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Back to Ecology: Reference Conditions as a Basis for Assessment, Restoration and Sustainable Management of Large Rivers

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Abstract: Under the EU Water Framework Directive, ecological assessment and management are based on type-specific reference conditions. In the EU it may be difficult to find sites in large rivers with at least near-natural conditions, though this is not the case in southeast Europe, where stretches of large rivers still exist with at least near-natural conditions, meaning that there is little or no disturbance from hydromorphological alteration, water quality, land use in the catchment and alien species. We examined benthic invertebrate assemblages in 45 samples collected from near-natural sites of several large rivers: Sava, Drava, Mura, Kupa and Una. The near-natural benthic invertebrate assemblages of large rivers contained several rare or remarkable species, especially among stoneflies, e.g., *Marthamea vitripennis*, *Xanthoperla apicalis*. We compared benthic invertebrate communities in river sections with fine and coarse substrates and in three eco-hydromorphological (ECO-HM) types of large rivers, reflecting habitat heterogeneity: lowland-deep, lowland-braided and intermountain. Multivariate analysis of variance (PERMANOVA) was used to statistically evaluate similarities among assemblages. It was found that the composition of benthic invertebrate assemblages varied by both ECO-HM types and substrate category. Similarity percentage (SIMPER) analysis showed that the average dissimilarity of benthic invertebrate assemblages was high between all ECO-HM type pairs and between fine and coarse substrate. We found that habitat heterogeneity and substrate independently influenced benthic invertebrate assemblages. To achieve ecological goals in the management of large rivers, in addition to functionality, a holistic view with at least near-natural assemblages, including the names of the taxa present, should also be considered.

Keywords: large rivers; reference conditions; near-natural conditions; eco-hydromorphological types; substrate; river-basin management; WFD; southeast Europe

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Citation: Urbanič, G.; Mihaljević, Z.; Petkovska, V.; Pavlin Urbanič, M. Back to Ecology: Reference Conditions as a Basis for Assessment, Restoration and Sustainable Management of Large Rivers. *Water* **2021**, *13*, 2596. https://doi.org/10.3390/w13182596

Academic Editor: Rui Cortes

Received: 10 August 2021
Accepted: 18 September 2021
Published: 20 September 2021

1. Introduction

Large rivers provide a plethora of utilities for humankind, such as a source of water for domestic, industrial and agricultural purposes, a means of energy production and waste disposal, routes for navigation, and space for recreational activities [1]. However, these human uses are also pressures and, in combination with land use changes in their catchments, pose a threat to the function and ecological integrity of aquatic ecosystems. The long history of intensive use of large rivers has led to the degradation of unique ecosystems, and today large rivers are among the most degraded ecosystems on Earth. Concerns about the ecological quality of aquatic habitats have led to legislation in most developed countries that sets the objectives (e.g., EU Water Framework Directive 2000, US

Clean Water Act of 1972, Australian Water Reform Framework [2]). The European Union in its Water Framework Directive set 2015 as the year by which good ecological status should also be achieved for rivers [3], though these aspirations are yet to be achieved, due primarily to hydromorphological alterations that were identified as the main reason for water bodies being unable to achieve the environmental objectives of Water Framework Directive [4]. The European Green Deal was developed and published at the EU level [5] as a holistic document that aims to improve the wellbeing and health of citizens and future generations. It contains a proposal for legally binding EU nature conservation targets for restoring degraded ecosystems in the framework of the EU Biodiversity Strategy by 2030 [6]. The importance of "semi-natural" rivers was recognized through the restoration of river continuity and reinforced by a target to transform at least 25,000 km of rivers into free-flowing rivers by 2030, mainly through the removal of obsolete barriers and restoring floodplains and wetlands. In addition, according to the strategy, Member State authorities are also required to review water abstraction and impoundment permits with the aim of restoring ecological flows.

Due to the understanding that altering the hydrology, water chemistry and biology of rivers has unintended consequences on human lives, an increasing number of restoration projects were initiated [7–10]. A prevailing paradigm in ecological restoration is that increasing habitat heterogeneity promotes biodiversity restoration, which is reflected in stream restoration projects through the common practice of reconfiguring channels by adding meanders and physical structures. A scientific review of river restoration projects found that most projects successfully improved physical habitat heterogeneity, though very few showed a statistically significant increase in biodiversity, increasing their similarity to reference reaches or sites [11]. Improved ecological conditions are a true measure of river restoration success, where observed conditions should be compared to reference conditions. The reference conditions approach, which implies past historical conditions, is widely used in ecological assessment and current legislation now widely depends on the establishment of reference standards (e.g., Water Framework Directive). In Europe, type-specific reference conditions have been established in a variety of ways, often taking a pragmatic approach [12]. The latter is particularly true when sites with at least near-natural conditions are lacking, as is the case for most large rivers in the EU. Regardless of whether reference conditions are based on true reference sites or near-natural sites, their selection is crucial for the development of useful measures of ecological status [13]. The characteristics of biota occurring at reference sites are used to develop metrics and indices that serve as a basis for comparison with impacted sites of the same river-type to assess their ecological status. Therefore, biota representing reference conditions are a key element of river ecological assessment, restoration and sustainable river basin management. The key question is whether reference sites or at least near-natural sites are available for large rivers and what the differences are in the biota between large river types.

In Europe, river engineering began several centuries ago, and today most European large rivers are channelized and highly fragmented by dams, with few catchments still containing free-flowing large rivers [14]. In large rivers, human impact often increases along the river course, i.e., the lower reaches are the most disturbed [15,16]. The Dinaric Western Balkan and the Pannonian Lowland (Ecoregions 5 and 11) [17,18] are areas in Europe where free-flowing large rivers still exist. According to water quality results, the impact of pollution is relatively low (e.g., [19,20]); thus, near-natural large river stretches can still be found. The Kupa and Una are in near-natural conditions along their river courses. The Drava is one of the few rivers in Europe that is not regulated in its middle and lower sections. The lower Drava in Hungary has been protected as a national park since 1996 [21]. Large parts of the Sava River still show a relatively natural geomorphic structure and hydrological regime, lined by large, protected wetlands. The mainstream has been channelized for flood control only in a few, short sections (e.g., in Zagreb); the mainstream has been channelized for flood control [14]. There are two Ramsar sites and three Important Bird Areas [22] in the middle reaches of the Sava, and this river is a priority area in the Pan

European Biodiversity and Landscape Strategy programmes and a key site in the Danube River Basin programmes. The Sava, the lower Drava, Mura and Kupa also have significant floodplains. A good example of intact floodplains is the Lonjsko Polje Nature Park on the Sava [23]. However, these types of habitats are becoming increasingly rare as development fundamentally changes river courses and floodplain landscapes. Although all these rivers are located in the same region, certain sections differ in their natural habitat heterogeneity, which is reflected in the eco-hydromorphological types [19,20].

