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Contaminant-free cultivation of *Pfiesteria shumwayae* (Dinophyceae) on a fish cell line

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ABSTRACT: Geographically distinct strains of the heterotrophic dinoflagellate *Pfiesteria shumwayae* were cultivated on a fish cell line in the apparent absence of bacteria and other microbial contaminants. Cultures were established with a high rate of success by inoculating single purified *P. shumwayae* cells into fish cell cultures containing a simple saltwater medium suitable for both cell types, and resulting isolates were serially cultivated on fish cells for months without visible signs of abnormality or reduced viability. *P. shumwayae* fed phagocytically on the fish cells and exhibited higher cell production than reported using other culturing methods. Compared to previous methods of studying the interaction between *Pfiesteria* spp. and fishes, this system enabled closer and more direct observation of the dinoflagellates and was also more economical and sustainable as a culturing method. The absence of bacteria and other contaminating microorganisms should facilitate important physiological and biochemical investigations. The methods used were inadequate for cultivating strains of *P. piscicida*, suggesting a possible difference in nutritional requirements between the 2 *Pfiesteria* species.

KEY WORDS: *Pfiesteria* spp. · Fish cell line · Dinoflagellate culture

INTRODUCTION

The dinoflagellate genus *Pfiesteria* presently consists of *P. piscicida* and *P. shumwayae*, 2 heterotrophic species found in brackish coastal waters. Over the past decade, instances of fish disease and death in cultures and estuarine waters of the mid-Atlantic US coast have been attributed to *Pfiesteria* spp. (Burkholder et al. 1992, 2001a,b,c, Noga et al. 1993, 1996, Marshall et al. 2000, Gordon et al. 2002). Toxin production and/or phagocytic feeding on fish epithelial cells are reported and recently debated mechanisms by which *Pfiesteria* spp. may harm or kill fishes, based primarily on studies in which living fish were exposed to the dinoflagellates or culture filtrates (Burkholder et al. 1992, 2001a,b,c, Noga et al. 1993, 1996, Marshall et al. 2000, Gordon et al. 2002, Quesenberry et al. 2002, Lovko et al. 2003). Without exacting precautions (i.e. Noga & Bower 1987), the use of entire fish in such experimental systems inevitably includes associated bacterial, fungal and protistan contaminants. Among these, certain bacteria might influence or participate in the production of toxic compounds (Shilo & Aschner 1953, Bein 1954, Douglas et al. 1993, Bates et al. 1995, Gallacher et al. 1997, Hold et al. 2001), and contaminating microorganisms may also contribute directly to fish mortality as infective pathogens (Noga 2000). Thus, experimental systems that expose entire fish to *Pfiesteria* spp. also incorporate potential interactions with other microorganisms, which may complicate the investigation and restrict interpretations. Microbial contaminants also confound sustained cultivation of *Pfiesteria* spp. on living fish, and the differences of scale in size and motility between the dinoflagellates and fish can hinder direct microscopic observation. In an attempt to overcome these difficulties, a method was tested for culturing *Pfiesteria* spp. on an established fish cell line in the absence of bacteria and other cont-

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aminating microorganisms. A technique that proved successful for highly productive serial cultivation of *P. shumwayae* is described which might facilitate a more precise examination of some biological and biochemical interactions between *Pfiesteria* spp. and fishes.

MATERIALS AND METHODS

Dinoflagellate cultures. The *Pfiesteria piscicida* and *P. shumwayae* strains used in this study are listed in Table 1. Stock dinoflagellate cultures were maintained in 15 salinity synthetic seawater (Instant Ocean, Aquarium Systems) and were provided with cryptophyte cells (*Rhodomonas* sp. CCMP757; Provasoli-Guillard National Center for Culture of Marine Phytoplankton) as a food source (Parrow et al. 2002). The *Pfiesteria* spp. stock cultures had each been initiated with a single dinoflagellate cell, but were not bacteria-free. All culture manipulations and transfers were made under a positive-pressure laminar-flow hood using sterilized instruments, solutions and aseptic techniques (Hamilton 1973, Guillard 1995). All culturing procedures were conducted at 21 to 23°C.

Fish cell culture. A heterologous fish cell line derived from Chinook salmon *Oncorhynchus tshawytscha* embryo tissues (CHSE) was used (American Type Culture Collection CRL-1681). The CHSE cell line was reportedly free of microbial contaminants including mycoplasmas (Lannan et al. 1984) and grew as an adherent layer of epithelioid cells 1 or more cells thick. CHSE cells were grown in Eagle's minimum essential medium (MEM) (Sigma M4655) with 10% fetal bovine serum (FBS) (Sigma F0926) in horizontal 25 or 75 cm^2 polystyrene cell-culture flasks (Corning) with 10 or 50 ml of medium, respectively. Confluent CHSE cell layers were disaggregated for subculturing by treatment with 0.05% trypsin-EDTA solution (Sigma T3924) for 20 min, followed by tapping of the flask on the counter to dislodge the cells. The disaggregated cells were then suspended in fresh medium and cultures were split at a 1:5 ratio into new flasks. Sterile, filtered (0.22 µm pore size) air with 5% CO_2 was added as a gas-phase buffer prior to sealing each flask (Freshney 1986). Transferred CHSE cells grew to a confluent layer in 5 to 6 d and a subset of cultures was transferred for further propagation every 7 d.

