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Changes in Glutathione S-Transferase Enzyme Activity in *Ulva rigida* According to Abiotic Factors and Locations

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Abstract

In the present study, changes in glutathione S-transferase (GST) enzyme activity were investigated in *Ulva rigida* specimens, collected from four different depths at six sites in the southern region of the Sea of Marmara. GST enzyme activity showed significant increases and decreases at some sites according to depth. Different abiotic factors affecting all the sites were shown on the Principal Components Analysis axis. Canonical correlation analysis (CANCORR) was performed among abiotic factors the measured and GST enzyme activity in *U. rigida* specimens. CANCORR analysis results showed that ammonia nitrogen values were negatively correlated with dissolved organic nitrogen values while positively correlated with GST enzyme activity. The present data showed that GST enzyme activity in *U. rigida* was related to water pollution and abiotic factors.

Keywords: *Glutathione S-transferase,* Ulva rigida, *water pollution*.

Abiyotik Faktörler ve Bölgelere Göre *Ulva rigida* Türünde Glutatyon S-Transferaz Enzim Aktivitesindeki Değişimler

Özet

Bu çalışmada, Marmara Denizi'nin güney bölgesinde 6 istasyonda 4 farklı derinlikten toplanan *Ulva rigida* örneklerinde, glutatyon S-transferaz (GST) enzim aktivitesindeki değişimler incelenmiştir. GST enzim aktivitesi bazı istasyonlarda derinliğe bağlı olarak anlamlı artış ve azalışlar göstermiştir. Bütün istasyonları etkileyen farklı abiyotik faktörler ana bileşenler analizi ekseninde gösterilmiştir. *U. rigida* örneklerinde GST enzim aktivitesi ve ölçülen abiyotik faktörler arasında Canonik korelasyon analizi (CANCORR) yapılmıştır. CANCORR analizi sonuçlarına gore amonyum azotu değerleri, çözünmüş organik azot ile negatif korelasyon oluştururken, GST enzim aktivitesi ile pozitif korelasyon göstermiştir. Bulgularımız, *U. rigida* örneklerinde, GST enzim aktivitesinin su kirliliği ve abiyotik faktörlerle ilişkili olduğunu göstermiştir.

Anahtar Kelimeler: *Glutatyon S-transferaz, su kirliliği,* Ulva rigida.

INTRODUCTION

In the attempt to define and measure the effect of pollutants on an ecosystem, biomarkers have attracted a great deal of interest. The principle behind the biomarker approach is the analysis of an organism's physiological or biochemical response to pollutant exposure. According to Pennec and Pennec (2003), there has been increasing interest in the use of biochemical parameters or biomarkers for diagnostic tests to detect the effects of chemical pollutants, especially at low concentrations, on environmental quality during the last decade. GST is a biological marker of aquatic contamination (Rees 1993). An extensive literature exists on GST activity in different living organisms for physiological and toxicological investigations.

All organisms have developed mechanisms to defend themselves against all chemical compounds that are harmful to their survival. The GST enzymes are important for xenobiotic metabolism and antioxidative protection, and are well characterized and studied enzyme activities in bacteria, microalgae, crop plants, mollusks, insects, fish and mammals (Dixon et al. 1997, Tang et al. 1998, Lopes et al. 2001, Blanchette and Singh 2002, Petushok et al. 2002)

Macroalgal communities provide nutrition, reproduction, and an accommodating environment for other living organisms in marine ecosystems (McClanahan et al. 2002, Wilson 2002). Because they contain proteins, carbohydrates and other nutritional elements, macroalgae are one of the most important organisms. Macroalgae are also affected by the changing chemical composition of seawater. Many wastes, discharged into marine ecosystems, contain aromatic hydrocarbons, halogen and nitrogenous compounds. Because of this contamination, different experiments with macroalgae have been

done by many researchers (Haritonidis and Malea 1999, Guimaraens and Coutinho 2000, Hernandez et al. 2002, Dere et al. 2003, Yıldız et al. 2003a, b). Multiple GST enzyme activities have recently also been characterized in freshwater algae (Tang et al. 1998). However, few investigations have been made and little information is available on GST enzyme activities in macroalgae related to pollutants and xenobiotics (Pflugmacher et al. 2000, Pflugmacher and Sandermann 2000, Kirchhoff and Pflugmacher 2000).

