DAIMON Toolbox Fact Sheets:

Methods to Study the Impact of Dumped Munitions on Marine Biota

Assessment category: Biological Effects

Toolbox component: Genotoxicity

Fact Sheet 3.26: Micronucleus assay (MN)

Author: Thomas Lang, Thünen Institute of Fisheries Ecology

What is it?

In environmental genotoxicity indication systems, the micronucleus assay has served as an index of cytogenetic damage for over 30 years. According to Baršienė et al. (2012), 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). A micronucleus (MN) arises in cell divisions as a result of spindle-apparatus malfunction, the lack or damage of centromere or chromosomal aberrations (Heddle 1973, Schmid 1975, Fenech 2000; cited in Baršienė et al. 2012).

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 Baršienė et al. (2012). 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; cited in Baršienė et al. 2012).

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; cited in Baršienė et al. 2012). 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; cited in Baršienė et al. 2012).

The frequency of the observed micronuclei may be considered as a suitable index of accumulated genetic damage during the cell lifespan providing a time integrated response of an organism's exposure to contaminant mixtures. Depending on the life-span of each cell type and on their mitotic rate in a particular tissue, the micronuclei frequency may provide early warning signs of cumulative stress (Bolognesi and Hayashi, 2011; cited in Baršienė et al. 2012).

The micronucleus assay is amongst the techniques recommended by ICES and OSPAR for integrated marine environmental monitoring of chemicals and their effects (Davies and Vethaak 2012).

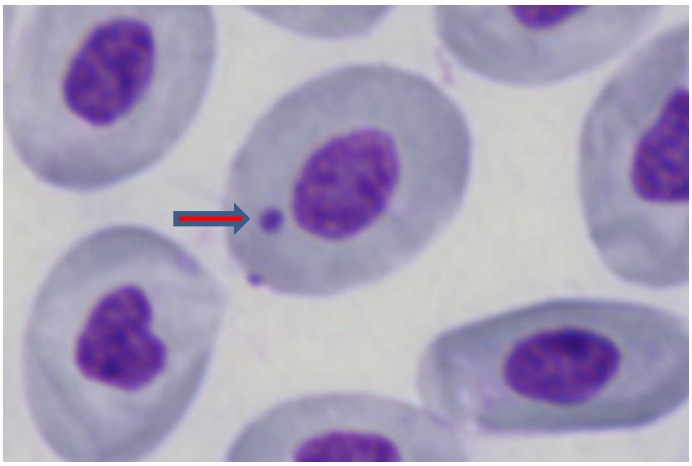

Fig. 1: Nucleated erythrocytes of common dab (*Limanda limanda*) (Blood smear stained with Giemsa). One cell with a micronucleus (arrow)

What does it tell you?

The induction of micronuclei is considered as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (Chaudhary et al. 2006, Udroiu 2006, Bolognesi and Hayashi 2011; cited in Baršienė et al. 2012). Baršienė et al. (2014) detected elevated MN levels in European flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) collected near chemical munitions dumpsites in the Baltic Sea.

Since MN is a regarded as a contaminant-specific indicator, it is applicable in a screening or detailed study on genotoxic effects of conventional or chemical munitions and warfare agents on fish. Because the indicator may also respond to non-munitions genotoxic compounds, it is not recommended to use it in isolation, but in concert with other biological effects indicators and chemical measurements.

Type of Indicator (tick box)

☐ non-specific stress indicator
☒ specific for groups of contaminants incl. CWA or explosives
☐ CWA-specific indicator
☐ specific for substances related to explosives (e.g. TNT)

How to measure it?

The MN assay involves the scoring of cells that contain one or more MN in the cytoplasm (Schmid, 1975). For MN quantification in fish, blood samples are taken from live fish in good condition and smears are prepared using clean and fat-free coded glass slides. Smears are air-dryed, fixed in 100 % methanol and stained with Giemsa solution. For MN analysis, the same samples can be used that are used for differential white blood cell counts (see DAIMON Fact Sheet 3.22, Straumer & Lang 2019).

