Section I: Aquatic Invertebrates as Experimental Models

CHAPTER 1

The Comet Assay in Aquatic (Eco)genotoxicology Using Non-conventional Model Organisms: Relevance, Constraints and Prospects

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1.1 Introduction

The integrity of the genome is the very foundation of the organism and all the complex downstream events that mediate the various levels of biological organization, from gene to protein, then cell and tissue, and from there to individual, population and ecosystem. Not surprisingly, the ''success of the fittest'' involves the ability to cope with agents that may interfere with the genome and its transcription. When this ability is overwhelmed (or led into malfunction) by any given agent, such as chemical or radiation, the genetic

Issues in Toxicology No. 33
Ecotoxicology and Genotoxicology: Non-traditional Aquatic Models
Edited by Marcelo L. Larramendy

Published by the Royal Society of Chemistry, www.rsc.org

material accumulates lesions that lead to metabolic dysfunction and then to cell death, or to the fixation of mutations if the cell does survive, thus propagating altered genetic material in somatic or germline cells. The latter case implies severe implications not only for the individual but also for the entire population since it may cause reproductive impairment, teratogenesis and, very importantly, tumorigenesis. Genotoxicity is therefore a phenomenon that affects all aspects of ecosystem functioning and may determine populational and species fitness in their changing habitat, rendering paramount the determination of its effects in ecologically relevant organisms outside the scope of the acknowledged laboratory model. The range of such ''unconventional'' models is increasingly wide, with particular respect to aquatic organisms, comprising many species of fish to molluscs (especially bivalves), to crustaceans, annelids and even echinoderms, cnidarians or macrophytes, whose exemplificative applications will be detailed in subsequent sections. On the contrary, the range of acknowledged model organisms holding some degree of ecological relevance is rather narrow. These include wild-type or genetically modified strains of the freshwater teleost zebrafish (*Danio rerio*) or the cladoceran crustacean *Daphnia magna*. In spite of their value in many fields of research (including biomedical, in the case of the former), these models are entirely laboratorial and cannot provide an entirely realistic insight into ecosystem function impairment by pollutants nor ensure the much needed long-term monitoring programs.

Not surprisingly, on account of the basic Paracelsian principle ''it is only the dose that separates benefit from poison'', toxicologists have long tried to understand, quantify and predict the effects of substances that may damage the genome of both humans and wildlife. As such, the first methods to detect and quantify DNA damage were adopted, adapted and improved from mammalian models (mostly *in vitro*) by environmental scientists from the start and successfully applied to a wide range of vertebrate and invertebrate organisms holding ecological and even economical relevance, thus giving birth to the domain of ecogenotoxicology. This implied, nonetheless, not only changes in protocols to harvest tissue and cells, for instance, but also in the interpretation of the findings *per se*, since genotoxicity is a complex biological phenomenon that depends on multiple pathways that likely differ between distinct taxa.

The methods to detect and quantify genotoxicity first focused on whole-chromosome changes, such as micronuclei and other nuclear abnormalities, which can be expeditiously scored, for instance, in whole-blood samples of non-mammalian vertebrates (since erythrocytes are nucleated), or the sister chromatid assay. These methods detect large-scale, irreparable, lesions that derive from clastogenic and aneugenic events. To this is added the widespread ^{32}P-postlabelling method for detecting DNA–xenobiotic adducts. Other methods, such as the Ames test, address mutagenesis by itself by detecting the reversion of his-mutant *Salmonella* strains back to bacteria able to synthetize this amino acid, by the action of mutagens. Even though

the adequacy and value of these methods is still beyond dispute, there was still a lack of a protocol that could efficiently detect alterations to the genome at the DNA strand level. A revolution thus took place when the single cell gel electrophoresis (SCGE) assay, or simply the ''Comet'' assay, was developed and rapidly incorporated within toxicological sciences, with emphasis on ecotoxicology and environmental toxicology. The common alkaline variant of the Comet assay, which stands as the workhorse of the protocol, originally settled by Singh *et al.*[1] and based on the ''neutral'' version developed by Östling and Johanson,[2] is nowadays little used. In fact, alkaline Comet assay or simply Comet assay are terms that are used almost interchangeably.

The principle of the assay is simple. Since DNA, like many organic molecules, is charged, when subjected to an electric field the smaller fragments will migrate faster towards one of the poles in a strong alkali environment, preceded by DNA denaturation in the same alkali buffer (~pH 13). Thus, the DNA of individual cells is exposed after embedding in an agarose matrix and the amount of fragment DNA migrating towards the positive electrode, *i.e.* the anode (since oligonucleotides are, essentially, anions), can be determined after staining and scoring using microscopy and imaging tools. The term ''Comet'' results from the typical shape of DNA after cell lysis and electrophoresis (the ''nucleoid''), since large oligonucleotides, *i.e.*, little or not at all fragmented, will be retained in the head whereas the smaller move toward the anode, forming the ''tail''. The migration of fragments, however, depends on several aspects that often tend to be overlooked. First, DNA is itself a supercoiled molecule formed by two oligonucleotide strands. Second, genotoxicants may or may not lead to direct strand breakage. In fact, one of the most critical factors of the assay is DNA denaturation under alkaline conditions since this will permit separation of the two strains and therefore allows the expression of single-strand breaks (if any), and the expression of double-strand breaks (if any) that were transformed into the single-strand after denaturation. To this is added the relaxation of altered DNA segments (loops) and expression of the so-called alkali-labile sites that consist essentially of altered nucleobases that, when DNA is loosened, may break upon electrophoresis (see for instance Tice *et al.*[3]). The intensity of the staining between the head and tail can then be extrapolated as the relative proportion between fragmented and unfragmented DNA as a simple metric among the several direct or derived measures that can be retrieved from analysis and that will be debated further on.

In other words, in spite of the many types of DNA damage that might occur, the alkaline Comet assay indiscriminately detects damage that may either result in strand breakage or contribute to relaxing the DNA molecule to the point of favouring migration towards the anode. As such, the Comet assay has been used to provide a measure of ''total strand breakage'', which, in spite of some bias, is evidently more accurate than ''total DNA damage''. There are, however, variants of the alkaline Comet assay that permit some discrimination of damage by type, which will be addressed later on.

