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FIELD AND LABORATORY STUDY OF THE PROCESS AND DYNAMICS OF *PERKINSUS MARINUS* INFECTION IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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EXECUTIVE SUMMARY

This study is the first to systematically examine the seasonality of *P. marinus* infection acquisition in oysters in relation to water column abundance of *P. marinus* cells, oyster mortality, and temperature. The timing and magnitude of seasonal peaks in environmental abundances of *P. marinus* cells and local oyster mortalities at an upper and lower Chesapeake Bay site were also contrasted. Lastly, a specific and sensitive immunoassay detection technique was employed to examine the process and site(s) of pathogen invasion into host oysters in both field and laboratory *P. marinus* exposures.

In the lower York River, VA, environmental abundance of *P. marinus* cells, infection acquisition by sentinel oysters, and mortality of *P. marinus*-infected oysters varied seasonally. Distinct peaks of all three parameters occurred during the month of August, following maximal summer temperatures. Water column parasite cell abundances, infection pressure, and oyster mortalities decreased from summer maximums as temperatures decreased in September and October, and remained at "wintertime" low levels from October through the termination of the study in March. Strong and significant positive correlations were found between water column parasite cell abundance and temperature, water column parasite cell abundance and oyster mortality, oyster mortality and temperature, and oyster mortality and *P. marinus* prevalence in sentinel oysters. These results support the currently accepted hypothesis that infective stages of *P. marinus* originate from dying oysters and are most abundant in August. The occurrence of elevated cell abundances in early summer, immediately before epizootic oyster mortalities, suggests that pathogen cells may also originate from other sources.

In the Tred Avon River, MD, maximum abundances of *P. marinus* cells in the water column were also observed during August. Environmental cell abundance was significantly correlated with temperature, but not with local oyster mortality rates. Abundance levels overall were generally higher than at the York River site but were still of the same order of magnitude. Local oyster mortality at the upper Bay site occurred later in the summer and was much lower than at the York river site.

Results of *P. marinus* initial infection studies suggest that the digestive tract may not be the only, or even the primary, site of infection. The localization of many parasite cells in gill and mantle epithelia may be indicative of alternate pathogen entry routes.

INTRODUCTION

Perkinsus marinus is the most widespread and destructive oyster pathogen in the mid-Atlantic region at the present time. Historically this protozoan pathogen was absent from a large proportion of the oyster grounds located in the upper reaches of Chesapeake Bay tributaries, but in the last decade, as a consequence of several drought years, the parasite's distribution has expanded (Andrews 1988). *Perkinsus marinus* is now present on nearly all oyster bars in Virginia and Maryland and has recently become established in Delaware Bay (Smith and Jordan 1993; Ragone Calvo and Burreson 1995). Since the initial report of its presence in the Chesapeake Bay in 1949, the pathogen has been extensively studied, but only recently, with the development of novel detection techniques, have researchers begun to examine the seasonality of parasite cell abundances in environmental water samples. The ability to quantify environmental abundances of *P. marinus* using flow cytometric immunodetection methods presents the means to rigorously address numerous questions regarding the natural transmission dynamics of the parasite and to clarify our understanding of this important aspect of the epizootiology of *P. marinus.*

The prevailing conceptual model of *P. marinus* transmission for Chesapeake Bay asserts that transmission occurs via the direct dissemination of water-borne infective cells from infected oysters (Andrews 1988). The abundance of disseminated cells is believed to be highest in August and September as a result of the death and decomposition of infected oysters during periods of epizootic mortalities. The rate of infection in nature is believed to be proportional to the abundance of water-borne infective pathogen cells and to be increased by close proximity to infected hosts (Andrews and Hewatt 1957). In the Gulf of Mexico, *P. marinus* may be transmitted by the ectoparasitic snail *Boonea impressa* (White et al. 1987). In Chesapeake Bay, no vectors have been identified, and transmission is considered to be direct from oyster to oyster. Evidence of direct transmission includes the establishment of infections in oysters exposed to crude minces of infected oyster tissues and to isolated cells of various pathogen life stages (Ray 1954; Mackin 1962; Volety and Chu 1994). Such investigations have also shown that transmission is dose dependent and that all known life stages of *P. marinus* are infective (Perkins 1988; Volety and Chu 1994).

While direct transmission is well established, the primary infective cell type and mechanism of infection have not been determined. Transmission is thought to occur through the digestive tract because foci of infection are most frequently observed there (Mackin 1951). Mackin (1951) proposed that hemocytes scavenge the epithelial surface of the oyster gut lumen, phagocytose ingested *P. marinus* cells, and then migrate through the epithelial layer, transporting the parasite into the oyster. An innovative study has demonstrated that inert particles introduced by intubation

of the oyster stomach, are assimilated to host connective tissues, and subsequently eliminated over time suggesting that such events may occur (Alvarez et al. 1992). However, Ray (1954) induced infections by exposing excised gill and mantle tissues to pericardial fluid from infected oysters, providing evidence that invasion may not be limited to entry through the digestive tract.

Investigations of disease transmission in nature have been limited to estimations of seasonal infection pressure in sentinel populations of uninfected oysters transplanted to endemic areas. Andrews and Hewatt (1957) found that uninfected oysters imported into endemic Chesapeake Bay waters in spring developed detectable infections in late summer, a time corresponding to epizootic deaths of oysters harboring existing *P. marinus* infections. Similarly, annual imports of sentinel oyster populations have been utilized to discern annual variability of *P. marinus* infection pressure (Burreson 1991; 1992; 1993). These studies rely on temporal disease prevalence and intensity data to make inferences concerning the timing and magnitude of transmission events. Although useful, such epidemiological studies have been limited by the low sensitivity of standard diagnostic techniques for detecting early infections (Bushek et al. 1994).

Development of a flow cytometric immunodetection method for the quantification of *P. marinus* cells in environmental water samples has provided the first opportunity to monitor environmental abundances of *P. marinus* cells and to test longstanding hypotheses concerning the sources and seasonal abundances of water-borne *P. marinus* cells. The flow cytometric immunodetection method utilizes antibodies that specifically bind to various *P. marinus* cell types to selectively fluorochrome-label *P. marinus* cells (Dungan and Roberson 1993a). Immuno-labeled *P. marinus* cells in water samples are then counted with a flow cytometer. This technique has been used to monitor environmental abundances of the parasite in the Tred Avon River, Maryland over a two year period (1992–1993) (Roberson and Dungan 1994). Major abundance peaks of *P. marinus* cells were observed during the months of June-August. The occurrence of a major peak in August supports the currently accepted hypothesis that relates *P. marinus* cell dissemination to disease-induced mortality of infected hosts, which typically occur during August and September. However, the observation of early summer abundance peaks suggests that a secondary source of infective cells may exist. Proposed alternate hypotheses link cell dissemination with oyster host spawning or excretory activities, alternate host or vector activities, possible heterotrophic environmental proliferation of *P. marinus*, and periodic resuspension of sediment-bound infectious cells (Dungan and Roberson 1993b). Now that a monitoring regime for disseminated *P. marinus* cells has been established the complexity of transmission dynamics can be investigated.

To elucidate the dynamics of *P. marinus* transmission it is necessary to examine disseminated cell abundances in relation to host oyster mortalities, infection acquisition, and environmental factors. In the present investigation, local oyster mortality, infection acquisition in biweekly

deployed sentinel oysters, and environmental abundances of *P. marinus* cells were monitored over a one year period. This research had four main objectives: (1) to determine the seasonal abundance of water-borne *P. marinus* cells, (2) to determine the timing and magnitude of host oyster mortalities, (3) to determine biweekly infection rates, and (4) to determine the strength of correlations between these three factors. By measuring temperature, salinity, parasite abundance and host oyster mortalities at two sites located in two different subestuaries of the Chesapeake Bay insight was also gained regarding spatial variability and the importance of environmental factors in regulating host parasite dynamics. In addition, separate laboratory experiments examined the process of infection, in particular the identification of the anatomical sites of initial *P. marinus* infections in oysters, i.e. portal(s) of entry.

METHODS

Enumeration of *P. marinus* cells in environmental water samples

Water sample collection. Water samples were collected three times each week from two separate subestuaries of the Chesapeake Bay, for the period 4-1-94 to 3-31-95. The northern Bay sampling site was the Cooperative Oxford Laboratory dock located in the Tred Avon River (TAR) at Oxford, Maryland. This sampling site extends over a populated, subtidal oyster sanctuary in an intermediate-salinity area of the Bay that is endemic for *P. marinus*. The southern Bay sampling site was a pier at the Virginia Institute of Marine Science located in the York River (YR) at Gloucester Point, Virginia. This area is characterized by high salinity and is also endemic for *P. marinus*. The area is no longer heavily populated by oysters but small aggregate populations of the bivalve are known to be present.

Water samples (1.0 liter) were collected at a depth of 1 m with a wastewater sampling device at approximately the same time each day to randomize tidal effects. Temperature and salinity were determined immediately after collection with a thermometer and hand-held refractometer respectively. Water samples were returned to the laboratory and immediately filtered through a 35 µm nylon sieve to remove coarse particulates. Filtered samples were then dispensed evenly into four 250 mL conical centrifuge bottles. Centrifuge-clarified formaldehyde (37%) was added to each bottle to a final concentration of 1.0% (v/v) and the samples were allowed to fix for 1 hr at room temperature. After fixation, fixed particles were pelleted by centrifugation at 1500 x g for 15 minutes and supernates were removed by aspiration. Pellets from all four bottles were pooled and resuspended in 10 mL 0.15 M phosphate-buffered saline containing 0.04% (w/v) sodium azide as preservative (PBS, pH 7.4). Samples were stored at 4°C until enumerated by flow cytometry.

Flow cytometric enumeration. Prior to analysis with the flow cytometer, sample cells were washed 3 times at 1250 x g in 10 mL PBS. From each washed, 10 mL sample

suspension concentrate, 1.0 mL was subsampled for staining and pelleted at 1250 x g, incubated for 1 hr with a 10^{-2} dilution of normal goat serum, and washed with PBS containing 0.05% Tween 20 by repelleting at 1250 x g. Subsample pellets were then incubated for 1-2 hr in 1.0 mL antiserum to cultured *P. marinus* (Dungan and Roberson 1993a) prepared in PBS at a dilution of 3.3 x 10^{-2} (~1 ug mL^{-1} rabbit IgG). Following primary antibody labeling, cells were washed three times by centrifugation in PBS with 0.05% Tween 20. Cell pellets were incubated for 1 hr in a 5 x 10^{-4} dilution of fluorescein isothiocyanate labeled (FITCL) goat $F(AB')_2$ anti-rabbit IgG (Cappel) made in PBS.

Stained cells were washed three times by centrifugation and finally resuspended in 0.4 mL PBS. Subsamples representing 10% of the original sample were resuspended and used for FITCL-discriminated counts at the rate of 200-400 counts sec^{-1} (cps).

Stained populations were analyzed with a Coulter EPICS 751 flow cytometer, using an argon laser tuned at 488 nm as the illumination source, and either a 76 µm or a 76 µm Sense-in- Quartz™ flow cell. Cells were analyzed for forward angle light scatter (FALS), a measure of size, 90° light scatter (90LS), a measure of surface texture and internal structure, green fluorescein fluorescence (525 ±10 nm), a measure of antibody binding, and red fluorescence (635±10 nm) a measure of red autofluorescence when nucleic acid stain is omitted. (The last channel was selected to separate *P. marinus* from a small autofluorescent particle that had previously resisted separation.)

Optical alignment of the flow cytometer was performed daily, using optical alignment fluorospheres (DNA-Check™, Coulter, Hialeah, FL). FALS was calibrated to particle diameter using latex beads, of 2, 6 (Polysciences, Warrington, PA),and 10 µm (Coulter) diameters. Two-parameter and 3-parameter counting and sorting maps were generated using modal FITCL, DNA, FALS and 90LS signal ranges generated by a pure standard of cultured *P. marinus* cells. Signal ranges for each parameter were recorded in list mode, using a 3-decade logarithmic scale mapped onto 256 channels, and downloaded to an EPICS Easy 88™ computer system for storage and analysis. After examining pure populations of cultured pathogen cells and then testing trial gating limits with a sample pool made from the previous year's water column samples, selected parameter ranges were adjusted to exclude non-*Perkinsus* contaminating particles, as judged by examining gated populations sorted onto 1.0 µm pore size black polycarbonate membranes (Poretics, Livermore, CA) by epifluorescent microscopy.

For each water sample, cell suspensions were mechanically agitated prior to analysis and analyzed until exhausted. Total *P. marinus* cell count $liter^{-1}$ was calculated from gated subsample counts after volumetric standardization.

Estimation of regional oyster mortality.

Captive populations (n=1000) of local adult oysters were deployed in four replicate retrievable racks (n=250 per rack) at the Tred Avon River and York River sites. The Tred Avon oyster population was collected on 14 April 1994 from an oyster ground located in the immediate vicinity of the sampling site and deployed at the site on 15 April 1994. Prevalence of *P. marinus* in these oysters at the time of collection as determined by Ray's fluid thioglycollate assay was 60%. Because of the scarcity of oysters in the vicinity of the York River site, oysters for the York River captive population were collected from Point of Shoal located in the James River, VA. The James River oysters were collected on 14 March 1994 and deployed at the YR site on 15 March 1994. No *P. marinus* infections were detected in Point of Shoal oysters at the time of collection as determined by Ray's fluid thioglycollate assays; however, oysters at this location are known to harbor subpatent infections (Ragone Calvo and Burreson 1993) .

