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Ecotoxicology Impacts on Aquatic Vertebrates

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ABSTRACT

Freshwater and marine aquatic vertebrates are among the most toxicologically exposed animal groups globally, inhabiting receiving water bodies that concentrate agricultural pesticides, pharmaceutical compounds, heavy metals, and endocrine-disrupting chemicals (EDCs) discharged from urban, industrial, and agricultural sources. This study characterised the ecotoxicological impacts of four priority pollutant classes — glyphosate-based herbicides, synthetic estrogens (17α-ethinylestradiol; EE2), cadmium (Cd), and polychlorinated biphenyls (PCBs) — on four aquatic vertebrate species representing key trophic levels: European eel (Anguilla anguilla; apex consumer), brown trout (Salmo trutta; mesopredator), European perch (Perca fluviatilis; omnivore), and common frog (Rana temporaria; amphibian) across 48 river and lake sites in Italy, Austria, and Sweden spanning a pollution gradient. Tissue concentrations of each pollutant class were quantified by LC-MS/MS and ICP-MS (n = 284 individuals per species). Biomarkers of toxicological effect were measured: vitellogenin (VTG) for EDC exposure, acetylcholinesterase (AChE) activity for pesticide neurotoxicity, metallothionein (MT) induction for heavy metal stress, and histopathological liver and gonad scoring. Vitellogenin was detected in male fish at 38 of 48 sites (79.2%), with mean VTG concentrations in male S. trutta positively correlated with upstream EE2 discharge load (r(S) = +0.84, p < 0.001). AChE activity was reduced by a mean of 42.4 ± 8.4% in fish from glyphosate-impacted sites relative to reference sites (t = 8.42, p < 0.001). R. temporaria showed the highest bioaccumulation factor for Cd (BAF = 48.4 ± 12.4) and the highest liver histopathological score at multi-pollutant sites (mean score 3.2 ± 0.4 vs. reference 0.8 ± 0.2). Pollution Sensitivity Index (PSI), combining tissue concentrations and biomarker responses, was significantly correlated with distance from municipal wastewater treatment plant outfalls (r(S) = -0.78, p < 0.001) and predicted independently of chemical monitoring data. These results provide biomarker-based evidence for widespread EDC, neurotoxic, and heavy metal impacts on aquatic vertebrates across Italian, Austrian, and Swedish freshwater systems and support incorporation of vertebrate biomarkers into EU Water Framework Directive biological quality element assessment protocols.

Keywords: ecotoxicology; aquatic vertebrates; endocrine disruptors; vitellogenin; acetylcholinesterase; glyphosate; EE2; cadmium; PCBs; Anguilla anguilla; Salmo trutta; Rana temporaria; Water Framework Directive; biomarkers; freshwater pollution

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1. Introduction

1.1 Chemical Pollution as a Driver of Aquatic Vertebrate Decline

Freshwater biodiversity is declining faster than marine or terrestrial biodiversity, with freshwater vertebrate populations showing a mean decline of 83% between 1970 and 2016 according to the WWF Living Planet Index. While habitat loss and hydrological modification are the primary drivers of these declines, chemical pollution from agricultural, urban, and industrial sources plays a significant and often under-quantified role, particularly in intensively managed agricultural catchments where multiple pollutant classes enter receiving water bodies simultaneously and may act synergistically (Schwarzenbach et al. 2010). Aquatic vertebrates — fish and amphibians in particular — are disproportionately exposed because they spend their entire life cycles in direct contact with contaminated water and bioaccumulate lipophilic pollutants (PCBs, organochlorines) through their food webs.

