Potential Estuarine Water Quality Improvement Via Marine Invertebrate Bioremediation.

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Abstract

Estuarine water quality has declined during the past 100 years due to the development of land and harbors by mankind. Increasing nitrogen and phosphorus from fertilizer run-off has enabled algal blooms with subsequent increases in chlorophyll *a* and turbidity. Shoreline modification has altered water circulation in some bodies of water, resulting in unhealthy bacterial concentrations. These factors have caused a reduction in water clarity. Regulations have had a favorable impact in reducing the input of nutrients. However, the utilization of native filter-feeding invertebrates for the bioremediation of estuarine waters contaminated with pathogenic bacteria, unwanted algae, and general turbidity has not been thoroughly evaluated. Tunicates, commonly found in estuarine waters throughout the world, are capable of indiscriminate filtration of organic and inorganic particles, making them potential bioremediators. Therefore, the subtropical rough tunicate *Styela plicata* was examined for its prospective reduction of bacterial particle concentration. Laboratory tests reveal one average size (~ 40 g) *S. plicata*, exposed to 10^5 and 10^6 bacteria ml^{-1}, can filter as much as 4.7 L hr^{-1} with 100% efficiency. From these results it is estimated that 200 rough tunicates could fully remove 10^5 bacteria ml^{-1} from 22,600 L each day. The size and concentration of suspended particulates, water flow, and temperature would affect the rate of bacterial removal. Controlled concentrations of filter-feeders, such as the rough tunicate, strategically placed in contaminated areas could substantially reduce unwanted bacteria and algae, thus improving water quality. Other tunicates or filter-feeders common to problematic areas warrant further investigation in bioremediation.

1. Introduction

Development and anthropogenic disturbance have negatively impacted estuarine benthic and ichthyofaunal communities with the destruction of mangrove, salt marsh, and seagrass habitats, considered nursery grounds for many organisms (Heck, Jr et al. 2003). The major causal factor of a worldwide decline in seagrass populations is eutrophication and reduced water clarity (Short & Wyllie-Echeverria 1996). Mangrove and salt marsh habitats are additionally stressed through anthropogenic disturbances and shoreline modification as coastal populations increase (Kennish 2002). It is estimated that six billion people will inhabit the coastal regions of the world by the year 2025, almost equal to the total world population today (Hameedi 1997). The increased discharge of pollutants and industrial wastes as the coastal population expands will exacerbate the problems of reduced water clarity, sediment loading, and eutrophication with the eventual loss of nursery grounds.

Loss of habitat correlates with a loss of filter feeding benthic invertebrates that inhabit those grounds (Bingham 1992). Filter feeders assist in providing water clarity by removing particulate from the water column (Jørgensen 1966; Bone et al. 2003). The loss or reduction of filtration benefits provided by suspension feeders has potentially contributed to declining water quality (Dame et al. 2002) with a direct correlation in the increase of harmful algal blooms, bacterial outbreaks, fish kills, beach closures, and oxygen depletion (Worm et al. 2006). A recent study of the Florida Bay revealed a direct correlation between phytoplankton and cyanobacterial blooms with the large scale decline in dominant sponge populations; the microbial blooms occurred without any increase in nutrient loads (Peterson et al. 2006).

Alterations in water current due to the construction of seawalls, bridges, locks, and marinas result in pockets with poor water circulation, often stagnant, producing elevations of potentially pathogenic bacteria such as fecal coliforms. Low levels of fecal coliforms are commonly found in natural waterways; however, high levels can indicate sewage contamination (Madigan et al. 2000) resulting in human health risks. Means of control of these problems vary from mechanical, such as cordoning and partitioning areas by barriers, to remediation by invertebrates. As cordoning does not result in over-all water quality improvements, the search for the ideal organism(s) for bioremediation is the target of this investigation.

Numerous studies have been conducted in recent years utilizing bivalves as indicators of metal contamination and other pollutants (Arnold et al. 2006; Inoue et al. 2006) as well as to improve water quality (Kohata et al. 2003; Nelson et al. 2004). These studies indicate effectiveness of bivalves in reducing large quantities of organic and inorganic particulate matter (Officer et al. 1982). However, there is concern that even larger volumes of inorganic nutrients may be excreted back into the water column in the form of pseudofeces. Bivalve excrement stimulates primary production of phytoplankton (Kohata et al. 2003; Orlova et al. 2004), a further cause of decreased water clarity. Asmus and Asmus (1991) discovered that phytoplankton biomass by mussels was reduced by 37 ± 20% between upstream and downstream areas of a mussel bed however the excreted nutrients released by the bed stimulated even higher primary production than the uptake. Additionally, bivalves are capable of particle selection based on size (Defossez & Hawkins 1997) or composition (Kennedy et al. 1996; Ward & Shumway 2004). With particles >4 µm, bivalves have a particle retention efficiency of approximately 100%; however, with bacterial sized particles the retention efficiency drops to 20% (Stuart & Klumpp 1984) making them ineffective against bacterial blooms.

Milanese et al. (2003) examined the sponge *Chondrilla nucula* as a potential

bioremediator for use in aquafarming. Their extrapolated results estimated that a patch of this sponge one meter square could retain up to 7.0 x 10^{10} *Escherichia coli* cells hr^{-1}.

