COMMISSION DECISION

of 16 May 1994

laying down the sampling plans and diagnostic methods for the detection and confirmation of certain mollusc diseases

(Text with EEA relevance)

(94/306/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 91/67/EEC of 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products [1], as amended by Directive 93/54/EEC [2] and in particular Article 15 thereof,

Whereas, in order to ensure the uniform application of the procedures laid down in Directive 91/67/EEC, it is necessary to establish sampling plans and diagnostic methods to be applied for declaring a coastal zone or farm free of diseases affecting molluscs, and for the examination of stocks where abnormal mortalities occur;

Whereas the Scientific Veterinary Committee established by Commission Decision 81/651/EEC [3] has been consulted;

Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

Article 1

The sampling and diagnostic methods for the detection and confirmation of bonamiosis *(bonamia ostreae)* and Marteiliosis *(marteilia refringens)* are laid down in the Annex.

Article 2

This Decision shall be applicable from 1 June 1994.

Article 3

This Decision is addressed to the Member States.

Done at Brussels, 16 May 1994.

For the Commission

René STEICHEN

Member of the Commission

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[1] OJ No L 46, 19. 2. 1991, p. 1.
[2] OJ No L 175, 19. 7. 1993, p. 34.
[3] OJ No L 233, 19. 8. 1981, p. 32.

ANNEX

I. SAMPLING AND TESTING PROCEDURES FOR THE MONITORING OF *BONAMIA OSTREAE* AND *MARTEILIA REFRINGENS* IN *OSTREA EDULIS*

1. Sampling

1.1. *Sampling points*

For each zone referred to in Annex B, point III of Directive 91/67/EEC, a number of sampling points shall be selected so as to maximize the chances of detecting *bonamia ostreae* and *marteilia refringens* where either or both are present. For that purpose, account shall be taken of parameters having an effect on the development of the pathogenic agents such as stocking density, water flows and the life cycle of the molluscs.

For a given zone at least three sampling points must be selected. The number of points shall be increased for large zones containing several discrete areas of cultivation of the susceptible species.

Whenever possible, at least one sample shall be taken from natural beds. Those molluscs showing abnormalities (abnormal growth, gaping shells) shall be selected.

1.2. *Sampling period and frequency*

The timing of the inspection referred to in Annex B under III B 2 of Directive 91/67/EEC is based on the period during which disease is manifest or detectable and inspections have to take place thereafter. The timing of the inspection shall also take account of the transfer of molluscs, which generally take place in spring and autumn. Therefore, sampling shall be carried out:

— once a year after the summer period for *marteilia refringens,*

— twice a year (spring/autumn) for *bonamia ostreae.*

1.3. *Sampling size*

During the initial two-year control period which preceeds the achievement of approved status, the sample size of each sampling point is 150 in order to ensure detection at a 95 % confidence level of pathogen carriers at a prevalence of 2 %.

During the subsequent years (maintenance of approved status), the sample size can be reduced to 30 to ensure detection at a 95 % confidence level of the pathogen carriers at a prevalence of 10 %.

2. Shipment of samples

All molluscs sampled must arrive at the approved laboratory within 24 hours after sampling. They must be packed in accordance with current standards in order to keep them in good condition. A label stipulating the place of sampling, the date of sampling and the history (if any) must be attached to the sample.

3. Macroscopic examination

The molluscs must be carefully opened so as not to damage the tissues, in particular the mantle, the gills, the heart and the digestive gland. Anomalies and lesions of the tissues shall be noted.

4. Preparation and examination of samples for bonamiosis

4.1 *Immunofluorescence*

4.1.1. Immunofluorescence can be carried out on larvae, small oysters and adult oysters. After drying of the samples, squash the larvae or make an impression smear of the heart tissue on a glass microscope slide. Dry in the air and fix by immersion in acetone for five to 10 minutes. These preparations can be used directly or be frozen at -20° C.

Prepare a solution of monoclonal antibodies in buffer solution (NaCl 8g/l, KH_2 PO_4 0,2g/l, Na_2 $HP0_4$ $12H_20$ 2,9g/l, KCI 0,2g/l, Azide Na 0,2g/l, Tween pH 7,4), using the dilution recommended by the supplier.

