Journal of Shellfish Research, Vol. 24, No. 4, 1013–1018, 2005.

A FLUOROMETRIC TECHNIQUE FOR THE *IN VITRO* MEASUREMENT OF GROWTH AND VIABILITY IN QUAHOG PARASITE UNKNOWN (QPX)

DEENIE M. BUGGÉ AND BASSEM ALLAM*

Marine Sciences Research Center, Stony Brook University, Stony Brook, New York 11794

ABSTRACT Quahog parasite unknown (QPX) is a protistan parasite that causes significant mortalities among hard clams, *Mercenaria mercenaria*, in the northeastern United States and Canada. This pathogen has been successfully isolated from clams from different geographic locations, and *in vitro* cultures are being used in investigations of the parasite's genetic makeup, virulence and environmental tolerances. Many of these investigations require an easily reproducible, quantitative method to rapidly measure QPX cell viability and proliferation. Therefore, a fluorometric microplate technique was developed using fluorescein diacetate (FDA) as an indicator of cell viability. The developed technique provides a good estimate of the biovolume of live QPX cells. Fluorescent signals are correlated with the number and the size of individual QPX cells. Optimal experimental conditions included an FDA concentration range of 30–50 μM with an incubation period of 15–30 min in the dark. This FDA assay was used to investigate the dynamics of *in vitro* growth of QPX. Results showed that the growth dynamic is different among QPX isolates. For instance, exponential growth lasted for 1 week in two QPX isolates cultured from clams collected from New York and Massachusetts; whereas a third isolate (from New York) grew exponentially during 2 weeks under similar experimental conditions. While there are limitations to *in vitro* studies that must be recognized, research using cultured QPX cells is indispensable and will probably lead to significant progress in our knowledge of the biology and physiology of this parasite.

KEY WORDS: *Mercenaria mercenaria*, northern quahog, clam, *in vitro*, fluorescein diacetate

INTRODUCTION

Quahog parasite unknown (QPX) is a protistan parasite of hard clams, *Mercenaria mercenaria*. In the late 1950s an unknown protistan disease was reported in hard clams in New Brunswick, Canada (Drinnan & Henderson 1963). This protist, later named quahog parasite unknown was again found in a clam hatchery on Prince Edward Island in 1989 (Whyte et al. 1994). Subsequently, QPX has also been documented to occur at locations in Massachusetts, Virginia, New Jersey, and New York (Smolowitz et al. 1998, Ragone Calvo et al. 1998, Ford et al. 2002, Dove et al. 2004).

Development of media formulations permitting axenic culture of marine pathogens has enabled scientists to study the growth of parasites, their virulence factors and host-parasite interactions. *Perkinsus marinus* is a strong example of how development of axenic culture and methods to measure cell viability and proliferation can lead to breakthroughs in the understanding of the biology of marine pathogens. La Peyre et al. (1993) and Gauthier and Vasta (1993) first demonstrated that *P. marinus* could be propagated *in vitro*. After this initial finding, different semiautomated methods to estimate cell proliferation have been developed. These methods include tetrazolium-based assays, which estimate *P. marinus* viability and proliferation by measuring mitochondrial hydrogenase activity in viable cells (Dungan & Hamilton 1995, La Peyre 1996, Volety & Fisher 2000, Elandalloussi et al. 2003). Others have used spectrophotometric measurements of the optical density of cell suspensions as a measure of *P. marinus* proliferation (Gauthier & Vasta 1994, 1995, 2002, Gauthier et al. 1995, Anderson & Beaven 2001). Each of these methods is a useful technique to rapidly measure *P. marinus* growth and proliferation *in vitro*. These semiautomated techniques have been used to develop a fully defined medium (Gauthier et al. 1995), optimize culture methodology and study the effects of temperature, salinity and pH on the *in vitro* growth of *P. marinus* (Gauthier & Vasta 1995). Additionally, similar methods have been used to investigate *in vitro* host-pathogen interactions, such as the effects of bivalve plasma on *in vitro* proliferation of *P. marinus* (Gauthier & Vasta 2002, Anderson & Beaven 2001) and *in vitro* killing of *P. marinus* by oyster hemocytes (Volety & Fisher 2000). As with *P. marinus*, the development of similar QPX cell proliferation assays will enable *in vitro* QPX studies that increase our understanding of the parasite.

