

Barbel species arrangement in a regional Natura 2000 network (Emilia Romagna, Northern Italy): An altitudinal perspective

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ABSTRACT

Southern Europe hosts a large number of critical catchments for freshwater biodiversity, including endemic fish species. Unfortunately, these areas are severely threatened due to direct and indirect anthropogenic effects. In this context, with the aim to improve the effectiveness of threatened fish protection, the Life project BARBIE (LIFE13 NAT/IT/001129) started in 2014 and focused on three congeneric species of the genus *Barbus*: two of “priority interest” *sensu* Habitats Directive [*Barbus caninus* (Bonaparte, 1839), and *B. plebejus* (Bonaparte, 1839)], and one alien [*Barbus barbus* (Linnaeus, 1758)]. Our main objective was to assess the contribution of a complex of protected areas included in the Natura 2000 network - located in the provinces of Parma, Piacenza and Reggio Emilia (Northern Italy) - to support the presence of the three barbel species in analysis. Additionally, we explored the role of a set of environmental variables (*i.e.*, physical, chemical, biological, and land-use descriptors) to drive the current conditions of the study sites and the responses of *Barbus* species. As a general rule, the present study confirmed a clear decline of the local native barbel populations, and confirmed the existence of a zonation pattern of the barbel *taxa*. Hence, we observed a strong altitude segregation between native *vs.* alien species, with the exotic *B. barbus* currently limited to plain and only sporadically present in the Apennine areas as genetic introgression. These evidences mirrored the altitudinal gradients of anthropogenic disturbance. The main causes were the progressive disappearance of well-structured riparian stripes, and the intense land use change, ranging from semi-natural patches (mountain and hill sectors) to land clearing for intensive agriculture (lowland sectors). This highlights the need to take into account the spatial dynamics of alien invasive species in programming recovery actions that could have unexpected impacts to the native fish populations, for example the reduction of the hydraulic fragmentation along rivers.

Key word: *Barbus* genus species; inland waters; endangered fish; Habitats Directive; Fluvial Functionality Index; physical and chemical drivers.

INTRODUCTION

Since streams and rivers have become some of the most endangered ecosystems worldwide, there is an urgent demand for comprehensive methodological approaches to evaluate their conservation status, and to monitor their rate of changes (Li *et al.*, 2010). Mediterranean regions represent key areas for freshwater fish endemism and introductions (Leprieur *et al.*, 2008). Indeed, a large portion of the European critical catchments for freshwater biodiversity is located primarily in southern Europe (Carrizo *et al.*, 2017). Unfortunately, these areas are severely threatened because of water scarcity and environmental degradation due to the high anthropic pressure (*e.g.*, hydropower, agriculture, public uses), and to the on-going climate change (Hermoso and Clavero, 2011; Hermoso *et al.*, 2011).

In inland waters, the natural fish stocks are generally over-exploited, and in the developed countries, uncontrolled larvae and juveniles' reintroduction have been carried out for recreational fishery purposes (Lewin *et al.*,

2008). Additionally, in last decades the distributions of many exotic fish species have extended worldwide further impairing the quality of autochthonous fish populations (Carosi *et al.*, 2017). This is especially true for Italy, particularly along the lower stretches of rivers throughout the Apennines (Italian Peninsula) that are characterized by rather warm and slow waters, and scarce quality conditions (Carosi *et al.*, 2017). All this translates into less than ideal conditions for the survival of endemic and threatened fish, and the conservation of the local population diversity.

