Arch. Environ. Contam. Toxicol. 44, 476–484 (2003)
DOI: 10.1007/s00244-002-1262-8

ARCHIVES OF
Environmental
Contamination
and Toxicology

Metal Accumulation and Binding Protein Induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Lagoon of Venice

P. Irato, G. Santovito, A. Cassini, E. Piccinni, V. Albergoni

Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy

Received: 20 December 2001/Accepted: 11 November 2002

Abstract. Heavy metal pollution is known to be widespread in the sediments of the Lagoon of Venice. Therefore, physiological parameters influenced by this form of contamination were examined. The bivalve molluscs blue mussel (*Mytilus galloprovincialis*), ark clam (*Scapharca inaequivalvis*), and Japanese littleneck (*Tapes philippinarum*) were sampled in two sites (Marghera, Chioggia) every 3 months for 1 year. The digestive gland and gills of each bivalve were analyzed. The concentrations of essential and nonessential metals (including chromium, manganese, iron, cobalt, nickel, copper, zinc, and cadmium) were determined. Because glutathione and metallothioneins (MTs) are involved in metal homeostasis and detoxification, their concentrations were evaluated in relation to metal concentrations. Results show that (1) all three studied species accumulate metals to a considerable extent, with some species-specific differences between the digestive gland and gills; (2) gills are a good tissue to evaluate pollution by examining the MT content. In particular, the correlation between Zn and MT levels in the gills indicates that *M. galloprovincialis* and *S. inaequivalvis* are sentinel organisms and can be used specifically for Zn pollution; (3) *T. philippinarum* accumulates Cu in the digestive gland more readily than the other two bivalves and therefore has the highest MT.

High metal concentrations in the environment are the result of both natural and anthropogenic sources. The accumulation of metals in waters and sediments affects various organisms in the environment, influencing their functions in several different ways (Duquesne *et al.* 1995; Regoli and Principato 1995; Temara *et al.* 1997).

As compared with the open sea, lagoons are more subject to pollution, particularly by heavy metals from industrial, agricultural, and urban origin. These sources contribute to the lagoon environment either directly or by means of watercourses discharging their contents into the lagoon. Numerous studies on heavy metal pollution from a wide spectrum of anthropogenic sources (industrial, urban, agricultural) in various areas of the Lagoon of Venice have been carried out over the years (Donazzolo *et al.* 1984; Bendoricchio *et al.* 1993).

It is well known that molluscs accumulate organic and metallic pollutants at concentrations several orders of magnitude above those observed in the field environment (Bryan *et al.* 1977, 1983). Among molluscs, mussels are extensively used in environmental monitoring studies, such as the Mussel Watch Program, as bioindicators of heavy metal pollution (Cantillo 1998; O'Connor 1998; Jeng *et al.* 2000). Most studies on heavy metals in bivalves have dealt with oysters and mussels, and only a few studies have included ark clams (*Scapharca inaequivalvis*) and Japanese littlenecks (*Tapes philippinarum*) (de Zwaan and Eertman 1996; Adami *et al.* 1997). In the past few decades these two species, native to Indo-Pacific areas, have been introduced into the Lagoon of Venice, where they have found a suitable environment for colonisation. *S. inaequivalvis* was introduced in 1960 by maritime traffic through the Suez Canal (Rinaldi 1972); *T. philippinarum* was introduced for commercial ventures in 1983 (Cesari and Pellizzato 1985).

The exposure of bivalves to these high environmental levels of metals can induce synthesis of metal-binding proteins and molecules, which are capable of sequestering and detoxifying excess intracellular metals. Glutathione (GSH) and metallothionein (MT) are considered to be important components involved in protecting cells, both as metal chelating agents and oxygen radical scavengers (Thornalley and Vašák 1985; Freedman *et al.* 1989; Albergoni and Piccinni 1998; Klaassen *et al.* 1999; Irato *et al.* 2001). It has been demonstrated that MT synthesis may also be induced by metals (such as Cd, Zn, and Cu) in aquatic animals, such as crustaceans and molluscs (Roesijadi 1992). As the result of their induction by metals, MTs have been proposed as biomarkers of metal contamination in field studies (Bordin *et al.* 1997; Mouneyrac *et al.* 1998).

Numerous laboratory studies also support the idea that aquatic organisms synthesize MTs as a defense against toxic metals. The occurrence of MTs or related proteins has been extensively reported in various species examined in laboratory assays in which exposure conditions differed greatly from those encountered in natural environments. MT contents have not been extensively studied in natural popula-

Correspondence to: V. Albergoni; *email:* biopd09@civ.bio.unipd.it

tions of environments subjected to metal pollution but in metal exposure experiments (Bebianno and Langston 1991; Bebianno *et al.* 1992; Pavicic *et al.* 1993). In particular, the metal concentrations that induce MT synthesis in laboratory experiments are often several orders of magnitude higher than those found in even the most contaminated aquatic systems (Cossons *et al.* 1991; Couillard *et al.* 1993).

In this paper, we aimed at evaluating the responses of heavy metal pollution in three species of bivalves sampled from polluted areas of the Lagoon of Venice (Casellato and Salmaso 2000). In particular, we focused on two different tissues with the goal of verifying characteristics of specific tissue responses to metal accumulation.

Materials and Methods

Sampling

Twenty individuals of each species (*M. galloprovincialis, S. inaequivalvis*, and *T. philippinarum*) were sampled in February, May, August, and November from two stations. These sites, Marghera and Chioggia (Figure 1), are heavily affected by anthropogenic pollution. The digestive glands and gills were removed, weighed, and immediately frozen in liquid nitrogen and stored at −80°C. Metal, GSH, and MT assays were performed; for each sampling 10 individuals from each species were used for metal assays and 10 for GSH and MT assays.

Tissue Heavy Metal Concentrations

For metal determination, samples were homogenized (1:4 w/v ratio) in 20 mM Tris HCl, pH 7.5, and digested with HNO_3 AristaR in PFA vessels in a CEM MD-2000 furnace microwave. This microwave is controlled by a computerized program, specially designed according to the type of sample, with specific pressure and temperature conditions providing optimal dissolution. Metal concentrations (Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd) were determined on an ICP-AES Spectroflame Modula sequential and simultaneous spectrometer equipped with a capillary cross-flow nebulizer (Spectro Analytical) to determine concentrations in the order of ppb. The instrument for the metal analysis was calibrated by standard addition methods and by reference to fresh standard salt solutions. Blank solutions on reagents and equipment revealed insignificant contamination. Data refer to dry weights (corresponding aliquots were dried at 130°C).

