



## Non-native minnows cause much larger negative effects than trout on littoral macroinvertebrates of high mountain lakes

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### ABSTRACT

Despite being naturally fishless, the widespread introduction of trout and minnows is threatening the conservation of high mountain lakes all over the world. Previous studies have reported that amphibians quickly disappear after trout introduction, followed by many conspicuous invertebrates. Here, we have studied the effects of minnows versus trout on the littoral macroinvertebrate community of 54 high mountain lakes from the Pyrenees, covering a gradient of environmental characteristics. The relative importance of fish compared to other variables in explaining macroinvertebrate communities was assessed using distance-based redundancy analysis (dbRDA) and multivariate regression tree (MRT) to find the main environmental thresholds. Both dbRDA and MRT approaches revealed that minnow density was the most important variable negatively determining community structure, followed by aquatic macrophytes, which increased taxa richness. The occurrence and abundance of relevant taxa was analysed in relation to fish densities and other environmental factors using binomial and gamma generalized linear models (GLM). GLM suggested that trout had an impact on the distribution of swimmer taxa and caused declines in the abundance of conspicuous clinger and burrower taxa. Minnows restricted the occurrence of more taxa than trout and negatively affected a wide variety of body sizes and functional groups. Indeed, we found that minnows were responsible for a dramatic biodiversity loss in the littoral macroinvertebrate community. The fast spread of minnows in high mountain areas is of great concern for the conservation of lake macroinvertebrates. Urgent measures to stop minnow introductions are strongly recommended.

### 1. Introduction

Invertebrate declines have been documented worldwide and are equally or more severe than in vertebrates (Cowie et al., 2022). Moreover, there exist important spatial and taxonomic biases in biodiversity assessments that are evidenced by the underrepresentation of this animal group in the IUCN Red List (only 2 % of the 1,305,250 species were assessed in 2018; Eisenhauer et al., 2019). Particularly, insects have been reported to suffer large losses in terrestrial and aquatic groups, and across ecological guilds, which could cascade onto ecosystem functioning and human well-being (Sánchez-Bayo and Wyckhuys, 2019; Wagner, 2020).

Among the biomes of the planet, freshwaters have suffered the strongest decline in biodiversity, as proved by the recent decline in the

number of vertebrate species (81 % in freshwaters compared to 38 % in terrestrial and 36 % in marine biomes; WWF, 2016). This great decline is a result, on a global scale, of factors related to habitat destruction, pollution, water-level reduction and invasive species among others (Collen et al., 2014). Fish are among the most common animal invaders threatening freshwaters (Hulme et al., 2009). Nowadays, many high mountain lakes around the world have suffered from the introduction of trout and other fish species for angling purposes (Wiley, 2003; Miró and Ventura, 2013; Ventura et al., 2017). Along with trout, minnows (mainly *Phoxinus* genus) have also expanded considerably throughout European mountains during the 20th century due to their use as live bait by anglers (Museth et al., 2007) and remain as the only fish when trout disappear (Miró and Ventura, 2015). The widespread presence of minnows has been documented not only in the north European lakes of

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Scotland and Norway (Maitland and Campbell, 1992; Museth et al., 2007), but also in the Pyrenees (Miró and Ventura, 2015) and the Alps (Tiberti et al., 2020).

The introduction of trout in high mountain lakes has been responsible for the decline or elimination of native fauna in many regions, including amphibians, large crustaceans, and conspicuous littoral macroinvertebrates (Knapp et al., 2001; Tiberti et al., 2014; Miró et al., 2018; Toro et al., 2020). As a result, the direct predation of more visible taxa can produce a series of indirect ecological effects (trophic cascade) affecting the entire ecosystem (Schindler et al., 2001; Eby et al., 2006). On the other hand, the effects of minnow introduction have scarcely been studied in comparison with those of trout. Regardless of their smaller size, minnows share a similar diet with trout, thus becoming the top predators when established in fishless lakes (Hesthagen et al., 1992). They seem to have a strong impact on both amphibians and zooplankton communities (Schabetsberger et al., 2006; Miró et al., 2018), and the evidence from boreal lakes suggest that a negative effect may be expected on macroinvertebrates, as minnows were found to feed on conspicuous taxa (Museth et al., 2010). However, there are no studies describing their effects on the conservation of the macroinvertebrate community of high mountain lakes.

In comparison with other freshwater ecosystems, high mountain lakes have littoral macroinvertebrate communities with relatively low species richness (Füreder et al., 2006). Their taxa, though, play an important role in lake food-web stability via their diverse feeding strategies and the magnified importance of the littoral zone in small lakes (high perimeter to surface area ratio) (Sánchez-Hernández et al., 2015). These communities comprise species well adapted to harsh environmental conditions, which are sensitive to local and global anthropogenic impacts. Indeed, groups like chironomids and caddisflies have been recognised as good indicators of climatic change in mountain areas (Ciamporová-Zaťovičová et al., 2010). The current study aimed to compare the effects of minnows versus trout introduction on the littoral macroinvertebrate community of high mountain lakes. Specifically, our objectives were: (i) to determine the relative importance of non-native fish introductions in explaining the macroinvertebrate community compared to other environmental factors, (ii) to evaluate how differently minnow and trout densities affect both macroinvertebrate community composition and abundance, and (iii) to ascertain whether the impact of minnows on the conservation of macroinvertebrate community is equally important as that of trout.

## 2. Materials and methods

### 2.1. Study area

We surveyed 54 high mountain lakes and ponds from the Central-Eastern Pyrenees (0°52'E–1°22'E, 42°46'–42°31'N), located within the area of the Aigüestortes i Estany de Sant Maurici National Park and the Alt Pirineu Natural Park, which were both designated as Sites of Community Interest by the European Commission's Natura 2000 conservation program (Fig. A.1). In addition, high mountain lakes are protected habitats listed in the European Directive 92/43/EEC for the conservation of natural habitats and of wild fauna and flora (Council of the European Communities, 1992). The lakes sampled were relatively small and shallow water bodies (area range: 0.03–8 ha, median area: 1.9 ha; maximum depth range: 0.6–51.4 m, median maximum depth: 7.1 m). A more detailed description of Pyrenean lake characteristics is provided in the online Appendix A.

We distributed the lakes into three nominal categories according to the fish species present in them: naturally fishless lakes ( $n = 22$ ), lakes with trout only ( $n = 15$ ) and lakes with minnows ( $n = 17$ ), of which six had both trout and minnows. The most common trout species found in the lakes was *Salmo trutta* ( $n = 14$ ), whereas *Salvelinus fontinalis* ( $n = 4$ ) and *Oncorhynchus mykiss* ( $n = 4$ ) were less frequent. The coexistence of two trout species, *S. trutta* and *O. mykiss*, was observed in only two lakes

(Lakes Llastra and Coveta). Selection of lakes was made to compose a set of sites as similar as possible to each other in their physical and geographic characteristics but at the same time, covering a gradient of small to medium size. As a result, the categories comprised similar replicates in terms of lake morphology and aquatic habitats availability for the different fish categories.