Since the beginning of 21st century, to counteract the above-mentioned critical issues many actions have been carried out to improve fish population, including mitigation of hydropower development, river fragmentation and hydropeaking (Premstaller *et al.*, 2017). In this context, the Natura 2000 network may represent a strategic tool to preserve both fish genetics and population resources, with the final goal of improving their local and regional survival chances. However, the designation procedures of the Natura 2000 sites often are driven by factors marginally

related to ecological criteria, thus reducing their effectiveness (Trochet and Schmeller, 2013). Furthermore, the biotic integrity of biological communities in rivers and lakes is considered a key predictor of colonized water bodies' quality suggesting a strong synergy between the Habitat Directive (HD; European Commission, 1992) and the Water Framework Directive (WFD; European Commission, 2000). However, unfortunately, these two fundamental legal issues proceed in isolation with negative effects on their relative efficiency (Bolgagni *et al.*, 2017).

In order to actively and mutually implement the HD and the WFD, and with the aim to improve their effectiveness in terms of threatened fish protection, the Life project BARBIE (LIFE13 NAT/IT/001129) started in 2014, focusing on three congeneric species of the genus *Barbus*. Two of them are of "priority interest" *sensu* HD: *Barbus caninus* (Bonaparte, 1839) and *B. plebejus* (Bonaparte, 1839). This two native species of *Barbus* are charismatic indicators for the estimation of the conservation status of water bodies (Angelini *et al.*, 2016). The third is an alien species, *Barbus barbus* (Linnaeus, 1758), that - in the last decades - showed an exceptionally fast spreading capacity within the Po River basin. This invasive species has also hybridized with the endemic *B. plebejus* (Meraner *et al.*, 2013), thus resulting in a widespread genetic introgression in the autochthon *Barbus* species.

In this context, to test the distinct distribution of the different species as per our hypothesis, we described the fish community structure in a representative array of 14 of the watercourses included in the Natura 2000 sites - and their surroundings - of the Parma, Piacenza and Reggio Emilia provinces (Emilia Romagna Region, Northern Italy). Our main hypothesis is that the exotic *taxon* (*B. barbus*) may be spatially limited to the lowland sectors, which are those with the highest human disturbance rates. On the contrary, prior barbel *taxa* (*B. caninus* and *B. plebejus*) are expected to be mainly distributed in mountain and/or hill areas. In other words, altitudinal segregation between these species may be hypothesized. To do this, we focused on the presence/absence and representativeness of the *Barbus* species, implementing the current data on their local spatial distribution. In this way, we were also able to assess the contribution of a complex of Natura 2000 sites to support fish communities. Additionally, we collected a set of environmental variables (including physical, chemical, biological, and land-use descriptors) to assess the current conditions of sites colonized by the different species in analysis.

METHODS

Study area

This study covered rivers and streams running along 14 sites of the Natura 2000 network of the Parma, Piacenza

and Reggio Emilia provinces (Emilia Romagna region, Northern Italy; Fig. 1 and Supplementary Tab. 1). The Köppen-Geiger classification includes both humid subtropical (cfa; plains and hill sectors) and oceanic climates (cfb; mountain sector), characterized by few extremes of temperature and pronounced precipitation in all months.

The study area includes a complex hydro-system that consists of several streams and rivers across an extended altitudinal range (about 600 m), stretching from the Tuscan-Emilian Apennine ridge to the Po River. Accordingly, the mean annual discharges and the main water chemical and physical conditions are extremely variable. Generally, the investigated water bodies showed a good chemical status, and fall into the sufficient or good quality classes of the ecological status *sensu* WFD (ARPA Emilia Romagna, 2013). The study area covers the entire distribution range of the two autochthonous target species in the Emilia Romagna region: canine barbel (*B. caninus*) inhabits mountain fast-flowing brooks and small rivers while the common barbel (*B. plebejus*) commonly occurs in hilly and lowland streams.

Biological, water quality and morphological characterization

Between July 2014 and December 2015, a total of 305 barbel individuals were collected by electric fishing (Macchio and Rossi, 2014), from 31 localities of the study area. Each animal was measured and fin-clipped and then released in the same location. Measurements included the total length and the weight. Fin fragment were preserved in 70% ethanol until DNA extraction. Starting from the visual based analysis of the fish community, the Index of the Ecological Status of Fish Communities (ISECI) was also calculated in each of the 31 study sites, according with Zerunian (2009) protocol.

