The Effects of Violacein-Producing Bacteria on Microzooplankton Grazing and Phytoplankton Growth

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Abstract

Microzooplankton are grazers that are less than 200 µm. They are important in regulating population levels of bacteria and other microorganisms in the ocean. Also they have an important role in the microbial loop transferring dissolved organic carbon and they are an important food source. Violacein pigment is a toxic pigment produce by bacteria as a chemical defense and it has shown to kill freshwater microzooplankton. We experimentally investigated study how the violacein-producing bacteria affected microzooplankton grazing and phytoplankton growth in estuarine plankton communities. Our results show that the addition of VPB has a negative effect on growth of some phytoplankton but little effect on growth than microzooplankton grazing.

Keywords: Violacein-producing bacteria, violacein, microzooplankton grazing

Introduction

Microzooplankton includes grazers less than 200 µm in size (Sierburth et. al 1978), mostly ciliates, dinoflagellates and heterotrophic and mixotropic nanoflagellates (Griford et al. 2000). Grazing by microzooplankton is believed to play important roles in planktonic foodwebs (Gallegos 1989) regulating population levels of bacteria and nanophytoplankton via grazing (Verity 1986, McManus & Fuhrman 1988). They play an important role transferring dissolved organic carbon excreted by phytoplankton via the microbial loop to higher tropic levels (Ducklow et al. 1986, Sherr et al. 1988). Microzooplankton are considered to be the main mortality factor controlling phytoplankton biomass (Calbet & Landry 2004, Irigoien et al. 2005). If this mortality control does not exist, an imbalance in the food chain and in the ecosystem is created. In addition, they are an important food source for marine organisms like copepods. Copepods prefer to consume microzooplankton over phytoplankton (Gifford and Dagg 1988; Merrell and Stoecker 1998; Rollwagen-Bollens and Penry 2003). Microzooplankton grazing appears to

dominate all other sources of nutrient regeneration in most environments studied (Harrison 1980).

In this study we investigated the effects of violacein-producing bacteria (VPB) on microzooplankton grazing. Violacein is a purple colored pigment characterized as an L-tryptophan-derived alkaloid, consisting of three structural units: 5-hydroxyindole, 2-pyrrolidone, and oxindole (Deines et al. 2009). It is produced by at least for six genera of aquatic bacteria as a chemical defense against bacteriovorous flagellates (Matz et al. 2004). This pigment is highly toxic and has been shown to kill freshwater microzooplankton that ingest VPB (Deines et al. 2009). In laboratory experiments, violacein had a dramatic impact on feeding rates, growth and survival of three common species of bacteriovorous nanoflagellates. In some cases just less than three violacein-producing cells was sufficient to cause death of nanoflagellates (Matz et al. 2004). We wanted to know if these violacein-producing bacteria also have the potential to kill estuarine microzooplankton that ingest them and inhibit microzooplankton grazing on plankton.

VPB are associated with particles and sediments and they can be more toxic under high nutrient conditions (Matz et al. 2008). Some experiments of this study were conducted at Horn Point Laboratory in Cambridge, Maryland on the Choptank River during June and July 2011 (Figure 1). The Choptank River has the conditions to increase the probability of toxic VPB in the water, because, like other Chesapeake Bay tributaries, the Choptank River delivers particles and nutrients from runoff into the bay, (Hearn & Robson 2001, Hagy et al. 2004), which may raise the abundance and toxicity of VPB. Additional experiments were conducted in the Cheasapeake Bay (Figure 2) during the “Dead Zone Zooplankton” (DeZoZoo) research cruise aboard the R/V Hugh R. Sharp in July 2011.

Microzooplankton are an important component of the planktonic webs in the Choptank River (Reaugh et al. 2007) and Chesapeake Bay. Violacein- producing bacteria have been isolated from the river in spring (Stoecker, unpub. data) but their impact on microzooplankton grazing had not been investigated.

We hypothesized that the addition of violacein producing bacteria to the natural microplankton communities would inhibit microzooplankton grazing on phytoplankton. Also, we hypothesized that the addition of the violacein producing bacteria would have a negative impact on microzooplankton abundance.

Methods & Materials

VPB Culture

For our experiments we cultured VPB, Pseudoalteromas tunicata (strain 4-1B) (Figure 3) which was isolated from the Choptank River in May 2010 and shown to produce violacein (Stoecker, D.K., Chen, F. and Hill, R.T. unpub. data). To culture the bacteria we used marine agar plus tryptophan made with 7.4 salinity water and the plates were incubated at ~19-20ºC. Then the bacteria were suspended in phosphate buffered saline (Kemp et al. 1993) and were counted using epifluorescent microscopy (Kemp et al. 1993), to determine the bacterial concentration in the stock.

