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Characterization of the cytosolic distribution of priority pollutant metals and metalloids in the digestive gland cytosol of marine mussels: seasonal and spatial variability

Authors: Željka Strižak[a*], Dušica Ivanković[a], Daniel Pröfrock[b], Heike Helmholz[b], Ana-Marija Cindrić[a], Marijana Erk[a], Andreas Prange[b]

[a]Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička c. 54, 10002 Zagreb, Croatia

[b]Helmholtz-Zentrum Geesthacht, Zentrum für Material und Küstenforschung, Institute of Coastal Research, Marine Bioanalytical Chemistry, Max-Planck-St., 21502 Geesthacht, Germany

*Corresponding author: Željka Strižak, Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička c. 54, 10002 Zagreb, Croatia. Tel.: +385 1 468 02 16; fax + 385 1 468 02 42. E-mail address: zstrizak@irb.hr

E-mail addresses: zstrizak@irb.hr (Ž. Strižak), Dusica.Ivankovic@irb.hr (D. Ivanković), daniel.proefrock@hzg.de (D. Pröfrock), heike.helmholz@hzg.de (H. Helmholz), Ana-Marija.Cindric@irb.hr (A.M. Cindrić), erk@irb.hr (M. Erk), andreas.prange@hzg.de (A. Prange)

Abstract

Cytosolic profiles of several priority pollutant metals (Cu, Cd, Zn, Pb) and metalloid As were analyzed in the digestive gland of the mussel (*Mytilus galloprovincialis*) sampled at locations with different environmental pollution levels along the Croatian coast in the spring and summer season. Size-exclusion chromatography (SEC) connected to inductively coupled plasma mass spectrometry (ICP-MS) was used to determine selected elements bound to cytosolic biomolecules separated based on their molecular size. Copper, cadmium and zinc eluted mostly associated with high (HMW) and medium molecular weight (MMW) biomolecules, but with a more prominent elution in the MMW peak at polluted locations which were probably associated with the 20 kDa metallothionein (MT). Elution of all three metals within this peak were also strongly correlated with cytosolic Cd as strong inducer of MT. Lead mostly eluted in HMW biomolecules range, but in elevated cytosolic Pb concentrations, significant amount eluted in low molecular weight (LMW) biomolecules. Arsenic, on the other hand eluted almost completely in LMW range, but we could not distinguish specific molecular weight biomolecules which would be predominant in detoxification mechanism. Seasonal variability in element abundance within specific peaks was present, although not in the same extent, for all elements and locations, especially for As. The results confirm the suitability of the distribution of selected metals/metalloids among different cytosolic ligands as potential indicator for metal exposure. Obtained findings can also serve as guidelines for further separation and characterization of specific cytosolic metal-binding biomolecules.

Keywords: *Mytilus galloprovincialis*, digestive gland cytosol, trace elements, SE-HPLC-ICP-MS

1. Introduction

Coastal environments are subjected to several forms of anthropogenic disturbances. In particular trace metal pollution is still of major concern. Although concentrations of priority metals in the seawater are regularly monitored worldwide, great effort is being made towards the application of biomarkers that indicate an early response in selected target organisms that finally provide evidence of the exposure to the chemical pollutants and may indicate a toxic effect. Especially effects based on the response at molecular and cellular levels represent the earliest warning signals of an environmental disturbance.

Metal toxicity arises predominantly from the binding of metals to essential biomolecules such as enzymes and transporter proteins or the role of certain metals in the formation of radicals (Mason and Jenkins, 1995). Therefore, the excess of metal ions needs to be removed from the vicinity of important biological molecules. The major types of internal sequestration are achieved by accumulation of metals in granules and lysosomes or by binding of metals to cytosolic proteins like metallothioneins (MT) (Vijver et al., 2004). In order to get a better insight not only into detoxification mechanisms but also in the mechanisms of metal toxicity, it is important to determine which biomolecules bind metals specifically in normal metabolism and whether the same biomolecules are involved in binding processes under exposure conditions.

Size-exclusion liquid chromatography (SEC) has been frequently used as the basis for the protein separation. The performance under mild, physiological chromatographic conditions keeps proteins as intact as possible which is important when analyzing natural, metal-binding proteins (De la Calle Guntiñas et al., 2002). Often, the subsequent step in characterizing metal-binding proteins is the analysis of metals in the separated fractions. In recent time the mass spectrometry as a metal detection system for different chromatographic separation techniques is being increasingly used due to the possible low detection limits and (hetero)element-specific detection (De la Calle Guntiñas et al., 2002, Pröfrock and Prange, 2012). Since cytosol is a complex biological matrix, SEC hyphenated to inductively coupled plasma mass spectrometer (ICP-MS) is a valuable tool for the screening of cytosolic metal

distribution in the biomolecules of different molecular size. Using this methodology, cytosolic molecules involved in the normal metal metabolism, detoxification mechanisms or metal toxicity could potentially be identified - a step towards defining biomarkers. In addition, quantitative and/or qualitative differences in the distribution of metals among different cytosolic ligands at different levels of environmental metal pollution could also be identified and used as a potential indicator of metal exposure.

Either the offline or online combination of SE-HPLC and ICP-MS for determination of the cytosolic metal distribution in various aquatic invertebrates and vertebrates has been used by various authors (Mason and Storms, 1993; Ferarrello et al., 2002; Li et al., 2005; van Campenhout et al., 2008; Krasnići et al., 2012). In our knowledge cytosolic distribution of metals was not investigated with reference to seasonality. Yet, it is known that the level of total tissue and cytosolic metals show a seasonal variability dependent on the abiotic and biotic factors even when the environmental concentrations of metals remain unchanged (Regoli and Orlando 1994, Raspor et al., 2004, Ivanković et al., 2005). Similarly, it is found that biomarkers are also affected by seasonality (Ivanković et al., 2005; Bocchetti and Regoli, 2006). Therefore, in order to use the cytosolic metal distribution as indicator of the metal exposure, it is necessary to identify cytosolic metal-bindind ligands that best reflects the environmental metal pollution level by applying this hyphenated approach of separation of biomolecules and detection of associated metals, but also to investigate the possible seasonal variability in cytosolic metal distribution.

This study was focused on the characterization of cytosolic profiles of several priority pollutant metals (Cu, Cd, Zn, Pb) and As in mussel digestive gland (*Mytilus galloprovincialis*) from coastal locations of different environmental pollution levels with reference to seasonal variability in two most distinct seasons (late winter/early spring vs. advanced summer) concerning reproductive cycle of mussels and abiotic factors.

Mussels are widely accepted as useful biological indicators of marine pollution on both the organism as well as the cellular level (Goldberg and Bertine, 2000, Narbonne et al., 2005, Zorita et al., 2007), while digestive gland represents the central detoxification organ of the

organism. Although, Cu and Zn are essential trace metals required for a wide range of metabolic processes of proteins, carbohydrates and lipids, for cell signaling, cell growth and respiration (Flemming and Trevors, 1989; Murakami and Hirano, 2008), they can also be toxic if present in a high enough concentration. Cadmium, on the other hand, is generally considered as a toxic trace element and identified as a priority hazardous substance in many EU countries (WFD, 2000) although some recent researches shown that Cd can be of benefit for some marine diatoms (Lane and Morel, 2001). Lead is not involved in any biological mechanism necessary for life (Gnassia-Barelli and Romeo, 1993) and because of a deleterious effect on the aquatic environment it is included in the list of the priority toxic pollutants within the European Water Framework Directive (WFD, 2000). Arsenic is a widespread metalloid in the marine environment existing in many chemical forms. Tissues of marine invertebrates and fish contain high concentrations of As, mainly in different organic less toxic forms (Neff, 2009), compared to a lower concentration of inorganic As species, which are considered of being more toxic (Del Razo et al., 2001; Thomas, 2007).

Among sources of coastal pollution with the investigated metals and metalloid in particular antifouling paints, used to prevent growth of the fouling organisms on the ships' hulls and submerged structures, play an important role. Since the ban of tributyltin, antifouling paints are mainly based on Cu(I) and zinc oxide boosted by other mostly organic co-biocides, while they also contain other metals as additives and non-biocidal pigments (Turner, 2010) represent an important threat on the marine invertebrates (Bellas, 2006). Around boatyards, besides Cu and Zn, elevated concentrations of numerous other metals such as Cd, and Pb were found (Tapinos et al., 2006; Turner, 2010), while constituents of prohibited paints (e.g. As, Sn and Hg) (Almeida et al., 2007) could also be released to the surrounding areas during the ship maintenance. Therefore, in this study we focused on the investigation of the cytosolic metal distribution in particular on organisms living sedentary in highly metal polluted aquatic environments such as marinas and harbors. In addition, the harbor selected for this research is located in a stratified estuary with frequent salinity and temperature changes, which could pose an additional stress for the organisms living in that place. Therefore, the

results obtained by this study will also provide information on the cytosolic metal distribution in organisms, which are in addition to the stress caused by metal pollution, also chronically exposed to the natural stress caused by salinity and temperature variations.

2. Materials and Methods

2.1. Sampling

Mussels *Mytilus galloprovincialis* were collected in two sampling periods (March and August, 2010) at three locations in the central part of the eastern coast of the Adriatic Sea. Since marinas and harbors are generally considered as sites of the metal exposure, the marina (M) located in the south west end of Kaštela Bay and the harbor (H) of the town of Šibenik were chosen as polluted locations. One location (village Zablaće in the vicinity of Šibenik) under less anthropogenic influence was selected as the reference location (R). Mussels ranging from 3.8 – 8.0 cm in length size were collected from the coastal rocks or concrete embankment structures between 0.5 m and 1 m below the sea surface. Digestive gland was dissected and frozen in liquid nitrogen immediately upon the sampling. Digestive glands of 6-8 individuals were pooled to form representative composite sample. Since it was not always possible to collect a sufficient number of similar size specimens, the samples were formed in such a way that they contain digestive glands of both larger and smaller mussels. Three composite samples per location were analyzed.

