Effects of pharmaceutically active compounds (PhACs) on fish body and scale shape in natural waters

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Background. In recent years, there are growing concerns about pharmaceutically active compounds (PhACs) in natural ecosystems. These compounds have been found in natural waters and in fish tissues worldwide. Regarding their growing distribution and abundance, it is becoming clear that traditionally used risk assessment methodologies and ecotoxicological studies have limitations in several respects. In our study a new, combined approach of environmental impact assesment of PHACs has been used. **Methods.** In this study, the constant watercourses of the suburban region of the Hungarian capital (Budapest) were sampled, and the body shape and scale shape of three fish species (roach *Rutilus rutilus*, chub *Squalius cephalus*, gibel carp *Carassius gibelio*) found in these waters were analyzed, based on landmark-based geometric morphometric methods. Possible connections were made between the differences in body shape and scale shape, and abiotic environmental variables (local- and landscape-scale) and measured PhACs. **Results.** Significant connections were found between shape and PhACs concentrations in several cases. Despite the relatively large number of compounds (54) detected, citalopram, propranolol, codeine and trimetazidine significantly affected only fish body and scale shape, based on their concentrations. These four PhACs were shown to be high (citalopram), medium (propranolol and codeine), and low (trimetazidine) risk levels during the environmental risk assessment, which were based on Risk Quotient (RQ) calculation. Furthermore, seven PhACs (diclofenac, Estrone (E1), tramadol, caffeine 17α-Ethinylestradiol (EE2), 17α-Estradiol (aE2), Estriol(E3)) were also categorized with a high risk level. However, our morphological studies indicated that only citalopram was found to

affect fish phenotype amongst the PhACs posing high risk. Therefore, our results revealed that the output of (traditional) environmental/ecological risk assessment based on ecotoxicological data of different aquatic organisms not necessarily show consistency with a “real-life” situation; furthermore, the morphological investigations may also be a good sub-lethal endpoint in ecotoxicological assessments.

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Abstract

Background. In recent years, there are growing concerns about pharmaceutically active compounds (PhACs) in natural ecosystems. These compounds have been found in natural waters and in fish tissues worldwide. Regarding their growing distribution and abundance, it is becoming clear that traditionally used risk assessment methodologies and ecotoxicological studies have limitations in several respects. In our study a new, combined approach of environmental impact assesment of PHACs has been used.
Methods. In this study, the constant watercourses of the suburban region of the Hungarian capital (Budapest) were sampled, and the body shape and scale shape of three fish species (roach *Rutilus rutilus*, chub *Squalius cephalus*, gibel carp *Carassius gibelio*) found in these waters were analyzed, based on landmark-based geometric morphometric methods. Possible connections were made between the differences in body shape and scale shape, and abiotic environmental variables (local- and landscape-scale) and measured PhACs.

Results. Significant connections were found between shape and PhACs concentrations in several cases. Despite the relatively large number of compounds (54) detected, citalopram, propranolol, codeine and trimetazidine significantly affected only fish body and scale shape, based on their concentrations. These four PhACs were shown to be high (citalopram), medium (propranolol and codeine), and low (trimetazidine) risk levels during the environmental risk assessment, which were based on Risk Quotient (RQ) calculation. Furthermore, seven PhACs (diclofenac, Estrone (E1), tramadol, caffeine 17α-Ethinylestradiol (EE2), 17α-Estradiol (aE2), Estriol(E3)) were also categorized with a high risk level. However, our morphological studies indicated that only citalopram was found to affect fish phenotype amongst the PhACs posing high risk. Therefore, our results revealed that the output of (traditional) environmental/ecological risk assessment based on ecotoxicological data of different aquatic organisms not necessarily show consistency with a "real-life" situation; furthermore, the morphological investigations may also be a good sub-lethal endpoint in ecotoxicological assessments.

Introduction

The first detection of pharmaceutical active compounds (PhACs) in aquatic ecosystems and drinking water dates back to the 1980s (Richardson and Bowron, 1985; Watts et al., 1983). Since then, an emerging number of studies have reported the distribution and the potential threat posed by these compounds (Boxall et al., 2012; Datel & Hrabankova, 2020; Dietrich, Webb & Petry, 2002). Selective Serotonin Reuptake Inhibitors (SSRIs), β-blockers and anti-inflammatories are considered to be the most abundant drug residuals occurring in surface waters (Boxall et al., 2012). These compounds can be released into natural waters via several ways. The main sources of pollution are Wastewater Treatment Plants (WWTPs) (after the excretion of human waste) (Subedi et al., 2012), the pharmaceutical industries and the excretion of drugs from animals used in agriculture (Boxall et al., 2012). The recent technologies of WWTPs cannot eliminate these compounds fully from wastewater (Golet et al., 2001; Ternes et al., 1998; Tsui et al., 2014; Yang et al., 2020). To minimize the potential environmental risk posed by PhACs, several regulations for ecotoxicological testing have been enacted (EMEA, 2006). In recent years, several weaknesses of these regulations have been reported in scientific articles (Ankley et al., 2007; Boxall et al., 2012) such as: (1) official tests usually use lethal endpoints, (2) little attention is paid to metabolites, (3) different regulations for human and for veterinary drugs, (4) tests for unique agents, (5) calculating the degradation of compounds and, (6) overabundant compounds (over 4.000 drug substances) to test all of them. These weaknesses and the resulting shortcomings in risk assessment procedures may cause uncertainties regarding their validity. If these points are not addressed and alternative, more adequate risk assessment techniques would not added to the regulations, then a false illusion of low risk may result in many cases. Therefore, the current shortcomings need to be examined in detail in order to better understand the problem. It is a well-known fact that several biotic and abiotic factors can influence the body shape of fish, such as food availability (Currens et al., 1989; Marcil, Swain & Hutchings, 2006; Park et al., 2001), food type (Day, Pritchard & Schluter, 1994), temperature (Beacham, 1990; Šumer et al.,

2005), and the presence or absence of predators (Brönmark & Miner, 1992). In addition, it has also been proven that environmental parameters can affect the shape of fish scales (Ibáñez, 2015; Staszny et al., 2013; Takács et al., 2016). The effect of basic chemical parameters (e.g. ion concentrations) of the water may also affect the phenotype of fish, however their effect on the shape (body or scale) is unclarified (Çoban et al., 2013; Franklin et al., 2005; Schlenk & Benson 2001). Due to the chronic, multigenerational exposure of fishes to PhACs, phenotypic alterations are possible, and there is evidence that progestogen contaminations can affect somatic indices (Maasz et al., 2017). Therefore, the aim of this study was (1) to find connections between the PhACs measured in small watercourses and the body and scale shape of selected fish species; and (2) to describe which type of PhACs or abiotic environmental factors are responsible for anatomical differences.

Materials & Methods

Ethics statement

This study followed all relevant national and international guidelines concerning the care and welfare of fish (Algers et al., 2009; Johansen et al., 2006). Fish samplings were authorized by the Minister of Agriculture (Permit no.: HHgF/298-1/2016) and fish collection for laboratory examinations was authorized by the Government Office of Pest county (Permit no.: XIV-I-001/2302-4/2012). During sampling, an effort was made to minimize the suffering of fish and all fish were anaesthetized with a lethal dose of clove oil after collection. No endangered species (according to the IUCN Red List of Threatened Species v. 13 [www.iucnredlist.org] and National Law Protected [www.termeszetvdelem.hu]) were caught during this study.

Study area

The study was performed in the suburban area of Budapest, which is the capital and the biggest city in Hungary and in the Carpathian Basin. Altogether, 22 points were sampled for chemical analysis during 2017-2018, and 420 specimens of three species (140 roach *Rutilus rutilus*, 180 chub *Squalius cephalus*, 100 gibel carp *Carassius gibelio*) were collected in 20 sampling points from 10 streams during 29 sampling occasions (Fig. 1).Body- and scale-shape data of 20 specimens/sites were included in the analyses, the number of sampling sites, where the necessary number of specimens were available has been indicated in Table 1.

Water sampling and chemical analysis

Water samples were taken during low water-level periods. General water chemical analysis was performed in the field (Hanna HI 98194 for dissolved O_2, electric conductivity, pH, total dissolved solids, temperature; Macherey-Nagel VisColor PF12 spectrophotometer for NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}). For further laboratory analyses (F^-, Cl^-, SO_4^{2-}, NO_2^-, NO_3^-, PO_4^{3-}, NH_4^+, Ca^{2+}, Mg^{2+}, Na^+, K^+) samples were collected in 500-ml borosilicate glass containers. Samples for total organic carbon (TOC) measurements were taken in white, borosilicate containers (50 ml sample with 500 µl 2M hydrochloric acid (VWR International, Pennsylvania, USA)). For the

elemental analysis, a 10-ml water sample was filtered through a 0.45 µm diameter syringe filter, into polypropylene centrifuge pipes free from metal pollutants, and 100 µl NORMATOM nitric acid (VWR International, Pennsylvania, USA) was added. TOC and total nitrogen (TN) concentrations were measured by using a Multi N/C 3100 TC-TN analyzer (Analytik Jena, Germany). For the determination of anions (F^-, Cl^-, SO_4^{2-}, Br^-, NO_3^-) and cations (NH_4^+, Ca^{2+}, Mg^{2+}, Na^+, K^+), a Dionex ICS 5000+ dual channel ion chromatograph (Thermo Fischer Scientific, USA) was used. PO_4^{3-}, NO_2^- concentrations, alkalinity as well as total hardness were measured by standard titrimetric and spectrophotometric methods (Eaton et al., 2005). The concentration of heavy metals was determined by using PlasmaQuant MS Elite inductively coupled plasma mass-spectrometer (Analytik Jena, Germany).

