assessed (ICES, 1995). The ICES (1995) Code of Practice is particularly useful for pearl oyster movements, since it aims at identification of unknown risks, unlike the OIE Code (2007) which concentrates on known disease agents. Currently, no pearl oyster diseases are listed in the OIE Code.

Ideally, a health profile (see Section 2.3) should be available for the source stock, however, such information may be rare for pearl oysters. Thus, one or more samples of the source stock should be examined for all pests and diseases before any movements to the pearl culture farm take place. Once the profile is obtained, the pearl farmer can determine whether there are any infectious agents or pests present which do not occur at the farm site. If so, the farmer is faced with the choice of finding another, more compatible, source or taking the risk of exposing the farm to potentially harmful additions. The methodologies used for health examinations are outlined in Sections 2.2 and 2.3.

## 2.3 DISEASE DIAGNOSTIC PROTOCOLS

### 2.3.1 Field collection of samples

#### Background information

Shellfish Health Questionnaire (Annex 2.1)

Field Data Sheet (Annex 2.2), note the following:

- hinge-lip length
- wet weight
- surface appearance (shell and soft-tissues)
- any damage to soft-tissues during opening of the shell.

Number of specimens collected (see Annex 2.3).Check the number required with the pathology laboratory and ensure each specimen is intact, i.e., no empty or mud-filled shells.

Shell surface, note the following:

- the presence of fouling organisms (barnacles, slipper limpets, sponges, polychaete worms, bivalve settlement, etc)
- shell deformities (shape, holes in the surface)
- obvious shell-fragility
- any abnormal colouration/smell
- any shell breakage or repair.

Inner shell, following removal of the soft-tissues note these observations:

- the presence of fouling organisms on the inner surface
- shell deformities (shape, holes in the surface, mud or water blisters)
- obvious shell-fragility
- abnormal colouration/smell
- pearls attached to the inner surface (cultured or wild).

Soft-tissues, note the following

- presence of abscess-like lesions, pustules or other tissue discolouration
- oedema (water blisters)
- overall transparency or wateriness
- any abnormal smell
- pearls (cultured or wild).

If fixing the tissue section on site, note the following:

- any worms or other organisms (small crabs, copepods, sponges) in the gut, mantle cavity or on the gills. Estimate numbers present (e.g. <50, >100, >1 000, etc.) and record.
- avoid fixation of tissues that contain pearls or sand or grit, where possible.

## Sample size and condition

Samples collected due to abnormal mortalities should consist of live individuals wherever possible, along with samples of unaffected shellfish, as available. Freshly dead shellfish may be collected if the soft tissues are intact and show no obvious signs of necrosis. Where *in vitro* culture is being used as the primary diagnostic tool for specific parasites (such as *Perkinsus* sp.), moribund shellfish with tissues in early stages of necrosis, may still be used.

It is important to emphasize that many disease agents of shellfish have carrier states which are impossible to detect using routine diagnostic techniques. Where there is the possibility of such a disease agent, sampling should be designed to maximize the opportunity for detecting the pathogen. This may involve:

- repeat sampling over one or more seasons
- transportation of samples to a diagnostic facility for live-holding and temperature stressing prior to analysis
- duplicate sampling for use of multiple detection techniques
- selection of appropriate specimen size/age.

The number of specimens collected for a disease outbreak may vary according to the extent of the problem (different genetic lines, sites, ages, etc.) and performance of tests to be applied. Samples for disease-screening of healthy populations, however, are usually based on a sampling regime designed to provide 95 percent confidence of detection of a single infected individual in a given population size at 5 percent prevalence of infection (see Annex 2.3, shaded column) (Ossiander and Wedermeyer, 1973).

## Sample preservation

Fixatives should be prepared and used with adequate ventilation to reduce inhalation of noxious gases.

1G4F (1 percent glutaraldehyde/4 percent formaldehyde)

1G4F is a fixative which can be used for light and electron microscopy examinations. Tissue samples should not exceed 2-3 mm in thickness. Following fixation, the tissue should be rinsed well in seawater before embedding or post-fixation for electron microscopy. Tissues can be stored in 1G4F at room temperature until ready for embedding or post-fixation and will tolerate long-term storage (months) in this fixative.

If glutaraldehyde is not readily available, or larger tissue samples have to be preserved, Davidson's fixative or 10 percent formalin (below) should be used. These fixatives are not suitable for electron microscopy or direct long-term storage. Both fixatives should be changed, either to 70 percent alcohol (ethanol or isopropanol) or fresh 10 percent formalin for long-term storage.

| | | |
|---|---|---|
| Stock solution: | formalin (37–40 percent formaldehyde solution) | 5 gal |
| | $Na_2HPO_4$ (disodium phosphate) | 284 g |
| | phenol red (pH indicator) | 0.5 g |
| | NaOH (sodium hydroxide) | 1.2 g |
| Working solution: | 37–40 percent buffered formalin stock | 120 ml |
| | 50 percent glutaraldehyde | 20 ml |
| | tap water | 360 ml |
| | filtered natural or artificial seawater | 500 ml |

The working solution should be prepared immediately prior to use. Seawater will cause flocculence and /or precipitate but this does not adversely affect fixation.

Carson's fixative
Similar to 1G4F except for the substitution of paraformaldehyde for formaldehyde.

