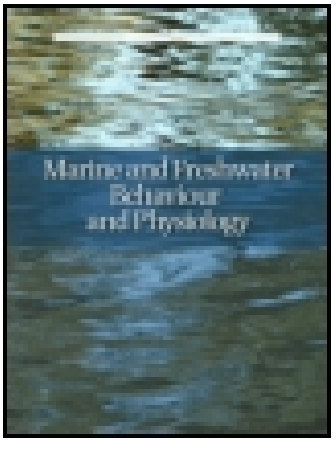

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Oxidative status of respiratory tissues of the bivalve *Mya arenaria* after exposure to excess dissolved iron

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The gills and mantle are the first tissues exposed to altered oxidative conditions during dissolved Fe uptake. The aim of this work was to characterize Fe affected oxidative metabolism on the respiratory tissues of *Mya arenaria*. After 9 days of exposure to elevated Fe levels (500 μM of Fe as Fe-EDTA), a significant increase in the total Fe and labile Fe pool content was found in the gills. Dichlorofluorescein diacetate oxidation rate was higher after 17 days of exposure as compared to controls. Thiobarbituric acid reactive substances content increased 1.9- and 3.7-fold over controls on day 9 and 17, respectively. Both, catalase activity and nitrate and nitrite content decreased significantly on days 9 and 17 compared with controls. Similar effects were observed in mantle, but catalase activity was not affected. The results showed that in respiratory tissues the labile Fe pool is critically controlled to avoid radical-dependent cellular deterioration, but when the endogenous protection mechanisms are overwhelmed, tissue injury was observed.

Keywords: bivalve; *Mya arenaria;* excess iron (Fe); gills and mantle tissues; oxidative stress

Introduction

Iron (Fe) is a micronutrient essential for growth and cellular functioning (Templeton & Liu 2003), but it is also involved in many toxic biochemical reactions. Catalysis of these reactions is not, however, achieved by Fe bound to electron-transferring enzymes or ferritin. Ferritin is known to be the main Fe storage protein which is also used as a detoxifying cellular component and has been identified as an Fe transporter in chitons (Kim et al. 1986), limpets (Burford et al. 1986; Webb et al. 1986), bivalves (Zhang et al. 2003), and snails (Bottke 1982). By contrast, the labile Fe pool (LIP) comprises a low molecular weight fraction of weakly chelated Fe which is catalytically active and satisfies the continuous need for new synthesis of Fe-containing proteins. This fraction potentially generates reactive O_2 species (ROS) by conversion of normal by-products of cell respiration, such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), to highly damaging radical species, such as hydroxyl radical ($^{\cdot}OH$).

Natural processes that increase Fe levels in coastal and oceanic areas are eolian dust deposition, river discharge of Fe-containing sediments, ground water discharge, glacial

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melting, volcanic activity coastal erosion, and weathering, as well as upwelling of Fe-rich deep waters over hydrothermal vents (Watson 2001; González, Wilhelms-Dick et al. 2012). Chemical and mining industries, disposal of waste metal, ports, and eolian deposition of atmospheric dust from polluted areas are further human activities bringing Fe and other metals to coastal and marine ecosystems. Coastal and open ocean waters from different regions thus differ with respect to dissolved Fe content due to these processes. Bioavailable reduced Fe can attain concentrations of several 100 μM in sediment pore water (Canfield et al. 1993). Water or pore water can therefore become an important source for transition metals, such as dissolved Fe and Mn, under reducing conditions (Poigner et al. 2013). Previous work has focussed on studying Fe overload in aquatic systems employing Fe concentrations even within the mM range (Gerhardt 1992; Estevez et al. 2001; Pino Chandia et al. 2012). The rationale and relevance of these studies are linked to the fact that in coastal waters Fe concentration may rise by natural or anthropogenic causes to the mM range (Winterbourn et al. 2000).

The soft shell clam *Mya arenaria* is an abundant infaunal species in the European Wadden Sea where it colonizes intertidal and subtidal areas. *M. arenaria* is a benthic filter feeder that burrows up to 40 cm into the sediment and takes up O_2 and food particles, such as algae and detritus containing Fe and other trace metals, from the sediment water boundary layer, and presumably from sediment pore water. The amount of trace elements absorbed into the tissues of filter feeders correlates with the amount of the bioavailable element fraction in their environment (Elder & Collins 1991).

