The Comet Assay in Toxicology

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The Comet Assay in Toxicology

Edited by

Alok Dhawan
Developmental Toxicology Division, Indian Institute of Toxicology Research, Lucknow, India

Diana Anderson
Division of Biomedical Sciences, University of Bradford, Bradford, UK

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The cover image shows photomicrographs of comets from (A) *Escherichia coli* (B) *Bacopa monerii L.* (C) *Drosophila melanogaster* (D) differential DNA damage lymphocytes (E) human lymphocytes (F) irradiated diploid human lymphocyte with FISH showing double hybridisation signals indicating strand breakage (G) comets in human sperm (H) human sperm showing double breaks (I) haploid human sperm with FISH showing single hybridisation signal.

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Preface

This book is the first of its kind to be devoted exclusively to the Comet assay and its applications as an important tool in current toxicology. This multi-author book will serve as both a reference and a guide for investigators in the biomedical, biochemical and pharmaceutical sciences. Specialists from the fields of genetic toxicology and human epidemiology, with first-hand knowledge of their chosen subspecialities, have contributed to this peer-reviewed scientific venture.

Simplicity, rapidity, versatility and ease of application of the Comet assay have made it a favourite amongst researchers and it is now also gaining acceptance amongst regulators. It can be used in all single cells from prokaryotes and eukaryotes, in plants and animals including humans, involving both somatic and germ cells. It is also a relatively inexpensive assay to perform.

The book is divided into different sections, reflecting the range of interest in the exploitation of this assay. It begins with an introductory section reviewing the genesis of the assay for those new to the technique, and details the various fields in which it finds wide acceptance. This sets the scene by explaining why the assay has become the most sensitive and sought after assay in modern toxicology.

There is a section that describes the protocols being followed to assess various types of DNA damage in different cell types. The third section brings together the specific applications of the assay in diverse areas ranging from genetic toxicity testing to human monitoring, and environmental toxicology. The last section considers strategies for the conduct of the assay using *in vitro* and *in vivo* systems, based on internationally accepted guidelines. The book draws to a close with an assessment of image-analysis principles and the statistics used for evaluating the data generated by the assay.

This book is a culmination of over fifteen years of active collaboration and friendship between the editors and provides a good basic understanding of issues relating to the assay.

The Editors

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GENESIS OF COMET ASSAY

CHAPTER 1

The Comet Assay: A Versatile Tool for Assessing DNA Damage

ALOK DHAWAN*, MAHIMA BAJPAYEE AND DEVENDRA PARMAR

Developmental Toxicology Division, Indian Institute of Toxicology Research (Formerly Industrial Toxicology Research Centre), P.O. Box 80, M.G. Marg, Lucknow, 226 001, India

1.1 Introduction

New chemicals are being added each year to the existing burden of toxic substances in the environment. This has led to increased pollution of ecosystems as well as deterioration of the air, water and soil quality. Excessive agricultural and industrial activities adversely affect biodiversity, threatening the survival of species in a particular habitat as well as posing disease risks to humans. Some of the chemicals, *e.g.* pesticides and heavy metals, may be genotoxic to the sentinel species and/or to nontarget species, causing deleterious effects in somatic or germ cells. Test systems that help in hazard prediction and risk assessment are important to assess the genotoxic potential of chemicals before their release into the environment or for commercial use as well as DNA damage in flora and fauna affected by contaminated/polluted habitats. The Comet assay has been widely accepted as a simple, sensitive and rapid tool for assessing DNA

*Corresponding author

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damage and repair in individual eukaryotic as well as some prokaryotic cells, and it has increasingly found application in diverse fields ranging from genetic toxicology to human epidemiology.

This review is an attempt to comprehensively encase the use of the Comet assay in different models from bacteria to man, employing diverse cell types to assess the DNA-damaging potential of chemicals and/or environmental conditions. Sentinel species are the first to be affected by adverse changes in their environment. Determination of DNA damage using the Comet assay in these indicator organisms would thus provide information about the genotoxic potential of their habitat at an early stage. This would allow for intervention strategies to be implemented for prevention or reduction of deleterious health effects in the sentinel species as well as in humans.

Ostling and Johanson[1] were the first to quantify DNA damage in cells using a microgel electrophoresis technique, known as the single-cell gel electrophoresis (SCGE) or Comet assay. However, the neutral conditions that they used allowed the detection of only double strand breaks in the DNA. Later, the assay was adapted under alkaline conditions by Singh *et al.*,[2] which led to a sensitive version of the assay that could assess both double- and single-strand DNA breaks as well as alkali-labile sites expressed as frank strand breaks in the DNA. Since its inception, however, the assay has been modified at various steps (lysis, electrophoresis) to make it suitable for various kinds of damage in different cells.[3,4] The assay is now a well-established, simple, versatile, rapid, visual, and a sensitive, extensively used tool to assess DNA damage and repair, quantitatively as well qualitatively in individual cell populations.[5] Some other lesions of DNA damage such as DNA cross-linking (*e.g.* thymidine dimers) and oxidative DNA damage may also be assessed using lesion specific antibodies or specific DNA repair enzymes in the Comet assay. It has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies,[4] genotoxicity testing[6] and human biomonitoring.[7,8]

Relative to other genotoxicity tests, such as chromosomal aberrations, sister chromatid exchanges, alkaline elution and the micronucleus assays, the advantages of the Comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 10^{10} Daltons of DNA[9]), requirement for small number of cells ($\sim 10\,000$) per sample, flexibility to use proliferating as well as nonproliferating cells, low cost, ease of application, and the short time needed to complete a study. It can be conducted on cells that are the first site of contact with mutagenic/carcinogenic substances (*e.g.* oral and nasal mucosal cells). The data generated at the single-cell level allow for robust types of statistical analysis.

A limitation of the Comet assay is that aneugenic effects, which may be a possible mechanism for carcinogenicity,[10] and epigenetic mechanisms (indirect) of DNA damage such as effects on cell-cycle checkpoints are not detected. The other drawbacks such as single cell data (which may be rate limiting), small cell sample (leading to sample bias), technical variability and interpretation are some of its disadvantages. However, its advantages far outnumber the

disadvantages and hence it has been widely used in fields ranging from molecular epidemiology to genetic toxicology.

The present review deals with various models ranging from bacteria to man used in the Comet assay for assessing DNA damage (Figure 1.1).

1.2 Bacteria

The first study to assess the genetic damage in bacteria treated with 12.5–100 rad of X-rays, using the Comet assay was conducted by Singh *et al.*[11] In the study, the neutral Comet assay was used for direct (visual) determination of DNA double-strand breaks in the single electrostretched DNA molecule of *Escherichia coli* JM101. A significant increase in DNA breaks was induced by a dose as low as 25 rad, which was directly correlated to X-ray dosage. The study supported a hypothesis that the strands of the electrostretched human DNA in the Comet assay represented individual chromosomes.

1.3 Plant Models

Plant bioassays are important tests that help detect genotoxic contamination in the environment.[12] Plant systems can provide information about a wide range of genetic damage, including gene mutations and chromosome aberrations. The mitotic cells of plant roots have been used for the detection of clastogenicity of environmental pollutants, especially for *in situ* monitoring of water contaminants. Roots of *Vicia faba* and *Allium cepa* have long been used for assessment of chromosome aberrations[13] and micronuclei.[14] During the last decade, the Comet assay has been extensively applied to plants (leaves, shoots, and roots) to detect DNA damage arising due to chemicals and heavy metals in polluted soil (Table 1.1).

