10 Background document: micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms

Janina Baršienė, Brett Lyons, Aleksandras Rybakovas, Concepción Martínez-Gómez, Laura Andreikenaite, Steven Brooks, and Thomas Maes

10.1 Background

Micronuclei (MN) consist of acentric fragments of chromosomes or whole chromosomes that are not incorporated into daughter nuclei at anaphase. These small nuclei can be formed as a consequence of the lagging of a whole chromosome (aneugenic event) or acentric chromosome fragments (clastogenic event; Heddle, 1973; Schmid, 1975). A micronucleus (MN) arises in cell divisions as a result of spindle-apparatus malfunction, the lack of or damage to a centromere, or chromosomal aberrations (Fenech, 2000).

Clastogens induce MN by breaking the double helix of DNA, thereby forming acentric fragments that are unable to adhere to the spindle fibres and integrate in the daughter nuclei, and are thus left out during mitosis. Aneuploidogenic agents are chemicals that prevent the formation of the spindle apparatus during mitosis. This leads to the generation of not only whole chromatids that are left out of the nuclei, thus forming MN, but also multinucleated cells in which each nucleus contains a different number of chromosomes (Serrano-García and Montero-Montoya, 2001). Thus, MN scoring during interphase provides a measure of genotoxicity both in the field and also specifically through genotoxic compound exposure in the laboratory as a result of clastogens and/or aneugens (Heddle *et al.*, 1991; Al-Sabti and Metcalfe, 1995). In addition, there are direct indications that MN may also be formed via a nuclear budding mechanism in the interphase of cell division. The formation of such MN reflects a reduced capacity of the organism to expel damaged, amplified, failed replicated or improperly condensed DNA, chromosome fragments without telomeres, and centromeres from the nucleus (Lindberg *et al.*, 2007).

The MN assay involves the scoring of cells that contain one or more MN in the cytoplasm (Schmid, 1975). The assay was first developed as a routine *in vivo* mutagenicity assay for detecting chromosomal mutations in mammalian studies (Boller and Schmid, 1970; Heddle, 1973). Hooftman and Raat (1982) were the first to successfully apply the assay to aquatic species when they demonstrated the induction of MN in erythrocytes of the eastern mudminnow (*Umbra pygmaea*) following waterborne exposure to the known mutagen ethyl methanosulfate (EMS). Following these initial experiments, other studies have validated the detection of MN as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (for review, see Chaudhary *et al.*, 2006; Udroiu, 2006; Bolognesi and Hayashi, 2011). In fish, most studies have used circulating erythrocytes (blood) cells, but samples from a number of tissues, such as liver, kidney, gill, or fin epithelium, can also be used (Arkhipchuk and Garanko, 2005; Baršienė *et al.*, 2005b, 2006a; Rybakovas *et al.*, 2009).

Environmental genotoxicity levels in organisms from the North Sea, Mediterranean, and northern Atlantic have been described in indigenous fish and mussel species inhabiting reference and contaminated sites (Wrisberg *et al.*, 1992; Bresler *et al.*, 1999; Baršienė *et al.*, 2004, 2008a, 2010a; Bagni *et al.*, 2005; Bolognesi *et al.*, 2006a; Magni *et al.*, 2006; Fernández *et al.*, 2011). Concerns about environmental genotoxicity in oil and gas industrial areas of the North Sea were raised when comparatively high

levels of MN incidences were detected in mussels (*Mytilus edulis*) and Atlantic cod (*Gadus morhua*) caged close to the oil platforms (Hylland *et al.*, 2008). Increased environmental genotoxicity and cytotoxicity have been described in an offshore Ekofisk oil extraction field (Rybakovas *et al.*, 2009). The Water Column Monitoring Programme indicated increased genotoxicity in caged mussels in sites that were close to the Ekofisk oil platform, indicating the ability to pinpoint source discharges with genotoxic endpoints in caged mussels (J. Baršienė, pers. comm.; Brooks *et al.*, 2011). Significant MN elevation in fish and mussels was found after exposure to the crude oil extracted from the North Sea (Baršienė *et al.*, 2006a; Bolognesi *et al.*, 2006a; Baršienė and Andreikėnaitė, 2007; Andreikėnaitė, 2010) and from Arctic zones (J. Baršienė, pers. comm.).

The frequency of the observed MN may be considered to be a suitable index of accumulated genetic damage during the cell lifespan, providing a time-integrated response of an organism's exposure to contaminant mixtures is available. Depending on the lifespan of each cell type and on their mitotic rate in a particular tissue, the MN frequency may provide early warning signs of cumulative stress (Bolognesi and Hayashi, 2011). Caged mussels in Genoa Harbour, which is heavily polluted by aromatic hydrocarbons, showed a continuous increase in MN in mussel gill cells, reaching a plateau after a month of caging (Bolognesi *et al.*, 2004). After 30 days of caging in the Cecina estuary on the Tyrrhenian coast, mussels showed a twofold increase of MN incidence in gill cells (Nigro *et al.*, 2006). A gradient-related increase in MN was found in haemocytes of mussels and liver erythrocytes of Atlantic cod caged for 5–6 months at Norwegian oil platforms in the North Sea (Hylland *et al.*, 2008; Brooks *et al.*, 2011). Furthermore, recovery was detected in the sinking zone of the oil tanker MT "Haven" using the MN test in caged mussels 10 years after the oil spill (Bolognesi *et al.*, 2006a). In this respect, an increase in MN frequency represents a time-integrated response to cumulative stress.

10.2 Short description of the methodology

10.2.1 Target species

The MN frequency test has generally been applied to organisms where other biological effects, techniques, and contaminant levels are well documented. That is the case for mussels and for certain demersal fish species (such as European flounder, dab, Atlantic cod, or red mullet), which are routinely used in biomonitoring programmes and to assess contamination in western European marine waters (see Figures 10.1 and 10.2; Table 10.1). However, the MN assay can also be adapted for alternative sentinel species using site-specific monitoring criteria.

