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Chemical occurrence of pesticides and transformation products in two small lentic waterbodies at the head of agricultural watersheds and biological responses in caged *Gasterosteus aculeatus*

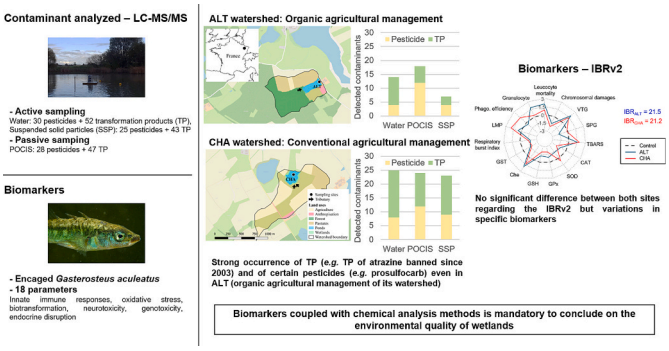
Sylvain Slaby [a,*,1], Audrey Catteau [b,2], François Le Cor [a,c,3], Amélie Cant [b], Vincent Dufour [a,4], Alain Iurétig [a], Cyril Turiès [b], Olivier Palluel [b], Anne Bado-Nilles [b], Marc Bonnard [d], Olivier Cardoso [e], Xavier Dauchy [c], Jean-Marc Porcher [b], Damien Banas [a,*]

[a] Université de Lorraine, INRAE, URAFPA, F-54000 Nancy, France
[b] Institut National de l'Environnement Industriel et des Risques (INERIS), UMR-I 02 SEBIO, Parc Technologique Alata, BP 2, 60550 Verneuil-en-Halatte, France
[c] ANSES, Nancy Laboratory for Hydrology, Water Chemistry Department, 40 Rue Lionnois, F-54000 Nancy, France
[d] Université de Reims Champagne-Ardenne (URCA), UMR-I 02 SEBIO, UFR Sciences Exactes et Naturelles, Campus Moulin de la Housse, BP 1039, 51687 Reims, France
[e] OFB, Direction de la Recherche et de l'Appui Scientifique, 9 avenue Buffon, F-45071 Orléans, France

HIGHLIGHTS

- Contamination of two headwater ponds was assessed with chemical and biological tools.
- Screening of 86 pesticides and transformation products, and analysis of biomarkers
- More contaminants were found in the pond surrounded by conventional agriculture.
- Prosulfocarb was quantified in all samples of both ponds.
- IBRv2 variations are similar on agricultural or conventional catchment basins.

GRAPHICAL ABSTRACT



* Corresponding authors.
E-mail addresses: sylvain.slaby@univ-lehavre.fr (S. Slaby), audrey.catteau@univ-reims.fr (A. Catteau), fcor@ecolimneau.fr (F. Le Cor), vincent.dufour@isa-lyon.fr (V. Dufour), alain.iuretig@univ-lorraine.fr (A. Iurétig), cyril.turies@ineris.fr (C. Turiès), olivier.palluel@ineris.fr (O. Palluel), anne.bado-nilles@ineris.fr (A. Bado-Nilles), marc.bonnard@univ-reims.fr (M. Bonnard), olivier.cardoso@ofb.gouv.fr (O. Cardoso), xavier.dauchy@anses.fr (X. Dauchy), jean-marc.porcher@ineris.fr (J.-M. Porcher), damien.banas@univ-lorraine.fr (D. Banas).
[1] Present address: Normandie Univ, UNILEHAVRE, FR CNRS 3730 SCALE, UMR-I 02 Environmental Stress and Aquatic Biomonitoring (SEBIO), F-76600 Le Havre, France.
[2] Present address: Université de Reims Champagne-Ardenne (URCA), UMR-I 02 SEBIO, UFR Sciences Exactes et Naturelles, Campus Moulin de la Housse, BP 1039, 51,687 Reims, France.
[3] Present address: Ecolimneau, 8 rue René Coty, 85,000 La Roche-sur-Yon, France.
[4] Present address: Univ Lyon, CNRS, Université Claude Bernard Lyon 1, Institut des Sciences Analytiques, UMR 5280, 5 rue de la Doua, F-69100 Villeurbanne, France.

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ABSTRACT

Recent monitoring campaigns have revealed the presence of mixtures of pesticides and their transformation products (TP) in headwater streams situated within agricultural catchments. These observations were attributed to the use of various agrochemicals in surrounding regions. The aim of this work was to compare the application of chemical and ecotoxicological tools for assessing environmental quality in relation to pesticide and TP contamination. It was achieved by deploying these methodologies in two small lentic water bodies located at the top of two agricultural catchments, each characterized by distinct agricultural practices (ALT: organic, CHA: conventional). Additionally, the results make it possible to assess the impact of contamination on fish caged in situ.

Pesticides and TP were measured in water using active and passive samplers and suspended solid particles. Eighteen biomarkers (innate immune responses, oxidative stress, biotransformation, neurotoxicity, genotoxicity, and endocrine disruption) were measured in *Gasterosteus aculeatus* encaged in situ.

More contaminants were detected in CHA, totaling 25 compared to 14 in ALT. Despite the absence of pesticide application in the ALT watershed for the past 14 years, 7 contaminants were quantified in 100 % of the water samples. Among these contaminants, 6 were TPs (notably atrazine-2-hydroxy, present at a concentration exceeding 300 ng·L^{-1}), and 1 was a current pesticide, prosulfocarb, whose mobility should prompt more caution and new regulations to protect adjacent ecosystems and crops. Regarding the integrated biomarker response (IBRv2), caged fish was similarly impacted in ALT and CHA. Variations in biomarker responses were highlighted depending on the site, but the results did not reveal whether one site is of better quality than the other. This outcome was likely attributed to the occurrence of contaminant mixtures in both sites. The main conclusions revealed that chemical and biological tools complement each other to better assess the environmental quality of wetlands such as ponds.

1. Introduction

In 2021, 469 active substances of pesticides were authorized in Europe and 61 were pending a decision (Baran et al., 2022). Assessing the concentrations and toxicological impacts of all these compounds and their transformation products (TP) is impossible in the current state of technical feasibility. However, recent articles have highlighted a multi-contamination of aquatic ecosystems likely to support a high level of biodiversity (Gaillard et al., 2016a, 2016b; Le Cor et al., 2021). Nowadays, managers of water resources and natural areas, scientists, and citizens are raising concerns about the widespread presence of such contamination and the impacts of these cocktails of contaminants, including TP, on the ecosystem health (Anagnostopoulou et al., 2022; Le Cor et al., 2021; Mahler et al., 2021).

The lack of knowledge about the effects of pesticide TP further complicates the assessment of risks to both ecosystems and human health. Consequently, establishing and implementing water quality laws and standards is challenging. For instance, recently acquired toxicological data on ESA and NOA TP of S-metolachlor, a widely applied herbicide in corn cultivation, previously classified as relevant by the French Agency for Food, Environmental and Occupational Health & Safety (Anses), allowed to downgrade it to "irrelevant" (Anses, 2022a, 2022b). These classifications have an impact on the acceptable limit values for TP in drinking water (100 ng·L^{-1} for relevant vs. 900 ng·L^{-1} for irrelevant). Shortly after considering the occurrence and concentrations of S-metolachlor-ESA, OXA, and NOA in groundwater, Anses recommended in February 2023 to withdraw the main uses of phytopharmaceutical products containing this herbicide (Anses, 2023). Such a situation underscores the need for scientists and regulation agencies to have rapid access to new data (i.e., toxicity and environmental occurrence) in order to assess the risks posed by TP to consumers, fauna, and flora. This urgency becomes even more relevant considering the introduction of numerous new active substances into the pesticide market, which in turn are sources of new TP (Kraehmer et al., 2014). Furthermore, agricultural practices implemented since World War II, heavily relying on the use of agrochemicals, can lead the agricultural profession to a deadlock when an active substance is prohibited. This situation leaves them without a solution against a crop pathogen, prompting them to actively seek involvement from policymakers, scientists, and agrochemical companies to provide new alternatives. However, such alternatives may subsequently reveal indirect toxicity and adverse effects on humans and biodiversity through their TP.

Environmental quality assessment can be conducted using chemical or biological tools, both presenting advantages and limits. Chemical methods provide the ability to determine precise contaminant concentrations within a matrix and are commonly employed in a regulatory context. Nevertheless, they are restricted by limits of quantification (LOQ) and detection (LOD) of analytical material. Additionally, these methods often require multiple rounds of sampling to assess temporal variations (Fonseca et al., 2019; Le Cor et al., 2021; Rousis et al., 2017). To avoid the rise in the number of samples and the subsequent increase in time and cost of analysis, passive samplers (e.g., polar organic chemical integrative samplers, POCIS) may represent a relevant alternative. They accumulate contaminants and assess the magnitude of the contamination (Bernard et al., 2019; Satiroff et al., 2021). However, it is impossible to search for all contaminants, and these studies do not provide information on toxicological impacts. Thus, we can then turn to biological tools to assess the state of ecosystems and biota. Among them, biomarkers have been used since the early 2000s. They are defined as measurable and/or observable changes in biological or biochemical responses, ranging from the molecular to the physiological level (including behavioral changes), that can be related to the exposure or toxic effects of environmental contaminants (van der Oost et al., 2003). The exposure to contaminant mixtures induces effects and responses at various biological levels, different from the impact of each substance taken separately. Thus, the assessment of a large set of biomarkers related to several biological functions is required to comprehensively integrate the variety of existing toxicity mechanisms. Furthermore, in order to minimize the variability induced by confounding factors (i.e., age, size, or sex of individuals), the transplantation of organisms from a reference site to the studied areas provides the advantage of standardizing individuals (Oikari, 2006). It also prevents biased results caused by the adaptation of organisms to the contamination (occurring in native populations) or even their disappearance due to contamination effects. Among the various species that can be used, *Gasterosteus aculeatus* (three-spined sticklebacks) gains interest in biomonitoring studies. Its tolerance to salinity, temperature variations (Wootton, 1984), and pollution (Pottinger et al., 2002) enables investigations of various types of aquatic hydrosystems in situ. A wide array of biomarkers has been adapted, developed, and validated for this species, including oxidative stress biomarkers, innate immune responses, endocrine disruption, neurotoxicity, and genotoxicity biomarkers (Bado-Nilles et al., 2013;

Cant et al., 2022; Katsiadaki et al., 2012; Sanchez et al., 2008b; Santos et al., 2016). The application of these biomarkers has already proven to be relevant in site discrimination (Catteau et al., 2020, 2021, 2022; Le Guernic et al., 2016a). However, when biomarkers are employed alone, establishing a connection between the measured biological effects and a precise chemical contamination remains challenging, given that only a few of them are specific (Lam, 2009).

