

## ABSTRACT

SKELTON, HAYLEY MOYNE. Nutritional Features and Feeding Behavior of the Heterotrophic Dinoflagellate *Pfiesteria shumwayae*. (Under the direction of JoAnn M. Burkholder and Daniel L. Kamykowski.)

Dinoflagellates are a diverse group of protists, and approximately half of the ca. 2000 extant species are obligate heterotrophs. Heterotrophic dinoflagellates are widespread and often abundant in aquatic ecosystems, contributing to the transfer of organic matter in aquatic food webs and representing a link between primary production and metazooplankton. Knowledge of heterotrophic dinoflagellate nutrition and feeding behavior is needed to better evaluate the role and impact of these ecologically significant protists in microbial communities.

The dinoflagellate genus *Pfiesteria* contains *P. piscicida* and *P. shumwayae*, two heterotrophic species commonly found in temperate estuarine and coastal waters of the U.S. Atlantic coast. *Pfiesteria* spp. are among the relatively few heterotrophic dinoflagellates that have been maintained in long-term culture, which has facilitated research on the biology and ecology of these species. Detailed studies on the physiology and biochemistry of *Pfiesteria* spp., however, have been complicated by the inability to culture these dinoflagellates in the absence of living prey and bacteria. The present research focused on one *Pfiesteria* species, *P. shumwayae*.

*Pfiesteria shumwayae* strains cultured bacteria-free on a fish cell line were used to investigate phosphatase enzymes in this dinoflagellate. Cellular localization of enzyme activity was determined with the fluorescent probe ELF-97. Phosphatase activity also was evaluated at three different pH values using traditional colorimetric methods. The

location of enzyme activity and supporting colorimetric measurements suggested that acid phosphatases predominate in *P. shumwayae* and have a general catabolic function.

Advanced culturing methods also were developed that permitted additional insight into *Pfiesteria shumwayae* feeding behavior and nutritional requirements. A complex, bi-phasic culture medium was formulated that supported the axenic growth of two *P. shumwayae* strains. A major component of the medium was chicken egg yolk, suggesting that this species may have a biochemical requirement for one or more lipids. Further, *P. shumwayae* cells ingested the yolk particles in the medium, permitting detailed microscopic examination of feeding behavior in this dinoflagellate.

Subsequently, a semi-defined medium was formulated that supported the axenic growth of three *Pfiesteria shumwayae* strains. The medium contained high concentrations of dissolved and particulate organic compounds, including amino acids and lipids, and permitted additional insight into the possible biochemical requirements of this heterotrophic dinoflagellate. Maximum cell yields attained in the semi-defined medium were up to 10 times higher than those obtained when *P. shumwayae* was cultured with living fish or microalgae in xenic cultures.

These investigations provided new information on the digestion, feeding behavior, and nutrition of the heterotrophic dinoflagellate *Pfiesteria shumwayae*. Information on the occurrence and role(s) of phosphatases in heterotrophic dinoflagellates is limited, and *P. shumwayae* is only the second heterotrophic dinoflagellate species examined for phosphatase activity. Development of first a complex and then semi-defined axenic culture medium represents significant progress toward a completely defined axenic culture medium and subsequent determination of

specific biochemical requirements of *P. shumwayae*, needed to advance understanding of the nutritional ecology of this species. Further, this culturing method provides a source of *Pfiesteria shumwayae* cells free from other metabolizing cells, permitting physiological and ecological investigations that would otherwise be complicated by the presence of prey and/or bacteria.

Nutritional Features and Feeding Behavior of the Heterotrophic Dinoflagellate  
*Pfiesteria shumwayae*

by  
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A dissertation submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the Degree of  
Doctor of Philosophy

Marine Earth and Atmospheric Sciences

Raleigh, North Carolina

2008

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## DEDICATION

This work is dedicated to my parents, who have always supported me and encouraged me to pursue my interests.

## BIOGRAPHY

Hayley M. Skelton was born at Elmendorf Air Force Base near Anchorage, Alaska to David and Anna Skelton. As a military child, she lived in many different locations, including Ohio, Germany, Florida (twice), and Georgia. It was in Germany that Hayley's mother first suspected that her daughter would become a biologist when, while in elementary school, Hayley spent endless hours collecting and studying the behavior of terrestrial snails. When Hayley was 9 years old, her family moved to Ft. Walton Beach, Florida. It was living in Florida and frequenting the beautiful beaches in the state that cemented her decision to become a marine scientist, a decision that she made during her sophomore year in high school. After 7 years in Florida, her family moved to Warner Robins, Georgia, where she graduated from Warner Robins High School in 1998. Hayley then returned to Florida and enrolled at Florida State University in Tallahassee where she studied biology with an emphasis in marine resource ecology. As an undergraduate, Hayley conducted research in several areas, including fisheries stock enhancement and microbial biogeochemistry. She received a Bachelor of Science in Biology with a minor in Chemistry in 2001 and move to Raleigh, North Carolina, to pursue her doctorate in the Marine, Earth, and Atmospheric Sciences Department at North Carolina State University. For her graduate research she studied the nutrition and feeding behavior of an estuarine heterotrophic dinoflagellate, *Pfiesteria shumwayae*. This dissertation presents those studies.

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the co-chairs of my advisory committee, Dr. JoAnn Burkholder and Dr. Daniel Kamykowski, for the opportunity to conduct this research and the guidance they both provided during my tenure as a graduate student. I also am genuinely grateful to the additional members of my graduate committee, Drs David DeMaster, Alan Lewitus, and Matthew Parrow. I could not have asked for a better advisory committee. Thank you for your enthusiasm, suggestions, and support over the years.

I would like to extend my appreciation to all of the students and employees of the NCSU Center for Applied Aquatic Ecology. It has been a pleasure and privilege to work with each of you. In particular, I would like to express my gratitude to Matthew Parrow who, while a graduate student and postdoctoral scientist at the CAAE, extended his friendship and support to me and offered many words of wisdom and encouragement. Thank you also to my wonderful friends and fellow graduate students, Susan Pate May and Kimberly Null. Our countless adventures together resulted in many fond and unforgettable memories. I also would like to thank Bradley Hollidge for entertaining conversations and assistance in maintaining the axenic *Pfiesteria* cultures.

I wish to express my appreciation to my amazing family, including my parents, David and Anna Skelton, my sister, Lyndsey Skelton, and my boyfriend, Francisco Flores. Your unconditional love, endless encouragement, and patience provided me with the strength needed to successfully complete my graduate studies.

Finally, I would like to thank Apple® for developing the iPod, which helped me maintain my sanity while counting *Pfiesteria* cells in the hundreds of samples generated during the course of this dissertation.

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1. PHOSPHATASE ACTIVITY IN THE HETEROTROPHIC DINOFLAGELLATE

*PFIESTERIA SHUMWAYAE*

Hayley M. Skelton, Matthew W. Parrow, and JoAnn M. Burkholder

*Harmful Algae* 5 (2006) 395-406

## 1.1 Abstract

The ELF-97 phosphatase substrate was used to examine phosphatase activity in four strains of the estuarine heterotrophic dinoflagellate, *Pfiesteria shumwayae*. Acid and alkaline phosphatase activities also were evaluated at different pH values using bulk colorimetric methods. Intracellular phosphatase activity was demonstrated in *P. shumwayae* cells that were actively feeding on a fish cell line and in food limited cells that had not fed on fish cells for 3 days. All strains, whether actively feeding or food limited showed similar phosphatase activities. *P. shumwayae* cells feeding on fish cells showed ELF-97 activity near, or surrounding, the food vacuole. Relatively small, spherical ELF-97 deposits also were observed in the cytoplasm and sometimes near the plasma membrane. ELF-97 fluorescence was highly variable among cells, likely reflecting different stages in digestion and related metabolic processes. The location of enzyme activity and supporting colorimetric measurements suggest that, as in other heterotrophic protists, acid phosphatases predominate in *P. shumwayae* and have a general catabolic function.

## 1.2 Introduction

Phosphatases are a general group of cellular enzymes that hydrolyze organic phosphorus compounds and include both alkaline and acid varieties with pH-dependent optima. Given their role in the remineralization of organic phosphorus, both alkaline and acid phosphatases are considered important enzymes in microbial ecology and the cycling of nutrients in aquatic food webs (Cembella et al. 1984, Chrost et al. 1984, Hoppe 2003). The term “phosphatase” mostly has been used in reference to phosphomonoesterases, a specific group of phosphatases that catalyze the hydrolysis of monoesters of orthophosphoric acid (Feder 1973, Cembella et al. 1984). Alkaline phosphatases typically are associated with cell surfaces or are secreted by organisms into the environment (Cembella et al. 1984) and play a role in phosphorus uptake (McComb et al. 1979, Jansson et al. 1988, Cotner and Wetzel 1992). Acid phosphatases generally are intracellular and have a catabolic function, aiding in the digestion of food and autophagy, the process by which cells degrade and recycle cytoplasm and organelles (Elliott and Clemmons 1966, Müller 1967, Cembella et al. 1984, Klionsky and Emr 2000). Production of alkaline phosphatases generally is repressed by high extracellular or intracellular phosphate concentrations, while most acid phosphatases are constitutively synthesized by cells (Kuenzler and Perras 1965, Wynne 1977, Cembella et al. 1984, Jansson et al. 1988).

Although phosphatases have been studied in aquatic organisms and ecosystems for decades, little is known about these enzymes or their activities in most aquatic heterotrophic protists (Hoppe 2003), in part because methodologies, until recently, have

been limited to bulk measurements at the community or population rather than cellular level. Research on phosphatase activity in aquatic protists has emphasized microalgae, especially phytoplankton, as important contributors to phosphatase activity in aquatic ecosystems (Jansson et al. 1988). Most phytoplankton research has focused on the association between alkaline phosphatase activity and phosphorus deficiency, with increased enzyme synthesis corresponding to low extracellular P concentrations (Berman 1970, Pettersson 1980, Smith and Kalff 1981, Chrost et al. 1984) or intracellular P concentrations (Fitzgerald and Nelson 1966, Wynne 1977, Pettersson 1980, Gage and Gorham 1985). The presence and role of acid phosphatases in phytoplankton has received less attention, but several species are known to have these enzymes (Talpasayi 1962, Antia and Watt 1965, Kuenzler and Perras 1965, Møller et al. 1975).

In contrast, phosphatase research on heterotrophic protists has focused on acid phosphatase localization in medically important parasites including *Acanthamoeba castellanii* (Douglas) Volkonsky (Wetzel and Korn 1969, Martin and Byers 1976, Ryter and Bowers 1976) and the trypanosomatid flagellates *Crithidia* spp. (Brooker 1971, McLaughlin et al. 1976), *Leishmania* spp. (Gottlieb and Dwyer 1981, 1982, Hassan and Coombs 1987, Wiese et al. 1996), and *Trypanosoma* spp. (Seed et al. 1967, Langreth and Balber 1975, Letelier et al. 1985, Grab et al. 1997). Phosphatase activity in free-living protists has been most studied in ciliates (Mueller et al. 1965, Dembitzer 1968, Karakashian and Karakashian 1973). Acid phosphatases appear to be more prevalent than alkaline phosphatases in heterotrophic protists due to the role of these enzymes in digestive processes (Müller 1967, Eeckhout 1973). Like phytoplankton, however, some

heterotrophic protists also have phosphatases that are active in the alkaline range (Blumenthal et al. 1955, Hunter 1957, Bowers and Korn 1973, Letelier et al. 1985).

Heterotrophic dinoflagellates have been increasingly recognized as important protists in aquatic systems (Lessard and Swift 1985, Jeong et al. 1999), and about half of the known dinoflagellate species appear to be entirely heterotrophic (Gaines and Elbrächter 1987). Nevertheless, research on dinoflagellate phosphatases has primarily emphasized photosynthetic species and the potential use of alkaline phosphatase as an indicator of cellular phosphorus limitation (Wynne 1977, Sakshaug et al. 1984, Boni et al. 1989, González-Gil et al. 1998, Dyhrman and Palenik 1999). Although several photosynthetic dinoflagellate species are known to have acid phosphatases (Kuenzler and Perras 1965, Schmitter and Jurkiewicz 1981, Wynne 1977, Jackson et al. 1989), the role of these enzymes in cellular metabolism remains unexplored. Furthermore, the occurrence and role(s) of alkaline and acid phosphatases in heterotrophic dinoflagellates are virtually unknown. At present, research on phosphatase activity in heterotrophic dinoflagellates has been limited to a single cytochemical study of acid phosphatase activity in *Cryptocodinium cohnii* (Seligo) Chatton (Barlow and Triemer 1986).

Recent advances in techniques that enable investigation at the level of individual cells have presented new opportunities to expand knowledge about heterotrophic dinoflagellates and other protists. Historically, alkaline and acid phosphatase activities have been quantified using colorimetric or fluorometric methodologies, yielding bulk measurements of enzyme activities. While these methods enable determination of the pH optima of active enzymes (through use of phosphatase substrates buffered at different pH

values), they do not allow examination of individual variation within populations and among taxa, or cytochemical localization of enzyme activity. Early cellular localization of alkaline and acid phosphatase activities was performed using cytochemical techniques (Gomori 1952) wherein the hydrolyzed enzyme substrate, in the presence of calcium (alkaline phosphatase) or lead (acid phosphatase) ions, forms an insoluble precipitate at the site(s) of enzyme activity that can be viewed using light microscopy or transmission electron microscopy. Disadvantages of this method include nonspecific staining and low resolution of precipitates with conventional transmitted light microscopy (Cox and Singer 1999). Within the past decade, the molecular probe ELF-97<sup>®</sup> (Enzyme Labeled Fluorescence; Molecular Probes, Inc., Eugene, OR) was developed for in situ fluorescence detection of both alkaline and acid phosphatases in individual cells (Huang et al. 1993, Larison et al. 1995, Cox and Singer 1999). The colorless, soluble substrate, 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4(<sup>3</sup>H)-quinazolinone produces an insoluble fluorescent precipitate at the site(s) of enzyme activity that can be visualized with fluorescence microscopy. The ELF-97 molecular probe has allowed detection of both extracellular and intracellular alkaline phosphatases (González-Gil et al. 1998, Dyhrman and Palenik 1999) and, more recently, acid phosphatases (Van Aarle et al. 2001, Al-Adhami et al. 2003, Štrojsová and Vrba 2005), with improved resolution of labeling patterns and enhanced cellular localization (Cox and Singer 1999, Al-Adhami et al. 2003).

The ELF-97 phosphatase substrate has successfully been used to examine alkaline phosphatase activity in several phytoplankton species, including photosynthetic

dinoflagellates (e.g. González-Gil et al. 1998, Dyhrman and Palenik 1999, Rengefors et al. 2001, 2003, Dignum et al. 2004a,b), but has not yet been applied to heterotrophic species. Here, we compared acid and alkaline phosphatase activities in the heterotrophic dinoflagellate, *Pfiesteria shumwayae* Glasgow et Burkholder (Marshall et al. 2006), using the ELF-97 phosphatase substrate, as well as the more traditional bulk colorimetric method. This species is a heterotrophic dinoflagellate that uses a feeding tube (often called a peduncle; Elbrächter 1991) to ingest the cytoplasm of other protists and fish cells (Burkholder et al. 2001).

### 1.3 Materials and Methods

*Pfiesteria shumwayae* cultures. Four strains of *Pfiesteria shumwayae* (Table 1.1) were cultured in the absence of bacteria on a Chinook salmon (*Oncorhynchus tshawytscha* Walbum) embryo cell line (CHSE) following Parrow et al. (2005). Briefly, CHSE cells were grown in 25 cm<sup>2</sup> polystyrene cell-culture flasks (Corning, Corning, NY) containing Eagle's minimum essential medium (Sigma, St. Louis, MO; M4655) and 10% fetal bovine serum (Sigma, F0926). When the CHSE cells reached confluence, the overlying medium was removed, 30 ml of 10 salinity synthetic seawater (Instant Ocean, Aquarium Systems) containing f/2 trace metals and 2 x f/2 vitamins (Guillard 1975) was added, and the dinoflagellates were inoculated. Strains of *P. shumwayae* were cultured in triplicate for phosphatase analyses. Sampling of cultures was conducted using aseptic techniques under a positive-pressure laminar flow hood (Guillard 1995), and all experimental solutions were sterile filtered (0.22 µm pore size).

*Pfiesteria shumwayae* cultures were examined daily using an inverted microscope (Olympus CK40; Olympus, Melville, NY, 40x - 200x) to monitor dinoflagellate feeding on CHSE cells. When the dinoflagellates were within ~24 h of depleting the CHSE cell line (5 - 7 days after inoculation), cultures were sampled for ELF-97 and colorimetric phosphatase analyses. At that time, *P. shumwayae* flagellate cells were actively feeding on fish cells, and most contained food vacuoles filled with fish cytoplasm. *P. shumwayae* cultures also were sampled for ELF-97 and colorimetric phosphatase analyses three days after depletion of the fish cell line, when the dinoflagellates were food limited and most cells lacked visible food vacuoles.

**Table 1.1** *Pfiesteria shumwayae* strains, source locations, and collection dates (CCMP = Provasoli-Guillard Center for Culture of Marine Phytoplankton).

Strain designation	Source location	Collection date
CCMP2089	Pamlico River, NC, USA	November 1999
CCMP2357	Carolina Pines, Neuse River, NC, USA	July 1998
CCMP2359	Marshall Creek, Chesapeake Bay, MD, USA	August 2000
CCMP2360	Tasman Bay, New Zealand	April 2000

*ELF-97 activity.* Cellular localization of acid and alkaline phosphatase activities in *Pfiesteria shumwayae* was determined using the ELF-97 Endogenous Phosphatase Detection Kit (Molecular Probes, E-6601) following methods modified from González-Gil et al. (1998). A sample (2 ml,  $\sim 0.7 - 3.0 \cdot 10^5$  cells ml<sup>-1</sup>) was removed from each *P. shumwayae* culture and fixed with 0.5% glutaraldehyde (final concentration). Initial experiments comparing several fixatives indicated that *P. shumwayae* cells preserved in glutaraldehyde, formalin, or ethanol equally retained the ELF-97 label, but glutaraldehyde was superior to formalin and ethanol in preserving cell structure, allowing for improved enzyme localization. Samples were stored at 4 °C in darkness and analyzed within 1 week. An aliquot (0.5 ml) was removed from each sample and set aside for negative controls (see below). Samples were centrifuged at 600 g for 10 min, followed by aspiration of the supernatant. Prior to use, the ELF-97 substrate was diluted 1:20 (v/v) in ELF detection buffer and filtered. ELF-97 solution (100 µl) was then added to each

sample and incubated overnight at 4 °C in darkness. An incubation time of at least 2 h at room temperature was necessary to adequately label *P. shumwayae* cells. Samples incubated for 2 h at room temperature were compared to those incubated overnight at 4 °C, and no difference was observed in the pattern of ELF-97 labeling. Thus, overnight incubations were chosen for ease of sample processing. Following incubation, the cells were washed once by centrifugation in 1 ml of sterile filtered 10mM phosphate buffered saline (PBS) and re-suspended in 500 µl PBS for microscopic viewing. Preliminary experiments indicated that multiple washes to remove residual substrate (e.g. González-Gil et al. 1998) were not necessary for these cultures, as evidenced by lack of diffuse, non-localized staining. Negative controls were treated identically but were incubated in 100 µl ELF-97 detection buffer without the ELF-97 substrate. An additional control of CHSE cells that were not inoculated with *P. shumwayae* was included to ensure that ELF-97 labeling of *P. shumwayae* cells was not from the ingested fish cytoplasm. Fish cells were disaggregated with 0.05% Trypsin-EDTA (Parrow et al. 2005), re-suspended in 10 salinity synthetic seawater, and fixed (0.5% glutaraldehyde, final concentration) for ELF-97 analysis.

Dinoflagellate and CHSE cells were examined for ELF-97 fluorescence with an Olympus AX-70 light microscope (Olympus, Melville, NY; 200x - 600x) equipped with a 100 W mercury lamp and an epifluorescence filter set with excitation at  $360 \pm 40$  nm and emission at  $535 \pm 50$  nm (#31060v2; Chroma Technology Corp., Rockingham, VT). The ELF-97 molecular probe has excitation and emission maxima of 360 nm and 530 nm, respectively (Singer et al. 1994, Larison et al. 1995). Photomicrographs were

captured with a DEI-750 cooled-chip CCD camera (Optronics Engineering, Goleta, CA) using a 60x 1.2 NA water immersion objective.

*Colorimetric assay.* Bulk phosphatase activity in *Pfiesteria shumwayae* cultures was measured colorimetrically as the rate of p-nitrophenyl phosphate hydrolysis to p-nitrophenol following procedures modified from Berman (1970) and Wynne (1981). From each culture, aliquots (2 ml) were removed for comparison of enzyme activities in both live and fixed (formalin, 0.5% final concentration) cell suspensions. Formalin-fixed cells yielded higher phosphatase activity than glutaraldehyde-fixed cells, perhaps due to poorer preservation of cell structure that allowed the phosphatase substrate to penetrate the cell membrane more easily. A sample (2 ml) was removed from each culture flask and filtered (0.2  $\mu\text{m}$  pore size) to assess dissolved phosphatases in *P. shumwayae* culture filtrate. In addition, a sample (1 ml) was removed from each experimental culture flask and preserved in acidic Lugol's solution, and the preserved cells were quantified using a Palmer-Maloney counting chamber (Wetzel and Likens 1991). Cell densities were used to calculate phosphatase activity on a per cell basis.

Enzyme activities in all samples were assayed under acidic (pH 5), neutral (pH 7), and alkaline (pH 9) conditions. Phosphatase assays were conducted in polystyrene cell culture plates using aseptic technique. The acid phosphatase reaction mixture contained 0.3 ml of sample (live, fixed, or filtrate), 1 ml of 10 mM p-nitrophenyl phosphate in 0.05 M sodium acetate buffer (pH 5), and 0.7 ml of 10 salinity synthetic seawater. Neutral and alkaline phosphatase activities were assayed similarly, except that 0.05 M Tris (hydroxymethyl)aminomethane buffer (pH 7 or pH 9) containing 0.01 M  $\text{MgCl}_2$  was used

instead of sodium acetate buffer. Samples were incubated for 5 h at 30 °C, after which 1 ml of 0.03 M NaOH was added to pH 5 and pH 7 samples to develop the p-nitrophenol color. The Tris buffer (1 ml, pH 9) was added to alkaline phosphatase samples to maintain a constant volume in all samples (3 ml). Sample absorbance was determined at 405 nm using a microplate reader (Elx800UV; Bio-Tek Instruments Inc, Winooski, VT). Controls included samples incubated without p-nitrophenyl phosphate, and the p-nitrophenyl phosphate substrate incubated alone. Phosphatase activity attributable to *Pfiesteria shumwayae* cells (live and fixed) was estimated by subtracting the filtrate activity from the activity of the whole cell suspension. Enzyme activity was reported as either nmoles p-nitrophenol released  $\cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$  (live and fixed cells), or nmoles p-nitrophenol released  $\cdot \text{ml}^{-1} \cdot \text{h}^{-1}$  (filtrate), and was calculated using p-nitrophenol as a standard.

The contribution of the fish cells to the bulk phosphatase activity measured in *Pfiesteria shumwayae* cultures also was assessed. Duplicate CHSE cell cultures were incubated for 5 days in 10 salinity synthetic seawater, followed by removal and filtration of the overlying medium for analysis of phosphatase activity in culture filtrate. The remaining CHSE cell layer was disaggregated from the bottom of the culture flask by incubation in a 0.05% trypsin-EDTA solution as above. The dislodged cells were re-suspended in 10 salinity synthetic seawater and fixed with formalin (0.5% final concentration). Phosphatase activity in CHSE cells and culture filtrate was assayed as described for *P. shumwayae* cultures.

To further resolve the source (*Pfiesteria shumwayae* or CHSE cells) of any dissolved phosphatases in *P. shumwayae* culture filtrate, a sample (5 ml) was removed from each *P. shumwayae* culture and placed in a sterile 15 ml centrifuge tube. Cells were centrifuged at 1,800 g for 5 min, the supernatant decanted, and the cell pellet re-suspended in 10 salinity seawater. The cell suspension was transferred to a polystyrene cell culture flask and cell condition examined. Although centrifugation caused some *P. shumwayae* cells to form temporary cysts, most cells had excysted and were actively swimming within 1 h. After 1 h, a sample (2 ml) was removed from each culture and filtered for phosphatase analysis. The remaining cell suspension was maintained without food for 3 days after which the filtrate was assayed for phosphatase activities.

