"OVIDIUS" UNIVERSITY OF CONSTANTA

DOCTORAL SCHOOL OF APPLIED SCIENCES

FUNDAMENTAL FIELD: BIOLOGY

ABSTRACT OF THE DOCTORAL THESIS

STUDIES ON THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE BIVALVE *Mytilus galloprovincialis*

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CONTENT

AIMS AND OBJECTIVES OF THE PAPER

This **thesis aims** to assess the impact of physico-chemical and biological factors and contaminants on the health status of the species *Mytilus galloprovincialis* in the Romanian Black Sea coastal area.

The **main objectives** of the thesis are:

1. Assessment of the physiological status of mussels in relation to physico-chemical parameters of water and biometric parameters of mussels;
2. To determine the food availability influence on the condition index and biochemical composition of mussels;
3. To investigate the degree of heavy metals and persistent organic pollutants contamination of the investigated areas;
4. To examine the degree of bioaccumulation of contaminants in mussels in relation to environmental factors;
5. Application of bioaccumulation factors and indices to assess the degree of heavy metals bioaccumulation in mussels and to evaluate the health status of mussels;
6. Investigation of the degree of microplastic contamination of mussels, determination of the relationship between mussel size and ingested microplastics and assessment of the impact on the physiological condition of mussels;
7. Evaluation of the condition index as a physiological biomarker of stress in heavy metal contamination of mussels;
8. Application of lysosomal membrane stability as a cellular biomarker in the assessment of sublethal effects of environmental contaminants.

Keywords: mussels, *Mytilus galloprovincialis*, heavy metals, organic pollutants, microplastics, physiological and cellular effects, condition index, lysosomal membrane stability.

The PhD thesis entitled "Research on the influence of environmental factors on the bivalve *Mytilus galloprovincialis*" is composed of two parts and is structured in ten chapters.

Part I covers the current state of knowledge and is structured into four chapters presenting information from the literature on the anatomy and physiology of marine bivalve molluscs, environmental factors influencing mussel physiology and behaviour, pollutants present in the marine environment and their impact on bivalve molluscs, and biomarkers as bioindicators of the effects of contaminants on bivalves.

Part II contains the personal contributions and is organised into six chapters. Chapter five describes the study areas, sample collection methodology and methods of analysing the collected samples. Chapter six presents the influence of environmental parameters on the condition index and biometric parameters of mussels as well as the relationships between the mussel condition index and mussel biometric characteristics. Chapter seven presents the seasonal variation in phytoplankton composition and abundance in the study areas and emphasises the influence of food availability on variations in the condition index and biochemical composition of mussels. Chapter eight highlights the degree of heavy metal contamination of seawater and sediments, the degree of bioaccumulation of heavy metals in mussels and the influence of heavy metals on the status index of mussels. Chapter nine highlights the degree of bioaccumulation of microplastics in the mussel tissues analysed, the characteristics of the microplastics identified (their type, colour and size) and the relationship between the abundance of bioaccumulated microplastics in mussels and the condition index. Chapter ten emphasises the degree

of contamination of water and mussels with heavy metals and organic pollutants, as well as the response in lysosomal membrane stability to environmental contaminant levels.

PART I. STATE OF KNOWLEDGE

Mussels belong to the class Bivalvia and are characterised by having a body protected by two calcareous valves, a sedentary lifestyle and filter feeding (Skolka, 2003). Mussels of the genus *Mytilus* are a group of marine bivalve molluscs belonging to the family Mytilidae and are found predominantly in the intertidal zone of exposed shorelines (Dailianis, 2010). The physiology and behaviour of mussels can be affected by various environmental factors such as temperature, salinity, air exposure, food availability, etc.

The primary pollutants in the marine environment are heavy metals, organic pollutants and microplastics. Mainly, the most significant amount of heavy metals present in the marine environment originate from human activities and are of concern due to their toxicity, persistence and non-biodegradability (Wu et al., 2016) and can have sublethal and lethal effects on marine organisms (Peters et al., 1997). Heavy metals, even essential heavy metals, can inhibit growth, filtering and feed uptake rates, respiration and metabolism, reproduction, gamete development and larval stages, and bivalve behaviour (Weis, 2014).

Polycyclic aromatic hydrocarbons (PAHs) are among the most widespread and toxic organic pollutants in the marine environment (Baussant et al., 2001). Bioaccumulation of PAHs leads to altered physiological processes of mussels, altered metabolism, inhibition of growth and reproduction (Weis, 2014). Polychlorinated biphenyls (PCBs) and organochlorinated pesticides (OCPs) are persistent organic pollutants that have been widely produced for various industrial purposes (Srogi, 2008) and are of exceptional importance due to their persistence, bioaccumulation and toxicity to wildlife and humans (Georgieva et al., 2016).

Exposure of bivalves to pesticides induces physiological (growth inhibition, reduced filtering and feed uptake rates, reduced oxygen uptake, stimulation/inhibition of ammonia excretion), behavioural (reduced valve activity) (Weis, 2014), and a variety of neurotoxic, hormone-modulating, immunological and tumourigenic effects (Androutsopoulos et al., 2013).

Plastics are synthetic organic polymers resulting from the polymerisation of monomers extracted from oil or gas (Cole et al., 2011). Microplastics are present in the water column, sediments and biota in all marine and freshwater environments (Coyle et al., 2020). Microplastics can be categorised as primary and secondary microplastics depending on their source (Cole et al., 2011). Primary microplastics and nanoplastics are deliberately produced at very small sizes for use in various cosmetic products and secondary microplastics result from the fragmentation of larger items as a result of physico-chemical processes (UV radiation, physical abrasion, chemical oxidation, etc.) (Miloloža et al., 2021). Exposure of bivalves to microplastics leads to physiological alterations (food uptake rate and respiration) (Sendra et al., 2021), alterations of bivalve life cycle (gametogenesis, embyogenesis, larval development and metamorphosis) (Sendra et al., 2021; Sussarellu et al., 2016), histopathological alterations (von Moos et al., 2012), genotoxicity (Sussarellu et al., 2016).

Marine mussels are commonly used as indicator organisms to detect environmental pollution in coastal waters due to their ability to accumulate various organic or inorganic contaminants (Livingstone, 1991). Simple changes in physiological and biochemical responses can predict the impact of pollutants (Dailianis, 2010). Biological indicators using molecular, cellular and physiological responses are commonly referred to as

biomarkers. Biomarkers are physiological, cellular, biochemical or behavioural changes that can be determined in tissues or fluids in the body or in the whole organism and that reveal exposure to and/or effects of one or more chemical contaminants (Depledge, 1993).

The condition index is used for two purposes: as an indicator of commercial meat quality (Orban et al., 2002) and as an ecophysiological measure of animal health. This index reflects the physiological activities of organisms (growth, reproduction, secretion, etc.) giving an insight into their general health status under specific environmental conditions (Lucas and Beninger, 1985). The main factors influencing the physiological (implicitly also the biochemical) condition of bivalves are: physical (temperature, salinity, organic matter), chemical (concentration of heavy metals and organic compounds) and biological (food availability, reproductive cycle and bacterial population) factors (Freites et al., 2003).

Lysosomal membrane stability (LMS) is used as a general biomarker of chemical pollution stress (Martínez-Gómez et al., 2015). Lysosomes are multifunctional organelles present in almost all eukaryotic cells, surrounded by a membrane containing hydrolytic enzymes involved in cellular processes such as digestion, defence and reproduction (Pipe, 1993). Destabilisation of the lysosomal membrane indicates physiological or pathological alterations induced by pollutants (Martínez-Gómez et al., 2015).

PART II. PERSONAL CONTRIBUTIONS

INTRODUCTION

Coastal environments are under increasing pressure on the environment due to population growth, urbanisation, industrialisation and tourism (Lay and Zsolnay, 1989). The high degree of urbanisation of the Black Sea coastal zone poses a permanent threat in terms of pollution of the marine environment. Although harbours are crucial for regional economic activity they are also significant sources of pollution (Catianis et al., 2016). The activities carried out in harbours have a major impact on the environment and consequently on marine organisms in ports (Knott et al., 2009).

Mussels, such as the species *Mytilus galloprovincialis* and other types of marine bivalves, are considered ideal indicator species for pollution monitoring due to their biological and ecological characteristics (Beyer et al., 2017). Mussels have the ability to efficiently accumulate chemical pollutants in seawater due to their water filtering behaviour, providing a comprehensive picture of the concentration and bioavailability of pollutants in the aquatic environment (Beyer et al., 2017). These organisms play a crucial ecological role, providing food and habitat for diverse species and, as primary consumers, serve as vectors for the transfer of anthropogenic pollutants from the abiotic phase and from the primary production level to higher trophic levels in the coastal marine food chain (Beyer et al., 2017). In addition, consumption of contaminated mussels represents a significant pathway for human exposure to chemical (Mititelu et al., 2022) and microplastic (Sangkham et al., 2022) contaminants.

In recent years, biomarkers have been included as a means to assess the biological impact of pollutants in marine pollution monitoring programmes (Beyer et al., 2017). An important role of biomarkers is to detect early signs of important biological changes. The presence of toxic substances in the ecosystem can disrupt complex relationships between organisms, which emphasises the need to enact laws and implement strategies to prevent negative impacts on aquatic environments, especially marine ecosystems (Dailianis, 2010).

VI. INFLUENCE OF ENVIRONMENTAL FACTORS ON MUSSEL CONDITION INDEX

6. 2. Material and methods

Study area

The study was carried out between November 2017 and November 2018, with seasonal frequency, in four locations in the coastal area of the Romanian coastline: Midia Port, Constanța Port, Mangalia Port and 2 Mai area.

Sample collection and processing

Physico-chemical water parameters (temperature, salinity, pH, dissolved oxygen and total dissolved solids (TDS)) were measured in situ at each sampling using the HANNA HI 98194 Multiparameter. For the laboratory analyses, three water samples (replicates) were collected for chlorophyll *a* (Chl *a*) and total suspended solids (TSS) concentrations.

The chlorophyll *a* concentration was determined by pigment extraction with 90% acetone and measured spectrophotometrically according to the SCOR-UNESCO method (1966). Determination of total suspended solids was carried out according to the method recommended by Grasshoff et al. (1999). For biometric parameters and condition index determination, 40-50 individuals (Mytilus galloprovincialis) were randomly sampled per station.

Biometric measurements and the condition index (CI)

Length, width and height of individuals were measured with a digital calliper. Total weight, wet weight of tissue, dry weight of soft tissues and valves after oven drying were weighed. The condition index (CI) was calculated using the formula: CI = (tissue dry weight (g)/valve dry weight (g)) × 100 (Davenport and Chen, 1987; Rainer and Mann, 1992).

Statistical data analysis

Statistical analyses of the data were performed using the JASP v0.19.0 and PRIMER v7.0.21 programmes. The Shapiro-Wilk test was applied to test the hypotheses of normal distributions of all data sets, and Levene's test to examine the homogeneity of variances. The non-parametric Spearman rank correlation test was applied and box plot (or Box-Whisker) plots were used. Principal Component Analysis (PCA) was performed on transformed and normalised data.

6. 3. Results and discussions

Some of the results presented in this chapter were published in Pantea et al. (2018).

The condition index recorded a maximum mean value in Mangalia harbour (16.67±9.04) and a minimum one in 2 Mai station (7.17±3.50) (Fig. 1A). Regarding the seasonal variation, the highest mean value was reached during the spring season and the lowest in the winter season (Fig. 1B).

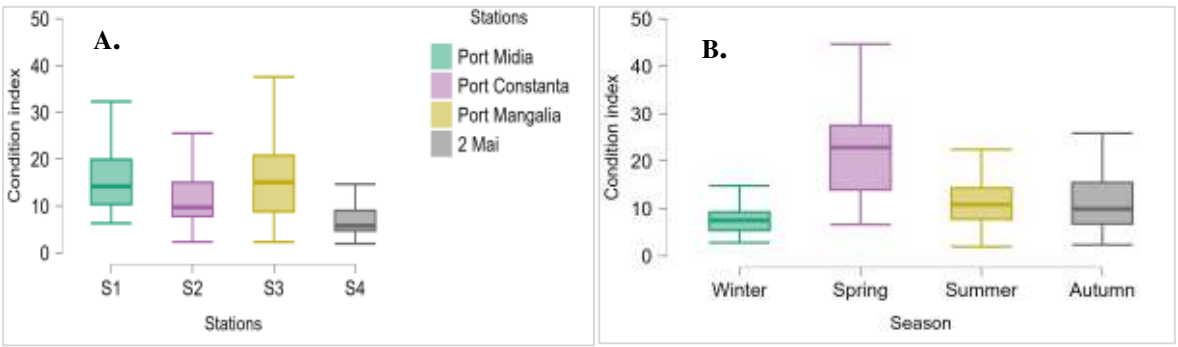

Fig. 1. Distribution of mussel condition index values by stations (A) and seasons (B)

The highest mean value of the condition index (24.80±6.33) was observed in mussels of the length class 6.1-9 cm (large mussels) in the spring season (Fig. 2). The lowest mean value of the condition index was recorded in mussels of the length class 4.1-6 cm (medium-sized mussels).

