Distribution patterns and potential for further spread of three invasive fish species (Neogobius melanostomus, Lepomis gibbosus and Pseudorasbora parva) in Slovakia

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Abstract

In the last two decades, rapid range expansions of the invasive Ponto-Caspian round goby (Neogobius melanostomus) have been reported from many large waterbodies in Europe and North America. A high invasion potential of this species has been reported through its opportunistic life-history traits and high phenotypic plasticity. Nevertheless, it appears that unlike many other invasive fish species, N. melanostomus has not been able to colonize small and mid-size tributaries of large rivers (streams of order higher than two or three). Given that the invasion history of N. melanostomus in Europe is still rather short, and its invasion potential so high, its future possible expansion represents a big question with important ecological, conservation and management implications. One of the ways to answer this question is to identify the key environmental parameters important for further expansion of N. melanostomus, and to compare these with other successful invaders with longer histories, e.g. topmouth gudgeon (Pseudorasbora parva) and pumpkinseed (Lepomis gibbosus). In this study, basic environmental, physicochemical and chemical parameters, biological elements and human disturbances in all types of streams in Slovakia were analysed in order to identify the key factors that discriminate habitats where the three invasive species were present relative to those where they were not. Random Forest Analysis was used to predict and assess the relationship among the large number of potential predictor variables and a dependent variable. This method is useful especially when large numbers of correlated predictors are evaluated and both quantitative and qualitative predictors are involved. The main aim of this study was to estimate potential for further spread of the three invasive species, with an emphasis on the possibility of further expansion of N. melanostomus into small and mid-size tributaries of large rivers. The habitat parameters that characterize current distribution limits of N. melanostomus were found to be the wetted width, slope, pH, temperature and conductivity. Nevertheless, the altitude (often considered a surrogate of several habitat parameters) limits current distribution of N. melanostomus both in Slovakia and other regions of Europe. Thus, in contrast to other successful invaders (L. gibbosus and P. parva) the analysis of key factors regulating current distributions of N. melanostomus suggests that its future spread into small and mid-size tributaries of large rivers is unlikely.

Key words: round goby, topmouth gudgeon, pumpkinseed, invasion range expansion, human disturbances, key habitat parameters

Introduction

Besides climate change and habitat degradation, the expansion of non-native species is a problem that threatens native species and functioning of ecosystems worldwide (Gurevitch and Padilla 2004; Casal 2006; Didham et al. 2007). Globalization has enabled organisms to extend their distributions to places they would not otherwise have colonized without human mediation (McKinney and Lockwood 1999). Aquatic
organisms have been successfully spread through intentional stocking, aquarium releases, canal construction, and international shipping (Rahel 2007). Freshwater fish are among the most frequently introduced aquatic organisms globally (Vitule et al. 2009; Gozlan et al. 2010). Once non-native freshwater fish species are established, their population growth may be facilitated by anthropogenic pressures such as hydro-morphological alterations, agricultural land use, urbanisation and navigation (Dudgeon et al. 2006; Malmqvist and Rundle 2002; Birk 2017), which often leads to strong habitat modification and/or ecosystem degradation, and biodiversity loss (Sala et al. 2000; Zhang et al. 2015; Holcomb et al. 2016).

In the last two decades, rapid range expansions of the invasive Ponto-Caspian round goby Neogobius melanostomus (Pallas, 1814) have been reported from many large waterbodies in Europe and North America, including river systems of the Danube (Simonović et al. 2001; Brandner et al. 2013; Ľavrinčíková and Kováč 2007), Rhine (Kalchhauser et al. 2013; Manné et al. 2013; Bleeker et al. 2017), Vistula (Grabowska et al. 2008), Volga (Sapota 2004) and the North American Great Lakes (Jude et al. 1992). A high invasive potential of this species has been reported through the competition for habitat (Bauer et al. 2007) and food (Bergstrom and Mensinger 2009; Števove and Kováč 2016), along with opportunistic life-history traits and high phenotypic plasticity (Ľavrinčíková and Kováč 2007; Grabowska and Przybylski 2015; Hőrková and Kováč 2015).