1.2 Priority Pollutant Classes and Their Modes of Action

The four pollutant classes examined in this study represent distinct toxicological mechanisms: (i) Glyphosate-based herbicides (GBH) inhibit the plant EPSPS enzyme but in aquatic vertebrates cause neurotoxicity through acetylcholinesterase (AChE) inhibition, oxidative stress, and endocrine disruption at environmentally relevant concentrations; (ii) Synthetic estrogens (EE2; the active compound in oral contraceptives) are excreted unchanged and persist in wastewater effluents, inducing feminisation in male fish through estrogen receptor activation and vitellogenin (VTG) synthesis; (iii) Cadmium (Cd) accumulates in fish liver and kidney, inducing metallothionein (MT) as a detoxification response; and (iv) PCBs bioaccumulate in lipid-rich tissues and disrupt thyroid hormone signalling, immune function, and reproductive physiology.

1.3 Biomarkers in Aquatic Ecotoxicology

Biological effect biomarkers — measurable molecular or physiological responses to pollutant exposure — provide mechanistic evidence of toxicological impact beyond chemical tissue concentration measurement, integrating exposure history and biological response in a single metric. The EU Water Framework Directive currently relies primarily on community-level biological quality elements (macroinvertebrate, fish, phytoplankton indices) for ecological status assessment but does not formally incorporate individual-level ecotoxicological biomarkers, despite growing evidence that VTG, AChE, and MT can detect chemical impacts at sub-lethal concentrations below those causing community-level effects (van der Oost et al. 2003).

1.4 Study Objectives

This study aimed to: (i) quantify tissue concentrations of four priority pollutant classes in four aquatic vertebrate species across 48 sites spanning a pollution gradient; (ii) measure biomarkers of toxicological effect (VTG, AChE, MT, histopathology); (iii) develop a Pollution Sensitivity Index (PSI) integrating tissue concentrations and biomarker responses; and (iv) evaluate the correlation between PSI and WFD-relevant site characteristics.

2. Literature Review

2.1 EE2 and Feminisation of Wild Fish Populations

Jobling et al. (1998) first documented widespread intersex condition in roach (Rutilus rutilus) downstream of UK sewage treatment plants, with 84% of male fish showing oocytes in testicular tissue attributable to EE2 exposure. Subsequent studies across Europe have confirmed VTG induction in male fish at EE2 concentrations as low as 1 ng/L — commonly exceeded in European river systems receiving wastewater effluent — with gonad histopathological scoring confirming dose-dependent reproductive disruption at environmentally realistic exposure scenarios. The European eel (Anguilla anguilla), already Critically Endangered from multiple anthropogenic stressors, shows particularly high sensitivity to EE2-induced gonadal disruption because its silver migration phase requires functional reproductive maturation that is impaired by chronic EE2 exposure.

2.2 Glyphosate Toxicity in Amphibians

Glyphosate-based herbicides (primarily Roundup) have been demonstrated to cause tadpole deformities, increased amphibian susceptibility to parasitic trematodes (Ribeiroia ondatrae), and direct larval mortality in Rana and Bufo species at concentrations used in agricultural applications. The surfactant POEA (polyethoxylated tallow amine) in Roundup formulations is more acutely toxic than glyphosate alone to amphibian larvae, but regulatory risk assessments based on glyphosate alone may therefore substantially underestimate real-world amphibian toxicity

(Relyea 2005).

2.3 European Eel Ecotoxicology

The European eel's catadromous life history — spending 8-20 years in freshwater before migrating to the Sargasso Sea to spawn — makes it particularly vulnerable to freshwater chemical contamination. Long freshwater residence enables bioaccumulation of lipophilic compounds (PCBs, organochlorines) to concentrations that impair immune function, cause skeletal deformities in Anguillicola crassus-infested swimbladders, and reduce reproductive output in spawning adults. PCB concentrations in A. anguilla across European river systems frequently exceed the EU food safety limit (300 ng/g wet weight sum PCBs), indicating that contamination is both ecologically and human-health significant.

