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Assessing teratogenic risks of gadolinium in freshwater environments: Implications for environmental health

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ABSTRACT

Gadolinium (Gd) is among the rare earth elements extensively utilized in both industrial and medical applications. The latter application appears to contribute to the rise in Gd levels in aquatic ecosystems, as it is excreted via urine from patients undergoing MRI scans and often not captured by wastewater treatment systems. The potential environmental and biological hazards posed by gadolinium exposure are still under investigation. This study aimed to assess the teratogenic risk posed by a gadolinium chelate on the freshwater cnidarian *Hydra vulgaris*. The experimental design evaluated the impact of pure Gadodiamide (25 μg/l, 50 μg/l, 100 μg/l, 500 μg/l) and its commercial counterpart compound (Omniscan®; 100 μg/l, 500 μg/l, 782.7 mg/l) at varying concentrations using the Teratogenic Risk Index (TRI). Here we showed a moderate risk (Class III of TRI) following exposure to both tested formulations at concentrations ≥ 100 μg/l. Given the potential for similar concentrations in aquatic environments, particularly near wastewater discharge points, a teratogenic risk assessment using the *Hydra* regeneration assay was conducted on environmental samples collected from three rivers (Tiber, Almone, and Sacco) in Central Italy. Additionally, chemical analysis of field samples was performed using ICP-MS. Analysis of freshwater samples revealed low Gd concentrations (≤ 0.1 μg/l), despite localized increases near domestic and/or industrial wastewater discharge sites. Although teratogenic risk in environmental samples ranged from high (Class IV of TRI) to negligible (Class I of TRI), the low Gd concentrations, particularly when compared to higher levels of other contaminants like arsenic and heavy metals, preclude establishing a direct cause-effect relationship between Gd and observed teratogenic risks in environmental samples. Nevertheless, the teratogenic risks observed in laboratory tests warrant further investigation.

1. Introduction

Freshwater ecosystems play a crucial role in providing various ecosystem services to humanity, such as water and food supply, climate regulation, water quality control, erosion prevention, recreation, and tourism activities (Vári et al., 2022). Currently, these ecosystems are among the most threatened globally due to both human activities and climate change (Flitcroft et al., 2019; Cera et al., 2022; Cesarini et al., 2023). To address these challenges, numerous legislative measures have been implemented, including the well-known European Water Framework Directive (WFD) (European Commission, 2000) which advocates for the use of monitoring tools to safeguard of aquatic ecosystems. One such tool is Teratogenic Risk Index (TRI) proposed by Traversetti et al. (2017) serving as an early warning system for detecting potential teratogenic effects in freshwater environments (Wilson, 1973) using the coelenterate *Hydra vulgaris* (Pallas, 1766), known for its ability to regenerate lost body parts. The *Hydra* test has proven valuable in assessing the impacts of physical, chemical, and biological agents on embryos or fetuses, including adverse effects on human embryonic development (Wilson, 1973). Furthermore, it facilitates the identification of synergistic effects of contaminant mixtures dissolved in waters which may be challenging to detect through chemical analyses alone,

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particularly when pollutants are present in trace amounts (Gosset et al., 2016; Marcheggiani et al., 2019; Pedrazzani et al., 2019).

Emerging contaminants pose a challenge in environmental detection due to their lack of regulation and incomplete understanding of their potential effects (Taheran et al., 2018). Among these contaminants, gadolinium (Gd) belongs to the of rare-earth elements (REEs) family known for their difficulty in detection due to their low concentrations, necessitating instruments with high sensitivity and low detection limits (Trapasso et al., 2021). The use of REEs escalating in parallel with advancements in medical and technological fields, and this trend is projected to continue in the coming years (Gwenzi et al., 2018). REEs find widespread application in electronic devices like mobile phones, flat screen displays, and optical fibers, as well as in industries such as nuclear and medical, where they are used in contrast media for magnetic resonance imaging (MRI) and new chemotherapy drugs (Resende and Morais, 2010; Eliseeva and Bunzli, 2011; Gwenzi et al., 2018; Trapasso et al., 2021). Specifically, Gd possesses paramagnetic properties, making it a commonly used contrast agent in MRI (Perazella, 2009; Telgmann et al., 2013). Various types of contrast agents exist, differing based on the molecules to which the gadolinium ion is chelated: linear contrast agents (e.g., Omniscan®, Magnevist®, Multihance®) and macrocyclic contrast agents (e.g., Dotarem®, Gadovist®, Prohance®) (Geraldes and Laurent, 2009). When administered to patients, gadolinium-based contrast agents (GBCAs) are in the chelated form, which is generally considered inert (Hanana et al., 2017). However, the transformation products of these compounds or the release of $Gd3+$ ions could potentially have toxic effects on human health and biota (Hanana et al., 2017). The stability of GBCAs is jeopardized by various factors, including the presence of competing molecules, UV radiation, pH levels, and temperature variations during sewage treatment process in wastewater treatment plants (WWTPs) (Brünjes and Hofmann, 2020; Trapasso et al., 2021). Among GBCAs, Omniscan® exhibits the lowest conditional stability, and despite no conclusive evidence of clinical implications, the European Medicines Agency (EMA) suspended its marketing as part of a risk mitigation strategy. Nevertheless, Omniscan® is currently available in various regions worldwide (Asia, South America, and the United States) (European Medicines Agency, 2017; Clases et al., 2018). It's important to note that following intravenous administration, Gd accumulates in the brain, bones, skin, and other tissues of healthy patients (Errante et al., 2014; Rogosnitzky and Branch, 2016; Quattrocchi et al., 2023).