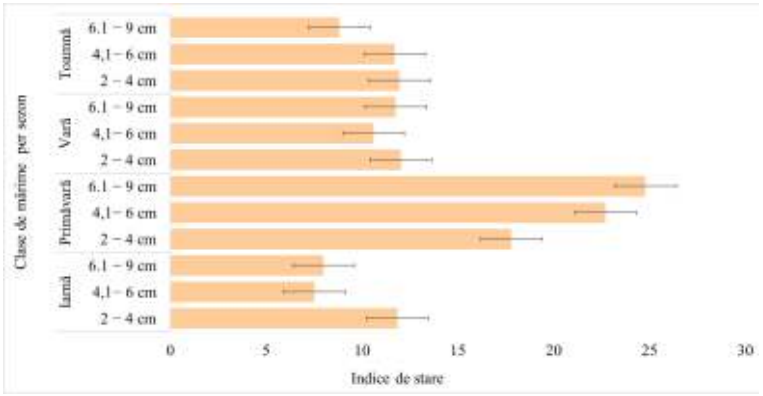

Fig. 2. Seasonal variation of condition index by size class (mean±std.dev)

The Spearman correlation test revealed statistically significant correlations between condition index, total dissolved solids and chlorophyll *a* (Table 1). Statistically significant correlations were also observed between temperature and dissolved oxygen, total suspended solids and chlorophyll *a*. Salinity correlated with total dissolved solids, dissolved oxygen with total suspended solids and total suspended solids with chlorophyll *a*.

Table 1. Spearman correlation matrix between environmental parameters and mussel (*Mytilus galloprovincialis*) condition index. Statistical significance level: * p < 0.05; ** p < 0.01; *** p < 0.001. T: Water temperature; S: Salinity; DO: Dissolved oxygen; TDS: Total dissolved solids; TSS: Total suspended solids; CHL *a*: Chlorophyll a; CI: Condition index.

Parameters		T	S	DO	pH	TDS	TSS	CHL a	CI
T	Coefficient rho	—							
	p	—							
S	Coefficient rho	0.021	—						
	p	0.940	—						
DO	Coefficient rho	**−0.785*****	0.013	—					
	p	<0.001	0.961	—					
pH	Coefficient rho	−0.035	0.075	0.203	—				
	p	0.900	0.782	0.450	—				
TDS	Coefficient rho	−0.297	**0.570***	0.109	−0.203	—			
	p	0.263	0.021	0.689	0.450	—			
TSS	Coefficient rho	**0.538***	0.127	**−0.544***	0.259	−0.300	—		
	p	0.034	0.640	0.032	0.332	0.258	—		
CHL a	Coefficient rho	**0.571***	0.046	−0.388	0.185	−0.438	**0.656****	—	
	p	0.023	0.867	0.138	0.491	0.091	0.007	—	
CI	Coefficient rho	0.488	−0.480	−0.382	−0.003	**−0.797*****	0.282	**0.553***	—
	p	0.057	0.060	0.145	0.996	<0.001	0.288	0.029	—

Applying PCA to these datasets, three principal components (PCs) with eigenvalue > 1 (eigenvalue) were extracted that together explained 80.7% of the total variability in the data. The eigenvalues of the factors were 3.45 (PC1), 1.61 (PC2) and 1.4 (PC3). PC1 explained 43.1%, PC2 20.1%, PC3 17.5% of the variability. From the contribution of variables by principal components, it was observed that both PC1 and PC2 were associated with different environmental factors. According to PCA, the condition index correlated positively with pH and chlorophyll *a* and negatively with total dissolved solids (Fig. 3). PCA analysis confirmed that the condition index showed significant seasonal variation. The multivariate analysis also confirmed that the condition index of mussels in port areas differed completely from that of mussels in the 2 Mai area, highlighting the influence of environmental parameters.

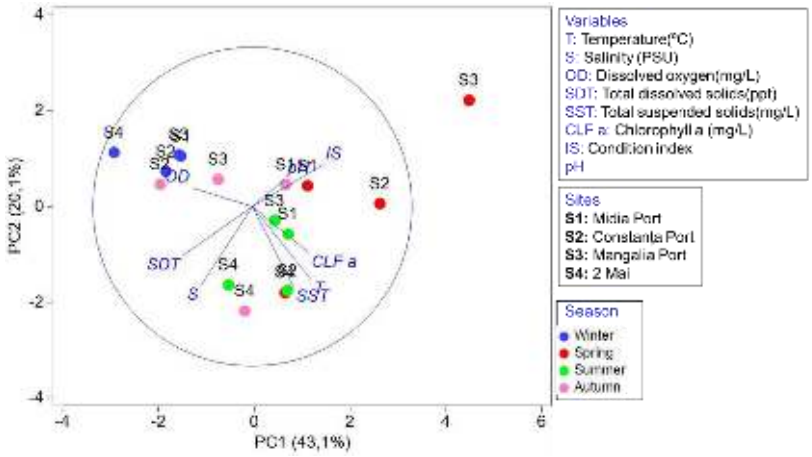

Fig. 3. Principal component analysis (PCA) showing the seasonal variation of environmental parameters and condition index per station

Statistically significant correlations were recorded between all investigated biometric variables and the condition index, although the correlation coefficient was weak to medium. The dependence between the biometric variables (length, width, height and weights) had high and medium correlation. The values of the correlation coefficients (rho) and the associated p-value are presented in Table 2.

Table 2. Spearman correlation matrix between biometric measurements and mussel (*Mytilus galloprovincialis*) condition index. Statistical significance level: * p < 0.05; ** p < 0.01; *** p < 0.001. SL: Shell length; SW: Shell width; SH: Shell height; TW: Total weight; STWW: Soft tissue wet weight; DTW: Dry tissue weight; VDW: Valves dry weight; CI: Condition index.

Parameters		SL	SW	SH	TW	STWW	DTW	VDW	CI
SL	Coefficient rho	—							
	p	—							
SW	Coefficient rho	**0.927*****	—						
	p	<0.001	—						
SH	Coefficient rho	**0.918*****	**0.928*****	—					
	p	<0.001	<0.001	—					
TW	Coefficient rho	**0.910*****	**0.856*****	**0.838*****	—				
	p	<0.001	<0.001	<0.001	—				
STWW	Coefficient rho	**0.773*****	**0.742*****	**0.732*****	**0.857*****	—			
	p	<0.001	<0.001	<0.001	<0.001	—			
DTW	Coefficient rho	**0.565*****	**0.561*****	**0.546*****	**0.687*****	**0.923*****	—		
	p	<0.001	<0.001	<0.001	<0.001	<0.001	—		
VDW	Coefficient rho	**0.898*****	**0.849*****	**0.825*****	**0.949*****	**0.737*****	**0.558*****	—	
	p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	—	
CI	Coefficient rho	**−0.237*****	**−0.202*****	**−0.199*****	**−0.148****	**0.297*****	**0.541*****	**−0.332*****	—
	p	<0 .001	< .001	<0.001	0.001	<0.001	<0.001	<0.001	—

The result of the principal component analysis (PCA) revealed two principal components (PC1 and PC2) with an eigenvalue greater than > 1 and which together explained 94.4% of the total variability in the data. The eigenvalues of the first two components were 5.36 (PC1) and 2.19 (PC2), respectively. PC1 explained 67.0% and PC2 27.4% of the data variability. Principal component analysis showed that condition index correlated positively with dry and wet tissue weight (Fig. 4). Positive correlations were also observed between morphological parameters (mussel length, width and height), total weight and dry weight of valves. The distribution of multivariate data confirmed that mussel biometric parameters were influenced by site-specific environmental conditions, with harbour mussels differing completely from those at the 2 Mai station.

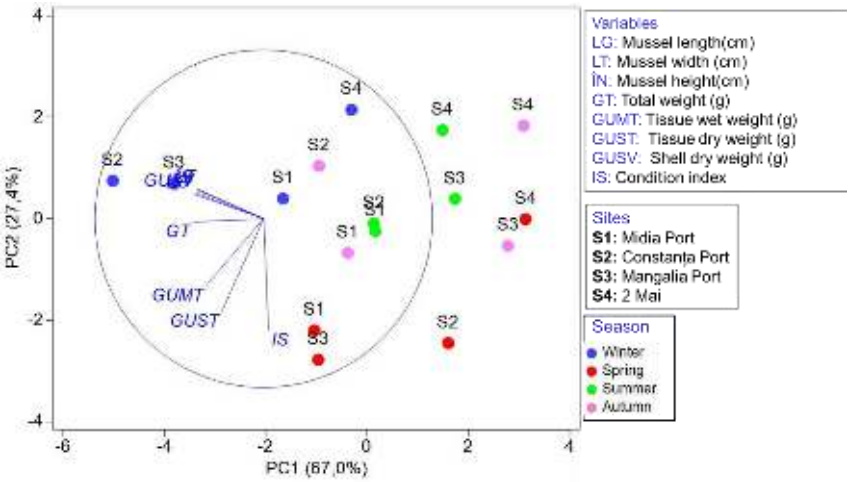

Fig. 4. Principal component analysis (PCA) showing the seasonal variation of biometric parameters and condition index per station

The variation of the condition index is influenced by the phases of the reproductive cycle and environmental factors (temperature, salinity and food) (Çelik et al., 2012). In the present study, the condition index of M. galloprovincialis mussels showed significant seasonal variation, closely following the gonadal development cycles. Strohmeier et al. (2008) argued that the amount of food induces an increase in the mussel condition index, which is also confirmed by the results of the present study. The positive correlation obtained between the condition index and chlorophyll *a* indicated that the variation of this index is closely related to food availability. In general, the condition index at the reference station (2 May) had lower values due to reduced trophic conditions and possibly the negative influence of total dissolved solids. In the present study, significant variations were observed among stations in morphometric and weight measurements of mussels, similar to the results of another study (Mendoza et al., 2023).

VII. INFLUENCE OF FOOD AVAILABILITY ON PHYSIOLOGICAL AND BIOCHEMICAL STATUS OF MUSSELS

7. 2. Material and methods

Study area and sample collection

Sampling campaigns were conducted between November 2017 and November 2018, along the Romanian Black Sea coast, in four sampling locations representing different environmental conditions, namely Midia Port (S1), Constanța Port (S2) and Mangalia Port (S3) and 2 Mai (S4). The locations were selected based on their different trophic conditions.

Seawater temperature and salinity were measured in situ using a multiparametric probe. Water samples were collected to assess food availability (phytoplankton quality and quantity). Samples were preserved immediately after collection with 20 ml of 37% formaldehyde.

Specimens of *M. galloprovincialis* (80-100 individuals) were randomly collected from different depths (0.5-2 m) using a metal rake or by hand. After collection, the samples were immediately transported to the laboratory and processed. Thirty individuals were randomly selected for condition index and the remaining mussels were stored at -20 °C for further biochemical analyses.

Qualitative and quantitative analysis of phytoplankton

Samples were sedimented in 10 ml Utermöhl sedimentation chambers for 24 hours (Edler and Elbrächter, 2010) and analysed under an inverted microscope at 200x and 400x magnification. Phytoplankton species were identified down to the closest possible taxonomic level (genus, species or subspecies), and then the cells of each species were counted. The abundance (cells/L) and biomass (mg/m3) of phytoplankton were calculated using the Ecology Database programme (source: NIMRD).

Condition index and biochemical analysis

Thirty mussels were randomly selected from each sampling location to measure condition index (SI), tissue moisture content (U) and ash (C). The index was calculated according to the method recommended by Davenport and Chen (1987) and Rainer and Mann (1992).Ash weight (C) and ash free dry weight (AFDW) were determined according to the AOAC method (1990).

To determine the biochemical composition of the soft tissues (e.g. protein, carbohydrate and lipid), 25-30 mussels were pooled in a single sample. Protein content was determined using the modified Lowry method (Razet et al., 1996). A calibration curve was performed using bovine serum albumin as standard. Carbohydrate was determined by the modified Dubois method (Razet et al., 1996) and lipid content by the Soxhlet method, which involves ether extraction in a Soxhlet apparatus.

Data analysis

All data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). Data were statistically analysed using the non-parametric Kruskal-Wallis test, Spearman correlation coefficients and multivariate statistical analysis (Principal Component Analysis, PCA). Data were also plotted using Shade Plot graphs. The statistical analyses were performed with XLSTAT 2023.3.1 and PRIMER v7.0.21. The significance level was set at $p < 0.05$ for all analyses.

7. 3. Results and discussions

The results presented in this chapter were published in Pantea et al. (2024).

