Environmental Research 236 (2023) 116817

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Employing molecular, chemical and physiological techniques using *Crassostrea virginica* to assess ecosystem health along coastal South Carolina and North Carolina, United States

Allisan Aquilina-Beck [a,*], Lou Ann Reed [b], Mary Rider [a], William Burdine [a], James Daugomah [b], Dennis Apeti [b], Pete Key [b], Marie DeLorenzo [b]

[a] CSS, Inc, Under Contract to National Centers for Coastal Ocean Science, National Ocean Service, National Oceanic and Atmospheric Administration, Charleston, SC, USA

[b] National Centers for Coastal Ocean Science, National Ocean Service, National Oceanic and Atmospheric Administration, Charleston, SC, USA

ARTICLE INFO

Handling Editor: Robert Letcher

Keywords:
qPCR
Oysters
Monitoring
Biomarkers
Zinc
Response

ABSTRACT

Natural and anthropogenic environmental impacts can introduce contaminants into sensitive habitats, threatening ecosystems and human health. Consistent monitoring of coastal areas provides critical environmental assessment data. Sediments and Eastern Oyster (*Crassostrea virginica*) tissues were collected at fourteen South Carolina (SC) and four North Carolina (NC) sites as part of the National Oceanic and Atmospheric Administration's Mussel Watch environmental monitoring program. Cellular and molecular techniques were employed to measure *C. virginica* stress response, specifically, Lipid Peroxidation (LPx), Glutathione (GSH), and qPCR techniques. Gene specific primers targeted for detecting oxidative stress and cellular death were developed in *C. virginica* to gauge response to current environmental conditions using gill and hepatopancreas (HP) tissue. In order to validate gene specific markers as additional assessment tools, a 96 h zinc (Zn) laboratory exposure was performed. Cellular biomarker data revealed tissue specific responses. Hepatopancreas data showed *C. virginica* exhibited stress through the lipid peroxidation assay amongst sampling sites, however, response was managed through glutathione detoxification. Gill tissue data had significantly lower levels of cellular biomarker response compared to hepatopancreas. Molecular biomarkers targeting these cellular stress pathways through qPCR analysis show upregulation of Metallothionein in hepatopancreas and gill tissue with a concurrent $>$ 2-fold upregulation in the detoxification marker Superoxide Dismutase (SOD) at three NC sites. SC sites displayed higher stress levels through LPx assays and down-regulation in GPx gene activity. Laboratory zinc exposure revealed no significance in cellular biomarker results, however, molecular data showed gills responding to zinc treatment through upregulation of Metallothionein, SOD and Cathepsin L, indicating an acute response in gills. Collectively, chemical, cellular and molecular methods clarify sentinel stress response of biological impacts and aid in evaluating environmental health in coastal ecosystems. This combined methodological approach provides a detailed analysis of environmental conditions and improves land-use management decisions.

1. Introduction

Anthropogenetic or weather impacts can alter sensitive coastal habitats, potentially threatening ecosystem stability, local economy, and/or human health. Extensive, reoccurring environmental monitoring provides informative assessments of local and regional ecosystems, results of which shape management decisions.

The National Oceanic and Atmospheric Administration (NOAA)'s Mussel Watch (MW) program, established in 1986, is a long-term chemical contaminants monitoring program in bivalve and sediment samples from designated sites spanning the coastlines and Great Lakes of the United States of America (USA) (Ankley, et al., 2010; Apeti et al., 2018). The data generated from yearly sampling aid in monitoring contaminants of historic and emerging concern (CEC), as well as influencing management and remediation decisions (Kimbrough, et al., 2008). For instance, tidal creek sediments within South Carolina (SC)

* Corresponding author. CSS under contract to NOAA, Ecotoxicology Branch, National Centers for Coastal Ocean Science, NOAA National Ocean Service, 331 Fort Johnson Road, Charleston, SC, 29412.
E-mail address: allisan.beck@noaa.gov (A. Aquilina-Beck).

https://doi.org/10.1016/j.envres.2023.116817
Received 1 June 2023; Received in revised form 10 July 2023; Accepted 1 August 2023
Available online 2 August 2023

have been extensively monitored for metals and organic contaminants since 1995 (Sanger, et al., 1999a,b). These data provide comparable contaminant records for coastal areas including those that undergo urbanization due to population growth along the coastlines as well as areas affected by extreme weather events such as hurricanes and flooding (Sanger, et al., 1999a,b; Holland et al., 2004; Kimbrough et al., 2008; Edwards et al., 2016). Historic MW sites both regionally and nationally, have been chosen to be representative, of background and standard contamination levels and therefore are not sited in close proximity to known contamination point sources in order to provide an accurate representation of the chemical makeup of the area (Lauenstein, et al., 2002).

Targeted monitoring of sentinel species provides essential spatial health assessments (Apeti, et al., 2018). Used along with chemical contaminant monitoring, bivalve health assessments could be a useful indicator for ecosystem change. Our study used an Adverse Outcome Pathway (AOP) approach whereby a molecular or chemical initiating event triggers a biological response and the results of which, could lead to a population response and relevant risk assessment (Ankley, et al., 2010). Cellular biomarkers targeting stress pathways provide a comprehensive analysis and awareness of influential environmental factors on organism and ecosystem health.

In this paper, we report biomarker and molecular data from oysters, *Crassostrea virginica*, collected at fourteen coastal South Carolina MW sites and four coastal North Carolina MW sites designated as urban, suburban, undeveloped or unknown. North Inlet Clambank (NICB) in South Carolina, was used as a reference site designated by the National Estuarine Research Reserve System (Sanger et al., 1999a). Physiological analysis through biomarkers such as Lipid Peroxidation (LPx) and Glutathione (GSH) provide information regarding cellular oxidative stress and detoxifying abilities within an organism. Specifically, LPx indicates lipid cell membrane damage as a result of free radicals measured by the release of malondialdehyde (MDA) (Ohkawa, et al., 1979; Khan et al., 2019). GSH, is part of the phase II enzyme system involved in chemical detoxification. It is the most abundant low molecular weight thiol in animal cells that, in the presence of reactive oxygen species, neutralizes free radicals to a less harmful form (Wu, et al., 2004). Additionally, gene specific primers were developed to examine gene regulation in two different tissue types, gill and hepatopancreas (HP), for the purpose of detecting: oxidative stress, stress response, and cellular death resulting from exposure to external conditions.

Specific primers used in this study were: Metallothionein (MET), Superoxide Dismutase (SOD), Glutathione Reductase (GR), Glutathione S Transferase (GST), Glutathione Peroxidase (GPx), B-Cell Lymphoma (BCL2), and Cathepsin L (CathL). MET is a group of cystine-rich proteins that bind to essential and toxic metals (Geffard, et al., 2005; Fasulo et al., 2008), thus it was used to indicate metal exposure. Cells have a complex antioxidant defense system which combine many enzymes to eliminate and protect against reactive oxygen species (Rashid, et al., 2013). The ones used in this paper have direct roles in detoxification. Both enzymes, GSH and GST are part of the Phase II detoxification enzyme family and are directly involved in reducing toxic reactive oxygen species into nontoxic forms to be eliminated by the cell (Wu, et al., 2004). SOD is responsible for converting O_2^- to H_2O_2; while Catalase further reduces this to water and O_2. (;(Erturk Gurkan and Gurkan, 2021; Rashid et al., 2013)). Free radicals and other reactive oxygen species are foraged by the oxidized form of GSH which is reduced by Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-dependent GR. GPx catalyzes the reduction of peroxide molecules into nontoxic forms (Wu, et al., 2004; Savaskan et al., 2007). Analyzing a suite of genes provides a comprehensive evaluation of changes that are occurring within a cell. In order to test the validity and specificity of genetic markers to determine *C. virginica* response, a controlled 96 h (h) zinc (Zn) laboratory exposure was conducted. Based on field chemistry results, zinc was selected as an anthropogenic metal to test at environmentally relevant concentrations. HP and gill tissue were processed and analyzed using the same cellular biomarkers and qPCR markers as described above along with the addition of three gene specific primers: Glutathione S Transferase (GST), B-Cell Lymphoma (BCL2), an anti-apoptosis gene, and Cathepsin L (CathL), a pro-apoptosis gene initiating cell death (Yadati, et al., 2020).

These data fill current knowledge gaps for evaluating stress impacts on coastal ecosystems. Employing specifically designed *C. virginica* genetic markers provides a comprehensive evaluation of sentinel response to environmental stressors. Joining physiological and molecular techniques together with chemistry, and laboratory experiments, provides repeatable and comparable levels of health for sentinel species and a complete approach to understanding how environmental exposures influence tissue responses.

Table 1
Site information from where *C. virginica* tissue and sediment chemistry were collected. Collection site full name, abbreviations, coordinates as well as the land use type are listed. Italicized site abbreviations were also used in molecular analyses. NICB is designated as a NERRS (National Estuarine Research Reserve's System Reference Site) and is used as a control site in biomarker and molecular assays. CHRT is listed as undeveloped in the 1990's. CHSF is an undeveloped site, however this site has high shipping and boat traffic associated with its location. NC sites are currently unclassified, however, surrounding BIPI is a boat launch and the CALO7 and 19 sites are an abandoned military base and town respectively.

