

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

Pierre Marmonier, Chafik Maazouzi, Nicole Baran, Simon Blanchet, Amy Ritter, Maritxu Saplairoles, Marie-José Dole-Olivier, Diana M.P. Galassi, David Eme, Sylvain Dolédec, et al.

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13	Marmonier Pierre <sup>1†</sup> , Maazouzi Chafik <sup>1</sup> , Baran Nicole <sup>2</sup> , Blanchet Simon <sup>3,4</sup> , Amy Ritter <sup>3</sup> ,
14	Saplairole Maritxu <sup>5</sup> , Dole-Olivier Marie-José <sup>1</sup> , Galassi Diana M. P. <sup>6</sup> , Eme David <sup>1</sup> , Dolédec
15	Sylvain & Piscart Christophe <sup>1,7</sup> .
16	
17	
18	Authors address:
19	<sup>1</sup> UMR-CNRS 5023 LEHNA, Université de Lyon, Université Lyon 1, 43 Boulevard du 11
20	Novembre 1918, 69622 Villeurbanne, France.
21	<sup>2</sup> Bureau de Recherches Géologiques et Minières, 3 Avenue Claude Guillemin, BP 6009,
22	45060 Orléans Cedex 2, France.
23	<sup>3</sup> Centre National de la Recherche Scientifique (CNRS) ; Station d'Ecologie Expérimentale
24	UMR 5321, F-09200 Moulis, France.
25	<sup>4</sup> Centre National de la Recherche Scientifique (CNRS), Université Paul Sabatier, École
26	Nationale de Formation Agronomique (ENFA); UMR5174 EDB (Laboratoire Évolution &
27	Diversité Biologique), 118 route de Narbonne, F-31062 Toulouse cedex 4, France.
28	<sup>5</sup> Bureau de Recherches Géologiques et Minières, 3 rue Marie Curie, B.P. 49, 31527
29	Ramonville-Saint-Agne, France.
30	<sup>b</sup> Department of Life, Health & Environmental Sciences, University of L'Aquila, 67100
31	L'Aquila, Italy.
32	' Present address: UMR-CNRS 6553 ECOBIO, Campus de Beaulieu, Bâtiment 14A, 263
33	Avenue du General Leclerc, 35042 Rennes, France.
34	Corresponding author: Pierre Marmonier
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#### 36 **ABSTRACT**

37 Ecological criteria are needed for a comprehensive evaluation of groundwater ecosystem health including biological components along with the physical and 38 39 chemical properties that are already required by European directives. We evaluated 40 the performance of two different methodological approaches for an ecological 41 assessment of groundwater ecosystems in two alluvial plains (the Ariège and Hers 42 rivers, southwestern France) varying in agriculture intensity (from grassland to crop rotation including corn and sunflower, and to corn monoculture). In the first approach, 43 44 we analysed the composition of invertebrate assemblages (only obligate-45 groundwater crustaceans, i.e. stygobite) sampled in 28 wells located in 6 sectors 46 differing in their agriculture contexts. Abundance, species richness, and assemblage 47 composition did not significantly vary at the sector scale, but significantly changed 48 with agriculture practices or urbanization around the well. These community metrics 49 are thus not always eligible as ecological indicators. In the second approach, we 50 tested an *in situ* exposure of sentinel organisms in the groundwater to guantify their 51 response to the environmental conditions. We used the epigean and local amphipod 52 species Gammarus cf. orinos as a sentinel species. Amphipods (30 individuals in 53 each of 10 wells) were exposed for one week to the *in situ* conditions at two seasons 54 with high and low transfer of pollutants. An EcoPhysiological Index (EPI) synthetizing 55 the survival rates and energetic storage decreased in wells with low oxygen and high 56 nitrate concentrations, but only during the high pollution transfer period. We also 57 found evidence that atrazine-related compounds may negatively impacted sentinel 58 health whatever the season. These results highlight that use of sentinel species in 59 experiments may have a major applied value for groundwater ecosystem 60 assessment.

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*Key words:* Subterranean ecosystems, Crustaceans, Stygobite, Sentinel organisms,
 Herbicides, Triazines.

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#### 66 **1. Introduction**

67 Facing the development of growing human populations and activities, a major challenge for the future will be to maintain groundwater ecosystem health, i.e. the 68 69 capacity of the groundwater ecosystem to sustain its structural and functional 70 integrity and sustainably provide appropriate services (Korbel & Hose, 2011). Among 71 the many services that groundwater ecosystems provide to humans (Tomlinson et 72 al., 2007; Boulton, 2009) the most valuable are drinking water supply (van der Gun, 73 2012) and water use for irrigation for both traditional and intensive agriculture (Burke, 74 2003; Zektser & Everett, 2004). Upwelling groundwater may also influence stream and wetland conditions through water level, temperature and water guality (Ramsar 75 Convention Secretariat, 2010). Groundwater quality is under threat in many areas 76 77 due to agriculture practices (Bohlke, 2002; Legout et al., 2005, 2007: Martin et al., 78 2006, European Environmental Agency, 2010; Lapworth et al., 2012; Di Lorenzo & 79 Galassi, 2013), industrial development and urbanisation (Datry et al., 2004, 2005; 80 Foulguier et al., 2010, 2011; Lerner & Barrett, 1996; Trauth & Xanthopoulos, 1997; Di 81 Lorenzo et al., 2012). Reliable evaluations of groundwater ecosystem health have 82 thus become essential for the proposal and the establishment of restoration 83 strategies (Boulton, 2005; Griebler et al., 2014).

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85 In Europe, groundwater quality is still evaluated based on physical, chemical and 86 hydrological characteristics (European Commission, 2006), although the inclusion of 87 ecological criteria has been repeatedly advocated (Danielopol et al., 2004, 2006b, 88 2008; Hancock et al., 2005; Hahn, 2006; Tomlinson et al., 2007; Steube et al., 2009; 89 Griebler et al., 2010). The few existing ecological indicators of groundwater 90 ecosystem health consist of the examination of parameters describing invertebrate or 91 microbial communities (Mösslacher, 2000; Danielopol et al., 2006a; Hahn, 2006; 92 Gutjard et al., 2013; Griebler et al., 2010; Stein et al., 2010; Korbel & Hose, 2011; 93 Gutjahr, 2013; Griebler et al., 2014) or biofilm growing on artificial substrates 94 (Williamson et al., 2012; Mermillod-Blondin et al., 2013). More recently, the use of 95 sentinel invertebrates caged inside groundwater wells has been proposed to avoid 96 confounding effects related to local characteristics (e.g. depth and characteristics of 97 the well, variability in the exchange of water between well of groundwater) and 98 biogeographical signature (e.g. defaunation linked to the last glacial extension) that may lead to misinterpretation of the relationships with ecosystem quality (Marmonieret al., 2013).

