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Screening potential toxicity of currently used herbicides in the freshwater amphipod *Gammarus fossarum* based on multi-level biomarker responses to field-realistic exposures

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Abstract

Herbicides are widely used to control weeds and maximize crop growth. Because of agricultural runoff, these chemicals are potentially hazardous to aquatic wildlife. However, their ecotoxicity and resulting disturbance in individual performance remain scarcely documented in freshwater crustaceans. This study aimed to screen the potential toxicity of currently used herbicides in the ecosystem engineer *Gammarus fossarum* using multi-level biomarkers. In microcosms, gammarids were exposed for 72 h to 12 herbicides individually (quinmerac, mesotrione, bentazone, isoproturon, chlortoluron, metazachlor, chloridazone, diflufenican, flufenacet, aclonifen, prosulfocarb and metolachlor) at a field-realistic concentration (i.e. 10 µg/L). The sublethal effects were assessed by monitoring several biochemical, physiological and behavioural traits. In exposed gammarids, alterations in behavioural activities were observed, i.e. increased locomotion and respiration as a general trend. Moreover, biochemical biomarkers suggested herbicide-dependent disruptions in moulting, antioxidant responses and cell integrity. Integrating multi-metric variations through statistical analyses allowed us to identify herbicide clusters likely to trigger common sets of biological responses. Depressed antioxidant defence at the cell level and impaired respiration at the individual level were the predominant toxic effects of herbicides, related to their hydrophobic feature. Furthermore, establishing relationships between sublethal alterations in gammarids and acute lethality or chronic toxicity values defined for regulatory purposes supports the relevance of these alterations as early warnings of toxicity. Our findings demonstrate that currently used herbicides have unexpected toxicological effects in a non-target wild animal, with possible long-term alterations in population dynamics and associated ecological functions, which constitute promising diagnostic tools for risk assessment in agricultural areas.

Keywords: Sublethal toxicity; Integrated biomarker response; Crustaceans; Pesticide; Behaviour; Bioassays

1. Introduction

To meet the demands of human population growth, pesticides are used intensively in agriculture worldwide to protect crops against diseases and pests so as to optimize food production. Although agronomically beneficial, the hydraulic transfer of these pesticides (e.g. agricultural runoff, drainage) from agrosystems to water bodies (e.g. rivers, streams, ponds) results in the degradation of water quality and organism habitats, hence threatening freshwater biodiversity (Tournebize et al. 2012, Lefrancq et al. 2017, Halbach et al. 2021). For instance, pesticides can lead to declines in populations and to disturbances in the structure of microbial and faunal communities as well as in associated ecosystem functions (e.g. litter decomposition) (Kohler and Triebskorn 2013, Rossi et al. 2018, Schäfer 2019). At the continental scale, almost half of European water bodies and their biodiversity are thus at risk from pesticides according to analyses of large datasets (Malaj et al. 2014, Szöcs et al. 2017). Early detection of the poisoning of wild organisms is therefore crucial to prevent severe ecological alterations in agriculturally impacted hydrosystems.

In the Northern Hemisphere, the predominant active ingredients applied to agrosystems are herbicides, which are used to control weeds. Consequently, these chemicals are frequently measured (i.e. from a few micrograms per litre to several milligrams per litre) in streams and wetlands (Tournebize et al. 2012, Szöcs et al. 2017, Bighiu et al. 2020) and they accumulate in fauna (Inostroza et al. 2016, Shahid et al. 2018). Despite their larger soil spread and higher occurrence in freshwaters compared with insecticides and fungicides, the toxic potential of herbicides in aquatic wildlife is still scarcely documented, glyphosate excluded (Kohler and Triebskorn 2013, Velisek et al. 2020). This is likely due to the presumed harmlessness of herbicides for animals according to their mode of action. Indeed, most herbicide groups are synthesized to target biological processes specific to plants such as photosynthesis as well as biosynthesis of carotenoids, chlorophylls or plant-growth regulators such as auxins. Besides, most toxicity data available for herbicides are based on lethal criteria obtained from test organisms, i.e. a lentic species of *Daphnia* genus. and/or a freshwater fish species, during controlled acute exposures (Malaj et al. 2014, Spycher et al. 2018). Nevertheless, lethality generally occurs at environmentally unrealistic levels that disregard biologically injurious events preceding mortality. Indeed, sublethal alterations have been reported in fish exposed to herbicide mixtures at environmental exposure levels, such as changes in immunity, antioxidant defences, cell integrity and swimming (Fatima et al. 2007, Shinn et al. 2015, Jacquin et al. 2019). Thus, the combined use of multiple-level biomarkers offers promising lines of enquiry for early screening of the unintended effects elicited by herbicides at different levels of biological complexity (from cell to population level) in aquatic non-target species (Jacquin et al. 2019, Lebrun et al. 2021).

As a ubiquitous biological component of water bodies, macroinvertebrates cover a broad range of trophic levels and ensure important ecological functions such as litter degradation and nutrient cycling. Among litter-decomposers abundant in European freshwaters, gammarids are easily sampled in situ and handled in the laboratory. Therefore, these ecosystem engineers are very popular for developing exposure biomarkers to address ecotoxicological issues regarding chemicals in the aquatic environment (Kunz et al. 2010). At the sub-individual level, biochemical biomarkers involved in vital functions of gammarids (e.g. digestion, moulting) or in cell stress have been proposed as early initiating events of toxicity for various organic contaminants, including pesticides (Lebrun et al. 2012, Gismondi and Thomé 2014, Demirci et al. 2018). At the individual/population level, pesticide exposure can elicit changes in behavioural endpoints, such as locomotion or mating (Cold and Forbes 2004, Beketov and Liess 2008). The relevance of combining such biochemical and behavioural biomarkers to detect sublethal impairments has been demonstrated in gammarids exposed to environmental concentrations of insecticides or fungicides (Lebrun et al. 2020, Lebrun et al. 2021).

