Graellsia, 75(2): e100
julio-diciembre 2019
ISSN-L: 0367-5041
https://doi.org/10.3989/graellsia.2019.v75.248

Notas / *Notes*

Unusual habitat for Bathynellacea (Crustacea, Malacostraca): first record of this groundwater crustacean in the mesovoid shallow substratum (MSS)

Ana I. Camacho[1,*] & Vicente M. Ortuño[2]

[1]Museo Nacional de Ciencias Naturales (CSIC). Dpto. de Biodiversidad y Biología Evolutiva. C/ José Gutiérrez Abascal 2. 28006 Madrid (Spain).ORCID iD: https://orcid.org/0000-0003-0596-7678

[2]Grupo de Investigación de Biología del Suelo y Ecosistemas Subterráneos. Dpto. de Ciencias de la Vida, Facultad de Ciencias, Universidad de Alcalá. Alcalá de Henares, Madrid (Spain); vicente.ortuno@uah.es; ORCID iD: https://orcid.org/0000-0001-5734-1621

* Corresponding author: mcnac22@mncn.csic.es

ABSTRACT

First world record of a crustacean (Malacostraca, Bathynellacea) that lives exclusively in groundwater in an unusual habitat, the mesovoid shallow substratum (MSS). The MSS is a terrestrial subterranean medium with high and constant relative humidity. Specimens of the family Parabathynellidae have been found in sampling devices set to collect terrestrial subterranean fauna in the MSS of Sierra de Guadarrama National Park (Madrid, Spain). Two species belonging to two different genera, *Hexabathynella nicoleiana* Camacho, 1986 and *Hexaiberobathynella mateusi* (Galhano, 1967), already known to occur in the province of Madrid, have been identified by morphological study, whereas their 18S gene sequences confirmed their generic ascription.

Keywords: MSS; groundwater fauna; Parabathynellidae; Sierra de Guadarrama; Spain.

RESUMEN

Hábitat inusual para Bathynellacea (Crustacea, Malacostraca): primer registro de este crustáceo de agua subterránea en el sustrato superficial mesovoide (MSS)

En este trabajo se documenta por primera vez el hallazgo de ejemplares de un crustáceo (Malacostraca, Bathynellacea) que vive exclusivamente en las aguas subterráneas de todo el mundo, en un hábitat inusual: el medio subterráneo superficial (MSS), un medio terrestre sin luz y saturado de humedad. Especímenes de la familia Parabathynellidae han sido encontrados en dispositivos de muestreo dispuestos para la recogida de fauna subterránea terrestre en el MSS del Parque Nacional de la Sierra de Guadarrama (Madrid, España). Se han identificado dos especies, de dos géneros diferentes, *Hexabathynella nicoleiana* Camacho, 1986 y *Hexaiberobathynella mateusi* (Galhano, 1967), mediante estudio morfológico. Las secuencias del gen 18S de varios ejemplares confirman su adscripción genérica. Estas especies eran ya conocidas en la provincia de Madrid.

Palabras clave: MSS; fauna acuática subterránea; Parabathynellidae; Sierra de Guadarrama; España.

Recibido/*Received*: 23/07/2019; **Aceptado/*Accepted***: 30/09/2019; **Publicado en línea/*Published online***: 30/10/2019

Cómo citar este artículo/*Citation*: Camacho, A. I. & Ortuño, V. M. 2019. Unusual habitat for Bathynellacea (Crustacea, Malacostraca): first record of this groundwater crustacean in the mesovoid shallow substratum (MSS). *Graellsia*, 75(2): e100. https://doi.org/10.3989/graellsia.2019.v75.248

The mesovoid shallow substratum ("milieu souterrain superficiel"; MSS or terrestial SSHs) (Juberthie *et al*., 1980, 1981; Uéno, 1980, 1981; Pipan & Culver, 2012) is a terrestrial habitat that consist of a network of voids and interstices located above the deep subterranean domain and immediately below the soil, lightless and highly humid (Mammola *et al*., 2016). It includes talus and scree slopes in both carbonate (soluble) and non-carbonate rocks, including volcanic rocks. Also are an aquatic SSHs that include epikarst and the hypotelminoheic realm (Pipan & Culver, 2012). The epikarst, uppermost layer of karst, may be air or water filled and occupies a similar vertical position to that of the MSS. The perched aquifers (isolated wetlands) (hypotelminorheic by Mestrov, 1962) are the most superficial of SSHs and together with epikarst and MSS expand the scope of subterranean habitats (Pipan & Culver, 2012). The characteristics of these environments are very different as different are the faunas that can inhabit them (Pipan & Culver, 2012). The common characteristics are absence of light, high relative humidity and attenuated fluctuations in temperature throughout the year. In the MSS there is a rich and diverse terrestrial fauna composed of both hypogean species with different degrees of adaptation to the subterranean environment as well as epigean and endogean species that transit between the surface and the subterranean environment (Pipan & Culver, 2012; Ortuño *et al*., 2013; Mammola *et al*., 2016). The MSS (terrestrial SSHs) has been hardly studied, mostly in the French Pyrenees and other (noncalcareous) areas from Europe, Japan and China (Gers, 1992; Juberthie & Decu, 1994; Ruzicka *et al*., 1995). Medina & Oromí (1990) extended the habitat to include volcanic terrain in the Canary Islands (López & Oromí, 2010; Pipan *et al*., 2011).

