FEATURE ARTICLE

Foliar DMSO:DMSP ratio and metal content as indicators of stress in *Spartina alterniflora*

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ABSTRACT: We evaluated 2 potential indicators of stress, viz. the ratio of dimethylsulfoxide to dimethylsulfoniopropionate (DMSO:DMSP) and foliar metals, in *Spartina alterniflora* collected from areas affected by 4 different disturbances (sudden marsh dieback, horse grazing, increased snail densities, wrack deposition) across 20 marshes in Georgia, USA. The DMSO:DMSP ratio was a stronger and more consistent indicator of stress than either DMSP or DMSO concentrations alone, with significantly higher ratios occurring in leaves and stems collected from affected compared to healthy areas in all 4 disturbance types. Foliar metal concentrations also differed in affected compared to healthy areas. Of 20 metals evaluated, concentrations of 19 were increased in leaves collected from edge and affected areas. Multidimensional scaling using the entire suite of metals showed separation between plants from affected and healthy areas, but no difference among disturbance types. In contrast, chlorophyll *a* concentrations were not significantly different between affected and healthy areas, and did not correlate with variation in either of the 2 indicators. These results suggest that the DMSO:DMSP ratio and foliar metal suite are sensitive indicators of sublethal stress in *Spartina*, capable of identifying stress before there are visible signs such as chlorophyll loss. The fact that both indicators were consistent across a variety of disturbance types suggests that they may be primarily responsive to general oxidative stress and thus, broadly useful tools for evaluating the health of salt marsh habitat in the field.

KEY WORDS: Smooth cordgrass · Dimethylsulfoniopropionate · Dimethylsulfoxide · Salt marsh · Disturbance · Sudden dieback

Spartina alterniflora salt marsh near St. Simons Island, GA, with a dieback area along the edge of the tidal creek.

Photo: C. McFarlin

INTRODUCTION

Spartina spp. are important wetland plants occurring in temperate salt marshes worldwide, with *S. alterniflora* dominant along the entire US Gulf and Atlantic coasts. These species play a disproportionately critical role in salt marshes by ameliorating chemical and physical stresses to other wetland plants and animals, functioning as essential habitat and refugia from predators, and providing a source of organic matter for associated fauna as a trophic base (Pennings & Bertness 2001). *Spartina* marshes are also important for storm protection, water purification, and erosion control. Salt marshes can be subject to a wide range of natural and anthropogenic disturbances, all of which can affect the services provided by *Spartina* spp.

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Two common disturbances that affect salt marshes are wrack deposition and herbivore overgrazing (Pennings & Bertness 2001). Wrack is capable of completely killing *Spartina alterniflora* when it becomes stranded on the marsh surface (often following storm surges) or when it is blocked by docks and other physical barriers (Valiela & Rietsma 1995, Alexander 2008). Overgrazing can also lead to bare patches in the marsh. These can result from the introduction of non-native species (e.g. nutria: Evers et al. 1998; feral horses: Turner 1987; feral cattle: Martin 2003), from the absence or reduction of predators which leads to increased herbivore populations (e.g. littorinid snails: Silliman & Bertness 2002; sesarmid crabs: Holdredge et al. 2009), and from agricultural subsidies that enhance food supply to herbivore populations (e.g. geese: Jefferies et al. 2004). More recently, sudden dieback has resulted in the loss of salt marsh vegetation in areas along the entire US Eastern Atlantic Seaboard and Gulf Coasts since 2000 (reviewed by Alber et al. 2008). The onset of sudden dieback is indicated by a rapid yellowing and browning of *S. alterniflora* in standing position followed by a complete loss of vegetation over the course of a few months (McKee et al. 2004, Alber et al. 2008). To date, no single factor has been linked to sudden dieback; rather, it has been described as a multi-stressor disturbance associated with drought (McKee et al. 2004, Silliman et al. 2005, Alber et al. 2008).

The most obvious and frequently studied response to disturbances in salt marshes has been the degradation and loss of plant biomass (Turner 1987, Baldwin & Mendelssohn 1998, Ewanchuk & Bertness 2003); however, physiological responses may occur long before there are visible signs of stress (Mendelssohn & McKee 1992). Moreover, the effects of many disturbances, such as increases in flooding frequency, pollutant contamination, and introduced species, can occur gradually and be difficult to detect (Mendelssohn & McKee 1992, Weilhoefer 2011). If we can identify early signs of stress in marshes, we will be in a better position to identify areas that are at risk and potentially preserve valuable habitat.

Currently, no consistent, sensitive measures are available that can be used to indicate *Spartina* stress under multi-stressor conditions that exist in the field. Numerous physiological indicators (adenine nucleotides, proline concentrations, CO_2 uptake, water use efficiencies, alcohol dehydrogenase activities, and leaf spectral reflectances) have been evaluated in *Spartina* spp. subject to individual stressors such as salinity, nutrient, and metal stress (Mendelssohn &

McKee 1992, Ewing et al. 1995a,b, Mendelssohn et al. 2001). However, these metrics have primarily been evaluated under manipulated greenhouse conditions and have translated poorly as consistent signals of stress in the field (Ewing et al. 1997). Many of the indicators are stressor-specific: glutathione is best suited for evaluating plants suspected of metal contamination (Mendelssohn & McKee 1992, Pennings et al. 2002), proline is better at identifying salinity stress, and nutrient stress may be identified via altered leaf spectral reflectance, CO_2 uptake, or adenine nucleotide levels (Ewing et al. 1995a,b). There have also been discrepancies in the ability of various indicators to respond to similar disturbances across different field studies. Padinha et al. (2000) found higher concentrations of metal-chelating thiolic proteins and lower photosynthetic efficiencies in *Spartina alterniflora* from polluted areas of the Ria Formosa lagoon in Portugal, whereas Pennings et al. (2002) found no differences in these same indicators in polluted versus healthy areas of South Carolina, USA, marshes.

