







## ORIGINAL ARTICLE OPEN ACCESS

# Impact of the Highly Invasive Signal Crayfish (*Pacifastacus leniusculus*) on Freshwater Macroinvertebrate Communities

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## ABSTRACT

1. Invasive non-native species threaten freshwater ecosystems, disrupting the functioning and structure of the food chain and potentially leading to biodiversity loss. As omnivores, crayfish can modify their environment, directly affecting organisms such as macroinvertebrates. Macroinvertebrates, intermediate consumers in food chains, are influenced by both top-down and bottom-up forces, playing fundamental functional roles within freshwater ecosystems.
2. The main aim of this study was to assess the effects of the signal crayfish *Pacifastacus leniusculus* on the freshwater macroinvertebrate community in a mountainous area with very low human disturbance, while controlling for other measured environmental factors (e.g., T°C, pH, TDS). To this end, 34 sites (18 invaded and 16 non-invaded) in the Rabaçal and Tuela River basins (Portugal) were sampled. Comparisons were made between basins and invaded and uninvaded sites regarding abundance, biomass, richness and indices of macroinvertebrate community diversity and functionality.
3. A total of 38,529 organisms from 133 freshwater macroinvertebrate taxa were identified. Our results showed a decline in the biomass, richness and diversity of macroinvertebrates with increasing crayfish abundance. Few differences in functional diversity were found, probably due to functional redundancy; however, crayfish did affect certain functional groups: drillers and periphyton-associated macroinvertebrates exhibited a negative correlation with crayfish abundance, whereas scrapers and gravel-associated macroinvertebrates demonstrated a positive response. In addition to the abundance of signal crayfish, temperature, total dissolved solids and pH showed some influence on the macroinvertebrate community.
4. Overall, these findings are fundamental for better understanding, predicting and managing crayfish impacts in low disturbance areas of upper catchments. It is important to continue monitoring not only non-native crayfish populations but also macroinvertebrate (and other) communities, as our findings clearly demonstrate the sensitivity of these organisms to crayfish presence. Future research should investigate the main mechanisms of this change in the macroinvertebrate community.

## 1 | Introduction

The introduction of non-native species, facilitated by human actions, poses a significant threat to biodiversity (Reid et al. 2019; Strayer 2010). These introductions can add a new functional

component to native communities (Gallardo et al. 2016), causing ecological, economic, health and social changes (Charles and Dukes 2008). Their presence disturbs native biodiversity and affects the structure and functioning of ecosystems (Ehrenfeld 2010; Sousa et al. 2009; Strayer 2012). In addition,

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these species end up being responsible for high monetary losses (Cuthbert et al. 2021; Diagne et al. 2021).

The impacts of non-native species often depend not only on their abundance, biomass and spatial distribution but also on their position in the food chain (Gallardo et al. 2016; Jarnevich et al. 2021). If they have a position in the upper levels, non-native species can exert top-down control, impacting the abundance and biomass of lower trophic levels (Pace et al. 1999). In contrast, changes in primary producers and nutrient input, triggered by non-native species, can lead to 'bottom-up' effects, influencing upper trophic levels as well (Heath et al. 2014). Furthermore, non-native species can also act as ecosystem engineers, modifying the habitat through changes in water clarity, stream hydrology, soil biogeochemistry and the concentration of nutrients and organic matter, or even providing habitat to other species (Gallardo et al. 2016; Sousa et al. 2009).

Many freshwater species have been introduced worldwide, including crustaceans (Strayer 2010). Notably, North American crayfish species like the signal crayfish *Pacifastacus leniusculus* were introduced to Europe, establishing viable populations due to releases or escapes (Charles and Dukes 2008). Despite being beneficial to humans in some aspects (e.g., source of protein), crayfish have become problematic, causing several ecological and economic impacts (Lodge et al. 2012; Twardochleb et al. 2013). The introduction of non-native crayfish to Europe also introduced pathogens like the crayfish plague (*Aphanomyces astaci*) that brought diseases to native crayfish populations, causing local extinctions (Charles and Dukes 2008). In addition, crayfish are ecosystem engineers that increase leaf decomposition and disrupt nutrient cycling, and their burrowing and grazing activities reduce bank stability and vegetation cover (Albertson and Daniels 2018; Creed and Reed 2004), transforming a habitat with clear conditions to one with more turbid waters dominated by phytoplankton in shallow lakes or wetlands (Geiger et al. 2005; Matsuzaki et al. 2009). Due to their omnivorous nature and rapid population growth, non-native crayfish directly and indirectly affect various trophic levels (Carvalho et al. 2022; Strayer 2010). They prey on amphibians and fish, compete for food resources and potentially become prey for predators such as birds and mammals (Correia 2001; Gherardi 2007). Nonetheless, they also affect macroinvertebrates through predation, as well as indirectly via competition and trophic cascades, reducing the diversity and abundance of native macroinvertebrate communities (Ercoli et al. 2015; Galib et al. 2021; Strayer 2010; Twardochleb et al. 2013).

With the invasion of crayfish, besides the expected decrease in diversity and abundance of macroinvertebrates, there has also been a change in the structure of the macroinvertebrate communities (Mathers et al. 2016). This change favours more agile taxa while diminishing less mobile organisms like molluscs and certain Trichoptera families (Dorn 2013; Galib et al. 2021; Hansen et al. 2013). These changes may have cascading effects across trophic levels, since with the decrease in herbivorous macroinvertebrates (gastropods) an increase in periphyton biomass in some crayfish-invaded sites was observed (Bobeldyk and Lamberti 2008; Scordo et al. 2023), which could free up resources benefiting other mobile herbivores (Mathers et al. 2016). Therefore, understanding the impacts of crayfish

on macroinvertebrates is crucial, since they occupy various trophic positions within the food chain, not only serving as food for other living beings (Klecka and Boukal 2013), but also play a key role in several ecosystem processes (Lamberti et al. 1989; Liboriussen et al. 2005; Swan et al. 2021). Moreover, they are organisms that respond to a wide variety of environmental conditions, being valuable indicators of human disturbance (Wallace and Webster 1996), and are easy to sample (González 2023).

Despite mounting evidence that non-native crayfish can modify trophic structure (Gherardi 2007) and ecosystem functions (Mathers, White, Guareschi, et al. 2020), the reality is that these impacts can be highly context-dependent due to environmental filtering, biotic resistance or even due to biological traits of the invasive population (Sousa et al. 2024). Therefore, it is crucial to increase the understanding and predictive power of the impacts generated by these species at the local level (Haubrock et al. 2024; Strayer et al. 2006). This study used the recent invasion of the signal crayfish in Montesinho Natural Park and its surrounding areas in Portugal. The signal crayfish, which is native to the Pacific Northwest region of North America, has spread into Europe and Asia (Lodge et al. 2012). This species is currently experiencing rapid population growth and spread in rivers in northeastern Portugal (Alves et al. 2025; Carvalho et al. 2022, 2025). To assess the effects of the crayfish on macroinvertebrates, several sites with and without signal crayfish were sampled along two river basins in the Montesinho Natural Park and surrounding areas to assess possible impacts on the taxonomic and functional diversity of freshwater macroinvertebrate communities.

