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Initial Research Activity

State-of-the art on epigenetics and general approach

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Executive Summary

This report presents the approach adopted in WP4 dedicated to “chronic low dose effects and risk assessment” and particularly, for the Initial Research Activity (IRA). This IRA is focused on a specific topic of common interest to radioecology and radiobiology: the possible contribution to long-term or transgenerational effects of mechanisms governing the activation or the repression of the epigenome of organisms exposed chronically to ionising radiations.

This report gives a state-of-the-art on epigenetic modifications seen from the radioecological point of view, with an overview of the major molecular pathways of epigenetic control and of the more recent tools that can be used. Consequences of epigenetic modifications are also described extensively through fundamental mechanisms essential for all stages of life, such as cancer, development, DNA repair and heritability.

Even if data on measured epigenetic changes are scarce in the radioecological field, the literature review on long-term effects of ionising radiation observed in Chernobyl indicates that in several cases, there is no direct evidence to link morphological or reproductive effects to genetic changes, suggesting a highly probable role of epigenetic mechanisms.

The final section of this report focuses on the Initial Research Activity, giving the preliminary list of the potential sets of selected species and endpoints, and of the methods and tools for establishment of dose (rate) - responses relationships. The global approach is presented, with laboratory studies dedicated to method development for epigenome characterization and to mechanistic studies aiming at understanding the link between DNA global methylation, gene expression and further transgenerational consequences. An overview of the planned field studies in the Chernobyl Exclusion Zone and in Fukushima is given, but the exact experimental protocols will be elaborated in more detail in the coming months, in particular through a workshop dedicated to field studies.

List of Acronyms

AFLP: Amplified Fragment Length Polymorphism

BER: Base Excision Repair

CERAD: Centre for Environmental RADioactivity

CEZ: Chernobyl Exclusion Zone

CMT3: Chromomethylase 3

DDR: DNA Damage Response

DNMT: DNA Methyl Transferase

DRM3: Domain Rearranged Methyltransferase 3

DSB: Double Strand Break

HAT: Histone Acetyl Transferase

HDAC: Histone Deacetylase

HMT: Histone Methyl Transferase

HR: Homologous Recombination

IRA: Initial Research Activity

LC/ESI/MS: Liquid Chromatography/ElectroSpray ionisation/Mass Spectrometry

MET1: Methyl Transferase 1

MMR: Mismatch Repair

NER: Nucleotide Excision Repair

NHEJ: Non Homologous End Joining

PCNA: Proliferating Cell Nuclear Antigen

PTM: Post Translational Modification

SAM: S-adenosyl-L-methionine

TET protein: Ten Eleven Translocation protein

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1. Introduction and general objectives

Evaluating the effects of low dose radiation on organisms is clearly a shared challenge in research on human and non-human species. This topic of common interest in radioecology and radiobiology is driven by the mutual need of knowledge in human and eco-toxicology of the impact of radioactive substances on man and the environment.

Understanding the role of primary mechanisms at the cellular and sub-cellular level when organisms are exposed to low dose radiation will help to explain the potential consequences on physiological functions and health at the individual and population level. Of equal importance to studying the immediate effects of exposure to low dose radiation is the need to understand a number of related issues such as: the combined effects of low dose radiation and other environmental and man-made stressors; the possible induction of long-term or transgenerational effects; the capacity of organisms to adapt to stress.

Within this general framework, the objective of WP4 is to increase our understanding of the effects of chronic low-dose radiation and the mechanisms provoking them, and this in a domain of interest to both radioecology and radiobiology.

An Initial Research Activity (IRA) is therefore planned in the following research domain: the possible contribution to long-term or transgenerational effects of mechanisms governing the activation or the repression of the epigenome of organisms exposed chronically to low-dose ionising radiation. The present document is focused on this IRA. It presents the approach that will be adopted by the COMET partners of WP4 to ensure a common set of core knowledge, experimental data and skills. After a comprehensive state-of-the art on mechanisms of epigenetic control, methodology of studying epigenetic changes and gaps in knowledge in the field of radioecology, selected common laboratory protocols and methods will be presented, including the rationale for model species, exposure conditions and endpoint selection. The onset to field experiments is discussed.

2. State of the art on approaches and tools for epigenetic changes assessment

2.1 Introduction

DNA forms the blueprint of all proteins of a cell using RNA as the intermediate messenger. This process is recognised as the central dogma of molecular biology and it provides an explanation of the flow of genetic information within organisms (Crick, 1970). It deals explicitly with the detailed residue-by-residue transfer of sequential information in a one way progression that can be stated as simply that "DNA makes RNA makes protein". As a framework for understanding the transfer of sequence information, it is identified that 3 major classes of biopolymers are involved: DNA and RNA (both nucleic acids), and proteins. There are 3×3 = 9 conceivable direct transfers of information that can occur between these. The general transfers between these polymers describe the normal flow of biological information: DNA can be copied to DNA (DNA replication), DNA information can be copied into mRNA (transcription), and proteins can be synthesized using the information in mRNA as a template (translation).

Since the identification of the role of DNA in protein production, it has become clear that the sequence variations in this biomolecule can provide the explanation for the heritable variation of adaptive traits from parents to offspring. As such, DNA sequence variations have been seen as the underpinning mechanisms of evolution and Mendelian inheritance. Within this role, it had been commonly assumed that all traits are passed on through the inheritance of DNA sequences and that those alone have an effect on the phenotype produced (Danchin et al., 2011). This paradigm has now, however, been shown to be an oversimplification and other factors such as the environment can have a hereditary effect on some organisms (Head et al., 2012). Recent work has begun to establish that modification to DNA and the proteins that are involved in its replication and transcription, collectively called epigenetic mechanisms, can play an important role in adaptation both within and across generations. Such mechanisms are the conduits for environmental influence on the genome. This realisation has led to the development of the field of epigenetics.

2.2 Definition of epigenetics

The first definition of epigenetics was introduced by Conrad Waddington (1939) to describe 'the causal interactions between genes and their products, which bring the phenotype into being', referring to any causal mechanism that acts on genes to govern a resulting phenotype. This definition was refined by Holliday (1994), stressing the importance of mitotic inheritance and explaining how the expression of a gene might be changed by any modification that does not mutate the nucleotide sequence itself: 'The study of the changes in gene expression which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression' and 'Nuclear inheritance which is not based on changes in DNA sequence'. Based on these definitions, a new definition is now widely adopted by many scientists in this field (Riggs et al., 1996 ; Allis et al., 2007 ; Berger et al., 2009 ; Vandegehuchte and Janssen, 2011) 'Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence'.

2.3 Description of molecular pathways

There are a number of known mechanisms of epigenetic control, including DNA methylation, histone modifications, and small interfering RNA (siRNA), and micro RNA (miRNA) processes (e.g. Dalmay, 2006). Recent findings indicate that these major epigenetic players are engaged in a network of interconnected 'cross-talk' (Irato et al., 2003), and that non protein coding RNAs (ncRNAs) may orchestrate the pattern of targeted gene expression that "underpins ... the differences between species, ecotypes and individuals" (Mattick et al., 2009). Epigenetic alterations are known to play important roles in the aetiology of human disease, including cancer (Huang et al., 2003). However, the significance of epigenetic mechanisms in toxicology (Szyf, 2007), ecology (Bossdorf et al., 2008), and evolutionary biology (Rapp & Wendel, 2005) has also begun to emerge (Kille et al., 2013). Within environmental biology and ecology, it has been suggested that epigenetics could define "… where the environment interfaces with genomics … and could provide a rapid mechanism by which an organism can respond to its environment without having to change its hardware" (Pray, 2004). Epigenetic systems are vitally important functional links between the detection of environmental change and regulation of gene expression (Bossdorf et al., 2008, Grativol et al., 2012). Determining the nature of analogous epigenetic regulatory relays in plants and

animals, especially in ecologically (and ecotoxicologically) relevant species that play important roles in delivering key ecosystem services, should therefore be seen as a matter of priority (Vandegehuchte & Janssen, 2011, Kille et al., 2013).

Recent studies have begun to provide the confirmatory evidence that epigenetic mechanisms are constitutively involved in the regulation of genes expressed in response to environmental cues and stimuli. These studies suggest that epigenetic responses can strongly influence the phenotypes that result following stress, including pollutant, exposure (Kille et al., 2013). Moreover some epigenetic changes have been proven to be stable and can lead to transgenerational heritable changes. This aspect has been well established in studies with both plants and animals and has been identified to include a potential role for heritable epigenetic change in the development of disease and responses to chemical exposure (McCue et al., 2012, Ruden et al., 2005). In contrast to the established role of heritability of epigenetic changes in plants and vertebrates, their potential for the trans-generational transfer of epigenetic factors in invertebrate species has been more controversial. Johnson and Tricker (Johnson & Tricker, 2010), however, have made a cautionary point that so far the majority of epigenomics research and population genomics has been performed on classic model organisms, and "it appears that many traditional model systems (e.g. *Drosophila melanogaster*) are atypical as regards the epigenetic control of their genomes". Other invertebrate species, from different taxa may, however, have different characteristics. For example, analysis of the levels of DNA methylation in genomes from a range of phyla, suggests that while some species may indeed have relatively low levels (e.g. nematodes, arthropods), there are other invertebrates (e.g. annelids and molluscs) that can have a substantively methylated genome (Regev et al., 1998). This observation has been confirmed in recent years as the role of epigenetic changes in heritable responses of a range of invertebrate species have begun to emerge (Morag et al., 2011, Furuhashi et al., 2010, Seong et al., 2011).

The regulation of gene expression by the epigenome is governed by different processes including DNA-methylation, modifications in histones, the core proteins of chromatin around which DNA is wrapped, and the interaction with small non-protein coding RNA sequences (small-interfering RNAs (si-RNA) and microRNAs (miRNA)). Although not completely elucidated yet information is rapidly coming available on the molecular mechanisms that underpin the establishment and regulation of the epigenome. The relative role of these mechanisms can vary between species. As highlighted above, in some species there is a relatively low level of methylated cytosines and hence in these species DNA methylation maybe secondary as a regulatory mechanisms. In others the range of histone modification can be variable in the way they are utilised. The mechanisms underpinning these process and their potential role in toxicity and heritable response to stress exposure are discussed in detail below.

2.3.1 Methylated DNA

DNA methylation occurs through the addition of a methyl group to the position 5 of a cytosine ring leading to the formation of 5methylcytosine. Methylation will lead to a repressed chromatin state, which in turn will lead to silencing of gene expression. As evolutionary methylation is an ancient biochemical process, it is well conserved in all

eucaryotic cells although some differences exist between animal and plant cells as reviewed by Law and Jacobsen (Law & Jacobsen, 2010). In animals, DNA methylation occurs mainly at CG sites, a cytosine followed by a guanine resulting in two methylated cytosine residues sitting diagonally to each other on opposing DNA strands (symmetric methylation). Methylation is in animals evenly spread across the DNA except for some CpG islands. In plants, however, methylation can in addition to cytosine followed by a guanine (CG) also occur on other sites such as CHG or even CHH (with H being either an A, T or C) (Vandegehuchte & Janssen, 2011, Klose & Bird, 2006). Unlike in animals, DNA methylation in plants predominately occurs on transposons and on other repetitive DNA elements (Law & Jacobsen, 2010).

De novo DNA methylation can be targeted by DNA-mediated DNA methylation and RNA-mediated DNA methylation the latter being the more common mechanism in plants (Wassenegger, 2000, Law & Jacobsen, 2010). The DNA-mediated DNA methylation can be regulated by the pairing of homologous DNA sequences, the influence of the flanking sequences, the capability of DNA to form specific structures and the recognition of some CpG or CpNpGp sites. In the RNA-mediated DNA methylation, RNA will bind to complementary DNA sequences which will lead to DNA methylation along the RNA-DNA complex (Wassenegger, 2000). *De novo* methylation of the DNA is mediated by enzymes of the DNA methyltransferase (DNMT) family, i.e. DNMT3a and DNMT3b (Auclair & Weber, 2012). Besides the *de novo* methylation, it is also important to copy the pre-existing methylation patterns so the information can be passed onto the new cell generation. This process is described as maintenance methylation. Those methylations are catalysed by DNMT1 (Klose & Bird, 2006, Bird, 2002) and will lead to the induction of long-term inheritable effects. In plants homologues of the DNMT3 and DNMT1 enzymes namely DOMAINS REARRANGED METHYLTRANSFERASE (DRM2, homologue to DNMT3) and DNA METHYLTRANSFERASE 1 (MET1 or DMT1 which is homologue to DNMT1) will fulfil similar roles in DNA methylation and its maintenance. In addition a plant-specific CHROMOMETHYLASE 3 (CMT3) will be involved in the methylation of CHG sites. The assymetric CHH methylation in plants on its turn is performed by the DRM2 (Law & Jacobsen, 2010).

Although the methyltransferase enzymes are the core proteins involved in the methylation process they are recruited and guided to their specific interaction targets by a protein complex: this complex on its turn again differs between plants and animals and if *de novo* methylation or maintenance is to be achieved. A good overview is given in Law and Jacobsen et al., 2010). As indicated above gene methylation will lead to transcriptional silencing of the genes. Two possible mechanisms have been suggested to underlie this silencing process: (1) methylation directly interferes with the binding of transcriptional activates to cognate DNA sequences and (2) proteins attracted by methyl-CpG bind to the DNA and recruit co-supressors to silence gene expression (Klose & Bird, 2006, Bird, 2002).

A further insight that has recently emerged is that DNA methylation represents only one part of a DNA methylation cycle. This includes on the one side addition of DNA methylation mark to cytosine residues at CpG sites as detailed above; but on the other hand both passive and active demethylation mechanisms. It was already recognised that DNA methylation could be

"diluted" by not replicating the methylated status to the newly synthesized strand during cell division. As a result, methylation at a specific locus would be lost in daughter cells upon further division. This process was termed as "passive DNA demethylation" and was thought to be a dominant mechanism governing any global demethylation responses. The recent identification of ten-eleven translocation (TET) proteins as 5-methylcytosine oxidases has provided researcher with a plausible pathways for the active removal of DNA methylation (Ko et al., 2013, Pastor et al., 2013). This mechanism was long assumed, since it was known that rapid loci specific demethylation was possible in certain cell types (Bruniquel & Schwartz, 2003). However, it is now well established. Its identification is consistent with observations of rapid global demethylation in response to a range of biological or environmental stimuli and provides a new perspective on how changes in DNA methylation are linked to cell differentiation, embryonic development, disease and stress.

Identification of the potential for active methylation and demethylation of the genome identifies the regulation of DNA methylation as a potential means to regulate gene expression in response to environmental stress as described on figure 1 (Guerrero-Bosagna et al., 2012, Manikkam et al., 2013). This may be particularly true when considering adaptation to environmental pollutant exposure. Metal and metalloid exposure in particular has been found to perturb epigenetic processes (Salnikow & Zhitkovich, 2008, Baccarelli & Bollati, 2009). Such responses include hypomethylation by Cd (Takiguchi et al., 2003), hypomethylation (Zhao et al., 1997) and hypermethylation (Jensen et al., 2008) by As, and targeted gene silencing via hypermethylation by Ni (Lee et al., 1995). In hemp and clover plants, exposure to Ni, Cd, or Cr exposures were found to induce significant dose-dependent changes (20-40%) in the global methylation status. Detailed analysis indicated that these changes were targeted at specific genomic sites, suggesting a role of DNA methylation in regulating responses to these metals (Aina et al., 2004). Reviews of the impact of Ni and As mechanism-of-action indicate that both act through modifying the epigenome impacting on m^5C, histone modification and miRNA expression (Salnikow & Zhitkovich, 2008, Baccarelli & Bollati, 2009). Specific histone modifications effected by Ni and As have been documented (Baccarelli & Bollati, 2009) allowing the proposed research to target these marks and developed specific predictions that may result for metal metalloid exposure. More expansive studies have linked the impact Ni on H3K3me3 to alterations in gene expression through genome-wide positional mapping of this epigenetic change (Tchou-Wong et al., 2011).

