Final Report on Fish Studies
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Canadian Nuclear Safety Commission

A Study of
The
Multigenerational Reproductive
and
Health Effects
of Chronic Lifetime Exposure of a Risk Model in
Fish and in Mammals to Ingestion of Alpha-Emitting
Radionuclides (Ra-226)

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Section a

Executive summary

This document reports the results of the study of the multigenerational effects of an alpha emitter (^{226}Ra) on fish. The species chosen was the fathead minnow (FHM) but some data are also presented for zebrafish (Zeb). The fish were exposed "from cradle to grave" to chronic doses of environmentally relevant levels of radium administered in the food. The FHM lifetime experiments were extremely difficult and required two years of carefully controlled and recorded exposure. In the end no breeding was achieved. The Zebs, established as a fall back due to the non-breeding of FHM, had a much shorter generation time and it was possible to get F0 and F1 fertility and fecundity data with and without continuation of ^{226}Ra feeding in the F1 cohort.

Despite the lack of breeding success, the FHM study is the first time a controlled laboratory experiment involving alpha radiation exposure has been done for the entire lifetime of FHM or any other fish species. The data were also generated following environmentally relevant low dose exposure. This is important because many studies using toxic or potentially toxic substances, use high doses and try to extrapolate back to low doses assuming a linear dose response. This rarely holds in the low dose range.

Our major findings are

1. Over 2 years of feeding 10mBq-10Bq we saw no greater mortality than in the controls.
2. Transient perturbations were seen in growth and fitness parameters. These peaked for all radioactive diets at 6 months. By 18-24 months all these effects had gone and we assume they were mainly homeostatic fluctuations.
3. No mutations were seen and if anything fish on the 10Bq diet looked healthier than the controls. We have no quantitative metric for this.
4. The dosimetry methods available for alpha particles in the very low dose range were developed as part of the contract and resulted in 3 theses and 2 papers (one still in prep.). The dosimetry was verified by Laval University (D. Lariviere) using the radon gamma generation system.
5. The dosimetry analyses reveal that ^{226}Ra is bio-accumulated initially but the radium is completely depurated in the 18 and 24 month samples. This was confirmed using liquid scintillation and autoradiography techniques where positive controls gave normal readings.
6. Experiments using injected radium as an acute dose showed than the radioactivity was rapidly removed from the fish body (within a few weeks) supporting the chronic depuration results.

7. Proteomic data showed that chronic low dose range ^{226}Ra had very similar effects to low dose acute x-ray exposure followed in other experiments on rainbow trout and medaka i.e. structural proteins, energy mobilising proteins and anti-oxidant proteins were induced strongly and once induced they remained up-regulated.
8. Low dose effects are usually regulated using the linear-non-threshold (LNT) model which assumes a linear dose response and adds in correction factors to account for more harmful types of radiation such as alpha particles. However because this is very likely a wrong assumption in the environment, we did a comparison of acute x-irradiation and chronic or acute ^{226}Ra exposure. The endpoints which showed effects in the 2 year study showed no difference in effect if x-rays or ^{226}Ra were given as acute one off doses. The RBE was one for 226Ra for our growth and stress signalling tests. There was no DDREF for acute v chronic 226Ra exposure either for these doses and endpoints.
9. Because of the concern about the validity of extrapolations to low dose from high dose effects data, experiments looking at mechanisms were done with the aim of finding new bio-indicators of harm or benefit. Apart from the stress signal or bystander assay, these are not discussed in detail in the report but papers are available on request.
10. Three more papers on this contract work are being prepared, dealing with the RBE data, the stress signal assay and the reproductive study. One more PhD thesis will result from the contract bringing the total to 2 Master's and 2 PhD's

Summary of conclusions and recommendations

We conclude that ^{226}Ra has no deleterious effects in FHM exposed either acutely by injection or chronically via their diet, to environmentally relevant activities or to activities up to 1000 times the relevant levels measured in fish. While our FHM fish did not breed, the dosimetric analyses show no ^{226}Ra was retained in the fish by the time they reached maturity despite continuous feeding the radium was depurated.

We conclude that in Zebs which did breed, there may be an effect on fertility and fecundity in the F1 generation of the fish maintained on the diet for F0 and F1 generations but analysis of the data suggest this may be due to an abnormally high control because all concentrations of radium in the F1 cohort in question, showed the same response and all the other control groups had lower total fecundity. Nevertheless the data are included in the report.

We conclude ^{226}Ra bio-accumulated in young FHM but then depurated. This result was confirmed several times in several ways and clearly means the models for radium bio-accumulation may be misleading.

We conclude that more lifetime studies of environmentally relevant levels of isotopes of concern are needed to test the predictions of models. These rely too

heavily on extrapolation and on acute data from a few high dose experiments on a limited number of species.

We recommend that anyone trying to do a repeat of this type of study should use a fish species with a short generation time so that reproductive data can be obtained more reliably.

We recommend that the zebrafish data be repeated and that attention is paid to the bioaccumulation of radium in these fish. We suspect fast generation time fish may not have time to turn on the depuration mechanism which took between 6 and 12 months to become active in FHM.

With hindsight we would probably have included a ^{226}Ra dietary dose known to be toxic even if never found in the environment as a positive control.

Section b

DESCRIPTION OF RESULTS ADDRESSING THE DELIVERABLES (DETAILED DISCUSSION FOLLOWS IN PART C OF THIS REPORT)

. 4.1 Develop the detailed work plan for the laboratory work (denoted as Phase 1 in the original contract).

This was done, submitted to CNSC and approved before the start of the project.

. 4.2 Study the multigenerational reproductive and health effects of chronic lifetime exposure of a risk model (e.g. fathead minnow) to ingestion of alpha-emitting radionuclides (e.g. Ra-226).

Figure 1. Fathead minnow. This image was provided by Marinco Bioassay Laboratory, Inc., Sarasota, Florida.

The species was chose because it is a native Canadian species. Activities of Ra-226 in the diet were based on actual activities and up to 1000 times greater than those found in the stomach contents of fish in contaminated lakes in the Elliot Lake region of Canada (Clulow and Pyle 1987). The activities were discussed with AECL and IRSN

(partners in the project and were agreed as the most relevant to use. The numbers of fish (100 per tank with two replicate tanks per concentration, solvent controls and absolute controls) were those agreed by the partners as permitting statistical power while giving a safety margin for mortalities and for interim sampling. The sampling was done at 1, 6, 12,15, 18 and 24 months. 12 and 15 month samples were non-invasive to conserve stocks. The results of the growth, morphology and biochemical indices are now published (copy of paper attached in Appendix 1 (paper 1). The detailed growth and morphology findings are not repeated here as they are all in the paper and the raw data are on the USB supplied with this report.

Transient small effects were seen which are likely due to homeostatic mechanisms. No adverse effects on growth, morphology or metabolism were observed. The fish did not breed and a repeat, started in late 2012, was unsuccessful due to an outbreak of fish dropsy which meant the fish had to be euthanized 6 months into the repeat experiment. A new cohort was established in February 2013. These were obtained at a growth stage where they were expected to become mature by July 2013 in the hope that Dr Richard Smith would be still employed to oversee the breeding. The fish were placed in breeding pairs in special tanks. No viable offspring were seen in any tank, although eggs were seen which did not hatch. Dr Smith was made redundant in August 2013 due to lack of funds and the experiment was terminated at Christmas 2013 with no breeding. A fallback set of zebrafish did breed and egg production and viability were obtained for the F0 and F1 generation. The F1 fish were reared with and without continued radium feeding. The results are contained in Table 1 and 2 in section c and on the excel spreadsheet (Table 3). There were no indications of any effects in any tank in the F0 fish or in the F1 groups where radium was discontinued. Statistical analysis of the F1 continuous feeding data showed significant impacts on reproduction resulting from the accumulated exposure (table 1b). More detailed analysis using other statistical software revealed an across the board reduction in both the number and viability of eggs which was not dose dependent but was fully expressed at the lowest dose used (Table 4). These data are discussed later in section c but the conclusion is that an aberrant control group accounts for the apparent effect.

In addition to growth, morphology and reproductive studies, a full proteomic analysis of changes in the proteome of the FHM gill was done. The data reveal changes in proteins similar to those seen in trout and medaka after xray exposure; repair, antioxidant and energy mobilization proteins are upregulated. These changes are consistent with adaptive and protective responses to these low dose exposures. The paper is currently with CNSC prior to being submitted to Int J Rad Biol (paper attached as paper 3 in the Papers appendix 1. Various mechanistic assays were also performed and these are detailed in part C of this report.

Figures 1 and 2 show the accumulated activities delivered to the FHM cohort of fish and to the zebrafish.

Fathead minnow. Cumulative dietary dose.

