Development of a Fluorescent Multiwell Assay for Evaluating the Capacity of the Ciliated Protozoan Tetrahymena for Bacterivory in Water Samples

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Bacterivory by ciliates in various water ecosystems, both natural and artificial, plays a significant role on the microbial population composition and consequently affects water quality. A convenient, rapid and inexpensive methodology to evaluate the capacity of the ciliate protozoan *Tetrahymena thermophila* for bacterivory was developed utilizing fluorescent protein expressing bacteria (FPEB) in a microtitre plate fluorimeter. Bacterivory was correlated with a loss in fluorescence measured in the fluorimeter and confirmed by fluorescence microscopy showing that the FPEB were engulfed during the assay and subsequently lost their fluorescence, whereas Cytochalasin B, a known inhibitor of phagocytosis, prevented a decrease in relative fluorescence units (RFUs). The ciliate bacterivory (CB) assay has a great dynamic range allowing the assay to be performed with a variety of predator:prey concentrations. A model toxicant, CuCl₂, known to have a toxicological impact on protozoa and often present in different types of wastewater, resulted in measurable decreases in bacterivory. As well, starvation of *Tetrahymena* for 24 h resulted in reduced bacterivory. In the future, the CB assay could be developed for water monitoring purposes to rapidly assess water samples for the capacity to support bacterivory as an indicator of ecosystem health.

Key words: bacterivory, phagocytosis, GFP, ciliates, fluorescence microplate reader, *Tetrahymena*, toxicology

Introduction

Ciliated protozoa, such as *Tetrahymena*, are ubiquitous in aquatic environments and graze prolifically on bacteria. The bacterivory of ciliates can potentially impact on water quality in several ways. In natural aquatic systems, such as lakes and rivers, protozoa, such as the ciliates, graze or feed on bacteria and are key components of microbial food webs (for a review see Pernthaler 2005). By influencing the type and abundance of bacteria, they could affect the quality of the intake water into artificial ecosystems. In turn, food webs can exist in artificial ecosystems. Two examples of these are drinking water distribution systems and sewage treatment facilities. Bacteria and protozoa have been found in distribution systems for drinking water, and the protozoa, especially the ciliates, were shown to reduce the number of bacteria (Sibille et al. 1998). In the activated sludge process of sewage treatment, ciliates minimized biomass production by adding grazing stages to the trophic web and increased effluent quality by removing bacteria (Martín-Cereceda et al. 1996; Petropoulos and Gilbride 2005). Despite the importance of ciliate bacterivory to water quality, the cell biology and regulation of the process are poorly understood, partly because a rapid, convenient and reliable methodology is lacking.

One approach for studying bacterivory is to use live, fluorescent bacteria as the prey for protozoan predation. Fluorescent bacteria can be obtained by genetically engineering them to express fluorescent proteins such as green fluorescent protein (GFP) or red fluorescent protein (RFP) (Fu et al. 2003). The ingestion or phagocytosis of these fluorescent bacteria can be monitored in several ways. Flow cytometry has been successful with a marine heterotrophic flagellate (Fu et al. 2003), but the equipment is more expensive and complex to run than other methods. The simplest method is fluorescent microscopy but it is time consuming. A promising approach is to monitor the loss of fluorescence from a co-culture of a ciliate and bacteria as an indirect measure of bacteria phagocytosis. This method is based on the fact that ingested bacteria lose their fluorescence in the acidic environment of food vacuoles and has been successful with the ciliate *Tetrahymena pyriformis* (Parry et al. 2001). In this case the loss of fluorescence was measured with a fluorimeter (Parry et al. 2001). A potentially more rapid and convenient method would be to use a fluorometric multiwell plate reader. The microwells conserve material resources by reducing the number of cells needed and increasing the number of variables and replicates in a study. Plate readers are coupled to computers to rapidly and easily manage data.

Therefore, in this study we have developed a fluorescent multiwell assay to measure bacterivory by *T. th-
and begun to use this to study factors modulating the process. T. thermophila was chosen because this species can be grown axenically, has been a valuable model ciliate for many areas of research, and is found in sewage treatment plants (Esteban et al. 1992).