The effect of *in vivo* dissolved Fe exposure on the oxidative metabolism in the digestive gland (DG) of *M. arenaria* has been studied previously (González et al. 2010). The results showed a significant increase in the total Fe content between 9 and 17 days of exposure. The oxidation rate of 2′,7′ dichlorofluorescein diacetate (DCFH-DA), ascorbyl radical (A$^{\cdot}$) content, CAT activity, and the oxidative damage to lipids detected as 2-thiobarbituric acid reactive substances (TBARS) was however affected and showed significant increases by days 2 and 9 of Fe exposure. The LIP content in the DG was elevated over control values by day 7 of the Fe treatment. This profile of response to Fe exposure suggested that at early stages of Fe supplementation the cellular control mechanisms, such as increase in CAT activity, were operative to limit oxidative damage but that progression of the treatment overwhelmed these protective mechanisms. Superoxide dismutase (SOD) activity did not, however, seem to be involved in this response since it only showed a significant decrease after 9 days of Fe exposure. Initial protection against Fe-dependent damage in DG was also associated with nitric oxide (NO) cellular network since its steady state concentration decreased significantly at day 2.

NO is a regularly occurring radical intermediate of many reactions mostly linked to its function as a signaling molecule (Davis et al. 2001). It is however also capable of binding to most transition metals (Thomas et al. 1997) including Fe (Cooper 1999). NO can readily diffuse across cell membranes and a postulated function of cellular NO flux is to bind and transport Fe out of the cell so avoiding Fe accumulation (Radi et al. 1995). NO can bind to Fe and endogenous thiols generating dinitrosyl-Fe, dinitrosyldiglutathionyl-Fe, or dinitrosyl–glutathionyl–Fe complexes among other nitrosyl–Fe complexes (Pedersen et al. 2007). The presence of these complexes was not, however, yet identified in aquatic animals. González, Abele (2008 first) were the first to show experimental evidence of physiological NO generation in DG of the clam *M. arenaria* and the involvement of nitric oxide synthase (NOS)-like enzymes in this NO generation.

Gills of bivalve filter feeders serve as a particle filter and as gas exchanger at the same time. The pair of gills separates the mantle cavity into a ventral inhalant chamber and a dorsal exhalent chamber. Sediment that enters with the inhalant water gets trapped by the ciliated gills and absorbed into secreted mucus. Periodically, the valves snap shut expelling the large sediment particle–mucus aggregates as pseudofaeces. Fine sediment particles, however, are transported over the gills toward the mouth and ingested (Hickman et al. 1994). O_2 is absorbed as the water flows dorsally over the gill surface. Toxic metals can interfere with gill function by inhibiting the cilia (Abel 1976). The mantle is moreover involved in gas and ionic exchange as well as nutrition in various bivalves (Henry & Saintsing 1983) and might also play a role in the uptake of dissolved contaminants. Gill and mantle function, as an exchange barrier, could be compromised by intoxication (Lemaire-Gony & Boudou 1997). They might therefore be prone to suffer both elevated ROS levels and oxidative damage related to continuous contact between these tissues and media from a contaminated surrounding environment. Gills and mantle, which are different in nature to DG, are the first tissues exposed to the oxidative conditions caused by dissolved Fe exposure so that their response is a critical issue to an understanding of global physiological effects.

The hypothesis that prompted the present work is that after *in vivo* Fe exposure, Fe increase in these respiratory tissues could be actively controlled to avoid extreme increases in catalytically active metal content leading to critical damage that could affect survival. The main aim of this study was to characterize oxidative metabolism, Fe and LIP content, damage to lipids, and CAT activity in the respiratory tissues during 17 days of exposure to an excess of Fe. Nitrosative metabolism was also evaluated.

Materials and methods

Animal collection and maintenance

Soft shell clams *M. arenaria* (75 ± 1 mm shell length and 47 ± 1 mm shell width) were collected on an intertidal sand flat at Dorum-Neufeld, near Bremerhaven, Germany. Animals were kept in two fully aerated aquaria at the Alfred Wegener Institute, Bremerhaven for at least 1 week prior to the experiments to ensure that they were healthy and not suffering from sampling stress. The natural seawater in the aquaria had a salinity of 23–26‰, Fe content of 10.7 ± 0.2 nM, and temperature of 10 °C. The bottom of the aquaria was covered with pea gravel to a depth of 10 cm. Animals were fed live phytoplankton every 84 h, using DT's live marine phytoplankton, premium reef blend consisting of *Nannochloropsis oculata*, *Phaeodactylum* sp., *Tricornutum* sp., *Chlorella* sp., between 2 and 20 μm particle size with no Fe added.