The Sea of Marmara has a two-level current system, because of the exchange of the Mediterranean and Black Sea waters (Tuğrul and Salihoğlu 2000). Because of the different sea-water density of the neighboring seas, the top 15-20 m level of the Sea of Marmara has low-salt Black Sea surface-water whereas the more salty Mediterranean Sea water is found beneath this level (Tuğrul and Salihoğlu 2000). Because of this interesting characteristic of the water of the Sea of Marmara, we have also attempted to collect *Ulva rigida* specimens from four different depths.

The aim of this experimental study was also to investigate the effects of abiotic factors on GST enzyme activity in *U. rigida*, as known biological markers of aquatic contamination, according to different depths and sites in the southern region of the Sea of Marmara.

MATERIALS AND METHODS

The Sea of Marmara (11.474 km^2), which is located in northwestern Turkey, is an inland sea, between Europe in the north and Asia in the south. 280 km long and 80 km wide, it is connected in the east to the Black Sea through the Bosphorus, and in the west to the Aegean Sea, part of the Mediterranean Sea, through the Dardanelles (Çanakkale Straits) (Fig. 1). The greatest depth is 1389 m.

Six sites are determined according to their industrialized location, population density and discharges of domestic and industrial wastes. Gemlik Bay-Kurşunlu is located at 40°19'N, 29°02'E; Narlı at 40°26'N, 29°01'E; Altıntaş at 40°19'N, 28°55'E; and Kapıdağ Peninsula-Tatlısu at 40°24'N, 27°56'E, Ocaklar at 40°27'N, 27°46'E, Ormanlı at 40°35'N, 27°52'E, in the Sea of Marmara (Fig. 1).

Uludağ University Sub-Aqua Club (USAT) took the *Ulva rigida* C. Agardh, (Chlorophyta) specimens (n= 7 for each depth) from four different depths: the surface layer (0-0.5 m), 5 m, 10 m and 15m by means of SCUBA diving in March and April 2002. *Ulva rigida* specimens were not found at 10 and 15 m at Ocaklar and Ormanlı sites, nor at 5, 10 and 15 meters at Altıntaş site. Specimens and water samples were taken to the laboratory by stable heated picnic-type containers. In addition, in the laboratory, dry and aqueous herbaria specimens were made to identify the macroalgae. They were cleaned, washed with distilled water and dried at 55°C to constant weight. Taxonomic identification was made by prior light microscope according to Bliding (1963), Chadefaud and Emberger (1960), Feldmann (1937) and Fritsch (1971).

A total of 119 specimens of *U. rigida* were used to determine GST enzyme activity. The samples were stored at -80°C for subsequent analysis. Samples of frozen *U. rigida* specimens were homogenized 15 minutes after the addition cold buffer (potassium phosphate) at pH 6.7 (1/3 mass/volume) using a porcelain mortar. The homogenate was centrifuged at 9000 g for 40 minutes at 4°C (Ferrat et al. 2003). The supernatant was separated using a micropipette. The supernatant was used for determining GST enzyme activities. Care was taken to achieve homogenization, centrifuging and all enzymatic studies at 0-4°C. Measurements were made using the method of Bowman et al. (1990) and Habig et al. (1974) with GSH and CDNB as substrates. GST enzyme activity was determined by tracing the formation of the thioether link between the GSH catalyzed by the enzymes and CDNB in Cecil 5000-Spectrophotometer. The activity was measured as the absorbance change per minute at 340 nm at 25°C. Protein determination was done according to Bradford (1976) using bovine serum albumin as protein standard. The spectrophotometric measurements were repeated three times.

