Metabolomics Reveals Cryptic Interactive Effects of Species Interactions and Environmental Stress on Nitrogen and Sulfur Metabolism in Seagrass

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ABSTRACT: Eutrophication of estuaries and coastal seas is accelerating, increasing light stress on subtidal marine plants and changing their interactions with other species. To date, we have limited understanding of how such variations in environmental and biological stress modify the impact of interactions among foundational species and eventually affect ecosystem health. Here, we used metabolomics to assess the impact of light reductions on interactions between the seagrass Zostera marina, an important habitat-forming marine plant, and the abundant and commercially important blue mussel Mytilus edulis. Plant performance varied with light availability but was unaffected by the presence of mussels. Metabolic analysis, on the other hand, revealed an interaction between light availability and presence of M. edulis on seagrass metabolism. Under high light, mussels stimulated seagrass nitrogen and energy metabolism. Conversely, in low light mussels impeded nitrogen and energy metabolism, and enhanced responses against sulfide toxicity, causing inhibited oxidative energy metabolism and tissue degradation. Metabolomic analysis thereby revealed cryptic changes to seagrass condition that could not be detected by traditional approaches. Our findings suggest that coastal eutrophication and associated reductions in light may shift seagrass-bivalve interactions from mutualistic to antagonistic, which is important for conservation management of seagrass meadows.

INTRODUCTION

Among the most integral tasks for ecologists are to investigate and understand the ways in which species interact with one another (e.g., mutualism, antagonism, competition) and the strength of these interactions. Species that physically or chemically modify environmental stressors or resource availability, such as ecosystem engineers, may simultaneously exacerbate and alleviate several stressors. This results in highly complex species interactions that vary depending upon environmental conditions. However, understanding the role of environmental context in mediating species interactions has historically been complicated by difficulties in quantifying sublethal physiological stress. Fortunately, advances in ecological metabolomics are providing novel solutions to this problem because stress-related effects are instantly reflected in the metabolic functioning of key organisms, providing new, practical analytical methods to assess sublethal stress in ecosystem engineers. Nevertheless, to date there have been no empirical evaluations of the potential for environmental conditions to mediate the metabolic mechanisms underlying species interaction.

Estuaries are ideal ecosystems to test this hypothesis because they support abundant and important ecosystem engineers, such as suspension-feeding bivalves, that can have context-dependent impacts on their surrounding community. For example, bivalves may have either positive or negative impacts on co-occurring seagrasses depending on environmental conditions (reviewed in Castorani et al.). Seagrasses are submerged marine angiosperms essential for coastal ecosystem
function by creating habitat for many species of invertebrates and fishes, providing food for larger resident and migratory species, increasing secondary production, enhancing biodiversity, stabilizing sediments, reducing coastal erosion, and recycling nutrients.\textsuperscript{10,11}

When plants face unfavorable environmental conditions, environmental and biological stress perturbs plant metabolism. Consequently, the metabolic network needs to be reprogrammed to adapt to the prevailing conditions and maintain essential metabolic pathways. Metabolomics aims to profile the entire set of low molecular weight metabolites, the metabolome, which in fact is determined by the physiological, developmental, or environmental state of an organism.\textsuperscript{12} Therefore, metabolomics represents an exceptional tool to assess the effect of environmental and biological stress on organisms by quantifying the capacity to react, acclimatize, and/or adapt to changing environments.\textsuperscript{5,13,14} Metabolomic techniques such as liquid chromatography–high resolution mass spectroscopy (LC-MS) and gas chromatography–accurate-mass mass spectroscopy (GC-MS) measure hundreds to potentially thousands of metabolites.\textsuperscript{15} Consequent data analysis of these metabolites allows rapid and accurate separation of samples according to their environmental exposure, yielding characteristic metabolic fingerprints directly related to the prevailing phenotype.\textsuperscript{16} Metabolic fingerprinting allows a rapid and holistic classification of the samples according to their origin and environmental or biological exposure,\textsuperscript{17} and does not require the identification of every metabolite present. The data can, however, also be screened for specific metabolic patterns or pathway types through exploratory data analysis (chemometrics),\textsuperscript{18} which prerequisites identification of the metabolites and provides in depth information about prevailing metabolic responses.\textsuperscript{14}