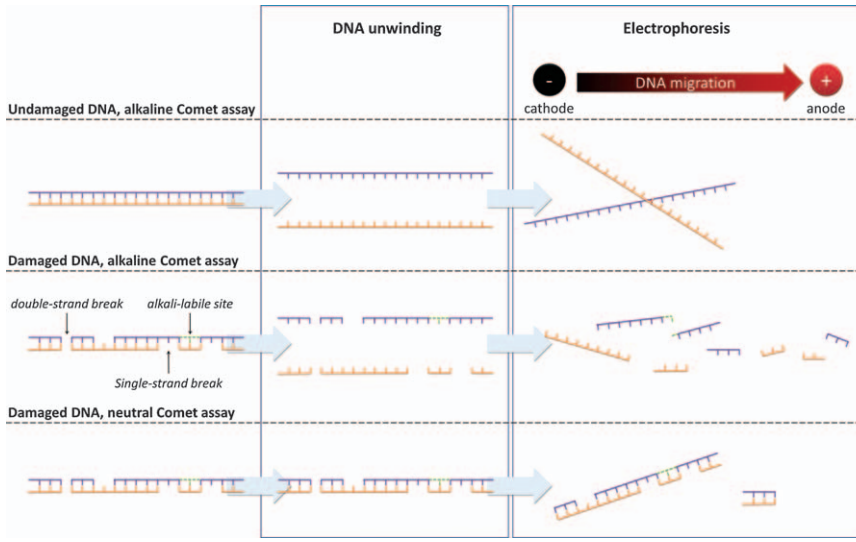

Figure 1.1 Illustration of the basic principle of the Comet assay. When subjected to a strongly alkaline buffer, DNA tends not only to unwind but also to separate the two chains, then exposing single- and double-strand breaks, as well as eventually some alkali-labile sites (*e.g.* altered nucleotides) that may break under the alkali electrophoresis. Compared to the ''neutral'' version, the alkaline Comet assay yields not only more fragments but also lower molecular weight fragments, whose speed of migration is therefore higher, thus producing longer ''tails''.

By comparison, the scantly used ''neutral'' Comet assay follows the exact same principle. However, the denaturation/electrophoresis buffer has a lower pH (10), therefore failing to efficiently separate the DNA strands, favouring only the migration of double-strand fragments (Figure 1.1).

There is thus an important difference in the meaning of DNA strand-breakage when compared to other genotoxicity assessment methods, especially the micronucleus test and its variants, often referred to as nuclear abnormalities (NAs), since the latter refers to whole-chromosome damage such as aneugenesis (chromosomes that are not integrated within the nucleus of a daughter cell) or clastogenesis (chromosomal fragmentation), which is commonly associated with faulty cell division. Unlike DNA strand-breakage, lesions at the chromosome level are most unlikely to be repaired (Fenech *et al.*[4]). Even though the relevance of scoring NAs in aquatic ecotoxicology is undisputable,[5] it has been shown, even in studies with non-model marine fish like sole and bass, that the two measures may not necessarily be correlated.[6,7] However, unlike assessing NAs, the Comet assay is not yet used regular biomonitoring approaches in aquatic ecotoxicology. It could be argued that the lack of standardization of protocols and its multiplicity could be hindering the value of the SCGE assay; however, in most cases, the logistics of field sampling greatly favour the high

cost-effectiveness of preparing blood smears when compared to a molecular method whose accurateness greatly relies on avoiding accessory strand breakage. Still, the Comet assay has been widely employed in both *in situ* and *ex situ* (laboratory) bioassays and, although to a lesser extent, in passive biomonitoring of marine and freshwater ecosystems, thus involving surveys with a broad range of unconventional model organisms, as debated below. Still, there are many technical aspects that render aquatic ecogenotoxicity with these species particularly challenging. As previously highlighted in the few reviews specifically dedicated to the topic, the application of the Comet assay cannot be based on the same assumptions of biomedical research and human-oriented toxicology that, to date, still dictate most protocols and guidelines.[8–10]

1.2 The Comet Assay in Aquatic Ecotoxicology: Role of Unconventional Models

1.2.1 Aquatic Ecosystems as the Ultimate Fate for Pollutants

When translated to ecotoxicology, the principle with which Paracelsus gave birth to toxicology simply stands as ''contamination does not necessarily mean pollution''. In other words, hazard and risk are two distinct concepts. Whereas some substances may be more hazardous than others (*e.g.*, we can compare the metals cadmium and copper), risk is the probability of adverse effects occurring. This means that the dose or concentration can turn a scarcely hazardous agent into a high-risk pollutant.[11] The ecotoxicologist must keep in mind that contamination occurs when the levels of one or more given agents surpass baseline environmental concentrations. If these concentrations cause deleterious effects to biota, then pollution is indeed occurring. The main challenges are, first, to detect deleterious effects and, second, to determine causality. In fact, ecosystem complexity is one of the major factors hindering the establishment of cause–effect relationships in this field of research. On the other hand, dealing with non-model organisms, quite often from ''unconventional'' taxa, poses additional challenges, albeit being crucial to understand how the ecosystem, and not just a species or a population, is affected by pollutants. Altogether, aquatic ecosystems hold many characteristics that render aquatic ecotoxicology as complex as it is important: (i) the aquatic environment is invariably the ultimate fate of environmental toxicants; (ii) areas adjacent to marine and freshwater ecosystems have always received the highest anthropogenic pressure; (iii) the sources of toxicants are multiple, natural or anthropogenic, and include aquatic transport, direct discharge, atmosphere, urban drainage and maritime/fluvial transport; and (iv) aquatic ecosystems, especially those of transitional waters, have peculiar characteristics that render them ideal for accumulation, transformation and long-term storage of hazardous substances, particularly in sediments.[11–13] Altogether, surveying the effects of

pollutants on aquatic organisms is paramount as a tool for the diagnosis of ecological status and as a means to understand how a toxicant can affect the functioning of an entire ecosystem. In other words, surveying aquatic organisms plays an important role in Environmental Risk Assessment (ERA), whether as a measure of exposure (effects-oriented research) or as a means to understand why and how a substance becomes toxic to aquatic biota (mechanism-oriented research). In either case, model organisms, such as laboratory strains of the zebrafish or *Daphnia,* are mere surrogates and are not realistic representatives of wildlife. Even though clear advantages of these model strains, such as reduced intraspecific variability and high genomic annotation, permit important basic toxicological research, extrapolation towards wild organisms must be cautionary.

While mechanistic research in ecologically relevant organisms is far from being as developed as in human toxicology, effect-oriented studies are of upmost importance to quantify exposure in these ''models'' since, unless the concentrations of toxicants are either too high or too low, chemical analyses of sediments, waters and biota may be insufficient. Furthermore, it has long been acknowledged that ERA should not rely on a single Line-of-Evidence (LOE), such as a on a single biomarker or chemical determination of toxicants, but rather it should be an integrative approach comprising several LOEs, often referred to as the Weight-of-Evidence (WOE) approach. Determining genotoxicity has been proposed as an active component for these approaches as a biomarker of effect. The reader may refer to the excellent reviews by van der Oost *et al.*,[14] Martín-Díaz *et al.*[15] and Chapman *et al.*[16] for a definition of biomarker practices in Aquatic Ecotoxicology.

