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Water quality — Biochemical and physiological measurements on fish —

Part 2: Determination of ethoxyresorufin-*O*-deethylase (EROD)

Qualité de l'eau — Mesurages biochimiques et physiologiques sur poisson —

Partie 2: Dosage de l'éthoxyrésorufine-O-dééthylase (EROD)

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 23893-2 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 23893 consists of the following parts, under the general title *Water quality — Biochemical and physiological measurements on fish*:

— *Part 1: Sampling of fish, handling and preservation of samples*

— *Part 2: Determination of ethoxyresorufin*-O-*deethylase (EROD)* [Technical Specification]

Introduction

The measurement of pollution biomarkers in fish, such as the measurement of biotransformation enzyme activities, is likely to provide information about exposure levels, bioavailability and the early biological effects of substances present in aquatic ecosystems. The measurement of the EROD enzyme activity allows the diagnosis of the exposure of fish to inducers of the P450 1A cytochrome, such as certain polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and dioxins. A large amount of research work bears witness to the extent of the studies conducted (see Bibliography).

An induction of EROD activity reflects the presence of inducers such as those mentioned above. On the other hand, the absence of induction does not necessarily reflect the absence of exposure of the fish to organic contaminants, account being taken of the inhibition phenomena of the EROD induction of possible modification of the bioavailability of the inducers or of low exposure concentrations.

The application of a standardised reference method is strongly advised within a monitoring programme. The intercalibration exercises on the measurement of the EROD enzyme activity undertaken since 1991 have revealed multiple sources of errors, which are very easy to avoid (dilution of resorufin, determination of proteins, calculation of the enzyme activity, etc.) once laboratories have become familiar with the analysis of enzyme activities and the possible error factors.

Water quality — Biochemical and physiological measurements on fish —

Part 2: Determination of ethoxyresorufin-*O*-deethylase (EROD)

1 Scope

This part of ISO 23893 specifies a method for measuring the ethoxyresorufin-*O*-deethylase (EROD) enzyme activity on a post-mitochondrial fraction of fish liver homogenate (subcellular fraction in which the EROD activity is located) employing a cell or microplate fluorimetric method.

It applies to fish that are sampled in their natural environment (fresh water or salt water) or exposed to substances or effluents in a laboratory.

This method is applicable for EROD values greater than or equal to 1 pmol/(min·mg) of proteins. A higher sensitivity may be achieved by using a cell (test tube) procedure.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 23893-1, *Water quality — Biochemical and physiological measurements on fish — Part 1: Sampling of fish, handling and preservation of samples*

3 Principle

Samples of fish are collected and dissected as described in ISO 23893-1 to obtain pieces of liver. Homogenates of fish liver are prepared by crushing (homogenisation) and the supernatant S9 fraction recovered by centrifugation. The EROD enzyme activity in the S9 fractions is determined by measurement of the increase in fluorescence due to the transformation of the 7-ethoxyresorufin into resorufin. The fluorescence is reported in quantities of resorufin by means of a calibration range (external calibration of resorufin or of rhodamine B). The EROD activity is related to the quantity of proteins in the S9 fraction.

4 Test environment

All of the tests and handling operations with the S9 fraction shall be carried out at a temperature close to 4 °C (e.g. handling in crushed ice), except the enzyme reaction which shall be performed at 20 °C ± 2 °C.

5 Reagents

Unless otherwise specified, use only reagents of recognised analytical grade.

5.1 Ultra pure water, having a conductivity below 1 µS/cm.

5.2 Potassium chloride, 150 mmol/l solution.

Dissolve 11,2 g of KCl (relative molecular mass 74,6) in 1 l of water (5.1). This solution is stable for 6 months at a temperature of 4 °C ± 3 °C.

5.3 Phosphate buffer, 100 mmol/l; pH 7,8 ± 0,1.

The ionic composition and pH can affect the EROD activity, and optimal conditions may vary between species. The following buffer is expected to provide measurable and comparable values of EROD activity for most species of fish.

Prepare the following two solutions, A and B:

— Solution A: dissolve 17,4 g of K_2HPO_4 (relative molecular mass 174,2 as the anhydrate) in 1 l of water (5.1);

— Solution B: dissolve 13,6 g of KH_2PO_4 (relative molecular mass 136,1 as the anhydrate) in 1 l of water (5.1).

Adjust solution A to pH 7,8 ± 0,1 with solution B.

This solution is stable for 6 months at a temperature of 4 °C ± 3 °C.

5.4 Glycerol (20 %) in phosphate buffer solution.

Add a mass fraction of 20 % of glycerol ($C_3H_8O_3$; relative molecular mass 92,1) to the phosphate buffer (5.3). The final concentrations are then 20 % glycerol and 100 mmol/l phosphate.

5.5 Resorufin (108 mg/l) stock solution.

Dissolve in the dark, shaking for 2 h, about 10,8 mg of resorufin (sodium salt $C_{12}H_6NNaO_3$; relative molecular mass 235,2) in 100 ml of dimethylsulfoxide (DMSO). Read the optical density at 572 nm on the spectrophotometer. Calculate the exact resorufin concentration, c, in millimoles per litre, using Equation (1):

$$c = \frac{D}{\varepsilon l} \tag{1}$$

where

D is the optical density (corresponding to the absorbance wavenumber in cm^{-1});

ε is the molar extinction coefficient {for resorufin, values of $\varepsilon = 73{,}2$ $(mmol/l)^{-1}$ cm^{-1} at 572 nm (Reference [17]) and 54,0 ± 1,1 $(mmol/l)^{-1}$ cm^{-1} (Reference [36]) have been reported};

l is the optical pathlength, in centimetres.