Studies in sudden dieback areas have reported 2 different types of physiological responses in *Spartina alterniflora* plants that may reflect general (oxidative) stress: lower concentrations of dimethylsulfoniopropionate (DMSP) and increased concentrations of metals (Fe, Al) were found in leaves of *S. alterniflora* collected near dieback areas as compared to healthy marsh areas in South Carolina and Louisiana, respectively (McKee et al. 2004, Kiehn & Morris 2010). DMSP is a secondary metabolite synthesized by *S. alterniflora* from the amino acid methionine, and has been speculated to be an herbivore deterrent/attractant, a sulfur detoxifying agent, and an antioxidant (Kocsis et al. 1998, Sunda et al. 2002, Husband & Kiene 2007). Recent work supports an antioxidant role of DMSP in *S. alterniflora*, in that there was direct conversion to its oxidation product, dimethylsulfoxide (DMSO), and thus a higher DMSO:DMSP ratio in yellowing and experimentally stressed plants compared to healthy plants (Husband & Kiene 2007, Husband et al. 2012). The increased concentrations of foliar metals in *S. alterniflora* near dieback areas may have been a direct consequence of altered environmental conditions caused by drought, leading to acidic and oxidizing marsh soil conditions (McKee et al. 2004). Metals often become more soluble and bioavailable to vegetation in drained, aerated marsh soils (Portnoy 1999) or when there has been a change in soil biochemistry (especially of pH and Eh; Kashem & Singh 2001). It is unclear whether these 2 potential indicators (metal concentration and the

DMSO:DMSP ratio) may also apply to other situations where *S. alterniflora* is stressed due to other disturbances.

It would be valuable to identify a generic indicator, capable of detecting plant stress in the field across a variety of conditions, even when the specific cause of 'stress' is unknown. An indicator that is responsive before visible symptoms appear (such as the loss of chlorophyll) would be particularly useful. Using a natural field experiment, we examined the DMSO: DMSP ratio, metal concentrations, and chlorophyll concentrations in *Spartina alterniflora* collected from sudden dieback areas, and in areas subject to 3 other salt marsh disturbances: wrack deposition, herbivory by littorinid snails, and herbivory by horses. Our goal was to test whether the DMSO:DMSP ratio and metal concentrations were useful as generic indicators of stress under varied field conditions, and whether the response was consistent, regardless of disturbance type.

MATERIALS AND METHODS

Study sites

In the fall of 2008 and 2009, we sampled 20 salt marshes along the Georgia coast that had areas experiencing a loss of *Spartina alterniflora*: 5 with a high snail density, 5 with wrack accumulation, 5 with damage by horses, and 5 sudden dieback sites. Sites were located on Sapelo Island, Cumberland Island, and in Meridian and Brunswick (Fig. 1). All disturbed areas were chosen based on the presence of an *S. alterniflora* monoculture. Snail sites had unusually heavy snail densities in the disturbed areas (overall site mean ± SE 452 ± 117 snails m^{-2}), which were close to the levels that had been previously reported to lead to loss of vegetation in Georgia (~600 snails m^{-2}, Silliman & Bertness 2002). Wrack sites were areas that had visible plant debris accumulated on the salt marsh surface (~5 cm thick), with no other known disturbance factors. Horse sites were located in areas identified as frequently grazed by horses at Cumberland Island National Seashore according to the observations of the National Park Service Rangers. Sudden dieback sites were locations that had been reported to the Georgia Department of Natural Resources, Coastal Resources Division (Brunswick) following the 2000–2001 droughts in Georgia, or were observed on Sapelo Island by Georgia Coastal Ecosystems – Long Term Ecological Research personnel.
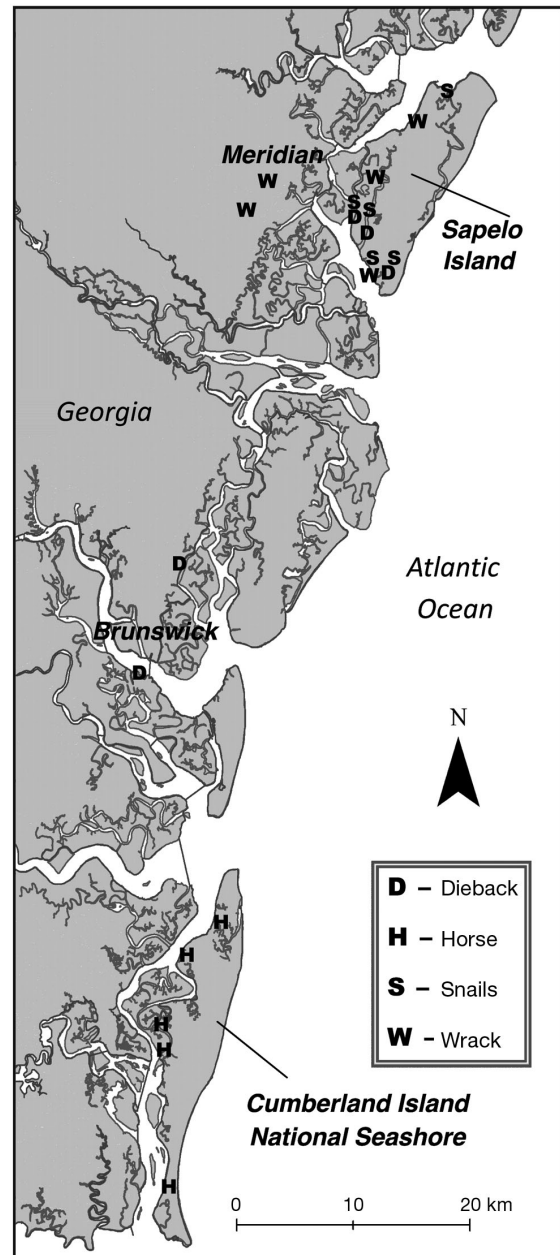

Fig. 1. *Spartina alterniflora*. Location of the dieback, horse, snail, and wrack sites along the Georgia coast (5 sites per disturbance type; see 'Materials and methods' for details of the disturbance types). At each site, plants were sampled haphazardly to test tissue DMSP, DMSO, chlorophyll, and metal concentration, from within the healthy, edge of affected, and affected marsh

Sample collection

We sampled plants in 3 zones at each site: the 'affected' zone, which included the areas where *Spartina alterniflora* was damaged (e.g. remaining rhizome stubble or standing dead plants, visible injuries due to

radulation or grazing) and visible agents of distur-
bance were often present (wrack, snails, horses);
along the 'edge' of the affected area; and a nearby
'healthy' area (generally ~10 m away from the edge of
the affected area, with no visible effects of distur-
bance). The rationale for including the 'edge zone'
was to examine *S. alterniflora* in an area that did not
appear visibly stressed, yet might still experience
negative effects from the nearby disturbed areas (e.g.
through rhizomes of *S. alterniflora* or through the loss
of neighboring plants that typically ameliorate eda-
phic stressors; Bertness & Shumway 1993). In addition,
S. alterniflora leaves and stems were already com-
pletely lost in the 'affected zone' at all dieback sites
(except for 1 stem at 1 site), and *S. alterniflora* leaves
were lost in the 'affected zone' at 2 of the snail sites, so
the 'edge zone' also provided an intermediate level of
a disturbance from which to obtain plant samples.

