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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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ABSTRACT

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

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

1. Introduction

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 capacity of the groundwater ecosystem to sustain its structural and functional integrity and sustainably provide appropriate services (Korbel & 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 agriculture (Burke, 2003; Zektser & 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 (Bohlke, 2002; Legout et al., 2005, 2007: Martin et al., 2006, European Environmental Agency, 2010; Lapworth et al., 2012; Di Lorenzo & Galassi, 2013), industrial development and urbanisation (Datry et al., 2004, 2005; Foulquier et al., 2010, 2011; Lerner & Barrett, 1996; Trauth & Xanthopoulos, 1997; Di Lorenzo et al., 2012). Reliable evaluations of groundwater ecosystem health have thus become essential for the proposal and the establishment 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 of the examination of parameters describing invertebrate or microbial communities (Mösslacher, 2000; Danielopol et al., 2006a; Hahn, 2006; Gutjard et al., 2013; Griebler et al., 2010; Stein et al., 2010; Korbel & Hose, 2011; Gutjahr, 2013; Griebler et al., 2014) 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 has been proposed to avoid confounding effects related to local characteristics (e.g. depth and characteristics of the well, variability in the exchange of water between well of groundwater) and biogeographical signature (e.g. defaunation linked to the last glacial extension) that

may lead to misinterpretation of the relationships with ecosystem quality (Marmonier et al., 2013).

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

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 studied sector of the Ariège alluvial plain (Fig. 1) is located between Varilhes (South) and 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. The Ariège River alluvium was deposited on the molasse in five distinct terraces with very similar grain size compositions. 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 much of the year, especially in summer. The unsaturated zone is generally less than 10 m thick.

The region is characterized by intensive agriculture, the major crop being corn and sunflower. The land use analysis (Fig. 1A) was established using satellite images 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; using MapInfo 8.5.1B®). The area of intense agriculture (red colour dominant in Fig. 1A) showed strong degradation in the groundwater quality. This degradation is documented for a long time by the Adour-Garonne Water Agency that manages the monitoring 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 this perspective, a preliminary large-scale study was performed in 2008 on groundwater chemistry, with a set of 144 wells and springs 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 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 corn and sunflower productions.

According to lithology, hydrogeology, and land use, the studied area has been divided into six sectors with distinct human activities and different degrees of groundwater alteration (Fig. 1B). The upstream part of the Hers (hereafter HUp) and Ariège (AUp) alluvial plains were dominated by grassland with forest patches (Table 1, percentages of grassland were higher in HUp and AUp than downstream sectors, ANOVA $F_{4,23}$=3.157, p=0.026, see below for statistics). In these two upstream sectors, groundwater was characterized by low to medium nitrate concentrations (Table 1). In the downstream part of the Hers (HDw) and a first intermediate sector of the Ariège plain (AInt1) where land use was strongly dominated by corn and sunflower productions ($F_{4,23}$=6.584, p=0.0007 for corn, $F_{4,23}$=3.282, p=0.023 for other culture such as sunflower), groundwater was characterized by high nitrate concentrations. A second intermediate sector occurred downstream of the Hers-Ariège confluence (AInt2) with a more diverse agricultural land use, but still very high 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.

2.2. Water quality and invertebrate assemblages

Invertebrate assemblages and water quality were studied in 28 wells (Fig. 1B) 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 distributed as evenly as possible in the 6 sectors defined on a land use basis. In the Hers alluvial plain, six wells were located in the upstream sector (H1 to H6 in HUp) and six others downstream (H7 to H12 in HDw). Along the Ariège River, 16 wells were distributed from upstream to downstream: A1 to A4 in the sector AUp, A5 to A8 in AInt1, A9 to A12 in AInt2, and A13 to A16 in ADw. Land-cover around each well (buffer of 3km^2) was established with MapInfo 8.5.1B using the 6 land-cover types mapped in Fig. 1A.

The fauna was sampled within these wells using a method derived from the Bou-Rouch technique (Bou & Rouch, 1967): 40 litres 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 reduced to *in situ* measures: temperature, electrical conductivity at 25°C, pH, and dissolved oxygen, using a HACH-LANGE portable apparatus (HQ40d Multiparameter, Düsseldorf, Germany) on the last litre of pumped water. The 40-L samples were 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 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, 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,
215 CA, USA) to perform PCR amplifications (recipes available upon request). PCR
216 products were revealed on an ABI PRISM™ 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).
229
230 Water quality was evaluated by field measurements (pH, electrical conductivity,
231 temperature, redox potential and dissolved oxygen) and laboratory analyses: water
232 samples were analysed by ICP-AES for Ca^{2+}, Na^{+}, K^{+}, Mg^{2+} (uncertainty 5%), ion
233 chromatography for Cl^{-}, SO_4^{2-}, NO_3^{-} (uncertainty less than 10%) and potentiometric