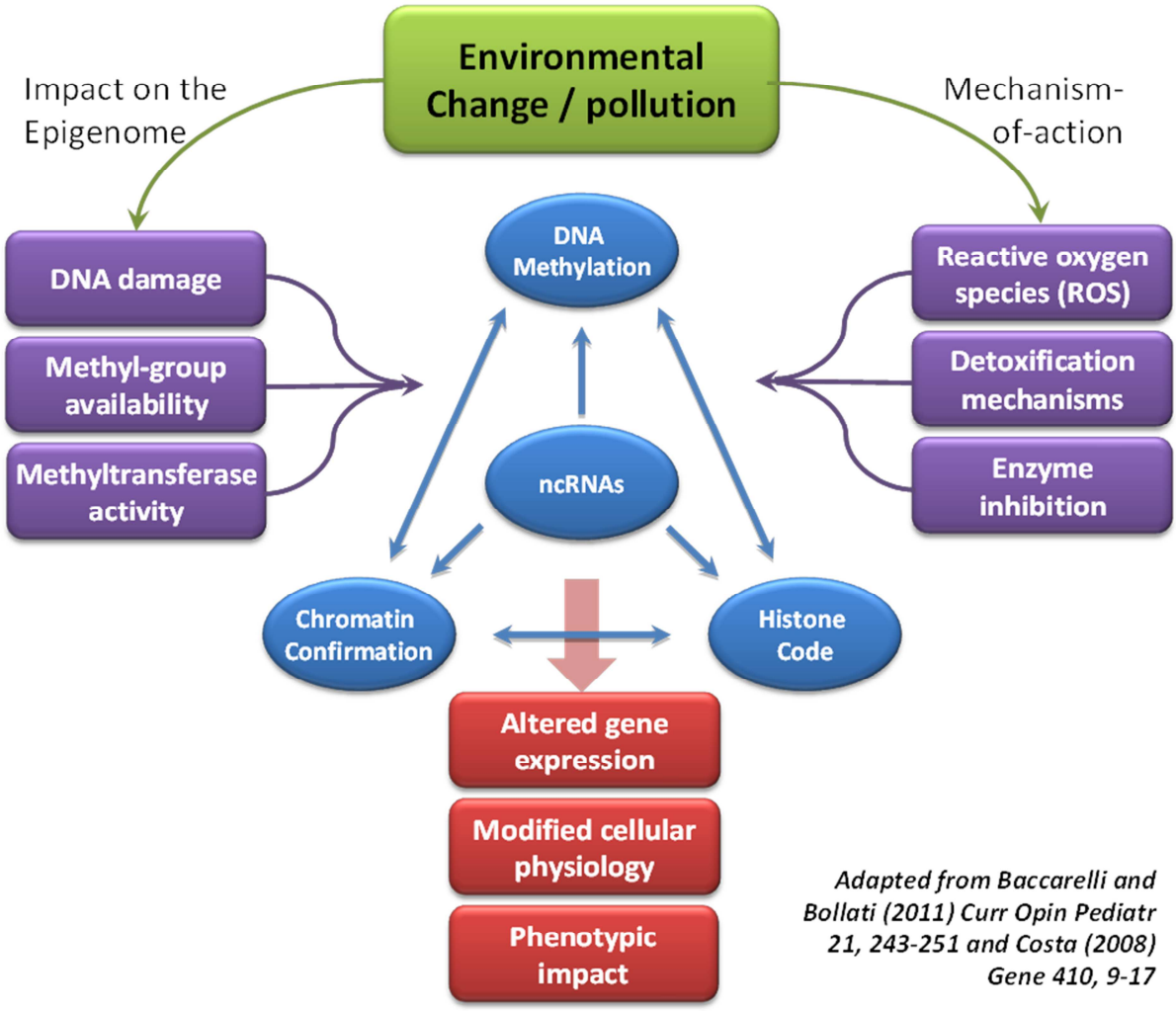

Figure 1. The environmental-epigenetic interface : mechanisms underlying mode of action. After Baccarelli and Bollati (2009) and Costa (2008).

2.3.2 Non-coding RNAs

Non-coding RNA are functional RNA molecules that are not translated into a protein. Non-coding RNA genes include a range of well-known and abundant RNAs like transfer RNA and ribosomal RNA that have well characterised roles in gene expression and protein translation. Genome sequencing has revealed that there are a range of additional categories on non-coding RNA that have been shown to have a regulating role in DNA transcription. The best known examples are microRNAs, small interfering RNA (siRNAs), small nucleolar RNA (snRNAs) and extracellular RNA. The number of non-coding RNAs encoded in any given genome is currently unknown. However recent transcriptomic and bioinformatic studies in humans have suggested the existence of thousands of non-coding RNA, the vast majority of which are of unknown function.

The most well-known group of non-coding RNA implicated in gene regulations are the small interfering RNAs and microRNA. These groups, which can be viewed as near synonymous, have been implicated in gene silencing. Indeed exogenously synthesised siRNAs have been

developed as specific tool for knock-down gene silencing based on mechanisms initially developed in the nematode *Caenorhabditis elegans* (Fire et al., 1998). The microRNAs are small single stranded RNA molecules, consisting of 21 to 24 nucleotides. Like all of the small non-protein coding RNAs, they are not able to lead to proteins directly, but instead are involved in the down regulation of gene expression by binding to the 3'-untranslated regions (UTR) of the target messenger RNA (mRNA) to allow the targeting of mRNA for destruction at the RNA-induced silencing complex. Although the generation of miRNAs differs between animals (Bushati & Cohen, 2007, Choudhuri, 2010) and plants (Xie et al., 2010) they play similar roles in the sequence specific regulation of gene expression.

The degree of complementarity between the target mRNA and the miRNA determines if the target will be cleaved (near-perfect complementarity) or if the translation will be repressed (imperfect complementarity). In the latter case, a bulge at the mismatched region is formed, preventing endonucleases from cleaving and hence degrading the target mRNA. Suppression of gene expression is in this case obtained by the interaction of the miRNA to the targeted mRNA strand that prevents different translational enzymes to bind on their turn to the mRNA (Choudhuri, 2010). In animals, most miRNAs have an imperfect complementarity with their target, leading to a repression of gene expression rather than degradation of the mRNA.

Advances in DNA sequencing technologies have opened up a new vista on the expression of non-coding RNAs at different developmental stage and as responses to environmental stimuli. Massive parallel sequencing techniques that generate millions of sequences from a transcriptome sample are particularly useful in this context. This is because they can allow transcript quantification following miRNA extraction, conversion to cDNA and sequencing. This profiling approach has already identified that pollution exposure influences the miRNA complement of exposed individuals indicating an important regulatory role of miRNA for within generation adaptation to stressor exposure (Kure et al., 2013, Wang et al., 2013, Song et al., 2012). Further non-coding RNAs have been linked to transgenerational epigenetic alterations in gene expression linked to chemical exposure (Hudder & Novak, 2008). For examples, congenital defects in cardiac and skeletal development associated with *in utero* exposure to xenobiotics such as valproic acid and dimethadione may be mediated through epigenetic modification of histones and possibly miRNAs (Weston et al., 2006).

2.3.3 Histone modifications

Within eucaroyotic cell nuclei genomic DNA is wrapped around histone proteins organised in octamer structures. Together these are called nucleosomes and are the fundamental units of chromatin (Berr, 2011). For years histones were regarded as merely structural proteins but now it has been recognised that they play an important role in regulating gene expression (Margueron et al., 2005). Nucleosomes are not static simple structures but are highly dynamic and can move, dis-/reassemble or be more or less stabilized. These changes are

triggered by specific environmental signals or by developmental cues and can be modified which will lead to a changed DNA accessibility and hence changed gene transcription. The dynamic nature of the nucleosomes is partly due to the different modifications that it can undergo. These modifications include posttranslational modification (PTM), ATP-dependent chromatin remodelling and replacement of canonical histones with histone variants (Berr, 2011). PTMs often occur on the N-terminal tails of the histone proteins that protrude from the nucleosomes and that are important in protein-protein interactions. As such histones and histone modifications orchestrate not only the accessibility of the DNA for gene expression but also the ability of transcription factors and other enzymes to bind and initiate gene expression. The best studied PTMs are histone acetylation, methylation and ubiquitination. A good overview of the enzymes involved in the different PTMs is given in Berr et al. (2011).

When a histone becomes **acetylated**, an acetyl group of acetyl-coA is added to the ε-amino group of lysine by histone acetyltransferases (HATs). This will lead to the neutralization of the positive charge of lysines, thereby modifying the interaction between histones and the DNA. As such, DNA will be relieved from its condensated state, leading to an activation of gene expression. A balance of the acetylation is maintained by histone deacetylases. (HDACs) (Berr et al., 2011, Berger, 2003). It has been shown that histone modifications and in particular histone acetylation might be very important in DNA damage and repair mechanisms and as such in reducing genotoxic stress (Drury et al., 2012).

Histone methylation is catalysed by histone methyltransferases (HMTs). They methylate lysine or arginine residues that are present on amino-terminal histone tails or in the nucleosome core by adding 1, 2 or 3 methyl groups from S-adenosyl-L-methionine (SAM). Methylation of histones is relatively stable possibly leading to long-term silencing, but it can be removed by histone demethylases. In addition, the methylase marks can also be removed by proteolysis of the methylated histone tails or by replacing the methylated histones (Berr et al., 2011, Berger, 2003).

An **ubiquitin** molecule can be covalently attached to the histone lysine residues in an ATP-demanding reaction involving different enzymes (Berr et al., 2011). If one ubiquitin molecule is attached to the histones, this will affect the subcellular localization and biochemical activity of the target protein. Poly-ubiquitination can lead to the degradation of the protein or to modified protein-protein interactions.

Changes in histone modification have been linked explicitly to chemical exposure in mammalian systems (Casati et al., 2012, Gadhia et al., 2012). In the nematode *C. elegans*, use of chromosome immunoprecipitation techniques has also identified the presence of conserved histone modifications in worms that pass through dauer as opposed to worms that by pass this resting stage. Further in the fruit fly *D. melanogaster* heat shock or osmotic stress has been shown to induce heterochromatin. When embryos were exposed to heat

stress over multiple generations, this condensed chromatin state was maintained over multiple successive generations, though gradually returning to the normal condition. These results suggest a mechanism by which the effects of stress are inherited epigenetically via the regulation of a tight chromatin structure, with modified histones likely to be involved in such responses. In this respect histone, like methylated DNA and non-coding RNAs, are potential mechanisms for transgenerational transfer of environmental effects on the genome.

2.4 Link with biological effects

Although the concept of epigenetics in biology has been recognized for more than 20 years, the last decade of research has demonstrated that epigenetics regulates a range of fundamental mechanisms essential for all stages of life. It also influences the way how organisms respond to life style factors and stressors encountered in the environment.

2.4.1 Cancer

An accumulation of genetic and epigenetic alterations in cells may lead to cancer disease. Epigenetic changes contribute to carcinogenesis and a mechanistic link exists between environmental and dietary exposures and cancer disease. Still today there are many questions remaining regarding the contribution of epigenetic changes to the development of cancer (Virani et al., 2012). From a clinical perspective, because epigenetic changes are thought to be reversible they may constitute possibilities for cancer treatment.

One of the most studied epigenetic mechanisms namely DNA methylation, is highly dysregulated in cancer cells. Loss of DNA methylation was one of the first epigenetic changes described in human cancer (Feinberg and Vogelstein, 1983; Gama-Sosa et al., 1983). At present most types of cancer have been shown to have an overall deficiency of 5mC compared to normal tissue which can increase genomic instability and promotes the progression of tumorigenesis.

Moreover, almost half of the genome consists of repetitive elements and they are normally heavily methylated. Hypomethylation of these repetitive elements constitute a major part of the 5mC loss in cancer cells (Ehrlich, 2009; Lander et al., 2001). At and near the centromere, tandem repeats are involved in keeping the DNA packaged into heterochromatin at the point of sister chromatin association resulting in chromosome stability (Eden et al., 2003). The most studied hypomethylated repetitive elements are centrometric tandem repeats, adjacent-centrometric tandem repeats, and short- (Alu) and long-interspersed elements (LINE-1). Hypomethylation of such tandem repeats unravels the structure of the genome and contributes to the tumorgenesis process (Kokalj-Vokac et al., 1993). Other regions in the genome that can be hypomethylated are promoter regions. Hypomethylation of such regions may lead to activation of normally silenced genes, aberrant gene expression, disruption of normal cellular processes, and genomic instability (Virani et al., 2012).

The genomes of cancers are globally hypomethylated, however, some regions have been found to be hypermethylated. This is achieved by overexpression of DNMT protein. DNMTs are the methyl donors to the cytosine residue and thus, regulate the DNA methylation process. A common feature in tumor cells are overexpression of DNMT, but only DNMT1 and DNMT3a and DNMT3b are involved in tumorgenesis (Issa et al., 1993). The genome in a cancer cell is recognized by hypermethylation of CpG islands in promoter regions (e.g. Edwards and Ferguson-Smith, 2007). CpG is a dinucleotide consisting of a cytosine paired with guanine and it has had a major focus in the epigenetic research because of its ability to directly silence gene expression. A CpG island is a 1000-kb stretch of DNA with GC content greater than 50 %. CpG islands occupy about 60 % of human gene promoter regions.

Hypermethylation of promoter regions is often an early step in the tumorgenesis process. It may seem that hypomethylation and hypermethylation in cancer cells are conflicting forces; however, they commonly coexist within the same tumor but come about in different genomic regions.

Histone methylation has been shown to regulate transcription and several enzymes involved in the process have been found to be dysregulated in cancer (Virani et al., 2012). More research is needed to fully understand the functional consequences of dysregulation of histone methylation but it has been shown that K-demethylases and K-methyltransferases play important roles in carcinogenesis.

2.4.2 Embryonic development

The embryogenesis is controlled by genetic and epigenetic mechanisms but development is by definition epigenetic (Reik, 2007). During the development of different organs and tissue generally no changes to the DNA occur. Genes in the genome are active in different tissues depending on the regulation by different combinations of transcription factors. In addition, changes in gene expression during the development are accompanied or caused by epigenetic changes such as DNA methylation at CpG sequences in vertebrates (Bird, 2002; Li, 2002), modification of histone tails (Turner, 2007), and the presence of non-nucleosomal chromatin-associated proteins (Ringrose, 2004).

Mammal gametes are differentiated and specialized cells that contain all the information that is needed for the initiation of a new life after fertilization. For a normal embryonic development to occur both maternal and paternal genomes are needed because they are programmed differently and they are functionally different (Surani et al., 1984; McGrath and Solter, 1984). In somatic tissue the gamete-derived methylation patterns of imprinted genes are kept during embryonic development, however, they are erased in primordial germ cells (Lee et al., 2002; Hajkova et al., 2002).

After fertilization rapid reprogramming occurs of the paternal and maternal genomes. First, demethylation happens in the paternal pronucleus. The zygote is formed and both maternal

and paternal chromosomes experienced methylation in the blastocyst stage during which most methylation marks are erased that were inherited from the gametes (Howlett and Reik, 1991; Kafri et al., 1992; Rougier et al., 1998; Aanes et al., 2011). However, on imprinted genes methylation marks are protected so parental imprints are preserved.

In the primitive ectoderm DNA methylation is rapidly increased and gives rise to the entire embryo. In contrast, methylation is inhibited or not maintained in the trophoblast that give rise to the placenta (Chapman et al., 1984) and in the primitive endoderm lineage that give rise to the yolk-sac membrane (Rossant et al., 1986). Both *de novo* and maintenance methyltransferase activities are needed to establish the embryonic methylation patterns. It has also been showed that maintenance of genomic methylation above a certain level is essential for a normal embryonic development (Li et al., 1992; Okano et al., 1999). In addition, histone acetylation and possibly histone methylation is required for regulation of imprinted genes during embryogenesis (Li, 2002).