Despite the analytical power of metabolomics to assess and understand the effect of environmental conditions on organisms, the field of seagrass metabolomics is still in development and only two studies have applied metabolomics to assess temperature\textsuperscript{16} and hypoxia\textsuperscript{1} stress on seagrasses, respectively. To date it is unclear what effect bivalves and light availability have on the metabolic functioning of seagrasses. Determining the influence of bivalves on seagrasses is important because seagrass distributions are estimated to be caused by coastal eutrophication.\textsuperscript{10,11} In oligotrophic systems, bivalves can facilitate seagrasses by increasing nutrient availability through deposition of feces and pseudofeces.\textsuperscript{17} However, bivalves can also inhibit seagrass growth by enriching sediments with organic matter and increasing concentrations of toxic sulfides.\textsuperscript{18} These variable interaction effects are modulated by environmental drivers such as light, hydrodynamics, and temperature and cannot be easily disentangled by traditional (nonmetabolomic) approaches.\textsuperscript{19,20} Using traditional plant metrics (e.g., growth, survival), a short-term study demonstrated a predictable influence of light on seagrass condition but failed to detect any effect of bivalves.\textsuperscript{19} However, cryptic seagrass-bivalve interactions might be discerned using new metabolomic tools. Thus, here we tested whether light, the primary factor of seagrass productivity,\textsuperscript{9,21} modulates bivalve-seagrass interactions through metabolic mechanisms. We used metabolomics to test for independent and interactive effects of light availability and bivalve presence on the seagrass metabolome and to relate environmental parameters to primary energy and nitrogen metabolism and to early apoptosis.

\section*{EXPERIMENTAL METHODS}

\textbf{Study System.} The epibenthic suspension-feeding blue mussel, \textit{Mytilus edulis} L., frequently co-occurs with eelgrass, \textit{Zostera marina} L., in estuaries and shallow coastal water of the temperate North Atlantic Ocean, North Sea, and Baltic Sea.\textsuperscript{22,23} In this study we used sediment, water, mussels, and plants from the Danish straits connecting the Baltic Sea with the North Sea. In this region \textit{Z. marina} and \textit{M. edulis} are widely distributed and often co-occurring.\textsuperscript{15} Danish coastal waters in this region are often eutrophic and turbid resulting in fluctuating light availability.\textsuperscript{24}

\textbf{Specimen Collection and Exposure.} To assess the role of light availability in mediating habitat modification by blue mussels \textit{Mytilus edulis} and impacts on \textit{Zostera marina}, we manipulated light availability (high vs low) and mussel abundance (present vs absent) in a factorial design in an indoor mesocosm experiment. Briefly, sediment, seawater, seagrass, and mussels were collected from coastal field sites in Denmark, transplanted into mesocosms, and maintained in a controlled recirculating seawater system (see Hasler-Sheetal et al.\textsuperscript{6} and Castorani et al.\textsuperscript{25} for details). Mesocosms (\textit{N} = 24) were established and the temperature (14.4 ± 0.8°C), salinity (13.4 ± 0.08), water column oxygen saturation (100%), and water column nutrients (11.5 ± 7.7 μmol NH₄⁺ L⁻¹, 0.293 ± 0.026 μmol NO₃⁻ L⁻¹) were maintained constant and similar in all mesocosms. To mimic low light conditions in eutrophic estuaries, half of the mesocosms were exposed to light levels slightly below 100 μmol photons s⁻¹ m⁻² (the light saturation level for \textit{Z. marina} is ∼100 μmol photons s⁻¹ m⁻²), limiting \textit{Z. marina} growth.\textsuperscript{25} The other half of the mesocosms received high light levels (∼550 μmol photons s⁻¹ m⁻²) that do not limit \textit{Z. marina} growth. During night (12 h) all systems were completely dark. To half of the mesocosms we added \textit{M. edulis} at densities observed in the field (891 m⁻²)\textsuperscript{26}. After 21 days of exposure the 24 mesocosms were harvested and processed as described in Hasler-Sheetal et al.\textsuperscript{6} In brief, plants were randomly harvested, separated in leaves, rhizome, and roots (total of 72 samples), snap frozen in liquid nitrogen, lyophilized for 48h, and homogenized for further analysis. Elemental sulfur in tissues, a proxy for sediment sulfide intrusion into seagrass tissues,\textsuperscript{27} was measured following Frederiksen et al.\textsuperscript{27} A detailed experimental description is presented in the Supporting Information.

