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Ecological status of the Istrian marine environment (NE Adriatic Sea, Croatia): insights from mussel *Mytilus galloprovincialis* condition indices, stable isotopes and selected elements

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Abstract

Ecological status of the marine environment in NE Adriatic Sea was estimated with indicator species Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819. The study was seasonally performed between years 2010 to 2013 at mariculture and local port locations. Condition indices of mussels were in the range from 13.3 to 20.9% at mariculture, and from 14.3 to 23.3% at ports. $\delta^{13}C_{DIC}$ in our study seasonally ranged from -10.9 to 0.7‰. Lowest $\delta^{13}C_{DIC}$ values in the Lim Bay indicate enrichment with ^{12}C due to fresh water input. Sewage sludge pollution was not confirmed because mean $\delta^{13}C$ and $\delta^{15}N$ in mussel soft tissue did not significantly differ between the mariculture and polluted locations ($p>0.05$) and was from -24.5 to –20.2‰ and from +0.4 to +8.3‰, respectively. Concentrations of selected chemical elements (Mn, Cu, Zn, Se, Cd, Pb) were significantly higher in the tissue of the mussels from the polluted locations (ports) while As showed little variation.

Key words: *Mytilus galloprovincialis*, Condition indices, Stable isotopes, Selected elements, As speciation, NE Adriatic Sea.

Introduction

Marine mussels have been recognized as useful tools for monitoring the environmental conditions, quality and/or pollution assessment (Viarengo and Canesi, 1991). Mussels are sedentary organisms filtering large amounts of water, allowing them to accumulate substances from the environment (Langston and Spence 1995; Andral et al., 2004; Saavedra et al., 2004; Mertense et al., 2005). With increase of seafood consumption in recent years marine mussels have become commercially more important aquaculture species worldwide (Perugini et al., 2007; Vizzini et al., 2010). The Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819 is usually aquacultured in the coastal seas (mariculture) and it is also used as sentinel organism in several biomonitoring programs under the patronage of UNEP in the Mediterranean Sea and OSPAR at the North and Baltic Sea (http://www.marbef.org). *M. galloprovincialis* live attached on hard substrata and as filter feeders exposed to ambient seawater accumulate high levels of different contaminants (pesticides, toxins, heavy metals and hydrocarbons) which have several impact on their physiology and immune system (Livingstone and Pipe, 1992). Some studies revealed that *M. galloprovincialis* is especially good indicator for heavy metal Pb, Zn and Cd pollution assessment (Puente et al., 1996; Juresa and Blanusa, 2003; Saavedra et al., 2004; Orescanin et al., 2006).

Important ecophysiological measure of the health status of mussels is a condition index, a ratio between soft tissue and whole mass of mussel (Pampanin et al., 2005). It summarizes the physiological activity of the organisms under given environmental conditions (Lucas and Beninger, 1985), including pollution (Viarengo and Canesi, 1991; Hamer et al., 2004). It is known that exogenous factors, pollution and environmental conditions (temperature, salinity and food availability) and endogenous factors (e.g. reproductive cycle) may influence the mussel condition index (Okumus and Stirling, 1998; Hamer et al., 2008; Pavicic-Hamer et al., 2016). When studying mussels collected from different habitats or seasons in relating to metal concentrations, different patterns have been reported (Savedra et al., 2004; Mubiana et al., 2006; Schiuntu et al., 2008; Ruane-Hacene et al., 2015). In general, metal accumulation is negatively correlated with condition index, but some studies reported that metal concentrations in molluscs can be independent of condition index (Saavedra et al., 2004).

Organisms contain substantial amounts of stable isotopes of light elements such as H, C, N and O. Variation in the isotope ratios of biogenic substances depends on the isotopic composition of diet, its metabolic pathways, and kinetic modes of reaction dynamics. Isotopic composition of an organism provides useful knowledge for diet analysis, such as the origin of nutrient sources and individual feeding behaviour, both of which determine organism's function and position in the material flow of an ecosystem (Wada et al., 1993). $\delta^{15}N$ is closely correlated with forms of nitrogen as well as organic growth rate (Wada, 1980). $\delta^{13}C$ and $\delta^{15}N$ of mussel soft tissue reveal their diets (Wada et al., 1993) and have been used effectively in ecological studies tracing the influence of different pollutants to marine ecosystem (Župan et al., 2014; Ezgeta-Balić et al., 2014; Žvab Rožič, 2014). It was also recognized that stable isotopes of carbon and nitrogen are useful tracers for determination of anthropogenic pollution (atmospheric, riverine and upwelling) to marine ecosystem (Killingley and Berger, 1979; Hellings et al., 2001; Rogers, 2003, Mook and Tan, 1991; Hellings et al., 1999; Bouillon et al., 2003; Gillikin et al., 2007). A lot of work on geochemistry and stable isotope geochemistry of *Mytilus* shells has been performed (Epstein et al., 1953; Mook and Vogel, 1968; Tanaka et al., 1986; Dettman and Lohmann,1995; Vander Kanduč et al., 2011) and it was recognized that bivalve shell geochemistry reflects environmental conditions under which mussels grew.

Mussels are an important nutritive source of Ca and Fe, vitamins such as niacin and thiamin, and proteins (Yap et al., 2004). Even though mussels are important food source, they can be potentially harmful since they can accumulate certain metal(loid)s such as Cr, Pb, Cd, Se, Hg and Cu, especially when growing in contaminated waters (Kljaković-Gašpić et al., 2007; Stanković et al., 2011). Among toxic elements, As takes a special place due to complexity of its chemistry in marine environment. Mussels as filter feeders receive most of arsenic from water and their diet, mainly particulate matter, phyto- and zooplankton. Phytoplankton and macro algae accumulate As from seawater and mainly convert it to arsenosugars. Zooplankton and organisms feeding on phytoplankton and algae normally contain some arsenosugars but mainly arsenobetaine (AsB) (Caumette et al., 2012), with lower amounts of trimethylarsine oxide (TMAO), arsenocholine (AsC), mono and dimethyl arsenic acid (MMA and DMA) and inorganic As. Normal As levels in seawater can be reflected in elevated levels of several As compounds in algae, phytoplankton and organisms at higher trophic levels, especially if accompanied by low phosphate levels (Caumette et al., 2012), as described in the Northern Adriatic Sea (Degobbis et al., 2005). Even at elevated levels, As normally doesn't present a risk for humans health due to prevalence of non-toxic AsB in molluscs, fish and crustaceans used as food.

The main objectives of this study were to seasonally and spatially trace the major seawater environmental parameters, the tissue contents of stable isotopic carbon and nitrogen composition, selected metal(loid)s levels (Cr, Mn, Ni, Co, Cu, Zn, As, Se, Cd and Pb) and As species of Mediterranean mussel together with its condition index from pristine locations (mariculture) and from polluted areas near the major ports of the Istrian peninsula, Croatia.

Based on measured parameters ecological status of marine environment at mariculture (5 locations) and ports (3 locations) from the NE Adriatic can be assessed.

Material and Methods

Study area and sample collection

Mussels were collected together with seawater from 5 mariculture and 3 port locations in the NE Adriatic Sea. In Istria (Istrian peninsula, Croatia), mussels are traditionally cultured in rafts supported by floats constructed of wood or steel. Locally sourced mussel seeds in mariculture areas are attached to the collecting ropes or as juveniles (2–3 cm) placed in net stockings and then hung at the floats, where they are fattened and grown to marketable size (6–8 cm) in less than 2 years depending on mariculture/location and ecological conditions. Fifteen individuals of mussel *M. galloprovincialis* were collected seasonally (February 2010, August 2010, April 2011, November 2011) from 5 mariculture areas (shellfish farms): Vabriga (VA, 45°16′24″N, 13°34′57″E), Lim Bay (LB, 45°08'00.9"N, 13°43'27.7"E), Pomer (PO, 44°49′06″N, 13°54′09″E), Budava (BU, 44°34′35″N, 13°59′28″E), and Raša Bay (RB, 45°01′13″ N, 14°03′ E) (Figure 1).

