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Ecology-based evaluation of groundwater ecosystems under intensive agriculture: A combination of community analysis and sentinel exposure

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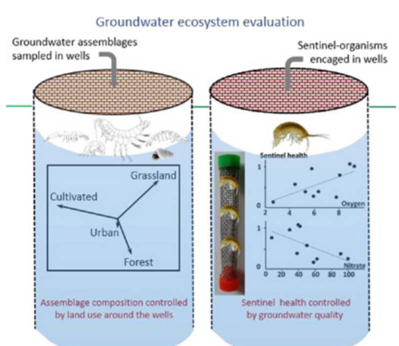
HIGHLIGHTS

- Two methods of ecology-based evaluation of groundwater are associated.
- Groundwater assemblages significantly changed with the land use types.
- Sentinel species were sensible to dissolved oxygen and nitrate concentrations.
- Atrazine-related compounds may negatively impact sentinel health.

GRAPHICAL ABSTRACT



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ABSTRACT

Ecological criteria are needed for a comprehensive evaluation of groundwater ecosystem health by including biological components with the physical and chemical properties that are already required by European directives. Two methodological approaches to assess the ecological status of groundwater ecosystems were combined in two alluvial plains (the Ariège and Hers Rivers, southwestern France) varying in agriculture intensity (from grassland to crop rotation including maize and sunflower, and to maize monoculture). In the first approach, the composition of invertebrate assemblages (only obligate-groundwater crustaceans, i.e. stygobionts) sampled in 28 wells differing in their land use contexts was analysed. Abundance, species richness, and assemblage composition significantly changed with agricultural land use or urbanization around the wells. In the second approach, we tested an in situ exposure of sentinel organisms to quantify their response to the environmental pressures. The epigean and native amphipod species *Gammarus* cf. *orinos* was used as the sentinel species. Amphipods (30 individuals in each of 10 wells) were exposed for one week to the in situ conditions at two seasons with contrasted concentrations of pollutants. The Ecophysiological Index (EPI) synthetizing the survival rates and energetic storage decreased in wells with low oxygen and high nitrate concentrations, but only during the

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Herbicides
Triazines

highest contamination period. Atrazine-related compounds negatively impacted sentinel health whatever the season. The combination of these two approaches may have major applications for orientating groundwater ecosystem management.

1. Introduction

More than 97% of the world freshwater reserves are stored in aquifers, making groundwater a critical resource on the planet (Smith et al., 2016). Facing the development of growing human populations and activities, a major challenge for the future will be to maintain the groundwater ecosystem health, i.e. the capacity of the groundwater ecosystem to sustain an appropriate structural and functional integrity that will sustainably deliver appropriate services (Korbel and Hose, 2011). Among the many services that groundwater ecosystems provide to humans (Tomlinson et al., 2007; Boulton, 2009) the most valuable are drinking water supply (Van der Gun, 2012) and water use for irrigation for both traditional and intensive agricultures (Burke, 2003; Zektser and Everett, 2004). Upwelling groundwater may also influence stream and wetland conditions through water level, temperature and water quality (Ramsar Convention Secretariat, 2010). Groundwater quality is under threat in many areas due to agriculture practices, industrial development and urbanization (Bohlke, 2002; Legout et al., 2005, 2007; Martin et al., 2006; European Environmental Agency, 2010; Lapworth et al., 2012; Di Lorenzo and Galassi, 2013; Datry et al., 2004, 2005; Foulquier et al., 2010, 2011; Lerner and Barrett, 1996; Trauth and Xanthopoulos, 1997; Di Lorenzo et al., 2012). Reliable evaluations of groundwater ecosystem health have thus become essential for the proposal, the establishment, and the use of restoration strategies (Boulton, 2005; Griebler et al., 2014).

In Europe, groundwater quality is still evaluated based on physical, chemical and hydrological characteristics (European Commission, 2006), although the inclusion of ecological criteria has been repeatedly advocated (Danielopol et al., 2004, 2006b, 2008; Hancock et al., 2005; Hahn, 2006; Tomlinson et al., 2007; Steube et al., 2009; Griebler et al., 2010). The few existing ecological indicators of groundwater ecosystem health consist in the examination of parameters describing invertebrate or microbial communities (Mösslacher, 2000; Danielopol et al., 2006a; Hahn, 2006; Griebler et al., 2010; Stein et al., 2010; Korbel and Hose, 2011; Gutjahr et al., 2013; Griebler et al., 2014; Mauffret et al., 2017; Dumnicka et al., 2017) or biofilm growing on artificial substrates (Williamson et al., 2012; Mermillod-Blondin et al., 2013). More recently, the use of sentinel invertebrates caged inside groundwater wells was suggested to avoid confounding effects related to local characteristics. Changes in depth and physical characteristics of the wells, variability in the rate of water exchange between the well and the surrounding aquifer and differences in biogeographical history of the region (e.g. defaunation linked to the last glacial extension) may lead to low densities and diversities and to a misinterpretation of the relationships between groundwater biota and ecosystem quality (Marmonier et al., 2013). Sentinel organisms caged in rivers and streams are now routinely used in the bioassessment of surface water ecosystems (Maltby et al., 1990, 2002; Xuereb et al., 2009). The taxa used in situ for the monitoring of surface water include molluscs (Schmitt et al., 2010; Taleb et al., 2009), crustaceans (Coulaud et al., 2011; Debourge-Geffard et al., 2009; Maltby, 1995; Maltby and Crane, 1994), insects (Custer and Burton, 2008) and fishes (Hanson, 2009). For groundwater ecosystems, Marmonier et al. (2013) proposed to distinguish the use of stygobionts, which may be resistant to long-term exposure (one month of starvation) and allow assessing diffuse pollution or providing a comprehensive evaluation of groundwater ecological quality, from the use of epigean species, which may resist to short-term exposure only (one week) allowing assessment of acute toxicity disturbances. Several health criteria have been used to evaluate the degree of environmental disturbance resulting from exposure: (1) survival rate (Brown, 1980; Gust et al., 2010), (2) feeding activity (Coulaud et al., 2011; Crane et al., 1995; Forrow and Maltby, 2000), (3) physiological parameters (e.g., respiration, Gerhardt, 1996; vitellogenesis, Xuereb et al., 2011) and (4) life-history traits (e.g., reproduction, Gust et al., 2011; Schmitt et al., 2010). To estimate sentinel health after exposure in a synthetic way, Marmonier et al. (2013) proposed to use an index that combines survival rates and changes in energetic stores.

For the first time two ecological approaches were combined for evaluating the ecological quality of groundwater ecosystems exposed to intensive anthropogenic activities (agriculture and urbanization) at the catchment scale. First, the composition and structure of stygobite crustacean assemblages was analysed in 28 wells along a gradient of human pressure from forest-dominated areas to grasslands, to maize monocultures and urban areas in the Ariège and Hers alluvial plains. We chose these two alluvial plains because they present two similar land use gradients. We hypothesized that intensive agricultural practices or urbanization should be significantly associated to decreases in abundance, species richness and changes in the composition of hypogean crustacean assemblages (long-term trends). In a second approach, we test the hypothesis that agriculture-derived contamination of groundwater should induce a significant decrease in sentinel health. We used a native epigean amphipod (*Gammarus* cf. *orinos*) as a sentinel caged in a subset of 10 wells, immediately after (July) and three months after (September) the end of maize herbicide spreading (mostly *S*-metolachlor). The survival rate and the level of energy consumption determined after one week of exposure and expressed as the Ecophysiological Index (EPI, Marmonier et al., 2013) allow the quantification of the stress endured by the sentinels during the exposure period (short-term trends).

2. Material & methods

2.1. Study area

The Ariège and Hers alluvial plains are located in southwestern France and encompass a 540 km^2 surface area, the Hers being the main tributary of the Ariège River. The studied sector of the Ariège alluvial plain (Fig. 1A) extends over 50 km from Varilhes (South) to Portet-sur-Garonne (North). The Hers plain is located between Mirepoix (South) and Cintegabelle (North) where the Hers River joins the Ariège River. The area is underlain by Aquitanian (Miocene) and Stampian (Oligocene) molasse deposits (Fig. 1B). The Ariège River alluvium was deposited on the molasse in five distinct terraces with very similar grain size composition, but differing by their degree of pebble weathering and silt pedogenesis (Mauffret et al., 2017). These slight differences have small consequences on the water chemical characteristics (i.e. changes in the ratio between the major ions, such as Na/Ca and Mg/Ca; unpublished data). The sand-and-gravel alluvium of the lower terrace and the lower plain is a continuous unconfined aquifer that is hydraulically connected to the Ariège and Hers Rivers, feeding them during most of the year, especially during late summer when river discharge is low with main flow direction from south south-east to north north-west (Fig. 1B). The unsaturated zone is generally <10 m thick.

