ACETYLCHOLINESTERASE ACTIVITY IN THE GILLS OF MUSSELS (*MYTILUS GALLOPROVINCIALIS*) FROM THE NORTH-EASTERN ADRIATIC COAST

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SUMMARY

The objective of the present work was to provide the data on acetylcholinesterase (AChE) activity in the gills of marine mussels *Mytilus galloprovincialis* from north-eastern part of Adriatic. In the first part of the study, the range and the pattern of AChE activity annual variation was established by monthly investigation of two populations of mussels originating from different natural habitats. No correlation of AChE activity and environmental parameters (temperature, salinity, oxygen) was found. Seasonal monitoring along the coast at sites with presumably different level and type of contamination was also performed in order to study the spatial and temporal trend of AChE activity in mussels in relation to possible exposure to neurotoxic compounds. Significantly lower enzyme activity in comparison to reference site was recorded at sites in the close vicinity to urban and industrial area (P and R). In addition, AChE activity decreased at site within the area of Lim Bay (L1). AChE reduction in the gills of mussels ~~implies the~~ that was recorded mostly in the polluted harbours, could be related to the presence of ~~neurotoxic compounds~~ common environmental pollutants. The results obtained in this study will be useful as background data for future biomonitoring surveys along the Adriatic coast using *M. galloprovincialis* as sentinel organisms.

KEYWORDS: AChE, Biomonitoring, *Mytilus galloprovincialis*, north-eastern Adriatic

INTRODUCTION

Organophosphorous pesticides (OP's) have been widely applied in agriculture and mariculture as protection against insects and parasites, and are recognised as potential threat for the health of aquatic organisms. Although elevated concentration of OP's can be present in chronically polluted areas characterised by poor circulation of water mass (estuaries, lagoons) [1, 2] it is generally considered that OP's are readily degradable in the aquatic environment and mostly difficult to detect by chemical methods. These compounds act primarily by inhibiting acetylcholinesterase (AChE; E.C.3.1.1.7.) a serine hydrolase essential for transmission of nerve signals [3]. The inhibition of AChE may persist for days to weeks, and thus, the measurement of AChE activity has been established as biomarker of exposure to OP's in aquatic environment [4].

Bivalve molluscs have been widely used as sentinel organisms in biomonitoring programmes in order to assess the quality of marine environment [5]. AChE activity is considerably reduced following exposure of bivalves to OP's [1, 6-8] and other classes of pollutants [9-15].

The north-eastern part of Adriatic is characterised by close vicinity of highly urbanised and industrialised zones that increase the sensitivity of marine ecosystem, and consequently affect economically important activities such as fishing, aquaculture and tourism. Monitoring of biological effect of contaminants in the tissues of mussels from north-eastern Adriatic coast enabled the identification of several hot-spots with high anthropogenic impact [16-19]. However, no study related to possible neurotoxic effect of contaminants has been conducted so far.

MATERIALS AND METHODS

Sample collection and preparation

Mussels from natural populations were sampled along the north-eastern Adriatic coast of Croatia (Fig. 1) within the frame of the Croatian National Monitoring Programme of the Adriatic Sea. Nine sites were chosen for the study. Sites L0 and L1 were located in the Lim Bay, a 10 km long, 30 meter deep, and (at its widest part) 600 m wide fjord–like bay on the western coast of the Istrian peninsula, important for mussels and fish farming. Site M was located in the south-eastern part of Rovinj harbour, close to the yacht and boat marina. Mussels were also sampled in Fažana (F) and Brestova (B), small ports and fishermen settlements. Site K was located in Kostrena, a beach resort not far from industrialized suburbs of Rijeka. Sampling site Ba was located in the vicinity of Bakar, a small port within Bakar Bay, known for the production of coke (abandoned 15 years ago). Finally, sites P and R were located in highly urbanised and industrialised harbours of Pula and Rijeka, respectively. The codes, geographical coordinates and brief description of sites are summarised in Table 1.

AChE activity was studied for its natural variability in the monthly sampled mussels at sites L0 and L1, whereas sites M, P, F, B, R, K and Ba were monitored seasonally (March, June, August and November). Site L0 was chosen as reference site far from known sources of agricultural, industrial and urban influence.