Wild mussels were collected seasonally in 2013 (January, April, June, November) at anthropogenic impacted sites, where the major ports/harbours of the NE Adriatic Sea are located (Rovinj, Pula, Rijeka) (Figure 1). At each sampling location, 15 individuals of wild mussels were sampled from the rocky shore (infralitoral) at 0.5 m depth. In Rovinj (RV, 45°05'10.2"N, 13°38'21.1"E), samples have been collected from the ferry pier near the Centre for Marine Research, Ruđer Bošković Institute. The main potential sources of pollution at this station are discharge from a nearby fish-processing factory and local city harbour. In Pula (PU, 44°52'22.7"N, 13°50'41.4"E), samples were taken in the area of the marina Pula "Torta" in the Pula port. The Pula port is extremely closed area due to the protective barriers, which prevent mixing of water masses, and reduces water exchange with the open sea. Several large industrial facilities like cement factory, shipyard, and additional 40 sewage discharges without purifier adding to organic pollution in the harbour. Such huge input of organic matter to the port is conducive to excessive growth and development of phytoplankton, and contribute to the low concentrations of dissolved oxygen in the port because of intense bacterial degradation. In Rijeka (RI, 45°19'35.6"N, 14°26'18.2"E) samples were taken from the first pier parking lot located on the coast of the Rijeka port. Here contamination is primarily of inorganic nature (heavy metals), originating from production process in the Rijeka shipyard and harbour-boat trafficking.

Mussel sampling and condition index determination

Immediately after sampling, mussels were transported to laboratory with ice blocks. Condition indices were measured on fresh mussels (10 mussels/sampling location), while mussels (5 specimen per sampling location) for elemental and stable isotope analyses were frozen until analyses.

The total weight of mussel, mussel weight without internal water, shells weight and wet meat/tissue weight were measured (0.01 g precision). Condition index was calculated as the ratio between soft tissue wet weight and total weight of whole mussel which include soft tissue, internal water and shell weight (Hamer et al., 2008; Pavičić-Hamer et al., 2016).

CI was calculated according to equation of:

$$CI = (\text{wet weight of soft tissue}) * 100 / (\text{whole mussel}) \qquad [\%; g/g] \quad (1)$$

Sample preparation and environmental conditions

For elemental and stable isotope analyses, mussels were unfrozen, soft tissue was scrapped from the shell, mixed, homogenized and freeze-dried (-54°C for a week in Christ, Alpha 1-4). After freeze-drying, samples were homogenized in a Pulverisette 7 mill (Fritsch) at a rotational speed of 18000 rpm. Dry mussel tissue was used for analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) compositions, selected metal(loid)s (Cr, Mn, Co, Ni, Cu, Zn, Se, Cd, Pb, As) and As speciation determination.

Over the investigated period, standard hydrographic parameters (salinity, pH, and temperature) were measured at sampling locations using a portable WTW Multimeter P4. Samples for total alkalinity analyses were collected in 30 mL HDPE bottles. Total alkalinity of the seawater was measured by Gran titration (Gieskes, 1974) with a precision of ± 1% within 24 h of sample collection. During the mussel collecting, seawater samples were taken for isotopic analyses of carbon (dissolved inorganic carbon - $\delta^{13}C_{DIC}$ and particulate organic carbon - $\delta^{13}C_{POC}$). Samples for determination of dissolved inorganic carbon were stored in glass serum bottles (volume of 12 mL) filled with no headspace and sealed with septa caps. Samples for stable carbon isotope analysis of particulate organic carbon were collected in LDPE bottles (2-3 L of sea water) (Schuster and Reddy, 2001)

Stable isotopic analyses

The stable isotope composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) was determined on an IsoPrime GV IRMS (isotope ratio mass spectrometer) coupled with a MultiflowBio preparation module. Phosphoric acid (100%) was added (100-200 µL) to a septum tube and then purged with pure He. The water sample (1 mL) was then injected into the septum tube and CO_2 was directly measured from the headspace. A standard solution of Na_2CO_3 (Carlo Erba and Scientific Fisher) with a known $\delta^{13}C_{DIC}$ value of -10.8 ± 0.2‰ and -4.8 ± 0.2‰ were used to calibrate $\delta^{13}C_{DIC}$ measurements (Spötl, 2005; Kanduč et al., 2007). After sampling, 2 - 3 litres of seawater sample was filtered through a Whatman GF/F glass fibre (0.7 µm) for determination of carbon stable isotope composition of particulate organic carbon ($\delta^{13}C_{POC}$). Filters were treated with 1M HCl to remove carbonate material and then they were dried at 60°C and stored until analyses. Approximately 1 mg of POC was scraped from the filter into a tin capsule. The isotopic composition of carbon was determined after combustion of the capsules in a hot furnace (temperature 1000°C) with a Europa Scientific 20-20 continuous flow IRMS ANCA - SL preparation module. NBS 22 (oil) reference material was used to relate the analytical results to the VPDB (Vienna Pee Dee Belemnite) standard.

Approximately 1 mg and 8 mg of soft tissue of mussel was weighed in a tin capsule for $\delta^{13}C$ and $\delta^{15}N$ analysis, respectively and measured the same way as described for $\delta^{13}C_{POC}$ analysis. Reference materials IAEA CH-3 with define value -24.7‰±0.1‰ and IAEA N-1 (ammonium sulphate) with define value 0.4±0.2‰ were used to relate the analytical results to the VPDB and AIR standards. The precision of both methods for $\delta^{13}C$ and $\delta^{15}N$ measurement was estimated to be ± 0.3‰.

Selectivity was calculated according to equation of Bouillon et al., 2003:

$$\text{Selectivity} = (\Delta\delta^{13}C_{tissue}\text{-}\Delta\delta^{13}C_{POC}/\Delta\delta^{13}C_{DIC}\text{-}\ \Delta\delta^{13}C_{POC})*100[\%] \qquad (2)$$

Determination of selected metal(loid)s in mussels

Freeze dried and homogenized mussel tissue (approximately 200 mg) was digested with a mixture of 3 mL concentrated HNO_3 (65%, Merck Suprapur) and 1 mL of H_2O_2 (30%, Merck Suprapur) in a microwave digestion system (Milestone, Ethos 1) for analysis of selected metal(loid)s. The following program was used: 10 min. of 1300 W at 140°C, then 10 min. of 1300 W at 210°C, and finally 20 min. of 1300 W at 140°C. The digested samples were diluted with MilliQ water (Milipore) to a final volume of 40 mL with additional dilution of 1 mL up to 10 mL prior measurements, when needed.

The concentrations of As, Cr, Mn, Co, Ni, Cu, Zn, Se, Cd, Pb were determined using a quadrupole ICP-MS - inductively coupled plasma-mass spectrometer (Agilent 7500ce) equipped with a concentric Micromist nebulizer, a Scott double pass spray chamber and a Fassel type quartz torch with an injector of inner diameter of 2.5 mm. The conditions used were as follows: RF power 1500 W, plasma argon gas flow 15 L min^{-1}, nickel sampler and skimmer cones, integration time 0.2 s and 0.3 s for Se. For all the measurements, helium gas (2 mL min^{-1}) was used as a reaction cell gas in order to avoid polyatomic interferences. The method was validated using NRC-CNRC (National Research Council Canada) Certified reference materials TORT-2 (Lobster Hepatopancreas Material for Trace Metals) and DOLT-4 (Dogfish Liver Certified Reference Material for trace Metals). The values obtained were in good agreement with the certified ones.

Determination of As species in mussels

Arsenic species were extracted into a methanol/water mixture (1:10) and evaporated to dryness using rotary evaporator. The dry residue was washed with aether and taken up in water (5 mL), filtered (0.45 µm) and kept frozen (-20ºC) until analysis. For each sample, two extracts were prepared. Ether fraction was evaporated to dryness and total arsenic as a measure for lipid-associated As in it was determined with instrumental neutron activation analysis.

HPLC, interfaced with UV decomposition, hydride generation and atomic fluorescence spectrometry (HPLC-UV-HG-AFS) was used for arsenic speciation in extracts (Šlejkovec et al., 2001). For the separation of arsenite (As(III), arsenate (As(V)), MA and DMA an anion exchange HPLC column (Hamilton PRP-X100, 250×4.1 mm) with KH_2PO_4 solution (15 mmol L^{-1}, pH 6.0) as a mobile phase was used and for the separation of DMA, AsB, AsC, TMAO and tetramethyl arsonium ion (TETRA) a cation exchange column Nucleosil SA with 10 mM pyridine/HCl in 0.2 M NaCl, pH 3.3 was used. The presence of arsenosugars was confirmed according to previously published procedure (Šlejkovec et al., 2006).

