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Master thesis in Biodiversity, Ecology and Global Change

Work made under supervision of:
Professor Doutor Ronaldo Sousa

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Impacto do lagostim de sinal (*Pacifastacus leniusculus*) nas comunidades de macroinvertebrados

Resumo

As espécies invasoras são uma das principais ameaças aos ecossistemas aquáticos. Os seus impactos são diversos, perturbando o funcionamento e a estrutura da cadeia alimentar, conduzindo potencialmente à perda da biodiversidade. Como omnívoros, os lagostins têm uma capacidade impressionante de modificar o seu ambiente a vários níveis, o que afeta diretamente grupos de organismos como os macroinvertebrados, que se tornam suas presas. Os macroinvertebrados são consumidores nos níveis intermédios das cadeias alimentares, sendo influenciados tanto por forças *top-down* e *bottom-up*, desempenhando funções fundamentais dentro dos ecossistemas aquáticos. Neste sentido, pretendemos investigar os impactos da recente introdução do lagostim-sinal (*Pacifastacus leniusculus*) no Parque Natural de Montesinho, uma área protegida com muito baixa perturbação humana. O principal objetivo deste estudo foi avaliar os efeitos do lagostim do sinal na comunidade de macroinvertebrados de água doce. Para isso, 34 locais (18 invadidos e 16 não invadidos) nas bacias dos Rios Rabaçal e Tuela foram amostrados no verão de 2022. Foram feitas comparações entre bacias e locais invadidos e não invadidos quanto à abundância, biomassa, riqueza e índices de diversidade e funcionalidade das comunidades. Ao todo foram identificados 38 529 organismos, pertencentes a 133 taxa de macroinvertebrados de água doce. Os nossos resultados demonstraram um declínio da abundância, biomassa, riqueza e diversidade dos macroinvertebrados, enquanto que o lagostim estava presente. Relativamente à diversidade funcional poucas diferenças foram encontradas entre locais invadidos e não-invadidos provavelmente devido à redundância funcional; contudo, o lagostim mostrou afetar determinados grupos funcionais (ex: os coletores de depósito, perfuradores e raspadores e herbívoros). Assim, o lagostim do sinal demonstrou uma grande influência sob os macroinvertebrados, demostrando a possibilidade de uma pressão seletiva. De um modo geral, os resultados deste estudo podem ser importantes para melhor compreender, prever e gerir os impactos do lagostim do sinal, sendo importante continuar a monitorizar não só as populações de lagostins, mas também as comunidades de macroinvertebrados, devido ao seu papel crucial nos ecossistemas de água doce.

Palavras-Chave: ecossistemas de água doce; espécies invasoras; índices de diversidade; diversidade funcional; *River Habitat Survey*

Impact of Signal crayfish (*Pacifastacus leniusculus*) on macroinvertebrate communities

Abstract

Invasive species are one of the main threats to aquatic ecosystems. Their impacts are diverse, disrupting the functioning and structure of the food chain and potentially leading to biodiversity loss. As omnivores, crayfish have an impressive ability to modify their environment, which directly affects groups of organisms such as macroinvertebrates, which become their prey. Macroinvertebrates are consumers at the intermediate levels of food chains and are influenced by both top-down and bottom-up forces, playing playing fundamental functional roles within aquatic ecosystems. In this sense, we intend to investigate the impacts of the recent introduction of the signal crayfish (*Pacifastacus leniusculus*) in the Montesinho Natural Park, a protected area with very low human disturbance. The main aim of this study was to assess the effects of the signal crayfish on the freshwater macroinvertebrate community. To this end, 34 sites (18 invaded and 16 non-invaded) in the Rabaçal and Tuela River basins were sampled in the summer of 2022. Comparisons were made between basins and invaded and non-invaded sites in terms of abundance, biomass, richness and indices of community diversity and functionality. A total of 38,529 organisms were identified, belonging to 133 freshwater macroinvertebrate taxa. Our results showed a decline in the abundance, biomass, richness and diversity of macroinvertebrates while crayfish were present. In terms of functional diversity, few differences were found between invaded and non-invaded sites, probably due to functional redundancy; however, crayfish were shown to affect certain functional groups (e.g., deposit feeders, drillers and macroinvertebrates associated with periphyton). Thus, the signal crayfish showed a large influence on macroinvertebrates, demonstrating the possibility of selective pressure. Overall, the results of this study may be important for better understanding, predicting and managing the impacts of signal crayfish, and it is important to continue monitoring not only crayfish populations, but also macroinvertebrate communities, due to their crucial role in freshwater ecosystems.

Keywords: freshwater ecosystems; invasive species; diversity indices; functional diversity; River Habitat Survey

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1. Introduction

Freshwater ecosystems correspond to a small area of the planet, hosting only 0.01% of the volume of all water on Earth, with lakes, rivers and reservoirs occupying 2.3% of the Earth's surface area (Reid et al., 2019). Yet, freshwaters provides habitat for more than a third of vertebrate species and is one of the most biodiverse (Dudgeon et al., 2006). Freshwater ecosystems, historically seen as islands, are isolated habitats and therefore have a great species richness and a high degree of endemism (Moyle & Leidy, 1992). Worryingly, declining populations and an increased risk of extinction have been noted in freshwater species (Collen et al., 2014). Between 1970 and 2018 The World Wide Fund for Nature showed a steeper decline in populations of freshwater species when compared to marine or terrestrial species (Almond et al., 2022). Freshwater ecosystems are affected by human activities that are responsible for habitat loss and fragmentation, pollution, overexploitation of resources and climate change (Dudgeon et al., 2006). In addition, freshwater ecosystems are highly affected by the introduction of invasive species (Strayer, 2010). Indeed, and with globalization, the distribution of species with invasive potential has been facilitated by human action (Charles & Dukes, 2008; Collen et al., 2014), leading to the homogenization of communities and putting the conservation of native species in peril (Reid et al., 2019).

The introduction of invasive species can thus add a new functional component to native communities (Gallardo et al., 2016), generating major interrelated ecological, economic and social changes (Charles & Dukes, 2008). The presence of these species thus leads to ecosystem disturbance, not only endangering native biological communities (through competition, hybridization, parasitism, infection and predation, among other possibilities), but also affecting the structure and functioning of ecosystems by altering hydrology, nutrient cycles, energy flows, habitat complexity and physical transport of materials, among other possibilities (Andersen et al., 2004; Charles & Dukes, 2008; Ehrenfeld, 2010; Sousa et al., 2009; Strayer, 2012). In addition, these species end up being responsible for high monetary losses (Cuthbert et al., 2021; Diagne et al., 2021; Mack et al., 2000).

The impacts of invasive species often depend not only on their abundance and spatial distribution, but also on their position in the food chain (Gallardo et al., 2016; Jarnevich et al., 2021; With, 2002). If they have a position in the upper levels, top-down control of the food chain is expected to affect the abundance and biomass of lower trophic levels (Pace et al., 1999). For example, the introduction of invasive predators such as rainbow trout (*Oncorhynchus mykiss*), alburnus (*Alburnus alburnus*) and spiny water flea (*Bythotrephes longimanus*) cause changes in the zooplankton community (decrease in abundance and richness), and this situation leads to the proliferation of phytoplankton (Gallardo et al., 2016; Ordóñez et al., 2010; Yan et al., 2002). In contrast, changes in primary producers and in the input of limiting nutrients, caused by the introduction of invasive species, affects the total energy available to the ecosystem, with 'bottom-up' effects propagating to the upper trophic levels (Heath et al., 2014). For example, the introduction of several invasive aquatic plants, which in some situations, despite providing food and habitat to other organisms, cause a decrease in the abundance of the native community in invaded habitats due to massive die-offs and consequent decomposition of high loads organic matter causing anoxia or hypoxia conditions highly detrimental for macroinvertebrates and fish (Gallardo et al., 2016).

Furthermore, invasive species can also act as ecosystem engineers, modifying the habitat by, for example, altering 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; Henn et al., 2016; Sousa et al., 2009). Some of these examples occur with invasive plants that manage to alter their habitat by affecting species of higher trophic levels or by competing with other native primary producers (Schultz & Dibble, 2012). For example, water chestnut (*Trapa natans*) creates huge dense masses, which with the combination of shade and high respiration on riverbeds, drastically reduces dissolved oxygen concentrations. This causes episodes of hypoxia or anoxia thus affecting the upper trophic levels (Strayer, 2010). Another example is the common reed (*Phragmites australis*) and killer algae (*Caulerpa taxifolia*), where their production of detritus and capture of fine sediments (Chisholm & Moulin, 2003; Rooth & Stevenson, 2000), affects the use of this habitat by other organisms such as fish and benthic invertebrates (Jayawardana et al., 2006). Another example is the invasion of the North American beaver (*Castor canadensis*) in Tierra del Fuego (Argentina) that caused major changes in the landscape by its construction of dikes and logging (Henn et al., 2016), introducing large amounts of plant litter into waterways creating a more lentic habitat, thus causing a habitat transformation where decomposer macroinvertebrates become more abundant than macroinvertebrates that feed on primary producers (Arismendi et al., 2020).

Several freshwater species have been introduced worldwide, both for bait and human consumption, such as crayfish (Strayer, 2010). Several crayfish with North America origin were introduced to Europe (*Pacifastacus leniusculus*, *Procambarus clarkii* and *Orconectes limosus*), some of which were released or escaped, which led to the establishment of populations in the wild and further dispersion (Charles & Dukes, 2008). However, despite being beneficial to humans in some aspects (e.g. source of protein), crayfish have become problematic invasive species, causing several ecological and economic impacts (Lodge et al., 2012; Twardochleb et al., 2013). With the introduction of invasive crayfish to Europe also came pathogens (*Aphanomyces astaci*) that brought diseases to native crayfish populations, causing local extinctions in some places (Charles & Dukes, 2008). In addition, crayfish are ecosystem engineers that increase leaf decomposition and disrupt nutrient cycling, and their digging and grazing reduce bank stability and vegetation cover (Albertson & Daniels, 2018; Creed & Reed, 2004), producing a change from a habitat with clear conditions to one with more turbid waters dominated by phytoplankton. This situation leads to a change in the composition and density of macroinvertebrate and fish communities (Albertson & Daniels, 2018; Matsuzaki et al., 2009), due to losses of trophic resources and habitat availability (Lodge et al., 1994). Nevertheless, since they are omnivores and quickly reach high densities, their introduction directly and indirectly affects various trophic levels of the native community (Carvalho et al., 2022; Strayer, 2010). Therefore, their presence will affect not only amphibians because they feed on their eggs and larvae, but also fish because they are also their prey and are indirectly competing for the same food resources (Gherardi, 2007). Yet, their trophic position in the middle of the food chain makes invasive crayfish a potential new form of food for predators such as birds and mammals (Correia, 2001). Nonetheless, in addition to the aforementioned groups, crayfish also affect macroinvertebrates through predation or other means, reducing the diversity and abundance of native macroinvertebrate communities (Ercoli et al., 2015; Galib et al., 2021; Strayer, 2010; Twardochleb et al., 2013). A general overview of the effects of invasive crayfish on aquatic communities can be seen on Figure 1.

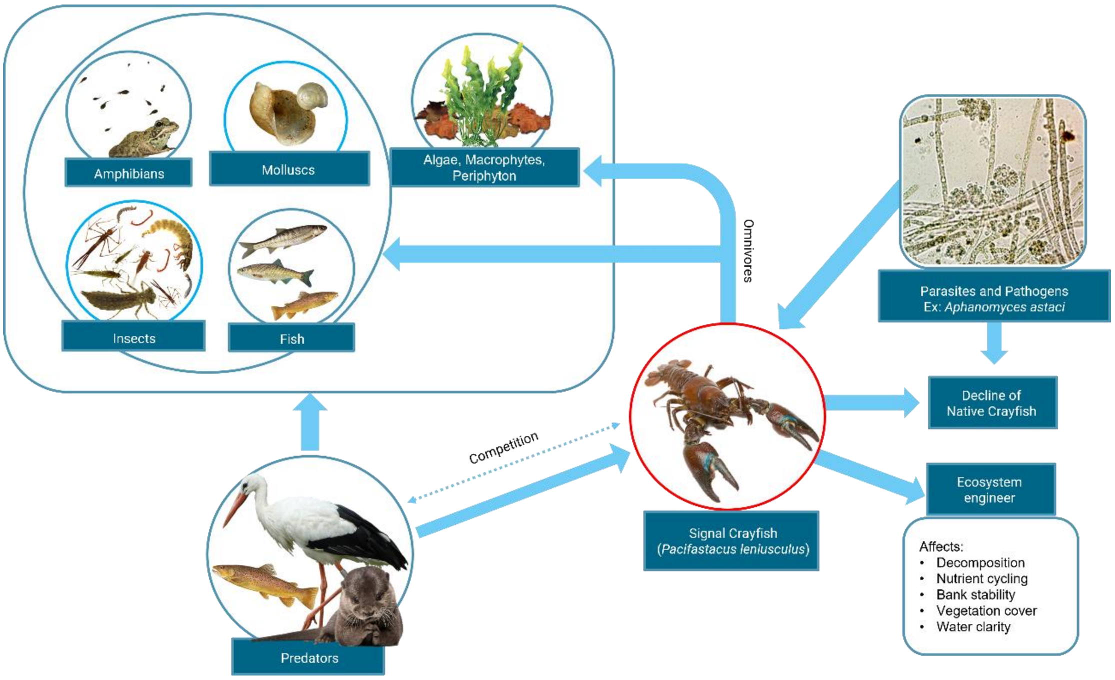


Figure 1 - Possible ecological effects of the signal crayfish *Pacifastacus leniusculus* in a hypothetical aquatic food web.

With the invasion of crayfish, besides the decrease in diversity and abundance, there has also been a transformation in the composition of the macroinvertebrate community where it seems that the communities have become richer in more agile taxa and poorer in less mobile organisms with less ability to escape crayfish predation, such as molluscs (especially gastropods) and some families of Trichoptera (Dorn, 2013; Galib et al., 2021; Hansen et al., 2013; Mathers et al., 2016). There are however some macroinvertebrate taxa that may have anti-predator responses, with changes in their life cycle and behaviour (Mathers et al., 2016), such as vertical migration and increased locomotion. For example, certain snail species have shown vertical migration in order to escape to crayfish predation (Haddaway et al., 2014; Lewis, 2001). With this, it is important to point out that the decrease and changes in the community can have cascading effects along several trophic levels. For example, with the decrease in herbivorous macroinvertebrates (gastropods) it was observed an increase in periphyton biomass in some crayfish invaded sites (Bobeldyk & Lamberti, 2008), which could free up resources benefiting other mobile herbivores (Mathers et al., 2016).

Given the above mentioned examples, and because the ecological effects may be highly context dependent, it is essential to understand the impacts that crayfish can have on macroinvertebrates, as these are organisms with key functions in freshwater ecosystems and include species with important roles as absorbers, deposit feeders, shredders, scrapers, filter-feeders, piercers, predators and parasites (Tachet et al., 2010). Therefore, because macroinvertebrates occupy various trophic positions within the food chain, they not only serve as food for other living beings (Klecka & Boukal, 2013), but will also play a key role in the ecosystem (Wallace & Webster, 1996). Such as in the translocation of materials (Wallace & Webster, 1996) and in primary productivity where macroinvertebrates will affect directly primary producers by feeding on them (Liboriussen et al., 2005; Rosemond et al., 1993), but also indirectly by bioturbation processes (Guo et al., 2022). In contrast, some studies have also observed the opposite, where macroinvertebrate grazers can increase primary productivity by removing dead cells, allowing greater penetration of light and nutrients into the algal film, and also changing the composition of the community towards more productive species (Lamberti et al., 1989). In addition, macroinvertebrates such as shredders, will also play an important role in nutrient cycling and decomposition, since they are responsible for converting and fragmenting organic matter (transforming coarse particulate organic matter, CPOM, into fine particulate organic matter, FPOM), facilitating decomposition and increasing the availability of nutrients and resources for other freshwater organisms (Lin et al., 2020; Santonja et al., 2020; Swan et al., 2021). Moreover, they are organisms that respond to a wide variety of environmental impacts, being valuable indicators of the degradation of watercourses (Wallace & Webster, 1996). Finally, sampling methodologies for these organisms are low cost and easy to implement, such as kick-net sampling (González, 2023).

In conclusion, invasive crayfish have been shown to have an enormous capacity to modify the trophic structure (Gherardi, 2007) and ecosystem functions (Mathers, White, Guareschi, et al., 2020). Therefore, is crucial to increase the understanding and predictive power of the impacts generated by these species (Strayer et al., 2006). This study will use the recent invasion of the signal crayfish (*Pacifastacus leniusculus*) in the Montesinho Natural Park to assess possible impacts on freshwater macroinvertebrates. The signal crayfish, which is native to the western Rocky Mountains of America, has already spread to other continents (Lodge et al., 2012). This species is currently experiencing a rapid growth and dispersal in rivers in north-eastern Portugal (Carvalho et al., 2022). To assess the effects of the crayfish on macroinvertebrates, several sites with and without crayfish were sampled along different rivers in the Montesinho Natural Park.

2. Study Objectives

As the introduction of the signal crayfish (*Pacifastacus leniusculus*) in the study area was recent (i.e., the first detection was made in the summer 2013 Carvalho et al., 2022), plus the fact that these invaded watercourses have a very low human disturbance, it was possible to select sites with the absence and presence of the crayfish. The macroinvertebrate community was sampled at these sites to assess the potential effects that crayfish may have on macroinvertebrates. The specific objectives of this study were:

1) Evaluate the abiotic conditions of the studied sites, using River Habitat Survey (RHS) and measurements of physico-chemical parameters;

2) To characterise the macroinvertebrate communities, comparing the data obtained with the presence and absence of crayfish in different river basins through a taxonomic approach (abundance, biomass, species richness, Shannon-Wiener index and Pielou's evenness index);

3) Evaluate macroinvertebrate communities in the presence and absence of crayfish in different river basins using a functional approach (Rao's quadratic entropy coefficient - FRAO and Community weighted mean - CWM), looking for impacts of crayfish on certain ecosystem functions.

Overall, the collected information will be used to assess the possible negative impacts of the signal crayfish in a key ecological group of organisms and can be important for future management actions aiming to control this invasive species in a protected area (i.e., direct possible management actions to the most affected areas).

3. Material and Methods

3.1 Study Area

The Montesinho Natural Park was created in 1979 and is located in the north-east of Portugal, in Trás-os-Montes, including the municipalities of Vinhais and Bragança. The protected area covers around 748 km^2 of wooded natural landscapes and traditional farming, with altitudes varying between 436 m and 1 477 m (Castro et al., 2010). The climate found in Montesinho is predominantly Mediterranean under the continental effect of the interior of the Iberian Peninsula with some Atlantic influence, with an average annual temperature of less than 12.5°C and rainfall ranging between 1000 and 1600mm, with the highest rainfall in the winter months and almost no rainfall in the summer months (Gonçalves, 1985; Sousa et al., 2015). This protected area was primarily designated for the conservation of birds, terrestrial vertebrates and plants, but its biodiversity has already suffered a great loss as exemplified by the decrease in the abundance of wolves and the spatial cover of native forests, but also with the disappearance of the bear and lynx several decades ago (ICNF, 2019).

Our study area comprised sites within and around the park, mainly on the Mente, Tuela, Rabaçal and Baceiro Rivers (Figure 2), tributaries of the Tua River. All rivers originate in Spain, belong to the Douro River basin, and have a total length of 57 km, 88 km, 102 km and 60 km, respectively. All the watercourses have low human disturbance and a similar climate in terms of rainfall and temperature (Nogueira, Teixeira, et al., 2021), making these rivers particularly interesting for studying the impacts of invasive species since there are no other relevant human disturbances (Bernardo et al., 2011; Sousa et al., 2019, 2020). They are also similar in that they all have a pool-riffle sequence, with a substrate dominated by pebbles, gravel, boulders and sand (Sousa et al., 2020). There are also some areas with lentic conditions, due to the presence of weirs and small reservoirs, where the substrate is predominantly mud (Sousa et al., 2020). The riverbanks have a landscape dominated by willows (*Salix* spp.), alders (*Alnus glutinosa*), ash trees (*Fraxinus angustifolia*) and poplars (*Populus nigra*) (Sousa et al., 2019).

Figure 2 - Rivers surveyed in this study: Mente (A), Rabaçal (B), Tuela (C) and Baceiro (D).

These rivers are rich in biodiversity, including species with higher conservation interest such as the dragonfly *Macromia splendens* (vulnerable), the pearl mussel *Margaritifera margaritifera* (endangered), the iberian desman *Galemys pyrenaicus* (endangered) and the southern water vole *Arvicola sapidus* (vulnerable) (IUCN, 2022; Oliveira et al., 2012; Sousa et al., 2015). However, invasive species such as the signal crayfish (*Pacifastacus leniusculus*), is now also present, having been detected for the first time in Portugal in the Maçãs River in 1997 (Bernardo et al., 2011). Since then, this species spread to other watercourses, namely those that were sampled in this study belonging to the Tua River basin. The first signal crayfish specimens were detected in the summer 2013 (Sousa et al., 2019).

3.2 Sampling strategies

Macroinvertebrates and crayfish were sampled during the summer of 2022 to minimise the seasonal influence on community composition and abundance. Thirty-four sites were sampled (18 invaded and 16 non-invaded) in the Mente (4 invaded), Rabaçal (6 invaded, 7 non-invaded), Tuela (4 invaded and 7 non-invaded) and Baceiro (4 invaded 2 non-invaded) Rivers (Figure 3). For abiotic characterisation, temperature (°C), oxygen (mg/L), conductivity (µS/cm), total dissolved solids (TDS) (mg/L) and pH were analysed *in situ* at all sites using a YSI EXO 2 multi-parameter probe.

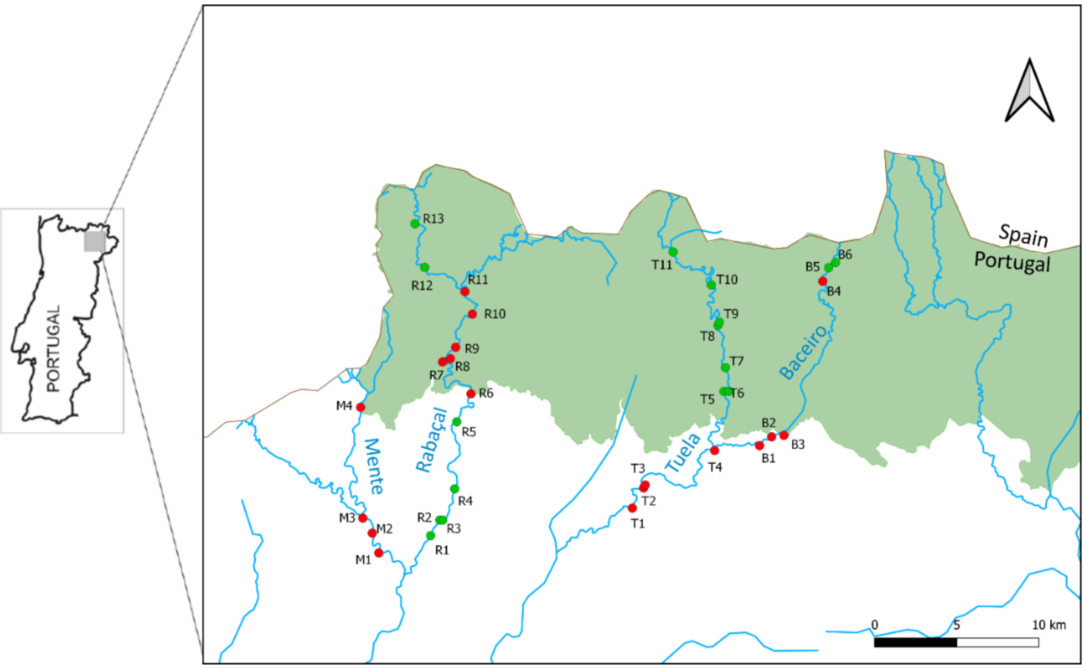


Figure 3 - Map of the surveyed area showing the location of the 34 sampling sites (18 invaded - red; 16 non-invaded - green) in Mente, Rabaçal, Tuela and Baceiro Rivers. Map produced using QGIS software (QGIS Development Team, 2022)

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: such as the type of substrate in the channels; the complexity of the structure of the vegetation on the banks; the type of aquatic vegetation; the characteristics of the habitat and the type of artificial modification found in the channel and on the banks (Raven et al., 1998). For this, the standard length of 500 metres of river channel was used for data collection at each of the sites sampled. Data on altitude, geology, slope and distance and height from the river source were also collected. All the features within the watercourse, on the banks and the adjacent river corridor were also noted *in situ*. To obtain information on the geomorphological processes at work in the various locations, the cross-section of the water and the width and height of the bank and the depth of the water were measured. The number of riffles, ponds and point bars on the site was also recorded. All this data was amassed in order to obtain the HMS and HQA indexes, which are important for ascertaining the degree of disturbance within the 34 sites. The RHS was carried out by certified person, namely the Prof. Simone Varandas (University of Trás-os-Montes and Alto Douro - UTAD).

