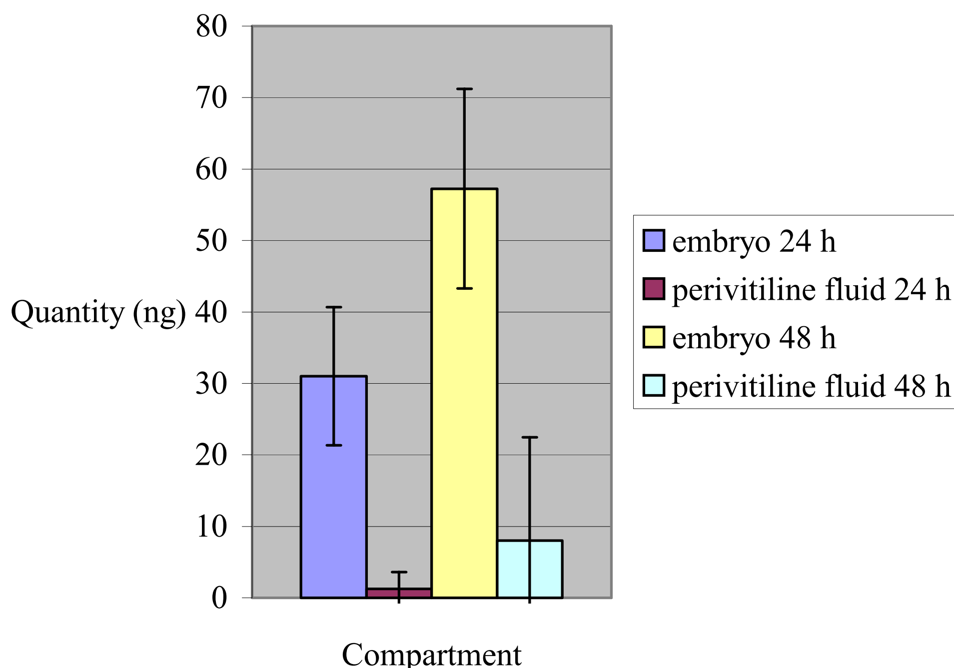
either substance into the embryo. However, this only informs about the permeability of the chorion and not whether the chemical has reached the actual target site of the embryo. For example, the chemical may have passed passively through the chorion and into the perivitiline fluid, but may have not been taken up into the embryo *per se*. In order to evaluate whether either of the chemicals actually partitioned into each of the different parts of the internal contents, the embryos were dissected, where possible, in to the three different portions (i.e. the perivitiline fluid, embryo or chorion). Where the dissections were not successful, the embryo was at least separated from the perivitiline fluid; however, it may have not been possible to separate the embryo from the chorion. Nevertheless, these dissections were still included, as previous work had shown that the quantity of radiolabelled chemical remaining on/in the chorion was normally minimal.

Experiment 4: Results

Uptake of [3-^{14}C]Fluoranthene into the different partitions of zebrafish embryos

The data obtained from these more detailed dissections of zebrafish embryos exposed to [3-^{14}C]Fluoranthene, after 24 and 48 hours, are shown in Figure 4.12. The mean (±SD, n=x) quantity of [3-^{14}C]Fluoranthene measured in the embryo after 24 hours was 31.01 (9.64, n=6) ng and the quantity measured in the perivitiline fluid was considerably lower at a level of 1.25 (2.33, n=6) ng. After 48 hours exposure, the mean quantity in the embryo had almost doubled by increasing to 57.24 (13.97, n=4) ng and the quantity in the perivitiline fluid had increased to 8.02 (14.44, n=4) ng, however, one of these samples was possibly contaminated with part of the embryo during the dissection process.

Figure 4.12. Mean uptake (±SD) of [3-^{14}C]Fluoranthene into the different partitions

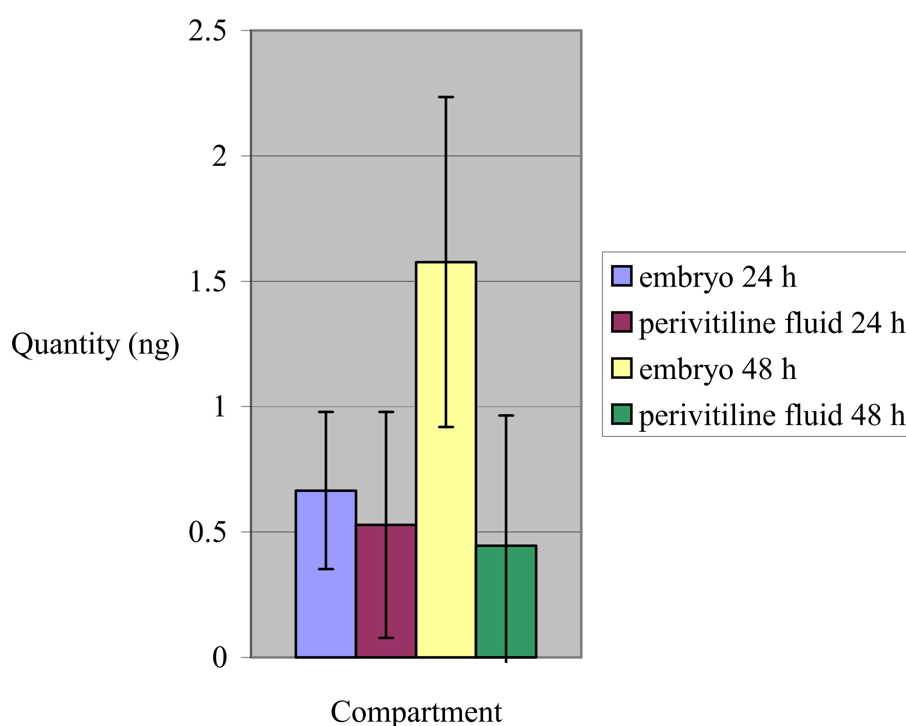


Uptake of [4-^{14}C]Estradiol into the different partitions of zebrafish embryos

In contrast, the uptake profile of [4-^{14}C]Estradiol into zebrafish embryos was slightly different. The results of the uptake of [4-^{14}C]Estradiol into the different partitions, of zebrafish embryos are shown below in Figure 4.13. The mean (±SD, n=x) quantity of [4-^{14}C]Estradiol measured in the embryos of zebrafish after 24 hours was 0.67 ng (±0.31, n=9) and in the perivitiline fluid it was quantified to be 0.53 ng (±0.45, n=8). It should be noted that these data are expressed as partitions of either the embryo or the perivitiline fluid, however, for ease of dissection, the majority of the embryos were analysed together with their respective chorions after the perivitiline fluid had been removed. This avoided any issues of destroying the embryo whilst attempting to tease the two partitions apart. The quantity of [4-^{14}C]Estradiol measured in the embryo together with the chorion were adjusted with the amount of radiolabelled compound determined in chorions that had been successfully separated from the

embryo without compromising the integrity of the dissection. As discussed earlier, this was considered acceptable as the level of [4-^{14}C]Estradiol was approximately the level of background radiation which would not have significantly affected the quantification in the embryo.

Figure 4.13. Mean uptake (±SD) of [4-^{14}C]Estradiol into the different partitions of the embryo after 24 and 48 hour exposure



It is also important to note that for these investigations, a larger number of dissected embryos were analysed. This was possible as data from 2 different experiments (run 1 and 2) were pooled together to obtain a greater number of dissected embryos. Figures 4.14 and 4.15 show the uptake profile of [4-^{14}C]Estradiol into zebrafish embryos and perivitiline fluid respectively, exposed for 24 and 48 hours on the two different occasions.

Figure 4.14. Uptake profile of [4-^{14}C]Estradiol into zebrafish embryos

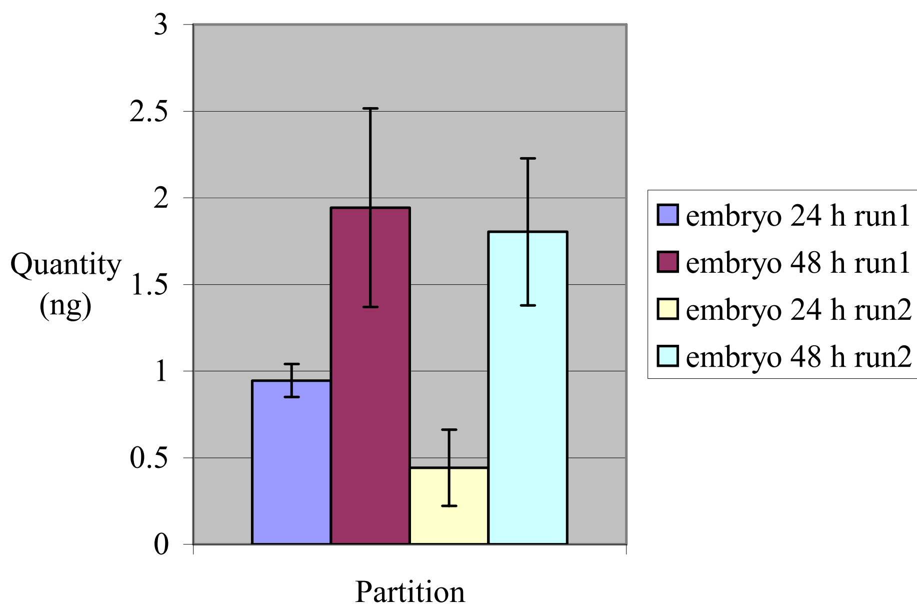
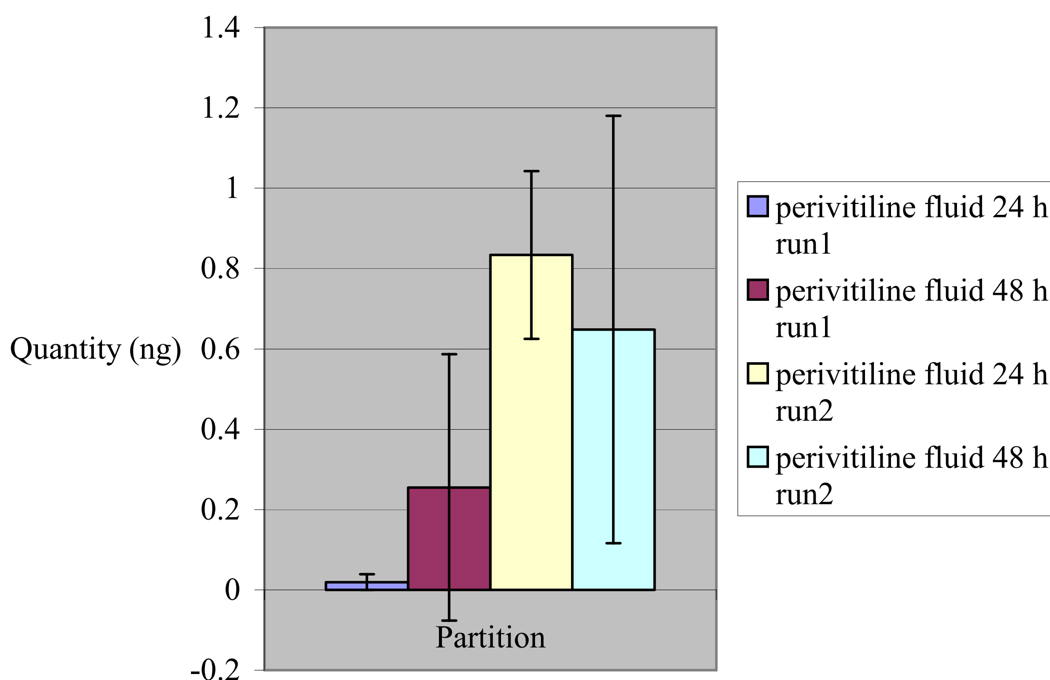


Figure 4.15. Uptake profile of [4-^{14}C]Estradiol into the perivitiline fluid of zebrafish embryos



Experiment 4: Discussion

As discussed earlier, the embryo and chorion were difficult to separate from each other once the perivitilline fluid had been extracted on to the filter paper. This was particularly the case for 2 of the embryos after 24 hours exposure and 1 after 48 hours where the quantity of test substance was measured based on the embryo plus the chorion. In order to provide an estimate of likely measured concentration in only the embryo, the level of [3-^{14}C]Fluoranthene, that had been previously determined in the chorions of 8 embryos from the previous experiments, was subtracted from the product of the 2 partitions in these 3 embryos. This was considered acceptable, as the quantity measured in/on the chorions was minimal and hence did not significantly affect the levels measured in the embryo. Plus, the important aspect to these dissections was whether the compound was partitioning into the embryo or was only present in the perivitiline fluid. Furthermore, the results confirmed the previous findings that [3-^{14}C]Fluoranthene passes through the chorion but more interestingly, these data also suggest that the radiolabelled compound transfers from the perivitilline fluid and in to the embryo. In addition, the [3-^{14}C]Fluoranthene appears to positively partition into the embryo from the perivitilline fluid.

With respect to the uptake of [4-^{14}C]Estradiol into the zebrafish embryos, the data also reconfirmed that the chemical can pass through the chorion and into the embryo, but the radiolabelled compound does not appear to be partitioning preferentially into either the embryo or the perivitiline fluid. Consistent with the uptake of [3-^{14}C]Fluoranthene after 48 hours exposure, the quantity of [4-^{14}C]Estradiol in the embryo had approximately doubled from the amount detected after 24 hours. The mean quantity (±SD, n=x) measured in the embryos after 48 hours was 1.58 (±0.66,

n=16) ng and in the perivitiline fluid it was measured to be 0.45 (±0.52, n=17) ng. Interestingly, the increase in quantity of [4-^{14}C]Estradiol measured in the embryo was not reflected in the quantity detected in the perivitiline fluid. This may have been due to the quantity of [4-^{14}C]Estradiol in the perivitiline fluid having reached equilibrium with the quantity of test substance in the test solution (i.e. internal concentration in the perivitiline fluid=external concentration). Furthermore, the increase in the quantity of [4-^{14}C]Estradiol in the embryos may have been because a steady state concentration had not been reached within the embryo within 24 hours. However, it was also possible that the quantity measured had increased because the embryo had also increased in size and therefore had adsorbed more test substance.

When the determination of the uptake of [4-^{14}C]Estradiol experiment was repeated, the data from the 2 different runs revealed that the quantity of [4-^{14}C]Estradiol in the embryos was consistent although the actual quantity in the embryos after 24 hours was slightly lower in the second run. Interestingly, this was in direct comparison to the quantity of perivitiline fluid in the two runs, which showed significantly higher quantities in the second run compared with run 1. However, the variability of [4-^{14}C]Estradiol measured in the perivitiline fluid of the embryos, analysed in both runs, was considerably higher than that observed in the embryos.