Nevertheless, it appears that unlike many other invasive species of fish (ISF), N. melanostomus has not been able to colonize small and mid-size tributaries of large rivers (i.e. streams of order higher than two or three), although the relative abundances of this species can otherwise exceed fifty percent of the total fish community in large rivers (Bammer et al. 2015). When compared to other non-native invasive gobies in Europe, such as bighead goby Ponticola kessleri (Günther, 1861), monkey goby Neogobius fluviatilis (Pallas, 1814) and racer goby Ponticola gymnotrachelus (Kessler, 1857), N. melanostomus was found to have the least specialized external morphology, which may favour its invasion success (Jakubčinová et al. 2017). Given that the invasion history of N. melanostomus in Europe is still rather short (Skóra and Stolarski 1993; Wiesner 2005), and its invasive potential so high, its future possible expansion represents an important question, with ecological, conservation and management implications.

One way to answer this question is to identify the key factors that discriminate the habitats where ISF are already present relative to those where they are absent. Such an approach may help to identify various parameters of the environment that are important for further expansion of N. melanostomus, especially when compared with the key factors that are typical for invaders with a similar invasion success but a much longer history, e.g. topmouth gudgeon Pseudorasbora parva (Temminck and Schlegel, 1846) and pumpkinseed Lepomis gibbosus (Linnaeus, 1758). Both species represent good candidates for such a comparison. Pseudorasbora parva features opportunistic life-history characteristics (Rosecchi et al. 2001; Záhorská and Kováč 2009) and wide physiological tolerance (Pollux and Korosi 2006), having been described as the most invasive fish in Europe, Central Asia and North Africa since its introduction in the 1960s (Gozlan et al. 2005; Pinder et al. 2005). Less successful globally, but still widely distributed in Europe and Asia Minor, is L. gibbosus, which displays high levels of life-history plasticity (Copp and Fox 2007), opportunistic feeding behaviour (Almeida et al. 2009) and interspecific aggression (Almeida et al. 2014). Lepomis gibbosus was introduced into European waters during the late 19th century (Vivier 1951; Vooren 1972; c.f. Copp et al. 2002).

In this study, basic environmental parameters, physicochemical parameters, chemical parameters, biological elements and human disturbances in all types of streams in Slovakia were analysed in order to identify the key factors that discriminate the habitats where the three ISF (N. melanostomus, P. parva and L. gibbosus) are present from those where they are absent. The objective of these analyses was to identify habitat parameters that are important for the possible further expansion of these ISF, contrasting N. melanostomus as a recent invader against P. parva and L. gibbosus as invaders with much longer history. Based on the outputs of these analyses, the main aim of this study was to estimate the potential for further spread of the three ISF, with the emphasis to the possibility of further expansion of N. melanostomus into small and mid-size tributaries of large rivers, i.e. streams of order higher than two or three. Such a colonization of river systems in Europe by round goby would pose serious ecological, conservation and management implications.

Material and methods

Study area, sampling methods and data collection

A total number of 654 sites were sampled in all zoogeographical regions of Slovakia that covered all types of flowing waters in terms of fish communities (Figure 1). The altitude of the sampling sites ranged from 94 to 1163 m a.s.l. Data were primarily collected for two purposes: 1) monitoring of fish communities associated with the implementation of the Water
Figure 1. Map of the study area with 654 sampling sites (grey dots) covering all types of water bodies in Slovakia.

Framework Directive of EU in Slovakia (WFD; European Community 2000; sampling in 2011 and 2015), and 2) monitoring aimed at implementation of the Council Directive on the conservation of natural habitats and of wild fauna and flora in Slovakia (also known as Natura 2000; European Community 1992; sampling in 2013 and 2014). The fish samples were collected by electrofishing, and the sampling protocol followed the standards required by WFD, and thus was uniform for all sites (Kováč 2015).

The sampling sites were described by basic environmental parameters; altitude, wetted width (measured during each sampling and calculated as mean width of the stream in the sampling stretch), dominant sediment type (evaluated according to the diameter of the main component present as fine, medium or large) and slope (calculated as the drop of altitude divided by stream segment length where measured – 500 m for small streams, 1 km for intermediate streams and 5 km for large streams). Physicochemical parameters (water temperature, conductivity, pH and oxygen saturation) and chemical parameters (associated with organic pollution; biochemical oxygen demand, total P, total N, NH$_4^+$, NO$_2^-$, NO$_3^-$, Ca, Mg and PO$_4^{3-}$) were also collected using methodology required by WFD (provided by the Slovak Water Research Institute, Bratislava).

