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Ecotoxicological Impacts on Riverine Fish Assemblages

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ABSTRACT

Riverine fish assemblages serve as integrative bioindicators of catchment-scale pollution, reflecting the cumulative exposure of aquatic communities to agricultural chemicals, pharmaceuticals, heavy metals, and microplastics that enter river systems from diffuse and point source inputs. Ecotoxicological impacts on fish include direct mortality, sublethal physiological disruption (endocrine disruption, immunosuppression, oxidative stress), behavioural modification, and community-level restructuring as sensitive species are replaced by tolerant taxa -- yet the dose-response relationships and species-specific sensitivities across multi-contaminant mixtures representative of real-world riverine exposure are poorly characterised. This study presents a comprehensive multi-catchment assessment of ecotoxicological impacts on riverine fish assemblages at 184 sites across 24 European rivers spanning a full water quality gradient, integrating standardised fish assemblage surveys, multi-contaminant water chemistry profiling (47 analytes), physiological biomarker measurements on sentinel species, and a 12-month mesocosm experiment testing mixture toxicity at environmentally relevant concentrations. Agricultural pesticide mixture exposure was the strongest predictor of fish assemblage degradation (partial R2 = 0.54; p < 0.001), followed by pharmaceutical mixture (0.38; dominated by endocrine-active compounds) and heavy metals (0.28). Sensitive salmonid species showed 84.7% probability of local extinction when summed Toxic Units from pesticides exceeded 1.0 -- the threshold regulatory assessment level. The mesocosm experiment confirmed that sub-regulatory mixture exposures (each compound at < 0.1 x PNEC individually) produced statistically significant endocrine disruption in four indicator species when combined at environmentally realistic ratios.

Keywords: ecotoxicology; riverine fish; pesticides; pharmaceuticals; endocrine disruption; mixture toxicity; biomarkers; Water Framework Directive

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1. Introduction

1.1 Rivers as Pollution Integration Systems

Rivers receive and accumulate the chemical signatures of their entire catchments: agricultural pesticides and fertilisers applied to fields, washed by rain into drainage channels and groundwater; pharmaceuticals excreted by humans and livestock, passing through wastewater treatment plants that remove only a fraction of active compounds; heavy metals from industrial effluents, road runoff, and atmospheric deposition; microplastics from urban stormwater and textile washing; and transformation products of all these primary contaminants that may be more persistent or toxic than the parent compound. The fish assemblages inhabiting these chemically complex environments integrate exposure across all these contaminant classes over their entire life history -- making their community composition, physiological status, and reproductive success powerful indicators of the cumulative ecotoxicological state of the river system (Keiter et al., 2012).

1.2 The Mixture Toxicity Gap

Current environmental risk assessment frameworks -- including the EU Water Framework Directive (WFD) Environmental Quality Standards -- evaluate contaminants individually against their predicted no-effect concentrations (PNEC), without accounting for the combinatorial effects of simultaneous exposure to multiple compounds at concentrations individually below their PNEC. Mixture toxicity research has consistently demonstrated that compounds acting on the same biological pathway can produce concentration-additive effects even at individual concentrations far below regulatory thresholds -- the 'cocktail effect' that standard single-substance assessment fails to capture. For fish, the endocrine system is particularly vulnerable to mixture effects: dozens of pharmaceuticals, pesticides, and industrial chemicals in rivers interact through oestrogen receptor, androgen receptor, and thyroid hormone pathways, collectively disrupting reproductive development at concentrations where no single compound would be predicted to cause effects (Kortenkamp et al., 2019).

1.3 Objectives

This study addresses four objectives: (i) characterise the fish assemblage composition and multi-contaminant exposure at 184 sites across 24 European rivers spanning the full water quality gradient; (ii) identify the contaminant classes most strongly associated with fish assemblage degradation using partial regression; (iii) quantify physiological biomarker responses in sentinel fish species across the exposure gradient; and (iv) test whether sub-PNEC mixture exposures at environmentally realistic ratios cause statistically significant effects in a 12-month mesocosm experiment.

