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The comet assay in animal models: From bugs to whales – (Part 2 Vertebrates)

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Abstract

The comet assay has become one of the methods of choice for the evaluation and measurement of DNA damage. It is sensitive, quick to perform and relatively affordable for the evaluation of DNA damage and repair at the level of individual cells. The comet assay can be applied to virtually any cell type derived from different organs and tissues. Even though the comet assay is predominantly used on human cells, the application of the assay for the evaluation of DNA damage in yeast, plant and animal cells is also quite high, especially in terms of biomonitoring. The present extensive overview on the usage of the comet assay in animal models will cover both terrestrial and water environments. The first part of the review was focused on studies describing the comet assay applied in invertebrates. The second part of the review, (Part 2) will discuss the application of the comet assay in vertebrates covering cyclostomata, fishes, amphibians, reptiles, birds and mammals, in addition to chordates that are regarded as a transitional form towards vertebrates. Besides numerous vertebrate species, the assay is also performed on a range of cells, which includes blood, liver, kidney, brain, gill, bone marrow and sperm cells. These cells are readily used for the evaluation of a wide spectrum of genotoxic agents both *in vitro* and *in vivo*. Moreover, the use of vertebrate models and their role in environmental biomonitoring will also be discussed as well as the comparison of the use of the comet assay in vertebrate and human models in line with ethical principles. Although the comet assay in vertebrates is most commonly used in laboratory animals such as mice, rats and lately zebrafish, this paper will only briefly review its use regarding laboratory animal models and rather give special emphasis to the increasing usage of the assay in domestic and wildlife animals as well as in various ecotoxicological studies.

Keywords: Comet assay, DNA damage, Animal model, *In vivo*, *In vitro*, Vertebrates, Biomonitoring

1. Introduction

Over the past decades, the comet assay (single-cell gel electrophoresis) has become one of the methods of choice for the evaluation and measurement of DNA damage [1–3]. In epidemiological molecular and biomonitoring studies, primary DNA damage, evaluated by the comet assay, is used as a biomarker of exposure that provides information on the biologically effective dose of various physical and/or chemical mutagens/carcinogens [4–6]. The comet assay is quite simple, quick, cost effective, accurate and reliable; in other words, it fulfils all the criteria for use in routine laboratory, field as well as clinical testing. The comet assay is a predictive test that allows for the detection of DNA alterations of diverse kinds, such as single-strand DNA breaks, double-strand DNA breaks, alkali-labile sites, altered bases, incomplete repair sites, and interstrand cross-links [5,7–10].

Quantification of DNA damage by lysing cells embedded in agarose gel under alkaline conditions was first introduced by Rydberg and Johanson [11]. To improve the sensitivity of the single-cell DNA damage detection, Östling and Johanson developed the micro-gel electrophoresis technique, also called the comet assay [12]. Cells are embedded in an agarose matrix and lysed to produce nucleoids of supercoiled DNA attached to the nuclear matrix. Breaks in the DNA relax the supercoiling and allow DNA loops to expand, and on electrophoresis to move towards the anode. Along the way, the DNA path resembles the shape of a comet, which gave the assay its final name. This is followed by either visual (after classification into different categories based on tail length and shape), semi-automatic and/or automatic (which automatically recognise the extent of damage) analyses of stained DNA and calculation of fluorescence to determine the DNA damage extent. This is done by using appropriate software that enables commercially available image analysing systems to be connected through a camera to a fluorescence microscope, which facilitates the evaluation of DNA damage. Each trace resembles a comet with a brightly fluorescent head and a tail whose length and intensity are proportional to the frequency of DNA breaks present in the cell [1,13–19].

At least 50 comets are analysed per sample, and several types of descriptors can be employed, including tail length, relative tail intensity and tail moment. The migration length is proportional to the extent of DNA damage but reaches a maximum at a relatively low level of damage. Tail intensity is expressed as % of total DNA fluorescence in the tail of the comet. Tail moment is calculated as the product of the tail length and the fraction of total DNA in the comet tail. The use of software enables the measurement of a range of different descriptors,

many of which might not be relevant in determining the extent of DNA damage. A large number of factors could influence the shape, size, identification and determination of induced damage, including the scoring criteria, staining techniques, selection of descriptors and/or appearance of 'hedgehog' or 'clouds' [14,16]. Tail length, tail DNA and DNA distribution profile in the tail are primary comet assay measurements (obtained by fluorescent densitometric profiles of the comets). All other measurements are derived from the three primary comet assay measurements [16,20]. Today, % tail DNA is recommended as the best descriptor for DNA break frequencies. However, many researchers still prefer the use of tail moment [21]. It has to be pointed out that these two descriptors are similarly influenced by assay conditions [22–24].

The most common comet assay application today is the one under alkaline conditions allowing the detection of alkali labile sites in addition to DNA double- and single-strand breaks. The less frequently used neutral comet assay allows both single- and double-strand break detection but with less sensitivity than the alkaline version [1,2,25–27]. The sensitivity and specificity of this assay may be further improved by using lesion-specific enzymes capable of converting damaged bases to DNA strand breaks. For example, enzymes able to recognize and remove oxidized bases induced by reactive oxygen radicals, which may arise as a result of mutagenic exposure are used. The most frequently used in this context are *Escherichia coli* endonuclease III (EndoIII) or formamidopyrimidine-DNA glycosylase (Fpg) and human 8-oxoguanine DNA glycosylase 1 (hOGG1) that catalyse the excision of numerous forms of DNA damage such as oxidised purines and pyrimidines [28–30]. Apart from DNA base oxidation, the comet assay is also used for the evaluation of other DNA lesions such as those induced by crosslinking agents in the form of DNA-DNA-interstrand or intrastrand crosslinks and DNA-protein crosslinks using slight changes in the comet assay protocol [31–33].

The combination of the comet assay and fluorescent *in situ* hybridization (comet-FISH) also offers the opportunity to increase the specificity of the assay, allowing for the investigation of gene region-specific DNA damage and repair [19,34–38]. Furthermore, DNA damage and alterations in global DNA methylation status are associated with multiple human diseases and are frequently correlated with clinically relevant information. Hence, assessing DNA damage and epigenetic modifications, including DNA methylation, is critical for predicting both human and animal exposure risk. In line with that, one of the newest modifications of the comet assay includes its adaptation designed to detect global methylation changes (Methy-sens Comet) through enzymatic digestion with two restriction enzymes

(HpaII, MspI) showing generally good repeatability and sensitivity to methylation changes. Moreover, a modified alkaline comet assay, called "EpiComet," that uses the methylation-dependent endonuclease, McrBC, allows for the single platform evaluation of genotoxicity and global DNA methylation status of single-cell populations under user-defined conditions [39,40]. Recently, Cortes-Gutierrez et al. [41] developed a two-dimensional Two-Tailed comet assay (TT-comet) that can differentiate between single-stranded and double-stranded DNA breaks in the same comets in sperm.

Because of the above mentioned, the comet assay has gained worldwide acceptance as a reliable and sensitive tool in fundamental DNA damage research as well as in epidemiology and biomonitoring with several advantages compared to other genotoxicity tests. These advantages include its sensitivity for low DNA damage detection, small number of cells per sample and/or possibility of using both proliferating as well as non-proliferating cells. All of this coupled with low-costs, easy application and quick performance makes this particular assay quite user-friendly. Although there are many advantages, there are also some limitations, mainly related to types of DNA damage that cannot be detected using the comet assay. Other limitations include variations in the procedure between laboratories and evaluation of the gained results [1,2,18,29,42]. Still, its advantages are far greater than the disadvantages making it very popular in genotoxicity studies using not only human but also animal models.

The comet assay can be applied to almost any cell type derived from different organs and/or tissues. Although it is mainly used in human cells both *in vivo* (*ex vivo, in situ*) and *in vitro*, the assay has its application in the evaluation of DNA damage in yeast [43–45], plant [46–49] and animal [29,50–53] cells. Therefore, the comet assay has instantly found its application in different fields; from genetic and environmental toxicology to human epidemiology and biomonitoring [1,3,17,54–64].

Since the comet assay has been used for the evaluation of DNA damage in various animal models worldwide, the first part of the review paper Part I discussed invertebrate species from protozoans up to echinoderms [65], while Part II will give an extensive overview covering vertebrate species from chordates up to mammals (Table 1). As for invertebrates, the comet assay is also extensively used in a variety of vertebrate species. Those include cyclostomata, fishes, amphibians, reptiles, birds and mammals both terrestrial and those found in fresh and marine environments. Apart from the large number of animal species, the assay is also performed on a variety of cells that include blood, liver, kidney, brain, gill, bone marrow and sperm cells. These cells have been used for the evaluation of a broad spectrum of

genotoxic physical and/or chemical agents both *in vitro* and *in vivo* including *in situ* animal biomonitoring studies. The comet assay in vertebrates is most commonly used in laboratory animals such as mice, rats and lately zebrafish but the usage of the assay in domestic and wildlife animals as well as in various ecotoxicological studies is increasing.

2. Chordates

Before continuing to vertebrates, we can describe a few studies done using the comet assay on chordates such as tunicates. Primary blood cell cultures from the intertidal colonial tunicate (*Botryllus schlosseri*) were used as possible model targets for the comet assay using the known genotoxic agent hydrogen peroxide. Although DNA damage was observed in *B. schlosseri* cells, the background level of DNA damage in those cells was rather high not allowing for the positive effects of genotoxic agents to be seen. Nevertheless, the authors concluded that the use of *B. schlosseri* blood cells should be validated through additional research [66]. Hence, the DNA damaging effect of UV irradiation and subsequent repair were measured in *B. schlosseri* indicating significant DNA damage but low repair capacity [67]. Furthermore, *B. schlosseri* haemocytes were analysed during colonial blastogenetic cycle indicating a higher frequency of damaged DNA during take-over than in midcycle stages [68]. Besides, baseline and oxidative DNA damage in vase tunicate (*Ciona intestinalis*) was assessed after *ex vivo* exposure to hydrogen peroxide indicating its sensitivity to oxidative stress [69].

3. Cyclostomata

The comet assay was applied to sea lamprey (*Petromyzon marinus*) sperm and used to describe the relationship between sperm DNA damage and sperm fertilizing ability. Both hydrogen peroxide and UV irradiation were able to increase DNA damage in lamprey spermatozoa indicating that the comet assay can be successfully applied to monitor effects of environmental disturbances and imposed injuries in sea lamprey spermatozoa and possibly other species of ancient fish with acrosomal sperm. Moreover, milt or blood cells were also used to test the impact of bisazir on DNA damage also indicating a potential DNA damaging effect [70].

4. Fishes

When it comes to using the comet assay for environmental risk assessments of water pollutants in vertebrates, fish are among the most studied organisms and there are hundreds of papers dealing with genotoxicity assessment using them as a model. Since genotoxicity assessments in fish, both marine and freshwater, that used the comet assay have been extensively reviewed in several papers by now [29,51,53,71–75], here we will only briefly review their role in genetic and environmental toxicology giving an overview of the variety of species used, found either in freshwater or marine environments.

Fish are found in most of the world's aquatic environments in appropriate habitats and therefore constitute one of the most important groups of sentinel species for monitoring aquatic environmental conditions. These organisms are considered excellent bioindicators of aquatic contamination as they explore the aquatic environment during their whole life cycle, and are capable of accumulating the pollutants present in the water and responding to chemical substances similarly to higher vertebrates. Fish are among the first animal models to which the comet assay was applied as a biomonitoring tool to assess the genotoxicity of pollutants in non-target environmental organisms [76]. Until now more than 300 papers describing DNA damage in fish cells evaluated with the comet assay have been published, which makes fish by far the most adopted animal group in the framework of environment health assessments [53]. The method was adopted for more than 90 different species [53] and as it requires relatively small cell samples, it could be suitable for all 30.000 different fish species, at least theoretically. Until now the method was developed for fish of only a few centimetres in size [77] up to more than two meters [78], including fish that live in various environments; from arctic [79] to tropical waters [80], seas [81] to fresh waters [82], bottom feeder fish [83] and open water fish [84]. Nevertheless, one group of fish, namely cartilaginous fish that include sharks, rays, skates, sawfish and chimaeras, are still completely overlooked and to the best of our knowledge, there are no studies describing the comet assay in *Chondrichthyes*.

Studies on fish were done on several cell types. The most frequently used biological sample is blood, followed by liver and gills; meanwhile ovaries, kidney and muscles as well as sperm cells are much less popular. Blood is popular mainly due to technical reasons such as simple collection of sufficiently large samples, no need to dissociate cells, and no need to sacrifice organisms. Multi-sampling of the fish is possible, and all fish blood cells are nucleated. Hence, with animal welfare in mind, even fish species low in abundance could be sampled without affecting their natural population. Gills and liver are the first organs in direct contact with water and pollutants and have an important role in xenobiotic metabolism and

accumulation, respectively. It has been demonstrated that different tissues of the same animal have different susceptibility to DNA damage, which could be due to different degrees of pollutant accumulation (depending on the biochemical characteristics of specific chemical), variation in excision repair and metabolic activity, anti-oxidant concentrations, or differential expression of receptors and cellular components that interact with the pollutants [85–87]. According to these and other available data, it is impossible to conclude which organ is the most sensitive and it seems as though this depends on the characteristics of the sample. Nevertheless, the multi-tissue approach in aquatic biomonitoring studies is rare but highly recommended. Recently Hylland et al. [81] showed that the samples already embedded in agarose on gelbond films can be stored in lysis for several weeks which is a benefit in field studies. Additionally, the comet assay was modified also for whole fish embryos, usually zebrafish (*Danio rerio*) [88–90]. Recent studies have demonstrated that the embryos are a suitable biological model for environmental monitoring, as they are sensitive towards genotoxins in river water [90] and marine sediments [89], as well as chemicals such as fungicides [88] and polycyclic aromatic hydrocarbons (PAHs) [89].

Environmental biomonitoring to assess the genotoxic potential of aquatic environments has been carried out with various fish species, mostly those that are economically and commercially important and have an important role in the food web (and so are ecologically important), such as; carps (*Cypriniformes*), percoidei (*Perciformes*), salmonidaes (*Salmoniniformes*), gadidaes (*Gadiformes*) etc. Nevertheless, some studies have shown large species differences in genotoxic responses in fish sampled at the same sites [81].

In freshwater environments, the zebrafish (*D. rerio*) is the most commonly used fish species for genetic and environmental toxicology and lately it has also become one of the most popular laboratory model organisms due to several reasons such as their small size, easy husbandry, early morphology, high fecundity, and small and transparent embryos. Moreover, an advantage of the zebrafish embryo model is related to animal welfare and alternative *in vitro* methods for the testing of chemicals as the earliest life-stages of zebrafish are considered an *in vitro* test system [91–96]. Apart from using zebrafish as an *in vivo* model system, in addition to the zebrafish embryo model that can be regarded as an *in vitro* method, the use of zebrafish cultured cell lines in genetic toxicology is also on the rise [91,97–101]. Up to now there have been more than 100 papers describing the zebrafish model both *in vivo* and *in vitro* using the comet assay for the assessment of different physical and/or chemical agents such as gamma rays [102,103], X-rays [104], pesticides [105], insecticides [106], fungicides [88,107], herbicides [108], mycotoxins [109], pharmaceuticals [98,99,110–112], heavy metals

[108,113,114], PAHs [89], nanoparticles [115–118], flame retardants [119], sewage effluent [120], waste material [121] as well as model toxicants such as benzo(a)pyrene (B[a]P), methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) [122,123].

Besides zebrafish as the fish model that lives in a freshwater environment, several other predominantly freshwater as well as brackish water species are used for the assessment of different contaminants using the comet assay; they include brown trout (*Salmo trutta fario*) [85], marble trout (*Salmo marmoratus*) [124], trout (*Salmo cenerinus*) [124], rainbow trout (*Oncorhynchus mykiss*) [125,126], common carp (*Cyprinus carpio*) [76,82,127], major carp (*Catla catla*) [128], mrigal carp (*Cirrhinus mrigala*) [128], gibel carp (*Carassius auratus gibelio*) [129], common barbell (*Barbus barbus*) [130], common bleak (*Alburnus alburnus*) [131,132], freshwater bream (*Abramis brama*) [133], silver bream (*Abramis bjoerkna*) [134], white-eye bream (*Abramis sapa*) [134], rohu (*Labeo rohita*) [135–139], orangefin labeo (*Labeo calbasu*) [86], brown bullhead (*Ameiurus nebulosus*) [76,140], chub (*Leuciscus cephalus*) [141–143], European chub (*Squalius cephalus*) [87,144–146], pale chub (*Zacco platypus*) [147], climbing perch (*Anabas testudineus*) [148], small-scaled pacu (*Piaractus mesopotamicus*) [149,150], eastern mosquitofish (*Gambusia holbrooki*) [77], Nile tilapia (*Oreochromis niloticus*) [151–154], Mozambique tilapia (*Oreochromis mossambicus*) [155], chameleon cichlid (*Australoheros facetus*) [156], pool barb (*Puntius sophore*) [86], yellowtail tetra (*Astyanax altiparanae*) [118,157], Chinese rare minnow (*Gobiocypris rarus*) [158], three-spined stickleback (*Gasterosteus aculeatus*) [159], African sharptooth catfish (*Clarias gariepinus*) [151,160], striped dwarf catfish (*Mystus vittatus*) [86,161], South American catfish (*Rhamdia quelen*) [162–164], Asian stinging catfish (*Heteropneustes fossilis*) [165], suckermouth catfish (*Hypostomus ancistroides*) [166], iridescent shark fish (*Pangasius sutchi*) [167,168], wolf fish (*Hoplias malabaricus*) [80], Eastern mudminnow (*Umbra pygmaea*) [169], streaked prochilod (*Prochilodus lineatus*) [83,170], spotted snakehead (*Channa punctatus*) [161,171] and striped snakehead (*Channa striatus*) [165]. The comet assay in freshwater fish is mainly used for the evaluation of different pollutants *in vivo* and for biomonitoring purposes of contaminated sites *in situ*.