### 2.2. Macroinvertebrate samples

Lakes were sampled once during July–August to make collected data comparable over the years, with 26 lakes sampled in 2014, 6 in 2015, 11 in 2016, 8 in 2017, and 3 in 2018. The one-time sampling approach is thought to successfully characterize the summer littoral macroinvertebrate community, as most mountain species have relatively long life cycles and larval development periods. As a result, species composition and richness change little over the summer (Knapp et al., 2001; Tiberti et al., 2014). Littoral macroinvertebrates were sampled from representative shore-accessible habitats at ca. 80 cm depth using a d-frame net with a mesh size of 250  $\mu\text{m}$ , employing commonly used sampling techniques (de Mendoza and Catalan, 2010; Tiberti et al., 2014). Collected material was pooled in a unique composite semi-quantitative sample per lake, and preserved in the field in absolute ethanol. Sampling methods are extensively described in the Supporting Information. Macroinvertebrates were counted under the stereo microscope (Olympus SZH, Japan) and determined to the lowest practical taxonomic unit (typically genus or species), with the exception of Hydrachnidia, Oligochaeta and Palpomyiini (Ceratopogonidae) specimens, that were counted only. For Chironomidae, the used taxonomic resolution was tribe or subfamily rank. Abundance per sample was expressed as total individuals  $\text{m}^{-2}$  preceding any statistical treatment.

### 2.3. Environmental data

The environmental descriptors measured to understand the factors shaping littoral macroinvertebrate community included water chemistry, morphometric, physical and biological lake variables, as well as catchment and geographical descriptors.

Water chemistry samples were taken from the lakes' outlet, or sub-superficially at the deepest point from an inflatable boat when there was no active outlet. Laboratory analyses of pH, conductivity, alkalinity, total nitrogen, dissolved inorganic nitrogen and total phosphorus were performed following the procedures described by Ventura et al. (2000). Water samples for chlorophyll-*a* (Chl-*a*) were taken from the deepest point of the lake at a depth of 1.5-times the Secchi depth, using a UWITEC-type sampling equipment. In those lakes where the Secchi disk bottomed out the samples were taken between 1 and 2 m above the sediment. From the collected samples, a known volume of water (between 1.5 and 2 L) was filtered using a manual vacuum pump and GF/F filters (47 mm in diameter). The filters were wrapped in aluminium foil and kept cold before freezing ( $-20\text{ }^{\circ}\text{C}$ ) within 3–6 h. Chlorophyll-*a* was determined using the spectrophotometric method of Jeffrey and Humphrey (1975).

Fish density in lakes was measured by the installation of multi-mesh gill nets (CEN 14757:2015). The number of nets placed in each lake was decided depending on its area (from 2 to 6 nets in a lake between 0.4 and 6 ha, distributed among different depths). They were kept in the lakes ca. 20–24 h, before counting and weighting all fish captured. The catches of trout (Salmonidae family) and minnows (*Phoxinus* sp.) in gill nets were used to produce the standardized values of catch per unit effort (CPUE) and biomass per unit effort (BPUE). CPUE was expressed as individuals  $\text{net}^{-1} \text{day}^{-1}$  for trout and minnows, whereas BPUE was expressed as  $\text{kg net}^{-1} \text{day}^{-1}$ . The density of minnows expressed as individuals  $\text{net}^{-1} \text{day}^{-1}$  can be roughly converted to individuals  $\text{trap}^{-1} \text{day}^{-1}$  using the regression equation  $Y = 0.77X + 39.22$  ( $R^2 = 0.32$ ), which resulted from fitting a linear model on data available from 18 lakes with minnows (data not shown).

Due to unavailability of data, CPUE and BPUE estimates of three lakes (namely Bassa Cloto de Baix-Minnow, Estanyet 4 Gargolhes Inferior-Trout, and Lõssa Mig-Minnow/Trout) were obtained from other similar lakes or averaging over similar lakes, according to expert criteria. Bassa Cloto de Baix values were obtained by averaging over the rest of lakes with minnows. The values of Estanyet 4 Gargolhes Inferior came from Gargolhes Inferior, while the ones of Lõssa Mig came from Xic, as both pairs of lakes share similar size and habitat diversity.

Water transparency was measured by estimating the light extinction coefficient in the water column ( $K_d$ ;  $m^{-1}$ ) from the Secchi disk depth measurement. In lakes where the Secchi disk reached the bottom, a constant extinction coefficient of  $0.2 m^{-1}$  was used (Buchaca, 2005). It was not possible to measure Secchi disk depth at Lake Lõssa Mig, where a  $K_d$  value estimated from the similar Lake Obago d'Amunt was used. Lake maximum depth, littoral substrate composition, littoral macrophytes percentage, and percentage of lithological and vegetation units in the catchment were measured in the field. The abundance of amphibians along the shoreline ( $ind. m^{-1}$ ) was estimated by visual encounter surveys (Miró et al., 2018). Additionally, the density of animal manure was counted in a 20 m wide area along the shoreline, obtaining a rough estimate of local livestock pressure (excrements  $m^{-1}$ ). Additional morphometric parameters (altitude, lake area, total catchment area) and geographical coordinates (easting and northing) were determined using cartographic information.

#### 2.4. Statistical methods

We used an exploratory non-parametric Kruskal-Wallis test and post-hoc tests with Bonferroni correction (level of significance was  $\alpha = 0.017$ ) to examine whether the richness of macroinvertebrates taxa differed among the three categories, i.e. fishless, trout, and minnow lakes. Lakes with both trout and minnows were included within minnow category since minnows are always present in the littoral zone either with or without trout. The same approach was used to compare for alpha, beta, and gamma diversity measures in the samples, which were calculated on rarefied subsets of lakes (30 permutations of nine randomly chosen lakes at a time) and employing the adaptation of common Shannon entropy index suggested by Jost (2007).

