Proteomic responses to metal-induced oxidative stress in hydrothermal vent-living mussels, *Bathymodiolus* sp., on the Southwest Indian Ridge

Catherine Cole[a,*], Ana Varela Coelho[b], Rachael H. James[c], Doug Connelly[c] and David Sheehan[d]

[a]*Ocean and Earth Science, University of Southampton, European Way, Waterfront Campus, Southampton, SO14 3ZH, UK*

[b]*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal*

[c]*National Oceanography Centre, University of Southampton, Waterfront Campus, European Way, Southampton, SO14 3ZH, UK*

[d]*School of Biochemistry and Cell Biology and Environmental Research Institute, University College Cork, Ireland*

*Corresponding author.

Address: Department of Ocean and Earth Science, University of Southampton, Waterfront Campus, European Way, Southampton, SO14 3ZH, UK

Telephone: +44 23 80596020

E-mail address: catherine.cole@noc.soton.ac.uk

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ABSTRACT

Bathymodiolin mussels are amongst the dominant fauna occupying hydrothermal vent ecosystems throughout the World's oceans. This subfamily inhabits a highly ephemeral and variable environment, where exceptionally high concentrations of reduced sulphur species and heavy metals necessitate adaptation of specialised detoxification mechanisms. Whilst cellular responses to common anthropogenic pollutants are well-studied in shallow-water species they remain limited in deep-sea vent fauna. *Bathymodiolus* sp. were sampled from two newly-discovered vent sites on the Southwest Indian Ridge (Tiamat and Knuckers Gaff) by the remotely operated vehicle (ROV) Kiel 6000 during the *RRS James Cook* cruise, JC 067 in November 2011. Here, we use redox proteomics to investigate the effects of tissue metal accumulation on protein expression and thiol oxidation in gill. Following 2D PAGE, we demonstrate a significant difference in intensity in 30 protein spots in this organ between the two vent sites out of 205 matched spots. We also see significant variations in thiol oxidation in 15 spots, out of 143 matched. At Tiamat, 23 protein spots are up-regulated compared to Knuckers Gaff and we identify 5 of these with important roles in metabolism, cell structure, stress response, and redox homeostasis. We suggest that increased metal exposure triggers changes in the proteome, regulating tissue uptake. This is evident both between vent sites and across a chemical gradient within the Knuckers Gaff vent site. Our findings highlight the importance of proteomic plasticity in successful adaptation to the spatially and temporally fluctuating chemical environments that are characteristic of hydrothermal vent habitats.

Keywords: Hydrothermal activity; Southwest Indian Ridge; *Bathymodiolus* sp.; Metals; Bioaccumulation; Oxidative stress; Detoxification; Proteome

1. Introduction

The circulation of conductively-heated seawater in tectonically active regions of the Earth's crust generates high-temperature hydrothermal fluids, which are highly enriched in volatile gases, sulphide and metals, and are discharged through focused and diffuse springs at the seabed (Von Damm *et al*., 1988). In the mixing zone between hydrothermal fluids and seawater, chemoautotrophic bacteria synthesise organic carbon using reduced compounds (sulphide and methane), supporting a highly productive ecosystem (Stewart *et al*., 2005; Fisher and Girguis, 2007). Bathymodiolin mussels are amongst the dominant vent fauna inhabiting the hydrothermal environment at the global scale (Miyazaki *et al*., 2010). These mussels host bacterial endosymbionts in their gills (Cavanaugh *et al*., 1987; McKiness and Cavanaugh, 2005; Stewart *et al*., 2005), but they can also feed heterotrophically on particulate organic matter (Page *et al*., 1991). This mixotrophic diet is an important adaptation to the spatially and temporally fluctuating supply of reducing agents. Adapting to survive in the chemically variable hydrothermal environment also requires an ability to cope with highly toxic concentrations of many metals. In these hydrothermal sites animals are exposed to metal concentrations of the order of a thousand times higher than in oceanic waters (Sarradin *et al*., 1999) and may have evolved specialised mechanisms of detoxification.

Elevated metal exposure can result in oxidative stress in an organism, as some metals lead to production of reactive oxygen species (ROS) which can exceed cellular antioxidant defences (McDonagh *et al*., 2005). In hydrothermal environments, metals catalyse the oxidation of sulphide to form a number of oxygen- and sulphur-based radicals. This initiates a chain reaction ultimately producing HO•, the most oxidising radical in biological systems (Fridovich, 1998; Tapley *et al*., 1999). The bulk of ROS are absorbed by proteins and prolonged exposure to metals can therefore cause damaging changes to proteins involved in

detoxification. Thiol oxidation is a well-known deleterious proteomic change resulting from the action of ROS produced in response to xenobiotics (Chora *et al*., 2008; Sheehan *et al*., 2010; Tedesco *et al*., 2010; 2012; Company *et al*., 2012). Proteins containing thiol groups (-SH) are critical components of the antioxidant defence system, and are important in enzyme catalysis and in control of the cellular redox environment (Eaton, 2006; Hansen *et al*., 2009). These groups are particularly susceptible to oxidation, leading to reversible or irreversible formation of a variety of sulphoxidation products. Many of the reversible reactions are integral to protein structure and cell signalling, and they may also provide temporary protection to key functional groups under conditions of oxidative stress (Schafer and Buettner, 2001). The irreversible formation of sulphinic ($R\text{-}SO_2H$) and sulphonic ($R\text{-}SO_3H$) acids are indicative of more severe oxidation (Hansen *et al*., 2009), and these changes can be detrimental to protein structure and function.

Fluorescent labelling of targeted functional groups of amino acid side chains provides a quantitative means of assessing oxidative damage to proteins. Iodoacetamidofluorescein (IAF) reacts with free –SH groups (but not with the oxidised variants) to form stable thioethers. These fluorescein-protein conjugates can be visualised as fluorescent bands/spots in electrophoretic separations (Ahn *et al*., 1987; Baty *et al*., 2002). This technique has proved to be a powerful indicator of oxidative stress in *Mytilus edulis* exposed to pro-oxidants (McDonagh and Sheehan, 2007; 2008), but studies in vent organisms are few (Fisher and Girguis, 2007; Mary *et al*., 2010; Company *et al*., 2011; 2012).

