Histological Techniques for Marine Bivalve Molluscs: Update

Cover photograph of trematode sporocysts completely occupying the mantle of a mytilid mussel, *Mytilus edulis*.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. GONADAL ANALYSIS

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ABSTRACT

This chapter describes the procedures for determining the reproductive stage of oysters, mytilid mussels, and dreissenid mussels collected for NOAA's National Status and Trends Mussel Watch Project. Analyses are conducted on paraffin-embedded tissues sectioned at a 5-µm thickness and stained using a pentachrome staining procedure. Each slide is examined microscopically to determine the animal's sex and stage of gonadal development. A semi-quantitative ranking is assigned.

1.0   INTRODUCTION

Assessment of the physiological state of bivalve populations requires an analysis of the state of gonadal development. Determination of reproductive stage is included as part of the Mussel Watch Project to give an indication of the amount of gametic material in bivalve tissues at the time of chemical analysis. Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Cunningham and Tripp 1975; Mo and Neilson, 1993). Because gametic material can account for 20% to 50% of body weight in target species of oysters and mussels (Sprung, 1991; Choi *et al.*, 1993), the relative proportion of gonadal to somatic tissue and the timing of spawns (an important depuration route for some contaminants) can significantly impact the body burden of contaminants.

This description updates the methods presented in Ellis *et al.* (1998b) and Powell *et al.* (1993). The original intent of the determination of reproductive stage was to assure that sampling was conducted at the same stage of the reproductive cycle so that analyses of the lipophilic organic contaminants and the trace element contaminants were not influenced by reproductive state. Unfortunately, the time required for sampling and the wide latitudinal range encompassed by the sites did not permit consistent recovery of individuals in similar stages of reproductive development at all sites. For example, typically oysters are undifferentiated in the winter. Gonads begin to develop in early spring and spawning occurs late spring through early fall. Most Gulf Coast oysters spawn at least twice during this time period. Single spawns tend to occur in the shorter summers of the mid-Atlantic region (e.g., Dittman *et al.*, 2001). The timing of the last spawn varies with latitude and with yearly variations in climate (e.g., Wilson *et al.*, 1990, 1992). Southeast Atlantic and Southern Gulf sites, for example, routinely yield oysters in reproductive development or that are ready to spawn in mid-winter during the period when Mussel Watch sampling occurs. Mid-Atlantic sites are typically characterized by individuals in an undifferentiated state and thus contain significantly less lipid-rich

gametic tissue than the southern animals. Mytilid mussels and dreissenid mussels have the same assortment of problems relating to latitude and interannual changes in climate (Newell, 1989; Seed and Suchanek, 1992; Borcherding, 1991). In addition, dreissenid mussels are typically collected during late August-September whereas the remaining Mussel Watch species are collected during winter. Thus, analysis of reproductive stage has proved important in identifying differences in tissue composition that might affect comparisons of contaminant data among sites and among years. Wilson *et al.* (1990, 1992) and Kim *et al*. (1999, 2001) discuss the influence of climate on reproductive stage and contaminant body burden in the Mussel Watch Project in more detail.

Oyster gonadal tissue is distributed around the body mass (Morales-Alamo and Mann, 1989). Gonads of dreissenid mussels also develop within the body, around the periphery of the viscera. In contrast to oysters and dreissenid mussels, gonadal follicles develop primarily within the mantle of mytilid mussels. Nevertheless, in none of these cases can the gonad be easily excised and weighed. Consequently, virtually all assays of reproductive stage use histological methods to recognize the changes in the germinal epithelium and germinal products that identify stages in gonadal development. The histological approach uses a semiquantitative numerical assignment to rank reproductive stage. Quantitative measures, such as egg protein content (Choi and Powell, 1993, Choi *et al*., 1993, 1994), remain expensive and time consuming, and do not permit a concomitant histopathological analysis. Therefore, a histological examination is still the single method of choice when only one method can be used.

For oysters, a dorsal-ventral slice of tissue is taken and fixed in Davidson's fixative. Dreissenid and mytilid mussels are preserved whole, and a dorsal-ventral slice is taken after fixation. Tissue slices are embedded in paraffin, sectioned, and stained using a pentachrome staining protocol. Stained sections are examined under a compound microscope, and sex and the state of gonadal development determined. Fixation follows the method described in Preece (1972). The staining procedure is an adaptation of Masson's (1928) trichrome procedure (Ellis *et al*., 1998b). Reproductive stage in oysters is determined using a semiquantitative scale adapted from Ford and Figueras (1988). The scale developed by Seed (1975, 1976) for determining gonad index in mussels was adopted for mytilid mussels and dreissenid mussels.

2.0   EQUIPMENT, REAGENTS, SOLUTIONS, AND SAMPLE PREPARATION

2.1.   EQUIPMENT

Cover slips - various sizes. Fisher Scientific, Pittsburgh, PA.

Disposable cassettes - HistoPrep tissue capsules, 15-182-218. Fisher Scientific, Pittsburgh, PA.

Disposable microtome blades, 12-634-11. Fisher Scientific, Pittsburgh, PA.

Drying oven, 13-254-1. Fisher Scientific, Pittsburgh, PA.

Embedding rings - HistoPrep embedding rings, 12-652-1OB. Fisher Scientific, Pittsburgh, PA.

Frosted microslides, 12-552. Fisher Scientific, Pittsburgh, PA.

Microscope, compound, Zeiss, 12-070-20. Fisher Scientific, Pittsburgh, PA.

Microtome, HM330. Microm GmbH. D-6900 Heidelberg, Germany.

Slide staining set, Tissue-Tek II. Miles Laboratories, Inc. Westmont, IL.

Slide staining holder, Tissue-Tek, 4466. Miles Laboratories, Inc. Westmont, IL.

Stainless steel molds, various sizes. Fisher Scientific, Pittsburgh, PA.

Tissue embedding system, Histocentre 2, 6400012. Shandon, Inc., Pittsburgh, PA.

Tissue processor, Citadel 2000, 69800006. Shandon, Inc., Pittsburgh, PA.

Vacuum infiltrator, Tissue-Tek II, 4613. Miles Laboratories, Inc. Westmont, IL.

Water bath - Fisher Tissue PrepModel 134 Flotation Bath, 15-464. Fisher Scientific, Pittsburgh, PA.

2.2.   REAGENTS

Acetone HistoPrep (CH_3COCH_3), [67-64-1], HC300-1GAL. Fisher Scientific, Pittsburgh, PA.

Acid fuchsin, certified stain ($C_{20}H_{17}N_3O_9S_3Ca$) [136132-76-8], A3908. Sigma Chemical Co.,St. Louis, MO.

Ammonium hydroxide (NH_4OH) [1336-21-6], A6899. Sigma Chemical Co., St. Louis, MO.

Aniline blue powder, certified stain [28631-66-5], A967-25. Fisher Scientific, Pittsburgh, PA.

Chromotrope powder 2R, ($C_{16}H_{10}N_2Na_2O_8S_2$) [4197-07-3], C3143. Sigma Chemical Co., St. Louis, MO.

Ethanol (C_2H_5OH) [64-17-5], R8382. Sigma Chemical Co., St. Louis, MO.

Fast green FCF, certified stain ($C_{37}H_{34}N_2O_{10}S_3Na_2$) [2353-45-9], F7252. Sigma Chemical Co., St. Louis, MO.

Ferric ammonium sulfate (Fe $NH_4(SO_4)_2 \cdot 12H_2O$) [7783-83-7], F1018. Sigma Chemical Co., St. Louis, MO.

Formaldehyde, 37% solution (CH_2O) [50-00-0], F1635. Sigma Chemical Co., St. Louis, MO.

Glacial acetic acid ($C_2H_4O_2$) [64-19-7], A0808. Sigma Chemical Co., St. Louis, MO.

Glycerin ($C_3H_8O_3$) [56-81-5], G7893. Sigma Chemical Co., St. Louis, MO.

Hematoxylin powder, certified stain ($C_{16}H_{14}O_6$) [517-28-2], H3136. Sigma Chemical Co., St. Louis, MO.

Orange G powder, certified stain ($C_{16}H_{10}N_2O_7S_2Na_2$) [1936-15-8], O7252. Sigma Chemical Co., St. Louis, MO.

Paraffin - Paraplast tissue embedding media (melting pt. 56 °C), 12-646-111, Fisher Scientific, Pittsburgh, PA.

Permount mounting media, SP15-500. Fisher Scientific, Pittsburgh, PA.

Phosphomolybdic acid ($20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) [51429-74-4], P7390. Sigma Chemical Co., St. Louis, MO.

Phosphotungstic acid ($12WO_3 \cdot H_3PO_4 \cdot H_2O$) [12501-23-4], P4006. Sigma Chemical Co., St. Louis, MO.

Sodium chloride (NaCl) [7647-14-5], S9625. Sigma Chemical Co., St. Louis, MO.

Sulfuric acid (H_2SO_4) [7664-93-9], S1526. Sigma Chemical Co., St. Louis, MO.

Tissue Clear III, SH3-4. Fisher Scientific, Pittsburgh, PA.

Tissue Dry, SH5-20. Fisher Scientific, Pittsburgh, PA.

Xyleness, histological grade ($C_6H_4(CH_3)_2$), X3S-4. Fisher Scientific, Pittsburgh, PA.

2.3.   SOLUTIONS

Ferric alum mordant: 25 g ferric ammonium sulfate dissolved in 500 mL distilled water.

Basic ethanol: 26 mL ammonium hydroxide in a solution of 3370 mL 95% ethanol and 630 mL distilled water.

Phosphomolybdic acid solution: 5 g phosphomolybdic acid crystals dissolved in 495 mL distilled water.

1% acetic acid: 20 mL glacial acetic acid in 1980 mL distilled water.

1% acid acetone: 20 mL glacial acetic acid in 1980 mL acetone.

Groat/Weigert hematoxylin working solution: 245 mL distilled water, 5 mL sulfuric acid, 5 g ferric ammonium sulfate, 245 mL 95% ethanol, and 2.5 g hematoxylin powder.

Acid fuchsin working solution: 1.5 g acid fuchsin powder dissolved in 495 mL distilled water, to which is added 5 mL glacial acetic acid.

Phosphotungstic acid solution: 10 g phosphotungstic acid crystals dissolved in 490 mL distilled water.

Orange G/Chromotrope: 4 g orange G powder and 1 g chromotrope powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Fast green/Aniline blue working solution: 5 g fast green FCF, 4 g aniline blue powder dissolved in 495 mL distilled water to which was added 5 mL glacial acetic acid.

Davidson's fixative solution: 1 part glycerin, 1 part glacial acetic acid, 2 parts 37% formaldehyde, 3 parts 95% ethanol, and 3 parts isotonic sodium chloride (usually 20 - 30‰).

3.0 SAMPLE COLLECTION AND FIXATION

3.1. SAMPLING

From 1986 to 1994, the same oysters were used for organic contaminant analysis and gonadal analysis at all Gulf coast sites (e.g., Powell *et al.*, 1993). The use of the same animals for gonadal analysis and for analysis of contaminant body burden potentially biases the latter analyses because digestive gland tissue and gonadal tissue, that contribute disproportionately to the tissue taken for histological analysis, may contain a higher than average body burden of certain contaminants. Sericano *et al.* (1993) showed that this source of error resulted in an underestimation of true body burden no greater than 10% when a 5-mm slice was removed from the large oysters normally sampled in the Mussel Watch Project. This potential error would be much larger for the smaller mytilids and dreissenids. To avoid this error, separate samples have always been obtained for gonadal analysis of mussels and East Coast oysters. Beginning in 1995, the same protocol was adopted for Gulf Coast oysters. The number of animals analyzed per site has also changed. Through 1994, 15 animals were analyzed per site. Thereafter, five were analyzed per site. Thus, the present sampling method requires the assumption that individuals collected from a common collection area will have experienced similar

chemical loading and that the five animals analyzed for gonadal analysis are representative of the animals pooled for contaminant analysis.

3.2. TISSUE PREPARATION

3.2.1. OYSTER TISSUE PREPARATION

Five of the 12 oysters processed for Dermo analysis (Ashton-Alcox *et al.*, this volume) are chosen randomly for gonadal analysis. A 5-mm thick cross-section of tissue is removed from the oyster using a scalpel or scissors. The determination of reproductive stage is based on a histological evaluation of the maturation stage of oyster gonads located within/around the visceral mass. The tissue section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gill tissue just posterior to the palps (Figure 1 in Ellis *et al.*, 1998). This aspect provides example sections of most oyster tissues for histopathological analysis (Kim *et al.*, this volume), while also providing a representative cross-section of oyster gonad. Each section is immediately placed in a tissue cassette and the cassette placed in a jar filled with Davidson's fixative for 48 hr. After 48 hr, the fixative is decanted, 70% ethanol added and the tissues are allowed to stand until processing.

3.2.2. MYTILID MUSSEL TISSUE PREPARATION

Determination of reproductive stage for mussels is based on a histological evaluation of the maturation stages of mussel gonads, most of which are located in the mantle (Newell, 1989). Five animals are analyzed but a few more are preserved for reasons discussed subsequently. The tip of a sharp knife is carefully inserted between the shells at the ventral lip and run dorsally between the shells until the posterior adductor muscle is cut so that the shells remain in an open position. Care is taken to cut no further than the adductor muscle to avoid cutting into the digestive gland immediately below the adductor muscle. Shucking of fresh mussels usually results in severe damage to the mantle tissue lying next to the shell. Therefore the mussels are placed whole in a wide-mouth jar filled with Davidson's fixative after the adductor muscle has been cut. Because the entire animal is being preserved, the specimens are left in fixative for at least a week to ensure preservation of all tissues. After this time, the fixative is decanted and 70% ethanol is added for storage until processing.

Once preserved, the tissue hardens and becomes easier to detach from the shell. To excise the preserved mussel meat from its shell, a knife is carefully run between the mantle and the lip of each valve, detaching the mantle from the shell. At this time, byssal threads are completely removed from the byssal gland to avoid later difficulties in tissue sectioning. Five specimens are chosen from each site and their anterior-posterior lengths are measured using a ruler. A 5-mm thick cross-section is then removed using a scalpel. The cross-section is obtained such that the dorsal-ventral aspect passes through the digestive gland and gills at an angle across the body and such that ventral edge of the cross-section is slightly towards the posterior-ventral margin. Each cross-section is placed in a tissue cassette and processed immediately after dissection. If the mantle tissue is damaged during the shucking procedure, the specimen is replaced by one of the additional

specimens preserved from the same site because the wound could result in the loss of gametic material and lead to an erroneous evaluation.

3.2.3. DREISSENID MUSSEL TISSUE PREPARATION

Most of the gonad of a dreissenid mussel is concentrated within the visceral mass (Borcherding, 1990). Due to their small size however, dissection of living tissue without destroying the gonads is difficult. Therefore, dreissenid mussels collected from each site are preserved whole in Davidson's fixative, without cutting the adductor muscle. They are left in fixative for one week to allow adequate time for tissue fixation. After this time, 20 to 30 mL of acetic acid is added to enhance decalcification of the shell. The shell is properly decalcified when it is no longer hard.

After decalcification, the Davidson's fixative is replaced with 70% ethanol according to the procedure followed for mytilid mussels and stored for later embedding. Prior to embedding, byssal threads are cut away from the byssal gland to minimize difficulty in sectioning the tissue. A 5-mm thick cross-section is taken from five individuals as described for mytilid mussels. Each section is placed in a tissue cassette and processed for embedding immediately thereafter.