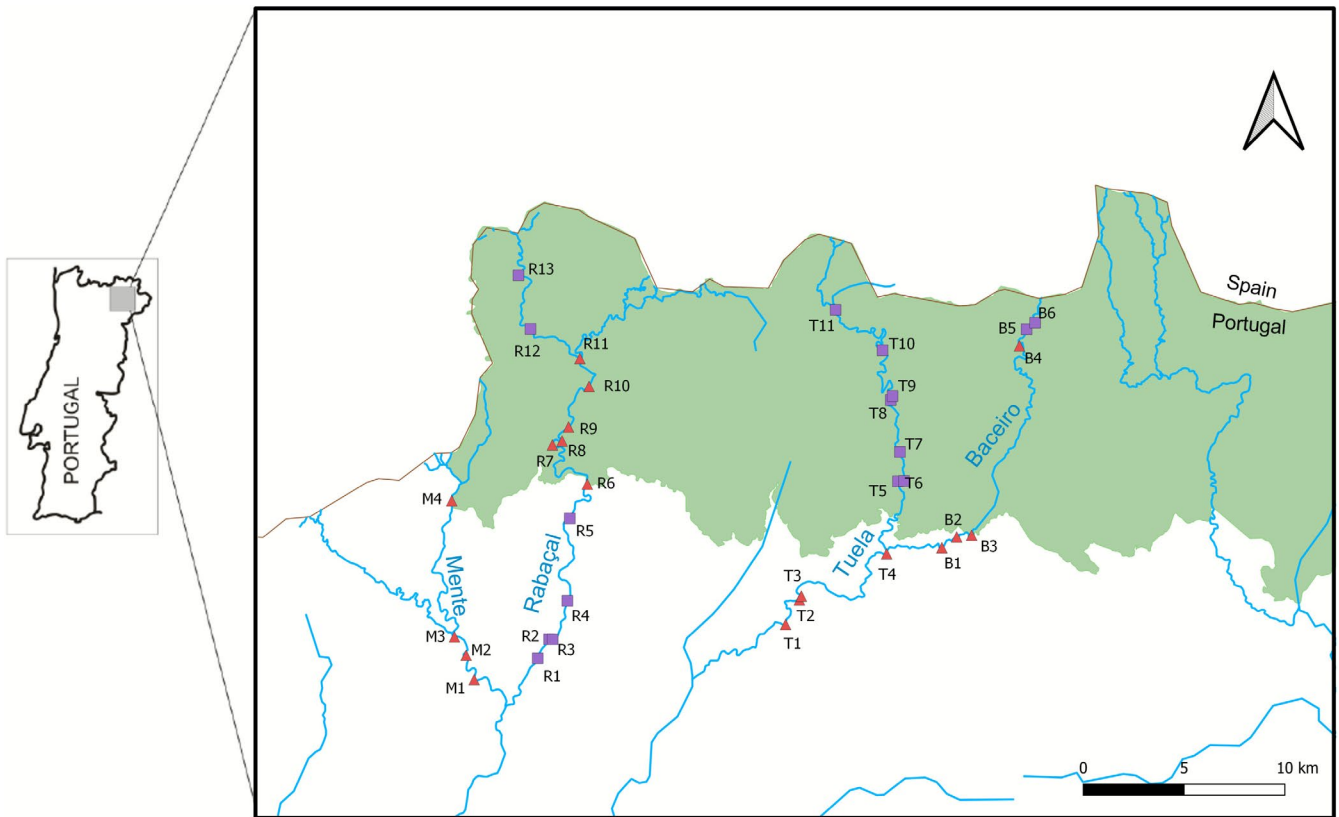
## 2 | Methods

### 2.1 | Study Area

The study area comprised sites within and around the Montesinho Natural Park, northeastern Portugal. This natural park and surrounding areas consist of wooded landscapes where traditional farming is practiced. Altitudes vary between 436 m and 1477 m (Castro et al. 2010), and the climate is characterised by high winter rainfall and dry summers (Gonçalves 1985; Sousa et al. 2015). Rabaçal (Mente and Rabaçal Rivers) and Tuela (Baceiro and Tuela Rivers) river basins are part of the Douro River basin and were sampled in this study. Both basins have low human disturbance and share a similar climate and habitat diversity (Nogueira et al. 2021; Sousa et al. 2019, 2020), making all four rivers particularly interesting for studying the impacts of non-native species since there are no other relevant human disturbances (Sousa et al. 2019, 2020). The studied area is rich in biodiversity, with several species of high conservation interest present (e.g., *Macromia splendens*, *Margaritifera margaritifera*, *Galemys pyrenaicus*) (IUCN 2022; Sousa et al. 2015). However, non-native species such as the signal crayfish are now also present, with first individuals detected in the summer of 2013 (Sousa et al. 2015).

### 2.2 | Sampling Strategy

Macroinvertebrates and signal crayfish were sampled in July 2022 to minimise seasonal variation in community composition



**FIGURE 1** | Map of the surveyed area showing the location of the 34 sampling sites (18 invaded—red/triangle; 16 uninvaded—purple/square) in Menté, Rabaçal, Tuela and Baceiro Rivers. The green shaded area represents the territory of the Montesinho Natural Park.

and abundance. Thirty-four sites (18 invaded and 16 uninvaded) were sampled in four rivers, which are grouped into two basins: the Rabaçal and Tuela basin. The Rabaçal basin includes the Menté (4 invaded sites) and Rabaçal Rivers (6 invaded and 7 uninvaded sites). The Tuela basin includes the Tuela (4 invaded and 7 uninvaded sites) and Baceiro (4 invaded and 2 uninvaded sites) Rivers (Figure 1). For abiotic characterisation, temperature (°C), oxygen (mg/L), conductivity ( $\mu\text{S}/\text{cm}$ ), total dissolved solids (TDS) (mg/L) and pH were measured in situ at all sites using a YSI EXO 2 multi-parameter probe.

These sites were also subjected to an in situ River Habitat Survey (RHS), making it possible to collect data related to the physical structure of the watercourses (Raven et al. 1998). For this, the standard length of 500m of the river channel was sampled and data on altitude, geology, slope and distance and height from its source were collected, along with in situ characterisation of the geomorphological features and processes in the watercourse, banks and adjacent river corridor. All this data was amassed to obtain the Habitat Modification Score (HMS), Habitat Quality Assessment (HQA) and the Riparian Quality Index (RQI); following Raven et al. (1998). The HMS and HQA indices were used to assess the degree of disturbance and/or naturalness in the sites under analysis (Raven et al. 1998). The HMS quantifies channel artificialisation using sub-indices to determine the HMS score and consequent classification (Habitat Modification Score Rules 2003). The HQA assesses habitat diversity and richness, with higher scores indicating better habitat quality. It includes several sub-indices that reflect the relevance of specific habitat features to biological communities (APA 2021). To carry out a

hydromorphological assessment based on HQA, it is necessary to have a database of reference sites (undisturbed), by river typology (APA 2021). After determining the HMS and HQA quality indices, the classification of the hydromorphological quality elements for the sampling sites corresponded to the most penalising class of the two. Additionally, the RQI assessed the naturalness, complexity and continuity of the riparian zone, covering the bank slope, top of the bank and the 5m buffer from the top of the bank. This assessment was made via the River Habitat Survey Toolbox (River Habitat Survey Toolbox 2023), rating riparian zone naturalness, complexity and continuity on a 5-class scale from 'Very low' (1st quintile) to 'Very high' (last quintile).

To assess signal crayfish abundance, crayfish were captured by placing eight funnel traps, five rectangular (50×30×20 cm; 0.5 cm mesh) and three cylindrical (43 cm diameter; 22 cm height; 1.5 cm mesh), per site for 24 h. All traps were baited with the marine dead fish, Atlantic horse mackerel (*Trachurus trachurus*). The abundance of crayfish per site was expressed as the total number of individuals per catch per unit of effort (hereafter ind. CPUE).

The macroinvertebrate community was sampled using a hand net with an opening of 25 cm in diameter and a mesh size of 500  $\mu\text{m}$ . The kick sampling technique was used in different microhabitats (banks, the centre of the channel, areas with different types of sediment, and with the presence/absence of macrophytes) and the collection was timed (5 min) to ensure the same sampling effort. Therefore, the abundance of macroinvertebrates per site was expressed as the total number of individuals per catch per unit of effort (hereafter ind. CPUE).