Following deployment, the captive oyster populations were retrieved biweekly and dead and live oysters were enumerated. Mortality was calculated as follows. Interval mortality, mortality occurring between enumeration dates, was determined for each replicate group by dividing the number of mortalities during the interval by the number of oysters that were alive at the beginning of the interval. Interval mortality was then multiplied by the proportion of survivors of the previous interval (1-cumulative mortality of preceding interval) to yield an adjusted interval mortality. Successive cumulative mortalities were determined by summing adjusted interval mortalities and preceding cumulative mortalities. Mortality rate, percent mortality per day, was calculated by dividing adjusted interval mortality by the number of days in the interval.

Determination of temporal patterns of infection acquisition.

Uninfected adult oysters (60–90 mm) from Damariscotta River, Maine were supplied monthly by Pemaquid Oyster Company, Damariscotta, Maine. Upon receipt, a sample (n=25) of oysters was processed for histological and Ray fluid thioglycollate disease diagnoses. The remaining oysters were immediately placed in temperature controlled (equivalent to Maine water temperature at time of collection) 1 µm-filtered York River water, and gradually adjusted to ambient York River temperature conditions. Filtration removed *P. marinus* cells from ambient York River water. During acclimation and holding periods the oysters were maintained in 40 L aquaria (20–25 oysters per aquarium) and fed daily (0.1 g algal paste oyster^{-1}).

Uninfected Damariscotta River oysters (n=30) serving as sentinels were deployed at the York River site in retrievable trays on approximately the 1st and 15th of each month for a period of 14-16 days from 15 April 1994 through 31 March 1995. During each exposure period a second group of Damariscotta oysters (n=25), serving as a control, was maintained in the laboratory at ambient York River temperature and salinity conditions in 1 µm-filtered York River water. After

the deployment period, five of the deployed oysters were sacrificed and preserved in Davidson's AFA fixative for histological/ immunoassay examination, and the remaining deployed and control oysters were maintained for a 30 day period in the laboratory in individual 1 L aerated tanks containing 1 µm-filtered York River water at 18–22 ppt and 23–27°C. It was assumed that the incubation period would provide time for subclinical early infections to develop to detectable levels. During the laboratory incubation period the oysters were fed 0.1 g algal paste daily (M–F) and water was renewed every other day (M–F).

Body-burden assays. Infection acquisition was assessed using a modification of the body-burden technique described by Choi et al. (1989). Oysters were shucked and tissues were removed from shells. Whole oyster meats were finely minced using a razor blade and added to a tube containing 20 mL of penicillin/streptomycin-fortified Ray's fluid thioglycollate medium (RFTM). The tubes were incubated in the dark for 7 days at room temperature. After incubation the samples were centrifuged at 1500 x g for 10 minutes and the RFTM supernate was removed. Pellets were resuspended in 30 mL of 2 M NaOH and incubated at 60°C for 1-3 hours. The samples were pelleted by centrifugation at 1500 x g for 10 minutes, supernates were removed, and pelleted cells were then washed three times in deionized water. After the final wash the pellets were resuspended in 1–5 mL of dilute Lugol's iodine solution. Subsamples were aspirated onto 47 mm diameter, 0.22 µm filter paper; stained *P. marinus* cells retained on the filters were counted using light microscopy at 50x magnification. When counts were greater than 200, samples were serially diluted and triplicate 100 µl aliquots were counted. Entire sample volumes were quantified when subsample counts contained fewer than 20 cells.

Immunoassay of histological sections. Five oysters were randomly selected from deployed groups and preserved for fluorescence immunoassay of histological sections. From each oyster, three parallel frontal crossections were cut that encompassed the majority of the visceral mass (Fig. 1). Oyster tissue sections were fixed in Davidson's AFA fixative and embedded in paraffin such that the specified sample face was exposed for sectioning. Blocks were systematically sampled by sectioning at 6 µm, and retaining sections 1–8 and 19–26 on individual microscope slides for subsequent immunoassay analysis. By this method, three tissue blocks from each selected oyster were assayed at 2 depths of the frontal plane. All sections from all blocks included the pallial and nacreal mantle epithelia, mantle and visceral connective tissues, the gonad, and the circulatory sytem. Block 1 (dorsal tissue block) sections always included labial palps, mouth, and esophagus, and frequently included stomach and digestive gland. Block 2 (mid-tissue block) sections always included stomach, digestive gland, and intestine, and fequently included gill. Block 3 (ventral tissue block) sections always included gill, intestine, pericardium, and kidney, and frequently included digestive gland, style sac and heart.

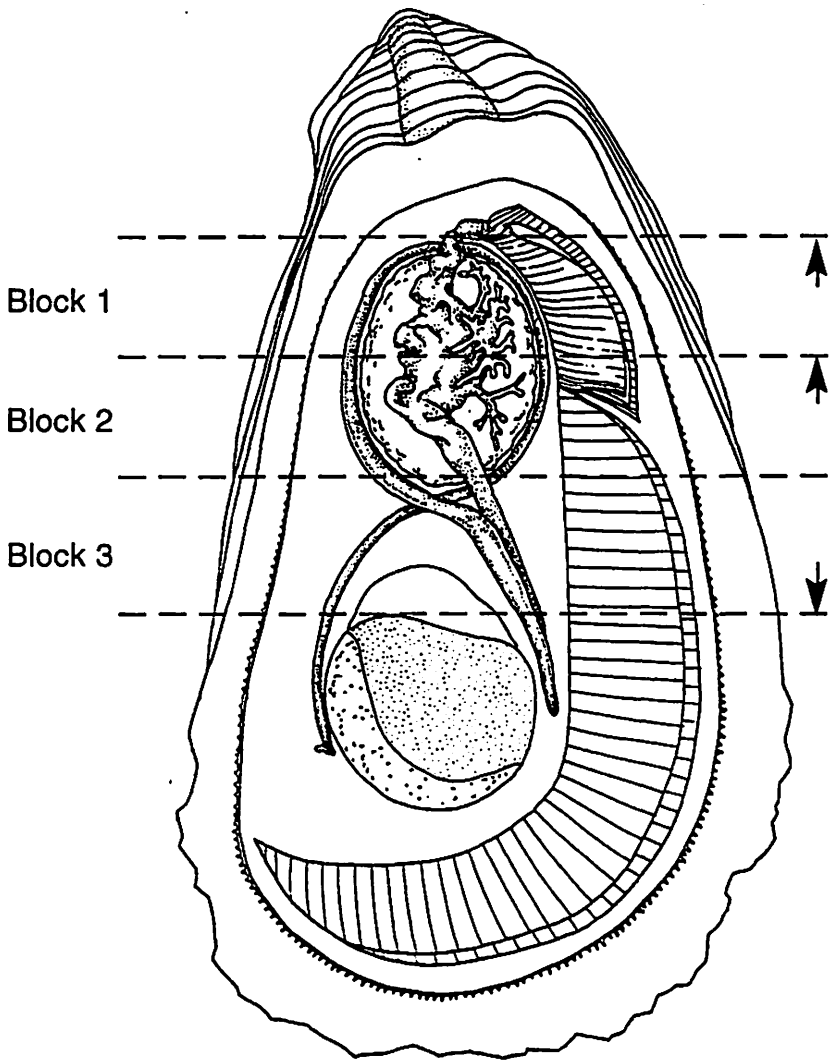


Figure 1. Frontal cross sections of oyster tissue for immunoassays. Each cross section was embedded as a separate block; arrows indicate face of each block where sectioning commenced.

Three adjacent sections (1–3; 19–21) from both sampled depths of each block were deparaffinized and rehydrated through a graded ethanol series. After equilibration in phosphate-buffered saline (PBS), sections were incubated for 1h in blocking buffer (BB) containing PBS + 0.05% (v/v) Tween-20 (PBST) supplemented with 2.0% (w/v) bovine serum albumin (BSA), 1.0% (v/v) normal goat serum, and 0.02% (w/v) sodium azide. Sections 3 and 21 served as autofluorescence controls and remained in BB until the counterstaining step. Other blocked sections were washed once in PBST, and incubated 1h in rabbit 1° antibody diluted to 10 $\mu g\ ml^{-1}$ in serum diluent (SD) containing PBST + 1.0% (w/v) BSA + 0.02% (w/v) sodium azide. Sections 2 and 20 served as nonspecific antibody binding controls, and were incubated in normal rabbit IgG 1° antibody. Sections 1 and 19 were incubated in Protein A-purified IgG fraction of polyclonal rabbit antiserum raised against *P. marinus* (Dungan & Roberson 1993a). Following 1° antibody incubation, sections were washed 3 times for 3 min in PBST, and incubated for 1 h in the dark with FITC-conjugated, affinity purified goat anti-rabbit IgG 2° antibody, diluted to $1\mu g\ ml^{-1}$ in SD. All sections were washed 3 times for 3 min with PBST, counterstained for 5 min in 0.1% (w/v) Evan's blue, rinsed in PBST, and coverslipped in buffered glycerol mounting medium. Each batch of immunostained sections included a known *P. marinus*-positive and -negative oyster tissue section to validate immunostaining.

Immunostained sections were analyzed by exhaustive overlapping parallel scans of the entire tissue section at 200x magnification, using epifluorescence microscopy with broad bandwidth (450–480) blue light excitation and ≥515nm (green) bandpass fluorescence image barrier filtration. All green-fluorescing objects were also immediately analyzed by switching dichroic filter cubes, to use U.V. excitation (330–385) and ≥ 420nm (blue) bandpass fluorescence image barrier filtration. Objects which were green-autofluorescent under blue light excitation generally showed equal or increased fluorescence intensity under U.V. excitation, along with a shift in fluorescence color toward blue. Fluorescein-conjugated antibodies labeling histological sections fluoresce weakly at green wavelengths, if at all, under U.V. excitation. Autofluorescence and nonspecific antibody binding control slides were employed to verify specific antibody labeling of selected other fluorescent objects. Cells in immunoassayed histological sections were scored as *P. marinus* only under the following conditions: (1) specific cellular immunofluorescence observed, (2) immunofluorescent cell(s) coplanar with tissue section, (3) intact cellular morphology observable. *Perkinsus marinus* cells detected by immunoassays were enumerated for each section assayed, and the external or tissue compartment location of each enumerated pathogen cell recorded. Selected pathogen cell morphologies and assay signals were recorded photographically at 413 or 165x magnification, using both color slide and black and white negative films.

In addition to immunoassay sections, two sections from each block at each sample depth (7-8 and 26-27) were processed by routine histological methods. Sections were cut at 6 μm,

deparaffinized and rehydrated through a graded ethanol series. Sections were then stained with Mayer's hematoxylin and eosin (MH&E) and examined using a light microscope. The MH&E sections provided a secondary assessment of *P. marinus* cell morphology and oyster histopathogy.

Statistical analysis. Correlation analyses of biweekly mean values of temperature, salinity, *P. marinus* cell abundance, percent mortality per day, and prevalence were conducted using Statview software on a MacIntosh computer. Percent mortality and prevalence values were arcsin transformed and abundance values were log transformed prior to analyses. Analyses were conducted on data split by site and on data pooled from both sites. Differences in *P. marinus* counts between sites, by month, were examined using t-tests. An analysis of variance was used to test the significances of monthly differences in cell counts at each site. Cell count data were log-transformed prior to analyses.

Portal of entry experiments.

Two separate laboratory experiments were conducted to examine *P. marinus* infection mechanisms and portal(s) of entry. The experiments differed with respect to *P. marinus* dose concentrations and sampling regimes.

Experiment 1.

Oysters. Uninfected oysters (n=60) from the Damariscotta River, Maine were obtained from Pemaquid Oyster Company. The oysters were received on 15 October 1994, placed in two 40 L aquaria, and gradually acclimated to 20 ppt and 24°C. Shell height ranged from 50–60 mm. At the initiation of the experiment oysters were placed in individual 1 L chambers containing 750 mL of aerated 1 µm-filtered York River water at 20 ppt and 23-25°C.

Tissue mince preparation and administration. Infected oysters were collected from Mobjack Bay, Virginia. Oysters with moderate to heavy intensity *P. marinus* infections were identified using fresh hemolymph smears. Whole tissues from five of the infected oysters were homogenized in filter-sterilized York River water (YRW) using an Ultra Turrax tissue homogenizer (Tekmar, Cincinnati, OH). The homogenate was subsequently filtered through a series of nytex filters—300, 75, and 53 µm and the total volume of the filtrate was raised to 600 mL with YRW. Immediately after preparation, 5 mL of the filtrate (tissue mince) and 5 mL of algal paste solution (0.05 g paste mL^{-1}) were added to each chamber. The number of *P. marinus* cells in the mince was estimated by culturing two 5 mL subsamples of tissue mince in 20 mL of antibiotic-fortified RFTM. Cultures were

incubated for 7 days and processed following the body-burden technique. The average dose concentration was determined to be 8.95 x 10^6 *P. marinus* cells $oyster^{-1}$.

The water in each container was replaced with fresh filtered water 18 hours after the tissue mince was added and once every 48 hr thereafter. The oysters were fed an algal paste suspension daily.

Determination of infection. Ten oysters were randomly selected and sampled at the initiation of the experiment and at 24, 48, 72, 96, and 120 hours after the tissue mince was added to each container. Five of the ten oysters were analyzed for total body-burden of *P. marinus* and the remaining five sampled oysters were preserved for immunoassays in Davidson's AFA fixative. Immunoassays were conducted on the three samples with the highest body burdens—24, 48, and 72 hr. Body-burden and immunoassay analyses proceeded as described above.

Experiment 2

Oysters. Uninfected oysters (n=30) from the Damariscotta River, Maine were supplied by Pemaquid Oyster Company, Maine on 14 March 1995. The oysters were gradually acclimated to, and maintained at 20 ppt and 24–25°C. At the initiation of the experiment the oysters were placed in 1 L-chambers containing 750 mL of aerated 1 µm-filtered York River water (20 ppt). During the experiment temperature was maintained at 25–27°C.

