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Hepatopancreas mitochondria of *Mytilus galloprovincialis*: effect of zinc ions on mitochondrial bioenergetics and metabolism

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Abstract: Oxygen uptake, respiratory complexes, and metabolic activities have been studied in mitochondria isolated from the hepatopancreata of *Mytilus galloprovincialis* collected in the "Mar Grande" of Taranto (Ionian Sea, Italy). Although exposure to 5.0 µg Zn^{2+}/L resulted in a significant increase of states 3 and 4 respiration with glutamate + pyruvate as respiratory substrate, it was found that the exposure of mussels to different concentrations of Zn^{2+} (2.5–7.5 µg/L) neither inhibited mitochondrial respiration nor exerted any inhibitory effect on representative mitochondrial dehydrogenases. It rather stimulates these activities, producing an extra synthesis of adenosine triphosphate by hepatopancreas mitochondria and possibly increasing its availability in the cytoplasmic compartment. This might be considered as a specific strategy utilized by the mussel to cope with variations in the heavy metals content of the marine environment, and it could be used to detect toxic effects.

Key words: Zinc, *Mytilus galloprovincialis*, mitochondria, respiratory enzymes

1. Introduction

Heavy metals are continually discharged into the marine environment by human activities and represent a very critical issue, causing numerous problems all over the world (Leblebici et al., 2011). Anthropogenic sources of metals include urban runoff, sewage, traffic emissions, coal and oil combustion, industrial production, mining, and the smelting of ores (Eisler, 1993; Qiao et al., 2007; Pote et al., 2008).

Marine organisms ingest heavy metal ions from their diet, and for many aquatic organisms, exposure to metals at above-threshold concentrations is extremely toxic (Grajeda y Ortega et al., 2011). Although the cellular effects of zinc ions have been extensively investigated in mammalian tissues (Frederickson et al., 2000), little is known about the effects of zinc ions on the cellular metabolism and bioenergetics of marine organisms.

Monitoring of the marine environment often includes the assay of metal loads in tissues of different test species. Zinc is measured at between 0.1 and 10 µg/L; these levels in biomonitor organisms reflect the quality of the aquatic environment. Cantillo (1998) suggested concentration levels for contamination in soft tissues of mussels at 200 µg/g. This metal can accumulate in mollusks and crustaceans, mainly in the hepatopancreas, gonads, and gills.

Recent studies of mitochondrial functions under a variety of physiological and stress conditions have highlighted that mitochondria are highly regulated and subject to malfunction in response to even moderate stress, rather than operating reliably in the background as was previously believed (Sokolova et al., 2000; Sokolova and Pörtner, 2001, 2003; Nicholls, 2002). Marine organisms, including mollusks, are exposed to a variety of environmental stress agents in their habitats, which can strongly affect their metabolism and bioenergetics. The mussels *Mytilus* spp. are sedentary good filter-feeders and bioaccumulators of some trace elements (George, 1980; Fabris et al., 1994).

The aim of the present study was to investigate the effects of Zn^{2+} on mitochondrial functions in *Mytilus galloprovincialis*, including respiration rate and enzymatic activities of hepatopancreas mitochondria.

2. Materials and methods

2.1. Chemicals

NADH, antimycin A, myxothiazol, oligomycin, horse heart ferricytochrome *c*, rotenone, safranin O (3,7-diamino-

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2,8,dimethyl-5-phenyl-phenazinium chloride), and FCCP (carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone) were obtained from Aldrich Chemical Co. All other reagents used were of commercially available analytical grade.

2.2. Animals

Adult specimens of commercial-sized (8 months old, 4 to 5 cm in length, average weight of 13.6 ± 2.0 g) mussel *Mytilus galloprovincialis* Lmk. were obtained from coastal culture plants in the "Mar Grande" (Ionian Sea, southern Italy) and transported alive in aerated seawater tanks to the laboratory. Water temperature at the time of collection was 12 ± 2 °C, in winter, with salinity of 38 ± 1‰. The collection site has very low background concentrations of heavy metals (Buccolieri et al., 2006), with an average Zn^{2+} concentration of 89.3 µg/g of dry sediment.

Animals were acclimated in the laboratory under controlled conditions (20 °C, salinity 37‰, and exposure to natural day/night cycles) for 2 weeks prior to experimentation. During the acclimation period, mussels were fed daily with an algal mixture containing *Tetraselmis suecica* and *Isochrysis galbana*, which was obtained from the culture collection of the Institute for Coastal Marine Environment, National Research Council (CNR IAMC) section of Taranto, Italy. Mortality was not detected during the preliminary acclimation period. The test individuals were starved for 1 day prior to the bioassay test.