Site Name	Site Code	Latitude	Longitude	Land Use Type
South Carolina				
Charleston Harbor Bull Creek	CHBL	32.8264	−80.0288	Suburban
Charleston Harbor Diesel Creek	CHDL	32.8155	−79.9633	Urban
Charleston Harbor Fort Johnson	CHFJ	32.7529	−79.8981	Suburban
Charleston Harbor Horlbeck Creek	*CHHB*	32.8657	−79.8222	Suburban
Charleston Harbor Metcalf's Creek	CHMC	32.7462	−79.9538	Suburban
Charleston Harbor New Market	*CHNM*	32.8062	−79.9401	Urban
Charleston Harbor Orange Grove Creek	CHOG	32.7997	−79.9772	Suburban
Charleston Harbor Rathall Creek	*CHRT*	32.8611	−79.8927	Undeveloped
Charleston Harbor Shem Creek	CHSM	32.7933	−79.8803	Urban
Charleston Harbor Shipyard Creek	*CHSH*	32.8392	−79.9452	Urban
Charleston Harbor Shutes Folly	*CHSF*	32.7753	−79.9138	Undeveloped
Charleston Harbor Vardell Creek	CHVR	32.8027	−79.9317	Urban
North Inlet Clambank	*NICB*	33.3342	−79.1929	NERRS Reference Site
Winyah Bay Lower Bay	WBLB	33.2435	−79.1979	Undeveloped
North Carolina				
Beaufort Inlet, Pivers Island	*BIPI*	34.7196	−76.6726	Unknown
Cape Lookout Site #7	*CALO7*	34.6122	−76.5376	Unknown
Cape Lookout Site #19	*CALO19*	35.0709	−76.0685	Unknown
Pamlico Sound, Cape Hatteras	*PSCH*	35.2028	−75.7162	Unknown

2. Methods

2.1. Field collection

C. virginica and sediment samples were collected at low tide from eighteen designated Mussel Watch (MW) sites (Supplementary Fig. 1, Table 1). All South Carolina samples were collected from October to November 2020 and North Carolina samples were collected in October 2021. Both collections followed detailed Mussel Watch sampling protocols outlined in National Status and Trends (NS&T) Program's

standard field protocols (Apeti, et al., 2012). At each site, 120 single whole *C. virginica* were collected for contaminant monitoring. *C. virginica* were placed in labeled bags on ice in a temporary travel cooler until transferred to a 4 °C refrigerator. Positional locations for each site were recorded via GPS and water quality was recorded using a YSI Pro plus handheld meter (Xylem YSI, USA): [temperature (23.9 °C ± 1.9), dissolved oxygen (5.5 mg/L ± 1.9), and salinity (20.7 ppt ±7.5)]. A Young modified Van Veen grab sampler (Aquatic Biotechnology, Spain) was used to collect 4 cm of surface sediment in triplicates at each site. The triplicate sediment samples were homogenized in the field with a plastic scoop, aliquoted into labeled glass jars, and frozen. Sediment tools were sanitized with 70% isopropanol wipes between sites. The *C. virginica* samples were preserved on ice and sent to the analytical laboratory where they were shucked for each site and the soft tissues of the 120 *C. virginica* composited to form a site sample.

Tissue and sediments were analyzed for the standard suite of chemical contaminants routinely monitored by the MW program. Specific methods for analysis of MW samples are outlined in the updated technical memorandum (Apeti, et al., 2018). Two independent labs conducted the analysis: NOAA/National Centers for Coastal Ocean Science (NCCOS) Charleston Chemistry Laboratory, South Carolina, USA and TDI Brooks Laboratories, Texas, USA. TDI Laboratories reported legacy organics (pesticides, Polychlorinated biphenyl (PCB)s, Polycyclic aromatic hydrocarbon (PAH)s) and Contaminants of Emerging Concern (CEC) from sediments, and reported whole tissue organic contamination. Both sediment and tissues were analyzed for metals, pharmaceuticals, and personal care products/alkyl phenol contaminants at NOAA/NCCOS Charleston Laboratory. Three additional *C. virginica* per site were collected and kept on ice until processing for biomarker and molecular activity by NOAA/NCCOS Charleston Laboratory, results of which are detailed in this paper.

2.2. *C. virginica* biomarker processing

Three *C. virginica* per field site were kept cold in a 4 °C refrigerator until processed (≤24 h) and were individually quickly rinsed with 20 ppt seawater to remove sediment and blotted dry. Seawater for rinsing was collected from Charleston Harbor estuary (32.7532, −79.899531), filtered (5 μm), UV sterilized and activated carbon filtered (5 μm), then diluted with deionized water to 20 ppt salinity. Shell length and width were measured and recorded: average length (85 mm ± 16 mm) and width (39 mm ± 7 mm). Dissections took place on ice. Gills and hepatopancreas (HP) were removed from each *C. virginica*, divided into separate 1 mL cryovials and immediately frozen in liquid nitrogen. Dissecting tools were cleaned with deionized water and wiped with 70% ethanol after each sample. Tissues were stored at −80 °C until processed for biomarker analyses and RNA extraction. Site CHVR had one *C. virginica* arrived dead, therefore, only two were processed.

2.2.1. Lipid peroxidation assay

The Lipid Peroxidation (LPx) assay measures the amount of malondialdehyde (MDA), an indicator of cellular membrane damage, by spectrophotometric thiobarbituric acid detection at 532 nm. Briefly, tissues from field sites and Zn laboratory exposures were homogenized at 250 mg tissue/mL in cold 50 mM potassium phosphate (K_2PO_4) buffer at pH 7.0 using a Pro Scientific model Pro 200 motor with a 20-mmx150-mm stainless steel rod for a minimum of 30 s. A 3200 μM MDA solution was used to prepare oyster serial dilutions from 800 to 25 μM using K_2PO_4 buffer. Tubes were prepared using 100 μL of either MDA dilution, sample homogenate or K_2PO_4 buffer as a blank. To these individual tubes, 1400 μL of 0.375% thiobarbituric acid/trichloroacetic acid and 14 μL of 2% butylated hydroxytoluene was added. All tubes were heated to 92 °C for 15 min, then centrifuged for 5 min at 13,000×*g* at room temperature. Supernatants were taken at 300 μL aliquots and plated in triplicate into a clear Corning 96-well plate and read using μQuant microplate Spectrophotometer (Bio-Tek Instruments Inc.) and KC junior software (Bio-Tek Instruments Inc.). Absorbance readings were subtracted from the blank value and the slope of the standard line was used to calculate MDA in nmol/g wet weight (w.w.).

2.2.2. Glutathione assay

The Glutathione Assay (GSH) measures the amount of Glutathione, a phase II enzyme, and is an indicator of whether or not detoxification is occurring within a cell. Methods followed those reported in Ringwood et al. (2003) for 5,5′-dithiobis (2-nitrobenzoic) acid-glutathione (DTNB-GSSG) recycling protocol. Tissues from field sites and Zn laboratory exposure experiment were homogenized in 10 times the volume of their weight in cold 5% sulfosalicylic acid (SSA) and centrifuged 13,000×*g* at 4 °C for 5 min. Supernatants of 100 μL were combined with equal volume of 5% SSA. L-glutathione reduced standards of 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM and 6.25 μM were serial diluted using 5% SSA. Aliquots of 25 μL of either sample, standard or blank (5% SSA) were combined with 0.208 mM of Nicotinamide adenine dinucleotide phosphate (NADPH), 10 mM of DTNB and brought to 1 mL total volume in deionized water and vortexed. A subsample of 900 μL of the mixture was added to a cuvette in addition to 50 U/L of GSSG reductase enzyme immediately before reading in an UltroSpec 5300 pro UV/Vis spectrophotometer (Amersham Biosciences). Readings were conducted at 15 second (s) intervals for a total of 90 s at a wavelength of 405 nm. Measurements were obtained using Swift II Reaction Kinetics software (Biochrom Ltd.). GSH data were expressed as nmol/g w.w.

2.3. Sample preparation for real-time quantitative PCR (qPCR)

C. virginica tissues from selected field sites (SC: CHSH, CHNM, CHRT, CHHB, CHSF, NICB) and (NC: PSCH BIPI CALO7, CALO19); were analyzed by qPCR with pathway specific molecular markers. Analyzed field sites were based on the largest measured differences calculated between tissue type within the cellular biomarker data. Field collected HP and gill tissue were extracted for RNA from each of the three *C. virginica* collected/site with the exception of SC sites (gill): CHSH, CHNM, CHRT, CHSF where only two *C. virginica*/site were examined due to low RNA yields.

2.3.1. Total RNA extraction and cDNA synthesis

Gill and HP tissue were kept frozen in liquid Nitrogen, weighed and extracted following protocol of Direct-zol RNA miniprep plus kit #R2072 (Zymo Research, Irving, CA, USA). Tissues were lysed using the TissueLyser 85300 (Qiagen, Hilden, Germany) for 1.5 min at 30Hz. RNA was quantified by NanoDrop® 2000 Spectrophotometer (Thermofisher ND-2000, Waltham, MA, USA), normalized to 100 ng/μL and processed for cDNA following Qiagen QuantiTect Reverse Transcription Kit (# 205311). The RNA 260/280 purity reads averaged 1.95 ± 0.07 for all examined HP and gill *C. virginica* tissues sampled.