In support of the results from the uptake of [4-^{14}C]Estradiol into the zebrafish embryo and to verify that the quantities which were being measured in the embryos was accurate, additional embryos were analysed on the same occasion, without being dissected. The purpose of this was to compare the dissected embryos with non-dissected embryos for mass balance analysis. The quantity of [4-^{14}C]Estradiol measured in 3 whole embryos from run 1 were 2.30, 2.38 and 2.50 ng respectively,

and the mean quantity of the dissected perivitiline fluid plus the embryos was 2.20 ng. If the theoretical quantity of [4-^{14}C]Estradiol in the chorion was approximately equal to background radiation, the quantity measured in the dissected embryos was comparable to that measured in the non dissected embryos. In run 2 the quantity of [4-^{14}C]Estradiol measured in the non dissected embryos was 2.04, 1.81 and 1.88 ng, whilst the quantity in the average embryo and perivitiline fluid added together was 244 ng. This suggests that the dissection methods were relatively precise in terms of managing to detect what was actually inside the embryo.

Experiment 5: Uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol into the different partitions of zebrafish embryos post pre-treatment with halomid

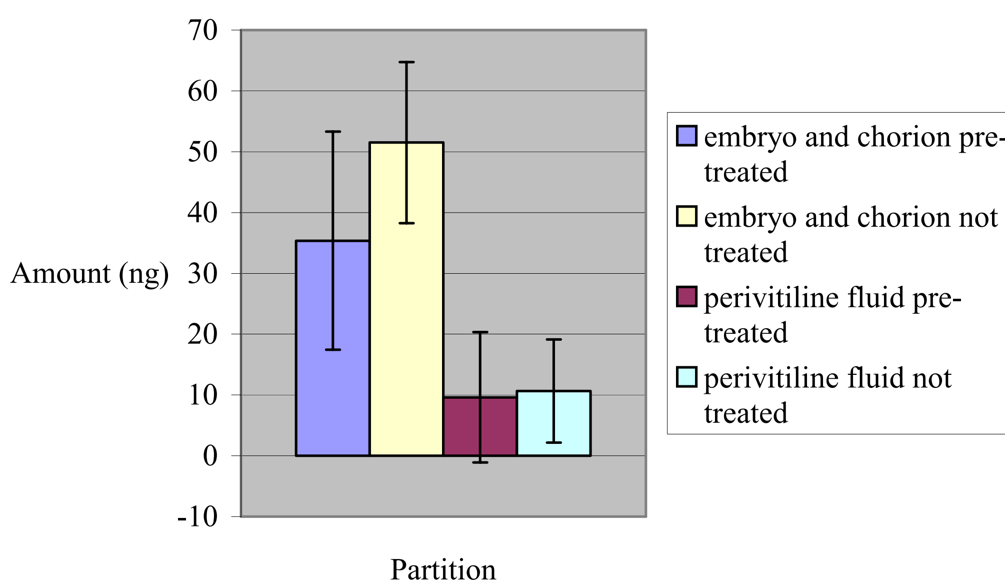
Experimental details

The results from experiment 3 showed that the uptake of either [3-^{14}C]Fluoranthene or [4-^{14}C]Estradiol into zebrafish embryos was not significantly affected by the use of the dilute solution of the sterilising agent. However, these investigations only focussed on whether the integrity of the chorion had been affected by the pre-treatment and hence uptake into the embryo. Furthermore, these experiments did not identify whether the sterilising agent had an affect on either of the internal partitions and consequently whether the halomid affected the uptake of the radiolabelled compounds into the embryo from the perivitiline fluid. To further investigate the use of the sterilising agent halomid, the different partitions of the internal contents of the zebrafish embryos were analysed with or without pre-treatment followed by 48 hours exposure to [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol.

Experiment 5: Results and discussions

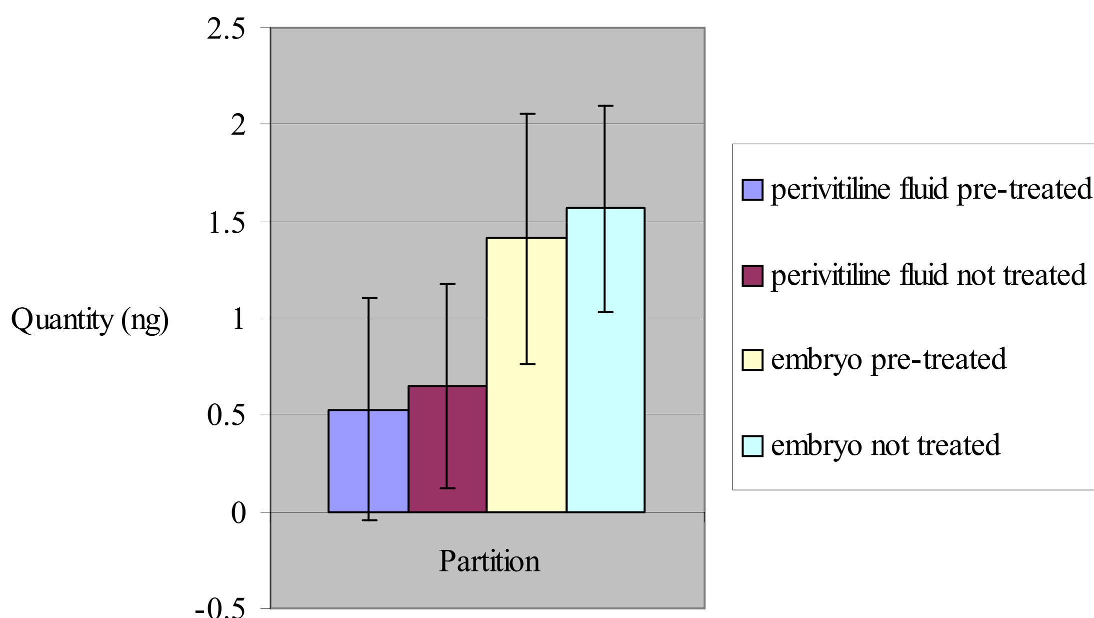
The results of the uptake of [3-^{14}C]Fluoranthene and [4-^{14}C]Estradiol from these experiments are shown in Figure 4.15 and 4.16 respectively below.

Figure 4.15. Uptake of [3-^{14}C]Fluoranthene into the different internal partitions of zebrafish embryos pre-treated and not treated prior to exposure for 48 hours



There was no statistically significant difference between the quantity of [3-^{14}C]Fluoranthene taken up into the embryo and chorion partitions for the different pre-treatments which confirms the results from the previous investigations that pre-treatment of zebrafish embryos with halomid does not significantly affect the uptake of [3-^{14}C]Fluoranthene into zebrafish embryos.

Figure 4.16. Uptake of [4-^{14}C]Estradiol into the different internal partitions of zebrafish embryos pre-treated and not treated prior to exposure for 48 hours



Statistical analysis of these data also showed that there was no significant difference between the quantity of [4-^{14}C]Estradiol taken up into the embryo and chorion partitions for the different pre-treatments which confirms the previous investigations that pre-treatment of zebrafish embryos with halomid does not significantly affect the uptake of [4-^{14}C]Estradiol into zebrafish embryos.

Conclusions

A method has been developed to quantify the uptake of radiolabelled compounds into zebrafish embryos, from aqueous solutions, after 24 and 48 hour exposure. Two different techniques, the Petri dish and the filter paper methods, were investigated for determining the uptake of radiolabelled chemical into the different partitions of zebrafish embryos. The main difficulties associated with the two different techniques

were the risk of cross contamination of the sample preparations, and ease of manipulation of the samples. It was possible that contamination of the partitions could occur with the Petri dish method during the transfer of the embryos into the clean water. For example, when an embryo was transferred using a plastic Pasteur pipette, some of the test substance was transferred into the clean water. For this reason, an additional wash stage was introduced to further dilute and reduce the amount of test substance transferred. In addition, after 24 hours of development, the chorion of zebrafish embryos becomes increasingly brittle, possibly due to the production of endogenous hatching enzymes. Consequently, on several occasions the mechanical action, caused by pipetting the embryos during the washing process, resulted in the chorions splitting open. These samples therefore, may have been contaminated during the transfer process. There was also the possibility that if the test substance was present in the perivitiline fluid, and subsequently dissolved in the wash water, that some of the test substance could have been transferred with the dechorionated embryo.

The manipulation of the embryos during the dissection process also proved to be difficult particularly using the filter paper method. For example, it was relatively easy to tear a hole in the chorion of the embryo causing the perivitiline fluid to be released on to the filter paper. However, the embryo and chorion appear translucent against the filter paper and hence were occasionally difficult to individually distinguish or select the different partitions once the perivitiline fluid had been released. Another limitation to these methods was the ability to quantify the concentration of compound in the perivitilline fluid, as the volume within the embryo was not quantified.

To further validate the initial findings regarding the uptake of radiolabelled compounds into zebrafish embryos, a further set of experiments were performed using [4-^{14}C]Estradiol and [3-^{14}C]Fluoranthene. Although, the initial results suggested that the level of radioactivity measured in the different dissected portions of the embryos was relatively consistent, it was also apparent that there were some issues with the numbers of embryos which were able to be dissected. This was either due to insufficient numbers of embryos being available, non viable embryos at the end of the exposure phase or due to unsuccessful dissections. Importantly, this indicates that the dissection process would need to be refined and practised by any individual intending on using this technique to quantify uptake of chemicals into zebrafish embryos.

Nonetheless, the results from these initial optimisation studies did indicate that both [4-^{14}C]Estradiol and [3-^{14}C]Fluoranthene could be detected inside the embryos after 24 and 48 hours exposure, and that the concentration inside the embryos increased approximately proportionally from 24 and 48 hours exposure. Therefore, this indicates that the chorion of zebrafish embryos is permeable to both [4-^{14}C]Estradiol and [3-^{14}C]Fluoranthene. In addition, it appeared that the level of [3-^{14}C]Fluoranthene had accumulated within the embryo to a level significantly higher than that of the [4-^{14}C]Estradiol. This was consistent with the fact the octanol water partition coefficient (log kow) of [3-^{14}C]Fluoranthene was higher (5.22) than that of [4-^{14}C]Estradiol (4.01) which suggests that the [3-^{14}C]Fluoranthene will have a greater propensity to partition into any of the lipids within the embryo.

Another significant finding was that the uptake of [4-^{14}C]Estradiol and [3-^{14}C]Fluoranthene into zebrafish embryos was not significantly affected by pre sterilising the embryos with halomid (1% $^{v}/_{v}$). However, it is not known if pre-

treatment with halomid would affect uptake of other substances, hence a specific case-by-case assessment should be considered for each substance if pre sterilising with halomid (1% $^{v}/_{v}$).

The final set of investigations involved dissecting the embryos more thoroughly to identify if either [3-^{14}C]Fluoranthene or [4-^{14}C]Estradiol was taken up specifically into any of the different internal partitions of the zebrafish embryos. The results for the uptake of [3-^{14}C]Fluoranthene into the different partitions indicated that the chemical was detected mostly in the embryo, with very little being found in the perivitilline fluid. These results suggest therefore, that the chemical is passing through the chorion and through the perivitilline fluid but is then partitioning into the embryo. This is again likely to be due to the lipophilicity of [3-^{14}C]Fluoranthene which could have an affinity for accumulating in the lipids and hence is being detected in the embryo rather than in the perivitilline fluid. It is also likely that it is partitioning into the yolk of the embryo, since this has a high level of lipid content.

The results for the uptake of [4-^{14}C]Estradiol indicated that the chemical could be detected in both the perivitiline fluid and the embryo itself. Interestingly, the amount of [4-^{14}C]Estradiol in the embryos was almost twice as high as that in the perivitiline fluid suggesting that the [4-^{14}C]Estradiol is partitioning into any lipids in the embryo. This would be consistent with the fact that the logkow of [4-^{14}C]Estradiol is greater than 4 which would indicate that the compound is likely to partition into lipids and hence bioaccumulate.

Investigations of partitioning within the embryo itself (i.e. blastoderm, yolk etc) were outside of the scope of these experiments as dissections of such small tissues may be

technically difficult. However, this highlights another area of research which could be addressed in the future to determine whether the radiolabelled isotope is taken up preferentially into the blastoderm or the yolk of the developing embryo. Nevertheless, the fundamental message that is evident from the research that has been carried out in this chapter is that the chorion is not the barrier of entry to either [3-^{14}C]Fluoranthene or [4-^{14}C]Estradiol. The following chapters look at whether the chorion of the embryo can be manipulated to become more permeable and whether molecular size has an effect on the uptake across the chorion of zebrafish embryos.

CHAPTER 5. INVESTIGATIONS INTO PERMEABILISING THE CHORION OF ZEBRAFISH EMBRYOS USING CHEMICAL MANIPULATION

Introduction

An important finding from the previous chapter was that both [3-^{14}C]Fluoranthene and [3-^{14}C]Estradiol were shown to be taken up into the zebrafish embryos. This indicated that the chorion is perhaps not a barrier that is impervious to entry for either of these compounds and therefore indicating that this envelope is a partially permeable membrane. This may not, however, be the case for all compounds. For example, research by Leonard (pers com), discussed previously in this thesis, has indicated that zebrafish embryos are not as sensitive as juvenile/adult zebrafish to certain cationic polymers and quaternary ammoniums. However, hatched forms of these embryos (eleutheroembryos) are more or less equally sensitive to the juvenile/adult life stages of zebrafish. This difference may arise because the embryos are less developed than the eleutheroembryos and have a reduced metabolic pathway. This could cause the embryos to be less sensitive if the chemical has a specific mode of action, requiring metabolic activation or deactivation to cause the chemical to be toxic. Alternatively, it could be because the embryos are not actually in direct contact with the chemical (i.e. the chorion is impervious to the chemical). Since neither of these groups of chemicals used by Leonard require metabolic activation or deactivation to cause toxicity, it seems likely that the main reason for lower sensitivity of embryos to the chemical is that they are unable to enter the embryo. For these chemicals therefore, the chorion may well act as a barrier for their entry. This uncertainty on how permeable the chorion is for chemical entry questions the efficacy

of using zebrafish embryos for ecotoxicological assessments. Indeed, this likely complicates assessments on chemical toxicity. One potential resolution to alleviate this problem of the possible different permeability of the chorion to different chemicals would be to enhance the permeability of this partially permeable membrane. Two approaches have been identified that can potentially increase the permeability of zebrafish embryos. The first (which was not investigated further in this chapter) is mechanical disruption, and the second is chemical manipulation. In terms of chemical disruption of the chorion, two compounds (pronase and saponin) were selected to investigate the possibility of increasing the permeability of the chorion prior to exposure of a test substance. These two chemicals were chosen because in published literature (Westerfield, 1995) pronase has been used successfully to dechorionate fish embryos, and saponins are recognised to reduce cholesterol, which may in turn disrupt the integrity of the chorion and hence increase the permeability to chemicals.

Investigations into increasing the permeability of zebrafish embryos

To develop a method for enhancing the permeability of the chorion with the two potential permeabilising agents and without causing overt adverse effect on the zebrafish embryos, a series of experiments were performed to determine the effective, but non-lethal, concentrations and associated treatment times required for both Saponin and Pronase. Having established non-lethal concentrations of both pronase and saponin for the treatment of embryos, an investigation was made into assessing whether such treatments enhanced the permeability of the chorion of zebrafish

embryos. This assessment was carried out using [3-^{14}C]Estradiol to determine uptake into zebrafish embryos.