Mussels (4.5 to 5 cm shell length), were collected at low tide, from depth of 0.5 to 1 meter at all sites. Temperature, salinity and oxygen saturation were measured *in situ*, at a depth of mussels sampling, using a Multiline P4 Universal Pocket Meter (WTW) at a depth of 0.5-1m. After sampling, mussels were transported to the laboratory in humidified plastic tanks within two hours of collection. Gills were rapidly excised out on ice-cold plate, weighted and stored at –80 °C. Gills tissue was homogenised in cold (4°C) 0.1M Tris HCl buffer, pH 8.0, using Teflon Potter homogeniser. Mass of the gills and buffer volume ratio was 1:4. Measurement of enzyme activity in *M. galloprovincialis* was performed in supernatant immediately after centrifugation of homogenate at 10000xg, for 30 min.

Enzyme activity was determined in duplicates in each of the five individuals per sampling site. Samples were assayed for enzyme activities using microplate reader (Labsystems, Multiscan Ascent ® and Ascent Software TM, 2.4. version). Tissue protein concentration was determined in homogenates by Lowry method [20] with bovine serum albumin (BSA) as protein standard.

AChE assay

AChE activity was measured by Ellman's method [21], adjusted to microtitar plates, as described in [22]. The reaction mixture (340 µl) contained 0.1M Tris HCl buffer pH 8.0, 4 µl of 5.5'-dithiobis-2-dinitrobenzoic acid (0.008 M) and the appropriate amount of gills' tissue homogenate. The absorbance increase at 415 nm was recorded every 30 seconds after starting the enzymatic reaction with 4 µl of substrate acetylthiocholine (0.045 M). AChE activity was expressed as specific activity in nmoles of hydrolysed acetylthiocholine per minute per mg of protein (nmol/min/mgP).

Data analysis

Results are presented as mean ± standard error (SE). Differences between groups were tested by non-parametric Kruskal-Wallis one-way analysis of variance on ranks. When significant, it was followed by non – parametric Mann-Whitney test. A value of $p<0.05$ was considered significant. Spearman's correlation coefficient was calculated to identify the relation between AChE activity and environmental parameters. The analyses were carried out using the STATISTICA software package (StatSoft, Tulsa, USA).

RESULTS

Temperature values measured monthly at sites L0 and L1 were in the range of normal oscillations typical for the north-eastern part of Adriatic Sea (Fig. 2a). Maximum values were recorded in summer (July-August, 26°C) and minimum values in January (7°C). The salinity ranged between 33 and 37 psu (Fig. 2b), and oxygen saturation was above 90 % throughout the