Here, we report for the first time the presence of Bathynellacean crustaceans in the MSS. These aquatic animals live exclusively in groundwater (stygobionts). To date they have been found in caves (gours, puddles, ponds, lakes, rivers, etc.), seepage springs, sources, artificial wells, aquifers (mine bores, bore holes, irrigation waters) and in the hyporheic habitat associated to epi- and hypogean rivers. Never before had they been found in a terrestrial environment like the MSS as defined.

Bathynellacea were collected with traps set to sample terrestrial subterranean fauna in the Sierra de

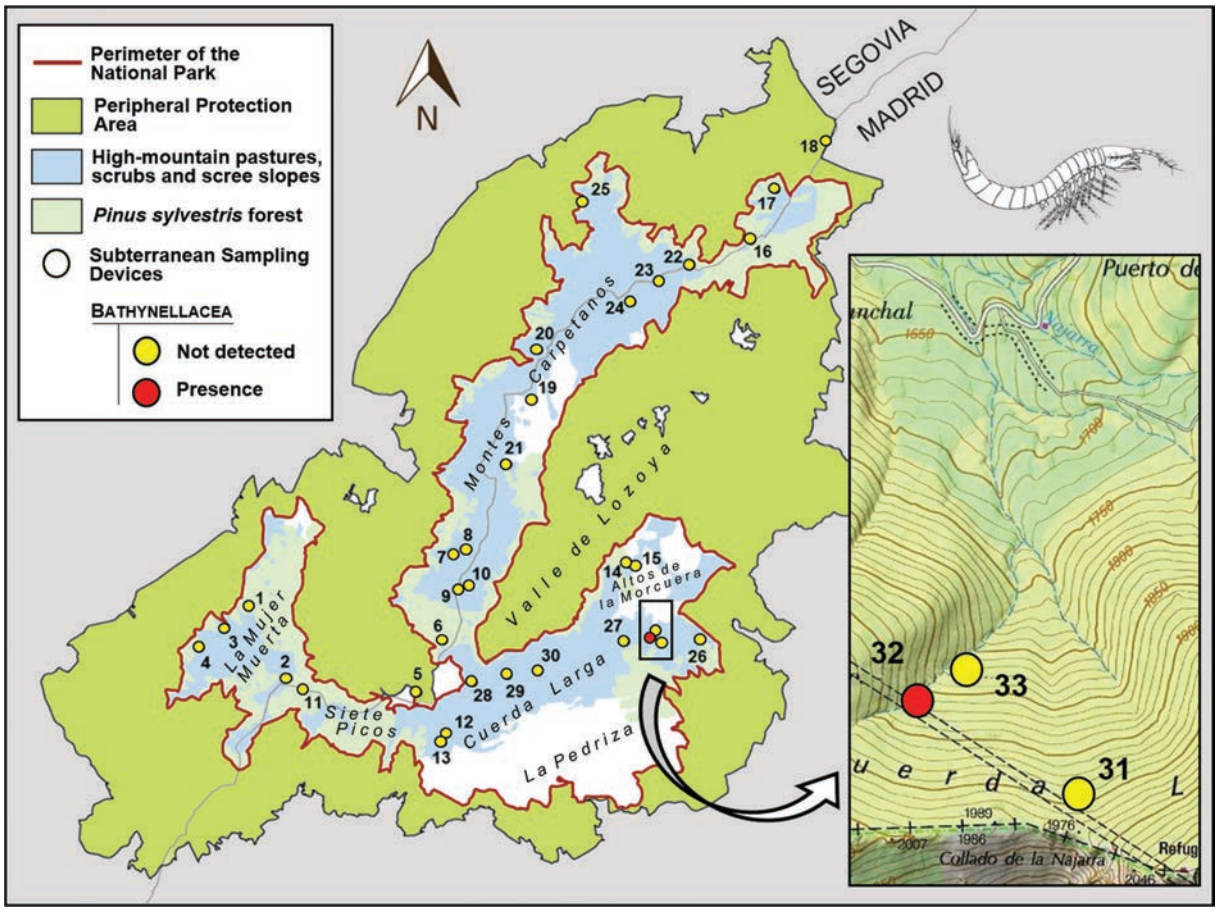

Fig. 1.— Distribution of subterranean sampling devices in the Sierra de Guadarrama National Park (Madrid, Spain) and (in red Bathynellacea specimens found).

