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# Yesterday's contamination – a problem of today? The case study of discontinued historical contamination of the Mrežnica River (Croatia)

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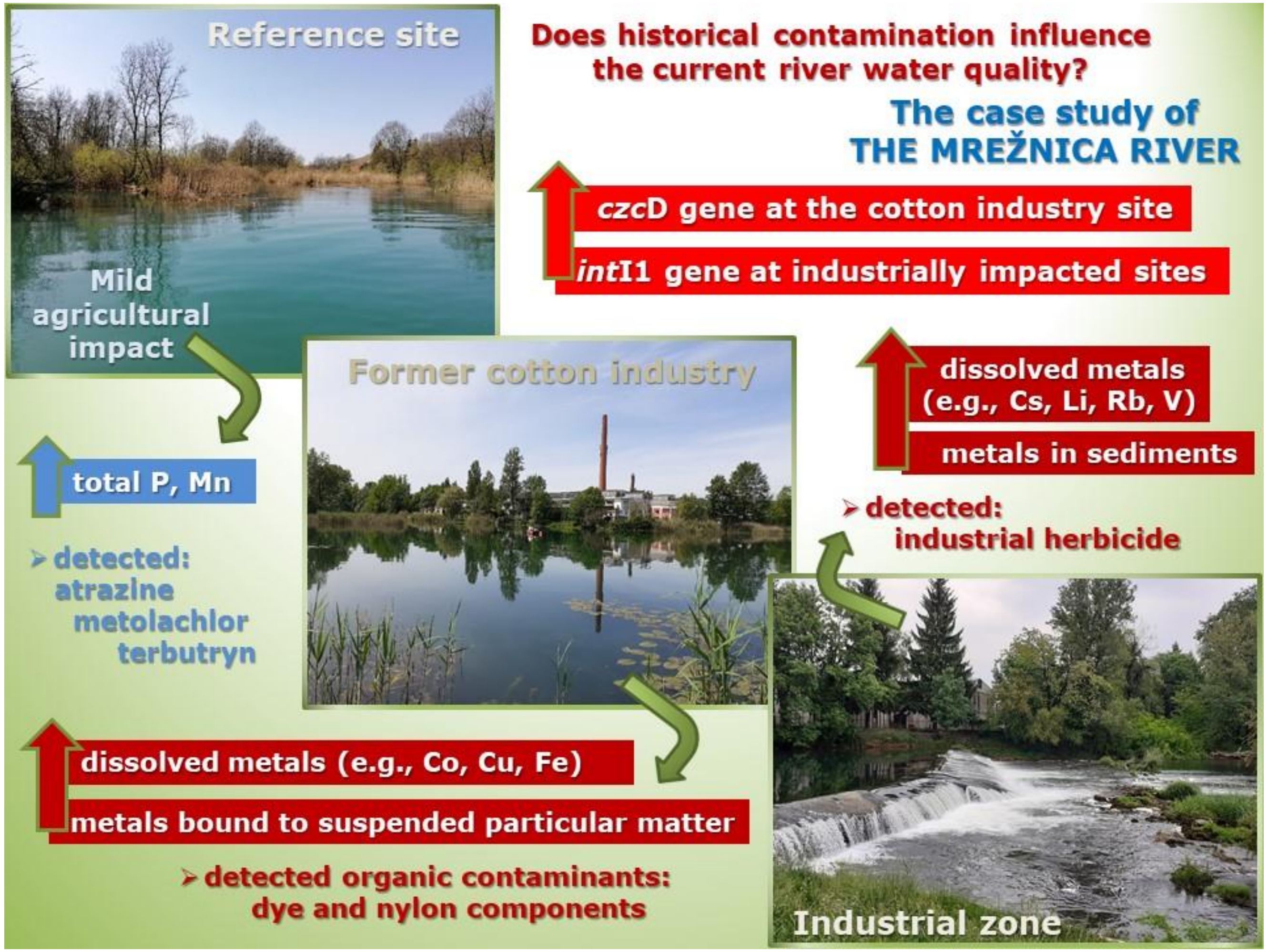
**Graphical abstract**



**Highlights**

- the remnants of historical contamination detected in freshwater ecosystem
- dye and nylon components found in river-water near former cotton industry facility
- high levels of industrial herbicide detected in water near urban industrial zone
- river mildly contaminated with metals at sites of past long-term industrial impact
- pollution biomarker, microbial gene *int*I1, higher at industrially impacted sites

## Abstract

The remnants of historical industrial contamination can be detected in many aquatic ecosystems worldwide even at present time. Mrežnica is a river in Croatia that has been, for more than a hundred years, continually exposed to effluents of various industries, which have, in modern time, mostly ceased to operate. Our aim was to establish the level of current contamination and pollution of the Mrežnica river-water and sediments. The study of river contamination at three sites (reference site; site nearby former cotton industry facility in Duga Resa – DRF; industrial zone of Karlovac town - KIZ) in three sampling campaigns (May 2020, April and September 2021) encompassed analyses of physico-chemical water parameters, screening of 369 pesticides, measurement of metal(loid) concentrations in the sediments, and in the dissolved and particulate phases of the river-water. The sediment pollution was assessed through the analyses of total bacteria abundance (by targeting 16S rRNA genes), and their associated metal resistance genes (*cnr*A, *pbr*T and *czc*D) and class 1 integrons (*int*I1). At the DRF site, industrial organic contaminants that can be traced to textile production were detected (dye and nylon components), as well as increased levels of some metals bound to suspended particulate matter and sediments. At the most downstream KIZ site, occasional high level of industrial herbicide neburon was measured in the river-water, and metal contamination of suspended particulate matter and sediments was evident. Although, based on the comparison with legislation and literature data, the level of contamination was rather mild, the effects on microbial communities were unquestionable, confirmed by increased abundance of the *czc*D gene at DRF site and the *int*I1 gene at both industrially impacted sites.  Obtained results indicated long-term sediment retention of some industrial contaminants at the places of historical freshwater contamination, and, thus, the necessity for their monitoring even after the termination of contamination sources.

**Key words**: freshwater, metal(loid), metal resistance genes, pesticides, sediment, textile industry

## 1. Introduction

The major contamination sources for the freshwater ecosystems are various industries (Dragun et al., 2009, 2011; Filipović Marijić et al., 2018; Domingues et al., 2020; Mijošek et al., 2020). Many industrial contaminants, such as metals, cannot be degraded, and thus, consequently, become available for accumulation in soil, water, bottom sediments, and living organisms (Samecka-Cymerman and Kempers, 2007). An important and easily recognizable source of aquatic contamination is certainly the textile industry because its effluents are highly colourful due to incomplete fixing of dye to the textile fibre, and thus, commonly cause the colouring of the receiving water-bodies (Domingues et al., 2020). Such was the case for a long time with the Mrežnica River in Croatia, which was exposed to the wastewaters of the large cotton textile factory in Duga Resa town (near Karlovac) (Frančišković-Bilinski et al., 2017). According to local residents, the colour of the river-water changed on daily basis during certain historical periods, depending on the colour of currently disposed effluent. However, textile industry effluents do not consist only of dyes, but also include various other chemicals (e.g., humectants and antifoam agents, pigments, and different compounds involved in the several dyeing stages; Domingues et al., 2020, and the references therein). Textile industry wastewaters are consequently rich in metals (such as Cu, Cr, and Zn), dyes containing sulphur compounds, fats, mineral oils, soaps, sulfuric and other inorganic acids, organic acids, $CS_2$, and different forms of nitrogen (Kabata-Pendias and Pendias, 1993; Samecka-Cymerman and Kempers, 2007). The textile industry is, actually, considered as primary polluter of the environment (Domingues et al., 2020, and the references therein). Recent reports imply that treatments used to process the textile effluents are not sufficiently effective, failing to degrade and remove cytotoxic or mutagenic substances (Domingues et al., 2020).

It is also critical to consider that some remnants of the historical industrial contamination can still be detected in our time, and thus, the extent of contamination that has persisted in aquatic ecosystems, particularly in modern riverbed sediments, should be established (Hurley et al., 2017). Hurley et al. (2017) have accentuated that many freshwater catchments reveal high metal(loid) concentrations although they are not subjected to intense present-day urban or industrial development; they have attributed it to releases from former industrial sites or to processing of historically contaminated floodplains and soils.

Considering the possibility of maintained water contamination even long after the cessation of industrial activities, we have set as our main goal to establish the consequences of diverse industrial activities in the lower section of the Mrežnica River in Croatia during the last century on its current water and sediment quality. The Mrežnica River is not only an important watercourse for Croatia, but also for Europe, being encompassed by the ecological network of European Union, NATURA 2000, which includes the most important areas for preservation of species and types of habitats. This river has been exposed to the effluents of the textile industry in Duga Resa town from 1884 to early 2000s; in addition to common textile industry contaminants, this factory also burned coal and discharged coal slag and ash directly into the Mrežnica River for 110 years, until 1994 (Frančišković-Bilinski, 2008). Frančišković-Bilinski et al. (2017) reported that magnetic susceptibility in the Mrežnica River sediments is still very high close to the pollution source and decreases downstream. Furthermore, Karlovac’s industrial zone has been built on both watersides of the Mrežnica River near its confluence with the Korana River, and some of the industrial facilities date as far back as 1930s. Although, about a decade ago, many of these facilities have been connected to a wastewater treatment plant that discharges its effluents into the Kupa River, a possibility of historical contamination should also be investigated at that part of the watercourse. Thus, this study specifically aimed at comprehensive examination of the quality status of the lowland terminal section of the Mrežnica River, including the assessment of physico-chemical water parameters and nutrients, determination of metal(loid) contamination of water and sediments, and screening for organic contaminants in the water. Various contaminants in sediments may select and enrich bacteria with specific genetic compositions (Chen et al., 2015; Koczura et al., 2016). Thus, our additional aim was to determine the distribution patterns of the gene encoding class 1 integrons as a bioindicator of anthropogenic contaminants in sediments of the Mrežnica River. We have also determined the distribution patterns of several other genes encoding metal resistances, as bioindicators of metal pollution, to define not only the levels of remnant river contamination, but also its still notable biological effects.

## 2. Materials and methods

### *2.1. Study area and period*

The study of the influence of the historical contamination sources on the current quality of water and sediments of a medium-sized watercourse was performed at the lowland section of the Mrežnica River in Croatia (Fig. 1). Mrežnica is a river 64 km long, and its water discharge ranges from ~2 to 160 $m^3$ $s^{-1}$ (Table SI.1). In its upper part, Mrežnica is a typical karst river with canyons, sedge barriers, and lacustrine areas, whereas in its lower part it enters the lowland region and becomes wider and shallower. It was precisely this lower part that for a long period was exposed to a high degree of industrial contamination. Our study was performed at three sampling points. The first one was located approximately 2 km upstream of the Town of Duga Resa; although the land in the vicinity of that site is cultivated, it was considered as a reference or the least contaminated site (i.e. the REF site, N 45° 26' 28,40" E 15° 30' 15,39"). The second sampling point was located in the Town of Duga Resa, directly in front of the former facility of textile industry which operated there since 1884 until about a decade ago (i.e. the DRF site, N 45° 27' 4,38" E 15° 30' 18,96"). A small hydropower plant with the belonging dam was built at the river at that location back in the 1884 for the requirements of then newly founded company, named Cotton industry Duga Resa; its presence only increased the possibility of retention and concentration of potential contaminants originating from industrial wastes in the accumulation area in front of the factory. At the location of the former textile industry (production disconnected in 2002), the factory of acrylic bathtubs and some other bathroom equipment is currently situated.  Our third sampling point was located approximately 2.5 km downstream of the Town of Duga Resa, within the limits of the industrial zone Mala Švarča of the Town of Karlovac (i.e. the KIZ site, N 45° 27' 49,05" E 15° 32' 30,09"), where numerous industrial facilities have been active for many years, some as far back as early 20th century. More than a decade ago, some of these facilities were connected to the wastewater treatment plant of the Town of Karlovac, and treated wastewater is now being discharged in the Kupa River downstream from Karlovac. However, several industrial facilities in that area still discharge their wastewaters into the Mrežnica River (e.g., heating plant, car wash, hospital, production of power plants, diesel engines, moulds and pumps, sliding bearings, textile, medical supplies made of cotton and personal care products). The fact that a large number of industrial facilities have been located on the small surface area for more than 70 years points to a possible significant burden for the river of the moderate dilution capacity, such as the Mrežnica River.

Our study has encompassed the period from May 2020 to October 2021. The majority of analyses have been performed within the three sampling campaigns, namely in May 2020, and in April and September 2021 (actual dates are presented in Table SI.1). For some parameters, additional water samplings were carried out, as indicated within the specific figures and tables. Although spring and autumn are usually known as the periods of maximal water-levels, due to heavy rains and snow melting, we have picked the warm days with the lowest achievable water discharges for our study, and the water-levels were either around the annual average or lower (Table SI.1); thus, the study can be considered as an investigation of the river contamination under baseflow conditions.

### *2.2. Analyses of physico-chemical characteristics of the river-water*

Several physico-chemical parameters were determined *in situ* in the river water at the sampling sites using the portable field meters, whose probes were calibrated before each sampling (Table SI.2 and Table 1). The pH, oxidation reduction potential (ORP), water temperature ($t_{water}$), dissolved oxygen (DO) saturation and concentrations, conductivity and total dissolved solids (TDS) were measured using portable digital meters SevenGo pro SG7 (Mettler Toledo, Switzerland-USA) different for each group of parameters (pH/ORP, DO/$t_{water}$, conductivity/TDS). For the laboratory measurements of several other physico-chemical parameters (Table SI.2 and Table 1), the river water was collected in the plastic bottles (1 L) and stored at +4 °C for the subsequent analyses (within 24 h). Water samples for chlorophyll-*a* determination where collected in dark plastic bottles (1 L), filtered through GF/F filter paper and stored in freezer at -24 °C until analysis. The following methods from Water Analysis Handbook (https://www.hach.com/wah) were applied for the examination of the river-water using UV/VIS spectrophotometer DR/6000 (Hach Lange, USA): for total phosphorus – Hach method LCK 348; for ammonium – Hach method 10023; for nitrates – Hach method 8192; for nitrites – Hach method 8507; for total nitrogen – oxidative digestion method (ISO 11905-1:1997); for turbidity – Hach method 8237; for chemical oxygen demand – Mn III method (ISO 8467:1993); and for chlorophyll-*a* – ISO 10260:1992. Moreover, several titration methods were applied that are based on Standard Methods for the Examination of Water and Wastewater (APHA, 1999): for dissolved $CO_2$ – phenolphthalein titration method; for m-alkalinity and carbonate hardness – methyl orange titration method; and for total hardness – EDTA titration method. The information about water-levels and water discharges (Table SI.1), measured at the station Mrzlo Polje indicated in Fig. 1, were obtained from the Meteorological and Hydrological Service of the Republic of Croatia.

### *2.3. Methods and analytical procedures for analyses of organic contaminants in the river-water*

Water samples for analyses of organic contaminants were collected as described in Stipaničev et al. (2017), and prior to analyses they were centrifuged (10,000 rpm for 20 min), and supernatants were used for the analyses. Analyses were done by online solid-phase extraction ultra high-performance liquid chromatography coupled to tandem mass spectrometry (SPE-UHPLC-MS/MS) and the results are presented in Tables 2 and 3, and Fig. 2. For method calibration, final working solutions of all standards (pesticide kit, Labinstruments, Italy; cybutrin (Irgarol 1051), AccuStandard, USA) were prepared in concentrations of 1 $\mu g\ L^{-1}$ (in methanol and ultrapure water). For creation of seven-point linear calibration line in the concentration range of 0.001-0.5 $\mu g\ L^{-1}$, calibration standards were extracted and treated by the online SPE procedure in the exactly same manner as the environmental samples (Shoemaker, 2016). Ultrapure laboratory water samples were always processed in parallel with the environmental water samples. Limits of detection (LODs) and limits of quantification (LOQs) were determined as the minimum detectable amounts of analytes, with a signal-to-noise ratio of 3 and 10, and theoretical LOQ ranged from 0.5 to 3.3 ng $L^{-1}$. Data processing was performed using LabSolutions Insight software (Shimadzu Corporation, Japan), with no manual correction. Accuracy and precision of the measurements were controlled after each twenty-samples series by injection of quality control samples at low level (LOQ) and intermediate level (50 ng $L^{-1}$). Acceptance criteria for accuracy were recoveries between 70 % and 125 %, and for repeatability relative standard deviations lower than 15 %.