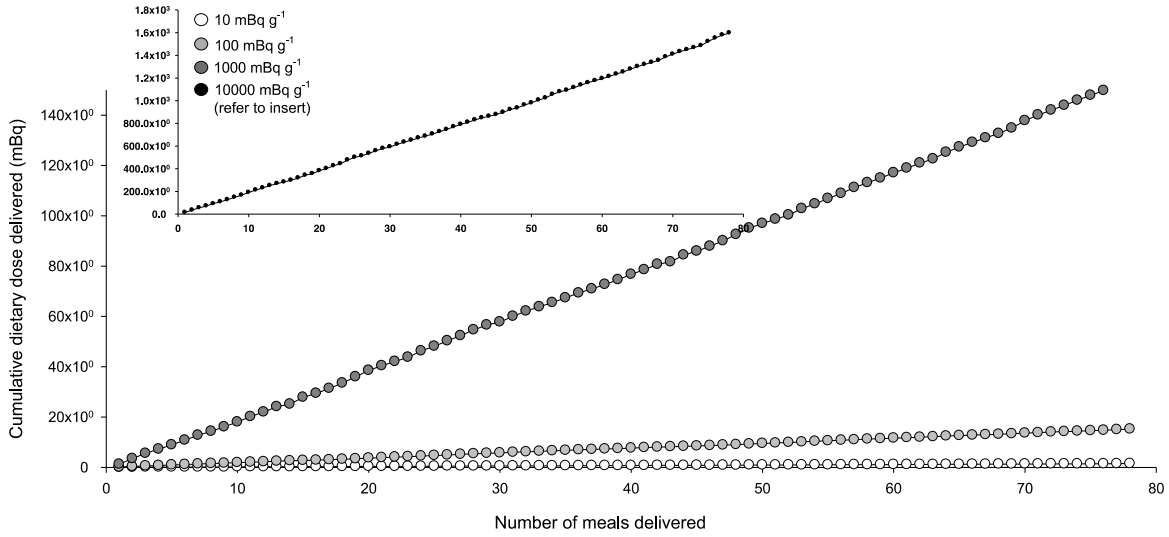

Figure 1a: Cumulative activity (mBq) delivered to the FHM during the breeding experiment

Fathead minnow. Mean daily food delivery.

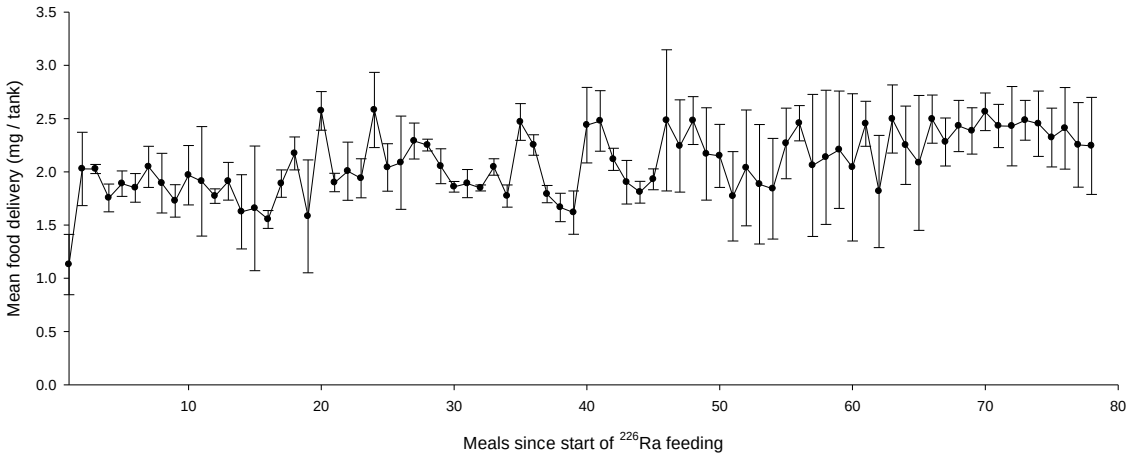

Figure 1B: Mean food delivery for FHM (mg/tank) maintained on a radium diet for 80 days

Figure 2 A and B

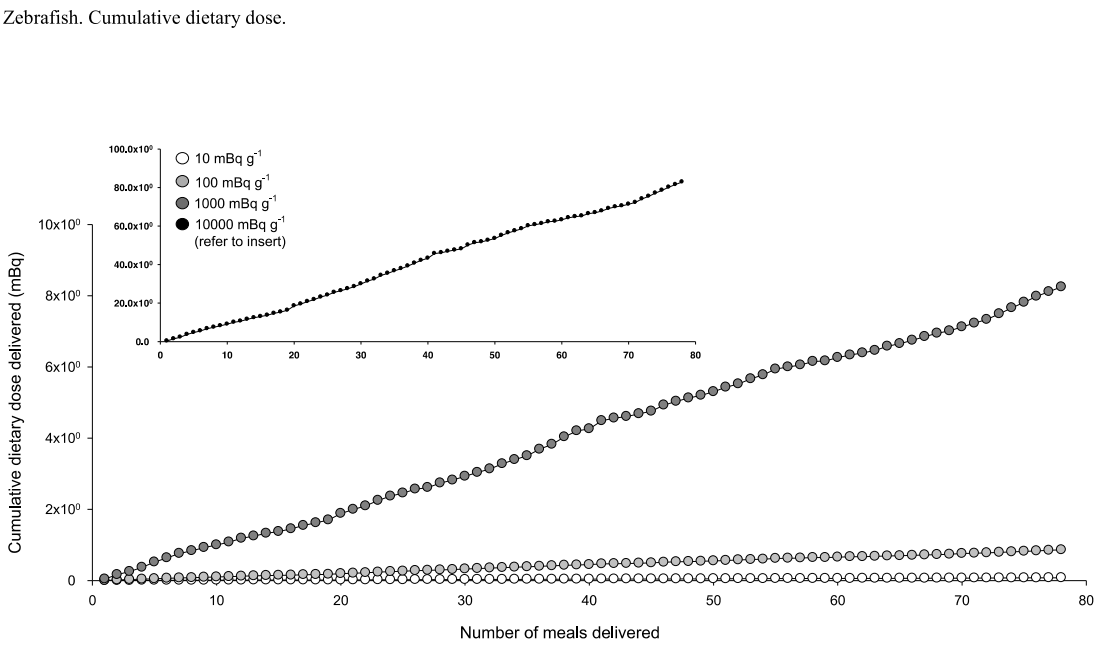

Figure 2A: Cummulative activity (mBq) delivered to zebrafish over the course of the breeding experiment

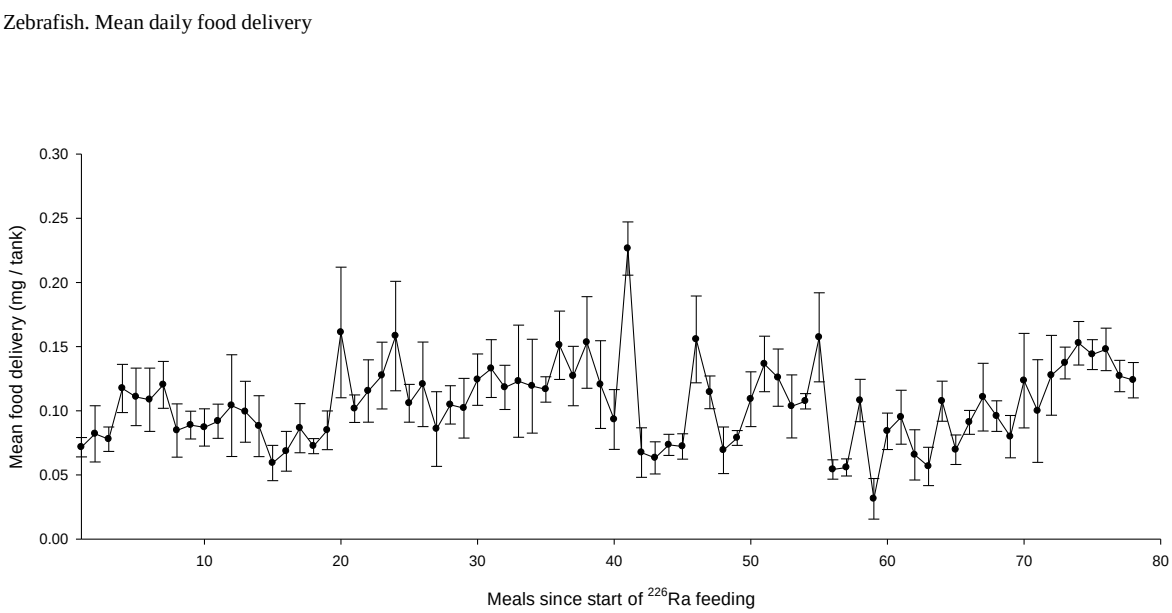

Figure 2B: amount of food delivered to zebrafish (mg/tank).

Relative biological effectiveness (RBE):

A further study aimed at determining the RBE was also conducted. Here Fathead minnow were given an acute injection of Ra-226 equivalent to the total dietary dose over 6 months to a fish on a chronic 100mBq/g diet. A second set of fish were given an x-ray dose equivalent to the Ra-226 dose. The effects are detailed in paper 4 of the series which has been prepared for submission to Int. J. Radiat. Biol. The RBE for the endpoints examined is 1. i.e acute radium injection and a similar x-ray dose had the same effect on specific growth rate and on the stress assay endpoints.

