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"Urbansied estuaries are among the most highly stressed marine environments in the world. Historical and continuing inputs mean that estuaries such as Sydney Harbour suffer significant chemical contamination. Molecular biomarkers in endemic species such as oysters are an efficient way of monitoring the biological impacts of such contamination. However, the suite of biomarkers available in oysters is relatively limited. In this article, we describe our studies of environmental stress responses in Sydney rock oysters. Those studies have identified a broad array of gene transcripts and proteins with the potential to act as efficient molecular biomarkers. Our work suggests that many of these transcripts and proteins contribute to a universal intracellular stress responses, and so may be useful for assessing many different environmental stressors in a broad range of species."

The Biology of Environmental Stress: Molecular Biomarkers in Sydney Rock Oysters (*Saccostrea glomerata*)

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Abstract

This review describes our recent work on environmental stress in Sydney rock oysters, focusing on the identification of molecular biomarkers for ecotoxicological analysis. We begin by describing the environmental pressures facing coastal estuaries in Australia, with particular reference to Sydney Habour. After providing that context, we summarise our transcriptional and proteomic analyses of Sydney rock oysters responding to chemical contamination and other forms of environmental stress. This work has shown that the intracellular processes of oysters are highly responsive to environmental threats. Our data agree with the broader literature, which suggests that there is a highly conserved intracellular stress response in oysters involving a limited number of biological processes. We conclude that many effective molecular markers for environmental biomonitoring are likely to lie within these biological pathways.

Introduction

Molecular biomarkers are increasingly valuable tools in aquatic ecotoxicology[1-7]. Assays measuring the expression of key stress response genes in endemic species offer a high throughput adjunct to more traditional ecotoxicological approaches . However, the development of effective transcriptional assays is often inhibited by a lack of detailed information on the intracellular systems that are affected by abiotic stressors in the endemic species to be used for biomonitoring. This hinders the identification of robust molecular biomarkers that can act as effective proxies of ecosystem health. The following article synthesises our work on the identification of intracellular systems that are affected by abiotic stressors in Sydney rock oysters (*Saccostrea glomerata*). The review begins with a brief rationale for our choice of experimental system, focusing on Australia's coastal waterways and the use of endemic oysters as biomonitors. We then describe transcriptional and proteomic analyses of Sydney rock oysters exposed to chemical contaminants in both the laboratory and the field as a way of identifying the intracellular pathways affected by pollutants. Finally, our data are compared to the broader literature to identify a common stress response pathway in oysters that points to the selection of ubiquitous biomarkers of environmental stress.

Australia's urbanised estuaries and Sydney rock oysters

Sydney Harbour and other urbanized estuaries

Estuaries are among the most productive aquatic environments in the world. They provide an array of ecosystem services, including water purification, nutrient recycling, nursery grounds, and essential habitats for many pelagic and benthic species. Urbanized estuaries are also hotspots of anthropogenic change[8]. They often suffer from significant chemical pollution, diminished water quality, and depleted natural resources[9]. As a result, aquatic species in urbanized estuaries are exposed to a suite of complex, interacting environmental stressors[10,11]. These multifaceted impacts often threaten the survival and functional diversity of local ecosystems[12].

Anthropogenic stress in urbanized estuaries is a major problem in Australia. In contrast to popular perception, Australia is, per capita, a highly urbanized country. The majority (65%) of the population lives in just five coastal cities, and over 80% live within 40 km of the coast[13]. Sydney is Australia's oldest and largest city, and so has suffered long term environmental change. The Sydney Harbour estuary supports a range of ecosystems with many resident species, including over 500 types of fish[14]. These ecosystems sit aside a metropolitan area with a population exceeding 4 million people[13]. Eight of the ten most densely populated communities in Australia are located adjacent to Sydney Harbour. The estuary has a relatively small catchment (480 km^2) surrounding a major port

that was established more than 200 years ago (ca. 1811). Historically, Sydney Harbour and the tidal rivers that feed into the waterway were the sites of substantial mineral refineries, chemical production, shipping, steel manufacture, ship building, whaling stations and urban construction[15]. Most industrial and shipping activities have now moved away from the estuary. However, continuing urbanization of the watershed (currently 86%) has led to persistent inputs of contaminants and sediments into the estuary, such that concentrations of some contaminants in Sydney Harbour remain amongst the highest in the world (Fig. 1)[16]. The shoreline still has more than 800 sewage overflows and many other point sources of urban runoff[17].

Not surprisingly, Sydney Harbour's ecosystem has been significantly impacted by anthropogenic change[14]. Its sediments are well known sinks for toxic contaminants (e.g., metals, PCBs, DDTs, and dioxins) that are responsible for significant trophic transfer of compounds to the estuary's food web[18,19]. Sediment transport models have shown that contaminants mainly enter Sydney Harbour by direct discharge in stormwater and terrestrial runoff[20]. These inputs are enhanced by the redistribution of historically contaminated soils and bottom sediments during high flow events[21,22]. Sediments in many areas of the estuary exhibit strong concentration gradients of organic contaminants, toxic metals and nutrients[21-26]. There are particularly strong gradients in metal concentrations in water adjacent to canals that discharge into numerous enclosed embayments of the harbour[22,27]. These discharges contain contaminant concentrations several orders of magnitude greater than in the open estuary[21,22,28].

