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Effect of gamma radiation on the production of bystander signals from three earthworm species irradiated in vivo

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Abstract
The effect of gamma radiation delivered over 24hrs on the induction of bystander signals of three earthworm species exposed in vivo was investigated: *A. chlorotica*, *A. caliginosa*, and *E. tetraedra*. Worms were exposed to external gamma irradiation (Co-60 source) for 24 hours and samples of head, body, and clitellum were dissected from exposed and control worms and placed in culture medium for 24hrs at 19C. The harvested medium was filtered and assayed for expression of bystander signals using both clonogenic and mitochondrial reporter assays. Different responses were observed in the different species and in the different tissues. *A. chlorotica* worm-treated reporters show insignificant mitochondrial response for all sections, yet a significant clonogenic reduction in survival for body sections. *A. caliginosa* worm-treated reporters show a significant mitochondrial response for some sections and insignificant mitochondrial response and insignificant reduction in clongenic survival for the rest. *E. tetraedra* worms from a control site show significant evidence of bystander signalling, measured by mitochondrial response in reporter cells, for all sections while those harvested from a contaminated site show insignificant changes in baseline signalling when exposed to the challenge dose. In vivo exposure of earthworm species shows evidence of bystander signalling using two different reporter assays. This effect varied between the different species and tissues. There is also evidence of attenuated bystander signalling in worms harvested from a site contaminated with radiation.

**Key Words:** Radiation protection, radiation-induced bystander effect, whole-organism irradiation, radioadaptive response, historic dose
1. Introduction

Radiation protection of the environment has undergone a paradigm shift in the last three decades from a position which regarded the protection of man to be sufficient to protect all other species to a situation where there is an acceptance that potential effects on non-human species need to be addressed explicitly (European Commission and others; ICRP 2008). The new approach recognized that a purely anthropocentric approach to radiation protection is insufficient not only for ethical reasons but because of the need to protect species living in environments where humans are largely absent, such as polar or marine areas (Ceballos et al., 2017; Higley et al., 2004; Mothersill et al., 2018a; Mothersill and Seymour, 2018; Bréchignac 2012). The current solution developed by the International Commission on Radiation Protection (ICRP) is to select representative generic species for aquatic and terrestrial habitats (so-called reference animals and plants, or RAPS). These are selected not only for biological reasons but also to represent physical dosimetric scenarios, such as flying in air or living in sediment, or generic shapes such as ellipses or cylinders to aid the estimation of dose (ICRP 2008). While it is clear that this system is an improvement on the old human-centric system, there are problems. One problem is lack of information about the effects of environmentally relevant radiation doses on many species, including RAPS. Another is the complexity of ecosystems and the interdependence of species within ecosystems which leads to effects due to radiation which are not just due to the dose received by any one organism. Further complications arise when laboratory and field data are compared (Coppleston et al., 2008; Garnier-Laplace et al., 2015, 2013) and where effects are being measured at legacy sites using ambient doses to determine chronic or legacy effects (Mothersill et al., 2018a; Omar-Nazir et al., 2018). This complexity probably means that in the long term it will be necessary to develop system level markers of ecosystem health (Mothersill et al., 2018a); given the knowledge gaps, it is important as a first step to build up information concerning the effects of low and chronic doses of relevant radiation qualities on non-human species.

This paper examines effects of in vivo irradiation on three species of earthworms where three different body parts were examined for evidence that radiation had had an effect. Earthworms are on the list of RAPS used to represent elongated cylinder-shaped dosimetry where the entire surface is exposed in sediment/earth. Previous studies with earthworms have established that reproduction is a sensitive endpoint for exposure to ionizing radiation, with exposures of 10 mGy/hr leading to greater than 50% reduction in cocoon hatching in *Esenia fetida*, and a 10% reduction in reproduction at dose rates of 4 mGy/hr (Hertel-Aas et al., 2007). Earthworms are also one of the organisms receiving high doses in contaminated environments due to their residence in soils, and reductions in populations were seen after the Chernobyl accident (Hinton et al., 2007). Effects on DNA have also been reported after the Fukushima accident (Fujita et al., 2014). Effects on cocoon hatching were also shown to be correlated with DNA damage in sperm and coelomocyte cells at dose rates of 4-10 mGy/hr, as measured by the COMET assay (Hertel-Aas et al., 2011).
1.1. Non-Targeted Effects