For the PhACs measurements, brown, a borosilicate glass container with Teflon faced caps (Thermo Fisher Scientific) was filled with a 2 l water sample, into which 2 ml of HPLC purity formic acid (VWR International, Pennsylvania, USA) was added. The samples were immediately stored in 4°C, and transported to the laboratory in a dark cooler box (Dometic CFX40W) within 4 hours, where they were then extracted.

Details of the sample preparation, extraction and analysis process for PhACs have also been described in our earlier papers (Jakab et al., 2020; Kondor et al., 2020; Maasz et al., 2019). Briefly, for sample quantification, the water samples were acidified with formic acid and spiked with the corresponding mass-labelled internal standard (IS). Because of the relatively low concentrations, analytes were isolated by an AutoTrace 280 automatic solid-phase extraction system (Thermo Scientific) using Strata X-CW cartridges (#8B-S035-FCH, Phenomenex). To reach the adequate sensitivity, dansyl-chloride was used in the derivatization of steroid agents. A supercritical fluid chromatography (ACQUITY UPC2 system, Waters) coupled with tandem mass spectrometry (MS/MS) (Xevo TQ-S Triple Quadrupole, Waters) was used to analyze and quantify the selected drug residues. Data were recorded by MassLynx software (V4.1 SCN950) in triplicates using TargetLynx XS software for evaluation. The compound separation was performed on an ACQUITY UPC2 BEH analytical column (#186007607, Waters) with 3.0 mm × 100.0 mm, 1.7 µm particle size.

Fish sampling

Fish were caught by electrofishing, and all sampling was undertaken based on the EU Water Framework Directive (EU WFD) (European Commission, 2009) and Hungarian Biodiversity Monitoring System (HBMS) protocols (www.termeszetvedelem.hu). Sampled watercourse sections belonged to River1 (bed width under 5 m, water depth < 1m) and River2 (bed width over 5 m, water depth < 2m) categories, therefore a battery-powered electrofishing device (HANS-GRASSL IG200/2) was used, with a 150-m section length wading in the water upstream. Two watercourses belonged to the River3 (bed width under 30 m, water depth > 2m) category; therefore an aggregator-powered electrofishing device (HANS-GRASSL EL63II) was used, with a 300-m section length leading from a rubber boat going downstream. At every

sampling point, 20 specimens comprised of common fish species (not endangered and not protected) were euthanized by using clove oil and stored at -20°C.

Environmental characterization of sampling sites

The most important environmental variables were recorded at two levels: local level and landscape level (Table 2). The two levels of environmental variables were analyzed separately.

Morphometric analysis

For body morphometrics, after defrosting, a high resolution digital picture was taken of the left side of all specimens using a NIKON D7200 DSLR camera, with a AF-S NIKKOR 35mm 1:1.8G objective, to avoid variability of side-effects (Takács et al., 2018). Standard length and wet weight were measured with an accuracy of 1 mm and 0.1 g, respectively. Sex was determined by dissection, after the digital photo was captured (Table S1). Five well-developed scales were removed from every individuals' left side from the flank. Scales were placed between glass slides and scanned using an upper-light scanner (EPSON Perfection V850 Pro) with high resolution (2400 dpi). One scale per specimen was used for the analysis. Body and scale shape were analyzed using landmark-based geometric morphometry (Zelditch et al., 2004). Ten landmarks were placed on fish body and seven landmarks on fish scales (Fig. 2). For further multivariate analysis, we used the MorphoJ software package (Klingenberg, 2011). To derive shape variables from the raw landmark coordinates, a generalized least-squares Procrustes superimposition (GLS) was applied to scale, translate and rotate the coordinates (Rohlf, 1990). To eliminate the variances associated with allometric growth, a regression analysis was performed between the logarithm of centroid sizes and the Procrustes coordinates. The regression residuals were used for further analysis (Zelditch et al., 2004). The Procrustes-distance (*Pd*) was used in Canonical Variates Analysis (CVA) for computing group differences, and permutations tests with 1000 iterations were performed to test for significance.

Ecological Risk Assessment

Ecological risk characterization for PhACs is usually performed by calculating and categorizing a risk quotient (RQ). RQ is a ratio of MEC/PNEC, in which PNEC (predicted no effect concentration) is the estimated highest concentration of an individual PhAC not affecting the aquatic ecosystem, and MEC is the maximum measured environmental concentration in the studied surface water. In general, $RQ < 0.01$ refers to a negligible risk, $RQ < 0.1$ denotes a low risk, $0.1 < RQ < 1$ indicates a medium risk, while $RQ > 1$ represents a high risk to the aquatic ecosystem.

PNEC derives from the ratio of available ecotoxicological data (e.g., NOEC, EC50, LC50, HC5) and an assessment factor (AF). When the PNEC value was not available in the literature, we used a selected ecotoxicological data/AF quotient keeping in mind the priorities between the raw data (e.g., applying experimental results instead of extrapolated modelled data, and chronic outcomes in place of acute test results). The magnitude of the AF varies between 1000 and 5, and it

198 depends on the available ecotoxicological information. The uncertainty (i.e. AF) of the data
199 decreases with expanding of the relevant data set. If PNEC can be calculated only based on acute
200 test results, then AF=1000. If PNEC can be derived from chronic data of a species, then AF=100.
201 Its value further decreases if ecotoxicological chronic test results are available at multiple
202 different trophic levels: AF=50 (2 levels) or AF=10 (3 levels). If PNEC can be determined
203 knowing of hazardous concentration for 5% of species investigated (HC5 based on
204 ecotoxicological results of at least 5 species), then AF=5. When data are available for each
205 trophic level, the lowest concentration was selected to determine PNEC since environmental risk
206 assessment is based on the most sensitive elements of the ecosystem (Molnar, Maász & Pirger,
207 2020). PNECs with raw ecotoxicological data and AFs are presented in Table S2.
208
209 **Statistical analysis**

210 Background variables were categorized into four groups: PhAC data, general water chemistry
211 data, local environmental variables data and landscape-scale environmental variables. All
212 variables were numeric and log10 transformed before further analyses. An unconstrained
213 Principal Component Analysis conducted on the shape datasets (x and y coordinates of the
214 regression residuals) was followed by the passive projection of the explanatory variables. The
215 number of permutations in a Monte-Carlo simulation were set to 1000. In the first model, body
216 shape data, while in the second model, scale shape data, were used with all the environmental
217 variables listed in the dataset. Where forward selection revealed significant effects, variance
218 partitioning was used to assess the relative contribution of the different variable groups (Borcard,
219 Legendre & Dapeau, 1992). Additional Mantel-tests were performed on shape-variables
220 (Mahalanobis- and Procrustes-distances) and PhACs concentrations, to assess the site-specific
221 component of differences.
222

223 ## Results

224 **PhAC data from sampling points**

225 Altogether 54 different types of PhACs were found in the water samples from the sampling
226 points (Table 3). Three compounds were detected in a µg/l concentration range in examined
227 samples, lamotrigine (maxMEC=14 338.3 ng/l), caffeine (maxMEC=13 635 ng/l), and
228 diclofenac (maxMEC=2 201.7 ng/l). The remaining 51 PhACs were measured in a few hundred,
229 a few tens, or a few ng/l concentration ranges each above the limit of detection. Twenty-seven
230 PhACs were used in analysis based on their RQ-values, from which eight showed high, eight
231 showed medium and the remaining eleven PhACs received a low risk classification based on the
232 environmental risk assessment (Table 3). To perform the risk assessment using relevant
233 ecotoxicological data, we used the AF and PNEC values of detected PhACs (see Table S2).
234

235 **Morphometric analysis**

236 Significant differences were found between the average shape of fish stocks in all three species
237 based on both fish body- and scale shape. In the case of roach body-shape, the differences based

on stream, as well as in scale shape (Fig. 3), significant differences and *Pd*-values are shown in Table 4 for body shape and Table 5 for scale shape.
Sampling points of Tápió-stream were discriminated from the others (Szent-László stream, Gerje-stream) along the first axis of CVA, according roach body shape. Significant differences were observed between GERTOS and every other points, based on Hotelling's t-test (Figure 3, Table 4). SZEBIC has been differed significantly only from TAPTAP. Scale shape of roach proved to be different in TAPUJS, than most of other sites.
In the case of chub body- and scale shape, there were no clear connections found with the stream (Fig. 4); significant differences and *Pd*-values are shown in Table 6 for body shapes and Table 7 for scale shapes. Figure 4 suggests negative correlation between the distance from the estuary and CV2 (HOSTOR < HOSKEL < HOSKAM; BUKTOR < BUKSZE < BUKIZB) in case of Hosszúréti-stream and Bükkös-stream also, however CVA-plot for scale shape not support this finding. In the case of gibel carp body shape, all sampling points differed significantly. In the case of gibel carp scale shape, there was a connection with stream, but there are similarities between the sampling points from different streams as well (Fig. 5); significant differences and *Pd*-values are shown in Table 8 for body shape and Table 9 for scale shape. Interesting pattern of sites could be observed in case of gibel carp body shape, since within-stream difference (GERTOR -GERCEG seems to be higher than between-stream (GERTOR – SZEBIC; GERTOR – HOSKEL) difference. Regarding gibel carp scale, GERTOR site have not been differed such harshly from others, like in case of body shape. BENBIA proved to be the most different site along CV1.