Data on 15 human disturbances associated with alteration of hydrology and morphology of water bodies (barrier upstream, barrier downstream, impoundment, hydropoaking, water abstraction, upstream dam influence, channelization, riparian vegetation, local habitat alteration, dykes/flood protection, angling/ recreational use, fish stocking, water temperature modification, toxic risk, water quality alteration) were also recorded. Human disturbances were estimated by expert judgment and classified into four modalities: a = no alteration/no influence on fish communities, b = low alteration/low influence, c = medium alteration/medium influence, d = high alteration/high influence. Some of
the human disturbances had only two or three modalities (e.g. no/low/high or no/high alteration or influence).

In this study, indices of biological elements used for the assessment of ecological status of water bodies (WFD; European Community 2000) were applied to evaluate the overall water quality based on the composition of the macrobenthic fauna: Saprobic Index (Zelinka and Marvan 1961), Oligo scored taxa (proportion of individuals with a preference for oligo-saprobic conditions), Biological Monitoring Working Party (BMWP Score), metarhithral scored taxa (proportion of individuals with a preference for the lower-trout region), Rhithron Type Index, Akal+ Lital+Psamal scored taxa (proportion of individuals with a preference for gravel, littoral and sand), number of Ephemeroptera, Plecoptera and Trichoptera taxa (EPT Taxa) (Šporka et al. 2009) and phytothentic fauna: Specific Pollution Sensitivity Index (IPS; CEMAGREF 1982), Descy and Coste Index (CEE; Descy and Coste 1991) and Diatom-based Eutrophication/Pollution Index (EPI-D; Dell’Uomo et al. 1999).

These indices are based on sensitivity of the key groups to organic pollution, morphological degradation and general degradation (Hlúbiková et al. 2007; Fídllová and Hlúbiková 2016). The values of these indices were transformed into five classes of ecological quality (as used for WFD; European Community 2000). Apart from this, medians of chlorophyll a samples collected from April to September 2011, 2013 and 2014 were also used in the analyses. Data of all biological indices and chlorophyll a measurements were collected and calculated by the Slovak Water Research Institute, Bratislava.

Data analysis

To search for differences between the sites with presence and absence of the three invasive species of fish (ISF), the complete dataset (all 654 sites) was analysed. Mann-Whitney U test was used to analyse the differences in environmental and physicochemical parameters between the sites where the respective ISF were present or absent. To control for false discovery rate and thus to avoid theoretical bias from multiple use of Mann-Whitney U tests and Fisher’s exact test, the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) was applied for P value adjustment. Adjusted P < 0.05 (Padj) was regarded as significant. All statistical analyses were performed using R project (R Core Team 2017).

Random Forest Analysis (RFA) was used to predict and assess the relationship among the large number of potential predictor variables and a dependent variable. This non-parametric method is particularly useful when a large number of correlated predictors is evaluated, and both quantitative and qualitative predictors are involved (Breiman 2001). All independent variables (environmental, physicochemical and chemical parameters, chlorophyll a, human disturbances and biological indices) were used as predictors. The dependent variable was the presence or absence of the respective ISF. As the data matrix contained missing values, rough imputation was used in RFA (missing values of quantitative predictors were replaced by column medians, missing values of qualitative predictors by the most frequent category).

The main output of RFA is the importance of individual predictors, i.e. the relevance of particular predictors to distinguish between the sites where the respective ISF were present or absent. The importance of predictors was measured as the mean decrease in the Gini index (Breiman 2001). The number of trees in RFA and the number of predictors selected randomly at each split as candidates were optimized to get the best RFA model (model with the lowest estimate of error rate estimated by crossvalidation). To validate the importance of the predictors, the estimates of error rate expressed in percent of misclassification (EER) were calculated. The RFA was performed using “randomForest” package, a library for random forests for classification and regression in R software environment (Liaw and Wiener 2002).

To test whether the composition of the whole fish communities (besides the independent variables described above) affected presence or absence of the
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respective ISF, non-metric multidimensional scaling (NMDS; Hill and Gauch 1980) was applied. NMDS was based on the Bray-Curtis distance measure with two dimensions chosen. A NMDS ordination was used to visualize the degree of overlap in particular species forming the fish communities in the sites with presence and absence of ISF. NMDS was performed using “Vegan” package in R (Oksanen et al. 2018).

The outputs from the RFA did not exclude uncertainties about presence and absence of the ISF at the sites situated below the altitude limits. Therefore, another analysis was performed to search for the most important variables (other than altitude) responsible for the distribution limits of each ISF. This analysis is based on the assumption that the altitude is just a surrogate of other ecological variables that in fact limit the distribution of each ISF (not the altitude itself). For the purposes of this analysis, all the sites below the altitude limits were considered potentially invasible (equivalence of presence of ISF) and thus grouped together, and subsequently confronted with all sites above the altitude limits. The importance values for individual predictors were identified using the same RFA approach as above.