A category of established methods for measuring cell culture viability and proliferation uses the latently fluorescent substrate fluorescein diacetate (FDA). Nonfluorescent FDA readily penetrates the cell plasma membrane and, once inside the cell, this molecule is broken-down by nonspecific esterases to form green fluorescent fluorescein (Bernhard et al. 1995). Fluorescein is hydrophilic, which inhibits the reduced molecule from leaking through a healthy cell membrane (Dayeh et al. 2004). The fluorescent signal of fluorescein indicates the amount of active esterases present in cells and is widely used as a proxy for estimating membrane integrity and cell viability (Schupp & Erlandsen 1987, Bernhard et al. 1995, Murphy & Cowles 1997, Bogaerts et al. 2001, Argüello-Garciá et al. 2004). This report describes the optimization and validation of an FDA microplate method for the measurement of the *in vitro* growth and viability of QPX cell populations.

MATERIAL AND METHODS

QPX Cultures

Two New York isolates of QPX, 8BC7 and 20AC6, were cultured from mantle nodules of infected hard clams collected in Raritan Bay, NY during October 2003 (Allam, unpublished). Isolation and subsequent subcultures were performed according to the methods described by Kleinschuster et al. (1998), and the identity of all isolates was confirmed using the polymerase chain reaction (PCR)/sequencing methods described by Stokes et al. (2002). A Massachusetts QPX isolate was obtained from the American type culture collection (ATCC 50749). QPX cultures were propagated in 25-cm^2 culture flasks at 23°C, using a minimal essential me-

*Corresponding author. E-mail: Bassem.Allam@stonybrook.edu

dium (MEM)-based growth medium. Isolates were subcultured on a weekly basis by transferring, under sterile conditions, 100 μl of culture suspension to a flask containing 4 mL fresh MEM medium. Phase-contrast microscopy was used to routinely monitor the cells.

Development of Assay

A stock solution of fluorescein diacetate (FDA, Sigma 101716) was made in dimethylsulfoxide (DMSO) at a concentration of 0.5 mg ml^{-1} and stored at −20°C. The stock solution was diluted in a range of volumes of MEM medium to form working solutions containing 6 μM, 12 μM, 30 μM and 50 μM of FDA. These working solutions were combined with a range of incubation periods to find optimal stain concentration and incubation period. First, exponentially-growing (typically 1-week-old cultures) QPX cells were evenly suspended in culture by passing the entire culture several times through a 3-mL sterile syringe (without a needle) before 100 μL aliquots were plated in a black 96-well microplate. For comparative purposes, a volume of QPX culture was killed by heating in a boiling water bath for 30 min. The cells were then allowed to return to room temperature before analysis. FDA working solution was added to microplate wells containing 100 μL QPX culture, including viable and heat-killed samples, with three replicate wells for each treatment. Replicate control wells containing MEM medium without QPX cells were included to measure any signal that was not caused by cellular activity, and additional control wells containing QPX cultures without FDA were included to control for auto-fluorescence. Microplates were then incubated in the dark at room temperature for a range of incubation times, before fluorescence was measured at 485 nm excitation and 535 nm emission (Wallac 1420 plate reader). For each sample, percent fluorescence was calculated as the ratio of fluorescence in that sample to the maximal fluorescence in the preparation ×100.

Assay Validation

After finding optimal FDA concentrations and incubation times, three experiments were conducted to validate the assay. First, a preliminary trial tested whether the fluorescent signal accurately reflects cell number. Two-fold serial dilutions of a QPX culture were plated in a 96-well microplate and incubated with a 50 μM final FDA concentration for 30 min. These experimental conditions were within the range of optimal conditions determined during preliminary trials. After incubation, the fluorescent signals were measured with the microplate reader as described earlier. A second validation step was to determine whether the fluorescent signal of fluorescein is a sensitive indicator of cell viability. To do this, mixtures were made containing different ratios of live and heat-killed QPX cells. Appropriate volumes of heat-killed culture were added to volumes of live culture to make mixtures containing 100% live cells, 50% live cells, 30% live cells and 10% live cells. For each mixture, 100 μL were plated in a 96-well microplate with three replicate treatment wells, FDA working solution was added to each well (50 μM final concentration), and the microplate was incubated in the dark for 30 min. A third validation step tested whether there are variations in the fluorescent signal associated with cell size. To test for these effects, QPX cultures of different average cell sizes were made by collecting cultures at different phases of growth because preliminary experiments showed that older cultures have larger average cell sizes than younger cultures. Measurements of cell diameters were made by taking a series of digital photographs under phase-contrast microscopy and using the spatial measurement function of Image-Pro Plus imaging software to measure the area of individual cells. Cell area was then converted to diameter by considering QPX cells as spherical. A minimum of 500 QPX cells were measured in each culture. Cultures that were 25 days old had an average cell diameter of 16.9 ± 6.2 μm (mean ± SEM), 19-day-old cultures had an average cell diameter of 10.9 ± 1.2 μm and 12-day-old cultures had an average cell diameter of 4.0 ± 0.4 μm. A small volume of each culture was washed and resuspended in filtered artificial seawater (FASW). Cell concentration in each culture was determined with a hemocytometer. The three samples were then diluted with appropriate amounts of FASW to make preparations with equivalent numbers of cells. Diluted samples were recounted to confirm equivalent cell concentrations. Two-fold serial dilutions of these three samples were made, 100 μL of each dilution was plated in three replicate microplate treatment wells, and the FDA assay was performed as mentioned earlier.