Simultaneously with the fish characterization, a water sample was collected by a plastic bottle just below the water surface. Directly *in situ* temperature, pH, conductivity and dissolved oxygen data were collected by a multi-parameter probe (YSI model 556 MPS). Samples for the biochemical oxygen demand (BOD) were transferred in glass bottles. Samples for NH_4^+ (ammonium), NO_3^- (nitrate), and NO_2^- (nitrite) determinations were filtered through Whatman GF/F glass fiber filters (\varnothing 47 mm, porosity 0.45 μm) and transferred to plastic vials. All water samples were kept to 4°C, and transferred to the laboratory. Total suspended solids (TSS) were measured by filtration through a pre-dried and weighed glass fiber filter GF/F (Whatman, UK, \varnothing 25 mm and 0.45 μm) (APHA, 2012). NH_4^+ , NO_3^- , NO_2^- , were determined with standard spectrophotometric methods APHA (2012), whereas BOD was calculated after incubation at $20\pm 1^\circ\text{C}$ for 5 days (= BOD₅) according to APHA (2012).

Each sampling sites was characterized by the applica-

tion of the Fluvial Functionality Index (FFI), that is devoted to investigate the functionality of a river stretch in terms of metabolic capacity (*i.e.*, fine and coarse particulate organic matter retention and cycling) (Siligardi *et al.*, 2000). This method is based on the analysis of riverbank vegetation, physical and morphological structure, the extent of the riparian area, the land use impact, the riverbed structure, and the key biological characteristics of river ecosystem. For further details, see Siligardi *et al.* (2000).

Molecular data analysis

Total genomic DNA was extracted from fin tissue using Wizard genomic DNA Purification kit (PROMEGA, Madison, WI, USA). DNA quality and concentration were tested by 1% agarose gel electrophoresis in 1% TAE buffer, by visual comparison with a DNA ladder mix and by spectrophotometry at 260 e 280 nm. The extraction procedure typically yielded not less than 40 ng/mL of HMW (high molecular weight) DNA. For each of 305 specimens we analysed 600 bp long region of the

cytb mtDNA and was amplified by polymerase chain reaction (PCR) using primer pair CYTB-Thr 5'-ACCTCCGATCTTCGGATTACAAGACCG-3' and CYTB-Glu 5'-AACCACCGTTGTATTCAACTACAA - 3' (Zardoya and Doadrio, 1998). A reaction volume of 25 μ l containing 1 U of GoTaq Polymerase (PROMEGA, Madison, WI, USA), Mg^{2+} 1.5 mM and dNTPs 0.2 mM, and 10 pmol of each primer were used. PCR was set as follows: 35 cycles of 45 s at 94°C, 1 min at 47°C, and 2 min at 72°C, after an initial 3 min denaturation step at 94°C and a final extension at 72°C for 10 min. Fragments sequencing was performed by MACROGEN Europe service (Amsterdam, the Netherlands). The obtained sequences were manually corrected using MEGA7.0 and were compared with those available in genomic databases using NCBI BLAST.

Statistical analysis

A principal component analysis (PCA) was performed on chemical and physical data. The interpretation of PCA