In vivo *Fe exposure*

Experimental bivalves were placed in 10 small aquaria containing 13 L each (13 animals per aquarium) of natural seawater of 23–26‰ at 10 °C, and 500 μM of Fe as Fe-EDTA complex (1:2). Fe-EDTA was used as the Fe source because of chemical nature of the complex and also because it is known to occur in natural waters (Hering & Morel 1988). In the Fe incubation treatments, the seawater was replaced every two days by a fresh solution to ensure water quality and to maintain a constant Fe level in the surrounding medium. The animals were fed every 72 h during experimentation. Feeding was stopped 48 h before animal sacrifice to avoid absorption of endogenous Fe

from the algae which could affect the Fe content in the organisms. Sampling was performed on day 0, 2, 7, 9, and 17 according to the findings of preliminary measurements of Fe enrichment. Animals were dissected, and gill and mantle were frozen and stored in liquid nitrogen.

Total Fe content

Total Fe content was analyzed by two independent techniques:

(1) According to Bralet et al. (1992) with modifications, approximately 40 mg of gills and mantle was homogenized in 1 mL of 150 mM KCl in 0.1 N HCl, pH 2.5, prepared with iron-free water (IFW). The samples were incubated for 24 h at 37 °C in the presence of 9 mg mL^{-1} pepsin, 4.5 mM 2,2′ dipyridyl, and 103 mM ascorbic acid, pH 7.0 prepared in IFW. For each sample both, a reactive blank and a sample blank without 2,2′ dipyridyl, were prepared. Absorbance was read at $\lambda = 520$ nm. To determine the Fe content, a standard curve was prepared with Fe in a concentration range from 0 to 120 μM.
(2) Using atomic absorption spectroscopy in an air–acetylene flame (Buck model 200 A, East Norwalk, Connecticut, USA). Liquid nitrogen frozen tissues were mineralized in a graphite muffle for 4 h at 500 °C. For Fe content assessment, a standard curve was prepared with Fe standard solutions of 1.8–5.4 μM. All materials used were treated with HNO_3 and IFW for 48 h to avoid Fe contamination.

LIP determination

The LIP content in tissues was determined by a fluorescence technique with the Fe sensor calcein (CA) according to Darbari et al. (2003) with modifications by Robello et al. (2007). Tissue homogenates were used here instead of cells, and the modification thus transformed this technique in a disruptive method that could be classified as an *ex vivo* determination. Disruptive methods have the disadvantage that they decompartmentalize Fe during fractionation and even disrupt some Fe-containing proteins or release Fe from storage proteins. Even though the protocol might have some limitations, non-disruptive techniques could not be applied to measure LIP in these tissues because of the nature of the preparations. Gills and mantle were treated as previously described in González, Abele et al. (2008). Tissues were homogenized in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4 (1:10). The homogenate was centrifuged at 8700 *g* for 15 min at 4 °C and the supernatant removed to Eppendorf tubes and centrifuged again at 8700 *g* for 15 min at 4 °C. The supernatant was filtered through filters with 30,000 nominal molecular weight limit (Centricon YM30). The filtered solution was then reduced with equal volume of 8% (v/v) thioglycolic acid. Fe in the reduced solution was measured using 1 μM CA solution in 40 mM potassium phosphate buffer, 120 mM KCl, pH 7.4. When Fe is added to CA solution, a fraction of the dye binds free Fe^{2+} leading to the generation of the Fe-bound (quenched) complex [CA–Fe], while another fraction remains free as unbound CA and provides the residual fluorescence. The fluorescence ($\lambda_{exc} = 485$ nm, $\lambda_{em} = 535$ nm) was recorded until stabilization of the signal (F_1), and then, deferoxamine (DF) was added to a final concentration of 1 mM. The fluorescence was monitored until a new stabilization of the signal (F_2) was reached. The magnitude

of the absolute change in fluorescence (F_2–F_1) is equivalent to the amount of Fe bound to CA. The fractional increase in fluorescence (ΔF) that reflects the LIP concentration was calculated according to Equation (1).

$$\Delta F = (F_2 - F_1)/F_2 \quad (1)$$

The LIP was assessed using Equation (2) and a dissociation constant (Kd) value of 0.46 μM (Robello et al. 2007).

$$LIP = (\Delta F \times [CA]) + [(Kd \times \Delta F)/(1 - \Delta F)] \quad (2)$$

