Run to the hills: exotic fish invasions and water quality degradation drive native fish to higher altitudes

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Abstract

While the significance of anthropogenic pressures in shaping species distributions and abundances is undeniable, some ambiguity still remains on their relative magnitude and interplay with natural environmental factors. In our study, we examined 91 late-invasion-stage river locations in Northern Italy using ordination methods and variance partitioning (partial-CCA), as well as an assessment of environmental thresholds (TITAN), to attempt to disentangle the effects of eutrophication and exotic species on native species. We found that exotic species, jointly with water quality (primarily eutrophication) and geomorphology, are the main drivers of the distribution of native species and that native species suffer more joint effects than exotic species. We also found that water temperature clearly separates species distributions and that some native species, like Italian bleak (*Alburnus alborella*) and Italian rudd (*Scardinius hesperidicus*), seem to be the most resilient to exotic fish species. We also analyzed the dataset for nestedness (BINMATNEST) to identify priority targets of conservation. As a result, we confirmed that altitude correlated negatively with eutrophication and nestedness of exotic species and positively with native species. Overall, our analysis was able to detect the effects of species invasions even at a late invasion stage, although reciprocal effects seemed comparable at this stage. Exotic species have pushed most native species on the edge of local extinction in several sites and displaced most of them on the rim of their natural distribution. Any potential site- and species-specific conservation action aimed at improving this situation could benefit from a carefully considered prioritization to yield the highest results-per-effort and success rate.

Introduction

The significance of anthropogenic pressures in shaping terrestrial and aquatic ecosystems at the global scale is undeniable (Foley et al., 2005; Halpern et al., 2008; Syvitski, Vörösmarty, Kettner, & Green, 2005). The scope and magnitude of these pressures has been investigated in several studies which attempted to find the thresholds of changes that lead to new dynamic states (Samhouri, Levin, & Ainsworth, 2010). From the worldwide effects of anthropogenic-driven climate warming (e.g. Behrenfeld et al., 2006) down to the local scale consequences of water abstraction or infrastructure construction (Jeppesen et al., 2015), some uncertainties still remain on the interplay between different pressures, as often they overlap in space and time and their effects on the ecosystem are not easy to disentangle.

A new factor that has been introduced in the equation of anthropogenic pressures is the introduction of exotic species, which enhances the complexity and the uncertainties of interpreting the individual role of specific stressors (Leprieur, Beauchard, Blanchet, Oberdorff, & Brosse, 2008; Pyšek et al., 2010). The assessment of invasion impacts could be accomplished in controlled experimental conditions (e.g. Johnson, Olden, Solomon, & Vander Zanden, 2009) or, where feasible, backtracking the effects in the sedimentary record (e.g. Milardi, Siitonen, Lappalainen, Liljendahl, & Weckström, 2016). Several studies have recently attempted to address the role of human-mediated species introductions in the loss of socio-economic values (e.g. Pimentel, Zuniga, & Morrison, 2005) or biodiversity (Wilcove, Rothstein, Dubow, Phillips, & Losos, 1998). It is generally recognized that exotic species constitute a relevant pressure on the environment at different scales (Meyerson & Mooney, 2007). However, while several authors pointed out that exotic species invasions could be a major driver of native species extinction and homogenization of ecosystems (Wilcove et al., 1998), others remained sceptic about the extent and intensity of their effects (e.g. Gurevitch & Padilla, 2004). This controversy could also be partially due to the fact that exotic species invasions are not a flash process: invasion impacts could be highest in the initial stages, but changes in native species abundance and distribution could take a longer time to complete. Furthermore, some studies might have failed to grasp the full effects of species invasions because overlapping factors (e.g. eutrophication levels) might jointly drive the interactions between native and exotic species, thus confounding the final outcome of invasions (Leprieur et al., 2008).

While there might remain a certain amount of uncertainty on their exact rank among pressures or their combined effects, it is undeniable that human-mediated species introductions played a role in homogenizing the worldwide biota (Clavel, Julliard, & Devictor, 2011; Rahel, 2000). This homogenization is particularly evident in the case of the fish fauna (Rahel, 2000), where a few species that are highly sought after for aquaculture or fisheries (e.g. the common carp, *Cyprinus carpio* or the rainbow trout, *Oncorhynchus mykiss*) have been introduced so widely that they can be now found in all continents. Most successful invaders are also generalist species which thrive in different environmental conditions, and could be favored by environmental degradation, even though patterns of invasion success might differ among taxa (Crawley et al., 1986). However, the environment plays a major role in regulating the distribution of species: therefore the success of species invasions might be mostly due to a synergic effect between the changing environmental conditions and the exotic species, regardless of the native biota (Moyle & Light, 1996). However, the reciprocal effect of native species on exotic ones has not yet been thoroughly investigated. Furthermore, albeit there is no agreement as to how long an invasion takes to reach a “late stage”, areas where exotic and native species interactions have had decades to play out could be most interesting to explore this issue.

The Mediterranean region has been the focus of many studies dealing with biodiversity loss due to species invasions (e.g. Didham, Tylianakis, Gemmell, Rand, & Ewers, 2007; Lloret et al., 2005). In this region, it has been argued that fish introductions constitute one of the major drivers of extinction, at least for fish species (Crivelli, 1995). However, the Mediterranean is one of the regions where anthropic modification of the environment has been undertaken at least since the Roman Empire, thus potentially creating degraded habitat conditions that could further favor the establishment of exotic species. However, more than a few challenges persist in disentangling and prioritizing biodiversity loss causes as well as in optimizing conservation efforts, due to the lack of a truly multidisciplinary approach and of adaptive management (Pooley, Mendelsohn, & Milner-Gulland, 2014). Methods of investigation that would be able to address these challenges could be very useful to.

To investigate the impact of exotic species invasions, and its relation to environmental conditions, we selected an area at a late stage (over 30 years) of exotic fish species invasion in the Mediterranean region, where native fish species were still present. We hypothesized that exotic species would be a major driver of native species distribution and that the reciprocal effects would be much smaller. We also hypothesized that water quality (primarily eutrophication) and geomorphological factors could play a role in both native and exotic fish distributions, but would favor the latter. We used ordination and variance partitioning methods to quantify the relative contribution of environmental gradients and exotic species in shaping the occurrence, distribution and abundance of native species. To identify priority targets of site-specific and species-specific conservation measures, we used a nestedness analysis to rank sites based on species population nestedness and rank species based on their nesting capacity.

Materials and Methods

Study area, surveys and data collection

The study area is located in Northern Italy and it is defined by the administrative boundaries of Emilia-Romagna Region with a total coverage of 22,446 km² (Figure 1). It is naturally bound north and south by the Po River and the Apennines Mountains, respectively, and has a Mediterranean continental climate. In this area, exotic fish species introduction date as far back as the XVII century (common carp), with few North American species (rainbow trout, brown bullhead, *Ictalurus melas*, and some Centrarchidae) arriving in the early '900s and most species being introduced around the '80s from Asia or East Europe (e.g. grass carp, *Ctenopharyngodon idella*, or wels catfish, *Silurus glanis*).