After acclimation to the laboratory conditions, the mollusks were transferred to aerated 30-L glass aquaria and divided into 4 groups of 10 specimens each. Mussels belonging to Group I were placed in chemical-free water and treated as the control. The mussels of Groups II, III, and IV were introduced into a medium with Zn^{2+} at concentrations of 2.5, 5.0, and 7.5 µg Zn^{2+}/L, chosen through a wider concentration range test in order to determine the lowest concentrations to which mussels showed the examined effects. For each group, 3 replicates were used. The test medium was freshly renewed every day to give a constant effect of Zn^{2+} on the mussels. Each exposure lasted 3 days, after which mitochondria were isolated from the mussels.

2.3. Isolation of mitochondria

After 3 days of zinc exposure, the hepatopancreata (average fresh weight: 1.5 ± 0.3 g) were separated from the control and test mussels with a plastic knife, blotted dry, and placed in 50 mL of ice-cold isolation medium containing 480 mM sucrose, 100 mM KCl, 50 mM NaCl, 70 mM HEPES, 3 mM EDTA, 6 mM EGTA, and 1% BSA (Ballantyne and Storey, 1984), modified by adding 1 mM PMSF to inhibit proteases released by the crystalline stylus. The pH was adjusted to 7.6 with KOH at 25 °C. The hepatopancreata were homogenized by 3 or 4 strokes of a Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle. Initially, the homogenate was centrifuged for 10 min at 1150 × *g*; the supernatant was collected and then centrifuged at 10,500 × *g* for 10 min. The resulting mitochondrial pellet was suspended in a small volume of isolation medium. All steps were carried out at 4 °C. The mitochondrial protein concentrations ranged between 18 to 24 mg/mL. Protein concentration was determined with Bradford's assay (Bradford, 1976), using BSA as the protein standard.

2.4. Mitochondrial respiration

Mitochondrial respiration (oxygen consumption rate) was determined in a closed thermostated chamber equipped with a Clark-type electrode (Rank Bros., UK). Incubations were performed at 25 °C in 1.5 mL of a standard medium at pH 7.6 containing 480 mM sucrose, 70 mM HEPES, 10 mM Pi, 100 mM KCl, 5 mM $MgCl_2$, and 1% BSA. Mitochondria were added to a final concentration of 1 mg protein/mL.

Maximal respiration rate (state 3), demonstrating the maximum adenosine triphosphate (ATP) synthesis capacity in mitochondria, was achieved by addition of 0.5 mM adenosine diphosphate (ADP). State 4 (in the absence of ADP) respiration was initiated by the addition of substrates: 5 mM succinate or 5 mM glutamate + 5 mM pyruvate. The respiration control ratio (RCR), a measure of mitochondrial coupling, was calculated by dividing the state 3 by state 4 respiration according to Estabrook (1967).

2.5. Mitochondrial membrane potential

Time-dependent mitochondrial membrane potential ($\Delta\Psi$) was determined fluorometrically with a PerkinElmer LS-5B fluorescence luminometer with the dye safranin O (3,7-diamino-2,8,dimethyl-5-phenyl-phenazinium chloride) (La Piana et al., 1998) at the wavelengths of 520 nm (excitation) and 580 nm (emission). Incubations were carried out at 25 °C in a standard medium at pH 7.6 containing 480 mM sucrose, 70 mM HEPES, 10 mM Pi, 100 mM KCl, 5 mM $MgCl_2$, and 1% BSA. Safranin at 10 µM was added to equilibrate for 3 min with mitochondria in 3.0 mL of the incubation medium, in order to avoid differences in the basal fluorescence of each sample. The samples contained 1.0 mg mitochondrial protein/mL.

2.6. Determination of enzyme activities

2.6.1. Malate dehydrogenase

Activity was determined following the oxidation of NADH by recording the absorbance decrease at 340 nm using a Varian (Cary 50) spectrophotometer. First, 100 µg of mitochondrial protein was added to 3.0 mL of a medium containing 50 mM Pi, 0.1 mM EDTA, 6 µM rotenone, 5 mM $MgCl_2$, 1 mM lauryl maltoside, and 0.5 mM NADH, pH 7.4. The reaction was then triggered by the addition of 0.5 mM oxaloacetate. The molar extinction coefficient for NADH was 6.22 mM^{-1} cm^{-1} (Bergmeyer and Bernt, 1987).

2.6.2. Cytochrome oxidase

Cytochrome oxidase activity was determined following the oxidation of ferrocytochrome *c* at the wavelength pair of 548-540 nm, using a Hitachi-PerkinElmer double beam–double wavelength spectrophotometer (model 557). First, 50 µg of mitochondrial protein was added to 3.0 mL of a medium containing 50 mM Pi, 0.1 mM EDTA, 6 µM rotenone, 6 µM myxothiazol, and 1 mM lauryl maltoside, pH 7.4. The reaction was then started by the addition of 50 µM ferrocytochrome *c*. The reaction was stopped by adding 1 mM KCN. The molar extinction coefficient for cytochrome *c* was 21 mM^{-1} cm^{-1} (Lofrumento et al., 1991).