Dilution Method

Choptank River

To carry out these experiments we used the dilution method (Båmstedt et al. 2000), which was used to determine the microzooplankton community grazing on phytoplankton. We

collected water at the Horn Point Laboratory dock on the Choptank River (Figure1). A YSI meter was used to record the temperature and salinity (Table 1). Time was also recorded. Using a similar method as Landry and Hasset (1982), we made three different fractions of whole sea water (WSW): 100% WSW, 20% WSW and 5% WSW. These dilutions were placed in 500 ml polycarbonate bottles for incubation. For each dilution experiment we ran two or three dilution series: the control, with no VPB added, and the experimental series which had the addition of VPB. For each treatment we had 3 replicates bottles (Table 2). VPB were added to each bottle in an equivalence of ~10% of the natural bacteria abundance (~10^{-5} VPB ml^{-1}, final concentration). Also in some experiments we tried smaller additions of the VPB (~ 10^4 VPB ml^{-1}, final concentration) to determine the threshold for effects on grazing.

The bottles were labeled and placed in mesh bags, then were suspended from the dock for 24 hours. At the beginning (t=0) and end (t=24) of the experiment we took three water samples for enumeration of microzooplankton from the 100% WSW fraction in both control and experimental dilution series. These samples were preserved in 125 ml bottles with 6.25 ml of Acid Lugol's solution. We measured chlorophyll as a proxy for the total phytoplankton in the sample using fluorometry (Parsons et al. 1984). The microzooplankton samples were used to compare changes in microzooplankton populations, to determine if the addition of VPB enhanced growth or caused mortality of microzooplankton. We used the chlorophyll measurements to compare the grazing on phytoplankton in the bottles with and without the addition of VPB.

Chesapeake Bay

For the experiments carried out during the DeZoZoo research cruise on the Chesapeake Bay, we sampled at two stations: one at the South of the bay and one in the North (Figure 2). Surface water was collected from the Niskin bottles at the surface and then the dilution method was performed as on the experiments on the Choptank River. The bottles were incubated in a water table on the deck of the ship, they were placed inside of screens bags to reduce the surface irradiance to 25%. Light, temperature and salinity measurements were taken for each experiment (Table 1).

Algal growth rate

This laboratory experiment was carried out to determine if VPB and what concentration have an effect on algal growth rate. We used two algae cultures grown using the F/2 medium (Guillard and Ryther 1962, Guillard 1975, Andersen 2005). We selected two algae: *Isochrysis galbana* (prymnesiophyte) and *Chaetoceros calcitrans* (diatom). We placed dilute algal cultures (7 ml) in 11 ml test tubes and then added different concentrations of VPB: 0 (Control) 10^3, 10^4 and 10^5 per ml and then incubated the tubes for five days at 15ºC with an average irradiance of 52.354 µmol/m2/sec. We had three replicate tubes. Using in vivo fluorescence (Brand et al 1981), we took measurements of chlorophyll everyday to calculate the growth rate. Also samples were taken at the initial time (t=0) and at the end time (t=96) for cells counts.

Results

Field experiments

Dilution Method

Choptank River

Four experiments were carried between May and July 2011 at the Choptank River (Table 1). The results for the dilution experiment at the Choptank River describe and measure the growth and grazing on algae. We used a regression line to obtain the linear equation (y=mx + b) where m is equal to the slope and b is the y intercept. The net changes in populations (K) is assumed to be due to growth ("μ") and mortality (m), which in this case we assume that is caused by grazing ("g"), so we have K=μ-g. Results for the ANOVA test (Table 3) shows there is not a statistically significant difference between the values of "g" and "μ" among treatments. In experiments 1 and 2 phytoplankton growth appeared to be lower in the 10^5 VPB ml^{-1} treatment than in the Control but there was not a statistical difference between growth. The grazing coefficient, g, also appeared to be lower in 10^5 treatment but there was no significant difference.

For experiment three we just compared net growth rates K, in the 100% WSW treatment. The One Way ANOVA test results (Table 4) showed there was a significant difference between the K from control and the 10^5 VPB treatment, with net growth, K, lower in the 10^5 VPB treatment addition the K value was lower than in the K value for the control treatment. So, the addition had a negative effect on the net growth rate (K). For the fourth experiment, we ran an incubation with just 100% WSW but with two different series, one with a 21 day old VPB culture and one with a 6 days old VPB culture using concentrations of 10^3, 10^4 and 10^5 ml^{-1} added VPB. The results show a significant difference between the K value for the treatments with 10^4 and 10^5 added VPB ml^{-1}. It also seemed that the 6 day old VPB culture had a more negative effect on K than did the older culture, but there was no difference between the VPB cultures of different ages.

Chesapeake Bay

South Station

For the experiment at the South station the results of the dilution experiment are shown in Figures 4 and 5. The apparent growth rates (K values) of the three treatments: Control, 10^4 and 10^5 VPB ml^{-1} were significantly different among the different treatments (Table 5). The "g" and "μ" values (Table 3) were compared. The "g" values were not significant different among treatments, but VBP treatment had a significant effect on phytoplankton growth (the "μ" values) (Table 3- Experiment 5). The growth coefficient for the control appeared to be higher than for 10^5 VPB treatment, but the difference was not statistically significant.

