

**Effects of increased nitrate concentrations
on *Zostera marina*
health and prevalence of
*Labyrinthula zosterae***

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Abstract

Seagrasses are facing a global population decline and bottom cover has grown sparser in many locations. Garrison Bay, in the San Juan Archipelago, was the site of a complete loss of eelgrass (*Zostera marina*) in 2003, and no recovery has occurred. Although the exact cause of the disappearance is unknown, one possible factor is nitrate loading caused by submarine groundwater discharge (SGD). This study was divided into two parts in order to investigate the causative factors behind the disappearance. First a field component focused on identifying SGD sites and determining the concentration of nitrate in the discharge. Second, in a mesocosm study *Z. marina* was exposed to ambient, 2X ambient, or 5X ambient concentrations of nitrate. Three SGD sites were identified, with the site in a highly modified area (the site of a hotel and marina established in 1886) containing elevated levels of nitrate. Eelgrass grown in mesocosms was exposed to increased nitrate concentrations (2X and 5X) introduced into the water column. Although shoot growth differences were not significant, there was a significant difference in the total number of lesions, associated with the marine pathogen *Labyrinthula zosterae*, found in each treatment with the fewest lesions in the 5X ambient nitrate concentration and 180% more lesions in the 2X ambient nitrate concentration. This finding may indicate that while growth differences between treatments were not statistically significant, the 5X nitrate treatment may have decreased the spread of *Labyrinthula zosterae* along shoots.

Introduction

Seagrasses are important and unique flowering plants that occupy and thrive in marine ecosystems (Chesworth et al. 2004). They typically grow in soft or sandy substrates in estuaries as well as in many coastal waters (Kenworthy et al. 2006). One

seagrass, *Zostera marina*, or eelgrass, is the most common species of seagrass worldwide and provides an important source of food and shelter to a large variety of organisms (Green and Short 2003). In the Pacific Northwest, *Z. marina* serves as a breeding ground and nursery for a variety of vertebrate and invertebrate species due to its physical structure and primary productivity (Phillips 1984). Before European contact, *Z. marina* had strong cultural significance as a food source to several Native American tribes (Wyllie-Echeverria & Ackerman 2003).

Eelgrass and other seagrass species are in decline globally (Nielsen & Dahllof 2007, Short and Wyllie-Echeverria 1996). With the expansion of artificial coastal modifications and structures, *Z. marina* has become more threatened due to human-induced habitat destruction and declining water conditions (Short & Wyllie-Echeverria 1996). Furthermore, recreational watercraft and small boats pose a threat to seagrass populations by scarring the plant habitat and causing fragmentation of populations (Wyllie-Echeverria and Ackerman 2003). Although there are government regulations protecting *Z. marina* in Washington State, *Z. marina* cannot be protected from all threats (Phillips 1984; Wyllie-Echeverria et al. 2003).

One major threat to *Z. marina* since the 1930s is the marine slime mold *Labyrinthula zosterae* (Muehlstein 1989). *Labyrinthula zosterae* was identified as the causative agent behind the wasting disease in both the 1930s and 1980s outbreaks (Muehlstein et al. 1991). *Labyrinthula zosterae* causes noticeable lesions on plant leaves allowing for easy identification of diseased short shoots.

In the San Juan Archipelago region of Washington State, *Z. marina* disappeared in Westcott and Garrison Bays in 2003. Within a single year, seagrass cover in Westcott

Bay dropped 24% and maximum depth of plant growth was reduced by over two meters (Wyllie-Echeverria et. al. 2003). Although the exact cause of the disappearance of *Z. marina* in these bays is unknown, there are several factors that may have contributed including disease, anoxia, or human-introduced factors, such as toxins or eutrophication. One possible transport mechanism for water-borne threats into the ocean is submarine groundwater discharge (Moore 2010).

Submarine Groundwater Discharge (SGD) is the flow of water from underground freshwater sources, such as an aquifer, into coastal marine waters (Moore 2010). SGD is recognized as an important part of the hydrological cycle and has similar amounts of flux to that from rivers (Moore 2010). Additionally, SGD may contain high concentrations of nutrients, carbon, minerals and metals that can be beneficial, even necessary to some marine ecosystems, but can also lead to ecological disturbances such as eutrophication and algal blooms (Moore 2010). It is possible that increased human activity has led to increased nitrogen concentrations in groundwater affecting the condition of *Z. marina* in Westcott and Garrison bays.