To assess the abundance of the signal crayfish the same 34 sites surveyed to characterize the macroinvertebrates communities (Figure 3) were also sampled in August 2022. Crayfishes were captured by placing 6 to 8 funnel traps, four-five rectangular (50 × 30 × 20 cm; 0.5 cm mesh) and one-three cylindrical (43 cm diameter; 22 cm height; 1.5 cm mesh), per site for 24 h. Therefore, abundance of crayfish per site was expressed as the total number of individuals per catch per unit of effort (ind. CPUE/24 h). The crayfishes collected were also measured from the rostrum tip to telson rear edge and their sex was determined following Sousa et al. (2013).

The macroinvertebrate community was collected using a hand net (Figure 4A) with the kick sampling technique in different microhabitats (banks, centre of the channel, areas with different types of sediment and with the presence/absence of macrophytes) and the collection was timed to ensure the same sampling effort. Therefore, abundance of macroinvertebrates per site was expressed as the total number of individuals per catch per unit of effort (ind. CPUE). This sampling was carried out in accordance with the Water Framework Directive (INAG IP, 2008). The hand net used has an opening of 25 cm in diameter and a mesh size of 500 µm. Afterwards, what was caught in the net was passed through a sieve (Figure 4B) and the organisms caught were stored in jars with alcohol to preserve them. In the laboratory, the samples were first sorted on white trays under good lighting conditions and separated from the rest of the sample using tweezers. After cleaning the samples, the organisms were taxonomically identified to family and when possible to species, 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 hours in an oven at 60°C to determine their biomass, following Sousa et al. (2006).

Figure 4 - A - Material used to collect macroinvertebrates (hand net, sieve, bucket, and jar). B - Sampling process, which involves passing the sample collected with the hand net through a sieve.

3.3 Data analysis

3.3.1 Abiotic data

The abiotic characterisation consisted of analysing the variables temperature, oxygen, conductivity, TDS, pH, altitude, HMS and HQA index. These variables were all normalised and analysed using a Principal Component Analysis (PCA) using Primer 6 software (version 1.0.3, Primer-E Ltd, Plymouth), which made it possible to organise the 34 sites according to the eight environmental variables.

The RHS was analysed using the Habitat Modification Score (HMS) and Habitat Quality Assessment (HQA) indices, therefore obtaining a methodology capable of assessing the degree of disturbance and/or naturalness in the sites under analysis. With the HMS it was possible to quantify and measure the degree of artificialisation of the channel by observing the presence and impact of artificial structures present in river habitats (dams, crossings, hydraulic crossings, spikes, bridges, reinforcement, resectioning, embankments, trampling of banks by livestock, cutting of riparian vegetation, dredging, artificial bottom material, among others) (Raven et al., 1998). To calculate HMS for each site, points were allocated for the presence and extent of these artificial resources (Habitat Modification Score Rules 2003). A high score is obtained if there are greater and more severe modifications. The total accumulated points provide the habitat modification score (HMS).

The Habitat Modification Class (HMC) allocates the condition of a site's channel into one of five modification classes, based on the total score (1 = almost natural; 5 = severely modified). Table 1 shows the different HMS sub-indices and Table 2 shows their respective scores and consequent classification.

Table 1 - HMS Index and its sub-indices.

HMS Outfall/Intakes
HMS Artificial berms and raised banks
HMS Bridges
HMS Culverts
HMS Ford passages
HMS Poached
HMS bank and bed reinforcement (Reinforced)
HMS resection of the banks and bed (Resectioned)
HMS dams and water diversion devices (Weirs/Dams/Sluices)
HMS score
HMS Class

Table 2 - Categories of artificialisation of the bed and banks of watercourses and respective HMS index score according to the Environment Agency (2003).

HMS Score	Category description	Quality classes
0 – 16	Pristine - Semi-natural	1
17 – 199	Predominantly unmodified	2
200 – 499	Obviously modified	3
500 – 1399	Significantly modified	4
≥ 1400	Severely modified	5

The HQA index measures the rarity, richness and diversity of river habitats and is made up of several sub-indices based on the relevance of certain habitat characteristics for biological communities. These sub-indices are related to substrate type, bank characteristics, channel characteristics, marginal vegetation structure, runoff, aquatic vegetation, riparian vegetation, land use, special features and overall habitat quality (Table 3).

Table 3 - HQA Index and its sub-indices.

HQA types of current
HQA bed substrate
HQA bed characteristics
HQA margin characteristics
HQA bank vegetation structure
HQA sediment accumulation in the inner zone of the meander
HQA bed vegetation
HQA land use
HQA characteristics associated with trees
HQA special characteristics
HQA Score

With the HQA index it was then possible to obtain an indication of the general habitat diversity provided by the natural features in the river corridor and channel. The sites sampled were scored for the presence of accumulation features in the channel, large woody debris, waterfalls and cascades, eroded cliffs, floodplain wetlands and backwater zones. Complementary points indicate the variety of flow types, channel substrates, vegetation in the channel and also the distribution of trees on the banks and the extent of land use close to the natural adjacent to the river. The points were then totalled to give the HQA score, where the higher the value, the higher the habitat quality of each site. As a way to carry out a hydromorphological assessment based on HQA, it is necessary to have a database of reference sites (undisturbed), by river typology (Table 4), which have similar physical characteristics (e.g., distance from the source, gradient and geology) to the sampled sites. The following table shows the border values for the typologies Small Northern Rivers (N1≤100) and Medium-Large Northern Rivers (N1>100), as these are the typologies corresponding to the sites studied. The lower the HQA scores, the greater the artificial intervention and modification of the river channel at a given site, affecting the quality of natural habitats.

Table 4 - Quality frontiers of the HQA index, applicable to rivers. Classification according to the Agência da Proteção do Ambiente (APA, 2021) criteria.

National type	HQA limits for the Excellent class
N1≤100	>68
N1>100	>60

After determining the HMS and HQA quality indices, the classification of the hydromorphological quality elements for the sampling sites will correspond to the most penalising class of the two.

In addition to these two indices, the Riparian Quality Index (RQI) was also calculated using the same software used for the other indices (The River Habitat Survey Toolbox). The RQI represents the naturalness, complexity and continuity of the riparian zone (Table 5). This zone covers the bank slope, the top of the bank and the 5m buffer from the top of the bank assessed during the RHS. The RQI is made up of 3 sub-indices relating to naturalness, complexity and that were calculated separately for each bank and then added together to obtain the final classification, a value that varies between 0 and 120. The final RQI score is classified into 5 equal classes that represent an increasing value of riparian quality, ranging from "Very low" (1st quintile) to "Very high" (last quintile).

Table 5 - RQI Index and its sub-indices.

RQI Naturalness
RQI Complexity
RQI Continuity
RQI Score

3.3.2 Biotic Data

We used a generalised linear model (GLM) to find possible differences in abundance of the signal crayfish between the Rabaçal and Tuela River basins. The data obtained after counting and identifying the macroinvertebrates was placed in a taxa-site matrix and used to calculate abundance (N), richness (number of families; 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 (version 1.0.3, Primer-E Ltd, Plymouth). With the richness data, a species accumulation curve was made to record how new taxa were added with the continuous sampling effort also comparing invaded and non-invaded sites (Thompson & Withers, 2003). In addition, the macroinvertebrate communities were analysed with Non-metric Multi-Dimensional Scaling (nMDS), where the abundance data was previously logarithmised and used to make a similarity matrix using the Bray-Curtis distance. 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 basin (two levels: Rabaçal and Tuela) as a fixed factor and crayfish presence (two factors: yes and no) as a random factor. The test considered 9,999 permutations, but when the number was lower than 150 the Monte Carlo test P-value was considered. Abundance data was previously subjected to a logarithmic transformation, where the Bray-Curtis similarity was also used. In addition, 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 (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 30 categories) (Table 6), which cover environmental preferences, life cycle and physiological and morphological characteristics, selected using Tachet et al. (2010) and other sources. These traits can be separated into two large groups: biological traits (body size, life cycle and feeding habits) and ecological traits (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, therefore covering the spatial and temporal variations that these groups can manifest in relation to their traits (Statzner & 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 turns possible the comparison of the macroinvertebrate communities in the different treatments based on the functional composition (categories of each trait that are less or more described). Calculating the FRAO was useful to get a general idea of the functional diversity of each community, helping to conclude which of the communities is the most diverse. These calculations were carried out using Excel Macro (Leps et al., 2006; http://botanika.bf.jcu.cz/suspa/FunctDiv.php).

Table 6 - Traits and categories of traits used to classify macroinvertebrate communities.

	Traits				
	Body size	**Life cycle**	**Feeding Habits**	**Habitats**	**Current Velocity**
Categories	< 0.25 cm	≤ 1 year	Absorbers	Coarse sediment	Null
	0.25-0.5 cm	>1 year	Deposit feeders	Gravel	Slow
	0.5-1 cm		Shredders	Sand	Moderate
	1-2 cm		Scrappers	Silt	Fast
	2-4 cm		Filter feeders	Macrophytes	
	4-8 cm		Piercers	Microphytes	
	> 8 cm		Predators	Twigs/Roots	
			Parasites	Organic detritus and litter	
				Mud	

To analyse the existence of significant differences between basins and the presence/absence of signal crayfish 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 not only visually through the residual distribution, but also using tests such as the Shapiro-Wilk test (normality) and Levene's test (homoscedasticity). For count data, namely abundance and richness data, GLMs were applied, for the remaining data (Shannon, evenness, biomass and functional diversity data - FRAO and CWM) ANOVAs (Analyses of Variance) were carried out and when these failed to meet the assumptions, even after being transformed, Kruskal-Wallis's tests were carried out. All statistical analyses were carried out using software Rstudio Team (2022).

4. Results

4.1 Abiotic characterization

The results of the abiotic characterisation can be seen in Table 7. Temperature varied between 16.1 (B5) and 23 °C (R7); dissolved oxygen between 7.98 (B1) and 9.47 mg/L (T8); water conductivity between 24.5 (R11) and 57. 7 µS/cm (T1); total dissolved solids between 5 (B6) and 28.9 mg/L (B1); pH between 6.45 (B4) and 7.1 (R6) and altitude between 385 (M1 and R1) and 843 m (B6).

Table 7 – Physical-chemical characterization of all the sampling sites.

	M1	**M2**	**M3**	**M4**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**
Temperature (°C)	19.9	19.6	20.8	19.7	21.4	20.6	21.5	21.4	22.4	22.5	23	23
Oxygen (mg/L)	8.21	8.46	7.99	8.17	8.44	8.39	8.28	8.27	8.27	8.24	8.67	8.45
Conductivity (µS/cm)	29.7	30.9	31.6	28.9	28.3	28.1	28.2	28.1	28.1	26.1	26.3	26.3
TDS (mg/L)	16.43	16.28	16.19	15.15	14.18	14.45	14.18	14.41	13.93	12.75	12.67	12.67
pH	7.01	7.07	6.89	6.78	6.78	7.04	6.95	6.53	6.48	7.10	7.08	7.08
Altitude (m)	385	395	398	452	385	395	398	409	459	471	487	490

	R9	**R10**	**R11**	**R12**	**R13**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Temperature (°C)	22.4	21.1	20.8	21	21.1	22	20.7	20.7	20.2	20.1	19.9
Oxygen (mg/L)	8.50	8.48	8.52	8.55	8.58	8.05	8.06	8.81	8.10	8.06	8.75
Conductivity (µS/cm)	26.5	25.0	24.5	25.6	25.8	57.7	48.6	48.6	42.6	41.3	39.1
TDS (mg/L)	13.3	12.42	12.3	12.93	12.89	28.80	25.00	25.00	21.50	21.50	20.78
pH	6.66	6.50	6.65	6.71	6.61	6.92	6.98	6.98	7.02	7.01	6.93
Altitude (m)	493	520	525	551	587	421	427	430	532	630	634

	T7	**T8**	**T9**	**T10**	**T11**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**
Temperature (°C)	19.5	20.3	22.2	21.4	19.2	18.9	18.1	17.2	16.2	16.1	16.3
Oxygen (mg/L)	8.89	9.47	9.33	9.34	9.46	7.98	8.02	8.05	8.12	8.18	8.20
Conductivity (µS/cm)	62.4	36.7	38.9	38	45.7	55.2	51.5	49.6	32.3	31.5	30.5
TDS (mg/L)	7.14	18.70	19.29	19.10	8.75	28.90	27.90	27.30	5.58	5.45	5.00
pH	6.96	6.79	6.72	6.69	6.79	6.75	6.74	6.83	6.45	6.69	6.60
Altitude (m)	643	655	656	684	750	594	608	612	831	835	843

With the PCA performed on the abiotic data for the 34 sites (Figure 5), it is possible to include sites into two large groups, which end up being divided by the Rabaçal (Mente and Rabaçal sites) and Tuela (Tuela and Baceiro sites) River basins. Even within the Tuela group, the division between sites of the two rivers is noticeable, mainly due to differences in altitude and conductivity. PC1 explains 53.4% of all the variation and PC2 explains 28.4% (Table S1 in Annex). Of all the variables analysed, the ones that explain most of the differences in PC1 are altitude, HMS index (positive values) and TDS (negative values), while for PC2 the variables were conductivity, TDS and altitude (positive values) and temperature and the HQA index (negative values, but in this case with a very low contribution) (Table S2 in Annex).

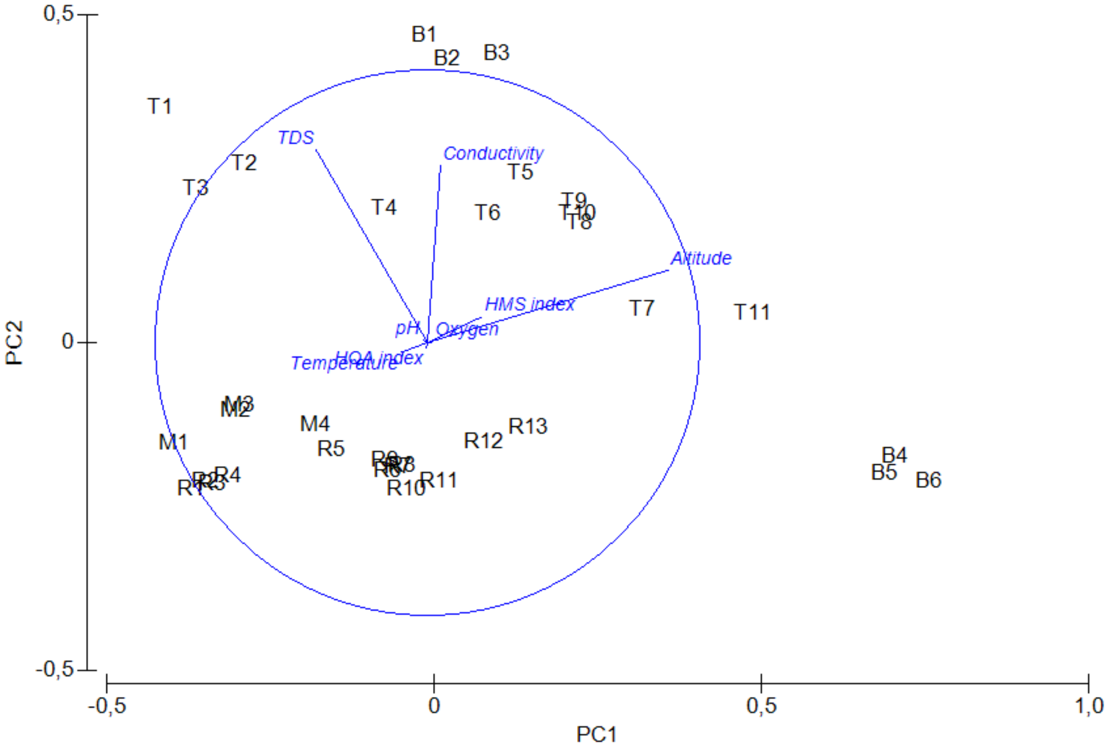


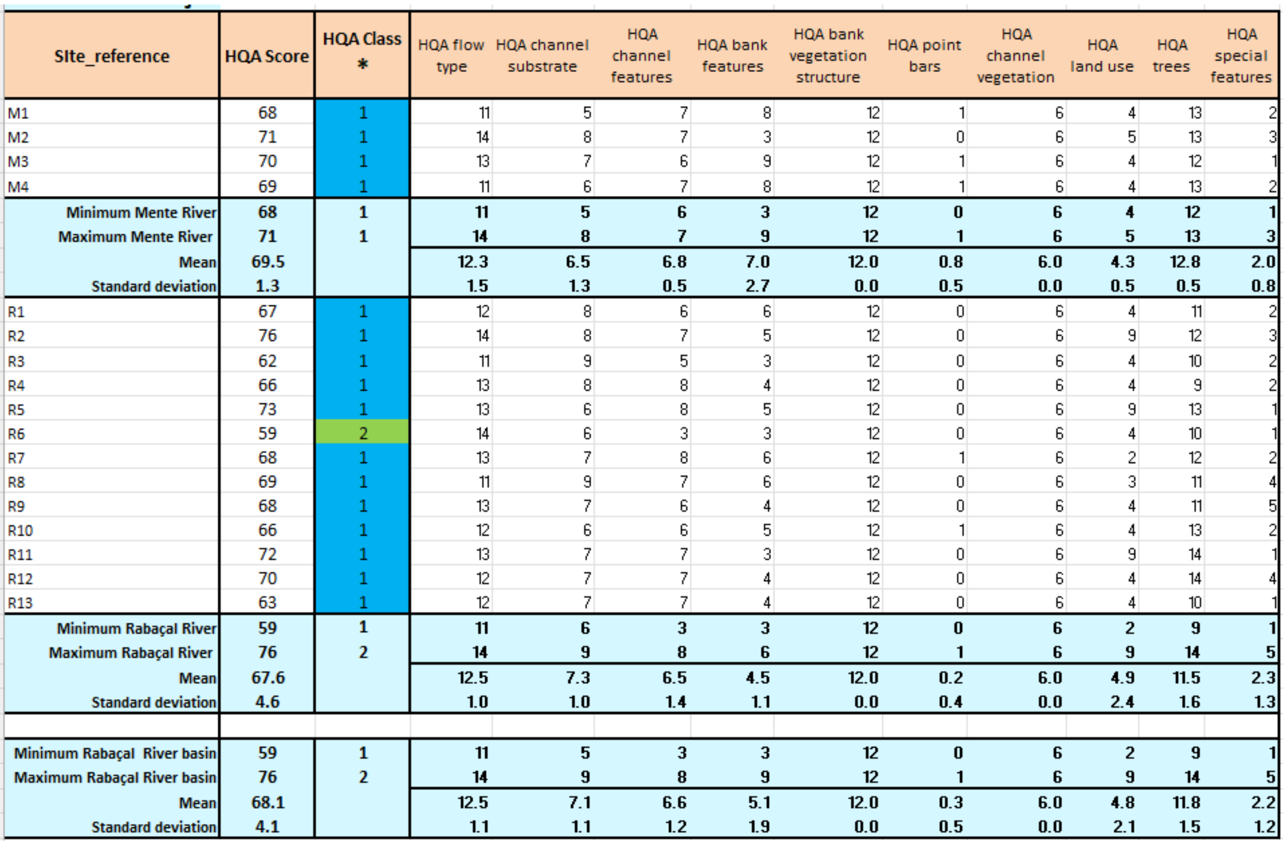
Figure 5 - Principal Components Analysis (PCA) showing the arrangement of the 34 sampling sites based on the abiotic factors measured. PC1 explains 53.4% of all variance and PC2 28.4%.

4.2 River Habitat Survey (RHS)

The data collected from the different sampling sites for the RHS are all summarized in Tables 7 to 12, relating to the HQA (Tables 7 and 8), HMS (Tables 9 and 10) and RQI (Tables 11 and 12) indexes.

In general, the HQA scores for the Rabaçal River basin were quite high with all the sites, except Rab 6 (class 2 - "Good"), obtaining the "Excellent" class (95% of the sites). Although the highest value was for Rab 2 (76), all the sites in the Mente River have higher values on average (69.5) than Rabaçal (67.6) (Table 8). All sites in the Tuela River basin had "Excellent" quality (Table 9). Overall, although both basins had practically "Excellent" habitat quality, it was in the Tuela River basin that the best and highest HQA values were achieved, with an average of 74.0.