These data are all discussed in the appropriate discussion section in part C but our conclusion is that Ra-226 at environmentally relevant levels as no significant effect on growth or reproduction in the species tested and that for the activity range and endpoints examined, the RBE for radium is 1.

4.4 Based on the results, derive Critical Toxicity Values (CTVs) and Expected No Effects Values (ENEVs) for fish in terms of daily intake rates, equilibrium tissue concentrations, and dose to critical tissues. Data sets, in the format of EXCEL spreadsheets, should he submitted with the final report. The quality and quantity of these data should follow the guidance outlined in Environment Canada (2005).

For the fathead minnow study, there was no toxicity at radium levels over 1,000 times greater than those seen in gut contents in fish from uranium mining areas in Canada. In fact, over the two years of the study, the fish appeared to adapt and selectively remove accumulated radium from their bodies. The repeat experiment confirmed this as did an early pilot experiment. The dosimetry data are now published in Int. J. Radiation Biology. A copy is attached (paper 2 in the paper appendix 1). To summarize we calculate the ENEV to exceed an intake by ingestion of 10Bq/g food over a fathead minnow's lifetime. CTV could not be estimated from these data but obviously also exceed 10Bq/g food and as this is 1000 times the level of radium in gut contents from fish in Canadian uranium mining lakes it is unlikely to be a problem. The significance of this is discussed in part c.

For the zebrafish study using similar concentrations of radium, there was a reduction in egg number and in egg viability in the F1 generation groups which were continuously fed radium but not in the fish where radium was discontinued after the F0 generation bred. The effect was not dose dependent and was seen to the same extent in the 10mBq and 10Bq groups. We suspect it is an aberrant result due to the abnormally high fecundity of the controls in that particular group which produced a total of 691 eggs compared with a mean of 533 eggs for the other 3 control groups. If the mean is used there is no significant effect in the F1 continuously fed fish (see tables 1 and 2 and excel chart contained in the 2013 FHM and Zeb folder). If the actual data are accepted we would estimate that the CTV for zebrafish multigenerational reproduction is <10mBq/g food if rejected as discussed then there is no effect and the ENEV and CTV are > 10Bq/g food

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. 4.6 Perform comparative dosimetry and radiochemistry of alpha-emitting radionuclides (e.g. Ra-226) in fish (micro vs. macro effects, including the behaviour of Rn-222). The focus should be on dose quantification for estimating and predicting higher-level organismal alpha effects.

There was no bioaccumulation after 6 months in fathead minnow and accumulated radium was depurated completely by 18 months. Autoradiography techniques (collaboration with AECL) and synchrotron techniques (collaboration with European Synchrotron facility at Grenoble in France) were used to determine microdosimetry of radium in the 24 month samples but no evidence of any radium tracks was found despite the use of very sensitive methods and autoradiographic exposure times exceeding 4 months. The positive control radium injected fish showed significant accumulation of radium using both LSC and autoradiography (Autorads shown in Appendix 3). The data are presented in the section on dosimetry and are being prepared for publication. The LSC data showing relative amounts of Ra-226 and radon daughters are contained in the Master's thesis produced by Manuela Thompson and appended to this report (Appendix 2 Theses). In regard to dose quantification, there is no dose accumulated even after ingesting the highest dietary activities. These data are discussed.

Section c

DETAILED REPORT AND DISCUSSION OF AS YET UNPUBLISHED DATA

Alpha feeding experiments:

A population of fathead minnows (FHM) ingesting a diet containing ^{226}Ra (10mBq-10Bq/g of food) was maintained from "cradle to grave". The fish were monitored at 3-month intervals for impacts on K factors, biochemical growth indices and stress/bystander signalling. The data reveal deviations from control values at 3 and 6 and 12 months which stabilised at 15 months. By 18 and 24 months a reverse trend was seen with evidence of adaptive responses. The growth data were submitted for publication to *International Journal of Radiation Biology* and are now published and the discussion is contained in the paper (Paper 1 in appendix 1).

Stress signalling and bystander effects were evident in all the ^{226}Ra fed fish and were most pronounced in the 100 and 10Bq/g fed groups at 6 months but remained stable in spite of the growth changes out to 24 months (Figure 1). The 1000mBq points are all suggestive of a growth inducing signal. We do not understand the biphasic response but suggest it may point to a transition point in the induction of a new "coping mechanism" These data are being prepared for publication and therefore are presented and discussed in detail.

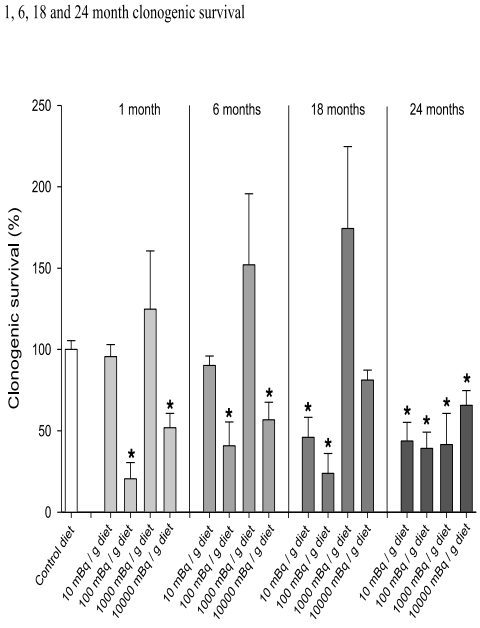


Figure 1 Bystander signal strength with time

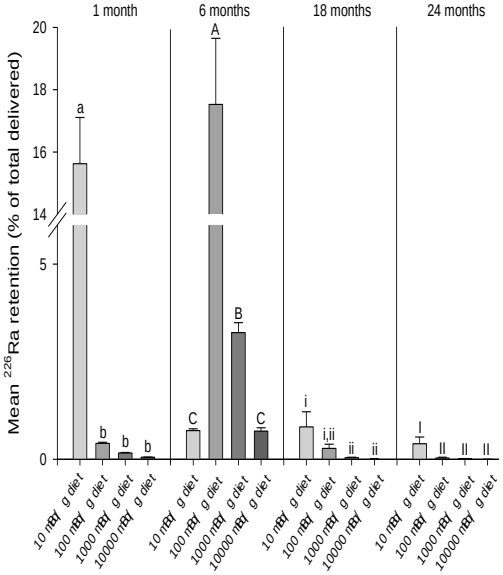


Figure 2 Radium retention with time

Our hypothesis is that the stress signalling is driving the generation of an adaptive response. The fish did not breed despite considerable efforts to induce them to. However in salmonids exposed to x-irradiation in a separate project, which resulted in successful breeding, the F1 but NOT the F2 generation showed evidence of stress signalling, this is discussed later in the RBE section. Another major result from the "cradle to grave" experiments is the individual variation within the fish cohorts even though these were obtained from the United States Environmental Protection Agency and are used for their routine toxicity testing. Because this variability in response to chronic low dose exposure is probably real and important in the environment, our data are presented as distribution curves as well as means with errors. We hope this approach will help the development of population- or system-based fitness indices which is a major goal of the regulatory bodies

concerned with the biota issue. The biggest surprise from the 24-month experiment is the dosimetric analysis result. This shows very clearly (Figure 2) that over the course of the experiment, the radium bioaccumulated for 6 months but was almost completely decorporated by 18 months and gone completely by 24 months in males and immature fish although there is some radium still measurable in the females (Table 1). This very important result suggests induction of a novel purging mechanism by the fish resulting in their eliminating already accumulated radium and preventing further uptake. These results have just been published (Paper 2 in Appendix 1) so are referred to here just to emphasise the importance of this paper.

Control diet

ID	Activity (Bq kg^{-1} wet)	Annual dose (mGy y^{-1})
Background (all fish)	2.7 ± 9.3	0.1 ± 0.3

10 mBq g^{-1} diet

ID	Activity (Bq kg^{-1} wet)	Annual dose (mGy y^{-1})
Sexually mature males	4.7 ± 5.6	0.12 ± 0.14
Sexually mature females	5.9 ± 7.1	0.15 ± 0.17
Immature fish	0.6 ± 1.6	0.02 ± 0.09

100 mBq g^{-1} diet

ID	Activity (Bq kg^{-1} wet)	Annual dose (mGy y^{-1})
Sexually mature males	3.1 ± 3.8	0.1 ± 0.1
Sexually mature females	0.0 ± 0.0	0.0 ± 0.0
Immature fish	4.5 ± 10.0	0.1 ± 0.2

1000 mBq g^{-1} diet

ID	Activity (Bq kg^{-1} wet)	Annual dose (mGy y^{-1})
Sexually mature males	11.8 ± 10.3	0.3 ± 0.3
Sexually mature females	10.1 ± 4.2	0.2 ± 0.1
Immature fish	23.1 ± 13.3	0.6 ± 0.3

10000 mBq g^{-1} diet

ID	Activity (Bq kg^{-1} wet)	Annual dose (mGy y^{-1})
Sexually mature males	39.2 ± 9.4	1.0 ± 0.2
Sexually mature females	14.9 ± 17.8	0.4 ± 0.4
Immature fish	55.0 ± 21.0	1.4 ± 0.5