2.6.3. NADH-cytochrome c reductase

The reduction of cytochrome *c* was followed by recording the absorbance increase at 548–540 nm. First, 50 µg of mitochondrial protein was added to 3.0 mL of a medium containing 50 mM Pi, 0.1 mM EDTA, 6 µM rotenone, 6 µM myxothiazol, 50 µM ferricytochrome *c*, and 1 mM KCN, pH 7.4. The reaction was initiated by the addition of 0.5 mM NADH (Marzulli et al., 1995).

2.6.4. Succinate–cytochrome c oxidoreductase

The activity was determined following the reduction of cytochrome *c* by recording the absorbance increase at 548–540 nm. First, 3 mg of mitochondrial protein was added to a medium containing 50 mM Pi, 0.1 mM EDTA, 6 µM rotenone, 1 mM KCN, 0.05% potassium deoxycholate, and 50 µM ferricytochrome *c*, pH 7.4. The reaction was then triggered by the addition of 5 mM succinate. After 2 min, 6 µM myxothiazol was added to account for complex III-independent reduction of cytochrome *c* (Lofrumento et al., 1991).

2.6.5. Duroquinol–cytochrome c oxidoreductase

This activity was determined by a spectrophotometer recording the decrease at 262–282 nm due to the oxidation of duroquinol (molar extinction coefficient for duroquinol was 13.0 mM^{-1} cm^{-1}); 40–50 µg mitochondrial protein was incubated in 3.0 mL of a medium containing 50 mM Pi, 0.1 mM EDTA, 6 µM rotenone, 1 mM KCN, 0.05% deoxycholate, and 20 µM ferricytochrome *c*, pH 7.4. The reaction was triggered by the addition of 200 µM duroquinol and, after 2 min, 6 µM myxothiazol was added to measure the complex III-independent oxidation of the quinol (Marzulli et al., 1985).

2.7. Statistics

Results are given as mean ± standard deviation. Statistical analysis used to test the effects of zinc exposure was performed using ANOVA after testing for normality of the data distribution and homogeneity of variances, and was followed by Tukey's test when significant differences ($P < 0.05$) were found. The minimal level of significance chosen was $P < 0.05$.

3. Results and discussion

Low concentrations of heavy metals might influence biological functions well before the metal can be detected in the tissues. Apart from a direct estimation in soft tissues of marine organisms, free metal ions might also be indirectly determined by measuring their effect on cellular metabolism. Mitochondria are targets of many xenobiotic substances that often interfere with either their bioenergetics or genetics (Wallace and Starkov, 2000).

In this paper, we have presented the results of a study to determine effects of Zn^{2+} on mitochondrial functions from isolated *Mytilus* hepatopancreas mitochondria.

The Zn^{2+} content in the soft tissues of our samples was not measured, although the average values of Zn^{2+} found in *M. galloprovincialis* from the "Mar Grande" was 89.6 µg/g (Cardellicchio et al., 2008), which is lower than the threshold concentration (200 µg/g) in soft tissues of mussels as determined by Cantillo (1998).

Isolated mitochondria from mussel hepatopancreas (MHM), incubated in an isotonic medium without any respiratory substrate, were able to generate a membrane potential, DY_m, due to the presence of NAD-dependent endogenous substrates (Figure 1).

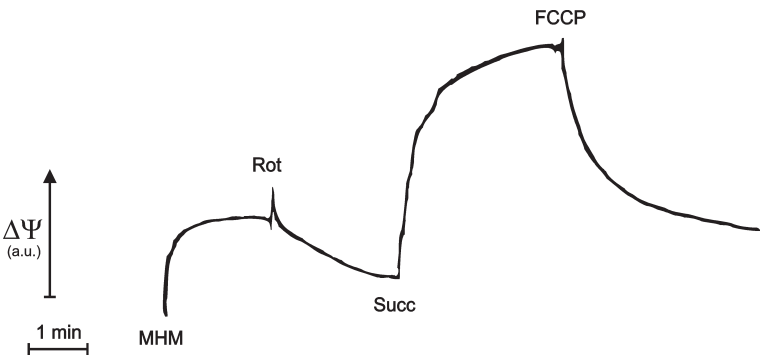

Figure 1. Mitochondrial membrane potential generated by the oxidation of succinate. Mussels' hepatopancreas mitochondria (MHM, 3 mg protein) were incubated for 3 min in 3 mL of standard medium (see Section 2). Further additions were, as indicated: 6 µM rotenone (Rot), 5 mM succinate (Succ), and 1.6 µM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone).