A Nexera X2 UHPLC was coupled to liquid chromatography mass spectrometry system LCMS-8060 high sensitivity triple quadrupole (Shimadzu Corporation, Japan). UHPLC analysis consisted of a 33 minutes gradient using mobile phases consisting of water, methanol, and acetonitrile, all supplemented with ammonium formate and formic acid (all chemicals LC/MS grade, J.T. Baker, Fisher Scientific, USA) to enhance ionization and improve chromatography in positive and negative ESI (electrospray ionization) mode. Online SPE column Evolute Express ABN 20 µm, 30 × 2.1 mm (Biotage, Sweden) and a large injection volume (1000 µL) were used. Separation was performed on an ACQUITY UPLC HSS T3 column, 100 Å, 1.8 µm, 2.1 mm × 100 mm (Waters Corp., UK). The column chamber was tempered to 40 °C. In the positive and negative ionization modes, the mobile phases were composed of solvent A (10 mM ammonium formate / water / 0.1 % formic acid), and solvent B (10 mM ammonium formate / methanol / acetonitrile 50:50 / 0.1 % formic acid). Mass spectrometry was done with a triple quadrupole fitted with an ESI interface and controlled by LabSolution software. Interface conditions were optimized for maximum intensity of the precursor ions, as follows: nebulising gas 3 L $min^{-1}$, heating gas 10 L $min^{-1}$, drying gas 10 L $min^{-1}$, interface temperature 300 °C, desolvation line 250 °C, and heating block temperature 400 °C. The ESI polarity ionisation mode was set individually for each target compound. Positive and negative polarity modes were used simultaneously during the same analytical run. MRM (multiple reaction monitoring) transitions were selected and tuned individually for each analyte. To optimize the mass spectrometer, a 500 µg $L^{-1}$ standard solution of each analyte was infused directly. The specific and intense product ions of each target analyte were used for quantification, and two products ions were used as qualifier ions for confirmatory purposes. Additionally, a non-target screening in surface water samples was performed on UHPLC system (1290 UHPLC, Agilent Technologies, USA) coupled to hybrid quadrupole time-of-flight mass spectrometry (6550 i-Funnel Q-TOF–LC/MS, Agilent Technologies, USA). Instrumentation, acquisition settings and software tools utilised for the processing and analysis of mass spectral data were done as described in Schymanski et al. (2015) and in Schulze et al. (2021). Identification confidence in high resolution mass spectrometric analysis of level 2, named also probable structure (Schymanski et al. 2014), was reached, but for unambiguous identification comparison to reference standard still remains to be performed.

### *2.4. Sampling and preparation of the water and sediments for the metal(loid) analyses*

For metal(loid) analyses, the surface river water was collected in triplicates in polyethylene plastic bottles (0.25-0.50 L), which were rinsed with nitric acid (v/v 10%, *p.a.*, Kemika, Croatia) and Milli-Q water before sampling. The samples were filtered through FilterBio® CA syringe filters (0.45 µm pore diameter, Labex Ltd., Germany) immediately after the sampling at the collection sites and acidified with nitric acid (v/v 2%; Normatom, 67-69%; VWR Chemicals, USA) to obtain only the dissolved metal(loid) fraction. Trace elements were measured directly in thus prepared samples, whereas the samples were ten times diluted for the measurement of the macro elements.

For the determination of suspended particulate matter (SPM), the river water was collected in two 1.5 L plastic bottles per site. The total volume of water collected at each site was subsequently filtered in the laboratory (0.45 µm cellulose acetate filter, Sartorius, Germany). Before and after filtration the filters were dried at 60 °C, weighed and then stored for elemental analysis. The samples were digested in 3 mL $HNO_3$ (TraceSELECT™, ≥69%, Fluka, Germany) in the microwave oven (Multiwave ECO, Anton Paar, Austria). The resulting solution was transferred to a volumetric flask (50 mL) and diluted to the mark with Milli-Q water.

Sediment samples (upper 10 cm) were collected in triplicates, as follows: from the middle of the riverbed with a grab sampler in May 2020, and from the lateral parts of the riverbed with a spatula in April and September 2021, and stored in plastic bags. In the laboratory, all samples were air-dried, homogenised using an agate mill, and stored until further analysis. Prior to analysis, sediment sub-samples (0.05 g) were subjected to total digestion in the microwave oven (Multiwave ECO, Anton Paar, Austria) using a two-step procedure consisting of digestion with a mixture of 4 mL nitric acid ($HNO_3$, 65%, *p.a.*, Kemika, Croatia), 1 mL hydrochloric acid (HCl, TraceSELECT, Fluka, Germany), and 1 mL hydrofluoric acid (HF, TraceSELECT, Fluka, Germany), followed by the addition of 6 mL boric acid ($H_3BO_3$, Fluka, Switzerland). The solution was then transferred to a volumetric flask and made up to 100 mL. In addition, the solution was diluted tenfold and acidified with 2% (v/v) $HNO_3$ (65%, TraceSELECT, Fluka Germany) prior the analysis. The indium was added as an internal standard for the SPM and sediment samples.

### *2.5. Analysis of sediment particle size distribution*

The granulometric analysis of the sediments (Table SI.5) was carried out using a laser diffraction particle size analyser (LS 13320, Beckman Coulter, Brea, CA, USA). The particle size distribution was calculated on the basis of the Mie theory of light scattering (optical parameters : refractive index = 1.53; absorption index = 0.1).

### *2.6. Metal(loid) measurements in water and sediment samples*

Four macro elements (Ca, K, Mg, Na) and 25-26 dissolved trace elements were measured in water (dissolved and particulate fraction) and sediments by high resolution inductively coupled plasma-mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2DX FAST (Elemental Scientific, USA). Specific details about the measurement were previously reported by Fiket et al. (2007; 2017). Indium (1 $\mu g\ L^{-1}$; Fluka, Germany) was added to all samples and calibration standards as an internal standard. Measurements of $^{7}Li$, $^{82}Se$, $^{85}Rb$, $^{95}Mo$, $^{109}Ag$, $^{111}Cd$, $^{120}Sn$, $^{121}Sb$, $^{133}Cs$, $^{205}Tl$, $^{208}Pb$, $^{209}Bi$, and

$^{238}$U were performed in low resolution mode, of $^{23}$Na, $^{24}$Mg, $^{27}$Al, $^{31}$P, $^{42}$Ca, $^{47}$Ti, $^{51}$V, $^{52}$Cr, $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{60}$Ni, $^{63}$Cu, $^{66}$Zn, $^{86}$Sr and $^{138}$Ba in medium resolution mode, and of $^{39}$K and $^{75}$As in high resolution mode. The external calibrations were applied using multielement standard solution for trace elements (100 mg L$^{-1}$, Analytika, Czech Republic) supplemented with standard solutions of Cs (1 g L$^{-1}$; Fluka, Germany), Rb and U (1 g L$^{-1}$; Aldrich, USA), and Sb and Sn (1 g L$^{-1}$; Analytika, Czech Republic), as well as standard solutions for macro elements (each 1.0 g L$^{-1}$; Analytika, Czech Republic). All calibration standards were prepared in Milli-Q water and $HNO_3$ (v/v 2%, Normatom, 67-69%; VWR Chemicals, USA; or TraceSELECT™, ≥69%, Fluka, Germany). Measurements were further performed in adequately prepared blank samples, and the corrections of the obtained results (i.e. concentrations in water and sediments) for the blank concentrations were made when needed. The limits of detection (LODs) were calculated as three standard deviations of trace and macro element concentrations measured ten times consecutively in the blank samples, and LODs are given within the supplementary materials (Table SI.3.). If any of the obtained concentrations was below LOD, LOD value was used instead for the purpose of statistical analyses. The accuracy control of the measurement in the water was performed using quality control samples acquired from UNEP/GEMS (Burlington, Canada), and recoveries were in the range from 95% to 108%. Analytical quality control of sediments was performed by simultaneous analysis of procedural blanks and certified reference materials of soil NCS DC 773902 (GBW 7410) and stream sediment NCS DC 73309 (GBW 07311), and recoveries were in the range from 90% to 102% (Fiket et al., 2017). The obtained recoveries indicated reliable measurements. The results are presented in Tables SI.4., 4 and 5 and Fig. 3.

### *2.7. DNA extraction from sediments and quantification of heavy metal resistance and integrase genes*

Total community (TC-) DNA was extracted from spring (April 2021) and autumn (September 2021) sediment samples (0.25 g fresh mass) using the DNeasy Powersoil kit (Qiagen, USA) according to the manufacturer's recommendations. DNA quality (260/280 ratio) was assessed using a Nanodrop spectrophotometer (BioSpec Nano, Shimadzu, Japan), and DNA quantity using a Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA). All extractions were stored at -20 °C until use.

Quantitative PCR (qPCR) was conducted with extracted TC-DNA to quantify the 16S rRNA gene as a marker for total bacteria, the class 1 integron-integrase gene *int*I1 as a biomarker for anthropogenic pollution and three genes encoding heavy metal resistance (*czc*D for Co, Cd and Zn; *pbr*T for Pb and *cnr*A for Co and Ni resistance) as markers for metal pollution. The primers targeting these genes and qPCR conditions are listed in Table 6. All qPCR assays were performed on the ABI 7300 Real-time PCR thermocycler (Applied Biosystems, USA) with Power SYBR® Green PCR Master Mix (10 µL, Applied Biosystems, USA), 1 µM of each primer (Table 6) and 2 ng of DNA template in a total volume of 20 µL. The plasmids pCR™-TOPO™ (Thermo Fischer Scientific, USA) with the corresponding inserts were used as quantification standards for the quantification of the genes *czc*D, *pbr*T and *cnr*A. The pNORM1 plasmid (Rocha et al., 2020) was used to quantify the *int*I1 gene, while the plasmid pGEM-T was used to quantify the 16S rRNA gene (Milakovic et al., 2019). Plasmid DNA was extracted with ExtractNowTM Plasmid Mini kit (Minerva Biolabs GmbH, Germany) and used after linearization to generate a standard curve ($10^2$-$10^8$). Both samples and standards were analysed in technical duplicates. Possible qPCR inhibition was assessed by conducting an inhibition test using 10- and 100-fold diluted samples, as previously described (Petric et al., 2011). The detection limit for all target genes was $10^2$ gene copies per reaction. Gene abundances were calculated per number of copies of the 16S rRNA gene (*rrn*) (relative abundance), and results were log transformed. The results are presented in Fig. 4.

### *2.8. Calculations and graphical data presentation*

All the calculations were done in Microsoft Office Excel. The graphs and statistics for metal(loid) results were done by use of statistical program SigmaPlot 11.0 for Windows. Comparisons among concentrations of metal(loid)s dissolved in the water or present in the sediment at three sampling sites were performed by Kruskal-Wallis one-way analysis of variance on ranks; subsequent pairwise multiple comparisons were done either by Dunn's method or Tukey's test. The statistical analyses on qPCR data were performed using GraphPad Prism version 9.3.0 for Windows (GraphPad Software, San Diego, California, USA). Log-transformed qPCR data were subjected to a Shapiro-Wilk test to assess their normality. Multiple comparison one-way ANOVA was then performed to compare the average abundance of genes between seasons and sampling sites.

## 3. Results and discussion

### *3.1. Physico-chemical characteristics of the Mrežnica River water*

The measurement of several physico-chemical parameters and nutrients in the surface water along the lower course of the Mrežnica River indicated its rather good quality (Table SI.2 and Table 1), which was mainly comparable at upstream reference site and two industrially impacted locations. In accordance with the samplings being performed during mild weather seasons (May 2020, April and September 2021), the water temperature ranged from ~11-18°C, and the lowest values were, as expected, measured in April 2021 (11-13°C) (Table SI.2). For few physico-chemical parameters the water quality status could not be determined since they were not covered by the legislation (turbidity, conductivity, TDS, ORP, m-alkalinity), but the comparison of the obtained results with the previously published information indicated low to moderate water contamination. For example, the highest turbidity was recorded at KIZ site (up to 7 FAU), which was comparable with moderately contaminated site (downstream from municipal sewage outlet) at the Krka River in Croatia (Filipović and Marijić et al., 2018). Conductivity (317-377 $\mu$S cm$^{-1}$) and TDS (158-188 mg L$^{-1}$) were lower than reported for moderately polluted Sava and Sutla rivers (Dragun et al., 2009, 2011) and comparable with the unpolluted section of the Ilova and Krka rivers in Croatia (Filipović Marijić et al., 2018; Mijošek et al., 2020); moreover, they were somewhat higher in the colder period (April 2021)

(Table SI.2). Dissolved oxygen concentrations ranged from 9.04 to 11.5 mg $L^{-1}$ and saturation from 96.6 to 112%, whereas, $CO_2$ concentrations were found in the range from 1.0 to 2.7 mg $L^{-1}$ (comparable to range of values reported throughout the Krka River by Filipović Marijić et al., 2018) with somewhat higher values at DRF site in all seasons and in September 2021 at all sites (Table 1).

pH values were slightly alkaline (8.05-8.38; Table SI.2) and indicated very good status according to requirements for pH (7.4-8.5) set in the Croatian Directive on water classification (GRC, 2019). The water hardness, which is also an important factor when considering metal contamination, was classified as fourth category (6.94-10.8 °dH or 124-193 mg $CaCO_3$ $L^{-1}$; Table SI.2) according to GRC (2019; 100 to <200 mg $CaCO_3$ $L^{-1}$). Chemical oxygen demand, which is used to evaluate the organic matter load in the river water (Mijošek et al., 2020), ranged from 3.6 to 6.4 mg $O_2$ $L^{-1}$ (Table 1) and mainly did not fulfil the requirement for good water status (<4 mg $O_2$ $L^{-1}$) at either site. Similar finding was previously reported for the Ilova River in Croatia, where COD was increased even at the otherwise unpolluted sites (3.8-6.5 mg $O_2$ $L^{-1}$; Mijošek et al., 2020). However, it was much lower compared to moderately contaminated site at the Krka River (17-21 mg $O_2$ $L^{-1}$; Filipović Marijić et al., 2018).

The levels of nutrients were also within the ranges recommended for good or very good status (GRC, 2019) with only few exceptions. Ammonium levels ranged from 0.02-0.16 mg N $L^{-1}$ (Table 1). Only the maximum value measured at KIZ site in May 2020 (0.16 mg N $L^{-1}$) surpassed the requirement for good water quality (0.12 mg N $L^{-1}$; GRC, 2019) and was somewhat higher compared to maximal value measured at the contaminated site of the Sutla River (0.12 mg N $L^{-1}$; Dragun et al., 2011). In April 2021, ammonium was even within the very good water quality limits (<0.04 mg N $L^{-1}$; GRC, 2019) at all three sites. Total nitrogen levels ranged from 0.20 to 1.75 mg N $L^{-1}$ (Table 1) and in several cases they surpassed the limits for good water quality (<1.5 mg N $L^{-1}$; GRC, 2019). This occurrence was observed at all three sites in various periods. In general, at all three sites the highest total nitrogen values were measured in May 2020, whereas the lowest values were recorded in September 2021 corresponding to very good water quality (<1 mg N $L^{-1}$; GRC, 2019). The concentrations of nitrates ranged from

0.01 to 0.29 mg N $L^{-1}$ (Table 1), with highest levels at all sites recorded in April 2021, but even then fulfilling the requirement for very good water quality (<0.7 mg N $L^{-1}$; GRC, 2019). The nitrite concentrations are not covered by legislation, but they also showed moderate levels (0.008-0.039 mg N $L^{-1}$; Table 1), comparable to reports for unpolluted site at the Ilova River (0.014-0.090 mg N $L^{-1}$; Mijošek et al., 2020) and lower than moderately contaminated site at the Krka River (~0.4 mg N $L^{-1}$; Filipović Marijić et al.). The nitrate and nitrite concentrations in the Mrežnica River were much lower compared to the values reported for the stream water impacted by the textile dyeing industry in Brazil (nitrate: 15.6 mg N $L^{-1}$; nitrite: 3.22 mg N $L^{-1}$) (Domingues et al., 2020). But they were still a bit higher than average levels reported for moderately contaminated Sutla River (0.001-0.026 mg N $L^{-1}$; Dragun et al., 2011). Somewhat higher nitrite concentrations were measured in all three seasons at KIZ site (0.015-0.039 mg N $L^{-1}$; Table 1) compared to the other two sites (0.008-0.022 mg N $L^{-1}$; Table 1). Also, nitrites showed similar trend to nitrates with the highest concentrations measured at all three sites in April 2021. Total phosphorus in general fulfilled the requirement for good (<0.06 mg P $L^{-1}$) or very good (<0.02 mg P $L^{-1}$) water quality (GRC, 2019) with the majority of measured values placed within the range from <0.010 to 0.041 mg P $L^{-1}$ (Table 1). The only exception was rather high value measured at REF site in May 2020 (0.266 mg P $L^{-1}$, Table 1), which was comparable to average values reported for the moderately contaminated Sutla River (0.17-0.37 mg P $L^{-1}$; Dragun et al., 2011), but still much lower than reported for moderately contaminated site at the Krka River (~1 mg P $L^{-1}$; Filipović Marijić et al., 2018). The water quality status according to additional requirements for indicators of eutrophication (nitrates, total phosphorus and chlorophyll *a*) was mainly very good, except for the highest level of total phosphorus which corresponded to good water quality (GRC, 2019).

Despite generally satisfactory water quality regarding the physico-chemical parameters, few observations should be pointed out, indicating the potential concerns at specific sampling sites: occasionally rather high concentration of total phosphorus at REF site; continually the highest $CO_2$ level at DRF site; occasionally increased ammonium level and continually the highest nitrite level at KIZ site. Also, chemical oxygen demand above the limits for good water quality recorded almost always at all sites indicated higher than acceptable presence of organic matter throughout the lower course of the Mrežnica River. Some of the parameters exhibited distinct seasonality, such as conductivity, TDS, nitrate and nitrite concentrations with highest values in April 2021; total nitrogen in May 2020; and $CO_2$ level in September 2021.