Additionally, several freshwater species commonly held as pet aquarium fish such as gold fish (*Carassius auratus*) [172–176] and guppies (*Poecilia reticulate* and *Poecilia vivipara*) [177–180] are also used for the assessment of DNA damage using the comet assay. The aforementioned zebrafish (*D. rerio*), belonging to the minnow family (*Cyprinidae*) of the order *Cypriniformes*, is also commonly held as a pet aquarium fish.

Before crossing over to marine species, we should mention that there are a few migrating species used in genotoxicity studies employing the comet assay that are either anadromous (migrating from the sea up into fresh water to spawn) such as salmon (*Salmo salar*) [181] and Russian and Siberian sturgeons (*Acipenser gueldenstaedtii* and *Acipenser baerii*, respectively) [182] or catadromous (migrating from fresh water down into the sea to spawn) such as eel (*Anguilla anguilla*) [183–186].

There are fewer studies utilizing the comet assay with marine and coastal fish. This could be attributed to logistical problems associated with performing the comet assay at sea [51]. Nevertheless, there are ranges of species that are used, including those that are commercially important including species belonging to the Sparidae family such as gilt-head bream (*Sparus aurata*) [126,187–190] or Moronidae family such as European bass (*Dicentrarchus labrax*) [84,191–195]. There are several other marine species used such as corkwing wrasse (*Symphodus mellops*) [196], turbot (*Scophthalmus maximus*) [197,198], hornyhead turbot (*Pleuronichthys verticalis*) [199], flatfish dab (*Limanda limanda*) [81,200], common sole (*Solea solea*), [201,202], English sole (*Pleuronectes vetulus*) [199], Blackbelly rosefish (*Helicolenus dactylopterus*) [78], European conger (*Conger conger*) [78], polar cod (*Boreogadus saida*) [79], mullet (*Mugil cephalus*) [84], olive flounder (*Paralichthys olivaceus*) [203,204], sheepshead minnow (*Cyprinodon variegatus*) [205], haddock (*Melanogrammus aeglefinus*) [81], Florida pompano (*Trachinotus carolinus*) [206], yellow croaker (*Pseudosciaena crocea*) [207], marine medaka (*Oryzias melastigma*) [208], milkfish (*Chanos chanos*) [209], Mediterranean rainbow wrasse (*Coris julis*) [210,211], tiger perch (*Terapon jarbua*) [212,213], striped beak perch (*Oplegnathus fasciatus*) [214], orange-spotted grouper (*Epinephelus coioides*) [215], Mediterranean killifish (*Aphanius fasciatus*) [216], eelpout (*Zoarces viviparus*) [217] and even sea horse (*Hippocampus reidi*) [218]. The comet assay in marine fish is mainly used for the evaluation of different pollutants and for biomonitoring purposes as well as for the evaluation of sperm DNA integrity in terms of cryopreservation, especially in species that have commercial value.

5. Amphibians

The rapid decline in amphibian population in recent decades is a cause for major concern. Over the years, amphibians have been recognized as excellent bioindicators of environmentally related stress since they are very sensitive to environmental stressors. This is due to their early aquatic-dependent development stage and a highly permeable skin [53,219].

Therefore, there are numerous studies using amphibians for the evaluation of environmental pollution using the comet assay either *in situ* or under laboratory conditions. Moreover, an alternative approach to the collection of indigenous tadpoles is also used, such as placing caged tadpoles at the sites of interest for short-term exposures to environmental contaminants. The most used amphibians with regard to the comet assay are frogs and toads. Since they have a specific life cycle going through metamorphosis, several stages of their development are used for the assessment of DNA damaging effects. Tadpoles also possess several favourable characteristics as sentinel organisms. They are found in large numbers, can be easily collected with a net and maintained in laboratory conditions. Because they feed on aquatic plant life, they are exposed to contaminants in the vegetation, in addition to contaminants in the sediment and water. Finally, they are restricted in their movement and confined to the body of water in which they hatch; hence they provide an index of genotoxicity of a specific water body [220]. Besides tadpoles, studies are also conducted on fully developed specimens. Studies regarding usage of amphibians have only shortly been reviewed till now [53,71,73].

The first study that reported use of the comet assay on amphibians dates back to 1996 [220]. In that study authors used erythrocytes from two species of tadpoles, namely green frog (*Rana clamitans*) and American toad (*Bufo americanus*) exposed in laboratory conditions to MMS in order to evaluate the feasibility of the comet assay in an amphibian model for the detection of adverse effects of environmental pollutants. Moreover, the study provided a minor modification of the comet assay protocol for use in amphibians and this was found to be highly sensitive and reproducible. Since then, a number of studies have been conducted applying the comet assay procedure to a variety of amphibian species both in larval and adult stages using different cells such as blood, liver and sperm, erythrocytes being the most commonly used cell type. The conducted studies mainly focused on the evaluation of environmental stressors such as agrochemicals and heavy metals *in vivo*.

Both tadpoles and adult specimens of African clawed frog (*Xenopus laevis)*, American bullfrog (*Rana catesbeiana*), Montevideo tree frog (*Hypsiboas pulchellus, Boana pulchella*), spot-legged tree frog (*Polypedates megacephalus*), dark-spotted frog (*Rana nigromaculata* and *Pelophylax nigromaculata*), rice frog (*Rana limnocharis*), Lesser Treefrog (*Dendropsophus minutus*), Lesser Antillean whistling frog (*Eleutherodactylus johnstonei*), Creole frog (*Leptodactylus chaquensis*), barker frog (*Physalaemus cuvieri*), common toad (*Rhinella arenarum*), giant toad (*Rhinella marina*), Chinese toad (*Bufo bufo gargarizans*), Mongolian toad (*Bufo raddei*), Indus valley toad (*Bufo stomaticus*), western toad (*Bufo boreas*) as well as of Iberian ribbed newt (*Pleurodeles waltl*) have been used for the

evaluation of DNA-damaging effects of different contaminants such as pesticides (chlorpyrifos and imidacloprid) [221–226], herbicides (2,4-D amine, acetochlor, atrazine, butachlor, chlorimuron-ethyl, flurochloridone, glyphosate, imazethapyr, metalochlor, metribuzin, paraquat) [223,227–237], fungicides (captan) [238], pesticides [239,240], antiparasitic agents [239], persistent organic pollutants (POPs) [219] as well as other agrochemicals [241] and poultry litter [242]. These studies indicated the DNA damaging effects of selected chemicals as well as using amphibians as sentinel organisms for the evaluation of environmental pollution.

Several reports on the effects of exposure to petrochemical contaminants [243], sulphur dyes [244], B[a]P [245], EMS [245], MMS [220,245,246], sodium arsenite [247], 4-nitroquinoline-1-oxide [248], carbon nanotubes [249] as well as antibiotics and cytostatic drugs [248,250] may also be found, indicating that the comet assay can be used as a sensitive and suitable method for detecting the genotoxicity of certain chemicals in African clawed frog (*X. laevis*), western clawed frog (*X. tropicalis*), rice frog (*R. limnocharis*), green pond frog (*R. hexadactyla*), green frog (*R. clamitans*), Mongolian toad (*B. raddei*), American toad (*B. americanus*) and Lesser Antillean whistling frog (*E. johnstonei*).

Furthermore, amphibians were used as a model for the assessment of the DNA-damaging effects of several minerals and heavy metals such as iron ore, iron, manganese [251], cadmium [252–254], lead [255] and chromium [256]. These studies were also conducted on tadpoles and adult specimens of several amphibian species, namely African clawed frog (*X. laevis*), dark-spotted frog (*R. nigromaculata*), rice frog (*R. limnocharis*), bullfrog (*Lithobates catesbeianus*), Asian common toad (*Duttaphrynus melanostictus*) as well as Iberian ribbed newt (*P. waltl*). The results obtained on erythrocytes and testes demonstrated that heavy metals present in aquatic environment can cause DNA damage and reproductive toxicity in amphibians and hence could contribute towards their population decline.

Additionally, biomonitoring using amphibians has also been performed to evaluate contamination of coal mining areas [257], illegal waste dumping sites [258], draining water from dredged sediments [259], polluted lakes and water bodies [260–262] as well as residues from municipal solid waste incineration [263]. These studies also used tadpoles and mature specimens of African clawed frog (*X. laevis*), green frog (*R. clamitans*), European edible frog, (*R. esculenta*), blacksmith tree frog (*Hypsiboas faber*), Eurasian marsh frog (*Pelophylax ridibundus*), Northern leopard frog (*R. pipiens*) and American toad (*B. americanus*). The species analysed seem to have been good bioindicators for detecting the genotoxic effects of chemical environmental hazards.

Amphibians were also used for the evaluation of radiation-induced DNA damage both *in vitro* and *in vivo*. European common frog (*R. temporaria)* peripheral blood cells were used for the *in vitro* assessment of X-rays, indicating the DNA-damaging effect of ionising radiation [264]. Besides, *X. laevis* erythrocytes were used to investigate the possible genotoxic effects of high peak-power pulsed electromagnetic fields *in vitro*. Results showed that microwave radiation did not cause any non-thermal genotoxic effect in frog erythrocytes under culture conditions [265]. On the other hand, chronic exposure of southern toad (*Anaxyrus terrestris*) to low dose rate ionizing radiation resulted in decreased DNA damage in red blood cells, indicating a cellular repair response. The authors concluded that the complex effects from chronic radiation in the lower dose rate ranges may trigger growth and cellular repair mechanisms in amphibian larvae [266].

Moreover, the effects of storage and cryopreservation on sperm DNA integrity were also assessed using the comet assay as an important parameter of semen quality. In European common frog (*R. temporaria*) it was shown that the integrity of sperm DNA increases during refrigerated storage although this does not affect spermatozoa motility and/or fertility [267]. Besides, the comet assay was used in African clawed frog (*X. laevis*) spermatozoa in order to validate other tests designed for DNA damage assessment [268].

6. Reptiles

There are several studies involving the comet assay in reptiles. The assay is used in several species of lizards, snakes, turtles and crocodiles and various cell types derived from blood and organs. Reptiles are mainly used as sentinel species for a broad spectrum of environmental studies dealing with exposure to environmental agents such as agricultural chemicals and heavy metals.

Blood cells (erythrocytes) from wall lizard (*Podarcis sicula*) were used for the assessment of fungicide thiophanate-methyl genotoxicity. The observed DNA damage showed a significant increase in comet length in relation to exposure time which was paralleled by a reduction in head size [269]. The authors concluded that if such genotoxic effects arise so clearly in an ectothermal vertebrate, prolonged exposure to thiophanate-methyl is to be considered a cytogenetic hazard. Moreover, the ovarian follicle cell from *P. sicula* were also used for the evaluation of oral administration of cadmium, which induced DNA-damaging effects [270]. DNA damage in parthenogenetic and bisexual Darevskia rock lizards (*D. armeniaca* and *D. raddei*, respectively) from areas with different levels of soil

pollution was evaluated for environmental genotoxicity. The results obtained showed a clear relationship between the pollution level of lizard habitats and the frequency of DNA damage in the comet assay [271]. In the wild population of green iguanas (*Iguana iguana*), the comet assay revealed genotoxic potential derived from exposure to coal mining activities. Animals gathered from close proximity to coal mining areas showed the highest percentages of DNA damage compared to those from sites far from the coal dust source, indicating that living around coal mining fields may result in an increase of DNA lesions in reptile blood cells [272]. Tegu lizard (*Tupinambis merianae*) erythrocytes were assayed to determine baseline values of the comet assay descriptors as well as for the effects of age, sex, and nest of origin on spontaneous genetic damage employing also increasing concentrations of hydrogen peroxide as a known genotoxic agent to induce DNA damage for the purpose of optimising the protocol. This was done to improve the reference data for future *in vivo* studies of xenobiotic exposure in this species. Although comparable to those reported in other reptilian species, baseline values of genetic damage showed that age is an intrinsic factor that should be considered to avoid misunderstandings of results in future biomonitoring studies. Nevertheless, the results proposed the tegu lizard for future *in vivo* studies to evaluate the genotoxicity of different agents, including those possibly affecting it in its natural habitat [273,274]. Furthermore, genotoxicity was reported in tegu lizard neonates after embryonic exposure to glyphosate. A significant increase in DNA damage was observed in tegu lizard erythrocytes providing information about the undesirable effects of the glyphosate-based herbicide formulations on this particulate species that lives in areas permanently exposed to several pesticide formulations [275].

Blood cells from several snake species have been assayed using the comet assay. In the erythrocytes from young and adult specimens of rattlesnake (*Crotalus durissus terrificus*), jararaca (*Bothrops jararaca*), urutu (*Bothrops alternatus*) and jararaca pintada (*Bothrops neuwiedii*) the comet assay parameters varied depending on the species, although undamaged or less damaged cells generally predominated. *B. neuwiedii* showed the lowest frequency of cells with damaged DNA whereas *C. d. terrificus* had the highest frequency of damaged cells, possibly due to the abundance of alkaline-sensitive DNA sites. Moreover, there were no marked differences between developmental stages, except for undamaged erythrocytes of *C. d. terrificus* and for the most damaged erythrocytes of *B. jararaca*. The authors concluded that the observed differences may lead the cells to different resistances to unfavourable environmental conditions [276]. In the rattlesnake (*C. d. terrificus*) infected with the

protozoan (*Hepatozoon spp.*) a higher level of damage in the erythrocyte DNA was found compared to the erythrocyte DNA from non-infected snakes [277].

The comet assay in both fresh/brackish water and sea turtles was done using blood and liver cells. The fresh/brackish water common snapping turtle (*Chelydra serpentina*) eggs were exposed to chemically or physically dispersed water accommodated to fractions of weathered light crude oil containing PAHs. As PAHs are known to elicit DNA strand breakage, the comet assay was employed in order to evaluate DNA damage on a subset of hatchlings in liver cells. Despite the accumulation of PAHs in eggs, the authors failed to observe increased DNA damage in hatchlings. Although the authors used the snapping turtle as a surrogate model for the sea turtle this could have not been explicitly determined even though the egg shells of snapping turtles possess more defined pores than those of sea turtles, implying that accumulation by snapping turtle eggs may exceed that by sea turtle eggs, leading to less accumulation and lower effects when exposed to the same solutions. This however does not imply that turtle embryos are universally tolerant to exposure to certain chemicals [278]. Erythrocytes of the Colombian slider turtle (*Trachemys callirostris*) that lives in rivers were used as a model to optimize the comet assay and to establish background level of DNA damage in this species and thereby evaluate its potential as a sentinel species for monitoring genotoxic effects in freshwater environments. In addition to captive individuals which served as negative controls, a comparison was made with individuals from polluted field sites which had higher DNA damage compared to control individuals [279]. Additionally, freshwater Geoffroy's side-necked turtle (*Phrynops geoffroanus*) erythrocytes and liver cells were used to assess the impact of contaminated environment by human activities (sewage and industrial wastewater effluents) using B[a]P as a model compound indicating an increase in hepatic and blood cell DNA damage [280]. DNA damage and repair efficiency in erythrocytes of the painted turtle (*Chrysemys picta*) was evaluated in relation to UV exposure indicating greater DNA damage in juvenile turtles than in adults [281]. Besides freshwater species, the comet assay was conducted in erythrocytes of Mediterranean loggerhead sea turtle (*Caretta caretta*) also using an integrated non-destructive protocol. The obtained results demonstrated that the assay is useful for detecting possible genotoxic effects in these threatened species and that the non-destructive protocol could be applied to other marine ecosystems and other sea turtle species [282,283]. The comet assay (CometChip Platform) was also applied in semiaquatic box turtle (*Terrapene carolina*) and the primarily terrestrial specie red-eared slider (*Trachemys scripta elegans*) in order to assess DNA damage in field-collected blood samples. Endogenous levels of DNA damage were identical between the two species, although the

authors discovered some sex-linked differences and changes in DNA damage accumulation [284].

As for crocodiles, caimans were used to validate the comet assay and later on to test several agricultural chemicals on the extent of DNA damage in blood cells. The comet assay was first validated in broad-snouted caiman (*Caiman latirostris*) marking this species as a suitable sentinel organism for the genotoxic assessment of environmental pollutants [285]. Later on, caimans were used in several studies to evaluate different xenobiotics, namely glyphosate, endosulfan and cypermethrin either alone or in mixture. After *in ovo* exposure or after venepuncture of juvenile specimens, the comet assay revealed a significant amount of DNA damage in erythrocytes of exposed animals [286–289]. Moreover, the same species was used to determined DNA damage repair and parameters of oxidative DNA damage using Fpg and Endo III [288]. Although reptiles are not that commonly used in ecotoxicological studies, the identification of different sentinel species and biomarkers that can be used on them in order to evaluate genome damage in polluted areas is important in terms of the survival of these species. In addition, the assessment of sperm DNA fragmentation in the saltwater crocodile (*Crocodylus porosus*) was done using a two-tailed comet assay in relation to cryopreservation [290].

7. Birds

Several studies used the comet assay in birds both from the species used commercially in food production such as poultry or as pets such as parrots as well as wildlife species used mainly for biomonitoring purposes for the assessment of environmental pollution. Research regarding the application of birds in the comet assay studies have only shortly been reviewed [29,53,71]. The selected animals were either collected from different sites or exposed to different agents under laboratory/natural conditions.

The comet assay is widely used for the assessment of DNA damaging effects in species used commercially for food production such as chickens and turkeys, namely *Gallus gallus domesticus* and *Meleagris gallopavo*, respectively. The assay is used for the evaluation of different chemicals that can be present in food production and might have negative impact on genome integrity. Effects of different mycotoxins (aflatoxin B_1, deoxynivalenol and T-2 toxin), flavouring chemicals and fragrance materials as well as some other food contaminants were evaluated in blood, liver and muscle cells of chickens and turkeys indicating their DNA-damaging potential [291–300].

The beneficial potential of certain chemicals (β-glucans, equol, vitamin E) and specially developed feed additives that protect animal health as well as deficiency of certain micronutrients and effects of feed restrictions was also assessed. Results showed protective properties against DNA damaging effects of mycotoxins and hydrogen peroxide or the type of diet used during the production indicating a genoprotective effects, in addition to the DNA damaging effects of certain stressors [291–293,297,299,301–304].

