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SHORT COMMUNICATION

EPIPHYTIC DIATOM PRODUCTION ON THE SEAGRASS *THALASSIA TESTUDINUM* IN NORTHERN GULF OF MEXICO§

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INTRODUCTION

Seagrasses provide coastal protection, habitat, and food resources for many economically important marine organisms and play a role in carbon sequestration (Sullivan and Moncreiff 1988). Seagrasses range from the tropics to polar environments and there are about 60 different species worldwide (Orth et al. 2006). Juvenile organisms have much greater growth rates in seagrass meadows than in other habitats (Heck et al. 2003). However, seagrass loss has increased over the last 40 years in both tropical and temperate regions (Orth et al. 2006).

Seagrasses can support epiphytic assemblages. Diatoms, a type of microalgae that can persist in the water column, on surface benthos, and as an epiphyte, can be found on most seagrasses (Borowitzka et al. 2006). Temperature and nutrient supply are key environmental drivers that control microalgal epiphyte growth and productivity (Fernández—González et al. 2022). Accordingly, epiphyte biomass can vary with depth for a wide range of seagrasses (Borowitzka et al. 2006). Seagrass epiphytes can also be ecologically important; for instance, they can provide food for grazers, which can facilitate predator activity through consumption of the grazers (Borowitzka et al. 2006). Some epiphytes, like diatoms, can also be significant in system carbon cycling (Sullivan and Moncreiff 1988). Water column diatoms are important because they contribute upwards of 20% to primary production globally (Nelson et al. 1995), but they can also be important contributors to primary production in the benthos and as epiphytes.

Six species of seagrasses have been identified in the northern region of the Gulf of Mexico (GOM; Handley et al. 2007). Epiphytic diatoms have been shown to contribute 70—80% of benthic net community production (NCP) in *Ruppia maritima* and *Halodule wrightii* seagrass beds in the GOM (Cox et al. 2020). However, for other regionally important seagrass species, such as *Thalassia testudinum* which typically have wider leaves per unit length and thus more surface area than *R. maritima* or *H. wrightii*, there are no data reporting whether there is a significant epiphytic diatom contribution to gross primary production or NCP. Therefore, we do not understand how spatially widespread or temporally persistent the epiphytic diatom contributions are to NCP in such systems, or what factors most control their production. Building on the earlier regional studies showing the importance of diatom epiphytes, we aimed to answer the following question: are epiphytic diatoms quantitatively significant to *Thalassia testudinum* NCP? We hypothesized there would be quantifiable and significant epiphytic diatom production in *T. testudinum* compared to *R. maritima* and *H. wrightii*, because of *T. testudinum*'s higher surface area which can facilitate diatom attachment.

MATERIALS AND METHODS

We used a one—factor experiment to examine epiphytic diatom production on the seagrass *T. testudinum*. We quantified NCP and community respiration via change in dissolved oxygen in a series of bottles containing seagrass either with active epiphytic diatoms (control) or without active epiphytic diatoms (Ge). We used germanium (Ge), which is a diatom—specific inhibitor, to arrest diatom activity among the seagrass epiphytic community and create our Ge (no diatom epiphyte activity) treatment following approaches in Brzezinski et al. (2011), Cox et al. (2020), and Scarratt et al. (2006). The NCP was determined in bottles receiving full sunlight. Respiration was quantified in the same size bottles and physical conditions but incubated in darkness (Figure 1A). The difference in rates between the control (all epiphytes) and Ge (no diatom epiphytes) treatments enabled detection of a diatom effect towards NCP and respiration. We collected seagrass on day 1, applied treatments on days 2—3 (for about 48 hours), and did our incubation on day 4. This sequence mirrors a prior study with other seagrasses in which a 48 h treatment duration was enough time for Ge to arrest diatom growth (Cox et al. 2020). We conducted our experiment 3 times and denote them in chronological order (Experiments 1, 2, and 3).

