Examining Variation in Response to No. 2 Fuel Oil Exposure Between Tall- and Short-Form Saltmarsh Cordgrass (*Spartina alterniflora*): Implications for Marsh Restoration After an Oil Spill

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ABSTRACT 279s1

Saltmarsh cordgrass, *Spartina alterniflora*, is a vital and ubiquitous component of many North American saltmarshes. Within saltmarshes, there are two genetically and phenotypically distinct forms of *S. alterniflora*: a tall-form (>1 m in height) that grows along the edge of tidal creeks and a short-form (<0.5 m) found further inland. Due to their locations, the two forms are exposed to different degrees of tidal inundation with tall-form plants in our research area (Charleston, SC, USA) experiencing two tidal cycles each day, while short-form plants experience immersive high tides less frequently. Marsh exposure to oil following a spill can inhibit growth and lead to eventual mortality of *S. alterniflora*. However, studies have yet to examine the impacts of oil on these two forms of *S. alterniflora* with a cyclical tidal exposure. This study used microcosm systems (12-gallon buckets with simulated tidal cycles) to examine the impact of No. 2 fuel oil on short-and tall-forms of *S. alterniflora* exposed to two tidal regimes. Half of the systems were dosed with marine diesel fuel to a 0.1 mm slick thickness, while half remained unoiled controls. Within each oiling condition, there were two different tidal simulations: 1) a daily simulation which received two high and two low tide events each day and 2) a weekly simulation with two high tides every Wednesday. Short- and tall-form plants were grown from seed to minimize environmental differences during development and placed into both tidal simulations. All treatments included four replicate microcosms resulting in 32 systems total. Measurements of plant growth, plant mortality, water quality, and water hydrocarbon concentrations were taken for up to three months following oil exposure. At the conclusion of the study, above- and below-ground *S. alterniflora* dry biomass was also measured. Results indicate that the fastest mortality rates among oiled treatments occurred for short-form plants exposed to weekly tides, while tall-form plants with daily tides exhibited the slowest mortality rates. This data elucidates the impact of No. 2 fuel oil spills on different areas of the marsh and may help inform future restoration efforts considering replanting short- or tall-forms in oil-contaminated environments post-spill.

INTRODUCTION

Saltmarshes are vital, intertidal environments home to many species of salt-tolerant vegetation and associated animals. These habitats also provide many ecosystem services, such as sequestering carbon and helping protect inshore areas from storm surges (Mitsch et al., 2013; Leonardi et al., 2018). However, saltmarshes are at risk due to anthropogenic causes such as oil spills (Culbertson et al., 2008; Fleeger et al., 2019). The National Oceanic and Atmospheric Administration's Office of Response and Restoration recorded over 100 notable oil spills in 2021 alone, but many spills are smaller and go unreported (Emergency Response Division, 2022).

No. 2 fuel oil is just one of the potential oils released during a spill. No. 2 fuel oil, commonly known as marine diesel or red diesel, is often used in recreational and commercial vessels, as well as in home heating applications. When released into the environment, this oil can quickly spread across the water's surface forming a film that can be carried into shore coating marsh organisms, such as *Spartina alterniflora*, and eventually partitioning into the sediment.

Along the Gulf and East Coasts of the United States, *Spartina alterniflora* is often the most abundant form of vegetation found in the lower marsh (Bertness, 1991; Pennings et al., 2005; Zengel et al., 2021). *S. alterniflora* is a vital ecosystem engineer that helps maintain the structure of saltmarsh habitats as well as provide food and shelter to other organisms (Currin et al., 1995). Within marshes, two forms of *Spartina alterniflora* are generally found: a tall-form (>1 m tall) that grows along the banks of tidal creeks and a short-form (<0.5 m tall) that grows further inland. There has been much debate concerning whether phenotypic variations between the two forms are due to genetic or environmental differences. However, it is likely a combination of both (Anderson and Treshow, 1980; Zerebecki et al., 2021).

A recent study by Zerebecki et al. (2021) showed significant genetic variation between the two forms at multiple sites across the East Coast of the United States. In addition, multiple common garden and reciprocal transplant experiments have shown that height differences persist when the plants are planted in different environments (Stalter and Batson, 1969; Gallagher et al., 1988; Zerebecki et al., 2021). However, other studies reported conflicting results in regards to the growth of both forms across different environments. Zerebecki et al. (2021) found that tall- and short-form height differences persisted when both forms were planted in the area where tall-form would normally grow but not when planted in the short-form area. Multiple years after a reciprocal transplant experiment, Shea et al. (1975) found no significant difference in height between transplanted plants and surrounding plants natural to that zone though differences in leaf width, with the tall plants having thicker leaves and stems, had persisted for some time. Therefore, differences between short- and tall-form *S. alterniflora* are complex and not fully understood. Literature suggests that the length of time a study takes place or the location of the study could impact the results due to the two forms potentially being genetically distinct in some areas but not others. Also, some habitat differences between the short and tall zones that could impact growth may be found in only some areas (Shea et al., 1975).

