



Stygoregions – a promising approach to a bioregional classification of groundwater systems

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Linked to diverse biological processes, groundwater ecosystems deliver essential services to mankind, the most important of which is the provision of drinking water. In contrast to surface waters, ecological aspects of groundwater systems are ignored by the current European Union and national legislation. Groundwater management and protection measures refer exclusively to its good physicochemical and quantitative status. Current initiatives in developing ecologically sound integrative assessment schemes by taking groundwater fauna into account depend on the initial classification of subsurface bioregions. In a large scale survey, the regional and biogeographical distribution patterns of groundwater dwelling invertebrates were examined for many parts of Germany. Following an exploratory approach, our results underline that the distribution patterns of invertebrates in groundwater are not in accordance with any existing bioregional classification system established for surface habitats. In consequence, we propose to develop a new classification scheme for groundwater ecosystems based on stygoregions.

Groundwater systems are diversely populated habitats. The world wide number of described obligate groundwater invertebrates, the so-called stygofauna, sums up to 7000 species¹. The true species richness though, is believed to exceed that number by far taking into account that groundwater systems have not been studied to a great extent and a multitude of species await detection and description²⁻⁵. The groundwater fauna is mainly composed of small crustaceans, oligochaetes, nematodes, acari, and molluscs, less than 1 mm to several centimeters in body size⁴ (Fig. 1 a–d).

The goods and services provided by groundwater ecosystems (i.e. ecosystem services) which include the purification of water by the breakdown of organic matter, nutrients and contaminants, are fundamental to both the integrity of the ecosystems and the sustaining of human life⁶⁻¹⁰. Intimately connected to microbiological remineralization processes, invertebrates are believed to play an important role in the purification of subterranean water and thus, the maintainance of good water quality^{11,12}. Feeding, burrowing and bioturbating activities of the fauna in highly active transition zones (e.g. hyporheic zone) contribute to maintain the hydraulic connectivity between surface systems and aquifers¹¹, a service groundwater dependant ecosystems (e.g. soils, wetlands, rivers) strongly rely on¹¹. Ecosystem services such as those mentioned above are only provided sustainably by groundwater communities with a structural and functional integrity^{13,14}.

Recently, the need for an ecologically sound management of groundwater resources has become increasingly recognised by national and international political authorities¹⁵⁻¹⁹. The European Union Groundwater Directive²⁰ (EU-GWD), released in 2006, emphasize the importance of protective measures for groundwater ecosystems proposing the conductance of more ecological research in order to provide better criteria for ensuring groundwater ecosystem quality in the future¹³. This triggered new scientific research on identifying and evaluating biological criteria related to groundwater ecosystem health and water quality, and *vice versa*^{13,14,19,21}.

Organisms integrate impacts over space (their habitat) and time, clearly extending the information obtained from abiotic monitoring²¹. Indeed groundwater invertebrates have been shown to be sensitive sentinels indicating the influence from surface water infiltration, changes of hydrological conditions, the impact of organic contaminants and nutrients, and were shown valuable biomonitors of heavy metal pollution as well as heat discharge¹⁴ and citations therein. Only recently, first integrative approaches for the assessment of groundwater ecosystem health, which define criteria, reference conditions and thresholds for individual physicochemical, microbiological and faunistic

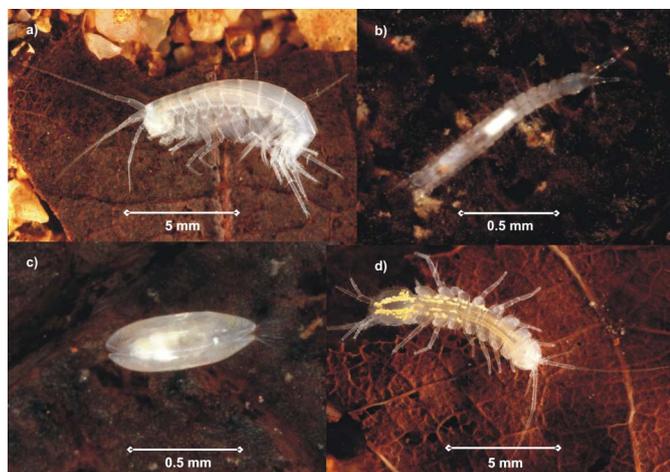


Figure 1 | Stygobiontic invertebrates are perfectly adapted to darkness, groundwater habitats. As a convergent evolutionary response to darkness, obligate groundwater animals are translucent, blind, exhibit enhanced tactile sense organs and lack circadian periodicity. Their bodies are elongated to vermiform facilitating locomotion in habitats with limited pore spaces. As an adaption to low and patchy food supply stygobites have slow metabolic rates, low reproduction rates and they exhibit longevity, compared to their surface water relatives. Shown are four frequently found representatives of the crustaceans; **a)** *Niphargus laisi*, Amphipoda; SCHELLENBERG, 1936 **b)** *Antrobathynella stammeri*, Bathynellacea; JAKOBI, 1954, **c)** *Fabaeformiscandona spec.*, Ostracoda **d)** *Proasellus slavus*, Isopoda, REMY, 1948) (Pictures: K. Grabow).

conditions, have been developed and indices are provided^{21–23}. However, a biogeoregional classification of groundwater habitats, as a necessary and solid basis for the standardized application of assessment schemes is still missing.

