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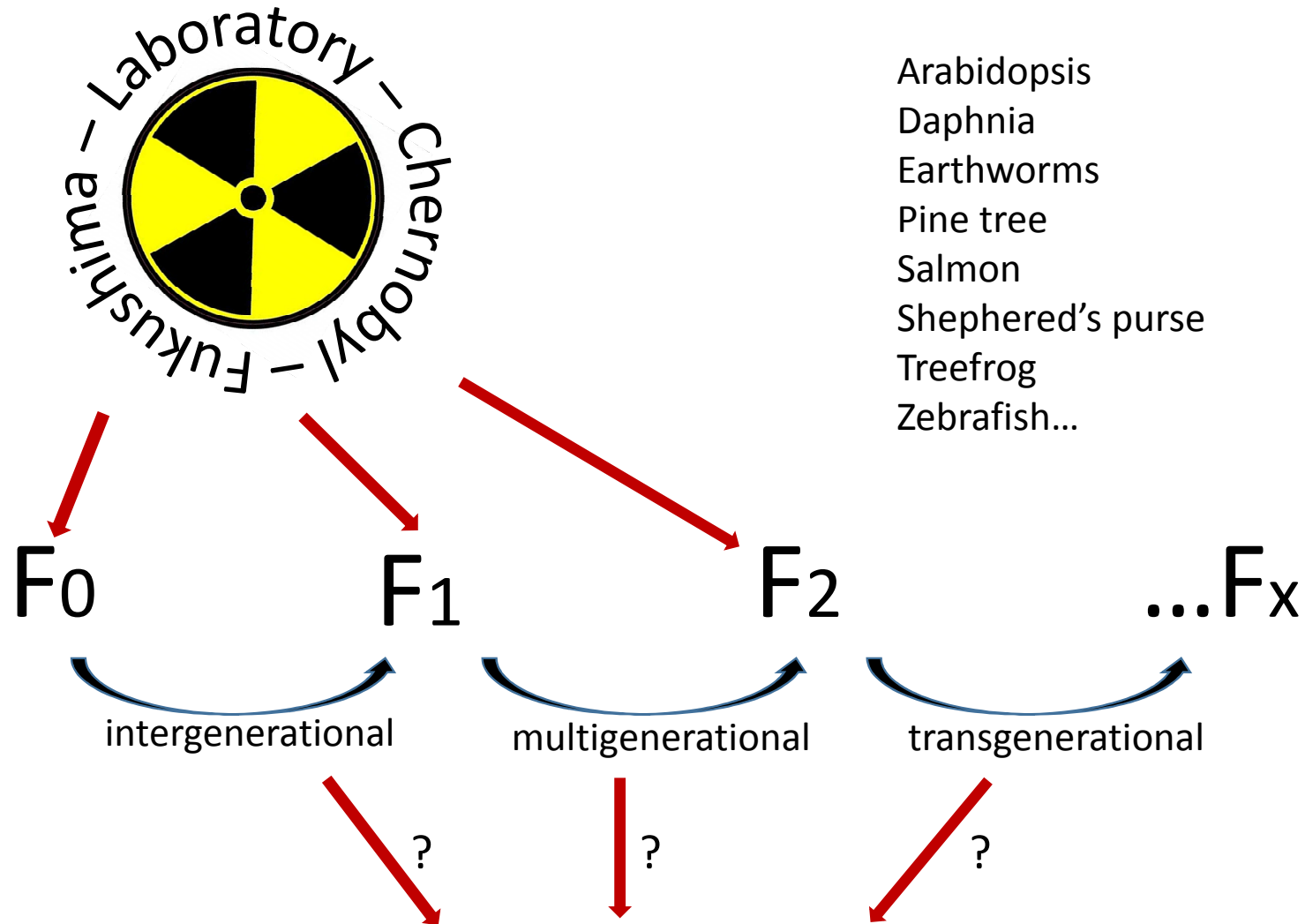
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Δ Epigenetic marks:

DNA methylation, histone modifications, miRNA expression

1 **Current evidence for a role of epigenetic mechanisms in response to ionizing radiation in**
2 **an ecotoxicological context**

3

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29 exposure; multi-transgenerational; Wildlife; Chernobyl; Chernobyl; Fukushima; Nuclear

30 accidents

31 **Abstract**

32 The issue of potential long-term or hereditary effects for both humans and wildlife exposed
33 to low doses (or dose rates) of ionising radiation is a major concern. Chronic exposure to
34 ionising radiation, defined as an exposure over a large fraction of the organism's lifespan or
35 even over several generations, can possibly have consequences in the progeny. Recent work
36 has begun to show that epigenetics plays an important role in adaptation of organisms
37 challenged to environmental stimulæ. Changes to so-called epigenetic marks such as
38 histone modifications, DNA methylation and non-coding RNAs result in altered
39 transcriptomes and proteomes, without directly changing the DNA sequence. Moreover,
40 some of these environmentally-induced epigenetic changes tend to persist over generations,
41 and thus, epigenetic modifications are regarded as the conduits for environmental influence
42 on the genome.

43 Here, we review the current knowledge of possible involvement of epigenetics in the
44 cascade of responses resulting from environmental exposure to ionising radiation. In
45 addition, from a comparison of lab and field obtained data, we investigate evidence on
46 radiation-induced changes in the epigenome and in particular the total or locus specific
47 levels of DNA methylation. The challenges for future research and possible use of changes as
48 an early warning (biomarker) of radiosensitivity and individual exposure is discussed. Such a
49 biomarker could be used to detect and better understand the mechanisms of toxic action
50 and inter/intra-species susceptibility to radiation within an environmental risk assessment
51 and management context.

52 **Capsule:**

53 Review: possible changes in epigenetic marks in wildlife exposed to ionising radiation
54 suggests DNA methylation changes as a key to transfer the response from one generation to
55 the next.

56 **1 Introduction**

57 Activities like ore mining and milling, nuclear accidents and production and testing of nuclear
58 weapons have resulted in enhanced concentrations of radionuclide pollutants in the
59 environment. This can lead to long-term or chronic exposures of organisms defined as an
60 exposure over a considerable fraction of the lifespan of the organism (IAEA 1992). The issue
61 of biological effects induced by chronic sub-lethal doses of ionising radiation along with the
62 question on the potential hereditary effects for both humans and wildlife is a topic of
63 considerable debate and concern. This has been reinforced after the Chernobyl and
64 Fukushima accidents, especially with respect to the quantification (and reduction if possible)
65 of the magnitude of risk to ecosystems when exposed chronically for multiple generations.
66 This concerns both short-term and chronic exposure over several generations and heritable
67 effects on unexposed progeny. To improve the scientific basis for risk assessment for both
68 human and environment in chronic exposure scenarios as observed e.g. in Chernobyl and
69 Fukushima exclusion zones (CEZ and FEZ), an enhanced understanding of the mechanisms
70 that underpin these responses is needed. This will lead to a better understanding of the
71 complex interplay between exposure, organism physiology and phenotypic response over
72 extended timescales (e.g., Marczylo et al. 2016). Comprehensive reviews of the observed
73 phenotypic effects observed in wildlife in CEZ and FEZ have been published e.g. by Hinton et
74 al. (2007), Geras'kin et al. (2008), Lourenco et al. (2016) Steinhauser et al. (2014), Strand et
75 al. (2014), Batlle (2016) and Beresford et al. (2016). The amounts of radionuclides released
76 into the environment after the Chernobyl accident (5300 PBq, excluding noble gases) were

77 about tenfold of those of the accident in Japan (520 PBq) (Steinhauser et al. 2014). Despite
78 this difference both exclusion zones have common features such as (i) for both areas the
79 exposure can be divided in 3 time-periods depending on the exposure rates as described in
80 paragraph 6, (ii) the degree to which spatial and temporal heterogeneity is present in the
81 distribution of the radionuclides (including the presence of hot particles); (iii) the presence
82 of other additional pollutants (e.g. from historical land use); (iv) the challenge of finding
83 comparable control conditions and (v) the difficulty to estimate the exact exposure dose
84 rates. Additionally and of importance for interpreting observations made in these
85 contaminated regions, both exclusion zones have undergone changes induced by the
86 removal of human presence and occupancy leading to specific ecological changes that are
87 hard to distinguish from the possible radiological impact (Beresford and Copplestone 2011).
88 The unique nature of these study areas means that the interpretation of field data from
89 these sites needs careful contextual consideration and have led to contrasting and
90 sometimes conflicting reports on effects observed in the CEZ and FEZ (Beresford and
91 Copplestone 2011; Garnier-Laplace et al. 2013).

