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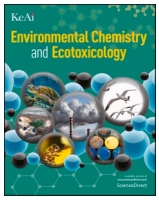

Research Paper

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ABSTRACT

Subsurface drainage increases pesticide transfer from intensive agriculture to surface waters. Their transfer to surface waters is facilitated by underground drainage networks, but the impact of seasonality still needs to be assessed *in situ*. This study aims to assess the effects of *in situ* pesticide on two gammarid species (*Gammarus pulex* and *Gammarus fossarum*) as well as the influence of confounding factors on their behavioural traits. A series of 18–19-day active biomonitoring campaigns were conducted during the 2022–2023 hydrological season at six sites in the Orgeval Observatory area (northeast France, Seine-et-Marne). The study area is subject to intensive drainage measures implemented through a dense network of sub-surface infrastructure. These sites were selected along a gradient of agricultural pressures, related to pesticide contamination within the hydrographic network, providing a comprehensive overview of the variability in environmental parameters (physicochemical, hydrological and physiographic) likely to affect gammarid behavioural traits. The results demonstrated significant temporal variations in pesticide concentrations related to application period and hydrological regime. Biomonitoring findings indicated sensitivity of both species, primarily driven by physicochemical factors, followed by contaminant levels, particularly those of transformation products and metals. The elevated responsiveness of these biomarkers (*e.g.* mortality, amplexus, locomotion, ingestion rate, biomass) to physicochemical parameters as opposed to chemical stresses (*e.g.* pesticides and metals) underscores the predominant influence of habitat parameters on the behaviour of both species. Consequently, it is imperative to consider these parameters when assessing the pesticide toxicity in agriculturally impacted aquatic systems to ensure accurate evaluations.

1. Introduction

Globally, pesticides are used extensively to protect crops from diseases and pests, thereby increasing yields to meet the needs of the human population. In France, which is the leading agricultural producer in the European Union, agriculture constitutes the predominant source of pesticide contamination, with annual usage estimated at 55,000–70,000 tons [1]. Runoff and leaching, processes which occur during precipitation and hydraulic transfers, are significant contributors to contamination of the hydrosphere. Implementation of subsurface drainage has become a widely adopted technique in contemporary agricultural practices in Northern Europe to remove excess water from waterlogged soils during winter. This approach facilitates the growth of crops and enhances agricultural productivity [2]. However, the implementation of artificial drainage systems facilitates water transfer from agricultural plots to ditches or rivers *via* networks of buried pipes [3]. In France, such drainage systems equip over 10% of the 27 million hectares of arable land, leading to an increased transfer rate of pesticides and nitrates into watercourses [4–6]. In artificially drained catchments, pesticides have been detected during initial flow events following application; conversely, their concentrations decrease in subsequent flow events [7].

In this context, aquatic organisms are continuously exposed to diffuse and chronic mixtures of pesticides, whose concentrations fluctuate seasonally. Notably, a previous study demonstrated that pesticides used in Europe are a contributing factor to the decline of aquatic invertebrate species in lotic environments [8–10]. These organisms are widely used in various experimental scenarios— such as *in vivo* [11], *in*

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mesocosms [8], and *in situ* [12,13] studies—to evaluate the impact of agricultural pollutants. The objective of these studies is to examine the impact of pesticides, focusing on the undesirable effects of certain pesticide molecules. However, the transformation products of these substances remain the subject of research. It has been demonstrated that the toxicity of transformation products is lower than the parent compounds, with some exceptions (*e.g.* the transformation products of tebuconazole) [14]. These transformation products can therefore have an effect on aquatic biodiversity. The use of macroinvertebrates in active biomonitoring has increased markedly, as the technique can effectively assess biological responses to environmentally realistic exposures. This approach comprehensively accounts for confounding factors—temperature, oxygen, pH, conductivity and temporal dynamics of contamination—that influence multi-contaminant toxicity and wildlife responses [15].

Macroinvertebrates, particularly gammarids, are considered ecosystem engineers and key indicators of aquatic ecosystem health due to their widespread distribution in watercourses and their multivoltinism life cycle [16,17]. Consequently, gammarids have frequently been used in active biomonitoring (*i.e.* caging standardized individuals). This approach limits biological variability, even at study sites lacking indigenous organisms, since the organisms are sampled from the same population with controlled exposure time [12], enabling assessment of the ecotoxicity of contaminants, particularly pesticides [18,19]. In European freshwaters, the two native predominant gammarid species are *Gammarus pulex* [20] and *Gammarus fossarum* [21]. These species demonstrate differential responses to abiotic pressures under *in situ* conditions. As demonstrated in the relevant literature, *G. pulex* displays a high degree of plasticity in its response to environmental stress [22,23]. By contrast, *G. fossarum* exhibits heightened sensitivity to elevated temperatures, organic pollution, and oxygen depletion, which are associated with environmental stresses [24–26]. A biomonitoring study using two species of gammarids can facilitate the assessment of inter-species variability and enhance our understanding of the early effects of pesticides on these organisms.

At the individual or population level, several biomarkers have been developed based on behavioural traits, as markers at the interface between the physiology and ecology of organisms, such as locomotion, amplexus and feeding [15,27]. Recent studies have demonstrated that exposure to pesticides, either *in situ* or in the laboratory at environmentally relevant concentrations (0.01–10 µg/L), can induce changes in biological traits such as locomotion, amplexus formation and ingestion rate [27,28]. For instance, the response to ingestion rate has been identified as a short-term sublethal bioindicator that is a precursor to water quality, indicating responses occurring over longer periods at the community and ecosystem levels [29]. While a number of studies indicated that pesticides have an impact on individual biomarkers [18], confounding parameters (pH, temperature, oxygen, conductivity, *etc.*) may also exert greater influence on biomarker responses [30,31], particularly in French biomonitoring programs with caged gammarids. Consequently, it is imperative to conduct multiple biomonitoring campaigns across diverse locations to account for the temporal and spatial variability of exposure dynamics, encompassing pesticides and environmental parameters under continuous monitoring.

However, experts have highlighted the lack of knowledge regarding the relationship between exposure to pesticides and their effects on biodiversity under *in situ* conditions. This gap in knowledge motivated the present study [1,32].

The aim of this study is to assess the impact of the seasonality of pesticide occurrences on non-targeted freshwater species exposed in rivers, focusing on two common gammarid species. Furthermore, the impact of confounding parameters on individual or population biomarkers in gammarids under *in situ* conditions is examined. This *in situ* study assesses (i) gammarid biomarker sensitivity to agricultural pressures, (ii) biomarker variability due to confounding parameters (physicochemical, hydrological, physiographic) and (iii) inter-species variability of these individual-population level biomarkers to identify the most suitable species for assessing the ecotoxicity of pesticide influxes in agricultural freshwaters.

2. Materials and methods

2.1. Experimental site: Orgeval catchment

The biomonitoring study was conducted at the ORACLE observatory (https://gisoracle.inrae.fr/) located in the Orgeval catchment area (France, Seine-et-Marne, 77). This catchment has been instrumented for hydrological monitoring since 1962 and for water quality monitoring since 1975. The catchment is mainly agricultural (81%) and is representative of the main cereal crop rotation in the Seine basin [33]. The present study focused on the Avenelles sub-catchment, *i.e.* the eastern part of the Orgeval catchment draining an area of 45 km^2 [34]. The average annual temperate is 9.7 °C and the annual rainfall is between 600 and 700 mm. The hydrogeological behaviour of the Orgeval basin is influenced by the aquifer system, which is composed of two main geological units: the Oligocene and the Middle Eocene. These two aquifer units are separated by a clayey aquitard composed of Priabonian mudstone and Bartonian marl [35]. Soils in the plateau regions are classified as predominantly luvisols. Approximately 80% of agricultural land is subject to subsurface drainage, a practice implemented to mitigate soil waterlogging during winter. The movement of pesticides into watercourses is facilitated by these hydraulic management systems, as observed 1 month after the application of pesticides [36]. The biomonitoring survey was conducted at six sites within the Avenelles catchment, encompassing two confluences of the Rognon River and its two tributaries (Étang and Loge streams) (Fig. 1).

The sites were selected on the basis of gammarid abundance, variability in physicochemical parameters and contamination gradients. These six monitoring sites were identified and instrumented to enable continuous tracking of the exposure dynamics of wild populations, including gammarids, following pesticide transfers into the environment. The six sites are hereafter referred to as follows: E (48.8628, 3.1541), M (48.8608, 3.1559), and its confluent site EM (48.8595, 3.1513), L (48.8494,3.1439), R (48.8517, 3.1472) and its confluent site LR (48.8425, 3.1431) (Fig. 1). For each site, land use was characterized by sub-catchment (Table 1).

2.2. Biomonitoring campaigns

2.2.1. Chemical monitoring

Each site is instrumented to continuously monitor the exposure dynamics of bioindicator organisms. Samplers (ISCO 3700) were used to collect daily composite water samples, at a pumping frequency of 3 h. For each campaign, a composite water sample was produced (a proportional mixture of each daily sample) at each station (except for collection sites where one-off sampling is carried out) to examine the dissolved concentrations of 575 pesticide molecules present (including transformation products). Samples were analysed by an independent COFRAC-certified laboratory (INOVALYS). Only 104 molecules were detected, *i.e.* 18% of the analysed molecules with an average LOQ of 0.01 µg/L. A total of 45 herbicides, 35 transformation products, 19 fungicides and five insecticides were quantified. Metals were also quantified because some of them are used as plant protection products. The concentration of dissolved metals (filtered at 0.2 µm and conditioned with nitric acid) was analysed by ICP-MS in an independent laboratory (Institut de Physique du Globe de Paris). To complete the list of analysed parameters, these composite water samples were also analysed for anions/cations (nitrite, phosphate, sulphate, sodium, ammonium, potassium, magnesium, calcium) and dissolved organic and inorganic carbon. Daily samples were collected for analysis of nitrate concentration, turbidity and total suspended solid (TSS). Additionally, at each station, multi-parameter probes (WTW Multi 3630) and