Table 1: Dosimetry broken down by gender

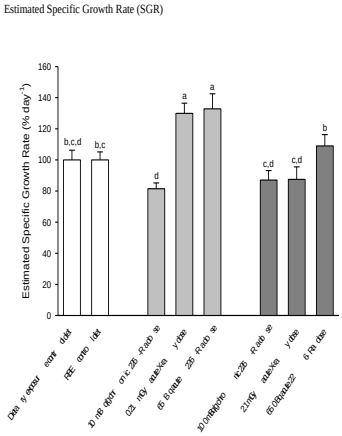


Figure 3a: α v γ SGR

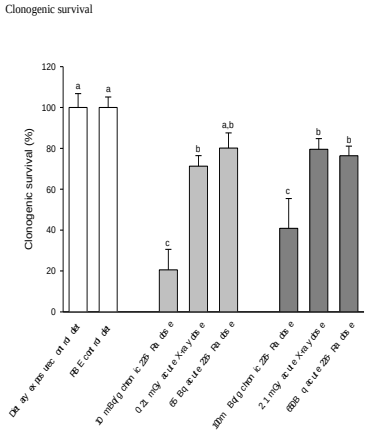


Figure 3b: ditto for bystander

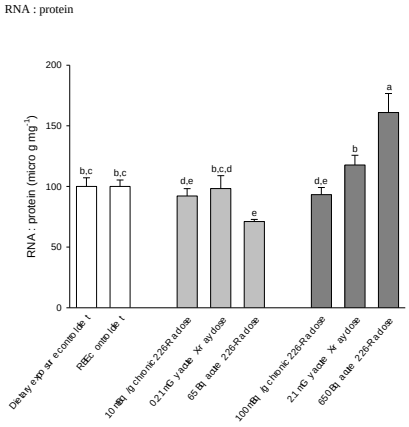


Figure 4a RNA:protein α v γ

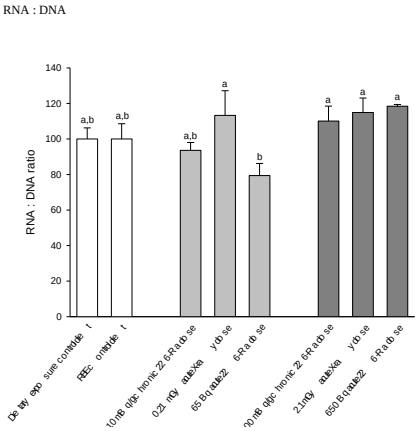


Figure 4b RNA:DNA for α v γ

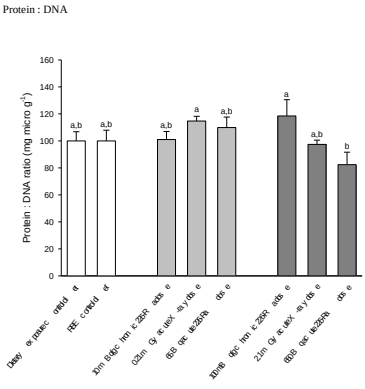


Figure 4c Protein:DNA for α v γ

Fish Dose Response:

Cumulative dose after two years

The side aim of these experiments was to establish a dose and dose rate effectiveness factor (DDREF) for alpha radiation exposure to non-human biota (fish) and also to establish an RBE (relative biological effectiveness) value. As stated earlier and in the published dosimetry paper, there was no radium in the fish bodies at 24 months despite evidence of activity related bioaccumulation at 6 months. The methods used included liquid scintillation counting, total body gamma accumulation, autoradiography using sections and

synchrotron analysis. All techniques confirmed that there was no detectable radium or radon daughters at 24 months when the fish were at the end of life stage and the experiment was terminated. This is in spite of earlier bioaccumulation and stable feeding throughout the two years with activities at or up to 1000 times the levels seen in gut contents of fish from lakes in the Elliot Lake region of Canada. This finding is a surprise and suggests the bioaccumulation models currently in use may need to be reviewed.

Relative Biological Effectiveness (RBE)

There is no good way of getting an alpha RBE – use of a gamma emitting isotope (e.g. ^{137}Cs) in a chronic feeding regime is problematic because of the chemistry associated with the gamma emitter which is different to the chemistry of radium. After much discussion we decided to give an acute x-ray dose equivalent to the projected lifetime radium dose to swim-up stage FHM and sample them at the times corresponding to radium fed cohorts. Data for 6 and 12 months show clear effects of both x-ray and radium treatment given as acute one off dose (Figure 3). The bottom line is however that for these endpoints and doses the RBE is 1.

Dose and dose rate effectiveness factor (DDREF)

For DDREF estimation, we compared the acute data from the x-ray and alpha fed fish with the corresponding time points in the chronic feeding study. The results in Figure 3 and Figure 4 suggest a DDREF which varies with dose, and with endpoint. This is not really all that surprising! Another important study was the assessment of the changes to the proteome associated with radium exposure. The data are summarised (Figure 5). Basically the proteome changes are very similar to those seen in trout and medaka exposed to external x-rays – i.e., the major proteins to change are annexins (associated with growth and, in mammals, carcinogenesis). Changes in structural and energy mobilising proteins are also seen. These data have just been accepted for publication in Int. J. Rad. Biol. (paper 3 in appendix 1)

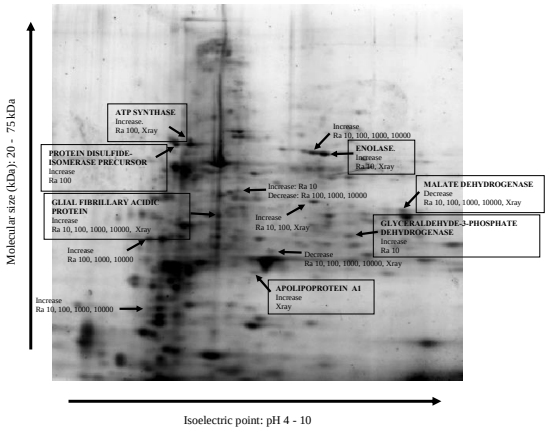


Figure 5: proteome showing ID's which changed.

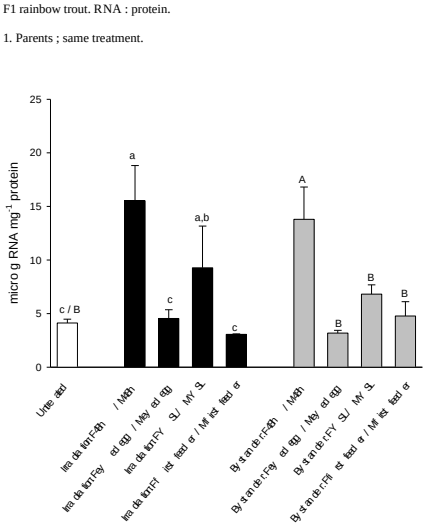


Figure 6: F1 generation of trout treated at Fo

Fish x-ray studies: In addition to the CNSC funded work with alpha particle exposure we also looked at the F1 generation of the trout established in the associated IRC research. We confirmed in that project that a very low dose acute exposure to x-rays at the early life stages, resulted in effects in the F1 generation (Mothersill, et al., *IJRB* 2010). We have since obtained data for other endpoints for F1 fish showing that the biochemical indices, particularly for the progeny of trout given a one off dose at 48hrs post fertilisation, show effects. Figure 6 shows examples of these data for RNA:protein ratios. These fish were bred two year ago and the F2 progeny were examined one year ago but did not show continued expression of radiation induced endpoints seen in F0 and F1 fish.

Zebrafish Reproductive Study

Eggs produced by zebrafish generation F0-F1

Zebrafish from the McMaster colony were established on the radium diets as soon as they started feeding. 100 zebrafish were initially established on each diet giving a total of 500 fish in the initial experiment. Food delivery averaged 20mg/fish/day over the duration of the experiment. The fish remained on the diets until egg laying began at 70 days post start of the radium diets. At this point 50 fish per tank were taken off the diets and returned to normal food. The other set remained on the diets throughout reproduction and the progeny were also maintained on the radium diets.

Breeding tanks were established as soon as the sex differences became obvious. 10 fish were placed in each breeding tank – 5 males and five females. The pairs of fish were placed in specially constructed 1.5l tanks fitted with a mesh, which allowed the eggs to pass through. The mesh was covered during the feeding time so food could not pass through. The fish sexes were separated using a plastic divider except once a week when they were allowed to mate using the protocol developed by Bresch et al (1986). This was to standardise the experiment and minimise the workload.

Eggs were collected each time the fish were given an opportunity to lay and were scored as viable using the criteria established in the literature (viable eggs remain clear after laying while non-viable eggs become opaque within 24hrs). Viable eggs were kept to determine hatchability and to provide fish for the F1 measurements. No significant effects of any diet were seen even though the dietary concentrations ranged from environmentally relevant levels seen in gut contents from radium contaminated lakes (Clulow and Pyle 1987) to 1000 times that level. The experiment was therefore stopped at the F1 stage. Carcasses have been preserved for autoradiographic analysis.

