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Source: Radiation Research, 168(5) : 515-526

Published By: Radiation Research Society

URL: <https://doi.org/10.1667/RR1012.1>

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## Effects of Chronic Gamma Irradiation on Reproduction in the Earthworm *Eisenia fetida* (Oligochaeta)

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Hertel-Aas, T., Oughton, D. H., Jaworska, A., Bjerke, H., Salbu, B. and Brunborg, G. Effects of Chronic Gamma Irradiation on Reproduction in the Earthworm *Eisenia fetida* (Oligochaeta). *Radiat. Res.* 168, 515–526 (2007).

*Eisenia fetida* were exposed continuously to <sup>60</sup>Co  $\gamma$  radiation during two generations (F<sub>0</sub> and F<sub>1</sub>). Adult F<sub>0</sub> reproduction capacity (i.e., number of cocoons produced, hatchability and number of F<sub>1</sub> hatchlings) in controls and at five dose rates (0.18, 1.7, 4, 11 and 43 mGy/h) was measured over a 13-week exposure period. Survival, growth and sexual maturation of F<sub>1</sub> hatchlings were observed for 11 weeks. F<sub>1</sub> adults were exposed for a further 13 weeks to determine their reproduction capacity. There was no radiation-induced effect on the cocoon production rate in either F<sub>0</sub> or F<sub>1</sub>. For F<sub>0</sub>, hatchability of cocoons produced during the first 4 weeks was reduced to 60% at 43 mGy/h (98% in controls), and none of the cocoons produced at 5–13 weeks hatched. At 11 mGy/h the cocoon hatchability was reduced to 25% at 9–13 weeks. In addition, the number of hatchlings per hatched cocoon was reduced at 11 and 43 mGy/h. Correspondingly, at these dose rates, the total number of F<sub>1</sub> hatchlings per adult F<sub>0</sub> was significantly lower than in the control. This number was also reduced at 4 mGy/h, but the effect was of borderline significance. For adult F<sub>1</sub>, the hatchability of cocoons at 11 mGy/h was reduced to 45–69% during the 13-week exposure period. The number of hatchlings (F<sub>2</sub>) per cocoon and the total number of F<sub>2</sub> individuals produced was also reduced. However, and in contrast to the results observed for F<sub>0</sub>, hatchability increased with time, suggesting a possible acclimatization or adaptation of the F<sub>1</sub> individuals. In conclusion, chronic irradiation reduced the reproduction capacity of *E. fetida*, but extensive exposure periods (13 weeks) were needed for these effects to be expressed. The lowest dose rates at which an effect was observed were 4 mGy/h in F<sub>0</sub> and 11 mGy/h in F<sub>1</sub>. © 2007 by Radiation Research Society

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### INTRODUCTION

Traditionally, radiological protection has focused on humans. In recent years the ecological impact of ionizing ra-

diation has emerged as an important research field. However, there are still considerable gaps in knowledge regarding the biological effects of chronic irradiation in wildlife, particularly for end points related to reproduction (1, 2). Reproduction is considered to be one of the most sensitive radiation-associated end points (3), and it not only determines the fate of the single organism but also may influence the population dynamics and the balance of higher ecological units. In most contamination situations, the majority of the radionuclide inventory in terrestrial ecosystems is found within soil; thus soil invertebrates can receive significant external and internal doses. A number of studies of the movement of radionuclides through invertebrate food chains have demonstrated that detritivorous species have higher concentrations of radionuclides (cesium, plutonium, americium) than herbivore and predatory species. Earthworms may receive increased exposure because of the passage of soil through their alimentary tracts, and they also lack the chitinous exoskeleton of some soil invertebrate species, which may reduce exposure from external radiation (1). Based on their radioecological properties and their important role in the soil ecosystem, earthworms have been identified by the ICRP (4) as one of the reference animals and plants (RAPs) to be used in environmental radiation protection.

The UK Environment Agency has recommended chronic irradiation studies using the earthworm *Eisenia fetida* (5). This species was chosen due to the ease with which it can be obtained and experimented upon, its use in standard soil toxicity testing (6, 7), and its short life cycle. Parameters such as growth rates of offspring and time of sexual maturation are recognized to be of great importance for population dynamics, and results from modeling indicate that earthworm populations are specifically sensitive to toxicants that retard maturation (8). A number of workers have therefore recommended that laboratory tests on the toxicity of chemicals should also include chronic tests for pre-adult growth (6, 8, 9).

Many of the data concerning the effects of radiation on earthworms are derived from field studies in which radionuclide activities in soil have been increased by artificial contamination (10–12) or by field monitoring after nuclear

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**TABLE 1**  
**Components of the Artificial Soil Used in the Exposure Experiment**

Soil component	Characteristics	Percentage (on a dry mass basis)
Sphagnum peat	Little to medium transformed, finely ground with no visible plant remains	10
Kaolinite clay	36% Al <sub>2</sub> O <sub>3</sub> and 48% SiO <sub>2</sub>	20
Quartz sand	Particle size: 5.6% ≤0.06 mm, 45.5% 0.06–0.25 mm, 48.9% 0.25–0.5 mm	70

accidents (3, 13–15). These studies have shown effects such as reduced population size, changes in the distribution of life cycle stages, and reduced number of species. The interpretation of results from field experiments is often complicated, and dose–response relationships are difficult to establish. Most laboratory experiments on earthworms involve acute irradiation and effects on mortality (16–19), and only two studies of reproduction end points have been published (19, 20). Recently, Hingston *et al.* (21) reported the results of a chronic experiment in which *E. fetida* was continuously exposed to  $\gamma$  radiation for 16 weeks. To our knowledge, there are no published data on exposures for more than one generation for *E. fetida* or other earthworms.

The standard OECD reproduction toxicity test for *E. fetida* lasts for 4 weeks, but when designing radiation experiments, various aspects of the reproduction and physiology of this species must be considered. Earthworms are hermaphrodites with separate testes and ovaries that function simultaneously. After copulation, the received spermatozoa are transferred to spermathecae and can be stored in these organs for relatively long periods (22). Thus, in short-term irradiation experiments, a significant portion of the ova can be fertilized by spermatozoa produced prior to exposure. This is important also because it is known that early stages of spermatogenesis are more sensitive to radiation than mature spermatozoa (20). The ova and the spermatozoa are discharged into the cocoon where fertilization takes place. *E. fetida* is a very prolific species, producing from two to five cocoons per worm per week. Most of the cocoons hatch 3 to 4 weeks after production, and it takes approximately 8 to 12 weeks before the hatchlings reach sexual maturity. Although the life cycle of *E. fetida* is short, its life span is rather long. Individuals of *E. fetida* have been kept in the laboratory for about 4½ years (23), and they can be reproductively active for more than 500 days (24). Thus there is a need for reproduction studies longer than 4 weeks when investigating effects of long-term exposure in *E. fetida*.

The experiment described in the present paper lasted for 38 weeks and included exposure of both F<sub>0</sub> and F<sub>1</sub> individuals. It was designed to study several end points related to growth, sexual maturation and reproduction, such as cocoon production rates, hatchability and number of offspring (F<sub>1</sub> and F<sub>2</sub>). End points were assessed after defined exposure periods, allowing determination of possible effects related to increasing accumulated doses at the different dose rates. The dose rates used varied 200-fold, from 0.18 to 43 mGy/h, covering the range recommended by the UK En-

vironment Agency for investigating effects on soil fauna: 1 mGy/h to 5 mGy/h. The two higher dose rates were included since it has been emphasized that doses should be sufficiently high to ensure that a dose–response relationship can be constructed (5).