Environmental concerns can arise when pharmaceuticals, either in their original form or as metabolites, enter water from various sources such as domestic and hospital wastewater treatment plants, or landfill leachate (Nikolaou et al., 2007; Caldwell, 2016). The use of Gd in the medical field has a significant impact on aquatic ecosystems, as they become reservoirs for GBCAs that are not metabolized and excreted by patients (Ebrahimi and Barbieri, 2019). Indeed, WWTPs are unable to intercept these compounds, leading to their release into the environment (Oluwasola et al., 2022). This type of Gd is of anthropogenic origin and is added to the naturally occurring gadolinium found in the Earth's crust (Le Goff et al., 2019). Although some impacts (e.g., biota accumulation and toxicological effects) of the pure gadodiamide (GDA) and its pharmaceutical formulation, Omniscan®, are known (Trapasso et al., 2021), the teratogenic risk of both the active substance and its commercial compound has not been assessed. GBCAs have been detected in both human and animal placental tissues and amniotic fluids (Silverstein et al., 2020), and specifically, gadolinium chelates have the potential to accumulate in amniotic fluid. There have been no reported cases of teratogenicity after intrauterine GBCA exposure (Nguyen et al., 2023). However, the American College of Obstetricians and Gynecologists and the American College of Radiology recommend avoiding GBCAs in the pregnant individuals and administering them only if their use significantly improves the diagnostic yield and/or can improve fetal and parental outcomes (Chen et al., 2008).

The aim of the present study is twofold: 1) to investigate, for the first time, the teratogenic risk of pure GDA compared to the pharmaceutical formulation (i.e., Omniscan®), in order to exclude any possible masking effect of the excipients present in the commercial drug; 2) to evaluate the teratogenic risk of freshwater samples (collected from Tiber, Almone and Sacco rivers) after performing chemical analyses to detect the concentration of 23 different chemical elements, including Gd.

To achieve these aims, the TRI was applied using *H. vulgaris* as an early warning system. Specifically, the TRI was used to assign a risk class to both laboratory solutions and environmental samples. Additionally, the reactivity of tentacles was tested, and the feeding assay was performed to investigate new ecologically significant functional endpoints that could potentially be overlooked (being morphologically undetectable). It is important sampling sites were selected based on the significant correlation between Gd concentration and the presence of urban/industrial WWTPs or hospital discharge (Gao et al., 2021; Liu et al., 2022).

2. Materials and methods

2.1. Hydra culture

H. vulgaris is a freshwater cnidarian used in ecotoxicological assays for its high sensibility to a wide suite of contaminants and its regenerative capacities (Trottier et al., 1997; Quinn et al., 2012; Traversetti et al., 2017; Cera et al., 2020).

The *H. vulgaris* organisms employed in this study were maintained in glass tanks (30 × 30 × 30 cm), each containing *Hydra* medium, a solution composed of distilled water (998 mL), sodium bicarbonate ($NaHCO_3$, 1 mL reaching the concentration of 1 mM), and calcium chloride ($CaCl_2$, 1 mL reaching the concentration of 1 mM), at the Department of Sciences, University Roma Tre. The laboratory culture was bred at 18 ± 1 °C with 18:6 h light-dark photoperiod and fed *ad libitum* with nauplii of *Artemia salina* (Linnaeus, 1758).

2.2. Regeneration assay

The regeneration assay was conducted following the protocol of Traversetti et al. (2017) to obtain the Teratogenic Risk Index (TRI). For the regeneration assay, hydras were decapitated by a sterile bistoury under a stereomicroscope (Leica MZ-8). The *hypostoma* (i.e. mouthpart, head and tentacles) was removed, while the *columna* (i.e. remaining body portion) was exposed to different exposure tests. Five *columnae* (i.e., organisms after decapitation) per petri were tested with 10 mL of exposure test; 3 replicates were conducted using a total of 15 *columnae* for each treatment (i.e., laboratory solutions, environmental samples and control). The exposure test was renewed every 24 h until the end of the study. Decapitated organisms were exposed for 96 h at the same maintenance conditions, the time necessary for the complete regeneration of an organism at control conditions (Wilby, 1988). During the exposure, morphological observations were conducted at 24, 48, 72, and 96 h to evaluate the regeneration. At the end of 96 h, regeneration rate (RR) and aberration frequency (AF) were evaluated following Traversetti et al. (2017) classification.