Total phytoplankton abundance (cells/L) and biomass (mg/m3) varied between seasons and location (Fig. 5). In general, total abundance and biomass follow a similar trend between seasons, with higher values at the sites of Midia Port, Constanța Port and Mangalia Port. Higher abundances and biomasses were observed in spring, summer and autumn. The highest abundance was recorded in Midia Port (autumn), followed by Constanța Port (summer) and Mangalia Port (spring). In terms of biomass, the Port of Constanța had the highest value in summer, followed by the Port of Midia in autumn and the Port of Constanța in spring.

Diatoms were the most dominant group in winter, spring, summer and autumn (Fig. 5A). Dinoflagellates were most abundant in summer (in Mangalia Port. Cyanobacteria were the most abundant group, especially in Mangalia Port and 2 May. Cryptophytes dominated the phytoplankton community in almost all seasons and seasons. In general, dinoflagellates were the most dominant group in terms of biomass (Fig. 5B). Diatoms had particularly high biomasses in spring, summer and autumn.

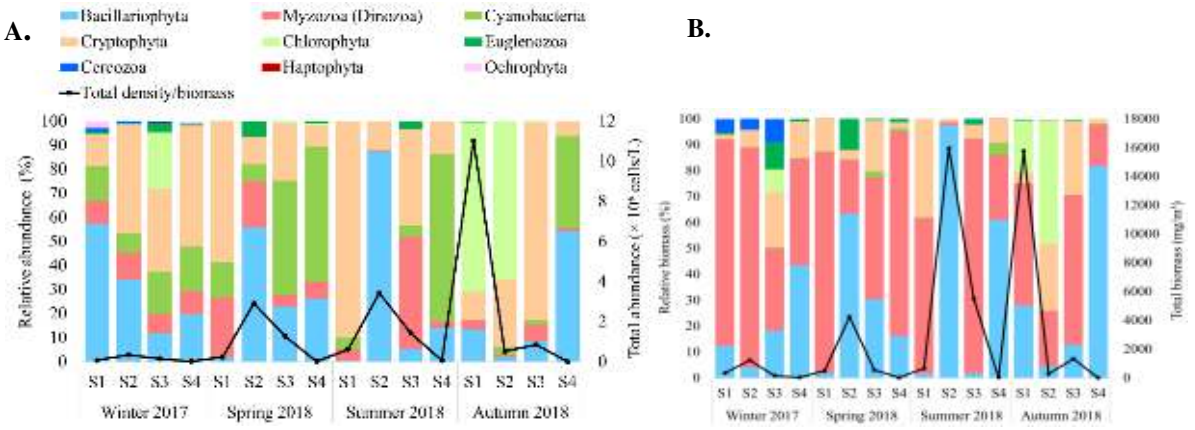

Fig. 5. Seasonal variation in phytoplankton relative and total density (A) and relative and total biomass (B)

Carbohydrate content ranged from 0.66±0.03% (at 2 May) to 20.31±0.60% (Port Mangalia), and lipid content from 1.29±0.59% (at 2 May) to 13.23±0.85% (in Port Midia) (Fig. 6A). The average carbohydrate content was highest in spring (18.55±1.22%) and lowest in summer (2.35±2.02%). The mean lipid content was highest in winter (9.23±3.84%) and spring (8.86±0.96%), and lowest in summer (3.76±2.63%) and autumn (2.57±1.21%) (Fig. The protein content in mussels ranged from 31.42±7.01% (Constanta Port) to 41.57±1.95% (Constanta Port) (Fig. 6C). The highest mean protein content was recorded in winter (40.38±1.01%) and autumn (35.53±1.65%), and the lowest in spring (34.09±3.14%) and summer (34.38±2.20%).

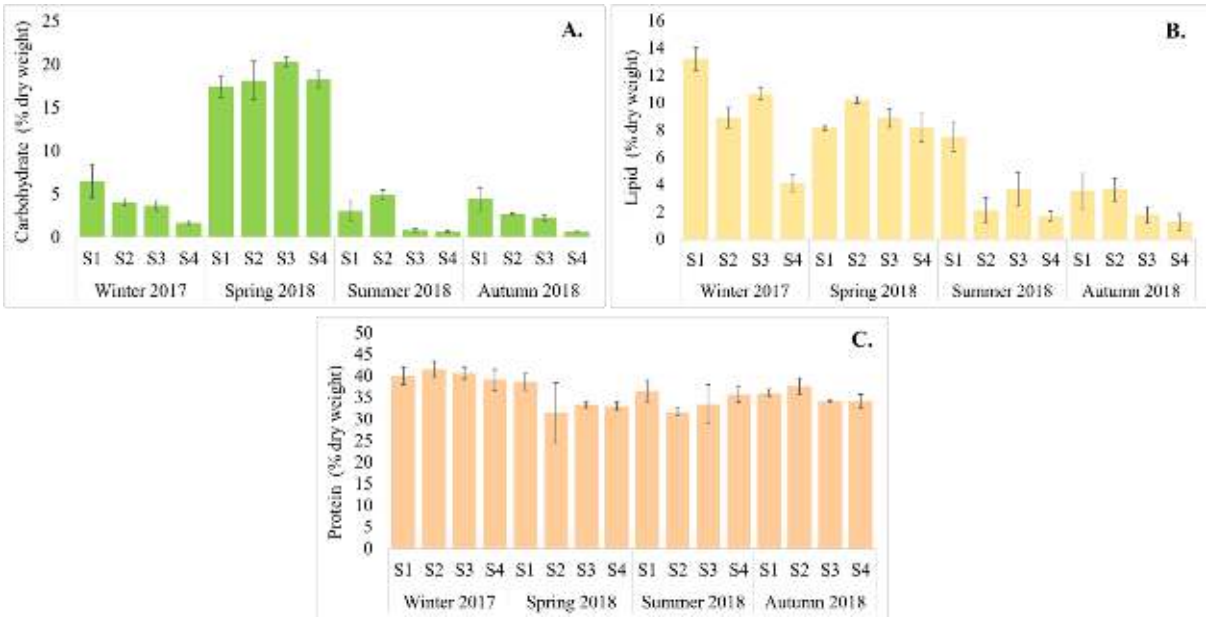

Fig. 6. Seasonal variation in carbohydrate (A), lipid (B) and protein (C) content in mussels *Mytilus galloprovincialis* (mean ± std. dev., n = 3) during 2017-2018. S1: Midia Port, S2: Constanța Port, S3: Mangalia Port, S4: 2 May.

Condition index was positively correlated with phytoplankton abundance and biomass, carbohydrate and tissue dry weight (Table 3). An inverse relationship between index and moisture was observed. Protein showed a significant correlation with moisture, tissue dry weight and seawater temperature. Lipids were correlated with carbohydrate, AFDW and ash. Carbohydrate showed a positive correlation with tissue dry weight and AFDW, and a negative correlation with moisture and ash. Tissue dry weight correlated with temperature, moisture, ash and AFDW. Moisture correlated with AFDW and temperature.

Table 3. Spearman's correlation coefficient between biological parameters assessed in *M. galloprovincialis*, phytoplankton abundance and biomass and environmental parameters (n = 16). L: lipid; C: carbohydrate; P: protein; CI: condition index; AFDW: ash-free dry weight; A: ash; H: humidity; TDW: tissue dry weight; SDW: shell dry weight; PA: phytoplankton abundance; PB: phytoplankton biomass; T: seawater temperature; S: salinity. Values in bold are statistically significant at $p < 0.05$.

Variables	L	C	AFDW	A	CI	M	TDW	T	P	PA	PB	S	SDW
L	**1**												
C	**0.650**	**1**											
AFDW	**0.647**	**0.871**	**1**										
A	**-0.647**	**-0.871**	**-1.000**	**1**									
CI	0.241	**0.688**	**0.744**	**-0.744**	**1**								
M	-0.003	**-0.591**	**-0.603**	**0.603**	**-0.791**	**1**							
TDW	0.003	**0.591**	**0.603**	**-0.603**	**0.791**	**-1.000**	**1**						
T	-0.182	0.215	0.132	-0.132	0.481	**-0.739**	**0.739**	**1**					
P	0.309	-0.229	-0.150	0.150	-0.447	**0.641**	**-0.641**	**-0.618**	**1**				
PA	-0.026	0.276	0.241	-0.241	**0.641**	-0.300	0.300	0.305	-0.394	**1**			
PB	-0.006	0.232	0.203	-0.203	**0.591**	-0.253	0.253	0.280	-0.303	**0.944**	**1**		
S	-0.124	-0.106	-0.379	0.379	-0.500	0.226	-0.226	0.001	-0.188	-0.391	-0.376	**1**	
SDW	-0.097	-0.041	-0.003	0.003	0.053	-0.074	0.074	0.230	0.288	0.018	-0.103	-0.141	**1**

The results of the Kruskal-Wallis test showed statistically significant differences between stations in phytoplankton abundance (KW = 9.715; p = 0.021), phytoplankton biomass (KW = 8.735; p = 0.033) and condition index (KW = 148.550; p = 0.0001). Statistically significant differences were also observed between seasons for condition index (KW = 182.475; p = 0.0001), protein content (KW = 9.419; p = 0.024), carbohydrate content (KW = 9.287; p = 0,026), lipid content (KW = 11,007; p = 0,012), moisture (KW = 11,228; p = 0,011), dry tissue (KW = 11,228; p = 0,011), AFDW (KW = 9,154; p = 0,027) and ash (KW = 9,154; p = 0,027).

The post hoc analysis (Dunn's test) showed that statistically significant differences in abundance and biomass and significant differences in status index were detected between 2 Mai station (low trophic level) and Port stations (high trophic level): Midia Port, Constanța Port, Mangalia Port. There were also significant differences between the index values between winter, spring, summer and autumn, but not between summer and autumn.

Principal Component Analyses (PCA) of the mean values of the biological parameters assessed in *M. galloprovincialis*, phytoplankton abundance and biomass, environmental parameters and associated variability in station distributions are presented in Figure 7. The first two principal components have eigenvalues of 3.89 and 2.31, respectively, and explain 68.9% of the total variance of the data matrix (Fig. 7A, B). Principal component 1 (PC1) explained 43.2% of the total variance and showed significant contribution of carbohydrate (0.42), condition index (0.48), AFDW (0.43) and phytoplankton abundance (0.32). Principal component 2 (PC2) explained 25.7% of the data variability and was mainly characterised by the contribution of proteins (0.46) and lipids (0.50).

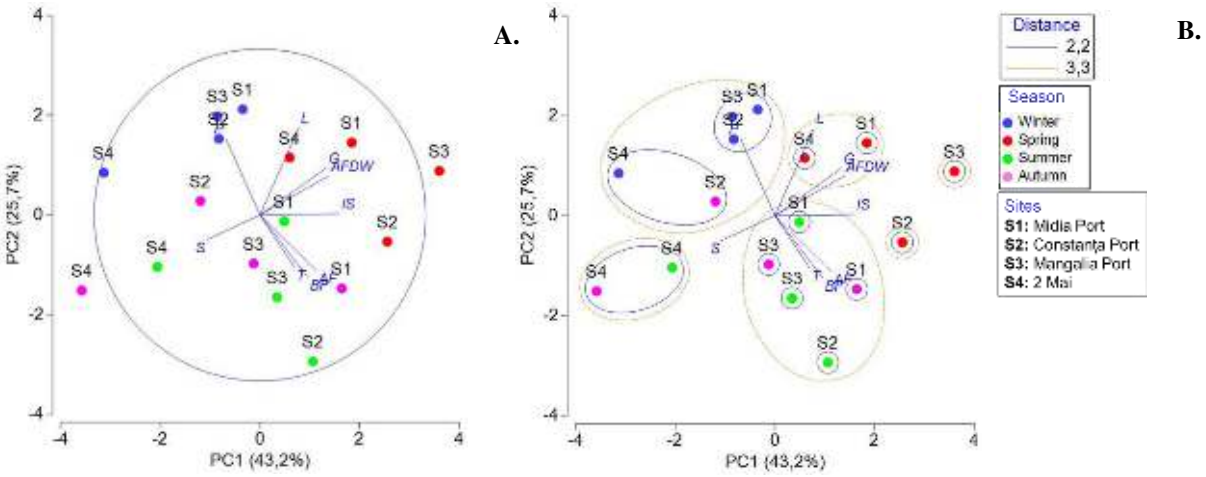

Fig. 7. Principal Component Analysis (PCA) of condition index, biochemical components, AFDW (ash-free dry weight), phytoplankton abundance and biomass and environmental parameters (temperature and salinity) (A) and station clustering (B), based on Euclidean distance matrix of the fourth-root transformed and normalised data

Phytoplankton dynamics influence food availability in water (Lok et al., 2010). Coastal waters are subject to significant variations in food availability, which are influenced by both food quantity and species composition (Lok et al., 2010). Phytoplankton composition varies spatially and temporally due to variation in various physical and biological factors (Bayne, 1993). The present study showed that the ports(Midia Port, Constanța Port and Mangalia Port) have higher species diversity and cell abundance than 2 Mai station (in all seasons).