In fact, the subsequent addition of rotenone dissipated this potential, inhibiting complex I (NADH dehydrogenase). The addition of succinate, in the presence of rotenone, generated DY_m values higher than the value generated by endogenous substrates. The reaction was stopped by the addition of the uncoupler agent FCCP, which dissipates the electrochemical potential across the mitochondrial membranes by abolishing the fluorescence signal of safranin O. These data confirm that the experimental approach already utilized to follow DY_m in isolated rat liver mitochondria (La Piana et al., 1998) is also suitable for MHM. It also suggests that the mitochondrial membranes are intact and therefore a membrane potential is generated by the activity of the respiratory chain.

In order to confirm these findings, the polarographic determination of the oxygen uptake of MHM, both with NAD-dependent substrates and with succinate, was performed (Figure 2A). These results also showed that the oxidation of glutamate + pyruvate (both at concentrations of 5 mM) has to be followed. The basal rate of respiration (state 4), obtained in the absence of ADP (10 mM Pi in the incubation medium), was stimulated by the subsequent addition of ADP in state 3. The respiration sensitivity of state 3 to the inhibition was determined by oligomycin, an ATP-synthase inhibitor, which proves that the mitochondrial membranes are intact and therefore the activation of respiration following the addition of ADP is linked to ATP-synthase.

Figure 2B shows that the respiratory activity of control mitochondria with succinate, as observed for the DY_m determination, doubles the value obtained with glutamate + pyruvate. This finding confirms the better efficiency of the first substrate for the respiratory chain. It must be noted that the substrate preference for MHM could depend on several experimental parameters like the composition of the incubation medium, pH, and temperature, as already reported by Ballantyne and Storey (1984) for *Mercenaria mercenaria* hepatopancreas mitochondria.

Figure 3A reports the respiration rates at states 3 and 4 obtained in experiments with mitochondria isolated from mussels and then exposed to different concentrations of Zn^{2+}. As can be seen, the addition of zinc at state 4 led to a significant increase in respiration rate after the treatment of the mitochondria with 5.0 μg Zn^{2+}/L. The highest concentration of Zn^{2+} used (7.5 μg Zn^{2+}/L) in this work showed similar values to the lowest (2.5 μg Zn^{2+}/L).

The RCR (Figure 3B), reported as the ratio between state 3 and 4 respiratory rates, ranged between 2.2 and 2.8, with the highest value for the 5.0 μg/L zinc-treated mussels. The rate of respiration increased up to 5.0 μg/L, but any further increases in zinc led to a sharp reduction in respiration rate. Effects of zinc at state 3 respiration were significant at all concentrations. In fact, ANOVA results and graphical analysis of the dose-dependent zinc effects support the conclusion of higher sensitivity of state 4 respiration to zinc levels when using succinate as the substrate. Higher concentrations of zinc (7.5 μg Zn^{2+}/L) resulted in a similar effect on state 3 and 4 respiration oxidizing succinate or glutamate to that of the lowest concentrations (2.5 μg Zn^{2+}/L).

