Environmental Science & Technology

Article

pubs.acs.org/est

Global Proteome Profiling of a Marine Copepod and the Mitigating Effect of Ocean Acidification on Mercury Toxicity after Multigenerational Exposure

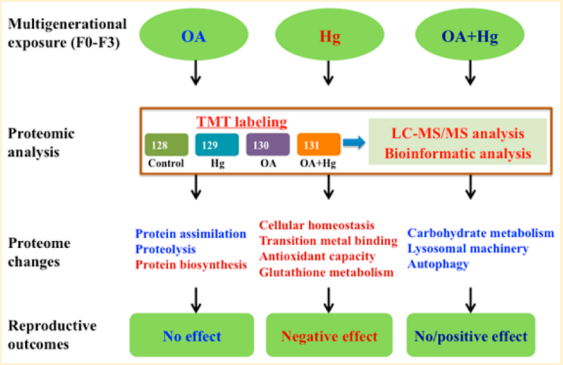
Minghua Wang,*[,†,‡] Jae-Seong Lee,[†,§] and Yan Li[†]

[†]Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems/College of the Environment & Ecology and [‡]State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, China

[§]Department of Biological Science, College of Science, Sungkyunkwan University, Suwon 16419, South Korea

Supporting Information

ABSTRACT: Previously, we found that ocean acidification (OA) mitigates mercury (Hg) toxicity to marine copepod *Tigriopus japonicus* under multigenerational exposure (four generations, F0−F3). To determine the response mechanisms of *T. japonicus* against long-term exposure to OA and Hg pollution, we investigated the proteome of F3 copepods after multigenerational exposure to four conditions: pCO_2 400 μatm + control; pCO_2 1000 μatm + control; pCO_2 400 μatm +1.0 μg/L Hg; and pCO_2 1000 μatm +1.0 μg/L Hg. Functional enrichment analysis indicated that OA enhanced the copepod's energy production mainly by increasing protein assimilation and proteolysis as a compensatory strategy, which explained its physiological resilience to reduced pH. Conversely, Hg treatment decreased many critical processes, including ferric iron binding, antioxidant activity, cellular homeostasis, and glutathione metabolism, and these toxic events could translate into higher-level responses, i.e., restrained reproduction in copepods. Importantly, the mediation of Hg toxicity in *T. japonicus* by OA could be explained by the enhanced lysosome-autophagy pathway proteomes that are responsible for repairing and removing damaged proteins and enzymes under stress. Overall, this study provided molecular insights into the response of *T. japonicus* to long-term exposure of OA and Hg, with a particular emphasis on the mitigating impact of the CO_2-driven acidification on Hg toxicity.



INTRODUCTION

Ocean acidification (OA), a continuous decrease in pH resulting from the absorption of increased anthropogenic CO_2, has become a major global threat to the fitness of marine ecosystems. Atmospheric pCO_2 has steadily increased from a preindustrial level (i.e., approximately 280 μatm) to a present-day concentration of ∼400 μatm.[1] Average ocean surface pH has been reduced by 0.1 units (a 26% increase in the hydrogen ion concentration) in comparison with levels during the industrial revolution,[2,3] and the atmospheric CO_2 level is projected to reach 1000 μatm by the end of 2100, leading to a decline of 0.3−0.5 units in seawater surface pH (7.6−7.9).[4] Also, some low-pH "hot spots" in coastal zones may already have experienced the pH values forecasted for the end of 2100 as a result of a multitude of drivers, e.g., upwelling of deeper acidified water along continental shelves[5] and high levels of heterotrophic respiration,[6,7] thereby subjecting the organisms in these zones to lower pH values than projected for the global sea surface. For instance, in the northwestern-northern nearshore areas of Bohai Sea, China, seawater pH values were 7.64−7.68 equal to that predicted for the end of 2100.[7] Elevated pCO_2 in seawater can cause hypercapnia and acidosis[8] and may subsequently result in redistribution of energy into growth and reproduction caused by the mobilization of energy-costly acid−base regulatory processes to counteract reduced pH. Accordingly, OA disturbs a multitude of physiological processes including calcification,[9] metabolism,[10] survival,[11] development,[12] and reproduction[13] in calcifying and noncalcifying organisms.

In addition to increasing global atmospheric CO_2 levels, human activities have also led to a mass of mercury (Hg) emission into the atmosphere, which is eventually deposited in marine environments especially coastal zones. Thus, OA and Hg pollution may co-occur in these environments. Indeed, Hg pollution is a severe problem for marine environments in China[14,15] because it contributes approximately 28% to the global Hg emission into the atmosphere. The maximum total Hg (T-Hg) has reached 2.7 μg/L in the seawater of Jinzhou Bay, Bohai Sea, about 3 orders of magnitude higher than the

Received: April 9, 2017
Revised: April 13, 2017
Accepted: April 17, 2017
Published: April 17, 2017

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

reference value.[15] The toxicity of Hg compounds is frequently caused by their high affinity for sulfur (i.e., the SH group), which subsequently induces efficient binding to cysteine residues in proteins and enzymes, causing their dysfunction (e.g., oxidative damage) and producing multiple toxicities in biota including human beings.[16−18] With some coastal zones already experiencing the low pH levels projected for the end of 2100,[5−7] marine organisms in coastal ecosystems are likely to have been co-exposed to multiple stressors, including OA and Hg pollution over many generations. Nonetheless, little information is available on the combined impacts of OA and Hg pollution on marine biota.

The harpacticoid copepod *Tigriopus japonicus* inhabits tide pools on rocky shores along the Western Pacific including Japan, South Korea, and China.[19] Given its ease of culture, rapid life cycle, and pedigree, this copepod is regarded as a good model species for marine stress ecology research, including OA-impacting assessments.[18−21] Therefore, using *T. japonicus* as a model organism, our previous work is the first to examine the effects of near-future OA (pCO_2 1000 μatm) and Hg exposure (a nominal concentration of 1.0 μg/L) on the development and reproduction of marine copepod under multigenerational life-cycle exposure (F0−F3).[22] We showed that OA obviously reduces Hg bioaccumulation in the copepods of each generation, and such decreased accumulation can account for the reduced inhibitory impact of Hg on the number of nauplii per clutch and total fecundity (Figures S1 and S2 and Table S1).[22] Thus, OA can potentially alleviate the negative effects of Hg on the reproductive performance of marine copepods, and this is in strong contrast to the synergistic interaction between OA and other metals (e.g., cadmium[23,24] and copper)[25,26] in marine animals. For example, OA strikingly increases copper toxicity to the copepod *Amphiascoides atopus*,[25] and the above observation is supported by the prediction that the toxic free copper ion concentration will be increased by as much as 115% in coastal seawaters by the end of 2100 as a result of reduced pH.[27] For Hg, OA does not influence its speciation in seawater because Hg can form strong complexes with chloride and also the concentration of which will not be affected by reduced seawater pH.[28] In our previous study, OA may modulate Hg toxicity to copepods by changing their physiological processes and reducing metal accumulation.[22] Nevertheless, the exact mode-of-action, particularly that of OA over Hg effects, is still not understood, although the molecular basis can be deciphered for marine animals as to how they regulate their tolerance limits under multistressors in marine environments.

Toxicoproteomics is a relatively new approach that applies global proteomic technologies to toxicology, and its purpose is to detect critical proteins/processes affected by environmental stressors.[29] In many previous studies, proteomic-based technologies are applied well to explore the molecular response mechanisms to environmental stressors, including OA and Hg pollution, in marine animals.[17,18,30−32] For example, in the copepod *T. japonicus*, tandem mass tag (TMT)-based quantitative proteomics, deciphering the molecular mechanism of Hg toxicity and tolerance under multigenerational exposure reveals that Hg toxicity inhibits many critical processes and pathways, such as protein translation, macromolecule metabolism, the cell cycle, and vitellogenesis.[18] However, the copepods can initiate several compensatory processes (e.g., increased carbohydrate metabolism and an enhanced stress-related defense pathway) to counteract metal toxicity.[18]

In our study, TMT-based proteome profiles were analyzed for the F3 copepods of *T. japonicus* after the exposure of four generations to OA (400 μatm, 1000 μatm) and Hg (control, 1.0 μg/L) pollution (alone or combined). Our objective was to address the questions "How does the copepod proteome respond to OA and Hg pollution (alone or combined) under a multigenerational exposure scenario?", "Which proteins or protein functional groups are involved in the response?", and, more importantly, "What is the molecular mechanism alleviating Hg toxicity caused by CO_2-driven acidification in this copepod?". This study should provide molecular insights into the individual marine animal's physiological response to multiple stressors in the future ocean.