Several classes of environmental contaminants that originate from urban and industrial inputs into Sydney Harbor (including metals, dioxins, polychlorinated biphenyls (PCBs) and other chlorinated hydrocarbons) are bioaccumulated by the endemic biota[19]. Tissue concentrations of these contaminants appear to be sufficient to incur toxic effects in resident fish and bivalves[29,30]. McCready et al.[18,31-32] found that 84% of sediment samples from 15 locations in Sydney Harbour were toxic to local invertebrates. Correspondingly, Stark et al.,[33] found that bays in the harbour that were polluted by metals had significantly different benthic assemblages when compared to unpolluted bays. In 2006, elevated levels of dioxins in fish and crustaceans led to a ban on commercial fishing in Sydney Harbour, and advisories against consuming fish caught in the harbour remain in place. Trends in contamination suggest such risks to biota are not diminishing[35].

Sydney rock oysters as environmental biomonitors

Bivalve molluscs (primarily mussels and oysters) are among the most frequently employed biomonitors of environmental stress. They closely reflect changes in water quality, providing time-sensitive information on impacts at the base of the food-web[36]. Most bivalve species feed on phytoplankton, sediment and detritus filtered from the water column[37,38]. As such, they are highly dependent on suitable water quality for survival and growth[39,40]. Any persistent water quality stressors or environmental modifications will have reverberating effects on bivalve populations[41]. The impacts of fluctuating environmental conditions on oysters is confirmed by genomic and transcriptomic analyses. The genome of the Pacific oyster (*Crassostrea gigas*) is notable for its proliferation of genes involved in intracellular stress responses, and the expression of genes within those intracellular systems is highly responsive to environmental perturbation[42]. The highly responsive nature of bivalves has supported the use of mussels and oysters as biomonitors of coastal waters around the world for over 30 years[43,44].

Sydney rock oysters (*S. glomerata*) represent an ideal bivalve for molecular biomonitoring in Sydney Harbour and other impacted estuaries on Australia's east coast. They are the key ecosystem engineers in many of these estuarine habitats, where they form the substrate for localized ecosystems with broad biodiversity[45]. *S. glomerata* is also the focus of a major aquaculture industry that supports fundamental research into their molecular biology. These features mean that Sydney rock oysters have already been used to assess water quality conditions and test responses to various stressors[46-49].

S. glomerata inhabit the intertidal zone to 3 m below the low water mark, placing them in direct contact with contaminants from terrestrial inputs or sediments. Even though their larvae are free swimming and spend up to 3 weeks in the water column, most data suggest that *S. glomerata* essentially live in restricted geographic locations for their entire life cycle, making them ideal models for assessing the long-term impacts of stress. They tend to closely reflect local water quality conditions and rely on suitable environmental conditions for growth and survival. Changes to hydrologic regimes (e.g., rainfall, water temperature, salinity, and sediment loads) have been associated with impacts on the growth, development and survival of *S. glomerata*[50]. One recent study of oysters transplanted to two Sydney estuaries contaminated with metals and PAHs also identified strong relationships between contaminant exposure, reproductive success, cellular biomarkers of oxidative stress and organismal health[51]. This suggests that Sydney rock oysters have the inherent responsiveness required for effective molecular biomonitoring.

Molecular biomarkers of environmental stress in Sydney rock oysters

Molecular biomarkers

The assessment of molecular biomarkers has become common in ecotoxicological studies of bivalves[52]. The molecular processes measured within cells responding to stress include alterations in DNA, mRNA transcription, or protein concentration/activity. The selection of appropriate biomarkers within bivalves has often been based on presumptions about the underlying cellular mechanisms responsible for stress responses. Common *a priori* biomarkers include lysosomal and autophagic reactions, antioxidant proteins, metallothioneins, vitellogenin, phenoloxidase enzymes and heat-shock proteins, all of which are thought to reflect physiological changes in bivalves responding to altered environmental conditions[53-58].

Data from such studies are often highly informative. However, assays based on *a priori* biomarker selection are often limited because they test just a few functional traits, such as antioxidant proteins or molecular chaperones. This excludes identification of other processes that may be modified under the same conditions. 'Omics' approaches have been adopted to address this need for more integrative strategies in the identification of relevant biomarkers[59,60]. The 'omics that have been used so far include broad scale analyses of mRNA transcript abundance (transcriptomics) and protein levels (proteomics). These techniques allow interactions between large numbers of genes and proteins to be studied simultaneously, building complex pictures of biological responsiveness. The majority of existing 'omics' studies in aquatic ecotoxicology have focused on the differential transcriptomes and proteomes of organisms responding to individual stressors-of-interest[60-62]. However, 'omics can also be used to investigate more complex scenarios. Chapman et al.,[63] provided an early example of the power of these techniques by integrating gene expression signatures in the Eastern oyster (*Crassostrea virginica*) with differing land use patterns in the South Atlantic Bight. Such analytic depth is key to investigating multiple stressor interactions in aquatic organisms.

Laboratory-based assessment of proteomic and transcriptional responses to environmental contamination in Sydney rock oysters

We have used both *a priori* marker selection and 'omics approaches to identify useful molecular biomarkers in *S. glomerata.* Both approaches require substantial existing nucleotide sequence data. So, these studies were preceded by next generation sequence (NGS) analysis of the transcriptome of *S. glomerata* exposed to a range of environmental stressors[64]. The goal of that NGS analysis was to provide a catalogue of cDNA sequences that could be interrogated with data from contaminant exposure experiments in order to identify differentially expressed genes and proteins, or to design

PCR primers for selected marker genes. The Sydney rock oysters used in this NGS analysis were transplanted into contaminated field sites (metals and organic contaminants) or exposed to stress (bacterial inoculation) under controlled laboratory conditions[64]. Transcriptome data were generated from gill tissues and digestive glands using 454 pyrosequencing technology. De novo assemblies yielded 28,685 contigs corresponding to 11,671 different genes. Prior to this work, there were less than 400 sequences for *S. glomerata* genes in publically accessible databases, so our NGS work provided a crucial new resource for gene discovery.

