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Interactive effects of cadmium and hypoxia on metabolic responses and bacterial loads of eastern oysters *Crassostrea virginica* Gmelin

Anna V. Ivanina [a], Brett Froelich [a], Tiffany Williams [a], Eugene P. Sokolov [b], James D. Oliver [a], Inna M. Sokolova [a,*]

[a] Biology Department, University of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC 28223, United States
[b] Department of General Surgery, Carolinas Medical Center, 1000 Blythe Blvd., Charlotte, NC 28203-5871, United States

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ABSTRACT

Pollution by toxic metals including cadmium (Cd) and hypoxia are important stressors in estuaries and coastal waters which may interactively affect sessile benthic organisms, such as oysters. We studied metabolic responses to prolonged hypoxic acclimation (2 weeks at 5% O_2) in control and Cd-exposed (30 d at 50 μg L^{-1} Cd) oysters *Crassostrea virginica,* and analyzed the effects of these stressors on abundance of *Vibrio* spp. in oysters. Hypoxia-acclimated oysters retained normal standard metabolic rates (SMR) at 5% O_2, in contrast to a decline of SMR observed during acute hypoxia. However, oysters spent more time actively ventilating in hypoxia than normoxia resulting in enhanced Cd uptake and 2.7-fold higher tissue Cd burdens in hypoxia. Cd exposure led to a significant decrease in tissue glycogen stores, increase in free glucose levels and elevated activity of glycolytic enzymes (hexokinase and aldolase) indicating a greater dependence on carbohydrate catabolism. A compensatory increase in activities of two key mitochondrial enzymes (citrate synthase and cytochrome c oxidase) was found during prolonged hypoxia in control oysters but suppressed in Cd-exposed ones. Cd exposure also resulted in a significant increase in abundance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* levels during normoxia and hypoxia, respectively. Overall, Cd- and hypoxia-induced changes in metabolic profile, Cd accumulation and bacterial flora of oysters indicate that these stressors can synergistically impact energy homeostasis, performance and survival of oysters in polluted estuaries and have significant consequences for transfer of Cd and bacterial pathogens to the higher levels of the food chain.

1. Introduction

Anthropogenic pollution and oxygen depletion are serious environmental problems in marine habitats worldwide. Reduced oxygen levels (hypoxia) or complete lack of oxygen (anoxia) typically occur in the near-bottom waters of the coastal zones and estuaries and are often associated with anthropogenic eutrophication fueling algal blooms, microbial respiration and oxygen depletion (Diaz and Rosenberg, 2008). In severe cases, such oxygen depletion may result in formation of the "dead zones" characterized by mass mortalities of benthic organisms. However, even moderate hypoxia may result in significant physiological and behavioral disturbances of the resident biota and lead to negative consequences for their growth, reproduction and long-term population survival (Baker and Mann, 1992; Diaz and Solow, 1999; Rabalais et al., 1999).

Periodical oxygen deprivation in estuaries and coastal waters often co-occurs with other stressors such as metal pollutants that can interactively affect physiology of benthic organisms. A trace metal cadmium (Cd) is an important persistent pollutant that is highly toxic, widely distributed in the environments and can adversely affect organisms at relatively low concentrations (Strydom et al., 2006). As a major water pollutant, it can accumulate in marine organisms and induce detrimental physiological effects such as metabolic or osmoregulatory dysfunction (Lionetto et al., 1998, 2000; Sokolova and Lannig, 2008). One of the key aspects of Cd toxicity is its strong inhibitory effect on aerobic metabolism and mitochondrial function that results in cellular energy deficiency and oxidative stress (Stohs and Bagchi, 1995; Sokolova, 2004; Lannig et al., 2006a, 2008; Cherkasov et al., 2007). Cd exposure can also suppress anaerobic metabolism in facultative anaerobes such as intertidal bivalves and impair their ability to recover after anoxic stress (Strydom et al., 2006; Kurochkin et al., 2009). The strong impact of Cd on energy metabolism makes it a prime candidate for interference with other stress tolerance mechanisms that critically

* Corresponding author. Tel.: +1 704 687 8532; fax: +1 704 687 3128.
E-mail address: isokolov@uncc.edu (I.M. Sokolova).

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depend on metabolic regulation and maintenance of energy homeostasis. Indeed, synergistic effects of Cd and temperature stress has been shown in a variety of aquatic ectotherms where Cd exposure led to a reduction in thermal tolerance and *vice versa* (review in: Sokolova and Lannig, 2008). In contrast, the interactive effects of Cd exposure and hypoxia on marine organisms are not well understood. Given the widespread coastal pollution and an alarming rate of increase of the coastal dead zones (5.54% year^{-1}) (Vaquer-Sunyer and Duarte, 2008), more research is urgently needed to understand the interactive impacts of hypoxia and metal pollutants on benthic organisms.

Eastern oysters *Crassostrea virginica* are ecologically and economically important species serving as ecosystem engineers in western Atlantic estuaries. Oysters are exposed to Cd via the contact with water, sediment and contaminated food (predominantly algae) in polluted estuaries and can accumulate high levels of Cd in their bodies making them susceptible to the toxic effects of this metal (Roesijadi, 1996; O'Connor and Lauenstein, 2006). Oysters play a key role in the dynamics of Cd in estuarine ecosystems serving as major vectors for accumulation and trophic transfer of this metal (Pigeot et al., 2006). They also serve as vectors for several important bacterial pathogens of humans and other vertebrates including highly pathogenic *Vibrio* spp. such as *Vibrio cholerae, Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (Thompson and Swings, 2006). Like other marine intertidal bivalves, oysters are among the animal champions of hypoxia tolerance. They are routinely exposed to oxygen-deficient conditions during air exposure at low tide as well as to prolonged periods of hypoxia or anoxia that can last from several days to several weeks in estuarine and coastal "dead zones" (Diaz and Solow, 1999; Greenway and Storey, 1999; Lenihan et al., 2001). The mechanisms responsible for a short-term endurance of hypoxia and anoxia are well studied in intertidal mollusks and involve reversible metabolic arrest, maintenance of the large reserves of fermentable fuels such as glycogen or aspartate, and alternative pathways of fermentative metabolism that increase ATP yield per unit metabolized substrate (Larade and Storey, 2002; David et al., 2005). In contrast, little is known about the potential effects of prolonged hypoxia and Cd exposure on oyster physiology and metabolism.

The goals of this study were to determine the interactive effects of prolonged hypoxia and Cd exposure on aerobic and anaerobic metabolism and tissue energy status (including levels of adenylates, free glucose and glycogen reserves) of eastern oysters *C. virginica*. We also determined mRNA expression and activity of key glycolytic and mitochondrial enzymes (hexokinase, aldolase, citrate synthase and cytochrome c oxidase) as well as mRNA expression of key regulatory genes involved in O_2 sensing and homeostasis (the hypoxia-inducible factor 1α or HIF1-α, and prolyl hydroxylase 2, or PHD2) in order to gain insight into the molecular mechanisms of metabolic responses of oysters to combined Cd and hypoxia stress. For hypoxic exposures, we selected 5% O_2 which is within the range of concentration found in the coastal "dead zones" (Baker and Mann, 1992; Diaz and Solow, 1999; Rabalais et al., 1999), is close to the critical partial oxygen pressure (P_cO_2) leading to metabolic rate depression during acute exposures but is not lethal to oysters (see "Results" below). For Cd exposures, we used 50 μg L^{-1} waterborne Cd (as $CdCl_2$); this concentration is within the range of Cd levels found in polluted estuaries (15–80 μg L^{-1} Cd; GESAMP, 1987; Crompton, 1997; Hackney et al., 1998). Our previous studies showed that this concentration of Cd elicits significant physiological response (but no acute toxicity) in oysters and results in physiologically relevant tissue Cd burdens similar to those found in oysters from polluted estuaries (Sokolova et al., 2005; Cherkasov et al., 2006a and references therein). Thus, this study provides insights into the effects of long-term exposures to sublethal, environmentally relevant levels of Cd and hypoxia stress and their combination in oysters. Understanding the impacts of Cd and hypoxia on energy metabolism is critical for assessing the organismal and population-level consequences of these stressors because stress-induced disturbances of energy homeostasis have direct consequences for the organism's fitness (Calow, 1989, 1991; Sibly and Calow, 2009). We also quantified tissue loads of commensal *Vibrio* spp. in oysters (focusing on human pathogens *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*) in order to test the hypothesis that prolonged exposure to hypoxia, Cd or their combination increases the abundance of these facultatively anaerobic bacteria and thus can affect the dynamics of these pathogens in polluted estuaries.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma Aldrich (St. Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Animal collection and maintenance

Oysters (*C. virginica*) were purchased from commercial oyster suppliers (Cuttyhunk Shellfish Farms, Cuttyhank, MA and Taylor Shellfish Farms, Shelton, WA), shipped overnight to the University of North Carolina at Charlotte and placed in recirculated aerated tanks with artificial seawater (Instant Ocean®, Kent Marine, Acworth, USA) at 20 ± 1 °C and 30 ± 1‰. Oysters were allowed to recover for 10 d. After this preliminary acclimation, half of the tanks were randomly selected, and Cd (as $CdCl_2$) was added to the nominal concentration of 50 μg L^{-1}. The remaining tanks were used as controls. To avoid pseudoreplication, at least three tanks were set for control or Cd exposure, and oysters were randomly sampled from these tanks for each experiment. Oysters were acclimated in tanks with or without Cd addition for 30 d prior to hypoxic exposures. Mortality during this acclimation period was less than 5% and did not significantly differ between control and Cd-exposed oysters.