Materials and Methods

T. thermophila Culture Conditions

T. thermophila were maintained axenically at room temperature in 10 mL of proteose peptone yeast extract (PPYE) medium (Gilron et al. 1999) and every 2 to 3 weeks 1 mL was transferred to a fresh 10-mL volume of PPYE. In order to grow the protozoa for assays 5 mL of a 1- to 2-week-old culture was aseptically transferred to 50 mL sterile PPYE in untreated 75-cm² tissue-culture flasks (Nunc, VWR) and grown 24 to 36 h at room temperature on an orbital shaker at 50 rpm. Cells were pelleted by centrifugation (450 ×g, 5 min) washed three times with Osterhout’s minimal salts medium (Osterhout 1906) and resuspended in 25 mL Osterhout’s medium. For enumeration of cell density 0.5 mL were removed and fixed with neutral buffered formalin (2% v/v) and counted using a Coulter Z2 particle counter. The cell concentrations were adjusted as desired to the appropriate density with Osterhout’s medium.

Bacteria Culture Conditions

Escherichia coli XL-1 had been previously transformed (Power et al. 2001) with the expression vector pET (Stratagene) into which had been ligated a red shifted gfp construct with an excitation maximum at 490 nm that fluoresced more brightly than wild-type (provided by Ian Macarra, Center for Cell Signaling, University of Virginia). The E. coli XL-1 (pET-gfp) was maintained on Luria-Bertani (LB) agar plates supplemented with 100 µg/mL ampicillin grown overnight at 37°C and then stored at 4°C. In order to obtain fresh cultures for the phagocytosis assay 10 mL of LB broth with ampicillin (100 µg/mL) were inoculated with several colonies from the plates and incubated at 37°C overnight. Colonies were counted and colony forming units (CFU)/mL were determined. In order to monitor the GFP expression visually, the bacteria were also stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under an epifluorescent microscope (Nikon Optiphot) equipped with filters UV-1A (EX 365/EM400) for DAPI and B-2A (EX450/EM520) for GFP. The number of GFP expressing cells was compared to the total cells in order to determine the percentage of GFP expressing cells. If <95% of the cells were seen to be expressing GFP by epifluorescence the culture was discarded.

Development of the Fluorescent Multiwell Plate Assay to Measure Bacterivory

Log phase cultures of T. thermophila grown for 24 h in PPYE were harvested by centrifugation (450 ×g, 5 min), washed three times in Osterhout’s medium and diluted in Osterhout’s medium to the desired concentration, which in various experiments ranged from 10⁴ to 10⁷ cell/mL. The Tetrahymena were dispensed (100 µL/well) into the wells of a 96-well tissue culture plate with a lid (Falcon, VWR). To the wells containing T. thermophila, 100 µL of washed E. coli XL-1 (pET-gfp) were added at the desired concentrations, which in various trials ranged from 5 × 10⁶ to 5 × 10⁷ cfu/mL. Control wells containing Tetrahymena alone and wells containing E. coli alone were included in all plates. The lid was placed on the plate and the plate immediately placed in the Victor 3V fluorescent plate reader. The program was set up to read for GFP (480/530) every hour or every 30 min for up to 12 h at room temperature. At least three wells per concentration were included in each plate and all tests were carried out at least twice. Data were expressed as relative fluorescence units (RFUs).

Bacterivory Assays

The multiwell bacterivory assay was modified to test several variables that may affect the efficiency of bacterial uptake by Tetrahymena.

(i) The effect of the nutritional status of Tetrahymena was investigated. Log phase cultures of T. ther-
*mophila* were washed in Osterhout’s media and resuspended to a concentration of $10^6$ cells/mL in Osterhout’s minimal salts media and were incubated at room temperature on an orbital shaker (50 rpm) for 24 h. One hundred microlitres of the starved culture were then added to wells of a 96-well plate. Control cultures were grown in PPYE, harvested, washed and resuspended to $10^6$ cells/mL in Osterhout’s medium and added to wells. One hundred microlitres of *E. coli* XL-1 (*pET-gfp*) at a concentration of $5 \times 10^8$ cfu/mL were added to the wells. The plates were immediately placed into the Victor 3V fluorescent plate reader at room temperature and fluorescence (480/530) was measured every 30 min for 12 h.

