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Water quality — Biochemical and physiological measurements on fish —

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

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

Partie 1: Échantillonnage des poissons, manipulation et conservation des échantillons

Reference number
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Foreword

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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.

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ISO 23893-1 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

Determination of biomarker responses can be used to detect toxicity of known as well as unknown pollutants, when they occur singly or in combination. Therefore, measurement of biomarkers is a cost-effective way to assess ecosystem health. In combination with determinations of occurring and suspected pollutants, determinations of biomarkers can facilitate the interpretation of cause-effect relationships in the environment, as well as in laboratory toxicity tests. Information on commonly used biomarkers and the interpretation of biomarker responses is given in Annexes A and B, respectively.

Biomarkers like ethoxyresorufin-*O*-deethylase (EROD), metallothionein and vitellogenin are used to detect and quantify sublethal effects of pollutants, especially in fish. However, many of the biochemical and physiological variables that are used as biomarkers are sensitive not only to disturbances by the pollutants of concern, but also by the normal biochemical and physiological adjustments made by the fish in response to seasonal variation, its normal development and sexual maturation. Some variables can also be affected by general stress to disturbances caused by the handling during fish and fish tissue sampling. Therefore, standardisation of procedures used for sampling and handling of samples prior to determination of the biochemical and physiological variables is important.

Sublethal responses at the individual level usually occur before effects are seen at the population and community level. In the aquatic environment, fish are suitable for detection of physiological effects of pollutants, because they are exposed both through the water and through their food organisms. Also, the physiology and biochemistry of fishes is rather similar to that of humans and other vertebrates, making comparisons with studies on mammals easier than for those with crustaceans and other invertebrates.

This part of ISO 23893 serves as guidance for sampling and a platform for determination of biomarkers in fish, making it possible to use the measurements to:

- describe the state of the environment regarding effects of anthropogenic compounds on the health of fish;
- perform time-trend surveillance (monitoring);
- provide reference data and material for assessment of effects from point sources;
- evaluate and assess environmental threats;
- provide background information for environmental measures;
- follow up and assess effects of environmental corrective measures;
- integrate the biomarker responses with other measurements (e.g. fish abundance, recruitment and pollutant residues) in order to facilitate the interpretation of environmental status or impact.

Water quality — Biochemical and physiological measurements on fish —

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

1 Scope

This part of ISO 23893 provides guidance on how to sample fish for determination of biochemical and physiological characteristics, such as the composition and enzyme activities of blood, liver, muscle and other tissues in order to asses the health of fish in the field as well as in the laboratory. The biochemical and physiological variables used for this purpose are often called biomarkers. This part of ISO 23893 includes recommendations and methods for:

obtaining a site-specific sample of a representative number of fish;

sampling fish tissues in the field and in the laboratory; and

handling and preservation of samples prior to analysis of biochemical and physiological variables.

2 Principle

Fish of a suitable species, age (size), and sex are sampled at selected sites at a suitable time of the year in order to reduce variability due to biological, geographical, and seasonal influences. Standardised sampling and measurement procedures, and qualified staff are used for collection of samples, transport, storage, and analysis. By these means, the results from time series of comparable data can be used to detect changes in the environment that are caused by anthropogenic compounds.

Necessary permits for fish and fish tissue sampling shall be obtained to comply with national legislation. This may include permits from the (land) owner of the fishing rights, regional environmental and fishery authorities, and ethical (animal rights) authorities.

The health of fish can be assessed by determination of biochemical, physiological, histological, and pathological methods. The subcellular and cellular variables are often called biomarkers. The primary toxic effect of pollutants, which occurs at the subcellular level, results in a biochemical or physiological change. This reaction is usually fast, and it can progress further and cause disturbances at higher levels of biological organisation within the organism, resulting in changes at the cellular and tissue (organ) level (histological changes). These can lead to disturbances of reproduction and growth, and can eventually cause death of the organism. Monitoring of fish health can, therefore, serve as an early warning system for anthropogenic disturbance. Through a combination with other measurements (integrated monitoring), it may be possible to correlate biomarker responses with for instance pollutant residues, distance from point sources, and ecological variables such as reproductive recruitment, which are known to be sensitive to pollutants.

In principle, this method can be applied to all species of fish from all types of environments (fresh, salt, brackish, cold, and warm water) and in shallow as well as reasonably deep water habitats. However, it is usually advantageous to restrict these methods to certain species of fish, which can be used as indicator species for fish health. These species shall be stationary, readily available (catchable in most locations) and reasonably resistant to handling stress. Their biology and physiology should be well known in order to make the interpretation of data easier. Examples of such species are the perch (*Perca fluviatilis*) and the eelpout (viviparous blenny, *Zoarces viviparus*), which are used for monitoring along the Swedish coast.

Preferably the fish species used in the field should be suitable for keeping in the laboratory for toxicological studies to investigate and confirm cause-effect relationships detected or suspected to take place in the field. Procedures for organ and tissue sampling are essentially identical in both field and laboratory studies. Procedures for collection of fish for field studies and for collection of organs are, therefore, described in separate sections.

