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Lysosomal membrane stability assessment in wild and farmed mussels (*Mytilus galloprovincialis* Lamarck, 1819) as biomarkers for environmental stress: winter and spring results

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Abstract. This study evaluated lysosomal membrane stability using the Neutral Red Retention Time (NRRT) assay in hemocytes of *Mytilus galloprovincialis* from wild and farmed populations, as a biomarker for environmental stress. Mussels were sampled during winter and spring to assess seasonal variability. NRRT values showed a significant reduction in both populations across both seasons, indicating compromised lysosomal integrity and exposure to environmental stressors. However, the wild mussels exhibited consistently lower NRRT values compared to the farmed individuals, suggesting a higher level of stress. The question of why is yet to be answered.

Key words: Black Sea, pollution, mussels.

Introduction

The Black Sea is considered one of the most polluted European seas and suffers from land-based pollution. Moreover, today, within the context of the Marine Strategy Framework Directive (MSFD), the Black Sea is among the four marine regions that have been subjected to careful observation. Bivalves are abundant filter-feeders, large enough for analysis, easy to collect, and significant from economic and ecological perspectives (Fabrello *et al.* 2023). Furthermore, assessing abiotic or anthropogenic effects relies on biomarkers that indicate molecular, biochemical, cellular, and tissue lesions, which reflect the health status of both the population and the ecosystem. Hence, the main goal of the present study was to assess the lysosomal membrane stability (LMS) in wild and farmed mussels from the southern Bulgarian Black Sea by applying the neutral red retention assay (NRRA), which is a common biomarker in mollusks. The hypothesis tested was that both mussel populations

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would experience cellular stress because of the ecological problems which the Black Sea is experiencing. Here we present and compare the results for two seasons - winter and spring of 2025.

Material and Methods

The wild mussels were collected during the winter and spring (around 15 kg in total) of 2025 from the region of the southern Bulgarian Black Sea coast, the town of Sozopol, with the help of local fishermen and boats, while the farmed ones (another 10 kg were purchased from a commercial farm. LMS (n = 40 individuals per season, 20 for each population) was evaluated by the neutral red retention time (NRRT; min) assay in hemocytes of mussels following the *in vivo* cytochemical method described by Viarengo *et al.* (2007) and Martínez-Gómez *et al.* (2015). The cells further were examined in parallel for lysosomal alterations and were given a score according to the severity of the effect: score 0 = no effects; score 1 = enlargement but no leakage; score 2 = leakage but no enlargement; score 3 = leakage and enlargement; score 4 = leakage and enlargement but colorless lysosomes; score 5 = rounded up fragmenting cells according to Martínez-Gómez *et al.* (2015). Lastly, the threshold values for the NRRT assay were as follows: no stressed organisms or healthy organisms if NRRT ≥ 120 min; stressed but compensating if 120 min ≥ NRRT ≥ 50 min; severely stressed and probably exhibiting pathology if < 50 min as explained by Pantea *et al.* (2023). All statistical analyses were conducted using Python 3.11 in the Jupyter Notebook environment. Data preprocessing, visualisation, and hypothesis testing were performed using the following Python libraries: pandas for data handling, matplotlib and seaborn for plotting, scipy.stats for statistical tests, statsmodels.stats.multitest for multiple testing correction. Normality of NRRT data was assessed using the Shapiro–Wilk test, and the Levene's test was applied to test homogeneity of variances. The data were not normally distributed, so non-parametric tests were used for group comparisons. Differences in NRRT and severity scores among mussel groups (season × origin: four groups in total) were tested using the Kruskal–Wallis H-test, followed by pairwise Mann–Whitney U tests with Bonferroni correction for multiple comparisons. Boxplots (for NRRT) and bar charts (for severity scores; mean ± SD) were generated to visualise group differences. Statistical groupings (denoted by letters) were added above the plots to indicate significantly different groups ($p < 0.05$). To assess the relationship between NRRT and lysosomal severity scores, Spearman's rank correlation coefficients (ρ) were calculated both overall (across all data points) and within each group. The significance level was set at $p < 0.05$.