After 30 d of acclimation, control and Cd-exposed oysters were randomly divided into four groups and exposed for 2 weeks to either normoxia or hypoxia. Cd concentrations in the water were maintained at the same levels as during the preceding 30-d acclimation period (i.e. control oysters were exposed to clean ASW and Cd-exposed oysters were kept in ASW with 50 μg L^{-1} Cd). This resulted in the following four experimental groups: (1) normoxia (21% O_2 or 100% air saturation) with no Cd addition (normoxic controls); (2) normoxia (21% O_2) exposed to 50 μg L^{-1} Cd (normoxic Cd-exposed oysters); (3) hypoxia (5% O_2 or 24% air saturation) with no Cd addition (hypoxic controls); (4) hypoxia (5% O_2) exposed to 50 μg L^{-1} Cd (hypoxic Cd-exposed oysters). For each experimental group, two replicate trays were set up, each containing 10–12 oysters in 5 L ASW. In normoxic treatments, water was aerated with ambient air. Hypoxia was achieved by bubbling ASW with a certified gas mixture containing 5% O_2, 0.04% CO_2 and balance of nitrogen (Roberts Oxygen, Charlotte, NC). Gas content of the mixtures was analyzed by the manufacturer and certified to be accurate within 10% of the target values (Roberts Oxygen, Charlotte, NC). Oxygen concentrations were tested periodically throughout the experimental exposures and were 97–100% and 20–30% of air saturation (corresponding to 7.3–7.5 and 1.5–2.3 mg O_2 L^{-1}, respectively) in normoxia and hypoxia, respectively. Water in each tray was changed every other day using clean or Cd-spiked (50 μg L^{-1}

Cd) ASW, as appropriate, equilibrated with the respective gas mixtures.

In all exposures, water temperature was kept at 20 ± 1 °C and salinity at 30 ± 1‰. Exposures were repeated twice with separate subsets of experimental animals to obtain sufficient amount of oysters for all analyses. Sample sizes for all analyses are given in Section 2.9: "Calculations and statistics". There was no mortality during the 2-week exposure period in normoxic or hypoxic treatments. Oysters were fed *ad libitum* on alternate days with a commercial algal blend (2 mL per oyster) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Premium Reef Blend, Sycamore, IL, USA) throughout the acclimation and experimental exposures.

2.3. Cadmium determination

Mantle samples were freeze-dried, weighed and fully digested with 1.5 mL of 50% nitric acid (trace metal grade, Fisher Scientific, Suwanee, GA, USA) using 3–4 cycles of microwave heating and cooling in Teflon bottles. Sample volume was adjusted to 10 mL with ultrapure water. Cd concentrations were determined with an atomic absorption spectrometer (PerkinElmer AAnalyst 400) equipped with a graphite furnace and deuterium background correction. National Institute of Standards and Technology (NIST) oyster tissue (1566b) was analyzed with the samples to verify the metal analyses. Tissue levels of Cd were determined in Cd-exposed and control oysters from normoxia and hypoxia and expressed as μg Cd g^{-1} dry mass.

2.4. Determination of bacterial loads

Following Cd and/or hypoxia exposure as described in Section 2.2, oysters (obtained from Cuttyhunk Shellfish Farms, Cuttyhank, MA) were rinsed with ethanol, patted dry with paper towels and shucked with a flame-sterilized oyster knife. Soft tissues were surface-washed with sterile ASW of 20‰ salinity, separated from the shell and placed in sterile test tubes to be weighed. The soft tissues were homogenized in 20‰ ASW at 1:1 w:v ratio (minimum 3 mL ASW) using sterile blender cups (Waring, Torrington, CT) and blended with three bursts of 15 s each, with a 5 s pause between the bursts.

After homogenization, samples were serially diluted in sterile phosphate buffered saline (PBS) and spread onto CHROMagar Vibrio (CHROMagar, Paris, France), a medium selective for *Vibrio* spp. and differential for *V. cholerae*, *V. vulnificus*, *V. alginolyticus*, and *V. parahaemolyticus.* Identification of these human pathogens was conducted according to the manufacturer's instructions, and total colony forming units (CFU) per gram of oyster tissue were calculated for each of these four species.

2.5. Whole-organism oxygen consumption

For determination of critical PO_2 (P_cO_2), a closed chamber respirometry was used. Normoxic control oysters were placed individually in 1.45 L closed chambers at 20 °C and 100% air saturation. To avoid interference with postprandial metabolism and feces excretion, animals were fasted for 24 h prior to the determination of oxygen consumption. Oxygen consumption was monitored continuously using a Clark type electrode (YSI, Yellow Springs OH, USA) for 22–24 h until all oxygen in the chamber was consumed. Two-point (0% and 100%) calibration of electrodes was performed prior to the experiment using oxygen-free saturated solution of sodium sulfite (Na_2SO_3) and air-saturated seawater at 20 °C. Continuous data acquisition was performed using a BIOPAC MP100 Data acquisition system (BIOPAC Systems Inc., Goleta CA, USA). A video recording was used to monitor the open vs. closed status of oysters, and only data for the periods when the oyster was open were used for MO_2 calculations. MO_2 rates were calculated for each 10% drop in the oxygen levels in the chamber (i.e. in the intervals of 100–90%, 89–80%, 79–70% etc. of air saturation). After the experiment was completed, oysters were dissected and soft tissue mass was determined. Respiration rate was calculated as:

$$MO_2 = \frac{\frac{dPO_2}{dt} \times \beta O_2 \times V}{M^{0.8}},$$

where MO_2 is the normalized oxygen consumption (μmol O_2 g^{-1} dry mass h^{-1}), dPO_2/dt the rate of oxygen decrease in the chamber (Torr h^{-1}), βO_2 the oxygen solubility in the seawater (μmol O_2 L^{-1} $Torr^{-1}$), V is volume of the respiratory chamber (L), and M is the dry tissue mass of the oyster (g).

To determine the effects of hypoxic acclimation and Cd exposure on standard metabolic rate (SMR), flow-through respirometry was used. For these experiments, we used oysters from all four experimental groups (control and Cd-exposed acclimated to either normoxia or hypoxia). Oxygen concentrations were monitored online by a needle-type fiber optic oxygen microsensor using TX-100 oxygen monitor with integrated temperature compensation (PreSens, Regensburg, Germany). Oxygen sensor was calibrated in oxygen-free saturated solution of sodium sulfite (Na_2SO_3) and air-saturated seawater for 0% and 100% air saturation, respectively. Sensor calibration and MO_2 measurements were conducted at 20 °C. Oysters were fasted for 24 h, placed into flow-through respiration chambers and allowed to recover overnight. Water flow of 20 mL min^{-1} was used so that O_2 concentrations were above 75% of air saturation at all times to avoid potential inhibitory effects of low O_2 levels on respiration rate. After measurements, oysters were dissected and tissue mass determined. MO_2 was calculated as follows:

$$MO_2 = \frac{\Delta PO_2 \times \beta O_2 \times V_{fl}}{M^{0.8}},$$

where MO_2 is the normalized oxygen consumption (μmol O_2 g^{-1} dry mass h^{-1}), ΔPO_2 the difference in partial pressure between in- and out-flowing water (Torr), βO_2 the oxygen capacity of water (μmol O_2 L^{-1} $Torr^{-1}$), V_{fl} the flow rate (L h^{-1}), and M the oyster dry tissue mass (g). All MO_2 data are presented as μmol O_2 g^{-1} dry mass h^{-1} and standardized to the individual dry mass of 1 g.

In order to compare differences in activity levels between different exposure groups, respiratory time activity (RTA) was determined as the percentage of time the oyster spent open and ventilating relative to the total experimental time (7–97 h) as described elsewhere (Lannig et al., 2006b).

2.6. Tissue metabolite concentrations

For determination of tissue metabolite concentrations, adductor muscle tissues of control and Cd-exposed oysters acclimated to hypoxia or normoxia were quickly excised and immediately shock-frozen in liquid nitrogen. Muscle tissue was powdered with mortar and pestle under the liquid nitrogen and homogenized with five volumes of ice-cold 0.6 M perchloric acid (PCA) with 150 mM EDTA for the maximum recovery of tissue ATP (Sokolova et al., 2000). Precipitated protein was removed by centrifugation. The extract was neutralized to pH 7.2–7.5 with 5 M potassium hydroxide. Precipitated potassium perchloride was removed by a second centrifugation. Extracts were stored at −80 °C.