### *3.2. Organic contaminants in the surface water of the Mrežnica River*

The quantity of certain categories of organic contaminants in the surface water of the Mrežnica River was also analysed at all three sites (REF, DRF and KIZ) and in all three sampling periods (May 2020, April and September 2021) (Fig. 2; Table 2). Total number of analysed compounds in each sampling was 369, and number of detected compounds per season was 15 (4.1%), 6 (1.6%), and 5 (1.4%), respectively. In both spring seasons, the highest total concentrations and number of detected compounds were recorded at KIZ site (Fig. 2) (May 2020: 0.722 μg $L^{-1}$ and 11 compounds; April 2021: 0.340 μg $L^{-1}$ and 6 compounds) compared to the other two sites, REF (May 2020: 0.258 μg $L^{-1}$ and 7 compounds; April 2021: 0.205 μg $L^{-1}$ and 2 compounds) and DRF (May 2020: 0.242 μg $L^{-1}$ and 6 compounds; April 2021: 0.240 μg $L^{-1}$ and 5 compounds). In autumn sampling, the highest total concentration of analysed organic contaminants was detected at DRF site (1.41 μg $L^{-1}$) compared to the two other sites (REF: 0.350 μg $L^{-1}$; KIZ: 0.580 μg $L^{-1}$). However, the number of detected compounds was comparable at all sites, and amounted to three (Fig. 2).

The analyses were focused on three groups of compounds, namely herbicides, insecticides and fungicides, and the portion of each group in total amount of measured contaminants is presented in Fig. 2. From spatial point of view, higher concentrations of herbicides were generally found at KIZ site (May 2020: 0.421 μg $L^{-1}$; April 2021: 0.029 μg $L^{-1}$; September 2021: 0.002 μg $L^{-1}$), compared to REF (May 2020: 0.051 μg $L^{-1}$; April 2021: 0.007 μg $L^{-1}$; September 2021: 0.002 μg $L^{-1}$) and DRF (May 2020: 0.038 μg $L^{-1}$; April 2021: 0.012 μg $L^{-1}$; September 2021: 0.004 μg $L^{-1}$) (Fig. 2). Seasonal differences were obvious, revealing much higher herbicide levels in the river water in May, consistent with the period of their use in agriculture and coinciding with the highest nitrogen level in the surface water as a sign of increased application of fertilizers (Ramani et al., 2014). However, there was a difference between rural and urban areas regarding the type of herbicides found. At upstream, more agriculturally developed sites (REF and DRF), the compounds that constituted the highest portion of detected herbicides were atrazine and its metabolites, and metolachlor (both commonly applied in corn cultivation), as well as terburtryn (commonly applied in wheat and barley cultivation), whereas, at KIZ site high level of herbicides in the first sampling period referred almost completely (97%) to neburon (Table 2), which is a herbicide usually applied in water-tanks and which could be associated to industrial application. Comparison of the highest herbicide levels at the agriculturally impacted section of the Mrežnica River measured in May 2020 (0.038-0.051 μg $L^{-1}$) with the herbicide levels reported for agriculturally impacted Bregalnica River in North Macedonia in May 2015 (0.84-1.09 μg $L^{-1}$) revealed much higher water contamination of the Macedonian river. However, the herbicide concentration found at urban KIZ site in May 2020 (0.421 μg $L^{-1}$) was notably higher compared to the urban section of the Vardar River downstream of Macedonian capital Skopje (<0.200 μg $L^{-1}$) (Stipaničev et al., 2017). Among detected herbicides, three were included in the list of contaminants set out by Croatian and European legislation (EPCEU, 2013; GRC, 2019), namely atrazine, diuron and terbutryn. However, the recommended annual average concentrations (AAC) of these compounds (EPCEU, 2013; GRC, 2019) were much higher than even their highest concentrations detected in the Mrežnica River: detected atrazine concentrations were up to 0.0025 μg $L^{-1}$, which is 240 times lower than AAC; detected diuron concentrations were up to 0.009 μg $L^{-1}$, which is 22 times lower than AAC; and detected terburtryn concentrations were up to 0.010 μg $L^{-1}$, which is 6.5 times lower than AAC.

In case of insecticides, in the first two sampling campaigns their quantity was rather uniform among sites and sampling periods, ranging from 0.203-0.262 μg $L^{-1}$ in May 2020 and 0.198-0.309 μg $L^{-1}$ in April 2021, with just slightly higher values obtained at KIZ site (Fig. 2). In September 2021, however, sharp increase of insecticide levels was observed, especially high at DRF site (1.40 μg $L^{-1}$), and somewhat more moderate at REF (0.347 μg $L^{-1}$) and KIZ (0.578 μg

L$^{-1}$) sites (Fig. 2). Predominant insecticide found at all three sites was nicotine (Table 2). The levels of insecticides in the Mrežnica River were much higher compared to previously published values for the rivers in North Macedonia, namely agriculturally impacted Bregalnica River and the river Vardar downstream from Skopje, which amounted up to 0.102 µg L$^{-1}$ and included a number of compounds different than found in this study (Stipaničev et al., 2017). Fungicides were the least represented group, occasionally found at some of the sites in rather low concentrations (usually below 0.005 µg L$^{-1}$) and referring to tetraconazole (commonly applied as agricultural fungicide). Only in May 2020 slightly higher fungicide concentration was found at KIZ site and referred to mepronil (0.039 µg L$^{-1}$), which is commonly used in agriculture, horticulture, and forestry. The concentrations of fungicides reported for Bregalnica and Vardar rivers in North Macedonia were somewhat higher, amounting up to 0.137 µg L$^{-1}$ (Stipaničev et al., 2017).

For the majority of the pesticides which can be found in the freshwaters, referring by this term to herbicides, insecticides and fungicides, the environmental quality standards were not set by the European Water Framework Directive (EPCEU, 2013), and thus, it is common to compare the actual environmental values with the permissible limits established for the drinking water in Europe (100 ng L$^{-1}$ for each compound individually, and 500 ng L$^{-1}$ for the sum of all pesticides found in the river water) (CEC, 1998; Papadakis et al., 2015; Stipaničev et al., 2017). In the Mrežnica River, the concentrations of individual compounds higher than 0.1 µg L$^{-1}$ were found for herbicide neburon at KIZ site in May 2020, and for insecticide nicotine almost always at all sites. The concentrations of total pesticides higher than 0.5 µg L$^{-1}$ were found at DRF site in September 2021 and at KIZ site in May 2020 and September 2021, and the main contributors were neburon and nicotine. Although the quantitative analyses revealed generally rather low levels of organic contaminants in the surface water of the Mrežnica River, the KIZ site, as an urban area, seemed to be slightly more contaminated with all pesticide categories than the upstream rural sites, but the presence of neburon additionally pointed to industry as a possible source of water contamination.

In May 2020, we have performed an additional qualitative analysis of organic contaminants at REF and DRF sites, to determine the possible presence of some other organic compounds that are not covered by standard screening method, especially those that could be associated to former cotton industry at DRF site. The 28 compounds detected by this screening are presented in Table 3, with 9 compounds (32%) found at both sites, 5 compounds (18%) found only at REF site, and additional 14 compounds (50%) found only at DRF site. Compounds found only at REF site (Table 3) included medicinal drugs, components of grill foods and food packaging contaminant, and polyester component, and pointed to probable contamination from households. Compounds found at both sites (Table 3) included polymerization component, ingredients used in cosmetics, veterinary antibiotic, urinary biomarker of meat consumption, as well as a number of compounds that indicated water contamination with dye components, namely azo-dyes. Furthermore, among 14 compounds found only at DRF site, five of them included marker of grilled meat, food packaging contaminant, components of home cleaning products, veterinary drug, and analgesics, whereas, nine compounds had possible industrial use (obsolete fungicide, a number of dye components, and nylon component). Although synthetic dyes, such as azo dyes, are extensively used in textile dyeing processes and released into the environment, and despite the reports about their possible mutagenic activity, the studies about their presence in aquatic ecosystems are still very rare (Carneiro et al., 2010). The findings of this study indicated that some possibly toxic remnants of the cotton industry contaminants are still present in the river even a decade after cessation of the running of the factory, perhaps in even higher concentrations in the sediments than found in the water.

### *3.3. The concentrations of dissolved trace and macro elements in the surface water of the Mrežnica River*

The concentrations of 30 dissolved elements, four macro and 26 trace, were determined in the surface water of the Mrežnica River at three sites (REF, DRF and KIZ) in overall six samplings, and the values obtained in each sampling at each site are given as supplementary information (Table SI.4).

Based on the gathered concentrations of all analysed elements during the entire sampling period, the Mrežnica River could be generally considered as only slightly contaminated. The concentrations of analysed macro elements were found in the following ranges: Ca (41.7-62.7 mg $L^{-1}$) > Mg (8.93-16.1 mg $L^{-1}$) > Na (1.05-2.11 mg $L^{-1}$) > K (0.314-0.553 mg $L^{-1}$). And the concentrations of analysed trace elements were found in these ranges: Sr (67.9-103 μg $L^{-1}$) > Fe (1.58-18.9 μg $L^{-1}$) > Ba (5.66-6.97 μg $L^{-1}$) > Mn (0.785-4.46 μg $L^{-1}$) > Al (LOD-2.86 μg $L^{-1}$) > Zn (LOD-1.05 μg $L^{-1}$) > Mo (0.370-0.978 μg $L^{-1}$) > U (0.474-0.662 μg $L^{-1}$) > V (0.364-0.569 μg $L^{-1}$) > Rb (0.205-0.450 μg $L^{-1}$) > Se (0.072-0.350 μg $L^{-1}$) > As (0.070-0.336 μg $L^{-1}$) > Cr (0.024-0.184 μg $L^{-1}$) > Cu (LOD-0.182 μg $L^{-1}$) > Ni (LOD-0.137 μg $L^{-1}$) > Li (0.038-0.100 μg $L^{-1}$) > Sn (LOD-0.078 μg $L^{-1}$) > Sb (0.025-0.046 μg $L^{-1}$) > Co (0.018-0.041 μg $L^{-1}$) > Cd (0.002-0.026 μg $L^{-1}$) > Bi (LOD-0.012 μg $L^{-1}$) > Tl (0.003-0.006 μg $L^{-1}$) > Cs (0.001-0.005 μg $L^{-1}$) > Ag (LOD-0.003 μg $L^{-1}$) > Pb, Ti (<LOD).

Among analysed elements only three are regulated by Croatian and European laws (EPCEU, 2013; GRC, 2019), and those are Cd, Ni, and Pb. Cadmium and its compounds were even categorized as priority hazardous substances by Water Framework Directive (EPCEU, 2013). The environmental quality standards for these elements refer to their dissolved, i.e. bioavailable, concentrations (recommended annual average: Cd, 0.15 μg $L^{-1}$; Ni, 4 μg $L^{-1}$; and Pb, 1.2 μg $L^{-1}$). Even the highest concentrations of all three elements measured in the course of our study were much lower than the recommended values (Cd, 6 times; Ni, 29 times, and Pb, >20 times).

Furthermore, all measured concentrations in the course of our study were lower compared to the values established by the Dutch Government in 1992 for dissolved polluting elements in unpolluted surface water (Cd, 0.06 μg $L^{-1}$; Cu, 1.3 μg $L^{-1}$; Ni, 7 μg $L^{-1}$; Pb, 25 μg $L^{-1}$; Zn, 30 μg $L^{-1}$) and to typical value of Mn in natural waters (20-60 μg $L^{-1}$) according to Kabata-Pendias and Pendias (1993) (Samecka-Cymerman and Kempers, 2007).

We have further compared the dissolved metal(loid) concentrations in the surface water of the Mrežnica River with several other variably contaminated rivers in Croatia. Metal(loid) concentrations in the Mrežnica River were generally much lower compared to moderately contaminated Sava and Sutla rivers (Dragun et al., 2009, 2011; Lučić et al., 2022). There were several exceptions: Mn, U and V were comparable in the Mrežnica and Sava rivers; Fe was occasionally higher in the Mrežnica River, especially at DRF and KIZ sites, even compared to industrially impacted site at the Sava River; and the concentrations of Cr, U, and V were placed between the values reported for unpolluted and highly polluted sites at the Sutla River (Dragun et al., 2009, 2011; Lučić et al., 2022). Furthermore, all measured dissolved metal(loid) levels in the Mrežnica River were either comparable or lower even compared to levels reported for generally unpolluted sites of the Ilova River (Mijošek et al., 2020). Finally, comparison was made with the karst Krka River, and the majority of metal(loid)s in the water of the Mrežnica River were either comparable or lower than values at the unpolluted sites of the Krka River, with the exception of Ba, Co and Mn, which were closer to the values reported for the contaminated section of the Krka River, and As and Fe, which were two times and 50% higher than values reported for that site, respectively (Filipović Marijić et al., 2018).

Despite rather low water contamination, we were still able to recognize some spatial patterns when we have considered our complete data set (Figure 3). Even at the REF site a couple of elements (Cd and Tl) were present in higher concentrations compared to downstream sites, probably as a result of either natural sediment composition or agricultural practices in the vicinity of that section of the river. The highest concentrations of Co, Cu and Fe were found at DRF site, and increased Cu was previously reported as contaminant characteristic for textile industry sewage (Samecka-Cymerman and Kempers, 2007). Also, it was reported that water contamination with Cu can occur due to waste effusions from the cooling systems which commonly use copper sulphate (Momčilović, 2004; Dragun et al., 2011). The largest number of elements had the highest concentrations at KIZ site, namely Al, Ba, Cs, Li, Na, Rb, and V. Copper and Fe were also higher at KIZ compared to REF site, but still somewhat lower than DRF. Some of the observed differences were statistically significant, and pointed to possible remnants of industrial contamination at DRF and KIZ sites.

### *3.4. Grain size distribution and trace and macro element concentrations in the bottom sediments of the Mrežnica River*

Sediments act like the main sinks for metal(loid)s in aquatic ecosystems (Fiket et al., 2019), and thus, it was important to perform the analyses of metal(loid) contamination of the bottom sediments of the Mrežnica River. During the first sampling campaign (in May 2020), sediments were collected from the middle of the watercourse, whereas, in the next two campaigns (in April and September 2021), the edges of the riverbed were sampled, resulting in differences in their grain size distribution as well as in multielement composition.

As shown in Table SI.5, in the sediments collected in May 2020 at REF and KIZ sites predominant fractions were gravel and sand. On the contrary, at the DRF site predominant fractions were silt (57.0%) and sand (35.3%), whereas, gravel was not observed. The sediments collected in April and September 2021 at all three sites were predominantly composed of silt (46.6% - 56.1%), and no gravel fraction was present. In general, fine-grained particles predominated in the samples collected at the edges of the riverbed, with average proportions of clay, silt, and sand of 9.1%, 52.5% and 38.4%, respectively (Table SI.5). As can be seen from the particle size distribution (Table SI.5), the studied locations exhibited certain variability if the sediments were sampled from the middle sections of the riverbed. Specifically, the sediment from the DRF site contained the highest percentage of silt, averaging 53.9%, a fraction which commonly contains the highest quantities of adsorbed metals (Quémerais et al., 1996). The reason for this specificity of the DRF site is the presence of the small dam in front of the factory which was built for the requirements of the textile production, and resulted in the formation of the stagnant water conditions, as well as diminished the washout of the river sediments. It can be expected that such hydrologic modification would impact the total levels of contaminants in the river sediments, and increase the possibility of their return to water column due to occasional resuspensions. Considering that the cotton industry was active at that site for more than hundred years, there is a possibility of higher availability of industrial contaminants, such as metals, specifically at the DRF site, and especially in the large area of stagnant water in front of the factory.

The concentrations of trace and macro elements in the sediments of the studied section of the Mrežnica River are presented in the Table 4 and could be generally ranked as follows: 0.1-0.5 μg $g^{-1}$ (Bi, Tl); 0.5-1.0 μg $g^{-1}$ (Cd, Sb); 1.0-5.0 μg $g^{-1}$ (As, Cs, Mo, Sn, U); 5.0-10.0 μg $g^{-1}$ (Co); 10.0-50.0 μg $g^{-1}$ (Cu, Li, Ni, Pb, Rb, V); 50.0-100 μg $g^{-1}$ (Cr, Zn); 100-200 μg $g^{-1}$ (Ba, Sr); 200-500 μg $g^{-1}$ (Mn); 500-1000 μg $g^{-1}$ (P); 1.0-5.0 mg $g^{-1}$ (Ti, K, Na); 5.0-50.0 mg $g^{-1}$ (Al, Fe, Mg); and > 150.0 mg $g^{-1}$ (Ca).

The obtained results (Table 4) confirmed that the concentrations of all elements were slightly to significantly higher at DRF and KIZ site than at REF site; some were even 3-12 times higher at DRF and 5-17 times higher at KIZ compared to REF site (e.g. Al, Cr, Cs, Li, Mo, and Ni). The sediment at the KIZ site also had concentrations of U, Sb, and Sn 3-20 times higher compared to the other two sites. A number of elements could be associated to industrial applications, including the use in textile production. The salts of Pb and Zn, for example, were applied throughout the history in the production of textile, for fabric softening and dyeing (Choudhury, 2006; Hurley et al., 2017). Copper and Cr could be linked to textile industry as components of dyeing and bleaching effluents, with Cu specifically associated to blue and green dyes (Hurley et al., 2017, and the references therein; Suteja et al., 2020). Accordingly, the previous studies of the Mrežnica River sediments revealed the highest concentrations of Fe and U at the sites close to the former textile factory, and the coals deposited directly into the Mrežnica River were probably the source of elevated U concentrations (Frančišković-Bilinski et al., 2017). The same authors have also reported that Cu concentrations in the sediments in the vicinity of the former textile factory exceeded the limit value of the USA federal criteria for heavily contaminated sediments (Frančišković-Bilinski et al., 2017).

