Sample processing in the context of mollusc mortality events

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Editions

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Sample processing in the context of mollusc mortality events

1. Scope

This procedure explains the different steps of sample processing in the context of mollusc mortality events. A proper sample processing will allow performing main recommended analyses to investigate the presence of pathogens potentially associated with mortalities.

2. Procedure

2.1. Sampling

To optimise the chance of detecting pathogens, **gaping animals** (still alive, but usually showing difficulties to close their shells) should be sampled in priority. Decaying animals should not be collected. In case of mortality of infaunal molluscs such as clams or cockles, animals having a tendency to rise to the surface should be chosen in priority.
At least 30 individuals should be collected. If sampled animals are < 1.5 cm in size, the sampling size should be increased to at least 70 individuals.

Once sampled, molluscs should be **maintained at 0-8** °C, ideally no more than 24 hours, and in any case no more than 72 hours before analysis. Animals should be placed in a plastic bag with information about the species, the geographic origin and the sampling date. Gaping molluscs should be maintained separately from other molluscs.

2.2. Sample processing

Samples should be systematically processed to allow performing the following methods: histology, bacteriology and molecular biology (general protocol). Optionally, additional methods can be used in case of suspicion a certain pathogen(s).

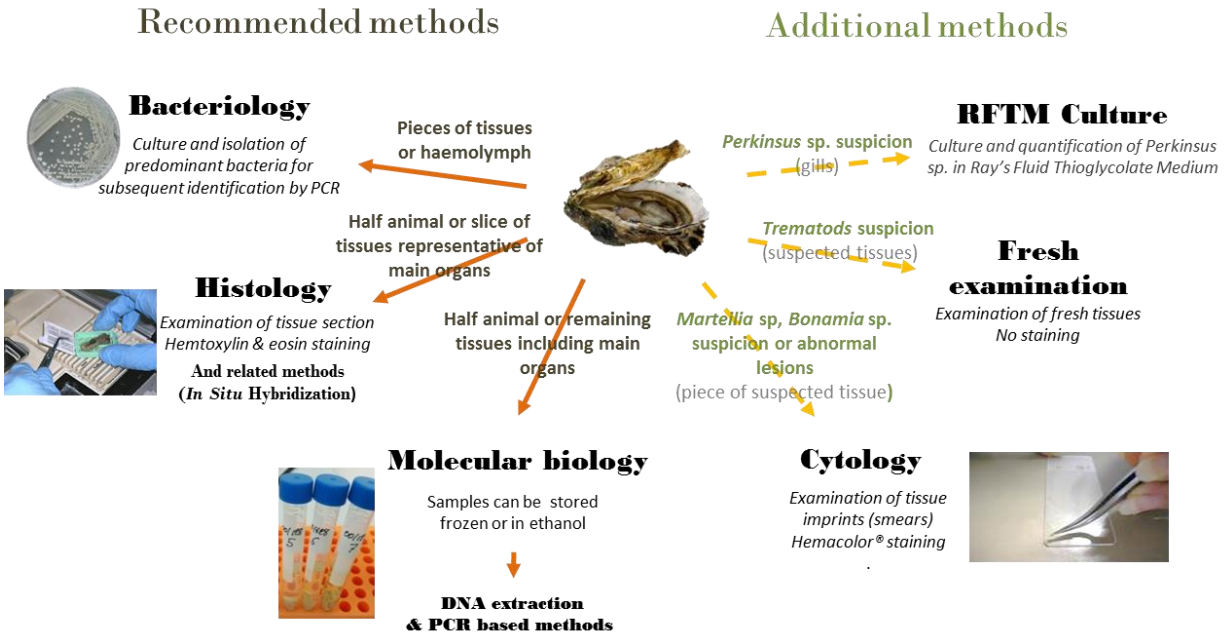

Fig.1: Main diagnostic methods used to investigate mollusc mortality events

Thirty animals should be stored for histology and molecular biology analysis, and 10 out 30 animals should be immediately processed for bacteriology. It is recommended to carry out the different analyses on the same individual. When animals are too small, each analysis is performed on different animals as follows: 10 animals analysed in bacteriology + 30 animals analysed in histology +30 animals kept for molecular biology = 70 animals.