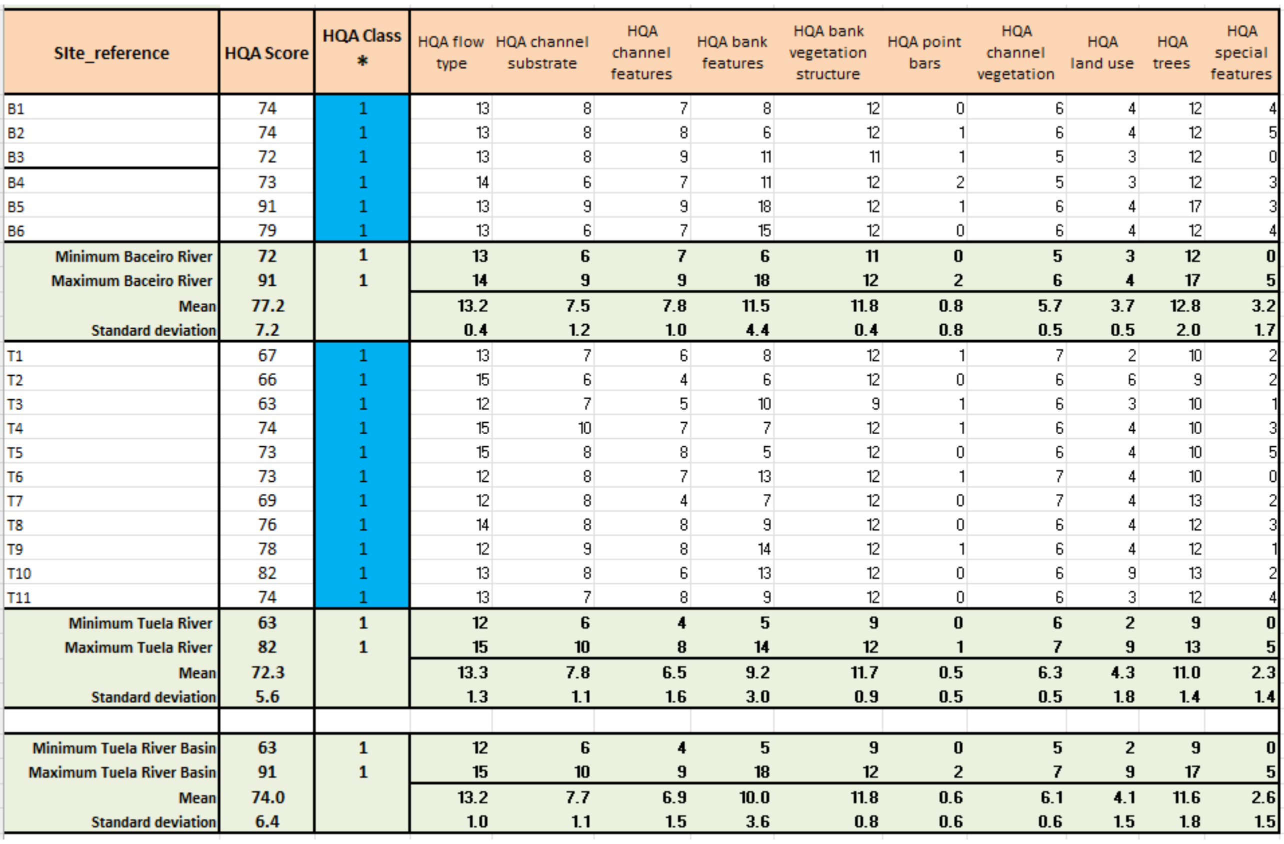
Table 8 - Partial HQA index values and respective sub-indices for the sites distributed throughout the Rabaçal River basin. Classification according to the Agência da Proteção do Ambiente (APA, 2021) criteria.



SIte_reference	HQA Score	HQA Class *	HQA flow type	HQA channel substrate	HQA channel features	HQA bank features	HQA bank vegetation structure	HQA point bars	HQA channel vegetation	HQA land use	HQA trees	HQA special features
M1	68	1	11	5	7	8	12	1	6	4	13	2
M2	71	1	14	8	7	3	12	0	6	5	13	3
M3	70	1	13	7	6	9	12	1	6	4	12	1
M4	69	1	11	6	7	8	12	1	6	4	13	2
Minimum Mente River	**68**	**1**	**11**	**5**	**6**	**3**	**12**	**0**	**6**	**4**	**12**	**1**
Maximum Mente River	**71**	**1**	**14**	**8**	**7**	**9**	**12**	**1**	**6**	**5**	**13**	**3**
Mean	**69.5**		**12.3**	**6.5**	**6.8**	**7.0**	**12.0**	**0.8**	**6.0**	**4.3**	**12.8**	**2.0**
Standard deviation	**1.3**		**1.5**	**1.3**	**0.5**	**2.7**	**0.0**	**0.5**	**0.0**	**0.5**	**0.5**	**0.8**
R1	67	1	12	8	6	6	12	0	6	4	11	2
R2	76	1	14	8	7	5	12	0	6	9	12	3
R3	62	1	11	9	5	3	12	0	6	4	10	2
R4	66	1	13	8	8	4	12	0	6	4	9	2
R5	73	1	13	6	8	5	12	0	6	9	13	1
R6	59	2	14	6	3	3	12	0	6	4	10	1
R7	68	1	13	7	8	6	12	1	6	2	12	2
R8	69	1	11	9	7	6	12	0	6	3	11	4
R9	68	1	13	7	6	4	12	0	6	4	11	5
R10	66	1	12	6	6	5	12	1	6	4	13	2
R11	72	1	13	7	7	3	12	0	6	9	14	1
R12	70	1	12	7	7	4	12	0	6	4	14	4
R13	63	1	12	7	7	4	12	0	6	4	10	1
Minimum Rabaçal River	**59**	**1**	**11**	**6**	**3**	**3**	**12**	**0**	**6**	**2**	**9**	**1**
Maximum Rabaçal River	**76**	**2**	**14**	**9**	**8**	**6**	**12**	**1**	**6**	**9**	**14**	**5**
Mean	**67.6**		**12.5**	**7.3**	**6.5**	**4.5**	**12.0**	**0.2**	**6.0**	**4.9**	**11.5**	**2.3**
Standard deviation	**4.6**		**1.0**	**1.0**	**1.4**	**1.1**	**0.0**	**0.4**	**0.0**	**2.4**	**1.6**	**1.3**
Minimum Rabaçal River basin	**59**	**1**	**11**	**5**	**3**	**3**	**12**	**0**	**6**	**2**	**9**	**1**
Maximum Rabaçal River basin	**76**	**2**	**14**	**9**	**8**	**9**	**12**	**1**	**6**	**9**	**14**	**5**
Mean	**68.1**		**12.5**	**7.1**	**6.6**	**5.1**	**12.0**	**0.3**	**6.0**	**4.8**	**11.8**	**2.2**
Standard deviation	**4.1**		**1.1**	**1.1**	**1.2**	**1.9**	**0.0**	**0.5**	**0.0**	**2.1**	**1.5**	**1.2**

When evaluating the two basins, the characteristics that contributed most to these high scores were those associated with current type (HQA flow type), characteristics associated with trees (HQA trees), bank vegetation structure (HQA bank vegetation structure), channel substrate type (HQA channel substrate), bank features (HQA bank features) and channel features (HQA channel features).

Table 9 - Partial HQA index values and respective sub-indices for the sites distributed throughout the Tuela River basin. Classification according to the Agência da Proteção do Ambiente (APA, 2021) criteria.

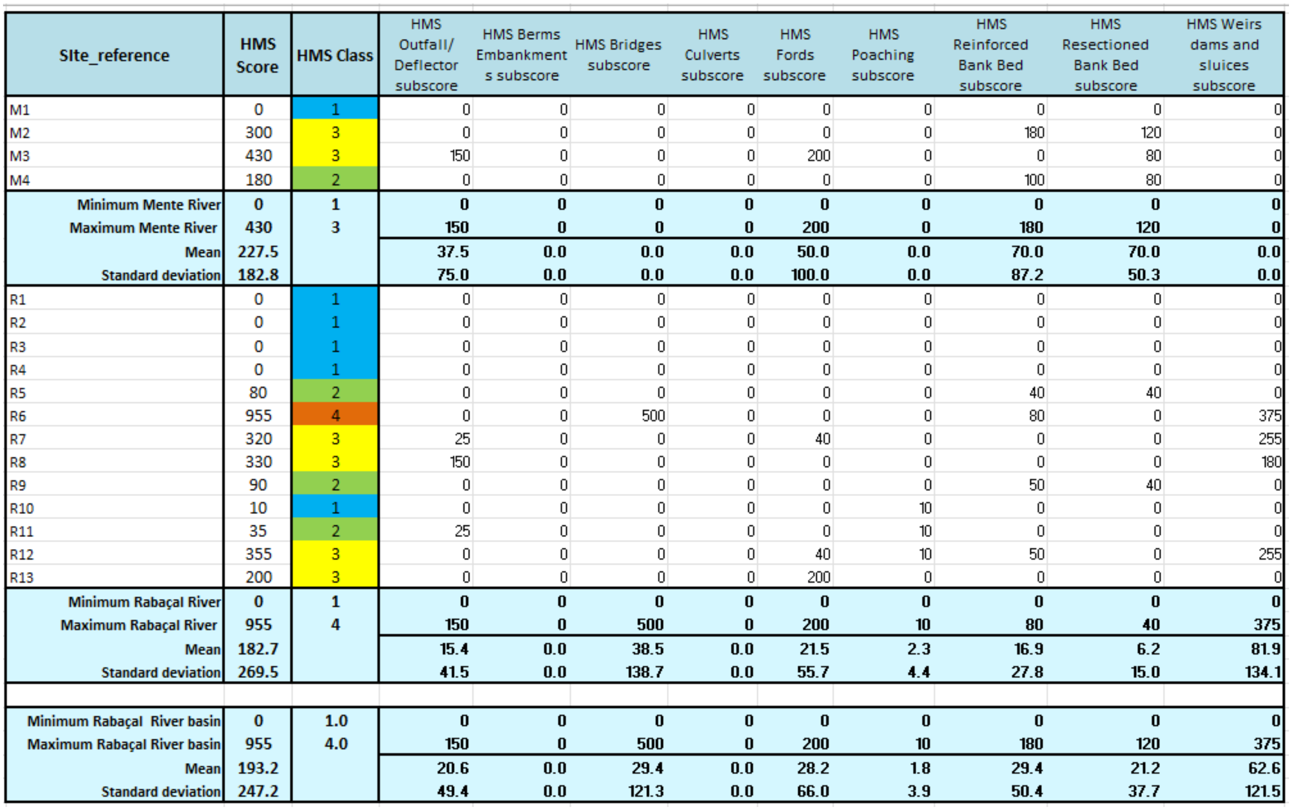


SIte_reference	HQA Score	HQA Class *	HQA flow type	HQA channel substrate	HQA channel features	HQA bank features	HQA bank vegetation structure	HQA point bars	HQA channel vegetation	HQA land use	HQA trees	HQA special features
B1	74	1	13	8	7	8	12	0	6	4	12	4
B2	74	1	13	8	8	6	12	1	6	4	12	5
B3	72	1	13	8	9	11	11	1	5	3	12	0
B4	73	1	14	6	7	11	12	2	5	3	12	3
B5	91	1	13	9	9	18	12	1	6	4	17	3
B6	79	1	13	6	7	15	12	0	6	4	12	4
Minimum Baceiro River	**72**	**1**	**13**	**6**	**7**	**6**	**11**	**0**	**5**	**3**	**12**	**0**
Maximum Baceiro River	**91**	**1**	**14**	**9**	**9**	**18**	**12**	**2**	**6**	**4**	**17**	**5**
Mean	**77.2**		**13.2**	**7.5**	**7.8**	**11.5**	**11.8**	**0.8**	**5.7**	**3.7**	**12.8**	**3.2**
Standard deviation	**7.2**		**0.4**	**1.2**	**1.0**	**4.4**	**0.4**	**0.8**	**0.5**	**0.5**	**2.0**	**1.7**
T1	67	1	13	7	6	8	12	1	7	2	10	2
T2	66	1	15	6	4	6	12	0	6	6	9	2
T3	63	1	12	7	5	10	9	1	6	3	10	1
T4	74	1	15	10	7	7	12	1	6	4	10	3
T5	73	1	15	8	8	5	12	0	6	4	10	5
T6	73	1	12	8	7	13	12	1	7	4	10	0
T7	69	1	12	8	4	7	12	0	7	4	13	2
T8	76	1	14	8	8	9	12	0	6	4	12	3
T9	78	1	12	9	8	14	12	1	6	4	12	1
T10	82	1	13	8	6	13	12	0	6	9	13	2
T11	74	1	13	7	8	9	12	0	6	3	12	4
Minimum Tuela River	**63**	**1**	**12**	**6**	**4**	**5**	**9**	**0**	**6**	**2**	**9**	**0**
Maximum Tuela River	**82**	**1**	**15**	**10**	**8**	**14**	**12**	**1**	**7**	**9**	**13**	**5**
Mean	**72.3**		**13.3**	**7.8**	**6.5**	**9.2**	**11.7**	**0.5**	**6.3**	**4.3**	**11.0**	**2.3**
Standard deviation	**5.6**		**1.3**	**1.1**	**1.6**	**3.0**	**0.9**	**0.5**	**0.5**	**1.8**	**1.4**	**1.4**
Minimum Tuela River Basin	**63**	**1**	**12**	**6**	**4**	**5**	**9**	**0**	**5**	**2**	**9**	**0**
Maximum Tuela River Basin	**91**	**1**	**15**	**10**	**9**	**18**	**12**	**2**	**7**	**9**	**17**	**5**
Mean	**74.0**		**13.2**	**7.7**	**6.9**	**10.0**	**11.8**	**0.6**	**6.1**	**4.1**	**11.6**	**2.6**
Standard deviation	**6.4**		**1.0**	**1.1**	**1.5**	**3.6**	**0.8**	**0.6**	**0.6**	**1.5**	**1.8**	**1.5**

Regarding the HMS, the scores obtained ranged from 0 (class 1 in both basins) to 955 in the Rabaçal River basin (class 4 - R6) and 1635 in the Baceiro River, a tributary of the Tuela river (class 5 - B3).

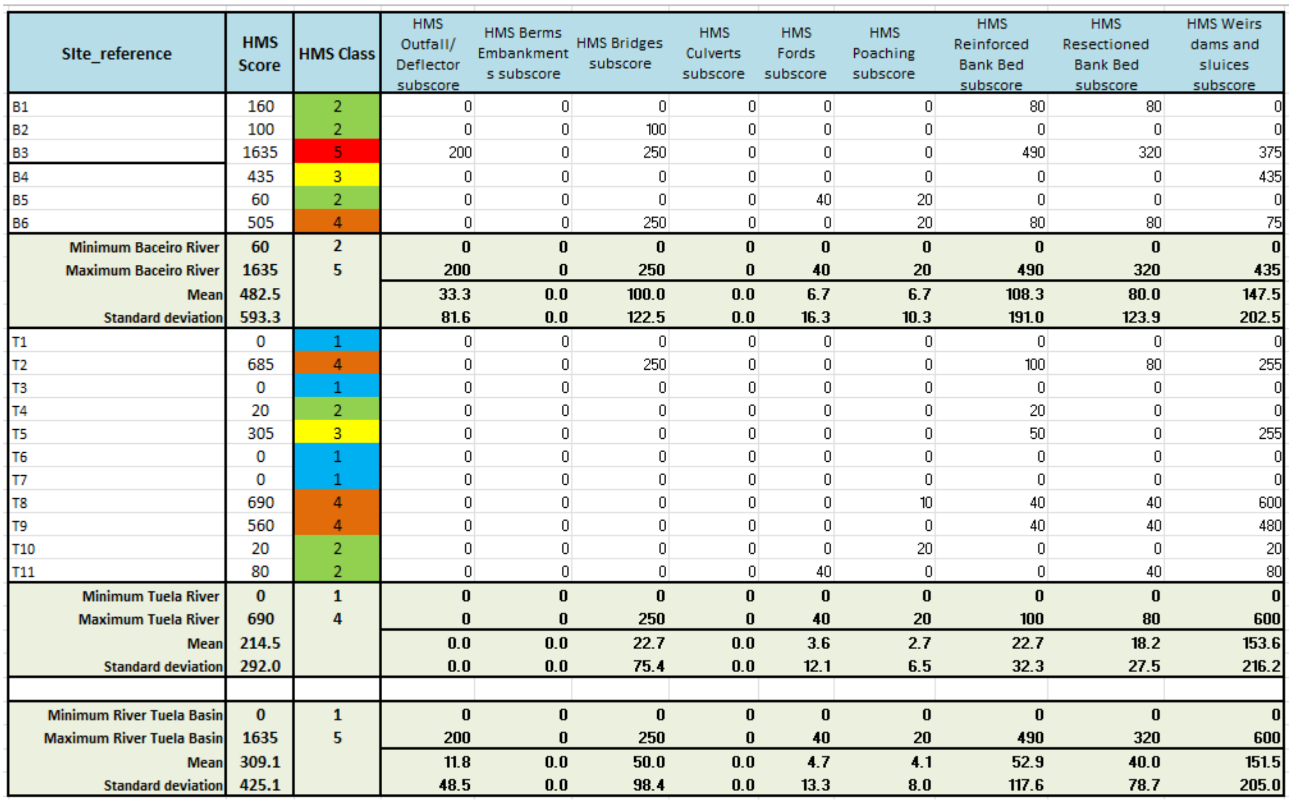
Sites in the Rabaçal River basin (Table 10) were mainly classified across 4 quality classes, that is, from "Pristine" (class 1) to "Significantly modified" (class 4). However, the "Pristine" and "Predominantly unmodified" classes comprised 62.5 % of the studied sites. The worst class was only reached by one site (R6, class 4), due to the presence of an impermeable weir and two bridges. The average percentage of sites with high HMS values (31.25 %) showed some anthropogenic influence on riparian habitats, through the construction of structures such as hydraulic crossings, bridges, reinforcement and resection of banks, and the presence of weirs/transverse barriers. This habitat alteration by human intervention was slightly worsen in the Tuela River basin (Table 11) where the number of "Obviously modified", "Significantly modified" or "Severely modified" sites represents 41.2 % of the total studied sites.

Table 10 - Partial HMS index values and respective sub-indices for the sites distributed across the Rabaçal River basin.



SIte_reference	HMS Score	HMS Class	HMS Outfall/ Deflector subscore	HMS Berms Embankments subscore	HMS Bridges subscore	HMS Culverts subscore	HMS Fords subscore	HMS Poaching subscore	HMS Reinforced Bank Bed subscore	HMS Resectioned Bank Bed subscore	HMS Weirs dams and sluices subscore
M1	0	1	0	0	0	0	0	0	0	0	0
M2	300	3	0	0	0	0	0	0	180	120	0
M3	430	3	150	0	0	0	200	0	0	80	0
M4	180	2	0	0	0	0	0	0	100	80	0
Minimum Mente River	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Maximum Mente River	**430**	**3**	**150**	**0**	**0**	**0**	**200**	**0**	**180**	**120**	**0**
Mean	**227.5**		**37.5**	**0.0**	**0.0**	**0.0**	**50.0**	**0.0**	**70.0**	**70.0**	**0.0**
Standard deviation	**182.8**		**75.0**	**0.0**	**0.0**	**0.0**	**100.0**	**0.0**	**87.2**	**50.3**	**0.0**
R1	0	1	0	0	0	0	0	0	0	0	0
R2	0	1	0	0	0	0	0	0	0	0	0
R3	0	1	0	0	0	0	0	0	0	0	0
R4	0	1	0	0	0	0	0	0	0	0	0
R5	80	2	0	0	0	0	0	0	40	40	0
R6	955	4	0	0	500	0	0	0	80	0	375
R7	320	3	25	0	0	0	40	0	0	0	255
R8	330	3	150	0	0	0	0	0	0	0	180
R9	90	2	0	0	0	0	0	0	50	40	0
R10	10	1	0	0	0	0	0	10	0	0	0
R11	35	2	25	0	0	0	0	10	0	0	0
R12	355	3	0	0	0	0	40	10	50	0	255
R13	200	3	0	0	0	0	200	0	0	0	0
Minimum Rabaçal River	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Maximum Rabaçal River	**955**	**4**	**150**	**0**	**500**	**0**	**200**	**10**	**80**	**40**	**375**
Mean	**182.7**		**15.4**	**0.0**	**38.5**	**0.0**	**21.5**	**2.3**	**16.9**	**6.2**	**81.9**
Standard deviation	**269.5**		**41.5**	**0.0**	**138.7**	**0.0**	**55.7**	**4.4**	**27.8**	**15.0**	**134.1**
Minimum Rabaçal River basin	**0**	**1.0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Maximum Rabaçal River basin	**955**	**4.0**	**150**	**0**	**500**	**0**	**200**	**10**	**180**	**120**	**375**
Mean	**193.2**		**20.6**	**0.0**	**29.4**	**0.0**	**28.2**	**1.8**	**29.4**	**21.2**	**62.6**
Standard deviation	**247.2**		**49.4**	**0.0**	**121.3**	**0.0**	**66.0**	**3.9**	**50.4**	**37.7**	**121.5**

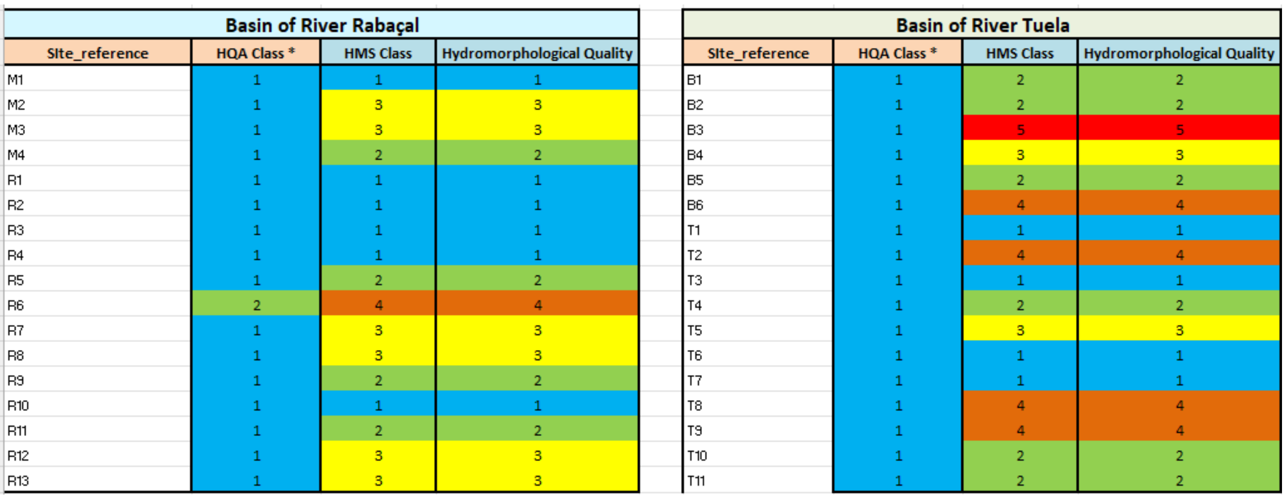
Table 11 - Partial HMS index values and respective sub-indices for the sites distributed across the Tuela River basin.



SIte_reference	HMS Score	HMS Class	HMS Outfall/ Deflector subscore	HMS Berms Embankments subscore	HMS Bridges subscore	HMS Culverts subscore	HMS Fords subscore	HMS Poaching subscore	HMS Reinforced Bank Bed subscore	HMS Resectioned Bank Bed subscore	HMS Weirs dams and sluices subscore
B1	160	2	0	0	0	0	0	0	80	80	0
B2	100	2	0	0	100	0	0	0	0	0	0
B3	1635	5	200	0	250	0	0	0	490	320	375
B4	435	3	0	0	0	0	0	0	0	0	435
B5	60	2	0	0	0	0	40	20	0	0	0
B6	505	4	0	0	250	0	0	20	80	80	75
Minimum Baceiro River	**60**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Maximum Baceiro River	**1635**	**5**	**200**	**0**	**250**	**0**	**40**	**20**	**490**	**320**	**435**
Mean	**482.5**		**33.3**	**0.0**	**100.0**	**0.0**	**6.7**	**6.7**	**108.3**	**80.0**	**147.5**
Standard deviation	**593.3**		**81.6**	**0.0**	**122.5**	**0.0**	**16.3**	**10.3**	**191.0**	**123.9**	**202.5**
T1	0	1	0	0	0	0	0	0	0	0	0
T2	685	4	0	0	250	0	0	0	100	80	255
T3	0	1	0	0	0	0	0	0	0	0	0
T4	20	2	0	0	0	0	0	0	20	0	0
T5	305	3	0	0	0	0	0	0	50	0	255
T6	0	1	0	0	0	0	0	0	0	0	0
T7	0	1	0	0	0	0	0	0	0	0	0
T8	690	4	0	0	0	0	0	10	40	40	600
T9	560	4	0	0	0	0	0	0	40	40	480
T10	20	2	0	0	0	0	0	20	0	0	20
T11	80	2	0	0	0	0	40	0	0	40	80
Minimum Tuela River	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Maximum Tuela River	**690**	**4**	**0**	**0**	**250**	**0**	**40**	**20**	**100**	**80**	**600**
Mean	**214.5**		**0.0**	**0.0**	**22.7**	**0.0**	**3.6**	**2.7**	**22.7**	**18.2**	**153.6**
Standard deviation	**292.0**		**0.0**	**0.0**	**75.4**	**0.0**	**12.1**	**6.5**	**32.3**	**27.5**	**216.2**
Minimum River Tuela Basin	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Maximum River Tuela Basin	**1635**	**5**	**200**	**0**	**250**	**0**	**40**	**20**	**490**	**320**	**600**
Mean	**309.1**		**11.8**	**0.0**	**50.0**	**0.0**	**4.7**	**4.1**	**52.9**	**40.0**	**151.5**
Standard deviation	**425.1**		**48.5**	**0.0**	**98.4**	**0.0**	**13.3**	**8.0**	**117.6**	**78.7**	**205.0**

In terms of hydromorphological quality, the Rabaçal River basin stands out for having better quality in general than the Tuela River basin (Table 12). The Rabaçal River basin ended up with a greater number of sites with "Excellent" quality (6), compared to the Tuela River basin with only 4 "Excellent" sites. It was also in the Tuela River basin that there was one site with the worst quality (B3) and 4 sites with "Significantly modified" quality (B6; T2; T8 and T9).

