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REVIEW

Micronucleus assay in aquatic animals

Claudia Bolognesi* and Makoto Hayashi[1]

Environmental Carcinogenesis Unit, National Institute for Research on Cancer, Largo Rosanna Benzi 10, 16132 Genoa, Italy and [1]Biosafety Research Center, Foods, Drugs and Pesticides, 582-2, Shioshinden, Iwata, Shizuoka 437-1213, Japan

*To whom correspondence should be addressed. Environmental Carcinogenesis Unit, National Institute for Research on Cancer, Largo Rosanna Benzi 10, 16132 Genoa, Italy. Tel: +39 010 573 7304; Fax +39 010 573 7304; Email: claudia.bolognesi@istge.it

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Aquatic pollutants produce multiple consequences at organism, population, community and ecosystem level, affecting organ function, reproductive status, population size, species survival and thus biodiversity. Among these, carcinogenic and mutagenic compounds are the most dangerous as their effects may exert a damage beyond that of individual and may be active through several generations. The application of genotoxicity biomarkers in sentinel organisms allows for the assessment of mutagenic hazards and/or for the identification of the sources and fate of the contaminants. Micronucleus (MN) test as an index of accumulated genetic damage during the lifespan of the cells is one of the most suitable techniques to identify integrated response to the complex mixture of contaminants. MN assay is today widely applied in a large number of wild and transplanted aquatic species. The large majority of studies or programmes on the genotoxic effect of the polluted water environment have been carried out with the use of bivalves and fish. Haemocytes and gill cells are the target tissues most frequently considered for the MN determination in bivalves. The MN test was widely validated and was successfully applied in a large number of field studies using bivalves from the genera *Mytilus*. MN in fish can be visualised in different cell types: erythrocytes and gill, kidney, hepatic and fin cells. The use of peripheral erythrocytes is more widely used because it avoids the complex cell preparation and the killing of the animals. The MN test in fish erythrocytes was validated in laboratory with different species after exposure to a large number of genotoxic agents. The erythrocyte MN test in fish was also widely and frequently applied for genotoxicity assessment of freshwater and marine environment *in situ* using native or caged animals following different periods of exposure. Large interspecies differences in sensitivity for MN induction were observed. Further validation studies are needed in order to better characterise the different types of nuclear alterations and to clarify the role of biotic and abiotic factors in interspecies and inter-individual variability.

Introduction

The aquatic environment is the ultimate recipient of an increasing amount of contaminants as a result of the discharge of industrial, agricultural and urban wastes. A large number of pollutants in this complex mixture are responsible for multiple effects at the organisms, including human beings, and ecosystem levels, affecting organ function, reproductive status, species survival, population size and ultimately biodiversity (1,2). Among these, carcinogenic and mutagenic compounds are the most problematic as their effect may exert a damage beyond that of individual and may be active through following generations. Epizootic neoplasms have been found in a variety of ectothermic species, such as shellfish, echinoderms, jawless fish and bony fish (3–5). A specific correlation between tumour incidence in aquatic species and concentration of chemical pollutants has been observed (6–8). In addition, the decline in frequency of liver tumours in fish population of inhabiting contaminated locations once the source of pollution was eliminated supports the relevance of the 'natural remediation effect' and the importance of an early identification of the sources of pollutants (9).

Environmental monitoring by direct chemical analyses of water and sediment is limited by the availability of the detection methods and by an inability to predict the toxicity of complex mixtures. The measurement of a wide range of xenobiotically induced variations in cellular or biochemical components, processes, structures or functions ('biomarkers') in sentinel organisms ('bioindicators') can reveal early responses to environmental stress (10). Routine biomonitoring programmes of aquatic environment involve at the present time the application of a number of biomarkers in different bioindicators with different strategies in order to evaluate the pollutant-induced stress syndrome (11). Genotoxic effects as main biomarkers in assessment of the pollution-related toxicity should be members of the battery. Large-scale biomonitoring programmes in marine environments have demonstrated the associations between genotoxicity and chronic health effects at the population level (6,12,13). The micronucleus (MN) test, due to its simplicity, is one of the most applicable techniques to identify genomic alterations in environmental animals. This procedure is technically easier and more rapid than the microscopic analysis of chromosomal aberrations in metaphase, considering also that many aquatic organisms have small chromosomes difficult to be analysed. This assay targets interphase cells of any proliferating cell population regardless of its karyotype. This is one of the reasons why this biomarker is widely utilised in environmental biomonitoring programmes.