2.3.2. Real-time quantitative PCR (RT-qPCR)

C. virginica candidate genes were chosen from NCBI nucleotide database (https://ncbi.nlm.nih.gov/) and primers were designed using Genome Compiler (https://openwetware.org/wiki/Genome_compiler) and Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/). A complete list of primer sequences used, accession numbers and information can be found in Supplementary Fig. 2. Expected base pair (bp) size and single amplicons were confirmed by PCR, agarose gel electrophoresis, Sanger Sequencing and qPCR single melt curves (data not shown). RT-qPCR set up was performed under a positive airflow hood. A 7500 Fast ABI system (Applied Biosystems, USA) and ABI Fast Sybr Green (Applied Bio Systems, USA) were used for qPCR reactions. Thermal cycle conditions used were as follows: (95 °C for 20 s, followed by 40 cycles of (95 °C for 3 s, 60 °C for 30 s)), immediately followed by a melt curve to confirm specificity. All reactions were performed in duplicate using diluted cDNA (1:8). Data were normalized to the housekeeping gene, Actin (Kuchovska, et al., 2021; Rahman and Rahman, 2021). Relative change

was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Efficiencies for all gene primer pairs used were within the acceptable range of 90–110%. Amplification factors, used in Fold Change calculations, were determined by equation 10^(−1/slope).

2.4. Zinc laboratory challenge test

C. virginica were collected from a commonly used control site (CS) at the mouth of Leadenwah Creek, a tributary of the North Edisto River on Wadmalaw Island, South Carolina (32.61775, −80.229056) (DeLorenzo, et al., 2006; Aquilina-Beck, et al., 2020). *C. virginica* shells were cleaned to remove any attached bivalves and dissected in the same manner as field collected *C. virginica* detailed above. Average length (81.1 mm ± 9.8 mm) and width (39 ± 4.7) were recorded. Eight *C. virginica* per treatment were used for this experiment: six *C. virginica* for biomarker analysis, in order to increase statistical power, and two *C. virginica* for tissue chemistry. *C. virginica* were allowed to depurate in carbon filtered recirculating seawater tanks for four days before the start of the experiment. *C. virginica* were kept at a 16 h light: 8 h dark cycle, 25 °C water temperature and fed Shellfish Diet 1800 ® (Reed Mariculture inc., Campbell, CA) during depuration. Seawater was collected from Charleston Harbor estuary (32.7532, −79.899531), filtered (5 μm), UV sterilized, activated carbon filtered (5 μm), and diluted with deionized water to 20 ppt salinity.

The range of zinc concentrations used in previous laboratory challenges with oysters varied widely (Jenny, et al., 2006; Mottin et al., 2012; Thompson et al., 2012; Liu et al., 2013). As reported in (Liu, et al., 2013), a 12% mortality was observed in oysters exposed to zinc at 4500 μg/L at day six. In order to avoid mortality in our experiments, we dosed at 4000 μg/L to represent a high dose and 10-fold less to represent a low dose, 400 μg/L. A $ZnCl_2$ stock solution at 0.1M was prepared in deionized water. Treatment solutions at: Control 0 μg/L, Low 400 μg/L or High 4000 μg/L were made in seawater. One *C. virginica* was placed in a clean plastic jar containing 1L of either Control, Low or High dose treatment solutions. There was a total of eight *C. virginica* per treatment. Each jar was equipped with a lid and was aerated with a plastic pipette attached to airline tubing in order to minimize loss of the toxicant throughout the duration of the experiment. Treatments were replenished daily for 96 h. Water quality during the experiment was monitored daily and was within acceptable range: dissolved oxygen (6.1 mg/L ± 1.3), pH (7.9 ± 0.2), temperature °C (24.6 ± 0.3), and salinity (ppt) (20.3 ± 0.6). Six 'Baseline' depurated *C. virginica* (prior to experiment) and all treatment groups (following the experiment) were dissected on ice, HP and gills removed, separated into individual cryovials, and flash frozen as described above for biomarker and qPCR analyses. For chemical analysis, two *C. virginica* for each treatment type (T = 96) and two *C. virginica* after initial field collection (T = 0) were dissected and ground in a glass homogenizer, whole composite soft tissues were analyzed for Zn concentration. HP and gill tissue were processed and analyzed with the same cellular biomarkers and qPCR markers as described above with the addition of three gene specific primers: GST, BCL2 and CathL. No *C. virginica* died during the experiment.

2.4.1. Inorganic analysis of Zn seawater samples

One mL composite water samples were taken from each treatment type at T = 0 and T = 24 h for chemical analysis to represent a one-day dose. Zn analysis in seawater for all treatment types at T = 0 and T = 96 h was conducted by GEL Laboratories, Charleston, SC. Seawater samples were filtered through a 0.45-μm filter, acidified with nitric acid, and diluted as appropriate prior to analysis by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES), AVIO500 (PerkinElmer, USA) at an emission wavelength of 213.856 nm. All analyses met the requirements of the National Environmental Laboratory Accreditation Conference (NELAC) standard. (U.S.EPA, 1992; 2014).

2.4.2. Inorganic analysis of C. virginica tissues

Individual *C. virginica* samples were homogenized and a portion of each were oven-dried for gravimetric estimation of dry fraction. The remaining *C. virginica* tissue was weighed and acid digested at 95 °C with an Environmental Express HotBlock SC100 Digestion System (Environmental Express, Charleston, SC) using ultrapure nitric acid (10 mL) followed by addition of ultrapure 30% hydrogen peroxide (2 mL) to provide complete digestion within a few hours (U.S.EPA, 1996). Resulting solutions were diluted to 50 mL with Millipore deionized water prior to further dilution with 2% nitric acid and analyzed by Inductively Coupled Plasma Mass Spectrometer (ICP-MS) trace element analysis (U.S.EPA, 1994). Samples were diluted as necessary for bracketing by calibration curves. A PerkinElmer Sciex NexION® 300D ICP-MS with an ESI Fast Pump/Autosampler (PerkinElmer, Inc., Waltham, MA) was used to measure the following 39 isotopes of 21 elements: Ag^{107}, Ag^{109}, A^{l27}, As^{75}, Ba^{136}, Ba^{137}, Ba^{138}, Be^{9}, Cd^{111}, Cd^{112}, Cd^{114}, Co^{59}, Cr^{52}, Cr^{53}, Cu^{63}, Cu^{65}, Fe^{54}, Fe^{56}, Fe^{57}, Li^{6}, Li^{7}, Mn^{55}, Ni^{60}, Pb^{208}, Sb^{121}, Sb^{123}, Se^{78}, Se^{82}, Sn^{117}, Sn^{119}, Sn^{120}, Tl^{203}, Tl^{205}, U^{235}, U^{238}, V^{51}, Zn^{64}, Zn^{66}, and Zn^{68}, with a multiple element internal standard (In^{115}, Lu^{175}, Rh^{103}, and Sc^{45}) added to each sample and calibration standard. Reported values are the average of listed isotopes for an element. For total Hg determination, samples were accurately weighed directly into nickel boats for analysis. A direct mercury analyzer, the DMA-80 (Milestone Inc., Shelton, CT), was used to determine the mass fraction of total Hg by pyrolytic decomposition of the sample, catalytic reduction to elemental Hg, trapping on a gold amalgamator, with Hg thermally desorbed and Hg atomic absorbance measured at 254 nm. Calibration curves were prepared daily using standard solutions purchased from High Purity Standards (Charleston, SC); correlation coefficients for all analyte calibration curves were ≥0.999. Data were collected using PE SyngistixTM for ICP-MS Software – version 2.5. Quality assurance for inorganic data included analysis of duplicate samples, matrix and reagent spikes, method blanks, standard reference material (SRM), National Institute of Standards and Technology (NIST) SRM 1566b – Oyster Tissue (NIST, Gaithersburg, MD), and Trace Metals in Drinking Water (TMDW), a certified reference material (High Purity Standards, Charleston, SC). Recoveries ranged from 83.8 to 96.4%.

2.5. Statistical analysis

JMP12 software (Statistical Discovery v.12) was used to perform an Analysis of Variance (ANOVA) on cellular biomarker and qPCR data with Dunnett's tests to determine differences from Control; alpha was set at 0.05 for all tests, *a priori*. All data were normally distributed. NICB was used as the control site for the SC/NC assessment assays and *C. virginica* (0 μg/L) were used as controls in the laboratory-based zinc metal exposure assays. Average percent of coefficient of variation was tested across all tissue types and genes for all qPCR experiments and was calculated at ≤ 1.3% (MW *C. virginica* and Zn experiments).

3. Results and Discussion

C. virginica tissue samples collected throughout the Carolinas were used to compare cellular and molecular gill and HP tissue data with whole *C. virginica* tissue chemistry data (Supplementary Fig. 1). North Inlet Clambank (NICB) is a historical NOAA Reference Site located within the NOAA National Estuarine Research Reserve System (NERRS) (Sanger, et al., 1999 a,b). Thus, NICB was used as a comparison to all other field sites in this paper. Sites were classified as either Urban, Suburban, Undeveloped or Unknown based on previous determinations of land use type data (Apeti, et al., 2018) (Table 1). Land use categories have not yet been established for NC sites at the time of this paper, and thus were designated as unknown. Designations of land use can change over time as relevant information such as increasing population growth and land use becomes available (https://coast.noaa.gov/states/north-carolina.html, https://coast.noaa.gov/states/south-carolina.html).