Table 12 – Hydromorphological quality for the sites distributed across the Rabaçal and Tuela River basins.



Basin of River Rabaçal

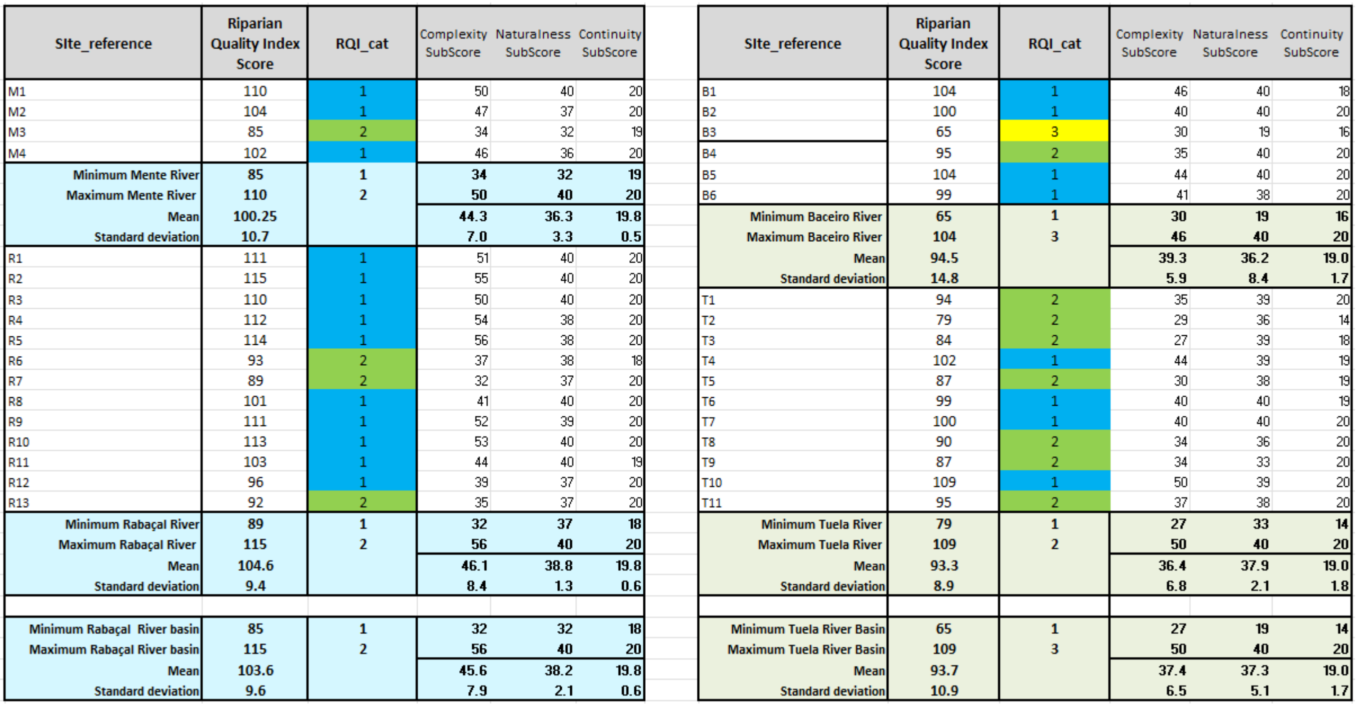
SIte_reference	HQA Class *	HMS Class	Hydromorphological Quality
M1	1	1	1
M2	1	3	3
M3	1	3	3
M4	1	2	2
R1	1	1	1
R2	1	1	1
R3	1	1	1
R4	1	1	1
R5	1	2	2
R6	2	4	4
R7	1	3	3
R8	1	3	3
R9	1	2	2
R10	1	1	1
R11	1	2	2
R12	1	3	3
R13	1	3	3

Basin of River Tuela

SIte_reference	HQA Class *	HMS Class	Hydromorphological Quality
B1	1	2	2
B2	1	2	2
B3	1	5	5
B4	1	3	3
B5	1	2	2
B6	1	4	4
T1	1	1	1
T2	1	4	4
T3	1	1	1
T4	1	2	2
T5	1	3	3
T6	1	1	1
T7	1	1	1
T8	1	4	4
T9	1	4	4
T10	1	2	2
T11	1	2	2

The results of the RQI index (Table 13) showed a similar pattern to the previous results, which means that the Rabaçal River basin is the one with the best riparian quality, where most of the sites had "Very high" quality (76.4%). In contrast, the Tuela River basin only had 47.1% of the sites with "Very high" quality being B3 classified with "Moderate" quality.

Table 13 – RQI index values and respective sub-indices for the sites distributed across the Rabaçal and Tuela River basins.



SIte_reference	Riparian Quality Index Score	RQI_cat	Complexity SubScore	Naturalness SubScore	Continuity SubScore
M1	110	1	50	40	20
M2	104	1	47	37	20
M3	85	2	34	32	19
M4	102	1	46	36	20
Minimum Mente River	**85**	**1**	**34**	**32**	**19**
Maximum Mente River	**110**	**2**	**50**	**40**	**20**
Mean	**100.25**		**44.3**	**36.3**	**19.8**
Standard deviation	**10.7**		**7.0**	**3.3**	**0.5**
R1	111	1	51	40	20
R2	115	1	55	40	20
R3	110	1	50	40	20
R4	112	1	54	38	20
R5	114	1	56	38	20
R6	93	2	37	38	18
R7	89	2	32	37	20
R8	101	1	41	40	20
R9	111	1	52	39	20
R10	113	1	53	40	20
R11	103	1	44	40	19
R12	96	1	39	37	20
R13	92	2	35	37	20
Minimum Rabaçal River	**89**	**1**	**32**	**37**	**18**
Maximum Rabaçal River	**115**	**2**	**56**	**40**	**20**
Mean	**104.6**		**46.1**	**38.8**	**19.8**
Standard deviation	**9.4**		**8.4**	**1.3**	**0.6**
Minimum Rabaçal River basin	**85**	**1**	**32**	**32**	**18**
Maximum Rabaçal River basin	**115**	**2**	**56**	**40**	**20**
Mean	**103.6**		**45.6**	**38.2**	**19.8**
Standard deviation	**9.6**		**7.9**	**2.1**	**0.6**

SIte_reference	Riparian Quality Index Score	RQI_cat	Complexity SubScore	Naturalness SubScore	Continuity SubScore
B1	104	1	46	40	18
B2	100	1	40	40	20
B3	65	3	30	19	16
B4	95	2	35	40	20
B5	104	1	44	40	20
B6	99	1	41	38	20
Minimum Baceiro River	**65**	**1**	**30**	**19**	**16**
Maximum Baceiro River	**104**	**3**	**46**	**40**	**20**
Mean	**94.5**		**39.3**	**36.2**	**19.0**
Standard deviation	**14.8**		**5.9**	**8.4**	**1.7**
T1	94	2	35	39	20
T2	79	2	29	36	14
T3	84	2	27	39	18
T4	102	1	44	39	19
T5	87	2	30	38	19
T6	99	1	40	40	19
T7	100	1	40	40	20
T8	90	2	34	36	20
T9	87	2	34	33	20
T10	109	1	50	39	20
T11	95	2	37	38	20
Minimum Tuela River	**79**	**1**	**27**	**33**	**14**
Maximum Tuela River	**109**	**2**	**50**	**40**	**20**
Mean	**93.3**		**36.4**	**37.9**	**19.0**
Standard deviation	**8.9**		**6.8**	**2.1**	**1.8**
Minimum Tuela River Basin	**65**	**1**	**27**	**19**	**14**
Maximum Tuela River Basin	**109**	**3**	**50**	**40**	**20**
Mean	**93.7**		**37.4**	**37.3**	**19.0**
Standard deviation	**10.9**		**6.5**	**5.1**	**1.7**

4.3 Biotic characterization

4.3.1 Crayfish Abundance

After sampling the crayfish, they were found in 18 (out of 34) sites. There were significant differences between the two basins (F=11.581; p<0.05) (Table 14), with the Tuela River basin showing higher abundance compared to Rabaçal (Figure 6). The Tuela River basin recorded an average abundance (±SD) of 28.3 (±26.4) ind. CPUE, compared to Rabaçal with 16.4 (±16.3) ind. CPUE. T2 and T3 were the sites with the highest average abundance of crayfish with maximums of 74 and 67 ind. CPUE, respectively. In the Rabaçal River basin, the site with the highest average abundance of crayfish was R7 with a maximum of 64 ind. CPUE (Table S3 in Annex).

Table 14- Summary of the analysis of deviance for the GLM applied to the abundance of signal crayfish in response to different basin (Rabaçal and Tuela). The asterisk and bold indicates significant values (P<0.05).

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			143	3434.1		
Basin	1	233.29	142	3200.8	11.581	**0.000866***

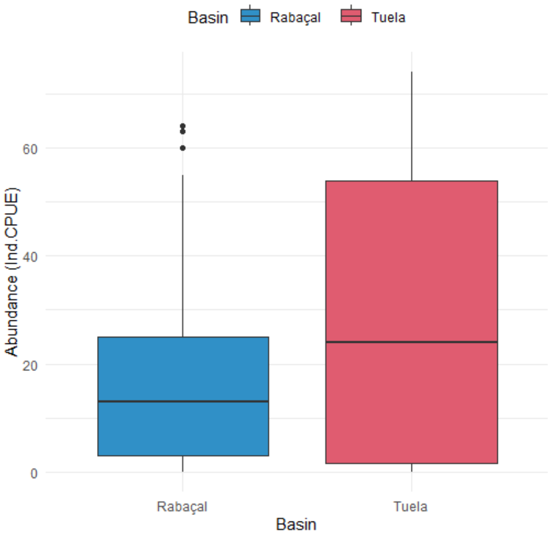


Figure 6 – Abundance of the signal crayfish (*Pacifastacus leniusculus*) in the Rabaçal and Tuela River basins. Boxplots show median values (central line), the range from the 25th to 75th percentile (box) and the largest and lowest value within 1.5 times interquartile range below and above the 25th and 75th percentile (whiskers) and dots represent extreme values.

4.3.2 Macroinvertebrate Communities

A total of 38 529 organisms were identified, belonging to 133 freshwater macroinvertebrate taxa (Table S4 in Annex). Of these 133, the majority belong to the phyla Arthropoda, Mollusca and Anellida. Arthropods were the most diverse group with 123 taxa from several different orders (Plecoptera, Diptera, Odonata, Tricoptera, Ephemeroptera, Hemiptera, Coleoptera, Megaloptera, Neuroptera and Decapoda), with 7 of these orders included in the Insecta class. The most abundant organisms were dipterans and ephemeropterans, with 9 178 and 9 035 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. In addition, it is worth noting the identification of some specimens of the dragonfly *Macromia splendens* (Vulnerable) and the bivalve *Margaritifera margaritífera* (Endangered), species of conservation interest. (IUCN, 2022).

The PERMANOVA results indicated that there are significant differences between the macroinvertebrate communities not only between the basins (Pseudo-F=2.56 p=0.0028), but also between sites invaded and not invaded by the crayfish (Pseudo-F=3.11 p=0.0354).

Table 15 - Results of the PERMANOVA analysis on macroinvertebrates communities along river basins and presence or absence of crayfish. The asterisk and bold indicate significant values.

Source	dF	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Basin	1	2003.6	2003.6	2.5558	**0.0028***	9900	**0.0082***
Crayfish	1	3321.3	3321.3	3.1085	0.4976	6	**0.0354***
Basin x Crayfish	1	1068.5	1068.5	1.3629	0.1531	9921	0.1772
Residual	30	23518	783.93				
Total	33	29762					

The nMDS of the macroinvertebrate community (Figure 7) showed great similarity between sites uninvaded by the crayfish, except for B5 and B6. On the other hand, the invaded sites appear to be grouped together mainly due to spatial proximity, although some exceptions to this pattern were found (e.g., M1, M4, R6, R9 and T4). By using 60% similarity, it was possible to group the sites into 5 groups, with the largest one showing most of the non-invaded sites. Sites in the Baceiro River turned out to be more different from the rest, where the sites ended up divided into two groups, one group with the most upstream sites and the other with the most downstream sites.

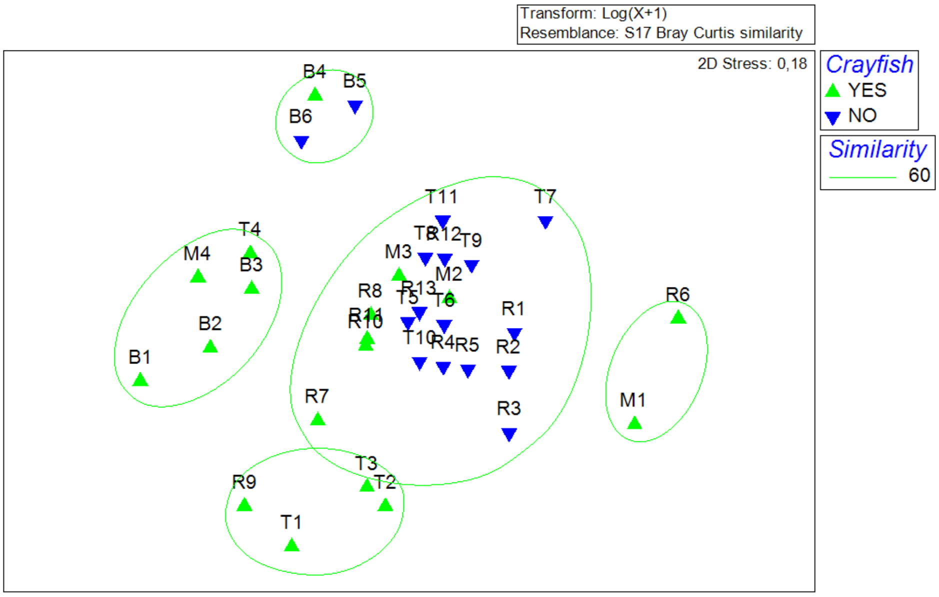


Figure 7 - Non-metric Multi-Dimensional Scaling (nMDS) of the macroinvertebrate communities showing the sampling sites in the four rivers basins and the presence or absence of the signal crayfish. Sampled sites grouped by 60% similarity.

The SIMPER analysis showed that the taxa that contributed most to the average dissimilarity between the Rabaçal and Tuela basins (41.25%) were *Chimarra marginata* (3.06%), *Habrophlebia/Habroleptoides* (2.50%), *Brachycentrus subnubilus* (2.29%), *Oligoneuriella rhenana* (2.18%), Simuliidae (2.15%) and *Setodes argentipunctellus* (2.06%), while between the absence and presence of crayfish (42.72%) were *Brachycentrus subnubilus* (2.75%), *Chimarra marginata* (2.58), *Aphelocheirus occidentalis* (2.41%), *Habrophlebia/ Habroleptoides* (2.18%), *Limnius* (2.12%) and *Oligoneuriella rhenana* (2.10%) (see Table S5 in Annex).

4.3.2.1 Diversity Indexes

Macroinvertebrate richness was higher in the sites not invaded by the crayfish, showing significant differences (t=-4.121 p<0.001), with a mean (±SD) of 53.4 (±5.5) in the crayfish-free sites, in contrast to a mean of 41.6 (±9.7) in the invaded sites (Figure 8A). However, there was no difference in richness between the two basins (t=-0.102 p=0.919). Sites R2, T7 and B5 had the highest macroinvertebrate richness with 62, 61 and 65 taxa, respectively. Sites M1, T1 and B1 had the lowest richness values with 33, 28 and 30 taxa, respectively.

The average abundance in the sites with no crayfish was also higher than in the invaded sites, with 1 348.0 (±539.7) ind. CPUE in the uninvaded sites and 942.3 (±516.3) ind. CPUE in the invaded ones (Figure 8B). This difference in abundance between invaded and uninvaded zones proved to be significant (t=-2.20 p=0.0352), in contrast to the differences between the Tuela and Rabaçal basins (t=0.341 p=0.735). Sites R12, T6, B5 and B4 had the highest abundances of macroinvertebrates with 1972, 2449, 1961 and 2181 ind. CPUE, respectively. It should be noted that B4 is a site invaded by crayfish despite the high abundance of macroinvertebrates. M1, R9, T1, and B2 were the sites with the lowest abundance, with 298, 258, 466 and 462 ind. CPUE, respectively.

On average, the Shannon-Wiener index values were also higher in the absence of crayfish than in their presence (Figure 8C), and their significant differences were detected (F=8.001 p=0.008). An average of 2.83 (±0.28) was obtained in the absence of crayfish and 2.59 (±0.21) in their presence. There were no significant differences across the two basins (F=0.001 p=0.977). Sites with the highest values of Shannon-Wiener diversity of macroinvertebrates were T5, T7, T10 and T11 with 3.04, 3.00, 3.03 and 3.22, respectively, all belonging to the Tuela River basin. In the Rabaçal River basin the site with the highest value was R1 with 2.95. The lowest index values were 2.14, 2.43, 2.32 and 2.41 from sites M4, R8, B1 and B3, respectively.

In the Pielou's evenness index (Figure 8D), no significant differences were detected either in the absence or presence of crayfish (F=0.283 p=0.598) or between basins (F=0.006 p=0.940). Sites R9, T5, T10 and T11 had the highest values with 0.805, 0.789, 0.797 and 0.808, respectively and M4, R8 and T6 had the lowest values with 0.596, 0.615 and 0.488, respectively.

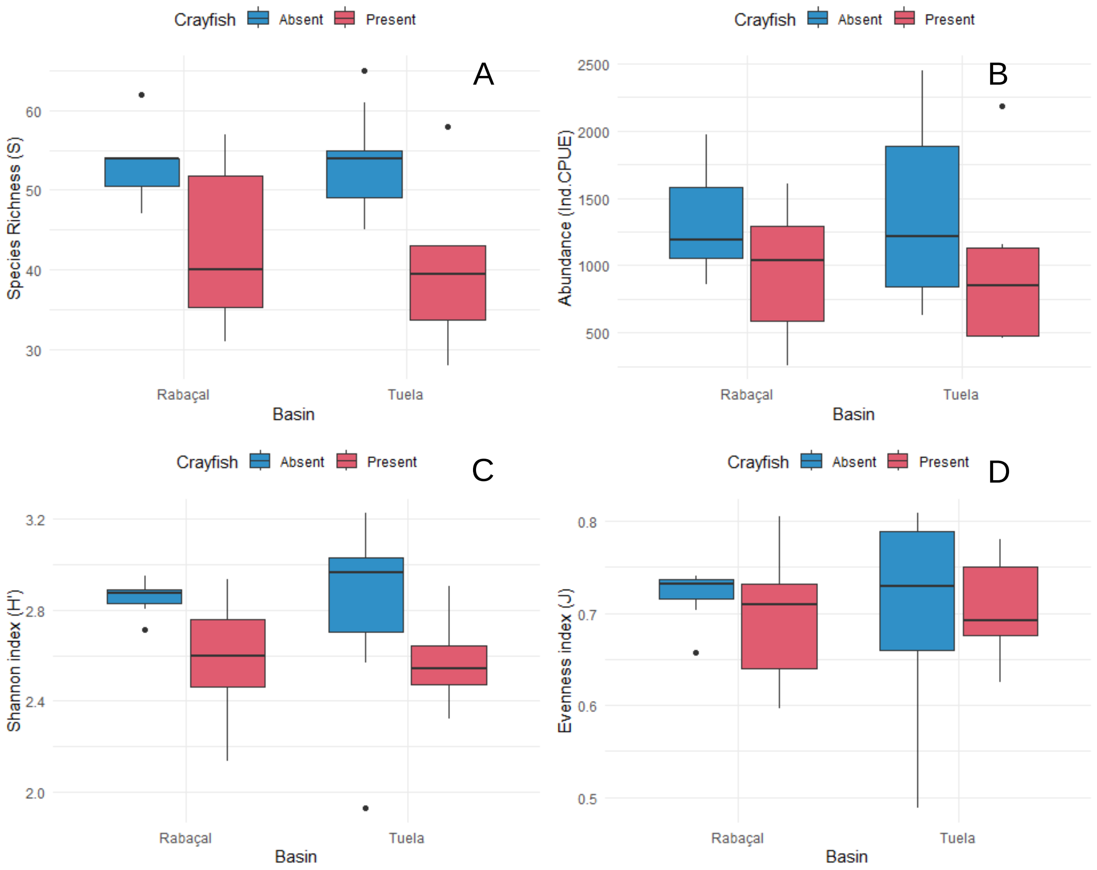


Figure 8 - Richness (A), Abundance (B), Shannon-Wiener diversity (C) and Pielou's evenness (D) of the macroinvertebrate communities in the Rabaçal and Tuela River basins with presence (red) and absence (blue) of signal crayfish. Boxplots show median values (central line), the range from the 25th to 75th 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.

In the biomass results (Figure 9), the average biomass of the uninvaded sites was 1.67 (±1.06) g CPUE, significantly higher than in the invaded sites, with 0.79 (±0.89) g CPUE (F=7.123 p=0.012). No significant differences were detected between basins (F=1.781 p=0.191). The highest biomass values were obtained in R12, T7, T9 and B4 with 4.61, 3.84, 2.03 and 4.18 g CPUE, respectively, all of which were in uninvaded areas, except for B4. Sites R9, R10, T1 and B3 were the sites with the lowest biomass, with values of 0.33, 0.35, 0.34 and 0.31 g CPUE, respectively.