Davidson's fixative
Tissue up to 10 mm in thickness can be fixed. Prior to embedding, tissues can be transferred either to 50 percent ethanol for 2 hrs (minimum) and then to 70 percent ethanol for an additional 2 hours (minimum), or directly to 70 percent isopropanol. Best results are obtained if the fixative is made up in the following order of ingredients.

| | | |
|---|---|---|
| Stock solution: | glycerin | 400 ml |
| | formalin (37-40 percent formaldehyde) | 800 ml |
| | 95 percent ethanol (or 99 percent isopropanol) | 1200 ml |
| | filtered natural or artificial seawater | 1200 ml |
| Working Solution: | dilute 9 parts stock with 1 part glacial acetic acid | |

10 percent formalin
Tissue up to 10 mm in thickness can be fixed using this solution. Wash in a buffered solution of ambient salinity for 30 min to 4 hrs prior to paraffin embedding.

| | | |
|---|---|---|
| Working Solution: | formalin (37-40 percent formaldehyde) | 10 ml |
| | filtered natural or artificial seawater | 90 ml |

*Note: The same flocculence and precipitate may occur as noted for the working solution of 1G4F. This does not adversely affect fixation of the tissue.*

## Label requirements

Storage jars
- Collection date (+ date of fixation, if different);
- Geographic location (as exact as possible);
- Scientific name of the shellfish or initials of genus and species;
- Number of specimens in each jar and number of jars in the sample;
- Name of fixative;
- Name and telephone number of sampler.

Tissue cassettes
- Date of collection (day: month: year);
- Initials of genus and species (as for the storage jars);
- Specimen No.;
- Code for geographic origin of the sample.

Specimen No. and Code for geographic origin should cross-reference to information on the questionnaire (Annex 2.1) and field data forms (Annex 2.2).

Shipping box or cooler
- The complete address of pathology laboratory doing the diagnostic testing;
- The complete address of the sender;
- An inventory of the contents (e.g., 10 jars containing American oyster tissue samples in 1G4F fixative, 2 data sheets, 1 completed questionnaire form).

### Mailing information

**Contact the laboratory before collecting the sample**

- to ensure the lab is ready to process the samples and that they are scheduled for examination;
- to verify mailing address, contact names and telephone numbers.
- to ensure that someone will be available to accept and properly store the parcel if arriving after normal working hours.
- to check number of specimens required by the pathology laboratory and determine whether or not live shellfish are necessary for tissue culture, bacteriology or other live specimen processing.

**Ensure that fixative solutions and shipping containers are labelled for compliance with any chemical transportation requirements**

All air shipments must meet International Air Transportation Association (IATA) regulations. Details can be obtained from the courier or airline company.

**Shipping instructions for live shellfish**

Pack shellfish in seawater soaked burlap, newspaper or paper towels with gelatine cold packs (ice packs are not allowed by airlines and freshwater leaks can affect the shellfish tissues) in a sealed watertight container. Label:

**"LIVE SPECIMENS, REFRIGERATE BUT DO NOT FREEZE"**

If being shipped by air also indicate:

**"HOLD AT AIRPORT AND CALL FOR PICK-UP"**

**For all shipments:**

- clearly indicate the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the lab.
- ship **early in the week** to avoid arrival during the weekend with possible loss of samples due to improper storage.
- inform the contact person as soon as the shipment has been sent and, where appropriate, give them the name of the carrier and waybill number.

### 2.3.2 Gross external observations

- All gross external clinical signs or abnormalities, which may indicate a disease problem should be noted (new growth, damage, fouling, hinge-ligament rupture). Note: Pea crabs (*Pinnotheres* spp.) are commonly found in the mantle cavity of *P. maxima*. Although fairly large, they do not appear to harm the pearl oyster (Fisheries Western Australia, 1997).
- If external shell material is to be tested, it should be collected following processing of the soft tissues which may be damaged by shell sampling.
- If organisms in the soft tissues require culture (e.g. bacteriology), samples should be removed prior to tissue collection for histology.
- Larval suspensions should be examined under a dissecting or light microscope, before being shipped to the diagnostic laboratory. Any abnormalities should be noted and submitted with the sample (e.g., reduced velar activity, bacterial "swarms", fouling by stalked ciliates).

### 2.3.3 Gross internal observations

#### Soft-tissue surfaces (abscess, oedema, mantle retraction, pearls)

Features which should be noted in the soft-tissues to ensure accurate interpretation of subsequent histology material include:

- abscess lesions – yellow/green spots in the mantle;
- water blisters or systemic oedema;
- mud blisters – formed due to perforation of the shell by boring sponges (*Cliona* spp.) or polychaetes (*Polydora* spp.) and susbequent invasion of the tunnels by mud or other irritants which come into contact with the soft-tissues;
- mantle retraction – acute retraction will show new shell growth and no inner surface fouling;
- chronic retraction may be associated with no new shell growth and fouling of the inner periphery of the shell;
- pearls – within the tissues or attached to the inner surface of the shell;
- gill deformities – convoluted edges, cysts, filament fusion;
- mantle-dwellers – nematodes; flatworms; pea crabs, etc.

#### Smears and tissue squashes

- blood samples can be taken either by heart imprint or by haemolymph suspensions in seawater. These can be air-dried, fixed and stained by a number of different commercial rapid stain kits or techniques. Blood samples should be examined for systemic infections by bacteria and protistans, or for neoplastic changes in cell morphology.
- gut contents can be smeared to check for internal parasites (protistans, helminths, copepods) as well as toxic algae. These can be examined fresh or fixed and stained.
- abscess lesion contents can be examined by preparing a smear or tissue squash, fixing and staining. Routine histological stains can be used, or Gram stain kits, depending on the suspected aetiology.

### 2.3.4 Laboratory protocols

If the shellfish are delivered live to the diagnostic laboratory, then the information given for field collections and gross observations apply to the first step of laboratory examination (Sections 2.2.1 and 2.2.2).

