**Croatian Science Foundation**

**Project: Accumulation, Subcellular Mapping and Effects of Trace Metals in Aquatic Organisms (AQUAMAPMET)**

# Wrap Up Meeting

## Bioaccumulation of metals, biomarker responses and the condition of caged freshwater mussels exposed to the effluent from a fertilizer factory in the Ilova River

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# OBJECTIVES

- to determine total and cytosolic concentrations of metal/metalloids in the digestive gland of freshwater mussels exposed in cages to effluents from a fertilizer factory, which are discharged into the Ilova River through the Kutinica wastewater channel

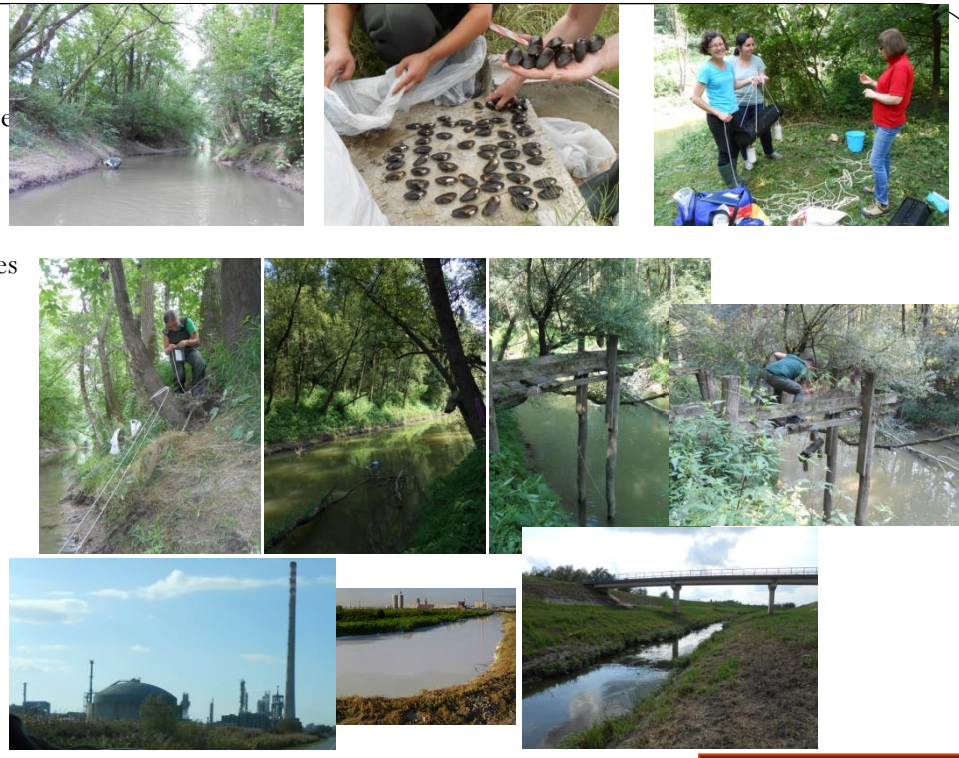
- to investigate the relationship between metals accumulated in the digestive gland and concentrations of dissolved metals in water,

- to assess the potential adverse impact of contamination on cage-exposed bivalves by determining a set of biomarkers of exposure and effects

# STUDY AREA, WATER SAMPLING, CAGE EXPOSURE OF MUSSELS

## MUSSEL CAGE EXPOSURE

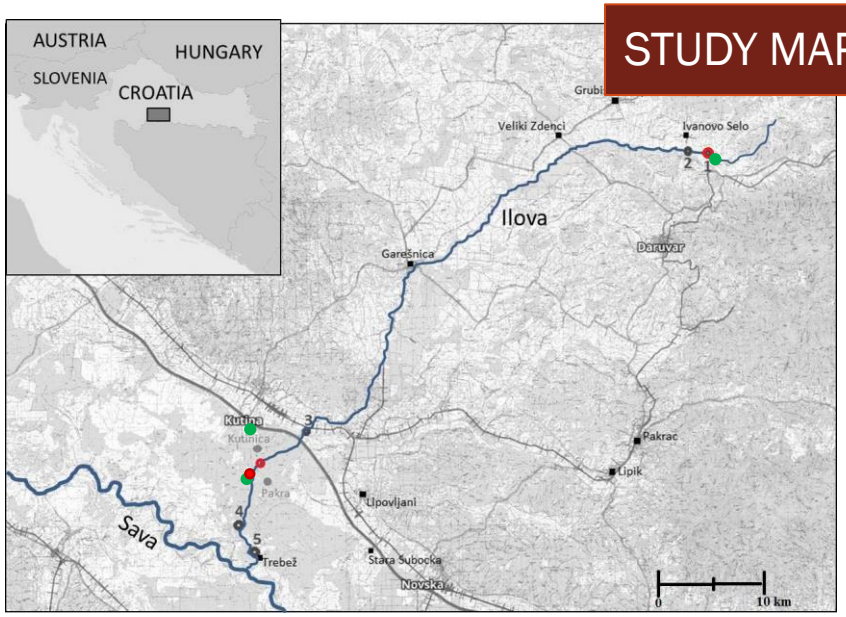
- Bioindicator organisms: mussels *Unio crassus* (Thick shelled river musse
- Mussels were collected by hand at the Ilova-MAS site, 20 mussels per cage were used
- The cages were deployed in the Ilova River at three location (red circles at the map):
  - **Ilova-MAS** (reference site)
  - **Ilova-LOP** (less exposed site )
  - **Ilova-near KUT** (most exposed site)
- Mussels were exposed for 40 days (July 12, 2018 - August 22, 2018)
- After exposure, mussels were purified for 24 hours, biometric measurements were performed, digestive gland dissected and tissue stored in liquid nitrogen.



