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Lila Ferrat, Christine Pergent-Martini, Catherine Fernandez, Michele Roméo. IS GLUTATHIONE TRANSFERASE (GST) ACTIVITY IN POSIDONIA OCEANICA A STRESS RESPONSE TO MERCURY EXPOSURE?. Bulletin of Marine Science, 2002, 71, pp.1183-1190. hal-01768843

HAL Id: hal-01768843
https://hal.science/hal-01768843

Submitted on 17 Apr 2018

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BULLETIN OF MARINE SCIENCE, 71(3): 1183–1190, 2002

IS GLUTATHIONE TRANSFERASE (GST) ACTIVITY IN *POSIDONIA OCEANICA* A STRESS RESPONSE TO MERCURY EXPOSURE?

L. Ferrat, C. Pergent-Martini, C. Fernandez and M. Roméo

ABSTRACT

Today, efficient monitoring of the environment is increasingly depend on the use of bio-indicator species. Marine phanerogams, and in particular *Posidonia oceanica*, would appear to be potentially valuable bioindicators of metal pollutants. Although correlations have been found between the mercury levels accumulated in the plant tissue and the concentrations of this metal in the water column, it would be of interest to identify early signs of the stress response induced by this xenobiotic. Thus, mercury concentrations and GST activity in *Posidonia oceanica* (L.) Delile from contaminated and pristine sites were measured. These results demonstrate that an increase in mercury level is correlated with an increase in GST activity, particularly in the sheaths of *P. oceanica* shoots. The sites contaminated by mercury were also those sites for which the highest enzyme activity was recorded. An even better correlation was found between the mercury levels and GST activity, if a 2 mo lag in the effect of mercury on GST activity is assumed.

It is becoming increasingly apparent that an efficient management of the environment is only possible through the use of tools that allow both a rapid and reliable evaluation of the environmental conditions and the monitoring of these over time. This 'biosurveillance' concept, which is based on environmental monitoring involving more than just the measurement of the classical physicochemical parameters, was introduced during the 80s (Blandin, 1986). It involves the identification of bioindicators, that is to say living organisms capable of providing information on the quality of, or any changes in, prevailing environmental conditions (Blandin, 1986).

To date, the most frequently used organisms in long term environmental monitoring programs have been bivalves mollusks and fish (e.g., "Mussel Watch Program" in Goldberg et al., 1983), although an increasing interest in marine macrophytes is currently observed. Indeed, in light of their worldwide distribution, marine phanerogams would appear to be potentially valuable bioindicators (Brix et al., 1983). Studies involving *Posidonia oceanica* (L.) Delile have confirmed this, revealing that this species is able to accumulate certain metal pollutants, notably mercury (Pergent-Martini, 1998). Mercury represents one of the most abundant marine pollutants and, within the Mediterranean, elevated mercury levels have been reported in certain regions (Maserti et al., 1991). In *P. oceanica*, correlations have been drawn between the mercury levels accumulated in the plant tissue and the concentrations of this metal in the water column (Pergent-Martini, 1998). These observations, however, only provide an indication of past environmental pollution, which limits the value of these results within the framework of an 'instantaneous' monitoring of the environment. It would thus appear of interest to seek more early-warning symptoms of pollutant action in the marine phanerogam *P. oceanica*. These early-warning symptoms, termed biomarkers, are usually defined as being molecular, biochemical and cellular changes brought on by chemical pollutants and which are measurable in biological mediums such cells, tissues and cellular fluids (McCarthy and Shugart, 1990). Recent studies (Ranvier et al., 2000) suggest that mercury increases the specific activity of the enzyme glutathione S-transferase (GST) in *P. oceanica* specimens sampled from areas

contaminated by or exposed to this metal. GST is generally considered to be a biomarker of exposure to organochlorides (Stien, 1998), although copper and mercury can also stimulate GST activity in mussels contaminated by these metals (Canesi et al., 1999). In light of this, it seems reasonable to consider the enzymatic activity of GST as a biomarker of exposure to metal pollutants. The GST family intervenes in the second phase/step of chemical pollutant metabolism (biotransformation). This metabolism does indeed usually occur in two phases/steps: the first phase/step is one of functionalization and consists in an oxidation, thereby increasing the polarity of the xenobiotic, and the second involves a conjugation which facilitates the bonding between the chemical pollutant and an endogenous compound (usually glucuronic acid, glutathione, sulfates). An increase in enzyme activity during either of these phases/steps is an indication of pollutant exposure. Phase/Step I is carried out by the cytochrome enzyme family. Those involved in phase/step II are generally transferases (e.g., uridinediphospho-glucuronosyl transferase, Glutathione S-transferase, sulfotransferases). Glutathione S-transferases (GST) can be found in both the animal and plant kingdoms (Mannervik and Danielson, 1988 in Gronwlad and Plaisance, 1998). They catalyze the conjugation of a number of electrophilic substrates to the thiol group of the endogenous GSH (reduced glutathione, Fitzpatrick et al., 1995).