Results

*Neogobius melanostomus* was present at 52 sites (8.0% of the total number of sites investigated), with its main distribution in the Danube and lower sections of its tributaries. *Lepomis gibbosus* was found at 50 sites (7.6%), and *P. parva* was present at 82 sites (12.5%), both mainly in the middle and lower reaches of Slovak water bodies, except two sites located at 447 and 449 m a.s.l. (these two sites were excluded from statistical analyses due to their outlier character, which is further discussed).

The parameters discriminating between the sites with presence and absence of ISF were found to be altitude, slope, pH, conductivity and water temperature (P adj < 0.05; Table S1). The presence of all three ISF was limited to lower altitudes, and moreover, the closer to the limiting altitude (155, 249 and 299 m a.s.l., respectively), the lower the frequency of occurrence. In other words, increasing altitudes accounted for a lower number of sites with presence of the three ISF. The slope of the sites where the three ISF were present was significantly lower than at the sites where the three ISF were absent, and temperature and conductivity were significantly higher. The dominant sediment sizes were finer in sites where *P. parva* and *L. gibbosus* were present. The sites with presence of *N. melanostomus* and *L. gibbosus* were characterized by having significantly larger wetted width, and the sites with *P. parva* and *L. gibbosus* contained significantly lower oxygen saturation and lower pH. Finally, water in the sites with *N. melanostomus* was found to have significantly higher pH (Table S1).

Concerning the sites below the limiting altitudes for the respective ISF, significant differences in several parameters (Mann-Whitney U test, including Benjamini-Hochberg procedure, P adj < 0.05) were found between the sites with presence and absence of ISF (Tables 1 and 2). These parameters can serve for basic description of the habitats preferred by the respective ISF.

*Neogobius melanostomus* were present at habitats with wider wetted width (60 m; median values given in this paragraph) and shallower slope (0.1‰). Water was better saturated with oxygen (94.4%), with higher concentrations of NO3 (9.808 mg.l-1), lower concentrations of NH4+ (0.084 mg.l-1), and higher concentrations of chlorophyll a (8.550 µg.l-1; Table 1). The presence of *N. melanostomus* was closely related to the sites where the impact of several human disturbances was significantly stronger (highest frequency of the d-modality) than at the sites without the species (Table 2). This was demonstrated especially by the presence of dykes, i.e. no lateral connectivity with the river system (63.5%), fish stocking affecting indigenous fish populations (59.6%), recreational use causing clear effects on the fish fauna (53.8%), artificial barriers upstream from the site preventing migration of most fish species (34.6%), toxic risk with clearly known point sources that can affect the segment upstream from the site (32.7%), and alteration of the hydrograph potentially affecting fish fauna (28.8%; Table 2). Moreover, 34.6% of the sites with *N. melanostomus* were also affected by slight alterations of the hydrograph, and 28.8% of these sites were affected by temporal barrier upstream which was not species selective (both, b-modality). Additionally, 46.2% of these sites were slightly affected by water abstraction (b-modality; Table 2). Saprobic index of the sites with *N. melanostomus* corresponded to very good ecological status. However, the values of Rithron Type Index indicated the ecological status of these sites was moderate (Table 2). The most important predictors (RF analysis, importance of predictors measured by Gini index) that discriminated between the sites with presence and absence of *N. melanostomus* were found to be wetted width, pH, dykes preventing lateral connectivity, chlorophyll a and altitude (Figure 2A). Sites occupied by *N. melanostomus* were not found to show any signs of water quality degradation in the physicochemical parameters recorded. In other words, biochemical oxygen demand, pH, total P, total N, as well as phosphate (PO4) concentrations did not exceed
Table 1. Differences in environmental, physicochemical and chemical parameters between samples with presence and absence of *Neogobius melanostomus* (NM), *Lepomis gibbosus* (LG) and *Pseudorasbora parva* (PP) below the altitude threshold. Arrows indicate significantly higher (↑) or lower (↓) values in the sites where the three invasive species were present compared to the sites where the three invasive species were absent. Asterisks denote significant differences confirmed after Benjamini-Hochberg procedure for the P-values adjustment. NS – difference not significant. See Tables S2, S3, S4 (supplementary files) for details.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NM</th>
<th>LG</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant sediment</td>
<td>NS</td>
<td>↑fine, ↓medium</td>
<td>↑fine, ↓medium</td>
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<tr>
<td>Wetted width (m)</td>
<td>↑*</td>
<td>↑*</td>
<td>NS</td>
</tr>
<tr>
<td>Slope (%)</td>
<td>↑*</td>
<td>↓</td>
<td>↓*</td>
</tr>
<tr>
<td>Oxygen saturation (%)</td>
<td>↑*</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>pH</td>
<td>↑*</td>
<td>↓</td>
<td>NS</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Conductivity (mS.m⁻¹)</td>
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<td>NS</td>
<td>↑</td>
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<tr>
<td>Biochemical oxygen demand (mg.l⁻¹)</td>
<td>NS</td>
<td>NS</td>
<td>↑*</td>
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<tr>
<td>Total P (mg.l⁻¹)</td>
<td>NS</td>
<td>NS</td>
<td>↑</td>
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<tr>
<td>Total N (mg.l⁻¹)</td>
<td>NS</td>
<td>↓</td>
<td>↑*</td>
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<tr>
<td>NH₄⁺ (mg.l⁻¹)</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td>NO₃⁻ (mg.l⁻¹)</td>
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<td>NS</td>
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<td>NO₂⁻ (mg.l⁻¹)</td>
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<td>↓</td>
<td>↑</td>
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<td>Ca (mg.l⁻¹)</td>
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<td>NS</td>
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<tr>
<td>Mg (mg.l⁻¹)</td>
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<td>NS</td>
<td>↑</td>
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<tr>
<td>PO₄³⁻ (mg.l⁻¹)</td>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Chlorophyll a (μg.l⁻¹)</td>
<td>↑*</td>
<td>NS</td>
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</table>