Four different values of RR were considered, from 0 indicating a lack of regeneration of the *hypostoma* to 4 indicating a fully regenerated hydra. The RR values were assigned to each organism and the average value of the 15 hydras per treatment was then calculated.

The AF was determined by calculating the ratio of the number of individuals with morphological aberrations to the total number of individuals assessed for treatment (i.e., 15). Any deviation from the control morphology has been considered an aberration, such as clubbed tentacles, tentacles arrangement of different planes, doubled tentacles, etc.

For each exposure test, the values obtained for the parameters RR and AF were cross-referenced in a double-entry table finding a TRI score. The horizontal entry is determined by RR, and the vertical entry by AF;

their match provided the TRI score. The TRI score was categorized into 5 risk classes: (I) no risk; (II) low risk; (III) moderate risk; (IV) high risk; (V) very high risk (see Traversetti et al., 2017).

2.3. Tentacles' reactivity and feeding assay

Tentacles' reactivity and feeding rate were evaluated as representative of the correct regeneration of the organisms' cell lines and their functionality. A nervous disorder can lead to a failure to catch the prey and feed, although not necessarily linked to a visible morphological aberration.

After 96 h of regeneration assay, reactivity of the tentacles and feeding assay were conducted both under stereomicroscope. For each organism, the reactivity of the tentacles was assessed by stimulating them with a pin. The frequency of reactive individuals (corresponding to the contraction of the stimulated tentacle) for each exposure test was observed under a stereomicroscope and recorded.

The feeding assay was carried out providing 10 nauplii of *A. salina* to each hydra and evaluating after 30 min the number of hydras fed (i.e. that had consumed at least one prey).

2.4. Experimental design

The biological test used to assess the teratogenic risk of gadolinium compounds was based on the regeneration of *Hydra vulgaris* tissues (Pallas, 1766). *H. vulgaris* was exposed to laboratory solution and environmental samples. *Hydra* assay, tentacles reactivity, and feeding rate were used to evaluate the teratogenic risk of both laboratory and environmental solutions (see follow).

The control solution was tested both for laboratory and environmental solutions in triplicate. The control solution was composed of $NaHCO_3$ 0.1 M, $CaCl_2$ 1 M e KCl 0.1 M in 997 mL of distilled water.

2.4.1. Laboratory solutions

Before starting with experiments, a literature search was carried out on concentrations of Gd considered environmentally significant. It is important to highlight that Parant et al. (2018), documented concentrations of Gd up to 80 μg/L in proximity to sewage treatment plants. Subsequently, concentrations of tested solutions were selected mostly based on this Gd value.

Laboratory solutions tested were pure GDA in free active form and GDA contained in Omniscan® formulation. Four different concentrations of pure GDA were tested starting from a stock solution at a concentration of 1 mg/L using GDA powder (Y0001875, Merck) and then proceeding with progressive dilutions in the control solution. The concentrations of pure GDA were 25 μg/L, 50 μg/L, 100 μg/L, and 500 μg/L, selected as considered environmentally relevant (Ferreira et al., 2020).

The Omniscan® solutions tested were 100 μg/L and 500 μg/L to compare the pure GDA and drug formulation, whereas 782.7 mg/L was the highest concentration used. This latter concentration was selected as a proxy for the attainable concentration in the blood of a 75 kg patient, with a volume of approximately 5.5 L of blood, given the standard dose of the drug (15 mL).

2.4.2. Field samples

The environmental samples were collected from rivers, chemically analyzed to establish Gd concentration (see Section 2.2), and tested.

Ten sampling sites were selected along 3 rivers, the Tiber River, the Almone River, and the Sacco River, in Central Italy in the city of Rome and neighboring areas (Fig. 1).

Sites were selected before (CGB1, TIB1, TDV1, COL1) and after (CGB2, TIB2, TDV2, COL2) a source of pollution such as either wastewater treatment plants or industrial area, except for the Almone River (ALMO) which was sampled in the last point before flowing in the Tiber River (Table 1). In addition, the Tiber River was also sampled at the mouth (CDRA).

2.5. Chemical analysis

Chemical analyses were carried out shortly after sampling to assess the presence of Gd; then *Hydra* assays were conducted.