This study focused on two fishponds located at the headwaters of different agricultural catchments (conventional and organic). Due to their small sizes, their environmental quality is poorly studied, despite their provision of valuable services such as water and food supply or biodiversity support (Lorenz et al., 2017; Meyer et al., 2003; Ulrich et al., 2022). The Water Framework Directive aims to protect all water bodies and requires a "good status" achievement concerning physico-chemical and ecological quality by the demanding deadline of 2027 (WFD, Directive 2000/60/EC). The French legislation on water and aquatic environments (French law no. 2006–1772), translating the WFD into French law, does not consider water bodies of <0.5 km^2 in a legal context, despite the fact that, collectively, they cover a significant portion of territories. For example, in France, ponds ranging from 0.001 to 1 km^2 cover an area of 2856 km^2, which is more than the cumulative area of those exceeding 1 km^2 (Terasmaa et al., 2019). The relevance of considering small water bodies in agricultural landscapes is increasingly recognized and recent studies reported their strong contamination by pesticides and TP at concentrations that pose environmental risks (Brodeur et al., 2021; Gaillard et al., 2016a; Le Cor et al., 2021; Slaby et al., 2022; Szöcs et al., 2017).

In both of the investigated sites, analyses of pesticides and TP in water were conducted using active sampling, passive sampling through POCIS, as well as in suspended solid particles (SSP). These analyses were combined with the measurement of several biomarkers (i.e., innate immune responses, oxidative stress, biotransformation, neurotoxicity, genotoxicity, and endocrine disruption) on *G. aculeatus* encaged in situ. This work aimed to establish a link between pesticide and TP contamination evaluated through three complementary approaches (direct water analysis + SSP analysis + POCIS) and biological effects on *G. aculeatus* in understudied wetlands. Furthermore, it enables a comparison of sampling protocols, exploring the complementarity of the methods and their respective limitations. At last, it examines the potential influence of the watershed management (conventional vs. organic) on the caged teleost fish, *G. aculeatus*.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile (ACN), formic acid, methanol isopropanol, and ultrapure water were purchased from Biosolve-chemicals (Dieuze, France) and were of LC-MS quality. Citrate buffer, glycerol, heparin, Leibovitz 15 medium (L15), magnesium sulfate, penicillin, phenylmethylsulfonyl fluoride, potassium phosphate buffer, streptomycin, tricaine methanesulfonate (MS222) were obtained from Sigma-Aldrich (Saint Louis, MO, United States). Phosphate buffered-saline solution was purchased from Fisher Scientific (Merelbeke, Belgium).

Individual solutions of analytical standards (>95 % grade of purity) were prepared in ACN (100 mg·L^{-1}), stored at −18 °C, and mixed before analysis to obtain a concentration of 5 μg·L^{-1}. They were purchased from Dr. Erhenstorfer (Ausburg, Deutschland), Neochema (Bodenheim, Deutschland), HPC standards GmbH (Cunnersdorf, Deutschland), Toronto Research Chemicals (North York, Canada), Honeywell (Seelze, Deutschland), and Cambridge Isotope Laboratories (Tewksbury, United States).

2.2. Studied sites

ALT and CHA are both dam fishponds with equivalent surface areas (ALT = 0.04 km^2, CHA = 0.05 km^2) situated along headwaters streams in the Grand Est region (North-Eastern France, GPS coordinates: 48°46′29.1"N 6°45′51.2"E and 48°45′18.0"N 6°44′17.9"E, respectively). Fig. 1 and Table 1 provide the composition of their respective catchments, primarily comprising forested lands for ALT (68.2 %), and a mix of arable lands and permanent pastures for CHA (91 %). Surface areas and compositions of the watersheds were determined using QGIS software (v. 3.6 Noosa, QGIS Development Team, 2019), the OCS GE2 ©GeoGrandEst (2019) database (background map: https://www.google.com/maps).

Crop cultures surrounding CHA follows conventional agricultural practices involving the use of synthetic pesticides. The management of arable lands is mainly composed of a rotation of silage corn, wheat, barley, and rapeseed, which is representative of the regional practices. Conversely, crops surrounding ALT are managed according to organic agriculture principles, and synthetic pesticides have not been employed since 2009. Both ponds are encircled by vegetated buffer strips of at least 5 m in width, which remain pesticide-free. Water flows into the ponds through their tributaries (2 for CHA, 1 for ALT), runoffs, and precipitations.

Both ponds support extensive aquaculture activities. In contrast to intensive production, fish consume the food naturally occurring in the ponds. No food is supplied except under extreme weather conditions (which was not the case during the study period). The production level is low, averaging around 125 kg·ha^{-1} per year (information sourced from fish farmers). Species like *Cyprinus carpio*, *Esox lucius*, *Rutilus rutilus*, *Scardinius erythrophthalmus*, and *Tinca tinca* are included in the production. Fish are not caged and can move freely within the pond. The production may be used for human consumption, for sale and introduction into other ponds to feed carnivorous fish, or for sale to fishing associations. Further details on the production cycle are available in Gaillard et al. (2016b). In a prior study conducted in CHA, it was determined that fish introduced into the pond in March 2019 were only contaminated by benzamide (not analyzed in the present article) among the 40 pesticides and TP which were analyzed in *C. carpio*, *R. rutilus* and *T. tinca* (Slaby et al., 2022). The introduction of fish is not considered to be a significant source of contamination.

The experimentations and the sampling period took place during 21 days, from October 28, 2019 (T0) to November 18, 2019 (TF). These dates were selected because they follow the major autumnal influx of pesticides into ponds (Gaillard et al., 2016a; Le Cor et al., 2021). During this period, the average daily rainfall and temperature were recorded at 2.2 ± 0.7 mm and 6.2 ± 0.7 °C, respectively (Infoclimat, 2019).

Sampling zones and caging locations were positioned close to the dam of each fishpond where the water level was the highest. Conductivity, pH, O_2, and temperature were measured in both ponds at T0 and TF (Table 2). In both ponds, pH, and temperature were very similar. Temperatures were particularly cold at TF in both ponds (around 3.1 °C) because of snowy weather during the last week of caging. Oxygen rate values were higher in CHA (O_2 = 97.6 to 98.7 % sat.) than in ALT (O_2 = 90.8–98.4 % sat.) but both remain high. pH values were slightly higher in CHA (8.5–8.7) than in ALT (8.2–8.4) Conductivity, which is relatively high in the study area characterized by a substrate rich in Keuper marl, was slightly higher in ALT (972–993 μS·cm^{-1}) than in CHA (743–784 μS·cm^{-1}).