4.0 SLIDE PREPARATION

4.1. TISSUE EMBEDDING

Individual tissue samples are prepared for embedding in paraffin using an established dehydration protocol (Table 1-1). The solutions used for dehydration, clearing, and infiltration are changed frequently to maintain solution purity. The tissue embedding sequence uses an automated tissue processor that processes tissue in plastic cassettes through the dehydration-clearing series and into paraffin. Embedding can also be done manually by moving the tissues through the sequence. The paraffin is melted in an embedding center with temperature set at 60°C. Newly melted paraffin should always be used in the final infiltration and embedding steps.

After the tissues are infiltrated with paraffin (Table 1-1), they are transferred to a vacuum infiltrator set at 60°C and placed under a vacuum for 30 min. Tissues are transferred to a holding tank of melted paraffin and removed singly to stainless steel molds. The tissues are oriented with the cross-sectional face down for sectioning, and a plastic mold embedding ring is placed on top. The ring is filled with paraffin and the mold moved to a cold plate of the embedding system. As the tissue/paraffin cools and hardens, the paraffin shrinks. Care must be taken to use sufficient paraffin to cover the tissue after this shrinkage. The mold is left on the cold plate until the tissue-paraffin block is removed. The block is then placed in a freezer until sectioning.

4.2.   TISSUE SECTIONING

The paraffin blocks are first cut at 20 μm to expose an entire tissue cross-section and then sliced at 5 μm using a microtome. Tissue sections may be cut singly or into contiguous sections. The sections are placed on the surface of a water bath maintained at 45-50°C and allowed to expand. Once the sections expand to full size, a microscope slide is held at an angle and slid under one or more of the tissue sections. The sections are then lifted out of the water and onto the slide. The sections are positioned on the slide in the orientation in which they will be stained and read. The slide is allowed to air dry until it can be placed in a slide rack. The slide rack is placed in a drying oven at 40°C. After drying overnight or longer, the slides are ready to stain.

Table 1-1. Tissue embedding sequence*.

Dehydration		Clearing	
Tissue Dry	60 min	Tissue Clear	60 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min	Tissue Clear	120 min
Tissue Dry	120 min		
Tissue Dry	120 min	**Infiltration**	
Tissue Dry	120 min		
Tissue Dry	60 min	paraffin	120 min
		paraffin	120 min
		paraffin (in vacuum infiltrator)	30min

* In cases where the sequential solutions are the same, each transfer is a transfer to a fresh solution.

4.3.   TISSUE STAINING

Sections are deparaffinized and hydrated using a xylenes-ethanol series (Table 1-2). Following hydration, slides are stained in a pentachrome series, dehydrated in a series of acetic acid dips followed by acetone, cleared in xyleness and mounted in Permount (Table 1-2). The pentachrome staining procedure is an adaptation of the trichrome stain of Masson (1928) as modified by Gurr (1956) (Ellis *et al.*, 1998b). The modifications include the addition of aniline blue to the fast green working solution, substitution of chromotrope 2R/orange G for Ponceau de Xylidene, and the addition of phosphotungstic acid prior to the orange G/chromotrope stain; the procedure is now a pentachrome technique. The addition of these stains yields better differentiation of tissue types and mucins. Times required for each step are flexible in both the staining procedures discussed here and in the previous embedding protocol. Different tissue types may require different times. All solutions, especially the xyleness and ethanol ones, should be changed frequently. Slides should not be allowed to dry during transfers. Solutions to

8

common embedding, sectioning, and staining problems are discussed in Preece (1972) and most other manuals of histological technique.

5.0 ANALYSIS

Each slide is examined microscopically to determine sex and stage of gonadal development. A histopathological examination can also be made at this time (Kim *et al*., this volume). Careful examination of early developmental stages is needed to positively distinguish males or females in early stages of development from individuals as yet undifferentiated. Occasional hermaphrodites will also be found (all target species normally have separate sexes). The stage in the gametogenic cycle is assigned based on the maturity of the follicles and gametes and a numerical value is assigned as described in Tables 1-3 and 1-4.

Cases of renewed gonadal development following spawning are common in oysters (stage 7), particularly along the Gulf of Mexico coast (Supan and Wilson, 2001). These animals typically have a few remaining large, mature ova and many developing ova that would normally be found in stages 3 or 4. Accordingly, for oysters, further data reduction can better be achieved by comparing the number of individuals with substantial gonadal development with those having little gonadal volume using an egg/eggless ratio, calculated as:

$$\text{Oyster egg/eggless ratio} = \frac{\text{the number of individuals at stages 3, 4, 5, 6 and 7}}{\text{the number of individuals at stages 1, 2 and 8}}$$

For mytilids and dreissenids, the analogous ratio is calculated as:

$$\text{Mussel egg/eggless ratio} = \frac{\text{the number of individuals at stages 3, 4 and 5}}{\text{the number of individuals at stages 0, 1 and 2}}$$

Abnormal gonadal development is commonly observed in mytilid mussels. This is often characterized by unusual development of gametes at the base of the follicles. The cells resemble those of a germinoma (Peters *et al.* 1994) and are differentiated from normal cells by being either enlarged or by appearing to have an enlarged nucleus. In other cases, underdeveloped, small gonadal follicles are observed. These occupy a smaller portion of the mantle tissue. Follicles may be filled with cellular debris (Figure 1-1; see also Figure 10 in Ellis *et al*., 1998a). Sometimes cells adhere to each other, forming accumulations and empty spaces among developing cells. Occasionally, fibrosis occurs, with proliferation of fibroblasts inside the follicles and in the interfollicular connective tissue. Abnormal gonadal development is often associated with degeneration of Leydig tissue around the follicles and hemocytic infiltration into the surrounding tissues (Figure 11 in Ellis *et al*., 1998). The approach used to score instances of abnormal gonadal

9

development uses a scale that rates the spatial coverage of the condition (e.g., fraction of follicles affected), but not the degree of effect in each follicle (Table 1-5). Normally, the entire follicle is completely affected or unaffected.

Table 1-2. Tissue staining sequence.

Deparaffinization
xylenes	5 min
xylenes	5 min
xylenes	5 min
100% ethanol	3 min
100% ethanol	2 min

Hydration
95% ethanol	2 min
10% ethanol	2 min
distilled water	2 min

Staining series
Ferric alum mordant	10 min
Running tap water	quick dip
Groat/Weigert Hematoxylin*	30-45 min
Running tap water	5 min
Acid Fuchsin stain	1.5 min
Running tap water**	5 min
Phosphotungstic acid	2 min
Orange G/Chromotrope stain	1.5 min
Running tap water**	5 min
Phosphomolybdic acid	2 min
Fast Green/Aniline Blue stain	3 min

Dehydration
1% acetic acid	20-25 dips
1% acetic acid	20-25 dips
1% acetic acid	20-25 dips
1% acid acetone	20-25 dips
1% acid acetone	20-25 dips
1% acid acetone	20-25 dips

Clearing
xylenes	5 min
xylenes	5 min

Mounting
Mounting in Permount	24 hr to dry

* A basic ethanol dip can be used to blue the hematoxylin, if necessary.
** At these steps, no stain should remain between the slides and holding grooves in the slide rack.

Table 1-3. Oyster development stages adapted from Ford and Figueras (1988) by Powell *et al*. (1993).

Developmental Stage	Value	Description
Sexually undifferentiated	1	Little or no gonadal tissue visible
Early development	2	Follicles beginning to expand
Mid development	3	Follicles expanded and beginning to coalesce; no mature gametes present
Late development	4	Follicles greatly expanded, and coalesced, but considerable connective tissue remaining; some mature gametes present
Fully developed	5	Most gametes mature; little connective tissue remaining
Spawning	6	Gametes visible in gonoducts
Spawned	7	Reduced number of gametes; some mature gametes still remaining; evidence of renewed reproductive activity
Spawned	8	Few or no gametes visible; gonadal tissue atrophying

Table 1-4. Mytilid and dreissenid development stages adapted from Seed (1975, 1976) by Hillman (1993).

Reproductive stage   Description

Resting/spent gonad

    Stage 0   Inactive or undifferentiated

Developing gonad

Stage 1	Gametogenesis has begun; no ripe gametes visible
Stage 2	Ripe gametes present; gonad developed to about one-third of its final size
Stage 3	Gonad increased in mass to about half the fully ripe condition; each follicle contains, in area, about equal proportions of ripe and developing gametes
Stage 4	Gametogenesis still progressing, follicles contain mainly ripe gametes

Ripe gonad

Stage 5	Gonad fully ripe, early stages of gametogenesis rare; follicles distended with ripe gametes; ova compacted into polygonal configurations; sperm with visible tails

Spawning gonad

Stage 4	Active emission has begun; sperm density reduced; ova rounded off as pressure within follicles is reduced
Stage 3	Gonad about half empty
Stage 2	Gonadal area reduced; follicles about one-third full of ripe gametes
Stage 1	Only residual gametes remain; some may be undergoing cytolysis

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Table 1-5. Semi-quantitative scale for abnormal gonadal development in mytilid mussels.

Score	Description
0	Normal gonad
1	Less than half the follicles are affected
2	About half the follicles are affected
3	More than half the follicles are affected
4	All follicles affected

6.0 CONCLUSIONS

The procedures described provide a semiquantitative ranking of reproductive stage but no quantification of the amount of gametic tissue present. The strengths of this approach are that it provides an assessment of sexual stage in the gametogenic cycle and allows for a concomitant histopathological analysis, with a single sample preparation protocol. The procedure cannot be performed on pooled samples. Thus, a direct correspondence between, for example, hydrocarbon body burden and stage in the gametogenic cycle may be difficult, because subsampling of individual animals will result in a certain degree of bias in the measurement of contaminant body burden, normally around 10% in adult oysters (Sericano *et al.*, 1993) and more for smaller individuals and species. This bias, therefore, will be size, contaminant, and time-of-year dependent**.**

If a quantitative gonadal/somatic index is desired, the technique of Choi and Powell (1993) should be used. The latter technique measures the concentration of egg protein present; however, it is not compatible with a concomitant histopathological analysis in that the standard histological preparation for assessing reproductive stage is not used in the quantitative analysis and tissue subsampling for histology cannot be done on the same individuals to be analyzed quantitatively for gonadal-somatic index. Choi *et al.* (1993) have further discussed the relative value of various approaches to gonadal evaluation. Overall, these authors found that the same general trends could be identified using either the semiquantitative or quantitative technique in most species, because normally an increase in gonadal volume occurs more or less simultaneously with advancement in reproductive stage and because gamete maturation occurs more or less evenly throughout the entire gonad.
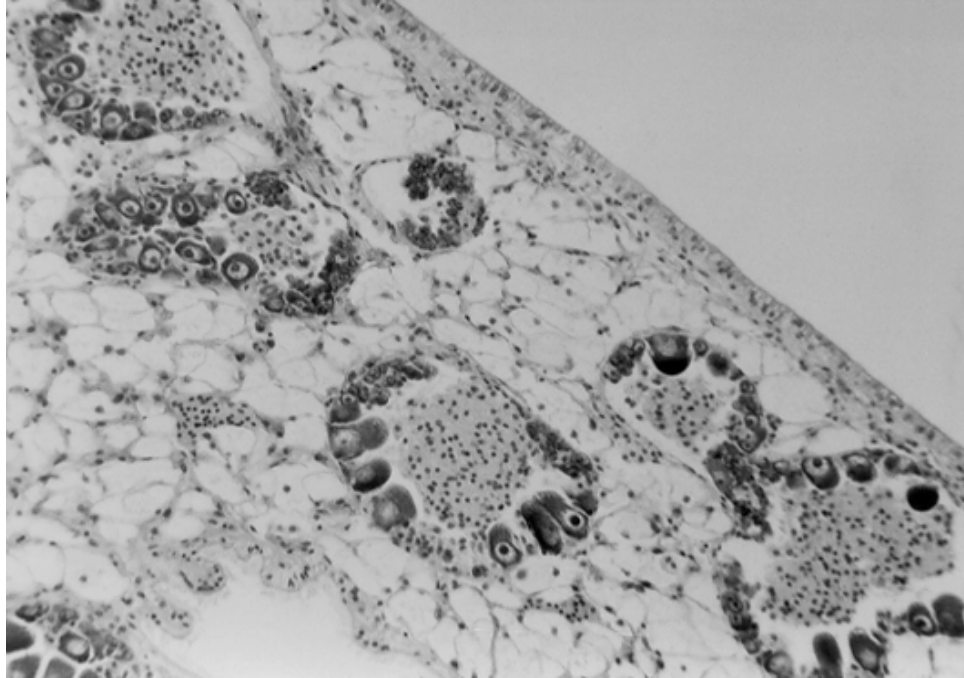
Figure 1-1. *Mytilus edulis* follicle with abnormal gametic tissue infiltrated with hemocytes.

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CHAPTER 2. HISTOPATHOLOGY ANALYSIS

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ABSTRACT

This chapter describes the procedures followed for histopathological analysis of oysters, mytilid mussels, and dreissenid mussels collected for NOAA's National Status and Trends Mussel Watch Project. Analyses are conducted on paraffin-embedded tissues sectioned at a 5-μm thickness and stained using a pentachrome staining procedure. The infection intensity of parasites, the occurrence and extent of tissue pathologies, and the intensity of diseases are recorded using quantitative or semi-quantitative measures.

1.0   INTRODUCTION

The use of bivalves in the National Status and Trends (NS&T) Program is predicated upon their reliability as environmental integrators of contamination. The influence of population health on body burden and, in turn, the influence of contaminant exposure on population health are, as yet, poorly understood. Clearly, certain diseases (normally caused by viruses or single-celled prokaryotes and eukaryotes) and non-disease causing parasites (mostly ciliates and multi-cellular parasites) produce tissue level changes that might be expected to affect contaminant body burden. Gonadal quantity, for example, can be dramatically altered by disease (Hofmann *et al.*, 1995; Barber, 1996; Ford and Figueras, 1988) and by parasites (Hopkins, 1957; Yoo and Kajihara, 1985). Certain contaminants are preferentially concentrated in gonadal tissue (Ellis *et al.*, 1993; Lee, 1993; Abbe *et al.*, 1994). Others are concentrated in non-gonadal tissue (Mo and Neilson, 1993; Cunningham and Tripp, 1975). Evidence, mostly by correlation, continues to mount for a relationship between certain tissue pathologies and contaminant exposure (Bowmer *et al.*, 1991; Weis *et al.*, 1995; Johnson *et al.*, 1992; MacKenzie *et al.*, 1995; Heinonen *et al.*, 1999) and the influence of contaminant exposure on the bivalve immune system has been described (Anderson *et al.*, 1992; Cheng, 1988; Winstead and Couch, 1988; Ashton-Alcox *et al.*, 2000). Besides contaminants, other environmental factors may facilitate disease or trigger the development of pathologies (e.g., Lee *et al.*, 1996; Landsberg, 1996; Zander, 1998) with significant consequences to tissue composition and, in all likelihood, subsequent contaminant retention. Thus, contaminant exposure and health, as they feed back upon each other, may have dramatic effects on monitoring programs that use sentinel organisms to define spatial and temporal trends in contaminant loading and contaminant gradients.

