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State of the Marine Environment along the Bulgarian Black Sea Coast as Indicated by Acetylcholinesterase Activity of Wedge Clam (Donax trunculus Linnaeus, 1758)

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Abstract. The aim of the study was to assess changes in the acetylcholinesterase (AChE) activity in the wedge clam (*Donax trunculus* L.), gathered in spring (March), summer (June/July) and early autumn (September) from shallow sublittoral sandy habitats at different localities along the Bulgarian Black Sea coast. It is well established that inhibition of AChE in marine organisms is mainly caused by the neurotoxic effects of organophosphate and carbamate pesticides, however recently it was found that a number of other chemical substances have similar effects including heavy metals, polycyclic aromatic hydrocarbons, detergents etc. The activity of AChE in the soft body of the clams in our study showed considerable variation among the studied localities. The most significant AChE inhibition was present in clams from localities close to coastal areas with intensive tourism, such as the big resorts Slanchev Bryag, Duni, Arkutino and Primorsko. Significant seasonal differences in AChE activity were also established. Higher activity of AChE was present in the wedge clams gathered in autumn than in the other seasons. Overall, our results demonstrated for the first time the presence of significant ecotoxicological effects of anthropogenic impact on the shallow sublittoral sandy habitats along the Bulgarian Black Sea coast where *D. trunculus* is a dominant. Hence, this clam species appears to be suitable bioindicator of marine environmental quality.

Key words: Acetyl choline esterase activity; Bulgarian Black Sea; *Donax trunculus* L

Introduction

Anthropogenic pressures are increasingly affecting marine ecosystems, causing significant changes in their state and functionality. The increase of urbanization and intensification of industry, agriculture and transport are leading to increased pollution of the marine environment (Gherras Touahri et al., 2016). The Black Sea is a unique semi-exclosed basin, which is accepted to be one of the highly polluted seas (Oguz & Velikova, 2010; Makedonski et al., 2017). Until recently, the main approach to marine environmental assessment has been the measurements of certain pollutants, both in the environment and in organisms. However, this approach does not provide sufficient information on the reaction of living organisms and their degree of adaptation to environmental changes and deterioration. Moreover, the number of possible pollutants is huge and they can have not only cumulative, but also synergistic effects (Canesi et al., 2014; Vernon et al., 2020), as the reported synergistic effects of global climate change and marine pollution (Cabral et al., 2019). Thus, assessment of the biological responses of susceptible organisms, along with the qualitative and quantitative analysis of chemical contaminants, is becoming a key tool in monitoring programs (Allan et al., 2006; Depledge, 2009). Such integrative approach has been also required to achieve Good Environmental Status in European marine waters as required by the Marine Strategy Framework Directive (MSFD, 2008/56/EC) (Lyons et al., 2010).

Currently, a number of biomarkers, as well their combinations, are used to assess the impacts of pollution, environmental risk and marine environmental management (Galloway et al., 2004; Viarengo et al., 2007). In modern ecotoxicology, two types of biomarkers are conventionally distinguished - those that reflect the exposure of organisms to environmental stressors (Exposure Biomarkers) and those that show the adverse effect of stressors on the state of organisms (Effect Biomarkers) (Hook et al., 2014). Inhibition of acetylcholine esterase (AChE) is used as a marker for both, exposure and effect, mainly of organophosphorus and carbamate pesticides in aquatic organisms (Bocquené et al., 1990; Magni et al., 2006; Blaise et al., 2017). AChE plays an important role in the functioning of the neuromuscular system by determining the nerve impulse transmission. When AChE is inhibited, it may cause continuous muscle contractions with depletion of energy resources, leading to serious dysfunction in organisms, e.g., paralysis and death (Fulton & Key, 2001). However, it has been established that despite pesticides, AChE may be inhibited also by a number of other substances present in the marine environment, i.e. heavy metals (Frasco et al., 2005; Sabullah et al., 2014), PAHs (Fu et al., 2018), PCBs, DDT, detergents (Lionetto et al., 2011; Tsangaris et al., 2011), algal toxins (Lehtonen et al., 2003), and recently microplastics (Tlili et al., 2020). AChE activity has been identified and biochemically characterized in many aquatic invertebrate species (Fulton & Key, 2001). In particular, high levels of AChE activity in the gills, digestive gland and haemolymph have been reported in different bivalves (Srivatsan, 1999; Escartín & Porte, 1997; Galloway et al., 2002), among which mussels from the order *Mytilida*.