Fig. 1.— Mapa del Parque Nacional de la Sierra de Guadarrama, Madrid, España y distribución de los dispositivos de muestreo del MSS (en rojo las trampas donde se han encontrado ejemplares de Bathynellacea).

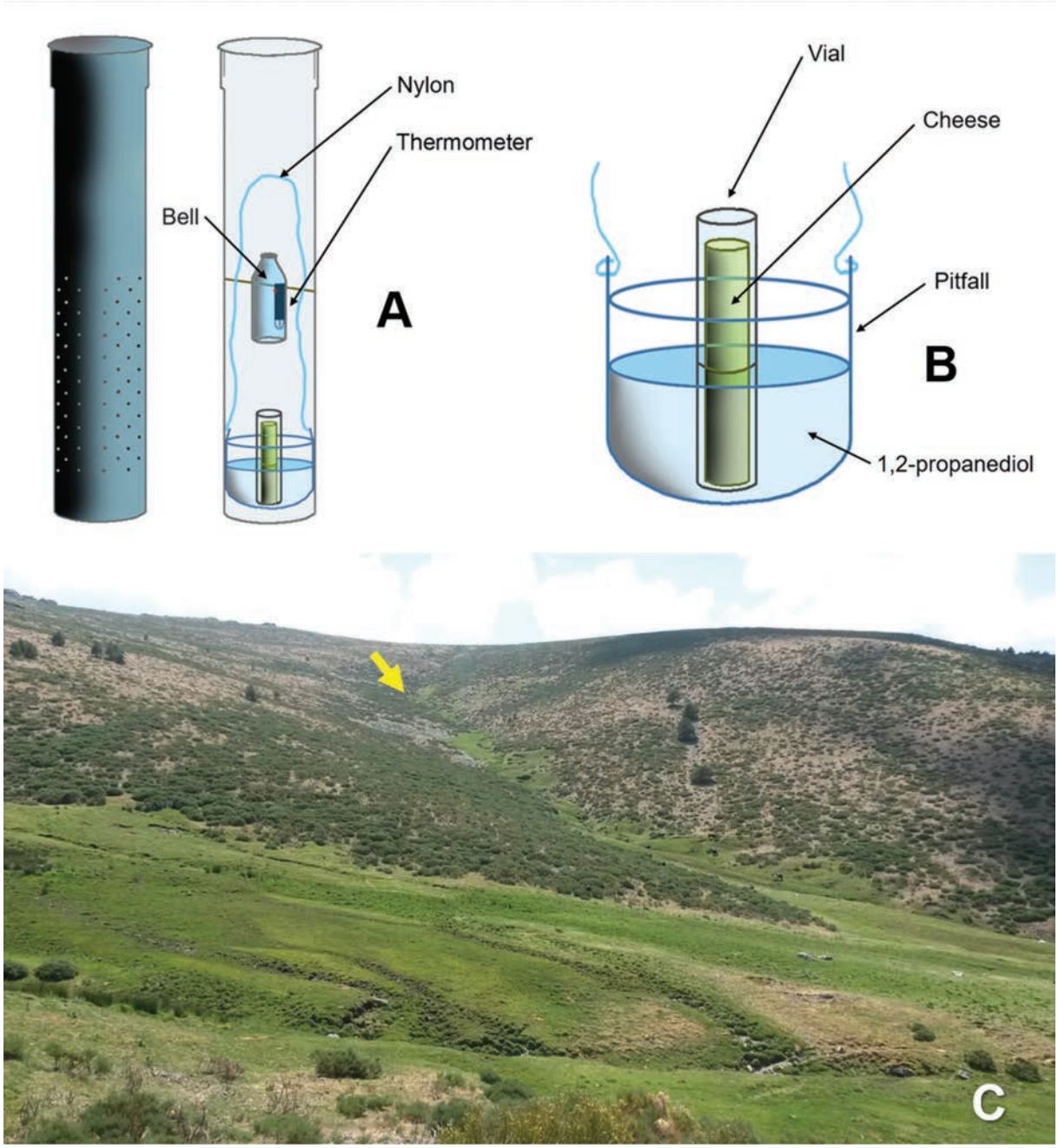

Fig. 2.— A) Sampling devices. B) Pitfall trap. C) Location of SSD-32 trap were the Bathynellacea specimens have been found.

