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REVIEW
Biomarkers in *Ruditapes decussatus*: a potential bioindicator species

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The clam *Ruditapes decussatus* is distributed worldwide and due to its ecological and economical interest has been proposed as a bioindicator in areas where mussels are not available. The accumulation of several anthropogenic compounds in their tissues suggests that they possess mechanisms that allow them to cope with the toxic effects of these contaminants. Besides pollutant uptake, the use of biomarkers is pointed out in this paper since it is a promising approach to monitor the effect of these contaminants in the marine environment. Biomarkers complement the information of the direct chemical characterization of different types of contaminants. Therefore, the aim of this paper is to review the role of several biomarkers: (metallothioneins (MT)), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx) (total and selenium-dependent), lipid peroxidation (measured as MDA, one of the final products of lipid peroxidation), glutathione S-transferase (GST) and acetylcholinesterase (AChE), measured in different tissues of the clam *R. decussatus*, in laboratory conditions and under various environmental stresses, in two ecosystems (Ria Formosa lagoon, Portugal) and Bizerta lagoon (Tunisia) in a perspective of a multibiomarker approach to assess environmental changes. Experiment and field studies are in good agreement since MT levels, especially in the gills, the first target tissue of these contaminants, can be used as biomarker of exposure to Cd. GPx and MDA may also be determined in this respect. AChE activity is inhibited by pesticide and, to a less extent, by metal exposure in the gills and whole soft body of clams. However, the induction of GST isoforms experimentally demonstrated is not observed in the field because only global GST activity was determined. The whole set of results opens new research perspectives for the use of this species to assess the effect of mixtures of pollutants in the aquatic environment.

Keywords: biomarkers, *Ruditapes decussatus*, metallothioneins, antioxidant enzymes, GST, AChE, endocrine disruption, Ria Formosa, Bizerta Lagoon.

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Introduction

The clam *Ruditapes decussatus* is widely distributed and has an important ecological and economical interest (ecosystem balance, important place in the trophic chain of lagoons and marine systems, fisheries and shellfish cultures). This species is distributed along the Atlantic coast, from Norway (61°N) to Congo

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(12°N). In the British Islands it is only present in the West and South coasts and in the North Sea only in Norway and Denmark. It is also found in the English Channel (from Southampton to Le Havre) and along the Atlantic Ocean through the Mediterranean Sea as well as in the Red Sea where this species migrated through the Suez Canal (Parache 1982).

Due to its economic importance it is heavily harvested in many countries, particularly in France, Portugal and Tunisia. In France, the Thau lagoon is the main site of clam production which reached 300 tons/year for *R. decussatus*; the production of *Ruditapes philippinarum* has been generally higher reaching 400 tons/year since the 90s until today (http://www.ifremer.fr). In Portugal, where there is a long tradition in bivalve culture, the production of *R. decussatus* is an important economic activity, mainly in the Ria Formosa lagoon in the South Coast of Portugal, with 1500 clam farms occupying a total of 1000 ha. Clam production reached around 8000 tons in 1993, 90% of which was exported, but decreased to about 3000 tons/year as a result of the deterioration of the water quality of the lagoon (Bebianno 1995). In Tunisia, *R. decussatus* is present along the coast but is especially abundant in the Bizerta and Tunis lagoons and in the Gulf of Gabès. The production in the Bizerta lagoon reaches 4.6 tons/year (mean values from 1989 to 1998, some reduction in production occurred in 1994 and 1997 due to microbial pollution) (Dellali 2001).

R. decussatus is a burrowing syphonate bivalve mollusc that lives in sand and muddy-sand sediments in the inter-tidal level of bays, estuaries and coastal lagoons. This mollusc dwells into the sediments, reaching a maximum depth of 10–12 cm, and moves with a speed of around 6 m per month. As many lamellibranches, *R. decussatus* can stand great changes in environmental conditions. This species is a primary consumer and is used for human consumption. Besides its economic value, these clams are rich in polyunsaturated fatty acids, particularly in eicosapentanoic acid which is investigated mainly for human health purposes to prevent cardio-vascular diseases (Beninger and Stephan 1985).

R. decussatus has two syphons, one of which is the inhalant syphon which allows filtration of water and suspended particles which is why it is considered to be a suspension-feeder and the other used for water and waste extraction. Clams have separated sexes without sexual dimorphism and the sexual cycle generally begins in March with external fecundation; two periods of reproduction may occur—one in June/July and another in September/October. Therefore this species has been selected as a bioindicator in areas where mussels or oysters are not available (Henry 1987, Serafim and Bebianno 2001).

Accumulation of several anthropogenic compounds, such as metals (Cd, Cu, Fe, Hg, Mn, Pb and Zn), organo-metallic compounds (tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT), hydrocarbons, radio-isotopes and herbicides in the tissues of *R. decussatus*, reflect the gradient of contamination in the environment (Coelho *et al*. 2002a,c; Bebianno and Serafim 2003, Smaoui-Damak *et al*. 2003) and consequently they become unsuitable for human consumption. Uptake experiments with *R. decussatus* exposed to a wide range of metals (Cd, Cu and Zn) revealed that metals are accumulated in different tissues (whole soft tissues, gills, digestive gland and remaining tissues) and uptake rate is metal concentration- and tissue-dependent as can be seen in table 1. This suggests that clam possess mechanisms, probably

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Table 1. Metal uptake rate in different tissues of *R. decussatus* after different time periods.

Tissues	Subcellular fractions	Metal	Conc. µg/l	Time days	Temp. °C	Uptake rate µg/g d	References
Gills	Total	Cd	4	28		0.5	Géret *et al*. 2002b
Gills	Total	Cd	40	28		2.5	Géret *et al*. 2002b
Gills	Total	Cd	100	28		4.2	Géret *et al*. 2002b
Gills	Total	Cd	100	30		3.6	Bebianno *et al*. 1994
Gills	Total	Cd	100	30		4.1	Bebianno and Serafim 1998
Gills	Heat-treated cytosol	Cd	100	30		2.4	Bebianno and Serafim 1998
Gills	Unsoluble fraction	Cd	100	30		1.2	Bebianno and Serafim 1998
Gills	Total	Cd	500	14	18±1	29	Roméo and Gnassia-Barelli 1995
Gills	Total	Cd	500	2	18±1	7.5	Roméo and Gnassia-Barelli 1995
Digestive gland	Total	Cd	100	30		5.8	Bebianno *et al*. 1994
Digestive gland	Total	Cd	500	14	18±1	21	Roméo and Gnassia-Barelli 1995
Whole soft tissues	Total	Cd	100	30		1.2	Bebianno *et al*. 1994
Whole soft tissues	Total	Cd	200		15	2.6	Vicente *et al*. 1988
Whole soft tissues	Total	Cd	200		26	13.8	Vicente *et al*. 1988
Whole soft tissues	Total	Cd	400	30		6.0	Bebianno *et al*. 1993
Whole soft tissues	Heat-treated cytosol	Cd	400	30	15	2.1	Bebianno *et al*. 1993
Whole soft tissues	HMW	Cd	400	30	15	1.0	Bebianno *et al*. 1993
Whole soft tissues	Total	Cd	500	2	18±1	6.36	Roméo and Gnassia-Barelli 1995
Whole soft tissues	Total	Cu	10	20	20±1	1.95*	Sobral and Widdows, 1997
Whole soft tissues	Total	Cu	10	20	20±1	1.54	Sobral and Widdows, 1997
Whole soft tissues	Total	Cu	150	2	18±1	2.95	Roméo and Gnassia-Barelli 1995
Gills	Total	Cu	150	2	18±1	1.24	Roméo and Gnassia-Barelli 1995
Digestive gland	Total	Cu	25	28		12.8	Géret *et al*. 2002a
Digestive gland	Total	Cu	150	7	18±1	5.2	Roméo and Gnassia-Barelli 1995
Remaining tissues	Total	Cu	150	7	18±1	1.1	Roméo and Gnassia-Barelli 1995
Gills	Total	Zn	1000	2	18±1	22.5	Roméo and Gnassia-Barelli 1995

*1st 24 hours.