Concentrations of all metabolites were measured in neutralized PCA extracts spectrophotometrically at 339 nm using standard NADH- or NADPH-linked enzymatic tests described elsewhere

(Grieshaber et al., 1978; Bergmeyer, 1985). Briefly, assay conditions were as follows:

ATP: 38.5 mM triethanolamine buffer, pH 7.6, 0.04 mM NADP, 7 mM $MgCl_2 \cdot 6H_2O$, 50 mM glucose, 0.462 U mL^{-1} glucose-6-phosphate dehydrogenase, 1.8 U mL^{-1} hexokinase.
ADP & AMP: 58 mM triethanolamine buffer, pH 7.6, 3 mM phoshoenolpyruvate (PEP), 0.09 mM NADH, 24 U mL^{-1} lactate dehydrogenase (LDH), 18 U mL^{-1} pyruvate kinase (PK), 16 U mL^{-1} myokinase (MK).
L-**Alanine:** 80 mM Tris buffer, pH 7.6, 7 mM 2-oxoglutarate, 0.24 mM NADH, 260 U mL^{-1} LDH, 10,000 U mL^{-1} alanine aminotransferase (glutamate-pyruvate transaminase).
Acetate: 100 mM triethanolamine buffer, pH 7.6, 0.2 M $MgCl_2$, 18 mM NADH, 91 mM ATP, 150 mM PEP, 5 U mL^{-1} PK, 5 U mL^{-1} LDH, 50 U mL^{-1} acetate kinase.
Succinate: concentration was measured with a succinic acid test kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.
D-**glucose:** 38.5 mM triethanolamine buffer, pH 7.6, 0.04 mM NADP, 7 mM $MgCl_2 \cdot 6H_2O$, 0.462 U mL^{-1} glucose-6-phosphate dehydrogenase, 1.8 U mL^{-1} hexokinase.

Tissue glycogen concentration was measured in PCA extracts of the muscle tissues after acid hydrolysis of glycogen to D-glucose by glucoamylase as described elsewhere (Keppler and Decker, 1984). Glycogen concentration was determined by the difference in the D-glucose levels in the tissue extract before and after glucoamylase treatment. Tissue metabolite concentrations were expressed as μmol g^{-1} wet tissue mass.

2.7. RNA extraction and quantitative real-time (qRT-) PCR

Total RNA was extracted from hepatopancreas tissues of individual oysters using Tri Reagent (Sigma–Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's protocol. Tissue to Tri reagent ratio was kept below 1:10 (weight:volume) yielding high purity total RNA with 280/260 nm absorbance ratio *ges*2.0. Single-stranded cDNA was obtained from 5 μg total RNA using 200 U μL^{-1} SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and 50 μM of oligo$(dT)_{18}$ primers.

Transcript expression of target genes was determined using quantitative real-time PCR (qRT-PCR) using a LightCycler® 2.0 Real-Time PCR System (Roche Applied Science, Indianapolis, IN) and QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. Specific primers were designed to amplify cDNA using *C. virginica* sequences (Table 1). Briefly, the qRT-PCR reaction mixture consisted of 5 μL of 2× QuantiTect SYBR Green master mix, 0.3 μM of each forward and reverse gene-specific primers, 1 μL of 10× diluted cDNA template and water to adjust to 10 μL. The reaction mixture were loaded into LightCycler 20 μL capillaries (Roche Applied Science, Indianapolis, IN) and subjected to the following cycling: 15 min at 95 °C to denature DNA and activate Taq polymerase; 50 cycles of 15 s at 94 °C, 20 s at 55 °C and 15 s at 72 °C. SYBR Green fluorescence (acquisition wavelength 530 nm) was measured at the end of each cycle for 2 s at the read temperature of 78 °C (to melt all primer dimers but not the amplified gene product). Serial dilutions of a cDNA standard were amplified in each run to determine amplification efficiency (Pfaffl, 2001), and an internal cDNA standard was included to test for run-to-run amplification variability. In pilot studies, we have also tested 18S rRNA mRNA as a possible housekeeping gene for normalization of the target gene mRNA expression. The qualitative mRNA expression patterns of the target genes were similar regardless of whether β-actin or 18S rRNA mRNA was used for normalization, but 18S rRNA transcript levels were more variable between tissues and exposure conditions than β-actin (data not shown). Therefore, in all further analyses the target gene mRNA expression was standardized relative to β-actin mRNA and against the internal standard as described elsewhere (Pfaffl, 2001; Sanni et al., 2008).

2.8. Enzymatic assays

Activities of two glycolytic enzymes (hexokinase (HK), EC 2.7.1.1 and aldolase, EC 4.1.2.13) and two mitochondrial enzymes (citrate synthase (CS), EC 2.3.3.1, prior to 2002 EC 4.1.3.7, and cytochrome c oxidase (COX), EC 1.9.3.1) were determined in hepatopancreas of control and Cd-exposed oysters after 2 weeks in normoxia or hypoxia. Tissues were thoroughly homogenized in enzyme-specific homogenization buffer (see below) using hand-held Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated 3 × 10 s (output 7, Sonic Dismembrator Model 100, Fisher Scientific, Suwanee, GA) to ensure complete release of the enzymes, with cooling on ice (1 min) between sonications. Homogenates were centrifuged for 10 min at 20,000*g* and 4 °C to collect debris, and supernatants were used to assay enzyme activities. Enzyme extracts were stored at −80 °C for less than 2 weeks before activity assays. Pilot studies have shown that activities of the four studied enzymes were not affected by storage at −80 °C or by up to three freeze–thaw cycles (data not shown).

Table 1
Primer sequences and PCR product characteristics used for real time quantitative PCR (qRT-PCR).

Gene	Primers	PL, bp	E	NCBI #
Hypoxia-inducible factor 1α (HIF-1α)	*HIF-1F326* CTGCTCCGATCTGATCAACA *HIF-1R* CATACGTGTCCCCCTGTCTT	150	1.95 ± 0.05 (4)	MGID89187
Prolyl hydroxylase 2 (PHD-2)	*PHD2-F* CGTCATGTCGATAATCCAAAC *PHD2-R* ATGTGGATTGCGTCTATCTGACCA	200	2.43 ± 0.02 (4)	HM441077
Hexokinase (HK)	*HK-F279* AACTTCTGCCCAGGGTTCACCAGTTC *HK-R432* TCCTTTCATCAGCTGGTATCGCCA	153	1.80 (1)	MGID93994
Aldolase	*ALD-F631* TACCAATCGTTGAGCCAGAGGTGT *ALD-R801* GACAACTCATTCCAGCTGTGACCA	170	2.14 (1)	MGID89960
Cytochrome c oxidase subunit 4 (COX 4)	*COX4-F293* TTCAAGGAGGTGGATGAGGAACTC *COX4-R456* TCTCCCAAGCATCGTTTCCAGTCA	163	2.25 (1)	MGID91924
Citrate synthase (CS)	*CS-F106* GACCTCCTCATCCTCCACAA *CS-R262* GCCTCTGATGCCTCTCATTC	156	2.24 ± 0.08 (2)	MGID89535
β-actin	*FW: Act-Cv-F437* CACAGCCGCTTCCTCATCCTCC *Act-Cv-R571* CCGGCGGATTCCATACCAAGG	134	1.99 ± 0.01 (4)	X75894.1

All primer sequences are given in 3′-to 5′-end orientation. NCBI accession numbers are given for gene sequences used to design specific primers. E – amplification efficiency as determined by RT-PCR, PL – expected length of amplified product (bp). Annealing temperature (T_{ann}) was 55 °C for all gene fragments.

For determination of enzyme activities, enzyme extracts were thawed on ice and immediately analyzed using standard spectrophotometric techniques as described elsewhere (Bergmeyer, 1985; Sidell et al., 1987; Birch Machin and Turnbull, 2001). Enzyme activities were measured at 20 °C using a UV–Vis spectrophotometer (VARIAN Cary 50 Bio, Cary NC, USA). The temperature of the reaction mixture was controlled within 0.1 °C of the set value using a water-jacketed cuvette holder. All assays were completed within 1 h during which enzyme extracts were maintained on ice. Activities of isolated enzymes did not change within this time frame (data not shown). Briefly, isolation and assay conditions for the studied enzymes were as follows (final concentrations are given):

CS: homogenization buffer: 75 mM Tris, pH 7.6; assay: 75 mM Tris pH 8.0, 0.25 mM 5,5′-dithio-bis-[2-nitrobenzoic] acid, 0.4 mM acetyl-Coenzyme A, 0.5 mM oxalacetate; acquisition wavelength: 412 nm;
HK: homogenization buffer: 75 mM Tris, pH 7.6; assay: 0.1 M triethanolamine, pH 7.6, 0.22 M glucose, 6.5 mM $MgCl_2$, 2.7 mM ATP, 0.83 mM NADH; acquisition wavelength: 340 nm;
Aldolase: homogenization buffer: 75 mM Tris, pH 7.6; assay: 100 mM Tris, pH 8.0, 5 mM D-fructose 1,6-biphosphate, 8.5 mM NADH, 300 U mL^{-1} *sn*-glycerol-3-phosphate: NAD^+ 2-oxidoreductase (GDH), 500 U mL^{-1} Dglyceraldehyde 3-phosphate ketolisomerase (TIM), 250 U mL^{-1}LDH; acquisition wavelength: 340 nm;
COX: homogenization buffer: 25 mM potassium phosphate, pH 7.2, 10 μg mL^{-1} phenylmethylsulfonyl fluoride (PMSF), 2 μg mL^{-1} aprotinin; assay: 20 mM potassium phosphate, pH 7.0, 16 μM reduced cytochrome c (II), 0.45 mM *n*-dodecyl-β-d-maltoside, 2 μg mL^{-1} antimycin A, acquisition wavelength: 550 nm.

2.9. Calculation and statistics

Adenylate energy charge (AEC) was calculated using formula:

$$\mathrm{AEC} = \frac{[\mathrm{ATP}] + 0.5 \times [\mathrm{ADP}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}]},$$

where [AMP], [ADP] and [ATP] are concentrations (μmol g^{-1} wet weight) of the corresponding compounds.