2.2. Analysis of total dissolved metals in seawater

The concentrations of dissolved Cu, Cd, Zn and Pb were determined in the filtered (0.45 µm) seawater using differential pulse anodic stripping voltammetry as described by Omanović et al., 2006 and Cukrov et al., 2008.

2.3. Isolation of cytosolic fraction

The digestive gland tissues were cut into the small pieces, diluted 6 times with a cold homogenization buffer (20 mM Tris-HCl/Base, Sigma, pH 8.6 at 4°C) supplemented with

reducing agent (2 mM dithiotreitol, Sigma) and protease inhibitors (0.006 mM leupeptine and 0.5 mM phenylmethanesulfonylfluoride, Sigma), then homogenized by using a Potter-Elvehjem homogenizer (Glass-Col) placed in a ice cooled tube at 4,000 rpm. The homogenates were centrifuged using an Avanti J-E centrifuge (Beckman Coulter) at 50,000 × g for 2 h at 4°C. Obtained supernatant (S50) which represents the water soluble tissue fraction (cytosol) was aliquoted and stored at -80°C for further analysis.

2.4. Analysis of the total metal concentration in cytosol and investigation of the cytosolic metal distribution

Distribution of the priority metals and the metalloid As in the digestive gland cytosol was studied by SE-HPLC (HPLC Agilent 1100) coupled to an ICP-MS (Agilent 7500cx). Separation of the cytosolic biomolecules by size was accomplished by using a prepacked Tricorn™ Superdex 75 10/300 GL column with a separation range 3 kDa – 70 kDa for globular proteins. Several protein standards (apo-Transferrin, Ovalbumin, Albumin, Superoxide dismutase, Chymotripsinogen A, Apomyoglobine, Metallothionein) were run under the same conditions as the samples to calibrate the column. Parameters of SE-HPLC-ICP-MS are listed in Table 1. Based on the elution time (t_e) of protein standards and the chromatographic profiles of metals, a high molecular weight range (HMW; t_e < 31 min), a medium molecular weight range (MMW; t_e = 31 – 49 min) and a low molecular weight range (LMW; t_e > 49 min) were defined (Figure 1).

Flow injection analysis (FIA) responses from three successive injections of 0.174 ng multielement standard (TraceCERT® No. V, Fluka) before and after each chromatographic run were used for the determination of the total element concentration by integrating areas of standards and chromatograms. For each metal characteristic peaks were identified and their areas expressed as a percentage of the total chromatogram area were calculated.

2.5. Data processing and statistical analysis

The concentrations of cytosolic elements, same as the peak areas were expressed as average ± standard deviation (SD). Statistical analyses were performed using the Statistica 6.0 software package (StatSoft, USA). To evaluate differences between the groups, t-test or one-way ANOVA followed by Tukey post hoc test of multiple comparisons were used. To examine the relationship between variables measured, a linear correlation was applied. Significant level was set at $p = 0.05$ in all statistical test.

3. Results

3.1. Metal concentrations in seawater and cytosol

Total concentrations of dissolved metals in seawater are summarized in Table 2, while cytosolic concentrations of the priority pollutant metals and As in the digestive gland are presented in Table 3.

In both seasons, seawater Cu concentration was higher in H and M locations than in reference location. Harbor location had the highest seawater Cd concentration in both seasons, especially in August. Seawater Zn concentration was the lowest in location R in both seasons. At all studied locations Pb concentrations in the seawater were higher in August than in March and slightly lower concentrations were observed at H location in both seasons (Table 2). Significantly higher concentrations of cytosolic Cu were observed at the M location in both seasons. In March, the R location showed higher cytosolic Cu concentration than H, but the difference was not significant (Table 3). In both sampling periods, cytosolic Cd concentrations were the lowest in samples derived from location R, but this difference was significant only in August (Table 3). Cytosolic Zn was significantly lower in samples from the reference location R than in those obtained from polluted locations M and H in both seasons. The difference in cytosolic Zn concentration between samples from location R and M as well as R and H was less obvious in March (Table 3), although in this season the difference in the seawater Zn concentration between those locations was higher than in August (Table 2). In August, cytosolic Pb concentration was significantly higher in the samples from location H than in those from location M and R, while in March such marked

difference was not present (Table 3). This was not in line with the concentration of Pb in the seawater which did not differ in such manner when comparing the different locations (Table 2). Total cytosolic As concentrations in samples from location H were significantly lower in both seasons compared to concentrations found in samples from location M and R, which was specially expressed in March (Table 3).

3.2. Cytosolic profiles of bound Cu

Cytosolic Cu was mainly distributed within the HMW and MMW biomolecules with pronounced elution in the MMW fraction when looking at samples from the polluted locations (Figure 2). In the Cu chromatographic profiles, four rather broad peaks appeared in the following ranges of elution time (t_e): 1) 22.5-31 min, 2) 31–35 min, 3) 35-46 min, 4) 48-55 min (Figure 2). Their areas were calculated and expressed as percentages (%) of the total chromatogram area (Figure 3). Although the cytosolic Cu pattern showed similar elution profiles (Figure 2), the peak areas and therefore the level of Cu-associated proteins did differ between the locations and the seasons. Seasonal differences were most pronounced in samples from the R location (Figure 3). On the other hand, samples from the M location showed no season-dependent peak area distribution (Figure 3) although total cytosolic Cu concentration between the seasons was significantly different (Table 3).

In both seasons studied, there was a remarkable difference between the reference and polluted locations with respect to the relative proportion of Cu bound to the separated cytosolic constituents (Figure 3). The most striking difference was the increase in the percentage of bound Cu in cytosols from polluted locations represented by peak 3 where this percentage was approximately two to three times higher compared to the data from the reference location (Figure 3). At the same time, a marked reduction of Cu bound to the constituents of the LMW proteins at peak area 4 and a less pronounced decrease in the HMW proteins at peak area 1 was observed in Cu profiles from contaminated locations (Figure 3). This observation was further supported by correlation analysis showing a significant negative correlation of peak 3 with peak 1 (March, r = -0.78, August r = -0.95) and

of peak 3 with peak 4 (March, r = -0.79, August r = -0.99), and a significant positive correlation between peak 1 and peak 4 (March, r = 0.96, August = 0.93).

3.3. Cytosolic profiles of bound Cd

Similarly to Cu, cytosolic Cd was also mostly bound to constituents of the cytosol eluting in the HMW and the MMW range, with expressed elution in the MMW range (Figure 2). Peak areas (%) from three ranges of t_e 1) 22.5-29 min, 2) 29–35 min and 3) 35-48 min (Figure 2) were calculated, although well resolved peaks were not always present. Peak 3 area of Cd distribution mostly overlapped with the peak 3 of Cu elution.

Seasonal variations in samples from location R and H were present in all peak areas (Figure 4). Peak areas 1 and 2 were enlarged, while peak 3 area was reduced in August. Samples from M location did not show seasonal variation either in total cytosolic Cd or in peak areas (Figure 4, Table 3).

Significant differences between the locations were observed for peak 2 and peak 3 with respect to the relative proportion of bound Cd (Figure 4). Peak 2 area was larger in samples derived from location R than in the polluted locations with the difference being significant comparing to samples from the H location in both seasons (Figure 4). Peak 3 area, which overlaps with peak 3 of Cu chromatogram was in both seasons the smallest in the reference location with striking difference in August when it was approximately three to four times smaller comparing to both polluted locations. The difference was also supported by correlation of total cytosolic Cd which was positive with peak 3 in both seasons at the significant level (March r = 0.79; August r = 0.93) and negative with peak 2 area in August only (r = -0.82) when the difference in total cytosolic Cd of the reference location comparing to the contaminated locations was more expressed (Table 3).

3.4. Cytosolic profiles of bound Zn

In the elution profiles of bound Zn we distinguished 3 peaks in t_e ranges: 1) 22.5 – 30 min, 2) 30 – 35 min, 3) 35 – 42 min (Figure 2). At least half of the cytosolic Zn eluted within the fraction related to the HMW biomolecules (Figure 2).

Regardless of the absence of the seasonal difference in total cytosolic Zn concentration for samples from location M and H (Table 3), the seasonal variation in the proportion of Zn bound to specific proteins represented by elution peaks was observed, especially for the samples from H location (Figure 5). Reference location samples showed seasonal variation in both cytosolic Zn (Table 3) and in the bound Zn-ratios (Figure 5). However, it has to be considered that peaks 1 and 2 were not well separated in any of the samples. Peak 3 was, however, well separated in all samples and more pronounced in those from March for all locations (Figure 2) although the significant difference between seasons of the same location was not always present (Figure 5). Spatial differences in the peak 3 were also present. Peak 3 area was smaller in samples from the reference location comparing to those from the polluted ones, but these differences were not always significant (Figure 5). Correlation analysis were used to indicate and evaluate seasonal fluctuations and the only correlation found analogue in both seasons was the significant negative correlation between peak 1 and peak 2 (March r = -0.76, August r = -0.86).

3.5. Correlation of Cu, Cd and Zn MT associated peaks with the total cytosolic metals

The area of Cu-MT peak showed positive correlation with cytosolic concentrations of Cd and Zn in both seasons (Table 4). The area of Cd-MT peak showed positive correlation with cytosolic Cd and Zn levels, which was significant with cytosolic Cd in both seasons and with cytosolic Zn in August only (Table 4). On the other hand, the area of Zn-MT peak showed positive correlation with the cytosolic Cd concentration with the significant level only in samples obtained in March. There was positive but not significant correlation of Zn-MT peak with cytosolic Zn, while the correlation with cytosolic Cu was negative in March and positive in August (Table 4).