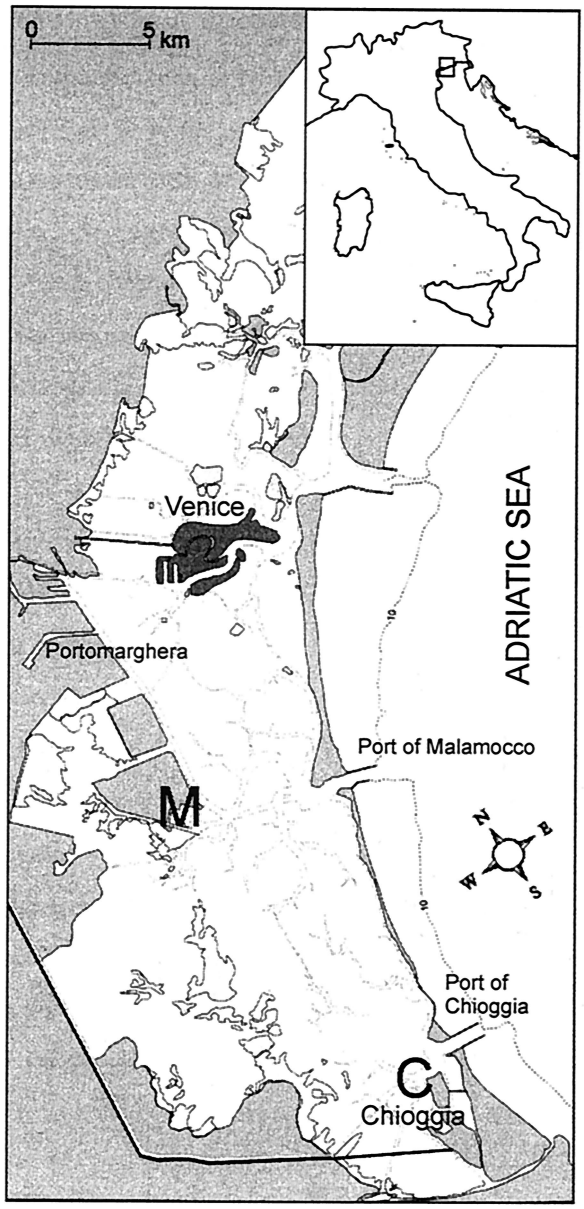

Fig. 1. Locality map of sampling sites in the Lagoon of Venice, Italy: Marghera (M); Chioggia (C)

Assay for Total GSH

For total GSH determination, samples were homogenized (1:5 w/v ratio) in 5% sulfosalicylic acid and centrifuged at 15,000 *g* for 30 min at 4°C. Total GSH contents were measured in the resulting supernatant by the Anderson (1985) method and refer to wet weight measurements.

MT Analyses

For MT assays, samples were homogenized (1:4 w/v ratio) in 20 mM Tris HCl, pH 7.5, adding 0.006 mM leupeptine, 0.5 mM phenylmethylsulphonylfluoride (as antiproteolitic agents), and 0.01% β-mercaptoethanol (as reducing agent) and centrifuged at 30,000 *g* for 60 min at 4°C. MT contents were measured in the resulting supernatant by the silver saturation method (Scheuhammer and Cherian 1991); values referring to total protein were determined using the Lowry *et al.* (1951) method.

Statistical Analyses

Values are shown as mean of 10 individuals ± SD. Statistical analysis was performed with the PRIMER statistical program. The Student *t*-test for comparison of pairs or ANOVA for different groups was followed by the Student-Newman-Keuls test for determination of significant differences ($p < 0.05$). Linear regression analysis was also performed using the mean values of 10 molluscs sampled in the reported months at the two stations.

Table 1. Metal concentrations (μg/g of dry weight) determined in the digestive gland and gills of *M. galloprovincialis*, *S. inaequivalvis*, and *T. philippinarum* sampled in May

			Marghera		Chioggia	
		n	Digestive gland	Gills	Digestive Gland	Gills
Cr	*Mytilus*	10	3.5 ± 1.7	2.7 ± 1.8	6.5 ± 3.9	2.8 ± 0.6**
	Scapharca	10	14.2 ± 6.8	46.5 ± 19.6***	18.1 ± 1.6	27.9 ± 10.9***
	Tapes	10	8.1 ± 5.4	5.3 ± 2.9	9.3 ± 8.8	10.4 ± 10.1
Mn	*Mytilus*	10	2.9 ± 1.1	1.7 ± 0.9**	19.6 ± 3.4	6.7 ± 1.6***
	Scapharca	10	2.4 ± 1.5	31.5 ± 15.1***	13.3 ± 6.6	40.4 ± 38.6*
	Tapes	10	8.1 ± 2.2	10.0 ± 2.6	12.0 ± 7.4	17.5 ± 6.6
Fe	*Mytilus*	10	101.7 ± 69.6	26.8 ± 16.9**	347.7 ± 135.4	138.5 ± 54.9***
	Scapharca	10	178.1 ± 84.0	514.0 ± 103.1***	230.1 ± 86.8	489.1 ± 27.7***
	Tapes	10	268.2 ± 84.9	251.0 ± 86.9	517.1 ± 208.1	405.0 ± 57.2
Co	*Mytilus*	10	6.2 ± 4.9	11.5 ± 5.5*	15.9 ± 14.9	4.3 ± 3.5*
	Scapharca	10	1.6 ± 0.6	26.9 ± 10.7***	4.7 ± 3.5	6.0 ± 4.4
	Tapes	10	15.7 ± 7.8	14.3 ± 7.2	54.0 ± 47.4	49.7 ± 25.4
Ni	*Mytilus*	10	9.8 ± 2.7	6.8 ± 2.0*	6.8 ± 2.5	7.5 ± 3.4
	Scapharca	10	4.1 ± 4.0	40.4 ± 32.2**	14.8 ± 8.0	15.0 ± 6.6
	Tapes	10	8.6 ± 7.9	4.7 ± 2.8	8.5 ± 8.2	9.5 ± 2.1
Cu	*Mytilus*	10	19.2 ± 3.9	14.5 ± 4.3*	16.2 ± 3.0	9.8 ± 4.3***
	Scapharca	10	19.8 ± 3.6	6.1 ± 3.0***	15.5 ± 4.5	13.6 ± 5.5
	Tapes	10	41.6 ± 11.6	10.9 ± 2.3***	25.2 ± 8.4	10.8 ± 3.5***
Zn	*Mytilus*	10	127.6 ± 20.0	115.5 ± 38.2	104.6 ± 15.9	71.4 ± 35.3*
	Scapharca	10	61.5 ± 8.6	229.8 ± 144.5**	59.6 ± 25.7	254.5 ± 113.7***
	Tapes	10	100.8 ± 21.1	101.2 ± 39.0	79.9 ± 32.4	108.6 ± 49.7
Cd	*Mytilus*	10	1.6 ± 0.4	0.6 ± 0.6*	0.9 ± 0.3	0.5 ± 0.3**
	Scapharca	10	0.8 ± 0.2	8.9 ± 8.0**	0.4 ± 0.4	7.7 ± 7.7**
	Tapes	10	0.7 ± 0.6	1.1 ± 0.6	0.4 ± 0.2	0.6 ± 0.4

Data are reported as mean ± SD. Statistical differences between the digestive gland and gills are reported: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

Results

Trace Metals

Metal concentrations, determined in the digestive glands and gills of *M. galloprovincialis*, *S. inaequivalvis*, and *T. philippinarum* are listed in Tables 1–4.

