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Relationship between anti-oxidant capacity and manganese accumulation in the soft tissues of two freshwater molluscs: *Unio pictorum mancus* (Lamellibranchia, Unionidae) and *Viviparus ater* (Gastropoda, Prosobranchia)

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ABSTRACT

Manganese is an element of great importance in the life cycle of plants and animals. For example, it plays an essential role as an activator of various enzymatic systems such as isoenzymes of superoxide dismutase. Freshwater Unionidae concentrate relatively large amounts of manganese in their tissues, but little is known about the physiological role of this metal. The aim of this research is to acquire a better knowledge of the role of manganese in molluscs which accumulate large amounts of this metal and in those with low manganese concentrations. As manganese is one of the metals present in the superoxide molecule, the possible relationship between manganese concentration in the soft tissues of molluscs and the antioxidant capacity of the metal can usefully be tested. To this end two species of molluscs were analysed: Unio pictorum mancus *(Lamellibranchia, Unionidae), which is very rich in manganese, and* Viviparus ater *(Gastropoda, Prosobranchia) which has a low manganese content. The adults of both species were analysed for manganese concentration by ICP, and for antioxidant capacity as RAC (Relative Antioxidant Capacity) by a superoxide dismutase method. The results clearly demonstrate the active role played by manganese against free radicals and consequently the important role of the metal in protecting* Unio *against oxidative stress. The low concentration of manganese in* Viviparus *may be the result of the effective excretion of this metal, as was found for ruthenium.*

Key words: metal bioaccumulators, superoxide dismutase (SOD), relative antioxidant capacity (RAC), isoenzymes, freshwater molluscs

1. INTRODUCTION

Manganese is an essential trace metal for plant and animal metabolism. For example, manganese plays an important role in the accumulation of nitrate and carbon dioxide assimilation in plants, and activates a number of enzymatic systems in different animal species (Malmstron & Rosenberg 1959). High concentrations of manganese have been measured in the soft tissues of freshwater mussels since the beginning of the 20th century (Bradley 1970a; 1970b).

The first data on many trace element concentrations in marine animals (including Mn concentration in Lamellibranchs) were reported by Vinogradov (1953), and amplified by Fukai & Meinke (1959).

Radiomanganese (Mn-54) is presumed to originate from the activation of Fe-54 and Mn-55 in nuclear weapons in the Pacific area after the 2nd World War. Lowman (1960) was the first to identify this artificial radioisotope in water, plankton, molluscs and fish near the Marshall Islands. Very high concentrations of Mn-54 were measured in the soft tissues of the Lamellibranch *Tridacna*.

In this context, an interesting question seemed to be whether the Mn-54 present in the fall-out from the Pacific weapons had also contaminated European aquatic environments. In September 1960, Mn-54 was measured in Unionidae (*Unio* and *Anodonta*) collected in Lago Maggiore and in nearby Lake Varese (Northern Italy); these mussels were found to have a great capacity for accumulating this radionuclide. At that time, Mn-54 could not be detected in fall-out, lake water, sediment, macrophytes, gastropods or fish samples collected in the same area, due to the extremely low activity of the radioisotope (Ravera & Vido 1961).

In Unionidae, the highest Mn-54 concentrations were found in the mantle and the gills, the lowest in the extrapalleal liquid. The distribution of Mn-54 in the mussel body was compared with that of stable manganese. Since the two distributions were strikingly similar, it is probable that the Mn-54 introduced into the lake was in a form similar to that for stable manganese, at least from the point of view of the availability to the mussels (Gaglione & Ravera 1964). Other molluscs (e.g. *Viviparus ater*) living in the same habitat as Unionidae concentrate such a low amount of Mn-54 that it could not be quantified by the commonly used methods (Gaglione & Ravera 1964).

The mean concentration of stable manganese in the soft tissues of *Unio pictorum* collected from 12 Northern Italian lakes was 7.5±6.3 $mg \cdot g^{-1}$ (dry weight) with a minimum of 2.23 $mg \cdot g^{-1}$ dw in Lake Mergozzo and a maximum of 15.26 $mg \cdot g^{-1}$ dw in Lake Montorfano (Ravera *et al*. 2003b). These values suggest that

Unionidae are useful indicators of stable and radioactive manganese.