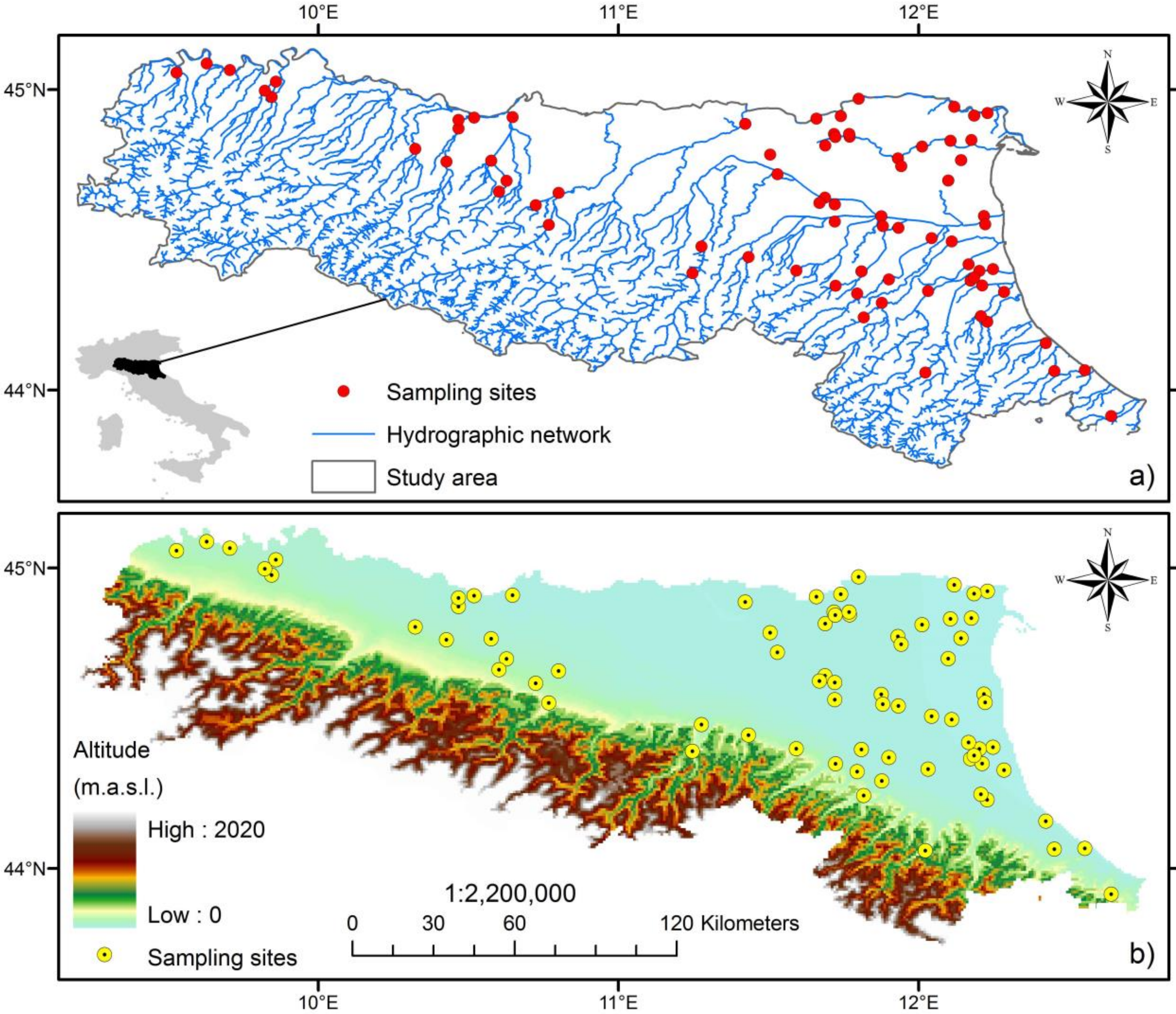

Figure 1. Location of the 91 sampling sites based on a) major rivers, streams and canals (source: http://www.eea.europa.eu/data-and-maps/data/european-river-catchments-1), and b) altitude profile of the area (source: https://lta.cr.usgs.gov/GTOPO30).

We selected 91 sampling sites within the study area, where both exotic and native fish species were present. The selected sites were mostly located in the lowlands, including both natural and artificial water bodies, over an altitudinal gradient of ~120 m (full range -1–389 m a.s.l.). Sites with fully native (at high altitudes) or fully exotic (at low altitudes) fish communities were excluded from the analysis, because the focus was to specifically investigate the area where native and exotic species distributions overlap. Further descriptions of sites, species and communities excluded from this analysis can be found in Lanzoni, Milardi, Aschonitis, Fano, and Castaldelli (2017, under review).

The sampling sites were located in waterways with section width ranging from 8 to 350 m (the maximum value corresponds to the Po River) (Figure 1). The sampling was performed in both natural (e.g. Po, Trebbia, Taro, Secchia, Panaro, Reno, Lamone, Fiumi Uniti, Bevano, Marecchia etc) and artificial (irrigation canals, mainly located in the lowlands, e.g. Po di Volano, Po di Primaro, Canal Bianco, Canale Circondariale) riverine habitats during the warm season (from April to September) of the period 1998-2004 as part of the institutional regional monitoring program for the compilation of the official Fish Inventories of the Emilia-Romagna Region (Regione Emilia Romagna, 2008). The dataset included information on each site location, altitude and main water quality parameters (e.g. nitrogen and phosphorus concentration, Biochemical Oxygen Demand - BOD, Chemical Oxygen Demand - COD, temperature and pH). Yearly LIM (*Livello di Inquinamento da Macrodescrittori*, Pollution Level from Macro-descriptors, in English) scores were measured during 1993–2002; LIM measures the environmental status based on the concentration of 7 different parameters representative of the chemical status of the water, sampled at monthly intervals. These parameters are dissolved oxygen, BOD, COD, *Escherichia coli*, phosphorus and nitrogen dissolved compounds, therefore LIM does not strictly measure chemical pollution, but rather provides a measure of the eutrophication level. Fish sampling was performed by electrofishing, adapting the standard guidelines to the particular conditions of waterway typologies, using direct current at 400–600 V and 4–5 A (Backiel & Welcomme, 1980; Reynolds, 1996). Sites were sampled once, during daylight, in an upstream zigzag direction by wading, when depth was less than 1 m, and by boat in deeper waters. The transect lengths were equivalent to 10 times the river width ensuring that the range of present macrohabitats of each site was fully surveyed (Hankin & Reeves, 1988). The duration of sampling was therefore quite variable ranging from half an hour to more than two hours, as in the case of the Po River. Electrofishing is considered the best quantitative method for fish sampling in shallow waters, up to a maximum of 1 m (Zalewski & Cowx, 1990) but its efficacy may be low in deeper waters, with big and mobile specimens, or with high conductivity. Such special conditions occurred in almost all the lower stretches of rivers and in the canals of the lowlands. For this reason, electrofishing in these sites was immediately followed by sampling with a standard set of trammel nets (with variable mesh size from 90 to 5 mm), with the support of professional fishermen (Backiel & Welcomme, 1980). Fish species were classified according to Kottelat and Freyhof (2007), taking into account recent taxonomical determinations and common names as listed in FishBase (Froese & Pauly, 2017). Site-specific fish abundances were expressed in Moyle classes (Moyle & Nichols, 1973), ranging from 1 (low abundance, 1-2 individuals per site) to 5 (high abundance, >50 individuals per site).

Ordination methods and variance partitioning

The data were used to form four groups of variables: Group 1 (native fish species - Ns), Group 2 (exotic fish species – Es), Group 3 (geographical variables – GeoTopo) and Group 4 (water quality parameters - WaterQ) (the latter two groups are summarized in Table 1). The data of abiotic environmental parameters (Table 1) were log-transformed before analysis to reduce normality departures (Aschonitis et al., 2016; Feld & Hering, 2007).

