Standard Operating Procedure (SOP)

Aim of SOP (tick box)

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

17. Lysosomal membrane stability

version 1.1

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Scope

This SOP describes the analysis method for assessing oxidative damage in biological samples exposed to hazardous substances, including substances derived from sea-dumped munitions. Lysosomes are cytoplasmic, single-membrane organelles ubiquitous to animal cells. Lysosome membrane stability (LMS) can be altered by physiological or pathological conditions induced by pollutants, resulting in the activation or liberation of hydrolytic enzymes to the cytosol. LMS is used as a biomarker of the general health status of an individual organism. A reduction in LMS is a non-specific stress response that can be caused by various factors, including exposure to pollutants. The methods a are based on references 1 and 2 and has been modified for mussel and fish samples by SYKE. The *in vitro* method has been used the EU-funded project Chemical Munitions, Search and Assessment (CHEMSEA) and the histochemical method in Decision Aid for Marine Munitions (DAIMON)[3].

Summary of the method/SOP

The stability of the lysosomal membrane can be assessed in histological samples of fish liver and mussel digestive gland, and *in vitro* in mussel haemocytes. Both methods study the integrity of the lysosomal membrane over time with visual inspection.

Measurements are recommended to be done in samples from both the suspected dumping site and a reference area.

Safety aspects

Normal laboratory safety rules should be applied. 2-methoxyethanol is hazardous if breathed, swallowed or in contact with skin. It is vey corrosive. 2-methoxyethanol can decrease fertility. Formaldehyde is hazardous if breathed, swallowed or in contact with skin. It is heavily corrosive, damaging to the eyes and carcinogenic. Very flammable.

Documentation

All samples should have a unique code and label. Possible errors or other anomalies that could affect the interpretation of the results should be recorded.

The test report should include the following results for each sample:

Time [min] of peak colour intensity (histochemical method) OR
Time [min] when 50% of the cells in an individual sample show the predefined alterations (NRR-method)

Methods

Matrix: Fish liver tissue, mussel digestive gland tissue, mussel haemocytes.

Equipment: Histochemical method: cryostat; equipment for the preparation of cryohistological slides; bright-field binocular microscope with ×10 and ×40 objectives and 580 nm green filter to enhance contrast of the dye product. *In vitro* method using haemocytes (Neutral Red Retention test, NRR): syringes and needles; slides; incubation chamber; bright-field binocular microscope with ×10 and ×40 objectives. For more details, see references. For reagents, see Tables 1 and 2.

Table 1: Reagents used in the analysis.

solution	**histochemical method**
calcium-formaline fixative	10% (v/v) formaldehyde + 2% (w/v) Ca^{2+}-acetate in water
citrate buffer	0.1M Na_3-citrate + citric acid + 0.46% NaCl in water, pH 4.5
sodium phosphate buffer	0.1M Na_2HPO_4 + 20mM NaH_2PO_4 in water, pH 7.4
physiological saline solution	0.9% NaCl in water
Polypep	7% (w/v) solution in citrate buffer
Naphthol AS-BI N-acetyl-β-D-glucosaminide (mussel) or Naphthol AS-BI phosphate (fish)	8% (w/v) solution in 2-methoxyethanol
Fast Violet B	0.1M Fast Violet B in phosphate buffer
	NRR-method
artificial sea water (brackish)	Hepes: 477mg/100ml water NaCl: 463 mg/100ml water $MgSO_4$: 486 mg/ml water KCl: 14 mg/100 ml water $CaCl_2$: 35 mg/100ml water pH 7.36

Neutral Red	stock solution: 28.8 mg/ml in dimethylsulfoxide DMSO working solution: 1:500 dilution of stock solution in artificial sea water

Table 2: Reagents used in the analyses.