MN are formed in the process of cell division and their expression can occur at different times after the DNA damage event, depending on the cell cycle kinetics and the mechanism of induction. The MN assay, originally developed with mammalian species (14), is today widely applied in fish and other aquatic organisms, including sea urchin, mussels, oysters, crabs and worms, and in wild and transplanted animals. The large majority of studies or programmes on the genotoxic effect

of the polluted environment have been carried out with the use of bivalves and fish. Bivalves such as mussels have been considered the ideal bioindicators for monitoring aquatic contaminants in coastal waters due to their wide geographic distribution, sessile lifestyle, easy sampling, tolerance to a considerable range of salinity, high accumulation of a wide range of chemicals and resistance to stress (15). Mussels (*Mytilus* sp), in particular, can be easily caged in adequate containers usually positioned few metres under the sea surface. The Mussel Watch Programs using caged animals were generally adopted in coastal monitoring, allowing the study of areas where mussels are not naturally present and reducing the influence of genetic and adaptive phenomena impairing the comparison among the animals from different stations (16). Although the mussel watch approach has been demonstrated as a useful strategy for biomonitoring aquatic pollution, the use of fish is relevant as environmental bioindicators, due to their role in the aquatic trophic chain.

MN test in bivalves

The MN assay has been widely applied in different species of bivalves exposed under both field and laboratory conditions. Haemolymph (haemocytes) and gill cells are the target tissues most frequently considered for MN determination in bivalves. Haemolymph constitutes a massive part of a mussel's soft tissue and, as circulating cells of an open vascular system, haemocytes are constantly exposed to waterborne pollutants. Moreover, since haemocytes are involved in processes such as the elimination of toxic substances and small particles, transport and digestion of nutrients and repair of tissue lesions (17), they represent cells of relevant importance in the mussel's response to toxicants. In addition, because haemolymph readily provides a single-cell suspension, it is easily collectable and its usage permits repeatable tissue sampling of the same individuals for the MN assay. On the other hand, one of the major limitations in using haemocytes is the complexity of the cell types. The haemocytes of *Mytilus edulis* include different cellular subpopulations, which can be divided into basophilic and eosinophilic groups, the former including hyaline (agranular) and granular cells and the latter only granular cells (18). The origin of the different subpopulations and their respective involvement in defence responses against xenobiotic agents are not completely known. Although the large majority of studies on MN frequency in mussel haemocytes do not refer to specific cell populations, some studies found granular haemocytes less sensitive to genotoxic damage compared with agranular haemocytes (19). Gills, as filter feeding apparatus and respiratory organ, are the first barrier of the potential contaminants and the ideal target in biomonitoring studies. Proliferating gill cells showed a more sensitive response to genotoxic agents compared with haemocytes. The main limitation for using gill cells in large-scale biomonitoring studies is that the cell preparation has not been adequately standardised. In addition, the cell suspension from gills is heterogeneous in composition including predominant larger epithelial cell type with large cytoplasm and well spread nuclear chromatin and smaller cells with higher nucleus:cytoplasm ratio. The presence of a variable percentage of large cells with cytoplasmic granule, impairing the MN detection, was described and was related to the extent of pollutant exposure.

Application of the MN test in mussels (*Mytilus* sp) under laboratory conditions

The validation process of the MN assay in the genus *Mytilus* started >20 years ago. Dose-related induction of MN by different pollutants have been reported in gill cells and haemocytes, namely mitomycin C, colchicine (20,21), ethyl methanesulfonate (EMS) (22,23), dimethylbenz[*a*]anthracene (24), benzo[*a*]pyrene (25,26), bisphenol A, diallyl phthalate, tetrabromodiphenyl ether (27), phenanthrene (28), heavy metals, such as zinc chloride (29), copper, cadmium and mercury (28,30), and tritiated water (23). Time-dependant increase in MN frequency was also observed with 5- to 6-fold increase over the control values after continuous exposure to genotoxic compounds (23,24,28) without any significant mortality effect. The persistence of MN frequencies in gill cells following a short-term treatment with genotoxic agents was shown to be >1 month (21,25). A number of studies compared the MN induction in gill and haemocytes of mussels and it appears that gill cells tend to have higher baseline and induced frequencies (28,31,32). The role of water temperature on the mitotic rate and consequently on the MN expression was evaluated in experiments simulating winter and summer thermic conditions (33).