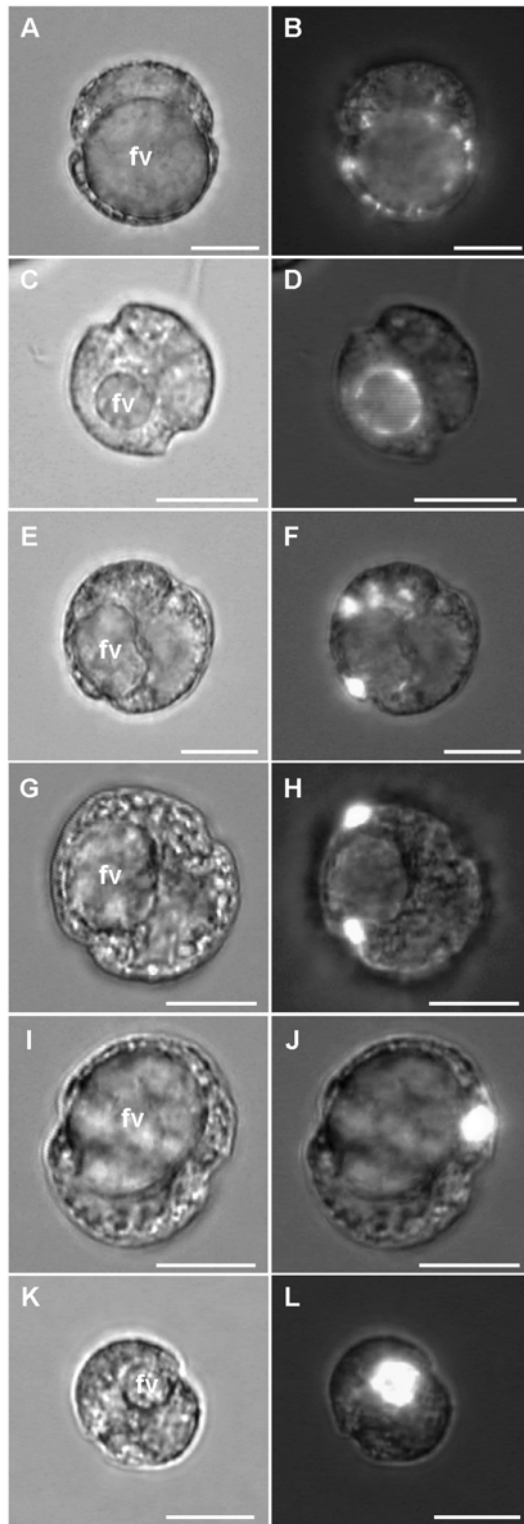
## 1.4 Results

*ELF-97 activity.* ELF-97 formed granular, bright yellow-green fluorescent deposits in *Pfiesteria shumwayae* cells (Fig. 1.1 – 1.4). Although the cells exhibited a slight green cytoplasmic autofluorescence, the bright ELF-97 labeling was easily distinguished, and diffuse non-localized staining was not observed in any *P. shumwayae* cells. ELF-97 labeling occurred in all *P. shumwayae* strains, both in cells that were actively feeding on the CHSE cell line (Fig. 1.1 – 1.3) and in cells that were without fish cells for 3 days (Fig. 1.4). ELF-97 fluorescence in both feeding and fish limited *P. shumwayae* cells was variable among individual cells, however, and some cells showed no activity (Fig. 1.2A,B; Fig. 1.3E,F).

The location of ELF-97 fluorescence also varied among the labeled cells. In actively feeding *Pfiesteria shumwayae* cultures, ELF-97 activity most often was observed surrounding the food vacuole (Fig. 1.1A-D). In other cells, ELF-97 labeling appeared as one to several relatively large bright deposits near the food vacuole (Fig. 1.1E-J). On occasion, the entire food vacuole displayed ELF-97 activity (Fig. 1.1K-L). In addition, ELF-97 activity in *P. shumwayae* cells was sometimes observed in close proximity to the cell membrane rather than near the food vacuole (Fig. 1.2A-D). These deposits appeared to be intracellular; however, it was not possible to determine the exact location (intracellular or extracellular) of enzyme activity. Some cells contained many bright ELF-97 deposits (Fig. 1.2E,F), while others exhibited ELF-97 activity that was associated with darkened areas (Fig. 1.2G,H). Reproductive cysts of *P. shumwayae* also frequently exhibited ELF-97 fluorescence (Fig. 1.3A-D). Large granular ELF-97 deposits like those

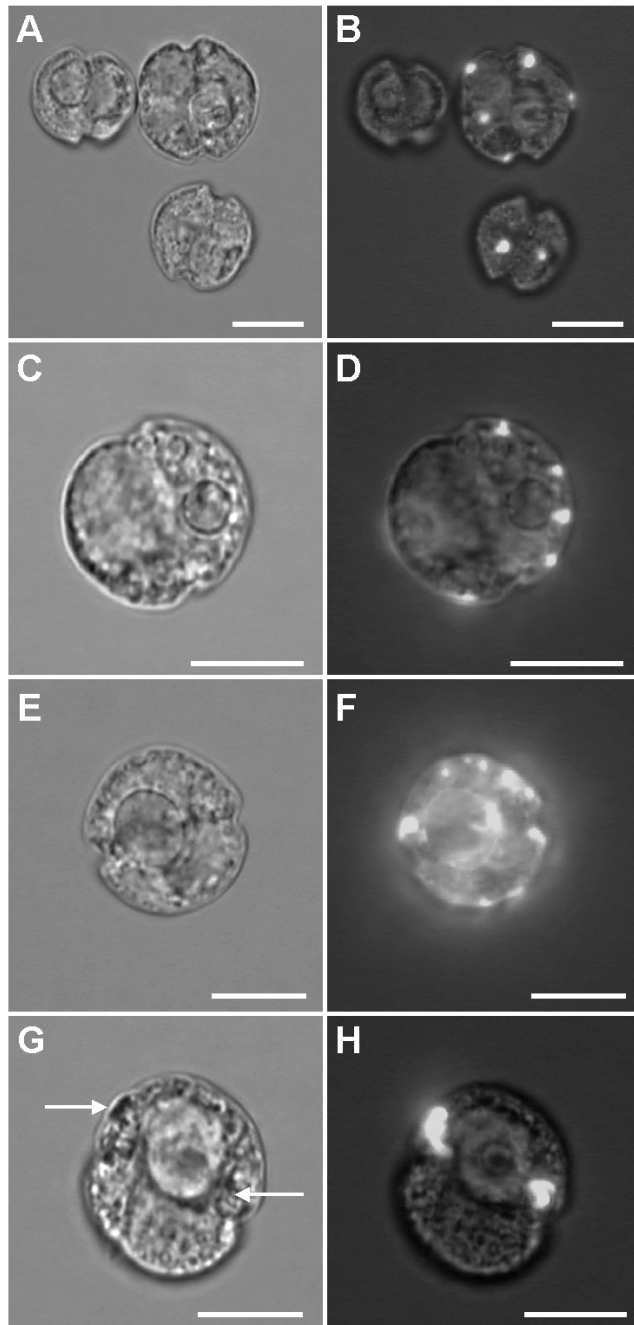
observed in the flagellate cells occasionally occurred within *P. shumwayae* cysts, but most ELF-97 labeling of cysts occurred as smaller, fine deposits that covered the entire cell (Fig. 1.3C-D). Several cell pairs fixed in the process of excystment were observed, and these cells displayed ELF-97 fluorescence while the remaining cyst wall did not show activity (Fig. 1.3E,F). ELF-97 labeling in food limited *P. shumwayae* cells was similar to that of feeding cells. ELF-97 fluorescence in food limited samples was highly variable among individual cells, and typically was characterized by one to many large bright deposits (Fig. 1.4A-D). Control CHSE cells did not exhibit ELF-97 activity (data not shown).

**Figure 1.1** Brightfield and epifluorescence micrographs of ELF-97-labeled *Pfiesteria shumwayae*: **A), B)** Flagellate cell (strain CCMP2357), showing ELF-97 fluorescence surrounding the food vacuole (fv). **C), D)** Flagellate cell (strain CCMP2089) with ELF-97 fluorescence completely surrounding the food vacuole (fv). **E), F)** Flagellate cell (strain CCMP2359), also showing several ELF-97 deposits surrounding the food vacuole (fv). **G) - J)** Flagellate cell (strain CCMP2357) with relatively large ELF-97 deposits near the food vacuole (fv). **K), L)** Flagellate cell (strain CCMP2360), with the entire food vacuole (fv) displaying ELF-97 activity. Scale bars = 10  $\mu\text{m}$ .

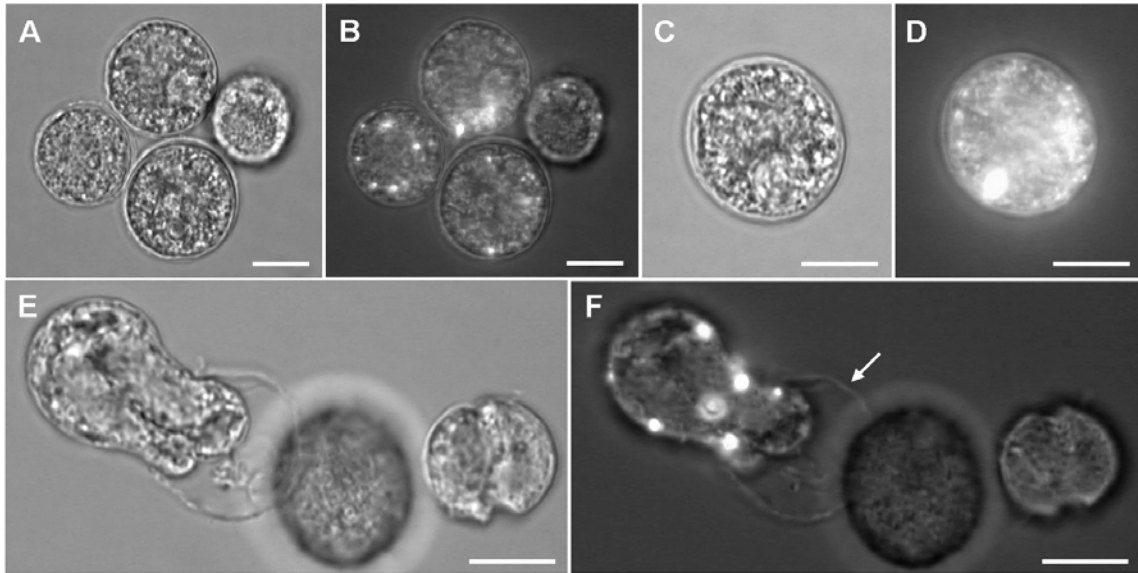


**Figure 1.1**

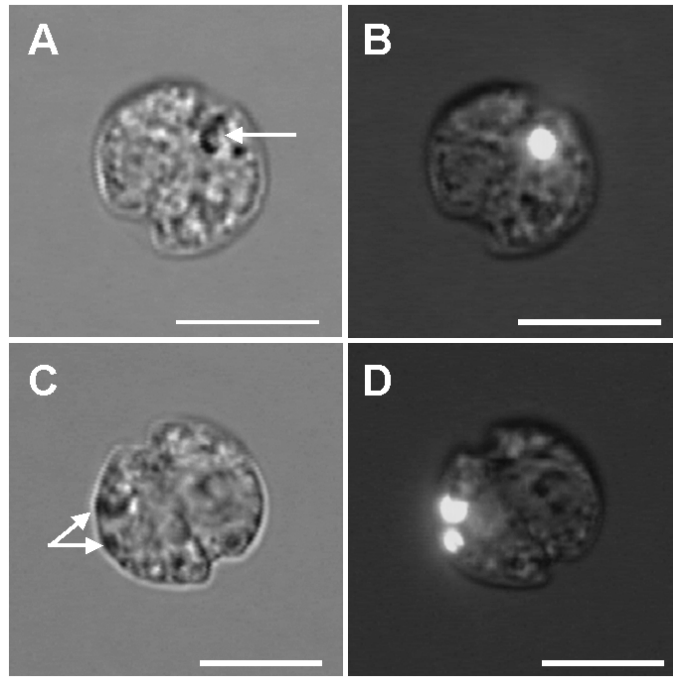
**Figure 1.2** Brightfield and epifluorescence micrographs of ELF-97-labeled *Pfiesteria shumwayae*: **A) - D)** Flagellate cell (strain CCMP2089) showing ELF activity near the cell membrane. **E), F)** Flagellate cell (strain CCMP2360) containing many ELF-97 deposits filling the entire cell. **G), H)** Flagellate cell (strain CCMP2089) with ELF-97 deposits associated with darkened areas of the cell (arrows). Scale bars = 10  $\mu\text{m}$ .



**Figure 1.2**



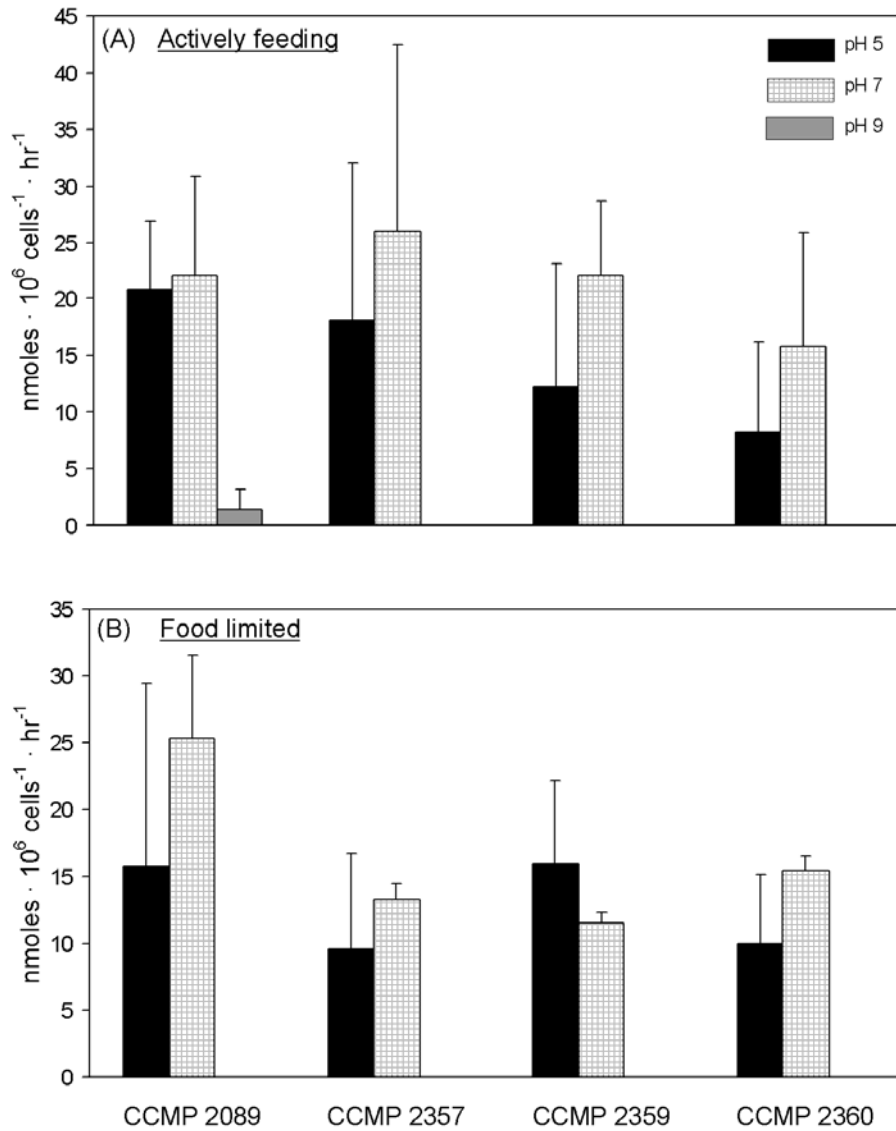
**Figure 1.3** Brightfield and epifluorescence micrographs of ELF-97-labeled *Pfiesteria shumwayae*: **A), B)** Cysts (strain CCMP2360) with ELF-97 fluorescence. **C), D)** Cyst (strain CCMP2357) showing ELF-97 activity. **E), F)** An excysting pair of flagellate cells (strain CCMP2357) with ELF-97 activity. The remaining cyst wall shows no ELF-97 activity (arrow). Scale bars = 10  $\mu\text{m}$ .



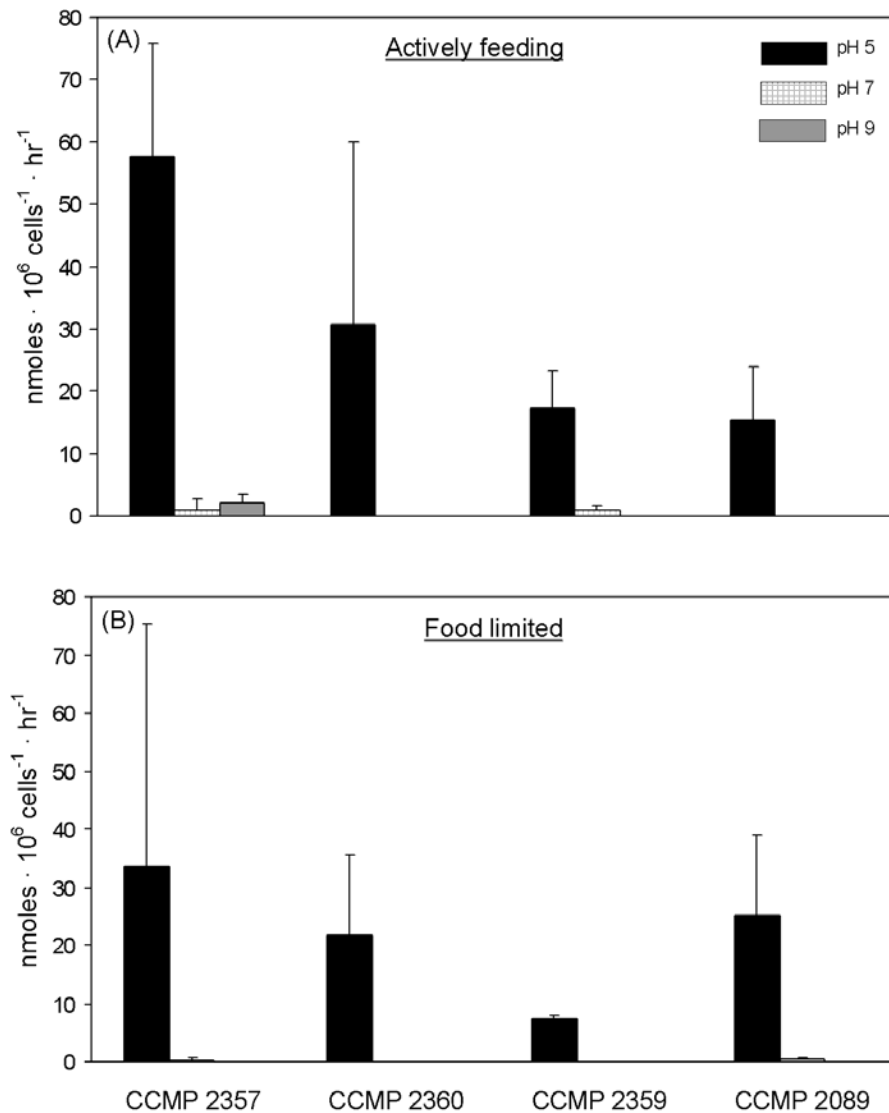
**Figure 1.4** Brightfield and epifluorescence micrographs of ELF-97-labeled *Pfiesteria shumwayae*: **A**), **B**) food limited flagellate cell (strain CCMP2360) showing a single ELF-97 deposit associated with a darkened area within the cell (arrow). **C**), **D**) food-limited flagellate cell (strain CCMP2089) showing two ELF-97 deposits associated with darkened areas within the cell (arrow). Scale bars = 10 $\mu$ m.

*Colorimetric assay.* Phosphatase activity as measured by hydrolysis of the phosphatase substrate p-nitrophenyl phosphate was detected in all *Pfiesteria shumwayae* strains, in both actively feeding and food limited samples (Fig. 1.5 – 1.7). Although the phosphatase activity appeared to differ among some *P. shumwayae* strains, the strains were not synchronous in population growth with respect to one another, precluding meaningful comparisons. Formalin-fixed cells of all *P. shumwayae* samples had phosphatase activity at pH 5 and pH 7 (Fig. 1.5), and the phosphatase activity was similar among strains, whereas there was negligible activity at pH 9 (Fig. 1.5). Live *P. shumwayae* cells of all strains showed phosphatase activity primarily at pH 5, and little or no activity at pH 7 or pH 9 (Fig. 1.6). As was observed in formalin-fixed *P. shumwayae*, live cells of all samples showed similar phosphatase activity at each pH. Microscopic observation following the 5-h incubations indicated that *P. shumwayae* cells were alive at pH 7 and pH 9, but were dead at pH 5. Filtrate from both feeding and food limited cultures contained dissolved phosphatases at all three pH values with the highest activity at pH 5 (Fig. 1.7).

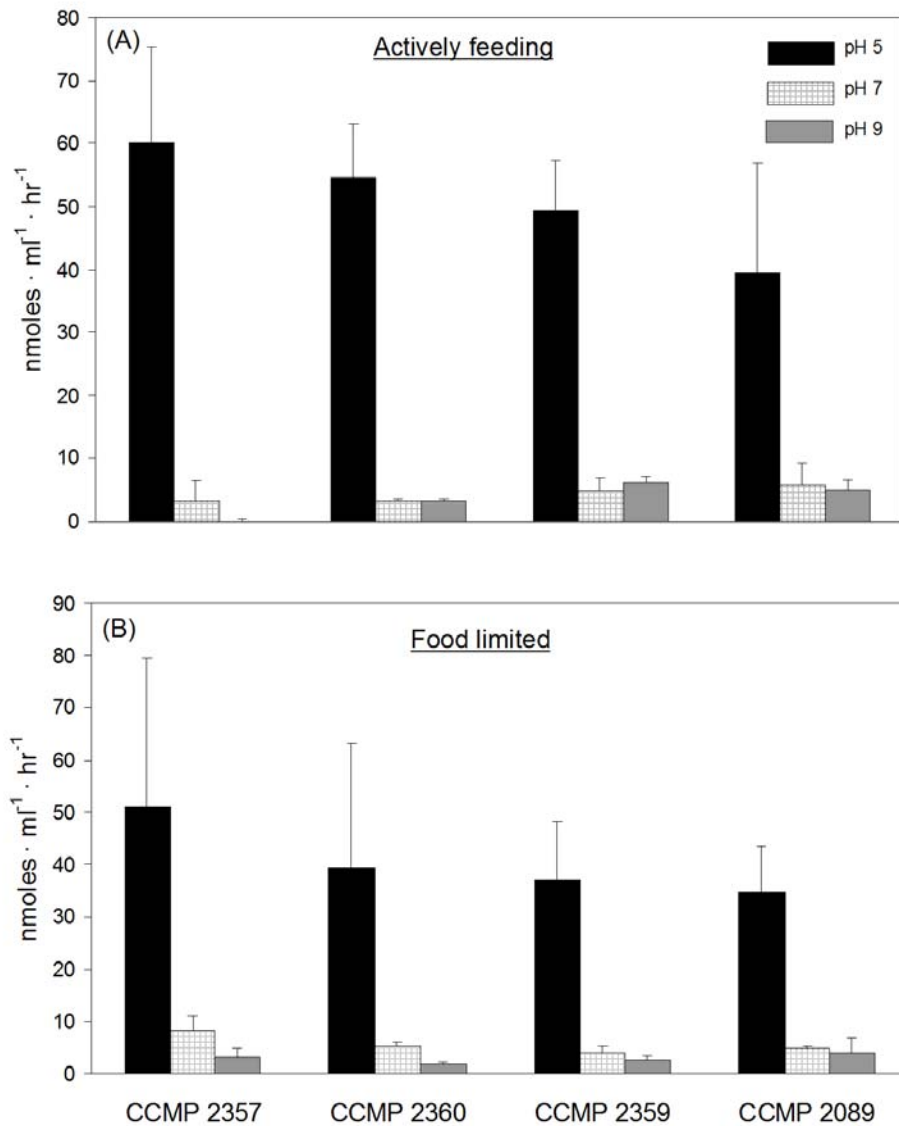
Phosphatase activity was detected in the CHSE cell controls, both in the filtrate and associated with formalin-fixed fish cells (Fig. 1.8). Enzyme activity associated with the filtrate was mostly at pH 5 and pH 9, whereas activity of formalin-fixed CHSE cells mostly was detected at pH 5 and pH 7 (Fig. 1.8). *Pfiesteria shumwayae* cells that were centrifuged and transferred to fresh 10 salinity synthetic seawater showed no increase in filtrate phosphatase activity at any tested pH value (data not shown).



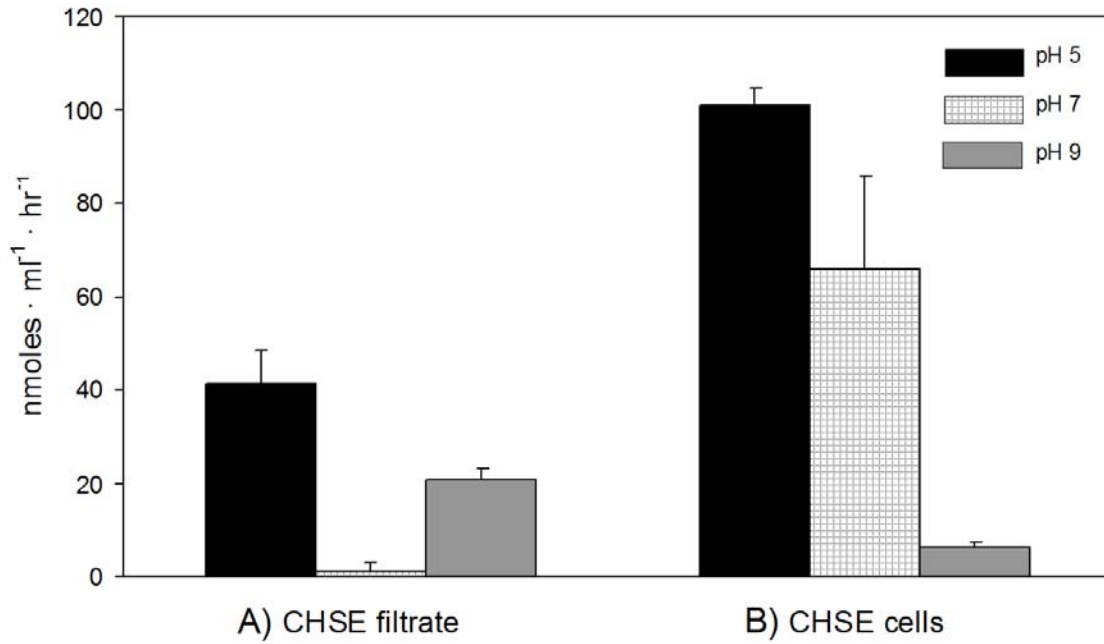
**Figure 1.5** Phosphatase activity, expressed as nmoles p-nitrophenol released, at different pH values in fixed (0.5% formalin, final concentration) *Pfiesteria shumwayae* samples: **A)** *P. shumwayae* cells that were actively feeding on a fish cell line. **B)** *P. shumwayae* cells that had not fed on fish cells for 3 days. Data are given as means ± 1 SE (n = 3).