For European groundwaters various classification systems are available, such as the classification into hydrogeological and geochemical units²⁴, defined by the type of aquifer (e.g. porous, karst), its petrographical or hydrochemical properties, its hydraulic permeability, groundwater recharge and productivity. Accordingly, for Germany 17 groundwater landscapes (hydrogeological units) have been refined by Kunkel et al.²⁵. Opposite to the majority of ‘abiotic’ classification schemes^{26–29}, only a few zoogeographical classifications are available, such as the ones suggested by Botosaneanu¹ or Husmann³⁰. However, these proved inapplicable in practice, due to their low biogeographical resolution¹ and enormous complexity in habitat types³⁰. More recent approaches, such as the pioneering PASCALIS project³¹ (Protocols for the Assessment and Conservation of Aquatic Life In the Subsurface), focussed on groundwater biodiversity delivering exciting diversity maps for Europe, but did not have the objective of a biogeographical classification of groundwater systems. Nevertheless, at that time the idea of a stygoregional classification has first emerged^{4,32} and was recently set into action by Stoch and Galassi⁵. Unfortunately, this study was limited to the edge of the Southern Alps comprising only one subterranean bioregion (stygoregion), i.e. ‘North Eastern Italy’. On a regional scale (100–10,000 km²) Hahn and Fuchs³³ proposed so called ‘georegs’ (regional geology = aquifer type + major physiographic unit: MPU) for the analysis of stygofaunal distribution patterns.

Indeed, the assessment of freshwater surface systems is based on bioregional references, strongly influenced by the zoogeographical classification from Illies³⁴, with the good ecological state being the key criterion³⁵. For freshwaters in Central Europe classification systems and assessment schemes, including biological criteria, are well developed, but do not take groundwater ecosystems into consideration. The spatial classification systems are based on biogeographical, geomorphological, hydrological, physicochemical, and zoogeographical aspects^{24,25,27,34,36,37}. On a biogeographical scale (10,000 to

100,000 km²) Central Europe encompasses at least three main ecoregions (Northern Lowlands, Central Uplands, Alps)³⁸ (Fig. 2a). However, most classification systems for Germany, including the water framework directive (WFD)³³, additionally comprise the Alpine foothills (Fig. 2b). On a regional scale, these ecoregions are refined into major physiographic units (MPU)³⁷ (Fig. 2b). This classification scheme, applied to Germany, but also to Austria, considers surface and subsurface features, including climate, geology, physical geography, and morphology³⁷. Examples for Germany are the Black Forest (Fig. 3, MPU No. D54) the Lower Rhine Valley (Fig. 3, MPU No. D35) or the Pfälzerwald Mountains (Fig. 3, MPU No. D51). In order to develop a first large scale classification for Central Europe’s groundwater systems, we evaluated the patterns obtained from our groundwater fauna data set for its agreement with existing classification schemes, following different approaches and levels of statistical analysis including selected spatial aggregation.

Results

Compared to sampling surveys in surface waters, groundwater studies very often reveal highly complex data sets, characterized by a large percentage of samples not containing animals or only a few species, which are often rare or endemic. Statistical analysis often require careful selective data pre-treatment. A promising strategy for the elucidation of meaningful spatial distribution patterns is a stepwise data aggregation starting with the highest possible resolution (level of individual samples) followed by pooling the data according to sampling sites (wells) and subsequently according to meaningful units such as the type of aquifer, GeoRegs, hydrogeological units (HU) or major physiographic units (MPU). Following this protocol, no clear distribution patterns for groundwater fauna were obtained analysing at the level of single samples and sampling sites, respectively, applying multivariate statistics (ANOSIM: $R < 0.09$). Pre-selective data aggregations on the level of aquifer types and HU did not reveal meaningful and reproducible patterns as well. In contrast, analysis of data aggregated at the level of MPU (ANOSIM: $R=0.896$) and GeoRegs (ANOSIM: $R=0.221$) offered reproducible distribution patterns, statistically most striking for MPU.

Thus, aggregating invertebrate community data within major physiographic units (MPU) best explained the groundwater faunal distribution on the biogeographical scale for the study area. The subsequent testing of the distribution patterns obtained by multi-dimensional scaling (MDS) analysis for agreement with existing bioregional classification schemes revealed that it neither matched the spatial units classified by the EU-WFD³⁵ nor the classification systems mentioned above^{25,33,34} (Fig. 3a, b). Our findings emphasize that existing classification schemes are not adequate for the fauna in groundwater. In contrary, data evaluation hints at four major clusters, in the following termed stygoregions, characterized by faunal communities significantly distinct ($p < 0.0001$) in their (aggregated) composition (Fig. 3 a, b, Table 1). The pairwise tests, corrected by a sequential Bonferroni test (see adjusted significance level in parenthesis) show significant differences between all four stygoregional assemblages (Northern Lowlands - Central Uplands: $p=0,0013$ ($p=0.0125$); Northern Lowlands - South-Western Uplands: $p=0,0003$ ($p=0.0083$); Northern Lowlands - Southern Uplands and Northern Alps: $p=0,0024$ ($p=0.025$); Central Uplands - South-Western Uplands: $p=0,001$; Central Uplands - Southern Uplands and Northern Alps: $p=0,0087$ ($p=0.05$); South-Western Uplands - Southern Uplands and Northern Alps: $p=0,0015$ ($p=0.016$)).