The aims of this study were (i) to define near-natural benthic invertebrate assemblages of large rivers, (ii) to examine difference in benthic invertebrate assemblage compositions between three eco-hydromorphological types of large rivers; lowland-deep, lowland-braided and intermountain and (iii) to explain some of the key drivers of benthic invertebrate variation across these river types. We hypothesized that (i) the composition of near-natural benthic invertebrate assemblages and contribution of taxa vary among large river types, and (ii) substrate and habitat heterogeneity independently influence the composition of benthic invertebrate assemblages.

2. Materials and Methods

2.1. Study Area

The study was conducted in two neighbouring countries: Slovenia with a total area of 20,273 km^2 and Croatia with a total area of 56,594 km^2. The rivers in each of these countries flow into either the Danube Basin or Adriatic Basin, but this study focused only on the Danube catchment, covering 16,381 km^2 (80.8%) of the Slovenian territory and 35,101 km^2 (62%) of the Croatian territory. This study was limited to five major rivers of the Danube Basin: Drava, Mura, Sava, Kupa and Una Rivers. Only stretches with a catchment area of 5000 to 64,000 km^2 at altitudes between 74 and 338 m were considered. These stretches belong to three eco-hydromorphological types [19,20]: intermountain, lowland-braided and lowland-deep (Table 1, Figure 1). The Sava and Drava are among the largest tributaries of the Danube River (first and fourth, respectively) and represent some of the most preserved rivers in Europe in terms of their biological and landscape diversity. Nature conservationists and scientists consider the Sava to be one of the “crown jewels” of European nature [24]. The lower Drava, together with the lower Mura, forms a 380 km long, free-flowing and relatively natural watercourse, representing one of the last remaining contiguous riverine landscapes in Central Europe [23].

Table 1. Main characteristics of the sampled rivers according to the eco-hydromorphological river type and the number of sampling sites and samples.

River	Eco-Hydromorphological Type	Catchment Size Range (km^2)	Altitude Range (m a.s.l.)	No. Sites (Samples)
Sava	Intermountain	4946–5203	191–222	3 (7)
Drava	Lowland-braided	13,189–31,038	122–236	7 (11)
Mura	Lowland-braided	9784–10,930	153–246	6 (12)
Sava	Lowland-deep	7151–64,073	74–191	7 (8)
Drava	Lowland-deep	33,916–39,982	81–100	3 (3)
Kupa	Lowland-deep	9184–9184	92–92	1 (2)
Una	Lowland-deep	9368–9368	94–94	1 (2)

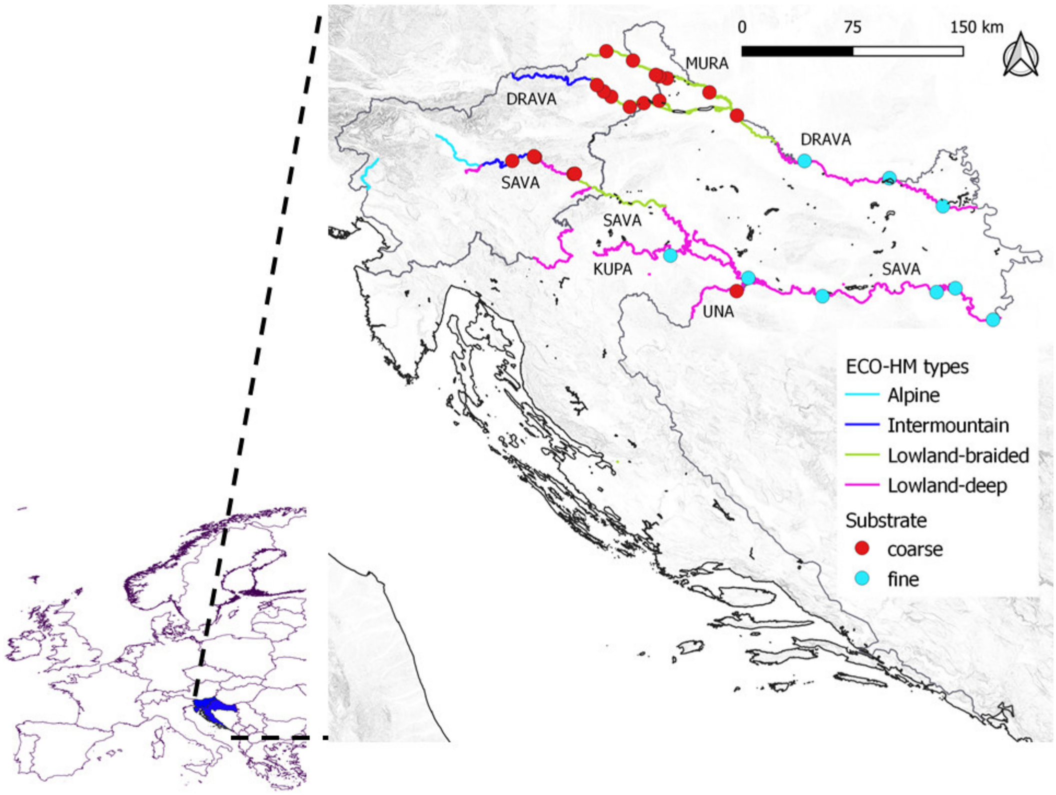

Figure 1. Study area with large river eco-hydromorphological (ECO-HM) types and near-natural sampling sites with prevailing natural substrate (closed circles).

2.2. Environmental Variables

The 28 sampling sites (Figure 1) selected cover "near-natural conditions", reflecting no or slight disturbance levels caused by hydromorphological alteration, water quality, catchment land-use and alien species (Table 2). Some variables were used as exclusion criteria for selection of suitable samples, or simply to represent values recorded at "near-natural" sites. The following criteria related to hydromorphology [19,25], water quality conditions [19,26–29], Corine land use/land cover [30] and alien species were used to select these near-natural sites:

(a) Hydromorphological quality and assessment index (HQM), reflecting the combined influence of habitat quality, habitat modifications and upstream and/or downstream barriers: HQM > 0.6 (at least class 2).
(b) Hydrological modification index (HLM) reflecting influence of upstream/downstream barriers (impoundments): HLM > 0.6 (at least class 2).
(c) Saprobic index (minimum normalised value–ecological quality ratio (EQR) for the Slovenian (SIG3) and Croatian version (SIHR): EQR > 0.6 (at least good ecological status).
(d) Percentage of CLC natural and semi-natural land use/land cover in the catchment at least 50% and CLC intensive agriculture + urban land use/land cover in the catchment <30%, where CLC urban land use/land cover in the catchment <5%.
(e) Alien species could be present but account for <35% of the benthic invertebrate assemblage.