When selecting an indicator fish species, consideration must be given to its karyotype, as many teleosts are characterized by an elevated number of small chromosomes (Udroiu, 2006). This means that in certain cases MN formed after exposure to clastogenic contaminants will be very small and hard to detect by light microscopy. This can be addressed to a certain extent by using fluorescent staining. After selecting target/suitable species, researchers should also ensure that other factors including age, sex, temperature, and diet are similar between the sample groups. If conducting transplantation studies, consideration needs to be given to the cell turnover rate of the tissue being examined to ensure sufficient cells have gone through cell division. For example, if using blood, the regularities of erythropoiesis should be known prior to sampling.

In general, indigenous, ecologically and economically important fish and mollusc species could serve as indicator species for biomonitoring of environmental genotoxicity levels, for screening of genotoxins distribution, or for assessments of genotoxicity effects from contaminant spills or effluent discharges. For monitoring in deep waters in northern latitudes (deeper than 1000 m), Arctic rockling (*Onogadus argentatus*) and amphipods (*Eurythenes gryllus*) are suitable species. In equatorial regions of the Atlantic, the indicator fish species *Brachydeuterus auritus, Cynoglossus senegalensis,* and *Cynoponticus ferox* are available for MN analysis (J. Baršienė, pers. comm.).

10.2.2 Target tissues

The majority of studies to date have used haemolymph and gill cells of molluscs and peripheral blood cells of fish for MN analysis (Bolognesi and Hayashi, 2011). There are other studies (albeit limited) available describing the use of blood cells of fish from other tissues, such as liver, kidney, and gills (Baršienė *et al.*, 2006b; Rybakovas *et al.*, 2009), and also other cells (e.g. fin cells; Arkhipchuk and Garanko, 2005) The application of the MN assay to blood samples of fish is particularly attractive as the method is non-destructive, easy to undertake, and results in an easily quantifiable number of cells present on the blood smears for microscopic analysis. However, studies must be undertaken to assess the suitability of any species or cell type analysed. For example, it is known that Atlantic cod have very low levels of MN in blood erythrocytes in specimens from reference sites or control groups in laboratory exposures to crude oil. Furthermore, it has been shown that MN induction in cod blood erythrocytes and erythrocytes from different haematopoietic tissues (liver, kidney, gill, and spleen) differ significantly after 3 wk of exposure to Stafjord B crude oil. In multiple laboratory exposures (108 exposure groups of cod), developing liver and kidney erythrocytes proved to be the most sensitive endpoint and most suitable approach for the assessment of oil pollution in the northern Atlantic and North Sea (Baršienė *et al.*, 2005a, 2006b). Liver can also be used as a target organ in *in situ* exposures with turbot and halibut (caged or laboratory; J. Baršienė, pers. comm.).

10.2.3 Sample and cell scoring size

The detected MN frequency in fish erythrocytes is approximately six- to tenfold lower than in mussels and clams. The large interindividual variability associated with the low baseline frequency for this biomarker confirms the need for the scoring of a consistent number of cells in an adequate number of animals for each study point. Sample size in most studies conducted with mollusc species has been 1000–2000 cells scored per animal (Bolognesi *et al.*, 1996, 2004, 2006b; Izquierdo *et al.*, 2003; Hagger *et al.*, 2005; Koukouzika and Dimitriadis, 2005, 2008; Magni *et al.*, 2006; Baršienė *et al.*, 2006b,c, 2008b, 2010a,b; Kopecka *et al.*, 2006; Nigro *et al.*, 2006; Schiedek *et al.*, 2006; Francioni *et al.*, 2007; Siu *et al.*, 2008), and previous reviews have suggested that when using fish erythrocytes, at least 2000–4000 cells should be scored per animal (Bolognesi *et al.*, 2006a; Udroiu, 2006). Previously, scorings of 5000–10 000 fish erythrocytes were used for MN analysis (Baršienė *et al.*, 2004). Since 2009–2010, the frequency of MN in fish from the North and Baltic Seas was mostly scored in 4000 cells. In stressful, heavily polluted zones, the scoring of 5000–10 000 cells in fish is still recommended.

In mussels, sample size in MN assays ranges from 5 to 20 mussels per site, as reported in the literature (Venier and Zampieron, 2005; Baršienė *et al.*, 2004, 2008a,b; Bolognesi *et al.*, 2004; Baršienė and Rybakovas, 2006; Francioni *et al.*, 2007; Siu *et al.*, 2008). Evidence suggests that a sample size of 10 specimens per site is enough for the

assessment of environmental genotoxicity levels and evaluation of the existence of genetic risk zones. In heavily polluted sites, MN analysis in 15–20 specimens is recommended, because of the higher individual variation of the MN frequency. MN analysis in more than 20 mussel or fish specimens shows only a minor change of the MN means (figure 1 in Fang *et al.*, 2009; J. Baršienė, pers. comm.).

10.2.4 MN identification criteria

Most of the studies have been performed using diagnostic criteria for MN identification developed by several authors (Heddle, 1973; Heddle *et al.*, 1991; Carrasco *et al.*, 1990; Al-Sabti and Metcalfe, 1995; Fenech, 2000; Fenech *et al.*, 2003):

- The size of MN is smaller than 1/3 of the main nucleus.
- MN are round- or ovoid-shaped, non-refractive chromatin bodies located in the cytoplasm of the cell and can, therefore, be distinguished from artefacts such as staining particles.
- MN are not connected to the main nuclei, and the micronuclear boundary should be distinguishable from the nuclear boundary.

After sampling and cell smear preparation, slides should be coded. To minimize technical variation, the blind scoring of MN should be performed without knowledge of the origin of the samples. Only cells with intact cellular and nuclear membranes can be scored. Particles with colour intensity higher than that of the main nuclei were not counted as MN. The area to be scored should first be examined under low magnification to select the part of the slide showing the best quality (good staining, non-overlapping cells). Scoring of micronuclei should then be undertaken at 1000× magnification.