3.6. Cytosolic distribution of bound Pb

Most of Pb eluted with the HMW biomolecules for all samples, but some well resolved peaks also occurred in the MMW and LMW range (Figure 6). Peak areas (%) from five ranges of t_e 1) 22.5-32 min, 2) 32–36 min, 3) 36-39 min, 4) 49-55 min, 5) 58.5-62 were calculated (Figure 7). Seasonal variability was the most evident for samples from location H with significant differences for almost all peaks, while seasonal variability in samples from location R and M were less pronounced (Figure 7).

Spatial differences in selected peak areas were not expressed consistently comparing two seasons. In March, significant spatial variability was found in areas of peak 2 and 4 (Figure 7). In that season, in the samples from location H with the lowest total cytosolic Pb (Table 3), peak 2 area was significantly smaller than in samples from location M and R (Figure 7). In the same season, samples from location M had the highest cytosolic Pb concentration (Table 3) and the smallest peak 4 area (Figure 7). Although significant positive correlation of the cytosolic Pb and peak 2 area ($r = 0.88$) was observed, peak 4 area did not correlate with cytosolic Pb ($r = -0.30$). The percentage of peak 5 area significantly correlated with peak 4 ($r = 0.88$) although this area itself did not show significant spatial variability.

In August, spatial differences occurred in peak 3, peak 4 and peak 5 areas comparing samples from location H with those from R and M, with differences being significant for areas of peak 3 and peak 4, but not for peak 5 (Figure 7). The cytosolic Pb concentration strongly correlated with peak 3 ($r = -0.83$) and with peak 4 ($r = 0.89$). Peak 4 showed negative correlation not only with peak 3 ($r = -0.84$), but also with peak 1 area ($r = -0.68$). Peak 5 area was increasing with peak 4 area ($r = 0.67$) at a significant level as in March. It was interesting that peak 2 area which strongly correlated with cytosolic Pb in samples from March showed no correlation with cytosolic Pb in samples from August.

3.7. Cytosolic distribution of bound As

Arsenic showed negligible elution within the HMW and MMW biomolecules with the most of As eluting within LMW fraction (Figure 8). Three well defined peaks in the LMW range eluted

in t_e ranges: 1) 48-56 min, 2) 56-60 min and 3) 60-66 min (Figure 8) and the areas (%) of these peaks were calculated and presented in Figure 9.

Seasonal differences in the peak areas were present for all peaks at the significant level with exception of peak 1 from samples derived from location H (Figure 9). Significant spatial differences in cytosolic As concentration from March (Table 3) were followed by significant differences in all peak areas between locations (Figure 9). In August, there were no significant spatial differences in cytosolic As concentration between R and M locations (Table 3), but spatial differences between these two locations existed in peak areas for peaks 1 and 3 (Figure 9). Regardless of the seasonal variability and of differences in total cytosolic As concentration, peak 3 area remained the lowest in the samples from reference location and the largest in the samples from harbor.

In both seasons cytosolic As negatively correlated with peak 3 area (March r = -0.78, August r = -0.74) at significant level. On the other hand peak 1 and peak 2 showed no such similarities. Peak 2 which had expressed differences between locations in March, showed no spatial difference in peak area in August (Figure 9), although cytosolic As in samples from location H significantly differ from the other two locations (Table 2). In August, peak 1 showed positive correlation with cytosolic As concentration (r = 0.93) and negative one with peak 3 (r = -0.92), both at significant level. However, in March this was not the case.

4. Discussion

Binding of metals to cytosolic ligands represents the important way by which the body removes excess metals from the vicinity of important biological molecules, thus preventing their toxic effects (Vijver et al., 2004). Therefore, it is very important to determine which cytosolic biomolecules bind metals specifically in normal metabolism as well as whether the same biomolecules are involved in binding processes under metal exposure conditions. In this study we related the distribution of several priority pollutant metals (Cu, Cd, Zn, Pb) and As in the cytosol of the digestive gland with metal exposure in native mussel populations in realistic environmental conditions taking into account the seasonal influence.

Beside the distribution of metals between various cytosolic ligands we also assessed the total cytosolic metal as soluble fraction of tissue metal that is more responsive to variations in metal concentrations in water. In general, higher cytosolic metal concentrations were obtained in M and H as polluted locations but because of the differences in geochemical behavior and bioavailability of particular metal that was not always the case.

Higher concentrations of Cu were expected particularly in M and H locations because of the various maritime activities. Prior to the nautical touristic season, fresh antifouling paint is usually coated on the ship hulls which caused an increase of Cu seawater concentration in the marina (Table 2) and consequently significantly higher cytosolic Cu concentrations in mussels sampled at the this location, which was particularly pronounced in March (Table 3). However, a different scenario was seen at the H location where higher concentrations of Cu in seawater were not accompanied by an increase in Cu concentration in the cytosol of mussel digestive gland (Tables 2 and 3). Lower cytosolic Cu concentration in samples from location H could be at least partially explained by the very low salinity at this location due to its position in the estuary. Owing to the fact that Cu binding to the dissolved organic matter increases when the salinity decreases (Lores and Pennock, 1998) a large proportion of Cu could have been bound to the organic matter which would decrease the Cu uptake (Lorenzo et al., 2005) and consequently the bioaccumulation in bivalves. Except for Cu dissolved organic matter has significant role also in Pb speciation in the seawater. Consequently, higher cytosolic Pb concentration in the samples obtained at location H could be explained by the probably more abundant organic matter in the water that can increase the Pb uptake as it has been found by Sánchez-Marín et al. (2007), although such situation was observed in August only. Contrary to Cu, low salinity contributes to higher accumulation of Cd because in such condition the Cd speciation changes from the predominance of Cd-chloro complexes in favor of free Cd ion (Cd^{2+}) as the easily bioavailable and more toxic form (Engel and Fowler, 1979). Although Cd concentration in the seawater was the highest at location H (Table 2), cytosolic Cd concentrations did not differ much when comparing with the samples from location M and H (Table 3). This could be the consequence of the physiological

response of the mussels to the salinity fluctuations. To help to reduce the rate of changes in cell volume associated with salinity fluctuations, mussels respond immediately by closing their valves (Bayne et al., 1976), consequently reducing the filtration rate and uptake of Cd. Total cytosolic As concentrations obtained in our study having lower values in samples from estuarine location H than in those from coastal locations (R and M) (Table 3) indicate that salinity also had an important role in As bioavailability. Generally, As concentrations in the estuaries are changeable as a result of fluctuating river inputs and salinity variations. In the Krka River estuary where H location is situated, Seyler and Martin (1991) observed a linear decrease in total As with decreasing salinity, ranging from 1.8 µg/L in seawater down to 0.13 µg/L in fresh waters. Also, accumulation of As, primarily in the form of arsenobetaine as a component of the mechanism of osmoregulation, was put in the proportional relation with salinity in *M. edulis* showing higher accumulation in higher salinities (Clowes and Francesconi, 2004). These findings were in line with the total cytosolic As concentrations obtained in our study having values in samples from harbor 3 - 13 times lower than in samples from locations R and M (Table 3).

Besides physico-chemical properties of the seawater, other factors related with the reproductive stage of mussels could also affect metal concentrations in the digestive gland (Regoli and Orlando 1994, Raspor et al., 2004, Ivanković et al., 2005). Therefore, the general trend of higher concentrations of cytosolic metals in March than in August as it was reported in this study for Cu, Cd and Zn (Table 3) could also be related with the possible influence of reproductive status. In addition, increased concentrations of essential elements in mussel tissues in spring are often reported probably as a consequence of increased food availability and higher requirements for essential metals due to gonad development (Raspor et al., 2004). Furthermore, discrepancy between the seawater and cytosolic concentration of the particular metal in this study could be caused by the fact that metals were analyzed in the soluble tissue fraction only, and it is known that some metals (e.g. Pb and Cu) are more accumulated in the insoluble tissue fractions such as granules or organelles.

Investigation of the whole cytosolic profile of particular metal showed clear differences between polluted and less polluted locations (Figures 2, 6 and 8). In these cytosolic metal profiles it was possible to observe a number of well resolved peaks (Figures 2, 6 and 8) and changes in their relative areas related to spatial and seasonal differences (Figures 3 to 5, 7 and 9).

