Ray's fluid thioglycollate medium (RFTM) assays for dermo disease in oysters, and *Perkinsus* sp. infections in other molluscs

Maryland DNR methods used at the Cooperative Oxford Laboratory
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Table 1. Ray's fluid thioglycollate medium components	Final RFTM volume	
	500 ml	2,000 ml
Fluid thioglycollate medium (FTM, Difco 225650)	14.6 g	58.4 g
Sodium chloride (NaCl)	10 g	40 g
Distilled water	485 ml	1,940 ml
2.5 % (w/v) chloramphenicol	10 ml	40 ml

Chloramphenicol, 2.5% (w/v) working solution (non-sterile, teratogen-hazard)

Chloramphenicol powder (Sigma C-0376)	0.25 g	1.0 g
Distilled water	10 ml	40 ml

Nystatin suspension, 4,000 units/ml (aseptic)

Nystatin powder (Sigma N-3503)	70 mg (5,800 U/mg)
Sterile distilled water	100 ml

Nystatin suspension, 4,000 units/ml (sterile)

Nystatin powder, gamma-irradiated (Sigma N-4014)	50 mg (3,970 U/mg)
Sterile distilled water	49.6 ml

I. RFTM preparation.

1. In a flask or beaker with a stir-bar, combine FTM powder, NaCl, and distilled water (Table 1).
2. In a well ventilated workspace (chemical hood ideal), heat suspension with constant stirring to near-boiling, to dissolve FTM powder and NaCl.
3. Cool briefly before adding chloramphenicol suspension with stirring. Cool additionally for handling comfort.
4. Fill screw-capped bottles (125 ml - 500 ml) at 75-80% capacity, to provide expansion room for heated RFTM. Leave bottle caps quite loose, to vent vapors during heating and cooling.
5. Autoclave RFTM at 15 psi (121 °C) for 15-20 min.
6. Allow sterilized bottles of RFTM to cool inside the autoclave, if possible, or in a sanitary environment. Once RFTM has cooled to room temperature, close bottle caps tightly.
7. Sterile RFTM can be stored at room temperature in the dark for 2-3 months before use. Refrigerated storage should be effective for a year.

II. RFTM inoculation with diagnostic tissues from mollusc hosts.

1. Aseptically aliquot sterile RFTM to sterile, capped tubes or lidded, sterile well-plates, at 1-3 ml/tube or well.

2. To the surface of the RFTM in each labeled sample tube or plate well, gently add 2-4 drops of 4,000 units/ml nystatin suspension to inhibit fungus growth. The chloramphenicol in the RFTM inhibits bacterial growth.

3. Aseptically excise a small piece (3-5 mm) of a selected inoculum tissue from each sample mollusc, and aseptically inoculate each tissue into a labeled RFTM tube or plate well. Rectum tissues are preferred inocula for RFTM assays of oysters; while labial palp or gill tissues are commonly used from several species of Chesapeake Bay clams.

4. Cap tubes or plates loosely, and incubate 7 days in the dark at 27 °C.
Expedited RFTM assays can be read after 2- to 4-day incubations at 27 °C.
Incubation temperatures of 20-30 °C are effective.

III. RFTM assay analysis

1. With a clean probe or pipet *, retrieve each RFTM-incubated mollusc tissue from its RFTM tube or plate well, and transfer it to a pool of 30% (v/v) Lugol's iodine solution on a labeled microscope slide.

2. With a clean probe *, macerate and tease each mollusc tissue apart in its pool of Lugol's iodine. Add another drop of 30% (v/v) Lugol's iodine solution if needed **, and mount a coverslip over the iodine-stained tissue-macerate.

3. If well plates and an inverted microscope are used together for RFTM incubations and subsequent assay evaluations, RFTM can be carefully aspirated from each well to leave tissues behind. Those tissues can then be macerated in Lugol's iodine that's added directly to tissues in plate wells (item 3. below), before microscopic analyses with an inverted microscope (item 4. below).

4. Examine iodine-stained tissue macerates microscopically (4x, 10x, 20x objectives) to detect and quantify spherical, blue-black *Perkinsus* sp. hypnospore cells with diameters of 10-100 µm among amber-stained mollusc host tissues (Fig. 1).

5. Based on RFTM assay results, score each host mollusc as infected or uninfected, and calculate the % prevalence of *Perkinsus* sp. infections among host molluscs in the sample: (number infected/number tested) (100).

6. Rank infection intensities for infected hosts, based on the modal or typical abundance of *Perkinsus* sp. hypnospores in the sample tissue. Uninfected tissue samples are scored as zeros, while categorical ranks for other infection intensities may be scored on one of several scales (Table 2).

* Rigorously and consistently avoid contamination of tissue samples, slides, and coverslips by contact with any potential source of *Perkinsus* sp. cells other than the specific sample being manipulated or processed. Carefully clean or sterilize dissection and manipulation instruments between samples.