The endpoints used to monitor radiation effects in the current study focussed on non-targeted effects (NTE). NTE is a term used to describe effects in cells or organisms where no direct deposition of ionizing radiation energy has taken place (Morgan, 2003a, 2003b). Bystander effects (RIBE) describe the detection of responses in cells, tissues, or organisms which received signals from other cells, tissues, or organisms and are thought to be due to signalling mechanisms coordinating damage responses at higher levels of organization (Mothersill and Seymour, 1997; Mothersill et al., 2017), while genomic instability or lethal mutation effects describe non-clonal events detected in distant progeny of irradiated progenitors (Lorimore et al., 2003; Mothersill and Seymour, 2001). These have been shown to predominate in the low dose region of the dose response curve and can be seen at doses below the threshold for adverse effects, such as mutation or impacts on mortality or morbidity (Kadhim et al., 2013; Little, 2010; Mothersill et al., 2002; Mothersill and Seymour, 2006; Prise et al., 2005; Rusin et al. 2018; Seymour and Mothersill, 2000). The endpoints of RIBE include a clonogenic reporter assay which reveals the presence of a bystander signal in medium samples harvested from the incubated worm parts. This assay has been used by our laboratory for over 20 years with many different species exposed both in vivo and in vitro to radiation (Fernandez-Palomo et al., 2016, 2015; Le et al., 2015a; M. Le et al., 2017; Mothersill et al., 2018b; Mothersill and Seymour, 1997; O’Reilly and Mothersill, 1997; Shi et al., 2016; Vo et al., 2017b, 2017a). The assay is particularly important because it allows system-level signalling to be identified (Mothersill and Seymour, 1997). Effects in progeny of cells receiving the signals can also be studied, meaning that genomic instability effects can be monitored (Kaup et al., 2006; Lyng et al., 2002; Seymour and Mothersill, 1997). The other NTE assay used in this paper detects mitochondrial membrane polarization (MMP) status changes. MMP is an indicator that stress signals have been detected by the reporter cells (Kam and Banati, 2013; Lyng et al., 2001, 2000; Nugent et al., 2007; Paz et al., 2008) and predicts changes in energy production and utilization via aerobic pathways such as OXPHOS (Le et al., 2017; 2018). The membrane can be depolarised relative to the control leading to apoptosis or hyperpolarised potentially leading to adaptive functions (Giovannini et al., 2002; Perry et al., 2011).

2. Materials and Methods

2.1. Sample collection, species, and irradiation

Earthworms are broadly defined as tube-shaped and segmented organisms, categorized under the phylum Annelida. Studies were carried out on three different species of earthworm, *Allolobophora chlorotica*, *Eiseniella tetraedra* and *Aporrectodea caliginosa* taken from research stock collections kept at the Norwegian University of Life Sciences (NMBU). *Allolobophora chlorotica* is a species of earthworm measuring approximately 50mm in length (Jones and Eggleton, 2014; Kalmus et al., 1955). The members of the species typically appear pink or green
and are native to various parts of Europe, Northern Asia, North Africa, and the Arabian Peninsula (Jones and Eggleton, 2014; Kalmus et al., 1955). They prefer a wide range of habitats, including farmland and woodland (Jones and Eggleton, 2014). *Aporrectodea caliginosa* is another species of earthworm that is found globally in all temperate zones. This species measures roughly 60mm in length and is typically pinkish in colour (Bart et al., 2018). *Eiseniella tetraedra* worms are typically found in waterlogged habitats as well as terrestrial environments (Terhivuo et al., 2002). These worms typically appear a pinkish-red and are usually found approximately 50mm in length (Terhivuo et al., 2002). In addition to the laboratory cultures of the worms, samples of *E. tetraedra* were collected from the Fen site in Norway two weeks before the start of the irradiation experiment. This site has naturally high levels of thorium radionuclides and decay products, with sediment activity levels of approximately 3000 Bq/kg and ambient dose rates of 4-7 μGy/hr at the sampling site. All specimens were kept at 20-22°C prior to irradiation and transferred to irradiation containers for acclimatization one week before irradiation. All specimens were stored and irradiated in their original soils.

Gamma irradiation of worms was carried out at the FIGARO ⁶⁰Co climate-controlled irradiation facility at NMBU, Ås, Norway (²⁶⁰Co source, activity ~ 420 GBq). Ten mature worms per irradiation replicate were placed in black Perspex boxes (9.8 x 11.9 x 12.9 cm, internal cross-sectional area: 103.5 cm²) containing approximately 500 g soil and irradiated for 21 hours at 10.1 +/- 1.2 mGy/hr (a total dose of 212 mGy) at 21°C. Field dosimetry of the FIGARO facility has been previously established using ionization chambers to allow placing of the boxes. A dose rate of 10 mGy/hr was chosen since this has previously been linked to organism level effects on reproduction (Hertel-Aas et al., 2007). Control boxes for all groups were placed in the same room in shielded areas (dose rates during irradiation were <0.5 mGy/hr). Additional dosimetry was carried out using NanoDots™ (Landauer, Glenwood, USA), with dosimeters placed at the front and back of all boxes in order to assess variation between boxes and attenuation across the soil. The variation in doses between boxes was less than 10%, but ranged from 6.9-13.3 mGy/hr from the front to the back of the box. Final doses to the worms were calculated as the average dose to water for a worm residing at the centre of the box.