2.2.1. General protocol

Sample processing depends on the size of sampled animals.

Individuals > 1.5 cm in size

- Open mollusc and quickly cut the adductor muscle(s) as close to the shell as possible.

- Look for any **macroscopic signs** that can be observed on the shell (for example: blister, boring sponge, brown ring, malformation, mud worm tunnel, pearl, pustules, scar) or on the soft tissues (for example: abscess, abnormal pigmentation, gill erosion, pustule, watery condition).

- **Bacteriology**: from the first 10 individuals, **take either:**
  - **around 10-50 mg of tissues including gills, mantle, adductor muscle and digestive gland** with scalpels, and crush tissues in 200µl of sterile seawater for bacterial culture.

  OR
  - **haemolymph** collected from the adductor muscle sinus or from the cardia cavity.

  Supernatant of crushed tissues or haemolymph should then be diluted (10^{-2} & 10^{-4}) and plated on Marine agar or Zobell on the day of sample reception. Tissue/ haemolymph sampling should be done with clean instruments under sterile conditions.
  Note: TCBS medium can be used to cultivate Vibrio bacteria, however it doesn't allow *V. aestuarianus* to grow.
  Refer to the SOP "isolation of predominant bacteria in marine mollusc" for further information.

- **Histology:** Gently remove the animal from the shell and place soft tissues on a paper towel. In case of adults, collect **slice of tissues including most of the organs** (Fig.2). In case of spat (1,5-3 cm), cut in half by sagittal plane (Fig. 3) and fix one half of the animal. Slice of tissues should never be thicker than 5 mm.

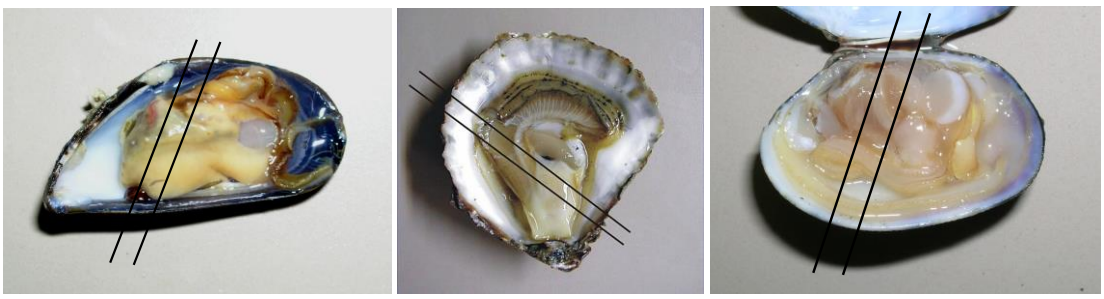

Fig 2: Example of slicing of different species of adult molluscs

Fig. 3: Spat slicing by sagittal plane

- Place animal slices in cassette and then in Davidson's fixative. This fixative is recommended for histological and *in situ* hybridization analysis; however it is also possible to use formaline 10% in sterile sea water or Carson fixative.
  Fixation should last 24h minimum and no more than a week. If samples can't be processed immediately, transfer the fixed tissues into 70% ethanol.

  Refer to the SOP "Molluscs processing for diagnosis by histology" for further information.

- **Molecular biology**: **store the remaining parts of each animal (or slice of tissues representative of most organs) individually** for further molecular biology analysis. Animal tissues can be stored:

  - In ethanol (70% or absolute ethanol)
  - Frozen at -80°C or -20°C

  Note: Storage at -80°C allows a better DNA storage for subsequent long fragment PCR and sequencing.

  Refer to the SOPs of each PCR assay for further information including the appropriate organs to be analysed.