North Station

This experiment shows a significant difference in the algae growth "μ" and microzooplankton grazing among VPB treatments (Figures 6 and 7; Table 3 –Experiment 6). The values of "g" and "μ" were compared, and show a significant difference between treatments (Table 3). The 10^5 treatment had lower "μ" compared with the other treatments. For "g" there was a significant difference between the 10^4 VPB and 10^5 VPB treatments, with g higher at 10^4 VPB. The K values for the three treatments: Control, 104 and 105 VPB were compared, and showed there was not a statistically significant interaction between dilution factor and treatment.

Effects on abundance of microplankton

The samples from the Choptank River were not counted. For the Chesapeake Bay dilution experiments, the abundance of dinoflagellates, diatoms and ciliates cells in the undiluted treatment was counted at t=0 and at the end of the incubation (t=1 day) in the control and 10^5 ml^{-1} VPB treatments (Figure 8). The results are in table 6 and show that the growth of

dinoflagellates appeared to be the most inhibited by the additions. After the 24 h incubation of south and north station samples, dinoflagellates abundances were higher than initial abundances, indicating significant growth, in the control treatments but not in the 10^5 ml^{-1} VPB treatments (Table 6). In the north station experiment, dinoflagellates densities in the control treatment were also significantly higher than in the VPB treatment at the end of the incubation (Table 6).

In the diatoms there was no inhibition of growth by VPB additions in the south station experiment, but at the north station significant growth occurred in the control treatment but not the VPB treatment (Table 6, Figure 8). There was significant increase in ciliate abundance in the control, but not VPB treatment, in the south station experiment (Table 6, Figure 8). In the north station experiment, ciliate growth also appeared to be inhibited by the VPB addition, but differences in abundance were not statistically significant (Figure 8, Table 6).

Algae growth rate

The algal growth coefficient, "μ", showed changes among treatments just with the *Chaetoceros calcitrans* cultures (Figure 9). For *C. calcitrans* the control treatment showed algae growth but with the VPB additions there was a significant negative effect on growth with mortality occurring in the 10^5 ml^{-1} VPB treatment. For *Isochrysis galbana* there was no difference between the algae growth of the control and the VPB treatments. This result is shown ion figure 9.

Discussion

Our results suggest that the addition of VPB to estuarine microplankton communities affected algal growth instead of inhibiting microzooplankton grazing. We had expected inhibition of grazing based on the results of Deines (2003) with freshwater microzooplankton. VPB doesn't have the same effect in all types of phytoplankton in natural assemblages or on cultures. In natural assemblages, dinoflagellate population growth was inhibited but diatom population growth was not always inhibited. In culture, the growth of the diatom *Chaetoceros calcitrans* was inhibited but there was no apparent effect on the prymesiophyte, *Isochrysis galbana*. We do not know why the diatoms were negatively affected in natural assemblages but the diatom culture was not affected by VPB additions.

If VPB have a high concentration (~10^5 cells ml^{-1}) in Chesapeake Bay, they would have a negative effect on phytoplankton growth. Upper trophic levels could be negatively affected by decreases in algal growth (primary production). Another effect could be on seasonal blooms of dinoflagellates. During some seasons VPB may increase in concentration and toxicity in the natural environment and this could inhibit algal growth, so in this manner blooms could be controlled. In the seasons that they are not present in significant concentrations there could be largest blooms. So VPB could have a negative impact on phytoplankton communities but there could also be a positive impact, the control of blooms the Chesapeake Bay and its tributaries.

Conclusion

We found that relatively high concentrations of VPB, ~10^5 ml^{-1}, are necessary to have a significant effect in 24 hours on microplankton. Additions of 10^4 cells ml^{-1} of our cultured VPB did not have a significant effect in 24 hours. Also, the addition of VPB at 10^5 cells ml^{-1} had a negative effect in some phytoplankton but not others and microzooplankton grazing was not

significantly inhibited. VPB had more negative effects on dinoflagellates growth than on ciliate or diatom growth in field experiments.

For VPB to have an effect in the natural environment, in estuaries it has to be at concentration range of ~10^5 ml^{-1}. In estuaries and coastal seas, estimates of total bacterial abundance range from ~ 1 x 10^6 ml^{-1} to 2 x 107 ml-1 (Azam et. Al. 1983, Coffin & and Sharp 1987). So, for there to be an effect at least ~10% of bacteria would have to be VPB. There is currently no evidence that VPB occur at these high densities in estuaries.