\textbf{Metabolomics.} Metabolites were extracted from 72 snap-frozen, lyophilized and homogenized seagrass samples (methanol/water 5:1 [v/v]), the extracts were dried and subsequently resuspended for LC-MS or derivatized prior GC-MS analysis, respectively. Metabolites were separated by reverse phase (RP), hydrophobic interaction chromatography (HILIC), and gas chromatography (GC), and detected by quadrupole time-of-flight mass-spectroscopy (qTOF-MS) in ES± (Electrospray ionization) and electron ionization, respectively. In total, we used five different analytical procedures to resolve the metabolome in leaves, rhizome, and roots of \textit{Z. marina}: RP, HILIC both in ES± qTOF-MS and GC-qTOF-MS. Data inspection, data mining, annotation, and interpretation were done in MassHunter, Profinder, and Mass Profiler Professional (Agilent Technologies, Santa Clara, California, USA). The LC-MS data was used to assess metabolic fingerprints and the GC-MS data to assess pathway analysis. Ceramide (validated as d18:1/12:0 with a mass of 481.4495)\textsuperscript{28} and sphingosine-1-phosphate (S1P) were assessed by RP-LC-MS and confirmed.

\section*{Supporting Information}

\begin{itemize}
\item EXPERIMENTAL METHODS
\item Study System.
\item Specimen Collection and Exposure.
\item Metabolomics.
\end{itemize}
by comparison with standards. A detailed description of the analytical setup and the metabolic profiling is given in the Supporting Information.

Data Analysis. Peak areas were standardized for sample weight and to the internal standard, missing values were imputed by k-nearest neighbor method32 and later log 2(x + 1) transformed and baselined by unit scaling (mean-centered and divided by the standard deviation of each variable). To exclude false positives, only entities with a coefficient of variation (CV) less than 35% and present in at least 80% of the quality control samples (QC) samples were used for analysis. The effects of light availability and mussel presence were compared using analysis of variance (ANOVA; α = 0.05) and Tukey’s posthoc test. We applied a false discovery rate correction using the Benjamini–Hochberg30,31 method, an adjusted p value of <0.05 was considered significant. To graphically visualize the data we used heatmaps illustrating Ward clustering of the Euclidian distances between the treatment groups and metabolites, respectively. In addition, we used principal component analysis (PCA) to visualize and summarize the metabolic response of Z. marina to light availability and mussel presence. To identify the most influential metabolites in separating the samples along the principal components (PC), covariance vs correlation plots (CC-plots) of all 3 components were inspected.32 We mined the metabolite matrix for correlations with ecological parameters like sediment biogeochemistry and plant performance by Spearman correlation. An alpha value of 0.05 was applied consistently.

RESULTS AND DISCUSSION

Metabolic Profiling and Multivariate Analysis. We detected 92,478 mass spectral features in Z. marina roots, rhizomes, and leaves and of these 5541 passed our quality control filters (present in 80% of the quality control (QC) samples and with a CV < 35%). The 5541 reproducibly detected metabolite entities represents a large number33 and suggest a good and robust coverage of the Z. marina metabolome and thus were used for metabolomic fingerprinting without further annotation. The visualization of the filtered and unannotated metabolome in a heatmap showed clear treatment-specific differences (Figure 1). Light intensity grouped the samples in two clusters and in each of these clusters mussel presence formed two subclusters (x-axis in Figure 1). The clustering of metabolites showed distinct and treatment specific metabolite clusters (y-axis in Figure 1). Light modified the effect of mussel presence on the metabolome indicated by the distinct condensed and mussel specific clustering under either low or high light conditions, suggesting interactive physiological responses.

To visualize treatment specific effects, a PCA was conducted and showed a clear treatment related clustering of the samples in all tissues and analytical conditions (Figure 2). Light exposure was the main variable observed between the samples, indicated by a light-dependent separation of samples on component 1 (accounting for 34−39% of the metabolic variation in the data set) and governed by metabolites of the energy metabolism. In particular, energy source and storage carbohydrates responded to light reduction (Figures 2 and S1 and Table S1).

