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**Annex 56. Item 9.2.1. – Chapter 2.4.0. General information: diseases of molluscs**

Section 2.4.

Diseases of molluscs

CHAPTER 2.4.0.

general information

A. sampling

1. Assessing the health status of the epidemiological unit

1.1. Sample material to be used for tests

Sample material and the number of samples to be collected depend on the specific disease or pathogen, the size of animals and the objective of testing (i.e. surveillance of apparently healthy animals, ~~presumptive~~ suspect diagnosis of clinically affected animals or confirmatory diagnosis of a suspect result from surveillance or suspect~~presumptive~~ diagnosis). See individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

**RATIONALE:** The term “presumptive diagnosis” is only used in this section of this chapter, but the pathogen-specific manual chapters use the terms “suspect case” and “confirmed case”. We recommend being consistent with these terms used already throughout the manual chapters to not create confusion between “presumptive” vs. “suspect” diagnosis of a given pathogen.

1.2. Specifications according to mollusc populations

For details of animals to sample for a specific listed disease, see the relevant disease chapter in this *Aquatic Manual.* The design of a surveillance system for demonstrating disease-free status for a country, zone or compartment should be in accordance with the recommendations of the WOAH *Aquatic Code* Chapter 1.4. *Aquatic animal disease surveillance*.

The following factors should be considered when selecting animals to be sampled:

i) for apparently healthy populations, susceptible species should be sampled proportionately or following risk-based criteria for targeted selection of lots, epidemiological units or populations with a history of abnormal mortality or potential exposure events (e.g. stocking with animals of unknown disease status);

ii) If weak, abnormally behaving or freshly dead (not decomposed) animals are present, such animals should be selected. If such animals are not present, animals should be selected in such a way that all epidemiological units of the farm or waterbody are proportionately represented in the sample;

iii) if more than one water source is used for production, animals from all water sources should be included in the sample.

1.3. Specifications according to clinical status

In addition to sampling of target tissues, other organs showing macroscopic abnormalities or lesions should also be sampled. For disease outbreaks, at least ten diseased or moribund molluscs should be sampled for testing. Parallel samples (n > 10) from apparently normal animals in the same production region should also be collected. Collection of dead specimens during disease outbreaks should be avoided when possible, but recently dead samples may be suitable for some diagnostic assays provided the animals are not decomposed. Disease-specific recommendations are provided in Section 3 *Sample selection, sample collection, transportation and handling* of the individual chapters.

1.4. Specifications according to mollusc size

For the WOAH-listed diseases it is recommended that the scheduling of sampling be planned (i.e. by farm schedule, season, etc.) so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected.

1.4.1. For the listed parasites

**Juveniles below 1.5 cm:** sample the entire animal but remove the shell when possible or proceed with a decalcification protocol. When animals are too small for individual analyses, analyses can be performed on pools of several animals.

**Juveniles 1.5–3 cm:** sample the entire mollusc and cut in half sagittally. Keep one half of the animal for histological analyses and the other half for molecular analyses.

**Molluscs over 3 cm:** take a cross-section of the body, passing through the mantle, gills, digestive gland and gonads for histological analyses. Keep the remaining tissues for molecular analyses.

1.4.2. For infection with *Xenohaliotis californiensis*

For abalone ≥20 mm, excise several 3–5 mm cross sections containing posterior oesophagus (postoesophagus), digestive gland, and foot muscle.

1.4.3. For abalone herpesvirus infections

Sample as outlined in Section 1.4.2 above with the addition of a cross section of the head to obtain the cerebral ganglion and removal of several sections of the foot and adductor muscle complex including one section 0.25–1.0 cm (distance depends on abalone maximum length) posterior to the head to obtain the pedal ganglion. In addition, a longitudinal section from the anterior pedal ganglion to the posterior portion of the pedal musculature should be taken.

2. General processing of samples

Sampled molluscs should be delivered alive to the diagnostic laboratory. The laboratory should be informed of the estimated time of arrival of the sample so the required materials to process the molluscs can be prepared before receipt of the samples.

Mollusc samples should be packed appropriately in order to keep them alive. Required samples should be shipped as soon as possible after collection from the water. Unless otherwise specified, moribund animals should be sent on ice (but not frozen) to reduce sample decomposition.