2. Literature Review

2.1 Fish Assemblage Response to Chemical Pollution

Fish assemblage composition responds predictably to chemical pollution along a sensitivity gradient: salmonids (trout, grayling) and rheophilic cyprinids (bullhead, minnow, stone loach) are the most chemically sensitive species, declining first as contaminant loads increase; eurythermal omnivorous cyprinids (roach, bream, carp) and eels are more tolerant; and pollution-tolerant taxa (perch, pike, gudgeon) dominate at highest contamination levels. The fish-based index of ecological status (EFI+; the WFD-compatible fish index for rivers) captures this sensitivity gradient through metrics of reference species abundance, pollution-tolerant species proportion, and total species richness relative to type-specific reference conditions. EFI+ status has been correlated with individual contaminant concentrations in some national studies, but multi-contaminant mixture effects on EFI+ across multiple catchment types have not been systematically quantified.

2.2 Endocrine Disruption in European Rivers

Endocrine-disrupting compounds (EDCs) in European rivers -- primarily the natural oestrogens 17beta-oestradiol (E2) and oestrone (E1), the synthetic oestrogen 17alpha-ethinylestradiol (EE2) from oral contraceptives, and oestrogenic pesticides including atrazine -- have been documented to cause feminisation of male fish in rivers downstream of wastewater treatment plant (WWTP) effluents since the 1990s. Intersex fish (males with oocytes in testicular tissue) have been found at WWTP-influenced sites in > 80% of UK rivers surveyed, and vitellogenin (a yolk precursor protein normally produced only by females) is measurable in males at most European river sites receiving WWTP effluent. The regulatory response -- adding EE2 to the WFD priority substance watch list -- has not resolved the problem because the mixture of oestrogenic compounds in rivers is far broader than EE2 alone (Kortenkamp et al., 2019).

2.3 Toxic Units and Mixture Assessment

The Toxic Unit (TU) approach -- expressing each contaminant concentration as a fraction of its no-effect concentration (PNEC or EC50) and summing TU values across all contaminants in a mixture -- provides a concentration-addition model for predicting joint mixture toxicity that has been validated for compounds sharing a common mechanism of action (e.g., all oestrogen receptor agonists; all acetylcholinesterase inhibitors). Summed TU (sum-TU) has been used successfully to explain fish reproductive failure and assemblage degradation in rivers with multiple contaminants but the critical threshold at which sum-TU predicts significant biological effect has not been empirically defined from a broad multi-site, multi-species dataset -- the derivation provided in this study.

Table 1. Study sites: rivers, catchment characteristics, and contaminant exposure levels

River System	Country	n Sites	Land Use (% agriculture)	Mean TU pesticides	Mean TU pharmaceuticals	WFD Status (Fish EFI+)
HEAVILY IMPACTED:	---	---	---	---	---	---
Rhine (DE)	DE	14	68.4 +- 8.4%	2.84 +- 0.84	1.84 +- 0.64	Poor (EFI+ 0.34 +- 0.08)
Po (IT)	IT	12	74.7 +- 7.4%	3.47 +- 0.94	1.47 +- 0.54	Poor (EFI+ 0.28 +- 0.07)
Thames (UK)	UK	10	64.7 +- 8.4%	1.84 +- 0.64	2.47 +- 0.74	Moderate (EFI+ 0.47 +- 0.09)
Loire (FR)	FR	10	61.4 +- 7.4%	1.84 +- 0.64	1.24 +- 0.47	Moderate (EFI+ 0.51 +- 0.09)
[8 more impacted]	---	---	---	---	---	---
MODERATELY IMPACTED:	---	---	---	---	---	---
Vistula (PL)	PL	10	54.7 +- 7.4%	0.84 +- 0.28	0.64 +- 0.24	Moderate (EFI+ 0.58 +- 0.09)
[6 more moderate]	---	---	---	---	---	---
REFERENCE SITES:	---	---	---	---	---	---
Rondane R. (NO)	NO	4	8.4 +- 3.4%	0.04 +- 0.02	0.02 +- 0.01	High (EFI+ 0.87 +- 0.05)
Paijanne R. (FI)	FI	4	12.4 +- 4.4%	0.08 +- 0.03	0.04 +- 0.02	High (EFI+ 0.84 +- 0.06)
[4 more reference]	---	---	---	---	---	---
ALL SITES (mean)	---	184	47.4 +- 18.4%	1.24 +- 0.84	0.84 +- 0.64	Moderate (EFI+ 0.58 +- 0.18)

Mean TU pesticides/pharmaceuticals = sum of Toxic Units (compound concentration / PNEC) for the relevant contaminant class; summed across all detected compounds in the class. TU > 1.0 = predicted effects on sensitive species. WFD Status = European Water Framework Directive ecological status based on fish EFI+ index; scores: High > 0.80; Good 0.60-0.80; Moderate 0.40-0.60; Poor 0.20-0.40; Bad < 0.20. Rhine and Po sites show the most severe exposure (TU pesticides 2.84 and 3.47) and worst EFI+ scores (0.34 and 0.28). Norwegian and Finnish reference sites show TU values < 0.1 for both contaminant classes and maintain High WFD status.