Fig. 2.— A) Dispositivo de muestreo. B) Esquema de la trampa usada. C) Área donde han sido encontrados los especímenes de Bathynellacea en la trampa SSD-32.

Guadarrama National Park (Madrid, Spain) (Fig. 1). The study area is located in the Central System mountain range of the Iberian Peninsula, which peaks from 1200 to 2428 m above sea level (a.s.l). Orthogneiss of metamorphic quartz-feldspathic origin are the geological substratum predominant in the Park (Vialet *et al*., 1987), and appears as colluvial and moraine deposits whose subsoil has been sampled as described elsewhere (Baquero *et al*., 2017; Ledesma *et al.*, 2019; Ortuño *et al*., 2019). Thirty-three subterranean sampling devices (SSD) set in 33 scree slopes covered most of the Park area (Fig. 1). Each SSD consisted of a PVC tube 1 m long and 11 cm in diameter with perforations of 8 mm in diameter arranged along 40 cm from midway of the cylinder to its base. The cylinders were inserted vertically into a previously excavated hole. A pitfall trap baited with very smelly cheese and filled with 1,2-propanediol was deployed within each cylinder and the whole set covered as shown (see Fig. 2A-B). The sampling period, as a whole, covered from May 20, 2015, to October 14, 2016, but the SSD-32 (Fig. 2C) worked from July 9, 2015 to October 28, 2016 (Fig. 3). In this SSD, 12 specimens of the family Parabathynellidae were collected (7 ♂♂, 4 ♀♀ and

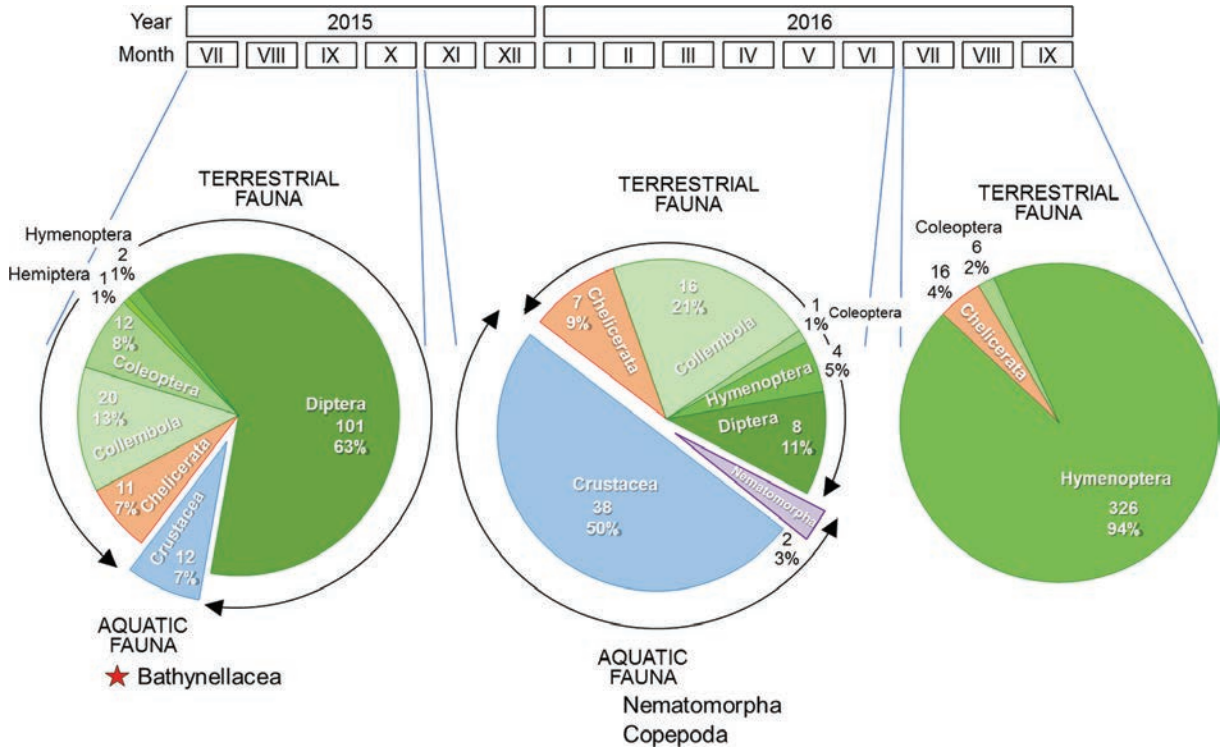

Fig. 3.— Outline of samples taken and % of fauna found at each period.