To further investigate the effect of either pre-treatment with pronase or saponin on the structure of the chorion of zebrafish embryos, a series of scanning electron microscopic (SEM) images were also obtained. The images obtained from both the pronase and saponin pre-treated embryos, were taken from the external surface of the chorion with the aim of determining if either of the pre-treatments significantly affected the structure and integrity of the membrane.

Materials and methods

Test substance

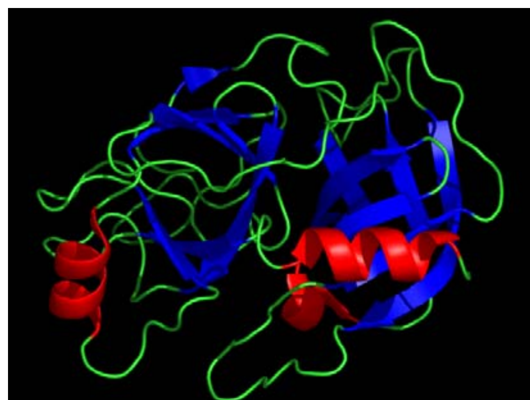
The two test substances (Pronase and Saponin) were supplied by Sigma-Aldrich Co. Ltd., Fancy Road, Poole, Dorset, BH12 4QH.

Chemical name: Pronase E
CAS no.: 9036-06-0
Chemical name: Saponin
CAS no.: 8047-15-2

Pronase (Figure 5.1) is a mixture of endo- and exo-proteinases, which cleave almost any peptide bond. The enzyme initiates protein catabolism by hydrolysis of the

peptide bonds that link amino acids together in the polypeptide chain. Since the chorion is constructed mainly from polypeptides, it is considered that pronase will affect these bonds and increase the permeability of the chorion.

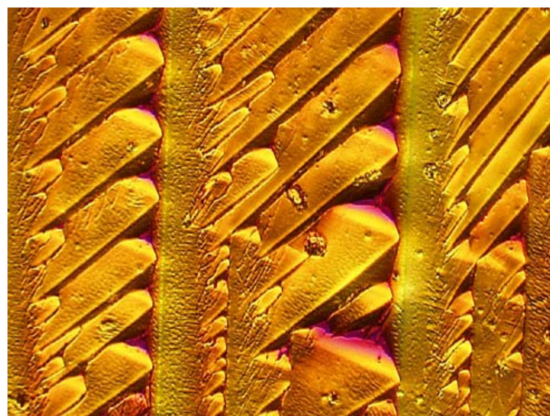
Figure 5.1. Diagrammatic representation of a pronase molecule



http://en.wikipedia.org/wiki/Serine_protease accessed 27.04.2007

Saponins (Figure 5.2) are natural surfactants, or detergents, which can be found in many plants, and are most abundant in the desert plants Yucca and Quillaja. Leguminous plants, such as peas and soybeans, also contain small quantities of saponins. Saponins are phytochemicals that have a wide spectrum of activity as antifungal and antibacterial agents, they lower blood cholesterol, and may potentially inhibit cancer cell growth (http://micro.magnet.fsu.edu/phytochemicals/pages/saponin.html accessed 20.09.2006). Saponins have also been shown to permeabilise cultured human intestinal epithelial cells (Jalal *et al*., 1992). Saponins may also be powerful emulsifiers that have haemolytic ability, which suggests that the use of saponins should be restricted to the early stages of embryogenesis when the circulatory system has not developed.

Figure 5.2. Photomicrograph of saponin



http://www.molecularexpressions.com/phytochemicals/images/saponin.jpg accessed 27.04.2007

The [3-^{14}C]Estradiol used for these investigations was the same standard reagent based radiolabelled chemical as detailed in chapter 4 and was chosen for these investigations to complement the work that was carried out previously.

Test conditions

For the embryo viability investigations post treatment with either pronase or saponin and for the [3-^{14}C]Estradiol uptake post treatment assessment, the dilution water used to prepare the test solutions was dechlorinated tap water set to a nominal temperature of 27 ± 2°C. The photoperiod was 16 hours light and 8 hours dark with 20 minute dawn dusk cycles between each transition. The embryos were obtained from the husbandry unit immediately prior to being used via the same methods as discussed in the previous chapter. The viability assessments were conducted in plastic 96 well microtitration plates (Linbro/Tiertek), with a well capacity of approximately 0.35 ml. The uptake experiments were performed in plastic Petri dishes (90mm diameter). All exposure work was carried out in a temperature-controlled laboratory (27 ± 2°C).

Procedure

Pronase and saponin viability assessments

A series of concentrations (DWC, 0.01, 0.32, 0.1, 0.32, and 1.0 g l^{-1}) of both test substances (Saponin and Pronase) were prepared by serial dilution from stock concentrates (1.0 g l^{-1}). All subsequent stock solutions were prepared in 50 ml glass volumetric flasks and dechlorinated water was used as the dilution water (27 ± 2 C). To prevent the enzyme (Pronase) from becoming deactivated through either interaction with the media, light or particularly the temperature the solutions were prepared on the day of use to prevent the protein from denaturing. For consistency, the saponin solutions were also prepared on the day of use. Any excess solutions were discarded after use. Approximately 20 embryos were exposed for periods of 2, 5 and 10 minutes in individual Petri dishes (90 mm diameter). Each Petri dish contained approximately 30 ml of each concentration of each test substance (3 replicates per treatment) and the amount of dilution water that was transferred with the embryos was kept to a minimum by carefully pipetting the embryos into the Petri dish using a 2 ml plastic Pasteur pipette.

Following exposure to the test substances, the embryos were rinsed. This was performed by carefully pouring the embryos onto a nylon-meshed container (mesh diameter of 0.1 mm gauge) followed by immersing the mesh in clean dilution water (27 ± 2°C). This process was repeated three times, replacing the dilution water each time, to ensure removal of the test substance from the outside of the embryos.

Following the exposure and cleaning procedure, the embryos were carefully rinsed into a clean Petri dish containing fresh dilution water (27 ± 2°C). From the Petri dish, the embryos were placed into individual cell wells of 96 well microtitration plates (Linbro/Tiertek) using a 2 ml plastic Pasteur pipette. Individual embryos were cultured in a volume of 200 µl dilution water per well.

Once the embryos had been transferred to the individual wells, observation of each embryo was performed to determine the approximate stage of development (according to methods described by Hisaoka and Battle, 1958) and any abnormalities were recorded. The incubation plates were then sealed, to avoid unnecessary evaporation, and the plate stored in a photoperiod controlled room (16:8 light dark) at 27 ± 2°C. Further observations of each embryo, to determine any gross malformations, stage of development and embryo mortality rates, were recorded at time intervals 24 and 48 hours post exposure.

Investigations into the effect of pronase or saponin on the uptake of [3-^{14}C]Estradiol into zebrafish embryos

All exposures were carried with approximately 20 embryos in 90mm diameter Petri dishes. The embryos that were pre treated were first exposed to either pronase or saponin at a concentration of 0.1 g l^{-1} for 2 minutes. The pronase and saponin solutions were prepared on the day of use with dechlorinated water tempered to approximately 27° C. The uptake investigations also used untreated embryos to serve as a control.

Following the treatment with either pronase or saponin, the embryos were cleaned thoroughly by rinsing in clean dechlorinated water to remove any residual chemical. The Petri dishes containing the embryos were then filled with approximately 30 ml of [3-^{14}C]Estradiol test solution at a concentration of 0.1 mg l^{-1}. This resulted in 3 Petri dishes: 1 pre treated with 0.1 g l^{-1} saponin, 1 pre treated with 0.1 g l^{-1} pronase and 1 control Petri dish containing embryos with no pre treatment.

Following exposure for 24 hours, the embryos were subsequently dissected (where possible) into their different tissue compartments (i.e. perivitiline fluid, embryo and chorion) and the different compartments were analysed separately. The filter paper method was used for the dissection of the embryos however, for the purpose of these investigations, the individual partitions were not used and the investigationwas based upon the total content of [3-^{14}C]Estradiol in all of the tissues was analysed (i.e. chorion+embryo+perivitiline fluid).

The quantity of [3-^{14}C]Estradiol in the different compartments of the embryos were analysed via liquid scintillation counting once the samples had been combusted using a Hewlett Packard Biological oxidiser. The protocol used for the combustions of the samples was as described in the previous chapter.

SEM images of zebrafish embryos post treatment with pronase or saponin

Twenty embryos were exposed to either 0.1 g l^{-1} solutions of pronase or saponin for 2 minutes, rinsed twice in clean dechlorinated water and then fixed in a 2% ($^{v}/_{v}$)

solution of glutaraldehyde prior to processing and observations using SEM. As detailed in the previous chapter, the embryos were the taken to the Bioimaging centre of the University of Exeter, where they were prepared for imaging by critical point drying using a series of alcohol solutions. Once prepared, the embryos were added to 12 mm diameter carbon tabs, attached to Jeol mounting stubs and then coated with osmium using a Fisons SEM coating system. The images were then observed using a Jeol JSM-6390LV scanning electron microscope.

Results

Results from the pronase and saponin viability assessments

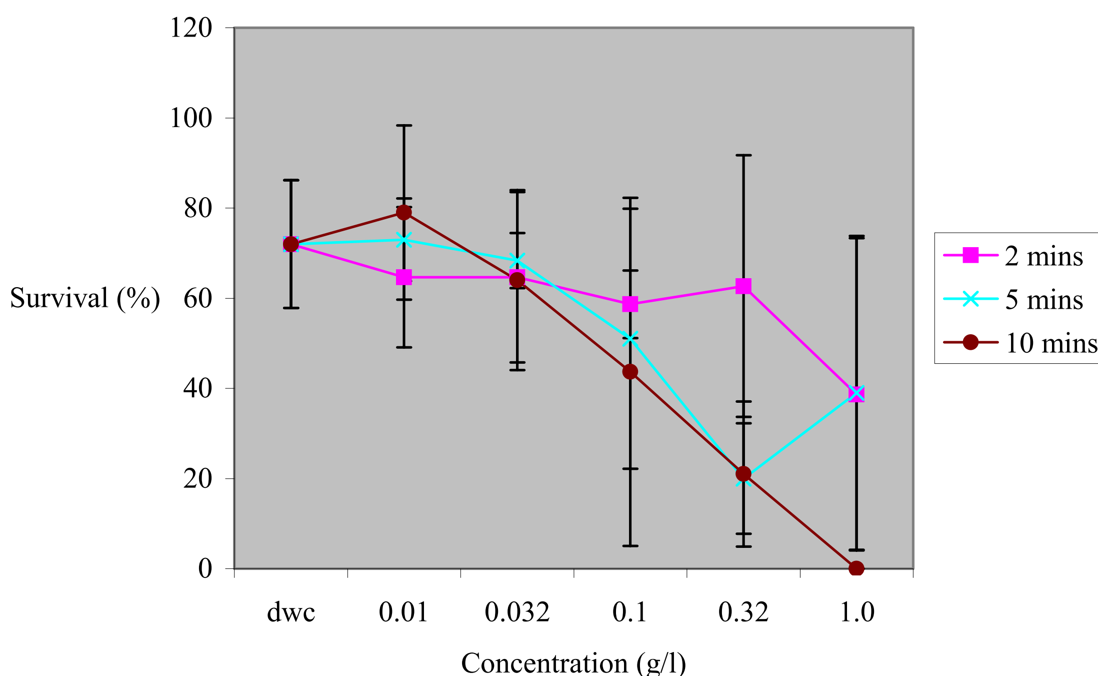
The percentage survivability of the embryos for the different time points of exposure (2, 5 and 10 minutes) after 48h are shown in Figures 5.3 and 5.4. The actual data obtained from the exposure assessments are presented in the appendices. The embryos exposed to pronase were significantly affected (i.e. the rate of survivability) at the concentration of 1.0 gl^{-1} when exposed for 2 minutes, however this may have been a function of specific sensitivity of the batch of embryos which were used, on run 2 which may have skewed the data.

Similarly, the survivability of the embryos exposed for 5 minutes to pronase were significantly affected at the highest concentration of 1.0 $g\ l^{-1}$ and also at 0.32 $g\ l^{-1}$. The data also indicated that there was a slight reduction in survivability at 0.1 gl^{-1}. Although it should be noted that the embryos used in run 2 appeared to be more sensitive than those used in runs 1 and 3.

Embryos exposed for 10 minutes to pronase were also significantly affected at the highest 3 concentrations studied (1.0, 0.32 and 0.1 gl^{-1}) when compared to dilution water control embryos. There was 100% mortality in embryos exposed to the highest concentration of pronase (1.0 $g\ l^{-1}$) for 10 minutes.

The mean control survivability for the dilution water control data was >70%.

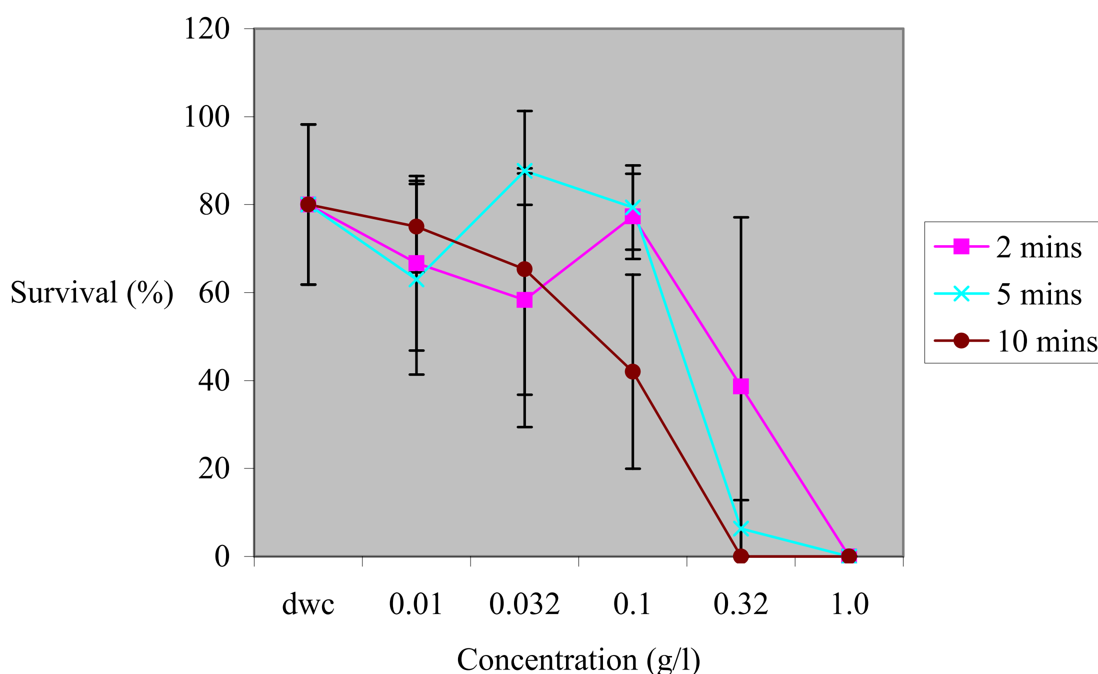
Figure 5.3. Graph showing percentage survivability of zebrafish embryos post exposure to a series of concentrations of pronase



For the saponin treatment data, the survivability of the embryos was significantly reduced at 1.0 and 0.32 $g\ l^{-1}$ after 2 minutes exposure. There was a slightly more pronounced affect at 0.32 $g\ l^{-1}$ test concentration when the embryos were exposed for 5 minutes compared with the embryos exposed for 2 minutes. Embryos exposed for 10 minutes showed a marked reduction in survivability at 0.1 $g\ l^{-1}$ and an LC100

(lethal concentration killing 100% of the population) was observed at concentration of 0.32 and 1.0 g l^{-1}. The mean control survivability for the dilution water control data 80%.