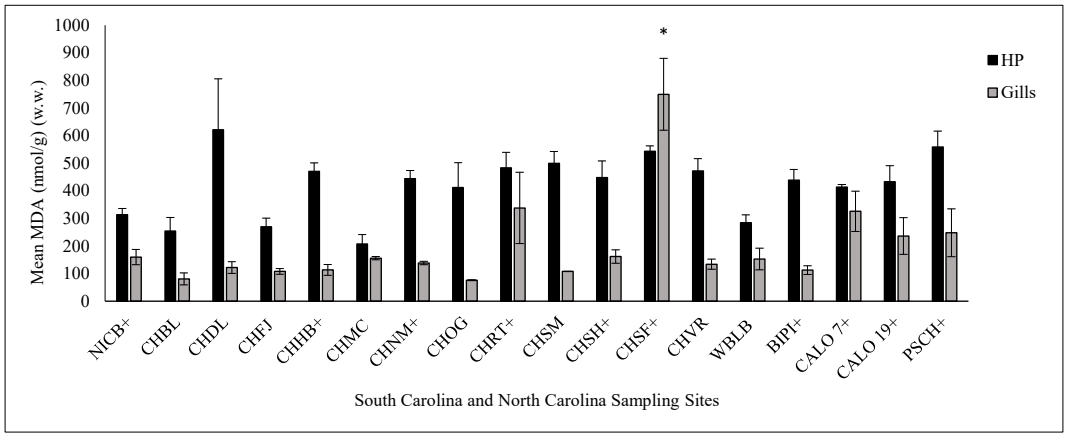

Fig. 1. Lipid Peroxidation in adult *Crassostrea virginica* collected along the South Carolina and North Carolina coasts. *C. virginica* from fourteen South Carolina Mussel Watch sites and four North Carolina sites had hepatopancreas (HP) tissue (black bar) and gill tissue (grey bar) analyzed for lipid peroxidation levels and recorded as MDA nmol/g tissue wet weight (w.w.). Higher levels of MDA in HP tissue are seen in most sites except for CHFJ, WBLB, CHBL and CHMC when compared to NICB, indicating cellular lipid bilayer damage. All sites except CHSF show higher levels of MDA in HP than Gill tissues. *C. virginica* collected from sites with (+) were used in both biomarker and molecular analysis. Statistical differences by one-way ANOVA analysis when compared to NICB is labeled by (*), $p = 0.05$. Bars represent mean ± standard error. $n = 3$ *C. virginica* per site, except CHVR $n = 2$.

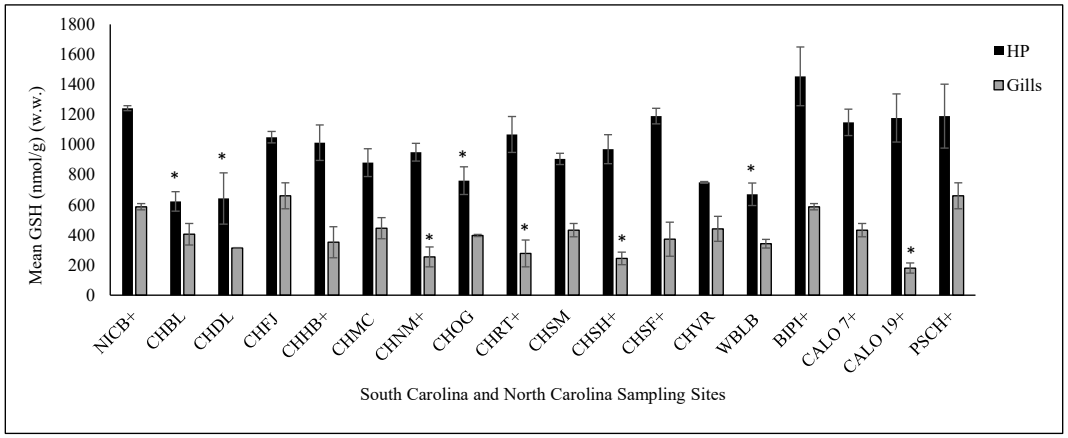

Fig. 2. Glutathione activity in adult *Crassostrea virginica* collected along the South Carolina and North Carolina coasts. *C. virginica* from fourteen South Carolina Mussel Watch sites and four North Carolina sites had hepatopancreas (HP) tissue (black bar) and gill tissue (grey bar) measured for glutathione activity as nmol/g tissue wet weight (w.w.). GSH levels for HP tissues were considered depleted if below 800 nmol/g. *C. virginica* collected from sites with (+) were used in both biomarker and molecular analysis. Statistical differences by one-way ANOVA analysis when compared to NICB is labeled by (*), $p = 0.05$. Bars represent mean ± standard error. $n = 3$ *C. virginica* per site, except CHVR $n = 2$.

Sites that are categorized as Undeveloped may have stressors such as shipping/boat traffic, higher concentrations of polycyclic aromatic hydrocarbons, contaminants of emerging concerns, or located downstream of industry waste run off (Lauenstein, 2002; Farrington et al., 2016). CHSM and CHHB for instance had site classification changes in 2018 as a result of increased urbanization in those areas (Sanger, et al., 1999a,b; Apeti et al., 2018). CALO7 site is near a historic military station and CALO19 site is near an historic abandoned town which is currently maintained by the United States National Park Service. (https://www.nps.gov/calo/learn/historyculture/portsmouth.htm). However, historic MW sites were strategically chosen to be distant from known point source contamination in order to give an accurate assessment of the natural area (Lauenstein, et al., 2002).

3.1. Biomarker analysis of field collected C. virginica

Two cellular biomarker assays were used to evaluate *C. virginica* tissue stress and detoxification in field collected samples: Lipid peroxidation and Glutathione activity. All eighteen South and North Carolina sites were evaluated for damage in *C. virginica* HP and gill tissues. Thirteen out of the eighteen sites showed mean HP levels of MDA higher than observed for the reference site, NICB (Fig. 1). Though not statistically significant, higher levels of MDA found in HP do reveal elevated levels of oxidative stress (>250 nmol/g) (Ringwood, et al., 2002). Mean gill MDA levels in CHSF *C. virginica* tissue was the only statically significant finding when compared to gill NICB levels and showed the most cellular damage when compared to all other sites. Qualitatively, four other (CHRT, CALO7, CALO19, PSCH) sites showed gill mean MDA levels higher than NICB, though not statically significant. Higher levels of measured MDA indicate that the gills of *C. virginica* at the CHSF site were negatively impacted by a stressor.

Detoxification activity was evaluated using the Glutathione (GSH) assay and measured in mean GSH nmol/g w.w. (Fig. 2). HP values between 800 and 1600 nmol/g are considered normal *C. virginica* HP levels (Ringwood, et al., 2002). *C. virginica* from thirteen sites fall within this range among HP tissues. Four SC sites: CHBL. CHDL, CHOG, and WBLB had statistically significantly lower mean values of GSH in HP, measured at < 800 nmol/g, signifying a depletion of this antioxidant. Compared to the reference site, four sites: CHNM, CHRT, CHSH and CALO19 had statistically significantly lower levels of gill mean GSH. However,