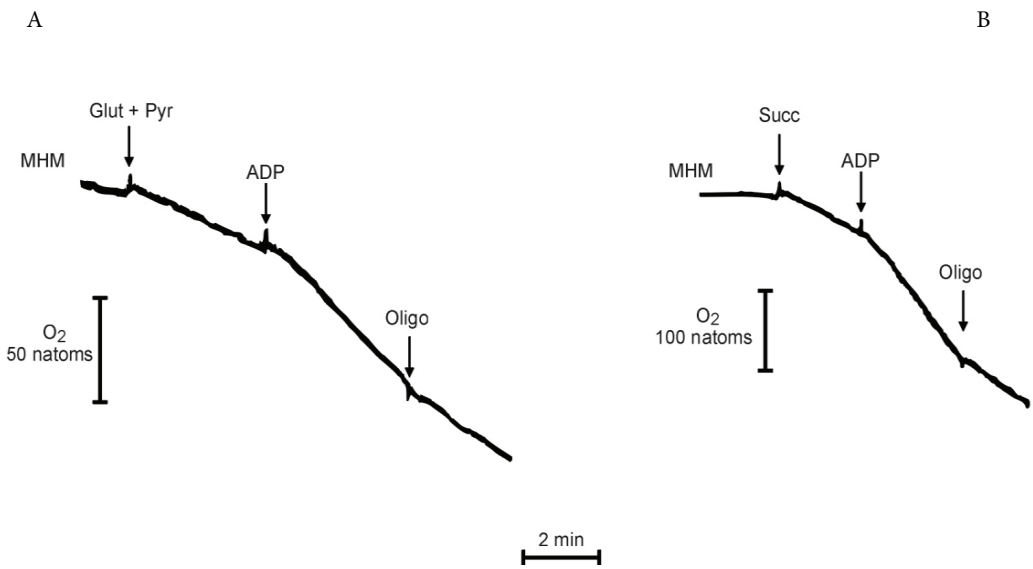

Figure 2. Oxygen uptake associated with the oxidation of glutamate + pyruvate (Glut + Pyr) and succinate (Succ) in control mitochondria. Mussels' hepatopancreas mitochondria (MHM, 1.5 mg protein) were incubated in 1.5 mL of standard medium (see Section 2). Glut + Pyr = 5 natoms min^{-1} mg^{-1}; ADP = 13 natoms min^{-1} mg^{-1}; Oligo = 8 natoms min^{-1} mg^{-1}; Succ = 17 natoms min^{-1} mg^{-1}; ADP = 44 natoms min^{-1} mg^{-1}; Olig = 21 natoms min^{-1} mg^{-1}.

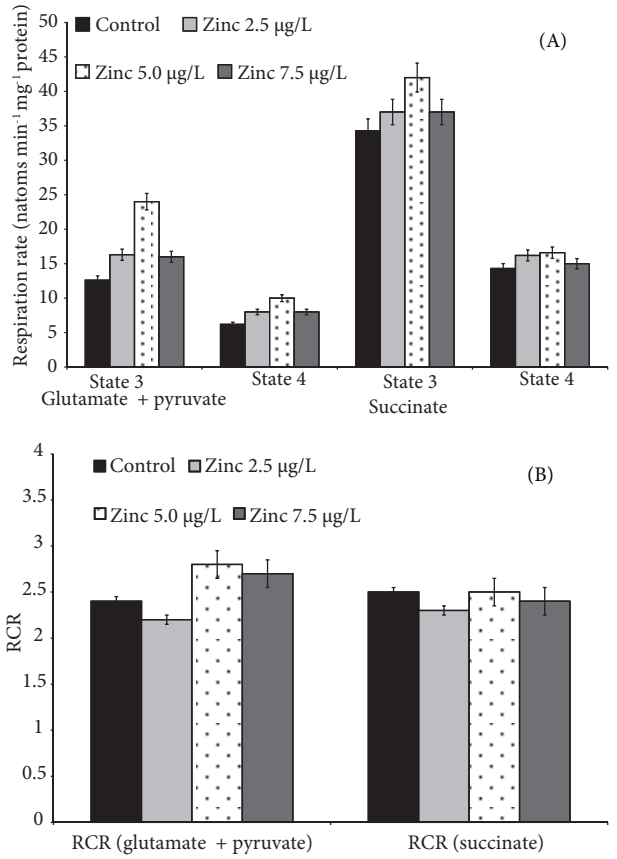

Figure 3. Effects of zinc on respiration rate (A) and RCR (B) of isolated *Mytilus galloprovincialis* hepatopancreas mitochondria in the presence of glutamate + pyruvate and succinate.

Zinc-induced stimulation of proton leakage can have important implications for the whole organism's basal metabolic rate (BMR), leading to an increased cost of mitochondrial maintenance and thus to a higher cost of basal metabolism in zinc-exposed mussels.

RCR values (Table) demonstrate that the exposure of mussels to Zn^{2+} at 3 different concentrations does not exert any significant effect on the respiration of isolated MHM, since similar values to those of control mussels were obtained both with glutamate + pyruvate and with succinate as respiratory substrates. These results agree with those obtained by Yamaguchi et al. (1982) on the single respiratory complexes of rat liver mitochondria, where a stimulation of succinate dehydrogenase, glutamate dehydrogenase, cytochrome *c* oxidase, and ATP synthase was inferred from the administration to rats of zinc sulfate in the water. This suggests that even control MHM are globally less coupled when isolated from Zn-treated mussels compared with routinely isolated rat liver mitochondria. This might be ascribed to the different medium and experimental protocol utilized to isolate mitochondria. However, the RCRs are in line with (or even higher than) those reported by other authors (Zaba et al., 1978; Yamaguchi et al., 1982).

Thus, the respiratory potential of an animal is one of the most important physiological parameters in assessing a toxic stress. Oxygen consumption has been identified as an indicator for sublethal stress in organisms exposed to toxic substances (Hughes, 1981). Changes in respiratory rates resulting from exposure to harmful chemicals indicate some abnormality or adaptive response in at least one of the biochemical pathways or physiological processes governing the metabolic rate in whole organisms (Watenpaugh and Beitinger, 1985). It is accepted that any change in metabolic rates depends on the nature, magnitude, and persistence of toxic effects. Therefore, studies on metabolic rates provide a clue to the chemical's mode of toxicity, in addition to revealing the importance of sublethal effect (Watenpaugh and Beitinger, 1985; Vijayavel and Balasubramanian, 2006).