Fe, always abundantly present, generally had higher levels than any of the other metals. Zn was also constantly present in high levels. The other metals were found at lower concentrations, especially Cd, which was very low. However, three bivalve species showed different characteristics of tissue-specific accumulation.

In *M. galloprovincialis* the heavy metals examined were generally more concentrated in the digestive gland than in the gills. In particular, the concentrations of Mn, Fe, and Cd were always statistically higher in the digestive gland. Cr and Cu concentrations were similar in the two tissues only in samples from Marghera in May (Table 1) and August (Table 2), respectively. Ni concentrations in the digestive gland were higher except at Chioggia in May (Table 1) and Marghera in August (Table 2). A few differences between the two tissues were found for Co and Zn.

In *S. inaequivalvis*, heavy metal concentrations were generally higher in the gills than in the digestive gland. This difference was always statistically significant for Cr, Fe, Zn, Cd. Mn and Ni concentrations were similar in the two tissues only in samples from Chioggia in August (Table 2) and May (Table 1), respectively, and Co concentrations were higher in the gills except at Chioggia in May (Table 1) and August (Table 2). Unlike other metals, Cu accumulated more in the digestive gland than in the gills in all specimens from Marghera. Specimens sampled at Chioggia showed this Cu tissue distribution only in November (Table 3).

In *T. philippinarum*, no differences were observed between the two tissues for all metals. Only Cu is more concentrated in the digestive gland, except at Chioggia in August (Table 2) and November (Table 3).

The statistical differences between the two sampling sites and among the four sampling periods seem to be random and are not constant.

GSH and MT

GSH concentrations are always higher in the digestive gland than in the gills in all three organisms (Figures 2A–D). The difference is up to 12-fold in *M. galloprovincialis* (sampled at Marghera in May; Figure 2A), up to 5-fold in *S. inaequivalvis* (from Chioggia in May; Figure 2A), and up to 17-fold in *T. philippinarum* (sampled at Marghera in November; Figure 2C).

MT concentrations in *T. philippinarum* are higher in the digestive gland than in the gills, almost always twofold higher. The opposite occurs in *M. galloprovincialis* and *S. inaequivalvis*, in which the difference is up to 12-fold in *M.*

Table 2. Metal concentrations (μg/g of dry weight) determined in the digestive gland and gills of *M. galloprovincialis, S. inaequivalvis,* and *T. philippinarum* sampled in August

			Marghera		Chioggia	
		n	Digestive Gland	Gills	Digestive Gland	Gills
Cr	*Mytilus*	10	2.3 ± 1.2	1.2 ± 0.6*	2.3 ± 1.3	1.3 ± 0.4*
	Scapharca	10	7.3 ± 1.4	34.2 ± 19.1***	4.9 ± 1.6	9.4 ± 1.5**
	Tapes	10	2.5 ± 1.2	4.9 ± 6.7	2.1 ± 0.7	1.2 ± 0.7**
Mn	*Mytilus*	10	16.7 ± 2.5	12.7 ± 4.2*	12.5 ± 1.9	8.5 ± 1.1***
	Scapharca	10	9.9 ± 5.4	20.9 ± 9.5**	7.7 ± 4.6	10.1 ± 1.4
	Tapes	10	10.4 ± 3.1	7.5 ± 2.8*	5.9 ± 4.7	5.7 ± 1.8
Fe	*Mytilus*	10	241.3 ± 33.0	103.3 ± 28.3***	201.1 ± 77.7	71.1 ± 12.9***
	Scapharca	10	289.3 ± 39.1	462.4 ± 35.0***	241.8 ± 41.5	469.5 ± 55.6***
	Tapes	10	341.5 ± 38.6	167.4 ± 45.4***	521.5 ± 301.8	311.2 ± 188.9
Co	*Mytilus*	10	7.1 ± 2.8	8.9 ± 5.2	16.4 ± 5.4	15.0 ± 4.1
	Scapharca	10	5.3 ± 1.6	31.9 ± 21.2***	10.3 ± 4.6	12.0 ± 8.8
	Tapes	10	9.7 ± 3.2	6.7 ± 1.9	21.0 ± 4.2	10.9 ± 5.8***
Ni	*Mytilus*	10	1.9 ± 1.0	2.5 ± 1.2	2.1 ± 1.2	0.2 ± 0.1***
	Scapharca	10	2.6 ± 1.3	28.1 ± 14.6**	0.6 ± 0.3	2.9 ± 1.7***
	Tapes	10	1.3 ± 0.9	4.6 ± 4.0*	1.9 ± 0.6	1.6 ± 0.1
Cu	*Mytilus*	10	10.5 ± 3.4	8.7 ± 2.6	12.1 ± 2.3	9.2 ± 2.3*
	Scapharca	10	13.9 ± 3.4	10.0 ± 1.6*	15.4 ± 2.6	15.3 ± 4.2
	Tapes		22.2 ± 4.8	14.3 ± 3.7***	17.8 ± 2.6	15.0 ± 3.5
Zn	*Mytilus*	10	218.2 ± 53.4	202.1 ± 110.1	146.5 ± 41.7	142.0 ± 61.2
	Scapharca	10	106.0 ± 63.6	422.3 ± 135.0***	65.4 ± 16.1	186.6 ± 41.1***
	Tapes	10	154.8 ± 76.9	182.9 ± 174.1	111.1 ± 15.5	129.7 ± 30.7
Cd	*Mytilus*	10	1.9 ± 1.7	0.7 ± 0.2*	0.9 ± 0.3	0.5 ± 0.4*
	Scapharca	10	0.8 ± 0.4	4.3 ± 2.1***	0.4 ± 0.3	5.7 ± 5.5*
	Tapes	10	0.4 ± 0.2	1.0 ± 0.9	0.5 ± 0.3	0.5 ± 0.5