Figure 5.4. Graph showing percentage survivability of zebrafish embryos post exposure to a series of concentrations of saponin

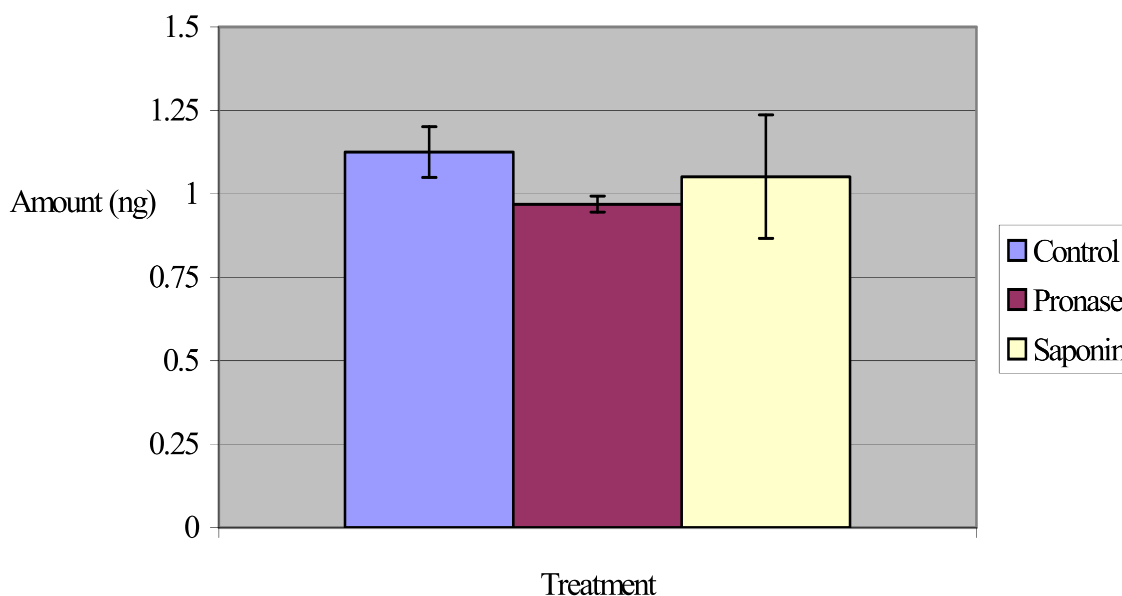


Results from the investigations into the effect of pronase or saponin on the uptake of [3-^{14}C]Estradiol into zebrafish embryos

Uptake measurements of [3-^{14}C]Estradiol in the embryos pre treated with the permeabilising agents pronase and saponin are shown in the following Graph (Figure 5.5). The actual data is presented in the appendices. Statistical analysis, using ANOVA indicated that there were no significant differences in the quantity of [3-

^{14}C]Estradiol taken up into zebrafish embryos following pre-treatment with either pronase or saponin.

Figure 5.5. Graph showing total (chorion, embryo plus perivitiline fluid) [3-^{14}C]Estradiol (ng) measured in individual zebrafish embryos exposed for 24 hours



SEM investigations into the effect of pronase on the structure of the chorion

The following images (Figures 5.6 and 5.7) are of a zebrafish embryo exposed to pronase (0.1 g l^{-1}) for 2 minutes prior to rinsing and fixing. These images reveal that the outer layer of the surface of the chorion appears to be significantly affected by the treatment and has started to slough considerably. Furthermore, it appears that the embryo has maintained its spherical shape in comparison to the untreated embryos. This indicates that the embryo had not collapsed during the dehydration process unlike the untreated embryos, which did collapse during the dehydration process.

Figure 5.6. Zebrafish embryo pre exposed to pronase (X 95 magnification)

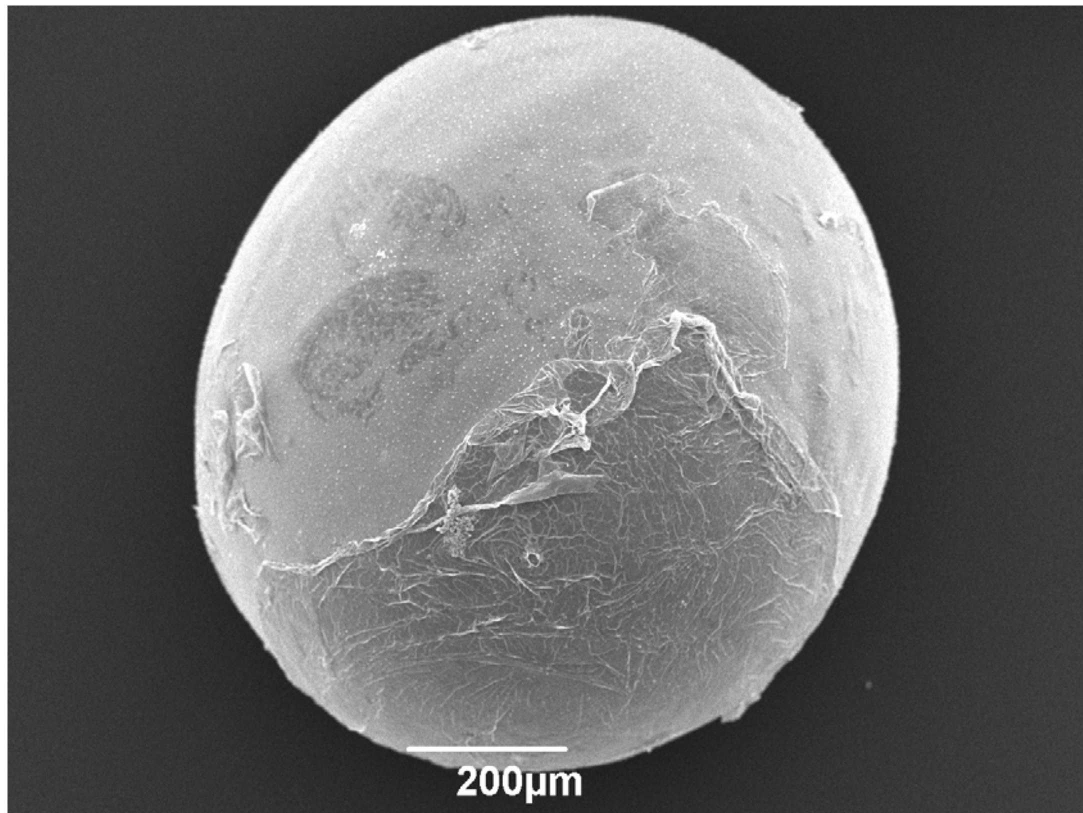
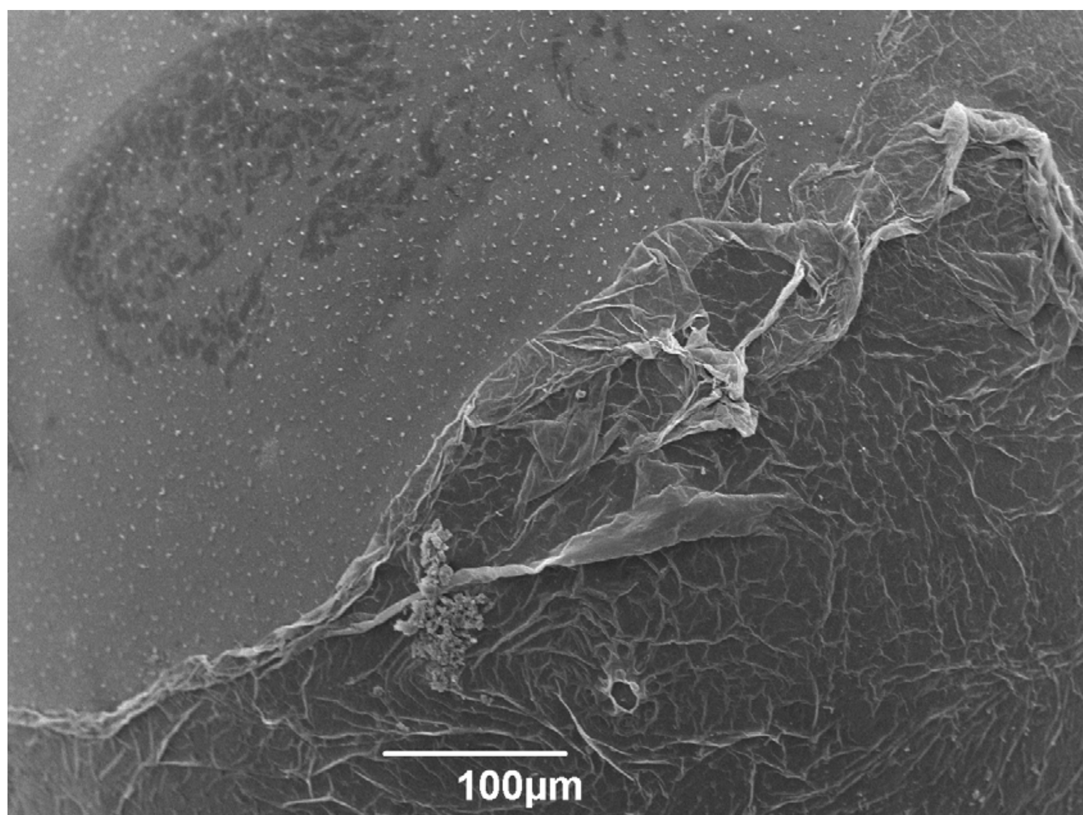


Figure 5.7. Zebrafish embryo pre exposed to pronase (X 220 magnification)



SEM investigations into the effect of saponin on the structure of the chorion

Figures 5.8 and 5.9 below are of a zebrafish embryo pre exposed to saponin (0.1 g l^{-1}) for 2 minutes prior to rinsing and fixing. As with the embryos exposed to pronase, there was some evidence of sloughing although it was not as pronounced as the sloughing observed on the embryos exposed to pronase. In addition, the embryos had collapsed and some had split open during the drying process.

Figure 5.8. Zebrafish embryo pre exposed to saponin (X80 magnification)

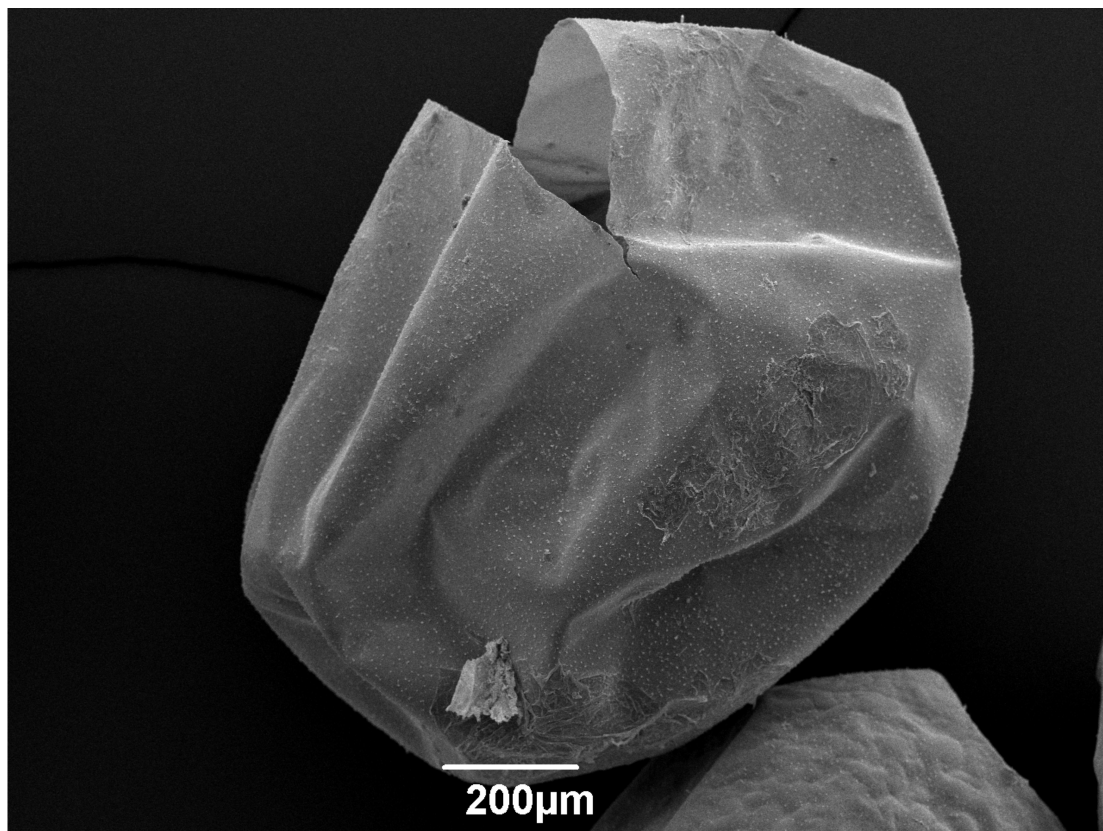
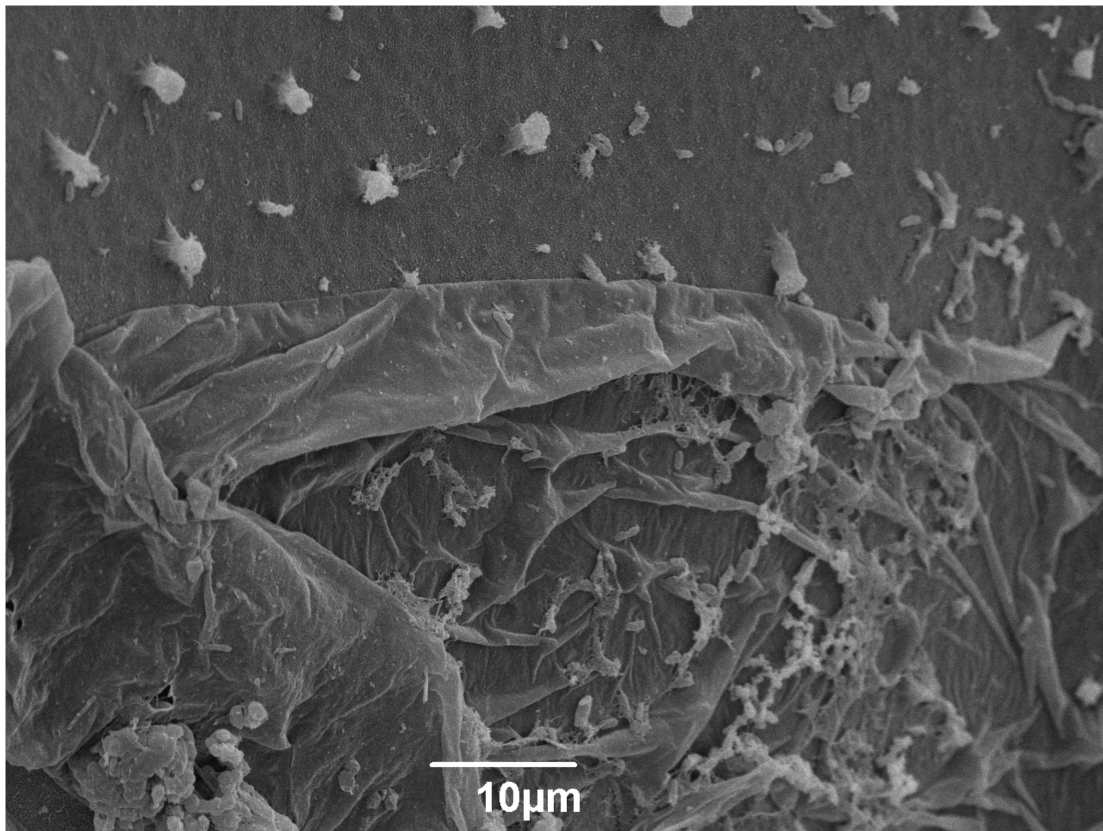