## WATER SAMPLING

- Water samples for metal analyzes were taken at the start and the end of mussel cage exposure at three locations (green circles at the map):
  - **Ilova-Maslenjača** (correspond to the Ilova-MAS site),
  - **Ilova-Lonjsko polje** (correspond to the Ilova-LOP site)
  - **Kutinica** (a location in the Kutinica wastewater channel)
- Samples were taken in acid cleaned plastic bottle, immediately filtered through 45 µm pore diameter cellulose acetate filter to obtain dissolved metal fraction and acidified with nitric acid

## STUDY MAP



# METHODS

## ANALYSIS IN WATER SAMPLES

- Concentrations of dissolved metals/metalloids
- 26 trace elements and 4 macroelements
- Measurement was performed on HR ICP-MS

## ANALYSIS IN DIGESTIVE GLAND

- Concentrations of total metals/metalloids in tissue homogenate
- Concentrations of cytosolic metals/metalloids in S50 fraction
  - Measurement was performed on HR ICP-MS
- Analysis of biomarkers
  - Metallothioneins were measured in S50 tissue fraction (cytosol)
  - MDA concentration was determined in S3 fraction
  - Activities of CAT and of AChE were determined in S10 fraction

## SAMPLE PREPARATION

TISSUE HOMOGENISATION

digestive gland : buffer = 1:4

TISSUE HOMOGENATE

- digestion using concentrated nitric acid and 30% hydrogen peroxide
- determination of total tissue metal/metalloid concentrations on HR ICP-MS

CENTRIFUGATION

50000xg, 2 h, 4 ºC

S50 FRACTION (CYTOSOL)

- digestion using concentrated nitric acid and 30% hydrogen peroxide
- determination of cytosolic metal/metalloid concentrations on HR ICP-MS

# Basic physico-chemical water parameters

| Site no. | Location | $O_2$ (mg $L^{-1}$) | $O_2$ % | pH | Conductivity (µS $cm^{-1}$) | $COD_{KMnO4}$ (mg $O_2$ $L^{-1}$) | N-$NO_3^-$ (mg $L^{-1}$) | P-$PO_4^{3-}$ (mg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | Maslenjača | 8.2 | 89 | 8.18 | 319 | 4.2 | 1.71 | 0.154 |
| 2 | Ilova-LOP | 7.5 | 88 | 8.01 | 425 | 5.14 | 2.28 | 0.27 |
| 3 | Kutinica | 13.28 | 166 | 8.60 | 630 | 1.25 | 2.91 | 0.503 |

| Site no. | Location | $O_2$ (mg $L^{-1}$) | $O_2$ % | pH | Conductivity (µS $cm^{-1}$) | $COD_{KMnO4}$ (mg $O_2$ $L^{-1}$) | N-$NO_3^-$ (mg $L^{-1}$) | P-$PO_4^{3-}$ (mg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1 | Maslenjača | 7.28 | 83.5 | 8.15 | 361 | 3.15 | 1.60 | 0.219 |
| 2 | Ilova-LOP | 4.10 | 48.6 | 7.83 | 660 | 6.07 | 5.6 | 0.275 |
| 3 | Kutinica | 3.86 | 49.4 | 7.95 | 483 | 6.46 | 2.14-11.688 | 0.560 |

Reference site Maslenjača – good water quality

Limiting values (HR-R_4):
pH 7.0 – 9.0
COD ≤ 5.5 mg $O_2$ $L^{-1}$
N-$NO_3^-$ ≤ 1.3 mg N $L^{-1}$
P-$PO_4^{3-}$ ≤ 0.1 mg P $L^{-1}$

Directive on Water Quality Standard. Official Gazette No.96(2019), Zagreb.

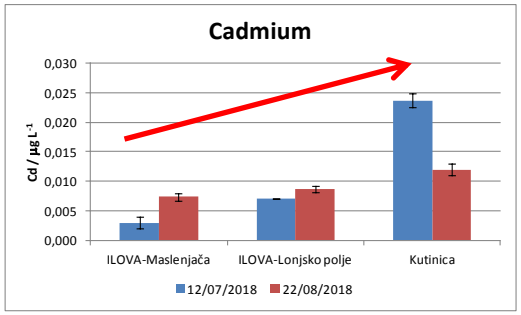
# RESULTS ON CONCENTRATIONS OF DISSOLVED METAL IN WATER

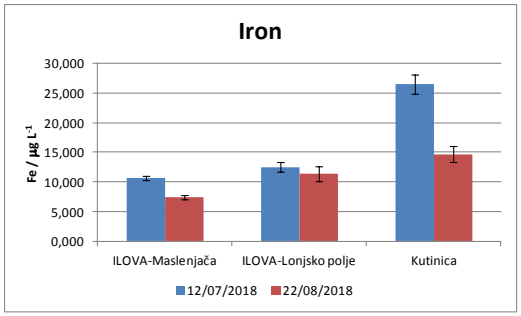
# Results: Concentrations of dissolved trace elements in water

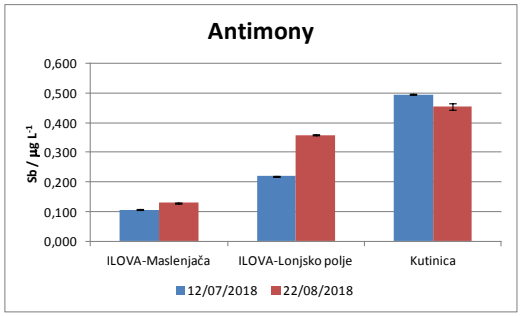
- < LOD:
  - Bi, Sn, Ag, Pb, Zn, Ti and Cr
- < 25 ng/L:
  - Cs, Cd and Tl
- hundreds ng/L – several µg/L:
  - Li, Rb, Mo, Sb, Ni, Co, Cu, Se and As
- Several tens µg/L – several hundred µg/L:
  - Al, V, Mn, Fe, Sr and Ba

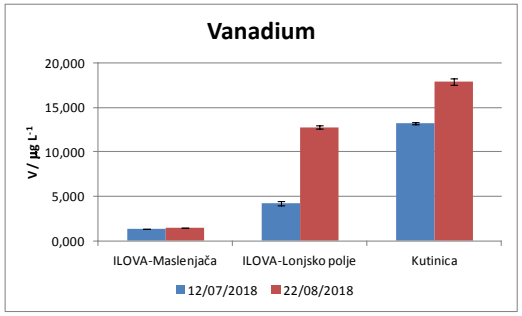
# Results: Concentrations of dissolved trace elements in water