Data are reported as mean ± SD. Statistical differences between the digestive gland and gills are reported: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

Table 3. Metal concentrations (μg/g of dry weight) determined in the digestive gland and gills of *M. galloprovincialis, S. inaequivalvis,* and *T. philippinarum* sampled in November

			Marghera		Chioggia	
		n	Digestive Gland	Gills	Digestive Gland	Gills
Cr	*Mytilus*	10	4.8 ± 0.9	0.9 ± 0.6***	2.7 ± 1.1	0.9 ± 0.6***
	Scapharca	10	7.8 ± 1.7	28.8 ± 27.4*	48.3 ± 1.2	76.9 ± 30.5**
	Tapes	10	0.9 ± 0.5	1.5 ± 1.1	1.8 ± 1.0	2.0 ± 1.0
Mn	*Mytilus*	10	30.1 ± 10.6	6.9 ± 1.1***	26.1 ± 4.6	6.9 ± 1.1***
	Scapharca	10	7.8 ± 3.8	27.9 ± 13.3***	18.9 ± 5.5	26.9 ± 5.4**
	Tapes	10	7.7 ± 3.1	6.8 ± 2.5	12.1 ± 8.7	10.0 ± 6.2
Fe	*Mytilus*	10	1,302.9 ± 543.9	86.2 ± 7.4***	1,223.3 ± 457.9	86.2 ± 7.4***
	Scapharca	10	237.0 ± 93.9	413.7 ± 59.6***	939.6 ± 49.5	1,179.0 ± 271.2*
	Tapes	10	390.7 ± 140.6	344.7 ± 62.4	263.7 ± 87.0	330.2 ± 294.5
Co	*Mytilus*	10	11.2 ± 2.9	12.6 ± 7.0	9.9 ± 1.1	12.6 ± 7.0
	Scapharca	10	4.0 ± 2.1	26.0 ± 13.9***	3.3 ± 0.9	5.1 ± 1.9*
	Tapes	10	13.9 ± 3.0	7.7 ± 2.5***	11.8 ± 1.5	9.4 ± 2.7
Ni	*Mytilus*	10	12.6 ± 5.1	1.6 ± 0.7***	9.3 ± 2.4	1.6 ± 0.7***
	Scapharca	10	2.5 ± 1.9	23.8 ± 21.3**	8.7 ± 0.2	24.7 ± 4.6***
	Tapes	10	1.5 ± 1.3	6.9 ± 6.6***	1.5 ± 1.1	2.0 ± 1.0
Cu	*Mytilus*	10	17.9 ± 4.7	10.9 ± 2.8***	31.2 ± 5.5	10.9 ± 2.8***
	Scapharca	10	16.4 ± 4.6	9.8 ± 5.1**	17.5 ± 1.4	13.9 ± 4.1*
	Tapes	10	32.7 ± 7.5	5.3 ± 2.5***	42.6 ± 10.5	38.4 ± 28.7
Zn	*Mytilus*	10	204.3 ± 35.6	158.4 ± 88.8	195.2 ± 96.5	132.0 ± 74.0
	Scapharca	10	76.0 ± 28.0	440.9 ± 110.5***	59.7 ± 43.3	449.8 ± 220.0***
	Tapes	10	156.4 ± 54.7	152.5 ± 54.3	100.3 ± 18.2	186.2 ± 55.3*
Cd	*Mytilus*	10	1.6 ± 0.4	0.7 ± 0.4***	2.0 ± 0.4	0.8 ± 0.7***
	Scapharca	10	1.1 ± 0.4	8.6 ± 6.0***	0.4 ± 0.5	15.4 ± 16.6**
	Tapes	10	0.5 ± 0.3	0.5 ± 0.3	0.6 ± 0.0	0.2 ± 0.1***

Data are reported as mean ± SD. Statistical differences between the digestive gland and gills are reported: *$p < 0.05$; $p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

Table 4. Metal concentrations (μg/g of dry weight) determined in the digestive gland and gills of *M. galloprovincialis*, *S. inaequivalvis*, and *T. philippinarum* sampled in February

			Marghera		Chioggia	
		n	Digestive Gland	Gills	Digestive Gland	Gills
Cr	*Mytilus*	10	1.8 ± 1.4	0.2 ± 0.1**	0.8 ± 0.7	0.2 ± 0.1**
	Scapharca	10	3.9 ± 1.8	5.6 ± 1.6*	1.4 ± 0.5	4.3 ± 2.6**
	Tapes	10	2.3 ± 1.3	3.9 ± 2.4	2.3 ± 1.6	2.0 ± 1.0
Mn	*Mytilus*	10	34.7 ± 3.3	5.9 ± 1.8***	12.4 ± 1.3	6.5 ± 1.1***
	Scapharca	10	10.9 ± 4.6	31.2 ± 15.4***	8.2 ± 1.5	15.6 ± 6.2**
	Tapes	10	12.5 ± 7.0	19.5 ± 13.8	8.7 ± 3.7	9.2 ± 2.2
Fe	*Mytilus*	10	404.1 ± 174.0	114.8 ± 58.6***	232.8 ± 52.6	94.6 ± 19.5***
	Scapharca	10	243.7 ± 119.6	396.9 ± 40.6***	130.4 ± 40.3	216.3 ± 60.8**
	Tapes	10	765.4 ± 160.2	596.0 ± 210.9	458.2 ± 212.4	268.7 ± 205.7
Co	*Mytilus*	10	12.1 ± 2.8	7.4 ± 3.3**	12.6 ± 3.7	12.4 ± 2.4
	Scapharca	10	5.3 ± 2.4	19.1 ± 9.7***	0.5 ± 0.3	1.0 ± 0.6*
	Tapes	10	3.8 ± 1.9	2.4 ± 1.3	12.9 ± 8.4	14.3 ± 3.0
Ni	*Mytilus*	10	2.4 ± 1.3	0.2 ± 0.1***	1.3 ± 1.2	0.2 ± 0.1**
	Scapharca	10	0.8 ± 0.3	2.9 ± 2.4*	1.0 ± 0.1	1.6 ± 0.3***
	Tapes	10	26.3 ± 16.5	16.3 ± 11.1	4.6 ± 3.2	2.2 ± 2.0
Cu	*Mytilus*	10	23.1 ± 10.6	5.9 ± 2.1***	11.8 ± 1.3	3.0 ± 1.0***
	Scapharca	10	19.5 ± 6.9	9.5 ± 5.5**	13.5 ± 5.7	11.6 ± 4.1
	Tapes	10	55.7 ± 17.2	15.7 ± 6.2***	34.9 ± 14.8	5.5 ± 2.5***
Zn	*Mytilus*	10	247.5 ± 64.7	218.4 ± 48.9	123.6 ± 33.4	120.6 ± 49.0
	Scapharca	10	60.5 ± 11.8	257.1 ± 26.1***	53.7 ± 17.8	161.3 ± 19.4***
	Tapes	10	152.1 ± 40.9	129.9 ± 36.2	84.0 ± 20.0	78.6 ± 43.1
Cd	*Mytilus*	10	2.1 ± 1.0	0.5 ± 0.3***	0.5 ± 0.2	0.2 ± 0.1***
	Scapharca	10	0.5 ± 0.2	12.5 ± 7.9***	0.4 ± 0.2	2.0 ± 1.1***
	Tapes	10	1.2 ± 0.8	0.3 ± 0.0**	0.2 ± 0.1	0.4 ± 0.3

