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SEASONAL CHANGES IN OXIDATIVE STRESS BIOMARKERS OF THE SNAIL *VIVIPARUS ACEROSUS* FROM THE VELIKA MORAVA RIVER, SERBIA

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Abstract - The river snail (*Viviparus acerosus*) from the Velika Morava River, Serbia was chosen in our study in order to determine seasonal changes in oxidative stress biomarkers between July (summer) and September (autumn). The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and the phase II biotransformation enzyme glutathione-S-transferase (GST), as well as the concentration of total glutathione (GSH), were examined in the whole body of the river snails. The obtained results show significantly higher activities of CAT, GSH-Px, GR and biotransformation phase II enzyme GST in September compared to July, while the GSH concentration was lower. There was no general trend in the seasonal changes in the activity of SOD. The presented data show that animals in their natural environment are exposed to constant fluctuations of environmental conditions that could cause antioxidants to exhibit seasonal variations. This fact should be considered as an important variable in the interpretation of results in biomonitoring studies.

Keywords: *Viviparus acerosus*, river snail, Velika Morava River, oxidative stress, antioxidant defense, season

INTRODUCTION

Studies of the link between biomarkers of oxidative stress biomarkers and seasonal changes in poikilothermic organisms have revealed their strong relationship with metabolic demands (Van der Oost et al., 2003). A lower metabolic rate is accompanied by lower antioxidative defense. The role of individual components in homeostasis is apparently different and is integrated in the antioxidative defense system. In aquatic animals, many natural and anthropogenic factors (xenobiotics) can induce an imbalance between the production of reactive oxygen species (ROS) and their removal, and as a result oxidative stress occurs (Halliwell and Gutteridge, 2007). The main enzymes that detoxify ROS in aerobic organisms are functionally divided into enzymes with antioxidant activity (superoxide dismutase-SOD, catalase-CAT, glutathione peroxidase-GSH-Px and glutathione reductase-GR) and phase II biotransformation components (e.g., glutathione-S-transferase-GST). In addition, nonenzymatic components (e.g. reduced/oxidized glutathione-GSH) contribute to the removal of ROS (Van der Oost et al., 2003). These parameters are widely used in biomonitoring studies as biomarkers and they were characterized in a certain number of aquatic organisms such as crustaceans (Antó et al., 2009), snails (Li et al., 2008), mussels (Vidal et al., 2002; Borković et al., 2005), annelidae (Geracitano et al., 2004) and fish (Aras et al.,

2009: Regoli et al., 2011; Oliva et al., 2012). Depending on the availability of nutrients, reproductive status, season-related growth rate and other factors, the activity of antioxidant defense enzymes and other biomarkers fluctuates significantly throughout the year (Sheenan and Power 1999). Some aspects of seasonal variations in antioxidant defense have been observed in the tissues of many aquatic organisms, such as thin-lip gray mullet (*Liza ramada*) (Pavlović et al., 2004), mussel (*Mytilus galloprovincialis*) (Borković et al., 2005; Bocchetti et al., 2008), horse mussel (*Modiolus modiolus*) (Lesser and Kruse, 2004), blue mussel (*Mytilus edulis*) (Manduzio et al., 2004) and in the digestive gland of brown mussels (*Perna perna*) (Wilhelm Filho et al., 2001). Seasonal variations have also been observed in the levels of pollutants and in natural exposure to oxidative stress (Sheenan and Power 1999).

Molluscs are one of the most important components of the macroinvertebrate community of the aquatic ecosystems in Serbia (Tomović et al., 2010). They are widespread and abundant in all types of ecosystems. Molluscs have limited ability to excrete contaminants via their excretion organs, metabolize organic chemicals and inactivate toxic heavy metals, thereby causing higher bioaccumulations of many toxicants (Foeckler et al., 2006; Oehlmann and Schulte-Oehlmann, 2002).

The river snail, *Viviparus acerosus* (Bourguignat, 1862) is a central European species originally inhabiting the Danube drainage system. Its dextrous shell grows to between 30 and 57 mm in length; it is a dirty grayish yellow to yellowish green and has three red-brown bands varying in width. Viviparity characterizes the reproductive pattern, and it is associated with iteroparity and parental care (Jakubik, 2006).

