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• Review Article

Epigenetic profiling to environmental stressors in model and non-model organisms: Ecotoxicology perspective

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Epigenetics, potentially heritable changes in genome function that occur without alterations to DNA sequence, is an important but understudied component of ecotoxicology studies. A wide spectrum of environmental challenge, such as temperature, stress, diet, toxic chemicals, are known to impact on epigenetic regulatory mechanisms. Although the role of epigenetic factors in certain biological processes, such as tumourigenesis, has been heavily investigated, in ecotoxicology field, epigenetics still have attracted little attention. In ecotoxicology, potential role of epigenetics in multi- and transgenerational phenomenon to environmental stressors needs to be unrevealed. Natural variation in the epigenetic profiles of species in responses to environmental stressors, nature of dose-response relationships for epigenetic effects, and how to incorporate this information into ecological risk assessment should also require attentions. In this review, we presented the available information on epigenetics in ecotoxicological context. For this, we have conducted a systemic review on epigenetic profiling in response to environmental stressors, mostly chemical exposure, in model organisms, as well as, in ecotoxicologically relevant wildlife species.

Keywords: epigenetics, ecotoxicology, transgenerational effect, environmental stressors, wildlife species

INTRODUCTION

Epigenetics refers to potentially heritable changes in genome function, such as gene activity or silencing, that occur without alterations to DNA sequence [1-3]. Commonly studied epigenetic mechanisms include chromatin remodeling (DNA methylation and histone modifications) and RNA-mediated modifications (e.g. non-coding RNA and microRNA) [4]. Environmental challenges can have immediate impact on epigenetic regulation in an individual or a population, and can be transmitted through mitotic and meiotic cell divisions and passed on to subsequent generations [3]. While certain epigenetic changes can result in increased risk of development of certain diseases, such as neurological disorders and cancers either immediately or at a later stage in life (adult onset of disease), others can be beneficial leading to plasticity and adaptation [2]. As a wide spectrum of environmental challenges (e.g. temperature, stress, diet, toxins) are known to impact on epigenetic regulatory mechanisms, epigenetics is an important component of ecotoxicology studies, it is however understudied so far.

Although the role of epigenetic factors in certain biological processes, such as tumourigenesis, has been heavily investigated [5-6], there are still areas that have either been neglected or have attracted little attention. For example, the impact of variation in the degree of methylation of otherwise identical genes (epialleles) on phenotypic and physiological endpoints in organisms is not yet broadly appreciated, in part because the phenomenon of epigenetics is not typically part of the design of physiological investigations. Furthermore, still enigmatic and somewhat ill-defined is the relationship between the overarching concept of epigenetics and interesting transgenerational phenomena (e.g. 'maternal/parental effects') that alter the physiological phenotype of subsequent generations. The lingering effect on subsequent generations of an initial environmental disturbance in parent animals can be profound, with genes continuing to be variously silenced or expressed without an associated change in gene sequence for many generations. Other areas that require further attention are: how natural variation in the epigenetic profiles of species

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alters their responses to environmental stressors, what evidence is available on the nature of dose-response relationships for epigenetic effects, and how to incorporate this information into ecotoxicology studies, such as chemical risk assessment.

CURRENT STATUS OF EPIGENETICS IN ECOTOXICOLOGY

Epigenetics parameters (markers) include DNA methylation, histone modifications (methylation, acetylation, phosphorylation, ubiquitinylation etc.), non-coding RNAs and newest member of this group is epitranscriptome [7]. Epigenetic mechanisms were first described as major agents in development and differentiation, but they appear as key components of the organism's response to environmental changes by modulating gene expression and other genome functions. Many organisms respond to environmental conditions by showing phenotypic plasticity, which means producing different phenotypes from the same genotype [8-9]. Increasing evidence shows that at least part of this phenotypic plasticity in animals exposed to environmental factors (such as diet, temperature, etc.) is mediated by epigenetics in a wide variety of organisms including vertebrates, insects and plants [10-14]. Environmental epigenetics investigates the cause-effect relationships between specific environmental factors that trigger adaptive responses in the cell and changes in epigenetic modifications. Given the dynamic and potentially reversible nature of the different types of epigenetic marks, environmental epigenetics constitutes a promising venue for developing fast and sensible biomonitoring programs. Indeed, several epigenetic biomarkers have been successfully developed and applied in traditional model organisms (e.g., human and mouse). Nevertheless, the lack of epigenetic knowledge in other ecologically and environmentally relevant organisms has hampered the application of these tools in a broader range of ecosystems. Fortunately, that scenario is now changing thanks to the growing availability of complete reference genome sequences and the development of high-throughput DNA sequencing and bioinformatics tools.

Toxico-epigenomics is the study of epigenomic changes caused by exposure to toxins/toxicants. Several studies (mostly in humans, primates and rodents) report epigenetic modifications related to exposure to chemicals and their relationship with the appearance of cancer [15]. Moreover, environmental exposures to chemicals can also affect the epigenetic status of ecotoxicologically relevant species of fish, plants and invertebrates [3]. Anthropogenic substances present in the environment have been found to not only chemically modify DNA and/or histones, but also affect (either by stimulation or inhibition) the enzymes activities involved in epigenetic control.

A summary of the recent published reports on applications of epigenetics to ecotoxicology studies can be found in Tables 1 and 2. These studies reinforce the idea that environmental stressors affect epigenetic markers, but the exact mechanisms of action are still unknown and further investigations are required. Environmental epigenetic analyses have extraordinary potential to advance our understanding of cellular and organismal responses to ecological challenges, and could serve as a promising source of rapid and sensitive tools for pollution biomonitoring of ecotoxicity species. The characterization of potentially hazardous substances may create a new field in ecotoxicology, but it would require the development of suitable epigenetic biomarkers both in mammals including humans, in other established model organisms and in environmentally-relevant species. In recent years, epigenetic tools have gained traction in ecotoxicology studies; the availability of genome assemblies for ecotoxicology-relevant species, and the wide conservation of known epigenetic components and mechanisms, will help drive further advances. In this regard, some researchers have already suggested that the "epigenetic foot-print" of an organism could be used as a tool to identify previous exposures to pollutants [16]. However, epigenetic knowledge and applications in ecologically relevant organisms still lags far behind mammalian and other model organisms. Importantly, we currently have a very limited understanding of the impact of environmental challenges on epigenetic mechanisms, subsequent phenotypic effects, and how often they are transmitted across generations. In order to fully achieve the promise of ecotoxicology studies, epigenetic mechanisms, in parallel with genetic mechanisms, should be considered in ecological risk assessments and sensible biomonitoring programs[16-17]

EPIGENETIC PROFILING IN RESPONSE TO ENVIRONMENTAL STRESS IN MODEL ORGANISMS

Tables 1 presented the available information on epigenetic profiling in response to environmental chemical exposure in model organisms. Most environmental epigenetic studies were conducted on zebrafish, *Danio rerio*, followed by water flea, *Daphnia magna*. Studies on the nematode, *Caenorhabditis elegans*, and fruit fly, *Drosophila melanogaster* were also conducted. Most of *D. rerio* and *D. magna* studies were on DNA methylation, whereas, most of *C. elegans* studies were on non-cording RNA and histone modifications.