At each site, 3 individual *Spartina alterniflora*
plants were haphazardly selected from within each
zone (except where plants were absent in the
affected zone) for the analysis of DMSP, DMSO, and
chlorophyll concentrations. Intact plants, which were
located at least 5 to 10 m apart, were removed with
root material, carefully transported upright in moist
soil to the laboratory, rinsed in deionized water to
remove bacterial and algal growth, and prepared for
immediate analysis or frozen at −80°C (for ≤6 mo)
within 48 h of collection. Plants were then clipped
into leaf, stem, and root sections for analysis. Because
the physical condition of plants varied, we clipped
samples from the best 2 of 3 plants based on color
and vigor and noted the leaf color. Leaves and stems
for measurements of DMSP, DMSO, and chlorophyll
concentrations were clipped from the same section of
each plant as follows: a small section of leaf (~0.5 cm
length) was clipped from the middle of the youngest
fully expanded leaf (typically the second or third leaf
from the top), and a small section of stem (~0.5 cm
length) was clipped at mid-height of the plant. Roots
were clipped for measurements of DMSP and DMSO
near the attachment to the rhizome.

For the analysis of metal content, only living leaves
(at least 75% green) were used, as previous studies
have shown that metals (Cu, Pb, Zn) accumulate as
Spartina alterniflora leaves get older and senesce
(Weis et al. 2003). Where possible, we used the por-
tion of leaf that remained after clipping for DMSO,
DMSP, and chlorophyll analysis, as well as the entire
length of the next youngest fully expanded green
leaf. Leaves were pooled across the 2 replicate plants
collected per zone at each site, and dried at 60°C
until they reached a constant weight.

Foliar DMSP, DMSO, and DMSO:DMSP ratio

Leaves and stems (10.0 to 50.0 mg pieces, weighed
to the nearest 0.1 mg) were weighed and placed into
30 ml serum vials (Wheaton, 37.4 ml of headspace),
sealed with gas-tight septa and aluminum crimp
tops. The concentration of cellular DMSP and DMSO
was measured by converting each to DMS gas, sepa-
rately. DMSP was converted to DMS gas by injecting
1 ml of 5 M NaOH into the serum vials, and incubat-
ing upside-down in the dark for a period of 24 h at
30°C (without shaking). The methods for DMSO con-
version were similar, except that 0.5 ml of 20% $TiCl_3$
was added to vials and incubation was for a period of
2 h at 50°C (without shaking). Following incubation,
0.2 ml (for DMSP analysis) or 0.5 ml (for DMSO
analysis) of headspace gas from the serum vials was
injected into a flame photometric detector gas chro-
matograph (SRI 8610-C with a Chromosil 330 column
with nitrogen as the carrier gas) and analyzed for
DMS area using the PeakSimple Program. In each
case, 2 subsamples each for DMSP and DMSO were
analyzed as independent analytical replicates from a
single plant sample.

Standard curves to relate peak area to DMS gas
were obtained by injecting the GC with DMS gas
liberated from known amounts of DMSP or DMSO
standard stocks that were converted to DMS gas using
similar volumes of NaOH and $TiCl_3$ as used for the
samples. It was assumed that all DMSP or DMSO was
converted to DMS gas, and that the DMS present as
headspace gas or dissolved in the liquid volume (based
on Henry's solubility coefficient constant) of the serum
vial was primarily due to direct liberation of foliar
DMSP and DMSO (DMS present in *Spartina* leaves
could also contribute a very minor proportion of the
DMS gas measured in the vial). The foliar concentra-
tion of DMSP and DMSO in µmol g^{-1} tissue (fresh
weight, FW) was determined by dividing the concen-
tration of DMS gas in the serum vial by the weight of
plant tissue. Blank controls for each were monkey
grass *Ophiopogon japonicus* and deionized water,
and positive controls of DMS gas were liberated from
DMSO standards. Controls were treated and injected
identically to samples.

Chl *a* and phaeo *a* concentrations

Leaves and stems (10.0 to 50.0 mg pieces, weighed
to the nearest 0.1 mg) were macerated with a tissue
grinder, extracted in 90% acetone, and centrifuged
at 2200 × *g* (10 min). The fluorescence of the super-

natant was measured before and after acidification with 10% HCl on a Turner Designs 10-AU fluorometer following US Environmental Protection Agency Method 445.0 (Arar & Collins 1997). High and low value liquid chlorophyll standards with certified spectrophotometer (Abs) readings were used to assign concentration values to fluorometer measurements. Fluorometer readings were converted to chlorophyll *a* (chl *a*) and phaeophytin *a* (phaeo *a*) per gram of tissue (FW). In each case, 2 subsamples were analyzed as independent analytical replicates from a single plant sample and then averaged.

Leaf metals

Dried leaves were ground in a Wiley mill (mesh no. 40), burned at 500°C for 4 h, and amended with a plant buffer solution (30% HCl, v/v; 10% HNO_3, v/v; and 20 ppm of molybdenum) at a ratio of 1:10, sample (ash):buffer. Samples were analyzed for a suite of 20 elemental constituents (Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Si, Sr, Zn) with an ICP spectrometer (Jarrell-Ash 965 Inductively Coupled Plasma-Optical Emission Spectrograph) at the University of Georgia's Chemical Analysis Laboratory using EPA analytical method 6010 C. National Institute of Standards and Technology (NIST) plant standards (apple leaves) were used to confirm the proper calibration for the matrix.