3 Equipment

3.1 Fish sampling equipment

3.1.1 Fishing boat, suitable for the area.

3.1.2 Clothing for outdoor work.

3.1.3 Lifejacket, of suitable size and buoyancy for each crew member.

3.1.4 Gill nets, made from textile or nylon fibres and of specified and suitable size for catching the desired species and size and their gentle release into the fish chest used for storage.

3.1.5 Other equipment for fish capture, e.g. electroshocker and fyke nets, shall be described in enough detail to allow interpretation and repeated sampling.

3.1.6 Global positioning system (GPS) instrument, for exact location of sampling sites.

3.1.7 Nautical map, for marking of sampling sites.

3.1.8 Knife and **pair of scissors**, for gentle removal of fish from gill nets.

3.1.9 Fish chest, made from wood or other inert material for storage of fish before tissue sampling.

3.1.10 Instruments for measurement of physical and chemical characteristics of water, e.g. **thermometer, pH meter, conductivity meter**.

3.1.11 Equipment for determination of water depth, an echo-sounder or a calibrated line can be used to determine the depth.

3.2 Tissue sampling equipment

3.2.1 Jetty, with easy access to fish chest and within 100 m of the field laboratory.

3.2.2 Landing net, suitable for the fish species and size.

3.2.3 Field laboratory, boathouse, garage or mobile laboratory supplied with electricity.

3.2.4 Stick (baton), for stunning the fish prior to sampling of blood.

3.2.5 Anaesthetic, to anaesthetise the fish (details on usage are given in 4.4.3).

3.2.6 Dissection equipment: forceps, scissors, scalpel, syringes, needles.

3.2.7 Ruler, for determination of body length.

3.2.8 Balance, for determination of body mass and tissue (liver, gonad, spleen) mass.

3.2.9 Centrifuge and **tubes**, for blood plasma.

3.2.10 Microscope slides, for preparation of blood smears.

3.2.11 Sample containers, of suitable sizes for tissue samples (e.g. of plastic with snap locks).

3.2.12 Marking pen, waterproof and freezeproof.

3.2.13 Vacuum flask with liquid nitrogen, for rapid freezing and temporary storage of samples.

3.2.14 Container with solid carbon dioxide, for transport of deep-frozen tissue samples between field laboratory and analytical laboratory.

3.3 Biomarker determination equipment for the field laboratory

3.3.1 Haematocrit tubes and **centrifuge**, if required.

3.3.2 Blood glucose meter, if required.

3.3.3 Haemoglobin meter, if required.

4 Fish sampling

4.1 Statistical aspects

Feral fish, like other wild animals, are affected by a number of natural factors besides those caused by anthropogenic load. Important natural factors for fish are climate, hydrology, oxygen and salinity (abiotic factors), as well as age, size, sex, maturation, nutritional status, parasites and diseases (biotic factors). All these factors can contribute to the overall variability of the measured response variables. In order to detect temporal changes in trend monitoring and geographical variation in mapping of potential disturbance, all the abiotic and biotic factors mentioned above shall be reduced in importance as much as possible.

4.2 Frequency and season for sampling

Fish should be sampled once a year during the autumn period in order to avoid the effects of rapid changes in physiological conditions due to the reproduction season. During the autumn, most species of fish are not reproducing, and the conditions to get enough fish by stationary gear like gill nets (3.1.4) and fyke nets (3.1.5) are still good because the fish are still active. More frequent sampling at other times of the year does generally not provide any new information in trend monitoring.

In Sweden, perch for fish-health monitoring is sampled by gill nets in September, and eelpout by fyke nets in November. The most suitable period differs between countries and regions due to differences in climate. Often only sexually mature fish of one sex (e.g. females for perch and eelpout, and males for chub and zebrafish) within a certain size interval are used for each species in order to minimise the influence of sex and size.

4.3 Selection of sampling sites

In fish-health monitoring, it is of utmost importance to have as much detailed information as possible about the anthropogenic load on sites to be used as reference locations. These sites should be monitored regularly, preferably each year, in order to detect any large-scale impact from diffuse sources of pollution.

Fish-health monitoring can also be applied on a local scale. The locations of the sampling sites should then be determined by the objectives, which are usually related to the location of point sources of pollution. A suitable number of sites should be placed in a gradient from the local discharge point, or at sites which should be protected from disturbances. A reference site with a biotope, which is as similar as possible to the recipient, should also be selected.

Another aspect to be considered in the selection of sampling sites is availability of fish and reasonably easy access to the sampling site, or at least to the site where the fish is to be killed for taking the samples [the fish chest site (3.1.9)].

4.4 Sampling procedures

4.4.1 General

The number of fish should be sufficient in order to detect a predetermined change in the response variable within a certain number of years. An experienced statistician can give advice on this. It should also be considered that an additional number of fish to be sampled does not necessarily add much to the total cost of the monitoring programme. For example for perch and eelpout, 25 females each, with a total length of 20 cm to 30 cm, are sampled at each station in the Swedish monitoring programme. This number fulfils the statistical need for determination of differences between stations for all the monitoring response variables used in that programme. These are presented in Annexes A and B. If more stations are used, as in mapping of disturbance from a point source, a lower number (10 to 20) should be used at each station. By these means, more sites can be included at the same overall cost. The sex of the fish shall be determined and recorded, and a sufficient number of the sex to be used shall be sampled. For most variables, females are the preferred sex, but in some studies males should be used (e.g. for determination of vitellogenin in blood plasma).