The region is characterized by intensive agriculture, the major crop being maize and sunflower. For the land use analysis (Fig. 1A) we used established using satellite images from 4 dates (winter, spring,

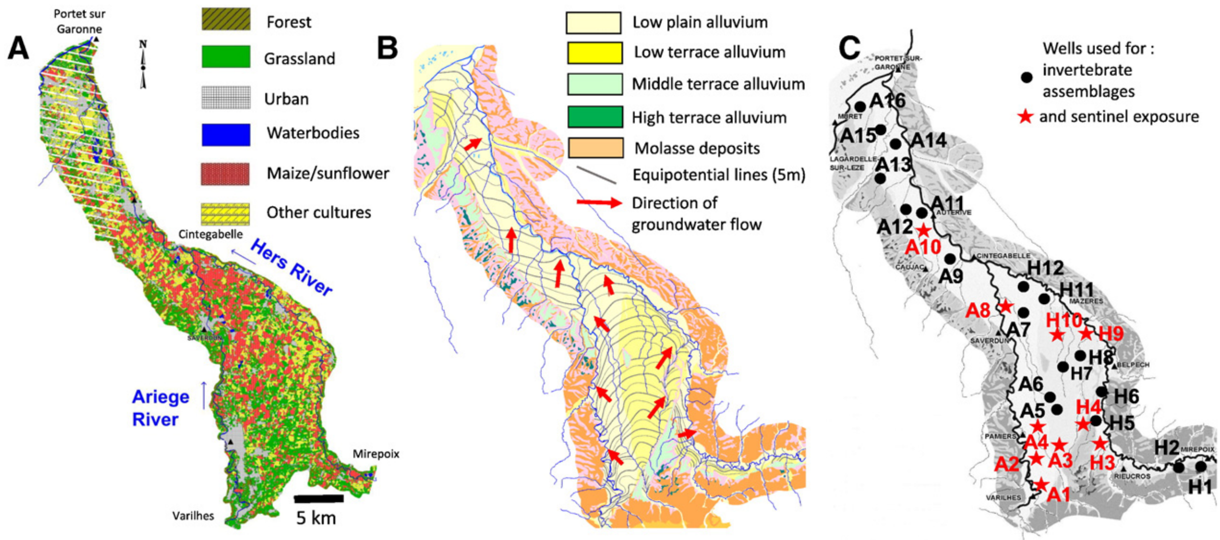

Fig. 1. Presentation of the studied area. Land use in the alluvial plains of the Hers and Ariège Rivers (A) groundwater flow patterns (B) and location of sampled wells for the survey of stygobite assemblages (all symbols) and both assemblages and sentinel exposures (red stars; C). The land use was established using four satellite images (one for each season), except in the northern part of the plain where spring image was not available (noted here with yellow stripes).

summer, and fall 2008) where six different land use types were defined (forest, grassland, maize-sunflower, other cultures, urbanized area and waterbodies; using MapInfo 8.5.1B®). In the urbanized areas (grey in Fig. 1A), domestic waste water treatment combines individual sanitation units with release in the soil and water treatment plants with release in rivers. The area of intensive agriculture (red colour in Fig. 1A) showed strong contamination of groundwater linked to the use of fertilizers and pesticides (herbicides, fungicides, and insecticides, see Appendix 1). This degradation has been documented for a long time by the Adour-Garonne Water Agency that manages the monitoring of water quality according to the Water Framework Directive (European Commission, 2000; especially for nitrate and pesticides concentrations), or by a reinforced monitoring (Amalric et al., 2013). In 2008, a large-scale study of groundwater nitrate concentrations using a set of 144 wells and springs showed that nearly half the wells were above the European standard limits (50 mg/L) established by the European Commission (2006), 22 of which (i.e. 15%) were over 100 mg/L. Wells with high nitrate concentrations were located in an area dominated by maize and sunflower productions.

2.2. Water quality and invertebrate assemblages

The groundwater fauna of a part of the studied area (i.e. the Ariège alluvial plain) was previously studied by Dumas (2000, 2002) and Janiaud (2004) in 15 wells. In the present study, the Invertebrate assemblages and water quality were sampled in 28 wells (Fig. 1C) in both Ariège and Hers alluvial plains, two sectors with similar gradients of land use and known to harbour diverse stygobiont communities (Dumas, 2002). Invertebrates were collected in January 2012, i.e. before the beginning of groundwater pumping for farming activities and two months before the beginning of water quality survey (see Section 2.3). Wells were distributed as evenly as possible in the studied sector: 12 in the Hers alluvial plain and 16 in the Ariège alluvial plain (8 above and 8 below the confluence of the Hers and the Ariège Rivers). Land use around each well (buffer of 3 km^2) was established with MapInfo 8.5.1B using the 6 land use types (see Fig. 1A and Table 1). Land use types were broad enough to persist between 2008 and 2012. Distances to the Hers and the Ariège Rivers, which informs on the potential influence of surface water on stygobionts, were measured as the shortest distance between the well and the river channel (Table 1).

The fauna was sampled within each well using a method derived from the Bou-Rouch technique (Bou and Rouch, 1967): 40 L of water and fine sediment were pumped by disturbing the bottom of the well with a long and weighted semi-rigid tube connected to a hand-pump (Malard et al., 2003). Water quality was assessed by in situ measures of temperature, electrical conductivity at 25 °C, pH, and dissolved oxygen, using a HACH-LANGE portable apparatus (HQ40d Multiparameter, Düsseldorf, Germany) on the last L of pumped water. The 40-L sample was then filtered through a 100 μm mesh net to retain fauna that was immediately preserved in 96° alcohol. The analysis of invertebrate assemblages was restricted to the stygobionts for comparison with previous studies performed in 1999 by Dumas (2000) and in 2003 by Janiaud (2004). Amphipods, isopods, copepods, and ostracods were identified to species level using morpho-anatomical criteria.

For amphipods, the morphological identification was completed by molecular barcoding to ensure tricky morphological identifications and to identify damaged/young individuals. Briefly, DNA was extracted from all individuals following standard protocols. Nuclear 28S rDNA fragments measuring about 830–870 bp were amplified using the primers 5′-CAAGTACCGTGAGGGAAAGTT-3′ and 5′-AGGGAAACTTC GGAGGGAACC-3′ from Fiser et al. (2008). Sequences were aligned using the Bio Edit Sequence Alignment Editor (Hall, 1999). Phylogenetic evolutionary analysis was conducted using maximum parsimony and bootstrapping with Mega version 5 (Tamura et al., 2011), and resulting phylogenetic trees were used to delineate the main species in the area. Because sequencing failed for most individuals (mainly due to poor DNA quality), we used a shorter fragment (180–215 bp) allowing the discrimination among the main species groups found in this area through genotyping. We used the primers 5′- TTGAGCCTGTGGGTGAC-3′ and 5′ GCCTGCACCAAGATTTAACC-3′ to amplify sequences of unique size for each species, which allowed identifying species based on the peak size of the peaks. We used the peak size of the peaks to distinguish a "*Niphargus kochianus*" species-group from the three other taxa (*N. ciliatus*, *N. foreli*, and *Niphargus* sp., smaller-sized fragments). These peaks were scored in the program Gene Mapper version 5 (Applied Biosystems, 2012). Although not ideal, this genotyping approach allowed for the validation of most morphological identifications. We used 5–20 ng of genomic DNA and QIAGEN® Core PCR Kits (Qiagen, Valencia, CA, USA) to perform PCR amplifications (recipes available upon request). PCR products were revealed on an ABI PRISM™ 3730 Automated Capillary Sequencer (Applied Biosystems, Foster City, CA, USA).

Table 1
Characteristics of the groundwater, the wells and the surrounding land use during the sampling period (February 2012). D.O.: dissolved oxygen (mg/L), T°: temperature, E.C.: electrical conductivity, Pz: piezometric level (m; nk: not known), Depth: well total depth (m), DistA: distance to Ariège River (km), DistH: distance to Hers River (km), Urb: urban land use (%), Gras: grassland (%), Cult: agriculture land use (%), For: forest (%), Wat: surface covered by water bodies (%), Unk: unknown land use (%).

Code	D.O.	T°	E.C.	pH	Pz	Depth	DistA	DistH	Urb	Gras	Cult	For	Wat	Unk
A1	4.6	12.8	168.6	6.99	5.3	8.3	0.24	10.14	17.8	37.2	15.5	24.5	4.9	0.0
A2	8.3	13.5	596	7.06	8.9[a]	16.2	1.55	8.51	30.1	30.1	35.9	3.0	0.8	0.0
A3	7.9	13.7	270	6.88	6.0	9.8	3.03	6.47	43.4	20.6	35.9	0.1	0.1	0.0
A4	8.32	11.5	621	7.00	9.2	22.5	2.45	7.46	20.7	11.2	64.7	3.2	0.2	0.0
A5	4.7	10.0	547	7.06	2.6	4.4	3.54	7.81	5.8	10.5	83.0	0.5	0.2	0.0
A6	4.78	9.6	452	6.85	2.2	nk	3.54	7.81	13.4	21.1	65.4	0.0	0.1	0.0
A7	6.9	11.7	466	6.94	7.6	6.5	2.13	3.85	5.8	16.1	73.4	4.5	0.1	0.0
A8	4.9	12.4	544	6.9	1.7[a]	4.1	0.58	5.37	4.4	15.7	71.3	4.9	3.7	0.0
A9	5.5	11.4	706	7.38	4.9	5.5	2.28	3.45	8.9	14.9	75.2	0.9	0.0	0.0
A10	3.6	9.0	723	7.35	3.2	5.4	0.13	8.0	29.2	10.2	50.1	0.2	0.5	9.7
A11	5.5	13.6	681	6.99	3.0	4.9	0.13	8.0	55.9	8.5	26.9	2.9	3.6	2.0
A12	6.95	11.5	812	7.18	3.5	4.4	3.24	13.19	20.9	15.6	44.3	5.6	0.2	13.4
A13	3.35	9.3	1200	7.24	3.3	5.5	3.82	17.81	25.7	15.5	40.5	2.0	0.1	16.1
A14	5.02	12.3	727	7.35	3.3	7.1	0.42	10.35	25.5	8.0	53.6	2.5	0.3	10.1
A15	1.85	13.4	886	7.49	9.2	9.1	1.83	20.26	70.3	3.3	22.6	0.9	0.0	2.9
A16	4.6	12.7	706	7.2	4.8	5.9	4.49	25.18	29.9	4.8	54.3	0.3	0.0	10.7
H1	2.98	11.3	630	7.2	3.7	9.4	19.34	1.16	0.0	30.5	15.7	0.0	0.0	53.8
H2	5.05	12.8	467	7.27	4.6	6.9	16.43	0.43	0.0	33.8	5.4	0.0	0.0	60.8
H3	4.7	15.6	669	7.29	4.1	4.6	9.61	0.47	4.4	16.7	62.4	14.1	1.6	0.9
H4	7.29	11.7	705	7.15	8.1	13.9	7.23	2.82	6.4	24.7	65.8	2.9	0.1	0.0
H5	4.9	12.3	740	7.02	2.1	5.0	8.66	1.42	3.7	9.6	79.1	7.6	0.0	0.0
H6	5.0	12.1	629	7.02	3.8	4.2	10.81	0.26	5.8	11.8	63.4	11.3	2.3	5.4
H7	3.2	13.1	435	7.2	4.3[a]	5.4	5.42	5.15	3.8	4.2	91.0	0.9	0.0	0.0
H8	1.91	12.8	810	6.58	2.8	7.0	7.80	2.76	24.3	9.6	64.3	1.8	1.5	0.0
H9	4.15	10.7	606	7.27	3.7	4.3	7.19	2.49	6.9	9.9	81.0	2.1	0.0	0.0
H10	3.12	11.9	431	7.23	4.7	6.7	5.03	2.46	8.2	21.3	70.3	0.1	0.0	0.0
H11	9.18	13.2	361	6.85	3.6	9.0	5.53	1.01	8.2	15.7	73.8	1.9	0.2	0.0
H12	7.5	12.2	787	7.16	3.7	7.1	2.96	0.93	10.5	9.5	76.7	2.2	1.1	0.0