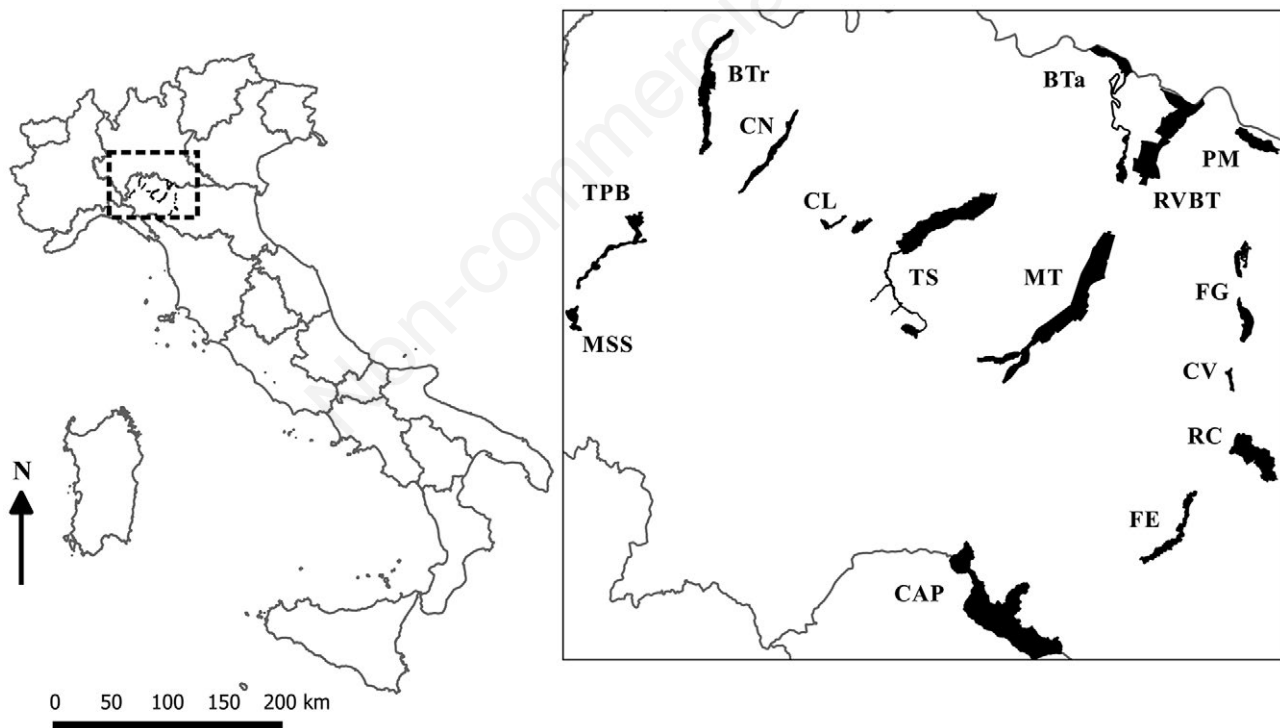


Fig. 1. Study area with the indication of the Natura 2000 network. MSS, Trebbia - Meandri di San Salvatore (IT4010006); TPB, Trebbia River - from Perino to Bobbio (IT4010011); BTr, Low Trebbia River (IT4010016); CN, Nure River - Conoide del Nure and Bosco di Fornace Vecchia (IT4010017); CL, Arada River - Castell'Arquato, Lugagnano Val d'Arda (IT4010008); TS, Stirone Stream (IT4020003); Bta, Low Taro River (IT4010017); RVBT, Lorno River - Aree delle risorgive di Viarolo, Bacini di Torrile, Fascia golenale del Po (IT4020017); PM, Parma Morta (IT4020025); MT, Medium Taro River (IT4020021); FG, Enza River - Fontanili di Gattatico (IT4030023); CV, Enza River - Cronovilla (IT4020027); RC, Cerezzola Stream - Rupe di Campotrera, Rossena (IT4030014); FE, Enza River - from La Mora to Compiano (IT4030013); CAP, Parma - Crinale dell'Appennino parmense (IT4020020).

ordination was limited to variables with loads higher than the vector representing a variable contributing equally to all the dimensions of the PCA space (Borcard *et al.*, 2011). The relationship between PCA ordination and the structure of *Barbus*' populations was assessed by means of the function `envfit` ("vegan" package) that fits vectors onto a multivariate ordination. Simple regression analysis was used to examine the relationships between FFI and altitude and BOD₅ values.