**Figure 1.6** Phosphatase activity, expressed as nmoles p-nitrophenol released, at different pH values in live *Pfiesteria shumwayae* samples: **A)** *P. shumwayae* cells that were actively feeding on a fish cell line. **B)** *P. shumwayae* cells that had not fed on fish cells for 3 days. Data are given as means  $\pm$  1 SE (n = 3).



**Figure 1.7** Phosphatase activity, expressed as nmoles p-nitrophenol released, at different pH values in *Pfiesteria shumwayae* culture filtrate: **A)** *P. shumwayae* cells that were actively feeding on a fish cell line. **B)** *P. shumwayae* cells that had not fed on fish cells for 3 days. Data are given as means  $\pm$  1 SE (n = 3).



**Figure 1.8** Phosphatase activity, expressed as nmoles p-nitrophenol released, at different pH values in CHSE cells: **A)** CHSE cells filtrate. **B)** fixed CHSE cells (0.5% formalin, final concentration). Data are given as means  $\pm$  1 SE (n = 3).

## 1.5 Discussion

This research is the first to use the ELF-97 phosphatase substrate to examine phosphatase activity in a heterotrophic protist. Intracellular ELF-97 labeling has been described in one photosynthetic dinoflagellate (*Prorocentrum minimum* (Pavillard) Schiller; Dyhrman and Palenik 1999), but the ELF-97 probe has not previously been used to examine acid phosphatases in heterotrophic dinoflagellates.

The ELF-97 molecular probe successfully allowed localization of intracellular phosphatases in both actively feeding and food limited *Pfiesteria shumwayae*. Phosphatases in actively feeding *P. shumwayae* cells likely aid in the digestion of prey cytoplasm, as indicated by strong localization in or around food vacuoles. The food vacuole is an organelle in which intracellular digestion occurs and is thought to contain hydrolytic enzymes with acidic pH optima (Müller 1967). The considerable variation in ELF-97 activity among individual cells probably represents different stages in the digestion process. Some *P. shumwayae* cells had fed shortly before samples were taken, evidenced by the relatively large size of the food vacuole, but did not show ELF activity. In these cells, sampling may have occurred just before the digestive process was initiated. Mueller et al. (1965) estimated a time of ~3 min from the formation of food vacuoles to the onset of acid phosphatase activity in digestive vacuoles of the ciliate, *Paramecium multimicronucleatum* Powers and Mitchell. Alternatively, the ELF-97 substrate may not have equally penetrated the vacuolar membrane of all *P. shumwayae* cells due to the lack of a permeabilization step in the method used; however, this possibility seems unlikely as the ELF-97 probe successfully labeled most cells.

In many *Pfiesteria shumwayae* cells, ELF-97 activity was localized in one to several relatively large spherical deposits near the food vacuole, whereas in other cells, ELF-97 activity surrounded the periphery of the food vacuole. Similar acid phosphatase labeling patterns have been observed in the ciliates *Tetrahymena* sp. and *Paramecium* spp. when actively feeding (Seaman 1961, Mueller et al. 1965, Elliot and Clemmons 1966, Karakashian and Karakashian 1973). The ELF-97 substrate can label phosphatases associated with organelles, including lysosomes and the Golgi apparatus (Paragas et al. 2002). The large spherical enzyme deposits may have been lysosomes that had not yet fused with the food vacuole. Alternatively, the ELF-97 deposits may have been associated with the endoplasmic reticulum (ER) or the Golgi apparatus, both of which are believed to be involved in enzyme production and subsequent dispersal throughout cells (Novikoff et al. 1964, Müller 1967). Barlow and Triemer (1986) observed acid phosphatase activity in the Golgi cisternae and ER of the heterotrophic dinoflagellate, *Cryptocodinium cohnii*. Acid phosphatase activity has also been detected in lysosome-like structures, the ER, and the Golgi complex of many non-dinoflagellate protists (Sommer and Blum 1965, Elliott and Clemmons 1966, Dembitzer 1968, Stoltze et al. 1969, McAlpine 1970, Brooker 1971, Gomez et al. 1974, Langreth and Balber 1975, Ryter and Bowers 1976, Grab et al. 1997). Smaller ELF-97 deposits occurred throughout the cytoplasm in some *P. shumwayae* cells. General cytoplasmic labeling also has been noted in other protists (Birns 1960, Klamer and Fennell 1963, Mueller et al. 1965, Cooper et al. 1974), but the role of phosphatases detected in the cytoplasm is unclear.

Clear evidence of surface phosphatase activity was not detected in any *Pfiesteria shumwayae* cells. Several cells showed ELF-97 activity near the cell membrane that appeared to be intracellular, but the exact location of enzyme could not be determined with certainty. In photosynthetic dinoflagellates, phosphatases apparently associated with the cell surface/membrane are believed to hydrolyze external organic substances, liberating phosphate ions for uptake (Kuenzler and Perras 1965, Rivkin and Swift 1979, Dyhrman and Palenik 1999, González-Gil 1998). Phosphatases with extracellular functions have not yet been detected in heterotrophic dinoflagellates, although information thus far is limited to *C. cohnii* (Barlow and Triemer 1986) and *P. shumwayae* (this study). It remains possible, however, that *P. shumwayae* may produce cell-surface phosphatases considering that phosphatase activity associated with the cell surface or plasma membrane has been described in other heterotrophic protists (trypanosomatid flagellates *Trypanosoma* spp. and *Leishmania* spp., Gottlieb and Dwyer 1981, Letelier et al. 1985, Nagakura et al. 1985, Fernandes et al. 2003). These protists are internal parasites of mammals, however, and are thought to feed by resorption or other uptake across or through the protist cell membrane (Dwyer and Gottlieb 1983), whereas *P. shumwayae* is a free-living, phagotrophic organism.

*Pfiesteria shumwayae* reproductive cysts often showed ELF-97 activity, and several contained the same dense granular deposits noted in the flagellate cells. Most *P. shumwayae* cysts had ELF-97 fluorescence consisting of fine crystal-like granules over the entire cell, but the location of enzyme activity (extracellular or intracellular) could not be determined. Since digestion and cell division occur in *P. shumwayae* reproductive

cysts (Parrow and Burkholder 2003), it is not surprising that these cells also have active phosphatases. *P. shumwayae* cell pairs fixed in the process of excysting showed no ELF-97 activity associated with the remaining cyst wall, indicating that the ELF-97 activity was not located on the cyst surface.

The relatively large spherical ELF-97 deposits observed in food limited *Pfiesteria shumwayae* cells were typically associated with unknown darkened cellular structures that could not be identified by light microscopy. This ELF-97 fluorescence may have been associated with remnants of small food vacuoles where digestion was not yet completed. The dinoflagellates had depleted the fish cells 3 days earlier, but apparent cannibalistic feeding was observed before sampling, and partially filled food vacuoles occurred in several *P. shumwayae* cells under food limited conditions. Alternatively, the unidentified darkened structures may have been autophagic vacuoles that functioned in the degradation of cellular constituents. Autophagy is believed to occur in all eukaryotic cells, and often increases during periods of cellular stress such as starvation (Dunn 1994, Klionsky and Emr 2000, Reggiori and Klionsky 2002). Presumed autophagic vesicles containing acid phosphatase(s) have been detected in many protists (Elliot and Clemmons 1966, Baker and Buetow 1976, Martin and Byers 1976, Swanson and Floyd 1979). Acid phosphatase activity associated with possible autophagic vacuoles has been detected in the photosynthetic dinoflagellates, *Alexandrium tamarense* (Stein) Dodge and *Lingulodinium polyedrum* (Lebour) Balech (formerly *Gonyaulax tamarensis* and *Gonyaulax polyedra*, respectively; Schmitter and Jurkiewicz 1981).

The colorimetric phosphatase assays supported the data obtained with the ELF-97 molecular probe, allowing interpretations about the operational pH of the detected phosphatases in *Pfiesteria shumwayae*. Phosphatases were detected in fixed cells of actively feeding and food limited strains at pH 5 and pH 7, providing further evidence that the phosphatases in this dinoflagellate have acidic pH optima. The activity may have been from a phosphatase with a broad pH distribution or, alternatively, from multiple phosphatases with different pH optima. Phosphatase activity in living cells was detected only at pH 5, yet the cells had apparently died after the 5-h incubation at that pH. Unlike ELF-97, the colorimetric phosphatase substrate did not penetrate the plasma membrane of living cells, but did penetrate dead cells, so that the enzyme activity detected at pH 5 was actually intracellular. *P. shumwayae* cells incubated at pH 7 survived the incubation period, and almost no phosphatase activity was detected in the live cells at this pH. In contrast, phosphatase activity was detected at pH 7 in fixed (i.e. permeable) cells, again suggesting that the enzymes involved were intracellular. The phosphatase activity at pH 7 in *P. shumwayae* may have reflected the presence of alkaline phosphatase(s); however, most alkaline phosphatases have an optimum between pH 8 and pH 10 (Kuenzler and Perras 1965), and no phosphatase activity attributable to *P. shumwayae* was observed at pH 9.

Phosphatase activity in the fish cell controls was also detected at pH 5 and pH 7, suggesting that some of the activity present in feeding cultures of *Pfiesteria shumwayae* could have come from ingested or unconsumed fish cell cytoplasm. The majority of the phosphatase activity in feeding cultures was considered to have come from *P.*

*shumwayae*, given that feeding cultures were sampled within 24 h of food depletion to minimize phosphatase activity contributed by fish cells, and phosphatase activity of food limited cultures of *P. shumwayae* was similar to that of feeding cultures. In contrast, the phosphatase activity in culture filtrates of *P. shumwayae* was not attributed to *P. shumwayae* cells, since filtrates of the CHSE cell controls showed a similar pattern of phosphatase activity. Also, washed *P. shumwayae* cells incubated in fresh medium for 3 days showed no net increase in filtrate phosphatase activity, providing further evidence that *P. shumwayae* did not produce extracellular dissolved phosphatases.

While most research on phosphatases in dinoflagellates has emphasized the extracellular alkaline phosphatases of photosynthetic species, the occurrence, function, and importance of intracellular acid phosphatases in heterotrophic taxa and microbial food webs are mostly unknown. This study represents a first step in understanding phosphatases in *Pfiesteria*. Additional research on the enzyme kinetics should examine the effects of different phosphatase substrates, buffers, and metal cations on enzyme activity. The enzyme optima within the acidic pH range also need to be determined. Further research also is needed to resolve the location and examine the role(s) of phosphatases in *P. shumwayae* flagellate cells and cysts, particularly in food limited cells where autophagic processes may be important. Comparisons with other heterotrophic taxa, including non-phagotrophic species, may also provide valuable information on the diversity and specialization of heterotrophic mechanisms in protists.

### Acknowledgments

We thank Z. Eyler for assistance. Funding support was provided by the U.S. EPA (no. 20040025.1), NOAA Ecohab (no. NA04NOS4780272), and the NC General Assembly.

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2. AXENIC CULTIVATION OF THE HETEROTROPHIC DINOFLAGELLATE  
*PFIESTERIA SHUMWAYAE* AND OBSERVATIONS ON FEEDING BEHAVIOR

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*Journal of Phycology* (submitted)

## 2.1 Abstract

*Pfiesteria shumwayae* is a heterotrophic dinoflagellate commonly found in temperate, estuarine waters. *P. shumwayae* can feed on other protists, fish, and invertebrates, but research on the biochemical requirements of this species has been restricted by the lack of axenic cultures. An undefined, biphasic culture medium was formulated that supported the axenic growth of two of three strains of *P. shumwayae*. The medium contained chicken egg yolk as a major component. Successful growth depended on the method used to sterilize the medium, and maximum cell yields ( $10^4$  ml<sup>-1</sup>) were similar to those attained in previous research when *P. shumwayae* was cultured with living fish or microalgae. Additionally, *P. shumwayae* flagellate cells ingested particles present in the biphasic medium, allowing detailed observations of feeding behavior. This research is an initial step toward a chemically defined axenic culture medium and determination of *P. shumwayae* metabolic requirements.

## 2.2 Introduction

Heterotrophic dinoflagellates are widespread in aquatic ecosystems and have been increasingly recognized as important components of aquatic food webs (Smetacek 1981, Lessard 1991, Jeong 1999). Knowledge of heterotrophic dinoflagellate feeding behavior and nutritional requirements is essential for assessing the role and impact of these organisms in microbial communities. Although detailed studies have shown that heterotrophic dinoflagellates use a variety of mechanisms to capture and ingest many different prey species (Schnepf and Elbrächter 1992, Hansen and Calado 1999), the nutritional requirements of most heterotrophic dinoflagellates are unknown (Gaines and Elbrächter 1987, Guillard and Morton 2003).

Laboratory cultures have been instrumental in increasing the understanding of heterotrophic flagellate biology (Guillard and Keller 1984, Caron 1993). Most heterotrophic dinoflagellates are phagotrophs (Schnepf and Elbrächter 1992, Hansen and Calado 1999), and laboratory cultures of these organisms generally are maintained with live prey. Research on physiology and nutrition often requires control of experimental variables that can be difficult to achieve with cultures containing multiple species (Caron 1993). Axenic cultivation, the growth of a single species in the absence of other metabolizing cells (Baker and Ferguson 1942, Droop 1959), allows the examination of cellular processes in the absence of potentially confounding activities of other living organisms (Caron 1993). In particular, axenic cultivation affords the most direct approach for both defining and quantifying the nutritional requirements of an organism in biochemical terms (Droop 1970). Once the nutritional requirements of phagotrophic

species are known, the relationships and interdependencies of these organisms with and on other members of the plankton community can be evaluated at the biochemical level (Droop 1970, Provasoli 1977).

Axenic cultures of phagotrophic organisms are not easily established, and success depends on finding a complete substitute for the living food (Droop 1959, 1970). The simplest method for attempting axenic cultivation is to first establish a monoxenic cultivation system in which the organism is cultured with one additional species as the food source (Droop 1959, 1970). If the food source is eliminated easily, monoxenic cultivation provides a continuous supply of axenic organisms that can be inoculated into various artificial media (Droop 1970, Provasoli 1977). Generally, the initial axenic culture medium is comprised of complex undefined biological products, as the aim is to provide a complete medium regardless of specific requirements (Provasoli 1977). Once the food is replaced by non-living material, it becomes possible to chemically define specific nutritional requirements (Droop 1970). Water-soluble requirements usually are identified first, followed by the lipid-soluble requirements (Droop 1970). Despite the prevalence of heterotrophic dinoflagellates in aquatic ecosystems, only four species, *Crypthecodinium cohnii* (Seligo) Chatton, *Gyrodinium lebouriae* Herdman, *Noctiluca scintillans* (Macartney) Kofoid et Swezy, and *Oxyrrhis marina* Dujardin, have been successfully cultured axenically (Pringsheim 1956, Droop 1959, Provasoli and Gold 1962, Gold and Baren 1966, McGinn and Gold 1969, McGinn 1971, Lee 1977).

The genus *Pfiesteria* contains *P. piscicida* Steidinger et Burkholder and *P. shumwayae* Glasgow et Burkholder (Marshall et al. 2006), two heterotrophic species

found in temperate estuarine and coastal waters (Ruble et al. 2005). *Pfiesteria* spp. are among the relatively few heterotrophic dinoflagellates that have been maintained in long-term culture, which has facilitated research on the biology and ecology of these species. These dinoflagellates are phagotrophic and use a feeding tube (often called a peduncle; Elbrächter 1991) to ingest other protists, fish cells, and shellfish tissues (Burkholder and Glasgow 1995, Glasgow et al. 2001, Springer et al. 2002, Parrow et al. 2005). The nutritional requirements of these dinoflagellates, however, have not been examined at the biochemical level, largely due to the lack of axenic cultures. Recently, *P. shumwayae* was cultured on an undefined dissolved organic medium in the presence of bacteria (Haas et al. 2005). The present research describes a culture medium that supports the axenic growth of *P. shumwayae*. Detailed observations of *P. shumwayae* feeding behavior also are reported.

### 2.3 Materials and Methods

*Preparation of culture media.* The axenic culture medium consisted of 10 salinity synthetic seawater (Instant Ocean, Aquarium Systems) supplemented with L1 trace metals, 2 x L1 vitamins (Guillard and Hargraves 1993), and a commercially available chicken egg yolk enrichment (MP Biomedicals #1003749). *Pfiesteria shumwayae* growth was tested in media with chicken egg yolk concentrations ranging from 0.025%-0.5% (final concentration). The effect of sterilization method on the establishment of axenic *P. shumwayae* cultures also was examined. Growth on the medium was evaluated after a) autoclaving, b) sterile-filtration (0.22 µm pore size), and c) neither (untreated). Untreated chicken egg yolk media were prepared by aseptic addition of the yolk enrichment to autoclaved, 10 salinity synthetic seawater. Although the manufacturer cannot sterilize the egg yolk enrichment by traditional methods, the product is aseptically prepared and must pass sterility tests prior to shipment (MP Biomedicals, *pers. comm.*). The L1 trace metals and vitamins were added to the media from sterile filtered (0.22 µm pore size) stock solutions following sterilization. Culture media developed for the axenic cultivation of the heterotrophic dinoflagellates *Cryptocodinium cohnii*, *Gyrodinium lebouriae*, *Noctiluca scintillans*, and *Oxyrrhis marina* (Droop 1959, Provasoli and Gold 1962, Droop and Doyle 1966, McGinn 1971, Tuttle and Loeblich 1975, Lee 1977) also were tested.

*Establishment of axenic cultures.* Three strains of *Pfiesteria shumwayae* (Table 2.1) were maintained in monoxenic culture on a Chinook salmon (*Oncorhynchus tshawytscha* Walbum) fish cell line following Parrow et al. (2005). Axenic culture

medium (10 ml) was added to sterile 25 cm<sup>2</sup> polystyrene culture flasks (Greiner Bio-One #690160), and dinoflagellate cells (~1.5 x 10<sup>4</sup> cells) were added from logarithmic growth phase cultures maintained on the fish cell line. Cultures were incubated in darkness at 21 °C and were examined microscopically daily for evidence of dinoflagellate proliferation. Cultures in which dinoflagellate growth occurred were subcultured every 7 days. The axenic media were made freshly prior to each transfer, and all culture manipulations were made under a positive-pressure laminar-flow hood using aseptic techniques (Guillard and Morton 2003).

**Table 2.1** *Pfiesteria shumwayae* strains, source locations, and collection dates (CCMP, Provasoli-Guillard Center for Culture of Marine Phytoplankton)

Strain designation	Source location	Collection date
CCMP2089	Pamlico River, NC, USA	November 1999
CCMP2357	Carolina Pines, Neuse River, NC, USA	July 1998
CCMP2360	Tasman Bay, New Zealand	April 2000

*Sterility Tests.* *Pfiesteria shumwayae* strains that were successfully cultivated in axenic culture media were tested for evidence of microbial contamination by culture in liquid broth and solid agar. A liquid sterility test medium comprised of 10 salinity L1 medium supplemented with bactopectone (0.5 g · L<sup>-1</sup>), yeast extract (0.5 g · L<sup>-1</sup>), and glucose (0.5 g · L<sup>-1</sup>) was used as a general test for bacteria and fungi. An additional liquid sterility medium of 10 salinity L1 medium enriched with bactopectone (1.0 g · L<sup>-1</sup>)

and methylamine · HCl ( $1.0 \text{ g} \cdot \text{L}^{-1}$ ) was used to test for methylaminotrophic bacteria (Guillard and Morton 2003). Solid medium contained 0.5x marine agar composed of Difco 2216-enriched agar ( $27.6 \text{ g} \cdot \text{L}^{-1}$ ) (Becton Dickinson), agar ( $7.5 \text{ g} \cdot \text{L}^{-1}$ ), bactopectone ( $5 \text{ g} \cdot \text{L}^{-1}$ ), and yeast extract ( $1 \text{ g} \cdot \text{L}^{-1}$ ) in deionized water. Duplicate samples (0.2 ml) from late logarithmic growth phase cultures were inoculated into 20 ml broth tubes and onto agar plates. Samples from xenic stock *P. shumwayae* cultures containing bacteria were inoculated into test media as positive controls. Un-inoculated test media served as negative controls. Sterility media were incubated in darkness at 21 °C and examined daily for 4 weeks for visible signs of contaminant growth. Sterility tests were repeated at 3-month intervals.

Axenic *Pfiesteria shumwayae* cultures also were examined for the presence of bacteria using epifluorescence microscopy after staining with the nucleic acid stain, SYTOX Green (Molecular Probes S7020). Samples (1 ml) from late logarithmic growth phase cultures were fixed with glutaraldehyde (0.5% final concentration), stained with SYTOX Green ( $5 \mu\text{m}$ , final concentration) in darkness for 30 min, and filtered onto 25mm GTBP black polycarbonate filters ( $0.22 \mu\text{m}$  pore size; Millipore). Each filter was placed on a microscope slide, covered by a drop of immersion oil (Cargille type FF) and a cover slip, and examined with an Olympus AX-70 microscope equipped with a 100 W mercury lamp, a triple-band pass filter set, and a 60x 1.2 N.A. water immersion objective. Samples from xenic stock dinoflagellate cultures containing bacteria were prepared identically and examined as positive controls.

*Dinoflagellate growth experiments.* Growth experiments were conducted on *Pfiesteria shumwayae* strains (CCMP2357, CCMP2360) that were cultured axenically on both autoclaved and untreated chicken egg yolk media (CYM) for 2 to 6 months. The axenic *P. shumwayae* strains were maintained in autoclaved 0.2% CYM and untreated 0.05% CYM (% refers to the concentration of chicken egg yolk). Experimental treatments consisted of autoclaved CYM (0.1%, 0.2%, 0.5%) and untreated CYM (0.025%, 0.05%, and 0.1%). Dinoflagellate cells were inoculated into triplicate, sterile 75 cm<sup>2</sup> polystyrene culture flasks containing the experimental medium (40 ml) at an initial density of ca.  $1.0 \times 10^3$  cells ml<sup>-1</sup>. Cultures were gently mixed and aliquots (1 ml) removed daily for 12 days. Samples were preserved with 2% (final concentration) acidic Lugol's solution, and cells were enumerated by light microscopy (100x) using a Palmer-Maloney counting chamber (Wetzel and Likens 1991). Population growth ( $k$ , divisions · d<sup>-1</sup>) was estimated over the exponential growth phase by computing the least-squares regression slope of semi-logarithmic daily cell count means (Guillard 1973). A Student's t-test assuming unequal variances was used to test for differences between strains in maximum cell abundance ( $\alpha = 0.05$ ). A one-way ANOVA was used to assess the effect of chicken egg yolk concentration on maximum cell abundance for each strain ( $\alpha = 0.05$ ).

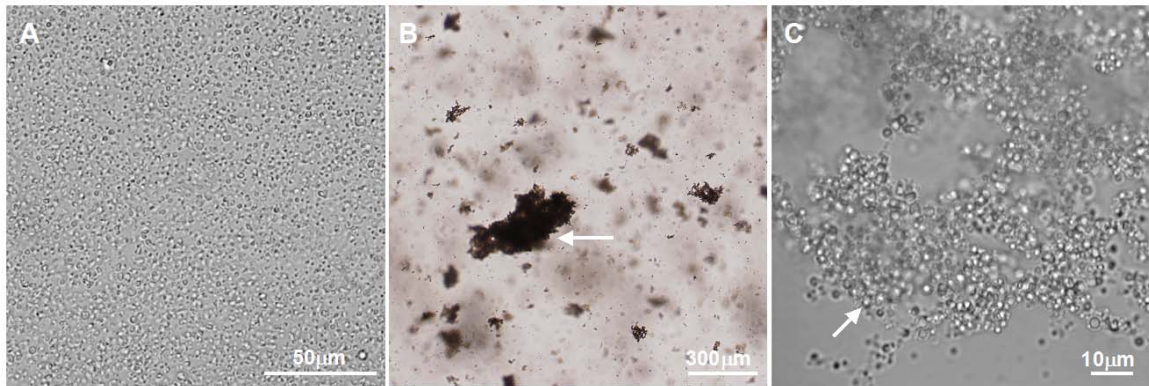
*Feeding behavior.* *Pfiesteria shumwayae* cultures were examined daily with an Olympus CK40 inverted microscope (40 - 200x). Detailed observations of feeding behavior were made in Petri dish subcultures with a Leitz Diavert inverted microscope equipped with a 0.60 N.A. long working distance condenser. Low magnification observations were made with a 6.3x 0.20 N.A. objective, and a 40x 0.95x N.A. objective

was used for observations at higher magnification. Images were captured with a DEI-750 CCD camera (Optronics Engineering, Goleta, CA, USA) and/or video-recorded using a Sony SVD-9500MD S-video recorder.

