

An Investigation into the Effects of Chronic Radiation on Fish

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This report is intended to broaden scientific knowledge on the comparative damaging effects of long-term exposures to alpha- or gamma-radiation with regard to reproductive output in a representative fish. It aims to gain further understanding on Relative Biological Effectiveness RBE in fish. It will provide an input to the development of future radiological assessment systems and criteria, including those that are underway through the EU FP5 "FASSET" project.

Keywords

Environment, Wildlife, Radioactivity, Relative Biological Effect, RBE, Alpha-radiation, Gamma-radiation, Fish,

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FOREWORD

There is increasing recognition, nationally and internationally, that the environment *in its own right* should be protected from the possible damaging effects of radiation. In a previous Environment Agency (EA) report it was shown that, in principle at least, there is in the UK a statutory basis for the establishment of criteria and application of controls for protecting the environment from incremental radiation exposure arising from radioactive waste management (Woodhead 1998). A further report discussed the ways in which possible effects of radiation on the environment might be assessed (Woodhead 2000). These included the need for a set of reference organisms (covering a range of environments and lifestyles) for which dosimetric models are available or could be developed, so that absorbed radiation dose rates and their likelihood of causing damage could be assessed. Since November 2000 a European Commission funded project (Framework for assessment of environmental impact, FASSET) has been working to produce a harmonised framework for future radiation assessments within the EU. This project will not be completed until October 2003 and in the meantime a report has been produced which sets out interim measures which may be used to assess the impact of ionising radiation on wildlife in England and Wales (Copplestone *et al* 2001).

While many of the data necessary for the assessment of the environmental impact of radiation are available, there are some areas in which they are crucially lacking. One of these, which has been pointed out in, amongst others, all of the reports mentioned above, is a knowledge of the relative damaging effects of different radiation qualities. Environmental organisms maybe exposed to radiation of several different types (α -, β -, γ -radiation). In order to assess the overall damaging biological effects of these radiations it is necessary to obtain a combined dose rate which takes into account their different damaging effects. α -radiation is known to be much more damaging than γ -radiation in humans and other mammals but no data is available for other species. In order to supply some much-needed data for aquatic organisms, the work described in this report has been to compare the damaging effects of α - and γ -radiations on fish, an important aquatic vertebrate.

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EXECUTIVE SUMMARY

Public concern for the effects of radiation on the environment have given rise to a worldwide movement towards the development of environmental guidelines for radiation protection. In Europe this has led to the setting up of the EU-funded "Framework for the assessment of environmental impact project" (FASSET, <http://www.fasset.org>) in which the Environment Agency is a major participant. In order to develop any environmental guidelines for radiation protection it is necessary to be able to estimate dose rates received by organisms and to know at what dose rates significant damage is likely to occur. In the context of animals or plants in the environment it is, with a few exceptions, damage likely to have a deleterious effect on the maintenance of the population rather than the individual which is important. Thus effects on reproduction are particularly relevant.

In the environment, organisms receive absorbed radiation doses from a range of different radiation qualities (eg α -, β - or γ -radiation). While it is often possible to estimate physical dose rates for each radiation quality, the determination of their combined biological damaging effects requires that the degree to which some radiation qualities are more damaging than others is known. In man and mammals it is well known that α -radiation is much more effective than γ -radiation, on an equal absorbed dose basis, in producing tissue damage and a conversion factor allows conversion of α -radiation dose rates to γ -equivalent rates. Although there is a considerable amount of data available on the effects of radiation (mostly γ -radiation) on aquatic organisms, there is none which allows comparison of the damaging effects of α - and γ -radiation for relevant endpoints such as reproduction. Therefore the purpose of the work carried out in this project has been to provide data on the comparative damaging effects of long-term exposures to α - or γ -radiation on reproductive output in a representative fish. As all fish species appear to have similar radiosensitivities, a small tropical freshwater fish the zebrafish has been used because of its suitability for experimental work. Polonium-210 (^{210}Po) was used as the source of α -radiation as it is known to accumulate to a high degree in soft tissues including the gonads that were a prime target in this experiment.

Groups of zebrafish were exposed from an early age to different dose rates of γ -radiation or α -radiation. γ -radiation was delivered from sealed external sources. α -radiation was delivered by feeding the fish meals spiked with ^{210}Po . There were three dose rate groups of γ -irradiated fish, four groups of α -irradiated fish and a control group. All were treated in the same way except for the radiation dose rates. The γ -radiation dose rates were 300, 1000, and 7400 microgray per hour ($\mu\text{Gy/h}$). These were chosen, on the basis of previous studies, to span the effect / no effect level for impairment of reproductive output. α -radiation dose rates were intended to span this same effect / no effect level but as the relative damaging effect of α - and γ -radiation was not known (it was the purpose of the experiment to find it) values for mammalian species were used as a guideline and dose rates assuming a 10 to 40 times greater damaging effect were chosen. Thus zebrafish were fed ^{210}Po -spiked meals at concentrations preliminary experiments indicated would deliver dose rates of 8, 25, 185 and 740 $\mu\text{Gy/h}$. γ - and α - irradiation started when fish were old enough to take spiked food readily. When old enough to distinguish sexes, pairs of fish were made up in special breeding tanks where they were allowed to breed once per week. All eggs laid were counted and 24 hours later non-viable eggs (which had become opaque) were counted. About every 4 weeks viable eggs were kept and the number which developed and hatched was recorded.

Among γ -irradiated fish only those in the highest dose rate group (7400 μ Gy/h) showed radiation damage. The number of non-viable eggs laid was significantly higher than in controls and by 20 weeks after the first mating all pairs had permanently ceased laying eggs. Histological examination showed some testes in this group were devoid of sperm-making tissue and others appeared to have reduced amounts, while ovaries appeared normal. Among α -irradiated fish no groups showed evidence of radiation-induced reduction in egg production or significant histological damage. Although autoradiographs demonstrated that ^{210}Po was present in testis and ovary the ^{210}Po concentrations in the fish at the end of the experiment gave calculated dose rates of 9.6, 19, 84 and 214 μ Gy/h. Thus the higher two rates were much lower than the intended 185 and 740 μ Gy/h. Reasons for this are uncertain but it could be that radiation damage caused reduced ^{210}Po uptake. As the highest α -dose rate of 214 μ Gy/h produced no effect, comparison with the γ -radiation dose rate of 7400 μ Gy/h which caused egg production to cease gives an upper limit to the relative damaging biological effect of α -radiation compared with γ -radiation ($\text{RBE}_\alpha = \text{Dose rate of } \gamma\text{-radiation causing an effect} / \text{Dose rate of } \alpha\text{-radiation causing the same effect}$) of < 35 (ie 7400 / 214). While ovary and whole body activities were similar in the lower three α -dose rate groups, in the highest one they were variable and much higher than whole body giving calculated dose rates of 376-1045 μ Gy/h and thus RBE_α upper limits of <20 to <7 . It is suggested that the value of 20 may be more appropriate for reproductive effects. Use of the upper limit RBE_α of 35 to convert α -dose rates to γ -equivalent dose rates is thus very conservative but even when applied to some of the highest α -dose rates to environmental aquatic organisms in the literature it gives γ -equivalent dose rates which are unlikely to cause detrimental effects. No other RBE_α for effects on fish are available. Thus the RBE_α derived from the present work, will provide the best available estimate for use in the development of standards for environmental protection.

This is the first time a study of this sort has been undertaken and not suprisingly it has indicated several ways improvements may be made in the future. Most important is the need to use animals in numbers which allow regular sampling to monitor ^{210}Po concentrations throughout the experiment rather than just at its termination. This will require small animals which can be kept in large numbers with low husbandry requirements and endpoints for radiation damage which are not labour intensive to measure.

A test of DNA damage was evaluated on γ -irradiated zebrafish larvae and allowed detection of damage after as little as 1 hour exposure to a dose rate of 1200 μ Gy/h, a rate similar to that of 1000 μ Gy/h which had no effect on reproduction in adults after exposures of more than 12 months. While the degree to which DNA damage might be repaired over long periods of continuous irradiation and the relationship of such damage to any endpoints which might affect maintenance of the fish population is unknown, such tests would undoubtedly be useful additions in future studies where, for instance, the RBE_α for DNA damage and for reproductive impairment could be compared

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1. INTRODUCTION

At present there are no standards that specifically provide for the protection of the environment (as opposed to man) from the impact of incremental radiation exposure resulting from radioactive waste disposal practices. The requirement for assessments of the possible damaging effects of radiation on the environment (in its own right) is increasing due to growing public concern for environmental protection issues and the integration of environmental impact assessments into the regulatory process. In Europe the EU-funded Framework for the assessment of environmental impact (FASSET) project is working to provide a formalistic framework for assessment of radiation effects on biota and ecosystems while in some other countries such as the USA and Canada, moves to implement environmental standards for radiation are already well advanced.

The development of standards to protect the environment from radiation requires data on dose rates received by environmental organisms and the relationship between the absorbed radiation dose rates and damaging effects to the organisms. Although much data is already available for aquatic organisms (eg Woodhead 1984, UNSCEAR 1996) there are key areas where it is lacking. In order to assess combined, biologically effective radiation doses from all sources (α -, β - and γ -emitters; internal and external) comparative factors for the relative damaging effects of the different radiation qualities are needed. Although a small amount of data are available for comparative effects of β - and γ -radiations (Hyodo-Taguchi 1980) none is available for the relative damaging effects of α - and γ -radiation on aquatic organisms (Pentreath 1998). It is well known that α -radiation is much more effective than γ -radiation, on an equal absorbed dose basis, in producing tissue damage in man and mammals but while it seems certain that the same will be true for aquatic organisms, there are no data available on this at present for relevant end points such as reproductive capacity. Reproductive endpoints have been chosen as most relevant because it is generally accepted that the population is the object of protection in the environment rather than the individual animal, and reproductive capacity is obviously of prime importance in the maintenance of populations. Therefore the purpose of the work carried out in this project has been to provide data on the comparative damaging effects of long-term exposures to α - or γ -radiation on reproductive output in a representative fish.

1.1 Overview of the experimental design

The purpose of the experiment was to find the dose rates of γ - and α -radiation which produce the same damaging effect on reproductive output in experimental zebrafish. Comparison of these allows the determination of a relative biological effect (RBE_{α} = dose rate of γ -radiation / dose rate of α -radiation, for the same damaging effect). Groups of fish were each exposed from a young age to a different radiation dose rate for a period of over a year. When they were sexually mature, fish were kept in pairs and various parameters of reproductive output were measured weekly throughout. There were 4 dose rate groups of α -irradiated fish, 3 of γ -irradiated and an unirradiated control group. Dose rates were chosen to span the effect / no effect level for egg production. γ -irradiation was carried out with sealed ^{137}Cs sources in the Lowestoft radiation facility where dosimetry measurement of absorbed dose rates to the fish was relatively simple. α -radiation was achieved by feeding fish food spiked with the α -emitting radionuclide ^{210}Po . The dosimetry of α -radiation was thus more complicated and the relationships between spiked food activity, feeding rate to the fishes, body radionuclide concentration and α -radiation dose rate had to be determined in preliminary experiments. At

the end of the experiment the ^{210}Po concentrations in samples of fish from all α -radiation dose rate groups were determined so that actual experimental absorbed dose rates (as opposed to those predicted from the preliminary experiments) could be calculated. In addition to obtaining the data for reproductive output, histological examination of some fish from all groups, including controls, was carried out at the end of the experiment.

2. METHODS

2.1 Experimental fish

The original choice of experimental animal was a small, freshwater tropical fish the guppy (*Poecilia reticulata*). It is apparent from the literature that fish are amongst the most radiosensitive of the aquatic organisms for which information is currently available (Woodhead 1998, UNSCEAR 1996) and all species (freshwater or marine, tropical or temperate) appear to have similar radiosensitivities (Woodhead 1977, Hyodo Taguchi 1980, Knowles 1999). Thus the results obtained for guppies were likely to be relevant to most fish species. The guppy offered particular practical advantages: it is relatively small, which means that the maintenance is straightforward and, more importantly from the radiological protection perspective, relatively less α -activity is required to deliver the necessary range of dose rates; it is a live-bearer, which means that the difficulties and uncertainties arising from hatching eggs and rearing larvae are avoided; and an earlier experimental study with this species at this laboratory had investigated the effects of γ -radiation (Woodhead 1977) so that information and experience was available from which to develop the experimental procedures.

Unfortunately, in spite of previous successful studies with guppies, those entered into the present experiment persistently showed high mortalities, even in untreated fish. Monitoring and testing for factors likely to be responsible were carried out over a long period without successfully revealing the cause of fish deaths and thus allowing corrective measures to be taken. Eventually, following consultation with the funding bodies, the decision was made to use another experimental fish.

Another freshwater, tropical fish, the zebrafish, *Danio rerio* was chosen as the experimental animal. They are small and easily kept in the laboratory in relatively large numbers and offer most of the advantages of guppies described above. They have been widely used as an experimental animal and a large body of data is available on their husbandry and biology (eg. Westerfield 1995). In addition some previous experience of exposing these fish to γ -radiation at this laboratory was available for use in developing experimental procedures (Rackham, Woodhead and Knowles unpublished). Unlike guppies, zebrafish are continuous egg layers. However, the eggs are easily counted and kept until hatching. Thus, while more labour intensive than counting guppy offspring, the ability to count fertile and non-fertile eggs and the hatching rate offers more experimental data for analysis.