** Never allow a Lugol's iodine dropper or reagent supply to contact any potential source of *Perkinsus* sp. cells, especially tissue samples.

Table 2. Selected RFTM assay infection intensity ranking scales, (reference), [used by]	Approximate alignments of numeric interval ranks for *Perkinsus* sp. infection intensities as described for several RFTM assay scoring systems (after Dungan & Bushek 2015)													
Mackin (Ray 1954a, 1954b) [HSRL]	0	0.5	1		2		3		4		5			
after (Mackin 1955) [VIMS, UMCES-CBL]	0	0.5	1		3				5					
Quick (1972)	0	1	2		3		4		5		6			
Farley (Gieseker 2001, Calvo et al. 1996) [MDDNR – COL]	0	1	2		3		4		5		6	7		
Bushek et al. (1994)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0			
Craig et al. (1989)	0	0.33	0.67 1.0 1.33		1.67 2.0 2.33		2.67 3.0 3.33		3.67 4.0 4.33		4.67 5.0			

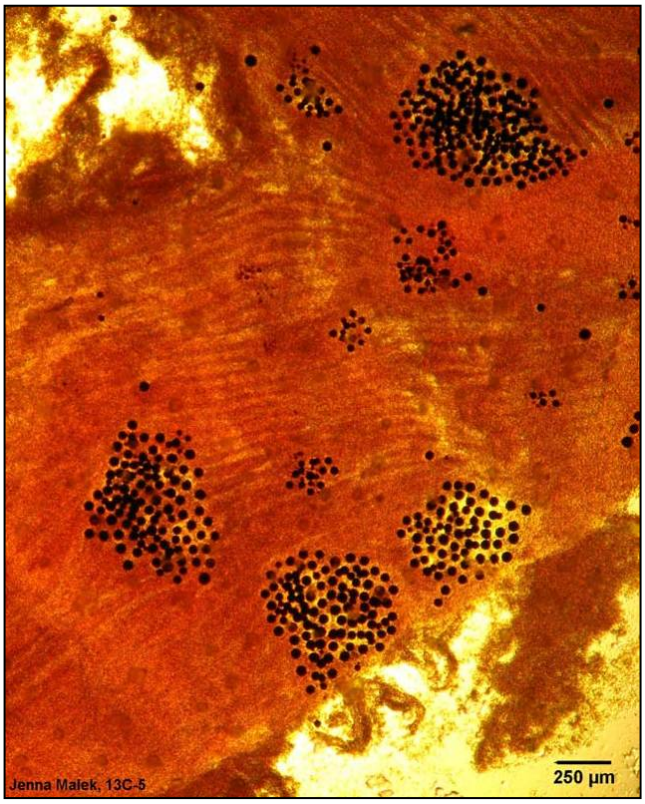

Fig. 1. Colonies of spherical, blue-black, Lugol's iodine-stained *Perkinsus* sp. cells among amber-stained gill tissues of an infected *Macoma balthica* clam. Individual parasite cells are also dispersed in the sample tissue. Image by Jenna Malek, SERC.

IV. Categorical ranking of *Perkinsus* sp. infection intensities.

A large and diverse number of numerical ranking systems for results from RFTM assays have been developed and used worldwide (Dungan & Bushek 2015, Table 2). Common features of those ranking systems for *Perkinsus* sp. infection intensities are that ordinal numeric ranks increase with increasing abundances of *Perkinsus* sp. cells in analyzed samples, that their numerical ranks are semi-quantitative and categorical, and that *Perkinsus* sp. cells are actually counted only for samples of the lowest infection intensity ranks, for convenience.

The microscopic and macroscopic ranking criteria for *P. marinus* infections of *C. virginica* rectum tissues by RFTM assays are listed below, as used by Maryland DNR with its 0-7 categorical ranking scale (Table 3). Table 4 shows those Maryland DNR ranks and criteria as they correlate approximately with similar ranking systems and criteria for RFTM assay results that are used at the Virginia Institute of Marine Science (VIMS), and at Rutgers Haskin Shellfish Research Lab (HSRL).

Table 3. *Perkinsus* sp. infection intensity ranking criteria for RFTM assays of oyster rectum tissues by RFTM assays conducted by Maryland DNR.