Significant background variables

Numerous significant background variables were found, which affect fish body shape and scale shape. Local- and landscape-scale environmental variables, water chemistry data and also PhACs were found to be significant. In case of roach scale shape, the significant variables were As (9%) and SO_4^{2-} (3%), and for body shape, TRIM (6%), and CITA (4%) were found to be significant (1% joint effect). In the case of chub scale shape, water chemistry data (significant variables: Mg, As, Ca) was responsible for 5% of the variance, local environmental variables (significant variables: emergent macrophytes, water depth) were responsible for 2% of the variance, while PhACs (significant variable: CODE) were responsible for 1% of the variance. The local environmental variables and CODE had 1% joint effect. In the case of chub body shape, only two variables were significant, Cd as water chemistry data and detritus as a local environmental variable, for 4% and 3% respectively, with 8% joint effect. In the case of gibel carp scale shape, the water chemistry variable Pb (2%) and the landscape scale environmental variable wetland (6%) were significant, with 1% joint effect. For gibel carp body shape, three different type of variables were significant, the PPCB PROP, the water chemistry variable Zn, and the landscape-scale environmental variable catchment size, for 6%, 11% and 2% respectively, with 4% joint effect for Zn and catchment size (Table 10).

Mantel tests did not show significant correlation among the site-specific shape variables and the significant background variables, in most of the cases (Table S3). In case of chub scale, Ca shows significant correlation with Procrustes-distances, although in case of Mahalanobis-distances the correlation was not significant. In case of roach scale, both As and SO_4^{2-} showed significant correlation with Mahalanobis-distances, although in case of Procrustes-distances the correlation was not significant.

Discussion

Our results indicated that PhACs can influence fish body shape and scale shape in natural environments and habitats. There are several studies that showed shape differences between fish stocks in natural waters (Ibáñez & Jawad, 2018; Takács et al., 2016). These studies usually explain the variations by different genetic background (Lõhmus et al., 2010; Staszny et al., 2013), phenotypic plasticity (Vasconcellos et al., 2008), or some basic environmental differences, such as food availability (Currens et al., 1989; Marcil, Swain & Hutchings, 2006; Park et al., 2001), temperature (Lõhmus et al., 2010; Šumer et al., 2005), flow-regime (Haas, Blum & Heins, 2010). These effects, and their combination have also affected the phenotype of fish included this study. Moreover, the observed impact of PhACs on shape is considered to relatively small, however it should be taken into consideration during the the studies, carried out in natural waters. In addition, the results of this study suggest that the mixtures of PhACs that occur in natural waters have different effects on different species and phenotypes such as body and scale.

Potential effects of environmental variables on shape

In the case of chub and gibel carp, significant environmental variables were found. The effects of local (section) level variables on chub scale shape could be explained by the life-history characteristics of the species. Different environmental characteristics of the given habitats may cause changes at the population level (Haas, Blum & Heins, 2010). Coverage of emergent macrophytes, water depth and the quantity of detritus were previously found to be connected to the life history parameters of chub (Bolland, Cowx & Lucas, 2008; Ünver & Erk-Akan, 2011), therefore these variables might affect the scale and body shape of the fish. In the case of gibel carp, significant environmental variables included landscape-scale variables, wetland (scale shape) and catchment size (body shape). There are several known examples regarding the shape-modification effects of environmental differences in fish. Species of the genus *Carassius* are characterized by a high level of phenotypic plasticity. In the case of crucian carp (*Carassius carassius*), the presence or absence of predators and the feeding behavior (zooplankton versus benthic chironomids) have a complex effect on body shape (Andersson, Johansson & Söderlund, 2006).

Potential effects of general water chemistry on scale shape

Water chemistry had a significant impact on roach and chub scale shape. The effects of arsenic (As) on muscle development in fish have already been reported (D'Amico, 2012), and this compound can accumulated in scales (Allen et al. 2004) as well, which might affect scale shape itself. Fliedner et al. (2014) studied the water chemistry, especially the heavy metal concentrations in rivers Rhine, Elbe, Danube, Saar, Mulde, Saale and in Lake Belau in Germany. Throughout the study As, Pb, Cu and Hg concentrations were measured from tissue samples of zebra mussel (*Dreissena polymorpha*) and bream (*Abramis brama*). Arsenic found to be the only compound, where increase in concentration was detectable while analyzing in bream muscle tissue samples from 1990s to 2014 (Fliedner et al., 2014). Mg^{2+} and Ca^{2+} significantly impacted the scale shape of chub. Ca^{2+} is an essential building component of fish scales (Sankar et al., 2008) while the Mg^{2+} content of water affects calcium uptake in fish (Dabrowska, Meyer-Burgdorff & Gunther, 1991, Van der Velden et al., 1991). Cadmium is a Ca^{2+} uptake inhibiting agent which was also shown to affect chub body shape. The presence of Cd has a negative effect on Ca^{2+} uptake through the gills (Franklin et al., 2005). Lead concentrations are also connected to gibel carp scale shape formation. This heavy metal cannot be excreted physiologically (via the gills or kidneys), and Pb impairs fish scale development to a greater extent than in other organs (Coban et al., 2013). Zinc also has a significant impact on gibel carp body shape, and is associated with higher (11%) variance. Zinc uptake is related to Ca^{2+} concentrations where high Ca^{2+} concentrations may decrease Zn uptake; excess Zn then accumulates in fish skin, muscle and bones (Hogstrand & Wood, 1996), and therefore might have an effect on body shape.

Potential effect of PhACs on shape

TRIM is a cytoprotective, anti-ischemic agent with a strong antioxidant effect (Sedky et al., 2017). In zebrafish (*Danio rerio*) TRIM can decrease the ototoxic effects of neomycin on hair-cell loss in the neuromasts (Chang et al., 2013). Phenotypic alterations have not been discussed previously, however, a significant effect was detected on roach body shape in this study. Citalopram, as a SSRI, have also been shown to significantly affect roach body shape. A strong anxiolytic effect has been reported in fish previously (Olsén et al., 2014, Porseryd et al., 2017), and alterations in behavioral patterns might also affect the phenotype as well, because the use of different habitats might alter the phenotype of different species (Faulks et al., 2015). Codeine, an opiate derivative, is used to treat rheumatic pain (Ytterberg, Mahowald & Woods, 1998), and significantly modulates chub-scale shape. There is evidence of the presence of codeine in fish tissues (Epple et al., 1993; Valdés et al., 2016), however, phenotypic alterations have not been detected. It might be in relation with the inhibition of the expression of receptors for vascular endothelial growth factor, which can affect the early life-stage development of fish (Karaman et al., 2017). PROP, a non-selective β-blocker, affected gibel carp body shape. It is used to treat heart diseases, and has proved to be the cause of decreased testosterone and estradiol levels in zebrafish, and has showed anxiolytic effects, and decreased growth (Mitchell & Moon, 2016). As we discussed in the case of roach and CITA, the anxiolytic effects of drugs might also alter phenotype. Based on RQ-values, CITA was ranked to be high risk, while CODE and PROP were

medium risk, and TRIM was low risk. These results also suggest that the widely used "traditional" risk assessment may have weaknesses when compared to a "real-life" measured effects.

Conclusions

In summary, our results suggest that PhACs in natural waters can affect the phenotypic characteristics of fish species. Although a relatively large number of PhACs (54 compounds) were found in the water samples, only 4 compounds were found to have significant effects on phenotype. This study did not aim to find clear cause and effect relationships between the given compounds, or to reveal the mode-of-actions; however, the individual-scale effect of PhACs was identified. The results of this study showed that differences in phenotype can be detected, therefore the morphometric analysis was suitable for an alternative, sub-lethal endpoint of environment-level toxicological investigation. However, in order to get a more accurate picture of the actual phenotypic effect of PhACs in the environment, a more detailed study with a larger sample size is needed. Since the effects of PhACs on scale shape have been observed, scale sampling may be a suitable, effective and ethically acceptable tool to extend studies on different river systems.

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References

Algers B., Blokhuis HJ, Bøtner A, Broom DM, Costa P, Domingo M, Greiner M, Hartung J, Koenen F, Müller-Graf C, Morton DB, Osterhaus A, Pfeiffer DU, Raj M, Roberts R, Sanaa M, Salman M, Sharp JM, Vannier P, Wierup M. 2009. General approach to fish welfare and to the concept of sentience in fish. Scientific Opinion of the Panel on Animal Health and Welfare. *The EFSA Journal* 954:1-27.

Allen T, Awasthi A, Rana SVS. 2004. Fish chromatophores as biomarkers of arsenic exposure. *Environmental Biology of Fishes* 71:7-11 DOI: 10.1023/B:EBFI.0000043145.58953.86.

Andersson J, Johansson F, Söderlund T. 2006. Interactions between predator- and diet-induced phenotypic changes in body shape of crucian carp. *Proceedings of the Royal Society B: Biological Sciences* 273:431-437 DOI: 10.1098/rspb.2005.3343.

Ankley GT, Brooks BW, Huggett DB, Sumpter JP. 2007. Repeating History: Pharmaceuticals in the Environment. *Environmental Science & Technology* 41(24):8211-8217 DOI: 10.1021/es072658j.

Beacham TD. 1990. A Genetic Analysis of Meristic and Morphometric Variation in Chum Salmon (*Oncorhynchus keta*) at Three Different Temperatures. *Canadian Journal of Zoology* 68:225-229 DOI: 10.1139/z90-033.

Bolland JD, Cowx IG, Lucas MC. 2008. Movements and habitat use of wild and stockedjuvenile chub, *Leuciscus cephalus* (L.), in a smalllowland river. *Fisheries Management and Ecology* 15:401-407 DOI: 10.1111/j.1365-2400.2008.00631.x.

Borcard D, Legendre P, Dapeau P. 2012. Partialling out the spatial component of ecological variation. *Ecology* 73:1045-1055 DOI: 10.2307/1940179.