Table 1a: Radium discontinued at start of egg laying

Activity mBq/g food	Total eggs F0	Viable eggs % F0	Hatchability of viable eggs %	Total F1	Viable eggs % F1
Control	550	50.1	98.1	520	47.4
10	670	49.3	96.4	575	45.6
100	583	51.4	95.2	586	43.9
1000	653	46.1	94.5	657	44.1
10000	667	44.5	94.8	602	47.1

Table 1b: Radium diet continued to F1 generation

Activity mBq/g food	Total eggs F0	Viable eggs % F0	Hatchability of viable eggs	Total eggs F1	Viable eggs % F1
Control	535	45.3	92.4	691	45.4
10	566	47.1	97.6	634	41.6
100	632	49.2	89.8	571	39.9
1000	604	46.3	92.1	603	42.6
10000	578	44.5	90.7	587	40.1

Table 2 Raw data of egg counts

Raw egg data: Radium discontinued at start of egg laying

Raw data: eggs collected per week from F0 fish

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	109	87	90	120	144	550
10	104	138	142	136	150	670
100	103	138	115	98	129	583
1000	145	106	163	185	54	653
10000	128	155	97	168	119	667

Raw data: eggs collected from F1 fish

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	110	136	84	106	84	520
10	121	112	156	90	96	575
100	65	81	123	76	141	586
1000	109	82	156	148	162	657
10000	74	128	181	95	124	602

Discontinued feeding

Viable from F0 fish

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	58	63	25	41	89	275
10	65	54	49	72	90	330
100	71	62	43	69	55	300
1000	64	81	80	53	23	301
10000	56	48	83	78	32	297

Viable from F1 fish

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	65	48	53	42	38	246
10	45	28	36	46	107	262
100	53	43	50	67	64	257
1000	71	55	55	70	39	290
10000	23	56	84	60	60	283

Radium continued throughout breeding

F0 data total egg counts

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	101	93	122	108	111	535
10	110	98	86	131	141	566
100	141	126	149	120	96	632
1000	91	132	110	139	132	604
10000	121	108	119	129	101	578

F1 data total egg counts

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	145	106	124	150	166	691
10	115	146	149	138	86	634
100	98	111	126	90	146	571
1000	125	131	96	128	123	603
10000	101	121	97	130	138	587

Radium continued throughout breeding

Viable from F0 fish

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	58	47	43	60	34	242
10	41	57	48	45	76	267
100	74	59	63	67	48	311
1000	49	61	55	40	75	280
10000	50	63	61	48	35	257

Viable from F1 fish

Activity (mBq/g food)	Week 1	Week 2	Week 3	Week 4	Week 5	Total
Control	74	48	42	56	94	314
10	39	47	61	58	59	264
100	43	49	56	39	31	228
1000	48	48	62	56	43	257
10000	37	39	48	54	57	235

Table 3

Statistical analyses

Two-way ANOVA analysis for Fish data

Summary Table 3a

Activity (mBq/g)	Generation	Discontinued Radium Mean Egg Count	Continued Radium Mean Egg Count	Discontinued Radium Mean Viability	Continued Radium Mean Viability
Control	F0	100.0 ± 4.5	100.0 ± 8.0	100.0 ± 19.5	100.0 ± 10.0
	F1	100.0 ± 7.6	100.0 ± 9.3	100.0 ± 9.6	100.0 ± 15.1
10	F0	105.8 ± 9.5	121.8 ± 6.0	119.6 ± 13.1	110.3 ± 12.9
	F1	91.8 ± 8.5	110.6 ± 11.2	106.5 ± 28.5	84.1 ± 6.7
100	F0	118.1 ± 8.6	106.0 ± 5.8	108.7 ± 9.2	128.5 ± 8.9
	F1	82.6 ± 7.3	93.5 ± 14.2	112.6 ± 9.1	69.4 ± 6.8
1000	F0	112.9 ± 8.3	118.7 ± 17.8	109.1 ± 19.3	115.7 ± 12.2
	F1	87.3 ± 4.6	126.3 ± 14.8	117.9 ± 12.0	81.8 ± 5.4
10000	F0	108.0 ± 4.6	121.3 ± 9.8	107.6 ± 19.3	106.2 ± 10.4
	F1	84.9 ± 5.8	115.8 ± 17.4	115.0 ± 19.8	74.8 ± 6.3

Table 3b: Statistic Summary

Variables	Endpoints	Significance (P-value) Discontinued Radium	Continued Radium
Activity (mBq/g)	Egg Count	0.263	0.982
	Viability	0.927	0.881

Generation	Egg Count	0.934	<0.001*
	Viability	0.894	<0.001*
Interaction between Generation and Activity	Egg Count	0.602	0.159
	Viability	0.968	0.081

*Significant difference ($p < 0.05$) from control by using a two-way ANOVA and Tukey's post-hoc.

Conclusion from the study

While there is a statistically significant drop in the F1 continuous feeding cohort, this is not activity dependent and appears to be due to a high control seen in the raw data in table 2 which is significantly higher than the mean of the other control groups (all should be the same). We are therefore inclined to flag this but do not think it is significant.

Mechanistic studies:

Low dose radiobiology is now known to involve novel mechanisms which do not occur or are masked following high doses. These include bystander signalling, protein induction/down-regulation and other active mechanisms aimed at re-establishing homeostasis. Understanding these mechanisms and how they behave in different species, environments, genetic backgrounds and through the life cycle is crucial for understanding risk of adverse effects from low dose exposure. The group put effort into this area and demonstrated a role for calcium, p53, TGFb, functional DNA repair processes and serotonin (5HT) in modulating low dose response. A key development during this project was the in vivo demonstration of effects occurring in cell culture which establishes the relevance of these studies for radiation risk estimation. We have now demonstrated that in vivo exposure of zebrafish to a serotonin inhibitor (reserpine) prevents inter-animal bystander signalling. We also confirmed a role for DNA repair in medaka fish, which we also demonstrated in vitro. Finally, the absence of signalling in neutron irradiated cells was confirmed in neutron irradiated fish, providing a tool with which to probe the mechanisms further. We also examined the issue of multiple stressors where radiation is one of many stressors to which biota are simultaneously exposed. Both depleted uranium and heavy metals including copper, aluminium and cadmium were studied in collaboration with Professor Salbu's group in Norway. Where the data have been published as papers or presented as posters they are also listed in the paper section and copies are attached. Another mechanistic development is the demonstration that irradiated cells emit UVA photons in a dose dependent manner. These photons induce bystander/stress responses in unirradiated cells. We have also demonstrated this in vivo in zebrafish. Two manuscripts have been submitted containing these results which strongly suggest the bystander signal may have a physical component. These papers can be supplied on request and although not directly funded under the CNSC contract they are important in understanding low dose effects.

Section d

Development of new Dosimetry Techniques

Radium dosimetry is very complicated. It involves estimations of uptake from the gut and biological half-life. There are established transfer factors relating food intake to bioaccumulation but in the case of fish these vary considerably depending on diet and habitat and as demonstrated in the preceding section, after about 12 – 18 months, levels actually decline. This puts the reliability of the theoretical transfer and biological half life figures in considerable doubt. To be really informative it also needs to measure radon daughter activity over time. **It must be stressed here that no group has ever done a laboratory experiment where fish were fed a single isotope for 2 years (effectively cradle to grave). Therefore the data generated by this project are of major interest to groups like the IAEA MODARIA project, which is trying to use published data to improve modelling**.

Development of the low level ^{226}Ra activity analysis:
In order to analyze the activities of ^{226}Ra and its daughters accumulated in fish, following the Liquid Scintillation Counting (LSC) study reported in the 16-month progress report, we are currently carrying out the 4π gamma-ray spectrometry study, which will enable us to determine the relative contributions of radium to radium daughters in live fish. An advantage of this technique in contrast to LSC is the capability to keep fish alive while a shortcoming is the low gamma emissions per decay. Along the ^{226}Ra decay chain, the relatively intense gamma lines are:
^{226}Ra: 262 keV (0.005 γ per decay), ^{214}Pb: 295 (0.21 γ), 352 keV (0.40 γ), ^{214}Bi: 609 keV (0.46 γ per decay)
Therefore, the total gamma emission per decay even in an equilibrium condition is only 1.08 while the total alpha emission per decay is 4.0. Another critical challenge in gamma spectrometry for low level analysis is the low efficiency of gamma detection. Conventional HPGe or NaI(Tl) gamma-ray detectors have very limited detection efficiencies mainly due to their small solid angles. In contrast, our 4π NaI(Tl) gamma-ray spectrometer offers an extremely high efficiency (about 70% peak efficiency at the gamma energies listed above). As our spectrometer required a significant upgrade in pulse processing system for ^{226}Ra measurements, we built a new digital pulse processing system in 2011. In 2013 and 2014, the gamma-ray spectrum analysis algorithm was optimized and the spectrometer was characterized for ^{226}Ra analysis (Figure 7). The ^{226}Ra detection limit for a small fish phantom is typically 0.99 Bq for 1 hr counting (0.21 Bq for 24 hr). For real live gamma-ray measurements, the correction for the gamma-ray self-attenuation effect within a fish and its water container is extremely complicated. In order to make the correction simple, extensive MCNP Monte Carlo simulations were carried out (Figure 8). Measurements for live fish injected with ^{226}Ra are planned in 2014 and 2015.