#### Biosecurity

All samples received for diagnostic examination should be treated as if they are positive for infection. Thus, the diagnostic laboratory is responsible for disposing of sample waste and materials in a manner which will ensure that nothing infectious is spread from the laboratory to the surrounding enviornment. All materials, including transport containers and water, shellfish remains, parasites, microbial cultures, contaminated equipment and instruments should be autoclaved, incinerated, or chemically decontaminated.

Storage of materials and clothing used for diagnostic examinations should be kept separate from all other laboratory areas and activities. All tissues and shells that remain after tissue samples have been collected should be labelled “biohazard” during the disinfection and disposal procedures. Laboratory effluent or liquid waste should also be disinfected before release (i.e. no direct flushing of chemicals or liquid waste into the municipal waste collection system).

All samples received for diagnostic testing should be clearly labelled on receipt by the laboratory and be tracked throughout the examination process (from necropsy to diagnostic report).

### Light microscopy

In general, bivalve molluscs are screened for diseases using histology, with individual specimens fixed separately. Duplicate tissue samples are stored in fixative to ensure tissue availability for additional sectioning, staining, electron microscopy, or molecular testing if required. Additional samples may be required if a disease agent is detected which requires live culture for specific identification.

### Fixatives

The same fixatives described under Section 2.2.1 for field preservation of samples are recommended for use in the laboratory. Frozen tissues are unsuitable for fixation and light microscopy.

### Tissue collection

Fouling organisms should be cleaned from the shell to prevent contamination of the soft tissues. Soft tissues should be examined for discolouration and deformity and any abnormal tissues preserved for histological examination, or collected for culture if bacteria or fungi are suspected.

Transverse body sections should contain samples of as many tissues as possible. Large specimens will require several sections to be cut from different areas of the body. This should be performed as quickly as possible and at cool temperatures to minimise processing-induced histology changes.

- Spat (< 20 mm in length) can be preserved whole in individual histology cassettes. This may require placing spat 1-3 mm in length inside a biopsy bag (or other commercially available cassette liner) to prevent the specimens slipping though the holes in the cassette. Once fixed and infiltrated with paraffin, the spat can be scraped out of the bag and embedded in a cluster in the paraffin block. Multiple specimens on a single slide provide sections through most planes of the body.
- Juvenile pearl oysters (< 20-40 mm hinge to lip length) can have a single cross section removed and placed within a single cassette. This is usually through the digestive gland behind the hinge, out towards the gills and mantle margin. The optimum orientation will include sections of the cardiac cavity, gonad, digestive gland, gills and mantle.
- Adult pearl oysters (> 40 mm) require multiple tissue sections to be removed for histological examination. Depending on the size of the oyster, these may fit within one or more cassettes, or each need an individual cassette.

Stressed pearl oysters can produce large quantities of mucous which adversely affect tissue collection and preservation. Tissues removed from oysters in this condition should be placed in the histology cassette and then rinsed quickly (seconds) in a dish of ambient temperature, clean, seawater to remove the surface coating of mucous before fixation.

Oysters in spawning condition also pose a problem for fresh tissue sampling and fixation. Fragile gonadal tissue and gamete release are difficult to keep intact for paraffin mounting on the microscope slide. Such oysters should either be removed from their shells or kept attached to the half-shell (cleaned of superficial fouling organisms) and placed in 10% buffered formalin or Davidson's fixative for 30 mins to 1 hour (depending on size) to allow fixation of the surface tissues. Following this treatment, tissue sections should be removed, as described above and fixed using the same fixative solution.

To achieve good preservation of tissues fixed within the shell, oysters should be anaesthetized prior to fixation. This can be achieved by adding propylene phenoxetol (1-3 ml/l) (Mills, Tlili and Norton, 1997) or magnesium chloride (14 gm/l) (Culloty and Mulcahy, 1992) to seawater containing actively swimming larvae or juveniles with their

valves agape[1]. Food may be provided for the oysters prior to adding the anaesthetic to encourage them to open their shells. Sufficient exposure to the anaesthetic is achieved when they do not close their shells on being disturbed (several minutes to several hours depending on species, size and water temperature). After fixation, the shells should be decalcified prior to embedding.

### Decalcification

Pearl oysters fixed within the shell (juvenile or spat), or which have tissues which contain calcified material, should be decalcified prior to being embedded.

- Fix tissue in fixative of choice.
- Rinse well in running water.
- Decalcify, using any commercially-available chelating agent, e.g. 5 percent trichloroacetic acid (TCA). If EDTA is used (10 g of ethylenediaminetetraa cetic acid (99.5 percent powder) in 100 ml distilled water), use the following procedure:
  - Place tissue in a solution of EDTA.
  - Change solution every 2-3 days to ensure optimum decalcification.
  - Retain tissues in EDTA solution until decalcification is complete (tissue no longer producing gas bubbles and shells are rubbery to the touch).
  - Specimens may be left in EDTA solution for up to 14 days without affecting the staining qualities of subsequent histological sections.
  - Rinse well in running water (30 min–1 hour, depending on the size of tissue).
  - Store in 70 percent ethanol until ready to process.

### Tissue storage

Duplicate tissue sections from all specimens should be kept in storage, either on-site, or at the diagnostic laboratory. The duration of storage depends on the purpose of the health examination, but should exceed the period required to do the initial diagnosis. Tissues kept in fixative should be stored away from points of combustion – 1G4F and 70 percent ethanol are combustible and should be stored with due caution. Tissues stored for electron microscopy should also be treated as toxic. No tissues or their fixatives should have access to open water or drainage points into open water, due to their toxicity. Consult the manufacturer's *Material Safety Data Sheets* for safe disposal of all chemicals used for fixatives and fixed tissues.

