Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Biologia da Conservação

Dissertação

Native species of freshwater amphipods in Portugal: species distribution and population's limiting factors.

Rui Diogo Sousa Marques

Orientador(es) | Pedro Manuel Anastácio
Filipe Banha

Évora 2023

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

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Abstract

The present thesis corresponds to a first look at freshwater Portuguese amphipods' distribution, population size, and potential threats to their conservation. A total of 63 locations were sampled between September 2021 and March 2022. Additionally, samples from past projects were used to fill the gaps left by the sampling campaign's gaps, totaling 1257 sites. Only 152 spots showed amphipod presence. Five native species of amphipods (*Echinogammarus lusitanus* Schellenberg, 1943; *Echinogammarus meridionalis* Pinkster, 1973; *Echinogammarus berilloni* Catta, 1878; *Gammarus chevreuxi* Sexton, 1913) were found, with one being a new record for Portugal (*Gammarus gauthieri* S. Karaman, 1935). The non-native *Crangonyx pseudogracilis* Bousfield, 1958 was also found, being present in almost every major river basin in the country, except the Guadiana. Distribution maps were made for each species, revealing a general closeness to the coastal areas, with *Gammarus gauthieri* being the only exception. Spearman correlation tests also revealed amphipods' presence associations with other variables, namely "annual average temperature", "altitude" and "ecologic quality of the water". Niche overlap analysis confirmed high levels of overlap between the exotic and almost every native. Possible explanations for the present distribution of the species were discussed. This study highlights the need to establish the conservation status of native amphipods.

Keywords: Amphipods; Crangonyx; Gammaridae; Invasive species; Niche overlap

Espécies nativas de anfípodes de água doce em Portugal: distribuição das espécies e fatores limitantes da população.

Resumo

A presente tese corresponde a uma primeira analise da distribuição, densidades e potenciais ameaças à conservação dos anfípodes dulçaquícolas de Portugal. Um total de 63 locais foram amostrados entre setembro de 2021 e março de 2022. Amostras de projetos anteriores foram utilizadas para preencher as lacunas deixadas pela campanha de amostragem, totalizando 1257 locais. Apenas 152 locais apresentaram presença de anfípodes. Foram encontradas cinco espécies nativas de anfípodes (*Echinogammarus lusitanus* Schellenberg, 1943; *Echinogammarus meridionalis* Pinkster, 1973; *Echinogammarus berilloni* Catta, 1878; *Gammarus chevreuxi* Sexton, 1913), sendo uma delas um novo registo para Portugal (*Gammarus gauthieri* S. Karaman, 1935). A espécie exótica *Crangonyx pseudogracilis* Bousfield, 1958 também foi encontrada, estando presente em quase todas as bacias hidrográficas do país, menos no Guadiana. Foram feitos mapas de distribuição para cada espécie, revelando uma proximidade geral às zonas costeiras, sendo *Gammarus gauthieri* a única exceção. Os testes de correlação de Spearman também revelaram associações com outras variáveis, nomeadamente "temperatura média anual", "altitude" e "qualidade ecológica da água". A análise de sobreposição de nicho confirmou níveis elevados de sobreposição entre a exótica e quase todas as nativas. Foram discutidas possíveis explicações para a distribuição atual das espécies. Este estudo torna clara a necessidade de avaliar o estado de conservação das espécies nativas de anfípodes.

Palavras-Chave: Anfípodes; Crangonyx; Espécie Invasora; Gammaridae; Sobreposição de Nicho.

Introduction:

Even though freshwater ecosystems cover only around 2.3% of the global land surface (excluding the polar ice sheets), they compose some of the richest and diverse living spaces on Earth, hosting 12% of all described species (Desforges *et al*., 2021; Reid *et al*., 2018; Sáez-Gómez & Prenda, 2022). Besides the clear contribution to global biodiversity, freshwater organisms are crucial for the maintenance of various natural phenomena, from production services to ecosystem regulation processes (Desforges *et al*., 2021; Ormerod *et al*., 2010).

Freshwater ecosystems have a high risk of species extinction and are one of the most threatened ecosystem types (Abellán *et al*., 2005; Desforges *et al*., 2021; Ormerod *et al*., 2010). The combination of increasing anthropogenic disturbances, overexploitation and climate change lead to an alarming reduction of freshwater biodiversity (Desforges *et al*., 2021; Ormerod *et al*., 2010; Sáez-Gómez & Prenda, 2022). An example of this is the recorded decline of freshwater vertebrates on the Living Planet Index 2020. reaching values of 84% diversity loss since 1970 (Desforges *et al*., 2021). The decrease of freshwater biodiversity is most prominent in drier regions, where water resources are more intensely explored. Therefore, freshwater ecosystems are a top priority for conservation actions, especially in areas like the Mediterranean with its high levels of freshwater endemic species (Abellán *et al*., 2005; Sáez-Gómez & Prenda, 2022).

The Iberian Peninsula, located at the southwestern end of Europe, shows both the most arid European ecosystem and one of the richest biotas in the globe, to the point it was classified as part of one of Earth's biodiversity hotspot areas (Abellán *et al*., 2005; Loidi, 2017). Geographically, the peninsula (comprising of both continental Spain and Portugal, Gibraltar and Andorra) acts as a bridge between two aquatic domains, the Atlantic Ocean and Mediterranean Sea, but also between two continents, those being Europe and Africa (Loidi, 2017; Santisteban & Schulte, 2007).

Due to its position between three tectonic plates (European, African and Atlantic), the Iberian Peninsula has a complex origin, where it was built around a smaller landmass created around the end of the Paleozoic through the successive addiction of land fragments to it (Loidi, 2017; Santisteban & Schulte, 2007). This consequently resulted in a variable lithological structure throughout the area, where three types of substrates compose the majority of the peninsula: siliceous (western side and Paleozoic cores of mountain ranges and plains); limestone (vast plateaus dissected by erosion); malt (great depressions) (Loidi, 2017).

The combination of the previously mentioned factors shaped the Iberian ecosystems, resulting in a large variety of landscapes and a high level of endemic species (Abellán *et al*., 2005; Loidi, 2017; Santisteban & Schulte, 2007). Iberian rivers are a good example of this effect since the adaptation to local and regional climatic and tectonic phenomena was different for each river system, contributing to their richness of rare and/or endemic freshwater species (Abellán *et al*., 2005; Santisteban & Schulte, 2007).

Arthropods represent around 75% of all described species on Earth, including freshwater areas (NHPBS, 2022). Although insects are by far the most numerous of the arthropods, crustaceans show a wider range of groups and have abundant populations throughout the world, especially in aquatic environments (NHPBS, 2022). One of such groups are the Amphipoda, an order of small crustaceans that are mostly detritivores or herbivorous (Eggers & Martens, 2001; NHPBS, 2022; Subida *et al*., 2005). Currently there are more than 6000 known species of Amphipoda, mainly from marine environments (Eggers & Martens, 2001).

Amphipods can be divided into five sub-orders: Gammaridea, Caprellidea, Cyamidea, Hyperiidea and Ingolfiellidea, of which only Gammaridea and Ingolfiellidea have some freshwater species representation (Chapman, 2007; Hou & Sket, 2016). Unfortunately, barely any information about Ingolfiellidea is available due to their subterranean lifestyle, which makes sampling of these organisms a challenge (Chapman, 2007; Hou & Sket, 2016).

The Gammaridea is the most common and diverse of all the five sub-orders, due to their ability to occupy a vast range of habitats, from pelagic to benthic environments in marine, freshwater or brackish water ecosystems (Chapman, 2007). Owning to its abundance on various aquatic ecosystem types, they are considered an invaluable food source to higher-level consumers on the food web, while also being capable of regulating populations of other invertebrates or fishes through processes of parasitism or competition (Chapman, 2007; Hou & Sket, 2016; Subida *et al*., 2005).

Despite playing an important role on aquatic ecosystems, this group of crustaceans is still not a common subject of scientific studies worldwide, especially in the case of freshwater amphipods (Chapman, 2007; Hou & Sket, 2016). Portugal is not an exception to this, since, only with the work of Marques & Bellan-Santini (1991), we got an increase on Portuguese marine amphipod fauna knowledge. Unfortunately, the same can't be said for Portuguese freshwater amphipods. Even though some species are known to science, like the Portuguese endemism *Echinogammarus meridionalis*, the Iberian endemism *Echinogammarus lusitanus*, and the only confirmed exotic species, *Crangonyx pseudogracilis*, the total number of existing amphipod species in Portugal is still uncertain (Grabowski *et al*., 2012; Pinkster, 1993; Mateus *et al*., 1979).

The lack of information about species richness and distribution in conjunction with the ever-increasing impact of climate changes and environmental degradation in freshwater areas brings about serious concerns about the conservation status of Portugal's native amphipod species. Since human pressure normally interferes with more than one environmental factor, a considerable number of threats were brought about (from changes in water quality to habitat degradation) that can impact native amphipod populations and allow the dispersal of exotic species (Ormerod *et al*., 2010; Reid *et al*., 2018). If established, some exotic species can become invasive and lead to the decline of native amphipod populations and distribution range. This problem is aggravated by the difficult differentiation between native and exotic, which, in conjunction with the lack of taxonomic knowledge, can result in a late detection and implementation of conservation measures (Chapman, 2007; Eggers & Martens, 2001; Reid *et al*., 2018).

Since freshwater invertebrates, including amphipods, are among the lesser-known group of animals to science, while simultaneously being among the most threatened ones (Reid *et al*., 2018), a first look at their distribution, population size, and their potential threats is needed. This study seeks to gather information about the existing species of amphipods and their habitats in mainland Portugal through a series of sampling campaigns on all river basins in the country. By the end of this study, it is intended to have a better idea of which factors affect the presence of native amphipods. The other goal of the study is to find out if the exotic amphipod, *C. pseudogracilis*, potentially impacts native species by overlapping with their areas of occurrence. It is hypothesized that native freshwater species will be mostly associated with low order rivers and river springs, where there's less human pressure, hence having better quality water. This country-wide assessment hopes to offer the first general insight on the conservation status of Portuguese freshwater amphipod species, providing valuable information on their distributions and threats while also highlighting knowledge gaps that need further investigation in the future.

Material and Methods:

1. Study area and occurence records

To study the distribution of native species of freshwater amphipods in Portugal, sampling throughout the whole continental area was required. To achieve that, information from the sampling points, in 2017 and 2019, for the Water Framework Directive macroinvertebrates monitorization (WFD) was obtained. Additionally, the study of Correia *et al*. (2021) provided information about 75 sampled locations for amphipods in the Tagus river basin in 2019.

A series of sampling campaigns were planned, based on the recorded presence of amphipods on the above-mentioned sampling points and their accessibility, so that at the end of the project each major river basin had at least five locations with amphipods sampled. In order to fill geographical gaps, other points were selected to be included on the previously established campaigns. The focus was on river springs and low order rivers, where the existence of exotic species and their potential impact on the native populations of amphipods isn't as extensive as downstream, where higher degree of human influence (Chapman, 2007; Eggers & Martens, 2001).

2. Field sampling

Samples were collected between late 2021 and the first half of 2022 on various occasions with approximately four to six locations described each day, depending on the distance between each other. Spring and winter were the preferred seasons for sampling so that there's enough rain to keep consistent water flow, especially in Southern Portugal where temporary rivers are common (Reis & Araujo, 2016). Additionally, some amphipod species are reported to have higher densities during this period (Correia *et al*. 2011), allowing for an easier detection.

In each of the sampling locations, a characterization of the overall habitat was made, where the types of substrate present were noted, using an adaptation classification of the Udden–Wentworth scale (silt, sand, gravel, stone, rock) (Wentworth, 1922). The general composition of the vegetation (macrophytes, hydrophytes, bryophytes) and filamentous algae was also noted, together with the amount of woody debris present on site. The characteristics of the water body were also measured, namely the type of current (no current, slow, moderate, fast), flow's velocity (m/s), estimates (%) of the total area occupied by pools, riffles and running water and its depth (cm). Using a multiparametric probe HI98194, water physical and chemical variables were also measured, namely temperature (ºC), conductivity (µS/cm), TDS "total dissolved solids" and pH (Sorensen scale). Complementarily, nitrates, nitrites and ammonia were also measured using 15 mL Sera test kits. Lastly, on this first description of the sampling sites, turbidity was measured in NTUs resorting to a Hanna HI 93703 turbidimeter.

In order to sample amphipods, a 1 mm mesh dip net was used to stir up the substrate on every type of micro-habitat found within each sampling location. The sampling area (m^2) and total time of the process (in minutes) was noted as well for the later determination of the CPUE ("catch per unit effort). All the collected amphipods, after being counted, were stored in small containers properly identified with the date and sampling locations, filled with 96% ethylic alcohol for posterior identification in the laboratory.

3. Laboratory measurements

From each locations, around 500 mL of the river's water was collected and then stored at 4ºC for further analysis. Before 48 hours have passed after the sampling, a measurement of the amount of calcium was made with a Calcium Hardness Checker® HC (0.00 – 2.70 ppm) using the Calmagite method.

4. Species identification

Besides the amphipods collected in 2021 - 2022 within the scope of this study, samples from projects in 2017 and 2019 were provided from other entities in order to provide a more complete outlook of the distribution of the native species in Portugal. Those entities being the Water Laboratory from the University of Évora (LAUE), with Marvin Freira, Superior Technician of the School of Science and Technology, being the person in charge, followed by Maria João Feio, Researcher from MARE the University of Coimbra (UCoimbra), and finally, Joaquim de Jesus, Executive Director of the River and Terrestrial Ecology Laboratory of the University of the University of Trás-os-Montes (UTAD).

The native species were identified using two identification keys, namely Pinkster (1993) for the genus *Echinogammarus sensu lato* and the work of Karaman & Pinkster (1977) for the genus *Gammarus*.

For this process a Leica S9D Stereomicroscope was used, and only the adult males in the samples were observed since the identifying characteristics are more predominantly shown in these individuals. All samples containing only unidentifiable amphipods due to a bad state of conservation or with only of females and/or juveniles were excluded from statistical analysis.

5. Data consolidation and mapping

The sampled points and the ones provided by the WFD were exported into ArcGIS software. Using the points' coordinates under the geographical coordinate system WGS_1984, several distribution maps were created. The distribution maps are of three categories: one to represent presence-absence of native amphipods in Portugal (the invasive appears as its own category); another to visualize all of the confirmed records of amphipods in the country; and lastly maps with only the presence sites for each species.

To the maps belonging to the last category, a buffer was applied to each of the presence points so that the map can accommodate a larger area with possible suitable locations for each species. Since buffer sizes don´t have standard protocols to determine, for the purposes of this project it was decided that a buffer range between 10 to 20 km was ideal (Elsseman & Allan, 2010; Graham & Hijmans, 2006). After a trial phase with buffer range, all the maps ended up with a fixed 15 km buffer as it is large enough to include more potential sites for the species but no so large to incorporate a variety of distinct ecosystems not relevant to the project (Elsseman & Allan, 2010; Graham & Hijmans, 2006).