The cytosolic profiles of Cu, Cd and Zn (Figure 2) could be grouped in a same category based on the changes in the area of elution range 3 related to the spatial differences. Cu and Zn homeostasis and Cd detoxification were found to be associated with the induction of and binding to MTs (Viarengo and Nott, 1993). In the current study these three metals showed strong elution in the MMW range marked as peak 3 area which might correspond to MT protein based on calibration of column and the used standards. Strong elution in this range was in line with the earlier findings of Ferrarello et al. (2002) who reported almost all Cu and Cd, and significant part of Zn eluted within the elution window of MT standards in SE-HPLC separated cytosol of mussels. However, in our study significant part of Cu and Cd (approximately 20%) and approximately half of Zn eluted with biomolecules of HMW and not specifically in the MT range. However in the polluted locations (M and H) the MT associated peak was certainly more pronounced for these three metals in both seasons (Figure 3, 4 and 5). In addition, MTs can be induced not only by metals, but also by other compounds such as hormones, cytokines, oxidative stress etc. (Andrews, 2000). Furthermore, it was shown that combined exposure to several metals had stronger effect to the MT induction in the mussel larvae compared to the individual metal exposure (Pavičić et al., 1994). Regardless of the cause of induction, MTs show high affinity for Cu, Cd, Zn. Consequently, in the current study correlations of some MT associated peaks of these metals with the total cytosolic concentrations were generally significantly positive (Table 4). Zinc is considered as the primer inducer of MT (Haq et al., 2003) which explains positive correlations of Cu-MT and Cd-MT peaks with the cytosolic Zn. The absence of the strong correlation of Zn-MT peak with the cytosolic Zn can be explained by an easy displacement of Zn from MT by Cu and Cd. Furthermore, as Zn is required for many biological processes it could have been

primarily bound to the proteins that require Zn for their function and not to MT. Positive correlation of all three metals associated with MT peaks with cytosolic Cd (Table 4) was in accordance with literature. Induction of MT biosynthesis by Cd in both freshwater (Malley et al., 1993, Marie et al., 2006, Ivanković et al., 2010) and marine (Lemoine et al., 2000, Ivanković et al., 2002, Dondero et al., 2005) bivalves was well confirmed and MT has strong affinity for this metal. On the other hand, MT induction by Cu in bivalves was put into question in several reports (Lemoine et al., 2000, Dondero et al., 2005; Ivanković et al., 2010). Therefore, the absence of significant positive correlation of metal MT peaks with the cytosolic Cu was in line with these findings. The fact that Cu-MT did not show correlation with the total cytosolic Cu at the significant level, but nevertheless Cu strongly eluted in this peak at polluted locations confirmed that various factors were responsible for Cu binding within the proteins of this investigated molecular range. Since MT has stronger affinity for Cu than for Cd and Zn (Amiard et al., 2006) it would bind Cu regardless of the source of the induction. Thus, the changes in peak elution range 3 of Cu chromatograms cannot be used as potential indicator of Cu exposure, but instead the assessment of the whole chromatogram could be made in such cases.

MTs exist in many isoforms. An MT isoform of 10 kDa was observed to be inducible in *M. galloprovincialis* by Cd, Zn, and Cu ions (Dondero et al., 2005), while particularly 20 kDa isoform but not 10 kDa isoform was found to be predominantly induced by Cd in *Mytilus* sp. (Mackay et al. 1993; Ivanković et al. 2002, Dondero et al., 2005). Our results showed that the majority of Cu was associated with the 20 kDa isoform and much less with the 10 kDa isoform. However, the binding of Cu to MTs does not necessarily mean its direct involvement in the induction of synthesis of this protein. The fact that the Cu peak 3 in samples derived from the M location in August remained very high although the cytosolic Cu dropped significantly suggested the possible induction of MT by some other factors such as the presence of other metals or oxidative stress as already mentioned earlier. It was found that mussel MT20 gene also responded to oxidative stress, while MT10 did not show any significant activation by e.g. hydroxyl radicals (Dondero et al., 2005). In Cd chromatograms

significantly positive correlation of peak 3 area with total cytosolic Cd was in accordance with the findings that elevated concentrations of Cd induce 20 kDa MTs in *Mytilus* sp. as already mentioned above.

In Zn chromatograms larger areas of the peak 3 in March can be explained by the enhanced need for Zn during the gonad development that occurs in spring. The role of MT in Zn homeostasis is well known (Viarengo and Nott, 1993) so more Zn could have been bound on MT in March to provide a sufficient amount of Zn, which is needed for various biological functions. Such correlation of increased concentration of MTs and essential metals in spring has been noticed also by other authors (Raspor et al., 2004). Besides MT as a possible ligand for Zn binding, another explanation of stronger Zn elution in samples from March obtained at location H compared to location M and R could be some Zn associated enzyme, possibly carbonic anhydrase (CA). The activity of CA can be linked with osmoregulation in organisms living in an environment of changing salinity (Henry and Saintsing, 1983; Henry et al., 2003) as was the case for the estuarine location of the harbor investigated in the current study. In particular in March the salinity was very low due to the river input. When Zn distribution was examined with more attention (Figure 2) it can be noticed that peak 3 eluted somewhat steeper in samples from location H than in those derived from R and M which would be the case with elution of CA (29 kDa) as a larger protein than 20 kDa MT. Although the values for the calculated peak area 3 did not correlate with cytosolic Zn concentration (Table 4) because of the above mentioned reasons, the elution of Zn in peak 3 was more pronounced in the polluted locations which were again common with Cu and Cd elution profiles.

The greatest differences in the whole cytosolic profiles of particular metal between polluted and reference locations were observed in Cu chromatograms (Figure 2). Contrary to the results obtained from mussels collected at polluted locations, the organisms at the R location had nearly 50% of cytosolic Cu associated with proteins represented by peaks 1 and 4, and only a smaller part in the peak 3 (Figure 3). This means that at the R location under conditions of low exposure to metals or other factors which could induce the *de novo*

synthesis of MT, the HMW and LMW biomolecules represented the main binding components of Cu in cytosolic fraction. However, in the case when, for any reason, there is an induction of MTs, most of Cu will be bound to MT proteins. Furthermore, it is also necessary to observe a significantly larger proportion of Cu in the peak 4 area at the reference location compared to the polluted locations. Another important biomolecule associated with Cu homeostatic control is the small tripeptide glutathione (Viarengo and Nott, 1993) with molecular weight of 307 Da. It may have eluted within peak 4. The fact that the elution of Cu in this peak was the largest at R location may indicate the importance of glutathione in the regulation of Cu at low level of exposure to the metal. Elution time of the peak preceding the MT peak (peak 2) corresponds to the molecular weight of superoxide dismutase (SOD, 37 kDa) as well as elution time of the used SOD standard. Therefore, the elution of Cu in this peak can probably be attributed to Cu bound to SOD. SOD is a Cu containing enzyme, usually associated with oxidative stress that can be induced by metals and various other contaminants (Lushchak, 2011) which are most probably present in a polluted environment such as marina and harbor. Our results showed that there was a small but significant decrease in the percentage of Cu bound in the peak 2 in samples from the location H in both seasons, as well as at samples from location M in August compared to the R location. At this stage of research, we cannot say whether this decrease was due to the possible replacement of Cu in the SOD molecule with other metals, as well as whether it had any adverse impact on the functionality of the enzyme.

The cytosolic profiles of Pb showed different features compared to the profiles of Cu, Cd and Zn and furthermore the Pb chromatograms from H location showed prominent difference compared to chromatograms from M and R locations (Figure 6). In Pb chromatograms about 50% of Pb eluted in the peak area 1 in the HMW range that might among other proteins contain 280 kDa enzyme δ-aminolevulinic acid dehydratase (ALAD). The activity of ALAD was proposed as a potential biomarker of Pb exposure and effect in mammals, birds and fish (Hylland, 2004) since it can be inhibited by Pb. However, there are only few researches for both freshwater (Company et al., 2008) and marine (Kalman et al., 2008) bivalves that

showed ALAD can be affected by the total Pb in tissues. The activity of ALAD was not measured in this study, but we did not find either spatial variability between the investigated locations within peak 1 which could contain ALAD (Figure 7, Table 2) or the correlation of peak 1 with total cytosolic Pb. In August, when total cytosolic Pb in samples from location H was the highest (Table 3), only small proportion of Pb was bound to the peak 3 (Figure 7), for which we believed, corresponded to the molecular weight range of MT. Although Pb has a weak affinity to MT (Amiard et al., 2006) very few studies have examined MT chelating Pb and only some researchers have found correlation between MT level and Pb concentration (Campana et al., 2003). Nevertheless, in our study it was obviously not a predominant factor. Besides HMW, a notable amount of cytosolic Pb eluted within the range of LMW biomolecules which showed interesting correlations in August when the biggest difference between the locations was found (Figure 7). Biotransformation of Pb through glutathione was recorded in rat brains (Struzyńska et al., 2002) and digestive glands and gills of green mussels (Yan et al., 1997). Presumably, if the level of glutathione with bound Pb was increased it would have eluted within these LMW peaks. Furthermore, in rat kidney cytosol 11.5 kDa proteins were reported which were also capable of reversing Pb-induced ALAD inhibition in the liver homogenates (Gonick, 2011). Also, low molecular weight Pb-binding proteins in human kidney have been identified as thymosin β4 (5 kDa) and acyl-CoA-binding protein (9 kDa) (Gonick, 2011). We found that occurrence of LMW peaks in the Pb chromatograms represented interesting starting points for further studies, and the seasonality should also be further investigated within this context. It appeared that the seasonal influence could play an important role in the Pb binding mechanisms. Different speciation of Pb in the presence of probably higher organic matter concentration in the H location situated in the estuary, could have contributed to the differences in the peak areas between the studied locations.

The cytosolic profiles of As showed entirely different features compared to the profiles of all other studied elements, namely As was completely eluted in the LMW range of chromatograms (Figure 8) meaning LMW proteins were of more significance for As binding

than the HMW proteins. Furthermore, spatial differences in the chromatograms clearly showed the influence of the physico-chemical behaviour of As in the coastal systems. Based on the spatial and seasonal differences of peak areas in As chromatogram (Figure 9), even for the samples that had closely equal concentration of total cytosolic As (Table 3) we assume the overall environmental conditions including speciation of As related to the salinity played an important role for As association to biomolecules. This is furthermore supported by the peak 3 area being always the lowest in the samples from R location and the largest in the samples from H location regardless of the seasonal variability (Figure 9) and of differences in total cytosolic As concentration (Table 3). However, we assume that biomolecules of this range were not important for As detoxification because their elution area negatively correlated with total cytosolic As in both seasons (March r = -0.78, August r = -0.74) at significant level. Since in March location H had very low salinity which influenced As uptake leading to exceptionally low cytosolic As concentration (Table 3) this could have caused the observed differences in cytosolic distribution and correlation parameters in these two seasons making it hard to find common link between As abundance in specific peak areas and locations. The link between As and stress proteins has been mostly identified for HMW stress proteins, but also for proteins of lower molecular weights such as MT (6-7 kDa) and ubiquitin (7-8 kDa) in different organisms (Del Razo et al., 2001). Unfortunately, based on the fact that the column we used has a separation range 3 kDa – 70 kDa we could not characterize a specific binding molecular weight range of the peaks in LMW range. In order to obtain better resolution in LMW range, the application of the size exclusion column that separates peptides and proteins of LMW would be the first choice for more detailed study of As binding biomolecules in *M. galloprovincialis*. The strong seasonal and spatial variability both were present regardless of the As cytosol concentration so more detailed study that would include more locations should be performed.