reagent	**CAS-number**
formaldehyde	200-001-8
Ca^{2+}-acetate	114460-21-8
Na_3-citrate	6132-04-3
citric acid	77-92-9
NaCl	7647-14-5
Na_2HPO_4	7558-80-7
NaH_2PO_4	7558-79-4
Polypep (Sigma registered trademark)	Sigma P5115
Naphthol AS-BI N-acetyl-β-D-glucosaminide	3395-37-7
Naphthol AS-BI phosphate	1919-91-1
2-methoxyethanol	109-86-4
Fast Violet B	14726-28-4
Hepes	7365-45-9
$MgSO_4$	10034-99-8
KCl	7447-40-7
$CaCl_2$	10035-04-8
Neutral Red	553-24-2
dimethylsulfoxide DMSO	67-68-5

Measurements and units: Histochemical method: Serial cryosections of the target tissue are prepared and treated for 50, 45, 40, 35, 30, 25, 20, 15, 10, 8, 6, 4, 2, and 0 minutes in 37℃ citrate

buffer in shaking water bath. Prepare the incubation solution by adding 2.5ml of the Naphthol AS-BI N-acetyl-β-D-glucosaminide (mussel) or Naphthol AS-BI phosphate (fish) solution to 50 ml of Polypep-solution. Decant the citrate buffer and replace it with the incubation solution. Incubate 20 min in 37℃ water bath with shaking. Decant the incubation solution, and rinse the slides with 37℃ physiological saline for 3 min. Decant the saline solution and add Fast Violet B -solution. Incubate 10 min in room temperature. Decant the Fast Violet B -solution and rinse with room temperature tap water for 10 min. Decant the water and add cold calcium-formaline fixative. Incubate in the fume hood for 15 min. Decant the fixative and rinse a few times with tap water and a final time with MQ-water. Let the slides dry in room temperature and add cover slips.

Slides are examined and/or photographed through a microscope and evaluated to determine the time (in min) of maximum colour intensity resulting from reactions involving the marker enzymes, and the time is used as the endpoint parameter. The light adjustments for the microscope and camera should be kept the same for all samples to allow comparison. If possible, the same person should evaluate all slides for more accuracy.

NRR: Living (mussel) haemocytes are observed at fixed intervals under a microscope after the introduction of the Neutral Red dye solution. When 50% of the cells in an individual sample show the predefined alterations, the observation time (min) is recorded as the endpoint parameter.

Sample size: Measurements are made from at least 15-20 individual specimens from each study site.

Conclusions (if applicable)

Histochemical method: Different species show slightly different baseline LMS levels and also different response sensitivity to pollutants. In the Baltic Sea, fish and mussels are considered to have no toxically-induced stress if LMS is >15 min, are stressed but compensating between 15 and 8 min, and severely stressed and probably exhibiting irreversible toxicopathic alterations if <8 min.

NRR: In Baltic Sea mussel populations, mean NRR times <50 min indicate a poor health status, values between 50 and 90 a moderate health status, and values >120 min a good health status.

Comparing lysosomal membrane stability values from the dump-site to samples from a reference site will help distinguish the effect of hazardous substances from the munitions from e.g. effects of seasonality. Statistical comparison of means (t-test, ANOVA) is suggested.

References

[1] Martínez-Gómez, C., Bignell, J. Lowe, D. *ICES Tech Mar Environ Sci 56,* **2015**.

[2] Moore, M.N., Lowe, D., Köhler, A., *ICES Tech Mar Environ Sci 36,* **2004**.

[3] Bełdowski, J., Fabisiak, J., Popiel, S., Östin, A., Olsson, U., Vanninen, P., Lastumaki, A., Lang, T., Fricke, N., Brenner, M., Berglind, R., Baršiene, J., Klusek, Z., Pączek, B., Söderström, M., Lehtonen, K., Szubska, M., Malejevas, V., Koskela, H., Michalak, J., Turja, R., Bickmeyer, U., Broeg, K., Olejnik, A. and Fidler, J. *Institute of Oceanology Polish Academy of Sciences, Gdańsk, Poland,* **2014**, ISBN: 978-83-936609-1-9, 86 pp

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