The area of the populations for the amphipod species was determined using the function "minimum convex polygon" under the "Minimum Bounding Geometry" feature in ArcGIS. That function was applied to the second category of maps created above (mainly to the map representing all the presences in Portugal) in order to extract the values of the total areas occupied by each species and also measure the percentage of overlapping area between two species. The overlapping values were obtained with the use of the "Intercept" function (on "Overlay" feature of Analysis Tools) between two of the species, repeating the process until every possible species combination was reached.

ArcGIS was also used to extract individual values at each of the points used in this project, from datasets from various sources (Table 1). These values were necessary for posterior statistical analysis.

Table 1 – Sources for the datasets used for extraction of values of the respective environmental variable (links for each of the source in supplements).

Variable	Dataset source
Altitude	WordClim – Historical climate data - Elevation (30s)
Water Body Type	SNIRH – Atlas da Água – Águas Superficiais - AlbufeirasPT
River Order	SNIRH - Atlas da Água – Águas Superficiais – hidcod.25k
River Spring	SNIRH – Base de Dados – Redes – Monitorização - Nascentes
Geology	Geoportal der Bundesanstalt für Geowissenschaften und Rohstoffe – IGME5000
Annual Average Temperature	WordClim – Historical climate data – Bioclimatic variables – BIO1
Annual Average Precipitation	WordClim – Historical climate data – Bioclimatic variables – BIO12
Ocean Distance	Natural Earth Data – Ocean - ne_10m_ocean
Ecological Quality	WISE Freshwater Information System for Europe – surfacewaterbody_river_line_PT
Chemical Quality	WISE Freshwater Information System for Europe – surfacewaterbody_river_line_PT
Human Density	GEOSTAT – Eurostat_Census_Grid_2021

This was achieved using the "Spatial Join" function under the "Overlay" feature of Analysis Tools, between the layer with all the points and each of the imported layers from the datasets. This was performed under "CLOSEST" in the optional "Match Option" of this function. The only exception to these procedures was for “Ocean Distance” where, instead of intersecting values from two different datasets, it was intended to calculate the minimal distance between the ocean and the study points. For this objective, the “Near” and “Generate Near Table” (inside “Proximity” of the “Analysis Tools”) were used, respectively, giving the distance values for each point to the ocean in Km.

All the resulting layers from this value extraction procedures were exported from ArcGIS into excel and then integrated together into a single excel file with all the point locations/coordinates (both sampled and provided by the WFD entities) and species found there.

6. Statistical analysis

With all the collected and registered values from various environmental variables, two separate Spearman rank correlations were performed to discern which variables are correlated with the presence of each species from a geographical and biological perspective.

For the first correlation, only the samples collected for this specific study during 2021 - 2022 were used, since these are the only points with all the habitat description variables, including the type of micro-habitats where the specimens were found. However, the total number of variables was around twenty, so a selection was performed beforehand. This was achieved using hierarchical cluster analysis in SPSS, resulting in a dendrogram representative of the independence of each variable in relation to the rest. For the selection, the variables with less connections were chosen to enter the analysis while the ones close to one another were put through the test again. This process was repeated until a dendrogram with a clear representation of the closeness between the last remaining variables was achieved. The choice was arbitrary to the goals of the study. By the end, of the initial twenty variables, only eight were used for the Spearman correlation test together with the presence of each species. Those variables were: “pH”; “Nitrates”; “Ammonia”; “Calcium”; “Macrophytes”; “Bryophytes”; “Current Type”; and “Sample Depth”. The other Spearman correlation was carried out with the extracted variables in the process above for all the amphipods recorded sites included in this study.

Finally, niche amplitude (Levins, 1968) was calculated and niche overlap between pairs of species was obtained according to a protocol adapted from Schoener (1970). This was done for the variables “Altitude”, “Annual Average Temperature”, “Annual Average Precipitation”, “Ocean Distance”, “Ecologic Quality of the Water”, “Chemical Quality of the Water” and “Human Density”. The relative frequency of each species for discrete intervals inside each variable was calculated.

After obtaining the frequency values P(i), niche amplitude (β) was obtained by equation 1.

$$\beta = \left(\sum_{i=1}^{n} \mathrm{P}_i^2 \right)^{-1}$$

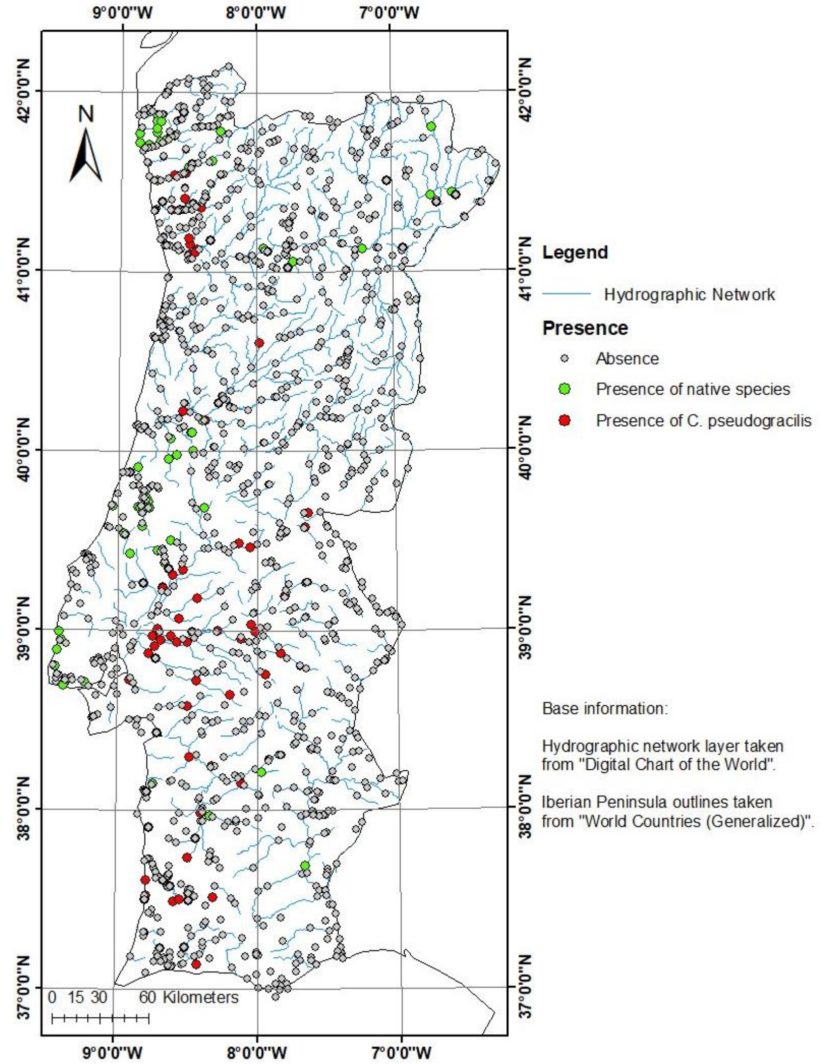
Lastly, equation 2 was applied to calculate the existing niche overlap (θ_{xy}) between species for a specific environmental variable.

$$\theta_{xy} = 1 - 0.5 \sum_{i=1}^{n} \left| \mathrm{P}_{xi} - \mathrm{P}_{yi} \right|$$

This process was repeated for all the possible combinations of two of the species identified in the samples and all the variables implemented in this analysis.

Results:

By the end of the field work, 63 locations were sampled in mainland Portugal between September 2021 and March 2022. In addition to this field work, 75 water framework directive macroinvertebrate samples from 2017 and 2019, were provided from three separate entities, introducing two new species into the analysis. In order to fully represent mainland Portuguese freshwater systems, 75 sample sites used for the work of Correia *et al*. (2021) about *Crangonyx pseudogracilis* on the Tagus basin and all of the monitoring sites the WFD were added to the analysis. The combination of all these sources together with all the absence sites throughout the country resulted in 1257 locations included into this study (fig. 1).



Base information:

Hydrographic network layer taken from "Digital Chart of the World".

Iberian Peninsula outlines taken from "World Countries (Generalized)".

Figure 1 - Occurrence of native freshwater amphipod species (in green) and the non-native, *Crangonyx pseudogracilis*, (in red) in all of the sample points throughout Portugal.

1. Species distribution and habitat description:

Of the total number of sites in this study, only 152 have recorded presence of any of the six amphipod species found (fig.2), hence representing 12.09% of the locations. Considering only the sites with amphipods, *Crangonyx pseudogracilis* is the most frequently found species (44.71% of the samples), while the native species of amphipods represent a total of 31.05%, with *Echinogammarus meridionalis* being the most representative (38% of the native species). The last 24.24% of the presences are composed of unidentifiable species, mostly due to poor state of specimens in samples with very few individuals.

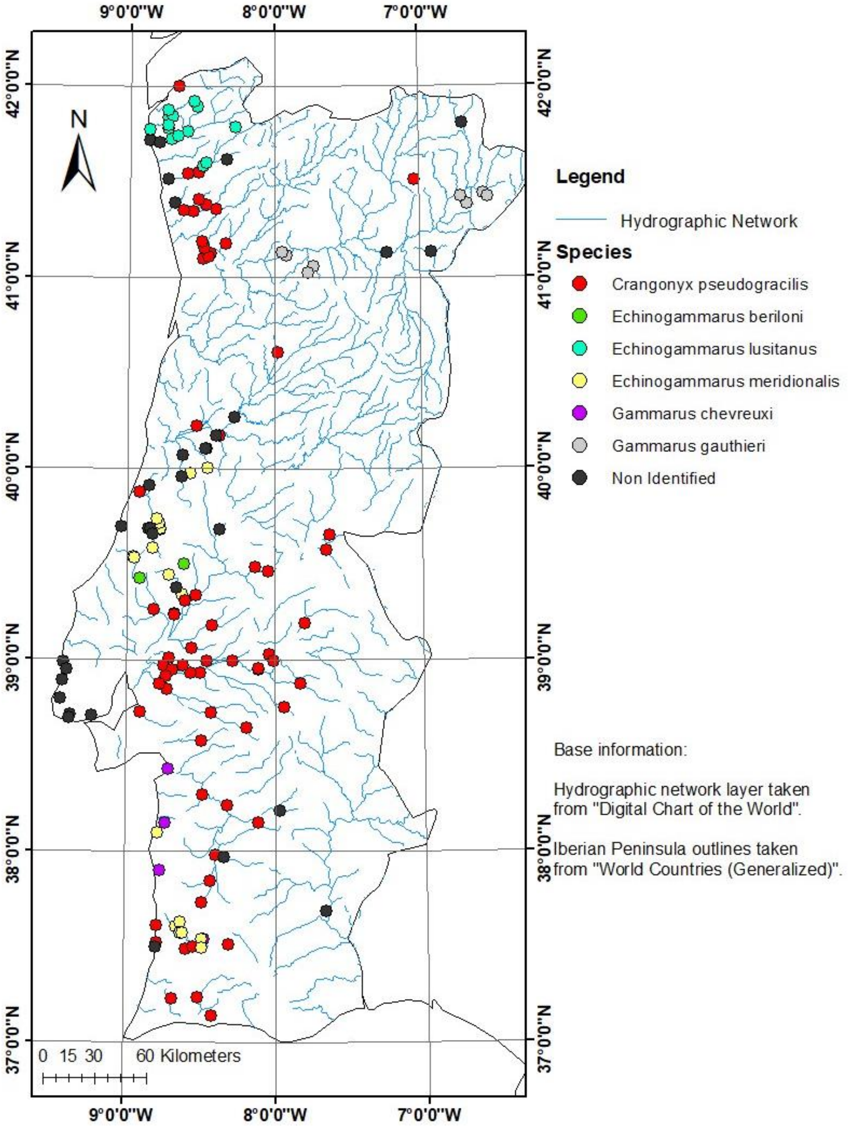


Figure 2 – Map of the amphipod species found in freshwater ecosystems in Portugal, with five native species and the non-native represented. Black dots represent the areas where identification of the samples was not possible.

For each of the six amphipod species, local description information gathered during the field sampling campaign of 2021 - 2022 to describe habitat characteristics is summarized in Table 2. However, there is incomplete information for *E. berilloni* and *E. lusitanus*, since they weren't found during those sampling campaigns.

Table 2 – Summary of the observed values of each habitat descriptive variables for every amphipod species ("---" meaning unable to verify).

Descriptive Variables		**Species**					
		Echinogammarus beriloni	*Echinogammarus lusitanus*	*Echinogammarus meridionalis*	*Gammarus chevreuxi*	*Gammarus gauthieri*	*Crangonyx pseudogracilis*
Water Properties	**pH**	---	---	6.80 - 7.58	7.98 - 8.28	8.41	6.54 - 8.85
	Nitrates (mg/L)	---	---	0.00 - 10.00	0.00	0.00	0.00 - 10.00
	Amonia (mg/L)	---	---	0.00 - 0.50	0.00	0.00	0.00 - 1.00
	Calcium (mg/L)	---	---	0.02 - 0.27	0.06 - 0.10	0.10	0.19 - 187.00
	Chemical Quality of the Water	Good (average = 1)	Good (average = 1)	Good (average = 1)	---	Good (average = 1)	Good to Bad (average = 1.40)
	Ecological Quality of the Water	Reasonable (average = 3)	Reasonable to Good (average = 3.47)	Mostly Reasonable (average = 3.10)	Mediocre to Reasonable (average = 2.50)	Reasonable to Good (average = 3.67)	Mediocre to Reasonable (average = 2.52)
Water Body Description	**Current Type**	Lotic	Lotic	Lotic (Reduced to Intermediate flow speed)	Lotic to Lentic (barely any flow speed)	Lotic (reduced flow speed)	Lotic (Reduced to Intermediate flow speed)
	River Order	min = 2; max =4; median= 3	min = 1; max =4; median= 2	min = 1; max =5; median= 2	min = 1; max =3; median= 2	min = 2; max =4; median= 3	min = 1; max =4; median= 2
	Percentage of Spring sites	0.00%	14.00%	20.00%	0.00%	0.00%	2.94%
Local Characteristics	**Ocean Distance (Km)**	2.36 - 41.68	0.56 - 36.61	2.36 - 35.74	0 - 28.35	58.88 - 180.49	1.08 - 140.61
	Anual Average Temperature (ºC)	15.36 - 16.22	12.22 - 14.71	12.55 - 16.62	16.11 - 16.55	12.46 - 14.20	13.13 - 17.11
	Anual Average Precipitation (mm)	586 - 747	1453 - 1649	538 - 1580	560 - 633	1005 - 1367	516 - 1533
	Geology	Only Sedimentary Rock	Between Plutonic and Metamorphic Rock	Mostly Sedimentary Rock	Mostly Sedimentary Rock	Between Plutonic and Metamorphic Rock	Mostly Sedimentary Rock
	Altitude (m)	4 - 132	3 - 588	4 - 461	0 - 118	66 - 630	3 - 455
	Human Density (nº individuals/km2)	2 - 202 (average = 70)	0 - 1460 (average = 257)	0 - 3120 (average = 292)	0 - 61 (average = 17)	0 - 251 (average = 97)	0 - 6316 (average = 338)
Number of captured individuals	**Minimum**	74	2	1	41	2	1
	Mean	99	31	347	350	225	44
	Maximum	141	133	1117	1238	845	306
CPUE (nº individuals/minute of sampling)	**Minimum**	---	---	1.71	1.78	7.00	0.33
	Mean	---	---	1229.58	134.19	7.00	14.61
	Maximum	---	---	5184.00	337.71	7.00	38.65

Being the most numerous of the native species with twenty confirmed records, *Echinogammarus meridionalis* has revealed to be a species closely associated with lowlands not far from the sea (annex I), with its farthest point being 35 km away from the ocean. The Spearman rank correlation results ($\rho = -0.067$; p-value = 0.017) indicates a likelihood of finding this species closer to the ocean, however the correlation coefficient (ρ) suggest a very low intensity association. Regarding the altitude, which ranges from 4 to 461 meters high, the resulting Spearman correlation was the same as with the ocean distance ($\rho = -0.067$; p-value = 0.017), meaning that the species is also very weakly associated with low altitudes to some degree.