The Velika Morava River and its tributaries constitute a major river system in Serbia. The riverbed consists mainly of gravel, sand and stones of different size. The Velika Morava flows through the most fertile and densely populated area in central Serbia. The main pollution sources are landfills that arise in areas of gravel exploitation, agricultural, municipal and industrial waste.

The aim of our study was to investigate the activities of antioxidative defense enzymes: superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px EC 1.11.1.9) and glutathione reductase (GR EC 1.6.4.2), as well as the activity of the phase II biotransformation enzyme glutathione-S-transferase (GST EC 2.5.1.18) and total glutathione (GSH) concentration in the whole body of river snails from the Velika Morava River (Bagrdan) collected in July (summer) and September (autumn).

Fig. 1. The geographical position of sampling site at the Velika Morava River.

MATERIALS AND METHODS

Sampling site and snail collection

The geographical position of the sampling site (Bagrdan, 44˚05'99" N and 21˚11'34" E) on the Velika Morava River is presented in Fig.1. Specimens of the river snail (*Viviparus acerosus* Bourguignat 1862) were collected in July (12 individuals with average length 29.87 ± 0.32 mm and average weight 7.00 ± 0.20 g) and September (12 individuals with average length 21.90 ± 0.43 mm and average weight 2.69 ± 0.14 g). These two periods were chosen because some abiotic parameters, such as water temperature, pH and oxygen saturation can modulate physiological processes in snails and could thus affect the changes of biomarkers. Live snails were brought to the laboratory, measured, weighed and immediately killed. The soft tissue of the snails was rapidly dissected out and frozen at -80°C for further biochemical analysis.

Biochemical analysis

The whole soft body of river snails *Viviparus acerosus* was minced and homogenized in 5 volumes (Lionetto et al., 2003) of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5 at 4°C, with an Ultra-Turrax homogenizer (Janke & Kunkel, IKA-Werk, Staufen, Germany) (Rossi et al., 1983). The homogenates were sonicated for 30s at 10 kHz on ice. One part of the sonicated material was used for determination of the concentration of GSH and centrifuged at 5000 rpm for 10 min in 10% sulphosalicylic acid. The concentration of GSH was detected according to Griffith (1980) and expressed as nmol/g of tissue. Another part of the sonicated material was centrifuged at 4°C at 100,000 *g* for 90 min (Takada et al., 1982), and the resulting supernatants were used for the measurement of enzyme activities. Total protein concentration was determined according to the method of Lowry et al. (1951). SOD activity was estimated by the epinephrine method (Misra and Fridovich, 1972), based on the ability of SOD to inhibit the autoxidation of adrenaline to adrenochrome. The activity of CAT was assayed by the method of Claiborne (1984), which is based on H_2O_2 degradation by the action of CAT contained in the examined samples. GSH-Px activity was evaluated following the oxidation of NADPH as a substrate with t-butyl hydroperoxide (Tamura et al., 1982). The activity of GR was estimated by measuring NADPH oxidation as described by Glatzle et al. (1974). The activity of the phase II biotransformation enzyme GST was detected by the procedure of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. All enzyme activities were measured simultaneously in triplicate for each sample using a Nicolet Evolution 600 UV-Vis spectrophotometer and expressed as specific in U/mg protein.

Statistical analyses

The data are expressed as mean ± S.E. (standard error). The non-parametric Mann-Whitney U-test was used to determine significant differences between means. A minimum significance level of $p<0.05$ was accepted. Principal Component Analysis (PCA) was employed to detect variables that significantly contributed to differences in the activity of the investigated oxidative stress biomarkers between the two examined seasons. Spearman correlation coefficients were used to confirm seasonal influence and the contribution of each of the investigated oxidative stress biomarkers. The analytical protocols described by Darlington et al. (1973) and Dineen and Blackesley (1973) were followed.