Boxall AB, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K, Staveley JP, Verslycke T, Ankley GT, Beazley KF, Belanger SE, Berninger JP, Carriquiriborde P, Coors A, Deleo PC, Dyer SD, Ericson JF, Gagné F, Giesy JP, Gouin T, Hallstrom L, Karlsson MV, Larsson DGJ, Lazorchak JM, Mastrocco F, McLaughlin A, McMaster ME, Meyerhoff RD, Moore R, Parrott JL, Snape JR, Murray-Smith R, Servos MR, Sibley PK, Straub JO, Szabo ND, Topp E, Tetreault GR, Trudeau VL, Van der Kraak G. 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? *Environmental Health Perspectives* 120(9):1221-1229 DOI: 10.1289/ehp.1104477.

Brönmark C, Miner JG. 1992. Predator-induced Phenotypical Change in Body Morphology in Crucian Carp. *Science* 258:1348-1350 DOI: 10.1126/science.258.5086.1348.

Chang J, Im GJ, Chae SW, Lee SH, Kwon S-Y, Jung HH, Chung A-Y, Park H-C, Choi J. 2013. Protective Role of Trimetazidine Against Neomycin-induced Hair Cell Damage in Zebrafish. *Clinical and Experimental Otorhinolaryngology* 6(4):219-225 DOI: 10.3342/ceo.2013.6.4.219.

Çoban MZ, Eroğlu M, Canpolat Ö, Çalta M, Şen D. 2013. Effect of Chromium on Scale Morphology in Scaly Carp (*Cyprinus carpio* L.). *Journal of Animal and Plant Sciences* 23(5):1455-1459.

Currens KP, Sharpe CS, Hjort R, Schreck CB, Li HW. 1989. Effects of Different Feeding Regimes on the Morphometrics of Chinook Salmon (*Oncorhynchus tshawytscha*) and Rainbow Trout (*O. mykiss*). *Copeia* 3:689-695 DOI: 10.2307/1445496.

D'Amico A. 2012. Arsenic Affects Muscle Development and Structure in *Fundulus heteroclitus*. *All Theses* 1491 https://tigerprints.clemson.edu/all_theses/1491

Dabrowska H, Meyer-Burgdorff KH, Gunther K-D. 1991. Magnesium status in freshwater fish, common carp (*Cyprinus carpio*, L.) and the dietary protein-magnesium interaction. *Fish Physiology and Biochemistry* 9(2):165-172 DOI: 10.1007/BF02265132.

Datel JV, Hrabankova A. 2020. Pharmaceuticals Load in the Svihov Water Reservoir (Czech Republic) and Impacts on Quality of Treated Drinking Water. *Water* 12:1387 DOI: 10.3390/w12051387.

Day T, Pritchard J, Schluter D. 1994. A Comparison of Two Sticklebacks. *Evolution* 48:1723-1734 DOI: 10.1111/j.1558-5646.1994.tb02208.x.

Dietrich DR, Webb SF, Petry T. 2002. Hot spot pollutants: pharmaceuticals in the environment. *Toxicology Letters* 131:1-3 DOI: 10.1016/s0378-4274(02)00062-0.

Eaton AD, Clesceri LS, Rice EW, Greenberg AE. 2005. *Standard Methods for the Examination of Water and Wastewater, 21st ed.* Washington, D.C.: APHA-AWWA-WEF

EMEA, 2006. Guideline on the Environmental Risk Assessment for Medicinal Products for Human Use, CHMP/SWP/4447/00. Available at: http://www.ema.europa.eu/documents/scientific-guideline/guideline-environmental-risk-assessment-medicinal-products-human-use-first-version_en.pdf (accessed 25 August 2020).

Epple A, Navarrol I, Horak P, Spector S. 1993. Endogenous Morphine and Codeine: Release by the Chromaffin Cells of the Eel. *Life Sciences* 52:117-121 DOI: 10.1016/0024-3205(93)90175-3.

European Commission, 2009. Common Implementation Strategy for the Water Framework Directive (2000/60/EC); Guidance document n.o 7. Monitoring under the Water Framework Directive. *Available at:* http://circabc.europa.eu/sd/a/63f7715f-0f45-4955-b7cb-58ca305e42a8/Guidance%20No%207%20-%20Monitoring%20(WG%202.7).pdf (accessed 25 August 2020).

Faulks L, Svanbäck R, Eklöv P, Östman Ö. 2015. Genetic and morphological divergence along thelittoral–pelagic axis in two common and sympatricfishes: perch, *Perca fluviatilis* (Percidae) and roach, *Rutilus rutilus* (Cyprinidae). *Biological Journal of the Linnean Society London* 114:929-940 DOI: 10.1111/bij.12452.

Fliedner A, Rüdel H, Knopf B, Weinfurter K, Paulus M, Ricking M, Koschorreck J. 2014. Spatial and temporal trends of metals and arsenic in German freshwater compartments. *Environmental Science and Pollution Research* 21:5521-5536 DOI: 10.1007/s11356-013-2487-y.

Franklin NM, Glover CN, Nicol JA, Wood CM. 2005. Calcium/cadmium Interactions at Uptake Surfaces in Rainbow Trout: Waterborne Versus Dietary Routes of Exposure. *Environmental Toxicology and Chemistry* 24(11):2954-2964 DOI: 10.1897/05-007r.1.

Golet EM, Alder AC, Hartmann A, Ternes TA, Giger W. 2001. Trace Determination of Fluoroquinolone Antibacterial Agents in Urban Wastewater by Solid-Phase Extraction and Liquid Chromatography with Fluorescence Detection. *Analytical Chemistry* 73(15):3632-3638 DOI: 10.1021/ac0015265.

Haas TC, Blum MJ, Heins DC. 2010. Morphological responses of a stream fish to water impoundment. *Biological Letters* 6:803-806 DOI: 10.1098/rsbl.2010.0401.

Hogstrand C, Wood CM. 1996. The physiology and toxicology of zinc in fish. In Taylor EW, ed. *Toxicology of Aquatic Pollution—Physiological, Molecular, and Cellular Approaches. Society for Experimental Biology Seminar Series 57*. Cambridge: Cambridge University Press, 61-84.

Ibáñez AL. 2015. Fish traceability: Guessing the origin of fish from a seafood market using fish scale shape. *Fisheries Research* 170:82-88 DOI: 10.1016/j.fishres.2015.05.016.

Ibáñez AL, Jawad LA. 2018. Morphometric variation of fish scales among some species of rattail fish from New Zealand waters. *Journal of the Marine Biological Association of the United Kingdom* 98(8):1991-1998 DOI: 10.1017/S0025315418000024.

Jakab G, Szalai Z, Michalkó G, Ringer M, Filep T, Szabó L, Maász G, Pirger Zs, Ferincz Á, Staszny Á, Dobosy P, Kondor ACs. 2020. Thermal baths as sources of pharmaceutical and

illicit drug contamination. *Environmental Science and Pollution Research* 27:399-410 DOI: 10.1007/s11356-019-06633-6.

Johansen R, Needham JR, Colquhon DJ, Poppe TT, Smith AJ. 2006. Guidelines for health and welfare monitoring of fish used in research. *Laboratory Animals* 40:323-340. DOI: 10.1258/002367706778476451

Karaman H, Tufek A, Karaman E, Tokgoz O. 2017. Opioids inhibit angiogenesis in a chorioallantoic membrane model. *Pain Physician* 20(2S):E11-E21.

Klingenberg CP. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* 11:353-357 DOI: 10.1111/j.1755-0998.2010.02924.x.

Kondor ACs, Jakab G, Vancsik A, Filep T, Szeberényi J, Szabó L, Maász G, Ferincz Á, Dobosy P, Szalai Z. 2020. Occurrence of pharmaceuticals in the Danube and drinking water wells: Efficiency of riverbank filtration. *Environmental Pollution* 265(Pt A):114893 DOI: 10.1016/j.envpol.2020.114893.

Lõhmus M, Sundström LF, Björkland M, Devlin RH. 2010. Genotype-temperature interaction in the regulation of development, growth, and morphometrics in wild-type, and growth-hormone transgenic coho salmon. *PLoS ONE* 5(4):e9980 DOI: 10.1371/journal.pone.0009980.

Maasz G, Zrinyi Z, Takacs P, Lovas S, Fodor I, Kiss T, Pirger Zs. 2017. Complex molecular changes induced by chronic progestogens exposure in roach, *Rutilus rutilus*. *Ecotoxicology and Environmental Safety* 139:9-17 DOI: 10.1016/j.ecoenv.2017.01.020.

Maasz G, Mayer M, Zrinyi Z, Molnar E, Kuzma M, Fodor I, Pirger Z, Takács P. 2019. Spatiotemporal variations of pharmacologically active compounds in surface waters of a summer holiday destination. *Science of the Total Environment* 677:545-555 DOI: 10.1016/j.scitotenv.2019.04.286.

Marcil J, Swain DP, Hutchings JA. 2006. Genetic and Environmental Components of Phenotypic Variation in Body Shape Among Populations of Atlantic cod (*Gadus morhua*). *Biological Journal of the Linnean Society London* 88:351-365 DOI: 10.1111/j.1095-8312.2006.00656.x.

Mitchell KM, Moon TW. 2016. Behavioral and Biochemical Adjustments of the Zebrafish Danio Rerio Exposed to the β-blocker Propranolol. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 199:105-114 DOI: 10.1016/j.cbpb.2015.10.009.

Molnar E, Maász G, Pirger Zs. 2020. Environmental risk assessment of pharmaceuticals at a seasonal holiday destination in the largest freshwater shallow lake in Central Europe. *Environmental Science and Pollution Research* 1-28 DOI: 10.1007/s11356-020-09747-4.