Overall, the species is mostly present in the basins of the Mondego and Mira rivers, while also occupying the upper limit of the Tagus River basin. In spite of its closeness to the ocean and lower altitudes, *E. meridionalis* was the species with more presence on river springs, corresponding to 20% of all its recorded sites. Additionally, the median of the river Strahler order was 2 from a range between 1 and 5, revealing a major presence in lower order water bodies.

The species was found to inhabit slightly acidic water, with some tolerance to nitrates (0 to 10 mg/L) and ammonia (0.0 to 0.5 mg/L). In terms of the calcium concentration, *E. meridionalis* was found in an interval of 0.02 to 0.27 mg/L. From the Spearman correlation results, the species is more likely to be present in water bodies with lower pH ($\rho = -0.278$; p-value = 0.012), lower concentrations of calcium ($\rho = -0.308$; p-value = 0.005) and higher concentrations of nitrates ($\rho = 0.244$; p-value = 0.028).

As for the habitat description, *E. meridionalis* was registered in lotic water bodies with currents varying from reduced (0.10 m/s) to intermediate (0.27 m/s) flow speeds. From the three types of vegetation sampled (bryophytes, macrophytes and filamentous algae), the species manifests a positive relation with the presence of aquatic bryophytes ($\rho = 0.228$; p-value = 0.040). In terms of geology, sedimentary rock was the most common type of primary rock, with limestone and sand being the most representative substrate. Lastly, *E. meridionalis* is uncorrelated with human density even though it shows the second highest average density, that being 292 people/km^2.

1.2 – *Echinogammarus berilloni:*

In contrast to the previous species, *Echinogammarus berilloni* is the species with the lowest number of confirmed records, being only detected in three sites making it impossible to calculate reliable correlations. The species has a somewhat fragmented distribution with two locations on the upper limit of the Tagus River basin and another in the downstream sections of the Alentejo rivers (annex II).

On the three sites, it was observed that the species was found on substrate composed of sedimentary rock, in lotic ecosystems. The species is found relatively close to the ocean (maximum distance of 41.68 km) at low altitudes, ranging from 4 to 132 meters high. Of the six species, it shows the second lowest average human density, 70 people/km^2.

1.3 – *Echinogammarus lusitanus:*

Echinogammarus lusitanus, the native species with the second highest number of confirmed records (15 registered sites), inhabits the basins of the Minho and Lima rivers up in the northwestern corner of the country (annex III). As its restricted to this portion of Portugal, the species seems associated to climate conditions normally seen in the region (de Lima *et al.*, 2015). This is observable through the results of Spearman correlation, with both annual average temperature ($\rho = -0.124$) and precipitation ($\rho = 0.182$) having a strong statistical significance (p-value under 0.001). From this, we can see that this species has higher chances of being present in lower temperatures areas, from 12.22 to 14.71 ºC, with higher precipitation levels (1453 to 1649 mm), which normally occur on the northern parts of Portugal (de Lima *et al.*, 2015).

The distance to the ocean was also negatively correlated with the species occurrence, with a p-value lower than 0.001, ($\rho = -0.103$) therefore demonstrating a small tendency to occur at low distances to the ocean. Even with a maximum distance of 36.61 km to the ocean, the species can reach altitudes as high as 588 meters, where river springs are found and compose 14% of its whole distribution.

The species is normally found in lotic ecosystems, inhabiting rivers with usually low Strahler order numbers as seen with a median of 2 (min = 1; max = 4). Regarding the geology of the sites, the substrate varies between acidic plutonic and metamorphic rocks, those being granite group rocks and phyllite specifically. Lastly, the species occurs in areas with an average of 257 people/km^2, the second highest human density among the native species, only surpassed by *E. meridionalis*.

1.4 – *Gammarus chevreuxi:*

Just like with *Echinogammarus berilloni*, this species has a low number of presences in Portugal, having only five confirmed sites. However, due to this very small sample size, it was impossible to analyze correlations with the variables in study.

In freshwater environments, *Gammarus chevreuxi* was observed mainly on the downstream sections of the Alentejo rivers, including the Sado and Mira, very close to the ocean (annex IV). In fact, it is the species with the closest range to the ocean, having a maximum distance of 28.35 Km from it.

The species was found at low altitudes, until 118 meters high, in ecosystems varying from lentic to lotic, with reduced speed current. Notably, it inhabited rivers with low Strahler order numbers (min = 1; median =2; max = 3) and appearing in low calcium concentrations, with values ranging between 0.06 and 0.10 mg/L.

On the sites *G. chevreuxi* was found, the substrate was mainly composed of sand or derivatives. Just like with *E. meridionalis*, the species seems to be mostly associated with aquatic bryophytes micro-habitats. Lastly, the species is present on areas with an average of 17 people per km^2. Therefore, it is the studied species occurring in areas with the lowest human population density.

1.5 – *Gammarus gauthieri:*

The last native species, *Gammarus gauthieri* is found in the northern region of Portugal, restricted to the Douro River basin, with nine confirmed presence sites (annex V). Compared to the other native species, that are relatively close to the ocean, *G. gauthieri* thrives upstream, being very weakly but positively correlated with distance from the ocean ($\rho = 0.090$; p-value = 0.001) and higher altitudes ($\rho = 0.098$; p-value < 0.001). It is associated with mountainous areas of the interior of the country where the Douro River runs, between the 66 and 630 meters high, with minimum distances of 58.88 Km from the sea. Very weak correlations with the annual average values of temperature (ρ = -0.116; p-value < 0.001) and precipitation (ρ = 0.070; p-value = 0.013) were also detected. In short, these results describe areas with lower temperatures (12.46 to 14.20 ºC) with higher precipitation (1005 to 1367 mm) at high altitudes, which fits the general climate found on mountainous areas (Pisani *et al*., 2019). Despite that, as seen from all the correlation coefficients, these variables don't have a high impact on the presence of this species.

The species occupied rivers with a higher Strahler number, from order 2 to order 4, with emphasis on rivers with order number 3 (median = 3). This preference seems to play a small role in this species presence through the Spearman's correlation result (ρ = 0.073; p-value = 0.010). It generally inhabited lotic ecosystems in higher order rivers with a percentage of calcium around the 0.10 mg/L, with good water quality even though some points are of unknown water quality.

Just like with *E. lusitanus*, the geology of the substrate is essentially of plutonic and metamorphic origin. As for the areas of presence in general, the human density isn't as high as other species, reaching an average of 97 people/Km^2, mostly due to the mountainous nature of the region.

1.6 – *Crangonyx pseudogracilis:*

Being the only confirmed freshwater invasive amphipod species in Portugal, *Crangonyx pseudogracilis* has, by far, the largest number of presence sites, spreading throughout most of the river basins in the country (annex VI). Of all the rivers, the Tagus contains the largest numbers presence of points the species, mostly detected by the work of Correia *et al*. (2021). In contrast, the Guadiana River and most of the rivers in the Algarve region show no presence of this invasive species.

With its bigger sample size, the Spearman correlation test results offered a clear relation of this species with a considerable amount of the variables used in this study.

For example, just like most of the species in this study, there is a weak negative correlation with ocean distance (ρ = -0.109; p-value < 0.001) and altitude (ρ = -0.134; p-value < 0.001). The species is more likely to appear at short distances from the sea and low altitudes, though it can be found 140.61 Km away from the sea and altitudes of 3 to 455 meters high. There are two more variables for which *C. pseudogracilis* presence shows a significance level of the correlation below 0.001. The annual average temperature is one of them, with a weak positive correlation coefficient ($\rho = 0.124$), indicating a preference for warmer areas, notably ranging from 13.13 to 17.11 ºC. The combination of these three conditions overlaps with areas where larger human settlements are found in Portugal, mainly coastal cities. In fact, this invasive species demonstrated a very weak but significant association with human density ($\rho = 0.065$; p-value = 0.021), with an average density of 338 people per km^2 along its distribution area.

Considering its close ties with human settlements, the quality of the water of those water bodies won't be as high as in springs, for example. This can be seen through the Spearman correlation, for which the likelihood of this species appearing is significantly associated with poor water quality, having a weak correlation with ecological quality ($\rho = -0.099$; p-value < 0.001) and a more intense correlation with chemical quality ($\rho = -0.213$; p-value < 0.001). In conjunction with the overall lower water quality, *C. pseudogracilis* thrives on water bodies with concentrations of ammonia, reaching 0.50 mg/L, as it can be seen from the correlation values ($\rho = 0.298$; p-value = 0.007). It was also detected a negative association with micro-habitats mainly composed of bryophytes ($\rho = -0.271$; p-value = 0.014).

This species shows a faint preference for lotic ecosystems with low current velocity ($\rho = -0.083$; p-value = 0.003), mainly inhabiting on the low depths of the river ($\rho = -0.220$; p-value = 0.048). It is generally found on low order rivers (median = 2), but it can also be found in river springs, composing 2.94% of all the confirmed presence sites. Lastly, in terms of the substrate, it was found on areas mainly composed of sand.

1.7 - *Population density:*

Referring now to the number of individuals in the sampling sites and CPUE for the sampling during this study, another perspective on these six species was obtained. Within the native species, even though it has the second highest number of presence sites, *E. lusitanus* has the lowest average per sample, with only approximately 31 total individuals per site. However, the CPUE for both this species and *Echinogammarus berilloni* wasn't possible to measure due to information gaps in the early stages of the project. Regarding *E. berilloni*, even with the lowest amount number of presences, it shows an average of 99 captured individuals per site.

E. meridionalis has both the highest number of presence sites and second highest number of individuals captured, with an average of 347 sampled individuals per site, capable of reaching values as high as 1117. It also has the highest CPUE values, with approximately 1230 individuals caught per minute of sampling. In certain areas visited during the sampling campaign, the population was so abundant that the CPUE reached a maximum of 5184 individuals/minute.

Despite the high values seen in *E. meridionalis*, the actual highest population density was observed in *G. chevreuxi*, one of the species with very few recorded sites. In its five confirmed locations, samples contained from 41 to 1238 individuals, with an average of 350 individuals. But, regarding the CPUE, the species comes right behind *E. meridionalis* with only an average of 134.19 captures/minute. After these two species at the top, there is *G. gauthieri,* with an overall mean of 225 sampled individuals per site and a CPUE of 7 amphipods/minute, as a result of only being found once along the campaign at low concentrations. Lastly, there is the invasive species, *C. pseudogracilis*, that has the highest range by far and still has the second lowest number of captures of the six species studied. Its samples reached a maximum of 306 captures on site, way much lower than the maximum values observed in the three species at the top, while only averaging a total of 44 sampled individuals. Pertaining to its CPUE, it has the second lowest average with a mere 14.61 individuals/minute, barely ahead of the species with the lowest number of records on this study.

2. Distribution areas and niche overlap:

The area of occupancy of each species was calculated using a minimum convex polygon (fig.3). As we can see, the invasive species *C. pseudogracilis* is the only species with a resulting area that ranges across the country, from north to south. Henceforth it shows the greatest area among the six species studied, that being 50723 km^2, intercepting all the other species occupancy areas. In contrast, *G. chevreuxi* shows the smallest area, with only 840 km^2.

From the “minimum convex polygon”, it can be seen that both *E. berilloni* and *G. chevreuxi* territory is completely inserted into the area of *C. pseudogracilis*, composing 3.72% (1889 km^2) and 1.66% (840 km^2) of its total area, respectively. These two species are also included into the occupancy area of *E. meridionalis*, which itself has 99% of its range overlapping with *C. pseudogracilis*. *E. berilloni* has its whole distribution inserted into the territory of *E. meridionalis*, occupying 22.10% of its range, while *G. chevreuxi* has most of its area overlapped with *E. meridionalis* (96.88% of its total area), even though it only fills in 9.52% of its total area.

While all the above-mentioned native species co-exist on the southwestern region of the country, *E. lusitanus* and *G. gauthieri* appear in the north side of Portugal without any kind of overlap, due to inhabiting different areas. Both species show similar occupancy area sizes, those being 1165 km^2 and 1234 km^2, respectively. The two species display 78.29% and 71.95% of its territory inside the invasive species occupancy area, corresponding to 1.80% and 1.75% of *C. pseudogracilis* territory, respectively.