Table 1. Ecotoxicology studies that include epigenetic approaches in model organisms

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Zebrafish *Danio rerio*	26°C±cadmium (200 μg/L) and 34°C±Cadmium (200 μg/L) for 7 days	Biochemical analysis, gene expressions	N/A	Gene specific methylation analysis of HSP70 (methylation specific qPCR after bisulfite modification)	The study reported that elevated temperature increased Cd toxicity due to several reasons. In particular, the expression levels of HSP70 were not significantly up-regulated by heat in Cd-exposed fish but increased by 117 times in Cd-free fish, hypermethylation of HSF binding motif in HSP70 promoter was observed during the combined exposure. This study provided the evidences that warmer temperatures can potentiate Cd toxicity, involved in the regulation of gene transcription, and DNA methylation.	[18]
Zebrafish embryo *Danio rerio*	0 to 72 h post fertilization (hpf) to bisphenol-A, diethylstilbestrol, 17α-ethynylestradiol, nickel, cadmium, tributyltin, arsenite, perfluoroctanoic acid, valproic acid, flusilazole, 5-azacytidine in subtoxic concentrations		N/A	Both global and site-specific methylation was examined. Genome wide locus specific analysis was performed using Digital Restriction Enzyme Analysis of Methylation (DREAM)	Global methylation was only affected by 5AC. Among the 3 selected (vasa, vtg1 and cyp19a2) site-specific gene promoter methylation tested, vasa (ddx4) was the most responsive. This analysis distinguished estrogenic compounds from metals by direction and sensitivity of the effect compared to embryo-toxicity.	[19]
Zebrafish *Danio rerio*	20 μg/L of depleted uranium for 6 days and 10 days	Gene expression and the appearance of histopathological damage in their progeny	Parental exposure and embryo diagnosis (P0-F1 embryo stage): Transgenerational effects	Global DNA methylation-2′-deoxycytosine (dC), 5-methyl-2′-deoxycytosine (5-mdC) and 5-hydroxymethyl-2′-deoxycytosine (5-hmdC) were detected and quantified by means of mass spectrometry (MS)	Increase in the global level of DNA methylation was observed in embryos (24 hpf) stage which was maintained at the 96 hpf, even though the DU had already been return to control level.	[20]
Zebrafish embryo *Danio rerio*	Hexabromocyclododecane (0, 2, 20, 200 nM)	Developmental analysis, histological analysis of cardiac morphology and defects	N/A	miRNA expressions (deep sequencing was performed on the Illumina Genome Analyzer II)	The exposure resulted in cardiac hypertrophy and arrhythmia, changed the miRNA expression profile associated with the cardiovascular system. The miR-1 was depressed by Nkx2.5 and mediated cardiac functions via its target genes in exposed conditions.	[21]
Zebrafish *Danio rerio*	Depleted uranium (2 and 20 μg/L) for 24 days		N/A	Global DNA methylation-2′-deoxycytosine (dC), 5-methyl-2′-deoxycytosine (5-mdC) and 5-hydroxymethyl-2′-deoxycytosine (5-hmdC) were detected and quantified by means of mass spectrometry (MS)	Sex and tissue specific differences in the methylation (hypo) level was evidenced. In males, these effects were present as early as 7 days after exposure while in females, significant effects were observed in the gonads after exposure for 24 days. In males, cytosine hypermethylation in the brain and eyes and hypomethylation in the gonads while in females, hypermethylation in brain, were observed.	[22]
Zebrafish embryos *Danio rerio*	Atrazine (0, 3, or 30 ppb) from 1 to 72 hpf		N/A	Global DNA methylation levels and dnmt expression and DNMTs enzyme activity analysis	Inhibition in DNMTs activity was observed thorough possible non-competitive Michaelis-Menten kinetics. Decrease in global methylation levels and the expression of dnmt4 and dnmt5 genes were also evident. Therefore, the atrazine exposure can decrease the expression and activity of DNMTs, leading to decreased DNA methylation levels.	[23]
Zebrafish *Danio rerio*	17α-ethinylestradiol		N/A	Gene specific methylation and mapping of vitellogenin I (pyrosequencing)	It was found that the methylation levels of all three CpG sites were higher in male liver than in female one. In brain, females and males showed similar high methylation levels in the analyzed CpG positions. Hypomethylation was found in the 5′ flanking region of vitellogenin I in the liver in both females and males.	[24]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Zebrafish embryo *Danio rerio*	Benzo[a]pyrene (24 ug/L) from 2.5 to 96 hpf	Global DNA methylation with ELISA (Epigentek) and bisulfite sequencing for CpG island mapping in vasa; related gene expressions with qPCR and DNMT1 activity	N/A	Global DNA methylation and gene specific CpG island mapping (vasa)	Significant reduction in global cytosine methylation (44.8%) and vasa gene promoter methylation (17%) was evident which was further supported though increase in vasa gene expression (33%). No change was found in CpG island methylation or gene expression in cancer genes (such as ras-association domain family member 1 (rassf1), telomerase reverse transcriptase (tert), c-jun, and c-myca) and DNA methyltransferase 1 (dnmt1) and glycine N-methyltransferase (gnmt). Overall, BaP is an epigenetic modifier for global and gene specific DNA methylation status in zebrafish larvae.	[25]
Zebrafish *Danio rerio*	Parental (continued to embryo) fish were water borne exposed to control (ethanol veichle) or BaP (50 μg/L) for 7 days before collecting eggs	methylation with ELISA (Epigentek) and PCR of sodium bisulfite-converted deep sequencing by RNA-seq (Illunina)	Mother and embryo (96hpf) duo exposure and continued to F4 generations (F1 to F4 is unexposed)	Global DNA methylation and gene specific methylation mapping	It was evident that at 96 hpf compared to 3.3 hpf, dazl, nqo1, sox3, cyp1b1 and gstp1 had higher methylation percentages while c-fos and cdkn1a had decreased CG methylation. BaP exposure significantly reduced egg production and offspring survival. Moreover, BaP decreased global methylation and altered CG, CHH, and CHG methylation both at 3.3 and 96 hpf. CG methylation changed by 10% or more due to BaP in six genes (c-fos, cdkn1a, dazl, nqo1, nrf2 and sox3) at 3.3 hpf and in ten genes (c-fos, cyp1b1, dazl, gstp1, mlh1, nqo1, pten, p53, sox2 and sox3) at 96 hpf. BaP also induced gene expression of cyp1b1 and gstp1 at 96 hpf which were found to be hypermethylated.	[26]
Zebrafish embryos *Danio rerio*	CuCl2 (0 and 325 μ g/L) from <1 hpf to 4 hpf at either 26.5˚C or 34˚C, followed by incubation in clean water at 26.5˚C till 96 hpf	Mortality/ Hatching failure, Global DNA methylation and related gene expressions	N/A	Global DNA methylation	Significant increased mortality rates and delayed hatching were observed. Both stressors, alone or in combination, significantly upregulated the expression of de novo DNA methyltransferase genes (dnmt3) along with no differences in global cytosine methylation level.	[27]
Zebrafish *Danio rerio* (4 months old)	Bisphenol A (15 and 225 μg/L); Sampling time point (7 d and 35 d after exposure)		N/A	Global DNA methylation (ELISA) together with the related gene expression (dnmt, gnmt, tets) in gonads (qPCR)	Global DNA methylation was significantly decreased in ovaries and testes. Besides the alteration of the global level of DNA methylation, varying degrees of transcriptional changes of dnmts, gnmt and tets were detected in gonads.	[28]
Zebrafish *Danio rerio* (3 months old male)	Di-(2-ethylhexyl) phthalate (0, 10, 33 and 100 mg/L) for 3 months	Reproductive ability and developmentaltoxicity (Histological testing, fertilized egg counting, sex hormone assays) and related gene expressions	P0 (gonads) and F1 (5 dpf, only for methylation analysis- short and specific transgenerational effects	Global and Gene specific DNA methylation(P0 male gonads and F1 5dpf)	A significant increase in global DNA methylation in both the male testes and their offspring larvae was observed. Spermatogenesis was inhibited which was associated decline in capability to fertilize untreated oocytes, decline of circulating testosterone (T) and an increase in the level of 17b-estradiol (E2), both of which were possibly derived from the downregulation of cyp17a1 and hsd17b3 genes and the upregulation of the cyp19a1a gene in the gonads. The DNA methylation statuses of these genes were altered within their promoter regions.	[29]
Zebrafish embryo *Danio rerio*	Tris (1,3dichloro-2-propyl) phosphate, tris (2-chloroethyl) phosphate , tris (1-chloro2-propyl) phosphate, and 2,2-bis (chloromethyl)propane-1,3-diyl tetrakis (2-chlorethyl) bis (phosphate) - toxicity ranges with various concentrations	Developmental toxicity (morphology)	N/A	Genome wide DNA methylation (restriction enzyme method)	TDCPP-induced delays in remethylation of the zygotic genome, a mechanism that may be associated with enhanced develop mental toxicity following initiation of TDCPP exposure at the start of cleavage.	[30]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Zebrafish *Danio rerio*	100 and 2,000 mg/L Bisphenol A (0.44 μM and 8.8 μM) to male and mating was conducted with untreated female	Phenotypic analysis of the F1, F2. Global DNA Methylation pattern of spermatozoa, testicular cells in F0 male and cardiac embryo development (myh6, cmlc2, atp2a2b, sox2 and insrb) related gene expressionsspermatozoa of P0 & F1	3rd generations (P0, F1, F2)	Global DNA methylation pattern of spermatozoa in F1 (ELISA)	An increase in the rate of heart failures of progeny up to the F2, as well as down-regulation of 5 genes involved in cardiac development in F1 embryos. Results reveal a paternal inheritance of changes in the insulin signaling pathway due to down-regulation of insulin receptor mRNAs. Nonetheless, only assumptions were made on the connection between DNA methylation and gene expressions changes and paternal inheritance for carcinogenesis.	[31]
Zebrafish *Danio rerio*	2, 3, 7, 8 TCDD (20 μg/kg), MeHg (10 mg/kg)for 47 days, or 5-aza-2′-deoxycytidine (10 mg/kg) , F0 adult females were exposed to the experimental diets for 47 days; Adult liver and embryo samples were collected	Gene expressions with qPCR	P0 (exposed), F1 & F2 (non-exposed)	Global DNA methylation determination by HPLC and genome wide CpG island mapping by MeDIP-microarray;	No significant effect on global DNA methylation levels in F1 (MeHg and TCDD) and F2 (MeHg) embryos were observed and only a limited number of genes were identified with altered methylation levels at their promoter regions.	[32]