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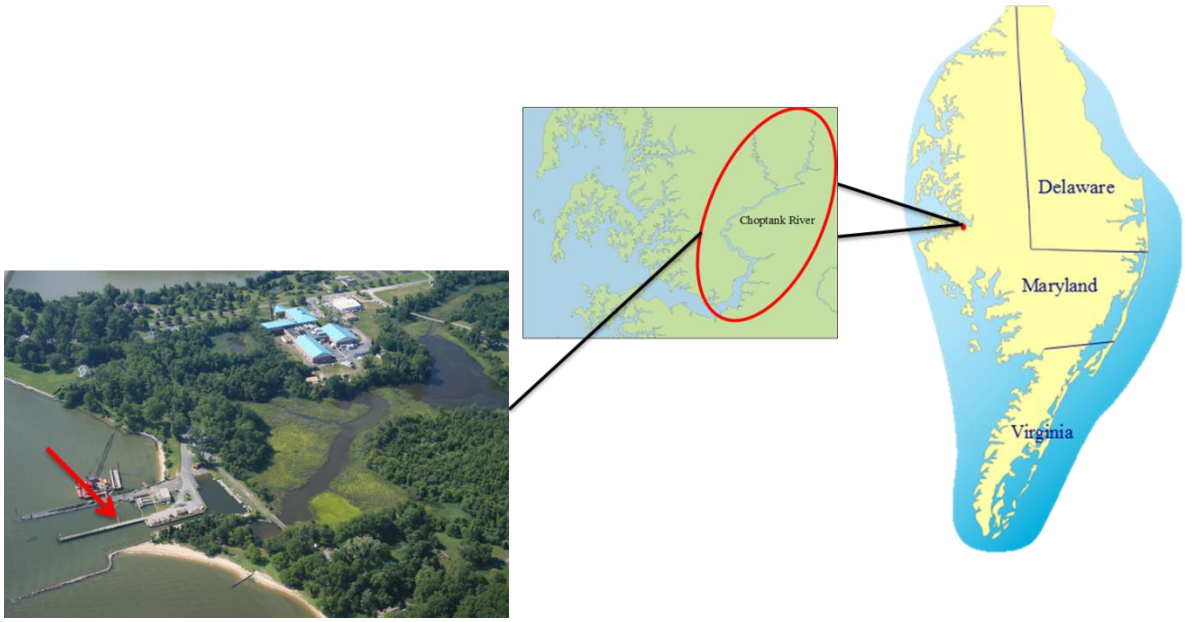

Figure1. Sampling location at Horn Point Laboratory, UMCES, Cambridge, MD, USA on the Choptank River, a tributary of Chesapeake Bay (Maps and photo provided by IAN).

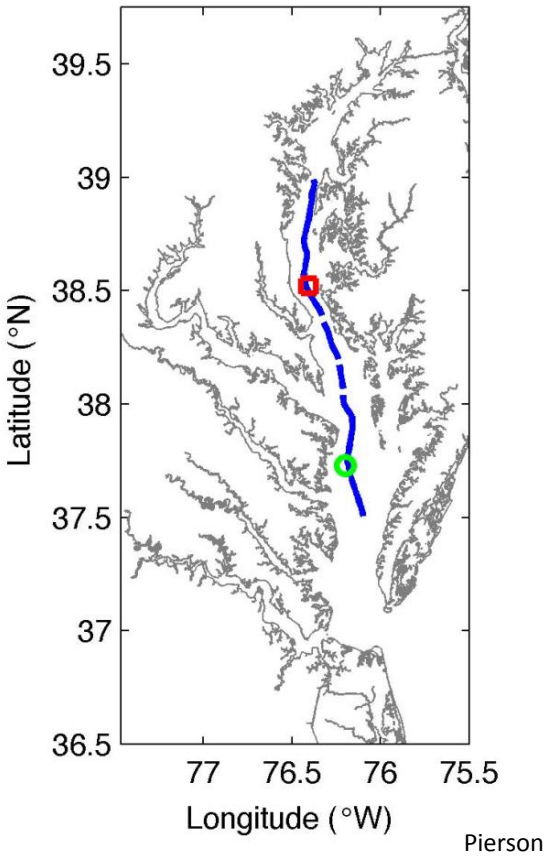

Figure2. Sampling stations on the Chesapeake Bay, the North station is indicated by the red circle and the South station by the green circle.

Figure3. Cultures of Violacein- producing bacteria, *Pseudoalteromonas tunicata* (strain 4-1B).