Status index and biochemical composition are closely related to seawater temperature, food availability and gametogenesis cycle (Beninger and Lucas, 1984; Strohmeier et al., 2008; Çelik et al., 2012). Temperature and food availability are the main factors affecting bivalve growth (Bayne and Newell, 1983). However, the influence of these variables is complex and depends on how each species acquires and consumes energy in its natural environment (Bayne and Newell, 1983).

Accumulated energy reserves, especially glycogen and protein, and gonadal development reflect good condition index values (Sahin et al., 2006). The metabolic activity of bivalves is characterised by phases of accumulation and consumption of body reserves (Moschino et al., 2023). This process is influenced by phytoplankton availability, environmental conditions and the gametogenesis cycle (Çelik et al., 2012; Orban et al., 2002). The results of this study showed that lipid and carbohydrate contents peaked in spring and decreased in summer, probably because they were utilised in the gametogenesis process. The high carbohydrate and lipid contents observed in spring may be related to increased food availability.

VIII. HEAVY METAL BIOACCUMULATION AND PHYSIOLOGICAL RESPONSE OF MUSSELS: CONDITION INDEX

8. 2. Material and methods

Sampling locations

The study was carried out between November 2017 and November 2018 in four stations in the southern Romanian coastline's southern sector, namely Midia Port, Constanța Port, Mangalia Port and 2 Mai (reference station).

Sample collection

The physico-chemical parameters (temperature, salinity, pH, dissolved oxygen and total dissolved solids) were measured in situ using a multiparametric probe. Water samples were collected for determination of total suspended solids (TSS) and heavy metal concentration in water. Approximately 50-60 mussels (Mytilus galloprovincialis) were randomly collected to determine the condition index and metal concentration in mussel soft tissues.

Chemical analysis

The procedure for analysing heavy metals in seawater, sediments and mussels was carried out according to the recommended methods for the marine pollution studies (IAEA - MEL, 1999; UNEP, 1995; UNEP, 1990; UNEP, 1993).

Determination of bioaccumulation factors and indices

The bioaccumulation factor (BAF) is the ratio of the metal concentration in mussels (Cm, µg/kg dry weight) to the metal concentration in the water column (Ca, µg/L) (Gobas and Morrison, 2000). The biota-sediment accumulation factor (BSAF) is the ratio of the metal concentration in mussels (Cm, µg/kg dry weight) to the metal concentration in sediment (Cs, µg/kg dry weight) (Szefer et al., 1999).

Bioaccumulation indices, i.e., individual multimetal mean bioaccumulation index (IMBI) and metal pollution index (MPI), were calculated according to Boudjema et al. (2022).

Condition index determination

Thirty mussels were analysed to determine the condition index. The condition index (SI) was calculated using the equation recommended by Davenport and Chen (1987) and Rainer and Mann (1992).

Statistical data analysis

The Shapiro-Wilk normality test and the Levene test were applied. Differences between data sets were tested using Analysis of Variance (ANOVA) or the non-parametric Kruskal-Wallis test as well as Post Hoc tests (Tukey's multiple comparison test or Dunn's test for pairwise comparisons). The Spearman correlation coefficient was used for all correlation analyses. Data were also plotted using Shade plots, Principal Component Analysis (PCA), Cluster Analysis and Non-Multidimensional Multidimensional Scaling (nMDS) Cluster Analysis, PCA, nMDS and Shade plots were performed using PRIMER v7.0.21 software and JASP v0.19.0.

8. 3. Rezultate și discuții

Some of the data presented in this chapter were published in Pantea et al. (2020).

The bioaccumulation potential of metals follows a decreasing sequence Cu > Ni > Cd > Cr > Pb (Fig. 8). Heavy metal concentrations accumulated in mussel soft tissues ranged from 0.65-5.44 µg/g g.um. Cu; 0.31-0.73 µg/g g.um. Cd; 0.01-0.33 µg/g g.um. Pb; 0.15-3.08 µg/g g.um. Ni; 0.08-1.31 µg/g g.um. Cr. The highest

concentration of Cu was recorded in the mussels of Midia Port (in summer), of Cd in the mussels of Mangalia Port (in winter), of Pb in the mussels of 2 Mai station (in winter), of Ni in the mussels of 2 Mai station (in spring) and of Cr in the mussels of Midia Port (in summer).

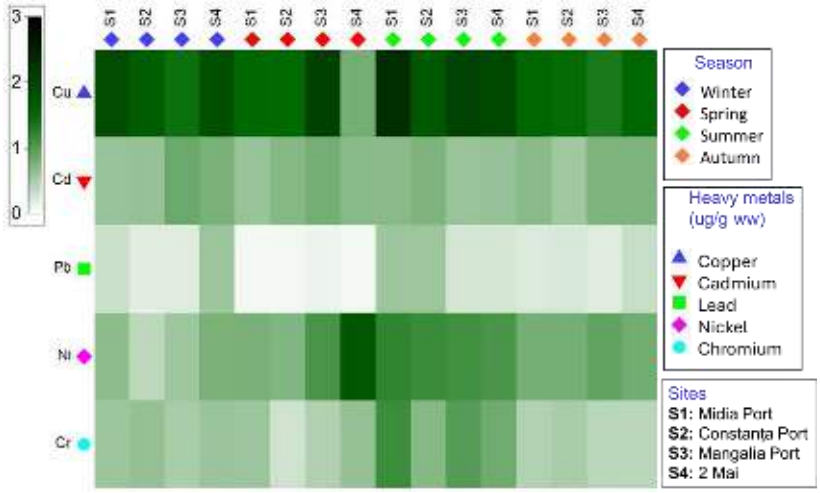

Fig. 8. Concentration of heavy metals accumulated in the tissue of *Mytilus galloprovincialis* (µg/g wet weight)

The ANOVA test showed no statistically significant differences in total metal concentration in mussel soft tissues ($F(3,12) = 3.688$; $p = 0.777$) between the stations investigated, but showed differences between seasons ($F(3,12) = 7.219$; $p = 0.005$). Significant differences were revealed by Post Hoc Tukey analysis between winter-summer, spring-summer and summer-autumn.

Principal Component Analysis (PCA) of heavy metal concentrations in M. galloprovincialis mussel soft tissue and environmental parameters showed that the two PCA components together explained 51.5% of the total variability in the data (Fig. 9). PC1 accounted for 26.8% of the variance and had an eigenvalue of 2.95, and PC2 24.7% and an eigenvalue of 2.72. PC1 was better accounted for by the contribution of temperature, dissolved oxygen, Ni and Cr, and PC2 by total dissolved solids and Pb.

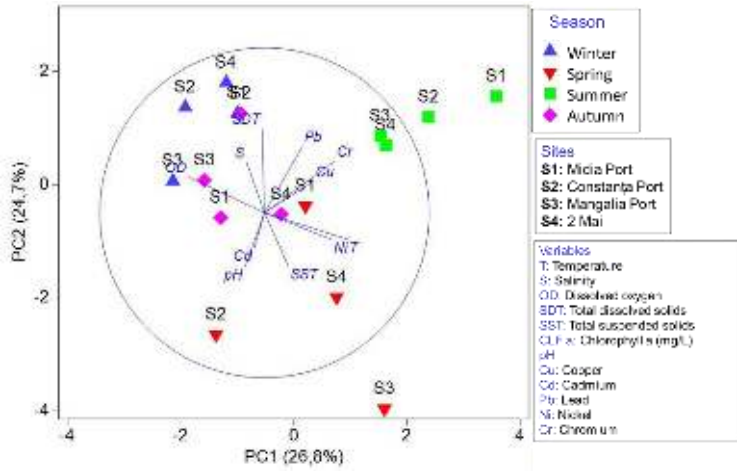

Fig. 9. Principal component analysis (PCA) showing seasonal variation in environmental parameters and heavy metal concentrations in mussel soft tissues

The Kruskal-Wallis H-test showed no statistically significant differences between stations ($H(3) = 0.624$; $p = 0.891$), in terms of heavy metal bioaccumulation factor (BAF). Significant differences were recorded between seasons ($H(3) = 8.843$; $p = 0.031$). Post Hoc Dunn's Dunn's analysis, showed significant differences between

winter - spring (p = 0.011), spring - summer (p = 0.039), spring - autumn (p = 0.011). There were no significant differences between winter - summer (p = 0.624), winter - autumn (p = 0.978) and summer - autumn (p = 0.644).

As for the Biota-Sediment Accumulation Factor (BSAF), the result of the Kruskal-Wallis H-test showed no statistically significant difference between stations ($H_{(3)}$ = 5.738; p = 0.125) or seasons ($H_{(3)}$ = 3.243; p = 0.356).

The highest IMBI value (0.71) was observed in Midia Port (S1) and was represented by cadmium (Cd) (Fig. 10A, B). However, as mean value, the highest value was recorded in Mangalia Port (S3) and the lowest in Midia Port (S1).

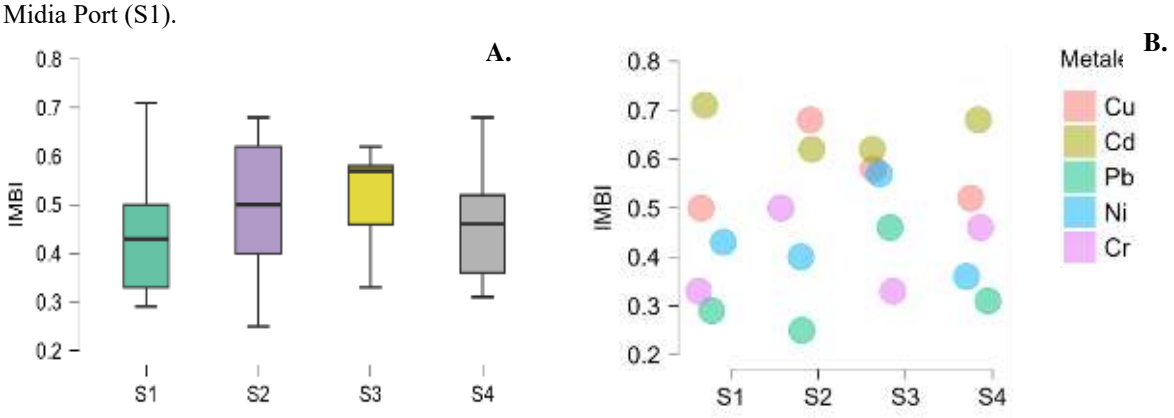

Fig. 10. Distribution of individual mean multimetal bioaccumulation index (IMBI) values by stations (A) and metals (B)

In general, the distribution pattern of IMBI per station follows the following sequence: Cd > Cu > Ni > Cr > Pb. The highest IMBI value (0.73) was observed in autumn and was represented by copper (Cu) (Fig. 11A, B). The highest mean value was recorded in autumn (0.62) and the lowest in spring (0.48). The distribution of IMBI values of metals by seasons varied widely without following any particular pattern.

The ANOVA test showed no statistically significant differences between stations (F(3,16) = 0.152; p = 0.927) or seasons (F(3,16) = 1.535; p = 0.244), in terms of the individual mean multimetal bioaccumulation index (IMBI).

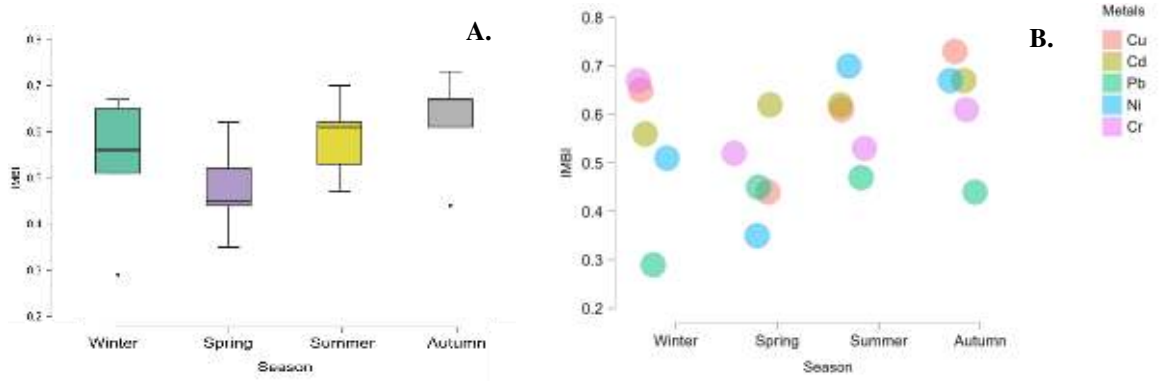

Fig. 11. Distribution of individual mean multimetal bioaccumulation index (IMBI) values by season (A) and metals (B)

The metal pollution index (IMP) values ranged from 0.05 to 319.41. The highest mean metal pollution index (MPI) value was observed in S1 - Midia Port (81.11) and the lowest in S3 - Mangalia Port (6.17) (Fig. 12A). The highest mean value of the index was recorded in summer (105.63) and the lowest in spring (0.53) (Fig. 12B).