During early mammal embryonic development an X-chromosome inactivation is also required. In this process one out of two X-chromosomes is silenced and converted from active euchromatin to transcriptionally silent and condensed heterochromatin involving epigenetic changes such as the coating of the X-chromosome by Xist RNA, DNA methylation, and histone modification (Li, 2002). However, this silencing process is reversible so that genes that are needed later in development can be reactivated. On the other hand, DNA methylation patterns that have been acquired during embryonic development are stable in somatic cells and during adult life (Reik, 2007).

2.4.3 DNA repair

DNA is continuously exposed to endogenous and exogenous insults inducing thousands of DNA lesions per cell per day. A number of DNA damage responses (DDR) that activate different repair pathways have evolved to recognize and repair specific DNA lesions, and most of these are highly conserved among species. In addition, the reinstatement of the original epigenetic state following DNA repair, is essential to preserve the integrity of the genome. Most types of DNA damage are repaired by one or a combination of multistep pathways that include: (1) base-excision repair (BER), (2) nucleotide excision repair (NER), (3) mismatch repair (MMR) and (4) double-strand break (DSB) repair. DSBs are generally considered as the most deleterious DNA damage induced by irradiation and these lesions are mainly repaired by homologous recombination (HR) and non-homologous endjoining (NHEJ). The following paragraph will therefore mainly focus on epigenetics in relation to DSB repair.

The DDR process requires multiple steps, including the initial signaling of the break, the opening of the compact chromatin to facilitate access for repair factors, and the restoration of the chromatin to its initial state (for review see Huertas et al., 2009; Hunt et al., 2013; Rossetto et al., 2010). It is now clear that a major aspect of the DDR damage response occurs through specific interactions with chromatin structure and its modulation. During the initial

steps, histones undergo a number of posttranslational modifications (PTMs) including phosphorylation, acetylation, methylation and ubiquitylation (for details see Hunt et al., 2013) and the complete removal of histones from DNA is achieved by many repair mechanisms for different DNA lesions. During chromatin restoration, the histones therefore have to be redeposited on the newly repaired DNA by the process known as chromatin reassembly. Histone redeposition takes place at DSB repair sites to replace those histones that have been evicted from the sequences closer to the breaks. In addition, in the HR repair pathway, strand invasion in the homologous donor sequence together DNA synthesis and branch migration also need the histones to be reincorporated during restoration (reviewed by Huertas et al., 2009). Furthermore, the pre-existing epigenetic marks, including locus-specific histone modification and DNA methylation patterns, should be reinstated. The mechanism by which the original histone modification pattern is reproduced in the new histones reassembled in repaired DNA remains unclear, although a range of possibilities has been envisaged from the employment of neighbouring chromatin regions, which are unchanged during the repair process, to the partial recycling of evicted old histones as epigenetic templates for the deposited new histones (Huertas et al., 2009). DNA methyltransferase DNMT1 is recruited to DNA repair sites through its interaction with PCNA (Proliferating Cell Nuclear Antigen). DNMT1 may preserve cytosine methylation patterns in the newly synthesized DNA, and so participates in the conservation of epigenetic information after repair of DNA damage. Faulty post-repair chromatin restoration leading to epigenetic changes may result in aberrant gene expression and genomic instability such as those found in cancer (Huertas et al., 2009).

There is increasing evidence that in addition to the post-translational modifications occurring after DNA damage, pre-existing chromatin modifications at the histone level ("histone codes") can directly affect the initial DNA damage response and subsequent pathway choice for NHEJ or HR and affect DSB repair efficiency. Examples of such pre-existing epigenetic markers are H4K16ac and H4K20me2 since their absence results in the loss of a DDR during DSB repair (reviewed by Hunt et al., 2013).

As discussed above, DNA repair pathways and efficiency can be influenced by epigenetic markers and faulty post-repair chromatin and DNA-methylation restoration could, on the other hand, lead to epigenetic changes. Furthermore, there are increasing evidence that active 5mC demethylation during cell differentiation and stress responses involves DNA repair, indicating that cellular DNA repair not only protects the genome from exogenous and endogenous DNA lesions, assuring genome stability, but also contributes to epigenetic gene regulation (reviewed by Niehrs and Schäfer, 2012). Both oxidative- and repair-based DNA demethylation mechanisms have been proposed and the latter involves three different ways to remove 5mC: (i) substitution of the methylated or oxidized base with unmethylated cytosine by base excision repair (BER) (Agius et al., 2006; Hajkova et al., 2010; He et al., 2011a; Morales-Ruiz, 2006; Kim et al., 2009), (ii) deamination of 5mC followed by BER (Rai et al., 2008; Metivier et al., 2008), and (iii) nucleotide excision repair (NER) removal of 5mC (Le May et al., 2010; Barreto et al., 2007; Schäfer et al., 2010; Schmitz et al., 2009). Although it is

still unclear what signals specify a particular mode of DNA demethylation, one possible factor is tissue-specific expression of demethylating factors and regulators (Niehrs and Schäfer, 2012).

As conferred in section 1.3.1 and 1.4.1, the genomes of cancers are globally hypomethylated, although some regions have been found to be hypermethylated. Epigenetic silencing of DNA repair genes by hypermethylation of promoter regions has been reported for several repair pathways including BER, NER, DNA MMR and several other DNA damage processing mechanisms in different tumor tissues. It is believed that such changes can be important events in cancer initiation and/or progression by reducing genomic stability leading to genetic aberrations at other important gene loci (reviewed by Lahtz and Pfeifer, 2011).

2.4.4 Heritability

This section will focus mainly on DNA methylation as this is by far the most studied epigenetic marker. As discussed in section 1.4.2, epigenetic processes, such as DNA methylation, are crucial in coordinating changes in gene expression leading to cell lineage differentiation during an organism's development. DNA methylation is, however, not exclusively influenced by intrinsic signals during development and there is increasingly evidence of spontaneous or environmentally induced changes in methylation profiles that can influence the phenotype (for review see Angers et al., 2010 and references therein). Such changes are stable at the time scale of an individual and present different levels of heritability.

The transmission of these marks within an individual's lifetime is called "mitotic epigenetic inheritance", "epigenetic maintenance" or "epigenetic stability" (Lange and Schneider, 2010). The transmission of DNA methylation is mediated by the action of DNA methyltransferase 1 (DNMT1). DNMT1 binds to the PCNA (proliferating cell nuclear antigen) complex as well as hemi-methylated DNA and catalyses the addition of a methyl group to the complementary unmethylated CpG during DNA replication (for details see Lange and Schneider, 2010). The transmission of histone marks through DNA replication is more complex as the parental nucleosomes, including modifications, are disassembled and in contrast to DNA methylation there is no template for the restoration of the correct histone modification pattern once the replication fork has passed (Lange and Schneider, 2010). Since the reproduction of DNA methylation occurs faithfully after DNA replication, it has been hypothesized that at least in mammals, DNA methylation could act as cornerstone to rebuild the epigenetic framework, recruiting histone modifiers, which in turn set anchors that orchestrate the higher order nuclear structure of the daughter cells. However, also the reverse direction of DNA methylation by histone modifications has been reported (for review see Lange and Schneider, 2010 and references therein).

For epigenetic variation to affect inheritance, however, meiotic transmission is also required (meiotic epigenetic inheritance). The transgenerational effects of DNA methylation therefore require either a direct or indirect alteration of methylation in the germ line. Inheritance of the DNA methylation variants appears to be highly variable among affected genes as well as among taxa. Depending on the genotypic context (Richards 2006) and the taxon, some methylation marks will be limited to the lifetime of an individual whereas others will be

transmitted across generations. DNA methylation has most extensively been studied in mammals, where methylation patterns are erased (to some extent) and reset twice during development. This reprogramming of DNA methylation takes place firstly on fertilization in the zygote, and secondly in primordial germ cells, which are the direct progenitors of sperm or oocyte (see section 1.3.2 and Seisenberger et al., 2013 for review). Erasure of methylation patterns has also been shown to occur during zebrafish development (Mackay et al. 2007), suggesting a common pattern in vertebrates. Therefore it is likely that the DNA methylation marks that these organisms acquire during their life will not be transmitted to their progeny. However, the extent of erasure of epigenetic marks has been found to vary among multicellular organisms. Even in mammals certain genomic regions escape DNA demethylation, such as pericentromeric heterochromatin, IAP retrotransposons and paternally methylated imprinted genes, allowing gametic transmission of epigenetic information to the next generation (Richards 2006; Lange and Schneider, 2010 and references therein). Transgenerational epigenetic transmission may be rare, but it has been reported in different mammalian species such as mice (e.g. Morgan et al., 1999) and rats (e.g. Anway et al., 2005; Chang et al., 2006; Guerrero-Bosagna et al., 2010; Skinner et al., 2012). Relatively little is known about epigenetic mechanisms in birds, and according to Frésard et al (2013) no transgenerational transmission of epigenetic marks has been reported. Epigenetic inheritability is best documented in plants (e.g. Petronis, 2010; Ou et al., 2012, Law & Jacobsen, 2010, He et al., 2011, Hauser et al., 2011). This probably relates to the fact that in organisms in which reproductive lineages or germ lines are derived from vegetative or somatic lineages late in development and epiallelle erasure is less extensive, the propensity for methylation mark transmission is higher (Richards, 2006). This is supported by the observation that most epiallelles have been detected in plants (Kalisz and Purugganan, 2004).

It seems that DNA methylation by itself is a process whose effects are in many cases limited to the lifetime of an individual. DNA methylation can, however, interact at several levels with other mechanisms that may indirectly promote its transmission, and it has been hypothesized that environmentally induced methylation could be indirectly propagated across generations via small RNA molecules that are transmitted in the cytoplasm of the gametes (Angers et al. 2010).

As discussed by Angers et al (2010) “epigenetic variation creates phenotypic differences that have an effect on individual fitness and therefore can be acted upon by natural selection. In addition, DNA methylation may allow individuals to use different strategies in fluctuating environments depending on its degree of inheritance. DNA methylation, unlike genetic modifications, may occur rapidly in response to environmental changes and could therefore represent a potential way to cope with environmental stress on very short time scales, possibly even during the lifetime of an individual (Rando and Verstrepen 2007). Because it could then allow individuals to produce alternative phenotypes in response to environmental change, DNA methylation would be a relevant process even in the absence of inheritance.

2.5 Relevance of epigenetics for the (radio)ecotoxicological field

A 2012 paper on epigenetics and ecotoxicology pointed out that while there were 1300 review papers on epigenetics in biomedicine, the application of epigenetics in ecotoxicology

was a comparatively young field with only a few review papers, and a handful of field ecological studies (Head et al., 2012). This has been attributed to the lack of methods available outside the typical human model organisms. However, it has been long recognized that epigenetic mechanisms and pathways exist across a variety of species, and the development and application of methods in ecologically relevant species has opened up the research field enormously. The links between epigenetic processes and exposure to environmental stress, hereditary and transgenerational effects, and not least the connection to adaptation, tolerance and resistance in exposed populations/species makes the topic one of clear relevance to environmental science and toxicology (Vandegehuchte and Janssen, 2011).

The fact that certain species (e.g., plants and invertebrates) can evolve resistance or tolerance to pollutants through transgenerational heritable adaptive processes is well established (Figure 2). On the other hand, hereditary transfer of effects might lead to reduced fitness or increased sensitivity of offspring to stress (Alonzo et al., 2008 ; Massarin et al., 2010) . Genetic and epigenetic mechanisms are thought to play a role in both cases (Table 1).

The combination of an environmentally induced modification, together with persistence and hereditability of that change makes the issue of importance for ecological risk assessment. Hence, questions of transgenerational and population level effects of epigenetic changes attract much attention, rather than the focus on disease in individuals found in many human directed studies. Recent papers on epigenetics and ecotoxicology have stressed the need for fundamental research on:

> *"(1) the occurrence of chemical-induced epigenetic modifications at environmentally realistic exposure concentrations in ecotoxicologically relevant species; (2) the phenotypic and population-level effects of these modifications; and (3) the transmission of these changes to subsequent non-exposed generations"* (Vandegehuchte and Janssen, 2011).

Table 1: Characteristics of genetic, epigenetic, and metabolic pathways by which environmental stressors can influence gene expression (Head et al., 2012)

	Susceptible to environmentally induced change	Reproducible	Reversible	Persistent after stressor is removed	Heritable
Genetic	Limited	Partially	No	Yes	Yes
Epigenetic	Yes	Yes	Yes	Yes	Yes
Metabolic	Yes	Yes	Yes	No	No

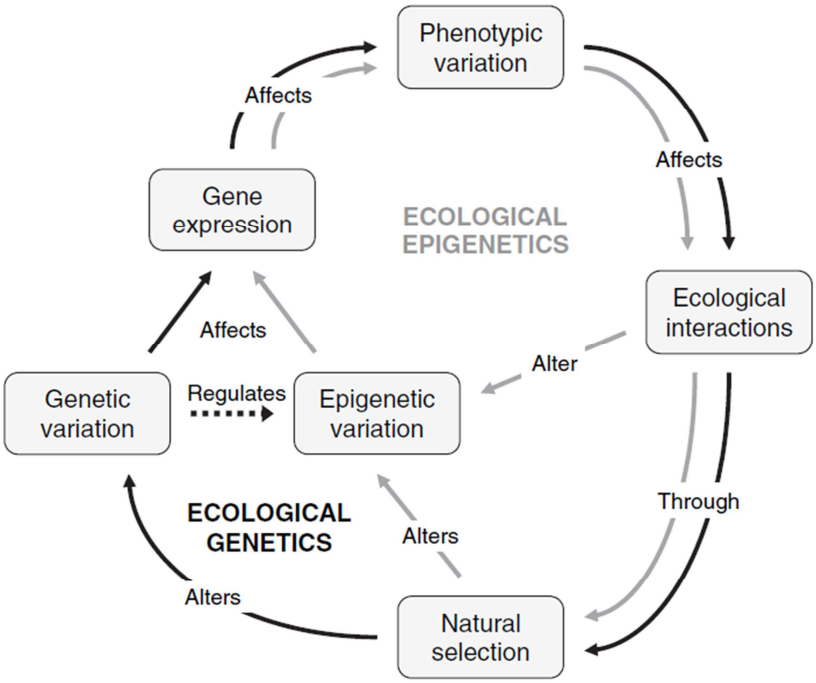

Figure 2. Adaptation mechanisms influenced by genetic and epigenetic variations (from Bossdorf et al., 2008).

Issues and Challenges for Ecotoxicology

The study of epigenetics across different species can offer valuable insight into underlying mechanisms and pathways, exploiting both the conservation of the mechanism across species as well as the variability. For example, different methylation distributions suggest that DNA methylation may have different functions in different organisms (Bird, 2002; Suzuki and Bird, 2008). These aspects are discussed in section 1.3 and 1.4. Other challenges for ecotoxicology and environmental risk assessment, and especially the design and interpretation of field experiments include :

1) *The temporal disconnection between cause and effect (e.g., that observed effects may occur in the offspring of exposed populations)*
2) *Supporting evidence and mechanisms for field observed effects*
3) *Applications in development of biomonitoring and biomarker assay (i.e., as a broad indicator of accumulated stress)*
4) *Relevance for DEB-tox models*
5) *Challenges for Risk Assessment and Regulation: population and community level impacts can be both positive and negative (e.g., acclimation/adaptation)*

2.6 Overview on methods available to characterize epigenetic changes

2.6.1 Methods to study DNA methylation

DNA methylation is essential for a properly functioning genome through its roles in the maintenance of chromosome stability and structure, and transcription. Methylation of cytosine outside of CpG is also found on plant genomes such as in Arabidopsis for which the non CG methylation has a significant biological function. Various methods were used for global methylation assay and some are listed and developed besides.