Table 1 – Geographical and water quality parameters and their grouping

Parameter	Abbreviation	Unit	Trasformation	Minimum	Maximum	Average	Standard deviation	Group
Latitude (WGS84 ellipsoid)	Lat	Dec. degrees	log(x+1)	43.91	45.09	44.62	0.28	GeoTopo
Longitude (WGS84 ellipsoid)	Long	Dec. degrees	log(x+1)	9.53	12.64	11.62	0.73	GeoTopo
Altitude	Alt	m a.s.l	log(x+5)	-1.00	389.00	28.80	54.69	GeoTopo
Ammonia Nitrogen	NH4+	N mg/L	log(x+1)	0.02	6.35	0.60	1.02	WaterQ
Nitrate Nitrogen	NO3-	N mg/L	log(x+1)	0.17	9.87	2.07	1.48	WaterQ
BOD5	BOD5	O2 mg/L	log(x+1)	0.63	10.50	4.44	2.10	WaterQ
COD	COD	O2 mg/L	log(x+1)	4.33	52.25	17.10	8.54	WaterQ
Electrical conductivity	EC	µS/cm	log(x+1)	334.50	3660.67	810.82	408.00	WaterQ
Total phosphorus	TP	P mg/L	log(x+1)	0.01	1.68	0.25	0.28	WaterQ
Total suspended solids	TotSS	mg/L	log(x+1)	0.50	297.83	52.51	49.26	WaterQ
Water temperature	Temp	°C	log(x+1)	12.75	24.57	20.24	2.41	WaterQ

Detrended Correspondence Analysis (DCA) was initially performed in order to select the most appropriate response model (between linear or unimodal) for gradient analysis (Lepš &

Šmilauer, 2003; Ter Braak & Smilauer, 2002) and Canonical Correspondence Analysis (CCA) (unimodal method) was finally used in all the studied cases instead of a linear method (e.g. Redundancy Analysis - RDA) because the dominant gradient length in DCA was always greater than 4 (Lepš & Šmilauer, 2003) (results not given). CCAs were performed either using Es group or Ns group or both groups as dependent variables versus the remaining groups of each case as descriptor variables. Each CCA was performed targeting either all the remaining groups (case of full-CCA) or one group after partialing out the effects of the parameters of the remaining ones, which were used as co-variables (case of partial-CCA). CCA was performed for each possible combination of targeted descriptor and co-variables using CANOCO 4.5, based on species correlations, and standardized species scores (Ter Braak & Smilauer, 2002). Significant descriptors for each group were identified using CANOCO's forward selection procedure and Monte Carlo permutation test (499 permutations) (Aschonitis et al., 2016; Feld & Hering, 2007). Collinear variables with a variance inflation factor VIF>8 were excluded before the CCA analysis (Zuur, Ieno, & Smith, 2007). Rare fish species (present in ≤1% of sites) were excluded from ordination analysis (Aschonitis et al., 2016). These species were southern pike (*Esox cisalpinus*), tench (*Tinca tinca*), flathead grey mullet (*Liza ramada*), European flounder (*Platichthys flesus)*, brown trout (*Salmo trutta*), roach (*Rutilus rutilus*), grass carp and pond loach (*Misgurnus anguillicaudatus*).

A variance partitioning scheme (Borcard, Legendre, & Drapeau, 1992; Liu, 1997) was applied for each group of variables based on the overall variance explained by the partial CCAs. This procedure allowed the distinction between unique effects (i.e. the variance explained by a single group of variables), joint effects (i.e. the variance jointly explained by variables of two or three groups), and unexplained variance. The proportion of variance explained by different groups of variables are expressed as the sum of all canonical eigenvalues of partial-CCA (or CCA) divided by the total inertia (Feld & Hering, 2007). Variance partitioning was also run with all variables to identify the marginal effects (λ-1) and the conditional effects (λ-A) of each descriptor variable. The marginal effect of a descriptor variable is equal to the eigenvalue of a partial CCA if the corresponding variable was the only environmental variable (additionally to the variance explained by covariables). The conditional effect of an environmental variable is equal to the additional amount of variance in species assemblages explained by the corresponding variable at the time it was included into the model during a selection procedure (additionally to the variance explained by covariables). Such effects were also examined to assess the relative contributions of environmental variables for predicting the community composition (Ter Braak & Smilauer, 2002).

Assessment of environmental thresholds – TITAN analysis

The main gradients identified through ordination methods were used to analyze the losses and gains of taxa along these gradients using the Threshold Indicator Taxa ANalysis (TITAN, Baker and King (2010)). Altitude (Group 3) and temperature (Group 4) were selected based on their ranking within variable groups for both native and exotic species. Additionally, crucian carp abundance (Group 2) was selected to identify thresholds solely for native species. TITAN uses indicator taxon scores (IndVal) to integrate occurrence, abundance and directionality of taxon responses along environmental gradients. The method identifies the environmental threshold (the optimum value of a continuous variable) that partitions sampling units and distinguishes negative (= losses: z−) and positive (= gains: z+) taxon responses. Thus, TITAN helps to identify taxon-specific change points along an environmental gradient at which the decline/increase in a given taxon's frequency and abundance is most prominent. Bootstrapping (500 repetitions) is used to estimate two important diagnostic indices (reliability and purity) as well as uncertainty around the location of individual taxa and community change points (Baker & King, 2010). Indicator purity is the proportion of change-point response directions (positive or negative) among bootstrap replicates that agree with the observed response. Pure indicators (e.g., purity≥0.95) are consistently assigned the same response direction, regardless of abundance and frequency distributions generated by resampling the original data. If bootstrap resampling substantially alters the probability of obtaining an equal or larger IndVal based on random permutations of the data, then that particular taxon is not a reliable indicator. Indicator reliability is estimated by the proportion of bootstrap change points whose IndVal scores consistently result in P-values below one or more user-determined probability levels (e.g., P ≤ .05). Reliable indicators (e.g., P ≥ 0.95) of the bootstrap replicates achieving P≤0.05 are those with repeatable and consistently large IndVal maxima (Baker & King, 2010). Similarly as for ordination analysis, the same species which were found in less than 3 sites were excluded also from TITAN analysis.

Nestedness analysis

The binary matrix nestedness temperature calculator (“BINMATNEST”; Rodríguez-Gironés and Santamaría (2006)) was used to quantify the level of nestedness in native and exotic species distributions. The calculator’s algorithm permutes rows (fish species) and columns (sampling sites) in such a way that matrix nestedness is maximized and a temperature T ranging from 0° (complete order) to 100° (complete disorder) is calculated. In an ordered dataset, every site contains a proper subset of the species at all of the sites above it. To determine the statistical significance of the observed T value, BINMATNEST provides three probability values, associated with different null models. The BINMATNEST is used in this study not for estimating nestedness temperatures of the populations but rather because it additionally provides two types of rankings through the final packed matrix: a) a ranking of species based on their nesting capacity and b) a ranking of sites based on species population nestedness.