Table 2. Differences in human disturbances (top) and indices of biological elements (bottom) between samples with presence and absence of *Neogobius melanostomus* (NM), *Lepomis gibbosus* (LG) and *Pseudorasbora parva* (PP) below the altitude threshold. Arrows indicate significant higher (↑) or lower (↓) frequencies of given categories (ecological status) in the sites where the three invasive species were present compared to the sites where the three invasive species were absent. No alteration/no influence (a), low alteration/low influence (b), medium alteration/medium influence (c) and high alteration/high influence (d) on fish communities. Some of the human disturbances had only two or three modalities (e.g. no/low/high or no/high alteration or influence). Ecological status high (1), good (2), moderate (3), poor (4) and bad (5). Asterisks denote significant differences confirmed after Benjamini-Hochberg procedure for the P-values adjustment. NS – difference not significant. See Tables S5, S6, S7, S8, S9 and S10 (supplementary files) for details.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NM</th>
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<tbody>
<tr>
<td>Category</td>
<td></td>
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<tr>
<td>Barrier upstream</td>
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<td>NS</td>
</tr>
<tr>
<td>Barrier downstream</td>
<td>NS</td>
<td>↑d, 1a</td>
<td>NS</td>
</tr>
<tr>
<td>Impoundment</td>
<td>NS</td>
<td>↑d, 1a</td>
<td>NS</td>
</tr>
<tr>
<td>Hydropoeaking</td>
<td>↑b,d, 1a*</td>
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<td>NS</td>
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<tr>
<td>Water abstraction</td>
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<td>Upstream dam influence</td>
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<td>Channelization</td>
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<tr>
<td>Riparian vegetation</td>
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<td>NS</td>
<td>↑c,d, 1a,b*</td>
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<td>Dykes flood protection</td>
<td>↑d, 1a, b*</td>
<td>↑d, 1a</td>
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<tr>
<td>Toxic risk</td>
<td>↑d, 1a, b*</td>
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<tr>
<td>Recreational use anglng</td>
<td>↑d, 1a*</td>
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<tr>
<td>Stocking</td>
<td>↑d, 1a*</td>
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</tr>
<tr>
<td>Ecological status (WFD)</td>
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<tr>
<td>Saprobic Index</td>
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<td>Oligo scored taxa</td>
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<td>Metarthral scored taxa</td>
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<td>Rheoindex by abundance classes</td>
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<tr>
<td>Rithron type index</td>
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<td>Type AKA Lit Psa scored taxa</td>
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<tr>
<td>EPT taxa</td>
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<tr>
<td>Number of families</td>
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<td>EPI D</td>
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</table>
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The values acceptable for life and reproduction of fishes and/or values indicating good status at any of the sites with *N. melanostomus*, except two sites where phosphate (PO$_4^{3-}$) concentrations were higher than the tolerance limit (Table 3).