There are many environmental factors that have been suspected to influence the heights of short- and tall-form *S. alterniflora*. One difference often mentioned between the short and tall zones is frequency of inundation. Tall-form plants can experience high and low tides as the tide changes in the creek, while short-form plants located further back from the water's edge and at higher elevations often experience immersive high tides less frequently (Shea et al., 1975; Valiela et al., 1978). Frequency of flooding and depth of flooding has been shown to impact the growth and asexual reproduction of *S. alterniflora* though the impact of inundation on short- and tall-forms was not specifically examined (Xue et al., 2018).

Nevertheless, both marsh habitats of the two *S. alterniflora* forms are susceptible to oil pollution following a spill. Exposure to No. 2 fuel oil has been shown to decrease biomass, height, and stem density of *S. alterniflora* with mortality occurring at higher levels of exposure (Lin et al., 2002; Culbertson et al., 2008). Many factors can contribute to a plant's response to oil such as the amount of oil that is spilled and the tidal height and frequency of the area which can impact the length of leaves that become coated in oil (Pezeshki and De Laune, 1993: Pezeshki et al., 2000; Lin et al., 2002). Intraspecific variation in oil response can also occur where certain genotypes are more impacted by oil exposure than others (Hester et al., 2000).

Despite the prevalence and importance of short- and tall-forms of *S. alterniflora* in marsh ecosystems, studies have yet to examine the response of these forms to oil. We first started to address this knowledge gap in 2022 with an unpublished pilot study that exposed mature, field collected short- and tall-forms to various doses of No. 2 fuel oil. We found that short-form appeared to be more vulnerable to the oil having a significantly lower LC50 (0.074 mm, 95% CI

0.071, 0.078), or oil slick thickness that would lead to 50% mortality in 32 days, than tall-form (0.133 mm, 95% CI 0.124, 0.143). For the follow-up study outlined in this paper, we sought to specifically examine whether there was an innate difference in response to No. 2 fuel oil by growing both forms from seed in a greenhouse prior to oil exposure to minimize environmental differences during the plants' development. We also examined how one environmental difference between the short and tall zones–frequency of tidal inundation–could impact the response of both forms to oil. Results from this study could help us better understand the role of genotypic and environmental differences in the response of *S. alterniflora* to oil spills.

METHODS

Seed Collection and Germination

In fall 2022, short and tall-form *S. alterniflora* seeds were collected from a long-term National Oceanic and Atmospheric Administration (NOAA) marsh reference site located on Wadmalaw Island, SC, USA (32°38'50.6"N 80°13'18.1"W). Seeds were collected haphazardly from plants within the zones where tall-form and short-form grow. Seeds from boundary areas between the two zones were not collected because of the potential to collect the wrong form. Seeds were immediately relocated to the laboratory and grown using methods inspired by the From Seeds to Shoreline® marsh restoration program (S.C. Sea Grant, 2023).

Within 24 hours of collection, the seeds were rinsed and stored in bags of tap water at 4℃. In January, seeds were drained and rinsed once again before being relocated to petri dishes with moist paper towels in the NOAA National Centers for Coastal Ocean Science (NCCOS) greenhouse, Charleston, SC. Each week, germinated seeds were planted in 2.5" plug pots with Scott's® Turf Builder® LawnSoil™ and moved into trays filled with tap water. At the beginning of April, all plants were moved into bins of seawater and fertilized once over the following week with Milorganite® (6-4-0) according to the fertilizers recommended application to aid growth and survival rates. One month later, all plants were replanted into 4” pots and fertilized again. Approximately two weeks before the start of the experiment on July 5th, 2023, plants were moved into microcosms to acclimate prior to the start of the experiment.

Tidal Microcosm Systems

Small Intertidal Microcosm PLant Exposure (SIMPLE) systems were built to expose the plants to oil (Figure 1). Each system consisted of upper and lower connected 12-gallon, plastic buckets. Four pots of either short- or tall-form *S. alterniflora* were placed into each upper bucket. The lower bucket held 25 ppt saltwater and contained a pump used to facilitate tides in the upper bucket. The pumps were connected to timers allowing half of the systems to be placed on a daily tide schedule and half on a weekly tide schedule. Plants in the daily tide systems experienced two high and two low tides each day for 6 hours at a time. Weekly tide systems remained at low tide except for two 6 hour high tides every Wednesday. High and low tides occurred at consistent times throughout the study. Saltwater was contained within each system, but deionized water was added each day to counter evaporation and maintain consistent water and salinity levels.