We initially used BINMANTEST to produce ranking of native, exotic and all species grouped to identify priorities for species-specific conservation actions. Species at the top of the rank should be the most nested and widespread, thus at the lowest risk of extinction, while species at the bottom of the rank should be the most vulnerable and at the highest risk of extinction. Furthermore we produced also site rankings to prioritize site-specific conservation actions. For each site, two different nestedness rankings were calculated based on native and exotic species, which were then combined according to the following formula:

$$Snest_i = Inv\left(NR_{Ei}\right) + NR_{Ni} \tag{2}$$

where NR_E and NR_N are the nestedness rankings of sites from the packed matrix of “BINMATNEST” based only on exotic species or native species, respectively, and i is the sampling site number. Both NR_E and NR_N get values from 1 (highest nesting) to x (lower nesting), where x is the number of sampling sites. Inv(NR_E) inverts the NR_E ranking values from 1, 2 … x to x, ... 2, 1. Thus, if a site i has Inv(NR_E) = x and NR_N = x means that it has the most nested exotic and the least nested native populations. Thus, higher values of $Snest_i$ indicate a both higher nested exotic and low nested native population in site i and is used to describe the highest potential threat of widespread exotic species on less common native species, for the purposes of species-specific conservation. Lower values of $Snest_i$, on the other hand, identify sites where least nested exotic species coexist with some of the most nested native species, therefore probable targets of site-specific conservation measures. Using the outcomes of ordination analysis, we tested correlations between ranks and $Snest_i$ values with the main environmental gradient (i.e. altitude) using generalized linear models. Trying to account for some of the general conditions of the sites prior to sampling, which could have contributed in shaping the current species distribution, we also used average LIM values to test dependencies between eutrophication level and nestedness rank, through a Spearman Rank correlation.

As the purpose of nestedness analysis was to identify priority species and sites for conservation measures all species records were used, including those of rare species.

Results

Ordination methods and variance partitioning

Native species distribution presented substantially higher joint effects associated to geographical parameters and exotic species than vice-versa (Figure 2a). Exotic species abundance and distribution, on the other hand, was almost twice as uniquely affected by water quality parameters (i.e. temperature) (Figure 2b). Overall, native species distribution and abundance were more affected by joint effects of other variable groups (21.1 %), when compared to exotic species (6.2 %).

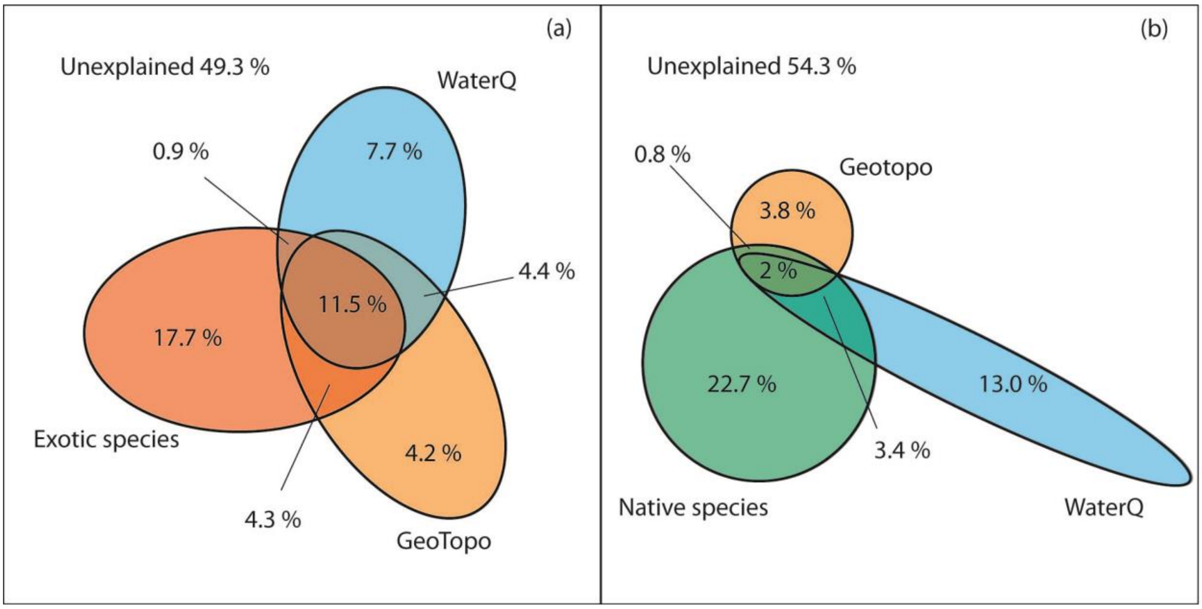

Figure 2 – Euler-Venn diagram of unique and joint effects of geographical (GeoTopo), water quality (WaterQ) and exotic species on native species distribution and abundance (a) and the reciprocal representation of the same effects for exotic species, using native species as explanatory variables (b). The numbers indicated the variance explained by each component.

Among geographical factors, CCA showed that altitude was a strong explanatory variable of both native and exotic species distribution (Figure 3). Marginal and conditional effects of exotic species on native species were still about one-third of the magnitude of altitude effects. Among water quality parameters, temperature was one of the strongest explanatory variables but other variables linked to eutrophication (COD and BOD) were also relevant. Ultimately, exotic species like crucian carp (*Carassius carassius*), common carp and common bream (*Abramis brama*) had the greatest negative effect on native species (see also Figure 4). Conversely, effects of native species were less directional but indicated that Italian rudd (*Scardinius hesperidicus*) and Italian bleak (*Alburnus alborella*) distribution and abundance had a positive effect on exotic species, whereas Italian chub (*Squalius squalus*) and Italian nase (*Protochondrostoma genei*) had a negative effect (Figure 3, Figure 4).