Excess nutrient levels, especially nitrates, have been attributed to decreased health in seagrass populations (Moore & Wetzel 1999). This is especially true when nutrient concentrations reach levels promoting algal growth that displaces or competes with seagrass (Moore & Wetzel 1999). In this study we investigated the potential effect of nitrate loading and if it contributed to the decline and persisting absence of eelgrass in Garrison and Westcott Bays. *Z. marina* was grown in outside mesocosms with increased nitrate levels in a contained environment and analyzed for differences in leaf growth, mortality, biomass and epiphyte biomass among different nitrate concentrations.

Mesocosms were used for easy manipulation of variables while minimizing the impact of external variables and conditions (Peralta et al. 2003). We predicted that nitrate treatments would cause a decrease in leaf growth rate and plant biomass while increasing mortality rates and as well as increased disease presence.

Methods

Sites

American Camp and English Camp, in San Juan National Historical Park, were chosen for sites based on *Z. marina* coverage. These sites are valued recreation sites and managed by the National Park Service (NPS San Juan Island). Detailed habitat and bathymetric maps were used to select specific sampling sites (Greene and Wyllie-Echeverria 2013). After initial surveys, three sites were chosen at English Camp (Garrison Bay) and two sites were chosen at American Camp (Griffin Bay) (Figures 1 & 2). Two sites at Garrison Bay (A and B) were chosen due to dense algal growth present near shore, with Site B located near a drainage ditch. The third site at Garrison Bay (Site C) was located near a public access dock with no algal growth or *Z. marina*, at the edge of the park. Site A at Griffin Bay had *Z. marina* beds and Site B had substantial algal growth and no *Z. marina* observed with a small submarine discharge site seen at an extreme low tide.

Sampling

Background sediment and water samples were taken from two sites at Griffin Bay (A and B) Garrison Bay (B and C). Sediment samples were collected at 30.5 and 45.7 m along a transect line placed perpendicular to the shore. Eelgrass density was sampled using a 0.5 m² quadrat at 30.5 and 45.7 m along the transect line and counting all plants

growing within the quadrat ($n = 2/\text{transect}$). Water samples were taken from each site in plastic and glass containers to test for nitrogen and phosphorus levels and organic contaminants, respectively. Different container types were used to prevent contaminants from adhering to the containers. During regular sampling, sediment and surface water samples (for organic contaminants) were taken from one transect point, 30-60 m from the shoreline. Water samples for nitrogen/phosphorus concentration analyses were collected at the shoreline for each transect. Additionally, three shoreline sites with submarine groundwater discharge (SGD) were identified, and water samples were taken directly from the water source and returned to the laboratory for nutrient analysis. Two SGD sites (Lime Kiln SGD 1 and 2) were in low-urbanized areas, whereas, the third site, Roche Harbor SGD, was in a highly urbanized area.

Total Nitrogen Analysis

Total Nitrogen analysis was performed by oxidizing alkaline persulfate to nitrate then analyzed using hydrazine reduction technique (APHA 1995). Samples were mixed with digestion solution then heated at 121°C for 30 minutes before borate buffer and sodium hydroxide were added to the solution. Sodium hydroxide reagent, reducing reagent and color reagent were then added and mixed before absorbance measurements were recorded at 535 nm using a spectrophotometer. Absorbance was compared to standard curve created using standards and same procedure.

Mesocosm Experiment

Mesocosms were set up in in three 568 l tanks, each tank was divided into to three sections by plexiglass sheets. Large plastic, Grainger trashcan liners were used to ensure water was not exchanged between treatments. Each tank had a dedicated inflow at the

bottom of the tank and outflow at the top of the tank with an average flow rate of 438.2 l/hr. Experimental tanks were made operational for several days to cleanse the system. Ambient light was measured and artificial lights were turned on during the day when sun was obscured behind clouds to ensure proper plant growth (light levels above $300 \mu\text{mol s}^{-1} \text{m}^{-2}$).

Each tank contained one control treatment (addition of regular seawater), one treatment of 2X ambient nitrate levels and one treatment of 5X ambient levels, with treatment sections selected at random in each tank. The 2X ambient nitrate treatment was selected based on *in situ* measurements at Fisherman's Bay (Takasue, pers. obs. 2014). The 5X ambient concentration was selected to investigate the effects, if any, of high nutrient loading on *Z. marina*. Nitrate solutions were made by creating a standard nitrate solution with 1 l deionized (DI) water and KNO_3 (APHA 1995).