This experiment consisted of eight treatments due to the use of a three-factor design with two plant-forms, two simulated tidal regimes, and two oil exposures. Four SIMPLE systems were used for each treatment resulting in 32 systems total. Half of the systems were dosed with 10 mL of No. 2 fuel oil on July 5th, 2023. The diesel fuel was pipetted onto the surface of the water in the upper bucket at high tide to form a 0.1 mm thick slick chosen based on the results of the pilot study. Following dosing, tides in all the systems were pumped down and then immediately up again to facilitate mixing of the oil. The systems then continued with their assigned tidal frequency for the next 12 weeks over which various measurements were taken.

Plant Growth and Health Measurements

Once a week, the number of stems in each pot and the height of every stem were measured. Stem chlorosis and mortality were measured daily for only 4 weeks after dosing because by that time all oiled systems had reached ~100% mortality. Chlorosis, a yellowing of the leaves due to a loss of chlorophyll, was recorded as a visual estimate of the percent of leaves with yellowing in each pot. Mortality was recorded as the percent of stems that had turned brown in each pot. For the purpose of statistical and graphical analysis, values were averaged across the pots in each bucket at each timepoint to calculate a singular data point for chlorosis, mortality, and number of stems, therefore treating each of the 32 buckets as independent replicates.

Statistical analysis was performed using R (R Core Team, 2023) and RStudio (RStudio Team, 2022) with the packages ggplot2 (Wickham, 2016) for graphing and viridis (Garnier et al., 2023) for graph color palettes. For mortality and chlorosis, the ecotox package (Hlina et al., 2021) was used to calculate time to 50% chlorosis (ET50) and 50% mortality (LT50), as well as their respective fiducial confidence limits for each oiled treatment using a probit analysis. Ratio tests were used to determine which treatments had significantly different ET50 or LT50 values.

At 12 weeks post-dosing, plants were harvested for biomass measurements. Above-ground and below-ground biomass for each system was separated and dried in a 70°C oven for 8 days until changes in weight had ceased. Samples were then weighed for dry biomass. Data were log-transformed to meet the assumptions of ANOVA, and then ANOVA was used to examine whether plant form, tidal frequency, presence of oil, and the interactions of those variables had a significant effect on above-ground and below-ground biomass. Post-hoc analyses were performed using Tukey’s tests to examine which treatments were significantly different.

Water Chemistry and Water Quality Measurements

556 Multi-Probe Systems (YSI) were used to monitor water quality (dissolved oxygen, pH, temperature, and salinity) in the lower bucket of each system throughout the study at low tide. Since measured water quality values did not differ substantially throughout the experiment between treatments, the mean and standard error (SE) were calculated for each parameter across all SIMPLE systems throughout the 12 week study.

Water samples were taken from the lower bucket before dosing as well as 1 h, 24 h, 14 d, and 28 d after dosing. Approximately 200 mL of water was removed from each replicate. Within each treatment, water samples were composited across the four replicate systems resulting in one ~800 mL sample per treatment at each timepoint. From these composite samples, 50 PAHs (parent and alkylated polycyclic aromatic hydrocarbons; tPAH50) and TEH (total extractable hydrocarbons) were extracted and measured according to procedures in DeLorenzo et al. (2017).

For each treatment at each timepoint, TEH values were below minimum detection limits, and therefore, were not analyzed further. Since tPAH50 values were highest 24-hours post-dosing, tPAH50 means, standard errors, and ranges were calculated for the four unoiled treatments combined and once again for the four oiled treatments combined at this time point. Samples were combined by oiling condition because differences were minimal between the treatments within each oiling condition.

RESULTS/DISCUSSION

Plant Growth and Health

Both genetic factors, in the case of short- and tall-form plants, and an environmental factor, tidal frequency, were shown to have an impact on the response of *S. alterniflora* to No. 2 fuel oil. Time to 50% chlorosis (ET50) values were significantly different between short-form and tall-form plants exposed to weekly tides (Figure 2a,3a). The ET50 for short-form/weekly tide plants was calculated to be 2.01 (fiducial confidence limits (FCL): 1.82-2.20) days, significantly lower than the ET50 for plants from the tall-form/weekly tide treatment (2.55, FCL: 2.41-2.68) days. Calculated measurements of stem mortality (analyzed as time to 50% mortality or LT50) were determined to be significantly different for all oil and tide treatments (Figure 2b,3b). Short-form/weekly tides once again had the shortest time at 11.60 (10.93-12.19) days compared to the longest time to mortality for tall-form/daily tides at 16.66 (16.20-17.11) days.