Thalassia testudinum was collected from Johnsons Beach

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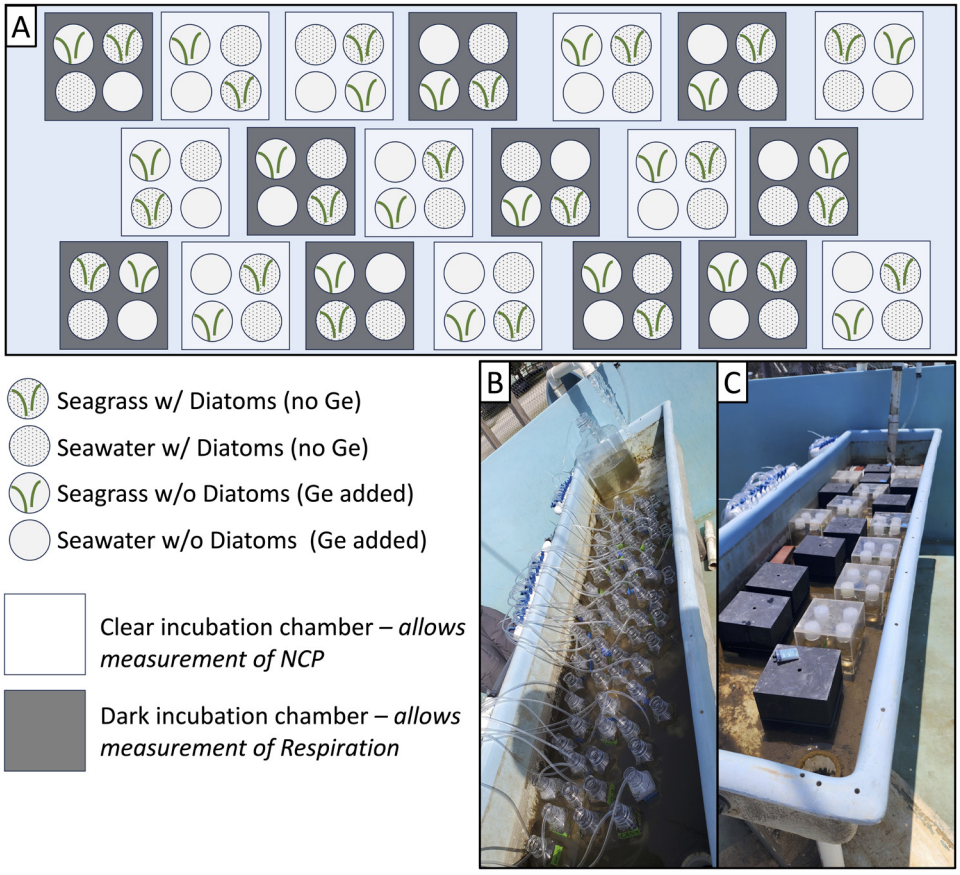

FIGURE 1. *Experimental design. A. Schematic of the setup of the one–factor experiments with +/– diatoms (manipulated by the absence or presence of Germanium (Ge), respectively) as the factor levels. Clear and dark chambers were used to measure net community production (NCP) and respiration, respectively, during a 3 h incubation. This schematic specifically represents experiments 2 and 3, when seawater only bottles were also included to account for NCP and respiration in the water column. B. All bottles were individually aerated while they received their treatments (no Ge, added Ge) for 2 d before the incubation. C. All bottles were sealed and placed into a clear or dark chamber during the 3 h incubation period to measure NCP and respiration.*

within the Gulf Islands National Seashore in Pensacola, FL. We collected seagrass shoots for our 3 experiments on 12 and 26 June and 3 July 2023. Within our collection site, we collected from a separate patch for each experiment (<100 m separation) to avoid damage to the local system by repeated sampling. We recorded ambient water temperature and salinity during seagrass collection using a YSI Sonde. Surface seawater was collected from our sampling site and used in our experiments (see below). After collection, we transported all seagrass and water samples to our laboratory on Dauphin Island, AL (160 km drive), and set up our experimental units (Figure 1). Meteorological conditions during the experimental incubations were examined using data from the Dauphin Island Alabama Real–Time Coastal Observing System (ARCOS, arcos.disl.org) monitoring station ~300 m from incubation location and near the mesocosm intake water.