Olsén KH, Ask K, Olsén H, Porsch-Hällström I, Hallgren S. 2014. Reprint of "Effects of the SSRI citalopram on behaviours connected tostress and reproduction in Endler guppy, *Poecilia wingei*". *Aquatic Toxicology* 151:97-104 DOI: 10.1016/j.aquatox.2014.02.011.

Park I-S, Im JH, Ryu DK, Nam YK, Kim DS. 2001. Effect of Starvation on Morphometric Changes in *Rhynchocypris oxycephalus* (Sauvage and Dabry). *Journal of Applied Ichthyology* 17:277-281 DOI: 10.1046/j.1439-0426.2001.00298.x.

Porseryd T, Kellner M, Caspillo NR, Volkova K, Elabbas L, Ullah S, Olsén H, Dinnétz P, Hällström IP. 2017. Combinatory Effects of Low Concentrations of 17α-etinylestradiol and

Citalopram on Non-Reproductive Behavior in Adult Zebrafish (*Danio Rerio*). *Aquatic Toxicology* 193:9-17 DOI: 10.1016/j.aquatox.2017.10.001.

Richardson ML, Bowron JM. 1985. The fate of pharmaceutical chemicals in the aquatic environment: A review. *Journal of Pharmacy and Pharmacology* 37:1-12 DOI: 10.1111/j.2042-7158.1985.tb04922.x.

Rohlf FJ. 1990. Morphometrics. *Annual Review of Ecology, Evolution, and Systematics* 21:299-316 DOI: 10.1146/annurev.es.21.110190.001503.

Sankar S, Sekar S, Mohan R, Rani S, Sundaraseelan J, Sastry TP. 2008. Preparation and partial characterization of collagen sheet from fish (*Lates calcarifer*) scales. *International Journal of Biological Macromolecules* 42(1):6-9 DOI: 10.1016/j.ijbiomac.2007.08.003.

Schlenk D, Benson WH. 2001. *New Perspectives: Toxicology and the Environment Volume 1. Target Organ Toxicity in Marine and Freshwater Teleosts*. London and New York: Taylor & Francis. p 426.

Sedky AA, El Serafy OMH, Hassan OA, Abdel-Kawy HS, Hasanin AH, Raafat MH. 2017. Trimetazidine potentiates the antiepileptic activity and ameliorates the metabolic changes associated with pentylenetetrazole kindling in rats treated with valproic acid. *Canadian Journal of Physiology and Pharmacology* 95(6):686-696 DOI: 10.1139/cjpp-2016-0263.

Staszny Á, Havas E, Kovács R, Urbányi B, Paulovits G, Bencsik D, Ferincz Á, Müller T, Specziár A, Bakos K, Csenki Zs. 2013. Impact of environmental and genetic factors on the scale shape of zebrafish, *Danio rerio* (Hamilton 1822): A geometric morphometric study. *Acta Biologica Hungarica* 64(4):462-475 DOI: 10.1556/ABiol.64.2013.4.6.

Subedi B, Du B, Chambliss CK, Koschorreck J, Rüdel H, Quack M, Brooks BW, Usenko S. 2012. Occurrence of pharmaceuticals and personal care products in German fish tissue: a national study. *Environmental Science & Technology* 46(16):9047-9054 DOI: 10.1021/es301359t.

Šumer S, Kováč V, Povž M, Slatner M. 2005. External morphology of a Slovenian population of pumpkinseed *Lepomis gibbosus* (L.) from a habitat with extreme thermal conditions. *Journal of Applied Ichthyology* 21:306-311 DOI: 10.1111/j.1439-0426.2005.00691.x.

Takács P, Vitál Z, Ferincz Á, Staszny Á. 2016. Repeatability, Reproducibility, Separative Power and Subjectivity of Different Fish Morphometric Analysis Methods. *Plos ONE* 11(6):e0157890 DOI: 10.1371/journal.pone.0157890.

Takács P, Ferincz Á, Staszny Á, Vitál Z. 2018. Effect of bodyside-specific data processing on the results of fish morphometric studies. *Fundamental and Applied Limnology* 192(2):137-144 DOI: 10.1127/fal/2018/1159.

Ternes T, Hirsch R, Mueller J, Haberer K. 1998. Methods for the determination of neutral drugs as well as betablockers and alpha2-sympathomimetics in aqueous matrices using GC/MS and LC/MS/MS. *Fresenius' Journal of Analytical Chemistry* 362:329-340 DOI: 10.1007/s002160051083.

553 Tsui MMP, Leung HW, Lam PKS, Murphy MB. 2014. Seasonal occurrence, removal
554 efficiencies and preliminary risk assessment of multiple classes of organic UV filters in
555 wastewater treatment plants. *Water Research* 53:58-67 DOI: 10.1016/j.watres.2014.01.014.
556 Ünver B, Erk'akan F. 2011. Diet composition of chub, *Squalius cephalus* (Teleostei:
557 Cyprinidae), in Lake Tödürge, Sivas, Turkey. *Journal of Applied Ichthyology* 27(6):1350-
558 1355 DOI: 10.1111/j.1439-0426.2011.01766.x.
559 Valdés ME, Huerta B, Wunderlin DA, Bistoni MA, Barceló D, Rodriguez-Mozaz S. 2016.
560 Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish
561 under field and controlled laboratory experiments. Evidences of carbamazepine
562 metabolization by fish. *Science of the Total Environment* 557-558:58-67 DOI:
563 10.1016/j.scitotenv.2016.03.045.
564 Van der Velden JA, Spanings FAT, Flik G, Zegers C, Kolar ZI, Wendelaar Bonga SE. 1991.
565 Growth rate and tissue magnesium concentration in adult freshwater tilapia, *Oreochvomis*
566 *rnossarnbicus* (Peters), fed diets differing in magnesium content. *Journal of Fish Biology*
567 39:83-91 DOI: 10.1111/j.1095-8649.1991.tb04343.x.
568 Vasconcellos AV, Vianna P, Paiva PC, Schama R, Solé-Cava A. 2008. Genetic and
569 morphometric differences between yellowtail snapper (*Ocyurus chrysurus*, Lutjanidae)
570 populations of the tropical West Atlantic. *Genetics and Molecular Biology* 31(1, Suppl.):308-
571 316 DOI: 10.1590/S1415-47572008000200026.
572 Watts CD, Crathorne M, Fielding M, Steel CP. 1983. Identification of non-volatile organics in
573 water using field desorption mass spectrometry and high performance liquid chromatography.
574 In: Angeletti G, ed. *Analysis of Organic Micropollutants in Water*. Dotrecht: Reidel Publ.
575 Corp., 120-131.
576 Yang H, Lu G, Yan Z, Liu J, Dong H, Bao X, Zhang X, Sun Y. 2020. Residues,
577 bioaccumulation, and trophic transfer of pharmaceuticals and personal care products in highly
578 urbanized rivers affected by water diversion. *Journal of Hazardous Materials* 391:122245
579 DOI: 10.1016/j.jhazmat.2020.122245.
580 Ytterberg SR, Mahowald ML, Woods SR. 1998. Codeine and oxycodone use in patients with
581 chronic rheumatic disease pain. *Arthritis & Rheumatology* 41(9):1603-1612 DOI:
582 10.1002/1529-0131(199809)41:9<1603::aid-art10>3.0.co;2-u.
583 Zelditch ML, Swiderski DL, Sheets HD, Fink WL. 2004. *Geometric morphometrics for*
584 *biologists: A primer*. New York: Elsevier Academic Press.

Figure 1

Sampling points with sufficient individuals. Red vertical arrows shows WWTPs introductions.

C – chub, R – roach, GC – gibel carp; 1 – MORVER, 2 – GOMVAC, 3 – BUKIZB, 4 – BUKSZE, 5 – BUKTOR, 6 – SZEBIC, 7 – BENBIA, 8 – HOSKAM, 9 – HOSKEL, 10 – HOSTOR, 11 – DTCDUN, 12 – VALBAR, 13 – TAPTAP, 14 – TAPSZE, 15 – TAPGYO, 16 – TAPUJS, 17 – GERCEG, 18 – GERTOR, 19 – GERKOR, 20 - GERTOS.