The zebrafish used in the experiment were the offspring of stock *wild type AB* fish supplied by the zebrafish facility at University College London. Details of husbandry and specially designed breeding tanks are given in Appendix A.

2.2 Exposure to α -radiation

The radionuclide Polonium-210 (^{210}Po) was chosen as the source of α -radiation. At first sight, it might appear that ^{241}Am or ^{239}Pu would be more directly relevant to environmental problems. It is the α -radiation dose rate to the specific organs and tissues that is of interest, however, and the radionuclide source of the α -radiation is of secondary concern provided that the radionuclide distributions are sufficiently well known to permit accurate dosimetric calculations. An additional problem relates to the fact that, although ^{241}Am and ^{239}Pu

contribute to the incremental radiation exposure in contaminated environments, neither is accumulated to any great degree into fish from either food or water. Relatively high concentrations would, therefore, have to be used in the experiments to obtain the necessary dose rates, with attendant problems of radiological protection of staff and potential contamination. ^{210}Po is well known to accumulate to a high degree in soft tissues including the gonads that were a prime target in this experiment (Carvalho 1988, Carvalho and Fowler 1994, Cherry *et al.* 1994). In addition, it is important in the environment both as a significant component of the natural background and as a contaminant from human activities (concentrated in wastes from phosphogypsum plants).

The delivery of radioactive ^{210}Po to zebrafish was carried out by feeding fish meals of spiked artemia larvae (brine shrimp). Details of the preparation of active meals are given in Appendix B. Preliminary experiments were carried out to determine the fraction of active meals ingested (entering the alimentary tract) and a mean of ~50% was obtained (Appendix C).

2.3 Exposure to γ -radiation

Zebrafish were exposed to γ -radiation from ^{137}Cs sealed sources in the CEFAS γ -radiation facility. Dose rates to tanks of experimental fish were determined using Lithium fluoride, thermoluminescent dosimeters (TLDs). Mean absorbed dose rates for each dose rate group of zebrafish were 7400, 1000 and 300 $\mu\text{Gy/h}$. On the basis of previous studies these dose rates of γ -radiation would be expected to cause marked effects on reproduction in fishes at the highest rate and little or no effect at the lowest rate (Woodhead 1977; Rackham and Woodhead 1984; Knowles, 1999). Further details of the radiation facility and dosimetry are given in Appendix D.

2.4 Estimation of feeding rates of α -radioactive food to zebrafish

The purpose of this experiment was to determine the relative damaging biological effects of α - and γ -radiation on reproduction in the zebrafish (RBE_α). However, some assumption about what it was likely to be had to be made in order to determine the experimental dose rates. Using primary oocyte survival in mice injected intraperitoneally with ^{210}Po as an endpoint, RBE_α as high as 50 - 90 were obtained at low estimated dose rates (Samuels 1966). Elsewhere an RBE_α of 20 has been used for biota, based on the radiation weighting factor of 20 recommended by the ICRP for use in radiation protection of humans (IAEA, 1992; Copplestone *et al.* 2000), though it has been suggested this value may be too high (Kocher & Trabalka 2000, Pentreath, 1998). Thus, in the present study RBE_α of 10 - 40 were used to obtain nominal dose rates of ^{210}Po α -radiation for the experimental groups of zebrafish. The dose rates of γ -radiation to be used were 300, 1000 and 7400 $\mu\text{Gy/h}$ (Section 2.3). The chosen dose rates of ^{210}Po α -radiation, were 8, 25 and 185 $\mu\text{Gy/h}$ (calculated using an RBE_α of 40) with an additional group irradiated at 740 $\mu\text{Gy/h}$, (based on an RBE_α of 10, Table 2.1).

If it is assumed that a ^{210}Po concentration steady-state exists and the isotope is distributed uniformly in the tissue, then the α -radiation dose rate from ^{210}Po is dependent on its concentration in the tissue and can be derived from

$$D_\alpha(\infty) = 5.76 \times 10^{-7} \times \bar{E}_\alpha \times C$$

where

$D_{\alpha}(\infty)$ is the α -radiation dose rate to the tissue in $\mu\text{Gy/h}$

^{210}Po $\bar{E}_{\alpha} = 5.4 \text{ MeV}$

C = tissue concentration of ^{210}Po in Bq/g

thus

$$D_{\alpha}(\infty) = 3.11 \mu\text{Gy/h} (\text{Bq/g})^{-1} \quad \text{Equation 1}$$

Using this relationship the tissue concentrations of ^{210}Po necessary to deliver the required α -radiation dose rates were calculated (Table 2.1). Estimates of the rate of feeding of ^{210}Po -spiked meals necessary to produce these concentrations in each group were obtained from pilot experiments, (described in Appendix F). They are given in Table 2.1.

It is important to remember that unlike γ -radiation dose rates to fish, ^{210}Po tissue concentrations and thus α -radiation dose rates could not be measured during the main experiment. They were measured at the end of the experiment (Section 3.1) and it is the dose rates derived from these terminal activity concentrations which represent most closely the actual dose rates received by experimental animals during the experiment and which must be used in any determination of RBE_{α} .

Table 2.1 The dose rates received by γ -irradiated zebrafish and the estimated α -radiation dose rates to be received along with tissue ^{210}Po concentrations and α -radiation meal activities required to deliver them.

Experimental Group	1	2	3	4
γ -radiation dose rate ($\mu\text{Gy/h}$)	300	1000	7400	
Estimated α -radiation dose rate. ($\mu\text{Gy/h}$)	8	25	185	740
Estimated tissue concentration of ^{210}Po required (Bq/g)	2.7	8	62	248
Estimated activity of ^{210}Po per meal (meals given 2 x weekly), (Bq/g body wt of fish)	7	20	155	620

2.5 Main Experiment: Long-term exposure of zebrafish to α -or γ -radiation

All fish entered for the main experiment were raised from eggs laid by stock zebrafish kept at Lowestoft. A more detailed description of the procedures used in setting up this experiment is given in Appendix G. After hatching the fry were raised on special fry food and then at 12 weeks of age, by which time all fish were taking adult fish flake and artemia as food, the irradiations were started. Fish in α -radiation groups commenced twice weekly feeding of active food at rates based on those given in Table 2.1 while those in γ -radiation groups were placed in the γ -radiation facility and continuous irradiation was started. After a further 10 weeks it was possible to determine the sex of fish from external features and male/female pairs were placed in tanks modified for breeding and egg collection (see Appendix A). Control fish were treated in exactly the same way as irradiated fish but received no ^{210}Po activity or exposure to γ -radiation. The first time male/female pairs of fish were allowed to mate was recorded as Lay opportunity 1. Eggs were collected after each lay opportunity and

counted using a stereomicroscope (Total eggs laid). After a further 24 hours when eggs which were not fertilised had become opaque and easily distinguished they were counted (Non-viable eggs). At approximately monthly intervals (every 4th lay opportunity) viable eggs were kept and the number of them hatching successfully was recorded. At termination of the experiment samples of zebrafish from all α -irradiated groups were analysed for ^{210}Po concentrations in their tissues. Whole fish, or gonads and body separately, were digested in acid and ^{210}Po activity counted by scintillation counting or a silicon surface barrier detector (Appendix E).

Zebrafish in γ -radiation Group 3 became functionally sterile and produced no eggs (Section 3.2). In a small additional experiment some irradiated females from non-laying pairs in Group 3 were paired with unirradiated males and irradiated males paired with unirradiated females. They were allowed 4-6 consecutive days together and checked daily for the total number of eggs and number of non-viable eggs laid.

2.6 Autoradiography and histology

In order to determine the distribution of ^{210}Po activity at the histological level and be sure that it was reaching the gonads, some fish which had been given activity but not used in making pairs for the main experiment were killed and processed for autoradiography. In addition samples of fish from all groups (α -irradiated, γ -irradiated and controls) were taken at termination of the experiment and processed for histology and autoradiography (α -irradiated only). In this way any radiation damage could be examined at the histological level and compared with controls and the distribution of α -activity could be examined in those fish fed ^{210}Po chronically throughout the main experiment.

Whole fish, or in a few cases gonads only, were fixed in formalin, decalcified and and processed for embedding in wax or plastic. Longitudinal sections were cut (5-7 μm) at 2-3 levels within the body and placed on slides. Sections for histology only were then stained with haematoxylin and eosin. Sections for autoradiography were coated in photographic emulsion (LM1, Kodak) and left in the dark in the refrigerator ($\sim 4^\circ\text{C}$) for several weeks to expose. The emulsion was then developed and the sections stained with haematoxylin and eosin.

2.7 Determination of DNA damage using the COMET assay

The main purpose of this project was to compare the effects of chronic radiation exposure to α - and γ -radiation on reproductive output. While the importance of this endpoint for fish populations is clear, the ability to detect more subtle changes after much shorter exposures to the same low dose rates may also be a useful tool in predicting possible damaging effects on reproduction or other important endpoints in the longer term. During this project the opportunity arose to evaluate a technique which allows such early changes to be detected. The COMET or single-cell gel electrophoresis assay allows early detection of single strand DNA breaks which can be produced by a range of genotoxic agents including radiation (Singh *et al.* 1995, Mayalpa *et al.* 1998).

This assay was evaluated using larval zebrafish exposed to γ -radiation only for relatively short periods. Larvae (5-6 d post laying, 2d post hatching) were exposed to γ -radiation for 24 hours at 400, 1200 or 7200 $\mu\text{Gy/h}$ and for only 1 hour at 400 or 1200 $\mu\text{Gy/h}$. Individual larvae were then macerated and their cells embedded in agarose gel on microscope slides. After

lysis to remove cell and nuclear membranes electrophoresis was carried out and the DNA then stained with ethidium bromide for visualisation under fluorescent microscopy. DNA damage (strand breaks) allows the migration of DNA strands and loops under electrophoresis and these appear as comet-like tails emerging from the nuclear DNA mass when viewed under the microscope. The size of the comet tail is dependent on the amount of DNA damage. Images of comets were captured with a low-light camera and a software package used to assess the amount of damage. The % DNA in the tail and the tail moment (%DNA in tail x length of tail) were used as parameters but as results were similar for both only results for %DNA in the tail are presented. Each irradiation and control group consisted of 5 zebrafish larvae and 50 comets were measured for each larva. Full details are given in a paper recently submitted for publication (Jarvis and Knowles, 2002)

3. RESULTS AND DISCUSSION

3.1 Tissue concentrations of ^{210}Po and α -radiation dose rates

The mean whole body (including ovary) and ovary alone ^{210}Po activity concentrations of some zebrafish not used for analysis of reproductive output and killed after 17 -23 weeks of spiked meals are shown in Table 3.1. Those for experimental fish at termination of the experiment are given in Table 3.2. The very small size of the testis in zebrafish (~1-3mg) meant that measurement of weights and ^{210}Po activity were prone to relatively high statistical uncertainties, compounded when activities per gram wet weight were calculated. For these reasons, testes activities are not presented, and as a best estimate they are assumed to be the same as for whole body. At 17-23 weeks whole body and ovary concentrations were similar within each group (Table 3.1) and for Groups 1-3 the calculated dose rates corresponded well to those expected from preliminary experiments and modelling (11, 16, and 186 $\mu\text{Gy/h}$ cf 8, 25, and 185 $\mu\text{Gy/h}$). However, in Group 4 the mean ^{210}Po concentration and dose rate were lower than expected (409 cf 740 $\mu\text{Gy/h}$). At the termination of the experiment (Table 3.2), while Groups 1 and 2 had ^{210}Po concentrations and dose rates similar to those expected (9.6 and 19 $\mu\text{Gy/h}$ cf 8 and 25 $\mu\text{Gy/h}$), Groups 3 and 4 both had concentrations and dose rates considerably lower than expected. The dose rate of 84 $\mu\text{Gy/h}$ in Group 3 is only 45% of the predicted 185 $\mu\text{Gy/h}$ while in Group 4 the rate of 214 $\mu\text{Gy/h}$ is only 29% of the predicted 740 $\mu\text{Gy/h}$. In Group 4 the ovary concentration of ^{210}Po showed great variation, the three ovaries analysed containing 336,187 and 121 Bq/g.

The reason why ^{210}Po concentration and dose rates in Groups 1-3 should initially correspond closely to those predicted (Table 3.1), and then subsequently those in Groups 3 and 4 should be so much lower (Table 3.2), is uncertain. The most likely reason for this would seem to be either that over a long period of exposure the excretion rate of ^{210}Po was stimulated to increase, or the rate of uptake of activity from the gut decreased. Neither possibility could have been foreseen by the model used to predict the relationship between feeding rate and tissue concentration of ^{210}Po , a model based on relatively short term preliminary experiments. The gut lining came into direct contact with active food ingested and autoradiography showed relatively high concentrations of α -tracks over it (Figure 3.1). Therefore dose rates to it must have been much greater than to other tissues and it seems possible that chronic radiation damage could have led to decreased ^{210}Po uptake. However, histological examination of the alimentary canal failed to show any evidence of any radiation damage such as ulceration and loss of the mucosa or fibrosis of the gut wall (Figure 3.1).