A procedure for processing fish is outlined below:

- Clean alive, well-being fish from slime.
- Take the blood from the caudal vein by a syringe, previously rinsed with heparin solution.
- Drop the blood on clean microscopic slide. The drop of the blood should not be bigger in diameter than 0.5 cm.
- Gently smear the blood drop with plastic spatula. Don't press the spatula, it should smear a very thin layer of blood (by its own pressure). Try to move the spatula at an angle of 45°.
- Code the slides with a pencil or waterproof permanent marker on the edge of slide.
- Air-dry the prepared slides and place them for 10-15 min into a slide-staining rack for the cell fixation in 100% methanol.
- Transfer air-dried slides into slide boxes for the transportation or storage before the staining.

The staining procedure is the same for fish and bivalve cells:

- Immerse the air-dried microscopic slides for 20-30 min at room temperature in staining rack containing 10% Giemsa solution.
- Check the efficiency of cell staining at 400x magnification.
- Wash the slides under tap water for some seconds
- Air-dry slides and place them into the slide boxes or the slide racks.
- Store the slides at room temperature.

Coded and stained slides should be scored by experts blindly without knowledge of the origin of sample. Slides are examined using light microscopy at x1,000 magnification. Slides should be analysed using high quality objectives without cover slips. At least 1,000-2,000 cells should be scored in bivalves and 4,000-5,000 cells in fish.

Using micronuclei identification criteria, the frequency of micronuclei or micronucleated cells is estimated:

- MN should be identified only as round or oval chromatin bodies located in the cytoplasm of the cell with diameter smaller than 1/3 of the main nucleus.
- The MN is non-refractive bodies located at the same optical plane as the main nucleus
- The MN is not connected, linked, touched chromatin body to the main nucleus

- MN chromatin structure and staining intensity is similar to that of the main nucleus

It should always be taken into account that parasitic or microbial infection could influence the formation of micronuclei. Infection of intracellular parasites, like *Trypanosoma* spp. etc. should be registered in protocols of MN analysis.

***Species*:** According to Baršienė et al. (2012), 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, common dab (*Limanda limanda*), Atlantic cod, or red mullet (*Mullus barbatus barbatus*), which are routinely used in biomonitoring programmes and to assess contamination in western European marine waters. However, the MN assay can also be adapted for alternative sentinel species using site-specific monitoring criteria. It can also be used for studies in bivalves. In principle, indigenous, ecologically and economically important fish and mollusc species could serve as indicator species for assessment of MN induction, as well as for screening of genotoxins distribution or for genotoxicity effects related to hazardous substances from dumped munitions.

***Matrix*:** In fish, most studies have utilized circulating peripheral erythrocytes (red blood cells), but erythrocytes can also be sampled from other tissues, such as liver, kidney or gills (Baršienė et al., 2006a; Rybakovas et al., 2009). In the DAIMON project, peripheral blood cells of fish (common dab and cod) were taken by means of puncture of the caudal vein.

***Equipment*:** General requirements relevant for wild fish sampling (see DAIMON Fact Sheet 3.1, Lang 2019), for dissecting and tissue sampling (see DAIMON Fact Sheet 3.16, Lang & Straumer 2019) and for blood sampling (see DAIMON Fact Sheet 3.21, Straumer & Lang 2019a) should be met. The following specific equipment is needed:

Blood sampling:

- 1 ml single-use syringes with needles, pre-treated with heparin solution
- Paper towels
- Microscope slides
- Plastic pipettes
- Plastic spatulas
- Microscope slide staining rack and cuvettes
- Boxes for slide storing

Microscopy:

- Microscope with excellent optical capacity for bright-field observation of slides at x1,000 magnification
- Immersion oil

***Measurements and units*:** A general sampling protocol should be used, including the following information: sampling cruise number, date, study station, trawling station (for fish), species, sex, biometrical and environmental variables (e.g., water temperature, salinity, oxygen concentration).