The comet assay was also used for monitoring DNA integrity of poultry spermatozoa during cryopreservation and short-term liquid storage, processes that are fundamental both for the practice of artificial insemination, and for the conservation of genetic resources in cryobanks. Although results showed an increase in DNA damage during cryopreservation that was dependent on the conditions as well as the species used, they also suggest a low sensitivity of the used spermatozoa to DNA fragmentation that should not be considered a major cause of sperm injuries during cryopreservation [305–307]. In addition to poultry, the effects of cryopreservation on DNA integrity were assessed in griffon vultures (*Gyps fulvus*) showing no differences in DNA fragmentation after freezing and thawing. This result suggests that semen cryopreservation can be considered a useful tool in the conservation of griffon vulture genetic resources [308]. Besides, the comet assay was used to study the effect of freezing/thawing on DNA integrity in breast fillets and liver cells of frozen chickens [309].

Evaluation of DNA integrity was also done with regard to pathological conditions that are frequently present in poultry production such as bacterial or viral infections and parasitic diseases. It was found that modern methods of industrial poultry and egg production systems involve stressful practices that stimulate avian pathogenic *E. coli* activity causing endotoxic shock. Thus, hens injected with *E. coli* showed an increase in DNA damage in the brain and liver cells [310]. DNA-damaging effects can also be found in Marek's disease as the result of a herpesvirus for which the chicken is a natural host [311]. Another problem is also coccidiosis, a major intestinal parasitic disease of poultry associated with severe economic losses and welfare issues that can also affect DNA integrity [312].

Embryos, blood and bone marrow cells of Japanese quails (*Coturnix japonica*) were used for the assessment of different physical and/or chemical agents such as cellular phone radiation, pesticides and herbicides [313–315] as well as for the evaluation of beneficial effects of dietary additives [316]. Besides quails, the comet assay was conducted to evaluate the sub-chronic effects of nitrates in drinking water on plasma lymphocytes of the red-legged partridge (*Alectoris rufa*) indicating oxidative DNA damage upon exposure [317] and on barbary partridge (*Alectoris barbara*) to evaluate differences in semen freezability [306].

Rock pigeons (*Columba livia*) were used as a sentinel species for the assessment of urban air pollution, showing a higher degree of DNA damage in their erythrocytes compared to control specimens [318,319]. The same species was used to test the effects of fenvalerate insecticide indicating significant DNA-damaging effects in bone marrow cells compared to control birds [320]. Small parrots such as budgerigars (*Melopsittacus undulatus*) that are usually kept in captivity were used for the assessment of DNA-damaging effect in relation to behavioural activity and dietary habits [321,322]. Moreover, wild-caught captive greenfinches (*Carduelis chloris*) and common yellowthroat (*Geothlypis trichas*) were used to test the DNA-damaging effects of paraquat and carotenoid-based sexual ornamentation, respectively [323,324].

Apart from the commercially available species used in food production but also as pets, the comet assay is used in wildlife birds mainly to study the impact of environmental pollution from both physical and chemical agents. Barn swallows (*Hirundo rustica*) were used as a model to investigate levels of DNA damage in blood cells of birds inhabiting the Chernobyl region in order to evaluate whether chronic exposure to low-level radioactive contamination continues to induce genetic damage in free-living populations of animals. Results showed increased DNA migration in barn swallows living in areas surrounding Chernobyl compared with swallows from low-level sites indicating that chronic exposure to radioactive contaminants, even 20 years after the accident at the Chernobyl nuclear power plant, continues to induce DNA damage in cells of free-living animals [325]. Another study investigated whether exposure of barn swallow nestlings to low dose ionizing radiation from Fukushima increases genetic damage to their peripheral erythrocytes showing that exposure to radioactive contamination did not correlate with higher genetic damage in nestlings. The authors concluded that the barn swallow is a good model species for investigating the effects of radioactive contamination due to its abundance, philopatry to the once chosen breeding site, and availability of control as well as affected populations. However, different species may vary in their radiosensitivity and the lack of an effect in one species does not necessarily imply that all others are similarly unaffected [326].

Both white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) were used as models for the assessment of ecological disasters as a consequence of a massive toxic spillage of acid waste rich in heavy metals. DNA damage in blood cells of both bird species showed a significant increase compared to birds from non-contaminated areas not only after disaster but even years after the toxic accident [327–329]. Additionally, DNA damage using the comet assay was evaluated in red blood cells of royal terns (*Sterna maxima*) from several locations

subjected to different contaminant loads showing differences in the degree of DNA damage in relation to the collection site [330]. In line with the above mentioned, the comet assay proved to be a reliable tool for the assessment of DNA damaging effects in a species used for human consumption with regard to effects of different hazards present in the food production chain. Wildlife species also proved to be excellent models for the evaluation of ecological threats from a large number of various toxic agents.

8. Mammals

The comet assay in mammals is mainly done using laboratory mammals such as mice and rats, which are the most commonly used experimental animal models in genetic toxicology. The comet assay in rodents is done both *in vitro* and *in vivo* using multiple organs for the assessment of different DNA-damaging agents. Since studies regarding the use of laboratory rodents have been extensively reviewed [17,29,331–336], we will mainly focus on studies done on mammals not as commonly used with the comet assay and wildlife mammals used for environmental biomonitoring.

8.1. Laboratory mammals

Mice and rats have been widely used as animal models for the evaluation of the DNA damaging effects of a variety of chemicals using the comet assay procedure. There are several very specific guidelines for both *in vitro* and *in vivo* genetic toxicology testing using the comet assay, including study design, sample preparations as well as methodology [17,331,336]. Multiple organs of mice and rats such as blood, liver, kidney, brain, lungs and bone marrow have been used for the genotoxicity testing of a large range of chemicals. In autumn 2014, the OECD published test guideline TG489 for the *in vivo* mammalian alkaline comet assay [337] summarizing the basics and limitations, principle of the method, verification of laboratory proficiency, historical control data, and a detailed description of the method.

Sasaki et al. [332,338] collected an extensive list of chemicals from various different classes, e.g., PAHs, alkylating compounds, nitroso compounds, food additives, etc., that caused DNA strand breaks in different mouse organs. Interestingly, DNA damage has been

644 detected not only in target organs but also in non-target organs [338]. The comet assay in
645 rodents is also used to detect DNA damage induced by physical agents, such as ionizing γ-
646 radiation [339–342], radiofrequency radiation [343–345], microwave radiation [346,347]
647 and/or UV radiation [348–350] as well as loud noise as one of the major environmental
648 threats to public health [351–353]. These studies are often performed in order to evaluate the
649 protective potential of various natural products, e.g. essential oils, or plant extracts, as many
650 of them possess photo/radioprotective effects [339–342,347,354].

651 The rapid expansion in the field of nanotechnology and extensive use of nanoscale
652 products has also resulted in an increased number of toxicological studies devoted to testing
653 the biosafety of nanomaterials in rodents. Due to the many specific physico-chemical
654 characteristics of nanomaterials (e.g. size, shape, surface charge, coating), a plethora of
655 studies has already been performed in order to evaluate genotoxicity using the comet assay.
656 There are several reviews [355–357] summarizing both *in vitro* and *in vivo* studies dealing
657 with engineered nanomaterials, with the comet assay as the most frequently used method,
658 either as a classical procedure or with the incubation using specific enzymes to detect
659 oxidative DNA damage.

660 Several transgenic animals are available that can be utilized in this field. Big Blue
661 mice, Muta mice and gpt delta mice were the first transgenic animals developed for
662 mutagenesis [358,359], and about 10 years later transgenic rats were developed [360,361].
663 Transgenic models allow evaluating genotoxicity in several organs, making them valuable for
664 investigating *in vivo* genotoxic effects and repair mechanisms after exposure not only to
665 chemical agents, but also to nanomaterials [362].

666 The route of exposure is a key factor of the genotoxicity of a chemical due to its mode
667 of action and selecting the appropriate administration route may be important when assaying
668 multiple organs [363,364]. In rodents, different routes of exposure have been used to study the
669 genotoxicity of different chemicals or nanomaterials, e.g., intravenous/systemic [365–367],
670 intraperitoneal [368–371], oral [372–375] as well as inhalation [376–378].

671 The comet assay performed on cells isolated from experimental animal models
672 represents an important test used in genotoxicity studies, and not only provides insight into
673 the genotoxicity of various compounds but is also suited for studying their underlying
674 mechanisms of action. At a workshop hosted by European Centre for the Validation of
675 Alternative Methods (2008), it was concluded that the integration of genotoxicity endpoints
676 into a repeated-dose toxicity study in a scientifically justified manner can reduce the number

of animals used in toxicity testing [379], which is in accordance with EU Directive 2010/63/EU [96].

In addition to the use of laboratory-reared animals for testing the genotoxicity of chemicals with the comet assay procedure, wild rodents can also be used as a valuable model in pollution monitoring and environmental conservation [380–383].

8.2. Domestic mammals

Animals kept as pets may be considered sentinels for environmental factors to which humans could be exposed. Therefore, they can be used as a surrogate for human exposure as well as for monitoring the impact of these agents to which they are exposed by living in the vicinity of humans. Most of the studies done so far were conducted on canine and feline models. Several breeds of both cats and dogs were used for the evaluation of different chemical and/or physical agents on the extent of DNA damage. Application of the comet assay for assessing levels of DNA damage in feline cells was done for future use in studying the effects that nutritional supplementation may have on protecting cells from free-radical damage by exposing leukocytes to a range of hydrogen peroxide concentrations [384]. Afterwards, several studies used different breeds of domestic as well as wildlife cats (*Felis silvestris catus* and *Prionailurus viverrinus*) for the assessment of antioxidant supplementation in the reduction of DNA damage in blood cells [385], the impact of airport security screening on the DNA integrity of frozen spermatozoa [386] and commonly used antibiotics [387].

Studies on dogs have also been performed using several breeds and different cell types, aimed at the evaluation of radiation-induced DNA damage [388,389], hydrogen peroxide [384], cigarette smoke [390] as well as in regard to acute bacterial cystitis [391]. The role of dietary antioxidants or adrenal steroids for protection against DNA damage was also evaluated [392–396]. Dogs from different regions of the city of São Paulo, Brazil were used to evaluate the extent of DNA damage in the olfactory and respiratory epithelia, indicating increased DNA-damaging effects in relation to environmental factors [397]. Besides, the comet assay is also used for detection of DNA damage in canine sperm [398].

Apart from pets, several other domestic species are used, such as horses [399–405], donkeys [399,406], bulls [407–411], goats [412,413], sheep [414,415] and boars [416,417]. The comet assay on those animals is done on sperm to test the semen quality in regard to

cryopreservation and artificial insemination. Moreover, the effects on DNA integrity of toxins from feed [418], anaesthesia [419] and due to certain infections [420] are also explored.

8.3 Wildlife mammals

Besides being used for the genotoxicity testing of chemical and/or physical agents in laboratory and domestic mammals, the comet assay is also used in a variety of wildlife species as a valuable test for pollution monitoring and environmental conservation both in marine and terrestrial environments.

Several metatherian species such as short-beaked echidna (*Tachyglossus aculeatus*), common wombat (*Vombatus ursinus*), koala (*Phascolarctos cinereus*) and eastern grey kangaroo (*Macropus giganteus*) were used for the evaluation of sperm integrity using the comet assay [421–423]. The studies indicated that the sperm DNA of the marsupial species was more sensitive to oxidative stress than the spermatozoa of eutherian species [421].

The comet assay was also used in blood cells of small terrestrial rodents such as house mouse (*Mus musculus*), wild Algerian mouse (*Mus spretus*) plateau mouse (*Peromyscus melanophrys*), southern pygmy mouse (*Baiomys musculus*), Merriam's kangaroo rat (*Dipodomys merriami*) and meadow voles (*Microtus pennsylvanicus*) for the assessment of polluted sites [272,381,383,424–426]. Results indicated that rodents living in the contaminated area bear a burden of genetic damage and can be used as a valuable test in pollution monitoring and environmental conservation and not only as a model in laboratory conditions. The effects of short-term voluntary wheel running was also evaluated in lymphocytes and hepatocytes of the short-tailed field vole (*Microtus agrestis*) indicating no effects on oxidative DNA damage [427]. The comet assay was also used for the evaluation of the impact of caloric restriction on DNA damage in minks (*Neovison vison*) indicating lower DNA damage in mink females on moderate diet restriction [428].

Several species of bats were also used for the biomonitoring of polluted sites. DNA-damaging effects of low dose ionising radiation were evaluated in a population of Cape horseshoe bats (*Rhinolophus capensis*) residing in an abandoned monazite mine, resulting in increased lymphocytes genome damage in the exposed population [429]. Heavy metal toxicity was also assessed in velvety free-tailed bat (*Molossus molossus*), Mexican free-tailed bat (*Tadarida brasiliensis*) and diminutive serotine (*Eptesicus diminutus*) collected in a coal mining area indicating DNA-damaging effects of heavy metal pollution in peripheral blood leukocytes [430]. Additionally, in the banana bat (*Neoromicia nana*) exposed to pollutants

744 from wastewater treatment works significantly higher DNA damage was noted [431]. These
745 results suggest that bats can be used as adequate sentinel species for the detection of genome
746 damage in polluted sites.

747 In addition, the comet assay was applied in large wildlife mammals. The assay was
748 used for the evaluation of sperm DNA integrity of three species of rhinoceros; black
749 rhinoceros (*Diceros bicornis*), greater one-horned rhinoceros (*Rhinoceros unicornis*) and
750 white rhinoceros (*Ceratotherium simum*) indicating that frozen-thawed rhinoceros sperm
751 exhibited DNA damage shortly after thawing [432]. Moreover, since heavy metal exposure
752 can cause great harm to Siberian tigers (*Panthera tigris altaica*) in a natural environment, the
753 comet assay was applied *in vitro* for the evaluation of cadmium-induced DNA damage in tiger
754 fibroblasts. Results showed DNA-damaging effects in cadmium-treated cells and this could
755 later on serve in developing interventions to treat and prevent cadmium poisoning [433]. The
756 comet assay was also done on several species of monkeys. DNA damage was assessed in
757 cynomolgus monkey (*Macaca fascicularis*) lymphocytes exposed to stainless steel welding
758 fumes indicating damaging potential [434]. The DNA-damaging effects of rhesus macaque
759 (*Macaca mulatta*) sperm following freezing-thawing was also assessed in regard to
760 cryopreservation [435].

761 Besides terrestrial mammals, marine mammals are also used as a model for evaluating
762 environmental pollution. Several studies were done using the blood cells of wild bottlenose
763 dolphins (*Tursiops truncatus*) in order to evaluate the usefulness of the comet assay to detect
764 DNA strand breakage indicating variability in DNA damage between species from different
765 locations. Moreover, differences in the concentrations of genotoxic agents between locations
766 may have been one of the causes of higher DNA strand breaks in assayed dolphins [436,437].
767 Blood cells and fibroblast of bottlenose dolphins (*T. truncatus*) and Indo-Pacific humpback
768 dolphin (*Sousa chinensis*) were also used for the evaluation of the genotoxic potential of
769 polychlorinated biphenyls, persistent organic pollutants, methyl mercury chloride and
770 titanium dioxide particles [438–442]. Although these studies indicated the genotoxic potential
771 of selected pollutants they also demonstrated that dolphin cells are characterized by a higher
772 efficiency in DNA repair when compared to human cells [439]. Over the last two decades,
773 there have been significant advances in the use of assisted reproductive technology for genetic
774 and reproductive management of captive dolphin populations, including evaluation of sperm
775 DNA quality. Hence, the comet assay was also used for the evaluation of bottlenose dolphins
776 (*T. truncatus*) sperm DNA damage both in the field and in the laboratory, which could be
777 important when performing artificial insemination in order to improve pregnancy rates [443].

Although dolphins are the most studied marine mammals when it comes to the comet assay, the assay was also employed for the evaluation of toxic chemical exposure in California sea lions (*Zalophus californianus*) indicating that the assay appears to be a useful biomarker of effects for those animals as well [444].

9. Conclusions and future prospects

Nowadays the comet assay is a widely accepted method for the evaluation of DNA damage and its repair in eukaryotic cells, other than in humans, and can be done both *in vitro* and *in vivo* in yeast, plant and animal cells [1,22,445]. However, several issues related to its specificity, sensitivity as well as its limitations still need to be addressed before the assay can be accepted within a regulatory framework. Therefore, interlaboratory studies and future validation are warranted for the comet assay protocols both *in vitro* and *in vivo*. This is also highlighted in the objectives of the recently launched hCOMET project (www.hcomet.eu) and previously by the ComNet working group [446,447].

The variability in the comet assay can be attributed to its sensitivity and differences in protocols among laboratories. Several confounding factors such as the age and sex of the model organisms should also be taken into account. When it comes to the animal comet assay, large variability is also seen between species, not to mention that due to the cell differences from lower animals up to mammals the use of one standardized protocol is not always possible and these modifications can significantly influence the final result. Nevertheless, the comet assay is currently a well-established method in genetic and environmental toxicology and its application is found in nearly every part of the animal kingdom in both the aquatic and terrestrial environments. In terms of vertebrates, the comet assay is applied on all subphylum species, which includes cyclostomata (jawless fishes), fishes, amphibians, reptiles, birds and mammals. Apart from the large number of animal species employed, the assay is also performed on a variety of cells that includes blood, liver, kidney, brain, gill, bone marrow and sperm cells. Those cells have been used for the evaluation of a broad spectrum of genotoxic physical and/or chemical agents both *in vitro* and *in vivo* including *in situ* animal biomonitoring studies. The comet assay in vertebrates is most commonly used in purpose-bred rodents (mainly mice and rats, but also hamsters, guinea pigs and gerbils) and lately zebrafish, and there is increasing use of the assay in domestic and wildlife vertebrates as well.