To shed light on the main patterns of variation in the community, we employed distance-based redundancy analysis (dbRDA) ordination (Legendre and Anderson, 1999). The response matrix consisted of Bray-Curtis distances on log-transformed taxa abundance. As a preliminary step, the explanatory variables that could potentially influence the community were classified into four groups, describing: i) spatial variation (space-dBRDA), which comprised 22 Moran's eigenvector maps (MEMs) with significant positive spatial autocorrelation and generated employing relative neighbourhood graph connectivity (Peres-Neto et al., 2006); ii) catchment properties (catchment-dBRDA), including 10 descriptors of catchment features, in addition to lake altitude and lake geomorphology, iii) in-lake habitat (inlake-dBRDA), consisting of 12 variables describing water physicochemical condition, littoral substrate, and littoral macrophytes dominance, and; iv) vertebrate interaction (vertebrate-dBRDA), composed by 6 variables describing predator and competitive interaction with vertebrates, among which CPUE and BPUE of trout and minnows, as well as tadpoles and newts abundance.

Using each group of explanatory variables, we firstly produced four dbRDA models, namely space-dBRDA, catchment-dBRDA, inlake-dBRDA, and vertebrate-dBRDA. All variables were normalised and standardised before entering the ordination algorithm. Searching for parsimony and preventing the problem of inflation of the overall type I error, we ran forward selection with the Blanchet et al. (2008) double stopping criterion on the above-mentioned models ( $P < 0.05$ , 9999 randomizations). The variables selected were incorporated into the full dbRDA model (full-dBRDA), that was examined using variation partitioning analysis by means of partial dbRDA to estimate pure and shared effects of groups of variables by computing adjusted  $R^2$ . Ultimately, a

forward selection applied on the full-dBRDA produced the parsimonious dbRDA (pars-dBRDA), which was explored for linear dependencies employing variance inflation factors (VIF).

To highlight local structures in macroinvertebrate community, we used a multivariate regression tree (MRT) on log-transformed taxa abundance (Bray-Curtis distance, 1000 multiple cross-validations) (De'Ath, 2002). Explanatory variables in the pars-dBRDA were passed to MRT as candidates to be explored. The number of clusters included in the tree were defined by the solution that minimized the cross-validated relative error (CVRE). We subsequently determined taxa significantly associated to groups of sites or combinations of those by means of the indicator species analysis suggested by De Cáceres et al. (2010).

We investigated the impact of fish density (minnows: Minnow\_CPUE; trout: Trout\_CPUE) on invertebrate taxa representative of different body sizes and functional groups according to their propensity and frequency of movement within the habitat (Table A.1) by generalised linear models (GLM). To control for additional sources of variability, we also considered other biologically relevant variables identified in pars-dBRDA, such as littoral macrophyte cover, altitude and lake area. The probability that a taxon was found in the samples was assessed by a binomial GLM (logit link function) on presence-absence data. Subsequently, the non-zero observations from the dataset were modelled using a zero truncated gamma GLM (log link), assessing the relevant variables driving its abundance when the taxon was present in lakes. Those taxa with low occurrences were aggregated to a higher taxonomic rank (always within the same functional group; Table A.1) to ensure sufficient cases. If a taxon was found in >48 of the 54 lakes (e.g. ubiquitous groups such as Oligochaeta and some Chironomidae taxa), only the gamma GLM was fitted, since there was not enough variation in the response variable to run the binomial model. All GLMs were initially fitted including the following predictors and interaction terms: Minnow\_CPUE, Trout\_CPUE, macrophyte cover, altitude, area, Minnow\_CPUE  $\times$  macrophyte cover, Trout\_CPUE  $\times$  macrophyte cover, Minnow\_CPUE  $\times$  Trout\_CPUE. Macrophyte cover was previously transformed using the inverse-sine function, and the square root transformation was applied on Trout\_CPUE and Minnow\_CPUE. A backward stepwise selection based on AIC (Akaike's Information Criterion) was used to find the best subset of terms for each taxon and GLM model separately. We determined the relative weight of terms by dropping them from GLM models and evaluating the resultant change in deviance.

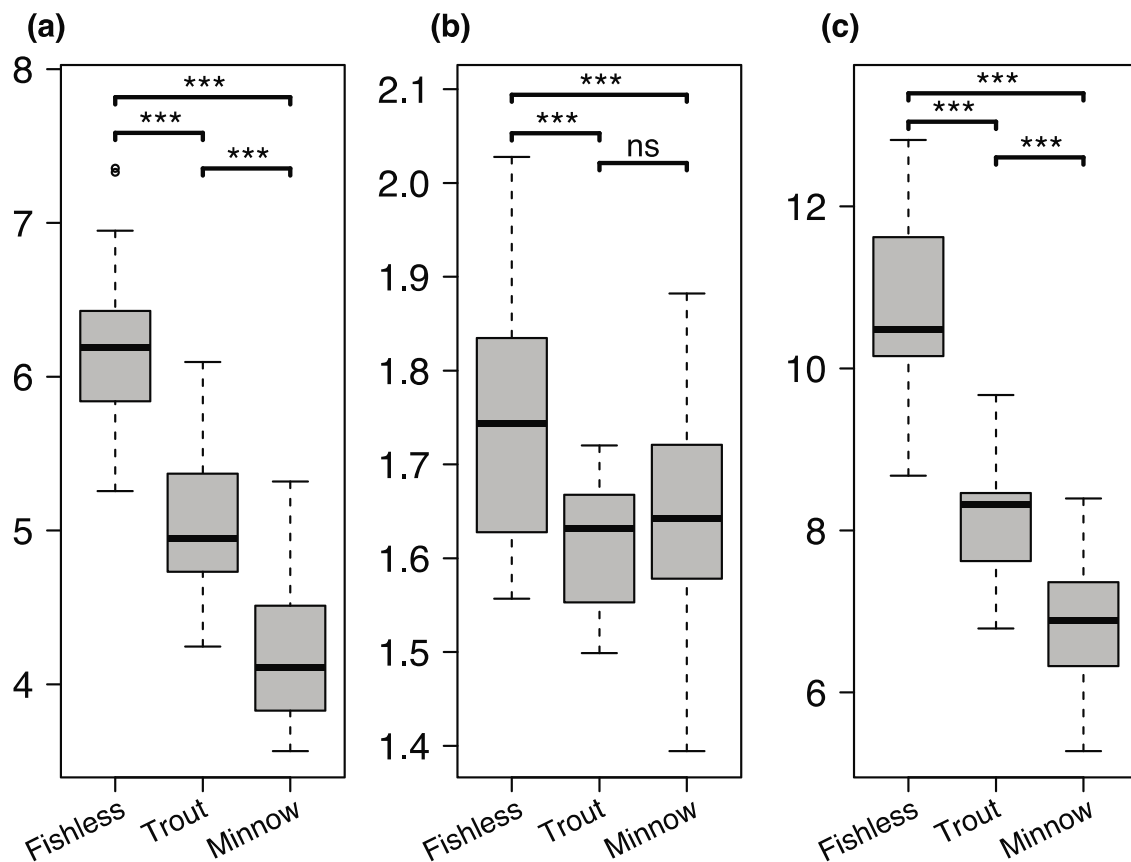
Statistical analyses were conducted within the R 4.1.0 statistical environment (R Core Team, 2021). R packages used are detailed in the Supporting Information. The level of significance adopted for all analyses was  $\alpha = 0.05$ .