Dilution Experiment at the South Station

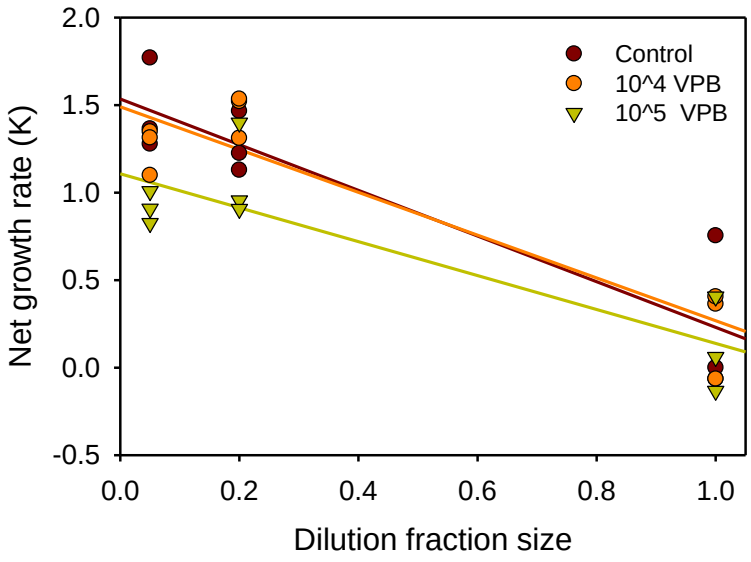

Figure 4. Linear regression for the dilution experiment at the South Station.

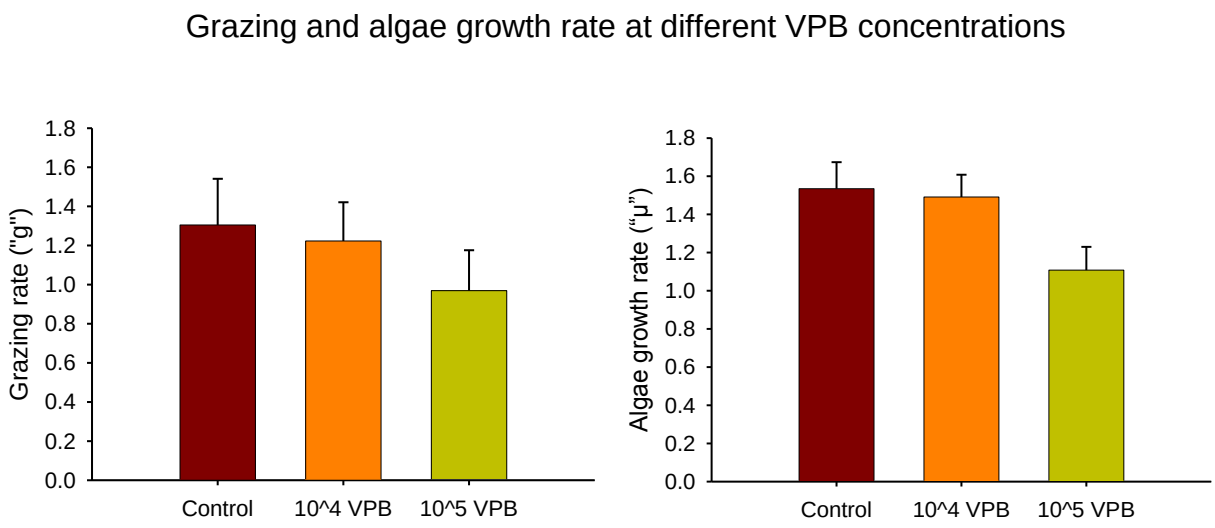

Figure 5. Grazing coefficients (*g*) and phytoplankton growth coefficients (μ) from dilution microzooplankton growth experiment with different VPB treatments at the South station.

Dilution Experiment at the North Station

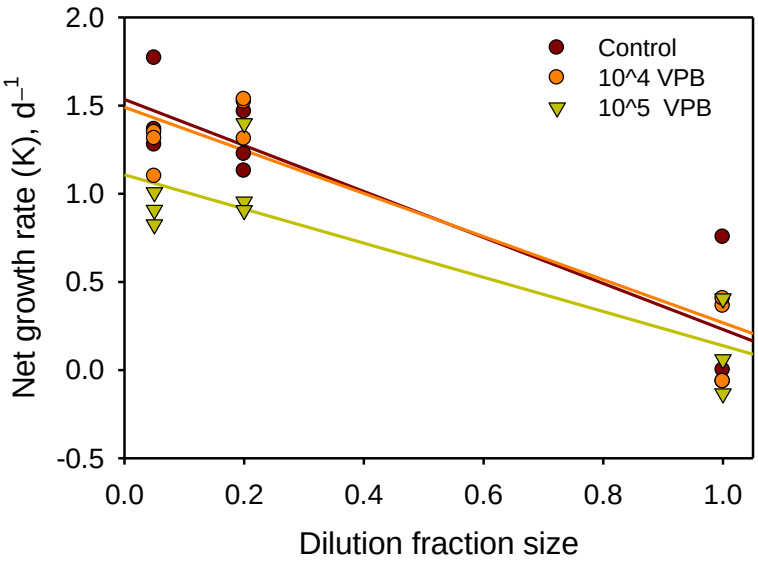

Figure 6. Linear regression for the dilution experiment at the North Station.