Figure 5.9. Zebrafish embryo pre exposed to saponin (X 1700 magnification)



Discussion

Pronase and saponin viability assessments

These experiments have reconfirmed the suitability of such test designs to conduct ecotoxicity evaluations on zebrafish embryos within 96 well plates and that this system could potentially be a suitable alternative to the use of whole fish particularly in terms of acute toxicity testing. In terms of the two potential permeabilising agents that were investigated, a lethal concentration has been determined based on the reduction in survivability. Therefore a suitable concentration and exposure duration for pretreating embryos with either pronase or saponin could be obtained. With

regards to pronase, the results suggested that there was no reduction in survivability at a concentration of 0.32 g l^{-1} when compared to the control. In order to ensure that an appropriate safety factor is incorporated into tests using pronase as a potential permeabilising agent, a concentration of 0.1 gl^{-1} would be recommended for a maximum period of 2 minutes. However, it is important to note that care must be taken when using any concentration of pronase to avoid damaging the embryos when rinsing, since the chorion of the embryos may become brittle following exposure to the enzyme. For example, it may be possible if the chorion rips open, following exposure and manipulation of the embryos into the 96 well plates, that the embryos become very vulnerable to damage by mechanical action. For this reason, the amount of physical manipulation should be kept to a minimum when using pronase and further investigations may benefit from an alternative test design from the 96 well plate design.

With regards to the saponin investigations, there was a similar effect pattern to the pronase experiments, although the saponin was slightly more toxic to the embryos. In addition, the embryos exposed to saponin did not appear to be as brittle as the embryos exposed to pronase. This suggests that embryos exposed to saponin can be easily manipulated following treatment without the possibility of causing mechanical damage. In terms of an appropriate dose of saponin to use as a permeabilising agent, the data indicates that a concentration of 0.1 g l^{-1} for 2 minutes should not compromise the survivability of the embryos when compared to the embryos in the dilution water controls. Using this concentration of saponins for all future permeabilising investigations would keep any comparisons with pronase consistent. It should be noted however, that any alterations in study design, to reduce the

possibility of mechanical damage of pronase treated embryos, must be incorporated for both substances for all subsequent comparisons to remain consistent.

The effect of pronase or saponin on the uptake of [3-^{14}C]Estradiol into zebrafish embryos

An interesting observation from the dissections of the embryos pre treated with Pronase was that it was not possible to dissect accurately the different partitions of the embryo. This was because the structure of the chorion had become very brittle and disrupted to such an extent that when the embryos were placed onto the filter paper, the chorion broke up into the embryo and perivitiline fluid. Therefore, the analysis of the results from the embryos, pre treated with pronase, could only be performed on total amount of [3-^{14}C]Estradiol in the combined partitions of the embryos and not the individual sections. Nevertheless, the data indicated that pre-treatment with either pronase or saponin did not have an effect on increasing uptake into zebrafish embryos. This may have been due to the fact that neither of the permeabilising agents actually had an effect on uptake into the embryos. Conversely, it may have been simply because the chorion of the zebrafish embryos is already freely permeable to [3-^{14}C]Estradiol and the chemical had already reached an internal equilibrium concentration with the external environment. Therefore, the level of [3-^{14}C]Estradiol would not have been affected by either of the permeabilising agents irrespective of if they had increased the permeability. The levels of [3-^{14}C]Estradiol measured in the control embryos are consistent with previous data from chapter 4

SEM investigations into the effect of pronase on the structure of the chorion

The treatment with the two permeabilising agents, pronase and saponin, revealed that the outer structure of the chorion was severely affected following an exposure regime of 2 minutes at a concentration of 0.1 gl^{-1}. However, from visual observations, the embryos treated with pronase appeared to be significantly more affected than those treated with saponin. For example, the level of sloughing was substantially more on the embryos treated with pronase. Furthermore, the embryos treated with pronase had not collapsed during the drying process. This indicates that during the dehydration process, water was removed from within the embryo without causing it to collapse. This could suggest that the pronase treatment actually increased the water permeability through the chorion, which would have been consistent with the visual appearance of the chorion (i.e. the sloughing of the outer layer), as shown in Figures 5.6 and 5.7. Conversely, although there was some evidence of sloughing on the outer surface of the chorions treated with saponin, these embryos had collapsed during the drying process and some had split open. This indicates that the chorion of these embryos were not as permeable to water as the embryos treated with pronase.

Conclusions

In this chapter, two potential chorion permeabilising agents (pronase and saponin) were investigated to determine non lethal concentrations of each substance to zebrafish embryos. The first substance (pronase) was considered as a potentially permeabilising substance as it is an enzyme capable of cleaving peptide bonds, such as those found in the chorion of fish. The second (saponin) is used to reduce

cholesterol with the assumption that this chemical might affect any lipid structure located on the outer membrane of the chorion. Subsequently, both substances were assessed to determine if pre-treatment, at a non lethal concentration, could affect the uptake of [3-^{14}C]Estradiol into zebrafish embryos. However, the uptake of [3-^{14}C]Estradiol into zebrafish embryos was not significantly affected by either of the pre-treatments. This may have been because the chorions of zebrafish embryos are already freely permeable to [3-^{14}C]Estradiol and therefore, it was not possible to ascertain if either compound was actually capable of increasing the permeability of zebrafish embryos.

From these investigations, it appears that the chorion is freely permeable to chemicals such as [3-^{14}C]Estradiol, and pre treatment with either pronase or saponin does not significantly increase the uptake of [3-^{14}C]Estradiol. However, it is still a possibility that the chorion may be impervious to molecules of a certain size, or to charged substances. This concept would be consistent with the findings of Leonard (pers com). For example, the substances that Leonard tested (e.g. amphoteric polymer, merquat 10 and luviquat HM 552) are highly charged and extremely large molecules with molecular weights in excess of 250,000. Therefore, to understand if there is a molecular weight cut off for chemicals to be taken up into zebrafish embryos, the following chapter focuses the uptake of different molecular weight molecules of poly ethylene glycol (PEG). This will assist in understanding if there is a molecular weight cut off value for substances to enter through the chorion of zebrafish embryos.

CHAPTER 6. UPTAKE OF DIFFERENT MOLECULAR WEIGHT [3-^{14}C]POLYETHYLENE GLYCOL (PEG) INTO ZEBRAFISH EMBRYOS

Introduction

Previous work in this thesis has indicated that zebrafish chorions appear to be more freely permeable to chemicals than indicated in the literature, with the exception of those with a large molecular weight or certain charged substances such as the quaternary ammoniums as detailed by Leonard et al. (2006). In the previous chapter, it was shown that chemicals such as [3-^{14}C]Fluoranthene and [3-^{14}C]Estradiol are measured in various internal partitions of zebrafish embryos after exposure in the water. This suggests that these chemicals are able to pass through the chorion and into either the perivitiline fluid or the embryo. However, as discussed previously, it is not known what the actual route for aqueous material to cross the chorion is, but there are several different possibilities. The first is that chemicals could enter, via gaps in the structure of the glycoprotein matrix; another possibility is that the micropyle is partially permeable post fertilisation; and finally the pores in the chorion may be the passage of entry for chemicals into the embryo. Regardless of the actual route of aqueous material into the embryo and across the chorion, there appears to be some properties associated with certain chemicals which limit uptake across the chorion. Therefore, this chapter investigates if the route for chemicals into the embryo through the chorion is limited by the size of the molecule of the chemical. To address whether chemicals with a large molecular weight, and hence potentially a large molecular size, can cross the chorion via either the micropyle, the pores or the gaps between the glycoproteins, an uptake experiment was performed using different size radiolabelled

polyethylene glycol (PEG). The experiments were performed using low to high molecular weight PEG (mw 400, 4000 and 40000) exposed to zebrafish embryos for 24 hours. Following exposure, the embryos were dissected and analysed for the quantity of radiolabelled material within the embryos. In addition, a supplemental investigation was also performed to assess if either of the two potentially permeabilising agents, pronase and saponin described in the previous chapter, would affect the uptake. For these investigations, embryos were pre-exposed to either saponin or pronase at a concentration of 0.1 g l^{-1} for 2 minutes prior to being rinsed in dechlorinated water followed by exposure to the different molecular weight solutions of PEG for 24 hours.

Materials and Methods

Test substance

Three different molecular weight variants of radiolabelled Polyethylene glycol (PEG) were obtained from American Radiolabelled Chemicals (ARC). Information and properties of the substances are shown below.

	PEG400	PEG4000	PEG40000
Molecular weight distribution			
Physical state	In ethanol	In ethanol	solid
Specific activity (MBq)	25.9	6.51	2.62

Polyethylene glycol was chosen as the test substance due to its availability in different varieties of molecular weight and PEG is widely used in a variety of products and has low toxic at relatively high concentrations (Sheftel, 2000). It was also considered that

the use of the same chemical, which was available with a variety of different molecular weight, would also avoid any chemical specific features that would complicate the analyses. In addition, radiolabelled PEG is readily available which avoided the high cost of specific chemical synthesis. The range of molecular weights of PEG chosen spanned low to high, and the largest chosen was in the molecular weight range of the quaternary ammoniums tested by Leonard *et al*. (2006). Adopting this approach, the intention was to be able to characterise the size of molecules capable of entering into the embryo.

Test conditions

The dilution water, photoperiod and methods for obtaining the embryos were the same as described in the previous chapters. Exposures were conducted in 90 mm diameter plastic Petri dishes. All work was carried out in a temperature-controlled laboratory (27 ± 2°C).

Procedure

Exposures were carried out in a similar manner to those detailed in the previous chapters. All analysis of the uptake of radiolabelled PEG was carried out by LSC following combustion using the methods outlined in the previous chapters. Initially, the test concentration, of each different molecular weight variant of the PEG, tested was 10 mg l^{-1}. However, it was realised subsequently that using this approach, in each of the different solutions there were a different number of molecules which were being exposed for the different molecular weight variations of PEG. For example, in the solutions that were prepared (10 mg l^{-1}), the PEG400 solution had 10 and 100

times more molecules than the solutions of PEG4000 and 40,000 respectively. Therefore, in the definitive investigations, the studies were conducted adopting a molar concentration (mM) approach, to expose the zebrafish embryos to the same number of molecules for each of the different molecular weight variants of PEG. All solutions were prepared using dechlorinated tap water as described in the previous chapters and the embryos were exposed for 24 hours. The embryos were all rinsed twice using fresh dechlorinated water prior to being dissected using the filter paper method as detailed previously. The embryos were dissected, where possible, into each of the respective partitions (i.e. embryo, chorion and perivitiline fluid) but for the purpose of these comparisons, the amounts were added together to allow for the comparisons to be based on the total amount of radiolabelled material in the whole of the embryo.

The supplemental investigation, performed to assess if either of the two potentially permeabilising agents, described in the previous chapter, would affect the uptake. For these investigations, embryos were pre-exposed to either saponin or pronase at a concentration of 0.1 g l^{-1} for 2 minutes prior to being rinsed in dechlorinated water followed by exposure to the different molecular weight solutions of PEG. All exposures were carried out using the same batch of eggs as the investigations on the untreated PEG uptake investigations above, and under the same conditions as described above.

Results

The measured exposure concentrations of the PEG solutions at the beginning and end of exposure are shown in Table 6.1

Table 6.1. Concentrations of PEG in the test solutions

	Concentration of PEG (mM)					
	PEG400		PEG4000		PEG40000	
	0h	24h	0h	24h	0h	24h
Mean	0.124	0.115	0.093	0.069	0.092	0.036
SD	0.0003	0.0003	0.0009	0.0007	0.0026	0.0007
% of initial concentration	-	93	-	74	-	40

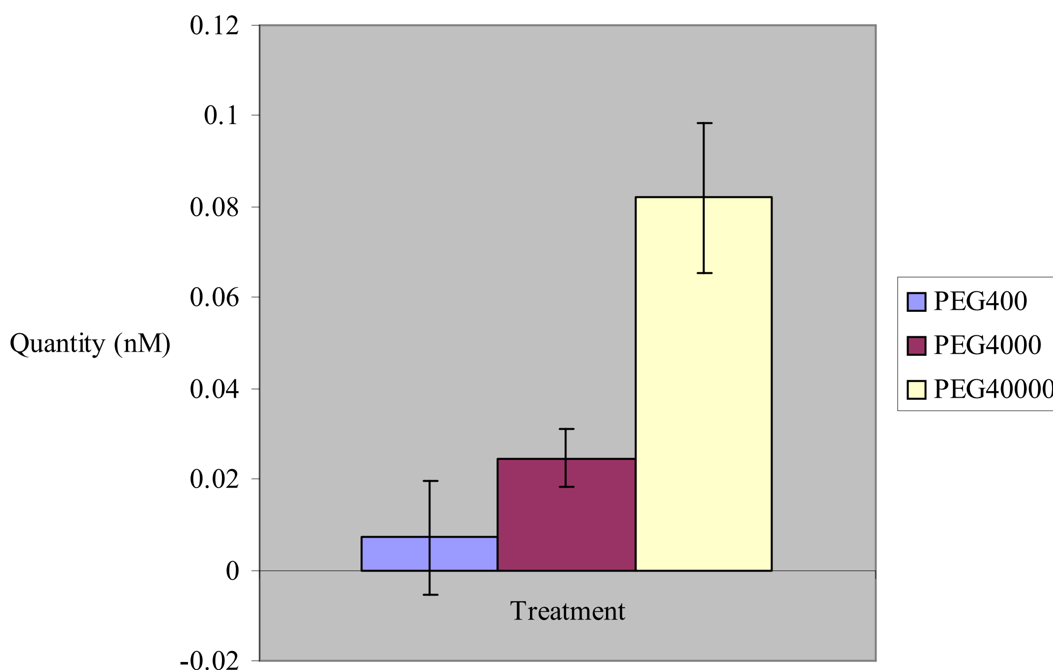
All samples taken for analysis at the start of exposure (0h) were measured in triplicate, and all samples taken for analysis at the end of exposure (24 h) were measured in duplicate. Interestingly, the concentration of PEG400 remained very consistent between 0 and 24 hours, there was a lowered measure concentration in the PEG4000.after 24 h, which was 74% of the initial concentration, and the concentration of PEG40000 was reduced considerably to 40% of the initial concentration after 24 h.