101

102 Here, we compared the performance of two ecological methods that evaluate the 103 quality of groundwater ecosystems exposed to intensive agriculture in southwestern 104 France. (1) The composition and structure of stygobite crustacean assemblages of 105 28 wells were studied in different agricultural contexts in the Ariège and Hers alluvial plains. We expected that water quality (influenced mainly by agriculture practices) 106 107 would be significantly associated to decreases in abundance, species richness or to changes in the composition of hypogean crustacean assemblages. We tested this 108 109 hypothesis for six different sectors of the plains with contrasted agriculture 110 characteristics and for each well considered individually. (2) We then used sentinel 111 crustacean species (a local epigean amphipod Gammarus cf orinos,) caged in a 112 subset of 10 wells in the same area, immediately after (July) and three months after 113 (September) the end of corn herbicide spreading (mostly S-metolachlor). The survival 114 rate and the level of energetic consumption were determined after one week and 115 expressed as the Ecophysiological Index (EPI, Marmonier et al., 2013) to quantify the 116 stress endured by the sentinels during the exposure period. We expected that 117 agriculture-derived contamination of groundwater would induce a significant 118 decrease in sentinel health.

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## 120 **2. Material & methods**

- 121
- 122 2.1. Study area

123 The Ariège and Hers alluvial plains are located in southwestern France and encompass a 540 km<sup>2</sup> surface area. The studied sector of the Ariège alluvial plain 124 125 (Fig. 1) is located between Varilhes (South) and Portet-sur-Garonne (North). The 126 Hers plain is located between Mirepoix (South) and Cintegabelle (North) where the 127 Hers River joins the Ariège River. The area is underlain by Aquitanian (Miocene) and 128 Stampian (Oligocene) molasse deposits. The Ariège River alluvium was deposited on 129 the molasse in five distinct terraces with very similar grain size compositions. The 130 sand-and-gravel alluvium of the lower terrace and the lower plain is a continuous 131 unconfined aquifer that is hydraulically connected to the Ariège and Hers Rivers,

feeding them during much of the year, especially in summer. The unsaturated zone isgenerally less than 10 m thick.

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135 The region is characterized by intensive agriculture, the major crop being corn and 136 sunflower. The land use analysis (Fig. 1A) was established using satellite images 137 from 4 dates (winter, spring, summer, and fall 2008) where six land use were defined (forest, grassland, corn-sunflower, other cultures, urbanized area and waterbodies; 138 using MapInfo 8.5.1B<sup>®</sup>). The area of intense agriculture (red colour dominant in Fig. 139 140 1A) showed strong degradation in the groundwater quality. This degradation is 141 documented for a long time by the Adour-Garonne Water Agency that manages the 142 monitoring according to the Water Framework Directive (European Commission, 143 2000; especially for nitrate and pesticides concentrations), or by a reinforced 144 monitoring (Amalric et al., 2013). In this perspective, a preliminary large-scale study 145 was performed in 2008 on groundwater chemistry, with a set of 144 wells and springs 146 where nitrate concentrations were measured. These results showed that 71 points (i.e. 49%) were above the European standard limits (50 mg/L) established by the 147 148 European Commission (2006), 22 of which (i.e. 15%) were over 100 mg/L. Wells with 149 high nitrate concentrations were located in an area dominated by corn and sunflower 150 productions.

151

152 According to lithology, hydrogeology, and land use, the studied area has been 153 divided into six sectors with distinct human activities and different degrees of 154 groundwater alteration (Fig. 1B). The upstream part of the Hers (hereafter HUp) and 155 Ariège (AUp) alluvial plains were dominated by grassland with forest patches (Table 156 1, percentages of grassland were higher in HUp and AUp than downstream sectors, 157 ANOVA F<sub>4.23</sub>=3.157, p=0.026, see below for statistics). In these two upstream 158 sectors, groundwater was characterized by low to medium nitrate concentrations 159 (Table 1). In the downstream part of the Hers (HDw) and a first intermediate sector of 160 the Ariège plain (AInt1) where land use was strongly dominated by corn and 161 sunflower productions ( $F_{4,23}$ =6.584, p=0.0007 for corn,  $F_{4,23}$ =3.282, p=0.023 for other 162 culture such as sunflower), groundwater was characterized by high nitrate 163 concentrations. A second intermediate sector occurred downstream of the Hers-164 Ariège confluence (AInt2) with a more diverse agricultural land use, but still very high 165 nitrate concentrations in groundwater. Finally, the downstream sector of the Ariège plain (ADw) was characterized by increasing urbanisation, decreasing agriculture,
 and decreasing nitrate concentrations in wells. These six sectors were used to
 determine the distribution of available sampling wells.

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#### 170 2.2. Water quality and invertebrate assemblages

171 Invertebrate assemblages and water guality were studied in 28 wells (Fig. 1B) 172 sampled in January 2012 before the beginning of irrigation and other farming activities but also before the beginning of the water quality survey. Wells were 173 174 distributed as evenly as possible in the 6 sectors defined on a land use basis. In the 175 Hers alluvial plain, six wells were located in the upstream sector (H1 to H6 in HUp) 176 and six others downstream (H7 to H12 in HDw). Along the Ariège River, 16 wells 177 were distributed from upstream to downstream: A1 to A4 in the sector AUp, A5 to A8 178 in AInt1, A9 to A12 in AInt2, and A13 to A16 in ADw. Land-cover around each well (buffer of 3km<sup>2</sup>) was established with MapInfo 8.5.1B using the 6 land-cover types 179 180 mapped in Fig. 1A.