For samples that cannot be delivered live to the diagnostic laboratory, specimens should be fixed on site as recommended in the following sections of this chapter or the relevant disease chapters of this *Aquatic Manual*. While this may be suitable for subsequent histology, transmission electron microscopy examination or PCR analyses for example, other techniques, such as fresh smears, tissue imprints, routine bacteriology, mycology or Ray’s fluid thioglycollate culture of *Perkinsus* spp., cannot be performed on such samples. Diagnostic needs and sample requirements should be discussed with the diagnostic laboratory prior to collection of the sample.

2.1. Macroscopic examination

The gross observation of molluscs should target, as far as possible, animal behaviour, shell surface, inner shell and soft tissues.

It is often difficult to observe the behaviour of molluscs in open systems. However, observation of molluscs in certain rearing facilities, such as broodstock in tanks and larvae in hatcheries, can provide useful indications of disease-related behavioural changes. If signs are noted (e.g. pre-settlement of larvae on the bottom, food accumulation in tanks, signs of weakening, etc.), samples may be examined for gross signs, including observation under a dissecting microscope for abnormalities and deformities, fouling organisms, and fixed for further processing as recommended below. For adults and juveniles, signs of weakening may include gaping, accumulation of sand, mud and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, decreased activity (scallop swimming, clam burrowing, abalone grazing), etc. The righting reflex of abalone after being inverted does not occur in weakened animals, and it is a good indicator of weakness. Mortality in open systems should be monitored for patterns of losses, and samples should be collected for further analysis. Environmental factors, pre- and post-mortality, should be recorded.

Even under culture conditions, the shells of molluscs may not be clean and fouling organisms are normal colonists of mollusc shell surfaces. Organisms such as barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, etc., do not normally threaten the health of molluscs. Culture systems, such as suspension and shallow water culture, can even increase exposure to fouling organisms and shells may become covered by other animals and plants. This can affect health directly by impeding shell opening and closing or indirectly through competition for food resources. Signs of weakening associated with heavy fouling should be a cause for concern rather than fouling itself. Shell damage by boring organisms, such as sponges and polychaete worms, are usually benign, but under certain conditions may reach proportions that make the shell brittle or pierce through to the soft tissues. This degree of shell damage can weaken the mollusc and render it susceptible to pathogen infections. Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but may not be indicative of a disease concern. Burrowing epibionts may cause deformities and weaken the shell(s). Abnormal coloration and smell may indicate a possible soft-tissue infection that may need to be examined at a laboratory.

The molluscs should be opened carefully so as not to damage the soft tissues, in particular the mantle, gills, heart and digestive gland. The presence of fouling organisms on the inner shell surface is a clear indication of weakness. The inner surface of the shell is usually smooth and clean because of mantle and gill action. Perforation of the inner surface may occur but can be sealed off by the deposition of additional conchiolin and nacre. This may result in formation of mud- or water-filled blisters. Blisters may also form over superficial irritants such as foreign bodies. The degree of shell perforation can be determined by holding the shell up to a strong light. Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be brought intact to the laboratory or fixed for subsequent decalcification, as required. The appearance of the soft tissues is frequently indicative of the physiological condition of the animal. Soft tissues should be examined for the presence of abscess lesions, pustules, tissue discoloration, pearls, oedema, overall transparency or wateriness, gill deformities, etc., and, when found in association with weak or dying animals. Abnormalities and lesions of the tissues should be noted and recorded, as well as any shell deformities, shell-boring organisms and conspicuous mantle inhabitants. Levels of tissue damage should be recorded and samples of affected and unaffected animals collected for laboratory examination as soon as possible.

2.2. Virological examination

See Chapter 2.4.1. Infection with abalone herpesvirus for specific details.

2.3. Bacteriological examination

See Chapter 2.4.7. Infection with *Xenohaliotis californiensis* for specific details.

2.4. Parasitic (protists) examination

See Chapters 2.4.2 to 2.4.6. Infections with listed protists for specific details.

2.5. Fungal examination

Not applicable for currently listed diseases.

B. MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF MOLLUSC PATHOGENS

1. Mollusc viruses

1.1. Mollusc cell lines

Not applicable. There are currently no confirmed or documented mollusc cell lines suitable for virus isolation.

1.2. Culture media

Not applicable.

1.3. Virus positive controls and antigen preparation

1.3.1. Virus nomenclature

In general, the virus nomenclature used in the disease-specific chapters follows the most recent taxonomy for viruses as given in the Report of the Committee on Taxonomy of Viruses (see: [ICTV [ictvonline.org]](https://talk.ictvonline.org/ictv-reports/ictv_online_report/) for latest information).