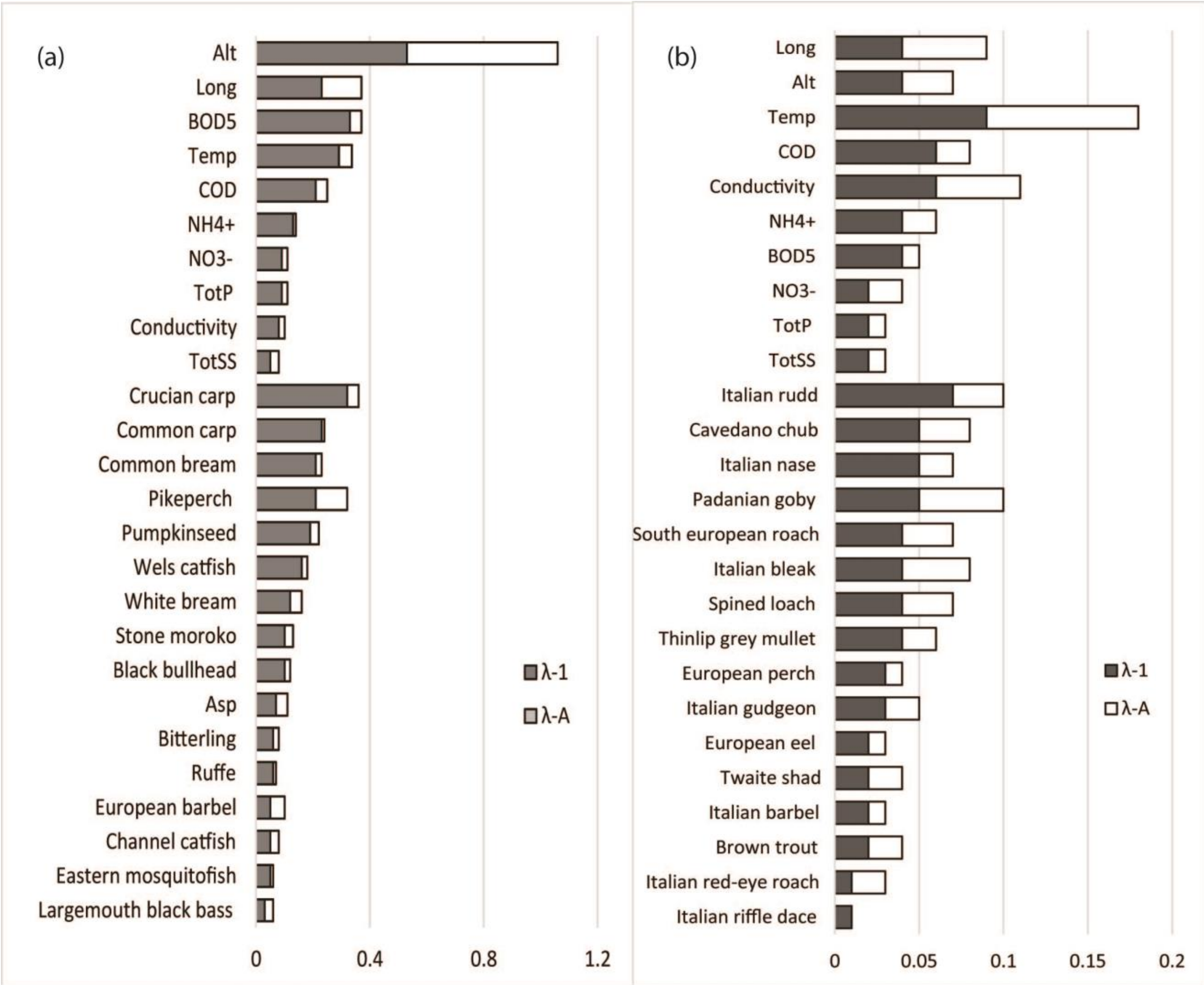

Figure 3 – Marginal (λ-1) and conditional (λ-A) effects of variables within variable groups that affect native (a) and exotic (b) species distribution and abundance. Bars are arranged according to a decreasing order of magnitude of marginal effects, by variable group (GeoTopo, WaterQ and exotic or native species).

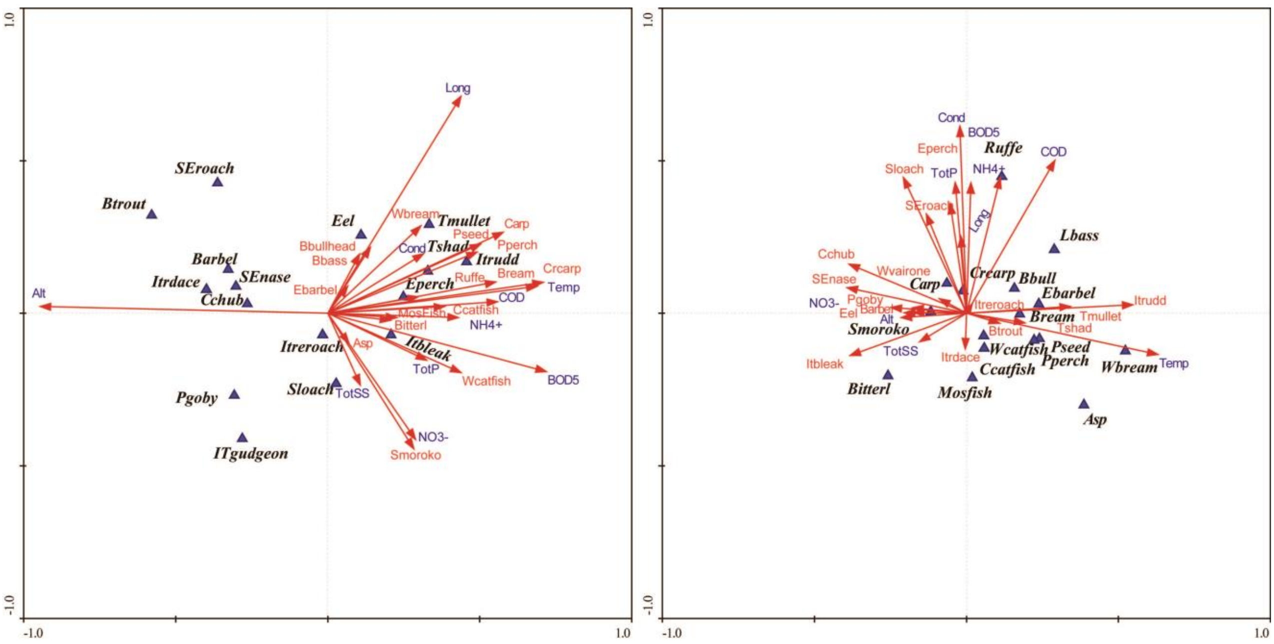

Figure 4 – Triplots of CCA ordination results showing the direction of combined effects of environmental variables (in blue) and exotic species on native species (in italics), on the left panel. The combined effects of environmental variables (in blue) and native species on exotic species (in italics) is shown on the right. Species are identified with codenames derived from contractions of their common names (see also Table 2).

TITAN

TITAN analysis revealed that several native species such as Italian bleak and Italian nase were distributed across the full range of altitudes in this sub-area (Figure 5a), in sympatry with equally widespread exotic species such as stone moroko (*Pseudorasbora parva*) and, to a lesser extent, other exotics (e.g. crucian carp, common carp, common bream, wels catfish). A temperature threshold was found close to 20 °C, with native species decreasing before the threshold and exotic species (with the exception of Italian rudd) preferring warmer waters, likely at lower altitudes (Figure 5b). Ultimately, TITAN analysis showed that only Italian bleak and Italian rudd coexisted with all densities of crucian carp, while a group of native species such as South European roach (*Sarmarutilus rubilio*), Padanian goby (*Padogobius bonelli*), Italian barbel (*Barbus plebejus*) and chub clearly decreased in presence of higher densities of crucian carp (Figure 5c).