Statistical data processing

Principal component analysis (PCA) based on a covariance matrix was used to examine the variation in the measured parameters from mussel tissues (metalloids, $\delta^{13}C$ and $\delta^{15}N$ stable isotopes) across the locations and seasons. The CANOCO software package was applied (Ter Braak and Šmilauer, 2002).

One-way ANOVA with location as a factor was carried out on 19 parameters measured in the seawater and mussel soft tissue to determine whether there are any statistically significant differences between the means of two groups of locations (mariculture, ports). Prior to the analysis, the data were tested for normality by applying the Sapiro-Wilk test in order to check if data are approximately normally distributed for each category of the independent variable. In a case of non-normality, the date were log (x+1) transformed. In addition, Levene's test for homogeneity of variances was conducted.

A correlation matrix between measured parameters was constructed with the statistical program Statistica 12. The correlation coefficients (r) between the two variables were calculated and the Pearson t-test was carried out to determine significant correlations (at significance level of 0.05). Cross-correlation matrix for 19 measured parameters in the seawater and mussel tissue was performed respectively for mariculture locations (ESM Table 4) and ports (ESM Table 5).

RESULTS AND DISCUSSION

Environmental conditions at investigated locations

Seawater parameters at mariculture locations and ports are given in Tables 1 and 2, respectively. At mariculture locations (in years 2010 to 2011), seawater pH ranged from 7.11 to 8.09, temperature from 11.0 (February) to 27.2°C (August) and salinity from 12.2 to 36.8‰ (Table 1). In ports, pH was from 7.02 to 8.22, temperature ranged from 10.7 (February) to 24.4°C (August) and salinity of seawater from 14.5 to 38.8‰ (Table 2). Total alkalinity ranged from 3.1 to 4.2 mM at both, mariculture and polluted locations, Salinity was the lowest in Lim Bay, seasonally ranging from 12.2‰ to 22‰, due to freshwater input. Also at Rijeka (port location) salinity was lower and ranged from 14.5 to 21.4‰ during investigated period. Lower salinity was also observed at Pula location in April 2013 (Table 2). This is due to submarine freshwater springs. We obtained good and significant positive correlation between salinity and $\delta^{13}C_{DIC}$ at mariculture (r=0.82) and port locations (r = 0.80) (ESM, Tables 4 and 5). Further, we obtained good and significant positive correlations between pH and salinity (r=0.91), pH and $\delta^{13}C_{DIC}$ (r = 0.88) and pH and CI (r=0.54), and significant negative correlation between pH and Cd (r=-0.61) at mariculture locations (Table 4, ESM).

Condition index (CI) of mussel *M. galloprovincialis*

There are several different approaches to calculate CI (Hamer et al., 2008). However, in our study we used percentage of whole soft tissue weight in relation to total mussel weight. The chosen CI [wet soft tissue weight (g) x 100/ total mussel wet weight (g)] is used because of easy measurement and simple, precise calculation allowing determination of stable discriminating values for the assessment of mussel growth (mariculture) and environmental conditions (pollution). Shellfish farmers commonly use CI for determining the quality of mussels and the best timing of mussel harvesting and placing on the market. Additionally, CI, followed by chemical contamination allows ranking of areas and sites according to pollution effects.

In our study, CI ranged from 13.3 to 20.9% at mariculture locations (on average 17.4%, Table 3) and from 14.3 to 23.3% in ports (on average 17.9%Table 3) (Figure 2). CI did not significantly differ between the mariculture and polluted locations (one-way ANOVA, $F_{1,26}=0.26$; $p>0.05$) and did not significantly change throughout the seasons(one-way ANOVA, $F_{mariculture(3, 12)}=1.473$, $p>0.05$; $F_{ports(3, 8)}=1.888$, $p>0.05$). Still, the lowest CI was observed during winter at both investigated years ($CI_{February\ 2010}=16.2\%$ at maricultures and $CI_{January\ 2013}=15.1\%$ at ports), while during summer and spring CI was close to 20% at both groups of locations. Thus, the environmental conditions in spring (April 2011) and summer (August 2010) were more favourable, particular at mariculture locations (e.g. high level of organic matter and food availability, low metal levels in tissues) for the physiological development and growth of mussels than in cold seasons (Ruane-Hacene et. al., 2015).

The CI in February was lowest in mussels maricultured in Lim Bay (13.3%) and highest in mussels maricultured in Pomer (20.9%) (Figure 2). The general increase in condition index in mussels collected in August was observed at all mariculture areas, with the highest value measured in mussels collected at Pomer (18.9%). In the mussels from polluted ports, the condition index was highest in mussels from Rijeka (23.3%) in July, and it was higher of observed at all mariculture areas.

At mariculture locations, CI of mussels was significantly positively correlated with salinity (r= 0.54), and pH (r=0.54) as was observed in many studies (Khan et al., 2006; Celik et al., 2009). In contrast, significant negative correlation was observed between CI and Cd (r=-0.60) at mariculture locations indicating negative effect of Cd on mussel fitness, which was also confirmed in Shiuntu et al. (2008) study. At polluted locations, the sensitivity of mussels to anthropogenic or natural stressors might increase, as organisms devoted more energy to defence mechanisms than to growth (Ruane-Hacene et al., 2015).

In general, mean CI of mussels from polluted locations were moderately higher than those of maricultured organisms, mostly due to high CI at port Rijeka, that seems that has favourable conditions for mussels despite the highest measured metalloid concentrations in mussel tissue (PCA analysis, Figure 6). Mariculture areas, which are considered as unpolluted locations, showed annual low selected heavy elements concentrations (Figure 4) and similar CI levels as at port locations (Figure 2), reflecting that physiological conditions for the mussel growth are similar at both locations.

Isotopic composition of seawater dissolved inorganic carbon ($\delta^{13}C_{DIC}$) and particulate organic carbon ($\delta^{13}C_{POC}$), and of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in the soft tissue of *M. galloprovincialis*

Isotopic composition of seawater dissolved inorganic carbon ($\delta^{13}C_{DIC}$) ranged from 13.4 to 0‰; the lowest values of $\delta^{13}C_{DIC}$ were observed at Lim Bay during all sampling seasons due to highest freshwater input. At all other locations $\delta^{13}C_{DIC}$ values around 0‰ were measured in all sampling seasons meaning that these are more or less marine locations (Table 1). We also obtained good significant correlations between salinity and $\delta^{13}C_{DIC}$ value at mariculture locations and at ports (r from 0.82 to 0.80, Tables 4 and 5 ESM) demonstrating that both measured parameters indicate the influence of freshwater input. At port locations, $\delta^{13}C_{DIC}$ values ranged from -11.1 to 0.7‰ (Table 2), which is similar as observed at mariculture locations (Table 2). The low $\delta^{13}C_{DIC}$ values (-11.1 ‰) were observed in April 2013 at port location Pula. The reason are most probably the submarine freshwater springs.

$\delta^{13}C$ values in mussel tissue ranged from -24.5 to -20.8‰ at maricultural locations and from -22.9 to -20.2‰ at ports and suggest that most of the samples investigated in this study are not impacted by sewage sludge pollution (Table 3, Figure 3). Still, significantly higher $\delta^{13}C$ values were measured in the mussels from the port locations (one-way ANOVA, $F_{(1,26)}=7.710$; $p<0.05$). It is known that mussels are 90% selective, assimilating their carbon primarily from phytoplankton, which in turn obtains its carbon from DIC pool (Bouillon et al., 2003). This is also confirmed in our study (equation 2), since we obtained results of $\delta^{13}C$ in mussel soft tissue that are characteristic for particulate organic matter, which is in our study on average of -25.0‰ at mariculture locations and on average of -25.1‰ at port locations. Selectivity of mussels was on average 86.9% at maricultures and 82.9% at port locations and ranged from 67 to 100%. Lower selectivity was observed at freshwater locations and is due to terrestrial input with different isotopic composition of POC (in the range from -25.0 to -32.0‰) (Kanduč et al., 2008) (Table 3).

The values of sewage effluent were estimated to be ca. -23.5‰ for $\delta^{13}C$ and between 1.8‰ to 2.5‰ for $\delta^{15}N$, and were taken from Rogers, 2003. The isotopic composition characteristic of Slovenian industrial sewage sludge is -23.8‰ for $\delta^{13}C$ and 2.6‰ for $\delta^{15}N$ (Kanduč, 2010). The soft tissue of *M. galloprovincialis* from different seasons from mariculture and polluted locations thus indicates an absence of influence of industrial sewage sludge on *M. galloprovincialis*.