Statistical analysis

Chl *a*, DMSP, DMSO, and the DMSO:DMSP ratio were compared among the 4 disturbance types and among the 3 marsh zones. In each case, we averaged the results from all subsamples of a given plant for statistical analysis. Each measure was analyzed using a 2-way split-plot (partially nested) analysis of variance (ANOVA), where disturbance type and zone were the between- and within (split)-plot fixed effects, respectively, and sites were considered the unit of replication. The significance of disturbance type was evaluated against the whole-plot error term (sites within disturbance type). The significance of zone and the interaction term zone × disturbance type were evaluated against the split-plot error term (sites within disturbance type × zone, i.e. the residual). The interaction term was used to evaluate the main (null) hypothesis in this study, which was that the effect of zone would be similar regardless of disturbance type (i.e. a non-significant effect supports

the hypothesis). Because the split-plot model requires a complete dataset with no missing values, there were a few cases where values were filled in. In the case of affected areas where no plants were collected (all dieback sites, 2 snail sites), we used the 'edge' zone value for the missing 'affected' zone. These were considered conservative in that concentrations of DMSP, DMSO, and chl *a* were typically lower in the affected than in the edge zones of the other 13 sites, and because plants had died in affected areas of dieback sites. Tukey's multiple comparison post hoc tests were used to evaluate pairwise differences among disturbance, zone, and disturbance × zone factors. Factors and pairwise differences were considered significant when $p \leq 0.05$.

In order to examine the full suite of elemental composition of *Spartina alterniflora* leaves, we used nonmetric multi-dimensional scaling (NMDS) (R statistical package, R Development Core Team 2011) to view how zones and disturbance types were separated based on Bray-Curtis distances. Analysis of similarity (ANOSIM) was used to detect whether there were significant overall differences in the group clustering of zone and disturbance type based on 1000 permutations of the data. A sequential Bonferroni significance post hoc test was used to examine differences within each factor (PAST statistical package, Hammer et al. 2001).

RESULTS

DMSP and DMSO

DMSP concentrations averaged 17.25 ± 1.02 (SE) and 10.86 ± 0.78 µmol g^{-1} FW in healthy leaves and stems, respectively, whereas DMSO concentrations were an order of magnitude lower, averaging 0.99 ± 0.14 and 0.87 ± 0.12 µmol g^{-1} FW in healthy leaves and stems (Table 1). These DMSP concentrations were within the range that has been previously reported in healthy *Spartina alterniflora* (Otte & Morris 1994, Husband & Kiene 2007, Kiehn & Morris 2010). The DMSO concentrations observed here were as much as 2 to 3 times higher than the only other study in which they were measured (leaves: ~0.60 ± 0.20 µmol g^{-1} FW; stems: ~0.36 ± 0.15 µmol g^{-1} FW; Husband & Kiene 2007). The DMSP and DMSO concentrations found in the roots of *S. alterniflora* were much lower than those of leaves and stems, with means of 1.26 ± 0.14 and 0.43 ± 0.13 µmol g^{-1} FW, respectively, in healthy plants. What is more important for this study, however, is how these constituents varied with stress.

Table 1. *Spartina alterniflora*. Mean (SE) of chlorophyll *a* (chl *a*), DMSP, and DMSO concentrations in leaves and stems collected in healthy, edge, and affected zones in dieback, horse, snail, and wrack disturbance types (see 'Materials and methods' for details of the disturbance types). The highest concentration of chl *a*, DMSP, or DMSO per zone is shown in **bold**, in order to highlight trends. Each mean represents N = 15 for chlorophyll and N = 10 for DMSP and DMSO in leaves and stems (except at wrack sites, where N = 9 for chlorophyll and N = 6 for DMSP and DMSO, and in the dieback affected area where '−' denotes no sample and [a] denotes 1 sample)

Disturbance type	Zone	Leaf chl *a*	Stem chl *a*	Leaf DMSP	Stem DMSP	Leaf DMSO	Stem DMSO
		—— (mg g^{-1} fresh wt) ——		——————————(μmol g^{-1} fresh wt)——————————			
Dieback	Affected	–	–	–	0.36 (0)[a]	–	0.11 (0)[a]
	Edge	0.389 (0.052)	0.073 (0.020)	13.45 (3.62)	8.35 (1.67)	**1.06 (0.30)**	0.68 (0.09)
	Healthy	**0.481 (0.055)**	**0.075 (0.010)**	**17.44 (2.41)**	**12.27 (2.14)**	1.05 (0.28)	**1.05 (0.29)**
Horse	Affected	**0.803 (0.045)**	**0.108 (0.024)**	11.32 (0.97)	4.37 (0.76)	1.25 (0.36)	0.61 (0.16)
	Edge	0.744 (0.054)	0.102 (0.017)	12.56 (1.53)	6.66 (1.36)	0.63 (0.09)	047 (0.11)
	Healthy	0.688 (0.078)	0.095 (0.013)	**21.74 (2.48)**	**9.43 (1.59)**	**1.66 (0.42)**	**0.86 (0.23)**
Snails	Affected	0.231 (0.069)	0.049 (0.013)	10.71 (3.48)	5.34 (1.79)	**0.90 (0.20)**	0.26 (0.06)
	Edge	0.43 (0.052)	0.059 (0.010)	**15.61 (1.27)**	8.04 (1.60)	0.50 (0.08)	0.37 (0.08)
	Healthy	**0.438 (0.036)**	**0.084 (0.014)**	14.97 (1.22)	**11.16 (1.58)**	0.57 (0.08)	**1.06 (0.30)**
Wrack	Affected	0.479 (0.042)	0.063 (0.010)	10.13 (1.64)	**12.21 (2.25)**	0.80 (0.26)	**1.93 (0.88)**
	Edge	0.422 (0.030)	0.058 (0.014)	**17.81 (3.04)**	11.99 (1.58)	**0.93 (0.37)**	0.76 (0.23)
	Healthy	**0.484 (0.030)**	**0.078 (0.014)**	15.22 (2.11)	10.30 (0.99)	0.67 (0.10)	0.51 (0.08)

DMSP concentrations in both leaves and stems varied significantly by zone (a proxy for stress), but not by disturbance type (Tables 1 & 2). We found significantly lower DMSP concentrations in the affected compared to healthy and edge zones in leaves (p = 0.0026) and stems (p = 0.0127), with the only exception being stems from wrack sites, which had higher DMSP concentrations in the affected zone. There was also no difference in the effect of zone × disturbance type, indicating that the effect of zone on the DMSP concentrations of leaves and stems was similar, regardless of disturbance type.