To obtain a more complete overview and to check the effect of zinc on the bioenergetic characteristics of MHM, the activities of representative complexes of the respiratory chain were determined. In particular, we followed succinate–cytochrome *c* reductase (complex II + III), duroquinol–cytochrome *c* reductase (complex III), and cytochrome *c* oxidase (complex IV) as complexes of the respiratory chain. Mitochondrial malate dehydrogenase, as a representative of mitochondrial dehydrogenases, was also determined, as well as rotenone-insensitive NADH–cytochrome *c* reductase. This last activity is strictly associated with the external mitochondrial membrane in animals even though it is not widely present in all tissues. For instance, rat heart mitochondria do not display this activity, while it is highly present in the liver (Schonheit and Nohl, 1996), where it can be induced by several xenobiotic substances (Wallace and Starkov, 2000). In this study, malate dehydrogenase was not significantly influenced by Zn^{2+}, even if it showed an increase of activity with an increase in concentration of Zn^{2+}.

Succinate dehydrogenase and duroquinol cytochrome *c* oxidoreductase activities were followed as the rate of reduction of exogenous cytochrome *c* following the addition of succinate or duroquinol in the presence of cyanide, to avoid the reoxidation of cytochrome *c*. The results are reported in the Table. Both activities were strongly stimulated in zinc-treated mussels, and for the duroquinol the stimulation was shown also at the lowest zinc concentration (ANOVA, $P < 0.05$).

Grajeda y Ortega et al. (2011) reported a significant decrease of succinate dehydrogenase activity in the oligochaete *Limnodrillus hoffmeisteria* compared to controls. Some enzymes found in mitochondria, such as NADH-dehydrogenase, cytochrome *c* oxidase, and succinate dehydrogenase, are associated with known

Table. Effect of zinc on the activities of various respiratory chain enzymes in mitochondria of *Mytilus galloprovincialis*. The activities and all experimental details are reported as U/mg protein. Data are expressed as a mean ± SD for 5 experiments. Mean values within the same column sharing a common letter are not significantly different ($P < 0.05$).

	Control	+ zinc 2.5 µg/L	+ zinc 5 µg/L	+ zinc 7.5 µg/L
Succinate cytochrome *c* oxidoreductase	0.05 ± 0.011[a]	0.048 ± 0.004[a]	0.08 ± 0.005[b]	0.08 ± 4E-3[b]
NADH cytochrome *c* (rotenone-insensitive)	0.108 ± 0.024[a]	0.118 ± 0.011[a]	0.14 ± 0.02[a,b]	0.16 ± 1.5E-2[b]
Cytochrome oxidase	1.014 ± 0.152[a]	1.335 ± 0.1[b]	1.4 ± 0.17[b,c]	1.652 ± 0.14[c]
Malate dehydrogenase	2.369 ± 0.8[a]	2.377 ± 0.75[a]	2.751 ± 0.77[a]	3.8 ± 0.8[a]
Duroquinol cytochrome *c* oxidoreductase	0.018 ± 1.4E-4[a]	0.02 ± 2E-4[b]	0.024 ± 1.8E-4[c]	0.04 ± 1.6E-4[d]

proteins as nonhematic ferroproteins, whose structure contains Fe (Korkmaz et al., 2011). Succinate dehydrogenase is an iron-dependent enzyme involved in ATP production via the Krebs cycle and in oxidative phosphorylation. Several studies have shown that succinate dehydrogenase activity decreases when the organism is exposed to heavy metals, such as Cd and Zn, by replacement of the iron in its molecule with the exogenous metal (Renella et al., 2003; Grajeda y Ortega et al., 2011).

The results of this study showed a significant increase of NADH cytochrome *c* activity at 7.5 µg/L zinc compared to the control and lower concentrations. The cytochrome *c* oxidase was significantly stimulated already at the lowest concentration of zinc.

The malate dehydrogenase activity did not show significant differences at any of the concentrations of zinc used (ANOVA, $P > 0.05$), even if an increase of activity can be observed (Table).