Oxidation of DCFH-DA by tissue homogenates

Gills and mantle samples were homogenized (1:5 w/v) in a buffer composed of 100 mM Tris-HCl, pH 7.75 with 2 mM EDTA, and 5 mM $MgCl_2$ (Gallagher et al. 1992). Measurements were conducted according to Lund Amado et al. (2009) and Viarengo et al. (1999) with modifications. Briefly, the homogenates were centrifuged at 4 °C for 20 min at 10,000 *g*, and the supernatants were used for the assay. The reaction was followed in a 30 mM HEPES buffer, pH 7.2 with 200 mM KCl, and 1 mM $MgCl_2$. The fluorescent probe DCFH-DA was added to the buffer at a final concentration of 40 μM. After addition of 5–10 μL of the supernatant for gills, or 10–20 μL of the supernatant for mantle, the reaction mixture was then incubated at 35 °C for 10 min. The fluorescent compound DCF, generated by radical-dependent oxidation of the probe, was detected spectrofluorometrically at λ_{exc} = 488 nm and λ_{em} = 525 nm.

Determination of TBARS content

TBARS content in tissue homogenates was measured according to Uchiyama and Mihara (1978) as described by Storch et al. (2001) and Abele et al. (2002). Tissues were ground in liquid N_2, and the powder was homogenized (1:5) (p/v) in 0.2% phosphoric acid. A 2% phosphoric acid solution was added to achieve a final concentration of 1.1% (v/v). An aliquot of 0.4 mL of the homogenate was supplemented with 0.4 mL of an aqueous solution of 1% (p/v) of 2-thiobarbituric acid (4,6-dihydroxypyridine-2-thiol) (TBA). Specific blanks were prepared replacing the TBA solution with 30 mM HCl. Samples and blanks were heated at 100 °C for 1 h. The samples were cooled at room temperature, and 2 mL of butanol was added. The samples were strongly shacked during 40 s, and the butanol phase was separated by centrifugation during 5 min at 1000 *g*. The supernatant was then centrifuged for 5 min at 14,000 *g*. The supernatant was separated, and the optical density was assessed at λ = 532 y 600 nm. The difference in absorbance between the two λ was quantified. Malondialdehyde standards were prepared from 1,1,3,3-tetraethoxypropane in the 0–50 μM concentration range.

Determination of CAT activity

Homogenates from gills and mantle were prepared in 30 mM potassium phosphate, 120 mM KCl, pH 7.4. CAT activity (EC 1.11.1.6) was assayed spectrophotometrically as the decomposition of H_2O_2 at λ = 240 nm in a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2 (Aebi 1984).

Determination of nitrate (NO_3^-) and nitrite (NO_2^-) content

The total content of NO_3^- and NO_2^- was assessed by the Griess reaction, according to Verdon et al. (1995) with modifications. The isolated tissues were homogenized in 14 mM sodium phosphate buffer, pH 7.1 (1:1.5 and 1:2.5 for gills and mantle, respectively). NO_3^- content in the extracts was reduced to NO_2^- by the addition of nitrate reductase enzyme and NADPH as cofactor. The sample was then incubated for 2 h at room temperature before addition of the Griess reagent followed by incubation for a further 10 min. The concentration of $NO_3^- + NO_2^-$ was measured spectrophotometrically at $\lambda = 540$ nm. Quantification was performed using a NO_3^- and NO_2^- standard curve in the range of 0–75 μM.

Determination of protein content

Protein measurements were performed according to Lowry et al. (1951).

Statistical analyses

Data in the text and tables are expressed as mean ± S.E.M. Statistical ANOVA one way tests were carried out using Statview for Windows, SAS Institute Inc., version 5.0.

Results

M. arenaria collected from their natural environment were maintained in the aquaria in fully aerated natural seawater for 2 months without occurrence of mortality. Gills of bivalves collected from the Wadden Sea sandflat had a total Fe content of 0.28 ± 0.05 nmol Fe/mg FW (fresh weight) as determined by atomic absorption and spectrophotometric measurements following enzymatic digestion (Figure 1).