Zebrafish *Danio rerio*	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin		N/A	Global methylation / hydroxymethylation (HPLC), gene specific methylation and CpG mapping (bisulfite sequencing), profiling of dnmts in different adult tissues (eye, liver, brain etc)	The results demonstrate that dnmt3b genes are highly expressed in early stages of development, and dnmt3a genes are more abundant in later stages. Upregulation of dnmt1 and dnmt3b2 expression, whereas down-regulation of dnmt3a1, 3b1, and 3b4 were evident. No changes were found in global methylation or hydroxymethylation levels, while AHR repressor a (ahrra) and c-fos promoters were differentially methylated.	[33]
Zebrafish embryos *Danio rerio*	Non-embryotoxic concentrations of the biologically active phthalate metabolite mono (2-ethylhexyl) phthalate (30 μM) and the DNA methyltransferase 1 inhibitor 5-aza-cytidine (10 μM) for 0 to 6 dpf	Morphology (length analysis), gene expressions and DNA methylation	P0 (exposed) ad analysis P0, F1, F2 Transgenerational effects	Direct, latent and transgenerational effects on DNA methylation were assessed using global (LC/MS), genome wide (reduced representation bisulfite sequencing (RRBS)) and locus-specific (BisPCR2) DNA methylation analyses	Multitude of differentially methylated regions, strongly enriched at conserved non-genic elements were evident from genome-wide analysis. Pathways involved in adipogenesis were enriched with the putative obesogenic compound MEHP. Exposure to 5AC resulted in enrichment of pathways involved in embryonic development and transgenerational effects on larval body length. Locus-specific methylation analysis of 10 differentially methylated sites revealed six of these loci differentially methylated in sperm sampled from adult zebrafish exposed during development to 5AC, including in first and second generation larvae, while with MEHP, consistent changes were found at 2 specific loci in first and second generation.	[34]
Zebrafish embryos *Danio rerio*	Fipronil (+, –) enantiomers (0, 100, 200, 400, or 800 μg/L) for 6-120 hpf (sampling and analyzing time: 120 hpf)	Acute toxicity and range finding for mortality, morphology, body length		DNA methylation (MeDIP-seq)	It was exhibited that S (+)-fipronil dysregulated a higher level of genomic DNA methylation than R (–)-fipronil. Compared with R- (–)-fipronil, S- (+)-fipronil significantly disrupted 7 signaling pathways (i.e., mitogen-activated protein kinases, tight junctions, focal adhesion, transforming growth factor-β, vascular smooth muscle contraction, and the hedgehog and Wnt signaling pathways) by hypermethylation of developmentally related genes.	[35]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Zebrafish embryos *Danio rerio*	B[a] P (5 and 10 μM) from 6 to 120 hpf and raised in chemical-free water until adulthood (F0)	Morphological, physiological and neuro-behavioral parameters were measured at two life stages, gene expressions	P0 (exposed), F1, F2 Transgenerational inheritance	Global DNA methylation	Juveniles of the P0 and F2 exhibited hyper locomotor activity, decreased heartbeat and mitochondrial function. Decreased global DNA methylation and reduced expression of DNA methyltransferases in wild type zebrafish were reported.	[36]
Zebrafish embryos *Danio rerio*	Lead (100 and 500 μg/L) from <1–2 hpf until 72 hpf		N/A	DNMTs enzyme assays (including kinetics), global DNA methylation, qPCR for DNMTs gene (dnmt1,3,4,5,6,7,8) expressions	Significant reduction in global DNA methylation as well as lower activity of maintenance DNMTs were found. Altered gene expression (dnmt3 and dnmt4) are also evident.	[37]
Zebrafish *Danio rerio*	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin; We exposed zebrafish embryos to DMSO (0.1%) or TCDD (5 nM) for 1 h at 30 hours post fertilization (hpf)		N/A	miRNA profiling (2platform-exiqon and agilent microarray) array and qPCR validation	Strong induction of CYP1A at 36 hpf and 60 hpf were identified and verified. The two microarray platforms yielded results that were similar but not identical; both showed significant changes in expression of miR-451, 23a, 23b, 24 and 27e at 60 hpf.	[38]
Zebrafish *Danio rerio*	Breeding groups of zebrafish to BPA (0.01, 0.1, and 1 mg/L) for 15 d.	Reproductive potentiality, gene expressions,	N/A	Global DNA methylation and dnmt1 gene expressions and gene specific promoter methylation (bisulfite pyrosequencing)	Significant increase in egg production, along with a decreased fertilization were observed. Further, significant alterations in the transcription of genes involved in reproductive functions were observed. Reduced expression of DNA methyltransferase 1 (dnmt1) along with a significant reduction in global DNA methylation, in testes and ovaries were evident.	[39]
Zebrafish embryos *Danio rerio*	A range of sodium arsenite (0–10.0 mM) between 4 and 120 hpf	Survival, development, cardiac development, apoptosis, Whole mount immunostaining (for neural system)	N/A	Global DNA methylation (5mc)	Embryos exposed to higher concentrations (0.5–10.0 mM) displayed reduced survival and abnormal development including delayed hatching, retarded growth and changed morphology. Alterations in neural development, abnormal cardiac function, altered cell proliferation and apoptosis status as well as abnormal genomic DNA methylation patterning (2.0 mM, 24 and 48 hpf) were detected in the arsenite-treated embryos.	[40]
Zebrafish adults *Danio rerio* (5-7 months old)	17α-ethinylestradiol (100ng /L) for 14 days		N/A	Vitellogenin I gene (CpG mapping by pyrosequencing)	Methylation levels in the 5' flanking region of vitellogenin I in the liver in both females and males were decreased significantly. This study suggest that induced expression of vitellogenin in fish following exposure to estrogens might involve alterations in DNA methylation.	[24]
Zebrafish embryos *Danio rerio*	AgNPs (40 nm 0.4 mg/L) from <4 hpf (sampling varies from 24 to 96 hpf)	Morphology, locomotion behaviors, and heartbeat rate, global transcriptomics (microarray)		Genome wide DNA methylation (bisulfite seq)	Shorter body, reduced heartbeats, and dysfunctional movements were observed. Less, loose, and unassembled myofibrils were detected. Genes in myofibrillogenesis and sarcomere formation were found to be down-regulated in treated embryos. Down-regulated calcium (Ca2+) signaling and loci-specific DNA methylation in specific muscle genes, such as bves, shroom1, and arpc1a, were also evident. Together, these might result in the down-regulated expression of myofibrillogenesis genes and muscle dysfunctions in the treated embryos.	[41]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Zebrafish *Danio rerio*	26°C and 34°C (preheating for 4days before exposure to Cd)± Cadmium (0 or 200 μg/L) for 1 week at 26°C.	Biochemical analysis (enzyme activity), gene expressions (qPCR)	N/A	Gene specific Methylation analysis (HSP70)	In the Cd-exposed groups, preheating decreased mortality and lipid peroxidation, increased activity of metallothioneins, inflammation-related genes, antioxidant enzyme levels, heat shock protein 70 (HSP70) and heat shock factor 2 (HSF2). Furthermore, preheating alone caused reduced methylation levels in the HSF binding motif of the HSP70 promoter. Together, preheating-induced accumulation of transcripts via demethylation might support the rapid defense responses at post-transcriptional levels caused by subsequent Cd exposure, indicating an adaptive mechanism for organisms exposed to one mild stressor followed by another.	[42]
Zebrafish *Danio rerio* (6 months old male)	Bisphenol A (500, 1000, or 1500 μg/L) for 21 d	Vitellogenin (VTG) analysis, plasma 17b-estradiol (E2) and testosterone measurement and related gene expression (hepatic esr1, gonadal cyp19a1a and cyp17a1)	N/A	Gene specific DNA methylation (hepatic esr1, gonadal cyp19a1a and cyp17a1) (bisulfite sequencing PCR)	The up-regulated expression of gonadal aromatase, mRNA levels of which were found to be negatively related to the methylation levels of both its promoter and one CpG site, were possibly the reason of increased E2 level. Further, hepatic esr1 mRNA levels were also negatively related to the methylation levels of both its promoter and one CpG site.	[43]
Zebrafish *Danio rerio*	7, 12-dimethylbenz [a] anthracene induced liver tumor		N/A	Genome-wide DNA methylation (MeDIP)	Comparative analysis found that the significantly hypomethylated genes in tumors were associated particularly with proliferation, glycolysis, transcription, cell cycle, apoptosis, growth and metastasis. While Hypermethylated genes included those associated with anti-angiogenesis and cellular adhesion. Of 49 genes that were altered in expression within tumors, and which also had appropriate CpG islands showed significant changes in both gene expression and methylation.	[44]
Zebrafish *Danio rerio* (Male)	Bisphenol S (0, 5, and 50 μg/L) for 21 d		N/A	miRNA profiling (microarrays and qPCR validation)	The GO term analysis revealed that miRNAs significantly affected were involved in hematopoiesis, lymphoid organ development, and immune system development. Among 14 miRNAs that were significantly deregulated, targeted cyp19a1b gene, suggesting the toxicity via the interference with the aromatization process.	[45]
Zebrafish *Danio rerio*	Fipronil (5-amino-1-[2, 6-dichloro-4-(trifluoromethyl) phenyl]-4-[(trifluoromethyl) sulfinyl]-1H-pyrazole-3-carbonitrile) and triazophos (3-(O, O-diethyl)-1-phenyl thiophosphoryl-1, 2, 4-triazol) and their mixture	miRNA expression	N/A	miRNA expression profiling	An association between these chemicals and the expression of 21 miRNAs was found. Among them, 14 miRNAs were differentially expressed due to the treatments with fipronil, triazophos and their mixture; 5 miRNAs showed altered expression level after treatment with formulations of these chemicals; miR-29b and miR-738 were differentially expressed after treatment with adjuvants. MiRNAs might be used as a toxicological biomarker.	[46]