### 3. Results

#### 3.1. Taxa diversity

A total of 77 macroinvertebrate taxa were found in 54 lakes. The order Diptera was the most diverse and abundant group (64 % of macroinvertebrate abundance), followed by Bivalvia, Ephemeroptera, Megaloptera, Hemiptera, and Coleoptera taxa. Oligochaeta were largely dominated by Tubificidae taxa. The list of all identified taxa and their frequency of occurrence in the sampled lakes are presented in Table A.1 of the Supporting Information.

There were significant differences in taxa richness among lake categories ( $\chi^2 = 11.2$ ,  $P < 0.005$ ). The richness of fishless lakes ( $16.3 \pm 2.5$ ) was significantly different from that of minnow lakes ( $11.7 \pm 5.3$ ). However, trout lakes ( $14.7 \pm 3.9$ ) did not significantly differ from the other categories. Significant differences arose among lake categories regarding alpha, beta, and gamma diversities (Fig. 1). Fishless lakes had significantly higher values of the three indices, whereas minnow lakes showed the lowest values of alpha and gamma diversity.



**Fig. 1.** Comparative boxplots of alpha (a), beta (b) and gamma (c) diversity indices among lake categories (Kruskal-Wallis  $\chi^2 = 69.6$ ,  $\chi^2 = 16.8$ , and  $\chi^2 = 71.8$ , respectively, and  $P < 0.001$  each). Diversity measures were calculated on rarefied subsets of lakes (30 permutations of nine randomly chosen lakes at a time) and employing the adaptation of common Shannon entropy index suggested by Jost (2007). Asterisks indicate the level of statistical significance of post-hoc tests with Bonferroni correction: \*\*\* $P \leq 0.001$ ; ns, not significant ( $P > 0.05$ ).

### 3.2. Overall community analysis

Five MEM variables (MEM-3, 13, 14, 19, 20) significantly described the spatial autocorrelation in the community (Table 1). The catchment-related variables best explaining the community were the percentage of meadows cover, the ratio total catchment/lake area (a surrogate of water renewal time), lake area, and lake altitude. Among in-lake variables, selected variables were littoral macrophyte cover, algal biomass (chlorophyll-*a*), dissolved inorganic nitrogen, total phosphorus and water conductivity. The most relevant vertebrate-related variables were the measures of catch per unit effort of minnows and trout, so the group was henceforth called fish-related variables.

The full-dBRDA was composed by the 16 variables listed above, explaining 54.3 % of macroinvertebrate variance (adjusted  $R^2 = 0.38$ ). The variation partitioning analysis revealed that fish-related variables had the highest fraction of pure effects, doubling the variation uniquely attributed to spatial and catchment influence, followed by in-lake variables (Table 1). In-lake variables showed the highest shared variance, most of it shared with fish variables (Fig. A.3 in Supporting Information). Most of the explanatory power of MEM variables (70.7 %) was shared with variables of other nature. The forward selection algorithm reduced the number of variables from the full-dBRDA to 10, which comprised the pars-dBRDA: Minnow\_CPUE (33.9 % of exclusive variance), total catchment/lake area (14.5 %), lake area (7.9 %), macrophyte cover (6.9 %), total phosphorus (6.6 %), altitude (6.5 %), conductivity (6.2 %), chlorophyll-*a* (6.0 %), MEM20 (5.8 %), and Trout\_CPUE (5.7 %) (Fig. 2a). Summary statistics of all but MEM variables are shown in Table A.2 in the Supporting Information. This model significantly accounted for 44.3 % of macroinvertebrate variance

(pseudo- $F = 3.4$ ,  $P = 0.001$ ), with the first three axes being significant (dBRDA1: pseudo- $F = 14.1$ , dBRDA2: pseudo- $F = 6.8$ , dBRDA3: pseudo- $F = 4.5$ ;  $P = 0.001$ , for all three). The variables Minnow\_CPUE, Chlorophyll-*a*, and MEM20 were negatively correlated with the first axis ( $r = -0.7$ ,  $-0.3$ , and  $-0.3$ , respectively). The second axis was negatively correlated with macrophyte cover, total phosphorus and chlorophyll-*a* ( $r = -0.6$ ,  $-0.6$  and  $-0.4$ , respectively), but positively correlated with lake area ( $r = 0.4$ ) and altitude ( $r = 0.3$ ). The third axis accounted for 13.1 % of the constrained variance, with total catchment/lake area and Trout\_CPUE as negatively correlated variables ( $r = -0.6$  and  $-0.4$ , respectively), and altitude positively correlated ( $r = 0.4$ ).

A tree with four 'leaves' resulted from the MRT analysis (Fig. 2b). Minnow density was responsible for the first split, with a threshold of 27.6 individuals  $\text{net}^{-1} \text{day}^{-1}$ . Macrophyte cover created two successive splitting nodes with similar threshold values (19 % and 18.5 % of cover). We found four taxa indicating significant association with lakes in leaves A, B, and C (Megaloptera, Bivalvia, and Chironomidae taxa). Five indicator taxa were shared between the sites with highest abundance of macrophytes (i.e. leaves B and C), including Hydrachnida and Odonata taxa. Five taxa were exclusively associated with leaf B (Corixidae, Hirudinea, Odonata), and two with leaf C (Diptera and Coleoptera taxa). No taxon was significantly associated with leaf D.

### 3.3. Factors explaining macroinvertebrates occurrence

A total of 14 taxa across different body sizes and functional groups were analysed by GLM. For nine of them, minnows had a significantly negative effect on their probability of occurrence, whereas four taxa were affected by trout (Fig. A.4). Exceptions were Gastropoda,

**Table 1**

Forward selection of variables in distance-based redundancy analysis (dbRDA) for spatial, catchment, in-lake and vertebrate variables explaining littoral macroinvertebrate community ( $P < 0.05$ , 9999 randomizations). Adjusted  $R^2$  of variables selected from each model to be incorporated in full-dbRDA is indicated in parentheses. Variation partitioning among groups of variables in full-dbRDA (total adj  $R^2 = 0.384$ ) is displayed below. Abbreviations stand for: TC/LA = total catchment area/lake area, Alt = altitude, Mac\_cov = percentage of littoral macrophyte cover, Chla = chlorophyll-*a*, TP = total phosphorus concentration, DIN = dissolved inorganic nitrogen concentration, Cond = water conductivity, Minnow\_CPUE = CPUE of minnows, Trout\_CPUE = CPUE of trout.