## MATERIAL AND METHODS

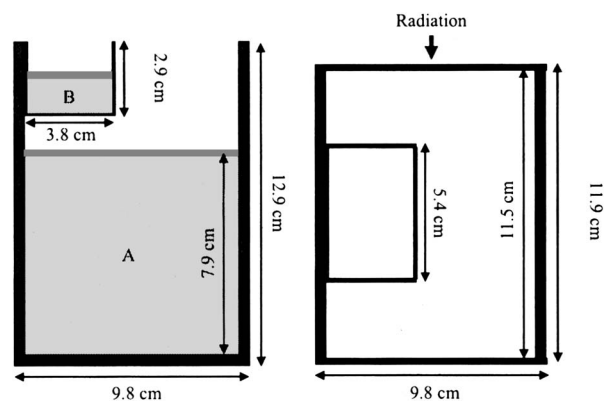
### Culture and Exposure Conditions

*E. fetida* (originally from the Norwegian Center for Soil and Environmental Research) were reared in synchronous cultures in commercial potting soil at room temperature in constant darkness for several generations. The worms were fed finely ground air-dried horse manure (pH 6.5–7.1) rewetted with deionized water, and the substrate (50:50, vol:vol, soil: manure) was changed every month. For the exposure experiment, adult earthworms (F<sub>0</sub>), 20–23 weeks old, with well-developed clitellum were used. Prior to exposure, all worms were acclimatized in artificial OECD soil for 14 days. After this time, the worms were sorted from the soil, washed in saline (0.85%), weighed individually, and added to the different test boxes at random. The average wet weight of the worms used was 346 ± 45 mg (range 281–505 mg).

The artificial soil was prepared according to OECD guideline 207 (25) and consisted of sphagnum peat (Tiur-torv, Nittedal torvindustri AS, Norway), kaolinite clay (China clay, 3309-25, WBB Minerals, England UK), and industrial quartz sand (Svelviksand AS, Norway) (Table 1). The dry constituents were passed through a 2-mm sieve to remove larger particles and mixed thoroughly. The pH of the soil was measured in 0.01 M CaCl<sub>2</sub> and adjusted to 6.2 ± 0.1 by adding CaCO<sub>3</sub>. One day before use, the soil was moistened by adding deionized water to 27% w/w (corresponding to 57% of the maximum water-holding capacity). Horse manure from the same horse (healthy and receiving no pharmaceuticals) was used as the feed supply. The manure was air dried and frozen at –20°C until use. The pH in each new batch was measured in 0.01 M CaCl<sub>2</sub> (range 6.8–7.3). Just before use, the manure was finely ground using a blender and rewetted with deionized water to 67%. To each test container (box A; Fig. 1), 677 g moist soil (corresponding to 50 g dry weight/worm) was added, giving a substrate depth of about 7.9 cm. Ten worms were placed in each replicate container, and horse manure, 0.5 g dry weight/worm, was spread on the soil surface. A perforated transparent plastic sheet was placed on top. The moisture content of the soil was maintained by reweighing the test containers once a week, and lost water was replenished. At the same time new feed (0.5 g dry weight/worm) was supplied. When the soil was replaced (see below), the pH and water content were determined in both the new and the old soil. The maximum changes in pH and water content in the boxes during the different exposure periods were –0.1 U and 3%, respectively.

### Irradiation

Irradiation was carried out in a thermostatically heated room with temperature logging at 21 ± 1°C (adult F<sub>0</sub> and F<sub>1</sub> growth and maturation). During F<sub>1</sub> reproduction, the temperature increased to 25 ± 1°C (weeks 12–16), 23 ± 0.5°C (weeks 17–20), and 22 ± 1°C (weeks 21–24) in



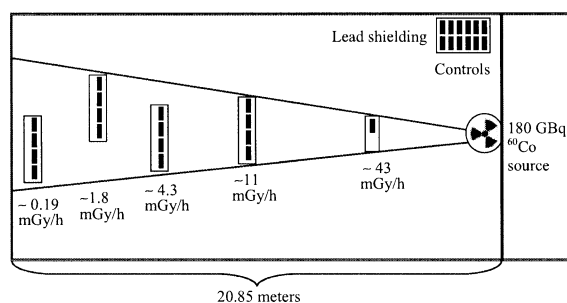
**FIG. 1.** The two types of experimental test containers used. Box A: Perspex;  $9.8 \times 11.9 \times 12.9$  cm, internal cross-sectional area:  $103.5 \text{ cm}^2$ . Box B (PVC;  $5.4 \times 3.8 \times 2.9$  cm, cross-sectional area  $18.36 \text{ cm}^2$ ) was placed inside box A as shown. Left panel, side view; right panel, top view.

periods of unusually hot weather. Illumination was 250–320 lx, with a cycle of 16 h light and 8 h dark.

External  $\gamma$  radiation was provided by a  $^{60}\text{Co}$  source (180 GBq) (Fig. 2). Field dosimetry was performed with an ionization chamber, and air kerma rates were measured. The dose determination is traceable to the Norwegian Secondary Standard Dosimetry Laboratory. The absolute dosimetry was supplemented with portable Mg-Ti thermoluminescence dosimeters (TLDs) that were placed at different positions outside and inside the filled box A. Based on the latter measurements, specific absorption coefficients for the soil were estimated and averaged. The dose rate at any position in box A can be calculated using this absorption coefficient and assuming linear absorption throughout the box. (Note that the latter approximation introduces a small but negligible error since in reality the absorption is exponential.) To calculate the dose received by the earthworms, it was assumed that the worm's tissue had the interaction characteristics of water and that the worms were distributed randomly throughout the box volume, with no systematic differences in their moving pattern. The geometric center of the box was used as the reference point and is assumed to represent the best estimate of the dose. The determination of the dose is based on the following equation:

$$D_{\text{worm}} = D_{\text{w.ref.i}} \times B \times k_{\text{att}} \times k_{\text{beh}}$$

where  $D_{\text{w.ref.i}}$  is the absorbed dose to water in the reference point at the  $i$ th box. The absorbed dose to water was determined from the measured air kerma by employing the mass energy absorption coefficient ratio for water and air, correcting for bremsstrahlung. The uncertainty in the reference absorbed dose to water is  $u_D = 7\%$ .  $B$  is the backscatter in the box ( $u_B = 2\%$ ),  $k_{\text{att}}$  is the correction for the attenuation of radiation from front to reference point ( $u_{\text{att}} = 9\%$ ), and  $k_{\text{beh}}$  is the correction for the behavior of the worm in the box. It was assumed that the earthworms would have received an average accumulated dose equivalent to that in the geometrical center of the box; hence  $k_{\text{beh}}$  was set at 1, with an estimated uncertainty  $u_{\text{beh}} = 10\%$ . [The unlikely situation that all worms stayed permanently in the box area closest to the source would have increased the dose rate by a maximum of approximately 25% (33% for the highest dose rate).] The overall uncertainty for the dose estimation was 15% (one standard deviation). The test boxes were placed at different distances from the source, at a slight angle to reduce variations due to the field geometry, giving absorbed dose rates in the range  $\sim 0.19$  to  $\sim 43$  mGy/h (Fig. 2). Due to the geometric limitations of the radiation beam, there was only one replicate at the highest dose rate. This was included with the primary intention of achieving a clear radiation-induced effect. Controls were placed in the same room, shielded by lead, and the external dose to these worms was measured to be approximately  $0.2 \mu\text{Gy/h}$  (air-to-water correction and soil attenuation have been taken into account).