Infection intensity rank	Abundance of *Perkinsus* sp. cells and related evaluation criteria
0	No *Perkinsus* sp. cells (hypnospores) in entire sample.
1	1 - 4 *Perkinsus* sp. cells in entire sample.
2	5 - 25 *Perkinsus* sp. cells in entire sample.
3	26 - 200 *Perkinsus* sp. cells in entire sample. Parasites may occur in isolated clusters of 10-20 cells, and/or be disbursed throughout to show 1-2 cells in each 100x field.
4	About 50% of the tissue sample is occupied by *Perkinsus* sp. cells. Each 100x field shows several cells. Dense masses of hypnospores may occur locally with uninfected tissues surrounding, but blue-black staining is not macroscopically evident.
5	*Perkinsus* sp. hypnospores are present in large numbers in all areas of the tissue sample. Uninfected tissues commonly occur between parasite cells. Less than half of the tissue sample area appears blue-black macroscopically.
6	*Perkinsus* sp. hypnospores are abundant in most areas of the tissue sample. Narrow areas of uninfected tissues occur between parasite cells. The majority of the sample appears blue-black or pigmented, macroscopically.
7	*Perkinsus* sp. hypnospores occur in enormous numbers throughout the tissue sample. Areas of uninfected tissues are rare or absent between parasite cells. The entire tissue appears blue-black macroscopically.

Table 4. Infection intensity ranks for RFTM assays of oyster tissues conducted by Maryland DNR at COL, and ranks assigned to similar results at VIMS and HSRL

MDDNR rank (0-7)	Nominal rank	Parasite abundance ranges and criteria Maryland DNR	VIMS rank (0-5) *	HSRL rank (0-5)
0	Absent	No *Perkinsus* sp. hypnospores in sample.	0	0
1	Rare	1 - 2 *Perkinsus* sp. hypnospores in sample.	0.5	0.5
1	Very light	1 - 4 *Perkinsus* sp. hypnospores in entire tissue sample.	1	0.5
2	Light	5 - 25 *Perkinsus* sp. hypnospores in entire tissue sample.	1	1
3	Light - moderate	26-200 *Perkinsus* sp. hypnospores in entire sample. Parasites may be in isolated clusters of 10-20 cells, or uniformly distributed throughout to show 1-2 in each 100x field.	3	2
4	Moderate	About 50% of the tissue sample contains *Perkinsus* sp. hypnospores. Each 100x field shows several hypnospores. Dense masses of hypnospores may occur locally, but blue-black staining is not macroscopically apparent.	3	3
5	Moderate-heavy	*Perkinsus* sp. hypnospores present in large numbers in all areas of the tissue sample. Less than half of the tissue sample appears blue-black macroscopically.	5	4
6	Heavy	*Perkinsus* sp. hypnospores present in enormous numbers in most areas of the tissue sample. The majority of the sample appears blue-black or otherwise pigmented, macroscopically.	5	5
7	Very heavy	*Perkinsus* sp. hypnospores present in enormous numbers throughout the tissue sample. The entire tissue appears blue-black macroscopically.	5	5

* VIMS currently scores RFTM assays with 9 nominal ranks, which are then pooled into 5 ordinal numeric ranks (0, 0.5, 1, 3, 5) before means are calculated. Such means are termed 'weighted prevalences' by regrettable historic convention.

Underlined rank numbers in Table 4 indicate where minor deviances from VIMS or HSRL specifications were required to force rank alignments with specified Maryland DNR rank criteria.

Discussion

Although data and statistics on numerical ranks for intensities of *Perkinsus* sp. infections estimated by RFTM assays are commonly reported, specific enumeration or estimation criteria used to assign those ranks to individual samples are relatively rare. Infection intensity ranks of the Mackin ranking scale that is commonly and historically used with RFTM assays (Ray 1954b, Table 2) were shown to be approximately base-10 logarithmic (Choi et al. 1989).

Despite the fact that calculating statistics by treating numeric ranks as numbers is marginally acceptable mathematically; especially where those ranks may reflect logarithmic intervals, users of RFTM assays have regularly calculated, reported, and compared trends for such means since the 1950s (Mackin 1955, Dungan & Bushek 2015). This is a common practice that Maryland DNR and others continue (Tarnowski 2017).

From results of RFTM assays of oyster rectum tissues, Maryland DNR calculates the four sample statistics listed below, using infection presence/absence and infection intensity rank (0-7) data for *Perkinsus* sp. infections.

1) Prevalence: Percent proportion of infected sample oysters (0-100%).

2) Percent lethal infections: Percent proportion of sample oysters with *Perkinsus* sp. infection intensities of ranks ≥5, which are anecdotally projected to be imminently lethal. (0-100%)

3) Mean infection intensity: The mean of infection intensity numerical ranks for all oysters in a sample (0.0 - 7.0). This is the same mean that's termed a *weighted prevalence* at VIMS, Rutgers HSRL, and other institutions (Dungan & Bushek 2015, Bushek et al. 1994).

4) Infection intensity index: The mean of infection intensity numerical ranks for only the infected oysters in a sample (1.0 - 7.0). Two credible authorities recommend the alternative term *mean infection intensity* (above) for this statistic (Margolis et al. 1982, Bush et al. 1997).

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