This sampling was carried out following the Water Framework Directive (INAG IP 2008). In the laboratory, organisms were first sorted and separated from the rest of the sample to be taxonomically identified by family and, when possible, to species level, with the aid of a binocular magnifier and dichotomous keys, such as Tachet et al. (2010). The macroinvertebrates were then separated taxonomically and weighed after drying for 24 h in an oven at 60°C to determine their dry biomass, following Stenroth and Nyström (2003).

### 2.3 | Data Analyses

We used a generalised linear model (GLM) to find possible differences in signal crayfish abundance associated with environmental factors and RHS indices across the two basins. To assess the influence of the signal crayfish and the two river basins on the macroinvertebrate community, a two-way PERMANOVA (Permutational multivariate analysis of variance) was used considering the crayfish presence (two factors: yes and no) as a fixed factor and basin (two levels: Rabaçal and Tuela) as a random factor. Abundance data were previously subjected to a logarithmic transformation, where the Bray–Curtis similarity was also used. The PERMANOVA analysis was performed with RStudio Team (2022) using the *adonis2* function (*vegan* package).

Additionally, a Canonical Correlation Analysis (CCA) was used to evaluate the influence of crayfish abundance and environmental variables on macroinvertebrate communities across sites. This analysis was conducted with RStudio Team (2022). Also, the environmental variables used in the statistical analyses were first normalised by *z*-score standardisation to ensure comparability and then selected on the basis of their low correlation and multicollinearity (e.g., pH, temperature, dissolved oxygen, total dissolved solids (TDS) and the RHS indexes (HMS and HQA)), using a correlation matrix (Figure S1) and the Variance Inflation Factor (VIF). Moreover, a similarity percentage (SIMPER) analysis with a cut-off of 90% was also used to verify the taxa that had the most influence on the dissimilarity between the two factors in question (i.e., basin and crayfish). These three analyses were carried out using the software Primer 6 (Clarke and Gorley 2006) (version 1.0.3, Primer-E Ltd., Plymouth).

For the macroinvertebrate community, the data obtained after counting and identifying the macroinvertebrates was placed in a taxa-site matrix and used to calculate abundance (N), richness (S), the Shannon-Wiener diversity index ( $H'$ ) and Pielou's evenness ( $J'$ ). These calculations were carried out using the DIVERSE function in the software Primer 6 (Clarke and Gorley 2006) (version 1.0.3, Primer-E Ltd., Plymouth). To study the effects of the signal crayfish on functional diversity, a matrix of functional traits was created, where the organisms identified were divided according to functional characteristics (5 traits and 29 categories), following Tachet et al. (2010) (Table S1). These traits were: body size, life cycle, feeding habits, habitats and current velocity. A fuzzy coding approach was used following Chevenet et al. (1994), where each value from 0 to 5 was assigned to each category of each trait, taking into account the taxon's affinity for a certain characteristic. The greater the affinity of a taxon for a

category, the higher the value given. In this way, the fuzzy coding technique is able to understand the variability in the affinity of a given taxonomic group for the various categories of a trait (Statzner and Bêche 2010). These values were then standardised on a scale of 0 to 1 to give the same weight to all the traits. The combination of the taxa-trait matrix and the relativised taxa-site matrix made it possible to obtain the community-weighted mean for each trait (CWM) and Rao's quadratic entropy coefficient (FRAO). The CWM enables the comparison of macroinvertebrate communities across different treatments based on their functional composition, while calculating the FRAO provides a general understanding of each community's functional diversity. These calculations were carried out using Excel Macro (Lepš et al. 2006; <http://botanika.bf.jcu.cz/suspa/FunctDiv.php>).

To investigate differences between the abundance of signal crayfish and environmental variables, while accounting for potential differences between basins, several statistical models and tests were used and chosen according to the nature of the data and whether it met the assumptions of normality and homoscedasticity, checked visually through residual distribution, but also using tests such as the Shapiro–Wilk test (normality) and Levene's test (homoscedasticity). The GLMMs included one of the macroinvertebrate indexes as the response variable, environmental variables and the abundance of crayfish as fixed effects. Basin was included as a random effect to account for spatial variability. Count data, namely abundance and richness data, were fitted with poisson and binomial negative distributions, respectively. The remaining data (Shannon, evenness, biomass and functional diversity data—FRAO and CWM) analyses were carried out with Gaussian distribution. However, when the data failed to meet the assumptions even after transformation, a Beta distribution was applied for indices ranging between 0 and 1, while a Tweedie distribution was used for datasets containing zeros. To assess the effect of the interactions between crayfish abundance and the different environmental factors on the macroinvertebrate community, a post hoc analysis was carried out, where the slope of the continuous predictors at different levels of crayfish abundance (minimum, median and maximum) was estimated using the *emmeans* function (*emmeans* package), followed by pairwise comparisons to evaluate differences in these trends. All statistical analyses were carried out in RStudio Team (2022), with the models constructed using the *glmmTMB* package.

## 3 | Results

### 3.1 | Environmental Characterisation

Temperature varied between 16.1 (B5) and 23°C (R7) (Figure S2A); dissolved oxygen between 7.98 (B1) and 9.47 mg/L (T8) (Figure S2B); water conductivity between 24.5 (R11) and 57.7  $\mu$ S/cm (T1) (Figure S2C); total dissolved solids (TDS) between 5 (B6) and 28.9 mg/L (B1) (Figure S2D); pH between 6.45 (B4) and 7.1 (R6) (Figure S2F) and altitude between 385 (M1 and R1) and 843 m (B6) (Figure S2E and Table S2).

Concerning RHS results, the HQA scores for the sites in both basins were generally quite high obtaining the 'Excellent' class 1,

**TABLE 1** | Results of the PERMANOVA analysis on macroinvertebrate communities along river basins and the presence or absence of crayfish.

Source	dF	SS	MS	Pseudo-F (df <sub>1</sub> , df <sub>2</sub> )	P_perm	Unique_perms	P_MC
Basin	3	0.826	0.275	1.79 (3, 63)	<b>0.046*</b>	999	0.006
Crayfish	30	4.522	0.15	2.33 (30, 36)	<b>0.02*</b>	999	0.015
Basin × Crayfish	33	5.348	0.162	1.83 (33, 33)	<b>0.003*</b>	999	0.011
Total	66	10.69					

Note: The asterisk and bold indicate significant values ( $p < 0.05$ ).

with the exception of the site Rab 6 (class 2—‘Good’) (Tables S3 and S4). HMS results showed that 58.8% of the Rabaçal River basin studied sites were classified as ‘class 1—Pristine’ and ‘class 2—Predominantly unmodified’ (Table S5). This situation worsened slightly in the Tuela River basin (Table S6), where 29.4% of the studied sites are classified as ‘class 4—Significantly modified’ or ‘class 5—Severely modified’, indicating a higher influence from man-made structures. In terms of hydromorphological quality, the Rabaçal River basin ended up with a greater number of sites with ‘class 1—Excellent’ quality (6), compared to the Tuela River basin with only 4 ‘Excellent’ sites (Table S7). The RQI index (Table S8) showed that the Rabaçal River basin is the one with the best riparian quality, where most of the sites had ‘class 1—Very high’ quality (76.4%). In contrast, the Tuela River basin only had 47.1% of the sites with ‘class 1—Very high’ quality.