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related to their feeding habit, that allow them to cope with the toxic effects of these contaminants. Like many other molluscs, *R. decussatus* has evolved a number of cellular responses for accumulation, regulation, excretion and immobilisation of these contaminants. Some of these responses known as biomarkers include: proteins such as metallothioneins (MT), enzymes such as glutathione S-transferases (GST) (soluble and insoluble forms), superoxide dismutases (SOD) (among them soluble Cu/Zn-SOD and unsoluble forms Fe- and Mn-SOD), glutathione peroxidases (GPX) (total and selenium-dependent), catalase (CAT), acetylcholinesterase (AChE) or products resulting from lipid peroxidation (LPO) (malonedialdehyde: MDA), etc. Parameters such as the condition index or the scope for growth (Sobral and Widows 1997) always give an indication of the physiological status of the animals and are very useful when considering biochemical markers. Endocrine disruption may also be caused by pollutants and corresponding biomarkers are being considered. This paper is a review of biomarkers (MT, GST, SOD, CAT, total and Selenium-dependent GPX, LPO, AChE inhibition) measured in different conditions (laboratory and field), under various environmental stresses, in the clam *R. decussatus*. EROD (ethoxyresorufin *O*-deethylase), considered as a defense biomarker, is not discussed in this paper since it does not give a satisfactory response in molluscs (Cajaraville *et al*. 2002). Pollutant data is included whenever available because it complements the response of biomarkers.

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Biomarkers

The use of biomarkers is a promising approach to monitoring the contamination of marine environment because they complement the information obtained from different types of contaminants. Usually defined as quantitative biochemical changes due to chemical pollutants measurable in biological media such as cells, tissues or body fluids (McCarthy and Shugart 1990), biomarkers respond to either (or both) exposure to and/or doses of xenobiotic substances. They constitute an early warning system of chemical stress in organisms. Biomarkers are different in their significance and terminology (i.e. biomarkers of exposure, effects, stress, alteration and susceptibility). Although a causal relationship must exist between exposure to contaminants and biological effects, such a causal link does not necessarily hold for all biomarkers, except if they share a common metabolic pathway (e.g. protein metabolism, see Stegeman *et al*. 1992). In order to categorize biomarkers according to their type of response, De Lafontaine *et al*. (2000) proposed to refer to them as biomarkers of 'defence' (e.g. the induction of MT or EROD response to specific classes of contaminants: the first to metals and the second to coplanar organic chemicals, particularly poly aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) is indeed a defence reaction by an organism) or as biomarkers of 'damage' (e.g. DNA strand breaks, AChE inhibition, LPO associated with oxidative perturbations, particularly within the polyunsaturated lipid-rich cell membranes (Chan 1987, Stegeman *et al*. 1992) or imposex induction). Non-specific biomarkers (such as catalase activity) are also important because they indicate that an organism was submitted to a particular environmental stress, for instance, to oxidative stress.

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Lagadic *et al.* (1997) underlined the importance of measuring several biomarkers at the same time, in the same organism, to evaluate the effects of pollutants on individuals. This multiparametric approach using different and/or complementary biomarkers will enable assessment of the effects of different contaminants in the aquatic environment.

Metallothioneins (MTs)

Metallothioneins (MTs) discovered, half a century ago, in mammals (horse kidney: Margoshes and Vallee 1957) and, more than 25 years ago, in marine molluscs (oysters, mussels and limpets, see Casterline and Yip 1975 and Noël-Lambot 1976), are a family of ubiquitous heat-stable, low molecular weight proteins with a high cysteine and metal content and two characteristic metal-thiolate clusters formed by the sulphur atoms of all cysteine groups of the protein (Dabrio *et al*. 2002). MTs are induced when organisms are exposed to high metal concentrations and are therefore considered a biomarker of metal exposure. In this case the term "biomarker of defence" is fully justified. Different stresses other than metals also induce MTs, nevertheless, this induction is less important. In molluscs, and particularly in *R. decussatus*, polymorphism is observed with the possibility of MT isoforms having different roles.

MTs are induced in the whole soft tissues, gills, digestive gland and remaining tissues of *R. decussatus* after exposure to excess of essential (Cu, Zn) or toxic metals (Cd, Ag) (Bebianno *et al*. 1993, 1994, Bebianno and Serafim 1998, Roméo and Gnassia-Barelli 1995). Chromatographic elution profiles in the whole soft tissues, gills and digestive gland, reveal that more than 80% of total Cd is bound to two MT isoforms (molecular weight around 10 and 20 KDa, named MT-10 and MT-20, respectively) induced after Cd exposure, confirming the existence of polymorphism in this species (Bebianno *et al*. 1993, Hamza-Chaffai *et al*. 2000). Four MT isoforms (CdMT-1, CdMT-2, CdMT-3 and CdMT-4) with an apparent molecular weight of 13,700 Da after gel filtration chromatography, or 7328 Da on calibrated gel SDS-PAGE electrophoresis, were identified in the digestive gland of *R. decussatus* after Cd exposure (100 $\mu g\ l^{-1}$ for 20 days). This discrepancy is largely reported in well-characterised MTs. Two of these isoforms were sequenced: CdMT-1 through residue 19 that included glycine at the NH_2-terminal and two Cys-XY-Cys sequences characteristic of MT and CdMT-2 through residue 5, which is probably an isoform of CdMT-1 in which the aspartic acid is substituted by a glutamic acid (Simes *et al*. 2003). The absence of methionine in these sequences is consistent with other aquatic invertebrate MTs (Roesijadi *et al*. 1989, Mackay *et al*. 1993) indicating that in this species, MTs are subject to NH_2-terminal modification that do not occur in mammalian MTs. A comparison between *R. decussatus* MT isoforms with those of other molluscs showed a higher degree of similarity with oysters (77% identity for *Crassostrea gigas* and 59% for *Crassostrea virginica*) than mussels (41% for *M. edulis* and 50% for *Perna viridis*) (Unger *et al*. 1991, Brouwer *et al*. 1989). Therefore it was concluded that *R. decussatus* MT belonged to class I MT, and recently to the mollusc MT family (Simes *et al*. 2003).

MT concentrations (MT-10 and MT-20), measured by differential pulse polarography (DPP), ELISA and UV spectrophotometry in different tissues, are

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Table 2. MT concentrations in different tissues of *R. decussatus* (experimental and field conditions).

Condition	Method	MT (mg g^{-1} ww)	References
Gills			
Bizerta: J (figure 1C)	ELISA	0.42 ± 0.08	Moraga *et al.* 2002
Bizerta: F (figure 1C)	ELISA	0.45 ± 0.08	Moraga *et al*. 2002
Bizerta:E (figure 1C)	ELISA	0.49 ± 0.08	Moraga *et al*. 2002
Cd (250 μg l^{-1})	Spectrophotometry	0.045 ± 0.001 0.042 ± 0.001 (exp)	Roméo and Gnassia-Barelli 1995
Cd (4 μg l^{-1})	DPP	0.50 ± 0.06 0.51 ± 0.06 (exp)	Géret *et al*. 2002b
Cd (400 μg l^{-1})	DPP	2–4*	Bebianno *et al*. 1993
Cu (75 μg l^{-1})	Spectrophotometry	0.058 ± 0.06	Roméo and Gnassia-Barelli 1995
Ria Formosa: 8	DPP	2.2 ± 0.4*	Bebianno and Serafim 1998
Ria Formosa: 8	DPP	1.03 ± 0.22*	Bebianno *et al.* 1994, 2000
Ria Formosa: 8 (figure 1a)	DPP	0.52 ± 0.05	Géret *et al*. 2003
Ria Formosa: 1 (figure 1a)	DPP	5.35 ± 0.25*	Bebianno and Serafim 2003
Ria Formosa: 2 (figure 1a)	DPP	0.60 ± 0.09	Géret et *al*. 2003
Ria Formosa: 3 (figure 1a)	DPP	0.54 ± 0.09	Géret *et al*. 2003
Ria Formosa: 10 (figure 1a)	DPP	0.50 ± 0.10	Géret *et al*. 2003
Zn (100, 1000 μg l^{-1})	DPP	0.50 ± 0.03	Géret et Bebianno 2004
Digestive gland			
Bizerta: J (figure 1b)	ELISA	0.78 ± 0.25	Moraga *et al*. 2002
Bizerta: F (figure 1b)	ELISA	0.91 ± 0.23	Moraga *et al*. 2002
Bizerta: E (figure 1b)	ELISA	0.74 ± 0.08	Moraga *et al*. 2002
Cd (250 μg l^{-1})	Spectrophotometry	0.14 ± 0.04	Roméo and Gnassia-Barelli 1995
Controls	DPP	1.41 ± 0.09	Géret *et al*. 2004
Controls	DPP	0.52 ± 0.17	Hamza-Chaffai *et al.* 1998
Cu (75 μg l^{-1})	DPP	0.62 ± 0.13	Hamza-Chaffai *et al*. 1998
Cu (75 μg l^{-1})	Spectrophotometry	0.12 ± 0.02	Roméo and Gnassia-Barelli 1995
Cu + lindane	DPP	0.42 ± 0.07	Hamza-Chaffai *et al*. 1998
Gulf of Gabès	DPP	2.08 ± 0.89	Hamza-Chaffai *et al*. 2001
Gulf of Gabès (Sidi Mansour)	DPP	2.12 ± 0.59	Hamza-Chaffai *et al*. 2003
Gulf of Gabès (Sidi Mansour) t = 62 d transplant exp.	DPP	1.43 ± 0.30	Hamza-Chaffai *et al*. 2003
Gulf of Gabès (Gargour) t = 62 d transplant exp.	DPP	3.29 ± 1.19	Hamza-Chaffai *et al*. 2003
Lindane	DPP	0.53 ± 0.16	Hamza-Chaffai *et al*. 1998
Ria Formosa: 1–7 (figure 1a)	DPP	7.3 ± 0.32*	Bebianno and Serafim 2003
Ria Formosa: 8	DPP	2.45 ± 0.38*	Bebianno *et al*. 1994, 2000
Ria Formosa: 8 (figure 1b)	DPP	1.60 ± 0.24	Géret *et al*. 2003