Data are reported as mean ± SD. Statistical differences between the digestive gland and gills are reported: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

Table 5. Correlation indexes between metals (Zn, Cu, and Cd) (values reported in Tables 1–4) and MT (values represented in Figure 3) in the digestive gland and gills of *M. galloprovincialis*, *S. inaequivalvis*, and *T. philippinarum*

		M. galloprovincialis				S. inaequivalvis				T. philippinarum			
		Digestive Gland		Gills		Digestive Gland		Gills		Digestive Gland		Gills	
	n	*r*	p	*r*	p	*r*	p	*r*	p	*r*	p	*r*	p
Zn	8	0.15	NS	0.88	< 0.01	−0.26	NS	0.90	< 0.01	−0.06	NS	0.61	NS
Cu	8	−0.14	NS	−0.08	NS	−0.06	NS	0.12	NS	−0.44	NS	0.40	NS
Cd	8	−0.14	NS	0.42	NS	−0.69	NS	0.53	NS	−0.09	NS	0.05	NS

The correlation indexes are obtained from the mean values of 10 molluscs sampled at the reported months at the two stations (see Materials and Methods).
NS, not significant.

galloprovincialis (sampled at Marghera in August; Figure 3B) and up to 9-fold in *S. inaequivalvis* (from Marghera in November; Figure 3C). Furthermore, in the gills of *M. galloprovincialis* and *S. inaequivalvis* there is a good correlation between Zn and MT concentrations ($r = 0.88$, $p < 0.01$ and $r = 0.90$, $p < 0.01$ respectively) (Table 5 and Figure 4A–B).

Even in the case of chelating molecules, the statistical differences between the two sampling sites and among the four sampling periods seem to be random and are not constant.

Discussion

Metals may be taken up bound to particulate material or in soluble form. Both the digestive gland and gills are involved in these mechanisms. In any case the major site of metal deposition is species-specific regardless of the metal source. To study metal accumulation, many studies have also examined the total soft tissue of bivalves (Cantillo 1998; O'Connor 1998; Jeng *et al.* 2000). In the present study, we considered two different tissues with the aim of identifying their role as sites of specific metal

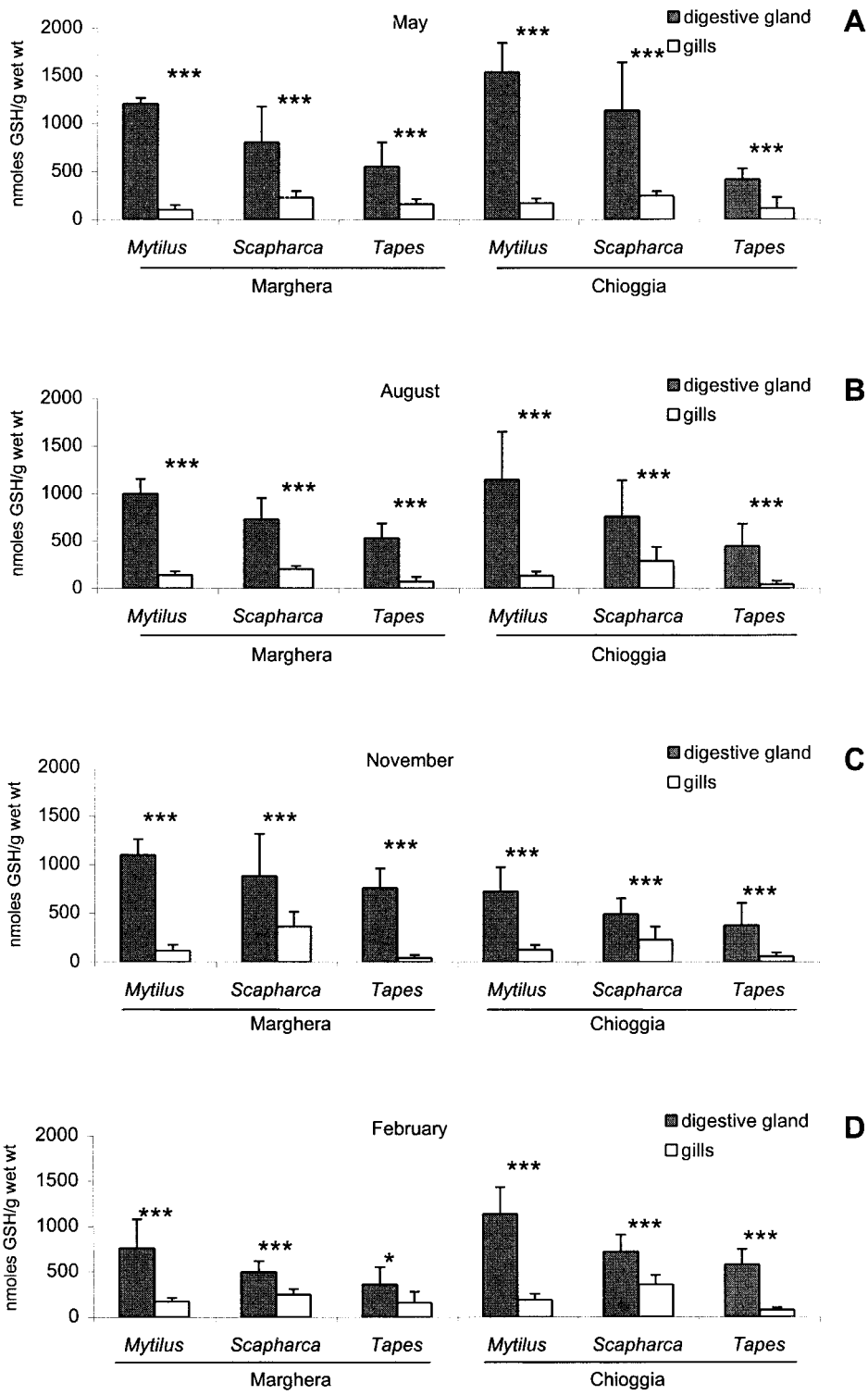

Fig. 2. GSH (nmoles/g of wet weight) determined in the digestive gland and gills of *M. galloprovincialis*, *S. inaequivalvis*, and *T. philippinarum*. (A) Sampling in May; (B) sampling in August; (C) sampling in November; (D) sampling in February. Data reported as mean ± SD (n = 10). Statistical differences between the digestive gland and gills are reported: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test).