Fig. 3.— Esquema de muestreos realizados y % de fauna encontrados en cada periodo.

a juvenile specimen displaying only 4 pairs of thoracopods) (Fig. 4A) during the first sampling period (09/07/2015 to 22/10/2015) (Fig. 3). They were preserved in 90% ethanol to carry out morphological and molecular studies.

We selected three whole specimens and the abdomen of another five to extract DNA (see Table 1). We succeed to extract DNA from six animals, and 18S rRNA gene sequences from three of them. We failed to sequence the COI gene in all the extracts. DNA extraction and amplification methods appear described in Camacho *et al*. (2018). The extracted DNA was deposited in the Tissues and DNA Collection of the MNCN (voucher numbers of the specimens shown in Table 1).

For the morphological study, nine specimens were completely dissected (Table 1) and the appendages preserved as permanent slides (special metal slides, glycerine-gelatine stained with methylene blue and paraffin as mounting medium; see Perina & Camacho, 2016). The morphological examination was performed using an oil immersion lens (at 1000x magnification) with a Zeiss interference contrast microscope equipped with a drawing tube. Photographs were taken with a Leica camera (LEICA MC170 HD) attached to the microscope with 400x magnification and 1000x. The specimens prepared on permanent slides are deposited in the Collection of Arthropoda of the Museo Nacional de Ciencias Naturales-ARTP/MNCN-, Madrid, Spain (see voucher in Table 1).

We identified two species of the Parabathynellidae family in the collected material: *Hexaiberobathynella mateusi* (Galhano, 1967) (9 specimens; 5 ♂♂ and 4 ♀♀) and *Hexabathynella nicoleiana* Camacho, 1986 (3 specimens; 2 ♂♂ and 1 juvenile) (Fig. 4B; Table 1).

Morphologically both genera are very different, but the differences are only observed in the dissected specimens and not when they are observed under the stereo-microscope (Fig. 4A). The species belong to the only two genera characterized by the display of only six pairs of thoracopods. The rest of known genera (85 currently; Camacho, 2019) display seven pairs of thoracopods. *Hexaiberobathynella* Camacho & Serban, 1998 display a 7-segmented antennule (AI) and a 3-segmented antenna (AII) (Fig. 4B); while *Hexabathynella* Schminke, 1972 has a 6-segmented AI and a 5-segmented AII, in addition to many other relevant differences (Table 2).

The molecular results have confirmed the morphological identification of both genera. We have succeeded in sequencing the 18S gene in extracts corresponding to three specimens (see Table 1). Comparison with sequences we have in our database (*Hexabathynella sevillaensis* Camacho, 2005, *Hexaiberobathynella hortezuelensis* Camacho & Serban, 1998 and *Hi. mateusi*) has shown that the new sequences correspond, without any doubt, to these two genera.

The confirmation of the identification to species of the studied material was not possible based on gene sequences since we do not have sequences of specimens from the type localities of both taxa: Douro River mouth (for *Hi. mateusi*) and Jarama River (for *H. nicoleiana*; Table 3). Furthermore, we failed to get

Fig. 4.— A) 12 specimens of Bathynellacea (7 ♂♂, 4 ♀♀ and a juvenile specimen) found in SSD-32 trap; B) *Hexaiberobathynella mateusi*, ♂.

Fig. 4.— A) Los 12 ejemplares de Bathynellacea (7 ♂♂, 4 ♀♀ y un juvenil) encontrados en la trampa SSD-32; B) *Hexaiberobathynella mateusi*, ♂.

COI sequences of the two species studied. Since cryptic species are frequent among Bathynellacea (Camacho *et al*., 2011) we cannot discard that although we have morphologically identified the specimens as belonging to these two species, it could be that they are sister species. What is unquestionable is the generic ascription of both species.

Both species had previously been found in Madrid, in the Jarama River Basin in Torrelaguna, Patones, and Talamanca del Jarama (Camacho, 1986, 1987), and *Hi. mateusi* also at Cueva del Reguerillo (Patones) and in other sites of the Iberian Peninsula (Soria, Guadalajara, Teruel, Toledo, Granada and Jaén, plus in Portugal (Galhano, 1967; Camacho & Serban, 2000; Camacho *et al*., 2000, 2014, 2017; Guil & Camacho, 2001; Camacho, 2003). Both species were recently discovered to occur at different points of the interstitial medium of the Tajuña river in

Table 1.— Specimens studied, voucher number of MNCN Collections (AIC voucher, author collections) and result of 18S sequenced.

Tabla 1.— Especímenes estudiados con los números correspondientes de la colección del autor, AIC y de las Colecciones de Artrópodos y de Tejidos y ADN del Museo Nacional de Ciencias Naturales (MNCN) de Madrid (CSIC) y resultados de la secuenciación del gen 18S.