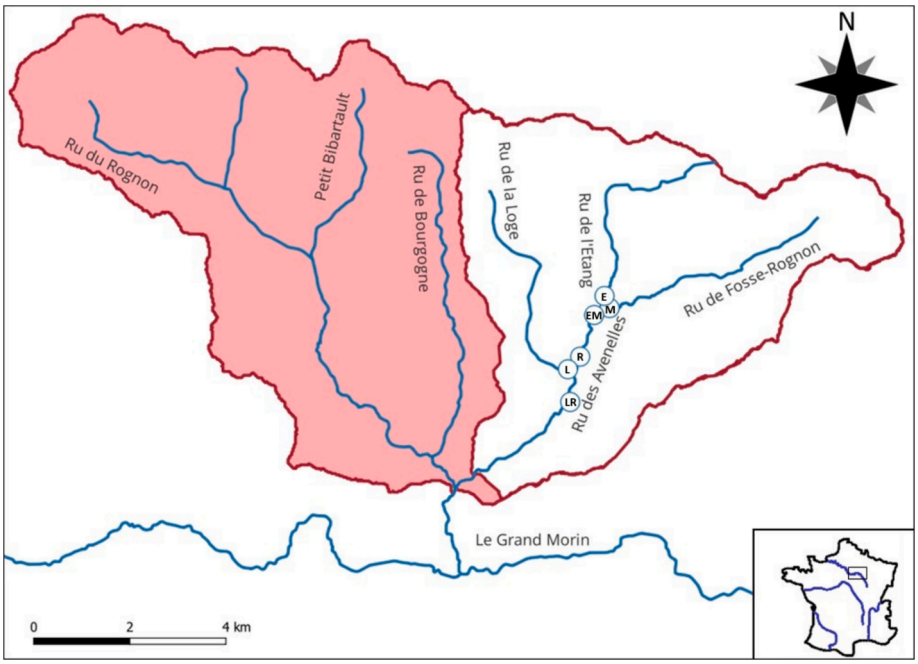

Fig. 1. Map of the Orgeval catchment and the six instrumented sites. The Avenelles catchment is the eastern sub-catchment (in white) of the Orgeval catchment. The six instrumented sites are E, M, EM, L, R, LR.

Table 1
Land use at the six instrumented sites expressed in percent or in km^2 for the total catchment area according to the Corine Land Cover inventory.

	Sites					
	E	M	EM	L	R	LR
Urban (%)	6.53	5.25	6.07	1.65	6.22	4.98
Arable (%)	88.20	92.31	90.76	44.16	90.63	77.10
Forest (%)	5.27	2.44	3.17	54.19	3.15	17.93
Total Surface (km^2)	6	15	22	10	23	36

dataloggers (CTD Diver) were used for continuous monitoring of the physicochemical and hydrological parameters at a 15-min acquisition frequency. The parameters continuously monitored were temperature, conductivity, dissolved oxygen, concentration, pH and water level. At each visit to the sites, spot measurements using multi-parameter probes were taken at the beginning and end of each biomonitoring campaign. Furthermore, hydrological monitoring data including rainfall, water level and discharge at the Orgeval catchment outlet were collected from the open database (BDOH ORACLE, https://bdoh.irstea.fr/ORACLE/) managed by INRAE.

2.2.2. Ecotoxicological monitoring

To integrate temporal variability into the physicochemical and chemical characterization of the sites (pesticide, nitrate and metal concentrations), combined analysis campaigns were conducted over 18- and 19-day periods from January 2023 to June 2023.

The two control populations of gammarids, *Gammarus pulex* (GP) and *Gammarus fossarum* (GF) were collected from two upstream sites of the Grand Morin basin (northern France upstream of Greater Paris): Ru de l'Etang, Doue (DOUE site) and Ru de Saint Blandin, Guérard (GUE site). These headwater sites have been selected as the reference sites for a number of studies. It is well documented that these sites are slightly contaminated by pesticides and metals, yet they demonstrate good physicochemical quality and a high density of gammarids [37,38]. Approximately 2000 individuals per species were collected and size-calibrated for each campaign, using a landing net deployed against the current to carry out transplantation. It was hypothesized that size selection serves as a proxy for life stage. Individuals measuring 1 ± 0.2 cm were selected using four successive sieves (from 5 to 0.5 mm) to allow gammarids measuring <1 cm (including juveniles) to remain in their environment, thereby ensuring the continued development of the local population. The organisms were transported in a cool box containing local water, which was circulated and aerated to maintain stable conditions until gammarids transplantation.

The transplantation experiment involved caging the two gammarid species at the six identified sites. The cages consisted of a transparent polyvinyl chloride tube (20 × 9 cm; length × diameter), closed with a 500-µm nylon mesh to allow water circulation inside the cage while preventing entry of native macroinvertebrates. Each cage contained 80 gammarids to have a representative part of the wild population and 2.5 g ± 0.02 of alder leaves (*Alnus glutinosa*). Alder leaves were collected, known for their attractiveness for detritivorous macroinvertebrates [39], were collected in autumn 2022 on the shore of a pond, located in the Orgeval area at Aulnoy (France). The leaves conditioned by selection and cleaning to remove other food sources (microorganisms), followed by initial drying at room temperature for 24 h and final drying for 24 h in an oven at 60 °C. At each monitored site, a weighted, large-mesh plastic box containing three cages (triplicate condition) per species was placed directly on the riverbed, aligned with the flow direction and at sufficient depth to remain fully submerged. The box was secured to the stream bed with steel poles and ropes. In addition, a smaller cage (9 × 7 cm; length × diameter), closed with a 500-µm nylon mesh and placed inside the box, contained only leaves to monitor natural decomposition in the absence of gammarids. These small cages enable monitoring of leaf degradation by microbial communities and/or by physical processes (in particular water flow); and to substitute this degradation for feeding rate to determine the degradation attributable to gammarids.

Five transplantation periods were established (*i.e.* five "campaigns", hereafter referred to as Ci, where *i* represents the campaign order) across the six study sites from 5 January 2023 to 27 June 2023 (Fig. 2). C1 took place during 6–23 January 2023, when drainage resumed, while C2

(from 17 February to 6 March 2023), C3 (from 22 March to 6 April 2023) and C4 (from 21 April to 5 May 2023) were subject to little or no drainage. Finally, C5 (from 9 to 26 June 2023) was subject to stormy rainfall resulting in significant peak flows. These different campaigns allow us to assess the seasonal nature of pesticide transfer and the natural variability of the monitored physicochemical parameters.

2.3. Individual and populational biomarkers

2.3.1. Mortality monitoring

At the end of each biomonitoring campaign, cages were retrieved with site water and transported to the laboratory, where they were opened and mortality was recorded. The daily mortality rate, used to standardize results across different campaigns (of variable duration due to climate conditions), was determined using Eq. 1:

$$\text{Mortality} = (100 - (100 \text{ x } N/n)/T \quad (1)$$

where N is the number of live gammarids, n is the number of totals gammarids at T0 ($n = 80$), and T is the duration of the campaign (in days).

2.3.2. Natural decomposition and ingestion rate activity

Leaves were collected from cages without gammarids, rinsed with cleaned water, dried at room temperature for 24 h, and finally oven-dried at 60 °C for 24 h. The natural decomposition was determined using the quotient C_L, which is the leaf change correction factor [27]:

$$\text{Natural decomposition } (C_L) = L_1/L_2$$

where L_1 is the dry weight of leaves fed initially (mg) and L_2 is the dry weight of leaves collected at the end of the campaign. The food material was collected to determine the ingestion rate (IR) using Eq. 2:

$$\text{IR} = (Li - Lf) \text{ x } C_L/W \text{ x } T \quad (2)$$

where L_i and L_f are the dry weight of leaves initially (L_i) supplied and recovered (L_f) (expressed in mg) at the end of the campaign, C_L is the leaf change correction factor, and W is the dry weight of livings gammarids (mg).

2.3.3. Amplexus activity

The percentage of amplexus among all surviving individuals was calculated to determine the proportion of the population in the first stage of the reproductive cycle, using Eq. 3:

$$\text{Amplexus} = (100 \text{ x } A)/N \quad (3)$$

where A is the number of amplexus and N is the number of livings gammarids.

2.3.4. Locomotor activity

Locomotor activity was assessed and expressed in movement/individual/min using a modified method from Lebrun et al. [37]. In total, 20 individuals (N) were randomly sampled in each cage and introduced into a cylindrical beaker (diameter: 9 cm) containing 250 mL of site water. Their locomotion was determined by counting the number of times that gammarids crossed for 30s a line drawn on the bottom of the beaker corresponding to a radius of the beaker. For each beaker, this measurement was repeated 4 times and averaged. Each beaker of 20 gammarids was first acclimatised for 5 min in their new environment."

2.3.5. Gammarid biomass

Finally, all remaining gammarids including the 20 from locomotion activity were collected and lyophilized for 24 h to determine their dry weight (required for ingestion rate determination, *cf.* Eq. 2).

2.4. Data and statistical analyses

For continuous monitoring (physicochemical and hydrological assessment), data were integrated to better characterize the spatial and temporal variability. Six descriptive statistics, namely minimum, 25th percentile (Q25), median, mean, 75th percentile (Q75) and maximum, were determined for each of nine monitored variables: temperature, conductivity, dissolved oxygen concentration, pH, nitrate concentration, anions/cations (5 anions and 4 cations) and dissolved organic and inorganic carbon. Among these six descriptors, only those that best explained variability (by PCA analysis) were retained to avoid redundancy that could bias the statistical analyses. For chemical monitoring, two main parameters were monitored: concentrations of pesticides and dissolved metals. For hydrology, three parameters were monitored: water level, turbidity and TSS; for physiography, three parameters were calculated (in surface (km^2) or in percent) depending on land use: urban, arable and forest based on Corine Land Cover. The available site information on physicochemical (1230 data points; 6 sites × 5 campaigns × 17 parameters × 6 descriptors; temperature, conductivity, dissolved oxygen concentration, pH, nitrate concentration), chemical (3000 data points; 6 sites × 5 campaigns × 100 molecules; 88 pesticides and transformations products, 12 ETM), hydrological (540 data points; 6 sites × 5 campaigns × 3 parameters × 6 descriptors) and physiographic (540 data points; 6 sites × 5 campaigns × 3 parameters × 2 descriptors) variables was summarized using Pearson's principal component analysis (PCA). From the PCAs, for each set of environmental data (physicochemistry, chemistry, hydrology, physiography) only F1 and/or F2 and/or F3 scores were retained if they explained more than 15%. These scores were used as integrated variables in subsequent statistical analyses.

The partial least squares (PLS) regression method was used to establish relationships between the environmental responses and multiple explanatory variables [41,42]. To identify the factors likely to influence the biological traits of the two gammarid species, PLS regression was performed using the biological traits as dependent variables and the F1 and/or F2 and/or F3 scores of the environmental data as explanatory variables. For pesticide concentrations, we used a concentration-based approach, which provides a higher percentage of explanation and cumulative variance (R^2Y) than concentration- and family-based approaches or even class- and family-based approaches (determination of concentration thresholds that allow molecules to be classified into five ranges).