MATERIALS AND METHODS

Copepod Maintenance. Copepods (*T. japonicus*) were collected from the rocky intertidal zone pools of Xiamen Bay, People's Republic of China (24°25.73′ N; 118°6.34′ E) in 2007. The copepods were raised at a temperature of 22 °C and a 12:12 h light/dark cycle in our laboratory. A total of three types of algae (*Isochrysis galbana*, *Platymonas subcordiformis*, and *Thalassiosira pseudonana*) were equally mixed at a density of 8 × 10^5 cells/L, and were supplied as food for the copepods. The seawater was obtained 20 km offshore in Xiamen Bay and was filtered through a 0.22 μm polycarbonate membrane. The reference value for T-Hg in seawater was measured as 3−4 ng/L. The seawater pH was 8.10, and other parameters are shown in Table S2.

Multigenerational Exposure. The copepods were exposed to different pCO_2 (400, 1000 μatm) and Hg (control and 1.0 μg/L) treatments for four generations (F0−F3). The copepods were subjected to one of four treatments: pCO_2 400 μatm plus control ("C" treatment); pCO_2 1000 μatm plus control ("O" treatment); pCO_2 400 μatm plus 1.0 μg/L Hg ("H" treatment); and pCO_2 1000 μatm plus 1.0 μg/L Hg ("OH" treatment). The pCO_2 levels of 400 and 1000 μatm were used to represent the present-day condition and the near-future value projected for the year 2100, respectively.[33] Different pCO_2 treatments were performed by continuously bubbling the ambient or CO_2-enriched air into the seawater using a CO_2 enrichment device (Ruihua, China). Correspondingly, the pH values were measured as approximately 8.1 and 7.7, respectively, for the present-day (400 μatm) and acidified (1000 μatm) seawater (Table S2). A 1.0 μg/L Hg concentration, which is environmentally relevant,[14,15] was chosen for the exposure, and this level was achieved by adding $HgCl_2$ (Sigma-Aldrich, 99.5%) to the seawater. To determine Hg levels in the seawater solutions during multigenerational exposure, we specifically performed a 2 day exposure for both Hg treatment and combined exposure, and the detailed procedure is available in Text S1. Also, seawater parameters such as salinity, pH, and total alkalinity were measured and adjusted as needed in each generation during multigenerational exposure (F0−F3). The detailed protocols are provided in Text S1.

To analyze the proteome profiles of the F3 adult copepods, a multigenerational test was conducted for *T. japonicus* subjected to the individual and combined stressors of OA and Hg for four generations (Figure 1). The detailed protocols are provided in Text S1. Briefly, 100 copepods were raised in polycarbonate bottles with 200 mL of working solution and were maintained under the above four treatments. Each treatment was repeated in four replicates. After the exposure, two replicates

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

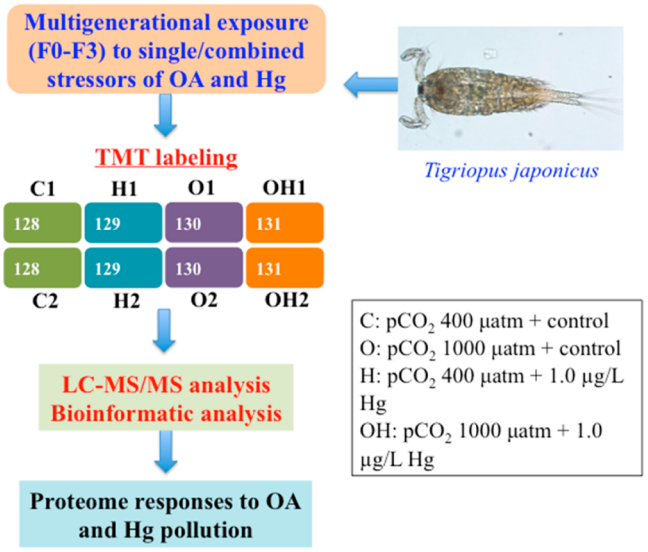


Figure 1. Experimental design and TMT workflow to investigate the impacts of individual and combined stressors of OA and Hg on the copepod proteome.

(approximately 200 copepods) were pooled for each treatment and immediately stored at −80 °C for proteomic analysis (two experimental replicates per treatment).

Proteomic Analysis. *Protein Labeling and Strong Cation Exchange Fractionation.* Proteins were extracted, quantified and labeled using the TMT-6plex Kit based on the manufacturer's instructions. Protein samples were labeled with the TMT tags as the followings (Figure 1): tag 128 was labeling one of two experimental replicates for the "C" treatment, 129 for the "H" treatment, 130 for the "O" treatment, and 131 for the "OH" treatment. The other replicates were treated in the same way. A pair of runs (with one set of experimental replicates per run) of liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis were performed, thus giving our study both biological and technical repeats. All four samples were pooled and dried using vacuum centrifugation. The peptide mixtures were fractionated using strong cation exchange chromatography. The detailed procedures are provided in Text S2.

LC−MS/MS Analysis and Database Searching. The peptides were analyzed using Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer coupled with an EASY-nLC 1000 UPLC system (ThermoFisher Scientific). The obtained peptide sequences were searched against the NCBI_Tigriopus proteome (1659 sequences) and *T. japonicus* transcriptome (i.e., a total of 46 369 sequences combined from a previous work[34] and our published data)[35] database. The detailed protocols are provided in Text S3.

Bioinformatic Analysis. The differentially expressed proteins (DEPs) were identified only if the normalized fold change was higher than 1.30 (up-regulated) or less than 0.77 (down-regulated), which was calculated as a 95% confidence level based on pair-wise analysis between two experimental replicates.[36] The DEPs were annotated into three categories based on Gene Ontology (GO) terms, i.e., biological process, cellular component and molecular function. Protein domain function was defined by InterProScan based on the protein sequence alignment method (http://www.ebi.ac.uk/interpro/). Protein pathway was annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Enrichment analysis was conducted for GO terms, protein domain and KEGG pathway using the Database for Annotation, Visualization and Integrated Discovery. The statistically significant enrichments were identified using the Fisher's exact test with a Benjamini-Hochberg's corrected p value being <0.05. Hierarchical clustering analysis was conducted for the DEPs based on the significant enrichments using Gene Cluster 3.0 software.

Biochemical Parameter Determination. To confirm the proteomic data, several biochemical parameters were specifically examined in the F3 copepods under four treatments. Namely, the enzymatic activities of superoxide dismutase [Cu−Zn], glutathione peroxidase, glutathione *S*-transferase, and xanthine oxidase were measured following the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The detailed protocols are available in Text S4.

RESULTS

Proteome Profiles. Analysis of the two LC−MS/MS runs produced 25 170 and 23 093 peptide spectra, which resulted in 3072 and 3120 proteins (Table 1). Subsequently, 3776 proteins were identified from the combined data, and an additional 2416 overlapped (Figure S3). OvsC represented the DEPs due to OA stress; HvsC for Hg treatment; OHvsC for the combined exposure of OA and Hg; and OHvsH for the impact of OA on Hg toxicity (Figure 2). The DEPs were quantified as 76, 70, 39, and 29, respectively, for OvsC, HvsC, OHvsC, and OHvsH, and they are listed in Tables S4−11. The reproducibility was analyzed for the two experimental replicates under each case, which was based on the ratios of the DEPs with regression coefficients greater than 0.95 (Figure S4). Meanwhile, the DEPs under each case were classified into several groups including metabolic process, cellular process, single-organism process, localization, biological regulation, response to stimulus, and other related functions (Figure 2).

Table 1. Summary for Quantitative Data in Protein Identity for the Two *T. japonicus* Proteome Sets via Mass Spectrometry Analysis

group name	total spectra	spectra	peptide	unique peptide	protein	FDR
Run1	221 465	25 170	13 810	11 761	3072	0.01
Run2	229 216	23 093	13 116	11 302	3120	0.01

Enrichment Analysis. To investigate the functional differences in up-regulated and down-regulated proteins, the quantified proteins were analyzed separately for GO terms, protein domain and KEGG pathway enrichment-based clustering analysis (Figures 3−5; the detailed information is also provided in Tables S12−15).

For the OA treatment, the up-regulated proteins were enriched in the biological process, molecular function, and KEGG pathway, where several important processes including serine-type peptidase activity (GO: 0008236), neuroactive ligand−receptor interaction (ko04080), and protein digestion and absorption (ko04974) were significantly increased. Also, the down-regulated proteins were mostly enriched in cellular component. Notably, the intracellular membrane-bound organelle (GO: 0043231) and mitochondrion (GO: 0005739) were markedly depressed by OA, suggesting that reduced pH could potentially target cellular organelles, especially the copepod's mitochondrion.