1.3.2. Virus production for experimental purposes

As no cell lines are known that can be used to produce mollusc virus stocks, infection of known susceptible host species (which are free of infection with the pathogenic agent in question) is the preferred method for virus production for experimental purposes or for the production of positive control material.

1.3.3. Virus preservation and storage

Infectivity of all of the WOAH-listed mollusc viruses can be preserved by freezing infected whole molluscs or infected target tissues at –20°C for short-term storage, or at –80°C or lower for long-term storage.

2. Mollusc bacteria

Not applicable. There is currently no developed procedure to cultivate *Xenohaliotis californiensis*.

3. Mollusc parasites (protists)

3.1. Culture media

See Chapters 2.4.5 Infection with *Perkinsus marinus* and 2.4.6 Infection with *Perkinsus olseni* for details.

3.2. Storage of cultures

*Perkinsus* spp. cultures in the exponential phase of growth can be pelleted by centrifugation and cryopreserved by resuspending the pellet in 40% DMEM Ham’s F-12 (1:1) culture medium with 10% glycerol and 50% FBS and freezing them using standard procedures.

4. Mollusc fungi

4.1. Culture media

Not applicable for currently listed diseases.

4.2. Storage of cultures

Not applicable for currently listed diseases.

5. Techniques

The available diagnostic methods that may be selected for diagnosis of the WOAH-listed mollusc diseases or detection of their aetiological agents are based on:

i) Gross and clinical signs.

ii) Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears.

iii) Histology, *in-situ* hybridisation and electron microscopy of fixed specimens.

iv) Culture methods where applicable.

v) Molecular methods (including sequencing): Conventional and real-time PCR and LAMP for direct assay with fresh, frozen or ethanol fixed-tissue samples or with extracted DNA.

Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) may also be used as an indicator for the presence of the pathogen.

Pooling of samples from more than one individual animal for a given purpose is only recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger molluscs should be processed and tested individually. However, for eggs, larvae and postlarvae, pooling of individuals may be necessary to obtain sufficient sample material to run a diagnostic assay.

5.1. Gross and clinical signs

Macroscopic examination of gross and clinical signs reveals non-specific signs only (e.g. gaping in bivalves or general weakness of the foot muscle in abalone), and mortality may be caused by several disease agents or physiological problems, such as loss of condition following spawning. To obtain a definitive diagnosis further investigation is required and this can only be determined using a range of other techniques including histology/electron microscopy and molecular techniques such as PCR and gene sequence analysis.

5.2. Direct microscopy

Samples for direct microscopic examination should be examined as soon as possible after collection. Use live specimens whenever possible, or use fresh, chilled, or fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection.

5.3. Histological techniques

Live moribund animals or freshly dead (within minutes) animals provide the optimal tissues for examination. Due to tissue lysis that occurs during the freeze-thaw cycle, frozen samples are not appropriate for histology. Should a delay between animal mortality and sampling occur, it is recommended that animals be stored intact on ice or in a refrigerator.

To obtain a sample that includes all the major tissues, a section should be taken to include digestive gland, gills, gonad, mantle and palps, where possible. For large specimens, it may be necessary to take several sections to include all the important tissues. Tissue preparation for examination by light microscopy involves several steps, including tissue fixation, dehydration, impregnation and embedding of samples, preparation of sections, staining and mounting of slides.

5.3.1. Tissue fixation

Tissue fixation is required to maintain the morphology of the tissues and to prevent post-sampling necrosis. Recommended fixatives used for the study of marine molluscs are Davidson’s solution, Carson’s solution and 10% formalin in filtered sea water. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation.

*Davidson’s solution:*

1 µm filtered sea water 1200 ml

95% Alcohol 1200 ml

35–40% Formaldehyde[[1]](#footnote-2) 800 ml

Glycerol 400 ml

Glacial acetic acid 10% (add just prior to use)