Multiple factor analysis (MFA) was used to examine associations between all biological traits and the different species, to assess the relevance of biomarkers from both species in evaluating the impact of pesticides *in situ*. MFA is a multivariate method for analysing data structured as sets of descriptive variables, such as biomarkers. It is used to highlight the structures that are common to groups of variables, to balance the influence of each set, to explore inter-group associations, and to compare group-based characterizations of observations [22]. MFA is derived from PCA (which identifies directions of maximum inertia) and canonical analysis (which seeks common factors and reduces dimensionality). All statistical analyses were performed using XLStat (Addinsoft) and RStudio for Windows (version 4.3.3).

3. Results and discussion

3.1. Dynamics of exposure

3.1.1. Hydrological dynamics

By relating the hydrological regime to the pesticide concentrations at each site, we characterized the dynamics of contaminant transfer more accurately and thus assess the impact on organisms more effectively. As demonstrated in Fig. 2, the hydrological season of 2022–2023 had a mean rainfall of 2.3 mm/d at the outlet of the Orgeval catchment and a corresponding flow discharge. The initial peak in flow discharge at the

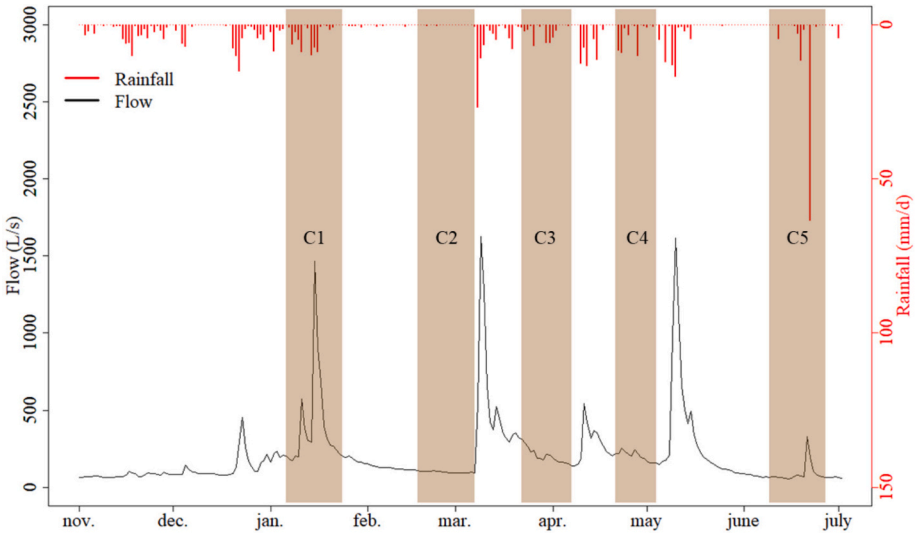

Fig. 2. Hydrogram of flow rates (L/s) and rainfall (mm/d) at the Orgeval catchment outlet during the study campaigns. Campaign: C1: 6–23 January 2023, C2: 17 February to 6 March 2023, C3: 22 March to 6 April 2023, C4: 21 April to 5 May 2023, C5: 9–26 June 2023.

catchment outlet was observed on 23 December 2022, prior to the commencement of the C1 campaign. This peak flow is concomitant with the initiation of drainage network flows. It can thus be concluded that C1 and C5 were the only two campaigns in which peak flow occurred. In summary, all campaigns adequately represent the range of typical hydrological conditions (Fig.2), which are characterized by: 1) low rainfall and low flow rates (C2, C3 and C4) and; 2) high flow events occurring in winter and spring (C1 and C5), with maximum daily rainfall of 65 mm and flow rates up to 1500 L/s.

3.1.2. *Metal concentrations in water*

To assess the potential toxicity of metals, dissolved metal concentrations were compared with the French Predicted No Effect Concentrations (PNECs) established for the protection of surface waters (INERIS, https://www.ineris.fr/fr). In Fig. 3, only the dissolved concentrations of Sb, Cu and Ni exceeded their respective PNECs at the outlet of our 6 monitored sites. Sb exceeded the PNEC during all campaigns, ranging from 1.15 µg/L (C4) to 1.25 µg/L (C3) showing limited seasonal variability. Cu exceeded its PNEC with a maximum concentration of 1.72 µg/L during C5 and exhibited marked seasonality, with higher concentrations during C1–C2 and C5, and lower concentrations during C3–C4 (0.97 and 0.86 µg/L, respectively). Ni exceeded the PNEC during all campaigns except C2, reaching a maximum concentration of 0.641 µg/L during C5. Overall, the highest dissolved metal concentrations were systematically observed during the C5 campaign (*e.g.* Sb, Cu, V, Ni, As and Cd). However, Lebrun et al. [40] demonstrate that metals at EQS-compliant concentrations can still induce sublethal effects, particularly on behavioural traits such as locomotion and respiratory activity in *Gammarus fossarum* questioning the relevance of these standards for the protection of aquatic life. In addition, concentrations of several metals measured in this study (notably Zn, Sb, Cu and Co) were higher than those reported by Lebrun et al. [43], in the context of constructed wetlands within a drained agricultural catchment with similar pedological conditions highlighting site-dependent variability. As Meite et al. [44] demonstrated, the transfer of metals, particularly the leaching of Zn and Cu, is significantly influenced by soil type and age. In fact, Zn leaching tends to be more significant in arable soils than in vineyard soils. However, the process of Cu leaching shows similar

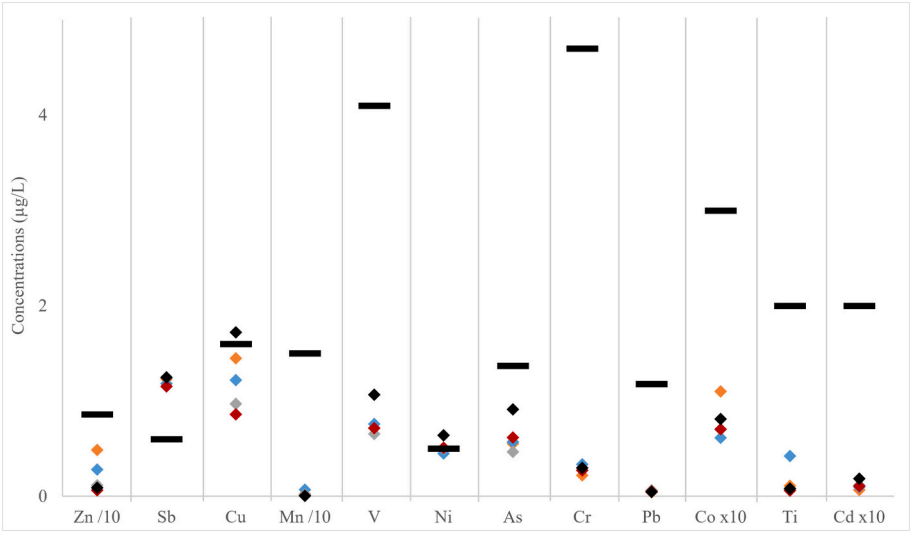

Fig. 3. Metal concentrations (µg/L) in dissolved fractions at the downstream site LR, for the five sampling campaigns. Campaign symbols: C1: orange; C2: blue; C3: grey; C4: red; C5: black. Black lines represent French quality standards for dissolved fractions. For more clarity, scaling factors are applied to Zn (/10), Mn (/10), Co (x10) and Cd (x10).

characteristics in both orchard and arable soils. Additionally, heavy precipitation at the onset of the agricultural season can promote the migration of metals from their original sources before soil consolidation occurs, thereby reducing their subsequent transfer. In summary, although dissolved metal concentrations at the study sites were generally below PNECs, the observed levels may still exert ecologically relevant effects on sensitive organisms such as gammarids. These results indicate that the studied sites are subject to multi-contamination (*e.g.* metals and pesticides).

3.1.3. Pesticide concentrations in water

Qualitative and quantitative measurement of pesticides at each site (including gammarid collection sites) across all campaigns enabled characterization of the chemical exposure level in organisms, capturing both spatial and temporal variability. As illustrated in Fig. 4, the concentrations of pesticides (both parent compounds and transformation products) at the two collection sites (DOUE and GUE sites) for the two gammarid species are lower (ranging from 0 to 3 µg/L) than at the monitoring sites (ranging from 3 to 12 µg/L). This result corroborates the hypothesis that these collection sites are less contaminated than the monitoring sites, resulting in low exposure of gammarids prior to their transplantation. However, it should be noted that the differences in concentrations may sometimes be minimal (*e.g.* C2).

In drained agricultural catchments, studies have demonstrated that, with the exception of three of the 24 analysed molecules, pesticides can be detected in drainage water within 1 month of application. This is attributable to the large quantities applied and their different chemical properties [36]. The findings obtained at the national level are also pertinent at the European and United States levels, as evidenced by Kladivko et al. [7]. It can therefore be deduced that the highest concentrations generally occur during the first drainage event(s) following pesticide application. However, in studies that span multiple years, meteorological conditions have been demonstrated to contribute to significant inter-seasonal variability in concentration peaks and overall pesticide losses [7,45].

Chemical analysis revealed that all sites exhibited elevated levels of contamination, with significant peaks observed during the first and fifth campaigns. C1 demonstrated a predominance of transformation product concentrations, with total pesticides and transformation product concentrations ranging from 2 to 8 µg/L across the various sites. However, the last campaign showed a dominance of parent compounds, with the highest concentration varying between 1.3 and 13.5 µg/L. As demonstrated in Fig. 1, during the C2, C3 and C4 campaigns, the concentration of transformation products is lower, with a maximum of 1.2, 2.9 and 1.8 µg/L, respectively. The highest concentration for each campaign was measured at site M, with a minimum concentration of 2 µg/L during C2 and a maximum concentration of 16 µg/L during C5. This phenomenon is likely associated with the land cover at site M, which has the highest proportion of arable land at 92.31% (see Table 1). Conversely, sites E and L exhibited minimal contamination levels, with concentrations as low as 1 µg/L during C2 and reaching a maximum of 6 and 4 µg/L, respectively, during C1. This phenomenon can be attributed to the strategic positioning of site E, which is situated at the head of the catchment area. This location is characterized by a source that functions as a diluent for pesticide transfers, thereby reducing their impact. The aforementioned factors resulted in lower pesticide transfer to watercourses upstream of site E. Furthermore, site L, which is 54.16% forested, exhibited the lowest pesticide concentration.