Uptake of different molecular weight PEG into zebrafish embryos exposed for 24 hours

The uptake of the different molecular weight PEG are shown in Graph 6.1, the data are also presented in the appendices. The data shows that PEG400 is only taken up minimally into zebrafish embryos, and for some of the embryos, the analysis showed that the levels of radioactivity were comparable to background radioactivity. This indicates that PEG400 could not be detected within these embryos and therefore was possibly not taken up through the chorion. The quantity of PEG4000 measured within

the zebrafish embryos was slightly higher than the quantity of PEG400 that was measured, in addition, all levels of radioactivity measured within the embryos exposed to PEG4000, were significantly higher than the background levels of radioactivity, indicating that PEG4000 had been taken up through the chorion and into the zebrafish embryos. With regards to the highest molecular weight PEG analysed, the quantity of PEG40000 measured within the zebrafish embryos was significantly higher than both the quantity of PEG400 and PEG4000 measured within the zebrafish embryos.

Graph 6.1. Uptake of different molecular weight PEG into zebrafish embryos exposed for 24 hours



The results from the supplementary investigations using the different pre-treatments of pronase or saponin as potentially permeabilising agents are shown in the following graphs (Graphs 6.2-6.4). The data is also presented in the appendices. The data are expressed as the uptake of PEG into untreated embryos, which represent the control

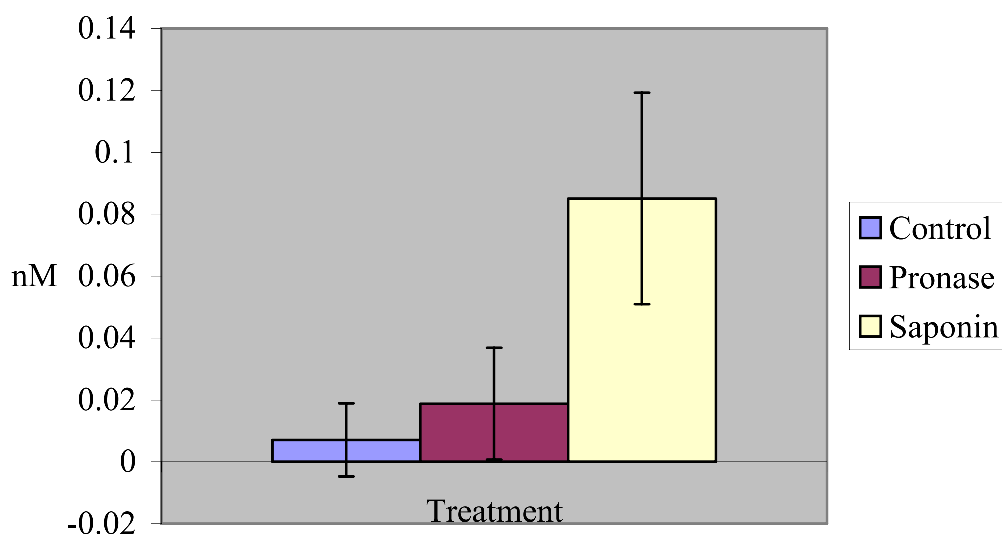
conditions, compared with uptake of PEG into embryos pre exposed using either saponin or pronase. All data are presented as the total quantity of radiolabelled material measured within the embryo i.e. the embryo plus the perivitiline fluid. The chorion data were excluded from these interpretations to avoid the possibility of any PEG sticking to the outside of the chorion skewing the data. Furthermore, it was considered that it was more important to understand how much PEG was actually taken up into the embryo and not what was associated with the chorion.

Uptake of PEG400 into zebrafish embryos

The data for the uptake of PEG400 into the zebrafish embryos is shown in Graph 6.2. These data indicated that the levels of radioactivity measured within the untreated embryos were either equivalent to or just above the background levels of radioactivity in the embryos after 24 hours exposure. This indicates that the quantity of PEG400 inside the untreated embryos was insignificant and therefore the chemical perhaps did not cross the chorion. Furthermore, pronase treatment did not significantly affect (enhance) the uptake of PEG400. In contrast, the embryos, pre treated with saponin, contained significantly higher quantities of PEG400 after 24 hours exposure. This suggests that saponin could be affecting the physical structure of the chorion hence enabling the passage of PEG400 through the envelope. However, it is also possible that the saponin could be affecting the physical structure or the chemical properties of the PEG400 molecule itself, which is subsequently affecting the uptake of the chemical into the embryo. Furthermore, the chemical analysis that was performed on the embryos was based on radioactive equivalents present in the sample and specific analysis (i.e. quantification of actual parent compound) was not performed to confirm the identity of PEG400 within the embryo. Therefore, it is possible that the

radioactivity measured within the embryos may have simply been the radio labelled isotope attached to part of a fragment of the PEG molecule.

Graph 6.2. Quantity of PEG400 in zebrafish embryos after 24 h exposure



Uptake of PEG4000 into zebrafish embryos

The data for the uptake of PEG4000 into the zebrafish embryos is shown in Graph 6.3. These data showed that the quantity of PEG4000 measured within the untreated and treated embryos, were higher than the levels measured in the control embryos exposed to PEG400. This indicates that the radioactivity measured within the embryos was higher than the background levels of radioactivity present and that PEG4000 could be measured within the embryos. However, the uptake response profile was very different for the embryos exposed to PEG4000 when compared to the embryos exposed to PEG400 (pre treated with saponin or pronase). For example, the mean quantity of PEG4000 measured in the embryos pre exposed to saponin, was not significantly different to the control (untreated) embryos. The data from the embryos,

pre treated with pronase prior to PEG4000, indicated that pronase had a significant effect on the uptake of PEG4000 which resulted in the quantity of PEG4000 being 3 fold higher than that of the uptake of PEG4000 in to the control embryos. Unfortunately, it was not possible to dissect the different components of the internal contents to assess if the pronase had affected the level of PEG4000 entering in to either the perivitiline fluid or the embryo. This was because the embryos had become very brittle post treatment with pronase. In addition, the chorion of these embryos also appeared to be ruptured when observed prior to being dissected (see Figure 6.1). Therefore, it was likely that the pre exposure of the embryos with the pronase had caused the chorions to become overly brittle and that they had probably ruptured in some way during the physical manipulation of the embryos, perhaps during the rinsing procedure. Therefore, the uptake of PEG4000 into the pronase pre-treated embryos may have been exacerbated by the damage caused to the chorion and less to do with simply increasing the permeability of the chorion with pronase. For example, if the chorion had ruptured, the PEG4000 could have entered through the chorion through the tear in the envelope rather than through any of the previously identified routes of entry (i.e. micropyle, pores etc). However, in the pronase treatment has increased the uptake of PEG4000 by potentially permeabilising the chorion all be it rather aggressively.

Graph 6.3. Quantity of PEG4000 in zebrafish embryos after 24 h exposure

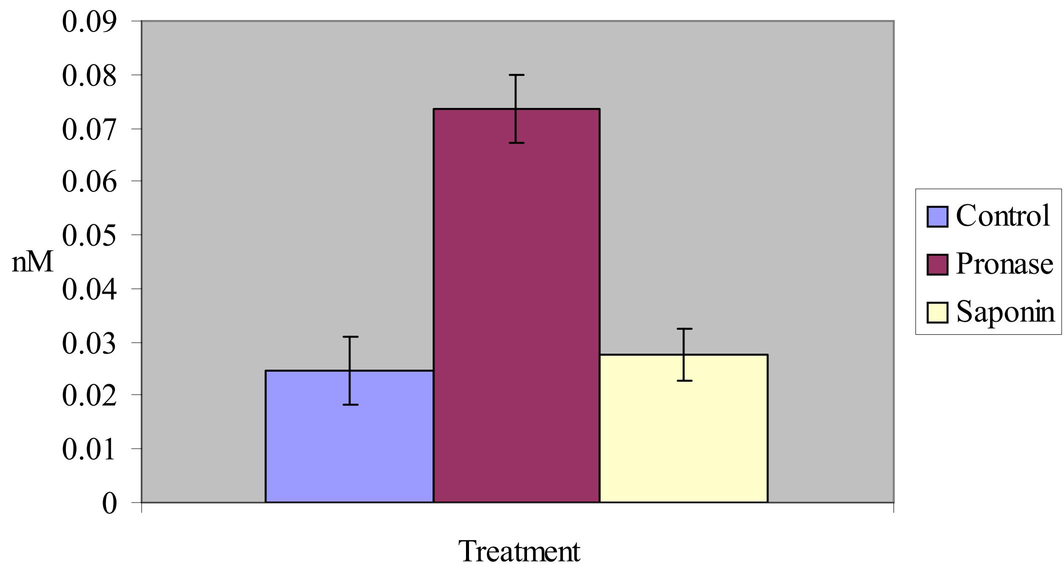
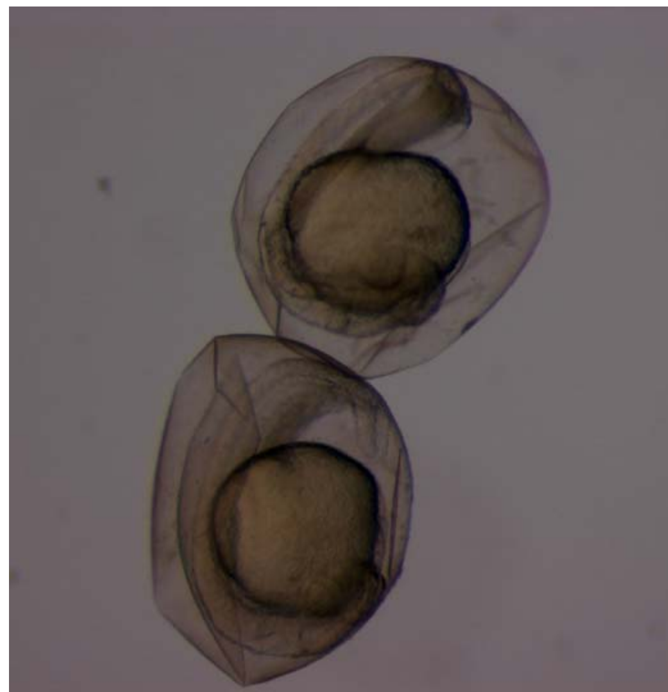


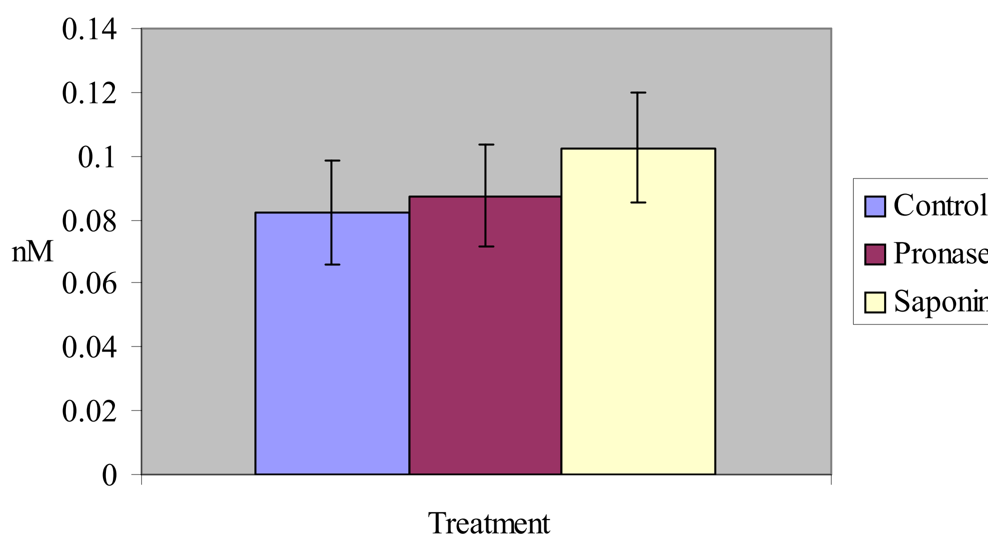
Figure 6.1. Embryos pre-exposed to pronase prior to being dissected



Uptake of PEG40000 into zebrafish embryos

The data for the uptake of PEG40,000 into the zebrafish embryos is shown in Graph 6.4 and followed a different uptake profile, compared with the previous uptake investigations with PEG400 and PEG4000. For example, there were no significant differences between the different treatments and the quantity of PEG40,000 taken up into the embryos. However, the quantity of PEG40,000 in the embryos, pre-exposed to the saponin, was slightly higher than that in the control and the pronase pre-treated embryos. Interestingly, the mean quantity of the uptake of PEG40,000, for the three different treatments (control, pronase and saponin), was similar to the highest quantities measured in the embryos exposed to PEG400 and PEG4000. This indicates that high molecular weight PEG is able to cross the chorion more easily than low molecular weight varieties. This contradicts the fundamental hypothesis presented within this chapter, that chemicals with a large molecular weight and hence potentially size, are restricted from crossing the chorion and entering into the embryo.

Graph 6.4. Quantity of PEG40,000 in zebrafish embryos after 24 h exposure



Discussion

The measured exposure concentrations of the different molecular weight test solutions of PEG indicated that there were differences between the exposure conditions for each of the different solutions during the exposure period. For example, the concentration of PEG400 remained very consistent between 0 and 24 hours. The concentration of PEG4000 was approximately 75% of the initial concentration after 24 hours, and the concentration of PEG40000 was reduced considerably to 40 % of the initial concentration after 24 h. The reason for the reduction in concentration of PEG4000 and PEG40000 after 24 hours exposure was possibly due to the PEG adhering to the surface of the Petri dishes. In addition, the reduction in PEG400 and PEG4000 in the test solution may have been due to the compounds being taken up into the embryo and hence reducing the concentration in the solutions. This was consistent with the findings that PEG4000 and PEG40000 were measured in the zebrafish embryos, which indicated that the radiolabelled material had been taken up into the embryos. As a result this would have caused the concentration of test material in the test solution to decrease. Similarly, the quantity of PEG400 measured in the embryos was close to the background levels of radiation, indicating that the quantity of test material taken up into the embryo was negligible. This was consistent with the fact that the concentration of PEG400 remained relatively consistent over the 24-hour period.