Following 100% mortality, no new live stems were produced in the oiled systems throughout the 83 day experiment (Figure 4). For above-ground biomass, ANOVA resulted in significant differences in both plant form ($p=0.011$) and exposure to oil ($p<0.001$), while tidal frequency and the interactions of each set of variables was not significantly different. Plant form ($p<0.001$), tidal frequency ($p<0.001$), presence of oil ($p<0.001$), the interaction of tidal frequency and presence of oil ($p<0.001$), and the interaction of form, tidal frequency, and oil ($p=0.011$) all had a significant effect on below-ground biomass. Significant differences between treatments for above-ground and below-ground biomass are shown in Figure 5a and 5b. Both below and above-ground biomass were lower in oiled systems compared to the unoiled treatments, and no significant differences were found between oiled treatments (Figure 5a,5b).

Water Chemistry and Water Quality

Throughout the study, differences in temperature (mean: 31.3°C, SE: 0.10), dissolved oxygen (134.6%, SE: 1.36), salinity (25.1 ppt, SE: 0.01), and pH (8.69, SE: 0.02) were not apparent between the various treatments. Further statistical examination of differences in water quality parameters between treatments throughout the study could be included in future publications. tPAH50 values indicated that oiled systems were dosed consistently with the planned amount of oil (Figure 6, Table 1). Concentrations remained consistent within the oiled treatments and the unoiled treatments at each timepoint, peaking for oiled treatments at 24 hours post-dosing and declining at the following timepoints. (Figure 6, Table 1).

CONCLUSIONS

To our knowledge, this is the first study showing that short- and tall-form *S. alterniflora* can exhibit different responses when exposed to anthropogenic contamination and in this case No. 2 fuel oil. Nevertheless, other studies have also examined intraspecific variation in oil response. When studying *S. alterniflora* on the edge of marsh sites after the *Deepwater Horizon* crude oil spill, Robertson et al. (2017) found genetic differences between contaminated and uncontaminated sites suggesting that differential mortality amongst different genotypes could have occurred. Hester et al. (2000) exposed 10 genotypes of *S. alterniflora* to crude oil and found differences in response across various measurements. Our study adds to this knowledge base of intraspecific variation in oil response by specifically showing that short-form S. *alterniflora* had significantly faster mortality rates than tall-form within two tidal regimes.

Within each form, plants exposed to weekly tides had faster mortality rates than those exposed to daily tides. This is possibly connected to the amount of time the stems spent covered in oil. As the tide falls in the buckets, oil is left coating the leaves of the plants. Therefore, stems in the weekly tide systems were covered in oil for most of each week. Oil coverage on stems can block stomatas and has been linked to reductions in photosynthesis and transpiration. This in turn could lead to mortality (Pezeshki and De Laune, 1993; Pezeshki et al., 2000).

Regardless of form or tidal condition, all oiled plants were dead within the first month of the study leading to no differences in dry biomass between those treatments. Because of the height of the plants, nearly 100% of the length of the stems were contained within the buckets and therefore coated in oil as the tides lowered. When studying plant mortality following the *Deepwater Horizon* spill, Silliman et al. (2012) noticed a turning point where most stems died after ~65% stem oil coverage. With No. 2 fuel oil, complete coverage of *S. alterniflora* stems has been shown to lead to near 100% mortality three weeks after dosing similar to what was seen in our study (Webb et al., 1985).

Though Webb et al. (1985) also found new stems produced months after oiling which varies from our study where no new stems were produced following 100% mortality. Surviving roots can asexually produce new stems in sediments contaminated with oil (Webb et al., 1985; Lin et al., 2002). However, high doses of oil can inhibit the production of new stems and lead to root mortality (Bergen et al., 2000; Lin et al., 2002). The lack of regrowth in our study even once tPAH50 water concentrations decreased below method detection limits could be an indication that the root systems were killed and not able to produce new stems following exposure to a 0.1 mm No. 2 fuel oil slick. In addition, unlike the Webb study, our study only continued for 12 weeks preventing the growth of new stems from seeds and did not occur in a field scenario. Water is recycled within each SIMPLE system throughout the experiment and therefore tidal removal of the oil could be limited compared to a field scenario. The retainment of water within each bucket could enhance root mortality and therefore limit the regrowth of plant stems.