The exceptions to this trend of increased metal(loid) concentrations at two industrially impacted sites were Ca and Cd, which were found in comparable or slightly higher concentrations at the REF site than at the DRF and/or KIZ site, and Mn which was higher at the REF site compared to DRF site, but lower compared to KIZ (Table 4). The Mrežnica River is a carbonate rich tufa-forming river which, at clean locations, has relatively high Ca concentrations in sediments; contrary, lower Ca levels were reported for contaminated locations (near textile factory) where the sediment is mixed with a lot of slag material (Frančišković-Bilinski et al., 2017). On the other hand, a slight increase of Mn in sediments at REF site can be attributed to the fact that Mn is a typical element not only for urban, but also for agricultural sewages, originating from fertilizers or disinfecting agents (Samecka-Cymerman and Kempers, 2007).

We have compared our results with existing sediment quality criteria for six elements (Cd, Cr, Cu, Ni, Pb, and Zn), summarized by Frančišković-Bilinski et al. (2017), which lead to conclusion that certain elements are present in the sediments in higher than desirable concentrations. Cadmium and Pb concentrations in the sediments of the Mrežnica River during our study never surpassed even the values for the lowest toxic effects of Canadian regulations (Cd: St. Lawrence R., 0.9 μg $g^{-1}$; Pb: British Columbia, 31 μg $g^{-1}$). However, Cr concentrations were always higher than Canadian lowest toxic effects value (British Columbia, 26 μg $g^{-1}$) at DRF and KIZ sites, and sometimes even at the REF site; at the KIZ site they have even approached the value for significant toxic effects (26 μg $g^{-1}$). Copper concentrations at the REF site were always lower than the limit for nonpolluted sediments (USA federal criteria; 25 μg $g^{-1}$), but at the KIZ site, and sometimes at the DRF site, they surpassed the value for the moderately contaminated sediments (USA federal criteria; 37.5 μg $g^{-1}$). Nickel concentrations at the REF site were always lower than the Canadian limit for the lowest toxic effects (St. Lawrence R.; 35 μg $g^{-1}$), but they were always above it at the KIZ site, and sometimes even at the DRF site; still, Ni was always below the Canadian value for the significant toxic effects (British Columbia; 75 μg $g^{-1}$). Zinc concentrations were mainly below the value for non-polluted sediments (USA federal criteria; <90 μg $g^{-1}$) at all three sites and only occasionally slightly surpassed it at the DRF and KIZ sites.

Our results were further compared to the values for bottom sediments of clean lakes in Poland given by Szymanowska et al. (1999; in μg $g^{-1}$: Co, 2.3-4.2; Cr, 1.1-1.9; Cu, 2-3; Fe, 3.0-9.9; Ni, 1.6-2.9; P, 6.8-8.5; Pb, 9.7-13) and in Ireland given by Bowman and Harlock (1998; Zn, 10-200 μg $g^{-1}$). With the exception of Zn, all the values in our study were much higher than concentrations reported for clean freshwaters. Furthermore, we have compared our results with the unpolluted and polluted sections of the Ilova River in Croatia (Mijošek et al., 2020). While we have observed that many elements were lower in the sediments of the Mrežnica River than in the unpolluted sediments of the Ilova River, several of them were either comparable or notably higher even compared to reports for the polluted sediments of the Ilova River. Namely, those were Cr, Ni, Sr and U at KIZ site, and Cu and Mo at both KIZ and DRF sites, which generally corresponded to metals that have surpassed above given sediment quality criteria (Frančišković-Bilinski et al., 2017). Thus, although the dissolved concentrations in the Mrežnica River have indicated rather clean river-water, the sediments pointed to certain level of contamination.

Similarly to our study, Hurley et al. (2017) reported that high metal concentrations in river sediments in the area of historical industrial contamination in Manchester region in UK were not accompanied by high levels of dissolved metals, which were even below the limits set by UK Environment Agency. According to them, the absence of significant relationship between dissolved metal concentrations and concentrations in bed sediments suggests that current contaminant inputs do not present the dominant influence on sediment contamination. Although metal(loid) concentrations in Manchester region sediments reported by Hurley et al. (2017) were much higher than observed in the Mrežnica River, the same principle could be applied. Thus, it could be presumed that both DRF and KIZ sites, as locations of long-term historical contamination, still reveal a certain degree of past sediment contamination with macro and trace elements, which can be especially hazardous for the ecosystem at DRF site due to high content of silt material in the middle of the riverbed.

### *3.5. The concentrations of trace and macro elements in the particulate fraction of the surface water of the Mrežnica River*

Suspended particulate matter (SPM) is a major carrier of inorganic and organic pollutants and nutrients in rivers; it originates from the weathering and erosion of soils and rocks, and from anthropogenic sources, thus being a complex mixture of minerals and organic solids, coated with organic substances (Renoldi et al., 1997, and the references therein). The results for particulate metal(loid) levels, i.e. metal(loid)s bound to suspended matter, in the water of the Mrežnica River further corroborated our hypothesis about the higher availability of silt fraction, and consequently also of metal(loid)s at the DRF site (Table 5). In April 2021, the observed concentration of SPM in river-water was higher at the REF site (1.77 mg $L^{-1}$), and somewhat lower at the remaining two sites (each 1.09 mg $L^{-1}$). The majority of analysed elements in the particulate fraction were then present in the highest concentrations at the DRF site, whereas few of them were comparable at DRF and KIZ sites (Ag, Pb, Se, Sn, Zn), or the highest at the KIZ site (Mn, Sr, Ca, Na). The differences were moderate, with only few elements two or three times higher at DRF compared to REF site (Ag, Ba, Fe, Mo, Ni, Se, and Zn).

In the September 2021, the observed concentration of SPM in river-water was the highest at the DRF site (6.23 mg $L^{-1}$; about five times higher than in the spring), and much lower at the remaining two sites (REF: 0.64 mg $L^{-1}$; KIZ: 0.56 mg $L^{-1}$; approximately two to three times lower than in the spring). Throughout our study, it was notably lower compared to Danube River near the Black Sea (20 mg $L^{-1}$; Guieu et al., 1998.), as well as to highly polluted rivers Lambro (33.7 mg $L^{-1}$) and Po (58.8 mg $L^{-1}$) in Italy (Renoldi et al., 1997, and the references therein). Moreover, it was comparable to Cornwall site at the St. Lawrence River in Canada (2.8 mg $L^{-1}$), and much lower compared to the other sites at that river (Port St. François, 16.2 mg $L^{-1}$; Québec, 19.9 mg $L^{-1}$) (Quémerais et al., 1996). Small amount of SPM, such as observed in this study, is typical for karst rivers like Mrežnica (Ivanić et al., 2020; Lučić et al., 2021). In September sampling, more notable differences in metal concentrations were observed between sites. The highest concentrations of the majority of analysed elements were again recorded at the DRF site, but now more elements were at least two times higher compared to REF site (As, Al, Co, Cr, Cs, Li, Rb, Tl, Ca), and some even three to six times (Bi, Cd, Cu, Ni, Pb). The concentrations of few elements at the KIZ site were again either comparable (Pb) or even higher compared to DRF site (Ag, Mn, Sn, Na), whereas, Mn was increased even at the REF site compared to DRF site.

Comparison of the maximal Cu, Cr and Fe particulate concentrations in the Mrežnica River with the reports for highly industrially contaminated Lambro River revealed 29 and 6 times lower Cu and Cr concentrations, respectively, in the Mrežnica River, whereas, Fe was 30% higher in the Mrežnica River; however, Fe levels in the Lambro River were typical for the natural drainage basin (Renoldi et al., 1997). Comparison of the Mrežnica River was further done with the St. Lawrence River in Canada (influenced by municipal, industrial and agricultural activities) and the Danube River (near the estuary) (Quémerais et al., 1996; Guieu et al., 1998), and here the differences between rivers were less pronounced. Several elements (Cd and Co in both rivers, Cu in St. Lawrence River, and Ni in Danube River) were only about two to three times higher than in the Mrežnica River at DRF site, whereas, Pb and Cu in the Danube River were ~3.5-5.5 times higher compared to the highest measured levels at the DRF site (Quémerais et al., 1996; Guieu et al., 1998). On the contrary, Mn at REF site of the Mrežnica River was 50% higher than reported for both St. Lawrence and Danube rivers, Fe at DRF site was 50% higher compared to St. Lawrence River, and Zn at DRF and KIZ sites of the Mrežnica River was comparable to values observed in the Danube (Quémerais et al., 1996; Guieu et al., 1998). Guieu et al. (1998) further suggested that, although particulate metal concentrations in the Danube River were similar to the major rivers of the world, the high pH of its river water (8.03-8.30) could have caused very low observed dissolved metal concentrations because under alkaline conditions a large fraction of many metals (e.g. Fe, Mn, Co, Al) precipitates in the hydroxide form or coprecipitates with thus formed particles. Similar explanation could be proposed in our study, since pH of the Mrežnica River water ranged from 8.05 to 8.38 (Table SI.2).

Although the actual concentrations of metal(loid)s bound to particulate matter can be perceived as only moderate, the obtained results have still confirmed that sediment at the DRF site, specifically in the middle section of the riverbed, definitely presents the reservoir of contaminants that can be released into the water-column under certain conditions and lead to increased exposure of local aquatic organisms. Moreover, continuously increased values of several elements in the particulate fraction at the KIZ site (Ag, Pb, Sn, Mn, Na) further confirmed the presence of certain level of contamination in the river section running through the Karlovac industrial area, even though many industrial facilities in that area have ceased to discharge their wastewaters into the Mrežnica River more than a decade ago.

### *3.6. The distribution patterns of total bacteria, heavy metal resistance genes and class 1 integrons in the sediments of the Mrežnica River*

We quantified the abundance of total bacteria (by targeting 16S rRNA genes), and their associated metal resistance genes (*cnr*A, *pbr*T and *czc*D) and class 1 integrons (*int*I1) as possible bioindicators of pollution in TC-DNA from sediment samples collected in two samplings (April and September 2021) at all three sites at the Mrežnica River using qPCR (Fig. 4). In spring, the average 16S rRNA gene copy number in sediment samples at two industrially impacted sites (DRF and KIZ; Fig. 4a) was similar to that at REF (~$6 \times 10^9$ copies $g^{-1}$), indicating similar abundance of bacteria at these three sites. However, in the autumn, the average 16S rRNA gene copy number was highest at the downstream DRF site ($8.8 \times 10^9$ copies $g^{-1}$) and was significantly different from the REF ($1.2 \times 10^9$ copies $g^{-1}$) and the KIZ site ($1.8 \times 10^9$ copies $g^{-1}$). There were seasonal differences in the copy number of the 16S rRNA gene in the sediment of the KIZ site. In spring, the copy number of 16S rRNA gene at that site was significantly higher than in autumn. In general, the average abundance of total bacteria in sediment samples at the three sampling sites was comparable to that found in river sediments under the influence of industrialization and agriculture (Chen et al., 2015; Koczura et al. 2016). The *cnr*A gene (Fig. 4b), which encodes an efflux pump that confers resistance to Co and Ni, was detected in spring 2021 only in sediment from the upstream REF site ($8.4 \text{ x} 10^5$ copies $g^{-1}$ dry sediment), but not at the industrially polluted downstream sites (DRF and KIZ) (Fig. 4b). No correlation was found between the *cnr*A gene and the amount of Co or Ni (Fig. S1). In contrast, the Pb-resistance gene *pbr*T (Fig. 4c) was only detected at downstream sites, such as the DRF site in spring 2021 or the KIZ site in autumn 2021; the latter being consistent with the highest Pb concentration measured at the KIZ site. Nonetheless, there was no correlation between the abundance of the *pbr*T gene and the amount of Pb (Fig. S1). Furthermore, Pb gene concentrations detected at downstream sites (~$10^5$ copies $g^{-1}$) were two orders of magnitude lower than those reported by Chen et al. (2019) for soils contaminated with heavy metals in a copper tailings dam area in China.

In contrast to the *cnr*A and *pbr*T genes, the *czc*D gene (Fig. 4d), which encodes an efflux pump that confers resistance to Co, Cd and Zn, was detected at all three sites in both sampling seasons, and a slight positive correlation was observed between *czc*D and Co levels (Fig. S1). Only in autumn 2021 was *czc*D significantly more abundant at the DRF site (~$10^7$ copies $g^{-1}$) than at the other two sites (~$10^4$ copies $g^{-1}$). This was unexpected because the highest Cd, Co, and Zn levels were measured in the sediment at the KIZ site. Still, concentrations of Cd and Co were the highest in the particulate fraction in water at the DRF site, and the difference was especially obvious in autumn, possibly indicating higher bioavailability of these metals to the microbiota in sediment at the DRF site. On the other hand, there was no significant difference in the abundance of the *czc*D gene in river sediments among the sampling sites in spring 2021, which may be related to the lower variation in bioavailability of the above metals (Cd, Co, and Zn) among the sites, as could be presumed based on their particulate concentrations (Table 5). Nevertheless, the *czc*D levels observed in river sediments in this study ($10^4$-$10^7$ gene copies $g^{-1}$) were higher than those reported for soils contaminated with heavy metals ($10^2$-$10^3$ gene copies $g^{-1}$) (Chen et al. 2019).

In addition, the class 1 integron-integrase gene, *int*I1, previously proposed as a genetic marker for anthropogenic pollution (Gillings et al., 2015), was found in sediment samples from all three sites (Fig. 4e), and was significantly more abundant at the industrially impacted sites than at REF site, indicating a contribution of industry to the increased abundance of class 1 integrons in the sediment microbial community. Indeed, the abundance of the *int*I1 gene was highest at the KIZ site compared to the other two sites in spring 2021, and at the DRF site compared to the REF and KIZ sites in autumn (~$10^7$ gene copies $g^{-1}$). The copy number of *int*I1 observed in this study was comparable to that found in Polish and Dutch rivers impacted by discharges from municipal wastewater treatment plants (Koczura et al. 2016; Sabri et al, 2018). The *int*I1 gene is typically associated with genes conferring resistance to heavy metals, synthetic organic compounds, and other environmental contaminants, and is found in a variety of environmental and clinical bacteria. The amount of *int*I1 can fluctuate rapidly due to the short generation time of host bacteria and horizontal gene transfer, reflecting variations in environmental contamination (Koczura et al. 2016).

## 4. Conclusions

The study on the Croatian river Mrežnica enabled the evaluation of the consequences that the continuous long-term historical contamination can leave on the freshwater ecosystems even a certain period (a decade or more) after the contamination have partially or completely ceased. First, we have observed slight influence of the agricultural practices at upstream rural river-section, revealed in occasional high concentration of total phosphorus, presence of agricultural herbicides atrazine, metolachlor and terburtryn in water, and detection of water contamination with some metals commonly present in fertilizers (e.g., Mn). However, a complex set of performed analyses has confirmed that the river section in the vicinity of the former textile factory, which has been polluting the river-water for more than hundred years incessantly, still demonstrates the signs of water contamination that in some cases can even be considered as troublesome (e.g., presence of industrial organic contaminants that can be traced to textile production; metal contamination of suspended particulate matter and sediments; lower Ca levels in sediments pointing to sediment being mixed with a slag material). The specificity of the former textile production location is that a small dam built in front of the factory for its requirements have created the section of stagnant river water and sediment rich in fine particles that abundantly adsorb metals, thus, presenting reservoir of contaminants that can be preserved for a long time. The river contamination at downstream industrial zone near Karlovac town have also been demonstrated, through increased ammonium and nitrite levels, presence of industrial herbicide in the water, and metal contamination of suspended particulate matter and sediments. According to the literature, the fact that the metal(loid) contamination of suspended particulate matter and especially sediments was far more notable than that of water at both industrial sites confirmed that historical contamination predominated over current contaminant inputs. More importantly, even mild sediment/suspended particulate matter contamination with metal(loid)s can affect sediment microbial communities. This was evident from increased abundance of the *czc*D gene at the former cotton industry site and the *int*I1 gene at both industrially impacted sites. The results of this comprehensive study emphasized the need for careful monitoring and long-lasting supervision of terminated sources of contamination.

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**Figure captions**

**Figure 1.** The map of the lower course of the Mrežnica River in Croatia with marked sampling sites: 1 – reference site (REF); 2 – Duga Resa factory (DRF); 3 – Karlovac industrial zone Mala Švarča (KIZ); * Mrzlo Polje – the location of the hydrological (i.e., gauging) station of the Meteorological and Hydrological Service of the Republic of Croatia.

**Figure 2.** Total concentrations and numbers of detected compounds in three categories of organic contaminants (herbicides, insecticides, and fungicides: H, I & F)) in river water at three sampling sites (REF – upstream reference location; DRF – Duga Resa factory; KIZ – Karlovac industrial zone) along the lower course of the Mrežnica River in Croatia: a) concentrations in May 2020; b) concentrations in April 2021; c) concentrations in September 2021; a) number of compounds in May 2020; b) number of compounds in April 2021; c) number of compounds in September 2021;. For each site in each season (May 2020, April and September 2021), the results of one screening analysis are present in a form of stacked vertical bars.

