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A One Health framework integrating teratogenic risk and ecological assessment in freshwaters

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ABSTRACT

Freshwater ecosystems face mixture-dominated pressures that often elude conventional monitoring. We assessed eight rivers in Latium (Central Italy) to jointly evaluate ecological status and teratogenic risk within a One Health perspective. We combined in-situ physico-chemical measurements and elemental profiling by inductively coupled plasma mass spectrometry (ICP-MS; including the rare-earth tracer gadolinium) with two biological lines of evidence: benthic diatom assemblages to derive the Intercalibration Common Metric Index (ICMi) and screen teratological valves, and the *Hydra vulgaris* regeneration assay summarized as the Teratogenic Risk Index (TRI), with behavioural endpoints. Environmental conditions were heterogeneous, with eutrophication and high organic load at some sites. ICMi classified Almone and Arrone as Poor, Marta and Sacco as Good, and Mignone, Aniene, Tevere and Ninfa as High. TRI indicated Very High teratogenic risk at Almone; High at Marta, Sacco and Tevere; Low at Arrone, Mignone and Aniene; and No risk at Ninfa. Diatom teratologies were detected at all sites and peaked at Tevere. ICMi showed a negative association with gadolinium ($r = -0.76$, $p < 0.05$), whereas TRI and ICMi were not correlated. These results demonstrate that ecological status and teratogenic hazard need not converge. TRI captured organism-level developmental and neuro-functional impairment at low doses and in complex mixtures, even where ICMi was Good/High. Integrating organism- and community-level indicators with targeted chemistry offers a sensitive, cost-effective framework to flag hotspots, prioritize monitoring of emerging contaminants, and support risk management under the Water Framework Directive.

1. Introduction

Freshwaters provide vital ecosystem services yet represent a vanishingly small share of Earth's water budget: only ~2.9% is freshwater, and rivers account for just ~0.0001% - a stark reminder of their vulnerability (Horn, 2017; Musie and Gonfa, 2023). Rivers are dynamic, spatially structured systems where community composition varies predictably along source–mouth gradients (Lucadamo et al., 2012), consistent with the River Continuum Concept and later four-dimensional views of lotic ecosystems (Vannote et al., 1980). We adopt a One Health perspective, recognizing that early alterations in sentinel taxa (*Hydra*, diatoms) can foreshadow impacts on aquatic fauna and ecosystem services and may signal potential human exposure pathways in basins used for drinking water, irrigation, and recreation.

In Europe, the Water Framework Directive (WFD 2000/60/EC; (Directive, 2000/60/EC, 2000)) reframed monitoring by integrating chemical, hydromorphological and biological quality elements to achieve good ecological and chemical status. Implementation relies on typology and intercalibration to ensure comparability across Member States (European Commission, 2003; Buffagni et al., 2008), but progress has lagged in places (Alberton, 2021). Chemical status hinges on priority substances and environmental quality standards, yet complex mixtures and non-priority contaminants can cause biological impairment without breaching thresholds, underscoring the need for bioindication and ecotoxicological tools (Malafaia, 2025).

Within bioindication, diatoms are cornerstone indicators thanks to well-established ecological sensitivity and distinctive siliceous frustules (two valves with species-specific ornamentation) which enable reliable identification and responsiveness to environmental gradients (Martin-Jézéquel et al., 2000; Masouras et al., 2021). Under the WFD, ecological status for rivers is assessed via the Intercalibration Common Metric Index (ICMi), an ecological quality ratio that averages Pollution

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Sensitivity Index (IPS; (Coste, 1982)) and Trophic index (TI; (Rott et al., 1999)) components relative to type-specific reference conditions (high status = 1). Beyond composition, teratological frustule deformities, e.g., altered valve outline, striation and raphe, offer early functional signals of stress (Cantonati et al., 2014); seven canonical types are recognized, with Types 1–2 most frequent across stressors (Falasco et al., 2009a).

Ecotoxicology complements community indices by testing biological responses across organizational levels (molecular to behavioural) using organisms exposed to real matrices, shifting from single toxicants to environmental samples (Van Gestel and Van Brummelen, 1996; Moulinec et al., 2025). Among organism-level assays, the Hydra regeneration assay (HRA) quantifies regeneration rate (RR) and aberration frequency (AF) after decapitation; protocol refinements extended observations to 96 h and simplified the stage scale to 0–4 to improve resolution (Traversetti et al., 2017). These endpoints are combined in the Teratogenic Risk Index (TRI) to classify risk into five classes (no–very high) and have shown low correlation with traditional biotic indices, revealing complementary information for management (Traversetti et al., 2017; Cera et al., 2019, 2020). These organism-level responses align with Adverse Outcome Pathways (Leist et al., 2017) related to development and neuro-function, and therefore constitute health-relevant biological effect signals in addition to indicators of ecosystem integrity.

The growing occurrence of emerging contaminants, such as pharmaceuticals, endocrine-disrupting chemicals, and micro- and nanoplastics, underscores the need for integrated lines of evidence (Quadroni et al., 2024; Boahen et al., 2025). In microcosms, environmentally realistic nano/microplastic exposures increased diatom teratologies (notably Type 1) and altered *Hydra* regeneration/behaviour; TRI summarized these effects across treatments (Cesarini et al., 2023a). Likewise, gadolinium (Gd) from medical uses is traceable by Inductively Coupled Plasma Mass spectrometer (ICP-MS) in urban rivers; laboratory and field applications pairing chemistry with *Hydra* Regeneration Assay (HRA) show that mixtures at low concentrations can still elicit teratogenic signals, highlighting TRI's early-warning value (Cesarini et al., 2024). These tracers and "new" emerging contaminants exhibit environmental behaviours (persistence, mobility, interactions with colloids and biofilms) that promote chronic low-dose exposures and complex mixtures in potamal reaches; this justifies the joint use of organism- and community-level indicators as functional screens where routine chemistry may be incomplete.

Such an integrative strategy is commonly formalized through Weight-of-Evidence (WoE) approaches, in which chemical information, effect-based tools and field ecological indicators are jointly interpreted to infer risk under mixture-dominated conditions. WoE therefore provides a practical operationalization of the One Health perspective adopted here, linking environmental exposure to biological effects relevant to ecosystem functioning and to human uses of river basins. WoE frameworks in aquatic science have a long tradition in the Sediment Quality Triad and related multi-LoE assessments integrating chemistry, ecotoxicological responses and field/community data, later formalized into decision-support and scoring systems (Chapman and McDonald, 2005; Chapman, 2007; Dagnino et al., 2008). More recent guidance has further standardized WoE principles for environmental assessments and uncertainty handling, reinforcing the value of synthesizing heterogeneous evidence (bioassays/biomarkers, field surveys, and chemical profiles) into an integrated judgement (Suter et al., 2017; US Environmental Protection Agency, 2016). In parallel, European initiatives on effect-based monitoring under the Water Framework Directive have advocated the integration of targeted chemical analysis with biological effect tools to better capture mixture toxicity and emerging contaminants (Wernersson et al., 2015; de Baat et al., 2019). From a One Health standpoint, WoE provides an operational bridge between environmental exposure and biological effects relevant to ecosystem and organism health, while supporting risk prioritization in human-adjacent waters; this alignment is also reflected in EU risk-assessment guidance that frames WoE for stressors affecting humans and/or the environment (SCHEER, 2026).The present study was carried out in the Latium region (Central Italy), a landscape-scale mosaic where strong environmental heterogeneity intersects with extensive anthropogenic alteration. Considering both rivers originating within Latium and those crossing it, the overall freshwater discharge to the Tyrrhenian Sea is ~12 billion m^3 yr^{-1} , and biotic assemblages vary markedly among basins (Traversetti and Scalici, 2014; Manfrin et al., 2013), as reported also by ARPA Lazio (https://www.arpalazio.it/documents/20124/53201/Relazione_annuale_balneazione_2019.pdf?utm; last accessed on October 20th, 2025).

The aim of this study was to assess whether ecological status and teratogenic risk converge or diverge across eight potamal rivers in Latium (Central Italy). To do so, we combined diatom-based ecological assessment (ICMi) and diatom teratology with the *Hydra vulgaris* regeneration assay summarized by the Teratogenic Risk Index (TRI), while using physico-chemical measurements and ICP-MS elemental profiling, including gadolinium, to contextualize site conditions and support interpretation of biological responses.