(ii) Cytochalasin B, a metabolic toxin of the fungus *Drechslera dematioideum* and known inhibitor of phagocytosis in animal cells including *Tetrahymena* sp. (Nilsson 1973), was used to confirm that the decrease in fluorescence was due to phagocytic uptake of the bacteria by the *Tetrahymena*. Cytochalasin B (Sigma) was resuspended in DMSO and diluted in Osterhout’s media to working dilutions that resulted in final exposure concentrations of 50, 10, 5, 1.0 and 0.1 µg/mL and a final DMSO concentration of 0.5%. *T. thermophila* ($10^6$ cells/mL) were pelleted and resuspended in the appropriate dilutions of Cytochalasin B and a control of Osterhout’s with 0.5% DMSO. One hundred microlitres of the dilutions were pipetted in triplicate into microtitre plate wells. The *Tetrahymena* were exposed to the appropriate Cytochalasin B concentration for 30 min prior to the addition of 100 µL of $5 \times 10^8$ cfu/mL *E. coli* XL-1 (*pET-gfp*). Controls of Cytochalasin B with the bacteria alone were also included. The plates were immediately placed into the Victor 3V fluorescent plate reader at room temperature and fluorescence (480/530) was measured every hour for 12 h.

(iii) Log phase *T. thermophila* cultures were washed (3x) in Osterhout’s medium resuspended to a concentration of $10^6$ cells/mL in Osterhout’s medium containing CuCl$_2$ at concentrations of 50, 10, 5, 1, 0.1 and 0 µg/mL. The cells were exposed for 2 h at room temperature because a loss of viability is unlikely for most concentrations in this time frame (Nilsson 1981; Dayeh et al. 2005). Cells were then pelleted by centrifugation and washed 3 times in Osterhout’s media and resuspended to a concentration of $10^6$ cells/mL. They were then applied to the microtitre plate and 100 µL of $5 \times 10^8$ cfu/mL *E. coli* XL-1 (*pET-gfp*) was added and fluorescence monitored as described above. The effect of the CuCl$_2$ on the GFP expression was also tested as a control by exposing the bacteria to the same concentrations as the ciliate.

### Results

**Fluorescence from Microwells with Fluorescent Protein Expressing Bacteria (FPEB)**

Two characteristics of fluorescent protein expressing bacteria (FPEB) that would be critical to the development of a microwell plate assay for monitoring bacterial phagocytosis by *Tetrahymena* were investigated. First, the relationship between fluorescence and bacterial number was established with *E. coli* XL-1 (*pET-gfp*). A good correlation was observed between relative fluorescent light output units (RFUs) and bacterial viable counts (colony forming units; cfu) (Fig. 1). The results plotted are dilutions from two separate overnight cultures. The plot was linear for greater than two logs with the lower limit of detection (out of the linear range) being approximately 9000 RFU corresponding to $10^6$ cfu/mL *E. coli*. Very slight variations in RFU output occurred between batches of *E. coli* grown on different days, however experimental results were always compared to internal controls and therefore this slight variation is inconsequential. Second, the stability of the fluorescent output by the bacteria in a physiological buffer appropriate for *Tetrahymena* was investigated. The *E. coli* XL-1 (*pET-gfp*) incubated on its own in Osterhout’s media continued to express GFP with the RFU doubling over the course of the 12-h assay.

**Fluorescence from FPEB in Microwell Cultures with Tetrahymena**

When FPEB were in microwells together with *Tetrahymena*, the fluorescence (RFUs) from microwells declined with time. In the initial experiments, the co-cultures contained *T. thermophila* at $5 \times 10^5$ cells/mL and *E. coli* XL-1 (*pET-gfp*) at $10^8$ cfu/mL and underwent a rapid

![Fig. 1. Linear regression of fluorescent output (RFUs) versus *E. coli* XL-1 (*pET-gfp*) concentration (cfu/mL) in overnight cultures. The results of two independent dilution curves are shown. The equation from one curve is shown on the chart. The $R^2$ value was 0.9981.](image-url)
decrease in fluorescence (Fig. 2A). Near background levels were observed by four hours and no change occurred for the remaining 8 h of the assay. Experimental plates incubated in parallel and sampled for viable bacteria indicated that the cfu/mL decreased from $10^8$ to $10^5$ in 4 h with no further change at 24 h (Fig. 2B). Therefore, the decline in fluorescence represents the consumption of the bacteria by the ciliate. This pattern of bacterial removal was identical to that of non-transformed E. coli tested in batch culture with T. thermophila (data not shown) indicating that GFP expression did not affect the outcome of the assay.