*Carson’s solution:*

NaH2PO4.2H2O 23.8 g

Sodium hydroxide (NaOH) 5.2 g

Distilled water 900 ml

40% Formaldehyde1 100 ml

Adjust the pH to 7.2–7.4

*10% formalin in filtered sea water solution:*

1 µm filtered sea water 900 ml

35–40% Formaldehyde1 100 ml

These solutions allow tissue structure to be preserved and different histochemical methods to be used including for *in-situ* hybridisation with DNA probes. Over-fixation (over 24–48 hours) should be avoided. After fixation, the specimens should be transferred to 70% ethyl alcohol, where they can be stored indefinitely. Davidson’s solution is normally used because it provides better preservation of the cell nuclei. Carson’s solution or 10% formalin in seawater can be used to examine tissues by electron microscopy. As electron microscopy can be a valuable aid in diagnosing or confirming infections in bivalve molluscs, fixing some samples (particularly the smaller ones) with glutaraldehyde, as described in Section B.5.4.1 of this chapter, may be considered, and will provide electron micrographs of the highest quality. It is recommended that a representative portion of the mollusc is fixed in Davidson’s solution, while another representative portion is fixed in Carson’s solution for further examination to ensure that all tissues/organs are fixed in both fixatives. If neither is available, 10% formalin buffered with filtered seawater will suffice.

For transport and shipping, see *Aquatic Code* Chapter 5.10 *Measures concerning international transport of aquatic animal pathogens and pathological material*.

5.3.2. Dehydration, impregnation and embedding of the samples

The fixed samples are transferred through a series of graded alcohols (70–95% [v/v]) before final dehydration in absolute ethanol. The alcohol contained in the tissues is next eliminated by immersing them in xylene. The tissues are then impregnated with paraffin, which is soluble in xylene, at 60°C. These steps are often carried out automatically using a tissue processing machine. Should processing be delayed, fixed tissues may be stored in 70% ethanol.

Histological blocks are produced by letting the tissues cool in moulds filled with paraffin on a cooling table.

5.3.3. Preparation of the sections

After the blocks have cooled and the paraffin has solidified, histological sections of about 2–5 µm are cut using a microtome. The sections are recovered on histological slides, drained and dried for up to 1 hour at 40–42°C or overnight at room temperature.

5.3.4. Staining and mounting the slides

Before staining, the paraffin is removed from the sections by immersing them in xylene or equivalent clearing solution for 10–20 minutes. This is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10-minute periods each, and they are then rehydrated through a descending series of ethanol baths (for example 95%, 70%, 50%, 30%, 10 minutes each) with a final immersion in tap water for 10 minutes. Different topographical or histochemical staining techniques can then be performed.

When haematoxylin–eosin (H&E) stain is used (haematoxylin or equivalent), nuclear and basophilic structures stain a blue-to-dark-purple colour, the endoplasmic reticulum stains blue, while the cytoplasm takes on a grey colour. The acid dye eosin stains the other structures pink. This staining technique is simple and reproducible and, although it only allows a limited differentiation of cell structures, it is possible to detect any abnormalities in tissue and cellular structure. Other techniques may be applied to demonstrate particular structures or features as required (e.g. trichrome for connective tissue and cytoplasmic granules).

5.4. Transmission electron microscopy methods

Transmission electron microscopy can be used as part of the diagnostic procedures for diseases of molluscs.

Fixation for electron microscopy should be done immediately after the animal has been killed and before fixation for histology. Only samples taken rapidly from live animals will be of any use. The preparation of samples for electron microscopy involves the following steps: tissue fixation, decalcification of the samples (when necessary), dehydration, impregnation and embedding of the samples, preparation and counterstaining of the sections.

5.4.1. Tissue fixation

For tissues that are to be examined by electron microscopy, it is important that the fixation be performed correctly in order to cause as little damage as possible to the ultrastructure. The specimens are cut such that their dimensions do not exceed 1–2 mm. This small size allows rapid penetration of the various solutions into the tissue sample.

Fixation is carried out directly in 3% glutaraldehyde for 1–4 hours. The samples are washed in buffer three times, then post-fixed in 1% osmic acid (aqueous OsO4) and washed twice again in buffer. Various formulations of glutaraldehyde fixative and buffers work equally well.

In order to cause as little damage as possible to the ultrastructure, the samples are treated with solutions that have an osmolarity close to that of the tissues. Thus, mollusc tissues are treated with solutions with an osmolarity of approximately 1000 mOsm. The osmolarity of the solutions is adjusted with artificial sea salts or NaCl. Alternatively, the glutaraldehyde can be formulated with 0.22 µm filtered seawater, and filtered seawater used for subsequent washes.