Although the harmlessness of herbicides for aquatic wildlife may be presumed through modes of action targeting plant-specific biological processes, we hypothesize that these chemicals are nevertheless likely to generate sublethal alterations at different levels of biological organization due to their internalization, induction of oxidative stress and unexpected interactions with biological components (Shahid et al. 2018, Velisek et al. 2020, Lee et al. 2021). Thus, this study aimed to screen the potential toxicity of currently used herbicides exhibiting different chemical properties, modes of action and target groups (i.e. aclonifen, bentazone, chloridazone, chlortoluron, diflufenican, flufenacet, isoproturon, mesotrione, metazachlor, metolachlor, prosulfocarb and quinmerac) in a non-target freshwater species, i.e. *Gammarus fossarum*. In microcosms, gammarids were exposed to herbicides for 72 h at 10 µg/L representing a field-realistic short exposure following runoffs (Lefrancq et al. 2017, Spycher et al. 2018). To explore impairments in performances and health of the individuals, various behavioural traits based on locomotion, reproduction and respiration were monitored in herbicide-exposed gammarids. In parallel, biochemical parameters involved in major biological functions– i.e. enzymes involved in digestive metabolism (β-glucosidase, β-galactosidase), growth and moulting (chitobiase) – and markers of cell stress (peroxidase, acidic and alkaline phosphatases) were also assessed to identify early disturbances caused by non-specific toxic interactions or induction of oxidative stress. All these behavioural and biochemical traits were selected to assess toxicant-induced sublethal changes in *Gammarus* spp. (Cold and Forbes 2004, Kunz et al. 2010, Gismondi and Thomé 2014, Lebrun et al. 2021).

2. Materials and Methods

2.1. Selected herbicides and environmental levels

The 12 herbicides were selected to cover different chemical classes and modes of action among active ingredients found in the aquatic environment, as presented in Table S1 (see Suppl. Info., SI). This selection was also supported by pre-existing data of routine monitoring programmes applied on large temporal and spatial scales in Europe. Table S1 summarizes the frequency of quantification and maximal concentrations recorded (*i)* locally in the Rampillon wetland constructed to collect water from a 355-ha catchment in an intensive agricultural landscape (northern France, approx. 20 composite samples/year during 2011–2018; (Tournebize et al. 2012), (*ii)* at the scale of the Seine River Basin (northern France, 77 freshwater sites periodically monitored during 2014–2015, 6–24 annual samples/site; Info'Phytos N°10, DRIEE website) and (*iii)* at the national scale for Sweden (32 streams from agricultural catchments monitored during 2015–2016, 6–24 annual grab samples/site; (Bighiu et al. 2020)) and for Switzerland (five freshwater sites monitored for 6 months in 2015, 34–60 composite samples/site; (Spycher et al. 2018)). Although this data extraction is not exhaustive, it highlights the occurrence of selected herbicides in freshwaters under agricultural pressure or not, and shows the maximal concentrations exceeding one µg/L for almost all chemicals, diflufenican excluded. Although the detection levels in surface water are not equal, the range of maximal concentration extracted from this dataset (i.e. 1.5–40 µg/L) led to our choice of a 10-µg/L exposure level for gammarids as an environmentally relevant concentration to be used for toxicity comparisons among active ingredients at equal amounts.

All pesticides, i.e. aclonifen (ACN), bentazone (BTZ), chloridazone (CLZ), chlortoluron (CLT), diflufenican (DFF), flufenacet (FFT), isoproturon (ISO), mesotrione (MST), metazachlor (MTZ), S/R-metolachlor (MTC), prosulfocarb (PRO) and quinmerac (QUI), were purchased from Sigma-Aldrich (PESTANAL® as analytical standards, purity ≥ 98%, except DFF with ≥ 95%.), diluted in acetonitrile/pure water 10/90 at 1 g/L and used as spiking solutions for experiments in microcosms. Note that metolachlor is supplied as predominantly *S*-metolachlor insofar as the product may contain up to 40% (*R*)-enantiomer.

2.2. Gammarid collection and experimental design

Gammarus fossarum individuals were collected in a forest stream (Ru St Blandin, France) defined as a reference site, i.e. freely contaminated by metals and pesticides (Urien et al. 2017, Lebrun et al. 2020). Adults were directly size-calibrated (1.0 ± 0.2 cm) in the field using a series of sieves and brought back to the laboratory in a cool box filled with local water. Because of logistic constraints and an important number of microcosms, two collections of gammarids were performed 3 weeks apart to achieve the toxicity screening of herbicides, i.e. the first collection was in February and the second in March 2021. In the laboratory, gammarids were acclimated for 4 days by switching gradually local water with Volvic® mineral water. For the following 3 days, females and males were sorted daily into two different aerated aquaria so as to separate newly copulating pairs by amplexus, as described by Lebrun et al. (2020). Amphipods were maintained at 13°C under a 9-h:15-h light:dark cycle to mimic the natural photoperiod at the moment of their collection, and were fed *ad libitum* with alder leaves (*Alnus glutinosa*) collected at the sampling site until their exposure in microcosms.

The first series of exposures included BTZ, CLT, ISO, MST, MTZ and QUI, and the second ACN, CLZ, DFF, FFT, MTC and PRO. All the exposures were carried out in microcosms (i.e. 800-mL Pyrex® beakers), in which 10 males and 10 females were introduced in 250 mL of Volvic water spiked with each of the 12 herbicides. For each exposure condition, four replicates were made (i.e. 4 microcosms × 20 gammarids) and a fifth microcosm was created without gammarids to determine oxygen consumption, as described below. All beakers were previously equilibrated for 4 days with contaminated water before introducing gammarids so as to prevent any interactions of the pesticides with the beaker walls during the exposures. All exposures lasted 72 h in order to mimic environmentally realistic short exposures resulting from periods of surface water runoff and/or drain flow (Lefrancq et al. 2017, Spycher et al. 2018, Schäfer 2019). During the 72-h exposures, the exposure media were renewed daily to ensure constant exposure and to reconstitute oxygen levels in each beaker. At each renewal, dead amphipods were removed and counted to monitor the mortality rate. Controls were performed for each of the two exposure series by adding only acetonitrile in the microcosm water in an amount equivalent to spiking by herbicides (i.e. 0.0001% or 1 ppmv). All microcosms were maintained at 13°C under a 9-h:15-h light:dark cycle and covered with cardboard to avoid photolysis from artificial lights.

In gammarid-free microcosms, water was sampled before and after each daily renewal to ensure the constancy of the exposure levels and the absence of abiotic effects on herbicides (e.g. photolysis, sorption on beaker walls, degradation). Water samples were analysed via liquid chromatography–mass spectrometry (LC/MS) following an analysis method developed in our laboratory (see Table S2 in SI).

At the end of the 72-h exposures, 3 males were randomly sampled in each beaker, gently dried between filter papers, weighed and stored at -80 °C for further biochemical analyses, as described below. Only males were used for biochemical assays because of the relative stability of their digestive metabolism, energy storage and reproduction stages when compared with females (Charron et al. 2014, Lebrun and Gismondi 2020).