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Table 2 (*Continued*)

Condition	Method	MT (mg g^{-1} ww)	References
Ria Formosa 12	DPP	$10.5 \pm 0.72^{\star 1}$ $12.8 \pm 2.8^{\star 2}$	Serafim and Bebianno 2001
Ria Formosa: 2 (figure 1a)	DPP	1.88 ± 0.10	Géret *et al.* 2003
Ria Formosa: 3 (figure 1a)	DPP	2.12 ± 0.12	Géret *et al*. 2003
Ria Formosa: 10 (figure 1a)	DPP	2.11 ± 0.30	Géret *et al*. 2003
Zn (1000 μg l^{-1}) T = 28 d experimental	DPP	1.55 ± 0.15	Géret et Bebianno 2004
Remaining tissues			
Ria Formosa	DPP	$1.96 \pm 0.72^{\star}$	Bebianno *et al*. 1994, 2000
Ria Formosa: 1–7 (figure 1a)	DPP	$2.82 \pm 0.27^{\star}$	Bebianno and Serafim 2003
Whole soft tissues			
Ria Formosa	DPP	$2.05 \pm 0.41^{\star}$	Bebianno *et al*. 1993

*mg g^{-1} dw; 1-males; 2-females.

present in table 2. MT concentrations in the whole clam (2.05 ± 0.41 mg g^{-1} dw) are similar to the proposed basal levels for MT in marine bivalves (2 mg g^{-1} dw) (Langston *et al*. 1998). Data in table 2 also demonstrates that MT concentrations determined by the spectrophotometric method is under-evaluated when compared with those determined by DPP and ELISA methods. This was already underlined by Roméo *et al*. (1997) in the liver of sea bass treated with copper, where MT was analysed by both DPP and the spectrophotometric method established by Viarengo *et al*. (1997).

Several laboratory experiments with *R. decussatus* exposed to Cd (100, 250 and 400 μg l^{-1}), Cu (75 μg l^{-1}) and lindane (34.5 μg l^{-1}) were carried out to assess MT induction. In unexposed clams, MT tissue distribution ranks from digestive gland > remaining tissues > gills = whole soft tissues. In metal exposed clams, MT induction is tissue- and metal-dependent.

Effect of Cd

Subcellular fractionation of Cd accumulated in clam tissues, indicates that the major part of Cd (35% in the whole soft tissues, 65–84% in the gills and 51–86% in the digestive gland) is associated with the heat-treated soluble proteins, where the metal binds to MT (Bebianno *et al*. 1993, 1994, Bebianno and Serafim 1998, Roméo and Gnassia-Barelli 1995). The induction of *de novo* MT in the whole soft tissues after Cd exposure (400 μg l^{-1}) is relatively low (Bebianno *et al*. 1993) in contrast with 3–4-fold MT induction in mussels—*Mytilus edulis* and *Mytilus galloprovincialis*, exposed to the same Cd concentration (Bebianno and Langston 1991, 1992). However, there is a net induction of MT synthesis in the gills (4-fold, 0.04 mg g^{-1} d^{-1}), digestive gland and remaining tissues (3-fold) after Cd exposure (100 and 400 μg l^{-1}) but the sequence of tissue MT concentrations remains unchanged. Depuration of Cd-exposed clams, results in a decrease of MT levels in different tissues higher in the other tissues than the gills, suggesting that MT turnover is tissue-dependent and faster than Cd (Bebianno *et al*. 1994,

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Bebianno *et al*. 2000). It seems that after binding to MT, Cd may therefore be displaced to the particulate fraction, mainly in membrane and intracellular granules (Roméo and Gnassia-Barelli 1995).

Effect of Cu

Similar MT induction is also observed in the gills after exposure to Cu (75 μg l^{-1}) confirming the potential role of MT as a biomarker of Cd and Cu exposure in clam gills (Bebianno *et al*. 1993, 1994, Roméo and Gnassia-Barelli 1995). In the gills and digestive gland, Cu subcellular distribution is, like Cd, also associated with the heat stable fraction ($>50\%$ in the digestive gland). However, in the gills of clams exposed to Cu (25 and 75 μg l^{-1} for 7 days), the excess of Cu triggers the induction of MT synthesis (58 ± 6 μg g^{-1} ww) confirming the potential role of MT in *R. decussatus* gills to assess the effects of dissolved and particulate metals in this species (Géret *et al*. 2002a, Hamza-Chaffaï *et al*. 1998). When *R. decussatus* is exposed to lindane (34.5 μg l^{-1}) separately or in combination with Cu (75 μg l^{-1}), MT levels in the digestive gland, determined by DPP, remained unchanged (table 2), suggesting that lindane does not induce MT (Hamza-Chaffaï *et al*. 1998). Therefore, the determination of MT in the gills seems preferable.

Antioxidant enzyme activities and lipid peroxidation

The cellular defence systems ("biomarkers of defence") directed against the toxicity of reactive oxygen species (ROS) include the activity of certain enzymes, namely SOD, CAT, and GPx (total and Se-dependent). SODs (Cu/Zn-SOD in the cytosol and Mn-SOD in the mitochondria) catalyze the dismutation of superoxide anion to hydrogen peroxide. CAT reduces the hydrogen peroxide to water, while glutathione peroxidases detoxify peroxides (hydrogen: Se-dependent GPx) or organoperoxides to stable alcohols using reduced glutathione (GSH) as a source of reducing equivalents to produce oxidized glutathione (GSSG) and therefore protect the cells from free radical damage, particularly lipid peroxidation. The induction of antioxidant systems reflects the adaptation or compensatory reaction to ROS formation and a deficiency in these mechanisms indicates a toxic effect of ROS, and the organisms become more sensitive to oxidative stress. Lipid peroxidation (considered as a "damage biomarker") may then occur. Metals such as Cd, Co, Cu, Hg, Ni, Pb, Fe, Sn, and V are known to stimulate peroxidation of membrane lipids that results in the production of lipid radicals and in the formation of a complex mixture of lipid degradation products (MDA and other aldehydes) extremely toxic for the cells, due to their high affinity for thiol and amino groups of peptides, enzymes, and nucleic acids (Knight and Voorhees 1990, Viarengo 1989). Elevated concentrations of MDA are an expression of lipid peroxidation (Sunderman 1987) and may be used as a biomarker since its biological significance is partly understood (Thomas 1990), although factors such as nutritional status and age can influence MDA formation (Wofford and Thomas 1988, Ribera *et al.* 1989, Viarengo *et al*. 1991).