Tissue mince preparation and administration. Tissue mince containing infective *P. marinus* cells was prepared and administered over a five day period. Infected oysters were collected from the James River, VA. Tissue from 5–7 infected live oysters or gapers was finely minced using a razor blade and subsequently homogenized in filter-sterilized York River water (FYRW) using an Ultra Turrax tissue homogenizer (Tekmar, Cincinnati, OH.). The homogenate was filtered through 300 and 75 µm nitex sieves. The total volume of the resulting filtrate was raised to 300 mL with FYRW. Each oyster was exposed to 5 mL of tissue mince preparation once each day for a period of 5 days. The tissue mince filtrate containing *P. marinus* cells was prepared on the day of administration (for dose 1, 3, and 5) or the day before administration (for dose 2 and 4). Immediately after the dose was administered, the oysters were fed a suspension of algal paste (0.1 g $oyster^{-1}$). The concentration of *P. marinus* meronts in tissue mince dose suspensions was estimated from two 5 mL subsamples of mince that were incubated in 20 mL RFTM and processed as specified for body-burden assays. Estimated *P. marinus* dose concentrations are shown in Table 1. The total dose over the 5 day challenge period was estimated to be 2.2 x 10^7 cells $oyster^{-1}$.

Table 1.
Perkinsus marinus dose concentrations for Portal of Entry Experiment 2. Oysters were dosed once a day for 5 days with tissue mince containing *P. marinus* cells. The number of *P. marinus* cells in each dose was determined from replicate tissue mince subsamples using a modification of the total body-burden assay.

Day/Dose	Replicate A No. cells x 10^6	Replicate B No. cells x 10^6	Average of Replicates No. cells x 10^6
1	4.9	3.5	4.2
2	3.1	3.8	3.5
3	2.2	2.9	2.6
4	4.2	5.9	5.1
5	6.5	7.2	6.9
	Average no. cells per day =		4.4×10^6 cells per day
	Total dose =		2.2×10^7 cells

Except during the dosing period water in each chamber was renewed three times each week. During the dosing period water in each chamber was renewed only on day 3. The oysters were fed an algal paste suspension daily.

Determination of infection. Oysters (n=5) were sampled immediately before exposure and at 1, 4, 7 and 21 days after the last dose. The sampled oysters were shucked and preserved in Davidson's AFA fixative. To verify the success of the infection experiment and immunoassay observations, an additional sample of 5 oysters was taken on day 21 and analyzed using total body-burden assays. Body-burden and immunoassay analyses proceeded as described above.

RESULTS

Temperature and Salinilty

Approximately 160 water samples were collected from each site during the period of study, 1 April 1994 through 31 March 1995. Temperature and salinity at the two study sites for the period of investigation are shown in Figure 2. Salinity ranged from 4.0–15.0 ppt at the Tred Avon River, MD (TAR) site and from 9.5–22.0 ppt at the York River, VA (YR) site. Minimum salinities were observed at both sites during the spring of 1994. Temperatures were very similar at TAR and YR ranging from 0.5–30.0°C at TAR and from 3.0–29.0°C at YR. Maximum temperature values occurred in July at both sites. Temperature at both sites consistently exceeded 20°C from the last week of May to the first week of October and consistently exceeded 25°C from mid-June through the first week of September.

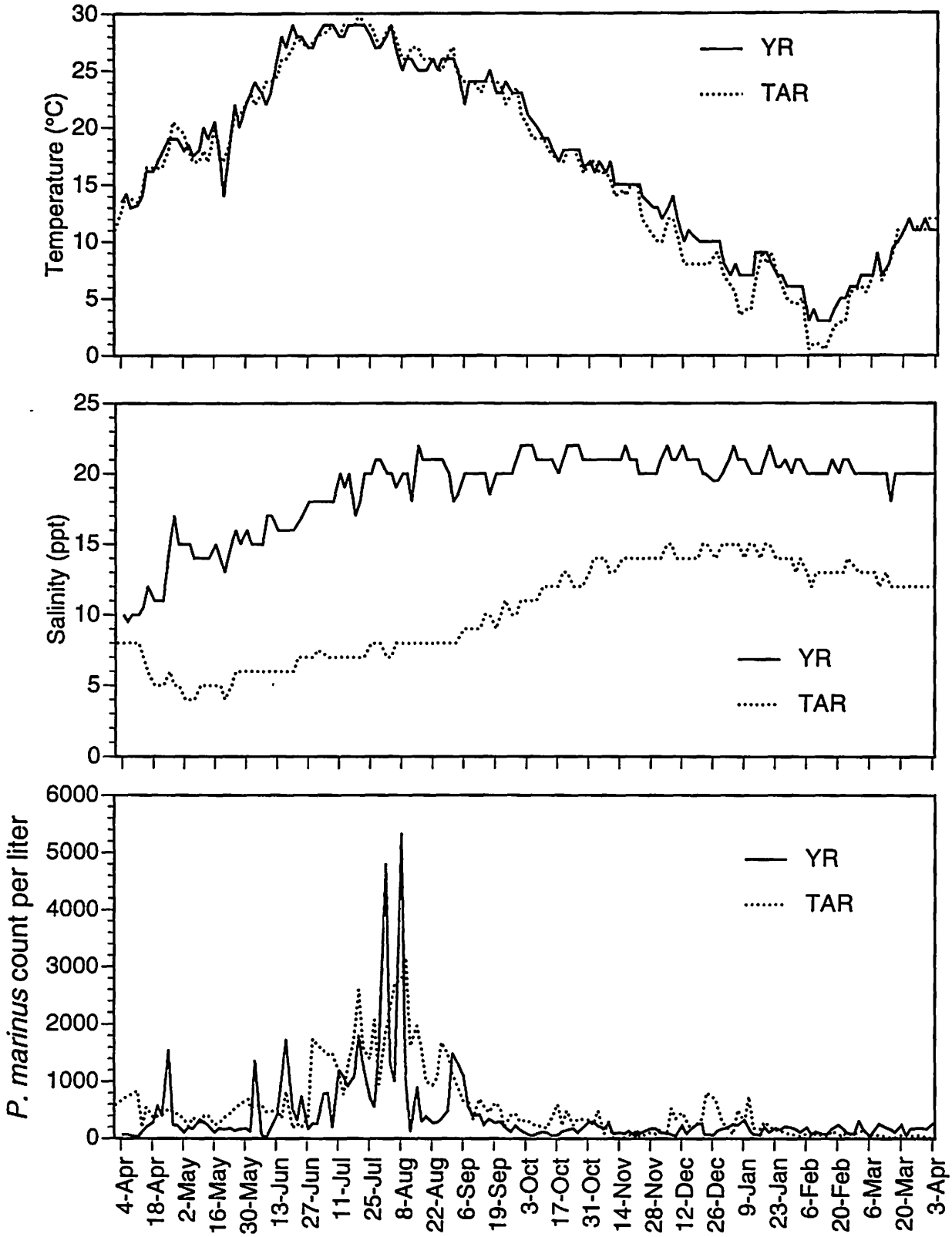


Figure 2. Temperature, salinity, and *P. marinus* cell counts in environmental water samples from York River, VA (YR) and Tred Avon River, MD (TAR).

Enumeration of *P. marinus* in water samples

Flow cytometric counts of *P. marinus* for individual water samples are shown in Figure 2. Major peaks in *P. marinus* counts were observed during the months of July and August at both the Tred Avon and York River sites. Maximum counts were observed in early August at both sites (Fig. 2). Peak abundance was higher at the York River site (5330 cells L^{-1}) than at the Tred Avon River site (3160 cells L^{-1}); however, cell abundances were generally higher at TAR than at YR. Statistical analyses of log-transformed cell counts indicated that differences between sites were significant for some months and across all months (Table 2). A distinct seasonal periodicity in cell abundance was observed at both sites. In order to better visualize seasonal trends, biweekly means, maximums, and minimums are presented in Figure 3. Abundances at YR during April and May were relatively low. During this time biweekly means did not exceed 600 cells L^{-1}, however, individual sample counts as high as 1550 cells L^{-1} were recorded. Biweekly mean cell count progressively increased during June and July and peaked in early August at 2000 cells L^{-1}. This summer increase in cell abundance closely corresponded with the period when temperatures exceeded 27°C (Fig. 2). The period of peak abundance in August was characterized by high variability in sequential individual sample counts. During the first two weeks of August counts ranged from 120-5330 cells L^{-1}. A sharp decline in *P. marinus* abundance was observed in late August and early September, corresponding to a decline in water temperature from 27°C to 20°C (Fig. 2). From late September 1994 through late March 1995 little variability in abundance was observed and cell counts remained low with fewer than 200 cells L^{-1}.

A similar seasonal trend was observed at TAR. Mean abundances ranged from about 300 to 600 cells L^{-1} during the period of 1 April through 15 June 1995 (Fig. 3). Biweekly cell count means progressively increased from late June to early August when an annual maximal biweekly mean of 2337 cells L^{-1} was observed. Throughout the summer, the variability in sequential individual cell counts at TAR was lower than at YR. Mean biweekly abundance declined during late August and September and remained at a winter baseline level of less than 200 cells L^{-1} from 1 October 1994 through 31 March 1995 (Fig. 3).

Analysis of variance tests indicated that log-transformed *P. marinus* cell counts significantly differed by month at both sites (at YR and TAR, P-value<0.0001). Results of Scheffe multiple comparison tests are shown in Table 3. In addition, statistical analyses indicated a strong positive correlation between biweekly temperature and abundance means at each site (Table 4 and Fig. 4). Cell abundance did not significantly correlate with salinity at YR but a significant negative correlation was observed at TAR (Table 4 and Fig. 4).

Table 2.
Mean monthly values of log transformed *P. marinus* cell counts at York River (YR) and Tred Avon River (TAR) sites and results of statistical comparison of site means using t-tests. Degrees of freedom (DF), t-value and P-value are shown. Asterisks denote significant differences between site means at an alfa value=0.05.

Month	YR	TAR	DF	t-Value	P-Value
Total	2.324	2.483	301	-2.239	.0259*
Apr	2.286	2.550	21	-1.338	.1952
May	2.239	2.563	24	-5.398	<.0001*
Jun	2.446	2.657	24	-1.144	.2640
Jul	2.904	3.159	23	-2.777	.0107*
Aug	2.846	3.225	26	-2.791	.0097*
Sep	2.528	2.692	24	-1.653	.1114
Oct	2.011	2.466	23	-5.782	<.0001*
Nov	2.108	1.896	22	1.303	.2060
Dec	2.050	2.483	22	-3.692	.0013*
Jan	2.201	2.274	23	-.680	.5031
Feb	2.059	1.744	21	3.735	.0012*
Mar	2.152	1.604	26	4.410	.0002*

Table 3.
Mean monthly log transformed *P. marinus* cell count ranked from highest to lowest at York River and Tred Avon River. Letters following month denote Scheffe multiple comparison test results. Months sharing like letters do not significantly differ from each other. Comparisons were made within sites only.

York River		Tred Avon River	
Month	Mean	Month	Mean
Jul	2.904 a	Aug	3.225 a
Aug	2.846 ab	Jul	3.159 ab
Sep	2.528 abc	Sep	2.692 bc
Jun	2.446 bc	Jun	2.657 bc
Apr	2.286 bc	May	2.653 c
May	2.239 c	Apr	2.550 c
Jan	2.201 c	Dec	2.483 c
Mar	2.152 c	Oct	2.466 c
Nov	2.108 c	Jan	2.274 cd
Feb	2.059 c	Nov	1.896 de
Dec	2.050 c	Feb	1.744 de
Oct	2.011 c	Mar	1.604 e