2.6.1.1 HPLC-MS

In 2002, Friso et al. (2002) developed a method for quantitative determination of 5-methyl-2'deoxycytidine using chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS). Mass spectrometry is a highly sensitive detection method that is, however, not quantitative. Using stable isotope-labelled reference compounds, which have the same chromatographic and ionization properties but different molecular weights, turns MS into a quantitative method (Taghizadeh et al., 2008). By comparing the integrals of the individual mass signals of the natural compound (amount to be determined) and labelled compound (known amount) and subsequent application of calibration curves, very precise quantification of the natural compound is achieved. This technique can be used also to quantify O6-methylguanine (Calmann et al., 2005) and 5-hydroxymethylcytosine (Globish et al., 2010).

2.6.1.2 AFLP

The Amplified Fragment Length Polymorphism (AFLP) technique is based on the amplification of subsets of genomic restriction fragments using PCR. The original methods from previous studies (Vos et al., 1995; Kronforst et al., 2008) were partially modified to study global methylation and do not need a sequenced genome to be processed. That is why, this methylation-specific AFLP analysis has been commonly used to survey patterns of methylation in a range of non-human species (Xu et al., 2009; Snell-Rood et al., 2012; Wang et al., 2013; Smith et al., 2012; Podio et al., 2012; Fu et al., 2013). This modified version of AFLP substitutes the MseI enzyme with a pair of cytosine methylation-sensitive isoschizomers, HpaII and MspI. This pair of enzymes recognizes the same target sequence (CCGG) but exhibit different sensitivities to the cytosine methylation status (McClelland et al., 1994).

2.6.1.3 Methylated DNA chips

Methylated DNA immunoprecipitation (MeDIP) is a recently devised method used to determine the distribution of DNA-methylation within functional regions (e.g., promoters) or in the entire genome robustly and cost-efficiently. This approach is based on the enrichment of methylated DNA with an antibody that specifically binds to 5-methyl-cytosine and can be combined with PCR, microarrays or high-throughput sequencing. All these methods can be combined with microarray hybridization to monitor DNA-methylation changes on selected genomic regions across several tissues or cell lines. The methods available are certainly comparable, but achieve different levels of coverage, sensitivity and accuracy (Suzuki et al., 2008, Zilberman et al., 2007 and Irizarry et al., 2008). The most widely used array-based

methods reported are: 1) methylation-sensitive or methylation-specific restriction (like CHARM, Irizarry et al., 2008) or 2) immunoprecipitation of methylated DNA (e.g., MeDIP, Weber et al., 2005; Keshet et al., 2006). This technique has emerged as one of the most widely used methods in the scientific community and has been used to derive methylation maps in different organisms such as in plants (Zhang et al., 2006) and some mammals (Weber et al., 2005), for epigenomic mapping in cancer (Keshet et al., 2006) and to monitor epigenomic changes upon differentiation of embryonic cells (Straussman et al., 2009).

2.6.2 Methylated histones

Chromatin is the physiological template of the genetic information and is composed of DNA, histones and other chromosomal proteins. The amino-terminal tails of histones are subjected to posttranslational modifications including acetylation, methylation, phosphorylation etc… In fact, genome-scale DNA methylation profiles suggest that in mammals, DNA methylation is correlated to histone methylation patterns (Meissner et al., 2008a and b). Furthermore, in zebrafish, Andersen and collaborators showed that a combination of DNA and histones methylation leads to a pre-patterning of developmental gene expression in embryos (Andersen et al., 2012). To this purpose, the authors used a ChIP-chip technique which combines a chromatine immunoprecipitation with a microarray technology to the aim of investigating interactions between proteins and DNA and in this case, the occupancy of promoters and 5' end of genes by H3K4me3, a permissive modification, H3K27me3, a mark associated with inactive promoters, H3K36me3, a mark of transcription elongation found on gene bodies, and H3K9me3, another modification associated with inactive promoters (Lindeman et al., 2011).

2.6.3 Sequencing of microRNAs

MicroRNAs (miRNAs) are members of non-coding RNAs. They are involved in diverse biological functions. MiRNAs are precisely regulated in a tissue- and developmental-specific manner, but dysregulated in many human diseases (Collotta et al., 2013). Transcriptional regulation, post-transcriptional regulation, as well as genetic alterations, are the three major mechanisms controlling the spatial and temporal expression of miRNAs. Emerging evidence now indicates that transcriptional and epigenetic regulations play major roles in miRNA expression such as methylation (Saito et al., 2009) and this status must be assessed by a MeDIP-chip technique described above. Another technique consists to profile gene and miRNA transcripts by the use of the sequencing of RNA (Rudgalvyte et al., 2013).

2.6.4 Next Generation Sequencing (NGS)

Recent advances in next generation sequencing (NGS) technology now provide the opportunity to rapidly interrogate the methylation status of the genome. DNA methylation is an important epigenetic modification involved in gene regulation, which can now be measured using whole-genome bisulfite sequencing. The method employs a specific chemical oxidation of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) to 5-formylcytosine, which subsequently converts to uracil during bisulphite treatment (Booth et al., 2013). Whole-genome bisulfite sequencing is being applied to organisms with larger genomes, including mammals (Lister et al., 2009; Laurent et al., 2010), but the prohibitive cost makes DNA methylation-based affinity enrichment and reduced representation

protocols followed by sequencing a favorable alternative (Meissner et al., 2008b; Gu et al., 2010). In fact, news techniques based on high-throughput sequencing of bisulfite-treated chromatin immunoprecipitated DNA (BisChIP-seq), can directly interrogate genetic and epigenetic processes that occur in normal and diseased cells (Pellegrini and Ferrari, 2012; Statham et al., 2012).

2.7 Data and knowledge gaps on epigenetic changes induced by radionuclides

Most of the published data within ecological epigenetics is from non-radiological studies with an extensive recent review of ecotoxicological data published by Vandegehuchte and Janssen (2011). In addition to the historical separation of radiation and other environmental stressors in environmental risk assessment in general, radiation is also seen as one of the classic genotoxins. This presents particular challenges when attempting to unravel the potential contribution of epigenetic and genetic processes in field and laboratory experiments. Nevertheless, there is a considerable amount of data on long term effects of ionizing radiation observed on plants and animals in the Chernobyl Exclusion Zone, and after other nuclear accidents (e.g., Kystym). Even if these studies were not originally focused on epigenetic changes, such non-genetic effects cannot be ruled out given the pattern of the observed effects (e.g. developmental abnormalities).

2.7.1 Data on long-term effects observed in the CEZ

Plants

Geras'kin et al. (2008) in their comprehensive review have compiled the Chernobyl data on effects of radiation to non-human species such as forest trees (pine and spruce), herbaceous plants, soil mesophauna, brown frog, silver carp, mouse-like rodents and cows. They identified the minimum doses (dose rates) at which certain effects were reported including death, reducing reproductive capacity, morphological changes, chromosome aberrations etc. Lots of papers of Geras'kin and coauthors relate to effects of radiation on plants, e.g. for spring barley (2005), allumium cepa (studies at USCB, Poland, 2011a), crested hairgrass (STS, Kazakhstan, 2012).

Studies of the radiation effects to the plant species in the Chernobyl Exclusion Zone (CEZ) started early after the accident as a reaction to the numerous observations of the abnormalities detected even at the morphological level. Diverse morphological defects that have been present in plants inhabiting the contaminated areas to date included changes in size, shape and color of individual organs and their location in the plants, in association with genetic alterations (Shevchenko and Grynikh 1995; Frolova et al. 1996; Geraskin et al. 2003a; Pozolotina 2003; Oudalova et al. 2005).However, for the CEZ the most studied species was Scots pine, for which numerous morphological defects have been detected (Kal'chenko et al. 1993a and b; Rubanovich and Kal'chenko 1994; Grodzinsky et al. 1995; Geraskin et al. 2003b).

Cytogenic effects were also observed in the same species, such as aberrant cells frequencies in the root meristem seedlings at various dose rates (Geras'kin et al. 2011b, Oudalova and Geras'kin, 2011). These studies were performed during several years at the same populations of the trees which allowed revealing the cyclic variation of the studied parameter in time.

Zelena et al. (2005) in studies in the CEZ showed the significant deregulation of twelve genes in dwarf needles of Scots pine as compared to normal needles at the close levels of ^{137}Cs activity in dwarf and normal needles. Kuchma et al. (2011) showed a radiation caused increase of mutation rate in Scots pine using AFLP. However, in the same time, they reported no increase of mutation rates for microsatellites due to radiation. Also, they did not observe any difference between the numbers of mutations in the trees from the Red Forest with strong vs those with no phenotypic effects.

Researches of Institute of General Genetics of RAS (Igonina 2010) studied the seedlings of *Pinus sylvesris* L. obtained from the irradiated trees at least for the period of 15 years after the Chernobyl accident. The offsprings of the irradiated pines did not accumulate genetic mutations and cytogenetic damages; however, they demonstrated the high level of the morphological abnormalities typical for the studied species in the CEZ. Hence, there is no direct evidence to link genetic changes to morphological effects.

A similar conclusion that there was no unequivocal dependence on radiation exposure was made in the study of frequency of loci of microsatellite DNA in two types of cattail: *Typha angustifolia* and *Typha latifolia* (Tsyusko *et al*. 2006). The data collected in Ukraine, including the CEZ, exhibited that variability of the genetic diversity of these plants was mostly due to the geographic latitude, characteristics of the region and their aqueous habitat, as well as the origin of the local population. However, the presence of a positive correlation between some genetic parameters (an effective quantity of alleles) and ^{90}Sr and ^{137}Cs specific activities in the plants tissues might imply an impact of the radiation related factors.

Interestingly, some studies highlighted the existence of an adaptation of specific communities of woody plants to polluted soils. Adaptive responses to growth in the contaminated environment were observed for the progeny of Scott pine (Arkhipov et al., 1994). The molecular mechanisms involved in these adaptive processes may include favourable mutations in essential genes. However, such an even would be rare – assuming a germline mutation rate in plants of about 10^{-5} to 10^{-6} per gamete - , one expect only one mutation in 500,000 plants (Kovalchuk et al., 2003). So, mechanisms explaining this rapid adaptation are more likely linked to epigenetic processes. To confirm this hypothesis, Kovalchuk et al. investigated the DNA methylation pattern in two generations of pine trees, using the AFLP method. They observed that the genomes of the young trees planted on the contaminated soil were the most methylated, while for trees grown in clean soil from exposed seeds were not significantly different from the control, suggesting that radiation levels received during the somatic development, but not as seeds, was governing the genome methylation. Moreover, the increase of methylation in pine trees was dependent on the received dose, as parental pine trees that received a higher total dose had a more methylated genome than the second generation.

In the progeny of *Arabidopsis sp.* sampled in three consecutive years in the CEZ from areas with different levels of contamination, a higher resistance in to mutagens compared to the control plants was observed (Kovalchuk et al., 2004). At a molecular level this difference in sensitivity could be attributed to differences in the expression of radical scavenging enzymes and DNA-repair enzymes and a higher level of global genome methylation in the CEZ-derived plants compared to control plants. It was hypothesised from these data that epigenetic regulation of gene expression and genome stabilization are involved in the complex

processes underlying the adaptation of *Arabidopsis* to ionizing radiation (Kovalchuk et al., 2004).

Animals

The International Radioecology Laboratory accumulated data confirming that, in spite of very high doses of complex radiation exposure (on the average, about 30 - 40 mGy d^{-1}), small mammals that had been inhabiting the most contaminated areas of the CEZ for tens of generations did not show any signs of depression and, in comparison with related populations from the control areas, had a higher, but not lower genetic diversity (Matson et al. 2000; Baker et al. 2001). At the same time, the radiation exposure that exceeded the recommended maximum allowable level of 1 mGy d^{-1} (IAEA 1992) for the wild fauna by factors of ten caused an increase in heteroplasmy of mitochondrial DNA, but this increase was insignificant relative to a spontaneous natural radiation level (Wickliffe et al. 2002). It would be logical to assume that either wild animal species (specifically, *Myodes glareolus*) acquired certain mechanisms of an adaptive response, successfully compensating a toxic impact of radiation, or they had an evolutionally preconditioned increased radioresistivity. However, using in similar experiments a laboratory mice strain with an increased radiosensitivity provided the same result (Wickliffe et al. 2003a). In addition, the laboratory mice as a well-studied test system of genotoxicological impact of radiation did not show a high frequency of point mutations being exposed to 3 Gy during 90 days under the conditions of the "Red Forest", the most contaminated area of the ChEZ (unlike an acute irradiation of the same dose) and did not differ from the control group (Wickliffe et al. 2003b).

Bank vole collected in 1997–1999 in Chernobyl exhibited increased mitochondrial DNA haplotype and nucleotide diversity (Matson et al. 2000; Baker et al. 2001). Samples from additional years (2000 and 2001) were incorporated into the study. Hypotheses that it was caused by increased maternal mutation rate and/or source-sink relationships were not supported by the study results. It appear to be explained by ecological dynamics in populations regardless radioactive contamination of the environment (Wickliffe et al. 2006).

A parallel assessment of cytogenic effects of *Myodes glareolus* species inhabiting the "Red Forest" showed that the frequency of micronuclei in their blood cells did not differ from that in a control group (Rodgers and Baker 2000). However, this fact did not result from any special mechanisms of reparation that this sub-population could have developed during tens of generations because an introduction of bank voles into the "Red Forest" did not cause any cytogenetic effects that would have exceeded the effects caused by natural spontaneous radiation levels (Rodgers et al. 2001a). Only an insignificant reaction of the adaptive response was detected, indicating an impact of the radiation stress, and that the body systems were being overcome by it. Similarly, exposure of a radiosensitive strain of laboratory mice under the conditions of the "Red Forest" during 30 – 40 days showed that the existing doses (up to 40 mGy/d) did not cause any unequivocal impact on the chromosomes and only resulted in an insignificant increase of frequency of the micronuclei (Rodgers et al. 2001b). In addition, no dependence between variations in the heterochromatin position and radiation levels was detected (Wiggins et al. 2002).

One more area of the studies devoted to the consequences of radiation influence was the assessment of an epigenetic indicator of development instability, i.e., a value of the fluctuating asymmetry (FA) for bilateral organisms. Since the FA is often used in ecotoxicological studies, it was decided to determine to what extent the development of small mammals deviated from the symmetrical one under the conditions of the CEZ. Study of *Myodes glareolus* and *Sylvaemus flavicollis* species as examples showed that, although deviation from a stable bilateral development took place in the entire territory of Ukraine, the FA values were significantly higher in those areas of the CEZ where the radiation doses exceeded 3 – 7 mGy d^{-1} (Oleksyk et al. 2002, 2004). However, on other areas of CEZ the FA values did not differ from the natural level. Since these two species were shown to have differences in their responses to the radioactive contamination of the environment, the contamination was unlikely the dominant factor causing an increase of the FA values. Frequency of DNA strand breakage in amphibians (*Rana terrestris)* inhabiting areas with various radioactive contaminations also appeared to be inconsistent (Jagoe et al. 2002). If short DNA strand breakage fragments prevailed in animals on the most contaminated areas, long fragments were found to prevail in relatively "clean" areas.

Since, on the one hand, the above mentioned studies did not meet wide spread expectations and did not demonstrate an unambiguous dependence of the frequency and type of genetic effects on the radiation conditions, it was decided to verify a hypothesis that the identified effects resulted from some other non-radiation ecological factors and/or the legacy of an earlier natural historical development of these populations; specifically, whether a higher level of the genetic diversity found in bank voles (*Myodes glareolus)* in the "Red Forest", resulted from their long-term immigration from the adjacent territories to the area where the original population had died due to a severe radiation exposure. In addition, the patterns of changes of their genetic diversity in a larger area were evaluated. It was indeed determined that indicators of the genetic diversity (frequency and uniqueness of haplotypes and frequency of nucleotides) were not a function of the radioactive contamination of the area, but they depended on a distance between subpopulations, geographic barriers and time of sampling of the genetic material during cycles of the population "life waves" (Meeks et al. 2007). The indicators of the genetic diversity reflected some intra-population processes and natural history of each population. Moreover, the genetic diversity of the *Myodes glareolus* species in Ukraine also appeared to vary significantly, and, consequently, genetic features of the vole's sub-population in the CEZ do not differ from those attributed to this species overall (Meeks et al. 2009).