Each experimental unit consisted of a polycarbonate bottle with filtered seawater, seagrass material, and associated epiphytic material. For each unit we included 2 seagrass leaves from the same seagrass shoot. We used the second and third youngest leaves, if the youngest leaf was ≥5 cm tall; if the youngest left was <5 cm tall, we used the third and fourth youngest leaves from the shoot. For each seagrass leaf we cut a 10 cm long segment measured downward from the tip. We removed any visible epifauna from seagrass leaves (e.g. snails or other mobile fauna which can disproportionately affect the results) before putting them into their incubation bottle (~300 ml brim volume) which was filled with 250 ml of the collected filtered seawater. Experiment 1 had 6 replicates (n = 6) per treatment (control, Ge) and per light/dark bottles, for a total of 24 incubation bottles. For Experiment 1, we filtered the collected seawater through a 0.6 µm capsule filter, which filters out all diatoms (and nearly all other main phytoplankton) in the water column. However, the turbidity and particle load of the seawater at our collection site limited the volume of water that could be filtered this way. Thus, for Experiments 2 and 3 we filtered the seawater collected from our field site through a 20 µm filter, which allowed smaller diatoms and other phytoplankton to remain in the water. To compensate, we also included seawater–only bottles in Experiments 2 and 3 which also received control or Ge treatments. These seawater–only bottles (i.e., no seagrass) accounted for NCP and respiration associated with the 20 µm filtered seawater added to each bottle. Thus, for Experiments 2 and 3 we had 4 bottle types (seagrass control, seagrass Ge, seawater control, seawater Ge), and we increased replication to 10 replicates per treatment and per light/dark bottle incubation for a total of 80 bottles per experiment (Figure 1C).

We randomly assigned bottles as control or Ge treatments and placed them in a flow–through water bath located in an outdoor mesocosm at the Dauphin Island Sea Lab (Figure 1). All units had air stones placed in them to ensure aeration and avoid potential hypoxia during acclimation (to account for heterotrophs also associated with the blades, Figure 1B). Bottles were acclimated overnight before 0.315 ml of saturated dissolved germanium (as $Ge(OH)_4$) was added to the Ge bottles (~3 µM final concentration). This final concentration was low enough to not affect producers other than diatoms (Brzezinski et al. 2011, Cox et al. 2020). Immediately prior to incubation, all bottles were filled to the brim with experimental seawater (300 ml total volume), sealed with parafilm (no air

bubbles), then capped tightly for the incubation. We assigned equal numbers of replicates as light or dark bottles for both control and Ge treatments. Dark bottles were contained in a black—painted polycarbonate cube (4 bottles per cube, Figure 1A, C) and corresponding light bottles were contained in a transparent polycarbonate cube (4 bottles per cube, Figure 1A, C). All polycarbonate cubes had drilled holes below the water line to allow for flow during the incubation (Figure 1C). Light and dark cubes were intermixed within the water bath (Figure 1C). Incubations commenced around 09:00 (local time) and were terminated after 3 h before daily solar and temperature maxima to capture the period of increasing production with sunlight during the day. Our incubations occurred on the mornings of 15 and 29 June and 6 July 2023.