Another group of organisms that warrants investigation for its members' abilities to remove particles from the marine environment is the tunicates (Chordata: Ascidiacea), also known as ascidians or sea squirts. Tunicates are filter-feeding animals capable of non-discriminate removal of inert, inorganic and organic particles, (Jørgensen 1966) including bacteria (Flood & Fiala-Médioni 1981) from marine waters. Some benthic solitary and colonial tunicates are capable of retaining particles as small as 0.6 µm with approximately 100% particle efficiency (Stuart & Klumpp 1984). Additionally, they do not create pseudofeces (Fiala-Médioni 1974), which make tunicates a more desirable organism for the bioremediation of estuarine waters.

The rough tunicate, *Styela plicata* (Lesueur, 1823), can be found in most subtropical waters of the world (Lambert & Lambert 1998; Thiyagarajan & Qian 2003) and has a more sophisticated branchial basket than other tunicates enabling higher volumes of filtered water (Fiala-Médioni 1978). For these reasons, it was investigated as a potential bioremediator for contaminated marine estuarine waters.

2. Materials and Methods

2.1 Collection and Acclimation of Organisms

Individual *S. plicata* specimens ranging in weight from 16.8-55.7g were collected from the Indian River Lagoon near Ft. Pierce, Florida, USA, in June and July, 2005. Each organism was carefully removed and placed in an aerated 19 L beaker of environmental seawater (28 ppt) and transported to the lab. Tunicates were manually cleaned of macro-fouling organisms and placed unsecured in 3.1 m x 0.66 m x 0.3 m troughs with flow-through treated seawater at 28 ppt for 48 hours to remove any remaining debris prior to experimentation. Treated seawater was ozonated, filtered (5 µm) and UV-treated to remove and reduce any micro-organisms present. During this time, tunicates were fed the microalgae *Isochrysis* aff. *galbana* (clone T-Iso) at a concentration of 1,000 cells ml^{-1} to keep gut contents to a minimum. Only healthy organisms exhibiting open siphons and consistent brown color were used for the experiments.

2.2 Experimental Design - Algal Filtration Rate

Nannochloropsis sp. 1 cultures (clone CCMP531 Bigelow Laboratory for Ocean Sciences, Maine, USA), were filtered twice through a 20 µm screen and placed in a water bath to be heat-killed at 45° C for 30 minutes. Immediately after heat shock, average cell counts from triplicate 10 ml samples were determined using a hemacytometer and phase contrast microscopy. *Nannochloropsis* sp. glows a consistent turquoise green color differentiating it from other particles present in the sample.

Fourteen tunicates were placed individually in 8 L beakers containing 6 L of treated seawater. Beakers were randomly placed in trough tanks of ambient flow-through water to provide water temperature stabilization. Specimens were secured by monofilament loop around the body and tied to 12 cm X 12 cm plastic grids and allowed to acclimate until each exhibited normal open siphon activity. Heat-killed

Nannochloropsis sp. was added to the beakers to obtain either 10^5 or 10^6 cells ml^{-1} (n=7 for each concentration). Six additional control beakers were established in the same fashion with the absence of a tunicate (n=3 for each algae concentration) to monitor any loss due to adherence to the plastic beaker or sedimentation.

Water temperature, salinity, and pH remained constant for the experimental period at 26° C, 28 ppt, and 8.5, respectively. Aeration was supplied by a pipette secured to the side of the beaker to prevent direct exposure of the tunicate to air bubbles. As microalgae was added, beakers were tapped lightly on the sides causing the tunicates to close their siphons so that a 10 ml water sample could be drawn at 'Time 0' prior to any filtration. All samples were drawn from the center of the beaker after stirring. Subsequent samples were taken at 1 hour intervals in this same fashion for a total of seven samples (six hours) per beaker. Samples were fixed with 1 ml of 10% buffered-formalin after completion of each hourly sampling and refrigerated at 4°C until processing. All samples were counted by hemacytometer within 96 hours. An average of three triplicate counts was taken from each hourly water sample.

2.3 Establishment of Bacterial Filtration Rate

2.3.1 Establishment of Bacterial Region for Flow Cytometry Analysis

Flow cytometry enables detection of a 'population of interest' by the demarcation of regions (i.e. gating) on a double parameter histogram. In three preliminary experiments we used a pure culture of *E. col*i grown in Luria broth to determine the gating region. Aliquots of this culture were collected, centrifuged at 10,000 rpm for 15 minutes, and the pellets resuspended in 0.85% NaCl. Dilutions of these samples were prepared as 1X, 1/100X, and 1/1000X. One ml samples of each dilution were analyzed with a Becton Dickinson Facs Calibur flow cytometer. A standard 10,000 events was collected for each sample. A control sample of buffer alone was also run. A double parameter histogram was established using Forward Scatter (FS) log vs. Side Scatter (SS) log channels. **Fig. 1 A–D** reveals a decrease in total events with increasing dilutions, indicating a properly established region. Further validation of the established region was achieved by staining *E coli* samples with SybrII green dye (Molecular Probes, Eugene OR). *E. coli* samples of 10^6 cells ml^{-1} were centrifuged at 10,000 rpm for 15 minutes, resuspended and washed twice in a solution of 0.85% NaCl followed by resuspension in 30mM potassium citrate and SybrII green dye at a dilution of 1:10,000 (Lebaron et al. 1998). One ml aliquots of both stained and unstained *E. coli* samples were analyzed using the same FS log vs. SS log region previously established and a FL1 histogram. Acquisition was stopped at 20 seconds in each case. A region was created on FL1 excluding the autofluorescence of the control unstained sample **(Fig. 2)**. The stained samples showed 91.35 % positive cells. These results were further confirmed by filtering the stained *E. coli* samples through a 0.2 µm filter, thus eliminating the bacteria **(Fig. 3).** As shown in **Fig. 3,** the gated events decreased from 91.35% to 2.5% after filtration. These three experiments indicate that the flow cytometry method developed for determining the *E. coli* population was valid.