	Spatial variables (space-dbRDA)	Catchment variables (catchment-dbRDA)	In-lake variables (inlake-dbRDA)	Vertebrate variables (vertebrate-dbRDA)
Selected variables (adj $R^2$ )				
MEM-3 (0.037**)		Meadow (0.051**)	Mac_cov (0.093***)	Minnow_CPUE (0.071***)
MEM-20 (0.03*)		TC/LA (0.028*)	Chla (0.032**)	Trout_CPUE (0.04**)
MEM-14 (0.03**)		Alt (0.028*)	TP (0.028**)	
MEM-13 (0.023*)		Area (0.026*)	DIN (0.023*)	
MEM-19 (0.02*)			Cond (0.021*)	
Variation partitioning on full-dbRDA				
Total (adj $R^2$ )	0.140	0.106	0.176	0.157
Pure (adj $R^2$ )	0.041	0.041	0.064	0.080
Shared (adj $R^2$ )	0.099	0.065	0.112	0.077

Asterisks indicate the level of statistical significance associated with each variable: \* $P \leq 0.05$ ; \*\* $P \leq 0.01$  and \*\*\* $P \leq 0.001$ .

Hirudinea, Hydrachnidia, and Odonata, which appeared unaffected by fish. The density of both minnows and trout was found to decrease the probability of occurrence of the coleopteran genus *Hydroporus* along with Dytiscidae and Corixidae families. The dytiscid genus *Agabus* was not found in any lake with fish presence, and both *Boreonectes* (Dytiscidae) and *Arctocoris* (Corixidae) genera were absent in lakes with minnows. The occurrence of Sialidae (Megaloptera) was negatively affected by minnows. Minnow density was the only significant and negative variable in the models of Baetidae (Ephemeroptera), Sphaeriidae (Bivalvia), Polycentropodidae (Trichoptera), Macropelopiini, and Procladiiini (both Chironomidae). Trout density was a negative predictor for Limnephilidae (Trichoptera).

Macrophyte cover increased the likelihood of occurrence of Odonata, Hirudinea, and Gastropoda, but had a negative effect on Limnephilidae. We found a positive interaction between minnows and macrophytes for Sialidae and Limnephilidae (the impact of minnow density on their probability of occurrence was less severe in lakes with high macrophyte cover) and between trout and macrophytes for Dytiscidae. See Tables A.3–A.21 for detailed GLM results and Fig. A.4 for visualization of taxa response (Supporting information).

For those macroinvertebrates significantly affected by fish, we compared the estimated fish density at which their probability of occurrence was decreased to 0.5 (Fig. 3). The relative sensitivity to minnow density was variable among taxa, but organisms differing in body size and functional group were impacted at low minnow densities, such as large and mid-sized clingers, large swimmers, and large and mid-sized burrowers. Although significantly affected, some burrower taxa like Sphaeriidae and Macropelopiini were particularly threatened at higher minnow densities. By contrast, trout density was mainly responsible for the impact on large swimmers and clingers.

### 3.4. Factors explaining macroinvertebrates abundance

The factors controlling the abundance of macroinvertebrates were assessed for 19 taxa (including five groups for which the probability of occurrence was not studied due to their ubiquitousness). Nine taxa were negatively affected by minnows, including large clingers, along with large, mid-sized, and small burrowers. Four taxa were negatively affected by trout, mainly large and mid-sized clingers, but also large and small burrowers (see Fig. 4 for relevant taxa). A negative relationship with minnows was found for Sialidae, Macropelopiini, Orthoclaadiinae (Chironomidae), Limnephilidae, Procladiiini, non-Tanytarsini Chironominae (Chironomidae), Polycentropodidae, Hydrachnidia, and Tanytarsini (Chironomidae). The impact of minnows on Oligochaeta was marginally significant. Oligochaeta, Sialidae, Baetidae and Limnephilidae displayed declines in abundance related to trout. Nevertheless, trout density had a positive effect on the abundance of the chironomids Orthoclaadiinae and Pentaneurini.

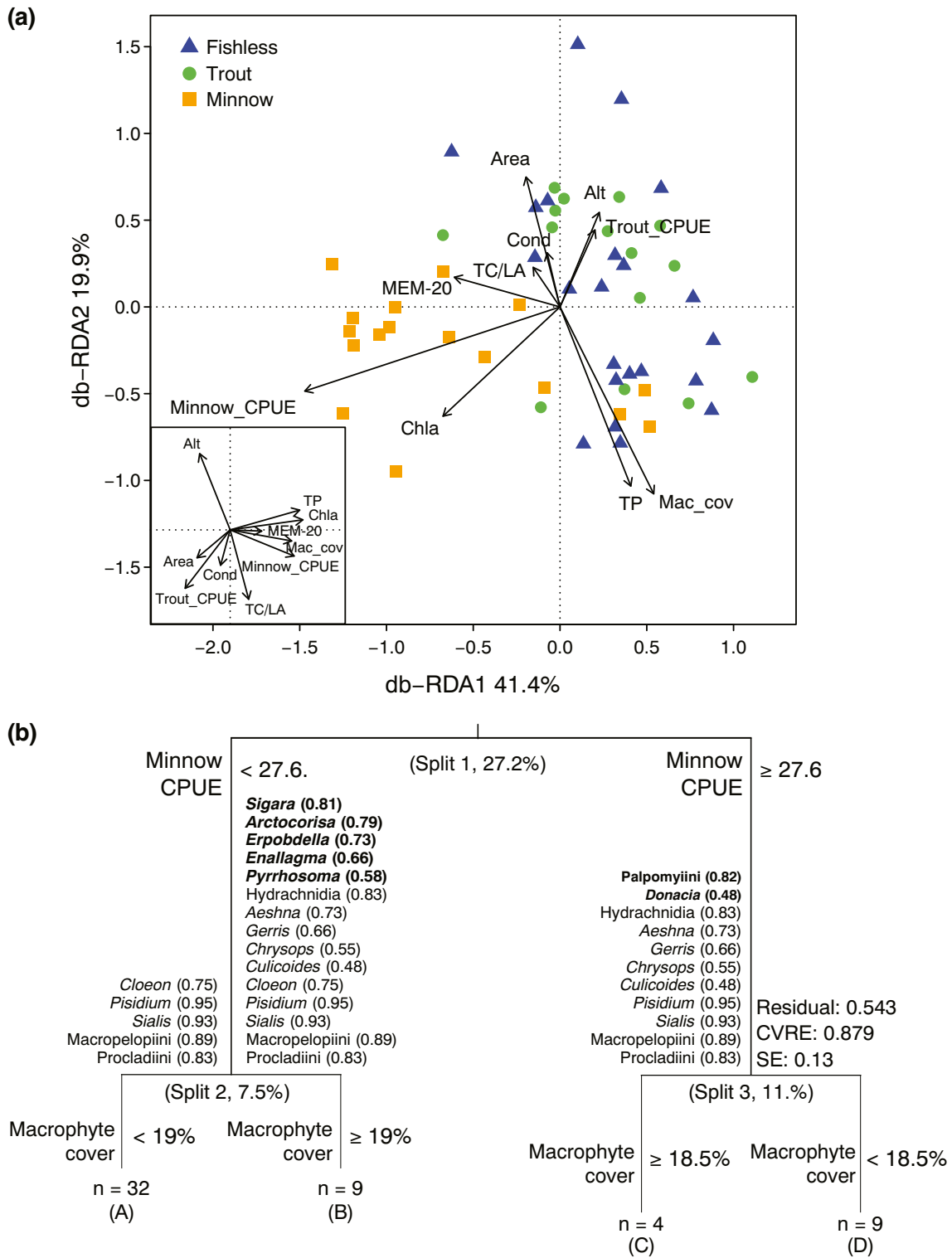
Macrophyte cover was a positive predictor of the abundance of Corixidae, non-Tanytarsini Chironominae, Pentaneurini, Tanytarsini, Hydrachnidia, Procladiiini, Gastropoda, Baetidae, Sphaeriidae, and Oligochaeta. Significant effects of lake altitude were detected for Corixidae and Tanytarsini, which were more abundant at higher altitudes. The abundance of Macropelopiini and Hydrachnidia increased with lake area, whereas Baetidae showed a negative relationship with that variable. Negative interactions were found between minnows and trout for Pentaneurini (the impact of minnow density on its abundance was more severe in lakes with low trout density), and between trout and macrophyte cover for Procladiiini (the impact of trout density on its abundance was more severe in lakes with low macrophyte cover). For further details on GLM results, see Tables A.3–A.21 in the Supporting Information.