## 3.2 | Biotic Characterisation

### 3.2.1 | Crayfish Abundance

When examining the influence of environmental factors and indexes on crayfish abundance, only the HQA index showed a significant relationship ( $z = -2.102$ ;  $p < 0.01$ ), with HQA decreasing as crayfish abundance increased (Table S9). The Tuela River basin recorded an average abundance ( $\pm$ SD) of 28.3 ( $\pm$ 26.4) ind. CPUE, compared to Rabaçal with 16.4 ( $\pm$ 16.3) ind. CPUE (Figure S3). T2 was the site with the highest average abundance of crayfish with a maximum of 57.3 ind. CPUE. In invaded sites, B4 was the site with the lowest average abundance with 0.7 ind. CPUE (Table S10).

### 3.2.2 | Taxonomic Diversity

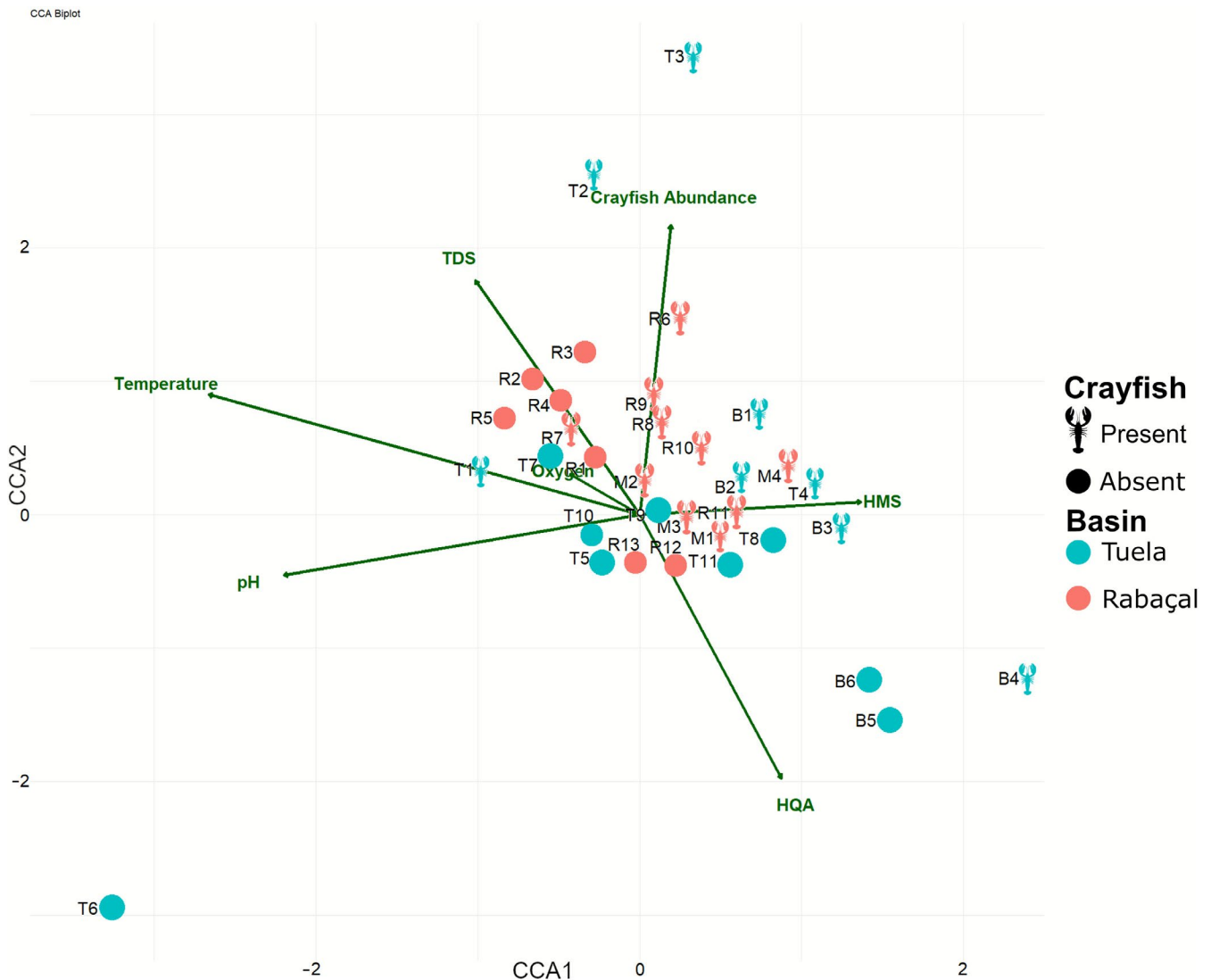
A total of 38,529 organisms representing 133 freshwater macroinvertebrate taxa were identified (Table S11). The majority of these taxa belonged to the phylum Arthropoda. Of these, arthropods comprised 123 taxa from several different orders. The most abundant organisms were dipterans and ephemeropterans, with 9178 and 9035 individuals collected, respectively. Overall, the most abundant taxa were Chironomidae, Simuliidae (Diptera), *Onychogomphus uncatus* (Odonata), *Leuctra* (Plecoptera), *Chimarra marginata*, *Hydropsyche* (Trichoptera), *Ephemerella*, *Baetis* and *Habrophlebia* (Ephemeroptera). A total of 46 small individuals of signal crayfish were also collected.

The PERMANOVA results indicated that there are significant differences in the macroinvertebrate communities between

basins (Pseudo-F<sub>(3,63)</sub> = 1.79,  $p = 0.046$ ), and sites invaded and uninvaded by the crayfish (Pseudo-F<sub>(30,36)</sub> = 2.33,  $p = 0.02$ ), which was further supported by pairwise comparisons. Additionally, there was a significant interaction between basin and invasion status (Pseudo-F<sub>(33,33)</sub> = 1.83,  $p = 0.003$ ) (Table 1).

With the CCA performed on the environmental data it was possible to separate the 34 sites into some larger groups (Figure 2), one that ends up being divided by the Rabaçal (Mente and Rabaçal sites are closer to each other) and Tuela (Tuela and Baceiro sites are more dispersed) River basins. The other key grouping is based on the presence or absence of signal crayfish, without disregarding the influence of other variables, such as TDS, temperature and pH. CC1 explains 31.32% of all the variation and CC2 explains 24.45% (Table S12). Of all the variables analysed, crayfish abundance ( $F = 2.49$ ,  $p = 0.013$ ), temperature ( $F = 3.83$ ,  $p = 0.001$ ), TDS ( $F = 2.22$ ,  $p = 0.007$ ) and pH ( $F = 2.00$ ,  $p = 0.007$ ) significantly explained the variations in macroinvertebrate community structure at the study sites. In contrast, oxygen concentration ( $F = 0.90$ ,  $p = 0.473$ ), the HQA index ( $F = 1.31$ ,  $p = 0.199$ ) and HMS ( $F = 0.79$ ,  $p = 0.584$ ) did not show a statistically significant effect.

The SIMPER analysis showed that the taxa that contributed most to the average dissimilarity between the Rabaçal and Tuela basins (41.25%) were *C. marginata*, *Habrophlebia/Habroleptoides*, *Brachycentrus subnubilus*, *Oligoneuriella rhenana*, Simuliidae and *Setodes argentipunctellus*, while between the absence and presence of crayfish (42.72%) were *B. subnubilus*, *C. marginata*, *Aphelocheirus occidentalis*, *Habrophlebia/Habroleptoides*, *Limnius* and *O. rhenana* (see Table S13). In biodiversity metrics, macroinvertebrate richness decreased significantly with the increase of invasive crayfish ( $z = -4.02$ ,  $p < 0.001$ ) (Figure 3A). Although abundance showed no significant relationship ( $z = -1.78$ ,  $p = 0.075$ ), it tended to be higher in uninvaded sites, with crayfish presence appearing to reduce their abundance (Figure 3B). The Pielou’s evenness index (J) (Figure 3C) showed no significant differences ( $z = -0.26$ ,  $p = 0.797$ ), while Shannon-Wiener index (H) (Figure 3D) was significantly lower with higher crayfish abundance ( $z = -2.40$ ,  $p < 0.01$ ). Macroinvertebrate biomass (Figure 3E) followed a similar pattern, being significantly higher in uninvaded sites, and increasing as crayfish abundance declined ( $z = -2.85$ ,  $p < 0.01$ ). Additionally, higher macroinvertebrate biomass was associated with significantly lower pH ( $z = -3.06$ ,  $p < 0.01$ ), oxygen concentration ( $z = -4.51$ ,  $p < 0.001$ ) and TDS ( $z = -3.49$ ,  $p < 0.001$ ). No significant changes in biodiversity metrics were observed in relation to environmental factors or their interactions with crayfish abundance (Table S14).