234 method according to N EN ISO 9963-1 for HCO_3^- and CO_3^{2-} (uncertainty 5%). All
235 pesticides and metabolites were determined with a Waters UPLC system coupled to
236 a Waters micromass MSMS (Waters Quattro-Premier XE/Q) after a solid phase
237 extraction. Chromatographic separation for neutral and ionic compounds was done
238 with a Waters Acquity UPLC BEH C18 column. Internal standards were used for
239 calibration. Method details are reported in Amalric et al. (2013) and the list of
240 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
247 height × 1.5 cm diameter; 200 µm mesh) and closed by two plastic caps at each end
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
260 assays were performed in an Uvikon 940 recording spectrophotometer (Kontron Inc.,
261 Germany) at 25°C.
262
263 *2.4. Data analyses*
264 Statistical analyses were performed at the scale of the six sectors defined in the
265 Ariège and Hers alluvial plains and at the scale of each individual well. At the sector
266 scale, species richness, total abundances of stygobionts and abundances of the
267 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
282 The Ecophysiological Index (EPI, Marmonier et al., 2013) was calculated in the
283 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
297 Linear correlations were calculated between EPI and water chemical characteristics
298 (oxygen, nitrate, and herbicide concentrations). We additionally ran a multiple
299 regression to specifically test (i) the chemical variables influencing this ratio and (ii)
300 whether or not these influences were constant over the two experimental sessions.
301 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 quality 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
308 Table 4 for correlation values between each actual variable and each PCA axis).
309 Then we built a mixed linear model with EPI ratio as the dependent variable and the
310 five synthetic variables and the experimental session (categorical) as explanatory
311 variables. To test for temporal consistency, two-way interactions between
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.
317
318 **3. Results**
319
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
332 At the sector scale, the abundances in wells located in the Hers and the Ariège
333 alluvial plains differed significantly ($F_{4,23}$=5.136, p=0.0028): the assemblages
334 sampled along the Hers (HUp and HDw) were more abundant than those of the
335 Ariège (except for wells A10 and A12 in the Aint2 sector). This difference was

336 observed whatever the dominant land use of the sector ($F_{1,8}$=35.32, p=0.0003
337 between HDw and ADw dominated by intense agriculture). Similarly, species
338 richness significantly differed among alluvial plains ($F_{4,23}$=3.56, p=0.0164): higher
339 species richness was measured in wells located along the Hers (Hup and HDw
340 sectors) than in wells distributed along the Ariège (except in Int2). Again this
341 difference was observed in all types of agriculture practices.
342
343 At the well scale, no significant correlation was detected between abundance or
344 species richness and the distance between wells and the two rivers (p>0.05 for both
345 distances to Ariège and Hers Rivers for A and H wells, respectively) nor with the
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
349 oxygen, temperature, or electric conductivity; in all cases r^2<0.08, p>0.05). In
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
353 slight decrease (r^2=0.158, p=0.018, Fig. 2B). This change in abundances was mainly
354 due to an increase in the number of *N. kochianus* (r^2=0.125, p=0.030). Inversely,
355 species richness decreased with the percentage of urban area around the well
356 (r^2=0.134, p=0.027, Fig. 2C). In all cases, the correlation coefficients obtained were
357 weak and a large dispersion of wells was observed for high values of abundances
358 and species richness.
359
360 The NMDS analysis (Fig. 3, stress=0.054) highlighted some differences in the
361 composition of the assemblages. A large number of wells were grouped close to the
362 origin of the NMDS1 and NMDS2 axes, because of high abundances of *N. kochianus*
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
368 because of the absence of stygobite crustaceans (reported in a box in Fig. 3A) were
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 sp*3,
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
383 to the river (r^2=0.4379, p=0.038): the well A11 located at 100m of the river and the
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
395 whereas an upstream-downstream gradient was evident during high transfer period
396 (Fig. 5). The EPI was close to 1 (i.e., similar to the uncontaminated laboratory
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
399 values were positively correlated to dissolved oxygen (r^2=0.420; p=0.021) and
400 negatively correlated to nitrate concentrations (r^2=0.370; p=0.031; Fig. 6). These
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

404 agriculture around the wells, the total pesticides concentrations nor each pesticide
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**
420 The need for ecological indicators for groundwater quality evaluation has been
421 advocated by several authors to warn about the sensitivity of these systems to
422 human activities (e.g. Marmonier et al., 1993; Danielopol et al., 2004; Griebler et al.,
423 2010). In our study, indicators based on crustacean assemblage composition were
424 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
429 activities. At the sector scale (when wells are grouped in land use based sectors), the
430 abundances, species richness and composition of assemblages were poorly related
431 to agricultural practices and the resulting water quality (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).
436

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

Two other factors may influence stygobite distribution in the studied area. Firstly, the major trend observed in the distribution of stygobite crustaceans in the studied sector was the significant difference in abundance and species richness between the assemblages sampled in the Ariège and the Hers groundwater systems. These differences are difficult to explain using the available 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 in January 2012 when assemblages were sampled. Similarly, no obvious differences in land use characteristics could be found between the Ariège and Hers alluvial plain (Fig. 1A). Other biogeographical or hydrogeological variables must be considered to understand this between-plain heterogeneity, such as sediment characteristics and permeability, or nutrient availability. Secondly, the distance to the river influences the composition of stygobite assemblages. This influence was already reported in 1999 using 15 wells 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 NMDS2 axis) was correlated to the distance to the river: *N. kochianus* decreased or disappeared in wells very close to the river. Such a distance may reflect the gradual river influence with buffered temporal variability at long distance (Dole & Chessel, 1986).