Although these mechanisms have been extensively studied in molluscs and fish (Monod, 1997), the detoxification mechanisms in plants remain poorly understood and the metabolism pathways of a given compound can differ from one plant to the next. In *P. oceanica,* the presence of both cytochrome P450 and its accompanying enzymatic activities have been demonstrated, notably the activity of cinnamate 4-hydroxylase (CA4H) that appears to be correlated to pollutant levels in the sediment (Hamoutène, 1995). In light of the important detoxification role played by GST in plants (Gronwald and Plaisance, 1998), it was deemed of interest to ascertain the role of these enzymes in *P. oceanica*.

Materials and Methods

The study was carried out over an annual cycle (June1999–June 2000), at two sampling sites at equal depths (−10 m): the first, just south of Livorno (Bay of Rosignano, Italy), has experienced industrial wastes rich in mercury since 1920. This first site (site A) is thus subject to anthropogenic wastes. The second, reference site (site R), located in the Bay of Cannes (Islands of Lérins, France), is free of any known industrial input.

Twenty shoots were sampled every 2 mo and dissected according to the protocol described by Giraud (1979). Only the sheaths and blades of adult leaves were analyzed, after removing all epiphytes using a glass slide. Half of the shoots were used to measure metal content, and the remainder were analyzed for enzyme activity. All shoots were analyzed individually.

Following lyophilization, both tissue types were ground to a powder and mineralized (0.05g dw, 100 mL teflon ACV reactor) in 5 mL nitric acid and 1mL hydrogen peroxide. Microwave mineralization (Mars 5, CEM®) was carried out using a temperature ramp of 8 min up to 200°C followed by a heating plateau of 20 min at 200°C. After mineralization, the samples were made up to 25 mL with ultrapure water and filtered. The mineralized samples were analyzed using flameless atomic absorption spectrophotometry (FIMS 100, Perkin Elmer®). A standard addition method was used to calibrate the protocol and the analytic procedure was verified using certified reference material (*Lagarosiphon major*, CRM 60).

The two tissue types were ground in liquid nitrogen using a mortar. The samples were then homogenized (ultraturrax®) in a pH 8.4 buffer, and subsequently centrifuged at 9000 g following the protocol described by Ranvier et al. (2000). The protein levels were determined for the supernatant, following filtration (Bradford, 1976). GST activity of the fraction obtained (supernatant)

with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) was measured spectrophotometrically at 37°C by following conjugation of the acceptor substrate with glutathione as described in Habig et al. (1974). Results are expressed as the formed conjugate per minute and per milligram of protein (nmoles min^{-1} mg $prot^{-1}$).

Data were processed by three-way ANOVAs, to allow comparisons between the GST or mercury levels and tissue, site and sampling period. As the interaction between the three factors is significant ($F = 4.7$ to 37.7 for mercury concentrations and $F = 21.3$ to 168.1 for GST activity; $P < 0.001$), one-way analyses followed by Tukey tests (for analyses over the annual cycle) or Student tests (for analyses of tissue and site factors at given months) were performed in order to eliminate these variations (Zar, 1984). Normality and homoscedasticity were verified by Shapiro Wilks and Bartlett tests, respectively (Zar, 1984). The relationships between GST and mercury level were assessed using correlation and regression analyses. The software Statgraphics plus (ver 3.1) for Windows was used.

Results

The mercury concentrations (Fig. 1) in each of the tissues examined confirms that contamination levels are significantly higher at station A (264 and 299 ng g^{-1} y^{-1} dw on average, for the blades and sheaths, respectively) as compared to station R (63 and 77 ng g^{-1} y^{-1} dw) this for all period considered (Tukey test, $P < 0.05$).

Conversely, there was a seasonal evolution in mercury levels at the two sampling stations. Station R exhibited maximum contamination levels in April (102 and 129 ng g^{-1} dw for the blades and sheaths, respectively) and minimum values in January (36 and 24 ng g^{-1} dw for the blades and sheaths, respectively). This was for both tissue examined (Table 1).