Table 2. Median, minimum and maximum values of the selected hydromorphology, water quality, land use variables and proportion of alien species at near-natural sites.

Variable	Median	Minimum	Maximum
Low annual discharge (m^3/s)	99	7	648
Mean annual discharge (m^3/s)	140	9	998
High annual discharge (m^3/s)	399	90	1530
Hydromorphological quality and modification index (HQM)	0.84	0.71	1
Hydrological modification index (HLM)	0.94	0.68	1
Water temperature—median (°C)	12.0	9.8	16.1
pH—median	8.0	7.7	8.3
Conductivity—median (µS/cm)	350	299	480
Total suspended solids—median (mg/L)	7	3	56
Total nitrogen—median (mgN/L)	1.60	0.69	2.34
Ammonium—median (mgN/L)	0.06	0.01	0.30
Nitrate—median (mgN/L)	1.40	0.50	2.01
Orthophosphate—median (mgP/L)	0.05	0.01	0.29
Biochemical oxygen demand (5 days)—median (mg/L)	1.2	0.8	2.4
Chemical oxygen demand ($K_2Cr_2O_7$)—median (mg/L)	6.15	2.5	19.6
Oxygen concentration—median (mg/L)	9.55	8.55	11.5
Oxygen saturation—median (mg/L)	90	80	104
Saprobic index (EQR)	0.78	0.63	1.00
CLC Natural (%)	71	55	78
CLC Agriculture (%)	25	19	43
CLC Intensive agriculture (%)	12	8	26
CLC Extensive agriculture (%)	12	11	25
CLC Urban (%)	4	1	5
Alien species (%)	0	0	33.3

Twenty-four environmental variables were measured or calculated: five hydromorphological, fourteen water quality, and five catchment land cover/land use variables. Details of how the variables were obtained or calculated are described elsewhere [20]; only a summary is provided here. Water flow data were obtained from available discharge data from the hydrological databases of the Slovenian Environment Agency (ARSO) and Croatian Meteorological and Hydrological Service (DHMZ). The mean daily discharges were used to calculate the mean annual discharge (MQ), the lowest annual discharge (daily mean, NQ) and the highest annual discharge (daily mean, HQ) of the sampling year. The Slovenian hydromorphological (SIHM) assessment method [19,25,31] was applied to examine habitat quality, habitat modifications and the influence of the main upstream barriers/impoundments. A river habitat survey [32,33] was conducted once between 2005 and 2012 in the benthic invertebrate sampling year or after sampling for each sampling site and the data were used to calculate the two morphological indices [25,31]: river habitat quality index (RHQ), and river habitat modification index (RHM). Normalised values (converted to a common scale of 0–1; RHQnor and RHMnor [19]) were used according to the eco-hydromorphological type of the considered river stretches according to Urbanič and Urbanič et al. [32,33] (Table 1, Figure 1). The data on impoundments recorded in the catchment of each sampling site in the year of benthic invertebrate sampling were used to calculate the hydrological modification index (HLM, [19,25]). The combination of the RHQnor, RHMnor and HLM indices was used to calculate the hydromorphological quality and modification index (HQM) [19,25,31]. Physical and chemical data were obtained monthly or at least four times per year (each season) from national surface water monitoring programmes during the sampling year. Only selected water quality parameters were used that were available for all selected sites: water temperature, conductivity, pH, oxygen concentration, oxygen saturation, water temperature, chemical oxygen demand ($K_2Cr_2O_7$), biochemical oxygen demand (BOD_5), orthophosphate, total nitrogen, ammonium and total suspended solids. The ecological quality ratio values were calculated based on the saprobic index according to the national standards [26–29]. The median of data collected for each parameter in the benthic invertebrate sampling year is shown in Table 2. In addition to the variables listed, for each site the naturally predominant substrate of each site was classified into two classes: fine (psammal) or coarse (lithal). The information on eco-hydromorphological river type was based on previous studies [19,20]. Land use variables were based on the proportion of land use categories at the catchment scale, extracted from Corine land cover (CLC) data [30] using ArcGIS version 10.2.1 (Esri Corp., Redlands, CA, USA). The categories were grouped into five land use variables: urban land use (CLC class 1), natural and semi-natural land use (CLC classes 3–5), extensive agricultural land use (CLC categories 2.3.1, 2.4.3, 2.4.4), and intensive agricultural land use (CLC categories 2.1, 2.2, 2.4.1, 2.4.2).

2.3. Benthic Invertebrates

Biological data were obtained as part of the WFD monitoring and assessment system development programmes in Slovenia and Croatia between 2005 and 2011. A total of 104 samples were collected at 56 sites [20], and of these 45 samples at 28 sampling sites met the criteria for "near-natural sites" as used in this study. Most samples (13) were collected in the lowland-braided sections of the Drava and Mura Rivers, 12 samples in the lowland-deep sections of the Sava, Drava, Kupa and Una Rivers, and seven samples were collected in the intermountain sections of the Sava River (Table 1). Some sites were sampled several times, but not more than once per year. Benthic invertebrates were collected at low to moderate flows using a multihabitat sampling approach. A detailed description of benthic invertebrate sampling is provided elsewhere [19,20]. Samples were collected in the wadeable part of the main channel using a hand net (frame 25 × 25 cm, mesh-size: 500 µm). Twenty subsamples were collected at each site with a total sampling area of 1.25 m^2 along a 100–250 m river stretch. The sampling procedure in Slovenia followed the standardized Slovenian river bioassessment protocol and is described in detail elsewhere [19,34,35]. Twenty sampling units were selected in proportion to the coverage of microhabitat types. Microhabitat types were defined as a combination of substrate and flow type with coverage of at least 5%. The channel substrates of each sampling site were classified according to [36], while the flow characteristics were classified according to [19,35,37]. Sampling units were pooled, preserved in the field with 96% ethanol and transported to the laboratory for further processing. Each sample was subsampled, and benthic organisms from one quarter of the total field sample were identified and counted. In Croatia, samples were collected according to the AQEM sampling strategy [36]. A total of 20 sampling units from representative substrates (i.e., substrates with a coverage >5% in the sampled reach) were sampled. In 2009, 10 subsampling units were collected from sampling sites (five sites) with homogenous substrates (sand and other soft sediments). In these cases, the sample was collected by pushing the hand net through the top (2–5 cm) layer of the substrate. Sampling units were pooled, preserved in the field with 96% ethanol and transported to the laboratory for further processing. In 2006, a more elaborate subsampling design was used: habitat (substrate)-specific subsampling units were pooled and analysed as separate samples. At least one-sixth of the sample was sorted in the laboratory until the target minimum number of 500 (habitat-specific samples) or 700 individuals (multihabitat samples) was reached. Benthic invertebrates were generally identified to the species and genus level but Oligochaeta and Diptera were identified to the (sub)family and genus level (Appendix A).