These findings are in line with previous studies showing major effects of light intensity on Z. marina energy metabolism.32 Remarkably, light exposure modified the effect of mussels on the metabolome as indicated by mussel dependent grouping of samples on component 2 under high light or respective component 3 under low light, explaining 27−24% and 22−16% of the variation in the data set respectively (Figure 2). Different metabolite sets were governing the separation on each component (Figure 2), illustrating divergent physiological responses of Z. marina to the presence of mussels under low and high light availability. Metabolites governing mussel-dependent separation under high light were associated with nitrogen metabolism (Figure 2; Table S1) where as under low light the mussel treatment was associated with metabolites of glycolysis and TCA cycle (Figure 2; Table S1). These light-dependent metabolic responses to mussel presence were not reflected in traditional plant performance metrics such as leaf growth rate, shoot density, and soluble sugars (data presented in Castorani et al.9 Table S3). The plant performance metrics were depressed by low light availability but, in contrast to the metabolome, were unaffected by mussels. This suggests that either the metabolic shift caused by mussel presence did not affect Z. marina performance or that the duration of the experiment (21 days) was not long enough to manifest traditional performance metrics. The latter explanation is likely because indeed high levels of sulfide intrusion26,34 were
observed in *Z. marina* grown in low light with mussels\(^9\) (Table S2) that typically lead to sulfide toxicity and impaired plant performance.\(^9,26\) To better understand the potential negative effects of mussels on *Z. marina* under low light, we explored the primary energy metabolism, associated nitrogen assimilation pathways, and entities of apoptosis.

**Growth Regulation and Early Apoptosis.** Several metabolites of the primary energy metabolism (glycolysis, TCA, and the associated amino acid pathways) were affected by one and two-way interactions of light and mussels (Figure 3). The general decrease of glucose and fructose under light depletion (Figure 3) indicates energy deprivation, most likely due to lower rates of photosynthesis. This was previously described in plants after prolonged periods of decreased photosynthesis.\(^3\) The even greater decrease of carbohydrates in *Z. marina* under low light and mussel presence (Figure 3) could further be related to increased glycolysis depleting glucose and fructose levels. Increased pyruvate and the decreased citrate levels (Figure 3) indicate that pyruvate is not sufficiently feeding the TCA cycle under low light and mussel presence, thus limiting the carbon flux via pyruvate, acetyl-CoA, and citrate into the TCA cycle, and ultimately leading to energy deprivation. The concomitant increase of lactate (Figure 3) indicates the presence of lactic fermentation leading to phytotoxic cytosolic acidification.\(^6,36\) These findings suggest (1) light-dependent energy deprivation in low light and (2) inhibition of oxidative energy metabolism in seagrasses under low light and co-occurred of bivalves. However, it has been proposed that seagrasses can compensate for energy deprivation by (1) increasing the glycolytic flux and (2) utilization of glutamine-derived carbon via the GABA shunt,\(^6,37\) which also shunts excess pyruvate to alanine and mitigates cytosolic acidification.\(^6\) In agreement with these mechanisms, we observed an associated increase of lactate and pyruvate as fructose and glucose both decreased with changes in alanine, GABA and glutamine levels (Figure 3). This indeed implies that increased glycolytic flux drained sugar pools, a non-functioning TCA-cycle caused energy deprivation, and a GABA shunt compensated for energy deprivation and mitigated cytosolic acidification.\(^38\)

The presence of mussels under low light induced accumulation of several amino acids (alanine, proline, threonine, valine) and depletion of the TCA cycle derived amino acids (glutamine, serine, tyrosine and glycine) in eelgrass tissues (Figure 3). This reprogramming of the amino acid profile is another indicator of the inhibition of oxidative energy metabolism.\(^39,40\) Overall, decreased levels of carbohydrates, increased lactic fermentation, and presence of mitigation pathways clearly indicate impaired energy metabolism of seagrasses that co-occur with mussels under low light.

