

## ***Perkinsus marinus* Assay**

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### ABSTRACT

This document 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. 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 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.* 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. 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<sub>2</sub>) [7553-56-2], I3380, purity 99%. Sigma Chemical Company, St. Louis, MO.  
Penicillin (C<sub>16</sub>H<sub>17</sub>N<sub>2</sub>O<sub>4</sub>SNa) [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<sub>21</sub>H<sub>39</sub>N<sub>7</sub>O<sub>12</sub>)<sub>2</sub>·(H<sub>2</sub>SO<sub>4</sub>)<sub>3</sub> [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* hyphospores 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. 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 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.

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

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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

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#### 4. 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* hypospores appear as blue/black spheres 5 to 300 µm in diameter when viewed through a microscope at 40 to 100x magnification (Fig. 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 2. For example, in Figure 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 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 hypospores present
VL	Very light	0.33	1-10 hypospores
L-	Light	0.67	11-74 hypospores
L		1.00	75-125 hypospores
L+		1.33	>125 hypospores but much less than 25% of tissue is hypospores
LM-	Light/moderate	1.67	<25% of tissue is hypospores
LM		2.00	25% of tissue is hypospores
LM+		2.33	>25% but much less than 50% of tissue is hypospores
M-	Moderate	2.67	>25% but <50% of tissue is hypospores
M		3.00	50% of tissue is hypospores
M+		3.33	>50% but much less than 75% of tissue is hypospores
MH-	Moderately heavy	3.67	>50% but <75% of tissue is hypospores
MH		4.00	75% of tissue is hypospores
MH+		4.33	>75% but much less than 100% of tissue is hypospores
H-	Heavy	4.67	>75% of tissue is hypospores but some oyster tissue is still visible
H		5.00	Nearly 100% of tissue is hypospores

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 hyphospores can vary from 5  $\mu$  to 300  $\mu$  (Fig. 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 hyphospores 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 infection. This means that a tissue sample with fewer large hyphospores 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):

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

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

## 5. 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 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 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. 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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