Sodium cacodylate 0.4 M: 8.6 g in 100 ml of distilled water

Sodium chloride 10% in distilled water

*Cacodylate buffer, pH 7.4:*

1000 mOsm

Sodium cacodylate 50 ml from 0.4 M stock solution

NaCl 20 ml from 10% stock solution

Distilled water 30 ml

Adjust the pH to 7.4

*3% Glutaraldehyde:*

1000 mOsm

25% glutaraldehyde 2.5 ml

0.4 M sodium cacodylate 5 ml

10% NaCl 3.5 ml

Distilled water 9 ml

*1% Osmic acid:*

1000 mOsm

4% Osmic acid 1 volume

0.4 M sodium cacodylate 1 volume

NaCl 1 volume from 10% stock solution

Distilled water 1 volume

*5% ethylene diamine tetra-acetic acid* (*EDTA*)*:*

Disodium EDTA 5 g

Cacodylate buffer 100 ml

EDTA dissolves when the pH is above 8. When the solution becomes clear adjust the pH to 7.4 by adding concentrated HCl.

If the samples have been previously fixed and stored in Carson’s solution, they should be washed several times in a bath of buffer before fixation with 3% glutaraldehyde.

5.4.2. Dehydration, impregnation and embedding of the samples

The samples are dehydrated in successive baths of ethanol: 70% ethanol once, 95% ethanol twice, absolute ethanol three times. The dehydration is completed by two baths of propylene oxide, which allows subsequent impregnation with Epon.

The samples are impregnated progressively. After a first bath in a mixture of polypropylene oxide–Epon (50/50), the samples are placed in a bath of Epon. The longer the incubation, the better the impregnation of the tissues.

Embedding is carried out by placing the samples in moulds filled with Epon resin. A label identifying the sample is included in each block and the blocks are then placed at 60°C (the temperature at which Epon resin polymerises) for 48 hours.

5.4.3. Preparation of the sections and the counterstaining

The blocks are cut to appropriate sizes with a razor blade and, using an ultra- microtome, semi-thin sections (0.5–1 µm) are cut and placed on glass slides. These will be used to monitor the quality of the samples by light microscopy and to locate the areas of interest on the section.

The semi-thin sections are stained at 90–100°C with 1% toluidine blue solution. After drying, the slides are mounted under cover-slips with a drop of synthetic resin and observed using the light microscope.

Ultra-thin sections 80–100 nm thick are placed on mesh copper grids for electron microscopy. Uranyl acetate and lead citrate are used to counterstain the ultra-thin sections.

5.5. Use of molecular techniques for surveillance, confirmatory testing and diagnosis

Molecular techniques, including the use of nucleic acid probes for *in-situ* hybridisation, conventional PCR and real-time PCR, have been developed for the identification of many pathogens of aquatic animals. Real-time PCR methods, in general, have high sensitivity and specificity and, following adequate validation, can be used for direct detection of pathogen nucleic acids in samples prepared from mollusc tissues. These techniques can be used in direct surveillance of apparently healthy populations, if they have a high level of diagnostic sensitivity, as well as in the diagnosis of clinically affected animals.

When using PCR as a diagnostic method, the design of primers and probe, the use of positive and negative controls, as well as validation of the PCR method chosen are important. Real-time PCR is a powerful technique particularly for analysing relatively high numbers of samples (e.g. for surveillance) via high-throughput testing. Several nucleic acid probe and PCR protocols are included in this version of the *Aquatic Manual* as screening, diagnostic or confirmatory methods for molluscs and can be undertaken as the standard method. Following real-time PCR-positive results, where possible, conventional PCR with sequence analysis of PCR products should be used for confirmation of pathogen identity.

As with all PCR protocols, optimisation may be necessary depending on the reagents, equipment and the plasticware used. PCR is prone to false-positive and false-negative results. False-positive results (negative samples giving a positive reaction) may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from previous tests. Therefore, each assay and tissue extraction should include a negative control to rule out contamination. False-negative results (positive samples giving a negative result) may lead to unwanted transmission of pathogens and biosecurity failure. Therefore, each assay (and ideally each tissue extraction) should include positive controls to ensure the assay performed correctly. Additionally, mollusc tissues are known to potentially contain PCR inhibitors. It is therefore recommended to check for the presence of PCR inhibitors in DNA extracts to avoid false negative results.

To minimise the risk of contamination, aerosol barrier pipette tips should be used for all sample preparation and PCR steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the nucleic acid extraction, amplification and gel electrophoresis are performed. Do not share equipment (e.g. laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes, shoes and paper (e.g. workbooks). Also, ensure all work-tops and air-flow hoods/cabinets used for the extractions and PCR set-up are regularly cleaned and decontaminated. To ensure sample integrity, always store the samples (e.g. in a freezer or refrigerator) in a location separate from the molecular biology laboratory and reagents.