Paraffin blocks should be stored in a relatively cool place to prevent meltdown. Ideally, temperatures should not exceed 20 °C for paraffin block storage and the duration of storage depends on the purpose of the examination, as for tissues in wet storage.

### Electron microscopy (EM)

Many intracellular and microscopic lesions cannot be identified directly using light microscopy, therefore, the increased magnification and resolution of electron microscopy is required. This is specialised technique, using toxic chemicals and highly sensitive equipment. Electron microscopes are not available in all diagnostic laboratories and analysis using this technique may, therefore, take longer than standard light microscopy. Electron microscopy can also be use as a confirmatory back-up to light microscope observations.

[1] N.B. These anaethetics have been used for adult pearl oysters and other oyster species - optimal anaethesia should not be fatal, which could potentially affect the histopathology. Conduct trials on larval juvenile recovery to determine optimum concentrations for the pearl oyster size, water temperatures, etc.

## Fixation for transmission electron microscopy (TEM)

*Note: all fixatives are toxic and should be handled in a fume cupboard/hood. The post-fixatives used for TEM tissue preparation are particularly dangerous and should only be used by laboratory personnel who have received training in their use.*

- The standard electron microscope fixative is 2 percent gluteraldehyde in ambient seawater. 1G4F may be used if 2 percent gluteraldehyde is unavailable. Formalin and ethanol fixatives are unsuitable, and frozen tissues cannot be fixed for electron microscopy.
- Post-fix tissue in 4 percent osmium tetroxide and embed in a resin matrix suitable for ultramicrotome sectioning.
- Stain with lead citrate and uranyl acetate or an equivalent EM stain.

## Negative stain

Tissues showing lesions, abscesses, granulomas, etc., but no obvious causative agent, can be processed for direct EM using negative-stain preparation[2].

Fresh tissues are ground down to a homogenous suspension in a buffer which is isosmotic with ambient seawater and supplemented with 2 percent gluteraldehyde. Some fragile viruses may be destroyed by this process, but if present in concentrations high enough to cause tissue pathology, virions should still be distinguishable.

- Drops from the tissue suspension are placed on clean Parafilm® and a carbon-coated 400 mesh copper grid is placed coated side down on top of the drop for 10 min.
- The grid is rinsed through two drops of phosphotungstic acid (PPTA) before being placed on a fresh drop of PPTA for a further 10 min.
- The grid is then air-dried and ready for examination.

## Bacteriology

For specimens which require bacterial examination, external tissues should be disinfected prior to collecting samples. Such disinfection should be noted for correlation to subsequent histology sections, which may show evidence of surface tissue irritation by the disinfection procedure. Examples of procedures used for bacterial infections in bivalves are given below:

## Smear preparations

Bacterial abscess lesions

Successful isolation of any single etiologic agent from such lesions has not been achieved to date. Gram-positive bacteria (*Micrococcus* sp.) and Gram-negative species (*Vibrio, Pseudomonas* and *Aeromonas*) have been cultured from abscess lesions in bivalves from Canada and the USA.

- Surface-sterilize tissues by wiping with 70 percent ethanol.
- Make a smear of abscess contents, air dry and stain with a commercial Gram stain kit, as per manufacturer's instructions.
- If bacteria are indicated by the Gram stain, attempt to culture the bacteria.

Non-fastidious marine culture media, such as marine agar (MA) or trypticase soy agar (TSA) and brain heart infusion agar (BHIA) have been used to culture bacteria from these abscesses, however, care is required to avoid contamination by surface or secondary bacteria.

---

[2] Note that the examination for viral particles in negative-stain preparations should be done by personnel with experience or training, because artifacts can easily be mistaken for viral particles, especially in bivalve molluscs.

Nocardiosis of oysters

Although not reported from pearl oysters, this bacterium is found in other oyster species and can be cultured, although cultures are not required for identification. Because infections involve Gram-positive bacteria – an unusual feature for the majority of marine bacteria – infections can be confirmed by routine Gram stains on histological sections or smears of suspected tissues to show the Gram-positive branching bacterial colonies.

## Media culture

Hinge ligament disease of juvenile bivalve molluscs

*Cytophaga* sp., the causative agent of hinge ligament disease of juvenile bivalve molluscs, belongs to a group of bacteria characterized by the following features:

- gliding motility resulting in colonies having a rhizoidal appearance on agar culture plate surfaces
- no flagellar appendages;
- long and variable cell lengths ranging from 2.5 microns to several hundred microns;
- flexible cell walls of typical Gram-negative structure; and
- ability to metabolize biomacromolecules.

*Cytophaga* sp. is isolated from infected hinges of most bivalves and grown on seawater *Cytophaga* agar, with a low nutrient concentration:

| | | | |
|---|---|---|---|
| 50 percent agar-tryptone | 0.5 g | 60 percent (enriched) agar -tryptone | 2.0 g |
| yeast extract | 0.5 g | yeast extract | 0.5 g |
| beef extract | 0.2 g | sodium acetate | 0.2 g |
| sodium acetate | 0.2 g | artificial seawater | 600 ml |
| artificial seawater | 500 ml | distilled water | 400 ml |
| distilled water | 500 ml | agar | 18.0 g |
| agar | 11.0 g | | |

Boil to dissolve solutes. Adjust pH to 7.2. Autoclave. Pour into Petri plates.