Prepare this solution at the time of determination and store aliquots of this solution frozen at −20 °C and sheltered from the light. These aliquots can be stored for 6 months.

NOTE Resorufin is very unstable under daylight conditions.

5.6 Resorufin working solution.

Dilute the stock solution (5.5) with DMSO to obtain approximately 10 ml of 11,5 µmol/l working solution. Prepare this solution at the time of determination.

5.7 Rhodamine B standard solution.

Dissolve 25 mg of rhodamine B ($C_{28}H_{30}N_2O_3$; relative molecular mass 442,55) in 250 ml of ethylene glycol monomethyl ether. Dilute this solution with ethylene glycol monomethyl ether, so as to obtain a standard 0,1 µmol/l solution to be stored in aliquots. This solution remains stable for 6 months in the dark and at a temperature of 4 °C ± 3 °C.

5.8 Nicotinamide adenine dinucleotide phosphate (NADPH).

For the microplate method, dissolve 19,2 mg of β-NADPH ($C_{21}H_{26}N_7Na_4O_{17}P_3$; relative molecular mass 833,35) in 2 ml of water (5.1) to obtain a concentration of 10 mmol/l.

Prepare this solution at the time of determination and keep it sheltered from light in an ice bath.

NOTE For the cell (test tube) method, 41,7 mg of NADPH are dissolved in 1 ml of water (5.1) to obtain a concentration of 50 mmol/l.

5.9 7-Ethoxyresorufin stock solution

Prepare a stock solution of concentrated 7-ethoxyresorufin ($C_{12}H_6NaO_3$; relative molecular mass 235,17), e.g. 5 mg/ml, in DMSO. Store this solution in the dark at room temperature for a maximum of 1 year.

5.10 7-Ethoxyresorufin (46 µmol/l) working solution

Measure the exact concentration of the stock solution (5.9) by spectrophotometry at 482 nm using Equation (1). At 482 nm, the molar absorption coefficient is $2{,}25 \times 10^4$ $(mol/l)^{-1}$ cm^{-1}.

Dilute the stock solution (5.9) with DMSO in order to obtain a 46 µmol/l working solution for the microplate method. Prepare this solution at the time of determination.

NOTE A 400 µmol/l working solution is used for the cell (test tube) method.

5.11 β-Naphthoflavone dissolved in peanut oil

For the injection of a dose of β-naphthoflavone ($C_{19}H_{12}O_2$, relative molecular mass 272,3) into the fish at a mass per body mass fraction of 50 mg/kg (injection of 10 µl of solution into the oil per gram of fish), prepare a solution of β-naphthoflavone in peanut oil at a concentration of 5 mg/ml. This solution is shaken and brought up to a temperature of 45 °C ± 5 °C using a water bath in order to improve the homogeneity.

NOTE A dose even 10-fold lower than 50 mg/kg can be sufficient for EROD induction. It is possible, therefore, that the exact dose to be used is of little importance.

6 Apparatus

Usual laboratory and dissection equipment and in particular the following.

6.1 Cryogenic tubes.

6.2 Liquid nitrogen container or **freezer**, set at a temperature below –70 °C.

6.3 Cell or **microplate spectrofluorimeter**, for 96-well microplates.

NOTE The use of white opaque microplates allows a significant reduction of the fluorimetric background noise.

6.4 Centrifuge.

6.5 Homogeniser, of Potter Elvehjem or equivalent type.

6.6 pH meter.

6.7 Spectrophotometer.

7 Sampling and preparation of samples

7.1 Sampling of fish

Sampling should be carried out in the natural environment by fishing or in a laboratory on fish exposed to substances or effluents as specified in ISO 23893−1.

Sample at least 10 fish of the same species and sex and of uniform size from each group to be examined for EROD activity.

Do not take samples during the spawning season because the habitat of the fish and their physiological activities may be modified by sexual activity.

Taking into account the factors likely to influence EROD activity, the following conditions shall be determined and recorded in the test report:

a) water temperature;

b) a general description of the health condition of each fish (sex, length, body mass, liver mass, gonad mass, presence of external and internal injuries) — this is usually reported in connection with sampling as described in ISO 23893−1.

Depending on the objectives of the study, ensure that the control fish (from the reference location or laboratory group) are taken from an environment of satisfactory ecological quality. Handle the control fish and their samples in the same manner as those from the examined or experimentally treated groups, except for exposure to the substance(s) of concern.

7.2 Killing of fish and dissection

After fishing or on completion of exposure, the fish are killed and the livers dissected one by one on removal from the water as described in ISO 23893−1. Sample a piece of less than 1 g from the same part of the liver from each fish as soon as possible after killing it. Be careful not to contaminate the liver with bile during dissection.

7.3 Storage

If the determination cannot take place on the day of sampling, the samples of liver shall be frozen immediately to below −70 °C, for instance by using liquid nitrogen or dry ice. The samples can thereafter be stored for 3 months in liquid nitrogen or at a temperature below −70 °C (6.2).

If measurements of EROD are to be made on the day of sampling, then the preparatory step (8.1) shall be started within 1 h and the liver samples shall be stored at below 4 °C.