2.3.2 Experimental Design – Bacterial Filtration Rate

Twelve tunicates were placed individually in 8 L beakers with 6 L of treated

seawater. Three control beakers with no tunicates were also established. The experimental design of the bacterial filtration experiment was the same as that described for the algae filtration experiment with changes only to numbers of replicates. Ethanol-killed *E. coli* (Oie et al. 1999) was added to each beaker to provide one of two treatment concentrations, 10^5 and 10^6 cells ml^{-1} (n=6 for each concentration). The *E. coli* strain was provided by Dr. Peter McCarthy of Harbor Branch Oceanographic Institution, Ft. Pierce, Florida. The control beakers only had 10^5 cells ml^{-1}. Hourly water samples (10 ml) were taken in the same fashion as in the algae experiment for a total of seven samples (six hours) per beaker. Water samples were fixed with 1 ml 10% formalin and were refrigerated at 4º C until preparation for flow cytometry.

2.3.3 Preparation of Bacterial Water Samples for Flow Cytometry

One ml was removed from each water sample and centrifuged at 10,000 rpm for 15 minutes. The supernatant was decanted and the pellet resuspended in 1000 µl 0.85% NaCl. This procedure was repeated, but the pellet was resuspended in 999.9 µl 30mM potassium citrate and 0.1 µl Syber Green II dye (1:10,000 dilution). Samples were analyzed with a Becton Dickinson Facs Calibur flow cytometer gated on the previously established region. Acquisition parameters were set to run a standard 20 seconds in each case. Triplicate acquisitions were obtained per water sample. The third acquisition was used in each case to assure uniformity and untainted results from cross contamination of sample tubes.

2.4 Calculation of Filtration Rate

Filtration rate is defined as the specific volume of water that has been completely cleared of suspended particles over a defined amount of time. With the algae filtration experiment, these particles were counted as cells ml^{-1} while the bacteria filtration experiment measured flow cytometer events ml^{-1}. Filtration rates were calculated following Quayles's formula that was modified by Willemsen for the adjustment of particle sedimentation as suggested by Coughlan (1969):

$$F=\left[\frac{V_0+V_t}{2nt}\right] \times \left[\ln\left(\frac{S^E_0}{S^E_t}\right) - \ln\left(\frac{S^{C2}_0}{S^{C2}_t}\right)\right]$$

where F = filtration rate in ml hr^{-1}; V_0 = volume of water remaining in experimental beaker after withdrawal of sample at time 0; V_t = volume of water in experimental beaker before withdrawal of sample at time t; n = number of animals; t = duration of experiment; S^E_0, S^E_t = suspension loads in cells or events ml^{-1} in experimental beaker at times 0 and t; S^{C2}_0, S^{C2}_t = suspension loads in cells or events ml^{-1} in control beaker with phytoplankton only at times 0 and t.

2.5 Statistical Analysis

All statistical analyses were done with *SAS* 9.1 software. Regression analyses were conducted on 1) dry organ weight (DOW) versus whole animal wet weight (WAW) , 2) mean hourly filtration rate of each individual tunicate vs. whole organism wet

weight in both the algae and bacteria experiments, and 3) dry organ weight vs. mean hourly filtration rate in the bacteria experiment. The *a priori* H_0 was that "weight does not have an effect on the volume of water cleared hr^{-1} by a tunicate". Differences were considered significant for probabilities *($P < 0.05$).* Repeated-measures ANOVA (critical alpha=0.05) was used to analyze filtration rates of the two food types and concentrations over time.

3. Results

3.1 Analysis of Weight Versus Filtration Rates.

Dry organs were found to represent a mean of 1.6% of the whole animal wet weight (n=15). Regression analysis revealed a positive relationship (r^2=0.7361) between whole animal wet weight (WAW) and the dry organ weight (DOW) with a resulting linear regression model of DOW = 0.0156(WAW) + 0.02426. However, no significant relationship was found between wet weight and average filtration rate of tunicates exposed to algae or bacteria (r^2= 0.0035 and 0.0036 respectively), nor with dry organ weight and average bacterial filtration rate (r^2=0.2637).