**Figure 3.** Concentrations of selected 12 dissolved trace elements that vary in river water among three sampling sites (REF – upstream reference location; DRF – Duga Resa factory; KIZ – Karlovac industrial zone) along the lower course of the Mrežnica River in Croatia: a) Al; b) Ba; c) Cs; d) Li; e) Na; f) Rb; g) V; h) Co; i) Cu; j) Fe; k) Cd; and l) Tl. For each site, the results encompass triplicate measurements in six samplings (April 2020 to October 2021) (n=18). The results are presented as box-plots whose boundaries indicate 25th and 75th percentiles; a line within the box marks the median value; whiskers (error bars) above and below the box indicate 10th and 90th percentiles; and the black dots present outliers. Comparisons among concentrations at three sites were performed by Kruskal-Wallis one-way analysis of variance on ranks. When differences among three sites were statistically significant (*p*<0.05; indicated within each figure), pairwise multiple comparisons were performed by Dunn’s method, and

the sites that differed statistically significantly from one another ($p<0.05$) were indicated by different letters (**a**,**b**).

**Figure 4.** Absolute abundance (gene copy number / g dry mass) of total bacteria (16S rRNA genes) (A), heavy metal resistance genes – *cnr*A (B), *pbr*T (C), and *czc*D (D), and class 1 integron-integrase gene (*int*I1) (E) in Mrežnica River sediments from three sites in two sampling seasons (spring and autumn 2021). Each value is the mean ± SD of three replicates. Different letters indicate significant difference ($p < 0.05$) using multiple comparison Fisher's LSD test.

**Figure 1.**

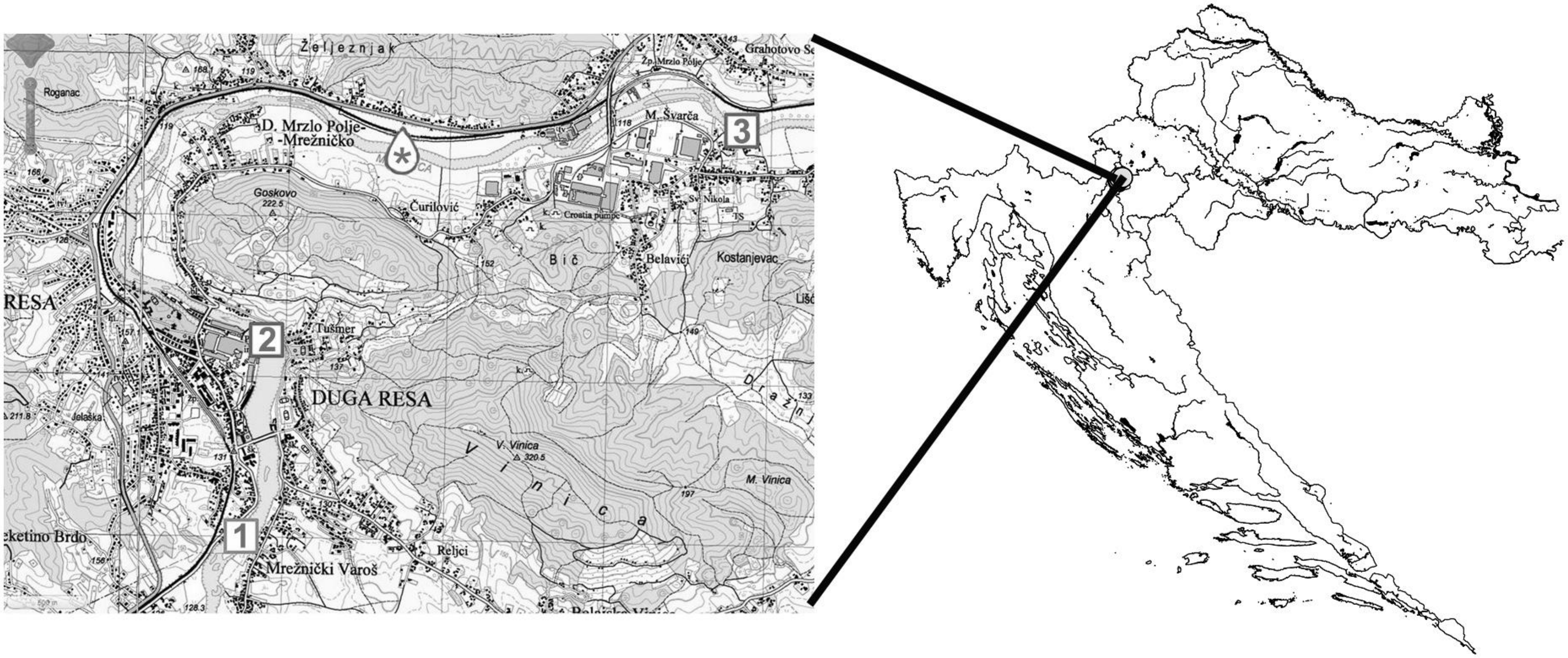
Željeznjak
Roganac
168.1
119
D. Mrzlo Polje-
-Mrežničko
166
126
Goskovo
222.5
Čurilović
Grahotovo Se
143
Žp. Mrzlo Polje
M. Švarča
118
3
Croatia pumpe
Sv. Nikola
TS
Bič
152
Belavići
Kostanjevac
Lišć
RESA
124
157.1
2
Tušmer
137
141
149
DUGA RESA
Dražni
133
211.8
Jelaška
131
V. Vinica
320.5
Vinica
M. Vinica
197
130
1
eketino Brdo
156
Mrežnički Varoš
Reljci
128.3
500 m

**Figure 2.**

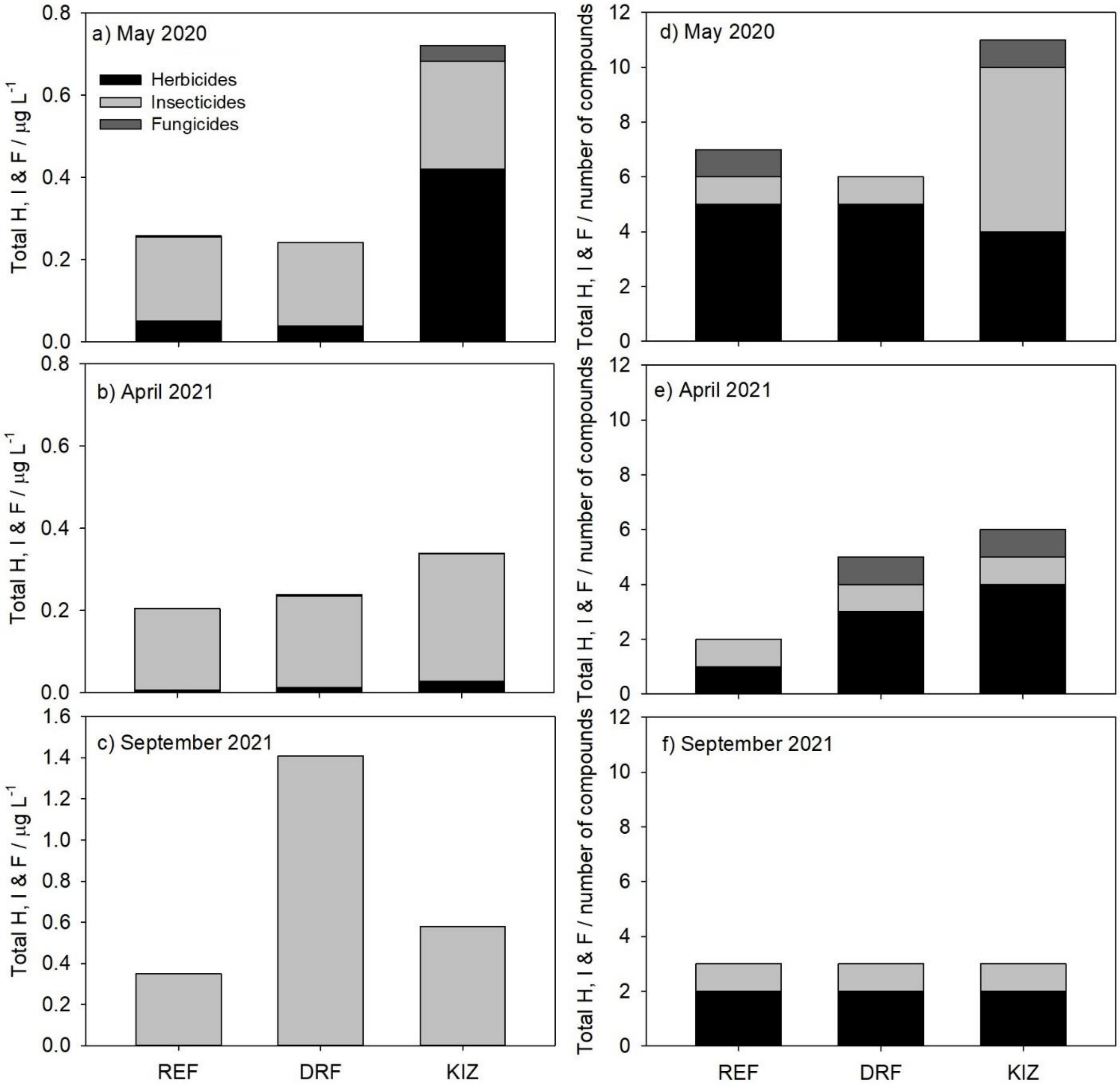
a) May 2020
Herbicides
Insecticides
Fungicides
Total H, I & F / μg L$^{-1}$
0.0
0.2
0.4
0.6
0.8
b) April 2021
c) September 2021
1.0
1.2
1.4
1.6
d) May 2020
e) April 2021
f) September 2021
Total H, I & F / number of compounds
0
2
4
6
8
10
12
REF
DRF
KIZ

**Figure 3.**

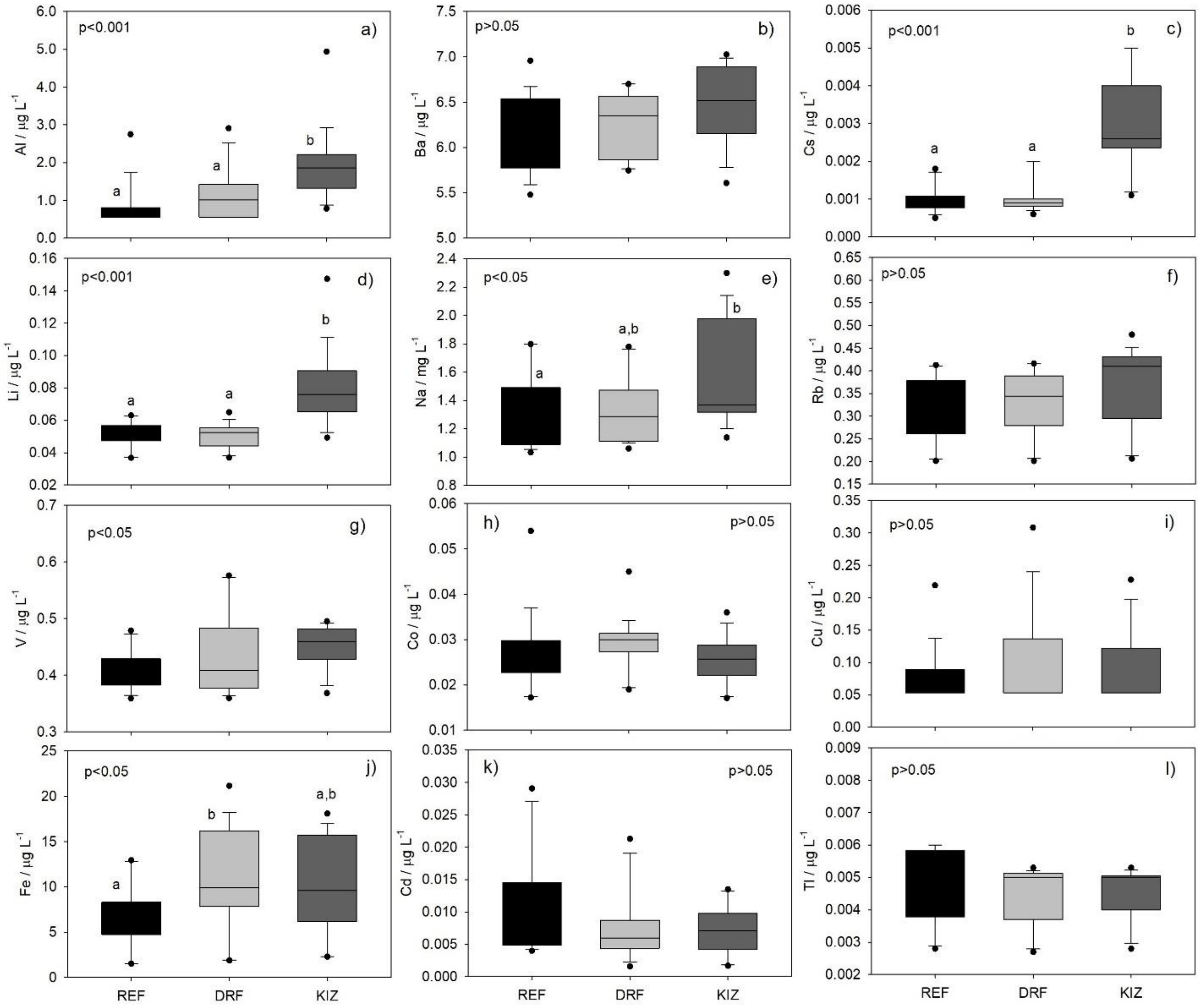
a)
p<0.001
Al / μg L⁻¹
b)
p>0.05
Ba / μg L⁻¹
c)
p<0.001
Cs / μg L⁻¹
d)
p<0.001
Li / μg L⁻¹
e)
p<0.05
Na / mg L⁻¹
f)
p>0.05
Rb / μg L⁻¹
g)
p<0.05
V / μg L⁻¹
h)
p>0.05
Co / μg L⁻¹
i)
p>0.05
Cu / μg L⁻¹
j)
p<0.05
Fe / μg L⁻¹
k)
p>0.05
Cd / μg L⁻¹
l)
p>0.05
Tl / μg L⁻¹
a
b
a,b
REF
DRF
KIZ

**Figure 4.**

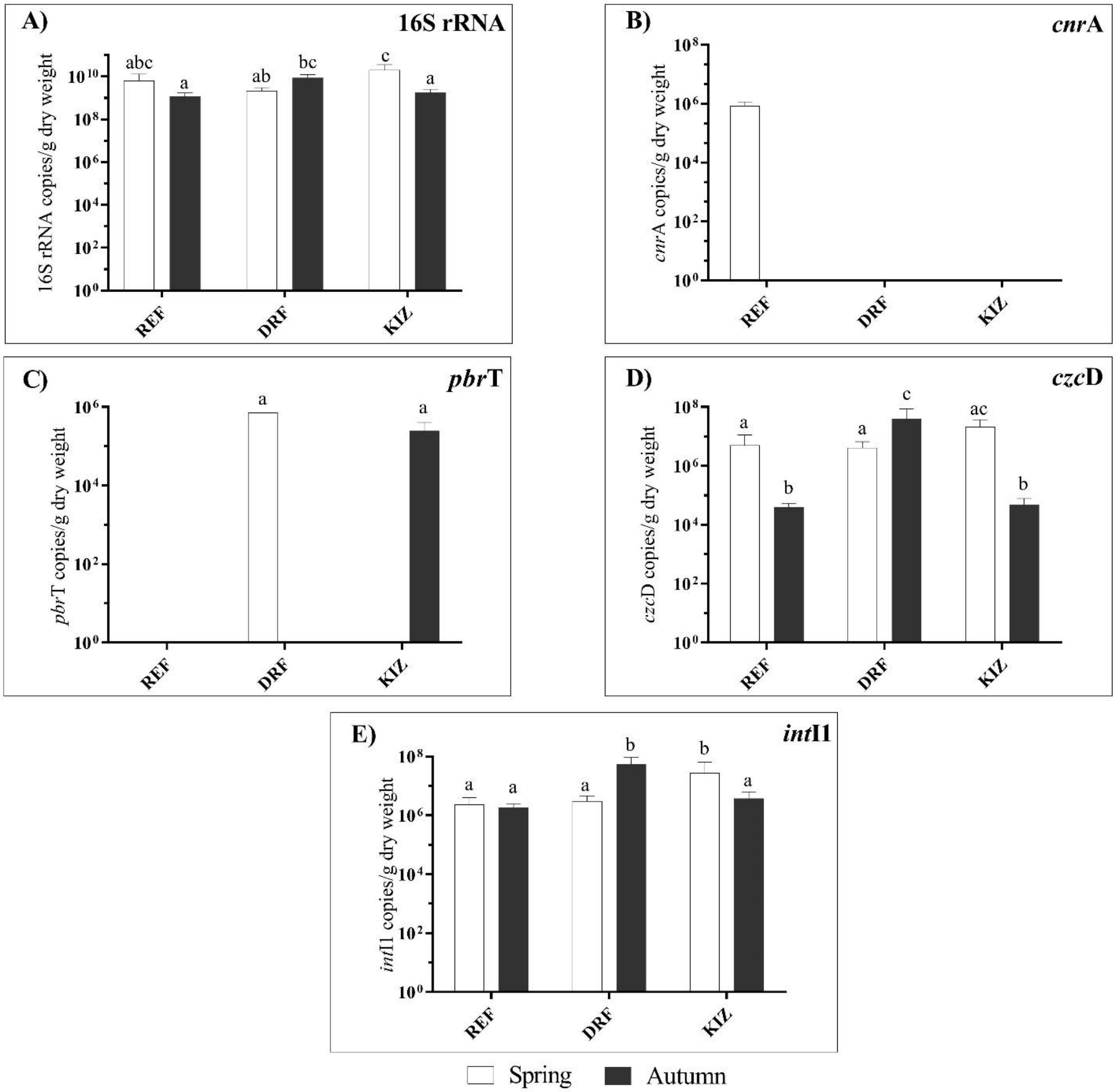
A)
16S rRNA
16S rRNA copies/g dry weight
10^10
10^8
10^6
10^4
10^2
10^0
abc
a
ab
bc
c
a
REF
DRF
KIZ
B)
cnrA
cnrA copies/g dry weight
10^8
10^6
10^4
10^2
10^0
REF
DRF
KIZ
C)
pbrT
pbrT copies/g dry weight
10^6
10^4
10^2
10^0
a
a
REF
DRF
KIZ
D)
czcD
czcD copies/g dry weight
10^8
10^6
10^4
10^2
10^0
a
b
a
c
ac
b
REF
DRF
KIZ
E)
intI1
intI1 copies/g dry weight
10^8
10^6
10^4
10^2
10^0
a
a
a
b
b
a
REF
DRF
KIZ
Spring
Autumn

**Table 1.** Selected physico-chemical parameters, nutrients and chlorophyll *a* measured in three samplings at three sites along the lower course of the Mrežnica River in Croatia (n=1).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Dissolved oxygen / mg L$^{-1}$** | May 2020 | 10.1 | 9.71 | 9.24 |
| | April 2021 | 11.4 | 11.5 | 11.3 |
| | September 2021 | 9.04 | 10.2 | 9.66 |
| **Oxygen saturation / %** | May 2020 | 108 | 102 | 98.3 |
| | April 2021 | 105 | 106 | 108 |
| | September 2021 | 96.6 | 112 | 104 |
| **$CO_2$ / mg L$^{-1}$** | May 2020 | 1.2 | 1.5 | 1.1 |
| | April 2021 | 1.1 | 1.2 | 1.0 |
| | September 2021 | 2.1 | 2.7 | 2.2 |
| **Chemical oxygen demand / mg $O_2$ L$^{-1}$** | May 2020 | 4.6 | 4.8 | 6.4 |
| | April 2021 | 4.0 | 3.6 | 4.8 |
| | September 2021 | 3.8 | 4.1 | 3.7 |
| **Ammonium / mg N L$^{-1}$** | May 2020 | 0.11 | 0.09 | 0.16 |
| | April 2021 | 0.02 | 0.03 | 0.03 |
| | September 2021 | 0.08 | 0.10 | 0.07 |
| **Total nitrogen / mg N L$^{-1}$** | May 2020 | 1.50 | 1.24 | 1.75 |
| | April 2021 | 0.80 | 1.50 | 0.40 |
| | September 2021 | 0.20 | 0.20 | 0.50 |
| **Nitrites / mg N L$^{-1}$** | May 2020 | 0.011 | 0.008 | 0.015 |
| | April 2021 | 0.021 | 0.022 | 0.039 |
| | September 2021 | 0.011 | 0.013 | 0.015 |
| **Nitrates / mg N L$^{-1}$** | May 2020 | 0.01 | 0.01 | 0.02 |
| | April 2021 | 0.28 | 0.25 | 0.29 |
| | September 2021 | 0.07 | 0.04 | 0.08 |
| **Total phosphorus / mg P L$^{-1}$** | May 2020 | 0.266 | 0.041 | <0.010 |
| | April 2021 | 0.017 | 0.021 | 0.022 |
| | September 2021 | 0.013 | 0.019 | 0.003 |
| **Chlorophyll *a* / mg L$^{-1}$** | May 2020 | - | - | - |
| | April 2021 | 0.757 | 0.516 | 0.871 |
| | September 2021 | 0.810 | 3.34 | 1.13 |

**Table 2.** Organic contaminants (herbicides, insecticides and fungicides) quantitatively detected in three samplings (May 2020, April and September 2021) at three sites along the lower course of the Mrežnica River in Croatia.

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Herbicides** | May 2020 | **Atrazine metabolites (43%)**, diuron (16%), metolachlor (37%), terbuthylazine (4%) | Atrazine and metabolite (6%), diuron (23%), **metolachlor (54%)**, terbuthylazine (17%) | Diuron (1.5%), metolachlor (1%), **neburon (97%)**, terbuthylazine (0.5%) |
| | April 2021 | **Metolachlor (100%)** | Diuron (15%), terbuthylazine (22%), **terbutryn (63%)** | Diuron (11%), metolachlor (33%), terbuthylazine (20%), **terbutryn (36%)** |
| | September 2021 | **Atrazine and metabolite (100%)** | **Atrazine metabolite (68%),** diuron (32%) | **Atrazine metabolites (100%)** |
| **Insecticides** | May 2020 | **Nicotine (100%)** | **Nicotine (100%)** | Bifenazate (22%), **methiocarb metabolites (30%)**, nicotin (28%), primicarb (1%), sulprofos (19%) |
| | April 2021 | **Nicotine (100%)** | **Nicotine (100%)** | **Nicotine (100%)** |
| | September 2021 | **Nicotine (100%)** | **Nicotine (100%)** | **Nicotine (100%)** |
| **Fungicides** | May 2020 | **Tetraconazole (100%)** | - | **Mepronil (100%)** |
| | April 2021 | **-** | **Tetraconazole (100%)** | **Tetraconazole (100%)** |
| | September 2021 | **-** | - | **-** |

**Table 3.** Qualitative QTOF-MS screening of water samples taken from the Mrežnica River in May 2020 at two sites, reference location upstream of Duga Resa (REF), and location in front of the former cotton industry facility in Duga Resa (DRF).

| Detected compound (n=28) | Known applications | Reference location (REF) | Duga Resa – factory (DRF) |
|---|---|---|---|
| **Compounds detected at both sites (n=9, i.e. 32%)** | | | |
| 1,4-Bis{2-[(2-methyl-2-propanyl)peroxy]-2-propanyl}benzene | polymerization initiator | + | + |
| o-Dianisidine | precursor to some azo dyes (Direct Blue) for drugs, textile, and personal care products | + | + |
| 3,3'-Dimethoxybenzidine (Dianisidine) | precursor to some azo dyes (Direct Blue) for drugs, textile, and personal care products | + | + |
| 2-Hydroxysebacic acid | urinary biomarker for the consumption of anatidaes, chickens, and domestic pigs | + | + |
| Lauryl diethanolamide | ingredient used in cosmetics | + | + |
| (2,2′-methylene-bis(6-tert-butyl-4-methylphenol)), BKF (Cyanox 2246) | polymer additive; antioxidant; food packaging contaminant; printing ink component | + | + |
| MNA / m-Nitroaniline (3-Nitroaniline) | precursor to some azo dyes | + | + |
| 4-Nitroaniline | precursor to some azo dyes, pharmaceuticals and pesticides | + | + |
| Ormetoprim | antibiotic for use in dogs and cats | + | + |
| **Compounds detected only at REF (n=5, i.e. 18 %)** | | | |
| 2-Aminopyridine | used in the production of the drugs | + | - |
| 2-Amino-9H-pyrido[2,3-b]indole (AalphaC) | found in grilled foods, in the pyrolysis products of proteins and in cigarette smoke | + | - |
| Cytarabine | cancer drug (for leukemia) | + | - |
| Eicosenamide | food packaging contaminant; printing ink component | + | - |
| Ethylene azelate | polyester polyol | + | - |

**Table 3.-continued.** Qualitative QTOF-MS screening of water samples taken from the Mrežnica River in May 2020 at two sites, reference location upstream of Duga Resa (REF), and location in front of the former cotton industry facility in Duga Resa (DRF).

| Detected compound (n=28) | Known applications | Reference location (REF) | Duga Resa – factory (DRF) |
|---|---|---|---|
| **Compounds detected only at DRF (n=14, i.e. 50 %)** | | | |
| 2-Amino-3-methyl-imidazo[4,5-f]quinoline (IQ) | food-derived carcinogen found in high temperature-cooked meats and tobacco smoke | - | + |
| Azelaic acid | food packaging contaminant; compound found in wheat, rye and barley; used in cosmetic products; printing ink component | - | + |
| Benodanil | obsolete fungicide | - | + |
| Benzoic acid | pH adjustor and preservative used in manufacture of perfumes, dyes, topical medications and insect repellents | - | + |
| Bisphenol A bis(2,3-dihydroxypropyl) ether | formed by degradation of dental composite material bis-GMA by salivary esterases; released from polymers of the inner coating of cans | - | + |
| Caprolactam cyclic dimer | impurity always present in industrial nylon 6 | - | + |
| 3,3'-Dimethylbenzidine | fragrances or odor agents used in home products (cleaners, laundry products, air fresheners) | - | + |
| Ditrimethylolpropane tetraacrylate | acrylate monomer for litho and screen inks; printing ink component | - | + |
| Hexaethyleneglycol dimethylether | used in numerous industrial applications, such as cleaning products, inks, adhesives and coatings, batteries and electronics | - | + |
| 4-Hydroxyantipyrine | metabolite of antipyrine, analgesic used for treatment of acute otitis media; also a veterinary drug | - | + |
| Irganox 1081 (Thioalkofen BP) | plastic additive - antioxidant; printing ink component | - | + |
| 4-MBC / 3-(4-methylbenzylidene)-camphor | ingredient in pain relief medications, including topical analgesics; sunscreen agent | - | + |
| Phthalic anhydride | plastic additive -retarder; printing ink component | - | + |
| 4,4'-bi-o-Toluidine | used in manufacture of dye (yellow) | - | + |

**Table 4.** Concentrations of trace and macro elements in sediments measured in three samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviation (n=3).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Al / $\mathrm{mg\ g^{-1}}$** | May 2020* | 5.14±0.35[a] | 23.6±0.4 | 28.0±4.1[b] |
| | April 2021 | 20.9±9.5 | 30.4±13.5 | 32.3±8.6 |
| | September 2021 | 19.0±2.2 | 20.3±0.9 | 26.3±2.6 |
| **As / $\mathrm{\mu g\ g^{-1}}$** | May 2020 | 4.81±0.40 | 4.30±0.32 | 5.25±0.53 |
| | April 2021 | 4.73±1.38 | 6.15±4.38 | 6.41±0.94 |
| | September 2021* | 3.41±0.47[a] | 3.79±0.20 | 7.24±0.67[b] |
| **Ba / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 43.4±4.6[a] | 110±3 | 141±16[b] |
| | April 2021 | 102±43 | 154±91 | 161±48 |
| | September 2021 | 82.2±9.3 | 85.9±3.4 | 148±5 |
| **Bi / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 0.044±0.003 | 0.152±0.004 | 0.047±0.004 |
| | April 2021 | 0.330±0.112 | 0.253±0.225 | 0.416±0.152 |
| | September 2021* | 0.254±0.030 | 0.226±0.039[a] | 0.405±0.052[b] |
| **Cd / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 0.710±0.074[a] | 0.492±0.031[b] | 0.483±0.022[b] |
| | April 2021 | 0.509±0.067 | 0.834±0.564 | 0.537±0.021 |
| | September 2021* | 0.516±0.047 | 0.387±0.020[a] | 0.582±0.058[b] |
| **Co / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 3.68±0.22[a] | 5.83±0.06 | 8.67±0.75[b] |
| | April 2021 | 6.23±2.00 | 8.85±5.55 | 10.0±2.5 |
| | September 2021 | 5.27±0.66 | 5.36±0.10 | 10.1±0.3 |
| **Cr / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 11.4±0.9[a] | 37.4±0.9 | 65.6±9.2[b] |
| | April 2021 | 52.6±17.5 | 71.1±37.7 | 81.0±13.9 |
| | September 2021* | 37.9±5.4[a] | 41.4±2.8 | 84.4±3.1[b] |
| **Cs / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 0.549±0.038[a] | 2.54±0.06 | 5.50±0.48[b] |
| | April 2021 | 2.02±0.85 | 3.53±2.15 | 4.13±0.94 |
| | September 2021* | 1.87±0.19[a] | 2.06±0.07 | 5.04±0.41[b] |
| **Cu / $\mathrm{\mu g\ g^{-1}}$** | May 2020* | 7.16±1.73[a] | 15.9±0.7 | 20.6±2.0[b] |
| | April 2021 | 19.4±6.0 | 38.4±29.9 | 31.7±8.9 |
| | September 2021* | 15.3±2.5[a] | 19.0±1.7 | 38.9±1.8[b] |

* statistically significant differences among sites in specific season according to Kruskal-Wallis one way analysis of variance on ranks ($p<0.05$)

[a,b] statistically significant differences between sites according to all pairwise multiple comparison procedures (Tukey Test)

**Table 4.-continued.** Concentrations of trace and macro elements in sediments measured in three samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviation (n=3).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Fe / mg g$^{-1}$** | May 2020* | 7.22±0.62[a] | 12.3±0.2 | 23.2±2.0[b] |
| | April 2021 | 11.7±3.7 | 17.2±11.6 | 21.9±5.4 |
| | September 2021 | 9.69±1.24 | 10.2±0.4 | 20.1±1.7 |
| **Li / µg g$^{-1}$** | May 2020* | 4.66±0.34[a] | 20.7±0.90 | 31.0±3.0[b] |
| | April 2021 | 16.7±7.2 | 27.8±16.3 | 26.0±5.4 |
| | September 2021* | 14.7±1.3[a] | 15.9±0.8 | 29.4±2.5[b] |
| **Mn / µg g$^{-1}$** | May 2020* | 405±24 | 286±5[a] | 458±10[b] |
| | April 2021 | 318±14 | 295±152 | 437±61 |
| | September 2021 | 220±24 | 210±7 | 395±20 |
| **Mo / µg g$^{-1}$** | May 2020* | 0.253±0.047[a] | 0.905±0.026 | 4.25±0.73[b] |
| | April 2021 | 0.935±0.207 | 1.77±1.06 | 2.46±0.39 |
| | September 2021* | 0.976±0.117[a] | 1.11±0.08 | 2.63±0.18[b] |
| **Ni / µg g$^{-1}$** | May 2020* | 9.34±0.86[a] | 27.4±0.3 | 51.0±8.9[b] |
| | April 2021 | 25.8±6.6 | 44.5±25.3 | 48.8±5.8 |
| | September 2021* | 23.6±3.0[a] | 26.4±1.8 | 54.2±8.3[b] |
| **P / µg g$^{-1}$** | May 2020* | 350±23[a] | 503±9 | 526±35[b] |
| | April 2021 | 518±124 | 882±577 | 649±163 |
| | September 2021* | 424±44[a] | 520±68 | 620±9[b] |
| **Pb / µg g$^{-1}$** | May 2020* | 17.7±1.3 | 21.4±0.3[a] | 15.3±2.2[b] |
| | April 2021 | 13.3±6.0 | 21.6±18.9 | 26.9±21.8 |
| | September 2021* | 9.95±0.86[a] | 12.5±2.7 | 20.6±1.7[b] |
| **Rb / µg g$^{-1}$** | May 2020* | 6.71±0.41[a] | 31.9±0.9[b] | 26.8±0.9 |
| | April 2021 | 28.0±12.7 | 46.9±27.8 | 38.8±11.7 |
| | September 2021 | 25.4±2.7 | 27.0±1.1 | 45.2±5.2 |
| **Sb / µg g$^{-1}$** | May 2020* | 0.283±0.008[a] | 0.607±0.032[b] | 0.456±0.057 |
| | April 2021 | 0.537±0.229 | 0.874±0.683 | 0.799±0.337 |
| | September 2021 | 0.434±0.062 | 0.422±0.029 | 4.19±5.66 |
| **Sn / µg g$^{-1}$** | May 2020 | 1.29±0.18 | 2.18±0.06 | 1.23±0.28 |
| | April 2021 | 1.52±0.68 | 3.56±3.38 | 1.89±0.55 |
| | September 2021 | 1.25±0.09 | 1.32±0.15 | 8.42±10.3 |

* statistically significant differences among sites in specific season according to Kruskal-Wallis one way analysis of variance on ranks ($p<0.05$)

[a,b] statistically significant differences between sites according to all pairwise multiple comparison procedures (Tukey Test, except Dunn's method for Pb in September 2021)

**Table 4.-continued.** Concentrations of trace and macro elements in sediments measured in three samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviation (n=3).

| | | Reference location (REF) | Duga Resa – factory (DRF) | Karlovac – industrial zone (KIZ) |
|---|---|---|---|---|
| **Sr / µg g$^{-1}$** | May 2020* | 81.6±7.0[a] | 111±2 | 217±22[b] |
| | April 2021 | 93.9±9.0 | 130±69 | 140±15 |
| | September 2021* | 81.2±9.4[a] | 128±5[b] | 115±12 |
| **Ti / mg g$^{-1}$** | May 2020* | 0.337±0.020[a] | 1.64±0.03[b] | 1.33±0.03 |
| | April 2021 | 1.65±0.89 | 2.37±1.43 | 2.07±0.70 |
| | September 2021 | 1.40±0.18 | 1.38±0.05 | 2.33±0.33 |
| **Tl / µg g$^{-1}$** | May 2020* | 0.191±0.010[a] | 0.446±0.006[b] | 0.258±0.007 |
| | April 2021 | 0.356±0.097 | 0.598±0.342 | 0.398±0.053 |
| | September 2021 | 0.348±0.035 | 0.333±0.014 | 0.538±0.070 |
| **U / µg g$^{-1}$** | May 2020* | 0.444±0.050[a] | 1.30±0.03 | 8.92±1.75[b] |
| | April 2021 | 1.18±0.48 | 1.99±1.33 | 3.75±0.76 |
| | September 2021 | 1.08±0.12 | 1.16±0.03 | 3.94±0.37 |
| **V / µg g$^{-1}$** | May 2020* | 17.3±1.2[a] | 47.6±0.8 | 84.1±12.8[b] |
| | April 2021 | 38.6±15.4 | 64.8±41.3 | 63.0±11.1 |
| | September 2021 | 36.6±4.8 | 37.3±1.3 | 71.1±6.5 |
| **Zn / µg g$^{-1}$** | May 2020 | 46.0±13.7 | 59.6±11.2 | 40.1±5.2 |
| | April 2021 | 49.1±19.9 | 94.6±73.1 | 93.2±30.2 |
| | September 2021* | 36.1±2.1[a] | 48.5±3.6 | 68.2±1.1[b] |
| **Ca / mg g$^{-1}$** | May 2020 | 368±21 | 265±4 | 263±9 |
| | April 2021 | 288±65 | 327±142 | 253±53 |
| | September 2021 | 279±33 | 280±19 | 188±10 |
| **K / mg g$^{-1}$** | May 2020* | 0.823±0.032[a] | 3.87±0.24[b] | 3.58±0.26 |
| | April 2021 | 3.65±1.88 | 5.80±3.45 | 5.14±1.89 |
| | September 2021* | 3.14±0.37[a] | 3.47±0.16 | 6.06±0.65[b] |
| **Mg / mg g$^{-1}$** | May 2020* | 2.36±0.13[a] | 4.99±0.11 | 5.45±0.42[b] |
| | April 2021 | 5.44±1.36 | 7.41±3.81 | 9.51±2.26 |
| | September 2021 | 5.08±0.47 | 5.23±0.07 | 7.21±0.78 |
| **Na / mg g$^{-1}$** | May 2020* | 0.326±0.016[a] | 0.975±0.039 | 1.70±0.13[b] |
| | April 2021 | 1.15±0.62 | 1.48±0.80 | 2.00±0.91 |
| | September 2021 | 0.887±0.123 | 0.928±0.066 | 1.86±0.05 |

* statistically significant differences among sites in specific season according to Kruskal-Wallis one way analysis of variance on ranks ($p<0.05$)

[a,b] statistically significant differences between sites according to all pairwise multiple comparison procedures (Tukey Test)

**Table 5.** The concentrations of particulate trace and macro elements measured in two samplings at three sites along the lower course of the Mrežnica River in Croatia (n=1).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Ag / µg g$^{-1}$** | April 2021 | 0.313 | 0.903 | 1.04 |
| | September 2021 | 0.165 | 0.113 | 0.240 |
| **Al / mg g$^{-1}$** | April 2021 | 28.7 | 49.9 | 32.6 |
| | September 2021 | 17.5 | 39.4 | 13.1 |
| **As / µg g$^{-1}$** | April 2021 | 8.04 | 13.5 | 9.57 |
| | September 2021 | 4.14 | 9.63 | 6.03 |
| **Ba / µg g$^{-1}$** | April 2021 | 80.8 | 159 | 68.7 |
| | September 2021 | 59.3 | 94.9 | 66.0 |
| **Bi / µg g$^{-1}$** | April 2021 | 0.314 | 0.434 | 0.310 |
| | September 2021 | 0.144 | 0.512 | 0.154 |
| **Cd / µg g$^{-1}$** | April 2021 | 0.278 | 0.390 | 0.241 |
| | September 2021 | 0.262 | 0.610 | 0.272 |
| **Co / µg g$^{-1}$** | April 2021 | 4.85 | 8.58 | 5.89 |
| | September 2021 | 6.64 | 13.0 | 5.07 |
| **Cr / µg g$^{-1}$** | April 2021 | 35.3 | 62.1 | 40.8 |
| | September 2021 | 23.2 | 46.7 | 20.6 |
| **Cs / µg g$^{-1}$** | April 2021 | 2.91 | 4.97 | 3.36 |
| | September 2021 | 1.79 | 4.57 | 1.44 |
| **Cu / µg g$^{-1}$** | April 2021 | 8.20 | 10.8 | 6.92 |
| | September 2021 | 3.91 | 21.1 | <LOD* |
| **Fe / mg g$^{-1}$** | April 2021 | 20.4 | 37.7 | 24.8 |
| | September 2021 | 18.4 | 31.1 | 18.0 |
| **Li / µg g$^{-1}$** | April 2021 | 25.1 | 41.9 | 28.3 |
| | September 2021 | 16.1 | 38.9 | 11.7 |
| **Mn / mg g$^{-1}$** | April 2021 | 0.389 | 0.482 | 0.556 |
| | September 2021 | 2.56 | 0.767 | 2.47 |
| **Mo / µg g$^{-1}$** | April 2021 | 1.04 | 1.93 | 1.22 |
| | September 2021 | 0.907 | 1.52 | 1.08 |
| **Ni / µg g$^{-1}$** | April 2021 | 30.9 | 57.1 | 46.0 |
| | September 2021 | 11.5 | 44.6 | 10.7 |

* LOD for Cu in particulate fraction.

**Table 5.-continued.** The concentrations of particulate trace and macro elements measured in two samplings at three sites along the lower course of the Mrežnica River in Croatia (n=1).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Pb / $\mu g\ g^{-1}$** | April 2021 | 12.8 | 18.6 | 19.1 |
| | September 2021 | 3.49 | 23.4 | 19.2 |
| **Rb / $\mu g\ g^{-1}$** | April 2021 | 21.4 | 36.1 | 24.3 |
| | September 2021 | 15.1 | 31.8 | 11.4 |
| ***Se / $\mu g\ g^{-1}$** | April 2021 | 0.648 | 1.36 | 1.47 |
| | September 2021 | - | - | - |
| **Sn / $\mu g\ g^{-1}$** | April 2021 | 0.814 | 1.18 | 1.16 |
| | September 2021 | 0.785 | 0.983 | 2.49 |
| **Sr / $\mu g\ g^{-1}$** | April 2021 | 35.0 | 39.6 | 50.3 |
| | September 2021 | 67.5 | 104 | 78.9 |
| **Ti / $\mu g\ g^{-1}$** | April 2021 | 525 | 813 | 613 |
| | September 2021 | 440 | 638 | 387 |
| **Tl / $\mu g\ g^{-1}$** | April 2021 | 0.464 | 0.780 | 0.507 |
| | September 2021 | 0.302 | 0.591 | 0.241 |
| **U / $\mu g\ g^{-1}$** | April 2021 | 0.720 | 1.23 | 0.903 |
| | September 2021 | 0.614 | 1.06 | 0.886 |
| **V / $\mu g\ g^{-1}$** | April 2021 | 62.1 | 110 | 73.8 |
| | September 2021 | 44.6 | 77.7 | 40.3 |
| ***Zn / $\mu g\ g^{-1}$** | April 2021 | 68.4 | 235 | 262 |
| | September 2021 | - | - | - |
| **Ca / $mg\ g^{-1}$** | April 2021 | 82.7 | 48.0 | 122 |
| | September 2021 | 71.8 | 152 | 70.4 |
| **K / $mg\ g^{-1}$** | April 2021 | 3.23 | 5.66 | 4.30 |
| | September 2021 | 3.00 | 4.19 | 2.98 |
| **Mg / $mg\ g^{-1}$** | April 2021 | 3.05 | 4.85 | 3.94 |
| | September 2021 | 4.43 | 5.20 | 4.80 |
| **Na / $mg\ g^{-1}$** | April 2021 | 0.501 | 0.688 | 0.849 |
| | September 2021 | 0.437 | 0.320 | 1.07 |

* Se and Zn concentrations in September 2021 are unavailable due to accidental sample contamination

**Table 6.** Primers and conditions used to quantify genes targeted in this study**.**

| Gene target | Primer sequence (5' → 3') | Size (bp) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|
| *czc*D | TCATCGCCGGTGCGATCATCAT<br>TGTCATTCACGACATGAACC | 272 | 55 | Roosa et al., 2014 |
| *pbr*T | AGCGCGCCCAGGAGCGCAGCGTCTT<br>GGCTCGAAGCCGTCGAGRTA | 488 | 55 | |
| *cnr*A | CCTACGATCTCGCAGGTGAC<br>GCAGTGTCACGGAAACAACC | 422 | 60 | Fierros-Romero et al., 2016 |
| *int*I1 | GATCGGTCGAATGCGTGT<br>GCCTTGATGTTACCCGAGAG | 196 | 57 | Rocha et al., 2020 |
| 16S rRNA | CCTACGGGAGGCAGCAG<br>ATTACCGCGGCTGCTGGCA | 196 | 60 | López-Gutiérrez et al., 2004 |

# Supplementary materials

**Table SI.1.** Average daily values of water-levels and water discharges for each sampling date at the station Mrzlo Polje in the lower part of the Mrežnica River watercourse.

| | **Water-level / cm** | **Water discharge / $m^3 s^{-1}$** |
|---|---|---|
| **20 May 2020** | 6 | 6.66 |
| **22 May 2020** | 47 | 17.3 |
| **7 July 2020** | -6 | 4.32 |
| **22 April 2021** | 42 | 15.8 |
| **23 April 2021** | 40 | 15.3 |
| **6 June 2021** | 12 | 7.65 |
| **22 September 2021** | 4 | 6.20 |
| **23 September 2021** | 3 | 5.88 |
| **3 October 2021** | -17 | 2.70 |
| **Minimum 2020** | -26 | 1.59 |
| **Minimum 2021** | -26 | 1.59 |
| **Maximum 2020** | 251 | 145 |
| **Maximum 2021** | 278 | 161 |
| **Annual average 2020** | 29 | 15.3 |
| **Annual average 2021** | 46 | 21.7 |

**Table SI.2.** Selected physico-chemical parameters measured in three samplings at three sites along the lower course of the Mrežnica River in Croatia (n=1).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Turbidity / FAU** | May 2020 | 3.0 | 1.0 | 7.0 |
| | April 2021 | 2.0 | 0 | 3.0 |
| | September 2021 | 3.0 | 4.0 | 1.0 |
| **Water temperature / °C** | May 2020 | 18.1 | 17.8 | 17.7 |
| | April 2021 | 11.5 | 11.2 | 13.1 |
| | September 2021 | 18.5 | 17.9 | 18.5 |
| **Conductivity / µS cm$^{-1}$** | May 2020 | 324 | 328 | 317 |
| | April 2021 | 375 | 377 | 366 |
| | September 2021 | 333 | 328 | 327 |
| **TDS / mg L$^{-1}$** | May 2020 | 164 | 162 | 158 |
| | April 2021 | 188 | 188 | 183 |
| | September 2021 | 167 | 164 | 164 |
| **pH** | May 2020 | 8.20 | 8.14 | 8.05 |
| | April 2021 | 8.30 | 8.30 | 8.38 |
| | September 2021 | 8.08 | 8.07 | 8.26 |
| **ORP / mV** | May 2020 | -67.8 | -67.6 | -59.4 |
| | April 2021 | -64.2 | -64.5 | -69.2 |
| | September 2021 | -58.5 | -58.2 | -69.4 |
| **m-alkalinity** | May 2020 | 3.21 | 3.85 | 3.67 |
| | April 2021 | 3.12 | 3.13 | 3.06 |
| | September 2021 | 3.26 | 3.16 | 2.48 |
| **Carbonate hardness / °dH** | May 2020 | 8.99 | 10.8 | 10.3 |
| | April 2021 | 8.74 | 8.76 | 8.57 |
| | September 2021 | 9.07 | 8.85 | 6.94 |
| **Total hardness / °dH** | May 2020 | 10.6 | 10.6 | 9.88 |
| | April 2021 | 9.63 | 9.73 | 9.81 |
| | September 2021 | 9.80 | 9.22 | 7.38 |

**Table SI.3.** Limits of detection (LOD) for metal(loid) measurement in dissolved river-water and in bottom river sediments.

| | **LOD for dissolved metal(loid)s in water** | | **LOD for metal(loid)s in sediment (Fiket et al., 2017)** |
|---|---|---|---|
| **Ag / µg L$^{-1}$** | 0.001 | **Ag / mg kg$^{-1}$** | 0.03 |
| **Al / µg L$^{-1}$** | 0.556 | **Al / mg kg$^{-1}$** | 25 |
| **As / µg L$^{-1}$** | 0.014 | **As / mg kg$^{-1}$** | 0.30 |
| **Ba / µg L$^{-1}$** | 0.012 | **Ba / mg kg$^{-1}$** | 0.30 |
| **Bi / µg L$^{-1}$** | 0.001 | **Bi / mg kg$^{-1}$** | 0.03 |
| **Cd / µg L$^{-1}$** | 0.002 | **Cd / mg kg$^{-1}$** | 0.03 |
| **Co / µg L$^{-1}$** | 0.001 | **Co / mg kg$^{-1}$** | 0.04 |
| **Cr / µg L$^{-1}$** | 0.002 | **Cr / mg kg$^{-1}$** | 0.40 |
| **Cs / µg L$^{-1}$** | 0.001 | **Cs / mg kg$^{-1}$** | 0.06 |
| **Cu / µg L$^{-1}$** | 0.053 | **Cu / mg kg$^{-1}$** | 0.30 |
| **Fe / µg L$^{-1}$** | 0.073 | **Fe / mg kg$^{-1}$** | 10 |
| **Li / µg L$^{-1}$** | 0.003 | **Li / mg kg$^{-1}$** | 0.14 |
| **Mn / µg L$^{-1}$** | 0.013 | **Mn / mg kg$^{-1}$** | 0.45 |
| **Mo / µg L$^{-1}$** | 0.005 | **Mo / mg kg$^{-1}$** | 0.15 |
| **Ni / µg L$^{-1}$** | 0.006 | **Ni / mg kg$^{-1}$** | 0.60 |
| **Pb / µg L$^{-1}$** | 0.060 | **Pb / mg kg$^{-1}$** | 0.45 |
| **Rb / µg L$^{-1}$** | 0.001 | **Rb / mg kg$^{-1}$** | 0.15 |
| **Sb / µg L$^{-1}$** | 0.001 | **Sb / mg kg$^{-1}$** | 0.15 |
| **Se / µg L$^{-1}$** | 0.026 | **Se / mg kg$^{-1}$** | - |
| **Sn / µg L$^{-1}$** | 0.003 | **Sn / mg kg$^{-1}$** | - |
| **Sr / µg L$^{-1}$** | 0.317 | **Sr / mg kg$^{-1}$** | 0.60 |
| **Ti / µg L$^{-1}$** | 0.140 | **Ti / mg kg$^{-1}$** | 0.60 |
| **Tl / µg L$^{-1}$** | 0.001 | **Tl / mg kg$^{-1}$** | 0.04 |
| **U / µg L$^{-1}$** | 0.001 | **U / mg kg$^{-1}$** | 0.03 |
| **V / µg L$^{-1}$** | 0.001 | **V / mg kg$^{-1}$** | 0.03 |
| **Zn / µg L$^{-1}$** | 0.519 | **Zn / mg kg$^{-1}$** | 1.5 |
| **Ca / µg L$^{-1}$** | 132 | **Ca / mg kg$^{-1}$** | 30 |
| **K / µg L$^{-1}$** | 28.7 | **K / mg kg$^{-1}$** | 25 |
| **Mg / µg L$^{-1}$** | 40.8 | **Mg / mg kg$^{-1}$** | 20 |
| **Na / µg L$^{-1}$** | 22.8 | **Na / mg kg$^{-1}$** | 45 |