Once the reference database of cDNA sequences had been assembled, we began a series of laboratory-based experiments in which different groups of oysters were exposed separately to four metals (cadmium, copper, lead and zinc) that are commonly associated with anthropogenic pollution in Sydney Harbour and other coastal estuaries on Australia's eastern seaboard[65-69]. Oysters were exposed to different concentrations of each metal (5 µg/l to 100 µg/l) for four days before their gills or hemocytes (blood cells) were collected for analysis. The doses of metals used are biologically relevant, representing low to relatively high concentrations found in contaminated environments[69]. Initially, we tested the transcriptional expression of a selected set of seven genes (superoxide dismutase, ferritin, ficolin, defensin, HSP70, HSP90 and metallothionein) in gill samples from oysters exposed to the 100 µg/l doses[65]. These target genes were chosen from our NGS database because changes in their transcript abundance had previously been reported in other bivalves responding to environmental contamination. Quantitative (real-time) PCR analyses of transcript abundance showed that each of the different metals elicited unique transcriptional responses. Significant changes in transcription were found for 18 of the 28 combinations tested (4 metals x 7 genes). Sixteen of these changes reflected down-regulation of transcript abundance. The molecular chaperone, HSP90, was the only gene to be significantly up-regulated by metal contamination.

A more extensive transcriptional analysis using a new set of 14 putative stress response genes showed that differential transcript abundance in response to metal contamination in the laboratory was dose dependent (Fig. 2)[69]. This expanded set of target genes included several that were identified by proteomic analyses (described below[68]) and fell into a range of intracellular functional pathways. The expression of all 14 genes was significantly affected ($P < 0.05$ vs. non-exposed controls) by at least one of the metals tested, and by at least one dose of that metal. Altered transcriptional expression of many target genes was most extreme at intermediate (rather than high) doses of metals. We concluded that such responses might be hormetic, reflecting adaptive

(acclimation) reactions in gene expression responding to low to intermediate doses of contaminants, followed by a decline in expression at higher doses that reflected morbidity.

We also analysed oyster samples from the laboratory based metal exposures using proteomics to broaden the scope of this early work[66-68]. Rather than focusing on selected sets of target genes, these proteomic analyses interrogated the entire proteomes of metal-exposed oysters in an effort to identify the broader suite of intracellular functions affected by contamination. Initially, oyster hemolymph proteins from metal-exposed oysters (100 µg/l of copper, lead or zinc) were compared to hemolymph from non-exposed controls using 2-dimensional electrophoresis (2DE) to identify proteins that differed significantly in relative concentration between the two treatments[68]. Tandem mass spectrometry was then used to assign identities to the differential protein spots. This process revealed unique proteomic profiles for each metal and identified 25 proteins that were differentially expressed after metal exposure. Only one of the differential proteins had significantly altered relative concentrations in response to all three metals. Eighteen of the 25 differential proteins were significantly affected by just one of the three metals. Mass spectrometry showed that the differential proteins contributed to only five distinct categories of intracellular function. Proteins affecting shell properties were the most common functional group, followed by molecules involved in cytoskeletal activity, energy metabolism and intracellular stress responses.

Subsequent 2DE analysis of oysters exposed to 100 µg/l, 50 µg/l and 5 µg/l doses of four metals (cadmium, copper, lead and zinc) revealed that substantially different sets of proteins were affected by each dose of metal (Fig. 3)[67]. Similarly, different sets of proteins were affected by the different metals. However, mass spectrometry again revealed that most of the differential proteins at each dose rate fell into the same broad categories of intracellular function, predominantly intracellular stress responses (including heat shock proteins), the cytoskeleton, and energy metabolism.

These 2DE studies benefited from relatively high throughput allowing substantial numbers of oyster samples to be analysed simultaneously. However, each 2DE gel could only resolve about 300 different proteins, relative to the predicted 2,000 or more proteins that are likely to be expressed in a particular tissue. We used label free shotgun proteomics to rectify this shortfall in analytical capacity[66]. Shotgun proteomics can identify far more proteins than 2DE, but is limited by lower throughput of samples. Hence, our shotgun analysis used a subset of the same oyster samples that were assessed by 2DE (oysters exposed to 100 µg/L of lead, copper and zinc for 4 days in closed aquaria). This meant that results of the two analytical techniques could be directly compared. Shotgun proteomics identified 84 proteins that were present at significantly different concentrations

in metal-exposed relative to control (non-exposed) oysters. Of these, 54 could be identified by reference to existing databases of oyster gene and protein sequences. Most of the identified proteins fell into the same functional categories as detected by 2DE, namely the cytoskeleton, energy metabolism and intracellular stress responses, with the addition of some involved in protein synthesis. This suggests that, while 2DE identified relatively less proteins, it still provided an effective snapshot of the functional systems within cells that are affected by metal contamination.

All of these laboratory-based studies suggested that oysters undergo substantial changes in their transcriptomes and proteomes in response to metal contamination. Not surprisingly, subsequent work showed that the observed changes in transcription and protein concentration were closely linked[70]. The transcript abundance of six genes encoding proteins identified as differentially expressed by 2DE (actin, ATP synthase, vasa, vitellogenin, myosin and tubulin) was also assessed by qPCR. This allowed relative transcript abundance to be directly compared to the relative abundance of the corresponding encoded proteins. Five of the six genes showed a positive association between mRNA transcription and protein concentration, with an increase in transcription corresponding to an increase in protein concentration. The only exception was the DEAD box protein, vasa. Vasa expression was up-regulated at the transcriptional level in metal-exposed oysters, whilst proteomics identified a significant reduction in the intracellular concentration of the encoded protein. This highlights a potential limitation of 'omics techniques. The abundance of transcripts for a particular gene may not translate into a proportionate concentration of the encoded protein, and protein concentrations may not have a proportionate effect on intracellular structure and/or function. Hence, it remains desirable that molecular biomarkers be benchmarked against quantifiable effects at the physiological, whole organism, or ecological levels.