Invertebrates

Due to high levels of exposure, soil invertebrates experience some of the highest doses of all organisms following nuclear accidents. Significant changes in population levels of soil invertebrates have been documented after both the Chernobyl and Khystym accidents (IAEA, 2006). Two months after the Chernobyl accident, the density of invertebrates living in the forest litter was reduced by a factor of 30 in the few kilometers around the NPP, and reproduction was strongly impacted (Krivolutsky and Pokarzhevskii, 1992; Krivolutsky et al., 1992). The high doses had dramatic effects on the invertebrate communities, including reproductive failure in adults. Within a year, the reproduction of soil invertebrates in the forest resumed, due in part to migration from less contaminated areas. After 2.5 years, their

immature/adult ratio and total biomass were not different from less or non-contaminated sites, but the species diversity remained markedly lower (Jackson et al., 2005). Compared to invertebrates within the forest-litter layer, those residing in arable soils inside the exclusion zone were not as strongly impacted. There, a four-fold reduction only in earthworm number was found. In parallel, it has been claimed that deposition in Belarus strongly affected the diplopods and the earthworms during the period 1986-2001 (Maksimova, 2002a; 2002b). Despite the clear documentation of effects, and the recovery of populations (IAEA 2006), there has been very little work looking at the mechanisms behind the observed changes in population densities and recovery of the populations.

More recently, the Fukushima Dai-ichi nuclear power plant (NPP) accident has impacted a large area in Japan, including a 80-km Nord-West area contaminated from radionuclide deposition. pale blue grass butterfly *Zizeeria maha* was used as an indicator species to evaluate the environmental impact of ionizing radiations (Hiyama et al., 2012). Mild morphological abnormalities were observed on some individuals of adult butterflies collected in May 2011 but an increase of the severity of these abnormalities occurred in the F1 generation, which were inherited in the F2 generation. These abnormalities may be explained by random mutation on important genes or by epigenetic mechanisms. None of these underlying mechanisms were studied by these authors.

Therefore, the studies presented in this paragraph reinstated a conclusion that the international communities have to revise their approaches to risk assessment of low dose exposures. The linear dose effect model developed in the experiments with an acute irradiation and at usually high doses (> 0.1 Gy) does not appear to reflect processes that occur in those biological systems that exist in conditions of a chronic complex radiation exposure, which is especially true for the wild biota and doses that do not exceed the natural background by factors of 100 – 1000. However, the mechanisms that make it possible for the biological systems to successfully exist in the conditions of such radiation exposure require further studies to provide a baseline for the development of new approaches such as epigenetics to the risk assessment for both wild biota and humans.

3. Initial Research Activity

3.1 Aim of initial research activity

The selected focus of the IRA is on the possible contribution of mechanisms governing the activation or the repression of the epigenome to long-term or transgenerational effects of organisms exposed chronically to ionising radiation, using hypothesis-driven research. Epigenetic modifications will be investigated among other more general biological parameters, as potential biomarkers for radiation exposure. The way these changes impact phenotypes and fitness for current and future generations will be analysed.

Following key research hypotheses will be addressed :

-H1: Exposure to gamma-irradiation results in a changed radiosensitivity (or resistance) within and over generations

-H2: Epigenetic mechanisms underlie changes in radiosensitivity (or resistance) observed in gamma-irradiated organisms

-H3: Epigenetic changes observed in gamma irradiated organisms grown under controlled lab-conditions are similar to epigenetic changes observed in areas affected by a nuclear accident

The IRA will be performed via (i) laboratory controlled exposure experiments (WP4 Subtask 4.1.1) to test hypotheses on the role of epigenetic changes in the alteration of physiological functions; this research will be conducted on laboratory models with fully sequenced genome and will include at least one species relevant to both ALLIANCE and MELODI; (ii) field studies on autochthonous species (WP4 Subtask 4.1.2) in part similar to the laboratory test species to investigate epigenetic changes in wildlife within contaminated areas (Chernobyl exclusion zone, Fukushima 100-km area).

3.2 Description of Initial Research Activity

The research outline presented above involves different species, both field and laboratory exposure conditions, and connection to human radiobiology. It can only be achieved by close collaboration between different groups in a multidisciplinary setting. The demonstration of the integration efficiency will be tested through the adoption of common protocols and methods for data acquisition and interpretation for both lab-conducted experiments and field studies. The global strategy adopted between the COMET partners to conduct these experiments is presented, together with a rationale for selection of exposure conditions, biological models and endpoints. The present reflection on the IRA is 'work in progress' and will also be submitted for review by external experts in field of epigenetics and transgenerational effects. A more detailed report on the exact protocols and experimental design will also be written for milestone MS4.1 (“proposition of biomarkers of epigenetic changes for *Arabidopsis*, zebrafish, earthworm, frogs”, delivery date month 18).

3.3 Experimental plans

The WP4 IRA covers both laboratory and field studies. While the overall aim is that the lines of research should be complementary, there are some distinct objectives, issues and challenges for the two types of studies. These are described below, together with an outline of the chosen approaches for the various study organisms and experiments.

3.3.1 Sets of selection

3.3.1.1 List of candidate species

Multiple criteria must be used to select the appropriate species to run this research program. These criteria were discussed in the first meeting of WP4 in Trømso in June 2013 (see Minutes in Annexe 2 and 3). They are presented in Table 2 and explained below:

Sequenced genome: organisms having a sequenced genome offer a range of advanced-methods to study epigenetic mechanisms in detail, as listed in section 2.6 (e.g. methylated DNA microarray, methylated histones, sequencing of miRNAs). Moreover, a comparison of the degree of homology between the human genome and the non-human organism genome can be done, which offers a link to the radiobiology community.

Methylated DNA (CpG islands): DNA methylation will be a common epigenetic mark to be measured in this research program, as global DNA methylation can be quite easily measured using methods such as AFLP, even in organisms whose genome is not sequenced. However, the levels of DNA methylation is not equal for all organisms and some organisms have undetectable levels of methylation (Tweedie et al., 1997). The extent of DNA methylation can be directly measured or it can be predicted - in organisms having a sequenced genome - from genomic analyses (e.g. frequency CpG islands for vertebrates).

Biosensor: mutant organisms are useful for many kinds of studies, such as e.g. unravelling mechanisms at basis of human diseases. Due to the high homology of some biological models with humans, hundreds of phenotypic mutants may be produced and applied as biosensors to assess real-time effect of pollutants (e.g. for the zebrafish see Lee et al., 2012).

Methods to study epigenetics applicable: this criterium is divided into three different categories: DNA methylation, histone methylation and miRNAs. These 3 methods refer to major factors/pathways involved in the epigenetic changes. Methods have already been developed to study these mechanisms for several model species.

Applicability to laboratory vs field studies: some model organisms can be very useful in the laboratory to unravel molecular mechanisms involved in epigenetic studies and transgenerational effects, but they may be more difficult to use in field studies.

Study of reproduction possible in the laboratory: as the research program is focused on the links between epigenetics and transgenerational effects, it is important to select model organisms that can reproduce easily under lab conditions. These issues may be less important for the field as it is more difficult to study transgenerational effects in the field, unless semi-field experiments are used with encaged organisms for example.

Table 2. Candidate organisms for IRA and their suitability according to the criteria. Field refers to Chernobyl or Fukushima.

Species	Sequenced genome	Methylated DNA (CpG islands)	Biosensor can be developed	Methods to study epigenetics applicable			Applicability to laboratory vs field studies	Study of reproduction possible in the laboratory	Possibility to study tissue sensitivity	Possibility to address gender specificity
				DNA methylation	Histone methylation	miRNAs				
Zebrafish (*D. rerio*)	Yes	Yes	Yes	AFLP/HPLC	Yes	Yes	Lab ~~field~~	Yes	Yes	Yes
Frog/toad (*H. Japonica, Rana/Bufo sp.*)	No	Yes	No	AFLP/HPLC in development for *H. Japonica* at IRSN	n.d.*	No	~~Lab~~ Field	No	Yes	Yes
Frog (*Xenopus tropicalis*)	Yes	Yes	No	AFLP, DNA transferases levels	n.d.*	n.d.	Lab	Yes	Yes	Yes
Birds (Great tit)	Yes	n.d.*	No	AFLP/HPLC in development at IRSN	Yes	Yes	~~Lab~~ Field	Yes	Yes	Yes
Rodent (Bank vole)	Yes	n.d.*	n.d.	AFLP/HPLC	Yes	Yes	Lab Field	Yes	Yes	Yes
Earthworm (*L. rubellus*)	Yes	Yes	No	AFLP/HPLC	Yes	Yes	Lab Field	Yes	Yes	No
Nematode (*C. elegans*)	Yes	No	Yes	no	Yes	Yes	Lab Field	Yes	No	yes (in some lines)

Table 2. Continued. Candidate organisms for IRA and their suitability according to the criteria. Field refers to Chernobyl or Fukushima.

Species	Sequenced genome	Methylated DNA (CpG islands)	Biosensor can be developed	Methods to study epigenetics applicable			Applicability to laboratory vs field studies	Study of reproduction possible in the laboratory	Possibility to study tissue sensitivity	Possibility to address gender specificity
				DNA methylation	Histone methylation	miRNAs				
Arabidopsis thaliana	Yes	Yes	Yes	AFLP	Yes	Yes	Lab Field	Yes	Yes	No

*n.d. : no data

Possibility to study tissue sensitivity: different epigenetic changes may be observed in tissues, having various implications for organ function and hence, biological function (e.g. reproduction for gonads or behaviour for brain).

Possibility to address gender specificity: different epigenetic changes were observed according to the model species sex (e.g. as shown in rats exposed to vinclozoline by Anway *et al*., 2005). This has important implications for transgenerational studies.

Based on this table, a first a priori-choice of model organisms can be done. For example, as no 5' cytosine methylation has been identified in the nematode *C. elegans* (although one recent paper has found DNA methylation in very discrete developmental stage of this worm, Gao et al., 2012), it jeopardizes the chances to observe any change in methylated DNA and hence, it cannot be used here as a model organisms for this research program.

A first set of biological models can be selected, belonging to the major groups of non-human organisms :

-vertebrates :

Zebrafish (*Danio rerio*) is a small tropical freshwater fish having a short generation time of approximately three to five months, allowing several generations to be studied within a reasonable time and a high fecundity (single female can lay up to 200 eggs per week). Adults are only approximately 3-5 cm long, so that they can be easily managed in large numbers in the laboratory at low costs and while minimizing the quantities of chemicals to deploy and the volumes of potentially hazardous wastes.

The development of the zebrafish is very similar to the embryogenesis in higher vertebrates, including humans, but, unlike mammals, zebrafish develop from a fertilised egg to an adult outside the female in a transparent egg so that developmental stages can be followed easily in vivo, unlike in mammals, where embryos have to be harvested.

Recently the whole genome of zebrafish has been sequenced (Sanger Institute, http://www.sanger.ac.uk/Projects/D_rerio). It is even more complex than the human genome, as zebrafish have two more pairs of chromosomes than humans. Moreover, due to some specificities of its genome, analyses of gene function in mutant zebrafish can be done that would be difficult to achieve in mutant mammals due to the associated embryo mortality (Hill et al., 2005; Spitsbergen and Kent, 2003). It is largely used in human biomedicine, as such, it could be a common biological model between ALLIANCE and MELODI platforms. Zebrafish is also a fish model for aquaculture, being closer to trout genome than other cyprinids because of evolutionary reasons (Volff, 2005). Besides the application of embryos, juvenile or adult zebrafish, cell lines may be used to completely avoid animal testing (e.g. embryonic cells such as ZF4).

Several frog and toad species have been studied to assess radionuclide contamination and effects, both in the field and in the laboratory (e.g. Panter, 1986; Jagoe et al., 2002; Audette-Stuart et al., 2011; Stark et al., 2012). The western clawed frog *Xenopus tropicalis*, an aquatic pipid frog species, is an important model for developmental biology and toxicology. It exhibits the experimental advantages of its relative the African clawed frog *Xenopus laevis* such as large eggs and transparent larvae but is smaller in size, thus requiring less laboratory space, and has a diploid genome as opposed to the allotetraploid genome of *X. laevis*. *X.*

tropicalis has a short generation time of 4-6 months which makes it possible to study reproduction and multigenerational effects of ionizing radiation within a reasonable amount of time. Recently, the genome of *X. tropicalis* was sequenced (Hellsten et al., 2010). The genome of *X. tropicalis* is relatively small with about 17 Gbp on 10 chromosomes and resembles the genomes of humans and chicken. The number of studies on DNA methylation in *X. tropicalis* is constantly increasing (Moffat et al., 2012). Thus, *X. tropicalis* constitutes an excellent laboratory vertebrate model organism for this IRA, however, for field studies another species would have to be chosen because *X. tropicalis* is native to Sub-Saharan Africa. The Japanese species *Hyla japonica* has been sampled in the Fukushima prefecture recently by IRSN and it can be easily found in rice paddy fields. Other possibilities is to study common species of *Rana* or *Bufo* that can be found in both the Chernobyl and Fukushima areas.

Birds have been studied in the field either in Fukushima or in Chernobyl. Among them, Great tit is particularly easy to catch in the field using nest boxes, which makes it a good field model. Its genome is also sequenced, allowing various tools to be studied. Finally, a review paper has been recently published highlighting the potential for epigenetic studies to improve the knowledge on environmental vs maternal factors (using cross-fostering studies) or to improve the performance of individual animals through changes in breeding conditions for birds (Frésard et al., 2013). Some issues were raised such as 1) how does the embryonic environment influence the adult phenotype in avian species? , 2) Does the embryonic environment have an impact on phenotypic variability across successive generations ?

Rodents such as the bank voles are also good field models because they are easy to catch using dedicated traps, and due to their life style in soils they are exposed to high dose rates. They have been largely studied in the CEZ, and adaptive effects have been evidenced, probably involving epigenetic changes rather than genetic ones (see section 2.5). Moreover, there is a collection of skulls of small rodents and shrews caught in Chernobyl exclusion zone over 1994–2011 period and referenced with description data (date, location, species, age, gender, generic morphological measurements, generic state, concentrations of ^{137}Cs and ^{90}Sr). They could be used to study instability of the skulls development (asymmetry, epigenetic markers) in conditions of multi-factorial influence (doses, seasons, gender, etc.). However, the study of this biological model requires researchers with specific skills adapted for rodents, and no one in the COMET WP4 group has got experience with molecular method development in rodents. But as a rodent species, this biological model could a common organism between the ALLIANCE and the MELODI platforms. This species could be proposed as a surrogate species in the framework of the call for proposals to be launched at the end of the year 2013.

-Invertebrates :

Earthworms

Earthworms are important test organisms in ecotoxicology, as well as a key species in terrestrial ecology. They have a high level of DNA methylation, the genome of *Lumbricus rubellus* has been sequenced, and a variety of methods for studying epigenetics have been developed (and applied by COMET partners). While not globally ubiquitous, *L. rubellus* is

often found at field sites, and adaptation of methods to other field species is feasible, as are other biomarker analysis, including tissue specific methods. Multi-generation studies are possible in the laboratory, and the variety of reproduction strategies in different earthworm species would allow for more focused, hypothesis driven, mechanistic studies. Living in soils, they are often exposed to high levels of pollutants, and population level effects were documented after both the Mayak and the Chernobyl Accidents (IAEA, 2006; Krivolutzkii *et al.*, 1992). Reproduction effects have been reproduced in laboratory studies on *E. fetida (*Hertel Aas *et al*., 2007, 2011). Field studies on metal polluted sites show that they can develop tolerance and adaptation to environmental stress, and recent studies have suggested that epigenetic mechanisms might play a role in phenotype adaptation to metal exposure (Kille *et al.*, 2012). This also opens for multiple stressor investigations.