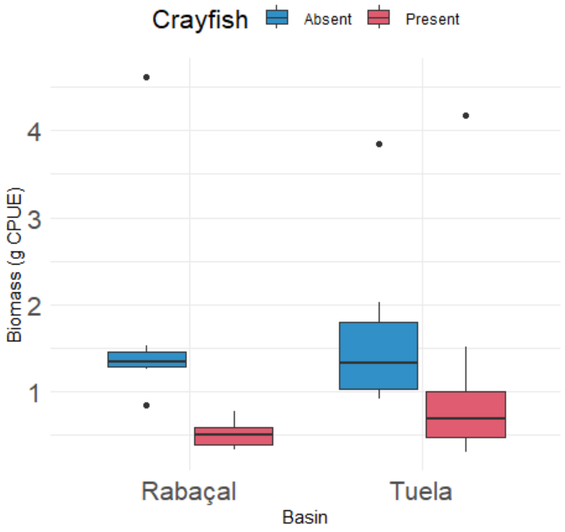


Figure 9 - Biomass of the macroinvertebrate communities in the Rabaçal and Tuela River basins with presence (red) and absence (blue) of signal crayfish. Boxplots show median values (central line), the range from the 25th to 75th percentile (box) and the largest and lowest value within 1.5 times interquartile range below and above the 25th and 75th percentile (whiskers) and dots represent extreme values.

The species accumulation curve of the macroinvertebrates sampled at the different sites invaded and not invaded by the signal crayfish showed a greater diversity of macroinvertebrates presents in non-invaded sites (Figure 10).

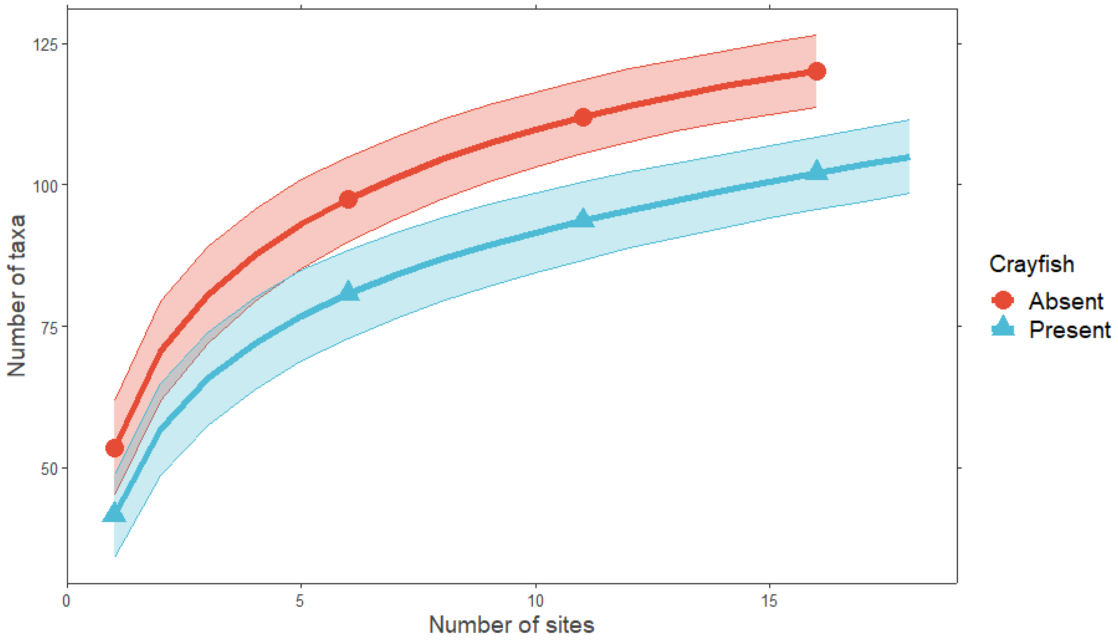


Figure 10 - Species accumulation curve of the number of macroinvertebrates taxa found with the presence or absence of the signal crayfish.

4.3.2.2 Functional Composition

4.3.2.2.1 Functional Diversity (FRAO)

When looking at the average FRAO functional diversity for the five categories, no significant differences were detected between invaded and non-invaded zones (F=2.111 p=0.156), although a tendency for the presence of crayfish to decrease functional diversity (Figure 11). Significant differences were almost detected between river basins (F=3.904 p=0.057), where the Rabaçal River basin showed greater functional diversity than the Tuela River basin.

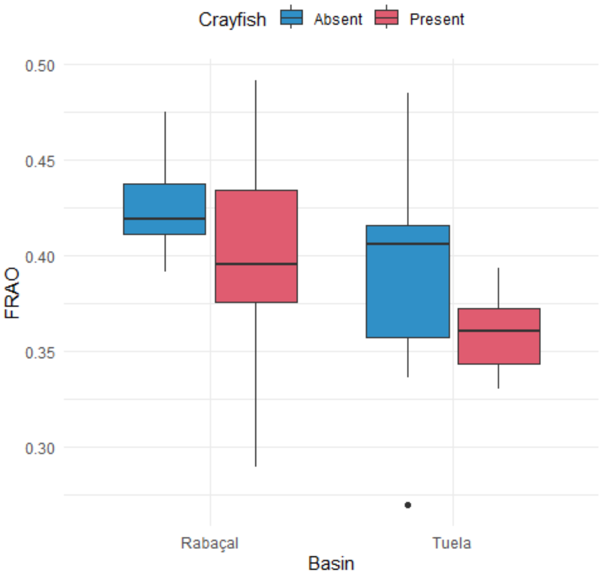


Figure 11 - Box diagram of the average value of FRAO functional diversity index of the macroinvertebrate fauna for the five traits for the Rabaçal and Tuela Tiver Basins considering the presence (red) or absence (blue) of the signal crayfish. Boxplots show median values (central line), the range from 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.

Regarding the body size trait, the FRAO results (Figure 12A) showed that the Rabaçal River basin has a significantly greater diversity of organisms with different sizes (X^2=3.922 p=0.048) than the Tuela River basin. In addition, the presence of crayfish also had a significant effect (F=5.539 p=0.025), decreasing the diversity of body sizes.

The results for the FRAO of the life cycle trait (Figure 12B) also showed significant differences, but only between basins (F=7.765 p=0.009), with no differences between the invaded and non-invaded sites (X^2=0.576 p=0.449). Again, the Rabaçal River basin had a greater diversity of macroinvertebrates with different lifecycle strategies and durations.

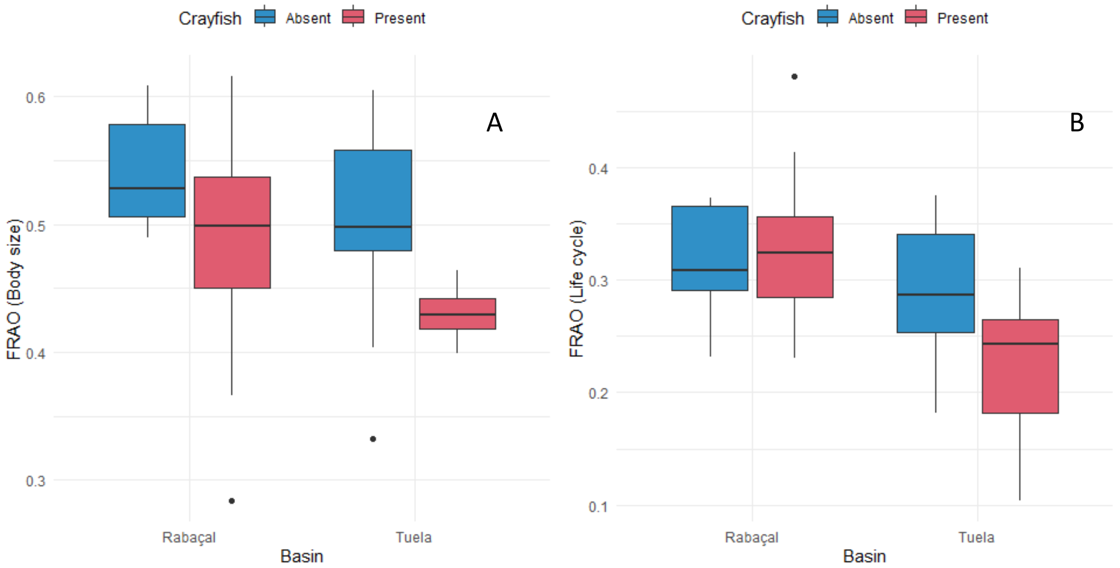


Figure 12 - Box diagram of the FRAO functional diversity index of the macroinvertebrate fauna considering the traits Body size (A) and Life Cycle (B) for the Rabaçal and Tuela River basins considering the presence (red) or absence (blue) of the signal crayfish. Boxplots show median values (central line), 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.

No significant difference was found in the trait of feeding habits (Figure 13A) between basins (X^2=0.964 p=0.326) and with the presence/absence of crayfish (F=0.259 p=0.614). The results of the FRAO index for the habitat trait showed almost significant differences between basins (F=3.162 p=0.085), having the Rabaçal River basin a higher functional diversity of organisms with the capacity to occupy a greater number of different habitats compared to the Tuela River basin (Figure 13B). On the other hand, for the presence/absence of crayfish no significant differences were detected (F=0.756 p=0.391). About the FRAO index data for the current velocity trait (Figure 13C), no significant difference was detected in the presence of crayfish (F=2.599 p=0.117) and between river basins (F=0.192 p=0.665). However, and despite non-significant statically, a greater diversity in this trait was detected in non-invaded sites (Figure 13C).

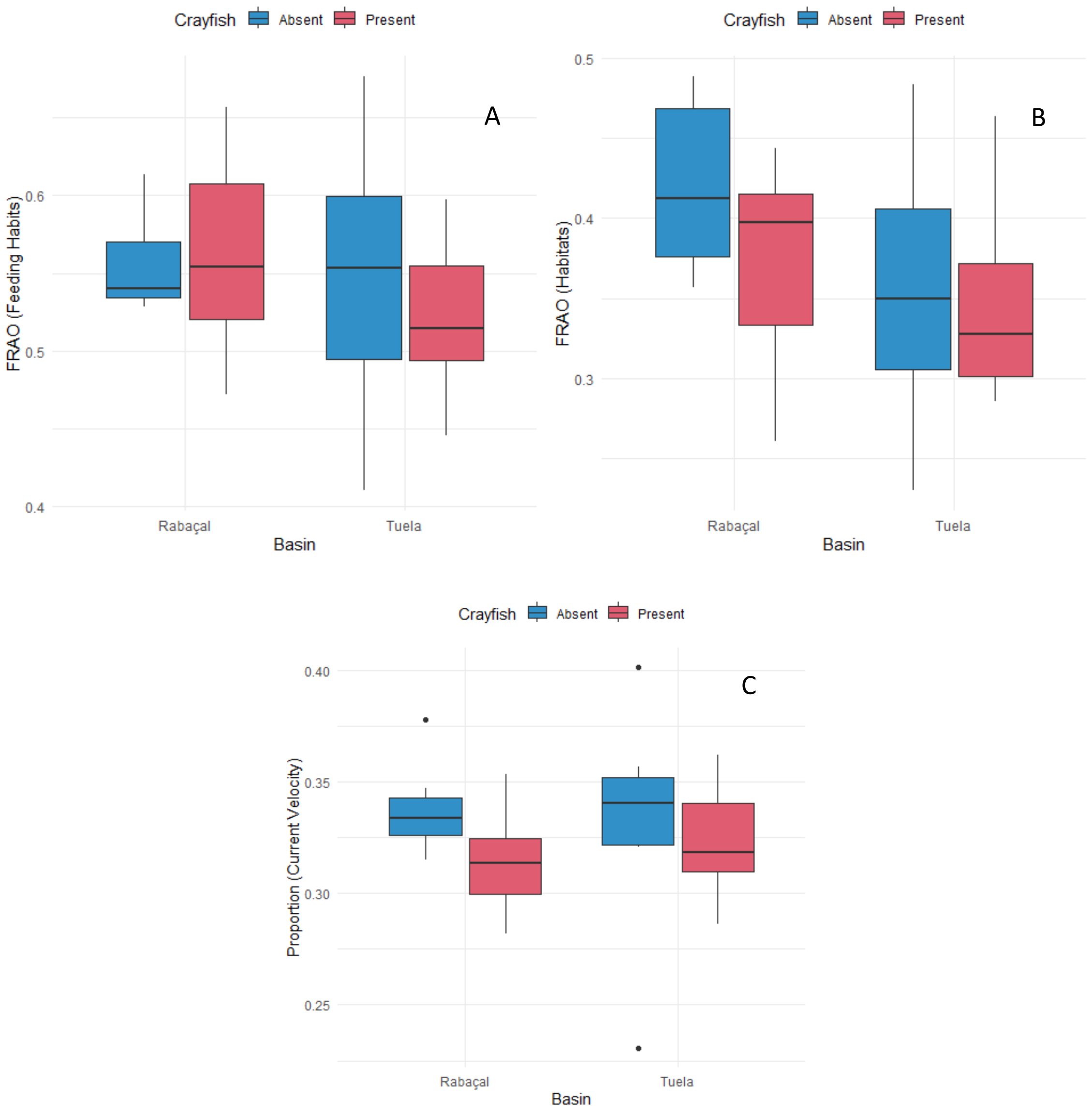


Figure 13 - Box diagram of the FRAO functional diversity index of the macroinvertebrate fauna considering the traits Feeding Habits (A), Habitats (B), and Current Velocity (C) for the Rabaçal and Tuela River basins considering the presence (red) or absence (blue) of the signal crayfish. Boxplots show 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.

4.3.2.2.2 Community-weighted mean trait value (CWM)

In general, the CWM values obtained for the body size trait showed a tendency for this community to have a greater proportion of organisms between 0.5 and 1.0 cm, compared to larger and/or smaller organisms (Figures 14 and 15). However, the class "less than 0.25 cm" was shown to vary significantly with the presence/absence of crayfish (X^2=5.844 p=0.016), where the uninvaded zones had a higher proportion of organisms smaller than 0.25 cm (Figure 14A). No significant differences were detected between basins (X^2=0.0003 p=0.986).

For the size classes "between 0.25-0.5 cm", significant differences were detected only for the presence and absence of crayfish (F=11.552 p=0.002), with a higher proportion of organisms between 0.25-0.5 cm in areas non-invaded by crayfish (Figure 14B). In contrast, no significant differences were found between basins (F=0.045 p=0.833). For the class "between 0.5-1.0 cm" (Figure 14C), no significant differences were detected for the presence of crayfish (F=1.365 p=0.251) and for the basins (F=0.174 p=0.679). Similarly, in the "between 1-2 cm" class (Figure 14D) there were no significant differences between basins (F=0.775 p=0.386) and between zones invaded and not invaded by crayfish (F=1.755 p=0.195).

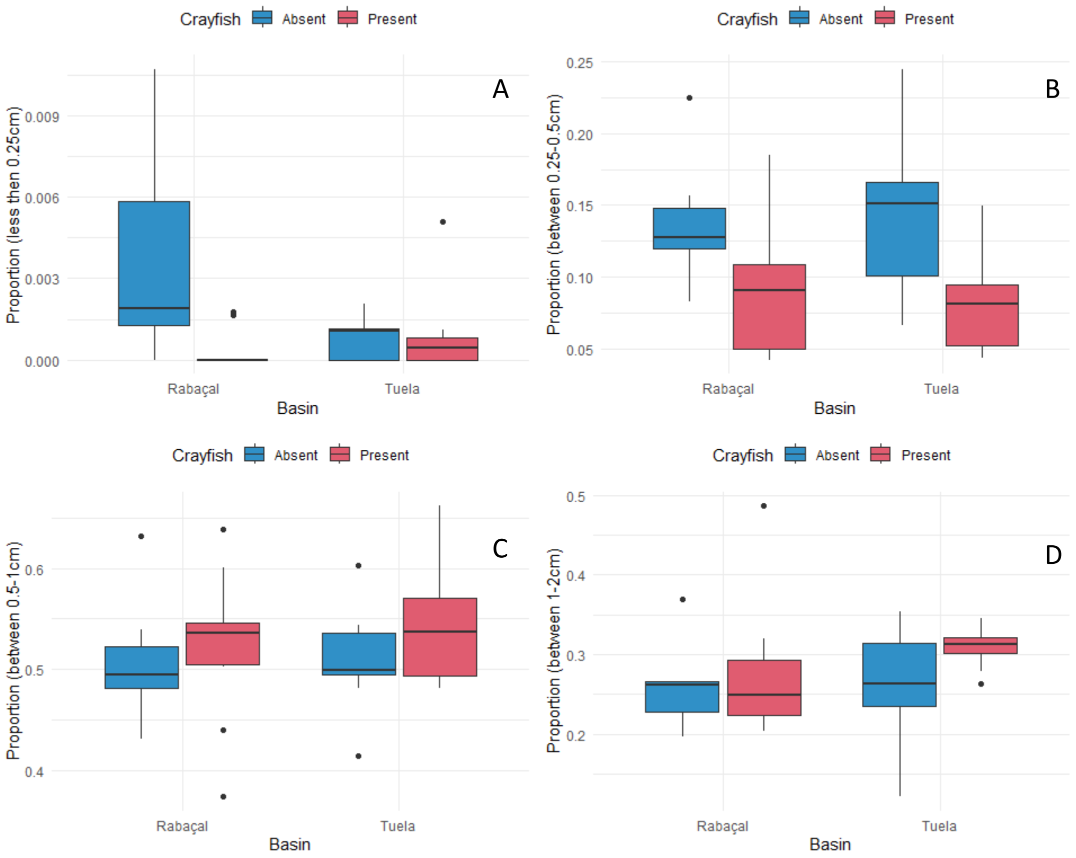


Figure 14 - Box diagram of the proportion of macroinvertebrates (CWM) for the Body size trait (< 0.25 cm (A); 0.25-0.5cm(B); 0.5-1.0cm (C) and 1-2cm (D)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median values (central 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.

In the "between 2-4 cm" class (Figure 15A), the CWM results showed no significant differences in both variables, crayfish presence (X^2=0.386 p=0.535) and basin (F=2.487 p=0.125). In contrast, the results for the class "between 4-8 cm" were significantly different between basins (F=5.007 p=0.032) but were not different between uninvaded and invaded zones (F=0.139 p=0.712). The Rabaçal River basin have a community with more organisms between 4 and 8 cm than the Tuela River basin (Figure 15B).

For the class "larger than 8.0 cm" (Figure 15C), no significant differences were detected between basins (X^2=0.493 p=0.482). However, significant differences were detected between invaded and non-invaded (X^2=17.52 p<0.001) but it was largely due to the presence of 46 signal crayfish individuals.

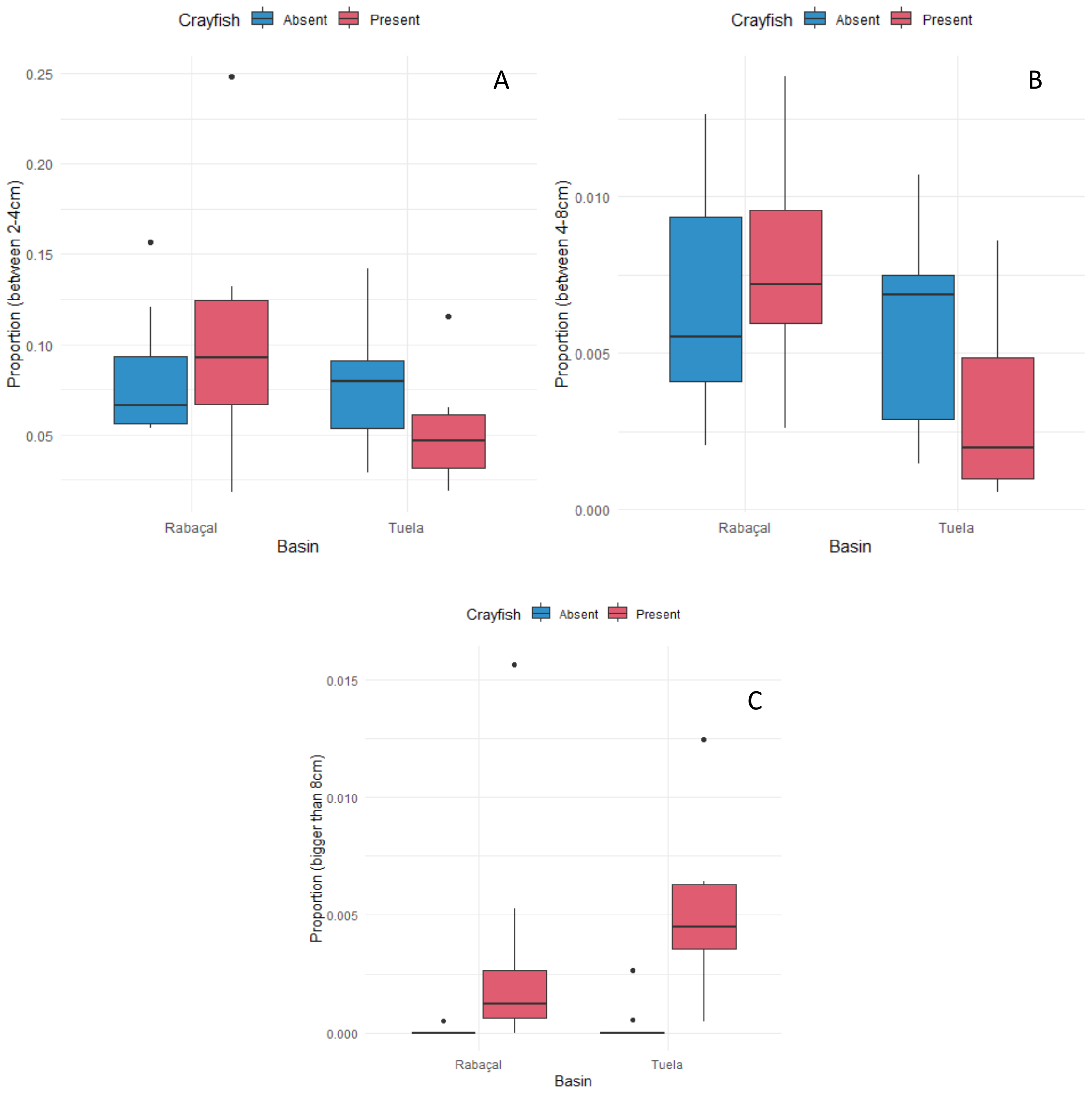


Figure 15 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Body size trait (2-4cm (A); 4-8cm (B); > 8cm (C)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median values (central line), the range 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.

For the life cycle trait, the CWM results showed no significant differences between the presence and absence of crayfish for the "less than 1 year" class and for the "more than 1 year" class (X2=0.525 p=0.469). However, between basins there were almost significant differences for both classes ("less than 1 year" - F=4.022 p=0.053; "more than 1 year" - F=3.925 p=0.056), with the Tuela River basin appearing to have a higher proportion of organisms with a life cycle lasting less than 1 year, compared to the Rabaçal River basin with more organisms with a life cycle longer than 1 year (Figure 16).