The differences between short- and tall-form *S. alterniflora* oil response could become more pronounced at lower doses of oil or with lower tidal heights where complete oil coverage on plants may not occur. Additional research could further examine the impact of different concentrations of oil as well as different tidal conditions on the response of short- and tall-form *S. alterniflora*. By examining both *S. alterniflora* form and tidal frequency, we showed that short-form plants with weekly high tides had both the fastest chlorosis and mortality rates, and therefore, short-form *S. alterniflora* could be most vulnerable following spills. When evaluating potential response efforts after spills or conflicting results from different oil spill studies, considering both the form of *S. alterniflora* and the tidal conditions could be important.

It has been suggested that replanting *S. alterniflora* after a spill could aid marsh recovery and reduce long-term environmental impacts, such as erosion connected to mortality of *S. alterniflora* (Bergen et al., 2000; Zengel et al. 2022). When planting, it might be better to plant specific genotypes since some can be less vulnerable to oil contaminated environments (Hester et al., 2000). When studying replanting following the *Deepwater Horizon* spill using genetically different sources of *S. alterniflora*, phenotypic differences and different erosion rates were found between the plants from different sources (Bernik et al., 2021). However, studies have not yet specifically examined replanting of short- and tall-forms.

Our results suggest that short-form may be more vulnerable in oil contaminated environments. However, the ecological roles of the two forms as well as potential differences in their growth following planting in the two zones should also be considered. Our study also found differences in growth in regards to number of stems produced, height of the stems, and below-ground and above-ground biomass between plants in unoiled treatments, and these results will be discussed further in a future publication. Further testing is needed to assess whether it may be beneficial to replant one form instead of the other after a spill, and whether the growth of the forms may differ depending on environmental conditions in the area of the marsh being replanted. Collectively, these data will benefit future oil spill response and salt marsh restoration decisions.

FIGURES AND TABLES

Table 1. Maximum composite water tPAH50 concentration means, standard errors, ranges, and sample sizes for oiled and unoiled treatments at 24 h post-dosing.

	Mean tPAH50 (µg/L)	Standard Error of Mean	Range	n
Oiled Treatments	4.71	0.11	4.52-4.96	4
Unoiled Treatments	0.066	0.0027	0.060-0.071	4

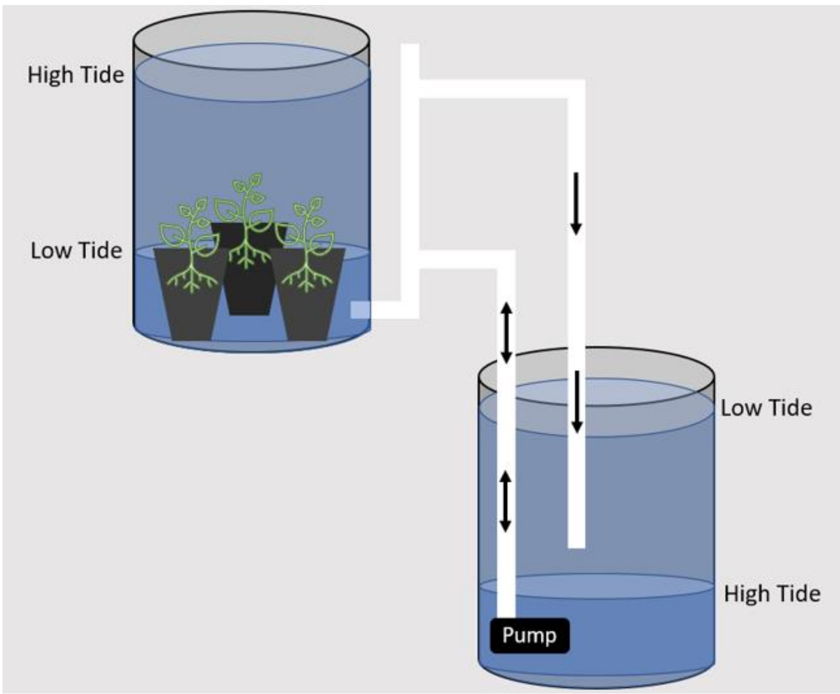
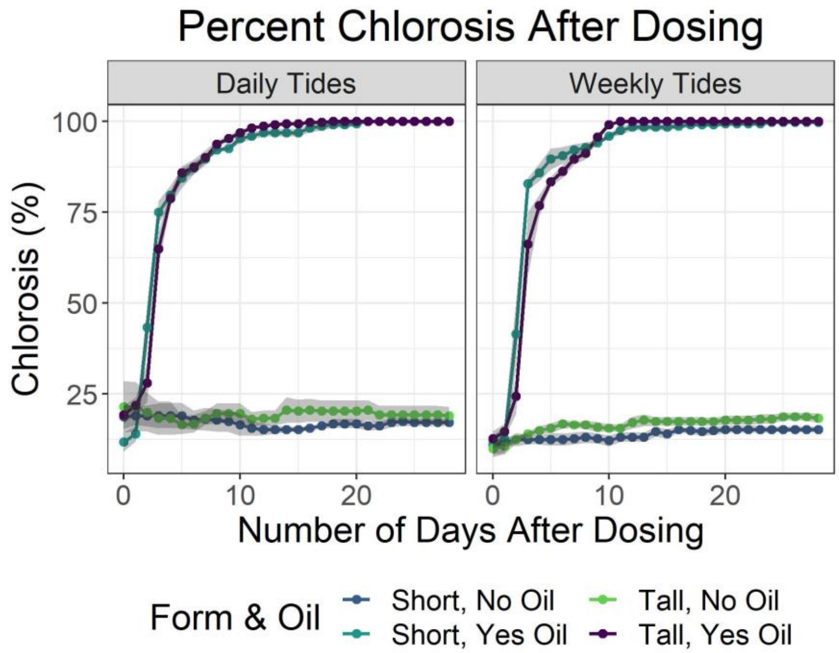