In conclusion, the study of the cytosolic distribution pattern and association to biomolecules of the priority pollutant metals (Cu, Cd, Zn, Pb) and As in the mussel's digestive gland from

two seasons and three locations of different environmental pollution levels confirmed cytosolic distribution as a potential indicator of metal exposure. Special accent should be put on the seasonal variability because it affected not only the total cytosolic metal and metalloid concentration, but also the element abundance within specific peaks of the cytosol separated by SE-HPLC. However, it should be emphasized the study is performed in two seasons only and the whole year seasonal cycle study would be encouraged. Further studies should include chromatographic matrices that can enable specific separation of both larger and smaller proteins and peptides. A clear identification and characterization of possible binding biomolecules within different fractions using complementary molecule - specific detection techniques such as electrospray ionization (ESI) or matrix-assisted laser desorption (MALDI) mass spectrometry would also be of great interest. The obtained findings provide guidelines for the specific fraction separation for precise characterization of metal binding biomolecules.

Acknowledgements

The financial support by the Ministry of Science, Education and Sport of Republic Croatia (project no. 098-0982934-2721) and Deutscher Akademischer Austausch Dienst (DAAD, project no. 50752021) are acknowledged. We wish to thank to the anonymous reviewers whose useful suggestions improved the quality of this paper.

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Figure captions

Figure 1

Chromatograms of Cu, Cd, Zn, Pb and As in samples from the location M as an example for defining molecular weight ranges (HMW – high molecular weight, MMW – medium molecular weight and LMW – low molecular weight).

Figure 2

Chromatograms of Cu, Cd and Zn cytosolic distribution in samples from the different investigated locations sampled in March. To ensure clear image for each element only one of three analyzed chromatograms per location is presented. Peaks selected for area calculations are marked within the chromatograms.

Figure 3

Peak areas of cytosolic Cu distribution from three sampling locations analyzed in two seasons (mean ± SD, n = 3). Different letters above bars indicate significant differences ($p < 0.05$) between locations investigated during the same season. Significant seasonal differences ($p < 0.05$) of a particular location are marked with the asterisk (*) inside the bars.

Figure 4

Peak areas of cytosolic Cd distribution from three sampling locations analyzed in two seasons (mean ± SD, n = 3). Different letters above bars indicate significant differences ($p < 0.05$) between locations investigated during the same season. Significant seasonal differences ($p < 0.05$) of a particular location are marked with the asterisk (*) inside the bars.

Figure 5

Peak areas of cytosolic Zn distribution from three sampling locations analyzed in two seasons (mean ± SD, n = 3). Different letters above bars indicate significant differences (p <

0.05) between locations investigated during the same season. Significant seasonal differences ($p < 0.05$) of a particular location are marked with the asterisk (*) inside the bars.

Figure 6

Chromatograms of Pb cytosolic distribution in samples from the different investigated locations in August. To ensure clear image only one of three analyzed chromatograms per location is presented. Peaks selected for area calculations are marked within the chromatograms.

Figure 7

Peak areas of cytosolic Pb distribution in samples from three sampling locations analyzed in two seasons (mean ± SD, n = 3). Different letters above bars indicate significant differences ($p < 0.05$) between locations investigated during the same season. Significant seasonal differences ($p < 0.05$) of a particular location are marked with the asterisk (*) inside the bars.

Figure 8

Chromatograms of As cytosolic distribution in samples from the different investigated locations sampled in March. To ensure clear image only one of three analyzed chromatograms per location is presented. Peaks selected for area calculations are marked within the chromatograms.

Figure 9

Peak areas of cytosolic As distribution in samples from three sampling locations analyzed in two seasons (mean ± SD, n = 3). Different letters above bars indicate significant differences ($p < 0.05$) between locations investigated during the same season. Significant seasonal differences ($p < 0.05$) of particular location are marked with the asterisk (*) inside the bars.

Table

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Table 1. Instrumental parameters for SE-HPLC-ICP-MS measurements used for determination of cytosolic metal and metalloid distribution in the digestive gland of mussel.

HPLC Agilent 1100	
SEC column	Tricorn™ Superdex 75 HR 10/300
precolumn	Security Guard Cartridges GCF 3000 4 x 3.0 mm
mobile phase	20 mM Tris, pH 7.4 (HNO_3), 20 µg/L Ge
injection volume	50 µL
flow rate	0.3 mL/min
ICP-MS Agilent 7500cx	
RF power	1600 W
carrier gas	0.91 L/min
makeup gas	0.3 L/min
extraction lens 1	5 V
extraction lens 2	-180 V
octopole bias	-19.6 V
quadrupole bias	-17.6 V
cell gas	4 mL/min He
integration time	0.1 s
measured isotope	^{63}Cu, ^{114}Cd, ^{66}Zn, ^{208}Pb and ^{75}As

Table 2. Seawater concentration of priority pollutant metals in investigated locations in two sampling seasons (measured values and 95% confidence interval (CI) for the method of standard addition with 5 analyzed points are presented).

Location/ season	METAL CONCENTRATION			
	Cu (ng/L)	Cd (ng/L)	Zn (µg/L)	Pb (ng/L)
March				
R	240 ± 24	8.7 ± 1.4	0.68 ± 0.04	10.5 ± 5.4
M	1291 ± 59	7.3 ± 0.2	1.89 ± 0.14	13.7 ± 1.6
H	484 ± 16	9.7 ± 0.7	2.60 ± 0.15	8.8 ± 1.5
August				
R	443 ± 67	8.8 ± 0.3	1.21 ± 0.30	41.4 ± 4.6
M	1605 ± 14	9.6 ± 1.1	2.46 ± 0.34	43.6 ± 3.7
H	2270 ± 235	17.9 ± 2.0	3.21 ± 0.42	37.3 ± 9.6

Table 3. Total cytosolic concentration of priority pollutant metals and metalloid As in digestive gland cytosol (mean ± SD, n = 3). Different letters (a, b, c) in superscript indicate significant differences ($p < 0.05$) between locations in the same season. The asterisk (*) indicates the significant seasonal difference ($p < 0.05$) at the particular location.

Location/ season	METAL CONCENTRATION				
	Cu (µg/L S50)	**Cd (µg/L S50)**	**Zn (µg/L S50)**	**Pb (µg/L S50)**	**As (mg/L S50)**
March					
R	450.87 ± 25.57 ^{a}	51.66 ± 20.46 ^{a}	708.57 ± 37.77 ^{a}	24.75 ± 0.01 ^{a,b}	1.42 ± 0.05 ^{a}
M	1431.21 ± 270.02 ^{b}	84.53 ± 18.33 ^{a}	982.38 ± 80.11 ^{b}	26.54 ± 3.46 ^{b}	2.03 ± 0.14 ^{b}
H	365.15 ± 5.08 ^{a}	87.87 ± 0.25 ^{a}	937.93 ± 117.35 ^{b}	20.57 ± 0.28 ^{a}	0.15 ± 0.01 ^{c}
August					
R	241.18 ± 9.13 ^{a,*}	18.39 ± 2.42 ^{a,*}	480.24 ± 9.59 ^{a,*}	23.91 ± 1.97 ^{a}	1.96 ± 0.16 ^{a,*}
M	406.53 ± 48.76 ^{b,*}	61.55 ± 6.77 ^{b}	837.59 ± 146.07 ^{b}	23.99 ± 3.05 ^{a}	1.80 ± 0.21 ^{a}
H	258.91 ± 42.50 ^{a,*}	67.47 ± 1.28 ^{b,*}	1073.12 ± 112.87 ^{b}	42.72 ± 8.93 ^{b,*}	0.65 ± 0.11 ^{b,*}

Table 4. Pearson correlation coefficients (r) between cytosolic Cu, Cd and Zn concentrations and peak areas of MT associated peaks of each metal (CuMT, CdMT and ZnMT). For each season, data of three pools from all three studied locations were grouped (n = 9). Bolded values indicate significant correlation of $p < 0.05$.