As analysis of ^{210}Po activity requires sacrifice of the fish it was not possible to monitor it during the experiment. However, the concentrations measured in fish at termination of the experiment offer the best estimate of those experienced by fish during the experimental period and it is the dose rates calculated from these (Table 3.2) which must be used when comparing α -radiation effects with those produced by γ -radiation.

Table 3.1 The activity concentrations of ^{210}Po in whole body and ovaries of zebrafish killed after receiving spiked meals for periods of 17- 23 weeks

α -radiation group	Nominal radiation dose rate $\mu\text{Gy/h}$	No. whole fish analysed (No. ovaries)	Mean whole body activity ($\pm st.dev.$) Bq/g wet wt	Mean ovary activity ($\pm st.dev.$) Bq/g wet wt	Radiation dose rate (from whole body activity) $\mu\text{Gy/h}$
1	8	11 (9)	3.5 ± 1.4	5.8 ± 3.8	11
2	25	6 (6)	5.2 ± 1.2	4.5 ± 1.7	16
3	185	5 (5)	60.1 ± 4.8	64.4 ± 36.0	186
4	740	4 (1)	131.0 ± 29.6	107.7	409

Table 3.2 The activity concentrations of ^{210}Po in whole body and ovaries of zebrafish killed at termination of the experiment after receiving spiked meals for 70-76 weeks (Group 4 for 51 weeks)

α -radiation group	Nominal radiation dose rate $\mu\text{Gy/h}$	No. whole fish analysed (No. ovaries)	Mean whole body activity ($\pm st.dev.$) Bq/g wet wt	Mean ovary activity ($\pm st.dev.$) Bq/g wet wt	Radiation dose rate (from whole body activity) $\mu\text{Gy/h}$
1	8	9 (3)	3.11 ± 1.63	2.14 ± 0.12	9.6
2	25	8 (4)	6.21 ± 1.67	6.17 ± 3.25	19
3	185	13 (5)	27.08 ± 4.42	39.85 ± 16.85	84
4	740	7 (3)	68.9 ± 13.45	$214.4^a \pm 110.36$	214

^aActual values 336,187 and 121 Bq/g

In zebrafish, ^{210}Po concentrations in the ovary showed more variation than those measured for whole fish but for Groups 1-3 mean values were similar (Tables 3.1 and 3.2). Only in Group 4 which received the greatest amounts of ^{210}Po were ovary concentrations considerably higher than for whole body (Table 3). In a selection of epi- and mesopelagic fish, concentrations of ^{210}Po in different organs varied considerably, with gut/liver and gonads having highest levels and skeleton and muscle the lowest (Carvalho 1988). In other bathypelagic and demersal fish, concentrations were much more uniform, though again muscle contained least activity, a finding in agreement with zebrafish where autoradiographs showed fewest tracks over muscle.



Figure 3.1 Autoradiograph showing the undamaged gutwall of an α -irradiation group 4 (214 μ Gy/h) zebrafish. A large number of α -tracks are seen over the gut mucosa (M) and a few are also seen over the contents in the gut lumen (C) and the liver (L).

At the microscopic level, autoradiographs clearly indicated that ^{210}Po does reach germinal cells in ovary and testis, and that dose rates to the whole organ represent reasonable estimates of those to the cells. In the testis, autoradiographs showed that ^{210}Po activity was distributed uniformly, and tracks were seen over all cell types (Figure 3.2). Single tracks had a range of only about one cell diameter for spermatogonia, while for the much smaller sperm and spermatids a single track could overlay 6 or more, with spermatocytes intermediate. In the ovary, tracks were seen over all stages of oocyte development but there were notable, occasional collections of tracks over areas where phagocytosis, possibly of an atretic oocyte, was occurring (Figure 3.3). It was not possible to infer that such radiation was implicated as the cause of phagocytotic activity (eg by killing an oocyte) or there as a result of it. In mammals, ^{210}Po concentrates in the follicle cells of the ovary but oocytes are easily within range and irradiated by the α -radiation, and tracks are seen over oocyte nuclei (Samuels 1966, Finkel *et al.* 1953).

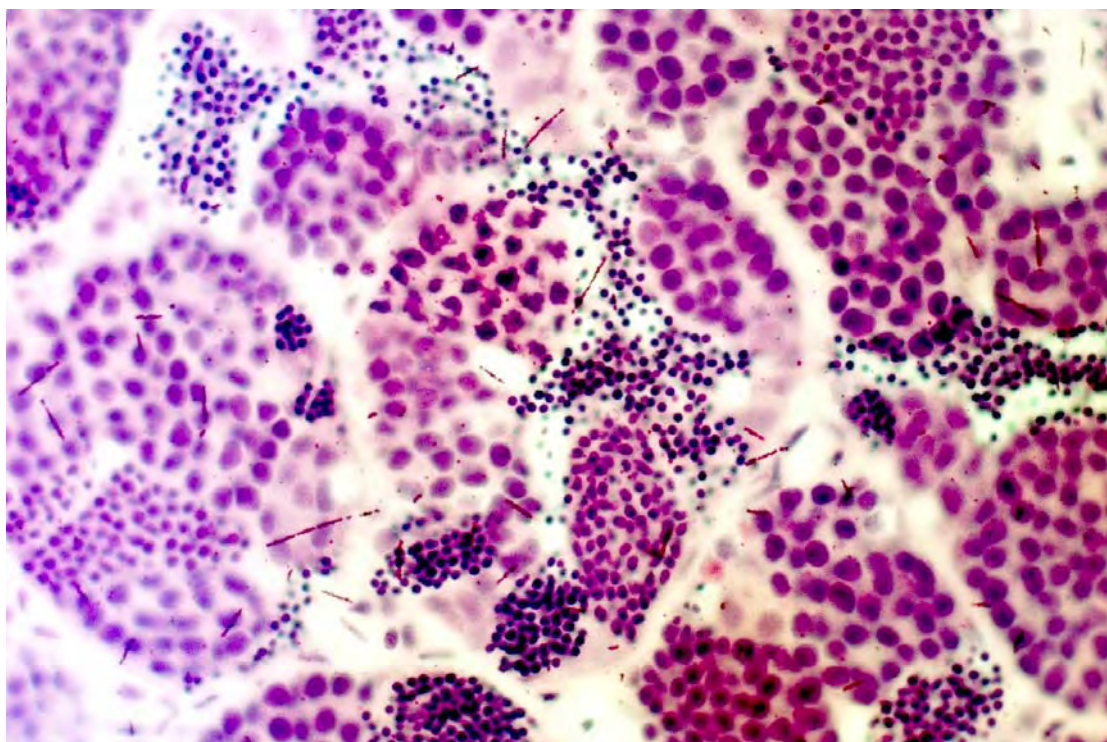


Figure 3.2 Autoradiograph of testis showing α -tracks spread randomly over all cell types. Tracks are more easily seen over paler areas of cyst walls and spermatocytes than over more darkly staining sperm and spermatidia.

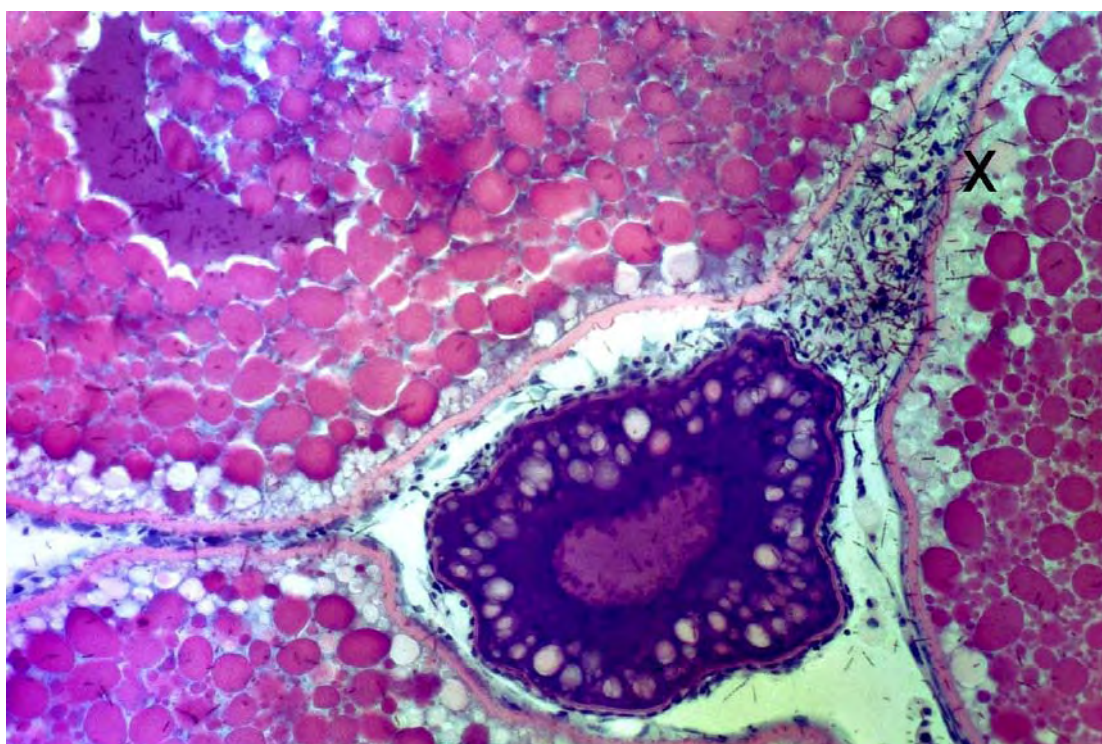


Figure 3.3 Autoradiograph of ovary showing a very high concentration of α -tracks over an area which shows evidence of inflammatory, phagocytic activity (X). Oocytes adjacent to this area also have increased α -tracks over them.

3.2 Egg production by irradiated zebrafish

The mean number of all - eggs per lay opportunity (ie total number of eggs including non-viable / total number of lay opportunities including those when no eggs were laid) was computed for each pair of zebrafish and the mean of these for each group obtained. In exactly the same way the mean number of viable eggs (ie those viable at 24 hours) per lay opportunity was computed for each pair and then the mean of these for each group obtained. Similarly, means of all - eggs per used lay opportunity (ie lay opportunities where no eggs were laid were excluded from the analysis) and viable eggs per used lay opportunity were calculated. Statistical tests of significance were all at $P < 0.05$. Results for mean all-eggs per opportunity and viable eggs per opportunity are shown in Figure 3.4, and results for mean all-eggs per used opportunity and viable eggs per used opportunity are shown in Figure 3.5.

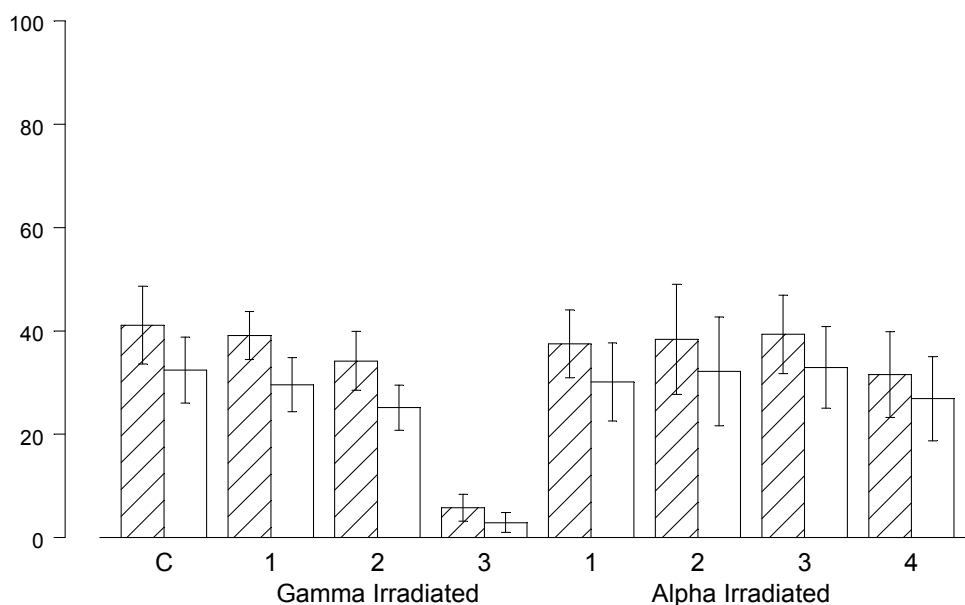


Figure 3.4 The mean number of all-eggs (viable and non viable, shaded columns) or viable eggs only (empty columns) per lay opportunity (including those where no eggs were laid). Error bars are 95% confidence levels

