

## The variation of stygofauna along a gradient of salinization in a coastal aquifer

M. Shapouri, L. Cancela da Fonseca, S. Iepure, T. Stigter, L. Ribeiro and A. Silva

### ABSTRACT

We examined the variation of stygofauna composition collected in wells, along a gradient in groundwater salinity/conductivity in a coastal aquifer from southern Portugal. The studied coastal aquifer is considered vulnerable to salinization due to seawater intrusion, caused by overexploitation of the aquifer. Knowing the response of the stygofauna species to present levels of groundwater salinity makes it possible to understand and measure the effects of saltwater intrusion on stygofauna, rendering them potential bioindicators of this environmental pressure. Biotic and abiotic sampling was conducted in six shallow wells located in the fresh–saltwater interface of the Arade estuary along the salinity gradient established in the groundwater from the estuary to inland locations (microSiemens/cm). Groundwater salinity was highly correlated with taxa distribution. The stygobites *Eucyclops hadjebensis* and *Acanthocyclops sensitivus*, the cosmopolitan *Megacyclops viridis* and *Cypria ophthalmica* were particularly associated with low-salinity conditions, whereas *Eucyclops graeteri*, *Megacyclops brachypus* and *Daphnia pulex* were related to high-salinity waters. Multivariate analyses indicated that stygofauna assemblages varied significantly with location. We suggest that stygofauna composition and structure can be a useful complementing tool for monitoring seawater intrusion into coastal aquifers, where reduction or deterioration in groundwater resources is predicted.

**Key words** | biomonitoring, coastal aquifers, overexploitation, salinization risk, stygofauna

**M. Shapouri** (corresponding author)

**T. Stigter**

**L. Ribeiro**

**A. Silva**

CERIS – Civil Engineering Research and Innovation for Sustainability, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal  
E-mail: [maryamshapouri@gmail.com](mailto:maryamshapouri@gmail.com)

**L. Cancela da Fonseca**

CTA – Centro de Ciências e Tecnologias da Água, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal  
and  
LMG/CO – Laboratório Marítimo da Guia/Centro de Oceanografia, Faculdade de Ciências, Universidade de Lisboa (FCUL), Avenida Nossa Sra. do Cabo, 939, 2750-374 Cascais, Portugal

**S. Iepure**

IMDEA-Water (Madrid Institute for Advanced Studies), Calle Punto Net 1, Edificio ZYE 2, Parque Científico Tecnológico de la Universidad de Alcalá, 28805 Alcalá de Henares, Madrid, Spain  
and  
'Emil Racoviță' Institute of Speleology, Romanian Academy, Clinicilor 5, 400006 Cluj Napoca, Romania

**T. Stigter**

Water Science and Engineering Department, Hydrology and Water Resources Chair Group, UNESCO-IHE, Westvest 7, 2611 AX Delft, The Netherlands

### INTRODUCTION

The change in precipitation and temperature regimes induced by climate change is predicted to be especially relevant in Mediterranean countries, translated into significantly decreased surface flows and aquifer recharge and discharge (Santos *et al.* 2002).

Coastal aquifers are more sensitive to disturbance compared to other aquifer types (Bear *et al.* 1999). Human

activities also induce alterations on groundwater (GW) resources in coastal aquifers through prolonged withdrawals, leading to alteration of aquifer habitats and the associated groundwater dependent ecosystems (GDEs) (Silva *et al.* 2012). In fact, the combined effect of prolonged and large extractions from the aquifer and reduced recharge in coastal areas can intensify seawater intrusion, which is

one of the most widespread phenomena of GW contamination in these areas (Kim *et al.* 2003; Somay & Gemici 2009; Wang & Jiao 2012). GW habitats display a high biodiversity, especially crustaceans (Gibert & Culver 2009; Deharveng *et al.* 2009). Hypogean faunal studies in Portugal have an old and rich history compiling more than 130 publications related to subterranean invertebrates (Reboleira *et al.* 2013). Based on the bibliographic analysis and literature survey by Reboleira *et al.* (2013), 67 stygobiont species and subspecies from 12 orders were introduced for this area. However, pioneer stygofauna data on composition and distribution have been collected mainly in caves (Reboleira *et al.* 2011, 2013) and very little information exists on aquifer stygofauna. The study of GW amphipods by Notenboom also reviewed a previous publication that pointed out the occurrence of GW amphipods in the Iberian area including the Portuguese ecosystem (Notenboom 1990).

The qualitative status of GW is generally based on its physical–chemical monitoring, which covers most of the contaminants relevant for human health (WFD 2000). However, to identify environmental impacts, it is required to broaden the monitoring to stygofauna and its associated ecosystems conditions. The use of stygofauna as biological and ecological indicators has been emphasized by many authors (Danielopol *et al.* 2004, 2006, 2007; Boulton *et al.* 2008; Thulin *et al.* 2008; Griebler *et al.* 2010; Stein *et al.* 2010; Steube *et al.* 2010; Korbel & Hose 2011) and environmental agencies to estimate GW ecosystem disturbance (Marnier *et al.* 2013). Stygofauna integrates impacts over space and time, and hence add more information than single use of abiotic criteria (Stein *et al.* 2010). Stygofauna are known to be sensitive to environmental changes in water conditions, particularly due to the enclosed nature of GW habitats (Notenboom *et al.* 1995). A precondition toward an integrative ecosystem assessment is to use a joint set of abiotic and biotic criteria enabling the detection of all physical, chemical and biological stressors and distinguish the cause of impact.

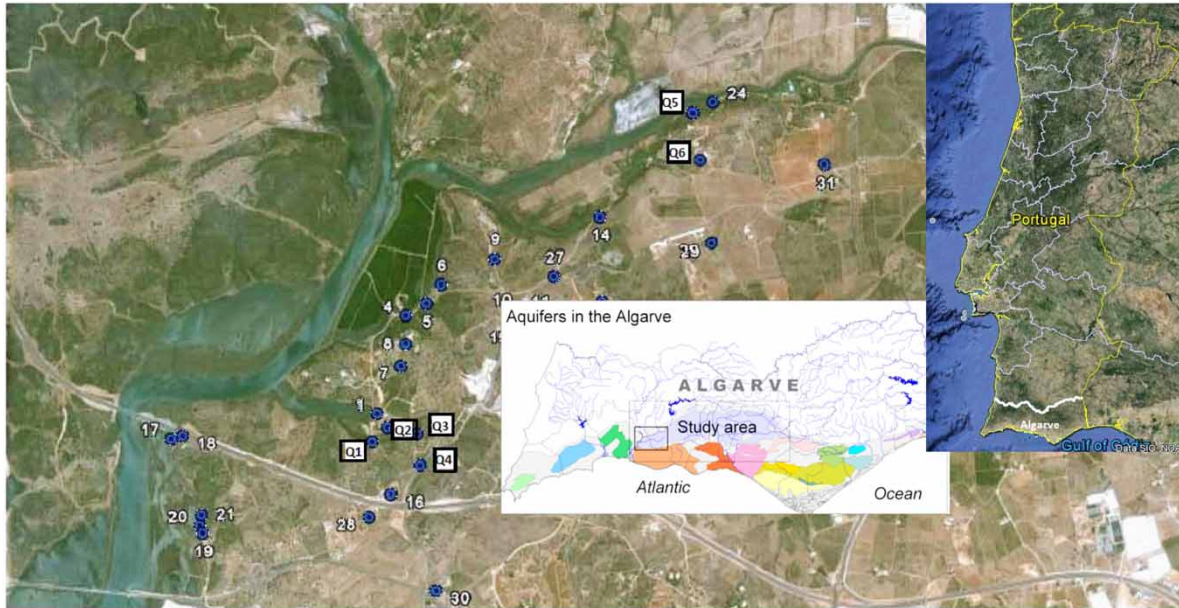
GW invertebrates, including micro- and macro-organisms, have broadly been used as bioindicators to explore different types of environmental disturbance such as heavy metal contamination (Canivet *et al.* 2001), surface water infiltration into aquifers (Malard *et al.* 1996; Dumas *et al.* 2001; Hahn 2006; Schmidt *et al.* 2007; Bork *et al.*

2009), organic matter pollution (Notenboom *et al.* 1995; Scarsbrook & Fenwick 2003) and oxygen and redox conditions (Mösslacher 1998, 2000). The present study aims to be one of the first examining the response of stygofauna to salinity shifts in coastal aquifers related to saline intrusion.