2. Material and methods

2.1. Study area

Between June–July 2025 we sampled the potamal reaches of eight rivers in Latium—Marta (MAR), Mignone (MIG), Arrone (ARR), Aniene (ANI), Tevere (TEV), Almone (ALM), Sacco (SAC) and Ninfa–Sisto (NIN), each georeferenced and mapped (Fig. 1). The Marta (Fig. 1-a) and Mignone (Fig. 1-b) flow through predominantly agricultural landscapes; at both sites riparian belts are simplified or asymmetric. The Arrone (Fig. 1-c), emissary of Lake Bracciano, was sampled upstream of a sluice where artificial banks give way to natural margins with shrubs and reeds. The Aniene (Fig. 1-d) transitions from protected uplands to the city of Rome; our reach in the Valle dell'Aniene Reserve is reed-dominated. The Tevere site (Fig. 1-e) lies just downstream of Ponte Marconi in central Rome, with a natural right bank versus an embanked left bank, abundant litter, and local Potamogeton. The Almone (Fig. 1-f) is short and heavily re-engineered; in the Appia Antica Park the reach is shaded by well-developed riparian trees and reeds, lacks aquatic macrophytes, and shows scattered refuse. The Sacco (Fig. 1-g) was sampled in an agricultural setting below a small dam with well-developed riparian vegetation. The Ninfa–Sisto (Fig. 1-h) drains the Pontine plain; in urban Pontinia the unarmored banks are dominated by herbaceous and reed formations, with regular mowing and *Potamogeton* present. Additional site descriptors are provided in Table S1.

2.2. Water sampling and analytical methods

Temperature (°C), dissolved oxygen concentration (mgL^{-1}), oxygen saturation (%), conductivity (mS cm^{-1}), salinity (%) and pH were measured *in situ* using a multimeter immersion probe (Q10; Hach Lange, Loveland, CO, USA). At each site, 200 mL of surface water were collected for chemical analysis (ICP-MS) and ecotoxicological tests (Hydra exposures) from the main flow whenever possible, at approximately 10–30 cm below the surface and 1 m from the nearest bank, avoiding sediment resuspension. Water was collected in pre-cleaned polypropylene bottles and transported to the laboratory in the dark in an insulated cooler at approximately 4°C. Upon arrival, samples were stored under the same conditions (i.e., dark, 4°C) and bioassays were started within 24 h from collection. Elemental concentrations (isotope included) were determined by Inductively Coupled Plasma Mass spectrometry (ICP-MS) using a 820-MS (Bruker, Bremen, Germany), following the analytical procedure described in (Cesarini et al., 2024) and references therein.

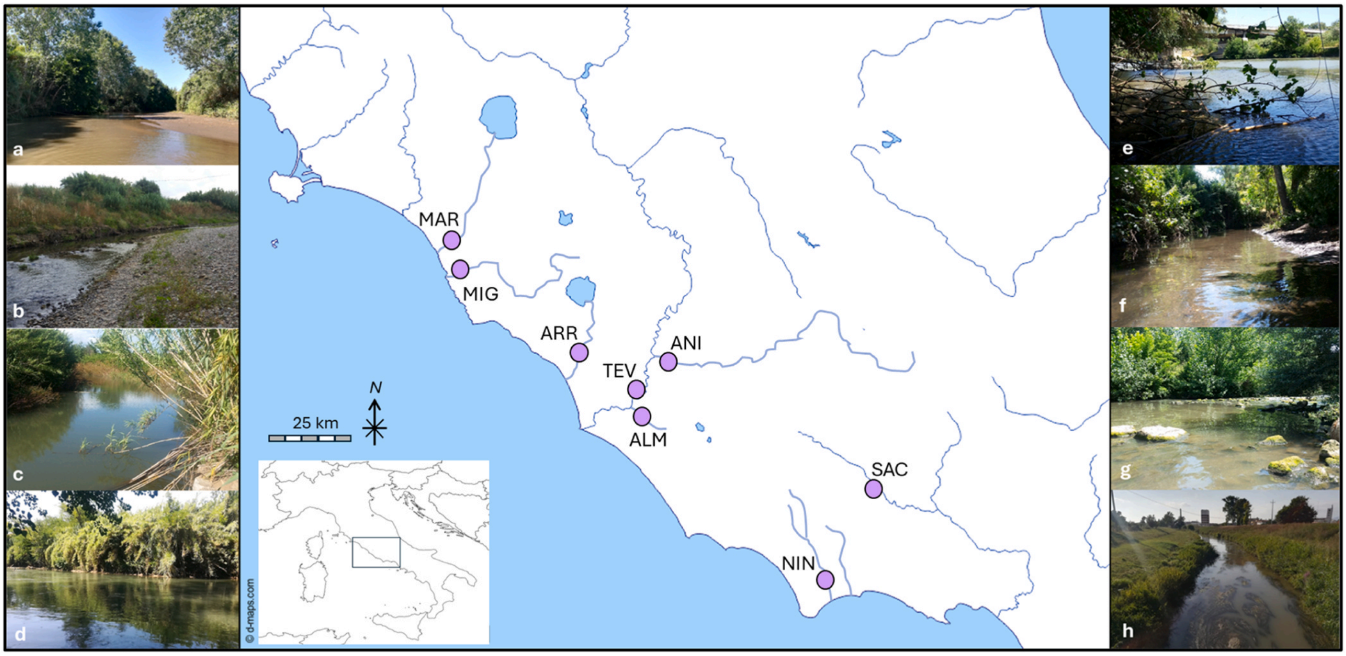

Fig. 1. Pictures and geographical distribution of the sampling sites on the eight rivers in the Latium region (Central Italy): a-Marta River; b-Mignone River; c-Arrone river; D-Aniene River; e-Tevere River; f-Almone River; g-Sacco River; h-Ninfa River.

2.3. Diatom sampling, preparation, and analyses

To sample diatoms, five pebbles, exposed to light and completely submerged, were collected along a 10 m transect. Each pebble was brushed with a toothbrush and the biofilm was rinsed into a Falcon tube pre-filled with 10–15 mL of field water and 1–2 mL of ethanol. This low ethanol addition was used as a field preservative following the national protocol for benthic diatoms. The total brushed area was standardized to 100 cm^2 (ISPRA, 2014a). In the laboratry, organic matter was removed by oxidation under a chemical hood by adding 20 mL of hydrogen peroxide (H_2O_2 30%) to 10 mL of sample on a hot plate set at 90°C. At the end of the reaction, 1 mL of hydrochloric acid (HCl 37%) was added to remove carbonates. This cleaning procedure yields isolated siliceous valves; therefore, morphological identification and teratological screening were based on the frustule (silica) features rather than on soft-cell traits potentially affected by preservation. Cleaned material was mounted on permanent slides using Naphrax resin (Naphrax, Brunel Microscopes Ltd), and taxa were identified using standard floras and atlases (ISPRA, 2014b; Ector and Hlúbiková, 2010; Taylor et al., 2007). Micrographs were analysed with ImageJ to measure valve length and width and to count striae in 10 μm, supporting species-level identification. For each slide, 400 valves were counted as required by the ICMi protocol (Directive, 2000/60/EC, 2000). Since nutrient concentrations were not measured, saprobic group percentages were calculated for each site based on the species composition obtained from 400 counted valves per slide. Each diatom species was assigned to a saprobic category (oligosaprobic, β-mesosaprobic, α-mesosaprobic, α-meso/polysaprobic, polysaprobic) according to the classification frameworks of (Dam et al., 1994) and also reported in (Suter et al., 2017). The relative abundance of each saprobic group was expressed as the percentage of valves belonging to that group relative to the total number of counted valves. Every specimen was assigned to one of the three *guilds* (motile, low profile, high profile) identified by (Passy, 2007), depending on information present in literature (Passy, 2007; Berthon et al., 2011; Stenger-Kovács et al., 2013). The ICMi, based on diatom species composition and abundance (Mancini and Sollazzo, 2009), was calculated to determine the corresponding ecological quality status for each sampling site. For every sampling site, the ICMi score was determined by integrating the Ecological Quality Ratio (EQR) of the two indices of Sensitivity to Pollution (IPS) (Coste, 1982), and Trophy (TI) (Rott et al., 1999). Each species is linked to a coefficient of sensitivity to pollution and reliability as bioindicators. Furthermore, during the counting process for species identification (i.e. 400 valves per slide) all valves showing visible morphological abnormalities were considered teratological and recorded, without applying a minimum abundance threshold. Each teratological valve observed was classified according to the morphological alteration framework proposed by (Falasco et al., 2009b), which distinguishes seven main types of teratologies: (i) deformed valve outline, (ii) alterations in striation pattern, costae and septae, (iii) changes in shape, size and position of the longitudinal and central areas, (iv) raphe modifications, (v) raphe canal modifications, (vi) unusual arrangement of cells forming colonies, and (vii) mixed types. Teratological valves were also subsequently assigned to one of the ecological guilds identified by (Passy, 2007).