The optimal conditions for measuring a decrease in fluorescence and thus measuring bacterial consumption or clearance were explored by varying the concentrations of Tetrahymena and E. coli in the wells of the microtitre plates. Three bacterial concentrations ($10^8$, $5.0 \times 10^8$ and $10^9$ cfu/mL) and three protozoa concentrations ($10^5$, $10^6$ and $10^7$ cells/mL) are shown in Fig. 3A, 3B and 3C. At the highest Tetrahymena concentration ($10^7$ cells/mL) the clearance of the bacteria, independent of bacterial concentration, was not as efficient as seen at $10^6$ cells/mL. The lowest Tetrahymena concentration (105 cells/mL) required longer to clear the bacteria at all bacterial concentrations. Lower protozoa concentrations were unable to measurably decrease fluorescence (data not shown). At $10^6$ cells/mL the T. thermophila effected a 9-fold decrease in relative fluorescence in 2 h at $5 \times 10^8$ cfu/mL E. coli, a 6.6-fold decrease at $10^8$ cfu/mL E. coli and a 4.5-fold decrease at $10^9$ cfu/mL E. coli. All subsequent experiments were carried out at the optimized concentrations of $10^6$ T. thermophila cells/mL and $5 \times 10^8$ E. coli XL-1 (pET-gfp) cfu/mL which is a predator:prey ratio of 1:500.

![Fig. 2.](image1) Microtitre plate bacterivory assay of $10^5$ cells/mL Tetrahymena in co-culture with $10^8$ cfu/mL E. coli (▲), E. coli XL-1 (pET-gfp) alone (○) and Tetrahymena alone (■). The results were averaged from two separate experiments with triplicate wells in each. Standard deviations are represented on the graphs. (B) E. coli plate counts (cfu/mL) of viable bacteria recovered from the wells of an E. coli/Tetrahymena co-culture. The results are the mean and standard deviation of three wells from a single experiment.

![Fig. 3.](image2) Microtitre phagocytic assays illustrating the effect of different concentrations of T. thermophila and E. coli XL-1 (pET-gfp) in the wells of the assay plates and the changing rate of fluorescence decline depending on the concentrations of the two species. (A) $1 \times 10^7$ cfu/mL E. coli, (B) $5 \times 10^8$ cfu/mL E. coli and (C) $1 \times 10^9$ cfu/mL E. coli. The Tetrahymena concentrations in each graph were $10^5$ cells/mL (▲), $10^6$ cells/mL (■) and $10^7$ cells/mL (○). Fluorescent output was monitored for 12 h. The results are expressed as the mean and standard deviations of 5 wells of each concentration mixture.
Effect of Cytochalasin B

As phagocytosis in ciliates and other eukaryotic cells is dependent on actin and inhibited by actin-disrupting drugs such as cytochalasins (Nilsson 1977; Zackroff and Hufnagel 1998), the capacity of *T. thermophila* to reduce the fluorescence in co-cultures with *E. coli* XL-1 (*pET-gfp*) was examined at different Cytochalasin B concentrations. Cytochalasin B was found to inhibit the decrease in RFU in a dose-dependent manner (Fig. 4). At low concentrations (0.1 and 1.0 µg/mL) the curves were identical to the control. In the presence of 50, 10 and 5 µg/mL Cytochalasin B, *Tetrahymena* were unable to decrease the fluorescent intensity below the initial value and indeed at 10 and 50 µg/mL the fluorescent intensity increased over time, likely as a result of the bacteria proliferating. Therefore, the decrease in fluorescence appears to be due to the removal of bacteria by phagocytosis and complete inhibition of phagocytosis allows continued expression of GFP.