Mussels from our study have $\delta^{15}N$ from 3.8 to 6.1‰ in samples collected from mariculture locations and from 0.4 to 8.3‰ in the samples from ports (Table 1). Enrichment of $\delta^{15}N$ through the trophic network is widely recognized among most animals, including invertebrates and vertebrates, leading to value of 3.4±1.1‰ (DeNiro and Epstein 1981). Mussels in our study have $\delta^{15}N$ on average value of 5.4‰, which is similar (range 4.4 to 6.5‰, Table 3) as in the case of the Gulf of Trieste study (Kristan et al., 2014). In our study, only Pula and Rijeka have lower $\delta^{15}N$ values. At mariculture locations, significant negative correlation was obtained between salinity and $\delta^{15}N$ (r = -0.61) (ESM, Table 4), meaning that lower $\delta^{15}N$ values are due to freshwater input.

Selected metal(loid)s in soft tissue of *M. galloprovincialis*

Mussels take up metals through the gills from the water column and through ingestion of food and particulates. Increased metal(loid)s concentrations (Cr, Mn, Co, Ni, Cu, Se, Cd, Pb, Zn, As) in mussel tissue can thus be attributed to natural metal levels in sea water (geological position), local point sources (anthropogenic activities), tide and current transport, and atmospheric deposition (Guevara et al., 2004). It has been recognized that the soft tissues of marine mollusks are generally more efficient accumulators of metals than the shells (Brown and Depledge, 1998) implying that soft tissue concentration of metal(loid)s reflects environmental conditions better than that of shells.

Our data (Figure 3, Table 3) show that mussels from mariculture locations (indicated in grey), intended for human consumption, contain on average lower concentrations of most of elements studied comparing to mussels from ports, a logical outcome to expect. Average levels of metal(loid)s are slightly higher in polluted sites for Cr (1.8 mg kg^{-1} compared to 1.3), Cd (0.6 mg kg^{-1} compared to 0.4) and Co (0.5 mg kg^{-1} compared to 0.3), about twice higher for Mn (7.5 mg kg^{-1} compared to 3.5), Ni (1.7 mg kg^{-1} compared to 0.9) and Se (4.2 mg kg^{-1} compared to 2.1), about 4-5 times higher for Cu (11.8 mg kg^{-1} compared to 2.3) and Zn (193.8 mg kg^{-1} compared to 51.7) and 12 times higher for Pb (6.2 mg kg^{-1} compared to 0.5) (Table 3). One-way ANOVA confirmed significantly higher concentrations of the following metalloids at port locations: Mn, Cu, Zn, Se, Cd, Pb ($p<0.001$), Co ($p<0.01$), and Ni ($p<0.05$) (Table 3). Similarly, results of PCA analysis clearly distinguish between a group of locations with less polluted mussels (in all months) and a group of mussel samples from clearly heavily polluted locations (ports) containing high concentrations of metalloids (Figure 6). The major differences between the two groups of samples were due to concentrations of Se, Zn, Cd and Co as seen from PCA ordination diagram.

The only exception is As, which shows uniform concentrations over all sites (17.2 ± 5.6 mg kg^{-1}) except Rovinj (35.8 ± 9.9 mg kg^{-1}, about twice higher concentration comparing to other sites). Rovinj is a site with fish-canning industry, which obviously influences local micro-location by returning its naturally arsenic-rich fish-parts containing waste back to the Sea. Obtained results for metal(loid)s fall in a range reported previously for *M. galloprovincialis* from the Mediterranean (Orescanin et al. 2006; Kljaković-Gašpić et al. 2007; Ščančar et al. 2007; Fattorini et al. 2008; Kristan et al. 2014; Maulvault et al. 2015) and Tagus estuary (Santos et al. 2014, Maulvault et al. 2015). Results for Ni, Co and Mn are considerably lower than in Boka Kotor Bay reported by Joksimovic et al. (2011). In the study of Kristan et al. (2014), conducted on *M. galloprovincialis* nearby in the Slovenian part of the Adriatic Sea (Bay of Koper, Bay of Strunjan, Bay of Piran in two different seasons: in March 2009, 2010 and in September 2009), intermediary values for Cu, Cd, Se, Zn, Pb and As were found, placing their values in between unpolluted and polluted values from present study (Table 3).

Literature data also suggest that concentrations of heavy metals in soft tissue vary mostly between seasons, where the biggest variations in metal levels comes from the reproductive cycle of mussels increasing it during the period before spawning (Burger and Gochfeld, 2006; Fattorini et al. 2008). According to the data of Ciocan (2002) spawning of *M. galloprovincialis* mostly happens in spring (April) and summer (July/August) thus increased metal concentrations before spawning would be expected at the end of winter (January/February). Indeed, in our study Cr, Co, Ni, Se and Cd showed some extent of seasonal variation with the highest concentrations found in February and the lowest concentrations detected in summer months (July – August). The same was observed in a study of Kristan et al. (2014) for nearby locations in the Slovenian part of the Adriatic Sea (Table 3). Seasonal fluctuations were smaller than fluctuations between unpolluted (mariculture) and polluted sites (ports).

At mariculture and port locations (ESM, Tables 4, 5), statistically significant negative correlations between temperature (T) and several metalloids (e.g., Se, r=-0.77 and -0.64) were observed indicating the importance of temperature conditions for the intensity of metalloid uptake by mussels. The temperature dependence of metal uptake by mussels was previously demonstrated by Mubiana and Blust (2007), who observed that fundamentally (i.e. at epithelial membranes), temperature-effects on uptake are largely due to changes in solution chemistry and physical kinetics, which favours higher uptake at high temperature. But, at whole organism level, complex physiological responses appears to mask this relationship that could result also in inverse effect of temperature. Significant positive correlation was also obtained between Cr and Ni (r = 0.96) and negative between Cr and Cd (r = -0.60). Also Co and Ni were significantly positively correlated (r = 0.73). Ni was positively correlated with Cr (r = 0.96) and Se (r = 0.80). Se was significantly positive correlated with Cu (r = 0.81) and Cr (r = 0.83). Cr is statistically positively correlated between Co (r= 0.60), Ni (r= 0.96), Cu (r=0.61) and Se (0.83). Cd is statistically significant negative correlated between pH (r = -0.61), temperature (r = -0.59), salinity (r = -0.43) and CI (r = -0.60) (ESM, Table 4).

At port locations (ESM, Table 5), significant correlation was observed between Cr and and Ni (r = 0.94). Significant negative correlation was observed between Mn and T (r = -0.69), Mn and salinity (r = -0.79), Mn and Cr (r = 0.79) and Mn and $\delta^{13}C_{DIC}$ (0.61). Significant positive correlation was observed between Ni and Co (r = 0.75) and Ni and Cr (r = 0.94). Also Cu and Pb (r = 0.62), Zn and Cd (r = 0.62) were positively correlated. Significant positive correlation between Se and Cd (0.73) and Cd and Zn (0.68) and Co and Cd (0.83) was also found. Significant positive correlation was also observed between Pb and Cu, Zn (0.62).

As speciation in soft tissue of *M. galloprovincialis*

Total As levels in mussel samples from 7 locations ranged from 12.2 – 32.6 (average 17.2 ± 5.6) mg kg^{-1} and in Rovinj (RV) they reached from 25.3 – 47.1 (average 35.8 ± 9.9) mg kg^{-1}. Arsenic extractability from mussel samples was moderate and 72.5 ± 22.6 % of total As in the samples was found in the extracts. Arsenic speciation of mussel extracts showed that AsB was the main As compound found in all cases, representing 83.3 ± 20.1 % of extractable As (Figure 5). Of other As compounds found in mussels only arsenosugar phosphate ribose (P. ribose, 11.2 ± 7.3 % of extractable As) and lipids-associated arsenic (5.6 ± 3.1 % of the total As) were present in considerable concentrations. Inorganic As(III) and As(V) and organoarsenic compounds MA, DMA, TMAO and an unknown cationic compounds were found at trace levels. Although total As concentrations in Rovinj port were considerably higher comparing to other (polluted and unpolluted) locations, no further differences in extractability and/or speciation were observed. Seasonal variations in neither total As nor in As speciation in any of locations were observed. Significantly higher values of lipids-associated As ($p<0.05$), were found in soft tissue of mussels from ports (means 0.87 vs. 1.42 mg kg^{-1}) and significantly higher values of As (III) ($p<0.05$), As (V) ($p<0.01$) and As(III)+As(V) (inorganic arsenic) were observed in the mussels from mariculture locations.