Measurement of in vitro *Growth*

After development and validation of the assay, the FDA technique was used to quantify the growth of different QPX isolates. One Massachusetts isolate and two different New York isolates were subcultured with three replicates in 2.5 mL MEM medium in a 24-well plate, and incubated for 4 weeks under standard culture conditions. The growth of the cultures was measured at T_0, and at 3, 7, 14, 21 and 27 days after initial culturing. To measure growth, QPX cells were first evenly distributed by passing the entire cultures several times through a 3-mL sterile syringe (without a needle), and 100-μL samples of each culture were processed in triplicate, according to the FDA assay described earlier.

RESULTS

Development of Assay

Measurements of the fluorescent signals produced by QPX cultures incubated with a range of FDA concentrations show that final concentrations between 30 and 50 μM FDA provide good staining and the greatest distinction between live and nonviable QPX cells (Fig. 1). An incubation period of 15–30 min is sufficient to allow adequate distinction between viable and nonviable cells (Fig. 2). Further incubation does not increase the sensitivity of the assay.

Assay Validation

Use of 50 μM FDA combined with a 30 min incubation period demonstrates that these are optimal staining conditions in which the fluorescent signal is directly proportional to cell number (R^2 = 0.887, Fig. 3). Fluorescence measurements of live and heat-killed QPX cells showed that FDA is a sensitive indicator of QPX cell viability (Fig. 4). For instance, there is a linear relationship between the concentration of viable cells in a sample, and the intensity of the fluorescent signal that it produces. Measurements of the fluorescent signal of 2-fold serial dilutions of QPX cultures showed that the fluorescent signal of fluorescein accurately reflects the number of QPX cells in a sample (Fig. 5). There is a clear linearity of the fluorescent signal with respect to cell concentration ($R^2 > 0.99$); however, there are also variations in the signal based on differences in average cell size in cultures. At equivalent cell concentrations, the strongest signal is measured in the culture with an average cell diameter of 16.9 ± 6.2 μm, followed by the culture