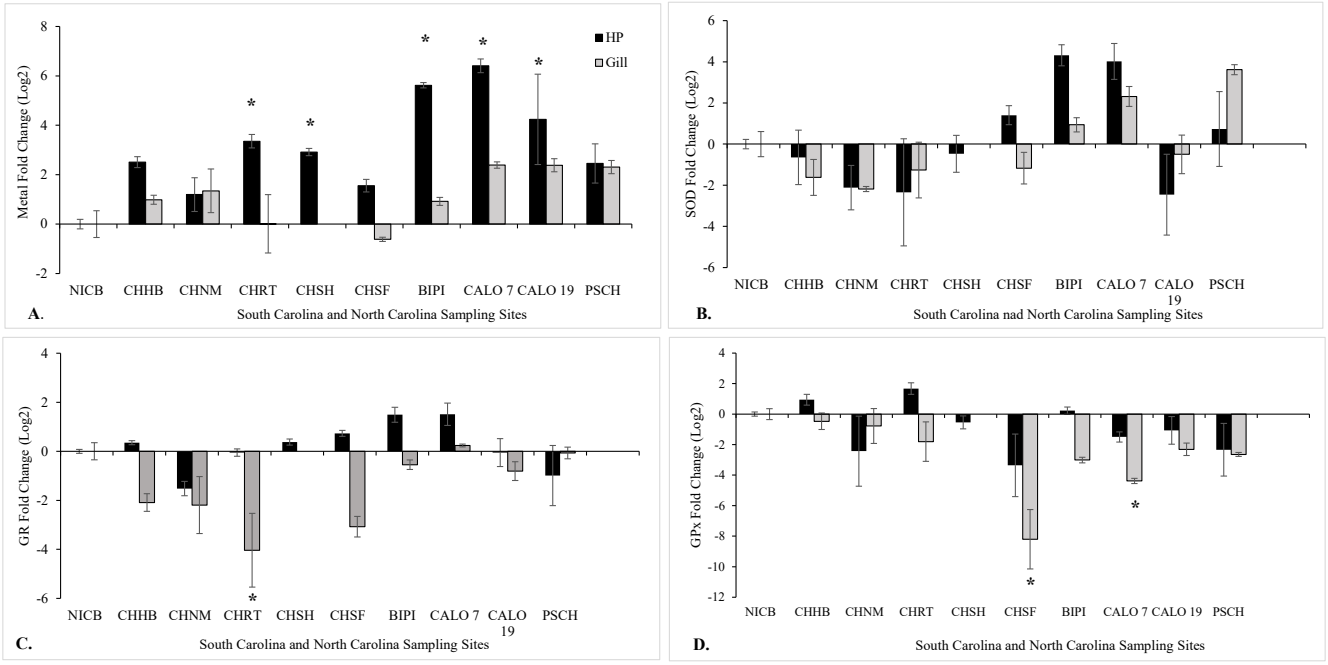

Fig. 3. Target gene fold change expression (log2) in adult *Crassostrea virginica* collected from Mussel Watch sites within South Carolina and North Carolina waters. Hepatopancreas (HP) tissue (black bar) and gill tissue (grey bar) were analyzed per gene tested. (A.) Metallothionein gene fold changes in both tissue types. Most sites show a significant gene upregulation in HP tissues when compared to NICB signifying a response to a metal exposure. (B.) Superoxide Dismutase displays differing gene expression based on location. Large upregulation is observed in NC sites. (C.) Glutathione Reductase gene suppression is most prevalent in gill tissue for SC sites, with CHRT gill expression showing significance. (D.) Glutathione Oxidase expression in gill and most HP tissue for all sites show suppression, with significance at CHSF and CALO7 gill tissue. Bars represent mean ± standard error. One-way ANOVA analysis determined statistical significance from control (*) $p = 0.05$. All sites HP $n = 3$, Gill sites: CHNM, CHRT, CHSF $n = 2$. CHSH gill data not included due to low RNA yields in gill samples.

fourteen sites in total had means lower than gill mean at NICB. Similar to our data, previous studies have shown baseline levels of GSH in HP tissues higher than those measured in gill tissues observed in filter feeding bivalves (Regoli and Principato, 1995; Regoli et al., 1997; McCarthy et al., 2013). The remaining sites showed normal levels of detoxification in the HP, including CHSF, which measured high levels of MDA in the HP and significantly high levels in the gills. These findings show most sites had *C. virginica* that were able to detoxify within normal ranges.

3.2. RT-qPCR analyzed field collected C. virginica

Genes involved in cellular damage (GPx), detoxification (SOD, GR), and general metal exposure (MET) were used to evaluate molecular changes in *C. virginica* tissues. MET gene expression (Fig. 3A) in HP tissue shows five sites with statistically significant upregulation among the ten sites analyzed. Two SC sites: CHRT (Undeveloped), CHSH (Urban), and three NC sites: CALO7 (Unknown), CALO19 (Unknown), BIPI (Unknown) had significant MET upregulation in HP tissues. Considering site classification (Table 1) CHRT is listed as undeveloped in the 1990's, though this area has been considerably developed since that designation. Likewise, the CALO7 and CALO19 sites are locations of a historic military base and a town, respectively. These site histories could have implications in adverse long-term effects within the environment.

SOD and GR are part of a group of antioxidants that protect against free-radical damage in bivalve tissues. Both genes have been used to evaluate stress in *C. virginica* within their natural environment and have important roles in the antioxidant pathway (Pie, et al., 2015; Shenai--Tirodkar, et al., 2017; Elia et al., 2020; Erturk Gurkan and Gurkan, 2021). Measured gene regulation associated with both SOD and GR (Fig. 3B and C) did not show any statistical differences in HP tissues, however SOD fold change showed a considerable >4-fold upregulation in CALO7 and BIPI tissue and a 2-fold upregulation in GR HP tissue for the same sites. The NC sites, CALO7 and BIPI, additionally had the greatest measured MET concentrations yet fell within normal ranges of LPx and GSH for HP tissue. This finding indicates that higher SOD gene activity could be complimentary to the detoxification process and could explain higher GSH levels for CALO7 and BIPI sites.

CHRT gill expression was significantly downregulated in GR gene expression and also showed a significant depletion of biomarker GSH in gill tissue (Fig. 2). Similarly, CHHB, CHNM, and CHSF in gill tissue all measured >2-fold down regulation in GR expression which corresponds with low GSH biomarker levels observed.

GPx has an important role in protecting against free radical damage in aquatic organisms (Elia, et al., 2020). All sites except two (CHHB and CHRT) were down regulated for both Gill and HP tissues, with two different sites (CHSF and CALO7) having significant downregulation in gill tissue. Likewise, (Fig. 1) shows CHSF and CALO7 also measuring the two highest mean levels of MDA recorded in gill tissue, respectively. The significant down regulation in gill tissue seen in these sites could be a response to control additional damage as observed in LPx gill biomarker results. This observed difference between antioxidation and gene regulation differences in GPx has also been documented in two scallop species, *Chlamys farreri* and *Patinopecten yessoensis* (Hlaing, et al., 2020).

3.3. Chemical tissue analysis on field collected C. virginica

Three metals: aluminum (Al), iron (Fe) and zinc (Zn) were the highest measured metals, in wet weight (w.w.), amongst the twenty-two metals tested within the Carolina sites (Supplementary Fig. 3 and Supplementary Fig. 7). CHBL and CHDL had the highest recorded Zn measurements of all sites examined. The elevated levels of Zn, in particular, is noted in our samples and further supports reported observations of high tolerance oysters have for this element (Luo, et al., 2017; Alexander et al., 2019). CHBL and CHDL also had significant depletion of GSH in the HP (Fig. 1). Of the MW subsites examined for molecular analysis:

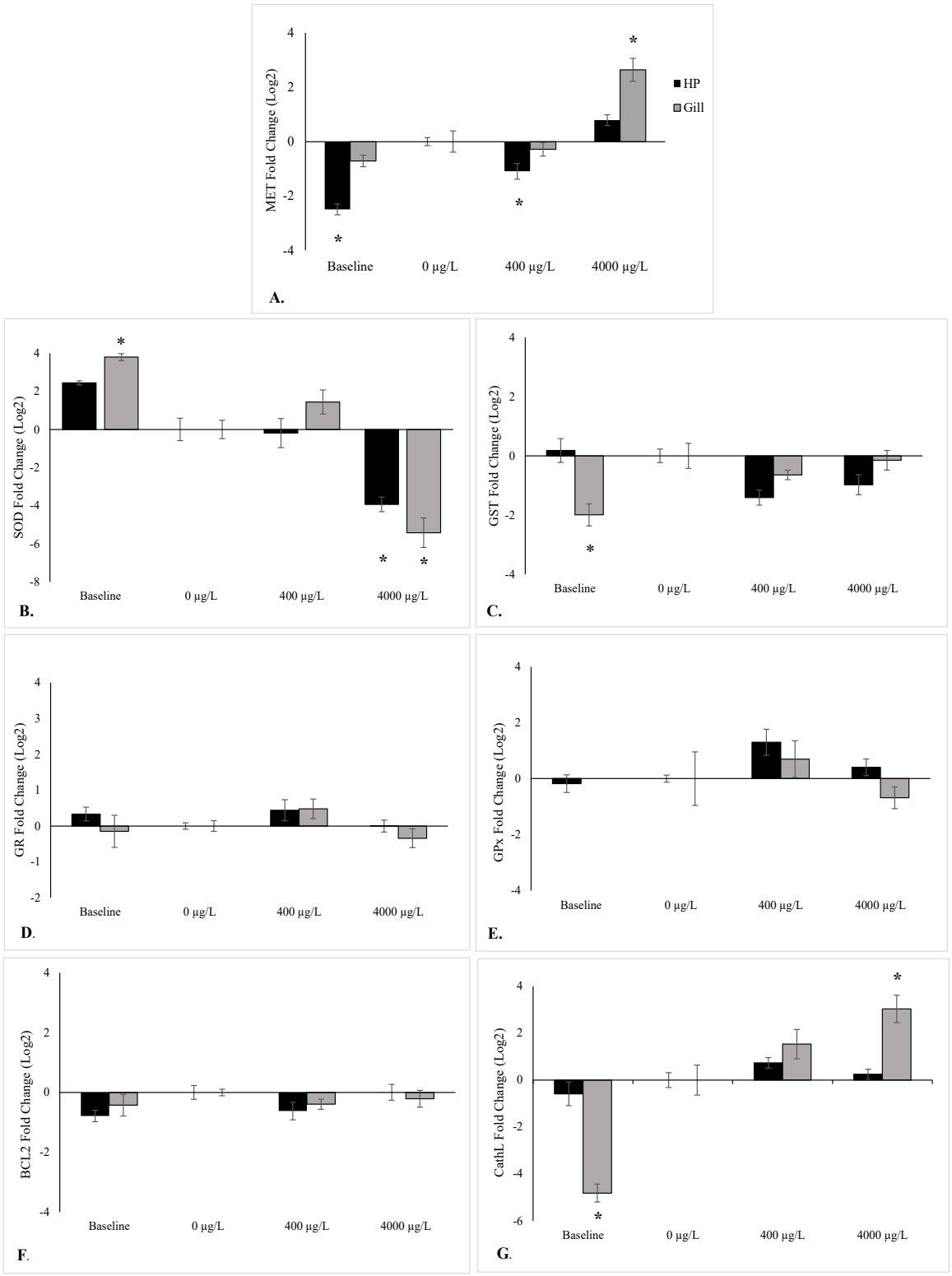

Fig. 4. Target gene fold change expression (log2) in adult *Crassostrea virginica* 96 h Zn exposure. *C. virginica* exposed to zinc treatment at 0 µg/L, 400 µg/L and 4000 µg/L. Baseline *C. virginica* dissected prior to experimental treatment. Hepatopancreas (HP) tissue (black bar) and gill tissue (grey bar) were analyzed per gene tested. Statistical outliers were removed from analysis. All treatment groups $n = 6$ unless otherwise noted. (A) Metallothionein HP: 4000 µg/L $n = 5$, Gill: Baseline, Control and 4000 µg/L groups $n = 5$ (B) Superoxide Dismutase HP: 4000 µg/L $n = 5$, Gill: Baseline and 4000 µg/L treatments $n = 5$,(C) Glutathione S Transferase Gill: Baseline and 400 µg/L treatments $n = 5$, Control $n = 4$ (D) Glutathione Reductase, Gill: Control $n = 4$, Baseline and 400 µg/L treatments $n = 5$ (E) Glutathione Oxidase HP: Baseline $n = 5$, Gill: 400 µg/L $n = 5$, Baseline removed due to high variability between samples (F) B Cell Lymphoma 2 (BCL2) Gill: Baseline and 400 µg/L treatments $n = 5$, Control $n = 4$, (G) Cathepsin L Gill: Baseline $n = 3$, Control and 400 µg/L $n = 5$. Bars represent mean ± standard error. One-way ANOVA analysis determined statistical significance from control (*).

CHHB, CHRT, and CHSH, and CALO19 further show a >2-fold up regulation in HP MET as well as elevated trace element concentrations (Fig. 3 and Supplementary 7).

3.4. Effects of zinc laboratory exposure

Due to complex chemical mixtures and natural environmental stressors that *C. virginica* live within, a single metal experiment was conducted applying all methods used in field monitoring: chemical analysis, cellular and molecular biomarkers.

Zinc was chosen as the exposure metal due to its abundance in field tissue data (Supplementary Fig. 3) and for its anthropogenic implications. Previous studies examining *C. virginica* along the East coast of the US reported *C. virginica* harboring more than ten-fold higher trace elements (Ag, Cu, Zn) compared to the mussel *Mytilus edulis* (Lauenstein, et al., 2002). Cellular biomarkers (LPx and GSH) and an extended set of qPCR markers were used to examine zinc exposed *C. virginica*. Baseline *C. virginica* were not exposed to experimental conditions and were dissected after all *C. virginica* completed the four-day deputation phase.

LPx HP levels showed no significance between treatment groups (Supplementary Fig. 4). Similar LPx baseline *C.virginica* HP levels have been reported (Aquilina-Beck, et al., 2020). Though the highest treatment (4000 μg/L) did exhibit HP MDA above >250 nmol/g, these levels were similarly recorded for the reference site NICB. Gill tissue showed no difference in mean values and were within the same values seen for NICB and previous control values reported in literature (McCarthy, et al., 2013). Baseline *C. virginica* showed a significantly lower level of MDA in HP tissue and therefore was not exhibiting stress within the lipid bilayer.

GSH results similarly yielded no significant difference between treatment groups for each tissue type (Supplementary Fig. 5). All treatments were within the normal accepted values for HP tissue.

Analysis of Zn seawater chemical concentrations corresponded with nominal dosing concentrations however, *C. virginica* tissue concentrations were not linear with increasing Zn concentrations (Supplementary Fig. 6). Though there was a decrease in concentrated Zn after 24 h in seawater, increased Zn concentration levels were not observed in tissues. Control field site collected *C. virginica* had measured Zn tissue concentrations of 210 μg/g (w.w.) which when compared to MW sites is higher than NICB, but lower than other SC sites examined (Supplementary Fig. 6 and Supplementary Fig. 7). Exposed *C. virginica* at all experimental Zn treatments had Zn tissue concentrations close to that of the field collected *C. virginica*.

Three additional molecular markers: GST, BCL2 and CathL, were added in order to gain insight into Zn exposure results (Fig. 4).

MET (Fig. 4A) showed a significant upregulation in gills at the highest dose and a small upregulation in HP tissue, indicating that a Zn concentration of 4000 μg/L was enough to elicit a gill tissue response in this gene. The low dose however, did not show any MET gene activation in either tissue type at 96 h exposure. Conversely, in the field collected MW data, MET showed significant upregulation in HP tissue (Fig. 3). This tissue specific response could be due to long term exposure effects in the field combined with the HP role for accumulating toxins (Ringwood, et al., 2009). MET in baseline *C. virginica* was significantly downregulated after depuration in clean seawater.

Zn is a known inhibitor of NADPH which results in lowering reactive oxygen species in a cell. Zn also is a cofactor in regulating the transcriptional induction of SOD (Prasad, 2014). At the low dose of 400 μg/L SOD shows a slight upregulation in gill tissue (Fig. 4B). However, there is a significantly strong downregulation response in both the HP and gill tissue at 4000 μg/L. It is possible that because of elevated concentrations of Zn in the tissues, the activation of SOD was not required due to the protective effects Zn has for lowering the amount of free radicals available in the cell. This same observation of decreased SOD levels with increasing Zn concentrations is documented in another study with *C. virginica* (Alexander, et al., 2019). Additional genes that compliment GSH and LPx markers: GST, GR and GPx all had no significant change from control values, further indicating the lack of oxidative stress available to generate a response. This absence of available reactive oxygen species in the tissues is confirmed with normal levels of biomarker results previously discussed. However, when BCL2 and CathL were analyzed in gill tissue, the high treatment group did show a significant upregulation in CathL activity. This upregulation of CathL is indicative of initiating cell apoptosis and therefore cell damage in gill tissue. Cathepsins are highly involved in maintaining cell homeostasis and are among the most abundant lysosomal proteases in a cell (Yadati, et al., 2020). Cathepsin L in particular, is found in both the cytosol and the nuclease of a cell, regulating the cell cycle and involved in Transcription Growth Factor beta (TGF-β) signaling (Yadati, et al., 2020). BCL2, also found in the cytosol, is involved in restricting apoptotic activity and preventing cell proliferation by initiating the G_0 phase of the cell cycle (Kulsoom, et al., 2018). These two proteases show opposing gene function. The apoptosis marker, CathL, shows significant upregulation at the high Zn treatment dose as BCL2 shows down regulation, demonstrating the importance of including these markers as tools in determining effects of a toxic exposure. Future experiments should include these additional genes to document apoptosis events in chronic exposures used in environmental monitoring among sentinel populations. While the acute Zn exposure experiment showed < 2-fold upregulation in both tissues for the SOD, GST, GR and GPx genes, the anti and pro cell death genes were valuable to determine detrimental effects in gill tissue. CathL and MET responses together confirm that the *C. virginica* were responding to and had adverse effects of Zn exposure in gill tissue.

The 96 h Zn exposure may have been too short of a time period to perceive decisive changes in gene regulation within in the HP. However, data obtained from this acute exposure demonstrates that gills have an immediate regulatory role, while consequences of longer environmental exposures are more apparent in HP tissues, demonstrated by the field *C. virginica* tissue data. Insights into exposure effects are observed when comparing sensitivities of gill versus HP tissues. Gills, the first tissue to be exposed to environmental stressors, are often the first responders to adverse conditions whereas the HP tissue is responsible for toxin accumulation, metabolism and immune defense (McCarthy, et al., 2013; Bouallegui et al., 2017; Shenai-Tirodkar, et al., 2017; Erturk Gurkan and Gurkan, 2021). As exposure progresses, the HP accumulates the toxins and either sufficiently removes these from the tissue or becomes overwhelmed depending on the length and toxicity of the stressor.

4. Conclusion

NOAA's MW program's objective is to document changes in chemical contamination status within the Great Lakes and coastal waters of the United States through environmental monitoring, assessment and related research (Kimbrough, et al., 2008). Repeat monitoring of the same coastal areas delivers temporal site information which provides valuable comparisons of changing environments, confirms clean reference sites, and evaluates land use classifications. The addition of bivalve health metrics allows comparisons of chemical contaminant levels to biological effects.