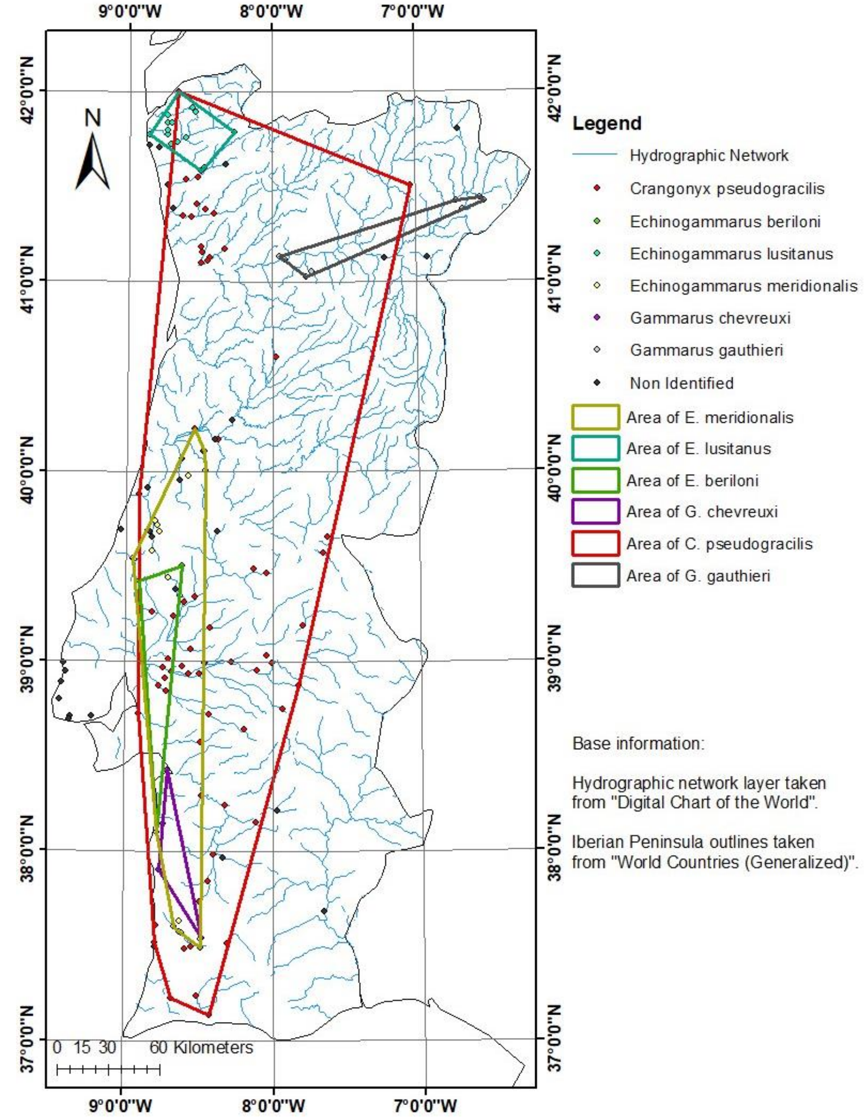


Figure 3 – Map of the amphipod species and the resulting area from "minimum convex polygon". Black dots represent the areas where identification of the samples was not possible.

Considering the overlap between species distribution areas, it was taken into consideration to what degree do the studied variables values coincide between the six species. To achieve this, a niche overlap analysis was performed for each variable in all possible combinations of two out of the six amphipod species, resulting in table 3.

Table 3 - Summary of the niche overlap values obtained for every possible combination between two species, with the mean value represented for the combinations and the descriptive variables. Green values represent low niche overlap while red represents high overlap. The grey areas on species and mean values area associated with the non-native amphipod.

Species	Altitude	Annual Temperature	Ocean Distance	Annual Precipitation	Ecologic Quality of the Water	Human Density	Mean value between species
Crangonyx pseudogracilis - Echinogammarus beriloni	0.813	0.447	0.422	0.624	0.967	0.821	0.682
Crangonyx pseudogracilis - Echinogammarus lusitanus	0.925	0.447	0.773	0.682	0.901	0.946	0.779
Crangonyx pseudogracilis - Echinogammarus meridionalis	0.979	0.425	0.875	0.753	0.883	0.964	0.813
Crangonyx pseudogracilis - Gammarus chevreuxi	0.961	0.425	0.622	0.860	0.695	0.821	0.731
Crangonyx pseudogracilis - Gammarus gauthieri	0.685	0.598	0.814	0.692	0.918	0.821	0.755
Echinogammarus beriloni - Echinogammarus lusitanus	0.782	1.000	0.649	0.538	0.898	0.871	0.790
Echinogammarus beriloni - Echinogammarus meridionalis	0.799	0.000	0.310	0.841	0.888	0.855	0.615
Echinogammarus beriloni - Gammarus chevreuxi	0.804	0.000	0.800	0.573	0.688	1.000	0.644
Echinogammarus beriloni - Gammarus gauthieri	0.716	0.173	0.395	0.593	0.944	1.000	0.637
Echinogammarus lusitanus - Echinogammarus meridionalis	0.907	0.000	0.661	0.663	0.883	0.982	0.683
Echinogammarus lusitanus - Gammarus chevreuxi	0.902	0.000	0.827	0.556	0.683	0.871	0.640
Echinogammarus lusitanus - Gammarus gauthieri	0.730	0.173	0.684	0.637	0.936	0.871	0.672
Echinogammarus meridionalis - Gammarus chevreuxi	0.973	1.000	0.510	0.713	0.800	0.855	0.808
Echinogammarus meridionalis - Gammarus gauthieri	0.665	0.778	0.703	0.668	0.943	0.855	0.769
Gammarus chevreuxi - Gammarus gauthieri	0.663	0.778	0.555	0.561	0.743	1.000	0.717
Mean value on each variable	0.820	0.416	0.640	0.664	0.851	0.902	

Low Niche Overlap	🟩	🟩	🟩	🟩	🟩	⬜	🟥	🟥	🟥	🟥	🟥	High Niche Overlap

As seen in table 3, the six variables most correlated to the species occurrences were used. Of these variables, the "annual average temperature" was the one with least niche overlap, having a mean of 0.416 between all its values. Even with that, it is observed a complete overlap between *E. berilloni - E. lusitanus and E. meridionalis – G. chevreuxi* (both with β = 1). Besides that, *G. gauthieri* temperature niche overlaps with both *E. meridionalis* and *G. chevreuxi* with a value of 0.778.

In contrast, the highest levels of overlap in all combinations were observed on the "human density" variable, where the frequency values range from 0.821 to 1.000, resulting in a high mean value of 0.902. In other words, for this variable every combination of species shows a high degree of overlap. Out of all combinations, three species show values of β = 1 between themselves, those being *E. berilloni* and the two species of Gammarus.

The "Ecologic Quality of the water", just like the "human density", has high relative frequency values for all species, ranging from 0.683 to 0.967 (mean = 0.851). For this variable, the highest overlap is between *E. berilloni* and *C. pseudogracilis* (0.967), with the non-native having overlap values above 0.880 with the other natives, except for *G. chevreuxi*. Between the natives, *Gammarus gauthieri* has high overlap with the species belonging to the genus Echinogammarus, with values above 0.930.

For the "Altitude" variable, with values between 0.663 and 0.979 (mean = 0.820), the higher overlaps are seen specially in *C. pseudogracilis* and *E. meridionalis*, followed by *E. meridionalis* and *G. chevreuxi* (0.973), since these are the species mostly associated with lower altitudes.

Now turning to the "annual average precipitation", with a mean of 0.664, the greater overlaps are seen between the native species present on the southwestern side of the country and the invasive species, where the precipitation reach lower levels. The highest values noted are between *C. pseudogracilis – G. chevreuxi* with 0.860. and *E. berilloni – E. meridionalis* with 0.841.

Lastly, for the "ocean distance" variable, that shows the second lowest mean at 0.640. The species with the most overlaps is *C. pseudogracilis* that has a broader range throughout the country. It shows high values between 0.773 and 0.875 with every native species, except with *E. berilloni* and *G. chevreuxi*, two species that are present very close to the ocean. With that, these two species also show an overlap with a frequency value of 0.800 due to that similarity in locations. Besides that, *G. chevreuxi* also shows overlap with *E. lusitanus* (0.827), another species that is restricted to an area close to the sea.

Overall, in terms of species overlap, it was found that the pair non-native species *C. pseudogracilis* and native *E. lusitanus* have the largest amount of overlaps. Considering the six variables studied, for five of them the overlap was over 0.600, with three variables presenting overlap values above 0.875. Those three variables are "altitude", "ecologic quality of the water" and "human density". Between only the native species, the highest levels of overlap are seen once again with *E. lusitanus* but now with *E. berilloni*. It has four variables over the value of 0.600, but only two of them go over 0.800, which is already way lower than we obtained in the previous case. The two variables with more overlap in this scenario are the "annual average temperature" and "human density" respectively. On the other hand, the combination with the lowest overlap is between *E. berilloni* and *G. chevreuxi*, with only three variables with high values (above 0.8). Those variables are "altitude", "ocean distance" and "human density".

Discussion:

Amphipod species were mainly present along the west coast of Portugal as seen by their negative correlation to "ocean distance", except *Echinogammarus berilloni*. However, it was in the southern region where more diversity of species was found, with three out of the five natives and the exotic present. This study confirmed in most cases the literature relating the environmental preferences of the six amphipod species, with only *C. pseudogracilis* being described in a contradictory way regarding temperature (Correia *et al*., 2021; Grabowski *et al*., 2012).

The southwestern side of the Iberian Peninsula is a region known for its long and complex tectonic evolution due to its proximity to the limits of the Eurasian and African plates (Fernàndez *et al*., 2004; Perea *et al*., 2016). The southern native species, as seen in annex II, are almost completely restricted to the existing Iberian massif of Cenozoic origin in Portugal when compared to the map present in the work of Osete *et al*., 2011 (figure 1b). Due to its transformation along the years, this region is a known catalyst to processes of speciation and diversification of freshwater organisms, having numerous cases of this in various fish groups, like the Leuciscinae (Perea *et al*., 2016; Sousa-Santos *et al*., 2018). However, since *E. meridionalis* is the only known Iberian endemism among the southern species, it would be more viable to believe that these species had their origin in the Atlantic coast after the establishment of the Iberian Massif in its smaller independent rivers (Gama et al., 2017; Karaman & Pinkster, 1977; Pinkster, 1993).

Echinogammarus lusitanus is one of restricted species on the northern region of the peninsula, present in the provinces of Orense, Lugo, La Coruna and Pontevedra (Galicia, Spain) and in the Minho and Lima river basins in northern Portugal (Hou & Sket, 2015; Margalef, 1953; Pinkster & Stock, 1972). A theory proposed by Pinkster (1973) translates to *Echinogammarus lusitanus* being the descendent of the first wave of colonization of the berilloni group in the peninsula and got isolated after the sea-arm formation in the valley of the Guadalquivir during the Burdigalian. After the isolation, it would develop into a the highly specialized amphipod existing today, capable of surviving in acidic waters (pH < 5) with low mineral content present in the region (Pinkster, 1973).

However, the species origin can be more ancient, predating the collision of the continents Gondwana and Balto-Laurentia in the late Paleozoic, which formed a mass of continental rock called the Variscian Belt (Fernàndez *et al*., 2004). It is possible that an ancestor of this species existed in that ancient platform and evolved into the present day after the Iberian plate isolated itself after the separation from the north American plate (120 million years ago) (Fernàndez *et al*., 2004; Vissers & Meijer, 2012). This assumption can be verified through the future genetic analysis of this species and comparison with north American amphipods.

The other native in the north, *Gammarus gauthieri*, is mostly associated with the upstream of the river Douro. This species is known to exist in Tunisia, Algeria, Morocco and in isolated areas of Spain (Karaman & Pinkster, 1977; Scheepmaker, 1990). With Portugal now confirmed to be part of its distribution area, its safe to assume that this species may had its origin in the Iberian Peninsula through an extension of its ancestor from northwestern Europe during the dried up phase of the Mediterranean sea, also known as the Messinian salinity crisis (5.97–5.33 Ma) (Krijgsman *et al*., 2018; Scheepmaker, 1990).

In Portugal, the river Douro basin corresponds to a river system that was composed of endorheic basins in the past, where rivers drainage occurred inland leading to the formation of lakes (Sousa-Santos *et al*., 2008). It formed two inland lakes during the Miocene, one being a large basin in the east at 700 m of altitude, and a small one in the west called "Atlantic Capture Zone" (Silva *et al*., 2017; Sousa-Santos *et al*., 2008). It is possible that *Gammarus gauthieri* found nowadays in Portugal originates from this small inland lake that existed between Zamora and Salamanca provinces in Spain. With the transition to a exorheic basin during the Pleistocene, the species started to colonize downstream until it reached the northeastern side of the country (Perea *et al*., 2016, Silva *et al*., 2017; Sousa-Santos *et al*., 2008).

As for the other Iberian endemic species, *Echinogammarus meridionalis* is limited to discontinuous patches inside the Cenozoic Iberian Massif in southwestern Portugal, at high density populations (Gama *et al*., 2017; Pinkster, 1993). With its overall presence in the basins of the Mondego and Mira rivers, and upper limit of the Tagus river basin, its highly likely that *Echinogammarus meridionalis* ancestor may have colonized one or more of the five endorheic lakes of the river Tagus in the upper Miocene (Sousa-Santos *et al*., 2008). Using the hypothesis proposed by Sousa-Santos *et al*. (2008) for endemic fishes of the genus Squalius, its more likely that the ancestor of this amphipod species used the northern route from the lower Tagus – primitive Sado basin for colonization after it opened to the Atlantic (Sousa-Santos *et al*., 2018). From there, it may have expanded southwards to colonize the Mira river through the existing coastal freshwater corridor established with the Sado river basin during the late Messinian and most of the Zanclean (Sousa-Santos *et al*., 2018). The expansion to the Mondego could have happened during the Piacenzian (3.6 – 2.6 million years ago), where the upper Tagus flowed towards west and united with the smaller endorheic lakes adjacent to the Mondego basin, allowing the passage of the ancestor to that area (Sousa-Santos *et al*., 2008).

This theory seems to be further supported by the fact that this species has a high tolerance to salinity, possibly due its relatively recent evolutionary process from a marine environment to a freshwater ecosystem (da Cruz, 2017). With the establishment of the current hydrological network in the early stages of the Pleistocene and increasing desertification in the southern areas of the Peninsula, the general connections between rivers ceased to exist, becoming independent, which could have lead to the isolation of this species populations (Perea *et al*., 2016; Scheepmaker, 1990; Silva *et al*., 2017).

Turning the focus to *Echinogammarus berilloni,* it appears as a widespread amphipod throughout southern Europe that mainly inhabits Atlantic affluents with some degree of salinity, which it can resist (Hou & Sket, 2015; Hupalo, 2020; Margalef, 1953). It is somewhat intriguing that it was only found in the affluents of the upper Tagus and downstream of the Alentejo river basins in Portugal, since its described as a native for the northeastern side of Spain and the Pyrenees (Hou & Sket, 2015; Margalef, 1953; Pinkster, 1973).

During the glaciations, the climate of the northern side of the peninsula was prevalently cold. Since the species is classified as a thermophile, which likes warmer environments, it may be possible to think that, in Portugal, these are the remnants of an ice age refuge for this species and got restricted to that area with the creation of the independent river basins seen today (Margalef, 1953; Pinkster, 1993; Oliva *et al*., 2016).

Focusing now on the last of the native species in this study, *Gammarus chevreuxi*, it is a common lagoonal species on intertidal habitats, ranging from estuarine environments to coastal rias, in the western Europe and north Africa (Cunha & Moreira, 1995; Margalef, 1953; Subida *et al*., 2005). In addition to its previous records on brackish environments in Portugal (like in Ria de Aveiro), the species was found downstream the Sado and Mira rivers, especially in the Mira basin since it has marine influence well over 7,5 km from the coast (Marques & Bellan-Santini, 1987; Subida *et al*., 2005).