The number of genotoxicant substances present in the aquatic milieu keeps increasing. Many of these pollutants are acknowledged to be effective or potential carcinogens to humans, as classified by the International Agency for Research on Cancer,[17] but their effects (including those from mixtures) on wildlife are mostly unknown. Among these substances are ''classical'' genotoxicants, such as many polycyclic aromatic hydrocarbons (PAHs), dioxins [such as tetrachlorodibenzodioxin (TCDD)], pesticides, and As and Cr compounds, just to state a few other examples. However, novel, ''emerging'', compounds are springing up, such as nanomaterials like carbon nanotubes and metal nanoparticles, whose genotoxic effects have been tested in freshwater snails.[18,19] In addition, complex mixtures of organic and inorganic sediment pollutants have been found to cause DNA strand breakage in marine fish even when the individual concentrations of the compounds would indicate reduced or null risk.[6,20] In a similar example from freshwater environments, crucian carp (*Carassius carassius*) exposed to flood water from agricultural grounds yielded significant DNA strand breakage in whole-blood cells, albeit yielding null effects when fish were exposed to ecologically relevant concentrations of pesticides found in the area, confirming not only the complexity of environmental samples but also the need to safeguard some measure of realism in ERA.[21]

It is also of upmost importance to understand that DNA damage may have multiple causes, depending on the substance (or mixtures), organism, route of exposure, concentration and even tissue and organ, if applicable. On the other hand, DNA damage results from the balance between aggression and repair, since evolution has favoured metabolic pathways that protect the integrity of the species' genetic heritage, which is far from being fully understood, especially in invertebrates, rendering data interpretation cautionary (Figure 1.2). Many organic compounds, like PAHs, which are hydrophobic and metabolically inert, are bioactivated by CYP (cytochrome P450) mixed-function oxidases (MFOs) and other enzymes into highly genotoxic metabolites that form bulky adducts with the DNA molecule. It is

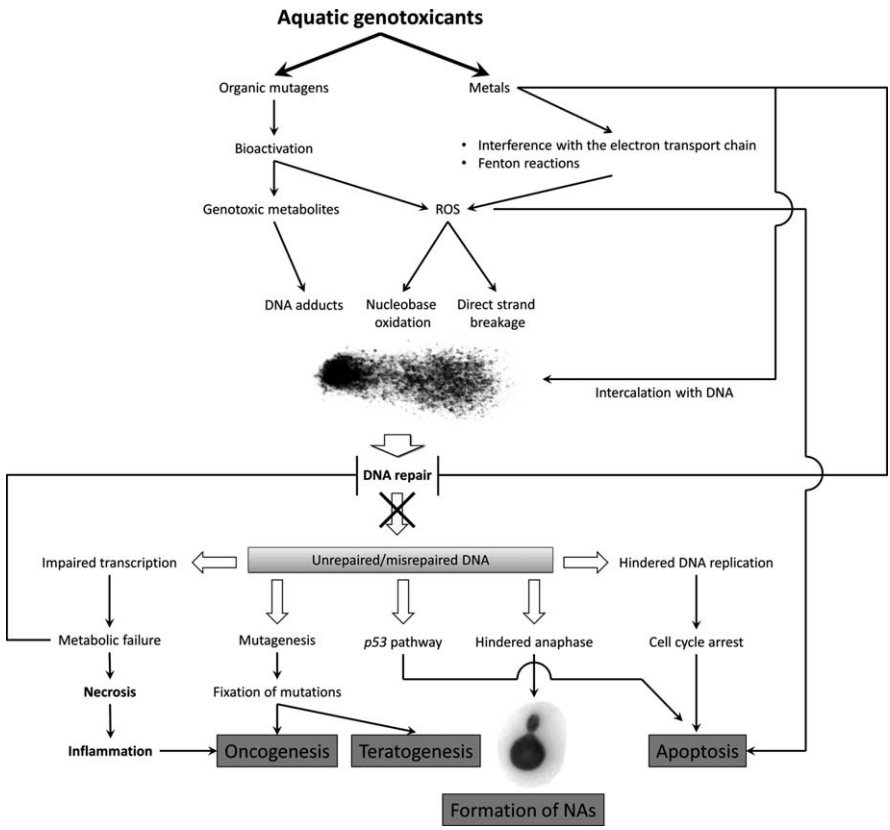

Figure 1.2 A simplified overview of the molecular pathways that link genotoxicity detectable by the Comet and nuclear abnormalities (NA) assays with oncogenesis, teratogenesis and other pathological alterations as expected to occur in vertebrates, exemplified here as in co-exposure to organic mutagens (such as some PAHs and dioxins) and metals to highlight some of the potential interactions between toxicants. These pathways, albeit originally described in higher-order vertebrates, have also been described in fish.

the case for diol-epoxides generated after activation of the potent mutagen and carcinogen benzo[*a*]pyrene (B[*a*]P). Nowadays, it known that the binding of B[*a*]P metabolites to DNA is not random as there is affinity towards sequences of proto-oncogenes (such as those of the *ras* family in vertebrates, with known homologues in fish), leading to their activation, overexpression and triggering of anaplastic transformation of cells. The reader may refer to the review by Xue and Warshawsky[22] for further details. The bioactivation process may generate reactive oxygen species (ROS) that, among other effects, can oxidise nucleobases, especially the highly reactive hydroxyl ($^{\bullet}$OH) radical (see Cadet *et al.*[23]). These single-strand lesions may be repaired by the nucleotide (NER) and base (BER) excision repair pathways, respectively. Furthermore, NER can be global genome (GG-NER) or transcription-coupled (TC-NER), involved in the removal of DNA and RNA polymerase blocking adducts, respectively, such as the bulky adducts formed by PAH metabolites.[24] Double strand breakage can also be repaired, albeit by the more complex processes of homologous recombination (HR) and non-homologous end-joining (NHEJ). The aforementioned pathways are the most familiar to toxicologists. Nonetheless, to these are added mismatch repair and interstrand crosslink repair. These repair mechanisms are well described in mammals and even in fish.[25] Nevertheless, little is known about DNA repair in invertebrates. In turn, metals (and some metalloids, like As) hold a very distinct mode-of-action as genotoxicants, as they enter the nucleus and intercalate with DNA only at high concentrations. The mechanism is then mostly indirect, through, for instance, the interference with DNA repair mechanisms, generation of reactive oxygen species (ROS) or general metabolic impairment. In the first case, metals like Cd (toxic) and Cu (essential) are believed to displace Zn from the active sites of Zn-finger enzymes involved in DNA damage detection and repair.[26,27] On the other hand, Se and its derived compounds may ameliorate damage, presumably though an anti-oxidative effect.[28] Interestingly, even apoptosis, which can be triggered intrinsically through the *p53* pathway when DNA repair is overwhelmed, can be blocked by toxic metals like Cd even in the presence of potent mutagens like B[*a*]P, which has been found to occur even in marine fish.[29] It may be inferred, therefore, that dealing with environmental samples and field-collected (or tested) animals will most likely imply interpreting results from interaction of toxicants. Moreover, mutagenesis and tumorigenesis are chronic effects that not only take time to occur as the fixation of the mutation may not necessarily correlate to the extent of DNA lesions determined by the Comet assay.[30] Still, in spite of its urgent demand, establishing cause–effect relationships between genotoxicants and neoplasia-related disease in wild aquatic organisms seldom occurs, with few exceptions, such as the notorious work by Myers *et al.*[31] that related PAH exposure to neoplasia-related disease in English sole (*Pleuronectes vetulus*) from the Puget Sound. This work resulted, nonetheless, from extensive sampling campaigns and statistical modelling. Genotoxicity was hitherto not assessed.