Nitrogen assimilation in plants is highly complex, being controlled by hormones and levels of sugars, organic acids, and amino acids.\(^41\) However, glutamate is a key source for amino acid synthesis, and acts as hub for nitrogen metabolism by...
shuttling reduced nitrogen into nitrogen assimilation, and is the primary product of ammonium and nitrate assimilation. We found that the presence of mussels increased glutamate and glutamine levels in high light, and decreased them in low light (Figure 3). This indicates that light can modify the effect of bivalves on seagrass nitrogen metabolism, enhancing nutrient uptake capacity with high light and reducing root nitrogen uptake with low light. This was clearly reflected in the levels of metabolites associated with nitrogen metabolism (i.e., serine, tyrosine, glycine; Figure 3).

When screening the metabolomic data for metabolites involved in apoptosis and cell degradation, we found increased levels of ceramide and decreased levels of sphingosine-1-phosphate (S1P) in Z. marina grown with mussels under low light (Figure 4). Ceramide and S1P play a central role in cellular processes governing growth, differentiation, apoptosis, and survival in eukaryotic organisms. While ceramide mainly induces apoptosis, reduces growth and increases in response to stress, S1P promotes survival and growth. The relative ratio between ceramide and S1P (referred as sphingo-lipid rheostat) determine the cell development and apoptosis. The latter is related with decreases in S1P levels and increases in ceramide.

In this experiment mussels presence under low light caused a shift in the relative ratios between ceramide and S1P from values around 1.7 to 0.6 (Figure 4). In addition, we found that low light increased phytol levels in the leaves and that mussels exacerbated this effect (Figure 4). Phytol is a chlorophyll degradation product accumulating under plant senescence. Based on the function of the sphingo-lipid rheostat and phytol in other organisms, we suggest that increased levels of ceramide, decreased levels of S1P, shifts in ceramide:S1P ratios and accumulation of phytol in seagrasses (Figure 4) under bivalve presence and low light indicate the onset of tissue degradation and propagated cell death.

Integration of Metabolomics and Environmental Parameters. To investigate the effect of sulfide intrusion on seagrass metabolism, we explored the metabolomic data matrix for relations between tissue elemental sulfur levels and metabolite concentrations. Elemental sulfur in the roots originates from oxidation of intruding gaseous sediment

**Figure 3.** Pathway analysis of metabolites in the leaves of the seagrass Zostera marina exposed to varying light and the blue mussel Mytilus edulis. Visualized are the primary energy metabolism (glycolysis and TCA cycle, left panel) and the associated nitrogen metabolism (right panel). Metabolites are denoted as the ratio in each treatment relative to high light conditions in the absence of mussels. The shaded area indicates low light conditions. Metabolites framed in green indicate significant ($p < 0.05$) increases, red indicate significant decreases, and black indicate no change relative to eelgrass grown under high light without mussels. Red bars indicate lower metabolite levels and blue bars indicate higher metabolite levels relative to eelgrass grown under high light without mussels. Solid arrows illustrate enzymatic reactions; dotted lines indicate the same metabolite presented in different and linked pathways. Abbreviations for experimental treatments are as follows: high light with mussels (H+); low light without mussels (L−); low light with mussels (L+). Modified and used with permission from Hasler-Sheetal et al. Copyright 2015 Springer.
sulfide and is a strong proxy for sulfide intrusion into seagrass tissues. Sulfide intrusion modulated (IRI > 0.6) levels of 77 metabolites (Table S1), suggesting Z. marina metabolism is strongly related to sulfide intrusion under stress from low light and mussels. This is a new discovery that corroborates prior work demonstrating the omnipresence and importance of sediment sulfides and sulfide intrusion in seagrass systems. Among the metabolites related to sulfide intrusion many play a role in metabolism under impaired oxidative energy metabolism. Lactate, pyruvate, GABA, alanine, glutamate, α-sitosterol, linoleic acid, and α-tocopherol were positively correlated to sulfide intrusion, whereas sucrose, proline, L-DOPA, glutamine, and glucanate were negatively correlated to sulfide intrusion (Table S1). This metabolic response is most likely related to sulfide toxicity or sediment hypoxia. Both would trigger similar metabolic responses by inhibiting the oxidative energy metabolism. Constant aeration of the water of the mesocosm throughout the experiment prevented hypoxic conditions in the water column. However, high respiration rates related to mussel presence (Table S2) likely caused local hypoxic conditions near the sediment surface, promoting sulfide intrusion into the plants. Sulfide toxicity inhibits oxidative energy metabolism but sulfide-induced formation of reactive oxygen species (ROS) formation can regulate sulfide toxicity. Eghbal et al. showed that ROS scavenging metabolites reduce sulfide toxicity and another study suggested that ROS formation in general stimulates plant antioxidant defense. This should be manifested in increased ROS scavenging antioxidants during stress acclimation. Indeed the ROS scavenging antioxidants α-tocopherol, β-sitosterol, L-DOPA and proline were correlated to apparent sulfide intrusion in our study (Table S1). These compounds also stabilize membranes and protect pigments, proteins, and fatty acids from oxidative damage. Consequently, the correlation of proline, β-sitosterol, L-DOPA, and α-tocopherol with sulfide intrusion may indicate increased membrane degradation caused by sulfide toxicity. Overall, the increased sulfide detoxification and concomitant increase in membrane degradation suggest sulfide toxicity in seagrasses was co-occurring with bivalves in low-light environments.