Field biomonitoring studies using bivalves

The MN test was successfully applied in a large number of field studies using bivalves from the genera *Mytilus* (19,34–47), *Mya* (48), *Perna* (49), *Crassostrea* (37) and *Dressena* (31,50,51) as indicator organisms for the assessment of genotoxic compounds and their effects on marine and freshwater environment. The results of a number of field studies using MN test in gill cells and/or haemocytes of mussels belonging to the genera *Mytilus* are reported in Table I. Biomonitoring studies were carried out with wild mussels in the Venice lagoon, where a number of sites were selected representing different contamination levels. Significant MN increases (1.4- to 5.3-fold compared with the related reference area) were found in haemocytes and gill cells of mussels from polluted sites associated with increased DNA adducts and with high levels of polycyclic aromatic hydrocarbon (PAH), polychlorinated biphenyl (PCB), heavy metals and organochlorinated compounds (19,34). A general stress condition was also detected in mussels from urban sites located in the canals of the Venice historic centre, where an increased MN frequency was significantly correlated with 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane, 1-dichloro-2,2-bis(p-dichlorodiphenyl)ethylene and PCBs (35).

Wild and transplanted mussels were sampled two times in a year along the west Ligurian cost in Italy (36). MN frequency in gill cells of animals showed a progressive increase associated with increased DNA damage and bioaccumulation of PAH and heavy metals. Seasonal effects were also observed with highest values in springtime. Wild mussels accumulated significant concentrations of chemicals and showed a higher induction of MN compared with caged mussels (36). This result was confirmed by another study carried out to evaluate the chemical impact of the estuary environment (Cecina estuary, Tyrrhenian coast, Italy), where the MN frequency in caged mussels doubled after 30 days of deployment, while in native mussels, it was more than four times those of control specimens (38). The MN test was also applied in caged mussels

Table I. Frequency of micronucleated cells (MN) in gills and haemocytes of mussels (*Mytilus* spp.) from different coastal areas