The aim of the present study was to obtain a preliminary assessment of the ecological and ecotoxicological risks associated with the contamination of the sublittoral sandy habitats using the wedge clam, *Donax trunculus* L. as a bio-indicator. The spatial and temporal variations of AChE activity were measured in wedge clams collected from different localities along the Bulgarian Black Sea coast.

Materials and Methods

Materials

The reagents acetylthiocholine iodide, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and Folin-Ciocalteu reagent were purchased from Sigma Aldrich, owned by Merck KGaA (St. Louis, USA). All other chemicals were of the highest commercially available purity.

Sampling

Adult clams (23-35 mm) were collected from their natural shallow sublittoral sandy habitats (depth 0.5 – 2.5 m) along the Bulgarian Black Sea coast (Fig. 1) in different seasons: spring (March), summer (June-July) and autumn (September) of 2020. A pooled sample of wedge clams from each locality was gathered by hand dredge and was transported to the laboratory in containers with sea water. In the laboratory, the clams from each location were put in dry ice for further transportation and thereafter were stored at –80°C. For the biochemical analyses subsamples of different number of clams (n=6-12) were randomly picked up from the corresponding pooled sample.

The biochemical analyses of the total clam soft tissues were carried out in the Laboratory of Free Radical Processes of the Institute of Neurobiology-Bulgarian Academy of Sciences.

Locality	N	E
Sv. Konstantin & Elena	43.2294	28.0146
Rodni Balkani	43.2026	27.9243
Pasha Dere	43.1212	27.9284
Shkorpilovtsi	42.9603	27.8970
Irakli	42.7498	27.8901
Sveti Vlas	42.7091	27.7595
Slanchev Bryag	42.6906	27.7137
Chernomorets	42.4468	27.6422
Dyuni	42.3662	27.6927
Arkutino	42.3334	27.7312
Primorsko	42.2738	27.7565
Tsarevo	42.1744	27.8504

Fig. 1. Sampling localities along the Bulgarian Black Sea coast with geographical co-ordinates of the sampling sites.

Tissue preparation

On the day of measurements, the soft tissue of each individual wedge clam to be studied was carefully extracted and homogenized, using a Potter Elvehjem homogenizer fitted with a Teflon pestle (Thomas Scientific, USA) in chilled potassium phosphate buffer (100 mM, pH 7.5). Homogenates were centrifuged at 10 000 rpm for 20 min at 4°C using a K-24 centrifuge (Janetsky, Germany). The obtained supernatants were used for the measurement of AChE activity.

Determination of acetylcholinesterase activity

The AChE activity was assayed by the method of Ellman (Ellman et al., 1961), based on the production rate of thiocholine. The reaction mixture contained 0.1M K-PO_4 buffer pH 8.0, 0.045 M acetylthiocholine iodide, 0.008 M 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) and the appropriate amount of clams' tissue homogenate. The thiocholine, resulted from acetylthiocholine hydrolysation by AChE, reacted with DTNB and yield a yellow colored product, 5-thio-2-nitro-benzoic acid. The rate of change of absorbance at 412 nm was recorded and calculated as U (μM ACh/min)/mg protein. Protein content was measured by the method of Lowry (Lowry et al., 1951) and was calculated from a standard curve, prepared with bovine serum albumin. The assays were performed using Spectrophotometer AE-450N (ERMA Inc., Japan).

Statistical analyses

All measurements are presented as mean ± standard error (SEM). Significance of differences between groups was estimated using t-statistic and ANOVA, at $p < 0.05$ significance level. The analyses were carried out using the STATISTICA 10 software package (StatSoft Inc., Tulsa, USA).

Results

There was significant variation in the measured AChE activity both among seasons and among localities. The activities of AChE in the soft tissue of *D. trunculus* gathered in March and in June were significantly ($p<0.05$) lower than the activities in the samples from July and September (Fig. 2).