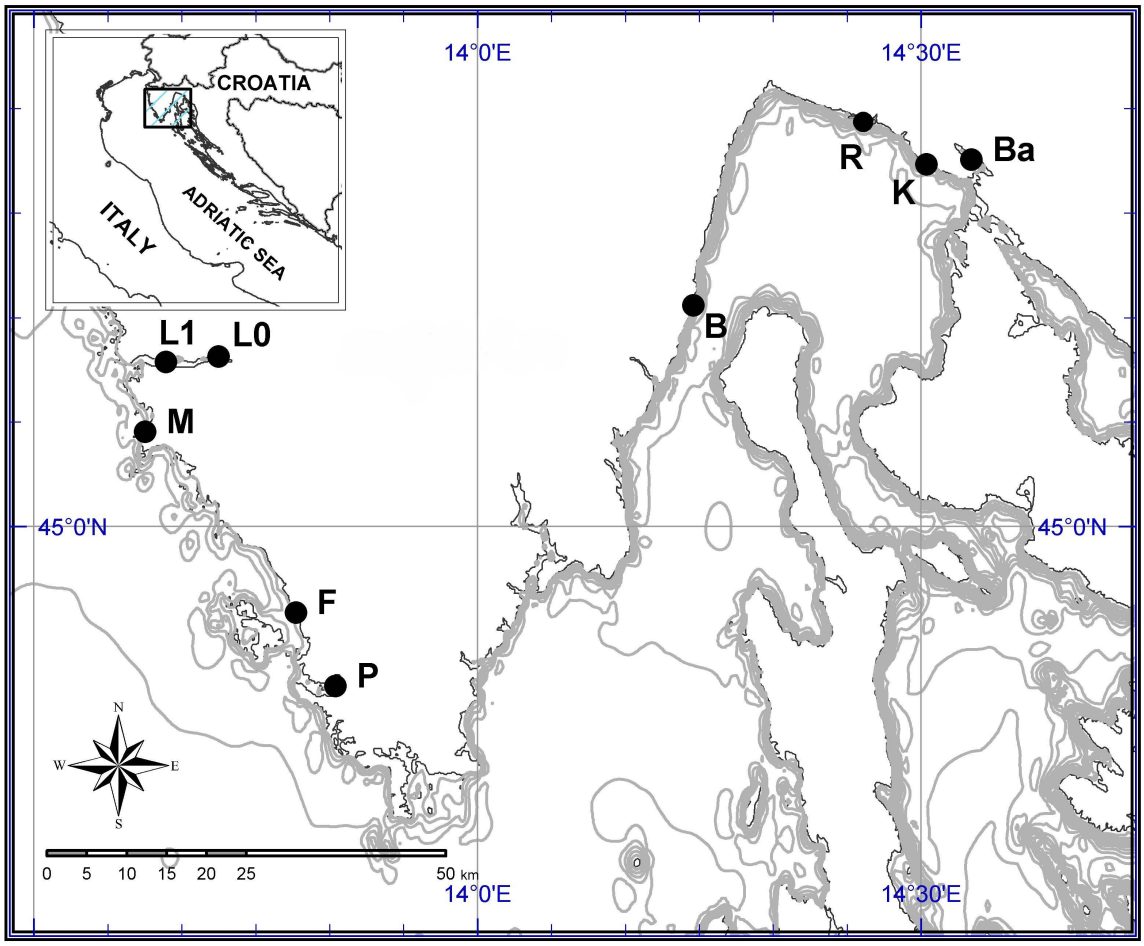


FIGURE 1 - Map of Northern Adriatic coast showing the sites of collection.

TABLE 1 - Sampling sites - codes, geographic positions and description. ~~of sampling sites.~~

Sampling site	Code	Latitude λ	Longitude φ	Description
Lim bay 0	L0	45º08' N	13º44' E	non - urban, mariculture
Lim bay 1	L1	45º08' N	13º41' E	non - urban
Marina	M	45º04' N	13º38' E	urban, marina
Fažana	F	44º56' N	13º48' E	urban, fishermen settlement
Pula	P	44º53' N	13º51' E	urban, harbor, industrial outflow, sewage
Brestova	B	45º09' N	14º14' E	non - urban, fishermen settlement
Rijeka	R	45º20' N	14º26' E	urban, harbor, industrial outflow, sewage
Kostrena	K	45º17' N	14º31' E	urban, recreational area
Bakar	Ba	45º18' N	14º33' E	urban, sewage

whole year (Fig. 2c). Environmental parameters at sites sampled seasonally were in accordance with the above values (not shown).

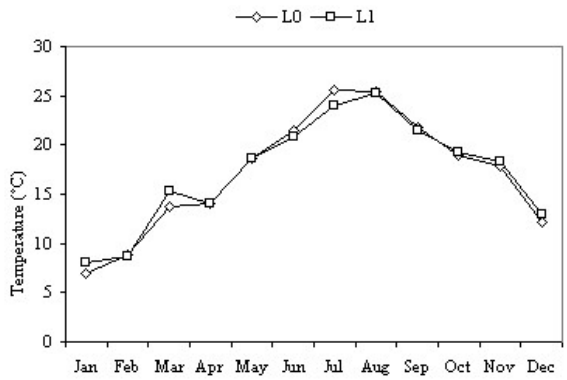
Monthly variations of AChE activity at sites L0 and L1

AChE activity detected in monthly sampled mussels at sites L0 and L1 is shown in Fig. 3. At site L0, the maximal AChE annual value of 39 nmol/min/mgP was recorded in April. Following the early spring peak, AChE activity gradually decreased to minimum value in July (12 nmol/min/mgP). During the rest of the year AChE activity ranged between 15 and 21 nmol/min/mgP. Likewise, the annual maximum at site L1 was detected in April (31 nmol/min/mgP) and it was followed by gradual decrease to the minimal annual value in August (11 nmol/min/mgP). From the late summer AChE activity again increased to 22 nmol/min/mgP in December. No correlations were found between AChE activity in the gills of mussels neither with temperature (R = 0.06 and 0.04 at sites L0 and L1, respectively) nor with other environmental parameters (not shown).