The specific aim of this study was to access the efficacy and use of gene specific primers using qPCR techniques as an additional tool for environmental monitoring. These markers showed a higher sensitivity and specificity to acute responses as well as providing metrics on tissue specific responses. These data are valuable for frequent environmental monitoring and are important in assessing impacts of environmental change, such as after an oil spill or hurricane. A complete assessment of MW SC/NC sites was beyond the scope of this paper, however incorporating the molecular data from this study in a subsequent complete report will aid in determining site specific health; especially in sites monitored for the first time this year such as North Carolina CALO7 and CALO19. For regional monitoring, molecular primers would need to be specifically developed for each sentential species examined, for

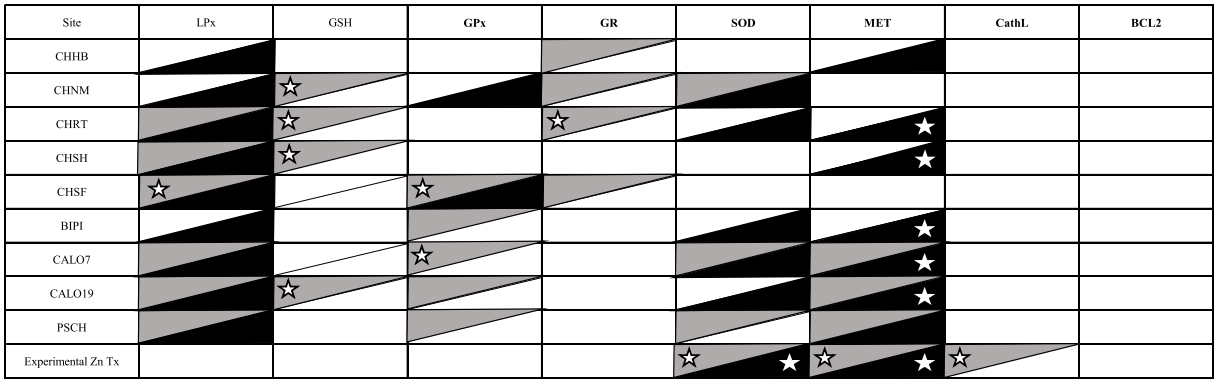

Fig. 5. Summary of combined cellular and molecular biomarkers data within subsampled sites and experimental Zn treatment. Black triangles represent HP tissue data. Grey triangles represent gill tissue data. Stars are data that are statistically significant. Biomarker LPx black triangles are values higher than NICB HP value of 300 nmol/g, and grey triangles are higher than NICB gill tissue value of 160 nmol/g. Biomarker GSH grey stared triangles are values statistically significantly from NICB tissue. Bold font represents qPCR data for which the shaded triangles are values that are above or below 2-fold (log2) gene expression change from either NICB values or control values in experimental Zn test.

instance: *Mytilus edulis* (North Atlantic, USA); *M. californianus* (California), *Chama sinuosa* (Florida Keys); *C. rhizophorae (Puerto Rico); Dreissena polymorpha* and *D. bugensis* (Great Lakes); and *Ostrea sandvicensis* (Hawaii) (Lauenstein, et al., 2002). However, once markers are developed, applying these to multiple PCR methods would provide valuable, gene specific data. Molecular technology is evolving and the use of advanced methods such as digital droplet PCR (ddPCR) provides absolute quantitative data which can then be specifically compared to similar regions and other monitoring data (Lopez-Landavery, et al., 2023). As this is the first use of qPCR techniques within Coastal Carolina MW sites, future subsequent monitoring within the same sites would be useful to compare with these presented results in order to reveal changing oyster community health. Some studies have shown the potential for oysters to develop tolerance and show genetic adaptation to changing environments (Yingprasertchai, et al., 2017; Wang et al., 2018). Future MW monitoring will be able to detect yearly differences within an oyster bed due to data presented in this paper.

We have shown that using specifically designed molecular markers to target stress response in the sentential specie *C. virginica* are sensitive enough to reveal slight changes in gene expression. Thus, gaining insight into subtle responses indiscernible using cellular biomarker techniques. Similar studies have shown molecular techniques enhance the identification of slight molecular change otherwise undetectable in traditional toxicity endpoints (Poynton, et al., 2008; Guo et al., 2021). Molecular endpoints provide numerical data for which consistent environmental monitoring can be compared. These additional metrics combined with physical, chemical and biological data can document changing environmental conditions. Fig. 5 summarizes the combined biomarker and molecular data discussed in this paper and demonstrates how molecular targets can further clarify sentinel bivalve shellfish tissue responses in both complex environments and acute exposures. Targeting molecular pathways can provide detailed information of organism health. Combining the data from multiple monitoring techniques identifies *C. virginica* response within a complex mixture environment, thereby, potentially revealing early warning signs of damage. Expanding the use of molecular biomarkers in field monitoring programs would be a valuable tool to improve assessments of chemical contaminant exposure and ecosystem health.

Author contributions

Allisan Aquilina-Beck: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data Curation, Writing- original draft, review and editing, Visualization, Project Administration; Lou Ann Reed: Investigation, Data Curation, Validation, Writing review; Mary Rider: Investigation, Resources, Visualization; William Burdine: Investigation; James Daugomah: Investigation; Dennis Apeti: Investigation, Writing Review; Peter Key: Methodology, Investigation, Writing Review, Supervision; Marie DeLorenzo: Conceptualization, Methodology, Writing Review, Supervision.

Funding

This research was funded in part by the US Department of the Interior, United States; National Park Service (Grant Number# P20PG00222) for work conducted in CALO, NC.

Ethics statement

Oysters were collected along the South Carolina and North Carolina coastline according to the SC Department of Natural Resources Marine Licensing and Permitting rules (Scientific Permit Number: ZZ1123000164), NC Division of Marine Fisheries (Permit number: 714162), and US Department of the Interior, National Park Service, Cape Lookout (Permit number: CALO-2021-SCI-0019).

Oysters collected for this research were handled according to the National Research Council's Guide for the Care and Use of Laboratory Animals. National Academies Press, Washington, D.C. (http://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to sincerely thank Dr. Thomas Kean (UCF, Orlando, FL) and Dr. Thomas Greig (NOAA, Charleston, SC) for their molecular troubleshooting assistance. Thank you also to the other NOAA, Charleston, SC staff: Dr. Paul Pennington for the much-appreciated statistical assistance; Dr. Phil Tanabe for assistance with *C. virginica* gill dissection, and Blaine West and Raven Ferguson for aid in *C. virginica* collection. Thank you to all the internal reviewers for their

time and suggestions. Finally, we acknowledge the scientists that help with the larger scope of Mussel Watch data generation: Dr. Ed Wirth, Emily Pisarski, Brian Shaddrix, and Katy Chung. Special thanks for the corporation of the US National Park Service specifically: Jeffery West, Jon Altman, and Tracy Ziegler.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.116817.

References

Alexander, T.C., Han, F.X., Arslan, Z., Tchounwou, P.B., 2019. Toxicity of as in Crassostrea virginica (Gmelin, 1791) from the Northern Gulf of Mexico at the presence of Zn and its antioxidant defense mechanisms. Ecotoxicol. Environ. Saf. 172, 514–522.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., et al., 2010. Adverse Outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–741.

Apeti, D.A., Johnson, E., Kimbrough, K., Lauenstein, G., 2012. National status and trends mussel Watch program. Sampl. Methods 2012 Update 134, 39.

Apeti, D.A., Wirth, E., Leight, A., Mason, A., Pisarski, E., 2018. An assessment of contaminants of emerging concern in chesapeake bay, MD and Charleston harbor. SC 240, 120.

Aquilina-Beck, A.A., Reiner, J.L., Chung, K.W., DeLise, M.J., Key, P.B., DeLorenzo, M.E., 2020. Uptake and biological effects of Perfluorooctane Sulfonate exposure in the adult eastern oyster Crassostrea virginica. Arch. Environ. Contam. Toxicol. 79, 333–342.

Bouallegui, Y., Ben Younes, R., Bellamine, H., Oueslati, R., 2017. Histopathological indices and inflammatory response in the digestive gland of the mussel Mytilus galloprovincialis as biomarker of immunotoxicity to silver nanoparticles. Biomarkers 1–11.

DeLorenzo, M.E., Serrano, L., Chung, K.W., Hoguet, J., Key, P.B., 2006. Effects of the insecticide permethrin on three life stages of the grass shrimp, Palaemonetes pugio. Ecotoxicol. Environ. Saf. 64, 122–127.

Edwards, A.C., Sinclair, A.H., Coull, M., Crooks, W., Rees, R.M., Lumsdon, D.G., 2016. Regional trends in Scottish advisory soil acidity and phosphorus results: significance of management history, land use and soil attributes. Soil Use Manag. 32, 44–53.

Elia, A.C., Burioli, E., Magara, G., Pastorino, P., Caldaroni, B., Menconi, V., Dorr, A.J.M., Colombero, G., Abete, M.C., Prearo, M., 2020. Oxidative stress ecology on Pacific oyster Crassostrea gigas from lagoon and offshore Italian sites. Sci. Total Environ. 739, 1398 1386.

Erturk Gurkan, S., Gurkan, M., 2021. Toxicity of gamma aluminium oxide nanoparticles in the Mediterranean mussel (Mytilus galloprovincialis): histopathological alterations and antioxidant responses in the gill and digestive gland. Biomarkers 26, 248–259.

Farrington, J.W., Tripp, B.W., Tanabe, S., Subramanian, A., Sericano, J.L., Wade, T.L., Knap, A.H., 2016. Edward D. Goldberg's proposal of "the mussel Watch": reflections after 40 years. Mar. Pollut. Bull. 110.

Fasulo, S., Mauceri, A., Giannetto, A., Maisano, M., Bianchi, N., Parrino, V., 2008. Expression of metallothionein mRNAs by in situ hybridization in the gills of Mytilus galloprovincialis, from natural polluted environments. Aquat. Toxicol. 88, 62–68.

Geffard, A., Amiard-Triquet, C., Amiard, J.C., 2005. Do seasonal changes affect metallothionein induction by metals in mussels, Mytilus edulis? Ecotoxicol. Environ. Saf. 61, 209–220.

Guo, H., Liang, Z., Zheng, P., Li, L., Xian, J., Zhu, X., 2021. Effects of nonylphenol exposure on histological changes, apoptosis and time-course transcriptome in gills of white shrimp Litopenaeus vannamei. Sci. Total Environ. 781, 146731.