Proteomic analyses of oysters exposed to environmental disturbance in the field.

As with our own early work, most laboratory-based studies of environmental change focus on the response of organisms to individual stressors (such as metals, temperature, or hypoxia) in isolation[71,72]. They usually do not assess the simultaneous effects of multiple stressors and are rarely conducted in conjunction with studies of natural abiotic variability in the field. This is probably because the presence of multiple stressors can confound observations. However, the interaction amongst multiple stressors requires careful consideration to realistically interpret biological effects as they would occur in the field.

Highly impacted environments, such as Sydney Harbour and other estuaries in eastern Australia, probably exert environmental stress on endemic biota through an array of interacting factors that are poorly understood[73]. Mixtures of stressors can exert synergistic or antagonistic effects on individual species. For instance, elevated temperature and pCO_2 have antagonistic impacts on energy metabolism and oxidative stress in oysters[74]. Matoo et al.[74] showed that altered biological activity was clearly evident when oysters were exposed to elevated temperature and CO_2 levels in isolation. However, responses were relatively indistinguishable from controls when the stressors were applied in combination. Conversely, extremes of temperature and salinity have been shown to exert a synergistic negative influence on the embryonic development of oysters[75].

We have undertaken field studies on oysters from a number of different estuaries in an effort to accommodate the potentially confounding influence of multiple environmental factors. The first of these studies investigated the effects of acid sulfate soil (ASS) runoff on the proteomes of Sydney rock oysters[76]. Runoff from ASS poses a serious threat to the ecology and biodiversity of estuaries around the world, and it impacts a number of estuaries on Australia's eastern seaboard[77-79]. ASS contain iron sulfides that oxidise to sulfuric acid when exposed to air during drainage or disturbance. This can release toxic quantities of heavy metal ions from sediments. After heavy rainfall, these oxidation products leach into nearby estuaries and can acidify adjacent waterways to as low as pH2–3[80]. The severe negative effects of ASS runoff on estuarine flora and fauna include large-scale fish kills, the weakening of mangrove pneumatophores, and reductions in the population sizes of oysters and gastropods at affected locations[81-85].

In our field study of ASS, we predicted that the proteomes of wild Sydney rock oysters would differ between populations exposed to recurrent episodes of ASS runoff compared with those that were unaffected by ASS[86]. 2DE was used to compare the gill proteomes of wild growing *S. glomerata* collected from two sites close to (acidified) and two sites away from (reference) ASS outflow drains in Port Stephens, a major urbanized estuary near Sydney. The data suggested that ASS runoff has substantial effects on oyster proteomes. Multidimensional scaling plots (supported by PERMANOVA analysis) showed a clear distinction in the proteomes of oysters from ASS-impacted compared to the reference sites (Fig. 4). Five proteins were significantly more abundant and one less abundant at the ASS sites, relative to reference sites. Another protein was present only in oysters from reference sites. We concluded that these altered proteomic profiles could reflect either short-term inducible responses to ASS runoff or genomically encoded adaption of gene expression resulting from recurrent (transgenerational) exposure of oyster populations to the stressor.

Interestingly, an adjunct to this study showed that oysters from ASS impacted sites were also more responsive to another form of environmental stress (elevated pCO_2)[86]. The responsive genes identified in this later study again fell primarily into intracellular functional categories involved in energy metabolism, cellular stress responses, the cytoskeleton, and protein synthesis. This is consistent with the hypothesis that environmental stress in oysters leads to a generic response involving increased mitochondrial energy production to maintain cellular homeostasis (see *Perspectives and Conclusions*)[87].

Similar results were obtained from a separate field study that used proteomics to assess the impacts of metal contamination in the field[88]. In this work, hatchery reared *S. glomerata* were transplanted into Lake Macquarie, an estuarine lagoon 100km north of Sydney. Industrial and urban development surrounding Lake Macquarie started in the 1890s. It included mining, a zinc smelter, a power station, and urbanization in the northern end of the lake[89]. These inputs (particularly from the decommissioned zinc smelter) have led to a strong north to south gradient of contamination with cadmium, lead, zinc, mercury and silver in the lake's sediment[89-91]. Our experiments used batches of hatchery-reared oysters that were transplanted for four days to replicated reference and metal contaminated sites along the north to south contamination gradient[92]. 2DE showed that the proteomes of oysters from the contaminated sites were clearly distinct from those at the reference sites. Principal components analysis attributed these differences to the combinations and concentrations of metals present, and to other environmental variables, such as salinity and pH. Mass spectrometric identification of the differential proteins again showed that they were primarily associated with cytoskeletal activity and intracellular stress responses. The data also revealed substantial temporal variability in these proteomic responses. The experiment was repeated three times over a two year period. Unique proteomic responses were evident for each of these different trials. Principal components analysis suggested that these differences over time reflected fluctuating levels of bioavailable contaminants and other environmental variables[88].