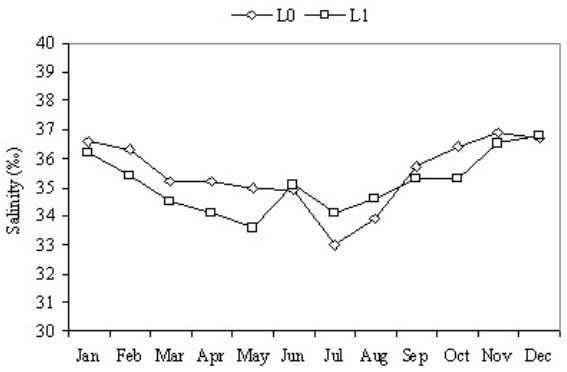
Seasonal variations of AChE activity

AChE activity in March at sites L1, P, R and Ba exhibited nearly equal level (between 12 - 14 nmol/min/mgP) (Fig. 4a). Statistically significant difference with respect to reference site L0 was recorded at sites L1 and R. Values above 20 nmol/min/mgP were observed at the rest of sites. AChE activity at site R (8 nmol/min/mgP) was significantly reduced in June with respect to reference site (Fig. 4b). Relatively homogenous values (16 - 17 nmol/min/mgP) were detected at sites L0, L1, F, P, B and Ba, whereas slightly higher values were recorded at sites K and M (19 and 21 nmol/min/mgP, respectively). AChE activity recorded in August at site P and L1 (7 and 11 nmol/min/mgP, respectively) were significantly lower than at reference site L0 (Fig. 4c). AChE activity at sites M, R and Ba was also markedly (but not significantly) reduced. At sites L0, F, B and K, AChE activity exhibited values between 15 and 17 nmol/min/mgP. In November, site R was characterised by the lowest AChE activity (10 nmol/min/mgP) that was significantly reduced with respect to reference site L0 (Fig 4d). Intermediate values (15 nmol/min/mgP) were recorded at sites L1, F and Ba. Relatively high activity was observed at sites K (17 nmol/min/mgP), L0 (19 nmol/min/mgP) and in particular at sites M, P and B (above 22 nmol/min/mgP).

a)



b)



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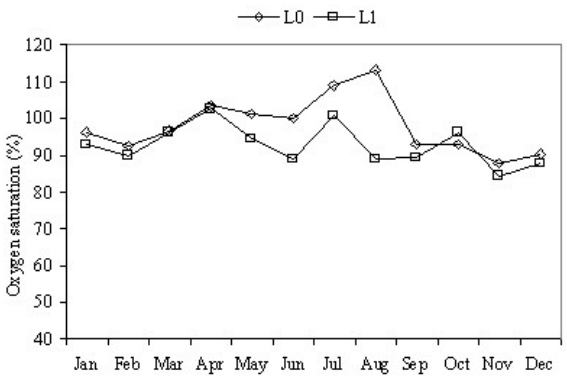

FIGURE 2 - Temperature (a), salinity (b) and oxygen (c) monthly values at sites L0 and L1.

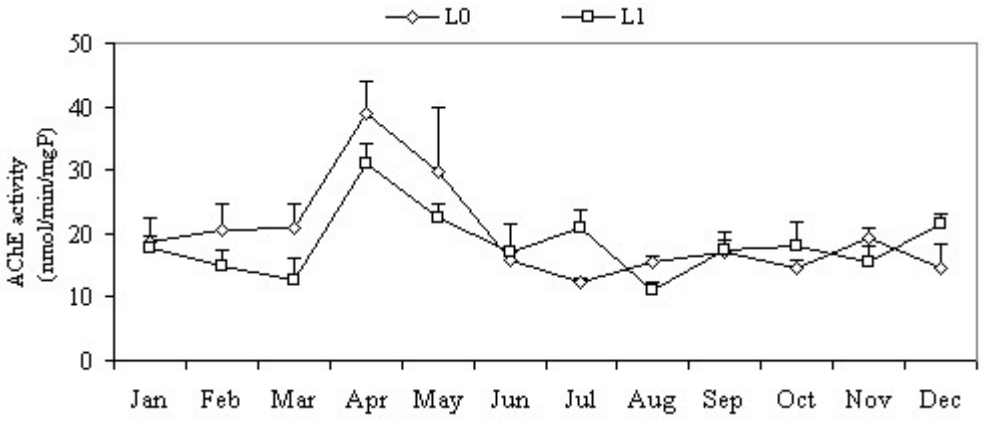

FIGURE 3 - Month-to-month variability of AChE activity (nmol/min/mgP) in the gills of *M. galloprovincialis* from sites L0 and L1 (Mean ± S.E.).