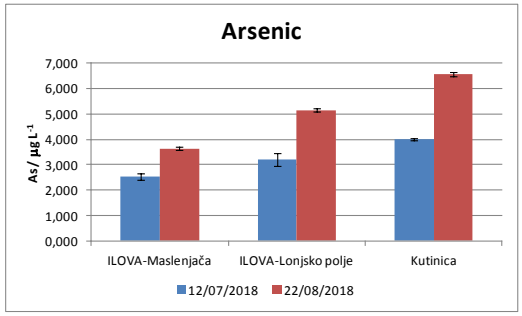
1. Elements showing concentration increase from reference to exposed sites in both samplings (Ilova-Maslenjača < Ilova-Lonjsko polje < Kutinica)

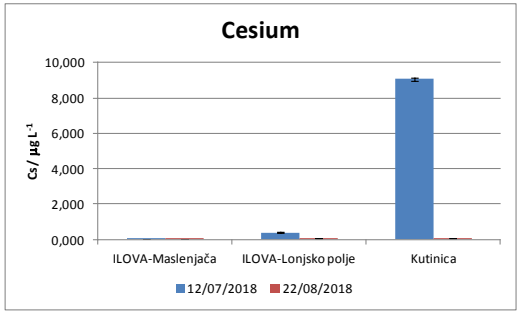


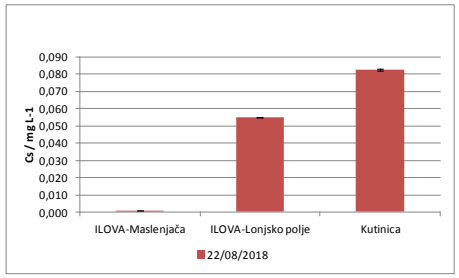



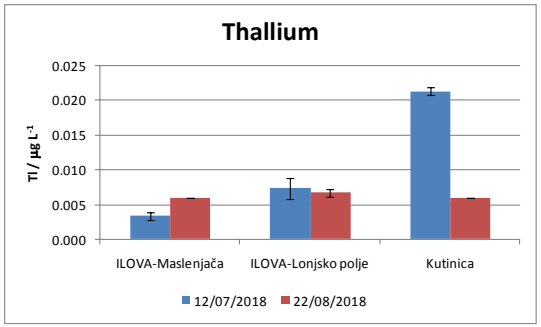



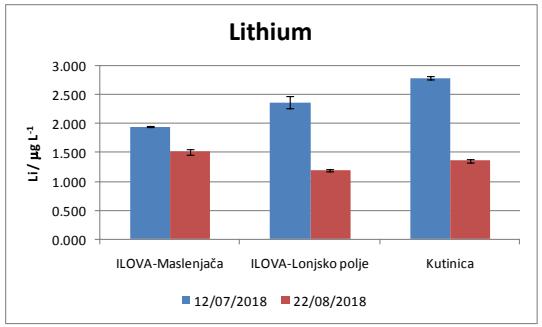



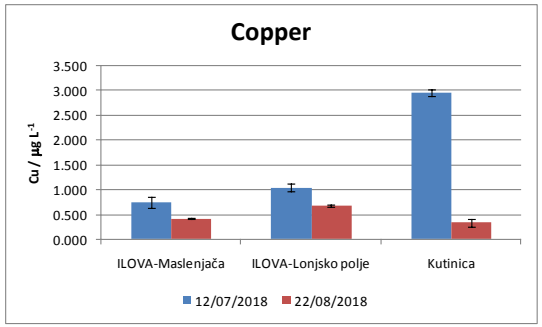

- Similar concentration gradient observed also for Al, Rb and Mo
- significant variations in concentrations between two samplings at the same location

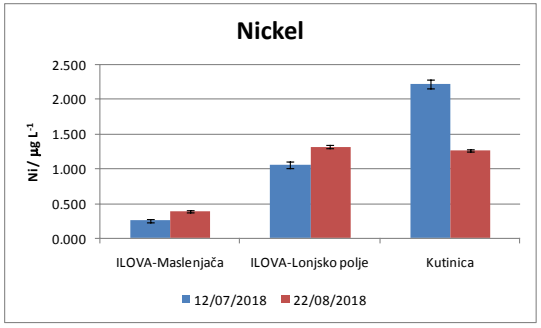
# Results: Concentrations of dissolved trace elements in water

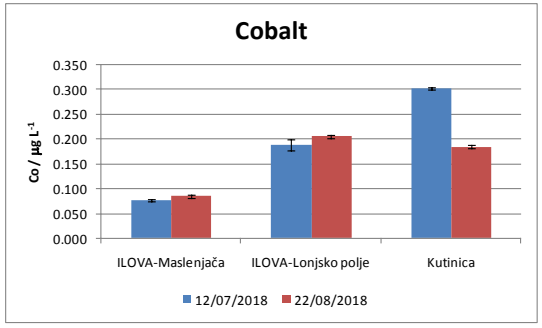
2. Elements showing clear concentration gradient from reference site to exposed sites in the first sampling