The in situ dissolved oxygen (DO) values were measured at the sampling site by using field-type YSI 55 model apparatus. In biochemical oxygen demand (BOD_5) analyses, the samples were immediately brought into the laboratory in order to determine for BOD_5. The in situ measurements of salinity, electrical conductivity (EC), total dissolved substance (TDS) and water temperature (T) were measured at the same time by using field-type EDT-FE 287 apparatus and the pH by Hanna HI 8314 apparatus.

Alkalinity (HCO_3^- and CO_3^{-2}) was determined

with titration methods (Anonymous 1995). Nitrate (NO_3-N) was determined with cadmium reduction colon method; Nitrite (NO_2-N) with Bendschneider and Robinson's method; Ammonia (NH_3-N) with phenate method; dissolved organic nitrogen (DON) with Solorzano and Sharp's method and orthophosphate (o-PO_4) with zinc chloral method (Parsons et al. 1984).

The significant differences among GST enzyme activities, depth and study sites were determined by Mann-Whitney U test to compare differences in more than two sets and Kruskal-Wallis in two sets of variables (Zar 1984).

Ordination of measured physicochemical variables in the water quality at six sites at different depths by Principal Components Analysis (PCA) was performed on standardized data, based on a correlation matrix (Fry 1996). Spearman rank correlation coefficients (Zar 1984) and Canonical Correlation Analysis (CANCORR) were used to determine the relations with physicochemical variables and the mean GST activities at six sites at different depths.

PCA was performed using Minitab version 13.1 and CANCORR was done by STATISTICA 5.0 statistical package programs. Mann-Whitney U, Kruskal-Wallis and Spearman rank analysis were performed using the SPSS 11.0 for Windows statistics program assessed at the 95% confidence level.

RESULTS

Sampling sites are given in Fig. 1. The changes in specific activity of GST enzyme according to depth and sites are given in Figs. 2 and 3.

According to Mann Whitney U and Kruskal Wallis tests results, we determined that only Narlı site ($Z= -3.14$, $P= 0.002$) was showed significant differences according to depths. However, significant differences were observed when the depths (surface, 5, 10 and 15 m) were compared at all sites.

GST activity in *U. rigida*, taken from Tatlısu in Kapıdağ Peninsula was lower in value than the specimens collected from the other sites in both Kapıdağ Peninsula and Gemlik Bay. When compared with statistical results at Tatlısu site, a significant decrease in GST enzyme activities was seen at all depths compared to the other sites ($P<0.05$) (Fig. 2). During the study, the second lowest mean enzyme activities were determined at the surface of Altıntaş site (9.25 ± 0.86 mg/protein 10^{-2}).

The highest mean of GST activity was observed samples taken from surface at Ocaklar site compared to the other sites surface values. It was observed that GST activities in *U. rigida* collected at Ocaklar were 2.5 times higher when compared with Kurşunlu, 1.8 times higher in comparison with Narlı.

It was seen that GST activity was the lowest at a depth of 5 m at Ocaklar in comparison with GST activities at a depth of 5m at the other sites (except Tatlısu) (Fig. 3). It was found that GST activity at Ocaklar site was lower than at the other sides; about 57.7% of Ormanlı, 46.7% of Kurşunlu, 33.5% of Narlı.

Similar values were observed in GST activities from Kurşunlu and Narlı sites at depths of 10m. However, at Tatlısu site this difference was statistically significant ($P<0.05$). Significant differences were observed in Kurşunlu, Narlı and Tatlısu sites at depths of 15 m ($P<0.05$).

The results of the physicochemical variables of each of the six sites at different depths are seen in Table 1. The PCA of measured variables is given in Figure 4. The first factor accounted for 46.6% of the total variance and salinity, EC, TDS, NO_2-N, NO_3-N, and HCO_3 weigh heavily on this axis. The second factor accounted for 18% of the total variation, related to CO_3, DO, BOD_5, DON and NH_3-N.