Relating to its origin, since its distribution is so widespread throughout western Europe and north Africa, not enough is known to pinpoint the exact place of origin and which event promoted its expansion. If it wasn't originated in the Iberian Peninsula it could either have descended from more northern regions through the glaciations and colonized by Atlantic sea routes (Krijgsman *et al*., 2018; Oliva *et al*., 2016). Until a focus is given to this thematic, a definite answer to this question isn't possible.

Crangonyx pseudogracilis, native to the eastern side of north America, was first detected as an exotic species in the Iberian Peninsula in a stream in Santarém during September 2011 (Grabowski *et al*., 2012). From that date on, the species extended its range throughout the entirety of Tagus river basin by the time the study of Correia *et al*. (2021) occurred. With this study, it was shown that, in 2022, *Crangonyx pseudogracilis* extends throughout the whole country, from north to south, resulting in an area of 50.723 km^2. This is equivalent to around 55% of the total area of the country.

Regarding the contradictory descriptions to the variable "temperature", Correia *et al*. (2021) proposed that, under higher temperatures, this amphipod unlocks a faster growth rate and sexual maturation in exchange for a shorter longevity. In conjunction with the recorded feminizing effect from *Fibrillanosema crangonycis*, a microsporidian parasite associated with the species, population growth would escalate due to the overproduction of female offspring (Correia et al., 2021; Galbreath et al., 2004). This hypothesis makes sense on the context of this work, where in significant associations with warmer areas, *Crangonyx pseudogracilis* presents the second lowest population density observed out of the all the amphipod species.

Combined with the broad range of resistance to salinity levels, pollution and being unaffected by native parasites, provided this species the necessary conditions for a successful invasion into previously unoccupied niches, and consequent expansion (Chapman, 2007; Grabowski *et al*., 2012). Moreover, the species shows a correlation with the most urban areas, due to its positive association with human density, implying a significant relation with its presence and dispersal, through pathways like artificial canals and fishing (Correia *et al*., 2021; Hupalo, 2020; Zhang & Holsinger, 2003).

The massive increase on the distribution area of *Crangonyx pseudogracilis* observed ever since the work of Correia *et al*. (2021) is of large concern since it also overlapped with the native species distribution areas, something that was never detected in earlier studies (Correia *et al*., 2021). The native species present more than 70% overlap with the non-native, with total inclusion (100% overlap) on *E. berilloni* and *G. chevreuxi*. There wasn't any short-term competition detected between one native and the non-native amphipods, mainly due to differences between micro-habitat preferences (Gama *et al*., 2017).

However the pressure of long-term competition might pose a threat to endemic species, like *E. lusitanus*, who might have lost the necessary tools to withstand competition due to its isolation (Correia *et al*., 2021; Gama *et al*., 2017; Pinkster, 1993). Urgent investigation is needed to ascertain the situation between *E. lusitanus* and *C. pseudogracilis*, so that conservation measures can be applied as soon as possible in case populations are declining. This might already be the case with *E. lusitanus* since it was found at low densities.

Overall, this study ended up answering the main questions proposed in the beginning, filling in the gaps pertaining to knowledge of freshwater amphipods existing in Portugal and their distribution along the country. The update of the distribution of *C. pseudogracilis* was also one of the main goals during this study, which was achieved, revealing the degree of invasion success this species had on the Portuguese territory and the first indications of overlap with the natives.

Conclusion:

By the end of this project, it was possible to determine which environmental factors played a bigger role in explaining the presence of the six species detected, while also detecting *Gammarus gauthieri* as a new record for Portugal. The species were mostly distributed over a single continuous area each, with *Echinogammarus meridionalis* being the only exception with its disjunct distribution along the upper Tagus and southwestern Portugal. So naturally, variables like "ocean distance", "temperature", "precipitation" and "altitude" played a major role on the presence of the native species. Species in the north, are mostly associated with lower temperatures and higher precipitation levels as expected, while in the south the inverse was seen. Only *Gammarus gauthieri*, the sole species found in the interior of the country, showed a positive correlation to the altitude, due to the more mountainous nature in that area of Portugal. The species occur close to the sea and at low altitudes. However some species were present on river springs, mainly the endemisms *E. lusitanus* and *E. meridionalis*, which could be found at high altitudes. Besides *Gammarus chevreuxi*, which is a species mostly related to brackish environments, native species were mostly related to lotic environments characterized by low flowing currents on the upper reaches of rivers, mainly Strahler orders of 2 or 3.

Relating to micro-habitats, bryophytes were the main type of vegetation where the natives were found, only having the non-native with a negative association with it. Macrophytes didn't show any kind of correlation to any of the four species detected during the sampling campaign. Overall, even though some natives have resistance to high salinity values and organic pollution, they are mainly associated with micro-habitat composed of bryophytes. These were found on the upper reaches of freshwater ecosystems in Portugal, supporting the proposed hypothesis at the beginning of this project. In contrast, human density results didn't show any signs of influence on the native species opposed to what was supposed.

Crangonyx pseudogracilis was found throughout Portugal, overlapping with the established ranges of the native species. It has considerably extended its distribution area since its discovery in 2011, possibly posing a threat to the natives, especially *E. lusitanus*. Being the only known non-native amphipod in the Iberian Peninsula, studies of environmental impacts on the natives are highly necessary in a near future. Even though the species wasn't legally classified as an invasive species to this day (Correria *et al.*, 2021), this exponential extension on Portuguese territory could help to officialize that status in a near future, being the first step needed to start controlling the spread of this amphipod on the freshwater ecosystems of the country.

Hopefully this study gives a better understanding on why and where the amphipod species in Portugal are, while also emphasizing the possible threats of long-term exposure to the non-native. Further studies will be required to fully understand the history and ecology of each species, with this project serving as possible stepping stone towards that goal.

It is also important to emphasize the benefit of this information to the future conservation of these species. Considering the generally restricted distribution of the native amphipods, it is of great interest to invest in the evaluation of these species according to ICUN criteria. Large scale alterations in those few locations, like the construction of dams for example, could cause the few existing populations to disappear.

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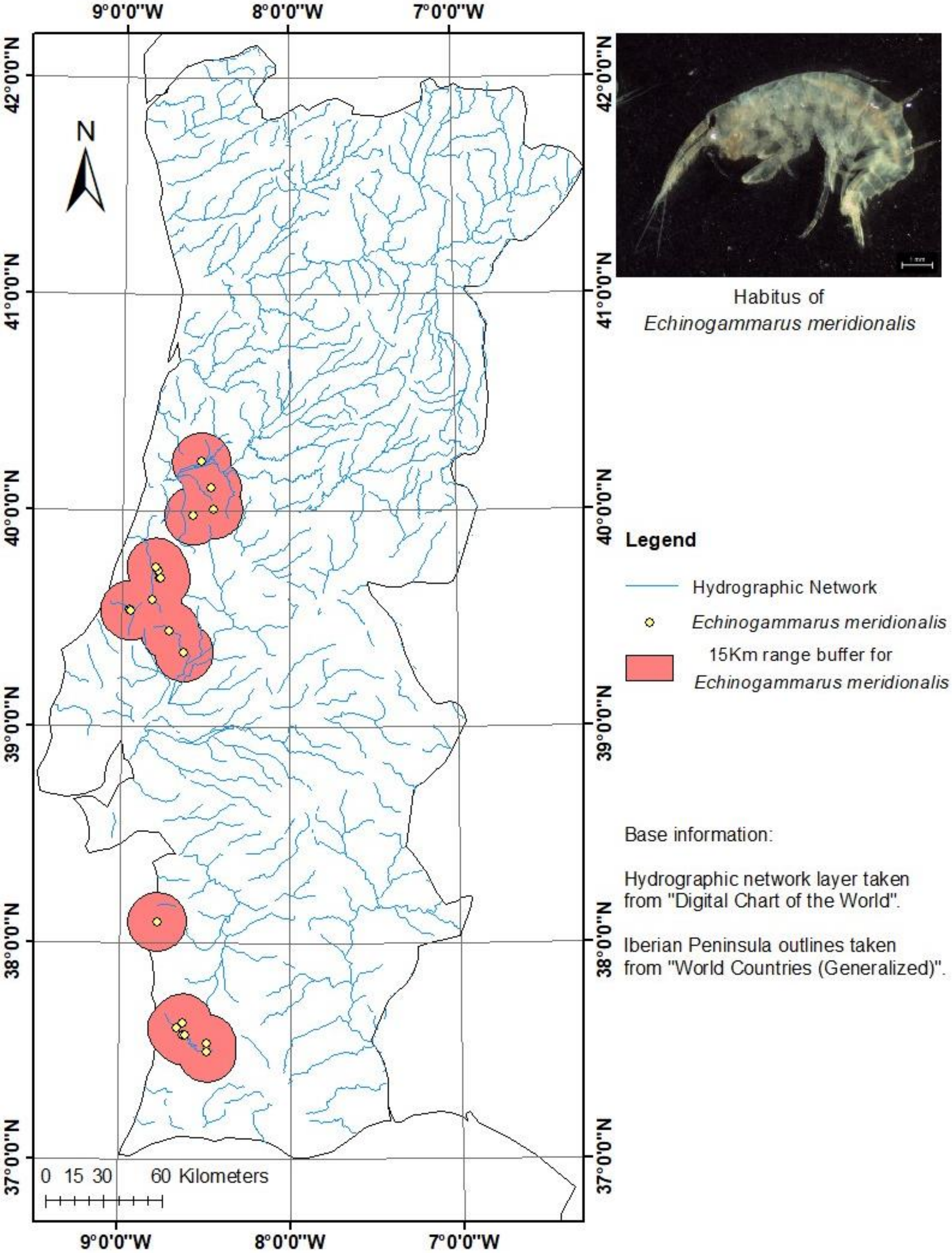
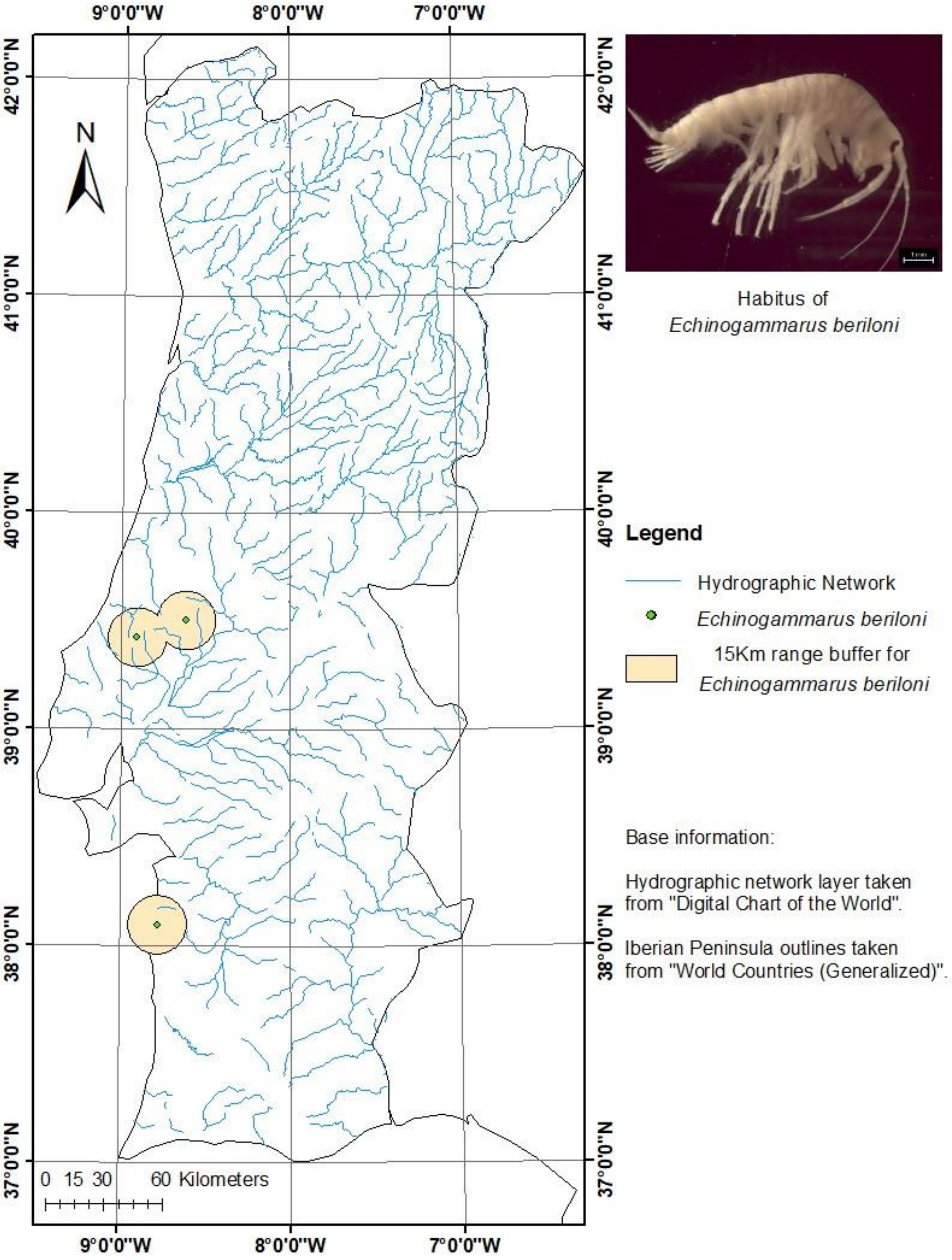
Annexes:

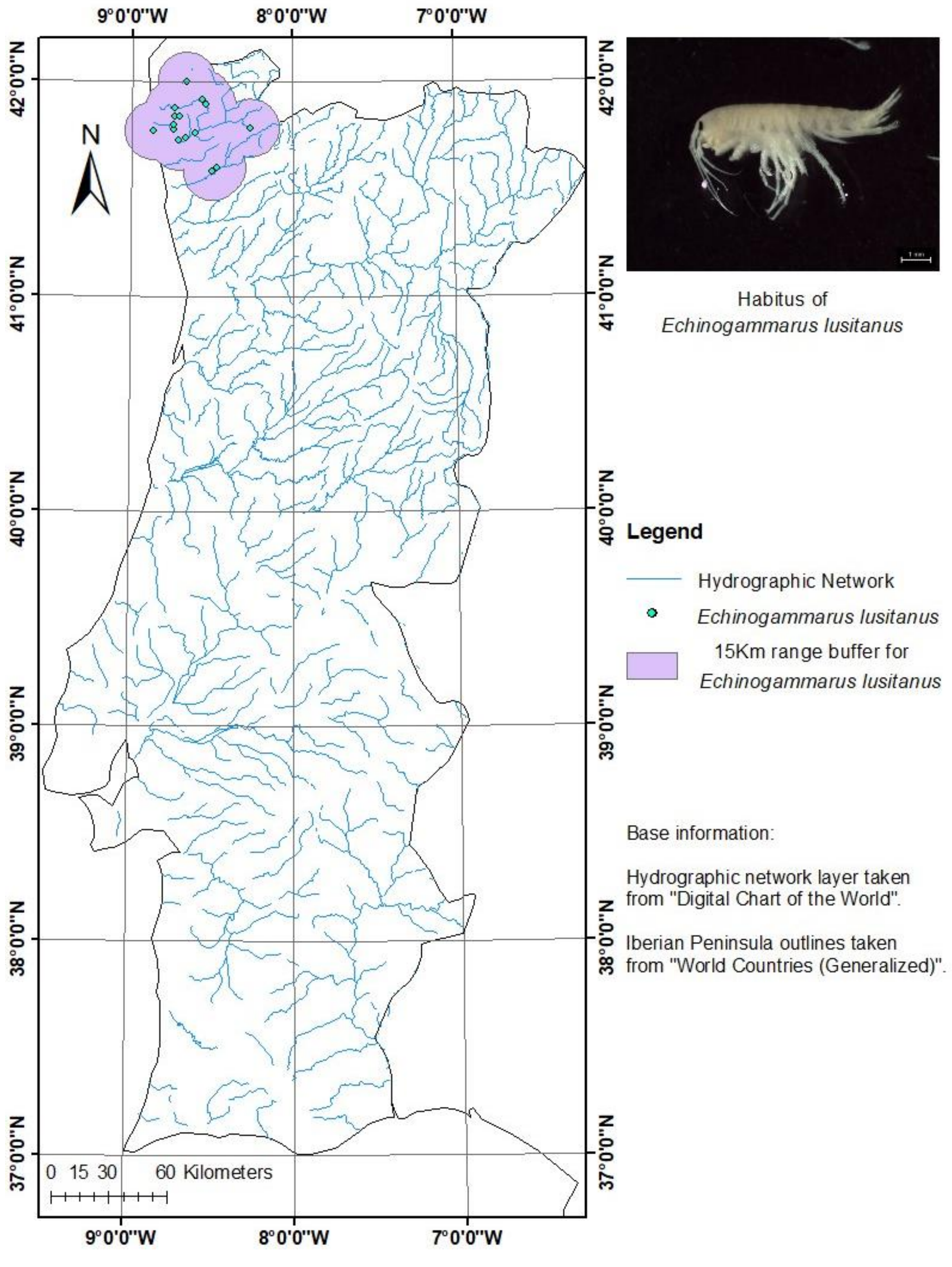
Annex I – Distribution map of the Iberian endemism *Echinogammarus meridionalis* in the freshwater ecosystems of Portugal (photo taken by the author).



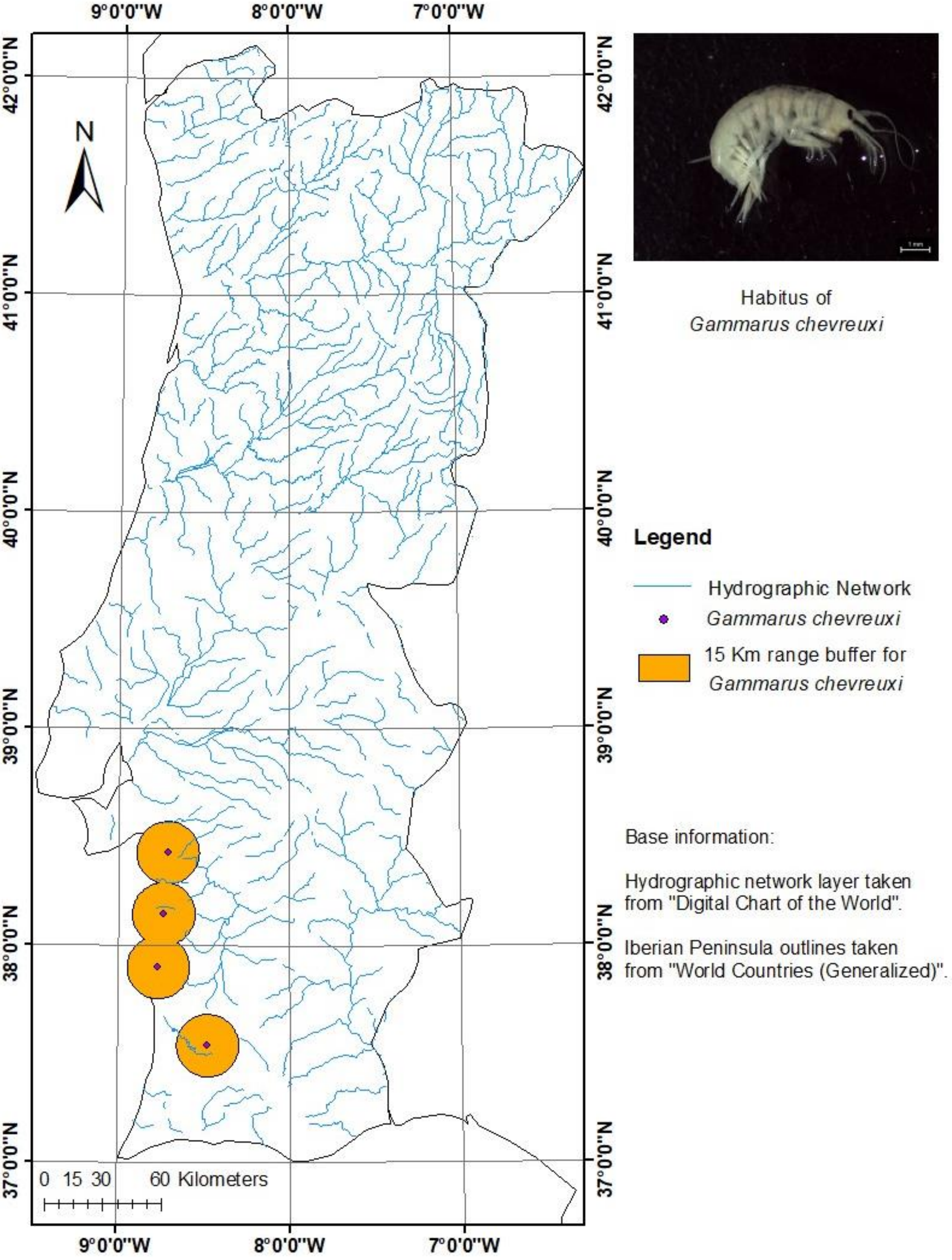
Annex II – Distribution map of the native amphipod *Echinogammarus berilloni* in the freshwater ecosystems of Portugal (photo taken by the author).



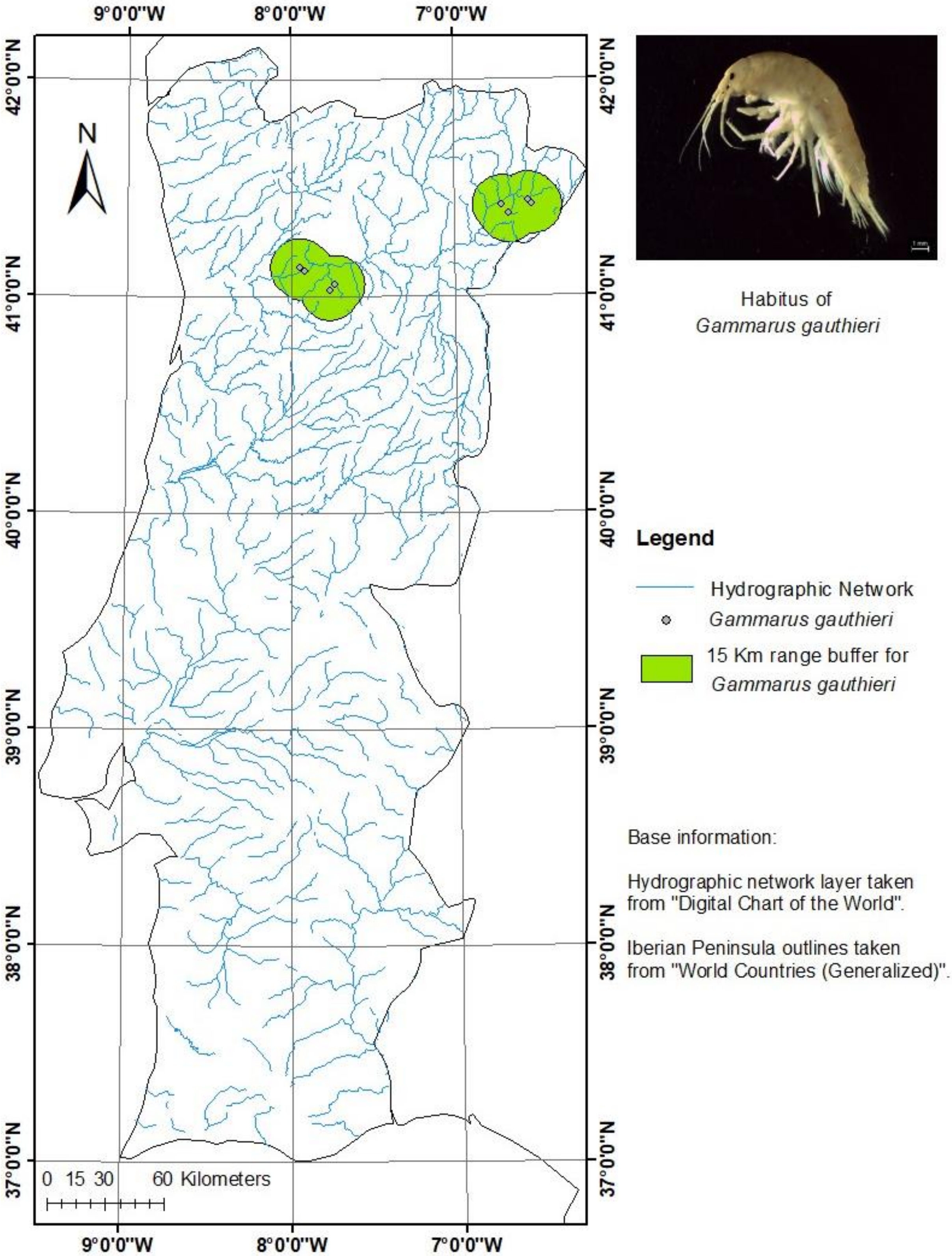
Annex III – Distribution map of the Iberian endemism *Echinogammarus lusitanus* in the freshwater ecosystems of Portugal (photo taken by the author).



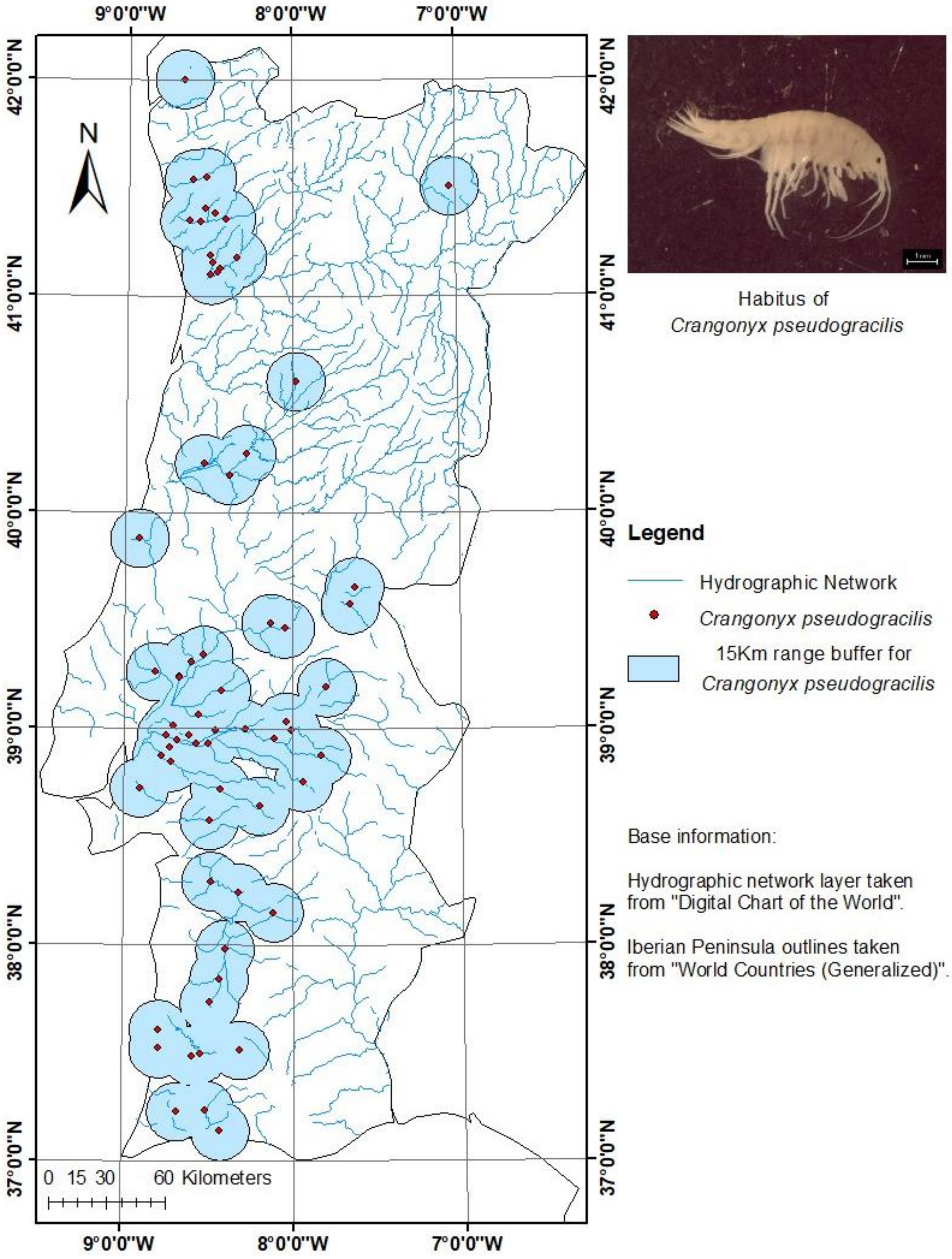
Annex IV – Distribution map of the native amphipod *Gammarus chevreuxi* in the freshwater ecosystems of Portugal (photo taken by the author).



Annex V – Distribution map of the new native amphipod *Gammarus gauthieri* in the freshwater ecosystems of Portugal (photo taken by the author).



Annex VI – Distribution map of the non-native amphipod *Crangonyx pseudogracilis* in the freshwater ecosystems of Portugal (photo taken by the author).