	March						August					
	Cu	CuMT	Cd	CdMT	Zn	ZnMT	Cu	CuMT	Cd	CdMT	Zn	ZnMT
Cu	1.00						1.00					
CuMT	0.65	1.00					0.57	1.00				
Cd	0.38	**0.91**	1.00				0.41	**0.94**	1.00			
CdMT	-0.22	0.56	**0.79**	1.00			0.29	**0.95**	**0.93**	1.00		
Zn	0.51	**0.85**	**0.80**	0.60	1.00		0.29	**0.90**	**0.86**	**0.93**	1.00	
ZnMT	-0.33	0.46	**0.68**	**0.98**	0.52	1.00	**0.73**	0.59	0.49	0.43	0.18	1.00

Figure1

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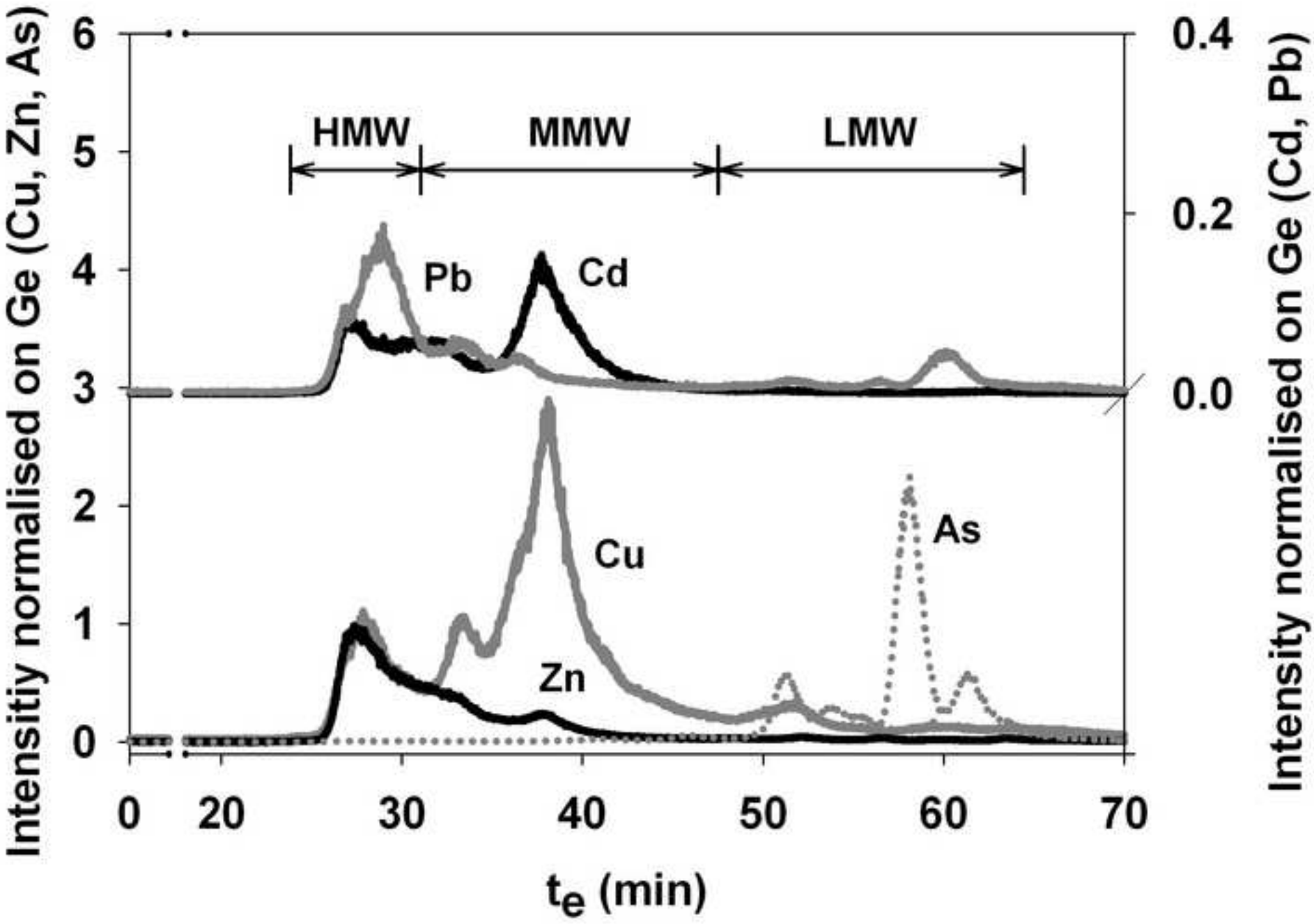

Figure2

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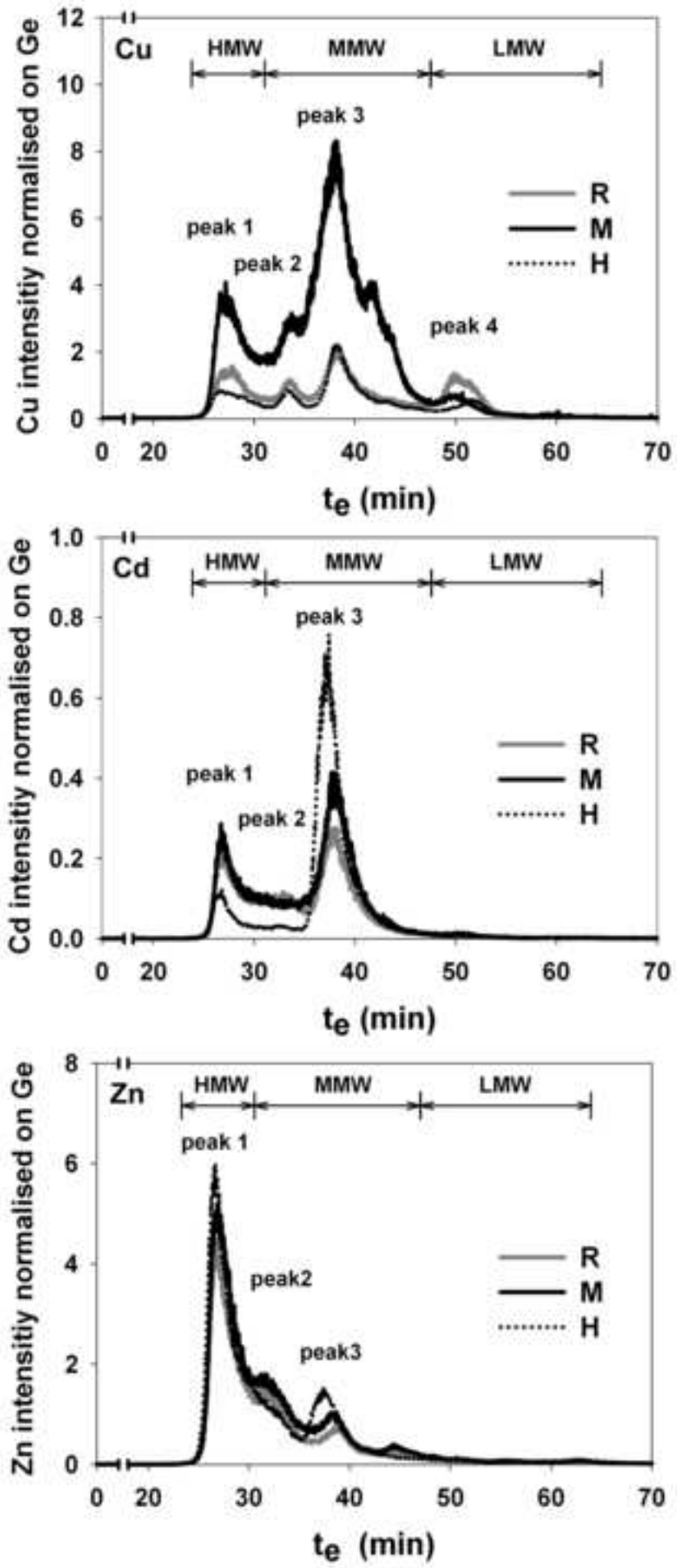

Figure3

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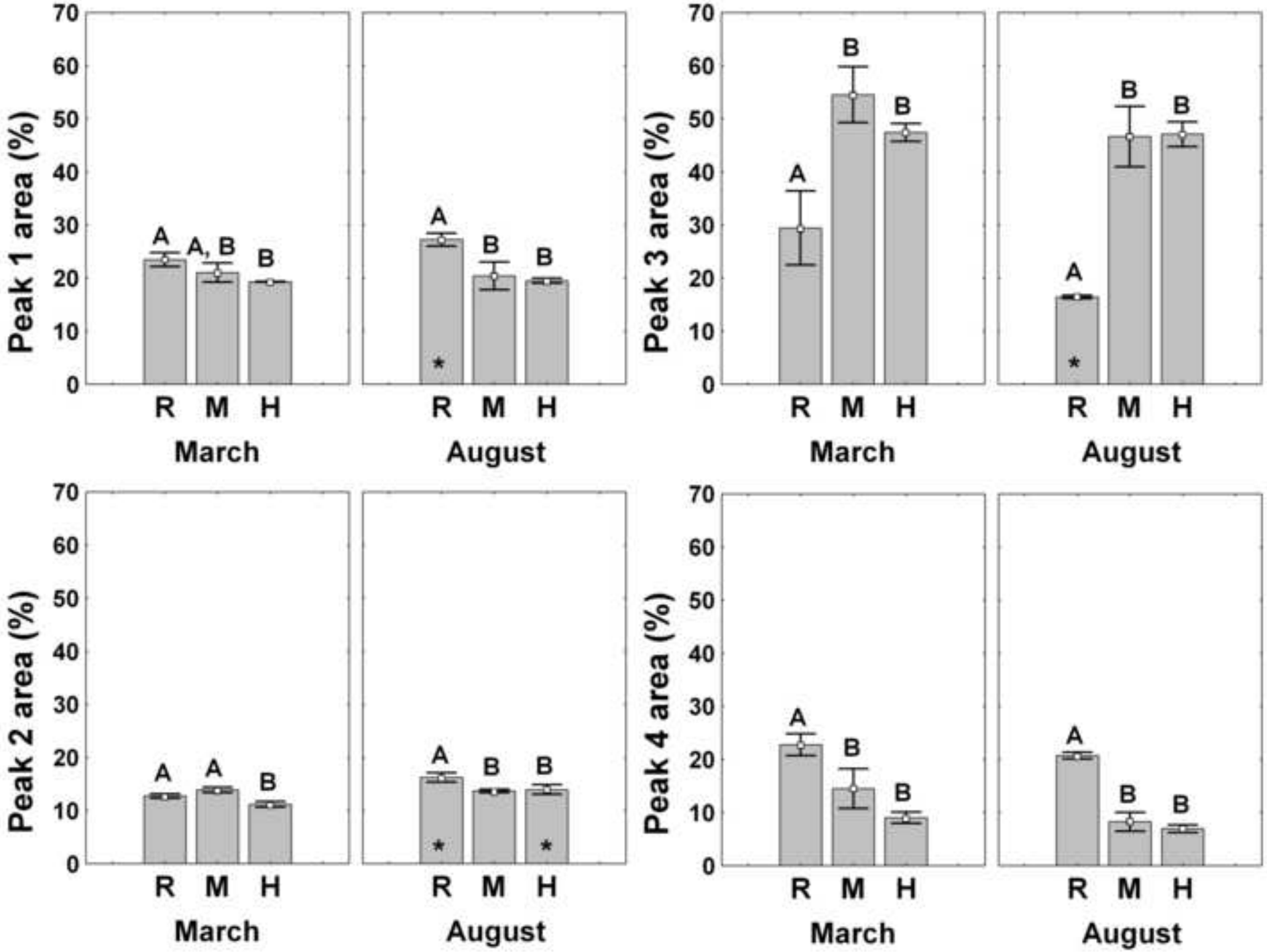

