Standard Operating Procedure (SOP)

Aim of SOP (tick box)

☐ Munition detection or identification	☐ Toxicity
☐ Sampling	☐ In situ exposure studies
☐ Chemical analysis	☒ Bioassays
☐ Bioindicators/biomarkers	

29. Micronucleus assay (MN)

version 1.1

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Scope

This Standard Operating Procedure describes the micronucleus assay, which has been used for over 30 years as a bioassay for the detection of genotoxic effects in different kind of cells. In addition, the micronucleus assay is the primary in vivo test in a series of genotoxicity tests and is recommended by regulatory authorities worldwide as part of assessment of product safety[1]. According to Baršienė et al.[2], micronuclei (MN) consist of acentric chromosomal fragments or whole chromosomes that are not incorporated into daughter nuclei in the anaphase. The assay detects chromosomal alterations caused by both clastogenic (acentric chromosome fragments) and aneugenic (lagging of a whole chromosome) event, when performed appropriately[3,4].

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 into the daughter nuclei and are therefore omitted in mitosis[2]. Aneuploidogenic agents are chemicals that inhibit the formation of the spindle apparatus during mitosis. This leads not only to the formation of whole chromatids, which are omitted in the nuclei and thus form MN, but also to multinucleated cells where each nucleus comprises a varying number of chromosomes[5].

The frequency of observed micronuclei can be considered as a suitable index for accumulated genetic damage during cell life, providing a time-integrated response to the exposure of an organism to pollutants. Depending on the lifespan of individual cell types and their mitosis rate in a certain tissue, the frequency of the MN can provide early warning signals for cumulative stress[6].

Summary of the method

The micronucleus assay is commonly applied in marine species and is one of the techniques recommended by ICES and OSPAR for integrated marine environmental monitoring of chemicals and their effects[7]. It has also been used to assess the biological effects on different fish species, such as European flounder (*Platichthys flesus*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*), caught in areas associated with dumped chemical warfare agents in the Baltic Sea[8]. In this framework, the method can be applied as a contaminant specific indicator for dumped munition compounds, especially when the contaminant is known to be genotoxic. However, it is recommended to use in combination with other bioindicators and chemical analysis because the MN assay may also respond to other genotoxic agent not related to dumped munitions.

Safety aspects

Depending on the chemicals to be tested, the corresponding risk assessments have to be considered before starting work. If further instructions are required, contact the local safety officer or the laboratory manager.

Documentation

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.

Methods

The MN quantification method described here refers to fish blood cells. Blood samples are taken from live fish in good condition and smears are prepared using clean and fat-free coded glass slides. The smears are air-dried, fixed in 100% methanol and stained with Giemsa solution.

Equipment:

General equipment relevant for wild fish sampling (see 'sampling of wild fish' SOP) and equipment relevant for dissecting and tissue sampling (see 'external visible fish diseases' SOP) is needed. Specific equipment for the MN assay:

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 1,000x magnification.
- Immersion oil.

Chemicals:

- Giemsa solution (contains methanol and glycerine).
- Methanol, CAS 67-56-1.

Species:

According to Baršienė et al.[2], the MN assay has generally been applied to organisms where other biological effects, techniques and levels of contaminants have been well documented. This applies to bivalve molluscs and certain demersal fish species such as European flounder, common dab (*Limanda limanda*), Atlantic cod or red mullet (*Mullus barbatus barbatus*), which are commonly used in biomonitoring programmes and for assessing 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 tests on bivalve molluscs. In principle, indigenous, ecologically and economically relevant fish and molluscs species could be used as an indicator species for the assessment of MN induction and for screening of genotoxic effects associated with 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[9]. For the method described here, peripheral blood cells of fish (common dab and cod) were taken by means of puncture of the caudal vein.

Sample size:

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

Procedure:

Fish processing procedure

- Clean the fish alive.
- 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. Do not 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.

The coded and stained microscope slides should be scored blindly by experts without knowing the origin of the samples. The slides are examined by light microscopy at 1,000x magnification. The slides should be analyzed using high quality objectives without cover glasses. At least 1,000-2,000 cells should be scored in bivalves and 4,000-5,000 cells in fish.

Assessment:

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 (Figure 1).
- The MN is a non-refractive bodies located at the same optical plane as the main nucleus.
- The MN is not connected, linked, touched 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.

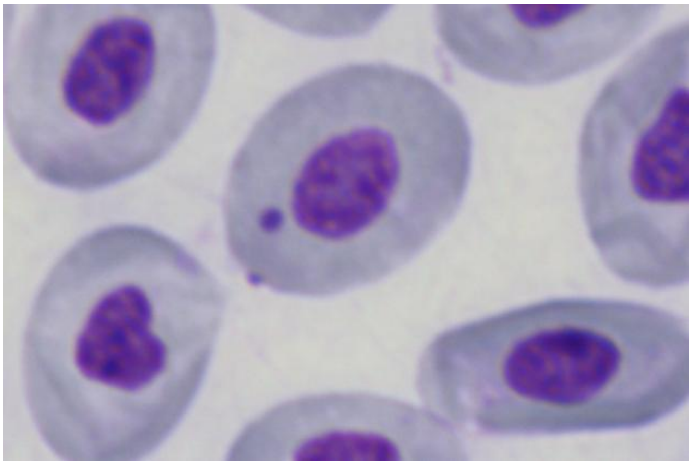

Figure 1: Nucleated erythrocytes of common dab (*Limanda limanda*), one cell contains a micronucleus. (Blood smear stained with Giemsa).

Data evaluation:

The result of the MN analysis is the frequency of MN per 1,000 cells analyzed. Mean values (median) can be calculated from the individual data. The non-parametric Mann-Whitney U-Test can be used to compare the observed frequency of MN in fish or shellfish from the reference and possibly contaminated sites. Pearson's correlation and regression analysis can be performed to illustrate possible relationships between the frequency of micronuclei and environmental variables or biometric 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[2], some of which are shown below. If MN frequencies exceed the ER values, there is indication for adverse genotoxic effects.

Fish species	BR	ER

	(MN per 1,000 erythrocytes)	(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

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