## 2. 4 Results

*Axenic culture media.* Preparation of the chicken egg yolk media (CYM) resulted in biphasic media containing numerous particles of various shapes and sizes. The untreated CYM mostly contained small, roughly spherical, yolk granules (~0.2 - 2  $\mu\text{m}$ ) that settled, but did not adhere, to the bottom of the culture flask (Fig. 2.1A). Larger spherical globules (~25 - 100  $\mu\text{m}$ ) and apparent semisolid masses (~30 - 60  $\mu\text{m}$ ) also were present in the untreated CYM but were less abundant than the granules. Autoclaving the CYM caused coagulation of the egg yolk proteins, resulting in large yolk aggregations. Vigorous shaking of the medium disaggregated the clusters into smaller aggregates with a wide range in size (0.2  $\mu\text{m}$  – 1 mm; Fig. 2.1B). The yolk aggregates were comprised of smaller adherent yolk particles (Fig. 2.1C). The autoclaved CYM contained small yolk granules like those present in the untreated CYM, but the granules were less abundant. The sterile-filtered CYM contained relatively few small yolk granules.

*Axenic cultures.* Two *Pfiesteria shumwayae* strains (CCMP2357, CCMP2360) were successfully cultivated axenically in both the autoclaved and untreated CYM, and these strains have been maintained in axenic culture for > 2 years. Both *P. shumwayae* strains could be cultured in autoclaved and untreated CYM at chicken egg yolk concentrations of 0.025% - 0.5%. The concentration that yielded the best growth varied with different manufacturing lots of the chicken egg yolk enrichment. Generally the range of chicken egg yolk concentrations that supported axenic *P. shumwayae* growth in the autoclaved and untreated CYM was the same for a single lot of yolk.



**Figure 2.1** Brightfield micrographs showing the yolk particles present in the CYM. **A)** Untreated CYM containing small, roughly spherical granules. **B)** Autoclaved CYM at low magnification containing large yolk aggregations (arrow). **C)** Autoclaved CYM at a higher magnification, showing the small particles that comprise the yolk aggregates (arrow).

Strains CCMP2357 and CCMP2360 could not be cultivated in sterile-filtered CYM at any concentration tested. Although multiple attempts were made, strain CCMP2089 could not be cultured axenically in any of the CYM, regardless of the sterilization method. CCMP2089 cells that were inoculated into autoclaved and untreated CYM gradually encysted over a 3 - 4 day period until no flagellate cells remained. Flagellate cells (CCMP2357, CCMP2360, CCMP2089) inoculated into sterile-filtered CYM did not encyst and could be transferred 2 - 3 times; however, the dinoflagellate cells gradually became smaller in size, and further efforts to transfer the cultures were unsuccessful. Media used for the axenic cultivation of other heterotrophic dinoflagellates did not support the growth of any *P. shumwayae* strain.

Transfer of axenic *P. shumwayae* cultures was most successful when media were freshly prepared and when transfer occurred during the exponential growth phase. Although the two *P. shumwayae* strains could be cultured in both the autoclaved and untreated CYM, they were easier to maintain in the untreated CYM. Flagellate cells in the autoclaved CYM gradually encysted as the culture reached the late exponential and stationary phases of growth, and transfer after this stage was often unsuccessful.

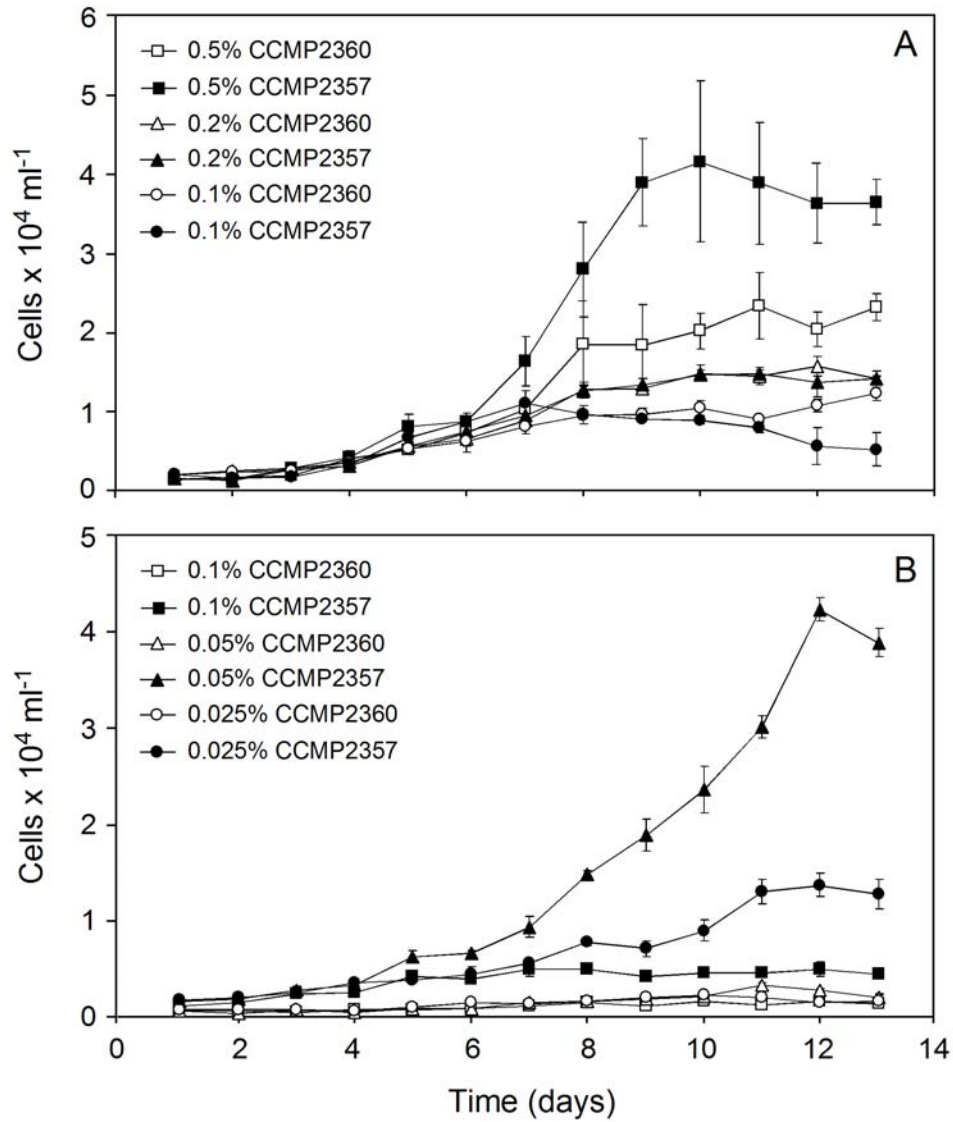
*Culture purity.* Detectable bacterial and fungal contaminants were not apparent in any axenic *Pfiesteria shumwayae* culture. Cultures tested in liquid and solid sterility test media showed no bacterial or fungal growth while contamination was apparent (cloudiness, colony formation) in all positive controls within several days. Samples from axenic cultures that were stained with SYTOX Green also showed no evidence of contamination. Positive controls contained fluorescent bacterial cells in every field of

view. Axenic *P. shumwayae* cultures have remained free of demonstrable microbial contaminants since establishment.

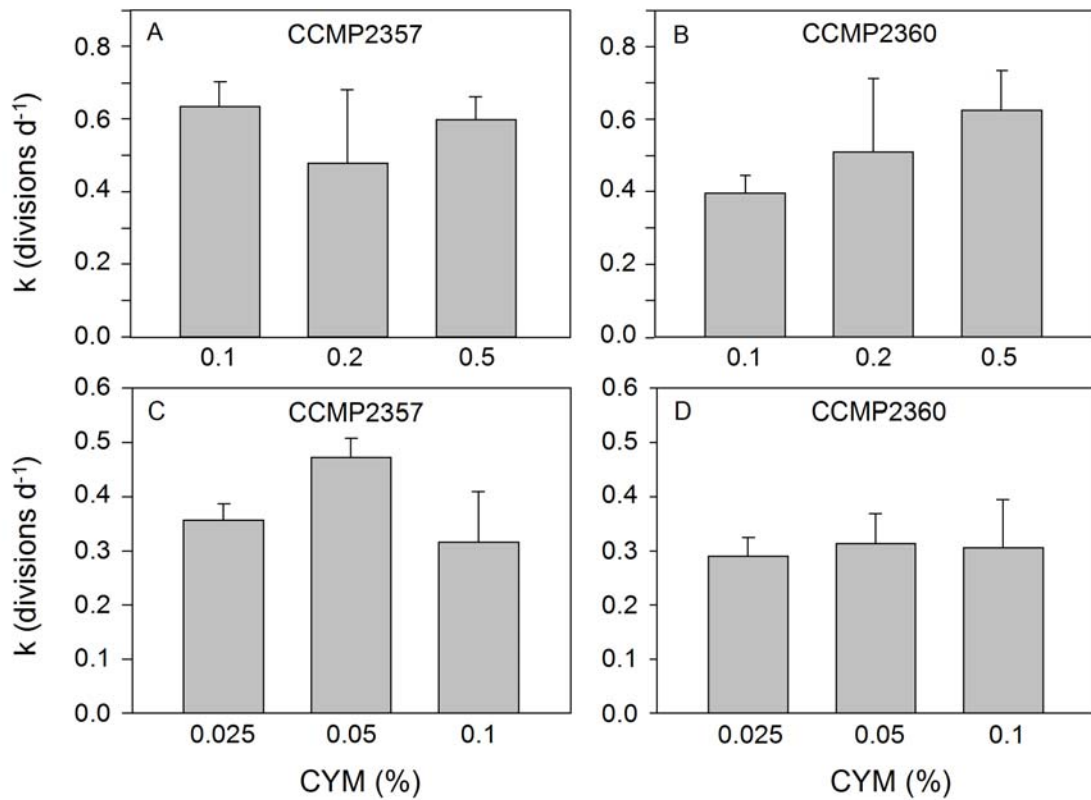
*Axenic growth.* *Pfiesteria shumwayae* strains cultivated on autoclaved CYM reached maximum cell densities of  $1.1 \times 10^4$  -  $4.2 \times 10^4$  cells ml<sup>-1</sup> in 9 to 11 days, and the highest cell yields for both strains were obtained in 0.5% CYM (Fig. 2.2A). Proliferation occurred in both strains at all three concentrations of autoclaved CYM tested, but the concentration of chicken egg yolk in the medium significantly affected the maximum cell yield. Cell yield of the two strains did not significantly differ at any concentration of autoclaved CYM; division rates ranged from 0.40 - 0.63 divisions per day (Fig. 2.3A,B).

The two *Pfiesteria shumwayae* strains that were cultured on untreated CYM reached maximum cell densities of  $1.6 \times 10^3$  -  $4.2 \times 10^4$  cells ml<sup>-1</sup> in 10 - 12 days (Fig. 2.2B). Although low cell yields were obtained for strain CCMP2360 in the untreated CYM, cultures in 0.025% and 0.05% CYM were transferable. Growth of both strains in untreated 0.1% CYM was limited, and the cultures were not easily transferred. Concentration significantly affected maximum cell densities of both *P. shumwayae* strains, and the highest cell yields were attained in 0.05% CYM. Maximum cell densities of the two strains were significantly different in all concentrations of chicken egg yolk. Division rates in the untreated CYM ranged from 0.29 - 0.47 divisions per day (Fig. 2.3C,D).

*Dinoflagellate feeding.* The biphasic axenic cultivation media enabled detailed observations of *Pfiesteria shumwayae* feeding behavior. *P. shumwayae* flagellate cells (CCMP2357, CCMP2360) cultured in the untreated CYM frequently were observed



**Figure 2.2** Time-course of cell densities for *Pfiesteria shumwayae* strains CCMP2357 and CCMP2360 cultured in **A)** autoclaved CYM (0.5%, 0.2%, 0.1% chicken egg yolk) and **B)** untreated CYM (0.1%, 0.05%, 0.025% chicken egg yolk). Data are given as means  $\pm$  1 SE (n = 3).



**Figure 2.3** Mean division rates ( $k$ , divisions  $d^{-1}$ ) of *Pfiesteria shumwayae* cells estimated during logarithmic growth phase for strain CCMP2357 and CCMP2360 cultured in **A**), **B**) autoclaved CYM (0.5%, 0.2%, 0.1% chicken egg yolk) and **C**), **D**) untreated CYM (0.1%, 0.05%, 0.025% chicken egg yolk). Data are given as means  $\pm$  1 SE ( $n = 3$ ).

swimming slowly in tight circles at the bottom of the culture flask. The cells generally were oriented so that the antapex faced toward the culture flask bottom. Movement of the transverse flagellum caused the egg yolk granules to spread away from the cell, creating a roughly spherical clearing that was easily viewed at low magnification (40x, Movie supplement 1). Multiple cells (2 - 13) often were observed in the same cleared area and up to 15 separate clearings could be observed in the same field of view. High magnification observations (400x) in Petri dish subcultures revealed that flagellate cells in the clearings each extended and attached the feeding tube (peduncle) to the bottom of the Petri dish. The attached feeding tube spread across the bottom of the Petri dish, and cytoplasmic extensions (pseudopodia) extended from the margin of the peduncle (Fig. 2.4A, Movie supplement 2). The pseudopodia ranged from ca. 2 - 30  $\mu\text{m}$  in length and up to 6 extensions originated from a single cell (Fig. 2.4). The flagellate cells slowly moved while attached to the bottom, and the peduncle and the pseudopodia were repeatedly extended and retracted along the bottom of the Petri dish in coordinated movements (Movie supplement 2). There was considerable variation in the time that each cell spent attached to the bottom (seconds to several minutes).

Trails of cytoplasmic extensions often were left behind the flagellate cells as they moved while still attached to the Petri dish (Fig. 2.4B-D). The extensions were adhesive and frequently remained attached to the bottom once a cell retracted its peduncle and moved away. Other flagellate cells, apparently attracted to the remnant cytoplasmic extensions, attached to and ingested the extensions (Movie supplement 3). When a flagellate cell detached from the Petri dish, the peduncle quickly was withdrawn into the

cell. Occasionally, cells were observed to detach and swim briefly with the peduncle extended until reattaching at a different location. Sometimes a cell appeared to have difficulty detaching its peduncle and extensions from the bottom and became deformed as it attempted to disassociate from the bottom (Fig. 2.4C).

Often flagellate cells were observed ingesting small egg yolk granules ( $\sim 0.2 - 2 \mu\text{m}$ ) through the peduncle (Fig. 2.5A, Movie supplement 4). The small particles flowed into the peduncle and entered the cell through a circular opening, which appeared to contract during the feeding process (Fig. 2.5B, Movie supplement 3). Large semi-solid masses ( $\sim 40 \mu\text{m}$ ) also were ingested through the peduncle (Fig. 2.6, Movie supplement 5). Cells that ingested large egg yolk masses often continued feeding. Particle ingestion, both large and small, occurred quickly ( $\sim 10 - 20$  seconds). Many cells contained apparent food vacuoles (Fig. 2.5C).

*Pfiesteria shumwayae* flagellate cells cultivated in the autoclaved CYM frequently attached to the large yolk aggregations with the peduncle and towed the particles while swimming. Often multiple cells (up to 10) simultaneously attached to a single aggregation. The epicone of attached dinoflagellates sometimes became deformed as the cells attempted to ingest the yolk particles. Ingestion of particles was not observed in the autoclaved CYM because the flagellate cells attached to the yolk aggregations usually moved out of the field of view, making detailed examination difficult. However, particle ingestion likely occurred, and most cells contained food vacuoles. Cells were not observed attached to the Petri dish in the autoclaved CYM. Cytoplasmic extensions also were not observed in these cells, but the large particles, combined with the movement of

the cells, would have made observations of any pseudopodia extremely difficult.

**Figure 2.4** Brightfield micrographs of *Pfiesteria shumwayae* cultured in untreated CYM.

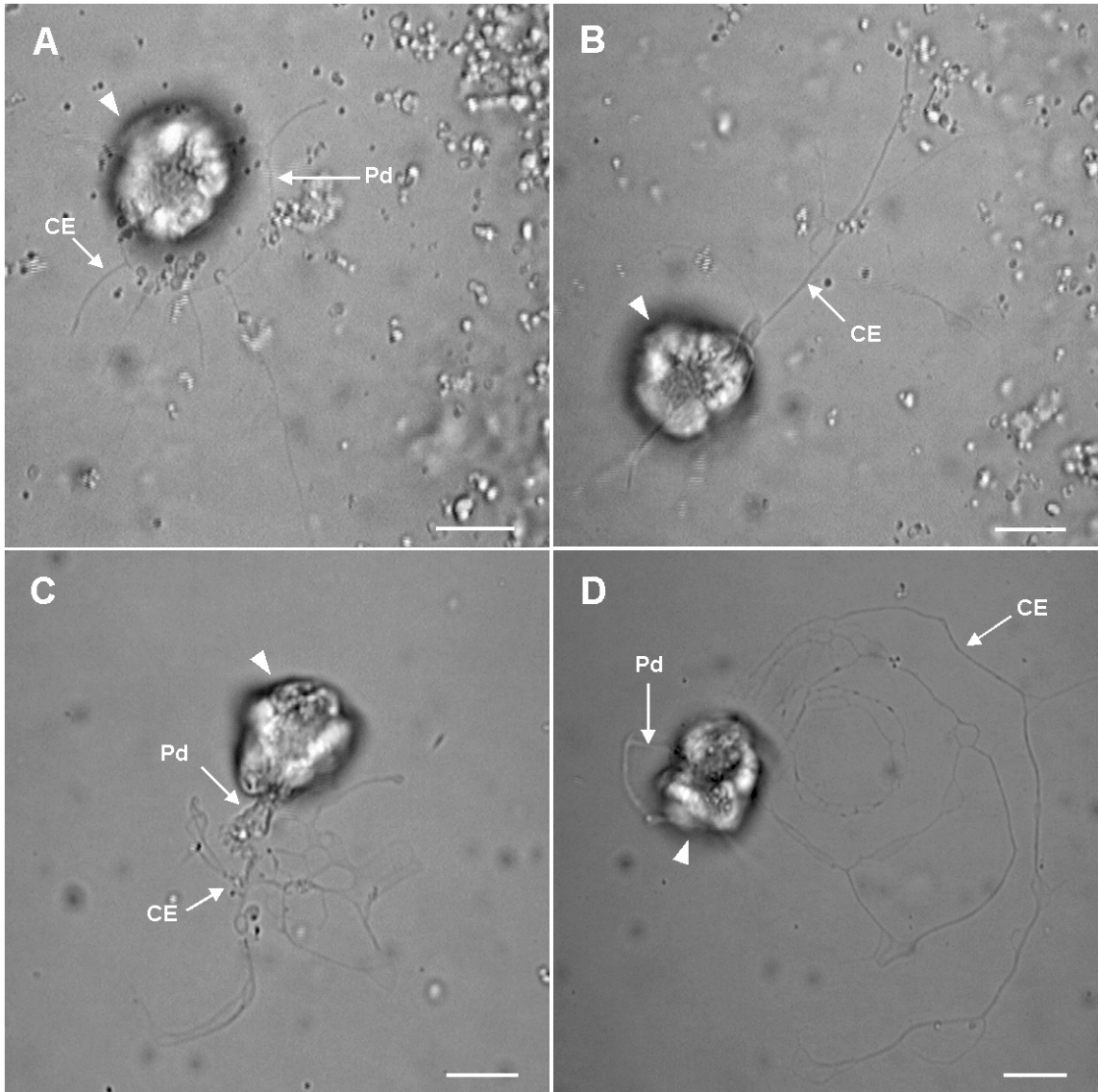
**A)** Flagellate cell (arrowhead) with peduncle (Pd) attached to the bottom of the culture dish. Cytoplasmic extensions (CE) are seen radiating from the margin of the peduncle.

**B)** Flagellate cell (arrowhead) with cytoplasmic extension (CE) trailing behind the cell.

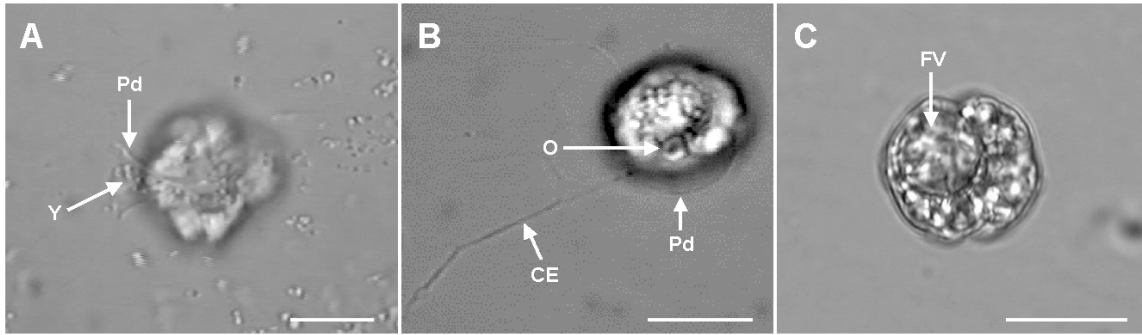
**C)** Flagellate cell (arrowhead) attempting to detach its peduncle (Pd) from the bottom of the culture dish. The cytoplasmic extensions (CE) adhered to the bottom of the culture dish.

**D)** Flagellate cell (arrowhead) moving across the bottom of the culture dish with the peduncle (Pd) attached. Note the cytoplasmic extensions (CE) that remained behind.

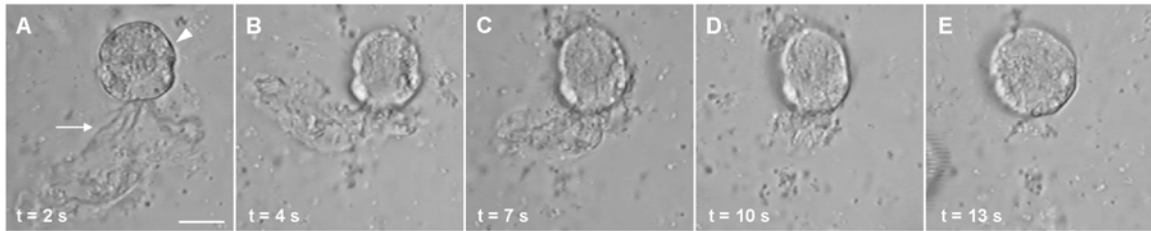
Scale bars = 10  $\mu\text{m}$ .



**Figure 2.4**



**Figure 2.5** Brightfield micrographs of *Pfiesteria shumwayae* cultured in untreated CYM. **A)** Flagellate cell ingesting small egg yolk particles (Y) through the peduncle (Pd). **B)** Flagellate cell with the peduncle (Pd) attached to the bottom of the culture dish. The entrance to the cell body (O) is visible, as well as a cytoplasmic extension (CE). **C)** Flagellate cell containing an apparent food vacuole (FV). Scale bars = 10  $\mu\text{m}$ .



**Figure 2.6** Sequential micrographs showing a time series of observations on a *Pfiesteria shumwayae* flagellate cell (arrowhead) ingesting a large, semi-solid yolk particle (arrow).

Scale bar = 10  $\mu\text{m}$ .

## 2.5 Discussion

*Axenic culture.* A culture medium containing chicken egg yolk supported the axenic growth of two *Pfiesteria shumwayae* strains (CCMP2357, CCMP2360). Chicken egg yolk is a common ingredient both in bacterial media (Atlas and Snyder 2006) and in media used for the xenic and axenic cultivation of certain protists (Boeck and Drbohlav 1925, Balamuth 1946, Chen 1950, Khouw et al. 1968, Zierdt and Williams 1974). *P. shumwayae*, however, is the first heterotrophic dinoflagellate to be cultured axenically on a chicken egg yolk medium.

Autoclaved and untreated CYM both supported the axenic growth of *Pfiesteria shumwayae*. Cell proliferation in the autoclaved medium suggested that the compounds required for growth were not heat-labile; however, the cultures were easier to maintain in the untreated CYM. High temperatures can alter the physical and chemical properties of egg yolk, resulting in changes in viscosity, solubility, and protein structure (Denmat et al. 1999). The lipids present in chicken egg yolk are bound to proteins, forming lipoproteins that comprise ~84% of the dry weight of egg yolk (Anton 1998). Untreated egg yolk is relatively resistant to lipid oxidation (Pike and Peng 1985), but autoclaving likely denatured a portion of the yolk proteins and increased the susceptibility of the yolk lipids to oxidation (Lea 1957, Pike and Peng 1988). Accumulation of lipid oxidation products (i.e. reactive oxygen species, secondary products) can damage cell membranes, inactivate enzymes, and inhibit DNA and protein synthesis (Frankel 1984, Esterbauer 1993).