Geographic location	Pollutants	Target tissue	MN per 1000 cells (reference area/polluted site)	Frequency ratio[a]	Reference
Venice lagoon (Italy)	Industrial discharges (PAH)				Dolcetti and Venier (19)
Wild mussels	1997	Gill cells	6.37/8.85	1.4	
		Haemocytes	1.42/4.59	3.2	
	1999	Gill cells	2.04/3.98	1.9	
		Haemocytes	0.73/3.74	5.1	
Venice lagoon (Italy)	Industrial discharges PAHs,	Gill cells	0.7/3.7	5.3	Venier and Zampieron (34)
Wild mussels	PCBs, HCBs	Haemocytes	2.0/4.0	2.0	
'Canals' of the Venice historic centre	Urban wastewater PAH, PCB, chlorinated pesticides, heavy metals	Haemocytes	0.44/2.66	6.0	Pampanin *et al*. (35)
Wild mussels					
Ligurian coast (Italy)	PAH, heavy metals	Gill cells			Bolognesi *et al*. (36)
Wild mussels		Spring	1.78/17.40	9.8	
Caged mussels (1 month of exposure)		Autumn	3.36/14.22	4.2	
		Spring	1.78/24.40	13.7	
		Autumn	3.36/10.54	3.1	
West Ligurian coast (Italy)	PAH, heavy metals	Gill cells	5.3/10.5	1.9	Bolognesi *et al*. (37)
Caged mussels (1 month of exposure)					
Estuary of the river Cecina (Northern Tyrrhenian sea coasts) Italy	Industrial activities and untreated urban wastewater discharge, heavy metals (Hg)	Gill cells			Nigro *et al*. (38)
Wild mussels			5.4/23.1	4.2	
Caged mussels (1 month of exposure)			5.4/9.3	1.7	
Central Adriatic Sea, Italy	Offshore gas platform	Haemocytes	1.0/8.0	8.0	Gorbi *et al*. (39)
Caged mussels (1 month of exposure)					
Harbour of Piombino, located along the Tyrrhenian coast	PAHs, heavy metals (Pb, Hg, Cd)	Haemocytes	0.27/2	7.4	Bocchetti *et al*. (40)
Caged mussels (1 month of exposure)					
Gulf of Oristano (Sardinia, western Mediterranean, Italy)	Industrial agricultural and dairy activities, heavy metals (Pb, Cd and Zn)	Gill cells	2.94/5.70	1.9	Magni *et al*. (41)
Wild mussels					
North Spain wild mussels 0–300 m distance from effluents discharge	Urban wastes	Gill cells	1.42/5.75	4.0	Izquierdo *et al*. (42)
Wild mussels					
Puerto Madryn (Patagonia, Argentina) from effluents discharge	Urban wastes	Gill cells	4.5/11.58	2.6	Izquierdo *et al*. (42)
Wild mussels	Industrial wastes		8.17/17.5	2.1	
Strymonikos gulf, northern Greece	Domestic and/or industrial wastes, wastewater treatment plant tube PAH, PCB, organochlorinated compounds	Gill cells			Dailianis *et al*. (43)
		Spring	1.30/2.70	2.1	
		Autumn	2.60/6.0	2.3	
Wild mussels		Haemocytes			
		Spring	0.30/1.70	5.6	
		Autumn	1.30/2.70	2.1	
Gulf of Patras (Greece)	Urban, industrial and agricultural wastewaters, heavy metals (Cu, Hg and Zn)	Gill cells			Kalpaxis *et al*. (44)
		Spring	2.5/11	4.4	
Caged mussels (1 month of exposure)		Summer	2.3/7	3.0	
		Autumn	2.4/12	5.0	
North Sea	PAHs, TBT and other organic pollutants	Gill cells	1.1/7.3	6.6	Barsiene *et al*. (45)
Wild mussels					
Baltic sea	Polychlorinated biphenyls, polynuclear aromatic hydrocarbons, chlorinated pesticides	Gill cells	0.37/6.7	18.1	Barsiene *et al*. (46)
Wild mussels					
Baltic sea	PCBs, DDTs	Gill cells	1.14/5.6	4.9	Schiedek *et al*. (47)
Wild mussels					

[a]Frequency ratio = MN × 1000 cells in polluted site/MN × 1000 cells in reference area.

in assessing the toxic impact associated with remobilised chemicals during dredging and disposal operations in a harbour area (Piombino, Tyrrhenian coast, Italy). Significant increased MN frequency was detected in haemocytes of mussels during and 3 months after the dredging activities in association with an increased level of lead and PAH in tissues (40).

The MN test applied in a multi-biomarker approach with caged mussels showed high sensitivity in revealing the impact of an offshore gas platform in the central Adriatic Sea (39). A recovery effect was shown by a study carried out with caged mussels in two stations in front of the Haven oilship sinking area (Italy), revealing a significant decrease of the MN frequency (from 10- to 2-fold compared to the control level) 10 years after the oil spill (37). This sensitivity of the MN assay to detect exposure to unknown mixture of pollutants was evaluated using caged mussel dislocated at regular distances from effluents with unspecific pollutants, such as the urban wastes in clearly different ecological and geographic locations (northern Spain and South Argentina). A statistically significant negative association was found between the MN frequency and the distance of sampling sites from the effluent (42). Two studies applying the MN test in biomonitoring polluted areas along the Greece coast with wild (43) or caged mussels (44) show an increased MN frequency in gill cells along a pollution gradient. Higher sensitivity and lower inter-individual variability of gill cells compared to haemocytes was observed in response to genotoxic damage (42,43).

Finally, a number of studies carried out in Baltic and North Sea using wild mussels showed increased MN frequencies in gill cells along a pollution gradient with significant inter-location and seasonal differences. Lower MN baseline levels were reported in these studies compared with those detected in mussels from the areas in the Mediterranean Sea. Water temperature and salinity are the main factors responsible for these differences. The baseline values of MN frequency in *Mytilus galloprovincialis* at the reference area ranged from 0.37 per 1000 cells at water temperature of 5°C to 3 per 1000 cells at temperature >20°C. Water temperature was shown to have a direct effect on the mitotic rate and consequently on the formation of MN (45,46).