Conclusions

We estimated ecological status of marine environment in the NE Adriatic Sea with the use of indicator species *M. galloprovincialis* and determination of following parameters: condition indices (CI), isotopic composition of carbon in seawater ($\delta^{13}C_{POC}$, $\delta^{13}C_{DIC}$) and isotopic composition of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) in soft tissue, selected elements (Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd, Pb) and As species at mariculture and polluted wildlife (ports) locations. According to all measured parameters in *M. galloprovincialis* we can conclude that marine environment in the NE Adriatic Sea is mildly polluted in ports under investigation while mariculture locations reflect unpolluted environment.

Interestingly, higher condition indices were detected of mussels from polluted locations then from mariculture. Mussels CI are on average 17.4% at mariculture and on average 17.9% in polluted locations. No significant difference was found among the CI of mussels collected at mariculture and polluted locations.

We obtained good regression (r=0.68 to 0.78) between salinity and $\delta^{13}C_{DIC}$. Lower $\delta^{13}C_{DIC}$ were observed at Lim Bay location due to more freshwater input. $\delta^{13}C_{DIC}$ has on average value around -1.2‰ (characteristic value for marine environment is 0‰) in period 2010 to 2011. $\delta^{13}C_{DIC}$ values at Lim Bay varies from -11.7 to -7.1‰, while at polluted locations an average $\delta^{13}C_{DIC}$ values are -2.7‰. Total alkalinity is on average 3.6 mM in year 2011 and 2013 sampling campagnas.

$\delta^{13}C$ in soft tissue of *M. galloprovincialis* is on average -22.5‰ at mariculture, while on average -21.4‰ at polluted locations. $\delta^{15}N$ is on average 5.0‰ at mariculture, while on average 5.9‰ at polluted locations. $\delta^{13}C$ and $\delta^{15}N$ reveal no sewage sludge pollution.

The highest concentrations of selected elements were found in following order: Zn >As> Cu> Mn>Pb >Se>Cr >Ni >Cd >Co. The highest As concentration was found at Rovinj location. Arsenobetaine was the most abundant species in soft tissue of *M. galloprovincialis*.

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List of tables:

Table 1: Seawater parameters (pH, temperature, salinity, total alkalinity, $\delta^{13}C_{POC,}$ $\delta^{13}C_{DIC}$) and stable isotopes in soft tissue of *M. galloprovincialis* at 5 mariculture locations (VA, LB, PO, BU, RB) during different sampling seasons (February 2010, August, 2010, April 2011, November 2011).

Table 2: Seawater parameters (pH, temperature, salinity, total alkalinity, $\delta^{13}C_{POC,}$ $\delta^{13}C_{DIC}$and stable isotopes in soft tissue of *M. galloprovincialis* at 3 polluted locations (ports RO, PU, RI) during different sampling seasons (January, April, July, November 2013).

Table 3: Mean values (±SD) of measured seawater and soft tissue parameters for mariculture and polluted (port) locations with the results of one-way ANOVA (mariculture and polluted locations as factor).

ESM Table 4: Cross–correlation matrix for 19 seawater and mussel tissue parameters measured from January 2010 to November 2011 at mariculture locations (5 locations). Marked (**bold**) correlations are significant at p<0.05.

ESM Table 5: Cross–correlation matrix for 19 seawater and mussel tissue parameters measured from January 2013 to November 2013 at polluted locations (3 locations). Marked (**bold**) correlations are significant at p<0.05.

Table 1: Seawater parameters (pH, temperature, salinity, total alkalinity, $\delta^{13}C_{POC}$, $\delta^{13}C_{DIC}$) and stable isotopes in soft tissue of *M. galloprovincialis* at 5 mariculture locations (VA, LB, PO, BU, RB) during different sampling seasons (February 2010, August, 2010, April 2011, November 2011).

			Seawater						**Soft tissue of *M. galloprovincialis***	
Season	**Location**		**pH**	**T (°C)**	**Salinity (‰)**	**Total alkalinity (mM)**	**$\delta^{13}C_{POC}$ (‰)**	**$\delta^{13}C_{DIC}$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**
February 2010	Lim Bay	LB	7.11	14.7	12.2	3.1	-24.6	-13.4	-20.9	5.6
	Vabriga	VA	7.89	11.0	25.0	3.4	-24.2	0	-20.8	5.5
	Budava	BU	7.85	11.0	29.0	3.6	-24.4	-0.6	-22.2	3.9
	Pomer	PO	8.01	11.1	34.5	4.1	-23.2	0	-23.2	4.2
	Raša Bay	RB	7.78	12.2	30.1	3.2	-25.4	-2.8	-21.8	3.8
Mean ± SD			**7.73±0.36**	**12.0±1.6**	**26.2±8.5**	**3.5±0.4**	**-24.4±0.8**	**-3.4±5.7**	**-21.8±1.0**	**4.6±0.9**
April 2011	Lim Bay	LB	7.38	16.0	16.0	4.1	-26.3	-12	-22.6	6.1
	Vabriga	VA	8.01	15.7	35.0	4.2	-24.1	-1.2	-22.2	5.1
	Pomer	PO	8.00	18.0	34.7	3.5	-24.6	-1.3	-24.5	4.9
	Raša bay	RB	7.85	16.3	30.6	3.6	-26.5	-1.4	-24.0	4.0
Mean ± SD			**7.81±0.30**	**16.5±1.0**	**29.1±8.9**	**3.8±0.4**	**-25.4±1.2**	**-4.0±5.4**	**-23.3±1.1**	**5.0±0.9**
August 2010	Lim Bay	LB	7.71	27.2	15.0	4.1	-26.1	-7.1	-22.8	6.1
	Vabriga	VA	8.00	26.0	34.2	3.1	-24.3	-1.5	-21.2	5.8
	Budava	BU	8.05	25.0	35.7	3.2	-24.6	-1.1	-23.5	4.5
	Pomer	PO	8.09	26.2	36.8	3.7	-23.8	-2.3	-21.7	4.9
	Raša Bay	RB	8.06	25.1	35.7	4.0	-27.4	-0.2	-24.4	4.4
Mean ± SD			**7.98±0.16**	**25.9±0.9**	**31.5±9.3**	**3.6±0.5**	**-25.2±1.5**	**-2.4±2.7**	**-22.7±1.3**	**5.1±0.8**
November 2011	Lim Bay	LB	7.55	15.0	22.0	3.3	-26.2	-11.7	-22.7	6.0
	Raša Bay	RB	7.70	13.3	25.6	3.4	-24.3	-1.4	-21.4	5.0
Mean ± SD			**7.62±0.11**	**14.2±1.2**	**23.8±2.6**	**3.4±0.1**	**-25.3±1.3**	**-6.6±7.3**	**-22.0±0.9**	**5.5±0.7**

Table 2: Seawater parameters (pH, temperature, salinity, total alkalinity, $\delta^{13}C_{POC}$, $\delta^{13}C_{DIC}$) and stable isotopes in soft tissue of *M. galloprovincialis* at 3 polluted locations (RO, PU, RI) during different sampling seasons (January, April, July, November 2013).