8 Procedure

8.1 Preparation of the fractions

Prior to conducting the determination, wash the liver sample in potassium chloride solution (5.2) and then homogenise it in 4 ml of phosphate buffer (5.3) by crushing in a homogeniser (6.5). If smaller (or bigger) liver samples are used, then the volume of the buffer should be reduced (or increased) to keep the 1 g:4 ml tissue:buffer ratio constant. Perform this operation at a temperature of about 4 °C by maintaining the

phosphate buffer at that temperature. Crushing shall occur within 0,5 min and 2 min, and the crushing conditions and time shall be identical for each sample. Adequate crushing results from five to 10 strokes for most liver samples.

Centrifuge the homogenates at 10 000g (about 90 000 m/s^2), at a temperature of 4 °C ± 3 °C for 10 min to 20 min. Recover the supernatant liquid (S9 fraction) and store it at a temperature of 4 °C ± 3 °C. Perform the enzyme determination within 1 h of supernatant recovery.

NOTE It is possible to freeze the liver homogenates rapidly in cryogenic tubes (6.1) to below −70 °C, using for example liquid nitrogen, under the conditions given in 7.3 provided that the liver samples (7.3) have not previously been frozen. In the case of prior freezing, the liver samples shall be homogenised beforehand as indicated above, but using a glycerol in phosphate buffer (5.4).

8.2 Determination of protein

For each S9 fraction, carry out a protein determination according to Reference [22] or Reference [2], for which methods commercial test kits are readily available. The concentration of protein standard [bovine serum albumin (BSA)] shall be checked using a spectrophotometer if in-house standards are used.

8.3 Determination of the EROD activity

8.3.1 Calibration

8.3.1.1 General

Carry out a calibration taking a resorufin working solution (5.6) or rhodamine B standard solution (5.7) as a basis. The fluorescence of rhodamine B is 1,2 times higher than that of resorufin at equivalent molar concentrations.

8.3.1.2 Calibration with resorufin

The linearity of the calibration curve shall be determined; samples should be measured in the linear part of the curve. Highly active samples have to be diluted to achieve this. The linear part of the curve is dependent on the apparatus used and its settings.

Establish the range by dilution of the resorufin working solution (5.6) with DMSO at the following dilution factors: 1; 5; 10; 50; 100.

Prepare the reaction mixture according to 8.3.2.1, replacing 7-ethoxyresorufin by resorufin and the S9 fraction by a protein solution (BSA or protein extract of the fish species under study). A concentration of protein which is as similar as practically possible to that of the S9 fractions being analysed should be used.

Measure the fluorescence according to 8.3.2.2. The measured fluorescence is proportional to the resorufin concentration. Ensure that only the linear part of the standard curve is used for quantification.

8.3.1.3 Calibration with rhodamine B

Introduce the rhodamine B standard solution (5.7) into the cell or into 3 wells of the microplate depending on the spectrofluorimeter (6.3) being used.

NOTE Taking into account the rhodamine B/resorufin intercalibration (under reaction mixture conditions), the rhodamine B solution is used as such as a standard. It is not necessary to add any reagent.

Measure the fluorescence according to 8.3.2.2. This fluorescence, divided by 1,2, is equal to the fluorescence of a resorufin solution having a concentration of 0,1 µmol/l.

8.3.2 Determination

8.3.2.1 Preparation of the reaction mixture

For each S9 fraction, prepare the reaction mixture by adding the solutions indicated in Table 1, always terminating with the NADPH solution, to a cell or microplate. Produce triplicates for the microplate method, and if possible also for the cell method.

Table 1 — Preparation of the reaction mixture

Solution	Volume of solution to be introduced into the		Concentration of solution		Final concentration in the reaction mixture
	Cell	Microplate	Cell	Microplate	
Phosphate buffer (5.3)	1,96 ml	200 µl	100 mmol/l	100 mmol/l	Approximately 100 mmol/l
7-Ethoxyresorufin (5.10)	10 µl	10 µl	400 µmol/l	46 µmol/l	2 µmol/l
S9 fraction (8.1)	20 µl	10 µl	5 mg to 10 mg of protein per ml	1,1 mg to 2,3 mg of protein per ml	50 µg to 100 µg of protein per ml
NADPH (5.8)	20 µl	11,5 µl	50 mmol/l	10 mmol/l	0,5 mmol/l

Record the time of addition of NADPH solution (5.8) as the start of the enzyme reaction. The reaction can either start directly or after 1 min to 2 min depending upon fish species. Finish recording after 2 min of the reaction period. The rate is determined from the reaction period, excluding the lag phase.

It is possible to mix the required quantity of 7-ethoxyresofurin into the phosphate buffer at the desired final concentration (2 µmol/l) before addition to the cells or microplate wells. For the calibration with resorufin (8.3.1.2), it is also possible to prepare the required quantity of phosphate buffer already containing the resorufin solution in the same way.

8.3.2.2 Measurement of the fluorescence

When a monochromatic spectrofluorimeter (6.3) is used, the fluorescence is measured at the maximum of the resorufin wavelengths, and when a filter spectrofluorimeter (6.3) is used, the fluorescence is measured at excitation and emission wavelengths from 530 nm to 535 nm and from 580 nm to 590 nm, respectively.

Calculate the slope of the time-dependent fluorescence increase at several measuring points using linear regression. If the spectrofluorimeter does not have this functionality, then calculate the slope on the basis of an initial and final point.