Statistical analysis was performed using generalized linear model analysis of variance (ANOVA) after testing for normality of the data distribution and homogeneity of variances, and was followed by post hoc procedures (Fisher's Least Significant Difference test for unequal N). Factor effects and differences between the means were considered significant if the probability of Type I error was less than 0.05. Data are presented as means ± standard errors unless otherwise indicated. Sample sizes were 8–13 for determination of tissue levels of metabolites, 8–9 for tissue Cd burdens, 4–13 for determinations of gene mRNA expression and enzyme activities, 9–17 for bacterial loads and 5–8 for oxygen consumption studies. Each sample represented an individual oyster.

3. Results

3.1. Cadmium accumulation

Exposure to 50 μg L^{-1} Cd resulted in a significant accumulation of Cd in mantle tissues during normoxia and hypoxia (Fig. 1A) (ANOVA for Cd effect: $F_{1,29} = 45.11$, $P < 0.0001$). Cd level was significantly higher in hypoxia compared to normoxia (ANOVA for hypoxia effect: $F_{1,29} = 17.16$, $P = 0.0003$).

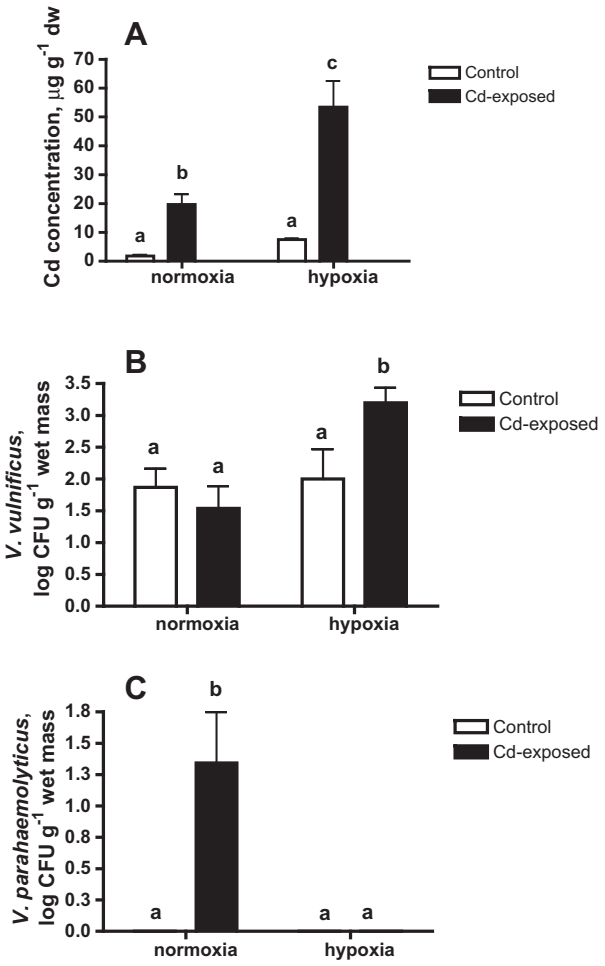

Fig. 1. Cd accumulation and *Vibrio* loads in oyster tissues during hypoxia and Cd exposure. Oysters were kept for 2 weeks in normoxia (100% air saturation) or hypoxia (24% air saturation) either in clean ASW (controls) or in ASW with 50 μg L^{-1} Cd (Cd-exposed ones). Vertical bars represent standard errors. Columns that do not share letters represent significantly different values ($P < 0.05$). (A) Cd accumulation in mantle tissue. (B) *V. vulnificus* loads (log-transformed number of colony forming units, or CFU per g wet body mass). (C) *V. parahaemolyticus* loads (log CFU, per g wet body mass). $N = 8$–9 for Cd accumulation and 9–17 for bacterial loads.

3.2. Vibrio loads

Two weeks of exposure to Cd and/or hypoxia had a significant effect on tissue loads of *V. vulnificus* and *V. parahaemolyticus* in oysters (Fig. 1B and C) but not on *V. alginolyticus* or *V. cholerae* ($P > 0.05$; data not shown). *V. vulnificus* was the most abundant in oysters simultaneously exposed to Cd and hypoxia compared to all other groups (Fig. 1B). In contrast, *V. parahaemolyticus* was below the detection limits of 1 CFU g^{-1} in all groups except Cd-exposed normoxic oysters that harbored on average 22 *V. parahaemolyticus* g^{-1} wet body mass (Fig. 1B). In a separate experiment with a different batch of oysters, we confirmed that Cd exposure (60 d in 50 μg L^{-1} Cd under normoxia) led to a significant increase in tissue burdens of *V. parahaemolyticus*: from 0.27 ± 0.27 log CFU g^{-1} wet mass in controls to 2.43 ± 1.00 log CFU g^{-1} wet mass in Cd-exposed oysters ($N = 5$–6, $P = 0.020$).

3.3. Aerobic and anaerobic metabolism

Acute exposure to declining oxygen levels (i.e. with a decrease in the oxygen levels from 100% to 0% air saturation within 22–

24 h) resulted in a significant decrease in oyster respiration rates when O_2 levels dropped below 30% of air saturation (Fig. 2A). Oxygen consumption rates (MO_2) were relatively stable in the range of ambient O_2 concentrations between 100% and 30% of O_2 saturation. At 10–20% air saturation, MO_2 decreased down to 20–30% of the normoxic levels (Fig. 2A). In contrast, when oysters were acclimated for 2 weeks in hypoxic conditions (24% air saturation), their MO_2 levels were not significantly different from the respective normoxic values (ANOVA for the effects of hypoxia: $F_{1,22} = 1.95$, $P = 0.176$; Fig. 2B). Cd exposure had no effect on MO_2 under normoxic or hypoxic conditions (ANOVA: $F_{1,22} = 0.37$, $P = 0.548$).

Relative time of activity (% of time that oysters remained open and actively ventilating) was significantly affected by hypoxic acclimation but not by Cd exposure (ANOVA: $F_{1,22} = 15.35$, $P = 0.001$; $F_{1,22} = 1.86$, $P = 0.186$ for hypoxia and Cd exposure, respectively). Both control and Cd-exposed oysters remained open longer in hypoxia (55–70% of the time) than in normoxia (30–35% of the time) (Fig. 2C).

There was no accumulation of anaerobic end products (L-alanine, succinate and acetate) during Cd exposure or hypoxia in oysters (Table 2). Tissue metabolite levels in normoxia and hypoxia, respectively, were: 16.22 ± 1.24 and 18.68 ± 1.14 μmol g^{-1} wet mass for L-alanine, 2.45 ± 0.73 and 1.82 ± 0.23 μmol g^{-1} wet mass for succinate, and 0.48 ± 0.07 and 0.53 ± 0.07 μmol g^{-1} wet mass for acetate ($P > 0.05$; $N = 8–15$).

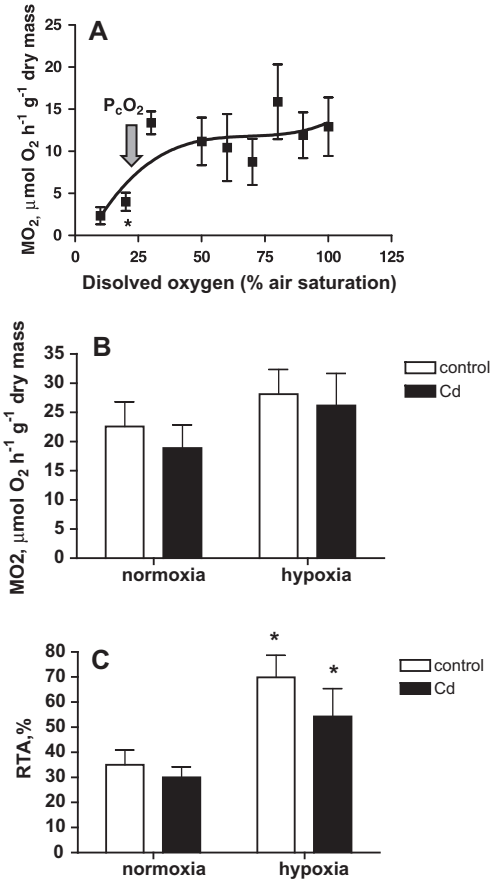

Fig. 2. Effects of hypoxia and Cd exposure on oxygen consumption rates (A and B) and relative time of activity (C) in *C. virginica*. (A) Estimation of the critical PO_2 (P_cO_2) of aerobic respiration in *C. virginica* during the acute decrease in the ambient O_2 concentration. Asterisk indicates a significant drop in MO_2 value, and the arrow indicates the putative P_cO_2 value (% air saturation). $N = 3–6$. (B) MO_2 of control and Cd-exposed oysters acclimated for 2 weeks in normoxia (100% air saturation) and hypoxia (24% air saturation). MO_2 was measured under the same O_2 conditions as during the acclimation $N = 5–8$. (C) Relative time of activity (RTA, %) determined as a percent of time that oysters stayed open. Asterisks indicate the values that are significantly different from the respective normoxic RTAs ($P < 0.05$). $N = 5–8$.

Table 2
ANOVA: Effects of hypoxia and Cd exposure on mRNA expression of target genes, enzyme activity and metabolite concentrations in hepatopancreas of eastern oysters *Crassostrea virginica*. Significant effects are highlighted in bold.