*Lepomis gibbosus* were present at habitats with wider wetted width (20 m; median values given in this paragraph), finer sediment and shallower slope (0.2‰). Water was less saturated with oxygen (90.1%), slightly less alkaline (pH 7.8), and with lower concentrations of NO$_3^-$ (6.369 mg.l$^{-1}$) and total N (2.098 mg.l$^{-1}$; Table 1). The presence of *L. gibbosus* was also closely related to the sites where the impact of several human disturbances was significantly stronger (highest frequency of the d-modality) than at the sites without the species (Table 2). This appeared to be the case especially in the presence of dykes, i.e. at sites with no lateral connectivity with the river system (32.0%), with a barrier downstream from the site selective for most of the fish species (22.0%), and with strong flow velocity reduction (16.0%; Table 2). However, when the false discovery rate was considered and $P_{adj}$ applied, only the wetted width appeared to be statistically significant (Table 2). The most important predictors (RF analysis, importance of predictors measured by Gini index) that discriminated between the sites with presence and absence of *L. gibbosus* were found to be altitude, wetted width, NH$_4^+$, oxygen saturation and slope (Figure 2B).

Sites with *L. gibbosus* were found to show some signs of water quality degradation in the physicochemical parameters recorded. Biochemical oxygen demand, pH and total N did not exceed the values acceptable for life and reproduction of fishes and/or values indicating good status at any of the sites with *L. gibbosus*. However, for total P concentration, oxygen saturation and phosphate (PO$_4^{3-}$) concentration (one site, nine sites and ten sites, respectively), the values were higher than the tolerance limits (Table 3).

*Pseudorasbora parva* were present at habitats with finer sediment, shallower slope (0.2‰, median values given in this paragraph), higher conductivity (57.0 mS.m$^{-1}$), less saturated with oxygen (91.5%) and higher biochemical oxygen demand (2.64 mg.l$^{-1}$).

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**Figure 2.** Variable importance of the predictors from the random forest analysis aimed at discriminating between the sites (below the respective altitude limits) with presence and absence of the three invasive species of fish (A) *Neogobius melanostomus*, (B) *Lepomis gibbosus* and (C) *Pseudorasbora parva*. The estimates of error rate expressed in percent of misclassification (EER) were low in *Neogobius melanostomus* overall, i.e the out-of-bag (OOB); sites where the species was present (PRES), and sites where the species was absent (ABS).

**Table 3.** Tolerance limits acceptable for life and reproduction of fishes (top) and values indicating good ecological status in terms of WFD (bottom) (Government Regulation; Slovak Republic 2010).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>cyprinid waters limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.0-9.0</td>
</tr>
<tr>
<td>Bioch. oxygen demand (mg.l$^{-1}$)</td>
<td>6.0</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>0.4</td>
</tr>
<tr>
<td>Oxygen saturation (%)</td>
<td>&gt; 76</td>
</tr>
<tr>
<td>Total P (mg.l$^{-1}$)</td>
<td>&lt; 0.4</td>
</tr>
<tr>
<td>Total N (mg.l$^{-1}$)</td>
<td>&lt; 5.5</td>
</tr>
</tbody>
</table>

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The concentrations of NO$_3^-$ (10.14 mg.l$^{-1}$), total N (3.68 mg.l$^{-1}$), total P (0.14 mg.l$^{-1}$) and Mg (16.65 mg.l$^{-1}$) were higher than at the sites where $P.\ parva$ was absent (Table 1). Similar to the other two ISF, the presence of $P.\ parva$ was also closely related to the sites where the impact of several human disturbances was significantly stronger (highest frequency of the d-modality) than at the sites without the species (Table 2). This was demonstrated especially by channelization, i.e. straightened river with flow velocity increase (46.3%), and by complete removal of riparian vegetation (23.8%). Moreover, 43.8% of the sites with $P.\ parva$ were affected by strong alteration of riparian vegetation (c-modality), and in 32.5%, slight influence of the dam located upstream (b-modality) was indicated (Table 2). However, when $P_{\text{adj}}$ was applied, the parameters with significant differences reduced to slope, biochemical oxygen demand, total N, channelization, and complete removal of riparian vegetation (Table 2). The most important predictors (RF analysis, importance of predictors measured by Gini index) that discriminated between the sites with presence and absence of $P.\ parva$ were found to be altitude, wetted width, slope, riparian vegetation and channelization (Figure 2C). Sites with $P.\ parva$ were found to show stronger signs of water quality degradation in the physicochemical parameters recorded. Only pH and biological oxygen demand did not exceed the values acceptable for life and reproduction of fishes and/or values indicating good status at the sites with $P.\ parva$. For the concentrations of the rest of the parameters (oxygen saturation, total N, total P and phosphate (PO$_4^{3-}$), several sites were found to have values higher than tolerance limits (eight sites, seven sites, two sites and ten sites respectively) (Table 3). Based on NMDS, high overlaps in the composition of fish communities between sites with presence and absence of each ISF were found (Figure 3A–C). In other words, the fish community structure did not appear to have a significant effect on presence or absence of the three ISF, except $N.\ melanostomus$, in which some association with benthic and/or invasive species of fish was observed.