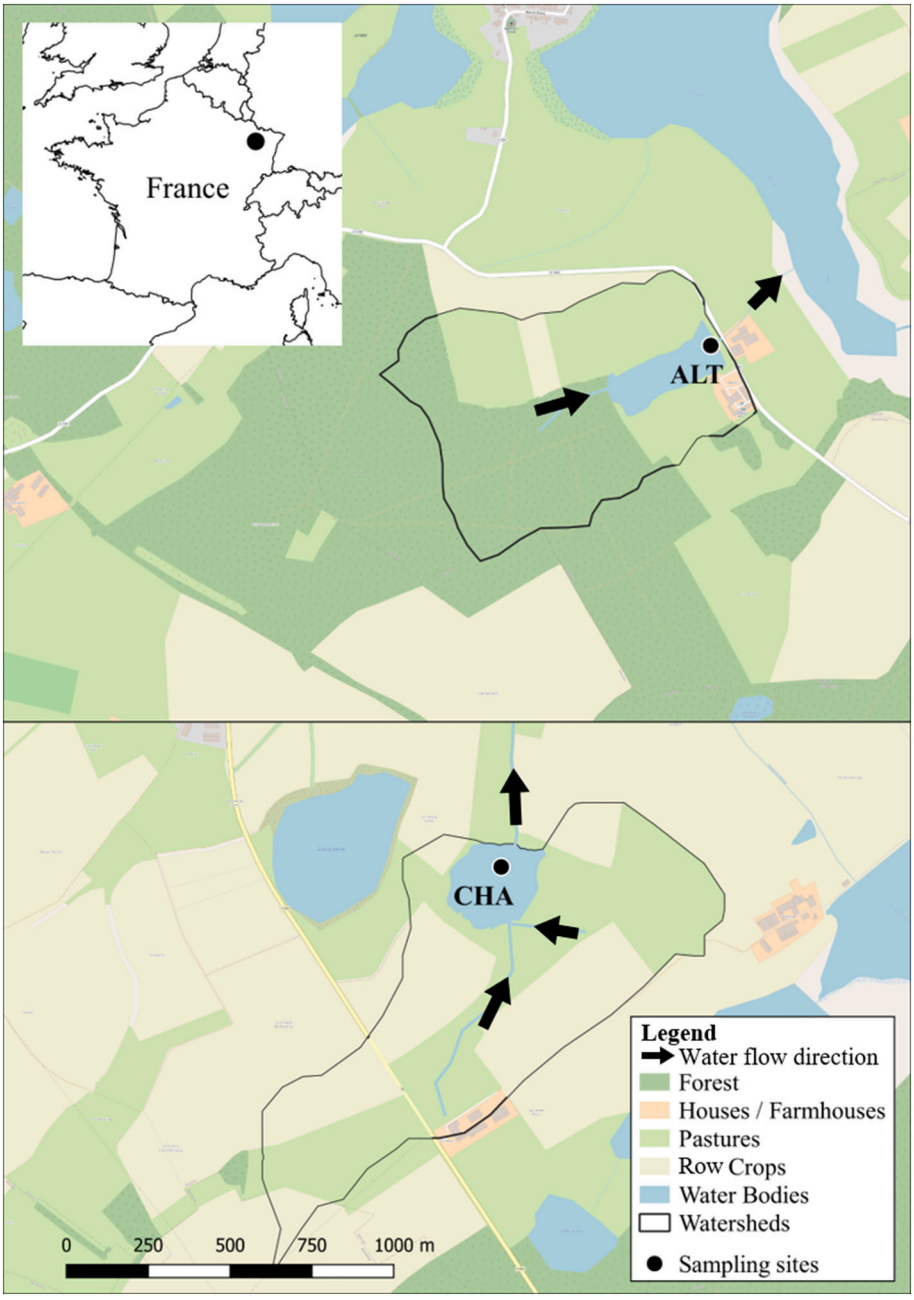

Fig. 1. Presentation of the studied ponds (ALT and CHA).

Table 1
Composition of ALT and CHA catchments.

	ALT	CHA
Catchment area (km^2)	0.944	0.807
Anthropized zones (%)	1.5	1.8
Arable zones (%)	2.1	42.2
Forest (%)	68.2	0
Fishpond (%)	4.5	5.7
Pastures (%)	23.6	48.8
Wetlands (%)	0	1.5

Table 2
Physicochemical parameters measured at TF in ALT and CHA.

	ALT		CHA	
	T0	TF	T0	TF
Conductivity (µS·cm^{-1})	972	993	784	743
pH	8.4	8.2	8.5	8.7
O_2 (% sat.)	98.4	90.8	98.7	97.6
Temperature (°C)	NA	2.9	NA	3.3

2.3. Chemical analysis

2.3.1. Water and SSP sampling and extract preparation

Pond water samples were collected near the tributary at least once a week between T0 and TF. For ALT, water samples were manually collected between T0 and TF (n = 4) using a polyethylene bottle (250 mL). In the case of CHA, as this pond was subjected to regular sampling for other research purposes, a daily automatic sampling process was used (n = 21, Sigma SD900, Hach, Düsseldorf, Deutschland). Samples were stored in polyethylene bottles and preserved at −18 °C until chemical analysis.

A volume of 1 mL of each sample was mixed with 10 μL internal standard solution (individual concentration between 50 and 250 ng·L^{-1} in ACN) into a polypropylene centrifuge tube, vortexed (10 s), and centrifuged (20,800 *g*, 18 °C, 10 min, 5810R, Eppendorf, Montesson, France). Supernatants were transferred into 2-mL glass vials to be analyzed using high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS).

SSP were obtained by using a laboratory-made system with a "honeycomb" structure (Fig. A.1). In each pond, a system was employed to collect SSP which sedimented from T0 to TF in a recipient. It consisted of polypropylene tubes of 1.6 cm diameter and 30 cm height placed perpendicularly to the water flow within the pond. The upper ends of these tubes were submerged 15 cm beneath the water surface and the area available for sampling was approximately 1255 cm^2. At TF, each SSP sample was freeze-dried for 92 h, sieved with a mesh size of 2 mm, and ground up. A total of 0.5 ± 0.01 g of SSP was mixed with 10 μL of the internal standard solution (individual concentration of 50–250 ng·L^{-1} in ACN). After the evaporation of the solvent under a fume hood (10 min), 5 mL of a solution of ACN and LC–MS quality water (90:10) was added and tubes were vortexed (10 s) and then centrifuged (3200 *g*, 15 min, 18 °C). Supernatants were collected into glass test tubes and submitted to a gentle nitrogen stream at 30 °C (Multivap 54, LabTech, Sorisole, IT) in order to remove ACN. Then, 500 μL of acidified water (0.1 % formic acid) was added and the mixture was transferred into a polypropylene centrifuge tube using a Hamilton syringe and vortexed (10 s). The volume was adjusted to 1 mL with formic acid (0.1 %) and after centrifugation (20,800 *g*, 10 min, 18 °C), the supernatants were collected into 2-mL glass vials to be analyzed with the HPLC-ESI-MS/MS.

2.3.2. Passive sampling and extract preparation

POCIS purchased from AFFINISEP (Petite Couronne, France) were constituted on the pharmaceutical conformation: 0.23 g of Oasis HLB sorbent phase between two semi-permeable membranes sealed by stainless steel rings. POCIS were immersed in both ponds in triplicates from T0 to TF. They were positioned 30 cm below the water surface within a stainless-steel cage enclosed by a metal rod stuck in the sediment. At TF, POCIS were rinsed with demineralized water, gently dried with a paper towel, packed up in an aluminum sheet, and stored at −20 °C until the extraction phase. The accumulative phase was collected in a solid phase extraction cartridge with demineralized water and dried under vacuum for a few hours (drying efficiency was mass controlled). Consecutive elutions were carried out with 3 mL of methanol, 3 mL of a methanol-dichloromethane mix (1:1 volume), and 3 mL of dichloromethane at 15 mL·min^{-1}. A volume of 500 μL of the eluate was aliquoted, and mixed with 20 μL of a 100 μg·mL^{-1} solution of internal standards, and evaporated under gentle nitrogen flow. Dried residues were dissolved in 500 μL ACN. The final extract was diluted 10 times in acidified ultrapure water (formic acid 0.1 %) for injection in HPLC-ESI-MS/MS.

Accumulative phases artificially spiked with natives were extracted in each sample series in order to control extraction efficiency and assess the quantification yields. A clean accumulative phase was also extracted and was considered as a protocol blank to control the potential contamination during the experimentation. Field blanks (not exposed to pond water) were extracted as described previously to characterize field contamination. All control and field blanks were submitted to the same extraction protocol.

2.3.3. Pesticide and TP analysis

Analysis of contaminants was performed with HPLC-ESI-MS/MS methods described in Le Cor et al. (2021) and Slaby et al. (2022). The list of analytes is given in Table A.1 according to the sampling procedure (water: 30 pesticides and 52 TP, SSP: 25 pesticides and 43 TP, POCIS: 28 pesticides and 47 TP). Among the molecules targeted in the water samples (the most complete list), 64 % were TP, and only 8 pesticides (e.g., boscalid, MCPA, omethoate, prosulfocarb, tebuconazole, terbutryn, and thiamethoxam) were found to lack corresponding TP analyses due to the difficulty of accessing standards.

Briefly, HPLC-LC20AD (Shimadzu, Marne-la-Vallée, France) coupled with a QTRAP® 5500 system (Sciex, Villebon-sur-Yvette, France) was used in both positive and negative modes and quantification was performed using internal standards (Tables A.2 & A.3). The process was validated according to the French standard NF T90-210 (AFNOR, 2018). Potential contamination during the analytical procedure was verified by using blank samples constituted by internal standard solution in LC-MS quality water in each series. Every ten samples and at the end of each series, a control solution (internal standard solution + analytes in ACN + quality LC-MS water with 0.1 % formic acid) was also used as quality control. Dilution was performed when the concentration exceeded (±10 %) the highest calibration point in order to reach the calibration rate. Recoveries were assessed by spiking one sample per injection series and are given in Table A.1. If the recovery was not included between 80 and 120 %, the quantified concentration was adjusted. LOQ was defined as the smallest tested concentration with an inter-day precision lesser than 30 % and LOD was obtained by dividing the LOQ by 2 (Gaillard et al., 2016a, 2016b; Le Cor et al., 2021; Slaby et al., 2022). LODs and LOQs are given in Table A.1 according to the sampling procedure. Data was interpreted with MultiQuant software (v. 3.0.1, Sciex, Villebon-sur-Yvette, France).

2.4. Biological analysis

2.4.1. Caging experiment

This experiment was conducted in accordance with the European directive 2010/63/UE concerning the protection of animals used for scientific purposes at the French National Institute for Industrial Environment and Risks facilities (registration number E60-769-02, INERIS, Verneuil-en-Halatte, France). The experiment was approved by the French ethics committee in animal experimentation (APAFIS project n° 20,760, approval number n° 096).

Adult *G. aculeatus* used in the caging experiment came from INERIS breeding facilities. The sex of fish was determined using the head morphology model (de Kermoysan et al., 2013). Males and females were kept separately for 8 weeks in outdoor ponds with natural vegetation and macroinvertebrate communities prior to the field experiment. These ponds received continuous renewal of tap water, which remained free of any chemical or bacterial contamination, with the exception of the occurrence of atrazine-desethyl at a low concentration of 14 ng·L^{-1} (Table A.4).