2.3. Behavioural traits

Behavioural traits measured in this study have been described in detail elsewhere (Lebrun et al. 2020). Briefly, locomotion and amplexus rate were directly determined by counting moves and copulating pairs in each microcosm after 24, 48 and 72 h of exposure. For respiration, the oxygen levels measured in the microcosms without gammarids were substituted to those measured in the presence of gammarids to establish the oxygen consumption inherent to the activity of amphipods.

2.4. Biochemical assays

After crushing the pools of frozen males in citrate/phosphate buffer, the cytosolic supernatants were used for enzymatic assays and total protein quantification in 96-well microplates, as detailed by Lebrun et al. (2021). Briefly, the activities of hydrolases were assessed by using their respective *p*-nitrophenyl-conjugated substrates (Sigma-Aldrich) on a microplate reader (Berthold Technologies, Germany). For peroxidase activity, the oxidation 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid was monitored in the presence of H_2O_2. All activities are given in units per g (wet weight) of gammarid (U/g_{ww}). One unit of activity corresponds to the amount of enzyme that catalyses 1 µmol of substrate in 1 min.

2.5. Calculations and statistical analyses

Significant differences in biochemical or behavioural levels between exposure conditions were determined with the Mann–Whitney test (U test; $P < 0.05$). For inter-series comparisons, all behavioural and biochemical deviations in exposed gammarids were related to levels measured in unexposed gammarids in each series separately, and expressed as percentage.

Multiple factor analysis (MFA) was used to study statistical links between the sets of behavioural and biochemical variables, and to assess their relative contributions to characterizing global stresses in gammarids exposed to herbicides. A hierarchical cluster analysis (HAC) was performed from the four first coordinates of the MFA-projected individuals (i.e. Dim1 to Dim4 as major contributors based on the squared-cosines of projections; see SI) to establish clusters of herbicides reflecting similarities in multi-metric responses at the threshold of $R > 0.5$. The data analyses were performed using XLStat (Addinsoft).

The MFA scores were plotted against the toxic units (TU) to assess the predictive potential of integrated sublethal responses developed in gammarids as early warnings of toxicity in wildlife. As toxic equivalence quotients, TUs were calculated as ratios of actual exposure concentrations to toxicity data established from acute or chronic exposures of standard organisms. For all herbicides, 48-h LC50 and 21-d NOEC were thus extracted from the Pesticide Properties Database or PPDB (Lewis et al. 2016) as reference values of acute and chronic toxicity in the crustacean *Daphnia magna* (except for ISO where $LC50_{48h}$ was derived from an unknown crustacean species).

3. Results and discussion

3.1. Mortality

Whatever the herbicide used, no increased mortality was observed in exposed gammarids when compared with controls, for which the mortality rates were 5.0 ± 2.4% and 4.2 ± 2.2% for series I and II, respectively. This was expected because of the modes of action targeting biological processes specific to plants and the low lethal potential of herbicides established in crustaceans (Table S1 in SI). Indeed, the concentrations in waters leading to 50% mortality obtained for the test organism *Daphnia magna* after 48-h exposure in standardized conditions ranged from 240 to 622,000 µg/L, with DFF–MST as the min–max values from the PPDB (Lewis et al. 2016). Although used in regulatory frameworks, this toxicity endpoint is not only environmentally unrealistic but also inappropriate for detecting sublethal changes potentially elicited by herbicides in animals. Consequently, changes in metabolic processes, individual performances and behaviour traits were investigated in exposed gammarids for an accurate assessment of the toxic potential of herbicides, according to an approach based on multi-level biomarkers (Kunz et al. 2010, Jacquin et al. 2019, Lebrun et al. 2021).

3.2. Physiological and behavioural traits

3.2.1. Base levels in unexposed gammarids

The base levels of physiological and behavioural traits monitored after 24, 48 and 72 h in gammarid controls from both experiment series are shown in Fig. S1 (see SI). For each series considered separately, statistical analyses revealed significant decreases in locomotion at 72 h when compared to 24 and 48 h. To a lesser extent, the respiratory activity and amplexus rate tended to decrease slightly over time. Such temporal variations most likely resulted from the acclimation of organisms to their new artificial media following their introduction in microcosms. Nevertheless, these variations are in compliance with ranges previously established in similar conditions (i.e. individuals collected in the same annual period and acclimated in microcosms) or during maintenance of gammarids for 10 weeks in Volvic mineral water (Lebrun et al. 2020, Lebrun and Gismondi 2020, Lebrun et al. 2021). Thus, this confirms the robustness of these behavioural tools for assessing the fitness of gammarids.

Concerning inter-series comparisons, no significant temporal differences were recorded between the controls of both experimental series for locomotion and respiration at each time point of monitoring and for amplexus formation after 72 h. Moreover, the analysis of variance confirmed the absence of an effect attributable to a series in the dataset of functional traits, hence supporting further inter-series comparisons, as discussed below.

3.2.2. Physiological and behavioural responses to herbicides

The effects of herbicides on amplexus rate, locomotion and respiratory activity are presented in Fig. 1 as percentages of functional gain or loss relative to controls inherent to each series. Regarding the amplexus rate, most herbicides had no effect on reformation of copulating pairs when compared with unexposed controls (Fig. 1A). Only MTC and DFF transiently stimulated the amplexus rate at 24 h by 50% and 63%, respectively, without lasting over the whole period of exposure. By contrast, ISO decreased the amplexus rate to 56% and 27% after 48 and 72 h of exposure, respectively. In the literature, alterations in mating are generally reported for gammarids exposed to insecticides following pulsed and short exposures (Cold and Forbes 2004, Kunz et al. 2010, Lebrun et al. 2020). Here, we shed light on early disturbances in the reproductive behaviour of gammarids elicited by a substituted urea herbicide with potential repercussions on population recruitment, providing that such effect is demonstrated in the long term. Nevertheless, this reduced amplexus rate cannot be extrapolated to the class of substituted urea synthesized to target plant photosystems. Indeed, the absence of an effect of chlortoluron on mating suggests an adverse effect inherent to the molecule isoproturon (Fig. 1A).