Unfortunately, the findings from these investigations into the uptake of the different molecular weight PEG did not follow the uptake profile that what was predicted to occur. For example, it was initially hypothesised that large molecules may not be able to pass through the chorion of zebrafish embryos. However according to the findings from these investigations it would appear that substances with a molecular weight of

up to 40000 could pass through the chorion. This may suggest that transport across the membrane for large molecular weight substances is important in understanding uptake through the chorion. Conversely, the chemical with the smallest molecular weight (PEG400) does not appear to cross the chorion of the embryo, as it was not possible to detect it in these investigations. This suggests that there may be other factors affecting the limitation on PEG400 from crossing the chorion. For example, the uptake could have been affected by the osmolarity of the test solution. Furthermore, compared with the PEG4000 and PEG40000, the PEG400 may have a significantly pronounced effect on the osmolarity of the test solution especially considering that the actual concentration of the PEG400 was 10 and 100 times higher than the PEG4000 and 40,000 respectively. This was because the investigations were based on molar concentrations to ensure that an equal number of molecules were used in each test solution.

On reflection, PEG is a highly water soluble polymer which is used in biochemistry to increase osmotic pressure but unfortunately the osmotic interaction was not considered when choosing the test substance. If the PEG400 was indeed affecting the osmolarity of the test solution, this could have explained why PEG400 was not taken up into the untreated embryos. Similarly, pronase did not appear to affect the uptake of PEG400 however, this does not indicate that the chorion was unaffected by the pronase treatment, and uptake may have been affected by the osmolarity of the test solution. With regards to the embryos pre-treated with saponin, it may have been possible that the pre-treatment affected the structure of the PEG molecule, which as a consequence would have affected the osmolarity of the test solution. This would provide some explanation why PEG400 was taken up in to the saponin treated

embryos. Regardless, the saponin must have affected either the substance or the chorion to enable the PEG400 to be taken up into the embryo.

In addition to the effect of osmolarity on the uptake, there is also the possibility that the PEG400 was not able to pass into the embryo because of physical limitations (e.g. molecular size and structure). Furthermore, as PEG is a surfactant, the molecule may have formed complex associations with the other molecules. This would have meant that the PEG400 complexes would have been too large to pass through the chorion via, for example, the pores or the micropyle.

Considering the embryos exposed to the PEG4000, a more likely solution to the increased level in the embryos pre-treated with pronase is the fact that the enzyme actually physically affected the chorion causing it to rupture. Therefore, the PEG4000 was able to pass freely into the embryo and there were no osmotic gradients between the inside and the outside. In comparison, the levels of PEG4000 in the control embryos and the embryos pre-treated with saponin were significantly lower. Therefore, although PEG4000 was measured within these embryos, is it possible that there was a reduced uptake because these molecules were also too large to pass through the chorion?

In terms of the embryos exposed to the PEG40000, it appeared that pre-treatment with either pronase or saponin did not affect the uptake when compared to the control embryos. Plus the quantities measured in the embryos of the three different treatments suggested that PEG40000 is able to cross the chorion and enter the embryos. A potential reason for this increased uptake is possibly due to the PEG40000 becoming smaller if the molecules folded in onto each other. Therefore,

although each molecule had the same molecular weight, the actual size of the molecules had been reduced. An additional explanation for the uptake of PEG40000 could be because the actual concentration was either an order or 2 orders of magnitude lower than the PEG4000 and PEG400 respectively. Therefore, at the lower concentration of PEG40000 may not have affected the osmotic potential in the solution and therefore, the PEG40000 was taken up into the embryos.

Furthermore, it is difficult to actually categorically specify whether the uptake profiles seen in these investigations was genuine as the analysis was performed using LSC following combustion which can only characterise the C14 as equivalents. Furthermore, there was no specific analysis performed on the embryos. In addition, it is difficult to know if it would be possible to carry out specific analysis to determine/characterise the parent compound within the embryo due to the sample size. Generally, for specific analysis, large sample volumes are required to perform thin layer chromatography. Therefore, for this PEG data to be further validated, a better understanding of the fate of the chemical within biological systems would also need to be investigated. For example, if the PEG molecule was breaking down within the test solutions into smaller fragments, then these smaller fragments may be small enough to pass through the chorion. The analysis by LSC is non-specific and would measure the fragments as whole molecules and would not be able to identify any degradates.

Finally, the question of whether fluid is able to pass through the pores is still completely unknown, but the possibility that these pores could be the route for fluid to enter or exit the embryo should be considered further. For example, if the size of the pores across the chorion are considered briefly, it is interesting to note that stomata

pores in some plants have a similar size diameter opening. Therefore, it is possible that the pores have some function in controlling the internal contents of the perivitiline fluid. Furthermore, there is a fundamental need for supplementary work to understand the function and properties of these pores across fish chorions. Additionally, there is a need for a thorough understanding of the structure and function of the micropyle as the potential route of chemical entry into fish embryos.

CONCLUSIONS

The structure and function of the zebrafish chorion has been investigated within this thesis, in addition to developing an understanding of the general ontogeny of zebrafish embryos and their appropriateness as an alternative life-stage for juvenile fish in ecotoxicity testing. With regards to the developmental ontogeny, it was shown that a subtle difference in temperature (e.g. 1-2°C variation) affects the developmental rate of the zebrafish embryos and retardation can occur at lower temperatures. For example, at a temperature of 30° C it was shown that zebrafish embryos begin hatching within 48 hours post fertilisation and at 25° C the embryos may take up to 96 hours post fertilisation to hatch. This has significant implications for their use as an alternative life-stage because if they have hatched within the exposure period then they will technically be classed as a protected organism and should be included in the Home Office returns for animal use. In addition, if the hatching period is extended or reduced, depending on the temperature that is used, this may have implications for standardising the test system in toxicity evaluations as toxicity is a function of exposure period and concentration. For example, if a lower temperature is used, and hatching takes up to 96 h the toxicity may be different than observed at a higher temperature. Therefore, if the use of zebrafish embryos is to be adopted as an alternative life-stage, it is recommended that the test design is strictly standardised for example, the temperature should be maintained at 27°C ± 0.5 and the exposure period should be 48 hours in duration. A major limitation to this test design however, is that a direct comparison between juvenile and embryo acute toxicity data may be restricted as juvenile fish toxicity data will probably be based on 96 hour LC_{50} values

rather than 48 hour LC_{50} values. Furthermore, the test temperature recommended in OECD technical guidance document 203 for acute toxicity tests with zebrafish is between 21 and 25°C. This may be sufficiently appropriate for juvenile fish, however, there may be issues with retardation if zebrafish embryos are used for testing purposes at these lower temperatures. Therefore, care should be taken when making comparisons between acute toxicity data from juvenile zebrafish and zebrafish embryos since the test temperature is likely to not be comparable which may affect the toxicity recorded.

Following the research into the developmental ontogeny of zebrafish embryos, the structure and function of the zebrafish chorions and its role as a protective envelope for zebrafish embryos was investigated. It was initially assumed that the chorion of zebrafish embryos prevents the transfer of external contaminants into the embryos hence offering some protection to toxicity of different substances. This assumption has been used to explain why fish embryos are intrinsically less sensitive than other more developed life-stages, e.g. eleutheroembryos or juvenile fish. However, the investigations, detailed in this thesis, indicated that the chorion of zebrafish embryos is permeable to simple chemicals such as fluoranthene or estradiol and that both chemicals could be quantified within the different partitions of the embryo (i.e. the chorion, the perivitiline fluid and the embryo itself). Furthermore, it was also shown that chemicals with a high molecular weight (e.g. PEG40,000) could pass through the chorion and could be quantified within the different internal partitions of the embryo. Therefore, if there is a molecular weight cut off value, for substances entering through the chorion of zebrafish embryos, it would need to be greater than 40,000. This would be consistent with the findings of Leonard *et al*. (2006) who found that certain

cationic polymers, with a molecular weight of approximately 450,000 (over an order of magnitude higher than the PEG40,000 used in this thesis), were only toxic to dechorionated embryos and not toxic to chorionated embryos. However, there was no chemical analysis to confirm presence or absence of test substance in the internal partitions of the chorionated embryos, but it is likely that the lack of toxic response in the chorionated embryos was because these substances were not passing through the chorion. Nevertheless, in terms of potential limitations for the use of zebrafish embryos as an alternative approach for ecotoxicity testing, certain cationic polymers may not pass across the chorion. Therefore, such substances, which form complex or large structures with other similar molecules, through chemical interactions such as ionic bonding, should not be assessed for ecotoxicity testing using fish embryos.

A supplementary part to the uptake investigations carried was to understand if the chorion of zebrafish embryos could be manipulated to be more permeable by using either pronase or saponin. The findings were that both chemicals had a substantial effect on the outer structure of the chorion, but that each technique may have been too invasive and would therefore need to be refined if its use was to be adopted as part of a testing regime using fish embryos. However, if the sensitivity of fish embryos is limited due to the chorion preventing test substances crossing the chorion (e.g. cationic polymers), it may be more pertinent to actually remove the chorion completely for such substances.

In attempting to understand how chemicals can actually cross through the chorions of zebrafish embryos, a series of SEM images were obtained to determine if there was

anything visible on the structure of the envelope, which could explain the passage of uptake. These images revealed a large number of pores, which were visible on the internal surface of the chorion. These pores were reported to be abundant at a rate of approximately 7.2×10^5 pores per chorion (Hart and Donovan, 1984). Furthermore, the pores could be seen from the outer surface, although the outer layer of the chorion was covering these openings. Hence, it is not clear if these pores are permeable or what the exact function of these pores is for. Interestingly, SEM images, obtained from fathead minnow embryos, revealed that the outer surface of the chorion is covered with clearly defined pores but these may also provide some function to enable the embryos to adhere to surfaces during spawning. Furthermore, the micropyle of a zebrafish embryo was also identified and it was considered that this may also have been a possible route for chemicals to enter into the perivitiline fluid through the chorion. Particularly considering that the diameter of the micropyle is approximately 25 µm which is much larger than most chemicals.

A significant finding, associated with these uptake investigations was that although these chemicals could be detected within the different partitions of the embryos, the technique used was difficult to perfect, took a long time to perform and was liable to cross contamination from the different partitions. Furthermore, the analysis of the different partitions was based on quantification of each test substance and not on actual concentrations (e.g. mg l^{-1}) of test substance within the different partitions. If the concentration of test substance were determined within the perivitiline fluid this would enable a better understanding of how much test substance was actually being exposed to the embryo. For example, in the investigations carried out in this thesis, it was shown the both fluoranthene and estradiol could be measured within the

perivitiline fluid. However, because the volume of perivitiline fluid was not determined a concentration could not been calculated. Therefore, it was not possible to determine whether the amount of test substance within the perivitiline was equivalent to the external concentration. To enable a truly representative comparison between the sensitivity of juvenile/eleutheroembryos with pre hatched embryos it would be necessary to determine the concentration of test substance within the perivitiline fluid. To be able to determine the concentration of test substance within a zebrafish embryo, it may be possible to remove the perivitiline fluid using a very fine needle attached to a micro syringe, but any analysis should be based on specific parent compound analysis and not solely on radioactive equivalents to ensure that what is being measured is actually the compound tested and not break down products or metabolites. Furthermore, if the concentration of test substance in the perivitiline fluid is not at equilibrium with the external medium, it may be more representative to calculate LC_{50} values based on the internal concentration of the perivitiline fluid.

Although the structure and function of the chorion has been investigated in this thesis, several other questions remain unanswered that have not been addressed. For example, there may be physicochemical interactions, which also have a major effect on uptake. Such physicochemical interactions may be related to the ionic content of the media, which may have an effect on the osmotic gradient between the inside and the outside of the embryo. Therefore, an area of further work may be to investigate uptake of chemicals into zebrafish embryos using media with different ionic contents (e.g. dechlorinated, deionised or embryo rearing media). Furthermore, the total organic carbon content of the test solutions, used in this thesis, was not investigated

and this may also have had an effect on the bioavailability of the test substances used which may also had an effect on the uptake.

Another area for future consideration is to understand how chemicals are taken up from the perivitiline fluid and into the embryo. For example, it was previously discussed that the uptake of chemicals from an aqueous medium through the chorion into the fish embryos is possibly via a passive process. This uptake may be via portals through the chorion, for example the micropyle or the pores, driven by the osmotic gradients between the internal and external mediums. However, the process governing the uptake of chemicals into the embryos, once the chemical has entered through the chorion, is unknown. It is possible that this uptake could be either a passive or an active process.

To understand why it is important for ecotoxicity testing to know the mode of uptake, the complexities regarding exposure and how this affects how embryos could be used to determine the toxicity of substances should be considered. For example, if the uptake of chemicals into fish embryos, from the perivitiline fluid, is a passive process then the issues regarding the size of molecules and any physicochemical properties affecting uptake across biological membranes, will apply. However, this will not apply for those chemicals with a mode of action that causes toxicity through a physical effect such as preventing gaseous exchange. Substances such as these will cause indirect toxicity without being taken up into the embryo and should be considered independently using fish at a more developed life-stage.

In addition to understanding whether uptake of chemicals into fish embryos is a passive or an active process, a further issue to consider is where in the embryo a chemical is likely to partition. For example, chemicals, that are highly lypophilic, are quite likely to preferentially partition into the yolk of the embryo. However, it is unknown whether the chemical passes from the perivitiline fluid and through the yolk syncitial layer into the yolk, or it is first taken up into the embryo and then later distributed to the yolk during the normal ontogeny of the embryo. If the chemical is passing through the yolk syncitial layer, it is possible that the passage through the membranes of the embryo is via a mixture of both active and passive mechanisms.

If the mechanism of uptake into either the body of the embryo or the yolk had been identified, it would be interesting to also carry out some comparative work to understand partitioning within the different parts of the body, for example, do highly lypophilic chemicals only partition into the yolk or are they also present in the body of the embryo? To understand the partitioning within the embryo, it might be necessary to dissect the yolk away from the body of the embryo, which would be considerably more difficult than dissecting the chorion away from the embryo. From experience with dechorionating embryos, what is apparent is that the yolk syncitial layer is extremely delicate, and can rupture easily. This is the major problem with manually dechorionating embryos for use in viability assessments, as any damage caused to the yolk results in impaired survivorship.