[a] Water depth below soil level estimated after March 2012 measures.

2.3. Water quality and sentinel exposure at the well scale

Ten wells were selected for the caging experiment exposing sentinel amphipods in groundwater (Fig. 1C, Table 1): 2 wells were located in the upstream part of each alluvial plain in landscapes dominated by forest and grassland (A1 and H3), 3 wells were located in landscapes with >20% of urban area (A2, A3 and A12) and the last 5 wells were located along a gradient of agricultural activities, from 65 to 83% of cultivated land (H4, H10, A8, H9 and A5).

To evaluate its sensitivity, the exposure experiment was carried out twice using identical approaches. Firstly, three months after the end of maize herbicide spreading, during the low groundwater level period of a rather dry year (September 2012) resulting in low transfer of pollutants and low contamination by pesticides and metabolites (hereafter 'low transfer period'). Secondly, just after the end of the maize herbicide application, during high groundwater level period of a rather wet year (July 2013) which generally results in high transfer of pollutants and high contamination (hereafter 'high transfer period').

At the beginning and the end of each cage experiments, water quality was evaluated by field measurements (pH, electrical conductivity, temperature, redox potential and dissolved oxygen) and laboratory analyses: water samples were analysed by ICP-AES for Ca^{2+}, Na^{+}, K^{+}, Mg^{2+} (uncertainty 5%), ion chromatography for Cl^{-}, SO_4^{2-}, NO_3^{-} (uncertainty < 10%) and potentiometric method according to N EN ISO 9963-1 for HCO_3^{-} and CO_3^{2-} (uncertainty 5%). All pesticides and metabolites were determined with a Waters UPLC system coupled to a Waters micromass MSMS (Waters Quattro-Premier XE/Q) after a solid phase extraction. Chromatographic separation for neutral and ionic compounds was done with a Waters Acquity UPLC BEH C18 column. Internal standards were used for calibration. Analytical details are available in Amalric et al. (2013) and the list of measured compounds with their detection limits are presented in Appendix 1.

As densities of stygobite amphipods were low in the studied area, we decide to use as sentinel an organism both frequent and living in close relation with subterranean water, i.e. at the surface/ground water interface, in springs and small streams. The selected species was an epigean amphipod of the genus *Gammarus* close to the species *G. orinos* (noted below *Gammarus* cf. *orinos*). The animals were captured in a neighbouring tributary of the Ariège River fed by groundwater, located at Artenac (43°25′28″N, 1°34′34″E, Maazouzi et al., 2016). They were kept for one week in the laboratory, fed with dead leaves and then caged in situ. Cages consisted of stainless iron netting cylinders (6 cm height × 1.5 cm diameter; 200 μm mesh) and closed by two plastic caps at each end (Marmonier et al., 2013). Six such cages, each containing five *G.* cf. *orinos* individuals, were placed in each well at 50 cm below the water level during both low and high transfer periods (i.e. 30 individuals per well and exposure period). The cages were retrieved after one week of exposure, a duration chosen to reduce the mortality due to starvation. Three ecophysiological parameters were measured on the specimens: (1) the survival rate, (2) glycogen and (3) triglyceride body concentrations. Three sets of three individuals (i.e. 9 individuals) found alive were frozen-dried at retrieval time and used for each biochemical analysis at each occasion (period/well). Glycogen and total triglyceride concentrations were measured by standard enzymatic methods using specific test combinations, according to Hervant et al. (1995, 1996). Ad hoc Sigma-Aldrich (France) glucose HK and GPO Trinder kits were used to determine glycogen and triglycerides, respectively. Glycogen concentration was expressed as mg of glycosyl, and triglyceride concentration was expressed as mg of glycerol. All assays were performed in an Uvikon 940 recording spectrophotometer (Kontron Inc., Germany) at 25 °C.

2.4. Data analyses

Species richness and abundances of stygobionts were compared between the Hers and Ariège alluvial plains using one-way ANOVA after log $(x + 1)$ transformation (significant level $p \leq 0.05$). Species richness, total abundances of stygobionts and abundances of the most frequent species were correlated to water chemistry (i.e. field measured parameters) and land use around the well, and regression curve was drawn when correlations was significant ($p \leq 0.05$). The assemblage composition was analysed by Non Metric Multidimensional Scaling and the Bray-Curtis dissimilarity index (NMDS, Legendre and Legendre, 1998). Chemical and land use characteristics around the well were fitted to the NMDS ordination to allow visualization of their contribution to the NMDS ordination. Analyses were performed using the R statistical software (R Development Core Team, 2013), using the vegan R package (Oksanen et al., 2012).

The Ecophysiological Index (EPI, Marmonier et al., 2013) was calculated in the sentinel study on surviving animals using the following equation:

$$\mathrm{EPI} = \mathrm{Survival\ rate} \times ([\mathrm{Gly}]/([\mathrm{Tri}] + [\mathrm{Gly}]))$$

where the sentinel survival rate (as %) is combined with the state of their energetic stores as a ratio between glycogen content (noted as [Gly]), which is the first energetic store substance used in response to acute stress (Maazouzi et al., 2011), and their total body stores, as estimated from the sum of triglyceride (noted as [Tri]) and glycogen concentrations. To avoid seasonal differences in animal energetic store before the experiment, we calculated the ratio between EPI of sentinel individuals exposed in wells and EPI of reference sentinels, i.e. stress-free animals caged at the laboratory in synthetic water (see Nogaro et al., 2008). The in situ/laboratory ratio for the EPI can vary between nearby 0 (high impact of well water on exposed sentinels) to nearly 1 or in some rare cases slightly above 1 (meaning no difference between exposed and reference organisms).

Correlations were calculated between EPI and water chemical characteristics. We additionally performed a multiple regression to specifically test (i) the chemical variables influencing this ratio and (ii) whether or not these influences were constant over the two experimental sessions (i.e. two periods of exposure with contrasting degree of water contamination). Given that the number of replicates (ten wells *per* two experimental sessions = twenty "replicates") was low compared to the total number of explanatory variables, we performed a regression on PCA axes (Jolliffre, 1982). Specifically, we ran a PCA on water quality variables (18 variables in total, see Table 4). We gathered the PCA coordinates of each well (*per* experimental session) along the first-five PCA axes, which summarized >82% of the total variation (see Table 4). This lead to a total of five synthetic explanatory variables correlated to the actual variables (see Table 4 for correlation values between each variable and each PCA axis). Then we built a mixed linear model with EPI ratio as the dependent variable and the five synthetic variables and the experimental session (categorical) as explanatory variables. To test for temporal consistency, two-way interactions between experimental session and each of the other variables was accounted for in the model. The well identity was included as a random effect to account for temporal pseudo-replication. This full model was reduced by building all possible models (i.e. all combination of variables) and ranking them according to their Akaike Information Criteria (AIC). The best-reduced model was that having the lower AIC.

3. Results

3.1. Stygobiont assemblages

2691 stygobite crustaceans, representing a total of 14 species were collected in situ among a total of 12,155 individuals and 36 taxa sampled. Six species were amphipods (from *Niphargus* and *Salentinella* genera, Table 2), four were copepods and four were ostracods. Molecular identifications reduced the number of otherwise unidentified specimens of amphipods by 40% (65 individuals remained unidentified because of small size, body degradation during pumping and DNA amplification failure). The spatial distribution of stygobionts was markedly heterogeneous. Abundances varied from 0 (A1, A2, A6, A13 and A15 wells) to >100 individuals per sample (in H8, Fig. 2), and taxonomic richness ranged from 0 to 5 species (in wells A12, H3, H5 and H6). The abundances in wells located in the Hers alluvial plain were significantly higher than those in the Ariège alluvial plain ($F_{1,26} = 15.94$, $p = 0.0004$; Table 2). In the same way, species richness was significantly higher in the Hers alluvial plain than along the Ariège River ($F_{1,26} = 8.39$, $p = 0.0075$; Table 2).

