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DETERMINATION OF MARINE POLLUTION BY COMPARATIVE ANALYSIS OF METAL POLLUTION INDICES

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Abstract – Due to the specific geographical and hydrological structure of Boka Kotorska Bay, that is characterized by a low flow of water through the bay, the anthropogenic impact is pronounced, exerting direct effects on this unique ecosystem. Trace metal (Pb, Hg, Ni, Co and Cd) concentrations were measured in the winter, spring and fall of 2008 in two marine organisms (*Posidonia oceanica* and *Mytilus galloprovincialis*) selected as biomonitors of trace metals in the Boka Kotorska Bay. These marine organisms have the ability to accumulate trace metals from their environment. Metal pollution indexes (MPI) for both species were compared, confirming that the most polluted was Tivat bay and the least Kotor bay.

Keywords: Mytilus galloprovincialis, *Posidonia oceanica*, trace metals, MPI, Adriatic Sea

INTRODUCTION

Standard chemical analysis methods do not provide information about the presence of metals in seawater. Thus, living organisms are used as water pollution biomonitors (Regoli, 1998; Schlacher-Hoenlinger and Schlacher, 1998; Campanella et al., 2001; Shahidul-Islam and Tanaka, 2004; Morillo et al., 2005; Usero et al., 2005; Angelo et al., 2007; Lafabrie et al., 2007; Lafabrie et al., 2008; Stanković and Jović, 2012), especially for identifying trace metals. Many microelements are found in trace amounts in seawater, often at elevated levels in aquatic organisms, making them very useful for biomonitoring (Morillo et al., 2005). Seagrasses and mussels are increasingly used as indicators of the chemical contamination of coastal regions (Lafabrie et al., 2007; Lafabrie et al., 2008; Jović et al., 2011).

The mussel *Mytilus galloprovincialis*, a native species of the Mediterranean, Black and Adriatic seas, is a sedentary, filter-feeding animal that through feeding not only assimilates the food necessary for growth and development, but also accumulates contaminants present in the water. These mussels have been studied extensively from both ecotoxicological and food safety points, i.e. as a potential bioindicator and also as an important seafood product (Martinčić et al., 1992; Besada et al., 2002; Klarić et al., 2004; Szefer et al., 2004; Lafabrie et al., 2007; Çevik et al., 2008; Desideri et al., 2009; Jović et al., 2011; Stanković et al., 2011, Jović et al., 2012; Stanković et al., 2012; Markovic et al., 2012).

Posidonia oceanica is an endemic species in the Mediterranean and can be found from the surface to a depth of 40 m. Its meadows serve as spawning area, hunting territory or permanent habitat for numerous plant and animal species. Furthermore, it plays an important role in the stabilization of soft bottoms and the protection of shores from erosion (Campanella et al., 2001). It can absorb trace elements directly from the water column or/and from interstitial water in sediments and has a high capacity for concentrating pollutants and accumulating trace elements occurring in a marine environment. P. oceanica has been studied as a bioindicator of trace metal contamination in various parts of the Mediterranean coast (Costantini et al., 1991; Schlacher-Hoenlinger and Schlacher, 1998; Campanella et al., 2001; Kljaković-Gašpić et al., 2004; Lafabrie et al., 2007; Lafabrie et al., 2008).

In the last decade, human and industrial activities in the coastal area of the southeastern Adriatic have increased and resulted in different types of pollutants. As the Montenegrin coastal area, especially Boka Kotorska Bay, receives a heavy influx of sewage and industrial effluents as well as domestic and agricultural wastes (Jović et al., 2011), it is an important entry point for a diverse array of polluting agents, including trace metals some of which are potentially toxic. Due to its specific structure, semi-enclosed bay system and low flow of water through the bay, the anthropogenic impact is more pronounced, exerting direct effects on this unique ecosystem and organisms. Boka Kotorska Bay was previously covered by large meadows of seagrass, *Posidonia oceanica*. However, a significant reduction in the surface covered by this plant has been observed along the entire coastal area of Montenegro, especially in Boka Kotorska Bay. Seagrass meadows in Risan bay have completely disappeared (Mačić, 2000; Mačić and Sekulić, 2001). Now, it is impossible to find this seagrass at depths of 20-25 m, while previously it was present even at the depth of 40 m. The exact reasons why the seagrass withdrew from this bay are still unknown; one of the most probable is certainly non-essential trace metal overload.