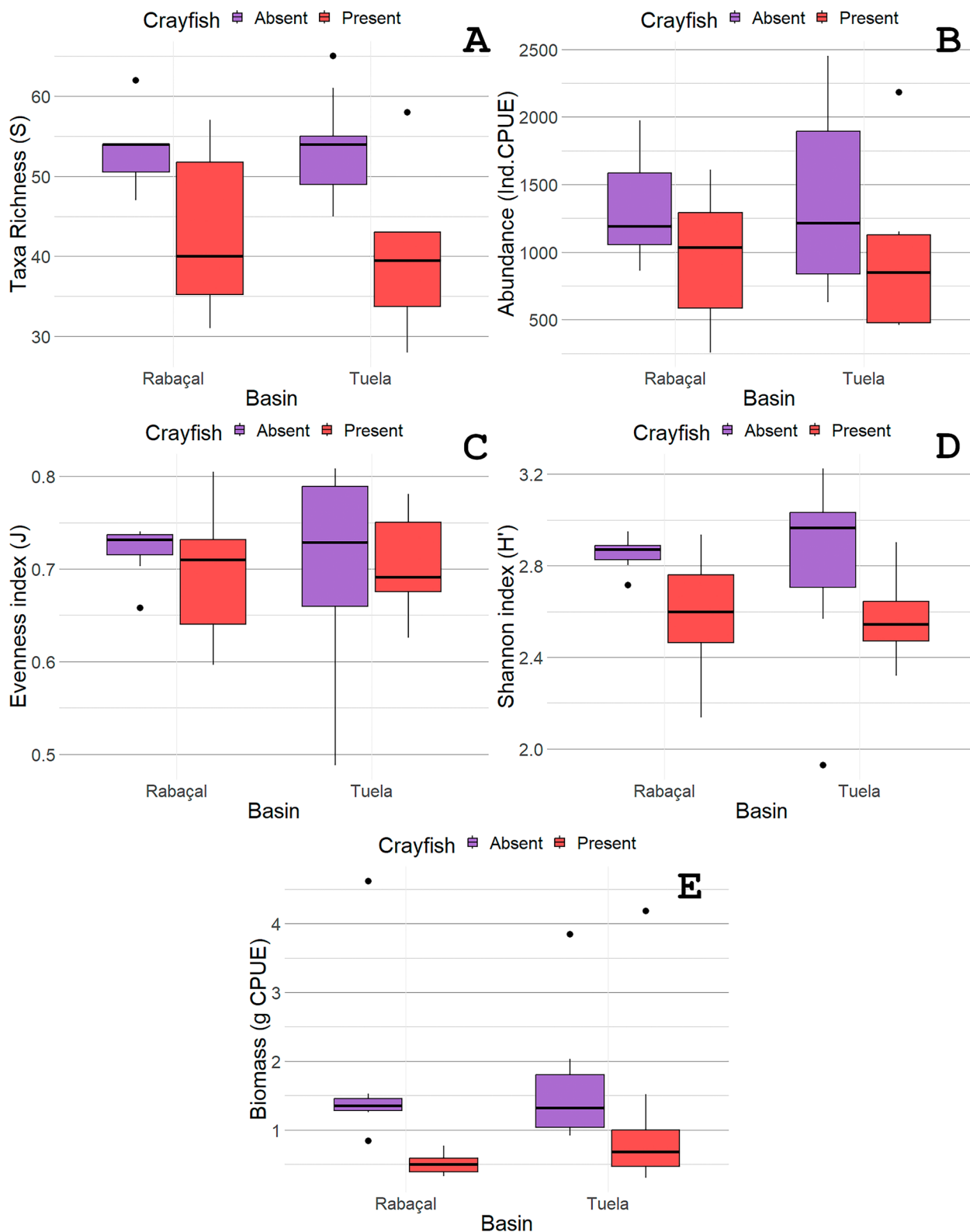
**FIGURE 2** | Canonical correlation analysis (CCA) showing the arrangement of the 34 sampling sites based on the abiotic factors measured and chosen. CCA1 explains 31.3% of the constrained variance, and CCA2 explains 24.5%. Together, these axes account for 55.8% of the total explained variation.

### 3.2.3 | Functional Diversity

Regarding the five traits used (Figure S4), the functional diversity (FRAO) revealed weaker, marginal and context dependent patterns (Table S15). In contrast, consistent responses to crayfish abundance were mainly detected through CWM metrics, especially for body size, feeding habits and habitat preferences. In general, the CWM values for the body size trait indicated that the macroinvertebrate community was typically dominated by organisms between 0.5–1.0 cm in body size (Figures 4A and S5). Higher crayfish abundance significantly decreased the proportion of smaller organisms (CWM '<0.5 cm';  $z = -6.17$ ,  $p < 0.001$ ) (Figure 4A), while increasing the proportion of those in the categories 2–4 cm (CWM '2–4 cm';  $z = 2.17$ ,  $p < 0.05$ ) and larger than 8.0 cm ( $z = 5.90$ ,  $p < 0.001$ ) (Figures S4C and S5E), the latter mainly reflecting the presence of signal crayfish in invaded sites. For the life cycle trait (Figure S6), higher crayfish abundance was negatively associated with taxa with longer life cycles (CWM '> 1 year';  $z = -3.00$ ,  $p < 0.01$ ) and positively with those with shorter life cycles than 1 year (CWM '< 1 year';  $z = 3.00$ ,  $p < 0.01$ ) (Table S16).

The CWM results showed a community with increased presence of shredder and scraper macroinvertebrates (Figures 4B,C, and S7), as well as more taxa associated with coarse sediments and macrophyte habitats (Figures 4D–F and S8). Regarding the trait feeding habitats, crayfish abundance was positively associated with scrapers ( $z = 2.82$ ,  $p < 0.01$ ) and negatively with piercers ( $z = -3.28$ ,  $p < 0.01$ ) (Figure 4B,C). The CWM results for the habitat trait showed that at higher crayfish abundance organisms associated with microphyte ( $z = -3.10$ ,  $p < 0.01$ ), silt ( $z = -2.67$ ,  $p < 0.01$ ), and mud ( $z = -2.92$ ,  $p < 0.01$ ) declined, whereas those linked to gravel habitats increased ( $z = 3.92$ ,  $p < 0.001$ ) (Figure 4D–F and S8F; Table S18). Similarly, the current velocity trait revealed that, as crayfish became more abundant, taxa adapted to fast-flow conditions ( $z = 3.13$ ,  $p < 0.01$ ) became more prominent, while those preferring slow ( $z = -3.01$ ,  $p < 0.01$ ) to null flow ( $z = -2.10$ ,  $p < 0.05$ ) declined (Figure S9; Table S19).