1.3.1 The Comet Assay in Lower Plants

1.3.1.1 Fungi

Schizosaccharomyces pombe has been used as a model organism to investigate DNA damage due to chlorinated disinfectant, alum and polymeric coagulant mixture in drinking-water samples.[15] The authors observed a significantly higher ($P<0.001$) DNA damage in chlorinated water (*i.e.* tap water) when compared to untreated (negative control) or distilled water (laboratory control). Hahn and Hock[16] used mycelia of *Sordaria macrospora* grown and treated with a variety of DNA-damaging agents directly on agarose minigels for the assessment of genotoxicity using the Comet assay. DNA-strand breaks were detected by an increase in the DNA migration from the nucleus. This model allowed for the rapid and sensitive detection of DNA damage by a number of chemicals simultaneously. *Saccharomyces cerevisiae* has also been employed for successful investigation of DNA damage at low concentrations of chemicals.[202]

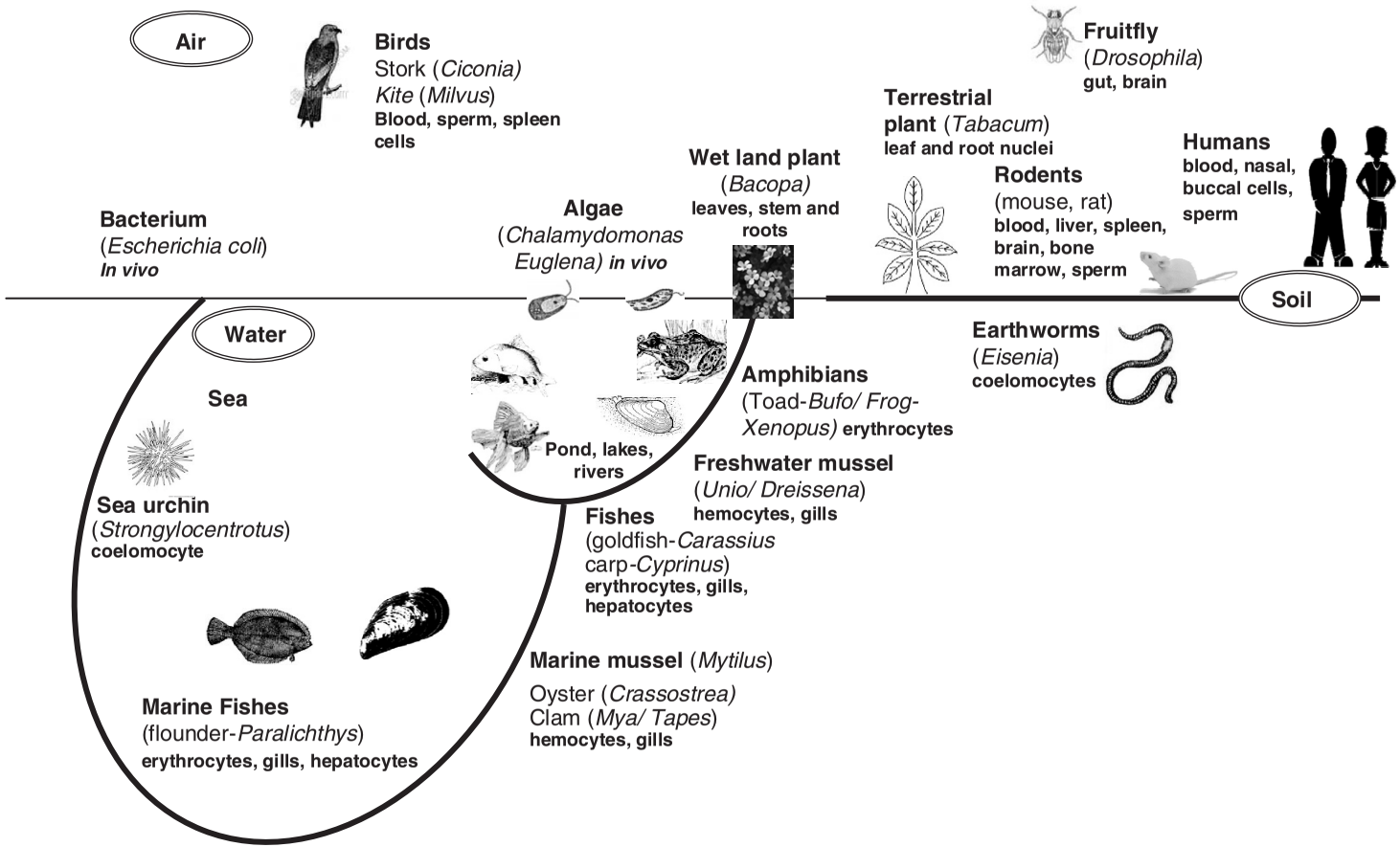

Figure 1.1 Schematic diagram of the use of the Comet assay in assessing DNA damage in different models from bacteria to humans.

Table 1.1 Comet assay for assessment of DNA damage – bacteria to humans.

Model	*Agent tested*	*Cell used*	*DNA damage*	*Ref.*
		Bacteria		
Escherichia coli JM101	X-rays	Whole organism *in vivo*	↑	11
		Plant Models		
Euglena gracilis	1-Methyl-3-nitro-1-nitrosoguanidine (MNNG), benzo[a]pyrene, mitomycin C and actinomycin D.	Whole organism *in vivo*	↑	18
Chlamydomonas reinhardtii	4-Nitroquinoline-1-oxide (4-NQO), N-nitrosodimethylamine, and hydrogen peroxide	Whole organism *in vivo*	↑	17
Rhodomonas	UV (UVA + UVB) radiation	Whole organism *in vivo*	↑	19
Vicia faba	N-methyl-N-nitrosourea (MNU) and methyl methanesulfonate (MMS)	Root tip meristematic cells	↑	21
Tobacco (*Nicotiana tabacum I*)	Ethyl methanesulfonate	Nuclei from leaf tissue	↑	22
	Age	Leaf nuclei		23
	Kinetics of DNA repair	Leaf nuclei		24
	Ethyl methanesulfonate (EMS) and N-ethyl-N-nitrosourea (ENU), maleic hydrazide (MH)	Whole roots *in vivo*	↑	25,26
	O-phenylenediamine (o-PDA), hydrogen peroxide and ethyl methanesulfonate (EMS).	Isolated root nuclei	–	27
	Heavy metal (Cd, Cu, Pb, and Zn)	Leaf nuclei	↑	28
	Polychlorinated biphenyls		↑	29
Potato plants (*Solanum tuberosum var. Korela*)	Heavy metal (Cd, Cu, Pb, and Zn)	Nuclei from leaf tissue	↑	28
Phaeseolus vulgaris	Uranium	Root or shoot cells	–	30
Impatiens balsamina	Cr^{6+} and airborne particulate	Stem, root and leaves	↑	31
Bacopa monnieri L.	Ethyl methanesulfonate, methyl methanesulfonate, cadmium	Nuclei isolated from roots and leaves	↑ dose- and time-dependent roots > leaves	32
		Animal models		
Tetrahymena thermophila	Phenol, hydrogen peroxide, and formaldehyde, influent and effluent water samples	Whole animal *in vivo*	↑	33

Table 1.1 (*Continued*).

Model	*Agent tested*	*Cell used*	*DNA damage*	*Ref.*
		Invertebrates – Bivalves		
Freshwater bivalve zebra mussel (*Dreissena polymorpha*)	Polybrominated diphenyl ethers (pbdes)	Haemocytes	↑↑	34
	Sodium hypochlorite, chlorine dioxide and peracetic acid		↑	35
	Pentachlorophenol		↑	36
	Varying temperatures		↑	37
	Polluted waters		↑	38
Mytilus edulis	Cadmium (Cd) and chromium (Cr)	Gills	–	39
	Styrene	Haemolymph cells	↑	40
	Tritium	Haemocytes	↑	41
	Marine waters (Denmark), French Atlantic Coast	Gill and haemolymph	↑ ↑	42
	Polycyclic aromatic hydrocarbons	Gill and haemocytes	↑	44
	Seasonal variation	Haemocytes		45
Freshwater mussels (*Unio tumidus*)	Polyphenols	Digestive gland cells	↑	46
Golden mussel (*Limnoperna fortunei*)	Guaíba Basin water	Haemocytes	↑	47
Bivalve mollusc (*Scapharca inaequivalvis*)	Organotin compounds (MBTC, DBTC and TBTC)	Erythrocytes	↑	48
Mytilus galloprovincialis	Environmental stress	Haemocytes	↑	49
	Heavy oil spill	Gills		50
	Cadmium	Digestive gland cells		51
Vent mussels (*Bathymodiolus azoricus*)	Hydrostatic pressure change	Haemocytes and gill tissues	↑	52,53
Green-lipped mussel (*Perna viridis*)	Benzo[a]pyrene	Haemocytes	↑	54
Freshwater mussel (*Utterbackia imbecillis*)	Chemicals used in lawn care (atrazine, glyphosate, carbaryl, and copper)	Glochidia	↑	55
Oyster (*Crassostrea gigas*)	Cryopreservation	Spermatozoa	↑	56