3.2 Filtration Rate - Algae

Initial cell counts taken at Time 0 were slightly lower than target concentrations. Algal cell concentrations fell below the threshold of reliable hemacytometer counting of 100 cells after only three hours in the 10^5 cells ml^{-1} treatment and four hours in the 10^6 cells ml^{-1} treatment. For this reason, statistical analyses of filtration rates are only from Time 0 through Hr 3 for both concentrations. Individual hourly filtration rates ranged from -2644 ml hr^{-1} to 5749 ml hr^{-1} (mean=2203, n=14). Within specimen filtration rates varied as much as 4677 ml from one hour to the next with no relation to tunicate weight as indicated above. Average cell count ml^{-1} hr^{-1} for each treatment was analyzed by regression analysis revealing a linear regression model of Cell count ml^{-1} = 79881 - 23571(hour), (r^2=0.7109) for the 10^5 cell ml^{-1} treatment (n=27) and Cell count ml^{-1} = 600265 -143162(hour), (r^2=0.8289) for the 10^6 cell ml^{-1} treatment (n=35) **(Fig. 4)**.

Hourly cell counts in the algae-only control beakers were highly variable. The 10^5 cell ml^{-1} control beakers revealed an apparent loss of 36% due to adherence or sedimentation between the onset of the experiment (Time 0) and Hr 1. However by Hrs 2 and 3 the average net loss had decreased to 30% and 21% respectively (**Fig. 5A**). The 10^6 cell ml^{-1} treatment indicated a net loss of 20% within the first hour followed by 6%, 10%, and 19% in the second, third and forth hours, respectively **(Fig. 5B).**

The average filtration rate per organism exposed to 10^5 algal cells ml^{-1} with controls factored in was 1663 ml hr^{-1} whereas the10^6 treatment filtered 2744 ml hr^{-1}. As the control beakers did not indicate a continuous net loss of algae, the filtration rates were recalculated without controls. Filtration rates without controls became more similar increasing to 3065 and 3252 ml hr^{-1} for the 10^5 and 10^6 treatments respectively **(Fig. 6)**.

3.3 Filtration Rate- Bacteria

Average hourly filtration rates for tunicates exposed to two concentrations of *E. coli* ranged from 750 ml hr^{-1} to 6068 ml hr^{-1} (mean 3439, n=12). Within specimen filtration rate ranges varied as much as 4174 ml from one hour to the next with no relation to weight. Regression analysis for the 10^5 cell ml^{-1} treatment revealed a significant relationship (r^2=0.8854) between time and flow cytometry events with a model of Events = 4018.3 -1325(hour). Linear regression analysis for the 10^6 cell ml^{-1} treatment similarly resulted in a significant relationship (r^2=0.6758) with a model of Events = 26418 -5998.6(hour). Less variability was found in the bacteria control populations from one hour to the next than was found in the algae controls, therefore very little difference was found in the filtration rate per organism with and without the controls. Those specimens exposed to the 10^5 bacteria ml^{-1} treatment filtered 4654 ml hr^{-1} when controls were factored in and 4353 ml hr^{-1} without controls. The 10^6 treatment resulted in filtration rates of 2225 ml hr^{-1} with controls and 2185 ml hr^{-1} without **(Fig. 6)**.

3.4 Analysis of Combined Food Types and Treatments

Repeated measures analysis of combined filtration rate data met the assumptions of normality of residuals (P=0.4136) and sphericity (P=0.2836). Wilkes-Lambda test indicates hourly filtration rates changed significantly with time ($F_{2,21}$ =10.69, P=0.0006) with the largest difference occurring between Time 0 and Hr 1 ($P > F$ = 0.0004). Slightly less difference was found between Hr 1 and Hr 2 (P = 0.0157). No interaction between time*treatment ($F_{2,21}$ = 0.37, P< 0.6921), time*food type ($F_{2,21}$ = 2.94, P= 0.0746) nor time*food type*treatment ($F_{2,21}$ = 2.71, P=0.0899) were found. Mean values of filtration rate averaged over all three sampling times were not significant between individual test subjects for the two concentrations ($P > F$ = 0.1389), however a significant difference in filtration rate averaged over all three sampling times was found between subjects for the two food types ($P > F$ = 0.0122) and food type*treatment ($P > F$ = 0.0015) indicating conflicting filtration responses to different food types and concentrations.

4. Discussion

Previous ascidian filtration rate studies have been based on volume filtered hr^{-1} g^{-1} dry organ weight (Fiala-Médioni 1978; Randløv & Riisgård 1979; Armsworthy et al. 2001). However, our findings indicate no relation between organism weight and filtration rate, suggesting filtration rates of *Styela plicata* should not be reported in weight-based units. Most of the specimens tested exhibited inconsistent filtration, often changing from a negative filtration rate, or expulsion of branchial contents, to a significant positive filtration in one hour. Because of this erratic behavior, the mean filtration rate for individual specimens over the course of the experiment was used in statistical analysis. Fiala-Médioni, (1978) reported a filtration rate of 3.5–5.0 (mean 4.1) L hr^{-1} for *S. plicata* exposed to the microalgae *Monochrysis lutheri* at a constant concentration of 2.0 x 10^4 cells ml^{-1} over the course of a 12 hour experiment. Her findings are slightly higher than our findings of a mean filtration rate of 3.1 L hr^{-1} when the tunicates were exposed to the 10^5 algal cells ml^{-1} treatment and 3.3 L hr^{-1} with the 10^6 treatment in our no control results. This discrepancy may be due to

differing algal species or concentration or her lower number of replicates (n=3) over a longer period of time.