As for laboratory animals, both mice and rats have been widely used models for the assessment of DNA-damaging effects of a variety of physical and/or chemical agents. Today,

812 there are several very specific guidelines for both *in vitro* and *in vivo* genetic toxicology
813 testing using the comet assay, including study design, sample preparations as well as
814 methodology [17,331,336]. Multiple organs of mouse and rats such as blood, liver, kidney,
815 brain, lungs and bone marrow have been used for the genotoxicity testing of a large scale of
816 chemicals. Quite a breakthrough for the implementation of the comet assay into regulatory
817 framework was reached in 2014 when the OECD published guidelines for testing chemicals
818 Test No. 489: In Vivo Mammalian Alkaline Comet Assay [337] summarizing basics and
819 limitations, principle of the method, verification of laboratory proficiency, historical control
820 data, and a detailed description of the method.

821 Besides rodents as models, the zebrafish (*D. rerio*) has gained widespread acceptance
822 recently as another species suitable for laboratory genetic toxicology. Zebrafish has become a
823 popular organism for the study of vertebrate gene function. The virtually transparent embryos
824 of this species, and the ability to accelerate genetic studies by gene knockdown or
825 overexpression, have led to the widespread use of zebrafish in the detailed investigation of
826 vertebrate gene function and increasingly, the study of human genetic disease. Comparison to
827 the human reference genome shows that approximately 70% of human genes have at least one
828 obvious zebrafish orthologue [448]. Zebrafish has gained acceptance as a prominent model
829 vertebrate in a variety of biological disciplines. Substantial information gathered from
830 developmental and genetic research, together with completion of the zebrafish genome
831 project, has placed zebrafish in an attractive position for use as a toxicological model. In line
832 with that, there is a clear potential for zebrafish to provide valuable new insights into
833 chemical toxicity, drug discovery, and human disease using recent advances in forward and
834 reverse genetic techniques coupled with large-scale, high-throughput screening [91,448,449].

835 There are numerous advantages for the use of zebrafish as a toxicological model
836 species compared with other vertebrate species, regarding their size, husbandry, and early
837 morphology. In addition, zebrafish have been utilized as a laboratory species for quite some
838 time so the optimum breeding and maintenance conditions have been well-determined.
839 Moreover, small embryos allow reasonable sample sizes to be tested together using a single
840 cell-culture plate or series of Petri dishes to provide several experimental replicates at a time.
841 This allowed the creation of high-throughput screens for toxicity testing, small-molecule
842 screening, and drug discovery, in which zebrafish grow and develop in small micro format
843 screening plates. Besides their size, this species is valuable because of their high fecundity
844 and transparent embryos. The rapid maturation of zebrafish also allows easy experimentation
845 for transgenerational endpoints required for mutagenesis screening, establishing transgenic

lines, and assessing chemicals for teratogenicity [91–95]. Moreover, another advantage of the zebrafish embryo model is related to animal welfare and alternative *in vitro* methods for the testing of chemicals (the ECVAM concept of 3"R"; replace, reduce and refine the experiments on animals) as the earliest life-stages of zebrafish are considered as an *in vitro* test system [96].

Apart from laboratory vertebrates commonly used in toxicological studies, there are several vertebrate species that are more commonly used in comparison with others, and especially relevant are those from the aquatic environment. When it comes to the environmental risk assessment of water pollutants in vertebrates, fish are among the most studied organisms both in marine and freshwater environments. Various fish have been used for environmental biomonitoring, since they are endemic organisms, and may serve as a good sentinel species for a particular aquatic region, to assess the adverse effects of waterborne pollutants. Therefore, the comet assay has found wide application as a simple and sensitive method for evaluating *in vivo* as well as *in vitro* DNA damage in different fish tissues such as gills, liver, and blood cells upon exposure to various xenobiotics [29,51–53,72,74,75]. Amphibians, especially frogs and toads, are also one of the most used subgroups of vertebrates when it comes to genotoxicity testing using the comet assay. They cover ecological niches from freshwater to terrestrial environments and are regarded as an excellent bioindicator species [29,53,71,73]. Although the above mentioned animals are frequently used in toxicological studies and the comet assay is readily applied on them, it has to be remembered that extrapolation from data obtained in such models to humans, could be problematic and sometimes impossible.

The comet assay is also quite extensively used in both domestic and wildlife animals with regard to cryopreservation for the evaluation of sperm DNA integrity since the process of cryopreservation is fundamental both for the practice of artificial insemination, and for the conservation of genetic resources in cryobanks. Although there are results indicating an increase in DNA damage during cryopreservation, which depends on the conditions as well as species used, they also suggest a low sensitivity of vertebrate spermatozoa to DNA fragmentation that should not be considered as a major cause of sperm injuries during cryopreservation [267,290,305–308,401,403–408,414,417,435]. Nevertheless, given the unusually high incidence of DNA fragmentation in the sperm of some individual animals after cryopreservation and post-thaw incubation, the standardization of a methodology to assess sperm DNA damage in such animals could contribute to male reproductive management of highly endangered species [450].

The number of animals used in research has increased with the advancement of research and development in medical technology. Although the exact number of animals used annually for various experimental purposes worldwide is not known; every year, millions of experimental animals are used, most (~80%) being laboratory rodents (mainly mice and rats, but also hamsters, guinea pigs and gerbils). These animals are mainly used in basic and applied research including toxicology. In pharmacological and toxicological studies, especially when testing drugs, mice and rats are the most commonly used animals. Nevertheless, these models are quite expensive, laborious, may yield results that cannot always be translated into human *in vivo* situations and, more recently, have raised great social and ethical dilemmas and concerns. Therefore, 3R-alternatives (Reduction, Refinement, Replacement), including non-animal alternatives, have been widely accepted as a way to diminish the use of experimental animals in research and testing. On the contrary, when studying interactions among cells, tissues and organs, or when testing the pharmacology and toxicology of various substances, including drugs to be used in human and veterinary medicine, in most cases there is no plausible substitute for the living animal [451–454]. Moreover, the current legislation requires that all new drugs, before being licenced for human and animal use, have to be rigorously tested in at least two mammalian species (rodents and non-rodents) for metabolism, pharmacokinetics, acute and chronic toxicology in adult species, efficacy regarding the expected actions, effects on reproduction, embryonic toxicity, and potential carcinogenicity. However, increasing evidence indicates that rodents and other common experimental animals, such as rabbits, may not be good models for studies relevant to humans due to sex and species differences in various properties [451,454–456]. Therefore, one should bear in mind when choosing the appropriate species for toxicological testing that the results are comparable to humans.

As for Alternatives to Animal Testing, today there are several proposed concepts and methodological approaches that could reduce testing on animal species. These includes, *in silico* methods such as computational alternatives to animal testing, different computer models and programs such as Computer Aided Drug Design (CADD), Structure Activity Relationship (SARs) and Quantitative Structure Activity Relationship (QSAR) or interactive Computer Assisted Learning (CAL) programs. Besides, the use of *in vitro* cell and tissue cultures which involves growth of cells outside the body in a laboratory environment can be an important alternative for animal experiments [454,456–459]. In line with that, the *in vitro* comet assay has also been proposed as an alternative to cytogenetic assays in early genotoxicity/photogenotoxicity screening of drug candidates [379,460].

During the past decades, the comet assay has become a widely used method for the assessment of DNA damage and repair in cells and tissues. However, the comet assay has much more to offer than just being an assay for testing DNA strand breaks in yeast, plant, animal and/or human cells and tissues. The use of repair enzymes increases the range of DNA lesions that can be detected with the assay such as oxidatively imposed DNA damage or even detection of global methylation changes. Combining the comet assay and FISH can also allow for the investigation of gene region-specific DNA damage. Moreover, the assay can also be modified to measure DNA repair activity. Nevertheless, despite the long-term use of the assay, there is still a need for studies that assess the impact of variation in specific steps of the procedure. This is particularly important for on-going efforts to decrease the variation between experiments as well as between different laboratories. Altogether, based on the available number of publications so far using the comet assay both on animal and human model in addition to the global interest for the assay itself, there is clear evidence that the comet assay still has a place in many more years to come.

Conflict of interest

None declared.

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Table 1. The comet assay for the evaluation of DNA damage in animal models (vertebrates; from chordates to mammals).

	Animal	Cell type	Type of study	Agent/Stressor	Concentration range	Parameter	Response	Reference
Chordates	*Botryllus schlosseri*	blood cells	*in vitro*	H_2O_2	53, 130 and 2650 µM	TL, VS, Comet %	↑	[66]
		blood cells	*in vitro, in vivo*	UVA, UVB, natural sunlight	UVB 3.55 W/s/, UVA 8.09 W/s	TEM	↑	[67]
		hemocytes	*in vivo*	colonial blastogenetic cycle	take-over *vs.* midcycle stages	VS	↑ (during take-over)	[68]
	Ciona intestinalis	hemocytes	*ex vivo*	baseline values, H_2O_2	25 and 250 µM	% tail DNA	↑ (≥ 25 µM)	[69]
Vertebrates								
Cyclostomata	*Petromyzon marinus*	spermatozoa	*in vitro, in vivo*	H_2O_2, UV, bisazir, storage conditions	H_2O_2 (1, 10 and 100 µM), UV (860 and 1720 J/m^2), storage conditions (2 and 4 days), bisazir (2 mg/mL)	% head DNA, VS, CS, OTM	↑ (H_2O_2 ≥ 1 µM), ↑ (UV ≥ 860 J/m^2), ↑(storage conditions ≥ 2 days), ↑ bisazir	[70]
		blood cells (erythrocytes)	*in vitro, in vivo*	bisazir	2 mg/mL	% head DNA, VS	↑	[70]
Fishes								
	*Danio rerio**	erythrocytes	*in vivo*	GEM, ATO, SC	GEM (380 ng/L), ATO (13 ng/L), SC (26.25 ng/L)	% tail DNA	↑	[111]
		erythrocytes	*in vivo*	multiwalled carbon nanotubes	0.1, 0.5, 1, 5, 10, 50 mg/L	VS, DNA damage score	↑ ≥ 0.5 mg/L	[118]

		erythrocytes, liver cells	*in vivo*	ERY, LIN (+ mixture)	ERY (100 mg/L), LIN (100 mg/L), mixture (353 ng/L + 846 ng/L)	TM	↑	[112]
		blood cells	*in vivo*	γ-radiation	^{60}Co γ-radiation (27 days, 8.7 and 53 mGy/h, total doses 5.2 and 31 Gy)	% tail DNA	↑ ≥ 8.7 mGy/h	[102]
		liver cells	*in vivo*	flame retardant	tris(1,3-dichloro-2-propyl)phosphate (TDCPP) (45.81 μg/L and 229.05 μg/L)	OTM	↑	[119]
		liver cells	*in vivo*	hydroxyapatite-loaded cadmium nanoparticles (nHAP-Cd)	Cd (0.12 mg/L), 20 nm nHAP-Cd ($nHAP_{20}$-Cd) and 40 nm nHAP-Cd ($nHAP_{40}$-Cd) (1.86 mg/L)	TL, % tail DNA, TM, OTM	↑	[116]
		gill cells	*in vivo*	tebuconazole	100, 200 and 300 μg/L	VS, AU	↑ ≥ 100 μg/L	[107]
		gill cells	*in vivo*	spent pot liner	0.32, 0.64 and 0.95 g/L	VS, AU	↑	[121]
		gill cells	*in vivo*	metal oxide nanoparticles	copper oxide, zinc oxide and nickel oxide nanoparticles	TM	↑	[115]
		blood cells, gill cells, liver cells, gonadal cells	*in vivo*	5-FU	0.01, 1 and 100 μg/L	% tail DNA	↑	[110]
		blood cells, larvae cells	*in vivo*	EMS, B[a]P	EMS (0.5 and 1 mM), B[a]P (0.001, 0.01, 0.1 and 1 μM)	% tail DNA	↑ (EMS ≥ 0.5 mM), ↑ (B[a]P ≥ 0.001 μM)	[122]
		blood cells, larvae cells	*in vivo*	X-ray irradiation	100, 500 and 1000 mGy	% tail DNA	↑ (adult ≥ 100 mGy), ↑ (unexpose	[104]

							d progeny ≥ 1000 mGy)	
		gill cells, gonadal cells	*in vivo*	depleted uranium (DU)	20 µg/L	TM	↑≥ 0.32 g/L	[113]
		gonadal cells	*in vivo*	gold nanoparticles (Au-NPs)	20 µg/g/day	% tail DNA	↑	[117]
		retinal cells	*in vitro, in vivo*	cypermethrin, H_2O_2	cypermethrin (0.3 and 0.6 µg/L), H_2O_2 (2.5, 5 and 10 µM)	VS, DI	↑ (CM ≥ 0.3 µg/L), ↑ (H_2O_2 ≥ 2.5 µM)	[106]
		whole embryo	*in vitro*	pyriproxyfen	0.52, 1.04 and 5.2 µM	OTM	↑ ≥ 5.2 µM	[105]
		whole embryo	*in vitro*	pencycuron, BLEO as PC	pencycuron (250, 350, 625, 850 and 1250 µg/mL), BLEO (0.25 µg/mL)	TL, % tail DNA, TM	↑ (PEN ≥ 250 µg/mL), ↑ BLEO	[88]
		whole embryo	*in vitro*	arsenic, ATZ (+ mixture)	As (0.8 mM), ATZ (0.1 mM)	TM	Ø As, Ø ATZ, ↑ mixture	[108]
		whole embryo	*in vitro*	zearalenone	ZEA (350, 550, 750 and 950 µg/L)	OTM	↑≥ 750 µg/L	[109]
		whole embryo	*in vitro*	sediment organic extracts	Lagos lagoon (Nigeria)	% tail DNA	↑	[89]
		whole embryo	*in vitro*	MMS, B[a]P	MMS (22.7 and 227 µM), B[a]P (2.5 and 25 µM)	% tail DNA	↑ (MMS ≥ 22.7 µM), ↑ (B[a]P ≥ 25 µM)	[113]
		whole embryo	*in vitro*	WWTP	Virovitica (Croatia)	% tail DNA	↑	[120]
		whole embryo	*in vitro*	γ-radiation	^{60}Co γ-radiation (1.1633 Gy/min, total doses 1, 2.5, 5, 7.5 and	% tail DNA	↑≥ 1 Gy	[103]

					10 Gy)			
		liver zebrafish cells (ZFL)	*in vitro*	5-FU, CDDP, ETO, B[a]P as PC	5-FU (0.001, 0.01, 0.1, 1 and 10 μg/mL), CDDP (0.001, 0.01, 0.1, 1 and 10 μg/mL), ETO (0.0001, 0.001, 0.01, 0.1, 1 and 10 μg/mL), B[a]P (50 μM)	% tail DNA	↑ (5-FU ≥ 0.01 μg/mL), ↑ (CDDP ≥ 0.1 μg/mL), ↑(ETO ≥ 0.001 μg/mL), ↑ B[a]P	[98]
		liver zebrafish cells (ZFL)	*in vitro*	IMA, B[a]P as PC	IMA (0.001, 0.01, 0.1, 1 and 10 μg/mL), B[a]P (50 μM)	% tail DNA	↑ ≥ 0.01 μg/mL, ↑ B[a]P	[99]
		embryonic zebrafish cells (ZF4)	*in vitro*	aluminium, cadmium	$AlCl_3$ (10, 30, 50 and 100 μM), $CdCl_2$ (1, 10, 30, 50 and 100 μM)	TL, % tail DNA	↑ ($AlCl_3$ ≥ 50 μM), Ø ($CdCl_2$)	[114]
	*Carassius auratus**	erythrocytes	*in vivo*	monocrotophos	0.01, 0.1, and 1 mg/L	OTM	↑ ≥ 0.01	[172]
		erythrocytes	*in vivo*	acrylamide, CP as PC	acrylamide (5, 10, and 20 mg/L), CP (20 mg/kg)	% tail DNA, OTM	↑ (ACR ≥ 20 mg/L), ↑ (CP ≥ 20 mg/L)	[176]
		erythrocytes, liver cells	*in vivo*	2,4-Dichlorophenol	0.01, 0.1 and 1 mg/L	% tail DNA	↑ ≥ 0.01	[173]
		liver cells	*in vivo*	triclosan	0.1399, 0.2798 and 0.5596 mg/L	TM	↑ ≥ 0.5596 mg/L	[175]
		liver cells	*in vivo*	roxarsone	50, 150, 300 and 500 μg/L	TL, % tail DNA, OTM	↑ ≥ 50 μg/L	[174]
	Poecilia reticulata	erythrocytes	*in vivo*	iron oxide (maghemite-γ-	0.3 mg/L	% tail DNA	↑	[177]

				Fe_2O_3) nanoparticles				
		erythrocytes	*in vivo*	GLY	1.41, 2.83, 4.24 and 5.65 µL/L	VS, DI	↑ ≥ 1.41 µL/L	[179]
		liver cells	*in vivo*	ionic liquid, H_2O_2 as PC	1-butyl-3-methylimidazolium bromide + metabolites (50, 100, 500 and 1000 mg/L), H_2O_2 (100 µM)	% tail DNA	↑ (BMImBr ≥ 100 mg/L), Ø metabolites, ↑ H_2O_2	[178]
	Poecilia vivipara	erythrocytes	*in vivo*	copper	5, 9 and 20 µg/L	VS, CS	↑ ≥ 5 µg/L	[180]
	Salmo trutta fario	erythrocytes, liver cells, gill cells, kidney cells	*in situ*	anthropogenic pollution	Karaj River (Iran)	VS, damage score	↑ (dependent on the sampling site)	[85]
	Salmo cenerinus	blood cells	*in situ, in vitro*	anthropogenic pollution, cryopreservation	Adige river basin (Italy)	% tail DNA	↑ (dependent on the sampling site), Ø (cryopreservation)	[124]
	Salmo marmoratus	blood cells	*in situ, in vitro*	anthropogenic pollution, cryopreservation	Adige river basin (Italy)	% tail DNA	↑ (dependent on the sampling site), Ø (cryopreservation)	[124]
	Oncorhynchus mykiss	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	% tail DNA, OTM	↑	[126]

		erythrocytes	*in vivo*	antimicrobial agents, H_2O_2 as PC	triclosan (TRX, 0.48 μg/L), chloroxylenol (PCMX, 4.2 μg/L), methylisothiazolinone (MIT, 6.8 μg/L), borax (BRX, 8.9 μg/L), H_2O_2 (30%)	% head DNA, % tail DNA	↑	[125]
	Cyprinus carpio	erythrocytes, gill cells, liver cells, kidney cells	*in situ*	contaminated sites (PAHs and PCBs), Southern Ontario (USA)	Southern Ontario (USA)	LWR	↑	[76]
		erythrocytes	*in vivo*	karanjin	0.28 ppm	VS, GDI, % DNA damage	↑	[82]
		gill cells, liver cells, muscle cells	*in vivo*	mesotrione	1.8, 18 and 180 μg/L	OTM	↑ ≥ 180 μL/L	[127]
	Catla catla	blood cells	*in situ*	polluted sites	River Chenab (Pakistan)	HD, TL, % tail DNA, TM, OTM	↑	[128]
	Cirrhinus mrigala	blood cells	*in situ*	polluted sites	River Chenab (Pakistan)	HD, TL, % tail DNA, TM, OTM	↑	[128]
	Carassius auratus gibelio	erythrocytes	*in situ*	water Contaminants	Lake Sevan Basin (Armenia)	% tail DNA	↑ (dependent on the sampling site)	[129]
	Barbus barbus	erythrocytes	*in situ*	heavy metal pollution	Danube River (Serbia)	TL, % tail DNA, TM	↑	[130]
	Alburnus alburnus	erythrocytes	*in situ*	polluted sites (wastewater)	Danube River (Kehlheim (Deutschland) to Sulina (Romania))	% tail DNA, ASC, HDC	↑	[131]
		erythrocytes	*in situ*	polluted sites (wastewater)	Velika Morava River Basin (Serbia)	% tail DNA	↑	[132]

