Ecotoxicology and Environmental Safety 205 (2020) 111367

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Tissues injury and pathological changes in *Hyla intermedia* juveniles after chronic larval exposure to tebuconazole

Ilaria Bernabò *, Antonello Guardia, Rachele Macirella, Settimio Sesti, Sandro Tripepi, Elvira Brunelli **

Department of Biology, Ecology and Earth Science, University of Calabria, Via P. Bucci 4/B, 87036, Rende, Cosenza, Italy

ARTICLE INFO

Keywords:
Amphibians
Fungicide
Tebuconazole
Hepatotoxicity
Nephrotoxicity
Gonadal differentiation

ABSTRACT

Tebuconazole (TBZ), an azole pesticide, is one of the most frequently detected fungicides in surface water. Despite its harmful effects, mainly related to endocrine disturbance, the consequences of TBZ exposure in amphibians remain poorly understood. Here, we investigated the adverse and delayed effects of TBZ chronic exposure on a native anuran species, often inhabiting cultivated areas, the Italian tree frog (*Hyla intermedia*). To disclose the multiple mechanisms of action through which TBZ exerts its toxicity we exposed tadpoles over the whole larval period to two sublethal TBZ concentrations (5 and 50 μg/L), and we evaluated histological alterations in three target organs highly susceptible to xenobiotics: liver, kidney, and gonads. We also assessed morphometric and gravimetric parameters: snout-vent length (SVL), body mass (BM), liver somatic index (LSI), and gonad-mesonephros complex index (GMCI) and determined sex ratio, gonadal development, and differentiation. Our results show that TBZ induces irreversible effects on multiple target organs in *H. intermedia*, exerting its harmful effects through several pathological pathways, including a massive inflammatory response. Moreover, TBZ markedly affects sexual differentiation also by inducing the appearance of sexually undetermined individuals and a general delay of germ cell maturation. Given the paucity of data on the effects of TBZ in amphibians, our results will contribute to a better understanding of the environmental risk posed by this fungicide to the most endangered group of vertebrates.

1. Introduction

Alterations of natural aquatic environments may severely impact both humans and wildlife, representing an issue of great public concern both in high-income and developing countries (Brühl and Zaller, 2019; Kumwimba et al., 2018; Stehle and Schulz, 2018). In recent years, the nonpoint-source pollution (NPS) in agricultural areas has increased owing to poor domestic wastewater management and the application of plant protection products (PPPs) (Davey et al., 2020) leading to the implementation of regulatory programs that restrict pesticide use at a global scale (Directive 2000/60/EC; Directive 2009/128/EC). Despite the growing need to bring together environmental protection and economic demand, PPPs remain among the major stressors for aquatic environments at continental-scale (EEA, 2018; Herrero-Hernández et al., 2013, 2016).

In the European Union (EU), fungicides accounted for more than 40% of the total pesticide sales over the whole period 2011–2018, and their use is forecasted to intensify in the next years (Eurostat, 2019). In the past, fungicides have received little attention compared with other PPPs, and the environmental hazards posed by this class of pesticides have not been adequately addressed (Zubrod et al., 2019). Besides, models used to derive predicted concentrations of fungicides could have underestimated the extent of their presence in water bodies (Morselli et al., 2018) and nowadays, fungicides are among the most frequently detected pesticides in freshwater ecosystems (Casado et al., 2019; ISPRA, 2018; Lefrancq et al., 2017).

Furthermore, as a result of frequent applications, low dilution potential, and persistence, fungicides may be present at elevated concentrations and for an extended period, especially in water bodies of reduced dimension (Le et al., 2017). In agricultural landscapes, small aquatic ecosystems represent important breeding sites for many amphibian species, and this may result in a chronic exposure during

* Corresponding author.
** Corresponding author.
E-mail addresses: ilaria.bernabo@unical.it (I. Bernabò), elvira.brunelli@unical.it (E. Brunelli).

https://doi.org/10.1016/j.ecoenv.2020.111367
Received 7 July 2020; Received in revised form 24 August 2020; Accepted 13 September 2020
Available online 21 September 2020

sensitive periods of embryonal and larval development (Wagner et al., 2016). Although there is no single cause responsible for the global amphibian decline, agricultural practices and anthropogenic pollution significantly contributed as a primary factor or interacting with other stressors (Hayes et al., 2010; Swanson et al., 2018), and an estimated 41% of amphibian species are currently listed as threatened with extinction (IUCN, 2020).

Tebuconazole (TBZ), a broad-spectrum azole pesticide, is the most frequent fungicide identified in surface waters and soils worldwide (de Souza et al., 2020; Silva et al., 2019) and is among the ten substances at the highest risk of inducing adverse effects in aquatic ecosystems (Zubrod et al., 2019). Due to its low soil solubility and high surface flow capacity (Silva et al., 2019), TBZ easily enters water bodies where it reaches concentrations ranging from 0.02 μg/L up to 81 μg/L, during outflow events (Bundschuh et al., 2016; Casado et al., 2019; Herrero-Hernández et al., 2013, 2016; Lefrancq et al., 2017).

Like other azoles, the interaction with the CYP51 enzyme, responsible for the biosynthesis of the ergosterol (an essential component of the fungal cell membrane), represents the mode of action through which TBZ exerts its antifungal activity (Zarn et al., 2003). Unfortunately, TBZ also affects many nontargeted cytochrome P450-dependent enzymes (e.g., CYP19 and CYP1A) interfering with steroidogenesis and xenobiotic detoxification in both mammals and aquatic species (e.g., fish) (Creusot et al., 2020; Matthiessen and Weltje, 2015). It has also been shown that exposure to TBZ might alter steroid hormone biosynthesis (Sanderson, 2006) and the thyroid system in mammals and fish (Li et al., 2019a; Lv et al., 2017). TBZ, therefore, can behave as an endocrine disruptor via several different mechanisms operating simultaneously, and it may affect survival, development, growth, reproduction, and behaviour of non-target organisms (Altenhofen et al., 2017; Creusot et al., 2020; Matthiessen and Weltje, 2015; Perez-Rodriguez et al., 2019).

Many detrimental effects have been reported in fish including bioaccumulation, oxidative stress induction (Clasen et al., 2018; Ferreira et al., 2010; Li et al., 2020; Toni et al., 2011a,b), genotoxicity (Castro et al., 2018), liver injuries (Ferreira et al., 2010; Li et al., 2020; Toni et al., 2011a,b), disturbance of respiratory and osmoregulatory gills functions (Macirella et al., 2019), and metabolic injuries (Li et al., 2020; Sancho et al., 2010; Toni et al., 2011a) while there is a paucity of information on the effects of TBZ on other aquatic vertebrates.

To date, TBZ toxicity mechanisms and its potential to induce both endocrine and nonendocrine effects have not been thoroughly investigated in amphibians. A few previous studies have shown that TBZ induces developmental toxicity in both embryos and larvae (Li et al., 2016; Wrubleswski et al., 2018) also affecting survival, development, and metamorphic traits (Bernabò et al., 2016) whereas available studies on adults mainly focused on bioaccumulation and steroidogenesis disruption (Battaglin et al., 2016; Hansen et al., 2014; Poulsen et al., 2015; Smalling et al., 2013). Furthermore, all these research deal with a small number of laboratory model species, thus introducing significant uncertainty in the predictive value of these results when assessing risks associated with exposure to TBZ in more sensitive native species.

Our study, taking a model the juvenile life stage of Italian Tree Frog (*Hyla intermedia*), will show the morphological and histological modifications resulting from exposure, over the whole larval development, to two environmentally relevant concentrations (5 and 50 μg/L) of TBZ.

The multiple mechanisms of action through which TBZ exerts its detrimental effects have been investigated using a multiorgan approach. We first evaluated several morphometric and gravimetric parameters. Then, in order to disclose the tissue-specific alterations, we investigated histological changes in three target organs highly susceptible to xenobiotic toxicity, liver, kidney, and gonads, also determining sex ratio, gonadal development, and differentiation. Given the scarcity of information on the effects of this fungicide in amphibians, the results presented here will provide a comprehensive overview of TBZ chronic toxicity in amphibians. To the best of our knowledge, no previous studies have investigated the effects of tebuconazole on these endpoints in amphibians.

2. Materials and methods

The experimental design and exposure conditions (selection of the dose, range-finding test, preparation of test solutions, and chemical analysis) have been previously reported in detail (Bernabò et al., 2016). Only a concise description will be given here.

2.1. Animal collection, experimental design and exposure conditions

H. intermedia egg masses (n = 10), freshly-laid, have been collected from natural ponds with no pesticide input (Calabria, Southern Italy; 39°21′36″N 16°9′3″E; elevation 387 m a.s.l.). In the laboratory, eggs were kept in 40 L glass aquaria (60 × 35 × 30 cm), filled with aerated and aged tap water, and renewed twice a week. The experiments started when tadpoles reached Gosner stage 25 (Gosner, 1960; feeding and free-swimming tadpoles). For each experimental unit, including control, tadpoles of comparable body dimension (n = 20) were assigned to 15 L aerated glass tanks (40 × 25 × 20 cm) filled with the appropriate test solution for 78 days (i.e., Gosner stage 46: end of metamorphosis and complete tail resorption). Each treatment was conducted in quadruplicate.

Two different realistic concentrations, at different orders of magnitude, were tested as a probabilistic exposure scenario that amphibians might encounter in aquatic habitats (5 μg/L is comparable to those detected in surface waters, whereas 50 μg/L represents peak contamination event).

The test solution was prepared by dissolving TBZ (purity 99.5%, Cas No: 107534-96-3, Sigma-Aldrich Chemie, Steinheim, Germany) in acetone (100 μL), and then diluting in dechlorinated tap water to reach the following nominal concentrations: 5 μg/L and 50 μg/L, referred as the low and the high respectively.

The control group was maintained in dechlorinated tap water and added with the same amount of acetone; therefore, acetone concentration in all treatment tanks was 9.3×10^{-5} mL in all treatment tanks. Since TBZ is stable in water and its levels remain relatively constant for at least one week (Bernabò et al., 2016), the water volume was renewed every seven days.

The analytical verifications of the actual concentrations were performed by an independent accredited analytical laboratory (Delvit Chimica, Cosenza, Italy) using gas chromatography with a nitrogen-phosphorus detector (GC-NPD) following the water analytical method APAT CNR IRSA 5060 Man. 29/03. The chemical analysis was undertaken to verify TBZ's actual concentrations and dispersion before and immediately after complete water renewal. A water sample was collected from a randomly selected tank (one of four tanks) for each treatment, before and after complete water renewal on day 0, 7, 42, and 49 of exposure (supplementary Table S1).

Animals were reared at a constant temperature (22 ± 1 °C), under a photoperiod of 15 L:9D; water quality parameters, in all holding aquaria, were regularly measured with a handheld multi-parameter PCE-PHD 1 (PCE InstrumentsUK Ltd.) and maintained constant (median pH 7.3, conductivity 300 mS/cm, dissolved oxygen 8 ± 1 mg/L, and hardness 180 mg). Tadpoles were fed every three days with boiled organic spinach, and food waste and debris were daily removed. At the beginning of metamorphic climax (Gosner stage 42), animals were moved into semi-aquatic tanks containing a thin layer of respective treatment water and dry areas to complete metamorphosis; throughout this period, animals were not fed. Mortality and completion of tail resorption in each experimental unit were checked at least once a day. Each newly metamorphosed (with a unique identification number) was housed for one week, singularly, in small plastic terrariums (18.2 × 11.5 × 14 cm) with a moist substrate, and fed *ad libitum* with *Drosophila melanogaster*.