Chemical analyses were performed at the Chemistry Department of the University "La Sapienza" in Rome using inductively coupled plasma-mass spectrometry (ICP–MS; 820-MS, Bruker, Bremen, Germany)

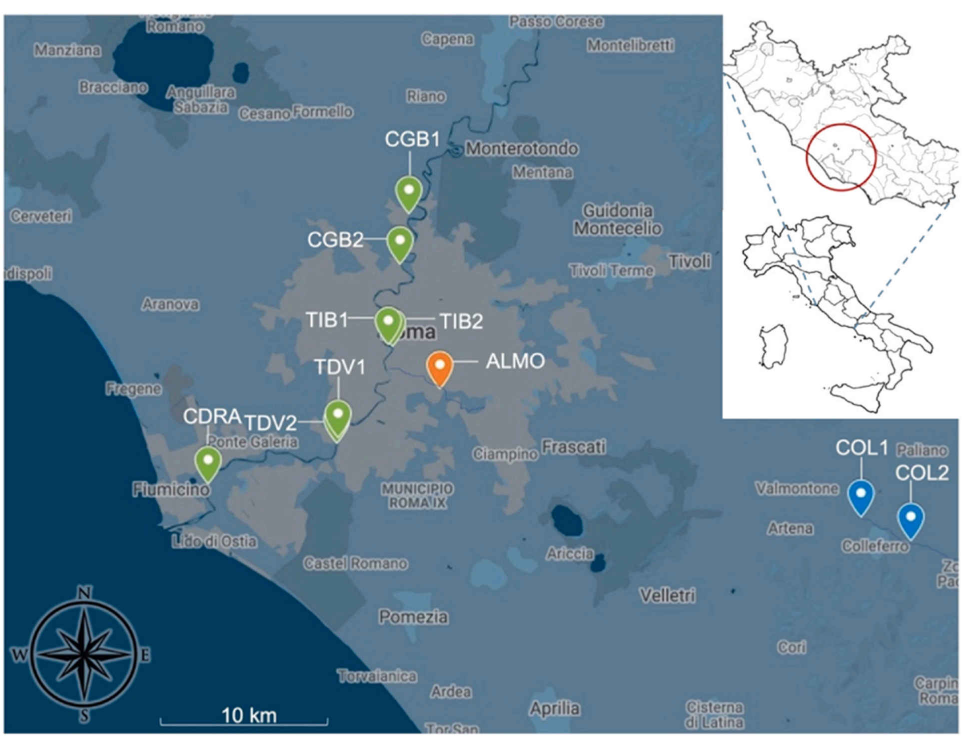

Fig. 1. Map of the sampling sites of the rivers Tiber, Sacco, and Almone located (red circle) in the Lazio region in Central Italy (blue dotted line).

Table 1
Characteristics of the sampling sites: river, site code, geographical coordinates, and a brief description of the location concerning the potential source of pollution. The number of the site code, where present, indicates the position considering the potential source of pollution: all numbers "1" are sites upstream of the polluting source, and numbers "2" are sites downstream of the polluting source.

River	Site	Latitude	Longitude	Description
Tiber	CGB1	41° 59' 52.1'' N	12° 29' 49.8'' E	Upstream of North Rome WWTP
Tiber	CGB2	41° 57' 24.2'' N	12° 29' 15.9'' E	Downstream of North Rome WWTP
Tiber	TIB1	41° 53' 27.5'' N	12° 28' 29.7'' E	Upstream of hospital discarge (Fatebenefratelli)
Tiber	TIB2	41° 53' 22.6'' N	12° 28' 45.4'' E	Downstream of hospital discarge (Fatebenefratelli)
Tiber	TDV1	41° 48' 54.7'' N	12° 25' 12.9'' E	Upstream of Tor di Valle WWTP
Tiber	TDV2	41° 48' 37.5'' N	12° 25' 08.7'' E	Downstream of Tor di Valle WWTP
Tiber	CDRA	41° 46' 37.9'' N	12° 16' 45.7'' E	Near to the river mouth
Almone	ALMO	41° 51' 17.5'' N	12° 31' 51.5'' E	Before the confluence with the Tiber River
Sacco	COL1	41° 45' 00.2'' N	12° 59' 17.8'' E	Upstream of Colleferro industrial area
Sacco	COL2	41° 43' 50.7'' N	13° 02' 33.3'' E	Downstream of Colleferro industrial area

equipped with a MicroMistTM (0.4 mL min^{-1} Analytik Jena AG, Jena, Germany) glass nebulizer and a collision reaction interface (CRI).

The instrumental conditions and methodology used are detailed in Ristorini et al., (2020). Briefly, each water sample, frozen at the time of sampling, was thawed and filtered with syringe filters (25 mm diameter, 0.45 μm pore size, GVS Filter Technology, Morecambe, England, UK) to remove sandy and clayey residues present in river waters before being analyzed. In addition to Gd, other 23 elements were quantified in standard mode (Al, As, Cd, Ce, Co, Cu, Fe, Ga, La, Mn, Mo, Nb, Ni, Pb, Sb, Sn, Ti Tl, U, V, W, Zn, Zr) after a 1:5 dilution with deionized water.

2.6. Data analysis

The normality of each data set (i.e., RR, AF, tentacles reactivity, and feeding rate both for laboratory and field samples) was checked by the Shapiro-Wilk test. The Analysis of Covariation (ANCOVA) was performed on the RR values collected at different time points (i.e., after 24, 48, 72, and 96 h), for both laboratory solutions and field samples. Two basic assumptions were verified before the ANCOVA: 1) independence of covariates and treatments, and 2) homogeneity of variance. The Tukey post-hoc was applied after the ANCOVA for pairwise comparisons. All previous steps of the data analysis were conducted with R libraries car (Fox and Weisberg, 2018) and multcomp (Bretz et al., 2016) and related functions.