Purification of *Pfiesteria* spp. cells. Dinoflagellate cells from the stock cultures were purified by 'washing' individual specimens through several drops of sterile medium using a finely pulled glass micropipette (Pringsheim 1946, Droop 1954, Caron 1993, Guillard 1995).

Micromanipulations were made by hand using an inverted compound microscope (Olympus CK40) placed in the laminar-flow hood; all contact surfaces and instruments used in the procedure were sterilized by heat if possible, or 95% ethyl alcohol. Micropipettes were equipped prior to use with a sterilized aspiration device modified from Guillard (1995), fitted with a 0.22 µm pore size in-line filter (Caron 1993). Individual *Pfiesteria* spp. flagellates were isolated from petri dish subcultures and dispensed into a drop of 0.22 µm filtered 12 salinity synthetic seawater (Instant Ocean) containing 0.5% methyl cellulose on a microscope slide. The isolated cell was allowed to swim for 2 to 3 min, during which time the micropipette was thoroughly rinsed with 95% ethyl alcohol and then refilled with sterile medium. Each cell was then re-isolated into a new drop, transferring as little medium as possible along with it, and this 'washing' process was repeated 8 to 12 times per cell. If the specimen became nonmotile (a temporary cyst) during the procedure, it was transferred from drop to drop as a nonmotile cell. The washed dinoflagellate cell was then deposited into a prepared culture flask containing 6 to 9 d old CHSE cells (see next subsection). At least 2 cells from each of the *P. piscicida* and *P. shumwayae* strains listed in Table 1 were isolated and purified in this fashion and deposited individually into separate flasks containing CHSE cells.

To provide a larger inoculum of purified dinoflagellate cells, a sample of CCMP2362 *Pfiesteria piscicida* cysts was purified using Percoll silica (Sigma P4937) density-gradient centrifugation and antibiotics (Oestmann & Lewis 1995, Cho et al. 2002). A stock CCMP2362 culture flask containing abundant cysts was rinsed with sterile deionized water (DI) to remove the swimming cells, and the cysts were then dislodged with a plastic cell

Table 1. *Pfiesteria* spp. Strains tested for growth on CHSE (Chinook salmon embryo) cells, geographical source locations, and collection dates

Strain designation	Source location	Collection date
Pfiesteria piscicida		
CCMP2362	Off Flanner Beach, Neuse River, NC, USA	Sep 1997
CCMP1832	Chicamacomico River, MD, USA	Jan 1998
CCMP2363	Marsh Side Pond, Hilton Head Island, SC, USA	Apr 2002
CCMP2423	Beard Creek, Neuse River, NC, USA	May 2002
Pfiesteria shumwayae		
CCMP2357	Carolina Pines, Neuse River, NC, USA	Jul 1998
CCMP2089	Pamlico River, NC, USA	Nov 1999
CCMP2359	Marshall Creek, Chesapeake Bay, MD, USA	Aug 2000
CCMP2360	Tasman Bay, New Zealand	Apr 2000

scraper, collected in 1 ml of DI in a centrifuge tube, and placed in an ultrasonic water bath (Fisher Scientific FS2OH) for 1 min to disaggregate the cysts from bacteria and debris. The cysts (ca. 3.0×10^4) were then placed on the surface of a stepwise 10, 20, 30, 40, 50% gradient of Percoll silica in DI composed of 1.5 ml layers in a 15 ml centrifuge tube, and pelleted ($2000 \times g$, 15 min). The pelleted cysts were then recovered, resuspended in DI, and the sonification and Percoll-gradient centrifugation steps were repeated twice. The cysts were incubated for 48 h in DI containing amphotericin B (0.50 µg ml^{-1}), penicillin (200 IU ml^{-1}), and streptomycin (0.2 mg ml^{-1}), washed twice by centrifugation, and incubated for another 48 h in DI containing amoxicillin (0.3 mg ml^{-1}), bacitracin (0.4 mg ml^{-1}), carbenicillin (300 µg ml^{-1}), erythromycin (0.3 mg ml^{-1}), and bactopeptone (0.2 mg ml^{-1}) to promote antibiotic activity through bacterial growth (Droop 1967). The cysts were then washed once in DI by centrifugation and added to a flask containing 6 to 9 d old CHSE cells (see next subsection).