2.2. Body measurements, histomorphology, and sex identification

Following one week, a sub-sample of froglets from the control group (n = 30) and all surviving individuals from the exposed group were euthanised by immersion in a solution of 0.1% tricaine methanesulfonate (MS-222, Sigma-Aldrich Chemicals Co., St. Louis, MO), and snout-vent length (SVL) and body mass (BM) were measured.

Preliminary sex identification was performed under a stereomicroscope (Leica MZ APO, Leica Microsystems, Wetzlar, Germany equipped with Canon camera), using a drop of Bouin's solution to enhance coloration; metamorphs were assigned to the phenotypic sex.

Target-organs were quickly excised and weighted in an analytical precision scale (0.01 mg) (Mettler Toledo AB 265-S); due to the small size of the gonadal tissue, the gonad-mesonephros was removed as a complex (GMC). Samples were immersed in Bouin's solution for 24 h at 4 °C, dehydrated in ethanol, cleared in xylene, embedded in paraffin wax, and cut at 7 μm. For the liver, step sections were taken at 50 μm intervals until the maximum diameter of the samples was attained. For GMC, serial sections from the dorsal, middle, and ventral regions were taken. All samples were stained with hematoxylin-eosin (Panreac, Barcelona, Spain), and slides were coded.

Qualitative and semiquantitative histological analyses were conducted blind by a light microscope (LM) equipped with a digital camera (Leica DME, ICC50 HD, Leica Microsystems, Wetzlar, Germany). For each individual (control n = 30, TBZ Low = 19, TBZ high = 13), three nonsequential sections were analysed. Each section has been fully observed using increasing magnification (20×, 40×, 100×) to disclose any histopathological changes. The degree and extent of alterations were evaluated semi-quantitatively by using the grading system, according to OECD (2015), appropriately modified. Grading system was: 0 = no pathological alterations in any field of the section, grade 1 = mild changes present in <25% of the section, grade 2 = moderate changes present in ≥25% but <75% of section; grade 3 = severe pathological alterations present in ≥75% of sections.

2.3. Immunohistochemistry

Deparaffinised sections of 7 μm were subjected to the indirect immunofluorescence technique (Coons et al., 1955). The slides were washed in phosphate-buffered saline (PBS, pH 7.4), and incubated for 10 min in a moist chamber with 20% normal goat serum to block non-specific sites. Unwashed sections were incubated overnight at 4 °C with a mouse monoclonal antibody to the inducible Nitric Oxide Synthase (iNOS; Sigma-Aldrich Chemical Co; working dilutions 1:100) or rabbit polyclonal antibody to Caspase 3 (Sigma-Aldrich Chemical Co; working dilutions 1:100). After several washes in PBS, the slides were incubated for 30 min at room temperature in the dark with the appropriate secondary antibody, fluorescein isothiocyanate-conjugated (goat anti-mouse or sheep anti-rabbit working dilutions 1:100, Sigma-Aldrich Chemical Co). The primary antiserum was substituted with non-immune goat/sheep serum at a dilution of 1:100 in PBS in the control sections to verify immunolabeling specificity. After several washes in PBS, the slides were counterstained with propidium iodide (1:200, Sigma-Aldrich), which labels cell nuclei. For each tested antibody, 10 immunolabeled slides for each experimental group (control, low, and high TBZ concentration) were randomly chosen, observed under a Leica TCS SP2 Confocal Laser Scanning Microscope.

2.4. Data analysis

All data were analysed using Graph Pad Prism 8.00 (GraphPad Software Inc., San Diego, CA) with a criterion for significance set at $P < 0.05$. Since no tank effect was detected, data from the four replicates were pooled into one data set per treatment group. Before analysis, assumptions of normality and homogeneity of variance have been tested with Shapiro-Wilks and Barlett's tests, respectively. Data were not normally distributed, and the Kruskal–Wallis test was performed for all variables.

For morphometric analysis, liver somatic index (LSI; [liver mass/body mass] × 100), and gonad-mesonephros complex index (GMCI; [GMC mass/body mass] × 100) were calculated.

Significant differences in morphometric parameters (SVL, BM, LSI, and GMCI) of juveniles among treatments were analysed using the Kruskal-Wallis test (KW), followed by Dunn's Multiple Comparison post hoc test to compare fungicide exposure groups with the control. Based on information reported by field monitoring studies, detected TBZ concentrations in freshwater ecosystems show a considerable variation depending, for example, on entry routes. Therefore, two different realistic concentrations, at different orders of magnitude were tested as a probabilistic exposure scenario in aquatic habitats (5 μg/L is comparable to those detected in surface waters, whereas 50 μg/L represents peak contamination event).

The sex ratio of froglets was determined based on both gonadal gross morphology and histology; because the genotypic sex of individuals considered undetermined/atypical was not known, this condition was categorised as sex distinct from normal males or females for sex ratio analysis. Sex ratio and deviation from the expected 50:50 sex ratio were calculated using the Chi-square test; two-tailed Fisher's exact tests were also performed for pairwise comparison between control and exposed groups.

Two-way ANOVA analysis has been conducted to test putative interactions between sex and gravimetric parameters (SVL, BM, LSI, and GMCI) in each experimental group. Post-hoc comparisons were performed with Tukey's multiple comparisons test.

3. Results

3.1. Survival, sex ratio, and gravimetric parameters

Survival was assessed from tadpole until the end of the experiment, one week after the completion of the metamorphosis, and TBZ exposure resulted in high mortality in a concentration-dependent manner during the metamorphic climax (supplementary Table S2). After one week of post-metamorphic development, no mortality was recorded in the control group, whereas low mortality was recorded in the TBZ exposed groups; abdominal edema was detected in one individual for each fungicide group (Table 1). No overall difference in SVL was detected among groups (KW = 1.39, $P = 0.497$); despite this, the froglets belonging to fungicide treatments were significantly heavier than the specimens from the control (KW = 12.72, $P < 0.001$ and $P = 0.046$, in the low and high TBZ concentration, respectively) (Table 1). Compared to control, no significant differences were detected in LSI (KW = 5.5, $P =$

Table 1
Summary table of evaluated endpoints and morphometrics measured.

Endpoint	Treatment		
	Control	TBZ Low	TBZ High
Percentage of one week surviving froglets	86.2 ($n = 69$)	33.8 ($n = 27$)	21.2 ($n = 17$)
Morphological abnormalities (%)	0	3.7	5.9
SVL (mm)	15.1 ± 1 ($n = 30$)	15.2 ± 1.3 ($n = 27$)	14.8 ± 0.8 ($n = 17$)
BM (g)	0.37 ± 0.02 ($n = 30$)	0.41 ± 0.06 ($n = 27$)**	0.39 ± 0.03 ($n = 17$)*
LSI	3.42 ± 1 ($n = 30$)	4.00 ± 0.9 ($n = 27$)	3.95 ± 0.9 ($n = 17$)
GMCI	3.51 ± 0.9 ($n = 30$)	3.78 ± 0.9 ($n = 27$)	3.42 ± 1 ($n = 17$)

SVL, snout-vent-length; BM, body mass; LSI, liver somatic index; GMCI, gonad-mesonephros complex index. The asterisk indicates a significant difference from the control, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test (*$P < 0.05$; **$P < 0.005$). Values are reported as means ± SD.

0.064) and GMCI (KW = 2.62, all $P > 0.05$) (Table 1).

Based on morphological and histological observations (see also below), we determined the sex ratio in all groups (Fig. 1). In the control group, the sex ratio (53% females and 47% males) was no significantly different from the theoretical expected 50:50 sex ratio (Chi-square test, $P > 0.05$).

In samples exposed to low and high TBZ concentrations, we revealed a percentage of 42% of females and 37% of males and 38.5% females and 23% males, respectively along with the appearance of individuals sexually undetermined (21%, and 38.5%) statistically significant in both groups (Fisher's test, $P = 0.018$ and $P = 0.001$ in the low and high concentration, respectively) (Fig. 1).

A two-way ANOVA analysis, with sex and treatment as factors, revealed no interaction between independent variables for any endpoints evaluated (all $P > 0.05$).

3.2. Histological and immunohistochemical analysis

The anatomical and histological arrangements of liver, kidney, and gonads of *H. intermedia* juveniles under basal conditions have been previously described (Bernabò et al., 2017), and only a brief general description will be provided. Histological alterations were observed in all specimens exposed to the two fungicide concentrations (Table 2).

3.2.1. Liver

In the control samples, the compact and homogeneous hepatic parenchyma was composed of large polygonal hepatocytes surrounding a sinusoid network. Hepatocytes showed round nuclei, usually located in the centre of the cells, and a granulated cytoplasm. In the parenchyma, a broad population of melanin-containing cells (also called melanomacrophages) were observed close or within sinusoids. In the portal area, the portal vein and hepatic artery profiles were recognisable along with minute bile ducts bordered by simple cuboidal epithelium (Fig. 2A).

Histological observations revealed, in all samples from TBZ exposed groups, severe liver injuries (Fig. 2B–F). After exposure to the low concentration, the histological architecture of the liver was altered. The intercellular spaces were dilated, originating poorly stained areas within the loose parenchyma (Fig. 2B). Moreover, wide zones of lysis were observed along with the infiltration of numerous mononuclear cells (Fig. 2B). Red blood cells often filled blood vessels, and sinusoids appeared dilated (Fig. 2C). Few melanomacrophages were scattered through the hepatocytes (Fig. 2B–C).

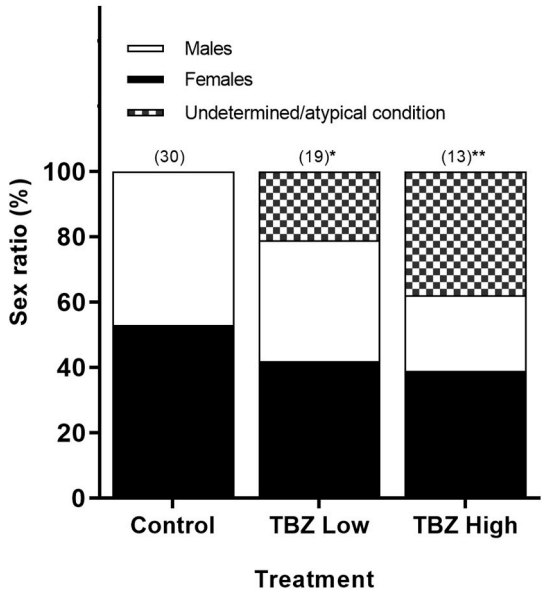

Fig. 1. Sex ratios (%) in *Hyla intermedia* juveniles from control and TBZ exposed groups. Numbers above bars indicate sample size used for phenotypic sex determination (based on both gross morphology and histology of gonads). Asterisks mark significant differences compared with the control group using Fisher's exact probability test (*$P < 0.05$, **P ˂0.005).

The emergence of both necrosis and apoptosis gave the liver tissue a dyschromic appearance. Pale necrotic cells were markedly swollen and with a poor vacuolated cytoplasm, which could be better appreciated with further magnification (inset). Apoptotic cells could be recognised by their hypereosinophilic cytoplasm and pyknotic or fragmented nuclei (Fig. 2B–C).