Since the 1960s, freshwater and marine mussels have been used as indicators for radioisotopes and toxic substances because of their capacity to accumulate these pollutants without evident consequences. The resistance of the mussel to these pollutants is due to their detoxification mechanisms, such as their capacity for concentrating pollutants in the shell, inactivating metals by binding them to thioneins, and sequestering them in calcareous granules distributed in their tissues. Bivalves were later used as indicators of persistent organic pollutants (e.g. aromatic hydrocarbons, biocides), which due to their low enzymatic activity are scarcely metabolised (Phillips 1977). Mussels are also used to assess the biological effects of pulp and paper mill effluent in marine environments (Gravato *et al*. 2005). Jorge & Moreira (2005) used *Perna perna* larvae to test, in laboratory conditions, the toxicity of various pollutants. The foremost example of this type of biomonitoring is the "Mussel Watch Programme", which uses bivalves (*Mytilus* and *Ostrea*) to monitor Pacific and Atlantic coastal zones of the U.S.A. (O'Connors *et al*. 1994). The advantages and disadvantages of such monitoring are discussed in other papers (e.g. Beone & Ravera 2003; Ravera 2004).

Little is known about the physiological role of manganese in the mussel. Pelseneer (1935) hypothesized that manganese in bivalves is the metal of a respiratory pigment called "acroglobulin", which had the same function as copper in hemocyanin and iron in haemoglobin. Vinogradov (1953) accepted this hypothesis, whereas Grassé (1960) did not agree that "acroglobulin" is a respiratory pigment.

According to Bowen (1950) the manganese concentration in Hymenoptera is too high to be explained by its role in enzymatic activity; more likely the manganese accumulation in these insects is a combined effect of their poor capacity for selecting manganese and the low excretion rate for the element.

The poor capacity of bivalves for selecting heavy metals has been clearly demonstrated for manganese, which is incorporated into their tissues through the same pathway as calcium, an element characterised by a similar ionic radius value (Markich & Jeffree 1994).

This study is an attempt to acquire better information on the physiological role played by manganese in *Unio pictorum mancus* by establishing a relationship between manganese concentrations and RAC (Relative Antioxidant Capacity) in the soft tissues of *Unio*. The same relationship was tested for a mollusc gastropod (*Viviparus ater*), which, unlike *Unio pictorum mancus*, is characterised by a low concentration of manganese in its tissues. This comparison was made to contribute to our understanding of the fundamental role played by manganese as a metal of superoxide dismutase in an antioxidant capacity.

2. MATERIALS

Two species of molluscs were used for this study: the polytypic bivalve *Unio pictorum mancus*, L. and the gastropod *Viviparus ater*, Crist. and Jan. *Unio pictorum mancus* is widely distributed in the coastal zone of Lago Maggiore; *Viviparus ater* was very common in the same habitat as *Unio* up to 30 years ago, but is now rather rare.

The life cycle of *Unio* is very different from that of *Viviparus*. Fecundation of *Unio* is external, and the larvae have to pass through a parasitic phase on a fish host to metamorphose into the young mussel. Fecundation of *Viviparus* is internal and the embryos complete their development in the uterus of the mother.

Both molluscs take up oxygen dissolved in the water through the gills. *Unio* can also survive for a long periods of time at a low oxygen concentration; for example, it may spend most of the cold season buried in hypoxic and anoxic sediments, reducing its metabolic rate. To overcome these conditions, the mussel lives anaerobically utilizing glycogen and producing succinic acid as a by-product; this acid is neutralised with calcium carbonate from the inner surface of the shell. In contrast, *Viviparus* is not resistant to anoxic conditions.

Adults of *Unio pictorum mancus* and *Viviparus ater* were collected from the littoral zone of Lago Maggiore (Northern Italy, Varese Province). Shortly after collection the specimens were placed on ice and rushed to the laboratory where they were kept at -20 °C until analyzed.