2.2. Dissection of worms in Norway and transport
All worms were rinsed in salt solution and dissected immediately after removal from the radiation source, and samples of head (containing reproductive organs), clitellum, and body segments (ca. 0.02 g) were taken directly to 6 mL culture medium for use in the bystander assays. Fresh medium was used to ensure that the dissection process itself would not allow any factors to leak into the medium. Head and clitellum were taken as whole segments and bodies as triplicate samples (*A. chlorotica* and *A. caliginosa*). The gut was not removed, as the aim was to process the samples as fast as possible. Nonirradiated controls were used to control for any potential transport, sacrifice, and dissection stress as well as any other factors released by the worm sections unrelated to radiation exposure.

Samples of the worm tissues described above were placed in sterile tubes containing 6ml of culture medium (Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin, and 100µL streptomycin sulphate). The tubes were
placed in an incubator set to 26°C for 24hrs. The medium was then decanted and filtered through a 0.22 micron filter (Acrodisc) into fresh sterile tubes. This conditioned medium harvested from the worm samples was brought from Norway to McMaster University, Canada. Samples were kept frozen upon arrival until they were used for the assay. A transport control was included along with the media samples. The transport control consisted of medium that was treated the same as the medium that contained the worm sections at every point in the experiment. This was done to control for any potential changes in the medium itself rather than factors produced by the worm sections. The transport control was also used to control the potential effects on the media as it was transported from Norway to Canada, as it was stored in the same conditions and along with the media samples at all times.

2.3. Cell Line and Subculture
An immortalized, non-transformed keratinocyte reporter cell line (HaCaT) was used for both assays in this study. This cell line was chosen because of its proven use as a reporter for RIBE experiments in previous studies in our lab and others (Fernandez-Palomo et al., 2016; Le et al., 2015b; Lyng et al., 2012; O'Reilly and Mothersill, 1997; Ryan et al., 2009; Vo et al., 2017c). The cell line was maintained using a protocol outlined in previous studies for the subculture technique (Le et al., 2015a). Briefly, HaCaT cells were cultured in (RPMI) medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin, and 100µL streptomycin sulfate in 75 cm² culture flasks. Cultures were incubated in 95% humidified air and 5% CO₂ at 37°C and received medium changes every three days.

Cells were subcultured every week when adhered at about 80-90% confluence into new 75 cm² flasks. Dissociation was accomplished using a 1:1 solution of 0.25% w/v trypsin and 1mM EDTA. Cells were typically incubated for approximately fifteen minutes until they visibly began to detach. Neutralization was accomplished by adding equal or greater amounts of culture medium to the trypsin stock solution.

2.4. Clonogenic Survival Assay
A clonogenic survival assay was performed to determine if the reporters would show differences in survival between control groups and worm medium treatment groups for the body sections in the *A. chlorotica* and *A. caliginosa* worms. This assay has a long history of use in bystander experiments, and its use as an endpoint is ubiquitous in both *in vitro* and *ex vivo* experiments (Mothersill and Seymour 1997; O’Reilly and Mothersill 1997; O’Reilly and Mothersill 1997; Liu et al. 2006; Mothersill et al. 2006; Fazzari et al. 2012; Mothersill and Seymour 2013; Smith et al. 2013; Le et al. 2015; Shi et al. 2016; Hanu et al. 2017; Hall and Hei, 2003; Sawant et al., 2002, 2001; Watson et al., 2000). Like the mitochondrial assay, the survival assay was done to determine if there were any signalling molecules present in the worm medium that could contribute to RIBE and specifically if such factors could cause reduced reporter cell survival. Prior to plating the clonogenic flasks, 80%-90% cells were detached using a 1:1 solution of 0.25% w/v trypsin and 1mM EDTA using the same procedure as subculture. The stock trypsin solution was neutralized using an equal volume of culture medium.
The concentration of the stock solution was determined using a Beckman Coulter Z2 particle count and size analyzer (Beckman Coulter LP, Mississauga, Canada), which had been calibrated using a hemocytometer. The stock solution was serially diluted in culture medium such that 500 cells could be seeded per 25 cm$^2$ culture flask easily. 500 cells were seeded in these flasks containing 5mL RPMI and incubated for 6 hours at 37°C. After 6 hours to allow for cell attachment, the RPMI was removed completely, the cells were washed with DPBS, and 3mL of worm medium was added onto the cells in each respective flask. The cultures were incubated for 8 days before they were stained with carbol fuchsin. Colonies containing at least 50 cells were counted according to the clonogenic survival assay developed by Puck and Marcus (1956). Due to limited worm-conditioned media volume, only the body sections of A. chlorotica and A. caliginosa were assayed.