Manila clam (*Tapes semidecussatus*)	Sediment-bound contaminants	Haemolymph, gill and digestive gland	↑	57,58
Clams (*Mya arenaria*)	Petroleum hydrocarbons	Haemocytes and digestive gland cells	–	59
		Invertebrates – Earthworms		
Eisenia foetida	Chemical-treated soil	Coelomocytes	↑dose dependent	60
	Soil from coke ovens	Coelomocytes	↑	61
	Soil from industrialised contaminated areas	Coelomocytes	↑	62
	Sediment from polluted river	Coelomocytes	↑	63
	Wastewater-irrigated soil	Coelomocytes	↑	64
	Commercial parathion	Coelomocytes	↑	65
	Imidacloprid and RH-5849	Sperm cells	↑	66
	PAH-contaminated soil and hydrogen peroxide, cadmium (*in vitro*)	Eleocytes	↑	67
	Nickel chloride	Coelomocytes	↑	68
Aporrectodea longa (Ude)	Soil samples spiked with benzo[a]pyrene (B[a]P) and/or lindane	Intestine and crop/gizzard cells	↑ intestine > crop	69
		Other Invertebrates		
Fruit fly (*Drosophila melanogaster*)	Ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-ethyl-N-nitrosourea (ENU) and cyclophosphamide (CP)	Gut and brain cells of first instar larvae	↑	70,71
	Cypermethrin		↑	72
	Lechates of industrial waste		↑	71
	Cisplatin		↑	73
Sea urchins (*Strongylocentrotus droebachiensis*)	Dispersed crude oil	Coelomocytes	↑ concentration-dependent	74
Grass shrimp, (*Paleomonetes pugio*)	UV, benzo[a]pyrene, and cadmium	Embryos	↑ damage and decreased repair	75
	Estuarine sediments	Hepatopancreas	↑	76
	Coal combustion residues			77
Sea anemone (*Anthopleura elegantissima*)	Hydrogen peroxide ethylmethanesulfonate (EMS) or benzo[a]pyrene (B[a]P)	Blood cells	↑ dose response	78

Table 1.1 (*Continued*).

Model	*Agent tested*	*Cell used*	*DNA damage*	*Ref.*
		Vertebrates – Fishes		
Chub (*Leuciscus cephalus*)	PAHs, PCBs, organochlorine pesticides (OCPs), as well as heavy metals	Hepatocytes	↑	79
	Exhaustive exercise	Erythrocytes	↑	80
Estuarine mullet (*Mugil sp.*) and sea catfish (*Netuma sp.*)	Organochlorine pesticides and heavy metals High temperature	Erythrocytes	↑	81,82
Fresh water teleost fish (*Mystus vittatus*)	Endosulfan	Gill, kidney, and erythrocytes	↑ in all cells	83
Eastern mudminnow (*Umbra pygmaea* L.)	Rhine water for 11 days	Blood erythrocytes	↑	84
Neotropical fish (*Prochilodus lineatus*)	Diesel water soluble fraction acute (6, 24 and 96 h) and subchronic (15 days) exposures,	Erythrocytes	↑	85
Freshwater goldfish (*Carassius auratus*)	Technical herbicide Roundup containing glyphosphate salt	Erythrocytes	↑↑ dose dependent	86
	ADDB and PBTA-6			87
Turbot (*Scophthalmus maximus* L.)	Sediment collected from polluted sites in Cork Harbour (Ireland)	Hepatocytes	↑	88
Brazilian flounder (*Paralichthys orbignyanus*)	Contaminated estuary waters	Blood cells	↑↑	89
Bullheads (*Ameiurus nebulosus*)	Polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyl (PCB) polluted waters	Erythrocytes	↑	90
Carp (*Cyprinus carpio*)	Polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyl (PCB) polluted waters	Erythrocytes	↑	90
Brown trout (*Salmo trutta fario*)	PCB77 (3,3′,4,4′-tetrachlorobiphenyl)	Erythrocytes	–	91
Marine flatfish	Ethyl methanesulfate	Blood, gill, liver and kidney	↑ in all tissues	92
Trout (*Oncorhynchus mykiss*)	Cryopreservation (freeze–thawing)	Spermatozoa	Slight ↑	93

European eel (*Anguilla anguilla*)	Benzo[a]pyrene, Arochlor 1254, 2-3-7-8-tetrachlorodibenzo-p-dioxin and beta-naphthoflavone	Erythrocytes	↑	94
Eelpout (*Zoarces viviparus*)	Oil spill (PAH)	Nucleated erythrocytes	↑	95
Gilthead sea bream (*Sparus aurata*)	Copper	Erythrocytes	↑↑	96
Dab (*Limanda limanda*)	PAHs and PCBs polluted waters of English Channel Gender and age	Blood cells	↑ in adults and males	43
Hornyhead turbot (*Pleuronichthys verticalis*)	Sediments collected from a natural petroleum seep (pahs)	Liver cells	↑	97
In vitro				
Carp (*Cyprius carpio*)	Organic sediment extracts from the North Sea (Scotland)	Leukocytes	↑	98
Trout (*Oncorhynchus mykiss*)	Cadmium	Hepatocytes	↑	99
	Oxidative stress and its prevention by indolinic and quinolinic nitroxide radicals	Erythrocytes	↑	100
	Tannins		↓	101
	Diaryl tellurides and ebselen (organoselenium)		↓	102
Zebrafish (*Danio rerio*)	Surface waters of German rivers, Rhine and Elbe	Hepatocytes and gill cells	↑	103
Rainbow trout hepatoma cell line (RTH-149)	Water samples from the polluted Kishon river (Israel)	Liver	↑	104
Rainbow trout gonad (RTG-2) cell line liver (RTL-W1) cell line	4-Nitroquinoline-N-oxide N-methyl-N′-nitro-N-nitrosoguanidine, benzo[a]pyrene, nitrofurantoin, 2-acetylaminofluorene, and dimethylnitrosamine, and surface waters	Gonad Epitheloid liver	↑ dose-dependent response	105
		Vertebrates – Amphibians		
Amphibian larvae (*Xenopus laevis* and *Pleurodeles waltl*)	Cadmium ($CdCl_2$) Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide)	Erythrocytes	↑concentration and time dependent	106,107
Amphibian larva (*Xenopus laevis*)	Benzo[a]pyrene, ethyl methanesulfonate methyl methanesulfonate, aqueous extracts of five sediments from French channels	Erythrocytes	– ↑	108,109

Table 1.1 (*Continued*).

Model	*Agent tested*	*Cell used*	*DNA damage*	*Ref.*
Toad (*Bufo raddei*)	Petrochemical (mainly oil and phenol) polluted area	Liver cells and erythrocytes	↑	110
Toad (*Xenopus laevis*, and *Xenopus tropicalis*)	Bleomycin-induced DNA damage and repair	Splenic lymphocytes	↑ DNA damage X. tropicalis > X. laevis DNA repair in X. laevis > X. tropicalis	111
Tadpoles of *Rana N. Hallowell*	Imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine] and RH-5849 [2′-benzoyl-l′-tert-butylbenzoylhydrazinel]	Erythrocytes	↑	112
Rana hexadactyla tadpoles	Sulfur dyes (Sandopel Basic Black BHLN, Negrosine, Dermapel Black FNI, and Turquoise Blue) used in the textile and tannery industries	Erythrocytes	↑↑	113
Bullfrog (Rana catesbeiana) tadpoles	Herbicides AAtrex Nine-O (atrazine), Dual-960E (metalochlor), Roundup (glyphosate), Sencor-500F (metribuzin), and Amsol (2,4-D amine)	Erythrocytes	↑↑	114
Tadpole *Rana clamitans* *Rana pipiens*	Agricultural regions, Industrial regions	Erythrocytes	↑ industrial regions > agricultural regions	115
In vitro				
Xenopus laevis	High peak-power pulsed electromagnetic field	Erythrocytes	↑ due to rise in temperature	116
	Vertebrates – Birds			
Wild nestling white storks (*Ciconia ciconia*)	Heavy metals and arsenic	Blood cells	↑ correlated with arsenic	117
	Toxic acid mining waste rich in heavy metals		↑↑	118,119,120
Black kites (*Milvus migrans*)	Heavy metals and arsenic	Blood cells	↑ correlated with copper and cadmium	117
	Toxic acid mining waste rich in heavy metals		↑ (2-10 fold)	118,119,120
Turkey	Short-term storage	Sperm	↑	121