2.4. Data Analyses

Variation in composition and structure of benthic invertebrate data were analysed using the Bray–Curtis similarity measure. Assemblage similarities were then visualized by nonmetric multidimensional scaling (NMS) plots, and statistically evaluated via permutational multivariate analysis of variance (PERMANOVA, [38]). In NMS plots, the samples vicinity to other samples in portrayable space—typically two dimensions—reflects their underlying similarity in multivariate space, and accordingly, this is the preferred method for illustrating relationships among ecological communities [39,40]. PERMANOVA is a multivariate, nonparametric permutation test for assessing differences in community composition or structure between two or more predefined treatment groups (eco-hydromorphological river types). We used a two-way PERMANOVA to test for significant structural differences between the benthic invertebrate assemblages of (a) the three eco-hydromorphological river types, and (b) the two substrate categories. When differences in benthic invertebrate assemblage structure were detected between the eco-hydromorphological river types and the substrate categories, similarity percentage (SIMPER) analyses were conducted to determine the contribution of specific taxa to these differences. The benthic invertebrate abundances were ln (y + 1) transformed prior to analysis. All statistical and graphical analyses were performed using the PAST data analysis package (v 3.14), (Natural History Museum, Oslo, Norway) [41].

3. Results

A total of 268,471 individuals representing 229 benthic invertebrate taxa were collected in 45 samples (Appendix A). Most of the taxa collected were autochthonous, but nine alien species were also recorded. Alien species were recorded in 16 benthic samples. Most alien species were detected in one to six samples (*Dugesia tigrina*, *Branchiura sowerbyi*, *Potamopyrgus antipodarum*, *Sinanodonta woodiana*, *Corbicula fluminea*, *Dikerogammarus villosus*, *Dikerogammarus haemobaphes* and *Jaera istri*), but *Chelicorophium curvispinum* was found in 11 samples. The recorded taxa included several species, e.g., the stonefly *Marthamea vitripennis* and *Xanthoperla apicalis*. *Marthamea vitripennis* was recorded in the Una River, while *Xanthoperla apicalis* was found in the Mura and Drava Rivers. Other large stonefly species from the Perlidae family were also recorded, e.g., *Dinocras cephalotes* and *Perla abdominalis (P. burmeisteriana)* in the Mura and Una Rivers. Several caddisfly species typical of large rivers were recorded: *Hydropsyche bulgaromanorum* in the Drava and Sava rivers, *H. ornatula* in the Sava, *H. modesta* in the Mura, Drava and Sava rivers, *H. contubernalis* was present in all five rivers, and *Setodes punctatus* in the Drava, Kupa and Una rivers.

Site grouping in the NMS showed that the benthic invertebrate assemblages differ among substrates (Figure 2A). The site grouping of eco-hydromorphological river types was distinct, but less clear, especially between intermountain and lowland-braided rivers (Figure 2B). PERMANOVA confirmed significant differences in assemblage structure between coarse and fine substrates ($F = 4.62$, $R^2 = 0.19$, $p < 0.0001$) and among ECO-HM types, where a slightly higher explanatory power was observed ($F = 4.62$, $R^2 = 0.22$, $p < 0.0001$). Despite the observed overlap of intermountain and braided samples in the NMS diagram, PERMANOVA revealed differences in assemblage composition between all pairs of ECO-HM types ($F = 3.67 - 7.85$, $p = <0.0001 - 0.0003$). The interaction between ECO-HM types and substrates was not significant ($p > 0.05$) (Table 3).

Similarity percentage (SIMPER) analysis indicated that the overall average dissimilarity of benthic invertebrate assemblages of all eco-HM type pairs was >50, with the highest dissimilarity between lowland-braided and lowland-deep rivers (69.21) and the lowest dissimilarity between intermountain and lowland-braided rivers (54.59). *Gammarus fossarum*, *Nais* sp., Orthocladiinae, *Stylodrilus heringianus*, *Lithoglyphus naticoides*, *Hydropsyche incognita*, Tubificidae (with and without hair chaetae), *Heptagenia sulphurea*, *Baetis rhodani*, *Hydropsyche* sp.-juv. and *Gammarus roeseli* were largely responsible for the observed differences among benthic invertebrate assemblages (Table 4). However, *Nais* sp. was the only taxon that was among the ten most influential taxa contributing to the observed differences between benthic invertebrate assemblages of all pairs of ECO-HM types. These taxa were also largely responsible for the observed differences between benthic invertebrate assemblages of coarse and fine substrates (Table 5), confirming that some differences between assemblages of ECO-HM types were also due to substrate differences. Most taxa with the highest percentage contribution to differences between coarse and fine substrates were found in higher abundance in rivers with coarse substrates, e.g., *Gammarus fossarum*, Orhocladiinae, *Stylodrilus heringianus*, *Nais* sp., *Hydropsyche* sp.-juv, *Baetis rhodani*, *Psycomyiia pusilla* and *Hydropsyche incognita*. The latter two species were not found in rivers with fine substrate. On the other hand, *Lithoglyphus naticoides* and Tubificidae without hair chaetae preferred river sections with fine substrate.

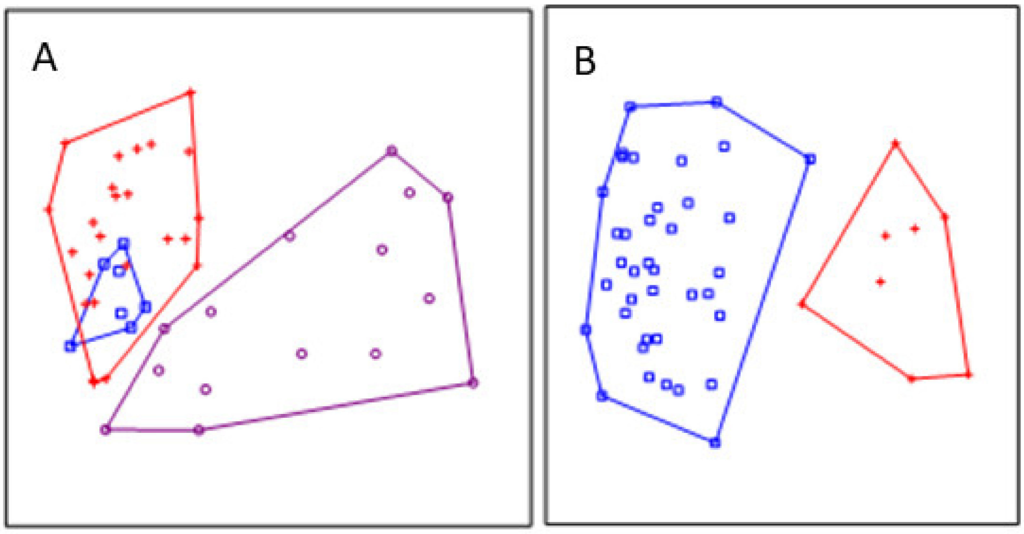

Figure 2. Nonmetric multidimensional scaling ordination diagrams of sampling sites. (**A**) Overlay indicates eco-hydromorphological types: intermountain (☐), lowland-braided (+), lowland-deep (o). (**B**) Overlay indicates coarse (☐) and fine (+) substrate. Stress (two-dimensional space) = 0.19.