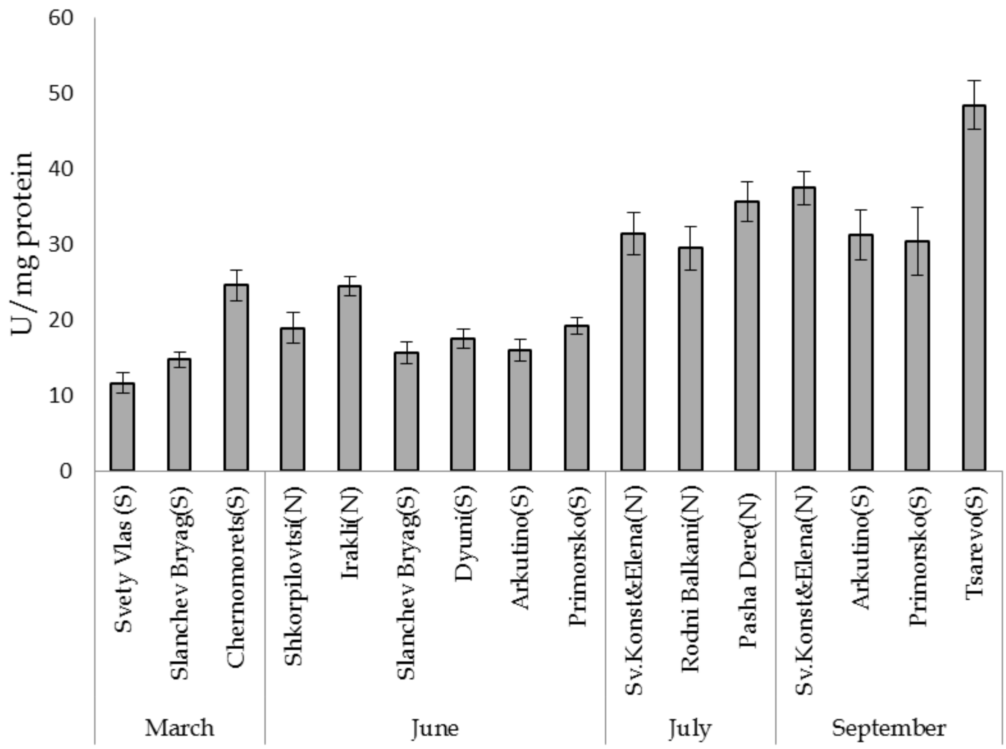

Fig. 2. Acetylcholine esterase activity in soft tissue of *D. trunculus* from different localities and seasons (S – Southern localities; N – Northern localities) in Bulgaria.

In spring the AChE activities, measured in the samples from Sveti Vlas and Slanchev Bryag were significantly lower than that from Chermomotrets. In summer, the lowest AChE activities were observed in wedge clams sampled from Slanchev Bryag and Arkutino (15.71±1.41 and 16.01±1.38 U/mg protein, respectively). It is noteworthy that the activity of the enzyme in the samples from July was almost twice as high as those from June. The AChE activities in the clams from the northern localities Sv. Konstantin & Elena, Rodni Balkani and Pasha Dere, were 31.48±2.84, 29.56±2.90 and 35.74±2.60 U/mg protein, respectively (Fig. 2). The AChE activity in the samples, collected during the autumn varied in the range from about 30 to 48 U/mg protein. The lowest average values were measured in the samples from Arkutino and Primorsko (31.30±3.27 and 30.48±4.54 U/mg protein, respectively) (Fig. 2). The highest activity of AChE was detected in the September samples from Tsarevo (48.49±3.19 U/mg protein).

It is well established that AChE activity in aquatic animals depends on the temperature of the environment. The enzyme activity in the wedge clams from

Arkutino and Primorsko was significantly higher at higher temperature of the marine water - the average water temperature in the localities for the time of sampling in June was +20.8°C, and in September +27.4°C (https://www.stringmeteo.com/synop/sea _water. php? year = 2020 # 03).

In order to confirm the observed overall patterns in AChE activity in the wedge clams from the studied localities in different seasons we applied factorial ANOVA analysis and the results are presented in Table 1. The main effect of “locality” alone was highly significant and confirmed the significance of the variations observed in the AChE activity in the wedge clams inhabiting the different localities studied (Table 1). In addition, the interdependent effect of ”locality” and “season” proved to be also highly significant (Table 1). This suggested that the effects of the state of the marine environment in a given locality on the AChE in the wedge clams living there can be different depending on the season.

Table 1. Results of ANOVA analysis of the effects of locality and season on the AChE in *D. Trunculus.*

Effects	df Effect	MS Effect	F	p
Locality	7	572.82	13.43	0.000000
Season	2	4028.19	94.47	0.000000
Locality* Season	8	564.59	13.24	0.000000

Concerning the localities, it is noteworthy that lower AChE activity was present in wedge clams from sites located near to sources of higher coastal anthropogenic impact, i.e. larger resorts. For instantce the lowest values of the enzyme were present in the samples from Sveti Vlas and Slanchev Bryag in March (Fig. 2). Similarly, lower AChE activities were measured in the tested individuals from Arkutino, Slanchev Bryag, Duni and Primorsko sampled in June, while in the clams from Irakli, an area falling within the protected sites "Emine" (for protection of wild birds) and "Emine-Irakli" (for conservation of natural habitats), significantly higher AChE activities were measured. A similar pattern was observed in the September samples, where the clams from Arkutino, Primorsko and Sv. Konstantin & Elena had significantly lower AChE activities than those from Tsarevo which, in turn, were over 30% higher than those from Arkutino and Primorsko, and over 20% higher than those from Sv. Konstantin & Elena. Although on average in the wedge clams gathered from the northern regions higher activities of AChE were found compared to those from the southern localities, the differences were rather individual and seemed to be due to the state of the marine environment at the concrete locality.