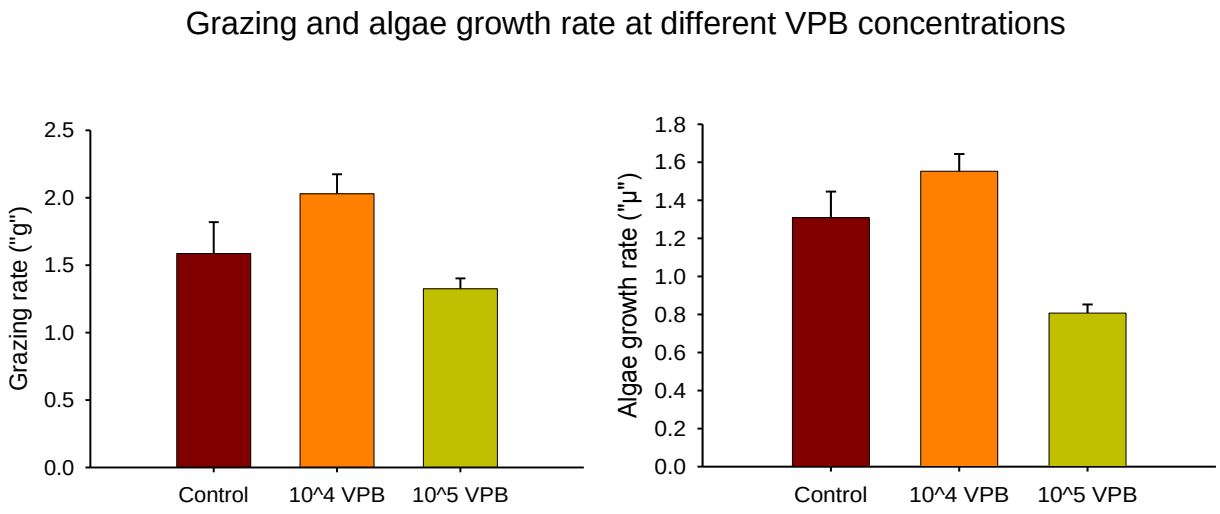

Figure 7. Grazing coefficients (g) and phytoplankton growth coefficients (u) from dilution microzooplankton growth experiment with different VPB treatments at the North station.

Microzooplankton and phytoplankton abundance (cells/ml) at t=0 and t=24h in incubations of whole seawater (<200 um fraction) at the North and South stations