Evaluation of the health of bivalves collected as a part of the Mussel Watch Project necessitates determining the prevalence and intensity of diseases, parasites and pathologies by histological examination. Certain pathological conditions recognized by

shell condition (Warburton, 1958; Lawler and Aldrich, 1987), ligament degradation (Dungan *et al.*, 1989), or periostracal abnormalities (Davis and Barber, 1994) will not be identified using this approach and some ectoparasites are lost during collection (e.g., odostomians, White *et al.*, 1985, 1989). However most common diseases, parasites, and pathologies can be evaluated by this method. Specific assays are available now for some organisms (e.g., Ko *et al.*, 1999; Stokes and Burreson, 2001), but a histological examination remains the best general approach for simultaneously evaluating a wide range of parasites, disease and pathologies (Ford, 2002).

Classically, histological examination involves evaluating samples for parasite prevalence and the occurrence of pathologies. Measures of prevalence or occurrence, however, do not give a true indication of the health of an organism. Wilson-Ormond *et al.* (2000) compared the usefulness of prevalence to semi-quantitative and quantitative measures of intensity in the Gulf of Mexico Offshore Operations Monitoring Experiment and found that most significant trends were observed from intensity data rather than prevalence. One reason for this is that prevalence depends on transmission, and transmission rate may be controlled by biological factors such as population density, encounter rates, and inherent differences in susceptibility, as well as factors acting as stressors directly on the individuals in the population (Kermack and McKendrick, 1991; Ackerman *et al.*, 1984; Hofmann *et al.*, 1995; Powell *et al.*, 1996). Thus, measures of intensity or extent of tissue alteration may more reliably correspond to measures of exposure. Accordingly, beginning in 1995, a histopathological analysis designed to evaluate population health was included in the Mussel Watch Project. The approach taken was to evaluate the intensity of diseases and parasites, and the extent of tissue pathologies, rather than simply prevalence, to better assess the health of sampled populations. This description updates protocols described earlier by Ellis *et al.* (1998).

A measure of overall health has not been applied, although a number of these have been suggested, based on tissue appearance (Quick and Mackin, 1971), histological grading (Bowmer *et al.*, 1991), or summation of total parasite load (Wilson-Ormond *et al.*, 2000). Although Laird (1961) argues on theoretical grounds for an underlying relationship between total parasite body burden and environmental quality, generally, these overall measures of health have not proven efficacious because the various parasites, diseases and pathologies originate in different ways and certain parasites, even if abundant, may not have a large impact on organism health.

2.0 EQUIPMENT, REAGENTS, AND SOLUTIONS

Preparation of samples for histopathological analysis follows the protocols established for gonadal analysis (see Kim *et al.*, this volume).

3.0   ANALYSIS

Prepared slides are examined individually under the microscope using a 10X ocular and a 10X objective. If any tissue needs to be examined more closely, a 25X or 40X objective may be used for closer examination of suspected pathologies or parasites. Major tissue types examined include gill, mantle, gonad and gonoducts, digestive gland tubules,

stomach/digestive gland ducts, and connective tissue. Thus, a proper tissue cross-section is essential (Kim *et al*., this volume). As the histopathological analysis is done in concert with gonadal analysis (Kim *et al*., this volume), mytilid mussels are usually examined beginning with the gonads and mantle tissue to determine sex and stage of gonadal development. The gills and the visceral mass are then examined. The gonads of oysters and dreissenid mussels are located within/around the visceral mass, so gonads are examined first, followed by scanning of the visceral mass and gills.

Parasites, diseases, or tissue pathologies are scored for intensity using either a quantitative or semi-quantitative scale, as described subsequently (Table 2-1). Conditions scored quantitatively are evaluated by keeping a running count of incidences of the condition as the slide is scanned to avoid re-examining each slide multiple times for each category. Conditions scored semi-quantitatively may require re-scanning portions of the tissue for each category to fully evaluate the scale of infection.

Listed subsequently are the common parasites and pathologies encountered during histopathological analysis including, in each case, the method of analysis, a short description, and a reference to published figures of the condition. The list is not intended to be inclusive of all known parasites and pathologies for Mussel Watch species or all conditions encountered in specimens obtained during the Mussel Watch Project. Frequently, in routine examination, we do not attempt to differentiate at a low taxonomic level between related parasites because infection intensities are low for the majority of parasite species and so, the information gained from taxonomic analysis does not warrant the time spent in identification. Rather, we have lumped the various species into higher categories (e.g., all cestodes, all ciliates). When further differentiation is needed, we first differentiate by tissue of occurrence because most species have distinct tissue preferences. In nearly all cases, this level of differentiation has been adequate for estimates of prevalence and infection intensity.

3.1. QUANTITATIVE CATEGORIES

Most parasites are counted quantitatively (Table 2-1). These include prokaryotic inclusion bodies, gregarines, ciliates of various types, *Pseudoklossia* (a coccidian), cestodes, trematode metacercariae, worms ectoparasitic or commensal on the gills, nematodes, copepods, pinnotherid crabs, and worms in the gonoducts. We also evaluate a number of tissue conditions quantitatively, including the number of ceroid bodies, incidences of tissue inflammation, and suspected neoplasms and tumors.

Table 2-1. List of quantitative and semi-quantitative categories for each bivalve taxon. In a number of cases, e.g., gregarines and ciliates, subcategories by tissue type and organism morphology are individually tallied, as described in the text.

	Oyster	Mytilid Mussel	Dreissenid Mussel
Quantitative Category			
Prokaryote inclusions	X	X	
Gregarines	X	X	
Ciliates	X	X	
Xenomas	X	X	
Coccidians		X	
Cestodes	X		
Trematode metacercariae	X	X	
Turbellarians and nemerteans		X	X
Nematodes	X		X
Copepods	X	X	
Pinnotherid crabs	X	X	
Echinostomes	X		
Unidentified organisms	X	X	X
Ceroid bodies	X	X	X
Neoplasms	X		
Tissue inflammation	X	X	X
Tissue necrosis	X	X	X
Semi-quantitative Category			
Haplosporidium nelsoni (MSX)	X		
Trematode sporocysts	X	X	
Digestive tubule atrophy	X	X	X
Gonadal abnormalities (Kim *et al.*, this volume)	X	X	
Unusual digestive tubules	X	X	X

Prokaryotic inclusions (Figures 2-1 – 2-2) [Additional photographs: Otto *et al*. (1979), p. 295, Figs. 2-7; Gulka and Chang (1984), p. 320, Fig. 1; Couch (1985), p. 63, Fig. 2; Gauthier *et al*. (1990), p. 112, Fig. 7; Murchelano and MacLean (1990), p. 9, Figs. 1-5, 1-6; Figueras *et al*. (1991b), p. 20, Fig. 2; Harshbarger *et al*. (1977), p. 667, Fig. 1; Robledo *et al*. (1994), p. 291 Fig. 3, p. 292 Fig. 4; Villalba *et al*. (1997), p. 130, Figs. 2-3; Powell *et al*. (1999), p. 2059 Fig. 2, p. 2060 Fig. 4], variously referred to as rickettsial bodies, chlamydial bodies or mycoplasms, are normally observed in the duct and tubule walls of the digestive gland. In Mussel Watch sites, prokaryote inclusions have been recorded in both mytilid mussels and oysters from the East, West, and Gulf coasts, but have not been observed in dreissenids from the Great Lakes area. Prokaryotic inclusions similar to those described by Harshbarger *et al*. (1977) have been observed within the epithelial cells of the digestive system and also occasionally occur in the lumen of the digestive tract in mytilids from West coast Mussel Watch sites and in oysters from East and Gulf coast Mussel Watch sites. In some cases, cysts containing prokaryotic inclusions are associated with the gill and the renal tissues in mytilids. Inclusions found in the digestive tract are usually roundish, whereas those in the gill and kidney are rather amorphous in shape. No apparent pathological effects or host responses to prokaryote infection have been detected, as is typical for most bivalves (Otto *et al*., 1979; Figueras *et al*., 1991a; Villalba *et al*., 1997). Each individual inclusion is counted.

Gregarines in the genus *Nematopsis* (Figures 2-3 - 2-5) [Additional photographs: Cheng (1967), p. 148, Fig. 29; Ford (1988), p. 218, Fig. 6G; Friedman *et al*. (1989), p. 136, Fig. 3; Gauthier *et al*. (1990), p. 110, Figs. 2, 3] are sporozoan parasites frequently found in oysters, and occasionally in mytilid mussels. Different *Nematopsis* species often show a tissue preference for either mantle or gill (Sprague and Orr, 1952). Kim *et al*. (1998) noted that gregarines were common in oysters from the southeastern and Gulf of Mexico coasts in Mussel Watch samples, and also observed gregarines in mytilid mussels from the West coast. Mud and stone crabs are known to be final hosts (Prytherch, 1940). Although gregarine infections are known to have low pathogenicity, mechanical interference by heavy infections has been suggested to have some harmful effects on the host (e.g., oyster) physiology (Sindermann, 1990). In Mussel Watch samples, gregarine spores typically occur in the connective tissue around the visceral mass of the body, in the gills, and in the mantle connective tissues of oysters and West coast mytilids. No host tissue reaction or discernible pathological effects have been observed, in agreement with Cheng (1967). Although, species identifications are not made, gregarines are routinely scored according to tissue occurrence; body, gill, or mantle, following Landau and Galtsoff (1951). Quantification is obtained by counting each individual in each tissue within one representative tissue section.
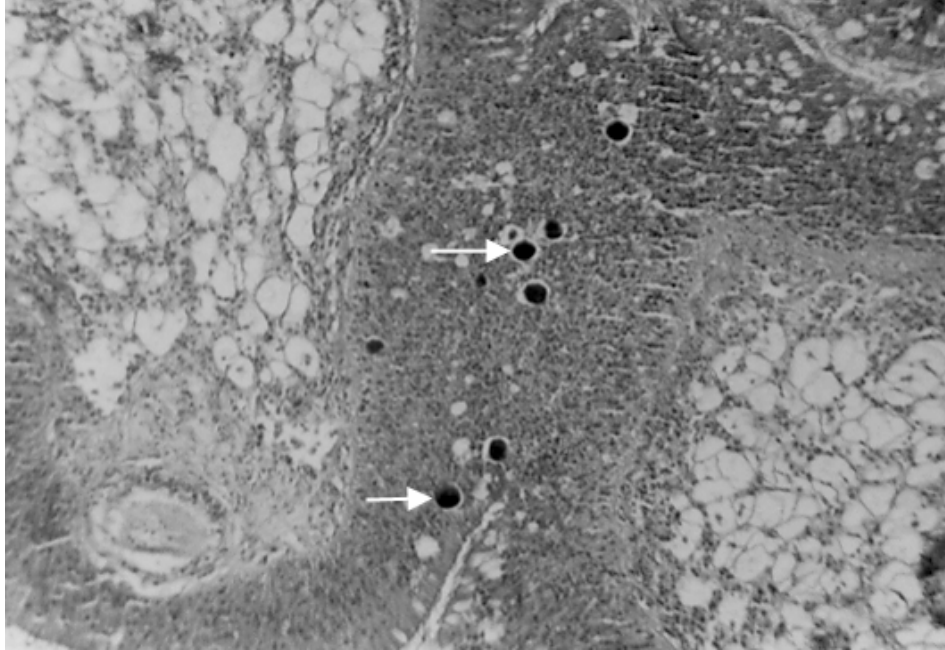
Figure 2-1. Prokaryotic inclusions present in digestive tract epithelium of an oyster, *Crassostrea virginica*. Arrows indicate examples. x 100.
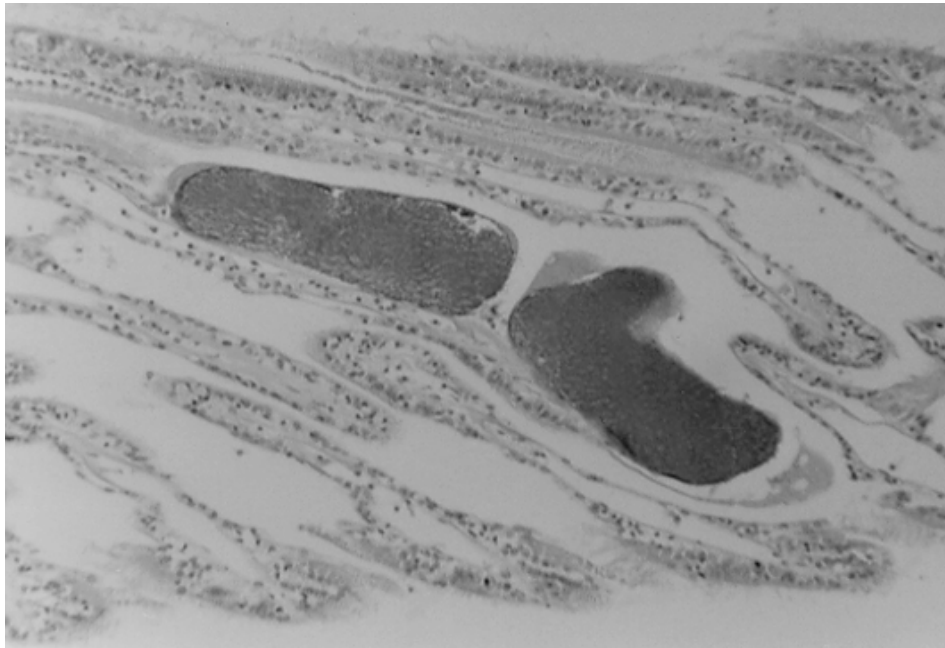
Figure 2-2. Cyst-like encapsulations of prokaryotic microorganism in gills of a mytilid mussel, *Mytilus edulis*. x 100.

Figure 2-3. *Nematopsis* sp. spores in connective tissue between digestive tubules of an oyster, *C. virginica*. Arrows indicate examples. x 100.
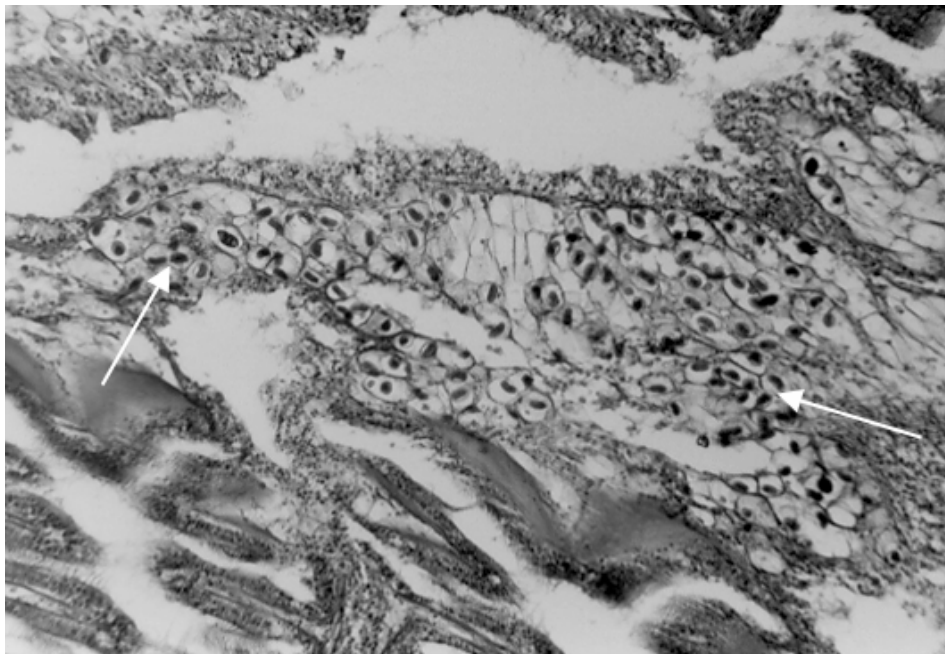
Figure 2-4. Numerous *Nematopsis* sp. spores in the gills of an oyster, *C. virginica*. Arrows indicate examples. x 100.
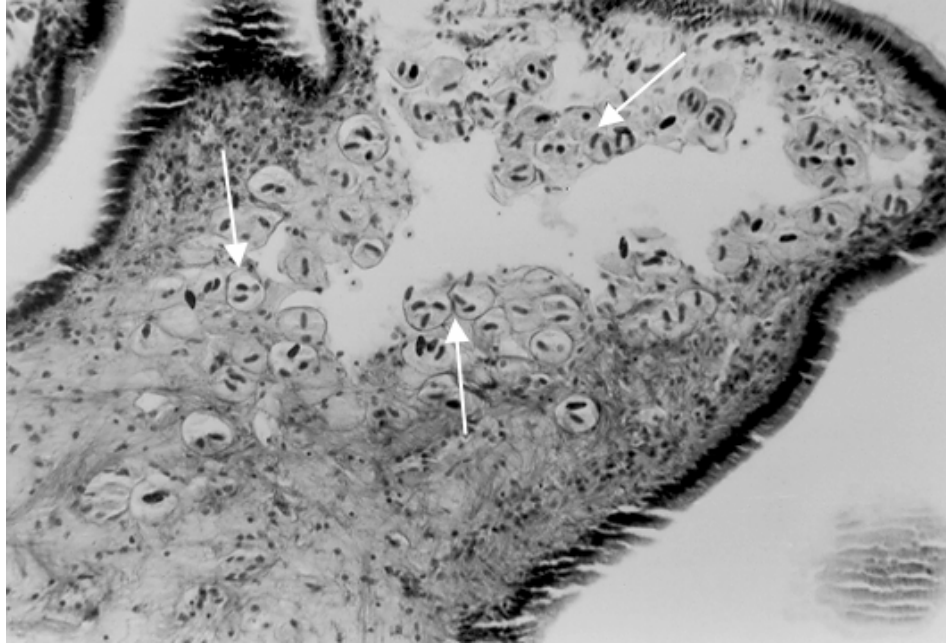
Figure 2-5. Gregarine-like spores near the tip of the mantle of a mytilid mussel, *M. californianus*. Arrows indicate examples. x 100.