-Plants :

Arabidopsis thaliana, commonly known as thale cress, is a dicotyledonous plant belonging to the mustard (*Brassicaceae*) family that also includes a number of important food and feed plants *e.g.* broccoli, cauliflower and oilseed rape. *Arabidopsis* is a relative small, self-fertilizing annual plant with a rosette forming leaves and flowering stems. It is native to Europe and Asia and ubiquitously present with a preference to more sandy soils. It is easy to grow under lab-conditions with standard protocols available (Smeets et al., 2008). It has a relative short life cycle of about 6 weeks from germination to seed setting and with prolific seed production enabling multigenerational studies. In 2000 its relatively small genome (~150 Mbp) was completely sequenced and since then completely annotated. In addition, a large number of specific mutant lines have been generated and are available from stock centres, enabling mechanistic studies. All genetic information is publicly available and can be searched on different genomic resource platforms such as “the Arabidopsis Information Resource” (TAIR, http://www.arabidopsis.org/index.jsp). All these have made *Arabidopsis* a model organism for studies of the cellular and molecular biology of flowering plants. Additionally, from an epigenetic point of view gene expression in *Arabidopsis* is known to be regulated by different epigenetic processes including methylation and protocols like AFLP but also methylation sensitive high throughput sequencing are available.

3.3.1.2 Common method to study epigenetics

In this COMET WP4 research program we want to demonstrate the integration efficiency through the adoption of common protocols. To ensure the consistency of used protocols for performing experiments in different laboratories, it has been decided to study DNA methylation using the AFLP method, as one common method, since this can be applied on a wide range of species including those without genome sequence. A check of repeatability and reproducibility of this method will be done intra-laboratory and experience of the different laboratories will be shared. Moreover, an inter-comparison exercise will be performed by sharing biological samples between laboratories. This will ensure robustness of the implemented method.

3.3.1.3 Radiation stressor

The radiation stressor chosen is gamma irradiation, as field sites in Chernobyl and Fukushima share a common contamination with gamma (and beta) emitters (^{137}Cs, ^{134}Cs), even though in Chernobyl other emitters may contribute to the total dose (^{90}Sr, alpha emitters). In addition, several laboratories within the COMET consortium and within WP4 have an external gamma irradiation facility for chronic low level exposure.

Experimentation design has to be appropriate for laboratory and field studies. Both types of experiments will deal with chronic exposure conditions, involving designs allowing continuous exposure of organisms during a significant length of their life or during particularly sensitive early life stages.

The exposure conditions will have to be chosen in order to be able to build dose(rates)-effects relationships or at least to compare a low and high dose(rate). These dose(rate) ranges do not have to be exactly the same for all the model species because of their different sensitivity but they should remain in the order of low doses (from background levels to a few hundreds of mGy/h), possibly including the generic screening value of 10 µGy/h recently proposed as a benchmark for ecological risk assessment (Garnier-Laplace et al., 2006).

External gamma irradiation offers the advantage of being able to expose organisms to a given dose(rate), without the complexity of integrating biokinetics. Dose(rates) are generally chosen after a first step of dose calculation (e.g. using MCNP) for the identification of the exact location of any experimental unit in the irradiation chamber to obtain the same dose(rate). UMB (Norway), SCK•CEN (Belgium) and IRSN (France) are equipped with adequate tools to specify the external irrradiation designs from *ca*. 0.04 to 40 mGy/h.

For the field experiments, sites will be chosen according to the habitat needed for the biological model (e.g. rice paddy field for frogs), the gradient of dose rates needed, the homogeneity in potential confounding factors. Despite this *a priori* choice of sampling sites, an important amount of work will be dedicated to dose assessment, as the total dose (internal plus external) may be different from background radiations. It must be underlined that this dose calculation must be performed in a homogenous way between the different laboratories.

3.3.2 Global strategy for the experiments

Experiments will be performed in the laboratory under controlled conditions and in the field. Laboratory controlled conditions allow to study the effect of the exposure to external irradiation while avoiding or limiting the number of other confounding variables. Different dose ranges can be chosen and laboratory model organisms can be used to unravel the molecular mechanisms involved in epigenetic changes and transgenerational effects. As regard to field studies, they are used for an application to more realistic conditions, but a real validation of laboratory studies using in situ experiments is not expected, due to differing exposure conditions, confounding factors, lower fitness of organisms in the natural environment and indirect effects. The laboratory studies are focused on a thorough understanding of molecular mechanisms underlying epigenetic and transgenerational

changes while field studies aim at observing those mechanisms occurring in a natural environment contaminated by radionuclides.

3.3.3 Laboratory studies

Laboratory studies can be divided into: 1) method development work and (2) mechanistic studies.

3.3.3.1 Method development

Method development will be done for each of chosen model organism selected. It will focus on global DNA methylation, and where appropriate supporting methods such as deep sequencing, barcoding, etc… The overall aim is to demonstrate the feasibility, applicability, robustness and sensitivity of the methods for laboratory and field studies. These studies may include focused new experiments as well as use of archived samples from previous exposure studies.

3.3.3.2 Mechanistic studies

The most relevant mechanistic studies into possible epigenetic markers and effects require model organisms with a sequenced genome, as well as sufficiently short life cycles to enable studies on multiple generations. Experiments could include studies of the persistence and hereditability of a variety of epigenetic markers (i.e. DNA methylation), the influence of life-stage, and correlation of epigenetic markers with other biomarkers of exposure or effect, such as reproductive output. These experiments will be performed at doses covering at least two doses (low and high), and spanning the range from expected field doses to doses known to induce effects in the selected species. Although such studies would be possible with several of the chosen model organisms, within the timeframe of the WP4 IRA, the most appropriate organisms for these types of studies are zebrafish and *Arabidopsis*.

3.3.4 Field studies

The first prerequisite to conduct field studies on epigenetic changes is to have a validated and robust method to measure epigenetic endpoints. That is the reason why the field studies have been planned later in the course of the program, in Chernobyl in 2014 and Fukushima in 2015.

The following issues will be of major relevance to the eventual field studies:

- Site descriptions
- Information on dosimetry and radionuclide concentrations (soil and if available site organisms)
- Site characterization (chemical, geochemical, biological, ecological and community level parameters)
- Available data on ecological status (e.g., population diversity studies, barcoding and metabarcoding)
- Other biomarkers to be included (e.g., cytogenetic, DNA damage, …)
- Dose response (gradient) and reference site
- Pre field study visit to check types and numbers of species (to check if there are the right kind of organisms, and enough of them)

- Type of biological and soil samples can be exported/imported
- Field sampling strategy to preserve DNA/RNA (flash freezing or AllProtect)
- Sampling timing (the best time can vary with species, so there will be a need to co-ordinate)

Site data will need to be gathered as part of the planning, including a prior site visit for both locations, necessary to gain insight into the types and abundance of test organisms, as well as scoping for study and reference sites. This would be combined where practicable with other missions or activities at the sites. Different dates are being chosen in order to share the partners experience on field studies in Fukushima and Chernobyl. A meeting between the WP4 members could be planned for the end of the year, in a place easy to reach (Paris, Brussels), or in April, in parallel of the Chernobyl training course.

Prior to that meeting, work must be done in link with the observatory sites that are being chosen within WP3 of COMET. This choice of observatory sites is highly linked with the choice of field sites for WP4. As such, a list of parameters governing the best choice of field sites has to be provided. To do that, a questionnaire will be sent in October 2013 to all the WP4 team in order to choose some *a priori* parameters to better focus field sites. This list will then be sent to the Ukrainian and Japanese partners. This will allow a first a priori choice of the candidate field stations, both in Ukraine and in Japan.

3.4 Experiments description

3.4.1 Vertebrate model : zebrafish

Laboratory studies

Husbandry conditions

Wild type zebrafish from AB (specific strain of zebrafish) background are raised and periodically purchased from a French supplier (Gis AMAGEN, Gif-sur-Yvette/France). Fish are kept in a dark room with a light:dark period of 16:8 h in aquaria (Aquatic Habitat system) containing 10 L of continuously renewed artificial water, i.e., a mixture of salts and of water treated by an osmosis system. The dilution rate is adjusted in order to reach a total hardness of 100 mg/L CaCO3 and a conductivity of 300 mS/cm, and a pH range between 7.5 and 8.5. The water temperature is kept at 26+/-1° C. Adult fish are fed 3 times a day, 7 days a week, with Tetramin flake food supplemented with live daphnids (*Daphnia magna*) each two day. For developmental studies, embryos are obtained from spawning of 2 males and 1 female. The genitors are placed separately in a specific spawning aquarium, equipped with a mesh bottom to protect the eggs from being eaten. Spawning is then induced on the morning when the light is turned on. Half an hour after, eggs from each aquarium can be collected and roughly observed with a dissecting microscope to select embryos from 2 groups.

External gamma irradiation

At IRSN, the irradiation system is made of Cs-137 sources installed in incubators, where embryos and small organisms can be irradiated. Five nominal dose rates of: 0, 0.7, 7, 70, 750 mGy/day can be delivered (Annexe 1). This system is not appropriate for larger organisms or a too large number of adult fish.

The FIGARO facility at UMB consists of a 12 Ci Co-60 source that provides a continuous dose rate field from 3 Gy/h (at source) down to 400 µGy/h (lower with shielding) and allows simultaneous, chronic exposure of samples over the whole dose-rate field (Annexe 1). Within the COMET IRA, the facility is planned to be used for zebrafish studies as part of mechanistic studies, as well as method development for earthworm studies.

The dose rates to be studied will be chosen according to the sensitivity of the methods used and of the biological models. Preliminary experiments will have to be done to choose these exposure parameters.

Endpoint measurements

External irradiation of zebrafish will be done in two different ways, either at the embryo stage only, with a further observation of consequences on adults (F0 and F1) ; and/or at the adult stage, in order to determine the effect of gender and of the life stages.

The fish, exposed at the early life stage or later, will be sampled at various exposure time in order to measure a change in epigenetic patterns. Different complementary methods will be used :

- **DNA global methylation** using **(1) MS-AFLP method (Methylation Sensitive - Amplified Fragment Length Polymorphism)**. It is a fast and cost-effective method to get the information on the global methylation profile of a DNA sample. Four successive steps are applied :

  -genomic DNA extraction,

  -digestion by EcoRI and one of the isoschizomers MspI/HpaII,

  -amplification of subsets of genomic restriction fragments using PCR,

  -analysis of the number and the size of the amplified fragments by sequencing.

- **DNA global methylation** using **(2) HPLC-MS method (High-Performance Liquid Chromatography-Mass Spectrometry).** It allows the detection of the different chemicals forms of cytosine (including 5-methyl-cytosine (5MeC)) and other bases in extracted DNA.
- **Methylated DNA immunoprecipitation sequencing** will uncover the DNA methylome using ME-DIP-Chips (Methylated DNA immunoprecipitation Chips) zebrafish specific. The following steps are used :

  -extraction of DNA enriched of methylated DNA with antibody anti-5MeC.

  -combination with PCR to increase the amount of DNA.

  -hybridization on microarrays to determine DNA-methylation within specific regions.

- The **consequences of DNA methylation on alteration of gene expression** will be assessed using **transcriptomic** analyses by RT-qPCR and Western blot.

-Other more general biological endpoints, measured routinely at IRSN, will be applied such as :

- Reproductive ability : male and females will be paired and eggs will be counted each day, together with the embryo survival, hatchability and growth.
- Development : embryo development will be assessed according to standard morphological criteria (Kimmel et al., 1995).
- Tissue ultrastructure will be assessed using a Transmission Electronic Microscope
- Genotoxicity : single and double DNA strand breaks will be assessed using comet assay and gamma H2AX

All these complementary methods have been tested on zebrafish exposed to depleted uranium (Adam-Guillermin et al., unpublished results). The measured endpoints will allow to study at the same time global DNA methylation and hypo/hypermethylation patterns in promoter regions of selected genes, and to compare with other non-targeted effects (ultrastructural alterations, genotoxicity). These global and specific epigenetic markers will give new knowledge on epigenetic mechanisms potentially occurring in non-human organisms, that will be usefull as baseline data for field studies.

Complimentary studies will be carried out at IRSN and UMB, allowing for both a check of reproducibility as well as inter-comparison of DNA methylation methods (particularly AFLP) as well as a more cost-effective division of other biomarker analysis.

Field studies

Zebrafish is only found naturally in small rivers in parts of India, Pakistan, Bangladesh, Nepal, and Burma, or in the United States where it has been introduced. It can be used as a field model but only for ex-situ exposure, as done by Orieux et al. (2011), who encaged zebrafish close to an old cadmium mine. This method may be difficult to apply in CEZ or in Fukushima, but water samples could be taken back from these sites into the laboratory and used as exposure media. Another solution would be to compare data obtained in the laboratory with another vertebrate field model such as frogs.

3.4.2 Vertebrate model : frogs

Laboratory studies

With the aim to develop DNA methylation and biomarker methods, a laboratory amphibian model, *Xenopus tropicalis*, will be used because autochthonous species are difficult to keep in the laboratory. The following radiation experimental procedures are planned at Stockholm University. To begin with, a dose response relationship will be established for *X. tropicalis* tadpoles covering a range of acute radiation doses from low (50 mGy) to high (3 Gy). Effects endpoints, such as DNA damage assessed by e.g. micronuclei or DNA strand breaks, oxidative base damage, and epigenetic effects in the form of global DNA methylation will be developed and tested to function for *X. tropicalis*. This will allow the methods to be tested in this biological model. Once a suitable effect endpoint method has been developed a chronic low dose exposure experiment could be conducted to study ecologically relevant doses. However, for such an exposure experiment a facility as the one at CERAD would have to be used.

When the timing is right (spring), the same methods developed for *X. tropicalis* will be tested on a common *Rana* or *Bufo* species collected from an uncontaminated Swedish field site. Possibly there might be a need for some adjustment regarding the endpoint methods.

Later on, the developed methods from the lab experiments will be tested for applicability in field studies conducted in observatory sites such as the Chernobyl and Fukushima area. In the field studies most likely a common *Rana* or *Bufo* species will be studied.

First Lab experimental set up :

For epigenetic method development, ten replicates of young tadpoles in each treatment will be exposed to an acute dose of 0, 50 mGy, 0.5, 3.0 Gy. For a subset of five replicates per treatment blood samples will be taken and, for example, DNA damage and oxidative base damage will be analyzed directly after exposure in erythrocytes. In addition, global DNA methylation will be analyzed. The remaining five replicates will be kept until metamorphosis for study of growth rates and body size.

Field studies

Molecular markers can be used to detect genetic structure patterns and provide an indirect way to study dispersal rates and patterns gene flow among populations. In a study of the Pyrenean brook newt *Calotritonasper* (Amphibia: Salamandriae) in the French Pyrenees the use of genome-wide AFLP revealed a pattern of isolation by distance indicating long-term restriction of gene flow among drainages, between sites within a drainage, and between populations separated by less than 4 km (Mila et al., 2010). In contrast, this pattern was not detected when using mitochondrial DNA (mtDNA) sequence data. Mila et al. (2010) highlight the importance of using multi-locus approaches to detect gene flow patterns in natural populations of amphibians.
In comparison, in Anurans, AFLP surveys have found lower levels of structure with little differentiation among frog populations at local scale (Curtis and Taylor, 2003; Measey et al., 2007; Lowe et al., 2008). A study in Northern Europe of *Rana lessonae* detected differentiation only at large scales but also overlaps among regions (Snell et al., 2005).
These methods may be applied along with other more general biological methods, to frogs sampled from CEZ or Fukushima Dai-ichi impacted sites, on a gradient of radioactive contamination.

3.4.3 Invertebrate model : earthworm

Laboratory studies

Three types of studies will be linked to method development to develop a better review of the role of methylation in radionuclide exposures: 1) laboratory exposure experiments with short term exposure of selected species; 2) analysis of archived samples from previous chronic irradiation studies; 3) analysis of field site samples (from ongoing projects at other field sites). The overall aim is to target the method development in order to optimize the application in field species.