Supplementation of seawater with 9 μM dissolved Fe for 9–17 days had no significant effect neither on Fe levels in the gills (0.23 ± 0.02 nmol Fe mg FW^{-1}) or in TBARS content. A greater increase of Fe content in the incubation seawater (500 μM) resulted, however, in a significant increase in Fe content in the gills after 9 days, and these enhanced values remained constant until day 17 (Figure 1). The LIP content in gill tissues increased correspondingly and significantly on day 17 following exposure to 500 μM Fe compared to control values (Figure 1 inset). The experimental protocol included a study performed to select the adequate time frame for Fe treatment (126 animals). Survival of the animals was registered in 6 independent sets of animals (12–40 animals in each). By the 17th day of exposure, there were either no deaths (4 experiments) or 1 death (2 experiments). On day 18 of Fe exposure, 20–25% of the treated animals died. To avoid the possible influence of an unknown factor or factors (e.g. toxic metabolites from cellular damaged components) when evaluating a Fe effect, the study employed a maximum incubation time of 17 days where a critical threshold that compromised cell survival was not reached.

Among other uses, DCFH-DA may be employed as a redox indicator probe that responds to changes in intracellular Fe signaling or peroxynitrite ($ONOO^-$) formation (Kalyanaraman et al. 2012). The data in Figure 2 indicate that dye oxidation rate in the sample homogenates was significantly higher after 17 days of exposure to high Fe overload when compared to values in gill homogenates from control animals. Oxidative damage in the lipid fraction increased during high Fe exposure. The increase of TBARS

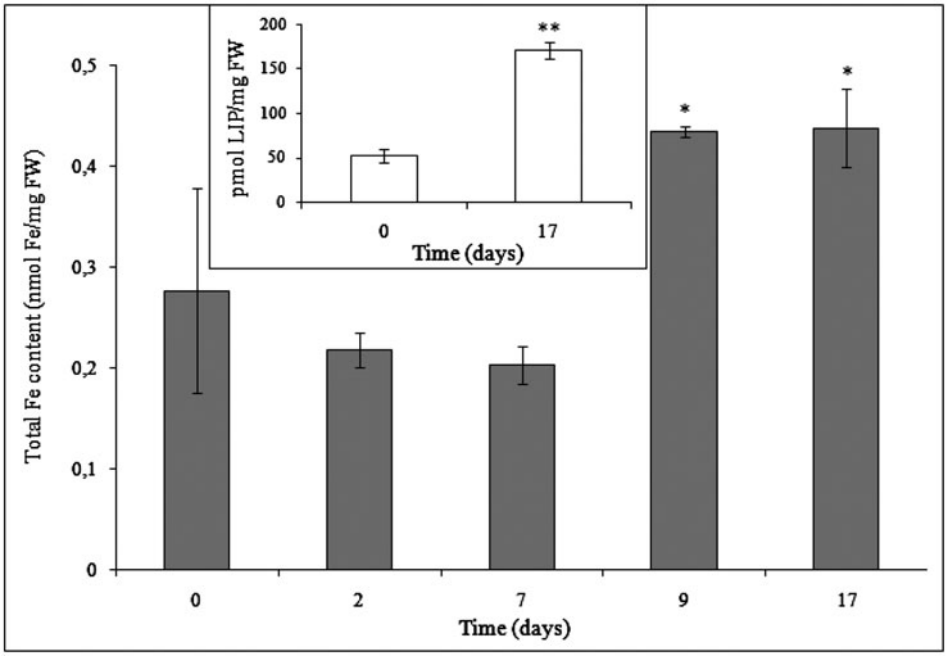

Figure 1. Kinetic profile of the total Fe content in gills from *M. arenaria* exposed to seawater supplemented with 500 μM Fe. Fe content was assessed spectrophotometrically, as indicated in the 2.3. Material and Methods Section. Fifteen animals (15) (3 animals per group) were employed in each experiment. Measurements were performed by duplicate. *significantly different from the value at day 0 with $p < 0.01$. ANOVA. **Inset:** LIP content in Gills from *M. arenaria* exposed to seawater supplemented with 500 μM Fe for 0 and 17 days. Twelve animals (12) (6 animals per group) were employed in each experiment. **significantly different from the value at day 0 with $p < 0.0001$. ANOVA.

content in gills was approximately 1.9-fold after 9 days of treatment and 3.7-fold on day 17 when compared to control values (Figure 2).

The exposure to Fe overload may alter the antioxidant capacity of the tissues. The activity of the H_2O_2 scavenging antioxidant enzyme CAT was constant over the initial period of 0–7 days of exposure but decreased significantly at days 9 and 17 of treatment compared to controls (Table 1). Moreover, the content of NO_3^- and NO_2^-, as indicator of tissue NO content, followed a pattern similar to that of CAT activity. There were no changes observed over the initial days of Fe exposure, but a significant decrease of putative NO content by 59 and 44% of control values was recorded on days 9–17, respectively (Table 1).