There was a lag in the variations at station A, however, with maximum concentrations in January for the blades (352 ng g^{-1} dw) and in April for the sheaths (415 ng g^{-1} dw). Minimum values were recorded in October for both of the tissues (Table 1; 174 and 119 ng g^{-1} dw for the blades and sheaths, respectively). Mean mercury concentrations were always higher in the sheaths as compared to the blades, regardless of the station consid-

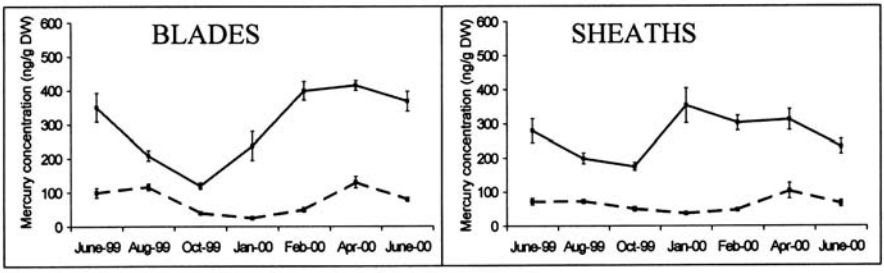


Figure 1. Mercury concentration in shoots of *Posidonia oceanica* from station A (full line) and R (dotted line).

Table 1. Results for one-way ANOVA on seasonality data ($P < 0.001$ for all values).

	Mercury		GST activity	
	Blades	Sheaths	Blades	Sheaths
Station R	15.98	66.35	17.27	109.89
Station A	17.11	72.49	8.00	77.70

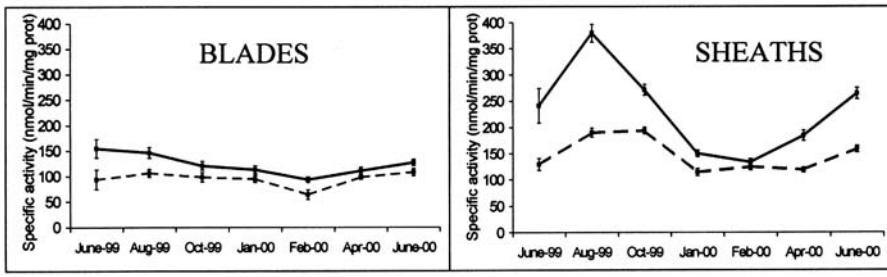


Figure 2. GST activity in shoots of *Posidonia oceanica* from station A (full line) and R (dotted line).

ered. The differences in mean concentrations between the two tissues was similar, however, throughout the year and at the two stations examined. This would seems to indicate that the allocation of mercury to the tissues is constant for a given contamination level and is thus in all probability the result of identical physiological mechanisms.

Analysis of GST activity in each of the tissues was observed to be significantly higher at station A (Tukey test, $P < 0.05$), regardless of the period considered (120 nmol min^{-1} mg^{-1} prot. and 178 nmol min^{-1} mg^{-1} prot., for stations R and A, respectively). GST activities were also seen to exhibit a seasonal trend (Fig. 2). For station A, minimum values were recorded in February (93 and 133 nmol min^{-1} mg^{-1} prot. for the blades and sheaths, respectively) and maximum values in June 1999 for blades (154 nmol min^{-1} mg^{-1} prot) and in August for sheaths (379 nmol min^{-1} mg^{-1} prot.), (ANOVA, Table 1). For station R, minimum values were recorded in January for the sheaths (114 nmol min^{-1} mg^{-1} prot.) and in February for the blades (64 nmol min^{-1} mg^{-1} prot.), whereas maximum values occurred in August for the blades (106 nmol min^{-1} mg^{-1} prot.) and in October for sheaths (193 nmol min^{-1} mg^{-1} prot.) (ANOVA, Table 1).

The enzyme activity in adult leaves was always significantly higher in the sheaths as compared to the blades, regardless of either sampling station or sampling period (110 nmol.min^{-1} mg^{-1} prot. for the blades compared to 190 nmol min^{-1} mg^{-1} prot. for the sheaths, all stations and sampling periods considered, Tukey test $P < 0.05$).

A significant correlation was observed (Variance analysis: $P = 0.0302$), between mercury levels and GST activities in both stations and tissue types (regression analysis; Fig. 3).

Discussion and Conclusions

Mean mercury levels recorded at station R for the blades and sheaths of adult leaves were higher than those recorded by Pergent-Martini (1998) at a site in Corsica (13 ng g^{-1} and 37 ng g^{-1} mercury, respectively), but remain comparable to values reported for similar sites subject to limited anthropogenic pressures (Maserti et al. 1991; Capiomont et al., 2001).