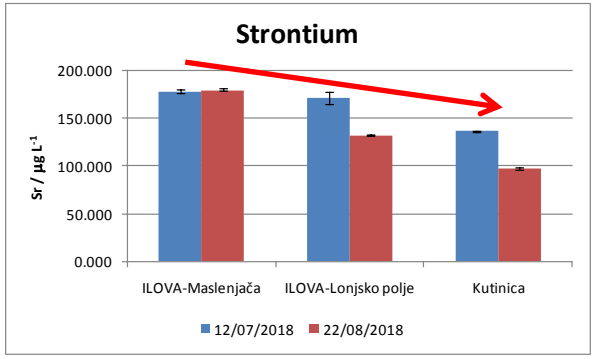



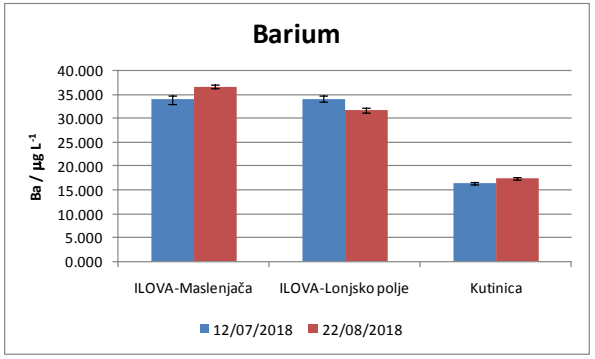



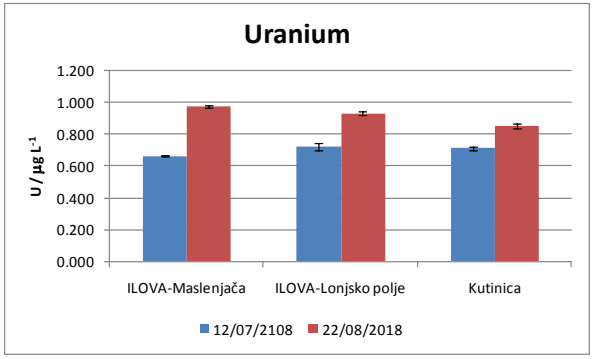

- At the same location, significant variations in concentrations between two samplings were observed
- In the second sampling, Tl, Li and Cu had even lower concentrations at exposed locations than in reference location

# Results: Concentrations of dissolved trace elements in water

## 3. Elements showing higher concentrations at the reference site

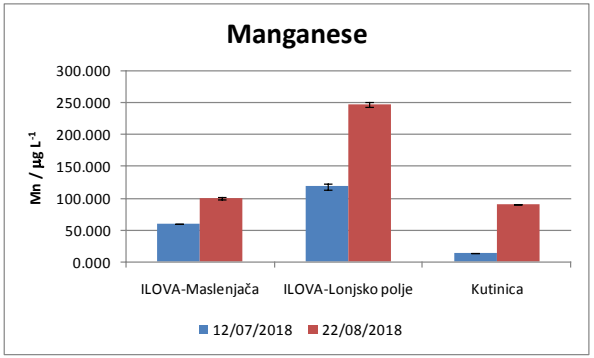


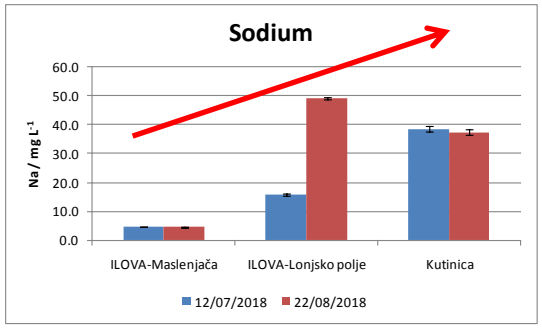

- Strontium and barium had the highest concentrations at the reference site, while uranium was present at similar concentrations at all three sites or had slightly higher values at reference site

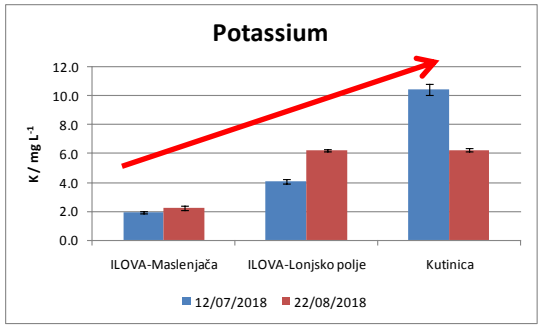
## 4. Element showing the highest concentration at the site Ilova-Lonjsko polje in both samplings