2.4. Hydra assay

To perform eco-toxicological test, 135 non-budding *Hydra vulgaris* specimen, were randomly selected from a laboratory colonyand maintained in *Hydra* medium ($CaCl_2 \cdot 2\ H_2O$, NaHCO3 and KCl) in a glass tank (30 ×30 x 30 cm) at 16.5–20°C, under a 16 h light/8 h dark photoperiod, and fed once a week with *Artemia salina* Linnaeus, 1758, nauplii previously rinsed in freshwater. Under a stereomicroscope, the oral regions was removed with a sterile bistoury and discharged; only the column (including the basal disc) was used for the regeneration assay. Exposures were carried out in sterile 6-well polystyrene plates (one well = one replicate). In each well, five columns were placed in 10 mL of test solution (field water) or control solution (*Hydra* medium). For each sampling site, three replicate wells were prepared ($n = 15$ columns per site; 5 polyps × 3 wells). A negative control (CTR) was run in parallel in three replicate wells ($n = 15$) containing 10 mL Hydra medium and maintained under the same environmental conditions as treatments.

To minimize bias due to handling, after introduction into the wells the columns were allowed to settle and attach to the well bottom, and their attachment status (attached vs floating) was checked during each 24 h observation, as floating individuals can show altered regeneration dynamics. Every 24 h, regeneration status was recorded under a stereomicroscope. Exposure solutions were renewed every 24 h by carefully removing and replacing the medium in the same well (i.e., polyps were not transferred to new wells) to avoid mechanical stress. Test solutions were renewed using the corresponding stored field water sample, and CTR wells were renewed using fresh Hydra medium.

At 96 h, regeneration rate (RR) and aberration frequency (AF) were calculated and inserted into the double-entry matrix to obtain the Teratogenic Risk Index (TRI) score for each sampling site (Traversetti et al., 2017; Cera et al., 2019). After 96 h from the start of the regeneration assay, two behavioral endpoints were evaluated under a stereomicroscope on the same exposed organisms (total $n = 15$ per group, i.e., 5 polyps $\times$ 3 replicate wells): tentacle reactivity and feeding performance. Tentacle reactivity was assessed by gently stimulating a single tentacle with a fine pin and recording a binary response (reactive = immediate contraction and/or withdrawal of the stimulated tentacle); the endpoint was expressed as the proportion of reactive polyps per exposure group (% reactive). Feeding performance was tested by supplying 10 *Artemia salina* nauplii per polyp and, after 30 min, counting polyps that had ingested at least one prey (classified as "fed"); the endpoint was expressed as the proportion of fed polyps (% fed). These behavioral measures serve as proxies for proper regeneration and functionality of neural/muscle cell lineages and can reveal functional impairment even in the absence of visible morphological aberrations (Cesarini et al., 2024).

2.5. Statistical analyses

Physico-chemical and chemical parameters were explored by Principal Component Analysis (PCA) after logarithmic transformation. Given the limited number of sampling sites ($n = 8$), PCA was used only as an exploratory ordination method to summarize multivariate patterns and visualize similarities among sites, rather than as an inferential tool.

The significance of differences between CTR and the sampling sites was assessed using a non-parametric analysis of variance (Kruskal–Wallis test) on RR values at each time point (24, 48, 72, and 96 h), as well as on AF, tentacle reactivity, and feeding capacity. When the Kruskal–Wallis test was significant ($p < 0.05$), Dunn's post-hoc pairwise comparisons were performed to identify which sampling sites differed from the CTR, applying a Holm correction for multiple testing.

Data resulting from the application of TRI Index on *Hydra* specimen were analysed by means of χ^2-*test*, with Yates correction, to test for significative differences between the CTR and every sampling site.

Spearman correlation between physico-chemical and chemical data, ICMi scores, the frequency of teratological valves TRI scores was investigated, considering each sampling site as an independent observational unit ($n = 8$). Correlations were computed using the site-specific point values for each variable; no site-level averaging across replicates was applied. The statistical significance level was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism software (version 8.0.1) and R version 4.3.1 (2023–06–16 ucrt).

3. Results

3.1. Environmental and ecological characterization

3.1.1. Physico-chemical parameters and water chemistry

The physico-chemical parameters recorded at each sampling site are summarized in Table 1. Among the investigated sites, ANI showed the highest conductivity value (1284 µS/cm), while relatively high conductivity was also recorded at TEV and ALM. Markedly low dissolved oxygen concentrations and oxygen saturation were observed at TEV (0.98 mg/L; 10.9%) and ALM (3.31 mg/L; 36.5%). The most negative redox potential values were recorded at NIN (-87.8 mV), ARR (-81.3 mV) and MIG (-81.0 mV). Elemental concentrations obtained through ICP-MS analysis are presented in Table 2. Most measured trace elements were below the Environmental Quality Standards (EQS) established by D.Lgs. 152/2006. Notably, arsenic concentrations exceeded the EQS at MAR (31 ppb), MIG (22 ppb), ARR (26 ppb), ANI (44 ppb), TEV (11 ppb) and ALM (14 ppb).

Table 1
Recorded values of temperature (°C), dissolved oxygen concentration (mg/L), oxygen saturation (%), pH, conductivity (µS/cm), and redox potential (mV) for each sampling site, ordered from north to south.

Site	T (°C)	O_2 (mg/l)	O_2 (%)	pH	Condutivity (µS/cm)	Redox Pot. (mV)
MAR	25.1	7.5	91	7.89	720	-75.5
MIG	27.2	8.18	102.5	7.96	752	-81
ARR	22.2	7.47	85.8	8	615	-81.3
ANI	23	6.39	86.2	7.79	1284	-68.1
TEV	21.2	0.98	10.9	7.69	889	-63.2
ALM	20.3	3.31	36.5	6.8	888	-12.6
SAC	25.8	6.85	86.4	7.68	588	-63.4
NIN	29.9	10.41	138.2	8.08	764	-87.8

Table 2
Concentrations (in µg/L = ppb) of selected trace elements (Ni, Zn, Mn, Fe, Cu, Gd, As) measured at each sampling site, ordered from north to south. Values in bold indicate exceedances of the Environmental Quality Standards (EQS) established by D.Lgs. 152/2006 (Part III) implementing the Water Framework Directive 2000/60/EC. The EQS reference limits for dissolved metals are: Ni = 20 µg/L, Zn = 3000 µg/L (indicative, WHO aesthetic criterion), Mn = 50 µg/L (national guideline), Fe = 200 µg/L, Cu = 1000 µg/L, Gd = n.a. (no standard established), As = 10 µg/L.