In their field study, Brown & Van Beinum [45] confirmed that the peak drainage event following pesticide application is characterized by high pesticide concentrations. However, if a significant amount of time elapses between application and drainage flow (more than 10 days), only certain pesticide residues are likely to be linked to an increase in the sorption strength of molecules over time [45,46]. For this reason, high concentrations of transformation products were observed during the C1 campaign, which corresponds to the first significant drainage event of the season. The highest concentrations were observed for soluble, poorly degradable or mobile molecules or certain herbicides applied in large quantities, such as bentazon (mean across all campaigns: 0.74 µg/L), metolachlor (mean across all campaigns: 0.17 µg/L), propyzamide (mean across all campaigns: 0.1 µg/L) [33].

3.1.4. Physicochemical dynamics

In Table 2, measures showed that the physicochemical characteristics were different for each site and campaign. The pH values ranged from 7.24 to 7.97, with minimal variation between campaigns

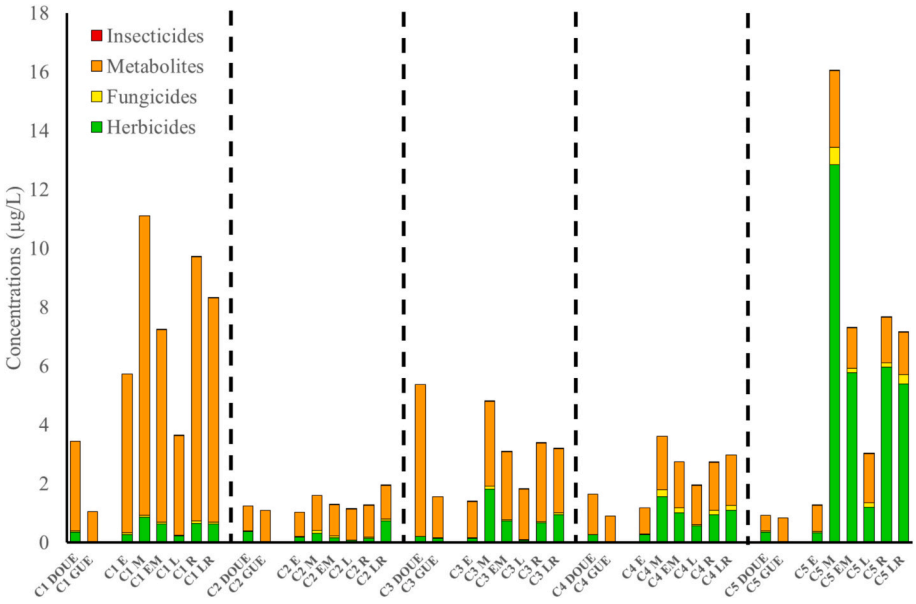

Fig. 4. Pesticide concentration (µg/L) in dissolved fraction (500 molecules analysed) grouped by family, site and campaign. Pesticide families: Insecticides (5 molecules quantified out of 104 detected); Metabolites: transformation products (35 molecules quantified out of 104 detected); Fungicides (19 molecules quantified out of 104 detected); Herbicides (45 molecules quantified out of 104 detected). Sites: DOUE (sampling of *Gammarus pulex*), GUE (sampling of *Gammarus fossarum*), E, M, EM, L, R, LR. Campaign: C1: 6–23 January 2023, C2: 17 February to 6 March 2023, C3: 22 March to 6 April 2023, C4: 21 April to 5 May 2023, C5: 9–26 June 2023.

Table 2
Physicochemical parameters in water at the six monitored sites (from 6 January to 26 June 2023; $n = 5$ campaigns). Values are presented as minimum ; **mean** ; maximum; values in italics represent the coefficient of variation in %. Significant differences between sites mean are indicated by letters (n = 5; Friedman *Post hoc* test; $P < 0.05$).

	Sites											
	E		M		EM		L		R		LR	
Temperature (°C)	8.3 ; **11.0** ; 15.0 *20*	a	6.7 ; **10.6**; 14.2 *46*	a	7.7 ; **11.0**; 14.1 *29*	a	7.3 ; **10.6**; 13.9 *33*	a	7.3 ; **10.5**; 13.7 *36*	a	7.3 ; **10.5**; 13.6 *40*	a
Oxygen (%)	65 ; **89** ; 137 *13*	a	70 ; **93** ; 128 *12*	a	57 ; **91** ; 120 *6*	a	63 ; **85** ; 109 *9*	a	61 ; **85** ; 103 *10*	a	77 ; **93** ; 115 *9*	a
Conductivity (µS/min)	445 ; **651** ; 683 *4*	a	375 ; **486**; 521 *3*	b	415 ; **586**; 695 *8*	ab	437 ; **659**; 746 *9*	a	376 ; **602**; 671 *9*	ab	452 ; **618**; 683 *8*	ab
pH	7.0 ; **7.2** ; 7.6 *2*	a	7.4 ; **7.6** ; 8.0 *5*	ab	7.3 ; **7.6** ; 8.0 *4*	a	7.5 ; **7.8** ; 8.0 *1*	ab	7.4 ; **7.8** ; 8.2 *3*	ab	7.6 ; **8.0** ; 8.4 *2*	b
Nitrates (mg/L)	61 ; **63** ; 68 *4*	a	43 ; **49** ; 60 *19*	b	53 ; **58** ; 65 *9*	ab	43 ; **49** ; 58 *17*	b	50 ; **54** ; 61 *11*	ab	50 ; **54** ; 62 *11*	ab

(coefficient of variation below 5%) and significative differences between E or EM and LR sites. Water electrical conductivity was somewhat stable over time (coefficient of variation less than 10%), and significative differences were detected between E or L and M sites.

Nitrate concentrations were stable over time at sites E and EM (coefficient of variation less than 10%), with mean concentrations of 63 mg/L and 58 mg/L, respectively. At the other four sites, the variation in nitrate concentration over time was slightly more significant (coefficient of variation between 10% and 20%), and significant differences were detected between M or L and E. Dissolved oxygen ranging between 85% and 93% but no significant differences were highlighted between sites. Finally, water temperature varied considerably between campaigns, ranging from lows of 6–7 °C in winter to highs of 14–15 °C in spring but no significant differences were highlighted.

Overall, we observed spatiotemporal physicochemical variability, with high coefficient of variation for nitrate concentration and temperature. The variations in nitrate concentration are directly related to the seasonal application of nitrogen fertiliser. However, seasonal variations in nitrate levels are also influenced by agricultural practices and the storage of nitrogen in the soil profile during periods of precipitation [3]. Water temperatures vary in response to hydroclimatic forcing, with low temperatures in winter and high temperatures in summer.

3.2. Interspecies variability

The biomarkers measured in both species showed marked spatiotemporal variability, as detailed in Appendix A. Although values generally fell within ranges reported in the literature [48], interspecific variability and temporal fluctuations suggest an influence of environmental confounding parameters and chemical pollution. As demonstrated in Fig. 5, multiple factor analysis (MFA) was conducted to evaluate the differences in individual/populational biomarkers linked to their variability in the two gammarid species. The dimensions of the MFA, F1 and F2, collectively account for 74% of the observed variability, indicating that the behavioural traits are adequately explained in this projection. As demonstrated in Fig. 5A, the F1 dimension of the MFA represents the predominant dimension of variability (45.89%), driven by two primary parameters: mortality and ingestion rate. The F2 dimension of the MFA, which explains 28% of the variability, is driven by two primary parameters: biomass and amplexus in *G. pulex*. As demonstrated in Fig. 5B, the data set is organized according to species, with each species distinguished by these specific biomarkers. Furthermore, Fig. 5B demonstrates a partial redundancy among biomarkers measured in both species. The F1 dimension was redundant for both species, being primarily driven by ingestion rate and mortality. This projection does not allow for a clear distinction between species, as they are all similarly positioned along this axis. Conversely, the F2 dimension differentiates the two species based on the biomass and amplexus of

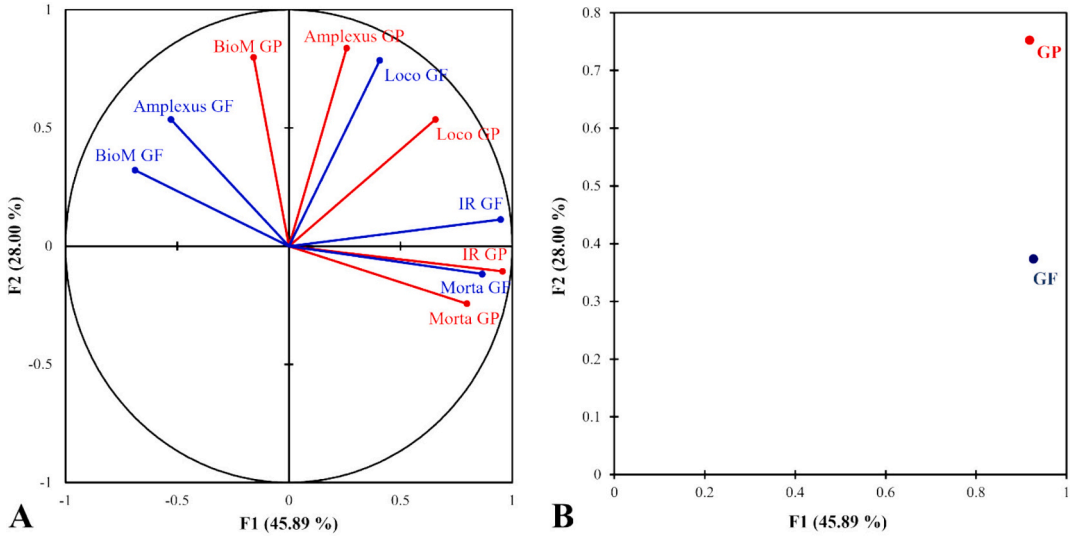

Fig. 5. Multiple factor analysis (MFA): (A) Pearson's PCA correlation circle of variables. (B) Projection of two species, *Gammarus pulex* (GP in red) and *Gammarus fossarum* (GF in blue), on the plane defined by F1 and F2 in the global MFA. Each species is represented by distinct individual and populational biomarkers: Morta (Mortality); Amplexus; Loco (Locomotion); IR (Ingestion rate); BioM (Biomass).