The main goal of this study was to determine the concentrations of trace metals (Pb, Hg, Ni, Co and Cd) in marine species, *P. oceanica* and *M. galloprovincialis*, from Boka Kotorska Bay. Based on the obtained trace-metal concentrations, a metal pollution index (MPI) was determined. This index represents the overall quality of environmental compartments with respect to metals, and has shown to be useful in evaluating pollution levels (Usero et al., 2005; Lafabrie et al., 2008). The metal pollution index (MPI) provides a comparison of the pollution statuses of different locations, i.e. the likely trace metal load.

In this work, we obtained the MPI of mussels in order to assess the state of the marine environment near the seagrass meadows. This approach can ensure a timely response in order to undertake the measures that could reduce the anthropogenic load of toxic metals in the marine environment and consequently prevent the decline of seagrass meadows.

MATERIALS AND METHODS

Study area

Boka Kotorska Bay is located in Montenegro (Fig. 1), and is naturally divided into four smaller bays: the Herceg Novi, Tivat, Risan and Kotor bays. It is a semi-enclosed bay system with more than 60 000 people living on the shores. This bay is an internationally protected area of nature under the UNESCO world natural and cultural heritage.

Samples were collected from five different locations in the winter, spring and fall of 2008, as indicated in Fig. 1.

These sites were selected for several reasons: (i) *Posidonia oceanica* can only be found at locations H1, T1 and K1 in the Boka Kotorska Bay; (ii) all five sampling sites are spread over the entire Boka Kotorska Bay, close to cities, ports and industries. Herceg Novi, Tivat and Kotor are the three largest cities of the Boka Kotorska Bay, in and around which are located the largest number of hotels, hospitals, ports and industries. The summer population of these towns increases because of tourism. In these towns, the major pollution problems are urban and industrial effluents: in Herceg Novi – shipyard, marine; in Kotor – metal, chemicals, petroleum storage and harbor; in Tivat – shipyard, airport, harbor. Tivat is the most exposed to pollution due to the proximity of the local airport, a military harbor, shipyards, oil tankers, agricultural land and the highest population density in all of Montenegro.

Sampling, sample preparation and analysis

The samples of *M. galloprovincialis* were collected at all five sampling locations. More than 2 kg of mussels of similar lengths (6.8-8.2 cm) were collected. The mussels were placed in polyethylene bags along with seawater and transported to the laboratory. 25-30 mussels from each station were pooled. They were cleaned and rinsed with deionized water, dissected fresh and the soft tissue was rinsed with Milli-Q water to remove sand and other particles.

At the same time but only from locations T1, K1 and H1, samples of *P. oceanica* were collected. The seagrass was handpicked at 6±1 m depth. The samples were washed with seawater, transferred to polyethylene bags and transported to the laboratory. Then the plant material was rinsed with Milli-Q water to remove any soil particles. The whole plant (leaf, rhizome and root) was sampled and analyzed.

Biota samples were subjected to lyophilization (CHRIST, Alpha 2-4 LD plus, Germany) under vacuum at a temperature of -40 °C for 48 h to remove any remaining water and then homogenized. Pooled samples were pulverized and homogenized using a mill.

Wet digestions were performed in triplicate by weighing approximately 0.5 g of biota sample with a mixture of 7 ml HNO_3 (65% Merck, Suprapur) and 2 ml H_2O_2 (30% Merck, Suprapur) in a microwave digestion system (CEM Corporation, MDS-2100). Digested biota samples were diluted to 25 ml with Milli-Q water and stored in polyethylene bottles until analyzed. A blank digest was performed in the same way.

Analyses of lead (Pb), cobalt (Co), nickel (Ni) and cadmium (Cd) were performed using a Graphite Furnace AAS (Perkin-Elmer, 4100ZL, with Zeeman background correction). Cold vapor technique was used for the analyses of mercury (Hg) (PerkinElmer, AAnalyst 200). The accuracy of the applied analytical procedure for the determination of trace elements in the mussels and seagrass were tested using SRM 2976 (Mussel homogenate; NIST) and SRM 140 (Fucus Sample; IAEA) certified reference materials. The recovery ranges for SRM were 2 976 – 96.8% for Ni, 106.8% for Co, 103.7% for Cd, 95.1% for Pb and 96.7% for Hg; and for SRM 140 – 94.4% for Ni, 105.9% for Co, 110.2% for Cd, 92.7% for Pb and 97.1% for Hg. Each measured and reported value is an average of five determinations.

Statistical analysis

For trace metal concentrations in *M. galloprovincialis* and *P. oceanica*, differences between organisms, locations and seasons were determined by a two-way analysis of variance (ANOVA) followed by the post-hoc Tukey test. Correlations between metals were performed by analysis of Pearson's correlations.