181

182 The fauna was sampled within these wells using a method derived from the Bou-183 Rouch technique (Bou & Rouch, 1967): 40 litres of water and fine sediment were 184 pumped by disturbing the bottom of the well with a long and weighted semi-rigid tube 185 connected to a hand-pump (Malard et al., 2003). Water quality was reduced to *in situ* 186 measures: temperature, electrical conductivity at 25°C, pH, and dissolved oxygen, 187 using a HACH-LANGE portable apparatus (HQ40d Multiparameter, Düsseldorf, 188 Germany) on the last litre of pumped water. The 40-L samples were then filtered 189 through a 100 µm mesh net to retain fauna that was immediately preserved in 96° 190 alcohol. The analysis of invertebrate assemblages was restricted to stygobionts for 191 comparison with previous studies performed in 1999 by Dumas (2000) and in 2003 192 by Janiaud (2004). Amphipods, isopods, copepods, and ostracods were identified to 193 species level using morpho-anatomical criteria.

194

For Amphipods, morphological identification was completed by molecular barcoding
to ensure 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'-AGGGAAACTTCGGAGGGAACC-3'

200 from Fiser et al (2008). Sequences were aligned using Bio Edit Sequence Alignment 201 Editor (Hall 1999). Phylogenetic evolutionary analysis was conducted using 202 maximum parsimony and bootstrapping using Mega version 5 (Tamura et al. 2011), 203 and resulting phylogenetic trees were used to delimitate the main species in the area. 204 Because sequencing failed for most individuals (mainly because of poor DNA 205 quality), we used a shorter fragment (180–215 bp) allowing discrimination among the 206 main species groups found in this area through genotyping. We used the primers 5'-207 TTGAGCCTGTGGGTGAC-3' and 5' GCCTGCACCAAGATTTAACC-3' to amplify 208 sequences of unique size for each species, which allowed identifying species based 209 on the size of the peaks. We used the size of the peaks to distinguish a "*Niphargus*" 210 kochianus" species group from the three other taxa (N. ciliatus, N. foreli, and 211 *Niphargus* sp., smaller-sized fragments). These peaks were scored in the program 212 Gene Mapper version 5 (Applied Biosystems 2012). Although not perfect, this 213 genotyping approach allowed for verification of most morphological identifications. 214 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 215 216 products were revealed on an ABI PRISM<sup>™</sup> 3730 Automated Capillary Sequencer 217 (Applied Biosystems, Foster City, CA, USA).

218

#### 219 2.3. Water quality and sentinel exposure

220 Ten wells were selected for the cage experiment with sentinel amphipods exposure 221 in groundwater (Fig. 1C). In order to evaluate the sensitivity of the method, the 222 exposure experiment was carried out twice with identical methods: (1) three months 223 after the end of corn herbicide spreading, during the low water period of a rather dry 224 year (September 2012) resulting in low transfer of pollutants (hereafter 'low transfer 225 period') and (2) just after the end of pesticide application, during high water period of 226 a rather wet year (July 2013) resulting in high transfer of pollutants (hereafter 'high 227 transfer period'). The 10 wells were distributed across 5 of the 6 sectors defined from 228 land use (Fig. 1C).

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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<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup> (uncertainty 5%), ion chromatography for Cl<sup>-</sup>,  $SO_4^{2^-}$ ,  $NO_3^{-}$  (uncertainty less than 10%) and potentiometric method according to N EN ISO 9963-1 for  $HCO_3^{-1}$  and  $CO_3^{-2^{-1}}$  (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. Method details are reported in Amalric et al. (2013) and the list of measured compounds with their detection limit are presented in appendix 1.

241

242 As local densities of stygobite amphipods were very low in the studied area, the 243 selected sentinel species was an epigean amphipod (Gammarus cf orinos) living at 244 the surface/ground water interface (in springs and small streams). Sentinels were 245 captured in the field in a neighbourhood brook, kept for one week in the laboratory 246 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 247 248 (Marmonier et al., 2013). Six such cages, each containing five G. cf orinos 249 individuals, were placed in each well in both low and high transfer periods (i.e. 30 250 individuals per well and exposure period). The cages were retrieved after one week 251 of exposure. Three ecophysiological parameters were measured on the specimens: 252 (1) the survival rate, (2) glycogen and (3) triglyceride body concentrations. Three sets 253 of three individuals (i.e. 9 individuals) found alive were frozen-dried at retrieval time 254 and used for each biochemical analysis at each occasion (period/well). Glycogen and 255 total triglyceride concentrations were measured by standard enzymatic methods 256 using specific test combinations, according to Hervant et al. (1995, 1996). Ad hoc 257 Sigma-Aldrich (France) glucose HK and GPO Trinder kits were used to determine 258 glycogen and triglycerides, respectively. Glycogen concentration was expressed as 259 mg of glycosyl, and triglyceride concentration was expressed as mg of glycerol. All assays were performed in an Uvikon 940 recording spectrophotometer (Kontron Inc., 260 261 Germany) at 25°C.

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#### 263 2.4. Data analyses

Statistical analyses were performed at the scale of the six sectors defined in the Ariège and Hers alluvial plains and at the scale of each individual well. At the sector scale, species richness, total abundances of stygobionts and abundances of the most frequent species were compared between the six sectors (with wells as 268 replicates) using one-way ANOVA (significant level of p=0.05) after the data were 269  $log_{10}$  (x + 1) transformed to reach a normal distribution. The quasi balanced design (n 270 = 6, 6, 4, 4, 4, Table 1) renders the ANOVA quite robust despite the low degree of 271 replication (Quinn & Keough, 2002; Zar, 2009). At the scale of the individual well, 272 faunal characteristics (stygobiont abundances and richness) were correlated to water 273 chemistry (i.e. field measured parameters) and land use around the well and 274 regression curve calculated when correlations were significant (p=0.05). The 275 assemblage composition was analysed using Non Metric Multidimensional Scaling 276 using the Bray-Curtis dissimilarity index (NMDS, Legendre & Legendre, 1998). 277 Chemical and land use characteristics around the well were fitted to the NMDS 278 ordination to allow visualization for testing which of them contributed significantly to 279 the NMDS ordination. Analyses were performed using the R statistical software (R 280 core Development Team, 2013), using the vegan R package (Oksanen et al., 2012).