Establishment and cultivation of *Pfiesteria* spp. on CHSE cells. Preliminary observations indicated that the MEM + FBS medium used to propagate the CHSE cells was not tolerated by the dinoflagellates. Therefore, the medium overlying the CHSE cell layer was replaced with an equivalent volume of 10 salinity synthetic seawater (Instant Ocean) supplemented with *f*/2 trace elements and $2 \times f/2$ vitamins (Guillard 1975) prior to adding the purified *Pfiesteria* spp. cell(s). This medium (SSF) approximated the osmolarity of the CHSE growth medium and was also suitable for the dinoflagellates. CHSE culture flasks that had been inoculated with *Pfiesteria* spp. were examined daily for dinoflagellate proliferation and evidence of microbial contamination. Cultures in which the dinoflagellates proliferated and microbial contaminants were not evident were subcultured after 11 to 14 d into new flasks containing CHSE cells in SSF medium. Once established, the dinoflagellate strains were subcultured every 7 d by transferring 100 µl (ca. 1.5 to 2.5×10^4 dinoflagellate cells) into 25 cm^2 flasks containing 7 d old CHSE cells in SSF medium. The gross impact of dinoflagellates on CHSE cell layers was assessed over time by examining these flasks daily during routine cultivation. Replicate CHSE cultures were incubated in SSF medium alone for 7 d as negative (comparison) controls.

Sterility tests. *Pfiesteria* spp. strains cultivated on CHSE cells were examined for microbial contaminants by test culture in enriched broths and on solid medium and by direct epifluorescence microscopic examination of culture subsamples stained with the DNA fluorophore DAPI. Enrichments of 10 salinity L1 medium were used as test broths: (1) bactopeptone (0.5 g l^{-1}), yeast extract (0.5 g l^{-1}), and glucose (0.5 g l^{-1}) as a general test for bacteria and fungi, and (2) bactopeptone (1.0 g l^{-1}) and methylamine · HCl (1.0 g l^{-1}) as a test for methylaminotrophic bacteria (Guillard 1995). Solid medium was 0.5× marine agar composed of Difco 2216-enriched agar (27.6 g l^{-1}) (Becton Dickinson), agar (7.5 g l^{-1}), bactopeptone (5 g l^{-1}), and yeast extract (1 g l^{-1}) in deionized water. Duplicate samples (1 ml) from late growth phase (7 to 10 d) cultures that visually appeared free of contaminants (400×, Hoffman modulation contrast) were inoculated into 20 ml broth tubes and onto agar plates. Test media were inoculated with samples from the original stock *Pfiesteria* spp. cultures (containing bacteria) as positive controls for contaminant growth, and un-inoculated test media served as negative controls. Test media were incubated at 21°C in darkness and checked daily over 4 wk for broth cloudiness and other visible signs of contaminant growth. These tests were repeated at 2 mo intervals.

Dinoflagellate cultures that had been serially transferred onto CHSE cells for at least 4 mo without visible contaminants or demonstrable contaminants using test media were examined for the presence of bacteria using epifluorescence microscopy after DAPI-staining and filtration (Hobbie et al. 1977, Turley 1993). Subsamples (2 ml) from 7 to 10 d old cultures were fixed with glutaraldehyde (1% final concentration), stained with DAPI (5 µg ml^{-1} final concentration) in darkness for 30 min, and filtered onto 25 mm 0.2 µm pore-size GTBP black polycarbonate filters (Millipore). Each filter was placed on a microscope slide, covered with a drop of non-fluorescent immersion oil (Cargille type FF) and a cover slip, and examined with an Olympus AX-70 microscope using mercury vapor lamp epi-illumination, a DAPI fluorescence filter set, and a 60× 1.2 NA water immersion objective. Samples from the original stock dinoflagellate cultures (containing bacteria) prepared in the same fashion were examined as positive controls for DAPI-stained contaminants. At least 30 fields of view per sample were examined.

Dinoflagellate population growth measurements. *Pfiesteria shumwayae* strains (CCMP2089, 2359, and 2360) that had previously been cultivated on CHSE cells for 3 to 9 mo were inoculated at an initial density of ca. 2.0×10^3 dinoflagellate cells ml^{-1} into triplicate 75 cm^2 flasks containing 8 d old CHSE cell layers overlain with 50 ml of SSF medium. The cultures were gently mixed and subsampled (1 ml) initially and at subsequent 24 h intervals for 12 d. Samples were preserved with ~1% (final concentration) acidic Lugol's solution and dinoflagellate cells were enumerated by light microscopy (400×) using a Palmer-Maloney counting chamber (Wetzel & Likens 1991). Reproductive cysts were each counted as 1 cell, regardless of cell divisional state. Population growth (k, divisions d^{-1}) was estimated from

successive counts over the exponential phase of growth by computing the least-squares regression slope of semi-logarithmic daily cell count means, as in Guillard (1973). *P. shumwayae* isolates in routine cultivation were randomly sampled for cell enumeration as above after 7 d growth on CHSE cells (prior to subculturing) to estimate cell yield among isolates. A Student's *t*-test assuming unequal variances was used to test for differences between treatments in maximum cell abundance and estimated division rate ($p < 0.05$).