Chicken	T-2 toxin and deoxynivalenol (DON)	Spleen leukocytes	↑	122
	Storage conditions (4 °C)	Liver and breast muscle cells	↑ liver cells > breast muscle cells	123
		Vertebrates – Rodents		
Aldh2 knockout mice	Ethanol	Hepatic cells	↑ oxidative damage	124
P53(+/–) mice	Melphalan	Liver, bone marrow, peripheral blood and the distal intestine	DNA cross-links in all cells tested	125
SKH-1 mice	UV A + Fluoroquinolones (clinafloxacin, lomefloxacin, ciprofloxacin)	Epidermal cells	↑↑ for fluoroquinolones	126
	UVA + 8-methoxypsoralene (8-MOP)		↓ for MOP	
Dyslipidemic ApoE(–/–) mice	Ageing	Aorta, liver, and lung	↑ Oxidative damage in liver	127,128
	Diesel exhaust particles		– in lung or aorta	
Balb/c mice	*Trypanosoma cruzi* infection	Peripheral blood, liver, heart and spleen cells	↑ in heart and spleen	129
CD-1 mice	Lead acetate	Nasal epithelial cells, lung, whole blood, liver, kidney, bone marrow, brain and testes	↑ in all organs on prolonged exposure – in testes	130
Swiss albino mice	Sanguinarine alkaloid, argemone oil	Blood, bone marrow cells and liver	↑ dose dependent in blood and bone marrow	131,132
	Cypermethrin	Brain, liver, kidney, bone marrow, blood, spleen	↑	133
	Steviol	Stomach cells, hepatocytes, kidney and testicle cells	↑	134
	Apomorphine, 8-oxo-apomorphine-semiquinone	Brain cells	–	135
	Ethanol, grape seed oligomer and polymer procyanidin fractions	Brain cells	↑ ↓ ethanol-induced protection by grape seed	136
Male CBA mice	Pesticide formulations (Bravo and Gesaprim)	Hepatic cells, bone marrow cells, spleen cells	↑↑	137
Isogenic mice	Sulfonamide, protozoan parasite *Toxoplasma gondii*	Peripheral blood cells, liver cells and brain cells	↑ in peripheral blood cells	138
Cirrhotic rats	Rutin and quercetin	Bone marrow cells	↑↑	139

Table 1.1 (*Continued*).

Model	*Agent tested*	*Cell used*	*DNA damage*	*Ref.*
In vitro				
FE1 muta mouse lung epithelial cell line	Carbon black	Lung epithelial cell line	↑	140
L5178Y mouse lymphoma cells	Ketoprofen, promazine, chlorpromazine, dacarbazine, acridine, lomefloxacin, 8-methoxypsoralen, chlorhexidine, titanium dioxide, octylmethoxycinnamate	Lymphoma cells	Positive with phototoxic compound	141
Murine primary cultures of brain cells and a continuous cell line of astrocytes	Xanthine/xanthine oxidase, hydrogen peroxide superoxide dismutase, catalase, or ascorbic acid	Brain cells	↓ by antioxidants	142
Chinese hamster ovary cell line (CHO)	Endosulfan	Ovary cells	↑	143
	Cypermethrin, pendimethalin, dichlorovous			144
		Humans – Clinical		
Breast cancer patients and controls	Radiosensitivity	Peripheral blood mononuclear cells	↑	145
Breast cancer patients and controls	Radiosensitivity	Peripheral blood mononuclear cells	↑ and reduced DNA repair	146
Normal individuals	Chlorhexidine	Buccal epithelial cells and peripheral blood lymphocytes	↑	147
Transitional cell carcinoma patients and controls	DNA-strand breaks	Exfoliated cells extracted from bladder washing	↑ in patients	148
Aaxia telangiectasia heterozygote	X-irradiation	Peripheral leukocytes	↑ (~3 times high) in patients	149

Nijmegen breakage syndrome (NBS) patients	X-irradiation	Peripheral blood mononuclear cells	↑ in patients	150
Alzheimer disease patients	–	Peripheral blood mononuclear cells	↑ in patients	151
Breast cancer patients	–	Peripheral blood mononuclear cells	↑ in patients	152
Type 2 diabetes mellitus	Oxidative DNA damage	Peripheral blood cells	↑	153
Cancer (testicular cancer, lymphoma and leukemia) patients	DNA integrity	Spermatozoa	Decreased DNA integrity	154
		Humans – Dietary intervention		
Healthy subjects	Tomato drink	Blood lymphocytes	↓	155
	Green vegetables			156
	Grape juice			157
Smokers	Vitamin C supplementation	Blood lymphocytes	↓	158
Technical anesthesiology staff	Vitamin E and vitamin C	Blood lymphocytes	↓ in oxidative damage	159
		Humans – Occupational		
Airport personnel	Jet fuel vapours, jet fuel combustion products	Exfoliated buccal cells and lymphocytes	↑	160
Agricultural workers	Pesticides	Lymphocytes	–	161
			↑	162,163
Rubber factory workers	Substances used in the rubber industry	Peripheral blood	↓ in exposed population	164
Outdoor workers in Mexico cities	Air pollutants	Blood lymphocytes	↑	165
Rickshaw pullers	Exhaustive exercise	Lymphocytes	↑	166
Nuclear medicine personnel	Ionising radiation	Peripheral blood leukocytes	↑	167
Workers	Polycyclic aromatic hydrocarbons (PAH)	Human T- and B-lymphocytes, and granulocytes	↑ B-lymphocytes > T-lymphocytes > granulocytes	168
	Benzene in printing	"	↑	169
	Lead (Pb) and cadmium (Cd)	Peripheral lymphocytes	↑	170
	Asbestos cement plant	"	↑	171
	Fenvalerate (FE) exposure	Sperm	↑	172
	Organic solvents	Peripheral blood	↑	173

Table 1.1 (*Continued*).

Model	*Agent tested*	*Cell used*	*DNA damage*	*Ref.*
	Coke oven emissions (coe)	Blood lymphocytes		174
	Welders (Cd, Co, Cr, Ni, and Pb)	Lymphocytes		175
	Pesticide formulators (organophosphorus pesticides)	Lymphocytes	↑	176
	Copper smelters (inorganic arsenic)	Leukocytes	↑	177
	Chrome-plating workers (chromium VI)	Lymphocytes	↑↑	178
	Workers in foundry and pottery (silica)	Lymphocytes	↑	179
Nurses	5-Fluorouracil, cytarabine, gemcitabine, cyclophosphamide, and ifosfamide	Lymphocytes	Slight ↑	180
	Humans – Lifestyle			
Normal individuals	Endurance exercise	Lymphocytes	↑	181
Active and passive smokers	Smoking	Lymphocytes	↑	182
Normal individuals	Smoking Diet (vegetarian or non-vegetarian)	Lymphocytes	↑	183–186
Rural Indian women	Biomass fuels	Lymphocytes	↑	187
Normal individuals	Benzo[a]pyrene, beta-naphthoflavone (BNF)	Human umbilical vein endothelial cells (HUVEC)	↑	188
In vitro				
Episkin	UV, Lomefloxacin and UV or 4-nitroquinoline-N-oxide (4NQO) and protection by Mexoryl	Skin fibroblast cells	↑ reduced by Mexoryl	189
Sperms	Reproductive toxins	Male germ cells	↑	190,191
Prostate tissues primary culture	2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP), its N-hydroxy metabolite (N-OH-PhIP) and benzo[a]pyrene (B[a]P)	Prostate cells	↑ dose related	192
Human keratinocytes	UVA or UVB	Skin cells	↑	193
MCF-7 cells	Estradiol	Breast cells	↑ concentration dependent	194
JM1 cells	Estradiol	Lymphoblast cells	–	194
HepG2 cells	Endosulfan	Liver cells		195
	Indirect acting genotoxins (cyclophosphamide)			196