Supplements:

Supplement S1 – Summary of the information pertaining to the 152 locations with presence of amphipods, with the sample origin and number of captures ("---" meaning unable to determine).

ID	Location	Y	X	Year	Altitude (m)	Species	Number of Captures	Origin
01F/50	Lomba do Rio	42,00223	-8,65758	2017	3	*Crangonyx pseudogracilis*	12	in: UTAD
01F/50	Lomba do Rio	42,00223	-8,65758	2017	3	*Echinogammarus lusitanus*	13	in: UTAD
02F/01	Mozelos	41,91929	-8,55546	2019	329	*Echinogammarus lusitanus*	14	in: UTAD
03D/04	Ribeira Pego	41,71907	-8,85820	2017	4	NA	2	in: UTAD
03D/05	Afife	41,77277	-8,85984	2017	9	*Echinogammarus lusitanus*	2	in: UTAD
03E/08	Vila Mou	41,72454	-8,70801	2017	9	*Echinogammarus lusitanus*	6	in: UTAD
03E/10	Portuzelo	41,70583	-8,79231	2017	1	NA	1	in: UTAD
03F/07	Foz do Trovela	41,76115	-8,59939	2019	5	*Echinogammarus lusitanus*	3	in: UTAD
03H/02	Germil	41,78583	-8,26417	2019	588	*Echinogammarus lusitanus*	5	in: UTAD
04E/15	Gemeses	41,51285	-8,72981	2017	11	NA	3	in: UTAD
04F/11	Pousa	41,54944	-8,51913	2017	50	*Crangonyx pseudogracilis*	5	in: UTAD
04G/06	Cerqueiral	41,58353	-8,49498	2017	15	*Echinogammarus lusitanus*	3	in: UTAD
04G/50	Prado	41,60100	-8,47110	2019	20	*Echinogammarus lusitanus*	4	in: UTAD
04H/13	Águas Santas	41,61869	-8,32356	2017	42	NA	1	in: UTAD
04O/50	Cortiços	41,50831	-7,03281	2019	455	*Crangonyx pseudogracilis*	5	in: UTAD
05E/01	Ponte Junqueira	41,38907	-8,68854	2019	31	NA	1	in: UTAD
05F/03	Ponte Trofa	41,34435	-8,55847	2019	24	*Crangonyx pseudogracilis*	1	in: UTAD
05F/04	Ponte Nova - Vizela	41,35850	-8,40281	2019	80	*Crangonyx pseudogracilis*	4	in: UTAD
05F/11	Eirado-Ferreiro	41,34904	-8,62458	2017	17	*Crangonyx pseudogracilis*	9	in: UTAD
05G/09	Ponte EN204-Pele	41,38118	-8,46754	2019	85	*Crangonyx pseudogracilis*	1	in: UTAD
05Q/02	Penas Roias	41,38118	-6,66678	2017	630	*Gammarus gauthieri*	490	in: UTAD
05R/04	Saldanha	41,43758	-6,54947	2017	484	*Gammarus gauthieri*	13	in: UTAD
05R/05	Gregos	41,42053	-6,52269	2017	579	*Gammarus gauthieri*	114	in: UTAD
06H/50	Irivo	41,17595	-8,33249	2019	156	*Crangonyx pseudogracilis*	1	in: UTAD
07G/01	Sousa Ribeira	41,09877	-8,49202	2017	9	*Crangonyx pseudogracilis*	2	in: UTAD
07J/08	Santinho	41,11604	-7,91381	2017	66	*Gammarus gauthieri*	2	in: UTAD
07K/18	Salzedas	41,05673	-7,72966	2017	494	*Gammarus gauthieri*	27	in: UTAD
07K/51	Tarouca	41,02598	-7,76360	2019	477	*Gammarus gauthieri*	504	in: UTAD
07P/54	Santa Marinha 1	41,12986	-6,91774	2019	285	NA	1	in: UTAD
10J/52	Fail	40,60640	-7,97620	2017	307	*Crangonyx pseudogracilis*	11	In: U. Coimbra
12G/02	Ponte Cabouco	40,17272	-8,37210	2017	63	*Crangonyx pseudogracilis*	4	In: U. Coimbra
12H/02	Ponte Penacova	40,27027	-8,27413	2017	37	NA	2	In: U. Coimbra
14D/07	Ponte Passagem	39,88060	-8,91450	2017	4	*Crangonyx pseudogracilis*	118	In: U. Coimbra
14D/52	Carreira	39,68910	-8,85215	2017	122	NA	1	In: U. Coimbra
14D/53	Canadas/Monte Redondo	39,91378	-8,85155	2017	11	NA	1	In: U. Coimbra
14F/01	Venda da Cruz	39,95717	-8,63152	2017	34	NA	1	In: U. Coimbra
14F/52	Redinha	40,22585	-8,52905	2017	6	*Echinogammarus meridionalis*	1117	In: U. Coimbra
15E/06	Fontes	39,68607	-8,77059	2017	89	*Echinogammarus meridionalis*	1104	In: U. Coimbra
15E/51	Vidigal de Baixo	39,71852	-8,78164	2017	48	*Echinogammarus meridionalis*	163	In: U. Coimbra
15E/52	Vale do Horto	39,68714	-8,83870	2017	64	NA	1	In: U. Coimbra
16D/03	Chiqueda	39,53705	-8,94684	2017	42	*Echinogammarus meridionalis*	567	In: U. Coimbra
16E/02	Batalha	39,65911	-8,82160	2017	62	NA	4	In: U. Coimbra
16E/51	Alcaria	39,73882	-8,79675	2017	27	*Echinogammarus meridionalis*	603	In: U. Coimbra
17F/01	Quinta d'Rei	39,34178	-8,52770	2017	12	*Crangonyx pseudogracilis*	18	In: LAUE
17F/03	Ponte Ribeira	39,38167	-8,66042	2017	22	NA	1	In: LAUE
18F/01	Ponte Borrado	39,31233	-8,60320	2017	10	*Crangonyx pseudogracilis*	2	In: LAUE
20G/01	Ponte Erra	38,99473	-8,45873	2017	21	*Crangonyx pseudogracilis*	72	In: LAUE
23E/01	Sado - Canal Arrozais	38,43080	-8,71025	2017	0	*Gammarus chevreuxi*	117	In: LAUE
24J/50	Ribeira do Malk Abraão	38,21381	-7,96977	2017	145	NA	1	In: LAUE
25E/01	Melides	38,14688	-8,73299	2017	26	*Gammarus chevreuxi*	297	In: LAUE
25E/50	Cerradinha	38,10069	-8,77808	2017	4	*Echinogammarus beriloni*	74	In: LAUE
25I/57	Barranco da Casa Branca	38,15186	-8,11095	2019	82	*Crangonyx pseudogracilis*	12	In: LAUE
26E/01	Alb. Morgavel	37,90234	-8,76323	2017	74	*Gammarus chevreuxi*	57	In: LAUE
26G/51	Ribeira da Gema	37,84464	-8,43195	2017	56	*Crangonyx pseudogracilis*	51	In: LAUE
26G/57	Rio Sado - Jusante Camp.	37,98212	-8,39708	2017	39	*Crangonyx pseudogracilis*	17	In: LAUE
26H/01	Nabos	37,96841	-8,33491	2017	41	NA	2	In: LAUE
27G/54	Riberia da Gema I	37,73522	-8,48774	2019	105	*Crangonyx pseudogracilis*	110	In: LAUE
27L/52	Ribeira do Freixiol	37,68761	-7,66280	2017	34	NA	1	In: LAUE
28E/50	Cerradinha	38,10069	-8,77808	2017	4	*Echinogammarus meridionalis*	151	In: LAUE
28E/53	Barranco da Zambujeira	37,52284	-8,78389	2017	10	*Crangonyx pseudogracilis*	10	In: LAUE
28F/23	Mira - Quinta Vale Palhete	37,60756	-8,65829	2017	6	*Echinogammarus meridionalis*	1	In: LAUE
28F/55	Torgal Jusante	37,63315	-8,62422	2017	16	*Echinogammarus meridionalis*	54	In: LAUE
28F/57	Jusante Santa Clara	37,57758	-8,62614	2019	8	*Echinogammarus meridionalis*	38	In: LAUE
28F/61	Jusante Santa Clara	37,57381	-8,61337	2017	14	*Echinogammarus meridionalis*	230	In: LAUE
28G/52	Santa Clara	37,53890	-8,48474	2017	68	*Echinogammarus meridionalis*	158	In: LAUE
29E/55	Barranco do Carvalhal	37,49941	-8,78967	2017	8	NA	1	In: LAUE
29F/50	Ribeira de Arredouças	37,48980	-8,59067	2017	57	*Crangonyx pseudogracilis*	82	In: LAUE
29F/51	Ribeiro de Foz de Casinhas	37,50008	-8,54275	2017	40	*Crangonyx pseudogracilis*	306	In: LAUE
29G/50	Santa Clara Saboia	37,49633	-8,48265	2019	40	*Echinogammarus meridionalis*	546	In: LAUE
Cav1N	Ribeira de Pontes	41,54144	-8,59795	2017	8	*Crangonyx pseudogracilis*	57	In: LAUE
DAN01	---	41,40801	-8,52385	2017	77	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN04	---	38,87597	-7,82676	2017	175	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN05	---	38,79820	-9,43726	2017	14	NA	---	in: Correia *et al* . (2021)
DAN07	---	37,61101	-8,78323	2017	60	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN08	---	38,71155	-9,36692	2017	39	NA	---	in: Correia *et al* . (2021)
DAN09	---	38,99058	-9,41558	2017	11	NA	---	in: Correia *et al* . (2021)

ID	Location	Y	X	Year	Altitude (m)	Species	Number of Captures	Origin
DAN10	---	38,94940	-9,39579	2017	5	NA	---	in: Correia *et al* . (2021)
DAN11	---	38,89216	-9,42603	2017	33	NA	---	in: Correia *et al* . (2021)
DAN12	---	38,71152	-9,22326	2017	16	NA	---	in: Correia *et al* . (2021)
DAN13	---	39,69642	-9,03302	2017	54	NA	---	in: Correia *et al* . (2021)
DAN18	---	39,46443	-8,04293	2017	37	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN20	---	39,48853	-8,12814	2017	43	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN22	---	39,03221	-8,03842	2017	136	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN23	---	39,68584	-8,37469	2017	146	NA	---	in: Correia *et al* . (2021)
DAN28	---	39,57540	-7,65321	2017	124	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN29	---	39,65750	-7,62821	2017	111	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN35	---	39,26449	-8,81500	2017	78	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN36	---	39,24092	-8,67242	2017	10	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN37	---	39,23915	-8,67276	2017	9	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN38	---	38,99528	-8,00843	2017	87	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN40	---	39,06486	-8,55535	2017	40	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN41	---	38,99600	-8,45765	2017	24	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN42	---	38,93526	-8,49927	2017	20	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN43	---	38,99750	-8,28094	2017	29	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN44	---	38,93514	-8,56658	2017	14	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN45	---	38,97347	-8,61298	2017	36	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN46	---	39,01279	-8,70591	2017	9	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN47	---	38,97215	-8,74555	2017	23	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN48	---	38,94974	-8,68518	2017	6	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN49	---	38,91595	-8,72788	2017	17	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN50	---	38,84619	-8,72073	2017	11	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN51	---	38,87637	-8,77200	2017	12	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN52	---	39,17971	-8,42236	2017	58	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN53	---	38,72807	-8,90440	2017	5	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN55	---	38,72366	-8,42897	2017	66	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN56	---	39,19203	-7,79599	2017	130	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN57	---	38,64463	-8,19202	2017	201	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN58	---	38,75388	-7,93623	2017	233	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN59	---	41,12868	-8,43336	2017	61	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN60	---	41,11081	-8,45099	2017	21	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN61	---	41,15701	-8,48411	2017	35	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN62	---	41,18681	-8,49431	2017	134	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN69	---	38,71338	-9,36721	2017	39	NA	---	in: Correia *et al* . (2021)
DAN70	---	38,69514	-9,37545	2017	6	NA	---	in: Correia *et al* . (2021)
DAN74	---	38,95351	-8,10835	2017	64	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
DAN75	---	38,95574	-8,11012	2017	52	*Crangonyx pseudogracilis*	---	in: Correia *et al* . (2021)
Dou23N	Rio Frio	41,80455	-6,69595	2019	513	NA	1	in: UTAD
Dou93N	Dízimos	41,13368	-7,94702	2017	192	*Gammarus gauthieri*	12	in: UTAD
Douro177N	Ribeira da Murça	41,12793	-7,22154	2017	127	NA	5	in: UTAD
Douro184N	Ribeira das Holas	41,42234	-6,71035	2019	380	*Gammarus gauthieri*	845	in: UTAD
Lima11N	Ribeira da Silvareira	41,73821	-8,66450	2017	6	*Echinogammarus lusitanus*	133	in: UTAD
Min2N	Agra de São João	41,87613	-8,73348	2017	47	*Echinogammarus lusitanus*	84	in: UTAD
PT_001	Rio Alviela	39,34604	-8,62851	2022	11	*Echinogammarus meridionalis*	2	by: R. Marques & J. Pinto
PT_005	Rio Almonda	39,50478	-8,61500	2022	79	*Echinogammarus berilloni*	81	by: R. Marques & J. Pinto
PT_006	Rio Lis	39,68497	-8,77311	2022	83	*Echinogammarus meridionalis*	206	by: R. Marques & J. Pinto
PT_007	Nascente Alviela	39,44558	-8,71216	2022	61	*Echinogammarus meridionalis*	31	by: R. Marques & J. Pinto
PT_008	Nascente Lena	39,58479	-8,81995	2022	135	*Echinogammarus meridionalis*	269	by: R. Marques & J. Pinto
PT_009	Nascente Alcoa - Alcobaça	39,54069	-8,95303	2022	30	*Echinogammarus meridionalis*	72	by: R. Marques & J. Pinto
PT_010	Ribeira de Alcobertas	39,42600	-8,90575	2022	132	*Echinogammarus berilloni*	141	by: R. Marques & J. Pinto
PT_013	Ribeiro de São João de Argã	41,83784	-8,73299	2022	426	*Echinogammarus lusitanus*	35	by: R. Marques & J. Pinto
PT_014	Regato da Fraga	41,84003	-8,70183	2022	474	*Echinogammarus lusitanus*	42	by: R. Marques & J. Pinto
PT_015	Rio Fulão - Cascata do Pereiro	41,77696	-8,73325	2022	206	*Echinogammarus lusitanus*	34	by: R. Marques & J. Pinto
PT_016	Nascente Âncora	41,79752	-8,73317	2022	289	*Echinogammarus lusitanus*	29	by: R. Marques & J. Pinto
PT_019	Ponte dos Cavaleiros	41,89239	-8,53319	2022	461	*Echinogammarus lusitanus*	58	by: R. Marques & J. Pinto
PT_026	Ribeira de Marateca	38,57810	-8,49092	2022	50	*Crangonyx pseudogracilis*	33	by: R. Marques & J. Pinto
PT_027	Ribeiro de Arcão	38,29644	-8,48056	2022	5	*Crangonyx pseudogracilis*	3	by: R. Marques & J. Pinto
PT_028	Ribeira de Melides	38,14968	-8,73316	2022	44	*Gammarus chevreuxi*	1238	by: R. Marques & J. Pinto
PT_030	Rio Xarrama	38,24392	-8,31990	2022	7	*Crangonyx pseudogracilis*	86	by: R. Marques & J. Pinto
PT_032	Ribeira de Morgavel	37,54266	-8,47477	2022	118	*Gammarus chevreuxi*	41	by: R. Marques & J. Pinto
PT_046	Ribeira da Vagarosa	37,14158	-8,42358	2022	27	*Crangonyx pseudogracilis*	70	by: R. Marques & J. Pinto
PT_047	Ribeira de Odiáxere	37,23099	-8,68015	2022	87	*Crangonyx pseudogracilis*	106	by: R. Marques & J. Pinto
PT_048	Ribeira de Odelouca	37,23825	-8,51258	2022	13	*Crangonyx pseudogracilis*	114	by: R. Marques & J. Pinto
PT_050	Ribeira de Santana	37,51575	-8,30808	2022	139	*Crangonyx pseudogracilis*	68	by: R. Marques & J. Pinto
PT_051	Rio Arunca	40,10646	-8,46525	2022	110	*Echinogammarus meridionalis*	432	by: R. Marques & J. Pinto
PT_053	Olhos de Água - Anços	39,97839	-8,57330	2022	67	*Echinogammarus meridionalis*	221	by: R. Marques & J. Pinto
PT_056	Alcalamouque	40,00615	-8,45655	2022	219	*Echinogammarus meridionalis*	980	by: R. Marques & J. Pinto
PT_057	Ponte Penacova	40,27027	-8,27413	2022	37	*Crangonyx pseudogracilis*	1	by: R. Marques & J. Pinto
PT_063	Ribeira de Tarouca	41,02598	-7,76360	2022	477	*Gammarus gauthieri*	14	by: R. Marques & J. Pinto
PT04MON0665	Conraria	40,17273	-8,39478	2019	22	NA	1	In: U. Coimbra
PT04MON0674	São Silvestre	40,22585	-8,52905	2019	6	*Crangonyx pseudogracilis*	29	In: U. Coimbra
PT04MON0689	Alcabideque	40,10672	-8,47134	2019	131	NA	394	In: U. Coimbra
PT04MON0694	Quinta das Nogueiras	40,07176	-8,61967	2019	13	NA	1	In: U. Coimbra