At the present time, the results from the biomonitoring studies carried out using bivalves from the genera *Mytilus* in different geographic locations show the sensitivity of the MN test as an early biomarker of exposure to genotoxic agents. A number of studies reported also the results related to the frequencies of nuclear abnormalities (NAs) (25,34,46), such as nucleoplasmic bridges, nuclear buds, binucleated or eight-shaped cells, showing pollution-related increases of these parameters. Despite the potential relevance of these parameters in environmental monitoring of the genotoxic impact, as shown in human studies, the lack of their characterisation and of specific criteria of scoring together with the high variability in the different studies and among the laboratories cannot allow any definite conclusion about these additional biomarkers.

Field studies reported higher variability in MN frequency compared with the evidence from laboratory experiments. Several factors could explain this apparent variability, including cytotoxicity and adaptation to polluted environments, which depend on the genetic make-up of the individual and populations. Higher variability and sensitivity were detected in heterogeneous wild populations of mussels applied in different studies (36,38). Increase in MN frequency represents a time-integrated response to cumulative stress. The different sensitivity between caged and native mussels can be interpreted considering that the MN becomes apparent after cell division and accumulates along cell generations. The use of caged mussels, following the Mussel Watch approach, is suggested in biomonitoring studies to reduce the influence of genetic/population differences and adaptive phenomena that can impair the capability to discriminate between different levels of environmental disturbance and to facilitate the investigation in areas where native organisms are absent.

Seasonal effects were also observed in many studies (36,43–47): a number of physical parameters, related to season, such as salinity, temperature, pH and food availability, influencing the cell turnover rate could modulate the extent of MN expression. Simultaneous presence of different kinds of biotic and abiotic stressors complicates the interpretation of subsets of data: multi-marker and repeated monitoring by the use of a battery of biomarkers allow to assess pollution-related effects and to evaluate the ecosystem status.

MN test in fish

Fish respond to toxic agents similar to higher vertebrates and can allow the assessment of substances that are potentially hazardous to humans. However, the low amount of DNA per cell, the large numbers of small chromosomes (52) and the low mitotic activity in many fish species impaired the metaphase analysis of chromosomal damage and sister chromatid exchanges. The MN test, due to its potentiality to be applied in any proliferating cell population regardless to the karyotype of the species used, was successfully applied in fish to evaluate the genotoxic activity of xenobiotic agents and of complex environmental mixtures in laboratory as well as in field studies. Different fish cell types were considered for the MN analysis: gill, fin, kidney and hepatic cells and peripheral erythrocytes (53–55). Branchial epithelium, as the primary target for all the water-borne contaminants, showed high sensitivity for the cytogenetic effects induced by environmental contaminants (54). However, the isolation of gill cells for the MN analysis needs complex experimental protocols and involves the killing of fish.

The use of fin cells that are in direct contact with the environment was also proposed (55). Fin tissue after its cutting is incubated to undergo regeneration, during which MN can be formed. The application of MN assay in fish hepatocytes has, as main limitation, the low mitotic index of liver cells. An *in vivo* hepatic MN assay with fish was (56) applied in a number of studies. The binucleated blocked technique was also successfully developed in fish hepatocytes (57), although the complexity of the protocols limited their application in the environmental monitoring. Nucleated erythrocytes are the most commonly used cells in the piscine MN test. The use of peripheral erythrocytes avoids the complex procedures associated with cell preparation and killing of animals. It was also proposed to analyse MN in nucleated erythrocytes from cephalic kidney, the main haemopoietic organ in fish, following the hypothesis that, at least in some species, the spleen could remove micronucleated erythrocytes from the peripheral circulation (58,59). The erythrocyte MN test firstly developed in *Umbra pygmea* (60) was validated in a number of studies (53,54,61).

Specific criteria for scoring MN and other NA were firstly described by Carrasco *et al.* (61). The size and the shape of MN in fish erythrocytes are different compared with the mammalian

species: round and frequently ovoid bodies with diameter ranging from 1/5 to 1/40 of the main nucleus are the structure commonly found. The use of DNA-reacting fluorescent dye is relevant to detect small MN. Different kinds of NAs are frequently observed in fish erythrocytes: buds, broken eggs, lobed, notched, vacuolated and karyolitic nuclei. Although the mechanisms responsible for NAs have not been fully explained, a number of them, such as buds, are considered to be indicators of genotoxic damage and, therefore, they may complement the scoring of MN in routine genotoxicity surveys. A correlation between MN frequency and nuclear buds was found in a number of studies (62–64). Other NAs, such as lobed and notched nuclei, are mainly associated to cytotoxicity and need to be separately recorded.