Bathymodiolin mussels inhabit hydrothermal vents in every ocean, and are therefore an ideal genus for enhancing our understanding of proteomic responses to the highly variable environmental stressors characteristic of vent habitats. This study uses redox proteomics to investigate the effect of tissue metal accumulation on protein expression and oxidation in a species of hydrothermal vent-living mussels, *Bathymodiolus* sp., sampled from newly-

discovered sites on the Southwest Indian Ridge (SWIR). Hydrothermal ecosystems hosted on the SWIR are of great importance to our understanding of vent-faunal biogeography, owing to the along-axis connections with the Atlantic and Pacific Oceans (German *et al*., 1998; Gamo *et al*., 2001; Gallant and Von Damm, 2006). Protein-based mechanisms of detoxification are relatively poorly understood in vent fauna, and may form an important piece in the puzzle of vent colonisation, and contribute to understanding the emergence of distinct faunal assemblages throughout the global mid-ocean ridge.

2. Materials and methods

2.1 Vent mussel sample collection and preparation

Hydrothermal vent mussels, *Bathymodiolus* sp. (6 – 9 cm), were sampled from two newly-discovered vent sites on the SWIR; Tiamat (37° 47.029' S, 49° 38.965' E, 2770m depth) and Knuckers Gaff (37° 47.030 S, 49° 38.967' E, 2785m depth) by ROV Kiel 6000 (GEOMAR) during the *RRS James Cook* cruise JC 067, in November 2011. Whole animals were flash-frozen in liquid nitrogen and stored at -80 °C. Onshore, animals were defrosted on ice and dissected for gill and digestive gland at University College Cork, where all proteomic work was conducted. Bacteria were not removed from dissected tissues. Due to the relatively limited number of animals available (n = 10), samples were not pooled but were homogenised individually in 10 mM Tris-HCl (pH 7.2), 0.5 M sucrose, 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF) using a motor-driven Teflon Potter-Elvejhem homogeniser, and centrifuged at 15,000 x *g* (60 min, 4 °C) to separate the soluble fraction from the pellet. Protein concentration in the supernatant phase was quantified in gill samples using the Bradford method (1976), using bovine serum albumin (BSA) as a calibration standard.

2.2 Analysis of metals in mussel tissues

A number of both essential (Fe, Mn, Cu, Zn) and toxic (Cd, Pb, Hg, As, Al) metals, known to be enriched in hydrothermal fluids relative to seawater, were analysed in gill and digestive gland tissues of *Bathymodiolus* sp. from the soluble and insoluble fractions generated through centrifugation as described in section 2.1. Solid pellets were freeze-dried, and corresponding supernatant fluids were heated to dryness at 130 °C. The dry-weight of the sample was then determined and an aliquot of ~100mg was dissolved in concentrated

105 thermally distilled (TD) HNO_3 by heating in a closed Savillex vial (15ml) on a hotplate at 60
106 °C for ~24 hours. The digested samples were then dried-down at 130 °C and re-dissolved in
107 3% TD HNO_3 spiked with Be (20 ppb), In (5 ppb) and Re (5 ppb) as internal standards.
108 Metal concentrations were determined by inductively coupled plasma mass spectrometry
109 (ICP-MS) (Thermo Scientific X-Series) at the National Oceanography Centre, Southampton.
110 External standards were prepared using 1000 μg ml^{-1} standard stock solutions (Inorganic
111 Ventures) in 3% TD HNO_3. The precision of the analytical procedure was confirmed through
112 digestion and analysis of certified reference material (CRM); lobster hepatopancreas TORT-1
113 (National Research Council of Canada), alongside the samples. The reproducibility of these
114 analyses was better than 8% for all metals, and measured values for the CRM were within
115 error of the certified values for all metals. The concentrations of metals in the Tris-HCl
116 buffer and HNO_3 were also determined and subtracted from the measured concentrations.
117 Metal concentrations are reported as the sum of the soluble and insoluble fractions, in μg g^{-1}
118 of the tissue dry tissue weight.

119 *2.3 Fluorescein labelling*

120 Protein thiols were labelled with 0.2 mM iodoacetamidofluorescein (IAF) from a 20
121 mM stock solution in dimethyl sulphoxide. Gill sample aliquots containing 25 μg protein
122 (1D PAGE) and 150 μg protein (2D PAGE) were incubated with IAF for two hours on ice in
123 the dark. Proteins were precipitated by incubating extracts in 10% (v/v) trichloroacetic acid
124 (TCA) for 5 min on ice, followed by centrifugation at 11,000 x *g* for 3 min. The resulting
125 pellet was washed in an excess of ice-cold acetone to remove TCA and any interfering salts
126 or non-protein contaminants. Protein extracts were re-suspended in 15 μl sample buffer for
127 1D PAGE (62.5 mM Tris-HCl (pH 6.8) containing 25% (v/v) glycerol, 2% (w/v) SDS, 5%
128 (v/v) β-mercaptoethanol and a trace amount of bromophenol blue) or 125 μl rehydration

buffer for 2D PAGE (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 4% (v/v) ampholyte (Pharmalyte 3-10), 1.2% (v/v) DeStreak reagent and a trace amount of bromophenol blue).

2.4 Polyacrylamide Gel Electrophoresis (PAGE)

2.4.1 1D PAGE

Gill samples (25 μg protein in 15 μl sample buffer) were heat-denatured and loaded alongside protein molecular mass markers (ThermoScientific, Dublin, Ireland) into wells embedded within a stacking gel of 4.5% (v/v) polyacrylamide in 0.5 M Tris-HCl, pH 6.8, set above a resolving gel of 14% (v/v) polyacrylamide in 1.5 M Tris-HCl, pH 8.8. Gel electrophoresis was carried out at 4 °C using an Atto AE-6450 mini PAGE system (BioRad; Hercules, CA, USA) at a constant voltage of 90 V until samples entered the resolving gel, then 120 V until the dye front reached the bottom of the gel. Fluorescently labelled bands were visualised using a Typhoon Trio+ Variable-Mode Imager (GE Healthcare, Little Chalfont, Bucks, UK) measuring excitation of Fluorescein at 532 nm and emission at 526 nm. Protein bands were visualised by colloidal coomassie-staining using the protocol of Dyballa and Metzger (2009).

2.4.2 2D PAGE

Gill samples (150 μg protein in 125 μl rehydration buffer) were loaded onto 7 cm non-linear immobilised pH gradient (IPG) strips (pH 3 – 10) and rehydrated for 18 hours in the dark at room temperature (Leung *et al*., 2011). Rehydrated IPG strips were focused on a Protean isoelectric focusing (IEF) cell (Bio-Rad) with linear voltage increases in the following sequence: 250 V for 15 min; 4,000 V for 2 hours; then up to 20,000 Vh. Prior to 2D PAGE, focused strips were incubated in equilibration buffer (6M urea, 0.375M tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol), first with 2% (w/v) dithiothreitol (DTT) to

ensure complete reduction of disulphide bridges and secondly with 2.5% (w/v) iodoacetamide (IAM) to reduce streaking. Equilibrated strips were loaded onto 14% SDS-polyacrylamide gels alongside a wick containing an unstained protein molecular mass marker, and sealed with agarose (0.5%) containing a trace amount of bromophenol blue. Gel electrophoresis was carried out as for 1D PAGE.

