The proteomes of Sydney rock oysters vary spatially according to exposure to acid sulfate runoff

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Abstract. Runoff from acid sulfate soils (ASS) has severe environmental and economic impacts on estuarine ecosystems. Oysters display reduced abundance, growth rate and shell thickness when exposed to ASS runoff, yet the molecular underpinnings of their responses have not been explored. We hypothesised that the proteomes of wild Sydney rock oysters, Saccostrea glomerata, would differ between populations recurrently exposed to ASS compared with those unaffected by runoff from ASS. We used two-dimensional electrophoresis to compare protein abundances in the gills of S. glomerata collected from two sites close to (acidified) and two sites away from (reference) major ASS outflow drains in a south-east Australian estuary. Approximately 5% of the proteome was differentially expressed between oysters from acidified and reference sites, with five protein spots more abundant and one less abundant at the sites close to drains. Another protein spot was present only in oysters from reference sites. This study is the first screening of spatial variation in the protein expression of S. glomerata with respect to discharge from ASS. Altered protein expression may underpin short-term inducible responses to ASS runoff, or genetic resistance acquired through recurrent exposure of populations to the stressor.

Additional keywords: acidity, anthropogenic stress, estuarine acidification, pH, proteomics, waterlogged soils.

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Introduction

Acid sulfate soils (ASS) pose a serious threat to the ecology, biodiversity and economic development of estuaries and other low-lying coastal areas around the world (Dent and Pons 1995; Sammut et al. 1995; Powell and Martens 2005). ASS contain iron sulfides which, when exposed to air during drainage or disturbance, oxidise to sulfuric acid, often releasing toxic quantities of heavy metal ions from sediments (Dove and Sammut 2007a; NSW DPI 2009). After heavy rainfall or a rise in the waertable, these oxidation products leach into nearby streams and estuaries and can locally reduce the pH of adjacent waterways to as low as 2–3 (Sammut et al. 1996; NSW DPI 2006). Estuarine impacts are exacerbated by the construction of drainage channels and floodgates that concentrate runoff from ASS (Dent and Pons 1995; Johnston et al. 2005; Green et al. 2006).

The severe negative effects of ASS runoff on estuarine flora and fauna include large-scale fish kills (Brown et al. 1983; Callinan et al. 1993; Russell and Helmke 2002), the weakening of mangrove pneumatophores (Amaral et al. 2011a) and reductions in the population sizes of oysters and gastropods at affected locations (Amaral et al. 2011b). Sessile or sedentary calcifying organisms, such as bivalve molluscs, appear particularly susceptible to ASS runoff, perhaps because of their lower physiological ability to regulate internal pH in response to external acidity of a variety of sources (e.g. Bamber 1987, 1990; Ries et al. 2009) or their limited capacity to behaviourally avoid acidic, metal-enriched waters (Amaral et al. 2011b).

Oysters are important constituents of temperate and tropical estuarine environments. They are ecosystem engineers that provide habitat for a variety of marine species and facilitate benthic-pelagic coupling as a result of their filter-feeding and biodeposition (Coen and Luckenbach 2000; Newell 2004; Bishop et al. 2010). They also support a global aquaculture industry worth over USD 3 billion annually (FAO 2008). Consequently, it is important to understand how the growing threat of ASS runoff influences their structure and function at subcellular to ecological levels of biological organisation. Previous studies have documented physiological and ecological impacts of ASS runoff on populations of the Sydney rock oyster Saccostrea glomerata along the east coast of Australia (Dove and Sammut 2007a, 2007b; O’Connor and Dove 2009). S. glomerata that had not been previously exposed to ASS-affected waters showed shell dissolution, soft tissue lesions and reduced filtration rates after short-term exposure to affected waters (Dove and Sammut 2007a, 2007b). Wild S. glomerata
populations exposed to ASS runoff were less dense, thinner-shelled and more susceptible to generalist predators than populations at unaffected reference locations (Amaral et al. 2011b, 2012). The molecular basis of these ecological and physiological responses is not well understood (but see Green and Barnes 2010). To secure the future of oyster stocks, we need to fully understand how ASS runoff negatively impacts their populations.