RESULTS AND DISCUSSION

Pfiesteria strains cultivated on fish cells

In this culturing system, an adherent layer of fish cells was provided to *Pfiesteria* spp. as a food source. Similar methods have been used to cultivate and study *Amyloodinium ocellatum*, a dinoflagellate ectoparasite of marine fishes (Noga 1987, Oestmann & Lewis 1996). The technique depended on use of a medium suitable for both the dinoflagellates and the CHSE cells. The euryhaline tolerance of *Pfiesteria* spp. (Sullivan & Andersen 2001) permitted use of a simple synthetic seawater medium approximating the osmolarity of the CHSE growth medium. The medium was supplemented with vitamins (B_{12}, thiamine and biotin) and trace elements in case any of these were required or stimulatory for growth of the dinoflagellates (Gaines & Elbrächter 1987, Lessard 1993). The CHSE cells tolerated this medium; replicate 7 d old CHSE cell layers incubated in SSF medium for 7 d appeared normal (but displayed increasingly granular cytoplasm over time), and could also then be transferred and subcultured in MEM plus FBS medium following the incubation. Thus, the SSF medium was not lethal to the CHSE cells, allowing living fish cells to be incubated with the dinoflagellates and subsequent damage to the cell layer to be directly attributed to dinoflagellate activity.

Isolates were established from each of the tested *Pfiesteria shumwayae* strains and have since been serially cultivated on CHSE cells for 6 to 12 mo (Table 2) without signs of morphological aberrancy or reduced viability. Of 10 individual purified *P. shumwayae* cells, 7 proliferated to produce sustained populations on CHSE cells, demonstrating a high rate of success with the methods employed. In contrast, the tested strains of *P. piscicida* were not successfully cultivated using these methods. Although some isolated *P. piscicida* cells were observed apparently feeding on the CHSE cells and limited proliferation occurred, the resulting low number of dinoflagellates did not grow well and could not be subcultured. The sample of *P. piscicida* cysts purified by density-gradient centrifugation and antibiotics produced numerous flagellates within 1 d of inoculation into CHSE culture, and flagellates were observed apparently feeding on the CHSE cell layer. However, as in the single-cell isolations, subcultured populations failed to proliferate sufficiently for serial cultivation. There are several possible causes of this apparent difference in culture requirements between the 2 *Pfiesteria* species. *P. piscicida* may have required some metabolite(s) that was (were) missing from the system (e.g. Droop & Doyle 1966), or there could have been a difference between the species in a feeding parameter related to the CHSE cells. For example, it was reported that 2 strains of *Amyloodinium ocellatum* required different types of fish cells for cultivation (Oestmann & Lewis 1996).

Table 2. *Pfiesteria shumwayae*. Strains cultivated on CHSE cells, number of contaminant-free isolates obtained from each strain, and duration in serial cultivation at the time of this report

Strain designation	No. of isolates	Duration (mo)
CCMP2359	2	12
CCMP2357	2	9
CCMP2360	2	6
CCMP2089	1	6

Purity of cultures

The 7 isolates of *Pfiesteria shumwayae* cultivated on CHSE cells (Table 2) were established without any visible bacterial or fungal contaminants. These cultures also did not elicit detectable bacterial or fungal growth in enriched test broths or on solid media, whereas all test media inoculated with samples from the original dinoflagellate stock cultures showed obvious contaminant growth (broth cloudiness and colony formation on solid medium) within days. Likewise, DAPI-stained samples from the original dinoflagellate stock cultures contained abundant fluorescent bacterial cells (rods and cocci) in every field of view, whereas no such contaminants were apparent in DAPI-stained samples from the 7 isolates of *P. shumwayae* cultivated on CHSE cells. Thus, the cultures were demonstrably free of microbial contaminants, and have remained so by these criteria since establishment. Representative CHSE culture flasks inoculated with single-washed *P. piscicida* cells also remained free of visible contaminants. Thus micropipette 'washing' of individual cells appeared successful as a technique for ridding *Pfiesteria* spp. cells of microbial contaminants, indicating that the isolated *Pfiesteria* spp. flagellate cells lacked tenaciously attached bacteria (Pringsheim 1946, Droop 1954). Endocytic bacteria within the *P. shumwayae* or

CHSE cells were unlikely unless they were uncultivable outside the eukaryotes using these methods. However, the possibility that poorly culturable bacterial contaminants occurred at such low abundances as to avoid detection by these methods could not be entirely excluded. Future tests for the presence of bacterial gene fragments could provide additional information on this possibility.