Difficulties encountered in serial subculturing of axenic *Pfiesteria shumwayae* cultures were avoided if transfers were made prior to the stationary growth phase and if

media were freshly prepared. Cultures in stationary growth likely were limited by one or several metabolites, and cells may not have been able to recover once transferred to a complete medium. Droop (1959) reported similar difficulties in transferring axenic cultures of *Oxyrrhis marina* whose growth had been arrested by nutritional depletion and speculated that the difficulty was due to the lipid requirements. The gradual oxidation of yolk lipids during storage may explain why the CYM medium had to be prepared immediately preceding transfer of the axenic cultures. Storage of lipids, even under sterile conditions, can lead to autoxidation (Lea and Hawke 1951, Lea 1957). Further, metals catalyze lipid oxidation (Lea and Hawke 1951, Schaich 1992), and the trace metal solution added to the CYM may have accelerated the oxidation process. The addition of a chelator, such as citric acid or ethylenediaminetetraacetic acid (EDTA) to the CYM might allow the medium to be stored for longer periods.

The autoclaved and untreated CYM growth experiments were conducted using two different lots of the chicken egg yolk enrichment, and the concentrations of chicken egg yolk that supported *Pfiesteria shumwayae* growth were slightly different for each lot. The differences in acceptable yolk concentration were likely due to slight variations in the chemical composition of the egg yolk received from the manufacturer. Changes in the axenic growth of protists have often been observed when complex animal products were obtained from different manufacturers and even individual lots from the same distributor can be different (Balamuth 1946, Diamond et al. 1978, 1995, Clark and Diamond 2002). Further, many animal products generally are not produced for protist culture and are tested only for the ability to support bacterial or fungal growth (Clark and

Diamond 2002). Variation among lots of the chicken egg yolk enrichment is not surprising as the biochemical composition of chicken egg yolk can vary depending on the age, diet, and breed of the hen (Stadelman and Pratt 1989). For the described culturing method, new lots of chicken egg yolk should be tested over a range of concentrations for the ability to support *P. shumwayae* growth.

The maximum cell yields attained for both *Pfiesteria shumwayae* strains cultured in autoclaved CYM and for strain CCMP2357 cultured in untreated CYM were comparable to maximum densities reported when *P. shumwayae* was grown with living fish or microalgae (Vogelbein et al. 2001, Parrow et al. 2002) but were much lower than yields obtained when this species was cultured monoxenically on a fish cell line ( $1.4 - 2.8 \times 10^5$  cells ml<sup>-1</sup>; Parrow et al. 2005). Division rates for both *P. shumwayae* strains cultured in autoclaved CYM approached the division rates reported by Parrow et al. (2005) for two of three *P. shumwayae* strains cultured on a fish cell line, but division rates in the untreated CYM were lower. Cell yields and/or division rates often are lower when a protist is cultured axenically (Droop 1959, Finley and McLaughlin 1965, Curds and Cockburn 1968, McGinn 1971). Although the compounds present in the CYM met *P. shumwayae* nutritional requirements, the medium may not contain some metabolite(s) in adequate concentrations or ratios to support high cell yields such as those obtained when cultivated on a fish cell line. *P. shumwayae* division rates and cell yields also may be influenced by the physical parameters of the medium. The fish cell line consisted of layers of non-motile, adherent cells that were phagocytized directly, providing all or most of the nutrients required for *P. shumwayae* growth in a single, packaged form. The exact

mechanism(s) of nutrient acquisition in the axenic culture medium are unknown, but at least some nutrients likely were obtained by resorption or diffusion across the cell membrane, a process that may not be as energetically efficient as phagotrophy.

Intraspecific variation has been documented in both autotrophic and heterotrophic protists (Wood and Leatham 1992, Burkholder and Glibert 2006). *Pfiesteria shumwayae* strain CCMP2089 could not be cultured on any of the axenic culture media tested although all three strains were successfully cultured monoxenically on a fish cell line (Parrow et al. 2005). Similarly, Provasoli and Gold (1962) reported differences in axenic growth of different strains of the heterotrophic dinoflagellate *Cryptocodinium cohnii*. When added to the CYM, the flagellate cells of strain CCMP2089 gradually encysted over several days, suggesting that the media lacked some biochemical compound(s) required for CCMP2089 proliferation. Alternatively, the CYM may contain all the metabolites required for CCMP2089 growth, but not at adequate concentrations or ratios. It also is possible that one or several compounds were present in the medium at concentrations that were inhibitory or toxic to CCMP2089. Chicken egg yolk obtained from a different distributor or chicken egg yolk concentrations other than those tested in this research may support the axenic growth of this strain.

The nutritional requirements or uptake processes of *Pfiesteria shumwayae* apparently are different from those of the other heterotrophic dinoflagellates that have been cultured axenically. Culture media used for the axenic cultivation of other heterotrophic dinoflagellates (*Cryptocodinium cohnii*, *Gyrodinium lebouriae*, *Noctiluca scintillans*, *Oxyrrhis marina*) did not support *P. shumwayae* growth. Egg yolk contains

approximately 32% lipid, 16% proteins, and 1% carbohydrates (Romanoff and Romanoff 1949, Li-Chan et al. 1995), and the axenic growth of *P. shumwayae* in a lipid-rich medium suggests that this dinoflagellate may have one or more lipid requirements. Several protists (e.g. *Uronema* sp., *Paramecium* spp. *Tetrahymena* spp.) are known to have lipid requirements, typically in the form of fatty acids and/or sterols (Conner and van Wagtenonk 1955, Miller and Johnson 1960, Holz et al. 1961, 1962, Soldo and van Wagtenonk 1967, Hanna and Lilly 1974). In dinoflagellates, lipid requirements are known thus far only for *Oxyrrhis marina*, which required a quinone (Droop and Doyle 1966, Droop and Pennock 1971). Certain sterols, including cholesterol, were stimulatory but not essential for *O. marina* growth (Droop and Pennock 1971). *O. marina*, however, could not be cultured on chicken egg yolk (Droop 1970). The nutritional requirements of most heterotrophic dinoflagellates are not known, and many species may require one or more lipids.

*Importance of particles.* The sterile-filtered CYM did not support the growth of these *P. shumwayae* strains at any of the concentrations tested. The filtration process removed egg yolk particles from the medium that apparently are required for *P. shumwayae* growth, at least in this culture system. The particles present in the untreated and autoclaved CYM likely induced phagotrophy and digestive vacuole formation, as flagellate cells in the untreated CYM frequently ingested the yolk particles, and cells in both the untreated and autoclaved CYM contained apparent food vacuoles. The presence or addition of particulate matter to media used for the axenic culture of other protists has been reported to stimulate phagotrophy and increase growth (Seaman 1961, Rasmussen

and Kludt 1970, Holst-Sørensen and Rasmussen 1971). The ciliate *Tetrahymena pyriformis* (Ehrenberg) Lwoff multiplied rapidly in an autoclaved peptone medium containing particles, but grew poorly if the particles were removed by sterile filtration (Rasmussen and Kludt 1970). The addition of various particulates to the sterile filtered *T. pyriformis* medium significantly increased division rates and cell yields (Rasmussen and Kludt 1970, Rasmussen and Modeweg-Hansen 1973). Similarly, the addition of particles to both complex and semi-defined media increased vacuole formation and enhanced the growth of *Paramecium caudatum* Ehrenberg (Reilly 1964). Of the other heterotrophic dinoflagellates that have been cultured axenically, only *Noctiluca scintillans* required particulate matter (McGinn 1971). *Noctiluca* cultures could not be maintained in axenic culture if particles were omitted from the defined medium or removed by filtration prior to autoclaving (McGinn 1971).

Induction of phagotrophy in the protists examined thus far can be achieved by the addition of inert particles that do not have any nutritional value (Reilly, 1964, Rasmussen and Kludt 1970, McGinn 1971, Rasmussen and Modeweg-Hansen 1973). The ingestion of these particles, however, allows the concurrent uptake of soluble substrates present in the medium, providing dissolved nutrients to the cell that may not be effectively transported across the cell membrane. Further, the formation of digestive vacuoles may be necessary for catabolism of certain macromolecules. Provosoli and Shiraishi (1959) hypothesized that the brine shrimp *Artemia salina* L. ingested soluble micronutrients while phagocytizing particles in an axenic culture medium. Similarly, the increase in cell yields of *Tetrahymena pyriformis* when particulates were added to the medium was

thought to be due to simultaneous uptake of dissolved nutrients along with particulate matter (Ricketts 1972). *Pfiesteria shumwayae* flagellate cells cultured in the untreated CYM ingested the surrounding liquid while phagocytizing small yolk granules and likely obtained at least some soluble dissolved nutrients in this manner.

*Pfiesteria shumwayae* flagellate cells became noticeably smaller in size over time when cultured in the sterile-filtered CYM and could not be maintained long-term in the medium, suggesting that some compound(s) present in the removed yolk particles was needed for growth or to stimulate phagotrophic uptake. Yolk granules are a major component of egg yolk and contain approximately 10% and 50% of the total yolk lipids and proteins, respectively (Burley and Cook 1961, Causeret et al. 1991). The brine shrimp *Artemia salina* could use soluble micronutrients but also needed particles to supply bulk nutrients (Provasoli and D'Agostino 1969). Although *P. shumwayae* likely obtained some required or stimulatory nutrients from the egg yolk particles, the particles were not all consumed, suggesting that growth in this culture system was limited by one or more solutes in the medium.

Phagotrophy may be necessary for growth of *Pfiesteria shumwayae* in axenic culture and should be considered when developing a chemically defined medium to examine the nutritional requirements of this species. *Pfiesteria* spp. are considered obligate phagotrophs in nature; however, the resorptive capabilities (“osmotrophy”) of these and other heterotrophic dinoflagellates are not well known (Schnepf and Elbrächter 1992). *Pfiesteria* spp. reportedly can take up dissolved inorganic and organic nitrogen compounds (Burkholder and Glasgow 1997, Lewitus et al. 1999, Glibert et al. 2006),

presumably by resorption, but it is not known if all of the biochemical compounds required for growth can be transported across the cell membrane. Knowledge of the biochemical compounds necessary for *P. shumwayae* growth would enable improved evaluation of the roles of resorption and phagotrophy in the transport of required nutrients into the cell.

*Feeding behavior.* The biphasic axenic culture medium allowed documentation of the feeding behavior of *Pfiesteria shumwayae* flagellate cells, including detailed observations of cytoplasmic extensions (pseudopodia) associated with the feeding tube. Pseudopodia are common in several protist taxa, including members of the Amoebozoa, Cercozoa, Foraminifera, Heliozoa, Heterolobosa, and Heterokonta (e.g. Parsons 1926, Droop 1962, Pittam 1963, Arnold 1972, Perkins 1973, Patterson and Hausmann 1981, Travis and Allen 1981, Moestrup and Sengco 2001, Wylezich et al. 2007). In dinoflagellates, pseudopodia are best documented in heterotrophic species of the genera *Oblea*, *Protoperidinium* and *Zygabikodinium*. The pseudopod or “pallium” (Jacobson and Anderson 1986) in these dinoflagellates is used in feeding, attaching to and enveloping prey cells (Gaines and Taylor 1984, Jacobson and Anderson 1986). When feeding on diatoms with setae, such as *Chaetoceros* spp. Ehrenberg, slender filopodia radiate from the pseudopodial envelope and enclose the setae (Jacobson and Anderson 1986). Digestion in pallium-feeding dinoflagellates occurs in an extrasomal vacuole, and only digested material is transported into the cell (Gaines and Taylor 1984, Jacobson and Anderson 1986, Elbrächter 1991)

Pseudopodia also have been observed in dinoflagellates with a peduncle, primarily in species that are ectoparasites or commensals of fish and cnidarians (i.e. *Amyloodinium ocellatum* Brown et Hovasse, *Crepidodinium* spp. Lom et Lawler, *Oodinium limneticum* Chatton, *Protoodinium chattoni* Hovasse; Brown 1934, Nigrelli 1936, Jacobs 1946, Cachon and Cachon 1971, Lom and Lawler 1973, Lom et al. 1993). In these dinoflagellates, however, the pseudopodia emerge from the peduncle and appear to function primarily in attachment to the host species (Brown 1934, Nigrelli 1936, Jacobs 1946, Cachon and Cachon 1971, Lom and Lawler 1973, Lom et al. 1993). Unlike pallium-feeding dinoflagellates, the extensions do not act as an extracellular vacuole for digestion (Jacobson and Anderson 1986), and in phagotrophic species, digestion is thought to occur in intracellular food vacuoles (Cachon and Cachon 1971, Lom and Lawler 1973).

The pseudopodia observed in *Pfiesteria shumwayae* have been reported previously (Vogelbein et al. 2002); however, the present research is the first to provide more detailed observations of the extensions. The cytoplasmic extensions in *P. shumwayae* appear to be most similar to pseudopodia reported in *Amyloodinium ocellatum* (Brown 1934, Nigrelli 1936), a species that shares similar reproductive characteristics (Parrow and Burkholder 2003) and a close phylogenetic relationship with *Pfiesteria* spp. (Litaker et al. 1999). In *Amyloodinium*, the pseudopodia also project from the peduncle and can be extended and retracted (Brown 1934, Nigrelli 1936, Lom and Lawler 1973). The extensions are embedded into the gill tissue of the host fish, and the tips become enlarged and function as an anchor once inside the host cell membrane (Lom

and Lawler 1973). Although the pseudopodia in *Amyloodinium* are best documented in the trophont stage, they also are present in the motile flagellate cells (Landsberg et al. 1994). Like *A. ocellatum*, the pseudopodia observed in this study of *P. shumwayae* likely function in attachment of the dinoflagellate to food items.

Other heterotrophic dinoflagellates also have structures associated with the feeding tube that are thought to aid in attachment to prey. Drebes and Schnepf (1982) described a “crook” associated with the feeding tube of the ectoparasitic dinoflagellate, *Paulsenella* sp. Chatton and suggested that it was used for attachment and penetration of the host cells. The attachment structure in *Paulsenella* was non-cytoplasmic (Drebes and Schnepf 1982, Schnepf et al. 1985), and therefore not a pseudopod. Similarly, *Peridiniopsis berolinensis* (Lemmermann) Bourrelly has a “hook” associated with the feeding tube that maintains contact with the prey cells (Calado and Moestrup 1997). Observations of attachment structures in flagellate cells of *P. shumwayae* and other tube-feeding dinoflagellates suggest that these structures might be relatively common; however, documentation likely has been restricted due to methodological difficulties.

The feeding tube typically is thought to function in a specialized form of phagotrophy called myzocytosis, in which the tube attaches to and pierces the prey cell membrane (Schnepf and Deichgräber 1984). The cellular contents are ingested, leaving the cell membrane behind (Schnepf and Deichgräber 1984). Exceptions have been documented, however, and some dinoflagellates can ingest detrital particles (Spero 1982) and entire cells (e.g. *Peridiniopsis berolinensis*, Calado and Moestrup 1997; *Amphidinium lacustre* Stein, Calado et al. 1998) through the feeding tube. Although

*Pfiesteria* spp. can feed myzocytotically on various microalgae (Burkholder and Glasgow 1995, Glasgow et al. 2001), Parrow et al. (2005) noted that a visible cell membrane was not left behind when *P. shumwayae* fed on fish cells. Similarly, in the present research, *P. shumwayae* ingested particles that were not associated with prey cells. Myzocytosis *sensu* Schnepf and Deichgräber (1984) applies to organisms feeding on cells and specifies that the cell membrane of the prey is not ingested; therefore, this term should not be used to describe the *P. shumwayae* feeding behavior observed in axenic culture.

Relatively little information exists on the prey size spectra for dinoflagellates that feed using a peduncle. It is known that peduncle feeders can feed on prey several times larger than their own volume, but this typically involves feeding on injured organisms, such as ciliates, rotifers, and nematodes, in which only a small portion of the prey is ingested (e.g. Spero and Morée 1981, Calado and Moestrup 1997). *Dinophysis* spp. Ehrenberg, however, apparently can ingest entire cells of the prostomatid ciliate *Tiarina fusus* (Claparède et Lachmann) Bergh through a feeding tube (Hansen 1991). The lower prey size limit for tube-feeding dinoflagellates is thought to be 2 – 4  $\mu\text{m}$  (Hansen and Calado 1999), suggesting that these dinoflagellates cannot feed on bacteria-sized particles (0.2 - 1 $\mu\text{m}$ ). Some reports of dinoflagellates feeding on bacteria-size prey (e.g. Lessard and Swift 1985, Porter 1988, Nygaard and Tobisen 1993) have been questioned (i.e. Jakobsen and Hansen 1997, Hansen 1998), and the ability of dinoflagellates to ingest particles the size of heterotrophic bacteria is not well known. Phagocytosis of bacteria by *Pfiesteria* spp. has been reported (Burkholder and Glasgow 1997), but was not described or documented. This study documented *P. shumwayae* flagellate cells ingesting

individual particles and semi-solids ranging in size from a  $\sim 0.2 - 40 \mu\text{m}$ . The feeding behavior observed in the axenic cultivation system broadens the known size range of particles that tube-feeding dinoflagellates can ingest and may have implications for the types of particles these dinoflagellates consume in the natural environment (e.g. bacteria, non-living colloids, detritus). Whether or not *P. shumwayae* actually does ingest bacteria-sized particles in the natural environment has not been studied; however, this research suggests that the possible significance of this feeding behavior in the natural environment should be considered.

The feeding behavior observed in *Pfiesteria shumwayae* cells cultivated in the untreated CYM may have been an artifact of the culture system. Phospholipids comprise approximately 37% of the total lipids in the yolk granules and primarily contain phosphatidylcholine (82%) and phosphatidylethanolamine (15%) (Li-Chan et al. 1995). These are also the major phospholipids in eukaryotic cell plasma membranes (Dowhan 1997). The *P. shumwayae* flagellate cells in the untreated CYM likely swam toward and attached to the bottom of the culture flask due to chemosensory attraction (Cancellieri et al. 2001) to the chicken egg yolk granules settled there. Thus, *P. shumwayae* may respond to chemical cues from egg yolk particles that mimic a eukaryotic cell membrane. *P. shumwayae* flagellate cells were not observed attached to the bottom of the culture flask in the autoclaved CYM. In that medium, the autoclaving process caused coagulation of the yolk granules into much larger particles that were not finely distributed on the culture flask bottom, and *P. shumwayae* cells attached to the large yolk particles. Similarly, *P. shumwayae* has not been observed attached to the bottom of the culture

flask when cultured with microalgae, even in the presence of bacteria and detrital particles (H. Skelton, *pers. obs.*)

Although the documented feeding behavior may be a culture artifact, the observations of *Pfiesteria shumwayae* feeding on egg yolk particles are of value, primarily because they support the premise that particulate components of the medium are required for growth, either by supplying nutrients or by inducing uptake of medium through the peduncle and possibly vacuole formation required for metabolism of dissolved compounds. Furthermore, this culturing system provided an ideal method for observing the fine details of the feeding process and allowed imaging by light and video microscopy of peduncle attachment and ingestion of bacteria-sized particles. The culture system also allowed detailed observations of the opening into the cell body through which the particles entered and may be useful in determining the mechanism by which particles are transported through the feeding tube into the cell.

The mechanism of particle ingestion in tube-feeding dinoflagellates is unknown and remains speculative. Spero (1982) suggested that membrane movements in conjunction with the microtubular system are responsible for particle movement into the cell, and suction is not involved. Evidence of membrane flow was not found, however, in the feeding tubes of *Paulsenella* sp. or *Peridiniopsis berolinensis* (Calado and Moestrup 1997, Schnepf et al. 1985), and the microtubules associated with the feeding tube likely have a cytoskeletal function (Schnepf et al. 1985). Alternatively, Schnepf et al. (1985) and Calado and Moestrup (1997) both proposed suction as the driving force for particle ingestion, but suggested two different mechanisms for generating the suction. The

feeding tube in *Paulsenella* sp. and *Peridiniopsis berolinensis* emerges from the cell through an electron-dense ring called the sphincter (Schnepf et al. 1985, Calado and Moestrup 1997). Schnepf et al. (1985) proposed a suction mechanism driven by hydrostatic pressure that is generated by ion pumping into a vesicle surrounding the internal part of the feeding tube and flagellar canals, along with coordinated contractions of the feeding tube sphincter and additional sphincters associated with the flagella. This hypothesis was questioned by Calado and Moestrup (1997), who suggested that mechanical generation of low pressure inside the food vacuole is responsible for the suction in *P. berolinensis*.

The feeding behavior of *Pfiesteria shumwayae* in the untreated CYM suggests that, as proposed for *Paulsenella* sp. and *Peridiniopsis berolinensis*, suction is involved in the transport of particles into the cell. The small, circular opening through which particles flowed into the cell appears to be analogous to the sphincter described in *Paulsenella* sp. and *P. berolinensis* (Schnepf et al. 1985, Calado and Moestrup 1997). Schnepf et al. (1985) speculated that the sphincter in *Paulsenella* sp. was contractile, and could restrict the flow of prey cytoplasm through the feeding tube and into and out of the food vacuole. Light microscope observations of the sphincter in *P. shumwayae* showed that this structure does contract during feeding, and this action likely controls the entry of fluid and particles into the vacuole. The processes regulating the contraction of the sphincter in dinoflagellates, however, are not known; in this study the exact mechanism of particle movement through the feeding tube could not be discerned and remains uncertain. The *P. shumwayae* axenic cultures may be of value in clarifying the details of

the feeding process through further examination of feeding by light microscopy in conjunction with an ultrastructural investigation of the feeding tube.

## 2.6 Conclusions

Few heterotrophic dinoflagellate species have been cultured axenically, preventing detailed examination of the nutritional requirements of these protists. The development of a complex culture medium that supports the axenic growth of two of the three tested *Pfiesteria shumwayae* strains represents an initial step toward a fully defined medium and subsequent determination of *P. shumwayae* biochemical requirements. Observations of *P. shumwayae* flagellate cells phagocytizing particles in the axenic medium suggests that phagotrophy may be obligate for growth of this species in culture and should be considered when developing a completely defined medium. Knowledge of *P. shumwayae* biochemical requirements will enable evaluation of how the concentrations and distributions of those compounds in the environment influence the abundance and distribution of this species.

The axenic culture medium or modifications of it may support the axenic growth of other heterotrophic dinoflagellates, particularly species that are closely related to *P. shumwayae*. The medium is inexpensive and easily prepared, making it a good method for the general maintenance of this dinoflagellate. Further, axenic cultures of *P. shumwayae* provide a dependable source of cells free from other living organisms, permitting physiological and biochemical research on this species that otherwise would be complicated by the presence of prey and/or bacteria.

## Acknowledgements

Funding support for this research was provided by the U.S. EPA Star program (Ecohab RD-83170401), the Park Foundation, and the North Carolina General Assembly. We thank Bradley Hollidge and Zachary Eyler for culturing assistance and the North Carolina State University Cellular and Molecular Imaging Facility for assistance converting the microscope videos into a digital format.

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3. AXENIC CULTURE OF THE HETEROTROPHIC DINOFLAGELLATE  
*PFIESTERIA SHUMWAYAE* IN A SEMI-DEFINED MEDIUM

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*Journal of Eukaryotic Microbiology* (submitted)

### 3.1 Abstract

A semi-defined, biphasic culture medium was developed that supported the axenic growth of three strains of the heterotrophic dinoflagellate *Pfiesteria shumwayae*. Maximum cell yields and division rates in the semi-defined medium ranged from  $1 \times 10^4$  -  $4 \times 10^5$  cell  $\cdot$  ml<sup>-1</sup> and 0.5 - 1.7 divisions  $\cdot$  day<sup>-1</sup>, respectively, and depended on the concentration of the major components in the medium as well as the *P. shumwayae* strain. The medium contained high concentrations of certain dissolved and particulate organic compounds, including amino acids and lipids. *P. shumwayae* flagellate cells were attracted to insoluble lipids present in the medium and appeared to feed on the lipid particles, suggesting that phagocytosis may be required for growth in axenic culture. Development of a semi-defined medium represents significant progress toward a completely defined axenic culture medium and subsequent determination of the biochemical requirements of *P. shumwayae*, needed to advance understanding of the nutritional ecology of this species. Further, this medium provides an economical, simplified method for generating high cell densities of *Pfiesteria shumwayae* in axenic culture that will facilitate controlled investigations on the physiology and biochemistry of this heterotrophic dinoflagellate.

### 3.2 Introduction

Heterotrophic dinoflagellates are widespread and often abundant in aquatic ecosystems (Smetacek 1981, Lessard 1991, Jeong 1999), contributing to the transfer of organic matter in aquatic food webs and representing a link between primary production and metazooplankton. Knowledge of heterotrophic dinoflagellate nutrition is needed to better evaluate the role and impact of these organisms in microbial communities; however, the nutritional requirements of most species have not been examined at the biochemical level (Gaines and Elbrächter 1987, Guillard and Morton 2003).

Most heterotrophic dinoflagellate species are phagotrophic (Schnepf and Elbrächter 1992), and nutritional studies are complicated by the need to culture these organisms with other living cells as a food source. Axenic cultivation, the growth of a single species in the absence of other living cells (Baker and Ferguson 1942, Droop 1959), allows the examination of metabolic processes in the absence of potentially confounding activities of other living organisms (Caron 1993). Specifically, axenic cultivation is the most direct approach for defining and quantifying the nutritional requirements of an organism in biochemical terms (Droop 1970). Once the biochemical requirements of phagotrophic dinoflagellates are known, it becomes possible to evaluate the relationships and interdependencies of these organisms with and on other members of the plankton community in biochemical terms (Droop 1970, Provasoli 1977).