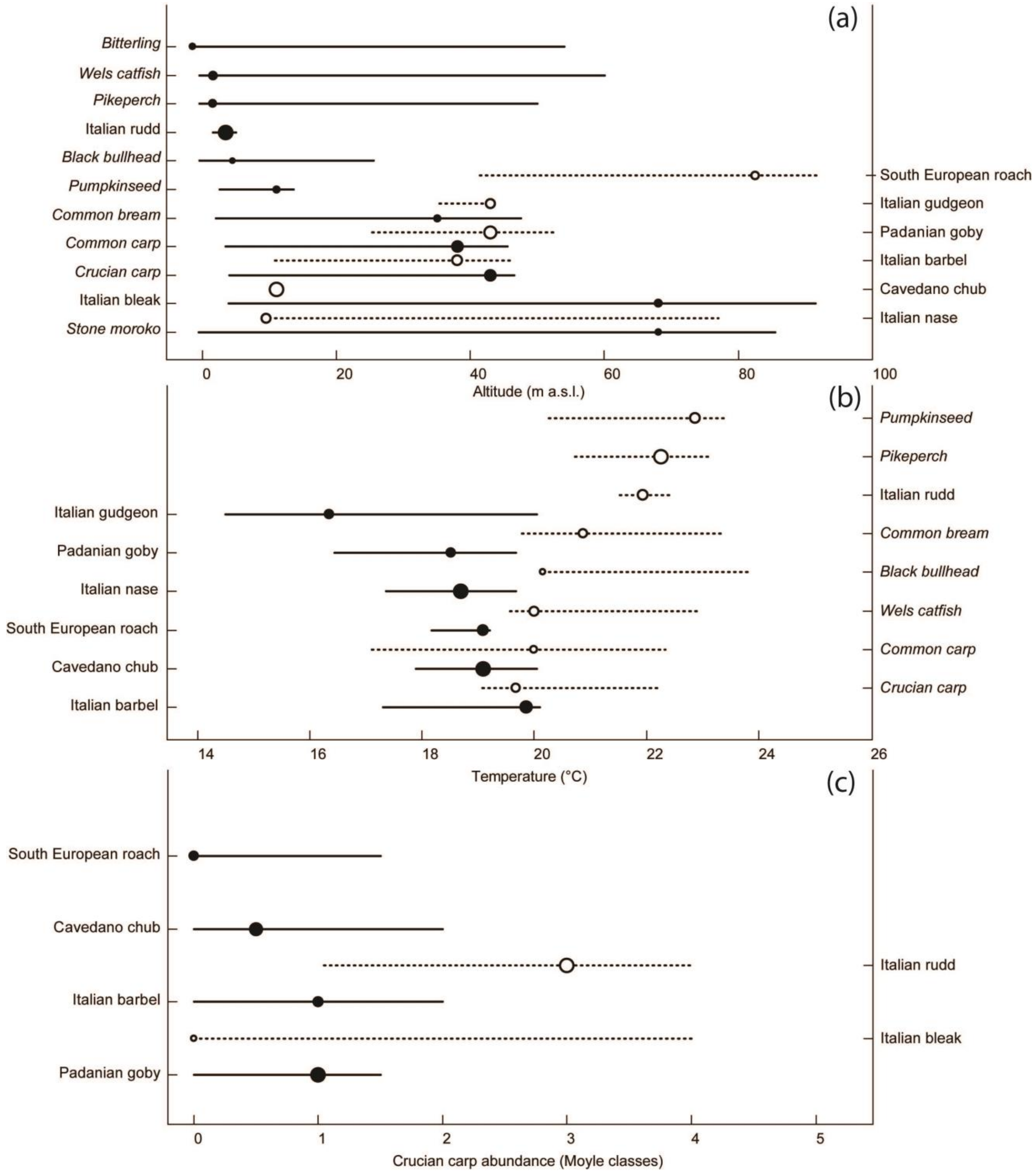

Figure 5 - Losses and gains of both exotic (in italics) and native species along altitude (a), and temperature (b), as well as losses and gains of native species along distribution/abundance of crucian carp (c), according to the TITAN analysis. Lines represent species distribution across the gradient, with circles identifying the distribution peak. Solid lines and circles represent species that decline along the gradient, while dashed lines and empty circles represent species which

distribution increases along the gradient. Species not shown in these figures have a distribution not predictable according to the environmental gradients examined.

Nestedness analysis

Overall, all-inclusive nestedness analysis revealed that Italian bleak, crucian carp, common carp, stone moroko and wels catfish were the most widespread species and coexisted in some sites (Table 2). Italian bleak was the native species the least affected by exotic species presence. Lowest ranking species were the least widespread, including occasional saltwater species (e.g. European flounder, *Platichthys flesus*), threatened native species (e.g. tench, *Tinca tinca*), least successful exotics (e.g. roach, *Rutilus rutilus*).

Among native species, Italian bleak, Italian rudd and chub were the most widespread and clustered together, while brown trout, thinlip grey mullet and European flounder where the least widespread and found only occasionally and never together. However, some of the most threatened native species (e.g. tench or southern pike) did not rank at the lowest places. Among exotic species, stone moroko, crucian carp and common carp were the most widespread and formed the backbone of exotic assemblages in several sites. At the other end of the scale, roach, white bream (*Blicca bjoerkna*) and grass carp (*Ctenopharyngodon idella*) were the least widespread.

Table 2 – Family, scientific name and common name of fish species in the sub-area where native and exotics overlap. The table includes their native or exotic status (N/E) and the number of sites within the sub-area where each species was present (#S). RankN represents the rank calculated for native species alone, RankE the rank calculated for exotic species alone and the OverallR value represents the global rank (considering all species). Bold values underline the first and last 5 species of each ranking.

Family	Species	Common Name	N/E	#S	RankN	RankE	OverallR
Anguillidae	*Anguilla anguilla* (Linnaeus, 1758)	European eel	N	9	8		17
Clupeidae	*Alosa fallax* (Lacépède, 1803)	Twaite shad	N	8	9		26
Cyprinidae	*Sarmarutilus rubilio* (Bonaparte, 1837)	South European roach	N	8	11		22
	Leucos aula (Bonaparte, 1841)	Italian red-eye roach	N	1	12		21
	Squalius squalus (Bonaparte, 1841)	Cavedano chub	N	32	**3**		6
	Squalius lucumonis (Bianco, 1982)	Toscana stream chub	N	2	**17**		31
	Telestes muticellus (Bonaparte, 1837)	Italian riffle dace	N	3	**18**		27
	Tinca tinca (Linnaeus, 1758)	Tench	N	1	16		**39**
	Scardinius hesperidicus Bonaparte, 1841	Italian rudd	N	30	**2**		8
	Alburnus arborella (Bonaparte, 1841)	Italian bleak	N	74	**1**		**1**
	Protochondrostoma genei (Bonaparte, 1841)	South-european nase	N	20	**4**		13
	Romanogobio benacensis (Pollini, 1816)	Italian gudgeon	N	9	10		19
	Barbus plebejus Bonaparte, 1839	Italian barbel	N	21	6		14
	Barbus barbus Linnaeus, 1758	European barbel	E	3		**16**	30
	Carassius spp. (Linnaeus, 1758)	Crucian carp	E	67		**3**	**4**
	Cyprinus carpio Linnaeus, 1758	Common carp	E	76		**2**	**2**
	Abramis brama (Linnaeus, 1758)	Common bream	E	30		6	10
	Blicca bjoerkna (Linnaeus, 1758)	White bream	E	4		**17**	29
	Rutilus rutilus Linnaeus, 1758	Roach	E	1		**19**	**40**
	Rhodeus sericeus (Pallas, 1776)	Bitterling	E	26		**5**	7
	Pseudorasbora parva (Temminck & Schlegel, 1846)	Stone moroko	E	70		**1**	**3**
	Ctenopharyngodon idella (Valenciennes, 1844)	Grass carp	E	2		**18**	33
	Leusciscus aspius (Linnaeus, 1758)	Asp	E	6		14	28
Cobitidae	*Misgurnus anguillicaudatus* (Cantor, 1842)	Pond loach	E	1		**15**	**36**
	Cobitis bilineata Canestrini, 1865	Italian spined loach	N	14	**5**		15
Siluridae	*Silurus glanis* Linnaeus, 1758	Wels catfish	E	43		**4**	**5**
Ictaluridae	*Ameiurus melas* (Rafinesque, 1820)	Black bullhead	E	18		9	12
	Ictalurus punctatus (Rafinesque, 1820)	Channel catfish	E	4		13	24

Esocidae	*Esox cisalpinus* Bianco & Delmastro, 2011	Southern pike	N	1	15		34
Salmonidae	*Salmo trutta* complex	Brown trout	N	2	**19**		32
Poeciliidae	*Gambusia holbrooki* (Girard, 1859)	Eastern mosquitofish	E	5		12	20
Centrarchidae	*Micropterus salmoides* (Lacépède, 1803)	Largemouth black bass	E	9		11	23
	Lepomis gibbosus (Linnaeus, 1758)	Pumpkinseed	E	23		7	11
Percidae	*Perca fluviatilis* Linnaeus, 1758	European perch	N	3	13		25
	Gymnocephalus cernua (Linnaeus,1758)	Ruffe	E	9		10	18
	Sander lucioperca (Linnaeus, 1758)	Zander or Pike-perch	E	27		8	9
Mugilidae	*Liza ramada* (Risso, 1827)	Thinlip grey mullet	N	3	**20**		**38**
Gobiidae	*Padogobius bonelli* (Bonaparte, 1846)	Padanian goby	N	16	7		16
Pleuronectidae	*Platichthys flesus* (Linnaeus, 1758)	European flounder	N	1	**21**		**37**

Sites with highest nestedness in terms of native species hosted communities formed by the most common and clustered species (Italian bleak, Italian rudd and chub) whereas the lowest ranking sites hosted only one native species, albeit common (i.e. Italian bleak). Sites on the top of the exotic species rank hosted the most widespread exotic species, whereas those at the bottom of the rank were those least invaded.