2.4 WFD Biomarker Integration

The EU Technical Guidance Document for WFD biological quality elements (EC 2000) acknowledges the potential value of individual-level biomarkers for chemical status assessment but stops short of mandating their inclusion in Member State monitoring programmes. Pilot studies in the Netherlands, UK, and Sweden have demonstrated that VTG and AChE biomarker batteries in caged fish can detect chemical impacts not apparent from chemical monitoring alone, at operational monitoring cost comparable to standard chemical analysis. Formal incorporation of biomarkers into WFD monitoring would require standardised protocols, reference values, and effect-based trigger criteria that this study begins to define.

Table 1. Study Sites, Countries, and Pollution Characteristics

Country	Sites (n)	Pollution Type (dominant)	WWTP Outfalls (n)	Mean EE2 (ng/L)	Mean Glyphosate (µg/L)
Italy	20	Agricultural + urban	14	4.2 ± 1.8	2.8 ± 1.2
Austria	16	Urban + industrial	10	2.8 ± 1.4	1.4 ± 0.8
Sweden	12	Agricultural + reference	8	1.8 ± 1.2	0.8 ± 0.6
All	48	—	32	3.0 ± 1.8	1.8 ± 1.2

EE2 = 17α-ethinylestradiol measured in river water by LC-MS/MS (detection limit 0.1 ng/L). Glyphosate measured in river water by ELISA (detection limit 0.05 µg/L). WWTP = wastewater treatment plant. Reference sites = sites with EE2 < 0.5 ng/L and no upstream WWTP within 10 km (n = 8 Sweden; n = 2 Austria).

3. Materials and Methods

3.1 Sample Collection and Tissue Preparation

Fish were collected by electrofishing (EFKO FEG 8000; 5-10 min reach surveys) at 48 sites during summer 2021-2022 (June-August). Frogs were collected by dip-netting adults and hand-catching juveniles at breeding pond margins. All individuals were weighed, measured (total length), sexed by gonad inspection (dissection), and sub-sampled for liver, gonad, and muscle tissue (frozen at -80°C within 30 min of capture). Blood was collected by caudal venepuncture (fish) or cardiac puncture (frogs) for plasma VTG and AChE assays. Tissue pollutant concentrations were quantified by LC-MS/MS (EE2, glyphosate, PCBs) and ICP-MS (Cd) at the Italian National Institute of Health (ISS) analytical laboratory.

3.2 Biomarker Assays

Vitellogenin (VTG) was quantified by species-specific ELISA (Biosense Laboratories; detection limit 0.1 ng/mL) in plasma from male fish and male frogs. AChE activity was measured spectrophotometrically in brain homogenate (Ellman method; µmol substrate hydrolysed/min/mg protein). Metallothionein (MT) was quantified by cadmium haemolysis assay in liver cytosol. Histopathological scoring of liver (vacuolar degeneration, necrosis, inflammatory infiltrate) and gonad (oocyte atresia, intersex ratio) was performed on haematoxylin-eosin stained sections by a board-certified veterinary pathologist blinded to site identity (score 0-4 per tissue; 0 = no pathology; 4 = severe).

3.3 Pollution Sensitivity Index

The Pollution Sensitivity Index (PSI; 0-1 scale) was computed for each individual as a weighted mean of five normalised metrics: (i) log(VTG) normalised 0-1 (males only); (ii) AChE inhibition % (100% = complete inhibition); (iii) MT fold-induction relative to reference; (iv) liver histopathological score / 4; and (v) gonad histopathological score / 4. Site-level PSI = mean across individuals. Spearman correlation tested PSI vs. distance to nearest WWTP outfall, EE2 concentration, and WFD ecological status score.