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

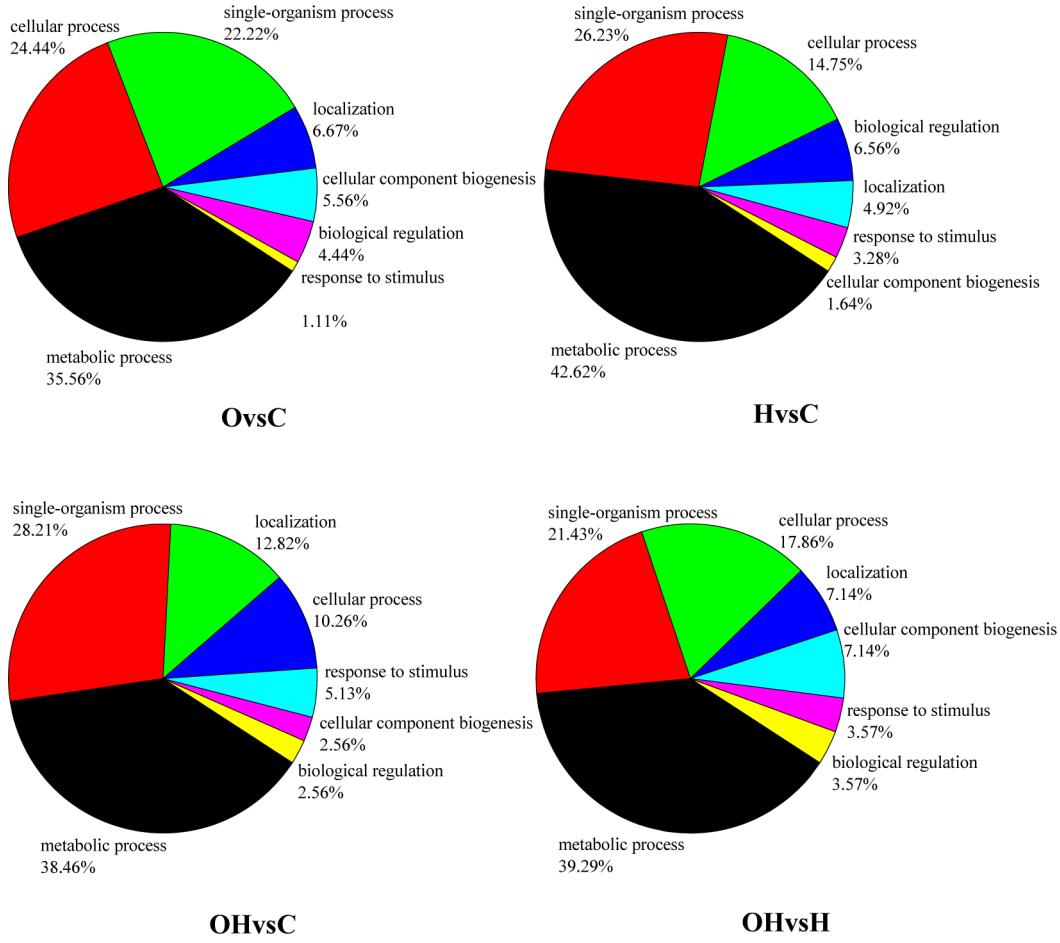


Figure 2. Functional classifications were performed for differentially expressed proteins (DEPs) in *T. japonicus* after four generations of exposure to OA and Hg pollution (alone or combined). OvsC represents the DEPs caused by OA stress, HvsC for Hg treatment, OHvsC for the combined effects, and OHvsH for the OA impact on Hg toxicity.

Hg exposure strikingly affected many crucial processes in copepods, highlighting the fact that this metal potentially exerted its toxicity to the cells on a large scale. Several processes, e.g., hydrolase activity, acting on glycosyl bonds (GO: 0016798) and carbohydrate metabolic process (GO: 0005975), were increased by Hg treatment. Interestingly, a KEGG pathway of neuroactive ligand−receptor interaction (ko04080) was up-regulated by Hg exposure. Despite the fact that these processes might be a defense mechanism against Hg toxicity, this metal ultimately caused toxicity to the copepod via a significant depression of transition metal ion binding (GO: 0046914), ferric iron binding (GO: 0008199), antioxidant activity (GO: 0016209), iron ion homeostasis (GO: 0055072), cellular homeostasis (GO: 0019725), response to oxidative stress (GO: 0006979), glutathione metabolism (ko00480), metabolism of xenobiotics by cytochrome P450 (ko00980), ferritin/DPS protein domain (IPR008331), and so on.

Also, many processes were strikingly impacted by combined exposure to OA and Hg. Nevertheless, more increased processes were observed for combined exposure in contrast to Hg treatment alone. For instance, hydrolase activity (GO: 0016787), carbohydrate metabolic process (GO: 0005975), starch and sucrose metabolism (ko: 00500), SNARE interactions in vesicular transport (ko04130), and lysosome (ko04142) were enhanced under combination treatment. As expected, combined exposure depressed some processes, e.g., mitochondrion (GO: 0005739), oxidoreductase activity (GO: 0016491), and antioxidant activity (GO: 0016209).

To explore the OA impact on Hg toxicity, the DEPs for combined exposure versus Hg treatment alone (i.e., OHvsH) were examined using functional enrichment analysis, indicating that several processes were significantly affected under this case. In particular, two important pathways, lysosome (ko04142) and phagosome (ko04145), were up-regulated, and thus, these two processes likely played a vital role in alleviating Hg toxicity in this copepod by reduced pH due to OA.

Biochemical Parameters. The proteomic data were confirmed using a biochemical assay for several enzymes in the copepods (Figures S5−8). Taking xanthine oxidase as an example, the expression and activity were increased by 1.53 and 1.27 times under Hg exposure, and similarly, 2.48 and 1.59 times for OA stress, 1.93 and 1.83 times for combined exposure, and 1.26 and 1.60 times for the combined exposure versus single Hg treatment (Figure S7). Moreover, a significant and positive linear relationship was observed between enzymatic activity and proteomic data for OvsC, HvsC, OHvsC, and OHvsH (R = 0.8039 and p = 0.000175; Figure S9).

DISCUSSION

Our study investigated the copepod proteomes under long-term multigenerational exposure to OA and Hg pollution,

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

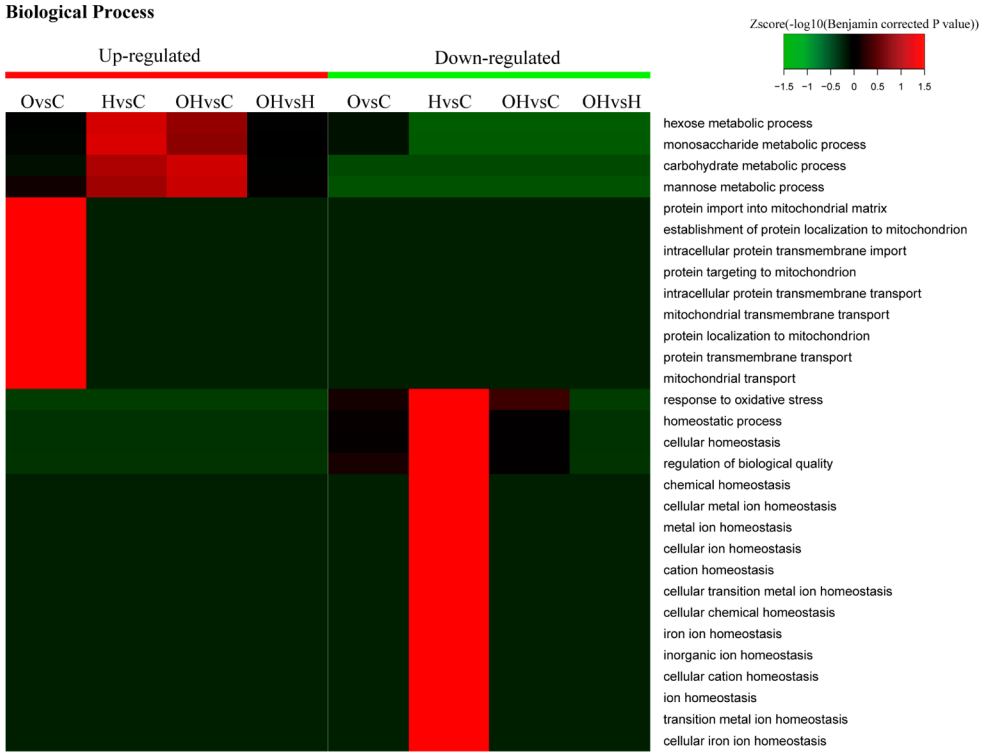


Figure 3. Hierarchical clustering analysis was conducted for the differentially expressed proteins (DEPs) in *T. japonicus* according to biological process based enrichment. The DEPs in response to each treatment were divided into two groups (i.e., up-regulated and down-regulated). OvsC indicates the DEPs caused by OA stress, HvsC for Hg treatment, OHvsC for the combined effects, and OHvsH for the OA impact on Hg toxicity. The *p* values were transformed into Z-scores for hierarchical clustering analysis. The Z-score is shown in the color legend, and the red color represents the significant enrichments.