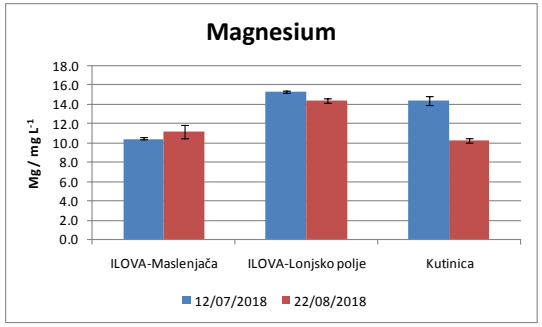


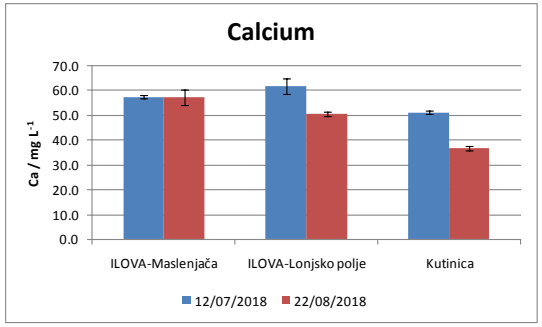

- significant variations in concentrations between two samplings

# Results: Concentrations of dissolved macroelements in water



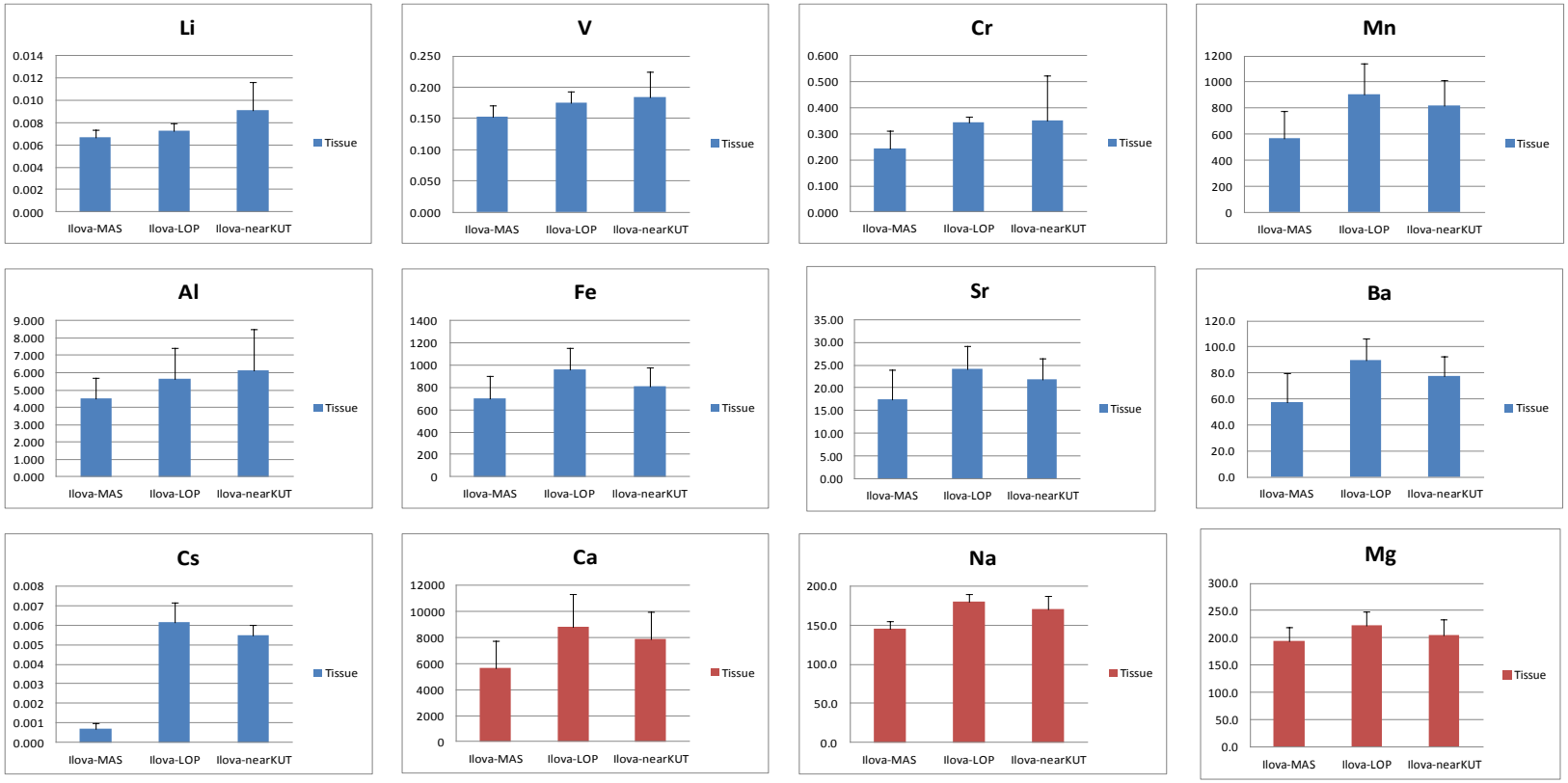




- mg/L concentrations
- K<Mg<Na<Ca
- Na, K (both samplings) and Mg (the first sampling) showed significant higher concentrations at both polluted locations

Metal concentrations in digestive gland of caged exposed mussels *Unio crassus*

# Results: Total concentrations of elements in digestive gland

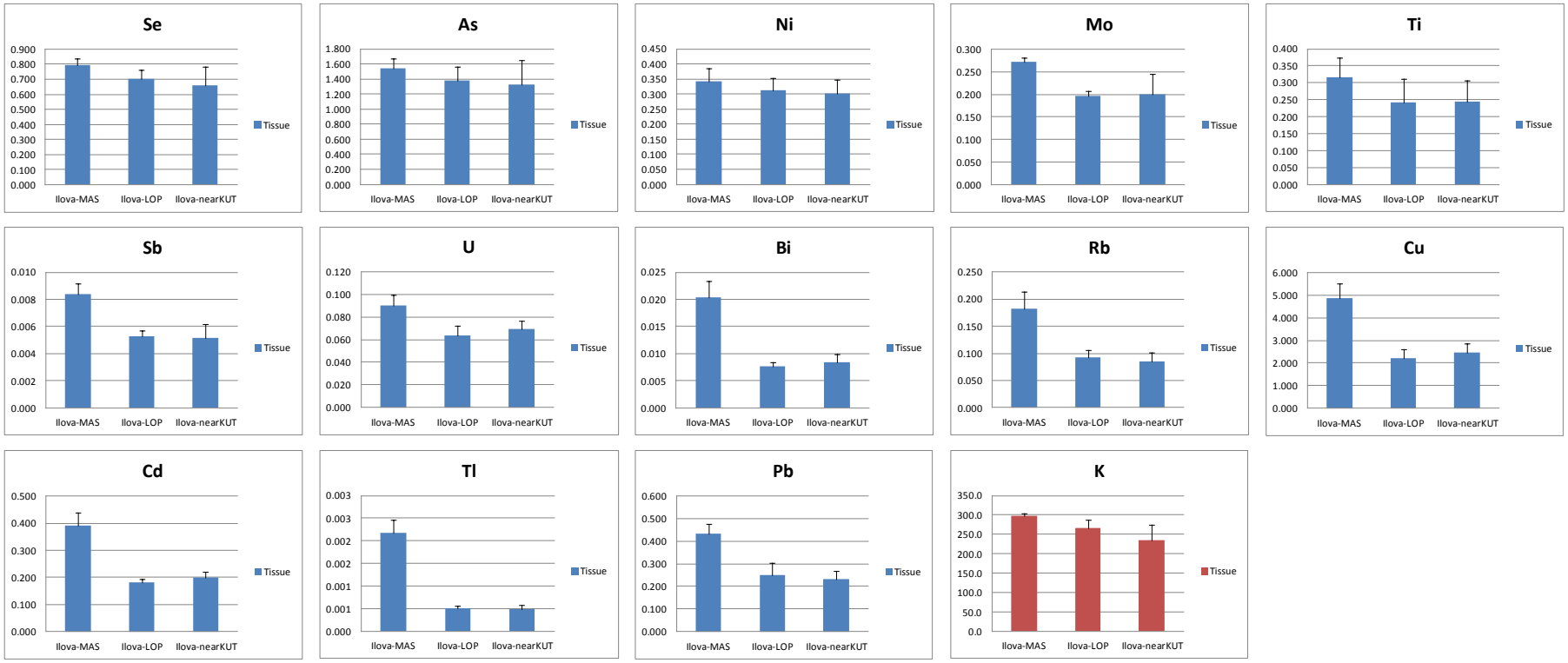
1. Elements whose total concentrations in digestive gland were higher in mussels at exposed locations