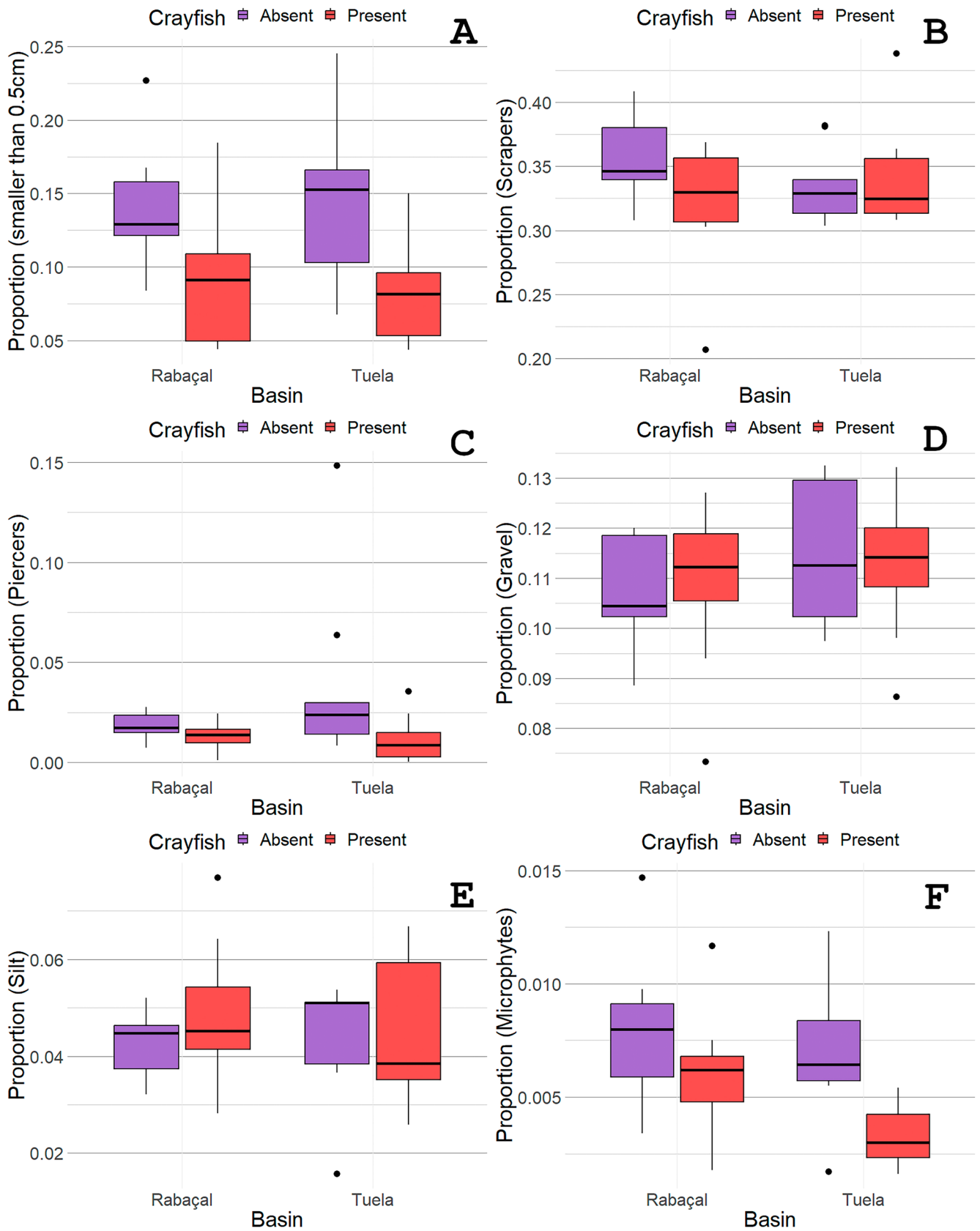
The analysis of functional diversity (FRAO and CWM) in response to environmental variables revealed some significant



**FIGURE 3** | Richness (A), Abundance (B), Pielou's evenness (C), Shannon-Wiener diversity (D) and Biomass (E) of the macroinvertebrate communities in the Rabaçal and Tuela River basins with presence (red) and absence (purple) of signal crayfish. Boxplots show median values (central line), the range from the 25th to 75th percentile (box) and the largest and lowest value within 1.5 times interquartile range below and above the 25th and 75th percentile (whiskers), and dots represent extreme values.

results and interactions with crayfish abundance, though no consistent trends emerged across traits (Table S15–S19). Some of the clearest trends involved HQA and pH. Higher HQA reduced

the proportion of mid-sized macroinvertebrates (CWM '1–2 cm';  $z = -2.41$   $p < 0.01$ ), while under high crayfish abundance, this trend shifted towards an increase in larger-bodied taxa [CWM



**FIGURE 4** | Box diagram of the proportion of macroinvertebrates (CWM) for the Body size [ $<0.5\text{cm}$  (A)]; the Feeding habits [Scrapers (B) and Piercers (C)] and the Habitats traits [Gravel (D), Silt (E) and Microphytes (F)] for the Rabaçal and Tuela River basins considering the presence (red) or absence (purple) of the signal crayfish. Boxplots show median values (central line), the range from the 25th to 75th percentile (box), and the largest and lowest value within 1.5 times interquartile range below and above the 25th and 75th percentile (whiskers), and dots represent extreme values.

'1–2 cm' ( $z = -2.07$ ,  $p < 0.01$ ); CWM '2–4 cm' ( $z = 2.16$ ,  $p < 0.01$ ) and '> 8 cm' ( $z = 2.07$ ,  $p < 0.01$ ). Feeding habits were also influenced, with higher habitat quality favouring deposit feeders ( $z = 2.99$ ,  $p < 0.01$ ), scrapers ( $z = 3.05$ ,  $p < 0.01$ ), while reducing the prevalence of predators ( $z = -4.27$ ,  $p < 0.001$ ); these effects were amplified under high crayfish conditions (Table S16). On the other hand, higher pH values, together with crayfish abundance, promoted an increase of organisms associated with slow-flowing habitats [CWM 'null' ( $z = 2.59$ ,  $p < 0.05$ ) and 'slow' ( $z = 4.15$ ,  $p < 0.001$ )] and finer substrates such as silt ( $z = 2.75$ ,  $p < 0.01$ ) and mud ( $z = 2.80$ ,  $p < 0.01$ ) (Tables S18 and S24). Both pH and the HQA index also influenced the life cycle trait, with crayfish abundance reinforcing these effects. Higher pH favoured taxa with longer life cycles (> 1 year) ( $z = 3.41$ ,  $p < 0.001$ ), while higher HQA promoted those with shorter life cycles (< 1 year) ( $z = 4.57$ ,  $p < 0.001$ ) (Table S16).

## 4 | Discussion

Our results highlight that the recent introduction of the signal crayfish in the Montesinho Natural Park and surrounding areas is reducing the biomass, richness and diversity of macroinvertebrate communities. Some functional groups responded negatively (e.g., piercers and periphyton-associated macroinvertebrates) and others positively (e.g., scrapers and gravel-associated macroinvertebrates) to the presence of the signal crayfish. Additionally, some environmental variables (HQA, pH) appeared to amplify or reinforce the crayfish-related changes in the macroinvertebrate community, rather than acting as independent drivers of distinct community patterns.

### 4.1 | Environmental Characterisation

Although the Rabaçal and Tuela basins share several environmental characteristics, there are some differences, mainly in altitude, temperature, conductivity and TDS. In general, the Tuela River basin has a higher altitude (mainly in more isolated sites in the upstream part of the Baceiro River) and higher conductivity and TDS values (mainly downstream of the Tuela River). These slightly higher conductivity and TDS values could be related to a greater human disturbance (Carvalho et al. 2022; Sousa et al. 2019).