Stygofauna communities in coastal aquifers are highly vulnerable to abrupt changes in their environment due to their special nature: narrow distribution, high endemism, resource specific (e.g. food and O<sup>2</sup> levels), poor dispersal capacities and less competitive than more tolerant epigean species ('surface waterspecies') (Stock 1994; Culver & Sket 2000). The variation of stygofauna groups, mainly abundance, has been shown to respond to environmental changes such as excessive pumping and habitat modification (Rouch 1977; Longley 1992). In the Kolbental Valley, Germany, stygofauna on a surface GDE revealed to be much more sensitive indicators to the effects of GW pumping than hydrochemistry parameters (Hahn 2007).

The Querença-Silves aquifer is located in south-west Portugal (Figure 1). This aquifer is the most productive and important GW reservoir in South Portugal due to its large area and significant recharge. Previous studies ruled with different climate scenarios were used to predict the future climate change impact on this aquifer net recharge and, consequently, the output to GDEs and water quality conditions in different wells (Stigter *et al.* 2013). The used models predicted a significant increase in the mean temperature for future years and, consequently, an expected increase in GW demand for crops. At the same time, and despite the inherent uncertainty, an overall decreasing trend in aquifer recharge towards the end of the century is predicted by the models. The combined effect of decreasing recharge and increasing extraction is foreseen to lead to an overall decreasing trend in GW levels, which is likely to cause recurrent saline intrusion into the aquifer, threatening its ecosystem stability (Stigter *et al.* 2013).

The aims of the present study were two-fold: (i) to examine the variation in stygofauna composition with differing levels in water salinity/conductivity; and (ii) to evaluate their potential use as salinity biomonitoring tools in coastal aquifers. Specifically, reliable assessments of GW biodiversity response to stressors are considerably useful to help resolve current and future issues relating to coastal aquifer sustainable management. The present study also adds



**Figure 1** | Location of the inventoried wells. Six wells were selected for sampling; wells Q1–6.

novel data on Portuguese stygofauna which remains largely unknown.

## STUDY AREA

The study area is located in the discharge zone of the Querença-Silves aquifer on the coastal fringe of the central Algarve, in southern Portugal (Figure 1). It covers an irregularly E-W elongated area of 324 km<sup>2</sup>. The aquifer is mainly used for irrigation, particularly of citrus orchards (31 × 10<sup>6</sup> m<sup>3</sup>/year) and for public water supply (around 10 × 10<sup>6</sup> m<sup>3</sup>/year). In extremely dry years as in 2005, the total abstractions of the aquifer reached 60 × 10<sup>6</sup> m<sup>3</sup>/year, representing more than half of the annual aquifer recharge rate (Stigter *et al.* 2013). This region is characterized by a warm Mediterranean climate with dry and warm summers and cool and wet winters. The mean annual temperature and precipitation for the 1980–2010 period were 17.5 °C and 739 mm, respectively (Stigter *et al.* 2009).

### Hydrogeology, land use and future climate scenarios

The Querença-Silves aquifer was studied and it is the largest in the Algarve region. The aquifer is built up of carbonate

sedimentary rock, has a total extent of 324 km<sup>2</sup> and a mean annual recharge of 100 × 10<sup>6</sup> m<sup>3</sup> (Monteiro *et al.* 2007; Stigter *et al.* 2009).

Surface stream flow in this region is restricted to a small number of temporary rivers except over the large karstified carbonate rock aquifer known as Querença-Silves, where it is significantly dependent on base flow in effluent reaches (Stigter *et al.* 2013). Estômbar springs are among the most important GW discharge from the aquifer into the Arade estuary, with approximately 25% of total outflow, though this percentage varies between seasons and years. This surface/GW interface zone is ecologically important and some ecotones are classified as protected area. The results of future climate modeling scenarios for rainfall (*P*) and temperature (*T*) suggest a significant increase for *T* and strong decrease for *P* in the studied zone. These changes in climate will likely have an influence on total and net recharge, both by the higher potential evapotranspiration due to higher *T* (which also leads to higher irrigation needs) and by lower precipitation (Stigter *et al.* 2013).

Land use at the study zone is mainly defined by irrigated citrus culture in the western sector overlying the aquifer, and in the eastern sector, it is dominated by extensive dry farming (olive, carob, almond and fig trees) (Stigter *et al.* 2013).

GW is the main water source for the required large amounts of irrigation. The crucial role of this aquifer system for the support of Algarve's water supply in urban areas was clearly revealed during the severe drought that affected Portugal in 2004 and 2005, causing the depletion of the surface water reservoirs and making the aquifer the only available water source. Currently, 30% of the mean annual recharge of aquifer is exploited for irrigation needs (Stigter *et al.* 2009). The combined effect of the decrease in recharge and increase in irrigation demand will stimulate GW exploitation and pumping rates, leading to likely declines in GW levels and consequently increased impact of saline intrusion also related to sea level rise.

## METHODS

### Study design

The stygofauna survey was made through sampling in shallow (water depth) and wide wells in December 2010. An *a priori* inventory of the shallow wells was made in order to select wells with distinct degrees of water salinity to obtain a gradient. The presented study considered the well salinity and location as factors, with location nested within salinity. Wells with higher salinities were located near the estuary and wells with lower salinities were located inland (Figure 1).

In total, six shallow wells were sampled for water physico-chemical parameters and stygofauna community composition, categorized as follows:

- (i) Wells with high salinity corresponding to electrical conductivity (EC)  $>6,000 \mu\text{S}/\text{cm}$ . These included wells number Q1, Q2 and Q3 located very near the Estômbar channel of the Arade estuary, as well as number Q5 located next to the Arade river where it still receives saline water due to the tidal influence (Figure 1).
- (ii) Wells of low salinity with EC  $<1400 \mu\text{S}/\text{cm}$ , including well numbers Q4 and Q6 (Figure 1), several hundred meters further away from the Arade estuary/river.

We considered our salinity categorization suitable as it incorporates the amplitude of the 31 inventoried wells, also giving a wide value gap between the categories, maximizing the potential to identify community responses. This

gap limited the number of potential well replicates for each category.

Wells Q1 (3 m depth) and Q2 (4 m depth) are located in the *Parque Municipal das Fontes de Estômbar* (the Municipal Park of the Springs of Estômbar, important discharge points of the Querença-Silves aquifer). Well Q3 (4 m depth) is a private well for domestic use located a few meters away from the park. It is a shallow well with around 4 m depth. Well Q4 is a large diameter well with more than 10 m depth. Wells Q5 and Q6 are also abandoned wells with 2 m and more than 30 m depth, respectively.

From the total of six wells, at least two wells per salinity category were sampled once, stygofauna and water. Three replicates per well were considered for fauna assessment. No temporal patterns were examined.

### Water and faunal sampling

Well-dissolved oxygen (DO) ( $\text{mg L}^{-1} \text{O}_2$ ), pH and depth (m) were measured directly in the field with portable meters at each sampling occasion. Temperature ( $^{\circ}\text{C}$ ), hydraulic head (m) and EC ( $\mu\text{S}/\text{cm}$ ) were measured with fixed data loggers in a long-term (2009–2010) survey for wells Q1, Q2, Q4 and Q5. The probes recorded the EC values every 6 minutes. The average value of the entire measurement was considered for mentioned wells. Wells Q3 and Q6 did not have probe surveys, but the average value of five *in situ* temperature and EC measurements were calculated. All the measured parameters are key in GW characterization and therefore, were deemed relevant to relate to stygofauna distribution.