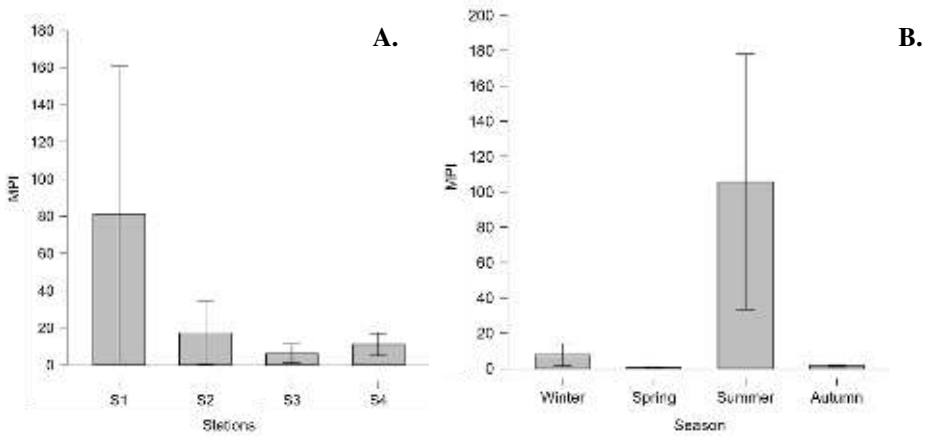

Fig. 12. Average Metal Pollution Index (MPI) (mean±st.dev.) per station (A) and per season (B)

The Kruskal-Wallis H-test showed no statistically significant differences between stations (H(3) = 0.926; p = 0.819), in terms of the metal pollution index (IMP). Significant differences were recorded between seasons (H(3) = 9.419; p = 0.024). Post Hoc Dunn's Dunn's analysis showed significant differences only between spring and summer season (p = 0.002). There were no significant differences between winter - spring (p = 0.158), winter - summer (p = 0.102), winter - autumn (p = 0.824), spring - autumn (p = 0.235) and summer - autumn (p = 0.063).

The condition index of *M. galloprovincialis* ranged between 2.79-18.31 (winter), between 6.61-44.66 (spring), between 1.94-22.46 (summer) and between 2.33-25.86 (autumn) (Fig. 13). The highest mean value of the index (29.49±6.02) was reached in the Port of Mangalia (spring) and the lowest at the station 2 Mai (4.96±1.60) in winter.

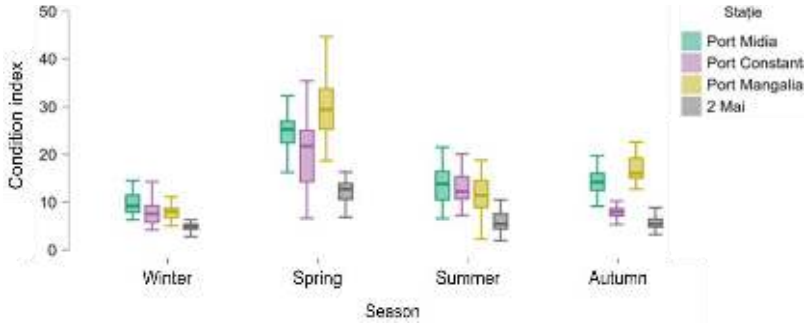

Fig. 13. Seasonal variation of the condition index of mussels *Mytilus galloprovincialis*

The non-metric multidimensional scaling (nMDS) analysis that was calculated based on the 2017-2018 condition index value data imposing station and season as a factor, is shown in Figure 14. From the nMDS analysis, it can be seen that the stations were grouped into four clusters (Fig. 14) according to the similarity of the condition index value. As a result, it can be observed that the first cluster is represented by the ports(S1 - Midia Port and S3 - Mangalia Port), stations that had the highest state index in spring. In general, by plotting nMDS, it can be observed that the stations in the ports(S1 - Midia Port, S2 - Constanta Port and S3 - Midia Port) tended to cluster together due to the similarity between the values.

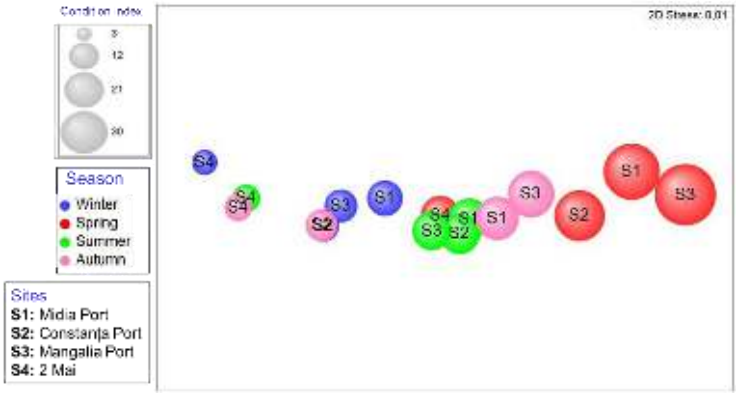

Fig. 14. Non-metric Multidimensional Scaling (nMDS) ordination plot of Bray-Curtis dissimilarities between stations based on the transformed mean (√) values of the noodle state index (2D stress value = 0.01)

The Kruskal-Wallis H-test showed a statistically significant difference between the different stations ($H(3) = 165.532$; $p < 0.001$). Post Hoc Dunn analysis showed significant differences between the condition index of Port Midia - Port Constanta, Port Midia - 2 May, Port Constanta - Port Mangalia, Port Constanta - 2 May and Port Mangalia - 2 May. There were no significant differences between Midia Port and Mangalia Port.

Significant differences were also revealed by the Kruskal-Wallis H test for the variation of the condition index between seasons ($H(3) = 182.391$; $p < 0.001$). Post Hoc Dunn Dunn's analysis showed significant differences between winter-spring, winter-summer, winter-autumn, spring-summer, and spring-autumn. There were no significant differences between the summer and autumn seasons.

The result of the Spearman correlation test between mussel condition index, heavy metal concentration in water, sediment and tissue, bioaccumulation factor and biota-sediment accumulation factor is presented in Table 4. Statistically significant correlations were observed only between the condition index and Pb ($R = -0.564$; $p = 0.023$).

Table 4. Spearman correlation between mussel condition index, heavy metal concentration (water, sediment and tissue), bioaccumulation factor and biota-sediment accumulation factor. Statistical significance level: * $p < 0.05$. Cu: Copper; Cd: Cadmium; Pb: Lead; Ni: Ni: Nickel; Cr: Chromium; CI: Condition index; BAF: Bioaccumulation factor; BSAF: Biota-sediment accumulation factor.

Parameters	Component		Cu	Cd	Pb	Ni	Cr
CI	WATER	Coefficient rho	0.150	0.330	0.126	0.218	0.458
		p	0.579	0.211	0.641	0.417	0.075
CI	SEDIMENTS	Coefficient rho	-0.271	-0.453	-0.206	0.432	0.224
		p	0.310	0.080	0.443	0.096	0.404
CI	TISSUE	Coefficient rho	-0.029	0.059	**-0.564***	0.233	-0.252
		p	0.917	0.828	0.023	0.386	0.346
CI	BAF	Coefficient rho	-0.280	-0.470	-0.299	-0.291	-0.567
		p	0.293	0.066	0.261	0.273	0.022
CI	BSAF	Coefficient rho	-0.164	0.449	-0.357	-0.293	-0.436
		p	0.543	0.081	0.174	0.270	0.091

PCA of the index of mussel status and metal concentrations in water, sediment and mussel revealed five principal components (PCs) with eigenvalue > 1 that together explained 81.8% of the total variability in the data (Fig. 15). PC1 explained 26.1%, PC2 20.6%, PC3 14.8%, PC4 12.6% and PC5 7.7% of the variability. Graphical representation of the PCA, showed that the condition index was influenced by the concentrations of heavy metals in the water, especially Cu, Pb, Ni and Cr (Fig. 15). The multivariate analysis also revealed the spatial distribution

of the variables by separating the stations into four groups (clusters), well delimited seasonally. Thus, it can be observed that heavy metals in sediments were better represented in winter, those in seawater in spring, and those in mussel tissues in summer and autumn.

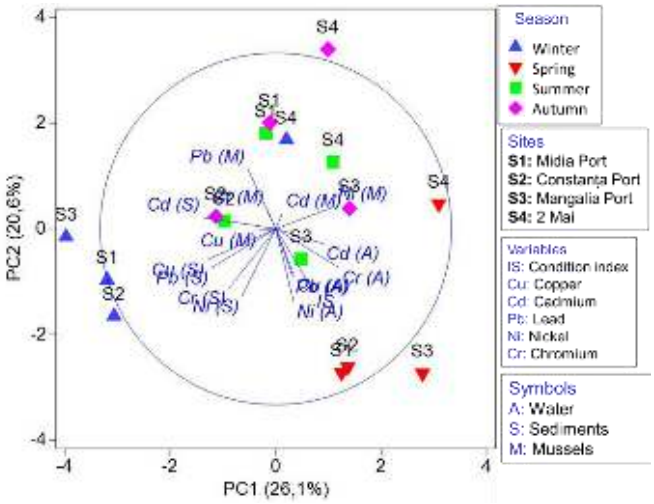

Fig. 15. Principal component analysis (PCA) showing the seasonal variation of status index and heavy metal concentrations in water, sediments and mussel soft tissues

The physico-chemical properties of water show a large variability depending on season and location (Rouane-Hacene et al., 2015). Physico-chemical parameters of water affect the availability of contaminants and therefore influence the bioaccumulation and biological responses of organisms to them (Rouane-Hacene et al., 2015). Water contamination exhibits seasonal and spatial variations in the type and concentration of contaminants and can be considered a possible cause of the seasonal variation in the bioavailability and bioaccumulation of heavy metals in mussel tissues (Rouane-Hacene et al., 2015). In general, the highest concentration of metals in the water was observed mainly in ports, similar to other studies (Oros et al., 2017; Lazăr et al., 2021). In contrast, high values of Cu and Pb were also recorded in the reference area (2 May). Exceedances of Environmental Quality Standards (EQS) for marine waters were only observed for Cd (EQS_{Cd} = 1.5 µg/L) in Mangalia Port (in autumn) (European Union, 2013).

In the present study, the highest concentrations of heavy metals were observed in sediments at all investigated locations and in all seasons, an aspect confirmed by other studies (Lazăr et al., 2021). Portswere the most contaminated locations in terms of metal concentration in sediments, an aspect also highlighted by other studies (Lazăr et al., 2021).

Physiological condition is one of the main factors able to control the internal distribution and retention of contaminants in mussels (Windows and Donkin, 1992). The concentration of metals detected in mussel tissues were the result of the net balance between uptake-storage and excretion processes as a result of exposure to metals through water, food, sediment and air (Abderrahmani et al., 2000). Referring to the concentration of heavy metals in mussels, a higher tendency of heavy metals bioaccumulation in portswas observed as a result of exposure to higher metal concentrations. The main pathway of heavy metal uptake being food ingestion (Abderrahmani et al., 2000). In the present study, the relationship between condition index and tissue metal concentration was inversely proportional.

IX. BIOACCUMULATION OF MICROPLASTICS IN MUSSEL TISSUES AND THEIR INFLUENCE ON THE CONDITION INDEX

9. 2. Material and methods

Study areas

In order to investigate the level of microplastic pollution and the physiological response (condition index) of wild mussels Mytilus galloprovincialis (Lamarck, 1819) from the Romanian Black Sea coastal area, four sampling locations exposed to different levels of anthropogenic pressures and varying environmental conditions were selected. The study was conducted from May to November 2018, at a seasonal scale (spring, summer and autumn). The sampling locations were selected considering the main potential sources of microplastics in the coastal zone, such as: domestic and industrial wastewater treatment plants, maritime traffic, tourism and fisheries.

Sampling

Approximately 60 mussels (*Mytilus galloprovincialis*) were randomly collected from different depths and different substrates (artificial and natural). Specimens collected for the determination of microplastic accumulated in the tissues were stored at −20 °C until further analysis, and the remaining individuals were placed in glass Berzelius beakers (3 L) and kept at 4 °C for approximately 24 h.

Microplastics analyses

The mussels were thawed at room temperature, measured and grouped into three size classes (n = 3): small (2.0-4 cm), medium (4.1-6 cm) and large (6.1-9 cm). A total of 108 mussels were analysed in the study. Soft tissue was also weighed to obtain the wet weight of the analysed sample (wet weight, g).

The microplastic extraction method involved hydrogen peroxide digestion of the soft tissues according to the protocol recommended by Li et al. (2015). A concentrated saline solution was used for the flotation separation of microplastics. Filters were examined using a stereomicroscope for visual identification of microplastics based on their physical characteristics. The microplastics were classified into five morphotypes (fibres, fragments, pellets, sheets and foam), grouped by colour (transparent, black, blue, red, green and black) and measured. Microplastics were visually identified based on the physical characteristics of the particles according to Barrows et al. (2017). The condition index was determined according to the method recommended by Davenport and Chen (1987) and Rainer and Mann (1992).