Table 2
Abundances (mean number of individuals per sample ± standard deviation) of stygobite species collected in the wells of the Ariège and Hers alluvial plains.

Groups	Species	Hers alluvial plain (n = 12)	Ariège alluvial plain (n = 16)
Amphipoda	*Niphargus kochianus*	12.8 ± 15.5	6.9 ± 11.3
	Niphargus ciliatus	2 ± 3.1	0.1 ± 0.3
	Niphargus foreli	2.3 ± 5.4	0
	Niphargus sp.	0.1 ± 0.6	0
	Salentinella major	2.8 ± 5.5	1 ± 2.4
	Salentinella petiti	1.3 ± 3.3	1 ± 2.8
Copepoda	*Ceuthonectes gallicus*	0	0.1 ± 0.5
	Diacyclops paolae	0.1 ± 0.3	0.4 ± 1.5
	Diacyclops clandestinus	0.1 ± 0.3	0
	Diacyclops belgicus	6.5 ± 23.0	0
Ostracoda	*Pseudocandona* sp. 1	0	1.4 ± 5.5
	Candonopsis boui	1.9 ± 4.6	0
	Pseudocandona sp. 2	2.2 ± 7.5	0
	Pseudocandona sp. 3	13.0 ± 40.5	0
Abundances		48.1 ± 40.3	9.4 ± 11.3
Richness		2.8 ± 1.4	1.1 ± 1.1

Abundances and species richness varied strongly between wells in both Hers and Ariège Rivers (see standard deviations in Table 2). This variability between wells was not linked to the distance of wells to the river since no significant correlation was detected between species abundance or species richness and the distance to the river ($p > 0.05$ for both distances to Ariège and Hers Rivers for A and H wells, respectively) nor with the upstream-downstream location of the wells along the rivers ($p > 0.05$ for both Ariège and Hers Rivers). In the same way, no significant correlation was found between these assemblage characteristics and the water characteristics (i.e. dissolved oxygen, temperature, or electrical conductivity; in all cases $r^2 < 0.08$, $p > 0.05$). In contrast, the abundances and species richness of stygobionts significantly changed with the human activities around the well. Abundances were highest at intermediate values of agriculture practices (between 30 and 60%) and lower outside this range ($r^2 = 0.158$, $p = 0.018$, Fig. 2B). This change in abundances was mainly due to an increase in the number of *N. kochianus* ($r^2 = 0.125$, $p = 0.030$). Inversely, species richness decreased with the percentage of urban area around the well ($r^2 = 0.134$, $p = 0.027$, Fig. 2C and D). In all cases, the correlation coefficients were weak and a large dispersion of wells was observed for high values of abundances and species richness (Fig. 2B and D).

The NMDS analysis (Fig. 3, stress = 0.054) highlighted some heterogeneity in the assemblages composition. A large number of wells were grouped close to the origin of the first and second axes (noted NMDS1 and NMDS2 respectively on Fig. 3), because of high abundances of *N. kochianus* and, to a lesser extent, of *N. ciliatus* in most of the wells (Table 2 and Fig. 4). This group of wells underwent very different anthropogenic activities, from traditional agriculture with dominant grasslands (A3 and A4 along the Ariège, H2, H5, H6 along the Hers alluvial plain) to intensive agriculture with maize as monoculture (A14, H7 to H12). Similarly, the six wells excluded from the analysis because of the absence of stygobite crustaceans (reported in a box in Fig. 3A) were located in sectors dominated by either traditional (A1, A2) or intensive agriculture (A9, A13, A15).

Two wells were located out of this central group on the most negative side of NMDS axis 1 (H8 and H9) because of the absence of *N. kochianus* and the occurrence of two rare species (*Diacyclops clandestinus* and *Pseudocandona* sp. 3, Figs. 3B and 4). On the opposite side of NMDS axis 1, the well H4 was isolated because of the occurrence

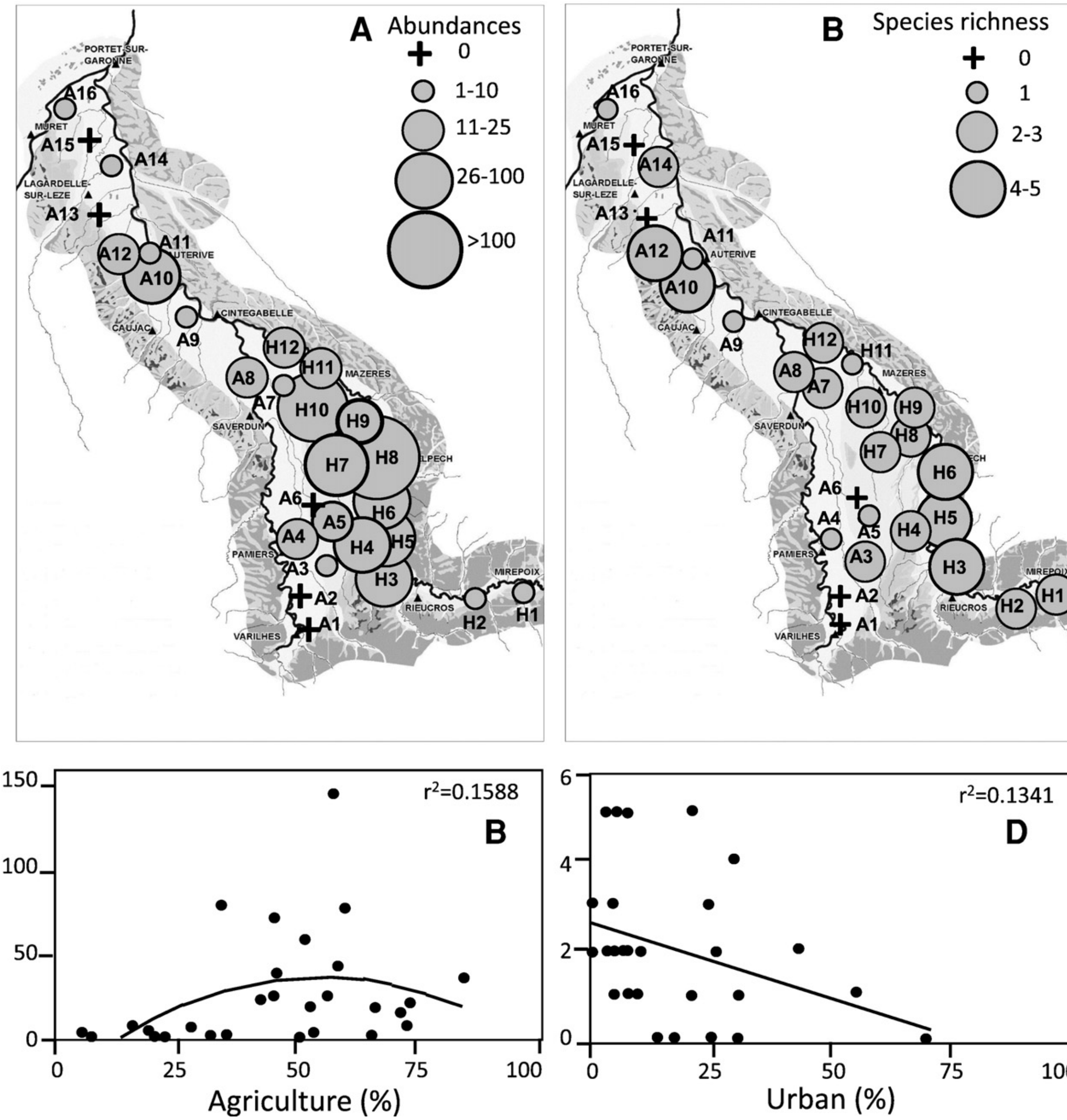

Fig. 2. (A) Abundances of stygobites as number of individuals per sample transformed in classes plotted on the sampling area (the size of the circle is proportional to the abundance). (B) Relation between abundances and the percentage of agriculture around the wells ($y = -0.019 \times ^2 + 2.095x - 22.05$, $p = 0.018$). (C) Species richness as the number of species transformed into classes plotted on the sampling area (the size of the circle is proportional to the richness). (D) Relation between richness and the percentage of urban area around the wells ($y = -0.0343x + 2.63$, $p = 0.027$).

of the rare *Diacyclops belgicus* (Figs. 3B and 4). Considering environmental factors, NMDS axis 1 was correlated with the percentage of grassland on the positive side and the percentage of cultivated land on the negative side (Table 3).

Similarly, three wells (A7, A8 and A11) were outside of the central group and located on the negative side of the NMDS axis 2 because of low abundances of *N. kochianus* and high abundances of *Salentinella* (both *S. major* and *S. petiti*, Fig. 3B). *Salentinella* species also occurred in wells A12, H1, H3 and H6, all located on the negative side of the NMDS axis 2. This second axis was structured by wells surrounded by high percentage of forest (Fig. 3C), although this correlation was not statistically significant (Table 3). Finally, the scores on the NMDS axis 2 of the 16 wells located in the Ariège alluvial plain were positively correlated to the distance to the river ($r^2 = 0.438$, $p = 0.038$): the well A11 located at 100 m of the river and the well A8 at 600 m yielded assemblages with only few or no *N. kochianus* nor *N. ciliatus*, despite the presence of the amphipod *Salentinella* (Fig. 4). Conversely, no significant correlation between the distance to the river and NMDS scores nor the abundances of amphipods was found for the Hers River (all $p > 0.05$), but here no wells were located at less of 300 m of this river.