The RHS results indicate low human disturbance and good longitudinal and lateral connectivity in the river corridor (Raven et al. 1998), with a great diversity of habitats available (Teixeira et al. 2010). However, the lower scores in the Tuela River basin still demonstrate the existence of some anthropogenic influence on riparian habitats through the presence of human structures (Raven et al. 1998). Despite the environmental differences, the studied sites have proved to be very similar and still provide excellent conditions for many macroinvertebrate species. The very low human disturbance detected is crucial for interpreting the main findings, as it reduces potential bias from varying environmental conditions, facilitating the comparison of invaded and uninvaded sites and the detection of signal crayfish impacts on macroinvertebrate communities. This pattern is further supported by multivariate analysis, which is discussed in more detail below.

### 4.2 | Taxonomic Diversity

From the 34 sampling sites, signal crayfish were found in 18, with higher abundance in the Tuela River basin. The lower abundance of signal crayfish in most Baceiro River sites (except B1) indicates a more recent and ongoing invasion in the upstream direction, resulting in a lower ecological impact observed in this watercourse (Sousa et al. 2019). Macroinvertebrate communities differed significantly between basins, as well as between invaded and uninvaded sites, with the CCA highlighting the influence of the signal crayfish, temperature, TDS and pH on community composition. When examining the group of invaded and uninvaded sites, the strong alignment of invaded sites along the crayfish abundance vector emphasises the significant impact of crayfish on macroinvertebrate communities. The CCA highlighted the strong influence of invasive crayfish on community composition, with invaded sites aligning closely with the crayfish abundance vector. Although some environmental variables (e.g., temperature, TDS and pH) also showed statistical significance, their influence appears secondary. Sampling was carried out in mountain rivers with low human disturbance, where natural variation in altitude, geology and vegetation cover likely drives local differences in physicochemical conditions (Bonacina et al. 2023; Maqbool et al. 2012). These differences may, in turn, affect the metabolism, development and composition of the community (Bonacina et al. 2023; Füreder et al. 2006; Olson and Hawkins 2017). For instance, the proximity of TDS to crayfish abundance in the ordination may reflect indirect effects of crayfish activity, such as sediment resuspension through burrowing and feeding (Angeler et al. 2001; Souty-Grosset et al. 2016). Nonetheless, the dominant pattern observed remains the shift in community composition associated with crayfish invasion.

The increase of signal crayfish abundance significantly decreases the biomass, richness and Shannon index of the macroinvertebrate community; this observation is in line with several previous studies (Carvalho et al. 2022; Galib et al. 2021; Twardochleb et al. 2013). Although the abundance decreased in invaded sites, the change was not significant. However, the lower biomass suggests that invasive crayfish may be altering community composition rather than simply reducing abundance (Albertson and Daniels 2016; Mathers et al. 2016). This implies that larger organisms are more affected, as crayfish predation is size-selective, targeting larger, more vulnerable taxa while favouring the remaining taxa (Stenroth and Nyström 2003). So, this decrease in taxonomic diversity can be caused by multiple factors, including predatory pressure, as suggested by the lower macroinvertebrate biomass (Stenroth and Nyström 2003), but also indirectly by loss of habitat complexity, trophic cascades or changes in decomposition processes and nutrient cycling (Bondar et al. 2005; Jackson et al. 2014; Nyström et al. 1996; Ruokonen et al. 2012). Some studies argue that the decrease in diversity and density of macroinvertebrates is related to the abundance of crayfish (Nyström et al. 1996; Szydlowski et al. 2023). Our results show a similar pattern, with sites with a recent invasion showing a lower abundance of crayfish but a higher abundance of macroinvertebrates (1152 and 2181 ind. CPUE in B3 and B4, respectively) compared to other invaded sites. However, some studies, such as Albertson and Daniels (2016),

have shown that in certain cases the presence of invasive crayfish can increase macroinvertebrate abundance. Nevertheless, these authors do not refute the impacts of crayfish on macroinvertebrates but argue that these may be context dependent.

### 4.3 | Functional Diversity

In terms of functional diversity (FRAO), no major differences were found for the presence of signal crayfish, despite the tendency for functional diversity to decrease in invaded sites, as shown in other studies (Carvalho et al. 2022; Mathers, White, Fornaroli, and Chadd 2020; Mathers, White, Guareschi, et al. 2020). This may be due to the high richness of the macroinvertebrate community, where several taxa share similar niches or ecological functions, leading to functional redundancy (Schmera et al. 2012). In such cases, species can compensate for each other (Schmera et al. 2012), which makes it difficult to detect significant differences in functional diversity between invaded and uninvaded sites, especially in ecosystems where the invasion is still very recent. Notably, in a manipulative field study in the Montesinho Natural Park, Carvalho et al. (2022) showed that functional diversity decreased with the increase in crayfish abundance. This decrease may also affect the functioning of the ecosystem via a reduction in productivity, nutrient cycling and litter decomposition (Guareschi et al. 2021).

Although FRAO showed no significant differences, character-based analyses (CWM) revealed changes in specific macroinvertebrate groups, highlighting potential functional changes masked by redundancy (Guareschi et al. 2021; Schmera et al. 2012). Decreases or increases in specific CWMs suggest selective pressures, possibly caused by crayfish, despite no overall changes in FRAO. Regarding the 'Body size' trait, CWM showed a lower proportion of taxa smaller than 0.5 cm in invaded sites, the Coleoptera order (principally the Elmidae family) contributing most to these differences. The SIMPER analysis also corroborates this difference, with the genus *Limnius*, a riffle beetle, proving to be a main contributor to the differences, with lower abundance observed in invaded sites. Ruokonen et al. (2016) found that the density of riffle beetles decreased in the presence of crayfish and Guan and Wiles (1998) demonstrated that signal crayfish feed on Elmidae, although predation pressure varies seasonally and with the signal crayfish age. Riffle beetles are especially vulnerable to predation because of their semivoltine life cycle, which is mostly aquatic, and also show limited locomotion (Aroviita and Hämäläinen 2008; Elliott 2008; Ruokonen et al. 2016).

In this study, as the abundance of crayfish increased, there was a decrease in organisms with longer life cycles (> 1 year), with an increase or replacement of macroinvertebrates with shorter life cycles (< 1 year). This pattern may occur because short-lived organisms are more resilient to the direct or indirect effects of crayfish, as they recover faster (Mathers, White, Guareschi, et al. 2020). In contrast, species with longer and more complex life cycles, such as Odonata, may be more vulnerable to these pressures (Siesa et al. 2014).

Significant differences were also found for the piercer group, with a lower proportion in invaded areas. When observing

which organisms had the greatest affinity, most were from the suborder Heteroptera. SIMPER analysis identified *A. occidentalis* as one of the key taxa contributing to the differences detected, as it was almost absent in invaded sites. Although Heteroptera is typically agile and less affected by crayfish predation (Gherardi 2007; Nyström et al. 1996, 1999), studies have shown some predation by crayfish species such as *Astacus leptodactylus* and *Faxonius limosus* on this group (Šidagyte et al. 2017; Vojtkovská et al. 2014). While crayfish predation affects these organisms, competition for food must also be considered, as *Aphelocheirus* preys on mayflies and caddisfly larvae (Carbonell et al. 2011).

The proportion of scrapers was surprisingly higher with the increase in the abundance of the signal crayfish. This could be possibly related as some crayfish-invaded sites have shown an increase in periphyton biomass (Bobeldyk and Lamberti 2008; Scordo et al. 2023). The increase in periphyton by the crayfish may be related to both the removal of senescent algal cells and nutrient resuspension (Stenroth and Nyström 2003). Additionally, the SIMPER analysis revealed an increase in Baetidae (*Centroptilum* and *Procloeon*) and Heptageniidae (*Epeorus* and *Rhithrogena*) scrapers at invaded sites. These organisms are more adept at escaping predation (Peckarsky 1980), thus benefiting from reduced predation pressure and increased food resources. Additionally, the greater abundance of some mayfly taxa likely contributed to the higher proportion of macroinvertebrates adapted to fast currents. This pattern could be caused by less predation pressure in high-flow habitats, given that adult crayfish show a preference for pool habitats over fast-flowing sections (Clark et al. 2013; Harrison et al. 2006) and their upstream movement could be limited by the current direction and intensity (Kerby et al. 2005). As a result, these microhabitats may be serving as refuges for these mayflies.