2.5 Image Analysis

Coomassie-stained gels were scanned with a calibrated imaging densitometer (GS-800; Bio-Rad). Background subtraction and optical density quantification of protein bands in 1D PAGE gels was performed using Quantity One image analysis software (Bio-Rad). For each gel lane, intensity of fluorescence (counts) was normalised against protein content (optical density) to correct for differences in sample loading and enable the extent of thiol oxidation to be compared between samples. Progenesis SameSpots image analysis software (Version 4.5; Nonlinear Dynamics, Durham, NC, USA) was used to align gels, match spots, and quantify spot volumes in 2D PAGE gel images of coomassie-stained, and thiol-labelled protein separations. Spots with a significant change in expression intensity (determined by a fold change of > 1.5; $p < 0.05$; student's t-test) between mussels from Tiamat and Knuckers Gaff were selected for protein identification.

2.6 Protein digestion and identification

Proteins were manually picked from 2D PAGE separations, lightly stained with colloidal coomassie. Following in-gel tryptic digestion, extracted peptides were loaded onto a R2 micro-column (RP-C18 equivalent) where they were desalted, concentrated and eluted directly onto a MALDI plate using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix solution in 50 % (v/v) acetonitrile and 5% (v/v) formic acid. Mass spectra of the peptides

were acquired in positive reflectron MS and MS/MS modes using a MALDI-TOF/TOF MS instrument (4800*plus* MALDI TOF/TOF analyzer) with exclusion list of the trypsin autolysis peaks (842.51, 1045.56, 2211.11 and 2225.12). The collected MS and MS/MS spectra were analysed in combined mode by Mascot search engine (version 2.2; Matrix Science, Boston, MA) and the NCBI database restricted to 50 ppm peptide mass tolerance for the parent ions, an error of 0.3 Da for the fragments, one missed cleavage in peptide masses, and carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. No taxonomy restrictions were applied as the genome has not been fully sequenced for species within the *Bathymodiolus* genus. The identified proteins were only considered if a MASCOT score above 95% confidence was obtained ($p < 0.05$) and at least one peptide was identified with a score above 95% confidence ($p < 0.05$). This analysis was conducted by the Analytical Services Unit, Instituto de Tecnologia Química e Biológica (ITQB), New University of Lisbon, Lisbon, Portugal.

2.7 GST assay

Glutathione transferase (GST) activity was quantified in gill tissues ($n = 8$) from sample aliquots containing 15 μg of protein diluted to a volume of 50 μl. Samples were loaded into a 96-well microtitre plate with 100 μl of 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) (from a 40 mM stock in ethanol) in 0.15 M potassium phosphate buffer (pH 6.5). GST activity was measured spectrophotometrically by adding 50 μl of 20 mM reduced glutathione (GSH) and measuring absorbance at 340 nm immediately and every 15 seconds for 5 minutes.

GST activity was calculated from the following equation (Habig *et al.*, 1974):

$$\text{GST activity } (\mu\text{mol/min/mg}) = (\Delta A_{340}\ V) / (\varepsilon\ l\ M) \qquad (1)$$

198 Where, ΔA_{340} represents the blank-subtracted initial rate of reaction between CDNB
199 and GSH (min^{-1}); V is the volume of reaction (0.2 ml); ε is the extinction coefficient of the
200 reaction product at 340 nm (9.6×10^{-3} μM^{-1} cm^{-1}); *l* is the path length (0.524 cm); and M is
201 the mass of protein (15 μg).

202 *2.8 Statistical Analyses*

203 The distribution of the tissue metal concentrations was significantly different from a
204 normal distribution, so a non-parametric statistical test was required to analyse the variation
205 between groups. Kruskal-Wallis multiple comparison tests (K-W) were applied together with
206 Dunn's post-hoc analysis to quantify the significance of the variation in metal content
207 between gill and digestive gland tissues, both within each vent site and between the two sites.
208 Correlation analyses were conducted to evaluate any relationship between metal
209 concentration and IAF fluorescence counts at each of the two sites. Significant differences in
210 the means of global fluorescence intensity (1D), GST activity, and of coomassie-stained and
211 IAF-labelled spot volumes (2D), in mussel gills between the two vent sites were analysed
212 using the student's t-test after testing for normality in the data. In all cases, significant
213 relationships are reported at the 95% confidence level where $p < 0.05$.

3. Results

3.1 Tissue metal concentrations

Tissue concentrations for essential (Mn, Fe, Cu, Zn) and toxic metals (Al, As, Cd, Hg, Pb) in gill and digestive gland of the hydrothermal vent-living mussel, *Bathymodiolus* sp., for both vent sites are shown in Figure 1. Where significant differences between tissues were observed, gill was found to have higher concentrations of each metal than digestive gland, with the exception of Fe. At the Tiamat vent site, Mn, Zn, Cd, Hg and Pb were all significantly enriched in gill compared with digestive gland at the 95% confidence interval (K-W, $p < 0.05$), whilst Cu was also enriched in gill and Fe enriched in digestive gland at the 90% confidence interval (K-W, $p < 0.1$). At the Knuckers Gaff vent site, gill tissues were significantly enriched in Cu, Zn, Cd, Hg and Pb compared with digestive gland (K-W, $p < 0.05$). Between the two vent sites, metal concentrations in tissues were consistently higher in mussels sampled from the Knuckers Gaff site compared with those from Tiamat. Whilst large biological variation and small group size, inherent in deep-sea vent sampling, hinders statistical confirmation of these differences, Fe concentrations were found to be significantly higher in gill tissues of mussels from Knuckers Gaff compared with Tiamat (K-W, $p < 0.05$). Aluminium and arsenic showed no statistical variation between tissues or between sites (K-W, $p > 0.05$).

