Standard Operating Procedure (SOP)

Aim of SOP (tick box)

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

6. Acetylcholinesterase inhibition

version 1.1

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Scope

This SOP describes the analysis method for assessing neurotoxicity in biological samples. Acetylcholinesterase (AChE) is a key neurotransmitter enzyme functioning especially in neuromuscular junctions. Inhibition of AChE can lead to serious neuromuscular disorders, leading to tetanus and death. The method is based on Bocquené and Galgani[1], but has been modified for mussel and fish samples by SYKE. Originally used for the detection of neurotoxic effects of organophosphates and carbamates AChE has in later studies been shown to be affected also by many other ubiquitous pollutant groups. AChE is a non-specific stress indicator. AChE has been used in the monitoring and ecological risk assessment of dumped munitions in two EU-funded projects: Chemical Munitions, Search and Assessment (CHEMSEA) and Decision Aid for Marine Munitions (DAIMON)[3].

Summary of the method/SOP

Acetylcholine esterase (AChE) activity is measured spectrophotometrically from tissue homogenates of mussel gills or fish muscle (see separate SOP ”Gill and muscle 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. Acetylcholine iodide is an irritant to the eyes and respiratory system.

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]
AChE activity [nmol mg^{-1} protein min^{-1}]

Methods

Matrix: Fish muscle and mussel gill tissue homogenates (see SOP " Homogenization of fish muscle and mussel gill tissues"[4]).

Equipment: Spectrophotometer/ microplate reader able to measure absorption kinetics at 412 nm wavelength; basic laboratory equipment (pipettes, microplates, decanters), water purification equipment. Reagents needed for the analysis, see Table 1.

Table 1: Solutions used in the analysis.

solution	details
20 mM phosphate buffer	20 mM Na_2HPO_4 + 20mM NaH_2PO_4 in purified water, pH 7.0
100mM phosphate buffer	100 mM Na_2HPO_4 + 100mM NaH_2PO_4 in purified water, pH 8.0
DTNB-solution	0.01M 5,5´-dithiobis 2-nitrobenzoic acid in cold 100mM phosphate buffer
ACTC-solution	0.1M acetylthiocholine iodide in cold purified water

Table 2: Reagents used in the analysis.

reagent	**CAS-number**
Na_2HPO_4	7558-80-7
NaH_2PO_4	7558-79-4
DTNB (5,5´-dithiobis 2-nitrobenzoic acid)	69-78-3
ACTC (acetylthiocholine iodide)	2260-50-6

Measurements and units: Reaction buffer should be room temperature for the measurements. Pipette 20 µl/well of the sample homogenate in four replicates in a 96-well plate. Keep the plate chilled e.g. over ice. In the first column, pipette 10 µl /well of the buffer to be used as blank

references. Add 300 µl of 20mM phosphate buffer to each well. Add 20 µl of DTNB-solution to each well. Add 10 µl of freshly prepared ACTC-solution to each well and start measurement in the spectrophotometer. Measure the absorbance at 412 nm for 8-10 minutes, in 15-20s 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 reference 1. The activity of AChE is adjusted to the protein concentration of the sample, measured with, e.g., the Bradford method[2]. Calculations: Based on absorbance measurement data, 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. AChE activity is calculated with the formula

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

where the molar attenuation coefficient of DTNB is 1.36 * 10^4 M^{-1} cm^{-1}

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

Conclusions (if applicable)

Data evaluation: Samples from a contaminated site should be compared with samples from a clean, uncontaminated reference site with similar hydrological properties. A lowered AChE activity level indicates a negative health effect. If the mean activity level is lower for more than one standard deviation (SD) of the mean values measured in the reference area, stress is considered moderate. If the level is more than two SDs lower, stress is severe. AChE is ubiquitous in the animal kingdom and can be measured also in other species than those mentioned in this SOP.

References

[1] Bocquené, G. and Galgani, F., *ICES Copenhagen, Denmark,* **1998.**

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

[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

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

Change history

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