In addition to the use of fish embryos as an alternative life-stage for acute toxicity purposes, they have also been suggested as an alternative for assessing chronic

toxicity or as a tool for determining bioaccumulation. However, care should be taken when extrapolating data, based on the bioaccumulation of highly lypohilic substances, since fish embryos have a large yolk. The yolk is likely to be a source for significant levels of the accumulating compound and therefore, it would be necessary to lipid normalise any data obtained from fish embryos to ensure a representative bioaccumulation factor is determined. Furthermore, although it is unlikely that fish embryos will ever be accepted as a pure replacement for fish in chronic toxicity testing, it is possible that they could be used, at least, as an indicator of chronic toxicity. Therefore, the use of fish embryos should be further investigated using the sub lethal endpoints suggested by Schulte and Nagel (1994). These data could then be used as part of an intelligent testing strategy to drive appropriate testing requirements, or could reduce the number of organisms required through the use of limit tests.

Nevertheless, the use of fish embryos in ecotoxicity testing is a promising tool, and from the work carried out in this thesis it would appear that the chorion is not a major barrier for simple chemicals, other than cationic polymers, to enter into zebrafish embryos. However, there is still a fundamental need for supplementary work to understand the function and properties of the pores across fish chorions to develop an understanding of how chemicals can pass through the chorion. Furthermore, if the use of fish embryos is to be considered as an alternative approach to more developed life-stages of fish, care should be taken to ensure that appropriate safety factors are considered.

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APPENDICES

Table 4.4. Uptake of [3-^{14}C]Fluoranthene into zebrafish embryos after 48 hour exposure

Embryo	Partition	sample (Bq)	Quantity (ng)
1	Chorion	0.995	0.121
	Embryo and perivitiline fluid	391	47.5
2	Chorion	18.1	2.20
	Embryo and perivitiline fluid	489	59.4
3	Chorion	33.1	4.02
	Embryo and perivitiline fluid	371	45.1
4	Chorion	42.4	5.15
	Embryo and perivitiline fluid	385	46.8
5	Chorion	1.85	0.225
	Embryo and perivitiline fluid	577	70.1
Mean (±SD)	Chorion	23.9 (18)	2.90 (2.2)
	Embryo and perivitiline fluid	443 (89)	53.8 (11)

Table 4.5. Uptake of [3-^{14}C]Estradiol into zebrafish embryos after 48 hour exposure

Embryo	Partition	sample (Bq)	Quantity (ng)
1	Chorion	23.1	3.27
	Embryo and perivitiline fluid	22.5	3.19
2	Chorion	0.86	0.122
	Embryo and perivitiline fluid	21.4	3.02
3	Chorion	0.96	0.136
	Embryo and perivitiline fluid	17.7	2.52
4	Chorion	0.31	0.0439
	Embryo and perivitiline fluid	18.4	2.60
Mean	Chorion	6.3 (11.2)	0.9 (1.6)
(±SD)	Embryo and perivitiline fluid	20.0 (2.3)	2.8 (0.3)

Table 4.6. Uptake of [3-^{14}C]Fluoranthene into zebrafish embryos after 24 and 48 hours

Sample number	24 hour exposure			Sample number	48 hour exposure		
	Partition	Sample (Bq)	Quantity (ng)		Partition	Sample (Bq)	Quantity (ng)
1	chorion	1.01	0.123	7	Embryo + perivitiline fluid	14.5	1.77
	Embryo + perivitiline fluid	17.1	2.08				
2	chorion	0.25	0.0304	8	Embryo + perivitiline fluid	18.9	2.30
	Embryo + perivitiline fluid	15.4	1.87				
3	chorion	2.24	0.272	9	Embryo + perivitiline fluid	21.6	2.62
	Embryo + perivitiline fluid	12.4	1.50				
4	chorion	0.67	0.0814	10	Embryo + perivitiline fluid	14.3	1.74
	Embryo + perivitiline fluid	16.6	2.01				
5	chorion	0.20	0.0243	-	-	-	-
	Embryo + perivitiline fluid	17.8	2.17				
6	chorion	2.73	0.332	-	-	-	-
	Embryo + perivitiline fluid	15.0	1.82				

24 hour mean (± SD) quantity of [3-^{14}C]Fluoranthene in the embryo plus perivitiline fluid = 1.91 (0.237)

48 hour mean (± SD) quantity of [3-^{14}C]Fluoranthene in the embryo plus perivitiline fluid = 2.11 (0.429)

Table 4.7. Uptake of [3-^{14}C]Fluoranthene into zebrafish embryos after 24 and 48 hour exposure

Embryo number	24 hours		Embryo number	48 hours	
	Sample (Bq)	Quantity (ng)		Sample (Bq)	Quantity (ng)
1	351	43	5	689	84$^{\#}$
2	419	51	6	520	63$^{\#}$
3	308	37	7	636$^{\Phi}$	77
4	416	51*	8	753$^{\Phi}$	92
-	-	-	9	668$^{\Phi}$	81
-	-	-	10	559$^{\Phi}$	68
-	-	-	11	618$^{\Phi}$	75
-	-	-	12	632	77$^{\#}$
-	-	-	13	597	72$^{\#}$
-	-	-	14	766$^{\Phi}$	93
-	-	-	15	562	68
-	-	-	16	618	75
-	-	-	17	580	70

*This data point (embryo number 4) was derived from the average of 7 embryos that were analysed as a group. The data was corrected for the presence of chorions in the sample by subtracting the mean quantity of [3-^{14}C]fluoranthene measured in the chorion of the other embryos (mean [±SD] =1.65 [±0.41] ng).

$^{\#}$Data corrected for the presence of chorions in the sample by subtracting the mean quantity of [3-^{14}C]fluoranthene that was measured previously in experiment 3 and in experiment 4 (mean[±SD] =3.90 [±4.58] ng).

$^{\Phi}$Data corrected as the samples were contaminated with the 2nd wash solution during the dissection process ([3-^{14}C]fluoranthene measured in 2nd wash =0.057 Bq).

Table 4.8. Uptake of [3-^{14}C]Fluoranthene inside zebrafish embryos after 24 hours

Embryo number	Quantity in run 1 (ng)	Embryo number	Quantity in run 2 (ng)
1	52	5	43
2	53	6	51
3	49	7	37
4	39	8	51
Average = 48		Average = 45	
Stdev = 6.2		Stdev = 6.5	

Table 4.9. Uptake of [4-^{14}C]Estradiol into zebrafish embryos after 24 and 48 hours exposure

Embryo number	24 hour exposure			Embryo number	48 hour exposure		
	Partition	Sample (Bq)	Quantity (ng)		Partition	Sample (Bq)	Quantity (ng)
1	chorion	1.26	0.178	7	chorion	0.77	0.109
	embryo	7.09	1.00		embryo	3.45	0.489
2	chorion	0.63	0.089	8	chorion	0.37	0.052
	embryo	8.07	1.14		embryo	2.48	0.351
3	chorion	0.85	0.120	9	chorion	10.4	1.48
	embryo	7.07	1.00		embryo	15.7	2.23
4	chorion	0.79	0.112	10	embryo	17.9	2.53
	embryo	5.36	0.759	11	chorion	1.26	0.178
5	chorion	0.25	0.035		embryo	16.9	2.39
	embryo	6.25	0.885	-	-	-	-
6	chorion	0.2	0.028	-	-	-	-
	embryo	7.41	1.05	-	-	-	-

Table 4.10. Uptake of [3-^{14}C]Fluoranthene into pre/untreated zebrafish embryos (24 h)

Embryo number	Embryos not pre treated			Embryo number	Embryos pre treated		
	Partition	Sample (Bq)	Quantity (ng)		Partition	Sample (Bq)	Quantity (ng)
1	chorion	1.10	0.13	10	chorion	1.87	0.23
	embryo	276	33.6		embryo	143	17.4
2	chorion	4.20	5.10	11	chorion	12.2	1.49
	embryo	201	24.4		embryo	222	26.9
3	chorion	30.3	3.68	12	chorion	23.4	2.84
	embryo	199	24.2		embryo	276	33.5
4	chorion	12.5	1.52	13	chorion	39.3	4.78
	embryo	234	28.5		embryo	293	35.6
5	chorion	12.6	1.53	14	chorion	8.05	0.98
	embryo	280	34.0		embryo	286	34.7
6	chorion	30.5	3.70	-	-	-	-
	embryo	282	34.2		-	-	-
7	chorion	3.23	0.39	-	-	-	-
	embryo	315	38.3		-	-	-
8	chorion	36.9	4.49	-	-	-	-
	embryo	351	42.7		-	-	-
9	chorion	36.5	4.43	-	-	-	-
	embryo	234	28.5		-	-	-

Table 4.11. Uptake of [4-^{14}C]Estradiol into pre/untreated zebrafish embryos (24 h)

Embryo number	Embryos pre treated			Embryo number	Embryos not pre treated		
	Partition	Sample (Bq)	Quantity (ng)		Partition	Sample (Bq)	Quantity (ng)
1	chorion	5.13	0.726	6	chorion	0.49	0.069
	embryo	9.03	1.278		embryo	7.56	1.070
2	chorion	1.81	0.256	7	chorion	0.46	0.065
	embryo	6.20	0.878		embryo	5.95	0.842
3	chorion	1.70	0.241	8	chorion	0.89	0.126
	embryo	6.74	0.954		embryo	7.27	1.029
4	chorion	0.39	0.055	9	chorion	0.17	0.024
	embryo	7.32	1.036		embryo	6.60	0.934
5	chorion	2.02	0.286	10	chorion	0.55	0.078
	embryo	5.69	0.806		embryo	6.52	0.923

Table 5.3. Percentage survivability of zebrafish embryos post Pronase exposure

2 minutes exposure (concentrations shown are g l^{-1})

Run no.	DWC	0.01	0.032	0.1	0.32	1.0
1	88	63	81	63	88	75
2	67	81	69	50	31	6
3	61	50	44	63	69	35
mean (±SD)	72 (±14)	65 (±16)	65 (±19)	59 (±8)	63 (±29)	39 (±35)

5 minutes exposure (concentrations shown are g l^{-1})

Run no.	DWC	0.01	0.032	0.1	0.32	1.0
1	88	63	63	75	25	50
2	67	75	75	19	6	0
3	61	81	67	59	29	67
mean (±SD)	72 (±14)	73 (±9)	68 (±6)	51 (±29)	20 (±12)	39 (±35)

10 minutes exposure (concentrations shown are g l^{-1})

Run no.	DWC	0.01	0.032	0.1	0.32	1.0
1	88	100	81	31	38	0
2	67	75	42	13	6	0
3	61	62	69	87	19	0
mean (±SD)	72 (±14)	79 (±19)	64 (±20)	44 (±39)	21 (±16)	0 (±0)

Table 5.4. Percentage survivability of zebrafish embryos post Saponin exposure

2 minutes exposure (concentrations shown are g l^{-1})

Run no.	DWC	0.01	0.032	0.1	0.32	1.0
1	91	44	38	75	81	0
2	90	81	81	88	6	0
3	59	75	56	69	29	0
mean (±SD)	80 (±18)	67 (±20)	58 (±22)	77 (±10)	39 (±38)	0

5 minutes exposure (concentrations shown are g l^{-1})

Run no.	DWC	0.01	0.032	0.1	0.32	1.0
1	91	38	88	88	6	0
2	90	75	88	81	13	0
3	59	76	87	69	0	0
mean (±SD)	80 (±18)	63 (±22)	88 (±1)	79 (±10)	6 (±7)	0

10 minutes exposure (concentrations shown are g l^{-1})

Run no.	DWC	0.01	0.032	0.1	0.32	1.0
1	91	63	25	19	0	0
2	90	81	77	63	0	0
3	59	81	94	44	0	0
mean (±SD)	80 (±18)	79 (±19)	64 (±20)	42 (±22)	0	0

Table 5.5. Total (chorion, embryo plus perivitiline fluid) [3-^{14}C]Estradiol (μg) measured in individual zebrafish embryos exposed for 24 hours

Control	Pre treated with Pronase	Pre treated with Saponin
1.29	0.97	1.09
1.20	0.98	1.24
1.07	0.99	0.53
1.11	0.97	1.03
1.13	1.01	0.97
1.06	0.96	1.29
1.13	0.97	1.16
1.09	0.94	1.04
1.06	0.93	1.14
-	-	1.22
-	-	0.95
-	-	0.98
-	-	1.08
-	-	1.01
Mean (±SD) 1.13 (±0.08)	Mean (±SD) 0.97 (±0.02)	Mean (±SD) 1.05 (±0.19)

- no sample

Table 6.1. Quantity of PEG (nM) measured in zebrafish embryo exposed at a concentration of 0.1 mM for 24 hours

Embryo	PEG400	PEG4000	PEG40000
1	0.022	0.034	0.099
2	0.000[a]	0.029	0.052
3	0.039	0.019	0.08
4	0.007	0.025	0.068
5	0.006	0.022	0.077
6	0.000[a]	0.024	0.075
7	0.000[a]	0.018	0.085
8	0.005	0.037	0.105
9	0.000[a]	0.022	0.102
10	0.000[a]	0.023	0.077
11	0.000[a]	0.018	-
Mean	0.007	0.025	0.082
SD	0.012	0.006	0.016

[a] Only calculated to be at the level of background radioactivity

Table 6.2. Quantity (nM) of radiolabelled PEG400 in zebrafish embryos after 24 h exposure

Quantity of PEG400 (control)	Quantity of PEG400 (pronase)	Quantity of PEG400 (saponin)
0.022	0.045	0.086
0.000	0.024	0.142
0.039	0.014	0.083
0.007	0.002	0.045
0.006	0.005	0.081
0.000	0.002	0.076
0.000	0.040	0.071
0.005	-	0.044
0.000	-	0.136
0.000	-	-
0.000	-	-
*0.007 (0.012)	*0.019 (0.018)	*0.085 (0.034)

*Expressed as mean data (±SD)

- no sample

Table 6.3. Quantity (nM) of radiolabelled PEG4000 in zebrafish embryos after 24 h exposure

Quantity of PEG4000 (control)	Quantity of PEG4000 (pronase)	Quantity of PEG4000 (saponin)
0.034	0.077	0.035
0.029	0.066	0.031
0.019	0.071	0.025
0.025	0.080	0.033
0.022	-	0.026
0.024	-	0.024
0.018	-	0.030
0.037	-	0.021
0.022	-	0.021
0.023	-	0.030
0.018	-	-
*0.025 (0.006)	*0.074 (0.006)	*0.027 (0.005)

*Expressed as mean data (±SD)

- no sample

Table 6.4. Quantity of radiolabelled PEG40000 in zebrafish embryos after 24 h exposure

Quantity of PEG 40000 (control)	Quantity of PEG 40000 (pronase)	Quantity of PEG 40000 (saponin)
0.099	0.068	0.089
0.052	0.092	0.130
0.080	0.105	0.108
0.068	0.083	0.078
0.077	0.071	0.116
0.075	0.106	0.078
0.085	-	0.091
0.105	-	0.108
0.102	-	0.110
0.077	-	0.115
*0.082 (0.016)	*0.088 (0.016)	*0.102 (0.017)