As the dataset (i.e., RR, AF, tentacles reactivity, and feeding rate) was not parametric, the Kruskal-Wallis test was applied, followed by Dunn's post-hoc test to assess significant differences between different solutions (i.e. both for laboratory solutions and field samples, including control group). Statistical analysis was conducted using Past4.03. The significance for all statistical analyses was set to $p < 0.05$.

Moreover, the Principal Component Analysis (PCA) was performed on concentrations of chemical elements dissolved in water, to compare sampling sites and their distribution to metal concentrations, and to identify similar sampling sites in the chemical composition.

3. Results

3.1. Laboratory solutions

Results of 96 h regeneration assay using *H. vulgaris* exposed to Gd-based laboratory solutions are shown in Figs. 2a, 3a, 4a and 5a. Results of statistical analysis are reported in Supplementary Materials 1 and 2. Regarding the RR (Fig. 2a), a significant difference ($p < 0.05$) was found in the case of the highest concentration of pure GDA (RR = 3.53) compared to the control (RR = 3.93). At the lowest and intermediate concentrations of pure GDA, it was possible to see a similar regeneration trend for the control group (RR = 3.73 at 25 μg/L, RR = 3.6 at 50 μg/L, RR = 3.67 at 100 μg/L, RR = 3.53). In the case of Omniscan®, all different concentrations highlighted significant differences ($p < 0.05$) in RR compared to the control. Very similar RRs were found, specifically, RR = 3.27 at 100 μg/L, RR = 3.33 at 500 μg/L, and RR = 3.26 at 783 μg/L.

ANCOVA on RR values (collected after 24, 48, 72, and 96 h; Fig. 3a) and laboratory solutions of pure GDA and Omniscan® showed that while controlling time points, laboratory solutions are statistically significant ($p < 0.05$) because they had significantly contributed to the model. Being interested in knowing which laboratory solutions are different from each other, the Tukey post-hoc revealed that RR values are significantly different ($p < 0.05$) between Omniscan® at 100 μg/L and the control (see Supplementary Material 1).

No significant differences were observed in AF between laboratory solutions and control (see Supplementary Material 2 for more details). Pure GDA showed an AF of 20 % at 25 and 50 μg/L, 53 % at 100 μg/L and 40 % at 500 μg/L (Fig. 4a). Instead, different concentrations of Omniscan® highlighted the same percentage of AF (40 %). Overall, as concern types of aberration, "tentacle that occludes the mouth" was the

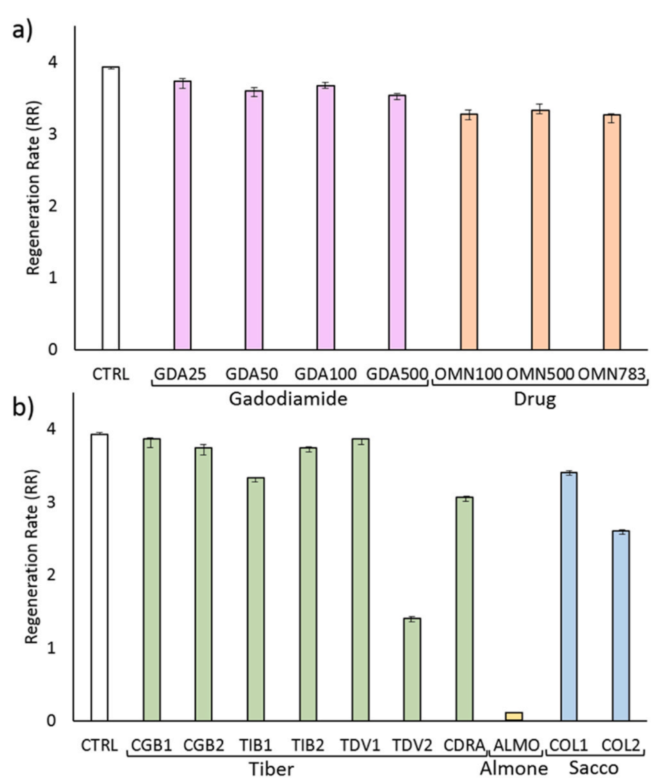

Fig. 2. Regeneration rate (mean ± s.d.) observed in *Hydra vulgaris* after 96 h in control conditions compared to: (a) pure gadodiamide (GDA, at different concentrations of 25, 50, 100, 500 μg/L) and Omniscan® (OMN, at different concentrations of 100, 500, 783 μg/L); (b) field samples from Tiber (CGB, TIB, TDV, CDRA), Almone (ALMO) and Sacco Rivers (COL), before and after (1, 2) a source of pollution.