Application of the MN test in fish erythrocytes under laboratory conditions

The MN test in fish erythrocytes was widely validated for laboratory testing with different species after exposure to a large number of genotoxic agents. A dose–response increase in MN frequency was observed after exposure to a range of doses of X-rays (65). Positive responses were also observed to a large number of experimental carcinogens, such as aflatoxins, benzidine, EMS, methylcholanthrene, chlorinated hydrocarbons (57), cyclophosphamide (66), to the most common carcinogenic pollutants, such as PAHs (57,67), pesticides (68,69), heavy metals (57,63) and ubiquitous environmental contaminants, e.g. bisphenol A, tetrabromodiphenyl ether (63) and domoic acid (70).

The analysis of the baseline MN frequencies reported by different authors (71–102) shows a large interspecies variability, ranging from 0 to 13 per 1000 cells, although the large majority of papers report data ranging from 0 to 1 (Table II). This variability can be related to an interspecies difference in metabolic competency and DNA repair mechanisms as well as cell proliferation in the target organ affecting the MN expression. A significant difference (up to one to two orders of magnitude) in the MN baseline frequency was recorded in the same species by different authors, e.g. *Cyprinus carpio* or *Oncorhynchus mykiss*, that can be related to different biotic factors such as age, sex, genetic make-up, conditions of treatment or different scoring criteria. Larger differences in the frequency of NAs were detected among the studies. A number of papers do not describe the criteria of scoring for MN and NA and in some cases only the total frequencies of NA including MN are reported.

Application of the MN test in fish erythrocytes: field studies

Erythrocyte MN test in fish was also widely applied for genotoxicity assessment of freshwater (95,103–113) and marine (12,45,114–116) environments *in situ* using native or caged animals following different periods of exposure. MN frequency in freshwater species was demonstrated to be a sensitive biomarker to detect genotoxic damage induced by urban or industrial discharges contaminated by different pollutant sources, such as petrochemical products (95,109), heavy metals (104,106) and pesticides (104,110), and it allowed detection of pollutant concentration gradients. The relevance of seasonal impact in the induction of chromosomal damage was also identified (107). Nevertheless, a number of studies applying the MN test in erythrocytes of freshwater fish species failed to reveal the impact associated to well known genotoxic mixtures, such as PAHs (105,108) or heavy metals (96). In fact, some fish species are less sensitive than others for analysing MN frequency, as it was shown in laboratories' studies (57,111). Many factors may be responsible for the

Table II. Spontaneous MN frequencies in erythrocytes (MN/1000 cells) from different fish species under laboratory condition and in the field