Oxygen and temperature were quantified during initial and final time points using PreSens oxygen DP—PSt8 dipping probes and PreSens Pt 100 temperature sensors. We also quantified the salinity (refractometer), seagrass and epiphyte biomass, and epiphyte chlorophyll from each seagrass experimental unit after incubation. A razor blade was used to scrape the epiphytes off both sides of the seagrass leaves in each unit. Contents were placed in a pre—weighed 20 ml glass scintillation vial and submerged in 90% acetone for 24 h at —20 °C in the dark to extract chlorophyll, which was quantified on a Trilogy fluorometer (Turner Designs) using an acidification method (Krause et al. 2021). After measuring chlorophyll, we dried the epiphytes at 60°C for at least 48 h to quantify epiphyte dry biomass. Scraped seagrass blades were also dried and quantified. Initial water column nutrients (nitrate+nitrite, ammonia, phosphate, silicate, and total dissolved nitrogen) from each collection day were quantified using a Skalar autoanalyzer following Cox et al. (2020).

Some experimental units were lost during the acclimation, e.g., bottles with air stones (Figure 1B) tipped over in the water bath prior to incubation, or grazers were detected on seagrass leaves post—incubation. We lost 2 units in Experiment 1, 4 in Experiment 2, and 5 in Experiment 3. We calculated NCP and respiration values, for light and dark bottles respectively, as (final oxygen — initial oxygen) / incubation time in mg O_2/L/h. For Experiments 2 and 3, we subtracted the NCP and respiration values of the seawater—only bottles from the seagrass bottles to obtain NCP and respiration values for only the seagrass and epiphytic material. The Dixon Q test (Dean and Dixon 1951) was used to objectively assess outliers in our NCP and respiration data, which excluded 1 NCP value for each experiment. There were no respiration values from any experiment that were identified as outliers. A Mann—Whitney U test was used to compare the amount of 1) NCP (light bottles) and 2) respiration (dark bottles) between our control and germanium treatments for each of our 3 experiments.

Results and Discussion

Hydrographic parameters showed expected trends. Water temperatures at the time of sampling were ~30°C among all 3 experiments (Table 1). Conditions were brackish but variable, with starting salinities of 26.2 (Experiment 1), 15.2 (Experiment 2), and 24.0 (Experiment 3, Table 1). While all experiments started similarly, the temperatures in the water feeding

TABLE 1. *Response of epiphytic diatom production on the seagrass* Thalassia testudinum *for the 3 experiments. A. Water temperature and salinity recorded in the field while collecting seagrass. B. The initial water column nutrients (nitrate+nitrite, ammonia, phosphate, silicate, and total dissolved nitrogen) from the collection day of each experiment. C. The final seagrass and epiphyte biomass and final epiphyte chlorophyll for each experimental treatment (control vs. +Ge) at the termination of each experiment, with Mann-Whitney U-Test and p values. Numbers are reported as means ± se when appropriate and single point measurements do not include any measure of error.*

	Experiment 1 12 - 15 June 2023	**Experiment 2 26 - 29 June 2023**	**Experiment 3 3 - 6 July 2023**
A) Collection Site Hydrology			
Salinity	26.2	15.2	24.0
Water Temperature (°C)	30.5	29.6	30.6
B) Initial Water Column Nutrients (μM)			
Nitrate+Nitrite (NO_3+NO_2)	0.40 ± 0.08	0.33 ± <0.01	0.18 ± 0.1
Ammonia (NH_4)	3.33 ± 0.08	0.42 ± 0.03	0.20 ± 0.03
Phosphate (PO_4)	0.17 ± 0.13	0.20 ± 0.04	0.17 ± 0.05
Silicate	36.1 ± 0.2	42.2 ± 0.1	48.7 ± 0.4
Total Dissolved Nitrogen	20.6	20.7	19.1
C) Final Biomass	Control +Ge	Control +Ge	Control +Ge
Seagrass (mg)	45.9 ± 3.0 40.9 ± 6 .2	39.5 ± 1.7 41.4 ± 1.4	62.2 ± 5.4 61.6 ± 3.6
U-Test, p-value	U = 67, p > 0.05	U = 143.5, p > 0.05	U = 151, p > 0.05
Epiphytes (mg)	7.4 ± 1.6 11.7 ± 2.5	8.1 ± 1.5 6.5 ± 1.9	7.2 ± 1.2 8.9 ± 1.9
U-Test, p-value	U = 50.5, p > 0.05	U = 116, p > 0.05	U = 146, p > 0.05
Epiphyte Chlorophyll (ng)	470 ± 74 886 ± 206	238 ± 49 397 ± 104	305 ± 58 303 ± 36
U-Test, p-value	U = 51, p > 0.05	U = 139, p > 0.05	U = 130, p > 0.05