The total Fe content in mantle of *M. arenaria* maintained in Fe-free seawater was 0.23 ± 0.01 nmol Fe mg FW^{-1} as evaluated by atomic absorption. As shown in gill tissue, the value obtained did not differ significantly from the value measured spectrophotometrically after enzymatic digestion (Table 2). Exposure to 500 μM dissolved Fe in natural seawater resulted in a significant increase in the total Fe content in mantle tissue after 17 days of exposure (Table 2). The LIP content in mantle tissues was not significantly different from the values in gills, neither under control nor after 17 days of exposure to Fe overload conditions. LIP values on day 0 and 17 in mantle were, however,

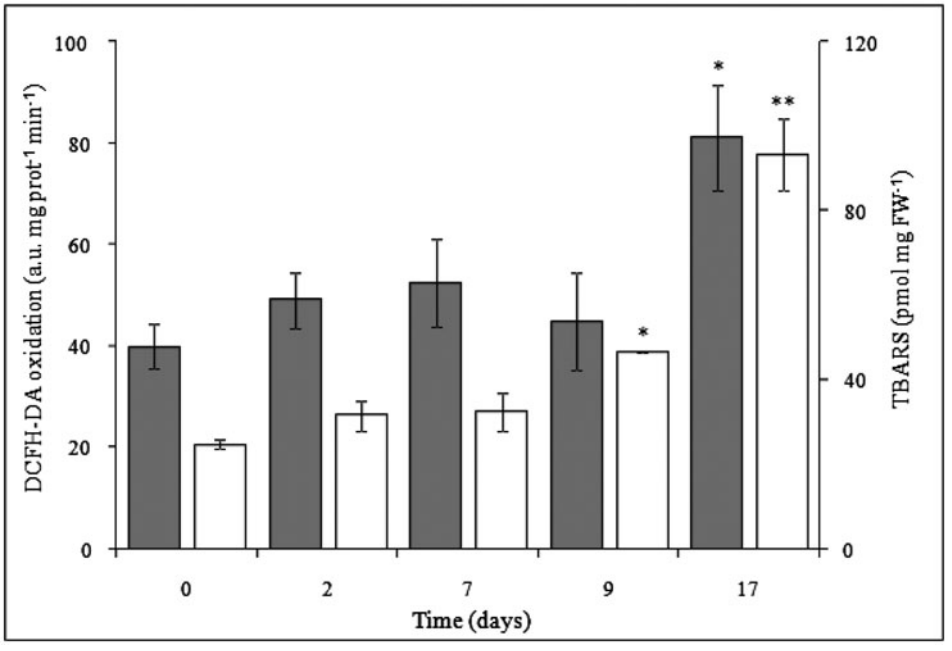

Figure 2. Kinetic profile of oxidative stress and oxidative damage indicators in gills from *M. arenaria* exposed to seawater supplemented with 500 μM Fe. ROS generation rate assessed as DCFH-DA oxidation rate (■). Twenty animals (20) (4 animals per group) were employed in each experiment. Measurements were performed by quadruplicate. TBARS content (□). Twenty animals (20) (4 animals per group) were employed in each experiment. Measurements were performed by triplicate. *significantly different from the value at day 0 with $p < 0.01$ and **$p < 0.0001$. ANOVA.

Table 1. CAT activity and $NO_2^- + NO_3^-$ content in gills from *M. arenaria* exposed to seawater supplemented with 500 μM Fe.

Time (days)	CAT activity (pmol mg prot^{-1}) (10^{-3})	$NO_2^- + NO_3^-$ (pmol mg FW^{-1})
0	397 ± 40	113 ± 13
2	310 ± 18	80 ± 20
7	372 ± 38	141 ± 45
9	234 ± 31**	46 ± 7*
17	249 ± 28**	63 ± 8*

Twenty (20) (4 animals per group) were employed in CAT activity determination. Twenty five (25) (5 animals per group) were employed in $NO_2^- + NO_3^-$ content determination. Measurements were performed by triplicate and duplicate for CAT activity and $NO_2^- + NO_3^-$ content, respectively.
*Significantly different from the value at day 0 with $p < 0.05$.
**$p < 0.01$. ANOVA.

significantly different (58 ± 9 and 195 ± 11 pmol mg FW^{-1}, respectively; $p < 0.0001$, ANOVA).