In rat liver mitochondria, zinc inhibits the oxygen uptake supported by the mitochondrial dehydrogenases α-ketoglutarate dehydrogenase and succinate dehydrogenase, and the oxidation of malate/glutamate. This general effect was ascribed to selective inhibition of complex III and α-ketoglutarate dehydrogenase, while the activities of succinate and malate dehydrogenase, when measured with detergent lysing mitochondria membranes, were not inhibited but were rather stimulated (Brown et al., 2000). However, these results were obtained by directly adding $ZnCl_2$ to isolated rat liver mitochondria. In fact, Yamaguchi et al. (1982) observed a stimulation of rat liver mitochondrial respiration and activities following the administration of zinc sulfate. A 60% increase was observed in the activities of succinate dehydrogenase, glutamate dehydrogenase, cytochrome *c* oxidase, and ATP synthase during the first 24 h following zinc administration. This suggests that zinc activates electron transport more efficiently than oxidative phosphorylation since it is able to release succinate dehydrogenase from the inhibition brought about by malonate, a specific inhibitor of the former enzyme. The effects observed might also result from a combination of concentration and time of exposure to zinc. In fact, it has been observed that the zinc content in the liver of rats given zinc sulfate in drinking water gradually declined after 24 h (Yamaguchi et al., 1982).

The concentrations of zinc used in this study are well above the values recorded in the Ionian Sea (0.5 ng/L); however, the Zn concentrations used are below those used by Geret et al. (2002). The addition of $ZnCl_2$ between 2.5 and 7.5 µg/L to the seawater of *Mytilus galloprovincialis* collected in the "Mar Grande" of Taranto appears to affect the metabolism and bioenergetics of mitochondria isolated from their hepatopancreata, in that it increases the activities of respiratory complexes and of mitochondrial enzymes. Succinate dehydrogenase, NADH-cytochrome *c* reductase, cytochrome oxidase, and rotenone-insensitive NADH-cytochrome *c* reductase were all stimulated by zinc ions. The same stimulation was also observed (although not significantly) with malate dehydrogenase, a representative dehydrogenase of the mitochondrial matrix. Therefore, not only does the low concentration of zinc used not inhibit mitochondrial functions, as previously observed for rat liver mitochondria with added $ZnCl_2$, but it stimulates them. This suggests that mussels under chemical stress activate mitochondrial respiration in order to obtain more ATP from oxidative phosphorylation and in some way to cope with the presence of zinc ions in the marine environment. Thus, the assessment of oxygen consumption and respiratory enzymes serves as a suitable tool for assessing zinc stress, which sheds light on the energy status of the mussel.

In conclusion, the results of this study support the hypothesis that Zn^{2+} can influence considerably the mitochondrial functions in marine invertebrates, and that this evaluation can be a useful tool to warn of harmful environmental conditions.

References

Ballantyne JS, Storey KB (1984). Mitochondria from the hepatopancreas of the marine clam *Mercenaria mercenaria*: substrate preferences and salt and pH effects on the oxidation of palmitoyl-L-carnitine and succinate. J Exp Zool 230: 165–174.

Bergmeyer HU, Bernt E (1987). In: Bergmeyer, HU, editor. Methods in Enzymatic Analysis, Vol. 3, 3rd ed. New York: Wiley, pp. 171–175.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brown AM, Kristal BS, Effron MS, Shestopalov AI, Ullucci PA, Rex Sheu KF, Blass JP, Cooper AJL (2000). Zn^{2+} inhibits α-ketoglutarate-stimulated mitochondrial respiration and the isolated α-ketoglutarate dehydrogenase complex. J Biol Chem 275: 13441–13447.

Buccolieri A, Buccolieri G, Cardellicchio N, Dellatti A, Dilee A, Maci A (2006). Heavy metals in marine sediments of Taranto Gulf, Ionian Sea, southern Italy. Mar Chem 99: 227–235.

Cantillo AY (1998). Comparison of results of Mussel Watch programs of the United States and France with Worldwide Mussel Watch Studies. Mar Pollut Bull 36: 712–717.

Cardellicchio N, Buccolieri A, Di Leo A, Giandomenico S, Spada L (2008). Levels of metals in reared mussels from Taranto Gulf (Ionian Sea, southern Italy). Food Chem 107: 890–896.

Eisler R (1993). Zinc hazards to fish, wildlife, and invertebrates: a synoptic review. Contaminant Hazards Reviews Report 26. Washington, DC: US Department of the Interior, Fish and Wildlife Service.

Estabrook RW (1967). Mitochondrial respiratory control and the polarographic measurements in mitochondria. Method Enzymol 10: 41–47.

Fabris JG, Richardson BJ, O'Sullivan JE, Brown FC (1994). Estimation of cadmium, lead and mercury concentrations in estuarine waters using the mussel *Mytilus edulis planulatus* L. Environ Toxicol Water 9: 183–192.

Frederickson CJ, Suh SW, Silva D, Frederickson CJ, Thompson RB (2000). Importance of zinc in the central nervous system: the zinc-containing neuron. J Nutr 130: 1471S–1483S.