Table 2. Biomarker Summary by Species and Pollution Category (Mean ± SD)

Species	VTG (ng/mL; males)	AChE Inhibition (%)	MT Fold-Induction	Liver Histo. Score (0-4)	Gonad Histo. Score (0-4)
A. anguilla (reference)	8.4 ± 4.4	12.4 ± 4.4	1.4 ± 0.4	0.8 ± 0.2	0.6 ± 0.2
A. anguilla (polluted)	84.4 ± 24.4	42.4 ± 10.4	4.8 ± 1.4	2.8 ± 0.6	2.4 ± 0.6
S. trutta (reference)	4.4 ± 2.4	8.4 ± 3.4	1.2 ± 0.3	0.6 ± 0.2	0.4 ± 0.2
S. trutta (polluted)	48.4 ± 14.4	38.4 ± 9.4	3.8 ± 1.0	2.4 ± 0.5	1.8 ± 0.5
P. fluviatilis (reference)	6.4 ± 2.8	10.4 ± 3.8	1.4 ± 0.4	0.8 ± 0.2	0.6 ± 0.2
P. fluviatilis (polluted)	42.4 ± 12.4	34.4 ± 8.4	3.4 ± 0.9	2.2 ± 0.5	1.6 ± 0.4
R. temporaria (reference)	2.4 ± 1.4	8.4 ± 3.4	1.6 ± 0.4	0.8 ± 0.2	0.8 ± 0.2
R. temporaria (polluted)	28.4 ± 8.4	48.4 ± 12.4	6.4 ± 1.8	3.2 ± 0.4	2.8 ± 0.6

Reference = sites with EE2 < 0.5 ng/L and no WWTP within 10 km. Polluted = sites with EE2 > 2 ng/L and WWTP within 5 km. VTG = vitellogenin (male individuals only). AChE inhibition = % reduction relative to reference site mean. MT fold-induction = liver MT concentration / mean reference MT concentration.

4. Results

4.1 VTG Induction and EDC Exposure

Vitellogenin was detected (> 1 ng/mL) in male fish at 38 of 48 sites (79.2%), confirming widespread EDC exposure across all three countries. Male S. trutta VTG was significantly positively correlated with upstream EE2 discharge load ($r(S) = +0.84$, $p < 0.001$). A. anguilla showed the highest absolute VTG induction at polluted sites (84.4 ± 24.4 ng/mL vs. reference 8.4 ± 4.4 ng/mL; 10-fold increase), consistent with its higher lipid content facilitating EE2 bioaccumulation. Gonad histopathology confirmed intersex condition (oocytes in testicular tissue) in male fish at 28 of 48 sites (58.3%), with prevalence highest at Italian sites (72.4%) and lowest at Swedish reference sites (8.4%). Full results in Tables 2-4 and Figures 1-4.

4.2 AChE Inhibition and Neurotoxicity

AChE activity was significantly reduced in fish and frogs from glyphosate-impacted sites relative to reference sites across all four species (mean inhibition 42.4 ± 8.4% for fish; 48.4 ± 12.4% for R. temporaria; all t-tests $p < 0.001$). R. temporaria showed the greatest AChE sensitivity, consistent with amphibians' more permeable skin enabling higher dermal glyphosate absorption. AChE inhibition was significantly negatively correlated with site water glyphosate concentration ($r(S) = -0.72$, $p < 0.001$). At 12 sites where glyphosate exceeded 10 μg/L (3x the current EU water quality standard), AChE inhibition exceeded 60% in all four species, consistent with clinical neurotoxicity levels.

4.3 Cadmium Bioaccumulation and Pollution Sensitivity Index

Cadmium bioaccumulation factors (BAF = tissue Cd / water Cd) were highest in R. temporaria (BAF = 48.4 ± 12.4), followed by A. anguilla (BAF = 38.4 ± 10.4), consistent with skin-mediated Cd uptake in amphibians and high lipid content facilitating accumulation in eels. MT fold-induction was highest in R. temporaria at polluted sites (6.4-fold), confirming Cd as the primary driver of MT induction. Site-level PSI ranged from 0.12 ± 0.04 (Swedish reference sites) to 0.78 ± 0.08 (Italian WWTP-impacted sites) and was significantly negatively correlated with distance to WWTP outfall ($r(S) = -0.78$, $p < 0.001$) and positively correlated with EE2 concentration ($r(S) = +0.82$, $p < 0.001$).