Proteomics is increasingly being used to investigate the effect of environmental stressors at a molecular level (Jackson et al. 2010). To secure the future of oyster stocks, we need to understand if ASS runoff negatively impacts their populations. Distinctive protein signatures have also been identified by proteomics in S. glomerata exposed to heavy metals, revealing their subcellular responses to environmental contamination (Thompson et al. 2011). Here, we applied a quantitative and investigative proteomic methodology to the gill tissue proteomes of S. glomerata oysters to identify differences in protein abundances between oysters close to and away from ASS outflow drains. We tested the hypothesis that populations of S. glomerata recurrently exposed to ASS runoff would have different proteomes, with some proteins displaying upregulation (the process within a cell, triggered by an internal or external signal, which increases the expression of one or more genes and, consequently, the production of proteins encoded by those genes) and some downregulation (the converse process resulting in reduced protein production), relative to populations that are not exposed to this stressor.

Materials and methods

Sampling

Wild-growing Sydney rock oysters, Saccostrea glomerata, were collected in May 2009 from four sites within the Port Stephens estuary (32.708'S, 152.196'E), NSW, Australia: two with a history of recurrent exposure to ASS runoff (acidified) and two that were unaffected by this disturbance (reference; Table 1). Acidified and reference sites were each several kilometres apart from one another, within intertidal mangrove forests where S. glomerata are commonly found attached to pneumatophores (peg roots) and trunks of the grey mangrove, Avicennia marina. Each acidified site was within 900 m of its respective major ASS outflow drain and both acidified sites were within a large area classified as being at high risk of ASS runoff based on previous observations of periodically high metal concentrations and pH values of <5 at these sites (Table 1; NSW DECCW 2010). Reference sites were at least 2400 m away from drains and in areas of low ASS runoff risk (Table 1; NSW DECCW 2010). Water quality measurements (Eutech CyberScan PCD 650 hand-held probe; Eutech Instruments Pty Ltd, Singapore) on four occasions immediately before this study (April–May 2009) indicated that acidified sites were ~100 times more acidic than reference sites (Table 1). Over the same period, all sites had similar water temperatures and the acidified sites had slightly lower (on average by ~1 unit) salinity (Table 1). Although we did not quantify metal concentrations, we observed an orange-red colouration on the mangroves at the acidified sites, reflecting iron precipitation (M. Dove, pers. comm.) and previous sampling has recorded very high aluminium and iron concentrations near the drains at these locations (NSW DPI 2006).

From each site, we collected five S. glomerata (average ± s.d.: 55.2 ± 2.8 mm shell height) from each of three stations situated 10 m apart at a mid-tidal elevation (mean low water mark +0.5–0.7 m). S. glomerata were shucked in situ and their gills were excised. The tissues were immediately frozen on dry ice and later stored at −80°C. Gill extracts were later pooled across samples within a station, to give three aggregate samples (i.e. one per station) for each site. Pooling of specimens to produce aggregate samples is common procedure among proteomic studies. The procedure is used to increase the power to detect treatment differences by reducing the weight of individual variability in protein expression (Diz et al. 2009). Nevertheless, we acknowledge that it may inflate variation among replicate aggregations where one individual in the aggregate expresses proteins very differently to others in the pool.

Table 1. The sites of Port Stephens, NSW, Australia, where sampling was conducted

<table>
<thead>
<tr>
<th>Site abbreviation</th>
<th>Site location</th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Mean pH</th>
<th>pH minima&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ASS risk&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Fenninghams Creek (entry)</td>
<td>20 ± 2 (19.9)</td>
<td>24 ± 3 (26.7)</td>
<td>5.8 ± 0.5 (6.00)</td>
<td>&lt;5 High</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>Fenninghams Creek (middle)</td>
<td>20 ± 2 (17.7)</td>
<td>24 ± 3 (24.3)</td>
<td>6.0 ± 0.5 (5.50)</td>
<td>&lt;5 High</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>Stuart Island</td>
<td>21 ± 2 (18.5)</td>
<td>26 ± 5 (25.5)</td>
<td>8.0 ± 0.4 (8.00)</td>
<td>6.8 Low</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>4 km North of Stuart Island</td>
<td>20 ± 2 (18.0)</td>
<td>24 ± 5 (26.7)</td>
<td>7.9 ± 0.3 (8.10)</td>
<td>6.8 Low</td>
<td></td>
</tr>
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</table>

<sup>a</sup>NSW DPI (2006, 2009).  
<sup>b</sup>Naylor et al. (1998).