Thus, our results suggest that classical metrics of groundwater crustacean assemblages, such as species composition, abundance and richness, may be of low value for the evaluation of the impact of intensive agriculture on subterranean ecosystems at the sector scale whereas better results may expected when wells are considered individually. Contrasted results can be found in literature about the relation between groundwater assemblages and land use. On one hand, Di Lorenzo & 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). It seems that the effect of chronic toxicity of pollutants used in agriculture is more obvious when considering the development rhythm of individuals instead of their mortality. For example, Di Marzio et al (2013) observed that chronic exposure of interstitial copepods to ammonium and herbicides (the carbamate pesticide Aldicarb) affected the developmental time spent to reach the adult stage.

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

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

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 monitoring 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 & Crane, 1994), insects (Custer et al., 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) 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. 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 locally abundant epigean amphipod, *Gammarus* cf *orinos* living at the interface between groundwater and surface water (e.g. springs and small spring-fed streams). The use of an epigean taxon also limited the exposure duration to one week because of high physiological needs of surface water organisms (Hervant et al., 1998).

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 & 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). In the present study, we used the EPI of Marmonier et al. (2013) that combines survival rates and changes in energetic stores expressed as a ratio between EPI measured *in natura* and obtained from reference individuals exposed in the laboratory (for comparisons between different periods of the year and different levels of energetic stores). Poor differences in the EPI score among crop-based sectors were observed during the period of low water transfer and low pollution (i.e. the dry season of a dry year), two months after the last potential pesticide application. In contrast, a strong upstream-downstream decreasing gradient in the EPI score occurred during the period of high water transfer and high pollution (i.e. a high water period of a wet year), during or just after a period of intensive agricultural activities (e.g. high and/or frequent pesticide applications). Chemical analyses of S-metolachlor confirmed these variable degrees of groundwater contamination between the two periods. This result suggests that sentinels caged in 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
545 that energetic stores, especially glycogen content, are valuable indicators of
546 environmental conditions for amphipods. The significant correlations of EPI with
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), S-
552 metolachlor as well as its major metabolites (ethane sulfonic acid and oxalinic acid)
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
564 Atrazine for *Daphina magna* is very high (35.5 ±9.2 mg/L, Palma et al., 2008) and
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_{50} 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 µg/L and DEA ranged from <LQ to 0.794 µg/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
580 considered by our sampling strategy) may have contributed to the observed
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
591 **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 sentinels were 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.

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Table 1: Physical and chemical characteristics of the groundwater sampled during the invertebrate survey of January 2012 in 28 wells located in the six sectors of the Ariège and Hers alluvial plains. Sectors are upstream (HUp) and downstream (HDw) of the Hers plain, upstream (AUp), intermediate (AInt1 and AInt2) and downstream (ADw) of the Ariège plain (n = number of wells sampled in each sector). Parameters include temperature (Temps. in °C), electric conductivity (EC in µS/cm), pH, oxygen concentrations (Oxy. in mg/L), and nitrate concentrations (NO_3 in mg/L; mean ± standard deviation). The land-cover around the 28 wells was used to characterize the six sectors with forest, grassland, urban area, total cultivated area (mainly corn) and oleaginous seed production (mainly sunflower, mean percentages ± standard deviation using wells as replicates).

	HUp (n=6)	HDw (n=6)	AUp (n=4)	AInt1 (n=4)	AInt2 (n=4)	ADw (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
pH	7.2 ± 0.1	7 ± 0.3	7 ± 0.1	6.9 ± 0.1	7.2 ± 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_3*	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

*Mean values (and SD) for nitrate were calculated on the basis of the monthly 2012-13 water 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).