Z. marina adult shoots used in experiments were selected from a tank containing seagrass growing for six years since removal from Picnic Cove, Shaw Island, Washington. Five adult shoots were placed in each tank, secured by zip ties to a PVC frame 0.5 m below the surface. The leaves of each shoot were examined for disease and disease was removed by clipping off affected areas. All plants were pierced in the meristem beneath the sheath to mark for growth measurements (Dennison 1990). *Z. marina* acclimated to the tanks for three days before treatment began. Treatments were applied twice daily for twelve days (Hosokawa 2011; Kentula and McIntire 1986). Nitrate solutions were drained from each intravenous bag by PVC tubes ending near the inflow spout. Tank flow were turned off for treatment application and then turned back on once all bags emptied 500 mL of nitrate solution to tank and were closed to simulate

tidal exchange. Growth of each shoot was determined by measuring distance between scar beneath sheath and scar on newest leaf (Dennison 1990). At the end of the trial, growth, biomass, mortality, and number of lesions were recorded.

Data Collection and Analysis

Twelve days after treatment, plants were removed from the mesocosms and epiphytes were scraped from all plant matter before measuring and drying (Russell 1990). Growth measurements were made by measuring the distance between original meristem punch and the one made after measuring initial growth following acclimatization. Each leaf was then examined for lesions, counted, if present, and the longest lesion was measured. The longest leaf containing lesions was then measured from leaf tip to first node above the rhizome. The rhizome was severed at this point and then wet weight of each shoot was recorded. Plants were dried at 60°C for 24 hours and dry weight was recorded for analysis (Ott 1990).

Results

Sample Site Nitrogen Concentrations

All sample sites in Garrison and Griffin Bays had statistically similar nitrate concentrations (chi-square goodness-of-fit, $X^2 = 0.0459$, $df = 5$, $p = 1.0$; Table 1). Nitrate levels at Lime Kiln SGD 1 were similar to those found at sample sites in Garrison and Griffin Bays (chi-square goodness-of-fit, $X^2 = 0.0459$ $df = 6$, $p = 1.0$; Table 1). Nitrate levels at Lime Kiln SGD 2 were 1.6 times the average sample site nitrate levels. Nitrate levels at Roche Harbor SGD site were 2.2 times the average sample site nitrate levels. Nitrate levels at Roche Harbor were similar to values previously recorded at Fisherman's Bay (Takasue, Pers. Obs. 2014).

Table 1. Sample site total nitrogen concentrations collected at two sampling sites and SGD stations, San Juan Island, Puget Sound, WA (6/19/14-7//07/14)

Site	Total N concentration (mg/L)
Garrison Bay	
English Camp A	0.8401
English Camp B	0.8710
English Camp C	0.9881
Griffin Bay	
American Camp A	0.9367
American Camp B	1.0016
FHL	1.0963
SGD	
Lime Kiln SGD 1	0.9556
Lime Kiln SGD 2	1.4812
Roche Harbor SGD	2.0962

Mesocosm experiment

Conditions were kept as similar as possible to reduce external variables. Temperatures in tank one ranged from 10.8 °C to 21.8 °C. Temperatures in tank two ranged from 10.9 °C to 21.6 °C. Temperatures in tank 3 ranged from 10.9 °C to 21.2 °C, not differentiated by day and night. Light measurements ranged from 330 $\mu\text{mol s}^{-1} \text{m}^{-2}$ to

1130 $\mu\text{mol s}^{-1} \text{m}^{-2}$. Growth rates were similar across both treatment groups and the control group (1-way ANOVA, $F = 0.553$, $df = 2$, 42 , $p = 0.58$; Figure 3). Additionally, growth per day was similar across both treatment groups and the control group (1-way ANOVA, $F = 0.553$, $df = 2$, 42 , $p = 0.58$; Figure 4). Each treatment contained individual shoots that had new leaves emerging from the sheath, indicating growth was occurring in the mesocosms. Although there was no statistical difference between dry biomass of treatments (1-way ANOVA, $F = 1.148$, $df = 2$, 42 , $p = 0.33$; Figure 5), there was a noticeable trend towards decreasing biomass with increasing nitrate treatment. There was no mortality in any of the treatments.