Dinoflagellate feeding and reproduction

Feeding by *Pfiesteria shumwayae* on living fish surficial tissues has been previously described (e.g. Burkholder et al. 2001a,b); this culturing technique enabled simple microscopic observation of the process. Individual *P. shumwayae* flagellates attached to fish cells by an extensible feeding tube (generally called a peduncle; Elbrächter 1991b), through which fish cytoplasm was aspirated into the dinoflagellate food vacuole (Figs. 1 & 2). The dinoflagellates often enlarged considerably during ingestion, sometimes doubling in length as the food vacuole swelled to occupy a majority of the cell. The feeding process only required seconds to minutes for completion (Fig. 2), after which the feeding tube was stretched thin, detached, and retracted as the dinoflagellate swam away. Based on observations of feeding and the amount of material ingested by some *P. shumwayae* cells, it appeared that the cytoplasm of more than 1 fish cell could be consumed in a single feeding act. No visible fish cell plasma membrane was left behind after feeding, as in true myzocytosis *sensu* Schnepf & Deichgräber (1983). Flagellate cells already containing food vacuoles full of fish cytoplasm were sometimes observed to feed again, and dinoflagellates full of ingested fish cytoplasm appeared to often swim for hours before becoming nonmotile (encysting) for further digestion and cell division.

Stages of *Pfiesteria shumwayae* reproductive cell divisions were readily observed on culture substrata (Fig. 3A). The protoplast of nonmotile reproductive

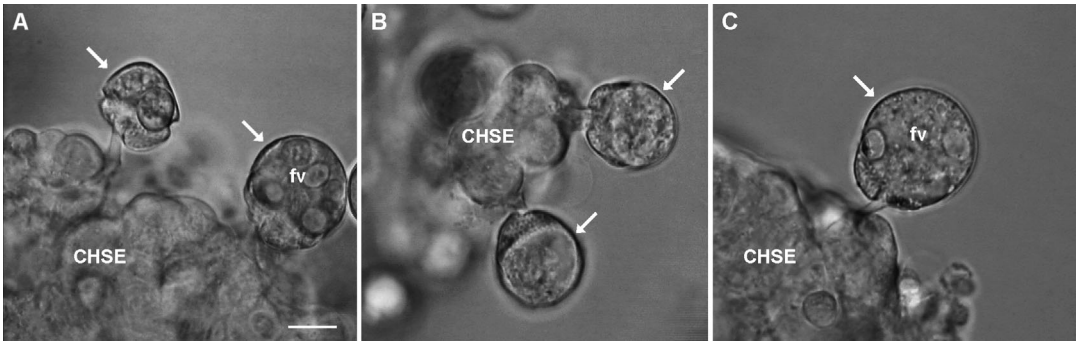

Fig. 1. *Pfiesteria shumwayae*. Photomicrographs of live flagellates feeding on *Oncorhynchus tshawytscha* (CHSE) cells. (A) Feeding cells (arrows); extended feeding tube of left cell can be seen attached to CHSE cells; cell on right has enlarged food vacuole (fv) containing ingested fish cytoplasm. (B) Feeding cells (arrows) attached to CHSE cells. (C) Feeding cell (arrow) attached to CHSE cells, with enlarged food vacuole (fv) containing ingested fish cytoplasm. Scale bar = 10 µm

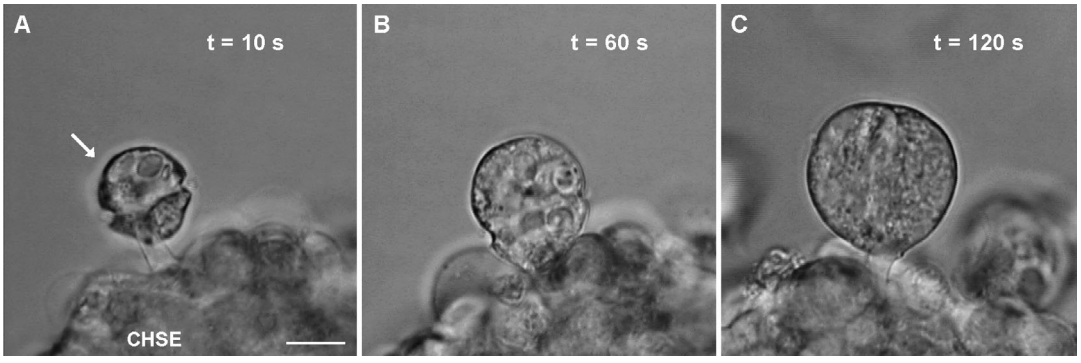

Fig. 2. *Pfiesteria shumwayae*. Sequential photomicrographs showing time series of observations on an individual flagellate cell feeding on *Oncorhynchus tshawytscha* (CHSE) cells. (A) Flagellate cell (arrow) 10 s after extension of feeding tube and attachment to CHSE cells; (B) flagellate cell 60 s later, now enlarged from ingestion of fish cytoplasm; (C) flagellate cell 120 s after attachment to CHSE cells, further enlarged from ingestion. Feeding tube was detached and the cell swam away <10 s after (C). Scale bar = 10 µm