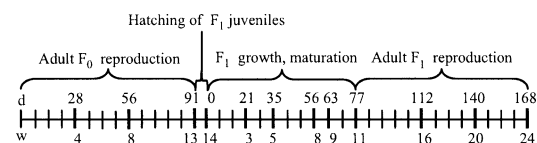


**FIG. 2.** Positioning of test containers and resulting dose rates. Replicate boxes A (see Fig 1), shown as filled squares, were placed at different distances and height relative to the  $^{60}\text{Co}$  source (i.e., none of the boxes were shielding each other). The resulting dose rates are shown and represent those received by worms in the geometrical center of the boxes at the start of the experiment (drawing not to scale).

### Adult $F_0$ Reproduction

The time schedule of the exposure experiment, and its different phases, is illustrated in Fig. 3. The adult  $F_0$  worms were continuously exposed to radiation for 13 weeks, interrupted only when the boxes were removed for weighing, registration of mortality (defined as missing individuals), and feeding. On return of boxes to the radiation beam, the positions of replicates were interchanged sequentially to reduce any effect of varying dose rates. After 4, 8 and 13 weeks, adult worms ( $F_0$ ) and test soil were removed, and the worms were washed in saline, blotted dry and weighed individually, after which they were transferred back to the test containers together with new soil and feed. At 13 weeks, all worms were removed and samples were taken for various cellular and molecular analyses, the results of which will be reported elsewhere. At each of the three sampling times, cocoons and hatchlings ( $F_1$ ) were hand sorted from the soil and counted. The cocoons were transferred to box B, supplied with 20 g soil and 2 g horse manure, covered with a transparent perforated plastic sheet, and placed inside the corresponding box A (Fig. 1). After 2 weeks, an additional 2 g horse manure was added to the B boxes. The hatchability of the cocoons and the number of hatchlings ( $F_1$ ) in box B were recorded 4, 7 and 9 weeks after removal from box A. Hatched cocoons were identified as those floating in 0.85% saline. Unhatched cocoons were transferred to 20 g new soil with 1 g horse manure. Normally cocoons hatch during the first 4 weeks after production, but the assessment period was extended to 9 weeks to determine whether hatching of cocoons was delayed or totally absent.

For each exposure period we estimated the number of cocoons produced per worm per week and the hatchability of the cocoons. Due to some migration of hatchlings from box B over to the corresponding box A, the average number of hatchlings produced per adult worm and the number of hatchlings per hatched cocoon could only be estimated at the end of the experiment. A reduced number of hatchlings per cocoon can be attributed either to reduced fertility of the cocoons or poorer health of the hatchlings, leading to death soon after hatching. The experimental protocol did not allow us to discriminate between these alternatives.



**FIG. 3.** Time schedule of the exposure experiment and its different phases. Each bar represents 1 week. Times for weighing of individual worms and counting of cocoons (as described in the Materials and Methods) are indicated in days (d) and weeks (w). Note the new time zero at the start of the  $F_1$  experiment.

### *F<sub>1</sub> Growth and Maturation*

*F<sub>1</sub>* hatchlings were taken from cocoons produced during weeks 9–13 that hatched during week 14. At the end of week 14 (or during the 3 following weeks as specified below for 11 mGy/h), ten hatchlings from each replicate were weighed together, transferred to box B, and supplied with 15 g soil and 2.5 g horse manure. Each box B was placed inside its corresponding box A, and irradiation was continued. The age of the hatchlings could be from 0 to 7 days at this point, which will be referred to hereafter as Day 0 of the *F<sub>1</sub>* growth and maturation study (Fig. 3). After 2 weeks, evaporated water was replaced and 1 g horse manure was added to each box. At 3 weeks, the juveniles were weighed individually and transferred to box A containing 677 g soil and horse manure. When mortality was observed, the amount of soil and horse manure was reduced to obtain equal densities and feeding conditions in all test boxes. New feed was added at week 5 and 7 and once per week thereafter. After 5, 8 and 9 weeks, worms were weighed individually, and viability, morbidity and development of clitellum were recorded.

### *Adult F<sub>1</sub> Reproduction*

After 11 weeks, *F<sub>1</sub>* worms were weighed individually and transferred to new soil and feed, and irradiation was continued. The reproduction capacity was recorded for 13 more weeks. Weighing of adult worms and counting of cocoons and *F<sub>2</sub>* hatchlings were performed at weeks 16, 20 and 24; cocoons were transferred to box B and re-examined for hatching as described above. At week 24, the experiment was terminated, except that four control boxes and 4 mGy/h boxes were exposed for a further 8 weeks. Worms from all the other boxes were weighed and various samples were taken for cellular and molecular analyses.

### *Histopathology*

Worms from the control and the higher-dose-rate groups (*F<sub>0</sub>*: 4.2, 11 and 43 mGy/h; *F<sub>1</sub>*: 11 mGy/h) were subjected to histopathological studies of the male reproductive organs. After weighing, worms were transferred to moist filter paper (0.85% saline) for 24 h to empty gut content from the anterior part. Worms were anesthetized in 12% ethanol and fixed in Bouin's fixative for 24 h at room temperature followed by 70% ethanol. Individuals were cut between the 7th and 8th segment and between the 15th and 16th segment. The eight resulting segments containing the reproductive organs were embedded in paraffin, and 5- $\mu$ m transverse sections were stained with hematoxylin and eosin.

### *Statistical Analysis*

Significant differences between dose-rate groups in the weights of worms, cocoon production rates, hatchability and numbers of offspring were calculated using Minitab software (MINITAB®Release 14, Minitab Inc.). Due to the unbalanced design of the experimental setup (i.e., different numbers of replicates), the general linear model (GLM) of ANOVA was used. The "per box" results were used as replicates, assuming that the experimental units (each box with ten worms) did not differ from each other by themselves. The treatment box means were compared at each separate time; when differences were found, Tukey's multiple comparison test was used to determine differences between specific groups at a 0.05 significance level (when specified, Dunnett's test was also used). The assumption of normality and of equal variance was checked using the Anderson-Darling test (0.01 significance level) and the Levene's test (significance level 0.05), respectively. The hatching of cocoons is a quantal response (a cocoon has either hatched or not), and hatchability is given as percentage, based on the fraction of hatched cocoons in each replicate. To achieve normal distribution, the fractions were transformed by taking the square-root arcsine.