Consequently, and following a similar profile to that described for gills, DCFH-DA oxidation rate in mantle homogenates showed a significantly higher value after 17 days of exposures compared to control values on day 0 (Table 2). TBARS content in mantle

Table 2. Total Fe content, DCFH-DA oxidation, and TBARS content in mantle from *M. arenaria* exposed to seawater supplemented with 500 μM Fe.

Time (days)	Total Fe content (nmol mg FW^{-1})	DCFH-DA oxidation (a.u. mg $prot^{-1}$ min^{-1})	TBARS (pmol mg FW^{-1})
0	0.22 ± 0.02	7 ± 2	26 ± 1
2	0.21 ± 0.02	5.6 ± 0.7	16 ± 4
7	0.20 ± 0.02	6.2 ± 0.5	39 ± 13
9	0.21 ± 0.02	10 ± 2	28 ± 7
17	0.37 ± 0.02***	12 ± 2*	54 ± 11**

Fifteen animals (15) (3 animals per group) were employed in total Fe and TBARS content determination. Twenty (20) (4 animals per group) were employed in DCFH-DA oxidation determination. Twenty five (25) (5 animals per group) were employed in $NO_2^- + NO_3^-$ content determination. Measurements were performed by duplicate except TBARS analysis which was performed by triplicate.
***Significantly different from the value at day 0 with $p < 0.001$.
**$p < 0.01$; *$p < 0.05$. ANOVA.

tissue was also significantly increased on day 17 by approximately twofold compared to control values (Table 2). The content of NO_3^- and NO_2^- decreased by approximately 77% on days 9 and 17 of the experimental period compared to control values which is similar to the change observed in gills (Table 3). Contrary to the results in gills, mantle CAT activity was not affected by high dissolved Fe treatment (Table 3).

Discussion

A high mortality on day 18 of the Fe overload during the preliminary experiments suggested that Fe overload exceeded tissue storage capacity and overwhelmed physiological protection mechanisms for dealing with toxic, catalytically active Fe in the gills and mantle of *M. arenaria*. Fe enrichment in both tissues caused an increase of the LIP that probably resulted in enhanced oxidation of DCFH-DA in homogenates at this time so that the animals died. The oxidative damage to lipids occurred earlier and was more pronounced in gills than in mantle, possibly owing to higher lipid content in the gills and differences in lipid composition between both tissues (Wenne & Polak 1989; Caers et al. 1999). The reduction of CAT activity by >38% in gills on days 9 and 17 of exposure may have contributed further to oxidative stress and damage as observed in SOD

Table 3. CAT activity and $NO_2^- + NO_3^-$ content in mantle from *M. arenaria* exposed to seawater supplemented with 500 μM Fe.

Time (days)	CAT activity (pmol mg $prot^{-1}$) (10^{-3})	$NO_2^- + NO_3^-$ (pmol mg FW^{-1})
0	14 ± 1	218 ± 32
2	19 ± 3	162 ± 51
7	18 ± 1	133 ± 52
9	15 ± 3	53 ± 12*
17	16 ± 2	47 ± 8*

Twenty (20) (4 animals per group) were employed in CAT activity determination. Twenty five (25) (5 animals per group) were employed in $NO_2^- + NO_3^-$ content determination. Measurements were performed by triplicate and duplicate for CAT activity and $NO_2^- + NO_3^-$ content, respectively.
*Significantly different from the value at day 0 with $p < 0.01$. ANOVA.

activity in DG (González et al. 2010). These results suggest that enzymatic antioxidant protection was not induced and, therefore, that these mechanisms were not strongly involved in the defense system during the treatment. In the DG of the mussels *Perna perna* exposed to 9 μM Fe, different effects were found for CAT, glutathione peroxidase, glutathione S-transferase, and phospholipid hydroperoxide glutathione peroxidase as compared to control animals (Alves de Almeida et al. 2004). The different profile of response seen in our study could be due to the fact that the high Fe load is extremely detrimental to the antioxidant enzymatic network so limiting its capacity. As suggested by Alves de Almeida et al. (2004), other protective mechanisms such as metallothioneins could, however, afford transient control of heavy metal toxicity during experimental exposure. Furthermore, it has been reported that CAT bound NO generates a rapidly and reversible inhibition of CAT with a K_i of 0.18 μM (Brown 1995). One possible interpretation of the parallel decline of the enzyme activity and the concentration of NO_3^- and NO_2^- may thus be the interaction between NO and CAT, leading to an inhibition of the enzyme in the gills. CAT activity may also be damaged by ROS and reactive nitrogen species (RNS). An even more dramatic decrease of Griess products did not lead to apparent CAT inhibition in the mantle which raises some doubts about this proposed direct interaction mechanism.