Miniorgan cultures of human inferior nasal turbinate epithelia	Sodium dichromate, N-nitrosodiethylamine (NDEA) and N-methyl-N-nitro-N-nitroso-guanidine (MNNG)	Nasal cells	↑ with sodium dichromate and MNNG – with NDEA	197
	Mono(2-ethylhexyl) phthalate (MEHP), benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), or N-methyl-N′-nitro-N-nitrosoguanidine (MNNG).		↑ with BPDE and MNNG – with MEHP	198
Human lymphocytes	Heterocyclic amine and prevention by monomeric and dimeric flavanols and black tea polyphenols	Lymphocyte	↓ in oxidative damage	199
	C_{60} Fullerenes		↑	200
	Municipal sludge leachates		↑	201

↑ significant increase in DNA damage; ↑↑ highly significant increase in DNA damage; ↓ decrease in DNA damage; – no DNA damage reported

1.3.1.2 Algae

Aquatic unicellular plants like algae provide information on the potential genotoxicity of the water in which they grow. Being single celled they can be used as a model for assessment of DNA damage and monitoring of environmental pollution utilising the Comet assay. Unicellular green alga *Chlamydomonas reinhardtii* was used for evaluation of DNA damage due to known genotoxic chemicals and also demonstrated that oxidative stress was better managed by the algal cells under light rather than dark conditions.[17] The Comet assay was found to be useful for evaluating chemically induced DNA damage and repair in *Euglena gracilis* and responses were more sensitive than those of human lymphocytes under the same treatment conditions.[18] The ease of culturing and handling *E. gracilis* as well as its sensitivity, makes it a useful tool for testing the genotoxicity of chemicals and monitoring environmental pollution. A modified version of the Comet assay was used as an alternative technique to assess DNA damage due to UV radiation in *Rhodomonas* sp. (*Cryptophyta*), a marine unicellular flagellate.[19]

1.3.2 The Comet Assay in Higher Plants

Vicia faba has been widely used for the assessment of DNA damage using the Comet assay. Strand breaks and abasic (AP) sites in meristematic nuclei of *V. faba* root tips were studied by the neutral and alkaline Comet assay.[20,21] The alkaline electrophoresis procedure was found to be most sensitive at low doses, while the neutral electrophoresis procedure yielded an optimal dose–response curve within a wider dose range. Angelis *et al.*[20] also suggested that the Comet assay was able to detect a phenomenon resembling clastogenic adaptation at the molecular level. Gichner and Plewa[22] developed a sensitive method for isolation of nuclei from leaf tissue of *Nicotiana tabacum*. The method resulted in high resolution and constant low tail moment values for negative controls, and hence it could be incorporated as a test for *in situ* plant environmental monitoring.[22]

The Comet assay has also been used to study the effect of age of plant on DNA integrity[23] as well as the kinetics of DNA repair[24] in isolated nuclei from leaves of tobacco plants. A small but significant increase in DNA damage compared to controls was noted in heterezygous tobacco and potato plants grown on soil contaminated with heavy metals.[28] The tobacco and potato plants with increased DNA damage were also found to be severely injured (inhibited growth, distorted leaves), which may be associated with necrotic or apoptotic DNA fragmentation. No DNA damage was observed in the root or shoot cells of *Phaeseolus vulgaris* treated with different concentrations of uranium.[30] The ornamental plant *Impatiens balsamina* was used as a model to understand the genotoxic effect of Cr^{6+} and airborne particulate matter,[31] which produced increased strand breaks in plant parts (stem, root and leaves). Thus, this plant could be used for environmental biomonitoring studies involving air pollution and heavy metals.

The major drawback with plant models was the fact that exposure needs to be given in the soil and it is difficult to say whether the result demonstrates synergies with other chemicals in the soil or nonavailability of the toxicant due to its soil binding affinity. Therefore, Vajpayee *et al.*[32] used *Bacopa monnieri L.*, a wetland plant, as a model for the assessment of ecogenotoxicity using the Comet assay. *In vivo* exposure to cadmium (0.01–500 μM) for 2, 4, and 18 h resulted in dose- and time-dependent increases in DNA damage in the isolated roots and leaf nuclei, with roots showing greater DNA damage than leaves. *In vitro* (acellular) exposure of nuclei from leaves of *B. monnieri* to 0.001–200 μM cadmium resulted in significant ($P < 0.05$) levels of DNA damage.

These studies revealed that DNA damage measured in plants using the Comet assay is a good model for assessment of genotoxicity of polluted environments since *in situ* monitoring and screening can be accomplished. Higher plants can be used as an alternative first-tier assay system for the detection of possible genetic damage resulting from polluted waters/effluents due to industrial activity or agricultural run offs.

1.4 Animal Models

To assess safety/toxicity of chemicals/finished products, animal models have long been used. With the advancements in technology, knockouts and transgenic models have become common to mimic the effects in humans. The Comet assay has globally been used for assessment of DNA damage in various animal models (Table 1.1).

1.4.1 Lower Animals

Tetrahymena thermophila is a unicellular protozoan, widely used for genetic studies due to its well-characterised genome. Its uniqueness lies in the fact that it has a somatic and a germ nucleus in the same cell. Therefore it has been validated as a model organism for assessing DNA damage using a modified Comet assay protocol standardised with known mutagens such as phenol, hydrogen peroxide, and formaldehyde.[33] The method was then used for the assessment of genotoxic potential of influent and effluent water samples from a local municipal wastewater treatment plant.[33] The method provided an excellent, low-level detection of genotoxicants and proved to be a cost-effective and reliable tool for genotoxicity screening of wastewater.

1.4.1.1 Invertebrates

Studies have been carried out on various aquatic (marine and freshwater) and terrestrial invertebrates (Table 1.1). The genotoxicity assessment in marine and freshwater invertebrates using the assay has been reviewed.[203–205] Cells from haemolymph, embryos, gills, digestive glands and coelomocytes from mussels (*Mytilus edulis*[42]), zebra mussel (*Dreissena polymorpha*), clams

(*Mya arenaria*), and polychaetes (*Nereis virens*), have been used for ecogenotoxicity studies using the Comet assay. DNA damage has also been assessed in earthworms[61,63] and fruit flies, *Drosophila*.[72,206] The Comet assay has been employed to assess the extent of DNA damage in organisms at polluted sites in comparison to those at reference sites in the environment. In the laboratory it has been widely used as a mechanistic tool to determine pollutant effects and mechanisms of DNA damage.[78]

1.4.1.2 The Comet Assay in Mussels

Freshwater and marine mussels have been used to study the adverse effect of contaminants in the aquatic environment as they are important pollution-indicator organisms. These sentinel species are adversely affected by the pollution of the water bodies and thus provide the potential for environmental biomonitoring. The Comet assay in mussels has been used to detect a reduction in water quality caused by chemical pollution.[41,42,49,207] *Mytilus edulis* has been widely used for Comet assay studies to evaluate DNA-strand breaks in gill and digestive gland nuclei due to polycyclic aromatic hydrocarbons (PAHs) including benzo[a]pyrene (B[a]P),[44] and oil spills with petroleum hydrocarbons.[59] The DNA damage was found to be elevated in the exposed mussels. However, the damage returned to normal levels, after continued exposure to a high dose (20 ppb-exposed diet) of B[a]P for 14 days. This was attributed to an adaptive response in mussels to prevent the adverse effects of DNA damage.[44] The green lipped mussels (*Perna viridis*) also showed a similar result on exposure to B[a]P in water.[54]

Significant levels of interindividual variability, including seasonal variations in DNA damage have been reported from some studies, both laboratory and field.[45,49,208,209] Baseline monitoring thus has to be carried out over long time intervals. Temperature-dependent DNA damage was observed in haemocytes of freshwater mussel *Dreissena polymorpha*[37] showing that the mussels are sensitive towards change in water temperatures. Thus, monitoring ecogenotoxicity with these species should take into account variations in temperatures. Findings have also suggested that antioxidant supplementation can improve the sensitivity of the Comet assay by lowering the baseline damage in untreated animals.[208]

Villela *et al.*[210] used the golden mussel (*Limnoperna fortunei*) as a potential indicator organism for freshwater ecosystems due to its sensitivity to water contaminants. The Comet assay in haemocytes of freshwater Zebra mussel, *D. polymorpha* Pallas, was used as a tool in determining the potential genotoxicity of water pollutants.[34–36,38] Klobucar *et al.*[38] suggested the use of the Comet assay in haemocytes from caged, nonindigenous mussels as a sensitive tool for monitoring genotoxicity of freshwater. DNA damage and repair studies in vent mussels, *Bathymodiolus azoricus*, have been carried out to study the genotoxicity of a naturally contaminated deep-sea environment.[52,53] The vent mussels demonstrated similar sensitivity to environmental mutagens as that of coastal mussels and thus could be used for ecogenotoxicity studies of deep sea waters using the Comet assay.