92 Long-term exposures to environmental stressors have been linked to lasting responses in
93 organisms within, but also over multiple exposed generations (Mirbahai and Chipman 2014;
94 Schultz et al. 2016; Jimenez-Chillaron et al. 2015; Marczylo et al. 2016; Hanson and Skinner
95 2016). Yet, the outcome of a long term-exposure to pollutants is not always predictable. For
96 example, chronic exposure to pollutants or adverse conditions has been shown to lead to
97 changed phenotypes (Singer et al. 2016; Gonzalez et al. 2016; Potters et al. 2007) resulting in
98 adaptation within a population (Costa et al. 2012; Coors et al. 2009; Bible and Sanford 2016).

99 In contrast, there is also evidence suggesting that long term exposures to environmental
100 stressors can lead to an increased population sensitivity (Parisot et al. 2015) that may result

101 in population declines (Vasseur and Cossu-Leguille 2006). This makes predicting the long-
102 term and/or transgenerational consequences of exposure to a stressor a particular challenge
103 for estimating risks to populations (Groh et al. 2015).

104 Selection has been recognised as a major mechanism through which adverse environmental
105 conditions can impact the phenotypes of successive generations. Selection of alleles
106 associated with tolerance can lead to changes in the phenotypic characteristics within a
107 population and, hence, is known to be a key driver of changes in population level sensitivity
108 to pollutant effects (Van Straalen and Roelofs 2007). Detailed studies of populations
109 inhabiting polluted sites have identified numerous cases of modified phenotypes and also of
110 specific genetic selection at loci that lead to biochemical changes that underpin adaptation.

111 Examples cover exposure to radionuclides, trace metals and persistent organic pollutants
112 and taxa such as cladocerans (Hochmuth et al. 2015; Jansen et al. 2015), collembola (Costa
113 et al. 2012; Nota et al. 2013), chironomids (Groenendijk et al. 1999; Loayza-Muro et al.
114 2014), terrestrial and freshwater annelids (Kille et al. 2013; Langdon et al. 2003; Levinton et
115 al. 2003), fish (Wirgin et al. 2011; Shaw et al. 2014; Reid et al. 2016; Theodorakis and Shugart
116 1997), plants, birds (Ellegren et al. 1997) and small mammals (Theodorakis et al. 2001).

117 Although selection for enhanced tolerance is a commonly observed phenomenon, some
118 data have shown that rapid adaptation towards heavy-metals or radionuclides in organisms
119 cannot be explained only by increased mutation rates, but could also be due to non-genetic
120 changes in the activity of functional genes and these might be heritable over generations
121 (Geras'kin et al. 2013; Kovalchuk et al. 2003; Mirbahai and Chipman 2014; Kille et al. 2013;
122 Wang et al. 2017). This has revealed further levels of complexity probably provided by
123 relevant epigenetic mechanisms relating to structure and regulation of gene expression and
124 splicing that have the potential to transfer information over generations.

125 In this paper an overview is given of epigenetic changes induced after long-term (within and
126 over generations) exposure to ionising radiation. Although different epigenetic mechanisms
127 will be discussed the main focus of the current review will be on comparing the evidence
128 from both lab and field studies on changes in DNA methylation.

129

130 **2. Overview of epigenetic mechanisms**

131 The first definition of epigenetics, as ‘the causal interactions between genes and their
132 products, which brings the phenotype into being’, was provided by Waddington (1939) long
133 before any mechanistic understanding of the relevant processes had developed. This
134 definition has since been refined. For example, Wu and Morris (2001) defined epigenetics as
135 ‘Nuclear inheritance which is not based on changes in DNA sequence’ or Bird (2007) as ‘the
136 structural adaptation of chromosomal regions so as to register, signal or perpetuate altered
137 activity states’. This reflects that epigenetics is now widely seen as ‘the study of the
138 landscape of mitotically and/or meiotically heritable changes in gene activity and transcript
139 architecture, including splicing variation, that cannot be explained solely by changes in DNA
140 sequence (Vandegheuchte and Janssen 2011; Allis et al. 2007; Berger et al. 2009).

141 The epigenetic landscape is shaped by three epigenetic marks; DNA methylation, histones
142 and its post translation modifications and small RNA interactions. Together they shape the
143 structure of the DNA called chromatin (Allis and Jenuwein 2016). These major epigenetic
144 players are engaged in a network of interconnected ‘cross-talk’ (Irato et al. 2003; Iorio et al.
145 2010) and orchestrate gene expression that “...underpins the differences between species,
146 ecotypes and individuals” (Mattick et al. 2009; Brautigam et al. 2013). Well established as a
147 key mechanism involved in the aetiology of human disease (Huang et al. 2003), it is only
148 relatively recently that the significance of epigenetic mechanisms in toxicology (Szyf 2007),

149 ecology (Bossdorf et al. 2008) and evolutionary biology (Rapp and Wendel 2005), has begun
150 to emerge. Within ecology, it has been suggested that epigenetics could define "... where the
151 environment interfaces with genomics ... (and could provide a) rapid mechanism by which an
152 organism can respond to its environment without having to change its hardware" (Pray
153 2004). Studies on plants have indicated that epigenetic systems provide functional links
154 between the detection of environmental change and regulation of gene expression
155 (Bossdorf et al. 2008; Grativol et al. 2012; Whittle et al. 2009; Rasmann et al. 2012;
156 Verhoeven et al. 2016; Sahu et al. 2013; He and Li 2018). Similarly in animals, the role of
157 specific components or changes of the epigenome in species responses to environmental
158 stress has been demonstrated (Vandegheuchte and Janssen 2014; Schott et al. 2014; Marsh
159 and Pasqualone 2014; Mirbahai and Chipman 2014; Wang et al. 2017; Marczylo et al. 2016).
160 Thus epigenetic mechanisms appear to play an important role in determining the
161 physiological responses of species to long-term multigenerational exposure, including to
162 persistent stressors such as radionuclides.

163 To integrate emerging understanding of epigenetic mechanisms with existing mechanistic
164 knowledge in radioecology, a clear understanding of long-term effects induced by ionizing
165 radiation exposure of non-human species and their potential (epigenetic) mechanistic basis
166 is needed. To provide this, we here give a brief overview of the evidence of trans- and
167 multigenerational effects in organisms exposed to ionising radiation. The potential role and
168 value of epigenetic analyses in site-specific studies in radioecology will be discussed,
169 including their relevance for future radiological risk assessment. As the most widely studied
170 mechanism and its potential to be transferred to the next generation, special attention will
171 be given to changes in DNA methylation (locus-specific or total) as a possible marker for
172 exposure to ionising radiation, including under field conditions.

173

174 **3. The biology of epigenetic mechanisms**

175 DNA methylation, histone modifications, and small non-protein coding RNA molecules are
176 the major known epigenetic mechanisms. DNA methylation is the addition of a methyl group
177 to the one of the DNA bases (cytosine or adenine). Most prevalent DNA methylation is on
178 the fifth position of the cytosine ring (5-methyldeoxycytidine, mC). In vertebrates this usually
179 but not exclusively located at in CpG sites. For example, in *Drosophila* methylation is mostly
180 found in the context of CpT dinucleotides (Feil and Fraga 2012), in honey bees there appears
181 to be a clear distinction of CpG sites in exons and non-CpG sites in introns (Cingolani et al.
182 2013) and in plants and embryonic stem cells also at CHG and CHH sites (H=A,T or C) in
183 addition to CpG (Feil and Fraga 2012; Cingolani et al. 2013).

184 In vertebrates, around 60% of genes are associated with CpG islands that occur at or near the
185 transcription start site of, particularly, housekeeping genes (Gardiner-Garden and Frommer
186 1987). The hypermethylation in CpG rich promoters can be associated with the repression of
187 gene expression (Bock 2012). In invertebrates, methylation is targeted more towards gene
188 body, potentially playing a role in alternative splicing and gene function diversification (Flores
189 et al. 2012; Asselman et al. 2016). Cytosines can be methylated via maintenance and *de novo*
190 methyltransferase enzymes (Law and Jacobsen 2010). In vertebrates, maintenance
191 methylation by DNMT1 occurs during the S-phase of mitosis, where the newly synthesized
192 DNA strand is methylated using the original strand as template. De novo DNA methylation is
193 undertaken DNMT3 family members, although recent insights have shown redundancy
194 between to two DNMT family members (Lyko 2018). *De novo* DNA methylation is
195 undertaken DNMT3 family members. In plants the homologues of DNMT3, DOMAINS
196 REARRANGED METHYLTRANSFERASE 1/2 (DRM1/DRM2) are responsible for the de novo

197 methylation whereas maintenance of CG methylation is conducted by DNA
198 METHYLTRANSFERASE 1 (MET1) which is a homolog for DNMT1 (Law and Jacobsen 2010;
199 Chan et al. 2005). In addition the plant specific CHROMOMETHYLASE 3 (CMT3) is responsible
200 for maintaining methylation in a context of CHG and together with DRM1/DRM2 for
201 methylation in a CHH context (Chan et al. 2005). Although the methyltransferase enzymes
202 are the core proteins involved in methylation, they are recruited and guided to their specific
203 interaction targets by proteins, such as UBIQUITIN-LIKE, CONTAINING PHD AND RING FINGER
204 DOMAINS 1 (URHF1) and PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) (Baubec et al.
205 2015). A further insight that has recently emerged is that DNA methylation represents only
206 one part of the DNA methylation cycle. Recently, Tet methylcytosine dioxygenases
207 (previously named ten-eleven translocation (TET) proteins) have been identified as crucial
208 proteins in putative demethylation pathways (Coulter et al. 2013; Scourzic et al. 2015).
209 Indeed, the dynamics between methylation and hydroxymethylation exemplifies the balance
210 of DNA methylation at specific regions as well as globally during early developmental
211 reprogramming (Wu and Zhang 2014).