The responsiveness of oyster proteomes to environmental contamination in the field was also reflected in our most recent field trials. These experiments focused on Sydney Harbour. They exploited gradients of contamination that occur within individual embayments of the harbour. Existing data showed that contamination in sediments is greatest at the landward ends of bays and decreases toward the mouths of bays connecting with the main channel of the harbour. In our study, wild growing *S. glomerata* were collected from four different bays with strong contamination

gradients[92]. Two sites were sampled (3 replicates per site) in each bay. These were "high-impact" sites at point sources of chemical contamination (storm drains, canals or legacy hotspots) and "low-impact" sites approximately 5km away from the point sources toward the mouths of the bays. Tissue burden analysis showed that polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyls (PCBs), tributyltin, lead, and zinc were present at significantly higher concentrations in the tissues of oysters from the high-impact sites relative to the low-impact sites. Water at the high-impact sites also had lower dissolved oxygen content and pH than the low-impact sites. 2DE of gill tissue detected 238 proteins. Between 27 and 50 of these proteins differed significantly in relative intensity between the high- and low-impact sites in each bay. The differences meant that nMDS analyses combining data from the entire proteome revealed clear distinctions between oysters from the high and low sites (Fig. 5A). In three of the four bays, the ordinates of differences between the high- and low-impact clusters were very similar, suggesting similar proteomic responses to contamination in these bays. A different pattern was evident in the fourth bay, which may reflect its different contaminant profile or water quality parameters. Eighty of the differential proteins could be identified by mass spectrometry. Again, half of these identified proteins fell into just two subcellular functional categories; energy metabolism and cytoskeletal activity (Fig. 5B).

Changes in the proteomes and transcriptomes of Sydney rock oysters reflect a universal intracellular stress response

All of the data from our transcriptional and proteomic analyses suggest that the intracellular processes of Sydney rock oysters are highly responsive to chemical contamination and other forms of environmental stress. The most notable feature of that responsiveness is that just a few intracellular systems are primarily involved (notably the cytoskeleton, energy metabolism, and intracellular stress responses). This meant that some key proteins were associated with responsiveness in most, if not all of our studies[65-69,86,88,92]. We were able to assign identities to 317 proteins in our six studies of Sydney rock oysters exposed to environmental stress. Molecular chaperones (heat shock proteins, HSPs) were the most commonly identified, representing 32 of the 317 identifications. The next most common proteins were the cytoskeletal proteins, actin (30/317) and β-tubulin (30/317), and the metabolic enzyme complex, ATP synthase (13/317). Among the 20 most frequently identified proteins, seven were involved in energy metabolism (including ATP synthase and NADH dehydrogenase), five were cytoskeletal proteins (actins, tubulins, tropomyosin and myosin) and two were involved in intracellular stress responses (HSPs and superoxide dismutases).

This is consistent with a repeating pattern found in numerous proteomic or transcriptional studies of oysters responding to environmental stress. We recently undertook a meta-analysis of 14 studies that have investigated the effects of environmental stress on transcription in a range of different oyster species[87]. These studies tested the transcriptional effects of numerous stressors, including chemical contamination, hypoxia and infection, as well as extremes of temperature, pH and turbidity. The meta-analysis showed that the abundance of transcripts for 586 genes changed significantly after exposure to these environmental stressors. The same repeating pattern that we had identified in our proteomic analyses of Sydney rock oysters was evident in these transcriptional responses. Again, many of the genes that responded to environmental stress encoded proteins involved in just three intracellular processes. The top 10 gene names associated with environmental stress were dominated by molecules involved in energy production by the mitochondrial electron transport chain (NADH dehydrogenases, cytochrome C's and ATP synthases; comprising 16% of the entire differential transcriptome), the cytoskeleton (actin and tubulin; 6%) and intracellular stress responses (metallothioneins, GSTs, HSP70s, and the antioxidant enzyme SOD; 6%). Moreover, the percentage of differentially expressed genes comprising the different functional categories was very similar between all four general classes of stressors (infection, temperature extremes, contamination and hypoxia). This suggests that many different types of stress elicit broadly similar subcellular responses.

A similar pattern is evident at the proteomic and genomic levels[42,93]. Tomanek[94,95] recently reviewed the proteomic responses of marine organisms to a range of different stressors. He again found that environmental stress responses primarily involve molecules contributing to energy metabolism, cytoskeleton and intracellular stress reactions (protein stabilization and turnover, and oxidative stress). Similarly, Zhang et al.[42] associated genomic adaptation of oysters to environmental stress with proliferation of genes encoding anti-oxidant enzymes and heat shock proteins, as well as inhibitors of apoptosis.

Perspectives and conclusions

We have put forward a consensus model of sub-cellular stress responses in oysters to explain the recent transcriptional and proteomic data (Fig. 6). This model complements the conclusions reached by a number of other authors[94,96,97]. It highlights the mitochondrial electron transport chain as the key cellular system impacted by environmental stress. As a corollary, the model suggests that

increased demand for cellular energy is the common and unifying response of oysters to environmental stress. Enhanced energy production in the mitochondria is taken to have beneficial outcomes in terms of powering adaptive cellular processes. However, it is also known to elevate the production of reactive oxygen species (ROS), which have cytotoxic effects on mitochondrial and cytoskeletal integrity. In our model, upregulation of anti-oxidant enzymes such as SOD and molecular chaperones (HSPs) is initiated to limit damage caused by ROS to prevent cellular dysfunction and programmed cell death.