Pipette 50µl of the solution into each well or onto slides to cover the smears. After incubation in a humid chamber for 15 minutes at 20° C, wash the slides with the abovementioned buffer and then cover with a solution of goat anti-mouse lg G antibody (50µl per well) conjugated with fluorescence isothiocyanate. Dilute the antibody solution in accordance with the recommendations of the supplier and add Evans blue (1 %) to the buffer used for dilution.

After incubation in a humid chamber for 15 minutes at 20° C, the preparations are placed in a glycerine buffer and examined by U/V microscopy.

The presence of fluorescent green spherical cells confirms *bonamia ostreae* infection.

Other tests developed in individual laboratories and yielding comparable results may also be used.

4.2. *Blood smears*

For larvae and oysters, after the samples have been dried in air, make a squash for larvae or make an impression smear of the cardiac tissues on a histological slide. The slides are dried in air, and then fixed in methanol.

The prepared larvae and oysters are stained in accordance with the usual standard methods for the particular stain. After staining, rinse with tap water and allow to dry completely with cold or warm air and mount using a synthetic resin.

The parasite (two micrometres) is identified by its blue cytoplasm and red nucleus. It may be observed inside or outside the haematocytes. An observation time of five minutes per slide is sufficient.

4.3. *Histology*

In order to perform the histological examination, cut a section ('steak') of the oyster through the heart, digestive gland and gills and place the sample in a fixative liquid such as Davidson's, Bouin's or Carson's, the last mentioned enabling the samples to be used for electron microscopy when necessary. The volume of the sample-fixative volume ratio of one to 10 must be respected.

Several non-specific stains, e.g. Haematoxylin-Eosin or Masson's trichrome method, enable *bonamia ostreae* to be visualized.

These examples are not limiting and other staining methods may be used. It is recommended that two sections per oyster are examined.

The parasite (two micrometres) is found free in the connective tissue or in the haematocytes.

5. **Preparation and examination of samples for marteiliosis**

5.1. *Cytological examination*

In order to prepare the smears : cut a section through the digestive gland and the branchiae, remove the excess water by placing the sample on blotting paper, then make an impression smear on the slide using the section which passes through the digestive tract. The slides which have been dried in air are then fixed in methanol (two to three minutes).

The prepared samples are stained in accordance with the usual standard methods for the particular stain. After staining, rinse using tap water and allow to dry completely with cold or warm air and mount using a synthetic resin.

The parasite, the size of which is five to eight micrometres for the early development stages, may reach up to 40 micrometres during sporulation. The cytoplasm of the cells stains blue of a greater or lesser intensity, the nucleus being intensely red. The secondary cells or sporoblasts are surrounded by a bright halo. An observation time of five minutes per slide is sufficient.

5.2. *Histological examination*

For histological sections, cut a steak through the digestive gland using small scissors, and place the sample in a fixative liquid such as Davidson's, Bouin's or Carson's, the latter enabling the samples to be reused for electron microscopy when necessary. The ratio of the volume of the tissue to the fixative volume must be no more than one to 10.

The samples are subsequently handled in accordance with the classical histological methods. Several stains allow *marteilia refringens* to be observed, e.g. Haematoxylin-Eosin or Masson's trichrome. These examples are not limiting and other staining methods may be used. It is recommended that two sections per oyster be examined.

The young stages of *marteilia* are present in the epithelium of the stomach : later developed stages can be found in the epithelium of the digestive diverticulata. Free sporangia can also be observed in the lumen of the intestine.

II. EXAMINATION OF STOCKS OF *OSTREA EDULIS* IN WHICH ABNORMAL MORTALITIES OCCUR

Whenever abnormal mortalities occur in stocks of *ostrea edulis* an urgent investigation must be carried out to determine the etiology.

The size of the sample taken must consist of 100 individual oysters and must be handled in accordance with the procedure defined for histological analysis in Section I. This technique must be used initially, before any other type of examination is used.

The samples are fixed preferably in Carson's fixative which also allows reuse of the sample for electron microscopy.