All the analyses and graphs were performed with the statistical software R (R Core Team, 2015), with base version, `ggplot2` (Wickham, 2009) and `vegan` (Oksanen *et al.*, 2016) packages.

RESULTS

Water quality and morphological characterization of sites

Physical, chemical, and morphological (*i.e.*, FFI outputs) data are reported in Supplementary Tab. 1. Results from water measurements highlight the variability between sites in terms of altitude and human impact gradients. In summer 2015, temperature, pH, dissolved oxygen, and conductivity were in the ranges 14.4-31.0°C, 7.30-8.91, 37-295% saturation, and 190-825 $\mu\text{S cm}^{-1}$, respectively. Similarly, the BOD₅ and TSS values varied from 0.0 (mountain sites, Trebbia River) to 20.8 mg L⁻¹ (lowland site, Parma Morta), and from 0.0 to 66.2 mg L⁻¹, respectively. At the same time, NH₄⁺ and NO₂⁻ concentrations exhibited only small variations, within the range 0.02-0.04 NH₄⁺ mg N L⁻¹, and 0.00-0.02 NO₂⁻ mg N L⁻¹. On the contrary, NO₃⁻ ranged between 0.08 and 5.00 mg N L⁻¹.

FFI ranged between 102 and 245, from poor/bad to good classes. It showed a clear spatial arrangement with a tendentially significant negative correlation with altitude ($r=0.45$, $P=0.07$; $n=17$). A progressive reduction in FFI values was recorded from mountain/hill sectors to lowlands. On the contrary, no significant relationship between FFI and BOD₅ values was recorded ($r=0.37$, $P>0.1$; $n=17$), although a gradual increase of the biochemical oxygen demand with the progressive loss of the functionality of riparian belt is generally expected.

Fish community and ISECI assessment

The number of individuals detected in each sampling station for the three investigated species is reported in Tab. 1. From a general point of view, considering the population size (expressed in terms of number of individuals), the observed data were quite low in the range 0-29 individuals. Considering species separation, mean values (\pm standard deviation) were 1.0 (± 3.5), 1.8 (± 6.1), and 7.1 (± 8.4) individuals for alien European barbel, canine, and

common respectively. Eight over 31 (26%) study sites showed complete absence of barbel specimens.

A structured population of canine barbel constituted of 24 and 25 specimens were retrieved in the streams Rio Cerezzola (Natura 2000 site IT4030014) and Rio Parmossa (bordering this Natura 2000 site area), respectively. On the contrary, common barbel peaked at Nure and Enza rivers with 29 and 25 individuals, respectively. The alien European barbel species colonized 7 (23%) study sites belonging to Arda, Trebbia, Taro, Ceno and Enza rivers, besides Naviglio channel (Tab. 1). In particular, the alien European barbel showed a representative population in Taro River, with 19 individuals, as a result of the only investigated plain river site.

The ISECI values varied from 0.72 (good ecological quality) at Nure River to 0.20 (poor ecological quality) at lowland site Parma Morta, with a mean value of 0.57 (± 0.14) (sufficient ecological quality) (Tab. 1). More specifically, 18 over 31 (58%) sites displayed "good quality" class, 10 (32) "moderate quality", 1 (3%) "poor quality", and 2 (7%) "bad quality".

Molecular analyses provided data on the percentage of different species at sampling site level (Tab. 1). Fragments of 600 bps were analyzed and compared to GenBank sequences. Samples displayed 100% identity with deposited sequences according to different species with alignment values $E=0.0$ and maximum identity in the range 97-100%. ClustalW assessment among investigated samples showed a total number of 230 polymorphic sites. From a taxonomy point of view, molecular analyses on mtDNA evidenced the following density values: 72% common barbel, 18% canine barbel, and 10% alien European barbel.