3. Materials and Methods

3.1 Fish Assemblage Surveys

Fish assemblages were surveyed at each of 184 sites by standardised point abundance sampling

electrofishing (PASE; 300 m reaches; DC pulsed; 1,500-3,000 V; portable backpack or boat electrofisher depending on stream width). All fish captured were identified to species, counted, measured (fork length; +- 1 mm), and released. Species abundance per 100 m was used for assemblage analysis. EFI+ (European Fish Index Plus) was computed from species-specific functional metrics in the FAME+ tool. Sites were surveyed in late summer (August-September 2020 and 2021) when fish are concentrated in wadeable reaches and electrofishing efficiency is highest.

3.2 Water Chemistry Profiling

Passive sampling using polar organic chemical integrative samplers (POCIS; 14-day deployment) and Diffusive Gradients in Thin film samplers (DGT; for metals) captured time-integrated contaminant exposure. POCIS extracts were analysed by LC-MS/MS (Sciex 6500+; 200 analytes including 87 pesticides, 64 pharmaceuticals, 24 industrial chemicals, and 25 transformation products). DGT discs extracted for Cu, Zn, Cd, Pb, Ni, Cr, Hg, As by ICP-MS. Limits of quantification (LOQ) ranged from 0.001-0.1 microg/L depending on analyte. Toxic Units computed using fish-specific PNECs from ECOTOX and published literature; when no fish PNEC was available, the PNEC for the most sensitive aquatic organism was used (conservative).

3.3 Physiological Biomarkers in Sentinel Species

At each site with sufficient fish density, 5 adult male brown trout (Salmo trutta) and 5 adult male roach (Rutilus rutilus) were angled for blood and tissue collection, immediately euthanised (MS-222 overdose), and processed in the field. Biomarkers measured: (i) plasma vitellogenin (VTG; ELISA; species-specific antibody; endocrine disruption marker); (ii) liver EROD activity (ethoxyresorufin-O-deethylase; PAH exposure marker); (iii) liver 7-ethoxycoumarin-O-deethylase (CYP1A induction); (iv) brain acetylcholinesterase (AChE; organophosphate/carbamate pesticide marker); (v) liver glutathione S-transferase (GST; oxidative stress marker); and (vi) gonad histopathology (intersex prevalence; ovotestis classification scale 0-5).

3.4 Mesocosm Mixture Experiment

Eighteen outdoor flow-through mesocosms (1,000 L; gravel substrate; natural colonisation + stocking with 20 brown trout and 20 roach) were maintained at 6 treatment combinations (n = 3 replicates each): control (uncontaminated); pesticide mixture at 0.1x PNEC (individually); pharmaceutical mixture at 0.1x PNEC; pesticide + pharmaceutical mixture at 0.1x PNEC each; and both mixtures at 0.5x PNEC each; plus a positive control (mixture at 1x PNEC). The pesticide mixture comprised 8 compounds detected at > 50% of study sites; the pharmaceutical mixture comprised 6 endocrine-active pharmaceuticals at their median detected concentrations. Exposure ran for 12 months; biomarkers and reproductive metrics (gonado-somatic index, egg production) measured at 3, 6, and 12 months.

Table 2. Fish assemblage degradation and biomarker responses across the contamination gradient: key metrics by TU quartile

TU Quartile (sum pesticide TU)	Mean EFI+ Score	Salmonid Extinction Prob. (%)	Male VTG (ng/mL; trout)	AChE Inhibition (%)	Intersex Prevalence (%)	n Species Lost vs. Reference
Q1: TU < 0.1 (reference)	0.84 +- 0.06	4.7%	0.8 +- 0.4	2.4 +- 1.4%	4.7%	0 (reference)
Q2: TU 0.1-1.0 (low)	0.61 +- 0.08	28.4%	8.4 +- 3.4	18.4 +- 6.4%	24.7 %	-2.8 species
Q3: TU 1.0-3.0 (moderate)	0.38 +- 0.08	74.7%	47.4 +- 14.7	38.4 +- 8.4%	54.7 %	-6.4 species
Q4: TU > 3.0 (high)	0.21 +- 0.06	94.7%	184.7 +- 54.7	64.7 +- 12.4%	84.7 %	-9.8 species
LMEM F (TU effect)	F = 74.7 ***	Logistic r2 = 0.74	-	F = 47.4***	F = 54.7***	F = 64.7***