In vitro Comet assay has also been used in cells of mussels. Dose–response increases in DNA-strand breakages were recorded in digestive gland cells[211] haemocytes[212] and gill cells[208,212] of *M. edulis* exposed to both direct (hydrogen peroxide and 3-chloro-4-(dichloromethyl)-5-hydroxy-2[*5H*]-furanone) and indirect (B[*a*]P, 1-nitropyrene, nitrofurantoin and *N*-nitrosodimethylamine) acting genotoxicants. Digestive gland cells of *Unio tumidus* were also used for *in vitro* studies of DNA damage and repair due to pro-oxidative effect of polyphenolic compounds.[46,213] Wilson *et al.*[208] demonstrated the potential application of the Comet assay to the gill cells of *M. edulis* as a potential *in vitro* screen for agents destined for release or disposal into the marine environment.

1.4.1.3 The Comet Assay in Other Bivalves

Coughlan *et al.*[57] showed that the Comet assay could be used as a tool for the detection of DNA damage in clams (*Tapes semidecussatus*) as biomonitor organisms for sediments. Significant DNA-strand breaks were observed in cells isolated from haemolymph, gill and digestive gland from clams exposed to polluted sediment.[57,58] The Comet assay was used for the assessment of sperm DNA quality of cryopreserved semen in Pacific oysters (*Crassostrea gigas*) as it is widely used for artificial fertilisation.[56] Gielazyn *et al.*[214] demonstrated the use of lesion-specific DNA repair enzyme formamidopyrimidine glycosylase (Fpg) to enhance the usefulness and sensitivity of the Comet assay in studying oxidative DNA damage in isolated haemocytes from oysters (*Crassostrea virginica*) and clams (*Mercenaria mercenaria*).

The studies in mussels have shown the Comet assay to be a sensitive, but nonspecific, molecular biomarker of genotoxicity. One of the drawbacks when applying single-cell gel electrophoresis to field populations may be the adapatability of the animals to high concentrations of contaminants (*e.g.* B[a]P), which may pose a major problem.[44] Also, seasonal variation and temperature altered both DNA damage baseline levels in untreated animals and cell sensitivity towards environmental pollutants under *in vitro* conditions.[37,58] The Comet assay detecting DNA-strand breaks has demonstrated that higher basal levels of DNA damage are observed in marine invertebrates, hence the protocol followed in these animals should be considered for biomonitoring the ecogenotoxicity of a region.[215]

1.4.1.4 The Comet Assay in Earthworms

The Comet assay applied to earthworms is a valuable tool for monitoring and detection of genotoxic compounds in terrestrial ecosystems[61,66] (Table 1.1). Since the worms feed on the soil they live in, they are a good indicator of the genotoxic potential of the contaminants present in the soil and thus used as a sentinel species. Verschaeve *et al.*[60] demonstrated a dose–response effect with the extent of DNA damage in coelomic leucocytes (coelomocytes) of

earthworms (*Eisenia foetida*) from soil treated with different chemicals as an indicator of soil pollution.

Coelomocytes from *E. foetida* demonstrated increased DNA damage when worms were exposed to soil samples from polluted coke oven sites,[61] or industrialised contaminated areas[62] and even sediment samples from polluted river system.[63] An insecticide, parathion, produced DNA-strand breaks at all time points and doses in the sperm cells of *E. foetida*[65] while dose-effect relationships were displayed by two pesticides, Imidacloprid and RH-5849 in the same species,[66] showing that pesticides could also have adverse effects on nontarget species. *In vitro* exposure of coelomocytes primary cultures to nickel chloride as well as whole animals either in spiked artificial soil water or in spiked cattle manure substrates exhibited increased DNA-strand breaks due to the heavy metal.[68] The eleocytes cells, a subset of coelomocytes produced increased DNA-strand breaks under both *in vitro* and *in vivo* conditions and could be used a sensitive biomarker for genotoxicity in earthworms.[67] Another earthworm, *Aporrectodea longa* (*Ude*), when exposed to soil samples spiked with B[a]P and/or lindane demonstrated genotoxicity in the intestinal cells to be more sensitive to the effect of the toxicants than the crop/gizzard cells.[69]

Fourie *et al.*[216] used five earthworm species (*Amynthas diffringens, Aporrectodea caliginosa, Dendrodrilus rubidus, Eisenia foetida and Microchaetus benhami*) to study genotoxicity of sublethal concentrations of cadmium sulfate, with significant DNA damage being detected in *E. foetida* followed by *D. rubidus* and *A. caliginosa*. The study showed the difference in sensitivity of species present in an environment and its influence on the genotoxicity risk assessment. Hence, for environmental biomonitoring, specific species have to be kept in mind to reduce false-negative results.

1.4.1.5 The Comet Assay in Drosophila

The simple genetics and developmental biology of *Drosophila melanogaster* has made it the most widely used insect model and has been recommended as an alternate animal model by the European Centre for the Validation of Alternative Methods.[217] Recently, *Drosophila* has evolved into a model organism in toxicological studies.[218,219] *D. melanogaster* has also been used as an *in vivo* model for assessment of genotoxicity using the Comet assay[70–72,206] (Table 1.1). Neuroblast cells of third instar larvae, DNA repair deficient in nucleotide excision repair (mus201) and a mechanism of damage bypass (mus308), have been used for mechanistic studies.[206]

Third instar larvae of *D. melanogaster* (Oregon R+) were validated for genotoxicity assessment using a modified Comet assay.[70,71] Since the cells of *Drosophila* are smaller than mammalian cells, modifications in the Comet assay were done, *e.g.* higher concentration of agarose (for the smaller size of *Drosophila* cells), removal of DMSO from lysing solution (DMSO is toxic to the cells) and lower electrophoresis time (for improved performance of the assay). This modified protocol was validated in gut and brain cells using

well-known alkylating agents, *i.e.* ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-ethyl-N-nitrosourea (ENU) and cyclophosphamide (CP) that were mixed in standard *Drosophila* diet and produced a significant dose-dependent response.[70,71] Cypermethrin, a synthetic pyrethroid, even at low concentrations (at 0.002 ppm) and leachates of industrial waste produced significant dose-dependent increases in DNA damage in the brain ganglia and anterior mid gut of *D. melanogaster*.[71,72] Results from the Comet assay have also shown a direct correlation between the concentrations of cisplatin adducts and DNA damage in somatic cells of *D. melanogaster*.[73]

In vitro studies using *Drosophila* S2 cells demonstrated that the ectopically expressed DNA glycosylases (dOgg1 and RpS3) reduced the oxidised guanosine (8-OxoG), but contributed to increased DNA degradation due to one of the constituents of the DNA repair system.[220]

The studies in *Drosophila* have shown it to be a good alternative to animal models for the assessment of *in vivo* genotoxicity of chemicals using the Comet assay.

1.4.1.6 The Comet Assay in Other Invertebrates

Nereis virensa, a polychaete, plays an important role in the distribution of pollutants in sediments due to their unique property of bioturbation. These worms are similar to earthworms in soil and can be used for genotoxicity assessment of sediments. Intracoelomic injection of B[a]P was given to the worms and the Comet assay was conducted on coelomocytes.[221] *Nereis* species was, however, not found to be suitable for assessing PAH genotoxicity probably due to its lack of metabolic capability to convert B[a]P to its toxic metabolite.[221]

DNA damage was assessed in neuroblast cells of brains of 1st instars of grasshoppers (*Chorthippus brunneus*) exposed to various doses of zinc from a polluted site to understand the mechanism of toxicity in insects due to industrial pollutants.[222]

The estuarine grass shrimp, *Palaemonetes pugio*, exposed to coal-combustion residues from coal-fired electrical generation, were studied for DNA damage using the Comet assay. Chronic exposure caused DNA damage in hepatopancreatic cells of adult shrimps as compared to the reference shrimp.[77] The Comet assay in planarians is an important test for environmental monitoring studies since these are simple organisms with high sensitivity, low cost and a high proliferative rate.[223] The genotoxic potential of water from Diluvio's Basin was evaluated in planarians, where an increase in pollutants towards the basin led to an increase in the DNA damage in these species.[223] A significant increase of primary DNA damage was observed in planarian cells due to a Norflurazon, a bleaching herbicide[224] and copper sulfate,[225] when compared to the control animals.