the mesocosm were higher and more variable among the latter experiments. Experiment 1 ranged from 29–31°C, whereas this range widened during Experiment 2 (27–33°C) and 3 (29–33°C, data not shown, accessed from ARCOS arcos.disl.org for the experimental dates).

The seagrass and total epiphyte biomass did not change significantly between the control and germanium treatments throughout all 3 experiments (Table 1), although seagrass biomass was higher in the Ge+ treatment in Experiment 2 while epiphyte biomass was higher in the Ge+ treatment in Experiments 1 and 3 (Table 1). The lack of difference supports that our +Ge approach did not have any detectable artifacts on the seagrass or epiphyte standing stocks during the experimental period; instead the Ge only affected the metabolism of the diatom epiphytes, as intended (see below). Seagrass biomass in control and Ge treatments were similar for each of our experiments (Table 1). Given the variability, there were also no apparent trends in the epiphyte mass or chlorophyll (Table 1) among experiments. However, Experiment 1 had the highest mean epiphyte chlorophyll and was the most variable.

Unlike biomass metrics, the initial concentration of dissolved nutrients, in particular nitrate+nitrite and ammonia, decreased throughout the 3 experiments, the latter substantially. However, there was no discernable trend in phosphate or total dissolved nitrogen among experiments. Dissolved silicate (Si) increased ~50% (comparison of Experiments 2 and 3 vs. 1) over the 3 experiments, which may have reduced the effectiveness of our +Ge treatments. Diatoms take up Ge (which has similar chemical properties to Si) when taking up Si, and diatom cell arrest occurs when Ge:Si ratios increase above 0.1 (Azam et al. 1973). Since we used the same Ge concentration in all 3 experiments (~3 µM, same as Cox et al. 2020), our Ge:Si ratios decreased across our 3 experiments (0.08, 0.07, 0.06, respectively).

We found the contribution of diatom epiphytes to NCP of *T. testudinum* communities is variable and may depend on environmental conditions. During 2 experiments (1, 3), NCP was higher in the control than Ge treatments (Figure 2A), suggesting an important diatom component to NCP. In Experiment 1 this difference was significant (Figure 2A, U—value = 1, $p < 0.01$), but not in Experiment 3 (U—value = 30, $p = 0.27$). In Experiment 2, NCP was higher in the Ge treatment than control, but this difference was not significant (U—value = 25, $p = 0.31$). For respiration, there were no significant differences between the control and Ge treatments for any experiment (Figure 2B, U—values = 14, 35, 26; p—values = 0.58, 0.44, 0.23 for Experiments 1 – 3, respectively). These results also support that the Ge addition did not affect the microbial community activity. Broadly, there was a decline in NCP from positive (Experiment 1, production > respiration) to negative (Experiments 2 & 3, respiration > production, Figure 2). The decline in NCP coincided with a decline in nutrients (ammonia, nitrate+nitrite, Table 1) and increased water temperature ranges during incubations in the latter 2 experiments. Thus, these environmental conditions may have influenced both overall NCP and the contribution of diatom epiphytes to NCP.