The results of site nestedness analysis based on native and exotic species correlated significantly with altitude, showing a positive correlation for native species (Correlation Coefficient = 0.605, P-value < 0.05) and a negative one for exotic species (Correlation Coefficient = -0.457, P-value < 0.05) (Figure 6a). The sites with lowest $Snest_i$ values had the highest native rank and the lowest exotic species rank; the $Snest_i$ values correlated negatively with altitude of the site (Figure 6b, Correlation Coefficient = -0.686, P-value < 0.05). Despite the relatively small altitude gradient, there was a significant negative correlation between eutrophication levels expressed through average LIM and altitude (Figure 6c, Correlation Coefficient = 0.522, P-value < 0.05).

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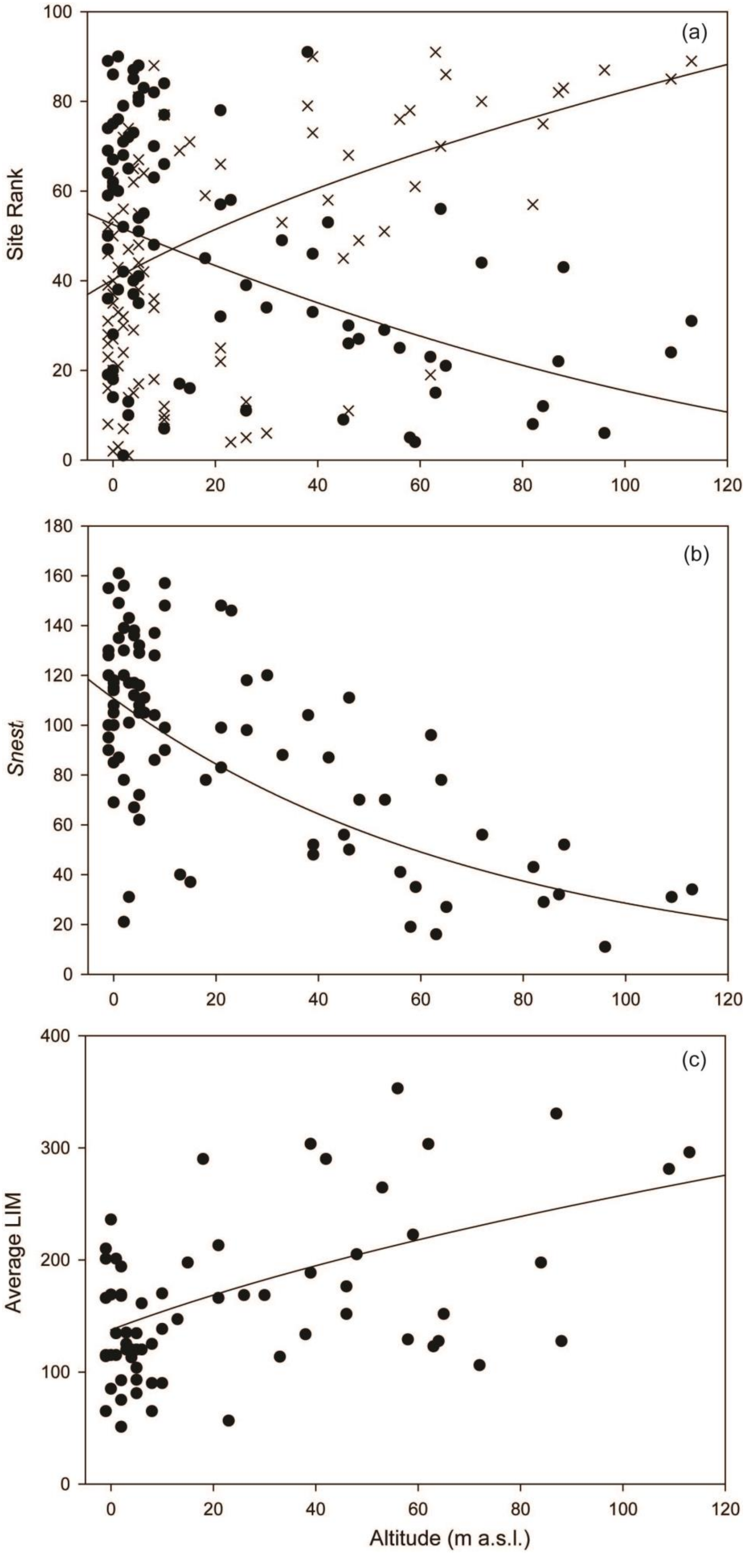

Figure 6 – Relations between altitude and (a) rank of sites based on native (full circles) and exotic species (crosses), (b) $Snest_i$ values, and (c) average LIM values. Solid lines represent significant regression curves.

The results of site nestedness analysis based on all species did not correlate with average eutrophication index LIM (P-value > 0.05). However, ranking of sites based on exotic species alone had a slight positive but not significant correlation with average LIM (Spearman rank corr = 0.1, P-value > 0.05) whereas ranking of sites based on native species alone had a significant negative correlation with average LIM (Spearman rank corr = -0.3, P-value < 0.05).

Discussion

At lower altitudes both native and exotic species coexist, but assemblages were dominated by exotic species, revealing a marked altitudinal effect despite the relatively small gradient (~ 120 m) while native fish species in the study area were mostly located at higher altitudes. Confirming our initial hypothesis, ordination methods revealed that exotic species still had strong effects on native species abundance and distribution but, perhaps counterintuitively, underlined a similar size of unique and joint effects of exotics on natives. This is likely because the very few native species which are still present in this area are those least affected by exotic species and eutrophication, which was confirmed by all analyses. Our nestedness analysis was useful in identifying sites which could be priority targets of conservation measures, but less so with species. Ultimately, careful consideration needs to be given to the outputs of this analysis prior to the elaboration of conservation plans. Before invasion, numerous native species were present in the area, including the lowlands (Castaldelli et al., 2013), but were pushed to higher altitudes, with cooler and less eutrophic water, by a combination of factors where exotic species likely played a major role.

Ordination methods and variance partitioning

Native species abundance and distribution seemed to be largely driven by the presence of exotic species jointly with water quality and geographical factors. The large joint effect of geographical factors (over 20% versus a 4.2% of unique effects) is very likely a product of exotic species pressure and water quality pressure that pushed several natives' distribution areas towards the upstream part of the rivers. However, our analysis focused on a late invasion stage, thus not detecting the full effects of exotic species at the invasion peak, but rather the product of those effects. Therefore, the results of our analysis underline the outcome of past interactions, which have driven down and away native species unable to cope with the presence of exotic ones, combined with other environmental factors. The few native species in this zone of overlap with exotic species, are clearly the most resilient to interactions with the exotics, which likely explains the similar size of unique and joint effects of natives on exotics (and vice-versa).