G. pulex compared to *G. fossarum*. This discrepancy can be attributed to the high spatiotemporal variability of biomass and amplexus in *G. pulex*. However, different biomarkers (projected on F1) such as ingestion rate, mortality and locomotion follow the same spatiotemporal variability, making it difficult to differentiate between the two species. It is evident that additional information is provided by F2 (28%), and consequently by *G. pulex*.

Furthermore, our results show variations in biomarker responses at both the individual and population levels in *G. pulex* and *G. fossarum*. For example, Maltby et al. [27] used ingestion rate as a robust biomonitoring assay to assess water quality parameters such as temperature, pH and alkalinity.

3.3. *In situ factors governing the sensitivity of biomarkers*

PLS regression was used to identify the environmental factors likely to modulate the biomarkers of the gammarids, namely chemical pollution (particularly pesticides and metals, expressed as substance concentrations), hydrological regime, physiography and physicochemistry of the six monitoring sites. All data of physicochemistry, hydrology, physiography, pesticides, transformation products and metals were summarized using PCA (Appendices B, C, D, E, F, G). For the physicochemical parameters, the first three dimensions (F1, F2 and F3) were retained, explaining 65% of the variability across the different campaigns. The first dimension (explaining 26% of the variance) accounts for the variability in temperature, dissolved oxygen, pH, potassium and calcium. The second dimension (explaining 23% of the variance) accounts for the variability in electrical conductivity, dissolved organic and inorganic carbon and anions (chloride and sulphate). Finally, the third dimension (explaining 16% of the variance) results from the combination of variables related to the concentrations of two ions: phosphate and ammonium. For the hydrological parameters, the first two dimensions (F1, F2) were retained, explaining 77% of the variability across the different campaigns. The first dimension (explaining 55% of the variance) accounts for the variability in water level. The second dimension (explaining 22% of the variance) accounts for the variability in turbidity and TSS. For the physiographic parameters, the first dimension (F1) was retained, explaining 67% of variability in land cover (agricultural, urban and forest) across the different sites. Regarding pesticides, including both parent compounds and transformation products, the first dimension explains 30% and 42% of the variability respectively, while the second explains 16% and 17%, respectively. Using these two dimensions, the variability in pesticide concentration is explained to the extent of 60% for parent compounds and 46% for transformation products. For metals, the first two dimensions (F1, F2) were retained, explaining 51% of the variability across the different campaigns. The first dimension (explaining 29% of the variance) mainly accounts for the variability in As, Cu, V, Cd and Pb. The second dimension (explaining 22% of the variance) accounts for the variability in Co, Ni, Cr and Ti.

The analysis of the PLS projection of the observations (6 sites × 5 campaigns), grouped by biomarkers and species (see Table 3), highlights several environmental parameters that influence these biomarkers. Statistical analysis demonstrated that physicochemical parameters can modulate (positively or negatively) the biomarkers monitored in the two gammarids. In particular, temperature and oxygen were found to positively modulate mortality (7.3% for *G. pulex* and 11.1% for *G. fossarum*) and ingestion rate (14.0% for *G. pulex* and 13.2% for *G. fossarum*). Consistent with the findings of the present study, the extant literature has documented that the two gammarid species differ in their habitat requirements [26]. *G. fossarum* exhibits a more limited ecological niche, demonstrating a marked preference for colder water (up to 12 °C) and water with higher oxygenation levels (at least 90% saturation), in contrast to *G. pulex* [26]. The effect of physicochemical parameters on various biomarkers (individual, cellular, molecular) has been extensively documented in numerous studies. These biomarkers include

Table 3
PLS-based contributions (%) of environmental data (physicochemistry (PC), chemistry (concentration of nitrates, pesticides grouped by molecules and metals), hydrology (Hydro) and physiography (PG)) to biomarkers. Bold values indicate statistically significant difference ($P < 0.05$) and signs represent positive or negative effects on variables. Italic values correspond to the cumulative explained variance for each dependent variable (R^2Y).

Variables	Species	Physicochemistry			Hydrology		Physiography	Chemistry						R^2Y
		F1	F2	F3	F1	F2	F1	Nitrates	Pesticides F1	Pesticides F2	Metabolites F1	Metabolites F2	Metals F1	Metals F2	
Mortality	*G. pulex*	**7.3**	3.7	−0.6	**6.1**	−1.6	1.1	0.0	6.8	**4.0**	−1.5	**5.3**	**6.9**	1.8	*47.0*
	G. fossarum	**11.1**	4.8	**−6.6**	**11.2**	4.8	1.7	2.2	4.5	**6.9**	−2.7	**3.6**	**7.3**	0.0	*67.4*
Amplexus	*G. pulex*	**6.6**	−2.3	−2.2	4.1	**11.5**	−2.0	−3.3	−1.8	2.1	**−7.8**	0.8	−1.8	−4.9	*51.2*
	G. fossarum	−2.7	**−10.0**	2.8	**−6.2**	−1.2	1.3	−2.8	**−4.8**	1.3	**−6.4**	**−4.2**	−3.3	**−7.1**	*54.2*
Locomotion	*G. pulex*	**9.2**	−4.3	**8.1**	1.0	6.2	−3.8	**−10.6**	**5.9**	0.8	**−13.0**	**8.3**	0.7	−4.1	*76.1*
	G. fossarum	**7.7**	−3.3	0.0	3.6	**13.8**	−3.5	−6.0	−1.3	1.2	**−10.4**	2.3	−2.9	**−5.7**	*61.6*
Ingestion Rate	*G. pulex*	**14.0**	4.0	−1.7	**10.8**	0.8	1.6	−1.3	**9.9**	**7.8**	**−6.0**	**8.2**	**10.6**	0.3	*76.9*
	G. fossarum	**13.2**	2.4	−2.9	**10.1**	7.7	−0.3	−2.3	5.6	**6.1**	**−7.6**	**6.1**	**6.0**	−1.9	*72.3*
Biomass	*G. pulex*	3.2	0.3	−2.2	2.9	**14.4**	−4.3	−2.9	−3.7	−2.1	−4.1	0.1	**−5.5**	−2.4	*48.2*
	G. fossarum	−5.5	−4.6	0.3	−5.1	−2.1	1.0	0.8	**−4.9**	−1.0	0.6	**−4.9**	−3.5	−2.5	*36.8*

mortality or ingestion rate in macroinvertebrates, such as gammarids, as well as immunotoxicity and genotoxicity in various species, including diatoms, fish and molluscs [27,30,31]. As shown in Fig. 6 and Table 3, no correlation was observed between physiographic factors and biomarkers. By contrast, hydrological parameters positively modulated mortality (6.1% for *G. pulex* and 11.2% for *G. fossarum*) and ingestion rate (10.8% for *G. pulex* and 10.1% for *G. fossarum*). The hydrological effect of water level, and indirectly of flow rate, on mortality may be related to the inability of caged organisms to drift, leading to increased mortality. These effects have already been observed in gammarids following run-off events [49,50]. Moreover, various hydrological events (*e.g.* subsurface drainage periods and extreme events) lead to changes in climatic variables such as temperature and oxygen, as well as to the transfer of contaminants that directly impact organisms [51].

For metals, as shown in Table 3, a significant positive correlation with mortality (6.9% for *G. pulex* and 7.3% for *G. fossarum*) and ingestion rate (10.6% for *G. pulex* and 6% for *G. fossarum*) was found. The observed correlation can be attributed in part to the variability in the concentrations of As, Cu, V, Cd and Pb. The combined effects of these metals at concentrations exceeding EQS by 2-fold (for Cu) to 10-fold (for Pb) have been shown to result in a decline in survival after 48 h in *G. fossarum* [40]. In addition, an *in situ* active biomonitoring approach using gammarids (*G. fossarum*) has shown that metal mixtures lead to a 10% increase in mortality and a reduction in ingestion rate, as reported in a previous study [52].

Regarding the use of pesticides, as illustrated in Table 3, a significant positive correlation is evident between mortality and ingestion rate, with 4% for *G. pulex* and 6.9% for *G. fossarum*, respectively. The observed modulation is driven by the presence of various herbicides, including ethofumesate and metobromuron (which are major components of F2 pesticides), as well as fungicides, such as bromuconazole (another major compound of F2 pesticides). However, these variations are not observed in molecules that are quantified most frequently or at high concentrations. The measured concentrations of insecticides ranged from 0 to 0.006 µg/L, which is 10 times lower than the concentrations tested (0.01–1 µg/L) in some studies. These studies demonstrated that low concentrations of insecticides have no effect on gammarid mortality [22]. Studies have shown that fungicide concentrations with EC50s between 20 and 200 µg/L lead to a 20% increase in *G. fossarum* mortality [47]. This is in contrast to the concentrations observed in our samples, which did not exceed 0.01 µg/L. Our study suggests that mixtures of contaminants found *in situ* (*i.e.* herbicides, fungicides and transformation products) can influence the behaviour of gammarids. However, identifying the specific effects of individual compounds, such as ethofumesate or bromuconazole, remains challenging in the context of *in situ* experiments. An *in situ* study reported that pesticide mixtures lead to increased mortality in *Gammarus spp.* [18]. Moreover, studies have shown that the ingestion rate of *G. fossarum* decreases when exposed to a mixture of pesticides (at concentrations of 1–40 µg/L) [11]. However, in the present study, an increase in ingestion rate was observed at concentrations 5 times lower (maximum of 8 µg/L). This observation indicates that the effects of pesticides on biomarkers *in vitro* (under controlled conditions) can be specifically linked to the concentration of a single molecule or a limited mixture of molecules. Nevertheless, it is challenging to correlate a specific effect with a broad array of pesticides (in this instance, 69 pesticides) acting simultaneously.

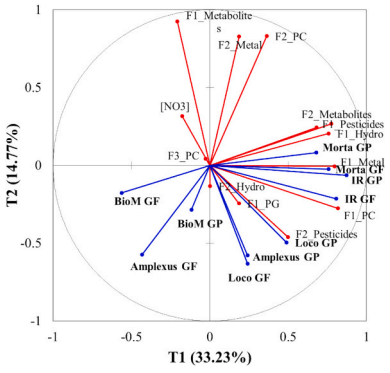

Fig. 6. PLS correlation circle of variables for gammarid biomarkers — Morta (mortality), amplexus, Loco (locomotion), IR (ingestion rate), BioM (biomass) — in two species (*Gammarus pulex* and *Gammarus fossarum*) and environmental integrated data of physicochemistry (PC), chemistry (concentration of nitrates, pesticide grouped by molecules and metals), hydrology (Hydro) and physiography (PG).