Analysis of similarities (ANOSIM) support this proposal by revealing strong differences between the faunal assemblages when tested for stygoregions ($R=0.896$, $p=0.001$ with $d=1$) while being less different when tested for the EU-WFD ecoregion classification ($R=0.505$, $p=0.002$ with $d=1$). To exclude that the introduction of a dummy species for integration of ‘zero value samples’ into the analysis (see Material & Methods section) may have caused these

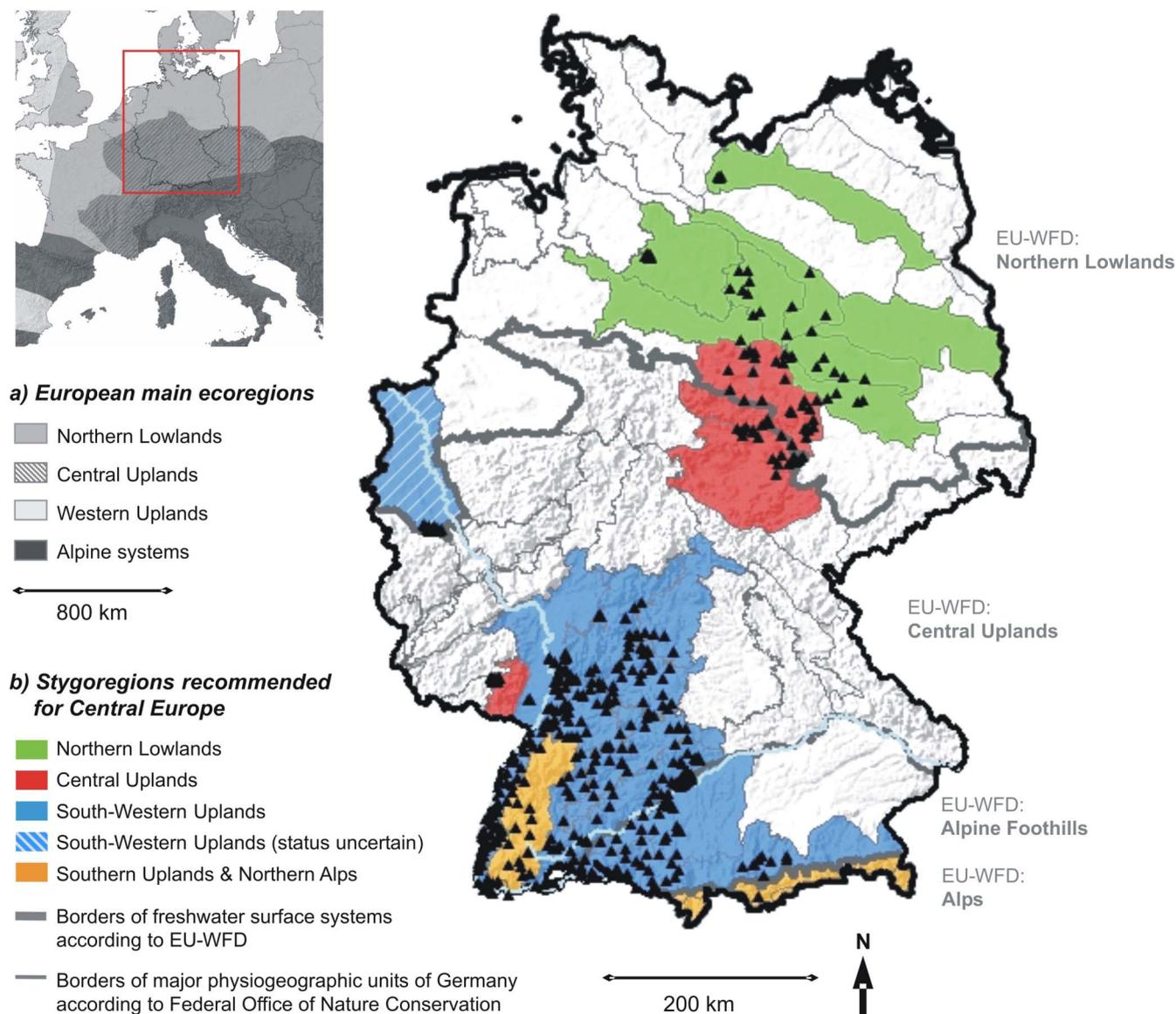


Figure 2 | a) European main ecoregions and b) a topographic map of Germany depicting the proposed stygoregions: Coloured areas show the major physiogeographic units³⁷ (MPU), which were studied. The white areas compile MPU where no data was available. The colours refer to the individual stygoregions, which were delineated according to invertebrate distribution patterns found in groundwater. The affiliation of the Lower Rhine Valley is under debate, indicated by the blue-white hatching. Topographic map/GIS: <http://www.eea.europa.eu/legal/copyright>.

patterns, the ANOSIM was re-calculated without the dummy. Now, the differences between the two classification systems were even more pronounced (stygoregions: $R=0.475$, $p=0.001$, EU-WFD classification: $R=0.196$, $p>0.05$). To further test the reproducibility and statistical quality of the distribution patterns obtained, the data set was split into two sets of different sampling times and the MDS was recalculated. Analysis of the two individual data sets revealed almost identical results (data not shown).

In the following, the four stygoregions proposed for Central Europe are described in brief:

1) *The Northern Lowlands* comprise groundwater systems that had been strongly affected by pleistocene ice shields and are characterized by fine porous sediments and low oxygen concentrations. As a result, these groundwaters are naturally unpopulated or stygofauna is scarce^{18,39}. Only 15% of the wells investigated were populated by invertebrates (Table 1).