Zebrafish *Danio rerio*	Fipronil (5-amino-1-[2,6-dichloro-4 trifluoromethyl) phenyl]-4 (trifluoromethyl) sulphinyl]-1H-pyrazole-3-carbonitrile) (0, 0.034, 0.068,0.137, 0.274, 0.548, ,1.098 μM)	cyb561d2 at gene and protein level	N/A	Targeted analysis of the mechanism of miR-155 with qPCR, western blotting (for cyb561d2), luciferase assays in 293T cells	The expression of miR-155 was downregulated, whereas cyb561d2 was upregulated in both mRNA and protein level in a dose-dependent manner upon stimulation of fipronil.	[47]
Zebrafish *Danio rerio*	Hypoxia	Analysis in zebrafish apoptosis, cell cycle analysis in ZF4 cells	N/A	Global miRNA expression profiling	It was evident that the up-regulation of the miR-462/miR-731 cluster in zebrafish larvae is induced by hypoxia. It was further validated that miR-462 and miR-731 are up-regulated in a Hif-1α–mediated manner under hypoxia and specifically target ddx5 and ppm1da, respectively. Overexpression of miR-462 and miR-731 represses cell proliferation through blocking cell cycle progress of DNA replication, and induces apoptosis.	[48]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Zebrafish embryo *Danio rerio*	Valproic acid (1 mM) or vehicle control (ethanol) starting from 4 hpf and sampled at 48 and 96 hpf.	Assessment of phenotypes;	N/A	miRNA profiling (microarray and qPCR validation)	Skeletal deformities, abnormal swimming behavior, and pericardial effusion was observed 96 hpf larvae. It was found that 13 miRNAs were differentially expressed at 48hpf and 22miRNAs were altered at 96hpf. Among them, six miRNAs (miR-16a, 18c, 122, 132, 457b, and 724) were common to both time points. Target prediction revealed that these miRNAs target several genes involved in the normal functioning of the central nervous system.	[49]
Zebrafish embryos *Danio rerio*	Ethanol (0.5, 1, 1.5, 2, or 3%) from 4 hpf to 24 hpf and embryos were allowed to grow until 96 hpf (sampling time: 96hpf)	Morphological changes, mortality, and deformations recorded at 24, 48, 72, and 96 hpf.; cell dealth assay (acridine orange staining)		miRNA profiling (microarray) (1 and 1.5% EtOH)	Significant upregulation of miR-153a, miR-725, miR30d, miR-100, miR-738, and miR-732, were identified. Putative gene targets of deregulated miRNAs are involved in cell cycle control, apoptosis, and transcription,	[50]
Zebrafish embryos *Danio rerio*	PFOS or DMSO control (1 μg/ml) from 6 hpf to 24 or 120 hpf.		N/A	miRNA profiling with microarray	It is found that a total of 39 and 81 miRNAs showed significantly altered expression patterns after PFOS exposure 24 and 120 hpf. These miRNAs were involved in development, apoptosis and cell signal pathway, cell cycle progression and proliferation, oncogenesis, adipose metabolism and hormone secretion etc.	[51]
Zebrafish embryos *Danio rerio*	Flame retardant, 2,20,4,40-tetrabromodiphenyl ether (BDE47) (0, 5, 500 μg/L) to 5–6 hpf until 144 hpf		N/A	miRNA profiling with RNA-seq (Hiseq2000 platform, Illumina)	miR735 was supposed to play essential roles in larval sensory development according to analysis results information.	[52]
Zebrafish *Danio rerio* (*female*)	Bisphenol A (5, 10, or 20 μg/L) for 3 weeks	Oocyte growth and maturation (histology), Egg collection and fertility, autophagy and apoptosis processes	N/A	Gene specific histone modifications (H3K4me3 & H3K27me3), and DNA methylation and related genes (dnmt1, 3,4, 5, 6, 7, 8) were assessed by Real-Time PCR(qPCR), (ChIP-qPCR).	Oocyte maturation-promoting signals down-regulated, likely through changes in the chromatin structure mediated by histone modifications, and promoted apoptosis in mature follicles. These data indicate that the negative effects of BPA on the female reproductive system may be due to its upstream ability to deregulate epigenetic mechanism	[53]
Caenorhabditis elegans (L1 to young adult)	Starvation	Life span	P0 (starved), F3 (fed)	Small RNA sequencing	It was demonstrated that the generation of endo-siRNAs that are inherited through at least three consecutive generations, significant increase in the lifespan of animals whose ancestors had experienced starvation	[54]
Caenorhabditis elegans (L1 to young adult)	Nicotine	-	P0 (exposed)-F2 (unexposed)	microRNAs profiling (qPCR-array)	Global expression profiles of miRNAs were found to be altered not only in the treated worms PF0 parent generation) but also in two subsequent generations. Target prediction and pathway enrichment analyses showed daf-4, daf-1, fos-1, cmk-1, and unc-30 to be potential effectors of nicotine addiction.	[55]
Caenorhabditis elegans	Graphene oxide (1, 10, 100, and 1000 mg/L) from L1 to young adult	Reproductive toxicity of GO on gonad development, apoptosis, DNA damage	N/A	microRNAs profiling	It is reported that the miRNA regulation mechanism activated in exposed condition to suppress its induced reproductive toxicity. A mir-360 regulation mechanism was activated by GO to suppress its induced DNA damage-apoptosis signaling cascade through affecting component of CEP-1.	[56]
Caenorhabditis elegans	Multi-walled carbon nanotubes (1 mg/L) from L1 to young adult	Reproductive toxicity, lipid level,	N/A	microRNAs profiling (RNA-seq)	It was reported that possibly the mir-355 regulate MWCNTs toxicity by inhibiting functions of its targeted gene of daf-2, suggesting that mir-355 may regulate functions of the entire insulin signaling pathway by acting as an upregulator of DAF-2 in exposed condition.	[57]
Caenorhabditis elegans	Nutrition (high dietary glucose)	Reproduction	P0-F3	H3K4 (mutant assay)	Heritable diminution of progeny from glucose exposure in the parental generation, Transgenerational inheritance of glucose phenotypes requires H3K4me3 components	[58]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Caenorhabditis elegans	MeHg (10 μM) from L1 to L4		N/A	Genome wide H3K4 trimethylation (H3K4me3) (ChIP-seq) followed by validation of the ChIP-seq hypothesis though RNAi and mutant animals	The histone modifications correspond to the locations of 1467 genes with enhanced and 508 genes with reduced signals. Among enhanced genes are those encoding glutathione-Stransferases, lipocalin-related protein and a cuticular collagen.	[59]
Caenorhabditis elegans	Worms were exposed to Iranian heavy crude oil under three different scenarios: partial early-life exposure, partial late-life exposure, and whole-life exposure	Growth, reproduction	P0 (exposed), F1-F3 (unexposed)	Histone trimethylation profiling (H3K4, H3K9, H3K27 and H3K36)	Reduced reproductive potential in the exposed P0 generation in the WE treatment group was reported. It was inhibited in the unexposed offspring generations of the P0 worms. This suggests that there was transgenerational inheritance of defective reproduction. Decreased methylation of histone H3 (H3K9) was found in the IHC-exposed parental generation. A heritable reduction in reproductive capacity occurred in wild type, N2, but was not found in a H3K9 histone methyltransferase mutant, met-2(n4256), suggesting a potential role for HMT in transgenerational toxicity.	[60]
Caenorhabditis elegans	Bisphenol A (100 μM) at L4 stage for next 48 h	Apoptosis, embryonic lethality (by monitoring numbers of embryos produced by each worm of each day of its reproductive life and subsequent larvae hatched from these embryos), genome wide transcriptome (RNA-seq)	P0 (exposed) to F1 to F3 (unexposed)	Genome wide H3K9me3 and H3K27me3 mark mapping and related enzymes (Jmjd-2 and Jmjd-3/UTX-1) levels and (ChIP-seq, quantification and mutant analysis)	It is demonstrated that exposure causes the derepression of an epigenomically silenced transgene in the germline for 5 generations, regardless of ancestral response. Experiments suggests that this effect is associated with a reduction of the repressive marks H3K9me3 and H3K27me3 in whole worms and in germline nuclei in the F3, as well as with reproductive dysfunctions, including germline apoptosis and embryonic lethality. Furthermore, targeting of the Jumonji demethylases JMJD-2 and JMJD-3/UTX-1 restores H3K9me3 and H3K27me3 levels, respectively, and it fully alleviates the BPA-induced transgenerational effects.	[61]
Caenorhabditis elegans	High temperature (25°C) exposure at embryo stage and rescue (shifting back to normal temperature -20°C at L4 stage)	Genome wide transcriptome,	P0 to F5 (high temp exposure) followed by F6 to F15 (normal temp exposed)	Histone di/tri methylation (H3K9, H3K4, H3K27, H3K36) (immunostaining)	Temperature-induced change in expression of heterochromatic gene array can endure for at least 14 generations. Inheritance is primarily in cis with the locus, occurs through both oocytes and sperm, and is associated with altered H3K9me3, before the onset of zygotic transcription. Long-lasting epigenetic memory of environmental change is therefore possible in this animal.	[62]
Drosophila melanogaster (Third instar larvae)	Cr (VI) (5.0–20.0 μg/ml) for 24 and 48 h.		N/A	miRNA profiling (microarray)	28 of the 36 differentially expressed miRNAs were found to be significantly deregulated targeting major biological processes viz., DNA damage repair, oxidation–reduction processes, development and differentiation. Down-regulation of mus309 and mus312 under DNA repair, acon to oxidation–reduction and pyd to stress activated MAPK cascade respectively belonging to these gene ontology classes concurrent with up-regulation of dme-miR-314-3p, dme-miR-79-3p and dme-miR-125p confirm their functional involvement against Cr(VI) exposure	[63]
Drosophila melanogaster	Dichloro-diphenyl-trichloroethane-resistant 91-R and -susceptible 91-C strains		N/A	miRNA profiling (RNA-seq)	Differential expression of 10 miRNAs between 91-R and 91-C were found which putatively target transcripts encoding proteins involved in detoxification mechanisms (based on Gene Ontology and pathway analysis).	[64]
Daphnia pulex	Cadmium: 20 μl/L CdCl2 (Cd-low, ~48 h LC_{01} value), 40 μl/L CdCl2 (Cd-high, ~48 h LC_{10} value)	Hypoxia	N/A	miRNA profiling (miRNA array)	The predicted miRNA target genes included oxidative stress, ion transport, mitochondrial damage and DNA repair, insulin-related network based on 22 and 21 differentially expressed miRNAs (under low and high exposure). It is reported that miR-210 is hypoxia-responsive and Cd and hypoxia induce miR-210 via a same HIF1α modulated pathway	[65]