Procedure

- Remove hinge ligaments from up to ten spat per sample, keeping the dorsal surface uppermost to prevent contamination with dissection products, use a pointed scalpel to sever the adductor muscle.
- Remove the soft tissues, separate the valves, and excise the hinge ligament into chilled sterile saline (1.5 percent w/v NaCl).
- Place ligaments in a chilled tissue grinder with 1.0 mL of ambient saline and homogenize.
- Ten-fold dilutions of homogenate may be prepared.
- Dispense 0.1 mL portions onto 50 percent *Cytophaga* agar[3] and spread with a glass rod.
- Incubate agar plates at 15 to 20 °C for 5-10 days.

Examination

- Examine agar surface for rhizoidal colonies.
- For further identification, rhizoidal colonies can be removed from agar surface, suspended in saline and subcultured onto enriched cytophaga medium or commercially-available marine agar (Difco).

[3] Note: *Cytophaga* bacteria are slow-growing and are quicky overwhelmed by other bacteria. Thus, initial isolation is made on the low nutrient medium, which reduces growth of other bacteria, and promotes gliding motility, enhancing recognition by behaviour and colony morphology.

*Vibrio tapetis* (Brown-ring disease) of Manila clams, *Tapes philippinarum*

*Vibrio tapetis* can be cultured on commercially-available marine agar or thiosulphate citrate bile salts sucrose agar (TCBS) supplemented to contain 2-3 percent NaCl.

Culture media:

- commercially-available marine agar (Difco)
- thiosulphate citrate bile salts sucrose agar (TCBS) (Difco) supplemented with 1 percent NaCl

Confirmatory tests:

- O/129® vibriostatic compound sensitivity disks (Oxoid), 10 and 150 mg
- Pathotec® cytochrome oxidase test strips
- Gram stain kit
- oxidative-fermentative medium, consisting of:

| | |
|---|---|
| phenol red broth medium | 16.0 g |
| glucose | 10.0 g |
| yeast extract | 3.0 g |
| sodium chloride | 25.0 g |
| agar | 3.0 g |
| distilled water | 1 000 ml |

Dissolve ingredients, adjust pH to 7.6, add 3 g of agar and boil. Dispense 10 ml into each test tube and autoclave. To conduct the oxidation-fermentation test, inoculate medium by making a stab in the medium using an inoculating loop or wire probe coated with bacteria from the colony. In a positive test, the lower pH of the acid formed from glucose fermentation changes the medium colour from orange to yellow.

Procedure

- Scrape material from the suspected site of infection using a sterile loop and smear onto marine agar or TCBS agar
  or
- Excise the suspected site aseptically and homogenize the tissue in about 1 mL of sterile seawater
  or
- If the animals are too small to be dissected, take a sample of packed larvae with a 1 ml syringe. Let larvae settle to the delivery end of the syringe and dispense 0.5 ml of larvae into 2 ml of sterile seawater or 1.5 percent (w/v) NaCl solution. Grind the sample using a tissue grinder. Allow the large particulate matter (such as large pieces of shell) to settle for 10 min. Using a sterile loop, smear a sample of the suspended material onto prepared Petri plates containing marine agar or TCBS agar.

Examination

- Incubate Petri plates at 10 to 20 °C. Check daily.
- If using TCBS media look for yellow colonies or a colour change in the medium from green to yellow within 48 hr.
- Characterize colonies which resemble the colony morphology and physiological characteristics described for *Vibrio* spp. in Bergey's Manual of Systematic Bacteriology (Holt and Krieg, 1984) (i.e. Gram-negative, cytochrome oxidase positive, motile, possessing the ability to ferment glucose (oxidative-fermentation test), and sensitivity to O/129® (Oxoid).

Note that *Vibrio* spp. are ubiquitous in the marine environment and most of the species associated with shellfish are thought to be facultative pathogens and, thus,

are not reportable disease agents. The only known mechanism for identifying *Vibrio tapetis* is to use a suspected isolate challenge to healthy Manila clams by injection into the pallial cavity. Development of clinical signs of the disease occurs four weeks after injection.

### Immunodiagnostics and nucleic acid probes

To date, there are no commercially available immunodiagnostic tools for any known disease agents of pearl oysters. Tools are under development for other shellfish, however, and may become available for rapid screening of microbial agents, such as *Vibrio harveyi* in the near future (Bachère *et al*., 1995).

### Record-keeping

An essential component of accurate disease diagnosis and efficient analysis of case submissions is meticulous record-keeping.

### Field collection records

The information outlined in the Shellfish Health Questionnaire (Annex 2.1) and Field Data Sheet (Annex 2.2) should be kept at pearl farm to ensure relevant information is readily available if a disease problem arises. Having data on conditions when the pearl oysters were healthy is an invaluable reference for comparing with field conditions associated with a disease situation.

### Case-tracking

Diagnostic submissions should be traceable from when they leave the farm to when they arrive at the laboratory via registered couriers. On arrival at the laboratory, they should be logged-in immediately to prevent accidental misplacement and ensure rapid processing for preservation. An example of a laboratory case-log is shown in Annex 2.4.