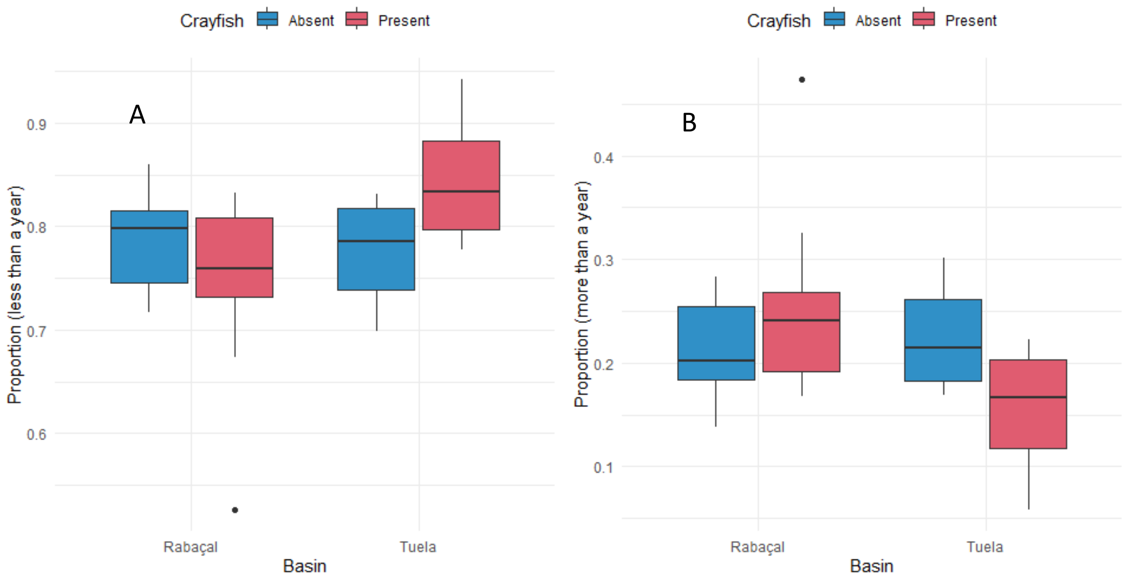


Figure 16 - Box diagram of the proportion of macroinvertebrates (CWM) belonging Life cycle trait (< 1year (A) and > 1year (B)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median values (central line), the range from the 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.

The CWM for the trait feeding habitats generally showed a community with a higher proportion of shredder and scraper macroinvertebrates (Figures 17 and 18).

The proportion of absorbers was not significantly different between invaded and non-invaded zones (F=0.074 p=0.787), but differences were detected between basins (X^2=8.877 p=0.003) since the Rabaçal River basin had a higher proportion of absorbers than the Tuela River basin (Figure 17A). It should be noted that all the absorbers identified were oligochaetes, which predominantly had more affinity for the deposit feeder's category. Regarding deposit feeders (Figure 17B), in the presence of crayfish a significantly higher proportion of deposit feeders was observed (F=4.505 p=0.042), while no significant differences were detected between basins (F=0.595 p=0.446).

The CWM data for shredders showed no significant differences either for the presence of crayfish (F=1.521 p=0.227) or between basins (X^2=0.897 p=0.343). Even so, it is possible to observe a tendency for invaded areas to have a higher proportion of shredders (Figure 17C). The proportion of scrapers (Figure 17D) did not appear to vary significantly between basins (X^2=1.144 p=0.285) and between invaded and non-invaded areas (F=0.986 p=0.329).

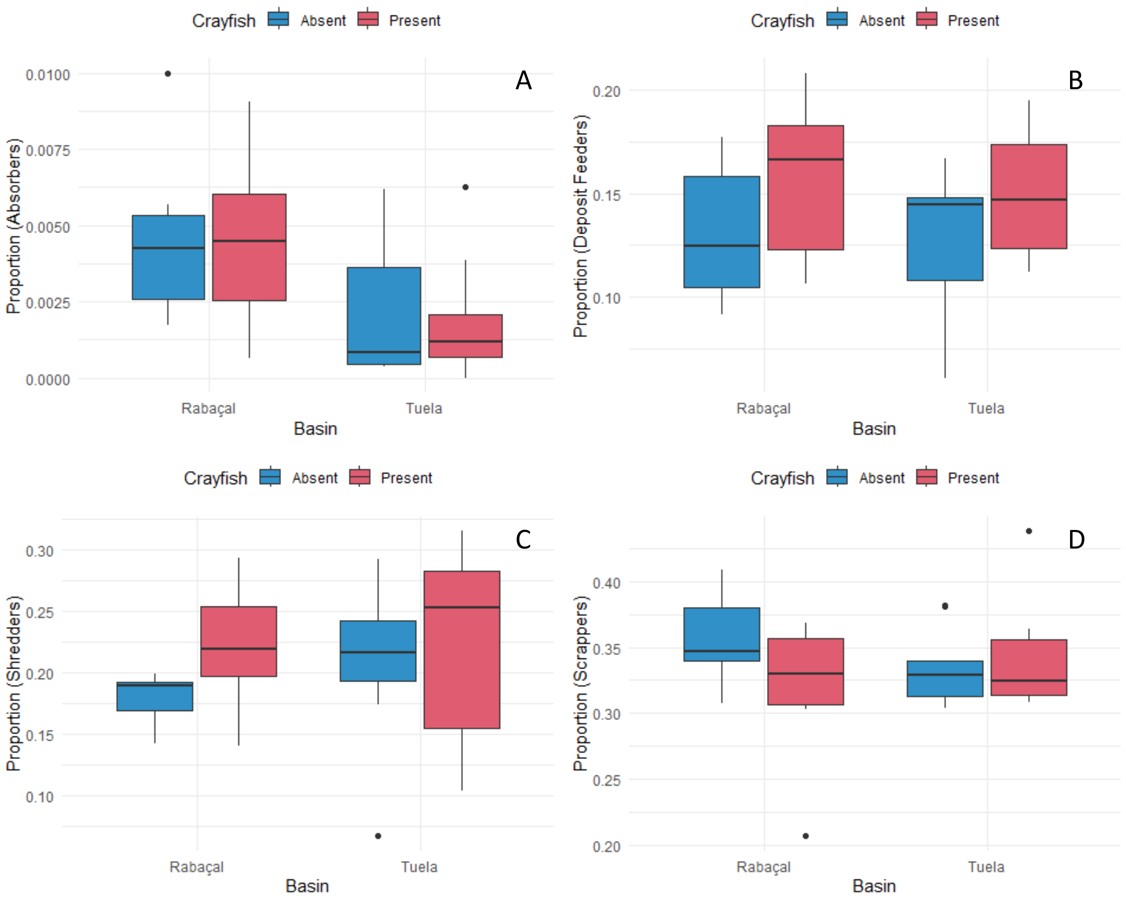


Figure 17 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Food habits trait (Absorbers (A); Deposit Feeders (B); Shredders (C) and Scrappers (D)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median 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.

In relation to the proportion of filter feeders (Figure 18A), the results did not show significant differences between basins (F=0.122 p=0.729), but there were almost significant in the presence/absence of crayfish (X^2=3.471 p=0.062), with the CWM of filter feeders showing to be relatively lower in the presence of crayfish. The piercers (Figure 18B) were significantly more present in the uninvaded sites (F=4.278 p=0.047) and showed no significant differences between basins (F=1.480 p=0.233).

In the group of predators (Figure 18C), the CWM results were close to showing significant differences, both for the crayfish (F=3.142 p=0.086) and for the basins (F=3.889 p=0.057). Higher proportion of predators were found in the presence of the crayfish and in the Rabaçal River basin. These results could be explained by the influence of the crayfish sampled, but also by the high abundance of the damselfly *Platycnemis* (N=200) at site R6.

For the parasite group (Figure 18D), no significant differences were found between basins (X^2=1.009 p=0.315) and invaded and non-invaded sites (X_2=1.401 p=0.237).

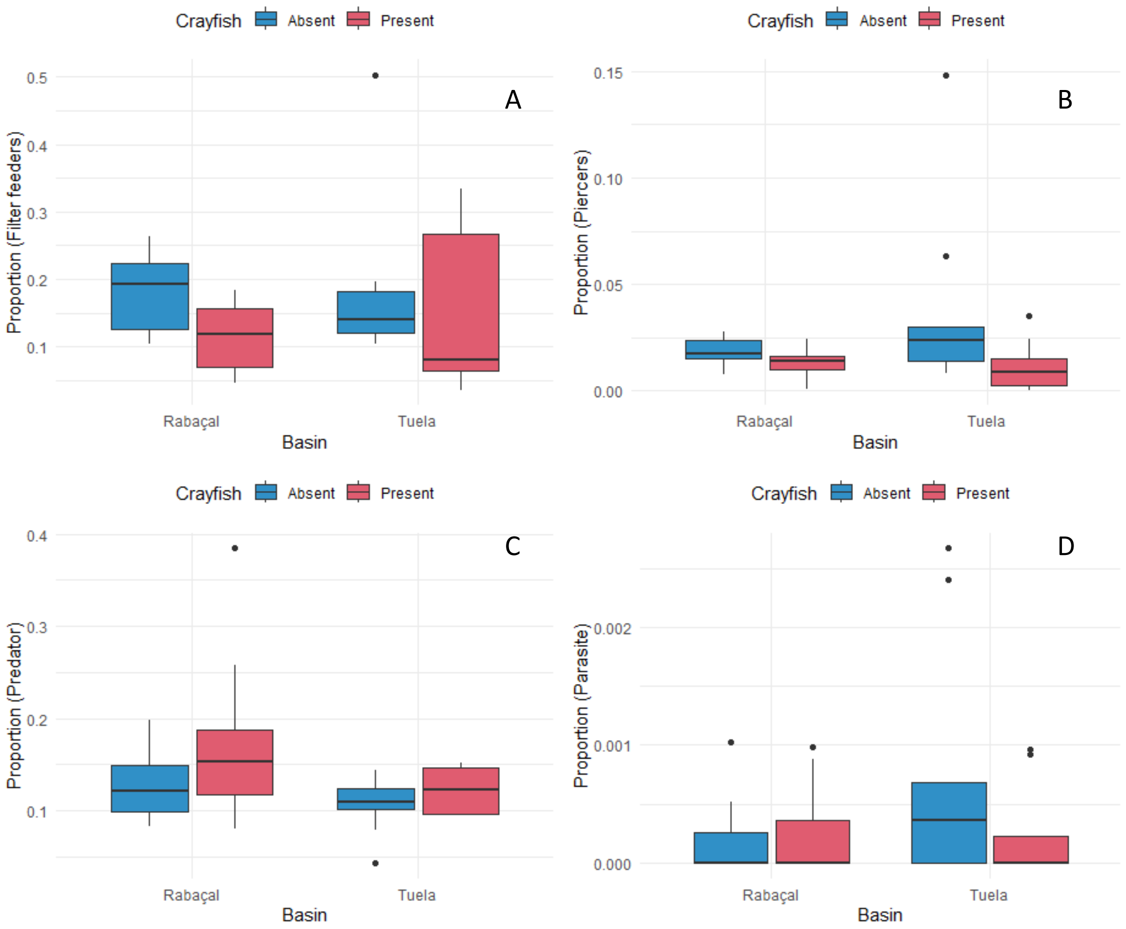


Figure 18 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Food habits trait (Filter feeders (A); Piercers (B); Predator (C) and Parasite (D)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median 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.

In general terms, the CWM results for the Habitat trait showed that the macroinvertebrate community sampled had a greater presence of organisms with a preference for coarse sediment and macrophyte habitats (Figures 19, 20 and 21). For coarse sediments (Figure 19A), no significant differences were identified for the presence of crayfish (F=0.294 p=0.591) and for the basins (F=0.084 p=0.774). Similarly, for the proportion of organisms inhabiting the gravel (Figure 19B), no differences were observed between basins (F=1.178 p=0.286) or between the presence/absence of crayfish (F=0.023 p=0.879).

For the "Sand" category (Figure 19C), no significant differences were detected between the invaded and non-invaded zones (F=0.200 p=0.658), but differences were observed between basins (F=4.191 p=0.049), with Rabaçal River basin having a higher proportion of sand-dwelling macroinvertebrates. For the CWM results of the silt-dwelling macroinvertebrates (Figure 19D), no significant differences were found (Crayfish - X^2=0.171 p=0.679; Basin - F=0.121 p=0.730).

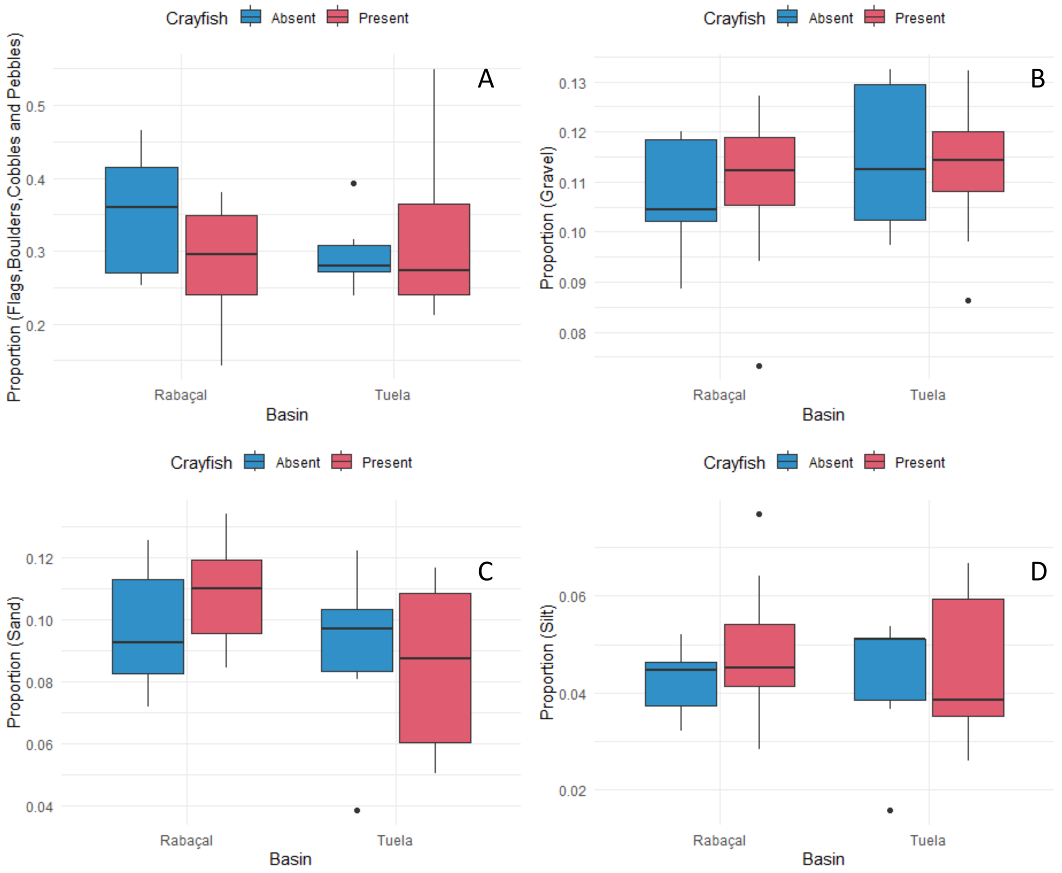


Figure 19 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Habitats trait (Flags, Boulders, Cobbles and Pebbles (A); Gravel (B); Sand (C) and Silt (D)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show 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.

For the "Macrophytes" category (Figure 20A), no significant differences were detected in the two factors under study (Crayfish - F=0.597 p=0.445; Basin - F=2.324 p=0.137). On the other hand, the CWM for the "Microphytes" category showed significant differences only in the presence of crayfish (F=6.741 p=0.014), showing that the existence of crayfish may be negatively affecting the macroinvertebrates that occupy this microhabitat (Figure 20B).

The results obtained for the proportion of macroinvertebrates occupying twigs and roots (Figure 20C) showed no significant differences (Crayfish - F=0.534 p=0.470; Basin - F=1.640 p=0.210) and the same was observed for the "Organic detritus and Litter" (Figure 20D) category (Crayfish - F=1.372 p=0.250; Basin - F=1.353 p=0.253).

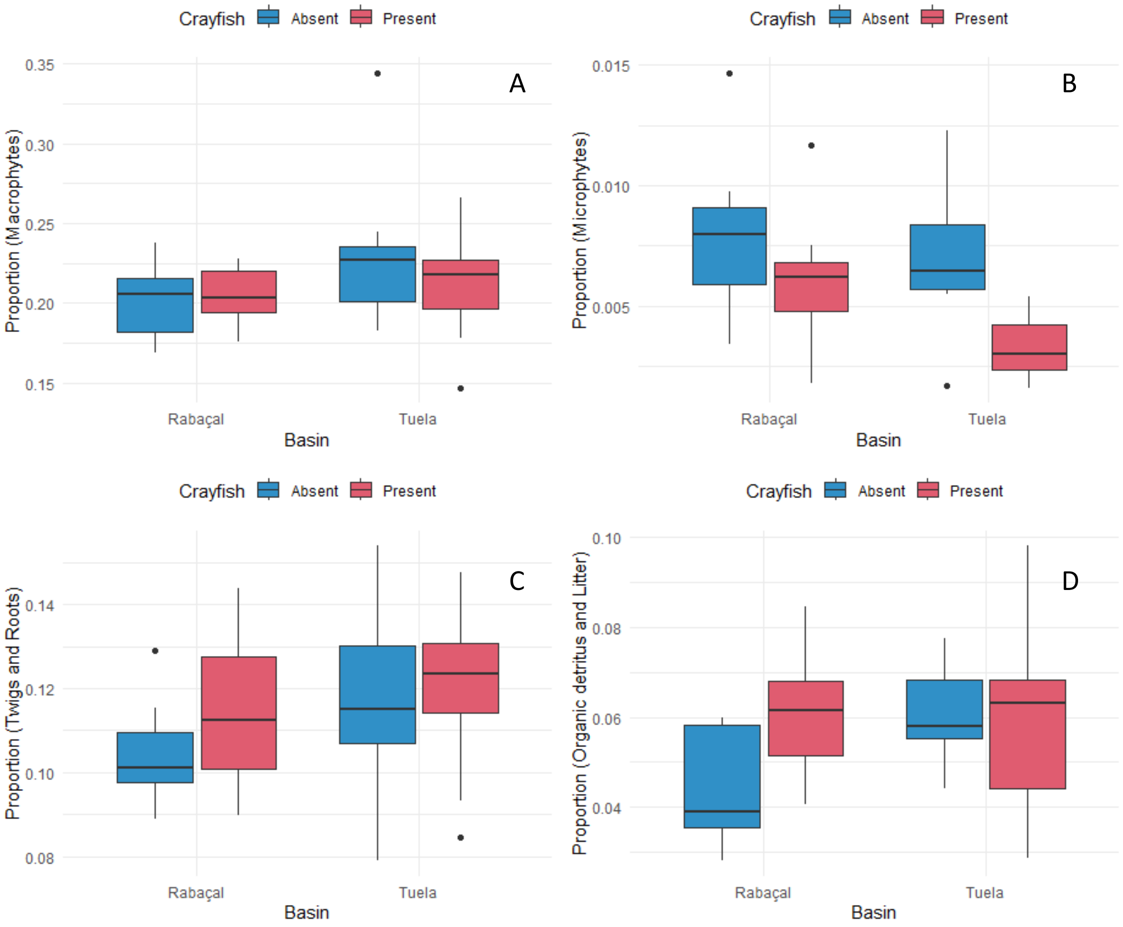


Figure 20 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Habitats trait (Macrophytes (A); Microphytes (B); Twigs and Roots (C) and Organic detritus and litter (D)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. 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.

In the "Mud" category (Figure 21), the proportion of organisms associated with muddy areas also showed no significant differences between the presence and absence of crayfish (F=1.151 p=0.291) and between the two basins (F=1.587 p=0.217).

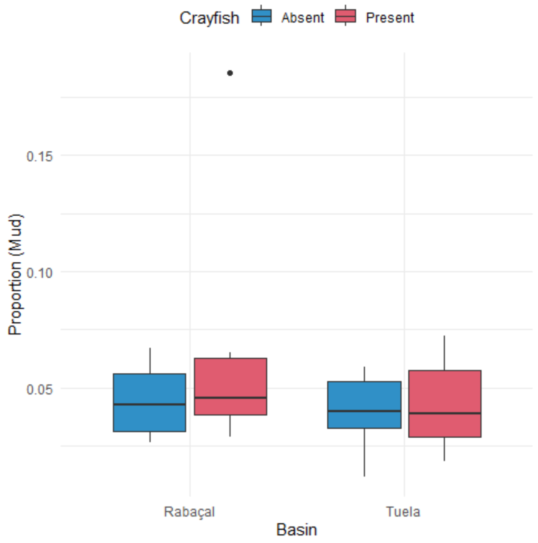


Figure 21 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Habitats trait (Category - Mud) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median values (central line), the range from the 25th to 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.

The CWM results for the trait "Current velocity" showed a community of macroinvertebrates that prefer slow and moderate water flow velocities. In the case of organisms that prefer lentic waters, no significant differences were detected between basins (F=0.887 p=0.353) and there were almost differences between the presence and absence of crayfish (F=3.336 p=0.077) (Figure 22A). In "Slow" current category, the data obtained from the CWM revealed no significant differences between basins (F=0.092 p=0.764), nor between invaded and non-invaded sites (F=0.106 p=0.747) (Figure 22B). In the remaining categories of the "Current velocity" trait, no significant differences were identified. This includes the "Medium" (Crayfish - F=2.586 p=0.118; Basin - F=0.364 p=0.551) (Figure 22C) and "Fast" (Crayfish - F=0.266 p=0.609; Basin - F=0.906 p=0.348) categories (Figure 22D).

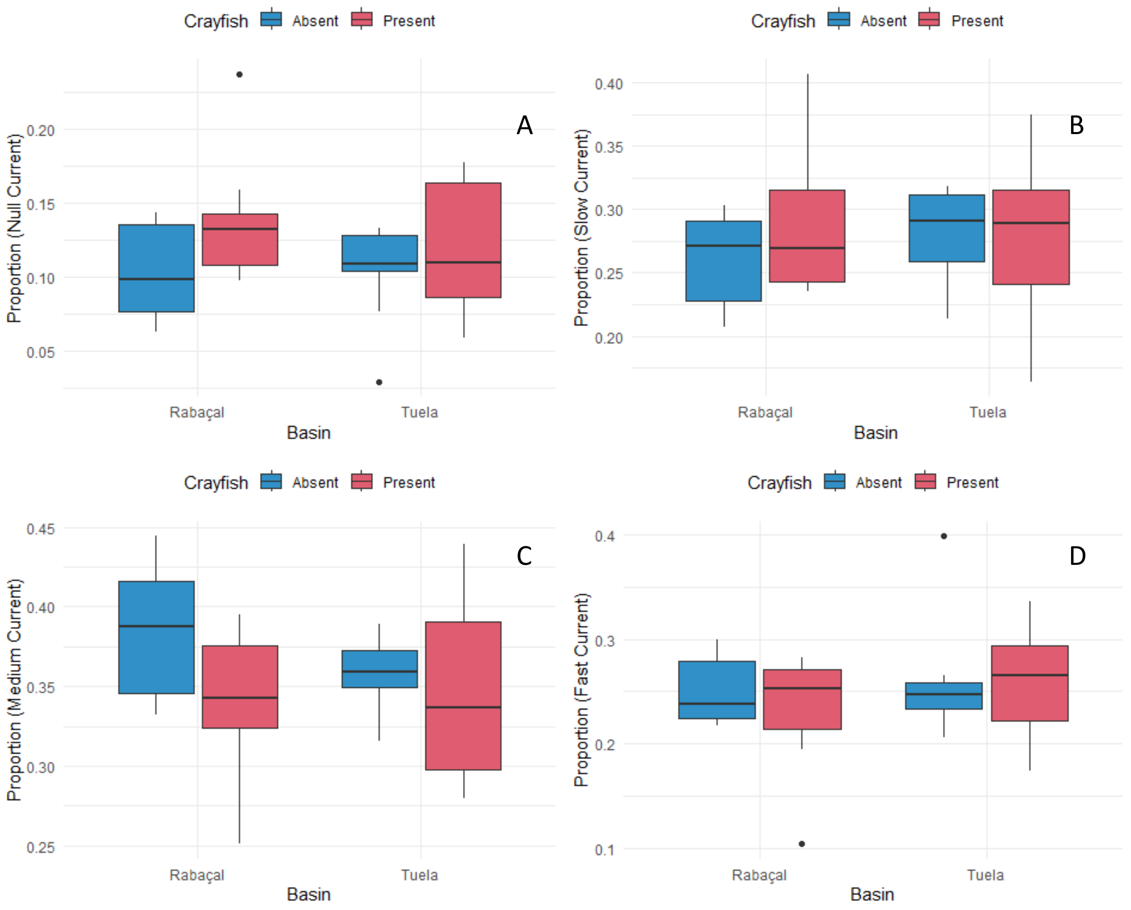


Figure 22 - Box diagram of the proportion of macroinvertebrates (CWM) belonging to the Current velocity trait (Null (A); Slow (B); Medium (C); Fast (D)) for the Rabaçal and Tuela River basins considering the presence (red) or absence(blue) of the signal crayfish. Boxplots show median values (central 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.