indicating that the phenotypic plasticity of the proteome could, to some extent, be charging for the regulation of tolerance limits in response to environmental stressors. Although such proteomic plasticity has been widely applied to explore the molecular mechanisms of how a multitude of marine biota modulate environmental tolerance limits,[37] ours is the first comparative and comprehensive proteomic study of marine invertebrates' responses to environmental stressors under multigenerational exposure. The present study also combined the application of quantitative shotgun proteomics and bioinformatics tools to analyze the proteome of an ecologically important marine copepod. Particularly, we used data generated from TMT-LC-MS/MS analysis and identified over 3500 proteins in *T. japonicus*, thereby expanding the proteomics database for this copepod and its usefulness as a model species in marine stress ecology. It should be noted that some information on the copepod's proteome might still be missing, as no public reference genome is available for this species *T. japonicus*. Nevertheless, using TMT-based shotgun proteomics combined with the transcriptomic database, we were able to identify the functional significance of several critical proteins and processes based on GO terms, protein domain, and KEGG pathway enrichment-based analysis. Also, by coupling this comparative proteome profiling with physiological observations (e.g., development and reproduction) presented in our previous work (Figures S2 and Table S1),[22] we demonstrated that proteomic alterations in this copepod were dependent not only on stressor type but also on the interaction between stressors.

Copepod Proteome Alteration Ensuring High Resilience to OA. Our previous study shows that exposure to the projected near-future OA insignificantly affects the development and reproduction of *T. japonicus* under multigenerational exposure, suggesting its high resistance in response to decreased pH at the physiological level.[22] However, the copepod proteomes in this study were significantly altered by reduced pH, which indicated the proteomic plasticity in response to OA stress. In this work, elevated pCO_2 exposure inhibited some processes, e.g., cytoplasm (10 proteins), intracellular membrane-bounded organelle (8 proteins), and mitochondrion (4 proteins). These large-scale inhibitory impacts on the cytoplasm and organelles indicated that the copepods appeared to conserve more metabolic energy, at least in part, via decreasing the synthesis of proteins (in particular mitochondrial protein biosynthesis) to maintain pH homeostasis under elevated pCO_2, which was in line with the results of several proteomic studies concerning OA effects in marine animals.[32,38,39] More importantly, two critical processes, serine-type peptidase activity (four proteins) and protein digestion and absorption (four proteins), were enhanced by increased pCO_2 exposure. A total of four proteins enriched into serine-type peptidase activity were related to protein degradation (i.e., proteolysis). In the copepod, protein digestion and absorption involve a series of digestive enzymes (e.g., trypsin and carboxypeptidase B in our study) that break down ingested proteins into components that can then be easily absorbed and directed into cellular metabolism. Collectively, the increased protein assimilation and proteolysis may reflect an enhanced digestive process in the copepods, probably resulting from compensatory feeding because the food was provided ad libitum during exposure. The exposed animals may have used compensatory feeding to increase the total energetic input for reallocating the same amount of energy into development and

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

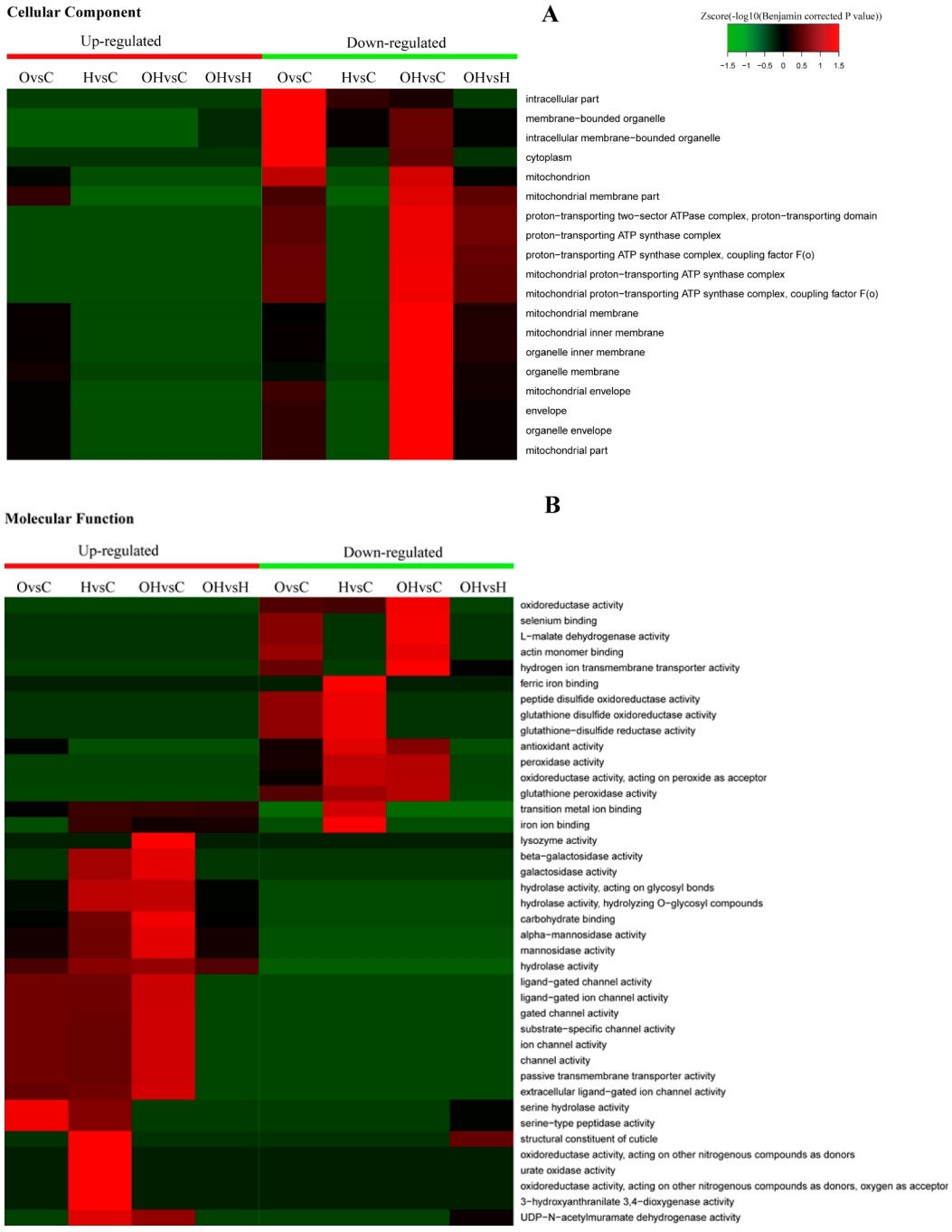


Figure 4. Hierarchical clustering analysis was conducted for the differentially expressed proteins (DEPs) in *T. japonicus* according to cellular component (A) and molecular function (B) based enrichment. In each category, the DEPs in response to each treatment were divided into two groups (i.e., up-regulated and down-regulated). OvsC indicates the DEPs caused by OA stress, HvsC for Hg treatment, OHvsC for the combined effects, and OHvsH for the OA impact on Hg toxicity. The *p* values were transformed into Z-scores for hierarchical clustering analysis. The Z-score is shown in the color legend, and the red color presents the significant enrichments.

reproduction, hence offsetting the negative impacts of reduced pH on the copepods, which has been reported for other copepods subjected to OA stress in several previous studies.[40,41] Taken together, our proteomic study demonstrated an altered but probably adaptive energy reallocation strategy, i.e., the increased energy input as well as a conserved energy utilization, employed by the copepods to display a physiological resilience to reduced pH.