			Seawater						Soft tissue of *M. galloprovincialis*	
			pH	**T**	**Salinity**	**Total alkalinity**	**$\delta^{13}C_{POC}$**	**$\delta^{13}C_{DIC}$ (‰)**	**$\delta^{13}C$**	**$\delta^{15}N$**
Season	**Location**			**(°C)**	**(‰)**	**(mM)**	**(‰)**		**(‰)**	**(‰)**
January 2013	Rovinj	RV	7.83	11.5	26.7	3.2	-23.8	-2.4	-21.0	6.4
	Pula	PU	7.95	12.3	35.7	3.9	-24.5	-2.3	-22.0	7.4
	Rijeka	RI	7.65	10.7	14.5	4.1	-24.6	-5.1	-21.0	8.1
Mean ± SD			**7.81±0.15**	**11.5±0.8**	**25.6±10.6**	**3.7±0.5**	**-24.3±0.4**	**-3.3±1.6**	**-21.3±0.6**	**7.3±0.9**
April 2013	Rovinj	RV	7.92	13.5	35.2	3.3	-25.4	-2.4	-21.9	8.3
	Pula	PU	7.62	15.3	14.5	3.2	-23.4	-11.1	-22.9	7.2
	Rijeka	RI	7.73	10.8	18.8	3.1	-27.0	-7.1	-21.2	8.1
Mean ± SD			**7.76±0.15**	**13.2±2.3**	**22.8±10.9**	**3.2±0.1**	**-25.3±1.8**	**-6.9±4.4**	**-22.0±0.9**	**7.9±0.6**
July 2013	Rovinj	RV	8.22	24.0	33.4	3.4	-25.8	-0.4	-20.2	7.3
	Pula	PU	8.00	24.4	38.8	3.5	-25.9	-2.6	-21.2	7.5
	Rijeka	RI	7.96	19.1	21.4	3.7	-26.0	-5.3	-21.2	7.1
Mean ± SD			**8.06±0.14**	**22.5±2.3**	**31.2±8.9**	**3.5±0.2**	**-25.9±0.1**	**-2.8±2.5**	**-20.9±0.6**	**7.3±0.2**
November 2013	Rovinj	RV	7.02	17.1	32.3	3.9	-25.9	-5.8	-21.0	4.0
	Pula	PU	7.93	17.8	37.3	4.0	-24.0	0.7	-21.5	1.8
	Rijeka	RI	7.65	13.2	-	4.2	-24.5	-7.4	-22.0	0.4
Mean ± SD			**7.53±0.47**	**16.0±2.5**	**34.8±3.5**	**4.0±0.2**	**-24.8±1.0**	**-4.2±4.3**	**-21.5±0.5**	**2.1±1.8**

Table 3: Mean values (±SD) of measured seawater and soft tissue parameters for mariculture and polluted (port) locations with the results of one-way ANOVA (mariculture and polluted locations as factor).

	Mariculture (n=16) mean±SD	**Port (N=12)** mean±SD	**One way ANOVA** F(1, 26)	P value	**Study of Kristan et al., 2014**
Seawater					
pH	7.8±0.3	7.8±0.3	0.053	0.820	8.1-8.3
T (ºC)	17.7±6.0	15.8±4.8	0.830	0.371	9.8-22.9
Salinity (‰)	28.3±8.1	27.0±9.6	0.149	0.702	36.3-37.5
Alkalinity (mM)	3.6±0.4	3.6±0.4	0.028	0.868	
$\delta^{13}C_{POC}$ (‰)	-25.0±1.2	-25.1±1.1	0.024	0.879	
$\delta^{13}C_{DIC}$ (‰)	-3.6±4.7	-4.3±3.3	0.164	0.688	
Soft tissue					
Cr (mg/kg)	1.3±1.6	1.8±1.0	1.000	0.326	
Mn (mg/kg)	3.5±1.6	7.5±3.1	19.378	0	
Co (mg/kg)	0.3±0.1	0.5±0.2	12.490	0.002	
Ni (mg/kg)	0.9±0.7	1.7±1.1	5.555	0.026	
Cu (mg/kg)	2.3±0.7	11.8±5.4	49.356	0	3.46-11.3
Zn (mg/kg)	51.7±15.7	193.8±60.5	81.977	0	73.4-172.3
As (mg/kg)	18.1±4.6	22.1±12.8	1.378	0.251	14.7-29.9
Se (mg/kg)	2.1±1.0	4.2±0.8	34.179	0	2.2-6.8
Cd (mg/kg)	0.4±0.1	0.6±0.2	29.088	0	0.5-1.2
Pb (mg/kg)	0.5±0.3	6.2±3.5	41.653	0	0.8-2.2
$\delta^{13}C$ (‰)	-22.5±1.2	-21.4±0.7	7.710	0.01	-21.4 to -22.0
$\delta^{15}N$ (‰)	5.0±0.8	6.1±2.6	2.747	0.109	4.4-6.5
Selectivity	86.9±8.9	82.9±7.1	1.677	0.207	
CI index	17.4±2.1	17.9±3.2	0.260	0.615	

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Figure 1: Investigated area of the NE Adriatic Sea (Istrian peninsula, Croatia) with sampling points showing mariculture (white spots): Vabriga (VA), Lim Bay (LB), Pomer (PO), Budava (BU), Raša Bay (RB) and polluted, anthropogenically impacted locations (local ports, black spots): Rovinj (RV), Pula (PU), Rijeka (RI).

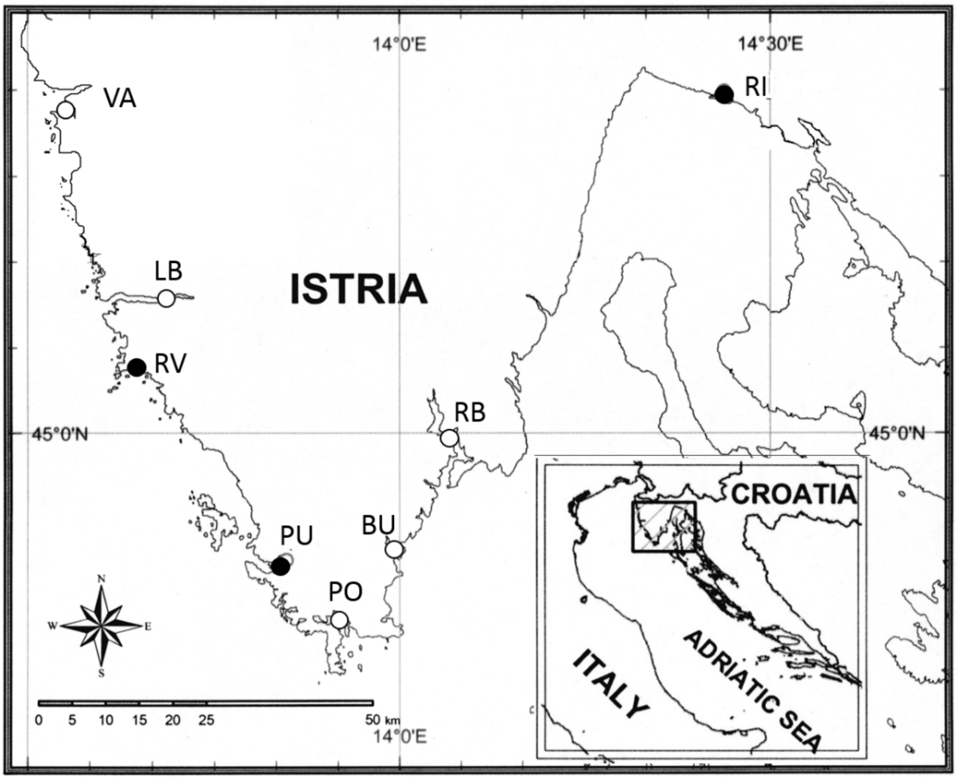
Figure 2: Condition indices of *M. galloprovincialis* at mariculture locations: Vabriga (VA), Lim Bay (LB), Pomer (PO), Budava (BU), Raša Bay (RB) and polluted locations: Rovinj (RV), Pula (PU), Rijeka (RI) during different sampling seasons.

Figure 3: Isotopic composition of carbon ($\delta^{13}C$) versus nitrogen ($\delta^{15}N$) of soft tissue of *Mytilus galloprovincialis* from NE Adriatic. VA- Vabriga, Lim Bay – LB, Pomer – PO, Budava – BU, Raša Bay – RB. The values for sewage effluent were estimated to be ca. -23.5‰ for $\delta^{13}C$ and between 1.8‰ to 2.5‰ for $\delta^{15}N$ and were taken from Rogers. 2003. Measured $\delta^{13}C$ and $\delta^{15}N$ values in sewage sludge in Slovenia are -23.8‰ and 2.6‰, respectively (Kanduč. 2010).

Figure 4: Average concentrations of selected elements (Cr, Mn, Co, Ni, Cu, Zn, As, Se, Cd, Pb all in mg kg^{-1} dry weight) in Mediterranean mussel *M, galloprovincialis* collected from mariculture: Vabriga (VA), Lim Bay (LB), Pomer (PO), Budava (BU), Raša Bay (RB) and polluted locations: Rovinj (RV), Pula (PU), Rijeka (RI) from N Adriatic Sea during different seasons. Error bars indicate seasonal variations at each site.