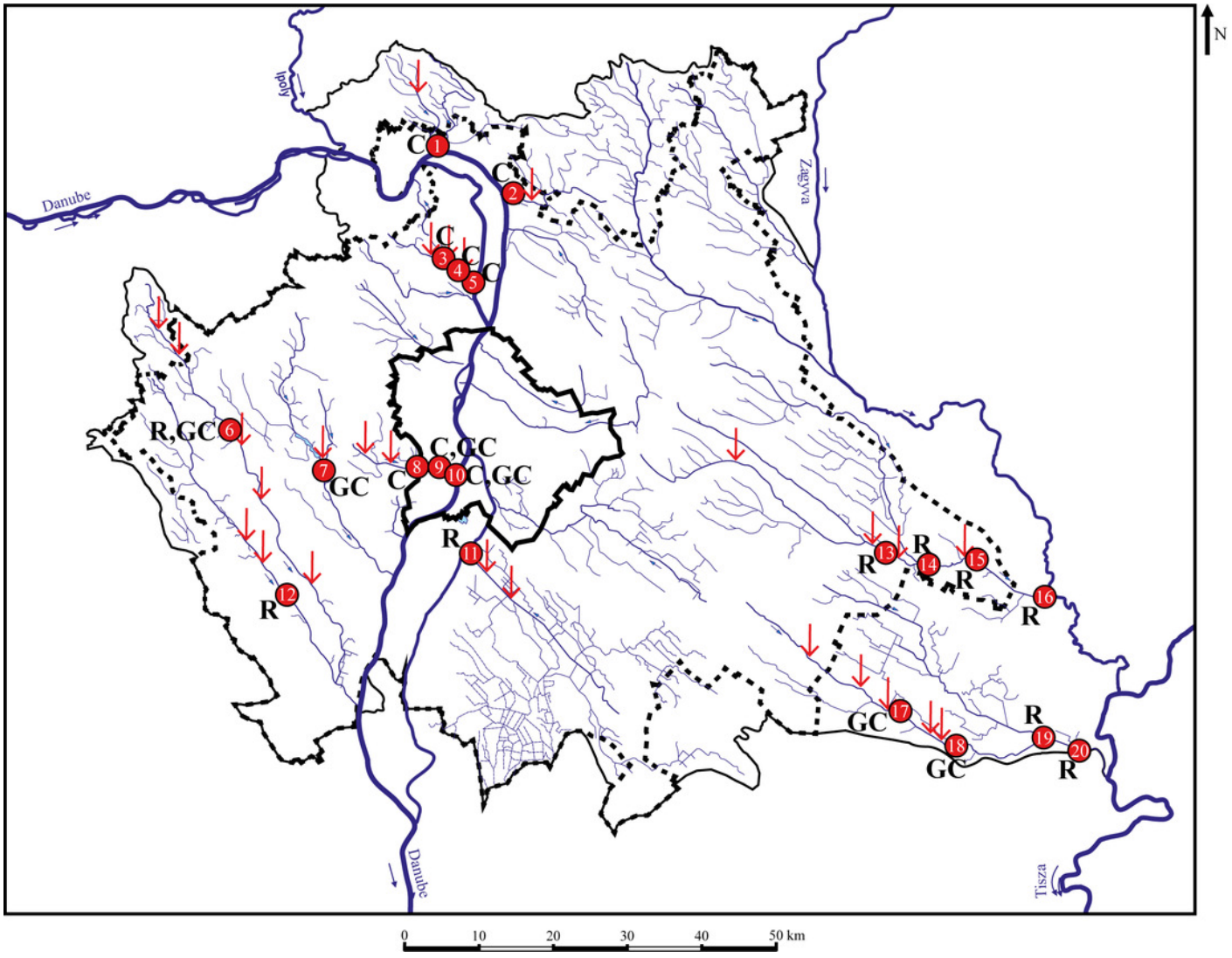

Figure 2

Morphometric landmarks on (A) a schematic gibel carp (*Carassius gibelio*) and a gibel carp scale, (B) a schematic roach (*Rutilus rutilus*) and a roach scale, (C) a schematic chub (*Squalius cephalus*) and a chub scale.

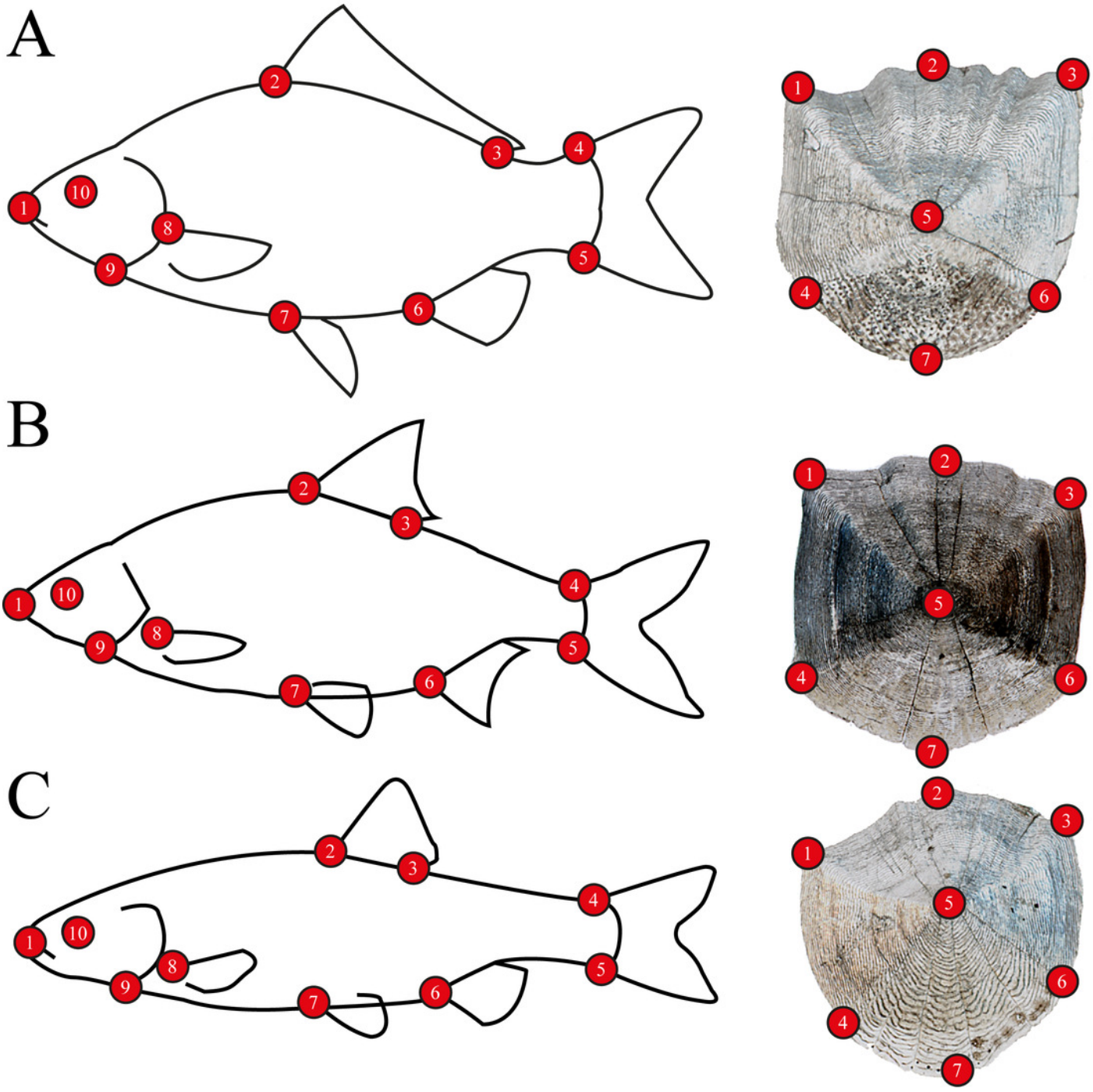

Figure 3

Canonical Variates Analysis (CVA) results of roach (*Rutilus rutilus*) body shape (A) and scale shape (B).

Small-case letters indicate significant differences based on Procrustes-distances, upper-case letters indicate the sampling points (first three letters indicates the stream). Symbols show the group centroids, crosshairs show the standard deviations.

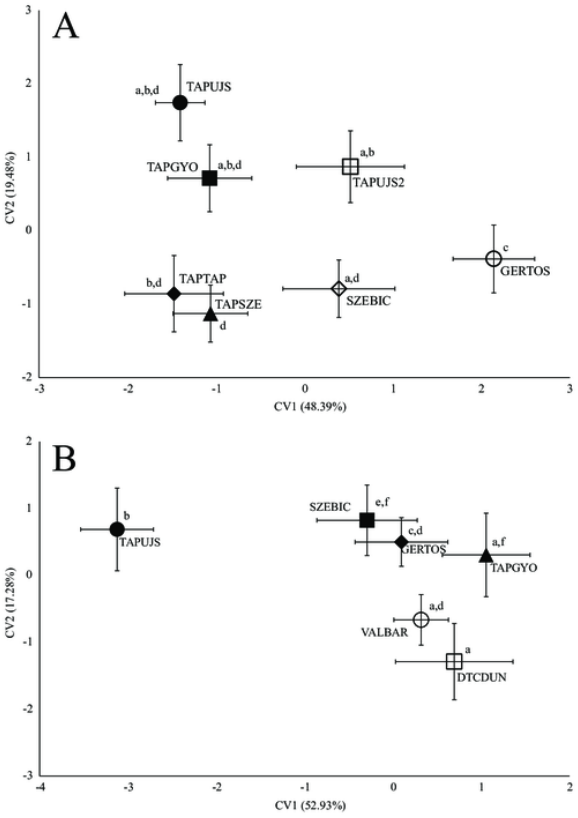
A
a,b,d
TAPUJS
TAPGYO
a,b,d
a,b
TAPUJS2
c
GERTOS
b,d
TAPTAP
TAPSZE
d
a,d
SZEBIC
CV2 (19.48%)
CV1 (48.39%)
B
b
TAPUJS
SZEBIC
e,f
c,d
GERTOS
a,f
TAPGYO
a,d
VALBAR
a
DTCDUN
CV2 (17.28%)
CV1 (52.93%)

Figure 4

Canonical Variates Analysis (CVA) results of chub (*Squalius cephalus*) body shape (A) and scale shape (B).

Small-case letters indicate significant differences based on Procrustes-distances, upper-case letters indicate the sampling points (first three letters indicates the stream). Symbols show the group centroids, crosshairs show the standard deviations.