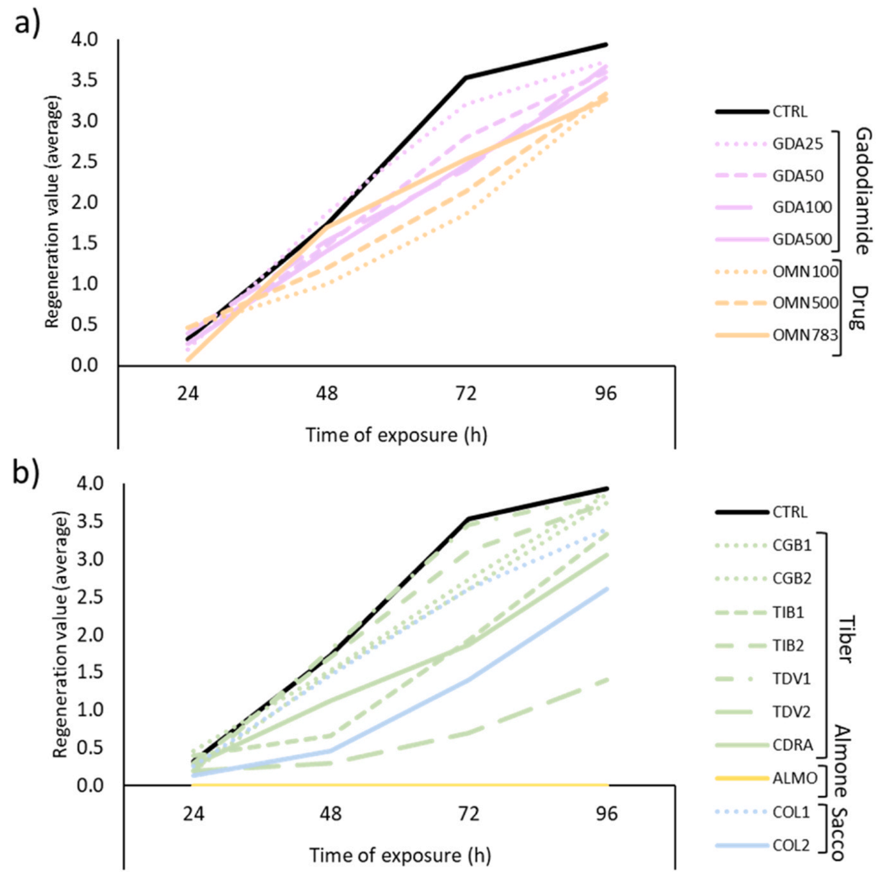

Fig. 3. *Hydra vulgaris* regeneration value (average) at 24, 48, 72 and 96 h of exposure to: (a) laboratory solutions of gadodiamide (GDA, at different concentrations of 25, 50, 100, 500 µg/L) and Omniscan® (OMN, at different concentrations of 100, 500, 783 µg/L) compared to control (CTRL); (b) field samples from Tiber (CGB, TIB, TDV, CDRA), Almone (ALMO) and Sacco Rivers (COL), before and after (1, 2) a source of pollution compared to control (CTRL).

most common aberration with 76 %, followed by "doubled tentacles" with 18 % and "tentacles arrangement on different planes" with 5 % testing laboratory solutions. The control solution reported a percentage of AF (13 %) with only one type of aberration ("tentacle that occludes the mouth"). Tests for tentacles' reactivity showed significant differences ($p < 0.05$) between the control and, (1) GDA at quite low concentration (50 µg/L); (2) Omniscan® at lowest and medium concentrations (100 and 500 µg/L). No significant differences were observed in the feeding rate between laboratory solutions and control, resulting in lower rates for pure GDA at quite low concentrations (50 µg/L) and Omniscan® at lowest and medium concentrations (100 and 500 µg/L).

Laboratory solutions with a concentration ≤ 50 µg/l had a low teratogenic risk, reaching a TRI value of 9. Laboratory solutions with concentrations ≥ 100 µg/l showed a moderate teratogenic risk with a TRI value of 6 (Fig. 5a).

3.2. Field samples

Results of 96 h regeneration assay using *H. vulgaris* exposed to field samples are shown in Figs. 2b, 3b, 4b and 5b. Results of statistical analysis are reported in Supplementary Materials 3 and 4.

Regarding RR (Fig. 2b), a significant difference ($p < 0.05$) was found in the case of Tiber River in the sites TIB1 (RR = 3.33), TDV2 (RR = 1.40), CDRA (RR = 3.06) compared to the control (RR = 3.93). In the case of Almone River, all the individuals of *H. vulgaris* were dead within 24 h of exposure (RR = 0). As regards Sacco River, a significant difference was found in COL2 (RR = 2.60) compared to the control (RR = 3.93).