In the present study filtration rates ranged from 1.6–3.3 L hr^{-1} with algae and 2.2 – 4.7 L hr^{-1} with bacteria. No selection based on particle size was noticed in our study; both particles are approximately the same size (1–2 µm). Algae filtration rates increased when the concentration of food increased as seen in **Fig. 6** both with and without the controls, however, the rate decreased with an increasing concentration of bacteria. These results are not expected if tunicates are non-discriminate filter feeders as claimed by Jørgensen (Jørgensen 1966). One possible explanation may be that the nutritional value of bacteria satisfies the metabolic needs of the tunicate more sufficiently than does algae. The result is that less water is filtered once this need is met. Another possibility is that tunicates are able to discriminate between particle types. Further testing is needed to determine if this is the case.

Extrapolating from our findings, 200 *S. plicata* would be able to filter as much as 940 L hr^{-1} or 22,560 L day^{-1} of bacterially contaminated waters at a concentration of 10^5 cells ml^{-1}. These same 200 organisms would filter 100% of the particulates in as much as 660 L hr^{-1} or 15,840 L day^{-1} of algae at a concentration of 10^6 cells ml^{-1}. One hundred percent clearance of particulates is not necessary for bioremediation. A two to three-fold reduction would restore the water to healthy standards in most cases. This filtration ability of *S. plicata* warrants further investigation for its potential positive impact on the environment.

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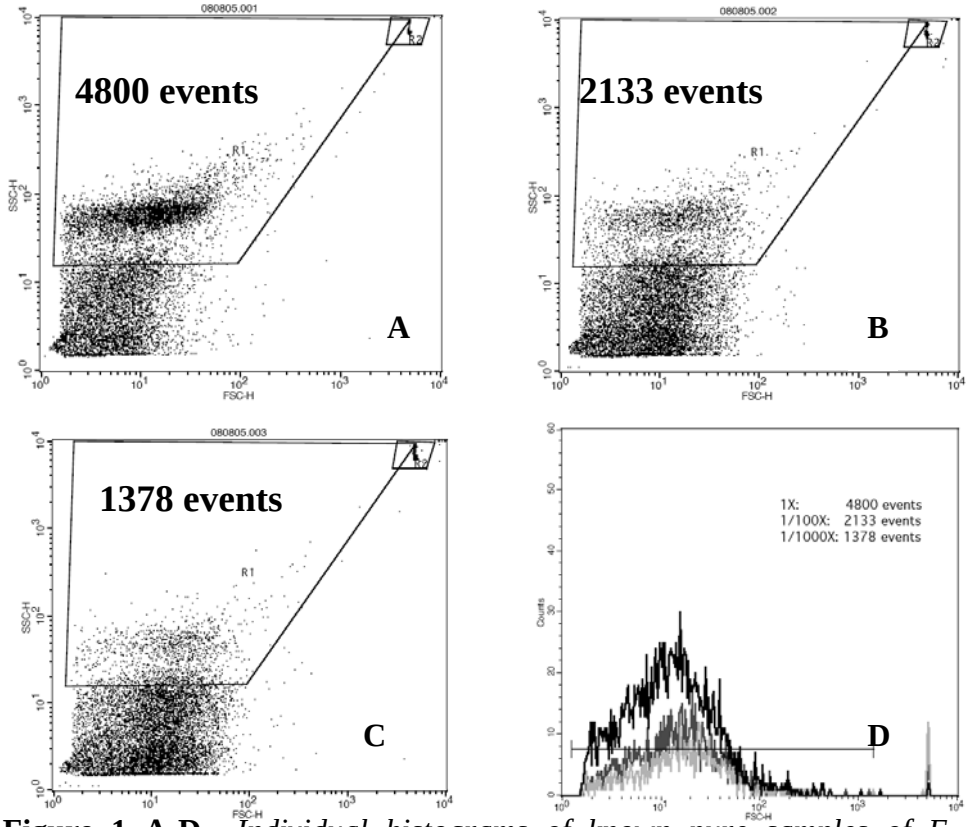

Figure 1 A-D *Individual histograms of known pure samples of E. coli prepared in dilutions to determine flow cytometric gating region.* **A** – 1X sample shows 4800 events within gated region. **B** – 1/100X dilution shows 2133 events in gated region. **C** – 1/1000X dilution shows 1378 events in gated region. **D** – Overlay histogram of the three dilutions. The small spike to the right is calibration microbeads (6µm) reflecting the small calibration gate visible in the upper right corner of histograms A – C.