**TABLE 2**  
**Dose Rates and Accumulated Doses, *F<sub>0</sub>***

Mean dose rate (mGy/h)	Accumulated dose (Gy)		
	Week 4 (619 $\pm$ 19 h)	Week 8 (1261 $\pm$ 4 h)	Week 13 (2030 $\pm$ 30 h)
0.18–0.19	0.11 $\pm$ 0.02	0.23 $\pm$ 0.03	0.37 $\pm$ 0.05
1.7–1.8	1.1 $\pm$ 0.2	2.2 $\pm$ 0.3	3.6 $\pm$ 0.5
4.2–4.3	2.7 $\pm$ 0.4	5.4 $\pm$ 0.8	8.6 $\pm$ 1.3
11.2–11.5	7.1 $\pm$ 0.9	14 $\pm$ 2	23 $\pm$ 3
42.4–42.7	26 $\pm$ 4	53 $\pm$ 8	85 $\pm$ 13

*Notes.* The mean absorbed dose rates are calculated for worms in the center of box A at the respective distances from the source and are given as ranges due to the decay of <sup>60</sup>Co during the 13 weeks of adult *F<sub>0</sub>* exposure. The accumulated doses are calculated by using the average dose rate of each week multiplied by the hours of exposure. Values are means  $\pm$  SD (including 15% uncertainty). Boxes were removed from the exposed positions once per week for feeding and/or weighing, counting of cocoons etc.; this process lasted for 2 to 8 h.

## RESULTS

### *Adult F<sub>0</sub> Reproduction*

Exposure times and accumulated doses at the end of the various periods for adult *F<sub>0</sub>* worms are shown in Table 2.

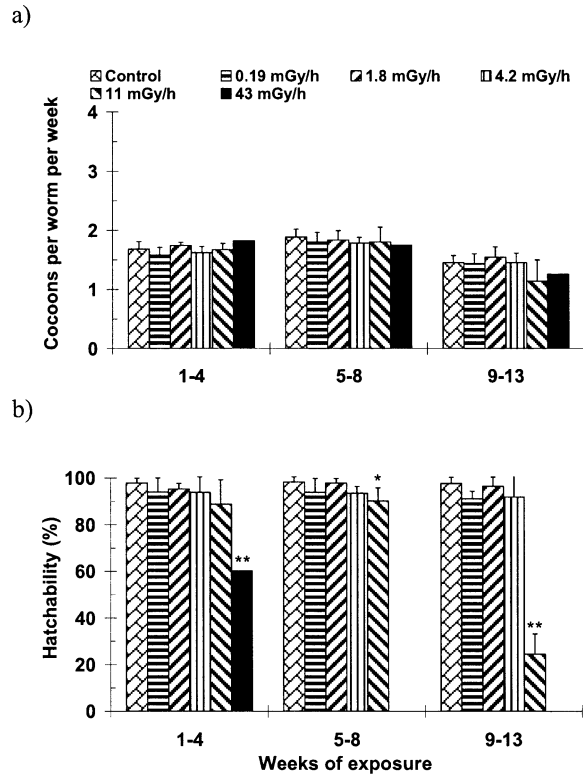
#### *1. Viability and growth*

The viability of the worms in all groups was high. Only one death was recorded during the 13-week exposure period, occurring between weeks 5 and 8 at 4.2 mGy/h. During the first 8 weeks of exposure, there was an increase in the average weight of worms in all groups (10–17%). This was followed by minor weight reductions during the subsequent period (9–13 weeks). None of these weight changes were significantly dependent on dose rate.

#### *2. Reproduction capacity*

The cocoon production rates in controls were 1.68  $\pm$  0.13, 1.89  $\pm$  0.13 and 1.46  $\pm$  0.13 cocoons/worm per week during exposure weeks 1–4, 5–8 and 9–13, respectively (Fig. 4a). These minor variations are most likely related to the use of different batches of feed. The cocoon production rate was not affected by the radiation exposure: There were no significant differences between the controls and the groups receiving various dose rates at any time. At weeks 9–13, however, the production rate was clearly reduced (50%) in one of the four boxes at 11 mGy/h (the standardized residual for this box was  $-3.2$ , and it could be considered as a potential outlier; whether this was omitted or not had no implication for the conclusions of the statistical analysis).

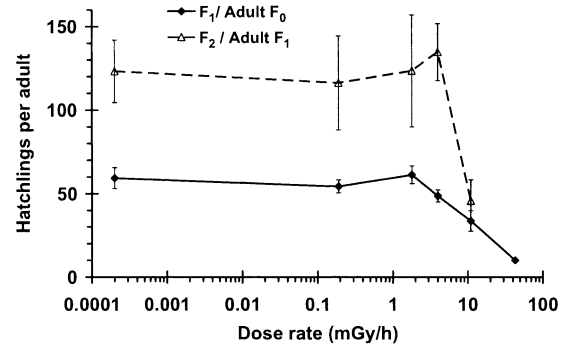
In the controls, hatchability was high during the whole exposure period (98  $\pm$  2%) (Fig. 4b). At the highest dose rate (43 mGy/h), the hatchability of cocoons produced during the first 4 weeks of adult *F<sub>0</sub>* exposure was reduced to 61% (these cocoons were also checked after 12 weeks, but no further hatching was observed). Lower dose rates had



**FIG. 4.** Adult  $F_0$  cocoon production rate and hatchability during different exposure periods. Panel a: Cocoon production rates. Panel b: Hatchability given as the accumulated percentages, recorded until 9 weeks after transfer of cocoons to box B. Means  $\pm$  SD of replicate boxes are shown for untransformed data. Control:  $n = 12$ ; 0.19–11 mGy/h,  $n = 4$ ; 43 mGy/h,  $n = 1$ . Significant difference from controls is indicated (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

no significant effect on hatchability during the first 4 weeks of exposure. It should be noted that a portion of these cocoons contained embryos produced by eggs and spermatozoa that developed before the radiation exposure had started. During the next exposure periods (5–8 and 9–13 weeks), none of the cocoons at the highest dose rate hatched. At the second highest dose rate (11 mGy/h), a slight but significant ( $P = 0.008$ ) reduction in hatchability was observed in cocoons produced during weeks 5–8 (to  $90 \pm 6\%$ ), with a marked decrease ( $P < 0.0001$ ) to  $25 \pm 9\%$  after 9–13 weeks of exposure. The large majority of the cocoons produced in every exposure group hatched during the first 4 weeks, with almost no further increase in the consecutive weeks (7 and 9 weeks). Some of the unhatched cocoons at the two highest dose rates were dissected, and no signs of maturing embryos were found.

There was a slight reduction in the average number of hatchlings emerging from each hatched cocoon compared to the control ( $2.81 \pm 0.25$ ) at 4.2, 11 and 43 mGy/h ( $2.53 \pm 0.10$ ,  $2.43 \pm 0.23$  and  $2.27$ , respectively), but this was significant only at 11 mGy/h ( $P = 0.035$ ). The total number of  $F_1$  hatchlings produced per adult  $F_0$  worm in each replicate box for the 13 weeks of exposure as a whole was significantly reduced ( $P < 0.001$ ) at 11 mGy/h and 43



**FIG. 5.** Production of hatchlings by exposed adult earthworms. The total number of  $F_1$  hatchlings per adult  $F_0$ , and  $F_2$  hatchlings per adult  $F_1$  produced during the whole 13-week reproduction exposure period in the two different generations. Note that external dose to controls is  $0.2 \mu\text{Gy/h}$ . Values are means  $\pm$  SD for replicate boxes. Control:  $n = 12$ ; 0.19–11 mGy/h,  $n = 4$ ; 43 mGy/h,  $n = 1$ .

mGy/h ( $33.7 \pm 6.2$  and 10, respectively) compared to the control ( $59.4 \pm 6.3$ ) (Fig. 5). Before a proper statistical analysis of the group exposed at 4.2 mGy/h could be carried out, the data had to be corrected for the death of one of the adult worms during weeks 5–8. We assumed that death occurred after 6 weeks and used the average values for the other worms in the box to generate the additional number of  $F_1$  hatchlings that this worm would have produced (this changed the average number of  $F_1$ /adult  $F_0$  for the four replicates, from  $48.6 \pm 3.6$  to  $49.4 \pm 4.6$ ). Analysis with the corrected value gave a significant reduction in  $F_1$ /adult  $F_0$  compared to controls when using Dunnett's test ( $P = 0.027$ ), but not with Tukey's test ( $P = 0.057$ ). When the uncorrected average was used, or when the replicate with the missing worm was excluded from the analysis, the reduction was significant with both Dunnett's ( $P = 0.014$  and  $P = 0.018$ , respectively) and Tukey's test ( $P = 0.031$  and  $P = 0.039$ , respectively).