4.4.2 Fish sampling

Fish can be caught by several methods (reviewed in Reference [3]) like angling and electric fishing gear (Reference [1]), if they are killed immediately on site, sampled directly and samples are handled appropriately. However, in most long-term monitoring programmes, adult fish are captured by gill nets (3.1.4), traps or fyke nets (3.1.5) in order to get a sufficient sample of fish of suitable size and sex.

In order to avoid unnecessary stress on the fish when they are caught and killed for tissue sampling, they should first be brought to a fish chest (3.1.9) and kept there for 2 days to 4 days before they are killed. This stabilises stress-sensitive response variables like blood glucose, blood lactate and haematocrit.

In the field, fishes should preferably be caught by gill nets or fyke nets and kept alive through frequent sampling carried out with the fishing equipment. However, other fishing techniques may also be used to collect fish. Reference to the method used or a detailed description shall be given in a report in such cases. The intention is that the fish are sampled from predetermined sampling sites by suitable fishing gear, e.g. gill nets for perch and fyke nets for eelpout. Gill nets shall be made from suitable material that facilitates the removal of fish with a minimum of damage. The mesh shall be adjusted to the species and size of the fish to be used in the study. For perch of 20 cm to 30 cm body length, a mesh size of 30 mm to 33 mm is suitable. The gill nets used for sampling of fish for population studies, as described in Reference [2], are multi-mesh gill nets, and these are not the same as the nets used in this part of ISO 23893. Ordinary fyke nets can be used to catch eelpout.

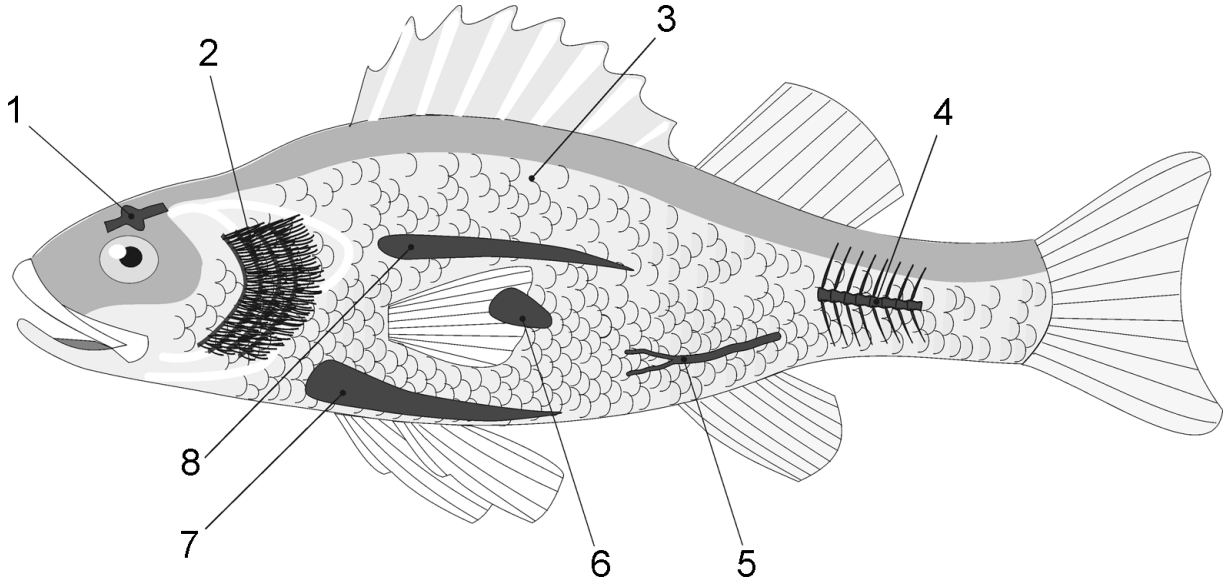
The nets should be set 3 days to 5 days before the fish tissues are to be sampled, in order to keep the fish in the fish chest for 2 days to 4 days prior to sampling the tissues. The gill nets shall be sampled frequently, and at least every 12 h, in order to collect as many live fishes as possible. They should be set at sunset and collected during sunrise. This also means that the laboratory staff sampling the tissues shall maintain contact with the local fishermen involved in the fishery to check that enough fish of suitable size is available before they arrive.

An example of a sampling form is given in Annex C.

4.4.3 Fish tissue sampling

The fish shall be collected from the fish chest (3.1.9) one by one by a landing net (3.2.2), taking care to disturb the remaining fish as little as possible. Tissue sampling shall be performed in a locality that is less than 100 m from the fish chest. The sampling locality (3.2.3) shall have electricity and adequate lighting, and be reasonably comfortable, so that the staff can operate safely and under suitable working conditions. A boathouse, a garage or a caravan is a suitable locality.