3. METHODS

3.1. Reagents and Chemicals

Xanthine (2,6-dihydroxy purine) sodium salt, ethylenediamine tetracetic acid (EDTA), superoxide dismutase 4980 U mg-1, dialysis membrane (art. D-9777), supplied by Sigma (Milan, Italy). Acetone RPE, cyclohexanone RPE, hydrochloric acid (37%) RPE, nitric acid (65%) RPE, hydrogen peroxide (40% m/v) RE, supplied by Carlo Erba (Milan, Italy). Xanthine oxidase 0.39 U mg^{-1}, cellulose acetate and kappa-carrageenan supplied by Fluka AG, (Buchs, Switzerland). Potassium chloride supplied by Riedel-de Haen (Seelze, Germany). Polyvinylacetate, supplied by Aldrich (Germany). KCl 3 mol l^{-1}/AgCl, buffer reference standard pH 4.0, pH 7.2, and pH 9.0, supplied by Crison.

3.2. Apparatus

Microwave Accelerated Reaction System 5 supplied by CEM Corporation, Matthews, (North Carolina, USA); electrode mod.4000-1 by Universal Sensor Inc. (New Orleans, LA, USA), coupled with an Amel potentiostat mod. 551 (Milan, Italy), connected to an Amel differential electrometer, mod. 631 and to an Amel analogical recorder, mod. 868; pHmeter CRISON GLP 22; Ultra-Turrax homogenizer mod. T8 by Ika

Labortechnik (Germany); magnetic stirrer mod. F20ST by Falc Instrument (Bergamo, Italy); analytical balance mod. METTLER AE 420; technical balance mod. METTLER PM 460, CCD-Simultaneous ICP-OES, VISTA-MPX, Varian.

3.3. Sample preparation

Unio pictorum mancus: two aliquots to be analysed (1 g) were sampled from each mollusc: one was mineralized using a suitable solvent for analysis by plasma emission atomic spectroscopy; the other was treated in 6.0 ml of phosphate buffer at pH 7.5, by a homogenizer at 10000 rpm for 5 min, and analysed by (SOD) superoxide dismutase biosensor.

Viviparus ater: sample was homogenized in as small a volume as possible of phosphate buffer at pH 7.5. The solution was divided into two parts: the first was analysed by plasma emission atomic spectroscopy, the second by (SOD) biosensor.

3.4. Plasma Emission Atomic Spectroscopy (ICP)

The biosensoristic response has to be confirmed by the results of plasma emission atomic spectroscopy. This technique is highly reliable, reproducible and easy to use, with no chemical or spectral interference. Samples to be analyzed were carefully weighed when fresh, then placed in a teflon cylinder with 9.0 ml of distilled water, 1.0 ml of HNO_3 (65%) and 1.0 ml of H_2O_2 (40% m/v). Each sample was submitted to the following mineralization program: 5 min at 50% power; 3 min at 0% power (cooling); 5 min at 50% power (100% power corresponds to 300 m^2 kg s^{-3}); 3 min at 0% power (cooling); 5 min at 50% power; last cooling for 10 min. All these solutions were filtered, diluted to 50 ml, and analyzed.

3.5. Superoxide dismutase (SOD) biosensor

The superoxide radical was determined by SOD biosensor obtained by coupling a transducer (amperometric Clark electrode for hydrogen peroxide) with superoxide dismutase enzyme immobilized in kappa-carrageenan gel. The gel containing the enzyme is sandwiched between a cellulose acetate membrane and a dialysis membrane (Campanella *et al*. 1999, 2000). Superoxide radicals were produced, in presence and in absence of the antioxidant, by reaction of oxidation of xanthine catalysed by xanthine oxidase, and the values of the recorded current were plotted versus xanthine concentration. The angular coefficients were calculated and the value of the anti-oxidant capacity was expressed by the following algorithm:

$$(RAC)\ \text{"Relative Antioxidant Capacity"} = 1 - (m_c/m_x) \qquad (1$$

m_x = angular coefficient of the straight line obtained through successive additions of xanthine (200 µl);

m_c = angular coefficient of the straight line obtained through successive additions of xanthine, but in the presence of the sample (500 µl) possessing anti-oxidant properties.