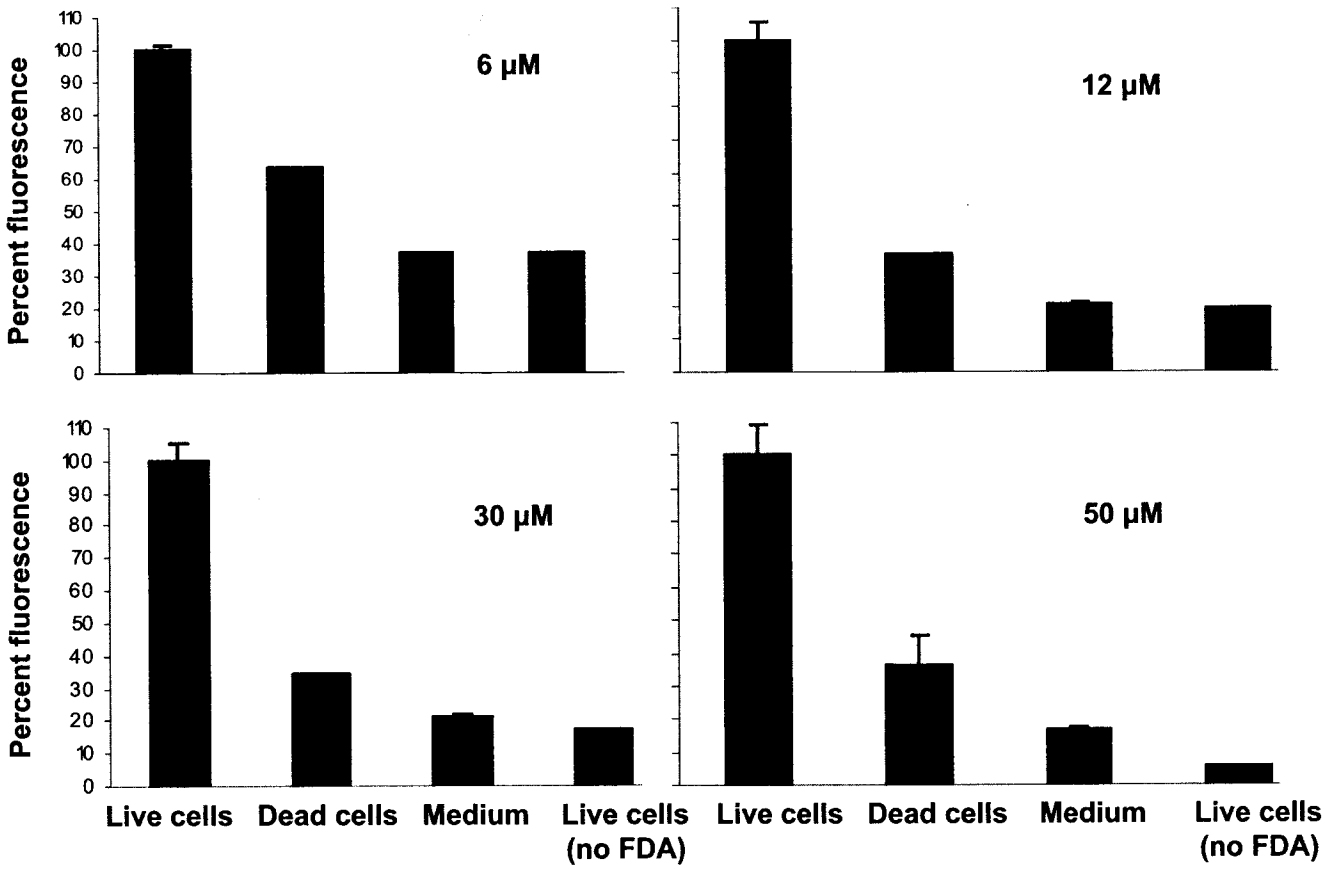

Figure 1. Fluorescent signals produced by incubating 100-µL QPX cultures with a range of FDA concentrations for 30 min. Concentrations between 30 and 50 µM FDA provide greatest distinction between live and heat-killed QPX cells. All data are presented as Mean ± SEM (3 replicates; error bars are sometimes extremely small because of the great precision of the assay).

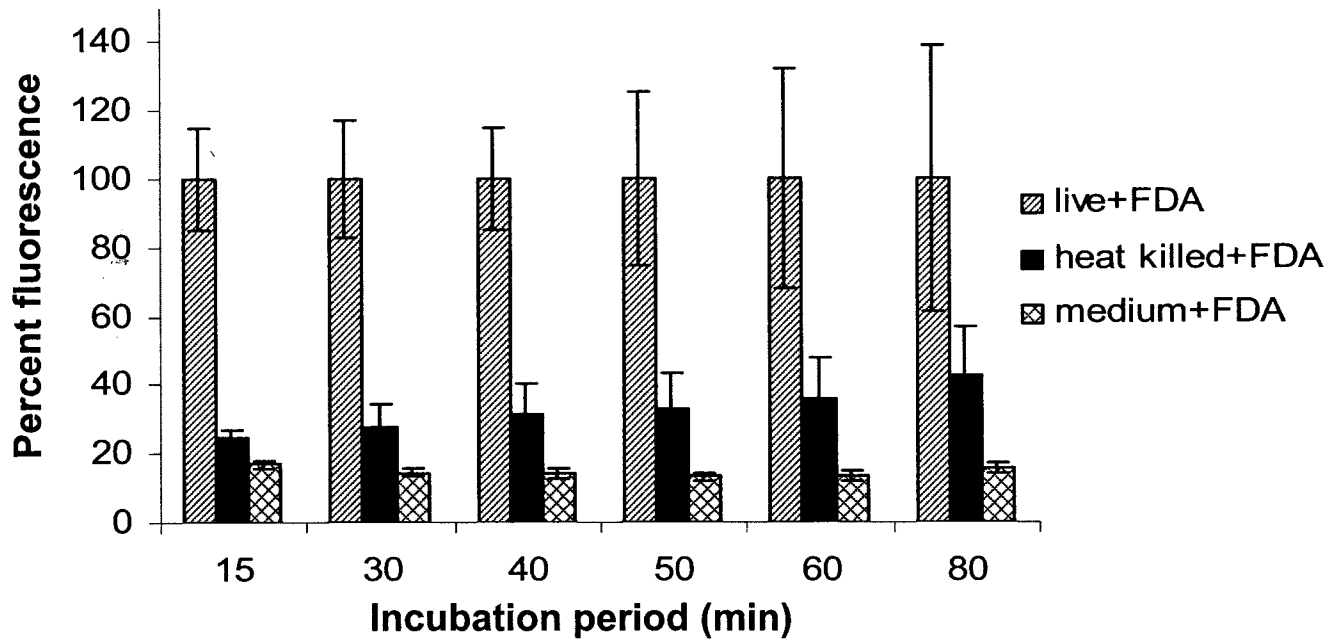

Figure 2. Fluorescent signals produced by incubating 100-µL QPX cultures with 50 µM FDA for different time intervals. An incubation period between 15 and 30 min provides good distinction between live and heat-killed QPX cells. Further incubation does not increase the sensitivity of the assay. All data are presented as Mean ± SEM (3 replicates).