With regard to locomotion (Fig. 1B), the activity was anecdotally and transiently decreased by about 50% only in gammarids exposed to QUI for 24 h and to DFF for 48 h. As a general trend, all of the herbicides excluding DFF significantly stimulated locomotion starting from 48 h of exposure until it more than doubled after 72 h (or even quadrupled for MTZ and ISO). This highlights the significant hyperactivity in exposed individuals when compared with controls. In the literature, opposite effects on locomotor activity are recorded for freshwater fish species, in particular according to the life stage. Thus, herbicides are reported to stimulate the locomotor activity of fish larvae, while hypoactivity is usually reported in juveniles/adults because of the altered perception of external stimuli (Shinn et al. 2015, Wang et al. 2018, Jacquin et al. 2019). Here, we suppose that the hyperactivity observed in gammarids is likely related to an avoidance strategy, i.e. an attempt to migrate towards uncontaminated habitats. For instance, avoidance was described in stream macroinvertebrates including gammarids exposed to aqueous drift-initiating toxicants or in freshwater crustaceans (i.e. isopods and amphipods) exposed to sediments contaminated by polycyclic aromatic hydrocarbons as organic pollutants (Beketov and Liess 2008, Szczybelski et al. 2018). Besides, initial hyperactivity was reported in gammarids exposed via the aqueous route to insecticides at field-relevant and physiologically acceptable concentrations before triggering a neurotoxic action leading to immobility (Lebrun et al. 2020).

Nine of the 12 herbicides tested in gammarids affected the respiratory activity, with opposite effects. In the statistical analysis, weak and transient inhibitions of respiration (< 30%) were observed after 24-h exposure to QUI and CLT, and after 72 h to DFF. By contrast, significant increases in respiratory activity with gains ranging from 34% to 85% were chiefly recorded after 72-h exposure to BTZ, ISO, FFT, MTC, ACN and PRO. For these latter herbicides, the increased oxygen consumption in exposed gammarids may, in part, be related to their hyperactivity in swimming (Fig. 1B vs 1C), as also documented in *G. fossarum* exposed to neonicotinoid and organophosphate insecticides at field-realistic exposure levels (Lebrun et al. 2020). However, other herbicides, i.e. QUI, MST, CLT, MTZ and CLZ, significantly stimulated the locomotion of gammarids without increasing oxygen consumption (Fig. 1B vs 1C).

Therefore, the effect of herbicides on oxygen consumption may instead reflect physiological adjustments to counterbalance respiratory distress in exposed gammarids. Although no data are available for *Gammarus* spp., some herbicides are indeed reported to cause histological and metabolic alterations associated with the respiratory system in various freshwater species. For instance, at the organ/histological level, MTZ exposure induced structural anomalies in the gills of decapod crayfish, likely to affect oxygen uptake (Velisek et al. 2020). Such histopathological alterations of the gills induced by direct contact are also reported in goldfish exposed to herbicide mixtures (Jacquin et al. 2019). As an example at the metabolic level, herbicides such as bi-pyridines or in particular the diphenyl ether ACN can promote mitochondrial dysfunction associated with alterations in basal respiration and spare capacity in zebrafish (Lee et al. 2021). In addition, excessive oxygen consumption could induce an overproduction of reactive oxygen species inherent to aerobic metabolism, which are deleterious for organisms when not counterbalanced by antioxidant defences (Lushchak 2011). However, such contact-histological or metabolic disruptions resulting from their internalization remain to be described in order to clarify the herbicide-elicited mechanisms of action in gammarids.

Considering that herbicide toxicity is still poorly documented in aquatic wildlife, the present study provides valuable information on the harmful effects in a non-target species widely distributed in European freshwaters. Indeed, we demonstrate sublethal disturbances in the behaviour of gammarids as a result of exposure to herbicides at environmentally relevant concentrations, which reflect alterations in individual performances. Insofar as behavioural endpoints are at the interface between physiological and ecological processes, the alterations in individual fitness and early stages of reproduction observed in this ecosystem engineer can affect its population dynamics in the long term with associated impairments in freshwater functions (e.g. shifts in food resources and chains, reduced litter decomposition and altered nutrient cycling, etc.) (Kunz et al. 2010, Kohler and Triebskorn 2013). For instance, a decline in the abundance of invertebrate communities was associated with decreased leaf-litter breakdown in pesticide-contaminated streams in various countries (Schäfer et al. 2007, Schäfer et al. 2012). Consequently, our results support the relevance of using gammarid-developed behavioural traits as early tools for assessing the ecotoxicity of pesticides and preserving aquatic wildlife in agricultural areas.

3.3. Biochemical responses to herbicides

The base levels of biochemical parameters in unexposed males are illustrated in Fig. S1 (see SI). Except for acidic phosphatase (PAC), no statistically significant difference was found between the controls of both experimental series. Besides, the natural variability of levels measured in the controls of the present study is included in ranges previously established elsewhere, i.e. in similar conditions of microcosm exposures or during 10-week maintenance of gammarids in aquaria (Lebrun et al. 2020, Lebrun and Gismondi 2020). Hence, this inter-study reliability validates the interest in using selected biochemical tools to screen metabolic changes potentially induced by herbicides at the sub-individual level.

The biochemical response to herbicides in *Gammarus fossarum* males exposed for 72 h is presented in Fig. 2, as percentages of gain or loss relative to controls inherent to each series. Concerning the energy-based biomarkers, all of the herbicides tested here had no statistically significant effect on digestive enzymes (i.e. β-glucosidase and β-galactosidase). Hence, these results suggest the absence of a direct impact of these plant-targeting chemicals at an environmental concentration of 10 µg/L on the carbohydrate metabolism and energy acquisition of gammarids, unlike neurotoxic insecticides or others toxicants such as metals (Lebrun et al. 2017, Lebrun et al. 2020).

The activity of chitobiase was stimulated by 21%, 23% and 48% in gammarids exposed to BTZ, MST and MTC, respectively. In the literature, alterations in moulting enzymes involved in exoskeleton degradation and recycling have also been recorded during the exposure of crustaceans, including gammarids, to organic chemicals such as polybrominated diphenyl ethers or pharmaceuticals (Richards et al. 2008, Gismondi and Thomé 2014, Gismondi 2018). Thus, this herbicide-elicited acceleration of moulting in gammarids could reflect endocrinal disruptions by inhibiting the release of the moult-inhibiting hormone or by interacting with ecdysteroid hormone receptors, for instance (Gismondi 2018). Although the mechanisms of action remain to be clarified, our results demonstrate that some herbicides, independently of their chemical class, induced sublethal changes in the moult cycle at field-realistic concentrations, which are likely to affect the growth and reproduction of gammarids with serious consequences in population dynamics in the long term (Gismondi and Thomé 2014).