1.2.2 Unconventional Models in the Biomonitoring of Aquatic Ecosystems

In the European Union, similarly to other industrialised nations, the need for monitoring the aquatic environment has been translated into policy and regulations, which implies more than mere substance testing, since it is critical that developed programmes consider passive sampling of local species for ERA. In the United States, for instance, monitoring programmes such as NOAA's (National Atmospheric and Oceanic Administration) National Status and Trends programmes (such as Mussel Watch) and the US Geological Survey's Biomonitoring of Status and Trends have been running for many decades for the purpose of ERA in marine and river basins, respectively, surveying a wide range of ecologically and economically relevant species, especially fish (*e.g.* salmonids) and shellfish (like mussels, cockles and clams). However, these approaches do not yet include genotoxicity assessment, favouring long-established methods such as histopathology and toxicant burden analyses. In the European Union, both the Marine Strategy Framework Directive (Directive 2008/56/EC) and the updated Water Framework Directive (Directive 2008/56/EC) mandate the development of effective monitoring programmes for marine and continental waterbodies, which include, as in the famous Descriptor 8 of the first, the need to ascertain the occurrence of deleterious effects to biota as a consequence of anthropogenic action (besides determining the contaminant burden in living marine resources of relevance for human consumption, as stated in Descriptor 9). This means, for an environmental toxicologist, to determine if contamination is rising towards pollution.[32,33] Still, some EU countries have their own long-established monitoring programmes for the aquatic milieu, such as the United Kingdom's Clean Seas Environmental Programme (CSEMP) and the transnational Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) to standardize biomarker methods. However, as with the aforementioned programmes, neither includes genotoxicity assessment, which is an important gap. In fact, some studies have disclosed the occurrence of genotoxicity in wildlife long after the removal of the stressor or accident clearance. It is the example of mussels (*Mytilus trossulus*) and clams (*Protothaca staminea*) collected from the Exxon Valdez accident area, in Alaska, that revealed an association between DNA damage (determined though the Comet assay) and PAHs.[34] In fact, the Comet assay has already been proposed as one of the main techniques to detect genotoxic effects in marine wildlife after oil spills, in large part owing to the known genotoxicity of many aromatic hydrocarbons.[35] It must be noticed, though, that the Comet assay has reduced or null specificity towards a specific toxicant of class or toxicant, mostly owing to the aforesaid complexity of genotoxicant action, which mandates caution when interpreting data obtained *in situ* or *ex situ* with intricate matrices such as natural waters and sediments. As such, careful planning of experiments, choice of biological model, complementary analyses (*e.g.* chemical determinations) and objective data interpretation are mandatory.[16,36]

1.3 Application of the Comet Assay in Non-conventional Aquatic Models

Non-conventional models are mostly employed as surrogates for wildlife or as indicators of ecosystem status in effects-oriented studies. Since different taxa have distinct molecular and physiological pathways to deal with a pollutant, the ''one measure fits all'' concept cannot possibly be applied to the biomarker approach in ERA. The choice of model or sentinel/indicator organism, and approach, is thus paramount and results from a careful balance between ecological relevance and the need to circumvent noise variables. It must be noted that the diversity of biological models in studies involving the Comet assay in marine or estuarine organisms is wide. However, the diversity of freshwater ''models'' keeps increasing, leading to applications in perhaps unsuspected organisms, from fish and molluscs to flatworms, leeches, and reptiles—without neglecting macrophytes, which have been receiving important attention in pesticide-related risk assessment.[37–40]

1.3.1 Fish and Other Vertebrates

Fish are unquestionably one of the most important sentinel organisms in biomonitoring, owing to their ecological and economical relevance and their similarity towards high-order vertebrates, *i.e.* mammals, for which toxicological mechanisms are far better described.[41,42] It must be noted that the zebrafish is, in fact, the only true acknowledged piscine biological model, holding high importance in many fields of research, from toxicology to cancer research, benefitting from high genomic annotation and availability of wild-type and genetically modified strains for high-profile biomedical research.[43] This model is thus out-of-scope of the present work.

Besides their abundance in the wild or availability from aquaculture facilities, the ease of collecting blood samples and performing the Comet assay on whole-blood (since all cells are nucleated, unlike in mammals) renders these models particularly appealing. Moreover, blood cells (more than cell lines) have been found to hold high responsiveness to the Comet assay. Kilemade *et al.*,[44] for instance, disclosed that blood, liver and gill cells were more sensitive than epidermis and spleen in turbots (*Scophthalmus maximus*) exposed *ex situ* to contaminated marine sediments. Still, gills may be an important target owing to the permanent contact with contaminants in waters or fine sediment particles. Della Torre *et al.*[45] revealed DNA strand breakage in conger eel (*Conger conger*) gills (but not liver, kidney, muscle or intestine) from a chemical weapon dumping site in the Mediterranean Sea, likely to be impacted by the old chemical warfare agent yperite (''mustard gas'').