This response seems to apply to a range of different environmental stressors. Hence, effective molecular biomarkers of environmental contamination in Sydney rock oysters are likely to lie within the suite of genes encompassed by our model. Some of those genes, such as HSPs, are already used extensively for molecular biomonitoring . Others are more unexpected. Whilst our data suggest that actins are highly responsive to environmental contamination, they have traditionally been used as reference (housekeeping) genes in transcriptional studies due to their presumed stability. Our work has also shown that the responses of individual genes and proteins can be relatively unpredictable over time and space, due in part to the stringencies that have to be placed on the definition of "significant" differential expression. However, responses viewed in the context of entire categories of subcellular function (such as the cytoskeleton or antioxidant systems) are predictable and consistent. This means that effective studies using molecular biomarkers should involve suites of genes or proteins that are amenable to multivariate statistical analyses. When taken with this proviso, our work and that of many others suggest that molecular biomarkers in oysters are a reliable and sensitive way of measuring the impacts of environmental contamination on biological systems.

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Figure Legends

Fig. 1**A.**The distribution of lead in sediments (fine fraction, <62.5µm) of the Sydney Harbour estuary. Units are µg g^{-1}. From[24.] **B.** Comparion of sediment concentrations for copper, lead and zinc (ppm) in various world estuaries. In this figure Sydney Harbour is shown as Port Jackson. Drawn from data in [24] and references therein.

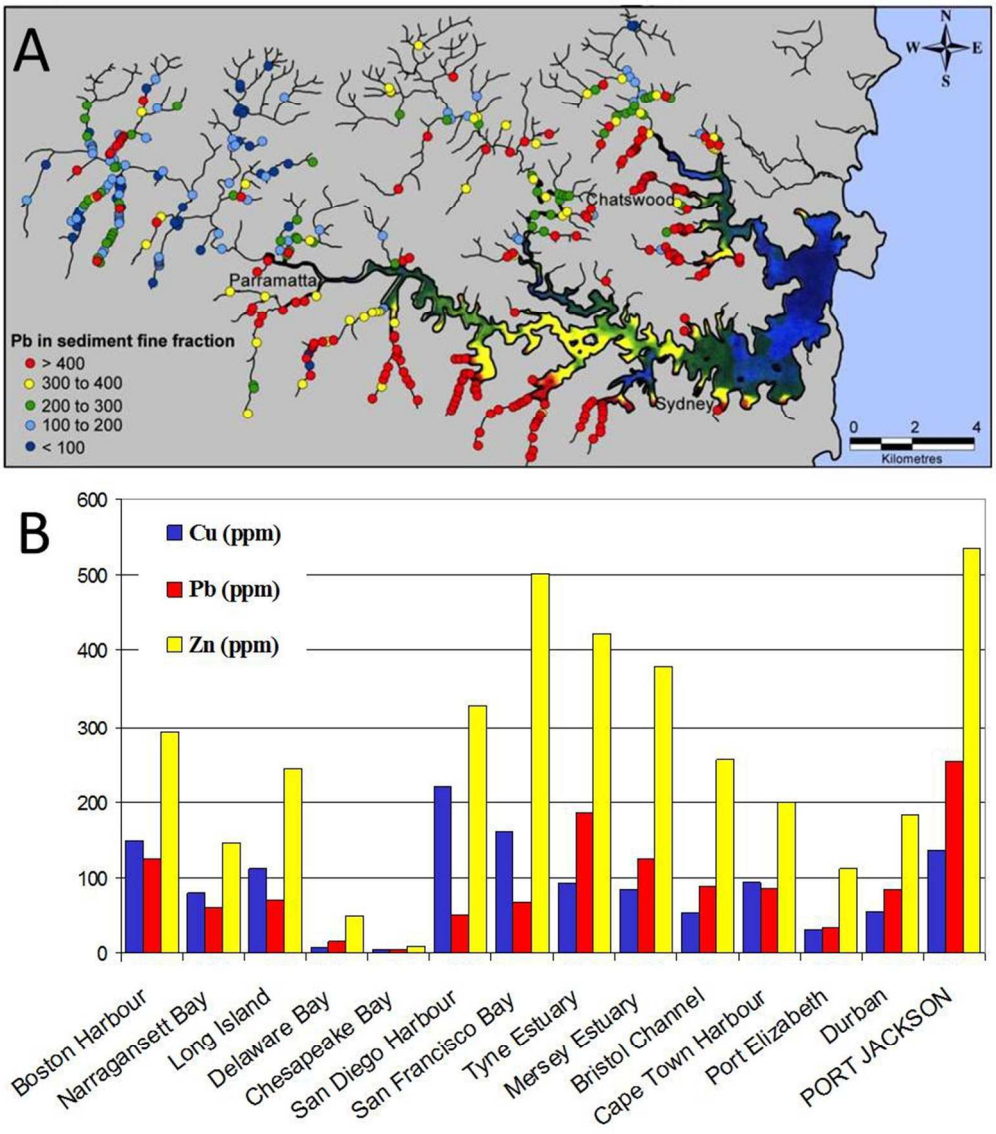
Fig. 2. Relative transcript abundance compared with nonexposed controls for three genes, superoxide dismutase (SOD), DNA Topoisomerase I (TOP1) and tubulin, in hemolymph of oysters exposed for 4 days to 5, 10, 25, or 50 µg/L of lead or zinc., *significant difference vs. non-exposed controls ($p<0.05$). Modified from[69]

Fig. 3 **A.** A proteome map showing the location of protein spots that exhibited significantly different intensities ($p < 0.05$) compared to controls when oysters were exposed to 100µg/l Cu, Pb, or Zn. The boxed areas are magnified in panels **B** and **C**, which also show the corresponding data for relative intensities of the differential protein spots (n = 3, bars – SEM). From[67].