The interesting thing is that the neuroactive ligand−receptor interaction pathway (four proteins) was up-regulated by OA stress. The proteins enriched in this pathway included ionotropic GABA-aminobutyric acid receptor BRL3−3b6a and neuronal acetylcholine receptor subunit α-3, highlighting that OA may have affected the copepod's behavioral performance via altered neurotransmitter function. Particularly, some earlier studies show that the GABA-aminobutyric acid receptor (GABA-A receptor, 1.72 fold increase in our study) is responsible for behavioral abnormalities in several marine animals under the projected near-future pCO_2 level; however, their behavior can be almost completely restored by treatment

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

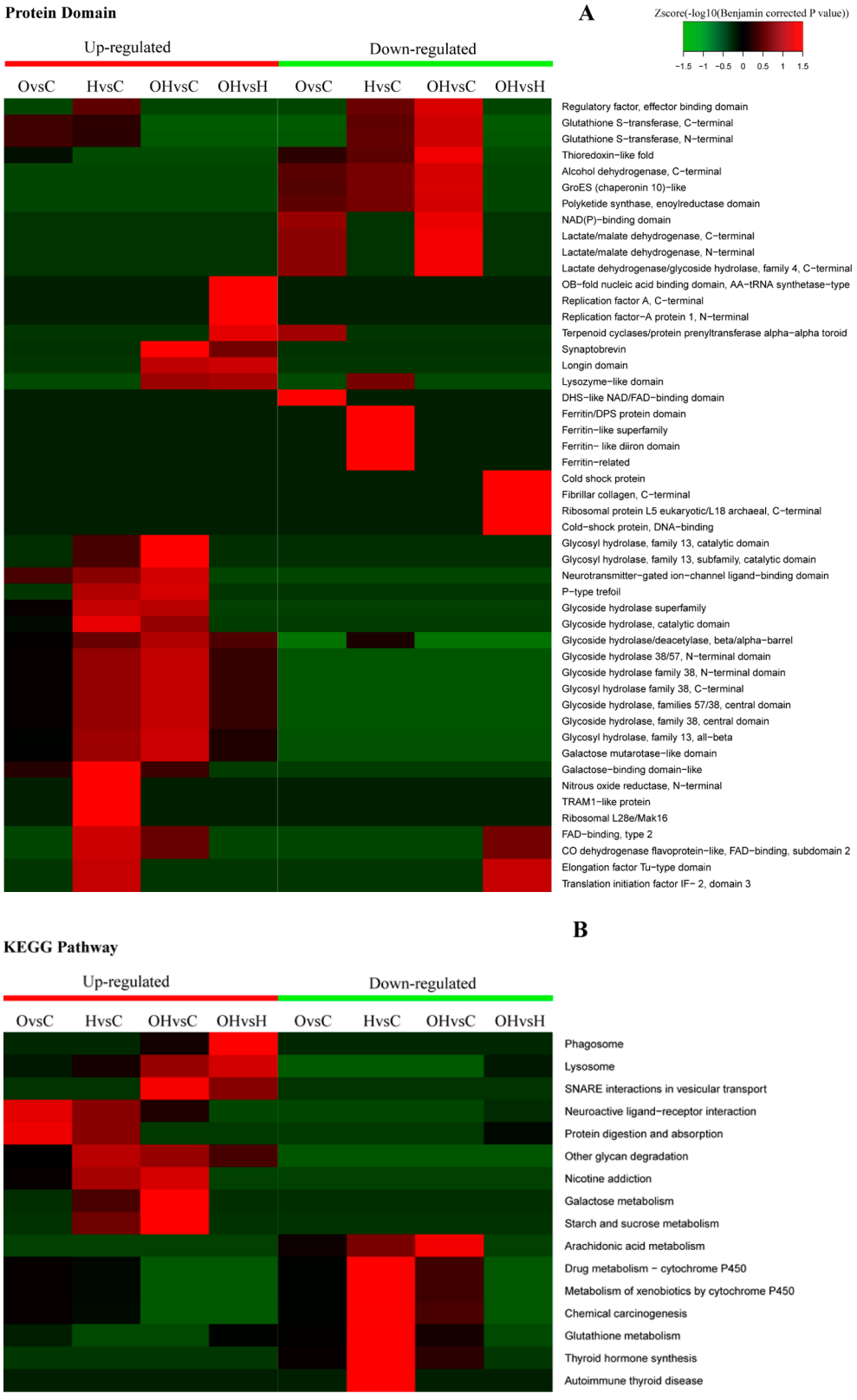


Figure 5. Hierarchical clustering analysis was conducted for the differentially expressed proteins (DEPs) in *T. japonicus* according to protein domain (A) and KEGG pathway (B) based enrichment. In each category, the DEPs in response to each treatment were divided into two groups (i.e., up-regulated and down-regulated). OvsC indicates the DEPs caused by OA stress, HvsC for Hg treatment, OHvsC for the combined effects, and OHvsH for the OA impact on Hg toxicity. The *p* values were transformed into Z-scores for hierarchical clustering analysis. The Z-score is shown in the color legend, and the red color presents the significant enrichments.

with a specific GABA-A receptor antagonist (gabazine).[42−44] The GABA-A receptor, the main inhibitory neurotransmitter receptor in the vertebrate brain,[45] has specific conductivity for Cl^- and HCO_3^-, both of which are likely to be affected by

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

OA.[42] Normally, an opening of the GABA-A receptor leads to Cl^- and HCO_3^- inflow, resulting in hyperpolarization and inhibitory neural activity. In response to elevated pCO_2, marine fishes facilitate their acid−base regulation mechanism to prevent acidosis by accumulating HCO_3^-, with concurrent reductions in Cl^- as a compensatory reaction.[46] These compensatory changes in Cl^- and HCO_3^- under increased pCO_2 conditions enable some GABA-A receptors to become depolarizing and excitatory, thereby leading to behavioral abnormalities in marine fishes.[42,43] The above hypothesis could be applied to marine copepods in response to reduced pH in this study because the GABA-A receptors are phylogenetically old in both primitive invertebrates and vertebrates, and, as inhibitory components, these receptors display a ubiquitous role in neural circuits as well as their formation.[47,48] Overall, our proteomic work is the first study to show that the projected near-future OA level could potentially cause behavioral changes in the copepod probably via disturbance of neuronal activity.

Proteome Response Explanation of Hg Toxicity in Copepods. Hg toxicity is normally attributable to its high binding affinity for the SH groups of endogenous biomolecules (e.g., proteins and enzymes), thereby disrupting cellular processes at the global scale.[18] High metal accumulation (i.e., 125.6 ng/g dry weight) is also observed in the F3 copepods of *T. japonicus* after multigenerational exposure to 1.0 μg/L Hg in our previous work.[22] Thus, one would expect this Hg treatment level to affect many important processes in the copepods in the present study. Several processes, particularly hydrolase activity (13 proteins), carbohydrate metabolism (8 proteins), and protein digestion and absorption (4 proteins), were increased by Hg treatment. Because most of the up-regulated enriched proteins were correlated with energy metabolism, the induced processes might function as a compensatory response to defend against metal toxicity. Nonetheless, Hg exposure ultimately caused its toxicity in the copepod, which was reflected by the dramatic depression of many crucial processes, including transition metal ion binding (five proteins), antioxidant activity (three proteins), iron ion homeostasis (three proteins), cellular homeostasis (four proteins), response to oxidative stress (four proteins), glutathione metabolism (four proteins), and metabolism of xenobiotics by cytochrome P450 (two proteins). Indeed, the global suppression of many critical processes after Hg exposure lends strong support to our previous study that Hg toxicity from multigenerational exposure leads to negative effects on development time and fecundity in the copepod *T. japonicus*.[20]

Our study showed that the processes of transition metal binding, iron ion homeostasis, cellular homeostasis, and such were inhibited by Hg treatment. A total of five proteins were enriched in the above processes, among which three proteins were common. These were the ferritin subunit, ferritin, and the ferritin light chain (oocyte isoform), suggesting that ferritin was likely an important target of Hg toxicity. Ferritin, produced by almost all living organisms, is a universal intracellular protein that stores and releases iron in a regulated fashion. Ferritin is reported to participate in iron metabolism[49] as well as other functions including, but not restricted to, immune defense[50] and stress response.[51] The other two proteins were leukotriene B4 12-hydroxydehydrogenase and metalloendopeptidase, both of which displayed zinc ion binding. Leukotriene B4 12-hydroxydehydrogenase is responsible for converting leukotriene B4 to the much less active metabolite 12-oxo-leukotriene B4, inhibiting the pro-inflammatory actions of leukotriene B4, which has been implicated in several allergic and inflammatory diseases.[52] Metalloendopeptidase is an enzyme that functions as a metalloproteinase endopeptidase, therefore taking part in protein degradation. In summary, our study indicated that Hg toxicity led to dysfunction in the copepod's homeostatic processes such as iron ion binding, metal ion homeostasis, and chemical homeostasis, among others, by disrupting several crucial proteins and enzymes via its substitution for functionally essential metals (e.g., iron and zinc).

Excessive Hg accumulation in the copepods could have resulted in a variety of oxidative effects related to the overproduction of reactive oxygen species, e.g., superoxide anion and hydrogen peroxide. Our proteomic analysis lends an evidence to the above assumption because the four proteins enriched in response to oxidative stress and glutathione metabolism were inhibited by Hg exposure: glutathione peroxidase, glutathione reductase, microsomal GST3 (Fragment), and glutathione *S*-transferase δ-ε 1. Meanwhile, glutathione peroxidase and glutathione reductase were enriched in antioxidant activity, and the other two proteins for metabolism of xenobiotics by cytochrome P450. Glutathione peroxidase is capable of converting lipid hydroperoxides (normally peroxided lipid yields) into their corresponding alcohols and of converting hydrogen peroxide into water. Glutathione *S*-transferase can detoxify damaged products (e.g., peroxidized lipids) and xenobiotics by conjugating them with the reduced glutathione (GSH). Glutathione reductase is responsible for reducing the oxidized glutathione into GSH, which is a crucial molecule in counteracting oxidative stress.[53] Therefore, a remarkable down-regulation of their expressions suggested that Hg exposure might cause depletion of endogenous sulfhydryl groups (e.g., GSH) and oxidative stress, consequently leading to oxidative damage in the exposed copepods.