A phreatobiological net of  $40 \mu\text{m}$  was used to sample the stygofauna associated with the well sediment and water column. This net is a standard stygofauna sampling method with semi-quantitative data (Cvetkov 1968; Gibert 2001; Malard *et al.* 2002). The net consists of a mesh cone mounted on a metallic circular frame of 40 cm diameter and length of 75 cm with a valve at the base, which allows the fauna to be captured when moving the net successively upward/downward and creating an ascending current in the well. The lower end of the net encompasses weights that disturb the sediment and shift this representative of adjacent aquifer (Hahn & Matzke 2005).

All six wells (Q1–Q6) were sampled with a phreatobio-logical net and three repetitions of sampling experiments were taken per well to estimate the variation of stygofauna conditions in each well. After each replicate collection, the net collecting glass was washed into a 50 µm mesh sieve. The sampled material was stored into sealed containers, fixed in 97% ethanol and stained with rose Bengal to simplify the sorting of colored organisms at a later stage.

There are several ecological classifications to categorize the stygofauna based on their morpho-physiological adaptation (Sket 2008). In the present work, stygofauna were classified as: (i) stygoxenes – surface water dwelling organisms that occurred in GW systems accidentally and have no affinities with GW; (ii) stygophiles – contain a number of species that live in the GW environment for part of their life cycle; and (iii) stygobites – exclusively subterranean dwelling organisms that are specialized subterranean forms that complete their whole life cycle exclusively in subsurface water (Gibert 2008). Most of the crustaceans collected in this survey were identified to the species or genus level where Cyclopoids, Ostracods, Branchiopoda and Amphipods were identified to species and genus level; while Isopods and Tanaidacea were only identified to the family level; gastropods (Mollusca) were identified mostly to species level. Other groups were identified to higher classification levels such as family or order.

### Data analysis

Multifactorial analyses were performed in the PRIMER+ package (Clarke & Warwick 2007; Anderson et al. 2008). The PERMANOVA test was used to evaluate whether there were significant differences in fauna assemblage between the examined factors. Two factors, location (Q1–Q6) and salinity (high and low salinity) were considered for this study. The similarity percentage breakdown procedure (SIMPER) was used to determine the contribution of individual taxa to the dissimilarity between and within the factors. Principal coordinates analysis (PCO) was used to graphically examine the species distribution and positioning specifically according to factor salinity. For all tests including biotic data, the Bray–Curtis similarity measure and 9999 permutations were used. The fourth-root transformation was applied *a priori* to balance

the abundance of rare species with the dominant ones. A set of physical variables including salinity measurement was analyzed in comparison to the abundance and diversity of fauna to explain the significant abiotic variable for faunal spatial similarities and differences. The BEST tool was used to calculate the most parsimonious model explaining the species distribution, in relation to the measured abiotic variables. The model with the highest Spearman correlation coefficient corresponds to the environmental variable set that better explained the variation of the biotic data.

## RESULTS

### Abiotic characteristics and faunal general characterization

Water physical characteristics are provided in Table 1. Although well Q6 was considerably deeper than the others, the water parameters were very similar between all, with the exception of EC, which reflected the salinity gradient aimed in our study.

A total of 896 organisms belonging to 51 taxa were sampled. The faunal assemblages were dominated (73%) by stygophiles/stygoxenes whereas the stygobites represented about 27% of the total number of the identified taxa. The highest taxa diversity and density was recorded in Q4, associated with low-salinity (freshwater) conditions, with stygofauna formed by a mixture of dominating stygoxene species and one stygobite (*A. sensitivus*). Conversely, the lowest diversity and density occurred in Q2 associated

**Table 1** | Abiotic characteristics (average values) and depths of wells of the Querença-Silves aquifer (southern Portugal) (Q1–6 refer to wells illustrated in Figure 1)

Well code	pH	Depth (m)	EC (µm/cm)	Dis. oxi. (mg/L)	Temp. (°C)	Hyd. head (m)
Q1	7.44	3	8,162	4.4	19.63	1.3
Q2	7.37	4	9,113	3.72	16.2	0.95
Q3	7.49	3.65	6,100	0.88	15.71	n.a.
Q4	7.03	>10	1,390	3.61	17.6	5.6
Q5	7.75	2	12,987	1.5	15.3	1.7
Q6	7.58	>30	848	6.15	15.43	n.a.

(n.a. = not available).

EC = electrical conductivity; Dis. oxi. = dissolved oxygen; Hyd. head = hydraulic head.

with high-salinity GW, where only stygoxene taxa were present.

Crustaceans dominated the stygofauna, accounting for 73% of the total of nine groups, followed in decreasing order by Mollusca (19%), Arachnida (2%), Annelida (2%), Insecta (1%), Platyhelminthes (1%), Hexapoda (1%), Cnidaria (0.01%) and Nematoda (0.01%).

Crustaceans were taxonomically distributed across seven orders, six families, nine genera and 13 species. The major represented families were Harpacticoida, Cyclopoida, Podocopida, Bathynellacea, Cladocera, Amphipoda and Isopoda.

Cyclopoida were the dominant crustacean taxa with 56%, followed by Ostracoda (36%), Cladocera (4%),

Amphipoda (3%), Harpacticoida (0.6%), Bathynellacea (0.2%) and Isopoda (0.1%). Four dominant crustacean species accounted for 69% of the total abundance, with higher abundance found for the cyclopoid *Eucyclops graeteri* (25%) and the ostracod *C. ophthalmica* (22%). All identified taxa to species and genus level were assigned to five ecological groups namely, brackish water (0.5–30‰), freshwater (<0.5‰), stygobite, stygophile and stygoxene (Table 2).

### Pattern of species distribution

The species distribution was statistically explained by the factor location reflecting the nested salinity gradient

**Table 2** | Identified taxa to species and genus level and their habitat type

Name	Ecology	Q1	Q2	Q3	Q4	Q5	Q6
<i>Eucyclops graeteri</i> (Chappuis 1927)	Hy	17				206	1
<i>Eucyclops hadjebensis</i> (Kiefer 1926)	Hy				11		
<i>Megacyclops brachypus</i> (Kiefer 1955)	Hy					2	
<i>Acanthocyclops sensitivus</i> (Graeter & Chappuis 1914)	Hy				1		
<i>Eucyclops serrulatus serrulatus</i> (Fischer 1858)	Epi (stx, stf)				2		2
<i>Eucyclops speratus</i> (Lilljeborg 1901)	Epi (stx, stf)				24		
<i>Megacyclops viridis</i> (Jurine 1820)	Epi (stx, stf)				2		
<i>Macrocyclus albidus</i> (Jurine 1820)	Epi (stx, stf)	10			8		53
<i>Acanthocyclops</i> sp.	Epi (stx, stf)					1	
<i>Cypria ophthalmica</i> (Jurine 1820)	Epi (stx)	2		30	171		
<i>Cypridopsis vidua</i> (Müller 1776)	Epi (stx)			1			1
<i>Bradleycypris oblique</i> (Brady 1868)	Epi (stx)					6	
<i>Myxas glutinosa</i> (Müller 1774)	F			1			1
<i>Potamopyrgus antipodarum</i> (Gray 1843)	F				5		
<i>Bithynia</i> sp.	F	2			28		
<i>Ancylus fluviatilis</i> (Müller 1774)	F				2		
<i>Theodoxus fluviatilis</i> (Linnaeus 1758)	F			1			
<i>Valvata</i> sp.	F				1		
<i>Physa acuta</i> (Draparnaud 1805)	F			2			
<i>Daphnia pulex</i> (Forbes 1893)	F					25	
<i>Gammarus lacustris</i> cf. (Sars 1863)	F	1					
<i>Gammarus</i> sp.	F		9				
<i>Chaetogammarus</i> sp.	F		3				
<i>Halicyclops</i> sp.	B	1					

B = brackish water; F = surface freshwater; Hy = hypogean (stygobite); Epi (stf) = epigean stygophile; Epi (stx) = epigean stygoxene.