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Table 1. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Daphnia magna (Two different genotypes)	Various biotic and abiotic stressors at specified exposure condition/ treatment, such as, predation (i.e., Triops), diet (i.e., Cryptomonas and Microcystis), and salinity; metal pollutants; pH; hypoxia etc.		N/A	Global DNA (5mc) methylation (UPLC)	A significant genotype effect, an environment effect, and a genotype- environment effect were observed Significant differences were found between the 2 genotypes when animals were exposed to predation (Tripos) cues, Microcystis, Cryptomonas, and sodium chloride. In particular, global cytosine methylation levels upon exposure to Triops exhibited a 5-fold difference between the genotypes (0.21% vs 1.02%). No effects were reported in response to arsenic, cadmium, fish, lead, pH of 5.5, pH of 8, temperature, hypoxia, and white fat cell disease.	[66]
Daphnia magna, Moina macrocopa	Humic substances, ubiquitous bio-geochemicals Humin-Feed® (either at a conc. of 15 mg/L DOC or 8.6 mg/L DOC)	Ephippia number per female until 3rd generations	3rd generations (P0, F1, F2) (only for ephippia counting)	Global DNA methylation (ELISA, epigentek) (only P0)	Increase of methylated DNA by a factor of 1.54 (Moina) and 1.73 (Daphnia) were observed. Alteration in epiphia production (considered as stress parameter) due to mother (P0) only exposed condition. The most sensitive generation is F1 (in respect of epiphia production). It is predicted that the methylation of DNA can alter the stress response.	[67]
Daphnia magna	Zinc (388±40 μg/L) (continuous and mother only exposed)	Reproduction (number of juveniles per organism)	3rd generations (P0, F1, F2)	Global DNA methylation (5mdc) (LC-MS)	The average [5mdC]/[dG] ratio ranged from 0.13% to 0.81%. A significant decrease in DNA methylation was found in the offspring (F1) in both multigenerational (continuous exposed) as well as transgeneral (mother only exposed) exposure. The effect did not observed into the 3rd generation (F2).	[68]
Daphnia magna	Cd (180 μg/L) for two generations (continuous)	Reproduction (for 2 weeks as per: OECD 211)	Two generations (P0 & F1)	Global DNA methylation status (CpG methylation by restriction-enzyme based method derived from the Amplification of intermethylated sites (AIMS)	Significant decrease in reproduction was observed. However, it was not demonstrated that Cd exposure altered DNA methylation. In addition, the homologs of human DNA methyltransferases (DNMT1, DNMT2 and DNMT3A) were identified in the D. magna genome (partially available)	[69]
Daphnia magna	Toxic cyanobacterium (*Microcystis aeruginosa*)		N/A	Genome wide DNA methylation patterns (Bisulfite sequencing)	The results exhibited that DNA methylation is modulated by environmental stress. Upon exposure to Microcystis sp., differential methylation patterns were observed primarily in exonic regions. These patterns were found to be enriched at serine/threonine amino acid codons and genes related to protein synthesis, transport and degradation. Moreover, it was found that the genes with differential methylation corresponded well with genes susceptible to alternative splicing in response to Microcystis stress.	[70]
Daphnia magna	5-azacytidine (continuous and mother only exposed)	Body Length	F0-F1	Gene specific DNA methylation (Bisulfite sequencing followed qPCR validation)	The results of this study suggests that epigenetic biomarkers have the potential to be used as indicators of past chemical exposure history of organisms. Significant changes in the methylome of target genes were observed in prenatal exposed condition. The continuous (pre- and postnatal) exposures caused the higher reduction in DNA methylation.	[71]
Daphnia magna	Chronic γ Irradiation (25-day γ irradiation) (6.5 μGy·h–1 or 41.3 mGy·h–1)	Survival, growth, and reproduction	P0 (exposed) followed by F1-F3 (unexposed)	Whole genome DNA methylation pattern (Bisulfite Sequencing)	Reproduction effects were observed only in P0 while no effect in the subsequent generations F1, F2, and F3. Interestingly, significant methylation changes at specific CpG positions, with a majority of hypomethylation. Were found in every generation. Common methylation changes between F2 and F3 clearly supports the idea of transgenerational inheritance of epigenetic modifications.	[72]