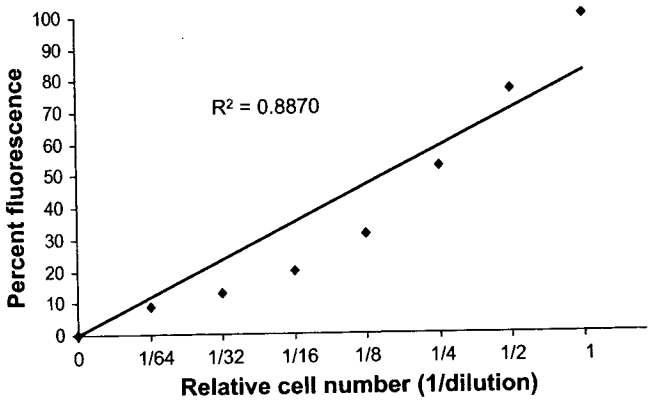

Figure 3. Fluorescent signal produced by 2-fold serial dilutions of QPX culture incubated with 50 µM FDA for 30 min. The fluorescent signal is directly proportional to relative cell number.

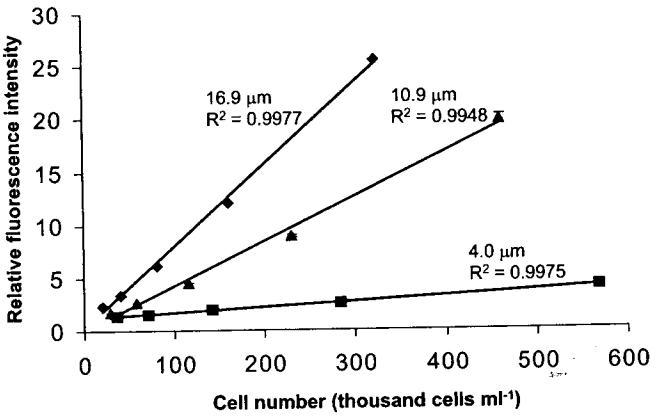

Figure 5. Linear regression of fluorescence produced by 2-fold serial dilutions of three different QPX cultures having different average cell diameters. Fluorescence intensity is linear with respect to cell concentration within each culture. However, the slopes of each regression line are different. All data are presented as mean ± SEM (3 replicates; error bars are sometime extremely small because of the great precision of the assay).

with an average cell diameter of 10.9 ± 1.2 µm, and the weakest signal was produced by the culture with an average cell diameter of 4.0 ± 0.4 µm. To determine whether differences in fluorescence at equivalent cell concentrations reflect differences in esterase activity associated with cell size, the data shown in Figure 5 were pooled, cell concentration was converted to cell biovolume (by considering QPX cells as spherical), and intensity of fluorescent signal was plotted against estimated cell biovolume (Fig. 6). Results show a strong relationship between fluorescence and QPX cell biovolume (R^2 = 0.878), indicating that the fluorescence of fluorescein is proportional to QPX biovolume, which varies as a combined function of cell number and average cell size.

In vitro Growth of Different QPX Isolates

Measurements of the fluorescent signal produced by different QPX isolates show a rapid increase in growth of the cultures from T_0 to day 7 (Fig. 7). During this period, all cultures appeared to be in the exponential phase of growth. However, after a 1-week period, the Massachusetts isolate and the New York isolate 8BC7 ceased incremental growth. The second New York isolate 20AC6 continued to grow exponentially through 2 weeks of incubation.

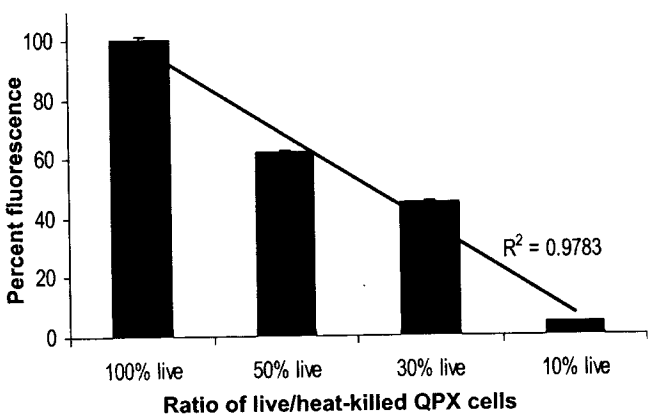

Figure 4. Fluorescence produced by samples containing combinations of live and heat-killed QPX cells. There is a close relationship between the percentages of total fluorescence and the percentage of live QPX cells present in a sample. All data are presented as mean ± SEM (3 replicates; error bars are sometimes extremely small because of the great precision of the assay).