In the habitats trait, the CWM results also showed significant differences for the Microphytes habitat, with a lower proportion of organisms associated with this microhabitat in the invaded sites. Mainly species of Trichoptera (Limnephilidae) and Gastropoda showed affinity for this microhabitat. The Limnephilidae are a group that responds negatively to the presence of crayfish, mainly due to predation, as shown by some studies (Laguer et al. 2014; Renai and Gherardi 2004; Šidagyte et al. 2017). Gastropods are particularly vulnerable to the invasion of several crayfish species (Galib et al. 2021; Mathers et al. 2016; Szydłowski et al. 2023; Twardochleb et al. 2013), as evidenced by our results with the absence of *Radix peregra* in invaded sites. Their low mobility and large size make them easy and profitable prey for crayfish (Stenroth and Nyström 2003). This decline in low-mobility organisms (gastropods) may benefit more agile taxa, namely the mayflies Heptageniidae and Baetidae (*Centroptilum*, *Procloeon* and *Cloeon*), which, as mentioned above, were more abundant in the invaded sites. Therefore, the mayflies could also be responding positively to the suppression of these invertebrates by the signal crayfish due to less competition for the same resources (Hansen et al. 2013; Hertonsen et al. 2008; Nyström et al. 1999). However, more studies are necessary to confirm these assumptions.

Previous studies suggest that signal crayfish contribute to sediment composition changes by altering the structure of substrates

through bioturbation/burrowing, increasing the amount of suspended sediment (Albertson and Daniels 2016; Usio and Townsend 2004). Our data support this pattern, showing an increase in macroinvertebrates associated with gravel and a decrease in those linked to silt and mud as crayfish abundance increases. However, it is worth noting that some studies have reported the opposite effect, with crayfish activity favouring finer substrates (Johnson et al. 2011; Mathers, White, Guareschi, et al. 2020).

Besides the impacts of crayfish, functional diversity was also analysed in relation to environmental variables. However, most of the effects found were inconsistent and often context dependent, with notable trends for habitat quality (HQA) and pH. Despite overall good habitat quality and minimal pH variation (6.45–7.1), high-crayfish abundances in higher-quality habitats favoured deposit feeders and scrapers, while reducing predators. Most of these patterns were observed with just an increase in crayfish abundance, suggesting that habitat complexity further supports macroinvertebrate populations that have already shown resilience to crayfish presence (Mathers, White, Guareschi, et al. 2020), while also buffering negative impacts through refuge availability (Usio and Townsend 2004).

In mountain rivers with little human activity, differences in water chemistry parameters such as pH are usually the result of geology (Füreder et al. 2006; Lewin et al. 2015), yet its interaction with crayfish abundance suggests a possible influence of crayfish bioturbation. Crayfish-induced sediment disturbance and alterations in organic matter dynamics may raise pH, which could lead to changes in habitats favouring taxa adapted to finer substrates and slower currents. While direct evidence of this relationship is limited, studies have shown that bioturbation can resuspend sediments and organic matter, influencing water chemistry (Angeler et al. 2001; Harvey et al. 2014; Sanders et al. 2023). Anyway, further research is needed to clarify these potential interactions.

These results of environmental variables on functional diversity compared to those of taxonomic diversity show a greater ability to detect small changes in the macroinvertebrate community (Statzner and Bêche 2010). Nevertheless, our results strongly support crayfish abundance as the main driver of change in this community. This follows previous studies in which the effects of non-native crayfish were able to override environmental effects (Kreps et al. 2016; Mathers et al. 2016; Twardochleb et al. 2013).

#### 4.4 | Management Implications

Understanding the potential effects of signal crayfish on macroinvertebrates is important because these organisms play several key roles in ecosystems, ranging from deposit feeders, shredders, scrapers, filter feeders, piercers, predators and parasites (Tachet et al. 2010). In this way, these organisms influence not only the functioning of the food chain (Klecka and Boukal 2013) but also other processes such as nutrient cycling, decomposition (Santonja et al. 2020; Swan et al. 2021) and primary and secondary production (Lamberti et al. 1989; Liboriussen et al. 2005). Given these functions, the decline in diversity, biomass and changes in community composition highlighted in this study

are alarming, as they may trigger cascading ecological effects, disrupting the dynamics of the food chain, reducing the resilience of the ecosystem and affecting nutrient cycling (Carvalho et al. 2022). These changes can also affect higher trophic levels (e.g., fish, amphibians and mammals) dependent on macroinvertebrates as a food source (Vaeßen and Hollert 2015).

The results reported here relate to a large spatial area and two different river basins, establishing a solid baseline for future studies to compare the progression of the signal crayfish and their ecological impacts in macroinvertebrate communities. Future studies could investigate some gaps left by this study, like whether the primary mechanism driving these changes is direct predation by crayfish or indirect effects (e.g., trophic cascades, habitat modification or alterations in nutrient cycling). For this, manipulative experiments should be carried out. Since this study only assessed a single summer period, future investigations should also consider seasonal and interannual variations, as crayfish activity fluctuates with temperature and environmental conditions. Despite these gaps, this study is also important to educate and increase the awareness of citizens about the ecological and economic problems mediated by freshwater non-native species such as the signal crayfish.

Given the negative effects described for macroinvertebrate communities, it is crucial to adopt management actions that prevent the spread and introduction of this (or other) non-native species in Montesinho Natural Park, an area of high conservation interest (Castro et al. 2010). Control measures like trapping programmes could help reduce the abundance of crayfish while promoting macroinvertebrate community recovery (Moorhouse et al. 2014). In addition, long-term monitoring should be implemented to follow the spread and evolution of the signal crayfish populations and their environmental impacts.

## 5 | Conclusion

This study highlights the significant impact of signal crayfish on key freshwater macroinvertebrate communities, where this non-native species negatively affects species richness, biomass and diversity, affecting groups such as scrapers, drillers and taxa associated with gravel and periphyton. These findings are consistent with patterns observed in other invaded freshwater ecosystems around the world, where non-native crayfish disrupt food webs, alter nutrient cycling and threaten biodiversity (Gallardo et al. 2016; Mathers et al. 2016; Vaeßen and Hollert 2015). Despite the low level of human disturbance in the Montesinho Natural Park and surrounding areas, the recent introduction of the signal crayfish could be a serious problem for macroinvertebrate communities and other organisms by jeopardising several key ecosystem functions. On the other hand, the low anthropogenic influence and the still high habitat diversity in the study area may provide functional redundancy, helping to mitigate, so far, the ecological impacts of the signal crayfish (Carvalho et al. 2022; Schmera et al. 2012). Therefore, the results reported here suggest that widespread and long-term ecological disruption of macroinvertebrate communities, with possible cascading effects on other taxonomic groups, is happening in the Montesinho Natural Park and surrounding areas. Preventing further introductions and

spread of non-native signal crayfish is crucial to mitigate the extent of those impacts.

## Author Contributions

Conceptualisation: A.B.N., A.T., R.S. Developing methods, conducting the research: A.B.N., B.O., S.V., A.T., R.S. Data analysis, data interpretation: A.B.N., J.P., S.V., R.S. Preparation of figures and tables: A.B.N., J.P. Writing: A.B.N., J.P., S.V., A.T., B.O., R.S.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** fwb70179-sup-0001-supinfo.pdf.