It should be emphasized that the pathway of neuroactive ligand−receptor interaction (four proteins) was also enhanced by Hg exposure. The related proteins contained ionotropic GABA-aminobutyric acid receptor BRL3−3b6a and nicotinic acetylcholine receptor α-2 subunit. Accordingly, both single OA stress and Hg treatment remarkably enhanced this pathway with analogue proteins, indicating that these two stressors probably impacted the copepod's behavioral capability via a similar mechanism (that is, interference with neurotransmitter function).

Proteome Changes in OHvsC and OHvsH and Indication of Alleviation of Hg Toxicity in Copepods under CO_2-Driven Seawater Acidification. Our earlier work shows that the projected near-future OA level significantly reduces Hg accumulation in *T. japonicus* under multigenerational exposure, e.g., 104.5 versus 125.6 ng/g for F3 under combined exposure compared with Hg treatment.[22] Additionally, Hg content analysis in the seawater (Table S3) suggested that there was little difference for metal levels in the testing solutions between Hg treatment and combined exposure during multigenerational exposure. It should be noted that, for both the above treatments, the average measured Hg levels were less than the nominal metal concentration (1 μg/L) in the seawater (Table S3), and the decrease was caused by several factors including algal uptake and copepod accumulation during exposure. Collectively, it seems pretty sure that the near-future OA condition potentially mitigates Hg toxicity to *T. japonicus* under multigenerational exposure, and this was also supported by our proteomic study. In short, in contrast to Hg treatment

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

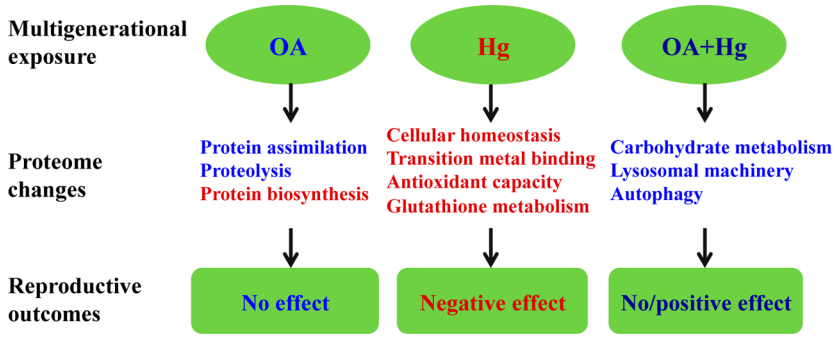


Figure 6. Schematic illustration showing the individual and combined impacts of OA and Hg pollution on the copepod proteome as well as their reproductive outcomes. The blue color represents the up-regulated processes under stress, and the red color represents the down-regulated processes.

alone (producing 70 regulated proteins), less proteomic response was observed for combined exposure (causing 39 regulated proteins). Meanwhile, more enriched processes (particularly biological process and KEGG pathway) were constructed by the up-regulated proteins under combination treatment. Specifically, carbohydrate metabolism (six proteins) was up-regulated by combined exposure, and the consequently increased energy metabolism might improve the ability of the exposed copepods to resist Hg attack. Additionally, the increased lysosome pathway would definitely facilitate the copepods under combined exposure to display a better fitness because this pathway could remove and repair the proteins and enzymes damaged due to stress. Nonetheless, with a striking Hg accumulation under combination treatment, it was not surprising that combined exposure did depress some processes such as mitochondrion (two proteins) and oxidoreductase activity (six proteins) in the copepods.

To provide a more-detailed molecular basis for the alleviative impact of OA on Hg toxicity, we further compared the proteomes between the combined exposure and single Hg treatment. The important finding was that two pathways, lysosome and phagosome, were enhanced, and they were probably responsible for OA impacting on Hg toxicity in the copepod. It should be underlined that the lysosome pathway was also increased by combined exposure. A total of three up-regulated proteins were enriched in the lysosome pathway: α-mannosidase, cathepsin i (Fragment), and palmitoyl-protein thioesterase 1 (Fragment). Meanwhile, cathepsin i (Fragment), putative tubulin α-1 chain, and vesicle-trafficking protein SEC22b-B were enriched in the enhanced phagosome pathway, suggesting that autophagy was induced in the copepods under combination exposure. α-Mannosidase can hydrolyze the α form of mannosidic linkages in endogenous and exogenous glycoproteins;[54] its deficiency leads to α-mannosidosis, i.e., a lysosomal storage disorder that is characterized by hyper-accumulation of mannose-containing oligosaccharides in the lysosome, and a range of pathological symptoms.[55] Cathepsins represent a range of lysosomal proteolytic enzymes and act as key modulators of cell death and inflammatory responses, participating in a variety of physiological and pathological processes, such as apoptosis, Alzheimer's disease, antigen processing, and atherosclerosis.[56] Palmitoyl-protein thioesterase 1 (PPT1) is an enzyme involved in the catabolism of lipid-modified proteins during lysosomal degradation by removing thioester-linked fatty acyl groups from cysteine residues.[57] Mutations in the *PPT1* gene cause a deficiency or complete lack of PPT1 activity, consequently resulting in infantile neuronal ceroid lipofuscinosis.[58] Tubulin is the major constituent of microtubules. Interestingly, the α-tubulin proteins are related to heavy metal tolerance.[59,60] Vesicle-trafficking protein SEC22b seems to interact with SNARE and play a role in ER-Golgi protein trafficking. In consequence, their up-regulation in expression enables the induction of the lysosome-autophagy system in the copepods. Autophagy is the most important intracellular process used by eukaryotic cells to sequester and degrade cytoplasm portions and organelles via the lysosomal pathway.[61] Additionally, autophagy is one of the most-useful pathways in the defense against cadmium toxicity.[62,63] Interestingly, the removal of oxidatively damaged organelles and proteins via the lysosome-autophagy pathway provides marine animals with an important defense strategy against oxidative stress.[64] Recalling that Hg exposure resulted in a series of toxic events, including oxidative stress, homeostatic dysfunction, and deregulation in glutathione metabolism, mainly by depleting sulfhydryl groups (e.g., GSH) and substituting for functionally essential metals such as iron and zinc, the toxicity outcome could be an uncontrolled accumulation of oxidatively damaged biomolecules especially proteins and enzymes in the exposed copepods. Correspondingly, the enhanced lysosome-autophagy system (which was supported by increased energy production mainly due to enhanced carbohydrate metabolism) was probably used by these copepods to remove accumulated abnormal proteins and enzymes as an effective compensatory strategy to counteract Hg toxicity. Taken together, the above declarations may, at the mechanistic level, account for the alleviating impact of OA on Hg toxicity in *T. japonicus*.

Implications. Our study clearly showed that the projected near-future OA condition would produce a significant proteome response despite the fact that the physiological traits (e.g., development and reproduction) are negligibly impacted by CO_2-driven acidification,[22] indicating strong proteome plasticity in *T. japonicus* in response to reduced pH. Many up-regulated proteins were enriched in protein assimilation and proteolysis, suggesting that the exposed copepods might engage in compensatory feeding under an ad libitum condition, with a concomitant increase in energy input, to offset the negative effects of a decreased pH (Figure 6). However, this hypothesis of compensatory feeding, which was based on proteomic findings, should be confirmed by measuring the grazing rate of this copepod under OA stress in a future study. Meanwhile, further work concerning the climate change effects (e.g., OA) may need to consider food quantity and quality during exposure and focus in particular on what impacts OA will

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

have on marine animals under food limitations, which may be a frequent scenario expected in the future oceans. Specifically, a common pathway of neuroactive ligand−receptor interaction (including a GABA-A receptor subunit) was increased by exposure to either OA or Hg. This result is particularly interesting because GABA-A receptor disturbances, which result in altered behavioral activities, have been evidenced in several other marine animals after exposure to elevated CO_2.[42−44] Our study is the first report suggesting that, at the proteomic level, OA is likely to alter the copepod's neuronal activity and behavioral performance, but this will require further confirmation through behavioral observations of copepods under elevated pCO_2 conditions.