2.5. MMP Assay
An assay measuring mitochondrial membrane depolarization was used as an additional endpoint to the clonogenic survival assay because it was a fast and an early sign that the bystander signal would be received in the test sample (Le et al., 2017; Mothersill et al., 2005). Specifically, the The MitoPT® JC-1 Assay (Immunochemistry Technologies, Bloomington, USA) was used. JC1 is a dye that assays MMP, defined as a dual-emission potential-sensitive probe. At low membrane potential, the probe exists in monomer form and fluoresces at a green wavelength ($\lambda_{ex}$ 520 nm). At high membrane potential, the probe forms aggregates that fluoresce at a red wavelength ($\lambda_{em}$ 596 nm). When added to a sample of cells in the 96-well plate, the resulting fluorescence can be measured at two different wavelengths using a plate reader. A full explanation of the dye and preparation of the dye solution used can be found in the JC1 assay manual (Immunochemistry Technologies, Bloomington, USA). Because transport medium controls were used in these experiments and all data were normalized to these baseline JC1 ratio values, an observed decrease in ratio is consistent with relative mitochondrial depolarization, while an observed increase in ratio is consistent with relative mitochondrial hyperpolarization.

Prior to assay with JC1, a stock cell suspension was used to set up the 96 well plate with 100,000 cells per well. This stock cell concentration was determined using a Bio-Rad TC20 automated cell counter (Bio-Rad Life Science Research Division, Mississauga, Canada). The cells were then left to adhere and grow for 24 hours in the same incubator with controlled temperature and CO$_2$ concentration before treatment with worm-conditioned media.

A brightfield microscope was used to determine if the reporter cells had adhered to and multiplied at the bottom of each well. Samples that were used in the assay were quickly thawed in a hot water bath at 37°C while all culture media was carefully removed with a micropipette from each well. The worm medium samples were then recorded and placed into each well at a volume of 100 µL, with each sample being assayed three times in three separate wells. The well plate was then placed back in the incubator for six hours. Previous studies have indicated that the mitochondrial membrane effects of bystander signals appear to peak after approximately
6hrs (Mothersill et al., 2005), so this time was chosen for this assay and kept consistent throughout each run.

The JC1 solution was prepared in DBPS buffer according to the instructions enclosed in the kit (Immunochemistry Technologies, Bloomington, USA). The worm medium was removed from the wells and the samples were then washed using DPBS. The JC1 solution was then pipetted into the wells and the plate was incubated again for 15 minutes in accordance with the JC1 manual. The JC1 solution was then removed and the cells washed three times with 200 µL DPBS. 200 µL of DPBS was then added in each well to prevent desiccation before the plate was read. Background measurements were also done using wells with only DPBS and incorporated into the statistical analysis. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) contained in the kit was used as a positive mitochondrial membrane depolarization control. The JC1 assay measurements were obtained from a Tecan Infinite® 200 PRO plate reader.

2.6. Statistical Analysis

Results are presented as means ± SEM for ten different biological samples unless otherwise indicated. Experiments were performed using triplicate measurements unless otherwise indicated. Significance was determined primarily using one-way ANOVA and Tukey’s Honestly Significant Difference test (HSD). Some tests were conducted using a Student’s t test at α = 0.05 where applicable. Potential outliers were determined using Grubb's Test for outliers.

3. Results

3.1. A. chlorotica worms

The survival assay showed a significant (p<0.0028) reduction in survival for reporters treated with the body section medium of the A. chlorotica worms. Figure 2a shows the clonogenic survival assay results for the A. chlorotica worms. A. chlorotica worm controls also show a significant reduction in survival, where a value of 1 is equivalent to baseline survival according to transport controls.

Figure 2b shows the mitochondrial membrane assay for the reporters treated with A. chlorotica worm media. The mitochondrial membrane assay showed no significant reduction or increase of mitochondrial potential in any section (Head p=0.38, Body p=0.98, Clitellum p=0.37, respectively). Each section group showed, much like the clonogenic survival assay, that there was a considerable reduction of MMP for worm medium compared to the transport controls (Figure 5a); this reduction was particularly apparent in the clitellum section group.

3.2. A. caliginosa worms
Figure 3a shows the survival results for the *A. caliginosa* worm reporters. The survival assay showed that there was no significant difference between the control and irradiated groups in the body sections of *A. caliginosa* worms (*p* = 0.43). Unlike the *A. chlorotica* worm samples, the transport controls were insignificantly different to the *A. caliginosa* controls.

The results for the mitochondrial assay (Figure 3b) showed a significant effect on MMP in the *A. caliginosa* worms for both the head and clitellum tissues (*p*<0.047 and <0.00021, respectively). Furthermore, the mitochondrial reporter assay in the body section showed no observed significant differences (*p*=0.91). Like the clonogenic survival assay, the body section trials were not statistically significant in comparison to the control groups. The effect that the test medium had on the worms that did show significant differences appeared to result in restoration of mitochondrial potential to basal levels. Both the clitellum average and the head average showed this increase. Like the *A. chlorotica* worms, the *A. caliginosa* worms also demonstrated a significant reduction between the transport controls and the control worm groups (Figure 5b).