Patterns of DMSO concentrations in leaves and stems across zone were often similar to those observed for DMSP concentrations (Table 1). This was especially true for stems, where DMSO concentrations in each of the 4 disturbance types were highest in zones where DMSP concentrations were also highest. However, DMSO concentrations in leaves and stems did not vary significantly by zone or disturbance type (Table 2). The concentration of DMSP was a significant predictor of the DMSO concentrations in leaves (p < 0.0001, r^2 = 0.17) and stems (p < 0.0001, r^2 = 0.17), but the relationships were much stronger when only the healthy zones were considered (healthy leaves: p < 0.0001, r^2 = 0.63; healthy stems: p < 0.0001, r^2 = 0.24).

The proportion of DMSO, and thus the ratio of leaf and stem DMSO:DMSP, varied significantly by zone but not by disturbance type (Table 2, Fig. 2). The ratio was significantly higher in the affected compared to healthy zones at horse, snail, and wrack sites both in leaves (p < 0.0001) and stems (p = 0.0056). In the case

of dieback, where no leaves were available in the affected zone for comparison, the ratio was increased in plants from the edge zone. This pattern was strongest in the leaves compared to stems, where DMSP concentrations tended to be highest (DMSO concentrations were similar in leaves and stems), and thus the ratio had a greater variation between zones. There was no effect of zone × disturbance type, indicating that the effect of zone (a proxy for stress) on the DMSP:DMSO ratio in leaves and stems was similar, regardless of disturbance type. Roots had relatively low concentrations of both DMSP and DMSO, and the patterns of DMSO:DMSP were not as strong or consistent (see Table S1 in the supplement at www.int-res.com/articles/suppl/m474p001_supp.pdf).

Chl *a*

The chl *a* concentrations measured in *Spartina alterniflora* ranged from 0.23 to 0.80 mg g^{-1} FW for leaves (Table 1). The chl *a* content in leaves from healthy zones was similar to field values reported previously (0.6 mg g^{-1} FW, Seneca & Broome 1972; 0.76 mg g^{-1} FW, Piceno & Lovell 2000), but slightly lower than those reported from plants grown in the greenhouse (Seneca & Broome 1972, Pezeshki & DeLaune 1993). Chl *a* content of stems was consistently 12 to 20% that of leaves of the same plant, ranging from 0.05 to 1.08 mg g^{-1} FW.

Chl *a* content was highest in stems and leaves in the healthy zones at all of the disturbance types, except for horse sites where concentrations were

Table 2. *Spartina alterniflora.* Statistical summary of split-plot analyses of variance for testing the main effects disturbance type (between-plot factor) and zone (within-plot factor) on the variation in chlorophyll *a* (chl *a*), DMSP, DMSO, and the DMSO:DMSP ratio in leaves and stems of plants at the 20 survey sites. Disturbance type was evaluated against the whole-plot error term (sites within disturbance type), whereas zone and zone × disturbance type were evaluated against the split-plot error term (sites within disturbance type × zone, i.e. the residual). Values in **bold** are significant (p < 0.05)

Source of variation			Leaves			Stems	
		df	*F*	p	df	*F*	p
Chl *a*	Disturbance type	3	10.99	**0.0004**	3	3.12	0.0555
	Whole-plot error term	16			16		
	Zone	2	0.61	0.5472	2	1.02	0.3704
	Zone × Disturbance type	6	1.44	0.2310	6	0.54	0.7757
	Split-plot error term	32			32		
DMSP	Disturbance type	3	0.29	0.8298	3	2.56	0.0917
	Whole-plot error term	16			16		
	Zone	2	7.22	**0.0026**	1	5.02	**0.0127**
	Zone × Disturbance type	6	1.19	0.3383	3	1.04	0.7561
	Split-plot error term	32			16		
DMSO	Disturbance type	3	1.76	0.1947	3	2.2	0.1280
	Whole-plot error term	16			16		
	Zone	2	1.45	0.2496	1	1.54	0.2308
	Zone × Disturbance type	6	1.58	0.1837	3	3.77	**0.0060**
	Split-plot error term	32			16		
DMSO:DMSP	Disturbance type	3	0.54	0.6622	3	1.02	0.4081
	Whole-plot error term	16			16		
	Zone	2	11.81	**<0.0001**	2	6.12	**0.0056**
	Zone × Disturbance type	6	1.27	0.2997	6	0.19	0.9771
	Split-plot error term	32			32		

Fig. 2. *Spartina alterniflora.* Ratio of DMSO: DMSP in leaves (top) and stems (bottom) in healthy, edge, and affected zones of dieback, horse, snail, and wrack disturbance types. The significance (p-value) of the split-plot ANOVA factors zone (Z), disturbance type (D), and zone × disturbance type (Z×D) are indicated by asterisks, where **p < 0.01, ****p < 0.0001, and ns: not significant, and different letters after the zone labels indicate pairwise differences among zones (Tukey's multiple comparison test). nd: no data, †: 1 sample (not included in statistical analysis). Error bars represent SE calculated based on N = 10 plants zone^{-1} at each disturbance type, except wrack sites where only 6 plants were available; ANOVAs were performed after averaging data from the 2 replicate plants analyzed at each site
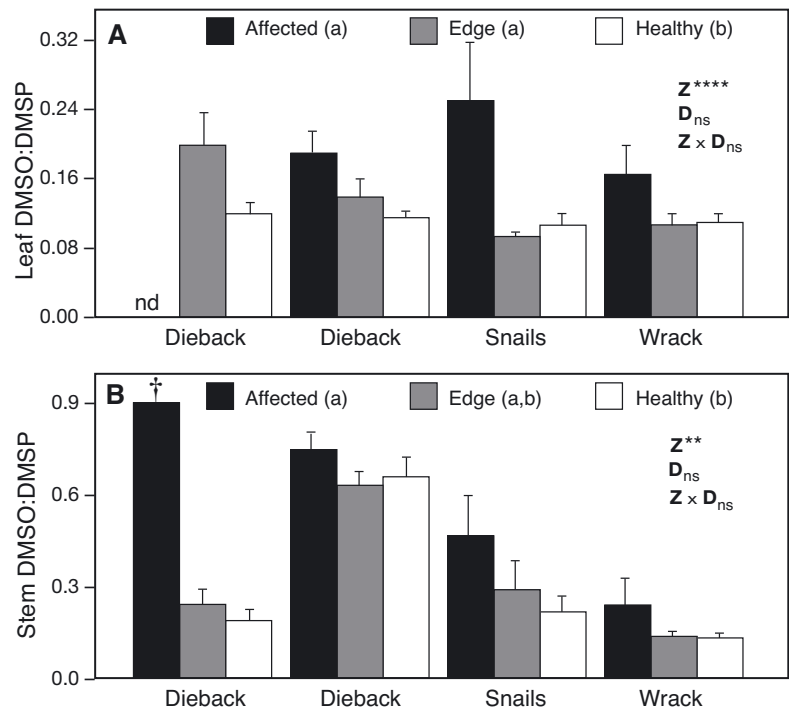
highest in the affected zone (no measurements were made in affected zones at dieback sites due to unavailability of plants). However, chlorophyll differences among zones within each disturbance type were typically fairly small, and zone was not signifi-