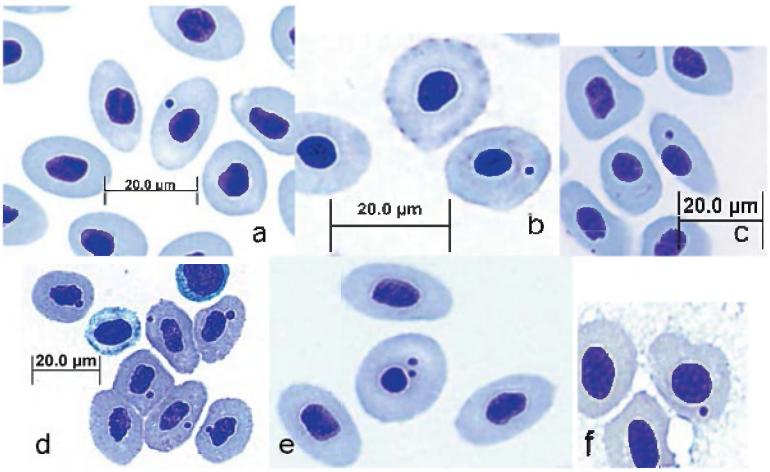

Figure 10.1. Micronuclei in blood erythrocytes of (a) *Platichthys flesus*, (b) *Limanda limanda*, (c) *Zoarces viviparus*, (d) *Clupea harengus*, (e) two MN in *Limanda limanda*, and (f) MN liver erythrocytes of *Gadus morhua*. Images from NRC (Nature Research Center) database.

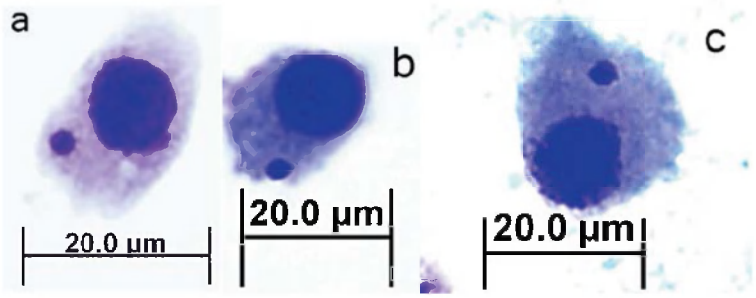

Figure 10.2. Micronuclei in gill cells of (a) *Mytilus edulis*, (b) *Macoma balthica*, and in (c) haemocytes of *Chlamys islandica*. Images from NRC database.

10.3 Confounding factors

Earlier studies on MN formation in mussels have disclosed a significant influence of environmental and physiological factors (Dixon *et al.*, 2002). Therefore, the role of the confounding factors should be considered prior to the application of MN assay in biomonitoring programmes, as well as in descriptions of genetic risk zones or ecosystem health assessments.

10.3.1 Water temperature

MN induction is a cell cycle-related process and depends on water temperature, which is a confounding factor for the mitotic activity in poikilotherm animals. Several studies have demonstrated that baseline frequencies of MN in mussels are related to water temperature (Brunetti *et al.*, 1988, 1992; Kopecka *et al.*, 2006). Baseline frequencies of MN are regarded as the incidence of MN observed in the absence of environmental risk or before exposure to genotoxins (Fenech, 1993). In fish, MN frequencies also showed seasonal differences in relation to water temperature, with lower MN levels in winter than in autumn (Rybakovas *et al.*, 2009). This was assumed to be an effect of higher mitotic activity and MN formation in response to high water temperatures in autumn (Brunetti *et al.*, 1988). In addition, it has been reported that increases in water temperature (4–37°C) can increase the ability of genotoxic compounds to damage DNA (Buschini *et al.*, 2003).

10.3.2 Types of cells

MN may be seen in any type of cell, both somatic and germ, and thus the MN test can be carried out in any active tissue. Nevertheless, there are some limitations to using different types of cells, for example, agranular and granular haemocytes in mussels. There are also differences between MN induction levels in mussel haemolymph and gill cells, mainly because gills are primary targets for the action of contaminants. The anatomical architecture of the spleen in fish does not allow erythrocyte removal in the spleen (Udroiu, 2006) as it does in mammals.

10.3.3 Salinity

The influence of salinity on the formation of MN was observed in mussels from the Danish coast located in the transitional zone between the Baltic and North Seas. No relationship between salinity and MN frequencies in mussels could be found for mussels from the North Sea (Karmsund zone), Wismar Bay, and Lithuanian coast. Similar results were found for *Macoma balthica* from the Baltic Sea—from the gulfs of Bothnia, Finland, Riga, and Lithuanian EEZ (J. Baršienė, pers. comm.).

10.3.4 Size

Because linear regression analysis of animal length and MN induction shows that size could be a confounding factor, sampling of organisms of similar size should take place (J. Baršienė, pers. comm.). It should also be noted that size is not always indicative of age; therefore, age could also potentially affect the genotoxicity response in fish.

10.3.5 Diet

Results have shown that MN formation was not influenced in mussels maintained under simple laboratory conditions without feeding (Baršienė and Rybakovas, 2006).

10.4 Ecological relevance

Markers of genotoxic effects reflect damage to genetic material of organisms and thus receive a lot of attention (Moore *et al.*, 2004a). Different methods have been developed for the detection of both double- and single-strand breaks of DNA, DNA adducts, MN formation, and chromosome aberrations. The assessment of chemical-induced genetic damage has been widely utilized to predict the genotoxic, mutagenic, and carcinogenic potency of a range of substances; however, these investigations have mainly been restricted to humans or mammals (Siu *et al.*, 2004). MN formation indicates chromosomal breaks, known to result in teratogenesis (effects on offspring) in mammals. There is, however, limited knowledge of the relationship between MN formation and effects on offspring in aquatic organisms. With growing concern over the presence of genotoxins in the sea, the application of cytogenetic assays to ecologically relevant species offers the chance to perform early tests on health in relation to exposure to contaminants.