The values observed at station A are typical of a highly contaminated site (4 times higher than at station R). These values are comparable to those of Maserti et al. (1991) for the same region.

Seasonal variations were observed at the two sampling stations for both the mercury levels and GST activities. Maximum mercury levels were recorded in the spring for station R and in the winter for station A, which is in agreement with data from the literature (Capiomont et al., 2001). Indeed, recent studies have demonstrated that the flux of mer-

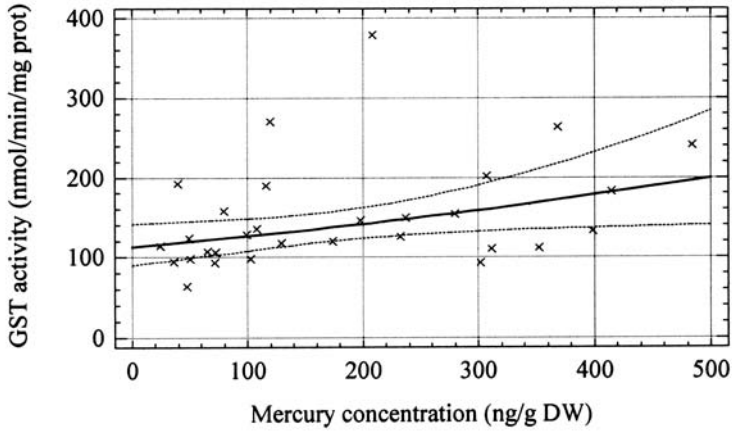


Figure 3. Correlation between GST activity and mercury concentration. Exponential equation model: GST = exp (4.74 + 0.001 mercury).

cury from the sediment to the water column appears to be more elevated in winter (Covelli et al., 1999; Gill et al., 1999). In light of the fact that contamination in *P. oceanica* would appear to occur mostly from the water column, a greater availability of mercury in the water could explain the winter maximums observed. A number of authors (Lyngby and Brix, 1982; Capiomont et al., 2001), however, have suggested that a 'dilution' of the metals may be occurring during the summer as the result of an elevated leaf biomass.

Although the GST activity also exhibits a seasonal trend (high summer values), there is a 2 mo lag between the mercury levels and enzyme activity fluctuations (Fig. 4). Although seasonal GST activity fluctuations have received very little attention in the literature, it is possible that a slowing down of the plant's physiological mechanisms during the winter (Caye, 1989) may explain the lower enzyme activity levels recorded.

As was seen for GST activity, mean mercury concentrations for any given shoot were higher in the sheaths than in the blades. This difference in the accumulation of metals has already been observed by a number of authors (see synthesis in Pergent-Martini and Pergent, 2000). This would seem to indicate that there exists either a basipetal translocation mechanism within the leaf (from the apex towards the sheath) or a greater abundance

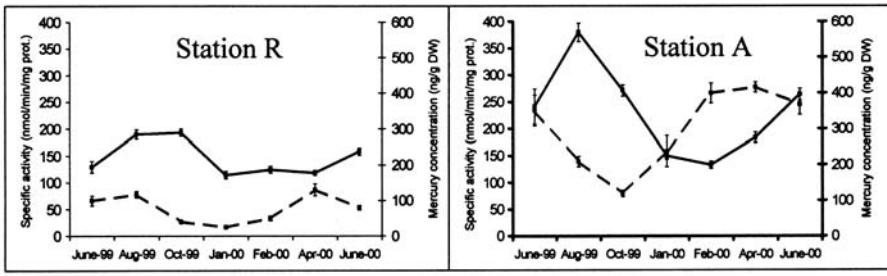


Figure 4. GST activity (full line) and mercury levels (dotted line) in sheaths of *Posidonia oceanica* from stations R and A.