### Specimen data-sheets

Specimen data sheets are spreadsheets designed to record all observations from individual oysters, including:

| Specimen No. | Haemocyte infiltration* | Viruses | Shell Sponges |
|---|---|---|---|
| Length | Diapedesis* | Intracellular Bacteria | Shell Polychaetes |
| Weight | Oedema | Extracellular Bacteria | Surface Fouling |
| Sex | Abscesses* | Surface Protistans | Shell Deformities |
| Maturity* | Hyperplasia | Internal Protistans | Abnormal Odour |
| Food content* | Metaplasia* | Intracellular Protistans | |
| | Neoplasia | Metacercariae | |
| | Ceroid* | Sporocysts/Rediae | |
| | Concretions* | Cestodes | |
| | Adipogranular* | Nematodes | |
| | Necrosis* | Surface Turbellarians | |
| | | Internal Turbellarians | |
| | | Copepods | |
| | | Decapods | |
| | | Fungi | |

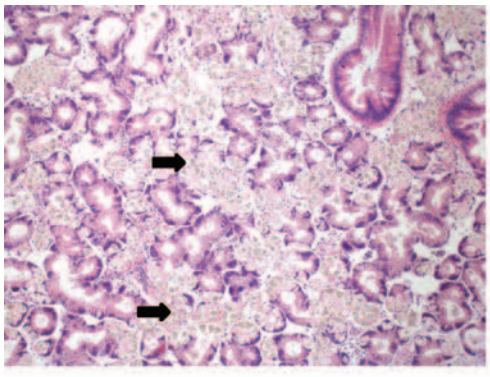
Most observations consist of counts per tissue section. The factors marked with an asterisk are scored by qualitative scale (Annex 2.5).

### Diagnostic report filing

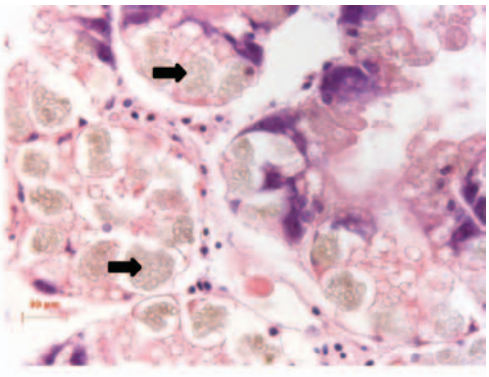
Diagnostic reports are sent directly to the client who submitted the samples for diagnosis. If the health check was for an introduction or transfer request, the diagnostic

## PLATE 2.1.1

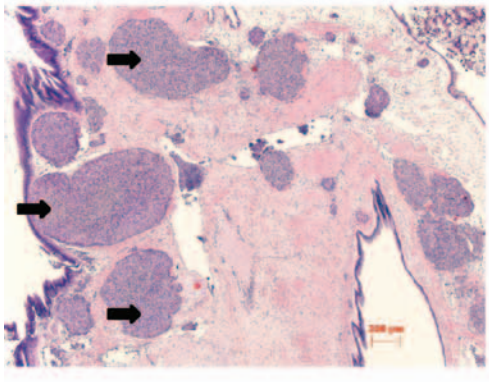
**Examples of histopathological observations (concretions, abcess-like lesions, granuloma, neoplasia) on molluscan species**



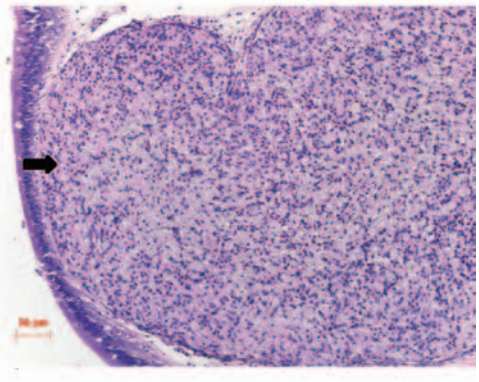
Low magnification of concretions (arrows) in the digestive gland of a scallop (*Placopecten magellanicus*)



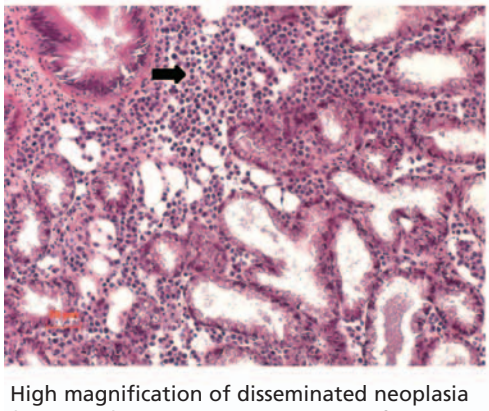
High magnification



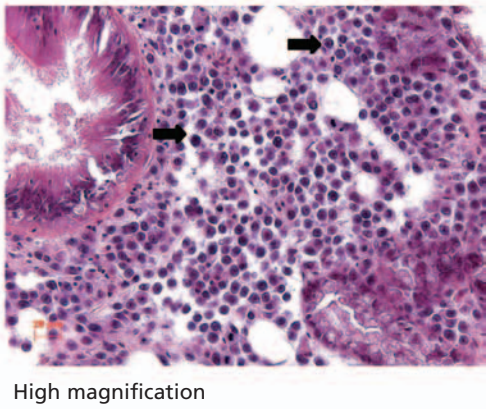
Low magnification of abcess-like lesion (arrows) in the mantle of a scallop (*Placopecten magellanicus*)



High magnification of a granuloma (arrows)



High magnification of disseminated neoplasia (neoplasia) in the connective tissue of the digestive gland of a mussel (*Mytilus trossulus)*