8.4 Checking the sensitivity of the biological reagent and compliance with the operating method

A check of the sensitivity of the batch of fishes used in the laboratory (positive control) is advised for each test of a substance or effluent concentration range. The number of fish to be induced with β-naphthoflavone (5.11) is identical to that used in the other experimental groups (at least 10). The exposure time shall be sufficient to allow an induction (e.g. 96 h).

Each fish is weighed and receives an intraperitoneal injection of β-naphthoflavone on the basis of its mass (e.g. 100 µl injected into a fish weighing 10 g).

For small fish, e.g zebrafish, water exposure to inducing agents (β-naphthoflavone dissolved in DMSO and test substances) can be used. Then 48 h exposure can be sufficient for induction.

For an injection of 50 mg/kg, a relatively high EROD induction is to be expected for the injected fish (positive controls) in comparison with the control fish (negative controls) and also compared with the inductions measured with most test substances and effluents or in most species of fish from the natural environment. For example, the induction rate (equal to the activity in fish injected with β-naphthoflavone divided by the activity in control fish) is usually around 10 (and always higher than 3) for cyprinids and in the region of 100 (and always higher than 30) for salmonids (Reference [11]).

9 Expression of results

9.1 Calculation of the EROD activity

For each S9 fraction, calculate the resorufin equivalent from the fluorescence slope of the sample. This fluorescence slope, in fluorescence units per minute, is related either to the resorufin range (8.3.1.2), by dividing the slope by the proportionality factor between fluorescence and resorufin, or to the rhodamine B standard (8.3.1.3). For each S9 fraction, calculate the EROD activity, A_{EROD}, in picomoles per minute per milligram of proteins, according to Equation (2):

$$A_{\mathrm{EROD}} = \frac{RV_{\mathrm{S9}}}{V_{\mathrm{r}}\rho} \tag{2}$$

where

R is the resorufin equivalent, in picomoles per microlitre per minute or nanomoles per microlitre per minute, as appropriate;

V_{r} is the total volume, in microlitres, of the reaction mixture;

ρ is the concentration, in milligrams per microlitre, of total proteins present in the S9 fraction;

V_{S9} is the volume, in microlitres, of S9 fraction present in the reaction mixture.

9.2 Statistical processing of the data

First test the distribution normality of the same batch (same fishing station or same concentration, same sex) and the homogenity of the variances (e.g. by a Levene test). A variable transformation may be needed to achieve this (References [1], [12]).

If the original or the transformed data are not normally distributed or do not have homogeneous variances, then use non-parametric tests (e.g. the Mann and Whitney U-test).

Then test the statistical differences between the EROD means of the different batches (ANOVA, the Student t-test), and in particular between the exposed batch and the laboratory or natural environment (field) control batch.

Present the results in graphical form and in table(s) (given as arithmetical or geometrical means for the different groups and with 95 % confidence intervals). Examples of laboratory and natural environment (field) results are presented in Annex A.

10 Test report

The test report shall contain at least the following information:

a) reference to this part of ISO 23893;

b) sampling date, water temperature, information about the fishing station (natural environment) or the batch of fish (laboratory), number of fish sampled (as specified in ISO 23893-1);

c) for each fish: size, mass, sex, gonadosomatic ratio, summary diagnosis of the health condition of the fish (as specified in ISO 23893-1);

d) for each fish, the EROD activity calculated according to 8.1;

e) values obtained on the negative control batch (unexposed fish) and the positive control batch (EROD-induced fish);

f) all operating details not specified in this part of ISO 23893, or considered as optional, together with details of any incidents which may have influenced the results.

Annex A
(informative)

Examples of results

A.1 Laboratory example

Rainbow trout (*Oncorhynchus mykiss*) juvenile fish were exposed for 96 h to a more or less concentrated urban effluent. In order to obtain a positive control, a batch of 10 trout was injected with β-naphthoflavone at a dose of 50 mg/kg. The raw data are listed in Table A.1 and statistical measures of the aggregated results for the different batches given in Table A.2.