	ANOVA factor effect		
	Cd exposure	Hypoxia	Cd x Hypoxia
mRNA expression			
HIF-1	$F_{1,40} = 21.22$	$F_{1,40} = 7.48$	$F_{1,40} = 4.93$
	$P <$ **0.0001**	**P = 0.009**	**P = 0.032**
PHD-2	$F_{1,40} = 1.31$	$F_{1,40} = 8.62$	$F_{1,40} = 0.08$
	P = 0.2585	**P = 0.006**	P = 0.780
HK	$F_{1,16} = 4.47$	$F_{1,16} = 2.86$	$F_{1,16} = 0.3$
	P = 0.050	P = 0.110	P = 0.594
Aldolase	$F_{1,16} = 2.20$	$F_{1,16} = 3.73$	$F_{1,16} = 2.72$
	P = 0.157	P = 0.071	P = 0.119
CS	$F_{1,35} = 2.56$	$F_{1,35} = 0.06$	$F_{1,35} = 3.52$
	P = 0.118	P = 0.806	P = 0.069
COX4	$F_{1,15} = 0.16$	$F_{1,15} = 7.35$	$F_{1,15} = 3.13$
	P = 0.698	**P = 0.016**	P = 0.097
Enzyme activities			
HK	$F_{1,16} = 20.92$	$F_{1,16} = 0.14$	$F_{1,16} = 1.51$
	P = 0.0003	P = 0.709	P = 0.237
Aldolase	$F_{1,16} = 19.23$	$F_{1,16} = 0.00$	$F_{1,16} = 3.43$
	P = 0.0005	P = 0.954	P = 0.083
CS	$F_{1,16} = 1.97$	$F_{1,16} = 0.27$	$F_{1,16} = 11.61$
	P = 0.179	P = 0.608	**P = 0.004**
COX4	$F_{1,15} = 0.11$	$F_{1,15} = 2.01$	$F_{1,15} = 3.64$
	P = 0.741	P = 0.177	P = 0.076
Metabolite concentrations			
Glycogen	$F_{1,42} = 7.50$	$F_{1,42} = 0.27$	$F_{1,42} = 0.20$
	P = 0.009	P = 0.608	P = 0.659
D-glucose	$F_{1,42} = 0.0$	$F_{1,42} = 0.49$	$F_{1,42} = 7.46$
	P = 0.955	P = 0.488	**P = 0.009**
L-alanine	$F_{1,45} = 0.59$	$F_{1,45} = 1.30$	$F_{1,45} = 0.83$
	P = 0.445	P = 0.260	P = 0.368
Succinate	$F_{1,16} = 2.12$	$F_{1,16} = 0.7$	$F_{1,16} = 0.49$
	P = 0.165	P = 0.414	P = 0.492
Acetate	$F_{1,44} = 3.06$	$F_{1,44} = 0.00$	$F_{1,44} = 0.87$
	P = 0.087	P = 0.947	P = 0.357
ATP	$F_{1,45} = 5.06$	$F_{1,45} = 5.8$	$F_{1,45} = 3.37$
	P = 0.029	**P = 0.020**	P = 0.073
ADP	$F_{1,45} = 0.95$	$F_{1,45} = 16.31$	$F_{1,45} = 0.29$
	P = 0.335	**P = 0.0002**	P = 0.590
AMP	$F_{1,45} = 1.68$	$F_{1,45} = 6.92$	$F_{1,45} = 0.42$
	P = 0.2013	**P = 0.012**	P = 0.520
Σ adenylates	$F_{1,45} = 3.94$	$F_{1,45} = 2.87$	$F_{1,45} = 1.8$
	P = 0.053	P = 0.097	P = 0.186
AEC	$F_{1,45} = 0.00$	$F_{1,45} = 17.74$	$F_{1,45} = 0.13$
	P = 0.978	**P = 0.0001**	P = 0.724
ADP/ATP	$F_{1,45} = 0.01$	$F_{1,45} = 25.25$	$F_{1,45} = 0.00$
	P = 0.987	**P <** 0.0001	P = 0.954

3.4. mRNA expression of oxygen-sensing genes

In normoxia, HIF-1α transcript expression was significantly lower in Cd-exposed oysters compared to their control counterparts ($P = 0.001$) (Fig. 3A). Acclimation under hypoxic conditions significantly decreased mRNA expression of HIF-1α in control oysters ($P = 0.010$) and but not in Cd-exposed ones ($P = 0.847$).

PHD-2 mRNA expression was not significantly different in control and Cd-exposed oysters in normoxia ($P = 0.557$). Hypoxic exposure resulted in elevated levels of PHD-2 transcripts in oyster hepatopancreas although this elevation was significant only in Cd-exposed oysters ($P = 0.033$), but not in their control counterparts ($P = 0.059$) (Fig. 3B).

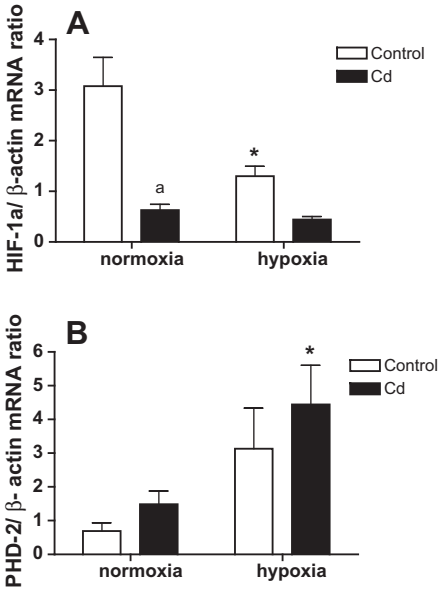

Fig. 3. Effects of hypoxia and Cd exposure on mRNA expression of genes of the oxygen sensing pathway in hepatopancreas of oysters *C. virginica*. HIF-1α (A) and PHD-2 (B) levels were measured using qRT-PCR and normalized to β-actin mRNA level. Vertical bars represent standard error. Asterisks indicate values significantly different from the respective normoxic values ($P < 0.05$). Letter a denotes the values that significantly differ between control and Cd-exposed oysters ($P < 0.05$). $N = 8$–13.

3.5. Transcript expression and activity of metabolic enzymes

In normoxia, HK mRNA expression was lower in Cd-exposed oysters compared to the respective controls ($P = 0.079$) (Fig. 4A). Acclimation to hypoxic conditions had no effect on HK mRNA expression in control or Cd-exposed oysters ($P = 0.283$) (Fig. 4A). In contrast, enzyme activity of HK was significantly higher in Cd-exposed oysters ($P = 0.001$) (Fig. 4B) and was not affected by hypoxic acclimation ($P = 0.557$ and $P = 0.272$ for control and Cd-exposed oysters, respectively). Overall, there was no correlation between HK mRNA expression and enzyme activity in oyster hepatopancreas (Pearson $R = -0.235$, $P = 0.319$, $N = 20$).

Expression of aldolase mRNA was significantly higher in Cd-exposed oysters compared to the respective controls in normoxia ($P = 0.042$). During hypoxic acclimation aldolase mRNA expression was significantly downregulated in Cd-exposed oysters ($P = 0.022$) but not in the controls ($P = 0.843$) (Fig. 4C). Aldolase enzymatic activity showed a similar pattern and was significantly higher in Cd-exposed oysters compared to the controls in normoxia ($P = 0.0004$), and tended to decrease during hypoxic exposure in Cd-exposed oysters although this trend was statistically non-significant ($P = 0.223$) (Fig. 4D). Aldolase enzyme activity was positively correlated with mRNA expression of this gene (Pearson $R = 0.522$, $P = 0.018$, $N = 20$).

CS mRNA expression was similar in control and Cd-exposed oysters during normoxia ($P = 0.847$) but significantly lower in Cd-exposed oysters compared to their control counterparts during hypoxic acclimation ($P = 0.020$) (Fig. 4E). In contrast, CS enzyme activity was significantly elevated in Cd-exposed oysters in normoxia compared to the respective controls ($P = 0.004$) (Fig. 4F). CS activity increased during hypoxic exposure in control oysters ($P = 0.013$) but not in their Cd-exposed counterparts ($P = 0.058$). Overall, CS mRNA expression and enzyme activity were not significantly correlated in oyster hepatopancreas (Pearson $R = 0.241$, $P = 0.305$, $N = 20$).

COX4 mRNA expression was not significantly different between control and Cd-exposed oysters during normoxia ($P = 0.503$) (Fig. 4G). During hypoxic acclimation, COX4 mRNA expression was downregulated in control ($P = 0.008$) but not in Cd-exposed oysters ($P = 0.135$) (Fig. 4G). COX enzyme activity was also similar in control and Cd-exposed oysters in normoxia ($P = 0.123$) (Fig. 4H). Hypoxic acclimation resulted in a strong increase in COX activity in control oysters ($P = 0.037$) but not in Cd-exposed ones ($P = 0.725$) (Fig. 4H). Similar to CS, COX4 mRNA expression and COX enzyme activity were not significantly correlated (Pearson $R = -0.374$, $P = 0.115$, $N = 19$).