In terms of environmental drivers of the observed barbel' species spatial distribution, FFI and oxygen saturation were the variables contributing most to respectively PCA axes 1 and 2, explaining the 88% of the total variance. Alien European barbel was significantly related to the PCA ordination ($R^2=0.67$, $P<0.05$), while this was not true for the other two barbel species: *B. caninus* ($R^2=0.15$, $P>0.05$) and *B. plebejus* ($R^2=0.14$, $P>0.05$) (Fig. 2).

DISCUSSION AND CONCLUSIONS

Our results confirmed a decline of native barbel populations in the study area compared with literature data (Nonnis Marzano *et al.*, 2003; Fish Charts Provinces of Parma, Reggio Emilia, and Piacenza). Barbel populations were historically present in several streams of the three provinces, both in the mid-Apennine and hillsides (Nonnis Marzano *et al.*, 2003). However, a well-structured population of the canine barbel was retrieved only in two sampling stations, highlighting a local high degree of rarity for this species. Additionally, in agreement with our initial hy-

potheses the present data confirmed the general existence of a clear zonation pattern among the barbel *species* analyzed, although a frequent overlapping of populations limited statistical significance of principal component analysis. A non-negligible altitude segregation between native vs. alien species was detected, with the exotic invasive *B. barbus* mainly limited to lowland watercourses. We also detected invasive *B. barbus* DNA in the native populations of the hill areas as a result of genetic introgression. These observations corroborate recent evidence on the rapid expansion of the European barbel in the Po basin (Meraner *et al.*, 2013), and indicate a higher level of vulnerability

for the autochthonous fish populations placed at lower altitudes, where the likely presence of alien barbels is wider. It is noteworthy observing that migration of *B. barbus* mitochondrial haplotypes reaching hill and mid-Apennines catchments could be referred to water heating due to global warming and water deprivation.

More specifically, the species spatial distribution mirrored the anthropogenic disturbance gradients. In fact, all the investigated sites are arranged along an altitudinal gradient stretching from the Tuscan-Emilian Apennine (up to 600 m asl) to the Po River (~25 m asl), that overlaps with human pressures, which significantly decreases

Tab. 1. Barbus' population size and ISECI results; for each sampled site, the indication of each watercourse and the Natura 2000 site it belongs, the number of individuals and their % distribution value (in brackets) of Canine (*B. caninus*), Common (*B. plebejus*), and alien European (*B. barbus*) Barbel, the values and quality class of ISECI were reported. In bold the sites without barbels.