Morphological changes became more severe after exposure to the highest concentration. The enlargement of intercellular spaces and numerous areas of inflammations were also observed. Numerous pales, foamy hepatocytes, were interspersed with hypereosinophilic hepatocytes enhancing the dyschromia of the hepatic parenchyma and confirming the increase in both necrotic and apoptotic phenomena (Fig. 2D–F). Both sinusoids and blood vessels were dilated, and modified endothelial cells were observed. The degeneration phenomena involved the cuboidal epithelium of the bile ducts that displayed severe disorganisation. Aggregates of highly pigmented melanomacrophages were sporadically observed along with apoptotic bodies and cellular debris (Fig. 2D–F).

The immunodetection for Caspase-3, a key mediator of apoptosis, confirmed the emergence of apoptotic phenomena in exposed groups (Fig. S1). An extensive and intense immunosignal could be observed in the whole liver parenchyma of all samples exposed to both low (Fig. S1B) and high TBZ (Fig. S1C) concentrations. In contrast, the liver samples from the control group showed no or extremely weak labeling (Fig. S1A).

3.2.2. Kidney

In samples from the control group, the typical histological arrangement of the adult kidney was observed. (Fig. 3A). The collecting duct system was made by proximal, distal, and collecting tubules; the filtration units were composed of the Bowman's capsule surrounding the glomerulus, better discernible at higher magnification (inset).

Examinations by LM revealed impairment of kidney architecture and histopathological modifications in all specimens of both experimental groups (Fig. 3B–F). Extensive haemorrhage areas and numerous mononuclear cell infiltrations altering the regular arrangement of kidneys were always recognised (Fig. 3B–F). Renal tubules were often dilated, and the epithelial cells appeared thinned and with a variable degree of alterations. At several point cells exhibiting a clear sign of degeneration were detected; cells with evident nuclear alterations could be observed along with cells with poor and pale cytoplasm. In the proximal tubules, destruction and loss of brush border were also visible (Fig. 3B–F).

A few renal corpuscles were observed, whereas necrotic areas characterised by loss of cellular details, and nuclear fragmentation were often identified. In the renal corpuscles, considerable dilation of Bowman capsules was observed along with shrinkage or loss of glomerular tuft (Fig. 3B, E, F).

The localisation of iNOS revealed the absence of immunoreaction in kidney sections from the control group (Fig. 4A). After exposure to TBZ, an increase in iNOS immunoreactivity was observed. In samples from the low concentration group, the iNOS expression was mainly located at the level of interstitial inflammatory cells, whereas epithelial cells were not labeled; a few positive cells were also detected in the glomerular area (Fig. 4B). After exposure to the high concentration of TBZ, the immunoreactivity is more intense compared to the low concentration group and involving the whole renal parenchyma. The signal was particularly pronounced in collecting duct system and was also detected in the infiltrating inflammatory cells and glomeruli (Fig. 4C). The localisation of Caspase 3 revealed an intense immunolabelling in all samples from exposed groups, whereas immunoreaction was not observed in samples from the control group (Fig. S2).

Table 2
Summary of the histological alterations found in the three target organs of *H. intermedia* juveniles: respective grade of severity and prevalence.

Organ	Description of the tissue damage	Median score (range)			Prevalence (% of *n*)			P-Value[a]	
		Control	TBZ Low	TBZ High	Control	TBZ Low	TBZ High	TBZ Low	TBZ High
Liver	Hepatic inflammation (blood congestion and dilation in sinusoids, inflammatory cell infiltrates)	0 (0–1)	3 (2–3)	3 (2–3)	3.3	100	100	<0.001	<0.001
	Cytoplasmic vacuolation and degeneration	0	3 (2–3)	3 (2–3)	0	100	100	<0.001	<0.001
	Necrosis	0	3 (2–3)	3	0	100	100	<0.001	<0.001
	Apoptosis	0	3 (2–3)	3	0	100	100	<0.001	<0.001
Kidney	Renal inflammation (dilations of tubules, haemorrhage, inflammatory cell infiltrates)	0 (0–1)	3	3	6.7	100	100	<0.001	<0.001
	Tubular degeneration	0	3 (2–3)	3	0	100	100	<0.001	<0.001
	Necrosis	0	3 (2–3)	3	0	100	100	<0.001	<0.001
	Glomerular atrophy and dilation of Bowman's space	0	3 (2–3)	3	0	100	100	<0.001	<0.001
Gonads	Inflammatory cell infiltrates	0	3 (0–3)	3 (2–3)	0	84.2	100	<0.001	<0.001
	Degeneration of the germ cells	0 (0–1)	3	3	10	100	100	<0.001	<0.001
	Apoptosis	0	3 (2–3)	3	0	100	100	<0.001	<0.001

Total *n*: control = 30, TBZ Low = 19, TBZ high = 13.
Note: (0) No histopathological manifestations; (1) mild histopathology; (2) moderate histopathology; (3) severe histopathology.
[a] Median score was determined for each histopathologic feature, and the Mann-Whitney test was performed to determine significant differences from the control.

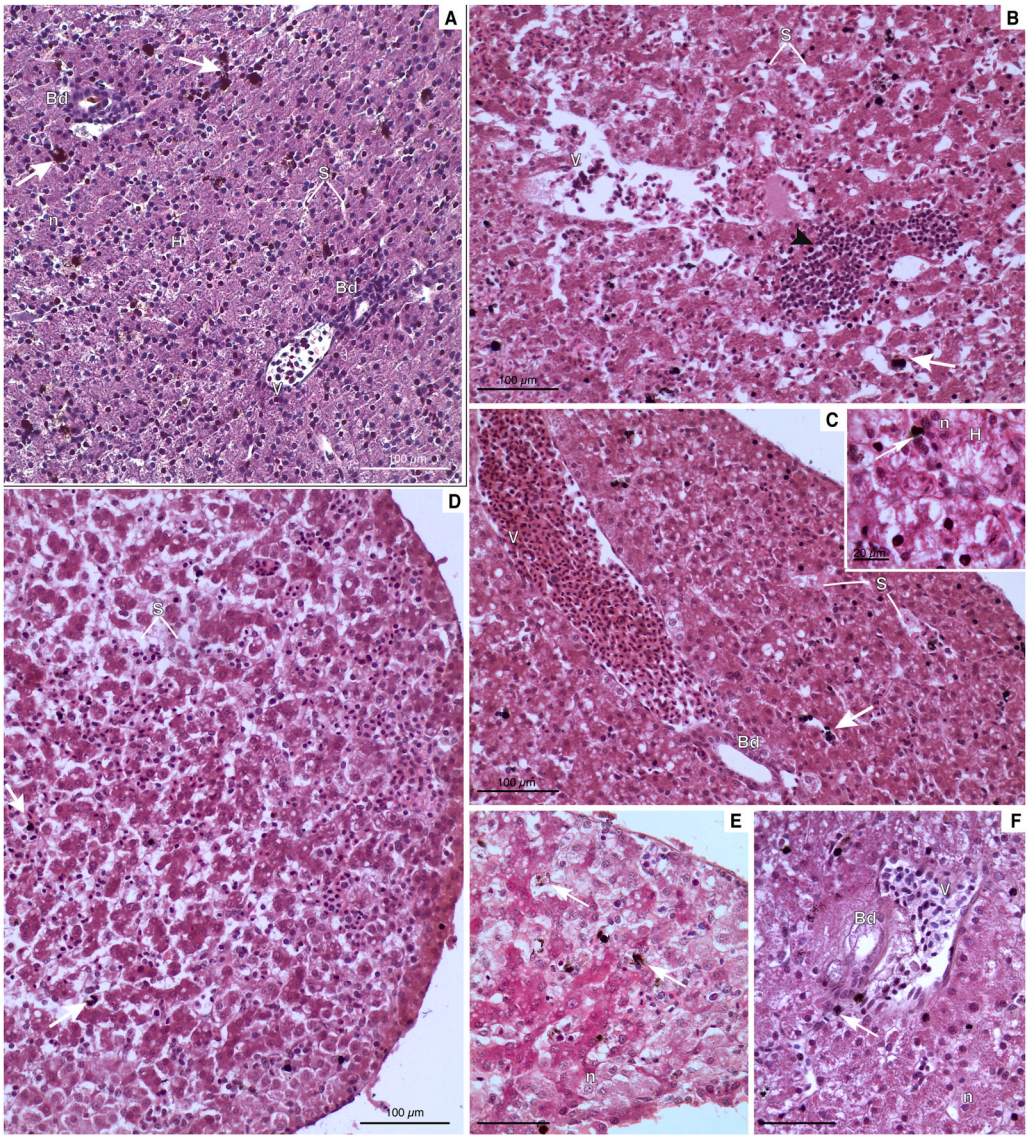

Fig. 2. Representative photomicrographs of liver from *H. intermedia* juveniles under basal conditions and after fungicide exposure. (A) Liver from control group showing the typical histological architecture with hepatocytes arranged in cords; note melanomacrophages, single or organised in a cluster, located in the sinusoidal lumen. (B and C) Histological sections from the low TBZ concentration showing an inflammatory response with congestion and dilatation of blood vessel and sinusoids, infiltration of mononuclear cells, and numerous necrotic hepatocytes; note nuclear condensation and fragmentation in the inset. (D–F) Extensive degenerative phenomena can be observed in the liver from the high TBZ group such as hyperemia with enlarged sinusoids (D), cytoplasm vacuolisation along with diffuse necrosis and apoptosis also involving melanomacrophages (E), and disorganisation in bile ducts (F). (H = hepatocytes; S = sinusoid; V = branch of the portal vein; Bd = bile duct; n = nucleus; arrows = melanomacrophages; arrowhead = mononuclear cells infiltration). All hematoxylin-eosin (H–E) stained sections. Scale bars equal 50 µm unless otherwise specified.