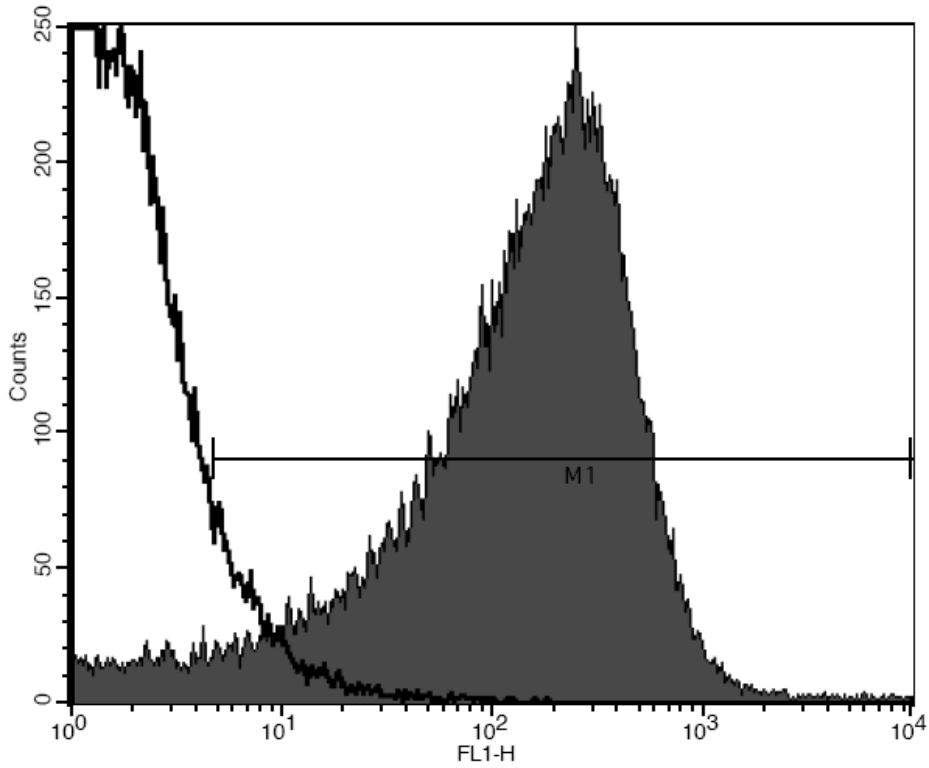

Figure 2 *Overlay histogram of FL1 region, gated on Escherichia coli*. Light peak is unstained control of sample buffer alone. Dark peak in region M1 is *E. coli* stained with SybrII Green bacterial stain. Histogram statistics indicate 91.35% positive cells in targeted M1 region.

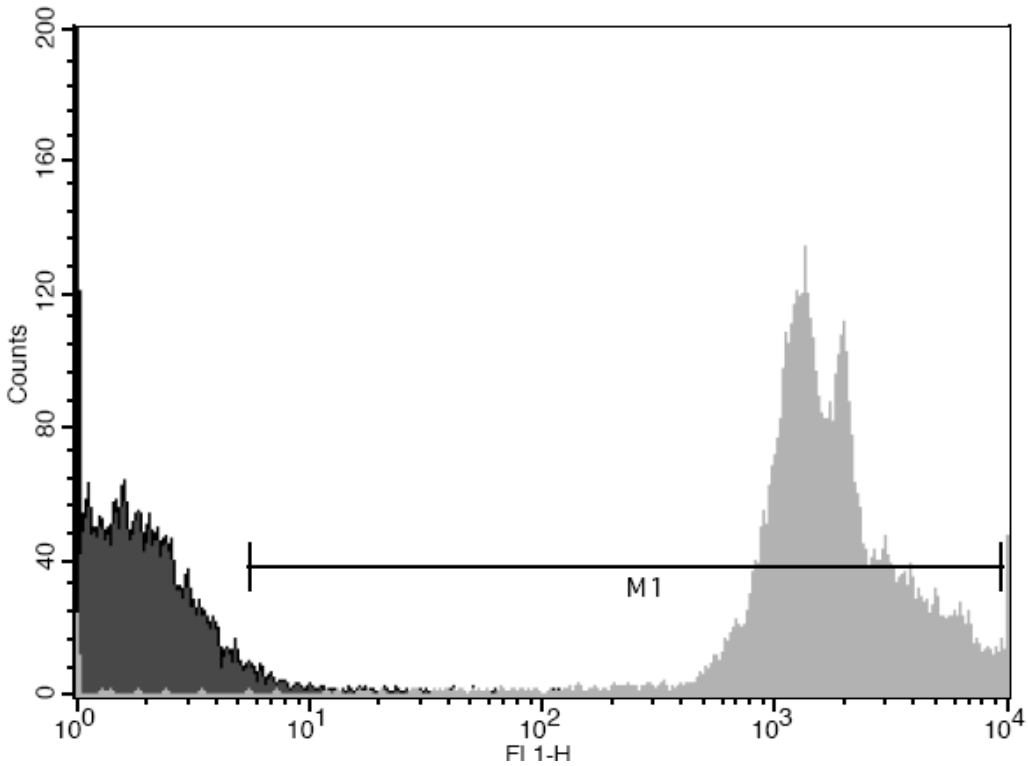

Figure 3 *Overlay histogram of stained unfiltered E. coli* (Region M1, light gray peak) *vs. E. coli filtered through a 0.2 µm filter* (dark gray peak) that eliminates bacteria. M1 gated peak indicates 91.35% positive cells prior to filtration and decreases to 2.5% after filtration.