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Effect of Cd

Although being a non-redox metal, and therefore unlikely to participate in Fenton-type reactions, Cd is known to enhance the formation of ROS and promote cellular oxidative stress, and therefore stimulate the lipid peroxidation process through oxidation of polyunsaturated fatty acids (Géret *et al*. 2002b). Cd is involved in changes in mitochondrial metabolism, membrane permeability, inhibition of oxidative phosphorylation and protein synthesis by binding to nucleic acid bases and phosporylate groups, affecting their structures (Géret *et al*. 2002b).

Laboratory studies conducted to evaluate the impact of Cd on the antioxidant-prooxidant systems in *R. decussates*, reveals changes on SOD, CAT and GPx activities. When clams are exposed to Cd (4, 40 and 100 $\mu g\,l^{-1}$ for 28 days) (Géret *et al*. 2002b), this metal stimulates the increase of O_2^- (through the replacement of Ca in the conversion of xanthine dehydrogenase to xanthine oxidase by calcain. Xantine oxidase then catalyses the oxidation of xanthine producing O_2^-) and the induction of SOD transforms superoxide anion into hydrogen peroxide which then activates CAT. One of the more striking effects of Cd in the gills is the exponential decrease of total GPx. Cd has strong affinity to form complexes with reduced glutathione used by GPx to remove both hydrogen peroxides and organic hydroperoxides (Regoli and Principato 1995). Cd also stimulates lipid peroxidation in the gills of *R. decussatus* (Géret *et al*. 2002b). An increase in basal lipid peroxidation is also observed in the supernatant of the gills of the clam *R. decussatus* incubated *in vitro* with 500 μg Cd ml^{-1} for 20 minutes (Roméo and Gnassia-Barelli 1997). Therefore GPx and LPO may be used, together with MT, as biomarkers to assess the effect of Cd in the gills (Géret *et al*. 2002b).

Effect of Cu

The induction of Cu/Zn-SOD and Mn-SOD was followed in the tissues of *R. decussatus* exposed to three environmental realistic Cu concentrations (0.5, 2.5 and 25 $\mu g\,l^{-1}$). SOD is predominantly detected in the cytosolic fraction (Cu/Zn-SOD), either in the gills or the digestive gland ($>70\%$ in the gills and $>85\%$ in the digestive gland). Compared to the cytosolic SOD, the mitochondrial SOD (Mn-SOD) activity in the gills and digestive gland is lower, but higher in the gills than in the digestive gland (Géret *et al.* 2002a,b, 2003, Géret and Bebianno 2004).

As many isoforms of SODs co-exist, partial *R. decussatus* cDNA encoding Cu/Zn-SOD, was isolated and amplified by PCR with degenerated oligonucleotide primers (derived from conserved amino acid sequence in Cu/Zn-SOD from several other organisms) with a length of 510 bp (GenBank AY377969) corresponding to the 5'end plus 390 bp of ORF sequence. The deduced amino acid sequence revealed high similarities ($>60\%$) with other molluscs (*Dreissena polymorpha, Bathymodiolus azoricus*), amphibian and mammalian Cu/Zn-SODs. Further comparison of the clam Cu/Zn-SOD with mammalian Cu/Zn-SOD showed that several residues maintaining the active site geometry are conserved: Gly-45, Gly-62, Pro-75, Gly-83 and metal binding sites His-47, 49, 64, 121 for Cu and His-64, 72, 81 and Asp-84 for Zn and Cys-58 involved in the intra chain disulfide bridge with Cys-147 among *R. decussatus* and other Cu/Zn SOD mollusc sequences (Géret *et al*. 2004).

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The response of antioxidant enzyme activities SOD, CAT, GPx (total Se-GPx), MT and MDA concentrations in the gills of *R. decussates* after Cu exposure (0.5, 2.5 and 25 $\mu g\, l^{-1}$) revealed a significant inhibition effect on CAT and GPx (total and Se-dependent) that was Cu-dependent. The Cu^{2+} ion reacts with ROOH while Cu^{+} reacts with H_2O_2, which is no longer available for CAT or GPx (Géret *et al.* 2002a). Meanwhile, a Cu-dependent activation of SOD in the cytosol occurs while mitochondrial SOD decreases showing that Cu is reactive in controlling Cu/Zn-SOD activity. The increase in lipid peroxidation in the gills of the clams, is also Cu and time-dependent (Géret *et al*. 2002a). Similar results were obtained for the same species exposed to Cu (30 $\mu g\, l^{-1}$ for nine days) (Roméo and Gnassia-Barelli 1997) showing that Cu induces an imbalance of oxygen metabolism.

Effect of Zn

As regards the impact of Zn in anti- and pro-oxidant systems, this metal has both catalytic and structural roles in enzymes and its antioxidant properties are independent of its metalloenzymatic activity (Maret 2000, Powell 2000). Zn is redox inert and, like Cd, does not interact directly with an antioxidant species but rather exerts its effects in an independent manner. Zn stimulates the activity of SOD and several other enzymes and is vital for the stabilization of DNA. The percentage of Cu/Zn-SOD and Mn-SOD found in the gills and digestive gland of *R. decussatus* is not disrupted by Zn exposure (100 and 1000 $\mu g\, l^{-1}$ for 28 days) (Géret and Bebianno 2004). In the same way, CAT activity in the digestive gland is 3-fold lower than that of the gills; therefore the effect of Zn exposure in *R. decussatus* depends not only on the tissue but also on the Zn concentration present (Géret and Bebianno 2004).

Glutathione S-transferases (GSTs)

Glutathione S-transferases (GSTs) are a multigene family of enzymes involved in metabolic processes such as bilirubin transport, and in detoxification processes by the conjugation of electrophile compounds with glutathione. A large number of isoforms have been described in mammalian organism (13 isoforms coexist in rats). Several criteria as kinetic or immunological properties and gene sequences have permitted their classification into nine classes (Sheehan *et al*. 2001). All native isoforms are constituted by two subunits with molecular weights between 20 and 35 KDa. The GSTs catalyze the conjugation of some molecules that they recognize according to their specificity with a ubiquitous molecule, such as the glutathione. The three-dimensional structure shows two distinct binding active sites for each subunit—the GSH site and the site for conjugation. The variability of xenobiotic fixation site between each isoform and the numbers of isoforms explain the ability of conjugation of a large number of compounds. In *R. decussatus*, at least seven GST isoforms have been described that catalyze the conjugation of the substrate, 1, chloro-2,4 dinitrobenzene (CDNB) with GSH; this *in vitro* co-substrate is currently used for the evaluation of the level of whole GST isoforms as biochemical biomarker. Among these isoforms, six are homodimeric and one heterodimeric, specifically expressed in certain tissues (Hoarau *et al*. 2002). In the gills, at least three subunits have been identified, two in the digestive gland and only one in both

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tissues (Hoarau *et al*. 2004). Mammalian criteria are not relevant enough to allow the classification of *R. decussatus* GSTs suggesting a putative mollusc GST class (Fitzpatrick *et al*. 1995, Hoarau *et al*. 2002).

In *R. decussatus* the GST-CDNB activity (table 3) ranged between 0.33 and 0.847 μmol min^{-1} mg $protein^{-1}$ in the whole organism, and follows the sequence gill (3.2–5 μmol min^{-1} mg $protein^{-1}$) > digestive gland (1.4–2 μmol min^{-1} mg $protein^{-1}$) (Hoarau *et al*. 2001, 2002, 2004). The exposure to 0.14 μM of pp'DDE (2,2-bis-(p-chlorophenyl)-1,1-dichlorethylene) and 0.14 μM of methoxychlor increases the GST-CDNB activity in gill extracts (more than 75% in comparison with controls) (Hoarau *et al*. 2004), whereas 25 μM of imidazole or 0.5 or 1 μM benzo[a] pyrene has no effect on GST activity (Hoarau *et al.* 2001). Similar results were obtained with pp'DDE in *M. galloprovincialis* (Khessiba *et al*. 2001). GST activites are much lower in individuals from the field then those kept in the laboratory.