3.2 Global thiol oxidation

Fluorescence intensity (IAF) measured over 1D PAGE separations, normalised to protein content to correct for any minor differences in sample loading, provides an indication of the global extent of thiol oxidation (Baty *et al.*, 2002) in gill tissues of *Bathymodiolus* sp. (Figure 2). We observed greater fluorescence intensity in IAF-labelled gill samples at Knuckers Gaff (163,000 ± 60%) compared with Tiamat (86,400 ± 9%), however this

238 difference was not statistically significant (t-test, $p = 0.16$). Global comparison of the redox
239 modifications to the proteome between the two sites studied is impaired by the extent of
240 biological variation in mussels from Knuckers Gaff. Video footage of the sample collection
241 indicates that individual specimens were collected over a wider area at the Knuckers Gaff
242 vent site compared with Tiamat. Hydrothermal vent habitats are characterised by steep
243 chemical gradients as high-temperature fluids mix with cold seawater, and mussels will be
244 exposed to a spatially variable chemical composition. Greater biological variation in thiol
245 oxidation at Knuckers Gaff may therefore reflect greater chemical diversity within this group
246 compared with Tiamat where individuals were sampled from a more tightly constrained area.
247 In figure S1 (supplementary material) the relationship between global thiol oxidation and
248 tissue metal content in gills of individual animals is analysed for each site. Correlation
249 analyses between metal concentration and coomassie-normalised fluorescence counts (1D
250 PAGE) reveal a number of significant relationships. IAF counts decrease significantly with
251 increasing As at Tiamat ($R = -0.895$; $p < 0.05$) and Fe at Knuckers Gaff ($R = -0.895$; $p <$
252 0.05), but increase significantly with increasing Pb at Tiamat ($R = 0.957$; $p < 0.05$).

253 *3.3 Protein expression profiles: 2D PAGE*

254 Whilst the 1D PAGE approach discussed in Section 3.2 provides a global indication
255 of thiol oxidation status in tissues, it does not readily distinguish effects at the level of
256 individual proteins. The response of individual proteins to oxidative stress can be better
257 assessed using 2D PAGE. As metal concentration in this study was found to be higher in gill
258 than digestive gland, we focused on 2D PAGE analysis of IAF-labelled proteins in gill tissues
259 (Figure 3).

260 Excluding smears and gel defects, a total of 205 well-resolved spots were matched in
261 coomassie-stained protein separations between the two groups. A significant difference in

signal level between the two sites was measured in 30 protein spots ($p < 0.05$), of which 10 were highly significant ($p < 0.01$). Of these, 23 spots were elevated at Tiamat and 7 were elevated at Knuckers Gaff (Figure 4). Successful protein identifications are presented in Table 1. In fluorescent scans of the same gels, a total of 143 IAF-labelled spots were matched in gill, and 15 of these showed a significant difference in spot volume between the two vent sites ($p < 0.05$). At Tiamat, 10 spots showed a reduction in fluorescence with IAF in comparison with Knuckers Gaff, whilst 5 spots showed an increase (Figure 4). These changes in fluorescence intensity occurred independently of any significant change in protein expression, suggesting redox modification to proteins present in all 15 spots.

3.4 GST activity

GST activity was found to be higher in gills from *Bathymodiolus* sp. at Tiamat (0.085 ± 0.03) compared to Knuckers Gaff (0.043 ± 0.03), though statistical comparison of the means of each group revealed that this difference was only significant at the 90% confidence level (t-test; $p = 0.094$).

4. Discussion

Bathymodiolin mussels are amongst the dominant fauna occupying hydrothermal vent ecosystems throughout the world's oceans (Fisher *et al*., 1988; Desbruyères *et al*., 2000; Cuvelier *et al*., 2009; Miyazaki *et al*., 2010). In these deep-sea vent environments, environmental stressors are manifold and extreme exposure to heavy metals may have necessitated the adaptation of specialised mechanisms of detoxification. In particular, metals trigger the production of ROS, which can disturb the cellular redox balance and lead to oxidative stress (Sheehan, 2006; Hansen *et al*., 2009). A ubiquitous cellular strategy for detoxification involves the binding of metals to specific low molecular mass, thiol-containing proteins known as metallothioneins (MT) (Viarengo and Nott, 1993), and MT expression has been well-studied in *Bathymodiolus* spp. (Geret *et al*., 1998; Company *et al*., 2006; 2010; Hardivillier *et al*., 2006; Martins *et al*., 2011). Oxidative stress also stimulates redox modifications to proteins involved in detoxification, and can detrimentally influence protein structure and function (Berlett and Stadtman, 1997). Whilst redox proteomics has frequently been employed in ecotoxicological studies with shallow-water animals (e.g. Manduzio *et al*., 2005; McDonagh *et al*., 2006; McDonagh and Sheehan, 2007; 2008; Chora *et al*., 2008; Tedesco *et al*., 2010), studies in vent fauna are comparatively rare (Company *et al*., 2011; 2012).

Mussels from Knuckers Gaff appear to have a higher metal load in their tissues, but a larger sample group would be needed to test whether this is significant, representing a considerable challenge for deep-sea, remote sampling. Statistical tests performed here indicate that tissue metal concentrations are generally similar between the two groups, with the exception of iron in gill. Higher concentrations of Fe in gill at Knuckers Gaff could indicate greater bioavailability of this element, potentially through a higher hydrothermal flux, or may reflect a slower rate of removal. Two-dimensional PAGE separations of gill

proteins revealed significant changes in intensity of 30 spots between the two vent sites, of which 23 were more intense at Tiamat and 7 were more intense at Knuckers Gaff. Whilst it is difficult to elucidate whether these changes indicate suppression of protein expression in response to greater xenobiotic stress at one site, or up-regulation of proteins involved in antioxidant defence at the other, it is clear that the proteome of *Bathymodiolus* sp. is highly sensitive to changes in chemical environment, as previously observed with *B. azoricus* on the Mid-Atlantic Ridge (Company *et al*., 2011). Significant differences in the thiol subproteome were also observed between the two groups, with 10 spots showing reduced intensity at Tiamat, and 5 showing greater intensity. Reduced IAF-associated fluorescence may reflect greater thiol oxidation, but may also indicate lower abundance of thiol-containing proteins. In this study, changes in spot intensity with IAF occurred independently of differences in intensity with coomassie, therefore it may be that the thiol subproteome of Tiamat mussels is more sensitive to oxidant attack than at Knuckers Gaff.