DISCUSSION AND CONCLUSION

In the present study, month-to-month oscillation of AChE activity in the gills of mussels from the north-eastern part of Adriatic coast was determined by investigation performed at sites L0 and L1, located within the area of Lim Bay. Fluctuating nature of AChE activity greatly enhance the possibility of erroneous interpretation of the biological response in natural environment. Thus, the choice of a suitable reference site and appropriate sampling seasons is necessary to recognize the enzyme activity variations induced by deleterious effect of contaminants.

When using mussels as sentinel organisms for biomarker studies, much importance is given to oscillations of abiotic variables, in particular temperature and salinity, both commonly related to AChE variability [1, 23, 24, 25]. ~~In this study, no correlation of AChE activity and temperature and salinity trends could be revealed~~ Although AChE activity gradually decreased with rising of water temperature no significant correlation could be revealed and our findings seem to be in accordance with the results reported previously [12]. However, mussels' AChE activity expressed particularly high amplitudes in spring and summer, indicating the possible involvement of internal factors, in particular nutritive stress and changes in the dynamics of reproductive cycle [23, 26, 27]. In fact, food availability supported by favourable environmental conditions such as the rising of water temperature in spring, determines the gonadal maturation cycle in mussels from Northern Adriatic and triggers the spawning activity [28]. Bearing this in mind, we assume that spring and early summer may not be appropriate for evaluation of AChE activity in mussels. However, a relation between dynamics of reproductive cycle and AChE activity oscillations in the gills of mussels still remains to be clarified with more detailed studies.

To investigate the exposure of mussels to neurotoxic compounds in the north-eastern Adriatic, the present study included sites with presumably different level of contamination, ranging from those located in recreational and protected area, to urban and industrial sites - hot spots [29]. AChE activity at two sites located in the vicinity of harbours of Rijeka and Pula was probably reduced by the presence of anti-cholinesterase compounds other than OP's, since both are located in the half - closed zones characterised by slow water exchange and continuous input of common environmental contaminants such as PAHs, petroleum, phenols, detergents and others originating from untreated wastewater discharges, runoff, urban and industrial sources [29, 30] whereas the presence of OP's was never registered. Moreover, recent study [31] confirmed a long term trend of elevated concentration of chlorinated hydrocarbons and heavy metals in the soft tissues of mussels sampled at sites P and R. Exposure to these compounds had deleterious effect on many histological and physiological parameters in mussels [16, 17] and furthermore, most of them are able to inhibit AChE activity in bivalves [9, 11, 12]. Interestingly, high level of AChE at site P