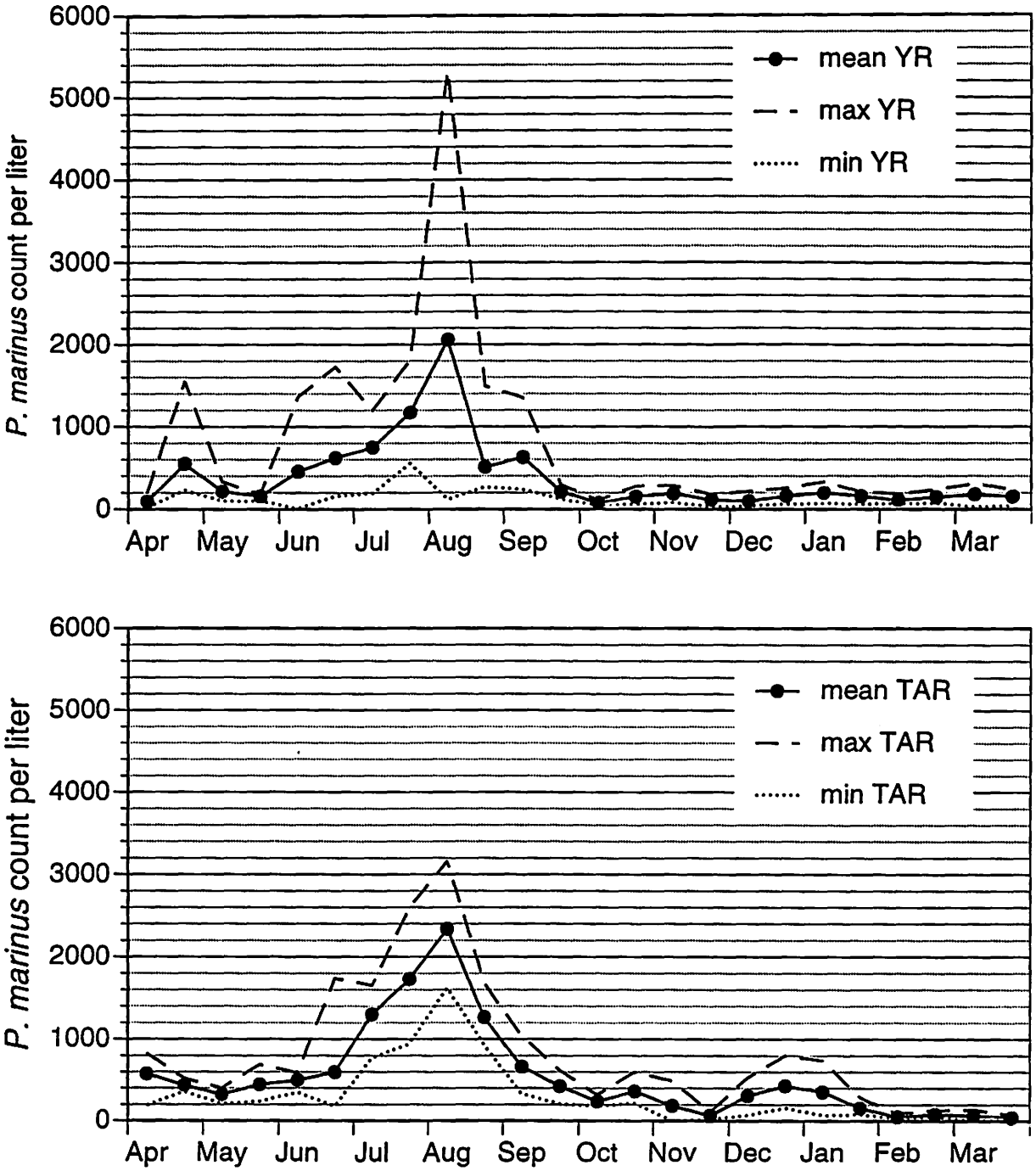


Figure 3. Biweekly mean, maximum, and minimum *P. marinus* cell count at York River (YR, top) and Tred Avon River (TAR, bottom) sites.

Table 4.
Correlation analysis of measured parameters—*P. marinus* cell counts, temperature, salinity, oyster mortality rate, and infection acquisition (prevalence). Analyses were conducted on York River data (top), Tred Avon River data (middle), and pooled data from York and Tred Avon Rivers (bottom). Data used in analyses were biweekly means. Correlation coefficients, number of observations considered in analysis (n), Fisher's Z-value, and P-values are shown. Asterisks note statistically significant correlations at an alpha level of 0.05.

York River Site

Parameters	Correlation	n	Z-Value	P-Value
P. marinus count, Temperature	.735	24	4.306	<.0001*
P. marinus count, Salinity	-.093	24	-.430	.6675
P. marinus count, Mortality	.712	23	3.985	<.0001*
P. marinus count, Prevalence	.376	23	1.768	.0770
Mortality, Temperature	.690	23	3.792	.0001*
Mortality, Salinity	.170	23	.769	.4419
Mortality, Prevalence	.585	23	2.997	.0027*
Prevalence, Temperature	.302	23	1.395	.1631
Prevalence, Salinity	.287	23	1.320	.1867

Tred Avon River Site

Parameters	Correlation	n	Z-Value	P-Value
P. marinus count, Temperature	.784	24	4.843	<.0001*
P. marinus count, Salinity	-.596	24	-3.148	.0016*
P. marinus count, Mortality	.314	19	1.301	.1934
Mortality, Temperature	.705	19	3.512	.0004*
Mortality, Salinity	-.335	19	-1.393	.1638

Tred Avon River & York River Site

Parameters	Correlation	n	Z-Value	P-Value
P. marinus count, Temperature	.749	48	6.518	<.0001*
P. marinus count, Salinity	-.340	48	-2.375	.0179*
P. marinus count, Mortality	.421	42	2.802	.0051*
Mortality, Temperature	.556	42	3.916	<.0001*
Mortality, Salinity	.263	42	1.683	.0924

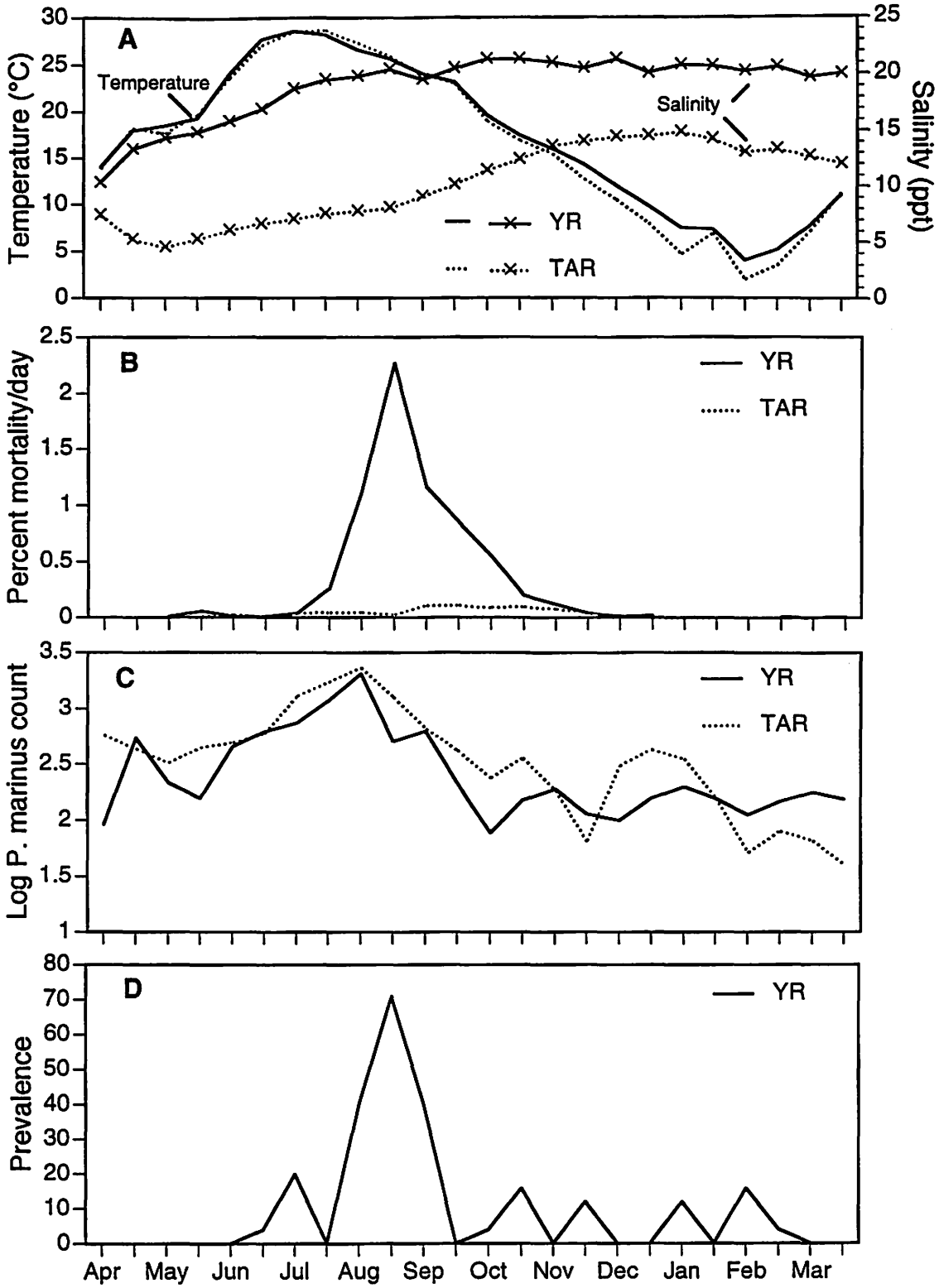


Figure 4. Biweekly mean temperature (A), salinity (A), and log-transformed *P. marinus* count (C) in relation to oyster mortality rate (B) and *P. marinus* infection acquistion, prevalence, (D) at the York River site.

Oyster mortality

Mortality of infected oysters differed dramatically between sites (Fig. 5). Mortality did not significantly differ between replicate trays at either site so the data were pooled for presentation and analysis. More than 95% of the oysters collected from the James River, VA and held at YR died during the period of 15 April 1994-3 April 1995 (Fig. 5). Most of the mortality occurred between 7 July and 26 October. During this time a single major peak in mortality rate, expressed as percent mortality per day, was observed. Rate of mortality increased rapidly from 0.26% d^{-1} during the interval 7–19 July to 1.12% d^{-1} for the interval 19 July–3 August and was highest during the interval of 3–17 August (2.27%) (Fig. 5 and Fig. 6). Disease diagnosis of retrieved dead oysters (gapers) indicated that the observed mortality was associated with infections of *P. marinus* and *H. nelsoni* (Table 5). Of the 25 gapers retrieved on 17 August, 17 had heavy infections of *P. marinus* and 12 had heavy infections of *H. nelsoni*. Overall, 72 of the 83 gapers examined between 19 July and 13 October had moderate or heavy *P. marinus* infections. The rate of mortality declined gradually during the months of September and October; however, relatively high rates were still observed in early September (1.16 and 0.85% d^{-1}). Between the months of November 1994 and April 1995, mortality rate did not exceed 0.05% and cumulative mortality increased by less than 2.0%. Maximum mortality rates at YR were observed during and immediately following maximal summer temperatures (Fig. 6). The increase in mortality rate during July and early August coincided with maximum *P. marinus* abundance in the water column; however, relatively high mortality rates in late August and early September were associated with relatively low *P. marinus* cell abundances in the water column (Fig. 6). Statistical analysis indicated that YR biweekly mortality rates, expressed as percent mortality per day, were strongly and positively correlated with biweekly means of temperature and *P. marinus* cell abundances (Table 4 and Fig. 4).

Table 5.
Parasite prevalence and intensity in gapers retrieved from the YR captive oyster mortality monitoring population. Diagnosis of *P. marinus* was by standard RFTM assays according to Ray (1966). Diagnosis of *H. nelsoni* was by routine histological methods. Infection intensity was categorized as light (L), moderate (M), and heavy (H).

	P. marinus			*H. nelsoni*		
Date	No. infected/ examined	Prevalence	Intensity L-M-H	No. infected/ examined	Prevalence	Intensity L-M-H
19 Jul	4/4	100%	1-1-2			
3 Aug	24/25	96%	5-6-13			
17 Aug	25/25	100%	3-5-17	17/25	86%	4-1-12
2 Sep	5/5	100%	1-0-4			
15 Sep	10/10	100%	0-3-7			
29 Sep	10/10	100%	0-0-10			
13 Oct	4/4	100%	0-1-3			

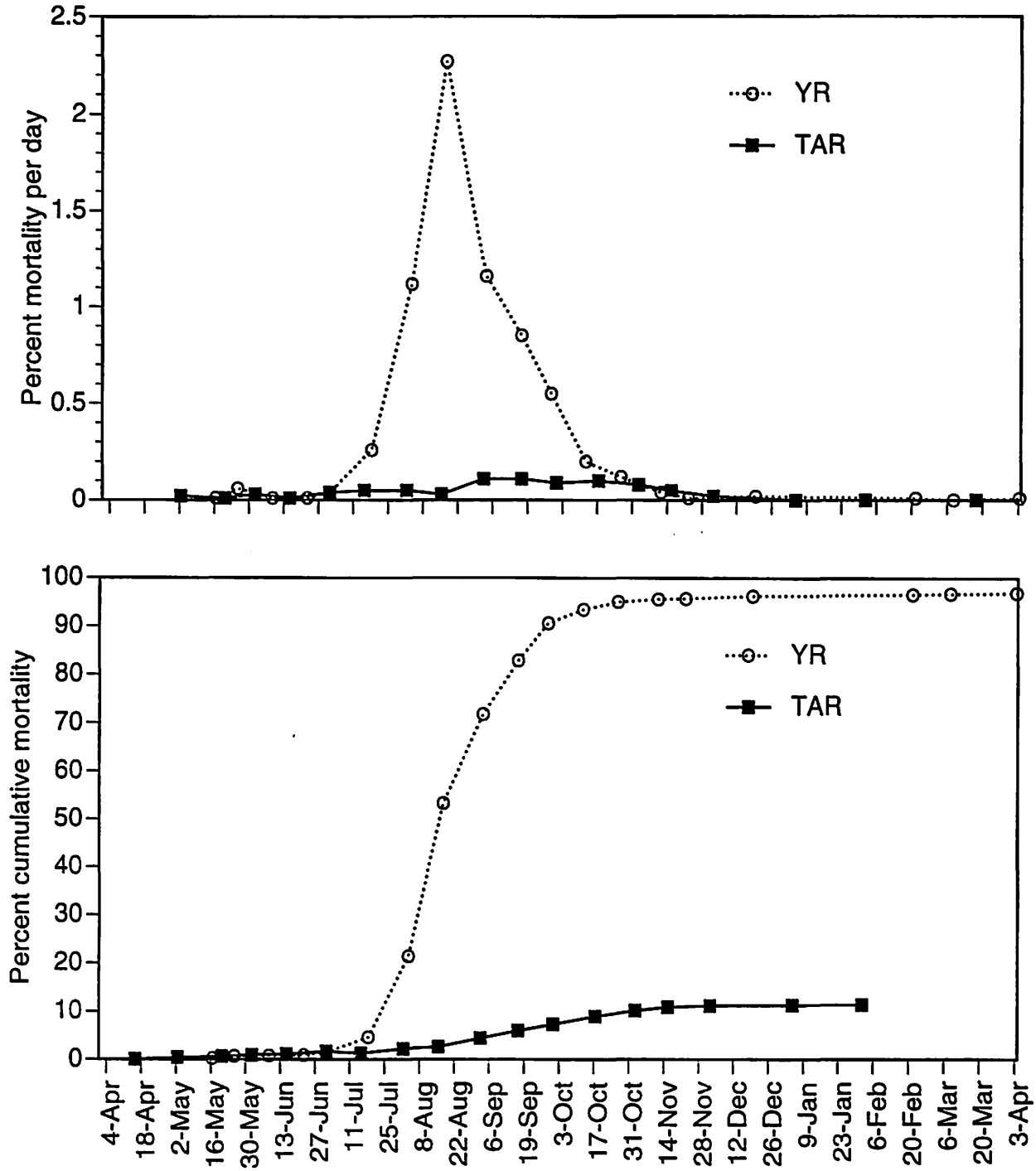


Figure 5. Mortality rate (percent mortality per day) and percent cumulative mortality of captive oyster populations at York River, VA (YR) and Tred Avon River, MD (TAR).