The caging experiment took place from T0 to TF, outside the *G. aculeatus* spawning period. Indeed, the male fish displays aggressive and territorial behavior during the breeding season, which prevents its use during the spring-summer period. Cylindrical tanks of 630 mm in length and 270 mm in diameter (volume = 36 L) were used as cages for field exposure. They had a mesh size of 5 mm allowing the water and particle flows, and also the natural entry of small benthic macroinvertebrates while preventing the escape of fish. The fish fed on natural prey and did not receive supplementary food during the experiment. In fact, a previous study has demonstrated that during periods outside of the breeding phase, an external food supply is unnecessary. The food that enters the cage is sufficient to maintain most of the biological functions (Catteau et al., 2019). Thirty adult *G. aculeatus* (1-year-old, 4.7 ± 0.3 cm, 1.4 ± 0.3 g) were caged in each pond. The sex ratio was set to 50:50 (15 male fish and 15 female fish per pond). The density of 1.17 kg·m^{-3} was based on previous studies demonstrating that biomarkers levels of *G. aculeatus* did not significantly vary within a density range of 1.08 kg·m^{-3} to 2.31 kg·m^{-3} (Le Guernic et al., 2016b). Thirty fish from the initial population (50:50 sex ratio) were kept in the initial stabling pond to be considered as the reference condition.

2.4.2. Sample collection and treatments

At TF, *G. aculeatus* from both ponds and the control site were anesthetized with MS-222 (100 mg·L^{-1}) before cervical dislocation. The biological samples (blood, liver, head kidney, muscle, and spleen) were isolated directly in situ to avoid transportation stress and preserved in liquid nitrogen until they were processed in the laboratory. Protocols for biological sample collection and biomarker measurements were detailed in previous publications (Catteau et al., 2019, 2021). All analyses were performed on each organism (Control site: 15 males and 15 females, ALT: 14 males and 15 females, CHA: 15 males and 14 females).

The spleen was pressed through sterilized nylon mesh (40 μm, Sigma-Aldrich, United States) and the obtained leucocyte suspension was kept in 1 mL of L15 at 4 °C supplemented with penicillin (500 mg·L^{-1}) and streptomycin (500 mg·L^{-1}) until the innate immune response measurements. The liver and a piece of muscle were stored at −80 °C respectively in 400 μL and 800 μL of a potassium phosphate buffer (0.1 M, pH 7.4) modified with glycerol (20 %) and phenylmethylsulfonyl fluoride (2 μM). Head kidneys were weighted and stored at −80 °C in a denaturation buffer solution (Tris-HCl 100 mM, EDTA 10 mM, urea 8 M, SDS 2 %, β-mercapto-ethanol 200 mM). Blood samples (5 μL) for the vitellogenin concentration (VTG) measurement were stored at −80 °C in 45 μL of phosphate buffered saline solution supplemented with 30 % heparin (100 mg·L^{-1}) and 20 % glycerol. Blood samples (2 μL) for chromosomal damages, were directly diluted in citrate buffer (Vindeløv and Christensen, 1990) after collection and were stored at −80 °C until analysis (Cant et al., 2022). In addition, standard length and total weight were recorded to calculate Fulton's condition index (K) indicating the general well-being of fish (Fulton, 1902):

$$K = \frac{Total\ body\ weight}{Standard\ length^3} \times 100$$

2.4.3. Biomarker measurements

Muscles and livers were ground with glass beads (diameter of 1 mm) using a FastPrep-24™ 5G (Millipore, France) and then centrifuged (10,000 *g*, 15 min, 4 °C). The supernatant of each sample (post-mitochondrial fraction) was recovered. Muscle supernatants were used for assessing the acetylcholinesterase activity (AChE) as a neurotoxicity marker. Liver supernatants were used to measure the thiobarbituric reactive substance concentration (TBARS), the total glutathione concentration (GSH), and the glutathione-S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase activity (GPx) and catalase (CAT) activities as biomarkers of oxidative stress. All these biomarkers were expressed by the total protein concentration, assessed using the Bradford method. The VTG was assessed in blood samples of both sexes of *G. aculeatus* and was expressed in ng·mL^{-1} of blood. The spiggin concentration (SPG) was measured in head kidneys after the dissolution process (ground up in boiling water) and was expressed in U·mg^{-1} of total fish weight. Specific competitive ELISA tests were used to measure VTG and SPG. All these biochemical biomarkers were adapted for *G. aculeatus* by Sanchez et al. (2005, 2008a, 2008b). The leucocyte suspensions from the spleen were used for innate immune biomarkers analysis following the protocols initially developed and described by Bado-Nilles et al. (2013, 2014) and Gagnaire et al. (2015). All analyses were carried out using a flow cytometer (MACSQuant X, Miltenyi Biotec, United States) with 96-well microplates and 200 μL of leucocyte suspension. The measured immune parameters were the cellular mortality percentage (apoptosis and necrosis rate), the leucocyte distribution (percentage of granulocytes and lymphocytes among the total leucocytes), the phagocytosis efficiency (the capacity of cells to internalize three or more fluorescent beads), and the respiratory burst index (the ratio of reactive oxygen species in PMA-stimulated cells = ROS A / the ROS in unstimulated cells = ROS B).

In addition, the chromosomal damage was determined on peripherical erythrocytes as recently described by Cant et al. (2022) following the procedure developed by Vindeløv and Christensen (1990) and adapted by Marchand et al. (2017) on *G. aculeatus*. Briefly, erythrocytes were adjusted at 40 × 10^6 cells·mL^{-1}. Treatments were applied for lysing the cytoplasmic and nuclear membranes to access DNA, to degrade RNA, stabilize amino acid, and mark DNA by the propidium iodide (1 mg·mL^{-1}, Invitrogen, Carlsbad, CA, United States). A sample of stabilized chicken red blood cells (Fitzgerald Industries, Acton, United States) was used as a standard and analyzed simultaneously with the fish blood samples (Vindeløv et al., 1983). All samples were analyzed by flow cytometry (Beckman Coulter, Brea, CA, United States). Each FL3 peak coefficient of variation (CV) corresponds to the nuclear DNA content variation and can reflect different types of genome damage, referred to as chromosomal damage, expressed at the chromosomal level. DNA damage corresponds to the CV of fish erythrocyte samples minus the CV of chicken red blood cell samples (Easton et al., 1997).

2.5. Statistical analysis and IBRv2 calculation

2.5.1. Statistical analysis

All statistical analyses were conducted using R software version 3.3.2. Regarding grab samples, the frequencies of detection (FOD) and quantification (FOQ) of each substance were determined. Caution must be taken when the compound is detected at a concentration below the LOQ (Hecht et al., 2018), in order to avoid an averaging based on a large set of unquantified values. Concentrations below the LOD were estimated as null, and those included between the LOD and the LOQ were set to LOQ/2 allowing to estimate an averaged concentration (Gaillard et al., 2016a; Le Cor et al., 2021). However, means were displayed only when FOQ > 90 % (Slaby et al., 2022). In other cases (FOQ < 90 %), mean concentrations were not presented.

Concentrations obtained in grab samples with FOQ > 90 % in at least one site were compared using the Wilcoxon test. In these cases, concentrations below LOD were estimated as null, and those comprised between LOD and LOQ were set to LOQ/2 when needed (Gaillard et al., 2016a, 2016b; Le Cor et al., 2021; Slaby et al., 2022). Two-way analysis of variance (ANOVA) followed by Tukey's HSD tests were applied for each biomarker to assess the effect of “Sex” and “Site” factors on the biomarker level. The normality of residuals (Shapiro-Wilk's test) and the homoscedasticity between groups (Levene's test) were verified to validate the use of ANOVA. Otherwise, the data were log-transformed, and if testing assumptions were not met despite the log transformation, Kruskal-Wallis tests followed by multiple comparisons of treatments were conducted. If the “Sex” factor was significant, male and female data were segregated, and one-way ANOVA associated with Tukey's HSD test were performed for each sex, following the method previously described (with “Site” as the single factor).

2.5.2. IBRv2 calculation

The IBRv2 (Integrated Biomarker Responses version 2) index was calculated following the methodology described by Sanchez et al. (2013). It is an indicator of the difference in biomarker responses between a study site and a reference value. To prevent redundancy in the IBRv2, certain biomarkers presented in Table 5 were consolidated. Leucocyte necrosis and leucocyte apoptosis were summed as a single parameter called “leucocyte mortality”. In the same way, only the respiratory burst index was kept whereas ROS B (Basal Reactive Oxygen Species) and ROS A (Reactive Oxygen Species when cells are Activated) were not taken into consideration, being already included in the calculation of the respiratory burst index.

Briefly, site averages were calculated for each biomarker (X_i). The calculated averages for the control group of fish were considered as the reverence values (X_0). Averages of the two sites (CHA and ALT) were divided by the reference values and a log transformation was applied ($Y_i = \log(X_i/X_0)$). The values were then divided by the general standard deviation (SD) of the log-transformed ratio (Y_i) to obtain the deviation index ($A = Y_i/SD$). This allowed the creation of the basal line and to represent biomarker variation from the reference values. For each site, A

Table 3
Detected compounds occurrence in POCIS and concentrations (ng.L^{-1}) in water samples from ALT and CHA.