The total number of *Labyrinthula* lesions increased as the nitrate level increased (chi-square goodness-of-fit, $X^2 = 28.7$, $df = 2$, $p < 0.001$; Figure 6). The control treatment had 180% times as many lesions as the high nitrate (5X) treatment, and the low nitrate (2X) treatment had 150% times as many lesions as the control. However, the number of lesions per shoot in each treatment was not statistically different (1 way-ANOVA, $F = 2.202$, $df = 2$, 42 , $p = 0.123$; Figure 7). There was a noticeable difference, with low nitrate treatments having more lesions per shoot than control or high nitrate treatments. The longest lesion identified was 15.5 cm and was present on a shoot in the 5X treatment, and in all but two shoots, with lesions; they were present on the longest leaf.

Discussion

Sampling

The total nitrogen concentrations at sample sites in Garrison and Griffin Bays were not significantly different from each other or in the seawater system used for the experiment. However, the total nitrogen concentrations from two of the submarine groundwater discharge sites were higher than the concentrations found at sample sites. This indicates that submarine groundwater discharge could be responsible for introduction of nitrogen, including nitrates, to aquatic systems. Whether or not the addition of nitrates by this discharge was the direct cause of observed algal blooms at sample sites remain unknown due to minimal differences in nitrogen concentrations across sample sites and known low rates submarine groundwater discharge (Bonsell 2011). Further analysis of seasonal variation in SGD and nutrient levels may be warranted to determine if nutrient loading of aquatic ecosystems occurs from SGD.

Mesocosm Experiment

Contradictory to our hypothesis and findings of previous papers, nitrate pulses had a negligible effect on leaf growth in *Z. marina* (Bonsell 2011, Moore & Wetzel 1999). Our results are more similar to those of Kaldy (2014) where increasing nitrate concentrations exhibited no significant effect on leaf growth. There was a minimal trend with decreased leaf growth with increased nitrate, however due to variation, this trend may be due to chance alone. There was a more noticeable, albeit not significant difference in average biomass between treatments and the control. Nitrate treatments may have decreased health of older leaves, causing them to slough off and decreasing overall biomass of the plants in higher nitrate concentrations.

The significant difference between the number of *Labyrinthula* lesions between the control and low nitrate concentration indicates that increasing the concentration of

nitrate in the water column may promote growth of *Labyrinthula* on *Z. marina*. However, this relationship does not appear to be linear due to the significant decrease in the number of lesions observed in the high nitrate treatment (Figure 4). While high nitrate loading to the surrounding environment may have slight effects on shoot health, it has significant effects on the health and spread of *Labyrinthula zosterae* along individual leaves. This could prove useful in future efforts to manage and control the spread of *Labyrinthula zosterae* by finding a management system of increasing nitrate levels periodically in order to control spread of disease. Although the number of lesions per shoot was not significant, a trend very comparable to total lesions per treatment was noticed.

While the scope of this project did not allow for further exploration of results, several questions did arise that could be answered by future studies. It would be interesting to examine the effect of a broader range of nutrient concentrations in order to further explore the tolerance of *Labyrinthula zosterae* to nitrate as well as find an LD50 of *Z. marina* when exposed to nitrate. The observed effect of nitrate treatments on *Labyrinthula zosterae* may be worth investigating by directly subjecting *Labyrinthula zosterae* grown on agar plates or small pieces of *Z. marina* to nitrate treatments. The effect of other nitrogen compounds on *Z. marina* health may be worth examining not only in terms of mortality and growth but also in terms of disease prevalence. Finally, I believe that it would be prudent to positively identify SGD sites at American and English Camp and analyze the organic and nutrient constituents they contain.