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 ± 12.7	16.2 ± 18.5	7.3 ± 12	6.5 ± 12.3	2.5 ± 3.8	0.5 ± 0.6
	Niphargus ciliatus	1 ± 2	3 ± 3.9	0	0.3 ± 0.5	0	0.5 ± 1
	Niphargus foreli	2.2 ± 5.3	2.5 ± 6.1	0	0	0	0
	Niphargus sp.	0.3 ± 0.8	0	0	0	0	0
	Salentinella major	5.7 ± 7	0	0	2 ± 3.4	4 ± 4.7	0
	Salentinella petiti	2.7 ± 4.4	0	0	2 ± 4	2.7 ± 2.4	0
Copepods	*Ceuthonectes gallicus*	0	0	0	0	0.5 ± 1	0
	Diacyclops paolae	0.2 ± 0.4	0	0	0	1.5 ± 3	0
	Diacyclops clandestinus	0	0.2 ± 0.4	0	0	0	0
	Diacyclops belgicus	13 ± 31.8	0	0	0	0	0
Ostracods	*Candonopsis boui*	3.8 ± 6.1	0	0	0	0	0
	Pseudocandona sp. 1	0	0	0	0	5.5 ± 11	0
	Pseudocandona sp. 2	4.3 ± 10.6	0	0	0	0	0
	Pseudocandona sp. 3	0	26 ± 56.7	0	0	0	0
Abundances		43.7 ±32.3	52.5 ±49.9	7.5 ±11.9	11.3 ±12.2	18.8 ±15.0	1.3 ±1.9
Richness		8.2 ±3.9	6.5 ±1.0	2.5 ±2.4	4.8 ±2.9	6.0 ±1.4	3.8 ±2.1

Table 3. Scores of land use and physical and chemical characteristics along the first (NMDS1) and second (NMDS2) 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	-0.0436	-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	-0.9758	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	0.1056	-0.9944	0.088	0.408
pH	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
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

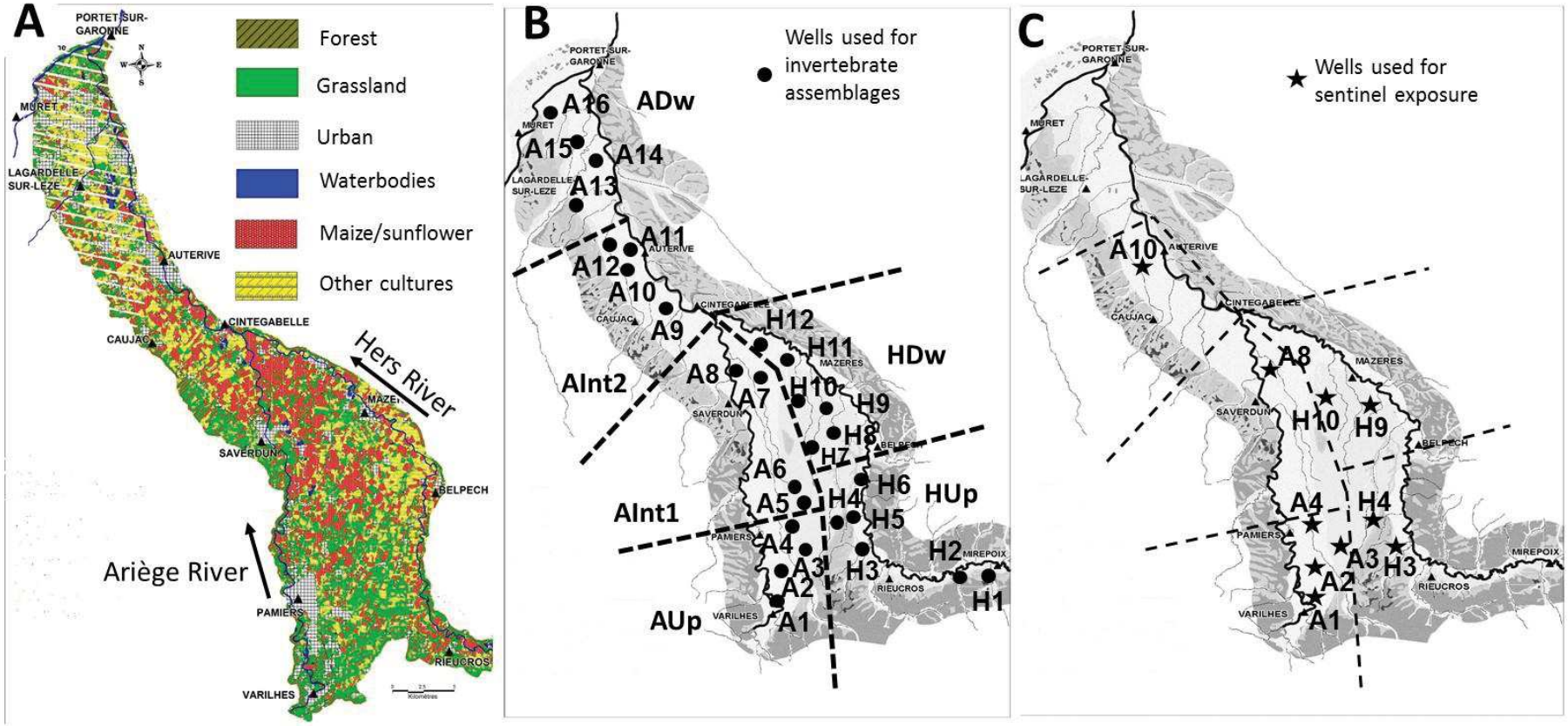

Figure 1. Land-use in the alluvial plains of the Hers and Ariège Rivers (A) and location of sampled wells for the survey of stygobite assemblages (B) and sentinels exposure (C). Dotted lines: limits of the six sectors based on PROMINENT agricultural crops and practices. The land use was established using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