Compounds	ALT						CHA						*p*-value[b]
	FOD	FOQ	Min	Max	Mean ± SEM[a]	POCIS	FOD	FOQ	Min	Max	Mean ± SEM[a]	POCIS	
3,5,6-trichloro-2-pyridinol	100	0	<20[c]	<20[c]	-	-	81	0	<10[d]	<20[c]	-	-	NA
Alachlor-acetochlor-ESA	50	0	<5[d]	<10[c]	-	-	14.3	0	<5[d]	<10[c]	-	-	NA
Atrazine-2-hydroxy	100	100	257	343	300 ± 37	X	100	100	43.5	61.5	51.9 ± 1	X	0.0002
Atrazine-desethyl-2-hydroxy	100	100	16.2	21	18.5 ± 2.1	-	0	0	<5[d]	<5[d]	-	-	<0.0001
Bentazon	0	0	<2.5[d]	<2.5[d]	-	X	0	0	<2.5[d]	<2.5[d]	-	X	NA
Boscalid	0	0	<2.5[d]	<2.5[d]	-	-	0	0	<2.5[d]	<2.5[d]	-	X	NA
Chloridazon	0	0	<2.5[d]	<2.5[d]	-	X	0	0	<2.5[d]	<2.5[d]	-	-	NA
Chlorotoluron	0	0	<2.5[d]	<2.5[d]	-	X	100	23.8	<5[c]	5.9	-	X	NA
Dimethachlor	0	0	<2.5[d]	<2.5[d]	-	X	81	0	<2.5[d]	<5[c]	-	X	NA
Dimethachlor-ESA	0	0	<2.5[d]	<2.5[d]	-	-	100	100	12.6	20	14.9 ± 0.4	X	0.002
Dimethachlor-OXA	25	0	<25[d]	<50[c]	-	-	81	19	<25[d]	68.9	-	-	NA
Dimethenamid	0	0	<5[d]	<5[d]	-	X	14.3	0	<5[d]	10[c]	-	X	NA
Dimethenamid-ESA	0	0	<2.5[d]	<2.5[d]	-	-	100	100	15.5	24.3	20.2 ± 0.6	X	0.002
Dimethenamid-OXA	0	0	<10[d]	<10[d]	-	-	100	100	74.4	107	85.9 ± 1.7	X	0.002
Flufenacet	75	25	<5[c]	5.8	-	X	100	100	6.3	124	67.1 ± 9	X	0.0002
Flufenacet-ESA	0	0	<2.5[d]	<2.5[d]	-	-	100	100	243	382	316 ± 8	X	0.002
Flufenacet-OXA	0	0	<5[d]	<5[d]	-	-	100	100	64.7	92.1	77.2 ± 1.7	-	0.002
Imidacloprid	25	25	<5[d]	121	-	-	0	0	<5[d]	<5[d]	-	-	NA
Isoproturon	0	0	<2.5[d]	<2.5[d]	-	X	0	0	<2.5[d]	<2.5[d]	-	X	NA
Isoproturon-monodemethyl	0	0	<2.5[d]	<2.5[d]	-	-	0	0	<2.5[d]	<2.5[d]	-	X	NA
Metazachlor	0	0	<2.5[d]	<2.5[d]	-	X	23.8	0	<2.5[d]	<5[c]	-	X	NA
Metazachlor-ESA	100	100	23.2	36.9	29.9 ± 5.9	X	100	100	107	145	128 ± 2	X	0.0002
Metazachlor-OXA	100	100	113	150	130 ± 17	X	100	100	187	467	351 ± 14	X	0.002
Prosulfocarb	100	100	14.4	35.9	27.1 ± 9.3	X	100	100	<5[c]	69.8	26.4 ± 3.7	X	0.44
S-Metolachlor	0	0	<2.5[d]	<2.5[d]	-	X	33.3	0	<2.5[d]	<5[c]	-	X	NA
CGA-357704	0	0	<10[d]	<10[d]	-	-	100	0	<20[c]	<20[c]	-	-	NA
Metolachlor-ESA	0	0	<2.5[d]	<2.5[d]	-	NA	100	100	26.8	44.3	33.8 ± 1	NA	0.002
Metolachlor-OXA	0	0	<10[d]	<10[d]	-	-	100	100	49.9	75.4	63.1 ± 1.5	X	0.002
S-metolachlor-NOA	0	0	<25[d]	<25[d]	-	NA	28.6	0	<25[d]	<50[c]	-	NA	NA
Tebuconazole	0	0	<2.5[d]	<2.5[d]	-	X	100	100	10.5	21.6	14.3 ± 0.6	X	0.002
Terbuthylazine	0	0	<2.5[d]	<2.5[d]	-	X	0	0	<2.5[d]	<2.5[d]	-	X	NA
Terbuthylazine-2-hydroxy	100	100	10.9	14.3	12.9 ± 1.4	X	61.9	0	<2.5[d]	<5[c]	-	X	0.0007
Terbuthylazine-desethyl	25	0	<2.5[d]	<5[c]	-	X	9.5	0	<2.5[d]	<5[c]	-	X	NA
Terbuthylazine-desethyl-2-hydroxy	75	0	<2.5[d]	<5[c]	-	X	0	0	<2.5[d]	<2.5[d]	-	X	NA
Thiamethoxam	50	0	<5[d]	<10[c]	-	-	0	0	<5[d]	<5[d]	-	-	NA

In grey: TP, FOD/Q: Frequency of detection/quantification in grab water samples (%), Min: Minimal concentration, Max: Maximal concentration, NA: Not assessed, X: Detected in POCIS, [a] Determined when FOQ = 100%,[b] Significant difference between ALT and CHA obtained only when FOQ = 100% in at least one pond and by substituting concentrations <LOD by NULL and <LOQ by LOQ/2, [c] LOQ in grab water samples, [d] LOD in grab water samples. Compounds never detected in both ponds are not presented. Mean concentrations significantly higher than in the other basin are underlined.

values were reported in a star plot representing the deviation of each biomarker from the reference value. Finally, the absolute values of these indexes were summed to obtain the overall IBRv2 of each site (IBRv2 = Σ |A|).

3. Results

3.1. Occurrence and concentrations of pesticides and TP in ponds

3.1.1. Grab sampling

Table 3 presents all concentrations of compounds detected in water and also the FOD and FOQ. Among the 32 pesticides and 54 TP analyzed in water, 14 were detected in ALT (including 10 TP) vs. 25 (including 17 TP) in CHA (substances with FOD > 0 %). In the same way, if only compounds with FOD = 100 % are considered, only 7 substances were found in ALT (including 6 TP) and 15 in CHA (including 11 TP). The sums of the average concentrations of these contaminants (i.e., the 6 systematically detected in ALT and the 13 systematically quantified in CHA) reached 518 ng·L^{-1} in ALT and 1250 ng·L^{-1} in CHA. In contrast, TP were found to be abundant, constituting 71 % and 68 % of detected molecules in ALT and CHA, respectively. Atrazine-2-hydroxy showed the highest mean (300 ± 37 ng·L^{-1}, in CHA: 51.9 ± 1 ng·L^{-1}) and maximum (343 ng·L^{-1}, in CHA: 61.5 ng·L^{-1}) concentrations in ALT (FOQ = 100 % in ALT and CHA). In CHA, the highest mean (351 ± 14 ng·L^{-1}, in ALT: 130 ± 17 ng·L^{-1}) and maximum (467 ng·L^{-1}, in ALT: 150 ng·L^{-1}) concentrations were determined for metazachlor-OXA (FOQ = 100 %). Concentrations of atrazine-2-hydroxy (*p*-value <0.001), atrazine-desethyl-2-hydroxy (*p*-value <0.001), imidacloprid (no statistical analysis), terbuthylazine-2-hydroxy (*p*-value <0.001) were higher in ALT than in CHA. No significant difference was detected for prosulfocarb. All other quantified contaminants showed a higher concentration in CHA (details are given in Table 3).

3.1.2. Passive sampling

With regards to POCIS, the compounds concentrations were not provided; instead, only the presence-absence of each molecule was indicated. Indeed, even if some studies provide these concentrations in POCIS, given that the accumulation in the phase depends on hydrodynamics and physicochemical factors such as temperature, we consider the concentrations measured in ponds with this tool as qualitative. As an indication, the concentrations measured in the POCIS phase are presented in Supplementary Information (Table A.5).

More substances were detected by POCIS in CHA (n = 24) than in ALT (n = 18). In both sites, detected contaminants by both sampling methods were not systemically similar. For instance, boscalid, chloridazon, isoproturon, isoproturon-monodemethyl, and terbuthylazine were only found using POCIS but never in grab water samples. In contrary, 3,5,6-trichloro-2-pyridinol, alachlor-acetochlor-ESA, atrazine-desethyl-2-hydroxy, dimethachlor-OXA, flufenacet-OXA, imidacloprid, CGA-357704, and thiamethoxam were not detected using POCIS but were already in water samples of ALT and/or CHA. All details results are given in Table 3.

3.1.3. SPP sampling

In SSP, a total of 7 and 23 compounds were detected in ALT and CHA, respectively (Table 4). The highest maximal concentration was quantified for prosulfocarb in both ponds (ALT = 5.06 ng·g^{-1} wet wt., CHA = 6.23 ng·g^{-1} wet wt.). Except for atrazine-2-hydroxy, benzamide,

Table 4
Concentrations (ng.g^{-1} wet wt.) of detected compounds in SSP of ALT and CHA.

Compounds	ALT	CHA
3,5,6-trichloro-2-pyridinol	<0.06[a]	0.162
Atrazine-2-hydroxy	0.9	0.19
Atrazine-desisopropyl-2-hydroxy	0.48	2.01
Boscalid	<0.015[a]	0.1
Chlorotoluron	<0.015[a]	0.65
Dimetachlor-ESA	<0.015[a]	0.06
Dimethenamid	0.08	<0.03[a]
Dimethenamid-ESA	<0.015[a]	0.05
Dimethenamid-OXA	<0.15[a]	0.13
Flufenacet	0.05	0.44
Flufenacet-ESA	<0.015[a]	0.24
Imidacloprid	<0.03[a]	<0.06[b]
Imidacloprid-desnitro	<0.015[a]	0.05
6-Chloronicotinic-acid	<0.015[a]	0.03
Isoproturon	<0.015[a]	0.28
Isoproturon-didesmethyl	<0.015[a]	0.06
Metazachlor	<0.015[a]	0.05
Metazachlor-ESA	<0.06[a]	0.13
Metazachlor-OXA	<0.06[a]	0.26
Prosulfocarb	5.06	6.23
S-Metolachlor	0.04	0.08
Metolachlor-ESA	<0.015[a]	0.07
Tebuconazole	<0.015[a]	1.22
Terbumethon-desethyl	<0.015[a]	<0.03[b]
Terbuthylazine-2-hydroxy	0.08	<0.015[a]
Terbuthylazine-desethyl-2-hydroxy	<0.03[b]	<0.015[a]

In grey: TP, [a] LOD, [b] LOQ. Compounds never detected in both ponds are not presented.

dimethenamid, terbuthylazine-2-hydroxy, and terbuthylazine-desethyl-2-hydroxy all found compounds were at higher concentrations in SSP from CHA.