Table 3. PERMANOVA test of eco-hydromorphological types of large rivers and substrates and their interactions. ECO-HM—eco-hydromorphological river type, Df—degrees of freedom, *p*—probability of statistical significance based on 9999 permutations of the data.

Scheme 2.	Df	Sum of Sqrs	Mean Square	F	R^2	p
ECO-HM type	2	2.22	1.11	2.64	0.22	0.0001
Substrate	1	1.94	1.94	4.62	0.19	0.0001
Interaction	2	−10.78	−5.39	−12.83		1
Residual	40	16.81	0.42			
Total	45	10.18				

Table 4. Results of SIMPER analyses indicating the contribution of benthic invertebrate taxa to the observed differences between benthic invertebrate assemblages of eco-hydromorphological (ECO-HM) types. Only taxa found in at least five samples were considered. Abbreviations refer to the ECO-HM types sampled: InterM—intermountain, L-braided—lowland-braided, L-deep—lowland-deep. For taxon abbreviations, see Appendix A.

ECO-HM Type A vs. B	Overall Average Dissimilarity	Ten Most Influential Taxa	Percent Contibution to Difference	Cumulative Percent Contibution to Difference	Average abundance (log10)—Type A	Average Abundance (log10)—Type B
InterM vs. L-braided	54.59	Nai_sp.	3.2	3.2	1.10	1.65
		The_dan	2.9	6.1	1.32	0
		Bae_f_s	2.7	8.8	1.26	0.30
		Hyd_inc	2.7	11.5	1.95	0.97
		Gam_roe	2.6	14.1	0	1.25
		Hep_sul	2.6	16.7	0	1.18
		Sim_sp.	2.5	19.2	1.38	0.78
		Hyd_spj	2.5	21.7	1.97	1.17
		Eis_tet	2.4	24.1	1.18	0.39
		Tubb_dae	2.3	26.4	0.96	1.68
InterM vs. L-deep	66.15	Gam_fos	5.4	5.4	3.20	0.85
		Hyd_inc	3.8	9.1	1.95	0.26
		Lith_nat	3.2	12.4	0	1.39
		Hyd_spj	3.1	15.4	1.97	0.62
		Psy_pus	3.1	18.5	1.80	0.54
		Orth_nae	2.9	21.4	2.46	1.67
		Bae_rho	2.8	24.2	1.57	0.69
		Sto_her	2.8	27.0	1.55	0.98
		Ant_sp.	2.7	29.7	1.39	0.15
		Nai_sp.	2.5	32.2	1.10	0.41
L-braided vs. L-deep	69.21	Gam_fos	5.4	5.4	3.12	0.85
		Lith_nat	3.3	8.7	0.03	1.39
		Orth_nae	3.3	11.9	2.62	1.67
		Nai_sp.	3.2	15.1	1.65	0.41
		Sto_her	2.8	17.9	1.63	0.98
		Gam_roe	2.4	20.3	1.25	0.13
		Tubb_dae	2.4	22.7	1.68	1.77
		Hep_sul	2.3	25.1	1.18	0.55
		Bae_rho	2.2	27.3	1.02	0.69
		Tubz_dae	2.2	29.5	0.75	1.13

Table 5. Results of SIMPER analyses indicating the contribution of benthic invertebrate taxa to observed differences between benthic invertebrate assemblages of coarse and fine substrate types. Only taxa found in at least five samples were considered. For taxon abbreviations, see Appendix A.

Substratum	Overall Average Dissimilarity	Ten Most Influential Taxa	Percent Contibution to Difference	Cumulative Percent Contibution to Difference	Average Abundance (log10)—Coarse	Average Abundance (log10)—Fine
Coarse vs. fine	74.91	Gam_fos	6.4	6.4	2.88	0.08
		Orth_nae	4.6	11.0	2.63	0.68
		Lith_nat	4.6	15.6	0.11	2.14
		Sto_her	3.3	18.9	1.65	0.22
		Nai_sp.	2.8	21.7	1.33	0.36
		Psy_pus	2.7	24.4	1.29	0
		Tubb_dae	2.5	26.9	1.48	2.19
		Hyd_spj	2.5	29.4	1.31	0.18
		Bae_rho	2.4	31.8	1.13	0.36
		Hyd_inc	2.3	34.1	1.07	0

4. Discussion

The selection of reference or near-natural sites and description of reference communities should be based on criteria associated with human disturbances that affect aquatic assemblages at a level of no or low impact. We chose stressors of different spatial scales describing different aspects of the aquatic environment (water quality, hydromorphology, land use in the catchment, alien species) that have been shown to be critical in shaping benthic invertebrate assemblages in large rivers [19,20]. Usually, the stressors applied in studies are a simplification of the true stressors to which biota are exposed. In this study, we performed river habitat surveys (RHS) once, which could bias the results because habitat condition changes over time. The hydromorphological quality and modification (HQM) index used in this study is partly based on the RHS conducted once in a given period. However, it proved to be very robust to natural change as it is based not only on river habitat survey data, but also on the presence of upstream and downstream impoundments considered in the year of benthos sampling. Environmental conditions in large rivers generally deteriorate over time and rarely improve (e.g., through restoration); thus, conducting RHS surveys after benthos sampling should not be an issue. We dealt with near-natural sites and all parameters were used as knockout criteria. In the case that habitat condition deteriorated prior to RHS was conducted, such sites would not be considered.