Figure 1. Diagram of SIMPLE system consisting of two buckets connected via pipes/tubing. The lower bucket uses a pump and timer system to simulate high and low tides in the upper bucket.

(a)



(b)

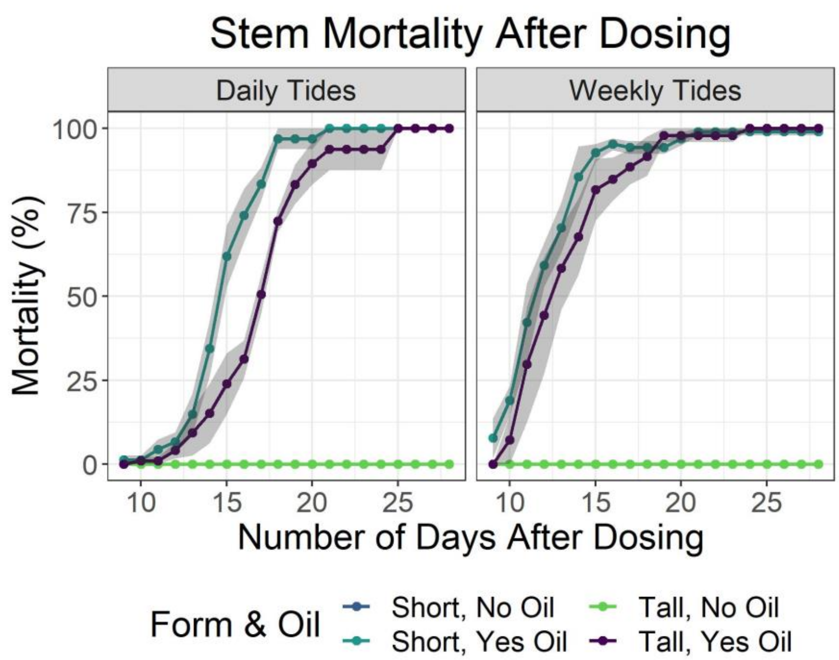
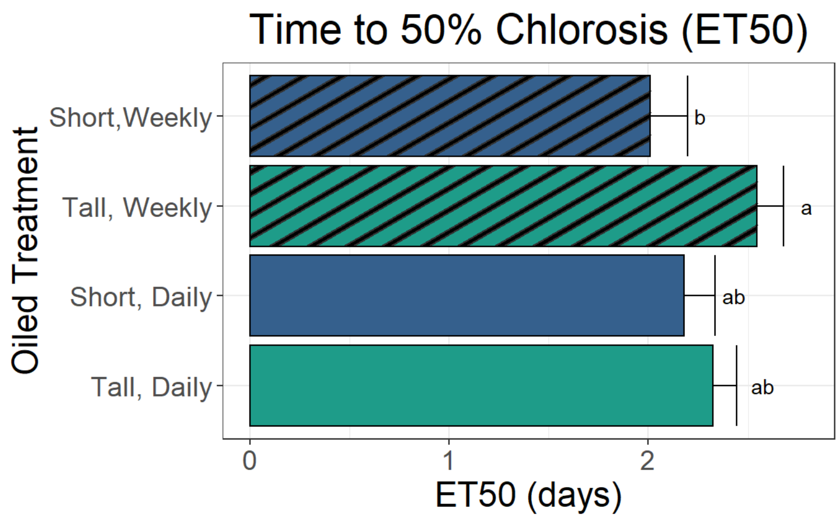


Figure 2. Mean percent chlorosis (a) and percent mortality (b) for 28 days following dosing with No. 2 fuel oil. All eight treatments are represented as distinct lines. For mortality, the tall-form, no oil curve overlaps the short-form, no oil curve. Gray shading indicates standard error.