Site	Ni60 ppb	Zn66 ppb	Mn55 ppb	Fe56 ppb	Cu65 ppb	Gd158 ppb	As75 ppb
MAR	0.87	2.3	3.9	4.1	1.76	0.011	**31**
MIG	1.33	4.1	9.4	1.5	3.67	0.004	**22**
ARR	0.6	2.5	33.2	4.2	2	0.053	**26**
ANI	1.73	1.4	4.2	1.5	1.69	0.057	**44**
TEV	1.41	1.9	10.6	1.5	1.6	0.01	**11**
ALM	0.59	5.2	17.8	9.3	1.57	0.08	**14**
SAC	0.67	4.9	1.3	5.3	1.65	0.052	8
NIN	0.72	1.9	4.9	7.2	2.11	0.025	3

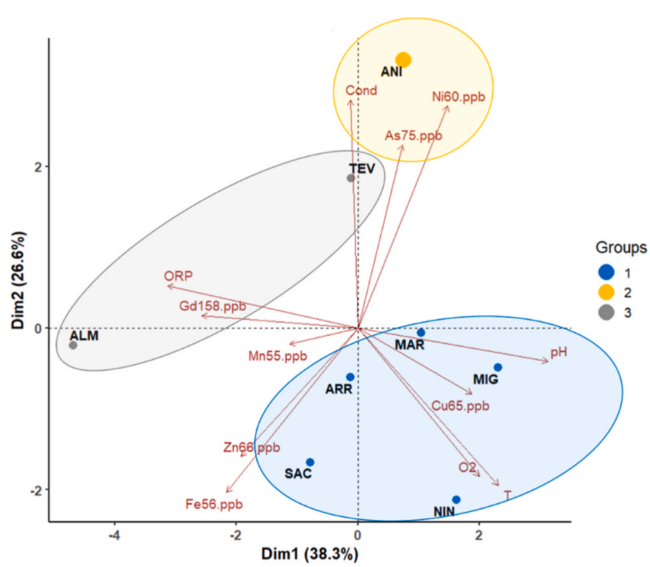

Fig. 2. Biplot graph of Principal Component Analysis (PCA) shows the distinction of 3 groups: 1 - Marta (MAR), Mignone (MIG), Arrone (ARR), Sacco (SAC) and Ninfa (NIN), 2 - Aniene (ANI), 3 - Almone (ALM) and Tevere (TEV). Groups are distinguished by the main physico-chemical parameters contributing to the separation, including pH, dissolved oxygen, temperature, copper, arsenic, nickel, conductivity, gadolinium, and redox potential.

The PCA biplot of physico-chemical parameters and water chemistry (Fig. 2) provided an exploratory visualization of site distribution, suggesting three main clusters (G1, G2, and G3) associated with different physico-chemical and chemical profiles. G1 included the agricultural sites (MAR, MIG, ARR, SAC and NIN), characterized by higher values of pH, dissolved oxygen, temperature, and copper. G2, corresponds to the urban site ANI, distinguished by elevated concentrations of arsenic, nickel, and higher conductivity. G3 comprises the remaining urban sites (ALM and TEV), which show higher gadolinium concentrations and more positive redox potential.

3.1.2. *Benthic diatoms assemblage*

The full list of diatom species recognized per each sample site has been reported in Table S2.

The number of species detected at each sampling site was relatively consistent, with the following counts: MAR (39 species), MIG (35 species), ARR (61 species), ANI (54 species), TEV (45 species), ALM (29 species), SAC (44 species), and NIN (59 species). The ten most abundant genera, listed in descending order of relative abundance, were *Nitzschia* (26.47%), *Cocconeis* (19.97%), *Achananthidium* (10.78%), *Gomphonema* (9.16%), *Navicula* (8.44%), *Eolimna* (5.69%), *Cyclotella* (4.13%), *Planothidium* (1.75%), *Sellaphora* (1.68%), and *Trybionella* (0.78%). Fig. 3 shows the most abundant species at each sampling site, including only those representing at least 5% of the total. In MAR the most abundant species was *Nitzschia frustulum* var. *incospicua*, in MIG *Achnantidium minutissimum*, in ARR *Nitzschia amphibia*, in ANI e TEV *Cocconeis placentula*, in ALM *Gomphonema parvulum*, in SAC *Eolimna subminuscula*, in NIN *Cocconeis pediculus*. The classification of saprobity according to Van Dam is presented in Table S2, while the relative abundance of each species at the sampling sites is reported in Table S3. Among the investigated sites, oligosaprobic taxa were generally scarce, reaching their highest relative abundance at NIN (21%), while remaining below 5% at all other sites (0–4%). β-mesosaprobic species dominated most assemblages, particularly at MAR (88%), MIG (80%), ANI (71%) and TEV (75%), indicating moderate organic conditions. In contrast, ALM showed a marked prevalence of α-meso-/polysaprobic taxa (54%) and polysaprobic species (28%), reflecting strongly impacted conditions. ARR and SAC displayed intermediate patterns, characterized by a mixed contribution of β-mesosaprobic (39% and 35%, respectively) and α-mesosaprobic taxa (45% and 34%, respectively).Ecological guild analysis, following the classification proposed by Passy (Passy, 2007), revealed distinct patterns among sites (Fig. 4). Motile taxa dominated the assemblages in MAR (92%), ARR (68%), ALM (59%), and SAC (63%). In contrast, low profile taxa were most abundant in MIG (77%), ANI (61%), TEV (62%), and NIN (72%). High profile taxa were generally less represented, with the highest proportion observed in ALM (38%).

According to the ICMi, four sites (MIG, ANI, TEV, and NIN) achieved a High ecological status, while two sites (ARR and ALM) were classified as Poor (Table 3). Sites belonging to the agricultural group (G1) showed more variable conditions, ranging from Poor (ARR) to High (MIG and NIN), whereas urban sites generally reached High status, with the exception of ALM.

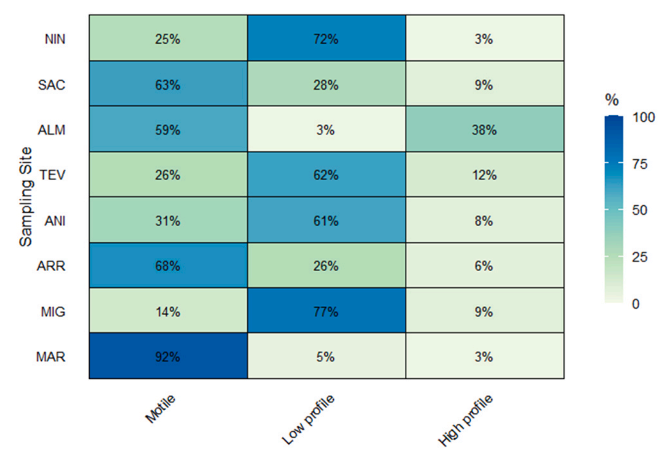

Fig. 4. Relative abundance (%) of the three ecological guilds: "Motile", "Low profile", and "High profile", across the sampling sites arranged from north to south. The heatmap illustrates the distribution of these guilds at each site, with color intensity representing the percentage abundance.

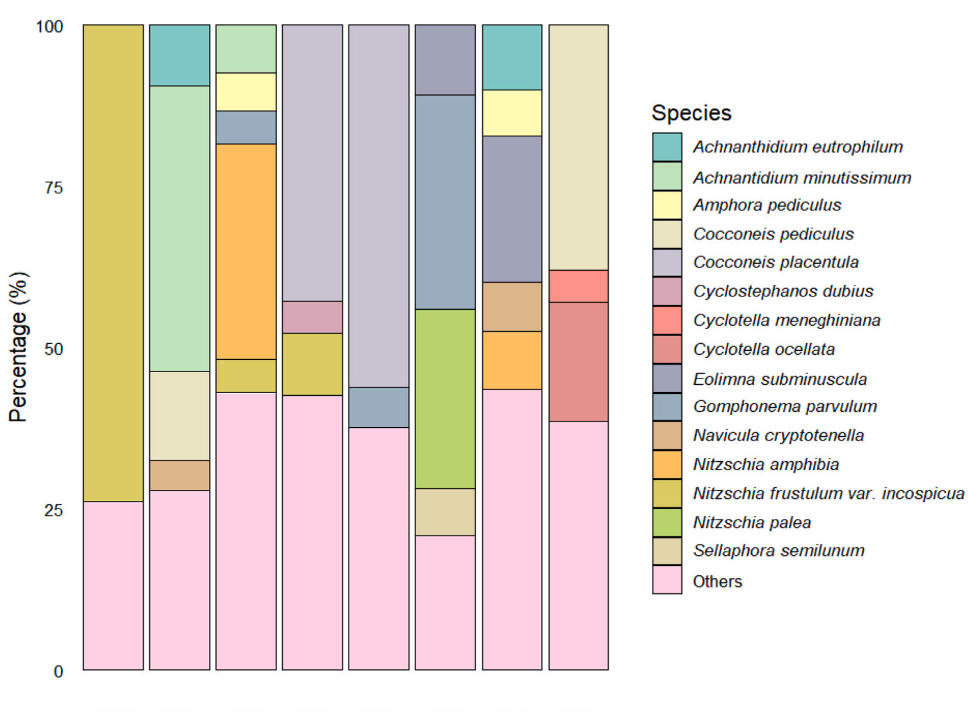

Fig. 3. The histogram reports the most abundant species (≥5%) observed at each sampling site from north to south over a total number of 400 specimens.

Table 3
River macrotype, ICMi value, corresponding color classification, and ecological status assessment for each sampling site ordered from north to south and classified by PCA groups.