While our hypothesis that *T. testudinum* would have higher diatom NCP contribution compared to other regional seagrasses (e.g. majority of NCP) was not supported, epiphytic diatoms were important to NCP during our first experiment. For a *R. maritima* and *H. wrightii* seagrass ecosystem (~80 km to west of study site), diatoms contributed up to 85.7% to benthic gross primary production (Cox et al. 2020). Similarly, previous studies suggest diatoms contribute to production in other regional plant—based systems, e.g., salt marsh grass, but do not report specific rates (Sullivan and Moncreiff 1988). Using a similar approach as prior studies (Cox et al. 2020), the contribution of the epiphytic diatoms to gross primary production (GPP) in Experiment 1 was estimated to be ~20% (calculated as: Control GPP 0.25 ± 0.02 mg O_2/L/h vs. +Ge GPP, 0.20 ± 0.07 mg O_2/L/h, thus, the diatom contribution 0.20 = *1* – (+Ge GPP:Control GPP) or 1 – (0.20/0.25)). The caveat to this estimated value is that the GPP propagated error (i.e., 0.02 and 0.07 mg O_2/L/h in the Control and +Ge, respectively) derived from the terms used to calculate GPP (NCP, respiration) for each treatment increases the proportional uncertainty in

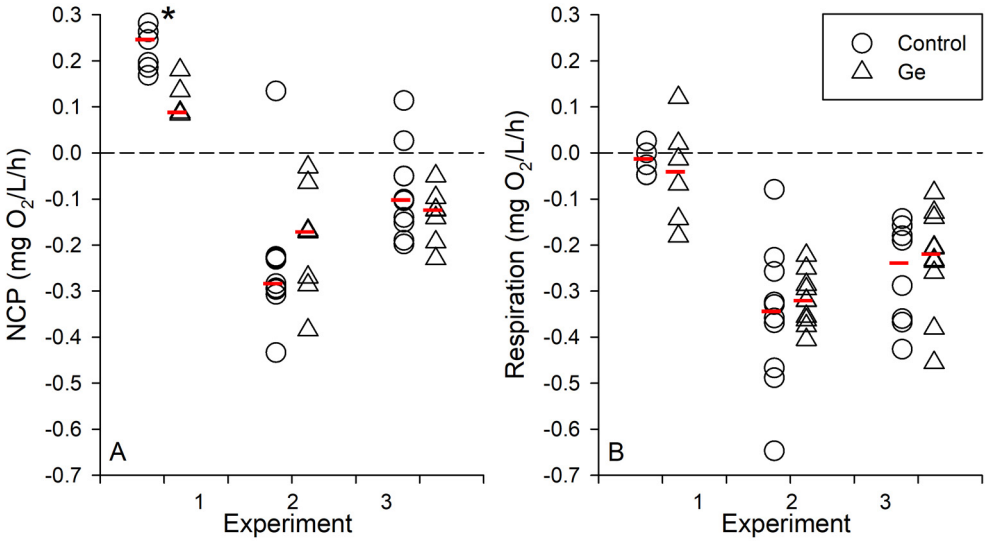

FIGURE 2. *The change in oxygen (ΔO_2) due only to the seagrass and epiphytes for each experiment and treatment. A. Light bottles to determine net community production (NCP). B. Dark bottles to determine community respiration. Circles and triangles are control and Germanium (Ge) treatments, respectively; red dash is the median. *Denotes significant difference between median values in treatments (Mann—Whitney, p<0.05).*

these values, and muddles the significant difference. However, the propagated error does not change the significant difference in these raw data between treatments. Water temperature variability could have played a factor in NCP decreasing during our study; however, Cox et al. (2020) did not observe a decrease in NCP even though they experienced similar temperatures and ranges. Thus, for our study, it is likely that diatoms were nutrient limited due to the significantly lower ammonium in Experiments 2 and 3.

While our trends show that epiphytic diatoms can be quantitatively significant producers on *T. testudinum*, the changing trend among our experiments demonstrates a potentially important shift in the contribution of various epiphytes to productivity that can occur on weekly time scales. This rapid shift of epiphytic diatom production toward production by other epiphytes or low total epiphytic production (both unexamined in this study) may affect the local seagrass food webs, specifically grazer nutrition in this ecosystem.

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