Figure4

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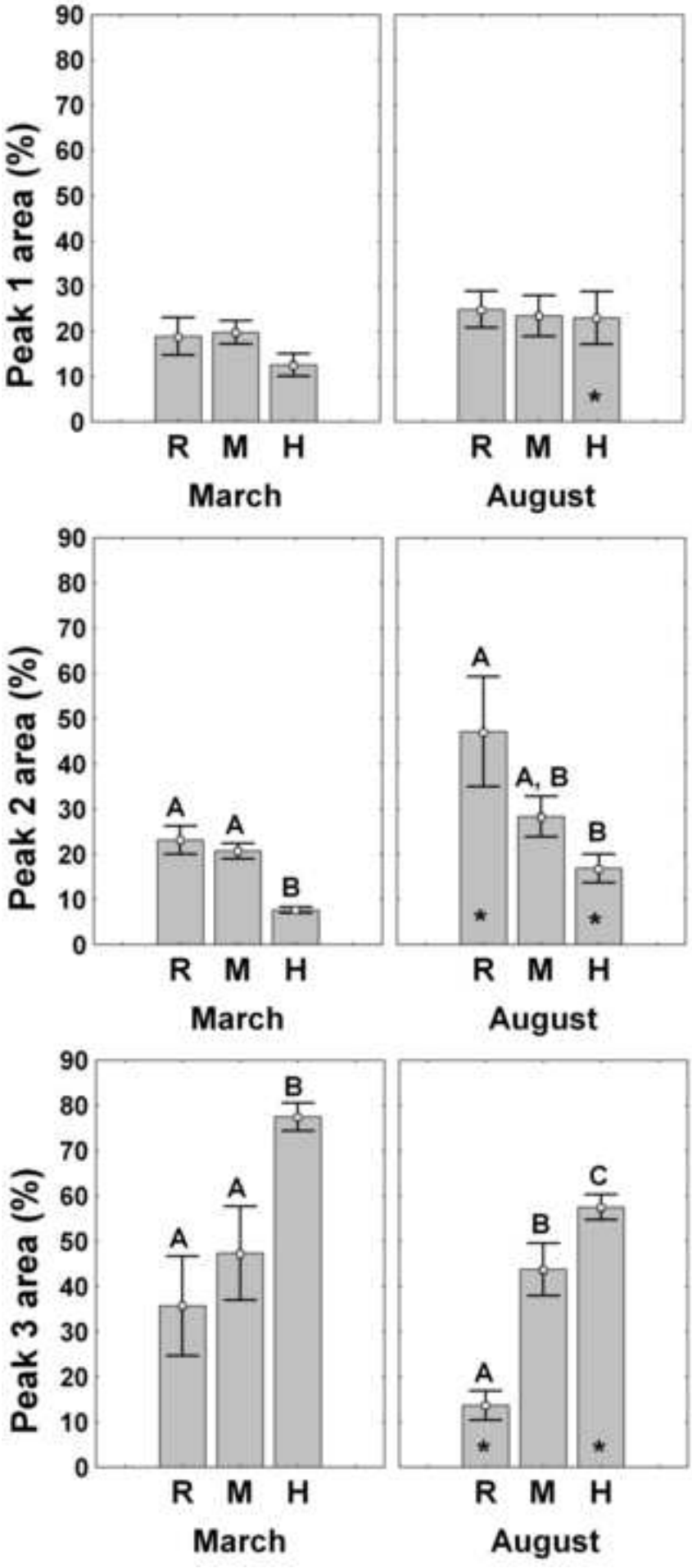

Figure5

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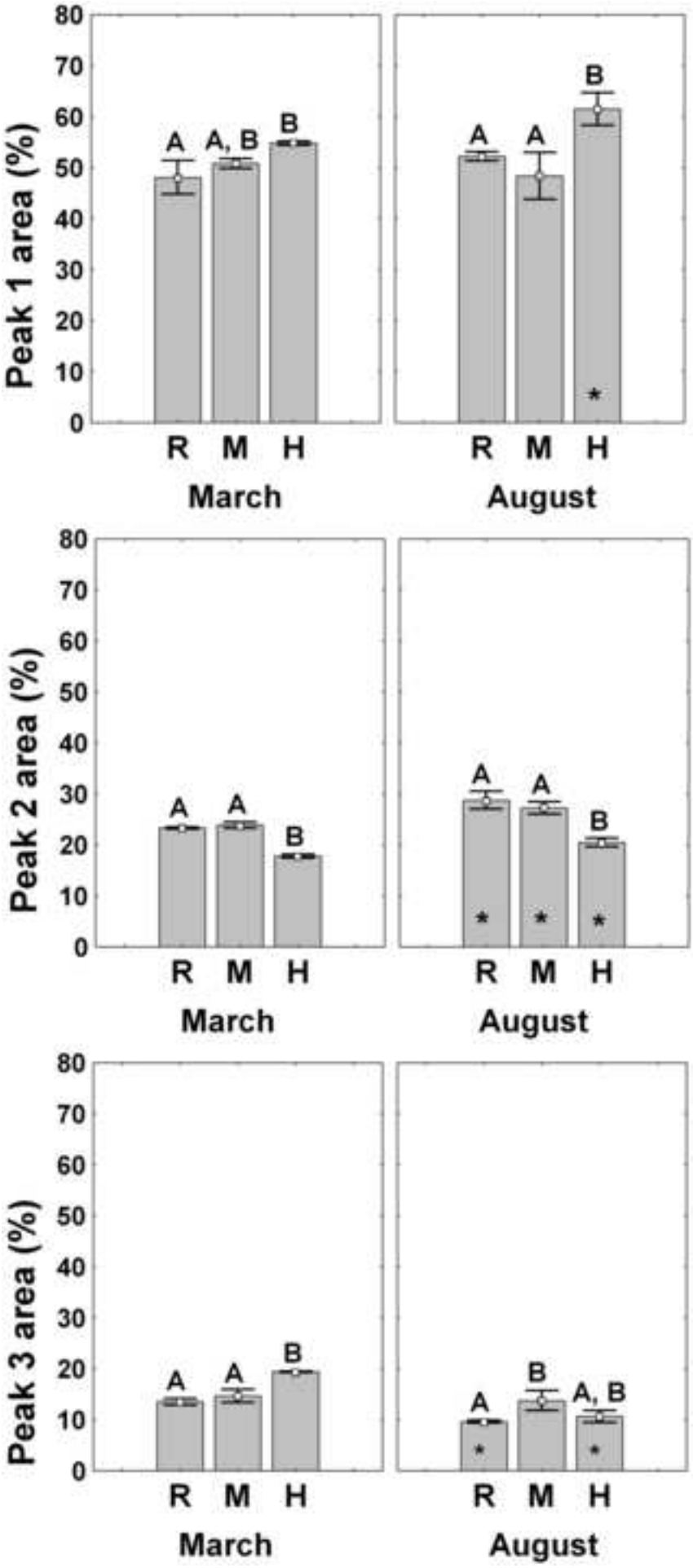