- Most of these elements follow the concentration trend observed in water
- Except for Cs, the increase was relatively low, generally ranging between 10and 50%
- Most of these trace elements belong to the group of elements that are predominantly bound to suspended matter particles - The elevation could indicate the presence of suspended matter particles due to incomplete purification of digestive gland

# Results: Total concentrations of elements in digestive gland

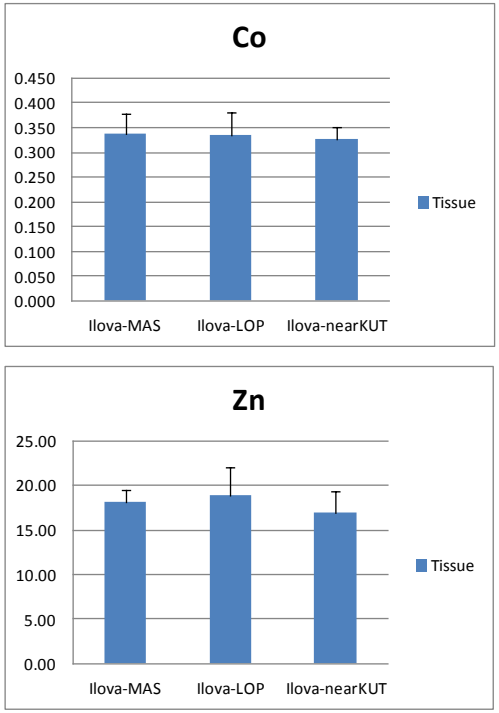
2. Elements whose total concentrations in digestive gland were higher in mussels at reference location




- Concentrations of Se, As, Ni, and macroelement K were slightly increased (10–25%) relative to the exposed sites
- An increase of 30-60% at the reference site for Mo, Ti, Sb and U was observed
- Differences between reference and exposed sites were most pronounced in the case of Bi, Rb, Cu, Cd, Tl, and Pb

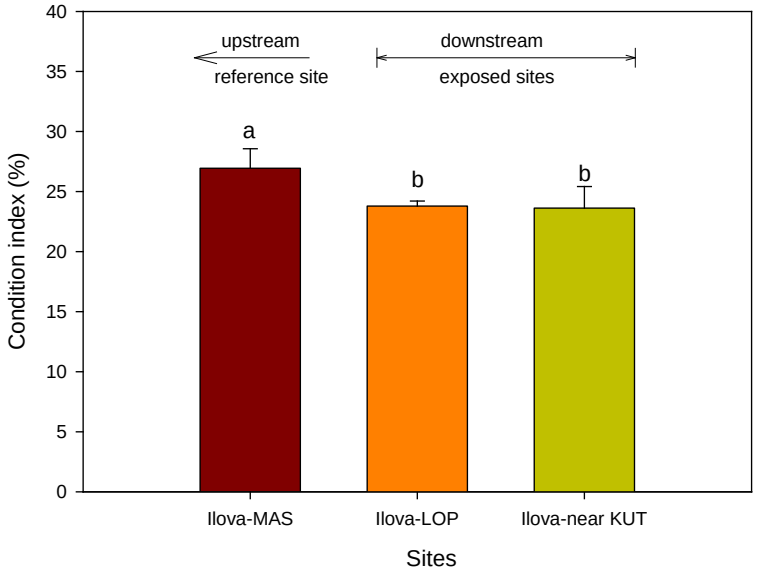
# Results: Total concentrations of elements in digestive gland

2. Elements whose total concentrations in digestive gland were comparable at all locations




- Essential elements
- Mussels can to some extent regulate the intake of certain essential elements

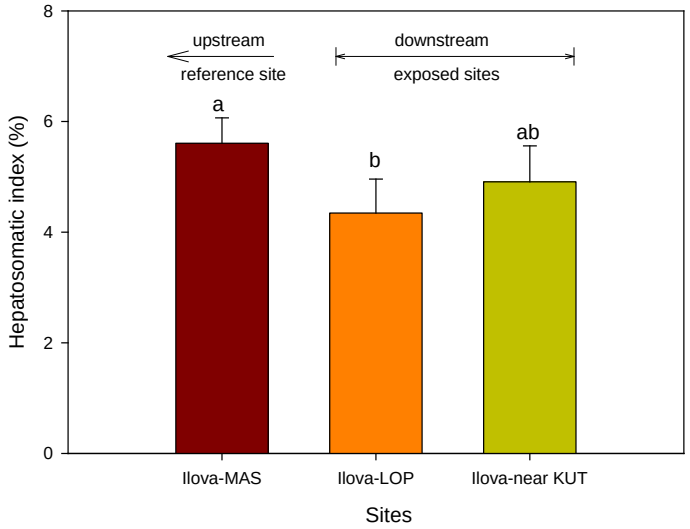
# CONDITION INDEX



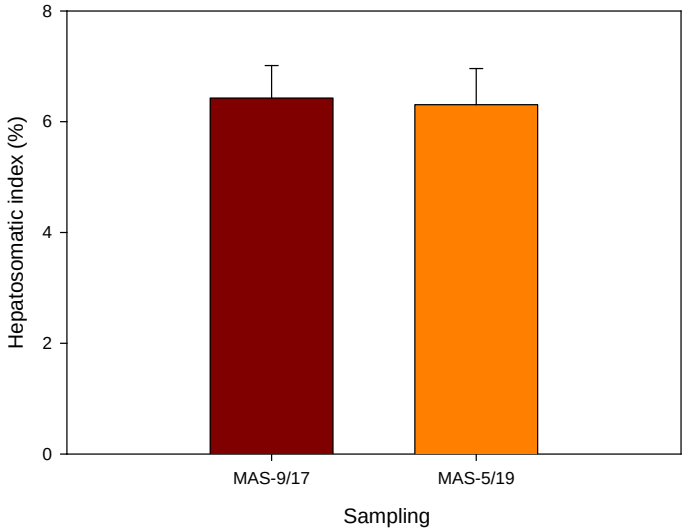

- an indicator of the physiological status of the mussels
- depends mainly on the reproductive cycle of bivalves, but also on food availability
- significant decrease of condition index in both exposed locations relative to the reference location