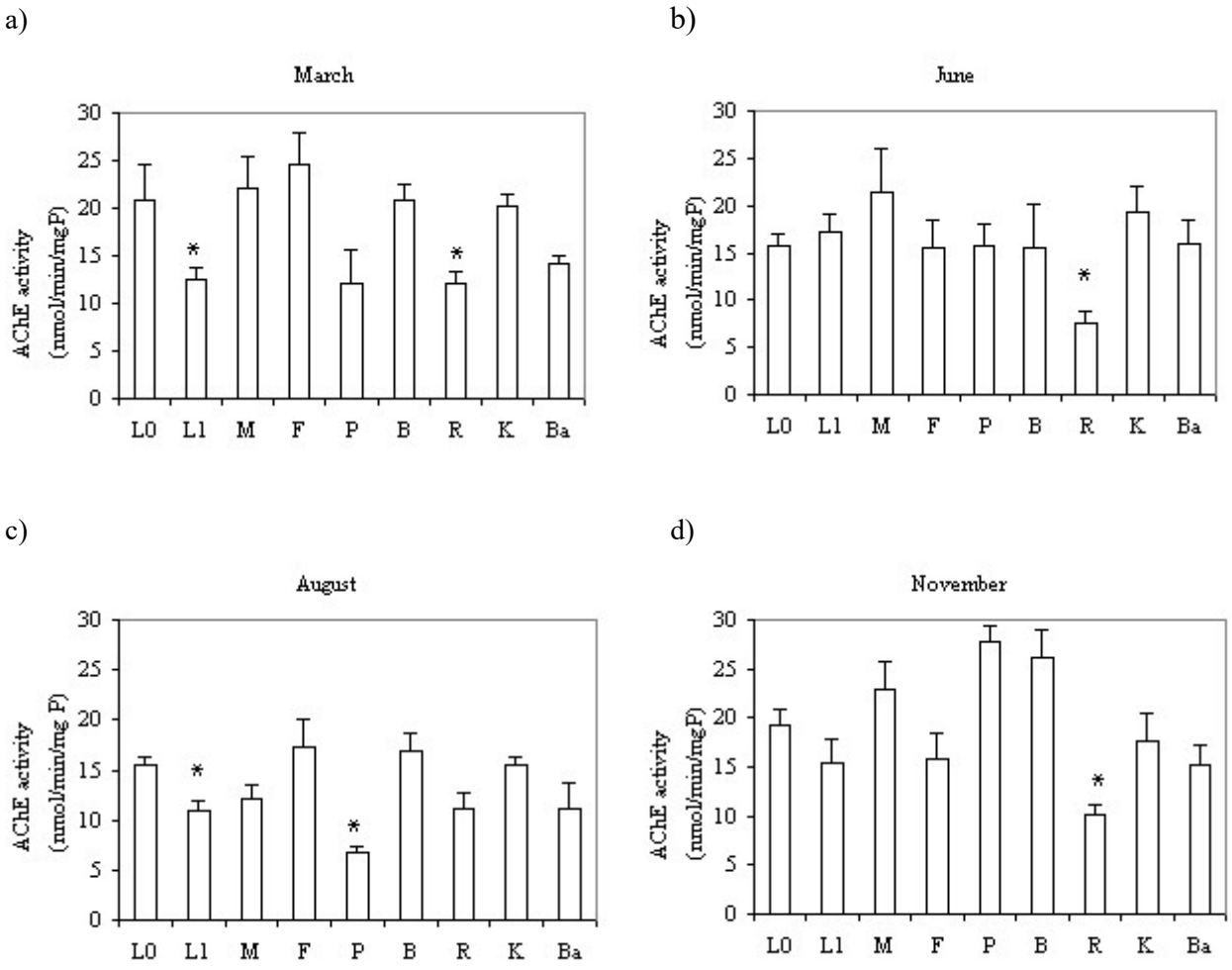

FIGURE 4 - Seasonal AChE activity (nmol/min/mgP) in the gills of mussels *M. galloprovincialis* from sites L0, L1, M, P, F, B, R, Ba and K sampled in March (a), June (b), August (c) and November (d) (Mean ± S.E). Asterisks indicate significant difference from reference site L0 ($p<0.05$).

was recorded in November. This effect could be associated with transient recovery of enzyme activity, similarly to what was observed following sub-lethal laboratory and field exposure to pesticides [32]. Reduced AChE activity in bivalve species at sites close to industrial and urban areas, which was not directly correlated with agricultural activity and OP's input, was also reported for the region of Venice Lagoon [33], Salento peninsula [34], Greek coast [24] and northern coast of Portugal [12]. High load of chlorinated hydrocarbons and heavy metals was previously found in the tissues of mussels from site Ba, in the vicinity of abandoned coke – plant [31]. However, AChE activity in the gills of mussels sampled at this site was not significantly affected. AChE activity at site located in the area of Lim Bay (L1), sometimes decreased to the level measured at sites at sites in harbours (P, R, Ba). Site L1 has been characterised by low toxic load and high seawater quality [35], and thus the observed disturbance could be explained by occasional input of anti–cholinesterase compounds possibly related to frequent boat traffic or other unknown sources. AChE activity at sites M, F, and K was relatively high, due to more effective exchange of water mass, currents and consequently dilution of pollutants.