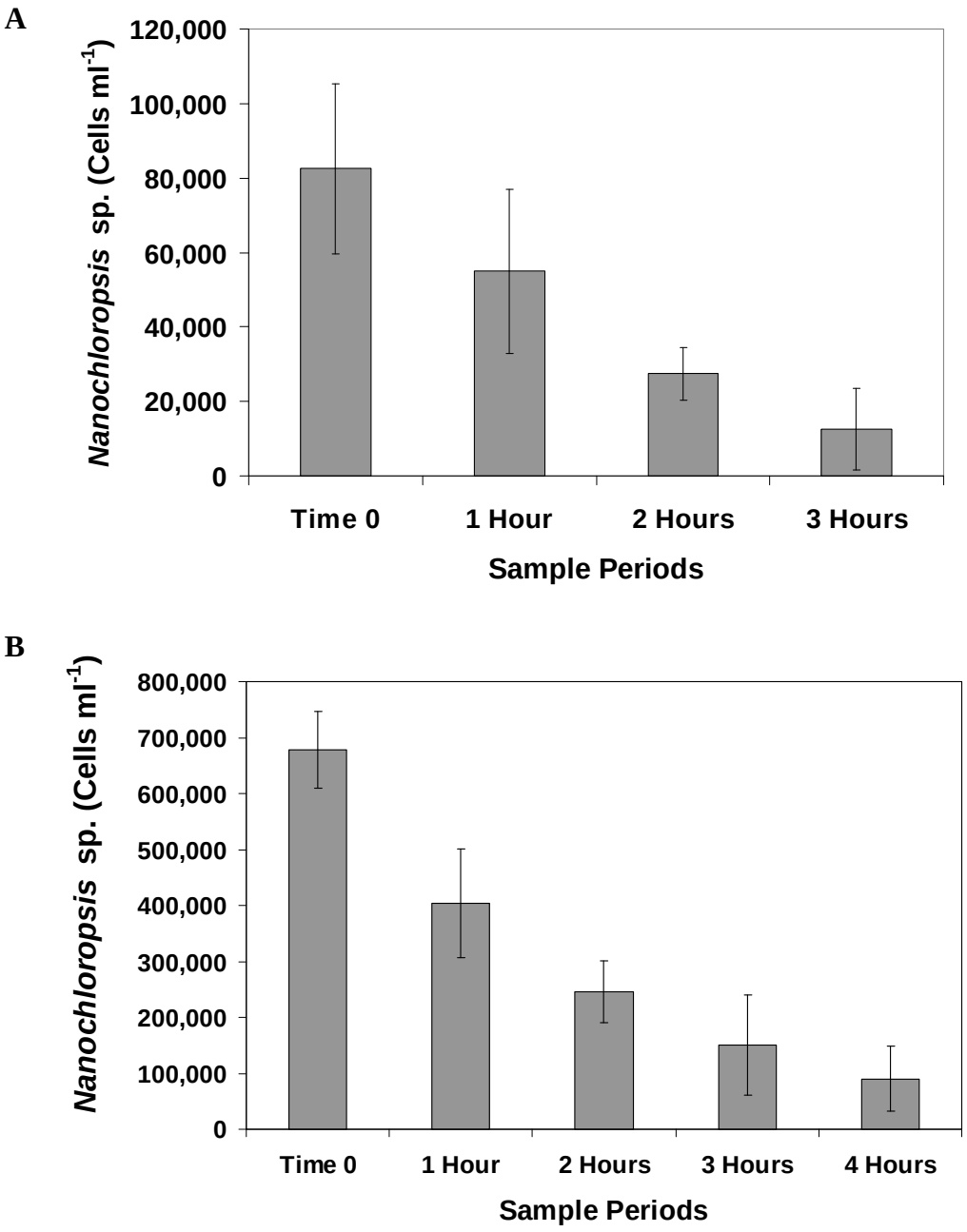

Figure 4 *Mean (± s.d., n=7) cell concentrations over time for tunicates, Styela plicata, exposed to two different concentrations of the microalgae Nannochloropis* sp. **A)** 10^5 algal cells ml^{-1} treatment. Linear decrease (y = 79881 - 23571(hour), (r^2=0.7109)) in the average hourly suspension load of algal cells, *(n=27).* **B)** 10^6 cells ml^{-1} treatment. Linear decrease (y = 600265 - 143162(hour), (r^2=0.8289)) in the average hourly concentration of cells remaining, *(n=35).*