As regards alkaline phosphatase, the activity was significantly decreased by 5 of the 12 herbicides studied here, i.e. CLZ, CLT, FFT, DFF and PRO, with losses ranging from 18% to 30% for PRO–CLZ representing the min–max values in 72-h-exposed males. As a polyfunctional brush border enzyme involved in transphosphorylation reactions, alkaline phosphatase is known to play a vital role in several important intracellular processes associated with cell integrity, signal transduction pathways, enzyme and phospholipid synthesis, secretion activity, metabolite transport across the cell membranes, spermatogenesis and non-specific immunity in crustaceans. Various organic toxicants including pesticides were reported to alter alkaline phosphatase in estuarine crabs and freshwater prawns (Omkar 1986, Vijayavel and Balasubramanian 2006, Dai et al. 2014). Consequently, herbicide-elicited changes in alkaline phosphatase levels can affect gammarids in a variety of ways, thereby generating physiological and metabolic abnormalities.

Concerning stress markers, no effect of herbicides on acidic phosphatase, a marker of intracellular cytotoxicity (e.g. destruction of lysosomal membranes, autolytic degradation of dead cells), was recorded. By contrast, most herbicides significantly affected the activity of peroxidase (PerOx), an antioxidant enzyme, with opposite effects on the function of their increasing hydrophobic feature (Fig. 2 and Table S1). Thus, only the herbicides with negative logK_{ow}, i.e. QUI and BTZ, stimulated the PerOx activity by 11% and 5%, whereas the others with positive $\log K_{ow}$ inhibited it significantly, with the maximal effect reaching –39% for ACN. Thus, we found a significant correlation between the $\log K_{ow}$ of herbicides and the decrease in PerOx activity (PerOx = $-7.4 \log K_{ow} - 0.1$; $R^2 > 0.78$ with $P < 0.01$; see Fig. S2 in SI). In accordance with our results, herbicides are often reported to decrease antioxidant enzyme levels in freshwater crustaceans, such as glyphosate in *G. pulex* or MTZ in crayfish during short exposures to environmental levels (Pala 2019, Velisek et al. 2020). Consequently, the tested herbicides seem to affect the defence system against oxidative stress without any associated alterations in the cell integrity of gammarids after 72-h exposure, since no PAC changes were observed. This suggests that gammarids are able to cope with internalized herbicides during short exposures despite a potential production of reactive oxygen species, as reported in crayfish exposed to MTZ (Velisek et al. 2020). Nevertheless, alterations in cell structure generated by oxidative stress cannot be excluded during exposures longer than 72 h because of accumulating levels of reactive oxygen species and reduced antioxidant defence in the long term. The inhibitive effect of herbicides on PerOx proportionally related to their hydrophobic feature could result in their internalization and accumulation in fatty tissues and organs, in particular in the hepatopancreas as the seat of enzyme production in gammarids. Further investigations are thus required to determine the internalization and intracellular fate of herbicides in gammarids and, thereby, to clarify their toxic mechanisms.

Here, our biochemical investigations shed light on sublethal alterations in various biological functions in a non-target freshwater species exposed to currently used herbicides at field-realistic levels. As early initiating events, these biochemical changes occurring at the cellular/individual level could generate deleterious alterations at higher levels of biological organization and in associated ecological functions (e.g. colony decline, shift in community structure and species richness, decreased litter decomposition and nutrient cycling, etc.), as supported by the concept of adverse outcome pathways (Kramer et al. 2011, Kohler and Triebskorn 2013). Although they offer promising lines of enquiry in population-level risk assessment, the establishment of such obvious links from cellular events to ecological repercussions remains, to date, a major challenge in predictive ecotoxicology.

3.4. Integrative analyses of multi-level responses

A multiple factor analysis (MFA) was performed to summarize multi-metric variations observed in exposed gammarids and to assess the complementarity of biochemical and behavioural biomarker sets in identifying sublethal alterations and in clustering herbicides exhibiting toxic modes of action that are potentially similar. As illustrated in Fig. 3, the first two dimensions of MFA, i.e. Dim1 and Dim2, explained 57% of the variability in multi-level responses. Dim1 was equivalently represented by F1_bioch and F1_trait as integrated factors derived from each of the two sets of variables, i.e. 51% and 49% respectively, (see Suppl. Info., Fig. S3). This partial redundancy of behavioural traits and biochemical biomarkers in characterizing the sublethal effects of herbicides suggests prospective links between metabolic/biochemical impairments and alterations in individual performance. By contrast, the behavioural set was the major contributor separating individuals in Dim2, i.e. 75% by F2 traits closely related to the respiration rate (Fig. S3). This supports the notion that altered oxygen exchanges provide additional and accurate information on herbicide toxicity because of physiological adjustments, mitochondrial dysfunctions or histopathological alterations of the gills, for instance, as discussed in Sect. 3.2.

As illustrated in Fig. 3, four clusters of herbicides exhibiting common multi-metric variations were established (HCA with $R > 0.5$), suggesting possible similar action sites and toxicity mechanisms. Nevertheless, these similarities were not implicitly associated with the chemical classes or inhibitory action site on plants (Table S1). Along Dim1 as the most informative dimension separating the individuals (35.2%), the individuals of clusters I with scores of Dim1 > 1 (namely BTZ, MTZ and ISO) were oppositely projected to those of cluster III (DFF, CLZ and CLT; Dim1 < –1), highlighting contrasting response trends at cellular and behavioural levels. Generally, clusters I and II tended to inhibit biochemical levels (mainly PAL, PerOx and proteins) and to stimulate the amplexus rate, unlike clusters III and IV (Figs. 1A and 2). Unsurprisingly, the positive and negative projections of clusters along Dim2 closely reflect global inhibitions or stimulations of herbicides on the respiration rate previously recorded in exposed gammarids (Fig. 1C). Only MTC was not clustered because it is essentially represented by Dim3 (see SI) for which chitobiase is the major contributor (33%), as shown in Fig. 2.

Unlike the partial points of behavioural traits, significant correlations were obtained between the projection coordinates of partial points derived from the biochemical dataset and $\log K_{ow}$ (F1_Bioch scores = $-0.56 \log K_{ow} + 1.26$ and F2_Bioch scores = $0.23 \log K_{ow} - 0.51$; $R^2 > 0.57$ and 0.50 respectively, with $P < 0.01$; see Fig. S4 in SI). This finding confirmed that the biochemical responses are closely related to the hydrophobicity of herbicides, as previously highlighted in the case of PerOx (Fig. S2 in SI). This is likely due to the facilitated uptake of hydrophobic/lipophilic herbicides and their internalization in biological tissues, hence generating toxic actions at the intracellular level. Indeed, relationships between bioaccumulation and $\log K_{ow}$ of a large panel of organic chemicals were reported in multiple algae, invertebrate and fish species (Arnot and Gobas 2006). Additional analyses will be performed to establish whether biochemical disturbances can be related to bioaccumulation levels of herbicides in exposed gammarids.