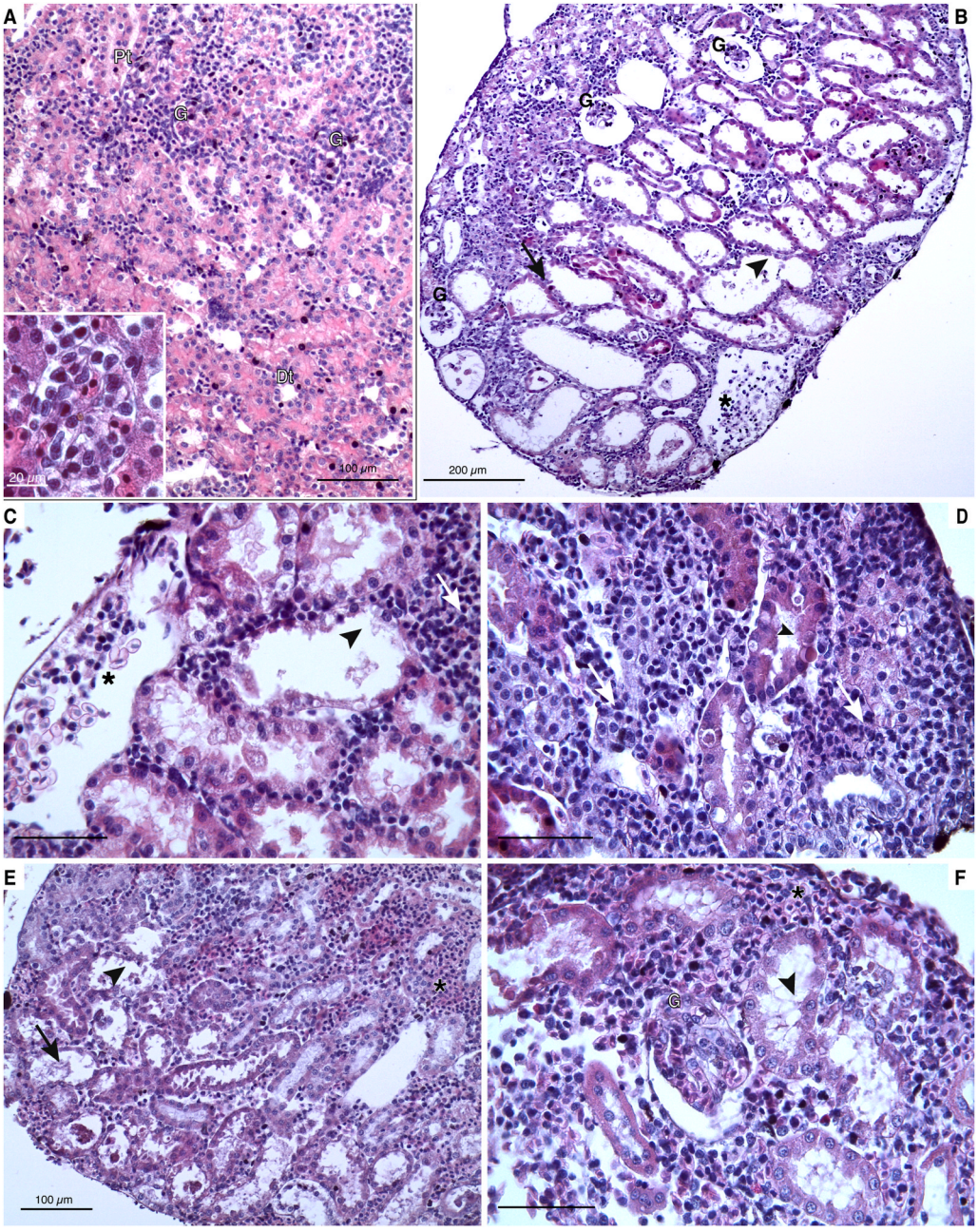

Fig. 3. (A) Kidney sections of *H. intermedia* juveniles from control group showing typical architecture with renal tubules and filtration unit; note capsule of Bowman surrounding the glomerulus in the inset. Representative micrographs of kidney tissue after chronic exposure to the low (B–D) and the high (E and F) TBZ concentrations showing severe histopathological alterations in both renal tubules and glomeruli; interstitial inflammatory infiltrates, and haemorrhage are always detected. (B and E) Note the renal tubule degeneration with dilation and thinning of epithelial cells. (C, D and F) at higher magnification tubules exhibiting a compromised epithelium with destruction of brush border, cell necrosis, nuclear changes and loss of cellular detail can be better visible. (B and F) Glomerular atrophy with dilated Bowman's space is evident. (G = glomerulus; Dt = distal tubule; Pt = proximal tubule; arrowhead = tubular degeneration; black arrows = renal tubular dilatation; white arrows = infiltration of inflammatory cells; * = haemorrhage). All H-E stained sections. Scale bars equal 50 µm unless otherwise specified.

3.2.3. Gonads

Examined for both morphology and histology, all froglets from the control group exhibited the typical features of juvenile ovaries (at stages VII-XI, according to Ogielska and Kotusz, 2004) or testes (at stages VI-VII following Haczkiewicz and Ogielska, 2013) The paired ovaria appeared as large and long sacs with lobulations; on the contrary, testes were recognisable by their granular appearance and the absence of external lobulations; besides, testes showed a different dimension with left testicle longer than the right one (Supplementary Fig. S3).

After a preliminary observation under the stereomicroscope, in froglets from TBZ exposed group, phenotypic sex was assigned based on the general arrangement of their gonads. Several individuals showed atypical gross morphology of the gonads, such as excessive folding in the left gonad (Fig. 5A–B), poor lobulation, absence of granular appearance or size differences (Fig. 5C–D). These individuals have been classified as undetermined/atypical.

Histological evaluation of all phenotypic females from the low concentration group confirmed anatomical sex determination; in ovaries, numerous diplotene oocytes filling the ovarian lumen were observed, while oogonia and germline nests were concentrated in the periphery (Fig. 5E–F). Notwithstanding this regular arrangement, it was possible to recognise mononuclear cell infiltrates, degeneration of the germ cells, and apoptotic bodies (Fig. 5E–F). Phenotypic males showed a preserved general arrangement of the gonad, but testes appeared poorly developed with seminiferous lobules barely or not recognisable; many spermatogonia and scattered somatic cells were distinguishable and degenerative changes were also visible along with mononuclear cells infiltration (Fig. 5G–H). The analysis of four undetermined/atypical samples always revealed a severely modified histological organisation. In two samples (Fig. 5I–J), signs of ovarian underdevelopment were observed, and morphological arrangement suggested a stage of development corresponding to stages V and VI (*sensu* Ogielska and Kotusz, 2004) as demonstrated by the persistence of meiotic oocytes and oogonia nests and the presence of few small diplotene oocytes. Signs of

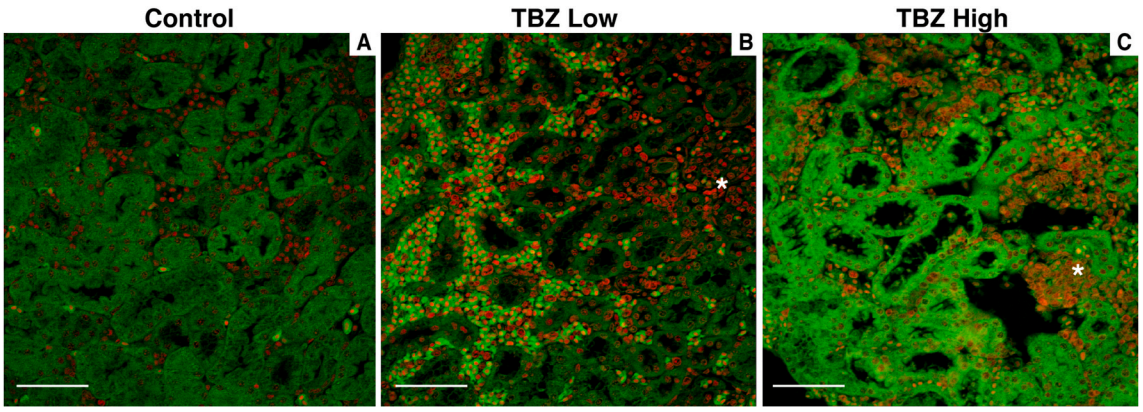

Fig. 4. Confocal microscopic images of *H intermedia* kidney sections labeled with a mouse monoclonal antibody against iNOS (green-FITC labeled); nuclei labeled with propidium iodide (red). (A) Control specimens reveal no iNOS immunoreactivity. (B) After exposure to the low TBZ concentration, iNOS-positive infiltrating inflammatory cells among tubules are visible. (C) In specimens from the high TBZ group, labeling is evident throughout the whole tissue, and strong immunoreactivity for iNOS is detected at the level of renal tubules. (*) indicates glomerulus; scale bars = 75 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

germ cell degeneration were also observed. The remaining displayed a histologically abnormal condition with the appearance of an ovarian-like cavity in the testicular tissue (Fig. 5K).

The histological evaluation of the phenotypic females from the high concentration group did not confirm the sex determination based on the gross morphology for all individuals. Despite the regular anatomical arrangement (Fig. 6A), one of the putative females revealed undetermined/atypical gonadal development. Only a few isolated diplotene oocytes were recognisable in a tissue widely affected by degenerative phenomena (Fig. 6B). Other samples under LM exhibited features of ovarian differentiation corresponding to stages from VII to IX (*sensu* Ogielska and Kotusz, 2004) as confirmed by the presence of large diplotene oocytes and a number of oogonia with nests of oocytes enter into meiosis restricted to the external layer of the cortex (Fig. 6C–D). In all samples, severe degeneration of germ cells was detected along with numerous mononuclear cell infiltrates, macrophages, and apoptotic bodies (Fig. 6C–E).

All phenotypic males under histological evaluation revealed some kind of alteration ranging from the presence of infiltrating inflammatory cells, observed in all samples (Fig. 6F) to an atypical gonadal differentiation. Underdevelopment and the presence of an ovarian-like central cavity were found in three samples (Fig. 6G). The individuals anatomically categorised as undetermined/atypical displayed the presence of oocytes within testicular tissue and an ovarian cavity (Fig. 6H–I).

Caspase 3 immunolocalization in gonads and testes (Fig. S4) showed the absence of the signal in all samples from the control group (Fig. S4 A, D). The extensive apoptotic phenomena, recognisable through the morphological analysis, were confirmed in all samples from both low (Fig. S4 B,E) and high (Fig. S4 C,F) TBZ concentration groups; interestingly an intense immunolabelling were also detected in undetermined gonads (Fig. S4 G,H).

4. Discussion

Because of the ability of azole fungicides to affect cytochrome P450-dependent enzymes, the available studies on TBZ have mainly focused on the oxidative metabolism disturbance, in both mammal and fish, thus giving a contribution to an already relevant body of evidence (Clasen et al., 2018; Ferreira et al., 2010; Li et al., 2020; Toni et al., 2010a, b). However, several potential effects of this fungicide remain still unclear, particularly in non-model species. Data from laboratory tests are mandatory to identify the causal relationship between the exposure to a specific contaminant and biological responses and are an essential starting point to evaluate toxicity mechanisms. Furthermore, when assessing the harmful potential of a substance, the experimental set-up should also be considered since the timing and duration of exposure are relevant to the onset of health effects. In this scenario, the histopathological evaluation of organs, characterised by a different degree of sensitivity and specificity, is essential to reveal the harmful effects of pollutants on the whole organism.

In the present study, we investigated for the first time the tissue-specific effects of TBZ exposure during morphogenesis in *H. intermedia* juveniles providing evidence of the systemic toxicity of this fungicide whether or not mediated by endocrine mechanisms. The doses of TBZ administered in our experiments were very low, but high enough to produce severe injury in key target organs susceptible to xenobiotic toxicity, such as liver and kidney, also affecting gonads differentiation and sex ratio.

This seems particularly important given that the concentrations tested here are very similar to those found in European aquatic environments, as reported in several field studies (Bundschuh et al., 2016; Casado et al., 2019; Creusot et al., 2020; Herrero- Hernández et al., 2013, 2016; Lefrancq et al., 2017). Therefore, our results suggest that wild populations can be affected by TBZ at concentrations that likely occur in aquatic habitats with profound implications in light of amphibian decline.

The morphological examination also revealed that for all considered organs, the intensity of injuries increased with TBZ concentration. To the best of our knowledge, this is the first report illustrating the histopathological alterations induced by TBZ on these target organs in amphibians.

4.1. Morphometry and gravimetry

In amphibians, both growth and developmental rate modifications in response to pollutant exposure are species-specific beings also highly variable depending on the chemical properties, concentration, and experimental conditions (Lavorato et al., 2013; Melvin et al., 2016; Sparling et al., 2010).

In our study, SVL resulted homogenous amongst groups, whereas the BM of juveniles from the TBZ exposed groups was significantly higher compared to control. A higher body mass has also been reported after exposure to other fungicides in anuran tadpoles (Brande-Lavridsen et al., 2010; Fioramonti et al., 1997; Hanlon et al., 2012).