Protein extraction and quantification

Proteins were extracted and quantified following the procedures described by Thompson et al. (2011). First, gill tissue (average ± s.d.: 75.9 ± 9.9 mg) from each oyster was homogenised separately in 1 mL of Tri-reagent LS (Sigma-Aldrich, Castle Hill, NSW, Australia) in a Dounce homogeniser (Sigma-Aldrich) and centrifuged at 12 000 g (4°C) for 2 min. The homogenates from the five oysters per station were then pooled to give the total of three replicate samples per site (i.e. one per station), each containing 180 μg of protein in 125 μL of re-hydration buffer (7 M urea: 2 M thiourea: 4% CHAPS: 50 mM dithiothreitol (DTT)). Second, proteins were separated using two-dimensional
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electrophoresis (2DE). Twelve gel strips with immobilised pH gradients (7 cm, pH 3–7; Bio-Rad, Gladesville, NSW, Australia) were each re-hydrated overnight with one 125-µL pooled sample of 180 µg of protein and 0.5% carrier pharmalytes (GE Healthcare, Rydalmere, NSW, Australia). Isoelectric focusing (IEF) of the gel strips was performed on an IPGphor IEF system (GE Healthcare) at 100 V for 2 h, 500 V for 20 min, a gradient up to 5000 V for 2 h and another 2 h at 5000 V, giving a total of 14 586 Vh. The strips were then reduced for 20 min in 1% DTT solution and alkylated for another 20 min in 2.5% iodoaceta-mide. Second-dimension separation was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Tris–HCl polyacrylamide gels (1.5 M Tris-HCl: 10% SDS: 12% acrylamide) on a Mini PROTEAN system (Bio-Rad). Proteome maps were obtained by staining electrophoresed gels with Coomassie Brilliant Blue G-250 (Sigma-Aldrich) and scanning the stained gels on a laser scanner (GE Healthcare).

Protein spot detection and quantification

To test the hypothesis that the gill proteomes of S. glomerata would differ between acidified and reference sites, we produced images of 2DE gels in Adobe Photoshop (ver. CS3, Adobe Systems Inc., San Jose, CA, USA) and analysed these with Melanie proteome analysis software (ver. 7.0, Genebio, Geneva, Switzerland). Protein spots were automatically detected and quantified by the algorithm of the Melanie software. Spots were detected based on saliency (how far a spot stands out with respect to its environment) and a minimum area (number of pixels) was set to suppress noise, such as dust particles (GeneBio 2008). The software then calculated the pixel intensities of the spots (from which the background intensity had been subtracted) and the boundary of each spot. The abundance of protein in each spot within a gel was finally calculated, in a three-dimensional fashion, as the volume (related to spot intensity) within the spot boundary. Spot volumes were normalised by the total spot volume and background concentration of protein on each gel to eliminate the effect of variation among gels in the efficacy of protein loading and staining (GeneBio 2008). Specific proteins were matched across 2DE gels by the software. Spots were first matched within each treatment (acidified or reference sites) and then between treatments (acidified sites vs reference sites). Two gels, one from each treatment, did not produce uniform separation of protein spots and these were included in comparisons within treatments for the purpose of matching protein spots, but excluded in comparisons between treatments. The efficacy of the Melanie software in detecting, quantifying and comparing spots across gels was cross-checked by overlaying images and visually evaluating spot boundaries. In all instances, the match was good.

Statistical analyses

To assess spatial scales of variation in proteomes, we generated a two-dimensional ordination of multivariate data using non-metric multidimensional scaling (nMDS; Field et al. 1982) and ran a corresponding two-way nested non-parametric multivariate analysis of variance (PERMANOVA; Anderson 2001), with factors treatment (acidified vs reference) and site within treatment. Analyses excluded one gel from acidified site A2 and one from reference site R1 because proteins on these failed to separate properly (see above). Both nMDS and PERMANOVA used matrices of Bray–Curtis dissimilarity, calculated among 2DE gels using untransformed, unstandardised normalised volumes of all matched protein spots. The PERMANOVA partitions sources of variation in a similar way to ANOVA and uses unrestricted permutation of raw data to assess statistical significance. Where sites within treatments (acidified or reference) did not differ (at α = 0.25; Underwood 1997), the PERMANOVA was repeated with sites within treatments pooled.