To assess the predictive potential of integrated sublethal responses developed in gammarids as early warnings of toxicity for wildlife, the MFA scores were plotted against the toxic units (TU) derived from acute or chronic exposure of the standard organism, *Daphnia magna* (Fig. 4). Unlike Dim1 scores, a significant linear relationship was found between Dim2 scores and TUs, whether acute or chronic (DFF excluded for acute TU with an inaccurate value of $LC50_{48h}$, i.e. >0.24 µg/L; Table S1). Since Dim2 was essentially represented by behavioural traits, in particular by changes in respiratory activity (> 60%), this suggested that the toxic action of herbicides would be essentially mediated by alterations impacting the respiratory process. According to the MFA results, MTC, ACN and PRO as hydrophobic molecules would thus be the most deleterious for gammarids among the tested herbicides (scores Dim2 > 1.0; Fig. S3). This assumption is consistent with the literature. Indeed, mitochondrial dysfunction associated with reduced respiration capacity was often reported in fish exposed to herbicides (Souders et al. 2018). For instance, MTC and ACN promoted dysfunction in the mitochondrial respiratory chain and oxidative stress related to impaired antioxidant defences, hence generating cell apoptosis and physiological anomalies in the vascular system of zebrafish or in the gills of the marbled crayfish (Velisek et al. 2020, Lee et al. 2021). Thus, respiration-based alterations observed in exposed gammarids appear to be early warnings of herbicide toxicity before the occurrence of mortality in individuals and possible deleterious outcomes at higher biological scales (population/community), providing that toxic mechanisms are clarified in this non-target species.

Conclusions

Considering that the toxicity of herbicides is still poorly investigated in aquatic wildlife, the present study provides valuable information on the harmful effects of herbicides in a non-target species widely distributed in European freshwaters. Indeed, we found sublethal alterations in various biochemical pathways, individual performances and behavioural traits of gammarids as a result of their exposure to currently used herbicides at field-realistic levels. The analysis of combined multilevel biomarkers allowed us to identify clusters of herbicides likely to trigger common sets of biological responses. Besides, reduced antioxidant defences at the cell level and impaired respiration at the individual level, both coupled with the hydrophobic feature of herbicides, were found to be the predominant toxic effects. Bioaccumulation investigations should thus provide additional information on the mode of action of herbicides. As early initiating events, these alterations occurring at the cellular/individual level in exposed gammarids could thus represent relevant early warnings of deleterious alterations at higher levels of biological organization and in associated ecological functions (e.g. colony decline, shift in community structure and species richness, decreased litter decomposition and nutrient cycling, etc.). Although such tools offer promising lines of enquiry in population-level risk assessment, further studies are required to establish clear links from cellular events to ecological repercussions.

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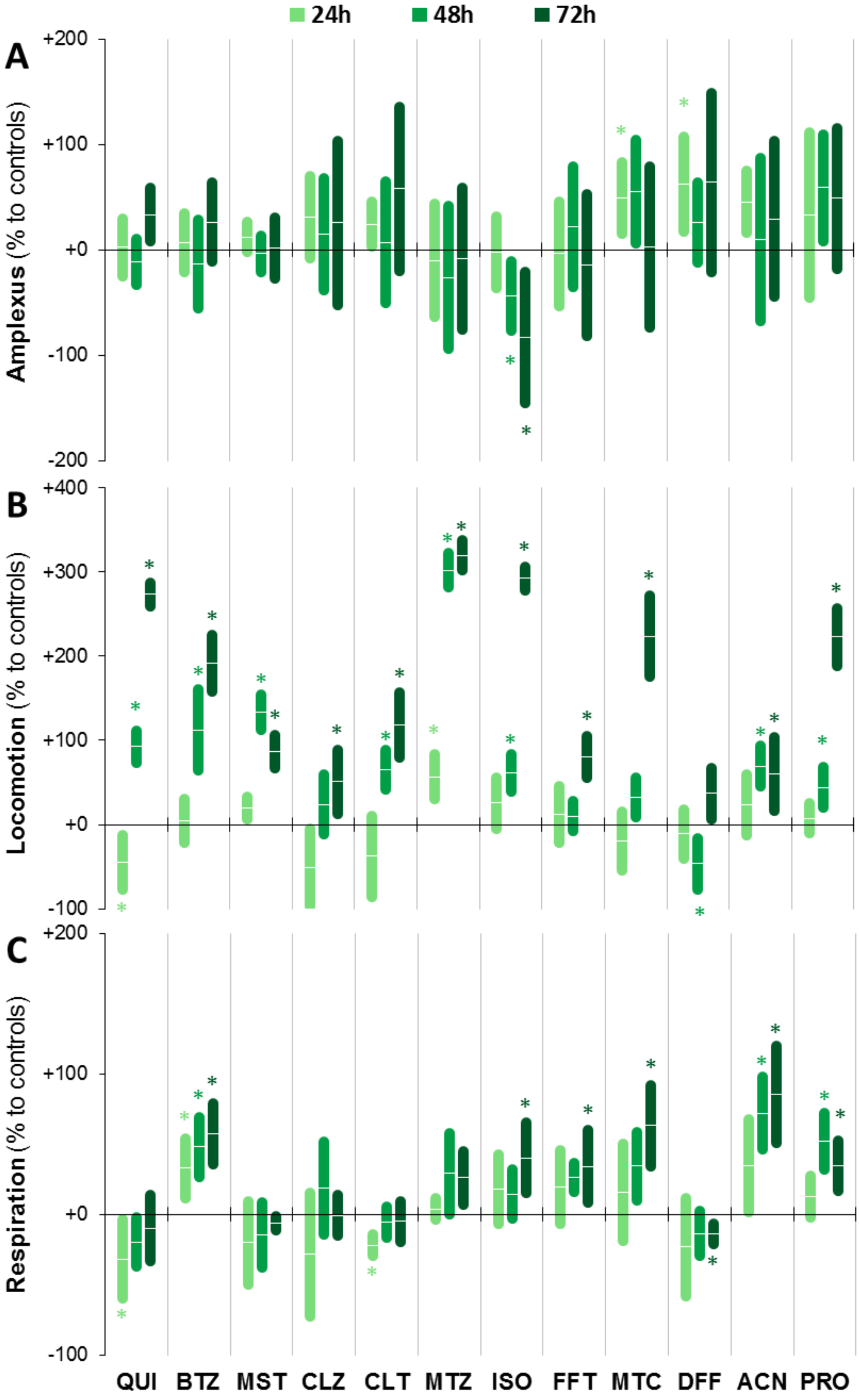