In addition, our proteomic study indicated that Hg caused a range of toxic events, e.g., oxidative stress, dysfunction in cellular homeostasis, and decreased glutathione metabolism, despite induction of some processes as a compensatory reaction. These toxic events ultimately produced adverse outcomes at higher levels, involving reduced reproductive capacity in the exposed copepods. However, the inhibitory effects of Hg on the copepod's reproductive performance could be reversed by OA, as indicated by our proteomic analysis, with the lysosome-autophagy machinery initiated to remove the oxidatively damaged biomolecules (e.g., proteins and enzymes) in the copepods. Consequently, the copepod's reproductive performance under combined exposure was significantly increased as compared with Hg treatment alone. In some cases, the total fecundity of the copepod under combination treatment exceeded that of ambient conditions (Figure 6). The outcomes of our proteomic study should facilitate assessment of how these molecular responses translate into population-level responses against multiple stressors (e.g., climate-change stressors and heavy-metal pollution) that will be encountered in future oceans.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b01832.

Additional details regarding multigenerational exposure, proteomic analysis, database searching, and biochemical parameter determination. Figures showing Hg contents in adult copepods, effects of pCO_2 and Hg, a Venn diagram showing identified proteins, experimental reproducibility, comparisons of enzyme activity and protein expression, and a linear regression of fold-changes. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86-592-2880219; fax: +86-592-2880219; e-mail: mhwang45@xmu.edu.cn.

ORCID

Minghua Wang: 0000-0002-9955-4745

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was kindly supported by the National Natural Science Foundation of China (no. 41476094), the National Key Research and Development Program (no. 2016YFA0601203), and the Natural Science Foundation of Fujian Province of China (no. 2017J01081). We are grateful to Professor John Hodgkiss for his assistance with the English in this manuscript.

■ REFERENCES

(1) Siegenthaler, U.; Stocker, T. F.; Monnin, E.; Lüthi, D.; Schwander, J.; Stauffer, B.; Raynaud, D.; Barnola, J.-M.; Fischer, H.; Masson-Delmotte, V.; Jouzel, J. Stable carbon cycle−climate relationship during the late Pleistocene. *Science* **2005**, *310* (5752), 1313.

(2) Brewer, P. G. A changing ocean seen with clarity. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (30), 12213−12214.

(3) Hoegh-Guldberg, O.; Bruno, J. F. The impact of climate change on the world's marine ecosystems. *Science* **2010**, *328* (5985), 1523.

(4) Caldeira, K.; Wickett, M. E. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J. Geophys. Res.* **2005**, *110* (C9), C09S04.

(5) Feely, R. A.; Sabine, C. L.; Hernandez-Ayon, J. M.; Ianson, D.; Hales, B. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science* **2008**, *320* (5882), 1490−1492.

(6) Wootton, J. T.; Pfister, C. A.; Forester, J. D. Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (48), 18848−18853.

(7) Zhai, W.; Zhao, H.; Zheng, N.; Xu, Y. Coastal acidification in summer bottom oxygen-depleted waters in northwestern-northern Bohai Sea from June to August in 2011. *Chin. Sci. Bull.* **2012**, *57* (9), 1062−1068.

(8) Melzner, F.; Gutowska, M.; Langenbuch, M.; Dupont, S.; Lucassen, M.; Thorndyke, M. C.; Bleich, M.; Pörtner, H.-O. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **2009**, *6* (10), 2313−2331.

(9) Langdon, C.; Takahashi, T.; Sweeney, C.; Chipman, D.; Goddard, J.; Marubini, F.; Aceves, H.; Barnett, H.; Atkinson, M. J. Effect of calcium carbonate saturation state on the calcification rate of an experimental coral reef. *Global Biogeochemical Cycles* **2000**, *14* (2), 639−654.

(10) Lannig, G.; Eilers, S.; Pörtner, H. O.; Sokolova, I. M.; Bock, C. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—Changes in metabolic pathways and thermal response. *Mar. Drugs* **2010**, *8* (8), 2318−2339.

(11) Talmage, S. C.; Gobler, C. J. Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (40), 17246−17251.

(12) Kurihara, H.; Shimode, S.; Shirayama, Y. Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). *Mar. Pollut. Bull.* **2004**, *49* (9−10), 721−727.

(13) Havenhand, J. N.; Buttler, F.-R.; Thorndyke, M. C.; Williamson, J. E. Near-future levels of ocean acidification reduce fertilization success in a sea urchin. *Curr. Biol.* **2008**, *18* (15), R651−R652.

(14) Gao, X.; Zhou, F.; Chen, C.-T. A. Pollution status of the Bohai Sea: An overview of the environmental quality assessment related trace metals. *Environ. Int.* **2014**, *62*, 12−30.

(15) Wang, S.; Jia, Y.; Wang, S.; Wang, X.; Wang, H.; Zhao, Z.; Liu, B. Total mercury and monomethylmercury in water, sediments, and hydrophytes from the rivers, estuary, and bay along the Bohai Sea coast, northeastern China. *Appl. Geochem.* **2009**, *24* (9), 1702−1711.

(16) Castoldi, A. F.; Coccini, T.; Ceccatelli, S.; Manzo, L. Neurotoxicity and molecular effects of methylmercury. *Brain Res. Bull.* **2001**, *55* (2), 197−203.

(17) Wang, Y.; Wang, D.; Lin, L.; Wang, M. Quantitative proteomic analysis reveals proteins involved in the neurotoxicity of marine medaka *Oryzias melastigma* chronically exposed to inorganic mercury. *Chemosphere* **2015**, *119*, 1126−1133.

(18) Xu, X.; Shi, L.; Wang, M. Comparative quantitative proteomics unveils putative mechanisms involved into mercury toxicity and