It should also be noted that size is not always indicative of age and therefore age could also potentially affect the response of genotoxicity in the fish. Thus, age should be recorded (age reading of otoliths) together with size and sex of the fish.

It is recommended to combine the study of MN with haematological studies, especially differential white blood cell counts (see DAIMON Fact Sheet 3.22, Straumer & Lang 2019b).

***Sample size*:** Ideally, MN should be recorded in 15-20 specimens per sampling site. Sampling of organisms with similar sizes is recommended.

How to analyse and assess the data?

As a result of the MN analysis, the frequency of MN per 1,000 cells scored is reported. From the individual data, means (median) can be calculated. The non-parametric Mann-Whitney U-test can be used to compare detected frequency of MN in fish or bivalves from the reference and contaminated sites. One way ANOVA with post Kruskal Wallis test can be applied to analyse the level of variance of micronuclei incidences in different fish or bivalve groups. Pearson's correlation and regression analysis can be performed to illustrate possible relations between micronuclei induction in studied organisms, or between micronuclei frequency and environmental variables, or biometrical measurements in studied organisms from different sites.

For the assessment of MN data, established background response criteria (BR) and elevated response criteria (ER) for Baltic Sea fish species can be used (Baršienė et al. 2012), some of which are shown here:

Fish species	BR (MN per 1,000 erythrocytes)	ER (MN per 1,000 erythrocytes)
Common dab (*Limanda limanda*)	<0.49	>0.49
European flounder (*Platichthys flesus*)	<0.23	>0.23
Atlantic cod (*Gadus morhua*)	<0.38	>0.38

If MN frequencies exceed the ER values, there is indication for adverse genotoxic effects.

References

Baršienė J, Butrimavičienė L, Grygiel W, Lang T, Michailovas A, Jackūnas T (2014). Environmental genotoxicity and cytotoxicity in flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) from chemical munitions dumping zones in the southern Baltic Sea. Marine Environmental Research 96: 56-67.

Baršienė J, Lehtonen K, Koehler A, Broeg K, Vuorinen PJ, Lang T, Pempkowiak J, Šyvokienė J, Dedonytė V, Rybakovas A, Repečka R, Vuontisjarvi H, Kopecka J (2006). Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipėda-Būtingė area (Baltic Sea). Marine Pollution Bulletin 53: 422- 436.

Baršienė J, Lyons B, Rybakovas A, Martínez-Gómez C, Andreikenaite C, Brooks S, Maes T. (2012). Background document: micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms. In:

Davies IA, Vethaak AD (eds.). Integrated marine environmental monitoring of chemicals and their effects. ICES Cooperative Research Report 315: 71-83.

Bolognesi C, Hayashi M (2011). Micronucleus assay in aquatic animals. Mutagenesis 26: 205-213.

Davies IA, Vethaak AD (2012). Integrated marine environmental monitoring of chemicals and their effects. ICES Cooperative Research Report 315: 84-93.

Fenech M (2000). The in vitro micronucleus technique. Mutation Research 455: 81-95.

Lang T (2019). DAIMON Ecotox Toolbox Fact Sheet 3.1: Sampling of wild fish. Interreg Project DAIMON.

Lang T, Straumer K (2019a). DAIMON Ecotox Toolbox Fact Sheet 3.25: Externally visible fish diseases (EVFD). Interreg Project DAIMON.

Straumer K, Lang T (2019a). DAIMON Ecotox Toolbox Fact Sheet 3.21: Hematology - erythrocytes, hemoglobin, hematocrit and leucocrit. Interreg Project DAIMON.

Straumer K, Lang T (2019b). DAIMON Ecotox Toolbox Fact Sheet 3.22: Hematology - differential white blood cell count (WBC). Interreg Project DAIMON.

Rybakovas A, Baršienė J, Lang T (2009). Environmental genotoxicity and cytotoxicity in the offshore zones of the Baltic and North Seas. Marine Environmental Research 68: 246-256.

Schmid W (1975). The micronucleus test. Mutation Research 31: 9–15.