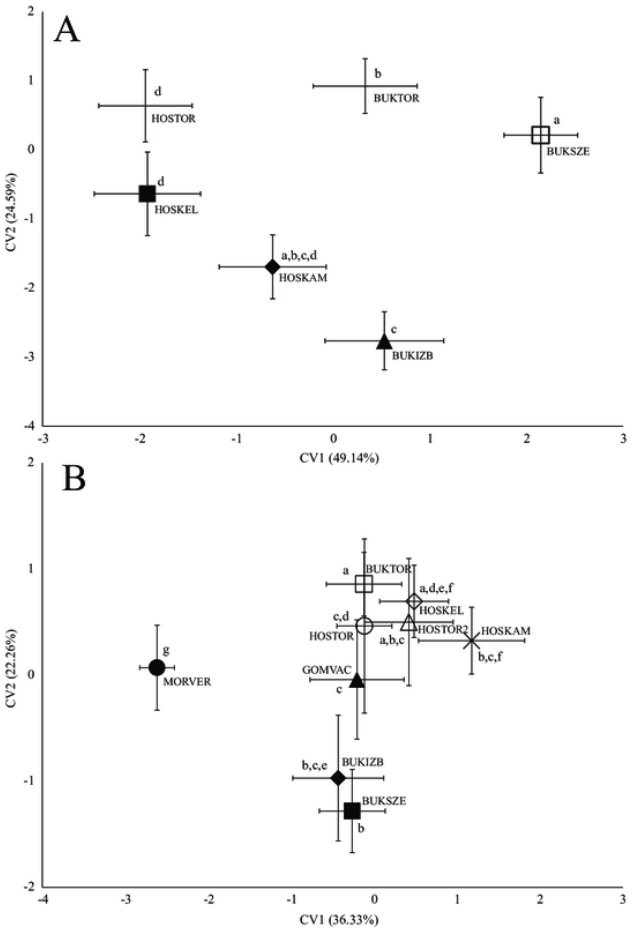
A
d
HOSTOR
b
BUKTOR
a
BUKSZE
d
HOSKEL
a,b,c,d
HOSKAM
c
BUKIZB
CV2 (24.59%)
CV1 (49.14%)
B
a
BUKTOR
a,d,e,f
HOSKEL
c,d
HOSTOR
HOSTOR2
HOSKAM
a,b,c
b,c,f
g
MORVER
GOMVAC
c
b,c,e
BUKIZB
BUKSZE
b
CV2 (22.26%)
CV1 (36.33%)

Figure 5

Canonical Variates Analysis (CVA) results of gibel carp (*Carassius gibelio*) body shape (A) and scale shape (B).

Small-case letters indicate significant differences based on Procrustes-distances, upper-case letters indicate the sampling points (first three letters indicates the stream). Symbols show the group centroids, crosshairs show the standard deviations.

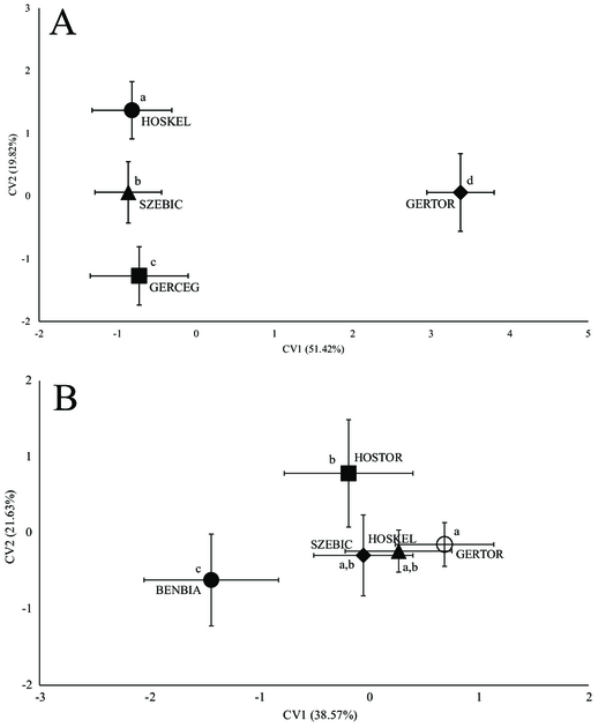
A
a
HOSKEL
b
SZEBIC
c
GERCEG
d
GERTOR
CV2 (19.82%)
CV1 (51.42%)
B
b
HOSTOR
SZEBIC
HOSKEL
a
GERTOR
a,b
a,b
c
BENBIA
CV2 (21.63%)
CV1 (38.57%)

Table 1(on next page)

Number of sampled species and sampling points.

1 **Table 1. Number of sampled species and sampling points.**

Fish species	No. of sampling points	No. of individuals / sampling points	Suitable data for analysis
roach (*Rutilus rutilus*)	6	20	scale
roach (*Rutilus rutilus*)	7	20	body
chub (*Squalius cephalus*)	9	20	scale
chub (*Squalius cephalus*)	6	20	body
gibel carp (*Carassius gibelio*)	5	20	scale
gibel carp (*Carassius gibelio*)	4	20	body

2

Table 2(on next page)

Local- and landscape-scale environmental variables used to characterize sampling points.

Table 2. Local- and landscape-scale environmental variables used to characterize sampling points.

	Name	Abbreviation	Measure
Local environmental characteristics	Woody stemmed coastal vegetation within 1 m from riverbed	wood 1m	Shoreline coverage (%)
	Woody stemmed coastal vegetation within 10 m from riverbed	wood 10m	Shoreline coverage (%)
	Soft stemmed coastal vegetation within 1 m from riverbed	soft 1 m	Shoreline coverage (%)
	Soft stemmed coastal vegetation within 10 m from riverbed	soft 10 m	Shoreline coverage (%)
	Riverbed width	width	m
	Water depth	depth	cm
	Flow rate	flow	m/s
	Sediment - detritus	detritus	Bottom coverage (%)
	Sediment – mud	mud	Bottom coverage (%)
	Sediment – sand	sand	Bottom coverage (%)
	Sediment– gravel	gravel	Bottom coverage (%)
	Sediment – stone	stone	Bottom coverage (%)
	Bottom– rock	rock	Bottom coverage (%)
	Bottom – concrete	concrete	Bottom coverage (%)
	Macrophyte coverage	macrophyte	Coverage (%)
Landscape-scale environmental characteristics	Catchment size over the sampling point	catch.size	km^2
	Inhabited area in the catchment	inhab.area	km^2
	Size of artificial surface in the catchment	art.surface	km^2
	Agricultural surface in the catchment	agri.surface	km^2
	Forest vegetation in the catchment	forest	km^2
	Non-forest vegetation in the	non-forest	km^2

catchment		
Wetland area in the catchment	wetland	km^2
Lakes above the sampling point	lakes	
Distance from estuary	distance	km
Distance from the nearest known wastewater discharge	wastewater.dis	km
Altitude of sampling point	altitude	m
Average altitude of the catchment	avg.altitude	m

3

Table 3(on next page)

Measured Pharmaceutically Active Compounds (PhACs) from the water samples of sampling points.

Compounds in bold were used in analysis based on their Risk Quotient (RQ), compounds in italics had a significant effect on fish shape, n.d. – no data.

Table 3. Measured Pharmaceutically Active Compounds (PhACs) from the water samples of sampling points.

PhACs	Abbr.	LOQ ng/L	No. of sampling points found	PNEC ng/L	maxMEC ng/L	RQ	Risk level
diclofenac	**DICL**	**0.5**	**20**	**2201.700**	**1.06E+01**	**207.708**	high risk
estrone	**E1**	**0.05**	**20**	**38.161**	**1.00E+00**	**38.161**	
tramadol	**TRAM**	**0.1**	**20**	**454.580**	**3.20E+01**	**14.206**	
caffeine	**CAFF**	**10**	**20**	**13635**	**2.32E+03**	**5.877**	
17α-ethinylestradiol	**EE2**	**0.05**	**7**	**2.241**	**4.40E-01**	**5.093**	
17α-estradiol	**aE2**	**0.05**	**1**	**8.491**	**2.00E+00**	**4.245**	
estriol	**E3**	**0.05**	**2**	**1.578**	**4.65E-01**	**3.394**	
citalopram	***CITA***	***0.1***	***20***	***20.942***	***1.00E+01***	***2.094***	
theophylline	**THEO**	**10**	**20**	**874.173**	**1.00E+03**	**0.874**	medium risk
temazepam	**TEMA**	**0.1**	**15**	**4.504**	**7.08E+00**	**0.636**	
17β-estradiol	**bE2**	**0.05**	**16**	**0.972**	**2.00E+00**	**0.486**	
metoclopramide	**MCLO**	**0.2**	**15**	**23.626**	**5.60E+01**	**0.422**	
propranolol	***PROP***	***0.1***	***20***	***14.870***	***4.11E+01***	***0.362***	
codeine	***CODE***	***5***	***1***	***20.030***	***6.00E+01***	***0.334***	
clozapine	**CLOZ**	**0.1**	**20**	**53.478**	**2.85E+02**	**0.188**	
trazodone	**TRAZ**	**0.05**	**3**	**1.032**	**9.00E+00**	**0.115**	
losartan	**LOSA**	**0.1**	**20**	**165.930**	**1.90E+03**	**0.087**	low risk
carbamazepine	**CARB**	**0.1**	**20**	**821.385**	**1.00E+04**	**0.082**	
propafenone	**PROF**	**0.5**	**20**	**80.350**	**1.02E+03**	**0.079**	
ketamin	**KETA**	**0.5**	**15**	**47.717**	**8.61E+02**	**0.055**	
lidocaine	**LIDO**	**0.1**	**20**	**133.910**	**2.61E+03**	**0.051**	
bisoprolol	**BISO**	**0.5**	**16**	**154.720**	**3.15E+03**	**0.049**	
alprazolam	**ALP**	**0.1**	**20**	**20.561**	**5.08E+02**	**0.040**	
trimetazidine	***TRIM***	***20***	***5***	***209.463***	***6.55E+03***	***0.032***	
tiapride	**TIPA**	**0.1**	**20**	**177.606**	**8.72E+03**	**0.020**	
naproxen	**NAPR**	**0.1**	**1**	**287.130**	**1.51E+04**	**0.019**	
midazolam	**MIDA**	**0.1**	**5**	**4.371**	**2.89E+02**	**0.015**	
paracetamol	PARA	20	1	550.820	5.72E+04	0.010	negligiable risk
cocaine	COCA	0.05	11	21.840	2.28E+03	0.010	
zolpidem	ZOLP	0.01	18	4.384	5.19E+02	0.008	
bupropion	BUPR	0.5	8	7.432	9.50E+02	0.008	