These studies have also shown the use of the Comet assay in biomonitoring diverse environmental conditions utilising sentinel species.

1.5 Higher Animals

1.5.1 Vertebrates

Studies of vertebrate species where the Comet assay is used include fishes, amphibians, birds and mammals. Cells (blood, gills, kidneys and livers) of different fishes, tadpoles and adult frogs, as well as rodents have been used for assessing *in vivo* and *in vitro* genotoxicity of chemicals, and human biomonitoring has also been carried out employing the Comet assay (Table 1.1).

1.5.1.1 The Comet Assay in Fishes

Various fishes (freshwater and marine) have been used for environmental biomonitoring, as they are endemic organisms, which serve as sentinel species for a particular aquatic region to the adverse effects of chemicals and environmental conditions. 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 tissues (gills, liver, blood) of fishes exposed to various xenobiotics in the aquatic environment (Table 1.1).

Environmental biomonitoring to assess the water quality in rivers has been carried out in hepatocytes of chub,[79] erythrocytes of mullet (*Mugil* sp.), sea catfish (*Netuma sp.*[81,82]), bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*[90,226]). The basal level of DNA damage has been shown to be influenced by various factors, such as the temperature of water in erythrocytes of mullet and sea catfish,[81,82] age and gender in dab (*Limanda limanda*[43]), and exhaustive exercise in chub.[80] Therefore, these factors should be accounted for during environmental biomonitoring studies. The sensitivity of the assay may be affected by high intraindividual variability.[43] The protocol and experimental conditions used for the Comet assay for monitoring marine ecosystems may lead to differences in the results obtained.[92] The use of chemical and mechanical procedures to obtain cell suspensions may also lead to DNA damage.[227] Anesthesia did not contribute towards DNA damage *in vivo* in methyl methanesulfonate (MMS) treated fishes and the anesthetic benzocaine did not alter the DNA damage in erythrocytes after *in vitro* exposure to MMS or H_2O_2.[228] Hence keeping in mind animal welfare, multi sampling in the same fish can be conducted.

In vitro studies on fish hepatocytes,[99] primary hepatocytes and gill cells[103] as well as established cell lines (with metabolic competence[229]) using the Comet assay have also been conducted to assess the genotoxicity of chemicals in water samples. The antioxidant potential of indolinic and quinolinic nitroxide radicals,[100] tannins[101] and low concentrations ($<10\,\mu M$) of diaryl tellurides and ebselen – an organoselenium compound[102] – in oxidative DNA damage has been studied in nucleated trout (*Oncorhynchus mykiss*) erythrocytes for use of these compounds in biological systems. Kammann *et al.*[98] demonstrated the Comet assay in isolated leukocytes of carp as an *in vitro* model for evaluating genotoxicity of marine sediment extracts and increased sensitivity of the

method with use of the DNA repair inhibitor, 1-beta-D-arabinofuranosylcytosine (ara C). The Comet assay with fish cell lines may be a suitable tool for *in vitro* screening of environmental genotoxicity, however, the metabolising capabilities of the cell line need to be taken into account.

Cryopreservation has been shown to induce DNA-strand breaks in spermatozoa of trout,[93,230] sea bass (*Dicentrarchus labrax*[231]) and gilthead sea bream (*Sparus aurata*[230]). The DNA damage was prevented by the addition of cryopreservants such as BSA and dimethyl sulfoxide.[231] These studies have demonstrated the sperm Comet assay as a useful model in determining the DNA integrity in frozen samples for commercially cultured species.

The above studies have shown the usefulness of the Comet assay in fishes as a model for monitoring genotoxicity of aquatic habitats.

1.5.1.2 The Comet Assay in Amphibians

The Comet assay in amphibians has been carried out at adult and larval stages for ecogenotoxicity of aquatic environments and studies since 1999 have been well reviewed by Cotelle and Ferard.[203] The animals chosen for the Comet assay act as sensitive bioindicators of aquatic and agricultural ecosystems (Table 1.1). The animals were either collected from the site (*in situ*) or exposed to chemicals under laboratory/natural conditions.

Erythrocytes from tadpoles of two species *Rana clamitans* and *Rana pipiens* have been used for the assessment of genotoxicity of water bodies as *in situ* sentinel organisms for environmental biomonitoring.[115] *R. clamitans* tadpoles collected from agricultural regions showed significantly higher ($P<0.001$) DNA damage than tadpoles collected from sites of little or no agriculture. Similarly *R. pipiens* tadpoles collected from industrial sites showed significantly higher ($P<0.001$) DNA-strand breaks than samples from agricultural areas. The higher levels of DNA damage may be due to the pesticides used in the agricultural region. Variation in DNA damage due to sampling time[115] and during various metamorphosis states[232] was also observed. Hence, for biomonitoring environmental genotoxicity using the Comet assay, pooling of early tadpole phases could be helpful. Studies have also been conducted on caged tadpoles in areas where the indigenous population is not present, due to ecological imbalance from pollution. *Rana clamitans* and the American toad (*Bufo americanus*) tadpoles were caged at the polluted reference site and demonstrated significant ($P<0.05$) increases in DNA damage, relative to control tadpoles in the laboratory.[233] These results demonstrated that caged tadpoles could be used for monitoring genotoxicity of water habitats that do not support the survival of tadpoles, *e.g.* large lakes and aquatic areas near high industrial activity.

Huang *et al.*[110] have shown the genotoxicity of petrochemicals in liver and erythrocytes of toad *Bufo raddeis*. DNA damage was found to be positively correlated to the concentration of petrochemicals in liver, pointing to the fact that liver is the site for metabolism and may be a good marker for studying genotoxicity of compounds that require metabolic activation. The effect of

polyploidy on bleomycin-induced DNA damage and repair in *X. laevis* (pseudotetraploid) and *Xenopus tropicalis* (diploid) was studied using the Comet assay.[111] The *X. tropicalis* was more sensitive with a lower capacity for repair than *X. laevis*, showing that polyploidy protects DNA damage and allows rapid repair, and hence these species may be used as a good model for DNA damage and repair studies.

1.5.1.3 The Comet Assay in Birds

There are few studies involving the Comet assay in birds (Table 1.1). Genetic damage due to a mining accident involving heavy metals has been reported in free-living, nestling white storks (*Ciconia ciconia*) and black kites (*Milvus migrans*) from southwestern Spain,[117–120] however, species-specific and intra-species differences were observed. Faullimel *et al.*[123] showed that the neutral Comet assay could be used to study the impact of freezing and thawing on DNA integrity in breast fillets and liver cells of frozen chicken. Frankic *et al.*[122] reported that T-2 toxin and deoxynivalenol (DON) induced DNA fragmentation in chicken spleen leukocytes that was abrograted by dietary nucleotides. Kotlowska *et al.*[121] have demonstrated increased DNA fragmentation in turkey sperm after 48 h of liquid storage which might be helpful in evaluating the DNA integrity for artificial insemination.

1.5.1.4 The Comet Assay in Rodents

Mice and rats have been widely used as animal models for the assessment of *in vivo* genotoxicity of chemicals using the Comet assay (Table 1.1). The *in vivo* Comet assay has been accepted by the UK Committee on Mutagenicity Testing of Chemicals in Food, Consumer Products and Environment[10] as a test for assessing DNA damage, and is recommended for follow-up testing of positive *in vitro* findings. A positive result in the *in vivo* Comet assay assumes significance if mutagenic potential of a chemical has already been demonstrated *in vitro*. Within a battery of tests, the Comet assay finds a place as a supplemental *in vivo* test that has been accepted by international guidelines.[234] There are specific guidelines for the performance of the Comet assay *in vivo* for reliable results.[235–237]

Multiple organs of mouse/rat including brain, blood, kidney, lungs, liver, bone marrow have been utilised for the comprehensive understanding of the systemic genotoxicity of chemicals.[133,134,238,239] The most important advantage of the use of Comet assay is that DNA damage in any organ can be evaluated without the need for mitotic activity and DNA damage in target as well as nontarget organs can also be seen.[239] Comprehensive data on chemicals representing different classes, *e.g.* PAHs, alkylating compounds, nitroso compounds, food additives, *etc.* that caused DNA-strand breaks in various organs of mice was compiled by Sasaki *et al.*[239,240] The mouse or rat organs exhibiting

increased levels of DNA damage were not necessarily the target organs for carcinogenicity. Therefore, for the prediction of carcinogenicity of a chemical, organ-specific genotoxicity was necessary but not sufficient.[240] The Comet assay can be used as an *in vivo* test apart from the cytogenetic assays in haematopoietic cells and also for those compounds that have poor systemic bioavailability.