2) *The Central Uplands* comprise the groundwater habitats of the Central Mountain Ranges and the adjacent sub-mountainous

forelands, including the Pfälzerwald Mountains. The Central Mountain Ranges were not covered by ice shields but were strongly affected by permafrost soils and low precipitations. The forelands mark the southern borders of the ice shields⁴⁰. Invertebrate groundwater communities are mainly characterized by ubiquitous species, so-called post-glacial recolonisers^{5,7,33}, with only few endemic species⁴¹ (Table 1). Invertebrates were found in around 65% of the wells sampled.

3) *The South-Western Uplands* are generally characterized by a highly diverse fauna in groundwater that have not been affected by the periods of glaciation. The proportion of stygobites (obligate groundwater species) is high and the invertebrate communities present generally reflect in their composition the pleistocenious Danube catchment area situation with a diverse amphipod and ostracod fauna³³ (Table 1). Because of major overlaps in species composition, so far, the Lower Rhine Valley was included in this stygoregion, although the groundwater dwelling invertebrate fauna of the Lower Rhine Valley exhibits a lower diversity. 76% of the wells investigated were populated by invertebrates.

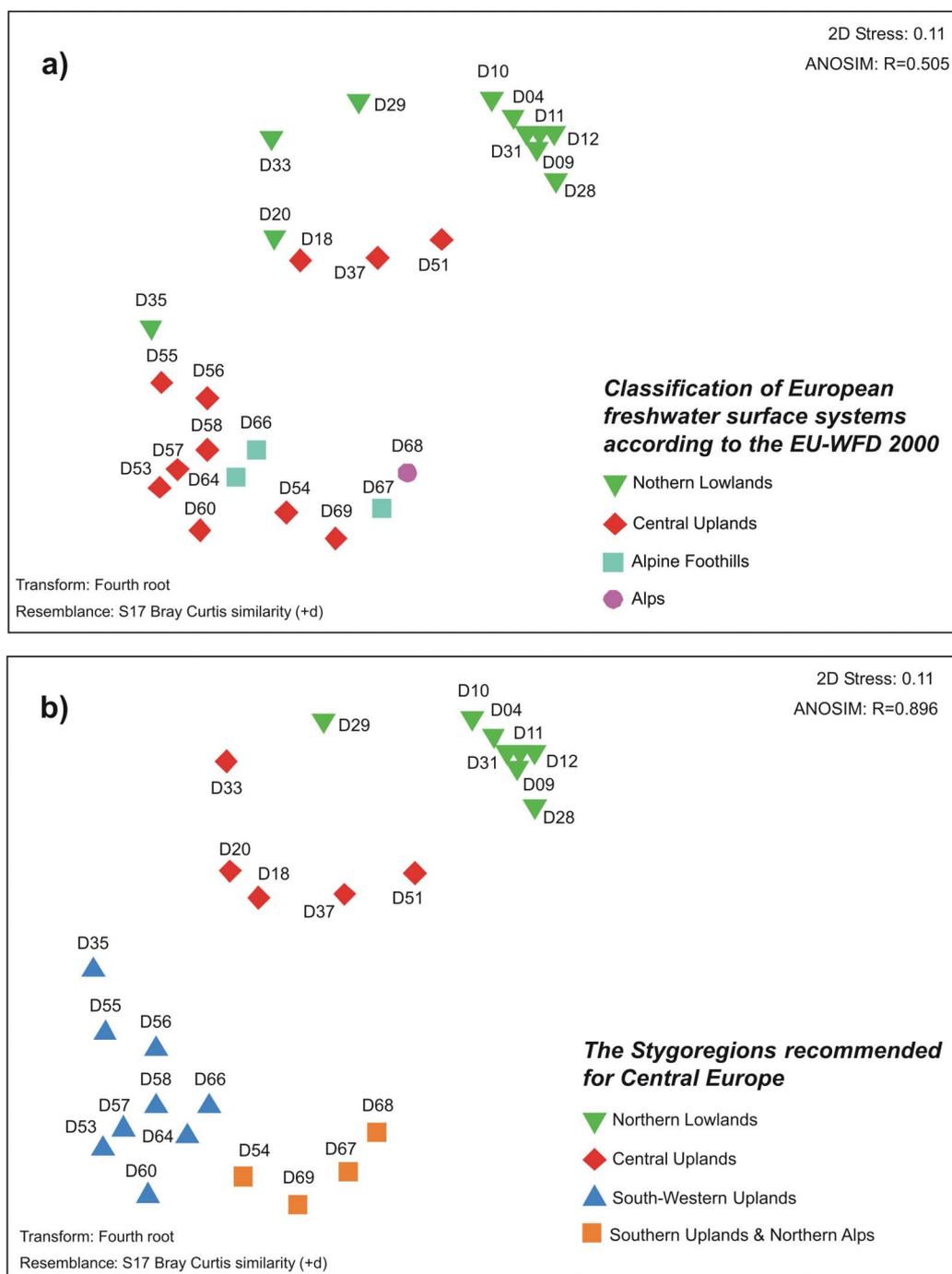


Figure 3 | Non-metrical multi-dimensional-scaling plot (MDS) depicting biogeographical distribution patterns of groundwater dwelling invertebrates. Symbols refer to a) freshwater surface systems according to the EU-WFD³⁵; b) stygoregions recommended for Central European groundwater systems. The labels of the symbols refer to the official German MPU³⁷ (not listed in detail). Plotted are Bray-Curtis similarities of faunal means that were pooled for the major physiographic units of Germany³⁷.