A variety of ciliate types (Figures 2-6 - 2-7) [Additional photographs: Cheng (1967), p. 184, Fig. 88, p. 193, Fig. 102; Murchelano and MacLean (1990), p. 11 Fig. 1-11, p. 15 Figs. 1-19, 1-20; Figueras *et al.* (1991a), p. 91, Fig. 2; Gauthier *et al.* (1990), p. 110, Figs. 4-6; Villalba *et al.* (1997), p. 132, Figs. 10-11; Laruelle *et al.* (1999), p. 254-256, Figs. 1-3; Moret *et al.* (1999), p. 36, Fig. 1] have been observed in bivalves from Mussel Watch sites (Kim *et al.*, 1998), normally at low prevalence and more frequently in mytilid mussels than in oysters. Ciliate infections are observed in mytilids from the East and West coasts and in oysters from the East and Gulf coasts. Gill ciliates are one of the most commonly observed parasites. Ciliates occur in between gill filaments or are attached to gill surfaces of mytilids and oysters. Ciliates are also found in the gut lumen or attached to the digestive tract epithelia. Otto *et al.* (1979), Figueras *et al.* (1991a) and Villalba *et al.* (1997) reported no notable pathology in bivalves parasitized by ciliates and ciliate infections do not appear to elicit any obvious pathological conditions or host responses in Mussel Watch samples. Ciliates are quantitated by tissue type (e.g., gut, digestive gland, gill). Xenomas (Figure 2-8), cells distended with maturing ciliates, are tabulated separately. If a large xenoma has burst, the individual small ciliates are counted.
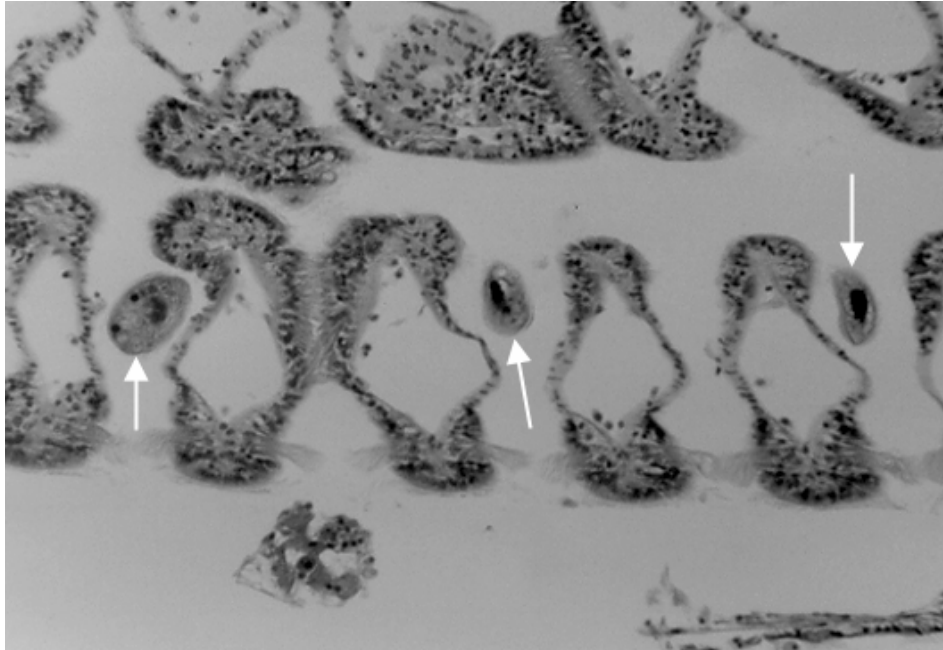
Figure 2-6. Ciliates between the gill filaments of a mytilid mussel, *M. edulis*. Arrows indicate examples. x 100.

Coccidians of the genus *Pseudoklossia* (Apicomplexa) [Photographs: Morado *et al.* (1984), p. 212 Figs. 7-10, p. 213 Figs. 11-16; Friedman *et al.* (1995), p. 35, Figs. 3-11; Villalba *et al.* (1997), p. 130, Figs. 5-6] are another protozoan parasite and are occasionally observed in the kidney of mytilid mussels at Mussel Watch sites. Each parasite is counted.

A variety of encysted larval cestodes (Figures 2-9 – 2-10) [Additional photographs: Cheng (1966a), p. 248 Fig. 6, p. 252 Figs. 1-6, p. 254 Figs. 1-2; Murchelano and MacLean (1990), p. 17, Fig. 1-21; Sindermann (1970), p. 128, Fig. 42] (for examples, see Cake, 1977; Cake and Menzel, 1980) have been observed. Encysted cestodes have been observed in either connective tissue around the digestive gland and gut or in the gills of oysters in Mussel Watch samples. None have been observed in mytilid mussels or dreissenids. From histological examination of their tissue location in oysters, cestodes presumably penetrate the gill or digestive epithelium of the host bivalve. Cestode infection does not seem to significantly damage the oyster. Cellular reaction to cestode cysts, characterized by encapsulation of larval cestodes by layers of connective tissue fibers (Cheng, 1966a; Sindermann, 1970), is routinely observed. Encapsulated larval cestodes normally appear to be disintegrating and to be in the process of resorption. Cestodes are quantified by tissue location (e.g., body, gill, mantle). Each occurrence observed is counted separately.
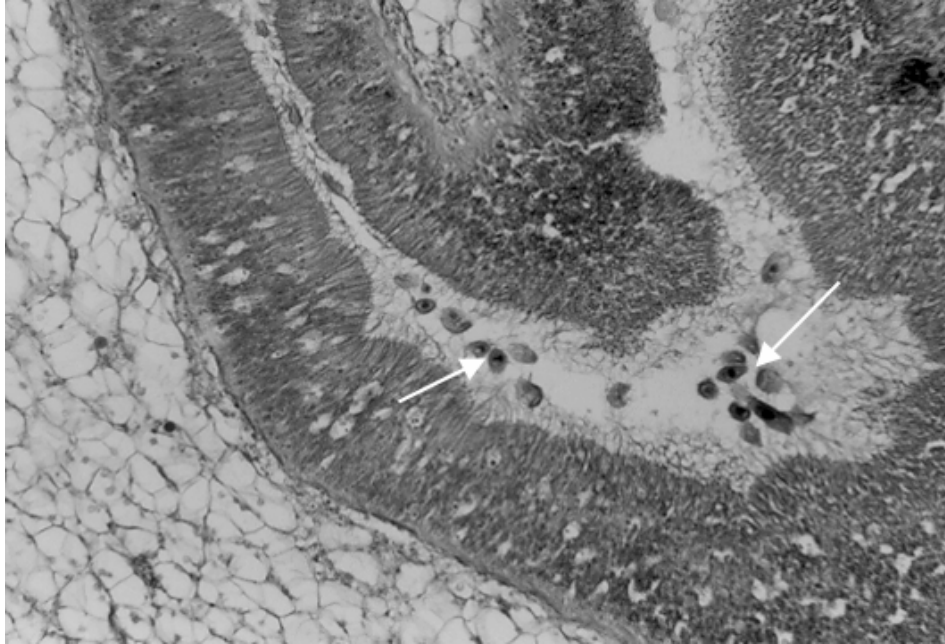
Figure 2-7. Ciliates in the lumen and attached to the epithelium of the intestine of an oyster, *C. virginica*. Arrows indicate examples. x 100.
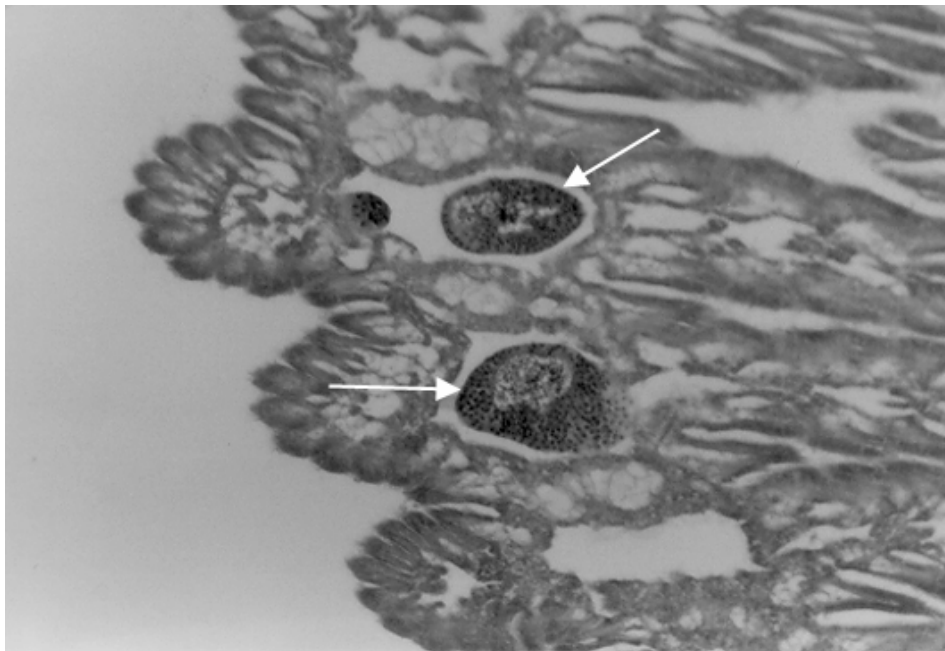
Figure 2-8. Ciliated xenomas on the gill surface of an oyster, *C. virginica*. Arrows indicate examples. x 100.

Metacercariae of trematodes (nearly all *Proctoeces* sp.) [Photographs: Little *et al.* (1969), p. 455, Fig. 1; Wolf *et al.* (1987), p. 380, Fig. 1; Tripp and Turner (1978), p. 77 Fig. 4, p. 79 Figs. 5-8, p. 81 Figs. 9-12; Winstead and Couch (1981), p. 297 Fig. 1, p. 299 Figs. 2-3] have been observed, normally at low prevalence, and occur in the mantle, foot, gonad/gonoduct and pericardial cavity of mytilid mussels and in the gonoduct of oysters at Mussel Watch sites. *Proctoeces* and occasional nematodes also found in the gonoduct should be distinguished from the echinostome metacercariae [Photograph: Ellis *et al.* (1998), p. 201, Fig. 1] observed in the gonoducts of oysters from the Gulf of Mexico (Winstead *et al.*, 1998). Encysted metacercariae (Figure 2-11) (presumably gymnophallids) [Additional photograph: Bower *et al.* (1994), p. 74, Fig. 62] are frequently observed in all tissues of mytilids: mantle, visceral connective tissue, foot, byssal gland and gill. In most cases, no conspicuous host response is observed. Hemocytes, however, occasionally infiltrate and surround the worms, especially those that are dead or dying. Each trematode occurrence is counted separately.

Gill nemerteans and turbellarians (Brun *et al.*, 1999) [Photographs: Villalba *et al.* (1997), p. 132, Figs. 12-13; Cáceres-Martínez *et al.* (1998), p. 218, Figs. 3-4] are occasionally seen between gill filaments. Whether these are commensal or ectoparasitic is unknown. Each cross-section observed is counted although one individual may be responsible for a number of tissue cross-sections. However, this method of quantification has proven effective even at high infection intensities (Wilson-Ormond *et al.*, 2000).
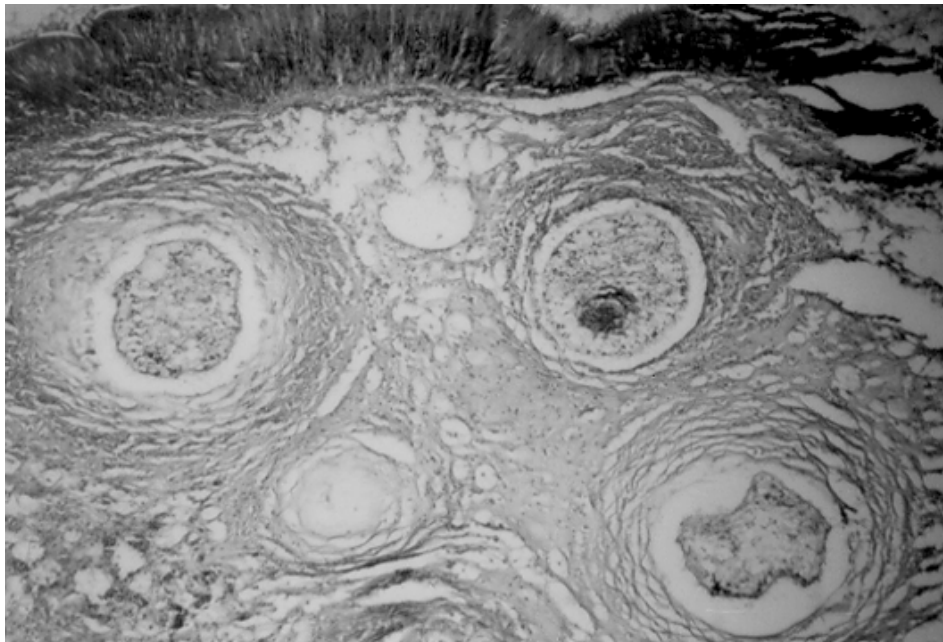
Figure 2-9. Encapsulated larval cestodes in the vesicular connective tissue surrounding the stomach of an oyster, *C. virginica*. x 63.
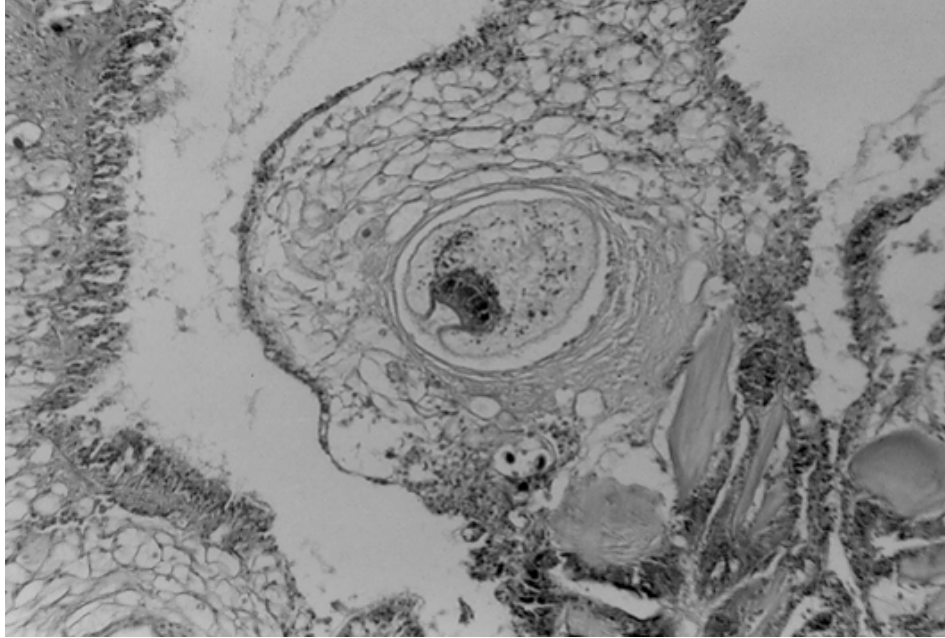
Figure 2-10. A larval cestode in the gill connective tissue of an oyster, *C. virginica*. x 100.