Figure 5: Average concentrations of arsenic compounds (mg kg^{-1} dry weight) in Mediterranean mussel *M. galloprovincialis* collected from mariculture: Vabriga (VA), Lim Bay (LB), Pomer (PO), Budava (BU), Raša Bay (RB) and polluted locations: Rovinj (RV), Pula (PU), Rijeka (RI) (color bars) from N Adriatic Sea. Error bars indicate seasonal variations at each site.

Figure 6. PCA ordination diagram indicating ranges of variation of measured parameters in mussel tissues (arrows) and differences in the samples from mariculture and polluted (port) locations collected during different seasons (points). Mariculture locations are Vabriga (VA), Lim Bay (LB), Pomer (PO), Budava (BU), Raša Bay (RB) and ports are: Rovinj (RV), Pula (PU), Rijeka (RI).



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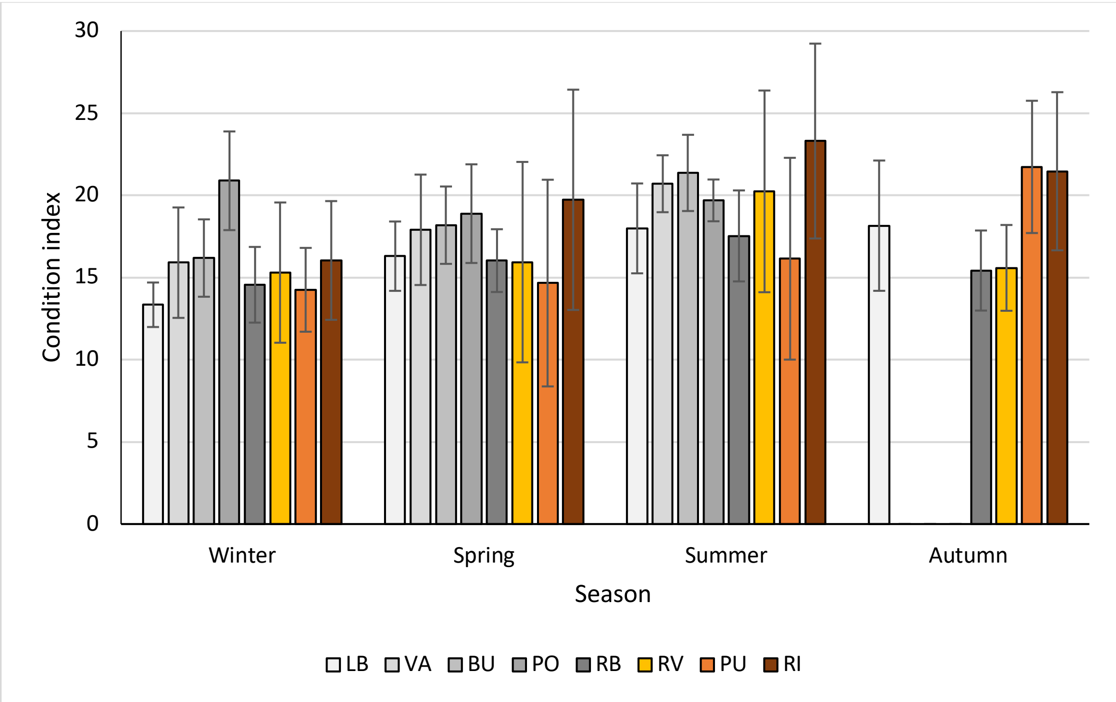

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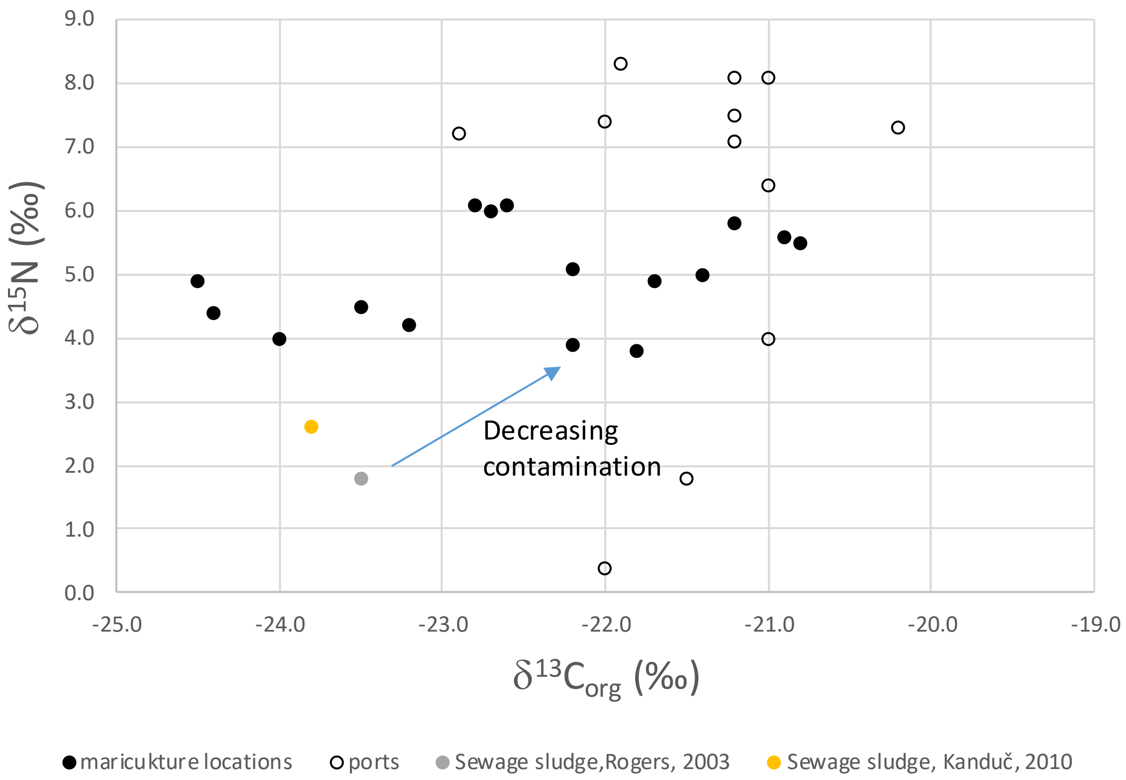

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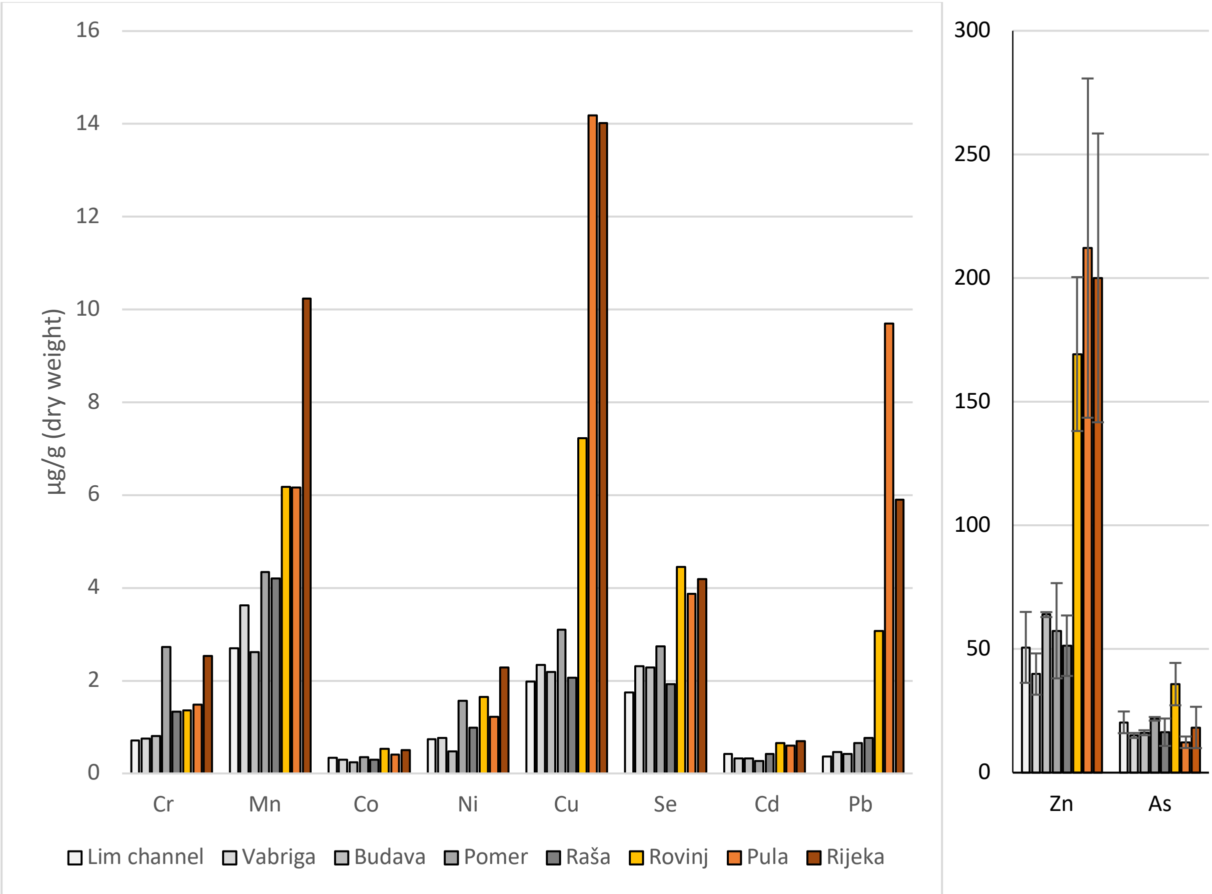