10.5 Applicability across the OSPAR maritime area

Large-scale and long-term studies took place from 2001 to 2010 at the Nature Research Center (NRC, Lithuania) on MN and other abnormal nuclear formations in various fish and bivalve species inhabiting sites in the North Sea, Baltic Sea, Atlantic Ocean, and Barents Sea. These studies revealed the relevance of environmental genotoxicity levels in ecosystem assessments. The NRC established a large database on MN and other nuclear abnormalities in 13 fish species from the North Sea, Barents

Sea, and Atlantic Ocean, and in eight fish species and in mussels, scallops, and clams (*Macoma balthica*) from the Baltic Sea. Fish and bivalve species were collected from 85 sites in the North Sea and Atlantic and from 117 coastal and offshore sites in the Baltic (Figures 10.3 and 10.4). Monitoring of MN and other nuclear abnormalities levels was performed (2–8 times) in many sites of the North and Baltic Seas. Data on MN levels in organisms inhabiting deep-sea and Arctic zones are also available (Table 10.1).

The validation of the MN assay was done with indigenous and cultured mussels (*M. edulis*), Atlantic cod, turbot, halibut, and long rough dab in multiple laboratory exposures to crude oil from the North Sea and Barents Sea, to produced-water discharges from the oil platforms, and to other contaminants. Additional active monitoring using mussels and Atlantic cod took place in the Ekofisk, Statfjord, Troll oil platform, oil refinery zones, some northern Atlantic sites, and in sites heavily polluted by copper or polycyclic aromatic hydrocarbons.

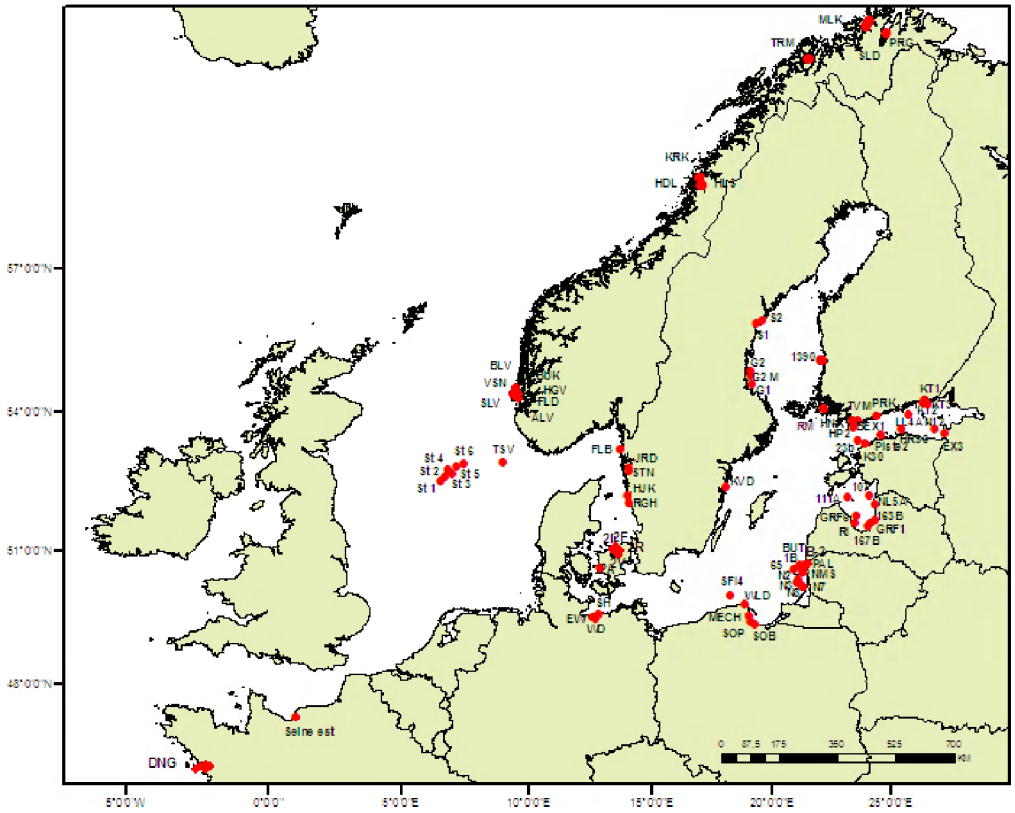

Figure 10.3. Sampling stations of bivalve molluscs for the micronuclei studies (NRC, Lithuania).