The immunoblot analysis of the effects of several PCBs in *R. decussatus* shows differential induction of GST isoforms as a function of the treatment (Hoarau *et al.* 2001). However, the molecular weights of the GSTs subunits are so close that the distinction between them has to be performed using other relevant characteristics than the molecular weight and the isoelectric points. The comparative analyses of the GST isoform pattern after treatment by pesticides compounds (pp'DDE, methoxychlor and imidazole) isolated an isoform of pI 5.2 over-expressed only in pp'DDE- and methoxychlor-treated animals. The induction of GST isoforms could be used as a biomarker of pesticide exposure together with the global evaluation of the CDNB activity to increase the sensitivity and the specificity of this "biomarker of defence".

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Acetylcholinesterase (AChE)

The inhibition of acetylcholinesterase (AChE) is linked directly with the mechanism of toxic action (irreversible or reversible binding to esterasic site, and potentiation of cholinergic effects) of organophosphorus and carbamate compounds used as pesticides. These chemicals are extremely toxic for a short period of time after application and are less hazardous to the environment than organochlorine chemicals due to their short half-life and short persistence in animal tissues. Nevertheless, they disrupt neurotransmitter processes in the central nervous system and therefore inhibit the AChE enzyme responsible for hydrolizing acetylcholine into choline and acetic acid. Cholinesterases are highly polymorphic enzymes in most species (Bocquené *et al*. 1997a). The number of genes coding for cholinesterases varies between species. Molluscs proved to be relatively insensitive to inhibitors when compared to vertebrate species, but some studies demonstrate the presence of isoforms of cholinesterases in mussels *M. edulis* and *M. galloprovincialis* (Mora *et al*. 1999) and oysters *C. gigas* (Bocquené *et al.* 1997b).

In laboratory experiments, described above (see Cu and MT responses), the AChE activity was measured in the gills and the remaining tissues of *R. decussatus* exposed to lindane or Cu, or to a mixture of both for five days. Lindane is not an organophosphorus or carbamate compound; nevertheless it acts as other neurotoxins (cyclodienes, toxaphene, picrotoxinin) through the nerve membrane. A

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Table 3. Biomarker levels (expressed per mg protein) in the gills, digestive gland (D.G.) and whole soft tissues of *Ruditapes decussatus* according to their origin.

Tissue	Condition (Lab. or field)	CAT activity $\mu mol\ min^{-1}\ mg^{-1}$	GST activity $\mu mol\ min^{-1}\ mg^{-1}$	AchE activity $nmol\ min^{-1}\ mg^{-1}$	MDA $nmol\ min^{-1}\ mg^{-1}$	Clam origin	References
Gills	Lab.	91.0 ± 5.0			0.381 ± 0.038	Ria Formosa (Portugal)	1
			3.20 ± 0.15			Thau lagoon	2
				4.87 ± 1.00		Thau lagoon	3
					0.643 + 0.09	Thau lagoon	4
D.G.	Lab.	85.6 ± 7.8			0.840 ± 0.058	Ria Formosa (Portugal)	1
			1.40 ± 0.32				2
				5.38 ± 2.44*			3
					0.656 ± 0.09	Thau lagoon	4
Whole soft tissues	Field						
	Site J	109.1 ± 0.01	0.36 ± 0.04	3.80 ± 0.70		Bizerta lagoon (Tunisia)	5
	Site F	115.5 ± 28.0	0.44 ± 0.04	3.14 ± 0.91			
	Site A	147.3 ± 13.0	0.33 ± 0.05	2.70 ± 0.74			

(*AChE determined in the remaining tissues).

1) Géret and Bebianno (2004); 2) Hoarau *et al.* (2004); 3) Hamza-Chaffaï *et al*. (1998); 4) Roméo and Gnassia-Barelli (1997); 5) Dellali *et al*. (2004).

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significant inhibition of AChE activity in both the gills and the remaining tissues occurs in the three groups of treated animals. The inhibition is greater in the case of lindane and copper and lindane treatments although no synergistic effect was noted (Hamza-Chaffaï *et al*. 1998). Heavy metals, and particularly Cu, have a pronounced preference for sulphur donor groups, and may therefore inhibit this enzyme by binding to SH residues of proteins, like those of MT (Viarengo 1989). The effect observed in the tissues of *R. decussatus* indicates that lindane may act on neurotransmission in molluscs in a way involving GABA receptors which function as Cl^- channels (Walker *et al.* 1996) or other ionic channels. The AChE activities determined for both *R. decussatus* (Hamza-Chaffaï *et al*. 1998) and *R. philippinarum* (Le Bris *et al.* 1995) are low (ca 80 U·mg protein^{-1} i.e. ca 5 μmol·min^{-1} mg protein^{-1}) and comparable between the two species and with field data from non-impacted areas (see below).

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Heat shock protein induction in relation with exposure to organotin compounds

Effects of organotin compounds (one of the most toxic compounds that man deliberately introduced into the aquatic environment) include shell malformation in oysters, imposex in neogastropods, reduced scope for growth and population decline in a variety of molluscs. Organotin compounds, particularly TBT, may become bioavailable to biota through a combination of different pathways (water, sediment and food). The effects of TBT on clam veliger larvae exposed to a range of TBT concentrations (25, 50, 75 and 100 ng Sn l^{-1}) in water, severely affects larval growth (reduction 3–6-fold in growth rate) and development (no further than D-larvae) (Coelho *et al*. 2001). Moreover, adult clams exposed to environmentally realistic TBT levels in water (100 ng Sn l^{-1}), sediments (0.8 ng Sn g^{-1} dw), separately or in combination, and to equivalent levels in food (^{14}C-TBT labelled phytoplankton *Isochrisis galbana)* revealed that the accumulation of TBT from water is the dominant pathway in this species (Coelho *et al*. 2002a,b). TBT tissue distribution also reflects these pathways. TBT accumulation from water is dominated by the gills, while absorption of TBT from sediments (and food) is dominated by the digestive gland at least initially (uptake rate of 6.5 ng Sn g^{-1} day^{-1}) and transported afterwards to the other tissues (Coelho *et al*. 2002b). Therefore, gills and digestive tract will accumulate TBT, preferentially, from water and food respectively (Coelho *et al*. 2002b). In clams exposed to several TBT concentrations (3.83, 81.4, 242, 740 2470 ng l^{-1} TBTCl) in water for 30 days, TBT is accumulated linearly in the whole soft tissues, followed by a steady state and is eliminated exponentially but faster in those exposed to the highest TBT concentrations. However, clams still contained twice the initial amount (300 ng g^{-1} dw) even after 100 days of depuration (Gomez-Ariza *et al*. 1999).

Body burdens of dibutyltin (DBT) represent only a small proportion (18–11%) of the total organotin burden, and reflect TBT accumulation pattern, suggesting that the source of DBT is from the internal metabolism of the parent compound, rather than accumulation from the external media.

Stress-proteins under normal conditions are involved in the transport, folding and assembly of newly synthesized proteins; however, under adverse environmental situations (such as TBT exposure) their synthesis is induced and acted to repair and

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protect cellular proteins and minimize aggregations. The most abundant and best studied are 60 and 70 kDa stress protein families that have been proposed as biomarkers of adverse effects at the cellular level (Sanders 1990). Evidence on HSP-60 induction occurred in the gills of TBT- exposed clams (91, 454 and 2268 ng l^{-1} as Sn for seven days). A TBT concentration of 53 ng g^{-1} w.w. is enough to induce a 2-fold increase in HSP-60, indicating a dose-dependent increase (up to 3.8-fold) in TBT exposed clams. However, when clams were transplanted to a TBT polluted marina, higher TBT burdens (230–290 ng g^{-1} ww) are needed to get a similar response. In contrast, HSP-70 in the gills did not reflect TBT exposure and the 2-fold increase detected in laboratory experiments, was not confirmed in the transplant experiment (Solé *et al*. 2000). In the digestive gland, TBT burden is dose related (from 6.8 ± 0.5 to 831.5 ± 77.5 ng g^{-1} Sn ww), it represents a 122-fold increase in this tissue and is responsible for cytocrome P_{450} breakdown. However, although P_{450} percentage decreases (from 78 to 73%), total P_{450} (P_{450} + '418 peak') is unaffected and in the digestive gland TBT does not affect the MFO components in this species (Solé 2000).

In clams transplanted to an environment contaminated with organotin (24 ± 5 and 7 ± 2 ng Sn l^{-1} of TBT and DBT, respectively, and n.d.- 9.7 ng g^{-1} ww of triphenyltin (TPhT)) and PAH (6.9–55.6 ng g^{-1} ww) compounds, total cytochrome P_{450} is directly related to TBT and PAH concentrations (Morcillo and Porte 2000, Solé 2000).