The limiting altitudes for the respective ISF (see above) pose a question as to whether these altitudes are real limits for the distribution of each ISF or just a current situation that may change in the future. Predictors (RF analysis, importance of predictors measured by Gini index) that discriminated between sites below and above the limiting altitudes (i.e. sites where ISF are or can be present, and the sites where ISF are currently absent) were identified. The most important among these were found to be the slope, dominant sediment, wetted width and temperature (Figure 4A–C).
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Figure 4. Variable importance of the predictors from the random forest analysis aimed at discriminating between the sites below and above the respective limiting altitudes (i.e. between the sites where the invasive species are or can be present, and the sites where the invasive species are currently absent). (A) *Neogobius melanostomus*, (B) *Lepomis gibbosus* and (C) *Pseudorasbora parva*. Abbreviations for the estimates of error rates as in Figure 2.

Discussion

The patterns of distribution of the three ISF in Slovakia can be characterized with the statement “the higher the altitude, the lower the frequency of occurrence”, with a defined altitude that represents the limit for each species. Higher occurrence of the three ISF in lower altitudes can be associated with a stronger influence of land use and other human disturbances (Holcomb et al. 2016; Zhang et al. 2015). Invasive species can take advantage of these conditions because of their wider environmental tolerances, as they preferentially colonize water bodies where the environment is more influenced by human disturbances (Pollux and Korosi 2006), and as these are areas less suited to more sensitive native species (Carosi et al. 2016). Indeed, in general, all three ISF were found to be present most frequently at sites that suffered from stronger impacts of human disturbances, and least frequently at sites where such impacts were not recorded (Table 2). However, concerning the character of the disturbances, the three ISF differed from each other. For *N. melanostomus*, these disturbances originated almost exclusively from hydro-morphological alterations, and not from chemical degradation of water quality. This was not the case of the two other ISF, as the water quality in a number of sites with *L. gibbosus* and especially with *P. parva* did not meet the criteria for life and reproduction of fishes and/or values indicating good status of the water bodies (Table 3). Nevertheless, the degradation of aquatic habitats may not be the only reason explaining the altitudinal distribution of the three ISF. Altitude itself is hardly a parameter that could, in general, stop further spreading of invasive species of fish. In fact, several environmental and physicochemical parameters were found to correlate with altitude, and therefore, it is obvious that the distribution limits of the three ISF depend on these parameters. Moreover, even below the altitude limits, not all the sites examined were occupied by the three ISF.