4. RESULTS

Table 1 reports the manganese concentrations in 10 soft tissue samples analysed by plasma emission atomic spectroscopy. The wide variability of Mn-concentrations is mainly due to the different parts of the mussel body analysed. This shows how heterogeneous the distribution of manganese is in the mussel body. In fact, there are very different concentrations of manganese in the organs of the same species from the same lake (Ravera *et al*. 2003a). The samples with the highest (N° 2) and the lowest (N° 6) Mn concentration were homogenised and analysed by superoxide dismutase (SOD) biosensor. The results are reported in table 2 and schematized in figure 1.

Tab. 1. Manganese concentration in *Unio pictorum mancus* samples determined by plasma emission atomic spectroscopy.

Sample	mg kg^{-1} ww
1	307.48
2	730.21
3	244.26
4	156.79
5	330.00
6	37.95
7	228.71
8	100.93
9	94.07
10	108.73

Tab. 2. Values obtained by plasma emission atomic spectroscopy and by superoxide dismutase biosensor (RAC units) in *Unio pictorum mancus*.

Sample	mg Mn·kg^{-1} ww	RAC units
2	104.23	0.600±0.007
6	176.61	0.696±0.010

The Mn concentration in *Unio* ranges from 100 mg·kg^{-1} (wet weight) to 200 mg·kg^{-1}, and appears to be correlated with the total antioxidant capacity according to the hypothesized central role and biological function of manganese against the radicals.

The relationship between total antioxidant capacity and Mn concentration was also evident in the soft tissues of *Viviparus ater*, although the Mn concentration in this mollusc ranged between 1.6 mg·kg^{-1} (wet weight) and 7.2 mg (Tab. 3, Fig. 2). The RAC until values of *Viviparus*, ranging from 0.53 to 0.86 (Tab. 3, Fig. 2), are of the same order of magnitude as those found for *Unio* (0.60÷0.70).

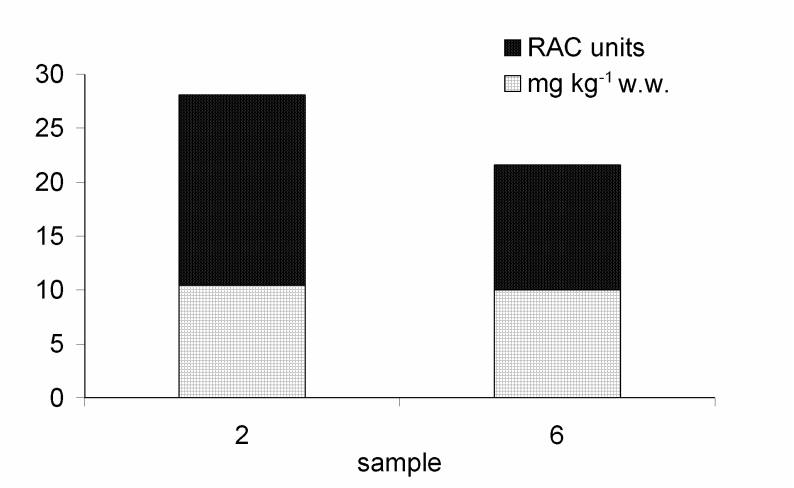

Fig. 1. Comparison between Mn concentration using plasma emission spectroscopy and total antioxidant capacity using SOD biosensor. To compare the experimental data graphically, the RAC units were multiplied by 100 and then divided by 6, while Mn values were divided by 10.