Water quality is a good example of the complex interactions having a strong unique effect on exotic species distribution. The most abundant and widespread exotic species thrive in eutrophic waters and some of these are known to affect water quality by increasing e.g. eutrophication and turbidity through sediment resuspension (Bonneau & Scarnecchia, 2015; Richardson, Whoriskey, & Roy, 1995) thus creating a positive feedback cycle. That different exotic species could mutually facilitate each other in the invasion is not a new hypothesis (Simberloff & Von Holle, 1999), but it remains somewhat controversial (Simberloff, 2006), and it could well be that exotic species would have complex interactions, both positive and negative, with native species and other exotics (Goodenough, 2010). In most invaded sites, exotic fish communities include predators such as pikeperch and wels catfish, and their prey:crucian and common carp, common bream and few other smaller bodied cyprinids. Pikeperch and wels catfish are adapted for predation in turbid waters (Ali, Ryder, & Anctil, 1977; Bruton, 1996) while common bream, crucian and common carp are ecosystem engineering species that increase turbidity and eutrophication and are likely to have co-evolved specific predator avoidance mechanisms (Bonneau & Scarnecchia, 2015; Castaldelli et al., 2013; Richardson et al., 1995). Ultimately, the outcome after almost 30 years of invasion is a homogenization of the fish fauna, with communities that occasionally include native

North-American species (introduced much earlier), but are mostly composed by species native of the Danube River drainage, similar to what underlined by Castaldelli et al. (2013) in a smaller section of this area. The “Danubification” of the Po River could be a good example of faunal homogenization and invasional meltdown in fish communities of which, so far, very few examples are known (e.g. the opposite effect found inBritton, Harper, Oyugi, & Grey, 2010).

Assessment of environmental thresholds – TITAN analysis

The results of ordination analysis cannot be interpreted without considering other factors, but can help direct further investigations. Considering a combination of the results of ordination methods and TITAN it was clear that altitude in itself could not be the sole limit for the expansion of exotic species, but temperature should be also accounted for. Water temperature generally correlates negatively with altitude and, to a limited extent, this was true even over the limited altitude gradient present among our sampling sites (see e.g. Figure 3a). However, it must be noted that native species distribution in the lowlands was not limited by temperature, before the exotic invasion (Castaldelli et al., 2013). Temperature might still play a relevant role, as some exotic species could lose part of their competitive edge at cooler temperatures (e.g. crucian carp, Roberts (1966); Vornanen, Stecyk, and Nilsson (2009)). Other factors, not accounted for in the analysis, such as the presence of migration barriers (i.e. weirs or dams), could further explain the distribution of alien and native species and likely contribute to the distribution pattern emerging from our data (Rolls, 2011). Furthermore, it could be worth to consider that our analysis worked on numerical abundances rather than biomass. Biomass is usually considered to give a stronger representation than numerical abundance, when analyzing ecological patterns in animal and plant communities, as it reflects more directly the allocation of energy among the species (Abrahamson & Caswell, 1982; Sprules & Munawar, 1986; Strayer, 1986), especially given the large size span of most fish communities. As an example, the native species coexisting with exotic in invaded sites were small bodied cyprinids, such as Italian bleak, which were not numerically abundant and therefore likely to constitute an even smaller part of the fish communities’ biomass. Overall, this further strengthens the loss of native biota (i.e. in terms of native biomass as well as number of species) revealed by our results.

Exotic species clearly distributed in sites with a wide range of eutrophication levels (summarized by the LIM index), showing that these species that were not significantly affected by eutrophication. This indirectly confirmed that the most widespread exotic species, if anything, could thrive in eutrophic habitats. While it is possible that native species were negatively affected by eutrophication, as some of them were poorly distributed in sites at higher eutrophication levels, it is also true that eutrophication levels correlated with altitude (Figure 6c). Therefore, it might not be possible to fully disentangle the interplay of these two factors. However, it is interesting to note that altitude had a stronger effect on native species distribution than any single parameter of water quality related to eutrophication (Figure 3a), which strongly suggests that eutrophication could be a lesser factor than exotic species in regulating native species distribution. This strengthens and broadens the conclusions drawn in Castaldelli et al. (2013), where water quality did not seem to play a strong role in native species decline. The notion that exotic species play a minor role in threatening biodiversity (e.g. Davis, 2003), seems to be inapplicable to our study area.

Nestedness analysis

The few native species still present in eutrophic sites were widely distributed and might not deserve species-specific conservation efforts. Conversely, species at the bottom of the native species ranking might be more plausible species-specific conservation targets, as these species were found only in few sites. However, to further implement a management strategy, further checks need to be performed on adjoining sites, both to verify whether these species could be surviving in adjoin areas and to identify surviving populations to use in artificial breeding and restocking. In our case, unfortunately, native species such as tench or southern pike could be found only in one site and are thus at the highest risk of regional extinction. However, they were not ranked among the lowest scoring species because brown trout (a species typical of higher altitudes, likely at the edge of its distribution area), thinlip grey mullet and European flounder (two species typical of transitional waters, and thus rare in freshwater) scored even lower. This signals that this analysis outputs could not be immediately used, but need further careful consideration as to the geographical range and number of species included. In fact, endangered native species ranked in the middle range when considering solely native species, because they co-occurred with very common native species (e.g. Italian bleak). A more appropriate ranking of these species for conservation purposes was obtained when considering exotic and native species together in the analysis.

In principle, nestedness analysis based on native species distribution should have identified sites where the least widespread and clustered native species can be found. If native species are also not abundant at these sites, these would be the next likely locations of local extinctions. However, as mentioned above, since the rarest native species (i.e. tench, southern pike, Toscana stream chub and Western vairone) often occurred together with some of the most widespread species (i.e. Italian bleak, Italian rudd or chub) these sites were not ranked at the bottom. Perhaps counterintuitively, the least ranked sites contained the most widespread and clustered species (i.e. Italian bleak). Yet these sites were correctly identified as some of the primary targets of site-specific conservation, as they will be easily lost to a full exotic community if, for any cause, that single native species is lost. Therefore, while the single native species is of no particular conservation value in itself, as it is in no danger of extinction on a large scale, the site probably deserves some attention because it is at higher risk of local total extinction of native species. This is what has already occurred in some of those sites since the last sampling in 2005 (Lanzoni, unpublished data). However, further consideration needs to be given also to this ranking, as there could be discrepancies and valuable lessons learned from a more holistic perspective.

In a world where conservation resources are limited and need to be optimally allocated to obtain the most significant results per effort, we further used $Snest_i$ as a measure of balancing conservation priorities taking into account both native and exotic species distribution. Sites with lowest $Snest_i$ could be identified as primary conservation targets, as they host robust native communities (highest native ranks) and weak exotic communities (lowest exotic ranks). These sites did not fully correspond to those identified using either native or exotic species alone and could be considered as an optimal sub-group. In these sites, site-specific conservation actions could be most effective and yield the highest success rate or results-per-effort. However, this is a method to devise a-posteriori actions, which face the struggle to reverse a situation that is already compromised and therefore might not be considered acceptable by natural resources managers and the public at large. Precautionary action could be always more advisable, bearing in mind the challenges of managing exotic species include also a time lag (Crooks, 2009). Furthermore, ecosystem services evaluations might help to underline that the gap between socio-economic and biodiversity conservation goals could be narrower than commonly thought (Nelson et al., 2009).

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