RESULTS

Metal concentrations depending of seasons and sampling locations

The metal concentrations of Pb, Hg, Ni, Co and Cd found in the marine organisms *M. galloprovincialis* and *P. oceanica* from the different locations and seasons are given in Fig. 2. Based on the results for all examined locations and seasons shown in Fig. 2, mean seasonal concentrations are calculated and are given in Table 1.

The mean concentrations of most metals were highest in *M. galloprovincialis* and *P. oceanica* in winter (Table 1). Mean seasonal concentrations in *M. galloprovincialis* for Co and Cd follow the order winter > fall > spring; for Hg and Ni: winter > spring > fall; and for Pb: fall > winter > spring. For *P. oceanica*, the mean seasonal concentrations for Pb and Hg follow the order winter > fall > spring; for Co and Cd: winter > spring > fall; and for Ni: spring > fall > winter.

Table 1. Range and mean metal concentrations (mg kg^{-1} dry weight) in *Mytilus galloprovincialis* (*M.g.*) and *Posidonia oceanica* (*P.o.*) in the winter, spring and fall of the year 2008.

	Pb	Hg	Ni	Co	Cd
M.g.					
winter	*3.60* (2.01-5.50)	*0.864* (0.610-1.19)	*5.74* (4.11-8.65)	*9.63* (8.02-12.0)	*3.31* (2.76-3.67)
spring	*3.11* (1.52-5.03)	*0.336* (0.121-0.850)	*2.51* (1.50-4.11)	*3.51* (3.05-4.81)	*1.26* (1.05-1.52)
fall	*5.10* (2.50-9.02)	*0.299* (0.202-0.570)	*1.99* (1.52-3.40)	*4.57* (3.05-6.55)	*1.65* (1.50-1.95)
	Pb	Hg	Ni	Co	Cd
P.o.					
winter	*15.4* (14.0-16.1)	*1.11* (0.870-1.31)	*32.5* (31.1-34.8)	*6.85* (6.40-7.55)	*8.61* (8.24-9.34)
spring	*6.84* (4.53-10.0)	*0.209* (0.252-0.270)	*39.6* (37.6-43.7)	*5.58* (5.42-5.95)	*1.85* (1.65-2.11)
fall	*7.02* (5.05-9.51)	*0.291* (0.261-0.329)	*33.0* (15.0-46.5)	*4.98* (2.45-8.85)	*1.78* (1.51-2.20)

Table 2. Mean metal concentrations (mg kg^{-1} dry weight) in *M. galloprovincialis* and *P. oceanica*.

	Pb	Hg	Ni	Co	Cd
M. galloprovincialis	3.94	0.499	3.41	5.91	2.07
P. oceanica	9.74	0.530	35.0	5.79	4.08

Metal content by sampling locations in a different part of the year

Fig. 3 provides data concerning metal concentrations in *M. galloprovincialis* and *P. oceanica* by location in the different seasons.

As regards mussels, the highest Pb concentrations are recorded at location T2 in all three investigated seasons and approximately the same distribution of Pb concentration by location was detected in the examined seasons. In the fall and winter, Hg concentrations at all sites were approximately the same, while during the spring only location T2 was distinguished by a slightly higher concentration. The highest variations of Ni and Co concentrations were obtained for *M. galloprovincialis* by location, and it is interesting that in every season the maximum concentration for these two elements was obtained at different locations (Fig. 3). Cd concentrations were uniform in all locations in relation to season and there is no location that stands out with extreme values.

A similar trend of metal concentrations in the seagrass *P. oceanica* was observed (Fig. 3). The highest Pb concentrations were recorded in Tivat bay – location T1; the most maximum concentrations of all investigated elements in *P. oceanica* were at this location; Hg concentrations at all sites were approximately the same in all three seasons and as in the mussels, the largest variation of Ni concentrations by location was obtained. In *P. oceanica* Cd concentrations were higher than Co concentrations in the winter period of the examined year at all three locations (Fig. 3).

Annual average metal concentrations in organisms

The two marine organisms had different amounts of metals in their tissues (Table 2). The concentra-