In conclusion, the data given here ~~represent the first evidence of neurotoxic effect of contaminants that was reflected~~ in helped to establish the range of AChE activity ~~reduction~~ in

native populations of mussels from north-eastern Adriatic. It was also highlighted that natural variations of AChE activity in the gills of mussels should be taken into account when using this biomarker in biomonitoring programmes. This study showed that evaluation of AChE activity as biomarker of exposure to anti-cholinesterase compounds contributes to more detailed evaluation of the risk for sensitive marine coastal areas.

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REFERENCES

[1] Escartín, E. and Porte, C. (1997) The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. Environmental Toxicology and Chemistry 16, 2090-2095.

[2] Kirby, M.F., Morris, S., Hurst, M., Kirby, S.J., Neall, P., Tylor, T. and Fagg, A. (2000) The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotixic contamination in UK estuaries. Marine Pollution Bulletin 40, 780 – 791.

[3] Massoulié J., Sussman, J., Bon, S. and Silman, I. (1993) Structure and functions of acetylcholinesterase and butyrylcholinesterase. Progress in Brain Research 98, 139-146.

[4] Fulton, M.H. and Key, P.B. (2001) Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environmental Toxicology and Chemistry 20, 37-45.

[5] Viarengo, A. and Canesi, L. (1991) Mussels as biological indicators of pollution. Aquaculture, 94, 225-243.

[6] LeBris, H., Maffart, P., Bocquené, G., Buchet, V., Galgani, F. and Blanc, G. (1995) Laboratory study on the effect of dichlorvos on two commercial bivalves. Aquaculture 138, 139 – 144.

[7] McHenery, J.G., Linley-Adams, G.E., Moore, D.C., Rodger, G.K. and Davies, I.M. (1997) Experimental and field studies of effects of dichlorvos exposure on acetylcholinesterase activity in the gills of the mussel, *Mytilus edulis* L. Aquatic Toxicology 38, 125-143.

[8] Dellali, M., Gnassia Barelli, M., Romeo, M. and Aissa, P. (2001) The use of acetylcholinesterase activity in *Ruditapes decussatus* and *Mytilus galloprovincialis* in the biomonitoring of Bizerta lagoon. Comparative Biochemistry and Physiology 130C, 227-235.

[9] Najimi, S., Bouhaimi, A., Daubèze, M., Zekhnini, A., Pellerin, J., Narbonne, J.F. and Moukrim, A. (1997) Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker of pollution in Agadir Marine Bay (South of Morocco). Bulletin of Environmental Contamination and Toxicology 58, 901-908.

[10] Hamza-Chaffaï, A., Roméo, M., Gnassia-Barelli, M. and El Abed, A. (1998) Effect of copper and lindane on some biomarkers measured in the clam *Ruditapes decussates*. Bulletin of Environmental Contamination and Toxicology 61, 397 - 404.

[11] Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T. and Narbonne, J-F. (2000) Enzymatic biomarker measurement and study of DNA adduct formation in benzo(a)pyrene-contaminated mussels, *Mytilus galloprovincialis*. Aquatic Toxicology 49, 269-287.

[12] Moreira, S.M., Moreira-Santos, M., Ribeiro, R. and Guilhermino, L. (2004) The 'Coral Bulker' fuel oil spill on the north coast of Portugal: Spatial and temporal biomarker responses in *Mytilus galloprovincialis*. Ecotoxicology 13, 619-630.

[13] Kankaanpää H., Leiniö, S., Olin, M., Sjövall, O., Meriluoto, J. and Lehtonen, K.K. (2007) Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. Chemosphere 68, 1210-1217.

[14] López-Galindo, C., Vargas-Chacoff, L., Nebot, E., Casanueva, J.F, Rubio, D., Mancera, J.M. and Solé, M. (2010) Sublethal responses of the common mussel (*Mytilus galloprovincialis*) exposed to sodium hypochlorite and Mexel®432 used as antifoulants. Ecotoxicology and Environmental Safety 73, 825-834.

[15] Solé, M., Shaw, J.P., Frickers, P.E., Readman, J.W. and Hutchinson, T.H. (2010) Effects on feeding rate and biomarker responses of marine mussels

experimentally exposed to propranolol and acetaminophen. Analytical and Bioanalytical Chemistry 396, 649-656.