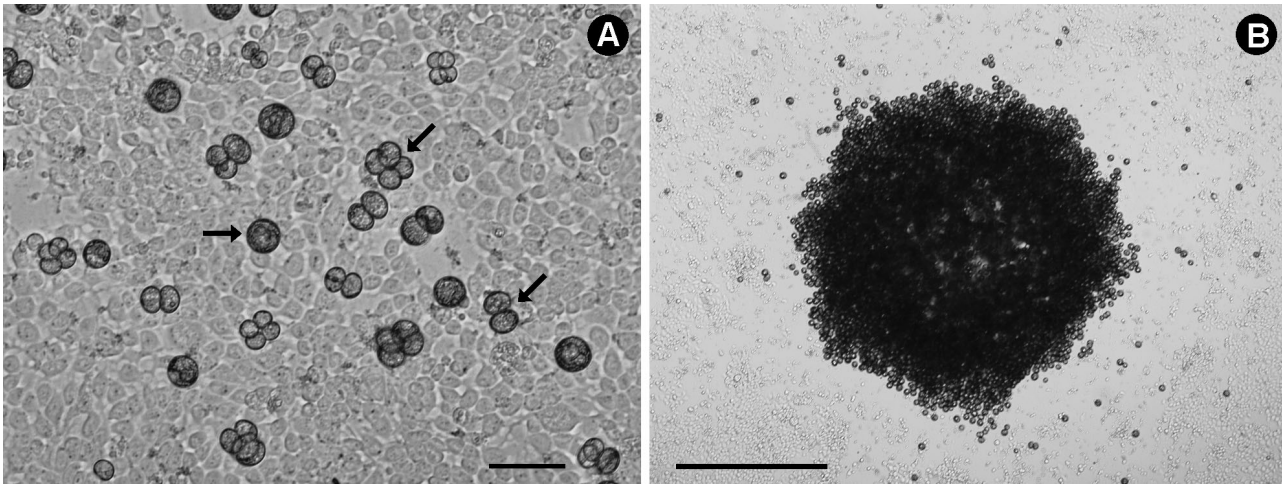

Fig. 3. *Pfiesteria shumwayae* and *Oncorhynchus tshawytscha* (CHSE). Photomicrographs of cultured cells. (A) *P. shumwayae* nonmotile cells (cysts) undergoing cell divisions (arrows), occurring on CHSE cell layer (background). Scale bar = 50 µm. (B) Representative aggregation of cysts found in CCMP2357 cultivated on CHSE cells. Scale bar = 500 µm

cells (division cysts) underwent 1 to 4 consecutive cell divisions that resulted in 2 to 16 flagellate offspring cells, confirming previous observations of *P. shumwayae* reproduction (Parrow & Burkholder 2003b). With the exception of CCMP2357, the *P. shumwayae* reproductive cysts were distributed and not adherent to the culture substratum; gentle agitation was sufficient to suspend the cysts. These observations in contaminant-free cultures suggest that the reported adherent nature of *P. shumwayae* cysts (Parrow & Burkholder 2003b) may be more related to an association with microbial biofilms than to an inherent adhesive capability. Unlike the other strains, the dinoflagellates in both isolates of CCMP2357 consistently formed concentrated, roughly circular aggregations of cysts up to 3 mm in diameter (Fig. 3B). These cyst aggregations also could be separated from the culture substratum by gentle agitation. The reason for this consistent difference in encystment behavior between the CCMP2357 isolates and the others was unknown. Active clustering of cells during temporary encystment occurs for unknown reasons and by unknown mechanisms in several genera of dinoflagellates (Lombard & Capon 1971, Ucko et al. 1997, Garcés et al. 1998, Parrow et al. 2002, Parrow & Burkholder 2003a).

Pfiesteria shumwayae population growth and cell yields

Pfiesteria shumwayae isolates grown on CHSE cells in 75 cm^2 flasks containing 50 ml of medium reached maximum densities of $1.4 \pm 0.3 \times 10^5$ to $2.8 \pm 0.6 \times 10^5$ dinoflagellate cells ml^{-1} (mean ± SD) within 7 to 10 d (Fig. 4). CCMP2089 exhibited a significantly higher division rate (Fig. 5) and maximum cell abundance (Fig. 4) than the other strains in this trial. Similar final densities of $2.4 \pm 0.7 \times 10^5$ dinoflagellate cells ml^{-1} were measured among isolates after 7 d of growth in the 25 cm^2 flasks (containing 10 ml of medium) that were used in routine cultivation (n = 27). *P. shumwayae* cell yield in this cultivation system was probably most dependent on the area of the fish cell layer (since layer thickness was relatively constant), rather than on the volume of medium overlaying it. The measured dinoflagellate cell densities corresponded to an estimated yield of $9.7 \pm 3.0 \times 10^4$ dinoflagellate cells produced cm^{-2} of CHSE cells in 25 cm^2 flasks. The maximum *P. shumwayae* cell densities obtained using this method were up to 10-fold higher than those reported when using living fishes or cryptophyte microalgae as the food source (Burkholder et al. 2001a, Vogelbein et al.