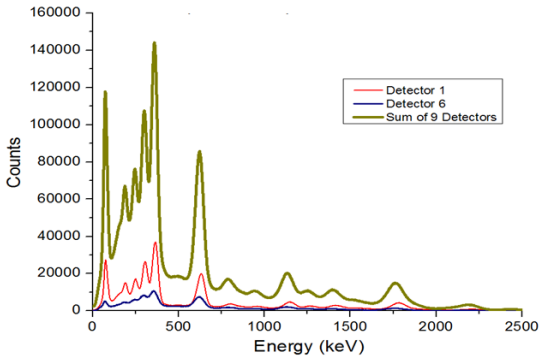


Figure 7 ^{226}Ra gamma-ray spectrum collected by the 4π NaI spectrometer.

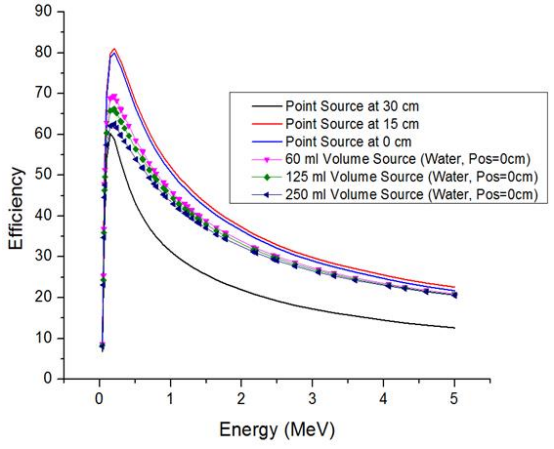


Figure 8 MCNP simulations: peak efficiencies for point and volume sources.

The radiation dosimetry development of optimized techniques

THGEM imaging detector development:
For two-dimensional alpha activity distribution measurements, we are developing an imaging detector based on a Thick Gas Electron Multiplier (THGEM). Although the original motivation was to image alpha particles, the detector can be applied for imaging X-rays and even neutrons when a neutron converter is incorporated.
As shown in Figure 9, THGEM is an insulator foil, copper clad on each side, and perforated with high density holes. When a high voltage is applied between the copper clads, a strong electric field is formed, making possible electron multiplication (i.e. accelerated electrons create additional electrons through ionization). Its outstanding feature in contrast to classical gaseous radiation detectors is the absence of wire electrodes. A unique feature of our THGEM over the standard gas electron multiplier (GEM) is a "thicker" insulator (typically 0.5 mm), which makes hole fabrication much easier. During the previous IRC term, we have successfully developed the world's first THGEM microdosimetric detectors, which is a solid foundation for both an imaging detector and an advanced neutron dosemeter developments.

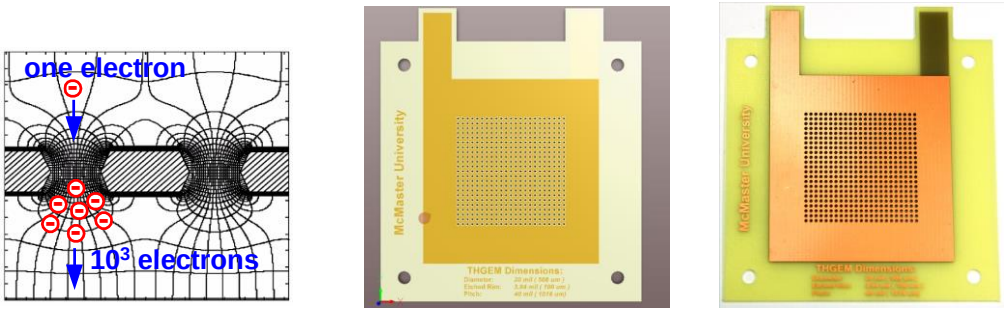


Figure 9 Electron multiplication principle of THGEM and a THGEM board (hole dia.: 0.5 mm, pitch: 1.0 mm) fabricated for the prototype imaging detector.

We fabricate THGEMs using the computerized drilling method. As this technique is common in electronics for printing circuit board (PCB) fabrication at inexpensive cost, we compared a number of PCB companies and found one with good quality as shown in the figure. To select good quality THGEMs, we established a quality control, which includes a visual hole inspection using a microscope and a signal strength observation using an alpha source.
The layout of the prototype THGEM imaging detector is shown in Figure 10. The detector consists of four regions. The sensitive volume and the induction region are filled with a proportional gas. For gas pumping and vacuum sealing, an aluminum chamber (not shown in the figure) encloses the detector assembly. The entrance window of the chamber is made of thin Mylar so that alpha particles or X-rays can penetrate. The top layer of the detector assembly is the cathode and biased negatively. Depending on imaging applications, either a cathode made of thin A-150 conducting plastic (for alpha imaging) or another one made of copper coated Mylar (for X-ray imaging) can be installed.
When a radiation particle is incident on the detector, gas molecules are ionized and the electrons drift toward the THGEM by the drift electric field produced by the potential difference between the cathode and the top conducting layer of the THGEM. Then electrons enter one of the THGEM holes and are multiplied within the holes. After multiplication, electrons are finally collected by the 2-D readout board.

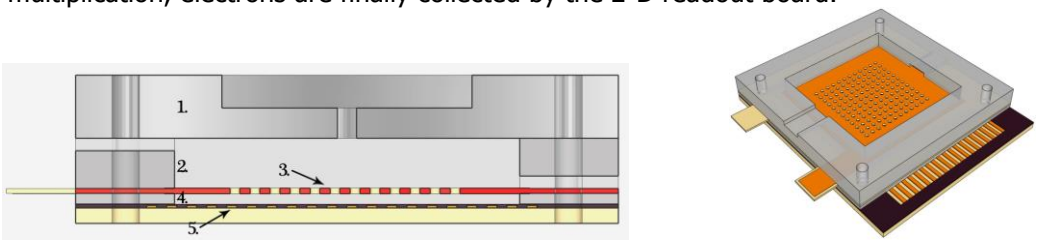


Figure 10 Layout of the prototype THGEM-based alpha imaging detector. 1) Cathode 2) Sensitive volume 3) THGEM 4) Induction region 5) 2-D readout board & delay-line

The core work of the THGEM imaging detector development is to devise a simple and efficient 2-D position readout board. We designed and fabricated a readout board based on the delay line principle, which is explained in Figure 11 (a) for the 1-D case. As shown in the figure, electrons collected on a readout pad will travel into one of the nodes of the delay line, which is coupled to the arrival position of the electrons. At this point, the signal will split and travel down the left and right side of the delay-lines. The signals which exit the delay-line will have experienced different amounts of time delay depending on the number of delay cells traversed. The original electron arrival position could then be extracted by measuring the time difference between the output signals of the delay-line.

We fabricated a 1-D delay-line board and its test results were successful. Following this promising result, we have developed a 2-D delay-line readout board during the 36-month reporting period. To identify the X and Y positions, we designed the readout board such that the arriving electrons are evenly divided into X and Y nodes as shown in Figure 11 (b). The X and Y nodes at each position are electrically separated and their axes are orthogonal to each other, which enables us to recognize the 2-D position.

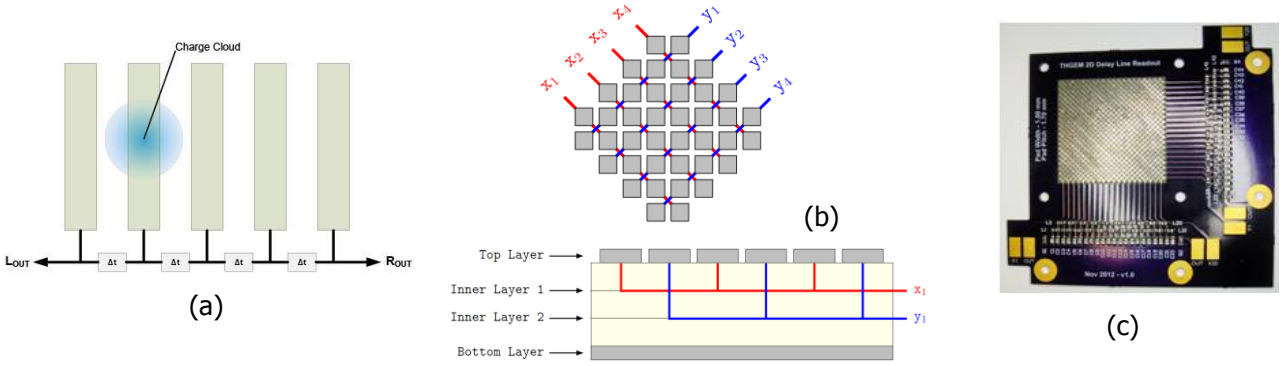


Figure 11 Delay-line position readout principle, 2-D readout board design and fabricated board.