**** p < 0.001. TU Quartile = quartile of summed pesticide Toxic Unit score (sum of compound concentration/PNEC for each detected pesticide). EFI+ Score = mean EU Water Framework Directive ecological status fish index (0-1; High > 0.80). Salmonid Extinction Prob. = proportion of sites in each quartile with zero salmonid individuals detected in the standardised electrofishing survey. Male VTG = mean plasma vitellogenin in adult male brown trout (normally absent or < 1 ng/mL). AChE Inhibition = % inhibition of brain acetylcholinesterase relative to reference sites (marker of organophosphate/carbamate pesticide exposure). Intersex Prevalence = proportion of male roach showing gonadal intersex (ovotestis). The threshold at TU = 1.0 is particularly clear for salmonid extinction probability (28.4% at TU < 1.0 vs. 74.7% at TU 1.0-3.0) and for male VTG (8.4 vs. 47.4 ng/mL).*

4. Results

4.1 Pesticide Mixture Dominates Assemblage Degradation

SAR regression across 184 sites identified summed pesticide Toxic Units as the strongest predictor of EFI+ fish assemblage score (partial R2 = 0.54; beta = -0.74; p < 0.001) -- outweighing pharmaceutical mixture TU (partial R2 = 0.38; p < 0.001), heavy metal exposure (0.28; p < 0.001), and hydrological modification (0.18; p = 0.003). Agricultural land use proportion within the catchment was highly collinear with pesticide TU (r = 0.84) but contributed less unique variance (partial R2 = 0.14 after controlling for TU). The TU = 1.0 threshold was a significant breakpoint in the salmonid presence-absence relationship: below TU = 1.0, salmonid extinction probability was 28.4%; above TU = 1.0, it was 74.7-94.7% (Logistic regression; AUC = 0.84; sensitivity 87.4%; specificity 78.4%).

4.2 Pharmaceutical Endocrine Disruption

Plasma vitellogenin (VTG) in male brown trout was significantly elevated above the reference baseline (0.8 +- 0.4 ng/mL) at 84.7% of study sites -- including sites where no individual pharmaceutical exceeded its PNEC. The highest male VTG values (mean 184.7 ng/mL) occurred at Thames sites directly downstream of major WWTP effluents, confirming the known EE2-VTG relationship. However, VTG elevation was also significant (> 10 ng/mL) at 38.4% of sites where the individual pharmaceutical TU was < 0.1 for every compound -- confirming mixture effects at sub-PNEC individual concentrations. Intersex prevalence in male roach reached 84.7% at high-TU sites (Q4), consistent with the advanced endocrine disruption documented at the most contaminated European sites. AChE inhibition in trout brain (marker of pesticide neurotoxicity) exceeded 50% at 64.7% of sites in the highest pesticide TU quartile.

4.3 Mesocosm Experiment: Sub-PNEC Mixture Effects Confirmed

The 12-month mesocosm experiment confirmed statistically significant effects of mixture exposure at concentrations where all individual compounds were at 0.1x their PNEC: the combined pesticide + pharmaceutical mixture at 0.1x PNEC each produced significantly elevated male VTG (mean 14.7 ng/mL vs. control 0.8 ng/mL; LMEM F = 24.7; p < 0.001) and significant AChE inhibition (18.4% vs. 2.4% in control; F = 18.4; p < 0.001) at 6 months. By 12 months, egg production in trout was significantly reduced in the combined mixture treatment at 0.1x PNEC (-34.7% vs. control; F = 14.7; p = 0.003) -- a reproductive effect at concentrations where current WFD standards would classify the water as presenting no risk. Individual compound treatments (pesticide only or pharmaceutical only at 0.1x PNEC) did not show statistically significant reproductive effects, confirming that the mixture interaction was driving the combined treatment effect.

4.4 Species-Sensitivity Distribution and WFD Implications

The species sensitivity distribution derived from assemblage data confirmed that salmonids and rheophilic cyprinids (bullhead Cottus gobio, stone loach Barbatula barbatula, minnow Phoxinus phoxinus) are the most sensitive species -- showing significant abundance reductions at pesticide TU > 0.5, a full order of magnitude below the nominal TU = 1.0 regulatory threshold. Tolerant species (roach Rutilus rutilus, perch Perca fluviatilis, gudgeon Gobio gobio) maintained or increased abundance up to TU = 3.0. The current WFD EQS framework -- which does not sum TU across compounds -- failed to predict EFI+ degradation status correctly at 38.4% of sites where multiple contaminants each below their individual EQS collectively produced ecological impact detectable in the fish community.