Moreover, the accumulation of organotin compounds also induces a disturbance of the *R. decussatus* endocrine system translated by an increase in testosterone titres (33%) (Morcillo *et al*. 1998, Morcillo and Porte 2000). While testosterone titres increase, estradiol levels are depleted (52–81% of the initial values) (Morcillo and Porte 2000). Therefore TBT exposure reduces in *R. decussatus* the ability of aromatization of testosterone to oestrone and estradiol suggesting an inhibition of P450-aromatase activity by TBT (Morcillo *et al.* 1998). The interaction of TBT with sex hormone metabolism has a potential risk for masculinization of clam physiology (Morcillo and Porte 2000).

Field studies

Two ecosystems (Ria Formosa (Portugal) and Bizerta lagoon (Tunisia)) characterized by the presence, production and economic relevance of *R. decussatus* and impacted with different contaminants are compared for biomarker responses (figure 1a). Clams from the Thau lagoon were purchased for some of the experiments carried out in the laboratory (Roméo and Gnassia-Barelli 1995, 1997; Hoarau *et al*. 2001, 2002, 2004) and control clams of these experiments are given in the tables with an indication of their origin.

Ria Formosa

The Ria Formosa lagoon (Portugal, figure 1b) is a coastal lagoon located in the south coast of Portugal that supports several economically important activities, particularly shellfish and aquaculture. It has a long tradition of bivalve harvesting, especially *R. decussatus*. Around 20% of the total area (1000 ha) of the lagoon is

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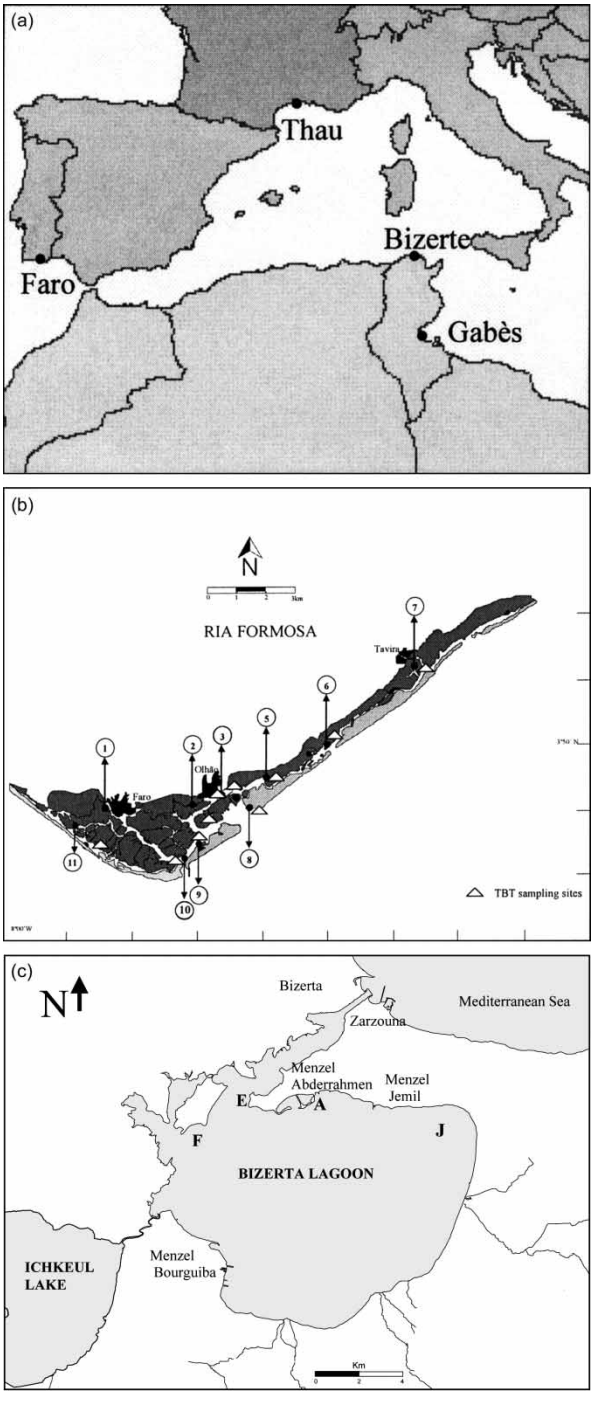

Figure 1. Localisation of *R. decussatus.* (a) Sampling sites of *R. decussatus* in the Ria Formosa lagoon. (b) Sampling sites of *R. decussatus* in the Bizerta lagoon. Clams were collected from: site A (Menzel Abderrahmen) receiving untreated domestic and industrial waste waters; site F (Farouah) impacted with agricultural contaminants via the Tindja wadi; site J influenced by contaminants; site, Echaraa, characterized by a high degree of pollution.

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occupied with on-growing banks of *R. decussatus*. Clam production is about 3000 tons/year, 90% of which is exported (Bebianno 1995, Coelho *et al*. 2002c). As an important edible species these clams constitute a potential risk for human consumption by accumulating pollutants transported to the lagoon through sewage, industrial effluents and agriculture runoff (Bebianno 1995). The lagoon does not receive any significant freshwater input. Temperature and salinity range between 16.4 and 28.4°C and 35.5 and 36.9 p.s.u., respectively. Water quality in the lagoon has deteriorated in recent years mainly due to unsustainable economic development with the consequent decrease in clam production (Coelho *et al*. 2002c).

Metals, organic and organotin compounds were measured in water, sediments and clam tissues in different sites of the Ria Formosa lagoon (Serafim and Bebianno 2001, Coelho *et al.* 2002c, Bebianno and Serafim 2003). The distribution of Cd, Cu and Zn concentrations in *R. decussatus* is tissue dependent (as in laboratory experiments). Among the three metals, Cd shows the most evident spatial variation decreasing from the inner parts of the lagoon towards the ocean. No significant spatial or seasonal variation occurred in clam tissues for the other two metals, though marginal elevated Cu concentrations are observed in the inner parts of the lagoon. In all the tissues, the highest subcellular concentration was in the cytosol for Cd (53–68%) and Cu ($\approx$50%) and in the insoluble fraction for Zn (47–67%) (Serafim and Bebianno 2001, Bebianno and Serafim 2003).

Mean TBT and DBT concentrations in the clam whole soft tissues ranged from 0.8 to 0.34 and 0.9–0.43 $\mu g\ g^{-1}$, respectively. The spatial variation of organotin (TBT and DBT) concentrations in water, sediments and biota is marked while seasonal variation is absent. The highest TBT concentrations are near the most important fishing harbour in the lagoon indicating that fishing vessels represent a continuous source of organotin contamination to the system. The lack of seasonal variation of TBT and DBT burdens in the whole soft tissues of *R. decussatus* also suggests a uniform input of organotin compounds throughout the year. TBT levels detected in water and clam tissue body burdens indicate that deleterious development and endocrine effects to this clam population may occur (Coelho *et al*. 2002c). The relationship observed between TBT concentrations in water and in *R. decussatus* whole soft tissues confirms laboratory results (see above) which indicates that water is the major vector for TBT uptake in this species. Furthermore, organotin body burdens in clam whole soft tissue can give an indication of the bioavailable organotin compounds present in this ecosystem (Coelho *et al*. 2002c).

Metallothionein (MT)

Factors other than contaminants may affect biomarker responses and their impact should be evaluated to validate biomarkers in the field. Biotic and abiotic factors such as seasonal variation, sex, age/size and development stage might interfere with MT synthesis and affect MT concentrations in clam tissues. Therefore, it is important to know how these factors affect MT expression in this species. To answer these questions, clams were collected during the period of sexual differentiation (from June to September) from two different sites (one directly

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influenced by anthropogenic inputs (site 1- figure 1b) and the other with no anthropogenic influence-site 11 – figure 1b) to evaluate the effect of sex on MT levels. No differences between MT levels occurred in the digestive gland of males and females from these sites (table 2). Therefore, sex did not affect MT concentrations in this species in this marine ecosystem so random samples can be used to assess MT concentration in the digestive gland (Serafim and Bebianno 2001). However, MT concentrations determined in the same species from the Gulf of Gabès during the same four-month period demonstrate that, in this population, reproductive effects on MT levels are less perceptible in males than in females (Hamza-Chaffai *et al*. 1999, 2000).