281

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

284

EPI = Survival rate x ([Gly] / ([Tri] + [Gly]))

285 where the sentinel survival rate (as %) is combined with the state of their energetic 286 stores as a ratio between glycogen content (noted as [Gly]), which is the first 287 energetic store substance used in response to acute stress (Maazouzi et al., 2011), 288 and their total body stores, as estimated from the sum of triglyceride (noted as [Tri]) 289 and glycogen concentrations. In order to avoid seasonal differences in animal 290 energetic store before the experiment, a ratio was calculated between EPI of sentinel 291 individuals exposed in wells and EPI of reference sentinels, i.e. stress-free animals 292 caged at the laboratory in synthetic water (see Nogaro et al, 2008). The in situ / 293 laboratory ratio for the EPI can vary between values close to 0 (high impact of well 294 water on exposed sentinels) to values close to 1 or in some rare cases slightly above 295 1 (meaning no difference between exposed and reference organisms).

296

Linear correlations were calculated between EPI and water chemical characteristics (oxygen, nitrate, and herbicide concentrations). We additionally ran 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. Given that the number of replicates (ten well *per* two experimental sessions = twenty

302 "replicates") was low compared to the total number of explanatory variables, we 303 performed a regression on PCA axes (Jollifre, 1982). Specifically, we ran a PCA on 304 water guality variables (18 variables in total, see Table 4). We gathered the PCA 305 coordinates of each well (per experimental session) along the first-five PCA axes 306 (these axes summarized more than 82% of the total variation, see Table 4). This lead 307 to a total of five synthetic explanatory variables correlated to the actual variables (see Table 4 for correlation values between each actual variable and each PCA axis). 308 Then we built a mixed linear model with EPI ratio as the dependent variable and the 309 310 five synthetic variables and the experimental session (categorical) as explanatory variables. To test for temporal consistency, two-way interactions between 311 312 experimental session and each of the other variables was accounted for in the 313 model. The well identity was included as a random effect to account for temporal 314 pseudo-replication. This full model was reduced by building all possible models (i.e. 315 all combination of variables) and ranking them according to their Akaike Information 316 Criteria (AIC). The best-reduced model was that having the lower AIC.

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#### **318 3. Results**

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### 320 3.1. Stygobite assemblages

321 2,691 stygobite crustaceans, representing a total of 14 taxa were collected in situ on 322 a total of 12,155 individuals and 36 taxa sampled. Six species were amphipods 323 (genera Niphargus and Salentinella, Table 2), four were copepods and four were 324 ostracods. Molecular identifications reduced the number of otherwise unidentified 325 specimens of amphipods by 40% (65 individuals remained unidentified because of 326 small size, body degradation during pumping and DNA amplification failure). The 327 spatial distribution of stygobionts was markedly heterogeneous. Abundances varied 328 from 0 (the A1, A2, A6, A13 and A15 wells) to more than 100 individuals per sample 329 (in H8, Fig. 2), and taxonomic richness ranged from 0 to 5 species (in wells A12, H3, 330 H5 and H6).

331

At the sector scale, the abundances in wells located in the Hers and the Ariège alluvial plains differed significantly ( $F_{4,23}$ =5.136, p=0.0028): the assemblages sampled along the Hers (HUp and HDw) were more abundant than those of the Ariège (except for wells A10 and A12 in the Aint2 sector). This difference was observed whatever the dominant land use of the sector ( $F_{1,8}$ =35.32, p=0.0003 between HDw and ADw dominated by intense agriculture). Similarly, species richness significantly differed among alluvial plains ( $F_{4,23}$ =3.56, p=0.0164): higher species richness was measured in wells located along the Hers (Hup and HDw sectors) than in wells distributed along the Ariège (except in Int2). Again this difference was observed in all types of agriculture practices.

342

At the well scale, no significant correlation was detected between abundance or 343 344 species richness and the distance between wells and the two rivers (p>0.05 for both distances to Ariège and Hers Rivers for A and H wells, respectively) nor with the 345 346 upstream-downstream location of the well along the rivers (p>0.05 for both Ariège 347 and Hers Rivers). In the same way, no significant correlation was found between 348 these assemblage characteristics and the water characteristics (i.e. dissolved oxygen, temperature, or electric conductivity; in all cases  $r^2 < 0.08$ , p>0.05). In 349 350 contrast, the abundances and species richness of stygobite crustaceans significantly 351 changed with the human activities around the well. Abundances increased with the 352 percentage of agriculture with highest values between 30 and 60% followed by a slight decrease ( $r^2$ =0.158, p=0.018, Fig. 2B). This change in abundances was mainly 353 due to an increase in the number of *N. kochianus* ( $r^2=0.125$ , p=0.030). Inversely, 354 species richness decreased with the percentage of urban area around the well 355 356 (r<sup>2</sup>=0.134, p=0.027, Fig. 2C). In all cases, the correlation coefficients obtained were weak and a large dispersion of wells was observed for high values of abundances 357 358 and species richness.

359

360 The NMDS analysis (Fig. 3, stress=0.054) highlighted some differences in the composition of the assemblages. A large number of wells were grouped close to the 361 origin of the NMDS1 and NMDS2 axes, because of high abundances of *N. kochianus* 362 363 and, to a lesser extent, of *N. ciliatus* in most of the wells (Table 2 and Fig. 4). This 364 group of wells suffered very different anthropic activities, from traditional agriculture 365 with dominant grasslands (A3 and A4 from the AUp sector, H2, H5, H6 from the HUp 366 sector) to intensive agriculture with corn as monoculture (A7, A14 from Aint sectors 367 or H7 to H12 from the HDw sector). Similarly, the six wells excluded from the analysis because of the absence of stygobite crustaceans (reported in a box in Fig. 3A) were 368 369 located in sectors dominated by either traditional (A1, A2) or intensive agriculture

370 (A9, A13, A15). Two wells were located out of this central group on the most negative 371 side of NMDS1 (H8 and H9) because of the absence of N. kochianus and the 372 occurrence of two rare species (Diacyclops clandestinus and Pseudocandona sp3, 373 Fig. 3B and 4). On the opposite side of NMDS1, the well H4 was isolated because of 374 the occurrence of the rare *Diacyclops belgicus* (Fig. 3B and 4). Considering 375 environmental factors, NMDS1 was correlated with the percentage of grassland on 376 the positive side and the percentage of cultivated land on the negative side (Table 3). 377 Similarly, three wells (A7, A8 and A11) were located on the most negative side of the 378 NMDS2 axis for similar reasons: low abundances of *N. kochianus* and high 379 abundances of Salentinella (both S. major and S. petiti, Fig. 3B). This second axis 380 was structured by wells surrounded by high percentage of forest (Fig. 3C), but this 381 correlation was not significant (Table 3). Finally, the scores of 16 wells located in the 382 Ariège alluvial plain along the NMDS2 axis were positively correlated to the distance to the river ( $r^2$ =0.4379, p=0.038): the well A11 located at 100m of the river and the 383 384 well A8 at 600m yielded assemblages with only few or no N. kochianus nor N. 385 *ciliatus*, but populations of *Salentinella* (Fig. 4). No significant relationship between 386 distance to the river and fauna was observed for the Hers River.