Table 2. Ecotoxicology studies that include epigenetic approaches in non-model organisms

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Brine shrimp *Artemia*	Daily nonlethal heat shock treatment of the parental (P0) generation (nonlethal heat shock at a rate of 7°C/min in a preheated and controlled water bath system)	Reproduction (nauplii & cysts), Thermo tolerance, *V. campbellii* challenge, Hsp70 protein expression (Western blot)	3rd generations (P0, F1, F2) (Only P0 exposure)	Global DNA methylation (UPLC) and total histone (H3 & H4) acetylation (ELISA & Western blot)	Upon exposure to nonlethal heat shocks, parental organisms exhibited an increase in levels of Hsp70 production, tolerance toward lethal heat stress, and resistance against pathogenic *V. campbellii*. Interestingly, it was also all these acquired traits were transmitted to next progenies (3 successive generations). Most importantly, the transgenerational inheritance of the acquired phenotypes was found to be associated with significantly altered levels of global DNA methylation and acetylated histones H3 and H4.	[73]
Brine shrimp *Artemia francis cana*	Non-lethal heat-shock treatment (30 min exposure to 37°C) followed by a recovery period of 6 h at 28°C. After recovery animals were exposed to either Cadmium (0-10-25-50-100-150-200-300 mg/L) and Zinc (0-0.5-1-4-10-25-60-150mg/L) for 48 h.	70kDa-HSPs protein expressions (western blot)	N/A	DNA methylation ([mdC]/[dG] detection in UHPLC) and total histone (H3 & H4) acetylation (ELISA)	This study suggest that the HSP production is a phenotypically plastic trait with a potential role in temperature-induced tolerance to metal exposure. Exposed organisms showed increased acute tolerance to Cd and Zn and different patterns of HSPs were observed between the two metal compounds. No epigenetic alterations were exhibited in response to heat shock or metal exposure.	[74]
Eastern oyster *Tegillarca granosa*	Cadmium (25, 250 and 500 mg/L)		N/A	miRNA profiling (RNA-seq)	Significant differential expressions of 16 miRNAs (5 up-regulated while 11 down-regulated) were found in the Cd exposed group.	[75]
Crassostrea virginica (collected at Rookery Bay National Estuarine Research Reserve, Naples FL)	Florida Red Tides (harmful Algal Blooms (HABs) - HAB simulation was conducted in laboratory conditions, exposing oysters to increasing concentrations of K. brevis (LC–MS as 5.0-6.8 pg/cell)	Gene expression analysis	N/A	Histone variants (protein extraction, separation and western blot analysis) and Genome-wide DNA methylation analysis (Methylation Sensitive Amplified Polymorphism (MSAP) protocol)	The histone variants, H2A.X, H2A.Z and macroH2A genes potentially involved in the maintenance of genome integrity during responses to the genotoxicity. An increase in H2A.X phosphorylation (γH2A.X, a marker of DNA damage) and a decrease in global DNA methylation were observed as the HAB simulation progressed.	[76]
Crassotrea giga	Diuron herbicide: At the start and the mid-course of gametogenesis, the oysters were exposed during two 7-day periods to pulses of diuron,(0.2 and 0.3 mg/l) respectively, and of 0.005% (50 ml/l) acetonitrile for the SC group	Related gene expression (qPCR) for validation of DNA methylation results	N/A	DNA methylation pattern (whole genome bisulfite sequencing (WGBS, BS-Seq))	It is demonstrated that parental diuron exposure has an impact on the DNA methylation pattern of its progeny. Conserved DNA methylation patterns in response to parental diuron exposure was observed, nonetheless, the the DNA methylation profile varied between individuals. This study suggest for new markers based on epimutations as early indicators of marine pollutions.	[77]
Crassostrea gigas	Embryos were exposed to 4 nominal copper concentrations (0.1, 1, 10 and 20 μg/L Cu^{2+}) during early development assays	Embryo toxicity (embryo-larval bioassay at the D-larva stage 24 hpf) and genotoxicity at 7 hpf. 15 genes expression (RT-qPCR)		DNA methylation proteins (RT-qPCR) was measured at three developmental stages (3 hpf, 7 hpf, 24 hpf). Global DNA content (5mc & 5hmc in HPLC) and gene-specific DNA methylation levels (Me-DIP-qPCR)	Significant larval abnormalities from 10 μg/L, significant genotoxic effects from 1 μg/L were observed. Impairment for some homeobox and DNA methylation genes (Notochord, HOXA1, HOX2, Lox5, DNMT3b and CXXC-1) was observed. In case global DNA methylation, no change in 5mc marker while significant changes (reduction) in 5-hmc marker were observed. This study suggests that the copper exposure cause embryotoxicity in oysters through homeotic gene expression impairment possibly by changing DNA methylation levels.	[78]
Sea Cucumber *Apostichopus japonicas* (collected from the coast of Weihai, China)	Hypoxia (analysis at different DO level)		N/A	miRNA profiling (RNA-seq)	A total of 26 differentially expressed miRNAs (12 upregulated and 14 downregulated) were found in severe hypoxia compared with mild and normoxic conditions. Moreover, gene ontology and pathway analyses of putative target genes suggest that these miRNAs are important in redox, transport, transcription, and hydrolysis under hypoxia stress. Notably, novel-miR-1, novel-miR-2, and novel-miR-3 were specifically clustered and upregulated in severe hypoxia.	[79]