After its capture, the fish is either stunned by a blow on the back of the head with a wooden rod or a rubber baton (3.2.4) or anaesthetised with a suitable anaesthetic (3.2.5) such as MS-222 (tricaine methanesulfonate or ethyl 3-aminobenzoate methanesulfonate)[1)]. Then the samples should be taken in the following order: blood, bile, liver, spleen, muscle, gonads, and other tissues (see Figure 1).



Key

1 brain
2 gills
3 muscle
4 backbone
5 blood
6 spleen
7 liver
8 kidney

Figure 1 — Sampling of blood from caudal blood vessels using a heparinised syringe

If some tissues are not needed then just proceed to the next item.

1) The body mass is determined to the nearest gram and the total body length is determined to the nearest millimeter.

2) Blood is taken by a heparinised syringe from the caudal vessels (see Figure 1).

3) The fish is decapitated.

4) The body cavity is cut open, taking care not to damage the gall bladder.

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1) MS-222 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 23893, and does not constitute an endorsement of this product by ISO.

Tricaine methanesulfonate is probably the most widely used fish anaesthetic, even if it is rather expensive. A dilution of 1:1 000 is lethal in 5 min to 10 min. Other commonly used anaesthetics for fish are quinaldine (2-methylquinoline), for which a dilution of 1:20 000 is lethal in 5 min to 10 min, and benzocaine (ethyl 4-aminobenzoate) which can be used in the field by dissolving 0,2 g in 5 ml acetone (to facilitate solubility) and adding it to 8 l of water (Reference [11]). It should be noted that the use of anaesthetics may affect certain biomarkers.

5) Bile is sampled using a syringe (no heparin needed).

6) The liver is removed and weighed to the nearest 0,1 g.

7) The gonads are removed and weighed to the nearest 0,1 g.

8) The spleen is removed and weighed to the nearest 0,1 g.

9) The intestinal tract is removed and weighed to determine somatic mass.

10) A piece of muscle tissue is removed from a predetermined place.

11) Otoliths or other tissues (opercular bone, scales) suitable for determination of age are taken.

12) Samples of tissues for histological examination are put into vessels with preservative.

13) The remaining part is sometimes stored frozen for later chemical analysis or for additional biochemical or physiological analysis.

All tissue samples taken for biochemical measurements (liver and muscle) shall be processed further immediately by taking subsamples for different analyses. The subsamples shall have been taken from the same part of the organ for each variable to be analysed. This is crucial because enzyme activities may differ within an organ (like the liver). The anatomy of the liver may differ between species of fish. This may require standardisation within national and regional monitoring programmes in order to reduce variations due to micro-anatomical variation. It is especially important for the liver, which is often used for determination of several biochemical variables and also for histological examination.

Blood samples shall be processed directly to determine haematocrit by centrifugation, haemoglobin by spectrophotometry, and to produce blood plasma by centrifugation. Blood smears for differential counts of blood cells shall be prepared directly. Determination of blood glucose and lactate may be determined on site, or samples of blood for glucose and lactate may be subsampled and processed further in an analytical laboratory.

Samples of blood plasma, bile and other tissues shall be placed in labelled containers (3.2.11) and frozen directly in liquid nitrogen or in contact with solid carbon dioxide. Labelling of containers shall be tested to be sure that they withstand the freezing and processing of samples. Samples for histological examination and determination of fish age shall be treated as required by the methods that are used.

The entire procedure, from taking the fish from the fish chest to processing of all samples, should not exceed 10 min. This requires some training of the team, which normally consists of two, three or four persons, when all tissues mentioned above are to be sampled. When fewer tissues are to be sampled, the number of staff may be reduced.

Examples of variables for monitoring of fish health mentioned above are described in more detail in Annex A and an example of a sampling form is given in Annex D.

4.5 Handling of samples and analytical procedures

All samples, which are not determined on site, and which are to be used for biochemical and chemical analysis, shall be kept frozen below −70 °C until analysed. If samples are to be stored for longer periods (decades, centuries), then they shall be sent to a certified tissue bank. Samples to be used for histological analyses can be stored in a bank at a lower cost.

If available, standard analytical procedures shall be used, and the sampling and handling of samples should refer to this part of ISO 23893 and be supplemented with details pertaining to the specific samples.

Raw data for all information relating to sampling and analytical data can be stored together in a spreadsheet program which allows for easy electronic transfer.

4.6 Background information

The evaluation of the data is more appropriate if information on concentrations of pollutants and stock assessment of the fish species used is available. Also information on meteorological conditions and hydrology at the sampling site can facilitate the interpretation, especially in time series of data used for trend monitoring.

When used for mapping of effects from point sources, interpretation can be facilitated if comparative data from external reference sites are available. In this respect, the use of comparable data regarding the fish (species, size and sex) and sampling time is crucial.

5 Quality assurance

5.1 General

For the results to be reliable, several factors shall be considered. In order to use determinations of biochemical and physiological measurements on fish in assessments of ecosystem health and environmental monitoring, the following factors shall be addressed:

— control or knowledge of influence of natural factors on the results;

— use of a stratified sampling design which minimises the variability caused by natural factors;

— adequate statistical basis for interpretation of differences and trends;

— determination of a fixed and optimised sampling time during the year in relation to the variables of concern;

— use of standardised methods for sampling (capture) of fish and tissue sampling, as described in this part of ISO 23893.