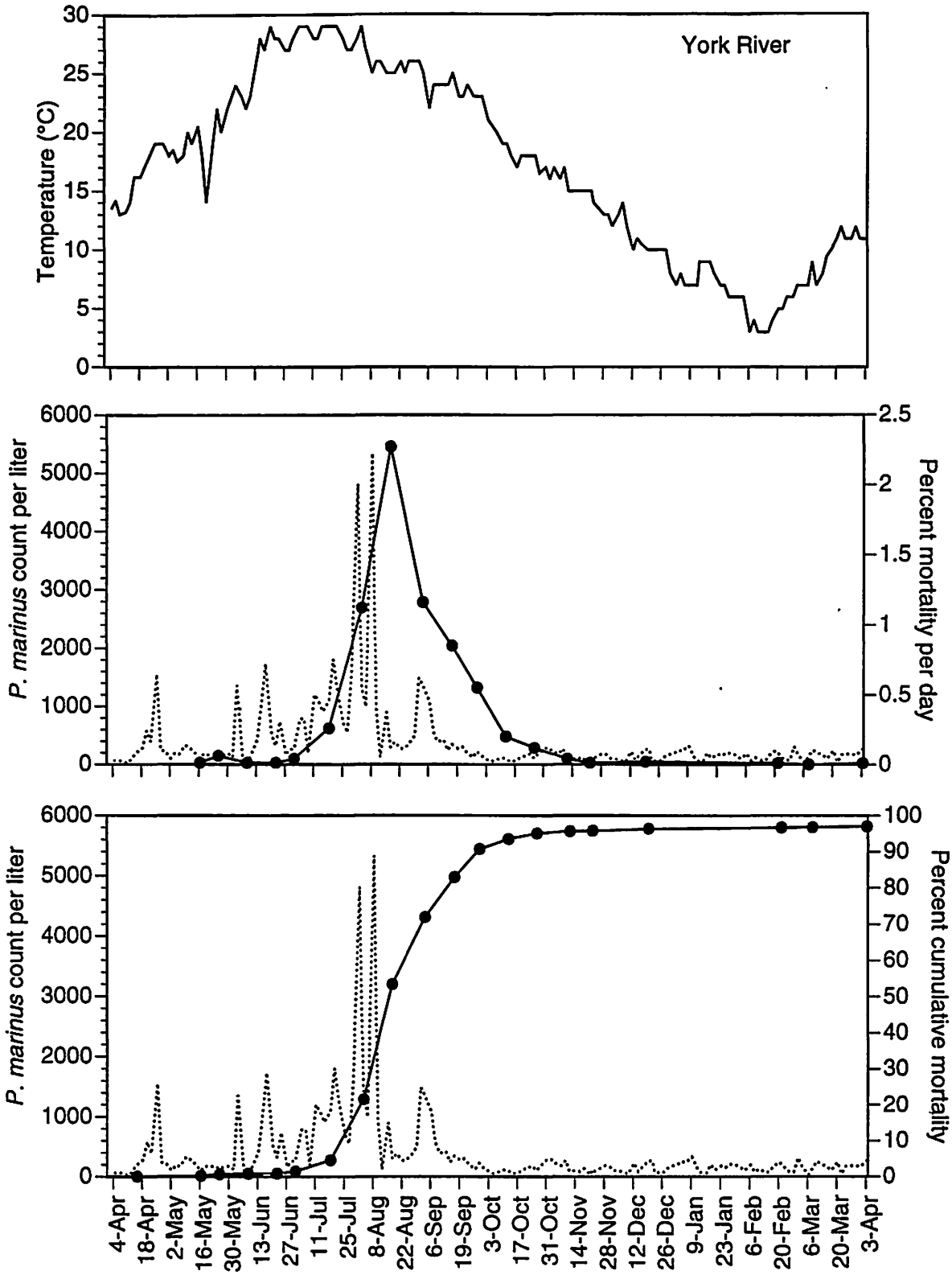


Figure 6. Temperature (top), and the relationship of *P. marinus* cell abundance (dashed lines) to oyster mortality rate (middle) and percent cumulative oyster mortality (bottom) at the York River, VA site.

Oyster mortality at TAR was much lower than at YR (Fig. 5). Cumulative oyster mortality of Tred Avon oysters for the entire study period was only 11.3%. Mortality progressed gradually from 1 April to 1 December. Maximal mortality rates, expressed as percent mortality per day, were observed from 15 Aug-1 Nov (Fig.7). The rate of mortality during this period ranged from 0.08–0.11% d^{-1}. Mortality at TAR occurred after maximal water column cell abundances and summer temperatures were observed. A statistically significant correlation was found between mortality rate and temperature but not between mortality rate and water column cell abundance (Table 4, Fig. 4).

Infection acquisition in sentinel oysters

Maximum rates of *P. marinus* infection in sentinel oysters deployed in the lower York River occurred during August (Fig. 8). Infections were not acquired by sentinel oysters deployed early (15 Apr–15 Jun 1994) or late (1–31 Mar 1995) in the study. Infections were observed sporadically in 11 of 17 sentinel groups deployed between 15 Jun 1994 and 28 Feb 1995. Generally, infections occurred in less than 20% of the oysters: however, relatively high prevalences (40–70%) were observed in oysters deployed between 1 August and 15 September (Table 6).

The peak infection acquisition period, 1 August–15 September, corresponded closely with the period of maximum oyster mortality rates (Fig. 8). A strong and significant correlation was found between infection acquisition (prevalence) and mortality rate; however, a significant correlation was not found between prevalence and temperature (Table 4). Although maximum prevalences occurred in oysters deployed at temperatures from 20–25°C, infections were also present in oysters deployed in mid-winter at temperatures as low as 4.0°C. Similarly, a relatively high prevalence (40%) was observed in oysters deployed during the period of peak cell abundance, 1–15 August; however, cell abundances declined precipitously during the period of maximal infection acquisition, 16–31 August (Fig. 8). The correlation between cell abundance and *P. marinus* prevalence in sentinel oysters was not significant (Tab. 4). Infected sentinel oysters generally exhibited light infections; 68% of positively diagnosed oysters had body-burdens of fewer than 10 parasite cells (Table 6). Maximum infection intensities were observed in oysters deployed from 1–15 July, 1–16 August and 16–31 August. Diagnosis of *P. marinus* using total body-burden assays was extremely difficult when infections were rare (fewer than 10 cells per oyster) because it is often difficult to distinguish stained non-*P. marinus* particulates from occasional parasite cells. Diagnoses reported as positive here are conservative as only definite positives, based on a combination of characteristics including stain coloration, shape, and cell wall morphology, were tallied.

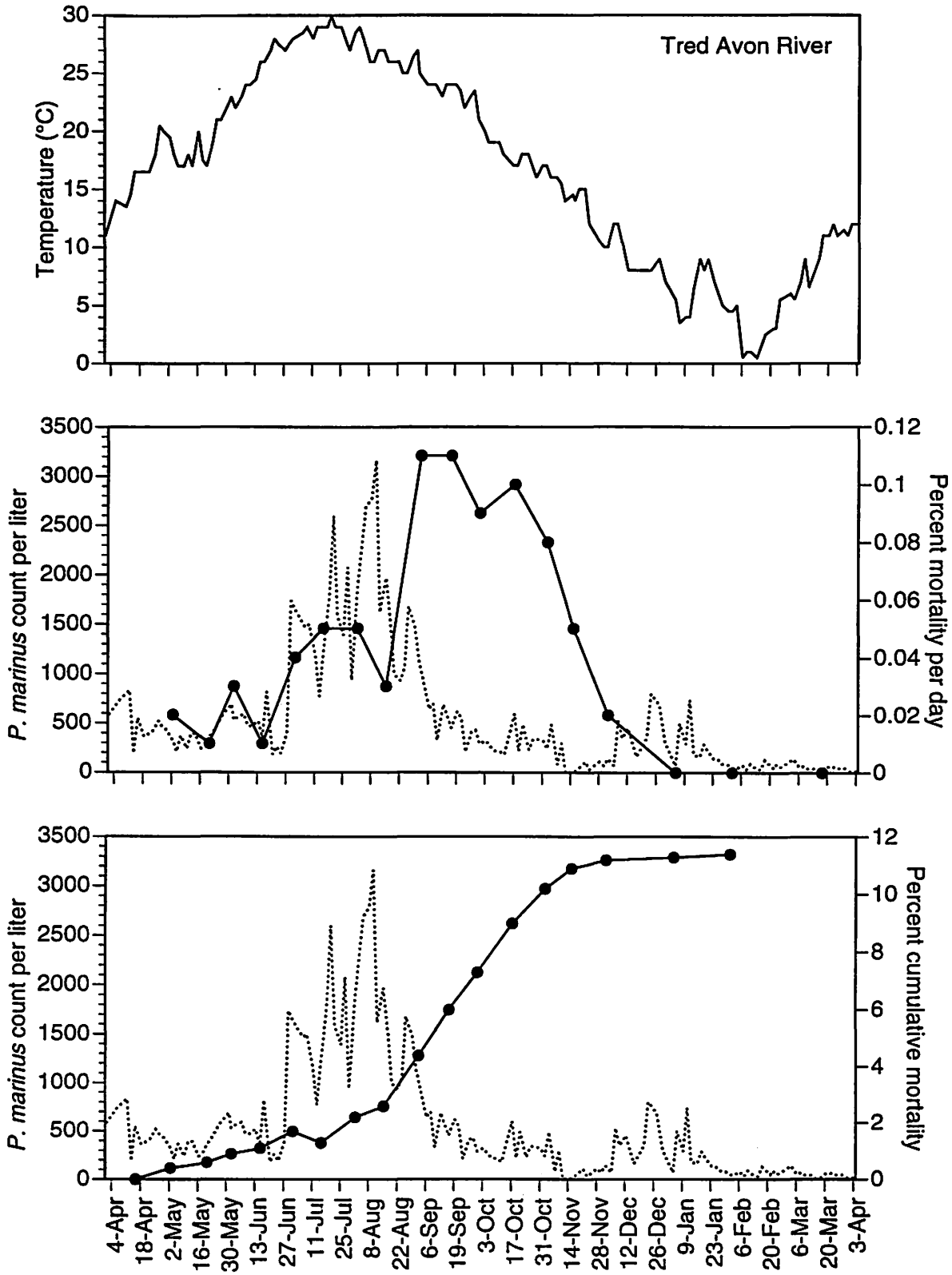


Figure 7. Temperature (top), and the relationship of *P. marinus* cell abundance (dashed lines) to oyster mortality rate (middle) and percent cumulative oyster mortality (bottom) at the Tred Avon, MD site.

