Recommended Operating Procedure (ROP)

Aim of ROP (tick box)

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

13. Lipid peroxidation

version 1.1

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Scope

This ROP describes the analysis method for assessing oxidative damage in biological samples exposed to hazardous substances, including substances derived from sea-dumped munitions. An overload of intracellular reactive oxygen species (ROS) can occur due to exposure to different groups of pollutants. If the antioxidant defense system (ADS) cannot effectively neutralize ROS, damage can occur to various cellular components and structures. Lipids are macromolecules readily damaged by ROS peroxidation and in cell membranes this can cause serious cellular dysfunctions. LPX is a non-specific oxidative stress indicator. Elevated levels of lipid peroxidation (LPX) indicate a breakdown of the ADS and damage to cellular membranes that may cause various negative effects on the individual's health status. The LPX method is based on references 1 and 2. It has been used the EU-funded project Decision Aid for Marine Munitions (DAIMON)[3].

Summary of the method/ROP

Lipid peroxidation is measured spectrophotometrically from tissue homogenates of mussel digestive gland or fish liver. Measurements need to be done in samples from both the suspected dumping site and a reference area.

Safety aspects

Normal laboratory safety rules should be applied. Methanol and cumene are very hazardous, and waste needs to be collected and treated accordingly. Triphenylphosphine is hazardous and ammonium iron (II) sulfate hexahydrate is hazardous and potentially carcinogenic.

Documentation

All samples should have a unique code and label. Pipetting schemes should be used to record the analysis procedure and order of samples, as well as possible pipetting errors or other anomalies that could affect the interpretation of the results.

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

Absorption of each sample at 570 and 590 nm [OD]
Sample weight used in the analysis [mg]
Lipid peroxidation as µM cumene-hydroperoxide equivalents per mg wet weight of sample [µM CuOOH-eq/mg ww]

Methods

Matrix: Fish liver and mussel digestive gland.

Equipment: Spectrophotometer/microplate reader able to measure at 570 nm and 590 nm, tissue homogenizer (e.g. Qiagen Tissue Lyser II), eppendorf centrifuge, other basic laboratory equipment (pipettes, microplates, decanters, fume hood). For reagents, see Table 1 and 2.

Table 1: Solutions used in the analysis.

solution	**details**
sulphuric acid	0.25 M in water
ammonium iron (II) sulfate hexahydrate	2.5mM in 0.25 M sulphuric acid
xylenol orange	0.1mM in methanol
triphenyl phosphine	2.6 mg/ml in methanol (1mM)
cumene hydroperoxide	0.002% (v/v) stock solution in methanol for fish, 0.001% (v/v) stock solution for mussels

Table 2: Reagents used in the analysis.

reagent	**CAS-number**
methanol	67-56-1
sulphuric acid	7664-93-9
ammonium iron (II) sulfate hexahydrate	12054-85-2
xylenol orange	3618-43-7
triphenyl phosphine	603-35-0
cumene hydroperoxide (technical grade)	80-15-9

Measurements and units: A 10-30 mg piece of tissue is homogenized in 200 µl (mussels) or 300 µl (fish) of methanol and centrifuged for 10 min at 5000g. The supernatant is divided into two tubes of 45 µl aliquots. The first tube is treated with 5 µl of triphenyl phosphine TPP (blocks lipid hydroperoxides interfering with the analysis) for 30min (add 5µl of methanol to the second tube). In the meantime, prepare the *reaction solution* by combining one part of the 2.5 mM ammonium iron (II) sulfate hexahydrate in 0.25 M sulphuric acid with 9 parts of xylenol orange solution. A standard curve is prepared with cumene hydroperoxide stock solution according to Table 3.

Table 3: Preparation of cumene standard curve.

STD	cumene stock (µL)	MetOH (µL)
1	0	50
2	2	48
3	4	46
4	8	42
5	16	34
6	24	26
7	32	18
8	40	10

Add 950 µl of the freshly prepared *reaction solution* to sample and standard tubes. Incubate 2 h at room temperature in the dark. After incubation, vortex the tubes and pipette standards and samples 200 µl/well in triplicate. Measure the absorbance with two wavelengths (570 and 590 nm). In order for the analysis to be reliable, he absorbances of the samples should fall in the standard curve range. If the sample absorbance is too high, the measurement for that sample has to be repeated with adjusted sample volumes to achieve a satisfactory result.

For more information, see references 1 and 2. Calculations: Absorbance difference between TPP-treated and untreated samples is compared to the absorbance of the standard curve. Lipid peroxidation is announced as µM of cumene-hydroperoxide equivalents per tissue wet weight (mg) used for homogenisation.

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

Conclusions (if applicable)

Compare the LPX levels measured from organisms collected from the target area to those from the reference area. An elevated or lowered activity level (bell-shape response) compared to the reference area indicate a negative effect. If the difference in mean activity level is more than one standard deviation (SD) of the mean values measured in the reference area, stress is considered moderate. If the level differs more than two SDs, stress is severe. LPX is ubiquitous in the animal kingdom and can be measured also in other species than those mentioned in this ROP.

References

[1] Vuori, K.A., Lehtonen, K.K., Kanerva, M., Peltonen, H., Nikinmaa, M., Berezina, N.A., Boikova, E., *Mar Ecol Prog Ser 538,* **2015**, 131-144.

[2] Ohkawa, H., Ohishi, N., Yagi, K., *Anal Biochem 95,* **1979**, 351-359.

[3] Brenner M., Schuster, R., Strehse, J.S., Ahvo, A., Turja, R., Maser, E., Bickmeyer, U., Lehtonen, K.K. *Marine Environmental Research,* **2020,** in revision.

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