accumulation. The data presented demonstrated that all three mollusc species studied accumulate metals, with some differences between their respective digestive gland and gills. The differences observed also seem to be species-specific. *M. galloprovincialis* accumulates metals especially in the digestive gland, whereas *S. inaequivalvis* accumulates metals mainly in the gills. *T. philippinarum* concentrates metals in both tissues, with the exception of Cu, which is mainly accumulated in the digestive gland.

As for tissue-specific accumulation, we observed that the digestive gland of *M. galloprovincialis* accumulates Mn, Zn, and Cd more than other species; the digestive gland of *S. inaequivalvis* accumulates more Cr; and that of *T. philippina-*

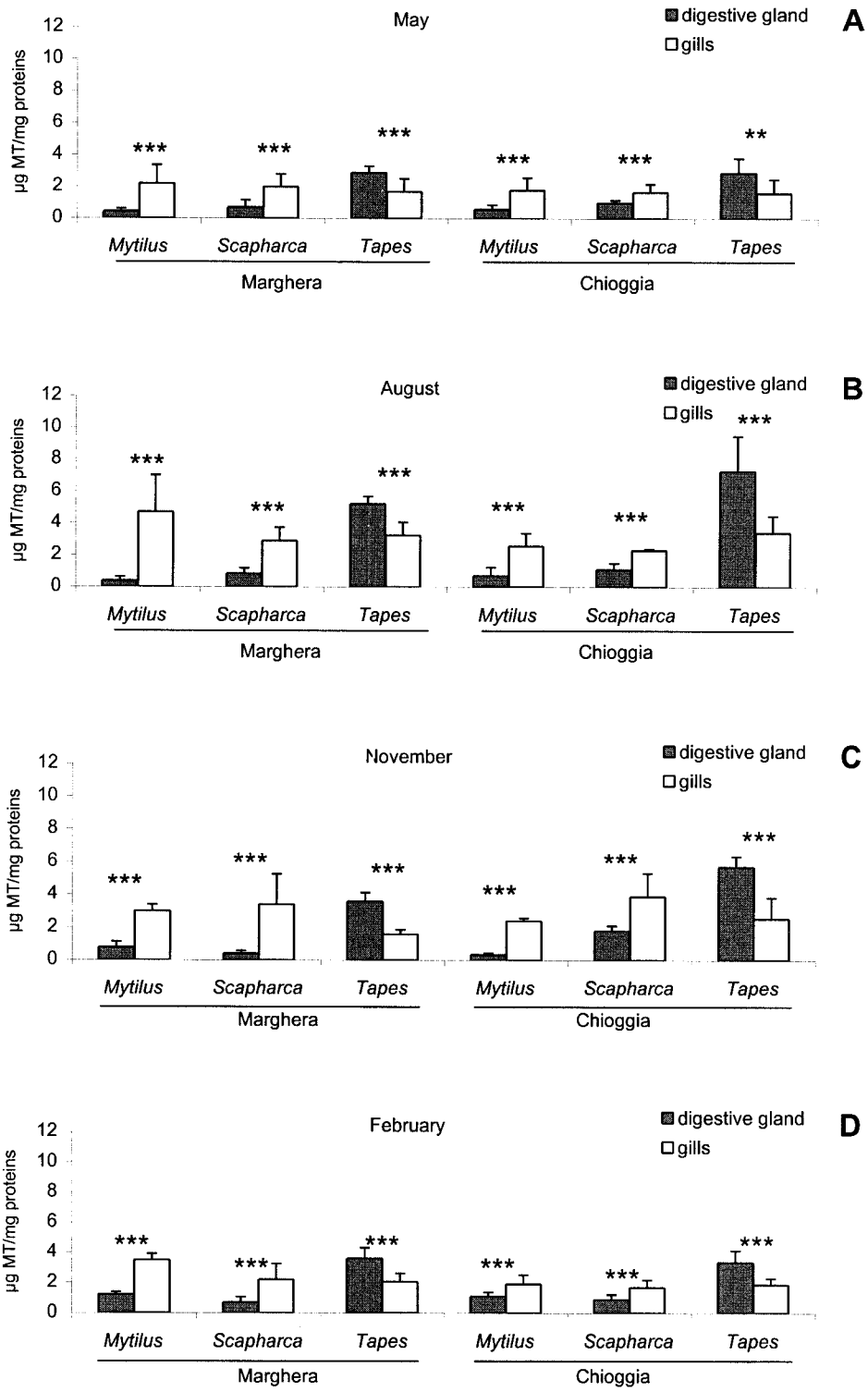

Fig. 3. MT (µg/mg of protein) determined in the digestive gland and gills of *M. galloprovincialis*, *S. inaequivalvis*, and *T. philippinarum*. (A) sampling in May; (B) sampling in August; (C) sampling in November; (D) sampling in February. Data reported as mean ± SD (n = 10). Statistical differences between the digestive gland and gills are reported: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's *t*-test)

rum accumulates more Fe, Co, and Cu. The three bivalves have similar concentrations of Ni.

S. inaequivalvis is the organism that accumulates Cr, Mn, Fe, Ni, Zn, and Cd in the gills at concentrations higher than the other species. The three bivalves have similar gill concentrations of Co and Cu.

