Recommended Operating Procedure (ROP)

Aim of ROP (tick box)

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

10. Glutathione peroxidase activity

version 1.1

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Scope

This ROP describes the analysis method for assessing oxidative stress in biological samples exposed to hazardous substances, including substances derived from sea-dumped munitions. Glutathione peroxidase enzyme (GPx) catalyzes the reduction of hydrogen peroxide to water and oxygen, as well as the reduction of peroxide radicals to alcohols and oxygen. GPx is a key enzyme in the antioxidant defence system (ADS). Elevated levels of GPx indicate that the cellular ADS has been activated due to an accelerated production of reactive oxygen species (ROS) that need to be neutralized to prevent oxidative damage to macromolecules. On the other hand, lowered GPx levels may indicate that the ADS is already overloaded and the organism cannot properly cope with the excessive amounts of ROS (the so-called bell-shape response). GPx is a non-specific stress response indicator. The GPx method is based on Vuori et al.[2], but has been modified for mussel and fish samples by SYKE. It has been used in the monitoring and ecological risk assessment of dumped munitions in the EU-funded project Decision Aid for Marine Munitions (DAIMON)[4].

Summary of the method/ROP

Glutathione peroxidase activity is measured spectrophotometrically from tissue homogenates of mussel digestive gland or fish liver (see separate SOP document for homogenization). 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. Sodium azide is fatally toxic to humans if swallowed or in contact with skin and very harmful to the environment, and waste should be separately collected and treated accordingly.

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 per minute of each sample at 340 nm [OD/min]
Sample volume in the analysis [µl]
Protein concentration [mg/ml]
GPx activity [nmol mg^{-1} protein min^{-1}]

Methods

Matrix: Fish liver, mussel digestive gland and gill tissue homogenates (see SOPs for homogenization[5,6]).

Equipment: Spectrophotometer/microplate reader able to measure at 340 nm in intervals; 96-well UV microplate or 96-well half area microplates; basic laboratory equipment (pipettes, decanters), laboratory grade water purification system. Commercial kit[1] (Glutathione Peroxidase Cellular Activity Assay Kit, Sigma CGP1-1KT). For other reagents, see Table 1 and 2.

Table 1: Reagents used in the analysis.

solution	details
NaN_3 sodium azide	0.1 µM dissolved in buffer provided by the kit
H_2O_2 -solution (shield from light)	2 mM H_2O_2 in purified water

Table 2: Reagents not provided by the kit.

reagent	**CAS-number**
NaN_3 sodium azide	26628-22-8
H_2O_2	7722-84-1

Measurements and units: Measurements is recommended to be perfomed using a commercial kit, e.g., Glutathione Peroxidase Cellular Activity Assay Kit (Sigma CGP1-1KT), modified for liver samples.

Prepare a 0.15-0.30 mM dilution of the H_2O_2 -solution.

Reaction buffer (provided in the kit) should be at room temperature for the measurements. Pipette 5 µl/well of blank and samples in a 96-well half-area plate in triplicate. Pipette 35 µl of buffer in each well. Pipette 5µl of NADPH-solution (from kit) in each well. Pipette 5µl of H_2O_2 -solution in each well and start measurement in the spectrophotometer. Measure the change in absorbance at 340 nm for at least 5 minutes, in 25 second cycles. Sample volumes in the analysis can and should be adjusted to achieve a satisfactory result (i.e. measurable activity). If the variance between sample replicate absorbances is too high, measurements for that sample should be repeated.

The activity of GPx is adjusted to the protein concentration of the sample, measured with, e.g., the Bradford method[3]. Calculate the change in absorbance (OD/min). Subtract blank measurement values from the sample measurements.

GPx activity is calculated with the formula

$$= \frac{-\,absorbance\ change\ [\frac{1}{min}] * \ analysis\ volume[ml]}{molar\ attenuation\ coefficient\ \left[\frac{1}{(\frac{mol}{l}) * cm}\right] * light\ path[cm] * \ sample\ volume[ml] * \ sample\ protein\ concentrat}$$

where molar attenuation factor of NADPH is 6.22 $mM^{-1}\,cm^{-1}$.

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

Conclusions (if applicable)

Data evaluation: Compare the GPx activity 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.

GPx is ubiquitous in the animal kingdom and can be measured also in other species than those mentioned in this ROP.

References

[1]Sigma-Aldrcih, Glutathione Peroxidase Cellular Activity Assay Kit Technical Bulletin, *Sigma-Aldrich Co, St. Louis,* ***2017***.

[2]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.

[3]Bradford, M.M., *Anal Biochem 72,* ***1976****, 248-254.*

[4] 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

[5]Ahvo, A. *DAIMON 2 PROJECT SOPs: Homogenization of fish muscle and mussel gill tissues,* **2020.**

[6]Ahvo, A. *DAIMON 2 PROJECT SOPs:* Homogenisation of fish liver and mussel digestive gland tissues **2020.**

Change history

1.0	11.2.2020	First edition
1.1	10.6.2020	
1.2	18.05.2021	Definition of the document was changed from SOP to ROP

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