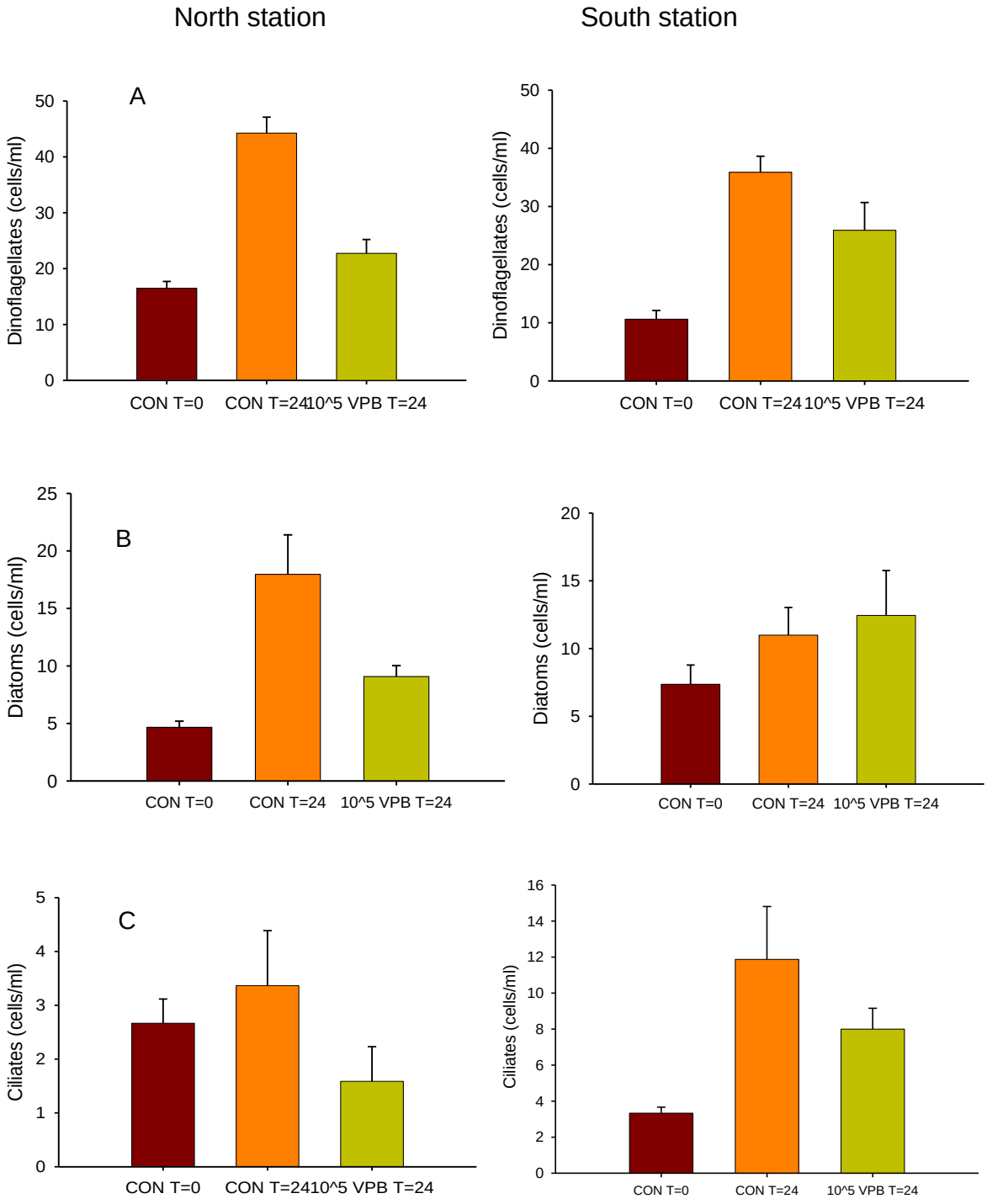

Figure 8. Abundances of cells in the different treatments: A. Dinoflagellates, B. Diatoms and C. Ciliates in the control at T=0 (red bar), control at T=24 (orange bar) and the experimental treatment (10^5 VPB ml-1) (green bar).

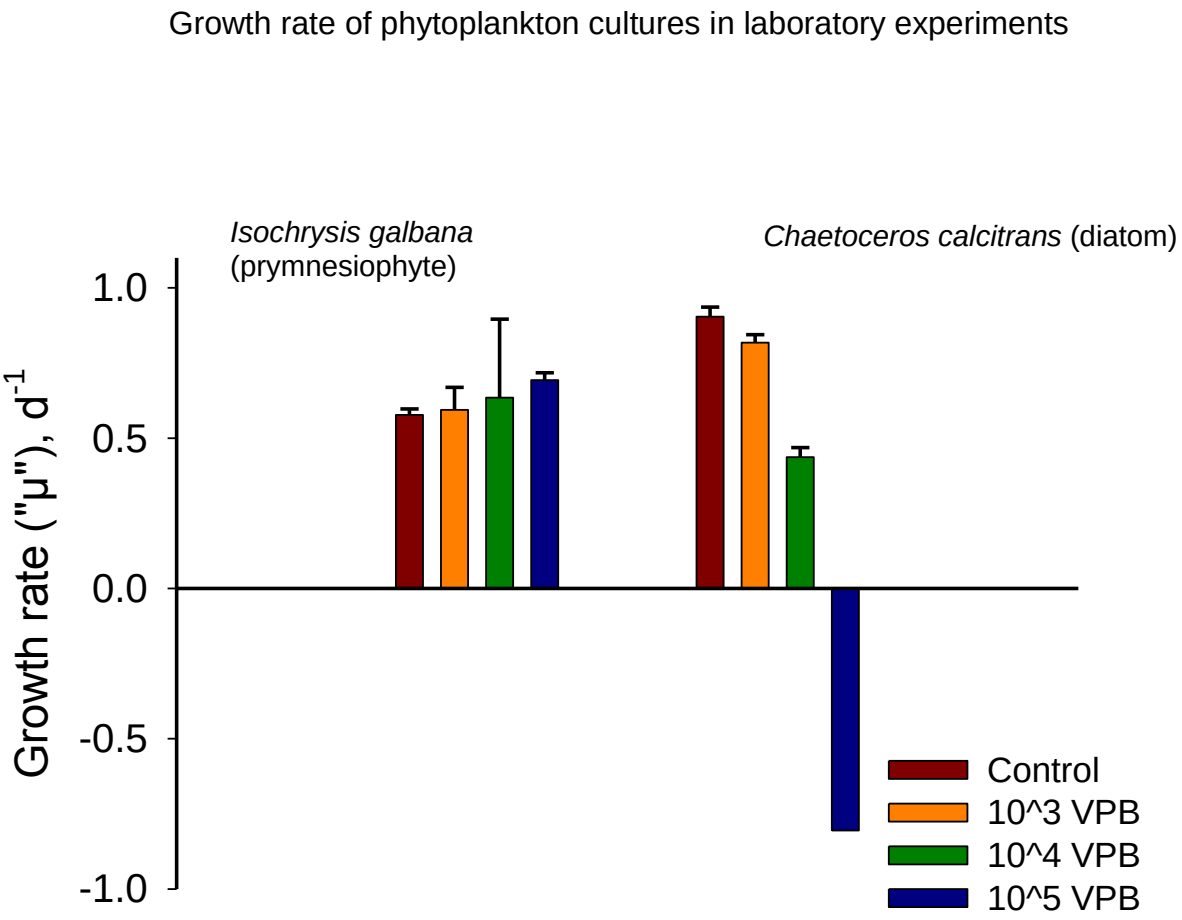

Figure 9. Growth rates for two types of algae: *Isochrysis galbana* and *Chaetoceros calcitrans* in culture with and without addition of VPB.