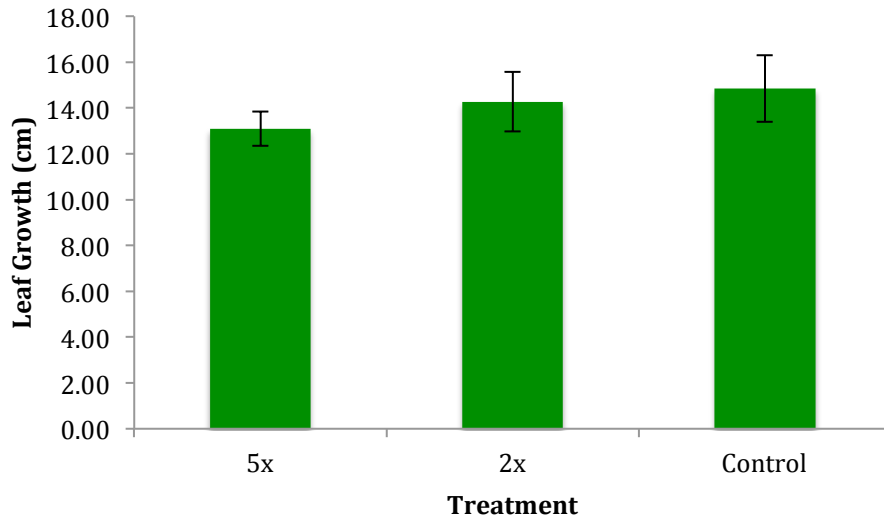


Figure 3. Average (\pm SE) leaf growth over the twelve day experiment period. Total leaf growth is compared among treatment groups ($n = 15$ plants per treatment). Nitrate concentration treatments had no significant effect on leaf growth ($p = 0.58$).

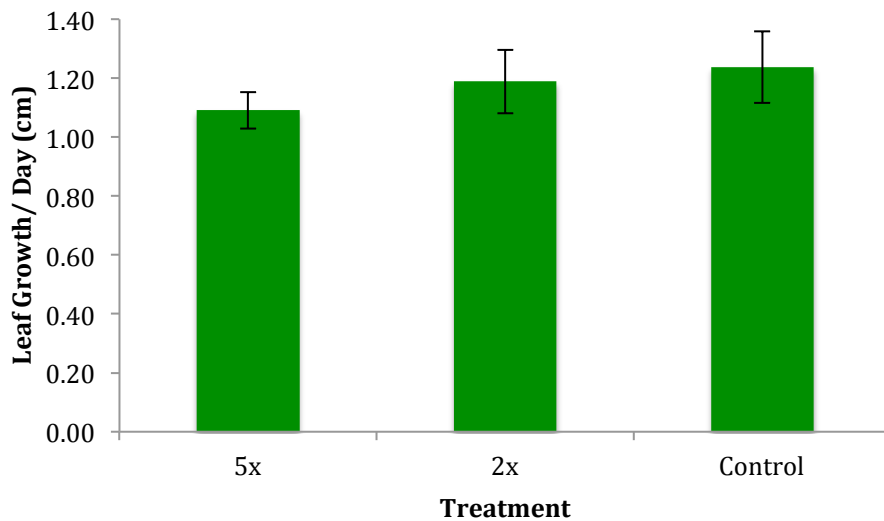


Figure 4. Average (\pm SE) leaf growth per day of *Z. marina* at different nitrate concentration. Nitrate concentration had no significant effect on leaf growth per day ($n = 15$; $p = 0.58$)

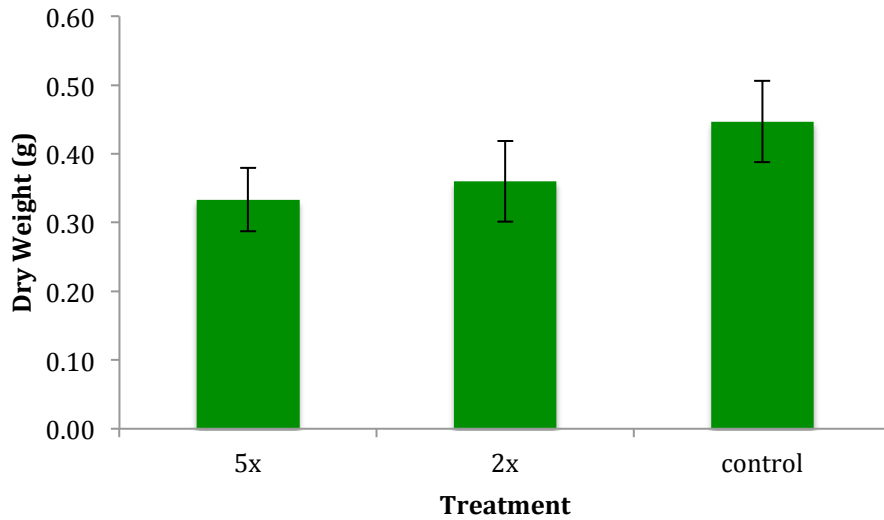


Figure 5. The effect of increased nitrate concentration on dry biomass of *Z. marina* at different nitrate concentrations is shown. Average (\pm SE) dry biomass was compared across both treatments and the control. Nitrate concentration had no significant effect on dry biomass ($n = 15$; $p = 0.33$).