Establishing successful axenic cultures of phagotrophic organisms depends on finding a chemical replacement for the living prey (Droop 1959). Monoxenic cultures, in which the phagotrophic organism is cultured with only one additional species as the food

source, usually are established first (Droop 1959, 1970). If the living food is easily eliminated, monoxenic cultures provide a continuous supply of axenic organisms that can be inoculated into various artificial media (Droop 1970, Provasoli 1977). The initial axenic medium often is comprised of chemically undefined animal products and/or a wide array of organic compounds, many of which may not be required for growth. The complex medium, however, is a starting point for identifying the active constituents of the medium, which usually is accomplished by stepwise substitution of natural materials for partially defined mixtures and commonly required compounds such as vitamins, metals, and known carbon sources (Provasoli and Pintner 1953, Droop 1970). Once each component of the medium is completely defined chemically, biochemical requirements can be identified by evaluating growth following the omission of individual compounds.

Despite the prevalence and recognized importance of heterotrophic dinoflagellates in aquatic ecosystems, only five species, *Cryptocodinium cohnii* (Seligo) Chatton, *Gyrodinium lebouriae* Herdman, *Noctiluca scintillans* (Macartney) Kofoid et Swezy, *Oxyrrhis marina* Dujardin, and *Pfiesteria shumwayae* Glasgow et Burkholder (Marshall et al. 2006) have been successfully cultured axenically (Pringsheim 1956, Droop 1959, Provasoli and Gold 1962, Gold and Baren 1966, McGinn and Gold 1969, McGinn 1971, Lee 1977, Skelton et al. submitted). *Pfiesteria shumwayae* is a heterotrophic dinoflagellate commonly found in temperate estuarine and coastal waters (Ruble et al. 2005). *P. shumwayae* is phagotrophic and uses a feeding tube (peduncle; Elbrächter 1991) to ingest other protists and fish cells (Glasgow et al. 2001; Parrow et al. 2005). Research on the biology and ecology of this dinoflagellate, in part, has been facilitated by

the ability to maintain long-term xenic cultures with microalgae or fish as a food source. The nutritional requirements of *P. shumwayae* however, have not been examined at the biochemical level due to the lack of axenic cultures. Recently *P. shumwayae* was cultured monoxenically on fish cells (Parrow et al. 2005) and then axenically in a biphasic culture medium containing chicken egg yolk as a major component (Skelton et al. submitted). The present research describes the development of a semi-defined culture medium that supports the axenic growth of three *P. shumwayae* strains, representing significant progress toward a completely defined medium and determination of the biochemical requirements of this heterotrophic dinoflagellate.

### 3.3 Materials and Methods

*Cultures.* The three strains of *Pfiesteria shumwayae* used in this research are listed in Table 3.1. Stock dinoflagellate cultures were maintained in axenic culture in a chicken egg yolk medium (CCMP2357, CCMP2360; Skelton et al. submitted) or in monoxenic culture on a Chinook salmon (*Oncorhynchus tshawytscha* Walbum) fish cell line (CCMP2359, Parrow et al. 2005). All culture manipulations were made under a positive-pressure laminar-flow hood using aseptic techniques (Guillard and Morton 2003).

*Preparation of the semi-defined medium.* The semi-defined axenic culture medium (PSD-IO; Table 3.2) consisted of 10 salinity Instant Ocean (75%, v/v; Aquarium Systems), a commercially available tissue culture medium, Medium 199 (25%, v/v; Sigma-Aldrich, St Louis, MO, USA, #M3769), ampicillin (0.5 g · L<sup>-1</sup>; Sigma #A2427), soy lecithin (0.5 g · L<sup>-1</sup>; MP Biomedicals, Solon, OH, USA, #102147), L1 trace metals and 2 x L1 vitamins (Guillard and Hargraves 1993). The Instant Ocean solution, ampicillin, and soy lecithin were combined and autoclaved, and the remaining components were added to the autoclaved medium from sterile-filtered (0.22 µm pore size) stock solutions. Fresh medium was made prior to use.

*Establishment of Pfiesteria shumwayae cultures in the semi-defined medium.* The PSD-IO medium (10 ml) was added to sterile 25 cm<sup>2</sup> polystyrene culture flasks (Greiner Bio-One, Monroe, NC, USA, #690160), and dinoflagellate cells (~1.0 x 10<sup>5</sup> cells) were added from axenic or monoxenic cultures in the logarithmic phase of growth. Cultures were incubated in darkness at 21 °C and were examined daily using an inverted

microscope (Olympus CK40; Olympus, Melville, NY, 40x - 200x). Cultures in which dinoflagellate growth occurred were subcultured every 7 days. Detailed examinations of the axenic cultures were made using an Olympus AX-70 light microscope (200x - 600x), and photomicrographs were captured with a DEI-750 cooled-chip CCD camera (Optronics Engineering, Goleta, CA).

**Table 3.1** *Pfiesteria shumwayae* strains, source locations, and collection dates (CCMP, Provasoli-Guillard Center for Culture of Marine Phytoplankton)

Strain designation	Source location	Collection date
CCMP2357	Carolina Pines, Neuse River, NC, USA	July 1998
CCMP2359	Marshall Creek, Chesapeake Bay, MD, USA	August 2000
CCMP2360	Tasman Bay, New Zealand	April 2000

*Medium modifications.* Adjustments were made to the initial semi-defined medium formulation (PSD-IO) to improve and/or further define the medium (Table 3.2). The 10 salinity Instant Ocean was replaced with the defined seawater bases SM-10 (PSD-SM) or AK (PSD-AK) (Oestmann and Lewis 1996, Guillard and Morton 2003, Table 3.2, 3.3). Further, the major components (amicase, soy lecithin, L1 vitamins, L1 trace metals, Medium 199) of the PSD-AK medium were individually omitted to determine which, if any, were not required for growth of *Pfiesteria shumwayae*. Efforts also were made to replace the Medium 199 with glucose ( $0.25 \text{ g} \cdot \text{L}^{-1}$ ; Sigma G5767) and glycerophosphate ( $0.5 \text{ g} \cdot \text{L}^{-1}$ ; Sigma G6251) (PSD-GG, Table 3.2). Growth of the three *P. shumwayae*

strains was assessed in each modification.

*Medium storage.* Although attempts were made to store the prepared semi-defined medium for prolonged periods, preliminary experiments indicated that the medium had to be freshly prepared immediately prior to each transfer. In an effort to increase the storage capacity of the medium, the chelator citric acid ( $40 \text{ mg} \cdot \text{L}^{-1}$ ) was added to the PSD-AK medium prior to autoclaving (PSD-CA, Table 3.2). Growth of the three *Pfiesteria shumwayae* strains was evaluated in this medium modification both after fresh preparation and after storage in darkness at  $4 \text{ }^{\circ}\text{C}$  for up to two months.

*Sterility tests.* *Pfiesteria shumwayae* strains that were successfully cultivated in the semi-defined axenic culture media were tested for evidence of microbial contamination by culture in liquid broth and solid agar. A liquid sterility test medium comprised of 10 salinity L1 medium supplemented with bactopectone ( $0.5 \text{ g} \cdot \text{L}^{-1}$ ), yeast extract ( $0.5 \text{ g} \cdot \text{L}^{-1}$ ), and glucose ( $0.5 \text{ g} \cdot \text{L}^{-1}$ ) was used as a general test for bacteria and fungi. An additional liquid sterility medium of 10 salinity L1 medium enriched with bactopectone ( $1.0 \text{ g} \cdot \text{L}^{-1}$ ) and methylamine  $\cdot$  HCl ( $1.0 \text{ g} \cdot \text{L}^{-1}$ ) was used to test for methylaminotrophic bacteria (Guillard and Morton 2003). Solid medium contained 0.5x marine agar composed of Difco 2216-enriched agar ( $27.6 \text{ g} \cdot \text{L}^{-1}$ ), agar ( $7.5 \text{ g} \cdot \text{L}^{-1}$ ), bactopectone ( $5 \text{ g} \cdot \text{L}^{-1}$ ), and yeast extract ( $1 \text{ g} \cdot \text{L}^{-1}$ ) in deionized water. Duplicate samples (0.2 ml) from late logarithmic growth phase cultures were inoculated into 20 ml broth tubes and onto agar plates. Samples from xenic stock *P. shumwayae* cultures containing bacteria were inoculated into test media as positive controls. Un-inoculated test media served as negative controls.

**Table 3.2** Semi-defined medium formulations tested for the ability to support axenic *Pfiesteria shumwayae* growth.

Component	PSD-IO	PSD-SM	PSD-AK	PSD-CA	PSD-GG
10 salinity Instant Ocean (75% v/v)	+	-	-	-	-
AK seawater base (75% v/v)	-	-	+	+	+
SM-10 base (75% v/v)	-	+	-	-	-
Medium 199 (25% v/v) <sup>a</sup>	+	+	+	+	-
Amicase (0.5 g · L <sup>-1</sup> )	+	+	+	+	+
Soy lecithin (0.5 g · L <sup>-1</sup> )	+	+	+	+	+
L1 vitamins (1 ml · L <sup>-1</sup> ) <sup>a</sup>	+	+	+	+	+
L1 trace metal solution (1 ml · L <sup>-1</sup> ) <sup>a</sup>	+	+	+	+	+
Glucose (0.25 g · L <sup>-1</sup> ) <sup>a</sup>	-	-	-	-	+
Glycerophosphate (0.5 g · L <sup>-1</sup> ) <sup>a</sup>	-	-	-	-	+
Citric acid (40 mg · L <sup>-1</sup> )	-	-	-	+	-

<sup>a</sup> Added after autoclaving from sterile-filtered stock solutions

**Table 3.3** Composition of the synthetic seawater bases, SM-10 and AK.

Component (per L)	SM-10 <sup>a</sup>	AK <sup>b</sup>
NaCl (g)	8.10	8.0
MgSO <sub>4</sub> · 7H <sub>2</sub> O (g)	1.62	1.67
MgCl <sub>2</sub> · 6H <sub>2</sub> O (g)	1.16	1.0
CaCl <sub>2</sub> · 2H <sub>2</sub> O (g)	0.39	0.13
KCl (g)	0.26	0.2
NaHCO <sub>3</sub> (g)	0.13	0.07
Na <sub>2</sub> HPO <sub>4</sub> · 7H <sub>2</sub> O (g)	0.023	-
KH <sub>2</sub> PO <sub>4</sub> (g)	0.015	-
NaBr (mg)	-	0.34
H <sub>3</sub> BO <sub>3</sub> (mg)	-	2.0
SrCl <sub>2</sub> · 6H <sub>2</sub> O (mg)	-	8.87
NaF (mg)	-	0.14
KI (μg)	-	11.0

<sup>a</sup> Modified from Oestmann and Lewis (1996).

<sup>b</sup> Modified from Guillard and Morton (2003) to attain a 10 salinity seawater solution.

Sterility media were incubated in darkness at 21 °C and examined daily for 4 weeks for visible signs of contaminant growth. Sterility tests were repeated at 3-month intervals.

Axenic *Pfiesteria shumwayae* cultures also were examined for the presence of bacteria using epifluorescence microscopy after staining with the nucleic acid stain, SYTOX Green (Invitrogen, Carlsbad, CA, USA, #S7020). Samples (1 ml) from late logarithmic growth phase cultures were fixed with glutaraldehyde (0.5%, final concentration), stained with SYTOX Green (5 µM, final concentration) in darkness for 30 min, and filtered onto 25 mm GTBP black polycarbonate filters (0.22 µm pore size).

Each filter was placed on a microscope slide, covered by a drop of immersion oil (Cargille, Cedar Grove, NJ, USA, type FF) and a cover slip, and examined with an Olympus AX-70 microscope equipped with a 100 W mercury lamp, a triple-band pass filter set, and a 60x 1.2 N.A. water immersion objective. Samples from axenic stock *P. shumwayae* cultures containing bacteria were prepared identically and examined as positive controls.

*Dinoflagellate growth experiments.* Growth experiments were conducted on *Pfiesteria shumwayae* strains that were successfully cultured axenically for at least 2 months in the various modifications of the semi-defined medium. Experimental treatments consisted of media prepared with 2 different artificial seawater bases (PSD-IO, PSD-AK), each tested at 3 different concentrations of ampicillin and soy lecithin (0.25, 0.5, 1.0 g · L<sup>-1</sup>). In addition, *P. shumwayae* growth was measured in the PSD-AK medium (0.5 g · L<sup>-1</sup> ampicillin, lecithin) containing different concentrations of Medium 199 (0%, 25%, 50%, 100%). Growth also was assessed in the PSD-GG medium and in the PSD-

CA medium both before and after storage at 4 °C for two months.

Dinoflagellate cells were inoculated into triplicate, sterile 75 cm<sup>2</sup> polystyrene culture flasks containing the experimental medium (40 ml) at an initial density of ca.  $5.0 \times 10^3$  cells · ml<sup>-1</sup>. Early experiments indicated that strain CCMP2360 did not grow in the semi-defined medium when inoculated at that initial density, and therefore this strain was added at a density of ca.  $1.0 \times 10^4$  cells · ml<sup>-1</sup>. Cultures were gently mixed and aliquots (1 ml) removed every other day for 18 days. Samples were preserved with 2% (final concentration) acidic Lugol's solution, and cells were enumerated by light microscopy (100x) using a Palmer-Maloney counting chamber (Wetzel and Likens 1991). Population growth ( $k$ , divisions · day<sup>-1</sup>) was estimated over the exponential growth phase by computing the least-squares regression slope of semi-logarithmic daily cell count means (Guillard 1973). Depending on the comparison, either a one-way ANOVA or a Student's t-test assuming unequal variances was used to test for differences between treatments and strains in maximum cell yields ( $\alpha = 0.05$ ).

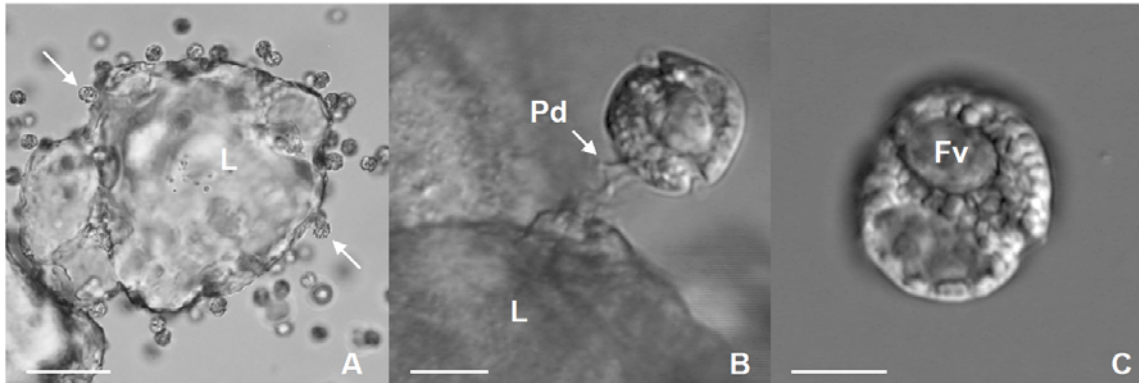
### 3.4 Results

*Media formulations.* Two of the three tested *Pfiesteria shumwayae* strains (CCMP2357, CCMP2359) could be cultivated in the PSD-IO medium. Despite repeated attempts, CCMP2360 was not cultured successfully in the semi-defined medium when Instant Ocean was used as the synthetic seawater base. All three *P. shumwayae* strains could be cultured in the PSD-AK medium, but none of the strains grew in the PSD-SM medium. When ampicillin, lecithin, L1 vitamins, L1 trace metals or Medium 199 was omitted from the PSD-AK medium, *P. shumwayae* growth (all strains) ceased within 1 - 3 transfers, depending on the component. Only one strain (CCMP2357) was successfully cultured in the PSD-GG medium. Addition of citric acid to the PSD-AK medium (PSD-CA) made it possible to store the medium in darkness at 4 °C for at least 2 months, and all three *P. shumwayae* strains were cultured in both freshly prepared and stored medium.

Soy lecithin is not water-soluble, and the semi-defined medium contained numerous lecithin particles ranging in size from ca. 3 µm - ≥ 1 mm in maximum dimension. *Pfiesteria shumwayae* flagellate cells frequently were observed aggregating around the particles (Fig. 3.1A), and individual cells extended the feeding tube and attached to the particle surface (Fig. 3.1B). Ingestion of the particles was not observed, but most cells contained apparent food vacuoles (Fig. 3.1C).

*Culture purity.* Bacterial and fungal contaminants were not detected in any axenic *Pfiesteria shumwayae* culture. Cultures tested in liquid and solid sterility test media showed no bacterial or fungal growth while contamination was apparent (cloudiness, colony formation) in the positive controls within 1 - 2 days. Axenic cultures that were

stained with SYTOX Green also showed no evidence of contamination. Positive controls contained fluorescent bacterial cells in every field of view. Axenic *P. shumwayae* cultures have remained free of demonstrable microbial contaminants since establishment.



**Figure 3.1** Brightfield micrographs of *Pfiesteria shumwayae* flagellate cells in the semi-defined medium. **A)** Flagellate cells aggregated around and attached (arrows) to a soy lecithin particle (L) in the medium. Scale bar = 50  $\mu\text{m}$ . **B)** Flagellate cell detaching the peduncle (Pd) from a soy lecithin particle (L). Scale bar = 10  $\mu\text{m}$ . **C)** Flagellate cell with a food vacuole (Fv). Scale bar = 10  $\mu\text{m}$ .

*Population growth.* *Pfiesteria shumwayae* strains (CCMP2357, CCMP2359) cultured in the PSD-IO medium attained maximum yields of  $3.4 \times 10^4$  -  $3.3 \times 10^5$  cells  $\cdot \text{ml}^{-1}$ , and division rates ranged from 0.47 - 1.67 divisions  $\cdot \text{day}^{-1}$  (Table 3.4 and Fig. 3.2). Population growth occurred in both strains at all three concentrations of amicase and lecithin tested ( $0.25 \text{ g} \cdot \text{L}^{-1}$ ,  $0.5 \text{ g} \cdot \text{L}^{-1}$ ,  $1.0 \text{ g} \cdot \text{L}^{-1}$ ), but growth of strain CCMP2359 in the

1.0 g · L<sup>-1</sup> treatment was poor and was not sustainable upon transfer (Fig. 3.2B). The concentration of amicase and lecithin in the medium significantly affected the maximum cell yields of both strains, with highest abundances in amicase and lecithin concentrations of 1.0 g · L<sup>-1</sup> and 0.5 g · L<sup>-1</sup>, respectively (Fig. 3.2A,B). Strain differences in maximum cell yield were significant at amicase and lecithin concentrations of 0.5 g · L<sup>-1</sup> and 1.0 g · L<sup>-1</sup>, but not at 0.25 g · L<sup>-1</sup>.

Maximum cell yields of *Pfiesteria shumwayae* strains cultured in the PSD-AK medium (CCMP2357, CCMP2359, CCMP2360) ranged from 1.2 x 10<sup>4</sup> - 3.9 x 10<sup>5</sup> cells · ml<sup>-1</sup> and division rates were 0.47 - 1.47 divisions · day<sup>-1</sup> (Table 3.4 and Fig. 3.3). Amicase and lecithin concentration significantly affected the maximum cell yield of all three strains. Strains CCMP2357 and CCMP2360 grew in the medium at all three amicase and lecithin concentrations (0.25 g · L<sup>-1</sup>, 0.5 g · L<sup>-1</sup>, 1.0 g · L<sup>-1</sup>), but for both strains the highest cell abundances were attained in the 1.0 g · L<sup>-1</sup> treatment (Fig. 3.3A,C). The cell yield of strain CCMP2359 was highest when the amicase and lecithin concentrations were 0.5 g · L<sup>-1</sup>, whereas growth was poor at 1.0 g · L<sup>-1</sup> (Fig. 3.3B). Maximum cell yields of strains CCMP2357 and CCMP2359 were not significantly different than those attained in the PSD-IO medium with the exception of CCMP2359 cultured at amicase and lecithin concentrations of 1.0 g · L<sup>-1</sup>. Cell abundances were not significantly different between strains in the 0.25 g · L<sup>-1</sup> and 0.5 g · L<sup>-1</sup>, treatments but were significantly different when the amicase and lecithin concentrations were 1.0 g · L<sup>-1</sup>, reflecting the poor growth of strain CCMP2359 at that concentration.

The concentration of Medium 199 in the PSD-AK medium significantly affected the maximum cell yields attained by all three *Pfiesteria shumwayae* strains (Fig. 3.4). Strains CCMP2357 and CCMP2359 exhibited some growth when Medium 199 was omitted from the semi-defined medium (0%; Fig. 3.4A,B). Strain CCMP2357 grew significantly better than strain CCMP2359 in the medium containing 0% Medium 199, but growth was not sustainable for either strain and ceased within 1 transfer. All *P. shumwayae* strains, however, grew in the PSD-AK media when Medium 199 concentrations were 25% or 50%, and maximum abundances ranged from  $1.8 - 2.3 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup> and  $2.3 - 3.5 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup>, respectively (Table 3.4 and Fig 3.4). Division rates were 0.9 - 1.0 divisions day<sup>-1</sup> (25% Medium 199) and 0.84 - 1.47 divisions day<sup>-1</sup> (50% Medium 199), depending on the strain (Table 3.4). All three *P. shumwayae* strains could not be cultured in the PSD-AK medium containing 100% Medium 199 (i.e. no AK; data not shown). The maximum cell yield of strains CCMP2357 and CCMP2359 cultured in the PSD-AK medium with 25% Medium 199 was significantly lower than the yield attained when the Medium 199 concentration was 50%. There was no significant difference in the maximum cell yield of CCMP2360 when cultured in the PSD-AK medium containing 25% or 50% Medium 199. Strains differed significantly in the maximum cell yield attained in the 50% Medium 199, and CCMP2357 and CCMP2359 reached higher densities than CCMP2360.

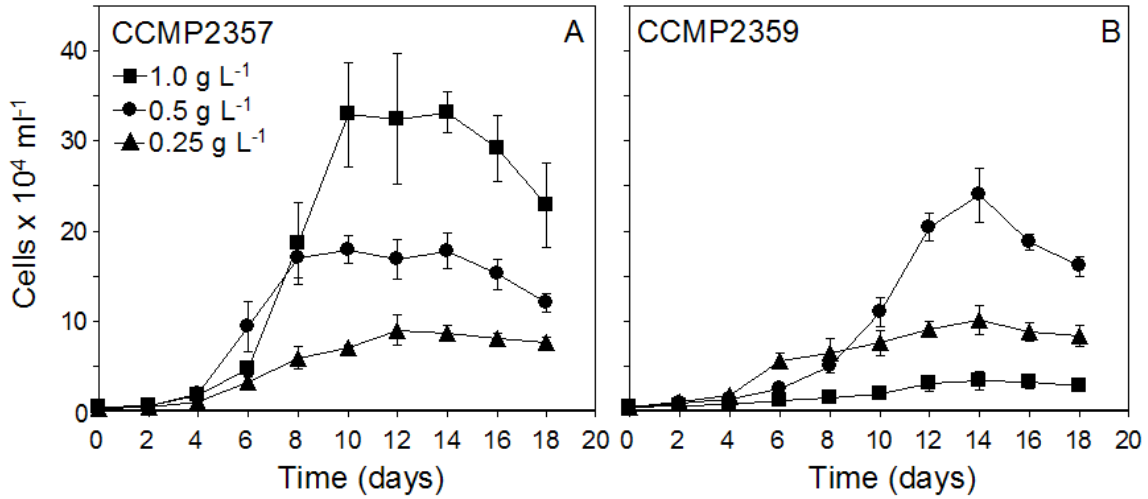
CCMP2357 was the only strain of *Pfiesteria shumwayae* that was cultured successfully in the PSD-GG medium, and the maximum yield attained was  $1.97 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup> (Fig. 3.5, Table 3.4). Division rates were 1.23 divisions  $\cdot$  day<sup>-1</sup> (Table 3.4).

Cell yields of CCMP2357 in the PSD-GG medium did not differ significantly from yields attained in the PSD-AK medium.

Maximum yields of the three *Pfiesteria shumwayae* strains cultured in the freshly prepared PSD-CA medium ranged from  $2.2 - 2.7 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup>, and division rates were 0.88 - 1.66 divisions  $\cdot$  day<sup>-1</sup> (Fig. 3.6, Table 3.4). Maximum cell yields reached after the PSD-CA medium was stored for 2 months ranged from  $1.95 - 2.1 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup>, and division rates were 0.6 - 1.55 divisions  $\cdot$  day<sup>-1</sup> (Fig. 3.6, Table 3.4). Storage of the PSD-CA medium had no significant effect on the maximum cell yield attained for any strain. Cell yields of the three strains also were not significantly different from those attained in the medium without the citric acid addition (PSD-AK). There were no significant differences in maximum cell yield between among the three strains cultured in that medium.

**Table 3.4** Maximum cell yields (cells ml<sup>-1</sup>) and division rates (*k*) for three *Pfiesteria shumwayae* strains cultured axenically in different formulations of the semi-defined medium. Standard deviation in parentheses.