Using novel applications of metabolomics, we have demonstrated that bivalves and light interactively structure seagrass metabolism. Under high light conditions, mussels stimulated nitrogen metabolism, but Z. marina performance did not benefit because growth was not limited by nitrogen (as shown in Castorani et al., Tables S2 and S3). Low light reduced the resilience of Z. marina by reducing growth and diminishing pools of stored sugars (as shown in Castorani et al., Tables S2 and S3). Consequently, Z. marina did not benefit from the increased nutrients. Strongly respiring mussels drained oxygen levels close to the sediment surface, thereby enhancing the potential for sulfide intrusion and leading to sulfide toxicity in less resilient, low light seagrass.

Eutrophication is accelerating in temperate estuaries worldwide, reducing light availability and increasing hypoxic conditions for seagrasses and other marine plants. Our results suggest that continued degradation of coastal and estuarine water quality will enhance sulfide stress on Z. marina and other seagrasses, especially where co-occurring with filter-feeding bivalves. This finding might to some extent contrast with two recent studies that predicted sulfide oxidation by bacterial symbionts within lucinid clams that could be critically important to seagrass persistence. In light of ongoing and future eutrophication of temperate estuaries, our results indicate a need for careful evaluation of suggestions that seagrasses and bivalves are strictly mutualistic. In some systems, the antagonistic effects of bivalves on seagrasses might overpower mutualistic interactions.

Our study also demonstrates that metabolomics may be an important tool for early identification of plant stress in coastal environments. Our approach (1) provides information on system shifts through heatmaps and PCA, (2) illuminates pathways affected by environmental drivers, (3) reveals in depth and otherwise hidden effects of environmental stress on seagrasses, and (4) provides potential candidates for early warning and stress-specific biomarkers (i.e., bioindicators), although such candidates must be thoroughly validated. Our novel metabolomics-based approach should be adapted for other systems to detect sublethal environmental stress far in advance of the physiological manifestations that are the focus of traditional methods. Continued developments and applications of metabolomics to community ecology will shed further light on the influence of environmental stress on species interactions.

### ASSOCIATED CONTENT

© Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04647.

Detailed methods, PCA plots of polar and apolar compounds, list of all putatively annotated metabolites reproducibly detected in all tissues and conditions, elemental sulfur (S0) and pore water ammonium levels for Zostera marina, and results of 2-way ANOVA (PDF)

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DOI: 10.1021/acs.est.6b04647

Environmental Science & Technology

11607

Author Contributions
All authors contributed equally to this study. The study was
designed by contribution of all authors. The experimental work
and data analysis was performed by M.C. and H.H.S.,
metabolomics were conducted by H.H.S. The manuscript was
written through contributions of all authors. All authors have
given approval to the final version of the manuscript.

Funding
H.H.S. was supported by the European Research Council
Advanced Grant program through the OXYGEN project (no.
267233-ERC). M.C.N.C. was supported by a U.S. National
Science Foundation (NSF) Graduate Research Fellowship and a
NSF—Danish National Research Foundation (DNRF) Nordic
Research Opportunity Fellowship.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
The authors thank E. Laursen, S. Møller, B. Christensen, M.
Flindt, A. Glud, K. Hancke, R. O. Holm, K. C. Kirkegaard, and
S. W. Thorsen for research assistance. We thank the three
anonymous reviewers for helpful and constructive comments.

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