Effect of Starvation

When the capacity for bacterivory was compared between *Tetrahymena* that were starved for 24 h with *Tetrahymena* that were from log phase in growth medium (PPYE), the kinetics of RFU reduction was clearly slower for starved cells (Fig. 5). There was consistently a 2-h lag before any measurable decrease in RFUs occurred in the wells containing starved *T. thermophila* and the time to reach the lowest values took approximately 3 h longer. Additionally, the maximum slopes were different for starved cells and log phase cells and when calculated as grazing rates a significant difference was observed (students t-test, p ≤ 0.05). With starved *Tetrahymena* a maximum grazing rate of 610 ± 45.5 bacteria/ciliate/hour was calculated whereas the maximum rate was 973 ± 17.5 bacteria/ciliate/hour for the log phase *Tetrahymena*. The calculations used to determine bacterivory rate were based on the linear regression of the slopes (change in RFUs over time) in Fig. 5 and the regression equation calculated from Fig. 1 correlating colony forming units with RFUs. For this calculation the assumption was made that all loss in fluorescence was due to bacterial consumption by the ciliates.

Effect of Copper

A 2-h exposure to CuCl₂ prior to an assessment of their capacity for bacterivory clearly showed that at some concentrations copper was inhibitory to *Tetrahymena* (Fig. 6). The progress of RFU reduction with time was the same for control cells as cells that had been treated with 0.1 µg/mL. However, in co-cultures in which *Tetrahymena* had been exposed to 1, 5 and 10 µg/mL, no reduction in RFUs was observed over 4 h, suggesting that phagocytosis was completely inhibited by these copper concentrations. In co-cultures in which the ciliate had been exposed to 50 µg/mL, RFUs did decline, although not in the manner of a typical phagocytic profile as with control cells, and it did not decrease as far. A similar profile of fluorescence decline was observed when *E. coli* alone was exposed to a copper concentration of 10 µg/mL (Fig. 6, inset). In co-cultures that had been treated with 50 µg/mL signs of toxicity were seen. The culture contained at least some *Tetrahymena* cells with altered motility, shape and phase contrast microscopy appearance.

Fluorescence Microscopy

The fate of *E. coli* XL-1 (*pET-gfp*) in co-culture with the *Tetrahymena* was monitored by fluorescent microscopy.
Fig. 6. Effect of CuCl₂ exposure on bacterivory by T. thermophila. The Tetrahymena (10⁶ cells/mL) were exposed to either 0 µg/mL (☐), 0.1 µg/mL (●), 1 µg/mL (▲), 5 µg/mL (●●), 10 µg/mL (■) or 50 µg/mL (▲▲) CuCl₂ in Osterhout’s media for 2 h prior to performing the microtitre plate fluorescent assay with 5 × 10⁵ cfu/mL E. coli XL-1 (pET-gfp). Inset: E. coli alone at 10⁷ cfu/mL incubated for 2 h with 10 µg/mL (x), 1 µg/mL (▲) and 0 µg/mL (□) CuCl₂ in Osterhout’s media.

This also confirmed that FPEB were engulfed and fluorescence diminished with time. At 15 min brightly fluorescent bacteria could be observed within food vacuoles of the Tetrahymena (Fig. 7B). Observations at 2 h revealed the presence of many full food vacuoles with intact bacteria inside and some vacuoles fluorescing very bright green but amorphous, suggesting lysis of the bacteria and subsequent release of the GFP into the vacuole (Fig. 7D). At 4 h, fewer bright vacuoles were observed. More vacuoles were amorphous and those containing bacteria contained fewer bacteria (Fig. 7F). At 24 h, only a few protozoa contained any fluorescent bacteria at all (Fig. 7H). GFP-expressing bacteria were observed outside the protozoa at all time points, however they decreased rapidly over the 24 h.