cant for either leaves or stems (Table 2). Disturbance type was a significant source of variation in leaf chlorophyll, with the horse sites having a higher overall mean concentration of chlorophyll (0.75 mg g^{-1} FW) compared to other disturbance types (which

were all ≤0.46 mg g^{-1} FW). There was no significant interaction effect of zone × disturbance type.

Because senescing/yellowing plants have higher ratios of DMSO:DMSP (Husband & Kiene 2007), we explored the relationship between chl *a* and DMSO: DMSP using linear regression. Variation in chl *a* concentrations did not predict leaf (N = 32; r^2 = 0.06, ns) or stem (N = 32; r^2 = 0.03, ns) DMSO:DMSP ratios.

Elemental composition (metals)

The elemental composition of green *Spartina alterniflora* leaves generally fell within range of previous reports (Table 3 and Table S2 in the supplement), except for B, Cd, and Co, which were approximately an order of magnitude higher than the limited number of previous observations.

The concentration of the 20 elemental trace metals examined exhibited a strikingly similar pattern among all of the disturbance types; the affected and edge zones had the highest concentrations for all 20 metal constituents in the dieback and horse sites, and for 19 of 20 metal constituents (all but K) in the snail and wrack sites (Table 3). The ratios of Al:K and Fe:K were examined in order to make a comparison to those reported as elevated by McKee et al. (2004)

in dieback areas of Louisiana (Al:K, ~0.04−0.07 and Fe:K, ~0.06−0.10). The Al:K ratios in our study (0.117− 0.237) were about 2- to 3-fold higher than theirs, whereas our Fe:K ratios (0.05−0.11) were similar. However, relative to control areas, McKee et al. (2004) found these ratios to be 4- to 6-fold higher in the dieback areas, whereas in our study the ratios in the affected zones were relatively similar to those in the healthy zones (only ~2-fold higher).

For the overall pattern of total metal composition in *Spartina alterniflora*, we used an NMDS to view how zones and disturbance types grouped (Fig. 3). Zones (especially the affected and healthy zones) were distinctly separate groups, whereas disturbance types were not (ANOSIM: zone, p = 0.002, R = 0.33; disturbance type, p = 0.14, R = 0.18). In post hoc multiple comparisons, the healthy zone was significantly different from the edge and affected zones, whereas the edge and affected zones were not different from one another.

DISCUSSION

This study examined the variation of DMSP, DMSO, chl *a*, and metal concentrations in *Spartina alterniflora* as potential measures of physiological

Table 3. *Spartina alterniflora*. Mean tissue elemental composition of leaves (µg g^{-1} leaf dry weight) in healthy, edge, and affected zones at dieback, horse, snail, and wrack disturbance types (see 'Materials and methods' for details of the disturbance types). The number of sites that were averaged per zone for each disturbance type is indicated (N). The highest concentration of each element per zone and disturbance type is shown in **bold**

| Disturbance type: | Dieback | | | Horse | | | Snails | | | Wrack | | |
Zone:	Healthy	Edge	Affected	Healthy	Edge	Affected	Healthy	Edge	Affected	Healthy	Edge	Affected
N (sites):	3	3	1	2	2	2	3	3	2	2	2	2
Element												
Al	1016	1623	**2368**	1351	**1901**	1596	1545	1580	**2399**	1486	**2493**	2307
As	0.21	0.29	**0.37**	0.27	**0.38**	0.33	0.28	0.28	**0.41**	0.24	**0.37**	**0.37**
B	43.8	**112.9**	99.1	46.1	**132.6**	121.0	43.1	59.8	52.3	43	35	**109**
Ba	0.00	0.00	**3.56**	1.94	**2.28**	2.14	1.00	0.42	**2.36**	0.00	0.18	**0.45**
Ca	2870	**3529**	2705	1583	**2493**	2418	3065	3088	**4192**	2471	2897	**4298**
Cd	2.82	**3.44**	2.84	2.78	**4.40**	4.03	2.94	3.15	**4.30**	2.28	2.89	**4.12**
Co	0.84	**1.05**	0.91	1.10	**1.75**	1.40	0.94	0.97	**1.39**	0.87	1.04	**1.50**
Cr	2.80	**3.59**	3.42	3.68	**5.85**	4.69	3.15	3.28	**4.24**	3.75	3.38	**4.44**
Cu	1.56	**2.98**	2.83	2.15	5.23	**7.73**	1.54	1.98	**2.43**	1.76	2.19	**3.21**
Fe	459	**690**	593	704	**1086**	842	589	613	**640**	552	**808**	542
K	8670	8588	**11040**	11027	9975	**12930**	**11825**	11247	9035	**11165**	11145	9733
Mg	2834	**3892**	3384	2503	**4163**	4022	3187	3826	**5678**	2132	3044	**5050**
Mn	46.1	**89.9**	46.0	25.8	**52.9**	48.5	17.6	19.8	**19.9**	22	36	**161**
Na	3720	3915	**6252**	3852	4930	**6405**	4456	4180	**7469**	4232	5843	**14925**
Ni	2.51	**2.96**	2.34	3.02	**7.24**	6.08	2.85	**5.58**	3.55	2.7	**11.4**	3.4
P	1377	1609	**1985**	1023	1707	**2258**	1235	**1285**	1106	1294	1448	**2358**
Pb	1.03	1.45	**1.65**	1.34	**2.28**	1.80	1.37	1.45	**2.36**	1.24	1.84	**1.92**
Si	2935	**4713**	4588	5187	**7815**	7603	4100	**4287**	3691	3326	**4079**	3578
Sr	38.1	**50.3**	43.0	20.1	32.3	**35.6**	43.6	44.6	**69.2**	33.9	44.0	**66.5**
Zn	6.3	11.7	**21.8**	8.1	12.6	**18.6**	5.9	6.8	**7.9**	4.7	6.4	**12.9**
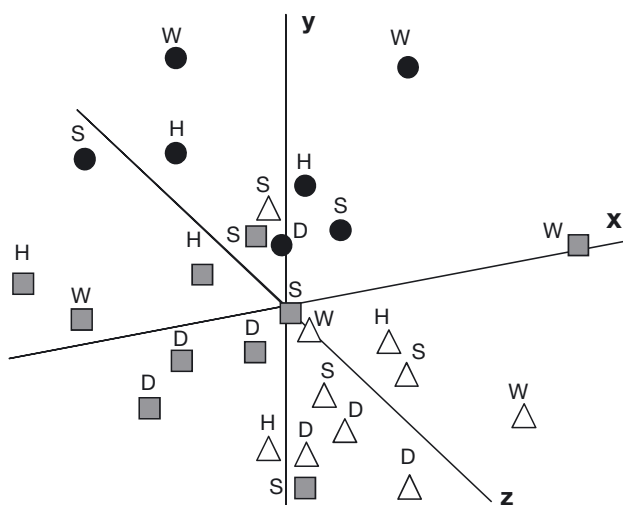
Fig. 3. *Spartina alterniflora*. Three-dimensional ordination (non-metric multidimensional scaling) grouped by salt marsh zone and disturbance site-type based on similarities (Bray-Curtis) in elemental composition in leaf tissues for the entire suite of constituents (20 elements). (●) Affected, (▪) edge, and (△) healthy zones; D: dieback, H: horse, S: snail, and W: wrack sites. Stress: 0.07