As reported for other PPPs, including azole fungicides (Lenkowki et al., 2010; Svartz et al., 2016), in amphibians the appearance of edema is a common sublethal effect, apparently related to histopathological lesions and the following disturbance in the osmotic balance and kidney

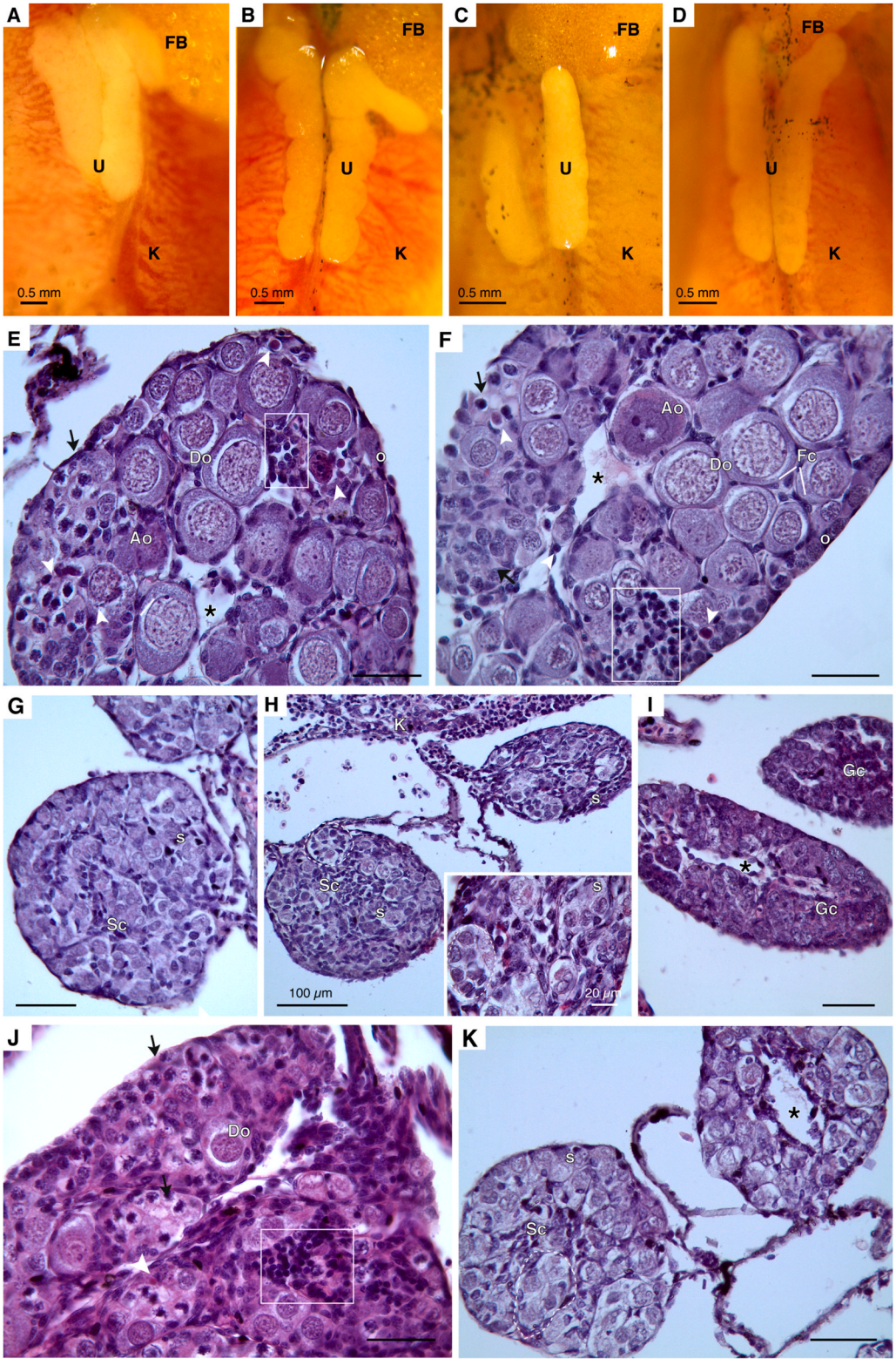

Fig. 5. Stereomicroscope images of the kidney-gonad complex of *H. intermedia* juveniles from the low (A–C) and high TBZ group (D). (A–D) In undetermined/atypical individuals, gonads could not be distinguished into either ovaries or testes based on gross morphology. (E–K) Light microscope photographs of gonads sample from the low TBZ group. Cross-sections of phenotypic females (E and F) showing newly formed and growing diplotene oocytes and areas containing oogonia and leptotene/pachytene oocyte (arrows); note diffuse degeneration affecting all germ cell types and presence of apoptotic bodies (pointed by arrowhead). (G and H) Phenotypic males showing the testicular appearance, with spermatogonia and somatic cells into barely organised seminiferous tubules. (I and J) Transverse sections of undetermined/atypical samples, shown respectively in (A) and (B), whit signs of ovarian underdevelopment and few small diplotene oocytes. (K) Abnormal condition with the gross testicular appearance and an ovarian-like cavity in one testis. (U = undetermined gonad; K = kidney; FB = fat body; Do = diplotene oocytes; o = oogonium; arrows = oogonia and nests of meiocytes; Ao = atretic oocytes; * = ovarian cavity; rectangle = mononuclear cell infiltrates; Gc = germ cells; Sc = somatic cells; s = spermatogonia). H-E stained sections. Scale bars equal 50 μm unless otherwise specified.

functions (see also below). The presence of an excess amount of fluids in the body's tissues can surely influence body weight. Besides, one may speculate that the recorded reduction in BM could be due to the selective mortality of smaller individuals during the entire exposure period (supplementary Table S2).

4.2. Histology

Liver - The liver is widely recognised as the most sensitive target organ to triazoles, and hepatotoxicity has been observed in several vertebrate models after TBZ exposure, both *in vitro* and *in vivo* (Li et al., 2020; Knebel et al., 2019).

In this study, we showed that all *H. intermedia* individuals displayed

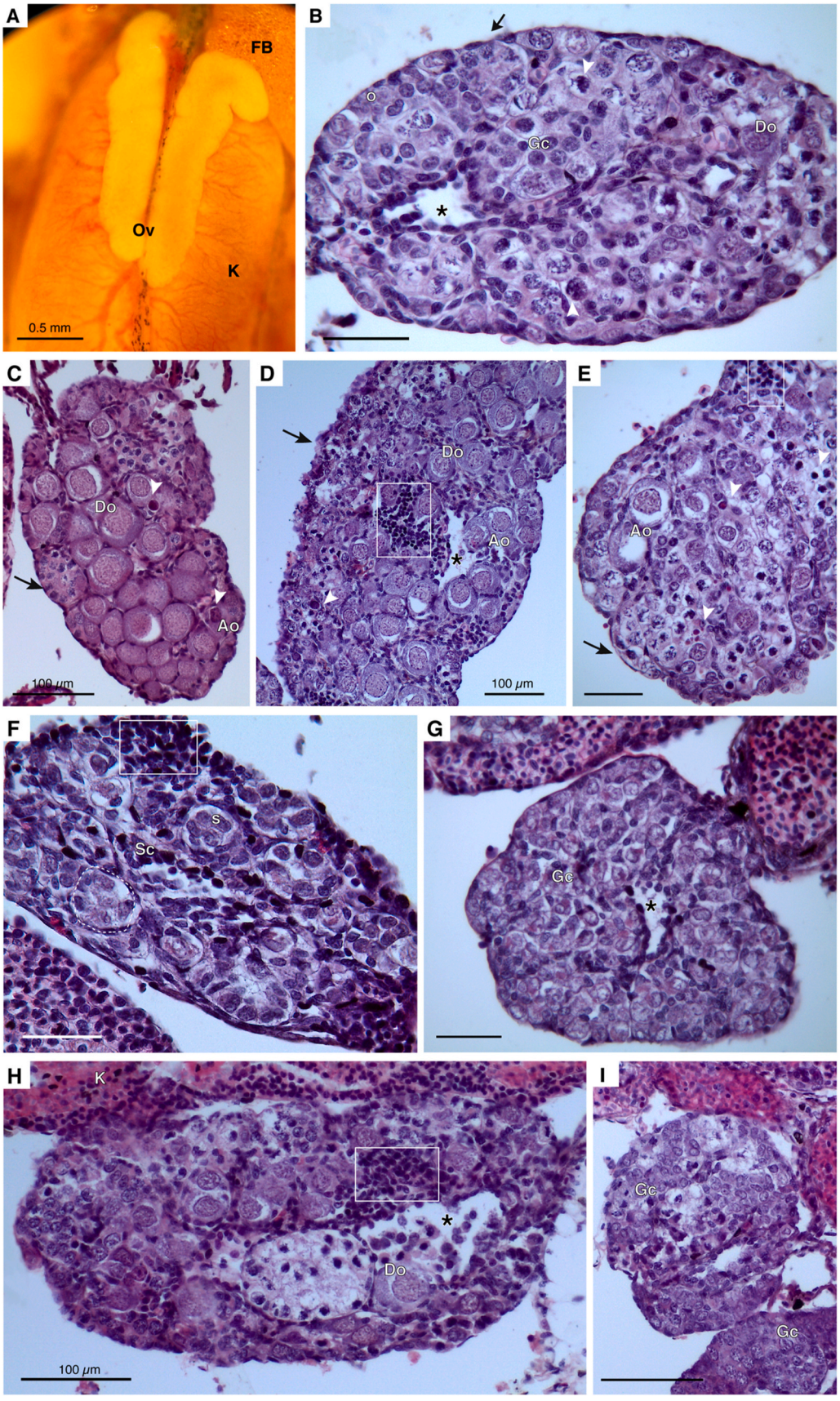

Fig. 6. Representative photomicrograph (A) and histological sections (B–I) of gonads of *H. intermedia* juveniles from the high TBZ group. The anatomical structure of one of the putative females (A) disclosing an undetermined/atypical gonadal differentiation even though the presence of isolated early diplotene oocytes and an ovarian cavity; note diffuse degenerative phenomena (B). (C–E) Cross-sections revealing typical ovarian differentiation modified by severe degeneration of all germ cells and the presence of macrophages and apoptotic bodies. Phenotypic males exhibiting clusters of proliferating spermatogonia interspersed with somatic cells and mononuclear cell infiltrates (F) or atypical gonadal differentiation such as underdevelopment and the presence of a central cavity in the medulla of the testes (G). (H and I) Transverse sections of undetermined/atypical sample shown in (Fig. 5D) displaying the presence of few oocytes only in the anterior portion and testicular architecture with an ovarian cavity in the median and posterior part. (Ov = ovaries; K = kidney; FB = fat body; Do = diplotene oocytes; o = oogonium; arrows = oogonia and nests of meiocytes; Ao = atretic oocytes; * = ovarian cavity; rectangle = mononuclear cell infiltrates; Gc = germ cells; Sc = somatic cells; s = spermatogonia). H-E stained sections. Scale bars equal 50 μm unless otherwise specified.

some hepatic alterations after TBZ exposure, and the most evident results of the fungicide application were a modified parenchyma architecture and the occurrence of extensive inflammatory response. The massive mononuclear infiltration, observed in both exposed groups, suggested a chronic inflammatory condition, often associated with haemostasis and dilation of both vessels and sinusoids. The enlargement of the intercellular spaces and the appearance of lysed areas changed the liver's structure and function. The uneven and dyschromic appearance of the liver parenchyma was enhanced by the simultaneous presence of both pale necrotic cells and hypereosinophilic apoptotic cells. Although the TBZ potential to accumulate in frog liver has been proven (Swanson et al., 2018), no previous information is available on TBZ-induced hepatotoxicity in amphibians for a direct comparison to our results. However, similar histopathological changes have been described in the same species after chronic exposure to another fungicide (Bernabò et al., 2017). This study represents the first evidence of the harmful effects of TBZ on liver histology in amphibians. Our findings can also provide useful insights on the toxicological physiology of this fungicide, suggesting that TBZ may either affect endocrine tissues or exert indirect adverse outcomes through alterations of homeostasis and activities of nonendocrine organs. Moreover, considering that the histopathological impact of fungicides in the amphibian liver is a topic somewhat neglected, our results will also contribute to filling a gap in the research literature.