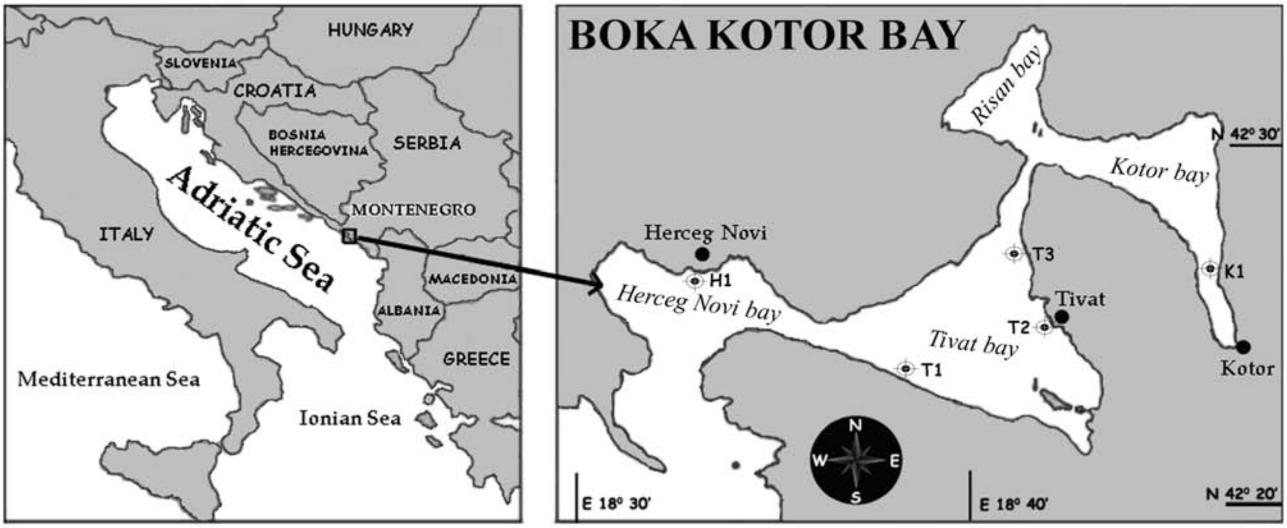

Fig. 1. Study area and sampling sites in the Boka Kotorska Bay: Tivat bay: T1, T2, T3; Kotor bay: K1; Herceg Novi bay: H1.

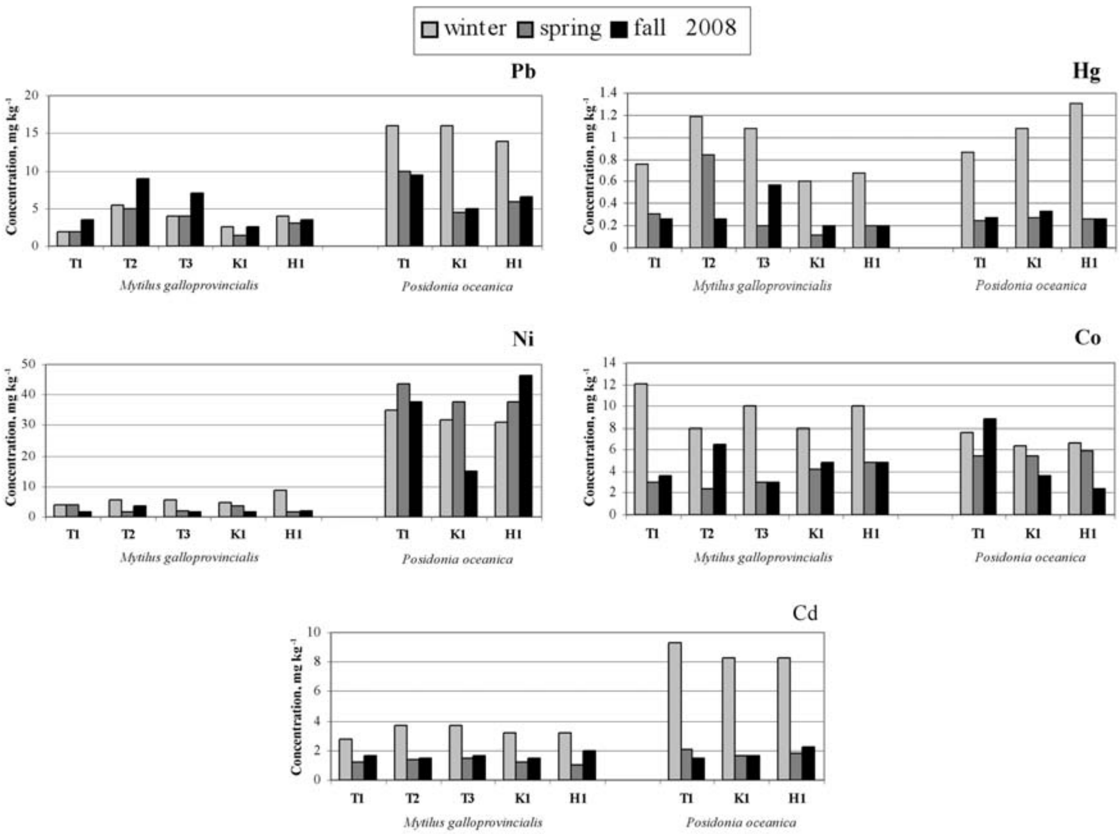

Fig. 2. Seasonal variations in metal concentrations (mg kg^{-1} dry weight) in *M. galloprovincialis* and *P. oceanica* from Boka Kotorska Bay in the winter, spring and fall of 2008.