3.6. Tissue energy status

In normoxia, levels of adenylates (ATP, ADP and AMP) did not significantly differ in adductor muscle tissues of control and Cd-exposed oysters ($P = 0.783$, $P = 0.7730$ and $P = 0.666$, respectively) (Fig. 5). In control oysters, hypoxic acclimation led to a significant increase in tissue ATP levels ($P = 0.002$), accompanied by a decrease in ADP concentrations ($P = 0.010$) while AMP levels did not change ($P = 0.131$) (Fig. 5A–C). In contrast, in Cd-exposed oysters hypoxic acclimation led to depletion of ADP ($P = 0.004$) and AMP ($P = 0.037$) and did not affect tissue concentrations of ATP ($P = 0.711$) (Fig. 5A–C). As a result, total adenylate concentrations were not changed by hypoxic acclimation in control oysters ($P = 0.7863$) but depleted in Cd-exposed oysters ($P = 0.053$) (Fig. 5D). Tissue ATP levels were also significantly lower during hypoxia in Cd-exposed oysters compared to their control counterparts ($P = 0.020$) (Fig. 5A). Energy-related indices (AEC and ADP/ATP ratios) were affected by hypoxia but not by Cd exposure in oyster muscle (Table 2), with ADP/ATP ratio significantly decreasing from 0.87 in normoxia to 0.31–0.32 in hypoxia ($P < 0.0001$) and AEC increasing from 0.51–0.52 to 0.66–0.67 during hypoxia ($P = 0.0001$).

Energy stores (measured as the tissue levels of glycogen) were significantly (by 30–40%) lower in the muscle of Cd-exposed oysters compared to their control counterparts ($P = 0.009$; Table 2, Fig. 5E). Hypoxic acclimation had no effect on tissue glycogen levels in oysters (Fig. 5E). ANOVA showed significant effects of the interaction between factors "Hypoxia" and "Cd exposure " on tissue levels of D-glucose (Table 2) indicating that hypoxia-induced changes in D-glucose levels differed between control and Cd-exposed oysters. Free levels of D-glucose tended to be higher in Cd-exposed oysters compared to the controls during normoxia ($P = 0.076$). In control oysters, hypoxic exposure resulted in a nearly twofold increase in free glucose levels ($P = 0.010$) but had no effect on the already elevated basal glucose levels of Cd-exposed oysters ($P = 0.614$) (Fig. 5F).

4. Discussion

4.1. Compensatory upregulation of aerobic metabolism during prolonged acclimation in hypoxia

Eastern oysters *C. virginica* showed a typical mixed strategy of MO_2 regulation during the acute decrease in ambient oxygen concentrations, with oxyregulation over a considerable range of ambient O_2 concentrations (30–100% air saturation) and transition to oxyconformity at low O_2 levels. The critical PO_2 (P_cO_2) beyond which the oysters switched to oxyconformity and experienced depression of the standard metabolic rate (SMR), was between 20 and 30% of air saturation (1.5–2.2 mg O_2 L^{-1}) at 20 °C. These values are somewhat lower than P_cO_2 previously reported for the Pacific oyster, *Crassostrea gigas* (43% air saturation or 3.4 mg O_2 L^{-1} at 20 °C) (Le Moullac et al., 2007) suggesting higher hypoxia sensitivity in the Pacific oyster. Critical O_2 levels similar to those found in our study (20–30% of air saturation) were reported as the hypoxia

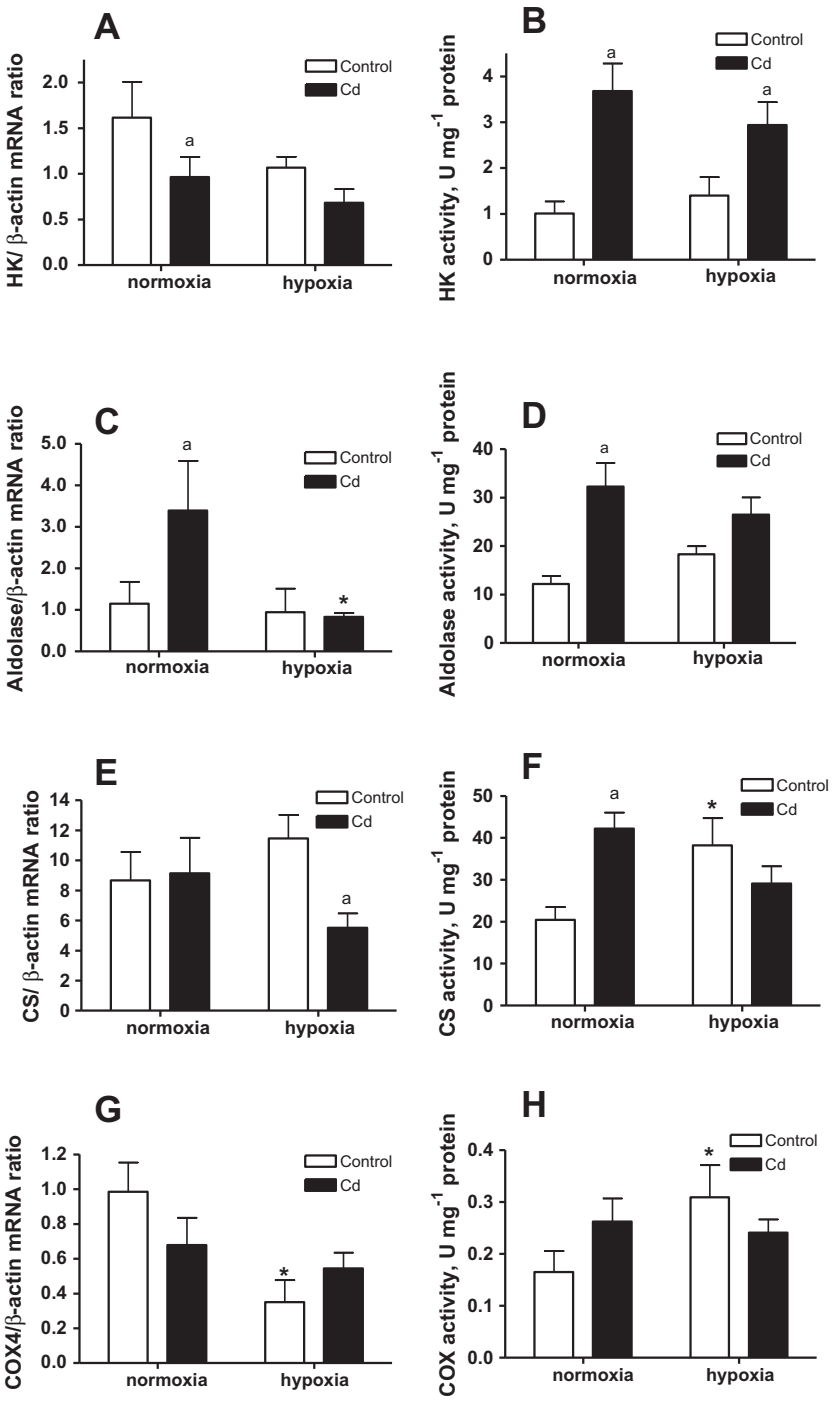

Fig. 4. Effect of hypoxia and Cd exposure on mRNA expression and activity of metabolic enzymes in hepatopancreas of *C. virginica*. Hexokinase (A), aldolase (C), citrate synthase (E) and cytochrome C oxidase subunit 4 (G) gene mRNA expression levels were measured using qRT-PCR and normalized to β-actin mRNA levels. Enzyme activities of hexokinase (B), aldolase (D), citrate synthase (F) and cytochrome C oxidase (H) were measured at 20 °C and presented as U mg^{-1} cellular protein. Vertical bars represent standard error. Asterisks indicate values significantly different from the respective normoxic values ($P < 0.05$). Letter 'a' denotes the values that significantly differ between control and Cd-exposed oysters ($P < 0.05$). $N = 4–5$.

threshold for behavioral escape responses and physiological stress for many marine benthic invertebrates (Diaz and Rosenberg, 2008; Vaquer-Sunyer and Duarte, 2008). Based on our results, the hypoxic conditions used in this study (24% air saturation or 1.8 mg L^{-1}) while representing moderate hypoxia, are close to the critical point (P_cO_2) for *C. virginica* when significant alterations of the energy metabolism can be expected.