The PCA analysis showed which abiotic factors are important at the sampling sites and the different depths. According to PCA analysis (Fig. 4), surface, 5 and 10m of the Tatlısu and Altıntaş-surface sites are displayed at the upper part of the right of the X axis with a combination of NH_3-N, salinity, EC and TDS. The sites, displayed at the upper left part of the X axis are related to BOD_5, DO and NO_2-N. Interestingly, the sites displayed on the whole upper part of the X axis are related to CO_3, displayed in the middle of the X axis. The sites displayed at the bottom left of the X axis are related to o-PO_4 and the sites displayed at the bottom right, however, are a combination of DON, NO_3-N and HCO_3.

The CANCORR analysis is highly significant with the first canonical variables of the variation in GST activity and measured environmental variables. The correlation coefficient associated with the first variable pair was 0.986 GST activity in *U. rigida* shows negative correlation with the first canonical

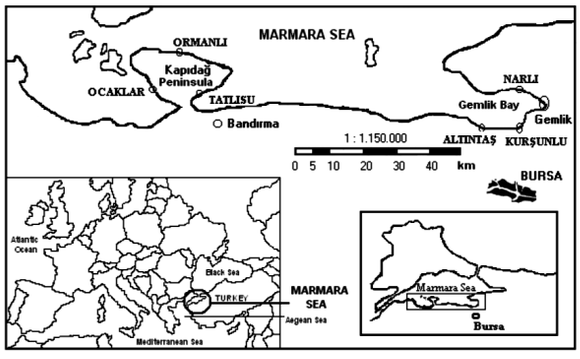

Fig. 1. Sampling sites (Ormanlı, Ocaklar, Tatlısu, Narlı, Altıntaş and Kurşunlu) in the Sea of Marmara.

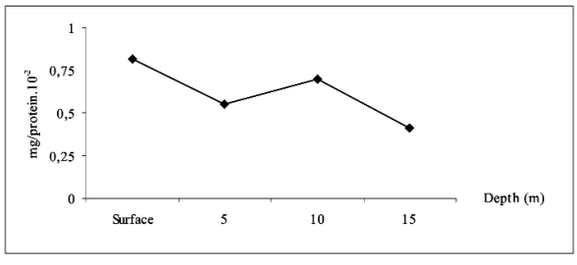

Fig. 2. Changes in GST specific activity according to depth at Tatlısu site.

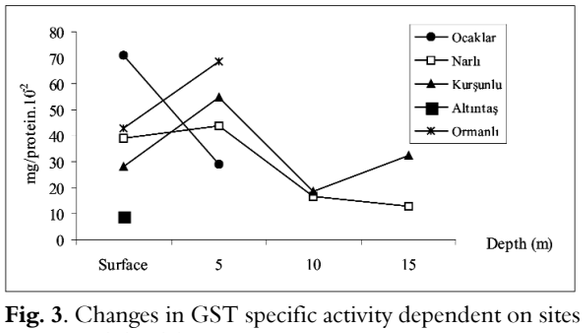

Fig. 3. Changes in GST specific activity dependent on sites and depth⋆.

⋆ *Ulva rigida* samples were not found at 10, 15 m at Ocaklar and Ormanlı sites, nor at 5,10 and 15 m at Altıntaş site.

variable (W1; -0.999). However, DON (V1; -0.556) and BOD_5 (V1; -0.282) displayed negative correlations and NH_3-N (V1; 0.605) and CO_3 (V1; 0.465) displayed positive correlations with the second canonical variables. In addition, according to Spearman rank correlation, GST enzyme activity in *U. rigida* is negatively correlated with NH_3-N (0.597; $P<0.05$) and positively correlated with DON (0.548; $P<0.05$).