Further, we observed a significant negative modulation of transformation products on F1, mainly driven by transformations product of metolachlor and metazachlor, affecting locomotion (−13% for *G. pulex* and −10.4% for *G. fossarum*), ingestion rate (−6% for *G. pulex* and −7.6% for *G. fossarum*) and amplexus (−7.8% for *G. pulex* and - −6.4% for *G. fossarum*) in both species. Conversely, metabolites on F2, which are mostly represented by AMPA (glyphosate transformation products), showed the opposite effect on ingestion rate. Additionally, metabolites on F2 modulate mortality by 5.3% for *G. pulex* and by 3.6% for *G. fossarum*. The correlation between pesticide transformation products and organisms such as gammarids remains poorly documented because of the difficulties associated with their presence, often in trace amounts, and their stability, as transformation products can continuously generate new products [14]. In addition, linear regression models (PLS) were tested based on pesticide concentration and family (herbicides, fungicides, insecticides and transformation products) as well as on concentration classes (varying from 1 to 5 depending on the concentration thresholds) and family (herbicides, fungicides, insecticides and transformations products). The results (presented in Appendices H and I) show there is no statistically significant difference between the two approaches, thus supporting the use of the more straightforward approach of pesticide concentration.

We found that biomass is a biomarker that is not well projected in our regression model, with more than 50% of its variation being unexplained (R^2Y). Biomass showed no inter-campaign variability, remaining stable over time within and between campaigns. However, interspecific differences in biomass were observed.

The biomarkers studied in the active biomonitoring were primarily modulated by physicochemical parameters known from other biomonitoring campaign studies [30,31], followed by transformation products and metal, and subsequently by pesticides (parent molecules). However, our biomonitoring campaigns did not include enough contamination spikes to potentially shift the biomarker response. Furthermore, we observed interspecies variability. The biomarkers of *G. fossarum* were modulated more by environmental parameters (physicochemistry, hydrology and physiography) than by chemical factors. This is in contrast to *G. pulex*, in which biomarkers were predominantly influenced by chemical factors, specifically transformation products, metals and pesticides. This observation can be partially explained by the ecological niches of the species and by its transposition to a catchment area not colonized by *G. fossarum*. In this particular case, *G. pulex* appears to be a more suitable subject for studying the impact of pesticides on aquatic organisms. However, this study has two major limitations: The first four campaigns were carried out mainly during low water periods, which limited the transfer of pesticides to watercourses. Thus, with the exception of campaign C5, only small variations in pesticide concentrations were observed between each biomonitoring campaign. It is clear that the linear models tested cannot be used to identify obvious ecotoxicological effects on gammarids. For future studies under *in situ* conditions, it is necessary to carry out more comprehensive monitoring over a full hydrological year, including different contamination patterns such as autumn or spring pesticide peaks. Another limitation of *in situ* experiments is the quantification of all sources of contamination. Indeed, we cannot exclude the impact of

pharmaceuticals, non-identified human pollution sources and microplastics, which may act in synergy (positively or negatively) with pesticides. The presence of these substances has been demonstrated to enhance the understanding of unexplained variability in the biomarker of interest within gammarid communities (where 50% of the R^2Y is unexplained for certain biomarkers).

4. Conclusion

This study aimed to assess the impact of pesticides and confounding environmental parameters on individual or population biomarkers in gammarids under *in situ* conditions. The relevance and usefulness of these biomarkers were confirmed at two organizational levels (individual and population) for identifying biological changes associated with multiple environmental pressures. These biomarkers proved to be sensitive first to confounding environmental parameters (temperature, oxygen), then to transformation products, and finally to pesticides. In the context of multi-contamination, as occurs *in situ*, it is challenging to ascertain the inherent impact of specific molecules (*e.g.* pesticides, metals). In the present study, analyses were conducted based on the concentration and type of pesticide family (*e.g.* herbicides, fungicides and insecticides) and concentration class (*e.g.* ranging from 1 to 5 depending on the concentration threshold). However, no significant analytical differences were found that would confirm the use of either approach. These sublethal effects highlight the impact of pesticide and metal mixtures on multiple biomarkers, showing an increase in mortality and decrease in amplexus, locomotion and ingestion rate in freshwater populations in natural environments. Finally, we have highlighted a difference in the variability and sensitivity of biomarkers to pesticides and various environmental factors between two gammarid species: *Gammarus fossarum* and *Gammarus pulex*. A distinct peak in pesticide concentration was detected during the final sampling campaign (C5), which coincided with flooding events that occurred between campaigns. This highlights the importance of incorporating actual seasonal variability through enhanced monitoring, such as continuous caging designs, to more accurately assess the effects of seasonality on the transfer of pesticides to gammarids. We found that *G. pulex* was a more suitable species for studying the inherent impact of pesticides. However, *G. pulex* was transplanted to sites that match its environmental requirements, facilitating its adaptation and promoting the identification of its sensitivity to other factors such as chemical contamination, in contrast to *G. fossarum* (which is primarily constrained by physicochemical factors).

This study provides novel insights into the relevance of combining individual- and population-level biomarkers in gammarids under realistic *in situ* multi-contamination conditions, allowing the disentanglement of chemical stress from confounding environmental factors. By comparing two gammarid species, our results demonstrate for the first time interspecific differences in sensitivity and variability of biomarker responses in relation to *in situ* pesticide exposure. Based on these findings, we propose a continuous biomonitoring strategy covering multiple hydrological seasons, in order to better capture pesticide transfer dynamics during hydrological events, rather than focusing on isolated pesticide or transformation product peaks. Integrating multiple biological levels, such as cellular or ecosystem functions, will also improve the assessment of the impact of pesticides on freshwater ecosystems under real environmental conditions.

CRediT authorship contribution statement

Léo Persat: Writing – original draft, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hocine Henine:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Julien Tournebize:** Writing – review & editing, Supervision, Funding acquisition. **Arnaud Blanchouin:** Writing – review & editing, Methodology, Data curation. **Cédric Chaumont:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Fatima Joly:** Writing – review & editing, Methodology, Data curation. **Romane Nespoulet:** Writing – review & editing, Methodology, Data curation. **Virginie Archaimbault:** Writing – review & editing, Supervision. **Jérémie D. Lebrun:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Ethical approval

Not applicable.

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Declaration of competing interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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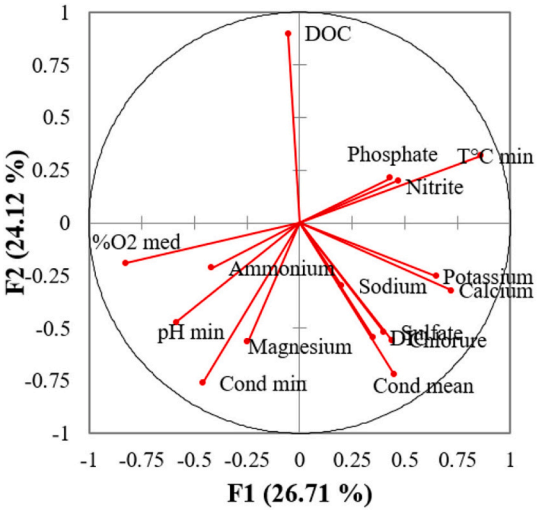
Appendix A. Appendix

Biomarkers of *Gammarus pulex* and *Gammarus fossarum* at the six monitored sites (from 6 January to 26 June 2023, $n = 5$ campaigns). Values are presented as minimum ; mean ; maximum; values in italics represent the coefficient of variation in %.

	Species	Sites					
		E	M	EM	L	R	LR
Mortality (%/d)	*G. pulex*	0.07 ; **0.27** ; 0.5 *61*	0.15 ; **0.36**; 0.76 *68*	0.12 ; **0.44** ; 1.25 *106*	0.02 ; **0.34** ; 0.83 *88*	0.13 ; **0.22** ; 0.42 *56*	0.05 ; **0.30** ; 0.86 *107*
	G. fossarum	0.17 ; **0.41** ; 1.10 *95*	0.02 ; **0.23**; 0.69 *115*	0.02 ; **0.26** ; 0.59 *88*	0.09 ; **0.44** ; 1.34 *114*	0.05 ; **0.32** ; 0.88 *107*	0.03 ; **0.36** ; 1.00 *106*
Amplexus (%)	*G. pulex*	17.72 ; **21.36** ; 24.86 *13*	2.65 ; **12.13** ; 20.43 *59*	8.60 ; **14.27**; 20.42 *31*	11.45 ; **17.09** ; 24.11 *29*	9.38 ; **16.04**; 22.93 *31*	11.66 ; **16.56** ; 26.67 *36*
	G. fossarum	13.62 ; **29.01** ; 37.55 *32*	7.62 ; **19.17** ; 35.18 *59*	10.34 ; **26.20** ; 46.03 *55*	26.70 ; **30.23** ; 34.32 *10*	11.85 ; **30.37** ; 45.36 *43*	23.59 ; **34.45** ; 49.34 *32*
Locomotion (mvt/ind/min)	*G. pulex*	1.95 ; **2.55** ; 3.01 *19*	1.05 ; **2.79**; 3.88 *39*	1.95 ; **2.60** ; 2.98 *16*	1.36 ; **2.42** ; 2.95 *27*	1.53 ; **2.64** ; 3.25 *26*	1.48 ; **2.79** ; 3.73 *30*
	G. fossarum	2.15 ; **2.58** ; 3.03 *13*	0.90 ; **2.19**; 2.93 *37*	2.04 ; **2.46** ; 3.06 *16*	1.87; **2.51**; 3.04 *18*	2.27 ; **2.65** ; 2.96 *11*	2.05 ; **2.80** ; 3.24 *17*
Ingestion rate (mg_{dw}/mg_{dw}/d)	*G. pulex*	0.11 ; **0.16** ; 0.24 *32*	0.11 ; **0.15**; 0.27 *43*	0.09 ; **0.14** ; 0.25 *43*	0.12 ; **0.17** ; 0.26 *33*	0.10 ; **0.15** ; 0.24 *38*	0.10 ; **0.15** ; 0.31 *59*
	G. fossarum	0.13 ; **0.17** ; 0.25 *30*	0.09 ; **0.14**; 0.23 *39*	0.07 ; **0.12** ; 0.20 *41*	0.08 ; **0.15** ; 0.26 *41*	0.12 ; **0.16** ; 0.24 *29*	0.011 ; **0.16**; 0.28 *45*
Biomass (µg)	*G. pulex*	5.2 ; **5.6** ; 6.3 9	4.8 ; **5.3** ; 6.3 12	5.4 ; **5.7** ; 6.1 6	4.9 ; **5.3** ; 6.1 9	5.1 ; **5.5** ; 6.4 9	5.3 ; **5.7** ; 6.5 9
	G. fossarum	5.5 ; **5.8** ; 6.0 3	5.0 ; **5.5** ; 5.9 7	5.5 ; **6.1** ; 6.5 8	5.6 ; **5.9** ; 6.1 4	5.4 ; **5.9** ; 6.1 5	5.4 ; **6.1** ; 6.5 7