Tables

Experiment #	Date	Salinity t=0 (psu)	Temperature t=0 (^{0}C)	Salinity t=24 (psu)	Temperature t=24 (^{0}C)	Depth(m)
1	6/21/11	6.6	26.6	6.8	27.2	~ 1
2	6/23/11	6.8	26.1	6.9	26.3	~1
3	7/5/11	7.1	30.9	7.4	28.6	~1
4	7/13/11	7.3	30.9	7.2	30.0	~1
5	7/21/11	/	29.4	/	31.1	~1
6	7/22/11	/	31.2	/	30.4	~1

Table1. Information recorded from the experiments at the Choptank River (1-4) and the Chesapeake Bay (5,6).

Control set	Experimental set
100% WSW	100% WSW + VPB
A B C	A B C
20% WSW	20% WSW + VPB
A B C	A B C
5% WSW	5% WSW + VPB

Table2. Experimental design for dilution experiments to measure microzooplankton grazing without (control) and with addition of VPB. WSW = whole sea water (< 200µm fraction).

A B C	A B C

Exp. #	Treatment	Grazing coefficient ("g")			Algal growth coefficient ("µ")		
		"g"	Standard error	ANOVA	"µ"	Standard error	ANOVA
1	Control	-0.0571	0.210	P = 0.828	-0.393	0.124	P = 0.239
	10^5 VPB	-0.129	0.249		-0.158	0.147	
2	Control	-0.325	0.186	P = 0.175	0.331	0.110	P = 0.807
	10^5 VPB	-0.966	0.411		0.397	0.242	
5 South Station	Control	-1.305	0.236	P = 0.522	1.535	0.139	P = 0.048
	10^4 VPB	-1.223	0.198		1.491	0.117	
	10^5 VPB	-0.969	0.207		1.108	0.122	
	Control	-1.586	0.233		1.309	0.137	
	10^4 VPB	-2.029	0.145	10^4 vs 10^5 VPB;	1.553	0.0906	Control vs 10^5 VPB;

6 North Station	10^5 VPB	-1.325	0.0771	P = 0.016	0.807	0.455	P = 0.004 10^4 VPB vs 10^5 VPB; P <0.001

Table 3. Summary of results. These are results for dilution microzooplankton grazing experiments in the Choptank River (Experiments 1 and 2) and for Chesapeake Bay (Experiment 5 and 6).

Exp. #	Treatment	"K"	Mean ± Standard error	ANOVA
3	100% (Control)		0.0109	P= <0.001 S.D.
	100% + 10^5 VPB/mL		0.0445	

Table 4. Summary of the analysis of data for Experiment 3 in the Choptank River. The net growth of phytoplankton (K) in 100% whole seawater was determined in 24 h incubations.

Exp. #	Tukey Test	ANOVA
4	Treatment	
	10^4 VPB vs 10^5 VPB	P < 0.0050
5 South Station	/	Different fraction of WSW; P <0.001 Different treatments; P = 0.041 Different fraction of WSW vs Different treatments P = 0.481
6 North Station	/	Dilution factor vs Treatment P = 0.008

Table 5. Results for the ANOVA analysis for experiment 4, 5 & 6.

	Category	Treatment	ANOVA
Exp. # 5 South Station	Dinoflagellates	Control T=0 Control T=24 10^5 VPB T=24	Control T=24 vs. Control T=0; P= 0.004 Control T=24 vs. 10^5 VPB T=24; P = 0.162 Control T=0 vs. 10^5 VPB T=24; P = 0.039
	Diatoms		P = 0.365
	Ciliates		Control T=24 vs. Control T=0; P = 0.038 Control T=24 vs. 10^5 VPB T=24; P = 0.358 Control T=0 vs. 105 VPB T=24; P = 0.248
			Control T=24 vs. Control T=0; P < 0.001

Exp. # 6 North Station	Dinoflagellates	Control T=0 Control T=24 10^5 VPB T=24	Control T=24 vs. 10^5 VPB T=24; P = 0.002 Control T=0 vs 10^5 VPB T=24; P = 0.212
	Diatoms		Control T=24 vs Control T=0; P = 0.010 Control T=24 vs. 105 VPB T=24; P = 0.052 Control T=0 vs 105 VPB T=24; P = 0.354
	Ciliates		P = 0.307

Table 6.Statistical Analysis of Changes in Microplankton Abundance in Results for the cells counts of the Chesapeake Bay Experiments. Data shown in Figure 8.