## 4. Discussion

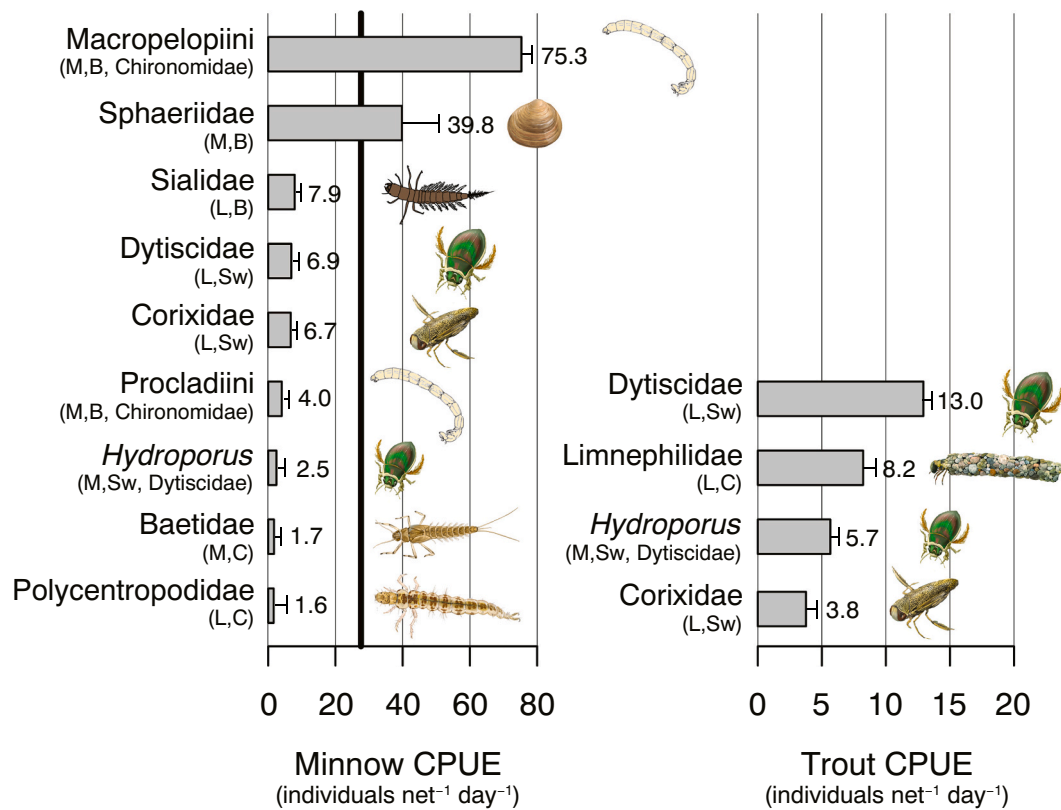
In this study, non-native minnows and trout were shown to play a strong role on shaping the littoral macroinvertebrate communities in high mountain lakes. Variation partitioning analysis indicated that the variation uniquely attributable to the density of fish was larger than the one explained by either spatial autocorrelation, catchment features, or in-lake habitat descriptors. Altitude-related and habitat variables have been usually defined as the most important factors affecting the macroinvertebrate distribution in high mountain lakes (Fjellheim et al., 2000; de Mendoza and Catalan, 2010; de Mendoza et al., 2015). Yet, in agreement with our results, the presence of predatory fish can also control the occurrence and abundance of many macroinvertebrate taxa and even surpass environmental and spatial factors in their influence on the community (Schilling et al., 2009; de Mendoza et al., 2012).

Trout have been described to threaten a variety of animal groups in high mountain lakes (e.g. Knapp et al., 2001; Tiberti et al., 2014; Miró et al., 2018). The effects of the presence of introduced trout on macroinvertebrate communities of naturally fishless lakes have been described previously across geographically disparate areas (Knapp et al., 2001; Tiberti et al., 2014; Toro et al., 2020). One contribution of this study, has been to take into account the effects of trout density (Figs. 3, 4 and A.4). In accordance with Knapp et al. (2001) and Tiberti et al. (2014), we found that trout caused a decline in the abundance of conspicuous Trichoptera, Ephemeroptera, Coleoptera, Hemiptera and Oligochaeta taxa. Conversely, Chironomidae groups such as Orthoclaadiinae and Pentaneurini benefited from higher trout densities. Evidence of enhanced emergence of chironomids caused by trout is reported in the literature (Tiberti et al., 2016). It seems very likely that the selective predation of trout on large-bodied and predator taxa is involved in the higher abundances of certain chironomids from interspecies competition and predation within the littoral invertebrate community (Mousavi et al., 2002), but other factors like an increase in nutrient concentration could also be involved (Leavitt et al., 1994).