An alternative role for NO as part of the line of defense triggered in *M. arenaria* against uncontrolled Fe uptake is the binding of NO to Fe leading to the outward transport of LIP (Radi et al. 1995). The formed nitro complexes could leave the cell through the membrane thereby avoiding metal accumulation under conditions of Fe excess. This mechanism could also be responsible for the decrease of measurable NO oxidation products on prolonged exposure (after 17 days). No chemical or experimental evidence to sustain this postulate is yet available. It could also be suggested that NO may have been removed from the system by its interaction with $O_2^{\bullet-}$. This reaction that would cause generation of the highly reactive $ONOO^-$ would support lipid peroxidation leading to increased TBARS accumulation in both tissues (González, Abele et al. 2012). The physiological decomposition of $ONOO^-$ into NO_3^- and NO_2^- has, however, a very short half-life (<0.1 s) which should cause an increase in the NO_3^- and NO_2^- concentration rather than a decrease (Koppenol et al. 1992; Miles et al. 1996; Fukuto et al. 2000). On the other hand, NOS-like enzymatic activity, as yet not isolated in molluscs, might have been affected by Fe leading to a reduced content of NO_3^- and NO_2^-. This would emphasize that RNS metabolism is endogenously linked to the catalytically active Fe that influences ROS metabolism.

Beninger et al. (1997) showed that the transport medium for particles on the gill was acid mucopolysaccharides in the form of mucus. The presence of this mucus surrounding the respiratory tissues could be, at least in part, responsible for the tissue-specific response to Fe excess. The gills and mantle in *M. arenaria* appear to be protected by the mucus which mediates against Fe incorporation into the tissues and the generation of H_2O_2. The early oxidative stress response reported for DG was thus not observed in gills or in mantle tissues. Even though the mechanism of Fe incorporation into cells has not been previously described for *M. arenaria*, similar pathways such as those described for mammalian cells (e.g. transferring receptors) could also be involved. Marigómez et al. (2002) also reported that Fe from the environment could be absorbed by pinocytosis in the gills of marine mussels. The higher numbers of transferring receptors in DG compared with those in respiratory tissues could be a sign of enhanced Fe incorporation into DG by comparison with the gills and mantle.

The LIP usually represents a minor fraction (<5%) of the total Fe in many organisms (Kruszewski 2004). The percentage of LIP in the gills and mantle of *M. arenaria* was 19 and 26% of the total Fe content at day 0. These higher values are consistent with previous reports in DG from the limpet *Nacella magellanica* assessed in its natural habitat (15%) (González, Malanga 2008). LIP in the DG was 10% of the total Fe content at day 0 which suggests that damage risk from catalytically active Fe was well controlled at this stage. After 17 days, Fe exposure LIP represented 39, 52 (present report), and 10% (González et al. 2010) for gills, mantle, and DG, respectively. In gills and mantle on the 17th day of exposure to high Fe, the percentage of catalytically active Fe was double the control levels and total tissue Fe was significantly enhanced. It was important to analyze these changes using absolute concentrations because the critical parameter for estimating the reactivity of loosely bound Fe is the absolute concentration of the LIP in the tissues. The basal LIP concentrations at day 0 were as follows: 0.050, 0.058, and 0.070 nmol/mg FW in gills, mantle, and DG (González et al. 2010), respectively. The values at day 17 of Fe exposure were as follows: 0.170, 0.195, and 0.190 nmol/mg FW in gills, mantle, and DG, respectively. It is evident that the absolute concentration of the fraction of Fe that is catalytically active for the generation of ROS is similar in the three tissues studied, both at day 0 and at day 17. The measured increase in the LIP in this period of exposure is also similar (around three fold) in the three tissues examined. The degree of protection appears therefore to be directly related to the ability of the metabolic control.

The findings presented here will be of particular relevance during consideration of pollution situations that affect marine invertebrates. The results demonstrated a way in which respiratory tissues can reduce catalytically active Fe and limit oxidative damage in molluscs exposed to elevated levels of dissolved Fe in seawater. It is also apparent that if the endogenous mechanisms regulating internal Fe levels are overwhelmed by very high levels of external Fe or extended exposure to lower levels, the physiological system fails. Contamination events involving elevated Fe can therefore have catastrophic outcomes for marine communities.

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