Table A.1 — Raw data from the laboratory example

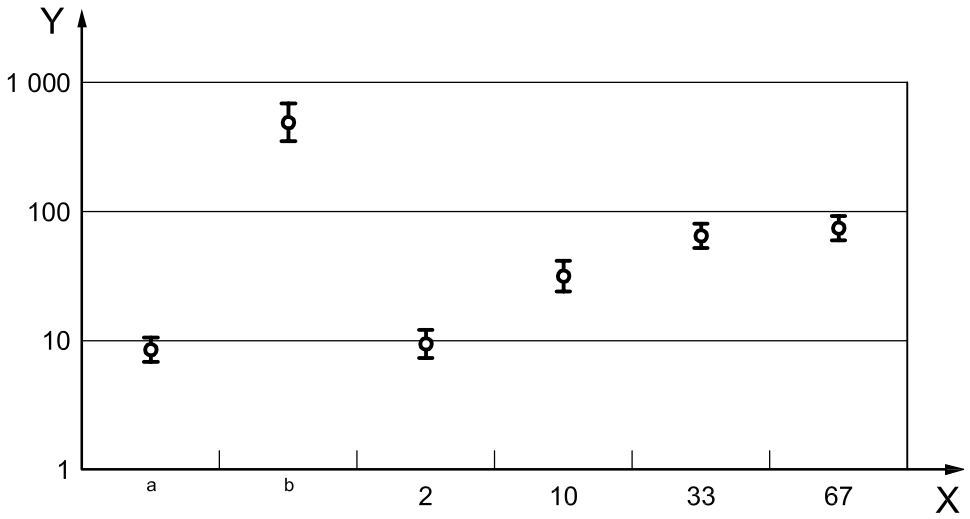
Code	Batch	Size	Weight	EROD
		mm	g	pmol/(min·mg) proteins
Trout001	negative control	86	7,9	11
Trout002	negative control	93	9,8	8
Trout003	negative control	90	9,4	7
Trout004	negative control	90	10,4	13
Trout005	negative control	102	12,8	9
Trout006	negative control	100	11,6	10
Trout007	negative control	100	12,7	9
Trout008	negative control	90	9,8	6
Trout009	negative control	91	8,6	5
Trout010	negative control	84	7,5	10
Trout011	positive control	98	12,1	205
Trout012	positive control	110	17	779
Trout013	positive control	92	9,7	285
Trout014	positive control	112	15,3	820
Trout015	positive control	105	13,7	730
Trout016	positive control	92	9,2	530
Trout017	positive control	98	10,8	616
Trout018	positive control	98	11,5	542
Trout019	positive control	93	11,7	347
Trout020	positive control	104	15,3	412
Trout021	2 % by volume effluent	100	11,7	8
Trout022	2 % by volume effluent	96	10,2	7
Trout023	2 % by volume effluent	102	12,3	10
Trout024	2 % by volume effluent	90	8,2	15
Trout025	2 % by volume effluent	82	6,7	15

Table A.1 *(continued)*

Code	Batch	Size mm	Weight g	EROD pmol/(min·mg) proteins
Trout026	2 % by volume effluent	92	9,7	14
Trout027	2 % by volume effluent	96	10,5	7
Trout028	2 % by volume effluent	92	9,8	6
Trout029	2 % by volume effluent	78	6,2	9
Trout030	2 % by volume effluent	92	10,4	9
Trout031	10 % by volume effluent	100	12	39
Trout032	10 % by volume effluent	92	9,7	26
Trout033	10 % by volume effluent	84	7,8	37
Trout034	10 % by volume effluent	100	12,5	26
Trout035	10 % by volume effluent	87	8,1	27
Trout036	10 % by volume effluent	110	14	40
Trout037	10 % by volume effluent	94	9,8	27
Trout038	10 % by volume effluent	92	9,5	22
Trout039	10 % by volume effluent	95	9,7	77
Trout040	10 % by volume effluent	102	12,7	25
Trout041	33 % by volume effluent	98	11,1	77
Trout042	33 % by volume effluent	86	7,9	68
Trout043	33 % by volume effluent	98	10,1	42
Trout044	33 % by volume effluent	92	8,7	83
Trout045	33 % by volume effluent	87	7,3	51
Trout046	33 % by volume effluent	94	9,5	80
Trout047	33 % by volume effluent	102	11,7	103
Trout048	33 % by volume effluent	94	9,6	46
Trout049	33 % by volume effluent	82	6,3	51
Trout050	33 % by volume effluent	87	7,3	68
Trout051	67 % by volume effluent	95	10,2	77
Trout052	67 % by volume effluent	87	8,4	59
Trout053	67 % by volume effluent	86	7,9	65
Trout054	67 % by volume effluent	105	14,6	102
Trout055	67 % by volume effluent	95	10	72
Trout056	67 % by volume effluent	102	12,9	49
Trout057	67 % by volume effluent	92	9,8	119
Trout058	67 % by volume effluent	104	13,2	69
Trout059	67 % by volume effluent	87	7,6	96
Trout060	67 % by volume effluent	100	11,3	62

Table A.2 — Statistical measures of the different batches from the laboratory example

Unit	Number of fish	Body length (mean ± SD) mm	Body mass (mean ± SD) g	EROD (mean ± SD) pmol/(min·mg) proteins	EROD geometrical mean (95 % confidence interval, CI95) pmol/(min·mg) proteins
Negative control	10	93 ± 6	10,1 ± 1,9	9 ± 2	8 (6, 11)
Positive control	10	100 ± 7	12,6 ± 2,6	527 ± 13	488 (347, 672)
2 % by volume effluent	10	92 ± 7	9,6 ± 2,0	10 ± 3	9 (7, 13)
10 % by volume effluent	10	96 ± 8	10,6 ± 2,1	35 ± 16	32 (24, 43)
33 % by volume effluent	10	92 ± 6	9,0 ± 1,8	67 ± 19	64 (51, 79)
67 % by volume effluent	10	95 ± 7	10,6 ± 2,4	77 ± 22	74 (60, 91)



Key

X effluent volume fraction, %

Y EROD [pmol/(min·mg) proteins] — logarithmic scale

[a] Negative controls.

[b] Positive controls.

Figure A.1 — Graphical representation of Table A.2 data

After conversion of the EROD data to logarithms to the base 10, the variances are homogeneous between the batches (Levene test). (See Figure A.1.)

The EROD activities of the positive controls are significantly greater than those of the control items (the induction rate between both batches is 488/8 = 61).

The EROD activities of the fish of the batch exposed to 2 % by volume effluent do not differ significantly from the EROD activities of the control fish (the Student t-test on the logarithms to the base 10 of the data gives $p = 0{,}43$). On the other hand, the EROD activities of the other batches are significantly greater than those of the fish of the control batch (the Student t-test on the logarithms to the base 10 of the data gives $p \leqslant 0{,}01$).

