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Toxic metals biomonitoring based on prey-predator interactions and environmental forensics techniques: A study at the Romanian-Ukraine cross border of the Black Sea

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ABSTRACT

Marine cross-border areas are ideal for monitoring pollutants so as to increase ecosystems protection. This study was conducted at the Romanian-Ukraine border of the Black Sea to reveal evidence of contamination with toxic metals based on biomonitoring of: cadmium, lead, total chromium, nickel and copper at different water depths and prey-predator interactions, combined with environmental forensics techniques of biological sampling and separation in witnesses size groups. The species used were *Mytilus galloprovincialis* L. and *Rapana venosa* V. collected at 17.5 m, 28 m and 35 m depth. An atomic absorption spectrometer with a high-resolution continuum source and graphite furnace was used for toxic metals quantification in various samples: sediments, soft tissue, stomach content, muscular leg, hepatopancreas. The best sample type, based on the pathology of metal location and bioaccumulation, is the hepatopancreas from *R. venosa* that proved a significant decrease of cadmium and lead at lower depths.

1. Introduction

Coastal areas provide unique habitats regions for wildlife sustainability (Constantin et al., 2015). Danube River contributes with the most important amount of freshwater from the north-western side of the Black Sea and it is the largest river from this basin (Gulin et al., 2014). Black Sea is almost a close marine ecosystem that was considered to be a freshwater lake from 22,000 to 9000 years before present (B·P) (Merey and Sinayuc, 2016), and below 180 m depth it is the largest natural anoxic environment (Makedonski et al., 2017). The water depth currents and the basin-scale circulation are counter clockwise (cyclonic) with kinetic energy different from one year to another, and that has an impact upon the entire ecosystem (Kubryakov et al., 2016). The Black Sea is surrounded by six countries (Bulgaria, Georgia, Romania, Russia, Turkey and Ukraine) that split the basin in territorial waters with borders, different environmental policies and administration. This may represent a threat for marine life preservation, pollutants management and implementation of the Marine Strategy Framework Directive (MSFD) which aims to protect the European marine environment and to achieve Good Environmental Status of the EU's marine waters by 2020 and to protect the resource base upon which marine-related economic and social activities depend (Loizidou et al., 2016; MSFD 2008/56/EC). Although many institutions (governmental and NGO's) are concerned about the involvement in research programs focused on the Black Sea, so far there so far there were not developed by the Black Sea regional partners strategic research collaboration programs, based on a shared mid-term vision and approved at governmental level (Teodosiu et al., 2013).

Marine cross-border areas are ideal monitoring sites (Fazio et al., 2014) of the pollutants so as to improve the management of human activities which supports the sustainable use of marine goods and services and ecosystems protection. However even if the legislation for the discharge of pollutants into territorial waters, might be slightly different from a country to another, the international directives prevail. There are no real borders to confine marine pollution and associated impacts due to the characteristics of this type of ecosystem. The only way to stop destruction and to preserve the marine integrity is the full cooperation between all the countries in finding the best ways and interest points to accept the challenges of Black Sea habitats protection.

In generally, mussels and snails are well-established bioaccumulators of toxic metals thus they are good bioindicators of aquatic pollution with toxic metals (Rzymski et al., 2014; Habib et al., 2016; Savorelli

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et al., 2017).

Mytilus galloprovincialis L., known as the Mediterranean mussel, is a widely distributed species (Torre et al., 2013a; Burgos-Aceves and Faggio, 2017) from temperate to subarctic coasts of Northern and Southern hemisphere. The studies proved that it is a very sensitive organism to any changes in the marine environmental equilibrium and anthropogenic pollutants such acoustic sounds (Vazzana et al., 2016), pharmaceuticals compounds (Mezzelani et al., 2016; Pagano et al., 2016), oxidative stress response to sodium dodecyl sulphate (Messina et al., 2014), metals pollution (Gonzalez-Rey et al., 2011; Torre et al., 2013b; Kristan et al., 2014; Richir and Gobert, 2014; Belivermiș et al., 2016; Barut et al., 2016; Yu et al., 2016; Pagano et al., 2017). This species may be used also in genotoxicity tests (Mohamed et al., 2014; Martinović et al., 2016; Touahri et al., 2016), cytotoxicity tests (Faggio et al., 2016) since it accumulates efficiently trace elements from the environment and was proposed as good indicator for water quality (Richir and Gobert, 2014). On the other hand, *Rapana venosa* V. (Asian whelk) is an invasive sea snail, from Asia, that settled down in the Black Sea > 60 years ago and became an abundant species (Kosyan, 2016). It threats the populations of Mediterranean mussel thanks to its predatory behaviour which is very aggressive and was considered responsible for extermination of oyster population from several areas (Janssen et al., 2014). This allows understanding the transfer of pollutants through trophic chain from the primary consumer to the higher rank predator. Furthermore this organism is fitted for biomonitoring of toxic metals contamination in the marine environment (Mülayim and Balkis, 2015).