Our findings confirm the tissue distribution of some metals (Mn, Fe, Cu, Zn) in *M. galloprovincialis* previously sampled in an unpolluted reference site in the Tyrrhenian Sea (Regoli 1998). Compared with our values, these prior studies also show that Mn, Fe, and Cu concentrations are similar, whereas the Zn concentration determined in our research was higher, indicating that heavy Zn pollution occurs at our specific sampling sites. In the gills of *M. galloprovincialis* and *S. inaequivalvis*,

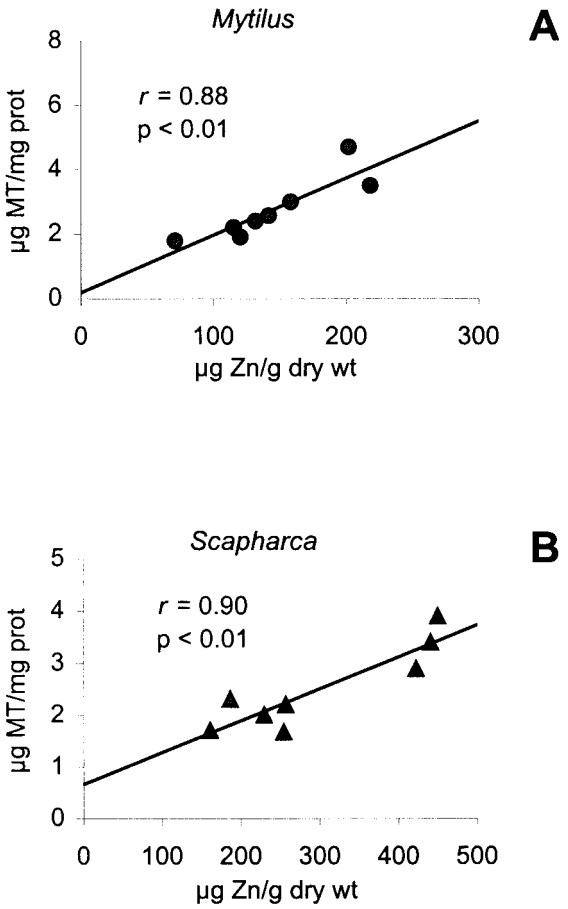

Fig. 4. Correlations between Zn and MT concentrations in the gills of *M. galloprovincialis* and *S. inaequivalvis*

the presence of a correlation between Zn and MT concentrations confirms this indication and also strongly validates MT as a biological indicator for metal pollution. Some authors have proposed MT measurements in mussels as an indicator of metal pollution, especially Cd, Hg, Ag, and perhaps Cu (Bebianno and Serafim 1998). Other authors have found highly significant correlations between metals (Cd, Cu, and Zn) and MTs in a bivalve mollusc (Hamza-Chaffai *et al.* 2000). However, it is necessary for MT analyses to be performed on appropriate tissues. Our results emphasize that these proteins shows differential tissue concentration in the various species, *M. galloprovincialis* and *S. inaequivalvis* having high MT concentrations in the gills and *T. philippinarum* in the digestive gland.

T. philippinarum had MT concentrations in the digestive gland higher than the other two bivalves, whereas MT levels in the gills were similar in all three molluscs. This observation, together with the presence of high Cu concentrations in the digestive gland of this species, may indicate the strong response of this organism to environmental conditions. Further investigation is warranted in the Lagoon of Venice as a result of the extensive farming of *T. philippinarum* at this site. Results suggest that using bivalve species as bioindicators requires knowledge of their individual tissue accumulation characteristics.

The higher GSH contents present in the digestive gland of all three bivalves are in agreement with published findings for *M. galloprovincialis* (Canesi *et al.* 1999) and suggest that the location of this tripeptide is probably a feature of this organism. Because GSH plays a primary protective role against the toxic effects of heavy metals and oxygen radicals (Freedman *et al.* 1989), its high content measured in the digestive glands of all three bivalves may be related to the high Cu concentrations measured in the same organs. In effect, Cu is a metal with redox properties and may be involved in Haber-Weiss and Fenton reactions producing free radicals (Fridovich 1978; Basaga 1990). GSH is known to form stable GSH-Cu(I) complexes (Freedman *et al.* 1989), preventing further redox cycling and the generation of free radicals; this may explain the complete protection afforded by GSH against the effects of Cu. The result is a decrease in the physiological GSH pool and alterations in the redox balance. Heavy metal accumulation in cells may result in decreased availability of reduced glutathione, due to both GSH binding and oxidation. Heavy metals (such as Cd, Hg, and Pb) have also been demonstrated to increase the concentration of GSH in both mammalian and fish tissues (Thomas and Wofford 1984; Thomas and Juedes 1992; Lash and Zalups 1996). In mammalian cells, the concentration of GSH is physiologically regulated by the γ-glutamyl cycle (Meister and Anderson 1983). The effects of heavy metals on glutathione metabolism in mussel tissues depend on the type of metal cation, their concentration in sea water, and the time of exposure. In Cu-exposed bivalves, a decrease in GSH tissue levels have been observed (Canesi *et al.* 1998; Conners and Ringwood 2000). In any case, at longer exposure times, the effects of Cu on glutathione metabolism showed a tendency to recover (Viarengo 1989).

Our GSH values seem consistent with those from unpolluted conditions. In fact, Regoli and Principato (1995) report analogous values for *M. galloprovincialis* sampled in April from Forte dei Marmi (Tyrrehnian Sea), an unpolluted site: 1,276 and 290 nmol/g wet weight in the digestive gland and gills, respectively.

In conclusion, although *M. galloprovincialis* is widely used as an indicator organism in research on environmental metal contamination, our data demonstrate that *T. philippinarum* and *S. inaequivalvis* may also be used as metal pollution bioindicators because they accumulate metals to a considerable extent. In particular, correlations between Zn and MT levels in the gills of *M. galloprovincialis* and *S. inaequivalvis* indicate that this tissue may be considered to be a good sentinel tissue specific for Zn pollution. Our results suggest that using bivalve species as bioindicators requires knowledge of their individual tissue accumulation characteristics, because the effect of metals may differ according to species. Several physiological responses have been revealed in both digestive gland and gills of bivalves exposed to metals in different laboratory conditions, but these investigations cannot substitute for field research when studying populations of organisms subjected or otherwise to anthropic pollution.

Acknowledgments. This research was supported by the Italian Ministero Università Ricerca Scientifica Tecnologica (MURST).