The effluent under test therefore induces the EROD activity in the trout as from a concentration of 10 % by volume in the water and for an exposure time of 96 h.

A.2 Natural environment example

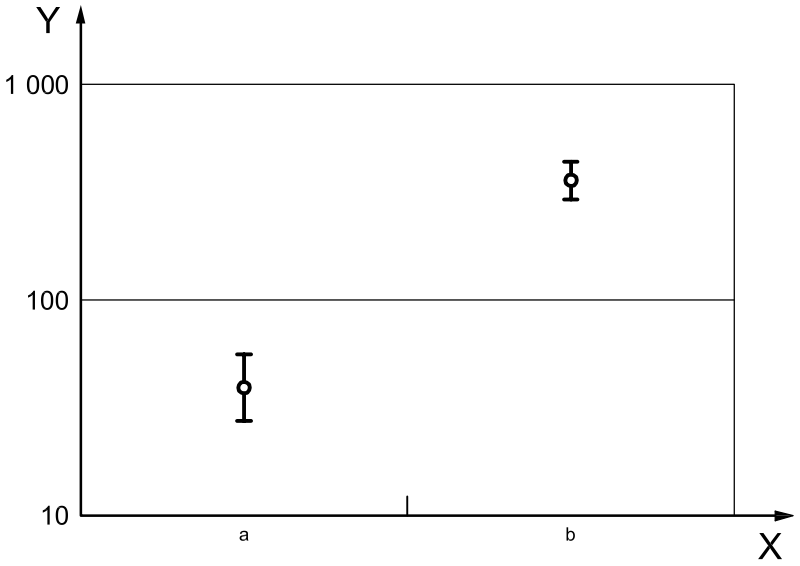
The natural environment example consists here in a comparison of the EROD activities measured in dab (*Limanda limanda*) sampled from two marine sites in the Seine bay: one barely contaminated site and one contaminated site. The raw data are listed in Table A.3 and statistical measures of the aggregated results for the different batches given in Table A.4.

Table A.3 — Raw data from the natural environment example

Code	Batch	Body length mm	EROD pmol/(min·mg) proteins
Dab001	barely contaminated site	265	58
Dab002	barely contaminated site	245	19
Dab003	barely contaminated site	235	49
Dab004	barely contaminated site	210	51
Dab005	barely contaminated site	240	31
Dab006	barely contaminated site	235	98
Dab007	barely contaminated site	235	27
Dab008	barely contaminated site	260	35
Dab009	barely contaminated site	230	25
Dab010	barely contaminated site	220	39
Dab011	contaminated site	290	396
Dab012	contaminated site	300	463
Dab013	contaminated site	290	372
Dab014	contaminated site	290	348
Dab015	contaminated site	290	483
Dab016	contaminated site	300	429
Dab017	contaminated site	260	344
Dab018	contaminated site	260	199
Dab019	contaminated site	290	330
Dab020	contaminated site	270	321

Table A.4 — Statistical measures of the different batches from the natural environment example

Unit	Number of fish	Body length (mean ± SD) mm	EROD (mean ± SD) pmol/(min·mg) proteins	EROD geometrical mean (95 % confidence interval, CI95) pmol/(min·mg) proteins
Barely contaminated site	10	238 ± 17	43 ± 23	39 (27, 55)
Contaminated site	10	284 ± 15	369 ± 82	358 (298, 446)



Key

X unit

Y EROD [pmol/(min·mg) proteins] — logarithmic scale

a Barely contaminated site.

b Contaminated site.

Figure A.2 — Graphical representation of Table A.4 data

After conversion of the EROD data to logarithms to the base 10, the variances are homogeneous between the batches (Levene test). (See Figure A.2.)

The EROD activities of the fish sampled from the contaminated site are significantly greater (the Student t-test on the logarithms to the base 10 of the data gives $p < 0{,}01$) than those of the fish sampled from the barely contaminated site (the induction rate between both batches is 358/39 = 9,2).

Bibliography

[1] BELIAEFF, B., BURGEOT, T. Sampling design optimization for EROD measurement in fish. *Mar. Ecol. Prog. Ser.*, 1997, **153**, pp. 239-46

[2] BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976, **72**, pp. 248-54

[3] BUCHELI, T.D., FENT, K. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Crit. Rev. Environ. Sci. Technol.*, 1995, **25**, pp. 201-68

[4] BURGEOT, T. L'ethoxyresorufine-*O*-deethylase, les adduits à l'ADN et les micronuclei dans les organismes marins: Application à la surveillance des effets biologiques sur les côtes françaises [Ethoxyresorufin-*O*-deethylase, DNA adducts and micronuclei in marine organisms: Application to biological effects monitoring along French coasts]. Doctoral Thesis, Nantes University, 1994, 253 p.