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Table 2. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Sea urchin *Glyptocidaris crenularis*	PFOS (0, 0.01, 0.1 and 1 mg/L) for 21 d, followed by a 7-d depuration period (behavioral characteristics: every day; antioxidant enzyme activities: 1, 3, 5, 7, 14, 21 and 28 d; DNA methylation status : 7, 14, 21 and 28 d)	Antioxidant enzyme activities, dropped spines, and lowered down the motor ability and feeding ability	N/A	Genome wide DNA methylation (MSAP method)	In gonad, the DNA methylation polymorphism, methylation/ demethylation rate increased following the prolonged exposure time followed by decreased (after the depuration period). The demethylation rates were lower than the corresponding methylation rates, therefore methylation events were dominant during the whole experimental period. This study suggest that sea urchin have possibly possess strong self-protection mechanisms against PFOS exposure.	[80]
Six species of corals (*Acroporahyacinthus, A. millepora, A. palmata, Pocillopora damicornis, Porites astreoides, Stylophora pistillata*)	Thermal stress and ocean acidification effects	N/A	N/A	DNA methylation (In silico annotation and prediction): In silico annotation and prediction (transcriptome analysis of CpG O/E, an estimate of germline DNA methylation that is highly correlated with patterns of methylation enrichment).	It was found that the genes differentially expressed in response to thermal stress and ocean acidification exhibited significantly lower levels of methylation, in three of the coral species. These results support a link between gene body hypomethylation and transcriptional plasticity which showed a potential pivotal role of DNA methylation in the response of corals to environmental change.	[81]
Chironomus riparius	Bisphenol A (3 mg/L), cadmium chloride (10 mM) and Benzyl butyl phthalate (1 mg/L) for 24 h	Immunocytochemistry of salivary glands, qRT-PCR	N/A	Long non-coding sequences were studied: telomeric repeats, Clarepetitive elements and the SINE CTRT1(RT-PCR)	Upregulation of telomeric transcripts was found after BPA treatments. Cadmium and BPA both exposure caused significant activation of Cla transcription. Transcription of SINE CTRT1 was not altered by any of the chemicals tested.	[82]
Chironomus riparius	Bisphenol A (1mg/L)	Basic ecotoxicity (development and reproduction) assays, DNA damage (comet assay), and global metabolomics (NMR based) approaches.		Global DNA and histone methylations	The reproduction failure, increase in DNA damage, global DNA hyper-methylation, and increased global histone modification (H3K36) status were evident. A potential cross-talk was proposed between altered epigenetics and metabolites, such as, increase in methionine and o-phosphocholine metabolites corresponds with the phenomena of global hyper-methylation in DNA and H3K36 mark.	[83]
Asian tiger mosquito *Aedes albopictus* (Larva of P0 were exposed)	Phytoestrogen genistein (5 mg/L), fungicide vinclozolin (3 mg/L)	Morality	Four generations (P0-F3) in a full life-cycle design with P0 exposed and F1-F3 is non-exposed generations	Global DNA methylation (5mc-HPLC/MS)	Exposure to the parental generation led to an alteration of the global DNA methylation level of the exposed individuals and their subsequent progenies.	[84]
Anguilla anguilla	Fish were sampled in two locations presenting a low or a high contamination level.	Gene expressions (qPCR); Histology of gonad; pollutants analysis of the sampling site water	N/A	Gene-specific DNA methylation of *aro, fshr* and *11β-hsd* genes (McrBC-digestion followed by qPCR)	The DNA methylation levels of the genes encoding for the aromatase and the receptor of the follicle stimulating hormone were higher in contaminated fish and positive correlation was found between hyper-methylation genes and decreased transcription level.	[85]
Atlantic killifish or mummichog *Fundulus heteroclitus*	Inhabiting a creosote-contaminated Superfund site on the Elizabeth River (VA, USA)		F0 & F1 (F0 is caught from superfund site and reared at lab to F1)	CpG analysis of CYP1A promoter region (bisulfite sequencing)	Cytosine methylation was not detectable at any of the 34 CpG sites examined, including 3 that are part of putative xenobiotic response elements.	[86]
Atlantic killifish *Fundulus heteroclitus*	PCB-contaminated Superfund site in New Bedford Harbor, MA, USA (NBH)		N/A	Gene specific methylation status of AHR1 & AHR2 (bisulfite conversion-followed by qPCR)	No significant differences in methylation profiles were observed in either AHR1 or AHR2 promoter regions between NBH and reference site fish.	[87]

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Table 2. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Atlantic cod *Gadus morhua*	Two temperature (9.5°C vs 4°C) condition with various gradient and exposure-recovery time followed		N/A	miRNA profiling (RNA-seq) and promoter specific methylation od selected miRNA (CpG sites methylation, 5′ upstream regions of pre-miRNAs	389 putative miRNA precursor loci, 120 novel precursor miRNAs, and 281 mature miRNAs were identified. Some miRNAs showed stage- or tissue-enriched expression, such as, the miR-17-92 cluster, myomiRs (miR-206), neuromiRs (miR-9, miR-124), miR-130b, and miR-430 showed differential expression in different temperature regimes. Inconclusive results were obtained regarding the effect of temperature on methylation status of selected miRNA promoter regions.	[88]
Small benthic fish *Mugilogobiusabe* (adult field collected fish)	Diclofenac (0.5, 5, 50, 500 mg/L) for 24 h and 168 h	Selected gene expressions	N/A	Selected miRNA sequencing on the basis of targeted genes	The negative correlation between miR-27a and p-gp mRNA expression under DCF exposure for 24 h indicated the role of miRNA in post transcriptional regulation on detoxification-related genes.	[89]
Goldfish *Carassius auratus* (purchased from farm and acclimatized in lab for 10 d)	2, 4-Dichlorophenol (0.01, 0.10, 1.0 mg/L) for 2 and 5 days			Global DNA methylation (5mc) levels, (HPLC and MspI/HpaII ethidium bromide assay), SAM and SAH contents (HPLC) the mRNA expression of DNMT1 and DNMT3 (qPCR).	Global DNA hypermethylation, elevated the methylation levels of CpG islands, increased the SAM and SAH contents, decreased the SAM/SAH ratio, and upregulated the mRNA expression of DNMT1 and DNMT3 were observed.	[90]
Adult wild thirteen-lined ground squirrels *Ictidomys tridecemlineatus*	Changing of external environmental conditions during hibernation		N/A	Global and gene specific DNA methylation (mef2c); changes in expression of DNA methyltransferases (DNMT1/3B) and methyl binding domain proteins (MBDs)	Alteration in global DNA methylation level in the liver and skeletal muscle along with changes in expression of DNA methyltransferases (DNMT1/3B) and methyl binding domain proteins (MBDs) were observed. A dynamic change in DNA methylation in the promoter of the myocyte enhancer factor 2C (mef2c) gene, a candidate regulator of metabolism in skeletal muscle, were also reported.	[91]
Thirteen-lined ground squirrels *Ictidomys tridecemlineatus*	Changing of external environmental conditions during hibernation		N/A	Phosphorylation and acetylation of histone H3, changes in HDAC (ELISA & immunoblotting) activity, and global DNA methylation (ELISA); related Gene expressions	An increase in global DNA methylation during long term torpor was observed. Acetylation of histone H3 (on Lys23) was found to be reduced by about 50% when squirrels entered torpor. A strong increase in histone deacetylase (HDAC1 and) and co-repressors of transcription (MBD1 and HP1) protein levels were found.	[92]
polar bear brains (in the lower brain stem region from 47 polar bears subsistence hunted in central East Greenland between 1999 and 2001)	Mercury associated exposure	.	N/A	Global DNA methylation (LUMA)	The average genomic DNA methylation was found as 57.9%±6.69. An inverse association was observed between genomic DNA methylation and brain mercury (Hg) exposure levels for the entire study population.	[93]