5. Discussion

The main aim of this study was to investigate the potential ecological effects of the signal crayfish *Pacifastacus leniusculus* on aquatic macroinvertebrate communities present in the Montesinho Natural Park region. The signal crayfish is an invasive species well known for its ecological and economic impacts on invaded ecosystems (Gherardi, 2007; Lodge et al., 2012; Momot, 1995; Rodríguez et al., 2005; Sousa et al., 2019; Strayer, 2010; Twardochleb et al., 2013; Vaeßen & Hollert, 2015), where its effects on macroinvertebrates are already well documented, with many of these effects considered negative. Several studies have shown that invasive crayfish have reduced not only the density, biomass and species richness, but also the functional diversity of macroinvertebrates (Albertson & Daniels, 2016; Carvalho et al., 2022; Galib et al., 2021; Mathers et al., 2016; Mathers, White, Guareschi, et al., 2020).

In this study, we also hypothesised that the recent introduction of the signal crayfish in the Montesinho Natural Park is affecting the macroinvertebrate community, reducing its abundance, biomass and diversity. This hypothesis was supported since we observed that the invaded sites showed a lower abundance, biomass, richness, and diversity of taxa. However, regarding the functional diversity, there was no clear reductions, but changes were observed between the density of some functional groups (e.g., deposit feeders, piercers and periphyton - associated macroinvertebrates). Therefore, the signal crayfish is affecting this community either by direct predation or by another indirect mechanism such as affecting the leaf litter decomposition or nutrient cycling (Galib et al., 2021; Jackson et al., 2014). In addition, potential differences in the macroinvertebrate communities between the Rabaçal and Tuela River basins were also investigated, and no significant differences were found between the diversity indices. Regarding functional diversity, differences were found between basins, with some functional groups showing a higher density in the Rabaçal River basin. During this study, the abiotic conditions of the two basins were also investigated, including carrying out an RHS, which showed that both basins still present almost pristine habitats, showing the Rabaçal River basin better habitat quality and less human disturbance. With this, we can deduce that the fact that the Rabaçal River basin has a higher density of some functional groups is possibly due to the better habitat quality available in this basin when compared to the Tuela River basin.

5.1 Abiotic characterisation

In general, the Rabaçal and Tuela River basins have similar abiotic conditions, with the differences between the basins being caused mainly by altitude, conductivity and TDS. In general, the Tuela River basin has a higher altitude (mainly the sites in the upstream part of the Baceiro River) and showed higher conductivity and TDS values. These conductivity and TDS higher values may be related to the lower hydromorphological quality and greater anthropogenic influence compared to the Rabaçal River basin. The higher conductivity and TDS values in the Tuela River sites may therefore be associated with this greater human disturbance (Harwell et al., 2008; Mustapha et al., 2013; Wu et al., 2020). However, we cannot ignore the possible influence of other factors on water composition such as small differences in the geology between both basins (Bhateria & Jain, 2016; Skoulikidis, 1993). In addition, increased conductivity is usually correlated with increased TDS, where a greater number of dissolved ions is an important factor affecting conductivity (Maqbool et al., 2012; Rusydi, 2018). Furthermore, it is possible to observe a greater difference in the upstream sites of the Baceiro River (B4, B5 and B6) compared to the rest of the sites sampled in the Tuela River basin. This difference is related due to the high altitude at which B4, B5 and B6 are located, which in turn led to the lower temperature observed, causing the TDS and conductivity to decrease (Maqbool et al., 2012; Rietman et al., 1985). In the same vein, these upstream sites of the Baceiro River are very isolated and with a very low human disturbance; this situation is possible contributing for the almost pristine conditions and very low TDS and conductivity values recorded in these three upstream sites.

Both the Rabaçal and Tuela River basins had high values for the HQA index with an average of 67.7 and 74.0, respectively, obtaining "Excellent" quality at all the sites except R6. These high scores demonstrate low human disturbance and the consequent natural character of the habitats in the channel and adjacent terrestrial areas, producing good longitudinal and lateral connectivity in the river corridor (Raven et al., 1998). It is also important to emphasise that the high scores of the HQA sub-indices demonstrate the great diversity of habitats available in the aquatic and adjacent riparian systems (Teixeira et al., 2010). The HMS score was higher in the Rabaçal River basin than in the Tuela, with the Rabaçal showing 62.5 % of sites classified as "Pristine" and "Predominantly unmodified", compared to 58.8 % in the Tuela River basin. The fact that some sites showed lower scores for the HMS, and this is very evident in the Tuela River basin, demonstrates the existence of some anthropogenic influence on riparian habitats, through the construction of structures such as hydraulic crossings, bridges, reinforcement and resection of banks and the presence of weirs/transverse barriers (Raven et al., 1998). Overall, the Rabaçal River basin stands out for having higher hydromorphological quality than the Tuela River basin.

The RQI index showed similar patterns to the HQA index, with Rabaçal River basin presenting 76.4 % of the sites with "Very High" quality, in contrast to Tuela with only 47.1 %. This difference can be explained by the complexity component related to the structure of the vegetation on the slope and in the first metre from the top of the bank (Del Tánago & De Jalón, 2011). The other components (continuity and naturalness) obtained similar average values in the two basins, but the standard deviations in the Tuela River basin showed greater variability in both the degree of continuity of the vegetation structure and the naturalness of the banks. In addition, the riparian vegetation in the Tuela River basin was more affected by the death of alder trees (caused by *Phytophthora lacustris* and *Phytophthora* x *alni*), resulting in greater gallery fragmentation and contributing for the lower scores reported concerning the RQI index (Raven et al., 1998).

In conclusion, despite the differences found between basins, the four studied rivers have proved to be very similar and still present almost pristine habitats, as also demonstrated by a previous study in the Montesinho Natural Park (Teixeira et al., 2010). This fact should be emphasised given the recent environmental deterioration of several tributaries of the Douro River basin and consequent biodiversity loss (Nogueira, Sousa, et al., 2021). Therefore, all the four studied rivers still present excellent conditions for the presence of many aquatic species with high conservation status and the preservation of these habitats should be a priority. Finally, and for the purpose of this study, the very low human disturbance detected is important because reduce the possible bias introduced by different environmental conditions when we compared invaded and on-invaded sites and in this way makes it easier to study and detect potential impacts of signal crayfish on macroinvertebrate communities.

5.2 Biotic characterisation

From the 34 sampling sites, signal crayfish was found in 18, having a higher abundance in the Tuela River basin. The lower abundance of the signal crayfish was registered in sites in the Baceiro River (except B1), being this an indication that the invasion of the signal crayfish in this watercourse is more recent and is still in progress in the upstream direction (Sousa et al., 2019).

The sampling and identification of macroinvertebrates managed to detect a high diversity of taxa, with 133 identified. Concerning the macroinvertebrate communities, it was possible to detect significant differences between basins and invaded and non-invaded sites. In the nMDS it was also possible to observe this influence with almost all the uninvaded sites being grouped close together. The exception was the Baceiro River sites, showing a similar pattern to the PCA results for the abiotic characterisation already discussed above. Therefore, and in the Baceiro sites, the environmental conditions have a major influence in the macroinvertebrate communities when compared to the possible influence of the signal crayfish. This may be explained due to the low abundance of the signal crayfish in the four invaded sites in the Baceiro River and a more recent introduction contributing to a lower ecological effect (Sousa et al., 2019).

The presence of signal crayfish significantly decreases the abundance, biomass, richness, and Shannon index of the macroinvertebrate community; this observation is in line with several previous studies (Carvalho et al., 2022; Charlebois & Lamberti, 1996; Galib et al., 2021; Twardochleb et al., 2013). This decrease can be caused mainly by direct predation of macroinvertebrates, but also indirectly by loss of habitat complexity, trophic cascades or due to changes in 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 density of crayfish, the higher the abundance of crayfish, the higher their impact (Nyström et al., 1996). Our results show a similar pattern, with sites with a recent invasion, namely those in Baceiro River (B3 and B4) showing a lower abundance of crayfish, but a higher abundance of macroinvertebrates (1152 and 2181 ind. CPUE, respectively) compared to other invaded sites. However, there have also been studies that have shown that in certain cases there may even be an increase in the abundance of macroinvertebrates in the presence of invasive crayfish (see for example Albertson & Daniels, 2016). Nevertheless, these authors do not refute the impacts of crayfish on macroinvertebrates but argue that these may be specific to each taxon and vary from ecosystem to ecosystem. The biomass of macroinvertebrates in invaded sites has also been shown to be lower, indicating the existence of predatory pressure by the crayfish as they feed on bigger taxa and organisms (Stenroth & Nyström, 2003).

In terms of functional diversity (FRAO), no significant differences were found for the presence of signal crayfish, despite the tendency to a decrease in functional diversity in the invaded sites, as some studies have already shown (Carvalho et al., 2022; Mathers, White, Fornaroli, et al., 2020; Mathers, White, Guareschi, et al., 2020). When looking at the FRAO results for each trait, it was only possible to find differences for the "Body size" category, with the invaded sites showing less diversity in body size. These FRAO index results show that despite the presence of crayfish, macroinvertebrate communities end up having similar functional diversity among the other traits analysed (life cycle, feeding habits, habitats and current velocity). However, it should be pointed out that we are dealing with a community with high richness, which can sometimes lead to several taxa having similar ecological niches or functions (Schmera et al., 2012). Therefore, when a community has functional redundancy, several 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, mainly in ecosystems where the invasion is still very recent. Anyway, in an earlier manipulative study, Carvalho et al., (2022) assessed functional diversity, taking into account the functional redundancy of the macroinvertebrate community, at sites in the PNM, showing that functional diversity decreased with the increase in crayfish abundance, making the communities less resistant to disturbances. This decrease may also affect the functioning of the ecosystem via a reduction in productivity, nutrient cycling and litter decomposition (Guareschi et al., 2021).

These FRAO results demonstrate the importance of also considering functional traits to facilitate the interpretation of biological community responses (Guareschi et al., 2021), such as the CWM. Regarding the "Body size" trait, the CWM showed that there is a lower proportion of taxa smaller than 0.5 cm in size in the invaded sites. When we look closer at the taxa x trait matrix, we can see that the organisms of the Coleoptera order are contributing most to these differences, being particularly important taxa from the Elmidae family. This difference can also be corroborated by the results of the SIMPER analysis, which showed the riffle beetle genus *Limnius* as one of the main taxa contributing to the differences (2.12 %) between invaded and non-invaded sites, presenting lower average abundance when the crayfish is present. Ruokonen et al. (2016) found that the density of riffle beetles decreased in the presence of crayfish and Guan & Wiles (1998) demonstrated that signal crayfish feed on Elmidae, although predation pressure varies seasonally and according to the age of the signal crayfish. In addition, riffle beetles are especially vulnerable to predation because of their semivoltine life cycle, which is mostly aquatic, and also show limited colonisation abilities (Aroviita & Hämäläinen, 2008; Ruokonen et al., 2016). For the other categories of the "Body size" trait, the CWM results showed no significant differences between sites, except for the category larger than 8 cm, but these differences were mainly due to the presence of 46 signal crayfish individuals.

The data obtained from the CWM showed no differences between the length of the life cycle trait. In contrast, Mathers, White, Guareschi, et al. (2020) observed that taxa that take a year or more to complete their life cycle may be negatively affected by the signal crayfish, making taxa capable of completing the cycle in less time more resilient to crayfish effects, allowing communities to recover faster when crayfish are less active, for example during the winter.

The proportion of deposit feeders was surprisingly higher in the presence of crayfish, this difference may be related to the fact that crayfish are also shredders, which can increase the decomposition rate of leaf litter, which in turn increases FPOM, as has been observed in studies with *Procambarus clarkii* (Carvalho et al., 2016; Jackson et al., 2014). It should be noted that all the four studied rivers have an excellent riparian cover and a high accumulation of leaf litter and other organic matter (Carvalho et al., 2022; Sousa et al., 2019, 2020). In other studies, it has also been observed that *Pacifastacus leniusculus* and *Orconectes rusticus*, in addition to macroinvertebrates, also consume large amounts of detritus and plant material when these are available (Bondar et al., 2005; Roth et al., 2006). Knowing that the main food of deposit feeders is FPOM (Cummins, 2016), the increase in its quantity could explain the higher proportions of this group of organisms in the invaded sites. Additionally, when comparing the SIMPER analysis and the organisms with an affinity for this functional group, there was an increase in Baetidae (*Centroptilum* e *Procloeon*) and Heptageniidae (*Epeorus* e *Rhitrogena*) in invaded sites. Curiously, these organisms have been shown to be better able to escape predation due to their higher mobility (Peckarsky, 1980), which gives them an even higher advantage in invaded sites, given the potential increase in food resources. The CWM in shredders did not show significant differences, although the pattern for invaded sites showed a higher proportion of organisms with this trait. It is important to emphasise the fact that the signal crayfish itself ends up being a shredder, as well as a predator, so its influence on the CWM results cannot be overlooked. The literature has shown different results for this functional group, where some were positively affected by the presence of crayfish (Mathers, White, Guareschi, et al., 2020) and others negatively affected due to predation (Lagrue et al., 2014).

Curiously, significant differences were 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, with SIMPER identifying the species *Aphelocheirus occidentalis* as one of the taxa contributing most to the differences detected, as it was almost absent in invaded sites. In contrast, the literature shows that Heteroptera are not usually among the taxa most affected by crayfish, even by predation, since they are usually very agile (Gherardi, 2007; Nystrom et al., 1999; Nyström et al., 1996). In the case of *Aphelocheirus*, earlier studies showed that crayfish species such as *Astacus leptodactylus* and *Orconectes limosus* showed some predation on Heteroptera (Šidagyte et al., 2017; Vojkovská et al., 2014). The effect of crayfish predation on these organisms cannot be ignored, but it is also important to consider for the possibility of competition for the same food resources, since *Aphelocheirus* are predatory piercers with a preference for mayflies and caddisfly larvae (Carbonell et al., 2011).

No significant differences were found in the CWM of the filter feeders group, but a pattern was observed in which the presence of crayfish caused a lower proportion of filter feeders. This observation is in line with the SIMPER analysis showing that some filter feeders contribute most to the differences detected between invaded and non-invaded sites, namely the caddisfly larvae *Brachycentrus subnubilus* and *Chimarra marginata,* with a contribution of 2.75 and 2.58 %, respectively. The lower density of *Brachycentrus* may be due to its lower mobility and feeding behaviour. In other words, this invertebrate clings to the surface of the rocks to filter suspended particles, exposing itself to a higher predation risk by the crayfish (Bobeldyk & Lamberti, 2008). In the case of the *Chimarra marginata*, it may also be subject to predation by the crayfish, since it does not move quickly and have poor camouflage capacity, making it easier to be recognised by potential predators (Lodge et al., 1994).

In the Habitats trait, the CWM results only showed significant differences for the Microphytes habitat, where organisms associated with periphyton, for example, had a lower proportion in the invaded sites. When we looked at which taxa had an affinity for this microhabitat, we mostly found species of Trichoptera (Limnephilidae) and Gastropoda. The Limnephilidae are a group that responds negatively to the presence of crayfish, mainly due to predation, as shown by some studies (Lagrue et al., 2014; Renai & Gherardi, 2004; Šidagyte et al., 2017). Gastropods are one of the groups most affected by the invasion of several crayfish species (Charlebois & Lamberti, 1996; Galib et al., 2021; Lodge et al., 1994; Mathers et al., 2016; Stenroth & Nyström, 2003; Twardochleb et al., 2013), and our results may also demonstrate a potential negative effect of the signal crayfish on gastropods, such as the complete absence of *Radix peregra* in invaded sites. These organisms have low mobility and large size, making them easy and profitable prey for crayfish (Stenroth & Nyström, 2003). In addition, it is important to point out that this decrease in gastropods and other organisms with low mobility may be facilitating other 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 may be responding positively to the suppression of these invertebrates by the crayfish, due to less competition for the same resources (Hansen et al., 2013; Hertonsson et al., 2008; Nystrom et al., 1999). However, more studies are necessary to confirm these assumptions.

Besides the impacts of crayfish, the macroinvertebrate communities were also compared between basins. The two communities were indeed significantly different using PERMANOVA, but showed no differences in diversity indices (richness, abundance, Shannon and evenness) and biomass. On the other hand, significant differences were detected in functional diversity. There were almost significant differences in the FRAO index, with the Rabaçal River basin showing greater functional diversity when compared to the Tuela River basin. The same pattern was observed in the "Body size" and "Life cycle" traits, with the Rabaçal River basin having a significantly more diverse community in size types and in life cycle strategies and durations. The CWM results followed the previous conclusions and showed significant differences for the categories "between 4-8 cm" (Body size); "less than a year", "more than a year" (Life cycle); "Absorbers" (Feeding Habits) and "Sand" (Habitats), having the Rabaçal River basin a higher proportion of organisms from these categories. These differences between basins may be related to the fact that the Tuela River basin is subject to a slightly higher human pressure than the Rabaçal, thus reducing the functional diversity of the macroinvertebrate communities (Carvalho et al., 2022). In addition, it is important to recognise macroinvertebrates as organisms that are susceptible to environmental impacts and are important indicators of the degradation of watercourses (Wallace & Webster, 1996). Anyway, it is not safe to associate these differences solely to human disturbance, because other factors not studied such as climate and biogeographical history may have some influence in the overall results. However, and given the geographical proximity and because both rivers (Rabaçal and Tuela) are tributaries of the Tua River, these climatic and biogeographic factors are probably less important.

In short, the effects of the signal crayfish (*Pacifastacus leniusculus*) on macroinvertebrate communities were clear, with a decrease in the abundance, biomass, richness and diversity of this community. In addition, the potential differences between taxonomic groups due to the selective pressure of the crayfish were evident, showing that the direct impacts of the crayfish largely depend on the lifestyle and behaviour of each taxon. On the other hand, the low anthropogenic influence, plus the still high habitat diversity present in the Rabaçal and Tuela River basins may contribute to some functional redundancy, and this mitigate the functional impacts of the signal crayfish on the Montesinho Natural Park. However, the situation could worsen in the future, showing the importance of continuing to monitor not only signal crayfish populations, but also macroinvertebrate communities, organisms that play a fundamental ecological role in freshwater ecosystems.

6. Conclusion and future directions

Macroinvertebrates are key organisms in aquatic ecosystems, responsible for a great diversity of functions and services and occupying various trophic positions. In fact, these organisms serve as food for other species, control primary productivity and are crucial in regulating nutrient cycling and decomposition (Wallace & Webster, 1996). Given these crucial roles of macroinvertebrates, the results obtained in this study are alarming, since sites invaded by the signal crayfish (*Pacifastacus leniusculus*) showed lower abundance, biomass, species richness and diversity compared to the uninvaded sites. Particularly affected were functional groups such as deposit feeders, piercers and periphyton - associated macroinvertebrates. This invasion becomes more worrying when we are referring to the Montesinho Natural Park, an area of high biodiversity conservation interest with almost pristine watercourses of high habitat richness and low anthropogenic influence. Despite the low level of human disturbance in the Montesinho Natural Park, the recent introduction of the signal crayfish could be a serious problem for macroinvertebrate communities and other organisms by jeopardising several key ecosystem functions. Therefore, this invasive species should be the target of management measures (control, for example) aimed at mitigating its negative impacts.

Bearing in mind the results and the impacts demonstrated here, in the future it will be important to understand whether the main mechanism for the differences found is direct predation by the signal crayfish or another indirect route, namely through trophic cascades or changes in nutrient cycling. In order to better understand these mechanisms, manipulative experiments could be carried out, giving more attention over certain variables. In our study, we only considered the presence and absence of crayfish, but comparing whether different densities of crayfish cause different outcomes on the macroinvertebrates is also important, since a higher density of crayfish can further aggravate the observed impacts. In addition, our study did not consider seasonal and annual variations as we only sampled at the summer of 2022. So, it would also be interesting to assess how the macroinvertebrate communities respond to the decrease in crayfish activity in the colder months or between different years subjected to distinct environmental conditions (i.e., years with different temperatures and precipitation regimes).

Since macroinvertebrates are a key community, it will also be important to find out which ecosystem functions are being affected (nutrient cycling, decomposition), or whether this community has enough functional redundancy and so their functions are not compromised. Furthermore, and since the abundance, biomass, richness and diversity of the macroinvertebrate communities were affected, it would be interesting to assess whether other taxonomic groups (fish, amphibians, mammals, fungi) are also being affected given their reliance in this taxonomic group.

Finally, and in order to mitigate the impacts found, it would be important to implement some management actions to the signal crayfish in the Montesinho Natural Park (e.g., control using traps), which in turn would allow us to study how macroinvertebrate communities respond to the reduction in crayfish abundance, and if this translates in a possible recovery of macroinvertebrate populations.

Even after this study, there is still a lot of work to be done to understand how to mitigate the impacts caused by invasive species. However, the results reported here relate to a large spatial area and four different rivers with very low human disturbance, establishing a solid baseline for future studies to compare the progression of the invasion of the signal crayfish and their ecological impacts in macroinvertebrate communities. We should also use this knowledge to educate and increase the awareness of citizens about the ecological and economic problems mediated by several aquatic invasive species. In addition, it is crucial to adopt management actions that prevent the spread and introduction of this (or other) invasive species in this important protected area in order to maintain the high conservation potential of the Montesinho Natural Park.

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Annex

Table S1 - Results of the Principal Components Analysis based on the abiotic data.

PC	Eigenvalues	%Variation	Cum. %Variation
1	0,102	53,4	53,4
2	5,42E-2	28,4	81,7
3	2,41E-2	12,6	94,4
4	8,05E-3	4,20	98,6
5	1,56E-3	0,80	99,4

Table S2 - Contribution of the abiotic variables to each of the five PCA axis.

Variable	PC1	PC2	PC3	PC4	PC5
Temperature	-0,099	-0,037	-0,053	-0,084	0,886
Oxygen	0,018	0,001	0,012	-0,040	0,342
Conductivity	0,048	0,649	0,367	0,655	0,099
TDS	-0,409	0,705	-0,245	-0,519	-0,060
pH	-0,015	0,009	-0,005	0,044	0,046
Altitude	0,883	0,266	0,063	-0,371	0,062
HQA index	-0,004	-0,021	-0,060	0,055	0,281
HMS index	0,201	0,095	-0,892	0,387	-0,005

Table S3 - Average abundance (ind.CPUE) of signal crayfish in the sampled sites of the Mente, Rabaçal, Tuela and Baceiro Rivers.