Our results suggest that minnows have a much larger negative effect



**Fig. 2.** (a) Ordination diagram showing the linear combination of variables describing the main gradients of variation in the littoral macroinvertebrate community (Bray-Curtis distances on log-transformed taxa abundance) along the first two axes of pars-dbrDA. A principal component analysis (PCA) of variables in pars-dbrDA is included as a subplot. (b) Multivariate regression tree (MRT) run on macroinvertebrate dataset (Bray-Curtis distances on log-transformed taxa abundance, 1000 multiple cross-validations) and using explanatory variables in pars-dbrDA as candidates to be explored. Percentage of constrained variance related to each split of the tree is displayed in parentheses. Taxa significantly associated with leaves are included along with their indicator value. Minnow CPUE is expressed as individuals  $\text{net}^{-1} \text{day}^{-1}$ . See Table 1 for a description of variable abbreviations.



**Fig. 3.** Estimated fish density at which macroinvertebrate probability of occurrence was equal to 0.5. Only macroinvertebrates significantly affected by either minnows or trout are presented. Fish density estimations and their standard errors are displayed (see Supporting Information for statistical procedures). The threshold of minnow CPUE suggested by MRT analysis (Fig. 2b) is indicated with a thick vertical line. The body size and functional group of taxa are codified as: L = large, M = mid-sized, S = small, Sw = swimmer, C = clinger, and B = burrower.

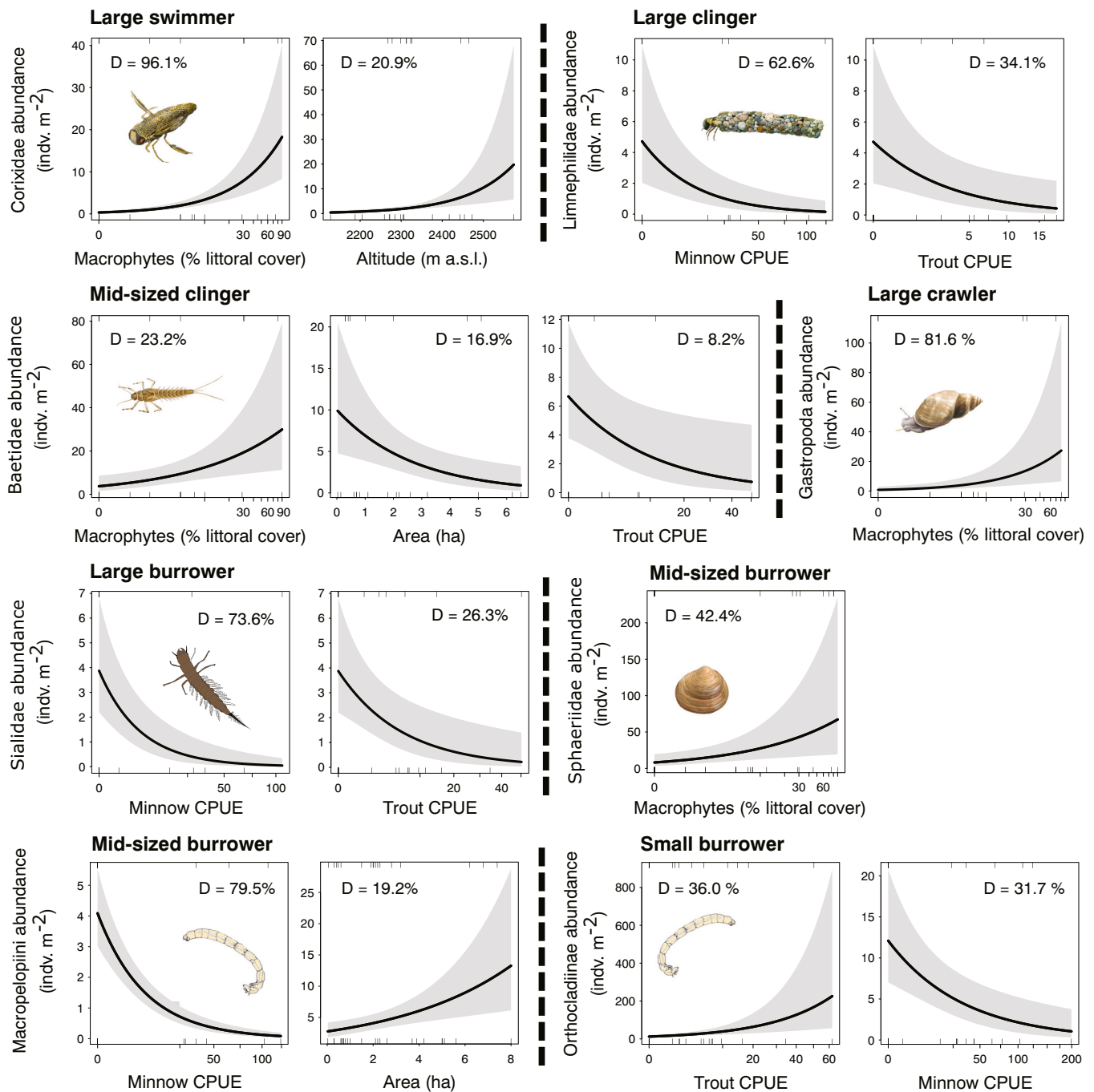
on the littoral macroinvertebrate community than trout. Both dBRDA and MRT approaches revealed that minnow density was by far the most important single variable determining community structure, with a threshold of 27.6 individuals net<sup>-1</sup> day<sup>-1</sup> (that corresponds to approximately 60.5 individuals trap<sup>-1</sup> day<sup>-1</sup>) beyond which the community was affected significantly. Only scarce literature exists on the role of minnows in high mountain lakes and their effects on littoral macroinvertebrates have remained so far unexplored. Previous studies in subalpine lakes revealed that minnow diet included Cladocera and large taxa, such as Gammaridae, Ephemeroptera, Plecoptera, and Trichoptera (Hesthagen et al., 1992; Museth et al., 2010). Yet, disagreeing results describing an increasing abundance of Ephemeroptera, Plecoptera and Trichoptera taxa in lakes with minnows have also been reported (Næstad and Brittain, 2010).