Discussion

Inhibition of AChE is considered as a specific indicator reflecting the pollution with organophosphorous and carbamate compounds either in aquatic or terrestrial environments. Recent evidence that the AChE activity depends not only on neurotoxins, but on a number of other factors (Fu et al., 2018), incl. oxidative stress (Lionetto et al., 2011), suggests that it may be more widely used as an indicator for assessing ecosystem health. In addition, it has been shown that apart from the catalytic function in neurotransmission and muscular contraction, different forms of AChE are involved in cell proliferation, differentiation, and responses to various stresses (Lionetto et al., 2011), which can affect the general condition of individuals.

Our data, although preliminary, clearly showed that AChE activity significantly varied among seasons. The enzyme activity in the clams sampled from Arkutino and Primorsko in September was significantly higher compared to the June samples. Furthermore, the activities of AChE in general were lower in the samples of March and June compared to those of July and September. These results strongly suggested that seasonal temperature differences could have played a significant role. There are published data, which show that the water temperature could affect the AChE activity (Bocquene & Galgani, 2004; Pfeifer et al., 2005). In *Mytilus* sp. from the south-western Baltic Sea seasonal differences in the AChE activity were observed with a maximum during the summer and minimum in the winter period, and these changes had a positive correlation with water temperature (Pfeifer et al., 2005). Presumably in wedge clams, as cold-blooded animals, lower ambient temperatures are associated with reduced vital functions and lower AChE activity.

Activity of AChE is proved to be sensitive to various types of pollutants in the natural environment, mainly organophosphorous and carbamate chemicals. Our preliminary results indicated that the enzyme activity was lowest in wedge clams sampled from localities adjacent to areas known as sources of relatively high coastal anthropogenic load such as the large resorts Slanchev Bryag, Sveti Vlas, Arkutino, Duni. Given that in the immediate vicinity of these complexes there is no intensive agriculture and industry, it is most likely that the wastewater influx in the sea from these resorts could produce the main effects on AChE activity in wedge clams. Similarly, it has been shown that in *Ruditapes decussatus*, exposed to treated municipal effluents (TME), the AChE activity has been reduced in both gills and digestive gland (Kamel et al., 2012). Exposure to TME was also found to affect the pro/antioxidant balance in clams' cells and oxidative stress indicators were shown to correlate with AChE activity (Kavitha & Rao, 2008; Lionetto et al., 2011). It should be noted that the tourist flow to the Black Sea resorts in Bulgaria in 2020 was extremely limited due to the Covid-19 pandemic, which reduced tourist numbers and consumption. Consequently, the inflow of wastewaters was reduced, so the differences between the AChE activities we established in this preliminary study may be not typical and very expressive. Another factor that can affect the results obtained is the constant high water input into the northern part of the Black Sea by the big rivers and the pollutants they carry (Dineva, 2011; Dar & Bhat, 2020).

In recent years, the problem of marine pollution with plastic waste, incl. microplastics (MPs) becomes obvious (Hidalgo-Ruz et al., 2012; do Sul & Costa, 2014). The small size (<5 mm in diameter) of the MPs makes them bioavailable to marine organisms and transferable through the food web. MPs have been found in the Black Sea and their concentration in the Bulgarian part is similar to that found in other parts of the Black Sea, as well as in the Baltic Sea, and the Mediterranean Sea (Berov & Klayn, 2020). MPs have been shown to affect AChE activity and inhibition of the enzyme was observed by exposure to them alone or in combination with organic/metallic pollutants (Oliveira et al., 2013; Ribeiro et al., 2017; Tlili et al., 2020). Preliminary studies we have carried out showed the presence of microplastics in *D. trunculus* from areas with intensive tourism – Slanchev Bryag and Gradina (unpublished data). It is highly possible that the presence of MPs could have also affected the AChE activity in the studied wedge clams.

Conclusions

The preliminary results, obtained from the present study, demonstrated that AChE activity is sensitive to environmental pressure by various types of pollutants and variations in the conditions of the wedge clam habitats. The reaction of AChE activity in *D. trunculus* towards the changes in the marine environmental quality suggested that this clam species, representative for the shallow sublittoral sandy habitats of the Black Sea, is a sensitive and suitable bioindicator for the state of the marine ecosystems.

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