Rabaçal Basin Sites	**Average Abundance (ind. CPUE)**
M1	8.0
M2	4.3
M3	31.7
M4	14.9
R1	0.0
R2	0.0
R3	0.0
R4	0.0
R5	0.0
R6	13.9
R7	40.5
R8	20.3
R9	19.9
R10	13.5
R11	9.6
R12	0.0
R13	0.0

Tuela Basin Sites	**Average Abundance (ind. CPUE)**
T1	47.3
T2	57.3
T3	54.5
T4	31.1
T5	0.0
T6	0.0
T7	0.0
T8	0.0
T9	0.0
T10	0.0
T11	0.0
B1	40.6
B2	7.0
B3	5.6
B4	0.7
B5	0.0
B6	0.0

Table S4 – Abundance (ind.CPUE) of macroinvertebrate taxa at the Mente, Rabaçal, Tuela and Baceiro sampling sites.

Taxa	**M1**	**M2**	**M3**	**M4**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**	**R11**	**R12**	**R13**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**
Isoperla	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	0	1	9	0	4	3	0
Perla	3	20	6	2	24	9	14	4	14	1	15	12	1	8	24	15	0	0	0	0	29	19	14	5	6	7	5	8	44	13	6	3	14	10
Leuctra	65	130	143	457	82	97	97	75	37	90	60	417	43	97	357	246	132	53	54	106	115	162	67	209	98	214	56	92	50	40	288	300	367	222
Protonemura	0	1	1	13	0	3	2	1	0	0	0	2	5	3	0	0	4	0	2	2	0	0	4	3	0	0	3	18	0	21	5	9	32	4
Chironomidae	83	307	255	246	317	214	76	125	115	51	30	185	23	365	141	454	204	43	141	135	170	76	129	149	113	134	71	173	45	90	281	575	535	381
Ceratopogonidae	0	3	2	3	2	5	1	1	1	3	0	2	0	1	1	2	0	0	0	0	2	0	0	0	1	0	2	0	0	0	1	3	7	3
Hexatoma	2	8	1	3	3	1	7	1	0	3	4	2	0	2	2	1	0	0	0	0	5	3	1	0	0	2	2	5	11	7	17	8	1	5
Dicranota	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	2	7
Atherix	1	1	9	1	0	0	2	2	1	2	2	9	1	2	7	6	6	0	0	1	0	4	4	4	4	4	0	75	0	1	2	33	33	4
Atrichops	1	0	6	0	0	1	1	0	0	1	0	7	0	3	2	6	0	0	0	0	0	0	0	1	1	0	0	34	0	1	1	1	6	0
Dixa	0	0	1	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	2	0	0
Dixella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Psychodidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Simuliidae	0	3	7	5	29	16	1	5	0	0	32	26	3	9	6	9	21	62	6	6	2	51	1406	39	0	0	6	13	2	22	25	10	57	109
Tabanidae	0	2	0	0	0	3	0	3	3	0	1	4	0	2	2	6	1	0	0	1	0	0	0	1	0	0	0	12	2	0	0	0	0	3
Wiedemannia	0	0	0	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0	0	0	0	0	2	0	0	1	0
Hemerodromia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	4	0
Tipulidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0
Chrysopilus	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0
Dolichopodidae	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sF. Anophelinae	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	1
Anthomyidae	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diptera pupae	0	7	13	14	11	7	0	3	3	0	2	5	1	4	4	11	3	0	3	4	4	0	9	2	2	2	5	8	0	5	17	19	20	8
Onychogomphus uncatus	10	61	84	6	23	14	15	37	37	6	110	28	24	72	62	145	129	6	2	16	5	33	21	21	18	26	26	39	4	2	54	1	13	2
Gomphus pulchellus	0	0	0	0	0	7	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Boyeria irene	7	11	32	1	4	3	1	3	1	8	0	5	2	1	3	13	5	0	1	2	1	6	7	8	9	15	5	4	1	1	1	4	4	3

Anax	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Calopteryx	0	3	0	0	23	1	0	0	0	3	0	3	0	1	0	2	0	1	0	0	0	0	3	75	0	16	0	0	3	0	1	14	8	2
Cordulegaster boltonii	1	0	0	1	0	0	0	0	2	0	0	0	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	5	0	1
Platycnemis	4	4	0	0	30	3	28	0	1	200	3	6	0	0	0	0	0	0	0	13	0	0	0	18	2	0	0	0	0	0	0	0	0	0
Erythromma lindenii	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
Sympetrum	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxygastra curtisii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	14	5	0	0	0	0	0	0	0	0
Chalcolestes viridis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Macromia splendens	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oxyethira	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4	0	1	5	5	0	0	0	0	0	0	0
Hydroptila	0	3	8	1	9	1	0	2	0	0	0	1	0	3	0	3	1	0	0	0	2	0	2	6	3	2	4	0	0	5	0	2	1	0
Orthotrichia angustella	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydropsyche	0	38	30	18	11	79	9	41	33	3	34	62	12	42	24	92	42	29	48	55	1	37	90	67	20	96	37	114	5	4	5	29	82	39
Cheumatopsyche lepida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	82	174	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Adicella	2	12	11	0	13	2	3	0	0	12	0	0	0	0	0	0	1	0	0	0	1	2	4	0	0	14	6	0	0	12	0	0	2	1
Mystacides	0	0	8	0	10	15	23	4	3	23	0	7	0	0	4	1	0	0	0	0	2	0	1	0	1	4	0	1	0	0	0	0	0	0
Setodes argentipunctellus	14	47	22	0	25	20	0	27	12	28	3	3	0	8	2	44	39	0	6	0	0	10	20	13	6	14	13	11	0	1	0	0	0	0
Oecetis	1	2	2	0	2	2	0	0	0	7	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Athripsodes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	4	2
Leptocerus lusitanicus	0	0	0	0	15	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chimarra marginata	0	166	24	22	55	556	198	250	137	7	68	247	31	107	40	57	39	24	329	161	1	14	48	401	74	65	21	64	0	1	1	0	0	0
Wormaldia	0	0	0	17	0	0	0	0	0	0	0	0	0	1	1	0	3	0	0	0	0	0	0	2	2	6	0	0	0	0	0	0	9	2
Brachycentrus subnubilus	4	7	9	0	96	108	27	48	256	9	2	2	6	3	0	5	29	54	22	0	0	49	46	60	0	19	27	5	0	0	0	0	0	0
Micrasema	0	8	8	1	3	7	6	2	0	0	0	2	0	2	0	0	2	2	0	0	0	1	1	1	0	1	0	0	2	1	0	1	2	0
Polycentropus	1	14	13	102	4	1	2	4	17	2	6	3	6	1	3	4	1	6	17	23	40	14	0	11	10	20	1	4	21	32	79	69	26	19
Pseudoneureclipsis lusitanicus	0	0	0	0	1	4	0	0	1	0	0	7	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
Rhyacophila	0	1	1	0	0	12	3	6	3	0	3	2	1	6	5	5	0	4	16	13	2	7	4	0	1	0	3	1	5	8	7	2	13	2
Psychomyia	0	12	6	6	10	4	2	2	8	0	0	7	0	5	4	5	1	3	1	4	4	5	5	0	5	0	2	0	0	0	0	0	0	0
Lype	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	0	0

Allogamus	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	15	7	0	0	0	0	0	0	0	3	0	0	0	0	0	0	27	3	3
Halesus radiatus	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	1	0	0
Potamophylax cingulatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Chaetopteryx lusitanica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	2
sF. Limnephilinae immature	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	0	0	0	0	0	0	0	0
Anomalopterygella chauviniana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1	3
Lepidostoma hirtum	0	1	0	0	0	0	0	1	7	0	0	0	0	0	0	6	1	0	0	0	0	3	3	0	3	8	3	0	0	0	0	0	1	0
Glossosoma privatum	0	0	1	0	0	0	0	0	0	0	0	1	0	1	2	36	0	0	0	0	0	1	0	1	0	1	2	0	0	0	0	2	11	24
Schizopelex festiva/ Sericostoma	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0	0	0	20	16	7
Sericostomatidae immature	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larcasia partita	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	3	2	0	1	0	0	0	4	4	6
Beraea	0	0	0	0	0	2	0	3	5	0	0	5	3	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	7	7	1	2	1
Thremma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	5
Calamoceras marsupus	1	0	0	0	0	25	1	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	10	0	1	0	10	0	0	0	0	3	0
Helicopsyche lusitanica	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Trichoptera pupae	0	1	0	0	0	11	0	3	7	0	2	4	1	2	0	2	1	0	12	21	0	2	2	0	3	0	1	3	0	0	0	4	2	0
Oligoneuriella rhenana	0	26	1	5	3	40	28	111	28	2	17	5	2	17	28	57	22	3	36	5	1	5	40	0	2	119	60	0	0	2	1	0	0	0
Ecdyonurus	6	25	11	56	10	11	18	13	40	6	31	32	20	2	6	5	14	27	30	43	40	43	12	13	27	16	5	31	47	22	52	25	18	38
Epeorus	0	2	1	8	2	24	9	22	2	0	12	12	8	52	16	37	13	14	50	39	2	4	12	16	1	2	5	7	1	8	13	0	0	0
Rhithrogena	0	0	3	17	0	1	2	6	0	0	13	14	4	9	17	0	0	0	0	0	0	2	5	0	0	1	0	0	4	2	3	9	8	21
Ephemerella	7	31	79	4	57	182	10	38	25	0	9	15	4	30	20	20	22	22	52	74	3	24	69	42	31	24	82	28	0	28	13	32	85	32
Baetis	20	146	93	377	126	184	38	121	56	15	73	321	35	107	118	253	107	81	88	109	69	79	228	73	66	118	66	87	170	71	121	109	54	185
Centroptilum	0	4	1	2	0	0	2	0	0	6	0	0	0	1	0	0	2	0	0	4	1	1	0	1	2	2	0	0	0	1	8	5	1	0
Procloeon	0	6	2	0	13	0	0	10	3	0	1	0	0	3	4	6	1	0	2	3	13	3	4	2	4	2	2	23	0	1	0	0	1	0
Cloeon	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	23	0	0	0	20	0	0	0	0	0	0	0	0	0	0
Choroterpes lusitanica	0	0	3	0	7	1	5	1	2	0	0	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thraulus bellus	2	3	0	0	1	2	0	1	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Habrophlebia/ Habroleptoides	7	20	32	56	4	0	0	2	10	25	7	9	3	1	12	66	34	0	0	4	50	30	10	4	74	33	8	57	28	4	73	509	140	158
Ephemera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	1	0	0	0	0	0

Caenis	3	5	1	0	12	6	18	4	20	18	0	0	0	1	10	1	6	0	9	13	9	5	3	7	3	1	2	3	1	0	2	0	0	0
Gerris	4	12	5	0	1	0	2	0	14	3	0	6	0	2	1	3	14	0	0	1	7	7	10	6	4	12	1	3	5	12	6	21	5	8
Hydrometra	0	0	0	0	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Micronecta	0	3	0	0	14	4	2	0	2	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	0	0	0	3	0	0	0	0	0	0
Naucoris	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Corixa	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Parasigara	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Velia	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microvelia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Aphelocheirus occidentalis	0	2	0	0	4	9	5	11	10	2	0	0	0	0	0	24	7	0	0	0	0	15	13	95	3	7	2	52	0	0	0	0	0	0
Ilybius	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Laccophilus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Hydroporus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Stictotarsus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Yola bicarinata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Meladema coriacea	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bidessus	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Limnius	14	71	14	0	34	106	108	97	34	18	4	5	3	13	4	91	11	2	19	15	3	26	31	27	11	11	22	51	8	0	0	46	21	17
Stenelmis canaliculata	9	28	5	0	9	27	31	24	52	0	0	3	0	0	0	7	0	0	0	1	1	5	9	7	9	8	0	1	0	0	0	0	0	0
Oulimnius	10	15	3	0	14	3	5	5	5	15	0	2	0	1	16	10	2	0	0	0	3	8	38	122	9	2	3	22	1	0	0	36	13	1
Dupophilus brevis	0	9	5	0	0	0	0	0	0	0	0	0	0	2	0	10	1	0	0	0	0	4	0	0	1	1	0	1	0	3	16	54	155	82
Elmis	2	2	7	0	0	3	0	1	0	0	1	2	0	1	5	1	8	2	1	0	1	0	2	63	1	8	7	27	0	3	8	11	49	8
Esolus	0	0	5	5	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	6	0	1	0	1	0	0	1	0	0	8	2	0	2
Normandia nitens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0
Hydraena	0	12	2	3	3	9	20	10	3	0	0	5	0	0	17	9	2	0	0	0	3	5	2	32	5	3	3	15	1	0	3	33	24	14
Dryops	0	1	1	0	0	2	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	2	1	3	0	0	0	1	2	0
Orectochilus villosus	0	0	4	0	0	0	0	1	1	0	0	0	4	0	0	4	1	0	0	1	0	0	0	14	0	7	0	6	0	1	0	0	0	0
Helodes	0	0	1	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Hydrocyphon	0	0	0	0	1	0	1	26	1	0	2	0	0	0	0	0	0	0	0	1	2	0	1	0	1	0	0	0	0	1	0	0	4	0

Laccobius	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Collembola	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Sialis	0	3	3	0	0	2	0	0	1	3	0	1	0	0	0	2	0	0	0	0	2	0	0	1	0	0	0	3	0	0	0	0	1	1
Sisyra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydrachnidia	1	6	1	0	9	6	0	5	2	0	3	1	0	10	0	8	9	1	0	0	1	3	38	14	14	8	15	10	0	5	2	10	60	3
Pacifastacus leniusculus	0	2	2	1	0	0	0	0	0	0	2	1	4	1	0	0	0	3	1	5	4	0	0	0	0	0	0	0	6	2	5	1	1	0
Pisidium	0	16	1	0	4	8	4	1	0	14	0	1	0	1	0	1	0	0	0	0	2	0	1	0	1	1	0	6	0	1	0	13	3	6
Margaritifera margaritifera	0	0	1	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gyraulus sp.	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Radix peregra	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	139	3	2	0	0	0	0	0	0	0	0
Galba sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Physella acuta	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ancylus	3	16	19	3	14	8	5	20	32	10	1	19	2	6	10	24	39	5	7	13	0	29	10	41	2	16	4	21	7	6	9	4	7	3
Valvata piscinalis	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oligochaeta	6	25	38	83	35	31	9	31	16	7	8	68	1	35	35	117	35	1	5	9	24	31	7	5	15	3	3	26	3	0	10	50	4	13
Erpobdella	2	1	0	0	4	35	8	3	2	0	4	18	3	8	1	2	0	0	2	0	0	1	1	3	1	0	5	2	0	1	0	6	7	5
Glossiphoniidae sp.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0
Nematoda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	2	1	0	0	0	0	0	0	0	0	0	0	1
Nematomorpha	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
Planariidae sp.1	0	1	2	0	0	0	0	0	0	0	0	0	0	1	0	0	6	2	0	10	5	3	1	1	4	1	3	1	0	1	1	1	1	0

Table S5 - SIMPER analysis results showing the macroinvertebrate taxa contributing the most to the average dissimilarity between basins (Tuela and Rabaçal) and Crayfish (Presence or Absence).

Rabaçal vs Tuela		Absence vs Presence	
Taxa	**%contribution**	**Taxa**	**%contribution**
Chimarra marginata	3,06	*Brachycentrus subnubilus*	2,75
Habrophlebia/ Habroleptoides	2,50	*Chimarra marginata*	2,58
Brachycentrus subnubilus	2,29	*Aphelocheirus occidentalis*	2,41
Oligoneuriella rhenana	2,18	*Habrophlebia/Habroleptoides*	2,18
Simuliidae	2,15	*Limnius*	2,12
Setodes argentipunctellus	2,06	*Oligoneuriella rhenana*	2,10
Polycentropus	2,01	*Setodes argentipunctellus*	2,05
Onychogomphus uncatus	1,90	Simuliidae	2,05
Oligochaeta	1,89	*Stenelmis canaliculata*	1,95
Epeorus	1,86	*Oulimnius*	1,92
Platycnemis	1,68	Hydrachnidia	1,84
Oulimnius	1,65	*Hydraena*	1,82
Limnius	1,64	*Hydropsyche*	1,70
Perla	1,63	*Epeorus*	1,67
Rhithrogena	1,60	*Ephemerella*	1,60
Ephemerella	1,59	*Onychogomphus uncatus*	1,53
Dupophilus brevis	1,56	*Perla*	1,53
Hydropsyche	1,55	*Caenis*	1,49
Stenelmis canaliculata	1,53	*Rhithrogena*	1,48
Caenis	1,53	*Atherix*	1,47
Elmis	1,50	*Polycentropus*	1,45
Gerris	1,49	*Dupophilus brevis*	1,42
Atherix	1,48	*Elmis*	1,42
Rhyacophila	1,47	Oligochaeta	1,39
Protonemura	1,45	*Platycnemis*	1,31
Mystacides	1,41	*Procloeon*	1,30
Diptera pupae	1,37	*Ancylus*	1,30
Psychomyia	1,34	*Boyeria irene*	1,29
Hexatoma	1,32	*Adicella*	1,28
Hydraena	1,31	*Protonemura*	1,27
Erpobdella	1,28	Diptera pupae	1,27
Adicella	1,26	*Gerris*	1,26
Hydrachnidia	1,26	*Calopteryx*	1,26
Cheumatopsyche lepida	1,25	*Erpobdella*	1,24
Calopteryx	1,24	*Rhyacophila*	1,22
Trichoptera pupae	1,22	*Pacifastacus leniusculus*	1,22
Procloeon	1,20	Trichoptera pupae	1,20
Boyeria irene	1,20	*Hexatoma*	1,19
Ancylus	1,20	*Mystacides*	1,14
Ecdyonurus	1,19	*Pisidium*	1,12
Chironomidae	1,17	*Psychomyia*	1,12

Pisidium	1,11
Planariidae sp.1	1,11
Leuctra	1,08
Baetis	1,03
Micrasema	1,01
Atrichops	0,98
Hydroptila	0,97
Beraea	0,95
Centroptilum	0,93
Ceratopogonidae	0,90
Tabanidae	0,89
Glossosoma privatum	0,84
Esolus	0,79
Aphelocheirus occidentalis	0,78
Orectochilus villosus	0,78
Choroterpes lusitanica	0,75
Wormaldia	0,74
Pacifastacus leniusculus	0,72
Allogamus	0,71
Schizopelex festiva/ Sericostoma	0,70
Micronecta	0,70
Isoperla	0,70
Hydrocyphon	0,67
Sialis	0,63
Oecetis	0,61
Larcasia partita	0,61
Cloeon	0,59
Calamoceras marsupus	0,57
Wiedemannia	0,55
Thraulus bellus	0,53
Cordulegaster boltonii	0,49
Lepidostoma hirtum	0,48

Chironomidae	1,07
Lepidostoma hirtum	1,04
Calamoceras marsupus	1,00
Glossosoma privatum	0,99
Leuctra	0,99
Hydroptila	0,98
Allogamus	0,95
Cheumatopsyche lepida	0,93
Ecdyonurus	0,93
Atrichops	0,91
Micrasema	0,85
Centroptilum	0,84
Baetis	0,84
Orectochilus villosus	0,83
Beraea	0,83
Tabanidae	0,83
Wormaldia	0,82
Ceratopogonidae	0,76
Micronecta	0,74
Hydrocyphon	0,74
Choroterpes lusitanica	0,72
Planariidae sp.1	0,70
Esolus	0,68
Radix peregra	0,66
Schizopelex festiva/ Sericostoma	0,65
Larcasia partita	0,59
Sialis	0,58
Oxyethira	0,56
Dryops	0,53
Isoperla	0,53
Cloeon	0,50
Wiedemannia	0,50
Cordulegaster boltonii	0,45

Table S6 – Results of the analysis of deviance of GLM models; ANOVAs and Kruskal-Wallis tests between the predictor variables (Presence of Crayfish and River Basin) with the response variables Richness (S), Abundance (N), Shannon-Wiener diversity (H'), Pielou's evenness (J'), Functional diversity (FRAO) and Community-weighted mean trait value (CWM). The asterisk and bold indicates significant values (p<0.05). The asterisk alone indicates values almost significant (p<0.1).

			Crayfish	**Basin**
Richness (S)			**t=-4.121 p<0.001***	t=-0.102 p=0.919
Abundance (N)			**t=-2.20 p=0.035***	t=0.341 p=0.735
Shannon Index (H)			**F=8.001 p=0.008***	F=0.001 p=0.977
Evenness Index (J)			F=0.283 p=0.598	F=0.006 p=0.940
Biomass			**F=7.123 p=0.012***	F=1.781 p=0.191
FRAO	**Average value**		F=2.111 p=0.156	F=3.904 p=0.057*
	Body size		**F=5.539 p=0.025***	**X^2=3.922 p=0.048***
	Life cycle		X^2=0.576 p=0.449	**F=7.765 p=0.009***
	Feeding Habits		F=0.259 p=0.614	X^2=0.964 p=0.326
	Habitats		F=0.756 p=0.391	F=3.162 p=0.085*
	Current Velocity		F=2.599 p=0.117	F=0.192 p=0.665
CWM	**Body size**	<0.25 cm	**X^2=5.844 p=0.016***	X^2=0.0003 p=0.986
		0.25-0.5 cm	**F=11.552 p=0.002***	F=0.045 p=0.833
		0.5-1.0 cm	F=1.365 p=0.251	F=0.174 p=0.679
		1-2 cm	F=1.755 p=0.195	F=0.775 p=0.386
		2-4 cm	X^2=0.386 p=0.535	F=2.487 p=0.125
		4-8 cm	F=0.139 p=0.712	**F=5.007 p=0.032***
		>8 cm	**X^2=17.52 p<0.001***	X^2=0.493 p=0.482
	Life cycle	< 1year	X^2=0.525 p=0.469	F=4.022 p=0.053*
		> 1year	X^2=0.525 p=0.469	F=3.925 p=0.056*

	Feeding Habits	Absorber	F=0.074 p=0.787	**X^2=8.877 p=0.003***
		Deposit Feeders	**F=4.505 p=0.042***	F=0.595 p=0.446
		Shredders	F=1.521 p=0.227	X^2=0.897 p=0.343
		Scrappers	F=0.986 p=0.329	X^2=1.144 p=0.285
		Filter feeders	X^2=3.471 p=0.062*	F=0.122 p=0.729
		Piercers	**F=4.278 p=0.047***	F=1.480 p=0.233
		Predator	F=3.142 p=0.086*	F=3.889 p=0.057*
		Parasite	X^2=1.401 p=0.237	X^2=1.009 p=0.315
	Habitats	Flags, Boulders, Cobbles and Pebbles	F=0.294 p=0.591	F=0.084 p=0.774
		Gravel	F=0.023 p=0.879	F=1.178 p=0.286
		Sand	F=0.200 p=0.658	**F=4.191 p=0.049***
		Silt	X^2=0.171 p=0.679	F=0.121 p=0.730
		Macrophytes	F=0.597 p=0.445	F=2.324 p=0.137
		Microphytes	**F=6.741 p=0.014***	F=2.020 p=0.165
		Twigs and Roots	F=0.534 p=0.470	F=1.640 p=0.210
		Organic detritus and litter	F=1.372 p=0.250	F=1.353 p=0.253
		Mud	F=1.151 p=0.291	F=1.587 p=0.217
	Current velocity	Null	F=3.336 p=0.077*	F=0.887 p=0.353
		Slow	F=0.106 p=0.747	F=0.092 p=0.764
		Medium	F=2.586 p=0.118	F=0.364 p=0.551
		Fast	F=0.266 p=0.609	F=0.906 p=0.348