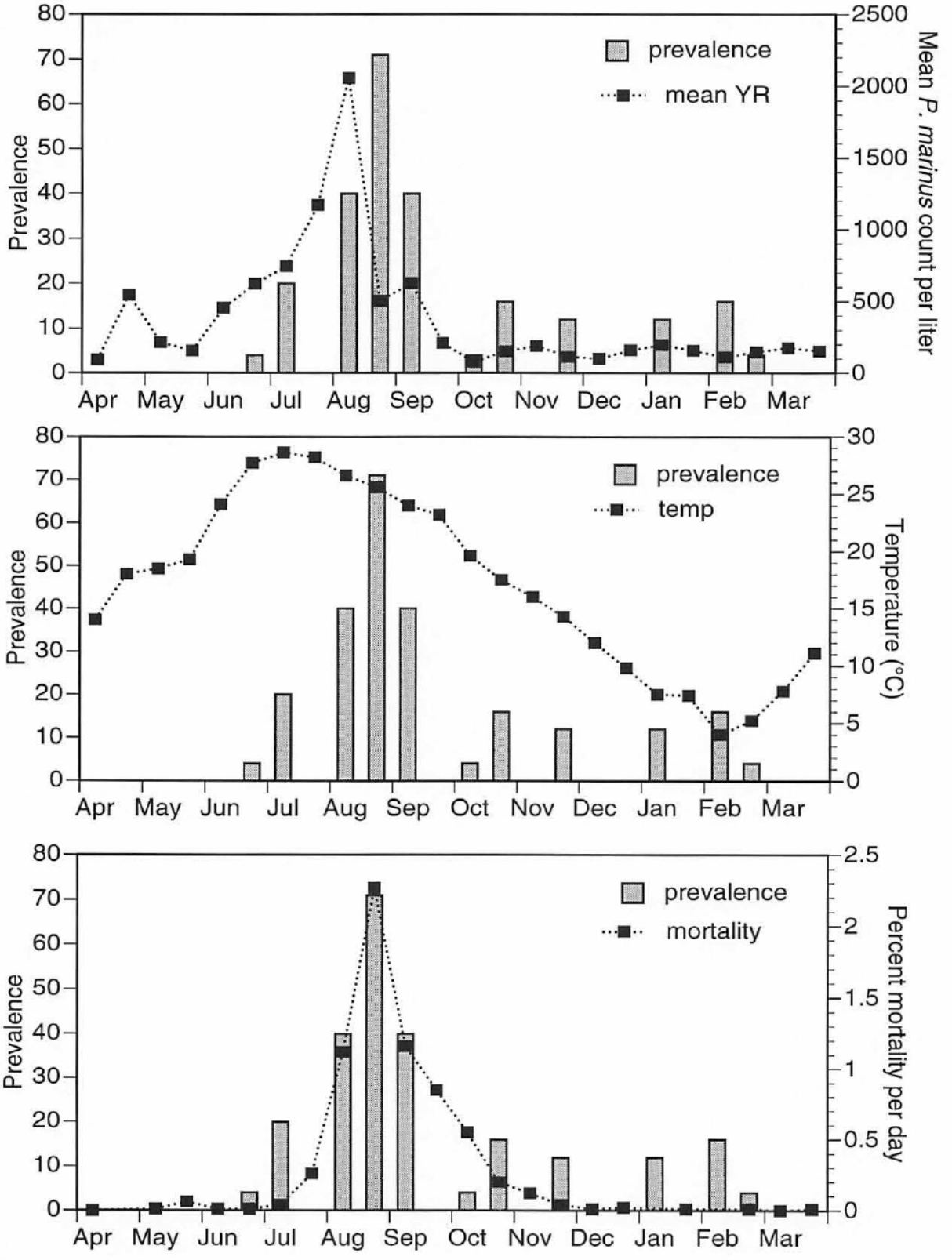


Figure 8. *Perkinsus marinus* infection acquisition (prevalence) in relation to biweekly mean cell counts (top), biweekly mean temperature (middle), and oyster mortality rate (bottom) at the York River site.

Table 6.
Perkinsus marinus infection acquistion in disease-free sentinel oysters deployed for 14-15 days in the York River, VA, and in laboratory-maintained control oysters. *Perkinsus marinus* prevalence and intensity were determined using total body-burden assays (n=23-25). Total body-burden intensities are categorized as rare (R) 1-10 parasite cells per oyster, light (L) 11-100 parasite cells per oyster, moderate (M) 101-1000 parasite cells per oyster, and heavy (H) >1000 parasite cells per oyster. The range in number of parasite cells detected in infected oysters is also shown.

	Sentinel Oysters			Control Oysters	
York River Exposure Period	Prevalence	Intensity R-L-M-H	No. parasites detected	Prevalence	No. parasites detected
1994 Apr 15-30	0%	0		0%	
May 1-15	0%	0		8%	1-3
May 16-31	0%	0		0%	
Jun 1-15	0%	0		0%	
Jun 16-30	4%	0-1-0-0	85	0%	
Jul 1-15	20%	4-0-0-1	1-2010	0%	
Jul 15-30	0%	0		0%	
Aug 1-16	40%	7-0-1-2	1-5025	8%	1-4
Aug 16-31	71%	8-8-0-1	1-1505	4%	120
Sep 1-15	40%	6-3-1-0	1-187	4%	1
Sep 16-30	0%	0		0%	
Oct 1-15	4%	1-0-0-0	1	0%	
Oct 16-31	16%	2-0-0-0	1-2	0%	
Nov 1-15	0%	0		0%	
Nov 16-30	12%	3-0-0-0	1-2	0%	
Dec 1-15	0%	0		0%	
Dec 16-2 Jan	0%	0		0%	
1995 Jan 2-16	12%	3-0-0-0	1-3	0%	
Jan 17-Feb 1	0%	0		0%	
Feb 2-16	16%	3-1-0-0	2-28	0%	
Feb 17-Mar 1	4%	1-0-0-0	2	0%	
Mar 2-16	0%	0		4%	1
Mar 17-31	0%	0		0%	

Perkinsus marinus was detected at extremely low prevalences (<8%) in five of the 23 groups of laboratory maintained control oysters (Table 6). Body-burden analysis of *P. marinus* infected control oysters revealed only 1-2 parasite cells in all but one case, which had 120 parasite cells. Disease diagnoses of Damariscotta River oysters sampled upon arrival to VIMS are shown in Table 7. *Perkinsus marinus* was not detected in any of the oysters sampled upon arrival. However, *P. marinus* diagnoses were based on standard RFTM assays, which are less sensitive than body burden assays for detecting rare infections. Hence, it is difficult to discern whether infections in control oysters existed in the Maine oysters upon arrival or were acquired at VIMS during the course of the experiment. *Haplosporidium nelsoni* was detected in 8% of the oysters sampled on 8 June, but was not detected in any other sample group. No other abnormalities were observed in the Damariscotta River oysters examined.

Table 7.
Prevalence of *P. marinus* and *H. nelsoni* in Damariscotta River, ME oysters examined upon arrival at VIMS. Diagnosis of *P. marinus* was by standard RFTM assays according to Ray (1966). Diagnosis of *H. nelsoni* was by routine histological methods. Infection intensity was categorized as negative (N), light (L), moderate (M), and heavy (H). All samples consisted of 25 oysters.

Sample Date	*P. marinus* prevalence	*P. marinus* intensity (H-M-L-N)	*H. nelsoni* prevalence	*H. nelsoni* intensity (H-M-L-N)
7 April 1994	0%	0-0-0-25	0%	0-0-0-25
9 May 1994	0%	0-0-0-25	0%	0-0-0-25
8 June 1994	0%	0-0-0-25	8%	0-0-2-23
7 July 1994	0%	0-0-0-25	0%	0-0-0-25
11 Aug 1994	0%	0-0-0-25	0%	0-0-0-25
13 Sept 1994	0%	0-0-0-25	0%	0-0-0-25
13 Oct 1994	0%	0-0-0-25	0%	0-0-0-25
10 Nov 1994	0%	0-0-0-25	0%	0-0-0-25
9 Dec 1994	0%	0-0-0-25	0%	0-0-0-25
11 Jan 1995	0%	0-0-0-25	0%	0-0-0-25
15 Feb 1995	0%	0-0-0-25	0%	0-0-0-25

Examination of sentinel oysters by immunoassay

Many *P. marinus* cells were observed at external epithelial surfaces (gill and mantle) and in stomach and intestinal lumina verifying exposure of oysters to the pathogen. The occurrence of *P. marinus* cells within epithelia or connective tissues was rare. When parasite cells were observed within host tissues, they occurred singly or in pairs, with little evidence of proliferation or colonization. Cells were detected in 5/5 of the August 2 group, and 2/3 of the September 1 group. The maximum number of cells observed in any individual was four. Cells were observed in tissues of the mantle, gill, stomach, heart, and intestine (Fig. 9).

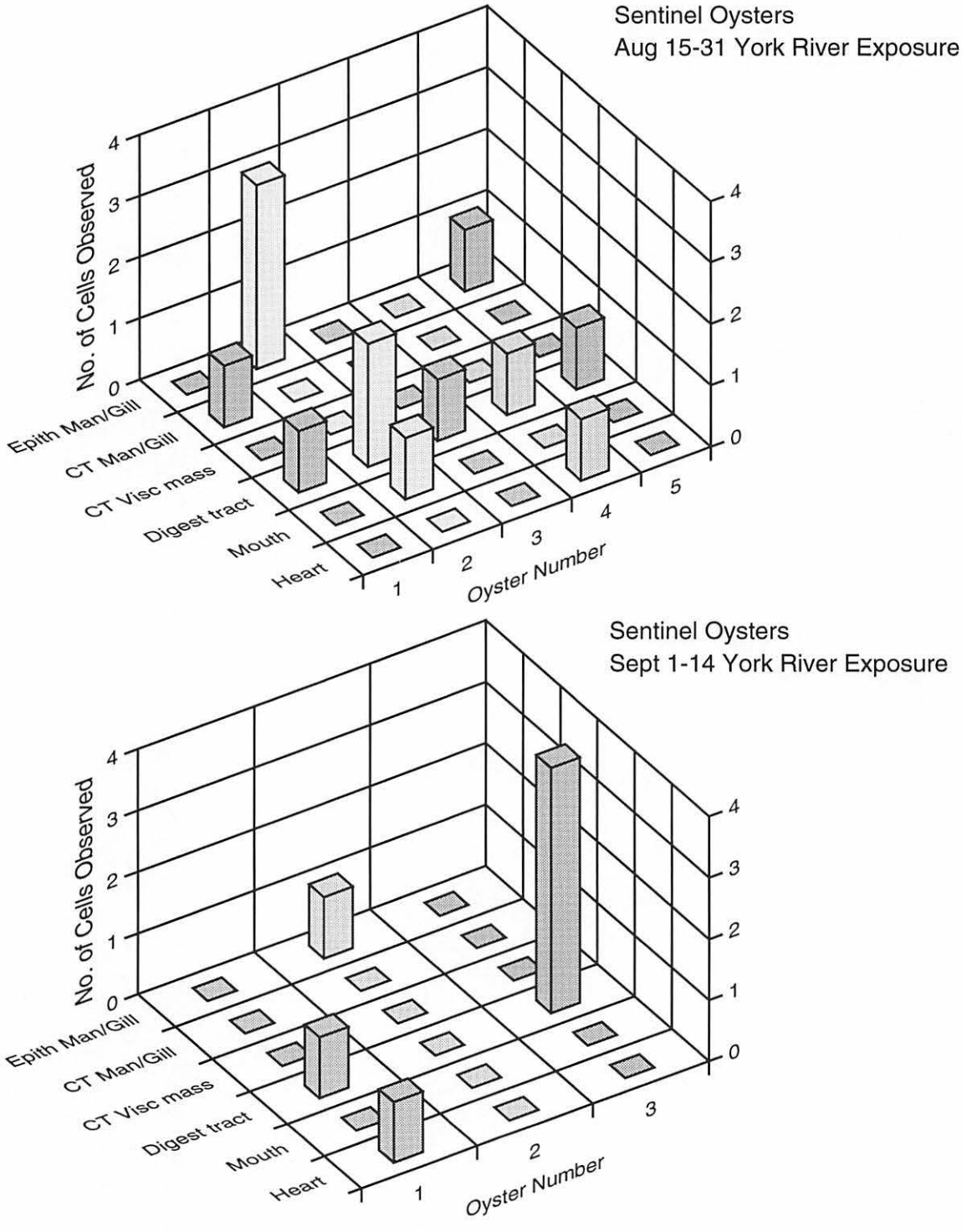


Figure 9. Distribution of *P. marinus* cells in sentinel oysters deployed at York River site. Localization is based on immunoassays. Infections were observed in 5/5 oysters deployed from 15-31 August (top, y-axis) and in 3/3 oysters deployed from 1-14 September (bottom, y-axis). Foci of parasite cells included mantle and gill epithelia, mantle, gill , and visceral mass conective tissue (CT), digestive tract (stomach, intestine and digestive gland), mouth epithelium, and heart (x-axis). Cells were not observed at any other tissue site examined. Frequency of parasite cells at specific sites for individual oysters ranged from 0-4 (z-axis). All observed lesions are shown.

Portal of entry

Experiment 1

Oysters sampled at the initiation of the experiment, prior to *P. marinus* exposure, were determined to be free of the parasite by both body-burden and immunoassay analyses. The results of body-burden analyses of oysters sampled 24, 48, 72, 96, and 120 hours after dose are shown in Table 8. Mean total body-burden progressively declined with time. *Perkinsus marinus* cells were detected in 5/5 oysters examined at 24, 48 and 72 hr, 4/5 oysters examined at 96 hr, and only 3/5 oysters analyzed at 120 hr. *Perkinsus marinus* cells were rare in oyster sections analyzed by immunoassays. Parasite cells were observed in tissues of only 2/5 oysters sampled at 24 hr, 3/5 oysters sampled at 48 hr, and 2/5 oysters sampled at 72 hr. Infection foci included mantle, gill, and stomach epithelia, and connective tissue of the mantle, palp and visceral mass (Fig. 10). In no case was more than a single *P. marinus* cell observed at a lesion site, so evidence of parasite proliferation, post invasion, was not obtained. While infections were rare, 87% of the assayed oysters showed *P. marinus* cells at mantle and gill epithelial surfaces and 100% of assayed oysters showed *P. marinus* in digestive tract lumina. Signet ring morphology was rare among *P. marinus* cells detected within epithelia, or associated with epithelial surfaces. Commonly observed *P. marinus* cell characteristics included diameters $\leq 3\mu m$ and pyriform shape, but no clearly flagellated cells (zoospores) were observed (Fig. 11). In one oyster, apparent schizogonic or zoosporogonic pathogen proliferation was recorded within the intestinal lumen (Fig. 11).

Table 8.
Number of *P. marinus* cells present in laboratory Portal of Entry Experiment 1 oysters sampled at time=0, and at 24, 48, 72, 96, and 120 hr after exposure to 8.95×10^6 *P. marinus* meronts. Counts are based on body-burden analysis. Mean and standard deviation (S.D.) are shown.