(a)



(b)

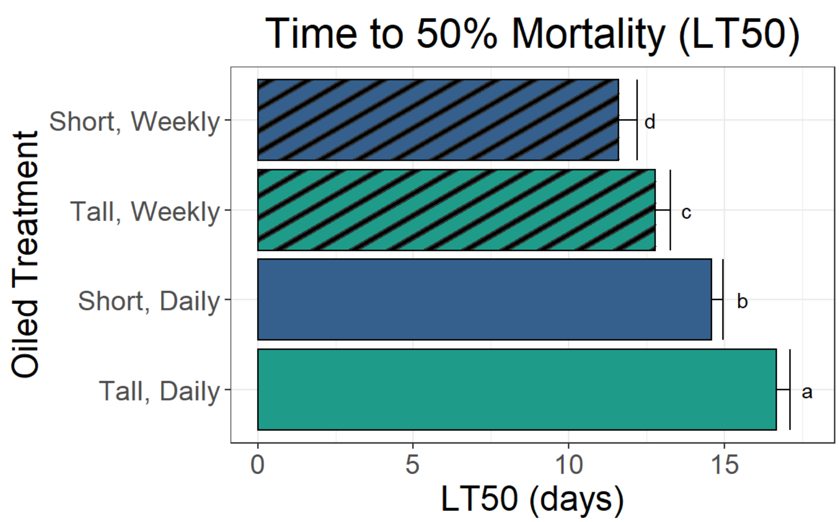


Figure 3. Mean time (days) for each of the four oiled treatments to reach 50% chlorosis (a) and 50% mortality (b). Bars with different letters are significantly different ($p<0.05$).

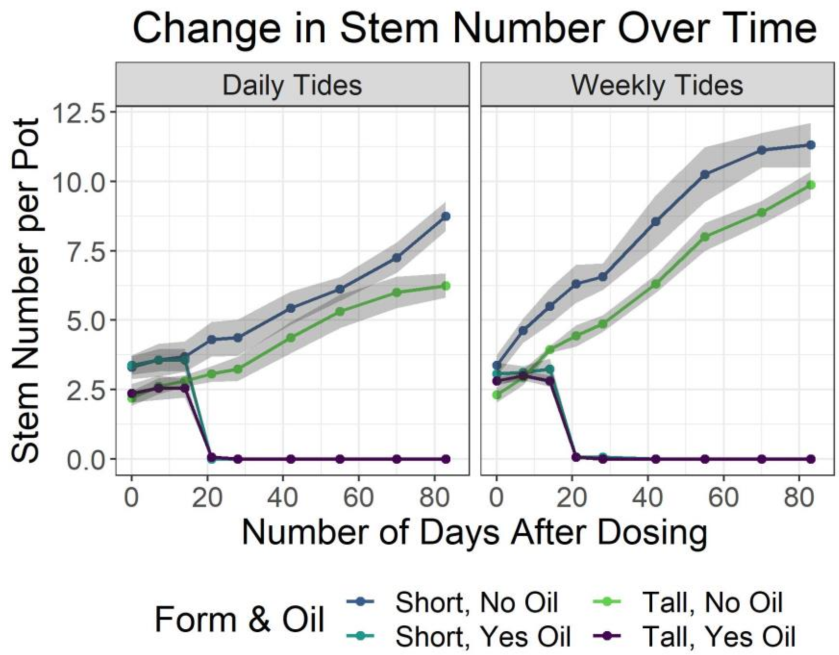
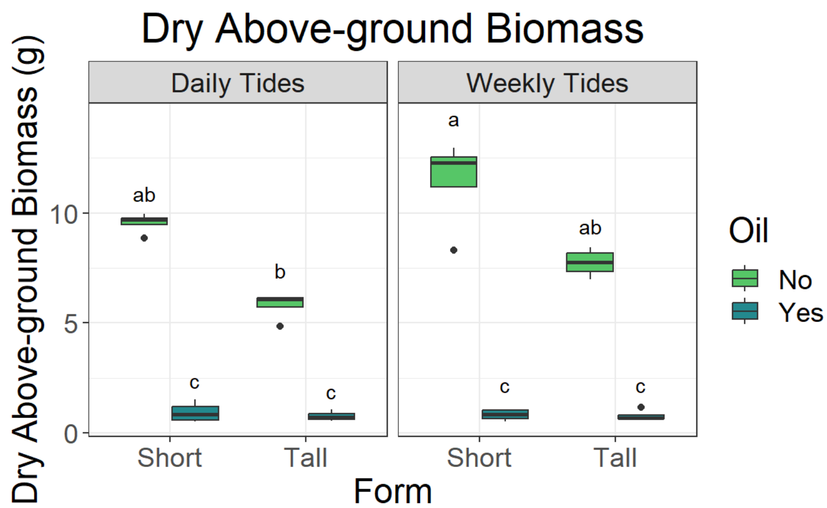