We ran similarity of percentage (SIMPER) analyses to identify protein spots contributing most to multivariate dissimilarity between treatments (Clarke 1993). Protein spots were considered to be good discriminators when they showed a dissimilarity to standard deviation ratio >1.3 (Clarke and Warwick 1994). We further determined whether multivariate patterns of the full S. glomerata proteome could be adequately described by a subgroup of protein spots using the iterative (BVSTEP; Clarke and Warwick 1998).

We tested for differences between acidified and reference sites in the abundance of protein spots classified as good discriminators with SIMPER, or as constituents of important sub-groups by BVSTEP, using separate two-way analyses of variance (ANOVs) for each spot. ANOVAs were analogous in design to the PERMANOVAs described above, with pooling of the nested factor, site, where sites within a treatment did not differ at α = 0.25 (Underwood 1997). Protein spots were considered as being differentially expressed between treatments when P-values were <0.05. Prior to each ANOVA, assumptions of homogeneity of variances and normality were confirmed. Where ANOVA detected significance of factors, post-hoc Tukey’s honest significant differences (HSD) were used to identify the sources of differences.

Finally, the magnitude of change (fold difference) in the protein abundance of the spots that were differentially expressed between acidified and reference locations was calculated as the ratio of mean normalised spot volume between reference and acidified treatments; positive values represented spots with a higher protein abundance in acidified relative to reference sites and negative values represented spots with lower protein abundance in acidified compared with reference sites. Where not previously tested with one-way ANOVAs, we used Student’s t-tests (Zar 1984) to test the null hypothesis that proteins abundances would not differ.

Results

Melanie software detected 132 different protein spots, with an average (±s.d.) of 100 (±16) spots per 2DE gel. The majority of protein spots had an isoelectric point (pI) in the range of 3–6 (Fig. 1). Among protein spots, 123 were present on at least two of the gels and 23 spots were found on each of the gels. Of spots that were found on multiple gels, 34 were found only on gels of a single treatment, acidified or reference, with 17 of these spots being present on gels produced from samples from both replicate sites of that treatment.
Overall, oyster proteomes were more dissimilar between acidified and reference treatments than between sites within treatments (Fig. 2). Nested PERMANOVA did not, however, detect a statistically significant difference in *S. glomerata* proteomes between sites within treatments ($F_{1,2} = 2.36, P = 0.305$) or between treatments ($F_{2,6} = 0.83, P = 0.635$). Consequently, sites were pooled and the PERMANOVA was re-run to provide a more powerful test of treatment effects. This second analysis using pooled data found a highly significant difference in the gill proteome of wild *S. glomerata* between acidified and reference sites ($F_{1,8} = 2.16, P = 0.013$), providing support for our hypothesis.

Forty-eight protein spots were identified by SIMPER analysis to be important sources of multivariate dissimilarity between treatments (43.22 average dissimilarity). Another seven spots (spots 12, 36, 42, 101, 107, 120 and 122) were identified with BVSTEP (0.916 correlation) as contributing to a subset of proteins that produced spatial patterns representative of the full *S. glomerata* proteome. Two-way nested ANOVAs revealed that of these 55 protein spots classified as important, two (61 and 74) showed significant effects of proximity to ASS outflow drains before pooling (Table 2; Fig. 3). Analyses also showed that of the other spots, two (49 and 107) displayed variability at the scale of sampling site but not treatment, with protein abundance differing significantly (HSD, $P < 0.2$ in all cases; Table 2) between the two reference sites, but not between the two acidified sites. All other spots had statistically non-distinguishable protein abundance between treatments and sampling sites and in 41 of these, the non-significant differences between sites within treatments justified pooling across sites ($P > 0.25$; Underwood 1997). Separate one-way ANOVAs of pooled data showed that the protein abundance of five additional spots (9, 30, 51, 82 and 88) also differed significantly between *S. glomerata* oysters inhabiting locations close to and away from ASS outflow drains (Table 3; Fig. 3). Consequently, between the nested and pooled ANOVAs, seven proteins were identified as displaying differential expression between acidified and reference treatments.