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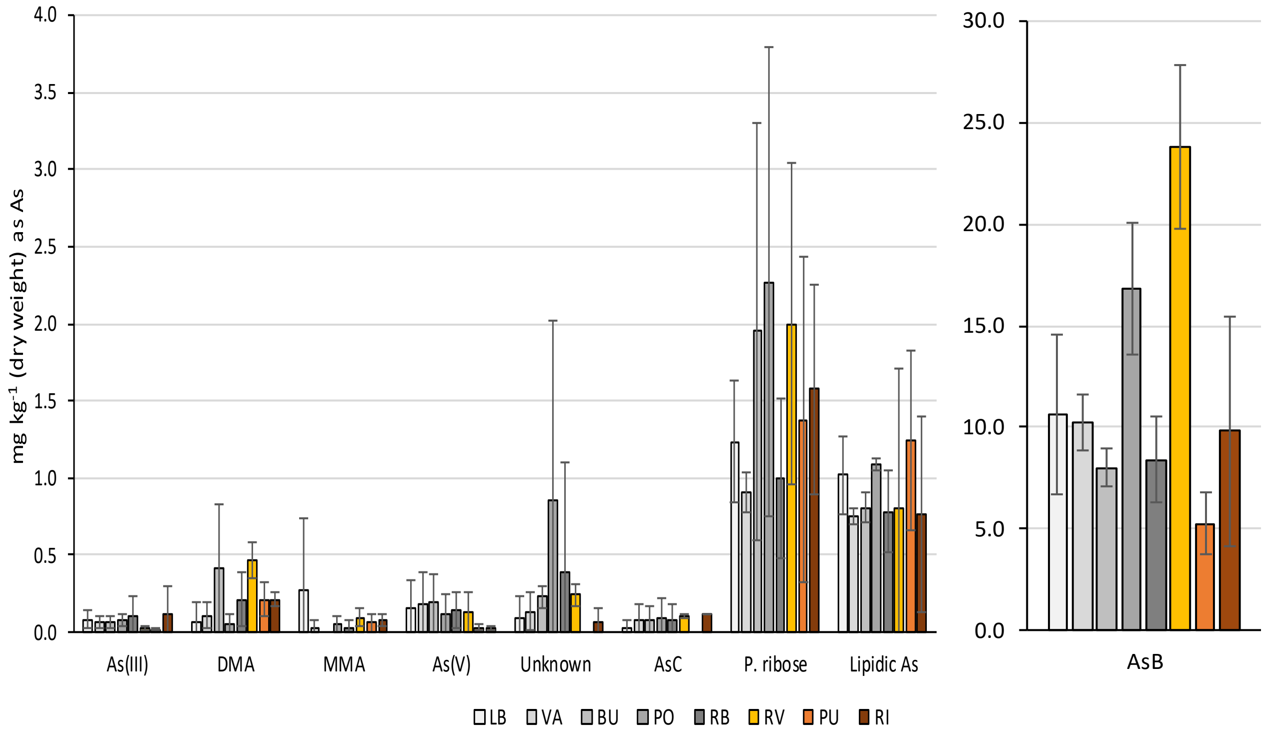

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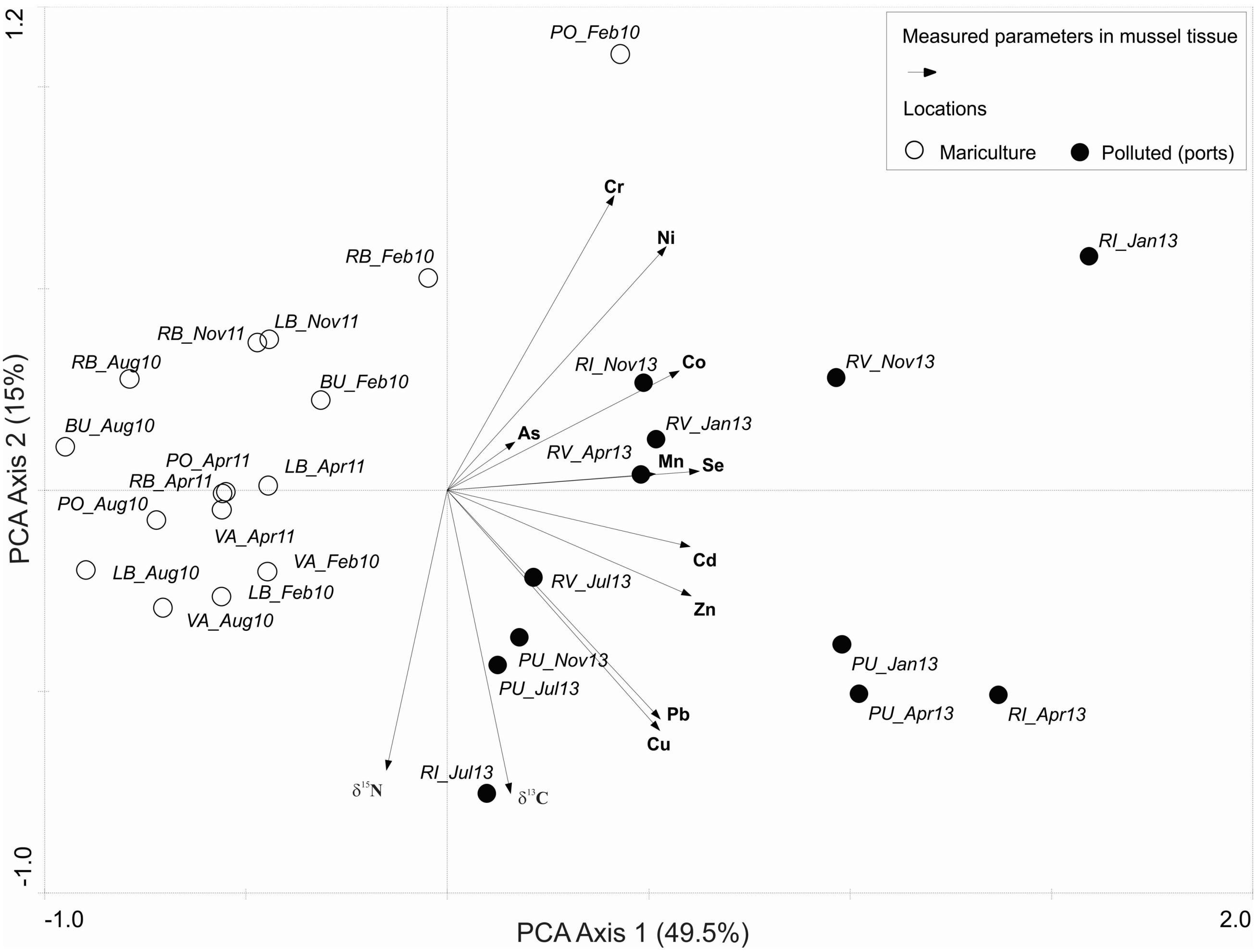

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