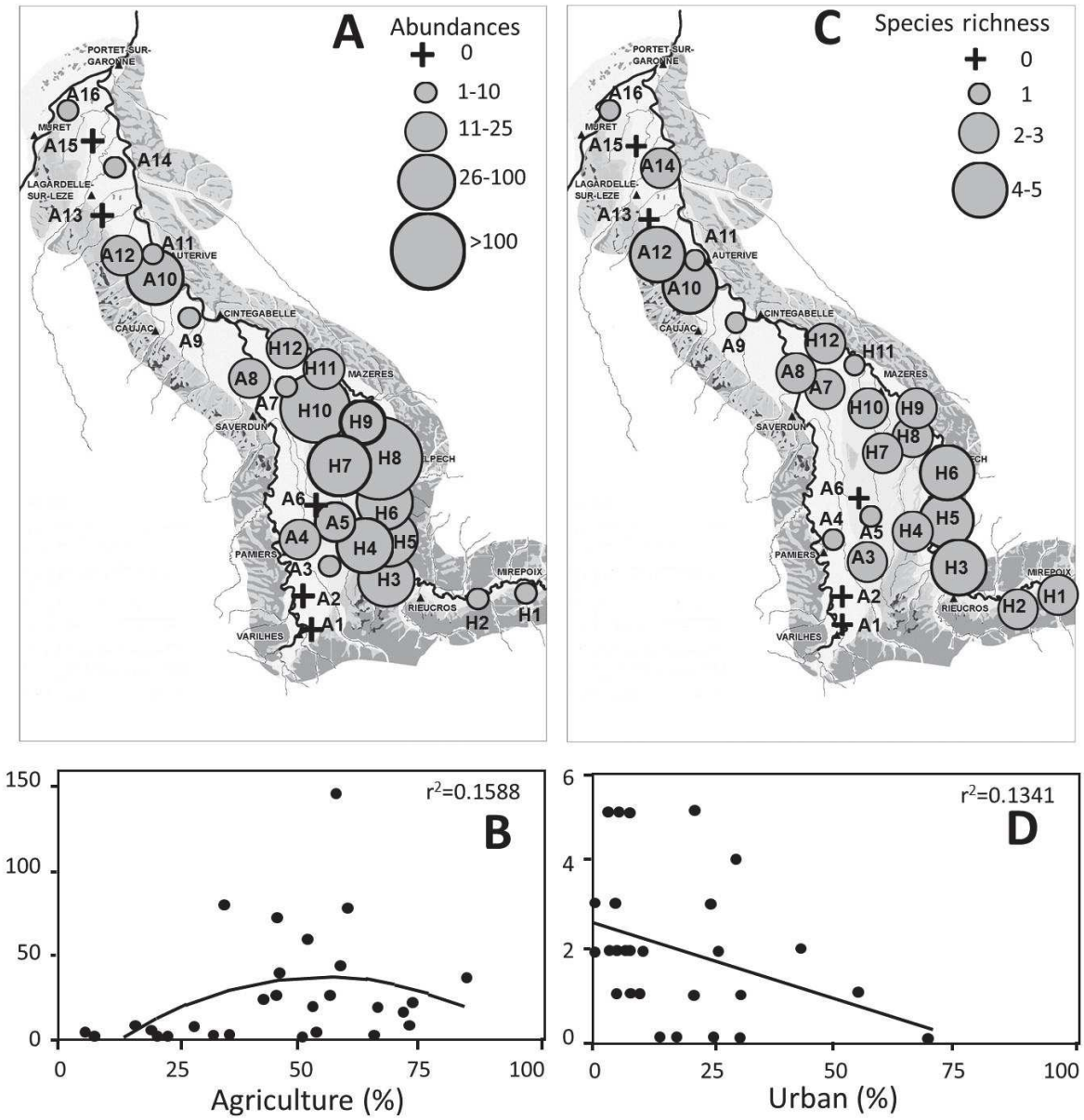

Fig 2. Abundances of stygobite as number of individuals per sample transformed in classes plotted on the sampling area (A) and relation between abundances and the percentage of agriculture around the wells ($y = -0{,}019x^2 + 2{,}095x - 22{,}05$, B). Species richness as the number of species transformed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