[16] Petrović, S., Ozretić, B., Krajnović - Ozretić, M. and Bobinac, D. (2001) Lysosomal membrane stability and metallothioneins in digestive gland of mussels (*Mytilus galloprovincialis* Lam.) as biomarkers in a field study. Marine Pollution Bulletin 42, 1373-1378.

[17] Petrović, S., Semenčić, L., Ozretić, B. and Krajnović - Ozretić, M. (2004) Seasonal variations of physiological and cellular biomarkers and their use in the biomonitoring of north Adriatic coastal waters (Croatia). Marine Pollution Bulletin 49, 713-720.

[18] Jakšić, Ž., Batel, R., Bihari, N., Mičić, M. and Zahn, R.K. (2005) Adriatic coast as a microcosm for global genotoxic marine contamination - A long-term field study. Marine Pollution Bulletin 50, 1314-1327.

[19] Bihari, N., Fafandel, M. and Piškur, V. (2007) Polycyclic aromatic hydrocarbons and ecotoxicological characterization of seawater, sediment, and mussel *Mytilus galloprovincialis* from the Gulf of Rijeka, the Adriatic Sea, Croatia. Archives of Environmental Contamination and Toxicology 52, 379-387.

[20] Lowry O.H., Rosebrough N.J., Fair A.L. and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

[21] Ellman, G.L., Courtney, K.O., Andrres, V. and Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88 – 95.

[22] Bocquené, G. and Galgani, F. (1998) Biological effects of contaminants: cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences, 22.

[23] Pfeifer, S., Schiedek, D., Dippner and J.W. (2005) Effect of temperature and salinity on acetylcholinesterase activity, a common pollution biomarker, in *Mytilus* sp. from the south-western Baltic Sea. Journal of Experimental Marine Biology and Ecology 320, 93-103.

[24] Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M. and Dimitriadis, V.K. (2003) Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Marine Environmental Research 56, 443-470.

[25] Cravo, A., Lopes, B., Serafim, A., Company, R., Barreira, L., Gomes, T. and Bebianno, M.J. (2009) A multibiomarker approach in *Mytilus galloprovincialis* to assess environmental quality. Journal of Environmental Monitoring 11, 1673-1686.

[26] Devier, M.-H., Augagneur, S., Budzinski, H., Le Menach, K., Mora, P., Narbonne, J.-F. and Garrigues, P. (2005) One-year monitoring survey of organic compounds (PAHs, PCBs, TBT), heavy metals and biomarkers in blue mussels from the Arcachon Bay, France. Journal of Environmental Monitoring 7, 224-240.

[27] Leiniö, S. and Lehtonen, K.K. (2005) Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. Comparative Biochemistry and Physiology 140C, 408-421.

[28] Hrs-Brenko M. (1971) The reproductive cycle of the *Mytilus galloprovincialis* Lamk. in the Northern Adriatic Sea. Thalassia Jugoslavica 7, 533-542.

[29] UNEP/WHO (1999) Identification of priority pollution hot spots and sensitive areas in the Mediterranean. MAP Technical Report Series No. 124, UNEP, Athens.

[30] UNEP/WHO (1994) Monitoring Programme of the Eastern Adriatic Coastal Area, Report for 1983–91. MAP Technical Report Series No. 86, UNEP, Athens.

[31] National Monitoring Programme Croatia, Report for 2007 (2008) Zagreb: Ministry of Environment and Physical Planning, 40-54. (In Croatian).

[32] Xuereb, B., Chaumot, A., Mons, R., Garric, J. and Geffard, O. (2009) Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda). Intrinsic variability, reference levels, and a reliable tool for field surveys. Aquatic Toxicology 93, 225-233.

[33] Matozzo, V., Tomei, A. and Marin, M.G. (2005) Acethylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarium* from the Lagoon of Venice. Marine Pollution Bulletin 50, 1686 – 1693.

[34] Lionetto, M. G., Caricato, R., Giordano, M.E., Pascariello, M.F., Marinosci, L. and Schettino, T. (2003) Integrated use of biomarkers

(acetylcholinesterase and antioxidant enzymes activities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. Marine Pollution Bulletin 46, 324-330.

[35] Fafanđel, M. and Bihari, N. (2007) Temporal and spatial variations of seawater toxicity along Adriatic coast, Croatia: A long - Term study. Fresenius Environmental Bulletin 16, 1457-1463.

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