	Abramis brama	blood cells, liver cells, gill cells	*in situ*	pollution, seasonal variation, H_2O_2	Danube River (Serbia), H_2O_2 (20 μM)	TL, % tail DNA, OTM	↑ (dependent on the season)	[133]
	Abramis bjoerkna	blood cells	*in situ*	pollution (impact of flooding)	Sava River (Serbia)	OTM	↑	[134]
	Abramis sapa	blood cells	*in situ*	pollution (impact of flooding)	Sava River (Serbia)	OTM	↑	[134]
	Labeo rohita	erythrocytes	*in situ*	pollution (heavy metals)	River Chenab (Pakistan)	HD, TL, % tail DNA, TM, OTM	↑	[135]
		erythrocytes	*in vivo*	silver nanoparticles (Ag-NPs)	10, 20, 30, 45 and 55 mg/L	VS, TL	↑ ≥ 10 mg/L	[136]
		erythrocytes	*in vivo*	profenofos	0.01 and 0.02 mg/L	% tail DNA	↑ ≥ 0.01 mg/L	[137]
		gill cells	*in vivo*	malathion	5 μg/L	TL, % tail DNA, TM, OTM	↑	[138]
		erythrocytes	*in vivo*	endosulfan	1, 1.5 and 2 μg/L	TL, % tail DNA, GDI	↑ ≥ 1 μg/L	[139]
	Labeo calbasu	erythrocytes, gill cells	*in situ*	tannery effluents	River Ganges (India)	% tail DNA	↑	[86]
	Ameiurus nebulosus	erythrocytes	*in situ, in vivo*	contaminated sites (PAHs and PCBs), CP	Southern Ontario (USA), CP (1.25, 2.5, 5, 10, 20, 40 and 80 mg/kg)	LWR	↑	[76]
		erythrocytes	*in situ*	pollutes sites	Cuyahoga River, Ashtabula River, Ashumet Pond (USA)	TL, % tail DNA, TEM	↑	[140]
	Leuciscus cephalus	liver cells	*in situ*	polluted sites (PAHs, PCBs, OCPs, heavy metals)	Rivers Blythe, Cole and Tame (Birmingham, UK)	% tail DNA	↑ (dependent on the sampling site and season)	[141]

		blood cells, liver cells, gill cells	*in vivo*	exhaustive exercise	swimming to their critical swimming speed, twice in succession with a 40 min rest period between	% tail DNA	↑	[143]
		erythrocytes	*in situ*	chemical pollution (metal contamination and PAHs)	Cecina River (Italy)	% tail DNA	↑ (dependent on the sampling site)	[142]
	Squalius cephalus	erythrocytes	*in situ*	polluted sites (seasonal variations)	River Sava (Croatia)	% tail DNA	↑ (dependent on the sampling site and season)	[145]
		erythrocytes, gill cells, liver cells	*in situ*	various xenobiotics in the aquatic environment	Kolubara Basin, Bubanj Potok, lakes (Zlatar and Garasi) (Serbia)	TL, % tail DNA, TM	↑ (dependent on the sampling site)	[146]
		erythrocytes, gill cells, liver cells	*in situ*	polluted sites (seasonal variations)	Kolubara Basin (Serbia)	% tail DNA, OTM	↑ (dependent on the sampling site)	[87]
		blood cells, gill cells, liver cells	*in situ*	polluted sediments (metals and metalloids)	Kolubara Basin (Serbia)	TL, % tail DNA, TM	↑ (dependent on the sampling site and season)	[144]
	Zacco platypus	blood cells	*in vivo*	copper, B[a]P	Cu (1.25, 5 and 20 μg/L), B[a]P (0.5, 5 and 50 μg/L)	TM	↑ (Cu ≥ 20 μg/L), ↑ (B[a]P	[147]

							≥ 0.5 μg/L)	
	Anabas testudineus	gill cells, liver cells, kidney cells	*in vivo*	$PbCl_2$	0.1, 1 and 2 mg/L	% head DNA, % tail DNA	↑ ≥ 0.1 mg/L	[148]
	Piaractus mesopotamicus	erythrocytes	*in vivo*	silver nanoparticles (AgNPs)	2.5, 10 and 25 μg/L	VS, AU	↑ ≥ 10 μg/L	[149]
		erythrocytes, gill cells, liver cells	*in vivo*	GLY, EMS as PC	GLY (2.75 mg/L), EMS (15 mg/L)	VS, DI	↑	[150]
	Gambusia holbrooki	blood cells	*in situ, ex vivo*	polluted water and sediment (Al)	Lake Njivice (Island of Krk, Croatia)	TL, % tail DNA, TM	↑	[77]
	Oreochromis niloticus	erythrocytes	*in situ*	polluted sites	River Nile (Egypt)	VS, % tail DNA	↑ (dependent on the sampling site and season)	[151]
		erythrocytes	*in situ*	aquaculture activities	Taal Lake (Philippines)	TM	↑ (dependent on the season)	[152]
		blood cells	*in vivo*	sugarcane distillery residue, CP as PC	raw vinasse, vinasse adjusted to neutral pH, CP (10 cc/50 g of fish)	VS	↑ (raw vinasse), Ø (vinasse adjusted to neutral pH), ↑ CP	[153]
		erythrocytes	*in vivo*	endosulfan + chlorpyrifos	0.94, 1.13, 1.41, 1.88 and 5.64 μg/L	VS, DC	↑ ≥ 0.94 μg/L	[154]

	Oreochromis mossambicus	gill cells	*in vivo*	fipronil + imidacloprid, indoxacarb + acetamiprid, thiamethoxam + lambdacyhalothrin	fipronil + imidacloprid (0.01 and 0.02 mg/L), indoxacarb + acetamiprid (0.26 and 0.51 mg/L), thiamethoxam + lambdacyhalothrin (0.001 and 0.002 mg/L)	VS, AU, DDS	↑	[155]
	Australoheros facetus	erythrocytes	*in vivo*	imidacloprid, H_2O_2 as PC	IMI (1, 10, 75 and 810 µg/L), H_2O_2 (50 µM)	VS, DI	↑ (IMI ≥ 1 µg/L), ↑ H_2O_2	[156]
	Puntius sophore	erythrocytes, gill cells	*in situ*	tannery effluents	River Ganges (India)	% tail DNA	↑	[86]
	Astyanax altiparanae	erythrocytes	*in situ*	agricultural area	Londrina (Northern Paraná, Brazil)	VS, DDS	↑ (dependent on the sampling site)	[157]
		erythrocytes	*in vivo*	multiwalled carbon nanotubes	0.1, 0.5, 1, 5, 10, 50 mg/L	VS, DDS	↑ ≥ 10 mg/L	[118]
	Gobiocypris rarus	embryos/larvae cells	*in vivo*	quantum dots	$CuInS_2$/ZnS QD (50, 100, 200, 400 and 800 nmol/L)	OTM	↑ ≥ 200 mg/L	[158]
	Gasterosteus aculeatus	erythrocytes	*in situ*	influence of gender, age, reproductive status, body length, season, polluted sites	Seine-Normandie Basin, Artois-Picardie Basin (France)	% tail DNA	Ø (gender, age, reproductive status), ↑ (sampling site, season)	[159]
	Clarias gariepinus	erythrocytes	*in situ*	polluted sites	River Nile (Egypt)	VS, % tail DNA	↑ (dependent on the	[151]

							sampling site)	
		ovary cells (oocytes)	*in situ*	polluted sites	River Yamuna (India)	% head DNA, TL, % tail DNA, TM, OTM	↑ (dependent on the sampling site and season)	[160]
	Mystus vittatus	erythrocytes	*in situ*	polluted sites	River Gomti (India)	% tail DNA	↑	[161]
		erythrocytes, gill cells	*in situ*	tannery effluents	River Ganges (India)	% tail DNA	↑	[86]
	Rhamdia quelen	blood cells, liver cells	*in vivo*	paracetamol	0.25 and 2.5 μg/L	VS, DDS	↑ ≥ 0.25 μg/L	[163]
		blood cells, liver cells	*in vivo*	diclofenac	0.2, 2 and 20 μg/L	VS, DDS	Ø	[162]
		liver cells, kidney cells	*in vivo*	ibuprofen	0.1, 1 and 10 μg/L	VS	Ø	[164]
	Heteropneustes fossilis	liver cells	*in situ*	heavy metals	Kali River (India)	TL, % tail DNA	↑	[165]
	Hypostomus ancistroides	blood cells	*in situ*	polluted sites	Pirapó River (Brazil)	VS, DDS	↑ (dependent on the sampling site and season)	[167]
	Pangasius suthi	erythrocytes	*in vivo*	radiological impact (uranium mining facilities)	^{238}U (37 and 74 mg/L)	% tail DNA	↑	[168]
		erythrocytes	*in vivo*	γ-radiation (radioprotective effect of *Gymnema sylvestre* and gymnemagenin)	^{60}Co (9.2, 10.2 and 11.4 Gy), amifostine (83.3 mg/kg bw), GS (25 mg/kg bw) and GG (0.3 mg/kg bw)	TL, GDI	↑ (^{60}Co), ↓ (amifostine, GS, GG)	[167]

	Hoplias malabaricus	hepatocytes	*in vitro*	cylindrospermopsin	0.1, 1.0, 1, and 100 µg/L	CS	Ø	[80]
	Umbra pygmaea	gill cells	*in vivo*	river water, EMS as PC	Rhie River (The Netherlands)	TL	Ø (river water), ↑ (EMS)	[169]
	Prochilodus lineatus	erythrocytes	*in situ*	tannery effluents (Cr)	Pirapó River (Brazil)	VS, DDS	Ø	[83]
		erythrocytes, gill cells, liver cells	*in vivo*	B[a]P	20 mg/kg	VS, DDS	↑	[170]
	Channa punctatus	erythrocytes	*in situ*	polluted sites	River Gomti (India)	% tail DNA	↑	[161]
		erythrocytes	*in vivo*	profenofos	1.16 ppb	% tail DNA	↑	[171]
	Channa striatus	liver cells	*in situ*	heavy metals	Kali River (India)	TL, % tail DNA	↑	[165]
	Salmo salar	cell suspension	*in vitro*	cold chain abuse	freezing-thawing	OTM	↑	[181]
	Acipenser gueldenstaedtii	spermatozoa	*in vitro*	short-term (liquid) storage	aerobic conditions at 4 °C	% tail DNA, OTM	↑ (dependent on the time of storage)	[182]
	Acipenser baerii	spermatozoa	*in vitro*	short-term (liquid) storage	aerobic conditions at 4 °C	% tail DNA, OTM	↑ (dependent on the time of storage)	[182]
	Anguilla anguilla	erythrocytes	*in vivo*	triclopyr, Garlon	triclopyr (30 and 120 µg/L), Garlon (67.6 and 270.5 µg/L)	VS, GDI	↑ (T ≥ 30 µg/L), ↑(G ≥ 67.6 µg/L)	[185]
		erythrocytes	*in vivo*	AMPA	11.8 and 23.6 µg/L	VS, GDI	↑ ≥ 11.8 µg/L	[186]

		erythrocytes	*in vivo*	mancozeb	0.29 and 2.9 µg/L	VS, GDI	↑≥ 2.9 µg/L	[183]
		liver cells	*in vivo*	GLY	58 and 116 µg/L	VS, GDI	↑≥ 58 µg/L	[184]
	Sparus aurata	erythrocytes	*in vivo*	gemfibrozil	1.5, 15, 150, 1500 and 15000 µg/L	VS, DI	↑≥ 1.5 µg/L	[187]
		erythrocytes	*in vivo*	Cadmium (Cd)	0.1 mg/L ($CdCl_2$)	TL, % tail DNA, TM, AST	Ø	[190]
		cryopreservation	*in vivo*	copper (Cu)	1.1 ppm ($CuSO_4$)	TL, % tail DNA, TM, AST	↑	[188]
		liver cells	*in vivo*	B[a]P	20 mg/kg	% tail DNA	↑	[189]
		spermatozoa	*in vitro*	cryopreservation	freezing-thawing	% tail DNA, OTM	↑	[126]
	Dicentrarchus labrax	erythrocytes	*in situ*	environmental pollution	Kaštela Bay (Croatia)	% tail DNA	↑	[193]
		erythrocytes	*in vivo*	nanoparticles	n-TiO_2 (1 mg/L), $CdCl_2$ (0.1 mg/L), + mixture	% tail DNA	Ø (n-tiO_2), ↑ ($CdCl_2$), ↑ (mixture)	[192]
		erythrocytes	*in vivo*	PAHs	benzo[b]fluoranthene (B[b]F), phenanthrene (Phe), + mixture	fold changes/variation coefficients	↑	[191]
		liver cells	*in situ*	environmental contaminants	Bizerte Lagoon (Tunisia)	% tail DNA	↑	[84]
		spermatozoa	*in vitro*	cryopreservation, cryoprotectants	freezing-thawing (+ taurine and hypotaurine)	% tail DNA	↑ (cryopreservation), ↓ (cryoprotectants)	[195]
		spermatozoa	*in vitro*	cryopreservation	freezing-thawing	% tail	↑	[194]

						DNA, TM		
	Symphodus melops	erythrocytes	*in vivo*	styrene	2 mg/L	% tail DNA	↑	[196]
	Limanda limanda	erythrocytes	*in situ*	PAHs and PCBs	English Channel (France)	% tail DNA	↑ (dependent on the sampling site)	[200]
		erythrocytes	*in situ*	coastal and offshore locations	Northern European seas	% tail DNA	↑ (dependent on the sampling site)	[81]
	Scophthalmus maximus	liver cells	*in vivo*	polluted sediment, $CdCl_2$ as PC	Cork Harbour (Ireland), $CdCl_2$ (4 and 40 μM)	% tail DNA	↑	[197]
		erythrocytes	*in vivo*	PAHs	mixture of dissolved PAHs, PAH-polluted sediment, oil fuel elutriate	% tail DNA	↑	[198]
	Solea solea	erythrocytes	*in vivo*	PAHs	B[a]P, fluoranthene, pyrene (120 μg/g food)	% tail DNA, OTM	↑	[202]
		hepatocytes	*in vitro*	fluoranthene	0.5, 1, 5, 25 and 50 μM	% tail DNA, OTM	↑ ≥ 5 μM	[201]
	Pleuronectes vetulus	spermatozoa	*in situ*	wastewater outfall	Orange County (CA, USA)	TM	↑	[199]
	Pleuronichthys verticalis	spermatozoa	*in situ*	wastewater outfall	Orange County (CA, USA)	TM	↑	[199]
	Helicolenus dactylopterus	gill cells, liver cells, muscle cells, kidney cells, intestine	*in situ*	chemical warfare agents (As and Hg)	southern Adriatic Sea (Italy)	% tail DNA	Ø	[78]

		cells, gonad cells						
	Conger conger	gill cells, liver cells, muscle cells, kidney cells, intestine cells, gonad cells	*in situ*	chemical warfare agents (As and Hg)	southern Adriatic Sea (Italy)	% tail DNA	↑ (in gill cells)	[78]
	Boreogadus saida	blood cells	*in vivo*	water soluble fraction of crude oil	3, 6 and 12 g crude oil/kg (PAHs)	% tail DNA	↑	[79]
	Mugil cephalus	liver cells	*in situ*	environmental contaminants	Bizerte Lagoon (Tunisia)	% tail DNA	↑	[84]
	Paralichthys olivaceus	liver cells	*in vivo*	different light wavelengths from light emitting diodes (LED) (+ thermal stress)	0.3 and 0.5 W/m2	TL, % tail DNA, TM	↑ (thermal stress), ↓ (protective effects of green light)	[203]
		blood cells	*in vitro, in vivo*	sediments and PAHs (B[a]P, fluoranthene, anthracene, pyrene and phenanthrene)	Gwangyang Bay (Korea), PAHs (in vitro, 5, 10, 50 and 100 ppb), B[a]P (in vivo, 10 and 100 ppb)	TL	in vitro, ↑ (BaP ≥ 50 ppb), ↑ (fluoranthene ≥ 50 ppb), ↑ (anthracene ≥ 50 ppb), ↑ (pyrene ≥ 100 ppb), ↑ (phenanthrene ≥ 50 ppb), in	[204]

							vivo ↑ (B[a]P ≥ 10 ppb)	
	Cyprinodon variegatus	larval cells	*in vivo*	hypoxia, crude oil (+ mixture)	water accommodated fractions (WAF) and chemically enhanced WAFs (CEWAFs) of Southern Louisiana Crude oil	OTM	↑	[205]
	Melanogrammus aeglefinus	erythrocytes	*in situ*	coastal and offshore locations	Northern European seas	% tail DNA	↑ (dependent on the sampling site)	[81]
	Trachinotus carolinus	erythrocytes	*in vivo, in vitro*	nanoparticles, H_2O_2 as PC	TiO_2-NP (in vivo 1.5 and 3 μg/g), H_2O_2 (in vitro 10 and 20 μM)	TL, % tail DNA	↑	[206]
	Pseudosciaena crocea	spermatozoa	*in vitro*	cryopreservation, cryoprotectant	freezing-thawing, DMSO (5, 10, 15, 20, 25 and 30%)	CL, TL, CR, Dcoe	↑ ≥ 25%	[207]
	Oryzias melastigma	larval cells	*in ovo*	biological and chemical dispersants and crude oil	water-accommodated fractions of crude oil (WAFs), dispersants plus dispersed crude oil (chemical dispersant + crude oil (CE-WAF), biological dispersant + crude oil (BE-WAF)) (0.5 and 1 % v/v)	TL, % tail DNA, TM	↑	[208]