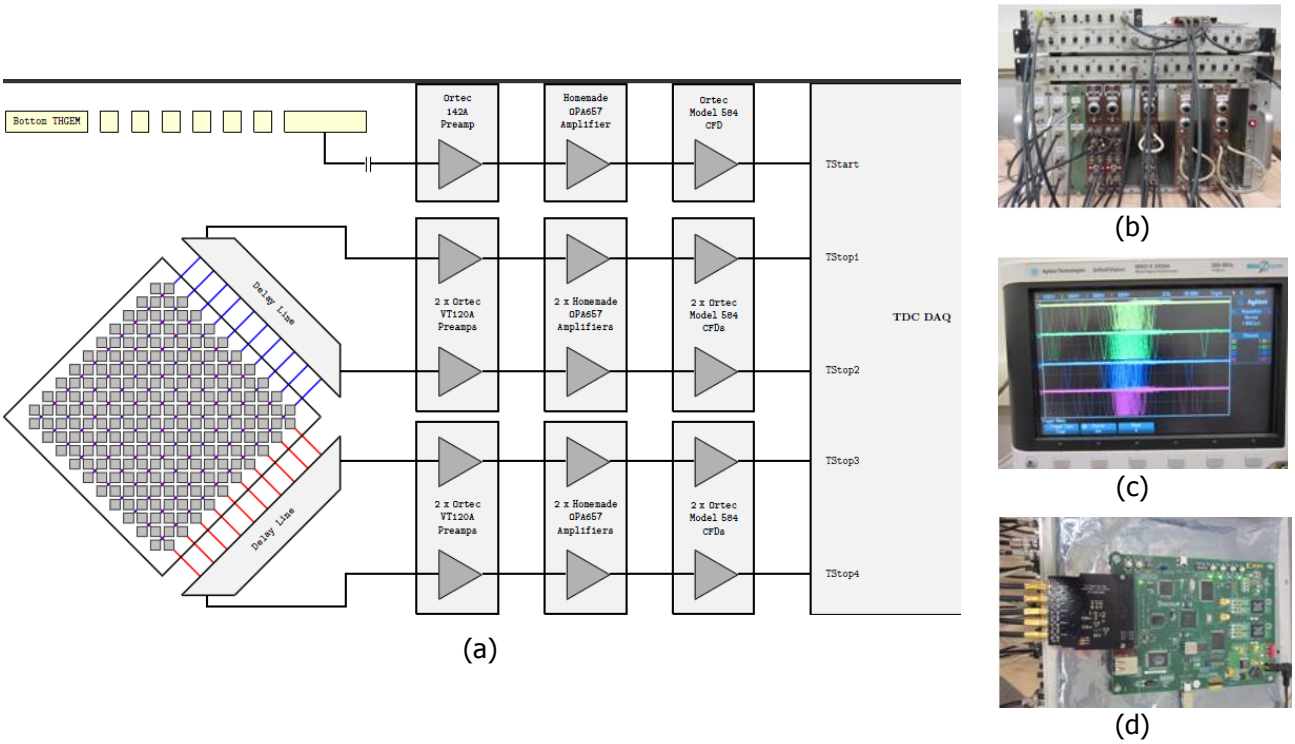


Figure 12 Block diagram of the signal processing for 2-D position readout (a), photographs of the signal processing electronics (b), the signals captured at the constant fraction discriminators which pick up the arrival times of the charges at both ends of each axes (c) and the assembly of the TDC and FPGA boards (d).

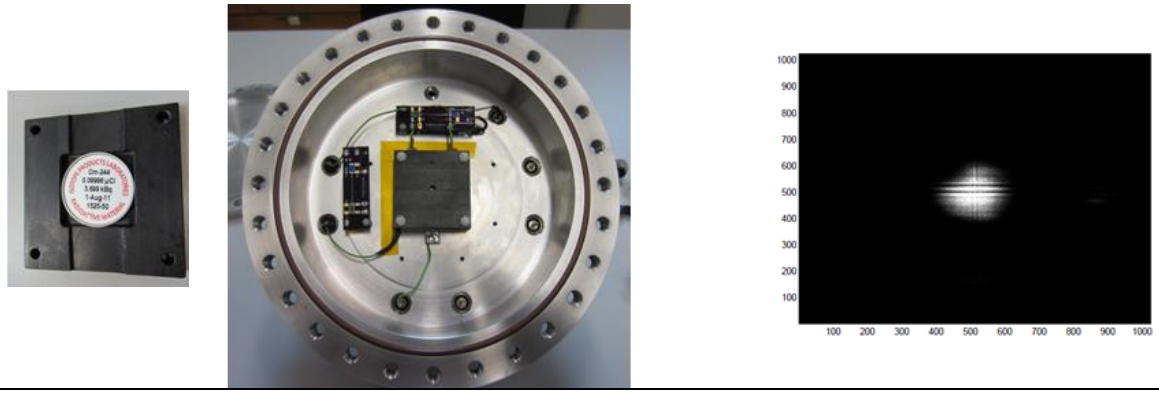
Figure 12 shows the block diagram of the signal processing. We designed the signal processing system in digital architecture. As shown in the figure, in addition to the four signals from the X and Y delay lines, there is another signal collected at the THGEM bottom. This signal functions as a trigger signal, which notifies the signal processing system of the occurrence of a detection event. In contrast to the other four signals, the trigger signal does not go through any time delay and therefore it arrives earlier than the other four signals and is used as a reference for arrival time measurements.

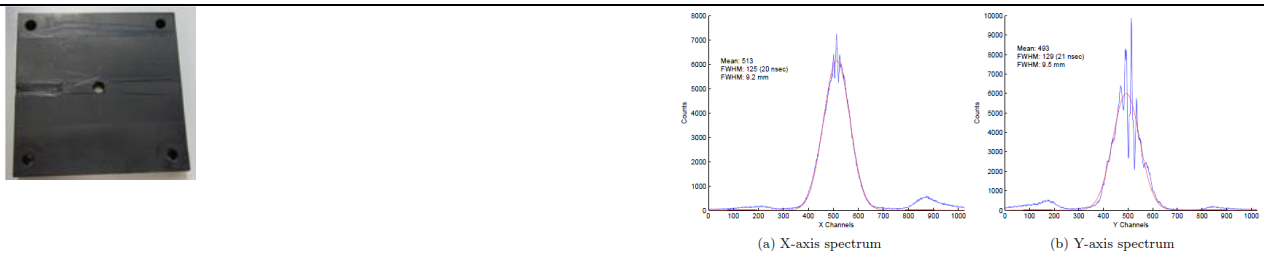
Each signal is picked up by a fast preamp, amplified by a wide band amplifier and then the timing output signal is generated by a constant fraction discriminator (CFD). The assembly of the electronics and the CFD output signals captured by an oscilloscope are shown in the figure. The signals are mostly distributed in the time range corresponding to the delay line circuit as designed.

The timing outputs of the four CFDs are digitized by a time to digital converter (TDC). We carefully reviewed TDC products available and chose the model TDC-GPX56 (Acam), which has four input channels. For each detection event, the CFD output of the trigger signal triggers the TDC clock and the arrival times of the other four CFD outputs from the X and Y delay lines are measured with respect to the trigger signal. TDC digitizes the arrival time of each CFD output and then send the digitized time difference data to the next step.

The simplest way of image data collection is to transfer the four TDC output data of a detection event to a PC each time and then do image reconstruction after data collection is completed. Although simple to implement, this data transfer method takes a significant time for each detection event and therefore, the throughput is quite limited. A more efficient way is to compute the arrival time difference using an external processor and save the data in a local memory, so that the data transfer traffic is minimized. To this end, we added a field programmable gate array (FPGA) processor (model Xilinx SP601) next to the TDC. In order to have the FPGA processor function as an image processor required for THGEM imaging, we programmed it to compute the time difference of the signals from each axis and then save them in its memory. The local memory of the FPGA was divided such that it has multi-channels for the X-axis time difference and Y-axis time difference. Hence, when a detection event happens, FPGA finds the memory location corresponding to the X and Y time differences, and adds a count to that location. Using an electronic pulser, the time difference in each axis is conveniently calibrated in terms of the spatial distance in each axis, so the data collected in the FPGA memory is the exact image data we want to collect. With this design, we can handle a counting rate of 10,000 counts/s without problem. The development of the TDC based signal processing system was submitted to Nucl. Instr. Meth. A (appendix H).