Exposure to metal-induced ROS has previously been shown to trigger up-regulation of numerous antioxidant enzymes in hydrothermal vent fauna (Company *et al*., 2004; 2006; 2010; Marie *et al*., 2006; Gonzalez-Rey *et al*., 2007). However, few studies have applied a redox proteomic approach to screen for changes in expression and oxidative transformations of individual proteins involved in key biological structures and processes (Boutet *et al*., 2009; Mary *et al*., 2010; Company *et al*., 2011; 2012). Identifying individual proteins in deep-sea vent fauna is challenging owing to the relative paucity in their genome information. Nevertheless, we report enhanced expression of S-adenosylhomocysteine hydrolase (SAHH), alpha enolase, glutamine synthetase type I, actin, and fumarylacetoacetate hydrolase (FAH) in gill tissues of *Bathymodiolus* sp. sampled at Tiamat compared to those sampled at Knuckers Gaff. These proteins occupy diverse roles in metabolism, cell structure, stress

response and redox homeostasis and may be variably regulated in response to conditions of oxidative stress.

SAHH is a cytosolic enzyme with an important antioxidant role owing to its involvement in regulating the synthesis of GSH via metabolism and regeneration of cysteine and methionine (Kloor *et al*., 2000; Martinov *et al*., 2010; Liao *et al*., 2012), and in regulating biological transmethylation (Turner *et al*., 2000). Enhanced expression of this enzyme has previously been linked to oxidative stress caused by metal exposure (Bagnyukova *et al*., 2007), and has been identified as a stress response in hydrothermal vent mussels, *B. azoricus* from the Mid-Atlantic Ridge (Company *et al*., 2011), and *B. thermophilus* from the East Pacific Rise (Boutet *et al*., 2009). Alpha enolase, a cytosolic enzyme, is both abundant and highly conserved in eukaryotic and prokaryotic organisms owing to its critical role in carbohydrate catabolism via the glycolytic pathway (Pancholi, 2001). Alpha enolase has also been found to protect cells from oxidative and thermal stress, functioning as a hypoxic stress protein (Aaronson *et al*., 1995) and a heat shock protein (Iida and Yahara, 1985), and can be considered as a marker of pathological stress with multiple stress response roles (Díaz-Ramos *et al*., 2012). Glutamine synthetase type I is exclusive to prokaryotes and must therefore derive from the bacterial symbionts hosted in gill tissue. Elevated expression of this bacterial protein may indicate a greater population of gill endosymbionts at Tiamat in response to a greater exposure to reduced substrates. Actin is an abundant cytoskeletal protein in eukaryotic cells, polymerising to form a network of microfilaments with numerous functions including cell motility, cell division, cell signalling and protein synthesis (Pollard and Cooper, 1986). Actin is highly sensitive to oxidant attack (Dalle-Donne *et al*., 2001) and has been shown in many studies to be a target of oxidative stress in bivalves inhabiting both shallow-water (Rodríguez-Ortega *et al*., 2003; Manduzio *et al*., 2005; McDonagh *et al*., 2005; McDonagh and Sheehan, 2007; 2008; Chora *et al*., 2009) and hydrothermal vent

environments (Company *et al*., 2011). Under moderate conditions of oxidative stress, the formation of disulphide bonds between cysteine sulphydryl groups in actin and those of GSH, prevents excessive intra-molecular polymerisation and enables microfilament preservation (Dalle-Donne *et al*., 2001). Thus, actin not only responds to ROS-induced stress but may be actively involved in buffering potential damage to cells. FAH is one of just ten enzymes known to have the capacity to hydrolyse carbon-carbon bonds in aromatic amino acids (Timm *et al*., 1999). It is involved in the catabolism of tyrosine and phenylalanine, catalysing the cleavage of fumerylacetoacetate in the final step of this essential metabolic pathway (Bateman *et al*., 2001). This enzyme is more abundant in mussels sampled from Tiamat compared with Knuckers Gaff, perhaps reflecting greater need for efficient break down of tyrosine metabolites which can further contribute to oxidative stress in cells (Fisher *et al*., 2008).

GST activity is also enhanced in gills of mussels from Tiamat compared with Knuckers Gaff, though this difference is only significant at the 90% confidence interval ($p < 0.1$). GST is a key enzyme involved in phase II detoxification, which catalyses conjugation of GSH to electrophilic centres on a range of xenobiotic substrates, facilitating their dissolution and subsequent excretion from the organism (Strange *et al*., 2001). Elevated expression of GST, and of proteins involved in diverse cellular processes (discussed above), suggests that mussels at Tiamat may have a greater battery of defences against xenobiotic substrates.

A recent study has shown that hydrothermal vent mussels from the Mid-Atlantic Ridge, *B. azoricus*, differ in their systems of antioxidant defence depending on the specific environmental conditions to which they are exposed (Company *et al*., 2012). *Bathymodiolus* sp. sampled from the SWIR in this study demonstrate significant proteomic variability between two vent sites. We measure enhanced expression of a number of proteins involved

in redox homeostasis at Tiamat, suggesting that chemical stress may in fact be greater at this site than at Knuckers Gaff. We also observe an increase in oxidative modifications to the thiol subproteome in mussels from Tiamat compared with Knuckers Gaff, further indicating a higher metal environment. Elevated exposure to metals is known to trigger the up-regulation of metal-binding proteins, facilitating excretion (Langston *et al*., 1998). It is possible that mussels from Tiamat experience greater exposure to metals, but maintain redox homeostasis through enhanced induction of GST and other antioxidant enzymes. Consequently, metal concentrations in tissues are regulated at a similar level to those in mussels at the Knuckers Gaff site.

Gill tissues of mussels from both Tiamat and Knuckers Gaff were found to contain higher concentrations of many of the metals analysed compared with digestive gland. This suggests greater exposure of gill to bioavailable metals in seawater and supports a primary feeding mechanism of chemoautotrophy via endosymbiotic bacteria (Fiala-Médioni *et al*., 2002; Duperron *et al*., 2006; Riou *et al*., 2008). Mussels in this study were sampled from active chimney structures where methane and sulphide are likely to have been in plentiful supply. Whilst Bathymodiolin mussels have a mixotrophic diet in which their energy requirements can be maintained both by symbionts in their gills and by suspension feeding on particulate organic matter (Le Pennec *et al*., 1990), filter feeding may be negligible as mussels increase in size and proximity to the vent (Martins *et al*., 2008; De Brusserolles *et al*., 2009). Gill tissue represents the direct interface between environmental metals and cellular physiology, and studies with *B. azoricus* collected from Mid-Atlantic Ridge vent sites have also shown a greater metal burden in gill compared with digestive gland (Geret *et al*., 1998; Kadar *et al*., 2005; Cosson *et al*., 2008). Whilst accumulation in digestive gland is considerably greater in vent mussels compared with non-vent fauna, reflecting long-term metal exposure (Cosson *et al*., 2008; Chora *et al*., 2009; Martins *et al*., 2011), higher MT

concentrations in this organ compared with gill enable greater metal regulation (Langston *et al*., 1998).