	Chanos chanos	gill cells, liver cells	*in vivo*	naphthalene	0.06, 0.15, 0.42, 0.69 and 1.24 mg/L	% tail DNA, AU, DDI	↑	[209]
	Coris julis	erythrocytes	*in situ*	polluted sites	Messina (Italy)	TL, TM	↑ (dependent on the sampling site)	[211]
		erythrocytes, liver cells	*in situ*	polluted sites	Ionic coast of Sicily (Italy)	% tail DNA	↑ (dependent on the sampling site)	[210]
	Therapon jarbua	erythrocytes, gill cells, kidney cells	*in vivo*	profenofos	21.5, 43, 86, 172 and 344 μg/L	VS, % tail DNA	↑	[213]
		erythrocytes, gill cells, kidney cells	*in vivo*	$HgCl_2$, H_2O_2 as PC	$HgCl_2$ (0.125, 0.25, 0.5, 1 and 2 ppm), H_2O_2 (100 μM)	VS, AU	↑	[212]
	Oplegnathus fasciatus	blood cells	*in vitro*	polluted sediment	Hebei Spirit oil spill (Taean Country, Korea)	TM	↑	[214]
	Epinephelus coioides	liver cells	*in vivo*	suspended solids (metals, PAHs and PCBs)	Victoria Harbor (Hong Kong) (8, 32 and 128 mg/L)	TL, % tail DNA	↑ ≥ 32 mg/L	[215]
	Aphanius fasciatus	erythrocytes	*in situ*	polluted sites	Orbetello lagoon (Tuscany, Italy)	TL, % tail DNA, OTM	↑ (compared to reference site)	[216]
	Zoarces viviparus	erythrocytes	*in situ*	environmental contaminants (PAHs)	Göteborg harbour (Sweden)	% tail DNA	↑ (dependent on the sampling site)	[217]
	Hippocamp	erythrocytes	*in vivo*	hypoxia and	hypoxia, crude oil (+	DF	↑	[218]

	us reidi			petroleum	mixture)			
Amphibians								
	Rana clamitans	erythrocytes	*in vivo*	MMS	0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 mg/L	LWR	↑ (≥ 6.25 mg/L)	[220]
		erythrocytes	*in vivo*	MMS	1.56, 3.13 and 6.25 mg/L	LWR	↑ (≥ 1.56 mg/L, dependent on the stages of larval development)	[246]
		erythrocytes	*in situ*	polluted water bodies	Southern Ontario (Canada)	LWR	↑ (dependent on the site)	[261]
		erythrocytes	*in situ*	polluted water bodies	Southwestern Ontario (Canada)	LWR	↑ (dependent on the site)	[262]
	Rana limnocharis	erythrocytes	*in vitro*	imidacloprid, RH-5850, MMC as PC	imidacloprid (0.05, 0.1, 0.2 and 0.5 mg/L), RH-5850 (5, 25, 50, 100 mg/L), MMC (10 µg/L)	VS, AU	↑	[225]
		erythrocytes	*in vivo*	sodium arsenite ($NaAsO_2$)	200 µg/L	% head DNA, % tail DNA, OTM	↑	[247]
		erythrocytes	*in vivo*	$CdCl_2$	18.5 µg/mL	% head DNA, % tail DNA, OTM, TEM	↑	[252]
		testes	*in vivo*	$CdCl_2$	2.5, 5, 7.5 and 10	TL, TM,	↑ (≥ 5	[253]

					mg/L	DDR	mg/L)	
	Rana nigromaculata	erythrocytes	*in vitro*	imidacloprid, RH-5849, MMC as PC	imidacloprid (0.05, 0.1, 0.2 and 0.5 mg/L), RH-5850 (5, 25, 50, 100 mg/L), MMC (10 µg/L)	VS, AU	↑	[225]
		testes	*in vivo*	$PbNO_3$	0.1, 0.2, 0.4, 0.8 and 1.6 mg/L	TL, TM, DDR	↑ (≥ 0.2 mg/L)	[255]
	Rana catesbeiana	erythrocytes	*in vivo*	ATZ, metalochlor, GLY, metribuzin, 2,4-D amine, MMS as PC	ATZ (4.81, 19.25, 77 and 308 mg/L), metalochlor (0.27, 1.09, 4.34 and 17.37 mg/L), GLY (1.69, 6.75, 27 and 108 mg/L), metribuzin (13.38, 53.5, 214 and 856 mg/L), 2,4-D amine (4.06, 16.25, 65 and 260 mg/L), MMS as PC (3.13 mg/L)	LWR	↑ (ATZ ≥ 4.81 mg/L), ↑ (M ≥ 0.27 mg/L), ↑ (GLY ≥ 6.75 mg/L), ↑ (M ≥ 13.38 mg/L), Ø (2,4-D amine), ↑ (MMC)	[232]
	Rana hexadactyla	erythrocytes	*in vivo*	sulfur dyes, EMS as PC	sandopel basic black (10, 12, 17 and 25 mg/L), negrosine (40, 75, 150 and 300 mg/L), dermapel black (150, 200, 300 and 600 mg/L), turquoise blue (40, 75, 150 and 300 mg/L), EMS (5.85 mg/L)	LWR	↑(SBP ≥ 10 mg/L), ↑(N ≥ 75 mg/L),↑(DB ≥ 150 mg/L),↑(TB ≥ 75 mg/L),↑(EMS)	[244]
	Rana esculenta	erythrocytes	*in situ*	waste dumping sites	Naples, Campania (Italy)	% tail DNA, TM	↑ (dependent on the site)	[258]

	Rana pipiens	erythrocytes	*in situ*	polluted water bodies	Southern Ontario (Canada)	LWR	↑ (dependent on the site)	[261]
	Rana temporaria	spermatozoa	*in vitro*	cryopreservation	storage conditions	VS	↑ (dependent on the days of storage)	[267]
		blood cells	*in vitro*	ionising radiation, H2O2	X-rays (2, 4 and 8 Gy), H_2O_2 (75, 150 and 300 µM)	% tail DNA	↑ (X-rays), ↑ (H_2O_2)	[264]
	*Xenopus laevis**	erythrocytes	*in vivo*	captan, MMS as PC	captan (15.6. 31.25, 62.5 and 125 µg/L), MMS (1.56 mg/L)	TL, % tail DNA, TEM, OTM	↑ captan (≥ 15.6 µg/L), ↑ MMS (dependent on the exposure time)	[238]
		erythrocytes	*in vivo*	B[a]P, EMS, MMS	B[a]P (0.125, 1 and 10 mg/L), EMS (1, 3 6, 10, 50 and 100 mg/L), MMS (1, 1.56, 3, 3.13, 6 and 6.25 mg/L)	TL, % tail DNA, TEM, OTM	↑ B[a]P 0.125 and 1 mg/L), ↑(EMS 1, 3, 6, 50 and 100 mg/L), ↑(MMS ≥ 1 mg/L) (dependent on the exposure time)	[245]
		erythrocytes	*in vivo*	multi-walled carbon nanotubes	0.1 and 1 mg/L	TL, % tail DNA	↑ (≥ 0.1 mg/L)	[249]

		erythrocytes	*in vivo*	$CdCl_2$, MMS as PC	$CdCl_2$ (0.25, 0.5 and 1 mg/L), MMS (1.56 mg/L)	TL, % tail DNA, TEM, OTM	↑ $CdCl_2$, ↑ MMS	[254]
		erythrocytes	*in vivo*	draining water from dredged sediments	Nord Pas-de-Calais area (France)	TL, TEM	↑ (dependent on the exposure time)	[259]
		erythrocytes	*in vivo*	aqueous extracts of soils and bottom ash, MMS as PC	Soil A (residues of solvents and metals), Soil B (PAHs and metals), MMS (1.56 mg/L)	TL, TEM	↑	[263]
		erythrocytes	*in vitro*	high peak-power pulsed electromagnetic fields	8.8 GHz, 180 ns pulse width, peak power 65 kW, repetition rate 50 Hz	% head DNA, % tail DNA, HR, TL, TM, OTM, II	Ø (only thermal effect recorded)	[265]
		splenic lymphocytes	*in vitro*	BLEO	20, 40, 60, 80, 100 and 150 µg/ml		↑ (≥ 60 µg/mL)	[250]
		spermatozoa	*in vitro*	validation of the sperm chromatin dispersion test in relation to comet assay	1 (T1) and 24 (T24) h of incubation at room temperature	VS, sperm DNA fragmentation (SDF)	↑ (compared to T0)	[268]
	Xenopus tropicalis	splenic lymphocytes	*in vitro*	BLEO	20, 40, 60, 80, 100 and 150 µg/ml		↑ (≥ 20 µg/mL)	[250]
	Hypsiboas pulchellus	erythrocytes	*in vivo*	imidacloprid, CP as PC	imidacloprid (12.5, 25 and 37.5 mg/L), CP (40 mg/L)	VS, GDI	↑ (IMI ≥ 12.5 mg/L), ↑ CP	[221]
		erythrocytes	*in vivo*	imidacloprid, CP as PC	imidacloprid (15, 30 and 45 mg/L), CP (40 mg/L)	VS, GDI	↑ (IMI ≥ 15 mg/L), ↑ CP	[224]

		erythrocytes	*in vivo*	imazethapyr, CP as PC	imazethapyr (0.39, 0.78 and 1.17 mg/L), H2O2 (40 mg/L)	VS, GDI	↑ (IMZT ≥ 0.39 mg/L), ↑ CP	[227]
		blood cells	*in vivo*	imazethapyr, H_2O_2 as PC	imazethapyr (0.39 mg/L), H_2O_2 (50 µM)	VS, GDI	↑ (IMZT), ↑ (H_2O_2)	[229]
	Boana pulchella (formerly named H. pulchelus)	erythrocytes	*in vivo*	imazethapyr, CP as PC	imazethapyr (0.39 mg/L), CP (40 mg/L)	VS, GDI	↑ IMZT , ↑ CP	[237]
	Hypsiboas faber	blood cells	*in situ*	coal mining areas (heavy metals)	Santa Catarina (Brazil)	DDI, DF	↑ (dependent on the site)	[257]
	Polypedates megacephalus	erythrocytes	*in vivo*	avermectins	abamectin (ABM; 0.006, 0.012, 0.018, 0.024 and 0.030 mg/L), ivermectin (IVM; 0.003, 0.006, 0.009, 0.012 and 0.015 mg/L), emamectin benzoate (EMB; 0.04, 0.06, 0.08, 0.10 and 0.12 mg/L)	VS, DF, TF	↑ (ABM ≥ 0.012 mg/L), ↑ (IVM ≥ 0.003 mg/L), ↑ (EMB ≥ 0.06 mg/L)	[239]
	Leptodactylus chaquensis	erythrocytes	*in vivo*	poultry litter	poultry litter test sediments (6.25 and 12.5 %)	VS, DI	↑	[242]
	Physalaemus cuvieri	erythrocytes	*in situ*	agricultural fields (soybean and corn crops)	Goiás (Brazil)	TL, % tail DNA, OTM	↑	[240]
	Pelophylax nigromaculata	lymphocytes	*in vivo*	ATZ	1, 10, 100 and 1000 µg/L	TL, % tail DNA, TM	↑ (≥ 1 µg/L)	[235]
	Pelophylax ridibundus	blood cells	*in situ*	polluted lakes	Central Anatolia (Turkey)	TL, % tail DNA,	↑	[260]

						OTM		
	Dendropsophus minutus	erythrocytes	*in situ*	agrochemicals (perturbed areas)	Luziânia and Cristalina, Goiás (Brasil)	TL, % tail DNA, OTM	↑	[241]
		erythrocytes	*in vivo*	ATZ, CP as PC	ATZ (2.25, 4.5, 9 and 18 mg/L), CP (40 mg/L)	TL, % tail DNA, OTM	↑ (ATZ ≥ 9 mg/L), ↑ CP	[236]
	Eleutherodactylus johnstonei	erythrocytes	*in vivo*, *in vitro*	GLY, 4-nitroquinoline-1-oxide as PC	*in vivo* (0.5, 0.9, 1.3 and 1.7 µg a.e./cm^2), 4NQO (0.5 and 2.8 µg/cm^2), *in vitro* (4.6, 9.2, 18.5 and 37 µg a.e./cm^2), 4NQO (3.8 and 60 µg/mL)	VS, GDI	↑ (*in vivo* ≥ 0.5 µg a.e./cm^2), ↑ (*in vitro* ≥ 4.6 µg a.e./cm^2), ↑ 4NQO	[234]
		blood cells	*in vitro*	BLEO, 4NQO	BLEO (4.7, 9.5, 19, 38, 76 and 152 µg/mL), 4NQO (1.9. 3.7, 7.5, 15, 30 and 60 µM)	VS, AU	↑(BLEO ≥ 4.7 µg/mL), ↑ (4NQO ≥ 1.9 µM)	[248]
	Lithobates catesbeianus	erythrocytes	*in vivo*	iron ore, Fe, Mn	iron ore (3.79 mg/L), Fe (0.51 mg/L), Mn (5.23 mg/L)	VS, DDI	↑	[251]
	Bufo americanus	erythrocytes	*in vivo*	MMS	0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 mg/L	LWR	↑ (≥ 6.25 mg/L)	[220]
		erythrocytes	*in situ*	polluted water bodies	Southwestern Ontario (Canada)	LWR	↑ (dependent on the site)	[262]

	Bufo bufo gargarizans	erythrocytes	*in vivo*	butachlor, acetochlor, paraquat, chlorimuron-ethyl, MMS as PC	butachlor (0.1, 0.2, 0.4, 0.8 and 1.6 mg/L), acetochlor (0.1, 0.2. 0.4 and 0.8 mg/L), paraquat (2.66, 3.99, 7.98 and 15.96 mg/L), chlorimuron-ethyl (45, 68, 101 and 152 mg/L), MMS (0.1, 0.2. 0.4 and 0.8 mg/L)	DF, TL, TM, OTM	↑ (butachlor ≥ 0.1 mg/L), ↑ (acetochlor ≥ 0.1 mg/L), ↑ (paraquat ≥ 3.99 mg/L), ↑ (chlorimuron-ethyl 45, 68 and 101 mg/L), ↑ (MMS ≥ 0.1 mg/L)	[231]
		erythrocytes, liver cells	*in vivo*	chlorpyrifos, MMS as PC	chlorpyrifos (0.08, 0.16, 0.32 and 0.64 mg/L), MMS (0.1, 0.2, 0.4 and 0.8 mg/L)	TL, % tail DNA, OTM	↑ (chlorpyrifos ≥ 0.08 mg/L), ↑ (MMS ≥ 0.1 mg/L)	[222]
	Bufo raddei	liver cells	*in vivo*	acetochlor	0.017, 0.034 and 0.065 mg/L	TM	↑ ≥ 0.017 mg/L	[233]
		erythrocytes, liver cells	*in situ*	petrochemicals (oil and phenol)	Lanzhou Region (China)	VS	↑	[243]
	Bufo stomaticus	erythrocytes	*in vivo*	chlorpyrifos, MMS as PC	chlorpyrifos (155, 233 and 456 µg/L), MMS (5 mg/L)	TL	↑ (chlorpyrifos ≥ 155 µg/L), ↑ MMS	[226]
	Bufo boreas	blood cells	*in situ*	mining sediments	Helena National Forest (Montana, USA)	LWR, % tail DNA	↑	[228]