4) The *Southern Uplands and Northern Alps* comprise those areas that were covered by the pleistocenious ice shields of the Alps and the Black Forest. The species composition is similar to those of the *South-Western Uplands*, but generally less diverse (Table 1). Characteristic for this stygoregion is some species (*i.e.* *Niphargus strouhali*; Amphipoda) which have so far only been recorded from Austria, the southeast neighbour state (Schellenberg 1942b). Invertebrates were found in almost 80% of the wells sampled.

Compared to bioregional classification systems for surface waters, groundwater stygofaunal distribution patterns differ most notably in the central and southern groundwater habitats: Here, the ecoregions

by the EU-WFD (Central Uplands, Alpine foothills, Alps) are very different from the stygoregions (Fig. 3a, b). The southern assemblages differed markedly from those of the surface aquatic fauna of the Central Uplands and the Alps (EU-WFD 2000) (Fig. 2a, b). Another striking difference to the surface aquatic fauna is the far outreach of stygofaunal species from the *Uplands* into the EU-WFD-boundary areas of the Northern Lowlands (Fig. 3a, b).

Discussion

The biogeographical distribution patterns found for groundwater dwelling invertebrates in Central Europe differ significantly from



Table 1 | Species matrix of groundwater dwelling invertebrates and their distribution in different stygoregions. Species are classified as stygobiontic and non-stygobiontic. Abbreviations: NL: Northern Lowlands, CU: Central Uplands, SWU: South-Western Uplands, SU & NA: Southern Uplands and Northern Alps. C. = Crustacea

Stygoregion		NL	CU	SWU	SU & NA
No. of GW-monitoring wells		40	60	376	38
No. of samples		116	223	821	81
Stygobiontic species	Taxonomic group				
<i>Parastenocaris phreatica</i>	Copepoda, C.	x			
<i>Parastenocaris phyllura</i>	Copepoda, C.	x	x		
<i>Bogidiella albertimagni</i>	Amphipoda, C.		x	x	x
<i>Crangonyx subterraneus</i>	Amphipoda, C.		x	x	x
<i>Niphargellus nollii</i>	Amphipoda, C.		x	x	x
<i>Niphargus fontanus</i>	Amphipoda, C.		x	x	x
<i>Diacyclops languidoides</i>	Copepoda, C.		x	x	x
<i>Graeteriella unisetigera</i>	Copepoda, C.		x	x	x
<i>Proasellus cavaticus</i>	Isopoda, C.		x	x	x
<i>Niphargus aquilex</i>	Amphipoda, C.		x	x	
<i>Microniphargus leruthi</i>	Amphipoda, C.		x	x	
<i>Parastenocaris germanica</i>	Copepoda, C.		x	x	
<i>Chappuisius singeri</i>	Copepoda, C.		x	x	
<i>Bathynella natans</i>	Syncarida, C.		x	x	
<i>Fabaeformiscandona breuili</i>	Ostracoda, C.			x	
<i>Fabaeformiscandona latens</i>	Ostracoda, C.			x	
<i>Fabaeformiscandona wegelini</i>	Ostracoda, C.			x	
<i>Parastenocaris psammica</i>	Copepoda, C.			x	
<i>Schellencandona belgica</i>	Ostracoda, C.			x	
<i>Schellencandona insueta</i>	Ostracoda, C.			x	
<i>Schellencandona triquetra</i>	Ostracoda, C.			x	
<i>Niphargopsis casparyi</i>	Amphipoda, C.			x	
<i>Niphargus kochianus</i>	Amphipoda, C.			x	
<i>Niphargus laisi</i>	Amphipoda, C.			x	
<i>Niphargus puteanus</i>	Amphipoda, C.			x	
<i>Niphargus tatrensis</i>	Amphipoda, C.			x	
<i>Acanthocyclops gmeineri</i>	Copepoda, C.			x	
<i>Acanthocyclops kieferi</i>	Copepoda, C.			x	
<i>Bryocamptus typhlops</i>	Copepoda, C.			x	
<i>Chappuisius inopinus</i>	Copepoda, C.			x	
<i>Echinocamptus pilosus</i>	Copepoda, C.			x	
<i>Elaphoidella elaphoides</i>	Copepoda, C.			x	
<i>Graeteriella laisi</i>	Copepoda, C.			x	
<i>Moraria fontinalis</i>	Copepoda, C.			x	
<i>Nitocrella omega</i>	Copepoda, C.			x	
<i>Parapseudoleptomesochra spec.</i>	Copepoda, C.			x	
<i>Parastenocaris c.f. glacialis</i>	Copepoda, C.			x	
<i>Anthrobathynella stammeri</i>	Syncarida, C.			x	
<i>Bathynella freiburgensis</i>	Syncarida, C.			x	
<i>Parabathynella c.f. ferdii</i>	Syncarida, C.			x	
<i>Pseudantrobathynella husmanni</i>	Syncarida, C.			x	
<i>Proasellus coxalis</i>	Isopoda, C.			x	
<i>Proasellus walteri</i>	Isopoda, C.			x	x
<i>Niphargus auerbachii</i>	Amphipoda, C.			x	x
<i>Niphargus bajuvaricus</i>	Amphipoda, C.			x	x
<i>Schellencandona schellenbergi</i>	Ostracoda, C.			x	x
<i>Niphargus inopinatus</i>	Amphipoda, C.			x	x
<i>Niphargus foreli</i>	Amphipoda, C.			x	x
<i>Niphargus kieferi</i>	Amphipoda, C.			x	x
<i>Parastenocaris c.f. moravica</i>	Copepoda, C.			x	x
<i>Cryptocandona kieferi</i>	Ostracoda, C.			x	x
<i>Fabaeformiscandona bilobata</i>	Ostracoda, C.			x	x
<i>Mixtacandona laisi</i>	Ostracoda, C.			x	x
<i>Acanthocyclops rhenanus</i>	Copepoda, C.			x	x
<i>Proasellus slavus</i>	Isopoda, C.			x	x
<i>Acanthocyclops venustus</i>	Copepoda, C.			x	x
<i>Acanthocyclops sensitivus</i>	Copepoda, C.			x	x
<i>Parastenocaris c.f. aedis</i>	Copepoda, C.				x
<i>Bathynella chappuisi</i>	Syncarida, C.				x
<i>Nitocrella hirta tirolensis</i>	Copepoda, C.				x
<i>Niphargus strouhali</i>	Copepoda, C.				x