Statistical data analysis

Statistical analyses were performed using JASP v0.19.0 and PRIMER v7.0.21. Data were assessed for normality and homogeneity of variances using the Shapiro-Wilk and Levene's tests, respectively. Because the data did not have a normal distribution and did not fulfil the assumption of homogeneity of variances, they were evaluated using the non-parametric Kruskal-Wallis test, followed by the post hoc Dunn test. Data were statistically analysed using the Spearman correlation test, Cluster Analysis and Principal Component Analysis (PCA).

9. 3. Results and discussions

A total of 4584 microplastics were identified in the analysed mussels (108 individuals). The highest mean microplastic count per individual was observed at 2 Mai station in summer (69.89 MP/ind.) and the lowest in Constanta Port in autumn (18.89 MP/ind.) (Fig. 16A). The Kruskal-Wallis H-test showed no statistically significant differences between stations ($H(3) = 4.744$; $p = 0.192$) or seasons ($H(2) = 0.346$; $p = 0.841$), in terms of microplastics (MP) abundance in mussel tissue per individual. Mean MP abundance per gram ranged from 4.87

MP/g (Midia Port, spring) to 57.76 MP/g (Mangalia Port, summer). Very high microplastic abundances were also observed at station 2 Mai in spring, summer and autumn (Fig. 16B).

The Kruskal-Wallis H test revealed statistically significant differences in microplastic (MP) abundance per gram between stations ($H(3) = 8.641$; $p = 0.034$). Post Hoc Dunn's analysis showed significant differences between Midia Port - Mangalia Port and between Midia Port - 2 May. Regarding the seasonal variation in microplastic abundance (MP) per gram, the result of the Kruskal-Wallis H test showed no statistically significant difference between seasons ($H(2) = 0.808$; $p = 0.668$).

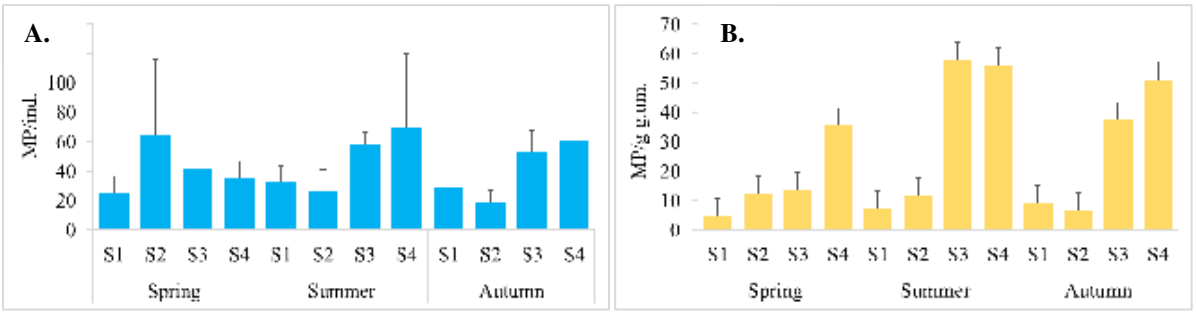

Fig. 16. Microplastic abundance (mean ± std. dev.) per individual (A) and per gram wet weight (g wet weight) in the investigated areas

The nMDS analysis based on the substrate factor revealed that the mussels with the highest abundances of PM were collected from plastic ropes and pontoon support pillars in their vicinity (at 2 Mai station - S4), from the plastic floats of floating pontoons (Mangalia Port - S3) and from the hull of a long-anchored vessel (Constanța Port - S2) (Fig. 17). The second group of stations, where the substrate was generally concrete quay, had lower values compared to the first group. It can also be observed that the station with the lowest mean value of microplastic abundance visibly delineated itself from the rest of the stations (Constanta Port - S2).

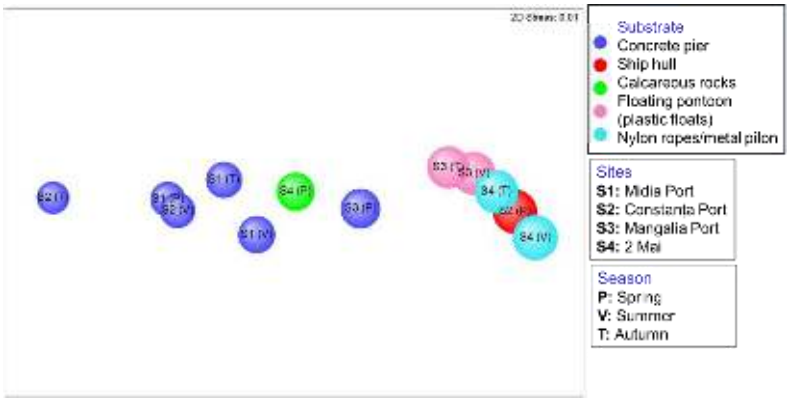

Fig. 17. Non-metric Multidimensional Scaling (nMDS) ordination plot of Bray-Curtis dissimilarities between stations based on the transformed mean (√) values of microplastic abundance as a function of the substrate factor (2D stress value = 0.01)

Concerning the nMDS analysis performed based on the "pollution sources" factor, it was observed that the abundance of PM abundance in mussels followed the same trends as the one performed based on the "substrate" factor (Fig. 18). The degree of availability of PM for mussels, due to their abundant presence in the water column, was higher at station 2 Mai - S4, Port of Mangalia - S3 and Port of Constanța - S2 due to domestic and/or industrial wastewater discharges and fishing/tourism activities. The Kruskal-Wallis H test did not reveal

statistically significant differences between stations ($H(3) = 4.744$; $p = 0.192$) or seasons ($H(2) = 0.346$; $p = 0.841$), in terms of total microplastic (MP) abundance in mussel tissue.

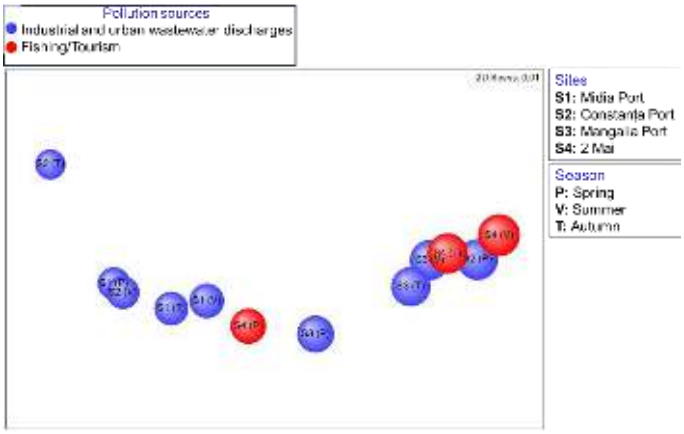

Fig. 18. Non-metric Multidimensional Scaling (nMDS) ordination plot of Bray-Curtis dissimilarities between stations based on the transformed mean (√) values of microplastic abundance, as a function of the "pollution sources" factor (2D stress value = 0.01)

Fibres were the most observed MPs in the investigated stations (77.22-99.21%), followed by fragments (0.79-21.10%). Pellets and foils represented 0.84% and 1.18% of the total MPs identified, respectively. Foam was the rarest MP identified in the analysed samples (0.59%) (Fig. 19).

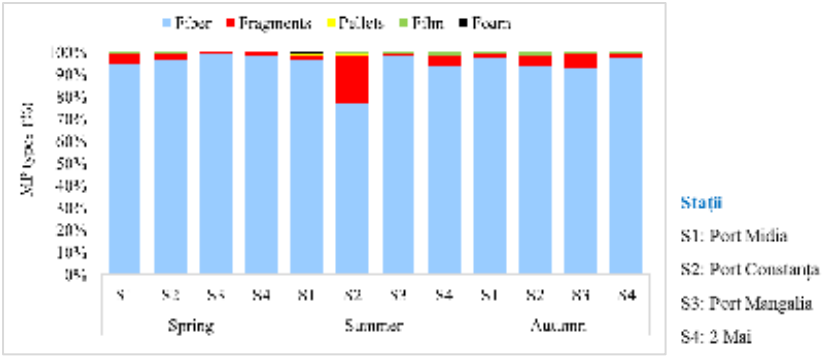

Fig. 19. Percentage composition of different types of microplastics identified in mussels by location and season

The observed microplastics had different colours, namely: black, transparent, blue, red and green (Fig. 20). The most common colour was transparent (86.29%), followed by black (21.52%), red (10.35%) and blue (9.41%). Green coloured microplastics were less represented (1.27%). In general, the most common colour observed in the fibres was transparent.

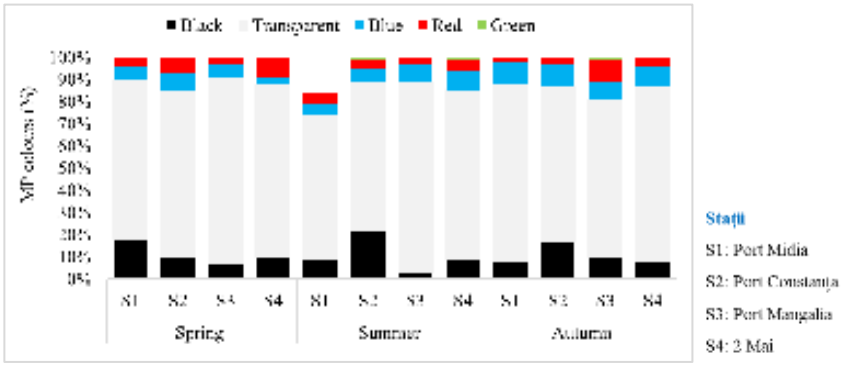

Fig. 20. Percent composition of different colours of microplastics identified in mussels by location and season

The size of the microplastics isolated from mussel tissue ranged from 0.01 mm to 5 mm (Fig. 21A). 49 plastic fibres larger than 5 mm (5.01-13.46 mm) were also found in the samples but were not included in the analysis as they were considered mesoplastics. Microplastics smaller than 1 mm were the most frequently found in the samples. The highest number of PM was found in the range 0.5-1 mm.

Regarding the abundance of MPs in mussel tissues by size class, the highest average abundance was observed in summer in mussels of size class 2.0-4 cm (198 MPs) and the lowest in autumn in mussels of size class 4.1-6 cm (76.50 MPs) (Fig. 21B). In spring and autumn, the highest proportion was observed in mussels of size class 6.1-9 cm (160.40 MPs and 166 MPs, respectively).

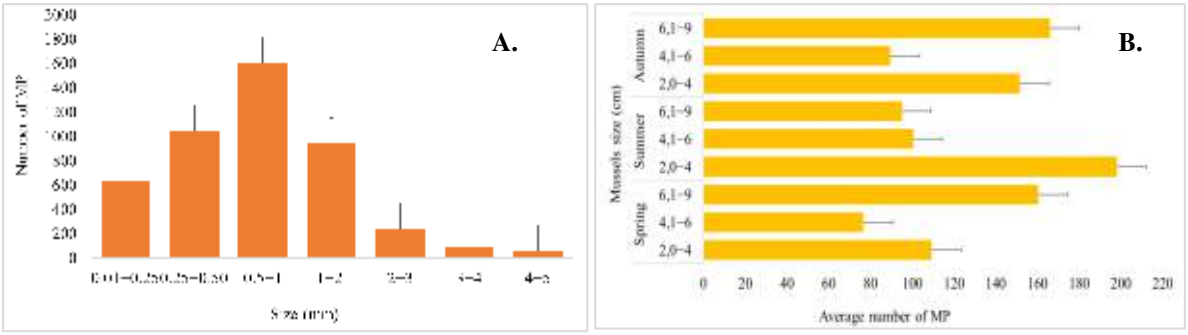

Fig. 21. Frequency of microplastics isolated from mussels by different size classes (A) and microplastics abundance (mean ± std. dev.) by mussel size classes in the investigated areas (B)

The Spearman correlation test revealed statistically significant correlations only between mussel length (LM), mussel tissue wet weight (GM), total microplastic count (MPT), microplastic count per individual (MP/ind.) and microplastic count per gram wet weight (MP/g) (Table 5). Negative correlations were recorded between ML - MPT, ML -MP/ind., ML- MP/g and MWW - MP/g. Positive correlations were observed between CI - MWW, MPT - MP/ind., TMP - MP/g and between MP/ind. - MP/g.

The nMDS analysis of the mean microplastics abundance data by size class overlaid with the cluster analysis performed based on the Bray-Curtis similarity between stations revealed that the stations were divided into two distinct groups according to the abundance of microplastics in mussels (Fig. 22).