Site	Watercourse	Code	Natura 2000 site code	Number of individuals			ISECI	
				Canine	Common	European	Value	Quality class
10006.TR.1	Trebbia	MSS	IT4010006	1 (7.5%)	15 (92.5%)	0	0.61	Good
10008.AR.1	Arda	CL	IT4010008	0	23 (95.8%)	1 (4.2%)	0.61	Good
10011.TR.1	Trebbia	TPB	IT4010011	2 (12.5%)	14 (87.5%)	0	0.64	Good
10016.TR.1	Trebbia	BTr	IT4010016	0	15 (75.0%)	5 (25.0%)	0.72	Good
10016.TR.2	Trebbia	BTr	IT4010016	0	0	0	0.69	Good
10017.NU.1	Nure	CN	IT4010017	0	29 (100.0%)	0	0.72	Good
20003.ST.1	Stirone	TS	IT4020003	0	13 (100.0%)	0	0.71	Good
20017.LO.1	Lorno	RVBT	IT4020017	0	0	0	0.66	Good
20020.PR.1	Parma	-	-	0	3 (100.0%)	0	0.40	Poor
20020.PR.2	Parma	-	-	1 (11.1%)	8 (88.9%)	0	0.52	Moderate
20020.PR.3	Parma	CAP	IT4020020	0	0	0	0.66	Good
20020.PM.1	Parmossa	-	-	25 (89.3%)	3 (10.7%)	0	0.66	Good
20020.FA.1	Fabiola	-	-	0	0	0	0.66	Good
20020.MO.1	Moneglia	-	-	0	0	0	0.63	Good
20020.BA.1	Baganza	-	-	1 (20.0%)	4 (80.0%)	0	0.49	Moderate
20021.TA.1	Taro	MT	IT4020021	0	5 (83.3%)	1 (16.7%)	0.63	Good
20021.NA.1	Naviglio	MT	IT4020021	0	8 (80.0%)	2 (20.0%)	0.52	Moderate
20021.CE.1	Ceno	MT	IT4020021	0	8 (88.9%)	1 (11.1%)	0.68	Good
20022.TA.1	Taro	BTa	IT4020022	0	0	19 (100.0%)	0.30	Mediocre
20025.PR.1	Parma Morta	PM	IT4020025	0	0	0	0.20	Bad
20027.EN.1	Enza	CV	IT4020027	0	7 (100.0%)	0	0.72	Good
30013.EN.1	Enza	FE	IT4030013	0	25 (100.0%)	0	0.55	Moderate
30013.CE.1	Cedra	-	-	0	2 (100.0%)	0	0.42	Moderate
30013.CE.2	Cedra	-	-	0	0	0	0.20	Bad
30014.RV.1	Rio Vico	RC	IT4030014	0	6 (100.0%)	0	0.50	Moderate
30014.RC.1	Rio Cerezzola	RC	IT4030014	1	0	0	0.67	Good
30014.RC.2	Rio Cerezzola	RC	IT4030014	24 (100.0%)	0	0	0.59	Moderate
30023.EN.1	Enza	FG	IT4030023	0	21 (91.3%)	2 (8.7%)	0.60	Moderate
30023.EN.2	Enza	FG	IT4030023	0	5 (100.0%)	0	0.53	Moderate
30023.EN.3	Enza	FG	IT4030023	0	5 (100.0%)	0	0.64	Good
30023.EN.4	Enza	FG	IT4030023	0	0	0	0.63	Good

ISECI, Ecological Status of Fish Communities.

as altitude increases. Hence, a progressive improvement of all monitored physical, chemical and morphological features was detected with the decreasing of elevation. The strong overlap between physical and chemical data reinforces the existence of an altitudinal zonation in term of barbel species representativeness among sites. At the site scale, with decreasing altitude the river functionality (e.g., FFI) varied from good to poor/bad, and BOD₅ from 0.0 to 20.8 mg L⁻¹.

Our results suggest that the progressive disappearance of well-structured riparian stripes, the increase of the riverbed incision - that actually encourages the progressive isolation of the watercourse from the local context surrounding - and the intense land use change, are the main reasons of the observed zonation. Consequently, the present results obtained through a multidisciplinary approach support the idea that the native and threatened priority barbel species were found preferentially in moderate to well-preserved watercourses, characterized by more expanded riparian areas, in terms of greater IFF values. Similar results were modeled for two Minnesota watersheds, where substantial changes in agricultural management, including an expansion of the riparian areas, would be expected to significantly improve local brook trout [*Salvelinus fontinalis* (Mitchill, 1814)] populations, by increasing streams shading up to 50% (Blann *et al.*, 2002). Hence, in lowlands and agricultural settings the destruction of riparian communities leads to a rapid physical and chemical deterioration of watercourses with dramatic effects on fish populations, as highlighted by Lorenzoni *et al.* (2006) for rivers in Central Italy.

Although the small number of sites under consideration in this work, a quite verified preference of the canine and common barbels for higher IFF values was verified,

as a proxy of well-developed and structured riparian contexts and higher altitude values. Marginal vegetated belts are able to efficiently offset the nutrient inputs generated at the basin scale, and to control the main local edaphic factors (Siligardi *et al.*, 2000). Hence, in presence of high FFI values, higher shading values, as well as lower water temperatures and reduced day-night temperature fluctuations are expected. Furthermore, watercourses with high FFI levels should have reduced primary producer rates with rather scarce macrophyte cover values, including algal mats. On the other hand, the alien European barbel was positively and significantly related to high levels of dissolved oxygen, which in turn was associated to higher levels of STT and BOD₅. This is not surprising, given that the above-mentioned high dissolved oxygen levels (up to 295% saturation) were due to the hyper-proliferation of microalgae, and were typical of lowland sites of Po plain where riparian belts/zones have been almost completely lost to land reclamation and by agricultural mechanization (Bolpagni and Piotti, 2015, 2016).