Nematodes (Figure 2-12) [Additional photographs: Cheng (1967), p. 264, Fig. 172; Lowe and Moore (1979), p. 140, Fig. 8; Gauthier *et al.* (1990), p. 112, Fig. 9; Murchelano and MacLean (1990), p. 19, Fig. 1-25; Sparks (1985), p. 375-376, Figs. 10-16] are occasionally observed parasitizing oysters from the East and Gulf coasts, and also dreissenids in Mussel Watch samples (Kim *et al.*, 1998). Nematodes reported in molluscs are usually larval stages (Cheng 1978; Lichtenfels *et al.*, 1980). Adults are found in predators of molluscs (Cheng, 1978), such as elasmobranchs (Millemann, 1963) and sea turtles (Berry and Cannon, 1981). Cheng (1966b) suggested that larval nematodes invade oysters via the digestive tract and migrate through tissues by way of blood vessels. In Mussel Watch samples, larval nematodes in oysters have been found localized in vesicular connective tissues around the region of the digestive gland, as described by Burton (1963) and Couch (1985), destroying adjacent host tissues. In some cases, a host cellular response, infiltration of hemocytes, is observed in association with the worm, as was reported by Couch (1985). Each individual cross-section is counted separately, although, like the nemerteans, a single individual may be responsible for a number of cross-sections.

Parasitic copepods [Photographs: Lowe and Moore (1979), p. 140, Fig. 7; Murchelano and MacLean (1990), p. 19, Fig. 1-26; Figueras *et al.* (1991b), p. 27, Fig. 6; Robledo *et al.* (1994), p. 295 Fig. 8; Villalba *et al.* (1997), p. 132, Fig. 15] are occasionally found in the gut lumen (Gee and Davey, 1986). Each occurrence is counted.
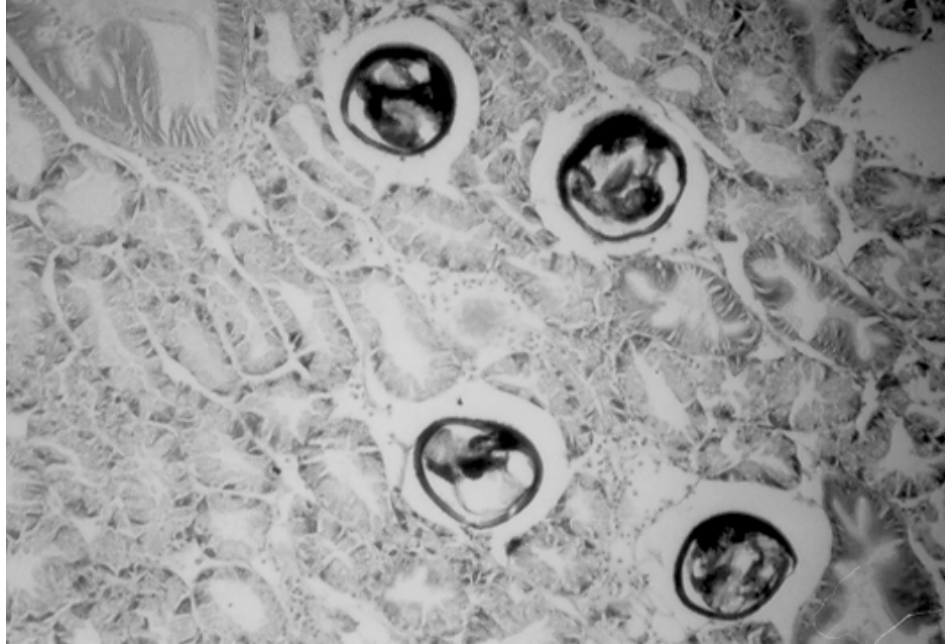
Figure 2-11. Cross-sections of metacercariae encysted in the visceral connective tissue of a mytilid mussel, *M. edulis*. x 63.

Figure 2-12. Sections of unidentified nematode larvae in the digestive gland connective tissue of an oyster, *C. virginica*. Arrows indicate examples. x 100.

Pinnotherid crabs [Photograph: Stauber (1945), p. 277, Fig. 23; Sandoz and Hopkins (1947), p. 257, Plate III] are occasionally found in the mantle cavity of oysters and mytilid mussels at Mussel Watch sites. Gill damage in infected hosts (Stauber, 1945; Christensen and McDermott, 1958; Haven, 1959) is frequently observed. Pinnotherid crabs also deprive the host of food (Stauber, 1945; Bierbaum and Shumway, 1988). Each occurrence is counted.

Ceroid bodies or brown cells [Photograph: Cheng and Burton (1965), p. 6, Fig. 5; Farley (1968), p. 590 Fig. 16, p. 592 Fig. 23; Murchelano and MacLean (1990), p. 11, Figs. 1-9, 1-12] are distinct brown-yellow aggregates that may occur in large clumps, and appear to be involved in metabolite accumulation and detoxification (Zaroogian and Yevich, 1993). Typically, they occur in greatest abundance in oysters, and in lesser numbers in mytilid mussels and dreissenid mussels. Quantification is obtained by counting each ceroid body. Occasionally, a ceroid body appears fractured or split; in this case only one fragment is counted.

Tumors and neoplasms (Figure 2-13) [Additional photographs: Murchelano and MacLean (1990), p. 19, Figs. 1-27, 1-28; Peters (1988), p. 81, Figs. C, E, F; Sparks (1985), p. 107 Fig. 27, p. 113 Fig. 42; Couch (1985), p. 69 Fig. 10, p. 70 Fig. 11; Figueras et al. (1991b), p. 30, Fig. 10; Villalba et al. (1997), p. 132, Fig. 16] are occasionally observed. Disseminated sarcomas, probably of hematopoietic origin, are particularly common in mytilid mussels in the Puget Sound region (e.g., Elston et al., 1990). The occurrence of neoplasms and tumors in oysters is extremely rare. Examples are described by Farley (1969, 1976), Harshbarger et al. (1979) and Ford and Tripp (1996). Neoplasms are occasionally observed in mytilids in Mussel Watch samples. Neoplastic cells with characteristic high nucleus-to-cytoplasm ratios (Ford et al., 1997) fill the vesicular connective tissues of the affected mytilids. All observed disseminated sarcomas have been seen in mytilids. For each specimen examined, neoplasms are recorded as either present or absent.

Cases of tissue inflammation (Figures 2-14 – 2-15) [Additional photographs: Farley (1968), p. 590, Fig. 17; Couch (1985), p. 65, Fig. 5; Figueras et al. (1991b), p. 28 Fig. 7, p. 29 Fig. 8; Murchelano and MacLean (1990), p. 11, Figs. 1-9, 1-10; Lowe and Moore (1979), p. 138, Figs. 1-4; Villalba et al. (1997), p. 132, Fig. 17; Sindermann (1970), p. 110, Fig. 34] characterized by intense infiltration of hemocytes may be focal or diffuse. The type of affected tissue and type of irritation responsible influences the nature of the cellular response (Ford and Tripp, 1996). Diffuse inflammation is differentiated from focal inflammation when the affected area does not appear to have a clear center or focal point of highest hemocyte concentration and hemocytes are abundant and distributed broadly over a large section of tissue. In Mussel Watch samples, most tissue inflammation, characterized by hemocytic infiltration, and most tissue necrosis, characterized by death or decay of cells and tissues, is observed in the visceral connective tissue and is sometimes associated with the presence of parasites. Granulocytomas (Figure 2-15), an inflammatory cellular condition characterized by clusters of hemocytes or the disintegration and sloughing of tissue, occur mainly in the digestive gland of mytilids, as was reported by Villalba et al. (1997). These tissue pathologies, focal

inflammation, diffuse inflammation and tissue necrosis, are tallied separately. Each affected area is counted.

3.2. SEMI-QUANTITATIVE CATEGORIES

Some conditions are assigned to a semi-quantitative scale related to the intensity or the extent of the affected area (Table 2-1). With one exception, so-called digestive gland atrophy, these are pathologies affecting large tissue areas, diseases characterized by systemic effects, or parasites for which individual counts are not feasible. Semi-quantitative categories include *Haplosporidium nelsoni* (MSX), trematode sporocysts, unusual digestive tubules, gonadal abnormalities (discussed in Kim *et al*., this volume), and digestive gland atrophy. *Perkinsus marinus*, an oyster parasite that is also assayed semi-quantitatively, is assayed by the more precise thioglycollate method, rather than by histology (Ashton-Alcox *et al*., this volume).
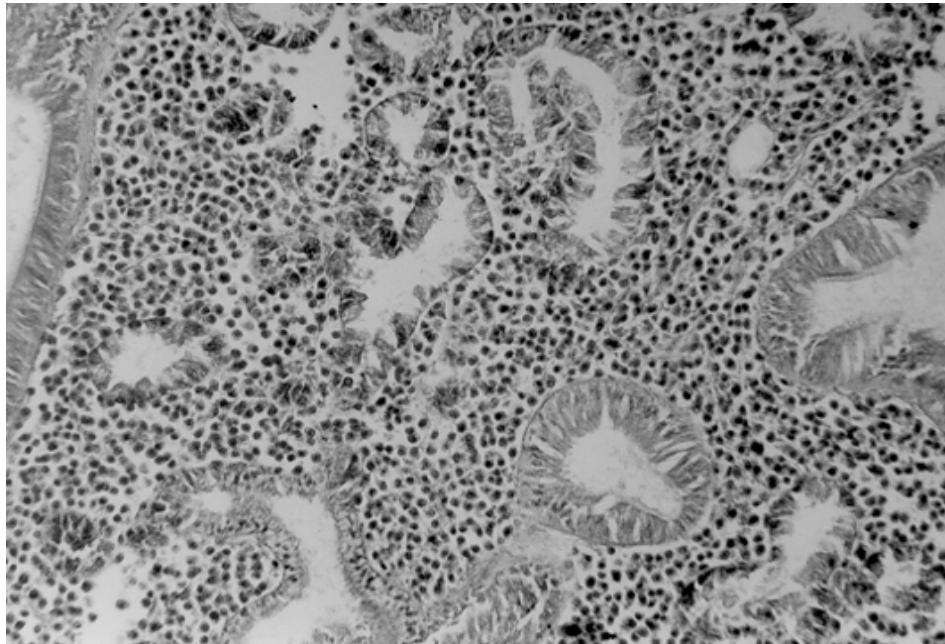
Figure 2-13. Neoplastic cells infiltrating the visceral connective tissue of a mytilid mussel, *M. edulis*. x 100.

Haplosporidium nelsoni (Figure 2-16) [Additional photographs: Farley (1968), p. 590 Fig. 13, p. 592 Fig. 21; Ford (1988), p. 214, Fig. 4; Ford and Tripp (1996), p. 617, Fig. 20], the haplosporidan protozoan responsible for MSX (multinucleated sphere X) disease in eastern oysters, was first reported in Delaware Bay oysters in the late 1950s (Haskin *et al*., 1965). *Haplosporidium nelsoni* was likely introduced from Japan (Burreson *et al*., 2000). It now ranges from Maine to Florida along the entire East coast (Kern, 1988; Ford and Tripp, 1996). Kim *et al*. (1998) observed *Haplosporidium nelsoni* in oysters from Delaware Bay to Georgia at Mussel Watch sites. Multinucleated plasmodia are observed in epithelial cells and the connective tissues of the gills and digestive tract.
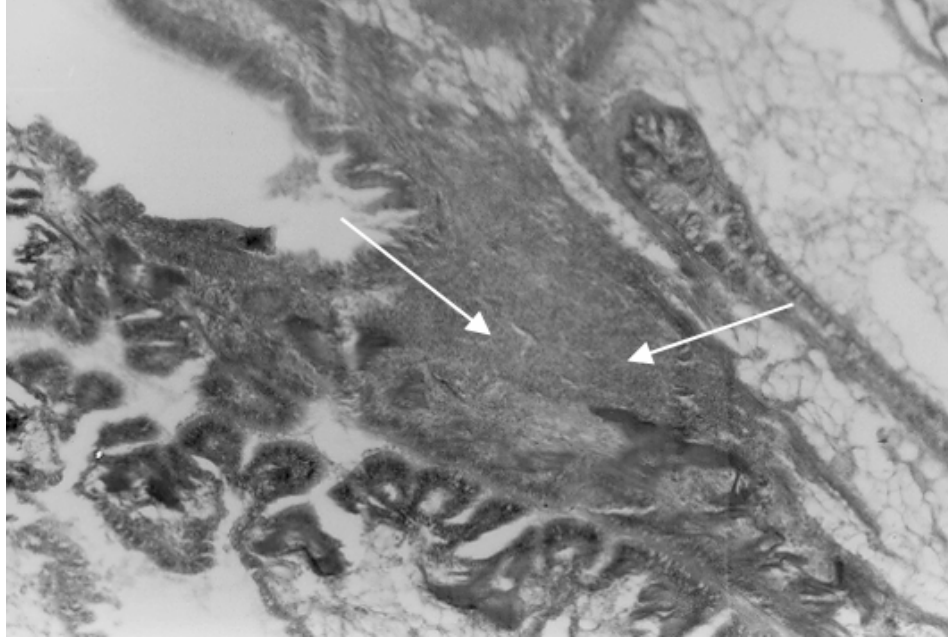
Figure 2-14. Hemocytic infiltration near the gill base of an oyster, *C. virginica*. Arrows indicate examples. x 100.
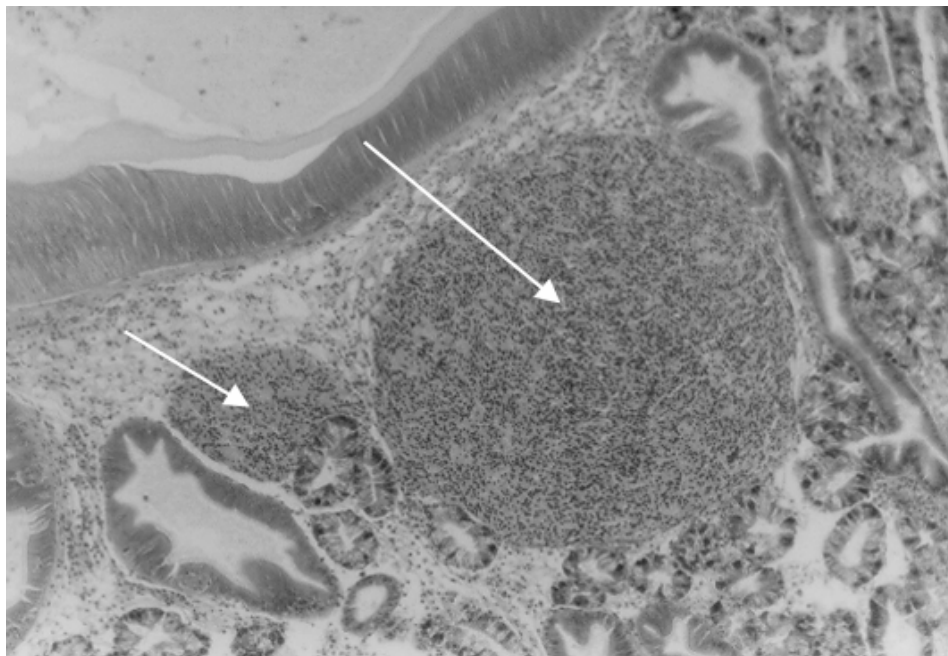
Figure 2-15. Granulocytomas in the digestive gland of a mytilid mussel, *M. edulis*. Arrows indicate examples. x 40.