387

#### 388 3.2. Sentinels exposure

389 The analyses of pesticides and metabolite concentrations confirm that pollution was 390 lower during the low transfer period (maximum total herbicide concentration of 2.89 391 µg/L and 2.18 µg/L for S-metolachlor, an herbicide widely used, and its ESA and 392 OXA metabolites, the predominant herbicide molecules) than during the high transfer 393 period (maximum total herbicide of 9.59 µg/L and 9.2 µg/L for S-metolachlor and its 394 metabolites). Weak EPI index differences were observed during low transfer period whereas an upstream-downstream gradient was evident during high transfer period 395 (Fig. 5). The EPI was close to 1 (i.e., similar to the uncontaminated laboratory 396 397 controls) in the upstream wells (A1, A2, A3, A4) whereas low values occurred in the 398 downstream wells (H9, H10, A8 and A10). During the high transfer period, the EPI values were positively correlated to dissolved oxygen ( $r^2=0.420$ ; p=0.021) and 399 negatively correlated to nitrate concentrations ( $r^2=0.370$ ; p=0.031; Fig. 6). These 400 401 correlations were not observed during the low transfer period (Fig. 6), despite a 402 similar range of variation in dissolved oxygen and nitrate concentrations. We did not 403 find any significant correlation between EPI and the percentage of land covered by

agriculture around the wells, the total pesticides concentrations nor each pesticide 404 405 considered separately (in all cases p>0.05). In contrast when all chemical parameters 406 were considered together, the best-reduced model (AIC = 7.4,  $W_i$  = 46.0%) included 407 PCA axes 1, 2 & 5 (Table 4). In the best-reduced model, the relationships between of 408 PCA axes 2 and 5 and EPI varied between the two experimental sessions as there 409 were significant two-way interactions between PCA axes 2 and 5 and experimental 410 session. These interactions suggest that the effects of the variables associated with 411 these two axes were not consistent over time. Contrastingly, the relationship between 412 PCA axis 1 and EPI was negative both during the first and second experimental 413 sessions, which demonstrated a highly consistent effect (Fig. 7). The strong 414 correlation of this PCA axis with atrazine-related compounds (i.e. atrazine, 415 deisopropylatrazine, and DEA with correlation to PCA axis 1 above 0.87, Table 4) 416 suggests a potential relationship between EPI decrease and the combination of these 417 pesticides and metabolites.

418

#### 419 **4. Discussion**

The need for ecological indicators for groundwater quality evaluation has been advocated by several authors to warn about the sensitivity of these systems to human activities (e.g. Marmonier et al., 1993; Danielopol et al., 2004; Griebler et al., 2010). In our study, indicators based on crustacean assemblage composition were combined with *in situ* sentinel exposure and brought contrasting results to the fore.

425

#### 426 4.1. Stygobite assemblages and water quality

427 The distribution of the crustacean assemblages was very heterogeneous in the two 428 alluvial plains and significantly but weakly related to water quality and human activities. At the sector scale (when wells are grouped in land use based sectors), the 429 430 abundances, species richness and composition of assemblages were poorly related 431 to agricultural practices and the resulting water guality (based on pH, dissolved 432 oxygen, electrical conductivity and nitrate concentrations). Similar patchy distribution 433 of groundwater fauna was already reported in less impacted environments, such as 434 the Danube and Rhône floodplains (Danielopol, 1989, Dole & Mathieu, 1984), or in 435 glacial streams (e.g. Malard et al., 2002, 2003) and springs (Fiasca et al., 2014).

When the wells were considered individually, no link was observed between faunal 437 438 characteristics and local water chemistry. During our first survey of the stygobite 439 assemblages in January 2012, the water quality assessment was limited to simple 440 parameters (i.e. temperature, pH, dissolved Oxygen, electric conductivity) and did not 441 include the total array of parameters as measured during the sentinel exposure. In 442 contrast, significant correlations were observed between abundances, species richness and the land use around the well (agriculture and urban area respectively). 443 444 NMDS analysis of the assemblage composition showed a rather similar trend (for the 445 percentages of cultivated land and grassland). Even if this statistically significant 446 trend was weak, the local human activities around the well explained at least a part of 447 the stygobite assemblage composition and our first prediction was thus partly 448 verified.

449

450 Two other factors may influence stygobite distribution in the studied area. Firstly, the 451 major trend observed in the distribution of stygobite crustaceans in the studied sector 452 was the significant difference in abundance and species richness between the 453 assemblages sampled in the Ariège and the Hers groundwater systems. These 454 differences are difficult to explain using the available set of environmental variables. 455 Similar ranges for pH (an important factor for crustacean moult), dissolved oxygen 456 (that can limit crustacean survival), and electrical conductivity (often linked to water 457 origin and circulation patterns) were measured in the two alluvial plains in January 458 2012 when assemblages were sampled. Similarly, no obvious differences in land use 459 characteristics could be found between the Ariège and Hers alluvial plain (Fig. 1A). 460 Other biogeographical or hydrogeological variables must be considered to 461 understand this between-plain heterogeneity, such as sediment characteristics and 462 permeability, or nutrient availability. Secondly, the distance to the river influences the 463 composition of stygobite assemblages. This influence was already reported in 1999 464 using 15 wells along the Ariège River (Dumas 2000, 2002). We found a similar 465 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 NMDS2 axis) was 466 467 correlated to the distance to the river: N. kochianus decreased or disappeared in 468 wells very close to the river. Such a distance may reflect the gradual river influence 469 with buffered temporal variability at long distance (Dole & Chessel, 1986).