Macroinvertebrate communities in lakes with minnows were composed of a very limited number of taxa, and dominated by small burrower organisms with semi-sessile habits. Chironomidae and Oligochaeta showed a substantial increase in their relative abundance in lakes with minnows (92 % of individuals counted in the samples on average), but simply as a result of the absence of other potential community members. Indeed, minnows restricted the occurrence of more taxa than trout, and negatively affected a wide variety of functional groups (i.e. swimmers, clingers, and burrowers). In contrast to trout, high minnow densities did not benefit any taxon, but rather caused population declines of Trichoptera and inconspicuous taxa, like Hydrachnidia and most of Chironomidae groups. Only Corixidae and Dytiscidae were both affected by minnows and trout, as these large and mobile insects are generally very vulnerable to visual predators (Tate and Hershey, 2003). The high densities in which minnows often occur may certainly be one of the reasons to their exacerbated impact on the community. The shore-based shoals of minnows perform a faster

location of patchy food than solitary trout and their efficiency increases with shoal size (Pitcher et al., 1982). Moreover, when living in sympatry with trout, minnows are confined to the shallow parts of the lake to avoid trout predation. Under these circumstances, minnows that would be otherwise consuming pelagic invertebrates may shift towards a heavier predation on littoral benthic fauna (Museth et al., 2010). Trout has a less flexible feeding strategy than minnows and is clearly influenced by the size frequency distribution of potential prey (Sánchez-Hernández and Amundsen, 2015).

Aquatic macrophyte cover was the only significant factor explaining the occurrence of groups like Hirudinea, Gastropoda, and Odonata. In fact, irrespective of minnow and trout density, we found higher taxa richness in lakes with more abundant macrophytes (Fig. A.5 of the Supporting Information). Aquatic vegetation is of great importance in providing food resources as well as increased habitat heterogeneity to macroinvertebrates (Carlisle and Hawkins, 1998). As a source of both competitive and predatory refuges, we observed that higher values of macrophyte cover increased the likelihood of occurrence and the abundance of taxa vulnerable to fish predation. Even inconspicuous taxa such as Oligochaeta, Sphaeriidae, and most chironomids, were favoured by submerged vegetation. On the other hand, Limnephilidae taxa were more common in lakes with low macrophyte dominance. This fact can probably be attributed to the higher availability of mineral grains required by dominant species of Limnephilidae (e.g. *Annitella pyrenaica*) to build their larval cases in lakes with low macrophyte dominance.

The results of this study show that non-native minnows pose a very serious threat for the conservation of littoral macroinvertebrates, being responsible for a dramatic biodiversity loss. Other studies have shown that conservation implications of minnow introduction extend far beyond the macroinvertebrate communities, as so far the survival of macrophyte populations and amphibians is also compromised by



**Fig. 4.** Estimated effect of significant variables ( $P < 0.05$ ) on the abundance of relevant macroinvertebrate taxa obtained by gamma GLM. The contour of the shaded areas corresponds to  $\pm 2$  SE (95% CI) relative to the main estimate, and hatch marks at the top and the bottom are descriptors of the frequency of data points along the gradient of continuous variables (above and below the main estimate, respectively). The increase in deviance (“D”, equivalent to variance for this type of analyses) resulting from dropping the selected variable from the model is indicated. Minnow and trout CPUE are expressed as individuals  $\text{net}^{-1} \text{day}^{-1}$ .

minnows. [Gacia et al. \(2018\)](#) described the massive uprooting of Pyrenean quillwort meadows related to minnow presence. The predation on primary consumers, together with the release of nutrients and increased turbidity due to the benthic scavenging activity of shoals, were proposed as the underlying causes. Amphibian declines are the first consequence of trout introduction ([Knapp et al., 2001](#); [Miró et al., 2018](#)), but minnow presence is also equally unfavourable for amphibians as is trout. Amphibians disappear from lakes when trout are introduced, but do not return when trout disappear and minnows remain as the only fish species present in the lake ([Miró et al., 2018](#)). [Miró and Ventura \(2015\)](#) did not find any lake where minnows disappeared once established in the

Pyrenees, whereas pre-existing trout populations have disappeared after minnow introductions in twenty lakes and ponds. Some of these lakes had brown trout introduced centuries ago while others were stocked recently. In addition, [Tiberti et al. \(2022\)](#) found that there is a strong negative relationship between trout and minnows, indicating that minnows have a direct role in the decrease of trout abundance. This suggests that minnows are a bigger conservation threat due to their higher resistance to adverse environmental conditions and their greater impact on littoral invertebrates than trout.

The conservation of invertebrates is at least as threatened as that of vertebrates worldwide ([Sánchez-Bayo and Wyckhuys, 2019](#)).



Invertebrate groups such as Coleoptera and Bivalvia, which were heavily impacted by minnows in this study, include very sensitive and endangered species that rely on the integrity of these habitats for their survival (Council of the European Communities, 1992; Verdú et al., 2011). The recovery of high mountain macroinvertebrate communities has been shown to be possible after trout eradication (Knapp et al., 2001; Tiberti et al., 2019). Although hard to accomplish, restoration of lakes invaded by minnows may be an effective conservation measure for impacted macroinvertebrate communities, as already proved for protected amphibians (Miró et al., 2020).

#### 4.1. Conclusions

We found that the negative effects of non-native minnow introduction prevailed over environmental and spatial factors in determining the littoral macroinvertebrate community of high mountain lakes. Minnows had a much larger negative effect on macroinvertebrates than trout and were linked to the elimination of many taxa from the studied ecosystems. Our results demonstrate substantial local effects at the lake scale, but uncontrolled expansion of minnows could also lead to regional and biogeographic changes in native aquatic biodiversity. As fish introduction makes water bodies unsuitable for the survival of vulnerable taxa, fragmentation and isolation of their populations is expected to occur. We conclude that given the ongoing and fast spread of non-native minnows in mountain ranges worldwide, there is an urgent need for incorporating restrictive criteria about trout stocking and the abolition of the use of minnows as live bait for angling. In addition, we suggest to implement restoration measures to remove non-native fish, especially minnows in early stages of introduction. In those lakes where minnows are already introduced and where conservation is not the main goal, we also recommend to establish more sustainable fishing methods such as fishing without death. This should prevent the disappearance of trout, and therefore a larger deterioration of the lake.

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#### CRedit authorship contribution statement

**Víctor Osorio:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **María Ángeles Puig:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition. **Teresa Buchaca:** Conceptualization, Investigation, Data curation, Writing – review & editing, Funding acquisition. **Ibor Sabás:** Investigation, Data curation, Writing – review & editing. **Alexandre Miró:** Investigation, Data curation, Writing – review & editing. **Federica Lucati:** Investigation, Data curation, Writing – review & editing. **Jongmo Suh:** Investigation, Data curation, Writing – review & editing. **Quim Pou-Rovira:** Conceptualization, Investigation, Data curation, Writing – review & editing, Funding acquisition. **Marc Ventura:** Conceptualization, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocon.2022.109637>.

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