### 3. Morbidity

We observed no exterior abnormalities in the adult ( $F_0$ ) worms in any of the exposed groups. Upon dissection and macroscopic examination, when tissue was taken for examination of other end points, spermathecae and seminal vesicles were clearly visible in the controls ( $n = 45$ , number of worms) as well as in the four groups with the lowest exposures ( $n = 16$  for 0.19 mGy/h and  $n = 29$  for 1.8, 4.2 and 11 mGy/h). However, at the highest dose rate (43 mGy/h), the male reproductive organs were atrophic, and only the spermathecae remained visible ( $n = 7$ ). These observations were confirmed by microscopy of stained transverse sections (Fig. 6). In *E. fetida*, the first spermatogonial divisions occur in the testis. When the spermatogonial morula (consisting of synchronously developing cells from the same spermatogonium) has reached the 8–32-cell stage, it leaves the testes and further spermatogonial divisions and spermiogenesis take place in the seminal vesicles (20). The mature spermatozoa pass quickly to the seminal funnels,



**FIG. 6.** Transverse sections showing male reproductive organs of  $F_0$  adults exposed for 13 weeks. Control (left), 11 mGy/h (middle) and 43 mGy/h (right). SV: seminal vesicles, SF: seminal funnels with darkly stained spermatozoa; SP: spermathecae with darkly stained spermatozoa.

where they remain until copulation occurs. During mating they pass through the *vas efferens* and the *vas deferens* to the exterior through the two male pores (in segment 15) (23). In controls ( $n = 3$ ), worms exposed at 4.2 mGy/h ( $n = 2$ ) and at 11 mGy/h ( $n = 3$ ), testes, seminal vesicles, spermathecae and seminal funnels with spermatozoa were seen. The worms exposed at 43 mGy/h ( $n = 3$ ) had atrophied testes and seminal vesicles, and the seminal funnels were empty. Although spermatogenesis had ceased, they all had spermathecae containing spermatozoa (received from other worms during copulation). This could be explained by the fact that spermatozoa can be stored and nourished in the spermathecae for several weeks after copulation (22).

#### $F_1$ Growth and Maturation

The  $F_1$  hatchlings used in the growth, maturation and reproduction experiment were taken from cocoons produced by  $F_0$  worms during the last exposure period (weeks 9–13). Due to the lack of hatching at 43 mGy/h, a study on the  $F_1$  generation at this dose rate could not be performed. The hatchability of the cocoons at 11 mGy/h was reduced, and sufficient hatchlings were therefore obtained only for one replicate box at the start of  $F_1$  exposure (Day 0; Fig. 3). Hatchlings for the remaining three boxes were obtained from cocoons hatched during the next 20 days. The subsequent weighing and treatment of these replicates were correspondingly delayed, but in the  $F_1$  reproduction study that followed, the boxes were examined in phase with the others.

Table 3 shows the doses accumulated by the  $F_1$  individuals, from fertilization in the cocoons to the end of the growth and maturation period. It should be noted that these doses do not include those received during germ cell stages. Assuming that the embryonic development lasts for 3 weeks and that cocoons are immobile and evenly distributed in the box volume, this will give absorbed doses in the range 0.07–0.11 Gy, 0.71–1.0 Gy, 1.7–2.5 Gy and 4.4–6.7 Gy for cocoons exposed at 0.18 mGy/h, 1.7 mGy/h, 4 mGy/h and 11 mGy/h, respectively.

*Viability, growth and maturation.* Some mortality was observed in the early period of the  $F_1$  experiment. Between weeks 3 and 5, two deaths were recorded among the controls and one at the lowest dose rate (0.18 mGy/h). At week 8, one more death was recorded among the controls. These deaths could be related to the small size of the hatchlings and the risk of mechanical damage during handling. The first part of the  $F_1$  growth curves (Fig. 7) was exponential as expected (26). When the data were fitted to Gompertz curves, it appeared that the model did not adequately describe growth during the late phases; i.e., weights increased although they were expected to reach a plateau. This could be associated with replenishing the substrate at week 11 (and also at 16 and 20) and the increase in temperature (weeks 12–20). There was no clear dose-related effect on the growth of the offspring ( $F_1$ ), except for some indication that worms at 4.0 mGy/h gained slightly more weight than the controls. When  $F_1$  juveniles were 5 weeks old, none had developed clitellum. At the age of 8 weeks, 35–55%

**TABLE 3**  
Dose Rates and Accumulated Doses,  $F_1$

Mean dose rate (mGy/h)	Accumulated dose (Gy)			
	Week 11 (2184 ± 28 h)	Week 16 (2971 ± 30 h)	Week 20 (3593 ± 41 h)	Week 24 (4161 ± 43 h)
0.17–0.18	0.39 ± 0.06	0.53 ± 0.08	0.63 ± 0.09	0.73 ± 0.11
1.6–1.7	3.7 ± 0.6	5.0 ± 0.8	6.1 ± 0.9	7.0 ± 1.1
3.9–4.1	9.0 ± 1	12 ± 2	15 ± 2	17 ± 3
10.5–11.1	24 ± 4	33 ± 5	39 ± 6	45 ± 7

*Notes.* Week 11 denotes the end of the growth and maturation period; weeks 16, 20 and 24 denote the end of each  $F_1$  reproduction period. The time given in parentheses represents the entire exposure period including the embryonic development in cocoons. See Table 2 for further details.

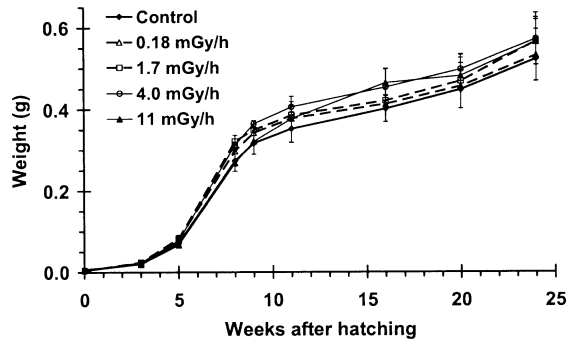


FIG. 7. Weight of  $F_1$  earthworms from hatching to termination of experiment. Means  $\pm$  SD of replicate boxes are shown. Control:  $n = 12$ ; 0.18–11 mGy/h,  $n = 4$ .

of the worms had fully developed this organ; i.e., they were sexually mature, and cocoon production had started. After 11 weeks, close to 100% of controls and worms at the three lowest dose rates had developed clitellum. This percentage was somewhat lower (85%) among earthworms exposed to 11 mGy/h, probably related to a delayed hatching of these  $F_1$  juveniles. At week 16 all worms except one at 11 mGy/h were sexually mature.