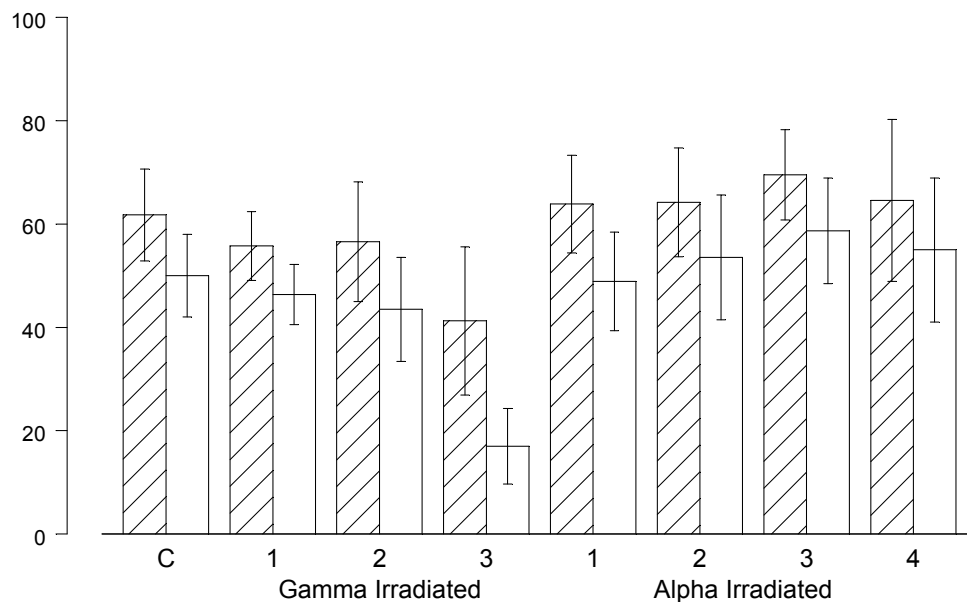


Figure 3.5 The mean number of all-eggs (viable and non viable, shaded columns) or viable eggs only (empty columns) per used lay opportunity (excluding those where no eggs were laid). Error bars are 95% confidence level

The values plotted in these graphs and number of pairs contributing to the means are shown in Tables 3.3 - 3.6. The results for all - eggs and viable eggs per lay opportunity (including those where no eggs were laid. Figure 3.4, Tables 3.3 and 3.4) both showed very significantly lower numbers per lay opportunity in γ -radiation Group 3 than in all others including controls. There were no significant differences between any other groups. The reason for the low values in γ -radiation Group 3 is that, by lay opportunity 20, all pairs had ceased laying, with the exception of a single lay by one pair at week 33 when all eggs laid were non-viable. The mean numbers of all-eggs and viable eggs per used lay opportunity (excluding opportunities where no eggs were laid) were also significantly lower in γ -radiation Group 3 (Figure 3.5, Tables 3.5 and 3.6) than in all others. The reduction in all-eggs was less marked than for viable eggs indicating that in γ radiation group 3, when egg laying did occur smaller clutches were produced and they had a decreased proportion of viable eggs.

Table 3.3 The mean number of all-eggs (viable and non-viable) laid per lay opportunity (including those where no eggs were laid)

Group	Number of pairs	Mean all-eggs per lay opportunity
Control	11	41.1
<i>γ-radiation</i>		
1	10	39.1
2	8	34.2
3	12	5.8 ^a
<i>α-radiation</i>		
1	12	37.5
2	11	38.4
3	13	39.3
4	10	31.6

^athis value is significantly different from all others

Table 3.4 The mean number of viable eggs laid per lay opportunity (including those where no eggs were laid)

Group	Number of pairs	Mean viable eggs per lay opportunity
Control	11	32.4
<i>γ-radiation</i>		
1	10	29.6
2	8	25.2
3	12	2.9 ^a
<i>α-radiation</i>		
1	12	30.1
2	11	32.2
3	13	33.0
4	10	26.9

^athis value is significantly different from all others

Table 3.5 The mean number of all-eggs (viable and non-viable) laid per used lay opportunity (excluding those where no eggs were laid)

Group	Number of pairs	Mean all-eggs per used lay opportunity
Control	11	61.8
<i>γ-radiation</i>		
1	10	55.8
2	8	56.6
3	12	41.3 ^a
<i>α-radiation</i>		
1	12	63.9
2	11	64.2
3	13	69.5
4	10	64.6

^athis value is significantly different from all others

Table 3.6 The mean number of viable eggs laid per used lay opportunity (excluding those where no eggs were laid)

Group	Number of pairs	Mean viable eggs per used lay opportunity
Control	11	50.0
<i>γ-radiation</i>		
1	10	46.4
2	8	43.5
3	12	16.9 ^a
<i>α-radiation</i>		
1	12	48.9
2	11	53.5
3	13	58.7
4	10	55.0

^athis value is significantly different from all others

The mean number of viable eggs per lay opportunity (including those unused) in γ -irradiated zebrafish are plotted against dose rate (on a logarithmic scale) in Figure 3.6. These values indicate the full effect of radiation on reproductive output (ie decreased numbers of viable eggs and of opportunities used due to cessation of egg laying). Dose- response relationships for biological effects often show a sigma-shaped curve and such a curve was the *a priori* expectation for zebrafish reproductive output. The fitted line is a logistic regression drawn on the basis of the data points and this expectation. From this it is apparent that significant radiation-induced reduction of viable egg production by zebrafish is unlikely to occur at radiation dose rates less than $\sim 400\mu\text{Gy/h}$. This dose rate is also given by UNSCEAR (1996) as the guideline rate below which detrimental effects on aquatic organisms are unlikely to occur.

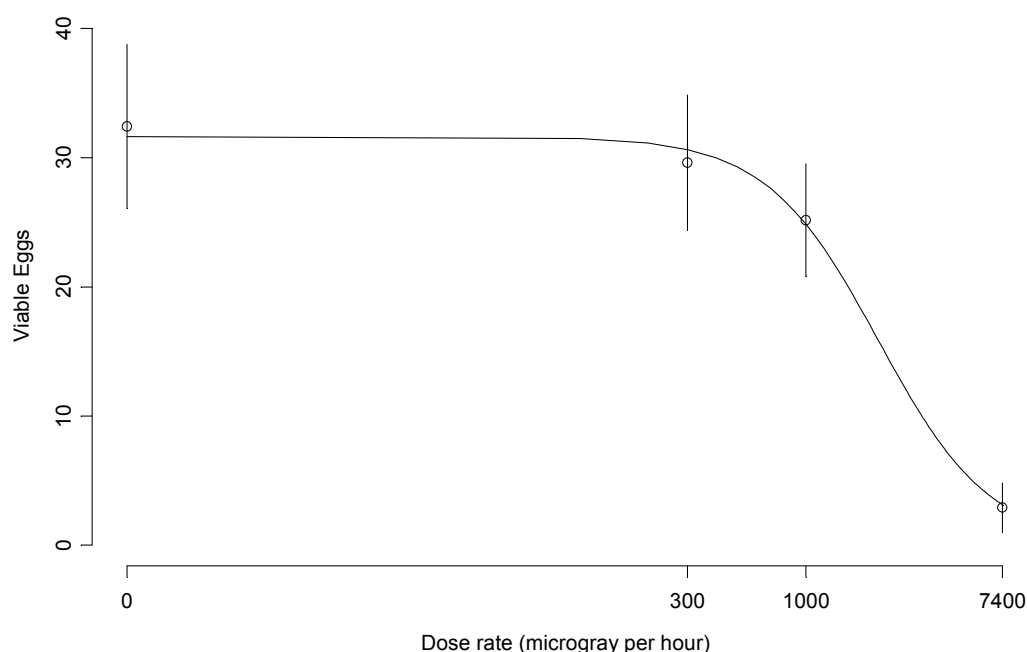


Figure 3.6 Dose - rate response curve for γ -irradiated zebrafish. Mean viable eggs per lay opportunity (including those unused) are plotted against dose rate (on a logarithmic scale). Error bars are 95% confidence limits. The fitted curve is a logistic regression drawn for illustration (see text)

The percentage of viable eggs (at 24 hours) which went on to develop and hatch was high in all groups (Table 3.7). The lowest hatch rate was in γ -irradiation group 3, the group in which all pairs ceased egg-laying (functional sterility). However even in this group the mean percentage of eggs which were viable at 24 hours post-lay which went on to hatch was 86%, not significantly different from controls. The early onset of functional sterility among pairs in this group meant that the number of clutches of eggs which were examined for hatchability was low and this is in part responsible for the relatively large standard deviation in this group.

Shortly before termination of the experiment, some female fish from γ -radiation Group 3 which had ceased producing any eggs for at least 20 weeks were paired with unirradiated males and their irradiated male mates were paired with unirradiated females. Some irradiated females did lay when paired with unirradiated male fish (Table 3.8). However, all the eggs that were laid were non-fertile. This must have been due to the fact that either the eggs failed to be fertilised (ie no sperm was released by the males or no sperm entered the egg), or that, even when fertilised by healthy sperm, the eggs were unable to develop and died within 24 hours. It is remarkable that even though they were non-fertile, eggs released when the females were paired with unirradiated males were the first to be laid for more than 20 weeks. This suggests some stimulation to lay eggs was provided by unirradiated male fish but not irradiated ones. Many of the paired irradiated male fish and unirradiated females produced eggs and although a high percentage were sterile some were fertilised and viable at 24 hours (Table 3.9). Viability was not examined at any later times. Thus at least some irradiated male fish were able to stimulate unirradiated females to lay eggs and to produce sperm capable of fertilising them.

Table 3.7 The mean number of viable eggs hatching successfully

Group	Dose rate (μ Gy/h)	Mean % of viable eggs hatching
Controls		98.0 $\pm$ 8.5
<i>α-radiation</i>		
1	9.6	96.3 $\pm$ 12.3
2	19	94.1 $\pm$ 12.9
3	84	98.4 $\pm$ 3.2
4	214	99.4 $\pm$ 2.3
<i>γ-radiation</i>		
1	300	98.2 $\pm$ 3.7
2	1000	93.4 $\pm$ 19.1
3	7400	86.0 $\pm$ 19.4

Table 3.8 Eggs laid when female zebrafish in γ -radiation Group 3 (7400 μ Gy/h) were mated with unirradiated male fish.

Lay opportunity	No. fish pairs	No. of pairs ^a laying	Total No. Eggs laid	Eggs sterile at 24 hours
1	5	2	72	72
2	5	0	0	
3	5	3	48	48
4	5	0	0	
5	5	2	40	40
6	5	1	16	16

^aTwo pairs failed to lay at any opportunity.

Table 3.9 Eggs laid when male zebrafish in γ -radiation Group 3 (7400 μ Gy/h) were mated with unirradiated female fish.

Lay opportunity	No.fish pairs	No.pairs ^a laying	Total Eggs laid	Eggs sterile at 24 hours	% sterile
1	10	5	321	253	79
2	10	5	104	84	81
3	10	3	112	76	68
4	10	3	36	9	25
5	5	3	40	21	53

^aThree pairs of fish did not lay at any opportunity.

3.3 Histological effects of radiation

In control zebrafish the testes exhibited all germinal cell types (spermatogonia, spermatocytes, spermatids and sperm). The relative proportion of the testis occupied by the cell types varied between individuals but generally spermatogonia were sparse while sperm and spermatidia were most numerous and spermatocytes were intermediate (Figure 3.7). Most control ovaries showed only very few examples of atresia (egg degeneration and loss) or any other pathological change and all stages of egg development from early perinucleolus stage oocytes through to mature yolky eggs were present. However, a few ovaries showed degenerative changes with atresia, accumulations of chorionic material and release of yolky material (eosinophilic fluid) into the ovarian cavity (Figures 3.8, 3.9). It seems likely these represent normal changes in zebrafish, possibly related to their continuous spawning at short intervals.

The findings for reproductive output (Section 3.2) were supported by the histological finding that only in γ -radiation Group 3 which showed severe effects on egg laying was there evidence of significant damage. Several testes contained no spermatogenic tissue with only the structure of outer lining and cyst walls remaining (Figure 3.10), while others did contain all spermatogenic cell types but in numbers which seemed to be depleted compared with controls. Perhaps surprisingly, most ovaries in the highest γ -dose rate Group 3 showed only moderate changes including a small area of atresia associated with a mass of stromal tissue (Figure 3.11) and other changes similar to those described for control fish. However, the ovary in one fish in this group did show marked damage with a large granuloma present,

indicating chronic degeneration and inflammatory reaction (Figure 3.12). Similar changes were seen in ovaries from one female in each of α -irradiated Groups 1 and 2, groups which received the lowest dose rates. In these, there was damage including granuloma formation and more widespread degeneration of yolky cells than seen in controls (Figure 3.13). No such changes were observed in other fish in these groups or in α -radiation Groups 3 and 4 which received higher α -dose rates. This suggests the changes in the ovaries, though marked, were probably normal in zebrafish, and, like the less severe changes observed in controls, they were possibly related to their continuous spawning at short intervals.

Testes in all α -irradiated zebrafish and γ -irradiation Groups 1 and 2 showed no notable changes in their testes. All spermatogenic cell types were present and in proportions similar to those observed in controls. The exception was one fish in γ -radiation Group 2 which exhibited ovotestis, with an oocyte present among the spermatogenic cells (Figure 3.14). This single example of ovotestis among all fish in the experiment was thought unlikely to be radiation-related.