[5] BURGEOT, T., BOCQUENÉ, G., TRUQUET, P., LE DÉAN, F., GALGANI, F. Induction of EROD activity in red mullet (*Mullus barbatus*) along the French Mediterranean coasts. *Sci. Total Environ.*, 1994, **142**, pp. 213-20

[6] BURGEOT, T., BOCQUENÉ, G., PINGRAY, G., GODEFROY, D., LEGRAND, J., DIMEET, J., MARCO, F., VINCENT, F., HENOCQUE, Y., OGER, J.H., GALGANI, F. Monitoring biological effects of contamination in marine fish along French coasts by measurement of ethoxyresorufin-*O*-deethylase activity. *Ecotoxicol. Environ. Saf.*, 1994, **29**, pp. 131-47

[7] BURGEOT, T., BOCQUENÉ, G., TRUQUET, P., LE DÉAN, F., POULARD, J.C., DOREL, D., SOUPLET, A., GALGANI, F. The dragonet *Callionymus lyra*, a target species used for evaluation of biological effects of chemical contaminants on French coasts. *Mar. Ecol. Prog. Ser.*, 1993, **97**, pp. 309-16

[8] BURGEOT, T., MÉNARD, D., *Mesure de l'activité enzymatique: Ethoxyrésorufine–O–déethylase (EROD) chez les poissons marins* [Measurement of enzyme activity: Ethoxyresorufine–*O*–deethylase (EROD) in marine fish]. Editions Ifremer, Plouzané, France, 2004. 28 p. (*Méthodes d'analyse en milieu marin*, No. 7.)

[9] BURKE, M.D., MAYER, R.T. Ethoxyresorufin: Direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.*, 1974, **2**, pp. 583-8

[10] DAVIES, J.M., BELL, J.S., HOUGHTON, C. A comparison of the levels of hepatic aryl hydrocarbon hydroxylase in fish caught close to distant from North sea oil fields. *Mar. Environ. Res.*, 1984, **14**, pp. 23-45

[11] FLAMMARION, P. Mesure *in situ* de l'induction du cytochrome P450 1A chez des cyprinidés d'eau douce: Optimisation de l'interprétation [*In situ* induction of cytochrome P450 1A in freshwater cyprinids: Assessment optimisation]. Thesis, Metz University, 1997, 107 p.

[12] FLAMMARION, P., GARRIC, J. A statistical approach for classifying the extent of EROD induction of fish sampled in clean and contaminated waters. *Water Res.*, 1999, **33**, pp. 2683-9

[13] FLAMMARION, P., GARRIC, J., MONOD, G. Utilisation de l'activité enzymatique EROD chez les poissons des hydrosystèmes continentaux [EROD enzyme activity use in fish from continental hydrosystems]. In: LAGADIC, L., CAQUET, T., AMIARD, J.C., RAMADE, F., editors. *Utilisation de biomarqueurs pour la surveillance de la qualité de l'environnement* [Biomarker use for environmental quality monitoring], pp. 57-75. Lavoisier Tec & Doc, Paris, 1998

[14] FLAMMARION, P., MIGEON, B., GARRIC, J. Statistical analysis of cyprinids: EROD data in a large French watershed. *Ecotoxicol. Environ. Saf. Sec. B: Environ. Res.*, 1998, **40**, pp. 144-53

[15] GALGANI, F., BOCQUENÉ, G., LUCON, M., GRZEBYK, D., LETROUIT, F., CLAISSE, D. EROD measurements in fish from the Northwest part of France. *Mar. Pollut. Bull.*, 1991, **22**, pp. 494-500

[16] GALGANI, F., PAYNE, J.F. *Biological effects of contaminants: Microplate method for measurement of EROD activity in fish*. International Council for Exploration of the Sea, Copenhagen, 1991, 11 p. (*ICES Techniques in Marine Environmental Sciences*, No. 13.)

[17] GOKSØYR, A., FÖRLIN, L. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquat. Toxicol.*, 1992, **22**, pp. 287-312

[18] HODSON, P.V., KLOEPPER-SAMS, P.J., MUNKITTRICK, K.R, LOCKHART, W.L., METNER, D.A., LUXON, P.L., SMITH, I.R., GAGNON, M.M., SERVOS, M., PAYNE, J.F. *Protocols for measuring mixed function oxygenases of fish liver*. Mont-Joli, Québec: Fisheries and Oceans Canada, 1991. 49 p. (*Canadian Technical Report of Fisheries and Aquatic Sciences*, 1829.) Also available (2007-10-30) at: www.dfo-mpo.gc.ca/Library/125000.pdf. ©1991 Minister of Supply and Services Canada

[19] KLOTZ, A.V., STEGEMAN, J.J., WALSCH, C. An aryl hydrocarbon hydroxylating hepatic cytochrome P-450 from the marine fish *Stenotomus chrysops*. *Arch. Biochem. Biophys.*, 1983, **226**, pp. 578-92

[20] LAFAURIE, M., GIUDICELLI, J., CARRIERE, S., LEMAIRE, P., MATHIEU, A., NÈGRE, Y. Pollutant biotransformation in marine teleost fish: Use in environmental health evaluation. In: *Advances in applied biotechnology*, Vol. 5: *Impacts on human health and the environment*, pp. 141-52. Gulf, Houston, TX, 1989

[21] LINDSTROM-SEPPA, P., OIKARI, A. Hepatic xenobiotic bio-transformation in fishes exposed to pulpmill effluents. *Water Sci. Technol.*, 1988, **20**, pp. 167-70

[22] LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 1951, **193**, pp. 265-75

[23] MINCHIN, A., MCINTOSH, A.D., DAVIES, I.M. BEQUALM workshop on P4501A1 activity in fish: Report on the training workshop. FRS MLA Report N° 03/02, Fisheries Research Services, Aberdeen, 2002