DISCUSSION

This study demonstrates that the developed FDA technique represents a valuable method by which *in vitro* QPX cell population growth can be measured. This fluorescence assay has many advantages over counting and spectrophotometric methods that have been used to quantify *in vitro* QPX growth. Unlike counting methods using a hemocytometer, this FDA assay requires minimal handling of QPX cultures. QPX cultures are very viscous and cells are highly aggregated, making counts of individual cells a relatively difficult task. To count QPX cells using a hemocytometer, the culture must first be treated to minimize the aggregation of cells caused by the mucus that is copiously produced by QPX (Brothers et al. 2000, Anderson et al. 2003). The FDA technique requires only minimal mixing of QPX culture before sampling and the fluorescent signal can be read directly from experimental treatment wells. The use of a syringe to evenly distribute QPX cells in a culture has been shown not to affect cell viability or subsequent growth of cells after culture in fresh medium (data not shown). Additionally, our laboratory is currently working to improve QPX

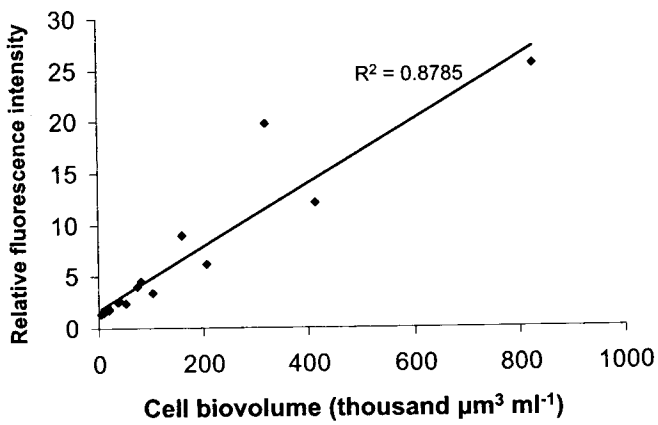

Figure 6. Linear regression of pooled data shown in Fig. 5 with cell densities converted to cell biovolumes. Fluorescence intensity is linear with respect to estimated cell biovolume.

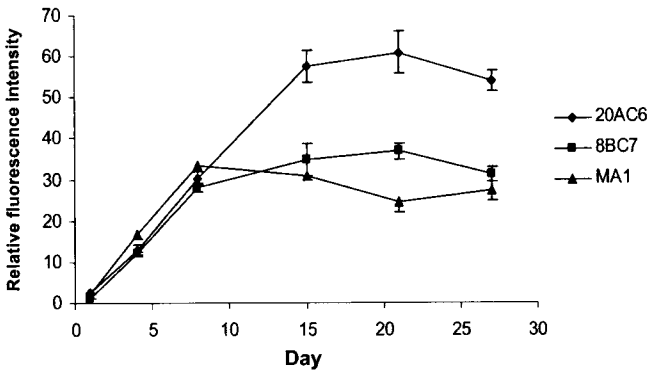

Figure 7. *In vitro* growth of three QPX isolates incubated under standard culture conditions for 4 weeks. Cultures 8BC7 and MA1 ended their exponential growth phase 1 week after the beginning of the experiment, whereas culture 20AC6 continued to grow exponentially through 2 weeks of incubation. All data are presented as mean ± SEM (3 replicates).

culture conditions that may potentially eliminate this mixing step, further decreasing the amount of handling required in the assay. Another advantage of this cell proliferation assay is that FDA staining is able to differentiate between live and dead cells, which cannot be accomplished with counting and turbidimetric methods (Elandalloussi et al. 2003). The FDA assay is also very rapid, which permits treatment replication and multiple assays in a short period.