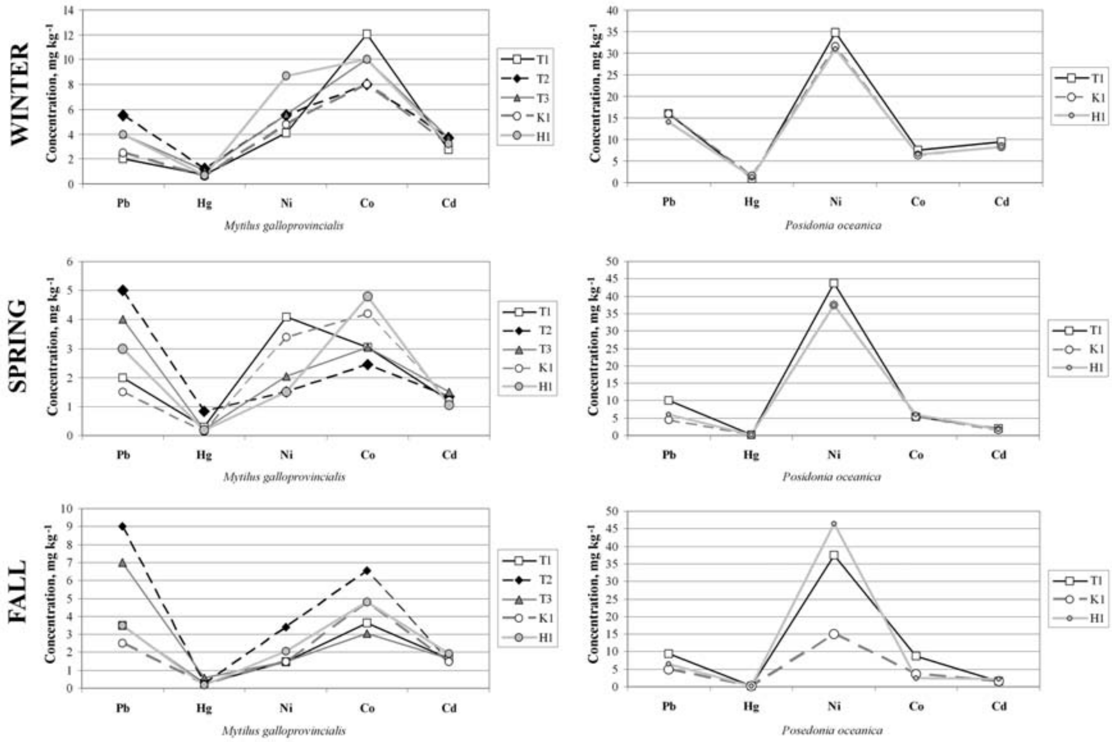

Fig. 3. Comparison of the metal concentrations (mg kg^{-1} dry weight) by locations in different seasons.

tions of Pb, Ni and Cd were significantly higher (p <0.05) in *P. oceanica* than in *M. galloprovincialis*. The remaining elements, Hg and Co, had approximately the same concentrations in these two organisms. In *M. galloprovincialis*, metal concentrations decrease in the order Co > Pb > Ni > Cd > Hg, while in *P. oceanica* the order is Ni > Pb > Co > Cd > Hg.

Metal correlation

Since data on metal concentrations suggested that some relation between trace metals may exist, they were tested for Pearson's correlations (Fig. 4). Highly significant positive correlations (p <0.05) in *M. galloprovincialis* were found between Pb-Cd, Pb-Hg and Hg-Cd in winter; Pb-Hg and Pb-Cd in spring; Ni-Co, Pb-Ni and Pb-Hg in the fall. Significant negative correlations (p <0.05) were found between CoCd, Pb-Co, Hg-Co in winter; Pb-Ni, Co-Cd, Hg-Co, Pb-Co in spring; Hg-Co, Co-Cd in fall. In the case of *P. oceanica* highly significant positive correlations (p < 0.05) were found between Ni-Cd, Co-Cd, Ni-Co and Pb-Ni in winter; Pb-Cd, Pb-Ni and Ni-Cd in spring; Pb-Ni, Pb-Co and Ni-Cd in fall. Significant negative correlations (p <0.05) were found between Hg-Co, Hg-Cd, Hg-Ni and Pb-Hg in winter; Hg-Cd, Pb-Hg and Hg-Ni in spring; Hg-Ni, Co-Cd, Pb-Hg and Hg-Cd in the fall (Fig. 4).