Laboratory irradiation studies Laboratory studies will include short term irradiation of *Lumbricus rubellus* to give samples for global DNA methylation and comparison with hypo/hypermethylation patterns in promoter regions of selected genes along with other

non-targeted effects. This will enable assessment of the sensitivity of the various techniques and quick test of the methods. Irradiation of up to one week at field relevant doses (to be decided when dosimetry data is available) and doses known to be linked to reproduction effects (ca 10 mGy/h). A similar irradiation scheme will then be applied to *E. fetida*. Providing sufficient replicates can be included, sub-samples of the irradiated worms can be kept for investigation of the persistence of DNA methylation. Analysis will include global DNA methylation analysis using biochemical or spectroscopic methods, low resolution genome wide DNA methylation assessment using a methylation sensitive AFLP method and some limited loci specific analysis. Another aspect that may be considered can be the role of short RNA molecules in gene regulation. This work is pending the improved assembly of a full genome sequence for *L. rubellus* which is being supported by a consortium involving NERC that is currently integrating long read single molecule sequence data with existing short-read data.

Archived Samples: Material is available from two long term exposure studies on *Eisenia fetida*. A variety of material is available, including different tissues/samples preserved with RNAlater @-20^{o}C.

a) A dose-response reproduction study (F0/F1). Total of 13 weeks continuous exposure at a range of 0.1 – 40 mGy/h. Clear reproduction effects (Hertel-Aas et al., 2007), and some additional studies on DNA damage using the Comet assay (Hertel-Aas et al., 2011a).
b) An adaptive response/recovery/adaptation study. Irradiation of 23 weeks covering F0/F1/F2. Reproduction effects induced at one of the dose rates used in (a) (Hertel-Aas et al., 2011b).

Investigation of global DNA methylation will start with samples from the second experiment, as it has samples from multiple generations. One of the F1 groups has irradiation of parents during embryogenesis but not during growth, maturation and reproduction, so there is material for studying the incidence of DNA methylation following irradiation, and its persistence in time.

Field studies

There was agreement for field studies in Chernobyl in 2014 and Fukushima in 2015. Site data will need to be gathered as part of the planning. However there was a consensus that a prior site visit for both locations would be necessary to gain insight into the types and abundance of test organisms, as well as scoping for study and reference sites. This would be combined where practicable with other missions or activities at the sites.

A first analysis of earthworm species at the COMET field sites has been done for the moment:

According to Blakemore (2012), there are 96 earthworm species belonging to eight families in Japan. The Megascolecidae is the dominant family of earthworms (58 species) in contrast to Europe where earthworms belonging to the lumbricid family dominates. Only 15 species of lumbricids have been reported in Japan, and *L. rubellus* is not included on the list (Blakemore 2012). *L. rubellus* have not been reported as found in the Chernobyl exclusion zone either. *Aporrectodeacaliginosa* (*Nicodriluscaliginosus*) and *Dendrobaeneaoctaedra*are two lumbricid species that have been found within 3 km from the Chernobyl nuclear power plant (Krivolutzkii *et al*., 1992; Krivolutzkii and Pokarzhevskii, 1992) and these species have also been reported to be present in Japan (Blakemore, 2012), although there is no available

information whether they can be found around the Fukushima nuclear power plant. In both cases, further information on available species should be gathered from the site specialists. In addition, field planning will include tests of the applicability of the epigenetic methods on the relevant field site species (see above).

3.4.4 Plant model: *Arabidopsis*

Lab experiments

All lab experiments will be performed on hydroponically grown *Arabidopsis* plants. It was chosen to do a 7 to 14-day gamma exposure with different dose rates (15-350 mGy/h) in the early life stages of every generation. Longer seed-to-seed exposures are at the moment not possible due to the internal demand for the gamma source. However, preliminary results indicated that the highest dose rate (350 mGy/h) induced significant growth changes (growth rate of shoot, number of flowering stems, number of flowers) within the mother generation. Hence, it can be expected that epigenetic changes will be available at this but also lower dose rates.

As gamma source ^{137}Cs source will be used. Within our experimental set-up *Arabidopsis* can be exposed to dose rates ranging from near background to maximally 350 mGy/h. Typically four different dose rate levels are used and a control. After exposure the recovery and further growth of the plants will be followed up and plants will be grown until seeding and seeds will be harvested as F1, F2 generation.

Seeds of maternal plants and F1 generation plants, recovered after a 2-week exposure (week 2 and 3 after germination) to different dose rates of gamma (15-350 mGy/h) are already available (Horemans *et al*. unpublished data). For gamma radiation at least one more (F2) generation of plants and seeds is foreseen, that will be used within this project. This will enable us to study the persistence of changes in methylation throughout generations.

Although AFLP has been described in the literature to study **global DNA methylation patterns** in *Arabidopsis*, implementation of this technique in the laboratories of SCK•CEN will be performed within this project. Additionally, we will study the expression of **specific miRNA's**, and if possible to identify histone modifications in primed and non-primed plants gamma exposed maternal plants or their offspring. We also wish to identify genes/proteins that show altered expression after exposure to radiation in order to pinpoint the mechanisms underlying the stress response. Recently developed techniques that enable identification of gene expression or proteins in a holistic way are rapidly becoming accessible. These techniques are based either on the partial purification and subsequent sequencing of methylated DNA or methylation sensitive next generation sequencing. Further analysis of the results and confirmation of the altered gene transcription through µ-array and/or quantitative RT-PCR will be performed. However implementation of these techniques will also be dependent on the available recourses. Finally, to further unravel the role of epigenetic changes in long-term effects induced by gamma radiation we can use the great number of knock-out mutants available in *Arabidopsis* that are deficient for example in post-translation histone modifications or in the production of miRNA or siRNA's. The sensitivity of

these mutants to radiation and the induction of long-term effects compared to the wild type will be assessed.
The epigenetic changes observed can also be compared to changes in **other endpoints** that we routinely measure in *Arabidopsis.* These include markers for growth, oxidative stress and DNA damage and repair:

- Growth will be analysed as biomass production.
- As oxidative stress markers different antioxidant metabolites (ascorbate and glutathione) and enzyme activities will be measured.
- To estimate the degree of DNA damage we will use
    - A cytological evaluation of Mitotic Index. This will be done in cooperation with the IUAR as they have the expertise to do these type of measurements
    - the ploidy of the nuclei, in plants a higher ploidy can indicate a cell cycle arrest a strategy known to be used by plant cells to allow for DNA repair mechanisms and limit DNA damage to daughter cells,
    - measuring the amount of oxidative DNA damage as 8-hydroxy guanine,
    - the degree of DNA fragmentation either by a comet assay or an in-gel system
    - and the expression levels of enzymes involved in DNA damage repair such as ligase IV, that performs ATP dependent ligation of DNA strands in the case of double strand breaks and Poly(ADP-Ribosyl)-polymerases (PARPs), enzymes that detect single strand breaks.

Field experiments

Site characterisation:

Field experiments are planned both in Chernobyl and in the Fukushima affected area with a time shift between them. The campaigns will start by doing a site characterisation. The objective here is to select a limited number of sampling sites that differ in degree of radiation but that are as similar as possible for other environmental parameters (soil type, vegetation, …). Site characterisation will include selection of a good reference site. This will be done by following a workshop as described in section 3.3.4 in order to further discuss the strategy among the partners.

Plant selection:

As indicated above (section 2.7.1 plants) *Arabidopsis* has been studied within the Chernobyl zone before and it has been suggested that the dose-dependent changes observed are epigenetic in nature (Kovalchuk et al., 2004). A dosimetry model specific for *Arabidopsis* was recently described that enables estimation of internal doses coming from e.g. ^{90}Sr in addition to the external dose (Biermans et al., 2013). Although this makes *Arabidopsis* the preferred species to perform lab as well as field studies within this project, due to its relative short generation time it might be difficult to sample it in the field. This especially for the Fukushima area where no previous studies on this species exist. Therefore we would consider additionally another commonly present flowering plant with a longer lifespan and known to be present both in Chernobyl as in Fukushima (for example a grass species or flowering primrose (*Oenothera*)) along with the lab exposure experiments to test global DNA methylation pattern and evaluate its use as plant species for the field campaign. *Oenothera*

could be a good complement to or alternative for (in case of non-presence of) *Arabidopsis* as experience with this plant is already available at IUAR were it was previously scored for DNA damage.

Sampling campaign:

Site characterisation will be followed with a sampling campaign to collect plant tissues from the different sites. It is the intention to process the plants as much as possible directly at the site or in a neighbouring lab. However, if needed some samples will be transported back to SCK•CEN to be further analysed e.g. for total DNA methylation patterns. The use of products to stabilize RNA and DNA (such as "AllProtect®, Qiagen) in order to preserve samples during shipping will be tested prior to the campaign. As only limited material will be available for testing and shipping is not always easy, not all endpoints can or will be estimated on the field samples. We will restrict ourselves to measure changes in global DNA methylation patterns in addition to biomass, and all necessary measurements for a good dosimetry. Additional endpoints will only be done if time, resources and samples allow.

3.5 Expected outcomes and alternative options

The expected outcome of these studies is to build a set of effect data on epigenetic changes observed in the laboratory under controlled exposure conditions or in the field on contaminated sites. Several outcomes are expected such as :

- Observation of epigenetic vs genetic effects in real (multi)exposure conditions, with animals in challenging conditions (nutrition, intra/interspecies competition …)
- Confrontation of results with the benchmark of 10 µGy/h (at least in situ)
- Comparison of laboratory and field studies
- Identification of a correlation between epigenetic tags and transgenerational effects

These studies will help the different teams involved in COMET to implement innovative molecular tools to radioecological biological models. The combination of skills, manpower and fieldwork logistics, will improve efficiency of the work planned.

There is a risk for the planned study to fail in generating such a dataset, due to the tests designs, the lack of sensitivity of the measured endpoints, or the effect of confounding factors in the field. In this case, alternative options could be :

- to increase the number of dose(rates) and/or of the number of tested replicates. To achieve this we will need to share the larger irradiation facility (e.g. irradiation facility before called CERAD, Norway).
- to examine other more sensitive endpoints
- to enhance the accuracy of endpoints measurements in order to decrease the variation due to individual variability
- to choose accurately the field sites by avoiding the more important confounding factors

4. Perspectives

This reports presents a comprehensive state-of-the-art on epigenetics and our first choices for the Initial Research Activity developed under WP4. Mechanistic experiments will be conducted aiming at unraveling the mechanisms and consequences of epigenetic changes, going from the study of global DNA methylation, gene and histone methylation, gene expression and physiological alterations. Two biological models have been chosen to conduct these experiments : one vertebrate model (zebrafish, *D. rerio*) and one plant model (*Arabidopsis thaliana*). The plant model will also be used for field studies in Chernobyl and in Fukushima. The other models that will be used in field experiments are vertebrate models (frogs and/or birds) and an invertebrate model (earthworm, *Lumbricus rubellus*).

The next step will be the meeting (Paris/Brussels at the end of the year or Chernobyl in April) to share the partner's experience with the chosen field sites and to begin the first pre-field studies, in order to go through the selection of field sites in CEZ and the site characterization process (KS4.2). The same approach has to be done for the Fukushima sites (KS4.5). A milestone (MS41) is also due to month 18, in the form of a report on biomarkers of epigenetic changes for *Arabidopsis*, zebrafish, earthworm and frogs, that will give first data on experiments performed in the laboratory.

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Annexes

Annex 1: Irradiation systems (IRSN & UMB)

Irradiation system of IRSN

At IRSN, the irradiation system is made of Cs-137 sources installed in incubators, where embryos and small organisms can be irradiated. Five nominal dose rates of: 0, 0.7, 7, 70, 750 mGy/day can be delivered Each source is installed in independent incubator and experimental units are placed around the source. During the test, temperature and moisture are continuously measured using data logger. The choice was made on the use of liquid and solid sources of Cs-137 of various activities. The experimental units (e.u.) of small size containing the samples to be irradiated are laid out in circle around the source of caesium. Table 3 gives the activities of the various sources used in the device of irradiation.

Table 3 : Activities of 137-Cs sources used in the irradiation facility

	Bq	Type of source
S1	1.6E+09	solid
S2	2.16E+08	liquid
S3	2.30E+07	liquid
S4	2.12E+06	liquid

To allow daily handling of the experimental units, a device allowing the lowering of a lead protection is dimensioned for the experiments. The support can accommodate two models different of protection:

• a cap thickness 20 mm, which could be used with the liquid sources of weak activity,

• a cap thickness 30 mm, intended for the handling of the liquid sources of strong activity as well as the solid sources. Figure 3 presents a diagrammatic view of the system of irradiation.

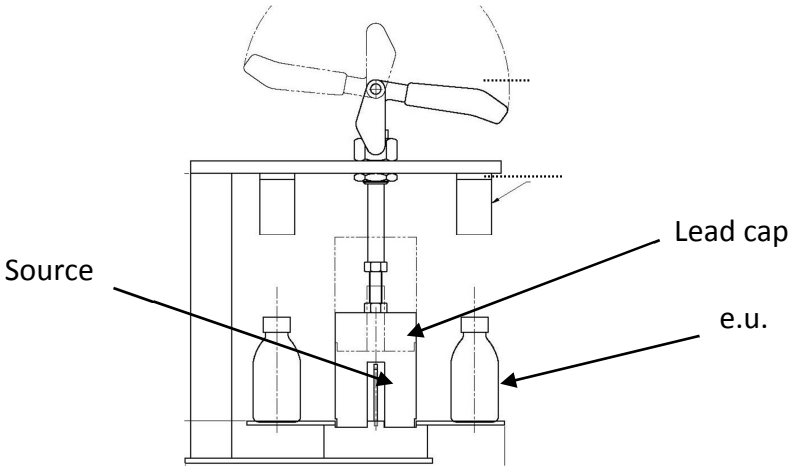

Figure 3: Scheme of the Irradiation facility

Thermoluminescent dosimeters (TLD) will be laid out along the lower, median and higher lines of the u.e for the various configurations given and the whole of the sources available.

The flows of Kerma were also calculated by digital simulation within the TLD in order to compare the calculation and results of measurement, and thus to validate the data on the activity of the sources. In complement, the isotropy of the radiation field on the level of the e.u. for the solid source was studied using TLD. Moreover, the absorbed dose rates inside the geometry of the e.u. were determined by calculation (modelling the transport and the interactions of the particles charged or neutral in the air. Only, the transport of the principal line of cesium 137 to 661.6 keV was taken into account).

Measurements will be realized with lithium fluoride powder doped Mg, Cu, P (GR207) conditioned in polypropylene tubes. For each configuration of irradiation and all the sources used, a series of dosimeters will be placed at the center of the e.u. on the various stages of the e.u. A number of e.u. identical to the use in routine was used so taking into account the radiation diffused by the adjacent samples of the site of the dosimeters.

Irradiation system of UMB

The FIGARO facility at UMB consists of a 12 Ci Co-60 source that provides a continuous dose rate field from 3 Gy/hr (at source) down to 400 µGy/hr (lower with shielding) and allows simultaneous, chronic exposure of samples over the whole dose-rate field (Figure 4). Within the COMET SRA, the facility is planned to be used for zebrafish studies as part of mechanistic studies, as well as method development for earthworm studies.