Nevertheless, variations of MT concentrations in the digestive gland of *R. decussatus* for both sexes were detected among sites and season. MT levels increased from June to August with a subsequent decrease in September at the site with anthropogenic influence (Serafim and Bebianno 2001). This highlights the need to evaluate changes of MT concentrations in other tissues of *R. decussatus* in the entire lagoon system. Therefore, spatial and seasonal variation of MT concentrations in different tissues (gills, digestive gland and remaining tissues) of the clam were determined along a metal contamination gradient, over a period of one year, from sites directly influenced by untreated and partially treated sewage (1–7) in the intermediate part of the lagoon (4, 9, 13) and in sites directly influenced by water exchange between the lagoon and the Atlantic ocean (8, 10, 11) (figure 1b). Tissue distribution of MT concentrations was similar to that obtained in the laboratory (table 2; see above). In the three tissues, MT binds most significantly to Cd and Cu whilst Zn, although binding to MT, is more strongly bound to other ligands. MT concentrations in these tissues differ among sites reflecting the different metal load, especially that of Cd, decreasing from the inner parts of the lagoon towards the ocean (Bebianno and Serafim 2003). Similarly a seasonal variation of MT concentrations is observed in the three tissues, with higher levels in the summer and winter (Bebianno and Serafim 2003). Therefore, MT concentrations in the Ria Formosa lagoon revealed that there are changes with site and season but not with sex (Bebianno *et al*. 2000, Serafim and Bebianno 2001, Bebianno and Serafim 2003). Similarly, results from a transplant experiment from two sites of the Gulf of Gabès: a contaminated site (Gargour) and another less affected site (Sidi Mansour) revealed that MT was induced in clams from the most contaminated site. At the other site (Sidi Mansour) neither metals nor MT levels changed (table 2). Moreover, MT levels in *R. decussatus* from the Gulf of Gabès (table 2) are of the same order of magnitude as those from Ria Formosa (Hamza-Chaffai *et al.* 1999) but in the population from the Gulf of Gabès the effect of size and reproduction is less perceptible in males than in females. Data suggests that metal contamination as well as other physiological factors, affect *R. decussatus* MT synthesis in this area. From the data available on *R. decussatus* from the Gulf of Gabès, MT in the digestive gland of males seems preferable as biomarker of metal contamination. A multiple regression analysis model that relates MT, metals and weight in the gills, digestive gland and remaining tissues of *R. decussatus* from the Ria Formosa lagoon, indicates that Cd is the metal that influences MT synthesis the most in all tissues. Cu is only related with MT in the remaining tissues, despite

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Table 4. Alterations of enzymatic activities, MT and LPO levels in *Ruditapes decussatus* exposed to contaminants (μg/l)

Contaminants	Tissues	MT	SOD	CAT	TGPx	Se-GPx	GST	AChE	LPO	References
Cadmium										
400	W.	+								Bebianno *et al*. 1993
4, 40, 100	Gills	+			−−				+	Géret *et al*. 2002b
100, 400	Gills	++								Bebianno *et al*. 1993, 1994
250	Gills	=								Roméo and Gnassia-Barelli 1995
5–500 (*in vitro*)*	Gills								+	Roméo and Gnassia-Barelli 1997
100,400	D. G.	++								Bebianno *et al*. 1993, 1994
100,400	R.	++								Bebianno *et al*. 1993, 1994
Zinc										
100, 1000	Gills	=	=							Géret and Bebianno 2004
100, 1000	D. G.	=	=							Géret and Bebianno 2004
Copper										
0.5, 2.5, 25	Gills	+		−	−	−				Géret *et al*. 2002a
75	Gills	+								Roméo and Gnassia-Barelli 1995
6.25–25 (*in vitro*)*	Gills								++	Roméo and Gnassia-Barelli 1997
75	D. G.	=						−***		Hamza-Chaffai *et al*. 1998
Cu+lindane										
34.5+75	D. G.	=						−−***		Hamza-Chaffai *et al*. 1998
Lindane										
34.5	D. G.	=						−−***		Hamza-Chaffai *et al.* 1998
BaP										
0.5–1**	Whole						+			Hoarau *et al*. 2001
pp'DDE										
0.14**	Gills						+			Hoarau *et al*. 2004
Methoxychlor										
0.14**	Gills						+			Hoarau *et al*. 2004
Imidazole										
25**	Gills						=			Hoarau *et al*. 2004

*μg/ml.
**μM.
***AChE measured in remaining tissues.

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the amount of Cu bound to MT in the other tissues (≈50%) and no relationship exists between MT and Zn in any of the tissues (Serafim and Bebianno 2001; Bebianno and Serafim 2003). A non-linear relationship between MTs and metals in clams from the Gulf of Gabès indicates that in this population, MT levels are affected first by Cd and then by Zn. For Cu, MT relationship was less evident (Hamza-Chaffai *et al*. 2003). Therefore, MT in the gills and digestive gland of *R. decussatus* can be used as an early warning marker for assessing the toxicological status of *R. decussatus* in metal-contaminated (Cd and Cu) environments and are a useful biomarker in the Ria Formosa lagoon (Bebianno and Serafim 2003).

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx) activities and Lipid peroxidation

Antioxidant enzymatic activities (SOD, CAT, GPx) and lipid peroxidation were analysed in the gills and digestive gland of clams from the four sites of the Ria Formosa lagoon mentioned above, and a control site (figure 1b) in an area with important water exchange with the ocean. SOD activity changes especially in site 2 where the cytosolic activity is lower in the gills. In site 3, the mitochondrial activity in the digestive gland increases, indicating the presence of different types of pollutants in these sites. CAT activity is higher in the gills of the clams from the three sites affected by contamination, while in the digestive gland, this is higher only in site 3 (table 3). GPx (total and Se-dependent) is inhibited in the gills from the sites directly affected by sewage and industrial wastes and by harbour activities (2 & 3), which is indicative of the precarious state of the bivalves in this area. Lipid peroxidation levels are also higher in the gills of the clams from these two sites and in the digestive gland from the third site (main navigation channel and exchange with the Atlantic Ocean). Clams from Ria Formosa have higher MDA concentrations in the digestive gland (0.840) containing more lipids than in the gills (0.381 nmol MDA mg^{-1} proteins, Géret and Bebianno 2004, table 3). This phenomenon is not found in clams from the Thau lagoon (0.656 in the digestive gland compared to 0.643 nmol MDA mg^{-1} proteins in the gills, table 3, Roméo and Gnassia-Barelli 1997).

In clam population from the Gulf of Gabés, MDA concentrations significantly increase in both transplanted sites and are strongly correlated with time which is related to the contamination pulse and the reproductive state of the animals (table 3). Despite the different metal exposure, MDA in this clam population is inversely related with size (Hamza-Chaffai *et al*. 2003).

Glycogen is the "fuel" for different metabolic and physiological processes. For glycogen concentrations, a marked inter-site and intra-site increase is observed with time in both sites. After 62 days of transplant, glycogen concentrations in *R. decussatus* increase by a factor 3.5 (Gargour site) and a factor 2.8 (Sidi Mansour) and differ among sites (Gargour > Sidi Mansour). Only Zn, size, and time, significantly affect glycogen concentrations. Glycogen decreases with the increase in Zn concentrations indicating a decrease in storage of energy. This is a predictive indicator of an ecosystem dysfunction provoked by anthropogenic influence and reinforces the need for the use of "biomarkers of health condition" (Hamza-Chaffai *et al*. 2003).

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There is also a relationship between lipid peroxidation and MT in the gills (Géret *et al*. 2003) which indicates that the production of free radicals is not removed, leading to fast and physiological consequences occurring (Pellerin-Massicotte 1994). These results suggest that the gills are more sensitive than the digestive gland and that CAT and GPx activities and MDA levels, along with MT, especially in the gills of the clams can be used as biomarkers to assess the impact of contaminants in the lagoon.