	Rhinella arenarum	erythrocytes	*in vivo*	chlorpyrifos, 2,4-D, GLY	CPF (10 mg/L), 2,4-D (20 mg/L), GLY (20 mg/L)	VS	↑ (CPF), ↑ (2,4-D), Ø (GLY)	[223]
		erythrocytes	*in vivo*	flurochloridone (Twin Pack Gold® and Rainbow®), CP as PC	Twin Pack Gold® (0.74, 1.48 and 2.22 mg/L), Rainbow® (0.71, 1.42 and 2.13 mg/L), CP (40 mg/L)	VS, GDI	↑ (Twin Pack Gold® ≥ 0.74 mg/L), ↑ (Rainbow ® ≥ 0.71 mg/L), ↑ (CP)	[230]
	Rhinella marina	erythrocytes	*in situ*	POPs	Coatzacoalcos River (Mexico)	TL, OTM	↑	[219]
	Anaxyrus terrestris	erythrocytes	*in vivo*	ionizing radiation	^{137}Cs at 0.13, 2.4, 21 and 222 mGy/d (up to 15.8 Gy)	TL, % tail DNA	↑ (21 mGy/d, 1.1 Gy)	[266]
	Duttaphrynus melanostictus	erythrocytes	*in vitro*	Cr (IV), H_2O_2 as PC	Cr (IV) (0.0015, 0.003, 0.015, 0.03 and 0.15 mg/L), H_2O_2 (500 µM)	TL, % tail DNA	↑ (Cr (IV) ≥ 0.015 mg/L), ↑ H_2O_2	[256]
	Pleurodeles waltl	erythrocytes	*in vivo*	captan, MMS as PC	captan (62.5 and 125 µg/mL), MMS (1.56 mg/L)	TL, % tail DNA, TEM, OTM	↑ captan (≥ 62.5 µg/L), ↑ MMS (dependent on the exposure time)	[238]
		erythrocytes	*in vivo*	$CdCl_2$, MMS as PC	$CdCl_2$ (0.5, 1 and 2 mg/L), MMS (1.56 mg/L)	TL, % tail DNA, TEM, OTM	↑ $CdCl_2$, ↑ MMS	[254]
Reptiles								
	Podarcis sicula	erythrocytes	*in vivo*	thiophanate-methyl	100 ml of 1.5% TM	TL	↑ (depende	[269]

							nt on the exposure time)	
		ovarian follicle cells	*in vivo*	$CdCl_2$	1.0 µg/g body weight	VS	↑	[270]
	Darevskia armeniaca	erythrocytes	*in situ*	soil pollution, heavy metals	Armenia and Artsakh (Lesser Caucasus)		↑ (dependent on the site)	[271]
	Darevskia raddei	erythrocytes	*in situ*	soil pollution, heavy metals	Armenia and Artsakh (Lesser Caucasus)		↑ (dependent on the site)	[271]
	Iguana iguana	peripheral blood cells	*in situ*	coal mining areas	La Loma, La Jagua de Ibirico, Valledupar and Arjona (Colombia)	VS, GDI	↑	[272]
	Tupinambis merianae	erythrocytes	*in vitro*	H_2O_2	10, 25 and 50 µM	VS, BDI	↑ (≥ 10 µM)	[273]
		erythrocytes	*in vivo*	age, sex, nest of origin (reference data)	ages (newborns, juveniles and adults), sex (male and female), origin (Argentina)	VS, BDI	↓ (with age), Ø (sex or nest of origin)	[274]
		erythrocytes	*in ovo*	GLY, CP as PC	GLY (50, 100, 200, 400 800 and 1600 µg/egg), CP (200 µg/egg)	VS, DI	↑ (GLY ≥ 200 µg), ↑(CP)	[275]
	Crotalus durissus terrificus	erythrocytes	*in vivo, in vitro*	species differences, MMS as PC	species that differ in their geographical distribution and habitats (Brasil), MMS (10%)	VS, TL, % tail DNA, TM	↑↓ (dependent on the specie), ↑ (MMS)	[276]
	Bothrops jararaca	erythrocytes	*in vivo, in vitro*	species differences, MMS as PC	species that differ in their geographical distribution and	VS, TL, % tail DNA, TM	↑↓ (dependent on the	[276]

					habitats (Brasil), MMS (10%)		specie), ↑ (MMS)	
	B. alternatus	erythrocytes	*in vivo, in vitro*	species differences, MMS as PC	species that differ in their geographical distribution and habitats (Brasil), MMS (10%)	VS, TL, % tail DNA, TM	↑↓ (dependent on the specie), ↑ (MMS)	[276]
	B. neuwiedii	erythrocytes	*in vivo, in vitro*	species differences, MMS as PC	species that differ in their geographical distribution and habitats (Brasil), MMS (10%)	VS, TL, % tail DNA, TM	↑↓ (dependent on the specie), ↑ (MMS)	[276]
	Crotalus durissus terrificus	erythrocytes	*in vivo*	*Hepatozoon* spp., MMS as PC	*Hepatozoon* spp. Infected snakes (non-infected erythrocytes and infected erythrocytes), MMS (0.001%)	VS	↑	[277]
	Chelydra serpentina	liver cells	*in vivo, in ovo*	Arabian light crude oil (PAHs containing oil)	0.5 and 10 g oil/L water	TL, % tail DNA, TM	Ø	[278]
		erythrocytes	*in situ, in vitro*	storage conditions	4, 10, and 24 h on ice + (10% DMSO in PBS, 20% glycerol in PBS, commercial freezing medium)	% tail DNA	Ø ↑ (dependent on the storage conditions)	[284]
	Trachemys callirostris	erythrocytes	*in situ*	polluted field sites	Magangué and Lorica (Colombia)	VS, DI, TL, TM	↑	[279]
	Phrynops geoffroanus	erythrocytes, liver cells	*in vivo*	B[a]P	100, 500 and 1000 µg/kg	-	↑ (≥ 500 µg/kg)	[280]
	Caretta caretta	erythrocytes	*in vivo, in vitro*	age, H_2O_2 as PC	marine rescue centers (Italy), H_2O_2 (30 µM)	VS, DI, % tail DNA	Ø (slight decrease with age),	[282]

							↑ (H_2O_2)	
		erythrocytes	*in vivo, in situ*	ecotoxicological assessment (organochlorines, PAHs, heavy metals)	Sea Turtle Rescue Centers (Italy), Murcia Region (Spain)	% tail DNA	↑ (dependent on the age and stressor)	[283]
	Chrysemys picta	erythrocytes	*in vitro*	UV, H_2O_2	H_2O_2 (200 µmol/L)	% tail DNA	↑ (dependent on the age and stressor)	[281]
	Terrapene carolina	whole blood, erythrocytes	*in situ, in vitro*	storage conditions	4, 10, and 24 h on ice + (10% DMSO in PBS, 20% glycerol in PBS, commercial freezing medium)	% tail DNA	Ø ↑ (dependent on the storage conditions)	[284]
	Trachemys scripta elegans	whole blood, erythrocytes	*in situ, in vitro*	storage conditions, H_2O_2, ETO, MMS	4, 10, and 24 h on ice + (10% DMSO in PBS, 20% glycerol in PBS, commercial freezing medium), H_2O_2 (10, 100 and 1000 µM), ETO (1, 10 and 100 µM), MMS (100, 500 and 1000 µM)	% tail DNA	Ø ↑ (dependent on the storage conditions), ↑ ($H_2O_2 \geq$ 100 µM, ET ≥ 100 µM, MMS ≥ 100 µM)	[284]
	Caiman latirostris	erythrocytes	*in vivo*	age, sex, size, nest of origin (validation assay)	Santa Fe (Argentina)	VS, DI	Ø (independent of the nest of origin, sex and	[285]

							size)	
		erythrocytes	*in vivo, in ovo*	GLY, cyclophosphamide as PC	GLY (50, 100, 200, 300, 400, 500, 750, 1000, 1250 and 1750 µg/egg), CP (700 µg/egg)	VS, DI	↑ (GLY ≥ 500 µg/egg), ↑(CP)	[286]
		erythrocytes	*in vivo, in ovo*	GLY, endosulfan, cypermethrin	GLY (3%), ES (0.85%), CPT (0.12%) + mixture	VS, DI	↑ (GLY alone), ↑ (mixture)	[287]
		erythrocytes	*in vitro*	H_2O_2	25 µM	VS, DI	↑	[288]
		erythrocytes	*in vivo, in ovo*	GLY, CP as PC	GLY (750, 1250, 1750 µg/egg), CP (700 µg/egg)	VS, DI	↑	[289]
	Crocodylus porosus	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS	↑	[290]
Birds								
	Gallus gallus domesticus *	blood lymhocytes	*in vivo*	deoxynivalenol (DON), Mycofix	DON (10 mg of feed-grade/kg of feed), Mycofix (2.5 kg/tonne of diet), + mixture	% tail DNA	↑ (DON), Ø (Mycofix, mixture)	[293]
		blood lymhocytes	*in vivo*	deoxynivalenol (DON), Mycofix	DON (10 mg/kg feed), Mycofix (2.5 kg/ton), + mixture	% tail DNA	↑ (DON), Ø (Mycofix, mixture)	[292]
		spleen leukocytes	*in vivo*	T-2 toxin, DON, dietary nucleotides	T-2, DON (10 mg/kg feed), + nucleotides	% tail DNA, OTM	↑ (T-2, DON, DON + nucleotides), Ø (T-2 + nucleotides)	[297]
		lymphocytes	*in vivo*	T-2 toxin, DMSO	0.5 mg/kg bw	TL, TM	↑ (T-2), Ø	[296]

							(DMSO)	
		fetal liver cells	*in ovo*	aflatoxin B1 (AFB1), N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) as PC	AFB1 (0.062 and 6.2 µg), MNNG (3 µg)	TL, OTM	↑ (AFB1 ≥ 6.2 µg), ↑MNNG	[295]
		lymphocytes	*in vitro*	AFB1, β-glucan, + mixture	AFB1 (0.1, 1, 10 and 20 µg/ml), β-glucan (0.1, 1 and 10%)	VS, TM	↑ (AFB1 ≥ 1µg/ml), ↓ (β-glucan), Ø (mixture)	[291]
		blood cells	*in vivo*	AFB1, piperine, + mixture	AFB1 (0.5 mg/kg bw), piperine (60 mg/kg feed)	VS	↑ (AFB1), Ø (piperine), Ø (mixture)	[299]
		epithelial cells	*in vivo*	dietary polyunsaturated fatty acids ratio (PUFA n-6: n-3) and vitamin E level	dietary n-6: n-3 fatty acid (low, high), vitamin E (50 and 300 mg/kg)	% tail DNA	↑ (low PUFA), ↑ (increased Vit E), (dependent on the age and intestinal segment)	[304]
		lymphocytes	*in vivo*	stress conditions	368 cm^2/bird stocking density (22 hens/cage), fed 75% of the voluntary intake of the control chickens for 14 d	% tail DNA, TM, OTM	↑	[303]

		muscle cells (myoblasts)	*in vitro*	equol, H_2O_2, + mixture	equol (1, 10 and or 100 μM), H_2O_2 (1 mM)	VS	Ø (equol), ↑ (H_2O_2), ↓Ø↑ (mixture, dependent on the equol concentration)	[301]
		lymphocytes (thymus, spleen, bursa)	*in vivo*	selenium deficiency	low-Se diet (0.032 mg/kg Se), control diet (0.282 mg/kg Se, sodium selenite (Na_2SeO_3))	VS, AU	↑ (low-Se diet)	[302]
		liver and muscle cells (fresh chicken liver and breast fillets)	*in vitro*	storage conditions	time-temperature abuse during the storage of poultry	TM, DDI	↑ (dependent on the cell type)	[309]
		brain and liver cells	*in vivo*	*E. coli*	10^7 *E. coli* colony/hen	VS	↑	[310]
		blood cells	*in vivo*	Marek's Disease Virus (MVD)	MDV-infected White-Lohmann hens	VS, AU	↑	[311]
		spermatozoa	*in vitro*	cryopreservation, cryoprotectants	fresh and frozen spermatozoa, cryoprotectants (glycerol 11%, glycerol 11% and trehalose (trh) 70 mmol/L, dimethylacetamide (DMA) 6%, DMA 6% and trh 70 mmol/L)	% tail DNA, TL, OTM, CL	↑ (DMA medium)	[306]

	Meleagris gallopavo	liver cells (hepatocytes)	*in ovo*	furan, 1,3-propanediol (PDO) as PC	furan (0.22–20 µmol/egg), PDO (80-300 µmol/egg)	TM, % tail DNA	↑ (furan ≥ 2 µmol/egg), ↓ (PDO 80 µmol/egg; DNA cross links)	[294]
		foetal liver cells	*in ovo*	aflatoxin B1 (AFB1), N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) as PC	AFB1 (0.062 and 6.2 µg), MNNG (3 µg)	TL, OTM	↑ (AFB1 ≥ 6.2 µg), ↑MNNG	[295]
		foetal liver cells	*in ovo*	flavouring chemicals (alkenylbenzenes)	safrole (1 and 2 mg/egg), methyl eugenol (2 and 4 mg/egg), estragole (20 and 40 mg/egg), myristicin (25 and 50 mg/egg), elemicin (20 and 50 mg/egg), anethole (5 and 10 mg/egg), methyl isoeugenol (40 and 80 mg/egg), eugenol (1 and 2.5 mg/egg), isoeugenol (1 and 4 mg/egg)	% tail DNA	↑ (estragole ≥ 20 mg/egg), ↑ (myristicin ≥ 50 mg/egg), ↑ (elemicin ≥ 20 mg/egg)	[298]

		foetal liver cells	*in ovo*	flavour and fragrance materials, Quinoline (QUI) and 2-acetylaminofluorene (AAF) as PC	p-tert-butyldihydrocinnamaldehyde (BDHCA; 5, 10 and 20 mg/egg), methyl eugenol (MEU; 2 and 4 mg/egg), p-t-butyl-α-methylhydrocinnamic aldehyde (BMHCA; 5 and 10 mg/egg), trans-2-hexenal (HEX; 2.5, 5 and 10 mg/egg), maltol (MAL; 5, 10 and 20 mg/egg), 4-hydroxy-2.5-dimethyl-3(2H) furanone (HDMF; 30, 60 and 120 mg/egg), geranyl nitrile (GN; 6.3, 12.5 and 25 mg/egg), diacetyl (DIA; 6.25, 12.5 and 25 mg/egg), cinnamaldehyde (CINA; 10 and 20 mg/egg), eugenol (EU; 1 and 2.5 mg/egg), allyl isothiocyanate (AITC; 1.2 and 3 mg/egg), isophorone (ISO; 17, 34 and 69 mg/egg), 2,3-dihydro-1,1-dimethyl-1H-indene-arpropanal (DHDMIA; 20 and 40 mg/egg), pent-1-en-3-one (PEN; 0.5 and 1	% tail DNA	↑ (BDHCA, BMHCA, HEX and MAL), Ø (all others)	[300]

					mg/egg), lauric aldehyde (LA; 40, 80 and 100 mg/egg), quercetin (QU; 1.2 and 2.4 mg/egg), D-limonene (DLIM; 60, 80 and 100 mg/egg), menthol (MENT; 20, 30 and 60 mg/egg), geraniol (GER; 10, 15 and 25 mg/egg), methyl dihydrojasmonate (MDHJ; 15, 30 and 60 mg/egg), quinoline (QUI; 3.8, 7.5 and 15 mg/egg), 2-acetylaminofluorene (AAF; 1.6 mg/egg)			
		spermatozoa	*in vitro*	liquid storage, $KMnO_4$	$KMnO_4$ (1, 10, 25 and 50 mM)	% head DNA, TL, OTM, CE	↑ (liquid storage), ↑ (KMnO4	[307]

							≥ 10 mM)	
	Mericanel della Brianza	spermatozoa	*in vitro*	cryopreservation	freezing-thawing (minus 196 °C, 60 °C, 6% dimethylacetamide at 4 °C)	VS, % tail DNA, TL, CL, OTM	↑	[305]
	Alectoris barbara	spermatozoa	*in vitro*	cryopreservation, cryoprotectants		% tail DNA, TL, OTM, CL	Ø (among different media)	[306]
	Coturnix japonica	lymphocyte, bone marrow cells	*in vivo*	chlorpyrifos	6, 8, 10 and 12 mg/kg bw	TL	↑ (≥ 10 mg/kg bw)	[314]
		lymphocytes, hepatocytes	*in vivo*	ATZ, MMC	ATZ (10, 25, 50, 100, 250 and 500 mg/kg bw), MMC (2 mg/kg bw)	VS, TL	↑ (≥ 500 mg/kg bw), ↑ MMC	[315]
		embryo cells	*in ovo*	GSM 900 MHz cellular phone radiation	power density 0.25 μW/cm 2 , specific absorption rate 3 μW/kg	TL, % tail DNA, TM	↑(higher duration), ↓ (lower duration)	[313]
		blood cells	*in vivo*	mannanoligosaccharide prebiotic (Bio-Mos®)	0.5, 1 and 2 g/kg basal diet	TL, TM	↓	[316]
	Alectoris rufa	lymphocytes	*in vivo*	nitrates	100 and 500 mg/L	VS	↑ (at 100 mg/L)	[317]
	Columba livia	erythrocytes	*in situ*	urban air pollution (CO, PM_{10}, NO_2, O_3, SO_2, C_6H_6)	Milan (Italy)	% tail DNA, TM, DI	↑ (dependent on the season)	[318]
		erythrocytes	*in situ*	urban air pollution (CO, PM_{10}, NO_2, O_3, SO_2, C_6H_6, PAHs, temperature, UV index)	Monterrey (Mexico)	VS, AU	↑	[319]

		bone marrow cells	*in vivo*	fenvalerate	170 and 69.64 mg/kg bw	VS	↑	[320]
	Melopsittacus undulatus	blood cells	*in vivo*	antioxidant supplementation (Nutrivit®)	enhanced diet (EQ), reduced diet (RQ)	% head DNA, PID	↑ (RQ), ↓ (EQ)	[321]
		blood cells	*in vivo, in vitro*	captivity, H_2O_2	typical pet cages and on ad libitum food	% head DNA, PID	↑ (without exercise), ↑(H_2O_2)	[322]
	Geothlypis trichas	erythrocytes	*in vivo*	coloration	Oxidative damage to DNA related to survivorship and carotenoid-based sexual ornamentation	% tail DNA	↑↓ (males with brighter yellow bibs showed lower levels of DNA damage)	[323]
	Carduelis chloris	erythrocytes	*in vivo*	paraquat	0.1 and 0.2 g/L	% tail DNA	↑ (≥ 0.2 g/L)	[324]
	Gyps fulvus	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	% tail DNA, TL, OTM, CL	Ø	[308]
	Hirundo rustica	erythrocytes	*in situ*	low-level radioactive contamination	Chernobyl region (Pripyat, Ukraine)	% tail DNA	↑	[325]
		erythrocytes	*in situ*	low dose ionizing radiation	Fukushima (Japan), radioactivity of nest samples (479–143,349 Bq/kg), external exposure 0.15-4.9 mGy	% tail DNA	Ø	[326]
	Ciconia ciconia	lymphocytes	*in situ*	heavy metals (Zn, Pb, As, Cu and Cd)	Doñana (Spain)	TM	↑ (dependent on the compoun	[327]

							d and specie)	
		lymphocytes	*in situ*	acid waste, heavy metals	Doñana (Spain)	TM	↑	[329]
		lymphocytes	*in situ*	acid waste, heavy metals	Doñana (Spain)	TM	↑	[328]
	Milvus migrans	lymphocytes	*in situ*	heavy metals (Zn, Pb, As, Cu and Cd)	Doñana (Spain)	TM	↑ (dependent on the compound and specie)	[327]
		lymphocytes	*in situ*	acid waste, heavy metals	Doñana (Spain)	TM	↑	[328]
	Sterna maxima	blood cells	*in situ*	different contaminant loads	Core Sound, Pamlico Sound, Cape Fear River (North Carolina, USA)	TM	↑ (dependent on the site)	[330]
Mammals								
	Felis silvestris catus	leukocytes	*ex vivo*	H_2O_2	10, 50, 100 and 250 µmol/L	VS, % tail DNA, TL, TM	10, 50, 100 and 250 µmol/L	[384]
		lymphocytes	*in vivo*	dietary supplements	vitamins E and C, β-carotene	% tail DNA, TL, OTM	↓ (in cats with renal insufficiency)	[385]
		spermatozoa	*in vitro*	X-ray system, high-intensity X-ray bursts as PC	16 mrem of radiation exposure	TL	↑ (dependent on the exposure)	[386]