In marine and estuarine environments, flatfish (Pleuronectiformes) have particular relevance in the ERA of ecogenotoxicants owing to their economical and ecological relevance and to their close contact with sediments. Furthermore, these fish tend to have relatively slow growth and attain

prolonged age, therefore being more prone to acquire chronic toxicopathological disease when subjected to moderate-low levels of toxicants throughout their lifetime. Although far from meeting the requirements as true biological models, their availability in the environment and from mariculture facilities is also an advantage for bioassay-based assessments. Another important aspect regarding these animals, especially when passive biomonitoring (field sampling) is involved, is that flatfish tend to be gregarious and relatively loyal to their habitats.[46] In addition, these animals often inhabit transition ecosystems, such as estuaries, which tend to be some of the most impacted aquatic habitats. The relevance of flatfish yielded attempts to develop specific Comet assay protocols for these animals.[47] Applications of the Comet assay on flatfish are wide and range from passive biomonitoring to field and laboratory assays, with mixed results, albeit with a tendency to yield a good link with the global pattern of environmental (sediment) contamination, especially metals and PAHs, which tend to be the main pollutants of concern in the marine environment. Species are often chosen accordingly to their regional relevance. As such, while soleids like *Solea senegalensis* (Figure 1.3B) tend to be sampled or tested in SW Europe and the Mediterranean,[10,20] while species like the dab (*Limanda limanda*), the English sole or the turbot (*Scophthalmus* spp.) are important targets in northern European countries, Canada and the USA.[43,48–50] Still, among coastal species of interest are also included bass (*Dicentrarchus labrax*), butterfish (*Pholis gunnellus*), wrass (*Symphodus melops*) and even eel (*Anguilla anguilla*).[7,51–53] Interestingly, common species like the gilthead seabream, *Sparus aurata* (Figure 1.3B), for instance, although well-known to ecotoxicologists more dedicated to substance testing[54] in SW Europe and the Mediterranean (mostly owing to its availability from mariculture facilities), have yet to be put to test in real biomonitoring scenarios using the Comet assay. Overall, virtually all of these species are able to produce convincing results in monitoring and substance-testing results and in the integration of the Comet assay with other biomarker techniques, even though the overall appraisal of these works yields the notion that there is much interspecific

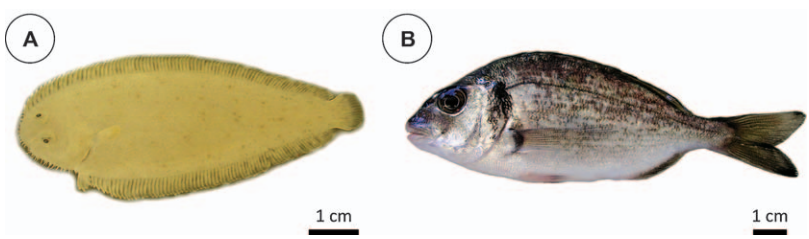

Figure 1.3 Examples of marine teleosts employed in ecotoxicology-related studies that used the Comet assay to determine genotoxicity. (A) Juvenile *Solea senegalensis* Kaup, 1858 (Pleuronectiformes: Soleidae). (B) Juvenile *Sparus aurata* L. 1758 (Perciformes: Sparidae). See [ref. 8, 20, 54], for examples.

variability, which calls for caution when selecting the target organism. Refer to Martins and Costa[10] for a detailed listing of published literature on marine fish.

In freshwater environments, the variety of target species for genotoxicity assessment is too wide to list here in full detail. Not surprisingly, freshwater fish are become increasingly important in the monitoring of tropical ecosystems, whose preservation is a priority worldwide. Tropical fish tend to be robust and easy to breed. More conventional models like the zebrafish, but also tilapia, carp and goldfish (all tropical cyprinids), for instance, greatly benefit from these characteristics. In addition, even in non-tropical countries worldwide these animals are deployed as laboratory models for a variety of subjects, toxicology (environmental or pharmacological) included. It is also the case of the tilapia (*Oreochromis* spp.), whose robustness improves the logistics of long term-assays like the one reported by Lima *et al.*[55] for the testing of chronic genotoxicity caused by animal farm effluents, which showed an increase in both oxidative stress biomarkers and DNA strand breakage. Southern American Characiforms (like *Prochilodus lineatus*) are also gaining ground, such as *Channa punctata* (=*Channa punctatus*) in SE Asia, for both ERA and substance testing.[56–58] The type of stressors that are studied are wide but there is a clear concern with the quality of effluents and the effects of pesticides upon the integrity of DNA (and other endpoints), thus reflecting the priorities of developing rural areas.

Amphibians, on the other hand, are far from being unconventional models in many fields of life science research, including substance or pollutant testing. Comet assay protocols are indeed available for model species such as the frog *Xenopus laevis*. The reader is thus diverted, for instance, to the review by de Lapuente *et al.*[9] However, biomonitoring approaches for ecogenotoxicants in freshwater ecosystems with wild amphibians are less common. These animals are very sensitive to pollutants and many are endangered species, which, in spite of increasing their relevance, may render extensive campaigns prohibitive. Still, there are a few works that should be mentioned as important examples. It is the case of the work by Gonçalves *et al.*[59] with tadpoles of the tropical frog *Dendropsophus minutus*. This work integrated both Comet assay and the micronuclei test (in blood, similarly to fish) and revealed the good sensitivity of both methods by comparing animals from impacted and reference sites. Maselli *et al.*[60] also stated the sensitivity of undisclosed anuran species collected in the wild for ecogenotoxicity assessment with the Comet assay. Furthermore, in the past there have been successful attempts to develop bioassays *in situ* with caged tadpoles, which may be an interesting alternative to passive biomonitoring.[61]

The application of the Comet assay in higher-order aquatic vertebrates is uncommon. As one of the few examples, Lee *et al.*[62] applied the Comet assay in the bottlenose dolphin (*Tursiops truncatus*) lymphocytes of dolphins from an impacted and a reference location, but yielded a stronger relation between susceptibility to acquire infection than environmental status. Caliani *et al.*[63] successfully surveyed DNA strand breakage in whole-blood of the

loggerhead turtle (*Caretta caretta*) collected in the Mediterranean Sea but might have neglected factors such as migration or signs of trauma in animals. Similarly, Zapata *et al.*[64] studied the application of the Comet assay and the micronucleus test in blood cells of the freshwater turtle *Trachemys callirostris* collected from several sites in Colombia. The authors claimed technical success and established a baseline of DNA damage for future endeavours. It must be noted, though, that these works with high-order vertebrates almost invariably suffer from constraints such as reduced number of specimens and high interspecific variability, which cannot thus be overcome by stratifying sampling into male/female or adult/juvenile, for instance. Furthermore, in spite of the many attempts to standardize the protocol (including the recent OECD guidelines for *in vivo* testing with mammals), the Comet assay is essentially comparative in biomonitoring studies, which requires adequate reference areas, some assurance that migration is limited and considering other noise factors such as natural disease.

1.3.2 Molluscs

Bivalves offer special advantages in ERA, from abundance and ecological relevance to the fact that they are easy to collect, handle and often even breed. In addition, these are fixed organisms and therefore they reflect the conditions of a given area throughout their lifecycle. The metabolization of organic toxicants like PAHs into genotoxic metabolites is, however, thought to be reduced, in comparison with vertebrates.[65] However, CYP-like proteins are known to exist in marine and freshwater bivalves and metabolization occurs that is able to generate PAH metabolite–DNA adducts.[66–69] Still, information regarding these pathways and their relation to DNA in molluscs and invertebrates in general is overall scarce, which further complicates the interpretation of results from the Comet assay since it is possible that lower or null metabolization may yield false negatives. On the other hand, differences in DNA repair, compared to their vertebrate counterparts, may result in increased sensitivity to genotoxicants and/or raising of the baseline levels of DNA damage by natural accumulation of lesions.