**Table 3** | PERMANOVA analysis results of the wells fauna for factors salinity (high and low) and different well locations. The number of permutations used was 9999

Source	df	SS	MS	Pseudo-F	p (perm)	perms	p (MC)
Sa	1	5,180	5,180	0.78	0.67	15	0.63
Lo(Sa)	4	26,545	6,636.3	23.365	0.0001	9,882	0.002
Res	12	34,083	2,840.3				
Total	17	65,808					

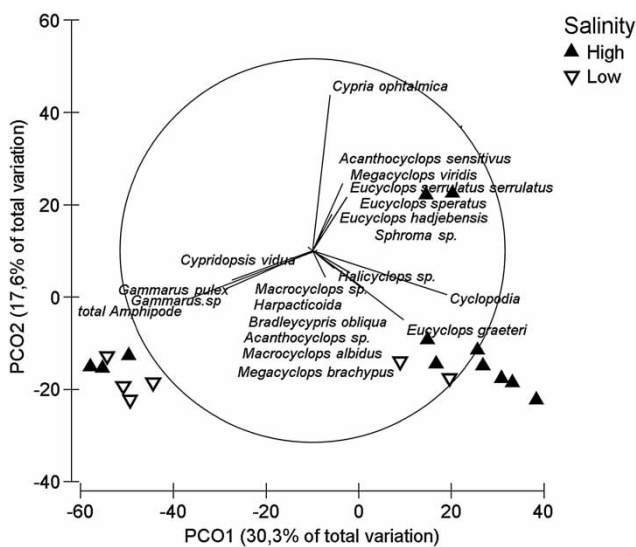
Sa = salinity; Lo = location.

(Table 3). We did not include pairwise tests for factor location in this study since location was designed as a random factor nested within high and low levels, therefore rendering it unimportant to prove differences between wells (i.e. locations) that 'belonged' or that were considered equal within each level. The 'location'  $p$ -value (Table 3) already proves that there are differences at the smallest sample scale – well within salinity level, but our level criteria related to the hypothesis was salinity concentrations, a much more interesting question from our point of view.

The PCO results displayed in Figure 2 were generated with all species and the taxonomic group of taxa which were not identified to species level. The PCO plot was ordinated according to factor salinity and it shows the fauna affinity to high- and low-salinity locations. The PCO ordination explained 48% of the taxa distribution, however there is no clear separation between high and low locations points

since the  $p$ -value for salinity is not zero. This means that there are some common species/abundances between high and low locations which justify the closeness of some points in the PCO.

The majority of the taxa with the exception of Amphipods and Gammarids were associated with high-salinity conditions. Three taxa clusters were identified accordingly to salinity conditions; high salinity (right bottom and right top sides of the plot) and low salinity (left side of the plot) (Figure 2). *Cyprina ophthalmica* distribution did not reflect salinity associations. The most heterogeneous assemblage was found in Q1, where a mixture of brackish water (*Halicyclops* sp.), obligate GW dwellers (*E. graeteri*) (Chappuis in 1927) and cosmopolitan epigeal species (*Macrocyclus albidus* (Jurine in 1820), *C. ophthalmica* (Jurine in 1820), *Bithinia* sp., *Gammarus lacustris* (Sars in 1863)) were present. Conversely, the most homogeneous assemblage was found in Q3, where only the cosmopolitan stygoxene species occurred (Table 2).

**Figure 2** | Principal coordinates analysis ordination for two groups of wells with high- and low-salinity conditions.

### SIMPER analysis I

The results of SIMPER analysis within location showed high dissimilarity between all wells (dissimilarity >60%, Table 4). The highest dissimilarity was observed between wells Q2 and Q5 with 100%, Q2 and Q3 with 96% and Q2 and Q4 with 96%.

Wells of low-salinity conditions (Q4 and Q6) presented a mixture of epigeal stygoxenes and three obligate GW dwellers (*E. graeteri*, *Megacyclops brachypus* and *A. sensitivus*). The dissimilarity between these two wells was related to the total number of amphipods and cyclopodia. Dissimilarity between wells Q1–3 and Q5, which correspond to high-salinity waters, short distance to the sea and lower water depth (<4 m), was high and this is probably due to presence of rare and endemic species at each location.

**Table 4** | SIMPER analysis results identifying the stygofauna taxa most significantly contributing to the differences between (I) six wells and (II) high- and low-salinity conditions

Taxon (I)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib.%	Cum.%
	Q1	Q2				
Cyclopodia	3.21	0.00	32.75	2.75	35.11	35.11
Total Amphipode	0.33	1.89	18.25	1.39	19.56	54.68
<i>Gammarus</i> sp.	0.33	1.28	11.18	0.93	11.98	66.66
<i>Macrocyclus albidus</i>	1.05	0.00	9.61	0.65	10.30	76.96
<i>Eucyclops graeteri</i>	1.37	0.00	8.54	0.66	9.16	86.12
Harpacticoida	0.47	0.00	4.30	0.65	4.61	90.73
	Q1	Q3				
Cyclopodia	3.21	1.94	33.35	1.72	39.31	39.31
<i>Cypria ophthalmica</i>	0.47	2.31	16.93	1.06	19.96	59.27
<i>Macrocyclus albidus</i>	1.05	0.00	9.51	0.62	11.21	70.48
<i>Eucyclops graeteri</i>	1.37	0.00	8.33	0.64	9.82	80.29
<i>Cypridopsis vidua</i>	0.00	0.33	5.51	0.53	6.50	86.79
Harpacticoida	0.47	0.00	4.25	0.62	5.01	91.80
	Q2	Q3				
Total Amphipode	1.89	0.33	28.88	1.46	30.19	30.19
<i>Cypria ophthalmica</i>	0.00	2.31	23.76	1.20	24.84	55.03
<i>Gammarus</i> sp.	1.28	0.00	16.15	0.99	16.88	71.91
Cyclopodia	0.00	1.94	12.68	0.66	13.26	85.17
<i>Cypridopsis vidua</i>	0.00	0.33	8.79	0.59	9.19	94.36
	Q1	Q4				
<i>Cypria ophthalmica</i>	0.47	6.25	29.48	1.07	38.23	38.23
Cyclopodia	3.21	3.15	10.76	1.71	13.96	52.20
<i>Eucyclops speratus</i>	0.00	1.63	6.36	0.65	8.24	60.44
<i>Eucyclops hadjebensis</i>	0.00	1.11	6.11	0.64	7.93	68.36
<i>Eucyclops graeteri</i>	1.37	0.00	5.19	0.66	6.73	75.10
<i>Macrocyclus albidus</i>	1.05	0.00	4.94	0.66	6.40	81.50
Harpacticoida	0.47	0.00	2.21	0.66	2.86	84.36
Total Amphipode	0.33	0.33	2.19	0.78	2.84	87.20
<i>Acanthocyclops sensitivus</i>	0.00	0.33	1.84	0.64	2.39	89.59
	Q2	Q4				
<i>Cypria ophthalmica</i>	0.00	6.25	37.66	1.20	39.44	39.44
Cyclopodia	0.00	3.15	16.81	1.31	17.60	57.04
Total Amphipode	1.89	0.33	8.61	2.03	9.01	66.05
<i>Eucyclops hadjebensis</i>	0.00	1.11	7.47	0.66	7.82	73.87
<i>Eucyclops speratus</i>	0.00	1.63	7.36	0.66	7.71	81.58
<i>Gammarus</i> sp.	1.28	0.00	6.87	1.04	7.19	88.77
<i>Acanthocyclops sensitivus</i>	0.00	0.33	2.25	0.66	2.36	91.13