	Number of *P. marinus* cells in replicate oysters						
Time	Oyster 1	Oyster 2	Oyster 3	Oyster 4	Oyster 5	Mean	S.D.
0	0	0	0	0	0	0	0
24	282	59	119	268	497	245	170.2
48	104	99	47	90	62	80.4	24.7
72	48	9	41	14	24	27.2	16.8
96	40	4	0	22	5	14.2	16.7
120	0	4	0	2	1	1.4	1.7

Experiment 2

Establishment of infections in experimental oysters was somewhat more successful in Experiment 2 than in Experiment 1. Oysters in Experiment 2 were exposed to a higher number of *P. marinus* cells over a longer exposure period. Parasite cells were rare in immunoassay sections of oysters sampled 1, 4, and 7 days after exposure. Cells were detected in only 4/15 oysters

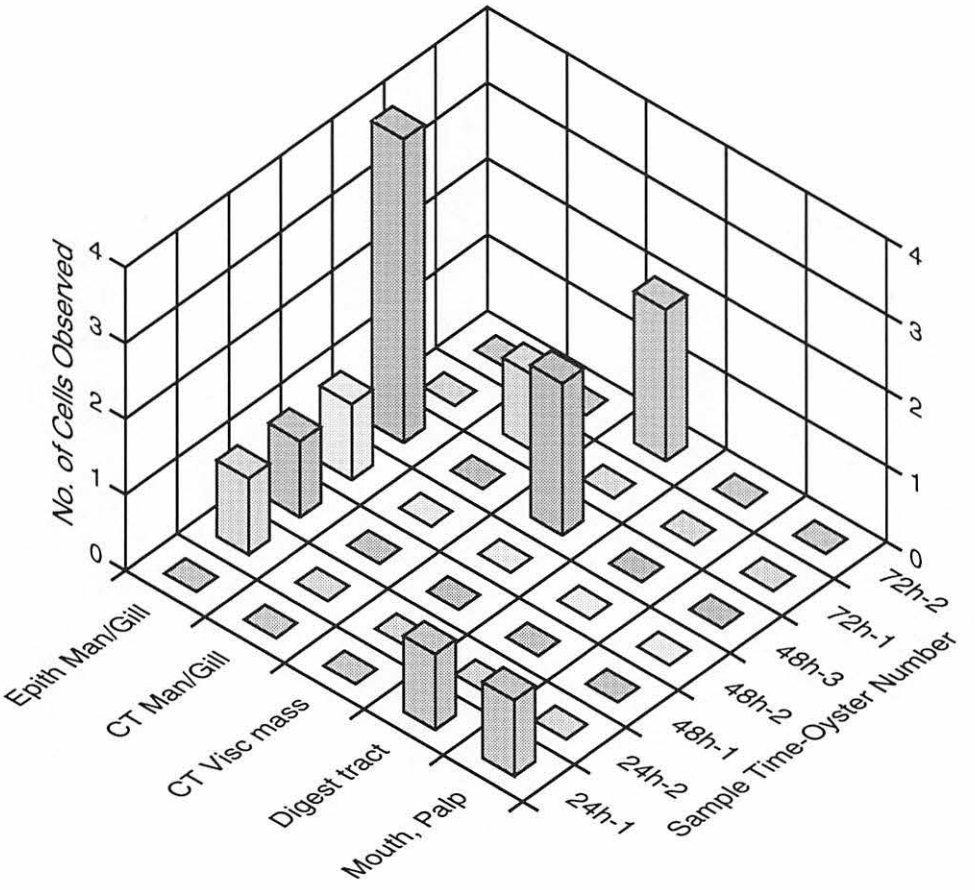
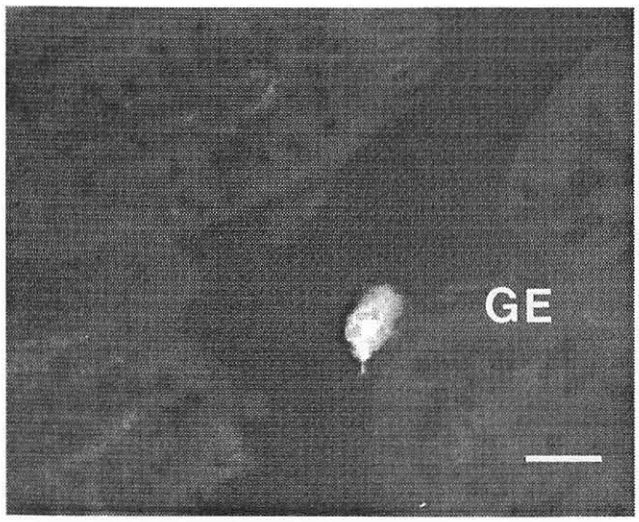
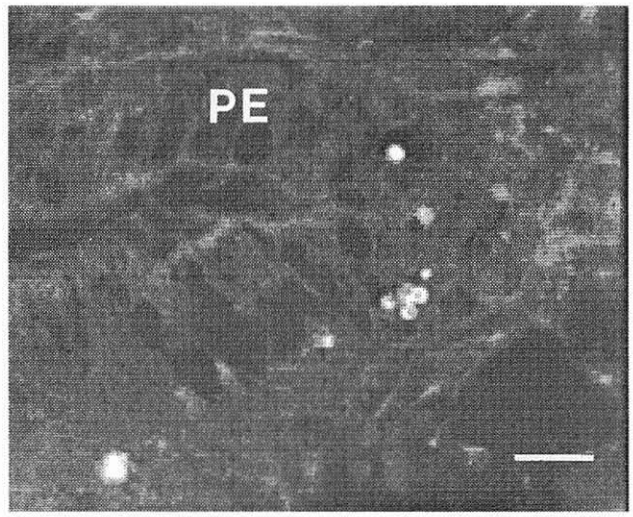
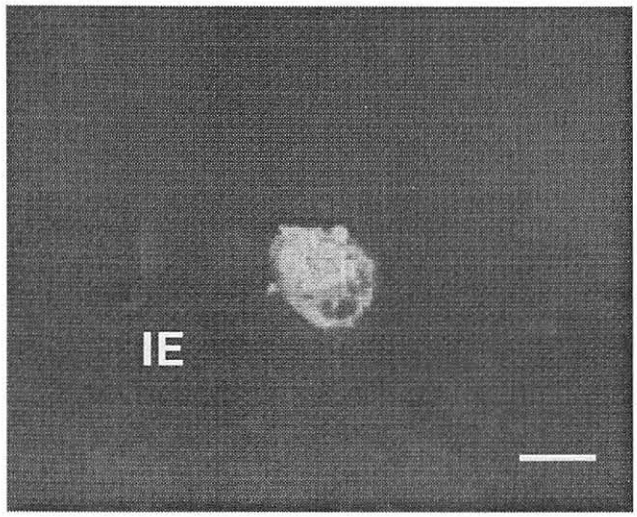


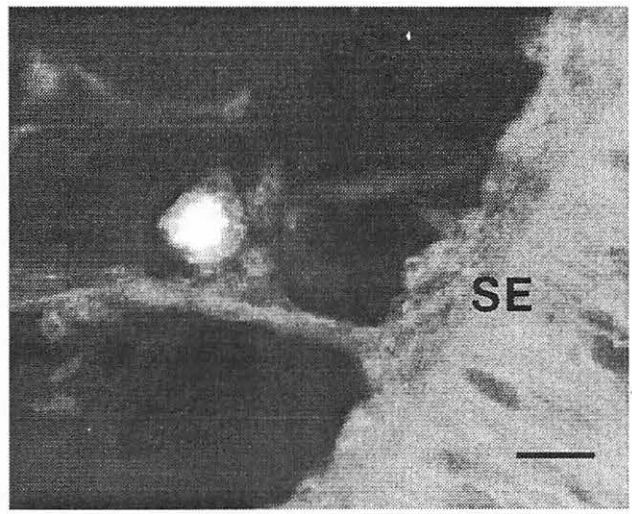
Figure 10. Distribution of *P. marinus* cells in Portal of Entry Experiment 1 oysters. Localization is based on immunoassays. Five oysters were sampled at 24, 48, and 72h post *P. marinus* challenge. Parasite cells were detected in only 7/15 oysters analyzed. All infected individuals are shown (y-axis). Foci of parasite cells included mantle and gill epithelia, mantle, gill, and visceral mass conective tissue (CT), digestive tract (stomach,intestine, and digestive glands), and palp conective tissue (x-axis). Cells were not observed in other tissues examined. The frequency of of cells detected at specific sites for individual oysters ranged from 0-4 (x-axis). All observed lesions are shown. Sample times and individual oyster number are shown on y-axis.

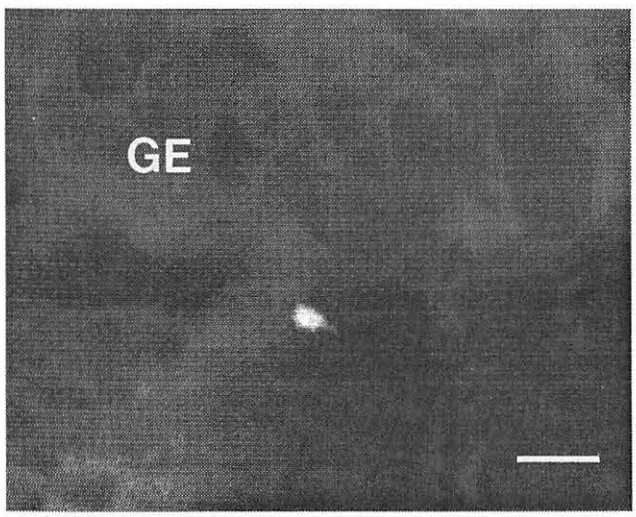
Figure 11. Immunoassay detection of *Perkinsus marinus* in histological sections for portal of entry experiments. Column 1 = experiment 1, column 2 = experiment 2. **Column 1, top:** cluster of immunopositive cells adjacent to gill epithelium (GE) (scale bar = 12 μm). **Middle:** immunopositive cell in intestinal lumen epithelium (IE) (scale bar = 6 μm); note 1–2 μm cells (zoospores?) apparently being released from larger cell. **Bottom:** immunopositive cell (1.2 μm) (zoospore?) closely associated with gill epithelium (scale bar = 4.5 μm). **Column 2, top:** scattered immunopositive cells in labial palp epithelium (PE) (scale bar = 25 μm). **Middle:** cluster of immunopositive cells within hemocyte in visceral connective tissue near stomach epithelium (SE) (scale bar = 10 μm). **Bottom:** single immunopositive cell within an oyster hemocyte; dark oval region at bottom of hemocyte is the nucleus (HN) (scale bar = 5 μm).

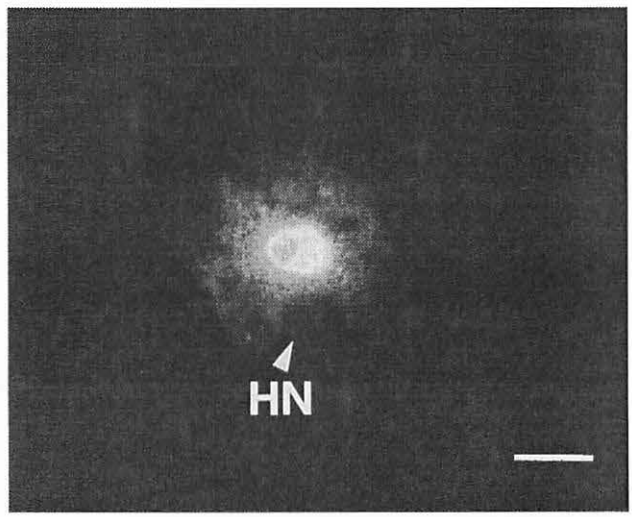












1

2

analyzed. Detected cells were located in gill and mantle epithelia, gill connective tissue, and within hemocytes in visceral connective tissue (Fig. 12). Lesions were not observed in tissues of the digestive tract. *Perkinsus marinus* lesions were much more common in oysters sampled 21d post-exposure—lesions contained many more cells and were present at some site in all (5) oysters. Foci of parasite cells, in order of decreasing frequency, included gill and mantle epithelia, visceral mass connective tissue, gill and mantle connective tissue, palp connective tissue, and digestive gland epithelium (Fig. 12). Pathogen cells were not observed in stomach or intestinal tissues. Lesions containing multiple pathogen cells were frequently observed, indicating post-invasion proliferation or multiple pathogen cell entries at lesion sites. Numerous pathogen cells were observed to be circulating within phagocytic hemocytes in the vascular system (Fig. 12). The presence of *P. marinus* cells in oysters sampled at 21d post-exposure was verified by body-burden analysis that showed relatively high parasite body-burdens (4000-52,000 cells per oyster)(Table 9) in 5/5 oysters analyzed.

Table 9.
Perkinsus marinus infection level in Experiment 2 oysters sampled 21 days following 5-day exposure to tissue mince containing infective *P. marinus* cells. Diagnoses were made using the total body-burden technique.

Oyster	Total number *P. marinus* cells	Number of *P. marinus* cells per gram tissue
1	12,400	2756
2	1,060	100
3	18,400	4089
4	52,500	6177
5	4,150	483
Average =	17,702	2721
S.D. =	20,616	2534

DISCUSSION

This study is the first to systematically examine the seasonality and site of *P. marinus* infection acquisition in oysters in relation to water column abundance of *P. marinus*, oyster mortality, and temperature and salinity. Results support the currently accepted hypothesis that infective stages originate from dying oysters, but suggest that the digestive tract may not be the only, or even the primary, site of initial infection.