212 Histone modifications occur as post-translational modifications predominantly to the N and
213 C terminal tails of histone proteins. Histone proteins are organised in octamer structures
214 forming nucleosomes as the fundamental units of chromatin (Berr et al. 2011). Initially
215 histones were thought of as primarily structural proteins. However, it is now recognised that
216 they play a pivotal role in regulating gene expression via structural changes of chromatin
217 (Jung and Kim 2012; Margueron et al. 2005). Major histone modifications include
218 acetylation, methylation, phosphorylation and ubiquitination (Bannister and Kouzarides
219 2011). A key role played by histone isoforms and post-translational modifications that is
220 highly relevant to ionising radiation exposure, is their involvement in DNA damage repair

221 (Hunt et al. 2013; Mondal et al. 2016). DNA repair requires multiple steps, including the
222 initial signalling of the break, the opening of the compact chromatin to facilitate access for
223 repair factors, and afterwards the restoration of the chromatin state (Hunt et al. 2013; for
224 details see Huertas et al. 2009). An authoritative overview of the post-translational
225 modifications in histones triggered in response to DNA damage is given by Méndez-Acuña et
226 al. (2010). Changes of histone modifications have also been linked to exposure to different
227 pollutants in both mammalian and non-mammalian species (Kim et al. 2012b; Mendez-
228 Acuna et al. 2010; Santos et al. 2011; Wang et al. 2017). Observations of heterochromatin
229 state maintenance over multiple successive generations following exposure to heat or
230 osmotic stress in *D. melanogaster* suggests a mechanism by which the effects of stress are
231 inherited epigenetically via the regulation of chromatin structure (Seong et al. 2011).

232 Short interfering RNAs and microRNAs are functional non-coding RNA molecules. They are
233 not translated into proteins and are involved in gene repression via RNA deactivation and
234 degradation (Castel and Martienssen 2013). Single microRNAs may on average interact with
235 ~400 different protein coding genes. Hence, changes in microRNA expression are proposed
236 to be a key component of organism response to stressor exposure (see e.g. for plant
237 responses Huang et al. 2016). Reduced expression of microRNA has been found in response
238 to insecticide and fungicide exposure (Qi et al. 2014; An et al. 2013). MicroRNAs have been
239 shown to be intimately involved in cellular response to metals such as cadmium and arsenic
240 (Liu et al. 2016; Meng et al. 2011; Gielen et al. 2012). Important roles of non-coding RNAs in
241 the epigenetic inheritance of DNA methylation through cell division and guiding de novo
242 methylation after meiosis indicate key interactions between epigenetic pathways (Calarco et
243 al. 2012; Larriba and del Mazo 2016). In plants e.g. DNA and histone methylation by DRM2
244 activity and subsequent gene silencing can also be mediated by siRNAs ARGONAUTE (AGO4)

245 and polymerase V (POLV) (Holoch and Moazed 2015; Neeb and Nowacki 2018). Hence
246 dynamic interactions of different epigenetic mechanisms would be expected in response to
247 environmental challenge.

248 The relative role of the different epigenetic mechanisms can vary between species. The
249 majority of eukaryotic phyla possess cytosine methylation ranging from <<1% in some taxa
250 (e.g. many arthropods) to >10% for annelids, molluscs and vertebrates, with species such as
251 *C. elegans* even proposed to lack cytosine methylation completely (Regev et al. 1998) or to
252 be very low (~0.0033%) (Hu et al. 2015). Because of those variations in DNA methylation
253 levels, it was initially uncertain how important cytosine methylation may be among those
254 phyla. However, evidence of the importance of DNA methylation in heritable responses in
255 invertebrates following stressor exposure has begun to emerge, as well as for other
256 epigenetic mechanisms (Seong et al. 2011; Schultz et al. 2016; Stern et al. 2014; Klosin et al.
257 2017). For some species, and particularly in *C. elegans*, a second DNA modification based on
258 methylation of the N-6 position on adenine may also act as an alternative form of DNA
259 methylation (Greer et al. 2015). In addition, the balance between DNA methylation, post-
260 translational modifications and types of microRNA molecules (both of which are species
261 specific and highly dynamic), presents a challenge to tease apart the roles that different
262 epigenetic mechanism play in gene expression dynamics and ultimately phenotypic
263 responses to stress including those in species exposed to radionuclides and other pollutants
264 over extended timescales (Lim and Brunet 2013).

265

266 **4. Main methods used to detect DNA methylation changes**

267 This review will mainly focus on the evidence for DNA methylation changes induced by
268 radiation in different animals and plants and this in both lab and field conditions. The

269 measurement of total DNA methylation levels is now routine using molecular genetic and
270 biochemical protocols. These analyses provide a useful picture of overall methylation states.
271 The methods have the advantages of reasonable cost per sample, established protocols,
272 sensitivity to overall methylation pattern change and rapid sample processing (Table 1). Two
273 global methylation methods that are commonly used are methylation sensitive amplified
274 fragment length polymorphisms (meAFLP) and measuring the % of methylated cytosine by
275 HPLC-MS/MS. The meAFLP technique is based on the use of two restriction enzymes, HpaII
276 and MspI. Both HpaII and MspI recognize a CCGG sequence. MspI is able to cut both
277 methylated recognition sites as well as unmethylated ones. In contrast, HpaII is unable to cut
278 at such locations when methylated (i.e. only unmethylated recognition sites are cut).
279 Methylation of these restriction sites can be assessed by electrophoretic recording bands cut
280 by MspI but not HpaII on a fragment analyser (e.g. capillary sequencer). The method has
281 been shown to demonstrate limited variability and has the benefit of an internal control
282 (EcoRI) to account for variability in the amount of DNA input. The detection of methyl groups
283 by HPLC-MS/MS allows highly sensitive quantification of methylated and hydroxymethyl
284 cytosines (5 mC and 5-hmC) present in a hydrolysed DNA sample. The specific ability to
285 detect and measure 5hmC is a specific advantage of this technique, given its recently
286 demonstrated roles in development (Pastor et al. 2011; Song et al. 2011; Xu et al. 2011).
287 Although useful, application of global methylation analysis methods do not allow analysis of
288 the specific methylation states needed to assess functional links between changes in site
289 specific methylation, gene expression changes and phenotypic changes to be made. The use
290 of methylation mapping techniques can provide improved resolution to identify and assess
291 specific genes/regulatory regions of interest that are differentially methylated under specific
292 treatment or exposure conditions. The number of options to study DNA methylation have

293 become more diverse and methods such as reduced representation or whole genome
294 bisulfite sequencing, are now considered close to routine. The value of these genome wide
295 methylation mapping techniques is that they go beyond the level of an overall change to
296 identify the gene associated sites of differential methylation. These methods are of course
297 limited when an organism reference genome is either not available or is poorly assembled or
298 annotated. Hence, significant effort needs to be given to genome resource development
299 before these methods can be used to study autochthonous species.

300

301 ***5. Laboratory evidence for multigenerational and transgenerational effects including those***
302 ***induced by ionising radiation***

303 The interest in understanding the effects of persistent pollutants, including radionuclides, on
304 population exposed for more than a single generation is ongoing. Therefore studies of
305 multigenerational and transgenerational stressor effects on apical phenotypes have become
306 more common. For multigenerational studies, exposure to the stressor in question is
307 maintained in a continuously cultured and exposed population for successive generations
308 (e.g., continuously exposed F0, F1, F2 etc.) to allow the consequences of multigenerational
309 exposure to be assessed. Phenotypes are observed in those generations directly exposed.
310 For these multigenerational cases, the simplest expectation is that the observed toxicity in
311 the offspring is not greater than that in parents exposed over their full life-span (i.e. embryo
312 until death), at least over initial generations, with possible development of tolerance over
313 longer time-scales. Transgenerational experiments, on the other hand, consider not just
314 effects on the exposed generation, but also effects on subsequent unexposed generation(s)
315 reared after hatching in stressor free conditions (Skinner and Guerrero-Bosagna 2009;
316 Skinner 2016; Groot et al. 2016). In such studies, stressor effects may be expected as a result

317 of exposure of the F0 mothers in F1 embryo and F2 germline, but not in later offspring. The
318 simplest expectation from transgenerational experiments is thus of physiological effects no
319 greater than those observed in F0s, only in F1s (and possibly F2s), with no further such
320 effects on the later (F3 etc.) generations.