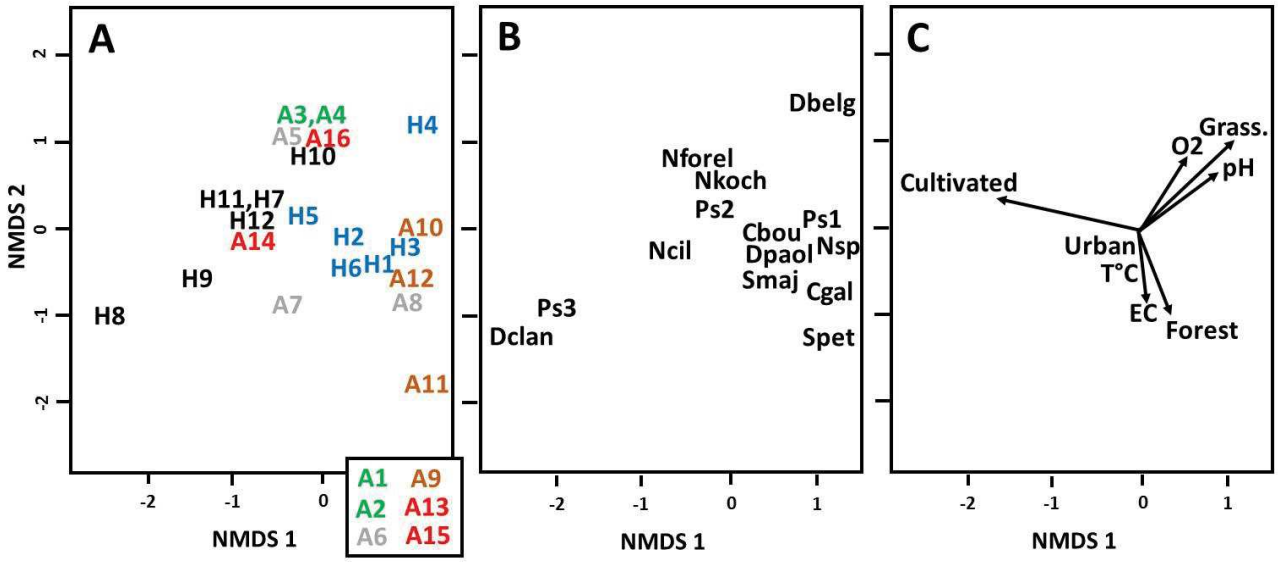

Fig. 3. NMDS of stygobite crustacean assemblages. The biplot of the first-two axes is separated into wells (A) and species (B). Environmental characteristics are fitted to this biplot (C). Wells noted in blue are located in HUp, black n HDw, green in AUp, grey in AInt1, brown in AInt2, red in ADw. The 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).

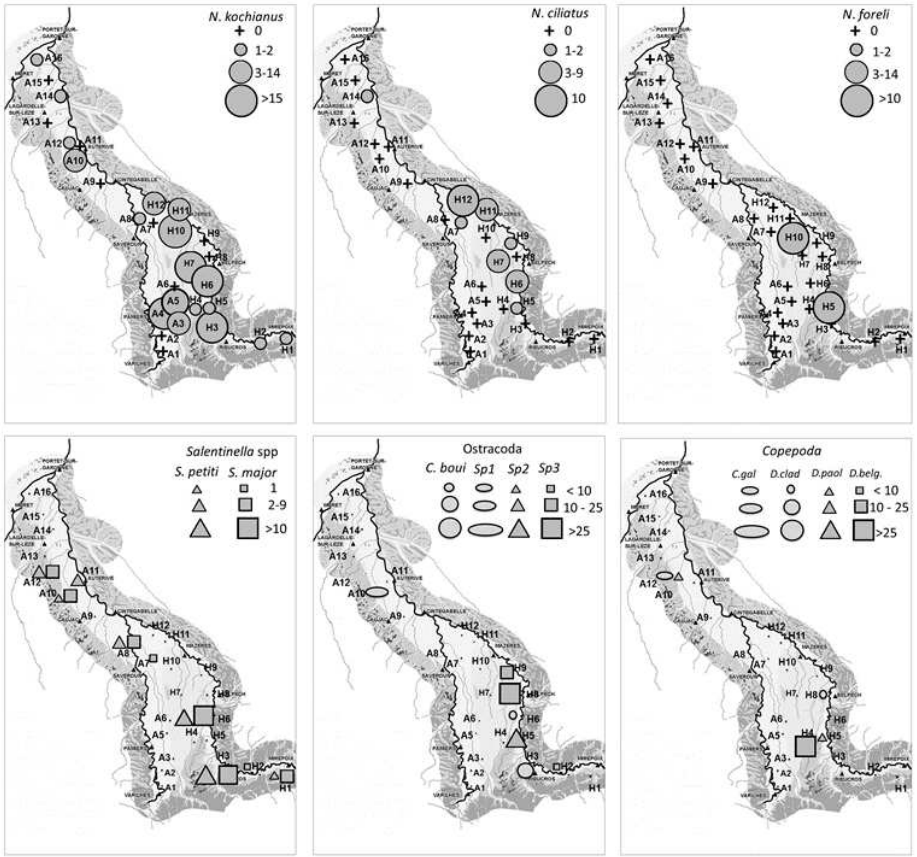

Fig. 4. Spatial distribution of stygobites crustaceans in the Ariège and Hers alluvial plains (as number of individuals per sample transformed in classes). Upper panels: *Niphargus* species. Lower panels: Salentinella spp; Ostracoda: *Candonopsis boui, Pseudocandona* sp. 1, sp. 2 and sp. 3; Copepoda: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