#### Adult $F_1$ Reproduction

Table 3 shows accumulated doses during the  $F_1$  reproduction experiment, starting at week 11 and continuing until week 24.

##### 1. Viability, growth and morbidity

One death occurred between weeks 21 and 24 in a box exposed at 1.7 mGy/h. The weight changes during the  $F_1$  reproduction study are shown in Fig. 7. Some exterior abnormalities were observed at the highest dose rate (11 mGy/h). Two of the worms (5%) developed an asymmetrical and segmented clitellum, which resulted in the production of “double cocoons” (Fig. 8). At week 16, the worm that failed to develop clitellum had lost its anterior part containing the reproductive organs. It survived throughout the rest of the experiment but was getting progressively smaller.

##### 2. Reproduction capacity

The numbers of cocoons produced by  $F_1$  adults in the various exposure groups were not significantly different (Fig. 9a), as was also the case for the  $F_0$  generation. The mean cocoon production rates in the control boxes were  $3.15 \pm 0.25$  and  $3.10 \pm 0.21$  cocoons/worm per week during weeks 12–16 and weeks 17–20, respectively, and dropped to  $1.90 \pm 0.21$  during weeks 21–24 (the double cocoons at 11 mGy/h were produced at a similar rate). The hatchability of  $F_1$  control cocoons was always above 96%. At 11 mGy/h, there was a significant reduction ( $P < 0.001$ ) of hatchability to  $46 \pm 13$ ,  $56 \pm 13$  and  $69 \pm 9\%$  in cocoons produced during weeks 12–16, 17–20 and 21–24,

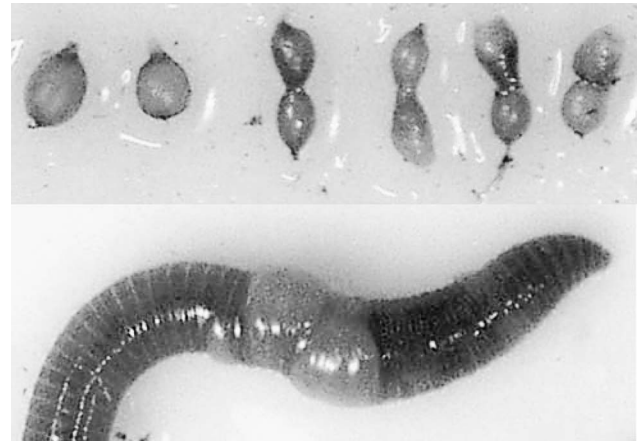


FIG. 8. Micrographs of abnormal earthworm and cocoons in  $F_1$  after exposure to 11 mGy/h. Upper panel, double and normal cocoons. Lower panel, worm with segmented clitellum.

respectively (Fig. 9b). It is noteworthy that the hatchability increased with exposure time at 11 mGy/h. This is in contrast to the results for  $F_0$  at the same dose rate, where the hatchability was slightly reduced during the first 1–8 weeks, with a marked drop in the last exposure period (weeks 9–13) (Fig. 4b). The hatchability of cocoons produced by  $F_1$

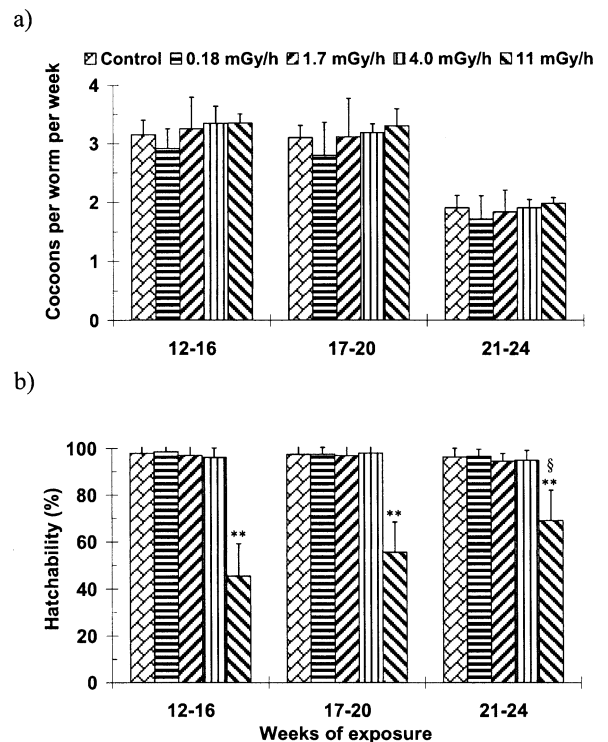


FIG. 9 Adult  $F_1$  cocoon production rate and hatchability during different exposure periods. Panel a: Cocoon production rates. Panel b: Hatchability is shown as accumulated percentages 9 weeks after counting. Means  $\pm$  SD of replicate boxes are shown for untransformed data. Control:  $n = 12$ ; 0.18–11 mGy/h,  $n = 4$ . Significant difference compared to controls is indicated by asterisks (\*\* $P < 0.01$ ). Significant difference compared to the first period (weeks 12–16) at 11 mGy/h is indicated with a paragraph symbol (§ $P = 0.047$ ).



adults during weeks 17–20 and 21–24 was significantly higher than that of cocoons produced by  $F_0$  adult during weeks 9–13 ( $P = 0.0097$  and  $P = 0.0006$ , respectively). This could indicate a radiation-induced acclimation and/or adaptation in  $F_1$ , but a number of confounders could also have contributed to these observations as further discussed below. No effects on the hatchability were observed at lower dose rates. At 11 mGy/h, there was also a reduction in the number of  $F_2$  hatchlings per hatched cocoon ( $2.24 \pm 0.46$  compared to  $3.53 \pm 0.38$  in the control;  $P = 0.0005$ ). The resulting total number of  $F_2$  hatchlings per  $F_1$  adult, at the end of the experiment was significantly reduced ( $46 \pm 13$  compared to  $123 \pm 19$  for controls;  $P < 0.0001$ ). Exposure to 4 mGy/h produced no such reduction, which is in contrast to the results from the previous generation at the same dose rate (Fig. 5).

At termination of the experiment, one worm exposed to 11 mGy/h was found to have an asymmetrical location of the male pores, one in segment 15 and the other in segment 16. Upon dissection and macroscopic examination, spermathecae and seminal vesicles were clearly visible in the controls ( $n = 35$ ) as well as in worms exposed to 0.18 mGy/h ( $n = 8$ ) and 1.7 mGy/h ( $n = 35$ ) and in most of the worms at 11 mGy/h ( $n = 35$ ). At this dose rate, three of 35 worms had very small seminal vesicles, and they were absent in one worm. Three control worms and four worms exposed to 11 mGy/h were subjected to microscopy of transverse sections. The worm with the most pronounced segmented clitellum had spermathecae containing spermatozoa, but the seminal vesicles seemed atrophied and the seminal funnels contained no spermatozoa. The other three worms at this dose rate seemed normal, with seminal vesicles, spermathecae and seminal funnels containing spermatozoa.