Results and Discussion

The neutral red retention time (NRRT) assay revealed significant differences in lysosomal membrane stability between the mussel groups across seasons and origins. The NRRT values were lowest in the winter wild group, indicating impaired lysosomal stability, and highest in the winter aquaculture group. The Shapiro-Wilk test indicated non-normal distribution for all groups, and Levene's test showed no significant variance heterogeneity. Consequently, non-parametric tests were applied. Kruskal-Wallis test revealed a statistically significant difference among the four groups ($H = 11.13$, $p < 0.05$). Post hoc Mann-Whitney U tests (Bonferroni-corrected) identified significantly lower NRRT values in the winter wild mussels compared to the winter aquaculture ($p < 0.01$) and spring aquaculture mussels ($p < 0.05$). Additionally, the spring wild mussels significantly differed from the winter aquaculture mussels ($p < 0.05$). Based on these results, the boxplot groups were annotated with the following statistical groupings: Winter Wild (A), Winter Aquaculture (B), Spring Wild (AC), Spring Aquaculture (BC) (Fig. 1). The corresponding boxplot (Fig. 1) clearly shows the lowest lysosomal stability in winter wild mussels, potentially reflecting a greater environmental stress load. The relatively high NRRT values observed in the farmed mussels,

particularly in winter, indicate better cellular health, likely due to controlled rearing conditions. The severity scores, which reflect lysosomal alterations, also varied significantly between the tested groups. Kruskal-Wallis test confirmed significant differences in severity scores ($H = 19.55$, $p < 0.001$). Pairwise Mann-Whitney U tests showed that the winter wild and spring wild mussels (Group A) had significantly higher severity scores than the winter and spring farmed mussels (Group B). This trend is presented in a bar chart (Fig. 2), where the farmed mussels consistently exhibited lower mean severity scores and standard deviations, suggesting less lysosomal damage. Spearman correlation analysis was performed to explore the relationship between the two biomarkers. The overall correlation ($n = 80$) between NRRT and severity score was moderate and statistically significant ($rho = -0.478$, $p < 0.00001$), confirming an inverse association between lysosomal membrane stability and morphological damage (Table 1). Group-wise correlations revealed a moderate but statistically significant correlation only in the spring wild group ($rho = -0.564$, $p < 0.01$). The other groups showed similar trends but did not reach statistical significance: Winter Wild ($rho = -0.419$, $p = 0.066$), Winter Aquaculture ($rho = -0.385$, $p = 0.093$), and Spring Aquaculture ($rho = -0.364$, $p = 0.115$). These findings suggest that the association between biomarker responses may be influenced by seasonal or environmental conditions, with wild populations in spring possibly being more sensitive to external stressors, including pollution.

Table 1. Spearman rank correlation between NRRT and lysosomal severity scores in mussels.

Group	Spearman Rho (ρ)	p-value
Winter Wild	–0.419	0.066
Winter Aquaculture	–0.385	0.093
Spring Wild	**–0.564**	**<0.01**
Spring Aquaculture	–0.364	0.115
Overall	**–0.478**	**<0.00001**

*Spearman's rank correlation coefficients (ρ) and corresponding p-values evaluating the relationship between NRRT (Neutral Red Retention Time; min) and lysosomal severity scores (0–5 scale) in each mussel group. Bold values indicate statistically significant correlations ($p < 0.05$).