5. Conclusions

This is the first study to incorporate a redox proteomics approach to investigate stress tolerance in hydrothermal vent mussels (*Bathymodiolus* sp.) collected from the Southwest Indian Ridge. Although, as yet, no environmental or fluid chemistry data are available for these sites, there is significant variability in the proteome between Tiamat and Knuckers Gaff, including the expression of proteins involved in a range of metabolic and detoxification processes, which is likely to reflect variability in response to environmental stressors. Gills were found to be significantly enriched relative to digestive gland in Mn, Zn, Cd, Hg and Pb at Tiamat, and Cu, Zn, Cd, Hg and Pb at Knuckers Gaff, indicating enhanced exposure of this tissue to bioavailable metals, and may indicate a greater reliance on the gill for nutrition via chemoautotrophic endosymbionts at both sites. At Knuckers Gaff, biological variation was very high in all analyses of metal content, protein expression and redox changes to the proteome. Mussels sampled at this site cover a wider geographic area, and consequently they are likely to have experienced more variation in their exposure to toxic compounds. This study highlights the variable proteomic response of *Bathymodiolus* sp. to a rapidly fluctuating and highly ephemeral chemical environment, and demonstrates the sensitivity of the redox proteomic approach to evaluating stress response between vent habitats within the same hydrothermal system. We identify five proteins as potential markers of oxidative stress in *Bathymodiolus* sp., and demonstrate the importance of proteomic plasticity in adaptation to the hydrothermal vent habitat.

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7. Author Contributions

C. C., R. H. J., D. C., and D. S. jointly conceived this study and designed the analytical approach. The proteomic analyses were performed by C. C. and A. V. C., and the metal analyses by C. C. All authors contributed to the writing of the manuscript.

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Table 1. Identifications of proteins significantly up-regulated in gill of *Bathymodiolus* sp. from Tiamat compared with Knuckers Gaff

Spot #	Identified Protein	Mw (Da)	Fold change	ANOVA *p*	GI Number	Mascot Score	Matched peptides	Sequence Coverage (%)	Function
479	**S-adenosylhomocysteine hydrolase**	47498	2.8	*0.016*	253769244	392	3	22	Cytosolic enzyme involved in cysteine synthesis and consequently glutathione-based redox homeostasis
461	**Alpha enolase**	40415	2.2	*0.007*	4416381	122	1	21	Enzyme involved in glycolysis, growth control, hypoxia tolerance, heat shock allergic responses
372	**Glutamine synthetase type I**	51968	2.0	*0.03*	345876672	205	2	24	Enzyme specific to prokaryotes, likely from bacterial endosymbionts in mussel gill
773	**Actin**	28947	1.8	*0.01*	8895877	566	3	53	Cytoskeleton maintenance; muscle contraction; cell motility; cell signalling; ROS target
478	**Fumarylacetoacetate hydrolase**	45979	1.6	*0.045*	157131060	158	2	18	Hydrolase enzyme involved in metabolism of aromatic amino acids