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

tolerance in *Tigriopus japonicus* under multigenerational exposure scenario. *Environ. Pollut.* **2016**, *218*, 1287−1297.
(19) Raisuddin, S.; Kwok, K. W.; Leung, K. M.; Schlenk, D.; Lee, J. S. The copepod Tigriopus: a promising marine model organism for ecotoxicology and environmental genomics. *Aquat. Toxicol.* **2007**, *83* (3), 161−173.
(20) Li, H.; Shi, L.; Wang, D.; Wang, M. Impacts of mercury exposure on life history traits of *Tigriopus japonicus*: Multigeneration effects and recovery from pollution. *Aquat. Toxicol.* **2015**, *166*, 42−49.
(21) Li, W.; Han, G.; Dong, Y.; Ishimatsu, A.; Russell, B. D.; Gao, K. Combined effects of short-term ocean acidification and heat shock in a benthic copepod *Tigriopus japonicus* Mori. *Mar. Biol.* **2015**, *162* (9), 1901−1912.
(22) Li, Y.; Wang, W. X.; Wang, M. Alleviation of mercury toxicity to a marine copepod under multigenerational exposure by ocean acidification. *Sci. Rep.* **2017**, *7* (1), 324.
(23) Shi, W.; Zhao, X.; Han, Y.; Che, Z.; Chai, X.; Liu, G. Ocean acidification increases cadmium accumulation in marine bivalves: a potential threat to seafood safety. *Sci. Rep.* **2016**, *6*, 20197.
(24) Ivanina, A. V.; Hawkins, C.; Sokolova, I. M. Immunomodulation by the interactive effects of cadmium and hypercapnia in marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Fish Shellfish Immunol.* **2014**, *37* (2), 299−312.
(25) Pascal, P.-Y.; Fleeger, J. W.; Galvez, F.; Carman, K. R. The toxicological interaction between ocean acidity and metals in coastal meiobenthic copepods. *Mar. Pollut. Bull.* **2010**, *60* (12), 2201−2208.
(26) Campbell, A. L.; Mangan, S.; Ellis, R. P.; Lewis, C. Ocean acidification increases copper toxicity to the early life history stages of the polychaete *Arenicola marina* in artificial seawater. *Environ. Sci. Technol.* **2014**, *48* (16), 9745−9753.
(27) Richards, R.; Chaloupka, M.; Sano, M.; Tomlinson, R. Modelling the effects of 'coastal' acidification on copper speciation. *Ecol. Modell.* **2011**, *222* (19), 3559−3567.
(28) Millero, F. J.; Woosley, R.; DiTrolio, B.; Waters, J. Effect of ocean acidification on the speciation of metals in seawater. *Oceanography* **2009**, *22* (4), 72.
(29) Alex Merrick, B. Toxicoproteomics in liver injury and inflammation. *Ann. N. Y. Acad. Sci.* **2006**, *1076* (1), 707−717.
(30) Thompson, E.; O'Connor, W.; Parker, L.; Ross, P.; Raftos, D. Differential proteomic responses of selectively bred and wild-type Sydney rock oyster populations exposed to elevated CO_2. *Mol. Ecol.* **2015**, *24* (6), 1248−1262.
(31) Dineshram, R.; Sharma, R.; Chandramouli, K.; Yalamanchili, H. K.; Chu, I.; Thiyagarajan, V.; Q, Q. Comparative and quantitative proteomics reveal the adaptive strategies of oyster larvae to ocean acidification. *Proteomics* **2015**, *15* (23−24), 4120−4134.
(32) Dineshram, R.; Chandramouli, K.; Ko, G. W. K.; Zhang, H.; Qian, P. Y.; Ravasi, T.; Thiyagarajan, V. Quantitative analysis of oyster larval proteome provides new insights into the effects of multiple climate change stressors. *Global Change Biology* **2016**, *22* (6), 2054−2068.
(33) Caldeira, K.; Wickett, M. E. Oceanography: Anthropogenic carbon and ocean pH. *Nature* **2003**, *425* (6956), 365−365.
(34) Kim, H.-S.; Lee, B.-Y.; Won, E.-J.; Han, J.; Hwang, D.-S.; Park, H. G.; Lee, J.-S. Identification of xenobiotic biodegradation and metabolism-related genes in the copepod *Tigriopus japonicus* whole transcriptome analysis. *Marine Genomics* **2015**, *24* (3), 207−208.
(35) Wang, M.; Jeong, C. B.; Li, Y.; Lee, J. S. Different transcriptomic responses of two marine copepods, *Tigriopus japonicus* and *Pseudodiaptomus annandalei*, to a low dose of mercury chloride ($HgCl_2$). *Aquat. Toxicol.* **2017**, *187*, 124−131.
(36) Ren, Y.; Hao, P.; Dutta, B.; Cheow, E. S. H.; Sim, K. H.; Gan, C. S.; Lim, S. K.; Sze, S. K. Hypoxia modulates A431 cellular pathways association to tumor radioresistance and enhanced migration revealed by comprehensive proteomic and functional studies. *Mol. Cell. Proteomics* **2013**, *12* (2), 485−498.
(37) Tomanek, L. Proteomics to study adaptations in marine organisms to environmental stress. *J. Proteomics* **2014**, *105*, 92−106.
(38) Dineshram, R.; Wong, K. K.; Xiao, S.; Yu, Z.; Qian, P. Y.; Thiyagarajan, V. Analysis of pacific oyster larval proteome and its response to high-CO_2. *Mar. Pollut. Bull.* **2012**, *64* (10), 2160−2167.
(39) Wong, K. K.; Lane, A. C.; Leung, P. T.; Thiyagarajan, V. Response of larval barnacle proteome to CO_2-driven seawater acidification. *Comp. Biochem. Physiol., Part D: Genomics Proteomics* **2011**, *6* (3), 310−321.
(40) Li, W.; Gao, K. A marine secondary producer respires and feeds more in a high CO_2 ocean. *Mar. Pollut. Bull.* **2012**, *64* (4), 699−703.
(41) Pedersen, S. A.; Håkedal, O. J.; Salaberria, I.; Tagliati, A.; Gustavson, L. M.; Jenssen, B. M.; Olsen, A. J.; Altin, D. Multigenerational exposure to ocean acidification during food limitation reveals consequences for copepod scope for growth and vital rates. *Environ. Sci. Technol.* **2014**, *48* (20), 12275−12284.
(42) Nilsson, G. E.; Dixson, D. L.; Domenici, P.; McCormick, M. I.; Sørensen, C.; Watson, S.-A.; Munday, P. L. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nat. Clim. Change* **2012**, *2* (3), 201−204.
(43) Hamilton, T. J.; Holcombe, A.; Tresguerres, M. CO_2-induced ocean acidification increases anxiety in Rockfish via alteration of $GABA_A$ receptor functioning. *Proc. R. Soc. London, Ser. B* **2014**, *281* (1775), 20132509.
(44) Watson, S.-A.; Lefevre, S.; McCormick, M. I.; Domenici, P.; Nilsson, G. E.; Munday, P. L. Marine mollusc predator-escape behaviour altered by near-future carbon dioxide levels. *Proc. R. Soc. London, Ser. B* **2014**, *281* (1774), 20132377.
(45) Bormann, J.; Hamill, O. P.; Sakmann, B. Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones. *J. Physiol.* **1987**, *385* (1), 243−286.
(46) Ishimatsu, A.; Hayashi, M.; Kikkawa, T. Fishes in high-CO_2, acidified oceans. *Mar. Ecol.: Prog. Ser.* **2008**, *373*, 295−302.
(47) Akerman, C. J.; Cline, H. T. Refining the roles of GABAergic signaling during neural circuit formation. *Trends Neurosci.* **2007**, *30* (8), 382−389.
(48) Tsang, S.-Y.; Ng, S.-K.; Xu, Z.; Xue, H. The evolution of GABAA receptor-like genes. *Mol. Biol. Evol.* **2007**, *24* (2), 599−610.
(49) Harrison, P. M.; Arosio, P. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta, Bioenerg.* **1996**, *1275* (3), 161−203.
(50) Sun, Y.; Zhang, Y.; Fu, X.; Zhang, R.; Zou, J.; Wang, S.; Hu, X.; Zhang, L.; Bao, Z. Identification of two secreted ferritin subunits involved in immune defense of Yesso scallop *Patinopecten yessoensis*. *Fish Shellfish Immunol.* **2014**, *37* (1), 53−59.
(51) Aguilera, V. M.; Vargas, C. A.; Lardies, M. A.; Poupin, M. J. Adaptive variability to low-pH river discharges in *Acartia tonsa* and stress responses to high pCO_2 conditions. *Mar. Ecol.* **2016**, *37* (1), 215−226.
(52) Lundeen, K. A.; Sun, B.; Karlsson, L.; Fourie, A. M. Leukotriene B4 receptors BLT1 and BLT2: expression and function in human and murine mast cells. *J. Immunol.* **2006**, *177* (5), 3439−3447.
(53) Couto, N.; Wood, J.; Barber, J. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. *Free Radical Biol. Med.* **2016**, *95*, 27−42.
(54) De Gasperi, R.; Daniel, P.; Warren, C. A human lysosomal alpha-mannosidase specific for the core of complex glycans. *J. Biol. Chem.* **1992**, *267* (14), 9706−9712.
(55) Robinson, A.; Crawley, A.; Hopwood, J. Over-expression of human lysosomal α-mannosidase in mouse embryonic stem cells. *Mol. Genet. Metab.* **2005**, *85* (3), 203−212.
(56) Conus, S.; Simon, H.-U. Cathepsins: key modulators of cell death and inflammatory responses. *Biochem. Pharmacol.* **2008**, *76* (11), 1374−1382.
(57) Saja, S.; Buff, H.; Smith, A. C.; Williams, T. S.; Korey, C. A. Identifying cellular pathways modulated by *Drosophila* palmitoyl-protein thioesterase 1 function. *Neurobiol. Dis.* **2010**, *40* (1), 135−145.
(58) Dearborn, J. T.; Ramachandran, S.; Shyng, C.; Lu, J.-Y.; Thornton, J.; Hofmann, S. L.; Sands, M. S. Histochemical localization

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831

of palmitoyl protein thioesterase-1 activity. *Mol. Genet. Metab.* **2016**, *117* (2), 210−216.
(59) Mattingly, K. S.; Beaty, B.; Mackie, R.; McGaw, M.; Carlson, J.; Rayms-Keller, A. Molecular cloning and characterization of a metal responsive *Chironomus tentans* alpha-tubulin cDNA. *Aquat. Toxicol.* **2001**, *54* (3), 249−260.
(60) Mireji, P. O.; Keating, J.; Hassanali, A.; Impoinvil, D. E.; Mbogo, C. M.; Muturi, M. N.; Nyambaka, H.; Kenya, E. U.; Githure, J. I.; Beier, J. C. Expression of metallothionein and α-tubulin in heavy metal-tolerant *Anopheles gambiae* sensu stricto (Diptera: Culicidae). *Ecotoxicol. Environ. Saf.* **2010**, *73* (1), 46−50.
(61) Klionsky, D. J.; Emr, S. D. Autophagy as a regulated pathway of cellular degradation. *Science* **2000**, *290* (5497), 1717−1721.
(62) Bargagli, R. Trace metals in Antarctica related to climate change and increasing human impact. *Reviews of Environmental Contamination and Toxicology* **2000**, *166*, 129−174.
(63) Chiarelli, R.; Martino, C.; Agnello, M.; Bosco, L.; Roccheri, M. C. Autophagy as a defense strategy against stress: focus on *Paracentrotus lividus* sea urchin embryos exposed to cadmium. *Cell Stress Chaperones* **2016**, *21* (1), 19−27.
(64) Moore, M. N.; Icarus Allen, J.; McVeigh, A. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Mar. Environ. Res.* **2006**, *61* (3), 278−304.

DOI: 10.1021/acs.est.7b01832
Environ. Sci. Technol. 2017, 51, 5820−5831