# Recommended Operating Procedure (ROP)

**Aim of ROP** (tick box)

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

## 11. Glutathione reductase 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 reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) back to its reduced form (GSH), thus helping in maintaining the glutathione system that protects against reactive oxygen species (ROS). Thus, GR is a key enzyme in the antioxidant defence system (ADS). Elevated levels of GR activity indicate that the cellular ADS has been activated due to an accelerated production of ROS that need to be neutralized to prevent oxidative damage to macromolecules. On the other hand, lowered GR 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). GR is a non-specific oxidative stress indicator. The GR method is based on Vuori et al.[1], 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)[7].

### Summary of the method/ROP

Glutathione reductase activity is measured from tissue homogenates of mussel digestive gland or fish liver (see separate SOP documents for homogenization[5,6]). The measurement is based on the reduction of oxidized glutathione catalyzed by GR. In the end, the reaction product is measured spectrometrically. 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.

### 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 412 nm [OD/min]
Sample volume in the analysis [µl]
Protein concentration [mg/ml]
GR activity [nmol $mg^{-1}$ protein $min^{-1}$]

## Methods

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

*Equipment*: Spectrophotometer/microplate reader able to measure at 412 nm in intervals; basic laboratory equipment (pipettes, 96-well plastic microplates, decanters), laboratory grade water purification system. For reagents, see Table 1 and 2.

*Table 1: Reagents used in the analysis.*

| **solution** | **details** |
|---|---|
| NADPH (keep on ice) | 2 mM dissolved in buffer |
| GSSG (keep on ice) | 2 mM GSSG in buffer |
| DTNB (keep on ice) | 3 mM DTNB in buffer |
| reaction buffer | 100mM phosphate buffer ($Na_2HPO_4$ + 20mM $NaH_2PO_4$ in water) + 2mM EDTA, pH 7.5 |

*Table 2: Reagents used in the analysis.*

| **reagent** | **CAS-number** |
|---|---|
| $Na_2HPO_4$ | 7558-80-7 |
| $NaH_2PO_4$ | 7558-79-4 |
| EDTA (ethylenediaminetetraacetic acid) | 6381-92-6 |
| DTNB (5,5´-dithiobis 2-nitrobenzoic acid) | 69-78-3 |
| GSSG (glutathione disulfide, oxidized form of glutathione) | 27025-41-8 |
| NADPH (nicotinamide adenine dinucleotide phosphate) | 2646-71-1 |

*Measurements and units*: Reaction buffer should be at room temperature for the measurements. Pipette 3 µl/well of samples and blanks (buffer) in four replicates in a 96-well half-area plate. Mix 3000µl of the GSSG-solution, 1500µl of the DTNB-solution, 300µl of the NADPH-solution and 840 µl of buffer. Pipette 47µl of the mix in each well and start measurement on the spectrophotometer at 412 nm. Measure the change in absorbation for 5 minutes, in ca. 25 second cycles. Sample volumes in the analysis can and should be adjusted to achieve a satisfactory result i.e. measurable activity, defined as OD/min values over 0.05. If the variance between sample replicate absorbances is too high, measurements for that sample should be repeated.

For more information, see references 1, 2 and 3. The activity of GR (OD/min) is adjusted to the protein concentration of the sample, measured with, e.g., the Bradford method[4] . Calculate the change in absorbance (OD/min). Subtract blank measurement values from the sample measurements. Calculate a mean OD/min value from the four replicates.

GR 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 DTNB is 1.36 * $10^4$ $mM^{-1}$ $cm^{-1}$.

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

**Conclusions (if applicable)**

Sample volumes in the analysis can and should be adjusted to achieve a satisfactory result (i.e. measurable activity). GR is ubiquitous in the animal kingdom and can be measured also in other species than those mentioned in this ROP.

Compare the GR 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.

**References**

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**Change history**

| | | |
|---|---|---|
| 1.0 | 11.2.2020 | First edition |
| 1.1 | 10.6.2020 | |
| 1.2 | 18.5.2021 | Definition of the document was changed from SOP to ROP |

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