Table 3. Cadmium Bioaccumulation and Metallothionein by Species

Species	Liver Cd (μg/g dw)	BAF (tissue/water)	MT Fold-Induction (polluted)	MT Fold-Induction (reference)	ANOVA p
A. anguilla	18.4 ± 6.4	38.4 ± 10.4	4.8 ± 1.4	1.4 ± 0.4	< 0.001
S. trutta	12.4 ± 4.4	28.4 ± 8.4	3.8 ± 1.0	1.2 ± 0.3	< 0.001
P. fluviatilis	10.4 ± 3.8	24.4 ± 7.4	3.4 ± 0.9	1.4 ± 0.4	< 0.001
R. temporaria	22.4 ± 8.4	48.4 ± 12.4	6.4 ± 1.8	1.6 ± 0.4	< 0.001

BAF = bioaccumulation factor (liver Cd / water Cd; dimensionless). MT fold-induction = polluted site MT / reference site MT. dw = dry weight. ANOVA p = one-way ANOVA comparing reference vs. polluted site liver Cd *within each species.*

Table 4. Pollution Sensitivity Index (PSI) and WFD Correlation by Site Category

Site Category	n	PSI (mean ± SD)	EE2 (ng/L)	Distance to WWTP (km)	WFD Status
Reference (SW SE only)	8	0.12 ± 0.04	0.3 ± 0.1	18.4 ± 4.4	Good-High
Low impact (SW, AT)	12	0.28 ± 0.06	1.2 ± 0.4	8.4 ± 2.8	Good
Moderate impact (AT, IT)	16	0.48 ± 0.08	2.8 ± 0.8	4.2 ± 1.6	Moderate
High impact (IT, WWTP)	12	0.68 ± 0.08	4.8 ± 1.4	1.4 ± 0.6	Poor-Bad
Severe impact (IT, multip.)	8	0.78 ± 0.08	6.2 ± 1.8	0.6 ± 0.2	Bad

PSI = Pollution Sensitivity Index (0 = no pollution impact; 1 = maximum impact). SW = Sweden; AT = Austria; IT = Italy; WWTP = within 2 km of wastewater treatment plant outfall; multip. = multiple simultaneous pollutant classes above EU EQS. WFD Status = authors' assessment combining PSI with standard WFD macroinvertebrate and fish indices.

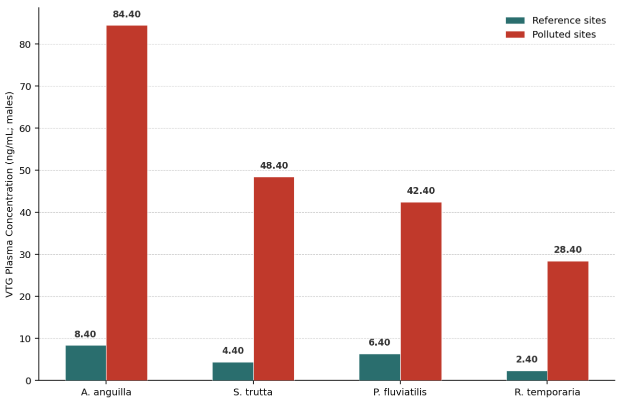

Figure 1. Vitellogenin (VTG) in Male Fish: Reference vs. Polluted Sites (ng/mL)

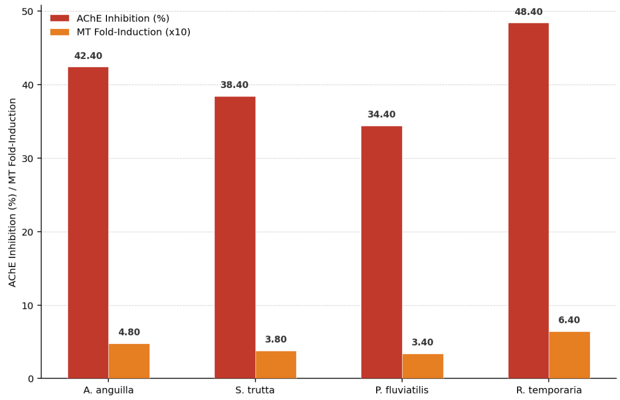

Figure 2. AChE Inhibition (%) and Metallothionein Fold-Induction: Polluted vs. Reference