321 There are cases where the simplest expectations of multigenerational and transgenerational
322 exposure are met, including examples for plants (Iglesias and Cerdan 2016; Groot et al. 2016;
323 Molinier et al. 2006), earthworms (Hertel-Aas et al. 2011), zebrafish (Baker et al. (2014)
324 (Schwindt et al. (2014) and mice (Ziv-Gal et al. (2015). However, critical analysis of reported
325 multigenerational exposures covering a range of stressor types including radionuclides,
326 metals, nanomaterials, organic chemical and antibiotics, suggests that, at least over the
327 durations used in the laboratory (usually < 10 generations) the simplest expectation of
328 similar sensitivity to F0 in later generations are not always be met. In a number of published
329 cases, an increasing sensitivity in later generations has been observed (see Table 2 and
330 examples below). While this prevalence may partly result from publication bias and from the
331 clonal organisms used, the high frequency of such responses does suggest that increased
332 sensitivity, at least over the initial generations of a multigenerational exposure, may be a
333 common phenomenon (see Table 2).

334 For exposure to radiation and radionuclides there are a number of multigenerational lab-
335 studies that have reported patterns of increased generational sensitivity for continuously
336 exposed populations (see Table 2 for exposure details). For daphnids it has been reported
337 that the progeny of organisms continuously exposed to gamma radiation, Am⁴²¹ (and
338 depleted uranium) show higher sensitivity in the F1 and F2 generations than that for parents
339 depending on the endpoint measured (Pane et al. 2004; Biron et al. 2012; Alonzo et al.
340 2008b; Parisot et al. 2015). Similarly, Zaka et al. (2004) exposed 5-day old *Pisum sativum*

341 plants over three generations to different acute doses of gamma radiation. Results indicated
342 that doses apparently harmless for the parental plants adversely affected the F2 generation.
343 *Arabidopsis thaliana* plants exposed to different dose rates of gamma radiation during the
344 vegetative growth stage for one or two generations also showed greater response in the
345 later generation. In this case, increased responses of antioxidative enzyme activity were
346 measured in multigenerationally exposed plants (van de Walle et al. 2016). This response
347 was accompanied by phenotypic changes, such as accelerated flowering after
348 multigenerational exposure (Horemans et al., pers. comm).

349 Transgenerational studies with radionuclides or after radiation-exposure have shown
350 responses not just in continuously exposed generations, but also in later unexposed
351 generations. A study of reproductive effects of gamma radiation in the nematode *C. elegans*
352 exposed from F0 to F2, either continuously or only at F0 generation also found
353 transgenerational effects in F2 organisms greater than in the initially exposed nematodes
354 (Buisset-Goussen et al. 2014). Daughter cells of chronically gamma-radiation-exposed *Lemna*
355 *minor* plants died off notwithstanding only a limited growth reduction in the exposed
356 mother colonies (10-30%) indicating that the effects were, thus, greater in the recovering
357 non-exposed plants than in the exposed F0s (Van Hoeck et al. 2017). These examples of
358 transgenerational effects leading to increased sensitivity of progeny match similar results
359 found for other stressors, suggesting a possible common mechanism (Schultz et al. 2016;
360 Moon et al. 2017; Annacondia et al. 2018; Groot et al. 2016).

361 The current multigenerational and transgenerational toxicity literature is dominated by lab-
362 studies with relatively high exposure dose rates (7-420 mGy/h, see table 2) and for
363 ecotoxicological relevant species like *C. elegans*, *D. magna* and zebrafish (Table 2). For *C.*
364 *elegans* and *D. magna*, the experimental populations that have been used in most

365 laboratories, multigenerational and transgenerational exposure studies are clonal. Hence,
366 the potential for selection of alleles that may lead to evolution of tolerance in later
367 generations in a multigenerational exposure experiment is limited. This is true especially
368 because the majority of such experiments are conducted over only a relative limited number
369 of generations (<10 and usually ≤ 3). Indeed, when nematodes were continuously exposed
370 for 22 generations to U, adaptation was shown to occur (Dutilleul et al. 2014). Although
371 many studies have shown generationally increased sensitivity and its transfer, the clonal
372 nature of species may be accentuated, because the limited genetic variation of the inbred
373 strains. In the study of Dutilleul et al. (2014) for nematodes discussed above, the population
374 used that showed adaptation composed of wild isolates with increased genetic diversity
375 above the clonal *C. elegans* strains used for previous multigenerational studies. Hurem et al
376 (2018b) showed effects on the transcriptome in offspring from irradiated zebrafish that were
377 even accentuated in offspring produced from the same parents does, however, indicated the
378 potential to identify epigenetic responses in a genetically diverse population.

379 Multigenerational exposure experiments by their nature involve continuous incubation of
380 populations with a toxicant or stressor, with generational phenotyping to allow detection of
381 changes in sensitivity. In such studies, increased sensitivity in the progeny could theoretically
382 arise if any toxicant induces “damage” that can be transferred to subsequent exposed
383 generations. Indeed Parisot et al. (2015) highlighted a possible role of DNA damage in
384 multigenerational effects by finding a correlation between increased sensitivity and the
385 transmission of DNA damage in daphnids exposed to gamma radiation. This possible role of
386 DNA damage and genome instability in multigenerational and transgenerational effects may
387 lead to hypotheses about the type of stressors that may cause such phenomena.

388 The role of both paternal and maternal effects has received much research attention in
389 ecology and toxicology (Frost et al. 2010; Wigle et al. 2007). Within these studies there is
390 strong evidence that indicate how the direct exposure of the developing embryo and
391 germline can be adversely affected as a result of exposures to environmental pollutants.
392 However, in addition to these more direct effects, there is evidence of a potential role of the
393 epigenome in the transfer of aberrant phenotypes to F1 offspring and indeed to generations
394 beyond (Bowman and Choudhury 2016; Chen and Baram 2016; Wang et al. 2017). For
395 example, exposing *C. elegans* to nanoparticles resulted in aberrant phenotypes, that were
396 persistent in future unexposed populations for 3 or more generations (Greer et al. 2011;
397 Katz et al. 2009; Rechavi et al. 2014; Schultz et al. 2016). When transgenerational effects
398 occur over these generation scales, germline exposures alone cannot be solely responsible,
399 with the potential that epigenetic mechanisms may be intimately involved.

400

401 ***6. Evidence for long-term effects induced by radiation on the environment coming from***
402 ***field studies***

403 The nuclear accidents of Chernobyl and Fukushima have made it possible to investigate
404 possible effects of radiation on a whole range of organisms exposed to radionuclides under
405 field conditions over extended timescales. The temporal changes that occurred in radiation
406 exposure in the CEZ and the FEZ, have resulted in a specific time course of responses among
407 non-human biota in the regions (IAEA 2006; Beresford et al. 2016; Beresford and
408 Copplestone 2011; IAEA 2015). The most pronounced biological effects were seen in the first
409 and second phases after the accident. In these early stages, the high doses experienced
410 shortly after the accident by the forest located to the west of the Chernobyl reactor, later
411 designated as the Red-forest. In this Red-forest massive death of pine trees was observed,

412 while deciduous species survived despite an early loss of leaves and damage to woody
413 tissues (Arkhipov et al. 1994; Kryshev et al. 2005). Similar morphological differences such as
414 loss of apical dominance were recently also reported in Japanese red pine in the FEZ
415 (Yoschenko et al. 2016). In the first phase after the nuclear accidents, direct effects such as a
416 decrease in numbers of small mammals as well as reduced development or survival of
417 embryos was also seen (Geras'kin et al. 2008) and the loss of specific groups of soil biota
418 were also recorded in the most contaminated areas (Krivolutsky 1996; IAEA 2006). These
419 effect could also be linked to the high levels of initial exposure that were experienced
420 following both nuclear accidents. Initial dose rates in the most contaminated areas of CEZ
421 were as high as 5mGy/h (IAEA 2006)

422 The second phase characterised by a decrease in dose rates due to disappearance of short-
423 lived radioisotopes and wash-out and run-off (IAEA 2006). This phase started from two
424 months after the accidents, was associated with reductions (up to a factor of 30) in the
425 density of invertebrates living in the forest litter experiencing greatest contamination. These
426 decreases were linked to radionuclide exposure effects on reproduction and recruitment
427 (Krivolutsky and Pokarzhevskii, 1992; Krivolutsky et al., 1992).