Marine mussels (*Mytilus* spp.) are one of the most important sentinel organisms for marine ERA for being abundant and sensitive, being applied in bioassays and passive sampling.[70,71] Nonetheless, sediment-burrowing bivalves like cockles and clams have also been surveyed through the Comet assay. As an example, Dallas *et al.*[71] disclosed good sensitivity towards DNA strand breakage in haemocytes of both mussels and cockles collected from an impacted estuary. However, differential sensitivity of marine bivalves has been reported, with sediment-burrowers potentially being more able to cope with ecogenotoxicants.[72] In another example, Martins *et al.*[73] disclosed DNA strand-breakage to occur in the marine clam *Ruditapes decussata* (=*R. decussatus*) subjected to sediment bioassays, inclusively in animals exposed to realistic concentrations of phenanthrene, a PAH regarded as non-carcinogenic, in a study involving the Comet assay adapted to gill cells.

Oysters are also of particular importance in biomonitoring programmes and have been deployed as target organisms in a range of studies, from basic substance testing to passive biomonitoring, as in the work by Bisset *et al.*,[74] who performed geospatial analysis of DNA strand breakage in the oyster *Crassostrea virginica* collected from an impacted bay in southern USA and disclosed good correlation with the proximity to industrial areas.

There are many species of freshwater bivalves that have been successfully applied in ecogenotoxicological studies with the Comet assay. There is, however, a trend favouring the zebra mussel (*Perna viridis*) for laboratory testing, whereas passive or active biomonitoring involves a multiplicity of species of regional significance, including invasive species such as the Asian clam, *Corbicula fluminea*.[75–78] It must be noted, though, that the heterogeneity of freshwater environments and the higher effects of seasonality are often noted to have a significant impact on Comet assay data.[76,77] Yet among aquatic molluscs, gastropods, especially freshwater, such as *Lymnaea* spp., have also been gaining some attention, albeit almost invariably in works related to the testing of various substances and even nanomaterials and not so much in true biomonitoring procedures.[18,19,79] Interestingly, Vincent-Hubert *et al.*[79] hypothesized that Cd may inhibit DNA repair enzymes in the freshwater snail *Potamopyrgus antipodarum*, similarly to what has been discussed for vertebrates. Overall, the full potential of these organisms for the ERA of ecogenotoxicants remains to be ascertained. Still, their ease to collect in the environment, breed and handle in the laboratory renders these animals interesting non-conventional models in the field of research. Among the rarest examples within molluscs are the application of the Comet assay in wild *Octopus vulgaris*, revealing differential baseline levels of DNA strand breakage between distinct organs, with the gonads yielding the lowest levels, followed by the ''kidney'', gills and digestive gland.[80]

1.3.3 Other Organisms: From Crustaceans to Algae

There are few reports on the use of the Comet assay to measure DNA strand breaks in crustaceans and they almost exclusively deal with toxicity testing rather than biomonitoring. The freshwater crustacean *Daphnia magna*, an acknowledged model species, may be considered the main target and has its own protocols for performing the Comet assay on the haemolymph-derived cells[81] that may be a good basis for other small-sized species. Among the few examples with unconventional species are works such as that from Hook and Lee,[82] who exposed embryos of the marine shrimp *Palaemonetes pugio* to Cr and B[*a*]P to study DNA repair among different developmental stages, disclosing no differences during exposure but showing that later stage embryos could recover faster from insult. In one of the scarce examples of biomonitoring approaches with crustaceans, Roberts *et al.*[83] investigated DNA strand breakage through the Comet assay in wild *Corophium volutator* (Amphipoda) to address the interaction between ocean acidification and metal toxicity.

Although widely employed in ecotoxicology, there are few applications of the Comet assay on Polychaeta, for which coelomocytes are the preferred target for the assay (as for their terrestrial counterparts, the Oligochaeta), most of which are related to substance testing.[84–86] Interestingly, there is some evidence in deposit-feeding Polychaeta of the ability to metabolize PAHs and generate DNA strand breakage in parallel.[87] Even though the mechanisms remain obscure, this makes these benthic animals interesting for ERA of aquatic sediments. Finally, a word must be provided for taxa that have been almost neglected from ecogenotoxicity, such as echinoderms and cnidarians. These organisms, so far subjected only to model toxicants in attempts to optimize protocols, have already yielded promising results with the Comet assay, revealing at least comparable sensitivity to other aquatic organisms such as mussels.[88,89]

Even though aquatic plants (marine included), have been proposed as indicator and sentinel organisms for ERA,[90] only a few studies have, so far, been conducted on ecogenotoxicity assessment. Overall, aquatic macrophytes and algae pose important problems for the Comet assay owing to the existence of a cell wall and subsequent impairment of lysis. However, there are a few interesting studies with freshwater macrophytes to address the effect of pesticides, which is an urgent problem at least in wetlands and other lentic ecosystems surrounding agricultural areas.[40] These promising works, which employ mechanical exposure of nuclei through tissue splicing, may indicate a novel path in ecogenotoxicity testing. Conversely, phytoplanktonic microalgae, in spite of the convenience of analysing cell suspensions, have produced null or scant results, in most part due to problems with cell lysis.[91] Table 1.1 summarizes some of the most significant examples on the application of the Comet assay in non-conventional aquatic model organisms.

1.4 Methods

1.4.1 The Comet Assay and its Modifications: Discriminating Type of Damage and Addressing DNA Repair in Unconventional Aquatic Models

The Comet assay has been widely applied *in vitro* and *in vivo* in model and non-model eukaryotic lifeforms. From the same basic protocol, several adaptations have been derived to fit cell cultures, whole blood or haemolymph, and cells harvested from solid tissues of diverse model and non-model organisms. However, the standard Comet assay is composed of basic eight steps: (i) collecting samples from live specimens; (ii) suspending cells in adequate buffer; (iii) embedding cells in a gel matrix; (iv) spreading the embedded cells onto pre-coated microscope slides; (v) lysing cells with detergents in a hypersaline buffer; (vi) promoting DNA alkali unwinding; (vii) subjecting the nucleoids to electrophoresis in an alkaline moiety and (viii) neutralizing, staining and scoring the slides. Besides proper collection

Table 1.1 Summary of example applications of the Comet assay with non-conventional models discriminated by main taxa and habitat of the species.