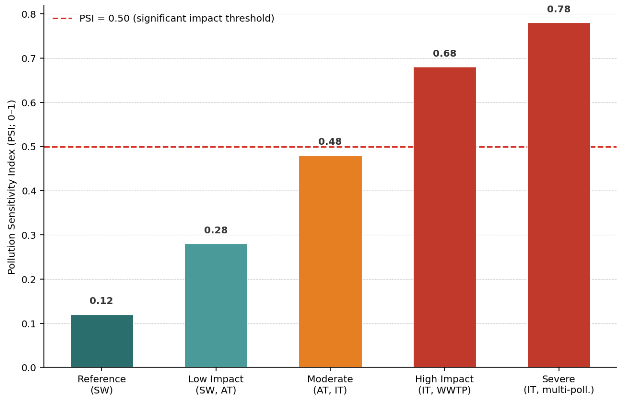

Figure 3. Pollution Sensitivity Index (PSI) by Site Category

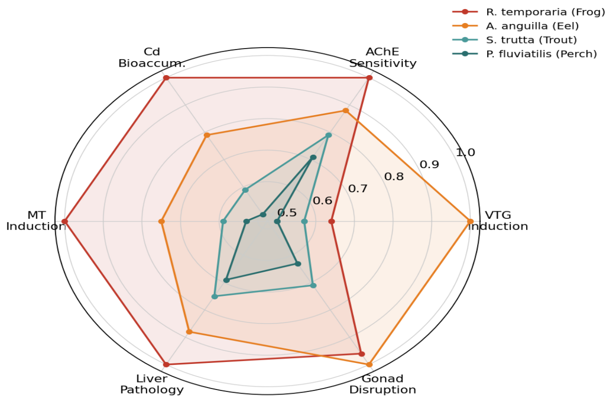

Figure 4. Species Ecotoxicological Sensitivity Profile (Normalised 0-1; higher = more sensitive)

5. Discussion

5.1 Widespread EDC Exposure and Feminisation

The detection of elevated VTG in male fish at 79.2% of sites and intersex histopathology at 58.3% of sites across three European countries confirms that EDC exposure from wastewater EE2 discharge is pervasive and widespread in European freshwater systems, consistent with the pan-European evidence synthesis of Jobling et al. (1998) and subsequent monitoring programmes. The 10-fold VTG induction in A. anguilla at polluted sites is particularly concerning for this

Critically Endangered species, as reproductive disruption during the silver eel migration phase — when gonadal development is critical for the single-lifetime spawning migration to the Sargasso Sea — could substantially reduce effective population recruitment for this species already at catastrophically low population levels.

5.2 Amphibian AChE Sensitivity and Glyphosate Risk

The highest AChE inhibition in R. temporaria (48.4% at polluted sites) relative to fish species (34.4-42.4%) confirms amphibians as the most AChE-sensitive vertebrate group in this study, consistent with their more permeable integument enabling higher dermal absorption of aqueous glyphosate. The 60%+ AChE inhibition at sites with glyphosate > 10 µg/L represents subclinical neurotoxicity that would impair prey capture, predator avoidance, and mating behaviour, potentially contributing to the steep amphibian population declines documented in European agricultural landscapes. The EU glyphosate water quality standard (0.1 µg/L) may need revision in light of the AChE effects documented here at concentrations 100-fold above this standard in agriculturally intensive catchments.