For individuals < 1.5 cm in size (including small spat and larvae), the protocol is adapted as follows:

- **Bacteriology**: For larvae and spat smaller than 6 mm, several whole animals (including the shell) can be pooled and crushed together in seawater. Pools should correspond to approximately 50 mg of animals. For spat between 6 and 15 mm in size, whole animal soft tissues should be individually sampled and crushed in sterile seawater. Refer to the SOP "isolation of predominant bacteria in marine mollusc" for further information.

- **Histology:** fix one or several whole animals, with or without the shell depending of animal size (see Table 1).

- **Molecular biology**: store remaining animals for molecular biology. In case of larvae and spat smaller than 6 mm, DNA extraction can be performed from pools of 25-50 mg of several animals. Shells should be removed when possible to avoid PCR inhibition. In case of bigger spat (6-15 mm), whole animal soft tissues should be stored and processed individually.

Table 1 summarizes of the different animal processes according to their size.

Animal size	**Bacteriology**	**Histology**	**Molecular biology**	**Total number of animals required**
Larvae & spat < 6 mm	Fresh **pools** of 50 mg of **whole animals,** (including the shell)	Fixed **pools** of **whole animals** (including the shell)	Ethanol fixed or frozen **pools** of **whole animals** (remove the shell when possible) (Use 25-50mg of animal tissues per DNA extraction)	**Approx. 500 mg** of animals, Note: It is recommended to perform each analysis on a minimum of three pools
Spat 6-15 mm	Fresh **whole animal soft tissues**, processed **individually**	Fixed pool of one to five **whole animal soft tissues** per cassette. (For example: 6 pools of 5 animals)	Ethanol fixed or frozen **whole animal soft tissues,** stored and processed **individually.** (Crush each whole animal separately and use 25-50 mg of tissues per DNA extraction)	**70**[a]
Spat 15-30 mm	10-50 mg of fresh tissues representative of the following **organs**: gills, mantle, adductor muscle, and digestive gland, **or haemolymph**, processed individually	**Half animal** (sagittal section), processed individually	Ethanol fixed or frozen **half animals**, processed individually. (Use 25-50 mg of the appropriate organs per DNA extraction)	**30**[b]
Animal > 30 mm		**Animal slice** including most of the organs, processed individually	Ethanol fixed or frozen **remaining tissues of the animals**, processed individually. (Use 25-50 mg of the appropriate organs per DNA extraction)	**30**[b]

a: 10 animals analysed in bacteriology, 30 animals fixed for histology and 30 stored for molecular biology
b: all analyses are performed on the same animals, bacteriology is performed on the first ten animals.

Table 1: Sample processing according to animal size

2.2.2. Additional methods

Additional methods can be used depending on the situation.

Imprints
Cytology allows quickly having a first result and can be useful when some **lesions** are visible on animals (such as pustules), or in case of **suspicion of some parasites (*Marteilia* sp.**, ***Bonamia* sp.**, ***Mikrocytos* sp.** …).
Refer to the following SOPs for further information:

Diagnosis by histo-cytopathology of *Marteilia* spp. in the flat oyster *Ostrea edulis* and the mussels *Mytilus edulis* and *M. galloprovincialis*
Diagnosis by histo-cytopathology of *Bonamia* spp. in flat oysters *Ostrea* spp.
Diagnosis by histo-cytopathology of *Mikrocytos* sp. in oysters

Fresh examination
Examination of fresh tissues under the microscope allows the observation of metazoan parasites and is recommended in case of **suspicion of trematodes.**

Ray's Fluid Thioglycollate Medium (RFTM) method
The RFTM method is the method of choice in case of **suspicion of *Perkinsus* sp.** Pieces of gills, mantle and rectum are typically used for RFTM culture on bivalves, and pieces of gills, mantle, and foot for abalone.
Refer to the SOP "Quantification of *Perkinsus* sp. infection intensity using Ray's Fluid Thioglycollate Medium (RFTM) Method" for further information.

Transmission Electron Microscopy
Parts of **abnormal tissues** can be cut and fixed separately in glutaraldehyde (or in Carson's fixative if not possible) for latter Transmission Electron Microscopy (TEM) investigation.