428 In the third exposure phase resulting from the Chernobyl accident, most strongly affected
429 populations of species of pine trees and soil invertebrates were shown to slowly start to
430 recover (Arkhipov et al. 1994; Zelena et al. 2005). Recovery from the initial negative effects
431 was also found in birch pollen, embryonic cells of herbaceous plants like evening primrose
432 embryonic cells (Boubriak et al. 2008) and *Arabidopsis thaliana* (Kovalchuk et al. 2004) and in
433 exposed birds (Galvan et al. 2014). In this phase Cs-137 and Sr-90 are the main contributors
434 to the dose with some additional Am-241 and Pu-isotopes for CEZ and Cs-137/134 for FEZ
435 (Horemans et al. 2018; Saenen et al. 2017). Ambient dose rates now measured are

436 maximally 0.5 mGy/h and these can be found in the forest western from the nuclear power
437 plant designated as the Red Forest (Beresford, personal communication).

438 In addition to changes observed at individual or population levels, the radiological impacts
439 within both the CEZ and the FEZ, have also been reported at the sub-organismal level.
440 Aberrant cell frequencies were found in the root meristem of plant seedlings (Geras'kin et al.
441 2011). Increased mutation rate (Kuchma et al. 2011) and gene deregulation (Zelena et al.
442 2005), have been seen in pine trees. Increased mitochondrial DNA haplotype and nucleotide
443 diversity have been reported in bank voles (Matson et al. 2000; Baker et al. 2001),
444 chromosomal aberrations in mice (Kubota et al. 2015) and in soil invertebrates, increased
445 DNA damage in earthworms (Fujita et al. 2014). Most of these studies so far have, however,
446 failed to find a link between these observed sub-organismal effects and impacts at higher
447 level of biological complexity such as radiation-induced phenotypical changes and long-term
448 effects on population dynamics (Meeks et al. 2009; Meeks et al. 2007).

449 The adaptive responses that have been indicated during the extended third phase of
450 exposure following the two accidents at Chernobyl and Fukushima are at least in part due to
451 the reduction over time in dose rates and, hence, exposure. Although a memory-effect of
452 the early high exposures cannot be excluded, the decreased exposure in the third phase
453 might allow both increased *in-situ* recruitment and survival leading to positive population
454 growth, as well as the survival of inwardly migrating individuals (Jackson et al. 2004;
455 Boubriak et al. 2008; Boubriak et al. 2016). Additionally it is also possible that increased
456 tolerance, through selection and as a result of favourable mutations may make a
457 contribution (Kovalchuk et al. 2003). However, in *Arabidopsis* no additional mutations
458 compared to plants collected in control sites were found in the CEZ (Abramov et al. 1992).
459 Ostensibly the probability of favourable mutations may be seen as unlikely. Assuming a

460 germline mutation rate in plants of about 10^{-5} to 10^{-6} per gamete, one would expect only
461 one mutation in 500,000 plants (Kovalchuk et al. 2003). Consequently it has been proposed
462 that rapid adaptation may be more strongly linked to epigenetic processes in the
463 development of locally adapted phenotypes at polluted sites (Kovalchuk et al. 2003).

464

465 ***7. Evidence for a role of epigenetics in long-term or transgenerational responses to***
466 ***radiation-induced stress***

467 Studies on the effects of stressors on the epigenome of organisms under environmentally
468 relevant exposure conditions have covered examples for ionising radiation exposure and for
469 a range of chemical and non-chemical stressors in different species. Within these studies, a
470 range of epigenetic mechanisms and endpoints have been considered (for review see e.g.
471 Aluru 2017; Bruce et al. 2007; Kim et al. 2012b; Mirbahai and Chipman 2014). Initial adaptive
472 changes resulting from exposure to these different stressors have been found for key
473 components of the epigenome, such as DNA methylation (Vandegheuchte and Janssen 2011;
474 Marczylo et al. 2016), non-coding RNAs (Kure et al. 2013; Wang et al. 2013; Song et al. 2012)
475 and histone modifications (Raut and Sainis 2012; Mondal et al. 2016). Changes in microRNA
476 expression have further been shown to be involved in metabolism following starvation and
477 the transfer of longevity (Greer et al. 2011; Katz et al. 2009; Rechavi et al. 2014). In plants,
478 small RNAs play an important role in chromatin remodelling and DNA methylation through
479 RNA-directed DNA methylation also in different abiotic stresses in plants (Hirayama and
480 Shinozaki 2010).

481 Although long a controversial issue and still not fully elucidated, recent evidence has
482 suggested that in plants, vertebrates and invertebrates, epigenetic marks induced by
483 adverse conditions encountered by the parents can be partly stable across generations (Uller

484 et al. 2015; Klosin et al. 2017; Whittle et al. 2009; Saze 2012; Pecinka and Mittelsten Scheid
485 2012; Sudan et al. 2018; Stassen et al. 2018; Norouzitallab et al. 2019). Such retention can
486 potentially lead to transgenerational heritable changes in offspring (Verhoeven et al. 2010;
487 McCarrey 2012; Guerrero-Bosagna and Jensen 2015; Guerrero-Bosagna et al. 2012).
488 Evidence has been accumulated for the transfer of DNA methylation patterns in the germline
489 (Verhoeven et al. 2010; Verhoeven et al. 2016). As an example of the link between
490 epigenetic mechanisms and transgenerationally altered phenotypes a study of
491 transgenerational response to temperature in *C. elegans* has identified altered
492 trimethylation of histone H3 lysine 9 as a mechanism for transgenerational inheritance
493 (Klosin et al. 2017). On the other hand, in *Arabidopsis*, nickel chloride caused a change in
494 DNA methylation patterns and some of this was inherited by the following generation (Li et
495 al. 2015). In the offspring of mechanically wounded *Mimulus guttatus* plants changes in
496 methylation could be associated with transgenerational plasticity (Colicchio et al. 2018).
497 Depending on the methylation context, CG or non-CG methylation, these changes were
498 found to be in gene coding regions or transposable elements, respectively (Colicchio et al.
499 2018). Dandelions (*Taraxacum officinale*) also showed altered DNA methylation that was
500 largely inherited by the next generation of the asexually reproducing plants when exposed to
501 a number of different stressors (Verhoeven and van Gurp 2012; Verhoeven et al. 2016).
502 A growing number of papers also indicate that exposure to ionising radiation will lead to
503 changes in epigenetic markers (Table 3). For example, scots pine trees present in the most
504 contaminated areas around the Chernobyl nuclear reactor have been found to have
505 hypermethylated DNA, with this hypermethylation directly (Kovalchuk et al. 2003) or
506 transiently associated with the radiation dose received (Volkova et al. 2018). Further work
507 established that the genomes of young trees planted on contaminated soil showed higher

508 levels of cytosine methylation than trees in uncontaminated soil. However, levels of cytosine
509 methylations in plants grown in clean soil from seeds taken from previously exposed plants
510 were not found to differ significantly from controls Kovalchuk et al., (2003). Hence these
511 results are suggestive of a within generation genome methylation effect, rather than of any
512 multigenerational or transgenerational mechanism, as a result of exposure during the
513 somatic development. However, since only overall levels of DNA methylation inheritance
514 was addressed, the potential for loci specific cannot be discounted.

515 In a study of the progeny of *Arabidopsis sp.* sampled in three consecutive years from areas
516 with different levels of contamination within the CEZ, higher resistance to mutagens in
517 progeny of plants from the most contaminated sites compared to unexposed plants was
518 identified (Kovalchuk et al. 2004). This difference in sensitivity could be attributed to higher
519 expression of free radical scavenging enzymes and DNA-repair enzymes and was associated
520 with global genome hypermethylation in the contaminated site plants. It was hypothesised
521 from these data that epigenetic regulation of gene expression and genome stabilization may
522 play a key role in the underlying processes that stabilise *Arabidopsis* genome architecture
523 under exposure to ionizing radiation exposure (Kovalchuk et al. 2004). A number of papers
524 have proposed a link between epigenetic effects and non-targeted effects (NTE) such
525 genomic instability and bystander effects (Schofield and Kondratowicz 2018). However,
526 while the existence of non-targeted effects is well established (Morgan 2002; Kadhim et al.
527 2004; Pouget et al. 2018; Burdak-Rothkamm and Rothkamm 2018), and studies have shown
528 an association between the two effects (e.g., Kaup et al. 2006; Xu et al. 2015), evidence of a
529 causal relationship is more elusive, since NTE could be either a mechanism or a consequence
530 of epigenetic changes (Schofield and Kondratowicz 2018). Changes in the level of DNA
531 methylation may be intimately linked with transcription remodelling in response to radiation

532 exposures, including changes to the pathways involved in antioxidant defence and DNA
533 repair. Confirmation of such effects would require the use of combined genome wide DNA
534 methylation mapping and transcriptomic approaches to allow loci specific methylation to be
535 associated with gene expression phenotypes in exposed plants.