Kidney - Amphibian kidney plays a major role in biotransformation and in maintaining a stable internal milieu, and it is susceptible to several xenobiotics (Bernabò et al., 2017; Fenoglio et al., 2011; Strong et al., 2016). Surprisingly, no information is available on the histological effects induced by TBZ on the kidney either in amphibians or in other vertebrates.

In our study, morphological alterations of both renal tubules and corpuscles have been detected in all juveniles from the TBZ exposed groups. The degree of lesions detected varied between the samples but remained at a reliably high level. Nuclear alterations, attributable to both apoptotic and necrotic phenomena, were often scattered within the renal tissue, even if the most evident effect was an extensive inflammatory response leading to the dilations of renal tubules, the appearance of wide haemorrhage and mononuclear cell infiltrations.

The evaluation of iNOS revealed an increase of this enzyme in the kidney of *H. intermedia* froglet, thus supporting the hypothesis of a severe inflammatory condition. The immunosignal was mainly detected in infiltrating inflammatory cells in the low TBZ group and extended to the whole renal parenchyma in the high TBZ group. According to Fenoglio et al. (2011), renal iNOS expression may reflect the degree of inflammation in tissue and provide a measure for assessing the inflammatory response to various chemicals (Kim et al., 2005; Mount and Power, 2006).

Comparable histological alterations have also been detected in *H. intermedia* juveniles after exposure to an anilinopyrimidine fungicide and in adults of *B. variabilis* and *Pelophylax ridibundus* after exposure to a systemic insecticide, a copper-based fungicide and a pyrethroid insecticide (Bernabò et al., 2017; Çakıcı, 2015; Păunescu and Ponepal, 2011; Păunescu et al., 2012). Our results are also consistent with histopathological changes reported in several anuran species after exposure to heavy metals (Jayawardena et al., 2017; Loumbourdis, 2003) and other pesticides (Çakıcı, 2015; Pă; unescu and Ponepal, 2011).

It seems that the histopathological effects on the kidney are largely independent of the category of substance applied, and the response of this organ to toxic stimuli is often a general inflammation. These results demonstrate that both morphological and physiological features of the kidney may be impaired by chronic exposures to TBZ, confirming that, for its high sensitivity, this organ is a suitable biomarker for ecotoxicological studies.

Gonads – Although much research has been focused on the effects of EDCs on reproductive traits, it is difficult to synthesise general patterns from such a diverse group of substances and experimental models. The EU has reported more than 553 substances as putative EDCs, and about 194 of these have been tested for their endocrine disruption potential in at least one study on a living organism (Ujhegyi and Bókony, 2020). TBZ, as other fungicides, has been identified and classified as endocrine-disrupting chemical (PAN Europe, 2015) exerting multiple endocrine effects in mammal models, both *in vivo* and *in vitro* (Creusot et al., 2020; Chen et al., 2019; Machado-Neves et al., 2018; Orton et al., 2011, 2014; Prutner et al., 2013; Taxvig et al., 2007). In amphibians, there is ample evidence and reviews that the reproductive system is one of the main targets of EDCs and a wide array of xenobiotics have been shown to alter gonadal tissues and to cause intersex or complete sex reversal (Bernabò et al., 2011; Hayes et al., 2010; Mackenzie et al., 2003; McCoy et al., 2008; Orton et al., 2018; Papoulias et al., 2013; Storrs--Mendez and Semlitsch, 2010). Specifically, in anurans, the presence of intersex represents the most common parameter used for evaluating reproductive perturbation (Orton et al., 2018; Mackenzie et al., 2003).

Here we showed that TBZ exposure throughout larval development markedly affects reproductive functions in *H. intermedia*, inducing the occurrence of a statistically significant number of intersex. Furthermore, even though we did not observe anatomical malformations of gonads or significant differences in gonad weight in exposed groups, a shift in sex ratio was observed, biased in favour of females. Although differences in sex ratio between groups did not achieve conventional threshold levels of statistical significance, this may likely be explained by the elevated mortality that greatly reduced the sample size (see above).

We also revealed that TBZ affects sexual differentiation by altering the maturation timing of gonads since all exposed individuals, both males and females, exhibited underdeveloped gonads with poorly differentiated tissues and delayed germ cell maturation compared to control. A similar delay has been reported after exposure to other fungicides in *H. intermedia* (Bernabò et al., 2017), *Rana temporaria* (Brande-Lavridsen et al., 2008), and *Rhinella arenarum* (Svartz et al., 2016).

Beside these well recognised pathological effects, one of the essential findings of this study is that TBZ might contribute to reproductive diseases through other mechanisms that include a general inflammatory response followed by the establishment of both apoptotic and degenerative phenomena. In *H. intermedia* juveniles, prominent signs of tissue injuries, closely linked to TBZ dose, were detected in all gonad sections, from males, females, and intersex. Indeed, morpho-histological alterations observed here might be a likely consequence of a direct toxic effect mediated or not by endocrine effects. The overall occurrence of histological injuries suggests considering histological changes of gonads as a relevant endpoint to assess reproductive impairment.

Altered gonadal development could have long-term impacts on fecundity and reproduction that are difficult to quantify or identify with consistency (Trudeau et al., 2020). It is increasingly recognised that phylogenetic divergence modifies endocrine disruptive vulnerability and that one cannot easily predict the sensitivity to EDCs studying model species only (Hoskins and Boone, 2018; Rozenblut-Kościsty et al., 2019; Tamschick et al., 2016a,b). Given the need to diversify taxonomic representation when investigating the effects of contaminants in amphibians, we confirmed *H. intermedia* as a good model to test putative EDCs effects. Moreover, our results in non-model species strongly support the concept that TBZ may affect reproductive success, thus resulting in compromised population dynamics. Our results also emphasise the importance of the exposure period when determining the adverse outcomes of EDCs as outlined by Slaby et al. (2019). In anurans, a critical window exists during which the gonads are sensitive to the activity of steroid hormones helping to shape gonads in a sex-specific manner; a perturbation during this sensitive window can alter sexual differentiation (Saidapur et al., 2001).

5. Conclusions

The present study concluded that exposure to environmentally relevant concentrations of tebuconazole during development could lead

to chronic multiorgan and multisystem dysfunction. Our results also indicate that the incidence of intersex is only one of the harmful consequences of TBZ exposure on the reproductive biology of amphibians, thus confirming the wide endocrine disruption potential of this fungicide in amphibians.

It seems that EDCs may exert their detrimental effects through a broad panel of mechanisms leading to different outcomes; therefore, studying histological features of target organs would be more meaningful than assessing a single parameter in determining the endocrine disruption potential of a substance.

Our findings may be predictive of the carry-over effects of TBZ exposure during aquatic developmental stages and advance our knowledge in the context of risk assessment of this poorly investigated fungicide.

Credit author statement

Ilaria Bernabò: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft. **Antonello Guardia:** Methodology, Investigation. **Rachele Macirella:** Methodology, Investigation, Visualization. **Settimio Sesti:** Data curation, Formal analysis. **Sandro Tripepi:** Funding acquisition, Resources, Validation, Visualization. **Elvira Brunelli:** Project administration, Conceptualization, Investigation, Writing - original draft, Writing - review & editing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111367.

Ethics

We performed all experimental procedures involving animals in compliance with ethical standards and under the authorisation of National Authorities (Ministero dell'Ambiente della Natura e del Mare: PNM-2011-0002086). All animals not used in the experiment were released in the place of collection.

References

Altenhofen, S., Nabinger, D.D., Wiprich, M.T., Pereira, T.C.B., Bogo, M.R., Bonan, C.D., 2017. Tebuconazole alters morphological, behavioral and neurochemical parameters in larvae and adult zebrafish (*Danio rerio*). Chemosphere 180, 483–490. https://doi.org/10.1016/j.chemosphere.2017.04.029.

Battaglin, W.A., Smalling, K.L., Anderson, C., Calhoun, D., Chestnut, T., Muths, E., 2016. Potential interactions among disease, pesticides, water quality and adjacent land cover in amphibian habitats in the United States. Sci. Total Environ. 566, 320–332. https://doi.org/10.1016/j.scitotenv.2016.05.062.

Bernabò, I., Gallo, L., Sperone, E., Tripepi, S., Brunelli, E., 2011. Survival, development, and gonadal differentiation in *Rana dalmatina* chronically exposed to chlorpyrifos. J. Exp. Zool. Part A 315, 314–327. https://doi.org/10.1002/jez.678.

Bernabò, I., Guardia, A., Macirella, R., Sesti, S., Crescente, A., Brunelli, E., 2016. Effects of long-term exposure to two fungicides, pyrimethanil and tebuconazole, on survival and life history traits of Italian tree frog (*Hyla intermedia*). Aquat. Toxicol. 172, 56–66. https://doi.org/10.1016/j.aquatox.2015.12.017.

Bernabò, I., Guardia, A., Macirella, R., Tripepi, S., Brunelli, E., 2017. Chronic exposures to fungicide pyrimethanil: multiorgan effects on Italian tree frog (*Hyla intermedia*). Sci. Rep. 7, 6869. https://doi.org/10.1038/s41598-017-07367-6.

Brande-Lavridsen, N., Christensen-Dalsgaard, J., Korsgaard, B., 2008. Effects of prochloraz and ethinylestradiol on sexual development in *Rana temporaria*. J. Exp. Zool. 309A, 389–398.

Brande-Lavridsen, N., Christensen-Dalsgaard, J., Korsgaard, B., 2010. Effects of ethinylestradiol and the fungicide prochloraz on metamorphosis and thyroid gland morphology in *Rana temporaria*. Open Zool. J. 3, 7–16.

Brühl, C.A., Zaller, J.G., 2019. Biodiversity decline as a consequence of an inappropriate environmental risk assessment of pesticides. Front. Environ. Sci. 7, 177. https://doi.org/10.3389/fenvs.2019.00177.

Bundschuh, M., Elsaesser, D., Stang, C., Schulz, R., 2016. Mitigation of fungicide pollution in detention ponds and vegetated ditches within a vine-growing area in Germany. Ecol. Eng. 89, 121–130. https://doi.org/10.1016/j.ecoleng.2015.12.015.

Cakıcı, O., 2015. Histopathologic changes in liver and kidney tissues induced by carbaryl in *Bufotes variabilis* (Anura: bufonidae). Exp. Toxicol. Pathol. 67, 237–243. https://doi.org/10.1016/j.etp.2014.12.003.

Casado, J., Brigden, K., Santillo, D., Johnston, P., 2019. Screening of pesticides and veterinary drugs in small streams in the European Union by liquid chromatography high resolution mass spectrometry. Sci. Total Environ. 670, 1204–1225. https://doi.org/10.1016/j.scitotenv.2019.03.207.

Castro, T.F.D., da Silva Souza, J.G., de Carvalho, A.F.S., de Lima Assis, I., Palmieri, M.J., Vieira, L.F.A., Marcussi, S., Machado, M.R.F., Murgas, L.D.S., 2018. Anxiety-associated behavior and genotoxicity found in adult *Danio rerio* exposed to tebuconazole- based commercial product. Environ. Toxicol. Pharmacol. 62, 140–146. https://doi.org/10.1016/j.etap.2018.06.011.