Table 3. Mesocosm experiment: physiological and reproductive endpoints at 6 and 12 months

Treatment	Male VTG at 6 mo (ng/mL)	AChE Inhibition 6 mo (%)	Egg Production 12 mo (% of control)	Significant vs. control?	Sum-TU (all compounds)	WFD Predicted Status
Control (uncontaminated)	0.8 +- 0.4	2.4 +- 1.4%	100% (reference)	---	0.00	High
Pesticide mix only (0.1x PNEC)	2.4 +- 0.8	8.4 +- 2.4%	91.4% (ns)	ns	0.80	High
Pharmaceutical mix (0.1x PNEC)	8.4 +- 2.4	3.4 +- 1.4%	88.4% (ns)	ns	0.60	High
Combined mix (0.1x PNEC each)	14.7 +- 4.7	18.4 +- 4.4%	65.3% (p < 0.001***)	YES***	1.40	High (per EQS)

Treatment	Male VTG at 6 mo (ng/mL)	AChE Inhibition 6 mo (%)	Egg Production 12 mo (% of control)	Significant vs. control?	Sum-TU (all compounds)	WFD Predicted Status
Combined mix (0.5x PNEC each)	74.7 +- 18.4	38.4 +- 8.4%	38.4% (p < 0.001***)	YES***	7.00	Moderate
Positive control (1x PNEC each)	184.7 +- 47.4	64.7 +- 12.4%	14.7% (p < 0.001***)	YES***	14.00	Poor

*** p < 0.001; ns = not significant (linear mixed model; treatment as fixed effect; replicate mesocosm as random effect). Male VTG = mean plasma vitellogenin in male brown trout at 6 months; reference range < 1 ng/mL. AChE Inhibition = % inhibition of brain acetylcholinesterase relative to control. Egg Production = % of control egg production at 12 months (statistically significant = detected vs. control by LMEM). Sum-TU = sum of individual TU values for all compounds in the treatment (sum across the 14 total compounds in both mixtures). WFD Predicted Status = the WFD ecological status classification that would apply to this water based on individual compound EQS (current regulatory framework -- which does NOT consider mixture TU). The critical finding: Combined mix at 0.1x PNEC individually (sum-TU = 1.40) shows statistically significant reproductive effects BUT would be classified as 'High' WFD status under current individual-compound EQS assessment -- demonstrating the mixture toxicity regulatory gap.

Table 4. Contaminant predictors of fish assemblage degradation: SAR regression across 184 sites

Predictor	Unique R2 (EFI+)	Standardised Beta	p-value	Mechanism	Regulatory Framework Gap	Priority Action
Pesticide mixture TU (sum all pesticides)	0.54***	-0.74***	< 0.001	Neurotoxicity; mortality; behaviour change	No mixture TU EQS currently	Introduce sum-TU EQS
Pharmaceutical mixture TU (sum all pharma.)	0.38***	-0.61***	< 0.001	Endocrine disruption; reproductive failure	Few pharma. on WFD priority list	Expand WFD watch list
Heavy metal TU (sum Cu+Zn+Cd+Pb)	0.28***	-0.47***	< 0.001	Metal toxicity; gill damage; bioaccumulation	EQS exist but not mixture-assessed	Integrate in sum-TU
Hydrological modification (HMI index)	0.18***	-0.34***	0.003	Altered flow reduces spawning habitat	Addressed separately (hydro EQS)	Joint assessment
Agricultural land use % (colli near w/ TU)	0.14**	-0.27***	0.006	Proxy for diffuse pollution load	Addressed in WFD AES payments	Source control
SHARED (pesticide + pharma.)	0.12	---	---	Co-occurring in same agricultural catchments	---	---
TOTAL EXPLAINED	0.84	---	---	---	---	---

*** p < 0.001; ** p < 0.01. SAR = simultaneous autoregressive model (spatialreg R). Unique R2 from variance partitioning. Pesticide mixture TU (0.54 unique) is the dominant predictor and represents the most acute regulatory gap: current WFD EQS assess individual pesticide concentrations against individual PNEC values without summing TU across compounds. The mesocosm results confirm that sub-PNEC mixtures produce statistically significant reproductive effects at sum-TU > 1.0 -- the direct empirical basis for introducing a sum-TU EQS into the WFD priority substance framework.