3.2. Biological analysis

At TF, the mortality rate of *G. aculeatus* was very low with only one dead fish per cage corresponding to 3.3 % of mortality. Organisms did not show external signs of stress nor injury. The Fulton's condition index was significantly reduced in males caged in CHA compared to the control group and males caged in ALT, but this was not observed for female individuals (Fig. A.2). Detailed results for all biomarkers are presented in Table 5.

Several innate immune parameters have presented statistical differences between the studied sites. Leucocyte apoptosis and granulocyte percentage were significantly higher in fish caged at ALT than at CHA or control. Conversely, phagocytic efficiency and lysosomal presence were significantly higher at CHA compared to both ALT and control which were similar. Basal ROS content was significantly highest at CHA, intermediate at ALT, and lowest at the control site. The respiratory burst index was significantly highest in control fish, intermediate in fish from CHA, and lowest in fish from ALT. ROS content after activation was also significantly higher in CHA and lower in ALT than in the control population (Table 5). For oxidative stress parameters in the liver, there were no differences between the ponds. However, fish at ALT and CHA had significantly lower GST activity and total GSH content and significantly higher TBARS content than the control fish (Table 5). Neurotoxicity, as measured by AChE activity was significantly higher in fish from ALT compared to the control, and intermediate in fish from CHA. No difference was detected regarding chromosomal damages (DNA content variation).

Regardless of the fish sex, no statistical difference between the three conditions (control, ALT, and CHA) was found for the leucocyte necrosis, the GPX, SOD, and CAT activities. A non-significant moderate VTG induction was measured in three males in ALT and two in CHA. In the same way, among females, a relatively high VTG induction was observed in seven individuals in ALT and six in CHA. In comparison, no male and only two females presented a VTG induction in the control population (Fig. 2). The SPG concentration in the head kidney was lower in CHA and ALT than in the control population, both in males and females.

The IBRv2 calculation result and the associated star plot are presented in Fig. 3. The IBR index was similar for both sites with 21.5 for ALT and 21.2 for CHA.

Table 5
Biomarker measurements (mean ± SEM, $n = 29–30$, $n_{♂} = n_{♀} = 14–15$).

Biomarkers	Control	ALT	CHA
Innate immune responses			
Leucocyte necrosis (%)	4.2 ± 0.3	5.7 ± 0.4	5.3 ± 0.5
Leucocyte apoptosis (%)	2.7 ± 0.2[a]	3.6 ± 0.3[b]	2.6 ± 0.3[a]
Granulocyte-macrophage subpopulation (%)	40.7 ± 1.5[a]	57.2 ± 1.4[b]	42.8 ± 1.8[a]
Phagocytosis efficiency (%)	12.1 ± 1.3[a]	10.5 ± 0.4[a]	17.7 ± 1.1[b]
Lysosomal presence (MFI)	325.2 ± 14.4[a]	360.9 ± 9.2[a]	468.1 ± 16.6[b]
Basal ROS content (MFI)	9.4 ± 0.7[a]	18.6 ± 2.4[b]	45.4 ± 1.7[c]
ROS content after activation (MFI)	28.3 ± 1.8[b]	16.4 ± 2.4[a]	52.6 ± 2.3[c]
Respiratory burst index (EU)	3.2 ± 0.2[c]	0.9 ± 0.05[b]	1.2 ± 0.03[a]
Oxydative stress			
GST activity (U.g^{-1} of total protein)	8599 ± 406.6[b]	7335.2 ± 282.3[a]	7077.6 ± 434[a]
Total GSH content (μmol.g^{-1} of total protein)	8.4 ± 1.1[b]	4.9 ± 0.8[a]	3.5 ± 0.6[a]
GPx activity ♂ (U.g^{-1} of total protein)	19.9 ± 2.3	17.4 ± 1.1	13.8 ± 1.5
GPx activity ♀ (U.g^{-1} of total protein)	49.5 ± 4.2	43.1 ± 4	40.4 ± 7.3
SOD activity ♂ (U.g^{-1} of total protein)	3857.9 ± 154.5	4109.5 ± 290.3	3926.9 ± 179.6
SOD activity ♀ (U.g^{-1} of total protein)	3740.3 ± 191.3	3268.8 ± 183	3779.8 ± 195.2
CAT activity ♂ (U.g^{-1} of total protein)	416,570 ± 48,729.3	419,095 ± 20,659.8	425,993 ± 57,947.6
CAT activity ♀ (U.g^{-1} of total protein)	929,818 ± 83,123.2	864,306 ± 76,147.7	841,083 ± 100,802.1
TBARS content (nmol.g^{-1} of total protein)	88.1 ± 8.5[a]	105.2 ± 5.2[b]	110.2 ± 5.9[b]
Neurotoxicity			
AChE activity (U.g^{-1} of total protein)	66.1 ± 6.9[a]	87.7 ± 4.4[b]	80 ± 4.4[ab]
Reproductive toxicity			
VTG concentration ♂ (ng. mL^{-1} of blood)	50 ± 0	152.6 ± 55.1	98.1 ± 33
VTG concentration ♀ (ng. mL^{-1} of blood)	411 ± 246.1	836.2 ± 261.8	751.9 ± 263.2
SPG concentration ♂ (U.g^{-1} of total fish weight)	14.9 ± 2.5[c]	0.7 ± 0.4[a]	11.5 ± 4[b]
SPG concentration ♀ (U.g^{-1} of total fish weight)	7.5 ± 1.4[b]	0.6 ± 0.3[a]	6.5 ± 0.8[b]
Genotoxicity			
Chromosomal damages (CV)	2.7 ± 0.2	2 ± 0.1	1.7 ± 0.09

CAT: Catalase, MFI: Mean fluorescence intensity, ROS: Reactive oxygen species, FU: Fluorescence Unit, GST: Glutathione-S-transferase, GSH: Glutathion, GPx: Glutathion-peroxydase, SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances, AChE: Acetylcholinesterase, VTG: Vitellogenin, SPG: Spiggin. Superscript letters indicate statistical difference (p-value <0.05).

4. Discussion

Our work highlighted a multi-contamination of the water and SSP of CHA (conventional agriculture in the watershed). We detected 25 among the 81 molecules analyzed in grab samples (Table 3) and 23

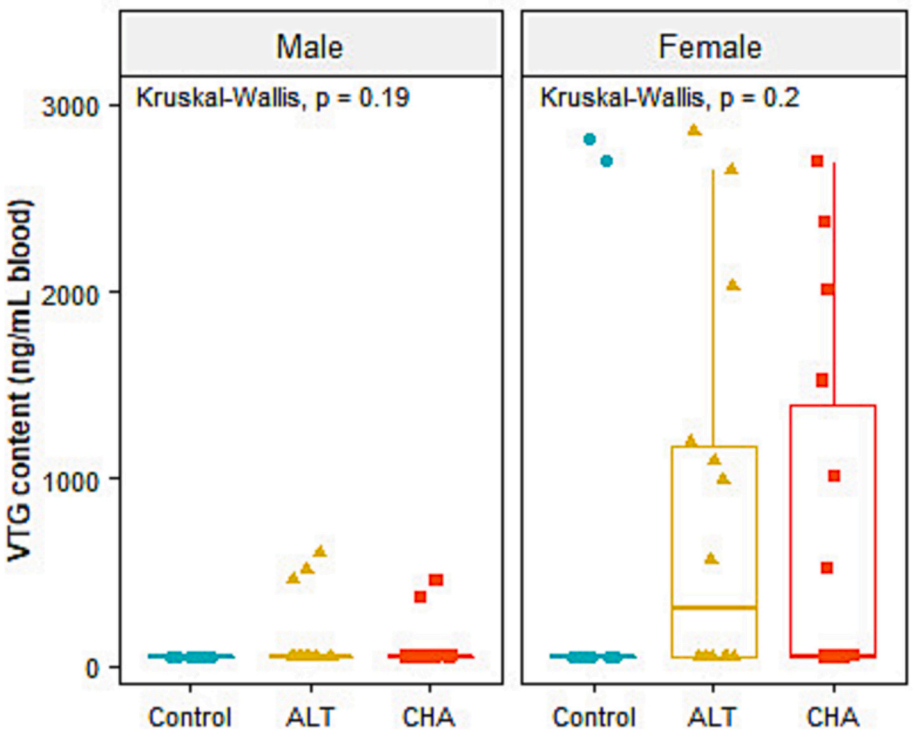

Fig. 2. Vitellogenin (VTG) concentration in male and female sticklebacks. Despite the absence of statistical signification, some male fish have presented an abnormal induction in the circulating VTG in both ponds. In the same way, half of the female fish have presented a high VTG concentration for this season.