Strain	CCMP2357		CCMP2359		CCMP2360	
Medium	Cells x 10 <sup>4</sup> ml <sup>-1</sup>	<i>k</i>	Cells x 10 <sup>4</sup> ml <sup>-1</sup>	<i>k</i>	Cells x 10 <sup>4</sup> ml <sup>-1</sup>	<i>k</i>
PSD-IO; 25% Med 199						
0.25 g · L <sup>-1</sup> amicase & lecithin	8.98 (1.68)	1.22 (0.05)	10.1 (1.62)	1.12 (0.20)	-	-
0.5 g · L <sup>-1</sup> amicase & lecithin	17.9 (1.57)	1.67 (0.05)	23.0 (2.97)	1.03 (0.10)	-	-
1.0 g · L <sup>-1</sup> amicase & lecithin	33.2 (2.28)	1.47 (0.05)	3.39 (1.02)	0.47 (0.08)	-	-
PSD-AK; 25% Med 199						
0.25 · g L <sup>-1</sup> amicase & lecithin	7.98 (1.37)	0.47 (0.02)	9.67 (1.31)	0.70 (0.03)	5.71 (1.36)	0.62 (0.19)
0.5 g · L <sup>-1</sup> amicase & lecithin	22.9 (4.10)	0.90 (0.07)	18.3 (4.93)	0.93 (0.06)	18.5 (1.94)	1.00 (0.07)
1.0 g · L <sup>-1</sup> amicase & lecithin	39.0 (5.58)	1.47 (0.12)	1.21 (0.18)	0.50 (0.11)	32.8 (2.88)	1.31 (0.13)
PSD-AK; 0% Med199	12.9 (0.65)	1.03 (0.02)	4.08 (0.79)	0.60 (0.02)	-	-
PSD-AK; 50% Med199	31.3 (3.78)	1.47 (0.20)	34.5 (2.69)	0.84 (0.03)	22.6 (3.87)	1.16 (0.15)
PSD-GG	19.7 (1.37)	1.23 (0.17)	-	-	-	-
PSD-CA; freshly prepared	26.8 (2.20)	1.66 (0.17)	21.6 (4.76)	0.88 (0.03)	22.5 (6.33)	1.24 (0.14)
PSD-CA; 2 month storage	20.7 (4.40)	1.55 (0.16)	18.8 (2.60)	0.60 (0.02)	18.5 (2.79)	0.92 (0.17)



**Figure 3.2** Time-course of cell densities from *Pfiesteria shumwayae* strains cultured in the PSD-IO medium with different concentrations of amicase and lecithin. 1.0 g · L<sup>-1</sup> (■), 0.5 g · L<sup>-1</sup> (●), 0.25 g · L<sup>-1</sup> (▲). Data points are replicate (n = 3) means ± SD. A) CCMP2357 B) CCMP2359.

**Figure 3.3** Time-course of cell densities from *Pfiesteria shumwayae* strains cultured in the PSD-AK medium with different concentrations of ampicillin and lecithin.  $1.0 \text{ g} \cdot \text{L}^{-1}$  (■),  $0.5 \text{ g} \cdot \text{L}^{-1}$  (●),  $0.25 \text{ g} \cdot \text{L}^{-1}$  (▲). Data points are replicate (n = 3) means  $\pm$  SD. A) CCMP2357 B) CCMP2359 C) CCMP2360.

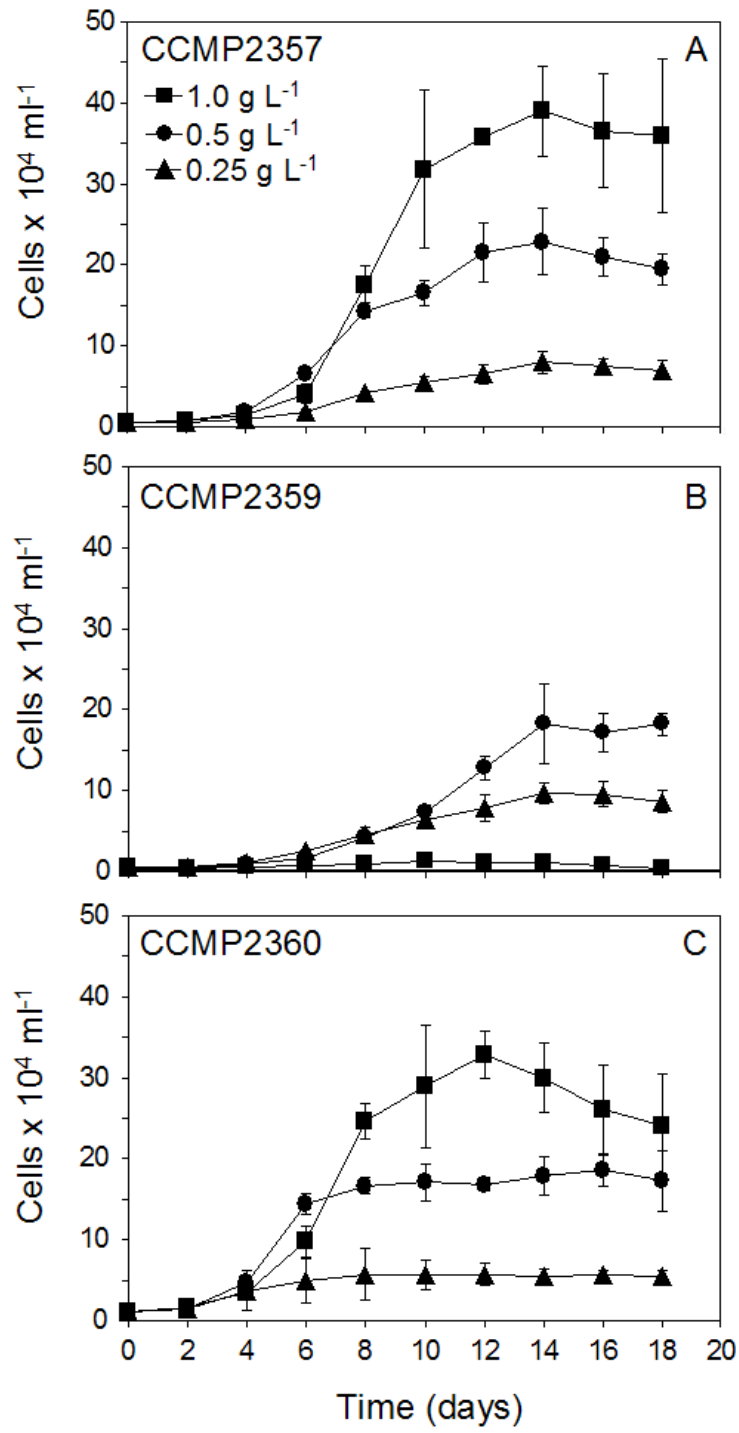





Figure 3.3

**Figure 3.4** Time-course of cell densities from *Pfiesteria shumwayae* strains cultured in the PSD-AK medium with different concentrations of Medium 199. 50% (  ), 0% (  ). Cell densities for *P. shumwayae* strains cultured in the PSD-AK medium with 25% Medium 199 (  ) are plotted from Fig. 5 for comparison. Data points are replicate (n = 3) means  $\pm$  SD. **A)** CCMP2357 **B)** CCMP2359 **C)** CCMP2360.

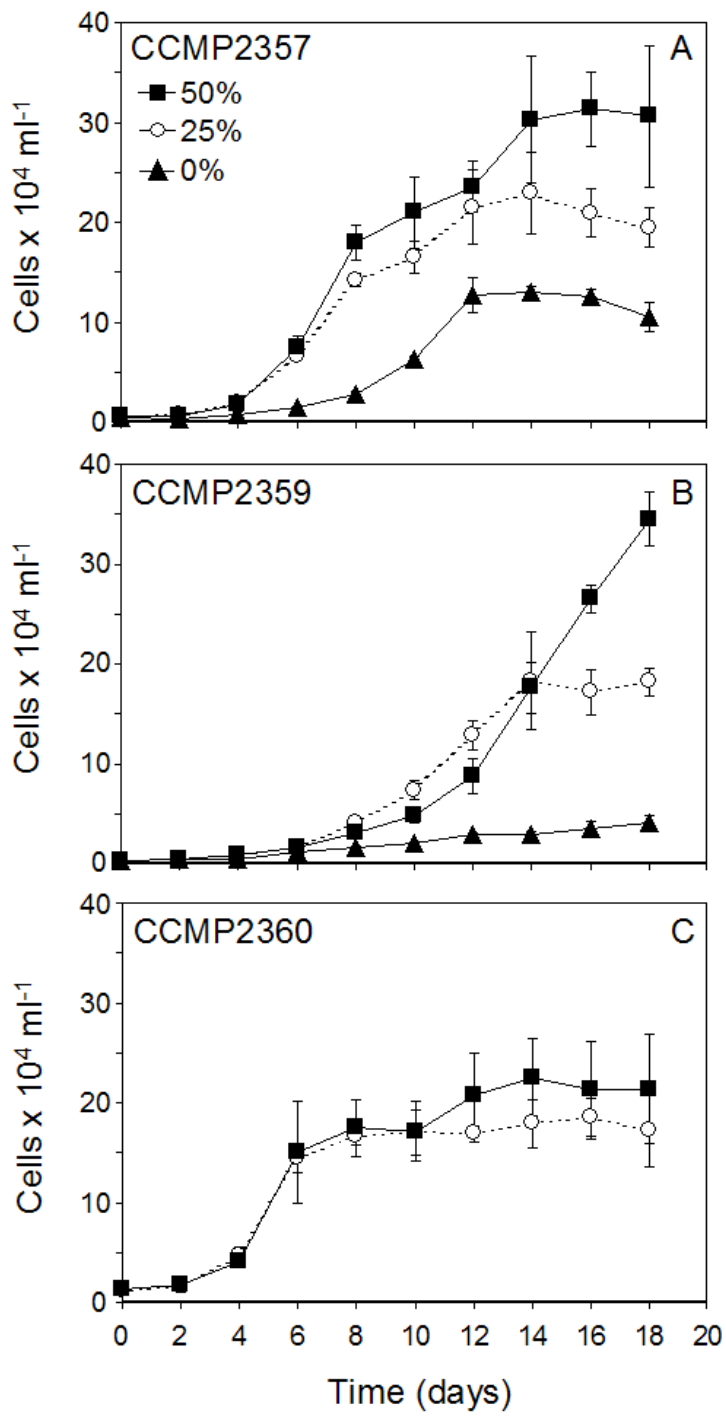
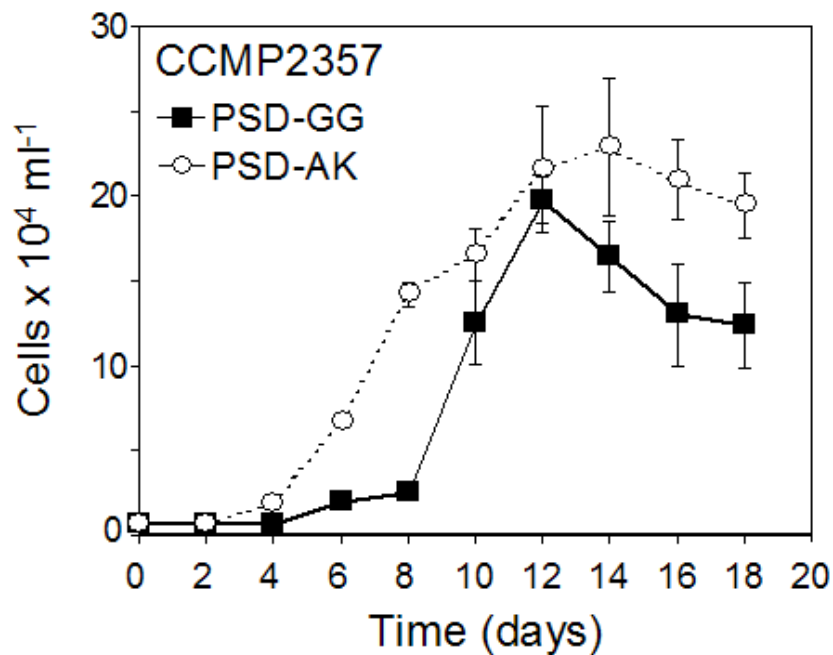


Figure 3.4



**Figure 3.5** Time-course of cell densities from *Pfiesteria shumwayae* strain CCMP2357 cultured in the PSD-GG medium (—■—). Cell densities for CCMP2357 cultured in the PSD-AK medium (—○—) are plotted from Fig. 5 for comparison. Data points are replicate (n = 3) means ± SD.

**Figure 3.6** Time-course of cell densities from *Pfiesteria shumwayae* strains cultured in the PSD-CA medium when freshly prepared (■) and after storage in darkness at 4 °C for 2 months (▲). Cell densities for *P. shumwayae* strains cultured in the PSD-AK medium (○) are plotted from Fig. 5 for comparison. Data points are replicate (n = 3) means ± SD. **A)** CCMP2357 **B)** CCMP2359 **C)** CCMP2360.

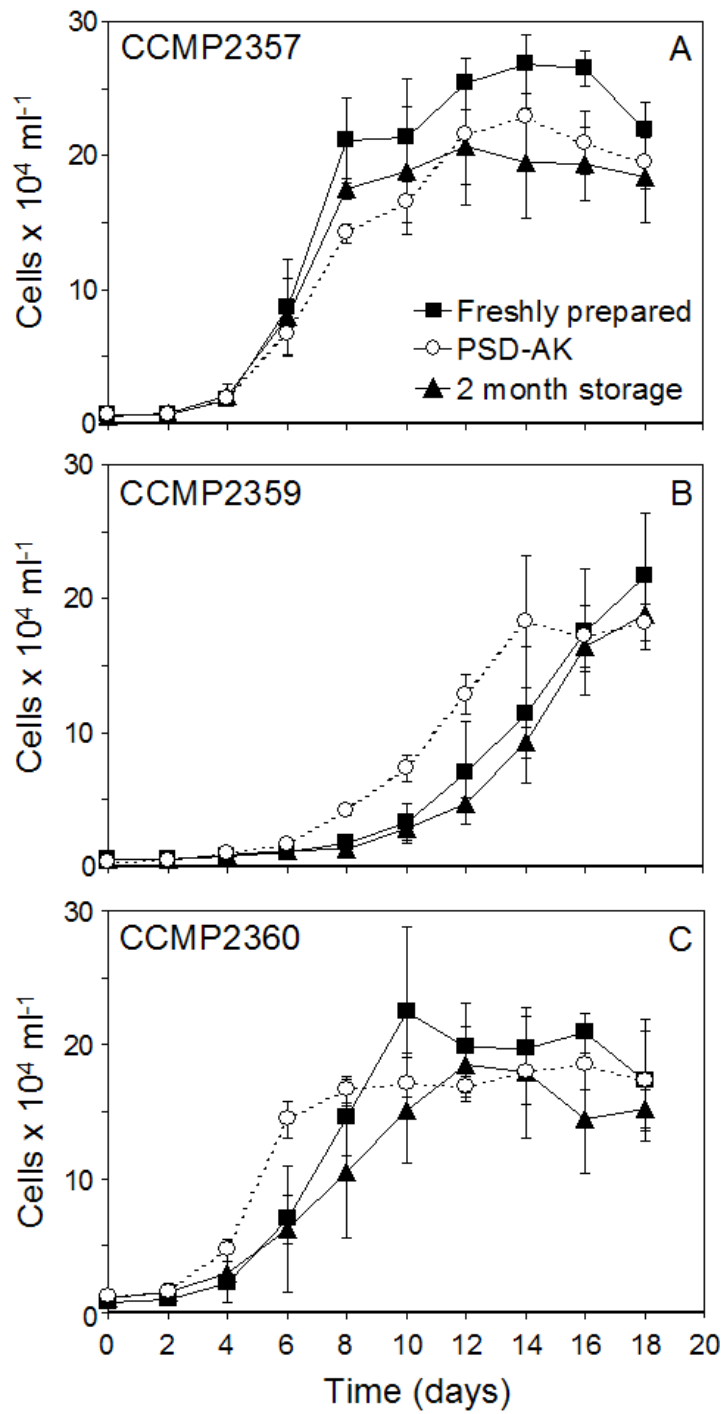


Figure 3.6

### 3.5 Discussion

*Media formulations and implications for nutritional requirements.* A semi-defined medium was formulated and subsequently modified to support the axenic growth of three strains of the heterotrophic dinoflagellate *Pfiesteria shumwayae*. The initial semi-defined medium (PSD-IO) only supported the growth of two strains of *P. shumwayae* (CCMP2357, CCMP2359); however, all three strains (CCMP2357, CCMP2359, CCMP2360) were cultured successfully in the medium when the Instant Ocean artificial seawater base was replaced with the defined base AK. It is unlikely that the Instant Ocean lacked compounds needed for CCMP2360 growth, as the chemical composition is similar to that of the synthetic AK base (Atkinson and Bingman 1998, Guillard and Morton 2003). It is possible, instead, that the Instant Ocean salts contained one or several contaminants that were inhibitory or toxic to CCMP2360 in the semi-defined medium. Concentrations of metals such as  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Pb}^{2+}$  are significantly higher in Instant Ocean than in natural seawater, likely as a result of industrial processing methods (Atkinson and Bingman 1998). However, in previous work strain CCMP2360 was maintained successfully in monoxenic culture on a fish cell line (Parrow et al. 2005) and axenically in a complex medium (Skelton et al. submitted) using Instant Ocean as the seawater base. The reason for this discrepancy is unknown and is difficult to adequately assess due to the complexity of both the monoxenic culturing system (i.e. presence of fish cells – Parrow et al. 2005) and the undefined components of the axenic medium (chicken egg yolk – Skelton et al. submitted). The metabolic activity of the fish cells present in the monoxenic cultures may have mitigated

the inhibitory effects of any trace contaminants present in the Instant Ocean salts. Similarly, the chicken egg yolk present in the complex axenic medium contains phosvitin, a protein that can form complexes with cations (Grizzuti and Perlmann 1975, Taborsky 1980), possibly reducing the negative impacts of contaminants in the Instant Ocean salts.

The PSD-SM medium failed to support the growth of any *Pfiesteria shumwayae* strain, suggesting that the SM-10 seawater base did not contain one or more compounds required for the growth of this dinoflagellate. The two defined seawater bases, SM-10 and AK are similar in chemical composition, but AK contains the additional constituents  $\text{Br}^-$ ,  $\text{B}(\text{OH})_3$ ,  $\text{Sr}^{2+}$ ,  $\text{F}^-$ , and  $\text{I}^-$ , which also are present in natural seawater (Pilson 1998). The lack of *P. shumwayae* growth in the absence of those ions/compounds suggests that one or more was needed for cell proliferation, at least when cultured axenically in the semi-defined medium. With the exception of Se (Harrison et al. 1988, Doblin et al. 2000) and certain metals (e.g. Fe, Cu, Zn, Mn; Brand et al. 1983, Peers et al. 2005), the possible importance of most trace elements to the growth of aquatic protists is not well known. Lewin (1966) reported a boron requirement for several diatom species, a prasinophyte, and one phototrophic dinoflagellate. Among the heterotrophic dinoflagellate species that have been examined for biochemical requirements, *Cryptothecodinium cohnii* and *Noctiluca scintillans* needed only the major ions present in seawater (Provasoli and Gold 1962, McGinn 1971, Tuttle and Loeblich 1975). Exact salt requirements were not examined for *Oxyrrhis marina* (Droop 1959) or *Gyrodinium lebouriae* (Lee 1977). A requirement for  $\text{Br}^-$ ,  $\text{B}(\text{OH})_3$ ,  $\text{Sr}^{2+}$ ,  $\text{F}^-$ , or  $\text{I}^-$  by *P. shumwayae*

would be the first such growth requirement reported for a heterotrophic dinoflagellate.

Evaluation of the major ingredients comprising the PSD-AK medium, as well as assessment of *Pfiesteria shumwayae* growth following the individual removal of those components, permitted additional insights into the possible biochemical requirements of this dinoflagellate. Nitrogen requirements in the semi-defined medium were met with organic nitrogen compounds, most likely amino acids. Amicase is a roughly defined casein acid hydrosalate from bovine milk and is comprised of 18 different amino acids, mostly in the form of free amino acids. The Medium 199 also contained those same 18 amino acids. In total, the PSD-AK medium contained amino acid concentrations of approximately  $0.48 - 1.14 \text{ g} \cdot \text{L}^{-1}$ , depending on the concentration of amicase ( $0.25 - 1.0 \text{ g} \cdot \text{L}^{-1}$ ) and Medium 199 (25 - 50%) in the medium. Medium 199 contained other nitrogen-containing compounds, including B vitamins and nitrogenous bases; however, the concentration of these compounds ranged from  $2.5 \mu\text{g} \cdot \text{L}^{-1} - 2.5 \text{ mg} \cdot \text{L}^{-1}$  in the culture medium and likely were too low to support high cell densities of *P. shumwayae*. Further, *P. shumwayae* did not grow when amicase was removed from the PSD-AK medium, indicating that amino acids were used to meet *P. shumwayae* nitrogen requirements, and that the concentration of amino acids in Medium 199 alone ( $0.25 \text{ g} \cdot \text{L}^{-1}$  at 25% Medium 199) was not high enough to support growth of this dinoflagellate.

The high concentration of amino acids in the culture medium strongly suggests that *Pfiesteria shumwayae* can take up (by resorption, endocytosis, or concurrent uptake of solutes during phagocytosis) and use nitrogen from amino acids for growth. Although studies using xenic cultures suggested that *P. shumwayae* also can take up radiolabeled

urea and inorganic nitrogen compounds (Glibert et al. 2006), it is not yet known whether those compounds can directly be used for growth. In general, most of the heterotrophic protists examined thus far have required nitrogen in the form of amino acids (e.g. *Oxyrrhis marina*, Droop 1959; *Paramecium aurelia* Ehrenberg, Soldo and van Wagtendonk 1969; *Noctiluca scintillans*, McGinn 1971, *Uronema marinum* Dujardin, Hanna and Lilly 1974), although the specific requirement(s) appear to be species-specific. Some protists, such as the heterotrophic dinoflagellates *Oxyrrhis marina* and *Cryptothecodinium cohnii* and the euglenoid *Peranema trichophorum* (Ehrenberg) Stein could only use certain amino acids (Droop 1959, Provasoli and Gold 1962, Allen et al. 1966) whereas other species apparently required many or all of the essential amino acids (e.g. *Glaucoma chattoni* Corliss, Holz et al. 1961c; *Paramecium aurelia*, Soldo and van Wagtendonk 1969; *Paraauronema acutum* Thompson, Soldo and Merlin 1977; *Tetrahymena setifera* Holz et Corliss, Holz et al. 1962). Interestingly, in several species (e.g. *C. cohnii*, *O. marina*, *N. scintillans*) axenic growth increased when multiple nitrogen sources were provided (Droop 1959, Gold and Baren 1962, McGinn 1971). It is possible that *P. shumwayae* required only one or a few of the amino acids present in the PSD-AK medium; however, the specific requirements (both compounds and concentrations) and the ability of this species to use various other nitrogen sources cannot be adequately evaluated until a completely defined medium is formulated.

Soy lecithin is an undefined mixture comprised mostly of phospholipids, including phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol (Hurst and Martin 1984). Lecithin also contains trace amounts of other lipids including

sterols (~5%; Van Nieuwenhuyzen 1976). The successful axenic culture of *Pfiesteria shumwayae* in a medium that contained soy lecithin as a major component indicates that this species may have one or more lipid requirements, likely in the form of fatty acids and/or sterols. The complex axenic culture medium formulated by Skelton et al. (submitted) also contained a lipid-rich component, chicken egg yolk, further suggesting a lipid requirement. Growth of the three *P. shumwayae* strains examined in this study ceased upon transfer into PSD-AK medium without lecithin, even though Medium 199 contained Tween 80 (Polyoxyethylenesorbitan monooleate), a complex lipid comprised mostly of the unsaturated fatty acid, oleic acid. It is possible that *P. shumwayae* could not use Tween 80 as a lipid source; alternatively, the concentration of Tween 80 in the medium ( $5 \text{ mg} \cdot \text{L}^{-1}$  at 25% Medium 199) was too low to support growth. The undefined nature of the soy lecithin makes it impossible to determine whether the speculated lipid requirement is for fatty acids, sterols, or both. Medium 199 also contained cholesterol, so the lack of *P. shumwayae* growth in the absence of lecithin might suggest a fatty acid requirement. It also is possible that the cholesterol contributed by the Medium 199 ( $50 \text{ } \mu\text{g} \cdot \text{L}^{-1}$  at 25% Medium 199) to the PSD-AK medium was too low to support growth, and the lecithin provided an additional source of sterols.

Fatty acid and sterol requirements have not yet been reported for any dinoflagellate species. A lipid requirement of any kind has been documented only for the heterotrophic species *Oxyrrhis marina*, which required a quinone (Droop and Pennock 1971). Several different sterols including cholesterol, stigmasterol, sitosterol, and ergosterol were stimulatory but not required for growth of *O. marina* (Droop and

Pennock 1971). Many heterotrophic protists including *Glaucoma chattoni*, *Hexamita inflata* Dujardin, *Miamiensis* spp., *Paramecium* spp., *Parauronema* spp., *Peranema trichophorum*, *Tetrahymena* spp., and *Uronema* spp. have been cultured axenically in media containing complex lipids (e.g. lecithin, asolectin, TEM-4T, Tween 80) and sterols (Holz et al. 1961a,b,c, Allen et al. 1966, Soldo et al. 1966, Khouw and McCurdy 1968, Soldo and Merlin 1972, 1977, Hanna and Lilly 1974, Allen and Nerad 1978, Keenan et al. 1978). Specific biochemical requirements for fatty acids and/or sterols were identified for several of those species (e.g. *Hexamita* spp., Khouw & McCurdy 1968, Biagini et al. 1998; *Paramecium* spp, Miller and Johnson 1960; Soldo and van Wagtenonk 1967; *Peranema trichophorum*, Allen et al. 1966; *Tetrahymena* spp., Holz et al. 1961a,b, 1962; *Uronema marinum*, Hanna and Lilly 1974), while lipids appeared to be stimulatory but not necessary for the growth of other species (*Glaucoma chattonii*, Holz et al. 1961c; *Parauronema acutum*, Soldo and Merlin 1977). Lipids may be required for or stimulatory to the growth of most phagotrophic protists.