Reference conditions are not only commonly used for ecological assessment, but are also important for restoration and river management in general, as improvement in ecological conditions is a true measure of management success. However, it can be difficult to find sites with at least near-natural conditions, as is the case with most large rivers in the EU. Southeast Europe still has stretches of large rivers with near-natural conditions where some rare benthic invertebrate taxa occur, as confirmed here. *Marthamea vitripennis*, rediscovered after 100 years in the Una River [42], once occurred in the rivers of central and southeast Europe, but is now largely extinct [43]. *Xanthoperla apicalis*, another stonefly species that is a potamal species, at least in central Europe, had disappeared for decades and was only recently rediscovered [43]. Other detected large stonefly species from the family Perlidae (*Dinocras cephalotes* and *Perla abdominalis* in the Mura and Una Rivers, respectively) confirm that remarkable large insect species can also occur in near-natural conditions of large rivers, playing an important role in the functioning of large rivers. We also found some other less endangered but typical potamal species typical for larger rivers, e.g., the caddisfly *Hydropsyche bulgaromanorum*, which still occurs in the lower Sava and the lower Drava. This was a common and dominant *Hydropsyche* species farther upstream (in the middle Drava near Maribor, Slovenia), prior to the construction of hydroelectric power plants, but it no longer occurs today (Urbanič, unpublished data). In addition to rare and typical potamal species, alien species are becoming increasingly common in near-natural sections of large rivers [44]. In the present study, we excluded sites dominated by alien species. However, in the near future, alien species might dominate river stretches with near-natural conditions, especially due to artificial connections between river catchments [45,46]. Most alien species recorded in this study are Ponto–Caspian elements that have spread upstream.

In the EU, type-specific reference conditions have often been established in the spirit of national individuality and with a distinct lack of stringency in defining reference states [47,48]. It is well known that river degradation leads to the homogenisation of aquatic communities [49]. Therefore, the use of communities from degraded sites could lead to a reduced number of taxa in reference communities, lower ecological expectations and a smaller number of recognised distinct river types. Abiotic factors, particularly hydromorphological characteristics, are often used for river typologies, which may lead to a lower number of river types [50] compared to the number of ecological river types. In Slovenia, a much smaller number of hydromorphological types compared to ecological types were recognised, although habitat diversity was taken into account. This is especially true for small rivers [25,34] and to a lesser extent for large rivers [19]. In the present study, the benthic invertebrate community composition differed significantly in all three eco-hydromorphological types that were considered for large rivers. This is even more remarkable because not all taxa were determined to species level. Nevertheless, *Nais* sp. was the only taxon that was among the ten most influential taxa contributing to the observed differences between the benthic invertebrate assemblages of all pairs of ECO-HM types. Thus, it is evident that not only the taxonomic composition but also the abundance of taxa differs between ECO-HM types. Habitat heterogeneity is important from the biodiversity point of view and was considered when delineating the eco-hydromorphological large river types [19]. However, the eco-hydromorphological types considered differ not only in habitat heterogeneity, but also in water depth associated with flow type and substrate size. We identified the latter as one of the key factors defining the benthic invertebrate communities of large rivers, as in many studies (see [51]). There were several taxa that preferred river types with coarse substrates, while some, such as the snail *Lythoglyphus naticoides* and Tubificidae without hair chaetae, preferred rivers with fine substrates. This is consistent with the finding that rivers with coarse substrates harbour more diverse communities compared to rivers with fine substrates [52].

A pragmatic approach has often been taken in setting reference conditions in the EU [12]. It has often been stated that reference conditions or near-natural conditions are also lacking in the case of most large river types in the EU. We have shown here that large rivers with near-natural conditions still exist or existed recently, and provide a description of the type-specific near-natural benthic invertebrate communities. Therefore, it might be possible to set ecological conditions that would serve as a true measure of assessment river restoration success and river management, especially in line with the EU Water Framework Directive. Following this pragmatic approach, the European Green Deal has set the restoration targets for rivers at EU level based only on the length of stretches that have been transformed back into free-flowing rivers by removing mainly obsolete barriers. Addressing one of the main pressures [4,53] could be a good starting point for river restoration. Parameters related to barriers have also been shown to be key factors for benthic invertebrate community composition and ecological status [19], together with fish composition in large rivers [54]. Fish communities may show a rapid response to dam removal [55]. However, although restoration is needed as river degradation is ongoing in Europe, it is evident that large rivers are affected by multiple stressors [4,35,56,57]. For benthic invertebrate assemblages in southeast European large rivers, water quality and land use effects are almost equally important as hydromorphological conditions [20], indicating that considering hydromorphological conditions alone in restoration may be insufficient. A more holistic view of restoration may be required to achieve ecological goals [58], particularly where the names of taxa present (reference communities) are important in addition to functionality.

5. Conclusions

Reference conditions, or at least near-natural conditions, are not only commonly used for ecological assessment, but are also important for restoration and river management, as improvement in ecological conditions is a true measure of management success. In most large rivers in the EU, it can be difficult to find sites with at least near-natural conditions, though southeast Europe still has stretches of large rivers with near-natural conditions where some typical potamal and rare benthic invertebrate taxa occur. We defined the near-natural benthic invertebrate assemblages of large rivers containing several rare and remarkable species among the stoneflies, e.g., *Marthamea vitripennis*, *Xanthoperla apicalis*, *Dinocras cephalotes*, *Perla abdominalis (P. burmeisteriana)*. We compared the composition of benthic invertebrate assemblages among three eco-hydromorphological (ECO-HM) types of large rivers (lowland-deep, lowland-braided and intermountain) and found that the composition of near-natural benthic invertebrate assemblages varied among large river types. Overall, the average dissimilarity in assemblage composition among all ECO-HM type pairs was high, with the highest dissimilarity between lowland-braided and lowland-deep rivers, and the lowest dissimilarity between intermountain and lowland-braided rivers. ECO-HM large river types were defined based on habitat heterogeneity, but also differed in water depth associated with flow type and substrate size. We found that habitat heterogeneity and substrate independently influence the composition of the benthic invertebrate assemblages. Habitat heterogeneity is often generally considered in river assessment, restoration projects or river management. However, it is evident that other factors should also be considered to maintain or restore ecological conditions with relevant benthic communities. For example, simple restoration goals for rivers, such as the length of stretches restored to free-flowing rivers, as stated in the European Green Deal, may not ensure sufficiency to meet all ecological objectives. To achieve ecological goals in the management of large rivers, a holistic view with at least near-natural assemblages, including the names of the taxa present, should be considered in addition to functionality.

Author Contributions: Conceptualization, G.U. and Z.M.; methodology, G.U., Z.M., V.P. and M.P.U.; investigation, G.U., Z.M., V.P. and M.P.U.; validation, G.U. and Z.M.; writing—original draft preparation, G.U., Z.M., V.P. and M.P.U.; writing—review and editing, G.U., Z.M., V.P. and M.P.U.; visualization, G.U.; supervision, G.U. and Z.M.; project administration, G.U. and Z.M.; funding acquisition, G.U. and Z.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was conducted within the Slovenian–Croatian bilateral project: Benthic invertebrate based ecological status assessment of large rivers with management goals focused on hydromorphological alterations (G.U. and Z.M.). The study was also supported by the Ministry of the Environment and Spatial Planning of the Republic of Slovenia as a part of the national program for the implementation of the EU Water Framework Directive conducted at the Institute for Water of the Republic of Slovenia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available on special request.