Table 1 | Continue

Stygoregion		NL	CU	SWU	SU & NA
No. of GW-monitoring wells		40	60	376	38
No. of samples		116	223	821	81
Non-stygobiontic species					
<i>Pristina proboscidea</i>	Oligochaeta	x			
<i>Tubifex tubifex</i>	Oligochaeta	x			
<i>Diacyclops crassicaudis</i>	Copepoda, C.	x	x	x	x
<i>Dorydrilus michaelseni</i>	Oligochaeta	x	x	x	x
<i>Marionina riparia</i>	Oligochaeta	x	x	x	
<i>Aelosoma hyalina</i>	Oligochaeta	x		x	
<i>Cernovsvitoviella atrata</i>	Oligochaeta	x	x	x	
<i>Bryocamptus minutus</i>	Copepoda, C.		x		
<i>Paracyclops poppei</i>	Copepoda, C.		x		
<i>Potamothenix ssp.</i>	Oligochaeta		x		
<i>Troglochaetus beranecki</i>	Polychaeta		x	x	x
<i>Paracyclops fimbriatus</i>	Copepoda, C.		x	x	x
<i>Diacyclops bisetosus</i>	Copepoda, C.		x	x	
<i>Diacyclops languidus</i>	Copepoda, C.		x	x	
<i>Mesenchytraeus armatus</i>	Oligochaeta		x	x	
<i>Bryochamptus echinatus</i>	Copepoda, C.			x	x
<i>Parastenocaris brevipes</i>	Copepoda, C.			x	x
<i>Aelosoma niveum</i>	Oligochaeta			x	x
<i>Haplotaxis gordioides</i>	Oligochaeta			x	x
<i>Tubifex ignotus</i>	Oligochaeta			x	x
<i>Tubifex species A</i>	Oligochaeta			x	x
<i>Tubificidae species B</i>	Oligochaeta			x	x
<i>Bythiospeum ssp.</i>	Gastropoda			x	x
<i>Acanthocyclops robustus</i>	Copepoda, C.			x	
<i>Acanthocyclops vernalis</i>	Copepoda, C.			x	
<i>Cyclops strenuus</i>	Copepoda, C.			x	
<i>Cyclops vicinus</i>	Copepoda, C.			x	
<i>Cypria ophthalmica</i>	Ostracoda, C.			x	
<i>Diacyclops bicuspidatus</i>	Copepoda, C.			x	
<i>Eucyclops serrulatus</i>	Copepoda, C.			x	
<i>Eudiaptomus gracilis</i>	Copepoda, C.			x	
<i>Macrocyclops albidus</i>	Copepoda, C.			x	
<i>Megacyclops viridis</i>	Copepoda, C.			x	
<i>Moraria brevipes</i>	Copepoda, C.			x	
<i>Moraria pectinata</i>	Copepoda, C.			x	
<i>Nitocra hibernica</i>	Copepoda, C.			x	
<i>Thermocyclops crassus</i>	Copepoda, C.			x	
<i>Tropocyclops prasinus</i>	Copepoda, C.			x	
<i>Candona weltneri</i>	Ostracoda, C.			x	
<i>Ancylus fluviatilis</i>	Gastropoda			x	
<i>Aelosoma hemprichi</i>	Oligochaeta			x	
<i>Aelosoma psammophylum</i>	Oligochaeta			x	
<i>Aelosoma quaternarium</i>	Oligochaeta			x	
<i>Amphichaeta leydigi</i>	Oligochaeta			x	
<i>Buchholzia appendiculata</i>	Oligochaeta			x	
<i>Eiseniella tetraedra</i>	Oligochaeta			x	
<i>Fridericia perrieri</i>	Oligochaeta			x	
<i>Marionina argentea</i>	Oligochaeta			x	
<i>Nais c.f. variabilis</i>	Oligochaeta			x	
<i>Phyllognathopus viguieri</i>	Oligochaeta			x	
<i>Potamothenix hammoniensis</i>	Oligochaeta			x	
<i>Pristinella bilobata</i>	Oligochaeta			x	
<i>Psammoryctides albicola</i>	Oligochaeta			x	
<i>Pseudocandona albicans</i>	Oligochaeta			x	
<i>Rhyacodrilus falciformis</i>	Oligochaeta			x	
<i>Uncinaiis uncinata</i>	Oligochaeta			x	
<i>Paracamptus schmeili</i>	Copepoda, C.				x
<i>Cryptocandona vavrai</i>	Ostracoda, C.				x
<i>Spirosperma velutinus</i>	Oligochaeta				x
<i>Vejdovskiiella comata</i>	Oligochaeta				x