Chen, X., Zhu, Q., Li, X., Huang, T., Wang, S., Wang, Y., Chen, X., Lin, Z., Ge, R.S., 2019. Pubertal exposure to tebuconazole increases testosterone production via inhibiting testicular aromatase activity in rats. Chemosphere 230, 519–526. https://doi.org/10.1016/j.chemosphere.2019.05.122.

Clasen, B., Loro, V.L., Murussi, C.R., Tiecher, T.L., Moraes, B., Zanella, R., 2018. Bioaccumulation and oxidative stress caused by pesticides in *Cyprinus carpio* reared in a rice-fish system. Sci. Total Environ. 626, 737–743. https://doi.org/10.1016/j.scitotenv.2018.01.154.

Coons, A.H., Leduc, E.H., Connolly, J.M., 1955. Studies on antibody. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. 102, 49–59.

Creusot, N., Casado-Martinez, C., Chiaia-Hernandez, A., Kiefer, K., Ferrari, B.J.D., Fu, Q., Munz, N., Stamm, C., Tlili, A., Hollender, J., 2020. Retrospective screening of high-resolution mass spectrometry archived digital samples can improve environmental risk assessment of emerging contaminants: a case study on antifungal azoles. Environ. Int. 139, 105708. https://doi.org/10.1016/j.envint.2020.105708.

Davey, A.J., Bailey, L., Bewes, V., Mubaiwa, A., Hall, J., Burgess, C., Dunbarb, M.J., Smith, P.D., Rambohul, J., 2020. Water quality benefits from an advice-led approach to reducing water pollution from agriculture in England. Agric. Ecosyst. Environ. 296, 106925. https://doi.org/10.1016/j.agee.2020.106925.

de Souza, R.M., Seibert, D., Quesada, H.B., de Jesus Bassetti, F., Fagundes-Klen, M.R., Bergamasco, R., 2020. Occurrence, impacts and general aspects of pesticides in surface water: a review. Process Saf. Environ. Protect. 135, 22–37. https://doi.org/10.1016/j.psep.2019.12.035.

EEA (European Environment Agency), 2018. European Waters-Assessment of Status and Pressures 2018. EEA Report No 7/2018. EEA, Copenhagen. https://www.eea.europa.eu/publications/state-of-water.

Eurostat, 2019. Pesticide Sales. Accessed 19 April 2020. http://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=aei_fm_salpest09&lang=en.

Fenoglio, C., Albicini, F., Milanesi, G., Barni, S., 2011. Response of renal parenchyma and interstitium of *Rana* snk. *esculenta* to environmental pollution. Ecotoxicol. Environ. Saf. 74, 1381–1390.

Ferreira, D., da Motta, A.C., Kreutz, L.C., Toni, C., Loro, V.L., Barcellos, L.J., 2010. Assessment of oxidative stress *in Rhamdia quelen* exposed to agrichemicals. Chemosphere 79, 914–921. https://doi.org/10.1016/j.chemosphere.2010.03.024.

Fioramonti, E., Semlitsch, R.D., Reyer, H.U., Fent, K., 1997. Effects of triphenyltin and pH on the growth and development of *Rana lessonae* and *Rana esculenta* tadpoles. Environ. Toxicol. Chem. 16, 1940–1947.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183–190.

Haczkiewicz, K., Ogielska, M., 2013. Gonadal sex differentiation in frogs: how testes become shorter than ovaries. Zool. Sci. 30, 125–134. https://doi.org/10.2108/zsj.30.125.

Hanlon, S.M., Kerby, J.L., Parris, M.J., 2012. Unlikely remedy: fungicide clears infection from pathogenic fungus in larval southern leopard frogs (*Lithobates sphenocephalus*). PloS One 7, e43573. https://doi.org/10.1371/journal.pone.0043573.

Hansen, M., Poulsen, R., Luong, X., Sedlak, D.L., Hayes, T., 2014. Liquid chromatography tandem mass spectrometry method using solid-phase extraction and bead-beating-assisted matrix solid-phase dispersion to quantify the fungicide tebuconazole in controlled frog exposure study: analysis of water and animal tissue. Anal. Bioanal. Chem. 406, 7677–7685. https://doi.org/10.1007/s00216-014-8207-8.

Hayes, T.B., Falso, P., Gallipeau, S., Stice, M., 2010. The cause of global amphibian declines: a developmental endocrinologist's perspective. J. Exp. Biol. 213, 921–933. https://doi.org/10.1242/jeb.040865.

Herrero-Hernandez, E., Andrades, M.S., Alvarez-Martin, A., Pose-Juan, E., Rodriguez-Cruz, M.S., Sanchez-Martin, M.J., 2013. Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region. J. Hydrol. 486, 234–245. https://doi.org/10.1016/j.jhydrol.2013.01.025.

Herrero-Hernández, E., Pose-Juan, E., Sánchez-Martín, M.J., Andrades, M.S., Rodríguez-Cruz, M.S., 2016. Intra-annual trends of fungicide residues in waters from vineyard areas in La Rioja region of northern Spain. Environ. Sci. Pollut. Res. 23, 22924–22936. https://doi.org/10.1007/s11356-016-7497-0.

Hoskins, T.D., Boone, M.D., 2018. Atrazine feminizes sex ratio in Blanchard's cricket frogs (*Acris blanchardi*) at concentrations as low as 0.1 μg/L. Environ. Toxicol. Chem. 37, 427–435. https://doi.org/10.1002/etc.3962.

ISPRA (Italian Institute for Environmental Protection and Research), 2018. Pesticides in Water Italian Monitoring 2016. Synthesis Report. ISPRA, Report 289/2018.