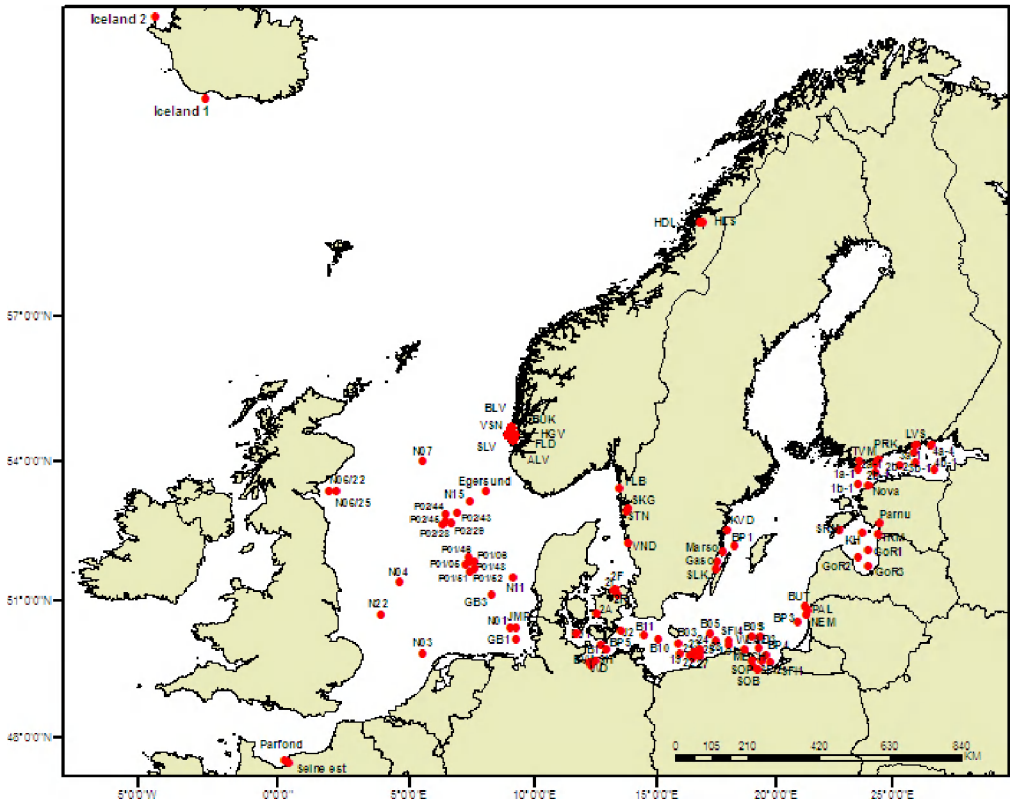

Figure 10.4. Sampling stations of fish species used for the micronuclei studies (NRC, Lithuania).

10.6 Background responses

Baseline or background frequency of MN can be defined as the incidence of MN observed in the absence of environmental risk or before exposure to genotoxins (Fenech, 1993). As mentioned above, several studies have demonstrated that MN baseline frequencies depend on water temperature. In fish, MN frequencies lower than 0.05‰ (the Baltic Sea) and lower than 0.1‰ (the North Sea) have been suggested by Rybakovas *et al.* (2009) as a reference level in the peripheral blood erythrocytes of the flatfish flounder (*Platichthys flesus*), dab (*Limanda limanda*), and also cod (*Gadus morhua*) after analysing fish from 12 offshore sites in the Baltic Sea (479 specimens) and 11 sites in the North Sea (291 specimens). For unpolluted sites in the Mediterranean Sea, baseline MN levels in gills of *M. galloprovincialis* have been set depending on water temperature to 1% at temperatures below 15°C, 2% between 15 and 20°C, and 3% above 20°C (Brunetti *et al.*, 1992).

The frequencies of MN in marine species sampled from field reference sites are summarized in Table 10.1. In addition, the frequencies of MN in blood erythrocytes of fish and in gill cells of mussels taken to uncontaminated sites are shown in Table 10.2).

Table 10.1. Reference levels of micronuclei (MN 1000^{-1} cells) in European marine species *in situ*

Species	Tissue	Location	Response MN 1000^{-1} cells	Reference
Mytilus galloprovincialis	Gills	Adriatic and Tyrrhenian Sea	1.0 at 15°C 2.0 at 15–20°C 3.0 at >20°C	Brunetti *et al.* (1992)
M. galloprovincialis	Haemolymph	Mediterranean coast	4.2 ± 0.7	Burgeot *et al.* (1996b)