Table 5. Spearman correlation matrix between mussel condition index, mussel length, mussel tissue wet weight, total microplastic count, microplastic count per individual and microplastic count per gram wet weight. Statistical significance level: * p < 0.05; ** p < 0.01; *** p < 0.001. CI: Condition index; ML: Mussel length; MWW: Mussel wet tissue weight; TMP: Total microplastic count; MP/ind: Number of microplastics per individual (*Mytilus galloprovincialis*); MP/g: Number of microplastics per gram wet weight (*Mytilus galloprovincialis*).

Variable		CI	ML	MWW	TMP	MP/ind.	MP/g
CI	Coefficient rho						
	p	—					
ML	Coefficient rho	0.266					
	p	0.404	—				
MWW	Coefficient rho	**0.811****	**0.671***	—			
	p	0.002	0.020				
TMP	Coefficient rho	0.217	**0.664***	0.434	—		
	p	0.499	0.022	0.161			
MP/ind.	Coefficient rho	0.217	**0.664***	0.434	**1.000*****	—	
	p	0.499	0.022	0.161	< .001	—	
MP/g	Coefficient rho	−0.434	**−0.601***	**−0.664***	**0.825****	**0.825****	—
	p	0.161	0.043	0.022	0.002	0.002	—

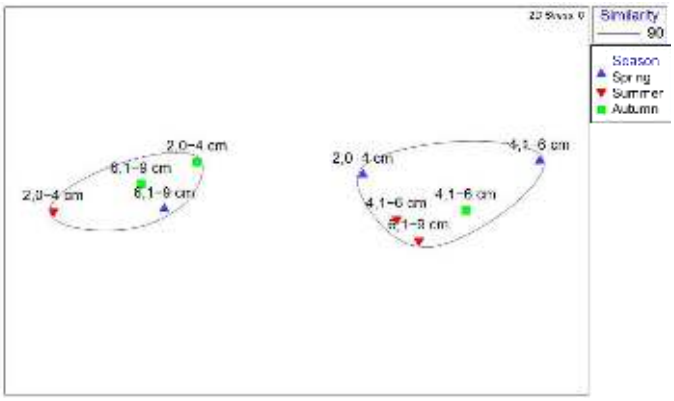

Fig. 22. Non-metric Multidimensional Scaling (nMDS) ordination plot of Bray-Curtis dissimilarities between stations based on transformed mean (√) values of microplastic abundance per size class, overlaid with cluster analysis (2D stress value < 0.01)

The Kruskal-Wallis H test revealed statistically significant differences in microplastid (MP) abundance ($H(2) = 7.853$; $p = 0.041$), with respect to mussel size classes. Post Hoc Dunn analysis showed significant differences between 2.0-4 cm and 4.1-6 cm size classes.

Cluster analysis showed a clear delineation of the data into four distinct groups (Fig. 23A). MP abundance by size class was more similar in the summer than in other seasons. Principal component analysis (PCA) of environmental parameters (temperature, salinity and chlorophyll *a*), mussel condition index and microplastic abundance by size class revealed two principal components (PCs) with eigenvalue > 1 that together explained 60.1% of the total variability in the data. PC1 explained 30.8% and PC2 29.3% of the variability of the data matrix (Fig. 23B). Because PC3, PC4 and PC5 had an eigenvalue < 1, they were not considered in the analysis.

Graphical representation of the PCA showed a positive relationship between condition index and chlorophyll *a* concentration in the water, but also between index and temperature. The analysis also showed a negative relationship between the mussel condition index and the amount of microplastics accumulated in the tissues (Fig. 23B).

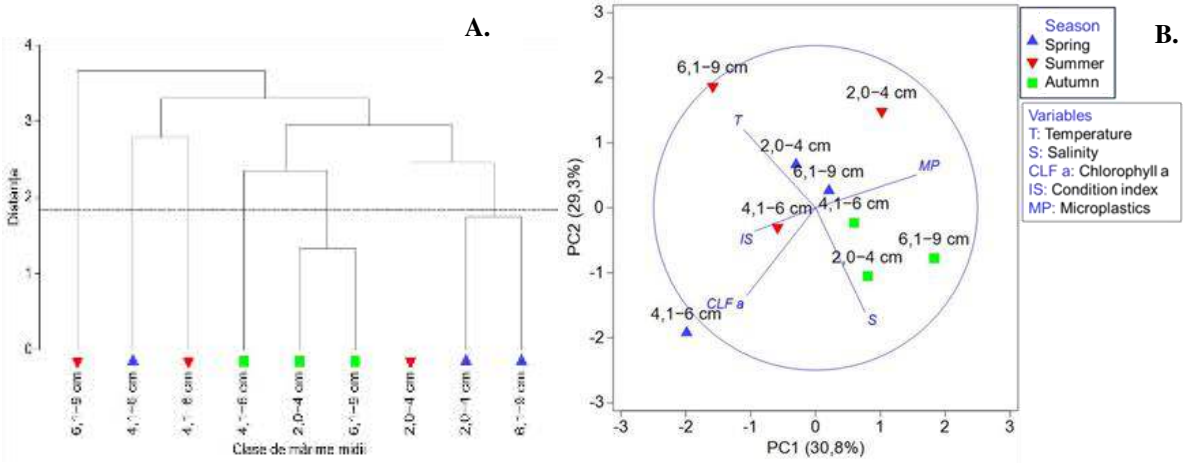

Fig. 23. Bray-Curtis similarity dendrogram (A) and principal component analysis (PCA) showing the variation of environmental parameters, mussel condition index and microplastic abundance by size class

This study demonstrates the presence of microplastics in bivalves M. galloprovincialis in the waters of the southern sector of the Romanian Black Sea littoral. The highest number of microplastics was found in the 2

Mai area (in summer) and the lowest in Constanta Port (in autumn). A possible explanation for these differences could be related to the proximity of different pollution sources associated with each location (sewage treatment plants, uncontrolled discharges of domestic sewage, fishing, influence of the Danube) (Pojar et al., 2022). Mussels have shown a high capacity to accumulate microplastics in soft tissues, most likely due to high amounts of microplastics in the environment (Mathalon and Hill, 2014). Qu et al. (2018) showed a strong positive linear relationship between the level of microplastics in water and mussels, suggesting that mussels ingest microplastics in relation to the amount present in the water.

In the present study, a total of 4584 microplastics were identified in 108 mussels analysed. A review of the literature on microplastics identified in M. galloprovincialis from the Black Sea shows that the quantitative results of the present study were significantly higher than those reported by Gedik and Eryaşar (2020), namely an average of 0.69 MP/individual.

Although there is no clear evidence in the present study that microplastics have a direct effect on mussel condition index, they may have an effect on feeding activities (Sussarellu et al., 2016), alter energy balance (Shang et al., 2021) and cause pathological alterations at the cellular and tissue level (von Moos et al., 2012). Insufficient food supply may exacerbate the negative effects of microplastics on mussel defence mechanisms, which could impact mussel survival and resilience under food-limited conditions, such as in winter, and in polluted coastal habitats (Shang et al., 2021). Shang et al. (2021) showed that exposure to microplastics and lack of food resulted in a significant decrease in mussel adhesive strength and the number of byssal threads produced.

X. CONTAMINANT BIOACCUMULATION AND CELLULAR RESPONSE OF MUSSELS: LYSOSOMAL MEMBRANE STABILITY

10. 2. Material and methods

Study area and sampling procedure

Four sampling expeditions were conducted along the Romanian Black Sea coastline in July 2022 to assess mussel lysosomal limb stability. Samples were collected from four locations (Midia Port , Mamaia Bay - Pescărie, Constanța Port, Mangalia Port), chosen based on their eligibility for assessing the biological effects of exposure to various anthropogenic contaminants associated with urban areas and Ports.

Seawater temperature was recorded in situ at each sampling with a calibrated glass thermometer (Termodensirom). Seawater samples were taken for subsequent laboratory analyses of abiotic factors (salinity, pH, dissolved oxygen) and contaminants such as total petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAHs), organochlorinated pesticides (OCPs), polychlorinated biphenyls (PCBs) and heavy metals (HMs). Seawater and biota (*M. galloprovincialis*) were sampled simultaneously from each location.

Fifteen mussels were selected for the mussel lysosomal membrane stability assay from each location (60 specimens in total) with a length range of 4-6 cm, and additionally, 20-25 mussels for chemical analysis.

Environmental data and chemical analyses

Salinity and pH were measured in the laboratory using the Mettler Toledo S479 multiparametric probe. Dissolved oxygen (DO) was determined by the Winkler method according to the method recommended by Grasshoff et al. (1999). Sixteen polycyclic aromatic hydrocarbons, nine organochlorinated pesticides and seven polychlorinated biphenyls were investigated in seawater and mussel tissue. The determination of organic

pollutants in mussels and seawater was carried out according to standardised methods (IAEA-MEL, 1995). Extraction of organic and persistent pollutants from mussel tissue was performed from 2 g of dried tissue using gas-chromatographic purity solvents. Heavy metal concentrations in seawater were determined by atomic absorption spectrometry using standard methods (IAEA-MEL, 1999; Grasshoff et al., 1999).

Assessment of lysosomal membrane stability

Lysosomal membrane stability was assessed by the neutral red retention time (NRRT; min) assay in mussel haemocytes according to the in vivo cytochemical method described by Martínez-Gómez et al. (2015). The principle of this assay is based on the ability of healthy lysosomes to retain the dye longer than damaged ones; lysosomal damage can cause leakage of the NR dye into the cytosol, potentially leading to cell death (Viarengo et al., 2007). NRRT was assessed against Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) (Martínez-Gómez et al., 2015).

Data analysis

The data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). The non-parametric Kruskal-Wallis test was applied, followed by the Dunn post hoc test. The significance level of the statistical results was set at $p < 0.05$. Multivariate statistical analysis (Principal Component Analysis, PCA) was also used.

10. 3. Results and discussions

The results presented in this chapter were published in Pantea et al. (2023).

Tissue organic pollutant concentrations are shown in Figure 24. The concentrations of ∑PAHs in mussels were low and ranged between 1.57 and 2.26 µg/kg g.us; the maximum value was recorded in the Mamaia Bay - Pescărie. Bioaccumulation of ∑OCP and ∑PCB in mussels was exceptionally high at all sampling locations. The highest level of ∑OCP (3952.39 µg/kg g.us.) and ∑PCB (8450.46 µg/kg g.us.) was measured in Midia Port.

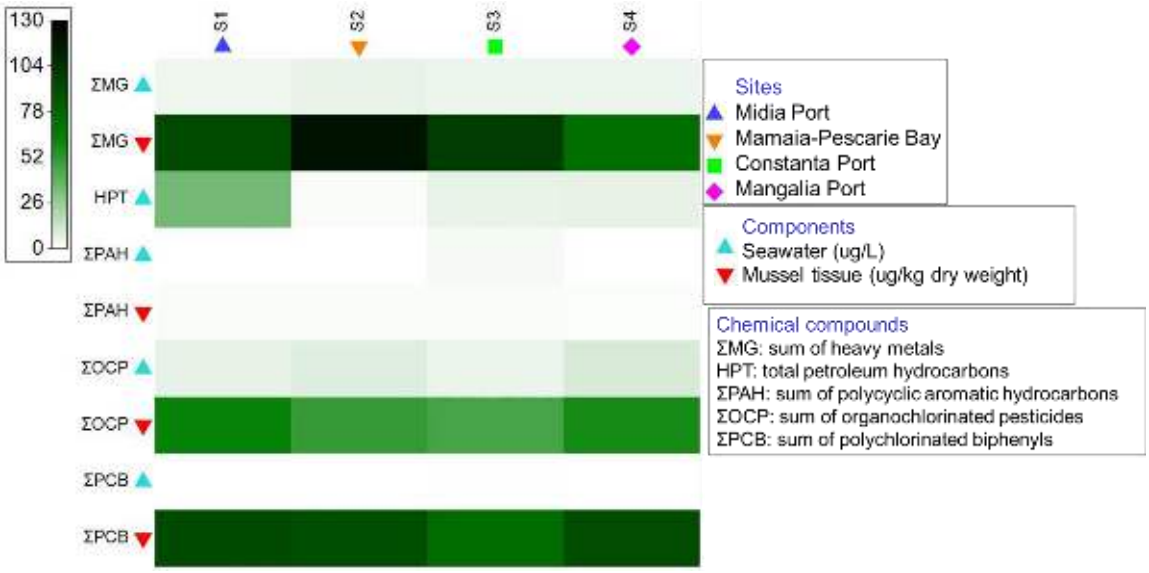

Fig. 24. Contaminant concentrations in seawater and mussel tissues from sampling stations. Data are expressed as square root transformed values (√)

The highest mean value (mean ± std. dev.) of NRRT was detected in the Bay of Mamaia - Pescărie (34 ± 18.34 min). The mussels from Constanța Port (11 ± 10.56 min), Mangalia Port (12 ± 10.14 min) and Midia Port (14 ± 10.56 min) showed an extremely low capacity to retain the colourant (Fig. 25).