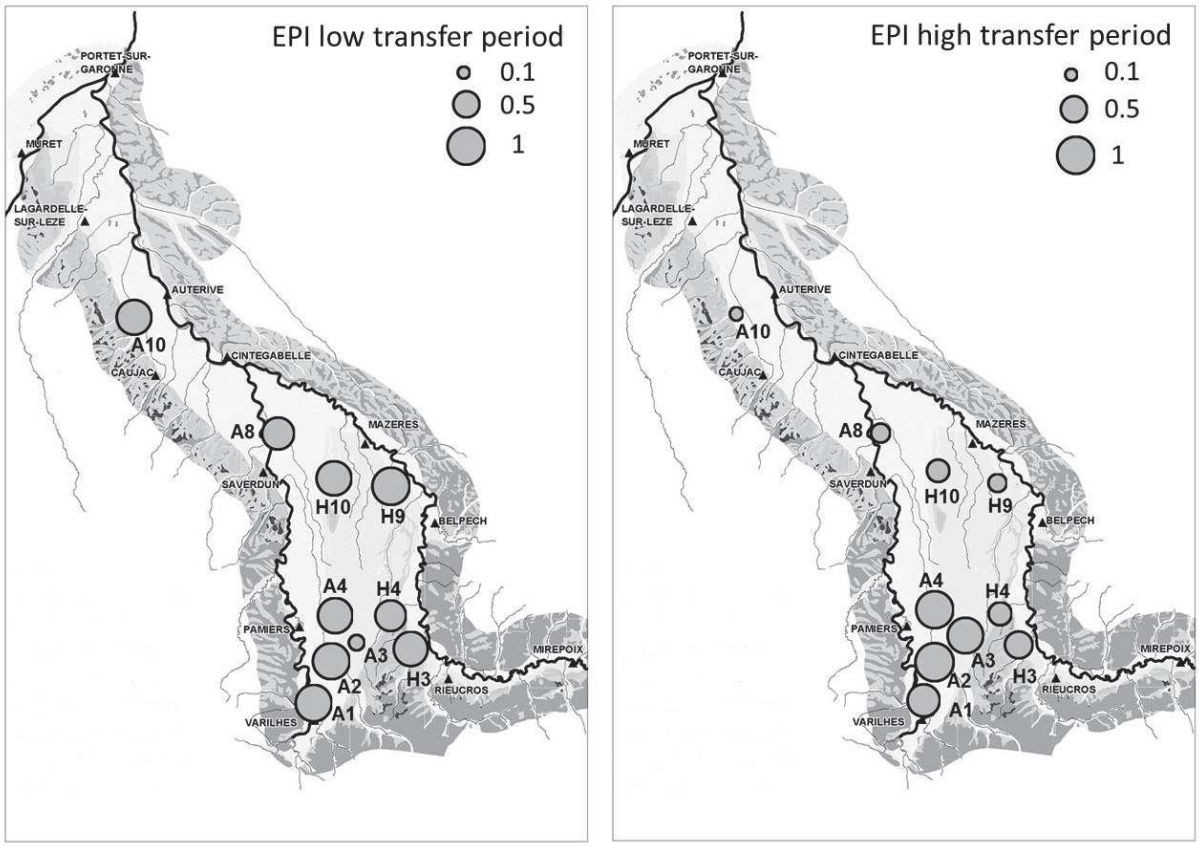

Fig. 5. Values of Ecophysiological Index (EPI) using *Gammarus* cf *orinos* sentinels after one-week exposure to *in situ* groundwater during low transfer period (September 2012) with low agricultural impact and during high transfer period (July 2013), withhigh agricultural impact. EPI values are expressed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