existing surface classification systems such as the aquatic bioregions by Illies³⁴ and the European ecoregions³⁵, implying the need of an independent classification system of groundwater habitats, *i.e.* sty-

goregions. In Central Europe, the distribution of groundwater fauna on the large scale is mainly a result of quaternary glaciations, which have severely affected species richness and composition^{5,42}. Our data



underline the general latitudinal gradient of species richness, declining from south to north, which was already observed in earlier studies and which is much less pronounced with organisms from surface freshwaters^{4,5,43}. Cumulative species curves (data not shown) indicate that continued sampling will definitely expand today's groundwater species richness in Central Europe, which is true for most regions worldwide^{33,44–46}. However, the distribution patterns obtained from the data collected in our study are robust, and the spatial distribution of individual groups, such as niphargids, isopods, and copepods match well with earlier studies^{47–51}.

Four stygoregions are now proposed, the *Northern Lowlands*, the *Central Uplands*, the *South-Western Uplands*, and the *Southern Uplands and Northern Alps*. The groundwater faunal impoverishment, which is considered typical of the *Northern Lowlands* (invertebrates were absent from almost 85% of the wells), is a consequence of species extinction during glaciation and inhibited post-glacial recolonization³³. It left a glacially shaped subsurface, mainly composed of sands and fine materials with limited pore space. Moreover, due to considerable amounts of organic matter and slow groundwater flow, oxygen is extremely low or absent²⁵. In comparison, the stygoregion of the *Central Uplands* was less affected by the periods of glaciation, and as such had served as refugial areas for some species – although many aquifers are assumed to have been dried out or have been affected by permafrost⁵². After the end of the ice age, the surviving groundwater species complemented by many ubiquitous species coming from the south recolonized the *Central Uplands* as well as the groundwater habitats of the formerly glacified sub-mountainous forelands, where environmental conditions were now appropriate (e.g. pore space and sufficient amounts of oxygen)^{33,42}. The low endemism in this stygoregion is a consequence of this post-glacial recolonisation.

The highly diverse groundwater fauna characteristic for the stygoregion *South-Western Uplands* is predominately composed of ancient stygobionts, which have not been affected by glaciation. As such, it is likely that this region provided refuges for stygobionts in the deep karst or in the alluvium of large streams³³. The affiliation of the Lower Rhine Valley groundwater communities to the ones of the *South-Western Uplands* is not fully clear yet (Fig. 2b, 3b). All classification systems, biological as well as geographical ones, consider the Lower Rhine Valley as part of the *Northern Lowlands*. From a groundwater faunistic point of view, however, the Lower Rhine Valley is very different from the impoverished *Northern Lowlands*. Our data indicate, that fauna is similar to the *South-Western Uplands*, implying that the groundwaters of the Lower Rhine Valley are connected via the middle Rhine valley to the Pleistocenious catchments of the rivers Danube and Main, which harbour a distinct fauna³³.

The status of the stygoregion *Southern Uplands and Northern Alps* is still under discussion. Although its fauna mirrors in general the *South-Western Uplands* fauna, species richness is lower and some species, such as *Niphargus strouhali*, are unique to this region and have so far been known only from groundwater habitats located further south and east, i.e. from Austria⁴⁷. We suppose, that recolonization of the *Southern Uplands and Northern Alps* took place both from the *South-Western Uplands* and from the east via the 'interstitial highways' of the Danube and its southern tributaries. These results corroborate to the earlier findings by Hahn & Fuchs³³, that indicate a high subsurface connectivity in that area.

Without doubt, the fauna data available for groundwaters in Germany are far from being complete. However, the study areas selected are representative of the main Central European landscapes. The delineation of stygoregions which has been proposed already earlier^{4,5,32} stands for a common step forward in coming up with an independent classification scheme for groundwater fauna and habitats⁵³. In dependence to the specific environmental condition of an area, the dimension of the stygoregions may vary considerably. According to our data from Central Europe, stygoregions encompass

up to 100,000 km². However, Stoch & Galassi⁵ describe a much smaller stygoregion for north-east Italy.

Although it is a drawback that there are many areas in Germany, Europe, and worldwide not yet sampled, we see this as a dynamic development. Individual stygoregions may still be expanded or diminished in size in the future and new stygoregions might be defined. This was true also for the various classification systems for surface ecosystems. Attempts should be made in the future to harmonize and merge data sets obtained by different sampling strategies, e.g. the European groundwater biodiversity data set produced in PASCALIS, for the review and extension of the stygoregion approach to the European scale. With our data set from Central Europe the delineation of stygoregions based on aggregation of community data at the level of MPUs proved to be an appropriate approach. Nevertheless, in other parts of the world other spatial subunits such as the type of aquifer, GeoRegs, HU or others might be more appropriate.