Prolonged acclimation to moderate hypoxia resulted in a compensatory metabolic response in oysters so that their SMR at 24% air saturation was the same as in control (normoxic) oysters at 100% air saturation. Notably, hypoxic acclimation resulted in a significant upregulation of activity of two key mitochondrial enzymes, CS and COX, in control oysters. These two enzymes are considered markers of mitochondrial density and capacity (Lucassen et al., 2003; Siu et al., 2003; Morley et al., 2009). Their elevated activities suggest that tissue aerobic capacity is upregulated to compensate for the reduced oxygen availability in hypoxia and may explain why SMR in hypoxia-acclimated oysters is similar to

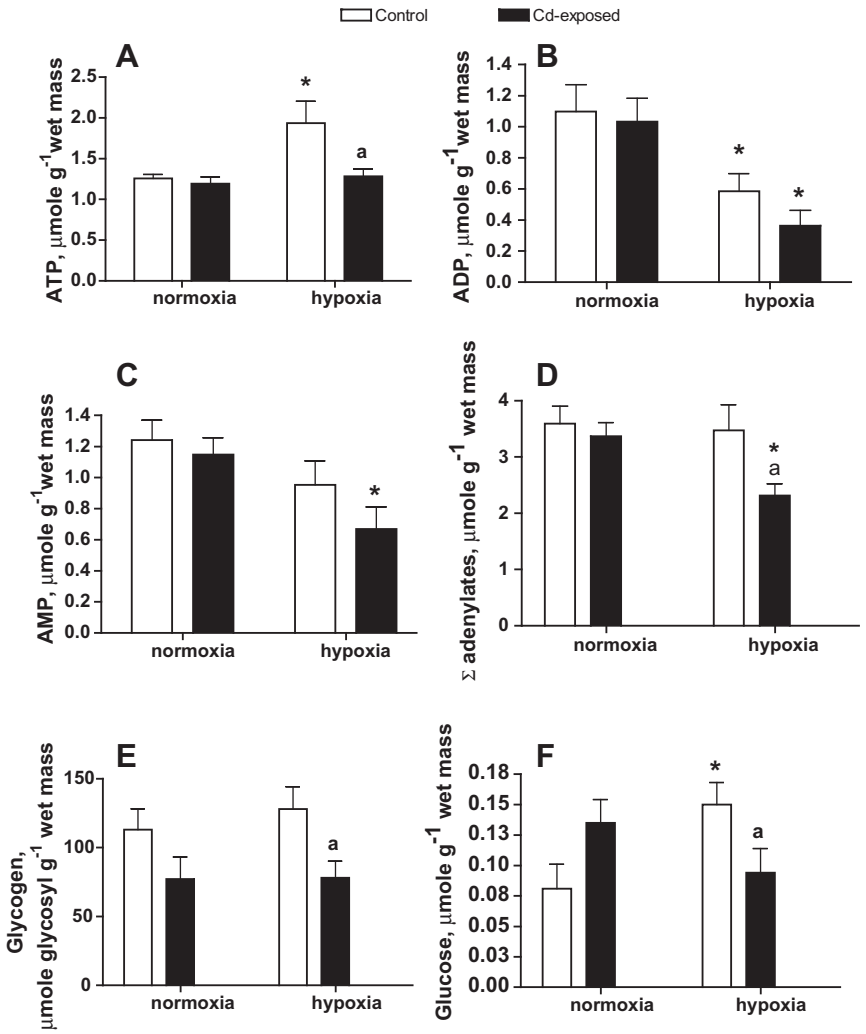

Fig. 5. Effect of hypoxia and Cd exposure on parameters of the energy status in muscle tissues of *C.virginica*. Concentrations of adenylates (A – ATP, B – ADP, C – AMP, D – sum of adenylates), glycogen (E) and free D-glucose (F) are given. Vertical bars represent standard error. Asterisks indicate values significantly different from the respective normoxic values ($P < 0.05$). Letter 'a' denotes the values that significantly differ between control and Cd-exposed oysters ($P < 0.05$). $N = 8–13$.

the normoxic values. While SMR did not significantly differ in normoxia- and hypoxia-acclimated oysters, hypoxic oysters spent significantly more time open and ventilating. Currently, it is unknown whether this increased ventilation activity reflects elevated tissue oxygen demand and/or reduced efficiency of oxygen delivery in hypoxia. Given that respiratory patterns in mollusks are directly regulated by hypoxia-sensitive peripheral chemoreceptor cells (Bell et al., 2008), increased ventilatory activity may also be a behavioral response directly triggered by low ambient O_2 levels rather than a reflection of elevated tissue oxygen demand.

Interestingly, acclimation to hypoxia affected parameters of cellular energy status in oysters. Unexpectedly, tissue ATP levels increased in hypoxia-acclimated oysters maintained in clean ASW while ADP and AMP levels decreased leading to suppressed ADP/ATP ratio and elevated AEC. Low ADP/ATP ratios typically reflect a decreased ATP consumption and are associated with suppression of the metabolic flux (Jeneson et al., 2000; Heijnen et al., 2004; Fridlyand et al., 2005). However, we observed no decrease in SMR of hypoxia-acclimated oysters. It is worth noting that in this study we have measured ADP/ATP ratios only in the adductor muscle. Thus, it is conceivable that if ADP/ATP ratios remained constant in other tissues, a decrease in the metabolic flux of the muscle tissue may not be detectable in the whole-organism SMR. Previous research showed that changes in adenylate levels are correlated between different tissues of oysters during prolonged anoxia (Kurochkin et al., 2009 and references therein); but the situation may be different during hypoxic acclimation and requires further investigation.

Hypoxic exposure resulted in increased Cd accumulation in oysters, likely as a side-effect of increased ventilatory activity. Cd burdens in mantle tissue of oysters were 2.7-fold higher when Cd exposure was combined with hypoxia compared to normoxia. Enhanced Cd accumulation under hypoxic conditions was also previously found in a freshwater clam *Corbicula fluminea* exposed to 30 μg L^{-1} Cd for 2 weeks (Legeay et al., 2005). In contrast to oysters, hypoxic *C. fluminea* did not spend greater proportion of time open, and Cd accumulation was increased by only 30% in hypoxia compared to normoxia. A parallel increase in ventilation rates and Cd accumulation was also found in hypoxic shrimps *Palaemon longirostris* and juvenile eels *Anguilla anguilla* (Pierron et al., 2007a,b). In contrast, in a hypoxia-tolerant carp *Cyprinus carpio* no increase in Cd or Zn uptake was observed during hypoxic exposure (Hattink et al., 2005, 2006). It is likely that respiratory patterns of different animals contribute to the species-specific differences in sensitivity of Cd uptake to hypoxia. In facultative anaerobes such as bivalve mollusks that periodically close their valves for prolonged periods of time, an increase in ventilation and the proportion of time spent open would directly translate into

a greater metal uptake due to the increased contact with contaminated environment. In contrast, in active animals such as fish and shrimp, gills are permanently in contact with the contaminated water, so that the enhancing effects of ventilation on Cd uptake are only pronounced at low Cd concentrations when simple diffusion is insufficient to fully saturate the uptake sites (Pierron et al., 2007a,b). Further studies on the effects of hypoxia on Cd uptake in other aquatic species are needed to test this hypothesis and determine whether this pattern can be generalized to all facultative anaerobes.

4.2. Cd-induced alterations of metabolic responses

In this study, Cd exposure had no significant effect on SMR of oysters in normoxia or hypoxia. This contrasts with previous studies where long-term Cd exposure (20–40 d) led to elevated SMR likely reflecting elevated costs of basal maintenance due to detoxification and damage repair (Cherkasov et al., 2006b; Lannig et al., 2006b). This variation of the metabolic response to metal exposure may be due to the modulating effects of other factors such as environmental temperature, population of origin and season (Cherkasov et al., 2006a, 2010; Sokolova and Lannig, 2008). Notably, previous studies that detected a considerable rise in SMR in response to Cd exposure were conducted in southern (North Carolina) oysters unlike the present work, which was done on oysters from northern (Massachusetts and Washington) populations. Our earlier research showed that oysters from southern populations (including Texas and North Carolina) are more sensitive to Cd toxicity and tend to accumulate higher Cd loads compared to their northern (Washington) counterparts (Cherkasov et al., 2010). This lower sensitivity to Cd in northern oyster populations may explain why no increase in SMR was observed in Cd-exposed oysters used for this study.

In contrast to control oysters, mitochondrial enzyme activity showed no compensatory response to hypoxic acclimation in Cd-exposed oysters, even though their SMR was fully compensated in hypoxia. Notably, activities of glycolytic enzymes (HK and aldolase) were significantly elevated in Cd-exposed oysters suggesting that their energy metabolism may be more dependent on glycolysis than that in the control ones. Higher dependence of Cd-exposed oysters on carbohydrate catabolism is also consistent with the elevated basal levels of free glucose in their tissues. A similar situation was previously described in a marine clam *Ruditapes decussatus* where Cd exposure inverted the order in which different energy reserves were used, with Cd-exposed clams preferentially using carbohydrate reserves to fuel their metabolism while control clams relied mostly on lipoproteins (Baghdiguian and Riva, 1985). Given that there was no accumulation of anaerobic end products in Cd-exposed oysters in normoxia or hypoxia, it is unlikely that the elevated glycolytic flux in Cd-exposed oysters indicates oxygen deficiency and transition to partial anaerobiosis. More likely, it reflects the elevated rate of tissue glucose metabolism either due to the higher demand for metabolic intermediates of glycolysis (e.g. for anabolic processes) or higher mitochondrial requirements for pyruvate. The latter scenario (i.e. if pyruvate derived from glycolysis is mainly used to fuel mitochondrial metabolism) may potentially represent an alternative compensatory mechanism allowing Cd-exposed oysters to maintain normal SMR in hypoxia despite the absence of upregulated mitochondrial capacity, and agrees with the previously reported higher reliance of Cd-exposed mollusks on carbohydrate catabolism for ATP production (Baghdiguian and Riva, 1985). However, upregulation of the glycolytic flux as a compensatory strategy to maintain SMR comes at a cost as indicated by a depletion of glycogen stores, an absence of a hypoxia-induced increase in tissue ATP levels and depletion of the total adenylate pool in Cd-exposed oysters. Similar depletion of glycogen stores during exposures to toxic metals including Cd has been previously reported in other aquatic ectotherms (review in: Sokolova and Lannig, 2008) and may represent a general mechanism for metal-induced energy stress.