536 A study of the pale blue grass butterfly *Zizeeria maha* within the FEZ has provided a further
537 indication of the potential for heritable epigenetic changes in a population exposed to
538 ionising radiation (Hiyama et al. 2012; Hiyama et al. 2013). Mild morphological abnormalities
539 were observed on some individuals of adult butterflies collected one month after the
540 accident, but an increase of the severity of these abnormalities occurred in the F1
541 generation that were further inherited by F2 progeny. These abnormalities and their
542 transgenerational transfer were proposed to be attributable either to random mutation on
543 important genes or through epigenetic mechanisms. As the underlying mechanisms of these
544 effects were not studied by the authors, leaving the mechanistic basis of the observed
545 effects and their inheritance remain an open question.

546 Recently a number of European research groups have combined research efforts to study
547 possible epigenetic changes in organisms exposed to ionizing radiation, in the laboratory or
548 *in situ* (Chernobyl or Fukushima), in a range of species (plants, earthworms, fish, frogs)
549 (Table 3). The focus of the combined efforts was to better understand the possible role of
550 these mechanisms in the induction of long-term/transgenerational effects and their
551 relevance as possible biomarkers of ionising radiation (Adam-Guillermin et al. 2013). The
552 organisms chosen were all reproductive non-clonal organisms. Hence the work addresses
553 multigenerational and transgenerational effects in genetically diverse populations. For
554 example, in offspring of zebrafish that were exposed to ionising radiation during
555 gametogenesis, a large number of differentially methylated regions were observed, with five

556 specific loci showing a persistent effect up to the third generation (Kamstra et al. 2018).
557 These methylation changes could be linked to changes in gene pathways and adverse
558 effects found in progeny (Hurem et al. 2017; Hurem et al. 2018b). In the same exposure
559 study, miRNA expression was measured in first filial offspring and histone marks H3K4me3,
560 H3K9me4 and H3K27me3 at 3 specific loci(Lindeman et al. 2019). There were 23
561 differentially expressed miRNAs indicating a multifaceted response to ionising radiation
562 exposure (Martin et al. 2019, in preparation). Differentially enriched histone marks were
563 observed as well at the three measures loci in F1 offspring, but interestingly these effects
564 were diminished in F2 offspring (Lindeman et al. 2019, submitted). Although only exposed
565 embryo's were analysed similar changes in histone markes were found for Atlantic salmon
566 (*Salmo salar*) at higher dose rates (Lindeman et al. 2019).

567 A dose-rate dependent induction of total methylation levels was observed in *A. thaliana*
568 plants exposed in the lab to different levels of gamma radiation for up to three generations
569 (Saenen et al. 2017)). Moreover triple methyltransferase mutants (*drm1drm2cmt3*) of *A.*
570 *thaliana* showed increased sensitivity to irradiation including an increased induction of
571 oxidative stress (Saenen et al. 2017).

572 In the clonal cladoceran *Daphnia magna*, transgenerational inheritance of DNA methylation
573 changes were studied using bisulphite sequencing, after irradiation of generation F0 to 6.5
574 $\mu\text{Gy/h}$ or 41.3 mGy/h (Trijau et al. 2018). Significant methylation changes at specific CpG
575 positions in every generation were found, independent of dose rate and with a majority of
576 hypomethylation. The total number of common differentially methylated regions was
577 greatest between generations F2 and F3, with three specific persistent loci associated to
578 genes known to play a role during exposure to ionising radiation. The results above suggest a

579 role of enhanced methylation induced by chronic exposure to radiation in lab-conditions and
580 indicate the multi- and transgenerational natures of these responses.

581 For earthworms, studies of DNA methylation in the laboratory and CEZ have shown effects
582 of ionising radiation exposure on DNA methylation pattern as measured by methylated AFLP
583 analysis (Saenen et al. 2017). There are, however, specific challenges in the interpretation of
584 the role of radionuclide exposure in these responses. Large differences in genetic diversity
585 that may occur between morphological similar earthworm “species” may, for example, make
586 it difficult to identify DNA methylation changes unless clades are assessed separately. Indeed
587 clades of the earthworm *Lumbricus rubellus* were found to differ in the nature of their
588 genetic and DNA methylation responses to soil contamination by copper and arsenic (Kille et
589 al. 2013). A similar response was found within an analysed laboratory experiment, where
590 both between and within species allelic differences precluded the identification of a clear
591 DNA methylation profile response to exposure. In CEZ collected earthworm from two species
592 *Aporrectodea caliginosa* and *Octolasion lacteum*, a clear site specific change in DNA
593 methylation status was found (Saenen et al. 2017) in *Aporrectodea caliginosa*, while only
594 limited separation was found for *Octolasion lacteum*. While these site specific changes in
595 DNA methylation patterning may indicate a response to radionuclide exposure, a caveat is
596 that the earthworms were collected from sites that differ in the prevailing ecosystem
597 characteristics (wetland and garden sites).

598 An *in situ* study of DNA methylation in frogs collected from a range of differently polluted
599 sites within the Fukushima impacted area indicated that DNA methylation measured as
600 methylated cytosines increased with total absorbed dose rate, up to 7 $\mu\text{Gy/h}$. This increase
601 was concomitant with increased levels of DNA damages (Saenen et al. 2017). As in the study
602 for *A. thaliana* in the CEZ (Kovalchuk et al. 2004), this finding of higher DNA methylation

603 associated with increased DNA damage and repair activity supports a functional role of the
604 epigenome in maintaining DNA integrity. These results are in agreement with previous work
605 done on zebrafish exposed to depleted uranium, where changes in DNA methylation
606 patterns both at specific restriction sites and across the whole genome, were observed in F₀
607 adults and F₁ at the same time as DNA damages (Gombeau et al. 2016; Gombeau et al.
608 2017). A transient increased methylation with the dose rate was also observed in needles of
609 *Pinus sylvestris* plants collected in radioactively contaminated areas of Belarus (Volkova et al.
610 2018). In contrast no dose dependent changes in total methylation levels were observed for
611 *C. bursa pastoris* plants sampled in spring 2016 in contaminated areas of FEZ. For *A. thaliana*
612 plants collected in CEZ a decrease in global DNA methylation was found in the highest
613 contaminated fields (Horemans et al. 2018).

614 Overall the range of studies of the epigenetic response of species to radionuclide exposure
615 in the laboratory point to a role of the epigenome in adaptive responses. The field studies
616 with plants (pine trees and *Arabidopsis*) showed the potential for ionising radiation to induce
617 changes in DNA methylation levels under field conditions (Georgieva et al. 2017; Kovalchuk
618 et al. 2003; Kovalchuk et al. 2004). For invertebrates, the laboratory and studies in the CEZ
619 and FEZ have partially supported a role of increased methylation in response to radiation
620 among the majority of species studied to date. The challenge from these field studies
621 remains to unequivocally link the observed effects on the epigenome to radiation exposure,
622 rather than to other aspects of environmental variation across the CEZ and FEZ. Studies that
623 specifically investigate changes in mutant lines with reduced DNA methyltransferase activity,
624 as outlined above for *Arabidopsis*, provide initial causal evidence on the validity of such as
625 link.

626

627 **8. Knowledge gaps on epigenetic changes induced by ionising radiation**

628 Although all three different epigenetic layers have been implicated as key mechanisms
629 involved in determining the long-term and transgenerational responses of species to
630 pollutant, including ionising radiation exposure, a majority of studies have to date focussed
631 on the role of DNA methylation (Norouzitallab et al. 2019; Sun et al. 2018; Meehan et al.
632 2018; Burgio et al. 2018). In cases where difference in DNA methylation response following
633 exposure to ionising radiation are observed, a number of aspects that need further
634 consideration in future work can be drawn.

635 (i) Global methylation alone may be too coarse a measure of epigenetic change to be able to
636 see all biologically relevant differences induced by exposure to low dose rates. As such,
637 differences in methylation might be located in specific sequences of the genome but cannot
638 be detected by global measurements. Therefore, it is important to also include other
639 techniques (e.g. whole genome or reduced representation sequencing) in order to identify
640 specific epigenetic changes and to link these observations to effects on gene expression and
641 physiological change (Paun et al. 2019).

642 (ii) Different DNA methylation response in function of cell type, tissues (as seen in the
643 depleted uranium exposure in zebrafish by Gombeau et al., 2015), or age (as seen in frogs
644 exposed at Fukushima (Saenen et al. 2017), could induce a mosaic of DNA methylation
645 response at the whole organism level, limiting the capability to identify a clear change in
646 methylation pattern. This argues for the analysis of more homogenous tissues or cell types.