(continued)



Table 4 | continued

Taxon (I)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib.%	Cum.%
	Q3	Q4				
<i>Cypria ophtalmica</i>	2.31	6.25	29.55	0.91	40.00	40.00
Cyclopodia	1.94	3.15	16.47	1.21	22.30	62.31
<i>Eucyclops hadjebensis</i>	0.00	1.11	7.29	0.64	9.87	72.18
<i>Eucyclops speratus</i>	0.00	1.63	7.16	0.65	9.69	81.87
Total Amphipode	0.33	0.33	2.55	0.76	3.45	85.32
<i>Cypridopsis vidua</i>	0.33	0.00	2.31	0.65	3.13	88.45
<i>Acanthocyclops sensitivus</i>	0.00	0.33	2.20	0.64	2.98	91.43
	Q1	Q5				
<i>Eucyclops graeteri</i>	1.37	7.85	27.04	1.95	43.26	43.26
Cyclopodia	3.21	7.90	18.20	1.77	29.11	72.37
<i>Macrocyclus albidus</i>	1.05	0.00	4.58	0.62	7.32	79.69
<i>Bradleycypris obliqua</i>	0.00	0.82	2.65	0.66	4.24	83.93
Harpacticoida	0.47	0.00	2.05	0.62	3.27	87.21
<i>Megacyclops brachypus</i>	0.00	0.47	1.75	0.65	2.80	90.00
	Q2	Q5				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib.%	Cum.%
Cyclopodia	0.00	7.90	37.81	8.62	37.81	37.81
<i>Eucyclops graeteri</i>	0.00	7.85	37.60	8.74	37.60	75.42
Total Amphipode	1.89	0.00	10.12	2.01	10.12	85.54
<i>Gammarus</i> sp.	1.28	0.00	6.38	0.94	6.38	91.92
	Q3	Q5				
<i>Eucyclops graeteri</i>	0.00	7.85	36.75	4.25	41.84	41.84
Cyclopodia	1.94	7.90	31.80	1.98	36.21	78.05
<i>Cypria ophtalmica</i>	2.31	0.00	9.82	1.07	11.19	89.23
<i>Bradleycypris obliqua</i>	0.00	0.82	2.92	0.65	3.33	92.56
	Q4	Q5				
<i>Eucyclops graeteri</i>	0.00	7.85	24.41	4.96	29.94	29.94
<i>Cypria ophtalmica</i>	6.25	0.00	21.42	1.17	26.27	56.21
Cyclopodia	3.15	7.90	16.11	1.71	19.75	75.96
<i>Eucyclops speratus</i>	1.63	0.00	4.69	0.65	5.75	81.70
<i>Eucyclops hadjebensis</i>	1.11	0.00	4.08	0.63	5.00	86.70
<i>Bradleycypris obliqua</i>	0.00	0.82	2.17	0.66	2.66	89.37
<i>Megacyclops brachypus</i>	0.00	0.47	1.39	0.66	1.71	91.07
	Q1	Q6				
Cyclopodia	3.21	2.47	28.34	1.62	36.44	36.44
<i>Eucyclops graeteri</i>	1.37	0.33	11.07	0.94	14.23	50.67
<i>Macrocyclus</i> sp.	0.00	2.13	9.72	0.65	12.49	63.16
<i>Macrocyclus albidus</i>	1.05	0.00	9.16	0.61	11.77	74.94
Total Amphipode	0.33	0.80	7.21	0.73	9.26	84.20

(continued)

Table 4 | continued

Taxon (I)	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib. %	Cum. %
Harpacticoida	0.47	0.00	4.10	0.61	5.27	89.47
<i>Cypria ophthalmica</i>	0.47	0.00	2.76	0.63	3.55	93.02
	Q2	Q6				
Total Amphipode	1.89	0.80	18.36	1.19	23.74	23.74
Cyclopodia	0.00	2.47	18.26	1.16	23.61	47.35
<i>Gammarus</i> sp.	1.28	0.00	15.50	0.96	20.04	67.39
<i>Macrocyclus</i> sp.	0.00	2.13	11.50	0.66	14.87	82.26
<i>Eucyclops graeteri</i>	0.00	0.33	6.76	0.62	8.74	91.00
	Q3	Q6				
Cyclopodia	1.94	2.47	24.30	1.32	27.07	27.07
<i>Cypria ophthalmica</i>	2.31	0.00	24.00	1.02	26.74	53.80
Total Amphipode	0.33	0.80	12.48	0.69	13.90	67.71
<i>Macrocyclus</i> sp.	0.00	2.13	11.19	0.65	12.47	80.18
<i>Cypridopsis vidua</i>	0.33	0.33	10.32	0.56	11.50	91.68
	Q4	Q6				
<i>Cypria ophthalmica</i>	6.25	0.00	35.57	1.11	40.47	40.47
Cyclopodia	3.15	2.47	15.91	1.39	18.10	58.57
<i>Macrocyclus</i> sp.	0.00	2.13	7.37	0.66	8.39	66.96
<i>Eucyclops hadjebensis</i>	1.11	0.00	7.05	0.63	8.02	74.98
<i>Eucyclops speratus</i>	1.63	0.00	6.96	0.64	7.92	82.90
total Amphipode	0.33	0.80	3.46	1.10	3.93	86.83
<i>Eucyclops graeteri</i>	0.00	0.33	2.15	0.65	2.45	89.28
	Q5	Q6				
<i>Eucyclops graeteri</i>	7.85	0.33	33.65	3.65	41.88	41.88
Cyclopodia	7.90	2.47	29.14	1.88	36.27	78.15
<i>Macrocyclus</i> sp.	0.00	2.13	6.87	0.64	8.55	86.70
Total Amphipode	0.00	0.80	3.69	1.05	4.60	91.30
Taxon (II)	High salinity	Low salinity				
	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib. %	Cum. %
Cyclopoda	3.26	3.25	12.89	1.13	14.81	14.81
<i>Cypria ophthalmica</i>	0.7	3.12	9.48	0.71	10.89	25.7
<i>Macrocyclus albidus</i>	0.26	2.12	8.76	0.76	10.07	35.77
<i>Bythinella</i> sp.	0.23	2.9	7.49	1.04	8.6	44.37
<i>Eucyclops graeteri</i>	2.31	0.17	7.08	0.71	8.13	52.5
<i>Bithynia</i> sp.	0.12	1.23	4.14	0.7	4.76	57.26
Oligochaeta	0.65	0.29	2.74	0.68	3.15	60.4
<i>Melitidae</i> sp.	0	0.24	2.48	0.37	2.85	63.25
<i>Gammarus</i> sp.	0.34	0	2.39	0.31	2.74	65.99

The stygofauna assemblage in well Q1 – high salinity – was represented only by brackishwater organisms, *Harpacticoids* sp. The low taxa number explained the low dissimilarity. Three true GW organisms, *E. hadjebensis*, *M. brachypus* and *A. sensitivus*, were absent in Q1 and accounted for dissimilarities between well Q1 and all other wells. Well Q2 did not harbor any stygofauna while two freshwater amphipods from Gammaridae family were recorded there, explaining part of dissimilarities between well Q2 and all other locations. Well Q4 showed the highest fauna density and diversity richness. The two stygobite species *E. hadjebensis* and *A. sensitivus* were found at this location (Q4) and were absent in all the wells with high-salinity conditions. Hence, the presence of stygobites in well Q4 accounted for the dissimilarity between this well and other high-salinity conditions (Q1, Q2, Q3 and Q5). However, the obligate GW species *E. graeteri* had the highest abundance at Q5 corresponding to the furthest location from the sea but still with high salinity. The time series of EC measurements for this well indicated a high amplitude of salinity variations, from 40,000  $\mu\text{S}/\text{cm}$  from August to mid-September and <2,000  $\mu\text{S}/\text{cm}$  in May. This wide range of salinity variation is linked to the movement of the fresh/saltwater interface, moving inwards in the dry season and pushed back by higher aquifer discharge during winter and spring. Well Q6 contained two individuals from the Melitidae family that contributed to dissimilarity between well Q6 and other wells. Generally, our results suggest that the samples dominated by non-stygobites are affected by a surface water.