betaxolol	BET	0.5	7	6.350	1.24E+03	0.005	
oxazepam	OXAZ	0.1	11	5.581	1.92E+03	0.003	
metoprolol	MPRO	0.1	20	150.161	6.15E+04	0.002	
nordiazepam	NORD	0.1	9	2.750	1.19E+03	0.002	
mirtazapine	MIRT	0.1	20	66.310	3.20E+04	0.002	
pethidine	PETH	0.1	13	1.218	6.89E+02	0.002	
risperidone	RISP	0.1	1	1.230	1.12E+03	0.001	
zopiclone	ZOPI	0.1	1	2.750	4.75E+03	0.001	
fentanyl	FENT	0.1	2	0.307	5.39E+02	0.001	
olanzapine	OLAN	5	13	54.071	1.41E+05	$3{,}83\times10^{-4}$	
verapamil	VERA	0.05	7	10.920	3.60E+04	$3{,}03\times10^{-4}$	
perindopril	PERI	0.1	20	285.461	9.90E+05	$2{,}88\times10^{-4}$	
diazepam	DIAZ	0.1	2	0.605	2.60E+03	$2{,}33\times10^{-4}$	
carvedilol	CARV	0.1	1	0.330	1.55E+03	$2{,}12\times10^{-4}$	
ethylmorphine	EMOR	0.5	12	15.869	1.33E+05	$1{,}19\times10^{-4}$	
lamotrigine	LAMO	5	20	14338.300	1.50E+08	$9{,}56\times10^{-5}$	
quetiapine	QUET	0.1	1	0.830	1.00E+04	$8{,}30\times10^{-5}$	
warfarin	WARF	0.1	3	0.880	1.20E+04	$7{,}33\times10^{-5}$	
methadone	METH	0.02	3	1.202	3.81E+04	$3{,}15\times10^{-5}$	
benzoyl-ecgonine	BEC	0.1	13	2.223	6.81E+06	$3{,}26\times10^{-7}$	
cinolazepam	CINO	0.1	20	394.197	n.d.	n.d.	
drospirenone	DROS	1	2	2.999	n.d.	n.d.	n.d.
lacosamide	LACO	0.5	18	82.549	n.d.	n.d.	

Compounds in bold were used in analysis based on their Risk Quotient (RQ), compounds in italics had a significant effect on fish shape, n.d. – no data.

Table 4(on next page)

Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on roach (*Rutilus rutilus*) body shape.

Significant differences are in bold.

Table 4. Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on roach (*Rutilus rutilus*) body shape.

		p-values						
		GERTOS	SZEBIC	TAPTAP	TAPUJS	TAPGYO	TAPSZE	TAPUJS2
Pd	GERTOS		**0.011**	**0.0003**	**0.0456**	**0.0074**	**0.0387**	**0.0337**
	SZEBIC	**0.0353**		**0.0216**	0.1186	0.0803	0.1031	0.7363
	TAPTAP	**0.0358**	**0.0302**		0.1444	0.1225	0.7136	**0.0269**
	TAPUJS	**0.0372**	0.0305	0.0288		0.5425	0.4209	0.6972
	TAPGYO	**0.0302**	0.0218	0.0197	0.0181		0.6884	0.5946
	TAPSZE	**0.0308**	0.0235	0.0138	0.0233	0.0131		0.3427
	TAPUJS2	**0.0298**	0.015	**0.0284**	0.02	0.0149	0.0213	

Significant differences are in bold.

Table 5(on next page)

Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on roach (*Rutilus rutilus*) scale shape.

Significant differences are in bold.

Table 5. Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on roach (*Rutilus rutilus*) scale shape.

		p-values					
		DTCDUN	GERTOS	SZEBIC	TAPUJS	TAPGYO	VALBAR
Pd	DTCDUN		**0.0213**	**0.0166**	**0.0012**	0.4392	0.3091
	GERTOS	**0.0408**		**0.0495**	**<.0001**	**0.0309**	0.0639
	SZEBIC	**0.0576**	**0.0344**		**0.0378**	0.1134	**0.044**
	TAPUJS	**0.0985**	**0.0753**	**0.051**		**<.0001**	**0.0006**
	TAPGYO	0.0289	**0.0332**	0.036	**0.0819**		0.4209
	VALBAR	0.0323	0.0312	**0.0432**	**0.0862**	0.0243	

Significant differences are in bold.

Table 6(on next page)

Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on chub (*Squalius cephalus*) body shape.

Significant differences are in bold.

Table 6. Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on chub (*Squalius cephalus*) body shape.

		p-values					
		BUKIZB	BUKSZE	BUKTOR	HOSKAM	HOSKEL	HOSTOR
Pd	BUKIZB		**0.0051**	**0.0052**	0.2253	**0.0441**	**0.0226**
	BUKSZE	**0.0292**		**0.0046**	0.085	**0.0001**	**<.0001**
	BUKTOR	**0.0285**	**0.018**		0.1404	**0.0014**	**0.0006**
	HOSKAM	0.0254	0.0235	0.021		0.2441	0.149
	HOSKEL	**0.0255**	**0.0361**	**0.0258**	0.023		0.374
	HOSTOR	**0.0253**	**0.0347**	**0.0237**	0.0238	0.0135	

Significant differences are in bold.

Table 7 (on next page)

Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on chub (*Squalius cephalus*) scale shape.

Significant differences are in bold.

Table 7. Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on chub (*Squalius cephalus*) scale shape.

		p-values								
		BUKIZB	BUKSZE	BUKTOR	GOMVAC	HOSTOR	HOSKAM	HOSKEL	HOSTOR2	MORVER
Pd	BUKIZB		0.8553	**0.0092**	0.1659	**0.0431**	**0.0417**	0.0673	0.6136	**0.0007**
	BUKSZE	0.018		**0.0001**	**0.0128**	**0.0002**	0.0552	**0.0018**	0.085	**0.0028**
	BUKTOR	**0.0426**	**0.0505**		**0.0106**	**0.0017**	**0.021**	0.219	0.3458	**0.0004**
	GOMVAC	0.0362	**0.0376**	**0.039**		**0.0003**	0.1365	**0.0293**	0.1222	**0.0265**
	HOSTOR	**0.0433**	**0.0501**	**0.0468**	**0.057**		0.459	0.549	0.5931	**0.0001**
	HOSKAM	**0.0523**	0.051	**0.0574**	0.0512	0.0369		0.4479	0.3201	**0.0237**
	HOSKEL	0.0378	**0.0443**	0.0264	**0.0417**	0.0242	0.0359		0.8486	**0.0003**
	HOSTOR2	0.0347	0.0462	0.0342	0.0505	0.0338	0.0524	0.0271		**0.0068**
	MORVER	**0.068**	**0.069**	**0.079**	**0.0617**	**0.0882**	**0.0917**	**0.0835**	**0.0914**	

Significant differences are in bold.

Table 8(on next page)

Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on gibel carp (*Carassius gibelio*) body shape.

Significant differences are in bold.

Table 8. Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on gibel carp (*Carassius gibelio*) body shape.

		p-values			
		GERCEG	GERTOR	HOSKEL	SZEBIC
Pd	GERCEG		**<.0001**	**0.0047**	**<.0001**
	GERTOR	**0.036**		**<.0001**	**<.0001**
	HOSKEL	**0.0261**	**0.0438**		**<.0001**
	SZEBIC	**0.0247**	**0.0475**	**0.0441**	

Significant differences are in bold.

Table 9(on next page)

Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on gibel carp (*Carassius gibelio*) scale shape.

Significant differences are in bold.

Table 9. Procrustes-distances (*Pd*) and p-values of Canonical Variates Analysis on gibel carp (*Carassius gibelio*) scale shape.

		p-values				
		BENBIA	GERTOR	HOSKEL	HOSTOR	SZEBIC
Pd	BENBIA		**0.0002**	**0.0175**	**0.0137**	**0.0038**
	GERTOR	**0.0676**		0.111	**0.0229**	0.3999
	HOSKEL	**0.0601**	0.0428		0.5836	0.2426
	HOSTOR	**0.0534**	**0.0475**	0.0346		0.0854
	SZEBIC	**0.0504**	0.0246	0.037	0.038	

Significant differences are in bold.

Table 10(on next page)

Proportion of significant background variables on fish body shape and scale shape.

Variable types: C – water chemistry data, PhAC – pharmaceutical active compound, LE – local environmental variables, LSE – landscape scale environmental variables.

Table 10. Proportion of significant background variables on fish body shape and scale shape.

Species	Analyzed shape	Variable category	Significant variable	Proportion of effect	Joint effect
roach	scale	C	As	9%	
		C	SO_4^{2-}	3%	
	body	PhAC	TRIM	6%	1%
		PhAC	CITA	4%	
chub	scale	C	Mg		
		C	As	5%	
		C	Ca		
		LE	macrophyte coverage	2%	
		LE	water depth		1%
		PhAC	CODE	1%	
	body	LE	detritus	3%	8%
		C	Cd	4%	
gibel carp	scale	LSE	wetland	6%	1%
		C	Pb	2%	
	body	C	Zn	11%	4%
		LSE	catchment size	2%	
		PhAC	PROP	6%	

Variable types: C – water chemistry data, PhAC – pharmaceutical active compound, LE – local environmental variables, LSE – landscape scale environmental variables.