	Sex	Voucher AIC Slide-DNA	Voucher MNCN/ARTP	Voucher MNCN/DNA	Gen 18S
Hexabathynella nicoleiana	juvenile	------- 1040	—	54698 (whole)	yes
	♂	------- 1044	—	54702 (whole)	yes
	♂	2688-1045	20.04/20128	54703 (abdomen)	no
Hexaiberobathynella mateusi	♂	2686-1041	20.04/20129	54699 (abdomen)	yes
	♂	2686-1041	20.04/20129	54699 (abdomen)	yes
	♂	------- 1043	—	54701 (whole)	no
	♂	2689- -------	20.04/20131	—	no
	♂	2690- ------	20.04/20132	—	no
	♀	2691- -------	20.04/20133	—	no
	♀	2692- -------	20.04/20134	—	no
	♀	2702-1055	20.04/20135	54713 (abdomen)	no
	♀	2703-1056	20.04/20136	54713 (abdomen)	no

Table 2.— Differences and similarities between the two species studied: *Hexabathynella nicoleiana* Camacho, 1986 and *Hexaiberobathynella mateusi* (Galhano, 1967).

Tabla 2.— Semejanzas y diferencias entre las dos especies estudiadas: *Hexabathynella nicoleiana* Camacho, 1986 y *Hexaiberobathynella mateusi* (Galhano, 1967)

	H. nicoleiana	*Hi. mateusi*
Antennule: number of segments	6	7
Antennal organ	Present	Absent
Antenna: number of segments	5	3
Labrum: number of teeth	10	8
Md: teeth pars incisiva	4-5	4-6
teeth pars molaris	5	7-8
distal spine modified	Y	N
Mx.I: teeth distal endite	4	6
teeth proximal endite	3	4
Mx.II: setae segment 1	2	0
setae segment 2	4	4
setae segment 3	13	14
Male Th. VIII: shape	Elongated	Almost square
Exopod	Long	Small
Female Th. VIII: size	Medium	Small
Spines	0	1
Uropod: sympod	5+1 spines	5-7+1spines
setae exopod	3 barbed	4 barbed
setae endopod	1+1 plumose	2 barbed
Furcal rami	3 spines	5-8 spines
Pleotelson: setae	1	1
Anal operculum	Large	Medium size

Table 3.— Populations of *Hexabathynella* Schminke, 1972 and *Hexaiberobathynella* Camacho & Serban, 1998 in the Iberian Peninsula (updated data of Camacho & Serban, 2000; Camacho, 2003, 2006, 2019; Camacho *et al*., 2013a, b, 2014, 2017 and new samplings). * Type locality; **Species confirmation by DNA analysis. Number in parentheses as figure 5.

Tabla 3.— Poblaciones de las especies de los géneros *Hexabathynella* Schminke, 1972 y *Hexaiberobathynella* Camacho & Serban, 1998 encontrados en la Península Ibérica (datos actualizados de Camacho & Serban, 2000; Camacho, 2003, 2006, 2019; Camacho *et al*., 2013a, b, 2014, 2017 y de nuevos muestreos). * Localidad tipo de cada una de las especies; **Especie confirmada mediante análisis de ADN. Los números entre paréntesis corresponden a los del mapa de la figura 5.