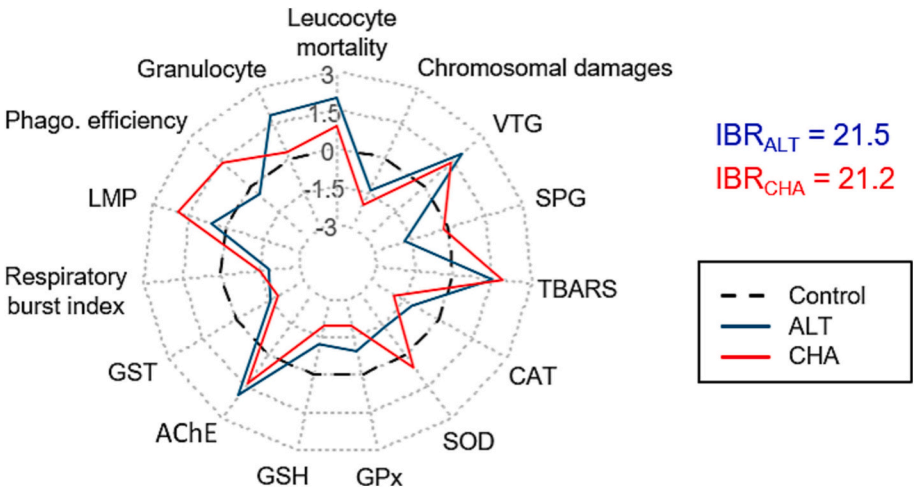

Fig. 3. Result of IBRv2 calculations and star plot of deviation index from the control group. VTG: Vitellogenin; SPG: Spiggin; TBARS: Thiobarbituric acid reactive substances; CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; GSH: total glutathione; AChE: Acetylcholinesterase; GST: glutathione-S-transferase; LMP: Lysosomale presence; Phago. efficiency: Phagocytosis efficiency.

among the 72 in SSP (Table 4). These findings are consistent with the study of Le Cor et al. (2021) which detected simultaneously up to 29 different compounds among 67 analyzed in the water of the river that supply this pond. In contrast, ALT is located on a watershed where no synthetic pesticides have been applied since 2009. Despite this, 14 molecules in grab samples and 7 in SSP were detected (Tables 3 & 4). Several studies have demonstrated airborne transport of pesticides may explain this contamination (Cech et al., 2023; Gavrilescu, 2005; Siebers et al., 2003; Zivan et al., 2016), which can be mitigate by new agricultural practices (e.g., use of nozzles or drift shields, windless application) have led to a reduction in drift phenomena (Prechsl et al., 2022).

The analysis of grab samples of water from ALT revealed permanent contamination throughout the study period (FOD = 100 %) with seven contaminants during the 21-day study, of which six were TP (i.e., TP of atrazine, chlorpyrifos, metazachlor, and terbuthylazine, Table 3). In only two cases (i.e., metazachlor and terbuthylazine), the corresponding parent molecules were detected by POCIS but at concentrations below 2.5 ng·L^{-1} (LOD in grab samples). Contamination by TP rather than parent molecules could reflect past contaminations, consistent with the fact that no pesticide has been applied upstream of the pond since 2009. Moreover, the molecules found in the highest concentrations in ALT are an insecticide (i.e., imidacloprid) and TP of pesticides widely used in agriculture in France until their ban. This encompasses two TP of atrazine and terbuthylazine-desethyl. These compounds are categorized as

persistent or even very persistent (Chiaia-Hernandez et al., 2017; Lewis et al., 2016; Neuwirthová et al., 2018). In their study, Riedo et al. (2021) observed the occurrence of pesticides and TP in soil from organically managed zones even 20 years after the cessation of pesticide application. Our findings showed that contaminants can also remain for years within aquatic non-target ecosystems, even after changes in land use practices within their catchment areas. Although fewer contaminants were detected in this zone, it is likely that the detoxification of soil and the aquatic environment requires an extended period to achieve (Schrack et al., 2009). This underscores the fact that the process of decontamination through changes in pesticide usage is successful, but it is a long road requiring efforts on a large geographical scale.

Prosulfocarb has also been detected in 100 % of the grab water samples and SSP in both ponds (Tables 3 & 4). This herbicide is frequently used in France (Devault et al., 2019). Organic farmers have raised concerns about this compound due to its tendency to cause contamination of untreated crops, sometimes several kilometers away from the application area. Anses has implemented regulatation for its use (i.e., anti-drift nozzle, treatment at >500 m from a neighboring crop, time restrictions for use under high humidity conditions). However, prosulfocarb remains volatile after application, once deposited on the crop. Prosulfocarb is subject to long-distance drift because of its high vapor pressure (Benzing et al., 2021). Contamination of different crops by prosulfocarb was observed in Germany and has been quoted to demonstrate the ubiquity of pesticides even in untreated fields (Rombach et al., 2020). A previous study conducted in our lab showed that this compound accumulates also in fish (Slaby et al., 2022). These findings further emphasize the need to pay particular attention to this herbicide and the risks it poses to humans and ecosystems. Prosulfocarb was analyzed but due to the cost of the analytical standard (i.e., > 90,000 € per gram in 2022) it was impossible to assess the contamination by its main TP, the prosulfocarb sulfoxide. Access to the analytical standard is also an obstacle to the acquisition of knowledge on the environmental fate of pesticides and in particular their TP.

TP represented a substantial proportion of systematically detected contaminants. Moreover, they were generally quantified at concentrations higher than their parent molecules, both in water and in SSP (Tables 3 & 4). For instance, metazachlor was either not detected or found below the LOQ, but its two main TP, metazachlor-ESA and metazachlor-OXA, were quantified in 100 % of grab water samples from both pond, reaching average concentrations of up to 351 $ng{\cdot}L^{-1}$ (metazachlor-OXA in CHA, Table 3). In the same way, the concentration of metazachlor in the SSP sample from CHA was approximately three and five times lower than those of metazachlor-ESA and metazachlor-OXA, respectively (Table 4). High detection frequency and worrying concentrations of metazachlor TP were also measured in small lentic water bodies located in agricultural areas in Northern Germany (Ulrich et al., 2022). Similarly, atrazine, which was banned since 2003 in France, was not detected while its TP were found in both ponds in water and SSP. Among them, the highest average concentration (300 $ng{\cdot}L^{-1}$) was observed for atrazine-2-hydroxy in ALT pond. The herbicide S-metolachlor, widely used on maize crops, was never quantified in pond water. However, metolachlor-OXA and metolachlor-ESA were consistently quantified in CHA. Limited toxicological data are available for these TP. Until February 2021, metolachlor-ESA was classified as a relevant metabolite by Anses due to the absence of data on its potential genotoxicity (Anses, 2019). In 2022, Syngenta provided the public authorities with new studies which concluded that there was no genotoxicity for metolachlor-ESA and S-metolachlor-NOA. Consequently, Anses re-evaluated these two metabolites as irrelevant in September 2022 (Anses, 2022b). Further data were expected during 2023 regarding S-metolachlor, which was under re-evaluated at the European level for reauthorization. On February 2023, due to the high occurrence and concentrations of three of its TP (metolachlor-ESA, metolachlor-NOA, and S-metolachlor-OXA) in groundwater, Anses decided to suspend the main uses of S-metolachlor (Anses, 2023). The conclusions released by the European Food Safety Authority (EFSA) supported the French decision. This scenario highlights the crucial need for toxicological and ecotoxicological data on pesticide TP, enabling regulatory agencies to make promptly the right decisions necessary for human and ecosystem safety.

Out of the 86 analyzed molecules, 14 and 25 were detected in grab samples of ALT and CHA, respectively (Table 3). The deployment of POCIS in the ponds during the study revealed the presence of 10 additional contaminants in ALT (i.e., bentazon, chloridazon, chlortoluron, dimetachlor, dimethenamid, isoproturon, metazachlor, S-metolachlor tebuconazole, and terbuthylazine) and 7 additional contaminants in CHA (i.e., bentazone, boscalid, chloridazon, isoproturon, isoproturon-monodemethyl, terbuthylazine, and terbuthylazine-desethyl-2-hydroxy). When both sampling methods were combined, 24 contaminants were detected in ALT and 31 in CHA. These substances detected by POCIS and never detected in water analyses were predominantly pesticides (only two TP) at trace concentrations below the LOD of grab water analytical methods. However, their effects at low concentrations and in mixture are unknown. In this context, a comprehensive analysis of the biotic compartment is essential to accurately assess risks to aquatic ecosystems.

Passive samplers have proven to be effective integrative tools for monitoring in streams (Corcoran et al., 2020; Gallé et al., 2020; Metcalfe et al., 2019; van Metre et al., 2017; Yabuki et al., 2018) and lentic water bodies (Satiroff et al., 2021). These tools are valuable for detecting contaminants that might remain undetected by traditional water testing methods. However, they are not intended to replace classical water analysis; rather, they serve as a complementary approach to enhance the accuracy of environmental monitoring. Indeed, among the 15 substances detected in all grab water samples in CHA (i.e., FOD = 100 %, Table 3), two of them (flufenacet-OXA and CGA-357704) were never detected by POCIS. Similarly, 2 compounds were detected in all grab samples but not in POCIS from ALT (i.e., 3,5,6-trichloro-2-pyridinol and atrazine-desethyl-2-hydroxy). The use of several analytical tools to assess the occurrence of contaminants brings real interest. Since the efficiency of analytical methods depends on the physicochemical properties of the compounds, it allows to better describe the actual contamination profile of an ecosystem. This finding aligns with previous research conducted in other aquatic environments (Bernard et al., 2019; Satiroff et al., 2021), emphasizing the complementary roles of both active and passive sampling methods in monitoring small lentic water bodies.