Figure6

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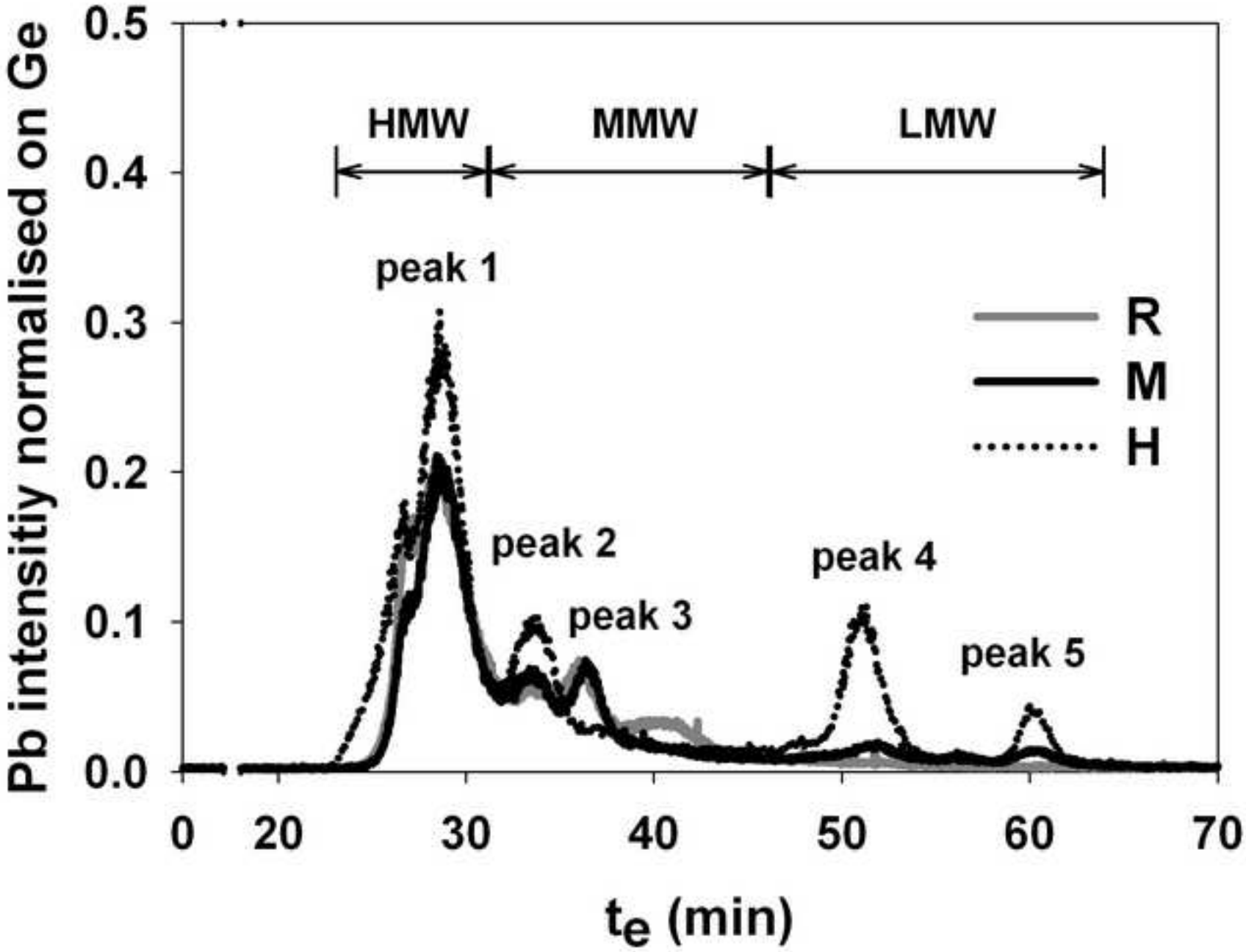

Figure7

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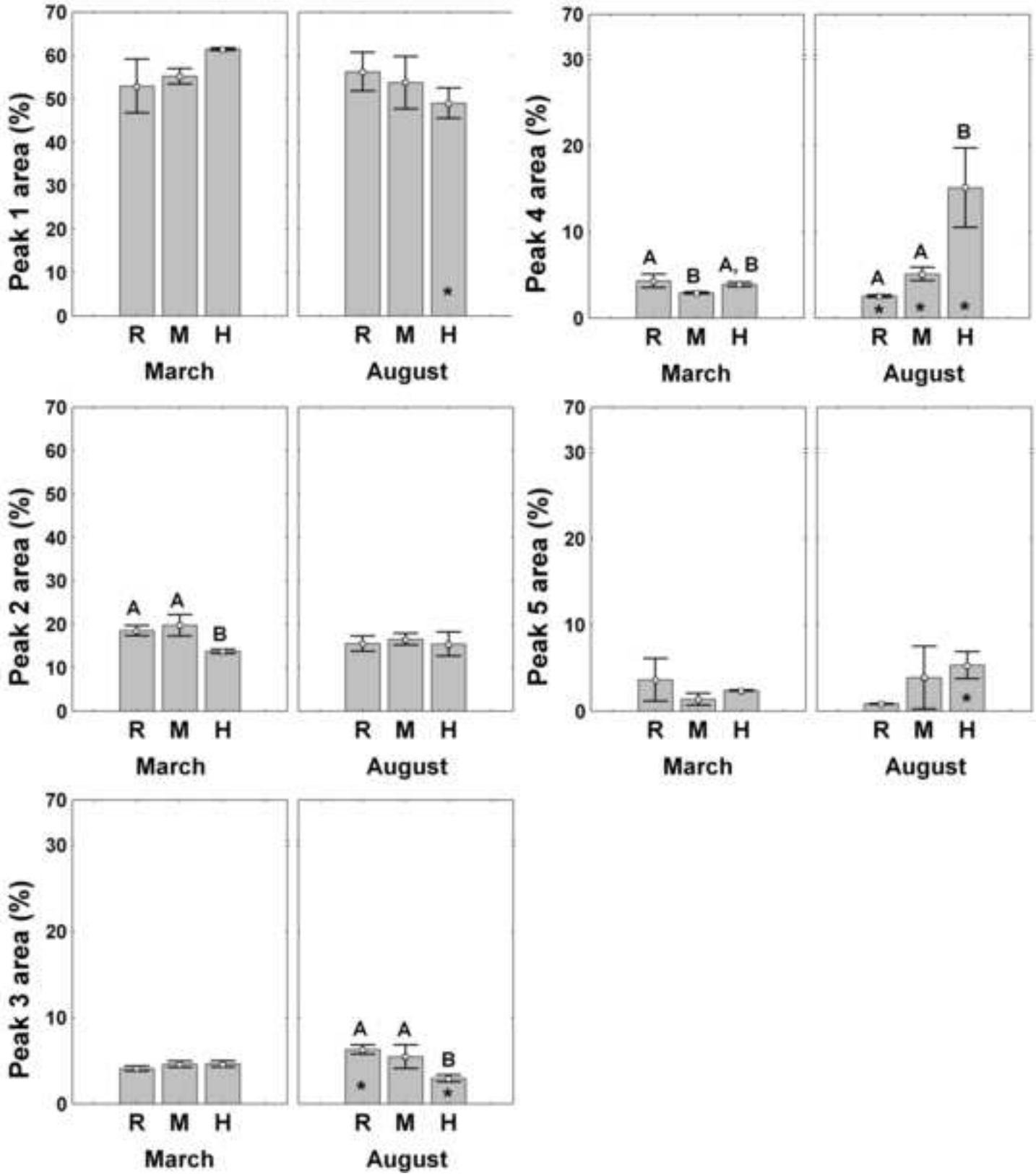

Figure8

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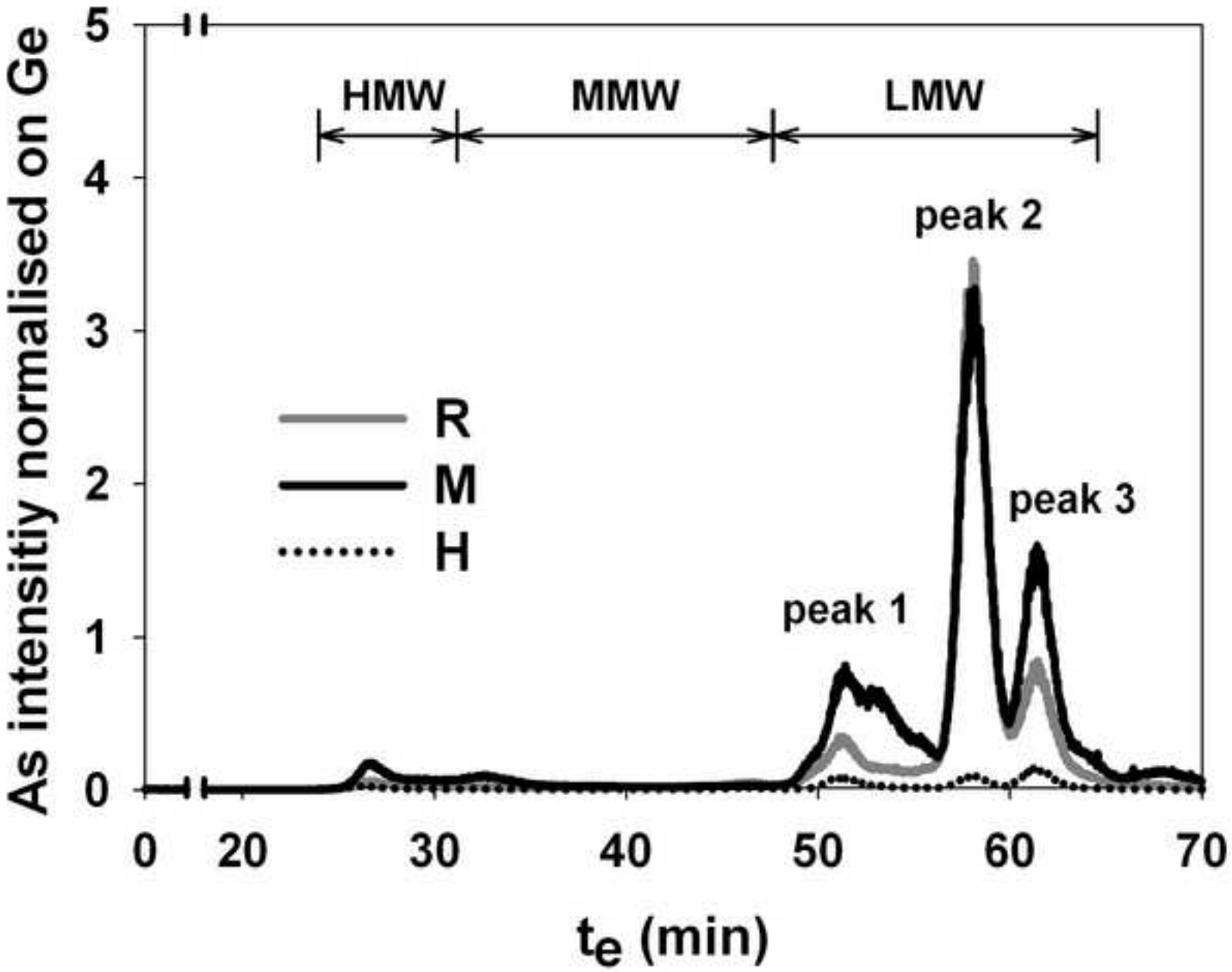

Figure9

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