The partial or total depletion of spermatogenic cells in γ -radiation Group 3 which received 7400 μ Gy/h is in agreement with findings for *Ameioba splendens* exposed chronically to a similar dose rate of 7300 μ Gy/h γ -radiation (Rackham and Woodhead 1984) and guppies exposed to 12,300 μ Gy/h γ -radiation (Woodhead 1977). The general lack of any significant histological damage in ovaries of fish in γ -radiation Group 3 (7400 μ Gy/h) contrasts with *Ameioba splendens* (7300 μ Gy/h) where all but large yolky oocytes were lost and guppies (12,300 μ Gy/h) where ovaries became devoid of all germ cells.

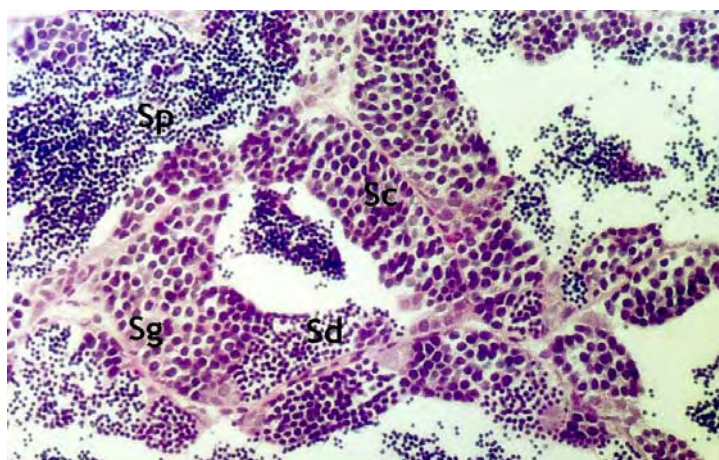


Figure 3.7 Control testis. Cysts contain groups of spermatocytes (Sc), and spermatids (Sd). Numerous sperm (Sp) are present in sinuses between cysts. A small number of large, pale spermatogonia (Sg) are present.

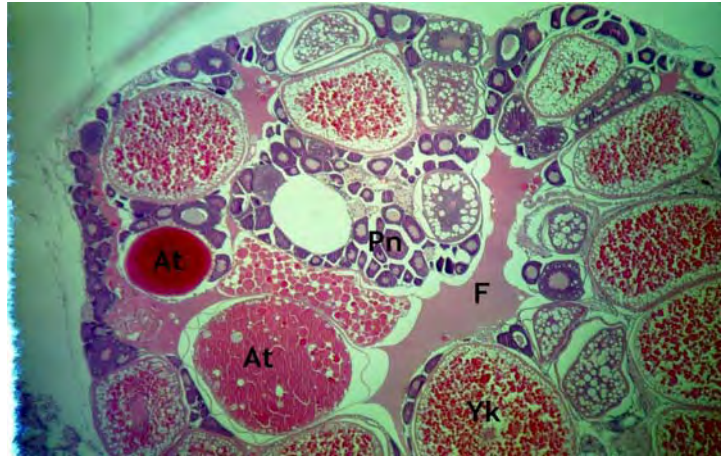


Figure 3.8 Control ovary. All stages of oocyte from early perinucleolar stage (Pn) to mature, yolky eggs (Yk) are present. Some eggs appear to be atretic (At) and there are areas of eosinophilic fluid (F).

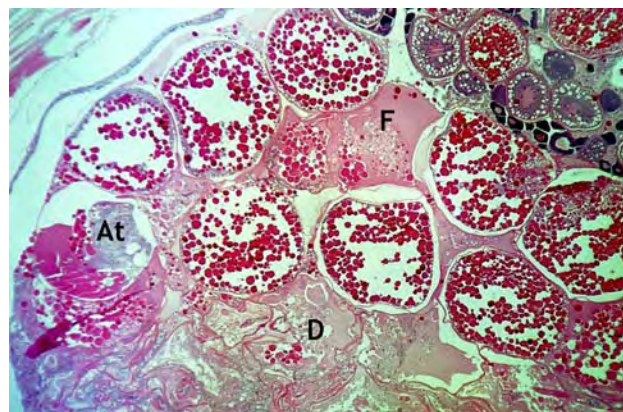


Figure 3.9 Control ovary showing more extensive degeneration. Atretic oocytes (At) and eosinophilic fluid (F) are present and there is an area of degenerate material (D) in which remains of egg and membranous structures (vitelline membrane, chorion) can be made out.

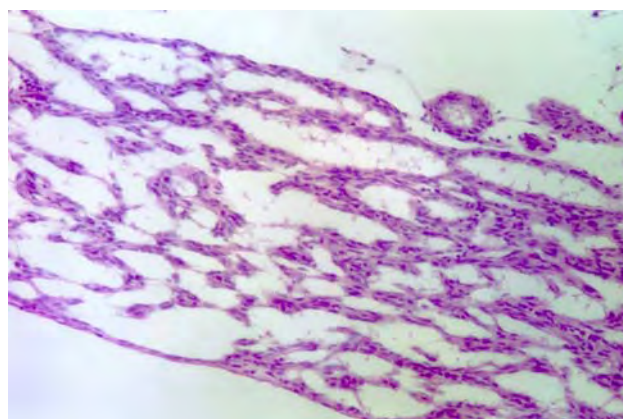


Figure 3.10 Testis from a γ -radiation Group 3 (7400 μ Gy/h) zebrafish. No spermatogenic tissue is present and only empty cysts remain.

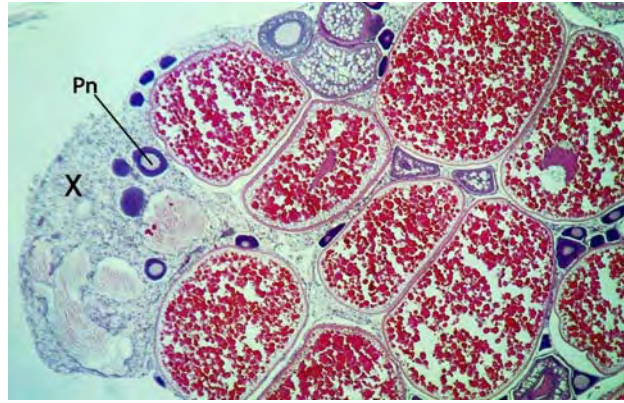


Figure 3.11 Ovary from a γ -radiation Group 3 (7400 μ Gy/h) zebrafish. Most of the ovary is normal. A small area of stromal tissue (X) contains remains of atretic oocytes as well as apparently normal perinucleolar stage oocytes(Pn).

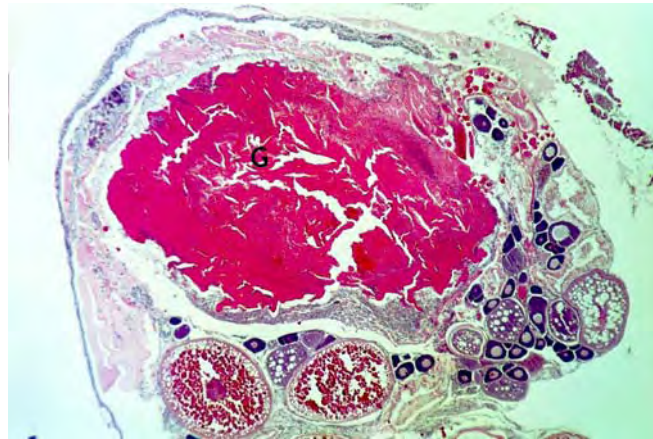


Figure 3.12 Ovary from a γ -radiation Group 3 (7400 μ Gy/h) zebrafish. A large granuloma (G) contains degenerate material (almost certainly oocyte). Adjacent oocytes appear normal.

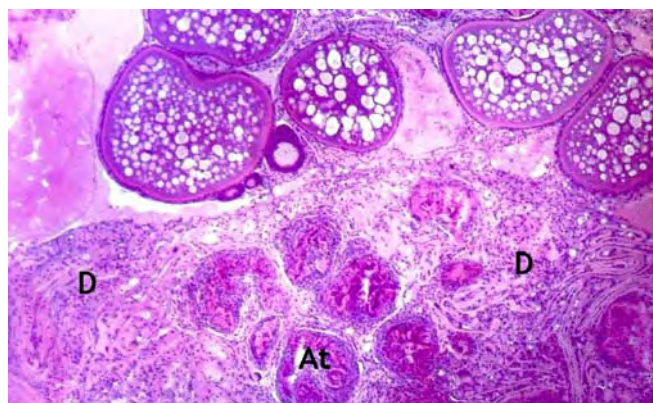


Figure 3.13 Ovary from an α -radiation Group 1 (9.6 μ Gy/h) zebrafish. Remains of degenerating oocytes are seen within a mass of tissue (D) containing inflammatory cells and membranous structures, probably remains of chorion.

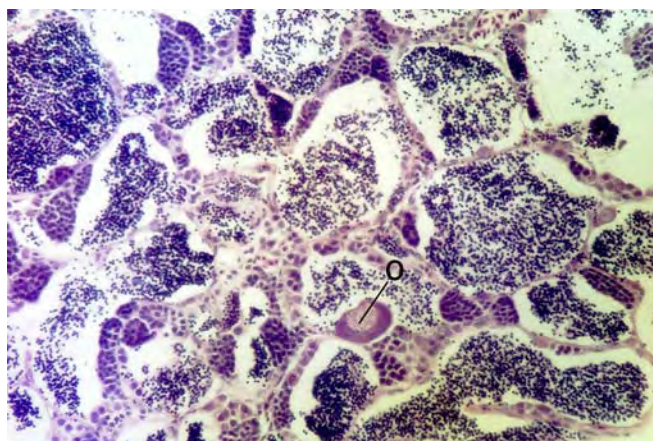


Figure 3.14 Testis from a γ -radiation Group 2 (1000 μ Gy/h) zebrafish. Within an apparently normal testis a developing oocyte(O) is present (ovotestis).

Though most ovaries from γ -irradiated females from Group 3 appeared histologically normal, the finding that some females from this group paired with unirradiated males did lay eggs but that all were sterile (Section 3.2, Table 3.8) indicates that radiation-induced damage had occurred. The decreased number of viable eggs per used lay opportunity (Figure 3.5, Table 3.6) also indicates radiation damage to oocytes. The fact that histological examination showed some male fish from γ -radiation Group 3 were devoid of spermatogenic tissue, while others had some present but in decreased amounts, correlates well with the finding that when some of these males were paired with unirradiated females, eggs were laid in some pairs and although large numbers were sterile some did develop (Table 3.9). Overall, these indicate a rather complicated picture which will require further experimentation to elucidate. The lack of any substantial pathological damage to ovaries and the presence of at least some spermatogenic tissue in testes of γ -radiation Group 3 fish indicates that γ -radiation-induced functional sterility, as exhibited by the lack of any eggs laid after about 20 weeks mating (~30 weeks radiation exposure), does not require complete destruction of germinal tissue in testes or even substantial destruction of ovaries. A similar conclusion was reached for γ -irradiated guppies (Woodhead 1977).

3.4 Comparison of α - and γ -radiation dose rates

The main purpose of this work has been to determine dose rates of γ - and α -radiation which produce the same damaging effects on reproduction in zebrafish and thus compute the relative biological effect for this endpoint ($RBE_{\alpha} = \text{Dose rate of } \gamma\text{-radiation producing an effect} / \text{Dose rate of } \alpha\text{-radiation producing the same effect}$). Unfortunately, it has only been possible to achieve this to a more limited and less precise degree than intended. There are two closely connected reasons for this. The first is that not even in the highest α -radiation dose rate Group 4 were any significant effects on reproduction detected (Section 3.2). The second is that in this group, the tissue activity concentration of ^{210}Po , and thus the α -radiation dose rate, was considerably lower than predicted from preliminary experiments (Section 3.1, Table 3.2) at 214 μ Gy/h rather than 740 μ Gy/h. In contrast, the γ -radiation dose rates chosen completely fulfilled expectations, with complete cessation of egg production in the highest dose rate group (7400 μ Gy/h). Although no significant effects were seen in the lower two dose rate groups (1000 μ Gy/h and 300 μ Gy/h). They did fit the sigmoid dose response curve which was an *a priori* expectation for irradiated fish (Figure 3.6, Section 3.2). Using the γ -radiation dose rate of 7400 μ Gy/h which produced this clear effect and the α -radiation dose

rate of 214 μ Gy/h which was the highest used and caused no effect, an $RBE_{\alpha} < 35$ (7400/214) was derived for the endpoint of ceased egg production. In the highest α -dose rate Group 4, ovary activities were considerably greater than the whole body activities but they also varied greatly between fish (Table 3.2). The range of dose rates to the ovary obtained using these activities is 376 μ Gy/h - 1045 μ Gy/h and RBE_{α} s derived by comparison with the γ -irradiated Group 3 fish (dose rate 7400 μ Gy/h) were $RBE_{\alpha} < 20$ to $RBE_{\alpha} < 7.1$. The Group 4 zebrafish, for which these ovary activities and dose rates were obtained, all laid eggs at least once during the last 4 breeding opportunities before the final kill, in one case that immediately before it. The variation in ovary ^{210}Po may be due to zebrafish being a continuous breeder which under the conditions of this experiment laid eggs every week or so.