Fig. 1. Changes in **A.** amplexus reformation, **B.** locomotion and **C.** respiratory activity in *Gammarus fossarum* exposed for 24, 48 and 72 h to each of herbicides at 10 µg/L. The functional changes are given as percentage, relative to unexposed controls. The bars are SD around means ($n = 4$). *Significant differences when compared with controls (U test; $P < 0.05$). The herbicides are classified according to their increasing hydrophobicity (LogK_{ow}). QUI: quinmerac, BTZ: bentazone, MST: mesotrione, CLZ: chloridazone, CLT: chlortoluron, MTZ: metazachlor, ISO: isoproturon, FFT: flufenacet, MTC: metolachlor, DFF: diflufenican, ACN: aclonifen and PRO: prosulfocarb.

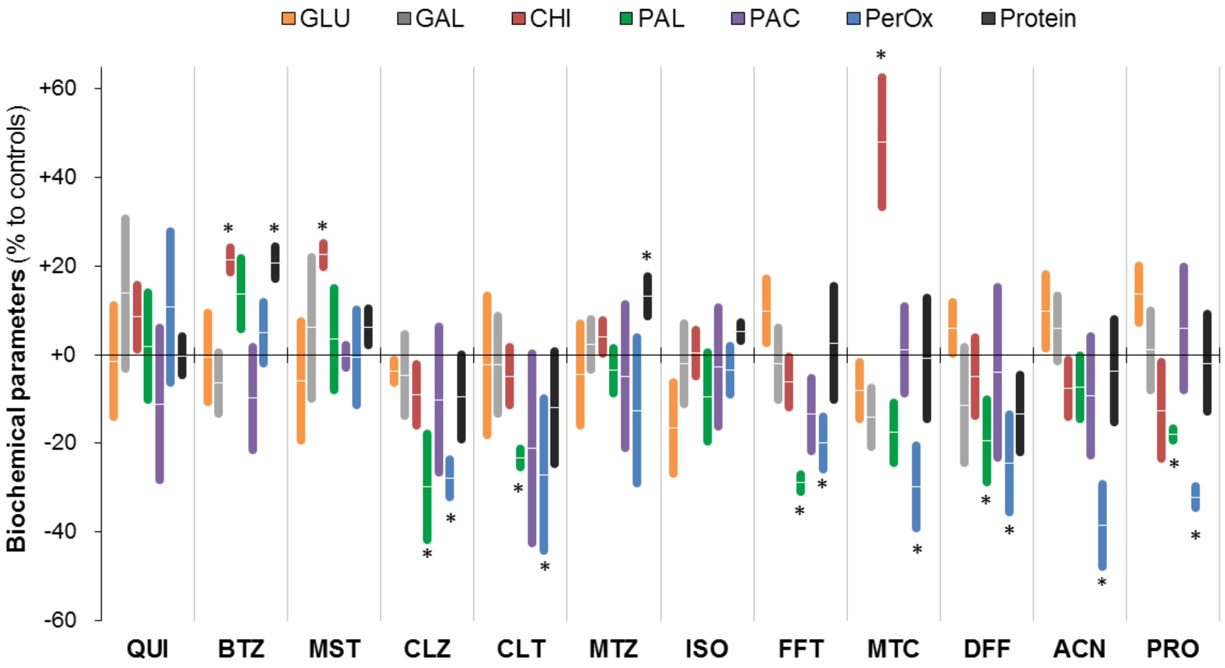


Fig. 2. Impacts of herbicides tested individually at 10 µg/L on biochemical biomarkers in *Gammarus fossarum* after 72 h of exposure. Biochemical changes are given as gain/loss percentage relative to unexposed controls. The herbicides are classified according to their increasing hydrophobicity (LogK_{ow}). The bars are SD around means (n = 4). *Significant differences when compared with unexposed controls (*U* test; $P < 0.05$). GLU, β-glucosidase; GAL, β-galactosidase; CHI, chitobiase; PAC, acidic phosphatase; PAL, alkaline phosphatase and PerOx, peroxidase