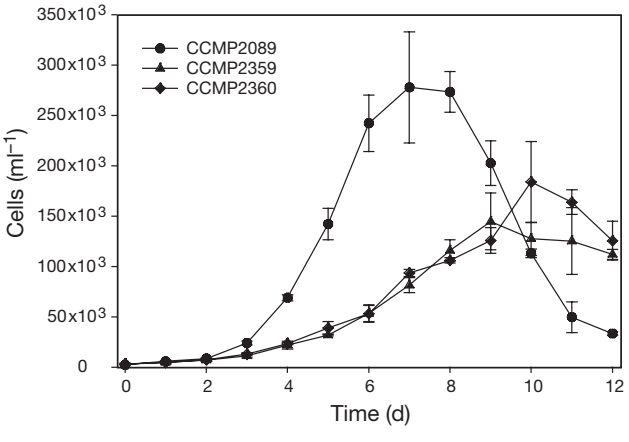

Fig. 4. *Pfiesteria shumwayae*. Time-course of cell densities for strains CCMP2089, 2359 and 2360 cultivated on *Oncorhynchus tshawytscha* cells in 50 ml batch cultures. Data points are replicate (n = 3) means ± SD

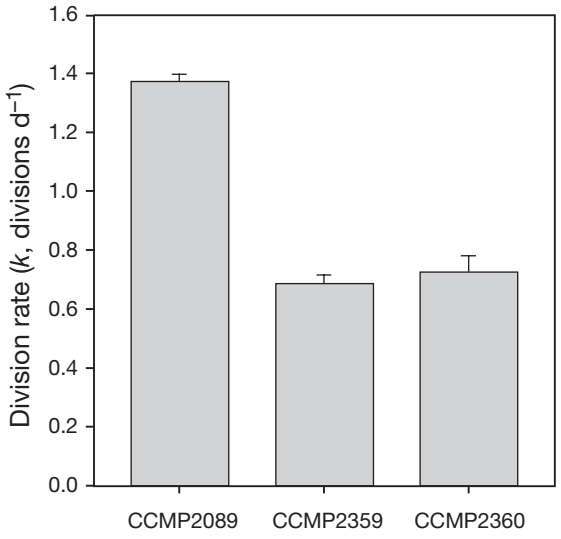

Fig. 5. *Pfiesteria shumwayae*. Mean (+SD) division rates (*k*, divisions d^{-1}) estimated during logarithmic growth phase for strains CCMP2089, 2359 and 2360 cultivated on *Oncorhynchus tshawytscha* cells in 50 ml batch cultures

2001, Parrow et al. 2002), suggesting that the culture conditions in this system were well-suited to dinoflagellate proliferation. More complete sampling of encysted cells compared to previous methods may have also contributed to higher measured cell abundance.

Effect of *Pfiesteria shumwayae* on CHSE cell layers

Initially the CHSE cell layer exposed to *Pfiesteria shumwayae* formed a confluent layer of fish cells covering the flask bottom (Fig. 6A). The CHSE cell layer became increasingly damaged and patchy over time as the dinoflagellates fed and reproduced, and within 4 to 5 d large areas denuded of fish cells were apparent (Fig. 6B). Once the dinoflagellate population reached maximum density (typically in 6 to 8 d) the culture flask was completely denuded of CHSE cells, and all that remained on the culture substratum were abundant *Pfiesteria shumwayae* cysts, empty cyst walls and small bodies that appeared to be egested food remnants (viz. dinoflagellate feces; Elbrächter 1991a) (Fig. 7). Exposure to higher abundances of *P. shumwayae* cells resulted in more rapid destruction of the CHSE cell layer. The absence of other microorganisms, together with negative controls, allowed the observed damage to the CHSE cell layer to be attributed directly to the activity of *P. shumwayae*.

Previous studies reported that filtrates from some *Pfiesteria* spp. cultures contained dissolved compound(s) capable of injuring or killing fishes and molluscs (Burkholder et al. 1992, Noga et al. 1993, Gordon et al. 2002, Springer et al. 2002). Other studies attributed fish mortality to feeding activity by *P. shumwayae* (Lovko et al. 2003), or reported apparent synergistic effects of feeding activity and dissolved toxin(s) (Burkholder et al. 2001a, Gordon & Dyer 2005). Unlike previous methods, the culturing system described here allows investigation of *P. shumwayae* as a fish pathogen without potential interference from other microorganisms. Other studies on the biology and ecology of the dinoflagellate might also be simplified by these conditions compared to those using entire living fishes. Disadvantages of this culture technique as an experimental system for estimating impacts of the dinoflagellates on living fishes include lack of a specific bioassay endpoint (i.e. fish death), and restricted ecological relevance due to system artificiality.