Acknowledgments: Authors gratefully acknowledge the members of the project teams for their assistance both in the field and in the laboratory. The Croatian Meteorological and Hydrological Service and Croatian Waters and Slovenian Environment Agency are thanked for providing hydrological and water physicochemical data. The authors would like to thank the anonymous reviewers for their useful comments to a previous version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Table A1. Benthic invertebrate taxa recorded at near-natural sites of large rivers.

Higher Taxon	Taxon	Abbreviations
Turbellaria	*Dendrocoelum album*	
Turbellaria	*Dendrocoelum lacteum*	
Turbellaria	*Dugesia lugubris/polychroa*	
Turbellaria	*Dugesia lugubris*	
Turbellaria	*Dugesia tigrina*	
Turbellaria	*Phagocata* sp.	
Turbellaria	*Planaria torva*	
Turbellaria	*Polycelis nigra/tenuis*	
Nematoda	Nematoda Gen. sp.	
Oligochaeta	Enchytraeidae Gen. sp.	
Oligochaeta	*Haplotaxis gordioides*	
Oligochaeta	*Eiseniella tetraedra*	Eis_tet
Oligochaeta	Lumbriculidae Gen. sp.	
Oligochaeta	*Lumbriculus variegatus*	
Oligochaeta	*Rhynchelmis* sp.	

Table A1. *Cont.*

Higher Taxon	Taxon	Abbreviations
Oligochaeta	*Stylodrilus heringianus*	Sto_her
Oligochaeta	*Stylodrilus* sp.	
Oligochaeta	*Dero* sp.	
Oligochaeta	*Nais* sp.	Nai_sp.
Oligochaeta	*Ophidonais serpentina*	
Oligochaeta	*Pristina* sp.	
Oligochaeta	*Stylaria lacustris*	
Oligochaeta	*Uncinais uncinata*	
Oligochaeta	*Propappus volki*	
Oligochaeta	*Aulodrilus pluriseta*	
Oligochaeta	*Branchiura sowerbyi*	
Oligochaeta	Tubificidae—without setae	Tubb_dae
Oligochaeta	Tubificidae—with setae	Tubz_dae
Hirudinea	*Dina punctata*	
Hirudinea	*Erpobdella nigricollis*	
Hirudinea	*Erpobdella octoculata*	
Hirudinea	*Erpobdella* sp.	
Hirudinea	*Erpobdella testacea*	
Hirudinea	*Trocheta bykowskii*	
Hirudinea	*Glossiphonia complanata*	
Hirudinea	*Glossiphonia concolor*	
Hirudinea	*Glossiphonia nebulosa*	
Hirudinea	*Helobdella stagnalis*	
Hirudinea	*Hemiclepsis marginata*	
Hirudinea	*Piscicola geometra*	
Gastropoda	*Acroloxus lacustris*	
Gastropoda	*Ancylus fluviatilis*	
Gastropoda	*Bithynia tentaculata*	
Gastropoda	*Borysthenia naticina*	
Gastropoda	*Lithoglyphus naticoides*	Lith_nat
Gastropoda	*Potamopyrgus antipodarum*	
Gastropoda	*Sadleriana* sp.	
Gastropoda	*Radix auricularia*	
Gastropoda	*Radix balthica/labiata*	
Gastropoda	*Radix balthica*	
Gastropoda	*Esperiana daudebartii acicularis*	
Gastropoda	*Esperiana esperi*	
Gastropoda	*Holandriana holandrii*	
Gastropoda	*Theodoxus danubialis*	The_dan
Gastropoda	*Theodoxus transversalis*	
Gastropoda	*Physa fontinalis*	
Gastropoda	*Physella acuta*	
Gastropoda	*Gyraulus albus*	
Gastropoda	*Valvata cristata*	
Gastropoda	*Valvata piscinalis*	
Gastropoda	*Viviparus viviparus*	
Bivalvia	*Dreissena polymorpha*	
Bivalvia	*Musculium lacustre*	
Bivalvia	*Pisidium* sp.	
Bivalvia	*Sphaerium corneum*	
Bivalvia	*Sphaerium* sp.	
Bivalvia	*Sinanodonta woodiana*	
Bivalvia	*Unio crassus*	
Bivalvia	*Unio pictorum*	
Bivalvia	*Corbicula fluminea*	
Arachnida	Hydrachnidia Gen. sp.	
Amphipoda	*Synurella ambulans*	
Amphipoda	*Gammarus fossarum*	Gam_fos
Amphipoda	*Gammarus roeseli*	Gam_roe

Table A1. *Cont.*

Higher Taxon	Taxon	Abbreviations
Amphipoda	*Corophium curvispinum*	
Amphipoda	*Dikerogammarus haemobaphes*	
Amphipoda	*Dikerogammarus villosus*	
Isopoda	*Asellus aquaticus*	
Isopoda	*Jaera istri*	
Ephemeroptera	*Baetis buceratus*	
Ephemeroptera	*Nigrobaetis digitatus*	
Ephemeroptera	*Baetis fuscatus/scambus*	Bae_f_s
Ephemeroptera	*Baetis lutheri*	
Ephemeroptera	*Baetis rhodani*	Bae_rho
Ephemeroptera	*Baetis scambus*	
Ephemeroptera	*Baetis* sp.-juv.	
Ephemeroptera	*Baetis vardarensis*	
Ephemeroptera	*Baetis vernus*	
Ephemeroptera	*Baetis buceratus/vernus*	
Ephemeroptera	*Centroptilum luteolum*	
Ephemeroptera	*Centroptilum* sp.	
Ephemeroptera	*Cloeon dipterum*	
Ephemeroptera	*Caenis* sp.	
Ephemeroptera	*Serratella ignita*	
Ephemeroptera	*Ephemerella notata*	
Ephemeroptera	*Ephemerella mucronata*	
Ephemeroptera	*Torleya major*	
Ephemeroptera	*Ephemera danica*	
Ephemeroptera	*Ephemera* sp.	
Ephemeroptera	*Ecdyonurus* sp.	
Ephemeroptera	*Epeorus sylvicola*	
Ephemeroptera	*Heptagenia* sp.	
Ephemeroptera	*Heptagenia sulphurea*	Hep_sul
Ephemeroptera	*Rhithrogena* sp.	
Ephemeroptera	*Habroleptoides confusa*	
Ephemeroptera	*Paraleptophlebia submarginata*	
Ephemeroptera	*Oligoneuriella rhenana*	
Ephemeroptera	*Potamanthus luteus*	
Ephemeroptera	*Siphlonurus* sp.	
Plecoptera	*Chloroperla* sp.	
Plecoptera	*Xanthoperla apicalis*	
Plecoptera	*Leuctra* sp.	
Plecoptera	*Nemoura* sp.	
Plecoptera	*Nemurella pictetii*	
Plecoptera	*Protonemura* sp.	
Plecoptera	*Dinocras cephalotes*	
Plecoptera	*Perla abdominalis (P. burmeisteriana)*	
Plecoptera	*Marthamea vitripennis*	
Plecoptera	*Isoperla* sp.	
Plecoptera	*Perlodes* sp.	
Plecoptera	*Brachyptera* sp.	
Plecoptera	*Taeniopteryx nebulosa*	
Odonata	*Calopteryx splendens*	
Odonata	*Enallagma cyathigerum*	
Odonata	*Ischnura elegans*	
Odonata	Coenagrionidae-juv.	
Odonata	*Cordulegaster heros*	
Odonata	*Gomphus* sp.	
Odonata	*Gomphus vulgatissimus*	
Odonata	*Gomphus flavipes*	
Odonata	*Onychogomphus forcipatus forcipatus*	
Odonata	*Platycnemis pennipes*	
Heteroptera	*Aphelocheirus aestivalis*	