**Table SI.4.** Concentrations of dissolved trace and macro elements in river water measured in six samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviations (n=3). The concentrations of Pb and Ti were always below detections limits (Pb: 0.060 µg L$^{-1}$; Ti: 0.140 µg L$^{-1}$).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Ag / µg L$^{-1}$** | May 2020 | <0.001 | <0.001 | <0.001 |
| | July 2020 | <0.001 | <0.001 | <0.001 |
| | April 2021 | <0.001 | 0.003±0.002 | 0.001±0.000 |
| | June 2021 | <0.001 | <0.001 | <0.001 |
| | September 2021 | <0.001 | <0.001 | <0.001 |
| | October 2021 | <0.001 | <0.001 | <0.001 |
| **As / µg L$^{-1}$** | May 2020 | 0.251±0.017 | 0.218±0.023 | 0.255±0.095 |
| | July 2020 | 0.266±0.034 | 0.335±0.035 | 0.336±0.030 |
| | April 2021 | 0.087±0.003 | 0.070±0.004 | 0.078±0.011 |
| | June 2021 | 0.162±0.004 | 0.173±0.008 | 0.159±0.010 |
| | September 2021 | 0.298±0.034 | 0.303±0.019 | 0.276±0.028 |
| | October 2021 | 0.285±0.015 | 0.326±0.010 | 0.310±0.034 |
| **Al / µg L$^{-1}$** | May 2020 | 1.91±0.74 | 1.65±0.72 | 2.32±0.08 |
| | July 2020 | 0.781±0.101 | 1.43±1.28 | 1.82±0.35 |
| | April 2021 | 0.566±0.017 | 0.919±0.628 | 0.861±0.070 |
| | June 2021 | <0.556 | 0.863±0.364 | 1.81±0.32 |
| | September 2021 | 0.695±0.043 | 1.13±0.20 | 1.77±0.24 |
| | October 2021 | 0.634±0.067 | 1.14±1.02 | 2.86±1.90 |
| **Ba / µg L$^{-1}$** | May 2020 | 6.20±0.17 | 6.21±0.42 | 6.48±0.49 |
| | July 2020 | 5.70±0.19 | 5.93±0.12 | 6.29±0.12 |
| | April 2021 | 5.66±0.05 | 5.77±0.02 | 5.84±0.24 |
| | June 2021 | 6.71±0.22 | 6.46±0.11 | 6.97±0.06 |
| | September 2021 | 6.38±0.19 | 6.44±0.09 | 6.52±0.01 |
| | October 2021 | 6.46±0.10 | 6.67±0.05 | 6.88±0.02 |
| **Bi / µg L$^{-1}$** | May 2020 | <0.001 | <0.001 | 0.003±0.003 |
| | July 2020 | 0.001±0.000 | <0.001 | <0.001 |
| | April 2021 | - | - | - |
| | June 2021 | 0.001±0.000 | 0.001±0.000 | <0.001 |
| | September 2021 | 0.012±0.009 | <0.001 | <0.001 |
| | October 2021 | <0.001 | <0.001 | <0.001 |
| **Cd / µg L$^{-1}$** | May 2020 | 0.006±0.001 | 0.005±0.001 | 0.007±0.003 |
| | July 2020 | 0.005±0.001 | 0.005±0.001 | 0.008±0.004 |
| | April 2021 | - | - | - |
| | June 2021 | 0.010±0.001 | 0.009±0.000 | 0.009±0.001 |
| | September 2021 | 0.026±0.003 | 0.018±0.003 | 0.010±0.003 |
| | October 2021 | 0.006±0.002 | 0.003±0.001 | 0.002±0.000 |
| **Co / µg L$^{-1}$** | May 2020 | 0.028±0.001 | 0.031±0.002 | 0.035±0.002 |
| | July 2020 | 0.041±0.011 | 0.035±0.008 | 0.028±0.002 |
| | April 2021 | 0.018±0.000 | 0.020±0.001 | 0.018±0.001 |
| | June 2021 | 0.030±0.001 | 0.030±0.001 | 0.028±0.001 |
| | September 2021 | 0.023±0.002 | 0.028±0.002 | 0.023±0.001 |
| | October 2021 | 0.025±0.001 | 0.031±0.002 | 0.025±0.000 |

**Table SI.4. – continued.** Concentrations of dissolved trace and macro elements in river water measured in six samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviations (n=3). The concentrations of Pb and Ti were always below detections limits (Pb: 0.060 µg L$^{-1}$; Ti: 0.140 µg L$^{-1}$).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Cr / µg L$^{-1}$** | May 2020 | 0.168±0.006 | 0.133±0.011 | 0.147±0.013 |
| | July 2020 | 0.080±0.006 | 0.063±0.007 | 0.053±0.012 |
| | April 2021 | 0.051±0.018 | 0.059±0.018 | 0.048±0.020 |
| | June 2021 | 0.143±0.003 | 0.184±0.055 | 0.179±0.044 |
| | September 2021 | 0.036±0.007 | 0.036±0.003 | 0.035±0.003 |
| | October 2021 | 0.030±0.004 | 0.024±0.013 | 0.049±0.010 |
| **Cs / µg L$^{-1}$** | May 2020 | 0.001±0.000 | 0.001±0.000 | 0.004±0.000 |
| | July 2020 | 0.001±0.000 | 0.002±0.000 | 0.005±0.000 |
| | April 2021 | 0.002±0.000 | 0.001±0.000 | 0.001±0.000 |
| | June 2021 | 0.001±0.000 | 0.001±0.000 | 0.003±0.000 |
| | September 2021 | 0.001±0.001 | 0.001±0.000 | 0.002±0.000 |
| | October 2021 | 0.001±0.000 | 0.001±0.000 | 0.003±0.000 |
| **Cu / µg L$^{-1}$** | May 2020 | 0.067±0.019 | 0.138±0.087 | 0.143±0.040 |
| | July 2020 | <0.053 | 0.058±0.008 | 0.054±0.002 |
| | April 2021 | <0.053 | 0.138±0.147 | <0.053 |
| | June 2021 | 0.144±0.065 | 0.119±0.019 | 0.182±0.050 |
| | September 2021 | <0.053 | 0.113±0.103 | <0.053 |
| | October 2021 | 0.071±0.031 | <0.053 | <0.053 |
| **Fe / µg L$^{-1}$** | May 2020 | 8.00±0.25 | 11.3±1.7 | 17.1±1.5 |
| | July 2020 | 12.6±0.4 | 18.9±2.0 | 16.2±0.7 |
| | April 2021 | 1.58±0.06 | 1.92±0.03 | 2.32±0.01 |
| | June 2021 | 4.68±0.64 | 7.87±0.47 | 6.21±0.15 |
| | September 2021 | 7.96±0.57 | 9.84±0.14 | 9.60±0.65 |
| | October 2021 | 7.49±0.26 | 16.1±1.4 | 9.68±0.66 |
| **Li / µg L$^{-1}$** | May 2020 | 0.061±0.002 | 0.057±0.003 | 0.096±0.008 |
| | July 2020 | 0.050±0.001 | 0.058±0.006 | 0.091±0.002 |
| | April 2021 | 0.055±0.007 | 0.050±0.007 | 0.055±0.007 |
| | June 2021 | 0.055±0.002 | 0.053±0.002 | 0.100±0.041 |
| | September 2021 | 0.049±0.004 | 0.044±0.003 | 0.066±0.002 |
| | October 2021 | 0.038±0.002 | 0.039±0.002 | 0.077±0.002 |
| **Mn / µg L$^{-1}$** | May 2020 | 1.89±0.03 | 2.27±0.26 | 4.46±0.40 |
| | July 2020 | 2.40±0.01 | 3.18±0.37 | 3.42±0.08 |
| | April 2021 | 1.48±0.02 | 1.65±0.05 | 1.18±0.06 |
| | June 2021 | 2.29±0.04 | 2.22±0.03 | 1.66±0.08 |
| | September 2021 | 1.80±0.05 | 1.60±0.02 | 0.785±0.002 |
| | October 2021 | 1.01±0.02 | 1.51±0.03 | 1.43±0.02 |
| **Mo / µg L$^{-1}$** | May 2020 | 0.732±0.004 | 0.752±0.043 | 0.711±0.071 |
| | July 2020 | 0.641±0.012 | 0.628±0.014 | 0.640±0.016 |
| | April 2021 | 0.380±0.003 | 0.385±0.006 | 0.370±0.014 |
| | June 2021 | 0.438±0.002 | 0.422±0.006 | 0.417±0.007 |
| | September 2021 | 0.906±0.011 | 0.939±0.027 | 0.874±0.009 |
| | October 2021 | 0.978±0.008 | 0.951±0.002 | 0.921±0.002 |

**Table SI.4. – continued.** Concentrations of dissolved trace and macro elements in river water measured in six samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviations (n=3). The concentrations of Pb and Ti were always below detections limits (Pb: 0.060 µg L$^{-1}$; Ti: 0.140 µg L$^{-1}$).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **Ni / µg L$^{-1}$** | May 2020 | 0.050±0.003 | 0.042±0.011 | 0.137±0.074 |
| | July 2020 | 0.088±0.040 | 0.061±0.034 | 0.053±0.006 |
| | April 2021 | - | - | - |
| | June 2021 | 0.067±0.006 | 0.072±0.018 | 0.067±0.008 |
| | September 2021 | <0.006 | <0.006 | <0.006 |
| | October 2021 | <0.006 | <0.006 | <0.006 |
| **Rb / µg L$^{-1}$** | May 2020 | 0.335±0.003 | 0.334±0.021 | 0.450±0.042 |
| | July 2020 | 0.335±0.014 | 0.346±0.005 | 0.407±0.008 |
| | April 2021 | 0.205±0.004 | 0.206±0.004 | 0.214±0.008 |
| | June 2021 | 0.262±0.003 | 0.281±0.005 | 0.298±0.004 |
| | September 2021 | 0.378±0.005 | 0.385±0.005 | 0.410±0.002 |
| | October 2021 | 0.411±0.002 | 0.416±0.000 | 0.443±0.002 |
| **Sb / µg L$^{-1}$** | May 2020 | 0.031±0.000 | 0.031±0.003 | 0.046±0.005 |
| | July 2020 | 0.033±0.001 | 0.035±0.002 | 0.034±0.001 |
| | April 2021 | 0.027±0.001 | 0.025±0.000 | 0.025±0.001 |
| | June 2021 | 0.027±0.001 | 0.027±0.001 | 0.030±0.001 |
| | September 2021 | 0.033±0.000 | 0.035±0.001 | 0.036±0.000 |
| | October 2021 | 0.032±0.002 | 0.035±0.002 | 0.036±0.001 |
| **Se / µg L$^{-1}$** | May 2020 | 0.099±0.012 | 0.125±0.024 | 0.135±0.059 |
| | July 2020 | 0.223±0.029 | 0.300±0.022 | 0.350±0.046 |
| | April 2021 | 0.084±0.007 | 0.106±0.005 | 0.092±0.008 |
| | June 2021 | 0.105±0.011 | 0.090±0.025 | 0.098±0.003 |
| | September 2021 | 0.072±0.038 | 0.108±0.012 | 0.110±0.007 |
| | October 2021 | 0.101±0.023 | 0.095±0.014 | 0.076±0.004 |
| **Sn / µg L$^{-1}$** | May 2020 | <0.003 | <0.003 | <0.003 |
| | July 2020 | 0.078±0.027 | 0.018±0.010 | 0.037±0.047 |
| | April 2021 | 0.013±0.011 | <0.003 | <0.003 |
| | June 2021 | <0.003 | 0.040±0.020 | 0.005±0.003 |
| | September 2021 | 0.004±0.002 | <0.003 | <0.003 |
| | October 2021 | 0.015±0.020 | <0.003 | 0.012±0.015 |
| **Sr / µg L$^{-1}$** | May 2020 | 83.4±1.1 | 85.3±4.6 | 84.2±7.5 |
| | July 2020 | 81.4±2.0 | 80.5±1.7 | 78.5±0.4 |
| | April 2021 | 69.2±0.3 | 69.0±1.0 | 67.9±2.9 |
| | June 2021 | 79.3±0.2 | 76.9±1.1 | 74.6±1.6 |
| | September 2021 | 99.0±0.6 | 98.2±0.3 | 95.7±0.7 |
| | October 2021 | 103±0.9 | 101±0.7 | 97.4±0.2 |
| **Tl / µg L$^{-1}$** | May 2020 | 0.005±0.000 | 0.005±0.000 | 0.005±0.000 |
| | July 2020 | 0.006±0.001 | 0.005±0.000 | 0.005±0.000 |
| | April 2021 | 0.003±0.000 | 0.003±0.000 | 0.003±0.000 |
| | June 2021 | 0.004±0.000 | 0.004±0.000 | 0.004±0.000 |
| | September 2021 | 0.005±0.000 | 0.005±0.000 | 0.005±0.000 |
| | October 2021 | 0.006±0.000 | 0.005±0.000 | 0.005±0.000 |

**Table SI.4. – continued.** Concentrations of dissolved trace and macro elements in river water measured in six samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviations (n=3). The concentrations of Pb and Ti were always below detections limits (Pb: 0.060 µg L$^{-1}$; Ti: 0.140 µg L$^{-1}$).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **U / µg L$^{-1}$** | May 2020 | 0.630±0.004 | 0.657±0.043 | 0.643±0.047 |
| | July 2020 | 0.481±0.029 | 0.528±0.009 | 0.525±0.003 |
| | April 2021 | 0.536±0.007 | 0.550±0.008 | 0.547±0.016 |
| | June 2021 | 0.487±0.006 | 0.474±0.009 | 0.490±0.007 |
| | September 2021 | 0.608±0.001 | 0.617±0.003 | 0.601±0.007 |
| | October 2021 | 0.662±0.005 | 0.650±0.003 | 0.654±0.004 |
| **V / µg L$^{-1}$** | May 2020 | 0.385±0.006 | 0.384±0.026 | 0.442±0.045 |
| | July 2020 | 0.410±0.011 | 0.391±0.017 | 0.455±0.008 |
| | April 2021 | 0.364±0.004 | 0.366±0.003 | 0.382±0.012 |
| | June 2021 | 0.425±0.005 | 0.405±0.006 | 0.454±0.003 |
| | September 2021 | 0.469±0.012 | 0.569±0.009 | 0.490±0.005 |
| | October 2021 | 0.419±0.009 | 0.481±0.005 | 0.483±0.007 |
| **Zn / µg L$^{-1}$** | May 2020 | <0.519 | <0.519 | <0.519 |
| | July 2020 | <0.519 | <0.519 | <0.519 |
| | April 2021 | <0.519 | <0.519 | <0.519 |
| | June 2021 | <0.519 | <0.519 | 0.640±0.209 |
| | September 2021 | <0.519 | 1.05±0.92 | 0.876±0.618 |
| | October 2021 | <0.519 | 0.812±0.507 | <0.519 |
| **Ca / mg L$^{-1}$** | May 2020 | 56.3±2.2 | 53.9±0.5 | 51.8±6.4 |
| | July 2020 | 44.7±0.4 | 45.1±3.2 | 41.7±2.2 |
| | April 2021 | 61.9±2.1 | 62.7±1.1 | 61.2±0.2 |
| | June 2021 | 57.9±0.5 | 56.8±1.4 | 56.1±0.6 |
| | September 2021 | 54.7±4.3 | 48.3±1.0 | 48.4±0.3 |
| | October 2021 | 56.9±3.6 | 55.0±1.2 | 48.3±4.2 |
| **K / mg L$^{-1}$** | May 2020 | 0.442±0.043 | 0.406±0.011 | 0.553±0.066 |
| | July 2020 | 0.314±0.014 | 0.337±0.035 | 0.361±0.030 |
| | April 2021 | 0.339±0.005 | 0.348±0.003 | 0.391±0.065 |
| | June 2021 | 0.333±0.009 | 0.329±0.014 | 0.347±0.006 |
| | September 2021 | 0.488±0.059 | 0.420±0.019 | 0.472±0.004 |
| | October 2021 | 0.494±0.020 | 0.513±0.011 | 0.481±0.044 |
| **Mg / mg L$^{-1}$** | May 2020 | 14.0±0.5 | 13.8±0.3 | 13.4±1.5 |
| | July 2020 | 10.9±0.4 | 11.2±0.9 | 10.8±0.5 |
| | April 2021 | 9.24±0.23 | 9.41±0.18 | 9.41±0.12 |
| | June 2021 | 9.24±0.02 | 8.93±0.23 | 9.19±0.03 |
| | September 2021 | 15.6±1.4 | 14.1±0.4 | 14.1±0.1 |
| | October 2021 | 16.1±1.0 | 16.0±0.5 | 14.2±1.3 |
| **Na / mg L$^{-1}$** | May 2020 | 1.50±0.06 | 1.47±0.02 | 2.11±0.17 |
| | July 2020 | 1.05±0.02 | 1.17±0.06 | 1.24±0.12 |
| | April 2021 | 1.78±0.04 | 1.76±0.03 | 2.01±0.08 |
| | June 2021 | 1.09±0.01 | 1.10±0.03 | 1.32±0.00 |
| | September 2021 | 1.24±0.12 | 1.18±0.08 | 1.33±0.00 |
| | October 2021 | 1.20±0.08 | 1.34±0.03 | 1.38±0.13 |

**Table SI.5.** Grain-size distribution of sediments in three samplings at three sites along the lower course of the Mrežnica River in Croatia, presented as average±standard deviation (n=2-3).

| | | **Reference location (REF)** | **Duga Resa – factory (DRF)** | **Karlovac – industrial zone (KIZ)** |
|---|---|---|---|---|
| **gravel (%) / fraction < 2 mm (%)** | May 2020 | 87/13 | 0/100 | 46/54 |
| | April 2021 | 0/100 | 0/100 | 0/100 |
| | September 2021 | 0/100 | 0/100 | 0/100 |
| **Particle size distribution within the fraction < 2 mm:** | | | | |
| **May 2020** | Clay / % | 3.9±0.9 | 7.8±0.2 | 4.3±1.2 |
| | Silt / % | 18.1±4.5 | 57.0±2.6 | 14.9±4.9 |
| | Sand / % | 78.0±5.4 | 35.3±2.4 | 80.8±6.2 |
| **April 2021** | Clay / % | 11.5±3.6 | 6.3±0.6 | 9.5±1.3 |
| | Silt / % | 54.3±5.5 | 50.8±4.7 | 46.6±2.7 |
| | Sand / % | 34.2±8.7 | 43.4±4.9 | 43.9±3.4 |
| **September 2021** | Clay / % | 9.5±1.1 | 6.4±1.1 | 11.9±2.3 |
| | Silt / % | 56.1±3.9 | 53.8±4.9 | 53.6±4.6 |
| | Sand / % | 34.4±4.7 | 39.7±6.0 | 34.6±6.9 |

**Figure SI.1.** Pearson’s correlatation analysis of absolute abundance (gene copies/g dry weight) of heavy metal resistance genes with heavy metals (mg/g) to which they show resistance. Cell colors from blue to red indicate positive and negative Pearson correlations. Asterisks indicate significant correlations (*, $p < 0.05$; **, $p < 0.01$; **, $p < 0.001$).