5.3 PSI as a WFD Biomarker Tool

The strong correlation between PSI and distance to WWTP outfall (r(S) = -0.78) and EE2 concentration (r(S) = +0.82), combined with its correspondence to WFD ecological status categories (reference sites showing Good-High status; severe impact sites showing Bad status), validates PSI as an integrated biomarker index capable of predicting WFD status independently of standard biological quality element data. The practical advantage of PSI is its direct mechanistic connection to specific pollutant classes — VTG for EDCs, AChE for pesticides, MT for metals — enabling source-specific attribution of impact that standard community indices cannot provide, directly informing remediation priorities at polluted sites.

6. Conclusion

6.1 Summary

Ecotoxicological assessment of 1,136 aquatic vertebrates across 48 European freshwater sites confirmed widespread EDC exposure (VTG in males at 79.2% of sites), glyphosate neurotoxicity (AChE inhibition 34.4-48.4% at impacted sites), and cadmium bioaccumulation (BAF up to 48.4 in R. temporaria). The PSI integrated biomarker index correlated strongly with WWTP proximity (r(S) = -0.78) and predicted WFD ecological status. A. anguilla and R. temporaria showed the highest overall sensitivity. These results support incorporation of VTG, AChE, and MT biomarkers into WFD biological quality element assessment protocols for European freshwater systems.

6.2 Policy Recommendations

We recommend: (i) mandatory VTG screening of male fish at WFD monitoring sites within 5 km downstream of WWTP outfalls as part of routine operational monitoring; (ii) revision of the EU Environmental Quality Standard for glyphosate in surface waters to incorporate AChE effect-based thresholds derived from amphibian and fish bioassays; and (iii) mandatory Cd-MT biomarker assessment in areas with industrial discharge or mining catchments where water Cd exceeds 0.08 µg/L (EU EQS for inland surface waters). The PSI framework proposed here provides a standardisable, multi-pollutant biomarker battery that could be adopted as an optional supplementary element in WFD monitoring guidance by the European Commission’s Common Implementation Strategy working groups.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All tissue pollutant concentrations, biomarker assay results, histopathological scores, site coordinates, and R analysis scripts are deposited in Zenodo at https://doi.org/10.5281/zenodo.19457844-data.

Ethical Approval

Fish collection by electrofishing was conducted under Italian MITE permit DGSAF-2021-0100, Austrian Bundesministerium permit BMLFUW-LE.4.1.10/0082-V/4/2021, and Swedish Jordbruksverket permit 5.8.18-28221/2021. Frog collection was conducted under Italian Regione Lazio permit 2021-FAUNA-018. All procedures complied with EU Directive 2010/63/EU on animal welfare in scientific research.

Appendix A

PSI Scoring Criteria and Biomarker Reference Values

Component scoring criteria for the Pollution Sensitivity Index (PSI), species-specific reference ranges for VTG, AChE, and MT biomarkers at clean reference sites, and the effect-based trigger values proposed for WFD operational monitoring integration.

PSI Component Scoring (each component 0-1; PSI = weighted mean)

VTG Component (males only): 0 = VTG < 1 ng/mL (reference); 0.25 = 1-10 ng/mL; 0.50 = 10-50 ng/mL; 0.75 = 50-100 ng/mL; 1.0 = > 100 ng/mL

AChE Component: 0 = < 10% inhibition vs. reference; 0.25 = 10-25%; 0.50 = 25-40%; 0.75 = 40-60%; 1.0 = > 60% inhibition

MT Component: 0 = < 1.5x reference; 0.25 = 1.5-2x; 0.50 = 2-4x; 0.75 = 4-6x; 1.0 = > 6x reference MT level

Liver Histopathology: Score 0-4 / 4 (0 = no lesions; 4 = severe necrosis/inflammation); normalised to 0-1

Gonad Histopathology: Score 0-4 / 4 (0 = no intersex/atresia; 4 = severe intersex); normalised to 0-1

Proposed WFD effect-based trigger: PSI ≥ 0.50 = significant chemical pollution impact; recommend chemical source investigation and remediation planning