Fig. 4 Non-metric multidimensional scaling ordination of the gill proteomes of oysters collected from ASS acidified (A1, A2) or reference (R1, R2) sites in Port Stephens, NSW. Ovals enclose sampling stations that were statistically indistinguishable (at $p<0.05$). From[76].

Fig 5 Proteomic analysis of Sydney rock oysters from Sydney Harbour. **A.** Non-metric multidimensional scaling ordination of proteomes in oysters collected from high-impact (black) and low-impact (white) sites in four different embayments of Sydney Habour. Each data point represents a replicate of 5 oysters. Ovals enclose replicates that were statistically indistinguishable at $p<0.05$. **B.** The percentage of 80 differential proteins identified by mass spectrometry falling into discrete categories of intracellular biological function. From[92].

Fig. 6 The consensus model of intracellular responses to stress in oysters proposed by Anderson et al.[87]. The model summarizes gene expression data from a range of studies to show the key intracellular processes that are affected environmental stress.



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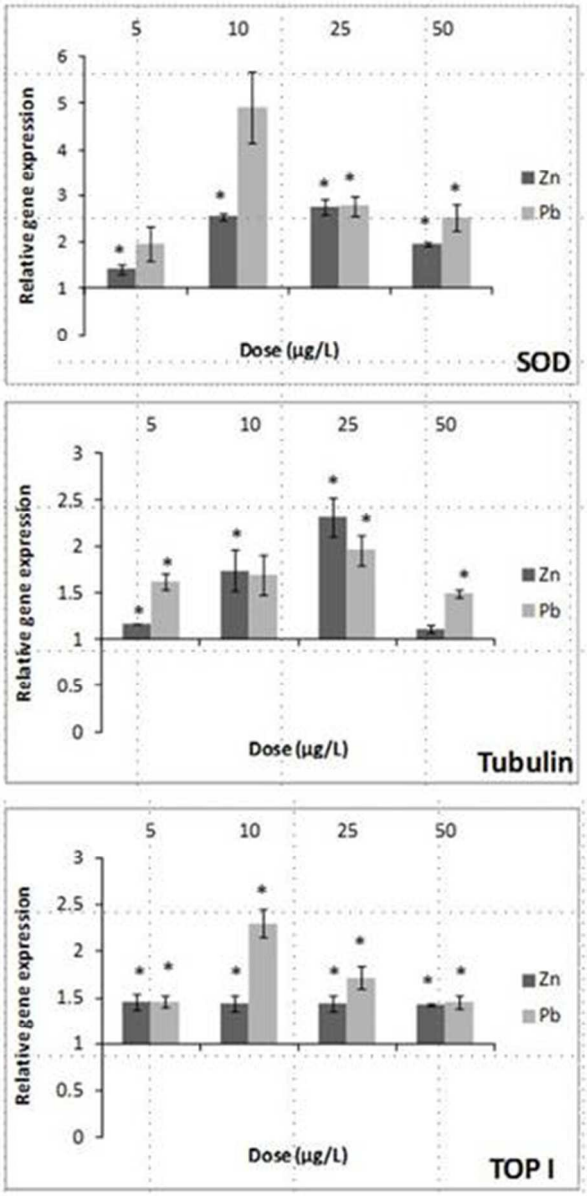

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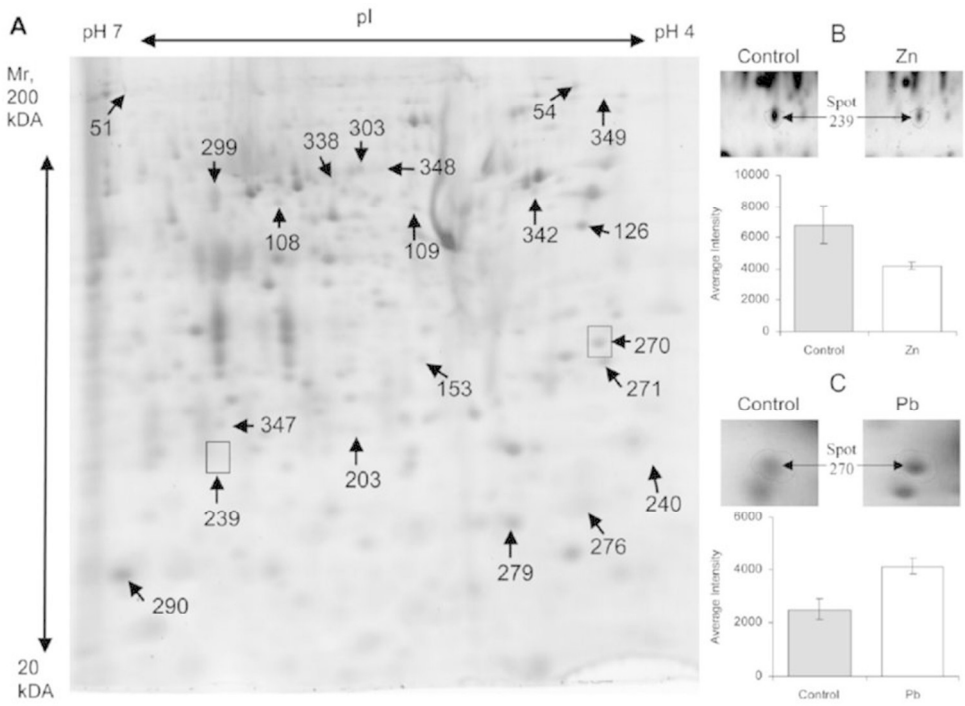