The $RBE_{\alpha} < 35$ seems likely to be a conservative upper limit. While the γ -radiation dose rate of 7400 μ Gy/h produced complete cessation of egg laying, the α -radiation dose rate of 214 μ Gy/h did not even reduce the numbers of eggs laid or their viability (Tables 3.3 - 3.6, Figures 3.4, 3.5). Although variable, the ovary concentrations of ^{210}Po were consistently greater than in whole body (Table 3.2) and the upper RBE of < 20 derived from these may be more appropriate. In Table 3.10, the ^{210}Po concentrations in gonads and some other organs of a range of fish from the literature are given along with absorbed dose rates calculated using Equation 1 (Section 2.4). The $RBE_{\alpha} < 35$ derived from present results was used to calculate γ -equivalent dose rates (ie the γ -radiation absorbed dose rate required to produce the same effect as the α -radiation absorbed dose rate). Although this RBE_{α} need not be limited for use to ^{210}Po exposures, this radionuclide is the major contributor to absorbed radiation dose to aquatic animals (UNSCEAR, 1996) and enhancement of natural levels of ^{210}Po may occur due to human activities (McDonald *et al.* 1999). It is the only α -emitter for which there are several data for gonad concentrations in the literature. The γ -equivalent rates to gonads vary considerably from 0.4 - 44 μ Gy/h (Table 3.10). Such rates are well below those which have been shown to affect fish reproductive organs (Woodhead 1977, Rackham and Woodhead 1984, Knowles 1999). The γ -equivalent rates to liver and intestinal organs were in some cases much higher than those for gonads. However, with the exception of *Sardinus pilchardus* pyloric caeca and intestine, they did not exceed about 150 μ Gy/h, a rate unlikely to cause significant damage. In *S. pilchardus* γ -equivalent dose rates of 400 to more than 1000 μ Gy/h were calculated. Although no specific experiments to examine gut damage from exposure to such dose rates have been carried out, there was no clear histological evidence of damage to the gut in zebrafish exposed to γ -radiation dose rates of 1000 or 7400 μ Gy/h in the present study.

Using measurements of radiation dose to Plaice in the Irish sea off Sellafield which had been tagged with TLDs, released and recaptured, it was estimated that dose rates to their gonads were a maximum of 18 μ Gy/h ($\beta + \gamma$ -radiation only, Woodhead 1973). Calculations based on more recent surveys of sediment activity indicate a maximum of 0.7 μ Gy/h (Greenwood and Knowles 1995). In order to use the endpoint of reproductive output, because of its relevance to possible effects on environmental fish populations, the dose rates chosen in the present study were considerably higher than those likely to be found in the environment except possibly under the conditions of a nuclear accident and in a few organisms where extreme concentration of ^{210}Po occurs, as described above in *S. pilchardus*. Although RBE_{β} for β -radiation compared with γ -radiation damage to fish gonads have been reported (Hyodo-Taguchi 1980), no RBE_{α} for α -radiation effects on fish are available. Thus the RBE_{α} derived from the present work, will provide the best available estimate for use in the development of standards for environmental protection.

Table 3.10 The mean concentrations of ^{210}Po and estimated absorbed dose rates in organs from marine fish. The γ -equivalent dose rates have been calculated using an RBE_α of 35

		^{210}Po concentration Bq/kg wet wt.	Absorbed dose rate $\mu\text{Gy/h}$	γ -equivalent dose rate $\mu\text{Gy/h}$
Epipelagic teleosts				
Clupeids ^{a,b}	gonad	40	0.12	4.20
	liver	391	1.22	42.70
<i>Sardinus pilchardus</i> ^a	gonad	319	0.98	34.30
	pyloric caecum	3980	12.2	427.00
	intestine	10379	32.3	1130.50
Mesopelagic Teleosts				
Strict mesopelagic ^{a,c}	gonad	405	1.26	44.10
	liver	552	1.72	60.20
<i>Triphoturus mexicanus</i> ^d	whole-body	27.8	0.086	3.03
	gonad	62.9	0.20	6.85
	viscera	444	1.38	48.33
<i>Myctophum nitidulus</i> ^d	whole-body	107.3	0.33	11.68
	gonad	136.9	0.43	14.90
	caecum	1258	3.91	136.93
<i>Lampanyctus ritteri</i> ^d	whole-body	50.3	0.16	5.48
	gonad	14.8	0.046	1.61
	viscera	278	0.86	30.26
<i>Argyropelecus pacificus</i> ^d	whole-body	40.7	0.13	4.43
	gonad	3.7	0.012	0.40
	viscera	370	1.15	40.27
Bathypelagic Teleosts				
<i>Aphanopus carbo</i> ^a	gonad	11	0.03	1.20
	liver	6	0.019	0.65
	intestine	43	0.13	4.68
Demersal Teleosts ^a				
	gonad	27	0.084	2.94
	liver	12	0.037	1.31
Elasmobranchs ^a				
	gonad	2	0.006	0.22
	liver	8	0.025	0.87

^aData from Carvalho (1988)

^b*Sardina pilchardus*, *Scomber japonicus*, *S. scombrus*, *Trachurus trachurus*, *T. picturatus*, *Belone belone*.

^c*Rouleina maderensis*, *Chiasmodon nigrum*

^dData from Hoffman *et al.* (1974)

3.5 Evaluation of an assay to measure DNA damage in zebrafish larvae

While it is of great importance to measure endpoints that are relevant to populations of fish in the environment, such as reproductive output examined in the present study, other more subtle changes at the cellular or molecular level may occur at lower doses or dose rates. These may be a useful tool in predicting possible damaging effects in the longer term. Although such endpoints have not been measured in zebrafish in the main experiment, during this project the opportunity arose to carry out some short experiments to evaluate the COMET assay which allows early detection of single strand DNA breaks. Zebrafish larvae exposed to γ -irradiation at rates of 7,200 μ Gy/h or 1200 μ Gy/h for 24 hours showed significant increases in %DNA in the comet tail compared with unirradiated control larvae, indicating significant DNA damage (Figure 3.15). Larvae exposed to 400 μ Gy/h showed no significant effect. Zebrafish larvae exposed to γ -radiation for only 1 hour at dose rates of 1200 μ Gy/hr, and 7200 μ Gy/hr also showed statistically significant, dose rate-dependent increases in %DNA in the comet tail (Figure 3.16) compared with controls. The lower dose rate is similar to that of Group 2 γ -irradiated fish (1000 μ Gy/h) in the main experiment which showed no significant reduction in egg production or histological change after exposures of a year or more.

In order to compare results for 1 hour and 24 hour exposures at the same dose rates, the data points for the 1 hour exposure were normalised to those of the 24 hour exposure. The percentage increase from the control values was determined for the 1 hour exposure experimental groups and these percentages were then used to multiply control values from 24 hour exposure experiment to obtain normalised 1 hour group results. The dose response curves for %DNA in comet tail after 1 hour and 24 hour exposures to 1200 μ Gy/hr and 7200 μ Gy/hr are shown in Figure 3.17. At both radiation dose rates the

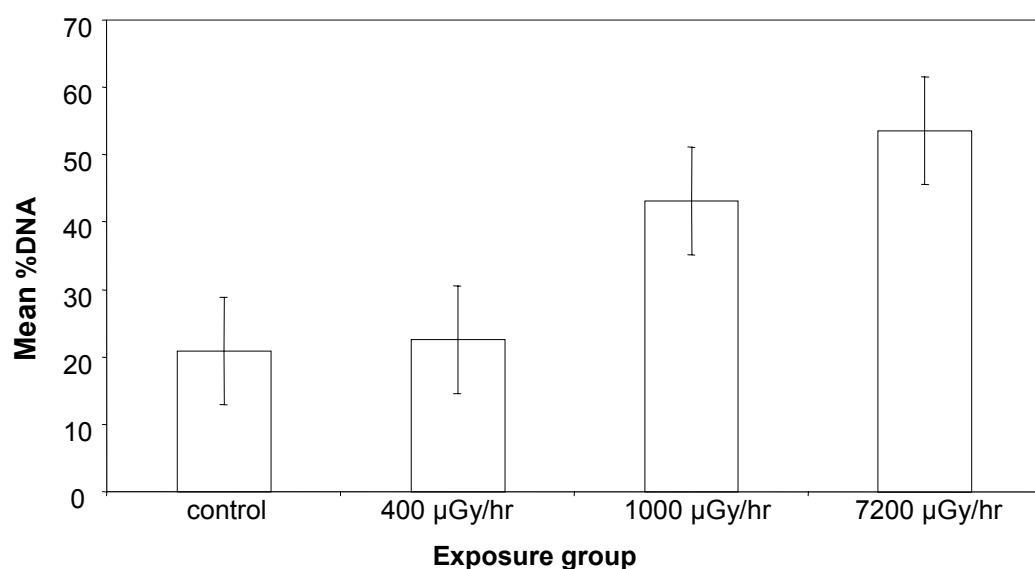


Figure 3.15 The mean %DNA in COMET tail of cells from zebrafish exposed to γ -radiation for 24 hours

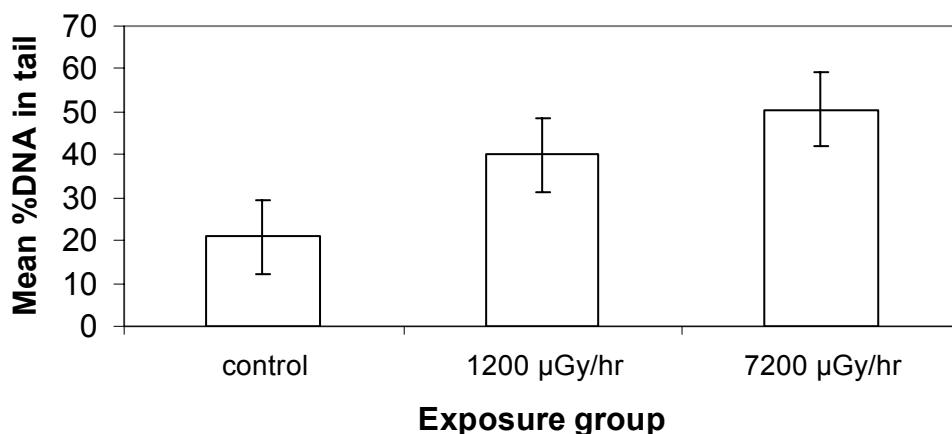


Figure 3.16 The mean %DNA in COMET tail of cells from zebrafish exposed to γ -radiation for 1 hour

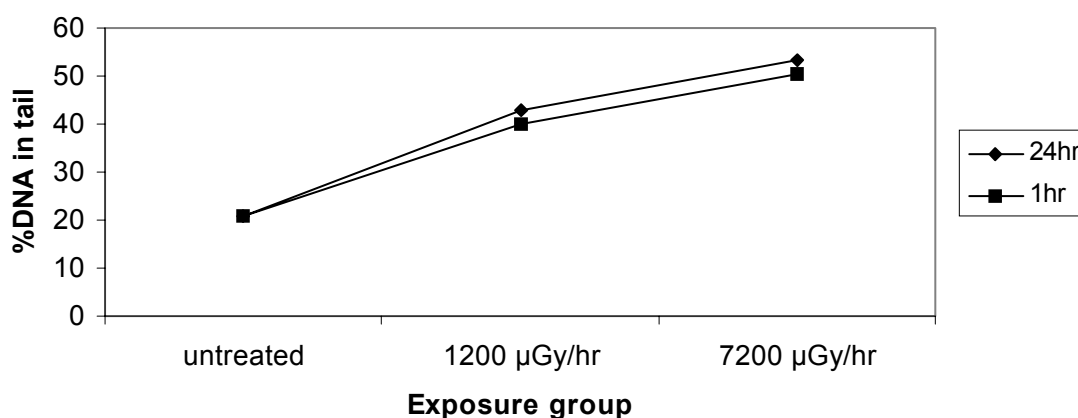


Figure 3.17 Dose-rate response curve for %DNA in the tail of zebrafish exposed for 1 hour and 24 hours to γ -radiation

%DNA in tail was significantly greater in the 24 hour exposure group than in the 1 hour group. However, it is clear that the increased DNA damage was not simply due to the 24 times greater total accumulated dose. For example, the mean %DNA in tail was lower in larvae exposed for 24 hours at 1200 μ Gy/hr (accumulated dose 28,800 μ Gy) than in those exposed for 1 hour at 7200 μ Gy/hr (Figure 3.17). DNA damage detected by the COMET assay can be repaired to some extent and an equilibrium between damage and repair might be expected in long-term irradiated fish but no information is available on this. For DNA damage, measured as DNA adducts, caused by chemical pollutants to fish in the environment there is some evidence that significant recovery can occur (Leonard *et al.* 1999). The relationships between such DNA damage and population-relevant endpoints such as reproduction or disease incidence are unknown, however, the success of this small experiment on zebrafish larvae suggests that the inclusion of such assays would prove a very useful addition when measured along with such endpoints as reproductive output.