Site	Riverine macrotype	Description	ICMi	Ecological status
MAR (G1)	M2	Medium lowland rivers	0.68	Good
MIG (G1)	M2	Medium lowland rivers	1.29	High
ARR (G1)	M2	Medium lowland rivers	0.47	Poor
ANI (G2)	M3	Very large lowland rivers	0.83	High
TEV (G3)	M3	Very large lowland rivers	0.85	High
ALM (G3)	M2	Medium lowland rivers	0.36	Poor
SAC (G1)	M2	Medium lowland rivers	0.63	Good
NIN (G1)	M2	Medium lowland rivers	0.88	High

3.2. Analysis of teratogen risk

3.2.1. Hydra regeneration assay

RR, AF, tentacle reactivity, and feeding capacity were assessed for *Hydra* organisms from each sampling site and the control. Observations were performed daily and at the end of the exposure period (96 h), and the results are summarized in Table 4.

For RR, significant deviations from the control were detected at multiple sites. ARR showed higher values at 24 h ($p < 0.05$) and reduced at 96 h ($p < 0.05$), whereas MIG and NIN displayed higher RR at 24 h ($p < 0.01$). ALM was the most affected site, with RR values significantly lower than the control from 48 h onwards ($p < 0.01$) (see Table 4).

AF was significantly higher in MAR (60%, $p < 0.01$) and in ALM (100%, $p < 0.01$) compared to the control (Table 4). The percentage occurrence and types of morphological malformations (Traversetti et al., 2017) observed at each site are shown in Fig. 5. Recorded abnormalities included tentacle arrangement on different planes (DP), duplicated tentacles (DT), clubbed tentacles (CT), tentacle-induced mouth occlusion (TO), tulip conformation (TP), and dead specimens. Among these, DP was the most prevalent overall. ALM exhibited the highest frequency of malformations (100%, $p < 0.01$), followed by MAR, SAC, and TEV, whereas MAR displayed the widest range of malformation types.

Tentacle reactivity was completely absent at ALM (0%, $p < 0.01$), while no significant differences were observed at the other sites. Feeding capacity was also significantly reduced at ALM (0%, $p < 0.01$) and at SAC (47%, $p < 0.05$).

At 96 h, the observed values of RR and AF were integrated to calculate the TRI score and to assign the corresponding teratological risk class (Fig. 6). Overall, MIG and NIN responded comparably to the control, while significant differences was revealed only at the ALM site (TRI score = 10, TRI class = 5; $\chi^2 = 8.1$, $p < 0.01$).

3.2.2. Teratological diatom forms

During the counting of 400 valves for sampling site, the presence and frequence of teratological valves was recorded. Overall, the most common was *Cocconeis placentula* (n. teratological valves = 33), followed by *Eolimna subminuscula* (n. teratological valves = 13), *Gomphonema parvulum* (n. teratological valves = 4), *Achnanthidium eutrophilum* (n. teratological valves = 3), *Planothidium lanceolatum* (n.teratological valves = 3), *Achnanthidium pyrenaicum* (n. teratological valves =1) *Eolimna minima* (n. teratological valves = 1), *Nitzschia frustulum* var. *inconspicua* (n. teratological valves =1), *Diatoma monofiliformis* (n. teratological valves =1), *Encyonopsis subminuscula* (n. teratological valves =1), *Navicula reinhardtii* (n. teratological valves =1), *Planothidiuum frequentissimum* (n. teratological valves =1), *Pinnularia subcapitata* (n. teratological valves =1), *Fragilaria virescens* (n. teratological valves =1),

Table 4
Observations of *Hydra* regeneration rate (RR) at 24, 48, 72, and 96 h, aberration frequency (AF), tentacle reactivity, and feeding capacity only at 96 h for the control and for each sampling site, ordered from north to south and classified by PCA groups.

Site	Regeneration Rate				Aberration Frequency (%)	Tentacles Reactivity (%)	Feeding capacity (%)	Teratogenic Risk Index
	24 h	48 h	72 h	96 h				
CTR	0.1	1.5	3.6	4	0	100	100	10
MAR (G1)	0.7	1.5	3.8	3.9	60**	67	87	4
MIG (G1)	1**	1	2.9	3.3	26	100	100	8
ARR (G1)	0.8*	1	2.9	3*	20	87	87	8
ANI (G2)	0.7	1.1	3.8	3.9	33	33	80	9
TEV (G3)	0.7	1.4	3.7	4	47	73	73	6
ALM (G3)	0.3	0.1**	0.1**	0.1**	100**	0**	0**	0**
SAC (G1)	0.6	1	2.8	3.3	47	33	47*	6
NIN (G1)	1**	1.1	3	3.2	7	100	100	10

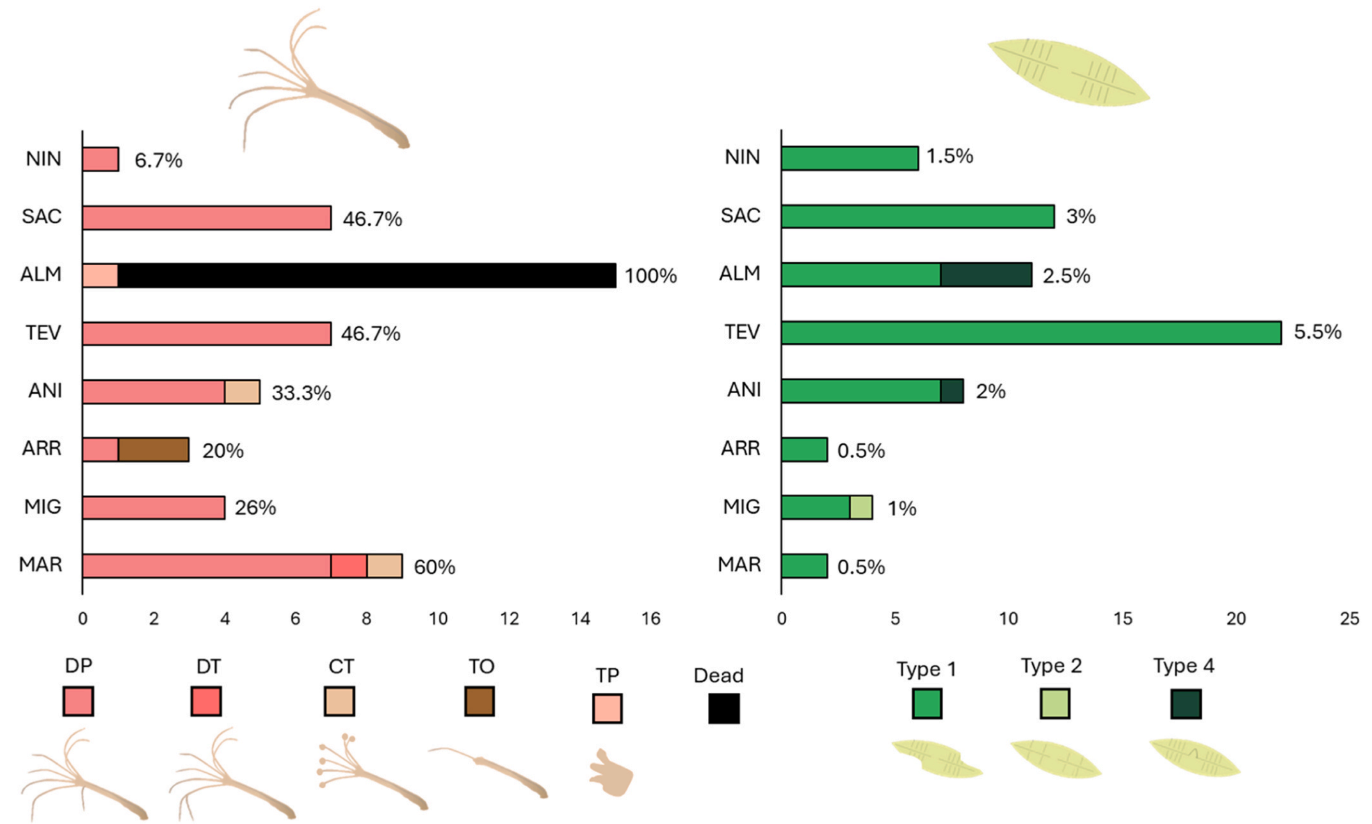

Fig. 5. Composition by teratological types for hydra (left) and diatoms (right) for each sampling site from north to south. The aberration frequency for the sampling site is reported as percentage. DP = tentacles arrangement on different planes; DT = doubled tentacles; CT = clubbed tentacles; TO = tentacle that occludes the mouth; TP = tulip conformation.