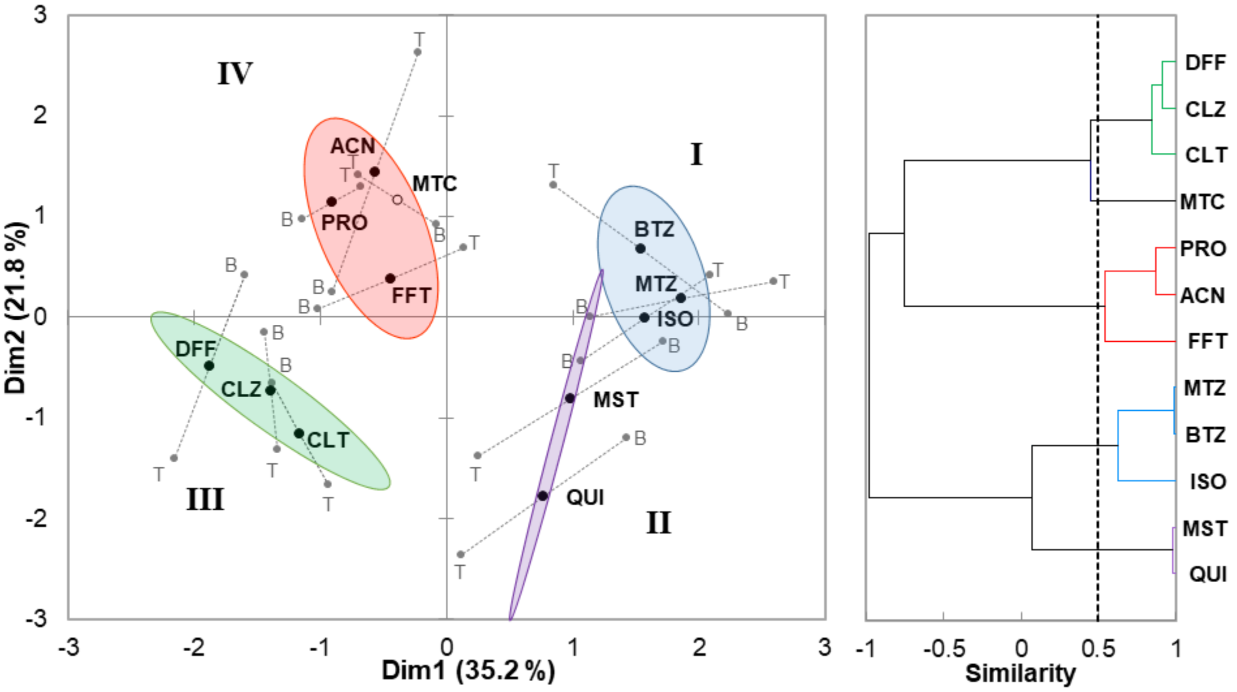


Fig. 3. Multiple factor analysis (MFA) and projection of individuals on the plane defined by Dim1 and Dim2. Each individual is at the barycenter of its partial points defined for a variable set, i.e. B for biochemical biomarkers and T for behavioural traits. Herbicide projections are clustered (from I to IV) according to their similarities established by hierarchical cluster analysis (with R > 0.5) in measured multi-metric variations. QUI: quinmerac, BTZ: bentazone, MST: mesotrione, CLZ: chloridazone, CLT: chlortoluron, MTZ: metazachlor, ISO: isoproturon, FFT: flufenacet, MTC: metolachlor, DFF: diflufenican, ACN: aclonifen and PRO: prosulfocarb.

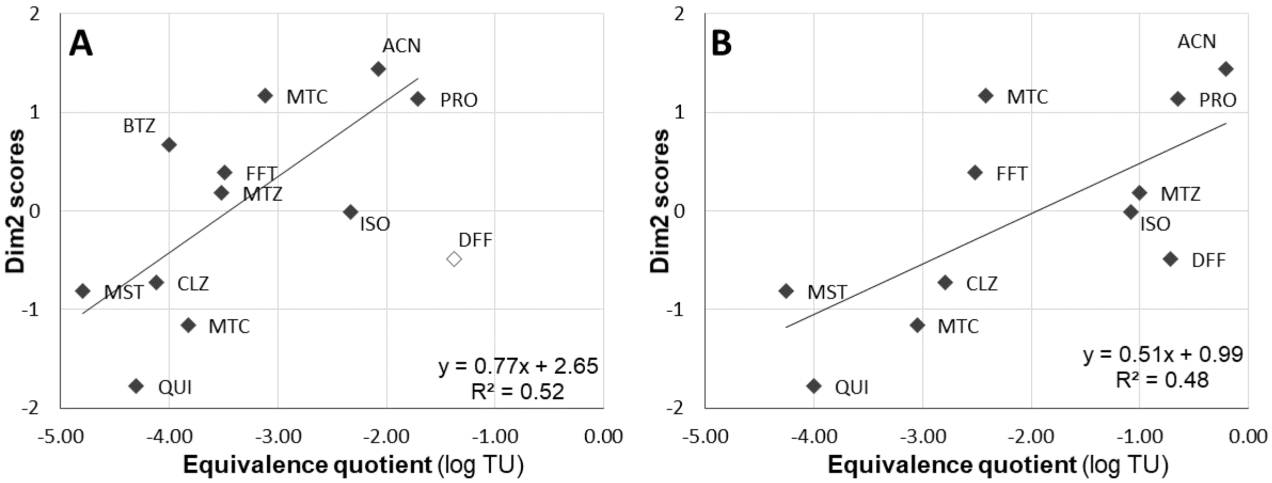
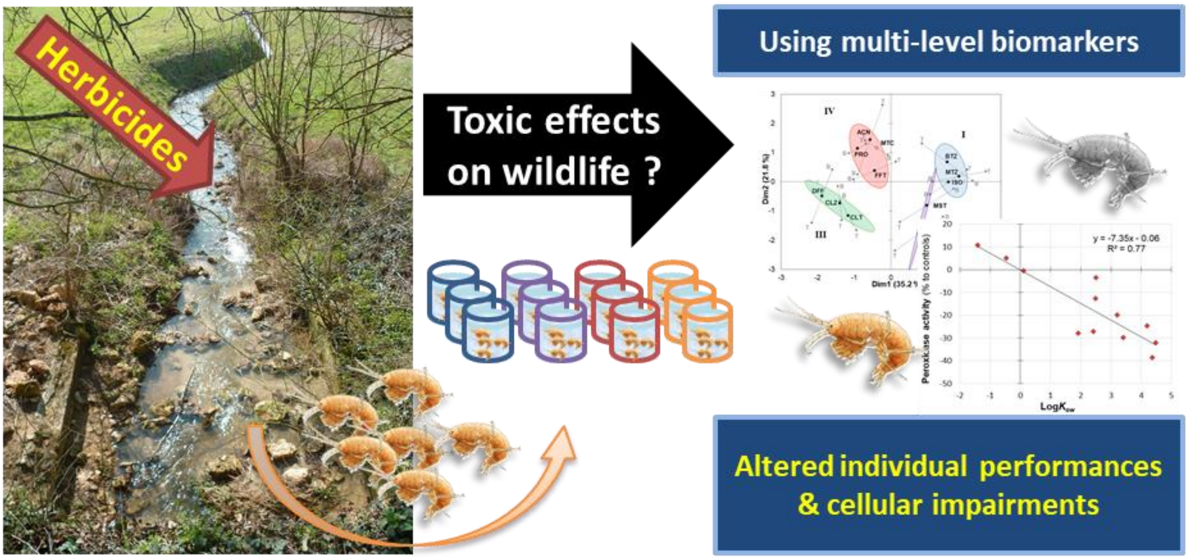


Fig. 4. Dim2 scores of MFA plotted against equivalence quotients of toxicity derived, respectively, from acute 48-h EC50 (A) and chronic 21-d NOEC (B) in the organism test *Daphnia magna* as toxic unit (TU). Note that DFF for acute toxicity is given for illustrative purposes (white symbol) and not considered in the linear fitting.


Herbicides
Toxic effects on wildlife ?
Using multi-level biomarkers
IV
I
III
ACN
MTC
PRO
FFT
BTZ
MTZ
ISO
MST
DFF
CLZ
CLT
Dim2 (21.8%)
Dim1 (35.2%)
y = -7.35x - 0.06
R² = 0.77
Peroxidase activity (% to controls)
LogKow
Altered individual performances & cellular impairments