There should preferably be one person (project leader) responsible for all steps in an investigation of fish health. This person should also be the one who is responsible for the interpretation in the primary report.

5.2 Fish sampling

Fish collection (the fishery) shall be performed by an experienced fisherman with good knowledge of gill net and fyke net fishery. He/she shall also have good knowledge of the area to be fished. The fisherman shall also be informed of the aim of the study, so that the fish are handled cautiously in order not to cause unnecessary damage.

5.3 Tissue sampling

Tissue sampling shall be performed by persons having the necessary skill and education for the task. Normally, two, three or four persons are necessary for sampling of all tissues from a single fish within 10 min. Training in sampling procedures should be done in advance, and routines for sampling, labelling, and transport of samples to the laboratory/laboratories for biochemical/chemical analyses shall be established and documented.

5.4 Biochemical/chemical analysis

Those analyses needed shall be performed by standardised or otherwise established methods. There shall be established routines for internal and external control of the laboratory work in order to guarantee the quality of the analytical data.

5.5 Evaluation

An evaluation of the results shall include a comparison with other similar studies. An in-depth knowledge of the literature in this area is therefore essential.

6 Report

6.1 General

Primary data from environmental monitoring are usually delivered to a data host, which is certified by an environmental authority. The data host describes how to deliver data (in spreadsheets electronically) and/or by mail. Data from environmental monitoring are also frequently reported at conferences and symposia and reported in scientific journals. This can guarantee that the results get a critical scientific evaluation.

Examples of reports for fish and fish tissue sampling are given in Annexes C and D, respectively.

6.2 Data logging, data hosting

The data should be delivered to the data host as primary data in formats suitable for statistical analyses.

6.3 Evaluation

Results are usually reported as mean values and standard deviation. The statistical evaluation usually includes parametric tests (ANOVA tests) or non-parametric tests if the data are not normally distributed. Data are usually compared with corresponding data for previous years and/or with other sampling sites, in order to establish differences and trends.

In studies on fish health, it is also essential to interpret functional disturbance, which can lead to a disturbance at the population level through effect on growth, reproduction, and/or survival. If indications of a disturbance at the individual level through biochemical and physiological variables have been found, this can lead to further investigation. A guide to the interpretation of various biomarkers in fish is given in Annex B.

Annex A
(informative)

Summary of variables used as biomarkers in fish

A.1 General

The following variables have been used as biomarkers in environmental studies on fish. Some of the methods for laboratory determination of these variables are also to be standardised in the future. References to scientific papers are given as information.

A.2 EROD activity in liver tissue

The excretion of lipid-soluble organic pollutants from fish and other vertebrates are facilitated by various enzyme systems, which convert lipophilic compounds into more hydrophilic compounds. Hydrophilic compounds can be more readily excreted in the bile or the urine. The first step in this detoxification is often catalysed by an enzyme system called cytochrome P450 monooxygenases (formerly also called mixed function oxidase, MFO). Cytochrome P450 exists in several forms. One of them, which is found in the liver of fish, is called cytochrome P450 1A. This enzyme is induced when the fish is exposed to substances like tetrachlorodibenzodioxins, planar polychlorobiphenyls (PCBs) and polyaromatic hydrocarbons.

The most commonly used method for determination of the activity of this enzyme and its induction is to determine the EROD (ethoxyresorufin-*O*-deethylase) activity. EROD activity have been used as a biomarker for both oil pollution and pulp mill effluents. In the latter case, effects have been detected as far as 40 km from the mill, which indicate the detection of large-scale or even regional effects.

A.3 White blood cells

Fish have several types of white blood cells. Those most studied are lymphocytes, neutrophilic granulocytes and thrombocytes. Lymphocytes are part of the immune system as producers and carriers of antibodies. Bacterial infections and other foreign objects activate neutrophilic granulocytes, which destroy them. Thrombocytes take part in the coagulation of blood.

Studies of blood cells in fish are a relatively easy method for detection of changes in the immune system. External factors, such as exposure to certain pollutants, can reduce the immunological defence system through a reduction of circulating lymphocytes. Changes in the number of white blood cells in fish blood have been seen in both laboratory and field studies with fish exposed to waste water from pulp mills and metal-processing industries.

A.4 Carbohydrate metabolism and stress

The most frequently studied effect of stress in fish is an increase of blood glucose and lactate and a decrease in liver and muscle glycogen. These are secondary effects from an increased secretion of stress hormones from the pituitary (adrenocorticotropic hormone) and adrenal and chromaffin tissue (cortisol and adrenaline) as a result of increased nervous activity. This is a natural response, which assists the animal in mobilisation of energy needed for attacking a rival, to catch a prey or avoid a predator. Many pollutants can also affect the carbohydrate metabolism in other ways than through secondary stress. Two examples of this are given below.

a) Changed contents of glycogen in liver and muscle have been seen in fish exposed to pulp mill effluents, and many chlorinated hydrocarbons [pentachlorophenol, PCBs, dichlorodiphenyltrichloroethane (DDT)] as well as some heavy metal species (cadmium, lead) can also affect the carbohydrate metabolism. The concentration of glycogen in liver and muscle tissue is measured enzymatically, and the method is fairly simple although time consuming.

b) Lactate in blood is the variable most sensitive to stress during sampling. Measurement of lactate is therefore often included in biomarker studies in order to be able to detect possible stress during sampling.