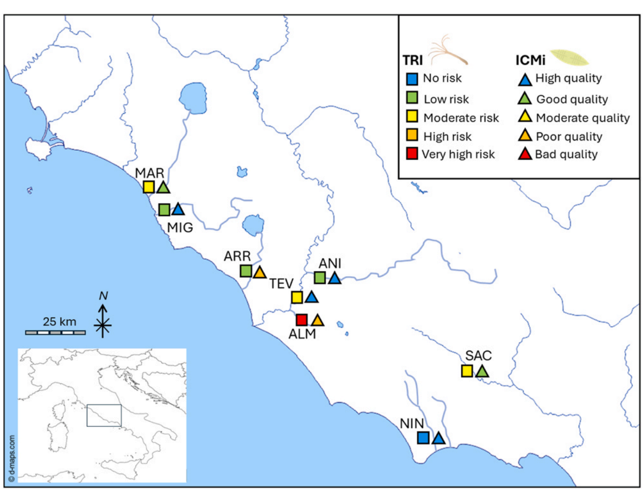

Fig. 6. For each sampling site, shown on the map, are indicated the class of teratogenic risk, highlighted by a rectangle of the respective colour, and the class of ecological quality based on diatom community, highlighted by a circle of the respective colour.

Sellaphora seminulum (n. teratological valves =1). Across sites, teratological valves represented 0.5–5.5% of the 400 counted valves per slide. Specifically: MAR 2/400 (0.5%), MIG 4/400 (1.0%), ARR 2/400 (0.5%), ANI 8/400 (2.0%), TEV 22/400 (5.5%), ALM 10/400 (2.5%), SAC 12/400 (3.0%), NIN 6/400 (1.5%), with the highest frequency at TEV. The type of morphological alteration (Falasco et al., 2009b) was recorded in Fig. 5. Most teratological valves fall into type 1 (deformed valve outline), while few fall into type 2 (changes in striation pattern, costae and septae) and type 4 (raphe modifications). No teratological valves fall into type 3 (changes in shape, size and position of the longitudinal and central area), type 5 (raphe canal modifications), type 6 (unusual arrangement of the cells forming colonies), and type 7 (mixed type).

The guild composition was also evaluated for the teratological valves. Overall, most aberrant individuals belonged to the "low motile" guild (63%), followed by "motile" (27%) and "high profile" (10%).

3.2.3. *Correlation analysis between parameters and comparison of indicators*

The ICMi showed a significant negative correlation with Gd^{158} concentrations ($r = -0.76$, $p = < 0.05$), suggesting that higher levels of this element may negatively affect diatom-based ecological quality. Notably, no significant correlation was observed between TRI and ICMi, indicating that the two indices respond differently to environmental stressors. A comparative assessment of the indicators (Fig. 6) shows that TRI displays higher spatial variability and appears more sensitive than ICMi in detecting potential environmental stress. Despite their different sensitivities, both indicators consistently identify the Almone River (ALM) as the site with the poorest ecological quality and the highest teratogenic risk, and the Ninfa River (NIN) as the site with the best ecological status and no teratogenic risk.

4. Discussion

We assessed river condition by integrating organism- and community-level evidence: *Hydra vulgaris* regeneration biomarkers summarized in TRI, and benthic diatoms used for ecological status (ICMi) plus the frequency of teratological valves. Considered together, these indicators offer a sensitive early-warning view under heterogeneous, mixture-dominated pressures. A key outcome is that ecological quality and teratogenic hazard need not overlap: TRI and teratologies target developmental/neuro-functional impairment, even at low doses or in complex mixtures, whereas ICMi reflects community shifts to nutrients, organic load, and hydromorphology. Thus, good or moderate ICMi can co-occur with elevated TRI (and vice versa). This non-covariance is informative, showing that the metrics probe distinct stress dimensions and should be interpreted jointly.

4.1. Environmental and ecological characterization

The combined analysis of physico-chemical and chemical parameters revealed marked spatial heterogeneity among the investigated river sites, reflecting the influence of land use and anthropogenic intensity. Agricultural sites (Group 1: MAR, MIG, ARR, SAC, NIN) generally showed higher dissolved oxygen and oxygen saturation and slightly alkaline pH, consistent with systems characterized by efficient organic matter turnover (Maier et al., 2025). Among them, the Ninfa River displayed the highest oxygenation, consistent with its protected status (*i.e.*, the proximity of Ninfa Natural Reserve) and minimal disturbance (Ronci et al., 2016). In contrast, urban sites (ANI, TEV and ALM) were characterized by higher conductivity and markedly lower oxygen concentration and saturation (particularly at TEV), suggesting eutrophic conditions and intensified microbial respiration (Arango et al., 2017). Redox potential values were negative at all sites (range: -87.8 to -12.6 mV), indicating overall reducing conditions that can influence contaminant speciation and bioavailability in the water column and biofilm. Such patterns are consistent with previous observations of organic pollution and poor ecological quality in the Almone and Tevere rivers across the urban area of Rome, as reported by ARPA Lazio (https://www.caffarella.it/dati-inquinamento-fiume-almone/; last accessed on October 14th, 2025). Urban sites also exhibited enrichment in trace elements, particularly arsenic, nickel, and gadolinium, indicating inputs from wastewater and medical effluents (Cesarini et al., 2024). Gadolinium, an emerging contaminant not yet regulated under the WFD, reached its highest values in Almone and Tevere rivers, corroborating evidence of anthropogenic sources and potential ecotoxicological relevance. Only arsenic consistently exceeded legal thresholds across most sites, primarily due to the geological characteristics of central Italy and the hydrogeological influence of its volcanic substrata (Aiuppa et al., 2003). These physico-chemical gradients provide an important context for interpreting both community patterns and ecotoxicological responses. Higher conductivity and reduced oxygen conditions are known to select for disturbance-tolerant and eutraphentic assemblages and may contribute to the dominance of tolerant taxa observed at the most impacted sites. In parallel, low oxygen and reducing conditions can co-occur with higher organic load and altered trace-element bioavailability, potentially amplifying organism-level stress responses. Therefore, the physico-chemical setting helps explain why the strongest toxicological impairment was observed at the most oxygen-depleted/high-conductivity sites, whereas well-oxygenated sites showed limited or no effects.

Benthic diatom communities showed clear structural and functional variability across rivers, with composition and dominance patterns closely mirroring environmental gradients, including nutrients, human pressures, and hydrology. Within agricultural rivers, benthic diatom communities were dominated by *N. frustulum* var. *incospicua* in Marta River, a species typical of mesotrophic waters (ISPRA, 2014b), and by *A. minutissimum* in Mignone River, which was also present at other sites, reflecting its broad ecological tolerance across varying nutrient and organic loads (Falasco et al., 2013). At Arrone River, *N. amphibia* prevailed, a taxon known to tolerate nutrient enrichment and fluctuating hydrological conditions, suggesting influences from agricultural runoff and periodic discharge (Cooper et al., 1999). The small motile species *E. subminuscula* dominated at Sacco River, consistent with elevated nutrient levels and fine sediment accumulation (Licursi and Gómez, 2009). In contrast, at the relatively pristine site Ninfa River, the cosmopolitan taxon *C. pediculus* prevailed, typically associated with well-oxygenated meso- to oligotrophic waters, aligning with the river's protected status and high-water quality (Falasco et al., 2013). Urban rivers exhibited distinct assemblages: *C. placentula* dominated Aniene and Tevere rivers, a low-profile species indicative of mesotrophic to slightly eutrophic conditions and relatively stable hydrology (Della Bella et al., 2012), while *G. parvulum* prevailed in Almone River, a taxon commonly reported in environments impacted by organic pollution and anthropogenic disturbance, including urban and agricultural inputs (Falasco et al., 2013). The distribution of saprobic groups across the sites further supports the interpretation of environmental gradients. Consistent with Van Dam (Dam et al., 1994), agricultural rivers were generally dominated by β-mesosaprobic taxa, indicative of moderate organic conditions, reflecting efficient organic matter turnover under low-to-moderate anthropogenic pressure. In contrast, Almone River supported a higher proportion of α-meso- and polysaprobic taxa, consistent with strong organic enrichment associated with urban wastewater inputs. Ninfa River, by contrast, exhibited a predominance of oligosaprobic species, in line with its well-oxygenated and minimally disturbed status. Intermediate patterns observed at Arrone and Sacco rivers highlight the gradient from low to moderate organic load across agricultural catchments.