Environmental forensics is considered to be a combination between science and the art of deduction that aims to investigate the pathways and history of the pollutants in environment (Aris et al., 2015). This field resulted in 1980 as a consequence to seek compensation when the environment is damaged or injured by the contaminants released by anthropogenic activities (Megson et al., 2016). Furthermore, the environmental forensics can identify who is responsible for the generation and from where the pollutants originate (Gallego et al., 2016). This technique of pollutant investigation requires much more attention and is more complex than the regular monitoring techniques.

This study was conducted at the border between EU jurisdiction (Romania) and non-EU jurisdiction (Ukraine) of the Black Sea to prove the possible environmental contamination with toxic metals that are often used in industries and the origins of this pollution. The aim of this study was to reveal the evidence of possible cross-border contamination with toxic metals based on the biomonitoring of cadmium (Cd), lead (Pb), chromium (Cr), nickel (Ni) and copper (Cu) at different water depths and prey-predator interactions (by using the species *Mytilus galloprovincialis* L. and *Rapana venosa* V.), combined with environmental forensics techniques of biological sampling and separation in size groups of witnesses. The equipment used for metal measurements was an atomic absorption spectrometer with a high-resolution continuum source and a graphite furnace (HR-CS GF-AAS), which was optimized and validated for the sample types used in this study (sediment, soft tissue, stomach content, muscular leg and hepatopancreas).

2. Materials and methods

2.1. Sampling area description

The samples were collected from the studied area in August 2013 during an expedition held on the Black Sea. The area of interest for this study was the maritime border between Ukraine and Romania. There were set three sampling sites located at different depths where the population density of Mediterranean mussel is high and it is a significant probability to find the Asian whelk which is feeding with them. In Fig. 1 there are presented the locations of the sampling sites coded as: S1_17.5 m depth, S2_28 m depth and S3_35 m depth. At S1 the sea bottom was rocky covered with shells, at S2 and S3 it was covered with sand and shells.

There were used two methods of sampling for the established sites. The sediments samples were collected with a marine Bodengreifer in 5 replicas per sampling site. There were used the first 7 cm of the sediment layer for each sample. These were preserved at −25 °C in PE labelled bags and transported in cool boxes from the research vessel to the laboratory. There they were kept at −25 °C until they were prepared. The biota was sampled with Charcot dredge that was immersed in water for 10 min per sampling in 3 replicas per site. A large number of specimens (Table 1) of Mediterranean mussel and Asian whelk were randomly sampled and preserved at −25 °C in PE labelled bags. These were transported in laboratory and kept at −25 °C until they were prepared for analyses.

2.2. Biometrical measurements of the samples

The samples that were collected from each depth were separated in different size groups to observe the exposure in time for the studied pollutants. In case of the molluscs, the separation in size groups is similar with the separation in age groups. The smaller size specimens are similar with the youngest one. In Table 1 there are presented the biometrical measurements, number of collected specimens and labels of each group. At the S1_17m the samples of Mediterranean mussel were separated in three size groups with significant differences between their biometrical measurements ($p < 0.05$, one-way ANOVA) and two groups of Asian whelk. It has been observed in this area that the predator *R. venosa* had the highest abundance as compared with the other sites. At S2_28 m the samples of Mediterranean mussel were separated in two different size groups ($p < 0.05$, one-way ANOVA) and *R. venosa* in one. It has been observed that at this sampling depth the predator had the biggest size specimens suggesting that the water pressure may be a limitation factor for them and the small ones cannot resist. This aspect is supported by the fact that at the sampling site located at 35 m depth (S3_35 m) the predator was not present among the Mediterranean mussel population.

2.3. Sample preparation and method validation

In laboratory the samples were unfrosted and prepared for the toxic metal analysis. Firstly, there were done biometric analysis and separation in groups labelled as presented in Table 1. The reagents used for the elements separation and measurement were high purity and quality certified by the producers. Water used for decontamination, sample preparation and reagents dilution was ultrapure, filtered by LaboStar™3/7 TWF (Siemens) purification system from double-distilled water. In the metal digestion process from the biological samples it was used high purity nitric acid 65% Suprapur (Merck, Germany) and hydrogen peroxide EMSURE 30% stabilized for higher storage temperature (Merck, Germany). The standard stock solutions for AAS used in calibration method were certified by Merck, Germany. The solutions necessary for calibration and quantification of the metals were: copper (1000 mg L^{-1}), cadmium (1000 mg L^{-1}), lead (1000 mg L^{-1}), chromium (1000 mg L^{-1}) and nickel (1000 mg L^{-1}).

For Mediterranean mussel samples it was used the following protocol in metal quantification. From each labelled group there were prepared 40 samples (a total number of 320) using only the soft body mass without the shell. This was several times washed with ultrapure water, finely chopped with PE tools, homogenized in agate mortar and weighted as 1 g mass per sample. This was mixed with 4 ml nitric acid 65% and 2 ml of hydrogen peroxide in decontaminated TFM pressure vessels that were inserted in Speedwave MWS-2 produced by Berghof. The digestion program for samples was in steps as follows: 145 °C for 5 min, 190 °C for 10 min, and 100 °C for 10 min (Strungaru et al., 2015). After the microwave digestion, the samples were transferred in 50 ml decontaminated flasks and filled up to volume with ultrapure water.

The Asian whelk samples were prepared in three different types: