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https://doi.org/10.3390/fishes9090364

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Metal Bioaccumulation in the Muscle of the Northern Pike (*Esox lucius*) from Historically Contaminated River and the Estimation of the Human Health Risk

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Abstract: The impact of the long-term historical contamination of the Mrežnica River with textile industry wastewaters on metal/metalloid bioaccumulation in the muscles of the young northern pike (*Esox lucius*) (0+ to 3+) was evaluated, as well as the association of bioaccumulated metal/metalloid concentrations in the muscle with fish physiology. Increased levels of several elements (As, Bi, Cs, Co, Cu, Zn), bioaccumulated in fish muscle, were occasionally found in front of the former factory, but the obtained metal/metalloid concentrations in muscle were in general either comparable or even lower than in the fish from moderately contaminated freshwaters. Calculated target hazard quotients indicated that the current risk for humans, arising from consumption of the northern pike meat originating from a historically contaminated section of the Mrežnica River, was negligible. The influence of biological factors, especially seasonal physiological changes, on metal/metalloid bioaccumulation was confirmed, indicating the importance of the involvement of these parameters in the freshwater pollution assessment, but also in the estimation of the human health hazard. The increased bioaccumulation of several metals/metalloids in fish muscle at a historically contaminated site pointed to the need for continuous supervision of fish health and biodiversity in freshwaters impacted by currently suspended long-term contamination sources.

Keywords: fish; freshwater; human health hazard; industry; physiology

Key Contribution: Historical freshwater contamination can cause observable effects in freshwater fishes even decades after its suspension. The notable influence of fish physiology on metal/metalloid bioaccumulation should always be considered in freshwater pollution assessment.

Citation: Dragun, Z.; Ivanković, D.; Tepić, N.; Filipović Marijić, V.; Šariri, S.; Pavin, T.M.; Drk, S.; Gjurčević, E.; Matanović, K.; Kužir, S.; et al. Metal Bioaccumulation in the Muscle of the Northern Pike (*Esox lucius*) from Historically Contaminated River and the Estimation of the Human Health Risk. *Fishes* **2024**, *9*, 364. https://doi.org/10.3390/fishes9090364

Academic Editor: David Janz

Received: 29 August 2024
Revised: 6 September 2024
Accepted: 12 September 2024
Published: 18 September 2024

1. Introduction

In many parts of the world, fish constitute a major part of the human diet [1]. And, in European, Asian, and North American freshwaters, the northern pike, *Esox lucius* Linnaeus, 1758, (Teleostei > Esociformes > Esocidae) is one of the most widely distributed fish species with an important role in commercial and recreational fisheries [2,3]. Aquatic organisms, including fish, were demonstrated to accumulate high levels of contaminants, such as metals, in their organs, which can then enter the human body through diet; thus, there is a need for strict monitoring of the metal contamination of fish meat [1,4,5]. In the case of northern pike, it is important to note that it is a carnivorous fish [4] and a common top predator [5], thus exposed to metal contamination from various sources originating from the entire aquatic food web (from contaminated sediments and water, to organisms at various levels of biological complexity).

The northern pike is an abundant fish species in the Mrežnica River [6,7], a Croatian river included in the European ecological network NATURA 2000, that was, however, until decade to two ago exposed to industrial contamination for more than a century [8]. Throughout the 20th century, the wastewaters of the cotton textile factory in Duga Resa town, which are known to be rich in metals (such as Cu, Cr, and Zn), were discharged directly into the river water, and several authors have confirmed that Mrežnica River sediments still contain increased concentrations of some metals (e.g., Bi, Cs, Cu, Fe, Zn) in the vicinity of the former factory [8,9]. Lead, Zn, Cu, and Cr are known as common ingredients used for fabric softening, dyeing, and bleaching [8], whereas Fe was associated with coal burning [9]. Many scientists consider that historical pollution can still pose a serious problem for freshwater ecosystems and the aquatic life inhabiting them, even though they are not subjected to current urban or industrial operations [10]. Thus, considering the nature of metals as contaminants, being non-biodegradable, sometimes highly toxic (e.g., Cd, Pb, Tl), having the tendency to bioaccumulate in the food chain, and high persistence in the environment [5], it is essential to assess the level of contamination of the fish meat that originates from the rivers characterized by long-term historical pollution. And the representative example is the northern pike from the Mrežnica River in Croatia.

Accordingly, the main goal of this study was to determine the concentrations of a set of elements (As, Bi, Cd, Co, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Pb, Rb, Se, Tl, and Zn) in northern pike muscle from the river section impacted by historical contamination, and to assess the potential risk for humans originating from its consumption, by calculating adequate target hazard quotients and hazard indices for the studied elements. Fish muscle was chosen for this study as a common indicator tissue for the assessment of human health hazards, as it is frequently used in the human diet [4,11]. The aim was to evaluate the pollution status of the historically contaminated section of the Mrežnica River, which has a great significance for the local population. On the other hand, the study also enabled the broadening of general knowledge on the dangers that historical pollution possibly presents even today for freshwaters and aquatic life worldwide. Furthermore, as it is a well-known fact that metal bioaccumulation in fish depends on numerous biotic factors (fish size, age, sex, diet, metabolism/feeding rates, reproductive cycle) [11–13], an additional aim was to recognize the biotic factors that can influence the metal bioaccumulation in the muscle of the northern pike, and to distinguish their impact from the impact of the environmental exposure.

2. Materials and Methods

2.1. Sampling of the Northern Pike and Muscle Dissection

A total number of 59 specimens of the northern pike were caught in two sampling campaigns (22/23 April 2021, 32 specimens (post-spawning period [14]); and 22/23 September 2021, 27 specimens (period of onset of gonadal growth [14])) at the lowland part of the Mrežnica River in Croatia. It is a 64 km long river with a water discharge in 2021 in the range from ~2 to 160 $m^3 s^{-1}$. Mrežnica is a typical karst river in its upper part, while in the lower section it becomes wider and shallower, which is a typical feature of lowland rivers [8]. The location situated in the vicinity of the village Mrežnička Varoš, about 2 km upstream of Duga Resa town and surrounded by cultivated land (N 45° 26′ 28.40′′ E 15° 30′ 15.39′′) was selected as the reference site (REF, Figure S1). The location situated in front of the former textile factory in Duga Resa town (N 45° 27′ 4.38′′ E 15° 30′ 18.96′′) was selected as the site of historical industrial contamination (DRF, Figure S1). The number of specimens caught per site and season was indicated in Figures 1–5 and Table S1. The fact that northern pike is usually a highly territorial, solitary species that does not undertake long migrations [5] allowed us to study two differently contaminated sites, separated by short distances. Electrofishing device Hans Grassl (EL63 II GI, 5.0 KW, 137 Honda GX270, 300/600V max., 27/15A max.) was applied for fish sampling as described by Dragun et al. [15], following the recommendations of the Croatian standard

HRN EN 14011 [16]. The fish were euthanized at the sampling location with unbuffered tricaine methane sulphonate (MS 222, Sigma Aldrich, St. Louis, MI, USA). In this procedure, the Ordinance on the protection of animals used for scientific purposes [17] was followed, whereas the exposure conditions matched those described in our previous paper (~50 mg L^{-1}, duration under 10 min [15]). Fish sex was determined by gonad examination at the macroscopic level, whereas the age was determined by counting the number of annuli (rings) on scales using an Olympus BM2 microscope (magnification 30×) (the results are presented in Figure 1 and Table S1).

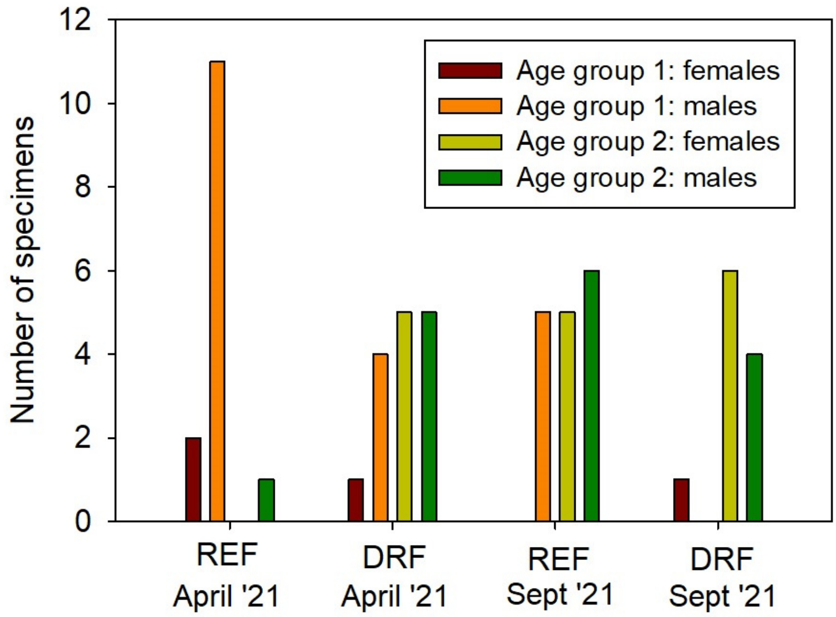

Figure 1. Presentation of age and sex of the northern pike specimens sampled from the Mrežnica River at two sites (REF—reference; DRF—contaminated) in two sampling campaigns (April 2021 and September 2021). The results are presented as vertical bars, each bar representing the number of specimens in a specific group, as indicated in the legend.

Several other biometric parameters were measured/calculated: the total fish mass, total length, gonadosomatic index (GSI) ((gonad mass in grams × 100)/total fish mass in grams) and the Fulton condition index (FCI) ((total fish mass in grams × 100)/(standard length in centimeters)^3), as described previously [18] (the results are presented in Figure 2 and Table S1). The total fish length was measured from the tip of the snout to the tip of the tail, whereas standard length was measured from the tip of the snout to the posterior end of the last vertebra or to the posterior end of the mid-lateral portion of the hypural plate. From each fish, one piece of epaxial musculature (about 2 g) was aseptically dissected from a standardized location anterior to the dorsal fin. The environmental exposure conditions (namely, the dissolved/particulate metal/metalloid concentrations in the river water and metal/metalloid concentrations in the river sediment) and physico-chemical characteristics of the studied section of the Mrežnica River in 2021, when fish were sampled, were previously analyzed and discussed in detail [8], and the excerpts relevant for this study are given in the Supplementary Information (Table S2).

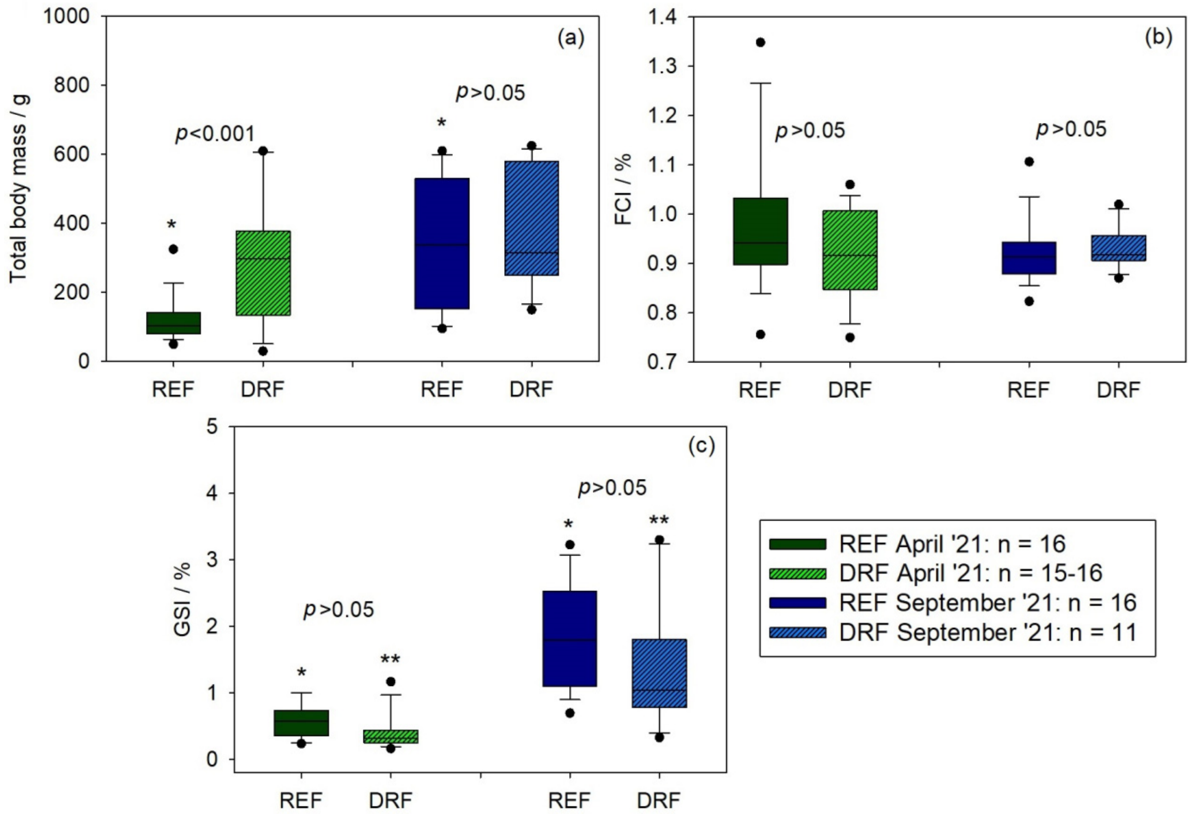

Figure 2. Biometric data for the northern pike sampled from the Mrežnica River at two sites (REF—reference; DRF—contaminated) in two sampling campaigns (April and September 2021): (**a**) total fish mass (g); (**b**) Fulton condition index (FCI, %); (**c**) gonadosomatic index (GSI, %). For each site in each period, the results were 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. The *p* values given within the figures refer to differences between the sites based on the *t*-test. Asterisks indicate the statistically significant differences ($p < 0.05$) between two periods at the REF site (*) and at the DRF site (**). The number of sampled fish per site/sampling period was also indicated within the Figure (for the DRF site in April 2021, n is 15 for GSI and 16 for total fish mass and FCI).

2.2. Fish Muscle Digestion

The procedure of the fish muscle digestion was derived from the previously described approach [19]. The samples of approximately 0.5 g of muscle tissue of each northern pike specimen were digested in duplicate in 3 mL of acid/peroxide digestion mixture (V_{HNO3}/V_{H2O2} = 2 + 1). The following chemicals were used: HNO_3 (*Normatom*® 67–69% for trace element analysis, VWR Chemicals, Radnor, PA, USA) and 30% H_2O_2 (*Suprapur*®, Merck, Taufkirchen, Germany). The digestion was performed at 85 °C in the laboratory dry oven for 3.5 h. Afterward, the obtained solutions were diluted with Milli-Q water five times for the measurement of trace elements and 20 times for the measurement of macro elements.

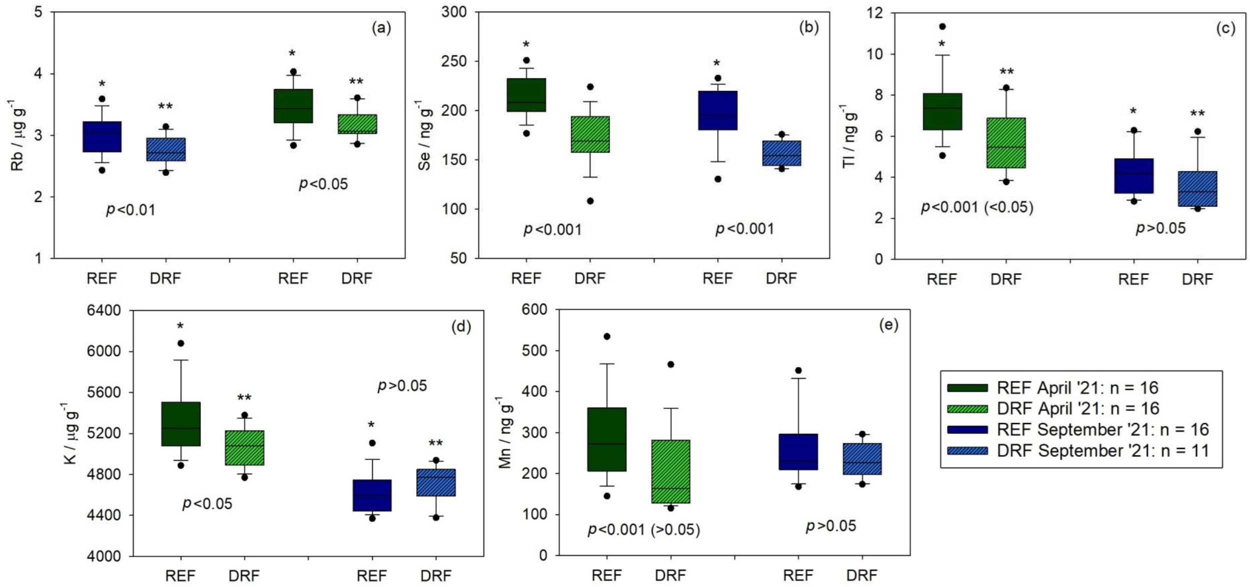

Figure 3. The concentrations of metals/metalloids in the muscle of the northern pike sampled from the Mrežnica River at two sites (REF—reference site; DRF—contaminated site) in two sampling campaigns (April and September 2021). Elements presented in this figure were characterized by higher bioaccumulation at the REF site: (**a**) Rb (µg g^{-1}); (**b**) Se (ng g^{-1}); (**c**) Tl (ng g^{-1}); (**d**) K (µg g^{-1}); and (**e**) Mn (ng g^{-1}). The data presentation and the statistical approach are the same as in Figure 2. In addition, the *p* values given within the brackets refer to differences between the sites based on the ANCOVA analysis with total mass as the covariate and are presented only when they differ from the results of the *t*-test.

2.3. Measurement of Trace and Macro Elements in Digested Fish Muscles

The concentrations of 14 trace elements (listed below) and three macro elements (K, Mg, and Na) were measured in the digested muscles of the northern pike applying high-resolution inductively coupled plasma mass spectrometry (HR ICP-MS; Element 2, Thermo Finnigan, Bremen, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, Omaha, NE, USA), cyclonic spray chamber Twister and SeaSpray nebulizer. Measurements were performed in three resolution modes. Low resolution was applied for ^{82}Se, ^{95}Rb, ^{98}Mo, ^{111}Cd, ^{133}Cs, ^{205}Tl, ^{208}Pb, and ^{209}Bi, medium resolution for ^{23}Na, ^{24}Mg, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn, and high resolution for ^{39}K and ^{75}As. Metal/metalloid concentrations were calculated based on the external calibration equations, which were made using adequate dilutions of multielement standard solution for trace elements (Analitika, Prague, Czech Republic) prepared in 2% (vol.) HNO_3 (*Normatom*® 67–69% for trace element analysis, VWR Chemicals) and supplemented with standard solutions of Cs (Fluka, Munich, Germany) and Rb (Aldrich, St. Louis, MO, USA). Separate calibrations were performed using standard solutions for macro elements (Analytika). To all the samples and calibration standards, In was added (Fluka) as an internal standard (1 µg L^{-1}).

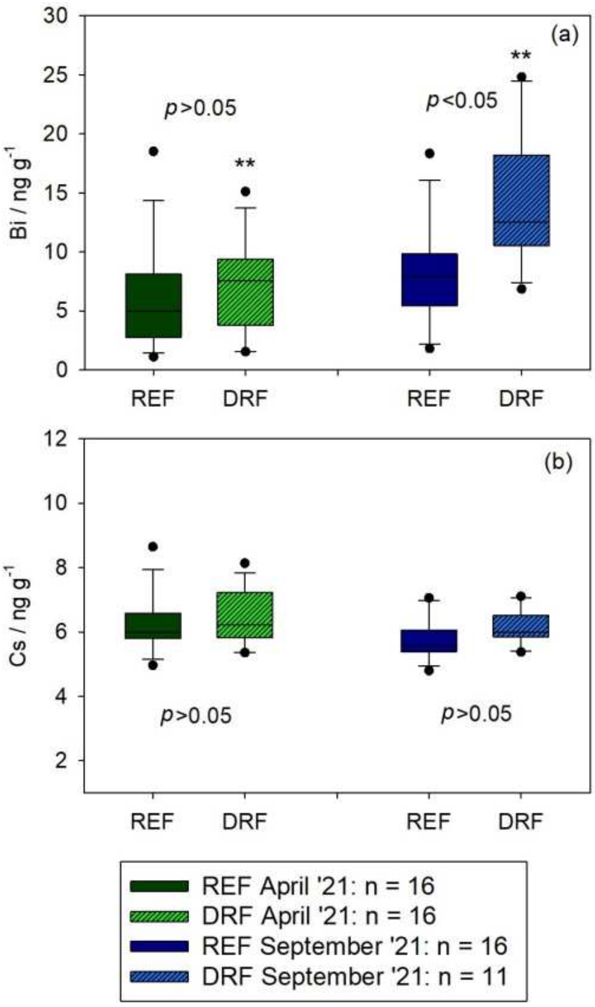

Figure 4. The concentrations of metals in the muscle of the northern pike sampled from the Mrežnica River at two sites (REF—reference site; DRF—contaminated site) in two sampling campaigns (April and September 2021). Elements presented in this Figure were characterized by higher bioaccumulation at the DRF site: (**a**) Bi (ng g^{-1}) and (**b**) Cs (ng g^{-1}). The data presentation and the statistical approach are the same as in Figure 2.

Limits of detection (LOD) for trace/macro elements in digested fish muscles were calculated based on three standard deviations of 10 successively measured trace/macro element concentrations in the blank sample (Mili-Q water), which was prepared, prior to measurement, using the same procedure as used for the fish muscles. The LODs were the following (in ng g^{-1}): As, 0.320; Bi, 0.337; Cd, 0.079; Co, 0.020; Cs, 0.014; Cu, 1.33; Fe, 22.2; K, 585; Mg, 97.3; Mn, 0.797; Mo, 1.22; Na, 498; Pb, 0.95; Rb, 0.090; Se, 3.46; Tl, 0.003; and Zn, 7.63. The accuracy of HR ICP-MS measurements was controlled by analyses of quality control samples (UNEP GEMS, Canada) on 2–3 separate occasions. The following recoveries (average ± standard deviation) were achieved (%): As, 99.6 ± 1.6; Cd, 102.8 ± 1.7; Co, 102.5 ± 4.9; Cu, 105.7 ± 1.7; Fe, 99.5 ± 4.2; K, 96.1 ± 3.6; Mg, 93.9 ± 0.2; Mn, 101.9 ± 2.1; Mo, 101.7 ± 3.3; Na, 97.0 ± 0.1; Pb, 100.3 ± 0.9; and Zn, 107.3 ± 5.9. The results are presented in Tables 1 and 2, and Figures 3–5, expressed on a wet mass basis. Literature values originally presented on dry mass were converted to wet mass by dividing by a theoretical factor of five [20].

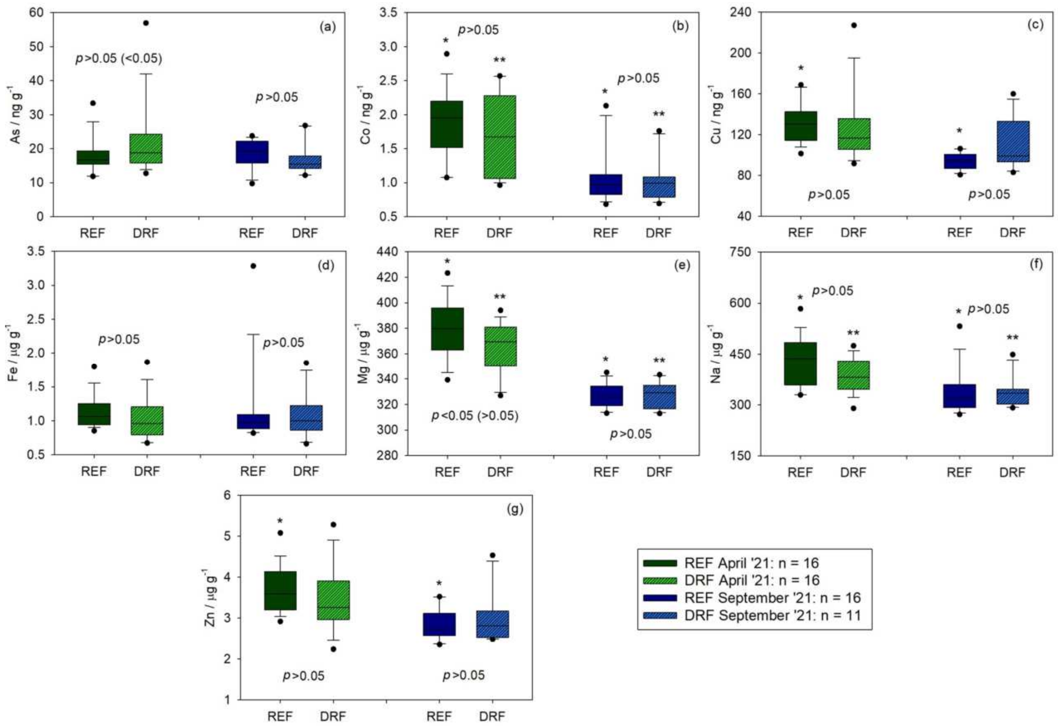

Figure 5. The concentrations of metals/metalloids in the muscle of the northern pike sampled from the Mrežnica River at two sites (REF—reference site; DRF—contaminated site) in two sampling campaigns (April and September 2021). Elements presented in this figure were characterized by the mainly comparable bioaccumulation at both sites: (**a**) As (ng g^{-1}); (**b**) Co (ng g^{-1}); (**c**) Cu (ng g^{-1}); (**d**) Fe (µg g^{-1}); (**e**) Mg (µg g^{-1}); (**f**) Na (µg g^{-1}); and (**g**) Zn (µg g^{-1}). The data presentation and the statistical approach are the same as in Figure 2.

Table 1. Between-site comparisons (*t*-test, when n > 2) of group means of fish total mass (g), gonadosomatic index (GSI, %), and the concentrations of metals/metalloids bioaccumulated in the muscle of the northern pike sampled at two sites of the Mrežnica River (REF—reference site; DRF—contaminated site), calculated separately for each campaign (April and September 2021). * $p < 0.05$.

		April 2021		September 2021	
		REF	**DRF**	**REF**	**DRF**
Total mass/g	**Age 1 [a]; Sex F**	113 ([c] n = 2)	260 (n = 1)	-	150 (n = 1)
	Age 1; Sex M	109 (n = 11)	122.0 (n = 4)	134 (n = 5)	-
	Age 2 [b]; Sex F	-	401 (n = 5)	452 (n = 5)	412 (n = 6)
	Age 2; Sex M	325 (n = 1)	390 (n = 5)	449 (n = 6)	345 (n = 4)
GSI /%	**Age 1; Sex F**	0.297	0.308	-	0.327
	Age 1; Sex M	0.665	0.673	1.93	-
	Age 2; Sex F	-	0.372	1.09	0.900
	Age 2; Sex M	0.277	0.280	2.38	2.47
As /ng g^{-1}	**Age 1; Sex F**	16.0	12.8	-	12.6
	Age 1; Sex M	* 19.0	* 34.2	18.1	-
	Age 2; Sex F	-	19.4	16.7	17.2
	Age 2; Sex M	12.4	16.5	20.2	18.6
Bi /ng g^{-1}	**Age 1; Sex F**	3.73	13.1	-	10.5
	Age 1; Sex M	6.29	3.90	7.56	-
	Age 2; Sex F	-	10.2	* 6.99	* 15.6
	Age 2; Sex M	12.5	6.03	9.52	14.9
Co /ng g^{-1}	**Age 1; Sex F**	1.70	1.01	-	1.06
	Age 1; Sex M	1.99	2.17	1.37	-
	Age 2; Sex F	-	1.25	0.939	0.844
	Age 2; Sex M	1.08	1.77	* 0.922	* 1.35
Cs /ng g^{-1}	**Age 1; Sex F**	5.99	5.90	-	5.95
	Age 1; Sex M	6.37	6.44	5.45	-
	Age 2; Sex F	-	7.10	5.70	5.99
	Age 2; Sex M	6.01	5.78	6.09	6.43

[a] age group 1 includes fish of age 0+ and 1+; [b] age group 2 includes fish of age 2+ and 3+; [c] number of samples per group is the same for all parameters as for total mass.

Table 1. *Cont.*

		April 2021		September 2021	
		REF	**DRF**	**REF**	**DRF**
Cu /ng g^{-1}	**Age 1 [a]; Sex F**	108 ([c] n = 2)	117 (n = 1)	-	106 (n = 1)
	Age 1; Sex M	132 (n = 11)	132 (n = 4)	96.5 (n = 5)	-
	Age 2 [b]; Sex F	-	131 (n = 5)	90.1 (n = 5)	99.8 (n = 6)
	Age 2; Sex M	140 (n = 1)	120 (n = 5)	95.5 (n = 6)	122 (n = 4)
Fe /µg g^{-1}	**Age 1; Sex F**	0.963	1.06	-	1.22
	Age 1; Sex M	1.18	1.15	1.40	-
	Age 2; Sex F	-	0.809	1.11	0.936
	Age 2; Sex M	0.934	1.10	1.12	1.25
Mn /ng g^{-1}	**Age 1; Sex F**	372	183	-	198
	Age 1; Sex M	276	260	326	-
	Age 2; Sex F	-	163	223	229
	Age 2; Sex M	348	178	230	253
Rb /µg g^{-1}	**Age 1; Sex F**	2.56	2.58	-	3.53
	Age 1; Sex M	3.13	2.79	3.35	-
	Age 2; Sex F	-	2.73	3.41	3.17
	Age 2; Sex M	3.23	2.71	* 3.59	* 3.01
Se /ng g^{-1}	**Age 1; Sex F**	196	153	-	142
	Age 1; Sex M	* 217	* 175	167	-
	Age 2; Sex F	-	163	* 204	* 153
	Age 2; Sex M	229	189	* 207	* 166

[a] age group 1 includes fish of age 0+ and 1+; [b] age group 2 includes fish of age 2+ and 3+; [c] number of samples per group is the same for all parameters as for Cu.

		April 2021		September 2021	
		REF	**DRF**	**REF**	**DRF**
Tl /ng g^{-1}	**Age 1 [a]; Sex F**	6.84 ([c] n = 2)	3.99 (n = 1)	-	4.79 (n = 1)
	Age 1; Sex M	7.41 (n = 11)	6.61 (n = 4)	5.19 (n = 5)	-
	Age 2 [b]; Sex F	-	5.55 (n = 5)	3.82 (n = 5)	3.66 (n = 6)
	Age 2; Sex M	8.16 (n = 1)	5.16 (n = 5)	3.87 (n = 6)	3.11 (n = 4)
Zn /µg g^{-1}	**Age 1; Sex F**	3.94	3.46	-	2.48
	Age 1; Sex M	3.62	3.83	3.12	-
	Age 2; Sex F	-	3.02	2.71	2.92
	Age 2; Sex M	2.92	3.38	2.72	3.29
K /µg g^{-1}	**Age 1; Sex F**	5366	5338	-	4710
	Age 1; Sex M	5255	5130	4681	-
	Age 2; Sex F	-	5017	4544	4768
	Age 2; Sex M	6078	5095	4624	4640
Mg /µg g^{-1}	**Age 1; Sex F**	374	356	-	316
	Age 1; Sex M	377	375	327	-
	Age 2; Sex F	-	355	324	330
	Age 2; Sex M	423	373	329	325
Na /µg g^{-1}	**Age 1; Sex F**	444	335	-	340
	Age 1; Sex M	427	389	307	-
	Age 2; Sex F	-	355	388	328
	Age 2; Sex M	442	420	321	347

[a] age group 1 includes fish of age 0+ and 1+; [b] age group 2 includes fish of age 2+ and 3+; [c] number of samples per group is the same for all parameters as for Tl.

Table 2. Comparison of metal/metalloid concentrations (on wet mass basis) accumulated in the muscle of the northern pike (*Esox lucius*) from the Mrežnica River (this study) with the previously published studies on the northern pike. Literature values originally presented on dry mass basis were converted to wet mass by dividing by theoretical factor of five [20] (ND—nondetectable; MAC—maximum allowed concentration; ML—maximum level).

	As ng g^{-1}	**Bi** ng g^{-1}	**Co** ng g^{-1}	**Cs** ng g^{-1}	**Cu** ng g^{-1}	**Fe** µg g^{-1}	**Mn** ng g^{-1}	**Rb** µg g^{-1}	**Se** ng g^{-1}	**Tl** ng g^{-1}	**Zn** µg g^{-1}	**K** µg g^{-1}	**Mg** µg g^{-1}	**Na** µg g^{-1}
Mrežnica, this study (means ± standard deviations)														
REF-Apr	17.9 ± 5.4	6.08 ± 4.53	1.88 ± 0.49	6.23 ± 0.91	132 ± 20	1.12 ± 0.24	292 ± 105	3.02 ± 0.32	213 ± 20	7.35 ± 1.58	3.66 ± 0.57	5323 ± 319	379 ± 23	428 ± 73
REF-Sep	18.5 ± 4.3	8.11 ± 4.28	1.07 ± 0.40	5.77 ± 0.64	94.1 ± 8.3	1.21 ± 0.64	258 ± 81	3.46 ± 0.35	194 ± 27	4.27 ± 1.07	2.84 ± 0.39	4617 ± 198	327 ± 10	337 ± 68
DRF-Apr	22.4 ± 11.0	7.11 ± 3.91	1.70 ± 0.57	6.48 ± 0.87	126 ± 35	1.03 ± 0.32	202 ± 97	2.75 ± 0.23	173 ± 28	5.77 ± 1.48	3.43 ± 0.79	5078 ± 198	365 ± 20	385 ± 50
DRF-Sep	17.3 ± 4.9	14.9 ± 5.7	1.05 ± 0.33	6.15 ± 0.52	109 ± 24	1.08 ± 0.33	235 ± 43	3.15 ± 0.24	156 ± 12	3.56 ± 1.17	3.02 ± 0.65	4716 ± 183	327 ± 11	336 ± 44
River Elbe, Germany [1]	67.5	-	-	-	-	-	-	-	-	-	-	-	-	-
River Danube, Serbia [2]	685 ± 807	-	151 ± 49	-	1690 ±1136	46.4 ± 25.4	3446 ±1326	-	1136 ±373	-	21.3 ± 6.96	-	-	-
Vizelj Channel, Serbia [3]	ND (<44.6)	-	ND (<3.80)	-	ND-1716	1.65–22.6	-	-	-	-	4.10–10.7	-	-	-
Međuvršje Reservoir, Serbia [4]	352 ± 74	-	ND	-	164 ± 4	3.66 ± 0.80	250 ± 20	-	-	-	9.72 ± 2.22	-	-	-
Flin Flon Lakes, Canada [5]	-	-	-	-	160 ± 20	-	-	-	-	-	5.6 ± 2.4	-	-	-
River Danube, Croatia [6]	6 ± 3	-	-	-	-	-	-	-	-	-	-	-	-	-
Sapanca Lake, Turkey [7]	-	-	-	-	342 ± 420	-	-	-	-	-	2.00 ± 0.36	-	-	-
Ińsko/Wisola lakes, Poland [8]	-	-	-	-	140/190	1.4/0.8	200	-	-	-	9.4/6.2	-	-	-
MAC-EU [a]/Croatia [c]	2000 [b]	-	-	-	-	30.0 [b]	-	-	-	-	-	-	-	-
ML-FAO [d]	100													

[1] long-term contamination, anthropogenic and geogenic sources [1]; [2] industrial contamination without any pretreatment, the city of Belgrade; additional elements: Cd—0.006 ± 0.003 µg g^{-1} [4]; [3] the suburban section of Belgrade, highly eutrophicated/polluted by nearby agricultural areas and urban wastewaters without treatment [5]; [4] untreated industrial and communal waters; additional elements: Cd—ND; Mo—0.488 ± 0.02 µg g^{-1}; Pb—ND [21]; [5] near smelter; additional elements: Cd—up to 0.015 µg g^{-1} [22]; [6] downstream from industrial/agricultural sources; additional elements: Cd—0.003 ± 0.001 µg g^{-1}; Pb—0.007 ± 0.004 µg g^{-1}; [23]; [7] sewage outlets, urban wastewater, agricultural runoff [24]; [8] without anthropogenic impact/receiving wastewaters from municipal sewage treatment plant [25]. [a] for fish, for additional analyzed elements: Cd—0.050 µg g^{-1}; Pb—0.30 µg g^{-1} [26]; [b] MACs for As and Fe were included in ordinance from 2007, but excluded from 2012 version [27]; [c] for fish, for additional analyzed elements: Cd—0.050 µg g^{-1}; Pb—0.30 µg g^{-1} [28]; [d] for edible fats and oils, including fish oil [29].

2.4. Calculation of Daily Intakes and Risk Quotients

To assess the potential human health risk originating from the consumption of fish meat, we have calculated several indices (Table 3) according to the formulas provided by Javed and Usmani [30]. Estimated daily intakes (EDI, mg kg^{-1} day^{-1}) of detected metals/metalloids were calculated as follows: EDI = ($M_c \times IR$)/B_m, where M_c was the average measured metal/metalloid concentration in the fish muscle (mg kg^{-1}, wet mass); IR was average ingestion rate of fish meat in Croatia which was presumed to be 13.4×10^{-3} kg day^{-1}, according to a study by Speedy [31]; and B_m was average human body mass for Croatians, presumed to be 74.5 kg; EDI was expressed as mg of metal/metalloid per kg of person's body mass introduced into the body per one day.

Target hazard quotients (THQs, dimensionless) were used to estimate the non-carcinogenic risk originating from the consumption of fish meat potentially contaminated with metals/metalloids. THQs were calculated as follows: THQ = ($M_c \times IR$)/(RfD $\times B_m$), where RfD is oral reference dose or tolerable daily intake, expressed as mg kg^{-1} day^{-1} for individual elements, and provided by U.S. Environmental Protection Agency (USEPA). A THQ above 1 indicates a potential non-carcinogenic risk to humans [30].

Hazard indices (HIs, dimensionless) were used to estimate the cumulative health risk caused by a larger number of metals/metalloids. HIs were calculated as follows: HI = $THQ_{M1} + THQ_{M2} + THQ_{M3} + \ldots + THQ_{Mi}$, where M_1–M_i represents all the metals/metalloids in the fish meat for which THQs were calculated. As for THQ, HI should be below 1 [30].

Target cancer risk (TR, dimensionless) was used to estimate the carcinogenic risk originating from consumption of fish meat potentially contaminated with metals/metalloids. TR was calculated as follows: TR = ($M_c \times IR \times CFS$)/B_m, where CFS is the oral cancer slope factor, expressed as mg kg^{-1} day^{-1} for individual elements, and provided by USEPA. TR categories are defined as follows: TR $\leq 10^{-6}$ represents very low risk; TR = 10^{-6}–10^{-4}, less low risk; TR = 10^{-4}–10^{-3}, moderate risk; TR = 10^{-3}–10^{-1}, high risk; and TR $\geq 10^{-1}$, very high risk [30].

2.5. Statistical Analyses and Graphical Data Presentation

Calculations were performed in Microsoft Office Excel (Version 16), whereas the graphs were created in the statistical program SigmaPlot 11.0 for Windows. The data analyses were performed by PROC GLM procedure from SAS® OnDemand for Academics software (SAS Studio 3.8 on SAS 9.4) with a significance level set at $\alpha = 0.05$. Association between fish mass and total length, as well as between fish mass and metal/metalloid concentrations at each site in each sampling campaign, was tested with linear regression, and the results were presented as the Pearson correlation coefficients, which were considered significant at $p < 0.05$ (Table S3). The comparisons between two different sites and between two different periods were performed by *t*-test; additional comparisons were performed between two different sites by ANCOVA with total mass as covariate (Figures 2–5), and the differences were considered significant at $p < 0.05$. The effect of age and sex on metal/metalloid concentrations and selected biometric parameters was tested by two-way ANOVA with estimation of interaction between main effects (age, sex) and the Tukey–Kramer method as an adjustment for multiple comparisons (Table S4). The effects were considered significant at $p < 0.05$. Between-site comparisons for each parameter, grouped by age and sex, were performed by *t*-test for each sampling campaign separately, and the differences were considered significant at $p < 0.05$. The logarithmic transformation (ln(x)) was used to achieve data normality and variance homogeneity where it was necessary.

Table 3. The estimation of potential risk of using muscle of the northern pike (*Esox lucius*) from the Mrežnica River in human diet: estimated daily intakes (EDI) of detected metals/metalloids, target hazard quotients (THQ), hazard indices (HI), and target cancer risk (TR).

	As	Bi	Co	Cs	Cu	Fe	Mn	Rb	Se	Tl	Zn
[a] **RfD/mg kg^{-1} day^{-1}**	0.0003 [c]	-	0.0003 [d]	-	0.04 [d]	0.7 [d]	0.14 [c]	-	0.005 [c]	0.00001 [c]	0.3 [c]
[b] **CSF/(mg kg^{-1} day^{-1})$^{-1}$**	1.5 [c]	-	-	-	-	-	-	-	-	-	-
EDI$_{REF\text{-}Apr}$/mg kg^{-1} day^{-1}	0.0000032	0.0000011	0.0000003	0.0000011	0.000024	0.000201	0.000053	0.000543	0.000038	0.0000013	0.000658
EDI$_{DRF\text{-}Apr}$/mg kg^{-1} day^{-1}	0.0000040	0.0000013	0.0000003	0.0000012	0.000023	0.000185	0.000036	0.000494	0.000031	0.0000010	0.000617
EDI$_{REF\text{-}Sep}$/mg kg^{-1} day^{-1}	0.0000033	0.0000015	0.0000002	0.0000010	0.000017	0.000217	0.000046	0.000622	0.000035	0.0000008	0.000511
EDI$_{DRF\text{-}Sep}$/mg kg^{-1} day^{-1}	0.0000031	0.0000027	0.0000002	0.0000011	0.000020	0.000193	0.000042	0.000566	0.000028	0.0000006	0.000543
THQ$_{REF\text{-}Apr}$ (HI = 0.1552)	0.0107	-	0.0011	-	0.0006	0.0003	0.0004	-	0.0077	0.1322	0.0022
THQ$_{DRF\text{-}Apr}$ (HI = 0.1276)	0.0134	-	0.0010	-	0.0006	0.0003	0.0003	-	0.0062	0.1038	0.0021
THQ$_{REF\text{-}Sep}$ (HI = 0.0982)	0.0111	-	0.0006	-	0.0004	0.0003	0.0003	-	0.0070	0.0768	0.0017
THQ$_{DRF\text{-}Sep}$ (HI = 0.0835)	0.0104	-	0.0006	-	0.0005	0.0003	0.0003	-	0.0056	0.0640	0.0018
THQ (brown trout, *Salmo trutta*) [e]	-	-	-	-	0.016	0.0002	0.0002	-	-	-	0.0017
THQ (Prussian carp, *Carassius gibelio*) [f]	-	-	0.0004	-	0.0007	0.009	0.0002	-	0.0040	-	-
THQ (common carp, *Cyprinus carpio*) [g]	-	-	-	-	0.0007–0.0008	0.036–0.044	0.0001	-	-	-	0.0020
THQ (Prussian carp, *C. gibelio*) [h]	-	-	-	-	0.008–0.010	-	-	-	-	-	0.017–0.030
TR$_{REF\text{-}Apr}$	0.0000048	-	-	-	-	-	-	-	-	-	-
TR$_{DRF\text{-}Apr}$	0.0000060	-	-	-	-	-	-	-	-	-	-
TR$_{REF\text{-}Sep}$	0.0000050	-	-	-	-	-	-	-	-	-	-
TR$_{DRF\text{-}Sep}$	0.0000047	-	-	-	-	-	-	-	-	-	-

[a] oral reference dose or tolerable daily intake; [b] oral cancer slope factor; [c] according to Integrated Risk Information System (IRIS), U.S. Environmental Protection Agency, Chemical Assessment Summaries, downloaded at 7 March 2023; [d] according to Javed and Usmani [30]; [e] uncontaminated mountain stream in Sardinia [32]; [f] isolated ponds—highway, agriculture and gravel factory, Serbia [33]; [g] Lake Mandra and Lake Bourgas across the coastal waters of Bulgarian Black Sea [34]; [h] Lake district—various anthropogenic influences, Turkey [35].

3. Results and Discussion

3.1. Biometric Characteristics of the Sampled Fish

Sampled fish belonged to the age groups from 0+ to 3+ and were classified for the purposes of this study into two age groups: age group 1 encompassed younger fish (0+ and 1+); age group 2 encompassed older fish (2+ and 3+); and according to sex, in females and males (Figure 1; Table S1). The REF site in April 2021 stood out, with predominantly younger fish and predominantly males. The remaining three data groups encompassed a comparable number of females and males in the older group of fish and differed slightly in the sex composition and number of specimens in the younger one (Figure 1; Table S1).

In the first campaign, in April 2021, 16 fish specimens meeting the age criteria were caught per site; at the REF site, their total length and mass were in the following ranges: 19.0–35.5 cm and 50.0–325 g, respectively. At the DRF site, they ranged from 17.0–45.0 cm and 30.0–610 g, respectively (Figure 2a; Table S1). In the second campaign, in September 2021, 16 and 11 fish specimens meeting the age criteria were caught at the REF and DRF sites, respectively; at the REF site, their total length and mass were in the following ranges: 24.5–46.0 cm and 95.0–610 g, respectively; at the DRF site they ranged from 28.5–45.0 cm and 150–625 g, respectively (Figure 2a; Table S1). Due to high positive correlations between fish mass and total length (r = 0.967–0.994; $p < 0.001$), fish total length was not further presented and analyzed. Since sometimes fish size can be a decisive factor influencing the metal concentration in fish tissues, it is important for metal bioaccumulation study that fish at different sites have comparable sizes, which was generally the case in our study. However, the exception was again fish caught at the REF site in April 2021, which were significantly smaller compared to the DRF site within the same sampling period, and compared to the same site in the September sampling period (Figure 2a; Table S1). Two-way ANOVA with age and sex as the main effects was applied to establish if these differences occurred due to biological or environmental reasons; the data for this analysis were taken from the DRF site in the April campaign and from the REF site in the September campaign, because the remaining two sets of data did not meet the criteria for this test (i.e., they did not contain both younger and older fish of both sexes) (Table S4). Only the statistically significant effect of age on the fish's total mass was recorded, with expectedly higher mass in the older fish; the age effect was not dependent on the fish sex. An additional between-site comparison performed for each season, separately for the younger and for the older fish of each sex, revealed that there were no statistically significant differences in total fish mass that could be attributed to the environmental conditions (Table 1), i.e., the observed smaller size of the northern pike at the REF site in April 2021 was obviously the result of their lower age.

FCIs were rather uniform at both sites in both campaigns, without any significant differences being observed; their medians ranged from 0.914–0.942%, with only slightly higher values in smaller fish at the REF site in April (Figure 2b; Table S1). For GSI, statistically significant differences between sites within each campaign were also not observed, although somewhat higher values were recorded at the REF compared to the DRF site in both campaigns. Namely, in the April campaign median at the REF site was 0.57%, and at the DRF site, 0.32%; and in the September campaign, the median at the REF site was equal to 1.80%, and at the DRF site, 1.04% (Figure 2c; Table S1). Two-way ANOVA indicated higher GSI values in males compared to females, and the difference was significant in September (Table S4), which could be associated with the fact that gonadal growth in male northern pike starts intensively already in summer, whereas in females it is much more intense in the winter [14]. Thus, somewhat higher GSIs at the REF site than at the DRF site, although not statistically significant, in both seasons (but especially in September) could probably be attributed to a higher number of male than female specimens at that site (Figure 1; Table S1). Moreover, significant differences in GSIs between the two sampling campaigns were observed at both sites, with higher percentages recorded in September (as presented above), indicating different stages of gonadal development. The spawning of the northern pike occurs in the period from March to May; for example, for the northern pike in Lithuania, it was established to occur from the middle of March until the end of April [3], and for the northern pike in Denmark, from the middle of March until the middle of May [36]. According to recorded GSI values, the northern pike sampled in April 2021 were caught immediately after the spawning, whereas in September 2021, gonad development and the preparation for the next spring spawning had already started, as previously reported for the northern pike [14].

3.2. Metal/Metalloid Bioaccumulation in Fish Muscle: Environmental Considerations

3.2.1. Overview of the Metals/Metalloids Concentrations in the Fish Muscle

The concentrations of 2 of the 17 analyzed elements (Mo and Pb) were always below their detection limits (1.22 ng g^{-1} and 0.95 ng g^{-1}, respectively) of the applied procedure. Molybdenum was thus markedly below the concentrations reported for the industrially impacted Međuvršje Reservoir (Serbia) (more than 400 times) ([21]; Table 2). Moreover, Cd concentrations were not determined for the April campaign due to instrumental issues, and the concentrations determined for the September campaign were also below/around their LOD (0.079 ng g^{-1}). They were, thus, lower even compared to Cd concentrations reported for northern pike from two lakes in Mazurian Lake District (Poland) (0.5–6.2 ng g^{-1}), characterized by good water quality (as stated by Łuczyńska et al. [13]). Cadmium and Pb concentrations in the muscle of northern pike from the industrially and/or agriculturally impacted Međuvršje Reservoir and Vizelj Channel (both in Serbia) were also reported as undetectable ([5,21]; Table 2). Cadmium concentrations in northern pike meat from freshwaters heavily burdened by industrial/smelting contamination were reported to be at least 75–200 times higher compared to our study ([4,22]; Table 2).

Even comparison to a rather unpolluted section of the Danube River in Croatia [23] indicated at least 7.5 times and 50% lower Cd and Pb concentrations (with respect to their LOD values), respectively, in the northern pike from the Mrežnica River.

The concentration order of the remaining metals/metalloids accumulated in the northern pike muscle was, independently of the sites and sampling campaigns, the following: 6 mg g^{-1} > K > 500 µg g^{-1} > Na, Mg > 10 µg g^{-1} > Zn, Rb > Fe > 500 ng g^{-1} > Mn > Se > Cu > 50 ng g^{-1} > As > Bi, Cs, Tl > 2 ng g^{-1} > Co > 0.5 ng g^{-1} (Table 2). Generally, with the exception of rather high Rb concentrations (~3 µg g^{-1}), the nonessential elements in the northern pike muscle were present, as expected, in much lower concentrations (up to ~25 ng g^{-1}) than the essential ones (with few exceptions, from 100 ng g^{-1} to a few mg g^{-1}). Rubidium, although nonessential, was previously reported to accumulate in rather high concentrations in the muscle tissues of various organisms due to its similarity and consequent coaccumulation with K; it was even reported that the skeletal muscle of rats accumulates Rb preferentially to K when exposed to comparable concentrations of both metals [37].

Comparison with available literature data for northern pike (only for seven elements: As, Co, Cu, Fe, Mn, Se, and Zn), as well as with legal recommendations (only for four elements: As, Cd, Fe, and Pb), indicated that accumulated concentrations of metals/metalloids in the northern pike muscle from the Mrežnica River were generally low (Table 2). The most pronounced difference between our results and the previously published data was observed in the case of the Danube River (in Serbia), heavily contaminated by industrial wastewater without any pretreatment.

Metal/metalloid concentrations in the northern pike muscle from the Mrežnica River were 5–80 times lower, depending on the element, compared to the report for the Danube River [4]. Comparison with the other available reports for northern pike indicated either comparable or slightly lower (with few exceptions, up to 5 times) concentrations measured in our study compared to pike from freshwaters exposed to various degrees of industrial contamination (Table 2). We can only point out somewhat increased concentrations of a few metals/metalloids. As concentrations were 3–4 times higher in the northern pike from both sites of the Mrežnica River compared to the Danube River section in Croatia, which is not directly influenced by contamination sources [23]. Zn concentrations were 40–80% higher in the northern pike from both sites of the Mrežnica River compared to

Sapanca Lake, which is influenced by urban and agricultural contamination sources [24]. And Mn concentrations were 30–50% higher in the northern pike from the reference site of the Mrežnica River compared to the Polish lakes mildly contaminated by municipal wastewater [25], which could be associated with higher sediment contamination with Mn in the Mrežnica River (200–400 µg g^{-1} wet weight [8]) compared to the Polish lakes (200–250 µg g^{-1} dry weight [25]). However, when compared to freshwaters exposed to industrial contamination, even As, Zn, and Mn were much lower in the northern pike in our study (Table 2).

Among analyzed elements, recommendations for maximum allowed concentrations (MACs; by European Commission, Croatian law, and FAO) were set only for As, Cd, Fe, and Pb [26–29]. All values obtained in our study were much lower than the legal recommendations: 4.5 and 90 times for As (depending on the authority), >600 times for Cd, 25 times for Fe, and >175 times for Pb.

3.2.2. Size/Age/Sex Influence on Bioaccumulated Metal/Metalloid Concentration Variability

The physiological characteristics of fish have often been reported to impact the bioaccumulation of metals/metalloids, for example, age, sex, size (i.e., total fish mass), and fish physiological condition [13,38]. Taking into consideration that biometric parameters of northern pike sampled in this study differed to a certain degree between sites/seasons (Figures 1 and 2), we have analyzed the potential influence of several fish key characteristics, namely the size (represented by total mass), age and sex, on metal/metalloid concentrations in the northern pike muscles (Tables S3 and S4). The influence of FCI was not considered since the differences in FCI between sites were negligible. Furthermore, it was previously reported that elemental accumulation and the condition of northern pike were not significantly correlated [5].