Figure 4. Change in mean number of stems following dosing with No. 2 fuel oil. All eight treatments are represented as distinct lines. Gray shading indicates standard error.

(a)



(b)

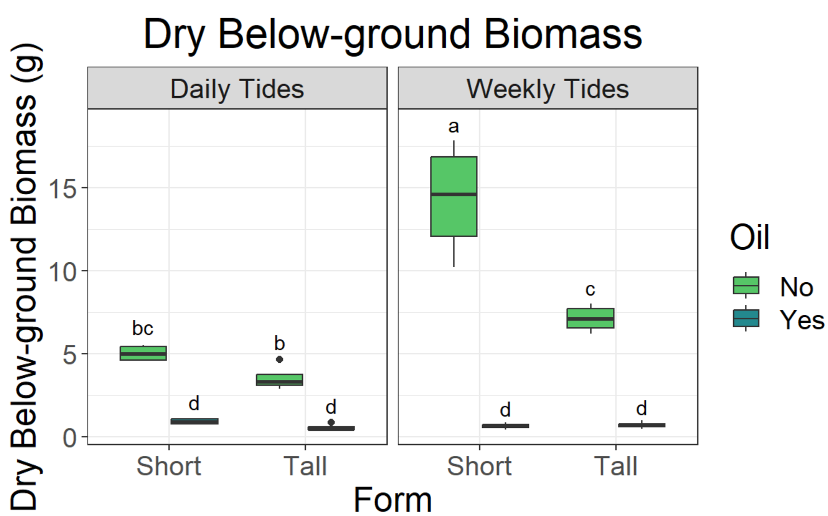


Figure 5. Above-ground (a) and below-ground (b) dry biomass for all treatments 12 weeks post-dosing. Top and bottom horizontal box lines indicate the third and first quartile values respectively. The horizontal line within each box represents the median. Boxes with different letters are significantly different.

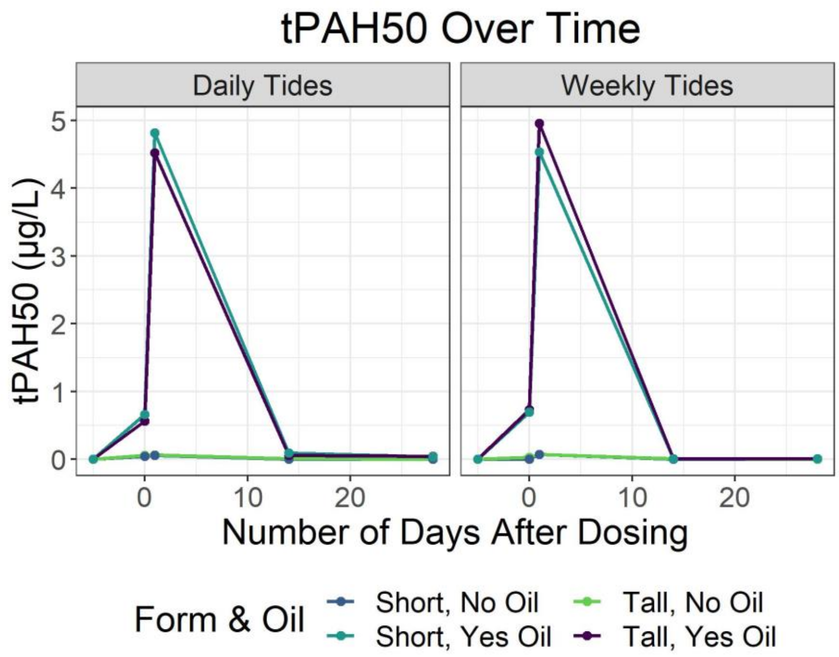


Figure 6. Composite tPAH50 concentrations at each timepoint. All 8 treatments are represented as distinct lines.

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