# HEPATOSOMATIC INDEX

Caged mussles *Unio crassus*




Resident *Unio crassus* at Ilova-Maslenjača



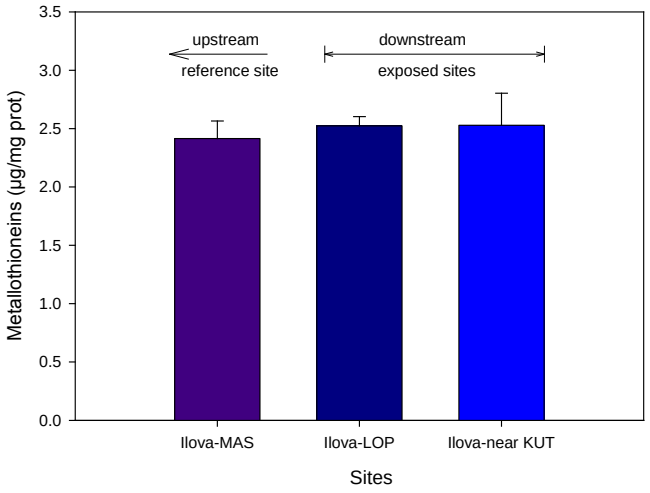

- Hepatosomatic index represents the proportion of digestive gland mass in total mass of soft tissue
- Its values may vary depending on food availability as well as on metabolic processes prevailing in digestive gland
- decrease of hepatosomatic index at exposed locations relative to the reference location
- decrease of hepatosomatic index in caged mussels relative to the resident mussels

# BIOMARKER RESPONSES

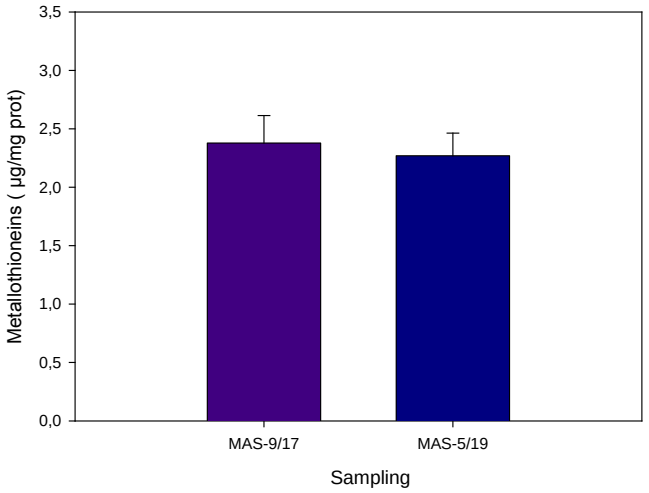
- Metallothioneins (MT)
  - Biomarker of metal exposure
- Catalase activity (CAT)
  - Biomarker of antioxidant capacity
- Acethylcholinesterase activity (AChE)
  - Biomarker of neurotoxicity
- Malondialdehyde concentration (MDA)
  - Biomarker of oxidative stress

# METALLOTHIONEINS

Caged mussels *Unio crassus*



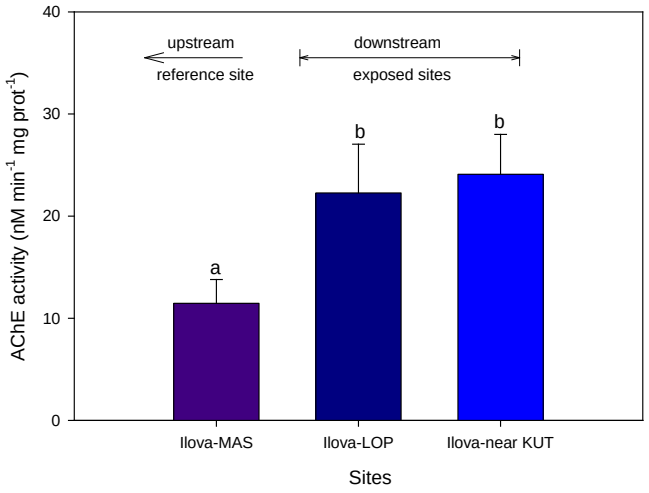

Resident *Unio crassus* at Ilova-Maslenjača



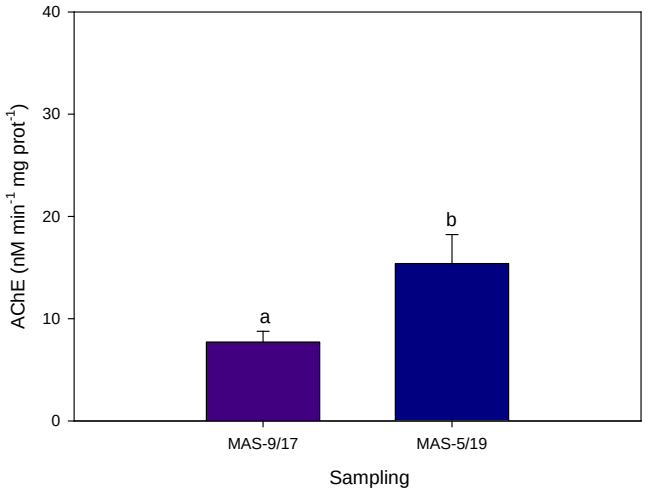

- generally used as a biomarker of metal exposure
- no significant differences were found between the sites
- MT values in caged mussels from the reference site were comparable to those of resident mussels
- there were no significant correlations with cytosolic metal concentrations