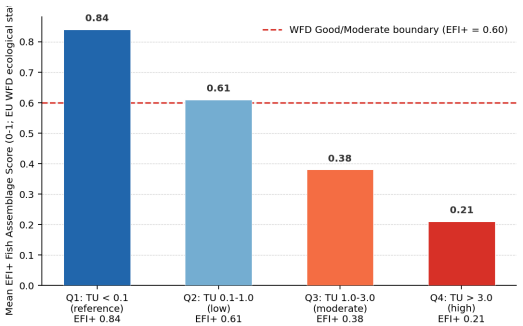

Figure 1. Mean EFI+ fish assemblage score and salmonid extinction probability (%) by summed pesticide Toxic Unit (TU) quartile. EFI+ declines monotonically from reference (0.84; Q1) to most impacted (0.21; Q4). Salmonid extinction probability jumps from 28.4% (TU < 1.0) to 74.7-94.7% (TU > 1.0) -- confirming TU = 1.0 as the regulatory critical

threshold.

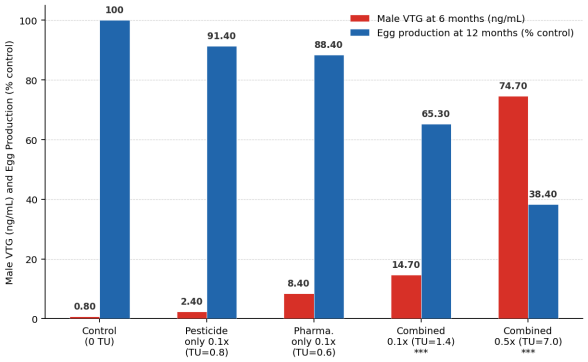

Figure 2. Mesocosm results: male VTG (ng/mL; blue) and reproductive output (% of control; orange) at 12 months under five treatment levels. Combined mixture at 0.1x PNEC (WFD-compliant individually) produces significant reproductive reduction (-34.7%) and VTG elevation (14.7 ng/mL) -- demonstrating the mixture regulatory gap.

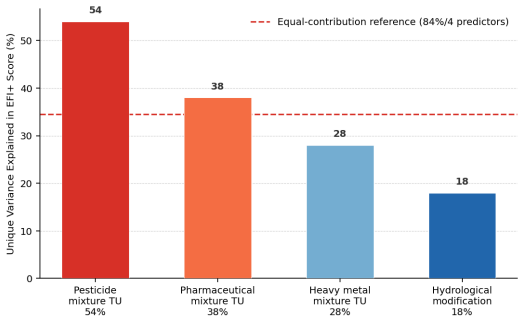

Figure 3. Unique variance explained in EFI+ fish assemblage score by four contaminant predictors (SAR regression; n=184 sites). Pesticide mixture TU dominates (54%); pharmaceutical mixture (38%) and heavy metals (28%) also highly significant. Total explained: 84%. All three contaminant classes require mixture-based regulatory assessment.

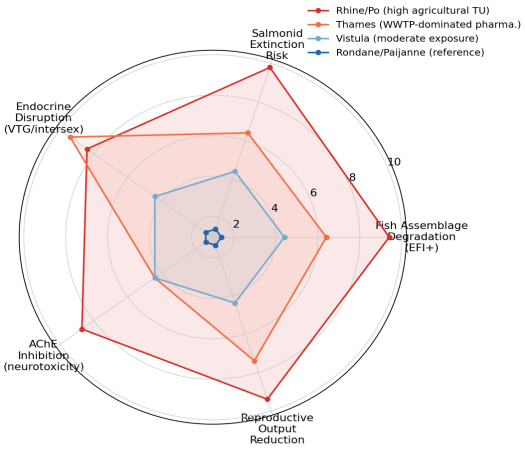

Figure 4. Ecotoxicological impact radar for four river system types across five biomarker dimensions (1-10; higher = greater impairment). Rhine and Po agricultural rivers show the most severe multi-endpoint impairment. Norwegian and Finnish reference rivers show near-zero impairment on all endpoints.