RESULTS

The physico-chemical parameters of the water in July and September in the Velika Morava River (Bagrdan) are presented in Table 1. The obtained results show that only the water temperature exhibited markedly different changes between July and September, with higher values in the summer period. Other parameters show similar values in July and September. In Table 2, the total protein concentration (mg/g wet mass) in the whole body of the river snail (*Viviparus acerosus*) is presented and no significant changes between the two seasons were observed. The specific activities of CAT (Fig. 2), GSH-Px, GR and phase II biotransformation enzyme GST (Fig. 3) were sig-

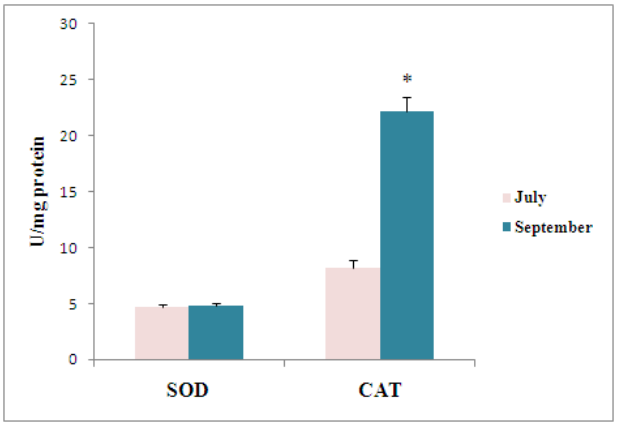

Fig. 2. Specific activities (U/mg protein) of SOD and CAT of the river snail (*Viviparus acerosus*) from the Velika Morava River in July and September. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. A minimum significance level of * $p<0.05$ was accepted.

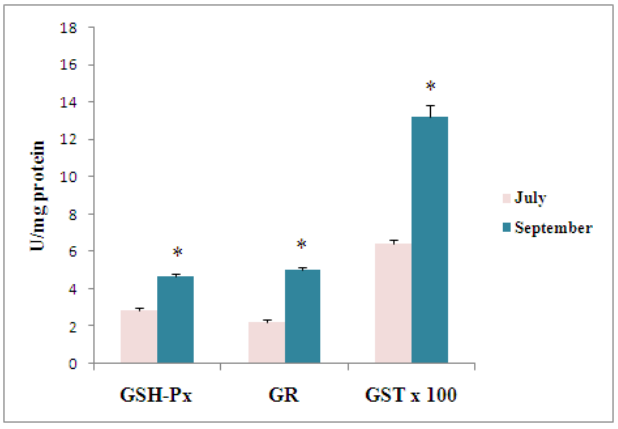

Fig. 3. Specific activities of GSH-Px, GR and phase II biotransformation enzyme GST in the river snail (*Viviparus acerosus*) from the Velika Morava River in July and September. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. A minimum significance level of * $p<0.05$ was accepted.

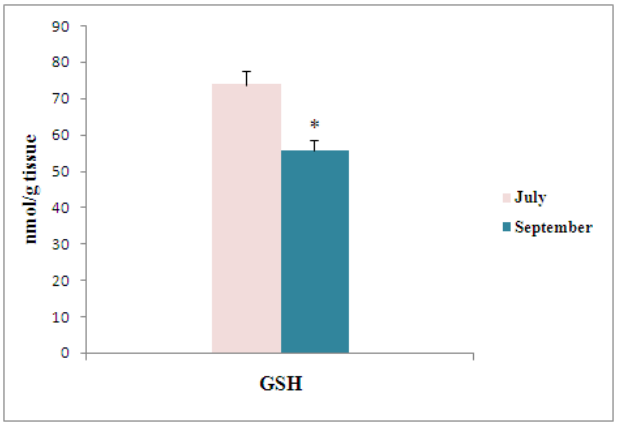

Fig. 4. The concentration of GSH in the river snail (*Viviparus acerosus*) in July and September in the Velika Morava River. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. A minimum significance level of * $p<0.05$ was accepted.