Bizerta lagoon

The Bizerta lagoon (figure 1c), due to its location between Lake Ichkeul and the Mediterranean, is characterized, as most Mediterranean lagoons, by a great variability of environmental conditions, in particular, in hydrological parameters, such as salinity (range: 31.2–39.3 p.s.u.), temperature (9.2–31.4°C) and dissolved oxygen levels (3.9–9.6 mg l^{-1}). In addition, this water body is subject to eutrophication during the summer. Anthropogenic disturbances of various origins increase the natural fragility of the lagoon (Dellali 2001). Four biomarkers: MT, CAT, GST and AChE activities were measured in clams from this area.

Metallothionein (MT)

Comparison of MT concentrations with three populations of *R. decussatus* from sites F (Faroua), J (Menzel Jemil) and E (Echaraa) of the lagoon (figure 1c) and also from the Bay of Brest (Atlantic coast, France) as well as with two other populations of *R. Philippinarum* from the same sites revealed that metals affect the genetic structure and MT concentrations of the five clam populations (table 2). The genetic structure has higher allelic diversity in metal-exposed clams. MT concentrations in the gills and digestive gland of field-collected *R. decussatus*, quantified by ELISA (table 2) using an antibody against a recombinant MT cloned in the Pacific oyster C. *gigas*, shows a relationship between MT levels and metal pollution and differential responses among these *R. decussatus* populations according to the degree of metal pollution, indicating that MT levels can be considered a specific marker of the biological effects of metals in theses sites. MT concentrations in *R. decussatus* are higher than in *R. philippinarum* suggesting a differential response of these two species in terms of cellular partitioning and detoxification of metals (Moraga *et al*. 2002). Clams originating from E, located in the channel between the Bizerta lagoon and the Mediterranean, seem to present the highest MT concentration in the gills (measured by ELISA) but not in the digestive gland where the highest MT concentrations were at F and the degree of pollution had increased from J to E (figure 1c).

Catalase (CAT), glutathione S-transferase (GST) and acetylcholinesterase (AChE) activities

Whatever site considered, CAT activity in clams (table 3) varied as a function of the season, with marked increases in September, when water quality was poor (Dellali *et al*. 2001a). Catalase activity measured in whole soft animals in Bizerta or gills of *R. decussatus* as in Ria Formosa may be used as a defence biomarker, since the activity in clam tissues increases when animals are submitted to environmental

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stress. A comparison of CAT activity between *R. decussatus* (whole soft tissues: 109.13 ± 40.01 μmol min^{-1} mg $protein^{-1}$, table 3) and mussels *M. galloprovincialis* (62.40 ± 16.50 μmol min^{-1} mg $protein^{-1}$) collected from the same place (station J) in the Bizerta lagoon (figure 1c), revealed a higher response of clams. Clams are located in the tidal zone and are therefore the first to be exposed to land-based pollutants while mussels live above the sediments and filter pollutants mainly from the water column (Dellali *et al*. 2001a).

Global GST activity (measured with CDNB as a substrate 0.36 ± 0.06 μmol min^{-1} mg $protein^{-1}$ at site J, table 3) does not give significant information about anthropogenic influence in field experiments. The activity was almost the same as for *Mytilus galloprovincialis* (0.33 ± 0.05 μmol min^{-1} mg $protein^{-1}$) from the same site.

The AChE activity is generally lower in the clams (3.80 ± 0.70 nmol·min^{-1} mg $protein^{-1}$) than in the mussels (5.53 ± 0.63 nmol·min^{-1} mg $protein^{-1}$) and more variable throughout the year. The differences of habitat mentioned above may explain the differences noted in AChE responses between both species indicating that the clams give a better response for AChE than mussels (Dellali *et al.* 2001b).

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Conclusions

This review highlights the developments performed in biomarkers of defense and damage in *R. decussatus* from laboratory experiments and impacted environments from Europe and Mediterranean regions. However, due to the lack of comparable data available, it is difficult to propose the most useful biomarkers to assess environmental pollution in these particular areas. Some conclusions can be drawn regarding which tissues and biomarkers are needed to evaluate the environmental quality of these areas in a perspective of a sustainable development.

Results point out that the gills and the digestive gland are the most "interesting" tissues from the ecotoxicological point of view. These two tissues reflect the bioavailability of contaminants present in the environment. The gills are in direct contact with the surrounding environment and reflect short-term metal exposure, while the digestive gland, where a certain number of metalloenzymes bind, metabolise and accumulate excess metal concentrations, act as a storage organ reflecting long term metal exposure. These results seem to indicate that the physiological function of each tissue is independent and, particularly for Cd, reflect metal concentrations in the natural environment (Bebianno and Serafim 2003).

The biomarker of defense MT was the only biomarker analysed in both field sites (table 2). Cd is bound to two MT isoforms (molecular weight around 10 and 20 KDa, named MT-10 and MT-20, respectively) in the gills, digestive gland and whole soft tissues of Cd-exposed *R. decussatus*, confirming that Cd in this species is mainly bound to MT (Bebianno *et al*. 1993; Hamza-Chaffai *et al*. 2000). Four MT isoforms have also been identified in the digestive gland after Cd exposure, with a weight (7328 Da) (Simes *et al*. 2003) similar to other molluscs *M. edulis* (Unger *et al*. 1991) and *C. gigas* (Roesijadi *et al*. 1989). It can be concluded that MT is the most dominant ligand for Cd contamination in the laboratory or in the field,

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principally in the gills of *R. decussatus*. Induction of MT also occurs as a function of Cu in the environment but not with Zn.

The oxidative stress induced by ROS, and particularly by those metals that participate at the Fenton reaction, is evaluated by SOD, CAT, GPX activities. The damage effects result in a perturbation in the oxygen metabolism translated by higher MDA levels and, sometimes, by MT induction. The interpretation of this experimental and field data is sometimes contradictory due to the delicate balance between pro-oxidant forces and antioxidant defences.

GST, like MT, has different isoforms in the gills and digestive gland of this species and these isoforms are differentially induced as a function of tissue and organic contaminants present. The development of bi-dimensional electrophoresis analysis and a better knowledge of the genes encoding for GST isoforms in *R. decussatus* are interesting venues to develop for a more relevant biomarker.

AChE isoforms are monomeric, dimeric and tetrameric in aquatic invertebrates but so far AChE isoforms have not been identified in *R. decussatus*. The expression of these different isoforms is linked to the effects of one or several types of pollutants. However, total AChE activity in *R. decussatus* is inhibited in the laboratory or in the field when pesticides (organochlorine, organophosphate and carbamate compounds) or metals are present.

In addition to the above mentioned biomarkers, others like SFG (scope for growth), condition index and particularly glycogen (an indicator of Zn contamination in this bivalve species) can be very useful as biomarkers of general stress.

Data obtained from transplant experiments (active biomonitoring) revealed that they are useful in the evaluation of the quality of the natural environment. Because in the field a mixture of pollutants is predominantly present, certain pollutants can mask a selected biomarker response and mislead the information on pollution sources. Although the comparison between these biomarkers in different tissues of *R. decussatus* from these ecosystems is difficult, due to the lack of simultaneous data for all the biomarkers listed above (table 4), besides MT, a multi-biomarker approach is envisaged for environmental risk assessment in these sites.

Experiment and field studies are in good agreement since MT levels, especially in the gills, can be used as biomarker of exposure to Cd. GPx activity (which is inhibited) and MDA levels (which increase) may also be determined in this respect. AChE activity is inhibited by pesticide and, to a lesser extent, by metal exposure in the gills and whole soft body of clams. However, the induction of GST isoforms experimentally demonstrated is not observed in the field because global GST activity is considered and not the induction of a specific isoform. The same conclusion will hold for SOD which presents different isoforms and which does not give significant results in the field. However, much progress will be expected with the use of nuclear probes (cDNA) of GST and SOD. At present there is a real need to go deeper from enzymatic tissue level responses to molecular responses such as cDNA of MT, SOD, GST. These are partially identified in *R. decussatus* and others are needed to complete a clam cDNA bank. As mentioned before, due to the presence of mixtures of contaminants in the environment, the combination of genomic and ecological research can provide new insights into the biological responses and new environmental challenges. In this perspective, complementary

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genomic and proteomic approaches might be promising technological tools to evaluate the relationship between toxicity and development of fingerprints of gene/protein changes of *R. decussatus* and we can use these as sensors in areas where mussels are not present and where this species is predominant.

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