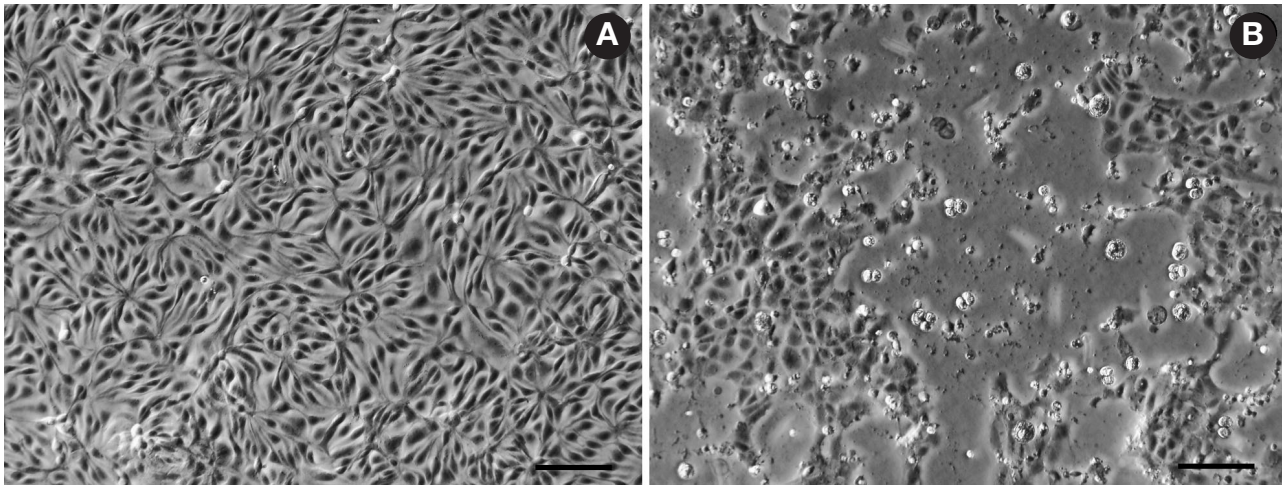

Fig. 6. *Oncorhynchus tshawytscha* and *Pfiesteria shumwayae*. Photomicrographs (Hoffman modulation contrast) of cultured cells. (A) Confluent fish cell layer of same age as (B), for comparison; (B) fish cell layer after exposure to growing *P. shumwayae* population for 4 d, showing damage caused by dinoflagellates. Both scale bars = 100 µm

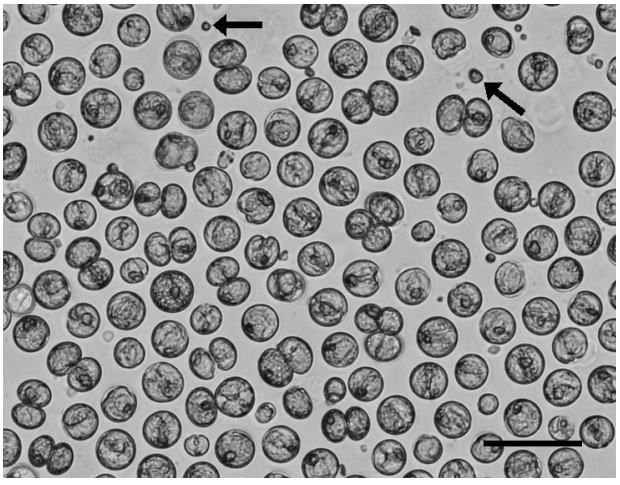

Fig. 7. *Pfiesteria shumwayae*. Photomicrograph of cysts and egested food bodies (arrows) remaining on culture flask substratum after consumption of the fish cell layer. Scale bar = 50 µm

CONCLUSIONS

Methods have been described for the establishment and sustained cultivation of *Pfiesteria shumwayae* strains on a fish cell line in the demonstrable absence of bacteria and other contaminating microorganisms. The techniques required minimal support equipment and reduced space, time and expense compared to previous methods of cultivating *P. shumwayae* on living fishes. The culture system was also more highly controlled and easily sustained due to the lack of microbial contaminants, and resulted in higher *P. shumwayae* cell densities than previously reported using other culture techniques. In addition, the culture-flask format enabled close microscopic observation of the dinoflagellates. *P. piscicida* was not successfully cultivated using these methods, indicating a possible difference in growth requirements between the *Pfiesteria* species that awaits further investigation. This culture system may facilitate studies that could advance understanding of the biology of *Pfiesteria* spp. and the potential impact of the dinoflagellates on living fishes.

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