Species	Habitat	Locality	Town	Province	Country
Hexabathynella					
H. minuta (1)	Interstitial	*Duero River	Zebreiros		Portugal
(2)	Interstitial	Rivera de Huelva Stream	Embalse de la Minilla	Sevilla	Spain
(3)	Interstitial	Pinhao Stream	Balsa		Portugal
H. nicoleiana (4)	Interstitial	*Jarama River	Torrelaguna	Madrid	Spain
(5)	Interstitial	Jarama River	Talamanca del Jarama	Madrid	Spain
(6)	Interstitial	Jarama River	Pontón de la Oliva	Madrid	Spain
(7)	Interstitial	**Tajuña Stream	Orusco	Madrid	Spain
(8)	**MSS**	****Ptº Morcuera**	**Sierra de Guadarrama**	**Madrid**	**Spain**
H. sevillaensis (9)	Cave	* **Santiago el Grande	Constantina	Sevilla	Spain
H. valdecasasi (10)	Interstitial	Torcón Stream	San Martín de Montalbán	Toledo	Spain
Hexabathynella sp (11)	Interstitial	Astillas Stream	Gredos	Ávila	Spain
Hexaiberobathynella					
Hi. mateusi (12)	Interstitial	*Duero River	Near Porto		Portugal
(13)	Interstitial	Mondego River	Coimbra		Portugal
(14)	Interstitial	Cavado River	Barcelos		Portugal
(15)	**MSS**	****Ptº Morcuera**	**Sierra de Guadarrama**	**Madrid**	**Spain**
(16)	Cave	Reguerillo	Patones	Madrid	Spain
(17)	Interstitial	Picnic Area	Talamanca del Jarama	Madrid	Spain
(18)	Interstitial	Bridge	Torrelaguna	Madrid	Spain
(19)	Interstitial	Pusa Stream	Santa Ana de Pusa	Toledo	Spain
(20)	Interstitial	Valdehornos Stream	Montes de Toledo	Toledo	Spain
(21)	Interstitial	Tajo River	Peñalen	Guadalajara	Spain
(22)	Interstitial	Tajo River	Zaorejas	Guadalajara	Spain
(23)	Interstitial	Hoz Seca Stream	Peralejo de las Truchas	Guadalajara	Spain
(24)	Interstitial	Sorbe Stream	----------	Guadalajara	Spain
(25)	Interstitial	Ucero Stream	Ucero	Soria	Spain
(26)	Well	Berlanga de Duero	----------	Soria	Spain
(27)	Well	FFC station	Hortezuela	Soria	Spain
(28)	Interstitial	Santos Stream	Sierra de Javalón	Teruel	Spain
(29)	Interstitial	Cinca Stream	Desfiladero de la Estada	Huesca	Spain
(30)	Interstitial	Mijares Stream	Montanejos	Castellón	Spain
(31)	Interstitial	Fardes Stream	Lanteira	Granada	Spain
(32)	Spring	El Baillo	Quesada	Jaén	Spain
(33)	Well	La Isla	Arganda del Rey	Madrid	Spain
(34)	Interstitial	Tajuña Stream	Luzón	Guadalajara	Spain
(35)	Interstitial	Tajuña Stream	Abanades	Guadalajara	Spain
(36)	Interstitial	Tajuña Stream	Loranca	Guadalajara	Spain
Hi. hortezuelensis (37)	Well	*FFC station	Hortezuela	Soria	Spain