[24] MONOD, G. Les biomarqueurs d'exposition. In: *Les variables biologiques*: *Des indicateurs de l'état de santé des écosystèmes aquatiques* [Biological variables: Some indicators of the state of health of aquatic ecosystems], pp. 145-8. Ministère de l'Environnement, Paris, 1997

[25] MONOD, G., DEVAUX, A., RIVIERE, J.L. Effects of chemical pollution on the activities of hepatic xenobiotic metabolizing enzymes in fish from the river Rhône. *Sci. Total Environ.*, 1988, **73**, pp. 189-201

[26] NARBONNE, J.F., GARRIGUES, P., RIBERA, D., RAOUX, C., MATHIEU, A., LEMAIRE, P., SALAUN, J.P., LAFAURIE, M. Mixed-function oxygenase enzymes as tools for pollution monitoring: Field studies on the French coast of the Mediterranean Sea. *Comp. Biochem. Physiol. C*, 1991, **100**, pp. 37-42

[27] PAYNE, J.F., FANCEY, L.L., RAHIMTULA, A.D., PORTER, E.L. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp. Biochem. Physiol. C*, 1987, **86**, pp. 233-45

[28] PAYNE, J.F., PENROSE, W.R. Induction of aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase in fish by petroleum. *Bull. Environ. Contam. Toxicol.*, 1975, **14**, p. 1126

[29] PLUTA, H.-J. International intercalibration and preliminary ICES standard protocol for measuring EROD activity in fish liver. *Z. Angew. Zool.*, 1995, **81**, pp. 85-111

[30] POHL, R.J., FOUTS, J.R. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem.*, 1980, **107**, pp. 150-5

[31] STAGG, R.M., ADDISON, R.F. An inter-laboratory comparison of measurements of ethoxyresorufin-*O*-deethylase activity in dab (*Limanda limanda*) liver. *Mar. Environ. Res.*, 1995, **40**, pp. 93-108

[32] STAGG, R., MCINTOSH, A. *Biological effects of contaminants: Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity*. International Council for Exploration of the Sea, Copenhagen, 1998, 16 p. (*ICES Techniques in Marine Environmental Sciences*, No. 23.)

[33] STEGEMAN, J.J., HAHN, M.E. Biochemistry and molecular biology of monooxygenases: Current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: MALINS, D.C., OSTRANDER, G.K. editors. *Aquatic toxicology: Molecular, biochemical and cellular perspectives*, pp. 87-206. Lewis, Boca Raton, FL, 1994

[34] STEGEMAN, J.J., TENG, F.Y., SNOWBERGER, E.A. Induced cytochrome P450 in winter flounder *(Pseudopleuronectes americanus)* from coastal Massachusetts evaluated by catalytic assay and monoclonal antibody probes*. Can. J. Fish. Aquat. Sci*., 1987, **44**, pp. 1270-7

[35] STEGEMAN, J.J., BROUWER, M., DI GIULIO, R.T., FORLIN, L., FOWLER, B.A., SANDERS, B.M., VAN VELD, P.A. Molecular responses to environmental contamination: Enzyme and protein systems as indicators of contaminant exposure and effect. In: HUGGETT, R.J., KIMERLE, R.A., MEHRLE, P.M., BERGMAN, H.L., editors. *Biomarkers: Biochemical, physiological, and histological markers of anthropogenic stress*, pp. 35-335. Lewis, Boca Raton, FL, 1992

[36] VAN DEN HEUVEL, M.R., MUNKITTRICK, K.R., STEGEMAN, J.J., DIXON, D.G. Second-round interlaboratory comparison of hepatic ethoxyresorufin-*O*-deethylase activity in white sucker *(Catostomus commersoni)* exposed to bleached-kraft pulp mill effluent*. Environ. Toxicol. Chem*., 1995, **14**, pp. 1513−120

[37] VIARENGO, A., LAFAURIE, M., GABRIELIDES, G.P., FABBRI, R., MARRO, A., ROMÉO, M. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. *Mar. Environ. Res*., 2000, **49**, pp. 1-18

[38] VINDIMIAN, E., GARRIC, J. Bio-essais et bio-indicateurs de toxicité dans le milieu naturel [Bioassays and toxicity bioindicators in the natural medium]. Ministère de l'Environnement, Paris, 1993, 53 p. (*Étude inter-agences*, No.17.)

[39] VINDIMIAN, E., NAMOUR, P., MIGEON, P., GARRIC, J. *In situ* pollution induced cytochrome P450 activity of freshwater fish: Barbel (*Barbus barbus*), chub (*Leuciscus cephalus*) and nase (*Chondrostoma nasus*). *Aquat. Toxicol*., 1991, **21**, pp. 255-66

[40] MONOD, G. L'induction du cytochrome P4501A chez les poissons. In: Lagadie, L., Caquet, T., Amiard, J.-C., Ramade, F., editors. *Biomarqueurs en écotoxicologie: Aspects fondamentaux* [Biomarkers in ecotoxicology: Fundamentals], pp. 33-54. Masson, Paris, 1997

[41] STIEN, X., RISSO, C., GNASSIA-BARELLI, M., ROMÉO, M., LAFAURIE, M. Effect of copper chloride *in vitro* and *in vivo* on the hepatic EROD activity in the fish *Dicentrarchus labrax*. *Environ. Toxicol. Chem*., 1997, **16**, pp. 214-9

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