4. CONCLUSION

Although the comparison between α - and γ -radiation effects on reproductive output carried out in this project has not quantified the RBE_{α} with the hoped-for precision, the data obtained represent a significant advance in an area where none was previously available. The $RBE_{\alpha} < 35$ inferred from this data represent an upper limit and the $RBE_{\alpha} < 20$ and < 7 obtained using ovary rather than whole body activities may represent closer estimates. Even using the higher value of $RBE_{\alpha} < 35$ it is unlikely that α -radiation dose rates to fish in the environment will give rise to γ -equivalent dose rates, which in combination with those from other radiations, exceed $400\mu\text{Gy/h}$, the guideline dose rate below which detrimental effects are unlikely to occur (UNSCEAR 1996). Rare exceptions to this are certain fish where some organs accumulate naturally occurring ^{210}Po to very high concentrations (Table 3.10). Other environmental situations may exist or arise where anthropomorphic discharges give rise to high tissue levels of α -emitters and thus γ -equivalent radiation dose rates. The development of standards to protect the environment will allow criteria with which to assess such situations. Data from the present work should be made available to help FASSET and other groups working towards the development of standards for environmental protection from radiation.

5. RECOMMENDATIONS

The need for RBE_{α} for relevant endpoints in environmental organisms is clear (Pentreath 1998), and, as the present study represents a first attempt, it is worthwhile examining it for ways which can lead to future improvements in the execution of these difficult experiments.

Dietary administration of ^{210}Po offers the only practical way of delivering α -radiation quantifiably over protracted periods. The use of only small organisms is necessitated by the need to keep amounts of ^{210}Po low enough not to cause problems of radiological safety. A major problem in the present study was the establishment of a reliable relationship between feeding rate of radioactivity and α -radiation dose rate in zebrafish, particularly at higher feeding rates. One way of improving this would be to carry out long-term preliminary experiments in which the relationship is measured empirically rather than inferred from modelling and short term preliminaries. This would be extremely costly in time and manpower. For smaller organisms, experiments in which large numbers were involved would allow subsamples to be taken for ^{210}Po analysis throughout the experiment allowing α -radiation dose rates to be calculated at intervals over the whole period. This would only be possible if, in contrast to zebrafish, the husbandry of the organisms and delivery of activity to a large number of them could be done simply and endpoints did not require frequent labour intensive measurement. While the importance of such relevant endpoints as reproductive output is undisputable, the use of other endpoints such as COMET to examine changes at subcellular or molecular levels over shorter time periods during the experiments would maximise the information obtained. It would allow for comparison of RBE_{α} at molecular and whole organism levels.

Taking into account the above considerations the Woodlouse offers itself as a good candidate for experiments and it has already been put forward by FASSET as a possible reference organism. It can be kept easily in colonies of sufficient size to allow in-experiment sub-sampling. ^{210}Po -spiking of the litter which it eats and in which it lives would allow relatively easy delivery of radioactivity. While there are several reproductive endpoints which can be measured for individuals, the output and survival of offspring from colonies as a whole can be also be used and do not require labour intensive high frequencies of measurement. In addition there are no apparent reasons why such assays as COMET should not be carried out on cells from the woodlouse.

6. REFERENCES

- Carvalho F P, 1988 *²¹⁰Po in marine organisms: a wide range of natural radiation dose domains*. Radiation Protection Dosimetry, **24**, 113-117
- Carvalho F P and Fowler S W, 1994 *A double -tracer technique to determine the relative importance of water and food as sources of polonium-210 to prawns and fish*. Marine Ecology Progress Series, **103**, 251-264
- Cherry R D, Heyraud M and Rindfuss R, 1994 *Polonium-210 in teleost fish and in marine mammals: Interfamily differences and a possible association between polonium-210 and red muscle content*. Journal of Environmental Radioactivity, **24**, 273-291
- Copplestone D, Toal M E, Johnson M S, Jackson D and Jones S R, 2000 *Environmental effects of radionuclides - observations on natural ecosystems*. Journal of Radiological Protection, **20**, 29-40
- Copplestone D, Bielby S, Jones S R, Patton D, Daniel P and Gize I, 2001 *Impact assessment of ionising radiation*. Environment Agency R&D Publication 128, Environment Agency, Bristol UK, 222pp
- Finkel M P, Norris W P, Kisielewski W E and Hirsch G M, 1953 *The toxicity of polonium-210 in mice: 1 The thirty day LD₅₀ , retention and distribution*. American Journal of Roentgenology, **70**, 477-485
- Greenwood L N and Knowles J F, 1995 *Effect of irradiation on the humoral immune response of a marine fish, the eelpout (Zoarces viviparus L.)*. International Journal of Radiation Biology, **67**, 71-77
- Hoffman F L, Hodge V F and Folsom T B, 1974 *Polonium radioactivity in certain mid-water fish of the eastern temporal pacific*. Health Physics, **26**, 65-70
- Hyodo-Taguchi Y, 1980 *Effects of chronic gamma-irradiation on spermatogenesis in the fish Oryzias latipes with special reference to the regeneration of testicular stem cells*. In Radiation Effects on Aquatic Organisms. (ed. N. Egami), pp. 91-104. Japan Scientific Societies Press, Tokyo / University Park Press, Baltimore
- International Atomic Energy Agency, 1992 *Effects of ionizing radiation on plants and animals at levels implied by current radiation protection standards*. Technical Report Series No 332. IAEA; Vienna
- Jarvis R B and Knowles J. F, 2002 *DNA damage in zebrafish larvae induced by exposure to low-dose-rate gamma radiation: Detection by the alkaline comet assay*. Mutation Research, Section: Genetic Toxicology and Environmental Mutagenesis, submitted paper.
- Knowles J F, 1999 *Long-term irradiation of a marine fish, the plaice Pleuronectes platessa: an assessment of the effects on size and composition of the testes and of possible genotoxic changes in peripheral erythrocytes*. International Journal of Radiation Biology, **75**, 773-782

Kocher D C and Trabalka J R, 2000 *On the application of a radiation weighting factor for alpha particles in protection of non-human biota*. Health Physics, **79**, 407-411

Leonard D R P, Law R J and Kelly C A, 1999 *Responding to the Sea Empress Oil Spill*. In Marine Pollution, Proceedings of the International Symposium held in Monaco 5 –9 Oct. 1998, pp 177 - 182. IAEA SM-354/86 IAEA – TECDOC - 1094

Malyapa S R, Bi C, Ahern E W and Roti Roti J L, 1998 *Detection of DNA damage by the alkaline comet assay after exposure to low-dose gamma radiation*. Radiation Research, **149**, 396-400

McDonald P, McKay K, John C, Jackson D and Leonard, D R P, 1999 *Pb-210 and Po-210 in Terrestrial Foodstuffs from regions of potential technological enhancement in England and Wales*. Journal of Environmental Radiology. **43**, 15 –29

Pentreath R J, 1998 *Radiological protection criteria for the natural environment*. Radiation Protection Dosimetry, **75**, 175-179

Rackham B D and Woodhead D S, 1984 *Effects of chronic gamma-irradiation on the gonads of adult Ameca splendens (Osteichthyes: Teleostei)*. International Journal of Radiation Biology, **45**, 645-56

Samuels L D, 1966 *Effects of polonium on mouse embryos*. International Journal of Radiation Biology, **11**, 117-129

Sinclair W K, 1985 *Experimental RBE values of high LET radiations at low doses and the implications for quality factor assignment*. Radiation Protection Dosimetry, **13**, 319-326

Singh N P, Graham M M, Singh V and Khan A, 1995 *Induction of DNA single-strand breaks in human lymphocytes by low doses of γ -rays*. International Journal of Radiation Biology, **68**, 563-569

UNSCEAR, 1996 *Effects of Radiation on the Environment*. In : Sources and effects of ionizing radiation, United Nations Scientific Committee on the Effects of Atomic Radiation, 1996 Report to the General Assembly, with Scientific Annex, pp. 8-86. United Nations, New York

Westerfield M, 1995 *The zebrafish book*. Institute of Neuroscience, University of Oregon.

Woodhead D S, 1973 *The radiation dose received by plaice (Pleuronectes platessa) from the waste discharged into the north-east Irish sea from the fuel reprocessing plant at Windscale*. Health Physics, **25**, 115-121

Woodhead, D S, 1977 *The effects of chronic irradiation on the breeding performance of the guppy Poecilia reticulata (Osteichthyes: Teleostei)*. International Journal of Radiation Biology, **32**, 1-22

Woodhead, D S, 1984 *Contamination due to radioactive materials*. Marine Ecology, **5**(3). Edited by : O. Kinne. (John Wiley, Chichester), pp. 1111-1287

Woodhead, D S, 1998 *The impact of radioactive discharges on native british wild-life and the implications for environmental protection*. R&D Technical Report P135, Environment Agency, Bristol, 80pp.

Woodhead, D S, 2000 *Environmental dosimetry: the current position and the implications for developing a framework for environmental protection*. R&D Technical Report P350, Environment Agency, Bristol, 48pp.

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10. GLOSSARY

Absorbed radiation dose	Quantity of energy imparted by ionising radiation to unit mass of matter such as tissue. Unit is the Gray (Gy). 1Gy = 1 joule per kilogram. Often shortened to radiation dose or dose when the context allows no ambiguity.
Absorbed radiation dose rate	Absorbed radiation dose received over a specific time period (eg Gy per hour). Often shortened to radiation dose rate or dose rate, when the context allows no ambiguity
Activity	Attribute of an amount of radioactivity. Describes the rate at which transformations occur in it. Unit is the Becquerel (Bq). 1 Bq = 1 transformation per second
Alpha-radiation	Radiation emitted by a radionuclide. Consists of α -particles which are atomic nuclei with two protons plus two neutrons
Artemia	The brine shrimp <i>Artemia salina</i> . A small crustacean used as food for pet and laboratory fish. Eggs may be stored and hatched when required
Atretic oocyte	<i>Oocyte</i> degenerating in the ovary
Autoradiography	The exposure of a photographic film to radiation from radionuclide(s) contained in a histological section. When developed the film allows the localisation of the radionuclide in tissue to be seen at a histological level
Bathypelagic	sub-division of <i>Pelagic</i> . "Lower pelagic"
Becquerel (Bq)	see <i>activity</i> . 1kBq = 10 ³ Bq
Beta-radiation	Radiation emitted by a radionuclide. Consists of β -particles, which are electrons.
COMET	The single-cell gel electrophoresis assay. Electrophoresis of single nuclei from treated cells allows damage to nuclear DNA to be assayed by the amount of DNA trailing from the nuclear mass, with an appearance like a "comet tail"
Demersal	Living in deep water on or near the sea-bed
DNA	Deoxyribonucleic acid. Chemical compound that controls the structure and function of cells and is the material of inheritance. Found especially in chromosomes in the cell nucleus

DNA damage	Damage to <i>DNA</i> can cause cell death. Cellular repair mechanisms can repair some DNA damage completely but incomplete or misrepair may cause mutations resulting in eg cancer, heritable defects
Epipelagic	sub-division of <i>Pelagic</i> . "Upper pelagic"
Gamma radiation	A discrete quantity of electromagnetic energy without mass or charge. Emitted by a radionuclide
Gray (Gy)	see Absorbed dose. $1\text{mGy} = 10^{-3}\text{Gy}$, $1\mu\text{Gy} = 10^{-6}\text{Gy}$
Guppy	<i>Poecilia reticulata</i> , a small, viviparous, freshwater, tropical fish
Oocyte	Cell which becomes the female gamete, the egg. Early perinucleolar oocytes increase in size and become filled with yolk to become mature yolky eggs.
Mesopelagic	sub-division of <i>Pelagic</i> . "Mid pelagic"
Ovotestis	The presence of one or more female <i>oocytes</i> in the testis of a male fish or <i>spermatogenic tissue</i> in the ovary of a female fish
Pelagic	Inhabiting the surface to mid depth waters of the sea. Sub-divided into <i>epipelagic</i> (upper), <i>mesopelagic</i> (middle) and <i>bathypelagic</i> (lower) layers. Used especially to describe fish.
Relative Biological Effectiveness (RBE)	The ratio of the <i>absorbed doses</i> of two different radiation types which produce the same biological effect. Radiation types and biological effect must be defined
Spermatogenic tissue	Cells which forms the the male gametes, the sperm. Consists of spermatogonia, spermatocytes , spermatidia and sperm
Thermoluminescent dosimeter (TLD)	Dosimeter made of a substance (eg Lithium fluoride) which when heated gives out light which is proportional in amount to the absorbed radiation dose it has received
Zebrafish	<i>Danio rerio</i> a small, egg-laying, freshwater, tropical fish.