DISCUSSION

According to CANCORR analysis, GST activity in *U. rigida* shows a strong negative correlations with NH_3-N and CO_3, and strong positive correlations with DON and BOD_5. The CANCORR results suggest that GST activity is strongly influenced by nitrogen concentrations, especially NH_3-N. The lowest GST activity in *U. rigida* was determined at Tatlısu and Altıntaş sites, displayed on the upper right part of the X axis in Fig. 4, and showed strong correlations with NH_3-N. Interestingly, the lowest DON values were also determined at Tatlısu. Rios-Gonzales et al. (2002) found that maize grown under ammonium nutrition showed highest GST activity in the leaves and roots. In addition, Pinchetti et al. (1998) explained that the green macroalga *Ulva* has been used widely as a biofilter because of its high efficiency in the removal of nitrogenous inorganic compounds. However, according to Lobban and Harrison (1994), when ammonia nitrogen concentration was over 30-50 μM, this effected an inhibition or toxicity in some macroalgae. Yıldız et al. (2003a, 2003b) also found that the pollution load at Tatlısu site was higher, using some biotic and abiotic factors. This supports our findings that the highest values of NH_3-N were noted at the same site. Nitrate and ammonium ions are the two major forms of nitrogen sources which are taken up by macroalgae. However, ammonia nitrogen is used as a primary nitrogen source by many macroalgae (Darley 1982). Rio et al. (1995) stated that *U. rigida* used ammonia nitrogen as a primary nitrogen source. Yıldız et al. (2003b) also found positive correlations between ammonia nitrogen and total protein amounts in *U. rigida*. This case seems to support the findings of Rio et al (1995) and Yıldız et al. (2003b).

The CANCORR analysis also shows one important consequence. An increase in NH_3-N values means a decrease in GST activity, but, interestingly, increasing DON and BOD_5 values also increases GST activity. Spearman rank correlation results show the same relations. DON-related sites (Ormanlı, surface and 5 m deep) are displayed at the bottom right of the X axis and the highest DON concentration was determined at Ormanlı at a depth of 5 m (Fig. 4). The second highest level of GST activity was also determined at this site. However, the highest level of GST activity was determined at Ocaklar-surface, which is displayed on the upper left part of the X axis, related to BOD_5. BOD_5 is generally a tool for measuring organic pollution. The positive relations of DON and BOD_5 with GST enzyme activity also suggest that an increase in GST activity is related to organic

	Axis 1	Axis 2
T	0.123	-0.115
DO	-0.078	0.516
BOD_5	-0.114	0.331
Salinity	0.392	0.043
EC	0.393	0.043
TDS	0.394	0.046
CO_3	0.022	0.556
HCO_3	0.33	-0.093
o-PO_4	-0.149	-0.248
NH_3-N	0.303	0.31
NO_2-N	-0.383	0.12
NO_3-N	0.364	-0.069
DON	0.004	-0.332

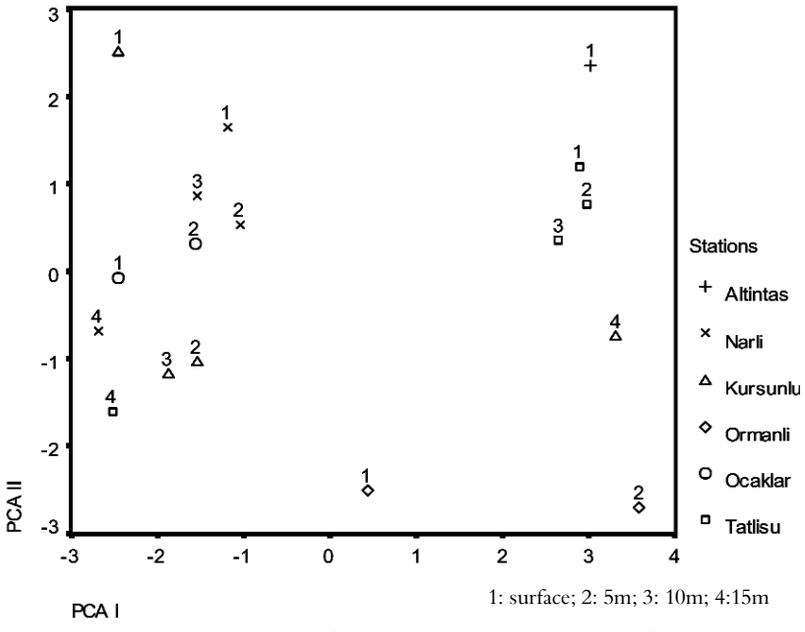

Fig. 4. Plot of the samples from each site according to their scores in the first two principal components of the abiotic factors.