Species	MN per 1000 cells	References
Carassius auratus	0.18; 0.0	Ueda *et al.* (71)
	0.26	Anitha *et al.* (72)
	2.26	Cavas (64)
	3.17	Cavas and Könen (73)
Carassius auratus gibelio	4.5–6.6	Al-Sabti (74)
	13; 10; 5.2	Al-Sabti *et al.* (75)
Carassius sp	1.8	Hayashi *et al.* (54)
Channa punctatus	0.02	Kumar *et al.* (76)
	0.028–0.048	Ali *et al.* (69)
	0.72–4.11	Farah *et al.* (77)
Cheirodon interruptus interruptus	0.0–1.0	Campana *et al.* (78)
Clarias batrachus	0.47–3.95	Ateeq *et al.* (79)
Clarias lazera	0.31	Odeigah and Osanyinpeju (80)
Cyprinus carpio	0.02	Gustavino *et al.* (65)
	0.03	Landolt and Kocan (81)
	0.3–0.5	Buschini *et al.* (82)
	0.52	Llorente *et al.* (83)
	0.9	Grisolia and Starling (68)
	1.2	Nepomuceno *et al.* (84)
	3.5	Kim and Hyun (85)
	6.2	Al-Sabti (86)
Esox lucius	5.3	Al-Sabti (87·88)
Genyonemus lineatus	0.8	Hose *et al.* (89); Carrasco *et al.* (61)
Ictalurus nebulosus	0.14	Metcalfe (90)
Misgurnus anguillicaudatus	1.6	Hayashi *et al.* (54)
Odontobutis oscura obscura	2.3	Hayashi *et al.* (54)
Oncorhychus mykiss	0.12	Castano *et al.* (91)
	0.181	Ayllon and Garcia-Vazquez (92)
	0.33	De Flora *et al.* (93)
	1	Schultz *et al.* (94)
	1.12	Marlasca *et al.* (95)
	2.5	Kim and Hyun (85)
Oreochromis niloticus	0.006	Hoshina *et al.* (96)
	0.21	Barbosa *et al.* (97)
	0.8	Grisolia and Starling (68)
	1.72	Cavas and Ergene-Gozukara (98)
	2.02	Cavas and Könen (70)
	15.8–17.2	Cavas and Ergene-Gozukara (99)
Paralabrax clathratus	0.8	Hose *et al.* (89)
Perca fluviatilis	10–9	Tuvikene *et al.* (100)
Pholis gunnellus	1	Bombail *et al.* (101)
Phoxinus phoxinus	0.3–0.7	Ayllon and Garcia-Vazquez (66)
Poecilia latipinna	0–0.4	Ayllon and Garcia-Vazquez (66)
Pseudopleuronectes americanus	0–1.30	Hughes and Hebert (12)
Rutilus rutilus	13–3	Tuvikene *et al.* (100)
Salmo trutta	0.4–2.3	Ayllon *et al.* (102)
Salmo trutta fario	2.8–0.75	Belpaeme *et al.* (103)
Umbra limi	0.14	Metcalfe (90)
Umbra pygmaea	0.0	Hooftman and de Raat (60)

interspecies sensitivity, such as metabolic capacity, DNA repair efficiency and defence mechanisms (112). These differences were related to cell removal kinetics (98) or to the development of adaptive mechanisms of tolerance to chemical stress that promote an increase in the replacement rate of dead or damaged cells to maintain normal physiological conditions or inhibition of nuclear division which is required for MN expression.

Biomonitoring studies were carried out in native marine fish species in different areas. Consistently, high MN frequency was shown in highly contaminated stations in mid-Atlantic coast (USA) (12). Increased MN frequency was detected on the basis of the pollution level in a number of stations along the south-eastern Mediterranean coast of Turkey (114). Biomonitoring studies involving multiple samplings of flat fishes in different offshore areas in North Sea and Baltic Sea showed the highest increase of MN frequency in animals from areas close to oil and gas platforms or related to intensive shipping (45,115,116).

Knowledge gaps and road map for future research and improvements

MN is one of the biomarkers that best correlate with contaminant load, as was demonstrated in a number of studies with different bioindicators, e.g. molluscs and teleosts. MN test was also applied to assess the baseline level of chromosomal damage in different species of marine mammals. Preliminary data are promising for the application of the assay in the evaluation of environmental contamination in these species as top predators in food chain (117).

MN frequency provides a useful index of accumulated genetic damage during the lifespan of the cells and it is an index of integrated response to the complex mixture of contaminants monitored or not that contributes to the genotoxic impact. The MN technique differs depending on the organism and cell types assayed and needs to be established based on the biology of the studied species. Some environmental species due to the low sensitivity are unsuitable for biomonitoring studies using MN test. MN expression strongly depends on the mitotic activity in the studied tissue, which in turn depends on a complex interaction of biotic and environmental parameters such as temperature, salinity and food availability.

The validation process of the MN in sentinel species involves the identification of the target tissues for the genotoxic effects through the evaluation of the kinetics of cytogenetic alterations under controlled conditions. Sensitivity and specificity of the assay, in revealing the exposure to the main classes of genotoxic pollutants, have to be determined. Sources of variability that may influence the expression of the MN have to be evaluated in field experiment. The MN is currently applied in mussels (*Mytilus* sp) as part of a battery of biomarkers in Mussel Watch Programs using caged animals. The test can be also applied in fish species in biomonitoring studies when a consistent homogeneous population of native animals can be collected in the different sampling stations. The MN assay in fish needs further investigation and validation in order to define the role of different factors responsible for the large interspecies and inter-individual variability. The establishment and the intercalibration of the experimental procedure for the MN test with special reference to the criteria for characterisation of the cell populations and scoring of MN and other NA is relevant in the application of the MN test both in laboratory testing and in field studies in environmental animals.

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