High magnification

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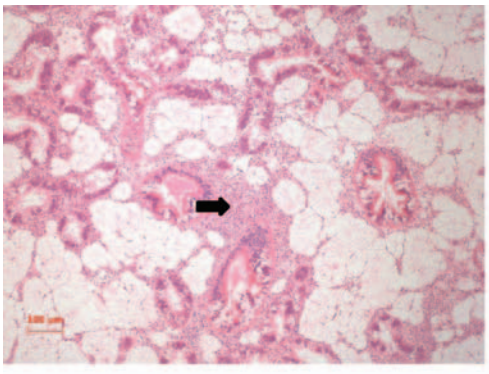
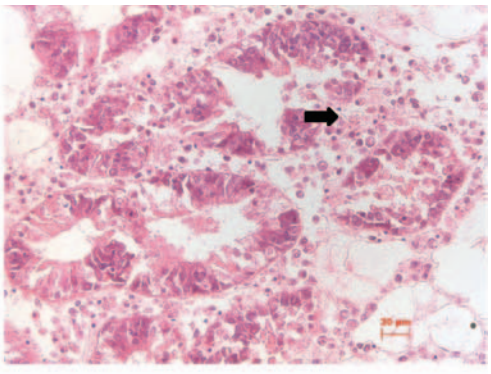
## PLATE 2.1.2

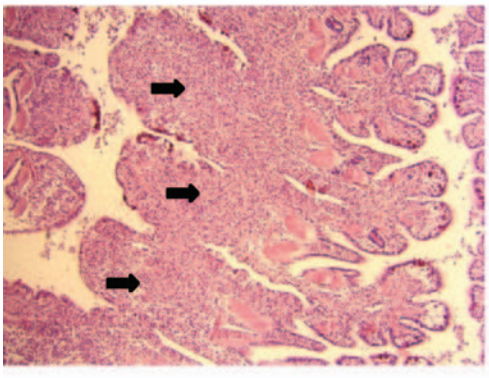
**Examples of histopathological observations (haemocytic infiltration, congestion) on molluscan species**



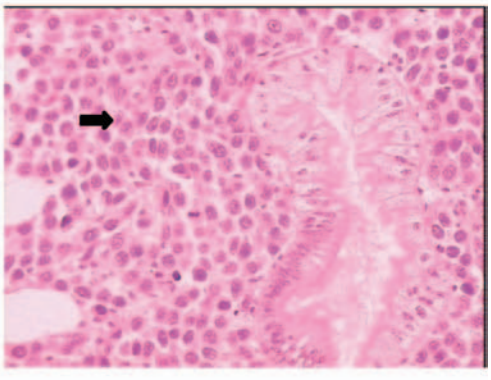
Low magnification of haemocytic infiltration (arrows) of the digestive gland connective tissue of a flat oyster (*Ostrea edulis*)



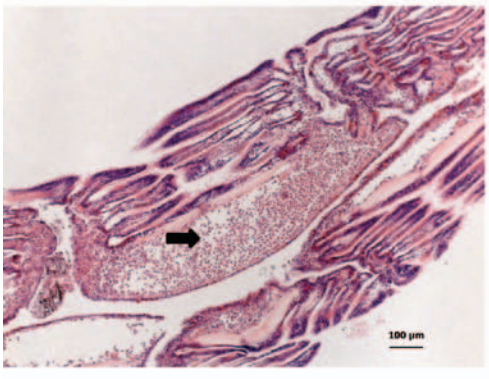
High magnification



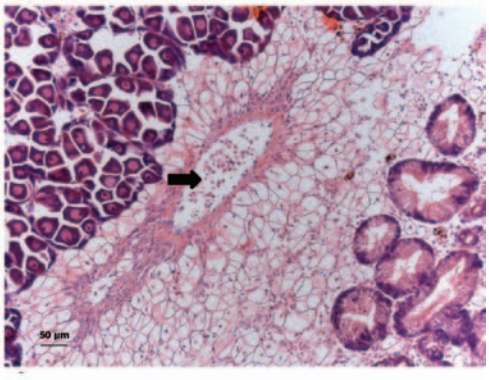
High magnification showing infiltration (arrows) of the gill filaments



Haemocyte infiltration associated with disseminated neoplasia (arrows) in the connective tissue of the digestive gland of a mussel (*Mytilus galloprovincialis*)




Congestion (arrow) of a vessel in the gill of an Eastern oyster (*Crassostrea virginica*)




High magnification of the connective tissue between digestive gland and gonad (arrow)

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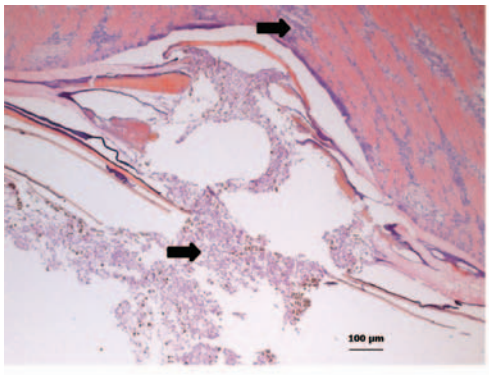
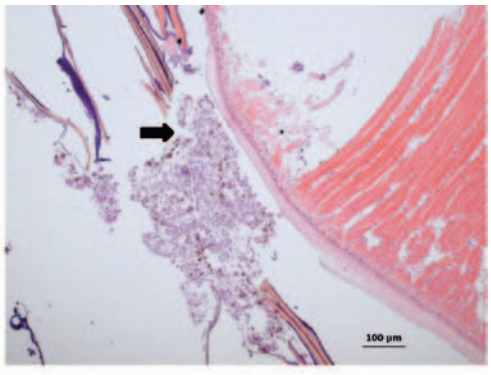
## PLATE 2.1.3