ANCOVA on RR values (collected after 24, 48, 72, and 96 h; Fig. 3b) and field samples of different rivers (see before) showed that while controlling time points, freshwater samples are statistically significant ($p < 0.05$) because they had significantly contributed to the model. Being interested to know which samples are different from each other, the Tukey post-hoc revealed that RR values are significantly different ($p < 0.05$) between (1) Almone River and most of the other sampling sites, including the control (but excepting for TDV2 and COL2); (2) the TDV2 sampling site of Tiber River and many of other sampling sites, including the control (but excepting for TIB1, CDRA, COL1, COL2) (see Supplementary Material 3).

Significant differences ($p < 0.05$) were observed in AF between field samples and control (AF = 13 %) and they are reported in Supplementary Material 4. Specifically, the significance was observed between CGB1 (AF = 53 %) and control, and between TDV2 (AF = 60 %) and control. In ALMO, all individuals died within 24 hours; as a result, no morphological aberrations could be observed. Instead, Sacco River (COL1 and COL2) didn't show any significant difference in AF between field samples and control. Concerning aberration types, "tentacle that occludes the mouth" was the most common aberration with 49 %, followed by "tentacles arrangement on different planes" with 37 %, "doubled tentacles" with 7 %, and "clubbed tentacles" with 5 %. It is mandatory to signal a new aberration for this test, never described before in literature: a "two-headed" hydra (Fig. 4d). It was observed with an incidence of less than 1 % (only 3 hydras overall) in sampling sites of CGB2 and CDRA.

Tests for tentacles' reactivity showed significant differences ($p < 0.05$) between the control and TIB1, TDV2, ALMO, and COL2 sampling sites. Significant differences were observed also in the feeding rate between control and TDV2, CDRA, and ALMO sampling sites.

The calculated TRI values show that 80 % of the sites analyzed

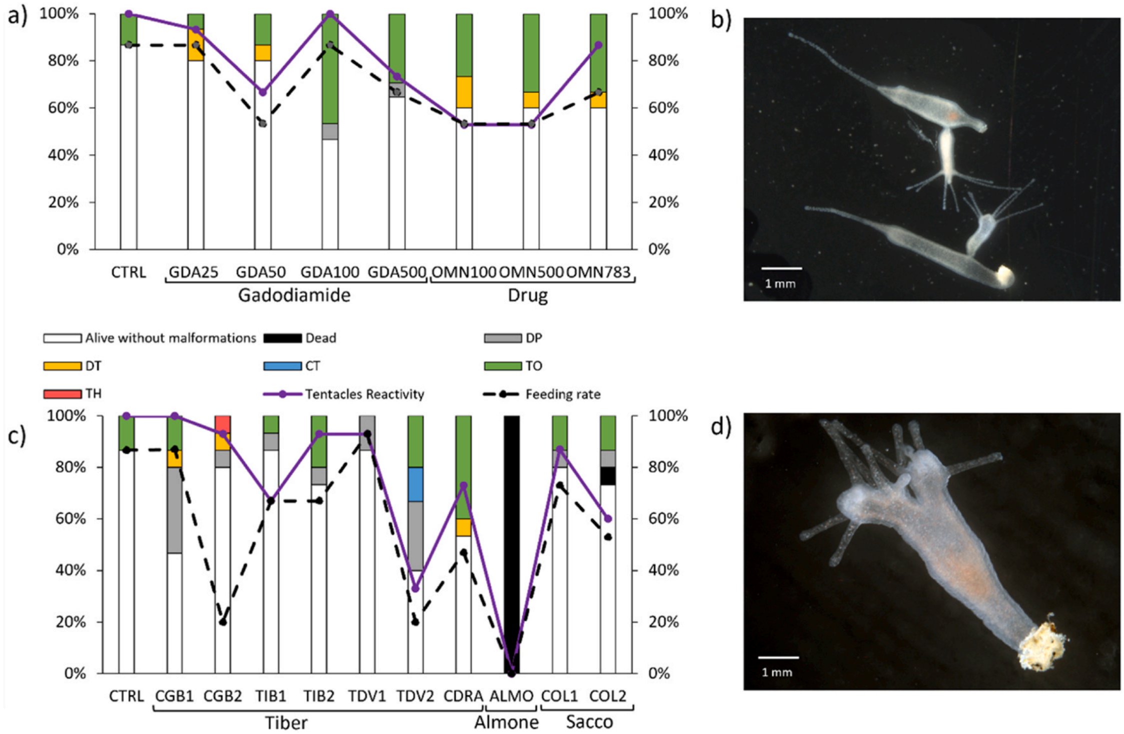

Fig. 4. Aberration frequency (bars), tentacles reactivity (lines) and feeding rate (dashed lines) observed after 96 h in *Hydra vulgaris* exposed to: (a) laboratory solutions of gadodiamide (GDA, at different concentrations of 25, 50, 100, 500 μg/L) and Omniscan® (OMN, at different concentrations of 100, 500, 783 μg/L) compared to control (CTRL); (c) field samples from Tiber (CGB, TIB, TDV, CDRA), Almone (ALMO) and Sacco Rivers (COL), before and after (1, 2) a source of pollution compared to control (CTRL). Data are shown as mean ± standard deviation. TH = two-headed; TO = tentacle that occludes the mouth; CT = clubbed tentacles; DT = doubled tentacles; DP = tentacles arrangement on different planes. Pictures showing some examples of teratological forms found: (b) single tentacle occluding the mouth; (d) doubled head.