Our results show that the average size of QPX cells vary with different culture conditions, namely the stage of growth of the culture. Differences have been observed in *in vitro* growth of *P. marinus* in which cell size varies with cell cycle (La Peyre & Faisal 1997) and growth rate (Gauthier & Vasta 1995), and with changes in medium osmolality (O'Farrell et al. 2000). The FDA-based assay described here has disadvantages similar to those of tetrazolium-based assays and assays based on optical density that are used for other protists. The signal of tetrazolium-based assays varies as a combined function of cell number, cell size and metabolic activity (Dungan & Hamilton 1995). Similarly, measurements of optical density vary as a combined function of cell density, cell size and aggregation of cells (Gauthier & Vasta 1995, La Peyre 1996). Like these assays, the fluorescent signal of fluorescein varies as a function of the size of the cells, the metabolic esterase activity and the number of cells in the culture being analyzed. Larger OPX cells possess a higher level of esterase activity than smaller cells, and thus produce a stronger fluorescent signal. Ultimately, the fluorescent signal of fluorescein is a function of QPX biovolume and thus, is a measure of the overall growth in a QPX culture, not a single measure of cell number or cell size. To specifically measure cell numbers with the FDA assay, an estimation of average cell size in a culture is required. However, without a measure of cell size, this assay can be used as a measure of cell biovolume to assess viability, relative proliferation and growth of a specific QPX culture.

In addition to differences in esterase activity associated with cell size, heat-killed QPX cells seem to retain some level of esterase activity which results in background fluorescence produced by nonviable cells. However, the level of these background signals is low and does not affect the applicability of the test.

Results from the assay in which we incubated different QPX isolates indicate that there are differences in growth rates among three of our QPX isolates. The New York isolate 20AC6 seems to grow exponentially for a longer period of time than the Massachusetts isolate and the New York isolate 8BC7. This coincides with slight morphologic differences among these isolates (Allam et al. unpublished) and may have implications for differences in virulence among the isolates. Further *in vivo* experiments may be useful in determining whether this difference in growth rate extends to differences in infection rate and proliferation within *Mercenaria mercenaria* host clams.

As with any semiautomated cell proliferation assay, there are advantages and disadvantages associated with the FDA assay. However, with an understanding of how the assay works and what limitations exist, steps can be taken to ensure that the assay is accurate and provides the desired information. The FDA assay, which provides sensitive, rapid and reproducible measurements of *in vitro* QPX growth, is a valuable tool that can be easily used for a range of *in vitro* growth experiments.

ACKNOWLEDGMENTS

This research was supported by the New York State Department of Environmental Conservation and is also a resulting product from project R/FBF-17 funded under award NA16RG1645 from the National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration, to the Research Foundation of State University of New York on behalf of New York Sea Grant. The statements, findings, conclusions, views and recommendations are those of the authors and do not necessarily reflect the views of any of those organizations. This work is part of the M.S. thesis of the first author.

LITERATURE CITED

Anderson, R. S. & A. E. Beaven. 2001. A comparative study of anti-*Perkinus marinus* activity in bivalve sera. *J. Shellfish Res.* 20(3):1011–1017.

Anderson, R. S., B. S. Kraus, S. McGladdery & R. Smolowitz. 2003. QPX, A pathogen of quahogs (hard clams), employs mucoid secretions to resist host antimicrobial agents. *J. Shellfish Res.* 22(1):205–208.

Argüello-Garciá, R., M. Cruz-Soto, L. Romero-Montoya & L. Ortega-Pierres. 2004. Variability and variation in drug susceptibility among *Giardia duodenalis* isolates and clones exposed to 5-nitroimidazoles and benzimidazoles *in vitro*. *J. Antimicrob. Chemother.* 54:711–721.

Bernhard, J. M., S. G. Newkirk & S. Bowser. 1995. Towards a non-terminal viability assay for foraminiferan protists. *J. Eukaryot. Microbiol.* 42(4):357–367.

Bogaerts, P., J. Bohatier & F. Bonnemoy. 2001. Use of the ciliated protozoan *Tetrahymena pyriformis* for the assessment of toxicity and quantitative structure activity relationships of xenobiotics: Comparison with the microtox test. *Ecotoxicol. Environ. Saf.* 49:293–301.

Brothers, C., E. Marks, III & R. Smolowitz. 2000. Conditions affecting the growth and zoosporulation of the protistan parasite QPX in culture. *Biol. Bull.* 199:200–201.

Dayeh, V. A., S. L. Chow, K. Schirmer, D. H. Lynn & N. C. Bols. 2004. Evaluating the toxicity of Triton X-100 to protozoan, fish, and mammalian cells using fluorescent dyes as indicators of cell viability. *Ecotoxicol. Environ. Saf.* 57:375–382.

Dove, A. D. M., P. R. Bowser & R. M. Cerrato. 2004. Histological analysis of an outbreak of QPX disease in wild hard clams *Mercenaria mercenaria* in New York. *J. Aquat. Anim. Health* 16:246–250.

Drinnan, R. E. & E. B. Henderson. 1963. 1962 mortalities and a possible

disease organism in Neguac quahaugs. Biological Station at St. Andrews, Annual Report B11, New Brunswick.