Organism	Environment	Approach	Toxicant(s)	Reference
Amphibians				
Bufo americanus	Freshwater	*In situ* bioassay	Undisclosed	61
Dendropsophus minutus	Freshwater	Passive sampling	Likely agricultural	59
Rana clamitans	Freshwater	*In situ* bioassay	Undisclosed	61
Annelids				
Arenicola marina	Marine	*Ex situ* bioassay	B[*a*]P	85
Capitella capitata	Marine	*Ex situ* bioassay	PAH (fluoranthene)	84
Hirudo verbana	Freshwater	*Ex situ* bioassay	Al compounds (water and sediments)	38
Nereis diversicolor	Marine	*Ex situ* bioassay	B[*a*]P, Ag nanoparticles	85,86
Nereis virens	Marine	*Ex situ* bioassay	PAH (fluoranthene)	84
Cnidarians				
Anthopleura elegantissima	Marine	*Ex situ* bioassay	B[*a*]P	88
Crustaceans				
Corophium volutator	Marine	*Ex situ* bioassay	Metals (sediment-bound)	83
Palaemonetes pugio	Marine	*Ex situ* bioassay	B[*a*]P and Cr	82
Echinoderms				
Asterias rubens	Marine	*Ex situ* bioassay	Model genotoxins	89
Fish				
Anguilla anguilla	Catadromous	*Ex situ* bioassay	Yperite	45
Carassius carassius	Freshwater	*Ex situ* bioassay	Pesticides (flood water)	21
Conger conger	Marine	Passive sampling	Yperite	45
Channa punctatus	Freshwater	*Ex situ* bioassay	Pendimethalin	56
Dicentrarchus labrax	Marine	*Ex situ* bioassay	PAHs (sediment-bound)	7
Limanda limanda	Marine	Passive sampling	Likely PAHs	48,50
Oreochromis niloticus	Freshwater	*Ex situ* bioassay	Livestock industry effluents	55
Pholis gunnellus	Marine	Passive sampling	Undisclosed	52
Platichthys flesus	Marine	Passive sampling	Mixed	92
Pleuronectes vetulus	Marine	Passive sampling	Mixed (potential endocrine disruptors)	49

Pleuronichthys verticalis	Marine	Passive sampling	Mixed (potential endocrine disruptors)	49
Prochilodus lineatus	Freshwater	Passive sampling, *ex situ* bioassay	Mixed (agricultural, urban and industrial), Cu	57,58
Scophthalmus maximus	Marine	*Ex situ* bioassay	Mixed (sediment-bound)	44
Solea senegalensis	Marine	*Ex situ*/*in situ* bioassay	Mixed (sediment-bound)	6,20
Sparus aurata	Marine	*Ex situ*	Cu	54
Symphodus melops	Marine	*Ex situ* bioassay	Styrene	51
Zoarces viviparus	Marine	Passive sampling	Mixed (oil spill)	93
Flatworms				
Dugesia schubarti	Freshwater	*Ex situ* bioassay	$CuSO_4$	37
Macrophytes				
Myriophyllum quitense	Freshwater	*Ex situ* bioassay	Azoxystrobin	40
Mammal				
Tursiops truncatus	Marine	Passive sampling	Unknown	62
Molluscs				
Cerastoderma edule	Marine	Passive sampling	Metals	71
Corbicula fluminea	Freshwater	*Ex situ* bioassay	Atrazine and Roundup	78
Crassostrea virginica	Marine	Passive sampling	Mixed	74
Dreissena polymorpha	Freshwater	*Ex situ*/*in situ* bioassay	Cd and B[*a*]P, PAHs	75,77
Lymnea luteola	Freshwater	*Ex situ* bioassay	Ag nanoparticles, carbon nanotubes	18,19
Mytilus edulis	Marine	*Ex situ* bioassay, passive sampling	Styrene, undisclosed environmental pollutants, PAHs (sediment-bound), metals	51,68,70,71
Mytilus trossulus	Marine	Passive sampling	PAHs (oil spill)	34
Octopus vulgaris	Marine	Passive sampling	Likely metals	80
Potamopyrgus antipodarum	Freshwater	*Ex situ* bioassay	Cd and bisphenol	79
Protothaca staminea	Marine	Passive sampling	PAHs (oil spill)	34
Ruditapes decussata	Marine	*Ex situ* bioassay	PAHs (sediment-bound)	73
Scapharca inaequivalvis	Marine	*Ex situ*	Cu	54
Scrobicularia plana	Marine	*Ex situ* bioassay	Ag nanoparticles	86
Sinanodonta woodiana	Freshwater	Passive sampling	Phosphates and metals	76
Reptiles				
Caretta caretta	Marine	Passive sampling	Unknown	63
Trachemys callirostris	Freshwater	Passive sampling	Unknown	64

of tissue and cells (a high percentage of viable cells should be achieved, typically 70% or more), one of the main points for the success of the Comet assay is to ensure that accessory DNA damage is avoided, *e.g.* by working under dim light and in the cold. Even though some cryopreservation protocols for cell suspensions have been developed, the Comet assay should be performed immediately after harvesting biological material to avoid DNA degradation. In addition, it is of great importance that the researcher keeps the protocol constant, once this is set, to safeguard the comparability of results. Table 1.2 provides a general protocol suitable for most circumstances.

Peripheral fluids such as blood and haemolymph from aquatic vertebrates and invertebrates, respectively, are suitable and logistics-friendly. In addition, collection may be performed in a non-destructive way. In fish, good quality blood samples may be collected from the caudal peduncle or immediately above the lateral line with a syringe treated (pre-washed) with an anticoagulant such as heparin or EDTA. Immediately after collection, the samples should be diluted in cold phosphate-buffered saline (PBS), typically between 1 : 100 and 1 : 1000. Too few or too many cells in the Comet field may pose problems upon scoring. On the other hand, haemolymph is usually more difficult to obtain, depending on species and location of puncture. In mussels and many other bivalves, haemolymph can be collected by an expert hand from the adductor muscles. Kenny's Balanced Salt Solution (KBSS) is the commonly recommended buffer to suspend cells harvested from most invertebrates, especially molluscs. In crustaceans, however, syringe puncturing may be more problematic, particularly in smaller animals. To circumvent this, Pellegri *et al.*[81] devised a protocol for *Daphnia magna* in which a quick step with an amalgamator device in presence of glass microspheres proved to be efficient for haemolymph extraction in this species and that PBS is the most appropriate buffer. Cells may be obtained from solid tissue by brief mechanical splicing (chopping) the material in adequate buffer, followed by soft pipetting.[73] Previous protocols for animal tissue included a collagenase step. However, this can usually be omitted, also avoiding accessory DNA damage. In order to precipitate the debris and damaged cells, centrifuging the cell suspension at low speed is an effective procedure in order to obtain the supernatant ready to be embedded in LMPA (low-melting point agarose). Plant material is more problematic owing to the presence of cell walls, but mechanical extraction of nuclei tends to be efficient in soft material.[90]

Recently, some modifications of the standard Comet protocol have been developed in order to detect different types of DNA damage and, eventually, to address mechanisms of DNA repair. These approaches are based on the excision of damage nucleotides by lesion-specific endonucleases after cell lysis, then generating breaks that are detectable after alkali unwinding and electrophoresis. As such, these enzyme-linked Comet assay approaches tend to improve the sensitivity of the method. Commercial forms of these restriction enzymes are now available, usually human forms obtained from recombinant bacteria. The most common are the BER enzymes oxoguanine

Table 1.2 Proposed consensus alkaline Comet assay protocol suitable for the majority of biological models. All steps should be conducted under controlled temperature and dim light or dark (immersion steps) whenever possible.