References

Adami G, Barbieri P, Predonzani S, Reisenhofer E (1997) Heavy metals in sediments of Marano Lagoon and their relevance on

clam hatcheries. A chemometric investigation. Ann Chim Rome 87:709–720
Albergoni V, Piccinni E (1998) Copper and zinc metallothioneins. In: Rainsford KD, Milanino R, Sorenson JRJ, Velo GP (eds) Copper and zinc in inflammatory and degenerative diseases. Kluwer Academic Publishers, Londra, pp 61–78
Anderson ME (1985) Determination of glutathione and glutathione disulfide in biological samples. Meth Enzymol 113:548–555
Basaga HS (1990) Biochemical aspects of free radicals. Biochem Cell Biol 68:989–998
Bebianno MJ, Langston WJ (1991) Metallothionein induction by *Mytilus edulis* exposed to cadmium. Mar Biol 108:91–96
Bebianno MJ, Serafim MA (1998) Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus.* Sci Total Environ 214:123–131
Bebianno MJ, Langston WJ, Simkiss K (1992) Metallothionein induction in *Littorina littorea* (Mollusca: Prosobranchia) on exposure to cadmium. J Mar Biol Assoc UK 72:329–342
Bendoricchio G, Di Luzio M, Baschieri P, Capodaglio AG (1993) Diffuse pollution in the Lagoon of Venice. Water Sci Technol 28:69–78
Bordin G, McCourt J Cordeiro Raposo F, Rodriguez AR (1997) Metallothionein-like metalloproteins in the Baltic clam *Macoma baltica:* seasonal variation and induction upon metal exposure. Mar Biol 129:453–463
Bryan GW, Langston WJ, Hummerstone LG, Burt GR, Ho YB (1983) An assesment of the gasteropod, *Littorina littorea*, as an indicator of heavy-metal contamination in United Kingdom estuaries. J Mar Biol Assoc UK 63:327–345
Bryan GW, Potts GW, Forster GR (1977) Heavy metals in the gastropod mollusc *Haliotis tuberculata* (L.). J Mar Biol Assoc 57: 379–390
Canesi L, Ciacci C, Piccoli G, Stocchi V, Viarengo A, Gallo G (1998) *In vitro* and *in vivo* effects of heavy metals on mussel digestive gland hexokinase activity: the role of glutathione. Comp Biochem Physiol 120C:261–268
Canesi L, Viarengo A, Leonzio C, Filippelli M, Gallo G (1999) Heavy metals and glutathione metabolism in mussel tissues. Aquat Toxicol 46:67–76
Cantillo AY (1998) Comparison of results of Mussel Watch programs of the United States and France with Worldwide Mussel Watch studies. Mar Poll Bull 36:712–717
Casellato S, Salmaso C (2000) Strategies of heavy metal detoxification in *Tubificoides swirencowi* (Jarosh, 1948): an estuarine species widespread in northern Adriatic area. Biol Mar Medit 7:662–665
Cesari P, Pellizzato M (1985) Molluschi pervenuti in laguna di Venezia per apporti antropici volontari o casuali. Acclimazione di *Saccostrea commercialis* (Iredale & Roughely, 1933) e di *Tapes philippinarum* (Adams & Reeve, 1950). Boll Malac 21:237–274
Conners DE, Ringwood AH (2000) Effects of glutathione depletion on copper cytotoxicity in oysters (*Crassostrea virginica*). Aquat Toxicol 50:341–349
Cossons RP, Amiard-Triquet C, Amiard JC (1991) Metallothionein and detoxification. Is the use of the detoxification protein for MTs a language abuse? Water Air Soil Pollut 57–58:555–567
Couillard Y, Campbell PGC, Tessier A (1993) Response of metallothionein concentrations in a freshwater bivalve (*Anodonta grandis*) along an environmental cadmium gradient. Limnol Oceanog 38:299–313
de Zwaan A, Eertman RHM (1996) Anoxic or aerial survival of bivalves and other euryoxic invertebrates as a useful response to environmental stress—a comprehensive review. Comp Biochem Physiol 113C:299–312
Donazzolo R, Orio AA, Pavoni B, Perin G (1984) Heavy metals in sediments of Venice Lagoon. Oceanol Acta 7:25–32
Duquesne S, Flowers AE, Coll JC (1995) Preliminary evidence for a metallothionein-like heavy metal-binding protein in the tropical marine bivalve *Tridacna crocea*. Comp Biochem Physiol 112C:69–78
Freedman JH, Ciriolo MR, Peisach J (1989) The role of glutathione in copper metabolism and toxicity. J Biol Chem 264:5598–5605
Fridovich I (1978) The biology of oxygen radicals. Science 201:875–880
Hamza-Chaffai A, Amiard JC, Pellerin J, Joux L, Berthet B (2000) The potential use of metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal exposure. Comp Biochem Physiol 127C:185–197
Irato P, Santovito G, Piccinni E, Albergoni V (2001) Oxidative burst and metallothionein as a scavenger in macrophages. Immunol Cell Biol 79:251–254
Jeng M-S, Jeng W-L, Hung T-C, Yeh C-Y, Tseng R-J, Meng P-J, Han B-C (2000) Mussel Watch: a review of Cu and other metals in various marine organisms in Taiwan, 1991–98. Environ Poll 110: 207–215
Klaassen CD, Liu J, Choudhuri S (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu Rev Pharmacol Toxicol 39:267–94
Lash LH, Zalups RK (1996) Alterations in renal cellular glutathione metabolism after *in vivo* administration of a subtoxic dose of mercuric chloride. J Biochem Toxicol 11:1–9
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
Meister A, Anderson ME (1983) Glutathione. Ann Rev Biochem 52:711–760
Mouneyrac C, Amiard JC, Amiard-Triquet C (1998) Effects of natural factors (salinity and body weight) on cadmium, copper, zinc and metallothionein-like protein levels in resident populations of oysters *Crassostrea gigas* from a polluted estuary. Mar Ecol Prog Ser 162:125–135
O'Connor TP (1998) Mussel Watch results from 1986 to 1996. Mar Poll Bull 37:14–19
Pavicic J, Raspor B, Martincic D (1993) Quantitative determination of metallothionein-like proteins in mussels. Methodological approach and field evaluation. Mar Biol 115:435–444
Regoli F (1998) Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. Arch Environ Contam Toxicol 34:48– 63
Regoli F, Principato G (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat Toxicol 31:143–164
Rinaldi E (1972) Osservazioni relative al mollusco appartenente al genere *Anadora* vivente in Adriatico. Conchiglie 8:121–124
Roesijadi G (1992) Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat Toxicol 22:81–114
Scheuhammer AM, Cherian MG (1991) Quantification of metallothionein by silver saturation. Meth Enzymol 205:78–83
Temara A, Warnau M, Jangoux M, Dubois P (1997) Factors influencing the concentrations of heavy metals in the asteroid *Asterias rubens* L (Echinodermata). Sci Total Environ 203:51–63
Thomas P, Juedes MJ (1992) Influence of lead on the glutathione status of Atlantic croaker tissues. Aquat Toxicol 23:11–30
Thomas P, Wofford HW (1984) Effects of metals and organic compounds on hepatic glutathione, cysteine, and acid-soluble thiol levels in mullet (*Mugil cephalus* L.). Toxicol Appl Pharmacol 76:172–182
Thornalley PJ, Vašák M (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophys Acta 827:36–44
Viarengo A (1989) Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. CRC Crit Rev Aquat Sci 1:295–317