647 (iii) Initial changes of DNA methylation resulting from an initial radiation exposure may be
648 lost in individuals exposed over generations of chronic exposure as found for pine trees by
649 Kovalchuk et al. (2003) and in the second generation of lab-exposed *A. thaliana* in a
650 laboratory exposure to gamma radiation. Such results suggest that DNA methylation may be

651 a transient acting potential as an intermediate state preceding later genetic selection and
652 adaptation.

653 (iv) Genetic diversity of species between isolated local populations within the CEZ and FEZ
654 may mean that populations exposed to different levels of radiation may show markedly
655 different epigenetic responses, precluding the identification of a clear exposure response
656 relationship. The presence of natural and man-made barriers to dispersal, which may result
657 in population isolation, across these two zones, may accentuate such differences (Meeks et
658 al. 2007).

659 (v) Although less commonly studied than DNA methylation, the work done to date on the
660 responses of other epigenetic mechanisms like microRNAs or histone modifications to
661 ionising radiation exposure, suggest that these complimentary epigenetic mechanisms may
662 play roles in the response to radiation that may even dominate over DNA methylation
663 changes (Putiri and Robertson 2011; Brautigam et al. 2013);

664 (vii) Long time exposure to radiation might result in selection of alleles linked to tolerance,
665 potentiated potentially by increased mutation (as is seen for frogs in FEZ) that may lead to
666 genetic adaptation that might negate differences in DNA methylation. An interplay between
667 epigenetic changes, notably DNA methylation, and the targeting of mutation has been
668 proposed mechanisms (Putiri and Robertson, 2011 ; Braütigam et al., 2013).

669 (vii) Confounding factors (habitat, soil type, water chemistry; climate etc.) may increase the
670 variability between the samples that may result in changes in DNA methylation that overlie
671 and obscure effects due to ionising radiation making it difficult to link epigenetic change to
672 exposure (see discussion, Garnier-Laplace et al. 2013).

673

674 **9. Differential DNA methylated regions as possible biomarkers for exposure or effect of a**
675 **pollutant and its use in risk assessment**

676 There is a strong interest in finding possible biomarkers for exposure and effects of radiation
677 and additionally those that can be markers for long-term effects. Loci specific changes of
678 DNA methylation have been proposed as possible biomarkers for different environmental
679 cues (Meehan et al. 2018) and could possibly be used as molecular fingerprints for e.g.
680 genotoxicity induced when exposed to ionising radiation. However, it is also recognised that
681 significant challenges related to the effects of genetic background and the influence of
682 confounding factors also exist (Pernot et al. 2012). Further studies at environmental realistic
683 doses are needed to assess the prevalence of such responses, including under field
684 conditions. In particular, the use of more targeted methods are needed that identify loci
685 specific changes in DNA methylation, histone modification and the expression of relevant
686 miRNAs.

687 A clear conclusion that emerges from past and ongoing studies concerning the role of the
688 epigenome in response to chronic radiation exposure, lies in the interpretation of changes in
689 methylation patterns from field collected samples in respect to attribution of the principal
690 driver of effects. Specific challenges relate to working with some autochthonous species for
691 which genome resources may be lacking and, the influence of confounding factors which
692 may mask the causal response between ionising radiation exposure and epigenetic changes.
693 In efforts to attribute changes to specific stressor effects, epigenetic approaches may be
694 more powerful indicators of effects when linked to known biomarkers using, for example,
695 transcriptional analysis. When used in conjunction with other mechanistic measurements,
696 epigenetic analysis has the potential to enhance the ecological relevance of molecular
697 biomarkers, as described in the Adverse Outcome Pathway concept (Groh et al. 2015). Given

698 the critical need to establish the nature of effect of prolonged low level exposures, this
699 integrated approach seems a promising way forward, building as it does on existing
700 mechanistic knowledge.

701 The risk assessment process for radiation and radionuclides is largely based on using results
702 from short-term bioassays to predict the effects of exposures in the field. The validity of this
703 laboratory to field extrapolation is one of the key uncertainties in risk assessment (Lourenco
704 et al. 2016). A comparison of field vs laboratory studies has indeed shown that species
705 sampled in the field were 8 times more sensitive than those studied under laboratory
706 controlled conditions (Garnier-Laplace et al., 2013) indicating the need for further thorough
707 lab to field studies. One of the largest differences between laboratory bioassays and field
708 exposures is exposure duration. This is true within a single generation (intergenerational
709 exposure), but even more so when subsequent generations are exposed to the same
710 stressful environment (multigenerational exposure) or when exposure of the parent
711 generation has a subsequent effect on the non-exposed offspring (transgenerational
712 exposure). When multigenerational exposures occur, these may result in effects in later
713 generations that match, and can even exceed those found in exposed F0s (see Table 2). The
714 biological response of species mediated through the genome and epigenome appear to play
715 a role in the development of such effects. Such findings may require a more refined
716 understanding to support and reduce the uncertainty in risk assessment for chronic low dose
717 exposures. Hence, the mechanisms that underlie differential responses within and over
718 generations to previous (sub-lethal) radiation-exposure require further studies to provide a
719 baseline for the development of new approaches such as Adverse Outcome Pathways on low
720 dose radiation exposure, to the risk assessment for both wildlife and human.

721

722 **10. Conclusions and Recommendations for further development and application.**

723 Work reported to date in both lab and field have indicated changes in DNA methylation
724 resulting from chronic exposure to low dose of ionising radiation. A common conclusion
725 from this work is that both laboratory and field studies have demonstrated changes in
726 overall methylation in organisms exposed chronically to ionising radiation. Generally a
727 chronic enhanced ionising radiation level induced hypermethylation or methylation pattern
728 change which could be taken as a response to induce DNA stability. The main advantage of
729 laboratory studies is the ability to set up controlled multi/transgenerational studies, and
730 avoid confounding factors like local difference in soil characteristics, microclimate. Together
731 with the use of homogeneous populations, this allows for greater insight into the underling
732 mechanisms and processes. Field studies can provide the increased environmental realism of
733 the responses studied. Although data suggest that methylation changes can be observed in
734 different organisms a lower dose rates than those seen in laboratory experiments. The
735 challenge remains to unequivocally link such observations to a specific cause. Furthermore,
736 processes linked to the potential for population adaptation and interactions with other
737 environmental stressors can add a further level of complexity as compared to laboratory
738 studies. Improvements could be made by increasing site coverage and further targeted work
739 on molecular mechanisms, as well as data on the background levels and variations in
740 methylation changes.

741 From the studies presented here, it can be concluded that DNA methylation might be the
742 key to transferring the response to ionising radiation from one generation to the next.

743 Whereas measuring total DNA methylation can be performed without any prior information
744 on genetic background of the species, the rapid technical evolution and the decreasing cost
745 of sequencing analyses will offer a wider comparison of radiologically induced DNA

746 methylation in different biological models and provide greater insight into the underlying
747 mechanisms. An important step will be to compare the sensitivity, reliance and above all
748 specificity of DNA methylation as a possible biomarker of ionising radiation exposure at
749 environmentally relevant levels, with other epigenetic mechanisms such as histone
750 modifications and microRNAs linked to responses at higher level biological complexity e.g.
751 changes in growth and reproduction.

Table 1. Pros and cons of DNA methylation methods. 5-mC (methylcytosine), 5-hmC (hydroxymethylcytosine), AFLP-MS (methylation specific amplification fragment length polymorphism), HPLC-MS/MS (high performance liquid chromatography coupled with tandem mass spectrometry), ELISA assay (enzyme-linked immunosorbent assay), MeDIP seq (methylated DNA immunoprecipitation coupled with next-generation sequencing), WGBS (whole genome bisulfite sequencing), RRBS (reduced representation bisulfite sequencing)

Method	Principle	Methylated base detected	Pros	Cons
AFLP-MS	Cut DNA with restriction enzymes and analyse on a fragment analyser	5-mC	Low cost per sample No need for sequenced genome Low DNA amount (250-500 ng) Low processing time	Detection of global methylation Specific equipment needed
HPLC-MS/MS	Detection of methyl groups on hydrolysed DNA sample	5-mC & 5-hmC	Medium cost per sample No need for sequenced genome Low processing time	Detection of global methylation High DNA amount (50-1000 ng) Specific equipment needed
5 mC ELISA assay	Use of monoclonal antibodies sensitive and specific for 5-mC	5-mC	Low cost per sample No need for sequenced genome No specific equipment needed Low processing time	Detection of global methylation High DNA amount (100-2000 ng)
MeDIP seq	Immunoprecipitation sequencing	5-mC	Detection of site specific methylation Low DNA amount (300 ng)	High cost per sample Need for sequenced genome Specific equipment needed High processing time
WGBS	Bisulfite conversion and DNA sequencing	5-mC & 5-hmC (oxBS-seq)	Detection of site specific methylation Low DNA amount (30 ng)	High cost per sample Need for sequenced genome Specific equipment needed High processing time
RRBS	Bisulfite conversion and DNA sequencing	5-mC & 5-hmC (oxBS-seq)	Detection of site specific methylation	High cost per sample Need for sequenced genome High DNA amount (1000 ng) Specific equipment needed High processing time