## SIMPER analysis II

The SIMPER analysis was also performed to measure dissimilarity for factor salinity. The results showed a high average dissimilarity (86.36%) between high- and low-salinity wells. The taxa that contributed the most to differentiating between the two groups of wells are presented in Table 4. Results of the Simper analysis in this study indicated that the taxa which contributed the most to dissimilarities between well groups were the total number of Cyclopoida (15% weight) which, together with the species *C. ophthalmica* (Ostracod) and *M. albidus* (Copepod), represented 35% of the dissimilarities.

## Stygofauna relationship with abiotic variables

The abiotic parameters EC, pH, depth, temperature, hydraulic head and dissolved oxygen were sampled to assess their influence on the distributional pattern of the stygofauna species from the Querença-Silves aquifer. The BEST analysis in Table 5 shows the correlation between the selected environmental parameters and spatial distribution of species. The most parsimonious model with highest correlation (0.80) included the variables EC, pH and the water depth. These three variables were thus considered the most suitable subset of the measured variables pool to explain the stygofauna distribution. The second best model differed only in replacing depth with well hydraulic head. All other models had much lower correlations (<0.15) and were thus not considered for interpretation. The relative high contribution of EC to the models shows the importance of this variable for explaining the pattern of stygobite distribution. Other variables, such as temperature and dissolved oxygen, did not show significant contributions, which can be partly explained by their small amplitudes of variation.

## DISCUSSION

### Stygofauna occurrence and distribution according to the salinity gradient

The distribution of stygofauna is significantly influenced by hydrological interactions and anthropogenic impacts at a regional scale (Humphreys 2009). The current study examined the potential use of GW associated organisms to detect anthropogenic impacts such as saline intrusion. Here, the regional survey within the shallow layers of the

**Table 5** | Biota and environmental matching results according to the BEST modeling

Best results		
Number of variables	Correlation	Selection
1	0.14	Hydraulic head
2	0.11	Depth; hydraulic head
2	0.11	pH; hydraulic head
3	0.80	pH; depth; EC
3	0.70	EC; hydraulic head; pH

Querença-Silves aquifer bordering the Arade estuary recorded stygofauna in all wells, with the dominance of crustaceans (73%), followed by gastropods (20%) and other less represented invertebrate groups. The aquifer hosts several Cyclopoids and Harpacticoids with a mixture of cosmopolitan and strict GW dwellers, but also of freshwater and marine origin. Hence, the results of our study show that the stygofauna assemblage carries informative signals to detect gradual salinity changes of the aquifer. In fact, the three true GW organisms (*E. hadjebensis*, *M. brachypus* and *A. sensitivus*) were absent in Q1, justified by the high-salinity conditions of the well. The epigeal *Gammarus lacustris* was only present in the upper layers close to the estuary (well Q1), potentially pointing towards the fresh/saline GW exchanges that occur here. The results of this study also suggest that salinization may reduce the diversity since the highest diversity richness and fauna density was registered for well Q4 with lowest salinity record. These results lead us to address the likelihood of applying these species for detecting salinity disturbance. A limitation of our work was the relatively low number of sampled wells, however, these reflected all available salinity levels – which was the purpose of the study – and a very high biological representation was obtained, with more than 50 taxa and 800 animals being collected.

### Taxa with high and low dispersal ability

The fauna distribution pattern may reflect the sensitivity of fauna to environmental factors such as EC. However, part of species distributions could be influenced by other factors such as patchy distribution of stygofauna, their low dispersal ability and also their occurrence in some wells could be justified by their high connectivity to adjacent surface water bodies. However, in this study, some taxa were not identified to species level and this might influence the interpretation of dispersal ability.

Oligochaeta groups are generally one of the most understudied invertebrate in GW, though they constitute a rich and diversified component of subsurface assemblages, able to inhabit a large array of subsurface habitats (Cook 1975; Creuzé des Châtelliers *et al.* 2009). Oligochaeta were present in five wells (Q1–Q5) within the Querença-Silves aquifer. Our findings indicate that Oligochaeta specimens do not

show any particular spatial distribution and density patterns, as they were present in almost similar abundances throughout the whole aquifer.

The Querença-Silves aquifer hosts a diverse group of gastropods. Within the collected samples, eight species belonged to seven different families. The two genera of *Bythinella* sp. and *Bithynia* sp. inhabit low-salinity waters (e.g. in Q4), and they were absent in all other locations. The results of SIMPER analysis also indicate the important roles of these two gastropods for differentiating the habitats with saline and freshwater conditions.

Ostracods were more numerous in low water salinity conditions and were dominated by *C. ophthalmica*, an epigeal benthopelagic species which can swim just above the sediment (Griffiths *et al.* 1993). The result of Simper analysis illustrated that this species had a large contribution to distinguishing high-salinity from low-salinity wells. *Cypridopsis vidua* and *Bradleyocypris obliquus* are new records for GW in Portugal (Bode *et al.* 2010). They are recognized as a potential indicator of freshwater conditions due to their restricted distribution (Bode *et al.* 2010).

*Daphnia pulicaria* had a very limited distribution pattern, significantly abundant in high-salinity waters only (i.e. well Q5).

Many cyclopoida species are true GW stygobite copepods (Galassi 2001) and four were present in our samples. The Cyclopoids with more than 200 stygobites prevailed in Q5, underling the patchy distribution of stygofauna and aquifer heterogeneity. Copepods are highly sensitive to alteration of water conditions due to anthropogenic impacts and are indicative of hydrological regimes in GW systems (Paran *et al.* 2005; Dussart & Defaye 2006), suggesting that they have great potential to be used as indicators for GW ecosystem conditions (Galassi *et al.* 2009).

Among the investigated abiotic variables, EC, pH, hydraulic head and water depth were found to be the variables that best explain the distribution pattern of stygobites in the Querença-Silves aquifer. However, the assessment of other environmental variables may also help provide additional explanations of the observed pattern of species diversity.

There are other factors controlling dispersal ability in stygofauna such as their elongated developmental cycles, low ability of productivity and lack of dispersal in larval stage (Dole-Olivier *et al.* 2009). In this regard, more detailed

species investigation is required to distinguish whether their distribution pattern is attributed to their biological characteristics or if it is their ecological tolerance that is the significant factor. Moreover, the irregular and patchy distortion of stygofauna sometimes limits their application as biological indicator thus, the improvement of data sets is required by increasing sampling frequency within an aquifer and focusing the investigations on distinct spatial scales, from a larger regional scale of GW bodies (>100 km<sup>2</sup>) to small local aquifers (few km<sup>2</sup>). A better understanding of the shifts in stygofauna patterns (diversity, distribution and turnover) in relation to changes in environmental conditions at distinct scales is also required.

## CONCLUSION

The main focus of this study was to identify the response of stygofauna communities to a water salinity gradient – one of the key elements to detect the effect of anthropogenic activities in coastal aquifers – and, to examine the predictive response of stygofauna to potential seawater intrusion phenomena resulting from adjacent inland aquifer overexploitation. As a result of seawater intrusion, the inhabiting stygofauna species may start to decline and/or show a shift in composition. Our results suggest that the two stygobite species *E. hadjebensis* and *A. sensitivus* can be monitored as ecological indicators of undisturbed freshwater conditions in coastal aquifer. The occurrence of these two common GW dwellers (Mioduchowska & Wojtasik 2009) only in low-salinity well supports the hypothesis of their sensitivity to salinity impact. The effect of surface water bodies to GW ecosystem can be detected by the survival rate of stygobite species as their occurrence decreases and conversely high frequencies and abundances of non-stygobites species occur (Griebler et al. 2010). However, in some karst aquifers which are in high connectivity with adjacent surface bodies, usually non-stygobite species can be expected (Bork et al. 2009). The occurrence of some epigeans (i.e. *C. ophthalmica*) and freshwater organisms (i.e. *Bithynia* sp.), as found in this study in wells with low-salinity conditions, can be explained by connectivity of aquifer to surface water bodies.