Haplosporidium nelsoni infections start in the gill epithelium and are limited to this area at light infection levels. As the disease worsens, it becomes systemic and is eventually found throughout the visceral mass. MSX disease, especially heavy infections, is

associated with host hemocyte infiltration into the site of infection and tissue necrosis as observed by Farley (1968) and Ford (1985).

Because of the small size of *Haplosporidium nelsoni*, oyster tissues may need to be examined at a higher power than 10X. MSX infection normally starts in the gill epithelium so the gill tissue must be carefully examined to score early infections accurately. In cases where the disease has become systemic, examination of the visceral mass is necessary to score infection intensity. Grading MSX infection is a two-step process. First, the intensity of the infection is graded according to a semi-quantitative scale (Table 2-2) that records the location, parasite numbers, epithelial or systemic, and extent of infection in the gill tissue or the body tissue separately. In the second step, the two separate ratings are composited into a 0-4 scale (Table 2-3). To get the composite rating, the semi-quantitative rankings for the gill and the body are applied to the matrix in

Table 2-2. Semi-quantitative scale for *Haplosporidium nelsoni* infection modified from Ford (1985, 1986), and Ford and Figueras (1988).

0	Uninfected, no parasites found in the tissue cross-section
1	Parasites confined to gill or digestive tract epithelial tissue, ≤ 10 plasmodia per 100X field of either gill or body tissue
2	Parasites restricted to gill or digestive tract epithelial tissue, Very light infection, $11 \leq$ plasmodia ≤ 100 per 100X field of either gill or body tissue
3	Parasites spreading into gill or digestive tract subepithelium, parasites restricted to epithelium and subepithelium area, > 100 plasmodia per 100X field of either gill or body tissue but < 1 per 1000X oil immersion field
4	Parasites more evenly distributed in gill or digestive tract subepithelium and scattered through systemic tissue, > 100 per 100X field of either gill or body tissue but 1 to ≤ 10 per 1000X oil immersion field
5	Moderate systemic infection, averaging 11 to ≤ 20 parasites per 1000X oil immersion field
6	Heavy systemic infection, averaging > 20 parasites per 1000X oil immersion field

Table 2-3. Composite rating matrix for *Haplosporidium nelsoni* infection.

Composite rating scheme

		Body						
		0	1	2	3	4	5	6
	0	0	1	1	2			
	1	1	1	1	2			
	2	1	1	1	2	2		
Gill	3	2	2	2	3	3		
	4	2	2	3	3	3	4	
	5	2	3	3	3	4	4	4
	6				3	4	4	4
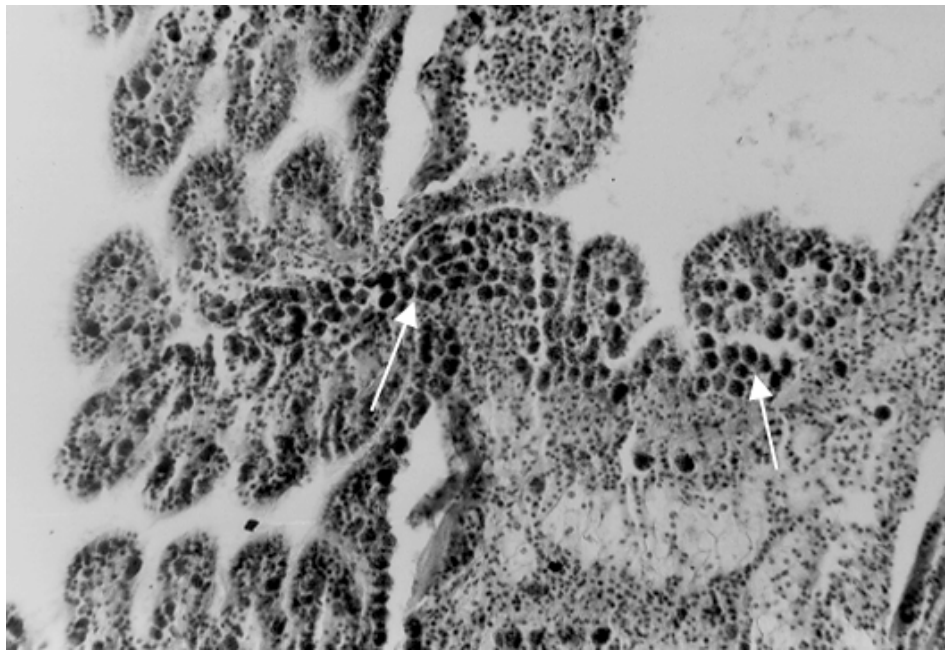
Figure 2-16. Numerous multinucleated plasmodia of *Haplosporidium nelsoni* in the gills of an oyster, *C. virginica*. Arrows point to example parasites. x 100.

Trematode sporocysts of the families Fellodistomidae and Bucephalidae (Figures 2-17 – 2-18) [Additional photographs: Ellis *et al*. (1998), p. 207-208, Figs. 7-9; Cheng and Burton (1965), p. 6 Figs. 1-4, p. 8 Figs. 10-12, p. 10 Figs. 14-17; Tripp and Turner (1978), p. 77, Figs. 1-3; Gauthier *et al.* (1990), p. 112, Fig. 8; Murchelano and MacLean (1990), p. 17, Fig. 1-23; Sindermann (1970), p. 125, Fig. 41; Figueras *et al*. (1991a), p. 92, Fig. 3; Davids and Kraak (1993), p. 751, Fig. 1; Robledo *et al*. (1994), p. 294, Fig. 7; Villalba *et al*. (1997), p. 132, Fig. 14; Powell *et al*. (1999), p. 2061 Fig. 5] occur principally in the gonadal tissue of oysters (Hopkins, 1957) and mytilid mussels. Kim *et al*. (1998) reported trematode sporocyst infections in East and West coast mytilid mussels and in Gulf oysters from Mussel Watch sites. Carnivorous fish are the final host of bucephalid trematodes (Hopkins, 1954). Fellodistomid trematodes of the genus *Proctoeces* can complete their entire life cycle in a single invertebrate host (e.g., mytilid mussels). Thus, they have a unique life cycle involving molluscs as regular final hosts and bottom fishes as alternative final or as post-cycle hosts (Stunkard and Uzmann, 1959). In Mussel Watch samples, trematodes that have invasive and ramifying sporocysts occur principally in the visceral connective tissues of the digestive gland and the gonadal tissue, destroying gametic tissue and often causing host sterilization. Sindermann (1990) noted that sterilization and tissue destruction are the principal result of the sporocyst invasion. Sterilization is normally observed in infected individuals in Mussel Watch samples (Hillman *et al*., 1988). In extensive and advanced stages of infection, sporocysts infiltrate the gill, mantle and other tissues (see also Cheng and Burton, 1965). Little or no apparent aggregation of host hemocytes around healthy sporocysts and no other evident host reactions have been observed in Mussel Watch samples, which concurs with Cheng and Burton (1965). However, infiltration of hemocytes is occasionally observed around dead or degenerating parasites in Mussel Watch samples, as was reported by Teia dos Santos and Coimbra (1995). The large branching sporocysts are difficult to quantify. Hence, infection intensity is scored on a semi-quantitative scale (Table 2-4). Pictorial examples of the rating scale include Figure 2-17 (scored 1) and Figure 2-18 (scored 2).

Table 2-4. Semi-quantitative scale for trematode sporocyst infection.

Score	Description
0	Uninfected
1	Present in the gonads only (some gametic tissue still present)
2	Completely filling the gonads (no gametic tissue present); may be present in digestive gland or gills in very limited amount
3	Completely filling the gonads; extensive invasion of the digestive gland and/or the gills
4	Completely filling the gonad; substantially filling the digestive gland or gill; individuals appear to be a sac of sporocyst
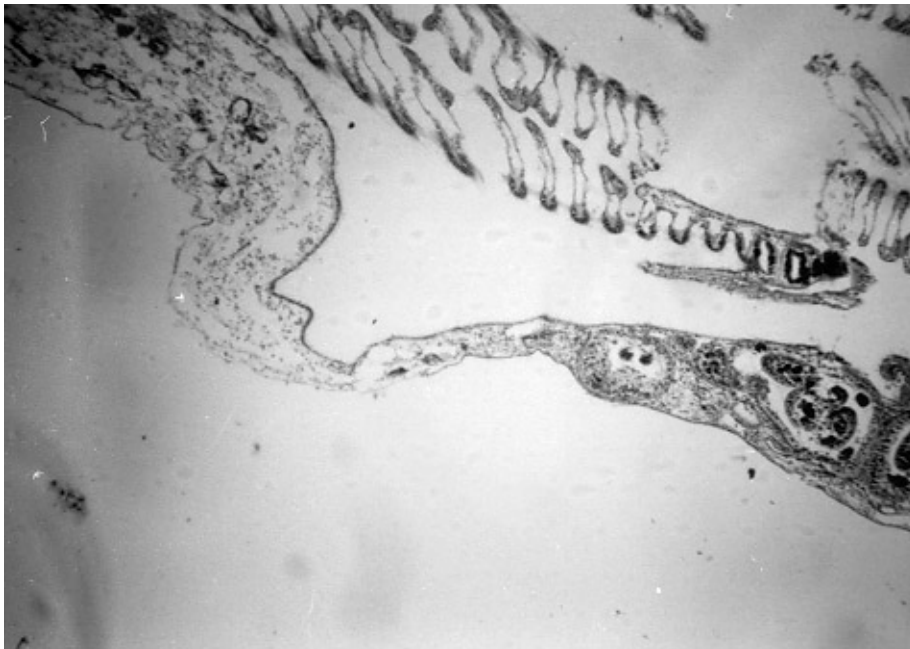
Figure 2-17. This trematode sporocyst infection in *Mytilus edulis* scored a 1 according to Table 2-4. Some gametic tissue is still present.
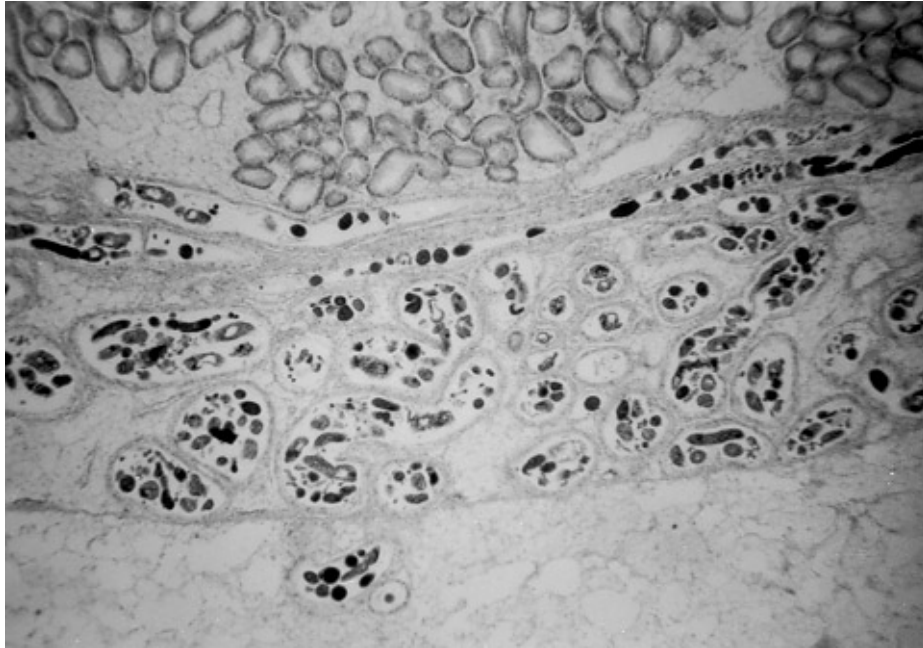
Figure 2-18. This trematode sporocyst infection in *Mytilus edulis* scored a 2 according to Table 2-4. No gametic tissue is present.

Digestive gland atrophy (Figures 2-19 – 2-21 reproduced from Figures 4-6 in Ellis *et al.*, 1998) [Additional photographs: Ellis *et al.* (1998), p. 205-206, Figs. 4-6; Couch (1985), p. 66, Fig. 6; Gauthier *et al.* (1990), p. 112, Fig. 10; Winstead (1995), p. 107, Figs. 3-4], a condition characterized by the thinning of the digestive tubule walls, has been described in a number of bivalve species (Bielefeld, 1991; Marigómez *et al.*, 1990; Axiak *et al.*, 1988). Causes of the condition have been ascribed to a variety of stressors including exposure to contaminants and variations in food supply. Winstead (1995) found that poor nutrition was a key element in producing the condition in oysters and that the digestive gland recovered to its normal state relatively rapidly once food supply improved. It is, therefore, not necessarily a pathology. The digestive gland is scanned for tubules showing evidence of epithelial thinning. The average degree of thinning is assigned a numerical rating (Table 2-5). The semi-quantitative assessment permits the reading of many samples in a short time. For increased accuracy, the ratio of tubule diameter to wall diameter (e.g., Winstead, 1995) or a direct measure of wall thickness (Marigómez *et al.*, 1990) can be used.

Table 2-5. Semi-quantitative scale for digestive gland atrophy.