Table A1. *Cont.*

Higher Taxon	Taxon	Abbreviations
Heteroptera	*Micronecta* sp.	
Hymenoptera	*Agriotypus armatus*	
Coleoptera	*Bidessus* sp. Ad.	
Coleoptera	*Elmis* sp. Ad.	
Coleoptera	*Elmis* sp. Lv.	
Coleoptera	*Esolus* sp. Ad.	
Coleoptera	*Esolus* sp. Lv.	
Coleoptera	*Limnius* sp. Ad.	
Coleoptera	*Limnius* sp. Lv.	
Coleoptera	*Normandia nitens* Ad.	
Coleoptera	*Oulimnius* sp. Ad.	
Coleoptera	*Oulimnius* sp. Lv.	
Coleoptera	*Stenelmis canaliculata* Ad.	
Coleoptera	*Orectochilus villosus* Lv.	
Coleoptera	*Haliplus* sp. Lv.	
Coleoptera	*Helophorus* sp. Ad.	
Coleoptera	*Hydraena* sp. Ad.	
Coleoptera	*Ochthebius* sp. Ad.	
Trichoptera	*Brachycentrus montanus*	
Trichoptera	*Brachycentrus subnubilus*	
Trichoptera	*Ecnomus tenellus*	
Trichoptera	*Agapetus delicatulus/ochripes*	
Trichoptera	*Agapetus laniger*	
Trichoptera	*Goera pilosa*	
Trichoptera	*Silo nigricornis*	
Trichoptera	*Silo piceus*	
Trichoptera	*Cheumatopsyche lepida*	
Trichoptera	*Hydropsyche bulbifera*	
Trichoptera	*Hydropsyche bulgaromanorum*	
Trichoptera	*Hydropsyche contubernalis contubernalis*	
Trichoptera	*Hydropsyche incognita*	Hyd_inc
Trichoptera	*Hydropsyche modesta*	
Trichoptera	*Hydropsyche ornatula*	
Trichoptera	*Hydropsyche pellucidula*	
Trichoptera	*Hydropsyche siltalai*	
Trichoptera	*Hydropsyche* sp.-juv.	Hyd_spj
Trichoptera	*Hydroptila* sp.	
Trichoptera	*Orthotrichia* sp.	
Trichoptera	*Lepidostoma hirtum*	
Trichoptera	*Athripsodes albifrons*	
Trichoptera	*Athripsodes* sp.	
Trichoptera	*Ceraclea annulicornis*	
Trichoptera	*Ceraclea dissimilis*	
Trichoptera	*Mystacides azurea*	
Trichoptera	*Mystacides nigra*	
Trichoptera	*Oecetis lacustris*	
Trichoptera	*Oecetis notata*	
Trichoptera	*Setodes punctatus*	
Trichoptera	*Oecetis* sp.	
Trichoptera	*Anabolia furcata*	
Trichoptera	*Halesus digitatus*	
Trichoptera	Limnephilinae-juv.	
Trichoptera	*Potamophylax rotundipennis*	
Trichoptera	*Philoptamus ludificatus/montanus*	
Trichoptera	*Polycentropus flavomaculatus*	
Trichoptera	*Lype reducta*	
Trichoptera	*Psychomyia pusilla*	Psy_pus
Trichoptera	*Rhyacophila* sp. s. str.	
Trichoptera	*Sericostoma* sp.	

Table A1. *Cont.*

Higher Taxon	Taxon	Abbreviations
Diptera	*Limnophora* sp.	
Diptera	*Lispe* sp.	
Diptera	*Atherix ibis*	
Diptera	*Ibisia marginata*	
Diptera	*Liponeura* sp.	
Diptera	Ceratopogoninae Gen. sp.	
Diptera	Chironomini Gen. sp.	
Diptera	*Chironomus obtusidens*-Gr.	
Diptera	*Chironomus thummi*-Gr.	
Diptera	*Chironomus* sp.	
Diptera	Corynoneurinae Gen. sp.	
Diptera	Diamesinae Gen. sp.	
Diptera	Orthocladiinae Gen. sp.	Orth_nae
Diptera	*Paratendipes* sp.	
Diptera	*Potthastia longimana*-Gr.	
Diptera	*Procladius* sp.	
Diptera	*Prodiamesa olivacea*	
Diptera	*Prodiamesa rufovittata*	
Diptera	Tanypodinae Gen. sp.	
Diptera	Tanytarsini Gen. sp.	
Diptera	*Thienemanniella* sp.	
Diptera	Dolichopodidae Gen. sp.	
Diptera	Clinocerinae Gen. sp.	
Diptera	Hemerodromiinae Gen. sp.	
Diptera	*Antocha* sp.	Ant_sp.
Diptera	*Hexatoma* sp.	
Diptera	Limnophilinae Gen. sp.	
Diptera	Limoniinae Gen. sp.	
Diptera	*Dicranota* sp.	
Diptera	*Pedicia* sp.	
Diptera	Psychodidae Gen. sp.	
Diptera	*Prosimulium* sp.	
Diptera	*Simulium* sp.	Sim_sp.
Diptera	Syrphidae Gen. sp.	
Diptera	*Chrysops* sp.	
Diptera	*Tabanus* sp.	
Diptera	*Tipula* sp.	

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