stress in response to various disturbances. In all 4 disturbance types evaluated (sudden dieback, horse overgrazing, high snail density, and wrack), both foliar DMSO:DMSP ratios and the metal composition of *S. alterniflora* were significantly higher in affected compared to the healthy zones. Because these responses varied by zone (a proxy for degree of stress) but not by disturbance type, the DMSO:DMSP ratio and metal composition both appear to be general stress response indicators in *S. alterniflora*. In contrast, the individual components (DMSP, DMSO, or single metal species alone) were not as consistently different among zones. Chl *a* concentrations, which are typically used as a visible sign of stress, were the least sensitive of all measures and were not significantly different among zones.

DMSP

The function of DMSP in *Spartina alterniflora* remains unclear. DMSP concentrations have not been found to vary consistently with salinity, sulfides, or nitrogen, suggesting that it is unlikely to function as either a compatible solute or a sulfur detoxicant (Otte & Morris 1994, Mulholland & Otte 2000, 2001). It is possible that DMSP functions as a methylating agent, an herbivore deterrent, an intermediate in the synthesis of acrylic acid (or other compounds), or any

combination of these (Otte & Morris 1994), but more recent evidence suggests that it may act as an antioxidant in *S. alterniflora*, which is similar to its role in phytoplankton (Sunda et al. 2002, Husband & Kiene 2007). Husband et al. (2012) observed increased oxidation of DSMP to DMSO in plants experimentally treated with the herbicides paraquat and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), each of which generates reactive oxygen species (superoxide and singlet oxygen, respectively).

Foliar DMSP was found at decreased concentrations nearest sudden dieback areas in South Carolina (Kiehn & Morris 2010) and in visibly stressed (yellowing) *Spartina alterniflora* in Alabama (Husband & Kiene 2007). We observed a similar pattern of DMSP concentrations at the Georgia dieback sites examined here: leaves and stems taken from healthy zones, located approximately 10 m from the dieback, had higher concentrations of DMSP than did those collected from the edge zones. DMSP concentrations in both leaves and stems were also significantly decreased in the affected zones of the other 3 disturbance types, except for stems at wrack sites (it is possible that *S. alterniflora* was not as stressed at the wrack sites). These results suggest that DMSP may decrease in response to generic (oxidative) stress, rather than to a specific stressor.

DMSO

DMSO concentrations alone were not effective indicators of stress because they occurred at a very low concentration and the variation among zones was inconsistent or insignificant. The one other study to measure DMSO concentrations in *Spartina alterniflora* reported even lower mean concentrations than those observed here (Husband & Kiene 2007). Less is known about the concentration of DMSO in *S. alterniflora*, and it may be that DMSO is highly variable across marshes. It is also possible that the conversion efficiency of DMSO to DMS was greater in the particular batch of TiCl$_3$ reagent that we used. Kiene & Gerard (1994) noted that when the reduction efficiency was low, TiCl$_3$ often yielded as much as 30% less DMS from DMSO standards. Methodological differences could also account for differences between studies: Husband & Kiene (2007) estimated DMSO from within the same serum vial (same plant sample) that was used to estimate DMSP, which required an additional degassing of DMS and neutralization (with HCl) of the NaOH reagent (used to oxidize DMSP) in order for DMSO reduction to take

place with TiCl$_3$. If the NaOH is not fully neutralized, then the TiCl$_3$ can react with it instead of reducing the DMSO, thereby leading to an underestimate of DMSO. Regardless of differences in the absolute value of DMSO, its concentrations were significantly related to those of DMSP in healthy *S. alterniflora* plants as was also noted by Husband & Kiene (2007). Because DMSO is an oxidation product of DMSP, it is not surprising that the 2 are related. We found that DMSO typically accounted for about ~3 to 8% of leaf DMSP and ~8 to 9% of stem DMSP. In contrast to healthy zones, DMSP was not well-correlated with DMSO concentrations in the edge and affected zones. This suggests that disturbances likely affect the proportion of foliar DMSP that gets converted to DMSO (Husband & Kiene 2007).

DMSO:DMSP ratio

If the proportion of DMSO varies with stress, then the ratio of DMSO:DMSP provides a useful way to make comparisons. In this study, 85% of the DMSO: DMSP ratios in leaves from the healthy zone were <0.14. Although there was overlap in the ranges of ratios from the healthy and affected zones, only the leaves from affected zones had ratios that exceeded 0.29. These potential cut-offs would need to be further explored at other sites.