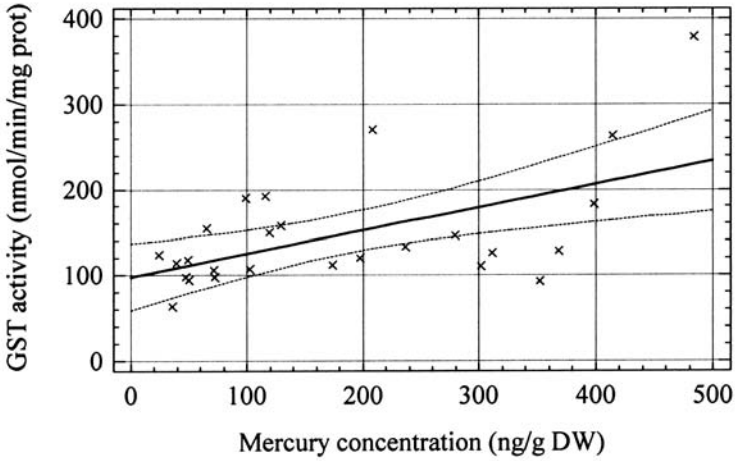


Figure 5. Correlation between GST activity and mercury with a 2 mo lag of GST activity. Linear model: GST = 98.06 + 0.27 mercury).

of fixation sites in the sheaths as compared to the blades. The fact that the ratio of accumulated mercury in the blades and sheaths remains constant for a given site would tend to confirm the existence of specific physiological mechanisms. The two mechanisms listed above have in fact been suggested by Malea et al. (1994), when these authors demonstrated the bidirectional translocation of cadmium and copper in this species.

As GST activity was observed to be higher in the sheaths, it would appear that an elevated metal content is responsible for higher GST activity. Ranvier et al. (2000) experimentally demonstrated that mercury increased GST activity in *P. oceanica*. GSTs are known to play a role in the protection of the cell against oxidative stress through the metabolism of glutathione (Canesi et al., 1999). According to these authors, metals may evoke a decrease in glutathione content which could be mainly related to a stimulation of GST activity. The increase in GST activity observed in *P. oceanica* may thus be considered as an indirect or secondary effect of mercury.

If we shift the GST activity values by 2 mo and compare these to the mercury levels (GST activity values for August 1999 are shifted to June, and so on) a more highly significant linear correlation is observed between these two parameters (Fig. 5) (Variance analysis: $P = 0.0034$). It would thus appear that the mercury levels are at least partly responsible for the GST activities recorded.

A large body of literature indicates that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals and that GST induction is part of an adaptive response mechanism to chemical stress that is widely distributed in nature (Jakoby, 1978, Armstrong, 1997 in Gadagbui and James, 2000).

Although aquarium experiments conducted by Ranvier et al. (2000) demonstrated that increases in GST activity in phanerogams occurred following exposure to mercury, which was thus probably responsible for an induction of this enzyme, this induction would appear to be very rapid (occurring in 48 hr). However, with the exception of these short-term reactions, it is possible that, within the context of a chronic contamination such as that observed at the Rosignano site, other regulatory mechanisms are deployed by the

plant. Perhaps short-term induction systems, and accompanying regulation systems, exist for GSTs, or even isoforms, whose role it is to respond to acute contamination, or isoenzymes responding in the long-term to chronic contamination of the environment.

Contrary to the sheaths, the blades exhibited very low activity levels. In addition, this last tissue provides very little information concerning seasonal variations in GST activities and only a very small difference in activity was observed between the two sampling, whereas a far greater difference was observed for the sheaths. Such low activities could be due to the presence of enzyme inhibitors such as phenols (Hamoutène, 1995), which are very abundant in the blade tissue. These substances are known to bond strongly proteins (Hamoutène,1995). Indeed, phenols may be released during the homogenization of our extractions despite the addition to the buffer of a chelator such as Polyvinylpyrrolidone (PVP).

In addition, we only tested the CDNB activity of GST, and it is therefore possible that only the isoform of GST possessing a high CDNB activity is present within the sheaths, and that another isoform exhibiting a low CDNB activity is present in the blades. GST activities are often assayed using 1-chloro-2,4-dinitrobenzene (CDNB), a relatively non-specific GST reference substrate. GST-CDNB activity reflects the integration of GST isoenzyme activities. In a given organism, the specific GST activities on a range of substrates (1,2-dichloro-4-nitrobenzene DCNB, ethacrynic acid ETHA, nitrobutyl chloride NBC and D5-androstene-3,17-dione ADI) may be used as a criterion for distinguishing GST iosenzymes (Fitzpatrick et al., 1995). In the future, the use of such GST substrates in conjunction with CDNB will allow for a more complete biochemical characterization of GST isozyme activities in *P. oceanica*.

Although the correlations drawn here reveal that GST activity may represent a valuable marker for mercury contamination, interpretation of the GST activities along with mercury concentration data does not allow us to formally conclude that this metal induces GST activity. A separation of the isoenzymes will be necessary if we are to determine whether or not the induction of one or several of these isoenzymes can be attributed to mercury contamination. It is also clear that future research efforts should be made to determine the value of GST activity as a biomarker of metal exposure.

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