A.5 Concentration of ions in blood plasma

Teleosts (bony fishes) have well-developed mechanisms for osmotic and ion regulation. Therefore, the concentrations of inorganic ions are controlled within rather narrow limits. Sodium ion (Na^+) and chloride (Cl^-) are the major ions in blood plasma and they have a crucial role in osmoregulation. Also, other ions such as potassium (K^+), calcium (Ca^{2+}) and phosphate (PO_4^{3-}) are controlled within narrow limits. A disturbed ion and osmoregulation can reduce the ability of the fish to maintain its normal body functions.

Many pollutants, like metals and chlorinated hydrocarbons, can affect ionic regulation and thus the ionic concentrations in blood plasma. The concentration of calcium decreases as a result of exposure to cadmium, and exposure to pulp mill effluents can reduce the concentration of chloride.

A.6 Gonadosomatic index (GSI), liver somatic index (LSI), and liver total index (LTI)

The gonadosomatic index (GSI) is the mass of the gonads (ovary or testes) expressed as a percentage of the somatic mass. The somatic mass is the total mass minus the mass of the intestinal tract and the gonads. The normal development of the reproductive organs in fish is controlled by cellular and molecular processes, which are regulated by the endocrine organs and other factors. This regulation can be affected by pollutants, and this may also disturb the reproduction in fish.

A crude but reliable indication of disturbance of fish reproduction is change in the size of its gonads. Gonad size has been reduced in fish in waters close to pulp mills and in fish exposed to PCBs and DDT in the laboratory.

The liver somatic index (LSI) is the mass of the liver expressed as a percentage of the somatic mass. An increased LSI has been seen in fish exposed to organic pollutants both in the field (pulp mills) and in the laboratory (chlorinated hydrocarbons). This increase of LSI can be a result of an increased storage of fat or glycogen and/or a stimulated protein synthesis in the liver. The mechanism behind the increased LSI has not been elucidated, but metabolic disturbances and/or an increased activity of pollutant-metabolising enzymes are probable.

The liver total index (LTI) is the mass of the liver expressed as a percentage of the total mass of the fish.

Table A.1 lists the variables chosen as biological markers in environmental studies relating to fish.

Table A.1 — List of variables used as biomarkers in fish

Determinand	Object(s)	Unit	Determination
Total length	Fish body	mm	By ruler (±1 mm)
Total mass	Fish body	g	By balance (±1 g)
Sex	Male or female		M or F
Gonad mass	Gonads	g	By balance (±0,1 g)
Somatic mass	Body without viscera (intestinal tract and gonads) but with the liver	g	By balance (±0,1 g)
Somatic condition factor	Somatic mass and total length	g/mm	Somatic mass/total length
Gonadosomatic index (GSI)	Somatic and gonad masses	%	100 × gonad mass/somatic mass
Liver somatic index (LSI)	Liver and somatic masses	%	100 × liver mass/somatic mass
Liver total index (LTI)	Total and liver masses	%	100 × liver mass/total mass
Age	Operculum, otholith or scale	year	Cutting of tissue and counting of rings
Histopathology	Liver, spleen, etc.	% of control	Fixation, mounting, staining, examination
White blood cells	Blood	% of red blood cells	Blood smears are stained and differentially counted
Haematocrit	Blood	% (volume)	Centrifugation and measurement of packed cell volume
Haemoglobin	Blood	g/l or mmol/l	Field instrument or spectrophotometer
Blood glucose	Blood	mmol/l	Field instrument or spectrophotometer
Blood lactate	Blood	mg/100 ml or mmol/l	Field instrument or spectrophotometer
Bile toxicity	Bile	%	Bioassay
Muscle glycogen	Muscle sample at selected site	mg/100 mg (%)	Sample stored < −70 °C
Cytochrome P450	Selected liver piece	nmol/mg protein	Sample stored < −70 °C
Ethoxyresorufin-*O*-deethylase (EROD)	Selected liver piece	nmol/mg protein per min	Sample stored < −70 °C
Protein	Selected liver piece	mg/ml of sample	Sample stored < −70 °C
Glutathione reductase (GR)	Selected liver piece	nmol/mg protein per min	Sample stored < −70 °C
Metallothionein (MT)	Selected liver piece	nmol/mg protein	Sample stored < −70 °C
DNA adducts	Selected liver piece	nmol/mol	Sample stored < −70 °C
Vitellogenin	Blood plasma	ng/ml plasma	Sample stored < −70 °C
Plasma chloride	Blood plasma	mmol/l	Sample stored < −70 °C

Annex B
(informative)

Guide to interpretation of biomarker responses with references

B.1 Introduction

A group of scientists from Sweden has developed a strategy for investigating environmental impacts of current pulp and paper mill effluents. This strategy includes the use of a battery of variables, reflecting both population characteristics and physiological functions of fish. In addition, the group has given recommendations for interpretation of the results and specified criteria for defining unacceptable environmental impact (Reference [7]).