471 Thus, our results suggest that classical metrics of groundwater crustacean 472 assemblages, such as species composition, abundance and richness, may be of low 473 value for the evaluation of the impact of intensive agriculture on subterranean 474 ecosystems at the sector scale whereas better results may expected when wells are 475 considered individually. Contrasted results can be found in literature about the 476 relation between groundwater assemblages and land use. On one hand, Di Lorenzo 477 & Galassi (2013) found stygobite species richness and abundance to be non-478 sensitive to nitrate concentrations (up to 150 mg/L) in the alluvial aquifer of the 479 Vibrata River in Italy, even if long-term effects could not be ruled out (Di Marzio et al., 480 2013). Similarly in Germany, Hahn (2006) and Griebler et al. (2010) found 481 inconsistent correlations between diversity and abundance of groundwater fauna and 482 physical and chemical variables. On the other hand, Di Lorenzo et al. (2015) 483 observed that groundwater assemblages sampled from bores in the alluvial plain of the River Adige were sensitive to  $NH_4^+$  concentrations (here  $\ge 0.032$  mg/L). It seems 484 485 that the effect of chronic toxicity of pollutants used in agriculture is more obvious 486 when considering the development rhythm of individuals instead of their mortality. 487 For example, Di Marzio et al (2013) observed that chronic exposure of interstitial 488 copepods to ammonium and herbicides (the carbamate pesticide Aldicarb) affected 489 the developmental time spent to reach the adult stage.

490

491 The community characteristics (i.e. composition, abundances, and species richness) 492 of stygobite crustaceans varied between wells according to the local context, but 493 were of poor value as ecological indicators at the sector scale compared to other 494 sites in Europe (Di Lorenzo et al., 2014) or North-Africa (Boulal et al., 1997). Future 495 research should include other groups of groundwater organisms in the assemblage 496 analyses, such as oligochaetes, molluscs, and bacteria. The microbes, and especially bacteria, represent a considerable "hidden" biodiversity now quantifiable 497 498 with molecular techniques that may be used for groundwater ecosystem evaluation 499 (Griebler et al., 2010, 2014).

500

### 501 4.2. Relevance of sentinels in the evaluation of groundwater ecosystem health

502 Sentinel organisms caged in rivers and streams are now routinely used in the 503 bioassessment of surface water ecosystems (Maltby et al., 1990, 2002; Xuereb et al., 504 2009). The taxa used *in situ* for monitoring surface water include molluscs (Schmitt et

505 al., 2010; Taleb et al., 2009), crustaceans (Coulaud et al., 2011; Debourge-Geffard et 506 al., 2009; Maltby, 1995; Maltby & Crane, 1994), insects (Custer et al., 2008) and 507 fishes (Hanson, 2009). For groundwater ecosystems, Marmonier et al. (2013) 508 proposed to distinguish the use of stygobionts, which may be resistant to long-term 509 exposure (one month) and allow assessing diffuse pollution or providing a 510 comprehensive evaluation of groundwater ecological guality, from the use of epigean 511 species, which may resist to short-term exposure only (one week) allowing 512 assessment of acute toxicity disturbances. Unfortunately, we did not find large 513 numbers of stygobionts in the studied area (300 living individuals of the same 514 species are necessary for 10 wells; Marmonier et al., 2013). So, we used a locally 515 abundant epigean amphipod, Gammarus cf orinos living at the interface between 516 groundwater and surface water (e.g. springs and small spring-fed streams). The use 517 of an epigean taxon also limited the exposure duration to one week because of high 518 physiological needs of surface water organisms (Hervant et al., 1998).

519

520 Several health criteria have been used to evaluate the degree of environmental 521 disturbance resulting from exposure: (1) survival rate (Brown, 1980, Gust et al., 522 2010), (2) feeding activity (Coulaud et al., 2011; Crane et al., 1995; Forrow & Maltby, 523 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; 524 525 Schmitt et al., 2010). In the present study, we used the EPI of Marmonier et al. 526 (2013) that combines survival rates and changes in energetic stores expressed as a 527 ratio between EPI measured in natura and obtained from reference individuals 528 exposed in the laboratory (for comparisons between different periods of the year and 529 different levels of energetic stores). Poor differences in the EPI score among crop-530 based sectors were observed during the period of low water transfer and low 531 pollution (i.e. the dry season of a dry year), two months after the last potential 532 pesticide application. In contrast, a strong upstream-downstream decreasing gradient 533 in the EPI score occurred during the period of high water transfer and high pollution 534 (i.e. a high water period of a wet year), during or just after a period of intensive 535 agricultural activities (e.g. high and/or frequent pesticide applications). Chemical 536 analyses of S-metolachlor confirmed these variable degrees of groundwater contamination between the two periods. This result suggests that sentinels caged in 537 538 the downstream-located wells were potentially exposed to high levels of stress.

539

540 Agriculture-derived human activities weakly altered sentinel health, with an effect 541 only on energetic stores but not on survival rates. Similarly, Marmonier et al. (2013) 542 found an effect of urban stormwater infiltration on EPI scores (mainly due to inputs of 543 DOC and related decreases in dissolved oxygen concentration, Foulquier et al., 544 2010) but only weak effects on sentinel survival rates. Maazouzi et al. (2011) showed that energetic stores, especially glycogen content, are valuable indicators of 545 environmental conditions for amphipods. The significant correlations of EPI with 546 547 dissolved oxygen (positive) and nitrate concentrations (negative) in the Ariège and 548 Hers alluvial plains during the high water transfer period suggest a link between 549 sentinel health and agricultural practices.