Table 2: Overview of lab-based studies in which ecotoxicological relevant model organisms were exposed to radiation, radioisotopes or other toxins for multiple generations; F0=Parental organism, F...= offspring with the number indicating the generation

Species	Chemical	Generations	Observed phenotype	Ref
<i>C. elegans</i>	Gamma radiation 7-42 mGy/h	F0-F2	Greater reproduction effects in multigenerationally and transgenerationally exposed F2s than F0 generation	Buisset-Goussen et al. (2014)
<i>D. magna</i>	Gamma radiation 0.007-35 mGy/h	F0-F2	Toxicity on multiple traits increased from F0 to F2	Parisot et al. (2015)
<i>D. Rerio</i>	Gamma radiation 9-53 mGy/h	F0-F1	Effect on DNA damage, transcription, lipid peroxidation and demographic endpoints in F1	Hurem et al. (2017), Hurem et al. (2018b), (2018a)
<i>D. Rerio</i>	Uranium 20-250 µg/L	F0-F1	Effect on DNA damage, transcription, DNA methylation and demographic endpoints in F1	Bourrachot et al. (2014), Gombeau et al. (2017)
<i>D. magna</i>	Americium 0.3-15 mGy/h	F0-F2	Threshold for effects on reproduction reduced from 1.5 mGyh ⁻¹ in F0 generation to 0.3 mGyh ⁻¹ in F2 and F3	Alonzo et al. (2008)
<i>D. magna</i>	Uranium 2-50 µg/L	F0-F1	Greater reduction in fecundity in F1 than F0 at 50 µg/L	Plaire et al. (2013)
<i>D. magna</i>	Nickel 42-85 µg/L	F0-F1	Greater reduction of ATP levels in F1 compared to F0	Pane et al. (2004)
<i>C. elegans</i>	Ag nanoparticles EC30-value	F0-F10	Greater (10 fold) sensitivity in F2, F5, F8 and F10 generations compared to P generation	Schultz et al. (2016)
<i>D. magna</i>	Ag nanoparticles EC10-EC50	F0-F10	Population growth rate at 10 µg/L reduced by 80% in F2s compared to 21% in F0 generation	Volker et al. (2013)
<i>D. magna</i>	Penta-chlorophenol 0.0002-2 µmol/L	F0-F3	Population growth rate reduction increases from 28.2% to 34.9% to 46.3% in F0, F1, F2 generations	Chen et al. (2014)
<i>D. magna</i>	Tetracycline 0.1-5 mg/L	F0-F1	NOEC decreased from 5 mg/L to 0.1 mg/L from F0 to F3	Kim et al. (2012)
<i>D. magna</i>	Enrofloxacin 13 mg/L	F0-F1	Reproduction NOEC decreased from 30 mg/L to 3.1 mg/L from F0 to F1 generation	Bona et al. (2015)
<i>C. elegans</i>	Uranium 4-50 µg/L	F0-F16	Greater maximal length but increased sensitivity to uranium across the generations	Goussen et al. (2015)
<i>C. elegans</i>	Uranium 4.6 µg/L	F0-F22	Increase of sensitivity from F0 to F6 and subsequent adaptation until F22	Dutilleul et al. (2014)

Tabel 3: Overview of studies in which changes in epigenetic mechanisms (DNA methylation, histone modifications or miRNA's) are measured in organisms exposed to radiation in a long-term set-up (within or over generations) either in laboratory or field conditions. F0=Parental organism, F...= offspring with the number indicating the generation, CEZ: Chernobyl Exclusion Zone, FEZ: Fukushima Exclusion Zone

		Organism	Experimental conditions	Epigenetic changes	Additional endpoints	Reference
Laboratory exposed	Plants	<i>A. thaliana</i>	F1, F2, multigenerational (F0 from CEZ, 1.8-4.4 μ Gy/h) methyl methane sulfonate (140 μ M) or Rose Bengal (10 μ M)	DNA methylation: hypermethylation in both F1 and F2	Higher resistance to mutagens, increased expression of ROS scavenging enzymes and DNA repair enzymes	Kovalchuk et al. (2004)
		<i>P. sylvestris</i>	F0, trans- and multigenerational set up, on contaminated soil both acute (~10Gy) and chronic (~80Gy) (F0 from CEZ, (absorbed dose 1986: >60Gy, 10-60Gy, 1-10, 0.1-10Gy),	DNA methylation: hypermethylation in exposed	-	Kovalchuk et al. (2003)
		<i>A. thaliana</i>	F1, F2 transgenerational, Progeny of plants collected at CEZ 1.8-4.4 μ Gy/h	DNA methylation: hypermethylation	-	Kovalchuk et al. (2004)
		<i>A. thaliana</i>	F0, F1, F2, mutligenerational, 14 day exposure during vegetative state, 22, 38, 86, 457 mGy/h	DNA methylation: dose-dependent hypermethylation, strongest in F2	Changes in ROS-scavenging enzymes, DNA repair and developmental traits, mutants in methyltransferases showed increased sensitivity to radiation	van de Walle et al. (2016), Saenen et al. (2017)
	Invertebrates	<i>D. magna</i>	F0, F1, F2 and F3 transgenerational, F0 exposed for 25 days, 6,5 μ Gy/h or 41.3 mGy/h	DNA methylation: hypomethylation but dose-rate independent	Reduction in fecundity in F0, no adverse effects in F1, F2, F3	Trijau et al. (2018)
	Vertebrates	<i>D. rerio</i>	F0, F1, F2, F3, transgenerational, exposure during gametogenesis, 8.7 mGy/h, 28 days	DNA methylation: Genome-wide in F1, locus-specific	Linked to gene pathways changes and adverse effects in progeny	Hurem et al. (2018b), Kamstra et al. (2018),

				regions up to F3		Hurem et al. (2017)
		<i>D. rerio</i>	F0, F1, multigenerational, exposure during gametogenesis, 8.7 mGy/h, 28 days	miRNA expression in F1 embryos	-	Martin et al., in prep
		<i>D. rerio</i>	F0, F1, F2 transgenerational, gametogenesis, 8.7 mGy/h, 28 days	Histone modifications (hypermethylation) at specific loci in F0 and F1 but no longer in F2	-	Lindeman et al. (2019)
		<i>S. salar</i>	F0-embryo's, exposure from one-cell fertilized eggs till early gastrula stage, 1, 10, 20 or 30 mGy/h	Histone modification (hypermethylation) at specific loci at highest dose rate	-	Lindeman et al. (2019)
Field collected	Plants	<i>P. sylvestris</i>	F0, (Belarus, Chernobyl affected area), annual absorbed dose: 10-158 mGy or 1-14 μ Gy/h	DNA methylation: transient with dose, hypermethylation	-	Volkova et al. (2018)
		<i>C. bursa pastoris</i>	F0, FEZ : total dose rates: 0.13-38 μ Gy/h	DNA methylation : no change	-	Horemans et al. (2018)
		<i>A. thaliana</i>	F0, CEZ : total dose rates : 0.1-160 μ Gy/h	DNA methylation : Hypomethylation at highest dose rates	-	Horemans et al. (2018)
		<i>G. max</i>	F0, after 7 generations CEZ, total accumulated dose : 1-132 mGy	DNA methylation: slight increase (10%) in radio-contaminated	Increased levels of single and double DNA strand breaks	Georgieva et al. (2017)

				samples		
Invertebrates	Earthworms (<i>A. caliginosa</i> , <i>O. lacteum</i>)	F0, CEZ, total dose rates 0.12-41 $\mu\text{Gy/h}$		DNA methylation: site-specific differences <i>A. caliginosa</i> . for no or limited changes found for <i>O. lacteum</i>	-	Saenen et al. (2017)
Vertebrates	<i>H. arborea</i>	F0, FEZ, total dose rate 0.38-41,7 $\mu\text{Gy/h}$		DNA-methylation: hypermethylation, dose- dependent	Concomitant with increased DNA damage	Saenen et al. (2017)

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- Review on long term effects of exposure of wildlife to chronic low dose radiation
- Inter-, multi- and transgenerational studies for both lab and field exposures
- Changes found in epigenetic marks induced by chronic exposure to ionising radiation
- DNA methylation possibly transfers the response from one generation to the next

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