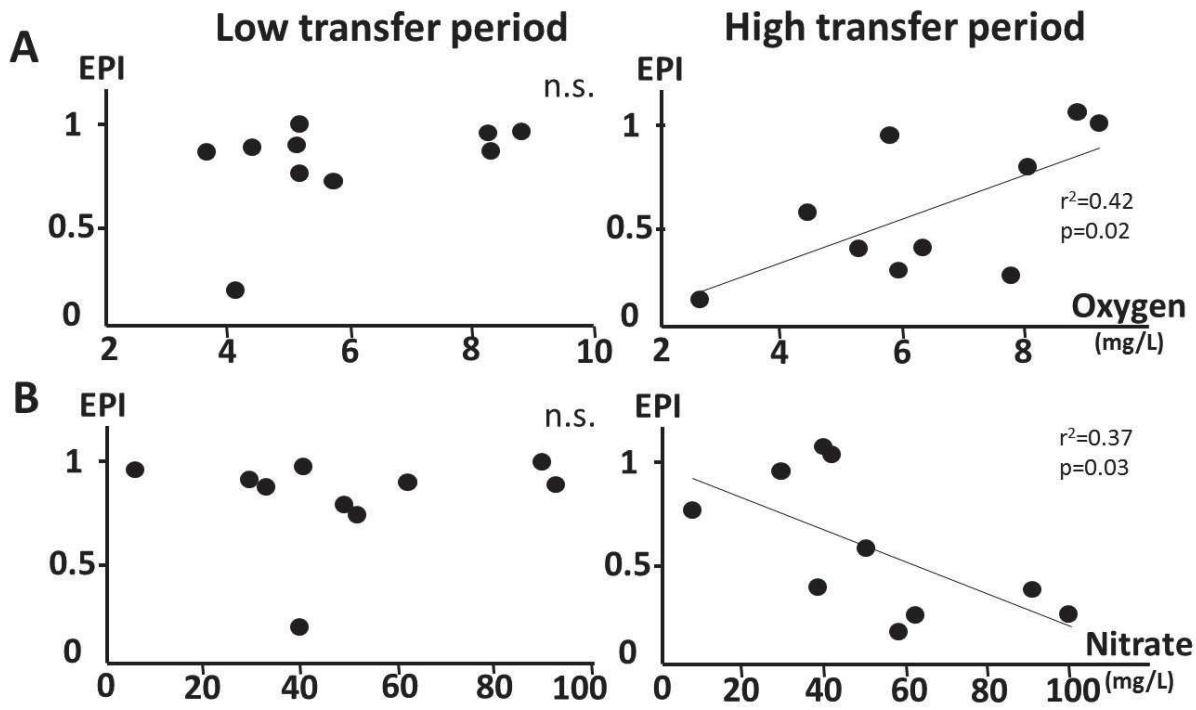

Fig. 6. Correlations between EPI and water chemical characteristics (A-Dissolved oxygen and B-nitrate concentrations) in period of low transfer (September 2012, left column, n=10) and high transfer periods (July 2013, right column, n=10). Regression line, r^2 and p-values are reported when significant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

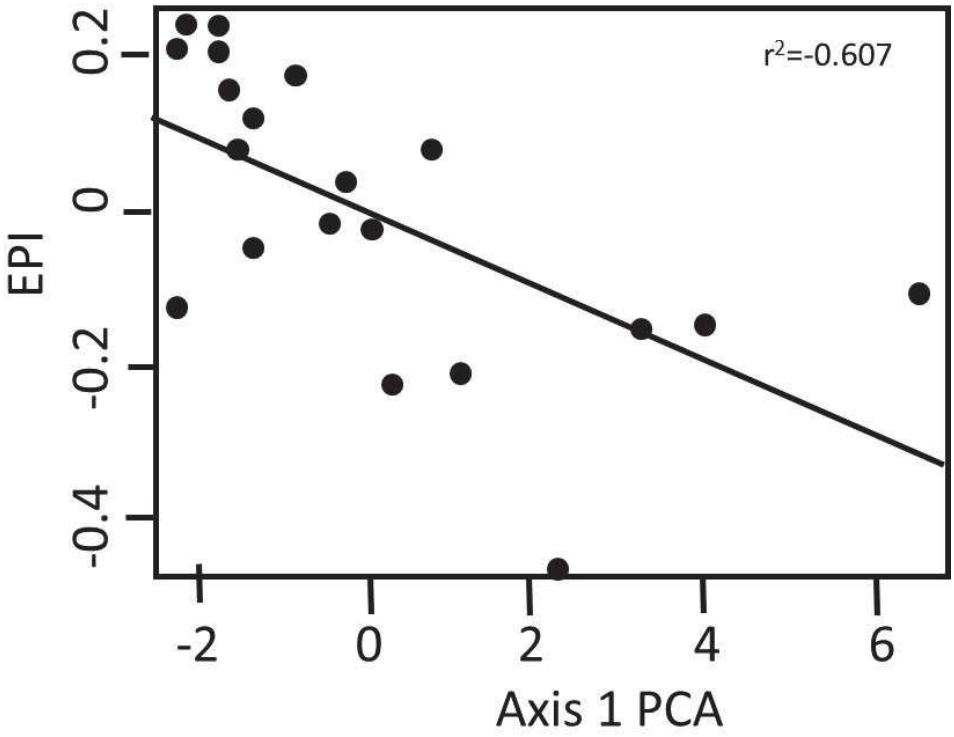

Fig. 7. Correlation between EPI and the first axis of a PCA performed on toxicants (period of high and low transfer cumulated, n=20). See Table 4 for variable loadings. EPI is represented as the residuals of a GLMM linking EPI to PCA axes 2 and 5, experimental sessions and the two resulting two-term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