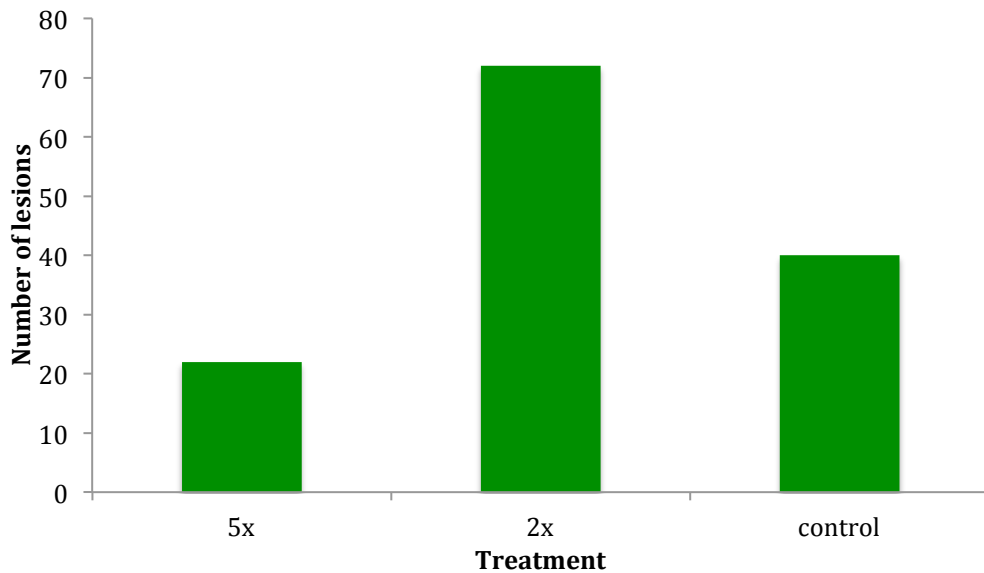


Figure 6. The influence of various nitrate concentrations on total number of lesions ($n = 15$). The control treatment had 1.8 times as many lesions as the high nitrate (5X) treatment, and the low nitrate (2X) treatment had 1.8 times as many lesions as the control ($p < 0.001$).

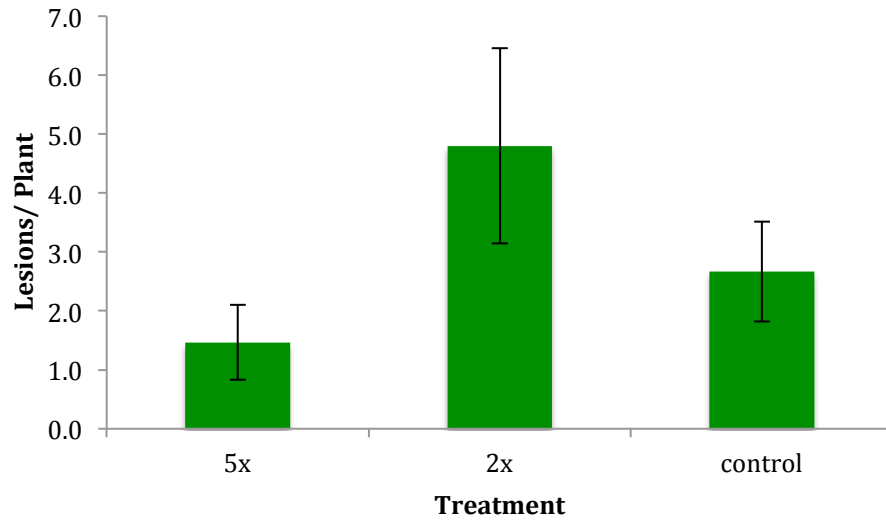


Figure 7. Nitrate had an effect on the average (\pm SE) number of lesions per shoot ($n = 15$). Nitrate concentration had no significant effect on the number of lesions per shoot ($p = 0.123$).

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