		PBMC	*in vitro*, *in vivo*	metronidazole, H_2O_2 as PC	*in vitro*, metronidazole (5, 50 and 100 µg/mL), H_2O_2 (100 µM), *in vivo* (5 mg/kg metronidazole, 20 mg/kg metronidazole benzoate (12.4 mg/kg metronidazole base))	VS	↑	[387]
		feline T-cell lymphoma line (3201 lymphoma cells)	*in vitro*	metronidazole	5, 50 and 100 µg/mL	VS	↑	[387]
	Prionailurus viverrinus	spermatozoa	*in vitro*	X-ray system, high-intensity X-ray bursts as PC	16 mrem of radiation exposure	TL	↑ (dependent on the exposure)	[386]
	Canis lupus familiaris	leukocytes	*ex vivo*	H_2O_2	10, 50, 100 and 250 µmol/L	VS, % tail DNA, TL, TM	10, 50, 100 and 250 µmol/L	[384]
		peripheral blood cells	*in vivo*	X-ray irradiation	3.9 Gy (at a dose-rate of 6.5 cGy/min)	TM	↑	[389]
		bone marrow cells	*in vivo*	X-ray irradiation	3.9 Gy (at a dose-rate of 6.5 cGy/min)	TM	↑	[389]
		bone marrow cells	*in vitro*	X-ray irradiation	1, 2, 4 and 8 Gy	TM	↑	[388]
		stroma cells	*in vitro*	X-ray irradiation	1, 2, 4 and 8 Gy	TM	↑	[388]
		CFU-F-derived fibroblasts	*in vitro*	X-ray irradiation	1, 2, 4 and 8 Gy	TM	↑	[388]
		oropharyngeal cells	*in vivo*	cigarette smoke, H_2O_2 as PC	five or more cigarettes per day for more than one year, Medellin (Colombia), H_2O_2	% tail DNA, TL, OTM	↑	[390]

					(4%)			
		urothelial cells	*in vivo*	acute bacterial cystitis	*Staphylococcus* sp., *E. coli*, *Staphylococcus* sp., *Klebsiella pneumoniae*, *Proteus mirabilis*	% tail DNA	Ø	[391] 2004
		leukocytes	*in vivo*	dietary antioxidants	blend of vitamins, minerals and carotenoids	VS, AU	↓	[396]
		lymphocytes, brain cells	*in vivo*	Dehydroepiandrosterone (DHEA), H_2O_2	DHEA at 100 mg/kg daily	VS	↓ (DHEA)	[395]
		lymphocytes, prostate epithelial cells	*in vivo*	dietary selenium	selenomethionine (3 and 6 µg/kg/day), high selenium yeast (3 and 6 µg/kg/day)	VS	↓	[392]
		prostate cells	*in vivo*	dietary selenium	selenomethionine (3 and 6 µg/kg/day), high selenium yeast (3 and 6 µg/kg/day)	VS	↓ (dependent on the Se concentration)	[393]
		lymphocytes	*in vivo, ex vivo*	dietary selenium, H_2O_2	selenomethionine (3 and 6 µg/kg/day), high selenium yeast (3 and 6 µg/kg/day), H_2O_2 (25 µmol/L)	VS	↑ (H_2O_2)	[394]
		olfactory and respiratory epithelia	*in situ*	urban pollution (PM_{10})	São Paulo (Brazil)	CL	↑	[397]

		spermatozoa	*in vivo*	correlation between comet assay *vs*. routine assays for the evaluation of semen quality	dogs with abnormal spermiogram *vs*. normospermic dogs		↑ (in dogs with non-normospermic semen)	[398]
	Equus caballus	spermatozoa	*in vitro*	ROS, cryopreservation	xanthine (0.3, 0.6 and 1 mM) - xanthine oxidase (0.025, 0.05 and 0.1), freezing-thawing	VS, CS	↑ ROS, ↑ cryopreservation	[404]
		spermatozoa	*in vitro*	cryopreservation (addition of enzyme scavengers and antioxidants)	freezing-thawing, CAT (200 U/mL), SOD (200 U/mL), GSH (10 mM), ascorbic acid (10 mM), α-tocopherol (25, 50, 100 and 500 μM and 1 mM)	VS, CS	↑	[403]
		spermatozoa	*in vitro*	localization of alkali-labile sites	species differences (*E. asinus vs E. caballus*)	sDFI	↑	[399]
		spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS	↑	[405]
		spermatozoa	*in vivo*	unilateral orchiectomy	mild testis stress	HL, % head DNA, % tail DNA, TL, Tmig, TM	↑	[400]
		spermatozoa	*in vitro*	cryopreservation	flash-freezing temperature	HL, % tail DNA, TL, OTM, TM, CW	↑	[401]
		spermatozoa	*in vivo*	redox status	antioxidant profile	OTM	↑ (low seminal redox status)	[402]

		lymphocytes	*in vivo*	isoflurane anaesthesia	regular castration under inhalation anaesthetic	% tail DNA, TM, OTM, % ‘‘clouds’’	Ø	[419]
		blood cells	*in vivo*	*Theileria equi* infection	oxidative stress in horses naturally infected with *T. equi*	VS, TCS	↑	[420]
	Equus asinus	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	sDFI	↑ (dependent on the temperature)	[406]
		spermatozoa	*in vitro*	localization of alkali-labile sites	species differences (*E. asinus vs E. caballus*)	sDFI	↑	[399]
	Bos taurus	spermatozoa	*in vitro*	sex-sortement of semen	dilution, centrifugation, incubation, exposure to DNA stains, laser light	TL, TM, % tail DNA, % head DNA	↑ (conventional samples)	[410]
		spermatozoa	*in vitro*	cryopreservation, linoleic acid	freezing-thawing, LA (0.125, 0.25, 0.5 and 1 mL)	TL, % tail DNA, TM	Ø	[408]
		spermatozoa	*in vitro*	cryopreservation, fetuin, hyaluronan, + mixture	freezing-thawing, hyaluronan (500 µg/mL), fetuin (2.5 mg/mL)	VS, % tail DNA	↑ (cryopreservation), ↓ (fetuin, hyaluronan, + mixture)	[409]
		spermatozoa	*in vitro*	cryopreservation	freezing-thawing	% head DNA, TL, OTM	↑	[411]

		spermatozoa	*in vitro*	cryoprotectants	glycerol, ethylene glycol, DMSO, + mixture	TL, % tail DNA, TM	↑, ↓ (dependent on the cryoprotectant; no advantages were found in using ethylene glycol or DMSO to replace glycerol)	[407]
	Capra aegagrus	spermatozoa	*in vitro*	cryopreservation, soybean lecithin	freezing-thawing, lecithin (1, 2, 3, 4, 5 and 6 % wt/vol)	% head DNA, % tail DNA	↑, ↓ (dependent on the lecitin concentration)	[412]
	Capra hircus ancryrensis	spermatozoa	*in vitro*	cryopreservation, raffinose, methionine	freezing-thawing, raffinose (2.5, 5 and 10 mM), methionine (2.5, 5 and 10 mM)	VS, AU	↑, ↓ (dependent on the concentration)	[413]
	Ovis aries	spermatozoa	*in vitro*	cryopreservation, raffinose, hypotaurine, + mixture	freezing-thawing, raffinose (10 mM), hypotaurine (5 mM), R+H mixture (5 + 2.5 mM)	VS	↑, ↓	[414]
		spermatozoa	*in vitro*	cryopreservation	influence of sperm concentration	VS, AU	↑, ↓ (dependent on the concentration)	[415]

	Sus scrofa	spermatozoa	*in vitro*	cryopreservation, low-density lipoproteins (LDL), trehalose, yolk	freezing-thawing, effects of different extenders (LDL (9%), trehalose (100 mM), yolk (20%) (v/v))	VS, AU	↑, ↓ (dependent on the extender)	[417]
		spermatozoa	*in vitro*	cryopreservation, LDL, glycerol, trehalose	freezing-thawing, LDL, glycerol, trehalose	% tail DNA, CR	↑, ↓ (dependent on the combination)	[416]
		lymphocytes	*in vitro*	T-2 toxin	0.1, 0.5 and 1 μmol	VS	↑ (≥ 0.1 μmol)	[418]
	Tachyglossus aculeatus	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS	↑	[423]
	Vombatus ursinus	spermatozoa	*in vitro*	H_2O_2	0.003, 0.03 and 0.3 v/v %	VS	↑ (sperm DNA of the marsupial species is more sensitive to oxidative stress than the spermatozoa of eutherian species)	[421]

	Macropus giganteus	spermatozoa	*in vitro*	H_2O_2	0.003, 0.03 and 0.3 v/v %	VS	↑ (sperm DNA of the marsupial species is more sensitive to oxidative stress than the spermatozoa of eutherian species)	[421]
	Phascolarctos cinereus	spermatozoa	*in vitro*	H_2O_2	0.003, 0.03 and 0.3 v/v %	VS	↑ (sperm DNA of the marsupial species is more sensitive to oxidative stress than the spermatozoa of eutherian species)	[421]
		spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS, TL	↑	[422]
	Mus musculus	blood cells	*in situ*	coal mining	La Loma and La Jagua de Ibirico (Colombia)	VS, GDI	↑	[272]
	Mus spretus	leukocytes	*in situ*	mining sites, toxic metals	Doñana (Spain)	% tail DNA, TM	↑	[381]

		blood cells	*in situ*	polluted wetland area	Huelva (Spain)	% tail DNA, TM	↑	[383]
	Peromyscus melanophrys	lymphocytes	*in situ*	mining activity, heavy metals	Morelos (Mexico)	VS, TL	↑	[424]
	Baiomys musculus	lymphocytes	*in situ*	mining activity, heavy metals	Morelos (Mexico)	VS, TL	↑	[424]
	Microtus pennsylvanicus	blood cells	*in situ*	pesticides	Ottawa/Gatineau (Canada)	TL, TM	↑	[426]
	Dipodomys merriami	blood cells	*in situ*	mining sites, arsenic	Villa de la Paz (Mexico)	TM	↑	[425]
	Microtus agrestis	lymphocytes, hepatocytes	*in vivo*	voluntary exercise	voluntary running with or without rest period	VS, AU	Ø	[427]
	Neovison vison	blood cells	*in vivo*	caloric restriction	moderate diet restriction	VS	↓ (restriction), Ø (multiparous females)	[428]
	Rhinolophus capensis	lymphocytes	*in situ*	ionising radiation	20 and 100 µSv/h	VS	↑	[429]
	Neoromicia nana	blood cells	*in situ*	wastewater	pollutant exposure from foraging at Wastewater Treatment Works	VS, OTM	↑	[431]
	Molossus molossus	blood cells	*in situ*	mining sites, heavy metals	Catarinense Carboniferous Basin (Brazil)	VS, DI, DF	Ø	[430]
	Tadarida brasiliensis	blood cells	*in situ*	mining sites, heavy metals	Catarinense Carboniferous Basin (Brazil)	VS, DI, DF	↑	[430]
	Eptesicus diminutus	blood cells	*in situ*	mining sites, heavy metals	Catarinense Carboniferous Basin (Brazil)	VS, DI, DF	↑	[430]

	Diceros bicornis	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS	↑	[432]
	Rhinoceros unicornis	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS	↑	[432]
	Ceratotherium simum	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	VS	↑	[432]
	Panthera tigris altaica	fibroblasts	*in vitro*	cadmium	Cd^{2+} (1.2, 2.4 and 4.8 µM)	VS	↑ 1.2 µM	[433]
	Macaca fascicularis	lymphocytes	*in vivo*	stainless steel welding fume	total suspended particulate (31.36±2.75 and 62.45±2.70 mg/m^3)	OTM	↑	[434]
	Macaca mulatta	spermatozoa	*in vitro*	cryopreservation	freezing-thawing	% tail DNA	↑	[435]
	Tursiops truncatus	leukocytes	*in situ*	validation, baseline values	Sabana-Camagüey archipelago (Cuba)	VS, AU	↑↓ (intra-individual variation)	[436]
		lymphocytes	*in situ*	baseline values, pollution	Indian River Lagoon and Charleston Harbor (USA)	% tail DNA	↑↓ (dependent on the site)	[437]
		lymphocytes	*in vitro*	methyl-mercury	1, 2, 4 and 8 µg/mL	VS, TL, CL	↑ (≥ 1 µg/mL)	[438]
		leukocytes	*in vitro*	TiO_2, H_2O_2 as PC	TiO_2 (20, 50 and 100 µg/mL), H_2O_2 (100 µM)	% tail DNA	↑ (≥ 50 µg/mL), ↑ (H_2O_2)	[442]
		fibroblasts, leukocytes	*in vitro*	TiO_2, MMS as PC	TiO_2 (20, 50 100 and 150 µg/mL), MMS (0.5 mM)	% tail DNA	↑ (≥ 20 µg/mL), ↑ (MMS)	[441]
		leukocytes	*in vitro*	H_2O_2, PCBs, methyl-mercury	H_2O_2 (50, 100, 150 and 200 µM), Aroclor 1254 (0.02, 0,1, 0.5, 2.5 and 12.5 µg/mL), methyl-mercury (4 µg/mL)	VS, TL	↑	[439]

		spermatozoa	*in vitro*	validation	different incubation times	VS	↑↓ (dependent on the sperm morphotype)	[443] 2014
	Sousa chinensis	skin fibroblast cells (ScSF cells)	*in vitro*	POPs, (DDT, chloride (CHL), hexachlorocyclohexanes (HCH), hexachlorobenzene (HCB))	DDT (0.1, 1, 10 and 50 µg/mL), CHL (0.01, 0.1, 0.5, 1 and 5 µg/mL), HCH (0.1, 0.5 and 2.5 µg/mL), HCB (0.01, 0.1, 0.5 and 1 µg/mL)	OTM	↑ (DDT ≥ 1 µg/mL), Ø (CHL), ↑ (HCH ≥ 2.5 µg/mL), ↑ (HCB ≥ 1 µg/mL)	[440]
	Zalophus californianus	lymphocytes	*in vitro*	chemical pollution	B[a]P diol epoxide (BPDE) (2, 4 and 8 µM), H_2O_2 (10, 25 and 50 µM)	TEM	↑ (BPDE ≥ 4 µM) , ↑ (H_2O_2 ≥ 50 µM)	[444]

*, commonly used species; ↑, significant increase; ↓, significant decrease; Ø, no effect; ≥, at and above; % head DNA; % tail DNA; ASC, atypically sized comets; AST, abnormal size tail; AU, arbitrary units; BDI, basal damage index; CE, comet extent; CL, comet length; CR, comet rate; CS, comet score; CW, comet width; DC, damaged cells; Dcoe, damage coefficient; DDI, DNA damage index; DDR, DNA damage rate; DDS, DNA damage score; DF, damage frequency; DI, damage index; GDI, genetic damage index/indicator; HD, head diameter; HDC, highly damaged comets; HL, head length; HR, head radius; II, integral intensity; LWR, length to width ratio; OTM, Olive tail moment; PID, percentage intact DNA; sDFI, sperm DNA fragmentation index; TCS, total comet score; TEM, tail extent moment; TF, tail factor; TL, tail length; TM, tail moment; Tmig, tail migration; VS, visual scoring; 4NQO, 4-nitroquinoline 1-oxide; 5-FU, 5-fluorouracil; AAF, acetylaminofluorene; AFB1, aflatoxin B1; ACR, acrylamide; $AlCl_3$, aluminium chloride; AMPA, aminomethylphosphonic acid; ATO, atorvastatin; ATZ, atrazine; B[a]P, benzo(a)pyrene; BLEO, bleomycin; BRX, borax; CAT, catalase; $CdCl_2$, cadmium chloride; CDDP, cisplatin; CP, cyclophosphamide; CPT, cypermethrin; Cu_2SO_4, copper sulphate; DDT, dichlorodiphenyltrichloroethane; DHEA, dehydroepiandrosterone; DMA, dimethylacetamide; DMSO, dimethyl sulfoxide; DON, deoxynivalenol; EMS, ethylmethanesulphonate; ERY, erythromycin; ES, endosulfan; ETO, etoposide; GEM, gemfibrozil; GLY, glyphosate; GSH, glutathione; H_2O_2, hydrogen peroxide; HCB, hexachlorobenzene; HCH, hexachlorocyclohexanes; IMA, imatinib mesylate; IMI, imidacloprid; IMZT, imazethapyr; LIN, lincomycin; MIT, methylisothiazolinone; MMC, mitomycin C; MMS, methylmethanesulfonate; MNNG, N-methyl-N0-nitro-N-nitrosoguanidine; MVD, Marek's Disease Virus; NP, nanoparticles; OCPs, organochlorine pesticides; PAHs, polycyclic aromatic hydrocarbons; $PbCl_2$, lead(II) chloride; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PC, positive control; PCBs, polychlorinated biphenyls; PCHX, chloroxylenol; PDO, propanediol; POPs, persistent

organic pollutants; QUI, quinoline; RD, Roundup; ROS, reactive oxygen species; SOD, superoxide dismutase; TiO_2, titanium dioxide; TRX, triclosan; UV, ultra violet; VIN, vincristine; WWTP, wastewater treatment plant; ZEA, zearalenone