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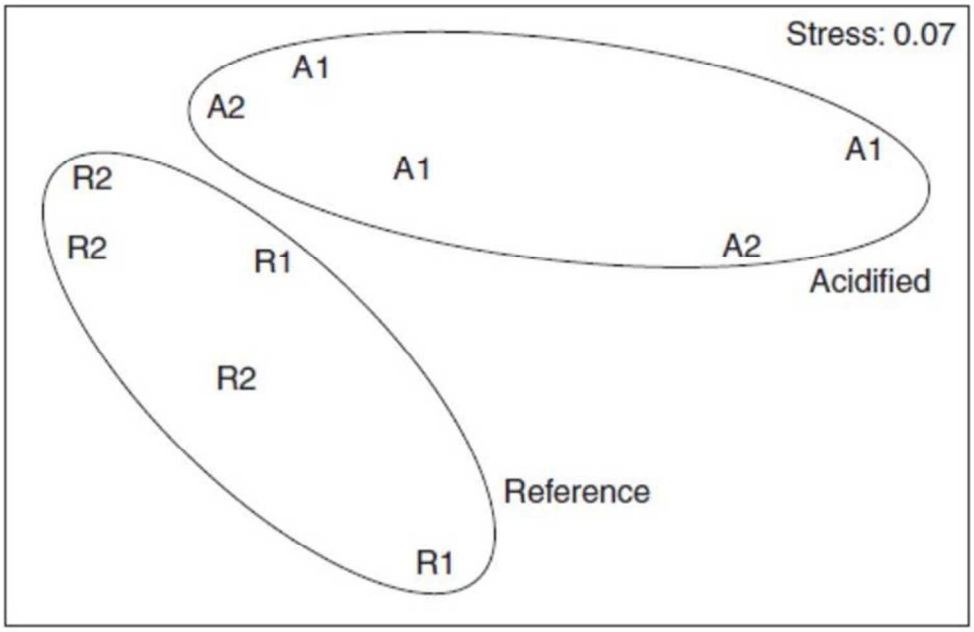

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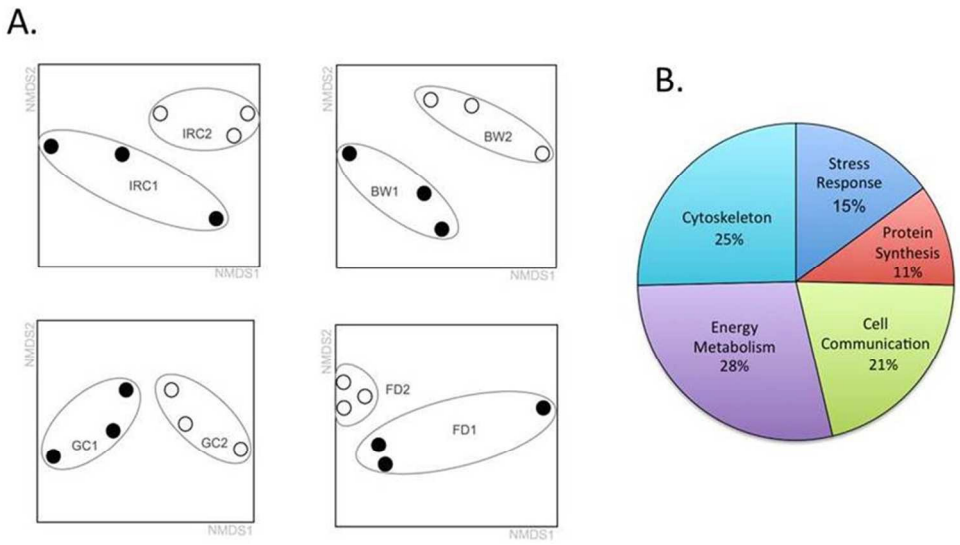
254x170mm (96 x 96 DPI)



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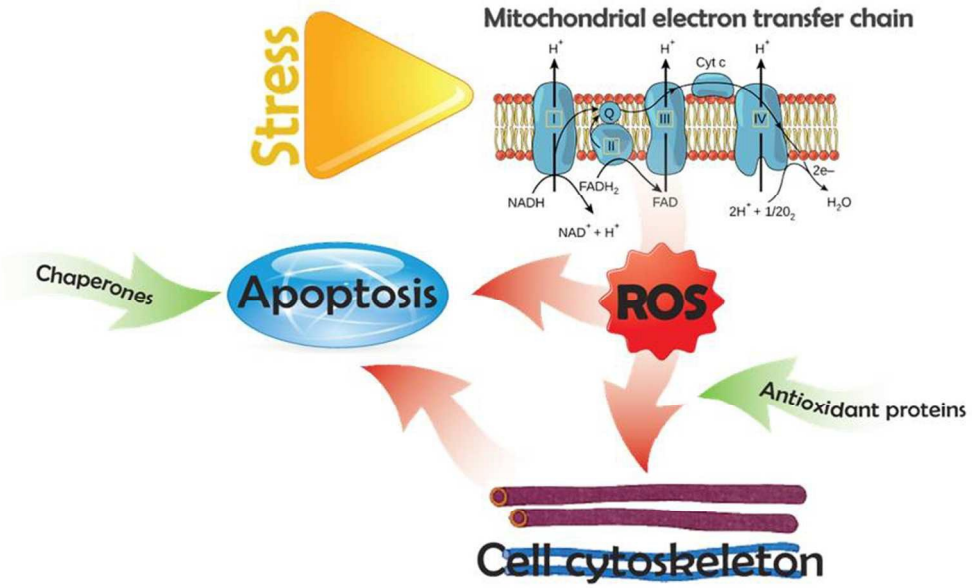
254x140mm (96 x 96 DPI)



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254x160mm (96 x 96 DPI)