Metal pollution index (MPI)

The overall metal content of mussels and seagrass at the locations investigated in this study was compared using the MPI. This is obtained with the following equation: $MPI = (C_1 \times C_2 \times \ldots \times C_n)^{1/n}$ where C_n is the concentration of the metal n in seagrass or mussels and n is the total number of metals (Usero et al., 2005). Values of the MPI for the two investigated species are shown in Table 3.

We calculated the MPIs for the mussel and seagrass separately, to compare their values and determine whether pollution levels correspond to the biota sample locations. As expected, the highest val-

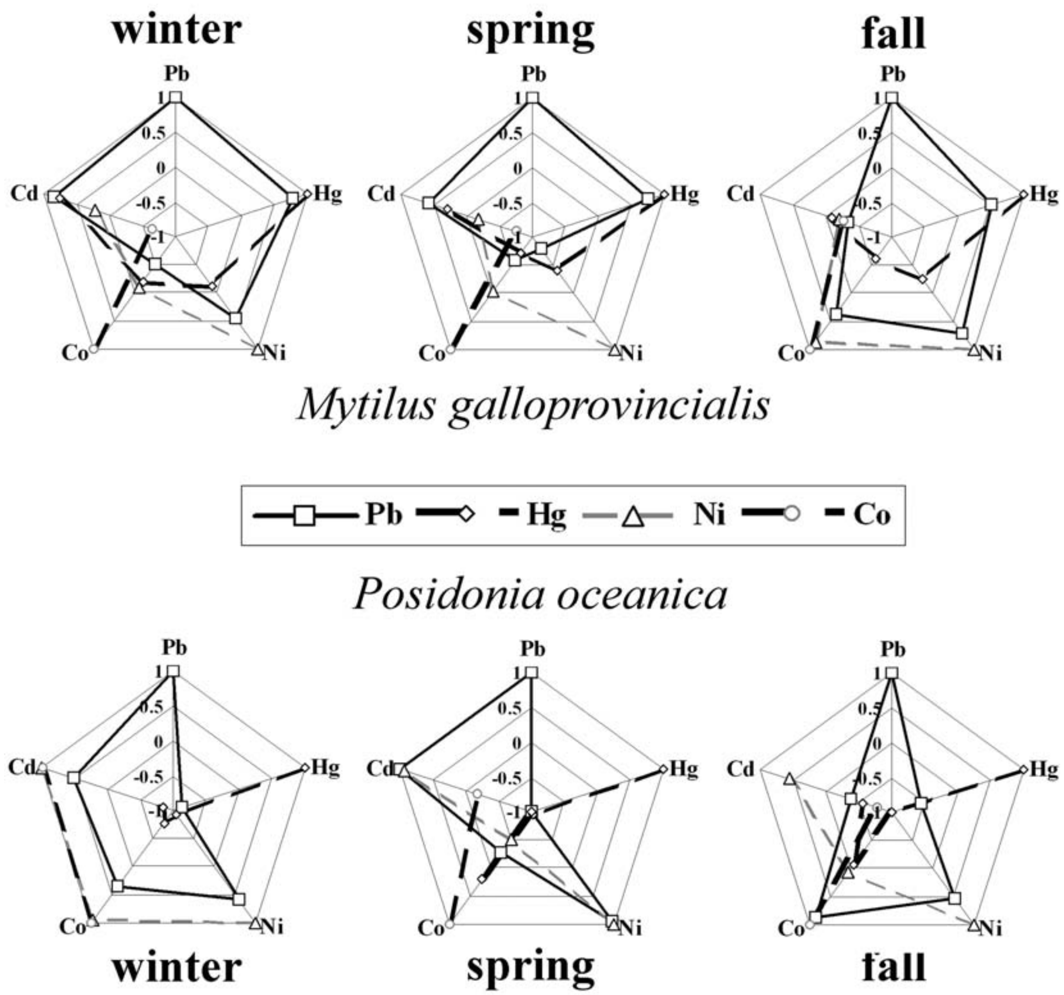

Fig. 4. Pearson correlation coefficients between metal levels in tissue of *M. galloprovincialis* and *P. oceanica*.

ues of MPI were obtained for the winter period for both examined species (Table 3). The location that was distinguished by the highest values of MPI for all studied seasons is the location T2 in the case of *M. galloprovincialis* and T1 in the case of *P. oceanica*. At locations T2 and T3, meadows of *P. oceanica* do not exist anymore.