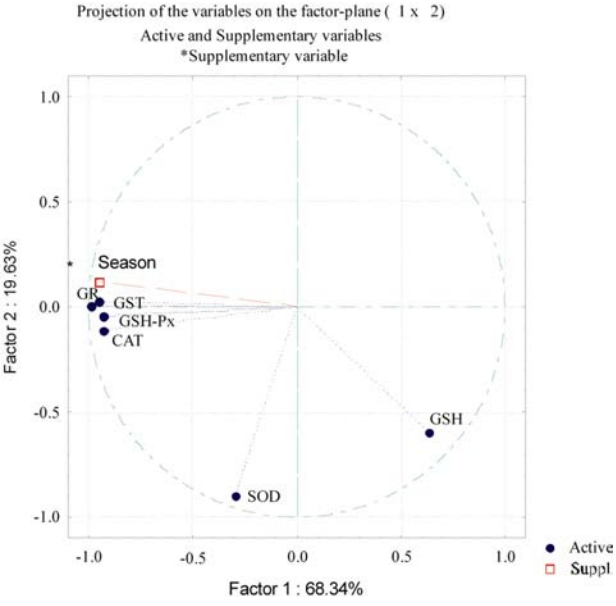

Fig. 5. Principal Component Analysis (PCA) represents the contribution of each individual antioxidant defense parameter of the river snail (*Viviparus acerosus*) from the Velika Morava River in response to seasonal pattern.

nificantly increased in September in comparison to July ($p<0.05$). No significant changes of SOD (Fig. 2) activity between the two investigated seasons were observed. GSH concentration (Fig. 4) was significantly decreased in September in comparison to July ($p<0.05$).

Principal Component Analysis (PCA) was applied to define statistically the differences in antioxidative defense enzyme activities between the two investigated seasons. The results of PCA for the antioxidative defense enzyme activities of SOD, CAT, GSH-Px and GR and phase II biotransformation enzyme GST, as well as GSH concentration in the river snail, are presented in Fig. 5. The treatment of overall data by PCA indicated that the season was

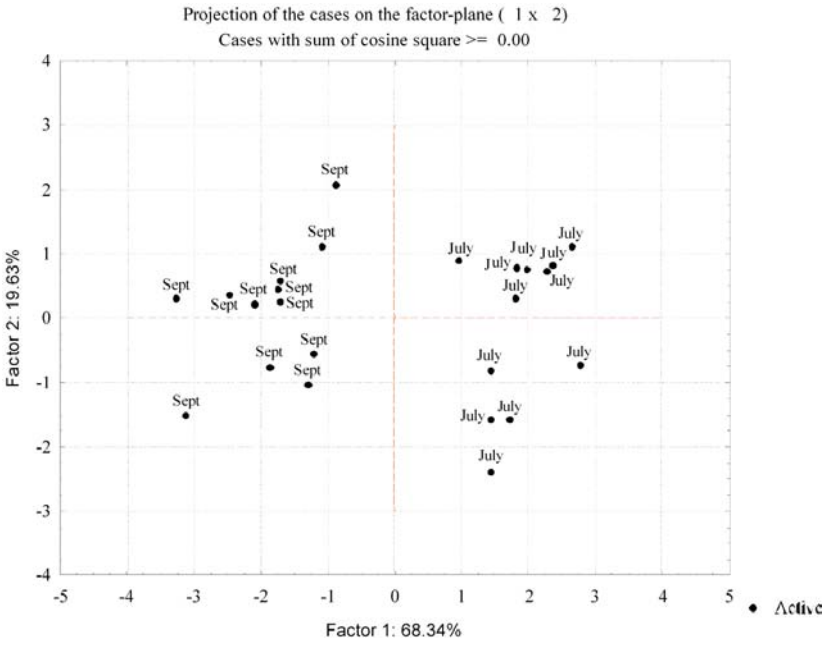

Fig. 6. Principal Component Analysis (PCA) of all investigated antioxidant defense parameters of each individual of the river snail (*Viviparus acerosus*) from the Velika Morava River in July and September.

a dominant factor in the differences between the investigated parameters (Factor 1: 68.34% and Factor 2: 19.63%). PCA of the investigated parameters is presented in Fig. 6. Another confirmation of seasonal effects is given in Table 3A as Spearman rank order coefficients with statistically significant positive correlations for CAT, GSH-Px, GR and phase II biotransformation enzyme GST, and a negative correlation for GSH ($p<0.05$). All investigated antioxidative defense enzyme activities, except SOD, correlate positively between each other, and negatively in comparison to GSH ($p<0.05$) (Table 3B). No correlation was observed between SOD activity and the values of the other investigated parameters (Tables 3A and 3B).

