SUPPLEMENTARY MATERIAL

Toxic mineral elements in *Mytilus galloprovincialis* from Sicilian coasts

(Southern Italy)

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Abstract

We assessed the relationship between V, Cr, Mn, Hg, As, Cd, Sn, Sb and Pb concentrations in *Mytilus galloprovincialis* samples from the coasts of Sicily and the expression of *metallothioneins*. The toxic mineral elements assessment was carried out by A.A. Spectrometry and ICP-MS. The *metallothioneins* expression was performed by q-PCR method. Low metals’ levels were found in the mussel samples examined, in comparison with what was reported in literature. The highest mean values of toxic mineral elements were found in Gela (Cr 0,178±0,03 mg/Kg, Mn 4,325±0,012 mg/Kg, As 3,706±0,009 mg/Kg, Sn 0,148±0,014 mg/Kg, Sb 0,009±0,004 mg/Kg e Pb 0,364±0,01 mg/Kg). Significant levels of Hg were found in samples from Catania (0,014±0,005 mg/Kg). Only vanadium and lead concentrations showed significant differences between sampling areas (p <0.05). Molecular analysis verified a basal expression of *Mt1* and the absence of over-expression of *Mt2*, confirming the low mineral’s concentrations found in the samples examined.

Keywords: mussels; biomarkers; toxic mineral elements; *metallothioneins*

Experimental

Sample collection

A total of 3180 samples of *Mytilus galloprovincialis* of similar size were sampled during 2016 from 10 large urban agglomerations, high industrial activities and national interest sites of Sicily (Barcellona Pozzo di Gotto, Catania, Gela, Licata, Messina, Milazzo, Palermo, Siracusa, Termini Imerese and Trappeto). Trappeto samples were considered as control group because of their distance from urban and industrial agglomerations. The *M. galloprovincialis* samples were pooled in 106 pools according to the sampling area and the date of sampling (Bolognesi et al. 1996; Catsiki and Florou 2006). The pools were homogenized by a vertical mixer B-400 (Büchi, Flawil, Switzerland). The homogenate was divided into 2 parts for the toxic mineral elements levels evaluation and for the metallothioneins expression by q-PCR.

Determination of the toxic metal concentration

The sample extraction for V, Cr, Mn, As, Cd, Sn, Sb and Pb assessment was carried out by a digestion procedure using a Multiwave 3000 (Anton-Paar, Graz) in accordance to UNI EN 13805:2002. The samples digested were made up to 50 ml with Millipore deionised water until the ICP-MS analysis. V, Cr, Mn, As, Cd, Sn, Sb and Pb levels were quantified by a 7700x series ICP-MS (Agilent Technologies, Santa Monica CA, USA). The operating conditions and instrumental settings are shown in table S1. The analysis were carried out on the basis of calibration curves,
constructed by the linear interpolation of at least 7 points corresponding to the readings of 7 standard solutions and white calibration, admitting a maximum error of 5% on the reading of the single standards and a correlation coefficient $r^2 > 0.999$. The repeatability limit was calculated by adding to 15 digested samples spiked 1.00 - 2.00 to 5.00 µg / L of all analytes. The repeatability within-laboratory is calculated with metrological approach and with the formula $\text{Horrat}_r$ where: observed RSDr divided by the RSDr value estimated from the modified Horwitz equation, using the assumption $r = 0.66 R$; reproducibility within-laboratory is calculated as $\text{Horrat}_R$ where: the observed RSD R divided by the RSD R value estimated from the modified Horwitz equation percentage variation coefficient (CV%); all the two values must be smaller than 2.

The validation of the analytical method allowed us to identify all the uncertainty contributions in order to calculate the expanded uncertainty. The Hg determination was carried out by the direct analyser DMA-80 (Milestone, Wesleyan University, Midletown, CT, USA). The quantitative determination was performed by atomic absorption spectroscopy at a fixed wavelength of 250 nm. The thermal parameters for the sample’s decomposition and the subsequent formation of the fumes with mercury are shown in table S2.