4.3. Enzyme activities and transcript expression profiles

Both hypoxia and Cd exposure had significant effects on the mRNA expression of two key genes of the hypoxia-sensing pathway, hypoxia-inducible factor 1α (HIF-1α) and prolyl hydroxylase 2 (PHD-2). PHD-2 and HIF-1α act in tandem as a cellular oxygen sensor regulating abundance of a transcription factor, the hypoxia-inducible factor 1 (HIF-1) that coordinates adaptive cellular response to hypoxia (Hu et al., 2003; Webster, 2003; Semenza, 2004). Cellular abundance of HIF-1α, the oxygen-sensitive subunit of the HIF-1 transcription factor, is post-translationally regulated by PHD-2, but transcriptional control also plays an important role in regulation of HIF-1α and PHD-2 levels in the cell (Brahimi-Horn and Pouyssegur, 2007; Hoogewijs et al., 2007; Ziello et al., 2007; Taylor, 2008; Soñanez-Organis et al., 2009; Xing and Bonanno, 2009). Thus, in the absence of specific antibodies that cross-react with PHD-2 and HIF-1α from oysters, determination of the changes in transcriptional profiles of these two genes was the only available approach allowing us for gain an insight into the involvement of HIF-1 pathway into responses to the combined Cd and hypoxia stress in oysters.

Consistent with previous studies of HIF-1α and PHD-2 in vertebrates and invertebrates, we found that transcripts of these two genes are expressed in oyster tissues under both normoxic and hypoxic conditions. Notably, acclimation under hypoxic conditions as well as Cd exposure resulted in a decline of HIF-1α transcript expression in oyster hepatopancreas. Although both stressors suppressed HIF-1α mRNA expression, they did not act synergistically, as indicated by the absence of significant factor interactions between hypoxia and Cd exposure (Table 2). Levels of HIF-1α and PHD-2 transcripts were inversely related so that a decreased mRNA expression of HIF-1α (i.e. in Cd-exposed oysters and/or in oysters acclimated to hypoxia) was accompanied by elevated expression of PDH-2 transcripts. Hypoxia is known to stabilize HIF-1α protein and to inhibit the activity of PHD-2 in a variety of organisms including vertebrates (such as mammals) and invertebrates (nematodes) (Hoogewijs et al., 2007). Possibly, a coordinated decrease in transcription of HIF-1α and elevated expression of PHD-2 in hypoxia-acclimated oysters is a compensatory response that allows them to maintain low levels of cytosolic HIF-1α during hypoxia despite the increased half-life of this protein. Elevated expression of PHD-2 transcript during hypoxia could also poise oyster cells for a rapid degradation of HIF-1α upon reoxygenation.

Interestingly, Cd exposure led to a decreased expression of HIF-1α mRNA in oysters in normoxia and, to a lesser degree, hypoxia. Earlier studies showed that Cd exposure stimulates proteosomal degradation of HIF-1α protein and prevents its nuclear binding to HIF-1β thereby reducing the levels of HIF-1 transcription factor and blocking the normal transcriptional response to hypoxia in mammalian cells (Chun et al., 2000; Horiguchi et al., 2000; Obara et al., 2003; Belaidi et al., 2008). Our study suggests that at least in some organisms, Cd also can inhibit HIF-1α expression at the transcriptional level further interfering with the signaling function of this protein.

Recognition sites for HIF-1 (called hypoxia responsive elements or HREs) are found in the promoters of most glycolytic genes including hexokinase and aldolase, as well as in mitochondrial genes such as COX (Webster, 2003, 2007; Moyes and LeMoine, 2005). In mammals, transcript expression of glycolytic genes is typically upregulated and that of mitochondrial ETC. and Krebs cycle genes – downregulated by HIF-1 (Semenza, 2004; Brahimi-

Horn and Pouyssegur, 2007; Ziello et al., 2007; Taylor, 2008). In this study, no upregulation of mRNA expression of HK and aldolase was found in hypoxia-acclimated oysters. COX mRNA expression was suppressed in hypoxia in control oysters similar to the situation found in mammals (Semenza, 2007) but CS mRNA expression was not affected. This suggests that transcriptional response to hypoxia may differ in hypoxia-tolerant animals such as oysters compared to the hypoxia-sensitive mammalian models.

Notably, out of the four studied metabolic enzymes (HK, aldolase, CS and COX), only aldolase activity was significantly correlated with mRNA expression of the corresponding gene. This suggests that activities of these enzymes are likely post-translationally regulated in oysters. Indeed, regulation by post-translational modifications (e.g. reversible phosphorylation) have been previously reported for a variety of metabolic (including glycolytic and mitochondrial) enzymes during exposure to anoxia and/or tissue hypoxemia caused by freezing in mollusks (Larade and Storey, 2002, 2009; Storey and Storey, 2004). Possibly, similar mechanisms may be responsible for the observed uncoupling between mRNA expression and enzyme activity in oysters. Irrespective of the exact molecular mechanisms, this uncoupling suggests that changes in mRNA expression levels are not necessarily a good predictor of relative activities of HK, CS and COX under different environmental conditions. In future studies, it would also be important to determine whether stress-induced changes in mRNA expression profiles in metabolic enzymes and/or genes involved in oxygen sensing and homeostasis are correlated with corresponding changes in the protein expression profiles. The lack of correlation between enzyme activity and mRNA expression for three out of the four studied enzymes indicates that such correlation may be absent in many cases, and that protein levels and/or activities of these enzymes need to be directly measured to infer the changes in metabolic phenotype during stress exposure of oysters.

4.4. Changes in resident Vibrio populations

The four *Vibrio* spp. studied in this work are gram-negative, halophilic bacteria that naturally inhabit estuarine and coastal waters worldwide and are part of the normal flora of *C. virginica* (Thompson and Swings, 2006). While not pathogenic to oysters, these *Vibrio* spp. can cause serious diseases in vertebrates including humans. Thus, *V. cholerae* is responsible for cyclical endemic and epidemic outbreaks of cholera, a virulent disease characterized by severe diarrhea and dehydration (Colwell, 2006), while *V. vulnificus* causes primary septicemia when ingested as well as severe wound infections (Oliver, 2006). *V. vulnificus* and *V. parahaemolyticus* both have the ability to cause gastroenteritis leading to acute diarrhea. *V. alginolyticus* is a less common pathogen, and is typically associated with superficial wound infections (Oliver, 2005). Of the four studied *Vibrio* spp., Cd exposure led to a significant increase in *V. parahaemolyticus* and *V. vulnificus* levels under normoxia and hypoxia, respectively. Presently, the mechanisms responsible for the Cd-induced increase in abundance of *V. parahaemolyticus* and *V. vulnificus* are not known. Overall, marine *Vibrio* spp. are tolerant to heavy metals including Cd in comparison to other bacteria (Kazmi et al., 1985; Suzuki et al., 1992; Bhattacharya et al., 2000; Fulladosa et al., 2005; El-Hendawy et al., 2009). This Cd resistance is likely due to the ability of *Vibrio* spp. to produce protective proteins (e.g. metallothioneins) after exposure to Cd, particularly after being previously stressed (Gauthier and Flatau, 1977; Nies, 1992; Toth et al., 1996). Moreover, as *Vibrio* species are facultatively anaerobic, they would have little problem dealing with reduced oxygen levels, whereas other competing organisms that comprise the oyster's normal microflora may perceive this as a stress. Thus, exposure to Cd and/or hypoxia may provide a competitive advantage to *Vibrio* spp. over other commensal bacteria of oysters, allowing them to expand their populations. Our data suggest that *V. parahaemolyticus* and *V. vulnificus* may especially benefit from such situations, possibly due to their higher tolerance of Cd. However, the relative tolerance of difference *Vibrio* spp. to heavy metals is not well understood and requires further investigation. Irrespective of the underlying physiological mechanisms, Cd-induced increase in the abundance of *V. parahaemolyticus* and *V. vulnificus* may have important implications for the dynamics of these pathogens in polluted estuaries, especially given that Cd exposure has been shown to provide *Vibrio* spp. with cross-protection against a variety of other stressors (including starvation, other pollutants such as tributyltin, antibiotics and heat stress) (Suzuki et al., 1992; Koga and Takumi, 1995; Toth et al., 1996; Bhattacharya et al., 2000).

4.5. Conclusions and significance

Despite the fact that acute hypoxic exposure results in metabolic rate depression in oysters, prolonged acclimation in low oxygen environment alleviates this response owing to a compensatory increase in ventilatory activity and upregulation of mitochondrial enzymes. In contrast to the energy conserving adaptive metabolic strategies that allow for only a time-limited survival (i.e. metabolic arrest and transition to partial anaerobiosis), compensatory metabolic responses observed in this study could provide long-time survival and minimize physiological stress of oysters under hypoxic conditions. Notably, exposure to Cd changes the profile of metabolic responses in oysters leading to a greater dependency on carbohydrate metabolism for ATP production and resulting in disturbance of tissue energy status during hypoxia. Thus, Cd exposure may limit the capacity of oysters to acclimate to periodical hypoxia and jeopardize their physiological performance (especially such energy-demanding functions primarily fueled by carbohydrate catabolism as reproduction) and survival in polluted estuaries. Cd exposure (with or without concomitant hypoxic stress) also selectively increases abundance of some *Vibrio* spp. pathogenic to humans and other vertebrates, thus potentially raising the human health risks in areas exposed to metal pollution and hypoxia.

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