M. galloprovincialis	Gills	La Spiezia Gulf, Ligurian Sea	3.0 ± 2.0	Bolognesi *et al.* (1996)
M. galloprovincialis	Gills, Haemolymph	Venice Lagoon	0.73–1.42	Dolcetti and Venier (2002)
M. galloprovincialis	Haemolymph	Strymonikos Gulf, Mediterranean Sea	0.30; 1.30	Dailianis *et al.* (2003)
M. edulis	Gills	Gijon coast, Spain	1.42	Izquierdo *et al.* (2003)
M. galloprovincialis	Gills	Strymonlkos Gulf, Mediterranean Sea	1.30	Dalllanls *et al.* (2003)
M. galloprovincialis	Haemolymph	Venice lagoon	0.44	Pampanin *et al.* (2005)
M. galloprovincialis	Gills	Tyrrhenian Sea	5.4	Nigro *et al.* (2006)
M. galloprovincialis	Gills	Gulf of Oristano, Mediterranean Sea	2.94–4.70	Magni *et al*. (2006)
M. galloprovincialis	Haemolymph	Adriatic Sea	1.0–1.5	Klobučar *et al.* (2008)
M. galloprovincialis	Haemolymph	Adriatic Sea	1.38–1.75	Pavlica *et al.* (2008)
M. galloprovincialis	Gills	Gulf of Patras	≈2.0	Pytharopoulou *et al.* (2008)
M. galloprovincialis	Gills	Algerian coast	0.0–1.18	Taleb *et al.* (2009)
M. galloprovincialis	Haemolymph	Algerian coast	1.6–2.47	Taleb *et al.* (2009)
M. galloprovincialis	Gills	Western Mediterranean	1.9–2.1	Fernández *et al.* (2011)
M. edulis	Haemolymph	Langesundfjord (Norway, rock)	0.90	Wrisberg *et al.* (1992)
M. edulis	Haemolymph	Store Belt (Denmark)	0.89	Wrisberg *et al.* (1992)
M. edulis	Gills	North Sea (Norwegian coast and Karmsund fjords)	1.05 ± 0.32	Baršienė *et al.* (2004)
M. edulis	Gills	North Sea (Göteborg coast)	0.71 ± 0.12	Baršienė *et al.* (2008a)
M. edulis	Haemolymph	North Sea	1.24 ± 0.37	Brooks *et al.* (2011)
M. edulis	Gills	Baltic Sea	0.37 ± 0.09	Baršienė *et al.* (2006c)
M. trossulus	Gills	Baltic Sea	2.07 ± 0.32	Baršienė *et al.* (2006c); Kopecka *et al.* (2006)
Macoma balthica	Gills	Baltic Sea	0.53–1.28	Baršienė *et al.* (2008b); J. Baršienė (pers. comm.)
M. balthica	Gills	Stockholm archipelago	0.4	Smolarz and Berger (2009)
Limanda limanda	Blood, kidney erythrocytes	North Sea	0.02 ± 0.01	Rybakovas *et al.* (2009)
Platychthys flesus	Blood erythrocytes	Atlantic Ocean	0.06 ± 0.04	J. Baršienė (pers. comm.)
P. flesus	Blood erythrocytes	North Sea	0.04 ± 0.03	Baršienė *et al.* (2008a)
P. flesus	Blood erythrocytes	Baltic Sea	0.15 ± 0.03	Baršienė *et al.* (2004)
P. flesus	Blood erythrocytes	Baltic Sea	0.0 ± 0.0	Köhler and Ellesat (2008)
P. flesus	Blood erythrocytes	Baltic Sea	0.08 ± 0.02	Napierska *et al.* (2009)
P. flesus	Blood erythrocytes	UK estuaries	0.27–0.66	B. P. Lyons (pers. comm.)
Zoarces viviparus	Blood erythrocytes	Baltic Sea	0.02 ± 0.02	J. Baršienė (pers. comm.)
Gadus morhua	Blood, kidney erythrocytes	North Sea	0.03 ± 0.02	Rybakovas *et al.* (2009)
G. morhua	Blood, kidney erythrocytes	Baltic Sea	0.03 ± 0.02	Rybakovas *et al.* (2009)
Clupea harengus	Blood erythrocytes	Baltic Sea	0.03 ± 0.03	J. Baršienė (pers. comm.)
Symphodus melops	Blood erythrocytes	North Sea	0.08 ± 0.04	Baršienė *et al.* (2004)
Scophthalmus maximus	Blood erythrocytes	Baltic Sea	0.10 ± 0.04	J. Baršienė (pers. comm.)
Perca fluviatilis	Blood erythrocytes	Baltic Sea	0.06 ± 0.02	Baršienė *et al.* (2005a); J. Baršienė (pers. comm.)
Mugil cephalus	Blood erythrocytes	Mediterranean coast, Turkey	0.82–2.07	Çavaş and Ergene-Gözükara (2005)
M. cephalus	Gill cells	Mediterranean coast, Turkey	1.84–2.91	Çavaş and Ergene-Gözükara (2005)
Mullus barbatus	Blood erythrocytes	La Splezla Gulf (Italy)	0.33[a]	Bolognesi *et al.* (2006b)
Dicentrarchus labrax	Blood erythrocytes	La Splezla Gulf (Italy)	0.75[a]	Bolognesi *et al.* (2006b)

Pagellus mormyrus	Blood erythrocytes	La Splezla Gulf (Italy)	0.4[a]	Bolognesi *et al.* (2006b)
Sargus sargus	Blood erythrocytes	La Splezla Gulf (Italy)	0.25[a]	Bolognesi *et al.* (2006b)
Seriola dumerili	Blood erythrocytes	La Splezla Gulf (Italy)	0.38[a]	Bolognesi *et al.* (2006b)
Serranus cabrilla	Blood erythrocytes	La Splezla Gulf (Italy)	0.0[a]	Bolognesi *et al.* (2006b)
Sparus auratus	Blood erythrocytes	La Splezla Gulf (Italy)	0.12[a]	Bolognesi *et al.* (2006b)
Sphyraena sphyraena	Blood erythrocytes	La Splezla Gulf (Italy)	0.25[a]	Bolognesi *et al.* (2006b)
Trachurus trachurus	Blood erythrocytes	La Splezla Gulf (Italy)	0.25[a]	Bolognesi *et al.* (2006b)
Mugil cephalus	Blood erythrocytes	Mediterranean Goksu Delte, Turkey	1.26 ± 0.40	Ergene *et al.* (2007)
Mullus barbatus	Blood erythrocytes	Western Mediterranean, Spain	0.10–0.16	Martínez-Gómez (2010)
Dicentrarchus labrax	Blood erythrocytes	Eastern Adriatic Sea	1.25 ± 1.97	Strunjak-Perovic *et al.* (2009)

[a]Number of MN 1000^{-1} studied erythrocytes.

Note: It is important to ensure that the data are normally distributed (e.g. Kolmogorov–Smirnov test) if the standard deviation is to be used to calculate MN frequency percentiles of the distribution, as this assumes that the data are normally distributed, which may not be the case.

Table 10.2. The reference levels of micronuclei (MN 1000^{-1} cells) in European marine organisms after caging in uncontaminated/reference sites *in situ*

SPECIES	TISSUE	LOCATION/EXPOSURE TIME	RESPONSE MN 1000^{-1} CELLS	REFERENCE
Mytilus galloprovincialis	Gills	Ligurian coast/30 days	1.78 ± 1.04[a]	Bolognesi *et al.* (2004)
M. galloprovincialis	Gills	Gulf of Patras/1 month	2.3–2.5	Kalpaxis *et al.* (2004)
M. galloprovincialis	Gills	Haven oil spill area/30 days	3.7 ± 1.62[a]	Bolognesi *et al.* (2006a)
M. galloprovincialis	Gills	Cecina estuary/4 wk	5.4	Nigro *et al.* (2006)
M. galloprovincialis	Haemolymph	Adriatic Sea/1 month	1.0	Gorbi *et al.* (2008)
M. galloprovincialis	Haemolymph	Tyrrhenian coast/1 month	0.27	Bocchetti *et al.* (2008)
M. galloprovincialis	Haemolymph	Algerian coast/1 month	1.6–2.47	Taleb *et al.* (2009)
M. galloprovincialis	Gills	Algerian coast/1 month	0.0–1.18	Taleb *et al.* (2009)
Mytilus edulis	Gills	Visnes copper site (Norway)/3 wk	1.87 ± 0.43	Baršienė *et al.* (2006d)
M. edulis	Gills	Karmsund (Norway)/4 weeks	1.40 ± 0.29	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	North Sea, oil platforms (Norway)/6 wk	2.13 ± 0.48	Hylland *et al.* (2008)
M. edulis	Haemolymph	Seiland site (Norway)/5.5 months	2.60 ± 0.21	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Ekofisk oil platform, North Sea/6 wk	1.24 ± 0.37 (2006) 3.34 ± 0.28 (2008) 2.78 ± 0.50 (2009)	Brooks *et al.* (2011)
M. edulis	Haemolymph	Oil refinery (France, 2004)	3.20 ± 0.36	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Oil refinery (France, 2006)	2.34 ± 0.37	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Oil refinery (Mongstad, 2007)/100 days	2.90 ± 0.40	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Sea Empress clean reference area (90 days)	0.75 ± 0.46	Lyons (1998)
M. edulis	Haemolymph	Sea Empress clean reference area (110 days)	0.81 ± 0.36	Lyons (1998)
Crassostrea gigas	Gills	MT "Haven" oil spill	1.49 ± 0.79[a]	Bolognesi *et al.*