In conclusion, the results provided by the current study suggest that the stygofauna assemblage carries informative

signals to detect gradual salinity changes of the aquifer. The pattern of stygofaunal community composition and distribution in the Querença-Silves aquifer varied among the wells and was linked to the variation in GW salinity, water depth and distance to the estuary. Stygobites species were revealed to be more suitable indicators of saline exposure while epigeans species may be used for more acute disturbance. Our findings showed that saline water intrusion into the Querença-Silves aquifer might be indicated by the population reduction of *E. hadjebensis*, *A. sensitivus*, *M. viridis* and *C. ophthalmica*. Changes in the stygofaunal community may reflect changes in aquifer dynamics, recharge, human interference or climate change which indicates that GW monitoring needs to be joined by monitoring stygofauna communities.

## ACKNOWLEDGEMENTS

The accomplishment of this work is owed to Paula Tavares who initiated the first and fundamental steps for this study. Thanks are also due to Margarida Machado, Gilda Silva, Maria José Caramujo and Paula Chainho for helping in the identification of some taxa. This work was part of the CLIMWAT project – Assessing and managing the impact of climate change on coastal groundwater resources and dependent ecosystems, funded by ERA-CIRCLE/0002/2007.

## REFERENCES

- Anderson, M. J., Gorley, R. N. & Clarke, K. R. 2008 *PERMANOVA For PRIMER: Guide to Software and Statistical Methods*. PRIMER-E Ltd, Plymouth.
- Bear, J., Cheng, A. & Sorek, S. 1999 *Seawater Intrusion in Coastal Aquifers Concepts, Methods and Practices*. Kluwer Academic Pub., Dordrecht.
- Bode, S. N. S., Adolfsson, S., Lamatsch, D. K., Martins, M. J. F., Schmit, O., Vandekerckhove, J. & Martens, K. 2010 [Exceptional cryptic diversity and multiple origins of parthenogenesis in a freshwater ostracod](#). *Mol. Phylogenet. Evol.* **54** (2), 542–552.
- Bork, J., Berkhoff, S. E., Bork, S. & Hahn, H. J. 2009 [Using subsurface metazoofauna to indicate groundwater–surface water interactions in the Nakdong River floodplain, South Korea](#). *Hydrogeol. J.* **17**, 61–75.

- Boulton, A. J., Fenwick, G. D., Hancock, P. J. & Harvey, M. S. 2008 Biodiversity, functional roles and ecosystem services of groundwater invertebrates. *Invertebr. Syst.* **22**, 103–116.
- Canivet, V., Chambom, P. & Gibert, J. 2001 Toxicity and bioaccumulation of arsenic and chromium in epigeal and hypogean freshwater macroinvertebrates. *Arch. Environ. Contam. Toxicol.* **40**, 345–354.
- Clarke, K. R. & Warwick, R. M. 2007 In: Plymouth Marine Laboratory (Ed.), PRIMER-E, Version 6 (UK).
- Cook, D. G. 1975 Cave-dwelling aquatic Oligochaeta (Annelida) from the eastern United States. *Trans. Am. Microsc. Soc.* **94**, 24–37.
- Creuzé des Châtelliers, M., Juget, J., Lafont, M. & Martin, P. 2009 Subterranean aquatic Oligochaeta. *Freshw. Biol.* **54**, 678–690.
- Culver, D. C. & Sket, B. 2000 Hotspots of subterranean biodiversity in caves and wells. *J. Cave Karst Stud.* **62**, 11–17.
- Cvetkov, L. 1968 Un filet phréatobiologique. *Bull. Inst. Zool. Mus. Acad. Bulgare. Sci. Sofia* **27**, 215–218.
- Danielopol, D. L., Gibert, J., Griebler, C., Gunatilaka, A., Hahn, H. J., Messana, G., Notenboom, J. & Sket, B. 2004 Incorporating ecological perspectives in European groundwater management policy. *Environ. Conserv.* **31**, 1–5.
- Danielopol, D. L., Gibert, J. & Griebler, C. 2006 Efforts of the European Commission to improve communication between environmental scientists and policy-makers. *Environ. Sci. Pollut. Res.* **13**, 138–139.
- Danielopol, D., Griebler, C., Gunatilaka, A., Hahn, H., Gibert, J., Mermillod-Blondin, G., Messana, G., Notenboom, J. & Sket, B. 2007 *Incorporation of Groundwater Ecology in Environmental Policy*. Groundwater Science and Policy, The Royal Society of Chemistry, RSC Publishing, London.
- Deharveng, L., Stoch, F., Gibert, J., Bedos, A., Galassi, D., Zigmajster, M. & Marmonier, P. 2009 Groundwater biodiversity in Europe. *Freshw. Biol.* **54** (4), 709–726.
- Dole-Olivier, M. J., Malard, F., Martin, D., Lefébure, T. & Gibert, J. 2009 Relationships between environmental variables and groundwater biodiversity at the regional scale. *Freshw. Biol.* **54** (4), 797–813.
- Dumas, P., Bou, C. & Gibert, J. 2001 Groundwater macrocrustaceans as natural indicators of the Ariège Alluvial Aquifer. *Int. Rev. Hydrobiol.* **86**, 619–633.
- Dussart, B. & Defaye, D. 2006 *World Directory of Crustacea Copepoda of Inland Waters. II–Cyclopiformes*. Backhuys Publishers, Leiden, 354 pp.
- Galassi, D. M. 2001 Groundwater copepods: diversity patterns over ecological and evolutionary scales. *Hydrobiologia* **453** (1), 227–253.
- Galassi, D. M. P., Huys, R. & Reid, J. W. 2009 Diversity, ecology and evolution of groundwater copepods. *Freshw. Biol.* **54**, 691–708.
- Gibert, J. 2001 Protocols for the assessment and conservation of aquatic life in the subsurface (pascalis): a European project. *Mapp. Subterr. Biodiv.* 19–21. Available from: [http://www.researchgate.net/profile/Fabio\\_Stoch/publication/267567541\\_Sampling\\_manual\\_for\\_the\\_assessment\\_of\\_regional\\_groundwater\\_biodiversity/links/54536d290cf26d5090a45849.pdf](http://www.researchgate.net/profile/Fabio_Stoch/publication/267567541_Sampling_manual_for_the_assessment_of_regional_groundwater_biodiversity/links/54536d290cf26d5090a45849.pdf).
- Gibert, J. & Culver, D. C. 2009 Assessing and conserving groundwater biodiversity: an introduction. *Freshw. Biol.* **54**, 639–648.
- Griebler, C., Stein, H., Kellermann, C., Berkhoff, S., Briemann, H., Schmidt, S. & Hahn, H. J. 2010 Ecological assessment of groundwater ecosystems – vision or illusion? *Ecol. Eng.* **36** (9), 1174–1190.
- Griffiths, D. S., Martin, A. J. & Shine, J. G. 1993 The ostracod fauna (Crustacea, Ostracoda) of the profundal benthos of Loch Ness. *Hydrobiologia* **254**, 111–117.
- Hahn, H. J. & Matzke, D. 2005 A comparison of stygofauna communities inside and outside groundwater bores. *Limnologica* **35**, 31–44.
- Hahn, H. J. 2006 The GW-fauna-index: a first approach to a quantitative ecological assessment of groundwater habitats. *Limnologica* **36**, 119–137.
- Hahn, H. J. 2007 Ökologische Bewertungsansätze für ein faunistisch begründetes Grundwassermonitoring: Bedeutung des hydrologischen Austauschs [Preliminary ecological evaluation for faunistically justified groundwater monitoring: Interpreting the hydrologic exchange]. In: *Proc. DWA Seminar 'Grundwasserökologie: Praxis und Forschung'*, November Hahn, Fulda, Germany.
- Humphreys, W. F. 2009 Hydrogeology and groundwater ecology: does each inform the other? *Hydrogeol. J.* **17**, 5–21.
- Kim, J., Yum, B., Kim, R., Koh, D., Cheong, T., Lee, J. & Chang, H. 2003 Application of cluster analysis for the hydrogeochemical factors of saline groundwater in kimje, Korea. *Geosci. J.* **7**, 313–322.
- Korbel, K. L. & Hose, G. C. 2011 A tiered framework for assessing groundwater ecosystem health. *Hydrobiologia* **661**, 329–349.
- Longley, G. 1992 The subterranean aquatic ecosystem of the Balcones Fault Zone Edwards Aquifer in Texas: threats from overpumping. In: *Proc. First Int. Conf. Groundwater Ecology* (J. A. Stanford & J. J. Simons, eds). American Water Resources Association, Bethesda, MD, pp. 291–300.
- Malard, F., Plenet, S. & Gibert, J. 1996 The use of invertebrates in ground water monitoring: a rising research field. *Ground Water Monit. Rem.* **16**, 103–113.
- Malard, F., Dole-Olivier, M. J., Mathieu, J. & Stoch, F. 2002 Sampling manual for the assessment of regional groundwater biodiversity. Protocols for the Assessment and Conservation of Aquatic Life in the Subsurface (PASCALIS). Available from: [www.pascalis-project.com/results/samplingmanual.html](http://www.pascalis-project.com/results/samplingmanual.html).
- Marmonier, P., Maazouzi, C., Foulquier, A., Navel, S., François, C., Hervant, F. & Piscart, C. 2013 The use of crustaceans as sentinel organisms to evaluate groundwater ecological quality. *Ecol. Eng.* **57**, 118–132.
- Mioduchowska, M. & Wojtasik, B. 2009 Copepoda-cyclopoida of water bodies of the coast of Gdansk. *Teka Kom. Ochr. Kszt. Srod. Przyn. OL PAN* **6**, 189–199.
- Monteiro, J., Ribeiro, L., Reis, E., Martins, J. & Matos Silva, J. 2007 *Modelling Stream-Groundwater Interactions in the Querença-Silves Aquifer System Groundwater and*