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Table 2. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Brain (cerebrum) tissues from mink (*Neovison vison*), chicken (*Gallus gallus*), yellow perch (*Perca flavescens*)	Methyl-mercury juvenile male mink were exposed daily to MeHgCl (0, 0.1, 0.5, 1, and 2 mg/kg in diet) for 3 months; leghorn chicken eggs were air-cell injected with MeHgCl (0, 0.62, 2.0, 3.2, and 6.4 μg/g egg injected) for 18 days; female yellow perch were fed pellets containing nominal MeHgCl concentrations of 0, 0.5, 5, and 50 μg/g and were fed twice daily for a period of 4 weeks.		N/A	Global DNA methylation (KUMA) and DNMT1 activity assay (ELISA)	Significant reductions in global DNA methylation and reduced DNMT activity was observed in mink. In chicken or yellow perch, no statistically significant effects of MeHg were observed. The variability in results across species may suggest inter-taxa differences in epigenetic responses to MeHg, or may be related to differences among the exposure scenarios.	[94]
Wild guinea pig males *Cavia aperea*	An increased ambient temperature (30°C) for 2 months (i.e. the duration of spermatogenesis)		P0 (exposed) father (liver as the main thermoregulatory organ) and F1 sons (liver and testes)	Genome wide methylation analysis (bisulfite conversion)	Differentially methylated regions (DMR) between P0 livers and F1 (sons) livers and testes were found to be shared which indicated a general response with ecological relevance. Thus, paternal exposure to a temporally limited increased ambient temperature led to an 'immediate' and 'heritable' epigenetic response that may even be transmitted to the F2 generation.	[95]
American alligator *Alligator mississippiensis*	Animals were selected at random from sites known to have either low, moderate, or high concentrations of mercury in the upper trophic levels (from 6 sites across Florida's north-south axis)	Trace element analysis	N/A	Global DNA methylation (5mc by LC/MS; extracted blood DNA used)	A relationship between Hg exposure and DNA hypomethylation was observed. Findings suggest that global DNA methylation levels are associated with mercury exposure.	[96]
Atlantic salmon *Salmo salar*	Acidic Alluminium rich water by addition of 100 μg/ L. The Al-solution added was a mixture of $Al_2(SO4)_3$ and H_2SO_4. The water. pH also decreased, i.e. from 7.87 in the control water to an average pH of 5.60 in the Al-enriched tank			Global miRNA profiling (RNA-seq) in muscle	A total of 18 miRNAs (4 down-regulated and 14 up-regulated) were significantly differentially expressed (FDR < 0.1) and 224 unique miRNAs were identified.	[97]
Earthworm (field collected)	Animals and the host soils were collected in September 2009 from the two sites in Mexico, close to silver mine and gold mine	ICP-MS for chemical speciation & conc.	N/A	Global DNA methylation (5mc) (HPLC-DAD)	Direct correlation was observed between soil and tissue for As, Se, Sb, Zn, Cu, Mn, Ag, Co, Hg, Pb ($p<0.05$). Inverse correlation was observed between the percentage of methylated DNA cytosines and total tissue As, As+Hg, As+Hg+Se+Sb, as well as inorganic As+Hg.	[98]
Marine polychaete *Spiophanes tcherniai,*	Sediment collected by SCUBA at the Intake Jetty off of McMurdo Station, Antarctica Cultured adult worms at two temperatures, -1.5°C (ambient control) and +4°C (warm treatment), for 4 weeks	Physiological measurement (respiration, citrate synthase)	N/A	Global DNA methylation (sequencing and CpG mapping-MSAP)	120,000 CpG sites in assembled contigs from both treatments were recovered and out of that 28,000 CpG sites were aligned as common between the two sample groups. In comparing these aligned CpG sites between treatments, only 3000 (11%) evidenced a change in methylation state, and >85% of changes involved a gain of a 5mc group on a CpG site (net increase in methyation).	[99]
Pacific oyster *Crassostrea gigas*	Thermal stress at different temperatures (18°C, 25°C and 32°C) Sample collection: 6 and 24 hpf		N/A	Global Hisotne methylation (H3K4, H3K9, H3K27) (ELISA) and related enzyme gene (JHDMs) expressions (qPCR)	When compared to the 25°C group, at 18°C H3K4, H3K9 and H3K27 residues were hypomethylated at 6 hpf and hypermethylated at 24 hpf. Conversely, at 32°C, 6 hpf animals present a hypermethylation of all examined residues. The mRNA expression of the nine oyster JHDMs, showed gene- and stage-specific temperature sensitivities through-out the early life. The results also indicate that temperature influences histone methylation	[100]

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Table 2. Continued

Species	Environmental stress (chemicals)	Endpoints (methods)	Multigenerational/ transgenerational effects	Epigenetic markers (methods)	Findings	Ref.
Sebastiscus marmoratus (fish)	Tributyltin (1, 10 and 100 ng/L as Sn), triphenyltin (1, 10 and 100 ng/L as Sn) and mixtures of TBT and TPT (TBT 0.5 ng/L+TPT 0.5 ng/L, TBT 5 ng/L+TPT 5 ng/L as Sn) Sampling: liver		N/A	Global methylation of DNA (HPLC), The S-adenosyl-l-methionine (SAM) and S-adenosyl-l-homocysteine (SAH) content analysis, DNMT1 expressions	Dose dependent DNA hypomethylation were observed in all the treatments (TBT, TPT, and a mixture). Strong linear correlations were identified between SAM to SAH ratio and the hepatic genome-wide 5-mc content of the DNA, while no correlation was found with DNMT1 expression. This study proposed that the organotins hypomethylation induced in the marine fish livers was due to altering the balance of the substrate and the product in transmethylation reactions.	[101]
Rare minnow *Gobiocypris rarus* (4 months old)	Bisphenol A at 15 ug/L (65.71 nM) for 7 and 35 days, gonads collected		N/A	Global DNA methylation (ELISA), gene specific methylation (bisulfite sequencing of 5′ flanking region of cyp19a1a gene), related gene expressions (dnmt1, dnmt4, dnmt6 and dnmt7 through 454 pyrosequencing	Increased global DNA methylation level was observed in testis and ovary. The alteration of the detected dnmts mRNA expression could affect the global DNA methylation levels following 15 ug/L BPA exposure. The methylation level of ovarian cyp19a1a gene was significantly suppressed and a significant negative correlation was found between cyp19a1a mRNA expression and methylation levels of the four CpGs at the 5′ flanking region of the same gene. So we hypothesize that there are some association between the reproductive toxicity of BPA and the global DNA methylation under BPA exposure.	[102]
Rare minnow *Gobiocypris rarus* (Adult male)	Bisphenol A (1, 15 and 225 ug/L) for two weeks.	The levels of glutathione (GSH), and enzyme levels for GSH synthesis (ELISA & qPCR) in the testes	N/A	The levels of (5mC & 5hmC) (ELISA) enzyme levels for DNA methylation (ELISA & qPCR)	Reduction of GSH level, TETs enzyme level, 5hmC levels (all treatments while significant increase of 5mC (225 μg/L) were evident. The results indicated that BPA exposure inhibited TETs mediated DNA demethylation and the declined DNA demethylation may result in DNA hypermethylation.	[103], [104]
Three-spine stickleback (*Gasterosteus aculeatus*)	Hexabromocyclododecane, 17-β oestradiol (E2), 5-aza 2′ deoxycytidine		N/A	Global DNA methylation (HPLC)	No significant differences in global DNA methylation were found in exposed liver of female fish. The methylation inhibitor, 5AdC, significantly lowered hepatic global methylation levels by 14% (Pb0.05). The E2 at 100 ng/L also decreased global DNA methylation levels in female liver (without statistical significance). In contrast, both E2 and 5AdC caused statistically significant ($p<0.001$ and $P<0.01$ respectively) global genomic hypermethylation in the male gonads.	[105]
Dab flatfish *Limanda limanda*	Captured from sampling sites in Irish Sea and Bristol Channel	Assessed for external diseases, sacrificed and livers were visually assessed for the presence of macroscopic lesions (nodules) followed by histopathology (hepatocellular adenoma tumors)	N/A	Genome-wide DNA methylation (MeDIP-seq)	Significant reduction of global methylation was observed in hepatocellular adenoma and non-cancerous surrounding tissues. Alteration of genes involved in pathways related to cancer, including apoptosis, wnt/β-catenin signaling and genomic and nongenomic estrogen responses, were evidenced both in methylation and transcription.	[106]
Bluegill sunfish *Lepomis macrochirus*	Benzo[a]pyrene		N/A	Global DNA methylation in liver (5mc- chromatography)	Trend of hypomethylation from day 2 to post-exposure	[107]

EPIGENETIC PROFILING IN RESPONSE TO ENVIRONMENTAL STRESS IN WILDLIFE SPECIES

Tables 2 presented the available information on epigenetic profiling in response to environmental chemical exposure in wildlife species, which are mostly ecological and ecotoxicological relevant non-model organisms. Most of studies using wildlife species were on DNA methylation.

FUTURE DIRECTION: EPIGENETICS IN ECOLOGICAL RISK ASSESSMENT

Various environmental factors, including chemical exposure, can modulate epigenome, the regulator of gene expressions, of several species, including model (Table 1) and non-model field organisms (Table 2). Some of the studies reported transgenerational inheritance of the exposure, i.e., epigenetic changes continued in non-exposed progeny. Real efforts are in progress to identify specific epigenetic marks which could provide 'signature of xenobiotic exposure' and linking epigenetic effects with toxicant induced altered phenotypes such as adverse outcomes, increased stress resistance, etc. Nonetheless, huge knowledge gap and challenges need to be overcome to integrate or organize toxicoepigeneics data in adverse outcome pathway (AOP) framework and/or risk assessment processes [7, 108-109].

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