DISCUSSION

River basins are among the most endangered ecosystems worldwide (Li et al., 2010). A number of natural and synthetic compounds (xenobiotics) enter rivers and spread through different routes of dispersion in aquatic ecosystems. Being the largest river in Serbia, the Velika Morava suffers the same endangerment.

Xenobiotics can cause an overexpression of the ROS that are constantly produced in the tissues of aerobic organisms, either directly as redox cycling compounds or through conversion in these compounds in the processes of biotransformation. This leads to changes in the activity of enzymes and other components of the antioxidative defense system that act together to achieve homeostasis. In a number of previous biomonitoring studies, antioxidative defense components were proposed as suitable biomarkers of oxidative stress (Regoli and Principiato 1995; Pavlović et al., 2004; Borković et al., 2005).

The interrelationship between the type and levels of xenobiotics, environment and living organisms is complex. Environmental conditions vary and they affect the contaminants, which complicates the interpretation of the results of biomonitoring studies (Sheehan and Power, 1999). In field studies, the oxidative destruction and activation of antioxidant defense enzymes are usually observed under con-

Table 1. Physico-chemical parameters of water in July and September in the Velika Morava River (Bagrdan).

	July	September
Temperature (°C)	24.60	20.50
pH	7.93	8.20
O_2 (mg/L)	7.90	8.55
O_2 (%)	96.00	95.60

Table 2. Total protein concentration (mg/g wet mass) in the whole body of the river snail (*Viviparus acerosus*) from the Velika Morava River in July and September.

	July	September
Protein concentration (mg/g wet mass)	5.07 ± 0.16	4.39 ± 0.13

Table 3 (A). Spearman rank order coefficients [RS (p-values)] between antioxidant enzyme activities and season of the river snail *Viviparus viviparus* from the Velika Morava River. Marked correlations are significant at * p<0.05.

	Season and SOD	Season and CAT	Season and GSH-Px	Season and GR	Season and GST	Season and GSH
Spearman R	0.204656	0.854740*	0.854740*	0.866778*	0.866778*	-0.674307*
p level	0.337414	0.000000	0.000000	0.000000	0.000000	0.000302

Table 3 (B). Spearman rank order coefficients (RS values) between antioxidant enzyme activities of the river snail *Viviparus viviparus* from the Velika Morava River. Marked correlations are significant at * p<0.05.

	SOD	CAT	GSH-Px	GR	GST	GSH
SOD		0.404348	0.307826	0.393043	0.354783	0.122200
CAT	0.404348		0.766957*	0.873913*	0.859130*	-0.593607*
GSH-Px	0.307826	0.766957*		0.835652*	0.837391*	-0.469232*
GR	0.393043	0.873913*	0.835652*		0.869565*	-0.599696*
GST	0.354783	0.859130*	0.837391*	0.869565*		-0.552729*
GSH	0.122200	-0.593607*	-0.469232*	-0.599696*	-0.552729*	

ditions of moderate pollution (Ferreira et al., 2005) or seasonal influences (Pavlović et al., 2004, 2009). In addition, the aquatic animals that are widely explored in biomonitoring exhibit the high seasonal dependence of these markers. They are therefore considered to be unreliable for monitoring purposes. Studies of aquatic animals have demonstrated that seasonal variations in different biomarkers were attributable to environmental and biological factors, mainly temperature and the metabolic status of the animals, rather than to the site (Leiniö and Lehtonen, 2005).

Snails are poikilotherms whose metabolism strongly depends on environmental factors. Seasonality in aquatic habitats is primarily reflected in the

changes in temperature and amount of dissolved oxygen. In our study, the difference in water temperature was prominent between July and September. An increase in temperature leads to elevated metabolic demands and this is accompanied by increasing enzymatic activities. According to our results, there were no differences in SOD activity in summer (July) and autumn (September), which can be explained by its high level of constitutive activity in *Viviparus acerosus*. Other authors have obtained controversial data. Lavarías et al. (2011) did not observe clear seasonal trends for SOD, CAT and phase II biotransformation enzyme GST. Contrary to this, Buchner et al. (1996) showed that the activity of SOD is affected by environmental temperature. Increased ambient temperature resulted in elevated SOD activity in the chloragog tissue of lugworms. In barnacles, a distinct seasonal pattern in SOD was shown by Niyogi et al. (2001), with a maximum activity in summer.