3.2. Sentinels exposure

The analyses of pesticides and metabolite concentrations confirm that pollution was lower during the low transfer period, several months after maize herbicide applications (maximum total herbicide concentration of 2.89 μg/L and 0.93 μg/L for *S*-metolachlor, the predominant herbicide molecules) than during the high transfer period, few weeks after maize herbicide applications (maximum total herbicide of 9.59 μg/L and 5.34 μg/L for *S*-metolachlor and its metabolites). At both

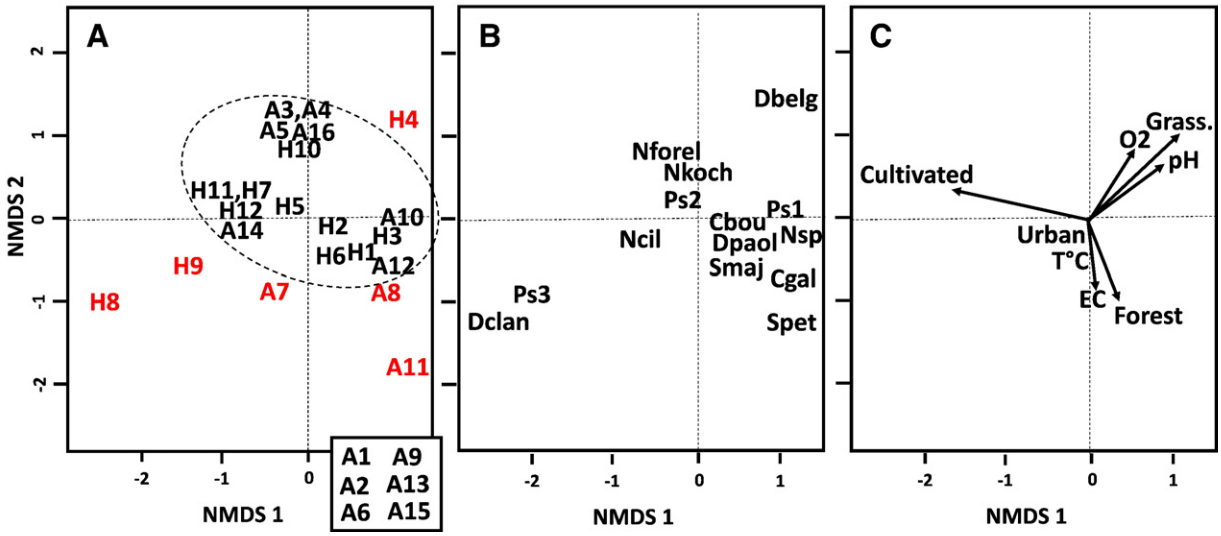

Fig. 3. NMDS of stygobite crustacean assemblages. The biplot of the first two axes is separated into wells (A) and species (B). Environmental characteristics are fitted to this biplot (C). Six wells listed in the box (A) were excluded from the analysis because of the absence of stygobites and 6 were noted in red because of their location out of the central group (noted in black). Cbou: *Candonopsis boui*; Cgal: *Ceuthonectes gallicus*; Dbelg: *Diacyclops belgicus*; Dclan: *Diacyclops clandestinus*; Dpaol: *Diacyclops paolae*; Ncil: *Niphargus ciliatus*; Nforel: *Niphargus foreli*; Nkoch: *Niphargus kochianus*; Nsp: *Niphargus sp.*; Ps1, Ps2, Ps3: *Pseudocandona* sp. 1, sp. 2 and sp. 3; Smaj: *Salentinella major*; Spet: *Salentinella petiti*. Land use types included are the percentages of forest, cultivated area, grassland, and urbanized areas. Chemical characteristics included are dissolved oxygen (O2), temperature (T°C) and electrical conductivity (EC).

periods, survival of sentinels was always high (86.4 ± 18.2% for one week), but EPI changed consistently (Fig. 5). Weak EPI differences were observed during low transfer period whereas an upstream-downstream gradient was evident during high transfer period (Fig. 5). The EPI was close to 1 (i.e. similar to the uncontaminated laboratory controls) in the upstream wells (A1, A2, A3, A4) whereas low values occurred in the downstream wells (H9, H10, A8, A10). During the high transfer period, the EPI values were positively related to dissolved oxygen ($r^2 = 0.420$; $p = 0.021$) and negatively related to nitrate concentrations ($r^2 = 0.370$; $p = 0.031$; Fig. 6). Such relationships were not observed during the low transfer period despite a similar range of variation in dissolved oxygen and nitrate concentrations (Fig. 6). In addition, we did not find any significant correlation between EPI and the percentage of land covered by agriculture around the wells, the total pesticides concentrations nor each pesticide considered separately (in all cases $p > 0.05$).

When all chemical parameters were considered together, the best-reduced model (AIC = 7.4, $W_i = 46.0\%$) included PCA axes 1, 2 & 5 (Table 4). In the best-reduced model, the relationships between of PCA axes 2 and 5 and EPI varied between the two experimental sessions (i.e. significant two-way interactions between PCA axes 2 and 5 and the experimental session). These interactions suggest that the effects of the variables associated with these two axes were not consistent over time. In contrast, the relationship between PCA axis 1 and EPI was negative both during the first and second experimental sessions, which demonstrated a highly consistent effect (Fig. 7). The strong correlation of this PCA axis with atrazine-related compounds (i.e. correlations between atrazine, deisopropylatrazine, and DEA and PCA axis 1 were above 0.87, Table 4) suggests a potential relationship between EPI decrease and the combination of these pesticides and metabolites.

4. Discussion

The need for ecological indicators that allow an appropriate assessment of groundwater quality has been advocated by several authors as a warning about the sensitivity of these systems to human activities (e.g. Marmonier et al., 1993; Danielopol et al., 2004; Griebler et al., 2010). In this study, indicators based on crustacean assemblage compositions were combined with in situ sentinel exposure yielding complementary results.

4.1. Stygobiont assemblages of the Hers and Ariège alluvial plain

The richness of the stygobiont assemblage sampled in the present study (i.e. 14 crustacean species) is high for the sampling effort (28 wells sampled at a single period). Alluvial plains of large European rivers harbour more diverse assemblages (22, 29 and 36 crustacean species in the Rhône, the Rhine and the Danube Rivers respectively, Dole-Olivier et al., 1994), but in all cases with a higher number of sampling stations and long term studies.

In comparison with previous studies conducted in the Ariège alluvial plain in 1999 (Dumas, 2000, 2002) and in 2003 (Janiaud, 2004), the composition of stygobite assemblages sampled during this study differed slightly. The richness was weakly higher with 22 species for 5 sampling occasions (Dumas, 2002), but the dominant species (e.g. *Niphargus kochianus-pachypus* group, *Salentinella petiti*) remained the same in all investigations and only few differences were observed for the less abundant species.

Moreover, some of these differences may be linked to recent changes in the systematics of the crustaceans or progress in species identification methods (e.g. *Diacyclops paolae* was not described when Dumas worked), to differences in sampled wells (some of the 15 wells studied by Dumas were not usable now) and to the number of sampling periods (4 in Dumas, 2000, a single one in this study). These differences prevent adequate comparison between studies.

4.2. Stygobiont assemblages and water quality

The distribution of the crustacean assemblages varied strongly in the two alluvial plains, as already observed in the Danube and Rhône floodplains (Danielopol, 1989; Dole and Mathieu, 1984) or in small streams (e.g. Malard et al., 2002, 2003; Fiasca et al., 2014). But contrary to these studies, no link was observed between faunal characteristics and water chemistry. When stygobiont assemblages were sampled, water chemistry was limited to few parameters (i.e. temperature, pH, dissolved oxygen, electrical conductivity) that may not describe correctly groundwater heterogeneity (Boulton et al., 1998). Analyses of the different forms of Nitrogen (ammonia for example, Di Lorenzo et al., 2015) may have bring explanations for the changes in stygobiont abundances. In contrast, significant correlations were observed between abundance or species richness and the land use around the well