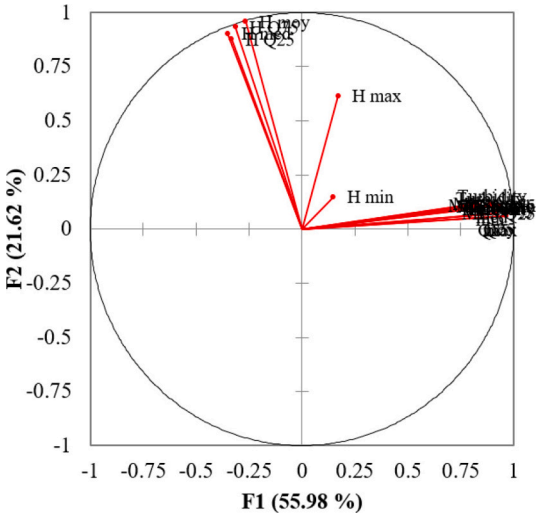


Contributions of variables for each factor (%):			
	F1	F2	F3
T°C min	**17.269**	2.600	0.279
%O2 med	**15.850**	0.961	3.265
Cond min	4.952	**15.011**	0.690
Cond mean	4.679	**13.398**	7.707
pH min	**8.029**	5.831	2.669
DIC	2.887	**7.693**	10.492
DOC	0.067	**20.930**	1.135
Chloride	4.503	**8.115**	8.319
Nitrite	5.127	1.042	7.261
Phosphate	4.342	1.180	**11.928**
Sulphate	3.769	**7.003**	3.888
Sodium	0.937	2.337	**23.447**
Ammonium	4.093	1.191	1.279
Potassium	**9.931**	1.683	10.829
Magnesium	1.418	8.332	0.234
Calcium	**12.147**	2.693	6.578

Appendix B. Appendix

Pearson's PCA correlation circle of integrated variables for physicochemical parameters and corresponding explanatory percentages. Parameters measured: T°C: Temperature (Celsius), [NO3]: Concentration in nitrates (mg/L), COD/CID: Concentration in carbon organic/inorganic dissolve, O2%: Concentration in dissolved oxygen (%), Cond: Conductivity (µS/min), Calculated values: min: minimum, Q25: first quartile, med: median, moy: mean,

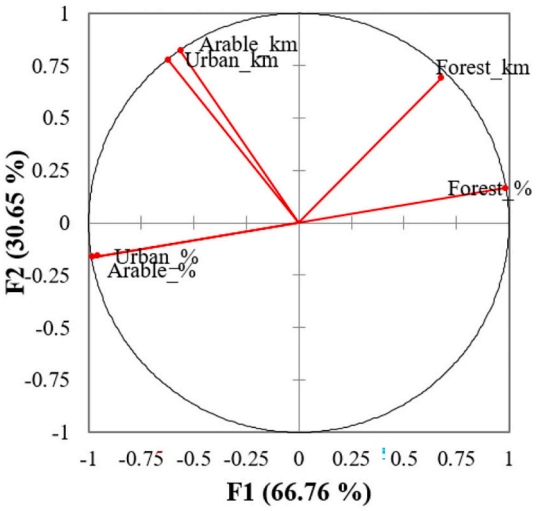
Q75: fourth quartile, max: maximum



Contributions of variables for each factor (%):		
	F1	F2
H Q25	1.093	**19.781**
H min	0.220	0.566
H med	1.219	**20.991**
H mean	0.703	**23.531**
H max	0.294	**9.701**
H Q75	0.973	**22.411**
Turbidity Q25	**8.407**	0.139
Turbidity min	**6.949**	0.302
Turbidity med	**9.200**	0.098
Turbidity mean	**8.685**	0.296
Turbidity max	**6.674**	0.361
Turbidity Q75	**7.834**	0.312
TSS Q25	**8.407**	0.139
TSS min	**6.949**	0.302
TSS med	**9.200**	0.098
TSS mean	**8.685**	0.296
TSS max	**6.674**	0.361
TSS Q75	**7.834**	0.312

Appendix C. Appendix

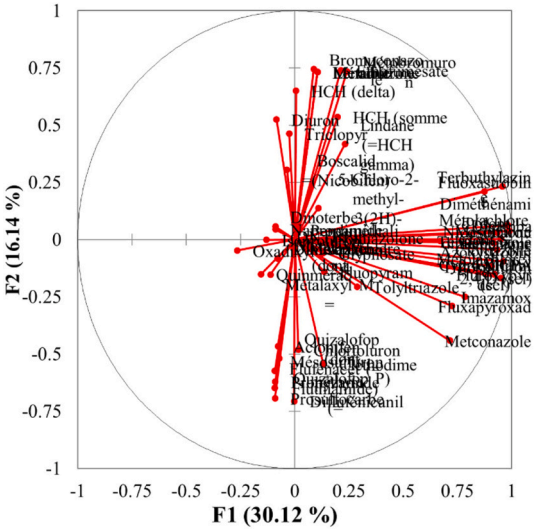
Pearson's PCA correlation circle of integrated variables for hydrological parameters and corresponding explanatory percentages. Parameters measured: H: water level (cm), Turbidity: Turbidity (Nephelometric Turbidity Unit), MES: TSS concentration (mg/L). Calculated values: min: minimum, Q25, first quartile, med: median, moy: mean, Q75: fourth quartile, max: maximum



Contributions of variables for each factor (%):		
	F1	F2
Urban_km	9.682	**32.884**
Arable_km	7.878	**36.686**
Forest_km	11.537	**26.118**
Urbain_%	**22.804**	1.371
Arable_%	**24.012**	1.469
Foret_%	**24.087**	1.471

Appendix D. Appendix

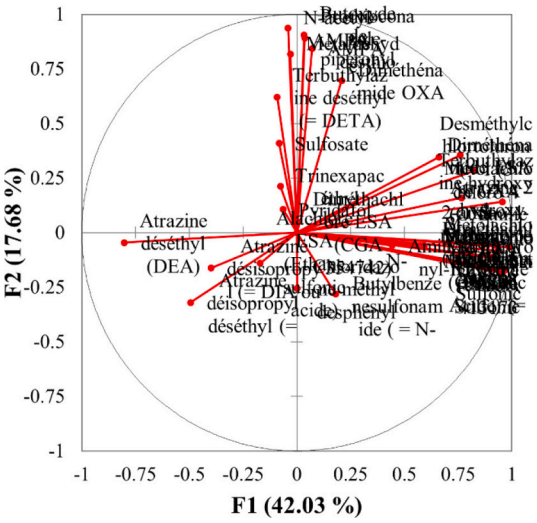
Pearson's PCA correlation circle of integrated variables for physiographic parameters and corresponding explanatory percentages. Parameters measured: Arable: area occupied by arable land (expressed in % land use or in km^2), Urban: area occupied by urban land (expressed in % land use or in km^2), Forest: area occupied by arable forest (expressed in % land use or in km^2)



Contributions of variables for each factor (%):		
	F1	F2
Prosulfocarb	0.052	**5.602**
Atrazine	0.019	0.016
Bentazon	**5.257**	0.003
Chlortoluron	0.002	**2.694**
2,4 D	**5.649**	0.324
Dinoterb	0.050	0.039
Diuron	0.044	3.223
Ethofumesat	0.282	**6.410**
Isoproturon	0.106	0.000
Metolachlor	**5.671**	0.000
Triclopyr	0.004	2.516
Pendimethalin	**0.000**	0.000
Propyzamide	0.050	4.492
Glyphosate	0.106	0.041
Napropamide	0.049	0.024
Metazachlor	0.000	0.015
Dimethenamid	**5.467**	0.011
Aclonifen	0.037	2.532
Diflufenican	0.000	**5.806**
Flufenacet	0.053	4.906
Quizalofop	0.032	3.136
Quinmerac	0.150	0.264
Mesosulfuron methyl	0.053	3.844
Clethodim	0.110	3.443
Imazamox	**3.863**	0.729
2,4 MCPA (sel)	**5.303**	0.265
Dicamba	**6.043**	0.047
Terbuthylazine	**5.739**	0.633
Mesotrione	**6.097**	0.017
Metobromuron	0.365	**6.322**
Nicosulfuron	**6.133**	0.016
Clomazone	**5.443**	0.022
Lenacil	0.069	**6.266**
Tritosulfuron	**5.150**	0.158
Metribuzin	0.069	**6.266**
Fluroxypyr	**5.303**	0.265
Metamitron	0.069	**6.266**
Cyprosulfamide	**5.303**	0.265
Oxadixyl	0.438	0.026
Tebuconazole	**2.821**	0.000
Metalaxyl	0.078	0.270
Bromuconazole	0.048	**6.489**
Metconazole	**3.198**	2.267
Azoxystrobin	**5.788**	0.105
Dinitrophenol 2,4	0.039	0.078
Boscalid	0.008	1.088
Tolyltriazole	0.521	0.491
Fluxapyroxad	**3.297**	0.983
Fluopyram	0.115	0.229
5-Chloro-2-methyl-3(2H)-isothiazolone	0.075	0.224
Fluoxastrobin	**4.802**	0.519
HCH (delta)	0.000	**4.945**
Lindane	0.335	2.035
HCH	0.246	3.373

Appendix E. Appendix

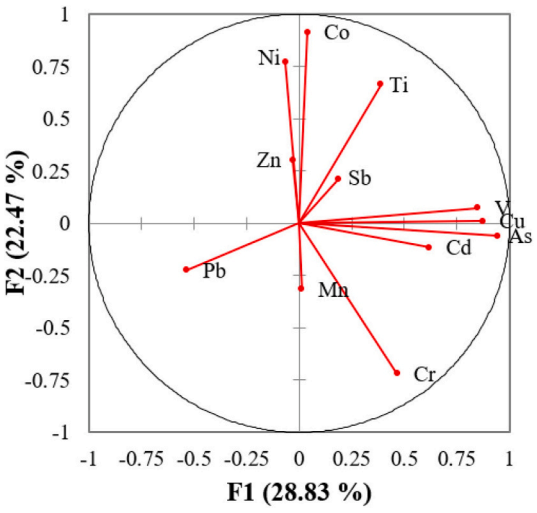
Pearson's PCA correlation circle of integrated variables for pesticide (parent compound) concentration (µg/L) and corresponding explanatory percentages. Molecules quantified: 5 insecticides, 19 fungicides, 45 herbicides