DISCUSSION

In this study, different amounts of trace metals (Pb, Ni and Cd) in the tissues of two marine organisms were obtained. Metal uptake occurs mainly from water, sediment and food but the effectiveness of metal uptake from these sources may differ in relation to the ecological needs and metabolism of organisms, the contamination gradients of water, food and sediment, as well as other factors such as salinity, temperature and interacting agents (Madkour et al., 2011). As *P. oceanica* may reflect both contaminations in the water column and in sediment (Joksimović and Stanković, 2012), the fact that nearly the same Hg and Co concentrations were found in the mussels and the seagrass may mean that both species primarily uptake these two elements from the water column. In relation to all the investigated metals in both species, the greatest differences were observed in Ni concentrations that were significantly higher in the seagrass. The concentrations of Ni in the *P. oceanica* are consistent with previously reported studies (Gos-

Table 3. Metal pollution index (MPI) of each site in different seasons of 2008 for two investigated species.

		Metal Pollution Index		
		winter	spring	fall
M.g.	T1	2.91	1.56	1.52
	T2	4.03	1.84	2.40
	T3	3.89	1.50	1.98
	K1	2.85	1.26	1.41
	H1	3.77	1.35	1.68
P.o.	T1	8.07	4.16	4.20
	K1	7.79	3.32	2.72
	H1	7.90	3.63	3.34

selin et al., 2006; Lafabrie et al., 2007; Joksimović and Stanković, 2012) confirming that this type of seagrass has the ability to accumulate large amounts of this metal, suggesting that Ni concentration in *P. oceanica* tissues reflects the Ni concentration in sediment.

In both species, the highest metal concentrations were measured in the winter for most of the studied metals (Table 1). An increased concentration of metals in the soft tissues of mussels in winter compared to other seasons has been found by several studies (Phillips, 1976; Latouche and Mix, 1981; Lobel and Wright, 1982; Regoli, 1998). An explanation for this could be the reduced dry weight of the edible part of *M. galloprovincialis* with respect to shell weight during the winter, i.e. due to decreased food assimilation in winter the edible body mass decreases and the metal content remains the same, but concentration expressed in relation to edible body mass therefore increases (Klarić et al., 2004). The distribution of Pb is normally controlled by atmospheric deposition and its concentration is directly linked to human activities (Besada et al., 2002). Because of cumulative land and sea traffic and the end of the tourist season, fall is the period of year with most anthropogenic activity in the bay, which can be a major source of Pb pollution of coastal seawater. Hence, it comes as no surprise that the highest Pb concentrations were found in mussels sampled in the fall. In general, metal concentrations in a plant population are the highest in the cold season, decreasing rapidly during the period of maximal growth (Ali et al., 1999). Such trends are, however, not always uniform among metal species, such as is the case with Ni concentrations in the examined seagrass (Fig. 3). Krivokapić et al. (2010) found that heavy rains contribute to the increased content of nutrients in the Boka Kotorska Bay during winter. Ni uptake by plants can be inhibited due to complex formation between nutrients and the metal ions in environments with high nutrient levels (Joksimović and Stanković, 2012). This can explain the lowest concentration of Ni in *P. oceanica* in the winter period of the year.

The highest variations of concentrations by location were obtained for Ni and Co in the case of *M. galloprovincialis* and for Ni in the case of *P. oceanica*. For these metals in every season, the maximum concentration was obtained for other locations. Variations in the metal concentrations during the year by location suggest that they are influenced by a number of variable environmental factors such as salinity, temperature, pH, oxygen content, nutrient level, precipitation, inflow of fresh water, currents, upwelling, etc., rather than by a constant source of pollution.

The positive correlations found in this study suggested that the correlated metals share a common accumulation process in organisms and/or may indicate common local inputs. Highly significant positive correlation coefficients for *P. oceanica* in all three seasons were obtained for Ni with Pb and Cd (p <0.05, see Fig. 4). The positive correlation found between Pb and Cd concentrations in *P. oceanica* and water (Joksimović and Stanković, 2012), and the positive correlation of Ni concentrations in the sediment and *P.* oceanica (Lafabrie et al., 2007), would suggest that Pb and Cd concentrations in the tissues of the seagrass primarily reflect their concentrations in the water column, and Ni in the sediment. However, the positive correlation found between Ni with Pb and Cd suggests that there could be some other uptake and distribution routes for these three elements, not only via leaves for Pb and Co, but also via the root system. Pb and Hg were significantly correlated with each other in *M. galloprovincialis* at all three seasons ($p < 0.05$, see Fig. 4). It was reported that particulate Hg is the most important source of this metal accumulation in bivalves (Cossa et al., 1997). The positive correlations found between the Pb concentrations in *M. galloprovincialis* and the water would suggest that Pb in the tissues of the mussels primarily reflects the Pb in the water column (Martinčić et al., 1992; Lafabrie et al., 2007; Joksimović and Stanković, 2012). This leads to the hypothesis that the positive correlation between Pb and Hg comes from the ability of Pb to be rapidly and very easily absorbed onto particles (Boisson et al., 2002), in this case particles containing mercury, and as such are taken by mussels through water filtration.