		area/30 days		(2006a)
Gadus morhua	Liver erythrocytes	North Sea, oil platforms (Norway)/5 wk	0.12 ± 0.05	Hylland *et al.* (2008)
G. morhua	Liver erythrocytes	North Sea, oil platforms (Norway)/6 wk	0.27 ± 0.13	J. Baršienė (pers. comm.)
Boops boops	Erythrocytes	Haven oil spill area/30 days	0.6 ± 0.7[a]	Bolognesi *et al.* (2006b)
Mullus barbatus	Erythrocytes	Haven oil spill area/30 days	0.7 ± 0.6[a]	Bolognesi *et al.* (2006b)
Uranoscopus scaber	Erythrocytes	Haven oil spill area/30 days	1.1 ± 0.5[a]	Bolognesi *et al.* (2006b)

[a]Number of MN per 1000 studied cells

In addition, the range of variation of the frequency of MN in blood erythrocytes of fish and gill cells of *M. galloprovincialis* is displayed in Table 10.3.

Table 10.3. The range of MN frequency fish (blood, liver, kidney erythrocytes), in mussels, clams, scallops, and amphipods (haemolymph, gill, and mantle cells) from different sites in the Atlantic Ocean, North Sea, Baltic Sea, and Mediterranean Sea

Species	Number of sites studied	Tissue	MN frequency range (‰)	Reference
Mytilus edulis	3	Haemolymph	0.89–2.87	Wrisberg *et al.* (1992)
M. edulis	2	Haemolymph	0.90–2.32	Wrisberg *et al.* (1992)
M. edulis	3	Mantle	≈3–7[a]	Bresler *et al.* (1999)
M. edulis	60	Gills, haemolymph	0.37–7.20	Baršienė *et al.* (2004, 2006c, 2008b, 2010a); Baršienė and Rybakovas (2006), Schiedek *et al.* (2006)
Mytilus trossulus	5	Gills	2.07–6.70	Baršienė *et al.* (2006c); Kopecka *et al.* (2006)
M. galloprovincialis	13	Gills	1.8–24	Brunetti *et al.* (1988); Scarpato *et al.* (1990); Bolognesi *et al.* (2004); Nigro *et al.* (2006)
M. galloprovincialis	3	Gills	2–12	Kalpaxis *et al.* (2004)
M. galloprovincialis	5	Haemolymph	1.38–6.50	Pavlica *et al.* (2008)
M. galloprovincialis	3	Gills	1.2–11.8	Taleb *et al.* (2009)
M. galloprovincialis		Gills	0–22	Fernández *et al.* (2011)
Macoma balthica	29	Gills	0.53–11.23	Baršienė *et al.* (2008b); J. Baršienė (pers. comm.)
Chlamys islandica	3	Haemolymph	3.50–5.83	J. Baršienė (pers. comm.)
Eurythenes gryllus	2	Haemolymph	0.35–0.52	J. Baršienė (pers. comm.)
Limanda limanda	3	Blood	≈2–5[b]	Bresler *et al.* (1999)
L. limanda	26	Blood, kidney	0.02–1.22	Rybakovas *et al.* (2009); J. Baršienė (pers. comm.)
Platychthys flesus	3	Blood	≈ 2–6[b]	Bresler *et al.* (1999)
P. flesus	53	Blood, kidney	0.08–1.45	Baršienė *et al.* (2004, 2005a, 2008a); Napierska *et al.* (2009); J. Baršienė (pers. comm.)
Zoarces viviparus	40	Blood	0.02–0.81	Baršienė *et al.* (2005a); J. Baršienė (pers. comm.)
Gadus morhua	19	Liver, blood,	0.0–0.64	Rybakovas *et al.* (2009); Baršienė *et al.* (2010a)
Symphodus melops	9	Blood	0.07–0.65	Baršienė *et al.* (2004, 2008a)
Clupea harengus	32	Blood	0.03–0.92	J. Baršienė (pers. comm.)
Melanogrammus aeglefinus	3	Liver	0.06–0.75	J. Baršienė (pers. comm.)

Scophthalmus maximus	4	Blood, liver, kidney	0.10–0.93	J. Baršienė (pers. comm.)
Perca fluviatilis	14	Blood	0.06–1.15	Baršienė *et al.* (2005a); J. Baršienė (pers. comm.)
Brachydeuterus auritus	3	Liver	0.28–0.85	J. Baršienė (pers. comm.)
Cynoglossus senegalensis	2	Liver	0.33–0.45	J. Baršienė (pers. comm.)
Cynoponticus ferox	2	Liver	0.13–0.96	J. Baršienė (pers. comm.)
Rhinobatos irvinei	1	Liver	0.50	J. Baršienė (pers. comm.)
Onogadus argentatus	2	Liver	0.23–0.47	J. Baršienė (pers. comm.)

[a]Frequency of MN in cells. [b]Frequency of MN in erythrocytes

10.7 Assessment criteria

Assessment criteria (AC) have been established by using data available from studies for molluscs and fish in the North Sea, northern Atlantic (NRC database), and Mediterranean area (Table 10.4). The background/threshold level of MN incidence is calculated as the empirical 90% percentile (P90). Until more data become available, values should be interpreted from existing national datasets. It should be noted that these values are provisional and require further validation as data become available from the ICES database.

The 90th percentile (P90) separates the upper 10% of all values in the group from the lower 90%. The rationale for this decision was that elevated MN frequency would lie above P90, whereas the majority of values below P90 belong to unexposed, weakly–medium exposed or non-responding adapted individuals. P90 values were calculated for those stations/areas which were considered reference stations (i.e. no known local sources of contamination or those areas that were not considered unequivocally as reference sites but were less influenced by human and industrial activity).

ACs in bivalves (*Mytilus edulis, Mytilus trossulus, Macoma balthica,* and *Chlamys islandica*; data from MN analysis in 4371 specimens) and in fish (*Limanda limanda, Zoarces viviparus, Platichthys flesus, Symphodus melops, Gadus morhua, Clupea harengus,* and *Melogrammus aeglefinus*; data from MN analysis in 4659 specimens) from the North Sea, Baltic Sea, and northern Atlantic have been calculated using NRC (Lithuania) databases with data from five or more reference locations (Table 10.1).

ACs for mussels (*Mytilus galloprovincialis*) and red mullet (*Mullus barbatus*) have been estimated using available data from the Spanish Institute of Oceanography (IEO, Spain). This dataset was obtained using *M. galloprovincialis* from reference stations along the northern Iberian shelf in spring 2003, namely Cadaqués and Medas Islands. In the case of red mullet, background values were derived from the results obtained in Almeria and Málaga areas (southeast Spain). Because significant sexual differences were not observed in red mullet, data of both genders were considered.

Table 10.4. Assessment criteria of MN frequency levels in different bivalve mollusc and fish species

Species	Size (cm)	Temperature (°C)	Regional Area	Tissue	BR	ER	*n*
Mytilus edulis	3–4	11–17	Atlantic–North Sea	Haemolymph, gills	<2.51	>2.51	1 280
M. edulis	1.5–3	8–18	Baltic Sea	Gills	<2.50	>2.50	1 810
M. edulis caged for 4–6 wk	3–4	7–9	North Sea	Haemolymph	<4.1	>4.1	44
M. edulis caged for 4–6 wk	3–4	9–16	North Sea	Haemolymph	<4.06	> 4.06	656
M. trossulus	2–3	3–15	Baltic Sea	Gills	<4.50	>4.50	230

Macoma balthica	1–3	13–18	Baltic Sea	Gills	<2.90	>2.90	330
M. galloprovincialis	3–4	13	Western Mediterranean	Gills	<3.87	>3.87	12
Chlamys islandica	4–5	2–4	North Sea	Haemolymph	<4.5	>4.5	65
Zoarces viviparous	17–30	15–17	North Sea	Erythrocytes	<0.28	>0.28	226
Zoarces viviparous	15–32	7–17	Baltic Sea	Erythrocytes	<0.38	>0.38	824
Limanda limanda	19–24	8–17	North Sea	Erythrocytes	<0.37	>0.37	544
Limanda limanda	18–25	8–17	Baltic Sea	Erythrocytes	<0.49	>0.49	117
Platichthys flesus	20–28	15–17	Atlantic-North Sea	Erythrocytes	<0.33	>0.33	62
Platichthys flesus	17–39	10–17	Baltic Sea coastal	Erythrocytes	<0.29	>0.29	828
Platichthys flesus	18–40	6–18	Baltic Sea offshore	Erythrocytes	<0.23	>0.23	970
Symphodus melops	12–21	13–15	Atlantic-North Sea	Erythrocytes	<0.36	>0.36	158
Gadus morhua	20–48	13–15	Atlantic-North Sea	Erythrocytes	<0.38	>0.38	340
Gadus morhua	20–48	13–15	Baltic Sea	Erythrocytes	<0.38	>0.38	50
Clupea harengus	19–25	5–10	Atlantic-North Sea	Erythrocytes	<0.32	>0.32	60
Clupea harengus	16–29	6–18	Baltic Sea	Erythrocytes	<0.39	>0.39	450
Melogrammus aeglefinus	27–44	8–14	North Sea	Erythrocytes	<0.30	>0.30	30
Mullus barbatus	12–18	17	Western Mediterranean	Erythrocytes	<0.32	>0.32	64

BR, background response; ER, elevated response; *n*, number of specimens analysed.

10.8 Quality assurance

The MN test was found to be a useful *in vivo* assay for genotoxicity testing. However, many aspects of its protocol need to be refined, knowledge of confounding factors should be improved, and interspecies differences need further investigation. In 2009, an interlaboratory comparison exercise was organized within the framework of the MED POL programme using *M. galloprovincialis*..

Intercalibration of MN analysis in fish was done between experts from NRC and Caspian Akvamiljo laboratories, as well as between NRC experts and the University of Aveiro, Portugal (Santos *et al.*, 2010). It is recommended that these relatively simple interlaboratory collaborations should be expanded to include material from all of the commonly used bioindicator species in 2011/2012.

10.9 Scientific potential

MN analysis in different marine and freshwater species of bivalves and fish is carried out in many European laboratories in Italy, Portugal, Spain, Turkey, Lithuania, the UK, Greece, Germany, Poland, Croatia, Estonia, Russia, Norway, and Ukraine. There are single laboratories in Hungary, Algeria, and Egypt. Highly qualified expert groups are working in Italy, Lithuania, Spain, Turkey, Portugal, and the UK and are able to perform analysis in both invertebrates and vertebrates.