Contributions of variables for each factor (%):		
	F1	F2
Atrazine desethyl	**4.656**	0.037
Atrazine desisopropyl	1.152	0.454
Metsulfuron methyl	**5.966**	0.213
Atrazine deisopropyldesethyl	1.761	1.755
Atrazine 2 hydroxy	**4.226**	0.433
AMPA	0.036	**12.210**
Terbuthylazine hydroxy	**4.918**	1.378
Terbuthylazine desethyl	0.060	**6.607**
Chloridazon methyl desphenyl	0.000	1.124
Dimethachlor ESA	0.000	0.000
Pyridafol	0.030	0.196
Alachlor ESA	0.209	0.337
2-Amino-4-methoxy-6-(trifluoromethyl)	**2.526**	0.062
Metolachlor OXA	**6.575**	0.341
Metolachlor ESA	**5.980**	0.236
Flufenacet OXA	**6.670**	0.493
Flufenacet ESA	**6.464**	0.562
Dimethenamid ESA	**4.166**	2.157
Metazachlor OXA	**6.765**	0.507
Metazachlor ESA	**6.641**	0.546
2-Aminosulfonyl-N,N	1.416	0.060
Dimethachlor CGA	**6.318**	0.119
S-Metolachlor NOA	**6.435**	0.569
S-Metolachlor CGA	**6.179**	0.058
S-Metolachlor CGA	**7.025**	0.077
Dimethenamid OXA	0.313	**8.313**
Desmethylchlortoluron	**3.150**	2.055
Prothioconazole-desthio	0.010	**13.602**
N-acetyl-AMPA	0.012	**15.018**
Trinexapac ethyl	0.042	0.775
Metaldehyde	0.007	**11.453**
Piperonyl butoxide	0.008	**14.014**
N-Butylbenzenesulfonamide	0.235	1.362
Sulphosate	0.049	2.879

Appendix F. Appendix

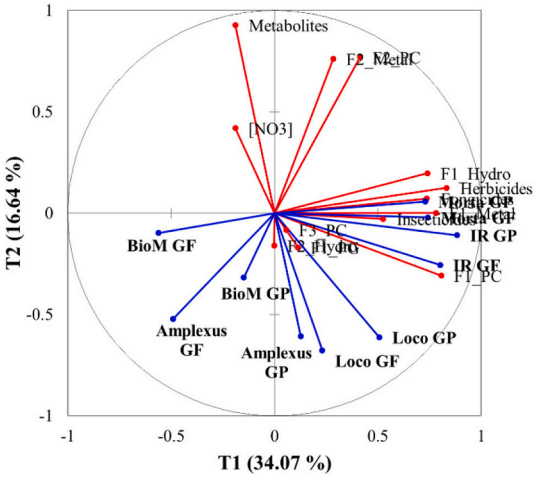
Pearson's PCA correlation circle of integrated variables for transformation product concentrations (µg/L) and corresponding explanatory percentages. Molecules quantified: 35 transformation products



Contributions of variables for each factor (%):		
	F1	F2
Zn	0.027	3.404
Sb	0.993	1.612
Cu	**22.066**	0.004
Mn	0.004	3.719
V	**20.951**	0.194
Ni	0.118	**22.165**
As	**25.732**	0.131
Cr	6.274	**19.164**
Pb	**8.298**	1.892
Co	0.046	**30.914**
Ti	4.390	**16.312**
Cd	**11.100**	0.491

Appendix G. Appendix

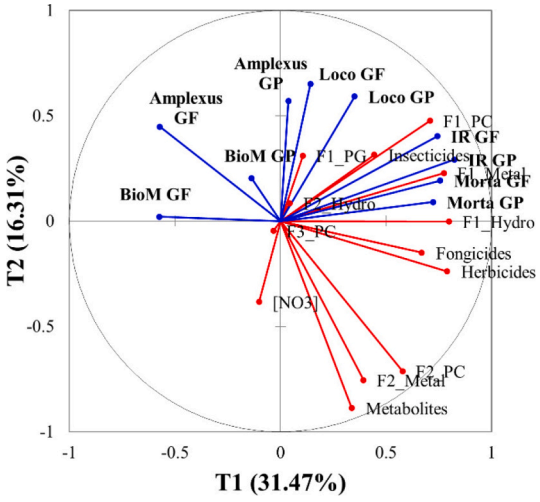
Pearson's PCA correlation circle of integrated variables for metal (trace elements) concentration (µg/L) and explanatory percentage



Variables	Species	Physicochemestry			Hydrology		Physiography	Chemistry							R^2Y
		F1	F2	F3	F1	F2	F1	Nitrates	Herbicides	Fungicides	Insecticides	Metabolites	Metals F1	Metals F2	
Mortality	*G. pulex*	**7.5**	3.4	−0.4	**6.0**	−3.3	1.6	0.4	**8.0**	**5.7**	6.7	−2.0	**7.8**	1.8	*54.7*
	G. fossarum	**11.5**	3.8	**−7.6**	**11.4**	4.2	2.4	2.7	4.0	**4.0**	4.2	−3.1	**−7.4**	−1.4	*67.9*
Amplexus	*G. pulex*	**7.4**	−2.7	−4.1	5.2	**11.9**	−0.9	−2.7	−2.8	1.4	−4.4	**−7.6**	−1.4	**−6.3**	*58.8*
	G. fossarum	−1.9	**−9.8**	2.2	**−5.6**	0.6	0.8	−2.9	**−5.4**	−4.1	−2.1	**−6.8**	**−3.8**	**−7.5**	*53.4*
Locomotion	*G. pulex*	**9.3**	−5.0	**7.7**	1.0	6.1	−2.9	**−10.7**	**6.6**	**9.0**	−0.4	**−14.1**	1.4	**−4.8**	*78.9*
	G. fossarum	**8.0**	−3.8	−0.9	3.9	**11.2**	−1.6	−5.2	−0.7	3.4	−3.9	**−10.0**	−1.1	**−6.5**	*60.4*
Ingestion Rate	*G. pulex*	**14.8**	3.1	−3.2	**11.4**	1.2	2.2	−0.5	**9.7**	**8.5**	**7.6**	**−7.0**	**10.7**	−1.1	*80.9*
	G. fossarum	**14.3**	1.5	−4.6	**11.0**	7.3	1.0	−1.5	**5.5**	**6.9**	3.0	**−8.3**	**6.9**	−3.6	*75.3*
Biomass	*G. pulex*	3.4	0.1	−2.5	3.1	**12.6**	−3.0	−3.0	**−3.4**	1.3	**−7.1**	−3.5	**−4.3**	−2.9	*49.9*
	G. fossarum	−5.3	−4.2	0.9	−5.2	−3.6	1.1	1.0	**−4.2**	−4.8	−0.5	0.8	−2.7	−1.9	*36.4*

Appendix H. Appendix

PLS regression (A) correlation circle of variables for gammarid biomarkers and environmental integrated data, including physicochemistry (PC), chemistry (pesticide concentrations grouped by family and metal concentration), hydrology (Hydro) and physiography (PG). (B) PLS-based contribution (%) of environmental data to biomarkers. Bold values indicate statistically significant difference ($P < 0.05$) and signs represent positive or negative effects on variables. Italic values correspond to the cumulative explained variance for each dependent variable (R^2Y)



Variables	Species	Physicochemistry			Hydrology		Physiography	Chemistry							R^2Y
		F1	F2	F3	F1	F2	F1	Nitrates	Herbicides	Fungicides	Insecticides	Metabolites	Metals F1	Metals F2	
Mortality	*G. pulex*	**10.8**	0.9	2.2	4.6	−6.0	−2.0	4.8	**7.0**	**6.2**	5.3	−2.6	**6.8**	1.2	*60.4*
	G. fossarum	**16.3**	1.3	−5.4	**11.2**	1.9	−0.3	6.5	**7.2**	4.2	4.2	−2.1	**7.8**	−2.4	*70.7*
Amplexus	*G. pulex*	**7.2**	−3.4	−3.9	4.8	**12.0**	−0.1	−3.9	0.3	−0.8	−3.6	−4.2	−1.2	**−7.0**	*52.5*
	G. fossarum	−0.2	**−9.5**	3.0	−5.3	1.0	−0.9	−2.4	**−5.4**	−3.3	−2.3	**−8.7**	−4.2	**−7.2**	*53.4*
Locomotion	*G. pulex*	**11.0**	−5.2	**8.3**	1.2	4.4	−3.5	**−9.7**	**6.3**	**7.7**	0.0	**−9.7**	2.2	**−4.9**	*74.2*
	G. fossarum	**8.5**	−4.2	−0.2	4.2	**12.8**	−0.8	**−9.0**	2.3	2.0	−3.4	**−6.4**	−0.5	**−7.4**	*61.7*
Ingestion Rate	*G. pulex*	**19.2**	−0.7	0.6	**9.2**	−3.2	−2.1	5.0	**9.8**	8.1	6.6	**−6.0**	**9.8**	−2.0	*82.2*
	G. fossarum	**19.0**	−1.8	−1.7	**10.1**	4.6	−2.2	2.1	**8.3**	6.2	2.7	**−6.4**	**6.8**	−4.9	*76.9*
Biomass	*G. pulex*	2.0	−0.7	−3.1	3.2	**15.3**	−1.0	−6.9	0.0	−0.9	−6.9	−0.4	−4.3	−4.4	*49.0*
	G. fossarum	−5.3	−3.1	0.1	−4.7	−2.7	0.1	2.5	**−5.2**	−4.2	−1.4	−0.9	−3.6	−1.1	*34.8*

Appendix I. Appendix

PLS regression (A) correlation circle of variables for gammarid biomarkers and environmental integrated data, including physicochemistry (PC), chemistry (pesticide classes grouped by family and metal concentration), hydrology (Hydro) and physiography (PG). (B) PLS-based contribution (%) of environmental data to biomarkers. Bold values indicate statistically significant difference ($P < 0.05$) and signs represent positive or negative effects on variables. Italic values correspond to the cumulative explained variance for each dependent variable (R^2Y)

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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