Pearson correlation analysis (Table S3) indicated, although not definitely, that there is a connection between the fish mass and bioaccumulated metal/metalloid levels. The concentrations of several elements showed negative correlations with total fish mass, in some cases statistically significant, namely for Co ($r = -0.10$ to -0.62), Cu ($r = -0.11$ to -0.59), Fe ($r = -0.16$ to -0.69), and Zn ($r = -0.32$ to -0.48), and this was independent of the sampling period or site (Table S3). A negative correlation of Fe and Zn concentrations in northern pike muscle with the length and mass of studied fish has already been reported [5]. The same was reported for bream, *Abramis brama*, with a negative association being observed between fish size and concentrations of Cu and Zn [11]. However, as a confirmation that it is important to consider the species when studying the variations of metal bioaccumulation, it should be noted that in some fish species, this metal–size association can be opposite, e.g., high positive correlations between fish size and concentrations of Cu, Fe, and Zn were previously reported for cyprinid fish *Leuciscus vorax* from Turkey [12]. In our study, high and sometimes statistically significant negative correlation with total fish mass was further observed for As (at both sites only in the April campaign; $r = -0.71$ to -0.78), for Mn (except at the REF site in April; $r = -0.29$ to -0.54), and for Tl (in two cases; $r = -0.24$ to -0.44) (Table S3). The observed negative association indicated that higher metal/metalloid concentrations bioaccumulated in the smaller fish, possibly as a result of their higher metabolic and feeding rates, and, in the case of essential elements, higher metabolic requirements since it is well known that feeding rate of many fishes decreases with their development, as observed for bream [11]. Another explanation can be found in inadequately developed detoxification systems in young fish [5] or in the metal dilution in bigger fish caused by the increase of their muscle tissue mass due to a higher percentage of lipids in older specimens [11].

A positive correlation with total fish mass, in some cases statistically significant, was, on the other hand, observed for Bi, independently of period and site ($r = 0.20$ to 0.47), for Se in three cases ($r = 0.09$ to 0.67), in two cases for K ($r = 0.20$ to 0.75) and Mg ($r = 0.47$ to 0.63), and in one case for Na ($r = 0.50$) (Table S3). Observed positive association pointed to higher metal/metalloid concentrations bioaccumulated in the bigger fish, possibly connected to a longer time of fish exposure to contaminants [5] or likely connected to differences in feeding habits between smaller and bigger fish. It is known that the younger/smaller pike feed on small invertebrates (from daphnia to the isopods or gammarids), whereas older/larger individuals feed mainly on fishes, frogs, and crayfish [39,40] and are accordingly exposed to metals from more variable sources.

The concentrations of Cs and Rb showed the weakest association with the fish mass, with the correlation coefficients (r) always below 0.35 (either positive or negative) (Table S3).

The dependence of metal/metalloid muscle bioaccumulation on fish age and sex was assessed by applying the two-way ANOVA (in the same way as described in Section 3.1) (Table S4). A statistically significant influence of age was obtained for three elements at the REF site in September 2021, namely for Mn, Se, and Tl. Higher concentrations in younger fish were observed for Mn (always) and for Tl (only in males), whereas higher concentrations in the older fish were observed for Se (always). Additionally, higher concentrations in younger fish, although not significant, were always observed for Fe, K, and Zn, and in the older fish for Na. Higher concentrations of Fe and Zn in muscles of younger specimens of northern pike were previously also reported by Nikolić et al. [5].

A statistically significant influence of sex, on the other hand, was obtained only for Bi and Co at the DRF site in April 2021, with Bi higher in females and Co higher in males. However, in addition to Co, a trend of always higher values in males, although not statistically significant, was further observed for Fe, Mg, Mn, Se, and Zn. Such differences can occur due to several reasons, including dietary preferences, foraging behavior, or specificities of the reproductive cycle [25]. Statistically significant interactions between the two effects (age and sex) were never obtained, and for all the remaining elements, clear trends regarding age and sex influence were not observed. Due to the limited number of data, the established influences of age and sex on the concentrations of several metals in the muscle of the northern pike are only indicative, and should be further confirmed by additional investigation.

3.2.3. Spatial Differences of Metal/Metalloid Concentrations in the Fish Muscle as the Result of the Differences in Environmental Exposure

Comparisons between reference (REF) and historically contaminated (DRF) sites were performed by series of *t*-tests, separately for each season (Figures 3–5). Additional tests were conducted to take into account the above-discussed influence of the physiological parameters on the spatial variability of metal/metalloid bioaccumulation (Figures 3–5, Table 1). Some elements, rather unexpectedly, revealed statistically significantly higher concentrations in the muscle of northern pike at the REF site (Figure 3), namely Rb and Se in both sampling campaigns, and Tl, K, and Mn in April sampling. If fish mass was applied as a covariate, only in the case of Mn, the difference was no longer significant, indicating that higher Mn levels measured at the REF site in spring were due to the small size of fish sampled at that site. The obtained differences were confirmed even when age and sex were considered, with special emphasis on mainly statistically significantly higher concentrations of Rb and Se in the muscle of northern pike at the REF site (Table 1). Increased bioaccumulation of three of these elements (Rb, K, Tl) could not be directly associated with exposure from water/sediment (Table S2; [8]), but based on their chemical similarity [37,41], it can be presumed that some common factor has influenced their intensified uptake at the REF site. In the case of Se, there are no data for sediment contamination, but hypothetically primary source of increased Se bioaccumulation could be the sediment.

Only two elements, Bi and Cs, showed a trend of higher concentrations in the muscle of the northern pike at the DRF site, but the difference was statistically significant only for Bi in the September campaign (Figure 4). The inclusion of fish mass as a covariate in the comparison did not change the outcome. Significantly higher bioaccumulated Bi concentrations in September sampling were confirmed even when age and sex were taken into account, whereas Cs variability between sites seemed almost negligible (Table 1). Water/sediment contamination of the Mrežnica River at the DRF site (Table S2; [8]) pointed to environmental exposure as the likely cause of slightly increased Bi and Cs bioaccumulation at the DRF site.

All the other elements, namely As, Co, Cu, Fe, Mg, Na, and Zn in the muscle of northern pike, were comparable at the two sites, i.e., the differences were generally not statistically significant (Figure 5). If fish mass was applied as a covariate, another statistically significant difference occurred, i.e., higher As concentrations at the DRF site, but only in April 2021, which was further confirmed when age and sex were considered (Table 1). Somewhat higher bioaccumulated Na concentrations at the REF site and bioaccumulated Cu, Co, and Zn concentrations at the DRF site were also occasionally observed when groups of the same age/sex were compared, but the differences were statistically significant only for Co in September 2021 for the older group containing only males (Table 1). In water/sediment of the Mrežnica River, higher concentrations at the time of fish sampling at the DRF site compared to the REF site were observed for several of the elements from this group (i.e., As, Co, Cu, Fe, and Zn; Table S2; [8]) some of which are known textile industry contaminants (e.g., Cu, Fe, Zn; [8]). This is consistent with the above-mentioned occasionally increased bioaccumulation of some of these elements in the muscle of the northern pike at this historically contaminated site.

As seen from the presented results, although some differences between sites were observed, they are much lower than could be expected based on differences in environmental contamination/exposure. However, since pollution associated with historical sources usually can be linked to river sediments, as the most important reservoirs of metals and other contaminants, more notable bioaccumulation could be expected in sediment-dwelling and benthic organisms than in carnivorous species [42], because fish feeding on benthic organisms are directly exposed to contaminated sediments [23]. Some mussel species, such as *Unio crassus*, were also described as good indicators of historical pollution of freshwater sediments [43]. Furthermore, it is well known that the muscle is commonly regarded as a tissue with a low potential for metal/metalloid accumulation, thus usually having the lowest metal/metalloid content of all fish organs [21]. Harrison and Klaverkamp [22] reported that metal concentrations in the muscle of the northern pike were poor indicators of concentrations in sediments, even in the heavily contaminated lakes, whereas Nikolić et al. [5] reported that muscle was least affected by pollution. Therefore, higher bioaccumulation in fish and more pronounced differences between sites could be expected in more metabolically active organs, such as the liver, gills, or intestine. This can be corroborated by higher levels of several metals/metalloids reported for liver and/or gills and/or the other fish organs of *E. lucius* from Sapanca Lake, the River Elbe, Mazurian lakes, Ińsko/Wisola lakes and the Međuvršje Reservoir compared to muscles of the same fish specimens [1,13,21,24,25]. Such occurrences can be attributed to the important role of the liver in metal detoxification [1] and to the gills being the first organ to come into contact with metals in water, as well as participating in their uptake [21]. Since high bioaccumulation of some metals can affect various physiological functions of fish (growth, reproduction) and even cause an increase in mortality and decline of the richness and variety of aquatic species [5], their bioaccumulation in metabolically active organs should be further investigated, especially knowing that some bioaccumulation was observed even in the muscle.