Available on the Web Site:. http://www.isprambiente.gov.it/it/pubblicazioni/rapporti/pesticidi-nelle-acque-2013-monitoraggio-nazionale-2016.
IUCN, 2020. The IUCN Red List of Threatened Species. Version 2020-1. Downloaded on 19 March 2020. https://www.iucnredlist.org.
Jayawardena, U.A., Angunawela, P., Wickramasinghe, D.D., Ratnasooriya, W.D., Udagama, P.V., 2017. Heavy metal-induced toxicity in the Indian green frog: biochemical and histopathological alterations. Environ. Toxicol. Chem. 36, 855–2867. https://doi.org/10.1002/etc.3848 d.
Kim, J.Y., Oh, K.N., Han, E.H., Kim, D.H., Jeong, T.C., Lee, E.S., Jeong, H.G., 2005. Methoxychlor-induced inducible nitric oxide synthase and proinflam- matory cytokines expression in macrophages via NF-jB, ERK, and p38 mitogen-activated protein kinases. Biochem. Biophys. Res. Commun. 333, 1234–1240.
Knebel, C., Heise, T., Zanger, U.M., Lampen, A., Marx-Stoelting, P., Braeuning, A., 2019. The azole fungicide tebuconazole affects human CYP1A1 and CYP1A2 expression by an aryl hydrocarbon receptor-dependent pathway. Food Chem. Toxicol. 123, 481–491. https://doi.org/10.1016/j.fct.2018.11.039.
Kumwimba, M.N., Meng, F., Iseyemi, O., Moore, M.T., Zhu, B., Tao, W., Jia Liang, T., Ilunga, L., 2018. Removal of non-point source pollutants from domestic sewage and agricultural runoff by vegetated drainage ditches (VDDs): design, mechanism, management strategies, and future directions. Sci. Total Environ. 639, 742–759. https://doi.org/10.1016/j.scitotenv.2018.05.184.
Lavorato, M., Bernabò, I., Crescente, A., Denoël, M., Tripepi, S., Brunelli, E., 2013. Endosulfan effects on *Rana dalmatina* tadpoles: quantitative developmental and behavioural analysis. Arch. Environ. Contam. Toxicol. 64, 253–262. https://doi.org/10.1007/s00244-012-9819-7.
Le, T.D.H., Scharmüller, A., Kattwinkel, M., Kühne, R., Schüürmann, G., Schäfer, R.B., 2017. Contribution of wastewater treatment plants to pesticide toxicity in agriculture catchments. Ecotoxicol. Environ. Saf. 145, 135–141. https://doi.org/10.1016/j.ecoenv.2017.07.027.
Lefrancq, M., Jadas-Hécart, A., La Jeunesse, I., Landry, D., Payraudeau, S., 2017. High frequency monitoring of pesticides in runoff water to improve understanding of their transport and environmental impacts. Sci. Total Environ. 2587–588, 75–86. https://doi.org/10.1016/j.scitotenv.2017.02.022.
Li, D., Liu, M., Yang, Y., Shi, H., Zhou, J., He, D., 2016. Strong lethality and teratogenicity of strobilurins on *Xenopus tropicalis* embryos: basing on ten agricultural fungicides. Environ. Pollut. 208, 868–874. https://doi.org/10.1016/j.envpol.2015.11.010.
Li, S., Wu, Q., Sun, Q., Coffin, S., Gui, W.J., Zhu, G.N., 2019a. Parental exposure to tebuconazole causes thyroid endocrine disruption in zebrafish and developmental toxicity in offspring. Aquat. Toxicol. 211, 116e123. https://doi.org/10.1016/j.aquatox.2019.04.002.
Li, S., Jiang, Y., Sun, Q., Coffin, S., Chen, L., Qiao, K., Gui, W., Zhu, G., 2020. Tebuconazole induced oxidative stress related hepatotoxicity in adult and larval zebrafish (*Danio rerio*). Chemosphere 241, 125129. https://doi.org/10.1016/j.chemosphere.2019.125129.
Loumbourdis, N.S., 2003. Nephrotoxic effects of lead nitrate in *Rana ridibunda*. Arch. Toxicol. 77, 527–532. https://doi.org/10.1007/s00204-003-0487-2.
Lv, X., Pan, L.M., Wang, J.Y., Lu, L.P., Yan, W.L., Zhu, Y.Y., Xu, Y.W., Guo, M., Zhuang, S. L., 2017. Effects of triazole fungicides on androgenic disruption and CYP3A4 enzyme activity. Environ. Pollut. 222, 504–512. https://doi.org/10.1016/j.envpol.2016.11.051.
Machado-Neves, M., Neto, M.J.O., Miranda, D.C., Souza, A.C.F., Castro, M.M., Sertorio, M.N., Carvalho, T.F., Matta, S.L.P., Freitas, M.B., 2018. Dietary exposure to tebuconazole affects testicular and epididymal histomorphometry in frugivorous bats. Bull. Environ. Contam. Toxicol. 101, 197-204. https://doi.org/10.1007/s00128-018-2377-6.
Macirella, R., Tripepi, M., Brunelli, E., 2019. Morphological and immunohistochemical modifications in zebrafish (*Danio rerio*) gills after short-term exposure to the fungicide tebuconazole. Zebrafish 16, 65–76. https://doi.org/10.1089/zeb.2018.1638.
Mackenzie, C.A., Berrill, M., Metcalfe, C., Pauli, B.D., 2003. Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. Environ. Toxicol. Chem. 22, 2466–2475. https://doi.org/10.1897/02-173.
Matthiessen, P., Weltje, L., 2015. A review of the effects of azole compounds in fish and their possible involvement in masculinization of wild fish populations. Crit. Rev. Toxicol. 45 (5), 453–467. https://doi.org/10.3109/10408444.2015.1018409.
McCoy, K.A., Bortnick, L.J., Campbell, C.M., Hamlin, H.J., Guillette, L.J., St Mary, C.M., 2008. Agriculture alters gonadal form and function in the toad *Bufo marinus*. Environ. Health Perspect. 116, 1526–1532. https://doi.org/10.1289/ehp.11536.
Melvin, S.D., Lanctôt, C.M., vande Merwe, J.P., Leusch, F.D., 2016. Altered bioenergetics and developmental effects in striped marsh frog (*Limnodynastes peronii*) tadpoles exposed to UV treated sewage. Aquat. Toxicol. 175, 30–38. https://doi.org/10.1016/j.aquatox.2016.03.010.
Morselli, M., Vitale, C.M., Ippolito, A., Villa, S., Giacchini, R., Vighi, M., Di Guardo, A., 2018. Predicting pesticide fate in small cultivated mountain watersheds using the DynAPlus model: toward improved assessment of peak exposure. Sci. Total Environ. 615, 307–318. https://doi.org/10.1016/j.scitotenv.2017.09.287.
Mount, P.F., Power, D.A., 2006. Nitric oxide in the kidney: functions and regulation of synthesis. Acta Physiol. 187, 433–446. https://doi.org/10.1111/j.1748-1716.2006.01582.x.
OECD, 2015. Guidance Document on Histopathology Techniques and Evaluation (Part 1) for the Larval Amphibian Growth and Development Assay LAGDA.
Ogielska, M., Kotusz, A., 2004. Pattern and rate of ovary differentiation with reference to somatic development in Anuran Amphibians. J. Morphol. 259, 41–54. https://doi.org/10.1002/jmor.10162.
Orton, F., Rosivatz, E., Scholze, M., Kortenkamp, A., 2011. Widely used pesticides with previously unknown endocrine activity revealed as *in vitro* anti-androgens. Environ. Health Perspect. 119, 794–800. https://doi.org/10.1289/ehp.1002895.
Orton, F., Ermler, S., Kugathas, S., Rosivatz, E., Scholze, M., Kortenkamp, A., 2014. Mixture effects at very low doses with combinations of anti-androgenic pesticides, antioxidants, industrial pollutant and chemicals used in personal care products. Toxicol. Appl. Pharmacol. 278, 201–208, 10. 1016/j.taap.2013.09.008.
Orton, F., Säfholm, M., Jansson, E., Carlsson, Y., Eriksson, A., Fick, J., Uren Webster, T., McMillan, T., Leishman, M., Verbruggen, B., Economou, T., Tyler, C.R., Berg, C., 2018. Exposure to an anti-androgenic herbicide negatively impacts reproductive physiology and fertility in *Xenopus tropicalis*. Sci. Rep. 8, 9124. https://doi.org/10.1038/s41598-018-27161-2.
Papoulias, D.M., Schwarz, M.S., Mena, L., 2013. Gonadal abnormalities in frogs (*Lithobates* spp.) collected from managed wetlands in an agricultural region of Nebraska, USA. Environ. Pollut. 172, 1–8. https://doi.org/10.1016/j.envpol.2012.07.042.
Perez-Rodriguez, V., Souders II, C.L., Tischuk, C., Martyniuk, C.J., 2019. Tebuconazole reduces basal oxidative respiration and promotes anxiolytic responses and hypoactivity in early-staged zebrafish (*Danio rerio*). Comp. Biochem. Physiol. Toxicol. Pharmacol. 217, 87–97. https://doi.org/10.1016/j.cbpc.2018.11.017.
Poulsen, R., Luong, X., Hansen, M., Styrishave, B., Hayes, T., 2015. Tebuconazole disrupts steroidogenesis in *Xenopus laevis*. Aquat. Toxicol. 168, 28–37. https://doi.org/10.1016/j.aquatox.2015.09.008.
Prutner, W., Nicken, P., Haunhorst, E., Hamscher, G., Steinberg, P., 2013. Effects of single pesticides and binary pesticide mixtures on estrone production in H295R cells. Arch. Toxicol. 87, 2201–2214. https://doi.org/10.1007/s00204- 013-1081-x.
Păunescu, A., Ponepal, M.C., 2011. Nephrotoxic effects of Champion 50WP fungicides in the marsh frog, *Pelophylax ridibundus*. Oltenia. Studii şi comunicări. Ştiinţele naturii 27, 119–122.
Păunescu, A., Ponepal, C.M., Grigorean, V.T., Popescu, M., 2012. Histopathological changes in the liver and kidney tissues of marsh frog (*Pelophylax ridibundus*) induced by the action of Talstar 10EC insecticide. Analele Universitatii din Oradea Fascicula Biologie 19, 5–10.
Rozenblut-Koscisty, B., Ogielska, M., Hahn, J., Kleemann, D., Kossakowski, R., Tamschick, S., Schöning, V., Krüger, A., Lutz, I., Lymberakis, P., Kloas, W., Stöck, M., 2019. Impacts of the synthetic androgen Trenbolone on gonad differentiation and development: comparisons between three deeply diverged anuran families. Sci. Rep. 9, 9623. https://doi.org/10.1038/s41598-019-45985-4.
Saidapur, S.K., Gramapurohit, N.P., Shanbhag, B.A., 2001. Effect of sex steroids on gonadal differentiation and sex reversal in the frog, *Rana curtipes*. Gen. Comp. Endocrinol. 124, 115–123. https://doi.org/10.1006/gcen.2001.7699.
Sancho, E., Villarroel, M.J., Fernández, C., Andreu, E., Ferrando, M.D., 2010. Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 73, 370–376. https://doi.org/10.1016/j.ecoenv.2009.09.020.
Sanderson, J.T., 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicol. Sci. 94, 3–21. https://doi.org/10.1093/toxsci/kfl051.
Silva, V., Mol, H.G.J., Zomer, P., Tienstra, M., Ritsema, C.J., Geissen, V., 2019. Pesticide residues in European agricultural soils – a hidden reality unfolded. Sci. Total Environ. 653, 1532–1545. https://doi.org/10.1016/j.scitotenv.2018.10.441.
Slaby, S., Marin, M., Marchand, G., Lemiere, S., 2019. Exposures to chemical contaminants: what can we learn from reproduction and development endpoints in the amphibian toxicology literature? Environ. Pollut. 248, 478–495. https://doi.org/10.1016/j.envpol.2019.02.014.
Smalling, K.L., Fellers, G.M., Kleeman, P.M., Kuivila, K.M., 2013. Accumulation of pesticides in pacific chorus frogs (*Pseudacris regilla*) from California's Sierra Nevada Mountains, USA. Environ. Toxicol. Chem. 32, 2026–2034. https://doi.org/10.1002/etc.2308.
Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K., 2010. Ecotoxicology of Amphibians and Reptiles. SETAC Press, USA.
Stehle, S., Schulz, R., 2018. The impact of pesticides on our freshwater Resources. In: Della Sala, Dominick A., Goldstein, Michael I. (Eds.), The Encyclopedia of the Anthropocene, vol. 5. Elsevier, Oxford, pp. 127–132.
Storrs-Mendez, S.I., Semlitsch, R.D., 2010. Intersex gonads in frogs: understanding the time course of natural development and role of endocrine disruptors. J. Exp. Zool. B 314B, 57–66. https://doi.org/10.1002/jez.b.21310.
Strong, R.J., Halsall, C.J., Ferenčík, M., Jones, K.C., Shore, R.F., Martin, F.L., 2016. Biospectroscopy reveals the effect of varying water quality on tadpole tissues of the common frog (*Rana temporaria*). Environ. Pollut. 213, 322–337.
Svartz, G., Meijide, F., Coll, C.P., 2016. Effects of a fungicide formulation on embryo-larval development, metamorphosis, and gonadogenesis of the South American toad *Rhinella arenarum*. Environ. Toxicol. Pharmacol. 45, 1–7. https://doi.org/10.1016/j.etap.2016.05.008.
Swanson, J.E., Muths, E., Pierce, C.L., Dinsmore, S.J., Vandever, M.W., Hladik, M.L., Smalling, K.L., 2018. Exploring the amphibian exposome in an agricultural landscape using telemetry and passive sampling. Sci. Rep. 8, 10045. https://doi.org/10.1038/s41598-018-28132-3.
Tamschick, S., Rozenblut-Kościsty, B., Ogielska, M., Lehmann, A., Lymberakis, P., Hoffmann, F., Lutz, I., Kloas, W., Stöck, M., 2016a. Sex reversal assessments reveal different vulnerability to endocrine disruption between deeply diverged anuran lineages. Sci. Rep. 6, 23825. https://doi.org/10.1038/srep23825.
Tamschick, S., Rozenblut-Kościsty, B., Ogielska, M., Kekenj, D., Gajewski, F., Krüger, A., Kloas, W., Stöck, M., 2016b. The plasticizer bisphenol A affects somatic and sexual development, but differently in pipid, hylid, and bufonid anurans. Environ. Pollut. 216, 282–291. https://doi.org/10.1016/j.envpol.2016.05.091.

Taxvig, C., Hass, U., Petersen, M.A., Dalgaard, M., Boberg, J., Andersen, H.R., Vinggaard, A., 2007. Endocrine-disrupting activities *in vivo* of the fungicides tebuconazole and epoxiconazole. Toxicol. Sci. 100, 464–473. https://doi.org/10.1093/toxsci/kfm227.

Toni, C., Ferreira, D., Kreutz, L.C., Loro, V.L., Barcellos, L.J.G., 2011a. Assessment of oxidative stress and metabolic changes in common carp (*Cyprinus carpio*) acutely exposed to different concentrations of the fungicide tebuconazole. Chemosphere 83, 579–584. https://doi.org/10.1016/j.chemosphere.2010.12.022.

Toni, C., Loro, V.L., Santi, A., de Menezes, C.C., Cattaneo, R., Clasen, B.E., Zanella, R., 2011b. Exposure to tebuconazol in rice field and laboratory conditions induces oxidative stress in carp (*Cyprinus carpio*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 128–132. https://doi.org/10.1016/j.cbpc.2010.09.008.

Trudeau, V.L., Thomson, P., Zhang, W.S., Reynaud, S., Navarro-Martin, L., Langlois, V.S., 2020. Agrochemicals disrupt multiple endocrine axes in amphibians. Mol. Cell. Endocrinol. 513, 1108612. https://doi.org/10.1016/j.mce.2020.110861.

Ujhegyi, N., Bókony, V., 2020. Skin coloration as a possible non-invasive marker for skewed sex ratios and gonadal abnormalities in immature common toads (*Bufo bufo*). Ecol. Indicat. 113, 106175. https://doi.org/10.1016/j.ecolind.2020.106175.

Wagner, N., Veith, M., Lötters, S., Viertel, B., 2016. Population and life-stage-specific effects of two herbicide formulations on the aquatic development of european common frogs (*Rana temporaria*). Environ. Toxicol. Chem. 9999, 1–11. https://doi.org/10.1002/etc.3525.

Wrubleswski, J., Reichert Jr., F.W., Galon, L., Hartmann, P.A., Hartmann, M.T., 2018. Acute and chronic toxicity of pesticides on tadpoles of *Physalaemus cuvieri* (Anura, Leptodactylidae). Ecotoxicology 27, 360–368. https://doi.org/10.1007/s10646-018-1900-1.

Zarn, J.A., Brüschweiler, B.J., Schlatter, J.R., 2003. Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14 alpha-demethylase and aromatase. Environ. Health Perspect. 111, 255–261. https://doi.org/10.1289/ehp.5785.

Zubrod, J.P., Bundschuh, M., Arts, G., Brühl, C.A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J.J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., Schäfer, R.B., 2019. Fungicides: an overlooked pesticide class? Environ. Sci. Technol. 53, 3347–3365. https://doi.org/10.1021/acs.est.8b04392.