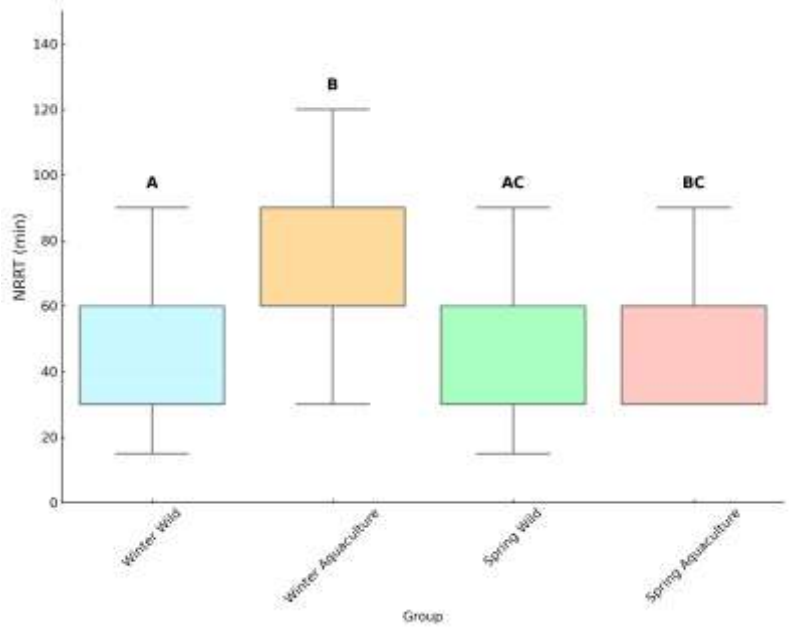

Figure 1. Lysosomal membrane stability in mussels assessed by the Neutral Red Retention Time (NRRT) assay. Boxplot showing the Neutral Red Retention Time (NRRT; min) in hemocytes of wild and farmed mussels collected during winter and spring 2025. Letters above the boxes indicate statistically significant differences between groups based on Kruskal–Wallis and post hoc Mann–Whitney U tests with Bonferroni correction ($p < 0.05$).

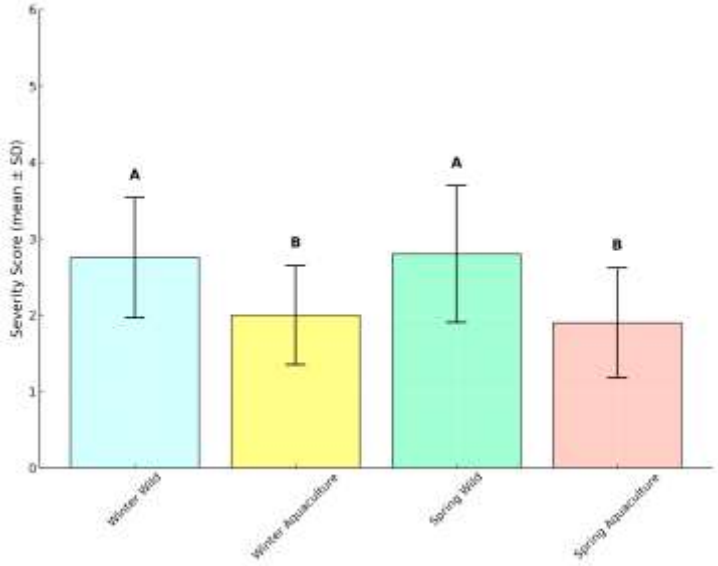

Figure 2. Lysosomal damage in mussels assessed by severity score analysis. Bar chart representing the mean ± standard deviation of severity scores (0–5 scale) in hemocytes of mussels from wild and farmed populations sampled in winter and spring 2025. Severity scores reflect morphological alterations in lysosomes. Statistical groupings (A, B) were assigned based on significant differences from Kruskal–Wallis and Mann–Whitney U post hoc tests ($p < 0.05$).

Our results are in agreement with the results of Pantea *et al.* (2023), who assessed the lysosomal membrane stability in wild *M. galloprovincialis* from the Romanian Black Sea and registered a significant reduction in NRRT in all studied locations. However, for our research, we suggest further chemical analyses of water and sediment samples.

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References

Fabrello, J., Ciscato, M., Munari, M., Vecchiatti, A., Roverso, M., Bogialli, S. & Matozzo, V. (2023) Ecotoxicological effects and bioaccumulation of BPA analogues and their mixture in the clam *Ruditapes philippinarum*. *Marine Environmental Research*, 192: 106228.

Martínez-Gómez, C., Bignell, J. & Lowe, D. (Eds.) (2015) Lysosomal membrane stability in mussels; ICES techniques in marine environmental sciences. *International Council for the Exploration of the Sea (ICES):* Copenhagen, Denmark, Volume 56, pp. 1-41.

Pantea, E.D., Coatu, V., Damir, N.A., Oros, A., Lazar, L. & Rosoiu, N. (2023) Lysosomal membrane stability of mussel (*Mytilus galloprovincialis* Lamarck, 1819) as a biomarker of cellular stress for environmental contamination. *Toxics*, 11(8): 649.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E. & Koehler, A. (2007) The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology Part* C, 146: 281–300.