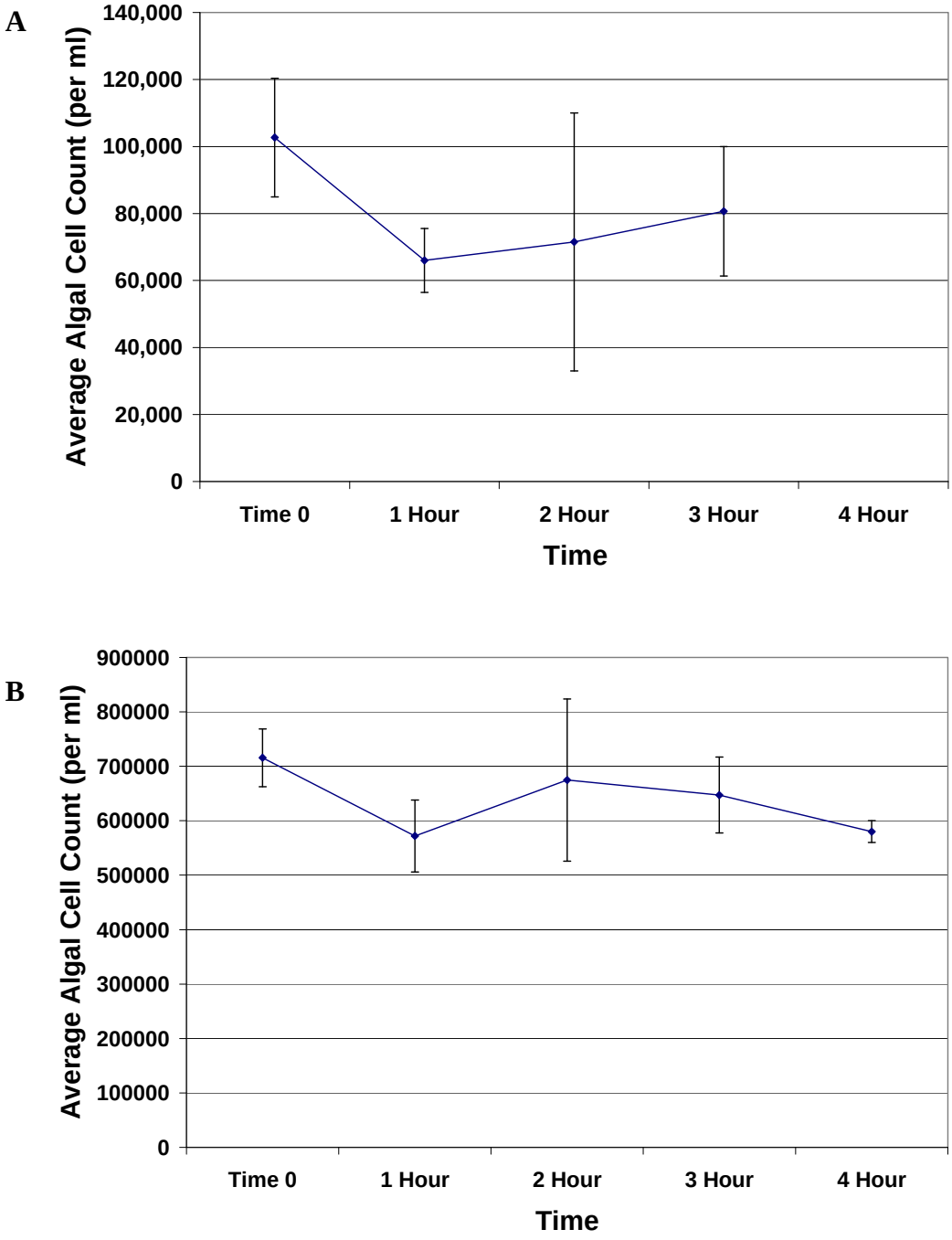

Figure 5 *Mean (± s.d., n=3) cell concentrations over time for control beakers without tunicates at two different concentrations of the microalgae Nannochloropis* sp. **A)** Mean for controls with 10^5 cells ml^{-1} treatment indicates a net loss of 36% due to settlement within the first hour. The second and third hours had a net overall loss of 30% and 21% respectively. **B)** Mean for controls with 10^6 cells ml^{-1} treatment indicates a net loss of 20% within the first hour. The second, third and fourth hours had a net overall loss of 6%, 10%, and 19% respectively.

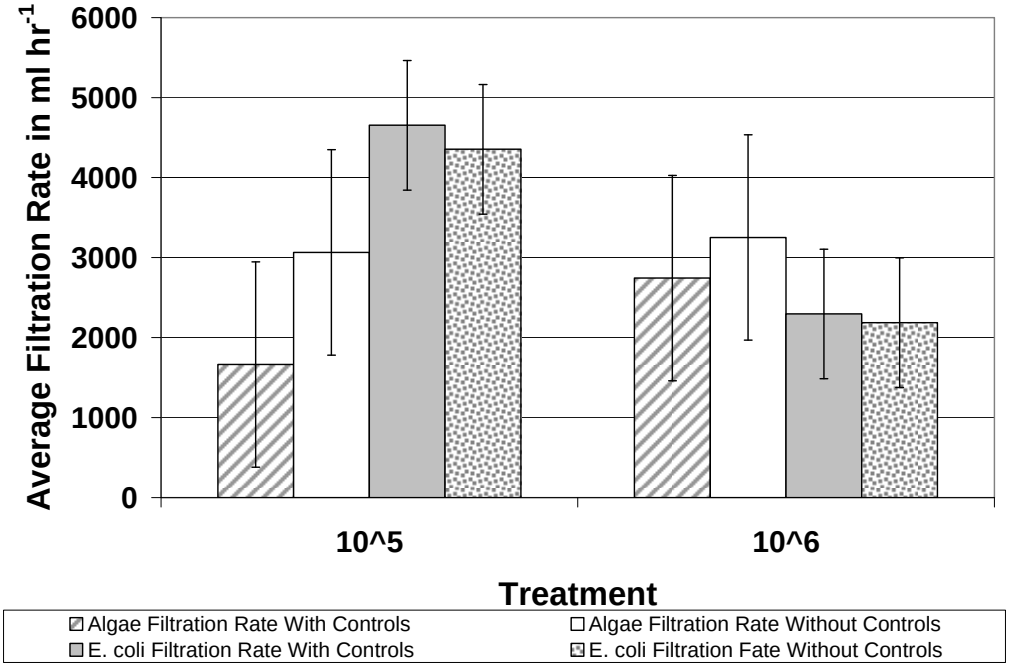

Figure 6 *Mean (± s.d.) filtration rates of Styela plicata exposed to two different concentrations of algae (n=7) and bacteria (n=6).* Algal filtration rates increased as food concentration increased whereas bacterial filtration rates decreased as food concentration increased.