3.2.4. Differences in Metal/Metalloid Concentrations in the Fish Muscle in Two Distinct Sampling Periods

Various factors (e.g., environmental conditions, food supply, the stage of the reproductive cycle), connected to different periods of the year, can also affect fish metabolism and, consequently, metal/metalloid bioaccumulation [44]. In our study, a clear seasonal trend with higher concentrations, mainly statistically significant, obtained at both sites in April than in September 2021, was observed for eight elements. Seven of these were essential (Co, Cu, K, Mg, Na, Se, and Zn), and their higher concentrations in the spring period could be related to typically higher feeding rates in fish after the winter inaction period. It is known that pike feeds more actively during the spring and summer, while in the study on stomach fullness, most of the empty ones were observed in winter [2]. One of the metals that increased in spring was nonessential, namely Tl, and its higher accumulation in the April campaign could be attributed to its chemical similarity with potassium in ionic radius and electrical charge [41], and thus concurrent concentration increase of both elements. Additionally, higher levels in the spring, immediately after the northern pike spawning, could be caused by the loss of the lipid percentage of muscle tissue during spawning [11], leading to higher concentrations of metals/metalloids in the fish muscle. It is consistent with the characteristic period of northern pike spawning between March and May [3,36], as well as with very low GSI in the spring sampling (Figure 2c; Table S1).

Four elements, namely As, Cs, Fe, and Mn, have not shown striking seasonal variability. However, two elements, i.e., Bi and Rb, showed higher concentrations in the September campaign, statistically significant for the first element only at the DRF site and for the latter one at both sites. As these elements are nonessential, and their autumn increase was isolated from the remaining elements, it could hardly be associated either with fish physiology or with chemical similarity with some other element. Thus, it could be hypothesized that the cause of Bi and Rb increase in late summer most likely was the transient increase in environmental exposure level at one site or in the entire river section, respectively.

3.3. Assessment of the Potential Human Health Risk from the Fish Meat Consumption

In Table 3, we have presented the estimated daily intakes (EDI) for 11 trace elements through the consumption of northern pike meat in the human diet. The estimation was made based on the average ingestion rate of fish meat in Croatia reported by Speedy [31]. Estimated values were considerably lower than tolerable daily intakes (RfD) of specific elements provided by regulative authorities [30]. The ratios of EDIs to RfDs are actually given in target hazard quotients (THQs, Table 3) for those elements with provided RfDs. A value equal to 1 indicates that EDI has reached the tolerance limit, whereas THQs above 1 indicate the potential non-carcinogenic risk for humans. In our study, THQs were always below 1, and the lowest risk was obtained for Fe (0.0003, or ~3000 times lower intake than tolerable), whereas the highest, but still not troublesome, was obtained for Tl (0.132, or ~7.5 times lower intake than tolerable). The THQ equal to 0.0004 for Cd in the muscle of northern pike from two Mazurian lakes, which are characterized by good chemical status [13], was comparable with THQ values obtained for Fe, Mn, and Cu in our study (Table 3), whereas THQ for Cd in our study could not even be calculated due to very low concentrations in the fish muscle, which altogether indicates that fish from waters of the Mrežnica River regarding analyzed metals/metalloids are safe for consumers.

Additional comparison was performed with reports on THQs for several other fish species (brown trout, *Salmo trutta*; Prussian carp, *Carassius gibelio*; common carp, *Cyprinus carpio*) from European freshwaters [32–35]. THQs in above mentioned studies were generally comparable to THQs obtained in our study (Table 3), although they referred to various freshwater environments, ranging from uncontaminated ones to those highly influenced by anthropogenic activities. There were few exceptions, having higher THQs compared to the northern pike from the Mrežnica River, and those were: 25 times higher THQ of Cu in brown trout from uncontaminated mountain stream; 10–15 times higher THQs of Cu and Zn in Prussian carp from anthropogenically impacted lakes in Turkey; and 30–130 times higher THQs of Fe in Prussian carp from anthropogenically impacted lakes in Serbia and in common carp from coastal lakes in Bulgaria (Table 3). Most likely, metal bioaccumulation in fish muscle depends, among other factors, on the fish species. Thus, in some cases, higher THQs of a particular element that reflect its higher bioaccumulation in muscle can be found in fish from uncontaminated environments (e.g., Cu in brown trout from mountain streams [32]) compared to some other fish species from the environment with higher degree of contamination (Prussian carp, ponds in Serbia [33]). However, all reported values were much lower than 1, indicating very low non-carcinogenic risk even in highly contaminated environments, probably due to low fish muscle tendency for metal accumulation [21], as discussed earlier.

Hazard indices (HIs; Table 3) were further calculated as an estimation of the cumulative health risk caused by ingestion of a larger number of metals/metalloids, and they were also always lower than 1 (0.0835–0.1552; or 6–12 times lower intake than tolerable). The highest contribution to HI at each site in each period was made by Tl. Furthermore, HIs were higher at both sites in the spring period, and in both periods at the REF site, which is consistent with Tl temporal and spatial variability.

Although non-carcinogenic risk from Tl consumption is still rather low, for people consuming more than the average quantity of fish in the diet, the risk can increase. The combined Tl intake from different sources should be assessed. This especially refers to the REF site, with higher bioaccumulation of Tl, not necessarily due to higher exposure but possibly due to coaccumulation with potassium. Thus, the coaccumulation of metals as a potential source of toxicity, especially for highly toxic elements, should be considered in monitoring studies.

Finally, estimation of the carcinogenic risk was performed by calculating target cancer risk (TR, Table 3), but only for As, since for the other studied elements there are no set regulations. The carcinogenic risk from As consumption through northern pike meat from the Mrežnica River can be classified as less low risk (the second lowest risk category [30]).

4. Conclusions

The study of metal/metalloid bioaccumulation in the muscle of northern pike from historically contaminated Mrežnica River in Croatia and of possible risk for human health due to fish meat consumption revealed that the bioaccumulated concentrations were still within recommended limits, comparable to moderately contaminated environments. The risk factors also indicated negligible risk from the use of northern pike meat in the diet. However, since previously published research indicated that the muscle of various fishes is generally a tissue with much lower potential for metal/metalloid accumulation compared to many other fish organs, the absence of pronounced metal/metalloid bioaccumulation in northern pike muscle did not necessarily demonstrate that there is no risk for the fish health and welfare. Certain differences between sites were observed: higher concentrations of several elements were recorded at the reference site that could not be connected to the environmental exposure, thus indicating a possible physiological impact on coaccumulation of several chemically similar elements (K, Rb, Tl); on the other hand, few elements were, in variable extent, present in higher concentrations in the muscle of northern pike from the historically contaminated site, namely As, Bi, Co, Cu, Cs, and Zn, and this observation could be associated to sediment and/or resuspended particulate matter contamination. Considering that even in fish muscle, a certain increase of metal/metalloid bioaccumulation was seen, more pronounced effects of historical contamination could be expected in organs such as the liver, thus emphasizing the need for regular monitoring of fish health status and biodiversity in areas previously exposed to long-term pollution. In addition, our study indicated the possibility that metal/metalloid bioaccumulation in fish muscle is associated with the physiological characteristics of fish, such as size, sex, age, and seasonal changes in metabolic and feeding rates, and pointed to the importance of incorporating these parameters in the monitoring studies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fishes9090364/s1, Table S1: Overview of the number of caught northern pike specimens and their biometric characteristics at the two sites in the two sampling campaigns; Table S2: The concentrations of selected 14 elements in the Mrežnica River water (dissolved and particulate) and in the sediments at two sampling sites in the two northern pike sampling campaigns; Table S3: The correlations between fish total mass and the concentrations of several metals/metalloids bioaccumulated in the muscle of the northern pike sampled at two sites of the Mrežnica River in April and September 2021; Table S4: The analysis of the influence of age and sex on fish mass, gonadosomatic index, and metal/metalloid concentrations bioaccumulated in the muscle of the northern pike sampled from the Mrežnica River; Figure S1: The map of the Mrežnica River in Croatia in the vicinity of the former textile factory in Duga Resa town, with marked sampling sites.

Author Contributions: Conceptualization, Z.D., D.I. and D.V.; methodology, V.F.M., S.Š., T.M.P., Z.K. and T.K.; software, N.T.; validation, Z.D. and N.T.; formal analysis, Z.D., V.F.M., S.Š., T.M.P. and S.D.; investigation, Z.D., D.I., V.F.M., S.Š., T.M.P., S.D., E.G., K.M., S.K., F.B., Z.K., T.K. and D.V.; resources, E.G., K.M., S.K., F.B., Z.K., T.K. and D.V.; data curation, Z.D.; writing—original draft preparation, Z.D.; writing—review and editing, Z.D., D.I. and D.V.; visualization, Z.D. and N.T.; supervision, Z.D. and D.I.; project administration, Z.D.; funding acquisition, Z.D., D.I. and D.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Croatian Science Foundation, grant number IP-2019-04-2636. The financial support of the Ministry of Science and Education of the Republic of Croatia for institutional funding of the Laboratory for Biological Effects of Metals is also acknowledged.

Institutional Review Board Statement: The authors state that the Ordinance on the protection of animals used for scientific purposes (NN 55/2013) issued by the Republic of Croatia was followed during this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available within the article or are available upon request.

Conflicts of Interest: Author Nataša Tepić was employed by the company Koios Consulting Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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