MAINTENANCE AND EXPERIMENTAL BREEDING OF ZEBRAFISH

The zebrafish used in the experiment were the offspring of stock *wild type AB* fish supplied by the zebrafish facility at University College London. Throughout this work all zebrafish, including eggs and larvae were kept in water purified by reverse osmosis. Conductivity was adjusted to 550 μ S using "Instant Ocean" and the water temperature was maintained at 26-28°C. A 12 hour light/12 hour dark regime was maintained throughout. Fish were fed routinely twice per day with flake food (once per day at weekends). During pilot experiments adult fish were maintained in 1.5 litre plastic tanks. During the main experiment, when egg production was monitored, pairs of fish were maintained in similar tanks modified to ensure the adults did not eat any eggs which were laid. This was achieved by placing an inner tank with the bottom removed and replaced by a stainless steel mesh inside the normal outer tank (Figure A1.1). Because of the way the tanks stacked the mesh lay about 3cm above the bottom of the outer tank. Any eggs laid sank and passed through the mesh to where they could not be eaten by the parents. The inner, mesh-bearing tank was easily lifted out and the adult fish in it transferred rapidly to clean water at tank changing. Eggs were then removed from the outer tank and placed in a plastic Petri dish for counting. In order that food (in particular meals of α -radioactivity) was not lost by passing through the mesh before it could be eaten a fitted perspex cover was placed over the mesh at all times except for when fish were allowed to breed.

Figure A 1.1 The experimental fish-tank showing outer normal tank (B), inner tank (A) with a stainless steel mesh bottom(D). The perspex cover (C) and tank divider (E) were removed during breeding (lay opportunities) so that eggs could fall through the mesh.



It has been shown that approximately the same number of eggs per week per female zebrafish were produced when breeding was allowed daily or once per week (Bresch *et al.*, 1986). Thus in order keep the amount of labour required during the main experiment to a manageable level pairs of fish were allowed to breed once a week with a perspex divider keeping them separate at all other times. For breeding the divider and perspex mesh cover (see above) were removed during the afternoon and the fish left together overnight until about 2 hours after the lights came on the next morning. This allowed plenty of time for laying to take place as it usually occurs within an hour or less of the lights coming on (Eaton and Farley, 1974; Ensenbach and Nagel, 1997). The tanks were then changed, the divider and mesh cover replaced and any eggs which had been laid were collected for counting.

References

Bresch H, Markert M, Munk R and Speiser O H, 1986 *Long term fish toxicity test using zebrafish: effect of group formation and temporary separation by sex on spawning*. Bulletin of Environmental Contaminants and Toxicology, **37**, 606-614

Eaton R C and Farley R D, 1974 *Spawning cycle and egg production of zebrafish, Brachydanio rerio in the laboratory*. Copeia, **1**, 195-204

Ensenbach U and Nagel R, 1997 *Toxicity of binary chemical mixtures: effects on reproduction of zebrafish (Brachydanio rerio)*. Archives of Environmental Contamination and Toxicology, **32**, 204-210

APPENDIX B

PREPARATION OF α -RADIATION-SPIKED EXPERIMENTAL MEALS

The delivery of radioactive ^{210}Po was carried out by feeding fish meals of spiked artemia larvae (brine shrimp). Batches of ^{210}Po -spiked artemia were prepared by incubating freshly hatched larvae overnight in a saline solution containing a known amount ^{210}Po activity. The larvae were then filtered off using fine nylon mesh and rinsed thoroughly before being resuspended in a known volume of saline. Aliquots of 1ml were taken into Ependorf tubes and frozen for storage until required. The activity per aliquot was determined by thawing a tube, centrifuging and removing the saline supernatant and processing the active artemia pellet for scintillation counting (Appendix E). The activity in the pellet (Bq/g) was used in determining activity delivered to the fish. The activity of different batches of artemia food was controlled by the amount of artemia and the concentration of ^{210}Po in the incubation solution. As far as possible the batches of artemia for the different experimental dose rate groups were prepared so that, while the activity of food for each group would give the desired tissue activity, the weight of artemia in a meal was approximately the same for each group. Analysis of batches frozen for periods of up to several months established that there was negligible loss of ^{210}Po activity (apart from physical decay, which was taken into account) during storage.

THE FRACTION OF ACTIVE MEALS INGESTED BY ZEBRAFISH IN SOLID-BOTTOMED AND GRID-BOTTOMED TANKS.

Four zebrafish were placed individually in solid-bottomed tanks and another 4 in tanks with a stainless steel grid at the bottom. Each fish was given a single meal of ^{210}Po -spiked artemia containing 280Bq and then killed 1 hour later, at which time it was assumed no food had passed all the way through the alimentary tract. Fish were then processed for analysis of whole body ^{210}Po content.

Table C 1.1 The whole body activity of zebrafish fed a single active meal of 280Bq and killed 1 hour after after feeding

Whole body activity of Zebrafish fed in solid bottom tanks (Bq)	Whole body activity of Zebrafish fed in mesh bottom tanks (Bq)
83	37
161	83
138	59
133	56
Mean 129 ± 33	Mean 59 ± 19

The mean whole body activity for fish in the solid bottom group was 129Bq which represents 46% of the activity in their meal. The mean whole body activity of fish in grid bottom tanks was 59Bq which represents 21% of their meal activity.

The stainless steel grid-bottomed tanks were used so that eggs could pass through and avoid being eaten by the parents. In order to ensure that the ingestion rate of all food, but in particular of ^{210}Po -spiked meals, was as high as possible a perspex cover was placed over the mesh bottom at all times except when breeding was allowed.

On the basis of this experiment an ingestion rate of 50% was used in calculations of meal activities to produce required α -radiation dose rates.

EXPOSURE OF ZEBRAFISH TO γ -IRRADIATION

Zebrafish were exposed to γ -radiation from ^{137}Cs sealed sources in the CEFAS γ -radiation facility. In this facility 3 sets of gamma sources (each set consisting of 3 sources) are arranged, each above a separate bench. Each set is collimated to deliver a uniform radiation dose over the surface of the bench and each contains a different amount of ^{137}Cs activity such that the dose rates at the 3 bench surfaces are different, allowing 3 groups of animals to be irradiated each at a different dose rate. The sources are remotely controlled to be in a "safe" position in lead-shielded containers when the facility is entered for inspection, fish feeding and husbandry etc. Dose rates to tanks of experimental zebrafish were determined by placing Lithium fluoride thermoluminescent dosimeters (TLDs) in waterfilled tanks with 12 dosimeters (in a 2 x 6 array) in each tank and 3 tanks per dose rate group. After exposure for about 70 hours the absorbed doses were obtained using a TLD reader (Harshaw 3000A). Mean dose rates for each dose rate group of zebrafish were calculated as 7400, 1000 and 300 $\mu\text{Gy/h.}$). Dose rates within tanks vary due to the shielding effect of the water and the calculated means assume zebrafish spend, on average, equal amounts of time in all parts of the tank. This assumption is supported by the almost continuous movement about the tank generally observed in zebrafish and its midwater position during "resting" periods.

APPENDIX E

PROTOCOL: ACID DIGESTION OF FISH MATERIAL AND PREPARATION FOR SCINTILLATION COUNTING

1. Weigh material (eg Whole fish, gonad alone) in a tared 100ml beaker. Add a spitzer (approx 3ml) of conc nitric acid and allow to digest. The initial digestion may be rapid and aggressive therefore do not heat to begin with. Reflux overnight with a watchglass over the beaker.
2. Remove the watch glass washing it with conc nitric acid into the beaker then take to near dryness and add approx 1.5ml conc nitric acid and then 1.5ml perchloric acid. DO NOT add perchloric acid to the dried residue but always add the nitric acid solution first. ENSURE there is no perchloric acid on the outside of the beaker when replacing it on the hotplate. Failure to follow these precautions could have explosive consequences!
3. Evaporate the 50/50 nitric/perchloric acid mixture to dryness. The nitric acid will evaporate off first followed by the white fumes of the perchloric acid.
4. To take the residue into the chloride form, add approximately 3 ml of conc hydrochloric acid and evaporate to insipient dryness at a low temperature.
5. Take up the residue into 6M hydrochloric acid by adding a few ml and warming gently. Pour into a volumetric flask (10, 25, 50ml). Rinse the beaker out into the volumetric with 6M hydrochloric acid and fill to volume with 6M hydrochloric acid.
6. Mix well and take 1ml of the diluted sample to a scintillation vial and add 3ml of distilled water. Make sure all dilutions are recorded to allow final calculations of Bq/g.
7. Add 15ml Ultima Gold AB scintillant, cap vial and shake to mix well. Allow to cool for 1 hour and count.

NOTE

²¹⁰Po recovery was checked periodically by spiking an untreated zebrafish with a known amount of activity before processing, while counting efficiency was measured by the method of addition. Recovery and counting efficiencies were both in the range 93 - 100%.

Low activity samples, principally some gonads from groups 1 and 2, were processed and counted on an α counter as well as by scintillation counting. Sub-samples of the 6M hydrochloric acid solution in the volumetric (see 5 above) were used for this. ²¹⁰Po activity was counted using a silicon surface barrier detector following spontaneous deposition on a silver disc (in 0.5M HCl in a plating bath at $90 \pm 5^\circ\text{C}$ for 4 hours)

APPENDIX F

THE DETERMINATION OF ACTIVE MEAL SIZES REQUIRED TO DELIVER EXPERIMENTAL TISSUE CONCENTRATIONS OF ^{210}Po AND α -RADIATION DOSE RATES

Estimates of the rate of feeding of ^{210}Po -spiked meals necessary to produce the body concentrations which would deliver α -radiation at the required dose rates for each experimental group of zebrafish were obtained from a mathematical model which was formulated from the results of pilot experiments. A group of 32 zebrafish were each given a single meal of α -spiked artemia (total meal activity 320 Bq /g fish wet weight of which approximately 160Bq/g was assumed to be ingested. See Appendix C) and then samples of 4 fish were killed at intervals of 4 hours, 1 day, 2 days, 4 days, 1 week, 2 weeks, 4 weeks and 6 weeks. Whole body ^{210}Po activities were determined by scintillation counting (Appendix E) and a clearance curve constructed. This could be resolved into three components, fitting the equation.

$$A_{(t)} = 75 \exp(-0.365t) + 85 \exp(-0.301t) + 3 \exp(-0.0116t)$$

where

$A_{(t)}$ is the activity in Bq/g at time t (in days) after ingestion of the active meal

The most rapidly clearing component ($t_{1/2} = 1.9\text{d}$) represents active food rapidly passing through the gut and voided as faeces. This was considered to be completed by 2d and was set at zero for times greater than this. The next most rapidly clearing component ($t_{1/2} = 2.3\text{d}$) was probably Due to activity in gut lining cells with a rapid turnover while the much more slowly clearing third component ($t_{1/2} = 59\text{d}$) seems likely to be most relevant to ^{210}Po activity concentrations in zebrafish tissues responsible for chronic radiation exposures. Using the clearance equation given above it was possible for the body burden from multiple meals of ^{210}Po -spiked food to be estimated as:

$$A_{(t)} = \sum_i [75 \exp\{-0.365 (t-t_i)\}] + \sum_i [85 \exp\{-0.301 (t-t_i)\}] + \sum_i [3 \exp\{-0.0116 (t-t_i)\}]$$

where

t_i are the individual meal times in days since the first meal

t is the total time since the first meal.

This model was used to estimate the amounts of ^{210}Po activity per meal, in Bq/g body wet wt of fish, needed to achieve the body burdens which would give exposure to α -radiation at the required dose rates to fish in the different experimental groups.

EXPERIMENTAL TREATMENT OF ZEBRAFISH

All fish entered for this experiment were raised from eggs laid by stock zebrafish kept at Lowestoft. After hatching the fry were raised in tanks of 50 or so and fed on special fry food (ZM systems Ltd). At 12 weeks of age, by which time all fish were taking adult fish flake and artemia as food, the irradiations were started. Fish were put 10 per tank and those receiving α -radiation commenced twice weekly feeding of active food. Weights of all ten fish in a tank were taken for several tanks in order to get the mean body weight for determination of meal size. At the same time fish for γ -radiation were placed in the γ -radiation facility and continuous irradiation was started with breaks only for fish husbandry and feeding. After a further 10 weeks it was possible to determine the sex of fish from external features and male/female pairs were placed in tanks modified for breeding and egg collection (Appendix A). Where possible 10 pairs were set up for each group. Radiation exposures continued with weights of several individual fish being taken at intervals and the means of these used to allow determination of feeding rates of active food. Control fish were treated in exactly the same way as irradiated fish but received no ^{210}Po activity or exposure to γ -radiation.

It proved impossible to start irradiation of all fish in all groups at the same time as in some groups a large excess of one sex or the other (presumably by chance) meant that there were not enough to make up the 10 pairs. To accommodate this more fish were raised and put in to pairs in the same way as described above. Several fish not used to make up pairs were killed and analysed for ^{210}Po concentrations in their tissues in order to compare these with the concentrations expected from modelling (Appendix F). Some pairs entered into the experiment were subsequently found to be single sex and swapping to give male/female pairs was carried out. The first time male/female pairs of fish were allowed to mate was recorded as Lay opportunity 1. Eggs were collected after each lay opportunity and counted using a stereomicroscope (Total eggs laid). After a further 24 hours when eggs which were not fertilised had become opaque and easily distinguished they were counted (Non-viable eggs). At approximately monthly intervals (every 4th lay opportunity) fertile eggs were kept and the number of them hatching successfully was recorded.