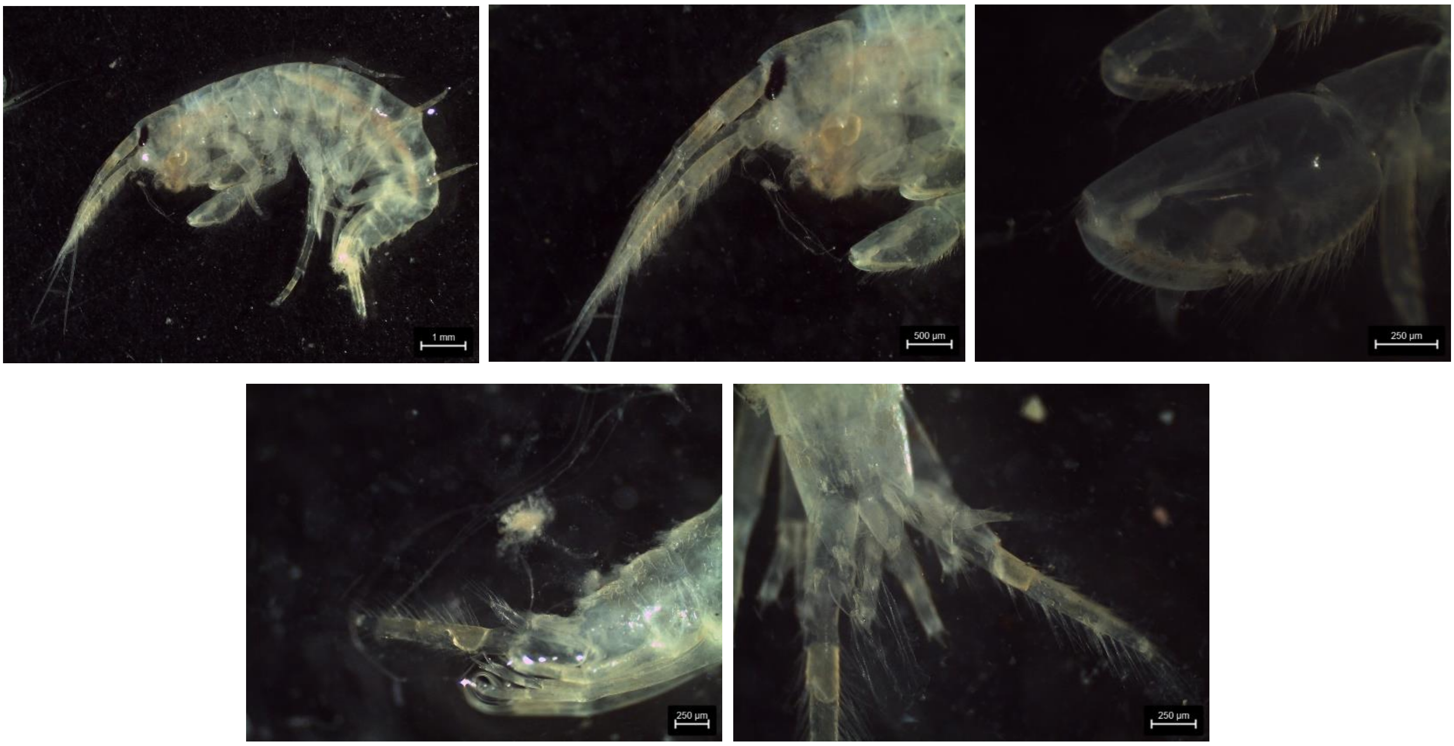
Supplement S2 – Photos taken for each species of the more distinct features of the species used during identification (1st - habitus, 2nd - second pair of antennae, 3rd - gnathopodes, 4th - urossome, 5th - telson respectively from right to left).

a) *Crangonyx pseudogracilis*:

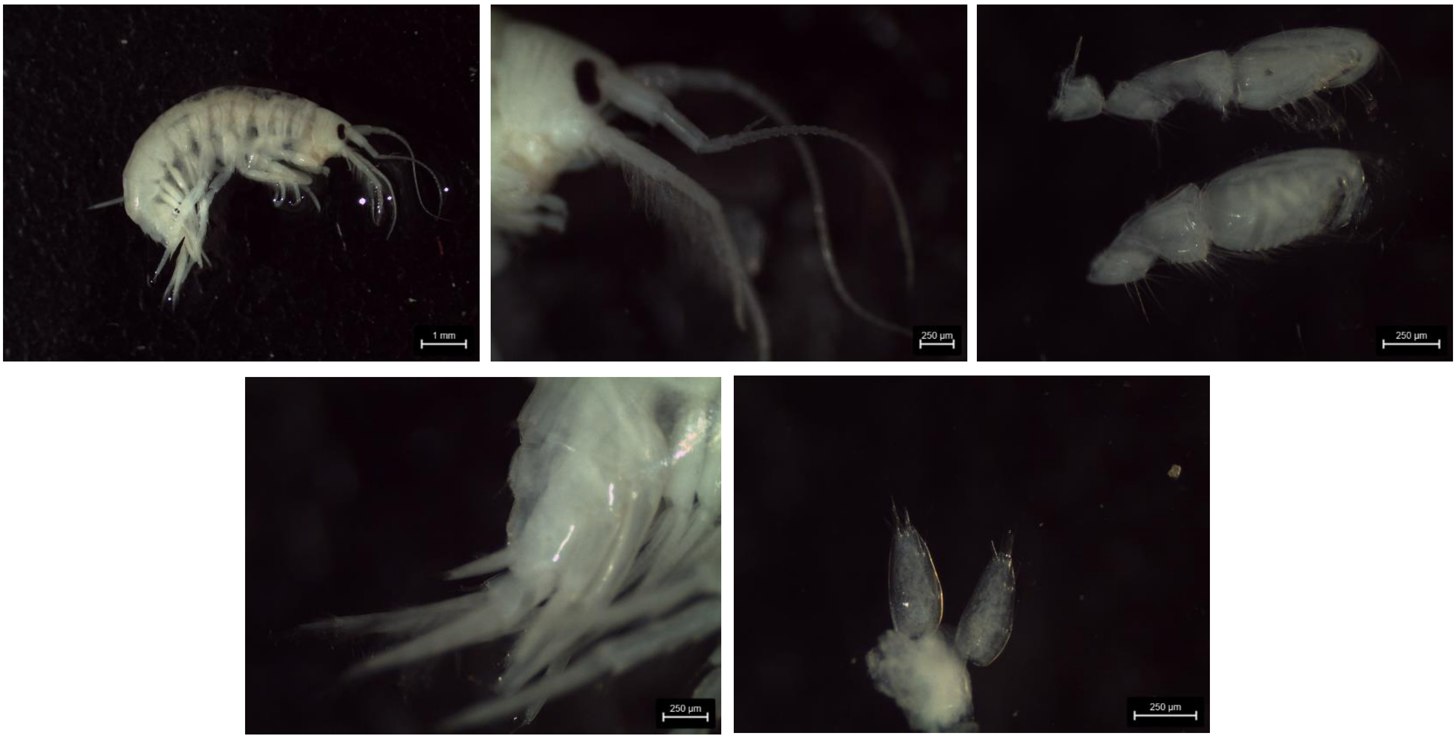
b) *Echinogammarus berilloni* (PT005):

c) *Echinogammarus lusitanus* (PT019):

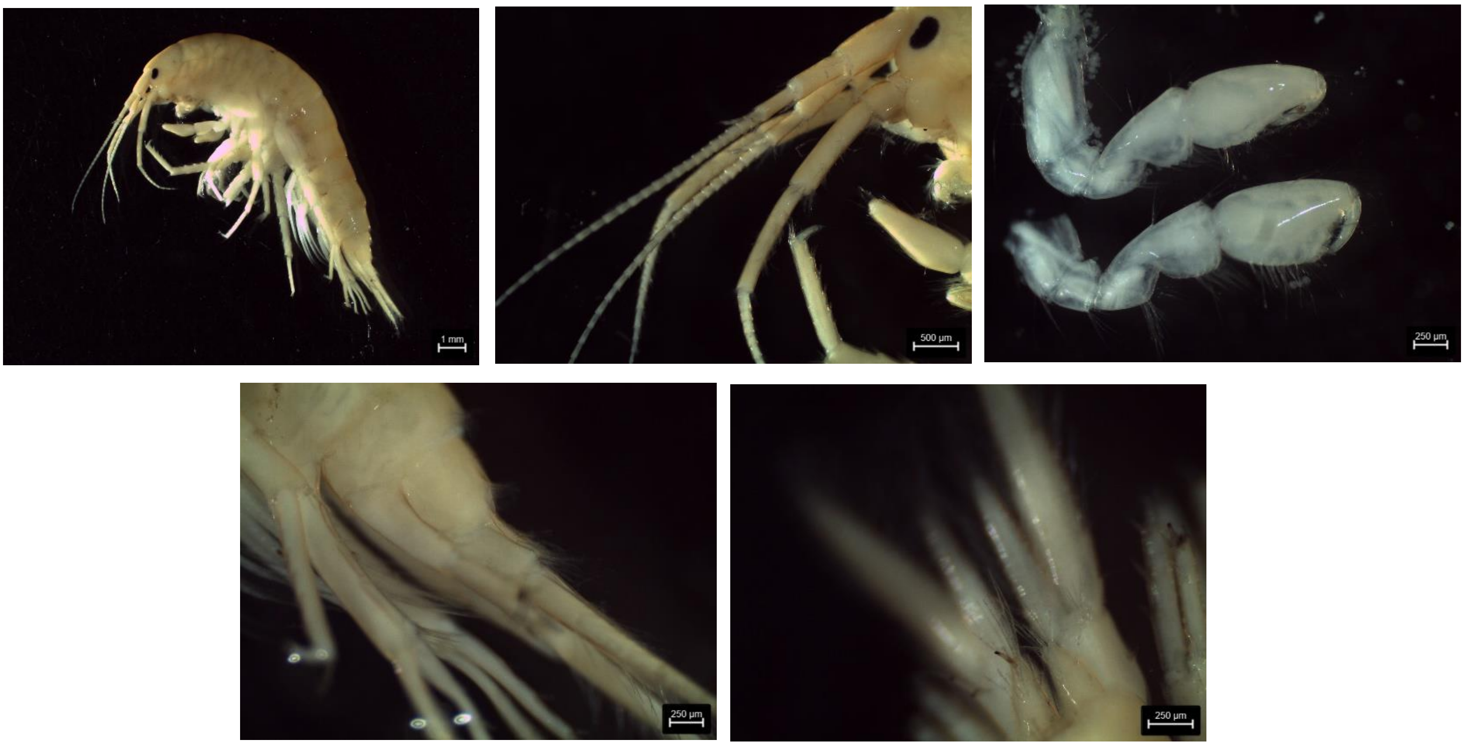
d) *Echinogammarus meridionalis* (PT001):



e) *Gammarus chevreuxi* (PT028):



f) *Gammarus gauthieri* (PT063 – gnathopodes from another individual):



Supplement S3 – Source Links for the datasets used for extraction of values of the respective environmental variable.

Variable	Dataset source link
Altitude	https://www.worldclim.org/data/worldclim21.html
Water Body Type	https://snirh.apambiente.pt/index.php?idMain=2&idItem=1
River Order	https://snirh.apambiente.pt/index.php?idMain=2&idItem=1
River Spring	https://snirh.apambiente.pt/index.php?idMain=2&idItem=1
Geology	https://geoportal.bgr.de/mapapps/resources/apps/geoportal/index.html?lang=en#/geoviewer
Annual Average Temperature	https://www.worldclim.org/data/worldclim21.html
Annual Average Precipitation	https://www.worldclim.org/data/worldclim21.html
Ocean Distance	https://www.naturalearthdata.com/downloads/10m-physical-vectors/10m-ocean/
Ecological Quality	https://water.europa.eu/data-maps-and-tools/water-framework-directive-surface-water-data-products
Chemical Quality	https://water.europa.eu/data-maps-and-tools/water-framework-directive-surface-water-data-products
Human Density	https://ec.europa.eu/eurostat/web/gisco/geodata/reference-data/population-distribution-demography/geostat