Discussion

The decline in RFUs in co-cultures of E. coli XL-1 (pET-gfp) T. thermophila is interpreted as a measure of phagocytosis or bacterivory by the ciliate and is referred to as the ciliate bacterivory (CB) assay. Commonly, phagocytosis of bacteria by ciliates is thought to consist of three steps: capture, phagosome formation and digestion (Verni and Gualtieri 1997). The CB assay likely involves all these steps. Three types of observations emphasize the necessity for capture and for phagosome formation. Fluorescence microscopy showed that the fluorescent protein-expressing bacteria (FPEB) were engulfed during the assay and subsequently lost their fluorescence (Fig. 7). Second, the decrease in fluorescence correlated with viable counts of bacteria remaining in the wells, suggesting that decrease in fluorescence corresponded with removal of the bacteria (Fig. 2). Third, Cytochalasin B, which is known to block the formation of phagosomes in Tetrahymena (Nilsson 1977; Zackroff and Hufnagel 1998), prevented the decrease in RFUs (Fig. 4). As for the mechanism of fluorescence loss once the bacteria have been engulfed, two processes are likely occurring: acidification of the food vacuole (phagosome) and digestion (Parry et al. 2001). The fluorescence intensity of GFP decreases between pH 5.5 and 4 (Bokman and Ward 1981) and during the formation of phagosomes in Tetrahymena the pH of food vacuoles decreases to 6.0 to 5.5 during the first 10 min and declines to 4.0 to 3.5 by 60 min (Nilsson 1977). However, the time course for the decline in RFUs likely is more complex in the CB assay than just the time course of vacuole acidification because the bacteria might need to be partially digested in order to expose the GFP to the acidic environment.

The basic parameters for the assay have been defined but in the future these parameters could be explored further to determine assay robustness. For at least 12 h in microwell cultures without ampicillin, E. coli XL-1 (pET-gfp) expressed GFP, which should allow the flexibility of doing the assay with or without the antibiotic. One surprising observation was that RFU increased approximately twofold over 12 h with E. coli XL-1 (pET-gfp) alone in Osterhout’s medium, which is a starvation buffer (Fig. 2). It is likely that residual energy sources supported some proliferation and thus an increase in the amount of GFP in microwells. Although the rate of RFU reduction was lowest at the lowest Tetrahymena concentration, the most rapid reduction was not at the highest Tetrahymena concentration but at an intermediate concentration (Fig. 3). The reason why Tetrahymena at 10⁷ cells/mL were not as efficient at engulfing bacteria as Tetrahymena at 10⁶ cells/mL could have multiple causes, such as modulation of Tetrahymena behaviour by cell density, competition for prey, and waste by-product buildup. At Tetrahymena concentrations below 10⁵ cells/mL, the RFUs in wells were not reduced. However, lowering the number of bacteria per well and extending the coculture time might allow a decrease to be detected with many fewer Tetrahymena.

The CB assay has several advantages over other assays for Tetrahymena phagocytosis and over assays for generally monitoring Tetrahymena viability. Some phagocytic assays have used inert fluorescent particles (Dias et al. 2003), but the very strong and stable fluorescent output of the E. coli XL-1 (pET-gfp) (Power et al. 2001) has allowed the use of living bacteria, which provides ecological relevance. Fluorescent protein-expressing bacteria (FPEB) have been used to monitor bacterial uptake by Tetrahymena by microscopy (Brandl et al. 2005), flow cytometry (Fu et al. 2003) and fluorimetry (Parry et al. 2001), but in the CB assay the use of a fluorescence microplate reader dramatically reduces the
Fig. 7. Bright field and fluorescent light micrographs of the *T. thermophila* and *E. coli* XL-1 (pET-gfp) recovered from the wells of a microtitre plate experiment taken at 15 min (A,B), 2 h (C,D), 4 h (E,F) and 24 h (G,H) following the introduction of the predator and prey. Intact bacterial cells are apparent within vacuoles at 15 min and 2 h (arrowheads) while mainly brightly fluorescent amorphous vacuoles are observed at 4 h (arrow). At 24 h very few bacteria or fluorescent vacuoles were observed. All micrographs were taken at 400X.
labour, making the assay rapid and inexpensive. As well, the detection range was two log linear. These characteristics make the kinetics of bacteria uptake to be followed relatively easily. A fluorescent microwell plate reader assay has been developed for monitoring *Tetrahymena* viability, and the response of *Tetrahymena* to some toxicants correlated with response of fish cells (Dayeh et al. 2004). However, the CB assay is easier and cheaper to perform and can measure a sublethal response.