In conclusion, distribution patterns of fauna in groundwaters of Central Europe on a regional and continental scale proved to be significantly different from any classification scheme related to hydrogeology, geochemistry, and surface fauna. If we intend to assess the ecological status of groundwater systems, as routine for surface aquatic systems, a reliable classification along with the definition of reference conditions is required. We propose the refinement of Europe's water saturated subsurface into stygoregions, which are bioregions that comprise both surface and subsurface features. Our investigations led to the delineation of four different stygoregions in Germany and Central Europe, to our opinion an indispensable initial step towards an ecological assessment scheme of groundwater ecosystems and a really sustainable groundwater management^{18,51}. In the near future we have to investigate the yet unexplored reaches to fill the groundwater fauna maps. Moreover, first attempts are already made to evaluate the synchronization of fauna data with those of microbial communities in groundwaters²³. An integrative and ecologically sound groundwater ecosystems assessment scheme will have to take faunal, and microbiological as well as physicochemical criteria into account.

Methods

Study regions. The faunal data presented originate from sampling surveys conducted in a wide variety of aquifers across Germany (Fig. 2b), including unconsolidated porous aquifers, karst and fractured aquifers. A total of 515 groundwater monitoring wells were repeatedly examined (2–5 times) between the years 2002 and 2009. Most samples were obtained from near-surface groundwater in approximately 10–80 m depth. The major selection criteria for the choice of sampling areas and wells were the geographic representativity and comparability of faunal data sets. While Hahn & Fuchs³³, frequently cited here, focused on the state of Baden-Wuerttemberg, this current paper encompasses samples from all over Germany.

Fauna sampling and taxonomic identification. We extracted the groundwater dwelling invertebrates from the bottom of monitoring wells by using a phreatic net sampler (75 µm mesh size), according to Hahn and Fuchs³³. All faunal samples were immediately stored in a refrigerator box. Following live observations, samples were fixed with 4% formaldehyde before further processing. The taxonomic identification was based on morphological characteristics. Crustaceans, oligochaetes, polychaetes and gastropods were major target groups and determined to species level. Specimens of other taxonomic groups were excluded from further analyses. Ecological characterizations of the species follow in general Schellenberg⁴⁷, Einsle⁴⁸, Janetzky et al.⁴⁹, Meisch⁵⁰, and Schminke⁵¹, but were simplified to a stygobiontic vs. non-stygobiontic classification according to Hahn²², Deharveng et al.⁴⁵, and Detry et al.⁵⁴.

Data analyses. Dealing with groundwater faunistic data means to analyze an extremely heterogeneous data sets with high proportions of faunistic zero values, low species numbers per site, many rare species and strongly varying abundances. Thus, for the biogeographical analyses pre-selective aggregation of the data was inevitable. The problem of aggregation though, is the loss of information and possible pre-determination due to the choice of aggregation units. To minimize these problems, the aggregation units should be as small as possible and aggregation should be proceeded stepwise. We first analysed our data prior to aggregation. Then we the data were aggregated on the level of sampling sites (wells), type of aquifer, geological region (GeoReg), hydrogeological unit (HU), major physiographic unit (MPU). The quality of the aggregates was tested with respect to a potential bioregional classification for groundwater. Using this iterative approach, best results for our data were



obtained by aggregating at the level of MPU from which the stygoregional classification derived. Since zero values (sample not containing animals) may also be a characteristic of certain areas, unpopulated wells were considered in the analysis as well.

Following the above mentioned tests, total counts of faunal populations collected in wells were averaged for each major physiographic unit (MPU) and subsequently fourth root-transformed. Data aggregation was conducted to reduce data scattering of individual wells, which is a result of the heterogeneous faunal distribution that is naturally found in groundwater. Since the faunal data, even after fourth root-transformation, did not show a normal distribution, exclusively non-parametrical methods were used for statistical analyses. Patterns in faunal community structure were explored by non-metric multi-dimensional scaling (MDS), Permutational multivariate analysis of variance (PERMANOVA) and analysis of similarity (ANOSIM) based on Bray-Curtis distance. The Bray-Curtis measure was chosen because it does consider zero values, but does not consider joint absences where both samples have zeroes⁵⁵. In this context, it is worth to mention, that in general around 30% of all wells are unpopulated²². Before generating the MDS, a dummy variable ($d=1$) was added to each group (MPU), for a better interpretation and a 2-dimensional graphical presentation of the community patterns found. This dummy variable suggests an additional virtual species that is shared by each group, and thus reduces the differences, without changing its proportions. The reliability of the MDS-plot is indicated by a stress value. Stress values < 0.2 indicate valid representations⁵⁶. In the PERMANOVA the number of permutations was set to 9999 using the reduced model and type III sums of squares⁵⁷ to obtain the P -values. For the pair-wise PERMANOVA test the level of significance was corrected using Holms sequential Bonferroni test⁵⁸. In addition, we used an Analysis of similarities (ANOSIM) to test the overall quality of the different spatial subunits (here based on faunal assemblages aggregated over the respective subunits) for the delineation of groundwater bioregions and the probability of patterns occurring by chance. The statistical quality of the ANOSIM is calculated by a test statistic (Global R) with values > 0.5 indicating a solid degree of separation of sample groups⁵⁶. This test is non-parametric analogue to the ANOVA and is adequate for data that do not show normal distribution⁵⁷. All statistical analyses were performed using the PRIMER v.6 software package⁵⁵ and the add-on package PERMANOVA+⁵⁷.

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Authors contributions

The design of this project was the result of discussions with all authors. H.S., D.M. & A.F. compiled the data set. H.S. performed the data analysis. H.S., H.J.H. and C.G. wrote the draft of the manuscript. S.E.B., H.S., C.G. & H.J.H. discussed the results & commented the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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