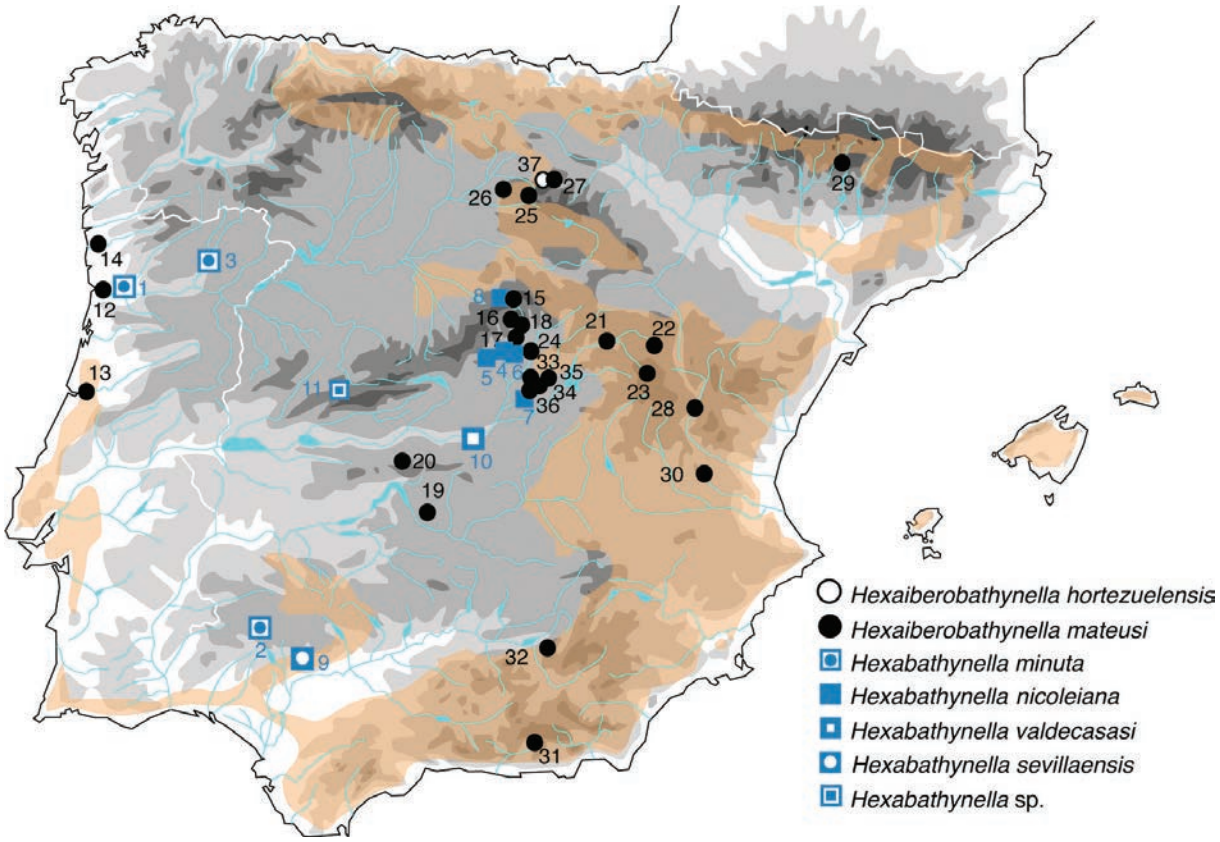

Fig. 5.— Distribution of species of *Hexabathynella* Schminke, 1972 and *Hexaiberobathynella* Camacho & Serban, 1998 genera in the Iberian Peninsula.

Fig. 5.— Distribución de especies de los géneros *Hexabathynella* Schminke, 1972 y *Hexaiberobathynella* Camacho & Serban, 1998 en la Península Ibérica.

the provinces of Madrid and Guadalajara (Camacho, 2019) (Table 3, Fig. 5). It is remarkable that in many occasions both species appear together. The type locality of *Hi. mateusi* is the hyporheic of the Duero River, 9km from the sea, near Oporto. However *H. nicoleiana* has not been found outside the provinces of Madrid and Guadalajara. *Hexaiberobathynella* is an endemic genus of the Iberian Peninsula that comprises only two species: *Hi. mateusi* and *Hi. hortezuelensis* (Soria). *Hexabathynella*, on the contrary, is the only cosmopolitan genus of Parabathynellidae and includes 23 species (Camacho, 2019). In the Iberian Peninsula, in addition to *H. nicoleina*, three more species are known to occur: *H. minuta* (Noodt & Galhano, 1969), found in several localities of Spain and Portugal (Table 3); *H. valdecasasi* Camacho, 2004 and *H. sevillaensis* Camacho, 2005, which are only known from their respective type localities, Arroyo el Torcón (Toledo) and Cueva de Santiago el Grande (Sevilla) respectively (Camacho, 2019) (Fig. 5).

It is interesting to remark the mutability of some MSS habitats, that can appear as a terrestrial underground environment and, temporarily, also as an aquatic underground environment. Thus, while the flooding process occurs, the MSS may also contain species which are typically aquatic. Therefore, similarly as to in the epikarst, the same spaces can, at different times, serve as a habitat for terrestrial and aquatic fauna, both epigean and hypogean (Ortuño *et al*., 2013). As seen in Figure 3, the SSD-32 collected aquatic fauna in two of the three sampling periods. In the first period, the aquatic fauna was limited to Bathynellacea (7% of the collection), and in the second period to Copepoda and Nematomorpha (50% and 3% of the collection, respectively). During the third period, the MSS was not flooded, and only terrestrial fauna was collected.

This discovery broadens the sampling horizon for this peculiar group of aquatic crustaceans. It will be necessary to consider looking for Bathynellacea in areas where there are shallow aquifers, more or less confined, and not as deep as those that have yielded such a large number of species as in the mining arid and remote areas of Australia (Perina *et al*., 2018, 2019).

Acknowledgements

This work was funded by the project "Study of the diversity and distribution of the animal species of the Mesovoid Shallow Substratum in enclaves of high Mountain (Sierra de Guadarrama National Park)", conceded by the Autonomous Organism of National

Parks of Spain. Ref. (1143/2014). We would like to thank the staff at the National Park who kindly helped us with the permission applications and other formalities, and to those who also helped us with the fieldwork, especially Patricia Riquelme, Pablo Sanjuanbenito, Juan A. Vielva, Javier Donés, Marisol Redondo, Ignacio Granados, Ángel Rubio, César Martín, José Carrillo, Miguel Ángel Palomar, Ángel Velasco, Germán Mato, Manuel Criado, Enrique Calvo, Federico Madejón, Montserrat Sanz, and forestry agents of Buitrago de Lozoya. Thanks also to our colleagues who collaborated in the design of the samplings and the fieldwork, such as Alberto Jiménez Valverde, Gonzalo Pérez Suárez, Enrique Baquero, Alberto Sendra, Pablo Barranco, Alberto Tinaut, Rafael Jordana, Luis Subías, Juan José Herrero–Borgoñón, Enrique Ledesma, José D. Gilgado, Douglas Zeleppelini and Javier Ledesma. We gratefully acknowledge C. Puch, I. Rey and A. Casado who helped us in different ways and many thanks to Damian Jaume who reviewed the English. This work has also been supported by CGL2015-66571-P, MINECO/FEDER projects.

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