Dungan, C. F. & R. M. Hamilton. 1995. Use of a tetrazolium-based cell proliferation assay to measure effects of *in vitro* conditions on *Perkinsus marinus* (Apicomplexa) proliferation. *J. Eukaryot. Microbiol.* 42(4):379–388.

Elandalloussi, L. M., R. Afonso, P. A. Nunes & M. L. Cancela. 2003. Effect of desferrioxamine and 2,2′-bipyridyl on the proliferation of *Perkinsus atlanticus. Biomol. Eng.* 20(4–6):349–354.

Ford, S. E., J. N. Kraeuter, R. D. Barber & G. Mathis. 2002. Aquaculture-associated factors in QPX disease of hard clams: density and seed source. *Aquaculture* 208:23–38.

Gauthier, J. D. & G. R. Vasta. 1994. Inhibition of *in vitro* replication of the oyster parasite *Perkinsus marinus* by the natural iron chelators transferrin, lactoferrin, and desferrioxamine. *Dev. Comp. Immunol.* 18(4): 277–286.

Gauthier, J. D. & G. R. Vasta. 1995. *In vitro* culture of the eastern oyster parasite *Perkinsus marinus:* Optimization of the methodology. *J. Invertebr. Pathol.* 66:156–168.

Gauthier, J. D., B. Feig & G. R. Vasta. 1995. Effect of fetal bovine serum glycoproteins on the *in vitro* proliferation of the oyster parasite *Perkinsus marinus:* development of a fully defined medium. *J. Eukaryot. Microbiol.* 42(3):307–313.

Gauthier, J. D. & G. R. Vasta. 2002. Effects of plasma from bivalve mollusk species on the *in vitro* proliferation of the protistan parasite *Perkinus marinus. J. Exp. Zool.* 292:221–230.

Gauthier, J. D. & G. R. Vasta. 1993. Continuous *in vitro* culture of the eastern oyster parasite *Perkinsus marinus. J. Invertebr. Pathol.* 62:321–323.

Kleinschuster, S. J., R. Smolowitz & J. Parent. 1998. *In vitro* life cycle and propagation of quahog parasite unknown. *J. Shellfish Res.* 17(1):75–78.

La Peyre, J. F., M. Faisal & E. M. Burreson. 1993. *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster *Crassostrea virginica. J. Eukaryot. Microbiol.* 40(3):304–310.

La Peyre, J. F. 1996. Propagation and *in vitro* studies of *Perkinsus marinus. J. Shellfish Res.* 15(1):89–101.

La Peyre, J. F. & M. Faisal. 1997. Development of a protein-free chemically defined culture medium for the propogation of the oyster pathogen *Perkinsus marinus. Parasite* 4:67–73.

Murphy, A. M. & T. J. Cowles. 1997. Effects of darkness on multi-excitation *in vivo* fluorescence and survival in a marine diatom. *Limnol. Oceanogr.* 42(6):1444–1453.

O'Farrell, C. L., J. F. La Peyre, K. T. Paynter & E. M. Burreson. 2000. Osmotic tolerance and volume regulation in *in vitro* cultures of the oyster pathogen *Perkinsus marinus. J. Shellfish Res.* 19(1):139–145.

Ragone Calvo, L. M., J. G. Walker & E. M. Burreson. 1998. Prevalence and distribution of QPX, quahog parasite unknown, in hard clams *Mercenaria mercenaria* in Virginia, USA. *Dis. Aquatic Org.* 33:209–219.

Schupp, D. G. & S. L. Erlandsen. 1987. A new method to determine *Giardia* cyst viability: correlation of fluorescein diacetate and propidium iodide staining with animal infectivity. *Appl. Environ. Microbiol.* 53(4):704–707.

Smolowitz, R., D. Leavitt & F. Perkins. 1998. Observations of a protistan disease similar to QPX in *Mercenaria mercenaria* (hard clams) from the coast of Massachusetts. *J. Invertebr. Pathol.* 71:9–25.

Stokes, N. A., L. M. Ragone Calvo, K. S. Reece & R. E. Burreson. 2002. Molecular diagnostics, field validation, and phylogenetic analysis of Quahog Parasite Unknown (QPX), a pathogen of the hard clam *Mercenaria mercenaria. Dis. Aquatic Org.* 52:233–247.

Volety, A. K. & W. S. Fisher. 2000. *In vitro* killing of *Perkinsus marinus* by hemocytes of *Crassostrea virginica. J. Shellfish Res.* 19(2):827–834.

Whyte, S. K., R. J. Cawthorn & S. E. McGladdery. 1994. QPX (Quahog Parasite X), a pathogen of northern quahog *Mercenaria mercenaria* from the Gulf of St. Lawrence, Canada. *Dis. Aquatic Org.* 19:129–136.