Ecological guild analysis further clarifies the functional responses of diatom communities to environmental gradients. Motile taxa dominated moderately impacted agricultural sites (MAR, ARR, SAC), reflecting their ability to relocate within biofilms and exploit microhabitats, which allows them to respond efficiently to intermediate nutrient availability and moderate physical disturbance (Passy, 2007). In contrast, low-profile taxa prevailed at both oligotrophic (NIN, MIG) and more strongly impacted urban sites (ANI, TEV), consistent with their tolerance to either nutrient limitation or elevated stress, and their ability to persist under conditions that favor epipsammic growth forms. High-profile taxa were generally rare, with notable dominance at ALM, where the predominance of the *Gomphonema parvulum* complex likely reflects adaptation to elevated organic load and heavy metal contamination rather than natural oligotrophic preference. Overall, the distribution of ecological guilds closely mirrors the physico-chemical and saprobic gradients observed: motile taxa dominate sites with moderate nutrient enrichment and intermediate disturbance, low-profile taxa occur under both low nutrient availability and strong stress conditions, and high-profile taxa persist mainly under nutrient-rich or localized tolerant conditions. These patterns, integrated with saprobic group distributions, reinforce the close coupling between water quality, organic load, and the functional composition of benthic diatom communities across the studied rivers

According to the ICMi classification, agricultural sites (G1) generally exhibited high or good ecological status, with the exception of Arrone River, which displayed poor quality. This discrepancy may be attributed to several factors. Firstly, this river could be subject to localized agricultural practices that lead to nutrient over-enrichment, promoting eutrophication and subsequent water quality degradation. Additionally, the site's hydrological conditions, such as reduced water flow or altered hydrodynamics, might exacerbate the accumulation of pollutants, further impairing water quality (Cesarini and Scalici, 2022). Urban sites generally showed high ecological status, except for Almone River, where poor quality likely resulted from wastewater inputs and organic pollution, which increase nutrient loads and contaminants, promoting eutrophication and ecological degradation (Cesarini et al., 2024).

These community patterns are consistent with regional studies on volcanic and siliceous streams of central Italy, where diatom assemblages and trophic indices have been shown to closely reflect nutrient concentrations (especially orthophosphate and nitrate) and to shift predictably under human pressure (Della Bella et al., 2012). Similarly, Pace et al (Pace et al., 2012). documented parallel changes in diatom and macroinvertebrate communities along anthropogenic gradients in Apennine rivers. Our findings therefore align with the broader regional pattern in which substrate composition, hydrological regime, and nutrient availability jointly determine benthic diatom structure and ecological quality in Mediterranean volcanic river systems (Della Bella et al., 2017; Falasco et al., 2016).

4.2. Teratogenic responses and risk level

The assessment of teratogenic responses through the *Hydra* assay and diatom morphological aberrations provided a sensitive and integrative measure of site-specific developmental risk, enabling early detection of sublethal contamination effects. The *Hydra* assay demonstrated that regeneration rate (RR), aberration frequency (AF), and behavioural endpoints such as tentacle reactivity and feeding capacity are highly sensitive indicators of waterborne teratogenic stress. Among all sites, the Almone River exhibited the most severe impairment, with complete inhibition of regeneration and 100% aberration frequency due to the death of exposed organisms. This ecotoxicological profile is indicative of osmotic lysis ("explosive death") likely triggered by ionic imbalance and membrane damage associated with exposure to complex mixtures of metals and organic pollutants (Zeeshan et al., 2017). Such effects are consistent with those reported in previous studies where *H. vulgaris* mortality occurred under metal concentrations similar to those measured in Almone River (Cesarini et al., 2024), confirming the extreme sensitivity of this model to contaminated freshwater environments (Singh and Nel, 2017). Milder yet indicative responses were observed at the Marta and Sacco rivers, suggesting combined agrochemical and organic contamination, whereas *Hydra* from the Mignone and particularly the Ninfa rivers exhibited complete regeneration and normal behaviour, reflecting preserved or well-managed ecological conditions.

The TRI effectively synthesized these organismal responses, classifying sites along a gradient from very high risk (Almone) to no risk (Ninfa). Unlike conventional endpoints (e.g., mortality or growth inhibition), this bioassay integrates morphological, regenerative, and behavioural biomarkers, providing mechanistic and ecologically meaningful evidence of sublethal developmental ecotoxicity (Cesarini et al., 2023a; Quinn et al., 2008, 2012). Moreover, the TRI represents a valuable tool for long-term monitoring of these river systems, as several stations coincide with those investigated in earlier research (Traversetti et al., 2017; Cera et al., 2019), allowing direct temporal comparison. Comparative evaluation with previous studies revealed both consistency and temporal evolution of risk across sites, suggesting persistent, low-level contamination in TEV and SAC (Traversetti et al., 2017; Cera et al., 2019). More recently, Cesarini et al (Cesarini et al., 2024). confirmed this trend, reporting in ALM a very high teratogenic risk linked to total metal and semimetal concentrations of 124.45 μg/L, including detectable gadolinium, highlighting the combined impact of trace metals and emerging contaminants in triggering teratogenic responses.

Identifying the specific compounds responsible for such effects remains challenging, as natural waters often contain complex mixtures that interact synergistically or antagonistically. This analytical complexity underscores the value of biological assays as functional early-warning systems (Wilby and Tesh, 1990). Chemical analysis alone may fail to capture biologically meaningful exceedances, as it quantifies only individual compounds and not their interactive toxicity (Ronci et al., 2016). Moreover, subthreshold concentrations of multiple contaminants can jointly trigger adverse developmental responses that would remain undetected in purely chemical surveillance (Cesarini et al., 2024). Only a few recent studies have used *Hydra* to test environmental samples rather than known contaminants (Cera et al., 2020). Indeed, even where the concentrations of chemicals were low, the *Hydra* regeneration assay revealed pronounced effects of pollution sources. Such findings highlight the diagnostic power of *H. vulgaris*, a model organism characterized by regenerative capacity and sensitivity to environmental stressors. Despite its proven potential for ecotoxicological testing, *Hydra* has not yet been incorporated into formal WFD monitoring frameworks, though recent reviews emphasize its suitability for standardized applications in aquatic toxicity assessment (Cera et al., 2020).

Parallel analysis of diatom teratological forms corroborated the occurrence of teratogenic risk at the same impacted sites, although the frequency of morphological anomalies was lower than that observed in *Hydra*. The most common abnormalities, valve outline deformities (Type 1) and striae pattern anomalies (Type 2), were detected primarily in *C. placentula* and *E. subminuscula*. These deformities, typically induced by metal and organic stress, reflect interference during frustule silicification and are widely recognized as indicators of chronic teratogenic exposure (Stenger-Kovács et al., 2013; Falasco et al., 2009b; Lavoie et al., 2017). Although overall frequencies were low, their consistent presence at disturbed sites supports the hypothesis of long-term exposure to low-level teratogens, in agreement with Falasco et al (Falasco et al., 2009a, 2021). As Lavoie et al (Lavoie et al., 2017). emphasized, even when a direct correlation with specific contaminants is not always demonstrable, the occurrence of teratological forms should be regarded as an early "red flag" of contamination, an effective screening signal that alerts water managers to potential degradation before it becomes ecologically evident. Accordingly, we did not expect the frequency of teratological valves to scale linearly with single-analyte concentrations measured at one time point, because teratologies reflect an integrated biological response influenced by exposure history, mixtures and contaminant bioavailability within the biofilm. In this perspective, diatom teratology should be viewed as a complementary screening line of evidence (rather than an interchangeable substitute for other biological quality elements such as benthic macroinvertebrates).