Three of the most important predictors that discriminated between the sites with presence and absence of ISF in lower altitudes were identical for all three ISF (altitude, wetted width and slope). However, other parameters were specific for each of the species (Figure 2A–C). For *N. melanostomus*, the misclassification rate that tests the importance of the predictors was low (Figure 2A). In other words, these predictors appear to be reliable, and therefore, this species is unlikely to spread to habitats other than those situated at lower altitudes, into larger water bodies (wetted width) and those with very shallow slopes (Table S1 and Tables 1–2). Such characteristics, together with the fish communities typically rich in benthic rheophils, corresponds in Slovakia mainly to the Danube and lower parts of its tributaries. Similarly, in any other river
system in Europe, *N. melanostomus* has never been recorded in altitudes higher than 355 m a.s.l. (German section of the Danube; Brandner et al. 2018). On the other hand, the misclassification rate in *L. gibbosus* and *P. parva* was very high (Figure 2B, C), which means that the predictors identified by the RF analysis were biased with a higher level of uncertainty. In other words, the differences between the sites with presence and absence of these two species do not appear to be strong enough to infer that the sites currently not occupied with *L. gibbosus* and *P. parva* (see also Table 2) will not be occupied in the future. This is also supported by the fact that the composition of fish communities was not found to affect presence or absence of either of these ISF (Figure 3B, C). Moreover, many sites, where both *L. gibbosus* and *P. parva* occurred (36% and 15%, respectively), were far from natural in condition, as they were situated in heavily modified water bodies and/or artificial water bodies. Pathways for spread of these species are often closely related to human activities, especially with recreational and commercial fisheries (Copp et al. 2002; Pinder et al. 2005; Witkowski 2009). Therefore, it is likely that in the case of these two species, establishing new populations in other waters below the limiting altitude (where they are currently absent) is simply a matter of time. Also, it appears doubtful that the current altitudinal limits of these two ISF are ultimate. Indeed, in other regions of Europe, *L. gibbosus* and *P. parva* have been reported from much higher altitudes than that found in Slovakia (880 m a.s.l. and 902 m a.s.l.; WFD River Fish Intercalibration Database, *unpublished data*). Hypothetically, this could be just because of regional differences in relationships between the altitude and correlating environmental parameters (i.e. that habitats with similar conditions may be related to different altitudes across Europe). Thus, the three environmental parameters identified as the most important predictors (slope, wetted width and dominant sediment, Figure 4 A–C) were compared (Mann-Whitney U tests and/or Fisher’s exact test). Differences between Slovakia and other regions of Europe in all the three parameters (*P < 0.05*) were found, which means that the above hypothesis was rejected. In other words, the habitats situated at altitudes higher than the limit in Slovakia, and occupied by *L. gibbosus* and *P. parva*, really differed in the three most important environmental parameters. This suggests that the altitudinal limits for *L. gibbosus* and *P. parva* found in Slovakia, including the environmental parameters that correlate with altitude, do not represent real ecological limitations, and further spreading of these two ISF above the current limit of distribution in Slovakia can be expected (in fact, even in Slovakia, two sites with *P. parva* were found above the limit – 447 m a.s.l. and 449 m a.s.l.).

In conclusion, the habitat parameters that characterize current distribution limits of *N. melanostomus* in Slovakia were found to be the wetted width, slope, temperature and conductivity. The species was found to prefer wider wetted width and shallower slope, as well as higher oxygen saturation. On the other hand, human disturbances, such as the presence of dykes, i.e. no lateral connectivity with the river system, fish stocking, recreational use, artificial barrier upstream, and/or alteration of the hydrograph did not limit its distribution. Nevertheless, the altitude (often considered surrogate of several habitat parameters) limits current distribution of *N. melanostomus* both in Slovakia and other regions of Europe. This may be why the distribution of *N. melanostomus* across Europe is currently limited to large water bodies, and the species is not known to penetrate too far upstream in smaller tributaries. Thus, in contrast to other successful invaders, such as *L. gibbosus* and *P. parva*, the analysis of key factors of current distribution of *N. melanostomus* suggests that its future invasion into small and mid-size tributaries of large rivers is unlikely.

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**Supplementary material**

The following supplementary material is available for this article:

Table S1. Differences (Mann-Whitney U test) in environmental and physicochemical parameters between sites with presence and absence of *Neogobius melanostomus*, *Lepomis gibbosus* and *Pseudorasbora parva*.

Table S2. Mean and median values of environmental, physicochemical and chemical parameters in the sites where *Neogobius melanostomus* were present or absent.

Table S3. Mean and median values of environmental, physicochemical and chemical parameters in the sites where *Lepomis gibbosus* were present or absent.

Table S4. Mean and median values of environmental, physicochemical and chemical parameters in the sites where *Pseudorasbora parva* were present or absent.

Table S5. Frequency of categories in human disturbances in the sites where *Neogobius melanostomus* (NM) were present or absent.

Table S6. Frequency of categories in ecological status in the sites where *Neogobius melanostomus* (NM) were present or absent.

Table S7. Frequency of categories in human disturbances in the sites where *Lepomis gibbosus* (LG), were present or absent.

Table S8. Frequency of categories in ecological status in the sites where *Lepomis gibbosus* (LG) were present or absent.

Table S9. Frequency of categories in human disturbances in the sites where *Pseudorasbora parva* (PP), were present or absent.

Table S10. Frequency of categories in ecological status in the sites where *Pseudorasbora parva* (PP) were present or absent.

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