Husband & Kiene (2007) first showed that the DMSO:DMSP value was higher in yellow and spotty, presumably stressed, leaves than in nearby healthy leaves. They suggested that the ratio may increase with senescence and the loss of plant pigment (yellowing). Although we found that the DMSO:DMSP ratio was significantly greater in the leaves and stems of affected zones, we did not find a significant relationship between the DMSO:DMSP ratio and chl *a* concentration, which suggests that the DMSO:DMSP ratio is responding to something other than senescence alone. The fact that the DMSO:DMSP ratio was significant across all disturbance types, however, shows that it is a potentially useful indicator no matter what caused the stress to *Spartina alterniflora*.

Foliar metal concentration

This study is among the first studies to view metal uptake as a symptom of stress, rather than the cause, and also among the first to examine the full suite of metals, rather than just focusing on a single or few specific metals of interest. Of the 19 metals (and P)

evaluated in foliar tissues of *Spartina alterniflora*, nearly all cases (77 of 80) were higher in either the edge or affected zone compared to the healthy zone. Only K was higher in the healthy zone. McKee et al. (2004) found increased Al and Fe accumulation in *S. alterniflora* in response to sudden dieback in Louisiana. Mobility and bioavailability of metals in the soil can increase with decreased soil pH (≤5) and an oxidizing environment (Portnoy 1999). McKee et al. (2004) suggested that the drought conditions in Louisiana could have led to the observed decrease in soil pH (~5), and that desiccation could have resulted in oxidizing conditions. However, no unusual pH or redox values were observed during sampling (the mean pH was 7.16 and the mean redox value was −180 mV in affected zones across all 4 disturbance types; McFarlin 2012). These results support the idea that metals were not a cause of stress, but rather were a symptom wherein plants accumulated metals under stressful conditions.

One possible scenario to explain the increased concentrations of metals in the disturbed areas is that under stress, plants often close their stomata (Maricle et al. 2007). *Spartina alterniflora* excludes metals through salt glands (Burke et al. 2000, Weis et al. 2002), but when stomata are closed, the reduced xylem pressure may inhibit the translocation and exclusion of ions from salt glands and thus increase metal concentrations in *S. alterniflora* tissues. It is also possible that there is increased metal translocation to leaves during stress. It has been documented that *S. alterniflora* leaves increase in metal concentration during senescence, which may also be a mechanism to reduce overall plant metal load as leaves are dropped (P. Weis et al. 2002, J. Weis et al. 2003). There is also the possibility that plants grow more slowly when stressed, yet accumulate metals at a similar rate to healthy plants, which could lead to an increased concentration (per unit weight) of metals in stressed plants.

Past studies have looked at increased foliar metals as a source rather than an indicator of stress. These have either reported the effects of metal toxicity on *Spartina alterniflora* in greenhouse studies (Mendelssohn et al. 2001, Mateos-Naranjo et al. 2008) or have examined metal accumulation in the field at polluted sites (Cambrollé et al. 2011, Salla et al. 2011). Across these studies, *S. alterniflora* was highly tolerant of soil metal contamination, able to hyperaccumulate metals, and capable of phytoremediation (Salla et al. 2011). Because our sites were located in pristine areas (Sapelo Island, a National Estuarine Research Reserve; and Cumberland Island, a National Sea-

shore), contamination was unlikely to be the cause of the observed increase in foliar metals. In fact, very few of the metal concentrations observed here (B, Cd, and Co) were elevated compared to other studies that have reported foliar metals in *S. alterniflora*, and none exceeded the amounts that would be expected to cause toxicity (Mendelssohn et al. 2001, Plank & Kissel 2011).

This study suggests that the metal suite is an indicator of stress in *Spartina alterniflora*. The NMDS analysis showed clear differences between the affected and healthy zones in their overall metal load, but there were no differences among disturbance types, suggesting that the response of *S. alterniflora* was similar, regardless of the initial cause of stress.

Chl *a*

Reduced chlorophyll content of *Spartina* spp. is often used in research studies as a symptom of stress (e.g. Mateos-Naranjo et al. 2008, Li et al. 2010). Although chlorophyll concentrations measured in the affected zones in this study were reduced by 47% in snail sites, it was surprising how little the chlorophyll concentrations were influenced by the other disturbance types. These results suggest that a reliance on chlorophyll content to indicate stress is not necessarily appropriate. This has also been observed in evaluations of salinity stress (Mateos-Naranjo et al. 2010), redox stress (Pezeshki & DeLaune 1993), and CO_2 stress (Mateos-Naranjo et al. 2010). We actually found that *S. alterniflora* plants disturbed by horses had increased chlorophyll content compared to the healthy zones at those sites, a trend opposite to that of the other disturbance types. Grazing is known to remove phenologically older plant material and stimulate regrowth of new, more photosynthetically active tissue from the meristem at the ground surface (Frank et al. 1998). In this case, it is likely that leaves collected from the affected zone were younger than those in healthy zones due to continuous grazing; younger leaves typically have greater chlorophyll content than older leaves (Šesták 1963). Piceno & Lovell (2000) also found a similar effect of increased chlorophyll content in *S. alterniflora* leaves that had been experimentally clipped.

CONCLUSIONS

Many indicators of stress that have been suggested for *Spartina alterniflora* have been stressor-specific and therefore of limited utility. However, indicators capable of detecting stress in many situations, as well as under multi-stressor scenarios, would be much more useful tools for identifying areas potentially at risk. The results presented here suggest that both the DMSO:DMSP ratio and the overall metal composition are good integrative indicators of generic stress in the field, in that both responded consistently to different disturbance types and across multiple field sites. Because the DMSO:DMSP ratio was more than just a simple function of chlorophyll concentration (a proxy for senescence), and the metal composition was responsive in otherwise apparently healthy (green) leaves, both were also sensitive early indicators of stress. It may be that both the DMSO:DMSP ratio and the metal composition of *S. alterniflora* are responding to oxidative stress that can be caused by a wide range of disturbances, but more research is needed to understand the underlying mechanisms of these stress responses. It would also be useful to use experimentally controlled levels of stress as a way to augment the results presented here (where we assumed that the edges of the disturbance zones were areas with lower levels of stress) to determine whether there is a threshold in either of these responses. Nevertheless, these results provide 2 potential early indicators of stress that can be used in the field under a wide range of conditions and deserve attention in future studies.

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