Score	Description
0	Normal wall thickness in most tubules (0% atrophy), lumen nearly occluded, few tubules even slightly atrophied
1	Average wall thickness less than normal, but greater than one-half normal thickness, most tubules showing some atrophy, some tubules still normal
2	Wall thickness averaging about one-half as thick as normal
3	Wall thickness less than one-half of normal, most tubules walls significantly atrophied, some walls extremely thin (fully atrophied)
4	Wall extremely thin (100% atrophied), nearly all tubules affected
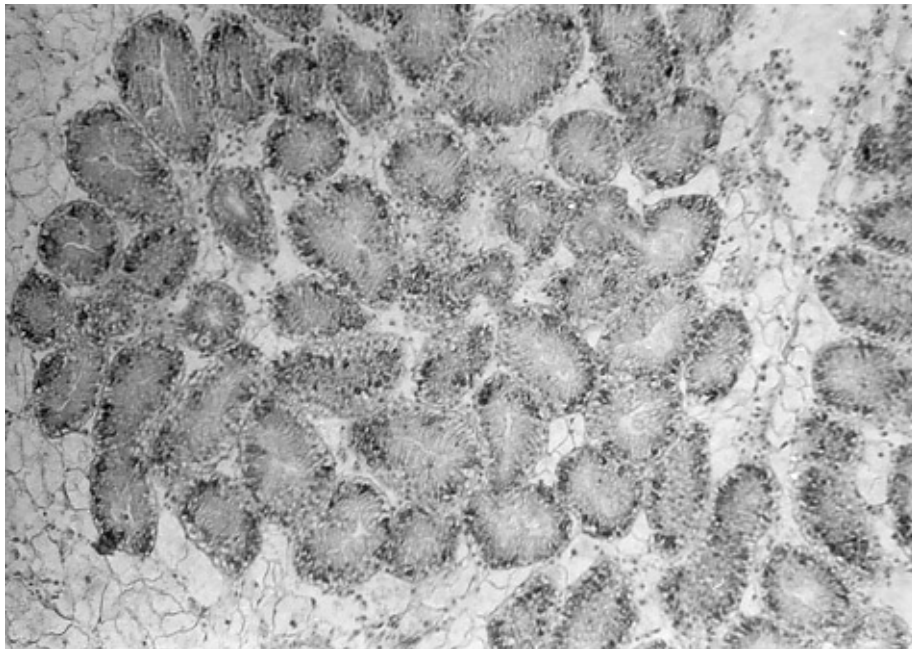
Figure 2-19. *Crassostrea virginica* normal digestive tubule, scored a 0 according to Table 2-5.
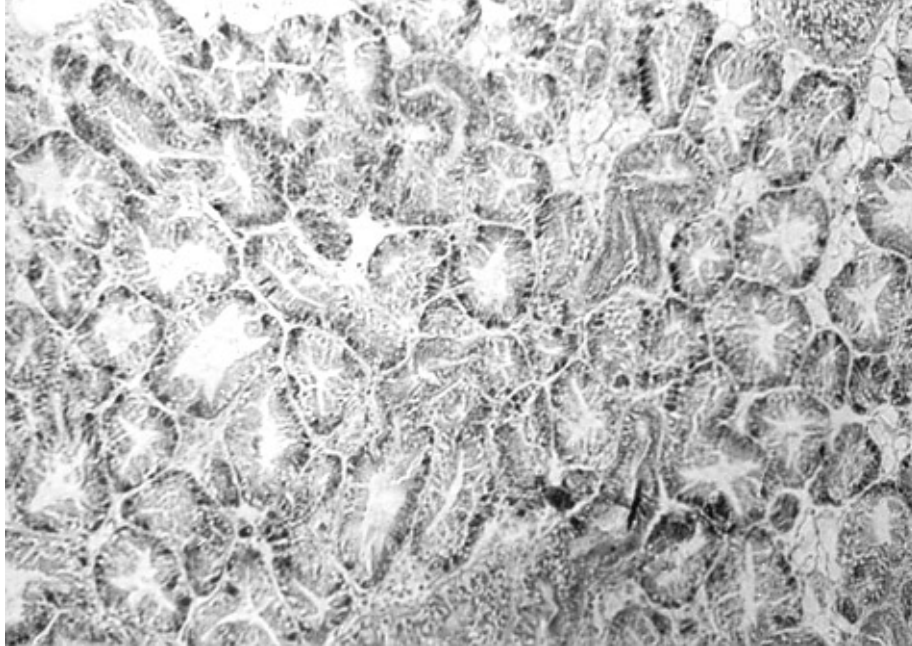
Figure 2-20. Digestive gland atrophy in *Crassostrea virginica* scored a 2 according to Table 2-5.
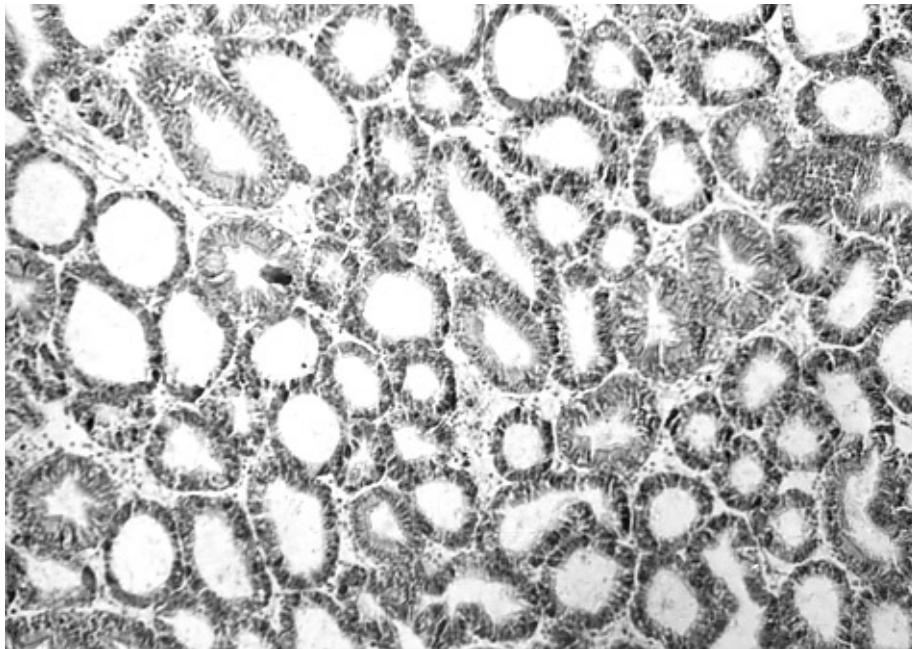
Figure 2-21. Digestive gland atrophy in *Crassostrea virginica* scored a 4 according to Table 2-5.

In Mussel Watch samples, degenerated and/or necrotic digestive glands (Figure 2-22) [Additional photograph: Couch (1985), p. 67, Fig. 7] were frequently observed, particularly in mytilids. This condition is characterized by digestive tubules in unusually poor condition with loss of their normal integrity and structure, and sometimes with vacuolated epithelium. Individual digestive tubules are sometimes not discernible from each other. For each specimen examined, unusual digestive tubules are recorded as either present or absent.

3.3.   SUMMARY STATISTICS

Three descriptions of parasite distribution are used: prevalence, infection intensity and weighted prevalence (Ford, 1988). Prevalence describes the proportion of individuals in the population that are infected by a specific parasite or pathology and is calculated as:

$$\text{prevalence} = \frac{\text{number of hosts with parasite or pathology}}{\text{number of hosts analyzed}}$$

Infection intensity is calculated as the average number of occurrences of the parasite or pathology in infected hosts. This is a measure of the intensity of infection in infected individuals.

$$\text{infection intensity} = \frac{\text{total number of occurrences of parasite or pathology}}{\text{number of hosts with parasite or pathology}}$$

Weighted prevalence or mean abundance (Bush *et al.*, 1997; Rózsa *et al.*, 2000) is the multiple of prevalence and infection intensity, and is a measure of the relative severity of infection within the population. Weighted prevalence is calculated as:

$$\text{weighted prevalence} = \frac{\text{total number of occurrences of parasite or pathology}}{\text{number of hosts analyzed}}$$
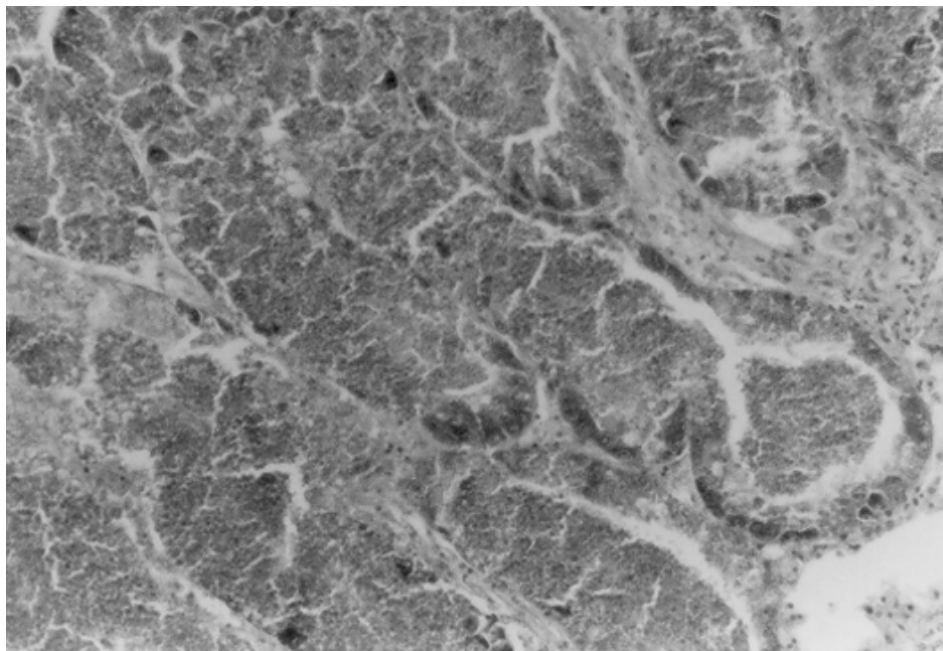
Figure 2-22. Unusual digestive tubules of a mytilid mussel, *M. edulis*. Note the absence of normal tubule structure as shown in Figures 2-20 and 2-21. x 100.

4.0 CONCLUSIONS

The described techniques provide the quantitative and semi-quantitative methods used to determine the prevalence and infection intensity of parasites, pathologies, and diseases affecting oysters, mytilid mussels, and dreissenid mussels in the Mussel Watch Project. The described histopathological method is an approach that targets a wide range of parasites and pathologies. Specific conditions are often better assessed by other methods [e.g., *Perkinsus marinus* infection in oysters (Ashton-Alcox *et al.*, this volume)]. The described method emphasizes the quantification of infection intensity. Prevalence rarely provides an adequate description of the population dynamics of disease and, in practice, often yields ambiguous results. Infection intensity as quantified by direct counts or the use of semi-quantitative scales consistently provides a more robust data set for statistical analysis comparing the spatial and temporal distribution of parasites, pathologies, and diseases to contaminant body burden.

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CHAPTER 3. *PERKINSUS MARINUS* ASSAY

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ABSTRACT

This chapter describes the procedures followed for the assay of *Perkinsus marinus* (etiological agent of Dermo disease) presence and infection intensity in oysters collected on the East and Gulf Coasts of the US and in Puerto Rico for NOAA's National Status and Trends Mussel Watch Project. Analyses are conducted on mantle tissue excised from the oyster and placed in Ray's Fluid Thioglycollate Medium (RFTM) to enlarge *P. marinus* present in the tissue. The enlarged cells are stained by the addition of Lugol's iodine solution. The tissue is examined microscopically and rated on a semi-quantitative scale.

1.0   INTRODUCTION

Perkinsus marinus is the most widespread pathogen of East and Gulf coast oysters and is the etiologic agent of Dermo disease [see Ford and Tripp (1996) for review]. Once considered a form of fungus (Mackin *et al,*. 1950), this protozoan parasite is now generally considered to be a member of the Apicomplexa (Levine, 1978). However, Reece *et al.* (1997) have recently emphasized the parasite's dinoflagellate affinities so that its phylogenetic placement remains controversial. Dermo is transmitted from oyster to oyster (Perkins and Menzel, 1966) and causes significant mortalities in oyster populations. Powell *et al.* (1992) and Kim *et al.* (1999) have considered the spatial distribution and temporal trend in *P. marinus* prevalence and infection intensity at Mussel Watch sites. Recent research continues to support the likely relationship of Dermo disease to environmental stress (Powell *et al.*, 1996; Lenihan *et al.*, 1999), including contaminant exposure (Chu, 1999; Fisher *et al.*, 1999; Chu and Hale, 1994; Wilson *et al.*, 1990; Winstead and Couch, 1988; Scott *et al.*, 1985). Most recently, Tall *et al.* (1999) have suggested a possible relationship between Dermo disease and *Vibrio vulnificus,* an important source of infection in susceptible humans eating raw oysters (Shapiro *et al.*, 1998).

The determination of the health of oyster populations requires an analysis of *P. marinus* prevalence and infection intensity. The standard method for monitoring purposes is the one developed by Ray (1966). Briefly, a section of mantle tissue is incubated in Ray's Fluid Thioglycollate Medium (RFTM) for 7 days under anaerobic, dark conditions. In the RFTM, the *P. marinus* cells enlarge to form thick-walled hypnospores but do not replicate (Ray, 1954; Stein and Mackin 1957). The hypnospores are stained with Lugol's iodine and a semi-quantitative (Craig *et al.*, 1989) assessment is made microscopically. A

quantitative estimate can be derived from the semi-quantitative designation using the formula found in Choi *et al.* (1989).

2.0   EQUIPMENT AND SUPPLIES

2.1.   REAGENTS

2.1.1. CHEMICALS

Denatured alcohol (for burner and sterilization), A962P-4. Fisher Scientific, Pittsburgh, PA.

Fluid thioglycollate medium (FTM), powder, T9032. Sigma Chemical Company, St. Louis, MO.

Iodine, (I_2) [7553-56-2], I3380, purity 99%. Sigma Chemical Company, St. Louis, MO.

Penicillin ($C_{16}H_{17}N_2O_4SNa$) [69-57-8], P3032. Sigma Chemical Company, St. Louis, MO.

Potassium iodide (KI) [7681-11-0], P8256. Sigma Chemical Company, St. Louis, MO.

Sodium chloride (NaCl) [7647-14-5] S-271. Fisher Scientific, Pittsburgh, PA.

Streptomycin sulfate ($C_{21}H_{39}N_7O_{12})_2$-($H_2SO_4)_3$ [3810-74-0] BP910-50. Fisher Biotech, Pittsburgh, PA.

2.1.2. SOLUTIONS

2.1.2.1.   Ray's (1952) Thioglycollate medium preparation

A mixture of 22 g NaCl, 29.3 g dehydrated Fluid Thioglycollate Medium (FTM), and 1 L distilled water is heated while stirring until the medium dissolves and the solution boils and becomes a transparent golden-yellow color. After cooling, the solution is dispensed, 5 mL at a time, into 15-mL culture tubes which are subsequently autoclaved and sealed. They are kept in the dark until use. Unused, autoclaved tubes of RFTM can be stored for many months in the dark without deterioration. Discard them if they become cloudy or the RFTM congeals.

RFTM maintains anaerobic conditions in the culture tube as well as providing needed nutrients and an appropriate osmotic environment. Therefore, tubes are sealed tightly and opened only briefly for addition of antibiotic and tissue as described below. After tissue is added, tubes are sealed and returned immediately to the dark for tissue incubation.

2.1.2.2.   Antibiotic solution

A 0.33 g quantity of Streptomycin sulfate and 0.159 g of Penicillin G are added to 500 mL of sterilized, deionized water and shaken lightly until the powder is dissolved. The

solution is kept refrigerated until use. The solution can be stored safely for several months if refrigerated.