Although undefined, the composition of lecithin is roughly known and permits insight about the fatty acids that may be required or stimulatory to *Pfiesteria shumwayae* growth, information that is essential when attempting to formulate a completely defined medium. The dominant fatty acid in soy lecithin is the unsaturated fatty acid, linoleic acid (18:2n-6; 60% of total fatty acids), but significant concentrations of palmitic (16:0; 18%), oleic (18:1n-9; 11%), and linolenic (18:3n-3; 9%) acid also are present (Schneider 1989). Similarly, the fatty acids in chicken egg yolk, a major component of the complex axenic culture medium formulated by Skelton et al. (submitted), are predominantly oleic

(40%), palmitic (22 -- 25%), and linoleic (14 - 20%) acid (Milinsk et al. 2003). Linolenic acid comprises only a small percentage of the total fatty acids in chicken egg yolk, while stearic acid (18:0) is more prevalent (8%) (Milinsk et al. 2003). The complex lipids in culture media used for the axenic culture of heterotrophic protists often have been replaced successfully with one or more fatty acids, including oleic, linoleic, linolenic, palmitic, and stearic acid (Miller and Johnson 1960, Soldo et al. 1966, Khouw and McCurdy 1968, Hanna and Lilly 1974). The fatty acid requirement of these heterotrophic protists usually was met with oleic or linoleic acid, although the addition of other fatty acids often proved stimulatory (Miller and Johnson 1960, Soldo et al. 1966, Khouw and McCurdy 1968, Hanna and Lilly 1974). The fatty acid profiles of lecithin and chicken egg yolk suggest that oleic and linoleic acid should be investigated further for the ability to support or stimulate *P. shumwaye* growth.

It also is possible that the lecithin in the semi-defined medium met not a biochemical requirement, but rather a feeding requirement. Chemical properties of the lecithin not only elicited a chemosensory response from flagellate cells of *Pfiesteria shumwayae*, but also induced extension of the feeding tube (peduncle) and subsequent phagotrophic feeding attempts. The phospholipids comprising soy lecithin also are the dominant lipids in eukaryotic cell membranes (Dowhan 1997), and the lecithin particles may have mimicked the living prey that *P. shumwayae* consumes in xenic culture and in the natural environment. The resorptive capabilities (“osmotrophy”) of *Pfiesteria shumwayae* and other heterotrophic dinoflagellates are not well known (Schnepf and Elbrächter 1992), and phagotrophy may be necessary for *P. shumwayae* cells to obtain

required compounds that cannot be transported across the cell membrane. The ingestion of particles in the semi-defined medium also could result in the simultaneous transport of dissolved nutrients into the cell, a mechanism that likely is more efficient than resorption. Provasoli and Shiraishi (1959) suggested that the brine shrimp *Artemia salina* L. obtained soluble micronutrients by phagocytosis of particles in the axenic culture medium. The lecithin particles may have been needed in the culture medium simply to induce phagotrophy. The heterotrophic dinoflagellate *Noctiluca scintillans* did not grow in axenic culture without the addition of inert particles to induce feeding (McGinn 1971). It has not yet been possible to induce feeding by *P. shumwayae* feeding on inert particles, suggesting that separating biochemical requirements from possible feeding requirements may prove difficult.

The Medium 199 contained all of the B vitamins except for B<sub>12</sub> (cobalamin); however, B<sub>12</sub> was supplied to the semi-defined medium by the L1 vitamin solution, which also contained vitamins B<sub>1</sub> (thiamine) and B<sub>7</sub> (biotin) (Guillard and Hargraves 1993). The lack of *Pfiesteria shumwayae* growth in the PSD-AK medium without the L1 vitamin solution added suggests that this dinoflagellate has a requirement for vitamin B<sub>12</sub>. A B<sub>12</sub> requirement is relatively common in heterotrophic and phototrophic protists (Droop 1954, 1955, Hutner and Provasoli 1964, Allen et al. 1966, Fulton et al. 1984). Among dinoflagellates, the heterotrophic species *Oxyrrhis marina* (Droop 1959) as well as several phototrophic species (e.g. *Glenodinium foliaceum*, *Scrippsiella trochoidea*, Droop 1958; *Peridiniopsis polonicum*, Holt and Pfister 1981) needed B<sub>12</sub> for growth. The removal of the L1 vitamins also may have resulted in insufficient concentrations of

thiamine and biotin in the PSD-AK medium for *P. shumwayae* growth. This seems unlikely, however, given the high concentrations of both vitamins in the Medium 199 (10  $\mu\text{g L}^{-1}$  at 25% Medium 199).

The dinoflagellate species studied thus far for vitamin requirements have only required B<sub>12</sub>, thiamine, or biotin, or some combination of the three vitamins (Droop 1959, Provasoli and Gold 1962, Provasoli and Carlucci 1974), but many heterotrophic protists need additional B vitamins (riboflavin, folic acid, pantothenate, nicotinamide, pyridoxine) for growth (Holz et al. 1961c, Allen et al. 1966, Hanna and Lilly 1974, Soldo and Merlin 1977). The growth of strain CCMP2357 in the PSD-GG medium indicated that at least this strain of *Pfiesteria shumwayae* does not have a requirement for those additional B vitamins. Future research likely can use the PSD-GG medium to evaluate the thiamine and biotin requirements of strain CCMP 2357 through omission experiments. Determination of specific vitamin requirements for the other two tested strains, however, first will require the ability to grow these strains in the semi-defined medium without Medium 199.

All three *Pfiesteria shumwayae* strains also did not grow when the L1 trace metal solution was removed from the PSD-AK medium, suggesting that this species required one or more trace metals. Requirements for various trace metals have been reported for several dinoflagellate species (Doucette and Harrison 1991, Doblin et al. 1999, Mitrovic et al. 2004). Among heterotrophic dinoflagellates Fe was required for growth of *Cryptothecodinium cohnii* (Provasoli and Gold 1962). In contrast, *Noctiluca scintillans* could not be depleted of trace metals in axenic culture (McGinn 1971). Trace metal

requirements were not examined for *Oxyrrhis marina* and *Gyrodinium lebouriae* (Droop 1959, Lee 1977). Trace metals requirements often are difficult to assess due to the presence of metals as contaminants of other compounds in the medium (Hanna and Lilly 1974, Tuttle and Loeblich 1975). The lack of *P. shumwayae* growth upon removal of the L1 trace metal solution suggests that it may be possible to evaluate the trace metal requirements of this species in the PSD-AK through omission of individual metals.

In the absence of Medium 199, the semi-defined medium lacked simple carbon and phosphorus compounds that likely were needed for *Pfiesteria shumwayae* growth. Medium 199 contained glucose and acetate, organic carbon sources that are effectively metabolized by many heterotrophic protists (e.g. Droop 1959, Holz et al. 1961c, Provasoli and Gold 1962, Allen et al. 1966, McGinn 1971, Lee 1977). The Medium 199 formulation also included inorganic and organic phosphorus sources in the form of sodium phosphate and two nucleotides (adenosine triphosphate, adenosine monophosphate). Inorganic phosphorus often is not used by heterotrophic species (e.g. Droop 1959), but phosphorus requirements often can be met with nucleotides (McGinn 1971, Hanna and Lilly 1974, La Peyre and Faisal 1997). When Medium 199 was omitted from the PSD-AK medium, *P. shumwayae* growth did not occur, indicating that the carbon skeleton of fatty acids and amino acids likely is not available as a carbon source. The lack of growth also suggested that the phosphorus contained in the complex phospholipids could not meet cellular phosphorus requirements.

It was hypothesized that glucose and glycerophosphate could replace the Medium 199 in the semi-defined medium (PSD-GG); however, only *P. shumwayae* strain

CCMP2357 grew successfully in this medium. The growth of strain CCMP2357 in the PSD-GG medium indicates that glucose and glycerophosphate met cellular carbon and phosphorus requirements, respectively. Among the heterotrophic dinoflagellates that have been cultured axenically, *Cryptocodinium cohnii*, *Gyrodinium lebouriae*, and *Noctiluca scintillans* used glucose as a carbon source (Provasoli and Gold 1962, McGinn 1971, Lee 1977) while *Oxyrrhis marina* required acetate or ethanol (Droop 1959). Glycerophosphate met the phosphorus requirements of *C. cohnii*, *N. scintillans*, and *O. marina* in axenic culture (Droop 1959, Provasoli and Gold 1962, McGinn 1977), but the phosphorus source in the medium used to grow *G. lebouriae* is unknown (Lee 1977).

The inability of the other two strains to grow in the PSD-GG medium suggests differences in the nutritional requirements of the three *Pfiesteria shumwayae* strains. Various types of strain differences appear to be common in protists (Wood and Leatham 1992, Burkholder and Glibert 2006), but significant differences in specific biochemical requirements among strains of a given species are unknown and may represent species-level rather than strain-level differences.

*Medium storage.* A major disadvantage of early formulations of the semi-defined medium (PSD-IO, PSD-AK) was the inability to store the media. Storage likely resulted in auto-oxidation of the lipids present in the medium (Lea and Hawke 1951, Lea 1957), a process that may have been accelerated by the trace metal solution (Lea and Hawke 1951, Schaich 1992). The addition of the chelator citric acid allowed storage of the PSD-AK medium for at least 2 months, improving the utility of the medium. Citric acid functioned as a chelator in the medium formulated for the axenic culture of *Oxyrrhis*

*marina* (Droop 1959) and is a relatively common component of media that contain lipids (Holz et al. 1961a,b,c, Allen et al. 1966). All three *Pfiesteria shumwayae* strains were grown to high cell densities in the PSD-CA medium, and storage of the medium resulted in no adverse effects on cell growth.

*Population growth and cell yields.* Cell yields and division rates of these strains of *Pfiesteria shumwayae* in the semi-defined medium were similar to or exceeded those reported for this species when cultured monoxenically on a fish cell line (Parrow et al. 2005). Further, the maximum cell densities obtained with this cultivation system were up to 10 times higher than those obtained when *P. shumwayae* was cultured with living fish or microalgae in xenic cultures (Vogelbein et al. 2001, Parrow et al. 2002) or in a complex axenic medium (Skelton et al. submitted). The highest cell yields of strains CCMP2357 and CCMP2360 were attained at amicase and lecithin concentrations of  $1 \text{ g} \cdot \text{L}^{-1}$ , and it is possible that increased concentrations of those components would result in higher yields. Poor growth of CCMP2359 at amicase and lecithin concentrations of  $1 \text{ g} \cdot \text{L}^{-1}$  may have resulted from toxicity of amino acids and/or fatty acids (Benevenga and Steele 1984, Borradaile et al. 2006). The lack of growth (all strains) in the formulation containing 100% Medium 199 likely was due to the absence of trace compounds (i.e.  $\text{Br}^-$ ,  $\text{B}(\text{OH})_3$ ,  $\text{Sr}^{2+}$ ,  $\text{F}^-$ ,  $\text{I}^-$ ) present in the AK synthetic seawater base. Similarly, *P. shumwayae* probably did not grow in the PSD-AK without Medium 199 (0%) because the medium lacked appropriate carbon and phosphorus sources (see above).

The reason for the lack of growth of strain CCMP2360 when inoculated into experimental treatments at an initial density of  $1 \times 10^4 \text{ cells} \cdot \text{ml}^{-1}$  is unknown and

requires further investigation. Similar results, however, have been reported for the ciliates *Tetrahymena* spp. when cultured axenically (Christensen and Rasmussen 1992, Christensen et al. 1993). It is possible that *P. shumwayae* cells produce and release a growth factor(s) that stimulate cell proliferation (Christensen and Rasmussen 1992). Monoxenic *P. shumwayae* cultures have been initiated from single purified cells (Parrow et al. 2005), but the fish cells used as the fish source in that method may have produced growth factors that permitted growth of the dinoflagellate.

If *Pfiesteria shumwayae* requires growth factors for cell proliferation, it may not be possible to establish axenic cultures from single cells. The ability to attain high cell yields of *P. shumwayae* monoxenically on a fish cell line circumvented this possible problem in the present study, demonstrating the value of monoxenic cultures. However, monoxenic cultures of *P. shumwayae* currently are not commercially available and attempts to generate bacteria-free cultures of *P. shumwayae* on microalgal prey might be unsuccessful. Growth of heterotrophic dinoflagellates on microalgae often is poor or fails in the absence of bacteria (McGinn 1971, Alavi 2001, Parrow and Burkholder 2003), making monoxenic cultures difficult to establish. In the absence of monoxenic cultures, a large inoculum of purified *P. shumwayae* cells might be generated from xenic cultures following the cyst purification method developed by Parrow et al. (2005).

*Nutritional Ecology.* Although the PSD-AK medium did not allow specific determination of *Pfiesteria shumwayae* biochemical requirements, the major components of the medium, along with insights gained previously from the development of xenic and monoxenic cultures, permit some speculations about the nutritional ecology of this

heterotrophic dinoflagellate. The high concentrations of lipids in the complex (Skelton et al. submitted) and semi-defined culture media strongly suggest that fatty acids and/or sterols are required for or stimulatory to *P. shumwayae* growth. A lipid requirement would be a disadvantage for any free-living protist that relies on resorption (Droop and Doyle 1966); therefore, species with lipid requirements most likely are obligate phagotrophs due to a demand for access to lipid-rich sources (i.e. cells) (Hutner and Holz 1962).

*Pfiesteria shumwayae* previously has been maintained in xenic culture with algal prey such as cryptophytes (*Cryptomonas* spp., *Rhodomonas* spp.) or chlorophytes (*Dunaliella* spp.) (Burkholder and Glasgow 1995, Burkholder et al. 2001). The cell densities attained in these cultures ( $2 \times 10^4$  cells  $\cdot$  ml<sup>-1</sup>; Parrow et al. 2002) were much lower than the yields attained in monoxenic (bacteria-free) culture on a fish cell line ( $1.4 \times 10^5$  -  $2.8 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup>; Parrow et al. 2005) and in the semi-defined axenic (up to  $4.0 \times 10^5$  cells  $\cdot$  ml<sup>-1</sup>) culture medium reported here, suggesting that microalgae are not ideal prey for *P. shumwayae*. The fatty acid composition of the fish cell line and the semi-defined medium is significantly different from that of most microalgae; therefore, the observed difference in cell yields between the cultivations systems might be attributable to the availability of fatty acids. In particular, we suspect that the unsaturated fatty acids oleic (18:1n-9) and linoleic (18:2n-6) acid may be required for or stimulatory to the growth of *P. shumwayae*.

Oleic acid is the dominant monounsaturated fatty acid in fish cells (26% of total fatty acids; Henderson and Tocher 1987, Tocher et al. 1988) and soy lecithin (11%;

Schneider 1989); however, this fatty acid only comprises 1 - 3% of the total fatty acids in cryptophytes and *Dunaliella* sp. (Volkman et al. 1989, Renaud et al. 1999). Further, soy lecithin contains high concentrations of the polyunsaturated fatty acid, linoleic acid (60%; Schneider 1989), which only is present at 1 - 10% in most microalgae (Volkman et al. 1989, Renaud et al. 1999). In contrast, the dominant unsaturated fatty acids in cryptophytes are  $\alpha$ -linolenic acid (18:3n-3; 12 - 25%) and stearidonic acid (18:4n-3; 20 - 30%) while  $\alpha$ -linolenic (44%) and hexadecatetraenoic (16:4n-3; 21%) acids predominate in *Dunaliella* sp. (Volkman et al. 1989, Renaud et al. 1999).  $\alpha$ -Linolenic and stearidonic acid comprises < 10% and 3% of the total fatty acids in fish cells or soy lecithin, respectively, and hexadecatetraenoic acid is not present (Kinsella et al. 1977, Henderson and Tocher 1987, Tocher et al. 1988). It is possible that *P. shumwayae* can use the other major unsaturated fatty acids found in cryptophytes and chlorophytes, but does so less effectively, resulting in low cell abundances. Alternatively, *P. shumwayae* may have a specific requirement for 18:1n-9 and/or 18:2n-6, and the low concentrations of those fatty acids in microalgae do not support high cell yields. The differences in fatty acid profiles and the associated cell yields obtained in the different cultivation systems suggests that fish or other aquatic animals, rather than microalgae may be more suitable prey for *P. shumwayae*.

The semi-defined culture medium also contained high concentrations of amino acids, and the availability of these compounds also may give some indication of prey nutritional quality. The amino acid composition of microalgae is similar across taxa, with aspartate and glutamate comprising the highest concentrations (7.6 - 12.4% of total

amino acids) and cystine, methionine, tryptophan, histidine, and ornithine found in the lowest concentrations (0.04 - 3.2%) (Brown 1991, Brown et al. 1997). Interestingly, the amino acid composition of fish tissue is very similar to that of microalgae (Kim and Lall 2000), suggesting that differences in lipid composition may be more important in determining the nutritional value of prey items. Protein does, however, comprise a higher percentage of the dry weight of fish (59 - 70%) than microalgae (12 - 34%), while the percent lipid is more similar (12 - 22% and 7.2 - 23% in fish and microalgae, respectively) (Brown 1991, Brown et al. 1997, Kim and Lall 2000). Fish not only may be a more suitable source of lipids, in terms of fatty acid composition, but also might be a richer source of amino acids for *P. shumwayae*. A difference in prey nutritional quality explain in part why *P. shumwayae* densities in estuaries generally are low except during fish kill events, when higher cell abundances have been documented (Burkholder et al. 2001). Although some speculation can be made at this point regarding the nutritional ecology of this species, knowledge will be greatly increased when a completely defined culture medium is developed that supports the axenic growth of *P. shumwayae*. Determination of specific nutritional requirements of *P. shumwayae* will strengthen assessment of how the occurrence of both dissolved and particulate compounds in the natural environment may influence *P. shumwaye* abundances and distributions.

### 3.6 Conclusions

*Pfiesteria shumwayae* is the first tube-feeding heterotrophic dinoflagellate to be cultured axenically, and certain nutritional requirements of this species appear to be different from those of the other heterotrophic dinoflagellates examined thus far. The semi-defined medium developed in the present study as well as the complex medium formulated previously both contained high concentrations of lipids, suggesting a requirement for fatty acids and/or sterols. In addition, *P. shumwayae* flagellate cells attempted to ingest insoluble lecithin particles in the medium, suggesting that for this dinoflagellate phagotrophy may be indispensable and needed to obtain all its required nutrients in axenic culture.

The development of a semi-defined medium that supports the axenic growth of *Pfiesteria shumwayae* represents significant progress toward the development of a completely defined culture medium and determination of specific biochemical requirements for this species. Future research should focus on replacing the ampicillin and lecithin components with defined amino acids, fatty acids, and sterols in an effort to fully define the medium. In addition, the semi-defined medium or modifications thereof should be tested for the ability to support the axenic growth of other heterotrophic dinoflagellates, particularly tube-feeding species that are closely related to *P. shumwayae*.

The semi-defined medium developed here is inexpensive, easily prepared, and can be stored for prolonged periods of time with no adverse effect on growth. Further, this medium produces high cell yields of *P. shumwayae*, making this cultivation system

superior to previous methods. Most importantly, this culturing method provides a source of *Pfiesteria shumwayae* free from other metabolizing cells, permitting physiological and ecological investigations that otherwise would be complicated by the presence of prey and/or bacteria.

#### Acknowledgements

Funding support for this research was provided by the U.S. EPA Star program (Ecohab RD-83170401), the Park Foundation, and the North Carolina General Assembly. We thank Bradley Hollidge for culturing assistance.

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## APPENDIX

**Table A1.** Chemical composition of Amicase (Sigma A2427)\*

Component	Concentration (mg/g)
Alanine	55.7
Arginine	35.7
Aspartic acid	50.1
Cysteine	0.2
Glutamic acid	187.5
Glycine	21.3
Histidine	28.4
Isoleucine	46.9
Leucine	75.6
Lysine	90.1
Methionine	22.0
Phenylalanine	41.3
Proline	83.5
Serine	24.6
Threonine	34.6
Tryptophan	< 0.5
Tyrosine	28.8
Valine	64.3

\* Product is 18-20% peptides and 80-82% amino acids.

**Table A2.** Chemical composition of Soy Lecithin (MP Biomedicals 102147) \*

Component	Percent composition
Phosphatidyl choline	25%
Phosphatidyl ethanolamine	22%
Phosphatidyl inositol	16%
Phosphatidic acid	7%
Other phospholipids & polar lipids	9%
Sterols and sterol glycosides (glycolipids)	6%
Carbohydrate (unbound)	8%
Minerals (not including phosphorus)	3%
Residual soybean oil	2.5%
Moisture and solvent insoluble	1.5%

\* A natural mixture of phospholipids; the predominant fatty acid is polyunsaturated linoleic acid (70%).

**Table A3.** Chemical composition of L1 vitamins\*

Component	Concentration (per L medium)
Vitamin B <sub>12</sub>	0.5 µg
Biotin (Vitamin B <sub>7</sub> )	0.5 µg
Thiamine · HCl (Vitamin B <sub>1</sub> )	0.1 mg

\* Guillard and Hargraves 1993

**Table A4.** Chemical composition of L1 trace metals\*

Component	Concentration (per L medium)
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	3.15 mg
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	4.36 mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	2.45 $\mu\text{g}$
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	19.9 $\mu\text{g}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	22.0 $\mu\text{g}$
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	10.0 $\mu\text{g}$
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.18 mg
$\text{H}_2\text{SeO}_3$	1.3 ng
$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$	2.7 $\mu\text{g}$
$\text{Na}_3\text{VO}_4$	1.84 $\mu\text{g}$
$\text{K}_2\text{CrO}_4$	1.94 $\mu\text{g}$

\* Guillard and Hargraves 1993

**Table A5.** Chemical composition of Medium 199 (Sigma M3769)

Component	Concentration (per L)
Calcium chloride (anhydrous)	0.2 (g)
Ferric Nitrate · 9H <sub>2</sub> O	0.72 (mg)
Magnesium sulfate (anhydrous)	97.67 (mg)
Potassium chloride	0.4 (g)
Sodium acetate (anhydrous)	50.0 (mg)
Sodium chloride	6.8 (g)
Sodium phosphate monobasic (anhydrous)	0.122 (g)
DL-alanine	50.0 (mg)
L-arginine HCl	70.0 (mg)
DL-aspartic acid	60.0 (mg)
L-cysteine · HCl · H <sub>2</sub> O	0.11 (mg)
L-cystine · 2HCl	26.0 (mg)
DL-glutamic acid	0.1336 (g)
Glycine	50.0 (mg)
L-histidine · HCl · H <sub>2</sub> O	21.88 (mg)
Hydroxy-L-proline	10.0 (mg)
DL-isoleucine	40.0 (mg)
DL-leucine	0.12 (g)
L-lysine	70.0 (mg)

**Table A5.** continued

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DL-methionine	30.0 (mg)
DL-phenylalanine	50.0 (mg)
L-proline	40.0 (mg)
DL-serine	50.0 (mg)
DL-threonine	60.0 (mg)
DL-tryptophan	20.0 (mg)
L-tyrosine 2Na · 2H <sub>2</sub> O	57.66 (mg)
DL-valine	50.0 (mg)
Ascorbic acid · Na (Vitamin C)	56.6 (μg)
D-biotin (Vitamin B <sub>7</sub> )	10.0 (μg)
Calciferol (Vitamin D)	0.10 (mg)
Choline chloride	0.50 (mg)
Folic acid (Vitamin B <sub>9</sub> )	10.0 (μg)
Menadione (sodium bisulfate) (Vitamin K)	16.0 (μg)
Myo-inositol	50.0 (μg)
Niacinamide (Vitamin B <sub>3</sub> )	25.0 (μg)
Nicotinic Acid (Vitamin B <sub>3</sub> )	25.0 (μg)
p-Amino benzoic acid (PABA)	50.0 (μg)
D-pantothenic acid (hemicalcium) (Vitamin B <sub>5</sub> )	10.0 (μg)
Pyridoxal · HCl (Vitamin B <sub>6</sub> )	25.0 (μg)

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**Table A5.** continued

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Pyridoxine · HCl (Vitamin B <sub>6</sub> )	25.0 (μg)
Retinol acetate (Vitamin A)	0.14 (mg)
Riboflavin (Vitamin B <sub>2</sub> )	10.0 (μg)
DL-a-tocopherol phosphate · Na (Vitamin E)	10.0 (μg)
Thiamine · HCl (Vitamin B <sub>1</sub> )	10.0 (μg)
Adenine sulfate	10.0 (mg)
Adenosine triphosphate · 2Na	1.0 (mg)
Adenosine monophosphate · Na	0.2385 (mg)
Cholesterol	0.20 (mg)
Deoxyribose	0.50 (mg)
Glucose	1.0 (g)
Glutathione	50.0 (μg)
Guanine · HCl	0.30 (mg)
Hypoxanthine	0.30 (mg)
Polyoxyethylenesorbitan monooleate (Tween 80)	20.0 (mg)
Ribose	0.50 (mg)
Thymine	0.30 (mg)
Uracil	0.30 (mg)
Xanthine · Na	0.344 (mg)

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