550

551 As observed in earlier investigations in the studied sector (Amalric et al., 2013), Smetolachlor as well as its major metabolites (ethane sulfonic acid and oxalinic acid) 552 553 were the predominant pesticides found in groundwater, followed by atrazine and two 554 metabolites (deisopropylatrazine and deethylatrazine, noted DEA). S-metolachlor, 555 one of the most commonly used herbicides in this area (Water Agency pers. comm.), 556 is prone to leach into groundwater (Hladik et al., 2008; Baran & Gourcy, 2013). 557 Atrazine widely used for decades was withdrawn from agricultural practices in 2003. 558 Nevertheless, atrazine and its major metabolite DEA have persisted in groundwater 559 (Baran et al., 2007). When all pesticides were considered together, the significant 560 correlation of EPI scores with PCA scores suggests that sentinel health can be 561 negatively impacted by atrazine and two of its metabolites deisopropylatrazine, and 562 desethylatrazine-DEA (Table 4). This herbicide is lowly to moderately toxic for 563 crustaceans. The median effective concentration ( $EC_{50}$  for immobility after 48h) of Atrazine for Daphina magna is very high (35.5 ±9.2 mg/L, Palma et al., 2008) and 564 565 reached 72 mg/L for the median lethal concentration ( $LC_{50}$  after 48h, Wan et al., 566 2006). For the amphipod, the  $LC_{50}$  of Atrazine is 7.5 mg/L for *Gammarus pulex* (for 567 48h, Lukancic et al., 2010) and 10.1 mg/L for G. italicus (for 96h, Pantani et al., 568 1997). To our knowledge the toxicity of deisopropylatrazine for amphipods is not 569 known, but we measure the acute toxicity of DEA for our sentinel species, G. cf 570 orinos, that reached 10.1 (±1.1) mg/L (LC<sub>50</sub> for 96h, Maazouzi et al., in prep.). In 571 contrast, Atrazine concentrations in the groundwater of the studied sector ranged 572 from below the limit of quantification (<LQ) to 0.205 µg/L, deisopropylatrazine ranged 573 from <LQ to 0.236  $\mu$ g/L and DEA ranged from <LQ to 0.794  $\mu$ g/L. These values were 574 far below the toxicity limit of these compounds, at least for Atrazine and DEA.

575

576 The decrease in EPI in downstream wells surrounded by agriculture may result from 577 the combination of several stressors, low values of dissolved oxygen, high nitrate 578 concentrations or occurrence of pesticides being the most obvious, but other 579 stressors not measured in our study (or peaks in herbicide applications not considered by our sampling strategy) may have contributed to the observed 580 581 decrease in sentinel health. In the future, it would be essential to test the use of 582 stygobite sentinels for longer exposure times (e.g. one month) that may integrate 583 several episodes of pollution (e.g. Di Lorenzo et al., 2014). We also advocate 584 repeated exposure experiments (i.e., at more than two periods) along a single year, 585 to account for changes in agriculture activities and related groundwater quality. 586 Finally, the EPI may be ameliorated and enriched with other potential 587 ecophysiological indicators such as reproduction activities, development rates, or by 588 proteomic analyses to determine the specific proteins indicative of particular 589 stressors (Armengaud et al., 2014).

590

#### **5**91 **5. Conclusion**

592

593 Three major conclusions arise from this study.

594 (1) The expected link between composition, abundance and diversity of stygobite 595 assemblages and groundwater quality (reflecting agriculture intensity) was significant 596 when wells were considered separately, but this relationship was highly variable. 597 When sectors were compared, these global indicators were of poor value for the 598 assessment of groundwater ecosystem health. Other variables must be included in 599 future evaluation strategies, such as other biological groups (e.g. oligochaetes or 600 microbes) or other biological characteristics (e.g. development rate, population 601 structure).

602 (2) The sentinel experiment gave consistent results with a decrease in their 603 ecophysiological status after one week of exposure in wells with low dissolved 604 oxygen and high nitrate concentrations. We observed correlations between the EPI 605 values measured on sentinels and occurrence of some organic molecules (e.g. atrazine and its metabolites, deisopropylatrazine and DEA) suggesting that sentinelswere submitted to a combination of several stressors.

- (3) This effect 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 area of intensive agriculture and
  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.
- 614

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940 Table 1: Physical and chemical characteristics of the groundwater sampled during the 941 invertebrate survey of January 2012 in 28 wells located in the six sectors of the Ariège and 942 Hers alluvial plains. Sectors are upstream (HUp) and downstream (HDw) of the Hers plain, 943 upstream (AUp), intermediate (AInt1 and AInt2) and downstream (ADw) of the Ariège plain (n 944 = number of wells sampled in each sector). Parameters include temperature (Temps. in °C), 945 electric conductivity (EC in µS/cm), pH, oxygen concentrations (Oxy. in mg/L), and nitrate concentrations (NO<sub>3</sub> in mg/L; mean ± standard deviation). The land-cover around the 28 946 947 wells was used to characterize the six sectors with forest, grassland, urban area, total 948 cultivated area (mainly corn) and oleaginous seed production (mainly sunflower, mean 949 percentages ± standard deviation using wells as replicates).

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	HUp	HDw	AUp	AInt1	AInt2	ADw
	(n=6)	(n=6)	(n=4)	(n=4)	(n=4)	(n=4)
Temp.	12.6 ± 1.5	12.3 ± 0.9	12.9 ± 1.8	10.9 ± 1.3	11.4 ± 1.9	11.9 ± 1.8
EC	640 ± 95	571 ± 193	413 ± 228	502 ± 50	730 ± 57	879 ± 228
рН	7.2 ± 0.1	7 ± 0.3	7 ± 0.1	$6.9 \pm 0.1$	$7.2 \pm 0.2$	7.3 ± 0.1
Oxy.	5.0 ± 1.4	4.8 ± 2.9	7.3 ± 1.8	5.3 ± 1.1	5.4 ± 1.4	3.7 ± 1.4
NO <sub>3</sub> *	34.9± 11.6	96.31± 36.6	28.6± 13.4	63.7± 21.4	87.3± 30.3	54.9± 14.2
Forest	6±5.9	1.5± 0.8	7.7±11.3	2.5±2.6	2.4±2.4	1.4±1.0
Grassland	21.2±10.3	11.7±5.9	24.8±11.3	15.8±4.3	12.3±3.5	7.9±5.4
Urban	3.4±2.7	10.3±7.2	20±11.5	7.4±4	28.8±19.9	37.9±21.7
Cultivated	48.6±30.2	76.2±9.2	38±20.2	73.7±7.3	49.1±19.9	42.8±14.8
Oleaginous	14.1±11.1	43.3±20.2	11.8±7	43.9±15.3	17.2±11.7	7.9±8.5

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<sup>952</sup> \*Mean values (and SD) for nitrate were calculated on the basis of the monthly 2012-13 water

953 survey (BRGM, in prep).

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. Sectors are upstream (HUp) and downstream (HDw) of the Hers, upstream (AUp), intermediate (AInt1 and AInt2) and downstream (ADw) of the Ariège (n = number of wells sampled in each sector).