- Ecosystems (Lisbon)*. IAH Congress, Groundwater and Ecosystems, Lisbon, pp. 17–21.
- Mösslacher, F. 1998 Subsurface dwelling Crustaceans as indicators of hydrological conditions, oxygen concentrations, and sediment structure in an alluvial aquifer. *Int. Rev. Hydrobiol.* **83**, 349–364.
- Mösslacher, F. 2000 Sensitivity of groundwater and surface water crustaceans to chemical pollutants and hypoxia: implications for pollution management. *Arch. Hydrobiol.* **149**, 51–66.
- Notenboom, J. 1990 Introduction to Iberian groundwater amphipods. *Limnetica* **6**, 165–176.
- Notenboom, J., Serrano, R., Morell, I. & Hernandezet, F. 1995 The phreatic aquifer of the ‘Plana de Castellón’ (Spain): relationships between animal assemblages and groundwater pollution. *Hydrobiologia* **297** (3), 241–249.
- Paran, F., Malard, F., Mathieu, J., Lafont, M., Galassi, D. M. P. & Marmonier, P. 2005 Distribution of groundwater invertebrates along an environmental gradient in a shallow water-table aquifer. World Subterranean Biodiversity, Proceedings of an International Symposium. University Claude, Lyon, France, pp. 99–105.
- Reboleira, A. S., Borges, P. A., Gonçalves, F., Serrano, A. R. & Oromí, P. 2011 The subterranean fauna of a biodiversity hotspot region – Portugal: an overview and its conservation. *Environ. Sustain.* **40**.
- Reboleira, A., Gonçalves, F. & Oromí, P. 2013 Literature survey, bibliographic analysis and a taxonomic catalogue of subterranean fauna from Portugal. *Subterr. Biol.* **10**, 51–60.
- Rouch, R. 1977 Considerations sur l’écosystème karstique [Considerations on the karstic ecosystem]. *CR Acad. Sci. Paris, Série D* **284**, 1101–1103.
- Santos, F., Forbes, K. & Moita, R. 2002 *Climate Change in Portugal: Scenarios, Impacts and Adaptation Measures: SIAM Project*. Gradiva, Lisbon, Portugal.
- Scarsbrook, M. R. & Fenwick, G. D. 2003 Preliminary assessment of crustacean distribution patterns in New Zealand groundwater aquifers. *N.Z. J. Mar. Freshw. Res.* **37**, 405–413.
- Schmidt, S. I., Hahn, H. J., Hatton, T. & Humphreys, W. F. 2007 Do faunal assemblages reflect the exchange intensity in groundwater zones. *Hydrobiologia* **583**, 1–19.
- Silva, A. C. F., Tavares, P., Shapouri, M., Stigter, T., Cancela da Fonseca, L. & Ribeiro, L. 2012 Estuarine biodiversity as an indicator of groundwater discharge. *J. Estuar. Coast. Shelf Sci.* **97**, 38–43.
- Sket, B. 2008 Can we agree on an ecological classification of subterranean animals? *J. Nat. Hist.* **42**, 21–22.
- Somay, M. A. & Gemici, U. 2009 Assessment of the salinization process at the coastal area with hydrogeochemical tools and geographical information systems (GIS): Selcuk Plain, Izmir, Turkey. *Water Air Soil Pollut.* **201** (1–4), 55–74.
- Stein, H., Kellermann, C., Schmidt, S. I., Briemann, H., Steube, C., Berkhoff, S. E., Fuchs, A., Hahn, H. J., Thulin, B. & Griebler, C. 2010 The potential use of fauna and bacteria as ecological indicators for the assessment of groundwater ecosystems. *J. Environ. Monit.* **12**, 242–254.
- Steube, C., Richter, S. & Griebler, C. 2010 First attempts towards an integrative concept for the ecological assessment of groundwater ecosystems. *Hydrogeol. J.* **17**, 23–35.
- Stigter, T., Monteiro, J., Nunes, L., Vieira, J., Cunha, M., Ribeiro, L., Nascimento, J. & Lucas, H. 2009 Screening of sustainable groundwater sources for integration into a regional drought-prone water supply system. *Hydrol. Earth Syst. Sci.* **13**, 1185–1199.
- Stigter, T. Y., Nunes, J. P., Pisani, B., Fakir, Y., Hugman, R., Li, Y., Tomé, S., Ribeiro, L., Samper, J., Oliveira, R., Monteiro, J. P., Silva, A., Tavares, P. C. F., Shapouri, M., Cancela da Fonseca, L. & El Himer, H. 2013 Comparative assessment of climate change and its impacts on three coastal aquifers in the Mediterranean. *Reg. Environ. Change* **14**, 41–56.
- Stock, J. H. 1994 Biogeographic synthesis of the insular groundwater faunas of the (sub) tropical Atlantic. *Hydrobiologia* **287**, 105–117.
- Thulin, B., Hahn, H. J. & Grundwasserökologie, A. 2008 *Ecology and Living Conditions of Groundwater Fauna*. SKB.
- Wang, Y. & Jiao, J. J. 2012 Origin of groundwater salinity and hydrogeochemical processes in the confined Quaternary aquifer of the Pearl River Delta, China. *J. Hydrol.* **438–439**, 112–124.
- WFD 2000 Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for Community action in the field of water policy. Joint text approved by the Conciliation Committee provided for in Article 251(4).

First received 25 August 2014; accepted in revised form 25 March 2015. Available online 28 April 2015