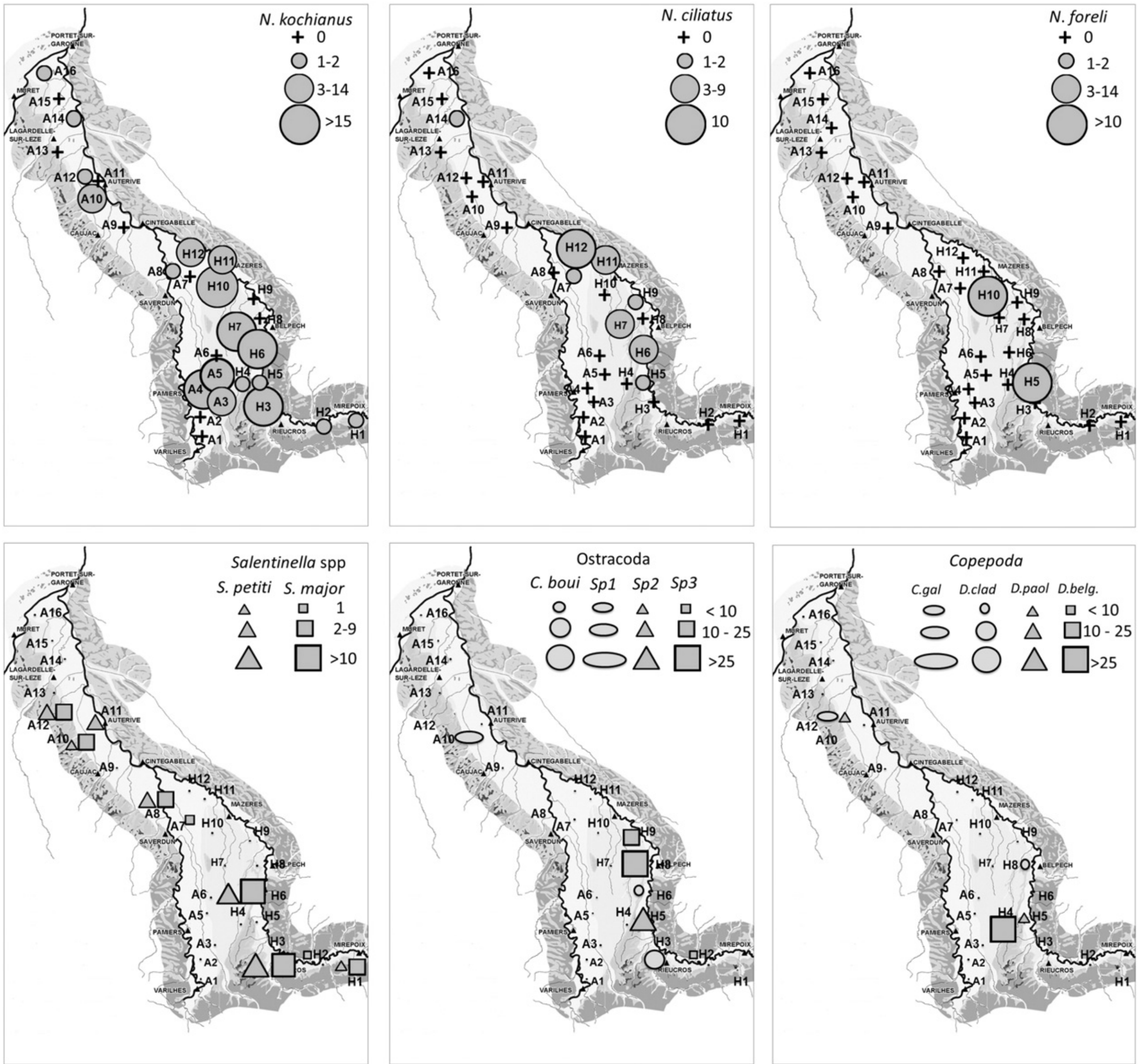

Fig. 4. Spatial distribution of stygobites crustaceans in the Ariège and Hers alluvial plains (as number of individuals per sample transformed in classes). Upper panels: *Niphargus* species. Lower panels: *Salentinella* spp.; Ostracoda: *Candonopsis boui*, *Pseudocandona* sp. 1, sp. 2 and sp. 3; Copepoda: *Ceuthonectes gallicus*, *Diacyclops clandestinus*, *Diacyclops paolae*, *Diacyclops belgicus*.

(agriculture and urban area, respectively) and the composition of the assemblages. Similar links between stygobiont assemblages and land use were already observed, but in most cases in the hyporheic habitat of rivers (Hahn, 2002; Sarriquet et al., 2006). The local human activities around the well explained thus a part of the stygobite assemblage composition and our first hypothesis was thus partly supported.

Table 3
Scores of land use and physical and chemical characteristics along the first (NMDS1) and second (NMDS2) NMDS axes. Goodness-of-fit statistics between variables and NMDS ordination is given by a squared correlation coefficient (R^2) and the significance of the fit is given by a p-value (noted with * when $p < 0.05$).

	NMDS1	NMDS2	R^2	p-Value
Urban	−0.044	−0.9990	0.005	0.949
Forest	0.3563	−0.9344	0.134	0.248
Grassland	0.7362	0.6767	0.285	0.046*
Cultivated	−0.976	0.2186	0.343	0.022*
Oxygen	0.5587	0.8294	0.105	0.362
Temperature	0.0052	−0.9999	0.019	0.840
Conductivity	0.106	−0.9944	0.088	0.408
pH	0.799	0.602	0.159	0.203

Two other factors may influence the distribution of groundwater fauna in the studied area. Firstly, the major trend observed in the distribution of stygobionts crustaceans in the studied sector was the significant difference in abundance and species richness between the assemblages sampled as well in the Ariège and in the Hers groundwater systems. These differences are difficult to explain using only the present set of environmental variables. Similar ranges for pH (an important factor for crustacean moult), dissolved oxygen (that can limit crustacean survival), and electrical conductivity (often linked to water origin and circulation patterns) were measured in the two alluvial plains when assemblages were sampled. Similarly, no obvious differences in land use characteristics could be found between the Ariège and Hers alluvial plain (Fig. 1A). Differences in hydrogeological characteristics may explain this between-plain heterogeneity, through changes in sediment characteristics, movement of water and pesticides, or nutrient availability, as recently observed in the same area for bacterial communities (Mauffret et al., 2017). Secondly, the distance to the river influenced the composition of stygobite assemblages. This influence was already

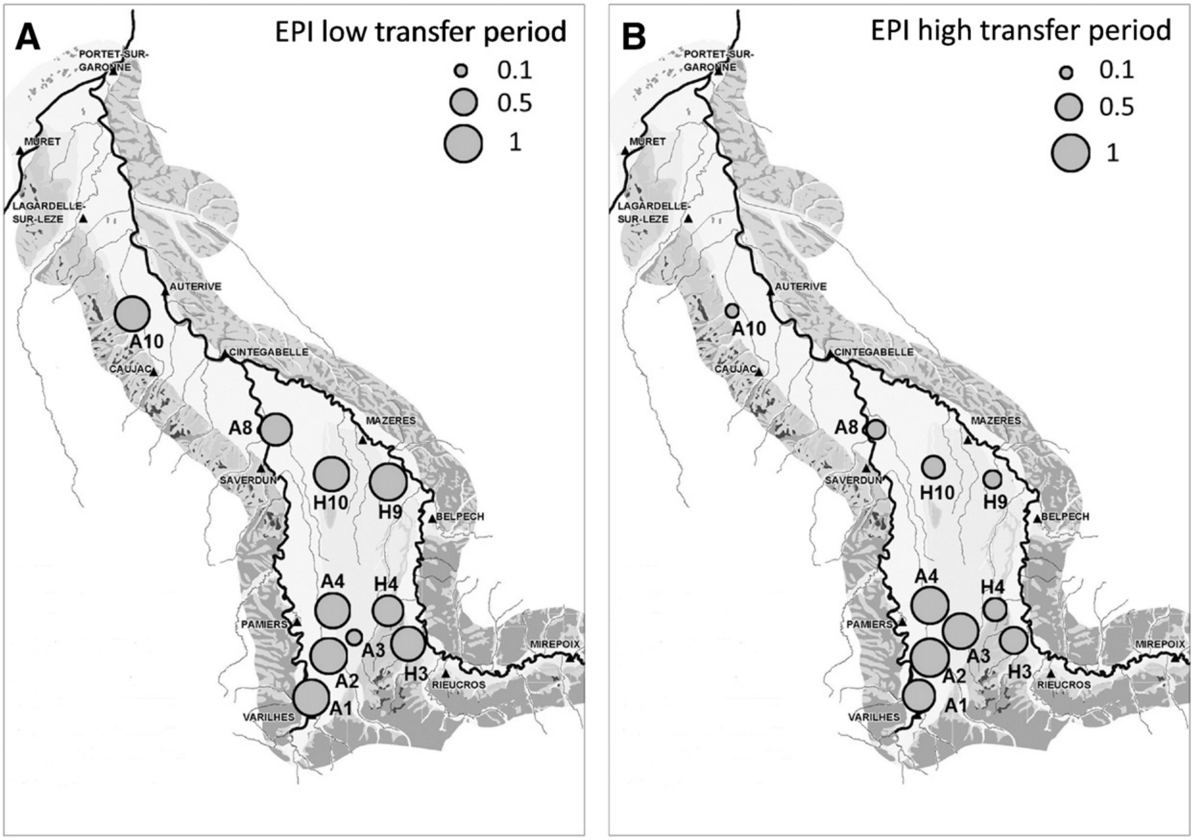

Fig. 5. Values of the Ecophysiological Index (EPI) using *Gammarus* cf. *orinos* sentinels after one-week exposure to in situ groundwater (A) during low transfer period (September 2012) with low agricultural impact and (B) during high transfer period (July 2013), with high agricultural impact. EPI values are expressed as the ratio between in situ exposure and laboratory control ratios, ranging from zero (maximum in situ impact on amphipod ecophysiology) to 1 (minimal impact).

reported in 1999 using 15 wells sampled along the Ariège River (Dumas, 2000, 2002). We found a similar influence of the distance to the river for the 16 wells located in the Ariège alluvial plain, where assemblage composition (i.e. the scores on the NMDS axis 2) was correlated to the distance to the river: *N. kochianus* decreased or disappeared in wells very close to the river. Distance to the rivers may reflect the gradual influence of Ariège water with buffered temporal variability at long distance (Dole and Chessel, 1986), but not in the case of the Hers alluvial plain where the nearest well was located at >300 m of the river.