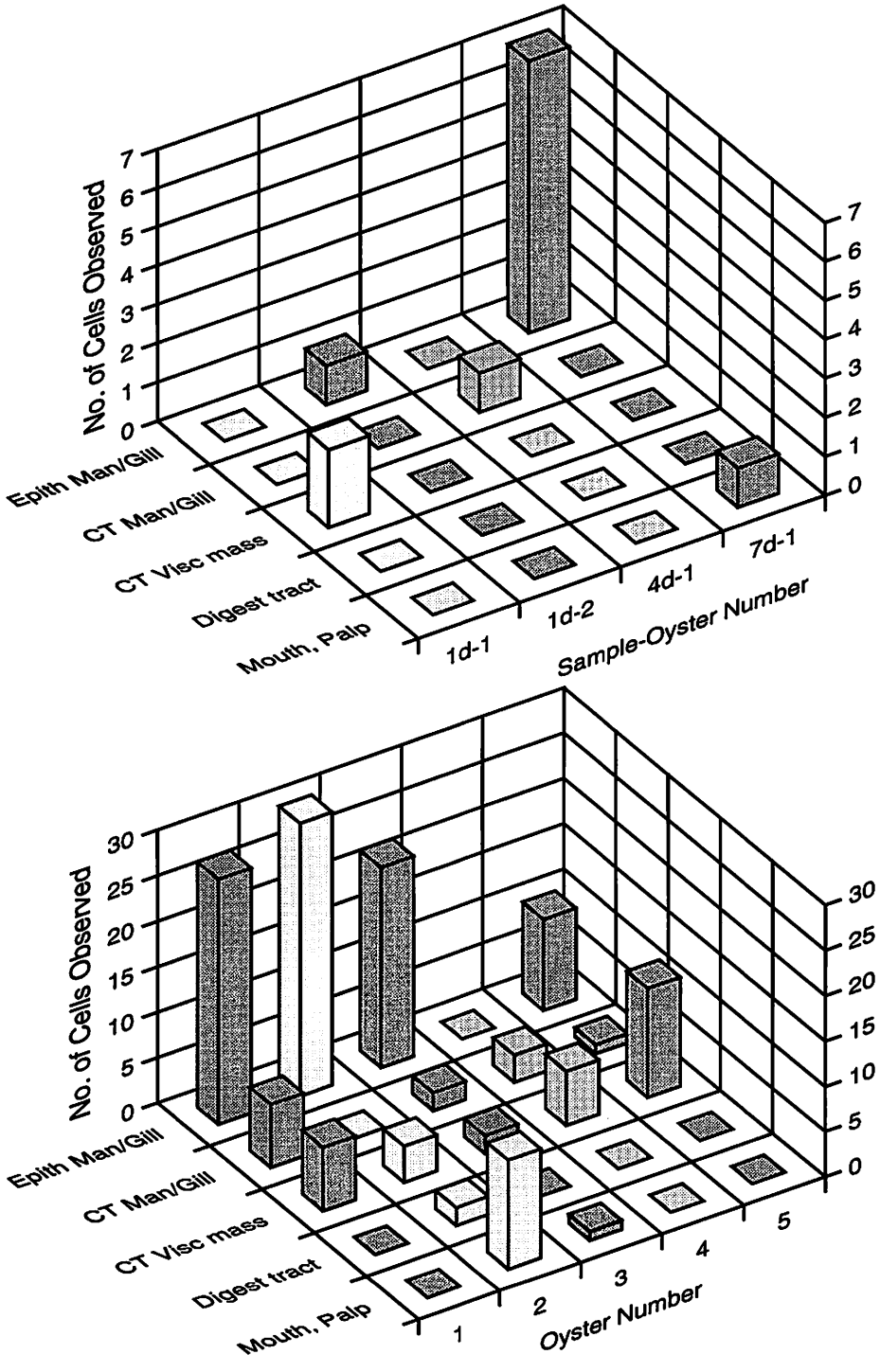


Figure 12. Portal of Entry Experiment 2 results. Distribution of *P. marinus* cells in infected oysters sampled at t= 1 d, 4 d, and 7 d (top) and at 21 d (bottom). Five oysters were sampled and examined by immunoassay at each time. Infections were observed in 2/5 oysters sampled at 1 d, 2/5 sampled at 4 d, 1/5 sampled at 7 d and 5/5 sampled at 21 d. All infected individuals are shown. Foci of parasite cells included mantle and gill epithelia, mantle, gill, and visceral mass conective tissue (CT), digestive tract (stomach,intestine, and digestive glands), and palp copnective tissue (x-axis). Cells were not observed in other tissues examined. The frequency of cells detected at specific sites for individual oysters ranged from 0-27 (z-axis). All observed lesions are shown. Sample times and individual and/or oyster number are shown on y-axis.

Perkinsus marinus antibody-labeled cells were observed in estuarine water samples collected throughout the year at both the upper and lower Chesapeake Bay tributary sites. Estimates of *P. marinus* cell abundances are believed to be conservative; however, the specificity of the antibody for staining *P. marinus* cells in water samples remains to be verified. In the absence of verification cell abundance data must be interpreted cautiously. Molecular genetic methods for estimating the sensitivity and specificity of the flow cytometric immunodetection technique are currently being developed.

Environmental abundances of antibody labeled *P. marinus* cells varied seasonally. Relatively low abundances were observed from mid-September through March. Abundance progressively increased during early summer and maximal counts were observed in early August. The distinct summer increase in abundance coincided with annual temperature maximums. This is reflected in the strong positive correlation between *P. marinus* cell count and temperature. Cell abundance declined as temperature declined from about 25 to 20°C during late August and September and varied little from October through March. At both sites, July and August cell abundances were significantly higher than October–March abundances.

Significant differences in *P. marinus* cell abundances were observed between sites. The highest individual cell counts were observed at the York River, VA (YR) site, but, overall, cell abundance was significantly higher at the Tred Avon River, MD (TAR) site. This result is surprising given the fact that salinities were always lower at TAR than at YR and that salinity has been shown to limit *P. marinus* prevalence, infection progression, and host oyster mortalities. Other factors contributing to between site differences in parasite cell abundance may include differences in hydrographic conditions and differences in the density of local native oyster populations.

Dramatic differences in host oyster mortality were observed between sites. Cumulative mortality of captive oysters at TAR did not exceed 12% during the entire study period while cumulative mortality at YR was nearly 95% in mid-September. The highest mortality rates were recorded in early August at YR and in early September at TAR. At YR a distinct pulse/peak of mortality was observed. About 50% of the captive oyster population died within a 30 day period beginning in mid-July. At TAR a distinct mortality peak was not observed, captive oysters died at low rates throughout the summer months. Differences between sites may be attributed to several factors. First, captive oysters at the two sites were from local sources each having different histories of infection exposure and hence different levels of infections at the initiation of the study. Second, higher salinities at YR were probably more favorable to infection development than the lower TAR salinities. Third, oysters maintained at YR were exposed to, and infected with, *H. nelsoni* as well as *P. marinus*. This additional stress probably exacerbated oyster mortality. Oyster mortality at YR occurred during and immediately after maximal summer temperatures. It is likely

that high summer temperatures enhanced parasite multiplication within host oysters and compromised host oyster defense capacities and physiological condition, thereby increasing oyster deaths. The timing of oyster mortality corresponded with that reported in previous studies in the lower Chesapeake Bay (Andrews 1965; Andrews 1967; Andrews and Wood 1967; Andrews 1988).

Abundances of disseminated *P. marinus* cells were strongly correlated with oyster mortality rates at YR, but not at TAR. The association of mortality and cell abundance at YR support the hypothesis that most pathogen cell dissemination occurs following disease-induced mortalities of infected oysters, but if this were true one would have expected higher abundances at YR than at TAR where little mortality occurred. It is possible that cells counted at TAR were transported from down river areas where oyster mortality was higher. Since the population of native oysters is much more extensive at TAR than at YR it is possible that even though oysters were dying at a lower rate the total number of oysters may have been higher. Alternatively, the association of mortality and cell abundance at YR may be coincidental. Given the fact that both factors correlated with temperature, both events may be triggered by temperature. The presence of elevated cell abundances early and late in the summer suggests that alternate sources of *P. marinus* cells may also be important. Alternative hypotheses suggest that cell abundances may be associated with host spawning and or excretory activities, alternate host or vector activities, heterotrophic parasite proliferation, or periodic resuspension of sediment-bound cells. Recent unpublished laboratory studies by Burreson and Schotthoeffer have demonstrated that living oysters with moderate to heavy *P. marinus* infections commonly shed hundreds to thousands of viable *P. marinus* cells in their feces each day. This finding supports the hypothesis that environmental cell abundances are associated with oyster excretory activities. Furthermore, the release of high numbers of pathogen cells from living oysters may obviate the need for oyster hosts to die in order for pathogen dissemination and transmission to occur.

The pattern of infection acquisition in sentinel oysters deployed at YR indicates that seasonal infection pressure is highest during August and early September, a period closely corresponding to epizootic oyster mortalities(Andrews and Hewatt 1957; Andrews 1965; Andrews 1967; Andrews 1988). This finding strongly supports previous studies and longstanding hypotheses that suggest infective cells arise from death and decomposition of infected hosts, which is maximal during August. Relatively high *P. marinus* water sample counts were recorded during the period of peak seasonal infection pressure. The correspondence of disseminated pathogen cell abundance with annual highs in infection pressure suggests that enumerated cells are infective. However, the maximum cell counts did not always coincide with high rates of infection acquisition. Environmental YR abundances of *P. marinus* cells in late July were nearly as high as in early August, but no infections were acquired by sentinel oysters deployed during that time.

Additionally, infection acquisition was highest in oysters deployed from 15–31 August, immediately after annual maximal cell counts were observed. The mismatch of the period of peak annual cell abundance and peak infection pressure may be explained by (1) undocumented daily high variability in cell abundance—if variability within sample dates is higher than between sample dates we may not have an efficient enough sampling design to unequivocally state that the maximum number of cells observed occurred only during the first week of August , (2) enumerated cells may not be infective, or only some fraction of enumerated cells are infective, (3) a lag time is required for disseminated cells to transform to an alternate stage, which is not enumerated, prior to infection, and (4) other environmental factors and/or differences in susceptibility between sentinel groups existed.

Surprisingly, a low percentage of deployed sentinel oysters acquired infections during the months of October to February, at temperatures of 15–4°C . Since infections were not observed in control oysters during this period it may be concluded that these infections were acquired during the York River exposure period. In previous studies sentinel oysters in Chesapeake Bay *P. marinus*-endemic waters did not acquire infections after about 1 November (Andrews 1965). The absence of new infections during the fall and winter months is thought to be associated with temperature-induced depressions of host oyster mortality which would limit the abundance of infective particles, and to more efficient host defense activities (Andrews 1988). More recently, in laboratory experiments where *P. marinus* meronts were injected into the mantle cavity of oysters held at various temperatures, infections were established at temperatures as low as 10°C (Chu and Peyre 1993). These laboratory results support the observations reported here and suggest that disease transmission may not be limited to the summer months. However, infection acquisition during the late fall and winter of 1994–95 may be atypical, as water temperatures were frequently 2-3°C above the long-term average (Ragone Calvo and Burreson 1995).

The periodic deployment of sentinel oysters has enabled the assessment of relative infection pressures throughout the year. The absence of infection during a specific period does not necessarily indicate that infections can not be acquired during that period. Duration of exposure may be an important factor, longer exposure periods would most likely result in higher rates of infection acquisition.

An additional objective of this study was to examine the infection portal of entry, or invasion site, of *P. marinus*, in the host oyster. Infections are believed to typically occur through the digestive tract (Mackin 1951). Infective cells are believed to be ingested with food, phagocytized in the stomach and carried into the digestive tract epithelium by host phagocytes. The phagocytic hemocytes facilitate transport to host connective tissues and to the blood sinuses through which parasite cells are distributed to all tissues of the body (Mackin 1951; Andrews 1988). The potential for infection to take place through any epithelial surface has also been suggested. In fact,

Ray (1954) demonstrated that infections could be established by placing excised oyster tissues in vials containing large numbers of *P. marinus* cells. In our investigation utilization of a specific antibody enabled the detection of low frequency initial infections. Infections were detected in 8/8 of the naturally infected sentinel oysters analyzed. The oysters, which were sampled immediately after a two week York River deployment period, exhibited infections of the mantle, gill, mouth, heart, and digestive tract. Nearly 90% of the oysters examined had parasite cells in stomach or intestinal epithelia and a high frequency of parasite cells were observed in lumens of the stomach and intestine. Cells were detected in gill and mantle tissue in 50% of the oysters analyzed and only 1–2 cases were observed in heart or mouth tissue. This result provides further evidence that the digestive tract is the primary portal of entry, but also suggests that other epithelia may be important sites of invasion. Few digestive tract infections were observed in either of our laboratory infection experiments. In contrast a high proportion of observed parasite cells were located in mantle and gill epithelia. At 21 d post-exposure, relatively high numbers of cells were observed in gill and mantle epithelia in 80% of the oysters examined and parasite cells were present in visceral connective tissues in 100% of the oysters, indicative of pathogen proliferation and dissemination through host tissues. The high prevalence of mantle and gill epithelia lesions suggests that these tissues may also serve as primary parasite entry routes. An investigation by Alvarez et al. (1992) showed that intubated polystyrene beads were transported through the gut epithelium to other tissues by motile hemocytes within a 2 hour period. The authors suggested bead-containing hemocytes exit the oyster through the palp and mantle epithelia. If a similar process occurs in response to *P. marinus* infections our observations could also be related to parasite expulsion processes. The observed progressive decline in parasite body burden in experiment 1 oysters sampled at 24, 48, 72, 96, and 120 hr after exposure to *P. marinus* cells may indicate that *P. marinus* cells were being eliminated from the host during that time. However, it is impossible to tell whether all cells detected in body burden assays were actually internalized—some cells may have been present on epithelial surfaces or in digestive tract lumena. Additional experiments that result in a greater number of invading *P. marinus* cells, and allow shorter sampling intervals, will be required to evaluate the merits of these alternative hypotheses.

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