Figure 13 A copper grid (1 mm diameter and 3 mm pitch) and its X-ray image collected by the THGEM detector





(a) X-axis spectrum (b) Y-axis spectrum

Figure 14 ^{244}Cm alpha source with a collimator and its image collected by the THGEM detector
(Spatial resolution: X-axis: 125 ch (= 20 nsec) ➡ 9.2 mm / Y-axis: 129 ch (= 21 nsec) ➡ 9.5 mm)

The THGEM imaging detector was extensively tested in 2013. Figure 13 shows an X-ray imaging example. As an imaging object, a copper grid (1 mm diameter and 3 mm pitch) was positioned in front of the detector and its X-ray image was collected. As shown in the figure, the collected image gives an overall feature of the grid. Another test result is shown in Figure 14 for an alpha source. As the alpha source activity is limited, we simply installed a circular slit collimator. As shown in the alpha image, the alpha beam is dispersed due to the thin slit collimator and the size of the image (FWHM: 9.2 mm X-axis and 9.5 mm Y-axis) is larger than the slit diameter. In both X-ray and alpha images, we can see a pair of artifact lines at the center of both axes. We carried out systemic tests to find out what caused this artifact and deduced the artifact is generated by cross-talk at the input stage of TDC. In order to get rid of the cross-talk problem, we are currently modifying the TDC input stage circuit.

While this is a final report on the dosimetry problem of measurement of very low alpha emissions in tissues, the work is being continued at McMaster using NSERC funding. CNSC will be given access to the data as it appears.

Section e

Overall Research Outcomes and Recommendations

Overall the results produced over the last four years help to reduce uncertainties concerning the impacts of low environmentally relevant doses of internal high LET radiation in fish.

1. The conclusions from the zeb and FHM experiments are that there are no significant biological effects associated with chronic exposure to ^{226}Ra over the fish lifetime and no impacts on reproduction.
2. Other data produced in the laboratory using low external x-ray exposure can be used with the alpha data to try to reduce uncertainties concerning the RBE. These data strongly suggest that in the dose range tested and for very sensitive endpoints such as biochemical indices of growth and stress signalling, the relative biological effectiveness (RBE) is 1 for ^{226}Ra. Currently 20-40 is used in risk estimates.
3. The data further suggest that a mechanism for depuration of ^{226}Ra is induced. Levels of bioaccumulation are extremely low but measurable up to 6 months but the later time points show no radium at all in the fish. It must be emphasised that a study of this duration has never before been done under controlled conditions in a laboratory. Most chronic lab studies only last for a month or two and field studies are difficult to interpret because of all the confounding factors.
4. All the signalling data suggest low doses induce protective and adaptive responses, which makes biological sense.
5. The proteomics data also support adaptive and restorative changes as time feeding on food containing ^{226}Ra increases.
6. In terms of furthering our understanding of low dose radiobiology, the research has validated the importance of mechanisms previously only known to operate in vitro, using live fish. For the first time a model has been developed by our group, which enables the stress signalling mechanism to be

studied in a whole animal no part of which ever received a direct dose of radiation. This is highly important and has shown that at least in fish, low dose exposure to stressors such as radiation induces mechanisms that are protective and not destructive.

7. The successful development of a THGEM imaging detector opened up a new gaseous radiation imaging detector technology. Particularly, both THGEM detector and digital signal processing electronics were accomplished at inexpensive costs and therefore, our new technology will be a good alternative to the current imaging detector technology which mostly relies on expensive scintillator or semiconductor detectors. Moreover, the THGEM imaging detector can be further developed into neutron imaging detector and a large area neutron detector to replace ^{3}He counters used in nuclear security applications.
8. The Monte Carlo simulation results for the multi-element high LET dosemeter designs accomplished a quantitative analysis on efficiency enhancement for various multi-element geometries for the first time. Thanks to this simulation study, detector physicists can make a reasonable judgement on optimizing their multi-element detector designs or estimating efficiencies for existing detectors. The GEANT4 based simulation code we developed will help health physicists to enhance the dose equivalent response even better. A unique feature of our THGEM based design has made multi-element detector fabrication much easier and flexible. Once THGEM multi-element detector development is completed, it is expected that multi-element neutron dosemeters will be more popular and readily available, which will greatly enhance the accuracy of neutron dosimetry.
9. One of the invaluable outcomes we accomplished is that we developed all detectors, signal processing systems and data acquisition software for ourselves. This will allow us a tremendous degree of freedom for further enhancement of the THGEM imaging detector and developments of advanced detectors required in other application areas.

Recommendations for further work

1. The uncertainty regarding the zeb reproduction data needs to be eliminated.
2. The mechanism underlying radium depuration needs to be understood both in terms of biochemistry and in terms of the time frame needed to induce the depuration mechanism.
3. Reproductive data still needs to be obtained for a native Canadian species but it is hard to see how this can be done in the laboratory due to the technical difficulties and the natural Canadian variables such as temperature and light intensity which cannot be simulated in the laboratory
4. Multiple stressor studies are needed because uranium mining and milling which are major generators of radium in the environment do not occur without significant use of toxic compounds such as sulphuric acid which is used as the leaching agent in acid leaching whereas alkaline leaching not only extracts uranium from the ore, but also several other constituents like molybdenum, vanadium, selenium, iron, lead and arsenic, the uranium must be separated out of the leaching solution and end up in the tailings. Therefore the radioactive effects of radium are only part of the complex effects in the environment resulting from uranium mining activity.

Recommendations for action on these findings

1. The findings of this contract research should reduce uncertainties around the harmful effects of radium on fish exposed to low activities of the isotope. This finding needs to be disseminated to bodies like ICRP, IAEA and UNSCEAR for consideration.
2. The depuration data should be independently confirmed as they suggest the ICRP/IAEA transfer and bioaccumulation dada may need to be reconsidered in the light of the finding of no bioaccumulation in fish by the time they reached maturity.
3. A CNSC or International multiple stressor group should be established to determine how to go forward with meaningful studies in complex environments where single species and single agent toxicity do not exist.

PAPERS AND THESES PUBLISHED	SUBMITTED	ACCEPTED/ PUBLISHED
REFERRED JOURNAL ARTICLES:		
C. Mothersill, D. Lariviere, R. W. Smith, M.P. Thompson, S.H. Byun, W. Prestwich, C. B. Seymour Dosimetric analysis of fathead minnow (Pimephales promelas, Rafinesque, 1820) exposed via ingestion to environmentally relevant activities of Ra-226 for two years. *Int J Radiat Biol*, 2014 Feb;90(2):169-78		X
Carmel Mothersill, Richard Smith, Dominic Lariviere, Colin Seymour Chronic exposure by ingestion of environmentally relevant doses of ^{226}Ra leads to transient growth perturbations in fathead minnow (Pimephales promelas, Rafinesque, 1820) *Int J Radiat Biol* 2013 Nov;89(11):950-64.		X
Smith RW, Seymour CB, Mothersill CE. Short and long term bystander effect induction by fathead minnows (Pimephales promelas, Rafinesque, 1820) injected with environmentally relevant whole body doses of 226Ra. J Environ Radioact. 2013 Dec;126:133-6.		X
Richard W Smith[1], Jiaxi Wang[2], Carmel E Mothersill[1], Lucy EJ Lee[3], Colin B Seymour, Proteomic responses in the gills of fathead minnows (*Pimephales promelas*, Rafinesque, 1820) after 6 months and 2 years of continuous exposure to environmentally relevant dietary ^{226}Ra. *Int. J. Radiat. Biol*. Accepted subject to minor revision		X
Publications related to the CNSC contract but not funded by CNSC		
Carmel Mothersill, Richard W. Smith , Lene Sørlie Heier, Hans-Christian Teien, Ole Christian Land, Colin B. Seymour, Deborah Oughton, and Brit Salbu. Radiation-Induced Bystander Effects In The Atlantic Salmon (*Salmo Salar L.*) Following Mixed Exposure To Copper And Aluminium Combined With Low Dose Gamma Radiation. *Radiation and Environmental Biophysics* 2014 Mar;53(1):103-14		X
Carmel Mothersill, Cristian Fernandez-Palomo, Jennifer Fazzari, Richard Smith, Elisabeth Schültke, Elke Bräuer-Krisch, Jean Laissue, Christian Schroll, Colin Seymour, Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. *Dose Response*, 2013 Aug 27;12(1):72-92.		X
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Pinho C, Wong R, Sur RK, Hayward JE, Farrell TJ, Seymour C, Mothersill C. The involvement of serum serotonin levels producing radiation-induced bystander effects for an in vivo assay with fractionated high dose-rate (HDR) brachytherapy. Int J Radiat Biol. 2012 Oct;88(10):791-7.		x
Fazzari J, Mersov A, Smith R, Seymour C, Mothersill C. Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect. Int J Radiat Biol. 2012 Oct;88(10):786-90.		x
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Mothersill C, Seymour C. Are epigenetic mechanisms involved in radiation-induced bystander effects? Front Genet. 2012;3:74.		x
Mothersill C, Seymour C. Changing paradigms in radiobiology. Mutat Res. 2012 Apr-Jun;750(2):85-95.		x
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Mothersill C, Bristow RG, Harding SM, Smith RW, Mersov A, Seymour CB. A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells. Int J Radiat Biol. 2011 Nov;87(11):1120-5.		x
Wang C, Smith RW, Duhig J, Prestwich WV, Byun SH, McNeill FE, Seymour CB, Mothersill CE. Neutrons do not produce a bystander effect in zebrafish irradiated in vivo. Int J Radiat Biol. 2011 Sep;87(9):964-73.		x
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