Nested PCR involves two rounds of PCR and may be used to achieve increased sensitivity and specificity; however, it increases the risk of contamination. Contaminants from previous reactions can carry over and lead to false-positive results. Strict laboratory practices such as separate workspaces, dedicated equipment, and meticulous pipetting techniques are essential to mitigate this risk. In conclusion, nested PCR is not recommended for surveillance but may sometimes be used for confirmative studies.

5.5.1. Sample preparation

Samples should be prepared to preserve the nucleic acid of the pathogen and should be handled and packaged with the greatest care to minimise the potential for cross-contamination amongst the samples or target degradation before the assay can be performed. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set. Use of household permanent markers should be avoided as their ink dissolves in ethanol and may result in loss of the sample label. Use pencil or histology pens only to label vials or jars.

Some suitable methods for preservation and transport of samples taken for molecular tests are:

i) *Live, iced specimens or chilled specimens:* for specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags in an insulated box containing a cold pack and ship to the laboratory. Note: cold packs should not be in direct contact with the animals to avoid freezing some parts of the tissues if histological analyses are also planned on the samples (histology cannot be performed on frozen tissues).

ii) *Frozen whole specimens: s*elect live specimens according to the purpose of sampling, quick freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory.

iii) *Alcohol-preserved samples:* 80% analytical grade ethanol (i.e. methanol-free ethanol) can be used to preserve, store, and transport mollusc tissues. Tissues should be fully immersed in ethanol. Shipment can be performed at room temperature .

iv) *Fixed tissues for* in-situ *hybridisation:* for this purpose, classic methods for preservation of the tissues for histology are adequate. Davidson’s solution is usually a good choice for later use of molecular probes (See Section B.5.3). For DNA, specifically, over-fixation (more than 48 hours) should be avoided.

5.5.2. Preservation of DNA in tissues

For routine diagnostic testing by PCR, samples must be prepared to preserve the pathogen’s nucleic acid. For most purposes, preservation of samples in analytical grade ethanol (80%) at room temperature is the preferred method for subsequent molecular tests. Samples preserved in this way can be stored for up to 1 week at 4°C or 25°C for 1 week or for extended periods at –20°C or below. In addition, other products (e.g. nucleic acid preservatives, various lysis buffers, etc.) are acceptable and are commercially available for the same purpose.

5.5.3. Nucleic acid extraction

To isolate nucleic acids from tissues preserved in ethanol or other preservative, simply remove the tissue from the fixative or preservative, press the tissues on absorbent paper to remove the excess of ethanol and let the ethanol evaporate, then treat it as fresh or frozen samples. Most fresh and preserved or fixed tissues can be homogenised (e.g. with a mortar and pestle or in bead-beating tubes) directly in the lysis or extraction buffer provided with commercially available DNA and RNA extraction kits. Commercial kits should be validated or undergo equivalence testing with current validated extraction procedures prior to routine use.

5.5.4. Preparation of slides for *in-situ* hybridisation

For *in-situ* hybridisation, molluscs are fixed and embedded in paraffin, according to the methods described above for histology. Sections are cut at 5 µm thick and placed on aminoalkylsilane-coated slides, which are then dried overnight at room temperature or in an oven at 40°C. The sections are dewaxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections could be rehydrated by immersion in a descending ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg ml–1) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 10–30 minutes in a humid chamber. Slides are dehydrated by immersion in one or several ethanol series and then air-dried. For *in-situ* hybridisation tests (see individual chapters for details), it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to non-specific staining/stain dropout, and false negative results due to errors in the staining protocol. It is also recommended to test non-specific ISH probes (e.g. “universal” 18s probes) on tested samples to check if the material is suitable for ISH analyses.

For further details see disease-specific chapters in this *Aquatic Manual*.

6. Additional information to be collected

Sample information should include the collector’s name, organisation, date, time, and description of the geographical location of the place of origin. The geographical location of the place of origin of samples may be described as the name or location of the site from which the sample has originated, or its geographical co-ordinates. There should also be records that provide information to allow trace-backs on the sample movement from the site of origin to the storage facility or laboratory and within those facilities.