Step	Duration	Temperature	Solution/buffer	Notes
Cell suspensions	—	4 °C	PBS (vertebrates and plant material) KBSS (invertebrates)	Dilute whole blood or haemolymph 1 : 100 to 1 : 1000. If working with solid tissue, rapidly mince in cold buffer and add sample and buffer to a tube in the proportion of 1 : 10 or greater. Release the cells by soft pipetting. Centrifuge briefly at low speed and use only the supernatant for the assay and for cell counting. Typically, No enzymatic steps (*e.g.* collagenase) are needed. For difficult plant material, refer to Costa *et al.*[94] and references therein.
Dilution in LMPA	—	~37 °C	0.5–1% LMPA (in PBS or KBSS)	Dilute cell suspension in molten LMPA (1 : 100). Typically, the *in vitro* Comet assay employs lower concentrations of LMPA (0.5–0.75%), whereas 1% is suitable for *in vivo* studies.
Preparation of slides	—	Room (~20 °C)	—	Place 2×75–80 μL of cells in LMPA per pre-coated slide. Use frosted or single-frosted slides for labelling.
Place coverslip			—	
Solidification	15 min	4 °C	—	

Table 1.2 (*Continued*)

Step	Duration	Temperature	Solution/buffer	Notes
Remove coverslip			—	
Lysis	30–60 min	4 °C	0.45 M NaCl (m/v); 40 mM EDTA (m/v); 5 mM Tris (pH 10).	The buffer has a short shelf-life even in the cold (1–2 weeks). Add 10% v/v DMSO and ~1% v/v Triton X-100 before use. The buffer cannot be re-used.
DNA unwinding	40 min	4 °C	0.1 μM EDTA; 0.3 M NaOH (pH ≈ 13)	
Electrophoresis	30 min	4 °C	0.1 μM EDTA; 0.3 M NaOH (pH ≈ 13)	Run electrophoresis at 25 V. Buffer can be re-used once.[a]
Neutralizing	15 min	4 °C	0.2 M Tris-HCl buffer (pH 7.5)	
[Fixation]	*10 min*	*4 °C*	*Absolute methanol*	*[Optional]*
Stain	5–10 min	Room (~20 °C)	Ethidium bromide (0.02 mg L^{-1} in water) or suitable replacement, such as SYBR Green	The staining time (to be done in the dark) allows the dye to bind to DNA, otherwise bleaching of the dye will occur immediately with exposure to UV. If working with pre-fixed, dry, slides, rehydrate in cold distilled water for at least 15 min. The slides are not to be washed after staining. Mount with coverslip. Caution: ethidium bromide is a suspected mutagen.[b]

[a]If the electrophoresis speed is too low, check the volume of the buffer in the tank (should just cover the slides).
[b]The slides can be washed in absolute methanol or ethanol after scoring, stored dry and re-analysed.

glycosylase (OGG) and formamidopyrimidine glycosylase (FPG), which convert sites containing oxidized nucleobases (such as oxoguanine) to single-strand breaks.[95] By contrasting the findings from the enzyme-linked and the standard Comet assay, a proportion of DNA oxidative damage can be derived. Although almost routinely employed in clinical research using mammalian cell lines,[96] with applications in ecotoxicology studies with human cell cultures,[97] the enzyme-modified Comet assay is just starting to be applied in ecogenotoxicology studies with unconventional models. It is the case, for instance, in the work by Gielazyn *et al.*[98] to detect oxidative DNA lesions in the oyster *Crassostrea virginica* and the clam *Mercenaria mercenaria*. Additionally, there is a modification of the Comet assay to detect alterations to the organisms' ability to repair DNA *per se*, which is based on treating suspended cells with a DNA-damaging agent like ethylmethylsulfonate (EMS), followed by a recovery period and then running the Comet assay.[99] Nonetheless, this protocol is not yet practiced *in vivo* in ecotoxicology. It must be noted, though, that alterations to the mechanisms of DNA repair in fish cell lines have already been proposed as potential biomarkers of genotoxicity.[25]

1.4.2 Final Remarks on Analysis and Interpretation of Data

There are many factors that influence the Comet assay performance, such as variations in LMPA concentration, alkaline incubation time, electrophoresis voltage (and duration) plus the scoring method, which were pointed out by Azqueta and Collins[100] as issues that can increase the variability of the results. As an example, small variations in electrophoresis speed may increase the size of tails without meaning increased DNA fragmentation, therefore rendering Comet length highly biased as a metric when compared to relative metrics. In addition, performing the Comet assay *in vivo* yields considerable variations in the levels of DNA damage within the same slide, since distinct types of cells at different stages of their life cycle are being surveyed. However, this hindrance can be overcome if at least 50–100 intact nucleoids per slide/individual were scored. In general, employing relative metrics, such as the %DNA in the tail or Olive Tail Moment, may avoid constraints created by variations induced by protocol or something as simple as genome size.[10,101]

Statistics and sampling or experimental planning are also paramount to guarantee data quality and have been discussed elsewhere.[10] Very importantly, the researcher must be aware that non-conventional models, whether reared in the laboratory or collected in the wild, offer high intraspecific variability that will invariably dilute statistical significance if replication and stratification by factors like age and gender are not properly taken into account. In addition, proper controls and references are mandatory in ERA in order to achieve the most important yet the most challenging goal in ecotoxicological studies in such complex areas like aquatic ecosystems—causality.

Abbreviations

B[*a*]P	benzo[*a*]pyrene
BER	Base excision repair
CYP	Cytochrome P450
ERA	Environmental risk assessment
HR	Homologous recombination
KBSS	Kenny's balanced salt solution
LMPA	Low melting point agarose
MFO	Mixed-function oxidase
NA	Nuclear abnormality
NHEJ	Non-homologous end-joining
NER	Nucleotide excision repair
NMPA	Normal melting point agarose
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis
TCDD	Tetrachlorodibenzodioxin

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