George SG (1980). Correlation of metal accumulation in mussels with the mechanism of uptake, metabolism and detoxification: a review. Thalassia Jugosl 16: 347–365.

Geret F, Jouan A, Turpin V, Bebianno MA, Cosson RP (2002). Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). Aquat Living Resour 15: 61–66.

Grajeda y Ortega MA, Ortiz OE, Favari L, Shibayama M, Silva OA, López LE (2011). Biochemical and mitochondrial changes induced by Cd, Fe and Zn in *Limnodrillus hoffmeisteri*. Int J Morphol 29 (2): 412–419.

Hughes GM (1981). Effect of low oxygen and pollution on the respiratory systems of fish. In: Pickering, AD, editor. Stress and Fish. New York: Academic Press, pp. 121–147.

Korkmaz EM, Budak M, Başıbüyük HH (2011). Utilization of cytochrome oxidase I in *Cephus pygmeus* (L.) (Hymenoptera: Cephidae). Turk J Biol 35: 713–726.

La Piana G, Fransvea E, Marzulli D, Lofrumento NE (1998). Mitochondrial membrane potential supported by exogenous cytochrome c oxidation mimics the early stages of apoptosis. Biochem Bioph Res Co 246: 556–561.

Leblebici Z, Aksoy A, Duman F (2011). Influence of salinity on the growth and heavy metal accumulation capacity of *Spirodela polyrrhiza* (Lemnaceae). Turk J Biol 35: 215–220.

Lofrumento NE, Marzulli D, Cafagno L, La Piana G, Cipriano T (1991). Oxidation and reduction of exogenous cytochrome *c* by the activity of the respiratory chain. Arch Biochem Biophys 288: 293–301.

Marzulli D, La Piana G, Cafagno L, Fransvea E, Lofrumento NE (1995). Proton translocation linked to the activity of the bi-trans-membrane electron transport chain. Arch Biochem Biophys 319: 36–48.

Marzulli D, Zanotti F, Lofrumento NE (1985). N-ethylmaleimide as oxidizing agent in biological and non-biological systems. B Soc Ital Biol Sper 61: 121–127.

Nicholls DG (2002). Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. Int J Biochem Cell B 34: 1372–1381.

Pote J, Haller L, Loizeau J, Bravo AG, Sastre V, Wildi W (2008). Effects of a sewage treatment plant outlet pipe extension on the distribution of contaminants in the sediments of the Bay of Vidy, Lake Geneva, Switzerland. Bioresource Technol 99: 7122–7131.

Qiao S, Yang Z, Pan Y, Guo Z (2007). Metals in suspended sediments from the Changjiang (Yangtze River) and Huang He (Yellow River) to the sea, and their comparison. Estuar Coast Shelf S 74: 539–548.

Renella G, Ortigoza ALR, Landi L, Nannipieri P (2003). Additive effects of copper and zinc on cadmium toxicity to phosphatase activities and ATP content of soil as estimated by the ecological dose (ED50). Soil Biol Biochem 35: 1203–1210.

Schonheit K, Nohl H (1996). Oxidation of cytosolic NADH via complex I of heart mitochondria. Arch Biochem Biophys 237: 319–323.

Sokolova IM, Bock C, Pörtner HO (2000). Resistance to freshwater exposure in White Sea *Littorina* spp. I. Anaerobic metabolism and energetics. J Comp Physiol B 170: 91–103.

Sokolova IM, Pörtner HO (2001). Physiological adaptations to high intertidal life involve improved water conservation abilities and metabolic rate depression in *Littorina saxatilis*. Mar Ecol-Progr Ser 224: 171–186.

Sokolova IM, Pörtner HO (2003). Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (*Littorina saxatilis*, Gastropoda: Littorinidae) from different latitudes. J Exp Biol 206: 195–207.

Vijayavel K, Balasubramanian MP (2006). Changes in oxygen consumption and respiratory enzymes as stress indicators in an estuarine edible crab *Scylla serrata* exposed to naphthalene. Chemosphere 63: 1523–1531.

Wallace KB, Starkov AA (2000). Mitochondrial targets of drug toxicity. Annu Rev Pharmacol 40: 353–388.

Watenpaugh DE, Beitinger TL (1985). Oxygen consumption in fathead minnows *Pimephales promelas* following acute exposure to waterborne selenium. Comp Biochem Physiol 80C: 253–256.

Yamaguchi M, Kura M, Okada S (1982). Role of zinc as an activator of mitochondrial function in rat liver. Biochem Pharmacol 31: 1289–1293.

Zaba BN, De Bont AMT, De Zwaan A (1978). Preparation and properties of mitochondria from tissues of the sea mussel M*ytilus edulis* L. Int J Biochem 9: 191–197.