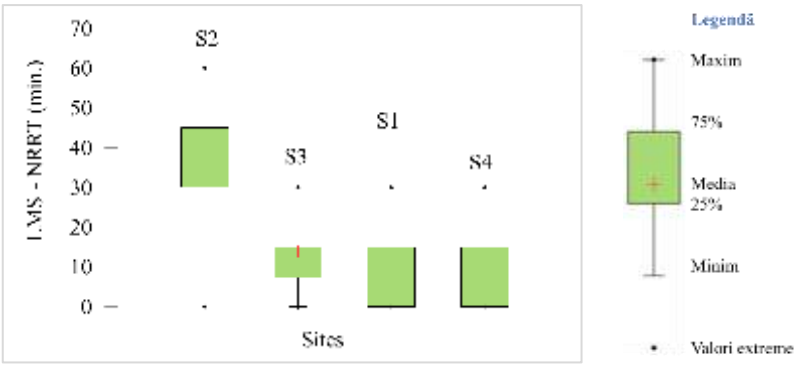

Fig. 25. Box-whisker plot of the lysosomal membrane stability (LMS) in mussel haemocytes, assessed by neutral red retention time (NRRT). S1: Midia Port; S2: Mamaia Bay - Pescărie; S3: Constanța Port; S4: Mangalia Port

The highest mean value of mussel lysosomal stability (49.16 ± 11.13%) was observed in Mamia Bay - Pescărie and the lowest (30.67 ± 6.96%) in Midia Port. In general, a high degree of lysosomal damage was observed in all analysed specimens (Fig. 26).

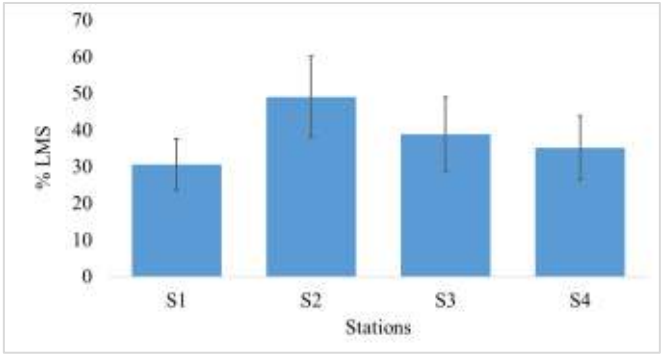

Fig. 26. Mean percentage of lysosomal damage (% SML ± std. dev.) in mussel haemocytes. SML: lysosomal membrane stability. S1: Midia Port; S2: Mamaia Bay - Pescărie; S3: Constanța Port; S4: Mangalia Port

The Shapiro-Wilk test showed that the retention time data deviated significantly from a normal distribution ($W(59) = 0.823$; $p = 0.0001$). The homogeneity of variances (Levene's test) showed that there were no differences between variances ($F(3, 56) = 2.76$; $p = 0.108$). Based on this result, the data were tested for differences between locations using the non-parametric Kruskal-Wallis test. The results of the test showed significant differences between mussel NRRTs in terms of sampling location ($p = 0.0003$). Dunn's post hoc test showed statistically significant differences between Mamaia - Pescărie and Port stations: Port Midia ($p = 0.002$), Port Constanța ($p = 0.001$) and Port Mangalia ($p = 0.001$).

To examine the associations between NRRT and bioaccumulated contaminants in mussel tissue, organic pollutants (∑PAH, ∑OCP and ∑PCB), heavy metals (Ni, Cu, Pb, Co, Cd and Cr), and NRRT in lysosomes were included in multivariate analyses. PCA showed that PC1 and PC2 explained 98.24% of the total variability in the data matrix (Fig. 27). PC1 explained 76.93% and PC2 21.31% of the variability in the data. The eigenvalues of the first two principal components were 7.69 (PC1) and 2.13 (PC2). PC1 was mainly characterised by the positive contribution of the variables Cu (0.97), Pb (0.97), Ni (0.97), Co (0.97), ∑PAH (0.97) and NRRT (0.89) and the negative contribution of the variables ∑OCP (-0.77) and ∑PCB (-0.77). PC2 was mainly represented by positive

contributions, particularly the loadings of the variables Cd (0.77), ∑OCP (0.63) and ∑PCB (0.63), and the negative contribution of the variable Cr (-0.63).

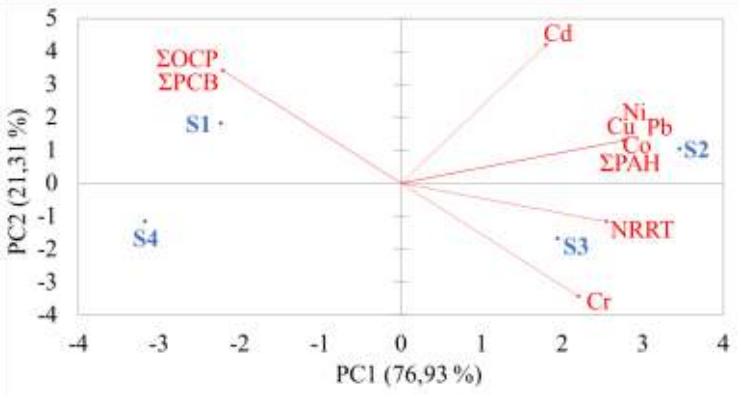

Fig. 27. Principal component analysis (PCA) of contaminants bioaccumulated in mussel tissue and neutral red retention time (NRRT). Cu: copper; Cd: cadmium; Pb: lead; Ni: nickel; Cr: chromium; Co: cobalt; ∑PAH: sum of polycyclic aromatic hydrocarbons; ∑OCP: sum of organochlorinated pesticides; ∑PCB: sum of polychlorinated polychlorinated biphenyls. S1: Midia Port; S2: Mamaia Bay - Fishery; S3: Constanța Port; S4: Mangalia Port

According to a previous study, concentrations of OCPs and PCBs in marine waters have shown a decreasing trend in recent years (Oros et al., 2016). In contrast, very high concentrations of OCPs and PCBs were measured in coastal waters in our study, mainly in the Mangalia Port and the Bay of Mamaia - Pescărie. The concentrations of lindane, heptachlor, cyclodiene pesticides (aldrin, dieldrin, endrin and DDT (p,p ′DDT, p,p ′DDE, p,p ′DDD) exceeded in all sampling stations the MAC value according to the European legislation (European Union, 2013),. An explanation for these results could be that the sampling stations were located close to sources of pollution, such as urban wastewater treatment plants, potentially polluted freshwater input and heavy maritime traffic. Heavy metal concentrations were below Environmental Quality Standards (EQS) for European marine waters (European Union, 2013) at all sampling locations.

In accordance with the evaluation criteria established for the neutral red retention test (Martínez-Gómez et al., 2015), none of the mussel groups in this study showed good health (NRRT ≥ 120 min). Mussels from all stations could be assessed as severely stressed (TRRN < 50 min) and showing pathologies (e.g. lysosome enlargement, intralysosomal fluid leakage, rounded fragmented cells) as described by Viarengo et al. (2007).

The decreased lysosomal membrane stability observed at all sampling locations could be mainly related to higher levels of OCPs and PCBs. Exposure to a diverse mixture of chemicals in the environment enhances toxic effects (Moore et al., 2018). In this study, effects on SML were observed even at low concentrations of some pollutants, suggesting that the complexity of the contaminant mixtures had a greater toxic effect regardless of individual pollutant concentrations. The low lysosomal stability observed in this study was similar to that observed in other studies carried out at the Romanian Black Sea littoral on M. galloprovincialis (Ciocan, 1997).

CONCLUSIONS

From the analysis of the data obtained in this doctoral thesis, judgements, comments, conclusions and recommendations can be made:

- The condition index of *Mytilus galloprovincialis* showed a significant seasonal variation, mainly influenced by variation in temperature, solids suspension and chlorophyll a. The highest value of the index was recorded in spring, which coincided with the maximum values of chlorophyll *a*.
- The environmental conditions in the Port areas seemed more favourable for the growth of M. galloprovincialis species due to higher trophic conditions, as demonstrated by the results of the biometric parameters and the condition index. In the reference area, the condition index was significantly lower compared to portsdue to lower trophic conditions.
- Increased food availability positively influenced the physiological condition of the mussels, which led to increased condition index values and accumulation of reserves, mainly in the form of proteins, carbohydrates and lipids. Mussels in the reference area (2 May), with low food availability, showed suboptimal physiological condition and low energy reserves.
- The seasonal cycle of *M. galloprovincialis* mussels from the Romanian Black Sea coast is marked by phases of accumulation and depletion of reserves, reflecting gonadal development and food availability.
- The dynamics and distribution of heavy metals in coastal waters and sediments exhibited significant temporal and spatial variations under the influence of natural and anthropogenic contributions.
- Exceedances of water quality standards for marine waters were observed only for cadmium, while copper, cadmium, lead, lead, nickel and chromium showed concentrations below the expected limits. In general, the highest concentrations of metals in water were recorded in the reference area (2 May), especially in the spring season.
- The most contaminated sediments with heavy metals were found in Port enclosures, with exceedances of marine sediment quality standards for most of the metals analysed, namely copper cadmium, lead and nickel, demonstrating the influence of anthropogenic factors.
- In general, the highest bioaccumulation of heavy metals in mussels was observed for mussels collected from sea Port enclosures, with the highest values being recorded in the summer season. The bioaccumulation tendency of heavy metals was higher in ports due to the higher bioavailability of metals in these areas.
- The Bioaccumulation Factor (BAF) of heavy metals showed high bioaccumulation tendencies of copper, cadmium, nickel and chromium and less lead due to their high bioavailability in the water column.
- The Biota-Sediment Accumulation Factor (BSAF) of heavy metals was higher for copper and cadmium, and lower for lead, nickel and chromium, demonstrating their lower availability for mussels.
- The Individual Multi-Metal Bioaccumulation Index (IMBI) showed a high degree of heavy metal bioaccumulation in the Port enclosures, especially for copper, cadmium and nickel, and a moderate one in the reference area.
- The assessment of the Metal Pollution Index (MPI) showed a very high level of contamination of mussels in Midia Port and Constanța Port, moderate contamination in Mangalia Port and high contamination in the reference area of 2 Mai.
- The relationship between the condition index and the degree of bioaccumulation of heavy metals was inversely proportional, a situation particularly noticeable in the spring and summer seasons.

- The mussels showed a high bioaccumulation capacity of microplastics in tissues, the results obtained being much higher compared to other areas of the Black Sea, but similar to a study carried out on the Romanian coast near Port areas.
- The high amount of ingested microplastics was influenced by the proximity of pollution sources (water treatment plants) but also by the mussel attachment substrate.
- Over 90% of the amount of microplastics ingested by mussels were represented by microfibres, followed by fragments, which indicated the high degree of microplastic microfibre contamination of the inverted areas.
- The results showed that the largest amount of ingested microplastics was very small in size (less than 1 mm), which poses a higher health hazard to the mussels due to the fact that their size allows them to bioaccumulate more rapidly in the tissues.
- In general, the highest amount of microplastics was identified in small mussels due to higher filtration rates, but also in very large mussels due to the larger gill surface area providing higher exposure.
- Lower food availability could induce a higher sensitivity of mussels to microplastic exposure. In order to explore possible adverse effects, further studies should focus on assessing sublethal effects, manifested at the cellular and tissue level.
- Although the present study did not demonstrate clear evidence that microplastics have a direct effect on the condition index of mussels, according to the results of the numerous studies carried out, microplastics may have a significant effect on mussel health.
- Increased food availability and stored reserves provide mussels with sufficient energy to withstand stressful conditions, even in environments heavily impacted by human activities such as Ports.
- This study emphasises the importance of monitoring changes in both the abiotic and biotic components of the ecosystem to understand potential impacts on mussel populations, providing valuable information for resource management.
- Mussels from all sampling locations showed severe stress conditions, indicating the presence of pathologies. The low value of mussel lysosomal membrane stability was mainly associated with higher levels of OCPs and PCBs, suggesting the toxic effects of contaminant mixtures.
- This study demonstrated the usefulness of lysosomal membrane stability (LMS) as a biomarker of mussel cellular stress upon contaminant exposure.
- This study provides important information on contamination levels and cellular responses of marine mussels from the Romanian Black Sea coast. The complexity of environmental contaminant mixtures may have a greater impact on lysosomal stability than individual pollutant concentrations.
- The results highlight the need for the application of a comprehensive set of biomarkers to assess the ecotoxicological impact of exposure to contaminants in the marine environment.
- Future research should investigate the mechanisms underlying lysosomal damage and explore additional biomarkers to provide deeper insight into the health status of marine organisms exposed to contaminants.

The originality of this PhD thesis lies in the fact that it emphasises the importance of monitoring changes in both abiotic and biotic components of the ecosystem to understand the potential impact on mussel populations, providing valuable information for resource management. This study has demonstrated the utility of lysosomal membrane stability (LMS) as a biomarker of mussel cellular stress upon exposure to contaminants.

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