Therefore, our results suggest that classical metrics of groundwater crustacean assemblages, such as species composition, abundance and richness, may be useful for the evaluation of the impact of land use (here intensive agriculture and urbanization) on groundwater ecosystems. Contrasted results can be found in literature about the relation

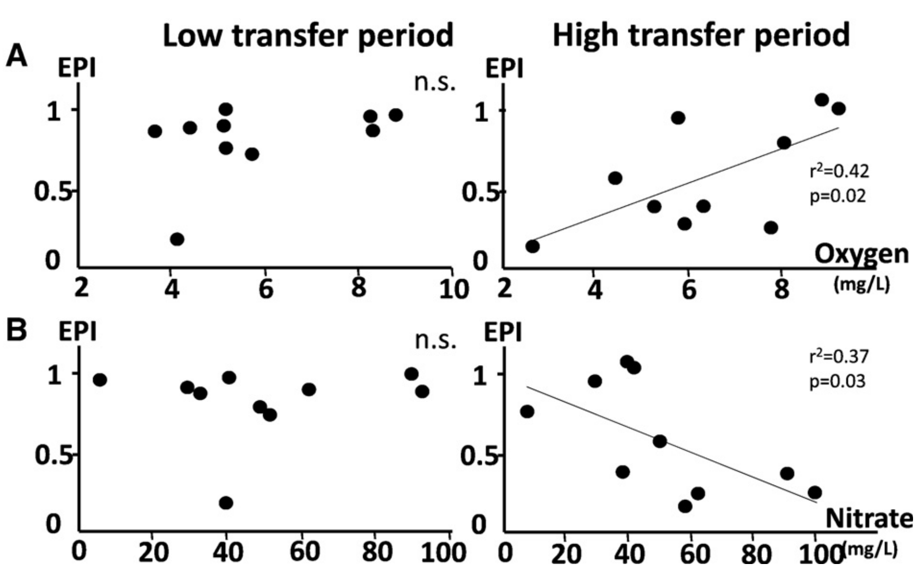

Fig. 6. Relationships between EPI and water chemical characteristics (A) dissolved oxygen and (B) nitrate concentrations in period of low transfer (September 2012, left column, n = 10) and high transfer periods (July 2013, right column, n = 10). Regression line, r^2 and p-values are reported when significant (n.s. non-significant).

Table 4
First-five axes of a PCA performed on chemical parameters and pollutants. Data below represent correlations between each PCA axis and each input variable. The percentage of variance explained by each axis is indicated under brackets. Bolded values indicate the most important variables loading along each axis using an arbitrary threshold of 0.5.

Variables	Axis 1 (30.11%)	Axis 2 (21.14%)	Axis 3 (13.70%)	Axis 4 (10.08%)	Axis 5 (7.63%)
Atrazine	**0.879**	−0.118	0.157	0.245	−0.018
Desethylatrazine	**0.934**	0.016	−0.003	0.097	0.053
Deisopropylatrazine	**0.960**	−0.056	0.015	0.098	0.115
Simazine	**0.762**	−0.205	0.066	0.207	−0.179
Metolachlor	−0.144	**0.738**	**0.568**	−0.258	−0.098
OXA Metolachlor	−0.162	**0.739**	**0.573**	−0.270	−0.113
ESA Metolachlor	**0.525**	0.365	**0.587**	−0.419	−0.042
Acetochlor Ethane sulfonic	−0.242	0.147	−0.227	−0.053	**0.763**
Alachlor Ethane sulfonic	**0.574**	0.219	−0.077	0.308	0.327
Conductivity	−0.024	**0.843**	−0.212	0.440	−0.022
pH	**−0.512**	0.321	0.289	**0.524**	0.105
Water temperature	0.099	−0.008	**−0.706**	−0.327	**−0.506**
Dissolved oxygen	**−0.553**	**−0.501**	0.198	0.384	0.088
Eh	0.205	**−0.671**	0.388	−0.001	−0.009
Nitrate (NO_3)	0.430	0.472	−0.254	−0.320	0.475
Natrium (Na)	**0.838**	0.284	−0.031	0.224	−0.164
Sulfate (SO_4)	−0.018	0.496	**−0.703**	−0.174	−0.057
Carbonate (HCO_3)	−0.228	**0.623**	−0.090	**0.612**	−0.285

between groundwater assemblages and land use. On one hand, Di Lorenzo and Galassi (2013) found stygobite species richness and abundance to be non-sensitive to nitrate concentrations (up to 150 mg/L) in the alluvial aquifer of the Vibrata River in Italy, even if long-term effects could not be ruled out (Di Marzio et al., 2013). Similarly in Germany, Hahn (2006) and Griebler et al. (2010) found inconsistent correlations between diversity and abundance of groundwater fauna and physical and chemical variables. On the other hand, Di Lorenzo et al. (2015) observed that groundwater assemblages sampled from bores in the alluvial plain of the River Adige were sensitive to NH_4^+ concentrations (here ≥0.032 mg/L). Similarly, Di Lorenzo et al. (2014) and Boulal et al. (1997) observed changes in abundances, species richness and composition of stygobite crustaceans with local pollution context. These contrasted results are difficult to explain. The first reason may be the differences in the sensitivity of stygobite crustaceans to agricultural pollutants (Marmonier et al., 2013; Maazouzi et al., 2016). Another reason is the reduce set of water characteristics available during the stygobiont assemblage study, especially the lack of ammonia and nitrite measurements (Di Lorenzo et al., 2015). Moreover, it seems that the effect of chronic toxicity of pollutants used in agriculture do not induce direct mortality (and changes in assemblage composition), while it alters the kinetics of the development of individuals. For example, Di Marzio et al. (2013) observed that chronic exposure of interstitial copepods to ammonium and herbicides (the carbamate pesticide Aldicarb) prolonged the developmental time before the adult stage. Future research may include measures of the development rate of organisms or population age structure.

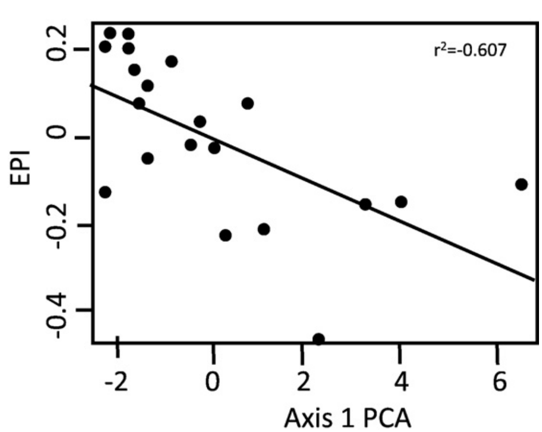

Fig. 7. Relationships between EPI and the first axis of a PCA performed on toxicants (period of high and low transfer cumulated, n = 20). See Table 4 for variable loadings. EPI is represented as the residuals of a GLMM linking EPI to PCA axes 2 and 5, experimental sessions and the two resulting two-term interactions.

4.3. Relevance of sentinels in the evaluation of groundwater ecosystem health

Sentinel organisms caged in groundwater is a recent strategy (Marmonier et al., 2013) with the use of stygobionts for long-term exposure assessing chronic pollution and the use of epigean species for short-term exposure assessing acute toxicity. Unfortunately, we did not find large numbers of stygobionts in the studied area (300 living individuals of the same species are necessary for 10 wells; Marmonier et al., 2013). So, we used a native and abundant epigean amphipod, *Gammarus* cf. *orinos* living at the interface between groundwater and surface water (e.g. springs and small spring-fed streams). However, the use of an epigean species limited the exposure duration to one week to reduce mortality during starvation because of higher physiological needs of surface water organisms (Hervant et al., 1998). Nevertheless, the use of sentinel was efficient: we observed differences in the EPI score among crop-based contexts during the period of high water transfer and high pollution, i.e. during or just after a period of intensive agriculture activities and pesticide applications.

Agriculture-derived human activities weakly altered sentinel health, with an effect only on energetic stores but not on survival rates. Similarly, Marmonier et al. (2013) found an effect of urban stormwater infiltration on EPI scores (mainly due to inputs of DOC and related decreases in dissolved oxygen concentration, Foulquier et al., 2010) but only weak effects on sentinel survival rates. Maazouzi et al. (2011) showed that energetic stores, especially glycogen content, are valuable indicators of environmental conditions for amphipods. The significant correlations of EPI with dissolved oxygen (positive) and nitrate concentrations (negative) in the Ariège and Hers alluvial plains during the high water transfer period suggest a link between sentinel health and water quality linked to agricultural practices.

As observed in earlier investigations in the studied area (Amalric et al., 2013), *S*-metolachlor as well as its major metabolites (ethane sulfonic acid and oxolinic acid) were the predominant pesticides found in groundwater, followed by atrazine and two metabolites (deisopropylatrazine and deethylatrazine, noted DEA). *S*-Metolachlor, one of the most commonly used herbicides in this area (Water Agency, pers. comm.), is prone to leach into groundwater (Hladik et al., 2008; Baran and Gourcy, 2013). Atrazine widely used for decades was withdrawn from agricultural practices in 2003. Nevertheless, atrazine and its major metabolite DEA have persisted in groundwater (Baran et al., 2007). When all pesticides were considered together, the significant correlation of EPI scores with PCA scores suggests that sentinel health can be negatively impacted by atrazine and two of its metabolites deisopropylatrazine, and desethylatrazine-DEA (Table 4). This herbicide is lowly to moderately toxic for crustaceans. The median effective concentration (EC_{50} for immobility after 48 h) of atrazine for *Daphnia magna* is very high (35.5 ± 9.2 mg/L, Palma et al., 2008) and reached 72 mg/L for the median lethal concentration (LC_{50} after 48 h, Wan et al., 2006). For the amphipods, the LC_{50} of atrazine is 7.5 mg/L for *Gammarus pulex* (for 48 h, Lukancic et al., 2010) and 10.1 mg/L for *G. italicus* (for 96 h, Pantani et al., 1997).