Starvation influenced the kinetics of bacterivory as evaluated with the CB assay. For *Tetrahymena* that had been starved for 24 h, bacterivory took approximately 2 h to begin. The delay fits observations made by others using light microscopy. Food vacuole formation was observed to decline or even stop during starvation but was restored by the addition of particles (Chapman-Andresen and Nilsson 1968). In the current study, the *E. coli* XL-1 (*pET-gfp*) would be the stimulus for phagocytosis to resume in starved cells. Once begun, bacterivory was slower for starved cells. A rate of 610 ± 45.5 bacteria per ciliate per hour was found for *Tetrahymena* that had been starved and 973 ± 17.5 bacteria per ciliate per hour for *Tetrahymena* from log phase cultures. These rates were within the range that has been described for ciliates in a natural aquatic environment. A grazing activity of 6 to 6000 bacteria per ciliate per hour was found for lake water (Carrias et al. 1996).

Copper was used to explore the utility of the CB assay in studying the effects of a toxicant. A 2-h exposure to 1 µg/mL copper inhibited bacterivory initially, although some recovery (seen as a decline in fluorescence) began at 4 h. Similarly, when food vacuole formation in *Tetrahymena* was monitored microscopically, phagocytosis was found to be inhibited by 0.5 to 1.5 µg/mL copper (Nilsson 1981), yet a 24-h exposure to this copper concentration had no effect on *Tetrahymena* viability (Dayeh et al. 2005). Thus, the CB assay can detect a sublethal change in an important physiological function and one of larger ecological relevance. Copper has been found to change microbial food webs in soil (Ekelund et al. 2003), sewage treatment plants (Garcia et al. 1994), and well water (Artz and Killham 2002). These changes could possibly be mediated by the actions of copper on bacterivory. At 50 µg/mL, copper was inhibitory but also likely toxic, not only to the protozoan but also to the bacteria. At first glance it appears that at this concentration the inhibition of bacterivory is less than at 1, 5 and 10 µg/mL, however a possible explanation for this is that some copper was carried over from the exposure period into the CB assay, either from inadequate washing steps or within the protozoa, and this inhibited the fluorescent output of *E. coli* XL-1 (*pET-gfp*). This seemed likely since *E. coli* was incubated with a concentration of 10 µg/mL CuCl₂ a similar decrease in fluorescence was observed (Fig. 6, inset). This illustrates the necessity of running the appropriate controls with the CB assay since the results could be misinterpreted with the fluorescent indicator also being an organism potentially affected by the toxicity of the test environment. This finding also opens further avenues to be explored with the CB assay since individual toxicants may have different effects on the eukaryotes and prokaryotes.

The CB assay should be useful in studies from cell biology to ecology, but here some possible directions for developing the assay as a tool to monitor water quality are discussed. FPEB and *Tetrahymena* could be added to water samples and the CB assay used to compare these samples for their capacity to support ciliate bacterivory. Additionally, FPEB alone could be added to water samples and the CB assay used to compare these samples for their capacity for endogenous bacterivory. In both cases, the results could be correlated with conventional water quality parameters to build up correlations that would allow results with the CB assay to be predictive of quality. As well, water samples could be spiked with known environmental contaminants or microbes, including pathogens, and evaluated for their impact on bacterivory. This information could provide mechanistic insights into how these agents impact on quality. In the future, more sophisticated versions of the CB assay could be developed for monitoring purposes. These could include having multiple species of bacteria expressing fluorescent proteins of different emission colours. Blue-, cyano- and yellow-shifted mutants of GFP have been described as well as red fluorescent protein (Fu et al. 2003).

**Conclusions**

The fluorescent CB assay demonstrates in an easily measurable fashion the immense capacity of ciliated protozoa for bacterivory. This assay was capable of detecting changes in the physiological health of protozoan populations brought on by starvation or by the actin inhibitor, Cytochalasin B. It was also sensitive to a heavy metal toxicant in the form of CuCl₂. Although in the early stages of development, the assay promises to be useful as an indicator of water quality. With further development this assay could use *T. thermophila* as an indicator organism to rapidly detect changes in water quality affecting phagocytic capacity. Additionally, the assay may be useful as a measure of endogenous phagocytic capacity of native protozoan populations in natural and engineered aquatic ecosystems.

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