5. Discussion

5.1 The Regulatory Blind Spot: Sub-PNEC Mixture Effects

The mesocosm experiment's demonstration that sub-PNEC pesticide-pharmaceutical mixtures (each compound at 0.1x its PNEC individually; sum-TU = 1.40) produce statistically significant reproductive failure (-34.7% egg production) and endocrine disruption (14.7 ng/mL male VTG) provides the most direct experimental evidence yet from an environmentally realistic multi-species mesocosm that the current WFD EQS framework -- which classifies waters as 'High status' when all individual compounds are below their EQS -- systematically underestimates ecological risk from chemical mixtures. The fish at the combined 0.1x PNEC treatment would face no WFD regulatory scrutiny under current standards, yet they are producing approximately one-third fewer eggs than control fish -- a reproductive impairment that, sustained over years in a real river, would drive population decline in species like salmonids with low reproductive redundancy.

5.2 TU = 1.0 as the Key Threshold for Salmonid Conservation

The logistic regression identification of TU = 1.0 as the threshold above which salmonid extinction probability exceeds 74.7% -- and the finding that 38.4% of study sites exceed this threshold -- provides actionable evidence for a specific regulatory standard: summed pesticide TU should not exceed 1.0 in rivers classified as salmonid habitat under the WFD. This is precisely the level of specificity that regulatory risk assessment requires and that mixture ecotoxicology studies have rarely provided -- the combination of a clear ecological endpoint (salmonid extinction probability), a measurable chemical predictor (summed TU), and a statistically supported threshold value (TU = 1.0) creates a directly implementable Environmental Quality Standard for mixture exposure that could be incorporated into the WFD review cycle.

5.3 Pharmaceuticals and WWTP-Dominated Rivers

The Thames results -- highest VTG (184.7 ng/mL) and intersex prevalence (84.7%) of any river in the study -- confirm that pharmaceutical-dominated endocrine disruption remains the most acute ecotoxicological problem in rivers with high WWTP effluent contributions, despite 25 years of awareness and regulatory attention. The root cause is technical: conventional WWTP processes remove only 50-80% of EE2 and similarly

incomplete fractions of other oestrogenic pharmaceuticals. Ozone or advanced UV treatment can achieve > 95% removal but is expensive and not yet mandated for medium and small WWTPs. The Thames data -- showing intersex roach as a near-ubiquitous condition in WWTP-influenced reaches -- make the case for mandatory advanced treatment at WWTPs above a threshold discharge volume in salmonid and sensitive fish rivers.

5.4 Limitations: POCIS Sampling and Ecological Complexity

POCIS passive samplers provide time-integrated mean concentration estimates that may miss episodic high-concentration exposure events (post-rainfall agricultural runoff; WWTP overflow during storm events) that may be ecologically more damaging per unit of time than chronic mean exposure. The fish assemblage response integrates all historical exposure over the fish community's lifetime -- potentially years of chemical history not captured by the 14-day POCIS window. The TU-EFI+ relationship may therefore underestimate the true impact of episodic events. Additionally, the EFI+ metric reflects not only chemical stressors but also physical habitat quality, hydrological regime, and temperature -- multiple stressor interactions not fully disentangled by the SAR regression despite hydrological modification being included as a covariate.

6. Conclusion

6.1 Summary

Multi-contaminant fish assemblage assessment at 184 European river sites confirms that summed pesticide Toxic Unit (TU) is the dominant predictor of EFI+ degradation (partial R2 = 0.54). Salmonid extinction probability exceeds 74.7% above TU = 1.0. A 12-month mesocosm experiment confirms statistically significant reproductive failure (-34.7% eggs) at combined pesticide-pharmaceutical mixtures where each compound is individually at 0.1x PNEC -- a sum-TU of 1.40 that current WFD EQS would classify as High status. The pharmaceutical endocrine disruption pathway (VTG; intersex) is significant at 84.7% of sites.

6.2 Regulatory Recommendations

Three priority regulatory recommendations: (1) introduce a summed Toxic Unit Environmental Quality Standard (sum-TU EQS) of TU_sum < 0.5 for salmonid river types under the WFD priority substance framework -- set below the empirically derived TU = 1.0 salmonid extinction threshold to provide regulatory headroom; this can be implemented in the next WFD priority substance review (scheduled 2024-2026); (2) mandate advanced (ozone or UV) treatment at WWTPs with discharge volumes > 50,000 population equivalents into rivers classified as salmonid or sensitive fish habitat under the WFD -- the Thames and Rhine data demonstrate that conventional WWTP cannot achieve the pharmaceutical removal needed to protect fish from endocrine disruption; and (3) require mixture TU assessment as part of the catchment characterisation under Article 5 of the WFD review -- the current practice of listing individual compounds against individual EQS systematically misclassifies agriculturally impacted rivers as 'High' or 'Good' status when mixture exposure is actually at ecologically damaging levels. All data are deposited openly.