One part of the strategy is to use sublethal toxicity in fish to assess unacceptable disturbances on fish health and the fish population. This is done by selecting important biological functions in fish, which are not allowed to be affected in a negative way, and giving priority to biomarkers and health indicators, which reflect these functions. During the last two decades, biochemical, physiological and pathological variables have been very useful as sensitive instruments for detecting health effects in fish exposed to pulp mill effluents, both in controlled laboratory experiments and in feral fish living in receiving waters (References [1], [5], [6], [7], [8], [9], [11], [12]). However, one crucial issue is how to interpret and evaluate the biological significance of observed deviations from the control values for single variables, and how to make an integrated assessment of adverse impact on vital biological functions in fish and finally on the sustainability of fish populations.

The present interpretation guide for use of biomarkers/health indicators for environmental risk assessment is based on studies on pulp and paper mill effluents (Reference [11], in which a proposal is given for a systematic approach to assess unacceptable disturbances of biochemical and physiological functions in fish).

B.2 Effect variables

A set of priority variables, complemented with some supporting variables, are grouped under the following five vital physiological functions:

— growth, condition and energy metabolism;

— liver function;

— reproduction;

— immune defence;

— pathology and haematology.

The variables selected for indicating adverse impact on these functions are presented in Table A.1.

In addition, recommendations are given indicating how many variables are required to describe effects on one particular physiological function. If these variables show statistically significant deviations from control values, it can be concluded that the function is subject to an unacceptable adverse effect or disturbance. Moreover, criteria are given for interpretation of the integrated impact on fish health and on the survival of a fish population, when physiological functions are adversely affected. The guidelines were developed for environmental risk assessment of pulp and paper mill effluents, but with minor and suitable modifications in the selection of variables, they can also be applied to other types of industrial and to municipal waste water effluents.

B.3 Strategy for interpretation and biological evaluation

B.3.1 General

When taking into consideration the present insufficient knowledge about “normal values” for biochemical and physiological variables in various fish species, and the large statistical variation, which exists for several variables, it is impossible to make a reliable evaluation of the magnitude of deviation from normal values required for establishing a serious effect or an unacceptable disturbance. Instead, a better strategy for evaluation of the results obtained is to consider all statistically significant deviations from the control fish (at the level $p < 0{,}05$) as proven effects and to make an assessment of the total effect pattern based on a joint evaluation of several variables. In the final evaluation and assessment of the joint results from a particular study, it is, however, reasonable to take into account the magnitude of deviations for the different response variables.

It should be strongly emphasised that, before the interpretation of biochemical, physiological, and pathological responses is done, the impact of different environmental factors (water temperature, food supply, etc.) should be considered. In addition, it should be verified that the test fish has actually been exposed to the pollutants or the industrial effluents (e.g. by analyses of substances in the bile).

The biological variables/biomarkers are chosen with the intention of reflecting important vital functions in fish. Some of these functions, such as growth and reproduction, have a high ecological relevance. This means that a significantly disturbed reproduction or a reduced growth, observed in laboratory experiments or field studies, should be regarded as an unacceptable environmental impact because such effects are expected to have serious consequences at the population and ecosystem level. For the same reason, significantly increased frequency of pathological alterations (e.g. fin erosion, skin wounds, tumours, skeletal deformations and other malformations) are not acceptable. Significant changes in single measuring variables can be accepted if they are not supported by significant responses in other variables reflecting the same physiological function. Based on the use of a battery of health indicators in fish for assessment of sublethal toxicity, the following systematic model is proposed for defining unacceptable disturbance of vital physiological functions as well as unacceptable impact on the integral fish health and ultimately an unacceptable environmental impact.

B.3.2 Assessment of unacceptable disturbance of a biological function in fish

The responses in all variables representing one particular physiological function in Table A.1 are considered jointly as follows.

— If one or two variables deviate significantly ($p < 0{,}05$) from the control values, this should normally lead to further investigation. This means that complementary studies are required in order to confirm if the biological function is affected or not.

— If three or more variables in the same functional group are significantly affected, this should be interpreted as an unacceptable disturbance of the function.

— Deviating variables, listed under two functional groups (e.g. liver size, liver glycogen, liver lipids) in Table A.1, are assessed only in one group.

There are the following exceptions.

— Growth: a strongly reduced growth, which can be logically connected to other serious disturbances (e.g. altered metabolism, disturbed liver function, tissue damage) should be assessed as an unacceptable effect on the fish population.

— Reproduction: significant effects on more than one of the end points, sexual maturation and gonad growth, should be interpreted as an unacceptable disturbance of the reproduction. In addition, an impairment of the reproduction observed in a separate reproduction test (e.g. egg/larval test with rainbow trout, reproduction test with eelpout, hatching test with perch eggs) is sufficient to establish a disturbed reproduction even though no or only one reproduction variable in Table A.1 deviates significantly.

— Pathological changes: increased frequencies of pathological alterations of the types mentioned above should be interpreted as an unacceptable effect on fish health and as an unacceptable environmental impact.

B.3.3 Overall assessment of unacceptable disturbance of the integral health status in fish

In general, an unacceptable disturbance of one of the physiological functions listed in Table A.1 should lead to further investigation in order to determine if effects which reflect other biological functions can be related to and strengthen the observed disturbance.