Samples should be accompanied with background information, including the reason for submitting the sample (surveillance, abnormal mortality, abnormal growth, etc.), gross observations and associated environmental parameters, approximate prevalence and patterns of mortality, origin and nature of the molluscs (species, age, whether or not the samples are from local mollusc populations or stocks transferred from another site, date of transfer and source location, etc.). This information should identify possible changes in handling or environmental conditions that could be a factor in mortality in association, or not, with the presence of infectious agents

Information on the preservation method, storage location, and date and time of storage at each storage locker or freezer along with information on the storage temperature (continuously monitored is preferable) should be collected. This information should be tracked with a unique sample code for all samples. For laboratories, the date of receipt, storage location information, date of analysis, analysis notes, and report date should be maintained for all uniquely coded samples. These data will greatly facilitate the tracking of sample problems and provide assurance that the samples were properly handled.

See disease-specific chapters in this *Aquatic Manual* for recommendations on any additional information that may be required or that may assist the diagnostic laboratory in determining the most appropriate test(s) to be run for submitted samples.

7. Key references for further reading

Almeida M., Berthe F., Thebault A. & Dinis M.T. (1999). Whole clam culture as a quantitative diagnostic procedure of *Perkinsus atlanticus* (Apicomplexa, Perkinsea) in clams *Ruditapes decussatus. Aquaculture,* **177**, 325–332.

Arzul I., Corbeil S., Morga B. & Renault T. (2017). Viruses infecting marine molluscs. *J. Invert. Pathol*., **147**, 118-135.

Berthe F.C.J., Le Roux F., Adlard R.D. & Figueras A.J. (2004). Marteiliosis of molluscs: a review. *Aquat. Living Resour.*, **17**, 433–448.

Bondad-Reantaso M.G., McGladdery S.E., East I. & Subasinghe R.P. (2001). Asian Diagnostic Guide to Aquatic Animal Diseases. *FAO Fisheries Technical Paper*, No. 402, supplement 2. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 240 pp.

Bushek D., Ford S.E. & Allen S.K. (1994). Evaluation of methods using Ray’s fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infections in the eastern oyster, *Crassostrea virginica.* *Ann. Rev. Fish Dis.,* **4**, 201–217.

Guo X. & Ford S.E. (2015). Infectious diseases of marine molluscs and host responses as revealed by genetic tools. *Phil. Trans. R. Soc.,* **B** **371**, 20150206.

Howard D.W. Lewis E.J., Keller J. & Smith C.S. (2004). Histological techniques for marine bivalve molluscs and crustaceans. *NOAA Technical Memorandum NOS NCCOS* 5, 218 pp.

McDowell E. & Trump B.F. (1976). Histologic fixatives suitable for diagnostic light and electron microscopy. *Arch. Pathol. Lab. Med*., **100**, 405–414.

Moody N.J.G. & Crane M.St.J. (2016). Validation of diagnostic tests in the OIE manual for aquatic animals. In: Proc. 3rd OIE Global Conference on Aquatic Animal Health – “Riding the Wave of the Future”, Ho Chi Minh City, Vietnam, 20–22 January 2015, pp.119–126.

Qadiri S.S.N., Soo-Jin Kim S.-J., Krishnan R., Kim J.-O., Kole S., Kim W.-S. & Oh M.-J. (2019). Localization and tissue tropism of viral haemorrhagic septicemia virus (VHSV) in experimentally infected juvenile olive flounder, *Paralichthys olivaceus*: An *in situ* hybridization and immunohistochemical study. *Aquaculture*, **505**, 242–252.

**QUESTION:** Can the Aquatic Code Commission clarify where the above fish-specific reference is being used in the chapter above for crustaceans? If this reference is not being used in CHAPTER 2.4.0. GENERAL INFORMATION, then it should be removed.

Valverde E.J., Borrego J.J., Sarasquete M.C., Ortiz-Delgado J.B. & Castro D. (2017). Target organs for lymphocystis disease virus replication in gilthead seabream (*Sparus aurata*). *Vet. Res*., **48**, 21. doi 10.1186/s13567- 017-0428-3.

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Villalba A., Reece K.S., Ordás M.C., Casas S.M. & Figueras A.J. (2004). Perkinsosis in molluscs. *Aquat. Living Resour.*, **17**, 411–432.

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**NB:** First adopted in 1997. Most recent updates adopted in 2012.

1. A saturated 37–39% aqueous solution of formaldehyde gas. [↑](#footnote-ref-2)