Different routes of exposure in rodents have been used, *e.g.* intraperitoneal,[131,133] oral[241,242] and inhalation[130,243] to study the genotoxicity of different chemicals. The route of exposure is an important determinant of the genotoxicity of a chemical due to its mode of action.[134] The *in vivo* Comet assay helps in hazard identification and assessment of dose–response relationships as well as the mechanistic understanding of a substance's mode of action. Besides being used for testing the genotoxicity of chemicals in laboratory-reared animals, the Comet assay in wild mice can be used as a valuable test in pollution monitoring and environmental conservation.[244]

The *in vivo* Comet assay in rodents is an important test model for genotoxicity studies, since many rodent carcinogens are also human carcinogens, and hence this model not only provides an insight into the genotoxicity of human carcinogens but is also suited for studying their underlying mechanisms.

1.5.1.5 The Comet Assay in Humans

The Comet assay is a valuable method for detection of occupational and environmental exposures to genotoxicants in humans and can be used as a tool in risk assessment for hazard characterisation[6,8,245,246] (Table 1.1). The DNA-damage assessed by the Comet assay gives an indication of recent exposure and at an early stage where it could also undergo repair[247] and thus it provides an opportunity for intervention strategies to be implemented in a timely manner. The assay can be conducted in the same population after removal of genotoxicant/dietary intervention to detect the extent of reduction in DNA damage. The assay is a noninvasive technique compared to other DNA-damage techniques (chromosomal aberrations, micronucleus), which require larger samples ($\sim$2–3 ml) as well as a proliferating cell population (or cell culture). Human biomonitoring using the Comet assay is advantageous since it is rapid, cost effective, with easy compilation of data and concordance with cytogenetic assays.[248]

The assay has been widely used in studying DNA damage and repair in healthy individuals,[3,194,249,250] in clinical studies[31,251,252] as well as in dietary intervention studies,[155,158,253–255] and in monitoring the risk of DNA damage resulting from occupational,[161,256–258] environmental,[187,259] oxidative DNA damage,[177,260] exposures or lifestyle.[185,261] White blood cells or lymphocytes are the most frequently used cell type for the Comet assay in human biomonitoring studies.[248,262,263] However, other cells have also been used, *e.g.* buccal cells,[264] nasal,[265] sperm,[191,266–268] epithelial[269–271] and placental cells.[272]

The Comet assay has been used as a test to predict the risk for development of diseases (renal cell carcinoma, cancers of the bladder, oesophagus and lung) due to susceptibility of the individual to DNA damage.[149,273–275] The *in vitro* Comet assay is proposed as an alternative to cytogenetic assays in early genotoxicity/photogenotoxicity screening of drug candidates[276] as well for neurotoxicity. Certain factors like age, diet, lifestyle (alcohol and smoking) as well as diseases have been shown to influence the Comet assay parameters and for interpretation of responses these factors need to be accounted for during monitoring human genotoxicity.[277,278]

Human biomonitoring studies using the Comet assay provide an efficient tool for measuring human exposure to genotoxicants, thus helping in risk assessment and hazard identification.

1.6 The Specificity, Sensitivity and Limitations of the Comet Assay

The Comet assay has found worldwide acceptance for detecting DNA damage and repair in prokaryotic and eukaryotic cells. However, there are issues relating to the specificity, sensitivity and limitations of the assay that need to be addressed by genetic toxicologists before it gets accepted in the regulatory framework including interlaboratory validation of *in vitro* and *in vivo* Comet assay.

The variability in the results of the Comet assay is largely due to its sensitivity and minor differences in the conditions of various laboratories as well as the effect of confounding factors in human studies (lifestyle, age, diet, inter-individual and seasonal variation). Prospective cohort studies have not been conducted to find the predictive value of the Comet assay in human biomonitoring, further limiting its application.[8] Cell to cell, gel to gel, culture to culture, animal to animal variability as well as use of various image-analysis systems or visual scoring[279] and use of different Comet parameters, *e.g.* Olive tail moment and tail (%) DNA, are the other factors contributing to inter-laboratory differences in the results.

The limitation of the Comet assay is that it only detects DNA damage in the form of strand breaks. The alkaline ($pH > 13$) version of the assay assesses direct DNA damage or alkali-labile sites, while specific classes of DNA damage including base oxidation DNA adduct formation cannot be measured. The specific and sensitive detection of these lesions requires the use of lesion-specific enzymes.[3] These enzymes are bacterial glycosylase/endonuclease enzymes, which recognise a particular type of damage and convert it into a break that can then be measured in the Comet assay. Hence, broad classes of oxidative DNA damage, alkylations, and ultraviolet light-induced photoproducts can be detected as an increased amount of DNA in the tail.[8] Oxidised pyrimidines are detected with use of endonuclease III, while oxidised purines are detected with formamidopyrimidine DNA glycosylase (FPG). Modifications have been made in the protocol to specifically detect double-strand breaks (neutral Comet

assay[280]), single-strand breaks (at pH 12.1,[281]), DNA crosslinking (decrease in DNA migration due to crosslinks[280]) and apoptosis.[280] The neutral Comet assay also helps to distinguish apoptosis from necrosis as evidenced by the increased Comet score in apoptotic cells and the almost zero Comet score in necrotic cells.[282] An adaptation of the Comet assay was also developed that enables the discrimination of viable, apoptotic and necrotic single cells.[283] Use of proteinase-K specifically removes DNA–protein crosslinking, leading to increased migration but would not affect the DNA–DNA crosslinking, thereby indicating a specific type of lesion.[280]

Tail (%) DNA and Olive tail moment give a good correlation in genotoxicity studies and since most studies have reported these Comet parameters, it has been recommended that both these parameters should be applied for routine use. Since the OTM is reported as arbitrary units and different image-analysis systems give different values, tail (%) DNA is a considered a better parameter.[285]

It is therefore required that the *in vitro* and *in vivo* testing be conducted according to the Comet assay guidelines, and appropriately designed multi-laboratory international validation studies be carried out.

Guidelines for the *in vitro* as well as *in vivo* Comet assay have been formulated.[235,236] Recently, issues relating to study design and data analysis in the Comet assay were discussed by the International Workgroup on Genotoxicity Testing (IWGT), where particular attention was given to the alkaline version (pH > 13) of the *in vivo* Comet assay and recommendations were made for a standardised protocol, which would be acceptable to international agencies.[237] It was decided that a single dose should be replaced with multiple dosing to avoid misinterpretation of data, isolated cells or nuclei could be used for the studies, cytotoxicity should be tested in the cells to prevent mechanisms of apoptosis/necrosis from interfering with the results, and scoring of comets could be carried out both manually as well as with image-analysis systems. Consensus was also reached on the need for an international validation study to stringently evaluate the reliability and accuracy of the *in vivo* Comet assay (as well as *in vitro* versions). These recommendations are also aimed at reducing the variability arising in interlaboratory studies.

Since *in vivo* Comet assay has been accepted as the first tier screening assay for assessment of DNA damage in rodents by the Committee on Mutagenicity, UK,[10] international validation studies are underway supported by the European Centre for Validation of Alternative Methods (ECVAM), Japanese Centre for Validation of Alternative Methods (JaCVAM), US Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM), US National Toxicology Program Interagency Centre for Evaluation of Alternative Toxicological Methods (NICEATM) and Japanese Environmental Mutagen Society.[237]

There has been only one multilaboratory validation study in the European countries that has been conducted to study the FPG sensitive sites and background level of base oxidation in DNA using the Comet assay, in human lymphocytes.[284] It was found that half of the laboratories demonstrated a