**Molecular analysis**

About 100 mg of samples at -20°C were immersed in tubes containing 500 µl of Trizol Reagent (Life Technologies, Carlsbad, California, USA) and vortexed for 5 s. The RNA quality was controlled by 1% agarose gel electrophoresis and then quantified by spectrophotometer.

Approximately 1 µg of total RNA was transcribed through the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germania) and the cDNA was used to perform a qPCR reaction in order to evaluate the Hprt region (internal control 5′GCTATAAATTCTTTGCTGACTGCTG3′ - 5′AATTACTTTTATGTCCCCTGGACTG3′) and Mt1 and Mt2 expression with the following primers: MT-1 5′AATGTGCCAAGGGCTGTGT3′ - 5′GCTGGGTTGGTCCGATCATT3′ and MT-2 5′TGTGCCTCCGATGGATCCT3′ - 5′GCAGCCCTGGGAGCCTT3′.

**Data analysis**

The data obtained were collected and divided by sampling area. A Shapiro-Wilk test was applied to verify the normality of distribution of toxic metals data groups. All the concentrations below the LOQ of the method were considered as the LOQ values for the statistical analysis, according to Helsel 2005. The assumption of normality has not been met therefore, a Kruskal-Wallis test was
carried out to verify significant differences in the of toxic mineral elements concentrations among sampling areas.

References

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Catsiki VA, Florou H. 2006. Study on the behavior of the heavy metals Cu, Cr, Ni, Zn, Fe, Mn and 137 Cs in an estuarine ecosystem using *Mytilus galloprovincialis* as a bioindicator species: the case of Thermaikos gulf, Greece. J Environ Radioact. 86(1):31-44.

Helsel DR. 2005. Nondetects and Data Analysis: Statistics for Censored Environmental data. 48, Wiley, Hoboken.
| Parameter                          | Set                      |
|-----------------------------------|--------------------------|
| RF-Power (W)                      | 1550                     |
| Reflected power                   | < 5                      |
| Carrier gas flow (mL/min)         | 1.2                      |
| Plasma gas flow (L/min)           | 15                       |
| Auxiliary gas flow (mL/min)       | 1.0                      |
| Spray chamber                     | Water cooled double pass |
| Spray chamber temperature (°C)    | 2                        |
| Lens voltage (V)                  | 4.5                      |
| Mass range (amu)                  | 6 - 209                  |
| Mass resolution                   | 0.7                      |
| Integration time points/ms        | 3                        |
| Points per peak                   | 3                        |
| Replicates                        | 4                        |

*Table S1. ICP-MS strumental conditions*
Table S2. Samples thermal decomposition parameters.

| Step | Time   | Temperature (°C) | Process       |
|------|--------|------------------|---------------|
| 1    | 00:00:10 | 200              | DRYING        |
| 2    | 00:01:00 | 200              |               |
| 3    | 00:01:00 | 650              | DECOMPOSITION |
| 4    | 00:01:00 | 650              |               |

Table S3. Detection and quantification limits of the method for some of the elements studied.

| Element | LoD (µg/kg) | LoQ (µg/kg) |
|---------|-------------|-------------|
| As      | 3           | 3           |
| Cd      | 3           | 3           |
| Pb      | 3           | 4           |

Table S4. Validation parameters of Hg determination method.

| Level | Concentration (mg/Kg) | Repeatability (mg/Kg) | Expanded measurement uncertainty | LOD (mg/Kg) | LOQ (mg/Kg) |
|-------|-----------------------|------------------------|----------------------------------|-------------|-------------|
| I     | 0.100                 | 0.021                  | ± 0.020                          |             |             |
| II    | 0.500                 | 0.174                  | ± 0.153                          | 0.041       | 0.050       |
| II    | 1                     | 0.380                  | ± 0.360                          |             |             |
| IV    | 2                     | 0.826                  | ± 0.710                          |             |             |
Figure S1. Average toxic metals concentrations obtained from mussel samples divided by sampling area.