### 3.3. *E. tetraedra* worms

Figure 4a shows the mitochondrial data from the *E. tetraedra* worms from the control site. The data show a significant increase in aggregate MMP for head, body, and clitellum sections (*p*<0.0001 for all tissue sections). The unirradiated worm controls also showed significant reductions in MMP when compared to the transport controls (Figure 5c).

The reporters treated with medium from *E. tetraedra* worms from the radioactive fen mirrored the *A. chlorotica* worms in for the body and clitellum section groups (Figure 4b); there was an insignificant effect (*p*=0.87 and 0.95, respectively). There was a significant (*p*<0.0001) reduction in MMP between the control and treatment group for the head sections. It should be noted that the control for this group elicited hyperpolarization in the reporter cells. Most of the control groups were observed to have a higher aggregate MMP than the transport medium controls (Figure 5d).

### 4. Discussion

#### 4.1. Evidence of the production of bystander signals *in vivo*

Results demonstrate, for the first time, measurement of bystander signalling in earthworms. The data are consistent with each worm species potentially producing a radiation-induced bystander signal *in vivo*, however the type of response observed in the reporters was varied. While the results are likely to be also influenced by different tissue responses to sacrifice and dissection stress as well as radiation exposure, the data suggest several trends that appear to be directly influenced by radiation exposure. There exist a number of reasons why these results can be used to evidence RIBE in earthworms *in vivo*. Firstly, this assay procedure has been
conducted before on human, mouse, fish, and worm samples, including studies involving no dissections and studies involving dissection post-irradiation (Mothersill et al. 2005; Seymour and Mothersill 2006; Marozik et al. 2007; Carmel Mothersill et al. 2007; C Mothersill et al. 2007; Smith et al. 2007; Salbu et al. 2008; Singh et al. 2011; Smith et al. 2011; Pinho et al. 2015); our procedure is an established one for assaying the presence of a bystander signal, both in vitro and in vivo. Moreover, clearly the modulation of the effect observed between the control and fen worms shows that a bystander signal exists and its effects on reporter cells can be modulated depending on whether or not the worm has adapted to radiation in vivo. This latter point is discussed further in Section 4.2. Previous studies have conducted whole-organism irradiation and have evidenced conserved RIBE signals that may be transmitted through ecosystems and between individuals of the same species (Smith et al. 2013). Future research is still needed to conclusively show bystander signalling in vivo between individuals in the environment; this is further discussed in Section 4.4.

*A. chlorotica* worms produced a signal detectable by clonogenic survival assay only. *A. caliginosa* worms showed that radiation treatment does not significantly affect the survival of reporters and produces restorative effect to MMP. *E. tetraedra* worms from the uncontaminated site showed a signal that resulted in mitochondrial hyperpolarization beyond basal levels in radiation-treated groups. This is evidence of a bystander signal that could be propagated in the environment between worms of the same species in vivo for these three species. Radiation-induced bystander effects have been extensively studied in vitro in human cell lines and others in previous research; additionally, there have been many papers that describe these effects in vivo in various organisms, including fish and human cancer patients, at various endpoints (Fernandez-Palom et al., 2016, 2015; Le et al., 2015a; M. Le et al., 2017; Mothersill et al., 2018b, 2007, 2005; Mothersill and Seymour, 1997; O’Reilly and Mothersill, 1997; Shi et al., 2016; Vines et al., 2009; Vo et al., 2017a, 2017b; Belyakov et al., 2003; Hanu et al., 2017, 2016; Harney et al., 1995; Vo et al., 2017c). This current research on earthworm species expands our knowledge of these NTE because only a handful of previous studies have discussed RIBE in the context of whole-organism irradiations. The differences in the signal produced between the different earthworm species, as evidenced by the two endpoints described in this paper, were expected; the major biological differences between the species have been explored in previous research using genetic analysis, indicating significant evolutionary divergence (King et al., 2008; Pérez-Losada et al., 2009). The differences in signalling could be attributed to these biological differences; many research groups have shown that these differences can result in differing responses to direct radiation exposure, such as varying radiosensitivity between different species (King et al., 2008; Mothersill et al., 2018a; Pérez-Losada et al., 2009). It is therefore likely that these biological differences can alter NTE as well.

4.2. Evidence of a potential adaptive response to chronic exposure in *E. tetraedra*
E. tetraedra worms from the contaminated site elicited a response in the reporters that was consistent with abolishment of the bystander signal. The results comparing the transport controls to the unirradiated worm controls show that incubating tissue in the culture medium can produce an appreciable effect on both reporter survival and MMP, resulting in both reductions in survival in some species and mitochondrial depolarization. This was expected because the media that the reporters were cultured in contained tissue that could have reduced MMP for a number of reasons, such as the release of other factors unrelated to RIBE yet inherent in the way that tissue can alter the physiological conditions of culture media, such as an increase or decrease in salinity due to the presence of said tissue (Li et al., 2010). These results were observed to be consistent with every worm group except the worms from the contaminated site, which show hyperpolarized mitochondria. Hyperpolarized mitochondria in these worms was also observed to be consistent with the E. tetraedra worms from the uncontaminated site receiving radiation treatment. Furthermore, exposure of these worms from the contaminated site to a challenge dose did not change the response of the reporters in most groups. This is evidence of a radioadaptive response, where the worms had most likely adapted over time to the low doses of radiation present in the contaminated fen and are therefore did not release the same signalling factors as those unaccustomed to chronic doses.

There are two primary reasons why the E. tetraedra results can be interpreted differently from the response by the other two worms and interpreted as evidence of an adaptive response to radiation. Firstly, the sham controls (Figure 5) from the contaminated fen show either hyperpolarized mitochondria or those that appear basal compared to the transport control, which is a different response than the A. chlorotica and A. caliginosa controls. Treating these worms in the lab with radiation did not appear to have an appreciable effect in both the body and clitellum sections. Secondly, comparing E. tetraedra worms from the control site and those from the contaminated fen (Figure 5) shows that the control groups had different effects on MMP. Further, the effect is also different in the irradiated groups: worms from the control site show hyperpolarization, what can be interpreted to be a response to the bystander signal, while fen worms show no effect, what can be interpreted to be a lack of a response to the bystander signal. Therefore, this appears to be an adaptive effect that is a direct result of the chronic radiation exposure in the Fen. What we see in E. tetraedra worms from the contaminated site is an abolishment of the bystander response compared to worms from the control site, and that is why this appears to be an adaptive effect.

It is important to note that the other two worm species could also potentially produce the same response, but we did not have sample worms from a contaminated site, and therefore we cannot draw any conclusions beyond the E. tetraedra species. The radioadaptive response a well-documented effect in radiation biology and has been assayed using a number of endpoints (Audette-Stuart et al., 2011; Bosi and Olivieri, 1989; Joiner et al., 1996; Maguire et al., 2007; Matsumoto et al., 2007; Mothersill and Seymour, 2006, 2004; Olivieri et al., 1984; Ryan et al., 2008; Smith et al., 2011, 2015b), however research into whole-organism in vivo radioadaptation in the context of NTE still requires further research. This data suggests that E. tetraedra worms
are capable of adapting the bystander signal they produce in response to chronic exposure to radiation and consequent radioadaptation. The importance of chronic dose has been explored in radiobiological research and has also more recently been proposed as a possible explanation in differences between observed and predicted data in radioecological research (Copplestone et al., 2008; Garnier-Laplace et al., 2015, 2013; Omar-Nazir et al., 2018). The *E. tetraedra* worm data shows that this effect could be extended to explain differences in NTE in organisms previously exposed to radiation. This modulation of radiosensitivity of worms is further evidence that using a more system-level approach is needed when doing radioecological research, including life history of the organism, environmental conditions, and other factors (Mothersill et al., 2018a).

4.3. Varied production of the bystander signal by tissue

Varied production of the bystander signal by tissue was also observed in some worms. Some *A. chlorotica* worms were observed to produce this difference in all except body sections in the MMP endpoint, however the results were not statistically significant. *A. caliginosa* worms however were observed to elicit significant increases in MMP in all sections except for the body. All tissue sections in the *E. tetraedra* worms from the control site showed mitochondrial hyperpolarization after irradiation, while this effect was abolished in the worms from the contaminated site. The head section control of the *E. tetraedra* worms from the unirradiated site caused mitochondrial hyperpolarization in reporters, and the challenge dose resulted in depolarization. Tissue-specific NTE have been extensively in vivo in previous studies (Fernandez-Palomo et al., 2015; Ilnytskyy et al., 2009; Koturbash et al., 2007; Smith et al., 2015a). This provides some evidence that in some species of earthworms, bystander signalling may be tissue-dependent as well as species-dependent.

4.4. Future directions

Various authors have criticized the RAPS system in the context of radioprotection for not taking into consideration biodiversity of the broad reference animal the system purportedly represents (Mothersill et al., 2018a). Accordingly, the differences observed in this study provides direct evidence that the RAPS reference worm in its current form is incomplete and not representative of the ostensibly uniform response that earthworms have to ionizing radiation exposure. This is further evidence that a more system-level approach is required when modelling organisms’ response to environmental stressors such as radiation, including recognizing biological differences between species and contextualizing organisms according to the environment they inhabit (Mothersill et al., 2018a).

It is possible that bystander signalling molecules could have been present internally in the worms that leaked into the medium after dissection. These putative signalling molecules may have persisted internally in the worms and contributed to stress signalling within the organism in response to ionizing radiation that could also have been detectable by both the survival and MMP assays due to dissection. While previous studies have used similar methods and
determined that bystander signalling does occur outside of worms by determining significant effects in fish (Smith et al. 2013) and that the present study provides some evidence for bystander signalling between earthworms, a future study should be performed to provide further evidence for bystander signalling between different individuals of the same species within their environment. It is recommended that follow-up studies perform experiments that confirm the findings of the present study, and there are a few tested methods to do this. For example, whole earthworms irradiated in vivo may be incubated in culture medium to allow signals to enter the medium without dissection. An explant tissue culture may then be established from unirradiated worm tissue from another individual of the same species. These explants may then be incubated with the irradiated worm-conditioned medium and assayed for potential effects.

The fact that the different worms appeared to produce different NTE signals raises a few questions about the nature of the signal being produced in the pursuit of gaining a better understanding of how to apply these findings to radioecological modelling. One might ask why the signal was only detectable by clonogenic survival assay in some worms. One possible explanation is that MMP reduction could have been transient and occurred at a specific timepoint as one endpoint of signal reception. Several papers have found that transient mitochondrial membrane depolarization plays a role in apoptotic effects (Duchen et al., 1998; Zhou et al., 2010). Another potential explanation is that the MMP assay used was not sensitive enough to detect further depolarization beyond basal levels in worm-infused media. This seems as though it may have been the case because the worm controls appeared significantly depolarized compared to the transport controls. This also provides evidence that though the MMP assay used in this paper may be applicable for field use, the clonogenic survival assay remains the gold standard for assaying RIBE and should be used alongside the MMP assay before the protocol is fully optimized. For future in vivo field studies, it is recommended that characterization of MMP should be conducted at various timepoints to elucidate the role of time in the response.

A second question, relating to the results from the A. caliginosa worms, concerns why some of the signal appeared to produce a restorative effect to MMP. The discussion of whether or not all effects of radiation exposure are necessarily negative has been a major topic of radiobiological research in the past (Mothersill and Seymour, 2014, 2005, 2004). Perhaps in these experiments the signal being produced in these worms conferred an adaptive effect to recipients of the signal. RIBE have been postulated to contain a potential protective component in previous research (Mothersill and Seymour, 2006, 2005; Zhou et al., 2004). Specifically, these publications argue that NTE killing of already damaged cells in a population and therefore removing them confers a protective effect on the population. This could have potentially occurred in the A. caliginosa worms, as the worm controls caused significant depolarization in the reporter cells. Through NTE killing of already damaged cells over the course of six hours, the various wash steps could have removed many detached dead cells from the wells, eliminating nonspecific binding of the probe present in the detached cells and causing a higher aggregate
MMP. Because this possibility was not foreseen, no conformational observation was made after the subsequent wash steps to verify this hypothesis experimentally. It is recommended that for future research this possibility is accounted for and incorporated into the methodology. In the context of radioecology, this effect could potentially prove beneficial to organisms receiving the signal. This finding is further evidence because the effects of NTE on living organisms may not be entirely detrimental, these factors must be incorporated into radioecological risk assessment and modelling.

A final question concerns the observation of mitochondrial hyperpolarization in reporter cells, which was not initially hypothesized in this study. Using results from the JC-1 probe as evidence for mitochondrial hyperpolarization has been done in previous research (Giovannini et al., 2002; Perry et al., 2011). However, results indicating mitochondrial hyperpolarization are somewhat ambiguous. According to the JC-1 manual (Immunochrometry Technologies, Bloomington, USA) the probe itself is mainly intended to assay mitochondrial depolarization. Given the data and considering that the probe and methodology is intended to be used specifically to assay depolarization and considering that similar research has also used JC-1 to conclude hyperpolarization, there exist two possibilities with the observation of an increase in MMP relative to the controls. The first possibility is that extranormal hyperpolarization had indeed occurred in the reporters. This itself is evidence of reception of a bystander signal that produced a deleterious effect in the reporters, potentially leading to cell death. That interpretation is consistent with many previous publications on the nature of the bystander effect in vitro (Mothersill et al., 2017). The second possibility is that the increase in MMP represents a beneficial stabilization of normal MMP represented as an aggregate ratio of polarized to depolarized mitochondria. This still represents reception of a bystander signal, however that signal produced a beneficial cell-killing response that removed already damaged cells from the population. This interpretation is also intuitively valid, as all of the A. caliginosa and E. tetraedra medium controls elicited depolarization in the reporters relative to the transport control, indicating cellular damage or an otherwise adverse response to the worm-infused media. Whether these results represent a novel hyperpolarization effect in the context of NTE or further evidence for a beneficial role for the bystander signal, it is recommended that future research incorporates the use of a secondary probe that specifically assays hyperpolarization, such as JC-9 (Molecular Probes/Invitrogen, Eugene, USA) alongside the JC-1 depolarization probe to confirm hyperpolarization.