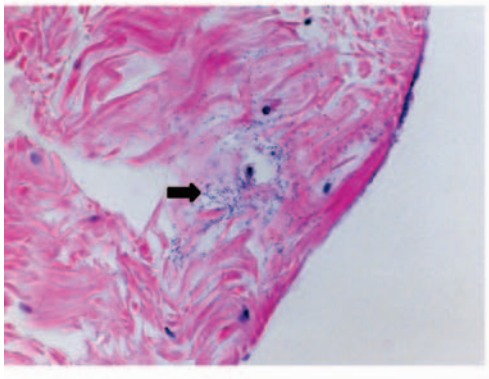
**Examples of histopathological observations (Cliona lesion, haemocytic infiltration, *Vibrio* sp. infection, oedema-type lesion, starving oyster) on molluscan species**



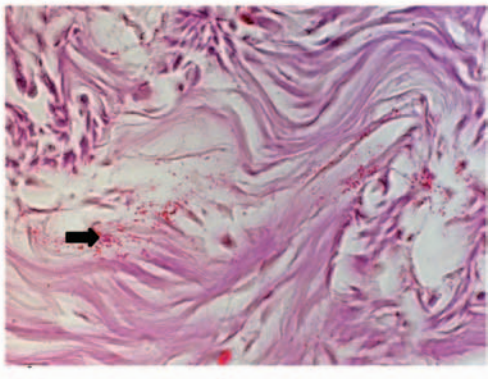

a) Low magnification of *Cliona* sp. lesion of the shell. Note haemocytic infiltration (arrows) of the adjacent muscular tissue (Eastern oyster, *Crassostrea virginica*)



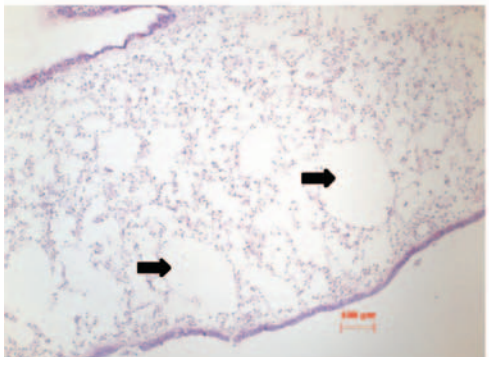

b) High magnification shows the breach of shell integrity (arrow)



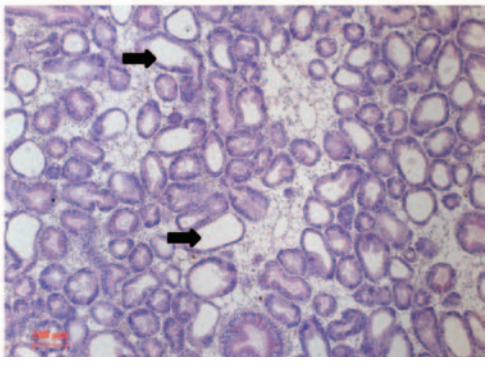
c) *Vibrio* sp. infection (arrow) of the foot of an abalone (*Haliotis asinina*)



d) Gram stain of *Vibrio* sp. infection (arrow)



e) Low magnification of oedema type lesion (arrows) in the mantle of Eastern oyster (*Crassostrea virginica*)



f) Low magnification of the digestive gland of a starving oyster (*Crassostrea virginica*) (arrows)

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laboratory should check with the client whether they want the health report sent directly to the transfer licensing authorities or the client will send a copy with their request. Copies of the diagnostic report should be kept on file as well as with the specimen data sheets.

## 2.4 HEALTH ZONATION

### Introduction

The concept behind zonation is to establish a reference health profile for a geographic area or facility which can be used as the basis for monitoring any changes in the health status of the culture stock and/or to assess the risk of disease transfers. This method of health management is recommended by the OIE (2006) and is applied on a regional basis for shellfish aquaculture in Canada, parts of the United States of America and the European Union. Stock from facilities or areas with identical health profiles present negligible health risks compared with those with different health profiles. Land-based facilities can be given a health status based on isolation from surrounding facilities. Since this is harder to assume with open-water, health zonation can be conducted on a geographic/hydrgraphic basis. With pearl oyster farms, the facility-based concept may apply more readily than with other mollusc culture systems, since there is little direct exchange between farms and the number of farms in any given area is usually limited. Where neighbouring farms undertake stock exchange (seedstock, broodstock, etc.), ,the zonation approach may be more applicable.

### Facility-based health status

In order to establish an accurate health profile, a facility (hatchery, culture production) should have at least four (4) consecutive health checks over a period of 18-36 months and at periods most likely to detect disease agents (e.g. post-transportation, post-spawning, post-surgery). Fewer health checks may be required for land-based facilities with a sterile water supply. Separate samples should be examined for different year-classes, species and stocks from different sources (unless they have been mixed). Numbers of oysters which need to be examined will vary with the number being held at the facility but should comply as closely as possible with 95 percent confidence of detecting an infectious organism at 2 percent prevalence, i.e. a maximum sample size of 150 animals (see Annex 2.3). Once the consecutive checks have been completed, a health status report can be compiled for the facility site. This can be used for requests to transfer stocks to other facilities or to assess risks from importations from other sites or facilities (based upon their health status records, if available).

### Geographic area

The same protocol for establishing a health profile can be applied to a geographic area. This is usually applied to discrete bays or areas with a well-established exchange of oysters, e.g. collection of local source stock or seedstock from a local hatchery, or hydrographic water exchange. Four consecutive health checks are recommended and samples of wild stock should consist of 150 oysters (unless stocks are limited or considered to be endangered). Where several facilities fall within a single geographic zone, the growers may decide to provide broodstock from one farm, seedstock from another, etc. The results from each can then be pooled to provide the health status for the zone.

### Health status maintenance

Following establishment of a health status profile for a facility or geographic zone, this can be maintained by annual samples of stocks.