## DISCUSSION

Earthworms are known to be relatively radioresistant, and the  $LD_{50/30}$  for adults is approximately 650 Gy for acute  $\gamma$  radiation (17–19). Hence, at moderate exposure levels, mortality is not expected to have any significant influence on earthworm population dynamics. For most species, reproduction is known to be far more sensitive to ionizing radiation than mortality (3). This is also the case for *E. fetida*. In the present study we found that reproduction in  $F_0$  was seriously affected by chronic irradiation at 11 and 43 mGy/h (accumulated doses of the order of 20 Gy), with indications of effects also at 4 mGy/h (9 Gy); this corresponds to 1/30–1/70 of the adult  $LD_{50/30}$  dose (19). In the next generation ( $F_1$ ), reproduction was seriously affected at 11 mGy/h, but no effects were observed at 4 mGy/h even when the exposure was extended by 8 weeks (accumulated dose 22 Gy; data not shown).

## Comparison of Chronic Laboratory Studies and Field Studies

There are few published laboratory studies on the effects of chronic radiation exposure on reproduction in earthworms and other invertebrates. Hingston *et al.* (21) reported that exposure of *E. fetida* to five dose rates ranging from 0.2 to 8.5 mGy/h for 16 weeks (accumulated doses 0.5–22 Gy, respectively) had no significant effect on reproductive capacity (number of cocoons and viable offspring). However, the production of cocoons and offspring was low, even in the background controls, and there was a high variability between the replicates at every exposure group. For other invertebrates, there are two relevant chronic studies with marine polychaete worms. Lifetime chronic exposure of adult *Neanthes arenaceodentata* (females are terminal spawners) caused a significant reduction in the number of larvae that hatched at dose rates as low as 0.19 mGy/h (cumulative lifetime dose at spawning, 0.55 Gy) (27). For another marine worm, *Ophryotroca diadema* (hermaphrodites, not terminal spawners), a multigenerational experiment was performed in which worms were continuously exposed to  $\gamma$  radiation (1.7–13.7 mGy/h), with reproduction being recorded in generations 1, 2, 3 and 7 (28). In the first generation, only the highest dose rate (cumulative dose 9.5 Gy) resulted in decreased production of larvae, while in the second generation a decrease was also observed at 3.2 mGy/h (cumulative dose 2.1 Gy). However, the latter dose rate had no significant effect in the third or seventh generation. For the last generation there was a tendency toward recovery at 7 mGy/h (at 13.7 mGy/h worms were followed for only two generations), and it was suggested that this was due to a selection of a radioresistant population. The lowest dose rate inducing a significant reduction in larvae is comparable with the lowest dose rate (4.2 mGy/h) associated with effects in our study.

In addition to the reproductive capacity, the growth and sexual maturation rate of the offspring are very important for the population dynamics. We found that the growth of the  $F_1$  juveniles was not inhibited by the radiation exposure, and there was no influence on the sexual maturation rate (maximum accumulated doses 24 Gy). This was also reported for the two marine invertebrates (polychaeta) at similar dose rates of chronic exposure (27, 28). In an acute exposure experiment, Suzuki and Egami (19) reported that growth and maturation of juvenile *E. fetida* (0–7 days old) were completely inhibited by 100 Gy, whereas 20 Gy had no apparent effect.

In our study and in those discussed above, dose rates are generally high compared to exposures expected in nature. At sites contaminated by regulated release of radionuclides, existing assessments indicate that in the UK the absorbed dose rates are generally no more than 0.1 mGy/h and always less than 1 mGy/h (29). However, there are situations after nuclear accidents in which dose rates in nature may be much higher. After the Chernobyl accident, the dose

absorbed by earthworms during the first 5 months after the accident was reported to be 86 Gy in the 3-km zone around the reactor (13). Reduced earthworm numbers were observed as well as changes in the composition of the population; overall, the data indicated reduced hatching and/or reduced juvenile survival. Two years later the earthworm population in the exposed zone had recovered. On the other hand, UNSCEAR has reported that even 30 years after the accident in Mayak, the total density and biomass of earth-living organisms were still depressed (3). Field studies from former Soviet Union sites have reported considerable damage in eggs and larva of invertebrates at 4 to 40 mGy/h (30), but effects have also been reported on earthworms at lower exposure levels, i.e., 100  $\mu$ Gy/h  $\alpha$ -particle radiation (3). Compared to experimental studies, conditions in nature are generally far more stressful, with variations in temperature, moisture content of the soil, food availability, and exposure to several contaminants at the same time. Organisms kept in a laboratory environment have been shown to be more resistant to radiation (31), and in a recent study, Daphnid neonates exposed to  $\alpha$ -particle radiation were shown to be less resistant to starvation compared to controls (32).

#### *Sensitive End Points and Possible Mechanisms*

In our experiment, the most sensitive end point was the number of hatchlings produced per adult worm, reflecting a reduced hatchability and reduced number of hatchlings emerging from each hatched cocoon. This could in principle be caused by a reduction in fertilization success, dominant lethal mutations in male or female germ cells killing the embryo, or radiation damage inflicted during embryogenesis. Since the hatchability of  $F_0$  cocoons at the two highest dose rates changed during the exposure periods, it is unlikely that the effect could be attributed to direct damage to the embryo. Further support for this interpretation comes from a pilot experiment in which cocoons from unirradiated parents were exposed for 21 days. Dose rates of 40 mGy/h had no effect on hatchability or number of hatchlings emerging from each cocoon (data not shown). This is also in accordance with results from *N. arenaceodentata* showing no effects on survival of larvae after irradiation at 17 mGy/h (cumulative dose 4.9 Gy) during embryogenesis (27).

A more likely mechanism for the reduction in hatchability is that damage is induced directly in male germ cells (i.e. spermatogenic cells) or that there is accumulation of damage in the testes and seminal vesicles. This could result in infertile sperm or a reduced or total lack of sperm production. Such effects were observed in the exposure experiments by Suzuki and Egami (19, 20). In *E. fetida* adults given one acute dose of 20 Gy, the maximum reduction in hatchability (50%) was observed in cocoons produced 20 days after the irradiation; at this time there were also few spermatozoa. The data indicated that cells in early sper-

matogenesis (i.e. spermatogonia) are the most radiosensitive, whereas the testicular stem cells (protogonia) are resistant. This is in agreement with what is generally found for other species, e.g. mice (33, 34). Further support for the male reproductive system being an important target for radiation is our observation that the seminal vesicles and testes in  $F_0$  adults were clearly atrophied after 13 weeks of exposure at 43 mGy/h (accumulated dose 85 Gy), as was also observed in adult *E. fetida* given a single acute dose of 80 Gy (20).