The MPI was calculated based on the concentrations of Ni, Pb, Co, Cd and Hg, some of the most commonly analyzed toxic trace metals in aquatic environments. These five selected trace elements appear quite sensitive to anthropogenic influences, e.g. urbanization, industrialization, agriculture (Jović et al., 2011). This index is easily evaluated in order to compare the pollution status of different places. As expected, the highest MPI values were obtained for the winter period for both examined species (Table 3). Comparing the concentrations of investigated elements and MPI values by location in both marine species, it can be observed that the highest concentrations and MPI values were recorded in Tivat bay. The location that was distinguished by the highest MPI values for all three studied seasons was T2 in the case of *M. galloprovincialis* and T1 in the case of *P. oceanica*. The location with the lowest MPI value is K1 for both examined species (Table 3).

Both investigated organisms indicated that the highest polluted area is Tivat bay, while the least polluted area is Kotor bay. It comes as no surprise that the highest concentrations were found in marine organisms from Tivat bay because this bay has the most anthropogenic activities (airport, military harbor, shipyards, oil tankers, agricultural, etc.) (Jović et al., 2011). According to the obtained metals concentrations as well as MPI value, Herceg Novi bay is the next most polluted part of the Boka Kotorska Bay. This bay also has pollution problems primarily with urban, touristic and industrial effluents. In the case of Herceg Novi bay, located near the entrance of the Boka Kotorska Bay, a major impact on reducing contamination is the mixing of the water from the bay with water from the open sea. In Kotor bay only the concentration of Hg in the samples of seagrass was higher than in the other two studied bays, but taking into account the MPI values, this bay showed the lowest contamination.

The MPI values for *M. galloprovincialis* follow the sequence winter > fall > spring, for all sites except for location T1, and for *P. oceanica*, winter > spring > fall, except for location T1. It is interesting to note that at T1 the MPI values for both species were almost the same in the spring and fall, i.e. the smallest differences were between the seasonal MPI values (Table 3). If we compare MPIs for mussels and seagrass at T1, the MPIs for seagrass were almost 3 times higher than for the mussels. This occurrence can explain the loss of seagrass meadows in Tivat bay, especially at locations T2 and T3. Namely, at these locations the MPI values for mussels in winter were around four, suggesting that at these locations the MPI values for seagrass would be higher than 11. Obviously, the seagrass meadows were not capable of surviving anthropogenic pollution at these locations in Tivat bay. That was not the case with the other two bays. Herceg Novi bay is connected to the open sea and it is certain that high seawater flux and water exchange with the open sea (Jović et al., 2011) significantly affects the seasonal variation of polluting agents in the water column and reduces the pollution of this bay. On the other hand, the freshwater influx from five small rivers, numerous streams and karstic submarine springs greatly affects the hydrological and chemical properties of the water column of Kotor bay (Milanović, 2007), influencing its seasonal pollution variation and reduction.

The MPI values for the investigated organisms correspond to the degree of pollution level of the individual sites. This offers the possibility of monitoring the metal burden of the environment near seagrass meadows via MPI values for mussels. Therefore, sampling is simplified and seagrass meadows should not hamper routine controls. Constant monitoring of MPI values for mussels will enable a clear insight into the degree of pollution of the selected locations and enable a timely response in the case of increased MPI values in order to prevent further destruction and disappearance of this unique and very important marine species.

CONCLUSIONS

The mean concentrations of Pb, Ni and Cd were significantly higher in *P. oceanica* than in *M. galloprovincialis*. Nearly the same Hg and Co concentrations found in the mussels and seagrass suggest that both species primarily uptake these two elements from the water column. In both marine species, the highest metal concentration and accumulation were measured in the winter period for most of the metals. MPI values for both investigated organisms in all three seasons correspond to the degree of pollution level of the individual sites and indicate that the most polluted area is Tivat bay and the least, Kotor bay. By constant monitoring of the mussel MPI value near seagrass meadows, the degree of pollution level of the selected locations can be established and the appropriate measures taken in case of increased MPI values. This approach can influence a reduction in the further destruction and disappearance of a unique and very useful marine environment species, *Posidonia oceanica*.

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