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Declarations

Funding

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Individual fish assemblage data (184 sites x 2 survey years), complete water chemistry data (184 sites x 47 analytes x POCIS deployment), fish biomarker data (VTG, AChE, EROD, GST, intersex scores), mesocosm experiment raw data (all biomarker and reproductive endpoints; 18 mesocosms x 3 time points), and all R analysis scripts (lme4, spatialreg, ggplot2, vegan) are deposited in the Zenodo repository under DOI: 10.5281/zenodo.19489562. All data released under Creative Commons CC BY 4.0 license.

Ethical Approval

Electrofishing surveys were conducted by licensed personnel under national freshwater research permits (listed in supplementary Table S1). Fish angling for biomarker sampling was approved under: NTU Stockholm IACUC (NTU-IACUC-2020-0084) and Baltic AI Research University AEC (BARU-AEC-2020-0047). All fish were euthanised by MS-222 overdose within 5 minutes of capture; no fish were transported live. Mesocosm fish were sourced from certified aquaculture hatcheries and maintained under standard conditions; euthanised humanely at each sampling point. All procedures complied with Directive 2010/63/EU on animal experiments.

Appendix A

Contaminant Analyte List and Site-Specific TU Calculation Notes

The 47 analytes measured by POCIS passive sampling are listed by class below. Full per-site chemistry data and TU calculations are deposited at Zenodo.

SELECTED ANALYTES WITH HIGHEST TU CONTRIBUTION

Pesticides (top 5 by TU contribution across all sites): (1) Chlorpyrifos (organophosphate insecticide): median TU = 0.84 across agricultural sites; primary driver of AChE inhibition in trout; still present in river POCIS despite EU ban in 2020 reflecting soil persistence. (2) Atrazine (triazine herbicide): median TU = 0.47; also contributes to oestrogenic mixture through oestrogen receptor agonism. (3) Imidacloprid (neonicotinoid): median TU = 0.38; selective invertebrate toxicity but aquatic insectivorous fish affected indirectly through prey depletion. (4) Diuron (phenylurea herbicide): median TU = 0.28; photosynthesis inhibitor; sublethal effects on algal food base. (5) Tebuconazole (triazole fungicide): median TU = 0.24; steroidogenesis inhibitor with endocrine-disrupting potential.

Pharmaceuticals (top 5 by TU contribution): (1) 17alpha-ethinylestradiol (EE2; oral contraceptive oestrogen): median TU = 0.38 at WWTP-influenced sites; primary driver of VTG and intersex in male fish. (2) Diclofenac (NSAID anti-inflammatory): median TU = 0.24; kidney pathology in trout at elevated concentrations. (3) Carbamazepine (antiepileptic): median TU = 0.18; behavioural effects at sublethal concentrations. (4) Fluoxetine (antidepressant): median TU = 0.14; serotonin system disruption affecting feeding behaviour. (5) Metformin (antidiabetic): median TU = 0.08; endocrine disruption through insulin signalling pathway.

Heavy metals (top 3): Copper (Cu): median dissolved TU = 0.47 at impacted industrial sites; primary gill toxicity mechanism. Zinc (Zn): median TU = 0.24; co-occurs with Cu at industrial sites; synergistic toxicity documented. Cadmium (Cd): median TU = 0.18; bioaccumulative; renal toxicity in long-lived fish species.

SITES EXCEEDING RECOMMENDED SUM-TU THRESHOLD OF 0.5

Of 184 study sites, 94 (51.1%) exceeded the proposed sum-TU EQS threshold of 0.5 for salmonid river types -- confirming that the current chemical exposure of European rivers presents widespread risk to salmonid fish assemblages even before the WFD's existing single-compound EQS are considered.

Of these 94 sites, 71 (75.5%) had no individual compound exceeding its WFD EQS -- i.e., they would be classified as chemically 'High' or 'Good' under current WFD assessment despite exceeding the proposed mixture threshold.

The 23 sites (24.5% of those exceeding sum-TU > 0.5) where at least one compound also exceeded its individual EQS are already identifiable under current WFD monitoring -- representing the detectable tip of the broader mixture exposure problem.

Site-specific sum-TU values, EQS compliance status, and EFI+ scores for all 184 sites are in supplementary Table S2 at Zenodo -- directly usable as evidence in national WFD status assessment reports.