To our knowledge the toxicity of deisopropylatrazine for amphipods is unknown, but we measured the acute toxicity of DEA and *S*-metolachlor for our sentinel species, *G.* cf. *orinos*, that reached 10.1 (±1.1) mg/L for DEA and 11.2 (±1.1) mg/L for *S*-metolachlor (LC_{50} for 96 h at 11 °C, Maazouzi et al., 2016). In contrast, atrazine concentrations in the groundwater of the studied area ranged from below the limit of quantification (<LQ) to 0.205 μg/L, deisopropylatrazine from <LQ to 0.236 μg/L, DEA from <LQ to 0.794 μg/L and *S*-metolachlor

from < LQ to 5.34 μg/L. These field values may impact microbial communities (Mauffret et al., 2017), but were far below the toxicity limit for Amphipods (for 96 h laboratory toxicity tests) and may explain why no increase in mortality was observed for sentinel organisms exposed to groundwater polluted by these pesticides even for a one week duration.

Contrastingly, the Ecophysiological Index decreased for the animals exposed during the high transfer period with higher water contamination. This decrease in EPI in wells surrounded by agriculture may result from the combination of several stressors, such as low values of dissolved oxygen, high nitrate concentrations or most obviously by the occurrence of pesticides. Thereby, the EPI represents an early indicator of field water toxicity compared to simple laboratory mortality test. In the future, testing the use of stygobite sentinels for longer exposure times (e.g. one month) that may integrate several episodes of pollution (e.g. Di Lorenzo et al., 2014) appears critical. In addition, we advocate for repeated exposure experiments (i.e. at more than two periods) along a single year, to account for changes in agriculture activities and related groundwater quality. Finally, the EPI may be improved and enriched with other potential ecophysiological indicators such as reproduction activity, development rates, or by proteomic analyses to determine the specific proteins indicative of particular stressors (Armengaud et al., 2014).

4.4. Comparison of the two methods

The two methods were used in the same 10 wells, but at very different periods: groundwater assemblages were sampled in January (i.e. before the beginning of farming activities), while the cage experiments and the measures of pesticides in groundwater took place in June and September (i.e. during and after pesticide applications). These differences prohibit any direct comparison between methods, but the different ways organisms perceived local disturbances may be compared.

Firstly, the two methods integrate groundwater characteristics at different time scales. On one hand, the sentinels were in contact with the studied environment for only short periods (here one week) and may be spared by short time peak pollution linked to temporary farming activities (Baran et al., 2007; Hildebrandt et al., 2008). This is clearly supported by the contrasted results obtained in this study, with poor decrease in sentinel health during low pollution period (September) compared to the marked gradient in EPI observed during the pesticide application period (June). On the other hand, stygobiont assemblages (here crustacean abundances and richness) integrate characteristics of the groundwater environment over a long period. Stygobionts have generally very long life duration (e.g. several years for most *Niphargus* species, e.g. Turquin, 1984, and several months for some Candonidae ostracods, Danielopol, 1980) and thus react to environmental characteristics over long periods. The different groundwater species have a wide range of sensitivity to various types of disturbances. For example, some stygobiont amphipod are known to resist to hypoxia better than epigean organisms (e.g. *Niphargus* species, Hervant et al., 1995, 1996), while some stygobiont copepods are more sensitive to pollution than surface dwellers (Di Marzio et al., 2009). This results in contrasted assemblage composition in wells with different environmental characteristics, as observed by Hahn (2006), Griebler et al. (2010), Stein et al. (2010), Boulal et al. (1997) and highlighted by this study where the composition of stygobiont assemblages was mainly related to land use type (i.e. a relatively long term characteristics).

Secondly, the evaluation of the potential water toxicity by the two method is based on contrasted range of sensitivity. The sentinel method focussed on the evaluation of water quality from the sensitivity of a single species (the epigean *Gammarus* cf. *orinos*), while stygobiont assemblages include several species (here 14 species) representing a wide range of sensitivity. In this study, very similar upstream-downstream gradient along the Ariège and Hers alluvial plains were observed for the sentinel health (at least during the high transfer period, Fig. 5) and the abundances of *Niphargus* gr *kochianus* and *Niphargus ciliatus* (Fig. 4), while the two *Salentinella* species did not follow this decreasing trend (Fig. 4). Differences in sensibility to disturbances were already highlighted between stygobiont species, for example for amphipods confronted to hypoxia (Hervant et al., 1998) or for hyporheic copepods facing pesticides (Di Marzio et al., 2009).

Thirdly, the way the organisms are in contact with potential toxic compounds differs in the two methods. The cages of the sentinel organisms are hanged inside the well at distance of the bottom sediment (in this study at 1 m from the well bottom). The animals are thus only in contact with the water flowing in the well and their health only impacted by fluxes of dissolved compounds linked to local human activities (here during pesticide application period). In contrast, the stygobiont assemblages are sampled by pumping at the surface of the sediment (Dumas and Fontanini, 2001). Here animals are in contact and fed directly on fine sediment that may be locally anoxic, generating reduced forms of Nitrogen (Dahm et al., 1998) or adsorbing a wide range of molecule (Baran and Gourcy, 2013). Stygobionts assemblages may thus integrate environmental characteristics on long period resulting in densities and richness more closely related to land use around the well than to water chemical characteristics.

When all these differences are considered together, it is clear that these two methods are more complementary than similar to assess groundwater ecosystem health. The combination of these two approaches may have major applications for orientating groundwater ecosystem management: combining long term and short term observations, single organism sensitivity or wide range of species with contrasted ecological requirements, evaluation of flowing groundwater alone or in association with the sediments.

5. Conclusions

Four major conclusions arise from this study.

(1) At the aquifer scale, the expected link between abundances, species richness and composition of stygobite assemblages and human activities in the vicinity of the studied wells (measured with the land use parameters) appears significant, but with a low predictive power. Other variables must be included in future evaluation strategies, such as other biological characteristics (e.g. development rate of organisms, population structure) or other organisms, such as oligochaetes, molluscs, and bacteria. The microbes, and especially bacteria, represent a considerable "hidden" biodiversity now quantifiable with molecular techniques (Voisin, 2017). As macroinvertebrates, the structure of microbial assemblages may be now used for groundwater ecosystem evaluation (Griebler et al., 2010, 2014).

(2) Consistent results with a decrease in the ecophysiological status of sentinel species can be obtained (i.e. after one week of exposure in wells with low dissolved oxygen and high nitrate concentrations in this study). The relationships between the EPI values measured on sentinels and the occurrence of some organic molecules (e.g. atrazine and its metabolites, deisopropylatrazine and DEA) suggests that sentinels are submitted to a combination of several stressors.

(3) The combination of approaches based on assemblage composition (long-term trends for community development) and sentinel exposure (short-term response to environmental constraints) yields consistent evaluation of groundwater ecosystem health.

(4) The effect of contaminants on sentinel health increased from the low- to the high-pollution transfer periods. Longer exposure times and repeated exposure along the year may help for a consistent evaluation of groundwater pollution in areas of intensive agriculture. More comprehensive sampling strategies for toxicants, such as Integrative Sampling Techniques, are needed together

with enriched ecophysiological indicators, such as individual development rates or proteomic analyses.

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Appendix 1. List of analysed compounds, their type (H = herbicides; MH = herbicide metabolites; F = fungicides), and their quantification limits

	Type	Quantification limit (μg/L)
Acetochlor	H	0.005
Alachlor	H	0.005
Ametryne	H	0.005
Atrazine	H	0.005
Chlortoluron	H	0.005
Cyanazine	H	0.005
Desethyl atrazine	MH	0.005
Desethyl terbuthylazine	MH	0.005
Desisopropyl atrazine	MH	0.005
Desmetryne	H	0.005
Diuron	H	0.01
Hexazinon	H	0.005
Isoproturon	H	0.005
Isoproturon-1CH3	MH	0.005
Isoproturon-2CH3	MH	0.01
Linuron	H	0.005
Metazachlor	H	0.005
Metolachlor	H	0.005
Prometryne	H	0.005
Propazine	H	0.005
Propyzamide	H	0.005
Sebuthylazine	H	0.005
Simazine	H	0.005
Terbuthylazine	H	0.005
Terbutryne	H	0.005
Flusilazole	F	0.005
Tebuconazole	F	0.005
Tetraconazole	F	0.005
OXA Metolachlor	MH	0.01
ESA Metolachlor	MH	0.01
OXA acetochlor	MH	0.01
ESA Acetochlor	MH	0.01
OXA Alachlor	MH	0.01
ESA Alachlor	MH	0.01
Glyphosate	H	0.05
AMPA	MH	0.05
Deschloro Metolachlor	MH	0.005
2-Ethyl 6-Methyl 2-Chloroacetanilide	MH	0.005
2-Hydroxy Metolachlor	MH	0.01
2-Ethyl 6-methyl aniline	MH	0.005
Metolachlor morpholinone	MH	0.01
2-Ethoxy metolachlor	MH	0.005
2-Chloro 2′,6′-diethyl acétanilide	MH	0.005
2,6-Diethylaniline	MH	0.005
Dimethanamid	H	0.005

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