Chronic irradiation could affect the testes and spermatogenesis by different mechanisms than acute irradiation. After a single acute dose, the spermatogonia (i.e. the most radiosensitive stage) would receive the whole dose, while at any time during chronic irradiation the differentiating spermatogonia will receive only a small fraction of the total accumulated dose. Since spermatogenesis lasts for approximately 30 days (20), the first 1–4 weeks of the reproduction study would comprise cocoons containing spermatozoa developed from unirradiated spermatogonia or those irradiated for a shorter time. At 43 mGy/h, this would explain why hatchability was reduced at 1–4 weeks and dropped to zero in the next periods (Fig. 4b). At 11 mGy/h, a large reduction in hatchability was observed only in the last 9–13-week period, with only a minor reduction in the 5–8-week period, even though the spermatozoa in cocoons at these stages were derived from spermatogonia receiving the same accumulated dose. The fact that these effects appeared so late suggests that other interactions are likely to be involved, in addition to direct effects on spermatogonia such as dominant lethal mutations. An additional mechanism that could apply to both chronic and acute radiation exposure may be that radiation affects spermatogenesis indirectly by accumulation of damage in other target cells that are important for the maintenance of this process. Highly radiation-sensitive mechanisms of this kind have recently been reported for rats and involve interference with androgen regulation in Sertoli cells (35). *E. fetida* has no Sertoli cell-like structures (B. G. M. Jamieson, personal communication), but a cerebral hormone has been shown to stimulate the production of testicular androgen, which maintains spermatogenesis (36). Removal of peri-esophageal ganglia causes changes in the cell population in the seminal vesicles (37) similar to those caused by radiation (20).

To our knowledge, nothing is known about the temporal development of oogenesis in *E. fetida* and the radiosensitivity of cells in the different stages. Since earthworms are hermaphrodites, such aspects are more difficult to study experimentally and were not given special attention in this study. It is likely however, that damage in female germ cells also contributed to the observed radiation-induced effects on reproduction.

#### *Time for Effects to Appear and Critical Load*

It is notable that the effects on hatchability from irradiation of  $F_0$  adults at the two highest dose rates appeared to

be related to a critical total accumulated dose of approximately 20 Gy (Table 2), and the effects were similar to those reported for acute irradiation [2.5 Gy/min, total dose 20 Gy (19, 20)]. Harrison and Anderson (27) also reported that chronic and acute irradiation (total dose 0.5 Gy) were equally efficient in reducing reproduction in *N. arenaceo-dentata*. It is generally accepted for somatic cells that protraction of a given total exposure generally reduces the extent of injury, which is traditionally explained as being related to processes such as DNA repair, the presence of protective substances, etc. However, the repair capability would be expected to vary with tissue, cell type and turnover.

#### *Differences between F<sub>0</sub> and F<sub>1</sub>: Possible Acclimatization or Adaptation?*

Similar to F<sub>0</sub>, F<sub>1</sub> showed a significant reduction in hatchability (Fig. 9b), number of hatchlings per hatched cocoon, and the total number of hatchlings per adult (Fig. 5) at 11 mGy/h. However, there were also a number of differences between the two generations. In contrast to what was observed for F<sub>0</sub>, in F<sub>1</sub>, the hatchability increased with exposure time and was higher than that seen in the 9–13-week period in F<sub>0</sub> (Figs. 4b and 9b). This could indicate acclimatization (physiological adjustments during the exposure history of individuals) or an adaptation (inheritable traits acquired during the exposure history of the population) in the F<sub>1</sub> generation. The total accumulated dose in F<sub>1</sub> at the start of reproduction (week 11) is comparable to the accumulated dose in F<sub>0</sub> at the end of week 13 (Tables 2 and 3), and it increased with time to a total dose of twice that received by F<sub>0</sub>. Thus the apparent critical dose seen in F<sub>0</sub> does not seem to apply to F<sub>1</sub>. The experimental protocol did not allow us to see whether there was a time-dependent trend in hatchlings per hatched cocoon and the total hatchlings per adult during the various periods. From Fig. 5 it is evident that the accumulated number of hatchlings per adult at 11 mGy/h is relatively more reduced compared to controls in F<sub>1</sub> than in F<sub>0</sub>, indicating that in F<sub>0</sub> the effects on hatchability took several weeks to appear (Fig. 4b). Finally, at 4 mGy/h, hatchlings were (borderline) significantly reduced in F<sub>0</sub> but not in F<sub>1</sub>.

Any comparison of results between the two generations should be made with care. There was an elevated temperature during the first two periods of the F<sub>1</sub> reproduction study. This was a likely cause of the increased cocoon production rates in all groups (Fig. 9a) compared to F<sub>0</sub> (Fig. 4a). The temperature optimum for *E. fetida* is close to 25°C, and there is a linear increase in the cocoon production rate in the temperature range 10 to 25°C (38). However, the cocoon incubation period, hatchability and the number of hatchlings produced per cocoon are reported to be negatively correlated with temperature (38, 39). In our experiment, we did not observe such a trend since the average number of hatchlings per hatched cocoon was higher in F<sub>1</sub>

controls and in all exposed groups compared to F<sub>0</sub> except at 11 mGy/h, where it was unchanged. It is difficult to see how the temperature changes alone could explain the observed differences between generations.

Interpretation of the difference between the two generations is further complicated by the variable exposure regimen. F<sub>1</sub> worms were exposed at all stages, i.e., as germ cells including critical stages of the spermatogenesis, during the embryonic development in the cocoons, during the growth and sexual maturation of the juveniles, and as adults. There are indications from the Chernobyl area that plants have acquired a higher resistance to mutagens (40); however, such apparent radiation-induced adaptation is somewhat controversial, and the phenomenon is complicated and difficult to study experimentally.

#### *Relevance of E. fetida in Ecotoxicological Risk Assessment*

In this study we used *E. fetida*, which is generally considered as the earthworm species of choice in risk assessment because it combines sensitivity, economic importance, and ecological relevance. However, the characteristics that make it a convenient species for use in toxicity testing (i.e. very prolific, high reproductive output, short life cycle) might also lead to a reduced susceptibility of the population to stresses (41) such as ionizing radiation. Earthworm species with a longer generation time may accumulate higher doses before they reach sexual maturity; furthermore, in species with low cocoon production rates, reduced hatchability may have more serious consequences for the population as a whole. It has been recommended that, ideally, a representative of each of the three ecological types of earthworms should be included in routine toxicity testing (9).

#### *Conclusions*

The present study shows clear evidence of effects on reproduction in *E. fetida* at dose rates that may occur in nature from anthropogenic releases. The variability in the response seen over time and generation presents a complex picture and underlines the need for extended exposure periods, over more than one generation, when radiation effects on population dynamics are to be analyzed.

The experiments described in the present paper are very time consuming (in total 18,000 cocoons and 55,000 hatchlings were examined), and their sensitivity may be limited since the number of adult individuals must be kept relatively low and only a few generations can be followed. In addition to the data reported here, samples were also taken from adult F<sub>0</sub> and F<sub>1</sub> worms for the estimation of DNA damage in spermatogenic and somatic cells (results will be published elsewhere). Molecular or cellular end points are needed to study underlying mechanisms and to aid in the extrapolation both between species and exposure conditions and in the elucidation of phenomena such as adaptation. In theory, such methods could provide convenient, quick and

inexpensive indicators of both exposure and effect (42). However, in radioecology, the ability of molecular biomarkers to accurately predict effects at the population level is still questionable and has not been validated.

### ACKNOWLEDGMENTS

The authors thank Dr. Ole Christian Lind for valuable help with designing the experimental setup at the  $^{60}\text{Co}$  source and Guro Holte for assistance in counting cocoons and hatchlings. This work was supported financially by the ERICA project [Contract Number: FI6R-CT-2004-508847, co-funded by the European Commission under the Euratom Research and Training Programme on Nuclear Energy within the Sixth Framework Programme (2002–2006)] and the Norwegian Radiation Protection Authority.

Received: March 2, 2007; accepted: July 16, 2007

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