An unacceptable disturbance of two or more physiological functions should be interpreted as an unacceptable disturbance of fish health and might imply an elevated risk for effects at the population and ecosystem level.

There is one exception: a disturbed reproduction, a strongly reduced growth or an increased frequency of serious pathological alterations should be interpreted as unacceptable effects on fish health and, thus, any one of these disturbances, singly, is sufficient to conclude that there might be unacceptable environmental impact.

B.4 Applications

The proposed interpretation guidelines and systematic model to assess environmental impact of pulp and paper mill effluents have been applied to results obtained in several fish investigations in the laboratory as well as in the receiving water of pulp mill effluents. These applications indicate that the developed strategy provides a good tool for making an integrated assessment of unacceptable disturbances of vital biological functions in fish and ultimately unacceptable impact on the sustainability of fish populations.

Annex C
(informative)

Suggested report for fish sampling

Location: _____ (e.g. Holmöarna, reference location in the Bothnian bay, Sweden)

Dates for fishery : XX-XX-XX--XX (yy-mm-dd-dd)

Fisherman (Name, address, phone number): _____

_____

Approximate total number of fish in the wooden fish chest: _____

Recorded mortality of fish during storage in wooden fish chest: _____

Sampling station	Sampling site	Latitude	Longitude	Water depth m	Sampling effort
1 V. Halörskatan	3	N63.41,30	E20.52,55	X m	2 gill nets
	9	N63.41,18	E20.52,60	X m	2 gill nets
	10	N63.41,05	E20.52,35	X m	2 gill nets
	11	N63.41,05	E20.52,65	X m	2 gill nets
	12	N63.40,96	E20.52,60	X m	2 gill nets
2 Ö. Halörskatan	13	N63.41,28	E20.54,10	X m	2 fyke nets
	14	N63.41,10	E20.54,05	X m	2 fyke nets
	15	N63.41,14	E20.54,35	X m	2 fyke nets
	16	N63.41,08	E20.53,90	X m	2 fyke nets
	17	N63.41,13	E20.53,75	X m	2 fyke nets

Location _____ Date (yy-mm-dd) _____

Signature _____

Annex D
(informative)

Suggested report for tissue sampling

Location:

_____

Dates of sampling (yy-mm-dd [to] dd):

Sampling team leader: Name

Address:

_____

Phone number:

Table D.1 — Tissue samples and results of measurements made during sampling

Variable, unit	**Sample or measured value**	**Fish number**									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Total length, mm	Measured										
Total mass, g	Determined										
Gonad mass, g	Determined										
Somatic mass, g	Calculated										
Somatic condition factor, g/mm	Calculated										
Gonadosomatic index (GSI), %	Calculated										
Liver mass, g	Determined										
Liver somatic index (LSI), %	Calculated										
Liver total index (LTI), %	Calculated										
Age, year	Operculum, otholit or scale										
Histopathology sample	Liver, spleen, etc. to fixation										
White blood cells	Blood smear										
Haematocrit, %	Determined										
Haemoglobin, g/l or mmol/l	Blood value										
Blood glucose, mmol/l	Blood value										
Blood lactate, mg/100 ml or mmol/l	Blood value										
Liver cytochrome P450 sample, ca 1 g [a]											
Liver EROD sample, ca 1 g [a]											
Glutathione reductase (GR) sample, ca 1 g [a]											
Metallothionein (MT) sample, ca 1 g [a]											
Liver DNA adduct sample, ca 1 g [a]											
Plasma vitellogenin sample, ca 0,5 ml [a]											
Plasma chloride sample, ca 0,1 ml [a]											

Table D.1 (*continued*)

Variable, unit	**Sample or measured value**	**Fish number**									
		11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Total length, mm	Measured										
Total mass, g	Determined										
Gonad mass, g	Determined										
Somatic mass, g	Calculated										
Somatic condition factor, g/mm	Calculated										
Gonadosomatic index (GSI), %	Calculated										
Liver mass, g	Determined										
Liver somatic index (LSI), %	Calculated										
Liver total index (LTI), %	Calculated										
Age, year	Operculum, otholit or scale										
Histopathology sample	Liver, spleen, etc. to fixation										
White blood cells	Blood smear										
Haemocrit, %	Centrifugation										
Haemoglobin, g/l or mmol/l	Field/lab. value										
Blood glucose, mmol/l	Field/lab. value										
Blood lactate, mg/100 ml or mmol/l	Field/lab. value										
Liver cytochrome P450 sample, ca 1 g [a]											
Liver EROD sample, ca 1 g [a]											
Glutathione reductase (GR) sample, ca 1 g [a]											
Metallothionein (MT) sample, ca 1 g [a]											
Liver DNA adduct sample, ca 1 g [a]											
Plasma vitellogenin sample, ca 0,5 ml [a]											
Plasma chloride sample, ca 0,1 ml [a]											

[a] Tissue sample deep frozen in liquid nitrogen (at −196 °C) and transported on solid carbon dioxide (at −78 °C); amounts are approximate and when several analytes are determined, the same original tissue sample may be utilised.

Location _____ Date (yy-mm-dd) _____

Signature _____

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