All the above clearly remarks the need for more efficiently designed long-term and wide-spatial scales actions to counteract the alien fish expansion, and furtherly focus on the sustainable management of river habitats and water flows. In this context, the reduction of the hydraulic fragmentation of watercourses is an essential paradigm to improve the survival prospects of a very large number of fish species of conservation interest. In fact, the progressive impairment of the longitudinal river continuity causes significant alterations in river dynamic processes and aquatic vegetation (Bolpagni *et al.*, 2016). However, it is of interest, and possibly of considerable practical importance, taking into account the spatial dynamics of the alien invasive species before operating management actions that

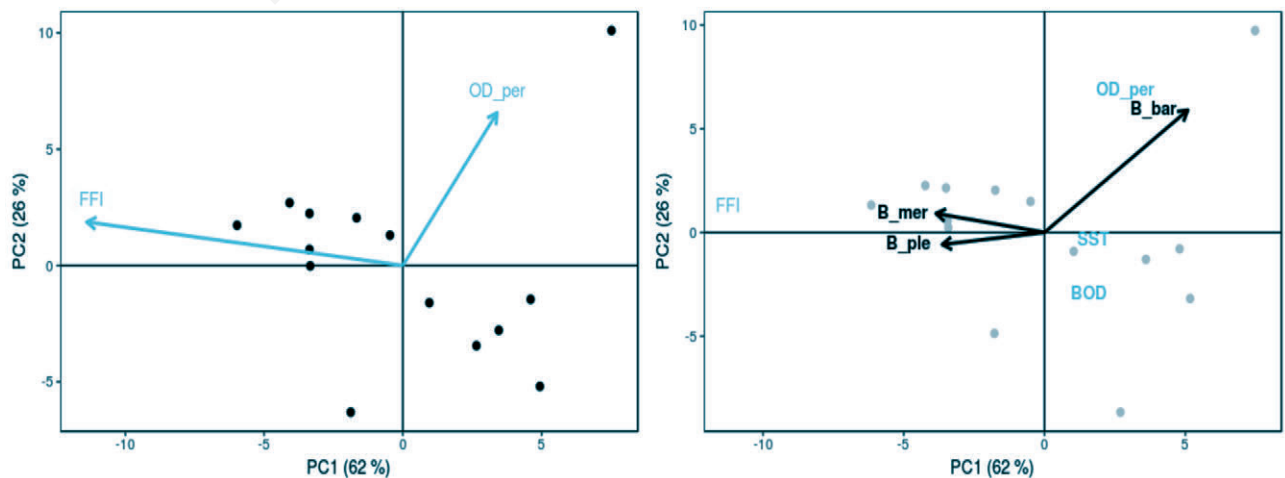


Fig. 2. Results of principal component analysis (left); vectors representing *Barbus*' population structure.

could later have an impact to the native fish populations. This critical issue must also be further discussed in the light of the on-going climate change that can affect barbel migration along the altitudinal gradient.

ACKNOWLEDGMENTS

The present work was performed in the frame of the LIFE project “BARBIE” (LIFE13 NAT/IT/001129) started in 2014. We would thank dr. Erica Racchetti and Marina Vaghi for the support in the laboratory analyses. The contribution of GB is part of her project within the joint doctorate program in Evolutionary Biology and Ecology (XXX cycle) at the Universities of Ferrara and Parma. AL and RB were supported by fellowships co-funded by the University of Parma.

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