The activity of CAT in the whole body of *Viviparus acerosus* was significantly higher in September than in July, indicating an increased presence of hydrogen peroxide (H_2O_2). In shallow coastal water, solar radiation can lead to H_2O_2 accumulation as a consequence of UV-driven oxygen radical formation. This process has a seasonal character and during the summer the concentration of H_2O_2 is 10 times higher (Buchner et al., 1996). We collected *Viviparus acerosus* from the water near the riverbank that showed a similar pattern to that of *Viviparus viviparus* in the forming of aggregations in some places of the shore during summer, as described by Jakubik (2006).

The antioxidant enzyme GSH-Px predominantly acts as a scavenger of organic hydroperoxides; we observed an increased activity of this enzyme in September. Sroda and Cossu-Leguille (2011) obtained similar results in the body of the freshwater species *Gammarus roeseli*. They explained that seasonal variability is in a relation with the reproductive status, with low enzymatic activity in periods without oogenesis. The reproductive process of *Viviparus viviparus* is influenced by water temperature and it can last for several months, from March until November/December (Jakubik, 2006).

The role of GR is to recycle GSH from its oxidized form GSSG. The GR activity was complementary to the GSH concentration. The lower concentration of GSH in September was accompanied by an increased activity of GR (Table 3B).

The phase II biotransformation enzyme GST has a crucial role in catalyzing the conjugation of electrophiles with GSH and modulating the toxicity of many exogenous compounds (Van der Oost et al., 2003). Correlations between phase II biotransformation enzyme GST activity and water temperature were investigated by some authors. Ronisz et al. (1999) showed a positive correlation of GST activity with temperature in female eelpout. On the other hand, Pavlović et al. (2004) obtained higher activities in winter than in spring in the liver and white muscle of *Liza ramada*, which was explained as the effects of the synergism between cold-stress and toxic effects of chemical pollutants. The phase II biotransformation enzyme GST activity in *Pomatoschistus minutus* was maximal in autumn and minimal in spring; however, according to Solé et al. (2006) there is no correlation between water temperature and GST activity.

The overall trend obtained in our work demonstrated an increased activity of the investigated enzymes in autumn (September) compared to summer (July). Multiple factors contribute to elevated levels of oxidative stress. The effects of certain types of pollutants that are present cannot be excluded. Other studies showed that changes in temperature and food availability induce oxygen consumption and cellular oxyradical generation, which are compensated by increasing the antioxidative defense (Borković-Mitić et al., 2011).

The metabolism of GSH is regulated by several enzymes. Cellular GSH content depends on the rates of synthesis, conjugation, oxidation and GR-dependent reduction GSSG to GSH. We observed a significant decrease in the GSH concentration in snails in September, which positively correlates with the increased activities of GSH-Px and GR in the same period (Table 3B). Viarengo et al. (1991) obtained the lowest values of GSH content in the diges-

tive gland of the mussel *Mytilus galloprovincialis* in winter, when there is less food and the gonads are in a state of rest.

In order to calculate the relative contribution of each investigated antioxidant defense component in the river snail, we performed Principal Component Analysis (PCA). The treatment of the overall data by PCA confirmed that season is a dominant factor for differences between the investigated parameters. At the same time, CAT, GSH-Px, GR and the phase II biotransformation enzyme GST significantly contribute to the observed seasonal variations in biomarker activities (Fig. 5). PCA of all investigated variables revealed a clear separation between individuals sampled in July and individuals from September (Fig. 6).

To prove the seasonal pattern of the investigated parameters, we performed Spearman non-parametric analysis. The Spearman rank order coefficients showed strong seasonal influences on all investigated enzymes, except SOD.

In conclusion, this study indicates the usefulness of integrating a set of oxidative stress biomarkers, which is important for environmental biomonitoring using the river snail *Viviparus acerosus* as a sentinel species. In addition, seasonality is a very important factor that could be included in the interpretation of results of biomonitoring studies.

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