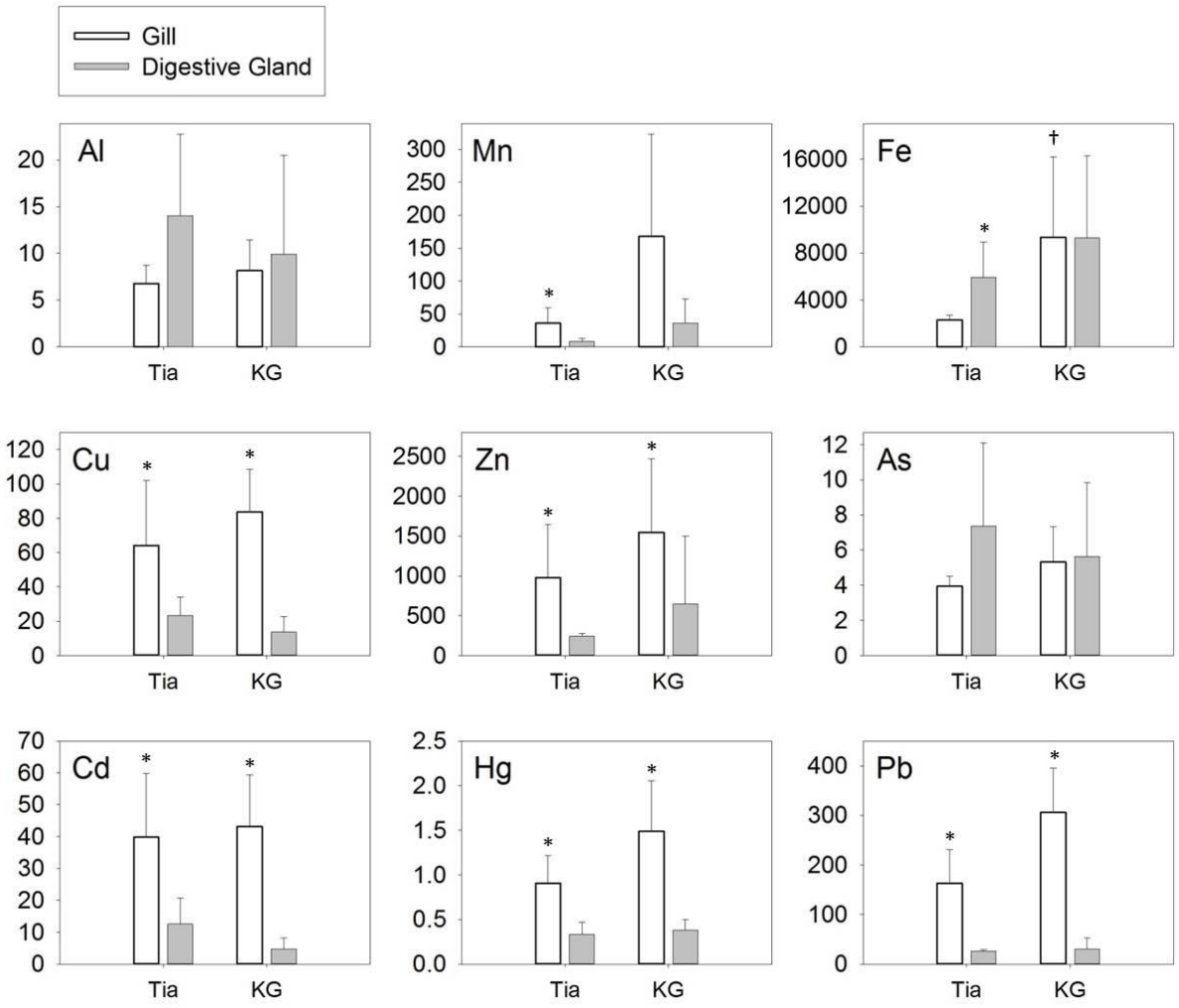

Figure 1. Mean concentration (μg g^{-1} dry weight; n = 5) of essential (Mn, Fe, Cu, Zn) and toxic (Al, As, Cd, Hg, Pb) metals in gill (white bars) and digestive gland (grey bars) tissues of *Bathymodiolus* sp. from SW Indian Ridge hydrothermal vent sites; Tiamat (Tia) and Knuckers Gaff (KG). Error bars indicate the standard deviation on the mean. Significant differences between gill and digestive gland are indicated by *, and between sites by †.

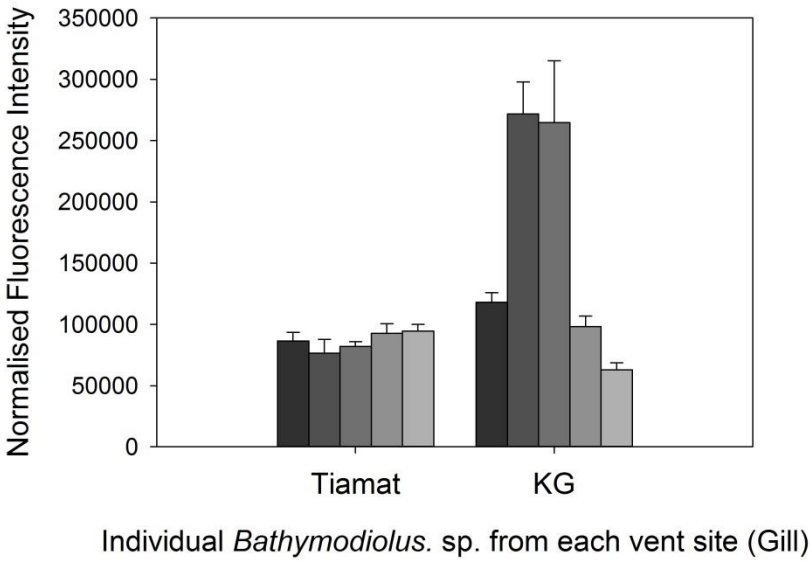

Figure 2. Intensity of fluorescence measured in IAF-labelled gill tissues of *Bathymodiolus* sp. individuals collected from Tiamat (n = 5) and Knuckers Gaff (n = 5) vent sites. Count values are normalised to protein content, as measured by optical density in coomassie-stained gels. Error bars indicate the standard deviation from the mean measured over four technical replicates.

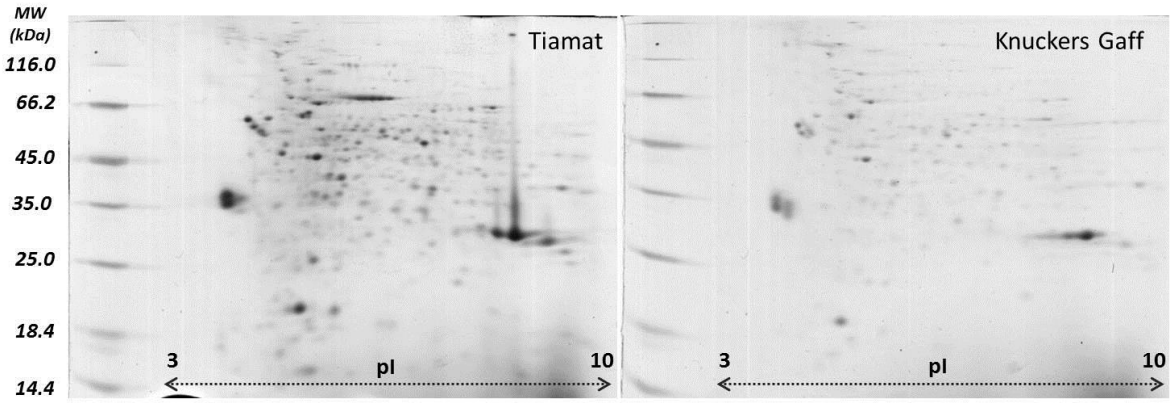

Figure 3. Representative images of electrophoretically separated, coomassie-stained protein spots for *Bathymodiolus* sp. gill tissues sampled from Tiamat and KG vent sites. A protein molecular mass marker ranging from 14.4 kDa (bottom) to > 116.0 kDa (top) is shown for size reference. Isoelectric point (pI) is indicated along the pH range (3-10).