The predominance of abnormal valves within low-profile taxa underscores the ecological relevance of diatom teratology as an early indicator of environmental contamination. According to the ecological guilds defined by Passy (Passy, 2007), low-profile species are slow-growing, firmly attached forms that dominate stable, oligotrophic habitats. Despite their position within the deeper, more protected biofilm layers, their limited mobility may increase susceptibility once contaminants penetrate the biofilm matrix. Notably, *C. placentula* (low-profile species) has been demonstrated to be highly sensitive under controlled laboratory conditions, exhibiting significant teratogenic responses to nanoplastic exposure at environmentally relevant concentrations (0.1 $\mu g\ L^{-1}$) (Cesarini et al., 2023a). This finding reinforces its suitability as a model diatom species for assessing teratogenic risk, due to its ubiquity, pioneer colonizing behavior, and easily identifiable teratological forms (Falasco et al., 2009a; Pandey et al., 2017; Marcheggiani et al., 2019).

4.3. Integration of indicators and management relevance

The comparative analysis between biological indicators and environmental variables demonstrates that TRI and ICMi respond to distinct but complementary ecological dimensions. The lack of a significant correlation between these indices confirms that they capture different temporal and functional scales of ecosystem response: ICMi reflects community-level structural changes integrated over long periods and driven primarily by trophic conditions, organic load and habitat constrains, whereas TRI detects short-term physiological and developmental impairments at the organismal level based on the dissolved and bioavailable fraction of the sampled water. Importantly, these metrics also target different biological compartments (benthic biofilm vs. water column exposure) and endpoints (community composition vs. regeneration/aberrations and behaviour), so divergence is not only expected but informative under mixture-dominated pressures. In addition, the present correlation analysis is based on a limited number of sites ($n = 8$), and relationships between community indices and effect-based endpoints may be non-linear (thresholds, hysteresis, and time lags), further reducing the likelihood of detecting simple monotonic associations. This complementarity helps explain the higher spatial variability and apparent sensitivity of TRI in detecting emerging contamination even where community structure appeared intact, and it supports the use of both indicators in a combined, management-oriented early-warning framework.

The strong negative correlation observed between ICMi and gadolinium concentrations supports the interpretation of Gd as a robust marker of wastewater/medical inputs and associated anthropogenic pressure that co-varies with environmental gradients shaping diatom-based ecological quality. At the same time, no significant relationship emerged between gadolinium and the teratogenic endpoints considered here (Hydra TRI and diatom teratology frequency), suggesting that Gd alone is not a reliable predictor of teratogenic hazard in our dataset. This does not exclude a potential biological relevance of Gd, since experimental studies have reported Gd-induced oxidative stress, metabolic impairment, and developmental anomalies in aquatic organisms (Goecke et al., 2017; Ebrahimi et al., 2019), but it indicates that any effect in field conditions is likely modulated by speciation/bioavailability (*e.g.*, chelated forms), exposure history and co-occurring stressors. Therefore, the observed teratogenic responses are more consistently interpreted as the outcome of complex mixtures and interacting pressures (including contaminants not quantified here), rather than being attributable to a single tracer element. Since Gd is not currently included among the WFD priority substances, its strong association with ICMi nonetheless highlights the value of integrating emerging tracersinto monitoring schemes to better capture evolving chemical pressures on aquatic ecosystems.

Overall, the lack of a clear relationship between TRI and individual chemical parameters reinforces the hypothesis that teratogenic responses may result from the combined and often synergistic action of multiple contaminants. Identifying specific compounds responsible for the observed effects is complex, as chemical analyses alone may not reveal exceedances of biological toxicity thresholds or the presence of interactive effects (Cesarini et al., 2024). Furthermore, previous biomarker studies in Italian rivers revealed consistent evidence of sublethal effects in aquatic fauna even under conditions meeting WFD chemical standards. Ronci et al (Ronci et al., 2016). reported increased genotoxicity in *Gammarus elvirae* from apparently compliant sites, demonstrating that legally "safe" contaminant levels can still induce measurable biological damage. In such cases, bioassays like the *Hydra* regeneration test serve as early-warning tools, detecting functional impairment even when contaminant concentrations remain below conventional limits. This reinforces the ecological and diagnostic value of integrating biological responses with chemical monitoring for a more realistic assessment of environmental quality. Interestingly, sites classified as having good or high ecological status (e.g., TEV, SAC) exhibited moderate teratogenic risk, indicating that compliance with WFD ecological thresholds does not necessarily imply the absence of developmental hazards.

From a management perspective, combining TRI and ICMi offers a cost-effective and ecologically meaningful toolset for prioritizing high-risk sites and guiding remediation strategies. This approach is further strengthened by the ease of data visualization, such as through derived maps, and by the presence of five corresponding assessment classes, which facilitate immediate interpretation. Incorporating *Hydra* assay into WFD surveillance and operational monitoring programs would enhance early detection of contaminant-induced developmental stress and further support the One Health approach, bridging ecosystem and human health.

4.4. Limitations and future perspectives

While this study provides strong evidence of teratogenic risk gradients across Central Italian rivers, some limitations must be acknowledged. The single sampling campaign limits temporal resolution and does not capture daily or seasonal variability, particularly in temperature. Moreover, because ecological status classification is inherently strengthened by repeated observations over time, the ICMi values reported here should be interpreted as an indicative ecological status under the surveyed conditions rather than a definitive classification. Integrating these results with historical and multi-seasonal monitoring data would improve the robustness of ecological status assessment and the interpretation of temporal variability and episodic events. This limitation constrains the interpretation of temperature–dissolved oxygen relationships, emphasizing the need for long-term monitoring to validate the observed patterns. Likewise, the chemical dataset, although comprehensive, did not encompass the full range of emerging contaminants, including pharmaceuticals, pesticides, and microplastics, known to induce teratogenic responses (Quinn et al., 2009; Pašková et al., 2011; Cesarini et al., 2023b).

Future research should address these gaps through multi-seasonal monitoring, expanded chemical profiling, and the inclusion of molecular biomarkers (e.g., oxidative stress genes, neurodevelopmental endpoints) to elucidate causal mechanisms underlying observed teratogenicity. In addition to *Hydra*, laboratory assays using the diatom *C. placentula* could be applied to evaluate teratogenic risk and compare organismal responses after the exposition to environmental samples.

Ultimately, integrating morphological, toxicological, and ecological indicators into a standardized early-warning framework could significantly strengthen freshwater biomonitoring. Such integration would not only improve diagnostic accuracy but also anticipate ecological degradation before irreversible shifts occur, offering decision-makers more proactive management tools for sustainable water resource protection.

Additionally, long-term field calibration of the TRI against established ecological and toxicological benchmarks would enhance its interpretative robustness and facilitate its standardization within European biomonitoring protocols. Coupling these approaches with hydrological, land-use, and wastewater treatment data could also help trace pollution sources, particularly for emerging contaminants like gadolinium, and support targeted mitigation actions.

5. Conclusions

This study provides an integrated evaluation of ecological quality and teratogenic risk across eight rivers in Central Italy, combining diatom-based ecological indices (ICMi) with bioassays on both uni- and pluricellular organisms (diatom teratology and *Hydra* regeneration, respectively). Results demonstrate that ecological status and teratogenic risk are not linearly correlated, revealing the presence of sublethal stressors undetectable by conventional monitoring.

The observed divergence between ecological quality (ICMi) and teratogenic risk (TRI) highlights a critical gap in current freshwater monitoring programs, which mainly rely on community structure and chemical thresholds established by the WFD. Sublethal and teratogenic effects, though biologically significant, often remain undetected in routine assessments. Integrating bioassays such as the *Hydra* regeneration test and diatom teratological analysis into existing monitoring frameworks to detect early, biologically relevant contamination that may escape detection through chemical or community-based assessments alone. The occurrence of teratologies should be regarded as a "red flag" for potential contamination: although not always directly correlated with contaminant levels, they serve as valuable screening indicators that can signal the need for targeted chemical analyses and help optimize monitoring resources. The detection of gadolinium enrichment in urban sites, together with arsenic exceedances of geogenic origin, underscores the importance of incorporating both natural and anthropogenic factors into freshwater management. These findings advocate for the inclusion of emerging contaminants in freshwater protection.

Overall, the combined use of *Hydra* and diatom models provides a sensitive and cost-effective framework for early detection of teratogenic hazards. Incorporating such bioassays into WFD-based assessments would strengthen predictive and preventive capacities, supporting ecosystem management and safeguard human health within a One Health perspective.

CRediT authorship contribution statement

Massimiliano Scalici: Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Giulia Cesarini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marco Colasanti:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Federica Spani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Conceptualization.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.120015.

Data availability

Data used are share as supplementary materials

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