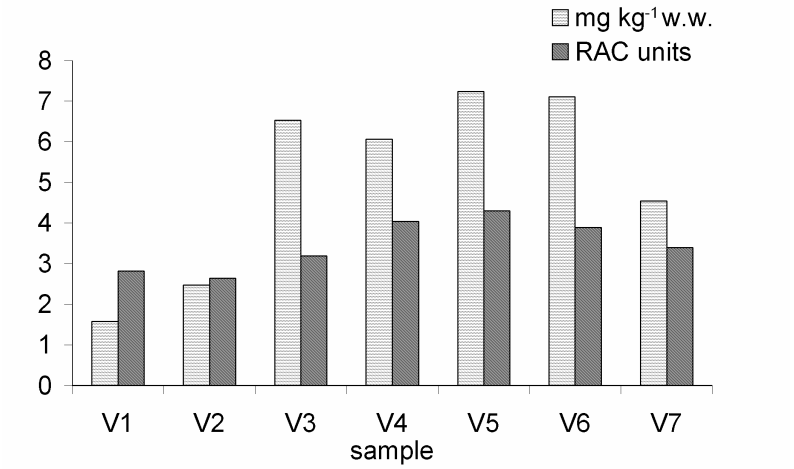

Fig. 2. Comparison between Mn concentration determined by plasma emission spectroscopy and total antioxidant capacity by SOD biosensor. To compare the experimental data graphically, the RAC units were multiplied by 5. V1÷V7 = order number of 7 individuals.

Tab. 3. Values obtained by plasma emission spectroscopy and by superoxide dismutase biosensor (RAC units) in *Viviparus ater*.

Sample	mg Mn·kg^{-1} ww	RAC units
V1	1.57	0.563±0.010
V2	2.47	0.528±0.005
V3	6.53	0.637±0.005
V4	6.06	0.807±0.006
V5	7.23	0.859±0.004
V6	7.10	0.777±0.004
V7	4.54	0.679±0.006

5. DISCUSSION

Aquatic animals take up metals from both water and food. Differences in concentrations of the same metal in different species living in the same habitat are due to different types of food and/or different metabolic processes, such as excretion-rate and detoxification mechanisms.

Unio pictorum and *Viviparus ater* specimens used in our research were collected from the same habitat, in which they use similar organic particles as food; the former species accumulates a high concentration of manganese in its soft tissues, whereas the latter concentrates a very small amount of the same metal.

These characteristics make *Unio pictorum* and *Viviparus ater* useful species for testing the relationship between anti-oxidant capacity and manganese accumulation in molluscs.

Manganese concentration in individuals from the same population varies widely in *Unio* as well as in *Viviparus*, but *Unio* values are generally of two orders

of magnitude higher than those found in *Viviparus*. Conversely, the order of magnitude of RAC (Relative Antioxidant Capacity) is the same for both molluscs; consequently, manganese efficiency as an antioxidant is much lower in *Unio*. Our initial question was answered by the interesting discovery that the antioxidant capacity in *Unio* and *Viviparus* increases with the increase of manganese concentration. The isoenzymes of the superoxide dismutase (SOD) catalyze the dismutation of the superoxide, assuming a central role in the organism's defence from the toxicity of oxygen by-products.

The prosthetic groups of these isoenzymes may be of different heavy metals, such as Cu, Zn, Fe and Mn; manganese is a component of an anti-oxidant enzyme which protects the mussels from oxidative stress (Luo 2001). The results obtained clearly demonstrate the role played by manganese against free radicals and highlight the probable relationship between manganese concentration and its role in protecting the molluscs from oxidative stress.

6. CONCLUSIONS

In conclusion, the low concentration of manganese in *Viviparus* may be the result of the effective excretion of the metal, as was found for ruthenium (Ravera 1964).

Manganese accumulation in *Unio* tissues does not have any obvious effects, due to the detoxification processes by thioneins (High *et al*. 1997) and the transfer of manganese from the soft tissues to the organic matter of the shell (Nyström *et al*. 1996) and to calcium phosphate granules (Byrne 2000). In addition, the isoenzyme of the superoxide dismutase reduces the toxicity of the oxygen by-product.

It may be interesting for future research to test the combined effects of Mn and carotenoids on the antioxidant capacity in some species of freshwater and marine mussels. Such a project might start from the results obtained by Tewary *et al*. (2001) on a marine bivalve *Perna perna*. These authors observed a relationship between the resistance of bivalves to high concentrations of toxic metals and high concentration of carotenoids in the bivalve.

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