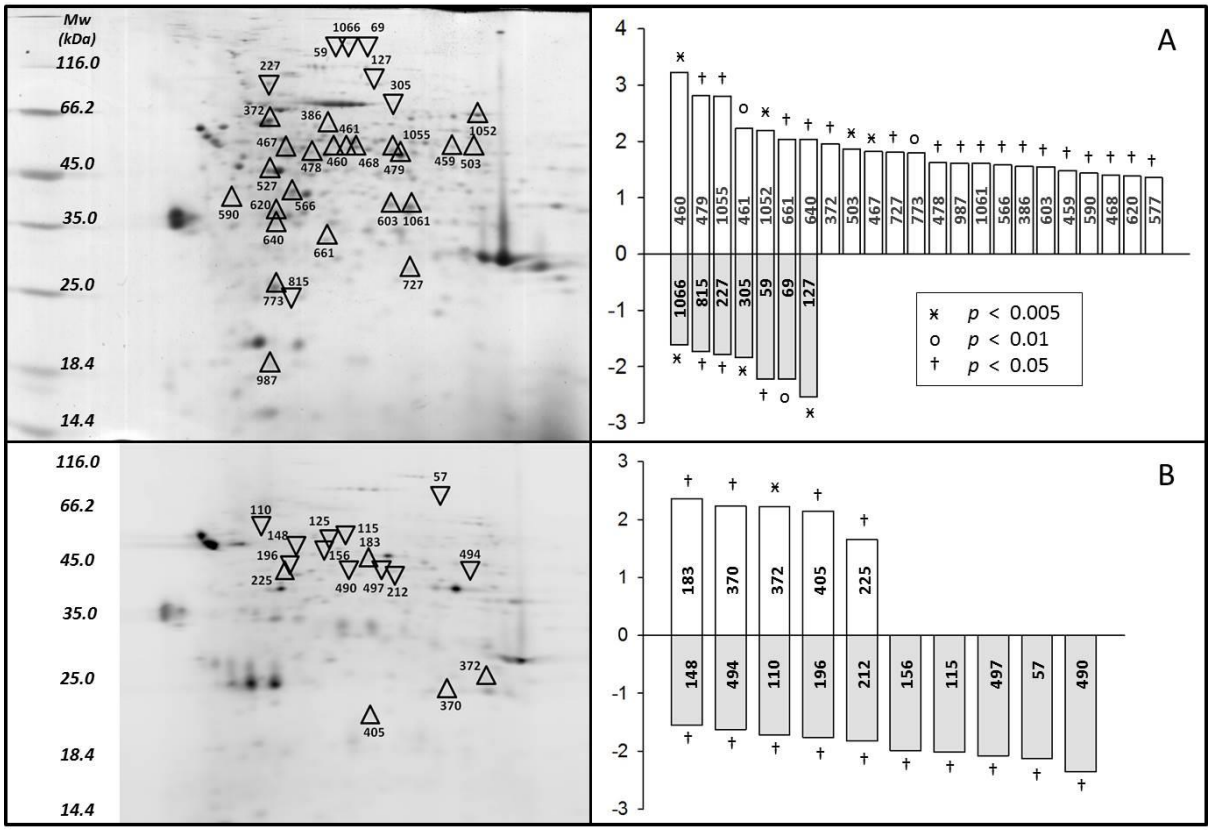

Figure 4. (**A:** Left) Coomassie-stained and (**B:** Left) IAF-labelled protein separations of a *Bathymodiolus* sp. gill tissue sample from the Tiamat site. Significant changes in spot intensity, compared with the Knuckers Gaff site, are indicated by upright triangles (greater at Tiamat) and inverted triangles (greater at Knuckers Gaff). A protein molecular mass marker ranging from 14.4 kDa (bottom) to > 116.0 kDa (top) is shown for size reference. (**A** and **B:** Right) Fold-differences in spot intensity at Tiamat, in comparison with Knuckers Gaff. Corresponding spot numbers are shown on each vertical bar, and the level of significance (t-test; *p* value) associated with each fold change is indicated by the symbols *, o, and †.

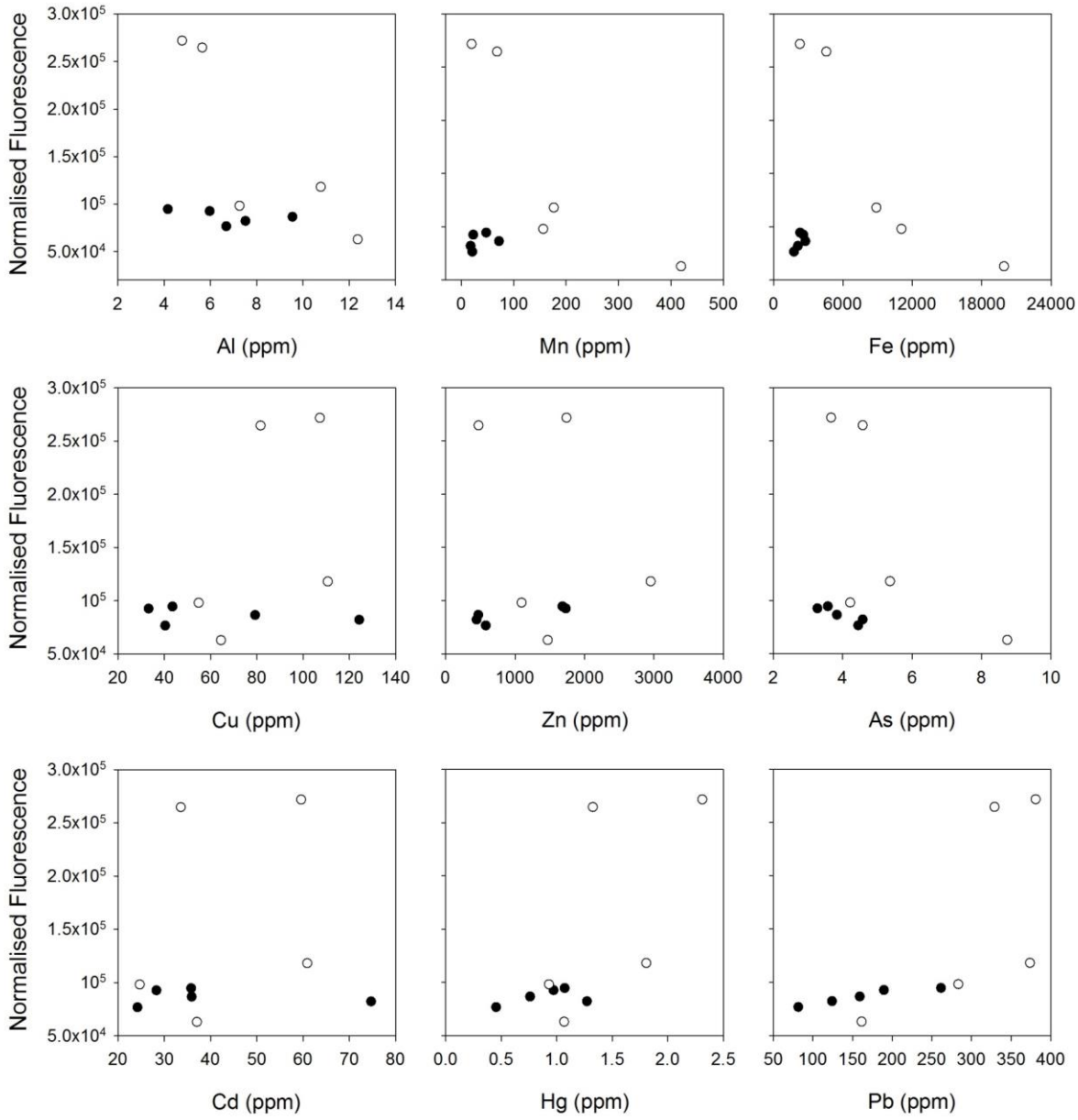

Figure S1. Coomassie-normalised fluorescence intensity for IAF-labelled proteins in relation to metal content in gill tissues of *Bathymodiolus* sp. individuals sampled from Tiamat (black circles) and Knuckers Gaff (white circles) hydrothermal vent sites.