Tablo 1. The changes in some abiotic factors depend on sites and depth.

Sites	Depth (m)	T (°C)	DO (mg L^{-1})	BOD_5 (mg L^{-1})	pH	Salinite (ppt)	EC (mS cm^{-1})	TDS (mg L^{-1})	CO_3^{-2} (mg L^{-1})	HCO_3^- (mg L^{-1})	o-PO_4 (mg L^{-1})	NO_2-N (mg L^{-1})	NH_3-N (mg L^{-1})	NO_3-N (mg L^{-1})	DON (mg L^{-1})
Tatlısu	Surface	16.8	10.50	0.10	8.46	18.5	30.10	20.70	30	160	0.011	0.014	0.648	0.49	0.002
	5m	14.5	10.55	0.15	8.41	22.5	36.60	24.40	20	140	0.013	0.014	0.548	0.45	0.001
	10m	12.9	9.60	0.20	8.29	20.9	34.10	22.70	20	135	0.015	0.010	0.538	0.46	0.001
	15m	12.7	8.58	0.18	8.16	6.2	11.05	7.36	0	75	0.020	0.086	0.254	0.22	0.372
Ocaklar	Surface	10.5	9.48	1.98	-	6.8	11.95	7.93	15	85	0.006	0.086	0.050	0.30	0.547
	5m	10.5	9.68	2.58	-	9.2	15.80	10.52	5	100	0.005	0.084	0.332	0.27	0.308
Ormanlı	Surface	16.8	8.47	1.07	8.23	12.2	20.70	13.78	0	145	0.025	0.006	0.166	0.41	0.805
	5m	15.0	8.78	2.18	8.25	25.6	40.80	27.20	0	220	0.025	0.0001	0.320	0.41	2.538
Kurşunlu	Surface	17.7	13.8	4.40	8.53	7.4	12.87	8.57	30	115	0.023	0.094	0.306	0.20	0.755
	5m	13.7	9.48	0.88	8.22	10.3	17.62	11.75	0	98	0.021	0.088	0.274	0.25	0.288
	10m	12.7	9.14	0.54	8.20	9.7	16.61	11.06	0	85	0.020	0.084	0.306	0.18	0.426
	15m	12.2	8.50	0.10	8.16	20.7	33.80	22.6	0	170	0.003	0.014	0.580	0.51	0.002
Narlı	Surface	10.0	10.89	2.89	-	13.0	21.70	14.48	20	105	0.012	0.092	0.420	0.20	0.378
	5m	9.4	10.12	2.72	-	14.6	24.00	16.12	15	135	0.021	0.086	0.248	0.18	0.318
	10m	9.5	10.81	1.71	-	11.7	19.35	12.89	25	140	0.023	0.086	0.248	0.17	0.408
	15m	8.9	8.98	1.58	-	7.1	12.44	8.29	20	95	0.035	0.088	0.274	0.22	0.884
Altıntaş	Surface	9.7	9.84	3.64	-	23.6	38.20	25.40	25	135	0.006	0.014	0.792	0.40	0.135

pollution. Pflugmacher et al. (2000) found that GST activity increased with a natural substrate cinnamic acid, which also supports our findings.

CO_3 values show a different result. CO_3-related sites are also displayed on the upper part of the X axis and interestingly, the site showing the highest GST activity, Ocaklar-surface, is displayed on the upper-left side, whereas the lowest GST activity sites are displayed on the upper-right side of the X axis of Fig. 4. This finding may suggest that CO_3 value or CO_3-related pH affect GST activity independently. Unfortunately, the missing measurements of pH do not show the pH relation directly. However, Blanchette and Singh (2002) found that a pH profile analysis of the GST isozyme-Q3 isolated from Northern Quahog indicated that the optimum catalytic pH is 7.6.