# AChE activity

Caged mussels *Unio crassus*



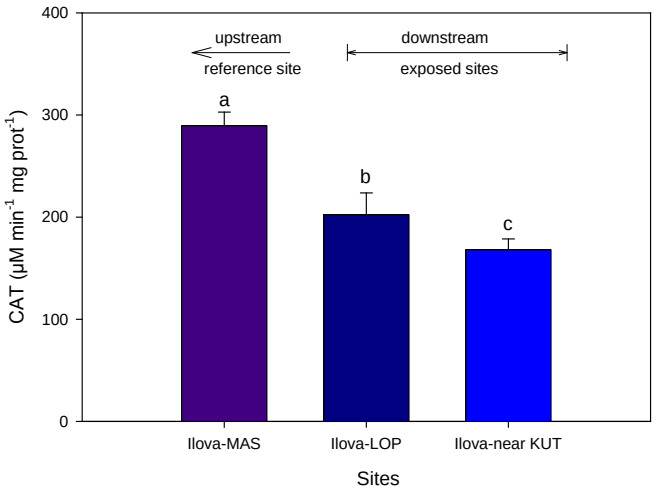

Resident *Unio crassus* at Ilova-Maslenjača



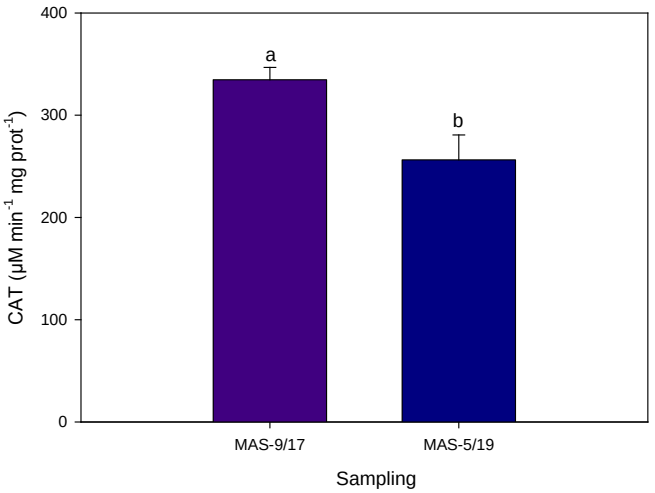

- Inhibition of AChE activity is commonly used as a biomarker of organophosphorous and carbamate pesticides exposure, but some metals, polycylic aromatic hydrocarbons or detergents can also cause inhibition of AChE
- a significant increase in AChE activity was observed at both exposed locations
- AChE activity in caged mussels at the reference site was in the range of AChE activities observed in wild mussels from this site

# CAT activity

Caged mussels *Unio crassus*



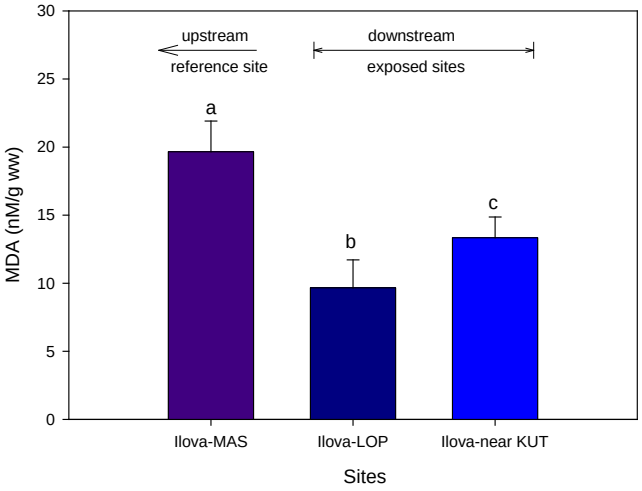

Resident *Unio crassus* at Ilova-Maslenjača



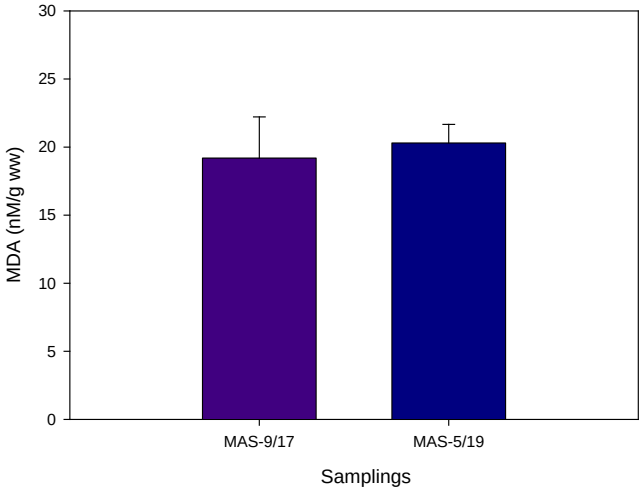

- used as a biomarker of antioxidative capacity
- a significant decrease in CAT activities was observed at both exposed locations, and the most pronounced decrease was observed at the Kutinica site as the most polluted site
- activity of CAT in caged mussels at the reference site was in the range of CAT activities observed in wild mussels from this site

# MDA concentrations

Caged mussels *Unio crassus*




Resident *Unio crassus* at Ilova-Maslenjača




- generally used as biomarker of oxidative stress -
- significant differences in MDA concentrations among sampling sites
- the highest concentration at reference site – ?
- MDA concentrations in caged mussels at the reference site were comparable to those of resident mussels
- MDA vs cytosolic metal concentrations:
  - significant ($r<0.05$) positive correlations with Rb, Cd, Sb, Bi, U, Ti, Cr, Ni, Cu, Tl, Pb and Fe

# CONCLUSIONS

- Both physico-chemical parameters and dissolved metal concentrations indicate a deterioration in water quality downstream of contaminated water inflow
- Among the 30 elements analyzed in the digestive gland of caged mussels, only Li, V, Cr, Mn, Al, Fe, Sr, Ba, Cs, Ca, Na and Mg showed a significant increase in concentrations at contaminated locations
- No significant differences were found in MT content, while a marked decrease in catalase activity at contaminated sites could indicate the toxic potential of surface water from these sites.
- In contrast to catalase, AChE activity was even increased at contaminated sites
- The significant decrease in MDA values at contaminated sites remains to be explained
- Declining condition index and hepatosomatic index could also indicate a deterioration of environmental conditions at the contaminated sites


Thank you for your attention!