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Groups	Species	HUp (n=6)	HDw (n=6)	AUp (n=4)	AInt1 (n=4)	AInt2 (n=4)	ADw (n=4)
Amphipods	Niphargus kochianus	9.5	16.2	7.3	6.5	2.5	0.5
	, 0	± 12.7	± 18.5	± 12	± 12.3	± 3.8	± 0.6
	Niphargus ciliatus	1	3	0	0.3	0	0.5
	, .	± 2	± 3.9		± 0.5		± 1
	Niphargus foreli	2.2	2.5	0	0	0	0
		± 5.3	± 6.1				
	Niphargus sp.	0.3	0	0	0	0	0
		± 0.8					
	Salentinella major	5.7	0	0	2	4	0
		± 7			± 3.4	± 4.7	
	Salentinella petiti	2.7	0	0	2	2.7	0
		± 4.4			± 4	± 2.4	
Copepods	Ceuthonectes gallicus	0	0	0	0	0.5	0
						± 1	
	Diacyclops paolae	0.2	0	0	0	1.5	0
		± 0.4				± 3	
	Diacyclops clandestinus	0	0.2	0	0	0	0
			± 0.4				
	Diacyclops belgicus	13	0	0	0	0	0
		± 31.8					
Ostracods	Candonopsis boui	3.8	0	0	0	0	0
		± 6.1					
	<i>Pseudocandona</i> sp. 1	0	0	0	0	5.5	0
						± 11	
	<i>Pseudocandona</i> sp. 2	4.3	0	0	0	0	0
		± 10.6					
	<i>Pseudocandona</i> sp. 3	0	26	0	0	0	0
			± 56.7				
Abundances		43.7	52.5	7.5	11.3	18.8	1.3
		±32.3	±49.9	±11.9	±12.2	±15.0	±1.9
Richness		8.2	6.5	2.5	4.8	6.0	3.8
		±3.9	±1.0	±2.4	±2.9	±1.4	±2.1

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964Table 3. Scores of land use and physical and chemical characteristics along the first965(NMDS1) and second (NMDS2) axes. Goodness-of-fit statistics between variables966and NMDS ordination is given by a squared correlation coefficient ( $r^2$ ) and the967significance of the fit is given by a P-value (noted with \* when P<0.05).</td>

970		NMDS1	NMDS2	r <sup>2</sup>	P-value
971	Urban	-0.0436	-0.9990	0.005	0.949
972	Forest	0.3563	-0.9344	0.134	0.248
973	Grassland	0.7362	0.6767	0.285	0.046 *
974	Cultivated	-0.9758	0.2186	0.343	0.022 *
975	Oxygen	0.5587	0.8294	0.105	0.362
976	Temperature	0.0052	-0.9999	0.019	0.840
977	Conductivity	0.1056	-0.9944	0.088	0.408
978	рН	0.7987	0.6018	0.159	0.203

**Table 4:** First-five axes of a PCA performed on chemical and toxicant parameters. 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
Metolachlor	-0.144	0.738	0.568	-0.258	-0.098
Simazine	0.762	-0.205	0.066	0.207	-0.179
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
рН	-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 <sub>3</sub> )	0.430	0.472	-0.254	-0.320	0.475
Natrium (Na)	0.838	0.284	-0.031	0.224	-0.164
Sulfate (SO <sub>4</sub> )	-0.018	0.496	-0.703	-0.174	-0.057
Carbonate (HCO <sub>3</sub> )	-0.228	0.623	-0.090	0.612	-0.285



Figure 1. Land-use in the alluvial plains of the Hers and Ariège Rivers (A) and location of sampled wells for the survey of stygobite assemblages (B) and sentinels exposure (C). Dotted lines: limits of the six sectors based on PROMINENT agricultural crops and practices. 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).



Fig 2. Abundances of stygobite as number of individuals per sample transformed in classes plotted on the sampling area (A) and relation between abundances and the percentage of agriculture around the wells ( $y = -0,019x^2 + 2,095x - 22,05$ , B). Species richness as the number of species transformed into classes plotted on the sampling area (C) and relation between richness and the percentage of urban area around the wells (y = -0,0343x + 2,63, D).



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). Wells noted in blue are located in HUp, black n HDw, green in AUp, grey in Alnt1, brown in Alnt2, red in ADw. The six wells noted in the box were excluded from the analysis because of the absence of stygobites. 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 electric conductivity (EC).



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*



Fig. 5. Values of Ecophysiological Index (EPI) using *Gammarus* cf *orinos* sentinels after oneweek exposure to *in situ* groundwater during low transfer period (September 2012) with low agricultural impact and during high transfer period (July 2013), withhigh 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).



Fig. 6. Correlations 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<sup>2</sup> and p-values are reported when significant (n.s. non-significant).



Fig. 7. Correlation 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.

# Appendix 1: List of analysed compounds and their quantification limit

	Quantification limit (µg/L)
Acetochlor	0,005
Alachlor	0,005
Ametryne	0,005
Atrazine	0,005
Chlortoluron	0,005
Cyanazine	0,005
Desethyl atrazine	0,005
Desethyl terbuthylazine	0,005
desisopropyl atrazine	0,005
Desmetryne	0,005
Diuron	0,01
Hexazinon	0,005
Isoproturon	0,005
Isoproturon-1CH3	0,005
Isoproturon-2CH3	0,01
Linuron	0,005
Metazachlor	0,005
Metolachlor	0,005
Prometryne	0,005
Propazine	0,005
Propyzamide	0,005
Sebuthylazine	0,005
Simazine	0,005
Terbuthylazine	0,005
Terbutryne	0,005
Flusilazole	0,005
Tebuconazole	0,005
Tetraconazole	0,005
OXA Metolachlor	0,01
ESA Metolachlor	0,01
OXA acetochlor	0,01
ESA Acetochlor	0,01
OXA Alachlor	0,01
ESA Alachlor	0,01
Glyphosate	0,05
AMPA	0,05
Deschloro Metolachlor	0,005

2-Ethyl 6-Methyl 2-Chloroacetanilide	0,005
2-Hydroxy Metolachlor	0,01
2-ethyl 6-methyl aniline	0,005
Metolachlor morpholinone	0,01
2-Ethoxy metolachlor	0,005
2-Chloro 2',6'-diethyl acétanilide	0,005
2,6-diethylaniline	0,005
dimethanamid	0,005