Hlaing, S., Lou, J., Cheng, J., Xun, X., Li, M., Lu, W., Hu, X., Bao, Z., 2020. Tissue-biased and species-specific regulation of glutathione peroxidase (GPx) genes in scallops exposed to toxic Dinoflagellates. Toxins 13, 21.

Holland, A.F., Sanger, D.M., Gawle, C.P., Lerberg, S.B., Santiago, M.S., Riekerk, G.H.M., Zimmerman, L.E., Scott, G.I., 2004. Linkages between tidal creek ecosystems and the landscape and demographic attributes of their watersheds. J. Exp. Mar. Biol. Ecol. 298, 151–178.

Jenny, M.J., Warr, G.W., Ringwood, A.H., Baltzegar, D.A., Chapman, R.W., 2006. Regulation of metallothionein genes in the American oyster (Crassostrea virginica): ontogeny and differential expression in response to different stressors. Gene 379, 156–165.

Khan, B., Adeleye, A.S., Burgess, R.M., Smolowitz, R., Russo, S.M., Ho, K.T., 2019. A 72-h exposure study with eastern oysters (Crassostrea virginica) and the nanomaterial graphene oxide. Environ. Toxicol. Chem. 38, 820–830.

Kimbrough, K.L., Johnson, W.E., Lauenstein, G.G., Christensen, J.D., Apeti, D.A., 2008. An assessment of two Decades of contaminant monitoring in the nation's coastal zone. NOAA Tech. Memo. NOS NGS 74, 105.

Kuchovska, E., Morin, B., Lopez-Cabeza, R., Barre, M., Gouffier, C., Blahova, L., Cachot, J., Blaha, L., Gonzalez, P., 2021. Comparison of imidacloprid, propiconazole, and nanopropiconazole effects on the development, behavior, and gene expression biomarkers of the Pacific oyster (Magallana gigas). Sci. Total Environ. 764, 142921.

Kulsoom, B., Shamsi, T.S., Afsar, N.A., Memon, Z., Ahmed, N., Hasnain, S.N., 2018. Bax, Bcl-2, and Bax/Bcl-2 as prognostic markers in acute myeloid leukemia: are we ready for Bcl-2-directed therapy? Cancer Manag. Res. 10, 403–416.

Lauenstein, G., 2002. Contaminant trends in US national estuarine research reserves. In: Quality NNSaTPfME, Editor. NS&T Silver Spring, MD.

Lauenstein, G., Cantillo, A.Y., O'Connor, T.P., 2002. The status and trends of trace element and organic contaminants in oysters, Crassostrea virginica, in the waters of the Carolinas, USA. Sci. Total Environ. 285, 79–87.

Liu, F., Rainbow, P.S., Wang, W.X., 2013. Inter-site differences of zinc susceptibility of th eoyster Crassostrea hongkongensis. Aquat. Toxicol. 26–33.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Lopez-Landavery, E., Galindo-Sanchez, C.E., Lopez-Galindo, L.L., Ramirez-Alvarez, N., Saavedra-Flores, A., Amador-Cano, G., Ventura-Lopez, C., San Martin-Del Angel, P., Hernanddez-Herrera, R.I., 2023. Hydrocarbons occurrence and transcriptomic response of oyster Crassostrea virginica from Lagoons of the Southern Gulf of Mexico. Front. Mar. Sci. 19.

Luo, L., Zhang, Q., Kong, X., Huang, H., You, W., Ke, C., 2017. Differential effects of zinc exposure on male and female oysters (Crassostrea angulata) as revealed by label-free quantitative proteomics. Environ. Toxicol. Chem. 36, 2602–2613.

McCarthy, M.P., Carroll, D.L., Ringwood, A.H., 2013. Tissue specific responses of oysters, Crassostrea virginica, to silver nanoparticles. Aquat. Toxicol. 138–139, 123–128.

Mottin, E., Caplat, C., Latire, T., Mottier, A., Mahaut, M.L., Costil, K., Barillier, D., Lebel, J.M., Serpentini, A., 2012. Effect of zinc sacrificial anode degradation on the defence system o fhte Pacific oyster, Crassostrea gigas: chronic and acute exposures. Mar. Pollut. Bull. 64.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95.

Pie, H., Schott, E.J., Mitchelmore, C.L., 2015. Investigating physiological, cellular and molecular effects in juvenile blue crab, Callinectus sapidus, exposed to field-collected sediments contaminated by oil from the Deepwater Horizon Inciden. Sci. Total Environ. 532, 528–539.

Poynton, H.C., Wintz, H., Vulpe, C.D., 2008. Progress in ecotoxicogenomics for environmental monitoring, mode of action, and toxicant identification. Adv. Exp. Biol. 2, 322–323.

Prasad, A.S., 2014. Zinc: an antioxidant and anti-inflammatory agent: role of zinc in degenerative disorders of aging. J. Trace Elem. Med. Biol. 28, 364–371.

Rahman, M.S., Rahman, M.S., 2021. Effects of elevated temperature on prooxidant-antioxidant homeostasis and redox status in the American oyster: signaling pathways of cellular apoptosis during heat stress. Environ. Res. 196, 110428.

Rashid, K., Sinha, K., Sil, P.C., 2013. An update on oxidative stress-mediated organ pathophysiology. Food Chem. Toxicol. 62, 584–600.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, Mytilus galloprovincialis, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat. Toxicol. 31.

Regoli, F., Nigro, M., Bertoli, E., Principato, G., Orlando, E., 1997. Defenses againstoxidative stress in the Antarctic scallop Adamussium colbecki and effects of acute exposure to metals. Hygrobiology 355, 139–144.

Ringwood, A.H., Hoguet, J., Keppler, C.J., 2002. Seasonal variation in lysosomal destabilization in oysters, Crassostrea virginica. Mar. Environ. Res. 54, 793–797.

Ringwood, A.H., Hoguet, J., Keppler, C.J., Gielazyn, M.L., Ward, B.P., Rourk, A.R., 2003. In: TCIfCaEE, Technology (Ed.), Cellular Biomarkers (Lysosomal Destabilization, Glutathione and Lipid Peroxidation) in Three Common Estuarine Species: A Methods Handbook, p. 49.

Ringwood, A.H., Levi-Polyachenko, N., Carroll, D.L., 2009. Fullerene exposures with oysters: embryonic, adult, and cellular responses. Environ. Sci. Technol. 43, 7136–7141.

Sanger, D.M., Holland, A.E., Scott, G.I., 1999a. Tidal creek and salt marsh sediments in South Carolina coastal estuaries: I. Distribution of trace metals. Arch. Environ. Contam. Toxicol. 37, 445–457.

Sanger, D.M., Holland, A.F., Scott, G.I., 1999b. Tidal creek and salt marsh sediments in South Carolina coastal estuaries: II. Distribution of organic contaminants. Arch. Environ. Contam. Toxicol. 37, 458–471.

Savaskan, N.E., Ufer, C., Kuhn, H., Borchert, A., 2007. Molecular biology of glutathione peroxidase 4: from genomic structure to developmental expression and neural function. Biol. Chem. 388, 1007–1017.

Shenai-Tirodkar, P.S., Gauns, M.U., Mujawar, M.W.A., Ansari, Z.A., 2017. Antioxidant responses in gills and digestive gland of oyster Crassostrea madrasensis (Preston) under lead exposure. Ecotoxicol. Environ. Saf. 142, 87–94.

Thompson, E., Taylor, D., Nair, S.V., Birch, G., Haynes, P.A., Raftos, D.A., 2012. Proteomic discovery of biomarkers of metal contamination in Sydney Rock oyster (Saccostrea glomerata). Aquat. Toxicol. 109, 202–212.

U.S. EPA, 1992. Method 3005A (SW-846): Acid Digestion of Waters for Total Recoverable or Dissolved Metals for Analysis by Flame AA or ICP Spectroscopy [Internet]. U.S. Environmental Protections Agency, Washington, DC. Available from: https://www.epa.gov/sites/default/files/2015-12/documents/3005a.pdfU.S.EPA. 1994.Method200.8.

U.S. EPA, 1994. Method 200.8 (SAM): Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma-Mass Spectrometry [Internet]. US Environmental Protections Agency, Washington, DC. Available from: https://www.epa.gov/sites/default/files/2015-06/documents/epa-200.8.pdf.

U.S. EPA, 1996. Method 3050B (SW-846): Acid Digestion of Sediments, Sludges, and Soils [Internet]. US Environmental Protections Agency, Washington, DC U.S. EPA. Available from: http://www.epa.gov/sites/production/files/2015-06/documents/epa-3050b.pdf.

U.S. EPA, 2014. Method 6010D (SAM): Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Revision 4. [Internet]. US Environmental Protections Agency, Washington, D.C. Available from: https://www.epa.gov/sites/default/files/2015-12/documents/6010d.pdf.

Wang, W.X., Meng, J., Weng, N., 2018. Trace metals in oysters: molecular and cellular mechanisms and ecotoxicological impacts. Environ. Sci. Process. Impacts 20, 892–912.

Wu, G., Fang, Y.Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492.

Yadati, T., Houben, T., Bitorina, A., Shiri-Sverdlov, R., 2020. The ins and outs of cathepsins: physiological function and role in Disease management. Cells 9.

Yingprasertchai, T., Yu, R.M.K., O'Connor, W.A., Hopwood, T., MacFarlane, G.R., 2017. Acclimatory processes are likely responsible for metal tolerance in oyster embryos. Mar. Environ. Res. 127, 49–61.