In parallel to chemical analysis, the environmental quality was determined with the use of several biomarkers measured in *G. aculeatus*. Above, we concluded that CHA was more contaminated than ALT (Tables 3 & 4). These results are consistent with the different land uses in their respective catchments (Table 2). The observation that male fish exhibited a lower Fulton's condition index in CHA than in ALT, suggesting a negative impact on the health of fish, was consistent with the findings from chemical analyses. However, when biomarker assessments are globally considered using the IBRv2, no clear difference between both sites could be underlined, and ALT appeared to have a slightly lower environmental quality than CHA (Fig. 3). However, separately, biomarkers revealed variations between both sites either to the advantage of CHA; or to the advantage of ALT. It could be explained by the fact that, despite the organic management of the watershed and its strong proportion of forest, ALT might be contaminated because of indirect contaminations from other conventionally managed fields and/or a legacy from historical pesticide use in the catchment. Also, the duration of exposure (21 d) could not be sufficient to reveal clear differences between sites using biomarkers. At last, it is important to remind that while 82 compounds were included in the study, they did not represent all the compounds potentially present in the environment. Thus, it is complicated to make a direct link between the chemical status and the biomarkers responses.

Some immune parameters exhibited differences between the two

ponds. Leucocyte mortality was higher in fish caged in ALT than in CHA and in the control site. This mortality was mainly driven by leucocyte apoptosis (Table 5) which is considered a normal mode of cell death. Additionally, a modification of leucocyte distribution was observed in ALT, with an increase in the percentage of granulocyte-macrophage subpopulation related to increased apoptosis in the lymphocyte subpopulation. This hypothesis was supported by the absence of stimulation phagocytosis activity in this station. In another way, activation of each other immune parameters was notified in CHA compared to ALT and the control suggesting the presence of a pathogen in this site (Table 5). In fact, the phagocytosis efficiency is an indicator of the ability of granulocytes to internalize pathogens, and the presence of lysosomes indicates the ability of these granulocytes to degrade these pathogens by enzymatic pathway after internalization. The induction of these two parameters may suggest an activation of the immune system, perhaps related to the presence of a pathogen in the environment. Even though the respiratory burst was not really high (lesser than in the control condition), the high basal ROS content suggests either oxidative stress or respiratory burst activation.

Regarding oxidative stress, a significant increase in the membrane lipoperoxidation (TBARS content) and a significant decrease in GST activity and total GSH in the liver were highlighted in both ponds compared to the control site (Table 5). Other parameters (GPx, SOD and CAT activities) did not differ significantly from the control site and between studied sites. As reviewed by Lushchak (2016) and Slaninova et al. (2009), such effects have been observed in fish exposed to pesticides and TP. A field study also reported a decrease in GSH content and an increase in lipid peroxidation associated with an inhibition of CAT and GPx activities in the liver and the adrenal gland in *Catostomus commersoni* sampled in the Yamaska River (Canada) in which various pesticides were quantified including dimethenamid and metolachlor at the minimal quantified concentrations of 40 and 330 $ng{\cdot}L^{-1}$, respectively (Dorval et al., 2005). Both of these pesticides were also detected in ALT and CHA but at lower concentrations (Table 3). In a rice-fish system in Brazil, which was contaminated with multiple pesticides including tebuconazole and thiamethoxam (minimal concentration: 1000 $ng{\cdot}L^{-1}$), an induction of lipid peroxidation was also found in *Cyprinus carpio* with the difference that it was followed by an increase in CAT and GST activities (Clasen et al., 2018). The concentrations were largely higher than those found in ALT and CHA (Table 3). However, such results are worrying as ALT and CHA are both intended for the production of various fish, notably *C. carpio*. Moreover, a previous work conducted in CHA at the same period as the present study demonstrated the accumulation of tebuconazole (along with prosulfocarb and benzamide) in this fish species (Slaby et al., 2022).

The AChE activity in fish is known to be inhibited by exposure to various compounds, including organophosphorus and carbamates, as well as neonicotinoids, pyrethroids, or organochlorines, for example (Santana et al., 2021). In the present study, this biomarker value was significantly higher in ALT compared to the control condition (Table 5). Two main reasons could lead to this difference between sites. First, the presence during the study period of non-monitored compounds with the ability to inhibit AChE activity in the control site cannot be entirely excluded. Conversely, compounds (whether included in our list of analytes or not) present in ALT might be responsible for the induction. Indeed, it has been proven in fish that certain polycyclic aromatic hydrocarbons can lead to an increase in AChE activity (Olivares-Rubio and Espinosa-Aguirre, 2021).

Although the VTG concentrations were not significantly different between the control individuals and those exposed in ponds, it is remarkable that the concentrations in the latter group are nearly twice as high as those in the controls. Indeed, it is important to note that a variation in VTG concentration is never expected in males and should remain stable in females outside of the breeding season. While these increases are not significant and should not be considered as a confirmed effect of contamination, they are abnormal enough to be reported as they could reflect exposure to estrogen-like endocrine disrupting chemicals (Sanchez and Porcher, 2009). Boscalid (0.1 and 1 $mg{\cdot}L^{-1}$ for 21 d, Qian et al., 2020) and tebuconazole (230 $\mu g{\cdot}L^{-1}$ for 7 and 14 d, Sancho et al., 2010) were recognized to induce a VTG in plasma of *Danio rerio* males in laboratory conditions (Qian et al., 2020; Sancho et al., 2010). Boscalid was only found in CHA (Tables 4 & 5), and tebuconazole was found in both ponds using POCIS but at a stronger concentration in CHA (Tables 3, 4 & 5). In *Gobiocypris rarus* exposed to thiamethoxam (0.5–50 $\mu g{\cdot}L^{-1}$ for 90 d), a slight increase but not significant in the VTG content in plasma was observed for both females and males (Zhu et al., 2019). The authors also measured variations in VTG mRNA expression in the testis, the ovary, and the liver of both sexes. Inversely, thiamethoxam (up to 12.3 $mg{\cdot}L^{-1}$ for 96 h) did not impact VTG content in *D. rerio* (Shen et al., 2021). During our study, thiamethoxam was not detected in CHA but was detected in 50 % of the water samples collected in ALT at low concentrations ($< 10\ ng{\cdot}L^{-1}$). At last, special attention can be given to atrazine TP found in both ponds (atrazine-2-hydroxy and atrazine-desisopropyl-2-hydroxy, Tables 2-4). This herbicide banned in European Union since 2003 (Sass and Colangelo, 2006), is still widely used in several parts of the world such as in Brazil (Tonelli Fernandes et al., 2018), in China (Liu et al., 2020) and in the United States (Mahler et al., 2017). Atrazine is recognized as a potent endocrine disruptor and such effects were observed in several aquatic species such as fish (Vasanth et al., 2015) and amphibians (Slaby et al., 2019). As some TP of this herbicide are occurring, the fish could be exposed to it at a concentration level below our limit of detection (LOD = 0.1 $ng{\cdot}L^{-1}$, Table A.2). Especially considering that atrazine was detected in the upstream rivers of CHA in another study conducted over all the fishpond production cycle (Le Cor et al., 2021) but not inside the pond (Slaby et al., 2022). The possibility of other estrogen-like endocrine-disrupting chemicals, not included in our analyte list, cannot be ruled out.

Depending on environmental conditions, SPG can be a marker of androgenicity (Sanchez et al., 2008a) or anti-androgenicity (Katsiadaki et al., 2012). In our study, SPG concentration decreased significantly in ALT compared to the control site (females) and CHA (males and females), but also in CHA in comparison to the control site (males). As the study was carried out outside the breeding season and involved short exposure times, interpretation is difficult. However, the presence of substances with anti-androgenic properties at the site of ALT can be suspected. The lack of effect at CHA can be explained either by the absence of pollutants interfering with androgen signaling or, more probably, by the presence of the mixture of substances interfering with the (anti)-androgen response.

5. Conclusions

Our work aimed to compare two small lentic water bodies through the application of chemical and ecotoxicological tools. The main findings revealed the presence of several pesticides and TP in both ponds, with a notably higher occurrence in the pond situated within the watershed managed under conventional agriculture. In the catchment principally consisting of forests and pastures and supporting organic agriculture, the results showed that despite farmers' efforts, a legacy of past contaminations and indirect pollution from other catchments resulted in the presence of various compounds, particularly TP. It seems of major importance to regulate the usage of certain pesticides (e.g., prosulfocarb, metazachlor) regarding their occurrence or the persistence of their TP, or at least to propose new efficient solutions to mitigate the transfer of contaminants to non-target areas.

However, the biomarkers did not reveal a higher disturbance in CHA, despite the detection of a greater number through chemical analyses. This additional result provided by the biological analysis suggested that in both ponds, the exposure conditions of the organisms were comparable and potentially influenced by the presence of historical and current contaminants. However, it is also possible that impacts on biomarkers could be induced by other contaminants or physicochemical parameters

not monitored in this study. This underscores that relying exclusively on analytical methods to evaluate the environmental quality of an ecosystem might be insufficient. Effect-based methods coupled with chemical analysis appear necessary to achieve a more precise assessment of the environmental quality of wetlands like ponds.

CRediT authorship contribution statement

Sylvain Slaby: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Audrey Catteau:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **François Le Cor:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Amélie Cant:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Vincent Dufour:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Alain Iurétig:** Investigation, Resources, Writing – review & editing. **Cyril Turiès:** Methodology, Investigation, Resources, Writing – review & editing. **Olivier Palluel:** Methodology, Investigation, Writing – review & editing. **Anne Bado-Nilles:** Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration. **Marc Bonnard:** Methodology, Writing – review & editing, Supervision, Project administration. **Olivier Cardoso:** Writing – review & editing, Funding acquisition. **Xavier Dauchy:** Methodology, Resources, Writing – review & editing, Supervision, Project administration. **Jean-Marc Porcher:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration. **Damien Banas:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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