5. Conclusion

Overall, these results suggest that all three worm species produce varied signals consistent with the radiation-induced bystander effect, that one of these species may be able to adapt to chronic doses of radiation given that other factors may also be involved in this response, and that the signal varies depending on tissue of origin. These results are evidence that these worm
species can produce these NTE in vivo in the environment they inhabit. These findings are of great importance to radioecological research because they provide evidence that a system-level approach is required when formulating policies on radioprotection due to the complexity of ecosystems and the complexity of the reference organisms contained therein. Similar future research should consider focusing on expanding the findings in this paper and potentially explaining the various effects observed using similar endpoints.

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Conflict of Interest

The authors declare no conflict of interest.

Figure 1: A flow-chart of the methodology described in the Methods section. (A) shows a flowchart depicting a flow-chart of the collection, treatment, and storage of the samples. (B) shows a flowchart depicting the assay procedures described in the methods section.

Figure 2: Results collected from the A. chlorotica worm media-treated reporters. (A) shows the clonogenic survival assay results represented as normalized survival fractions (nSF) for worm controls (CH-C Bo) and lab-irradiated worms (CH-10 Bo). (B) shows the results for the MMP assay in relative change in MMP (RΔΨ), where a value of 1.000 is equivalent to the transport control. Assay results from head (CH-C He, CH-10 He), body (CH-C Bo, CH-10 Bo), and clitellum (CH-C CL, CH-10 CL) are shown.

Figure 3: Results collected from the A. caliginosa worm media-treated reporters. (A) shows the clonogenic survival assay results represented as normalized survival fractions (nSF) for worm controls (CA-C Bo) and lab-irradiated worms (CA-10 Bo). (B) shows the results for the MMP assay in relative change in MMP (RΔΨ), where a value of 1.000 is equivalent to the transport control.
control. Assay results from head (CA-C He, CA-10 He), body (CA-C Bo, CA-10 Bo), and clitellum (CA-C CL, CA-10 CL) are shown.

Figure 4: Results collected from the *E. tetraedra* worm media-treated reporters. (A) shows the results from the MMP assay for control site worms (ETB). Assay results from head (ETB-C He, ETB-10 He), body (ETB-C Bo, ETB-10 Bo), and clitellum (ETB-C CL, ETB-10 CL) are shown. (B) shows the results from the MMP assay for contaminated site worms (ETF). Assay results from head (ETF-C He, ETF-10 He), body (ETF-C Bo, ETF-10 Bo), and clitellum (ETF-C CL, ETF-10 CL) are shown. As in previous figures, a value of 1.000 is equivalent to the transport control.

Figure 5: An analysis of the control worm media. Each control group from the *A. chlorotica* (CH-C He, CH-C Bo, CH-C CL), *A. caliginosa* (CA-C He, CA-C Bo, CA-C CL), and *E. tetraedra* (EFB-C He, EFB-C Bo, EFB-C CL; ETF-C He, ETF-C B, ETF-C CL) worms was compared to the transport control (TC). This statistical analysis was performed primarily to ensure a consistent response that the reporters had to unirradiated worm-condition media, and to detect any potential discrepancies in the worms from the irradiated fen (ETF).

Supplementary Figure 1: Results collected from reporters treated with the transport control (TC) and the positive control for mitochondrial membrane depolarization (CCCP). CCCP values were normalized to transport control within-plate for each experiment. The data represent three replicate measurements for *n*=6.
References


Fernandez-Palomo, C., Seymour, C., Mothersill, C., 2016. Inter-Relationship between Low-Dose Hyper-Radiosensitivity and Radiation-Induced Bystander Effects in the Human T98G


Clonogenic Survival

Reporter Assay

Plate 500 Reporter Cells

Incubate 6 hrs

Worm Medium Transfer

Carbol Fuchsin Stain

Incubate 8 days

Determine Survival

Mitochondrial Viability

Plate 100,000 Reporter Cells

Incubate 24 hrs

JC-1 Stain

Incubate 6 hrs

Determine Mitochondrial Polarization
Highlights

- Three earthworm species were irradiated using a gamma source *in vivo* and assayed for bystander signalling
- *A. chlorotica* and *A. caliginosa* worms show evidence of bystander signalling determined by both survival and mitochondrial reporter assays
- *E. tetraedra* worms also show evidence of bystander signalling and a potential adaptive response to the signal if exposed to chronic doses of radiation previously
- These results are evidence that the nature of radiation acting on living organisms is complicated and must be appreciated on a system-level in the context of radiation protection