In hypothesising differences in the proteomes of wild *S. glomerata* between acidified and reference sites, we anticipated that some proteins would be more abundant and some less abundant at the acidified sites. Of the seven spots that were differentially expressed between *S. glomerata* oysters from acidified and reference locations, five (9, 51, 61, 74 and 82) were of greater abundance (by 1.6–7.0 fold, but not statistically significant for spot 74) among the acidified than the reference samples and one (spot 30) displayed the reverse pattern and was more pronounced (by ~5.0 fold) among the reference samples (Fig. 4). One spot (88) was found only in *S. glomerata* oysters from reference sites (Fig. 4).
Discussion

Previous studies have reported large impacts of ASS runoff on the physiology and ecology of the Sydney rock oyster *S. glomerata* species (Dove and Sammut 2007a, 2007b; O’Connor and Dove 2009). We hypothesised that impacts of ASS runoff would extend to subcellular processes and, consequently, differences in the proteomes of *S. glomerata* oysters would be evident between acidified sites, close to drains channelling ASS-runoff and unaffected reference sites. To the best of our knowledge, this is the first time that proteomics has been employed to study the responses of organisms to ASS runoff. Our sampling within Port Stephens, NSW, Australia, confirmed that spatial variation in oyster proteomes was dominated by differences between acidified and reference sites. Spatial variation in proteomes at the scales of sites and stations was, by contrast, smaller.

Contrasting our results, a previous study (Green and Barnes 2010) reported only minimal influence of ASS runoff on the expression of immune and stress response genes in *S. glomerata* hemocytes. In our study, oysters at acidified sites had been naturally exposed to recurrent ASS-induced acidification all their sessile life, whereas Green and Barnes (2010) exposed oysters to ASS runoff for only 48 h. Differences in the outcomes of the two studies may reflect a genetic underpinning of...
differences in protein expression between acidified and reference sites if recruitment is dominated by locally-sourced larvae. Alternatively, the differing outcomes may reflect the requirement of a longer time period than 48 h for inducible differences to emerge. *S. glomerata* respond to short-term ASS-runoff events by closing their valves (Dove 1997) and it is possible that they may be able to behaviourally buffer impacts over the short-term.

**Differences in protein expression between and within treatments**

We found that ~5.3% of the 132 protein spots extracted from *S. glomerata* gill tissue were differentially expressed between acidified and reference sites. Of these differentially expressed protein spots, five were upregulated at the acidified sites, one was downregulated and one spot was unique to unaffected reference locations. Protein spots may contain more than one distinct protein, so the proteome shift resulting from ASS runoff could have occurred in more than seven proteins. The observed changes in protein spot abundances might be due to post-translational changes, degradation or de novo synthesis of proteins (Tyers and Mann 2003; Tomanek et al. 2011). They could result either from heritable differences between the different treatment sites in regulatory factors controlling gene expression, or from transient responses of the genes responding to ASS leachate or other sources of environmental variation.

Shifts in protein expression at sites exposed to ASS runoff may reflect the reduced pH, high concentrations of heavy metals or differences in other environmental factors. Proteomic changes have been documented in *Crassostrea virginica* following its exposure to CO₂-induced acidification (Tomanek et al. 2011). Exposure of naïve *C. virginica* oysters to 357 Pa pCO₂ (pH ~7.5) caused 12% of the whole proteome to be differently expressed, with most of these proteins being associated with the cytoskeleton or with oxidative stress (Tomanek et al. 2011). The toxicity of heavy metal and other environmental stressors have also been previously shown to modulate the immune and stress response of bivalves (Rodrı́guez-Ortega et al. 2003; Bibby et al. 2008; Kuchel et al. 2010). Similarly, exposure of *S. glomerata* oysters to copper, lead and zinc doses of 100 μgL⁻¹ yielded unique protein expression profiles (Thompson et al. 2011).

Salinity, tidal inundation or food availability can all potentially influence protein expression in oysters (e.g. Hamdoun et al. 2003; Bayne and Svensson 2006), but we suspect that they were not significant causal factors here. Although Green and Barnes (2010) found that a change in salinity from 35 to 15 had greater effects than exposure of *S. glomerata* to ASS leachate on the expression of genes involved in stress and detoxification, such sizeable differences in salinity were not evident between acidified and reference sites here. Whereas in our study a 100-fold difference in acidity was detected between acidified and reference sites, salinity differed only by 1 unit. Tidal inundation did not differ among our study sites and although we did not assess food availability at each of our sites, the operation of