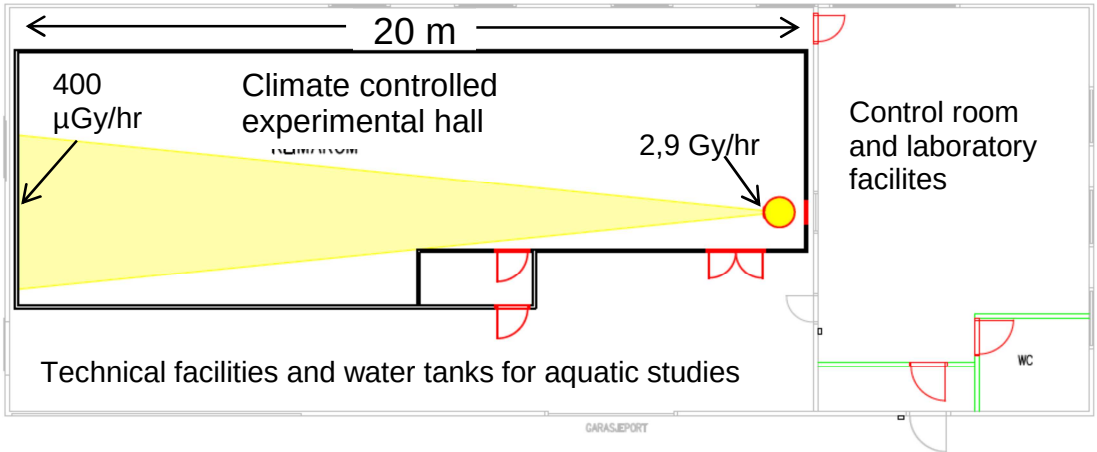

Figure 4. The Figaro Irradiation Facility at CERAD/UMB

The facility has been given approval for research on a number of organisms, including rodents, fish, aquatic and terrestrial invertebrates, as well as genetically modified organism (GMO) vertebrates. The facility is also authorized for radionuclide internal exposure (including alpha emitters), as well as other chemical stressors (e.g., metals, organics, nanoparticles) and UV exposure, thus permitting multiple stressor experiments. A

temperature and pH controlled flow–through system is available for aquatic organism exposures.

The climate control specifications for the experimental hall are:

Temperature: 4-37^{o}C (+/- 1 ^{o}C)

Light : ca. 50 - 300 lux with automatic dimmer (10 min)

Humidity: 45 – 65% (ScanClime)

Ventilation: 300 m^{3}/h

Annex 2: Minutes of WP4 Tromsø meeting, June 10 2013

MEETING MINUTES

WP4 MEETING

Tromsø, 06/10/2013

Organised by IRSN

ATTENDANCE

1. SCK•CEN	Hildegarde Vandenhove, Nele Horemans
2. STUK	
3. NRPA	
4. IRSN	Christelle Adam-Guillermin
5. NERC-CEH	Dave Spurgeon, Claus Svendsen
6. CIEMAT	
7. SU	Clare Bradshaw, Karolina Stark
8. BfS	
9. UMB	Deborah Oughton, Turid Hertel-Aas
10. GIG	
11. IRL	
12. NUBiP	
13. UnivFuk	

AGENDA

Discussion on the deliverable D4.1 from 17:30

AIM

To agree on the detailed outline of the deliverable and to begin to distribute the responsibilities for writing

MEETING MINUTES

Main issues which were discussed are related to the deliverable D4.1:

The detailed outline of the deliverable was proposed :

1 General context and objectives of the D4.1
2 State-of-the art on epigenetic changes
- Description of molecular pathways
- Link with biological effects

- Overview on methods available to characterize epigenetic changes
- Data and knowledge gaps on epigenetic changes induced by radionuclides

3 Aim of IRA
4 Description of Initial Research Activities
- Methods
  - Choice of methods to study DNA methylation
  - Choice of biological models : plants, animals
- Experiment description (lab, field studies)
  - Plants
  - Invertebrates
  - Vertebrates (fish, frogs)

5 IRA work plan
6 Add something on other researches in connection with the call for proposals ?

The discussion was developed according to this outline :

1. **General context and objectives** : presentation of the global context and aim of this task. Deborah Oughton (UMB/CERAD) proposed to add a separate section in the introduction part, on a comparison of the field of epigenetics as applied in biomedicine and human studies, with that in ecotoxicology.

Action : Deborah will send a summary of that section in order to include it in the introduction part

2. State-of-the art on epigenetic changes

Several reviews exist on this topic. Dave Spurgeon (CEH) proposed to summarize the main conclusions of this section in tables according to the sub sections of the 'state-of-the art' part. Based on this idea, at least 3 tables could be proposed.

Karolina Stark (SU) proposed to take part to the writing of the section 'link with biological effects'.

3. Aim of IRA

This section should describe the aim of initial research activity, and should highlight the main scientific issues to address such as to characterize the role of epigenetics in transgenerational effects and/or genomic instability and/or DNA repair and/or resistance-sensitivity-adaptation mechanisms. If the IRA will mainly be focused on epigenetic and transgenerational effects, the

other implications could be addressed in the framework of programs selected during the call.

4. Description of IRA

A discussion was launched on the exposure scenarios for laboratory studies. Some questions were raised such as :

-which dose range to study ?

-use of external gamma irradiation ?

-study of a whole dose-response curve or of at least 3 exposure treatments?

-should we try to identify a threshold level ?

-should we add other biomarkers than epigenetics, which one, and for what purpose ?

For field studies : as Chernobyl Exclusion Zone site characterization should begin after 12 month, and field studies after 25 month, it was decided to plan a workshop to share the knowledge on CEZ and Fukushima sites and to begin to organize field campaign by mutualizing efforts that can quickly become time and man month consuming. This could be done in parallel of one of the meetings planed at month 12 (in Ukraine, WP5).

Action: IRSN proposes a task allocation to everybody and a timing suggestion. These decisions will be made during the web conference on Thursday the 27th of June.

Table 1. Candidate organisms for IRA and their suitability according to the criteria. Field refer to Chernobyl or Fukushima.

Species	Sequenced genome	Methylated DNA (CpG islands)	Biosensor developable	Method applicable to study epigenetics			Applicability to lab vs field studies	Reproduction possible	Possibility to study tissular sensitivity	Possibility to adress gender specificity
				DNA methylation	Histone	miRNAs				
zebrafish	yes	yes	Yes	AFLP/HPLC	Yes	Yes	Lab ~~field~~	yes	yes	yes
Frog (*Hyla japonica*, rana species)	No	No	No	AFLP/HPLC?	?	No	Lab Field	?	yes	yes
Birds ? (Great tit)	yes	?	?	AFLP/HPLC	?	yes	~~Lab~~ Field	yes	Yes	yes
Earthworm (*L. rubellus*)	yes	yes	No	AFLP/HPLC	yes	yes	Lab Field	yes	yes	no
Nematode (*C. elegans*)	yes	no	Yes	no	yes	yes	Lab Field	yes	no	yes (in some lines)
Arabidopsis thaliana	yes	yes	Yes	yes	yes	yes	Lab Field	yes	yes	?

Table 2. Overview of data available on epigenetics in the field of ecotoxicology

Biomarker	Method/assay	Tested endpoint	Pollutant studied	Species tested	Exposure concentration Exposure time	Main result	Reference
DNA methylation	AFLP	Methylation of DNA using restriction enzymes					
…							

Table 3. Overview and advantages and drawbacks of different epigenetic approaches.

Techniques	Advantages	Limitations

Annex 3: Minutes of WP4 webmeeting, June 27 2013

MEETING MINUTES

WP4 MEETING

Webmeeting 08/28/2013

Organised by SCK-CEN

ATTENDANCE

1. SCK•CEN	Nele Horemans, Eline Saenen
2. STUK	
3. NRPA	
4. IRSN	Christelle Adam-Guillermin, Laureline Février, Sandrine Pereira
5. NERC-CEH	
6. CIEMAT	
7. SU	Francisco Nascimento
8. BfS	
9. UMB	
10. GIG	Bogusław Michalik
11. IRL	Sergey Gaschak
12. NUBiP	Vasyl Yoschenko ; Valery Kashparov
13. UnivFuk	

AGENDA

-Start of the meeting at 9:00.

-9:00 Presentation of WP4 (Christelle Adam-Guillermin)

-9:15 Presentation of each partner (organization, tools/facilities/field access)

-10:00 Discussion on the laboratory/field experiments

-11:00 Agree on timeline for D4.1 production and responsibility distribution for each section of the document

AIM

Presentation of WP4, of partners involved in WP4 and organization of the work to produce

MEETING MINUTES

Main issues which were discussed are related to the deliverable D4.1:

- ✓ **D4.1 outline :**

A presentation of WP4 was made by C. Adam-Guillermin summarizing the main aims of WP4 tasks, key-steps and deliverables and focusing on D4.1 (see file WP4 270613 Adam.ppt).

Table 1 : D4.1 outline and responsibility distribution

The detailed outline of the deliverable was proposed with the according propositions of responsibilities. These propositions were accepted by the different partners.

Section	Responsibility for section Nb.
1. General context and objectives of the D4.1	IRSN + UMB (1.)
1.1. comparison of the field of epigenetics as applied in biomedicine and human studies, with that in ecotox	
2. State-of-the art on epigenetic changes	
2.1. Link with biological effects	SU + UMB (2.1)
2.1.1. Cancer	
2.1.2. Embryonic development	
2.1.3. DNA repair	
2.1.4. Heritability	
2.1.5. …	
2.2. Description of molecular pathways	CEH+SCK-CEN (2.2)
2.2.1. Methylated DNA	
2.2.2. miRNAs	
2.2.3. Methylated histones	
2.2.4. …	
2.3. Overview on methods available to characterize epigenetic changes	IRSN+CEH (2.3)
2.3.1. HPLC-MS	

2.3.2. AFLP	
2.3.3. Methylated DNA chips	
2.3.4. Methylated histones	
2.3.5. Sequencing of miRNAs	
2.3.6. …	
2.4. Data and knowledge gaps on epigenetic changes induced by radionuclides	SU+IRSN+IRL+NUBiP (2.4)
3. Aim of IRA	IRSN+SCK-CEN (3.)
4. Description of Initial Research Activities	All (4.)
4.1. Methods	
4.1.1. Choice of biological models : plants, animals (in vitro/in vivo?)	
4.1.2. Choice of methods to study DNA methylation	
4.2. Experiment description (lab, field studies)	
4.2.1. Plants	
4.2.2. Invertebrates	
4.2.3. Vertebrates (fish, frogs)	
5. IRA workplan	All (5.)

✓ **D4.1 timing :**

The deliverable D4.1 is due to month 3, which goes to the 31st of August. It is proposed to start to write as soon as possible, between July and mid-August and to start to circulate the document in the beginning of August. A first complete draft version should be available from week 34 (19th of August).

✓ **Questions regarding the laboratory and field studies :**

- Regarding epigenetic methods to use :

DNA methylation (AFLP) was proposed as a common method to study epigenetic changes, as it can be applied to all types of model organisms, having or not a sequenced genome. Other methods could be used such as histone methylation or miRNAs.

It was proposed to organize an inter-comparison exercise of the AFLP method between the different laboratories in order to check the robustness of this method. This inter-comparison should be done after its optimization by the different partners (from month 36 ?).

- Regarding the strategy for laboratory studies :

Should we validate the chosen epigenetic biomarkers for a whole dose-response curve or for at least 3 doses? Should we address the question of transgenerational effects and on how many generations? Should we address only ionizing radiations or also another stressor ?

Bogusław Michalik pointed the importance of the choice of the dose and dose rate to study in the laboratory and in the field, in order to be able to compare both laboratory and field studies. This remark underlines the importance of having a good knowledge of dose rates to which organisms may be exposed in situ in Chernobyl and Fukushima. Nele Horemans noticed that laboratory and field studies do not have to be completely connected, as field studies will involve a different context (multicontamination by several radionuclides and stable pollutants). Moreover, laboratory studies are more focused on understanding the mechanisms in a simplified and controlled environment, as compared to observations in the contaminated environment including several confounding factors.

- Regarding the choice of the model species :

Vertebrates : IRSN is optimizing DNA methylation methods (AFLP and HPLC-MS in collaboration with the CEA Grenoble) on the zebrafish exposed in laboratory controlled conditions. The patterns of DNA methylation is being studied in two key tissues of zebrafish exposed to depleted uranium : brain and gonads. These tissues were chosen because they have been shown to be the most sensitive and because they are linked to major biological functions. This species allows to study the consequences of DNA methylation on gene expression; as such, a DNA methylated microarray is being conceived at the present time. These results will then be compared to those obtained in zebrafish exposed to gamma external irradiations. Other DNA damage biomarkers will

also be studied (DNA Single and Double Strand Breaks with the comet assay and □H2AX; ultrastructural abnormalities). In addition to having a sequenced genome, one of the strengths of this biological model is that the impact on different generations can also be studied in a reasonable time period ; it is also a largely used model in human toxicology.

For field studies, the frog was chosen as vertebrate model, due to their easy catching. Stockholm University is sampling different frog species at the moment to optimize biomarkers of epigenetic changes. They will then be used for Chernobyl field campaigns. IRSN has also sampled a frog species (Hyla japonica) as part of their Freebird program in Fukushima. DNA methylation will be studied on these samples.

Invertebrates : Earthworms. UMB has never measured DNA methylation, but has access to archived samples from sub-chronic exposure experiments (including studies with observed reproduction effects) from Turid's previous earthworm experiments. UMB should also have access to Komi fieldwork samples to be collected during the July 2013 expedition (providing fieldwork, export and import all goes to plan). These could be used to test/develop methods, prior to Chernobyl and Fukushima field trips. It is hence proposed to collaborate with CEH on pilot method development for DNA methylation in earthworms collected at Komi and to explore possibility of using archived samples from long-term, sub-chronic earthworm exposure experiments.

Other endpoints/biomarkers to be studied as part of the Komi expedition (as part of the EANOR/ERANET project): soil invertebrate macrofauna diversity (classical taxonomy and DNA barcoding); microorganism diversity and adaptation (to metal and gamma irradiation); plant diversity and biomarker analysis (Russian colleagues). RN analysis and soil chemistry. Soil samples will be collected for DNA metabarcoding (as part of field protocol development under CERAD).Biological samples from earthworms will be stored in AllProtect for DNA methylation analysis and other biomarkers, since we will not have access to liquid nitrogen in the Siberian field.

For laboratory studies, the proposed action plan is the following one, with some questions remaining :

- Literature review
- Biomarker calibration
- Links with community and transgenerational effects. Do we need to go to F3?
- Time for endpoints to kick in

- Adaption/adaptation studies ?
- Bystander and genomic instability studies ?

Plants : Arabidopsis (see attached presentation of Nele Horemans). Arabidopsis plants will be exposed to gamma external irradiation for two generations and transgenerational effects will be studied in terms of general stress (e.g. fresh weight, root length, seed production, gene expression…) and of DNA damage and repair (e.g. oxidative DNA damages, DNA DSBs…). The mechanisms underlying these transgenerational effects will then be studied by analysing : the degree of DNA methylation (in addition to classic methods like AFLP new techniques such as next generation sequencing and KO mutants will be used), microRNAs (q PCR), cytogenetic effects in collaboration with NUBiP (chromosomal aberrations, mitotic index), histone modifications (next generation sequencing). Next generation sequencing will be used to identify the genes that have e.g. changed methylation degree. It will only be applied if classic methods show a significant change in overall DNA methylation.

For field studies, it was underlined by NUBiP that some difficulties could arise to study arabidopsis in the field, due to their very short time appearance in Chernobyl. As NUBiP has got several data on the Scott pine (e.g. dynamics of DNA damages, morphological aberrations), this species could be used as a surrogate species.

- Regarding field studies: as Chernobyl Exclusion Zone site characterization should begin after 12 month, and field studies after 25 month, it was decided to plan a workshop to share the knowledge on CEZ and Fukushima sites and to begin to organize field campaign by mutualizing efforts that can quickly become time and man month consuming. A first workshop could be planed around month 6 or 7 (in parallel to Finland workshop ?), to share theoretical knowledge on field characteristics, field stations and gradients etc… It could be followed by a second one at month 12 or before, to begin Chernobyl field studies. UMB listed a strategy and key issues to address for field studies :
  - Site descriptions
  - Site characterization (chemical, geochemical, biological, ecological and community level parameters)
  - Measurements: e.g., population diversity studies, barcoding and metabarcoding
  - Other biomarkers ?
  - Gradient or reference site ?

- Pre field study investigation to check types and numbers of species (are there the right kind of organisms, and enough of them?)
- Sampling timing (the best time can vary with species, so need to co-ordinate)

As underlined by Sergey Gashchak, there is an optimal time slot for field studies in Chernobyl, which is around April and May. During the summer, lots of mosquitos and hot temperatures can be a problem.