According to PCA ordination, salinity seems to be the most important factor that affected the site features. We also expected strong correlations between GST activity and salinity, because of the large range of salinity values (Table 1). However, CANCORR analysis does not show strong correlations. Fitzpatrick et al. (1997) have said that salinity stress does not result in elevated GST specific activity in *Mytilus edulis* L., which supports our results. However, Rios-Gonzales et al. (2002) have found that GST activity increased in maize

roots under salinity.

We found a negative correlation (0.734; $P<0.01$) between total protein amounts in a study of Yıldız et al. (2003b) and GST activity determined in our study. We think that xenobiotics affected protein synthesis indirectly because of the negative correlations between total protein amount and GST activity. At the same time GST activity also increased due to the fact that these xenobiotics are substrates of GST enzyme. Toxic substrates affect enzyme activities both directly and indirectly. In a study by Pinchetti et al. (1998), changes in 1,5-biphosphate carboxylase/oxygenase and carbonic anhydrase in exist of NH_4^+ in *U. rigida* were observed. This case was explained changes in equilibrium among inorganic nitrogen, photosynthesis and carbon metabolism (Pinchetti et al. 1998). In *Daphnia magna* Straus, studies on a series of six chlorinated phenols have shown that there is a clear relationship between lipophilicity, toxicity, and inhibition of GST activity (LeBlanc et al. 1988).

In this study, the lowest GST activity was determined at Tatlısu site and activity was ten times lower at the second lowest concentration determined at Altıntaş (Figs. 2, 3). Tatlısu site is located in Bandırma port, which has a population of about 140.000 and is the second largest port on the Sea of Marmara. About 15% of the fertilizer (calcium, ammonium, nitrate) produced in Turkey is produced in fertilizer plants in Bandırma. Moreover, there are various acid plants together with a total of 66-industrial establishments in this bay. The port of Bandırma is also subjected to an intense maritime traffic (cargo ships). We consider that GST enzyme activity in *U. rigida* taken from Tatlısu site, near the important pollution sources, was inhibited by various pollutants coming from the factories.

These unstable changes in GST activity, related to depth and site differences, suggest that different pollutants affected GST activity at different locations. Moreover, we believe that the types and concentrations of pollutants are more important than changes in water depth in affecting GST activity.

In this study, we have measured the effects of some important environmental variables and nutrients on GST activity, and found the important relations described above. However, some important chemicals may affect GST activity highly and these kinds of toxic chemicals may be abundant at these sites because of the proximity of important ports, factories and urban sites. Rees (1993) has shown that GST activity increases with water pollution. According to Fitzpatrick et al. (1997) and Stien et al. (1998), GST reacts to polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Robillard et al. 2003). In addition, GST activity decreases with increases in total pesticide (Robillard et al. 2003) and heavy metal concentrations (Stien et al. 1998, Nagalakshmi and Prasad 2001). In other studies, cadmium caused an increase in GST activity in *Posidonia oceanica* (L.) Delile (Hamoutène et al. 1996), whereas copper increased GST activity in *Scenedesmus bijugatus* (Turpin) Kuetzing (Nagalakshmi and Prasad 2001) and copper and selenium lead to increased GST activity in freshwater fish (Lopes et al. 2001).

As a result, it is known that *U. rigida* in polluted water is more prevalent than in clean water although it is present in clean water. *U. rigida* is a biological marker of aquatic contamination in eutrophication and metal pollution (Haritonidis and Malea 1999). A change was shown in GST enzyme activity in *U. rigida* collected at different depths from Gemlik Bay and Kapıdağ Peninsula in respect to water pollution and some physicochemical variables. It seems to support the findings that GST activity increases related to environmental pollution in many living organisms (Sheenan et al. 1991, Leaver et al. 1992). However, Robillard et al. (2003) suggest that inhibition of in situ GST activity may be a more useful biomarker, but they added that more work is needed to assess to which pollutants this enzyme is more responsive.

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