2.1.2.3. Lugol's iodine solution

Four grams of potassium iodide and 2 g of iodine crystals are dissolved in 100 mL of distilled water, allowed to stand for 24 h, and filtered. The solution is stored in a dark bottle at room temperature to avoid particle precipitation. The solution remains stable for many weeks but should be filtered occasionally to remove particles that may precipitate. These particles may be confused with *P. marinus* hypnospores by less experienced slide readers although they are disks, not spheres; they are always black, never blue; and they are birefringent (Bushek *et al.,* 1994).

2.2. EQUIPMENT

Alcohol lamp

Autoclave

Automatic repipet, Barnstead/Thermolyne Repipet II (9010), 13-687-62B. Fisher Scientific, Pittsburgh, PA.

Coverslips, various sizes, eg. 12-545-B. Fisher Scientific, Pittsburgh, PA.

Culture tubes with Screw Cap, Pyrex, 14-932A. Fisher Scientific, Pittsburgh, PA.

Dissecting tools- scissors, scalpels, probes, forceps

Heater/stirrer, 11-500-7SH. Fisher Scientific, Pittsburgh, PA.

Microscope, Carl Zeiss Standard KF2, 12-070-273. Fisher Scientific, Pittsburgh, PA.

Microscope slides, Fisherbrand Frosted, 12-550-34. Fisher Scientific, Pittsburgh, PA.

Oyster knives Dexter/Russell "Sani-Safe" S134. Memphis Net & Twine Co., Inc. Memphis, TN.

Pasteur pipets, 13-678-20A. Fisher Scientific, Pittsburgh, PA.

Repipet, 1 mL capacity, adjustable volume, P-1000. Rainin Instrument Co., Woburn, MA.

Repipet tips, Redi-Tip Universal, 21-197-8F. Fisher Scientific, Pittsburgh, PA.

Vortex mixer, Vortex-Genie 2 Model G-560, 12-812. Fisher Scientific, Pittsburgh, PA.

3.0 TISSUE COLLECTION

Twelve oysters are randomly selected from each group of 20-25 collected per site. The anterior-posterior length (Morales-Alamo and Mann, 1989) of each oyster is measured with a ruler. Each oyster is opened with an oyster knife by cutting the adductor muscle at its connection with the upper (right) shell. A gross examination of each oyster is made and each oyster is given a numerical condition code according to the system described by Quick and Mackin (1971) (Table 3-1). Using sterile dissecting scissors and forceps, a 5 x 5-mm piece of mantle-edge tissue is excised from just over the palps. Sterile instruments must be used when going from oyster to oyster to avoid cross-contamination. An alcohol dip and flaming is sufficient. The tissue is placed in a culture tube containing 5 mL RFTM to which 0.5 mL of the Penicillin-Streptomycin solution has been added. The tube is recapped and inverted so that the tissue is submerged in the RFTM. When processing many samples, it is convenient to add the antibiotic solution to the vials prior to, but not earlier than 1 h before, opening the oysters. Of the original 12 oysters, 5 are randomly chosen and further processed for gonadal analysis and histopathology (see Kim *et al.*, this volume). The culture tubes are placed in the dark at room temperature and incubated for at least 5 days. If the tissue has not been analyzed by the end of 7 days, the tube is placed in a refrigerator in the dark. Tissues ready to be analyzed can be kept for at least 3 months without deterioration if the culture tubes are kept dark and refrigerated.

4.0 TISSUE ANALYSIS

After the incubation period, the oyster tissue is removed from the RFTM using a sterile probe and placed on a microscope slide. The tissue sample is teased apart using sterile probes to assure even staining with Lugol's iodine solution. One or two drops of Lugol's solution is added to the tissue with a Pasteur pipette, the tissue is covered with a cover slip, and then examined microscopically. (Note: Microscope slides are thoroughly cleaned and re-used although coverslips are disposed of after use)

Perkinsus marinus hypnospores appear as blue/black spheres 5 to 300 μm in diameter when viewed through a microscope at 40 to 100x magnification (Fig. 3-1 and Bushek *et al.*, 1994). An infection intensity is assigned to each sample based on the number or coverage of enlarged *P. marinus* observed in the tissue using the scale in Table 3-2. For example, in Figure 3-1: Photo 1 would be rated VL (.33) or L- (.67); Photo 2 would be rated L+ (1.33); Photo 3 would be rated M- (2.67); Photo 4 would be rated MH (4.00); Photo 5 would be rated H (5.00).

To maintain quality control, blind assays may be conducted among slide readers to correct for the technician bias that may be present with any semi-quantitative technique. Other laboratories are encouraged to standardize their analyses with laboratories already using the technique so that data are comparable (e.g., Fisher and Oliver, 1996).

Table 3-1. Oyster condition rating key (from Quick and Mackin, 1971).

| Condition index	Code no.	Oyster appearance
Very good	1	Animal firm and filling shell cavity; coloration creamy white and evenly textured; usually ready to spawn
Good	2	Not quite as firm or large as above; usually ready to spawn
Good minus	3	Coloration less opaque, often slightly yellow or gray
Fair plus	4	Animal distinctly not filling shell cavity; coloration often mottled, with blood vessels and muscle fibers showing through the more translucent epithelium
Fair	5	Oyster well-developed but not opaque or tending toward white; grayish and translucent; flesh flaccid
Fair minus	6	Translucency more pronounced
Poor plus	7	Oyster not well-developed, darker gray, often greenish; pericardial cavity clear; small portion of shell cavity filled
Poor	8	Negative qualities more accentuated
Very poor	9	Animal distinctly atrophied; coloration dark and uneven, very translucent; seldom more than third of shell cavity occupied; adductor muscle often discolored and transparent even in the normally white sector
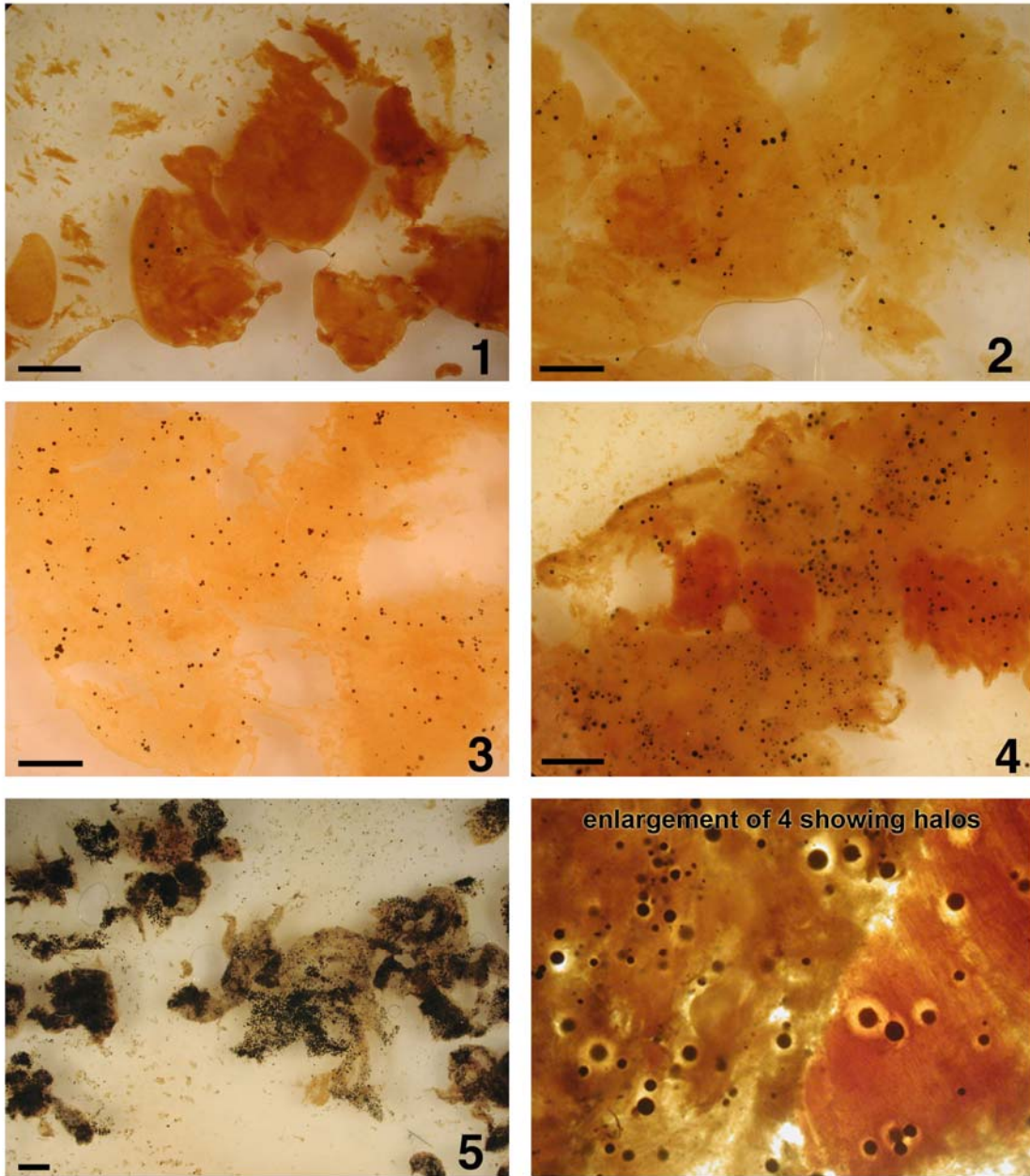
Figure 3-1. Enlarged and stained *Perkinsus marinus* hypnospores. Hypnospores appear as black dots in the photographs. Scale bars = 3 mm (courtesy of S. E. Ford, Haskin Shellfish Research Laboratory, Rutgers University, NJ)

Table 3-2. Semi-quantitative scale of infection intensity for *Perkinsus marinus* [adapted from Mackin (1962) by Craig *et al.* (1989)].

Letter designation	Infection intensity	Numerical value	Description
N	Negative	0.00	No hypnospores present
VL	Very light	0.33	1-10 hypnospores
L-		0.67	11-74 hypnospores
L	Light	1.00	75-125 hypnospores
L+		1.33	>125 hypnospores but much less than 25% of tissue is hypnospores
LM-		1.67	<25% of tissue is hypnospores
LM	Light/moderate	2.00	25% of tissue is hypnospores
LM+		2.33	>25% but much less than 50% of tissue is hypnospores
M-		2.67	>25% but <50% of tissue is hypnospores
M	Moderate	3.00	50% of tissue is hypnospores
M+		3.33	>50% but much less than 75% of tissue is hypnospores
MH-		3.67	>50% but <75% of tissue is hypnospores
MH	Moderately heavy	4.00	75% of tissue is hypnospores
MH+		4.33	>75% but much less than 100% of tissue is hypnospores
H-	Heavy	4.67	>75% of tissue is hypnospores but some oyster tissue is still visible
H		5.00	Nearly 100% of tissue is hypnospores

Alternative techniques have been developed for the assay of *P. marinus* prevalence and infection intensity in addition to Ray's (1966) tissue technique. Gauthier and Fisher (1990) used an adaptation of Ray's method to non-destructively follow the progression of *P. marinus* infection by sampling oyster hemolymph. Choi *et al.* (1989) and Fisher and Oliver (1996) describe quantification of the RFTM method by using a total body burden technique to assess total numbers of *P. marinus* cells present in an oyster. Whole body counts minimize false negatives that commonly occur at low *P. marinus* infection levels (Nickens *et al.*, 2002; Choi *et al.*, 1989). The accuracy and precision of the three RFTM-based methods is discussed in Bushek *et al.* (1994) and Oliver *et al.* (1998). Although the most accurate method for estimation of *P. marinus* infection levels in individual oysters

is total body burden, the removal of a small piece of mantle tissue is rapid, inexpensive, and can be done with little tissue loss from animals destined for other analyses. This method accurately reflects the prevalence and infection intensity in an oyster population (Bushek *et al.*, 1994; Oliver *et al.*, 1998). Recently described molecular techniques are not yet in general use but promise greater precision and accuracy (eg., Ottinger, *et al.*, 2001) although time and expense, again, may be prohibitive for monitoring purposes.

Although the RFTM method is recommended for survey and monitoring work (Bushek *et al.,* 1994; Oliver *et al*., 1998) and is widely used, some cautionary comments are necessary.

1. The relatively small size of the sample used in this method may lead to false negatives at less than 1000 *P. marinus* per gram wet weight of oyster tissue (Bushek *et al.*, 1994; Choi *et al.*, 1989; see also Ottinger *et al.*, 2001) or inaccurate sample ranking due to the non-uniform distribution of *P. marinus* within (Choi *et al.*, 1989) and between (Oliver *et al.*, 1998) oyster tissues. The use of the Ray (1966) FTM method has been shown, however, to accurately estimate the relative overall intensity of *P. marinus* in a sample, if not necessarily in individual oysters (Bushek *et al.*, 1994; Oliver *et al.*, 1998), and is considered to be adequate for survey and monitoring work.

2. All microscopically identifiable stages of *P. marinus* present in the oyster appear to enlarge in RFTM with minimal reproduction so that the number of cells present after incubation accurately represents the number of cells in the oyster tissue (Ray, 1954; Stein and Mackin, 1957). However, the mean diameter of hypnspores can vary from 5 μ to 300 μ (Fig. 3-1, see also Fig. 2 Bushek *et al.*, 1994; Ray, 1954). The degree of enlargement appears to vary seasonally and may be inversely related to infection intensity (Bushek *et al.*, 1994). The size of the hypnospores is not taken into account by the slide reader and, because the ranking scale is semi-quantitative, the numbers of cells are not counted except at the "Light" and lower levels of infecton. This means that a tissue sample with fewer large hypnospores may have the same numerical rank as a sample with many smaller cells. In cases where this source of error is of concern, calibration using tissue weights can be conducted (Choi *et al.*, 1989):

$$\text{Hypnospores (g wet wt.)}^{-1} = 1409.9 \,(10^{0.64296x})$$

where x is the semi-quantitative numerical value from Table 3-2 (Choi *et al.*, 1989).

5.0   DEFINITIONS AND CALCULATIONS

Three calculations of parasite distribution are generally used to describe Dermo disease in oyster populations: infection intensity, prevalence, and weighted prevalence (also termed mean abundance-Bush *et al*., 1997; Rosza *et al*., 2000). Infection intensity for each site sampled is calculated as the sum of the infection rankings from Table 3-2, divided by the number of oysters with infections. Prevalence describes the proportion of individuals in the sample that are infected and is calculated as the number of oysters infected by *P. marinus* divided by the total number of oysters analyzed. Weighted prevalence or mean abundance is the infection intensity multiplied by the prevalence. This gives a measure of

the relative severity of *P. marinus* infection in a population. Due to the truncated nature of the semi-quantitative scale in Table 3-2 and because infection intensity in a sampled population may not be normally distributed, the calculation of the median infection is often more desirable than the mean infection. Confidence intervals for the median can normally be calculated by bootstrapping (Efron and Tibshirani, 1986).

6.0   CONCLUSIONS

The described technique provides a semi-quantitative method to determine the presence and infection intensity of *P. marinus* in oysters. Despite its drawbacks, the Ray (1966) FTM method is currently the accepted method to determine population health in routine monitoring. This method is considerably more accurate than examination of tissue sections in routine histopathological analysis (Ray, 1954) and less expensive and time-consuming than the more accurate body burden method (Choi *et al.*, 1989; Bushek *et al.*, 1994; Oliver *et al.*, 1998).

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