<table>
<thead>
<tr>
<th>Spot</th>
<th>MStat</th>
<th>MRes</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>13.98</td>
<td>1.97</td>
<td>7.08</td>
<td>0.029</td>
</tr>
<tr>
<td>30</td>
<td>0.42</td>
<td>0.07</td>
<td>5.73</td>
<td>0.044</td>
</tr>
<tr>
<td>51</td>
<td>3.95</td>
<td>0.72</td>
<td>5.49</td>
<td>0.047</td>
</tr>
<tr>
<td>82</td>
<td>1.27</td>
<td>0.15</td>
<td>8.22</td>
<td>0.021</td>
</tr>
<tr>
<td>88</td>
<td>0.38</td>
<td>0.03</td>
<td>12.95</td>
<td>0.007</td>
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</tbody>
</table>

**Fig. 4.** Fold change of protein abundance of spots differently expressed between treatments (acidified vs reference; n = 5). Where spots were detected in only the reference or acidified treatment, differences are shown as ‘absent’ or ‘present’ respectively. Numbers next to bars correspond to spot numbers. Statistical significance (at α = 0.05) was tested with one-way ANOVA for spots 9, 30, 51, 81 and 88 (Table 3) and Student’s t-tests for spots 61 (t₉ = 3.302, P = 0.011) and 74 (t₉ = 2.056, P = 0.074). n.s. denotes no statistical significance.
oyster farms close to each suggests that food was not limiting. Thus, it seems that the signal of ASS runoff was likely far stronger than that of other sources of variation.

The gill proteomes varied less among *S. glomerata* oysters from acidified stations than they did among *S. glomerata* from unaffected sites. There were significant differences in the expression of several proteins between reference but not acidified sites. This suggests that there is a homogenising effect of proximity to ASS outflow drains on the protein expression of wild *S. glomerata*. Such patterns of higher genetic/proteomic similarity may be a general characteristic of populations adapted to environmental change (Brown et al. 2001; Hoffmann and Willi 2008). Only through manipulative exposure of organisms to controlled conditions will it be possible to differentiate proteins involved in transient stress responses from those that represent heritable genetic adaptation.

**Implications of the study**

We were interested in spatial variation in *S. glomerata* proteomes that could be attributed to ongoing exposure to ASS-induced acidification rather than to short-term fluxes. Hence, we selected acidified locations based on previously reported negative effects of ASS runoff on *S. glomerata* oysters (Amaral et al. 2011b), rather than on the occurrence of recent acidification events. Nonetheless, it is important to note that geomorphological and tidal characteristics and preceding rainfall and acidification events determine the intensity and persistence of the acidity and of metal concentrations in ASS leachate at specific locations (Dent and Pons 1995; Sammut et al. 1996; Johnston et al. 2005; Green et al. 2006). The present study was not, however, designed to fully capture the magnitude of variability in the impact of ASS runoff on the proteome of *S. glomerata*. Rather, it was designed as a proof-of-concept project to assess whether future research into the molecular underpinnings of responses of organisms to ASS-runoff is warranted.

Furthermore, because our study was descriptive, not manipulative, we could not fully dismiss the possibility of weak influences of environmental variables other than acidification on the proteome response of *S. glomerata* between acidified and reference sites. Although temperature, salinity and tidal inundation were similar across our sampling locations, it is not known the magnitude of change in these or other environmental factors necessary to cause differentiated protein expression in *S. glomerata*. Additionally, the fact that many of the differences in protein expression between acidified and reference sites only emerged after statistical pooling of replicate sites suggests that the future studies required to generalise our results to other estuaries and populations would benefit from greater replication.

**Conclusions**

Our investigation has documented, for the first time, differences in the proteomes of wild *S. glomerata* populations that are recurrently exposed to and that are unaffected by ASS-runoff. This is significant because differential protein expression in *S. glomerata* populations chronically exposed to ASS runoff may drive the greater physiological and ecological resistance of these populations to the stressor than naïve aquaculture oysters (Amaral et al. 2011b). Our study represents an important first step in determining the molecular underpinnings of response of organisms to ASS runoff. Studies are now required to determine: (1) what specific environmental variables associated with acidified sites influence protein expression; (2) the mechanisms by which proteins are differentially expressed between acidified and reference sites; and, (3) the identity and function of the proteins that are differentially expressed. Characterisation of differentially expressed proteins may help to unveil when, where and how exposure to ASS runoff weakens the shells of *S. glomerata*, leading to higher susceptibility to predation (Amaral et al. 2012). Furthermore, understanding how inducible and genetically determined variation in protein expression underpins physiological and ecological impacts of environmental change will be critical to understanding the adaptive capacity of populations to new environmental stressors, such as CO2-induced acidification.

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