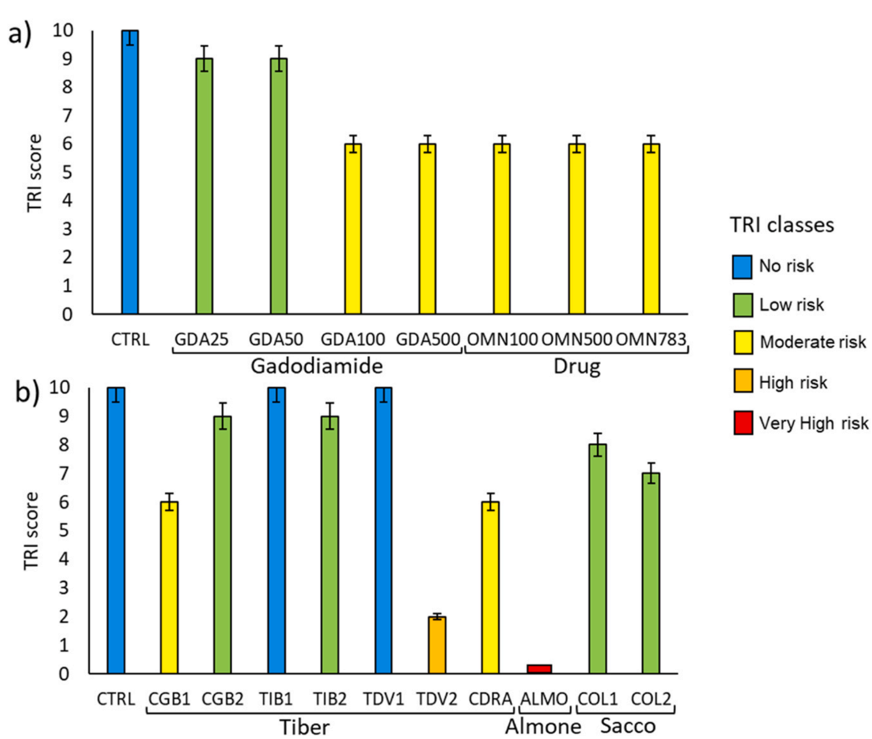

Fig. 5. Application of Teratogenic Risk Index (TRI) to (a) laboratory solutions of gadodiamide (GDA, at different concentrations of 25, 50, 100, 500 μg/L) and Omniscan® (OMN, at different concentrations of 100, 500, 783 μg/L) compared to control (CTRL); (b) field samples from Tiber (CGB, TIB, TDV, CDRA), Almone (ALMO) and Sacco Rivers (COL), before and after (1, 2) a source of pollution compared to control (CTRL). Colors indicate classes of Teratogenic Risk Index (TRI), from no risk (blue) to very high risk (red). Data are shown as mean ± standard deviation.

present a teratogenic risk. In particular, 40 % of stations have a low risk, 20 % a moderate risk, 10 % a high risk, and 10 % a very high risk (Fig. 5b).

3.3. Chemical analyses

Table 2 showed the main results of chemical analyses, reporting concentrations of gadolinium (being of interest for the present study) plus five heavy metals (known also as Potentially Toxic Elements – PTE) usually evaluated in ecotoxicological studies (i.e., copper, zinc, iron, nickel, and manganese) and the arsenic (a semimetal well known for its teratogenic effects and ubiquitous in the Latium rivers). Specifically, some comparisons could be operated in and between sampling sites, such as: (1) from CGB1 to CGB2, placed before and after a sewage treatment plant in northern Rome, respectively, there is an increase in manganese equal to 215 % and a decrease of copper and zinc; (2) from TIB1 to TIB2, placed before and after the Tiber Island, respectively, there is an increase in manganese equal to 175 %; (3) from TDV1 to TDV2, placed before and after a sewage treatment plant, respectively, there is an increase in copper equal to 1666 %, in zinc equal to 4810 %, and manganese equal to 226 %; (4) from COL1 to COL2, placed before and after an industrial area, respectively, there is an increase in manganese equal to 98 %, and a decrease in iron and arsenic; (5) in ALMO, placed after several domestic wastewater discharge channels in the southern Rome, manganese and iron showed the highest concentrations recorded in this study. For additional results on chemical analyses, please refer to Supplementary Material 5.

The result of PCA on chemical element concentrations is shown in Fig. 6. The first and second Principal Components (PC1 and PC2) explained more than 90 % of the sample variance (PC1 = 66.7 %; PC2 = 25.01 %). The biplot graph highlighted that most of the sampling sites were similar in chemical composition and were all placed in the down left of the graphical space. Otherwise, those results peculiar in the chemical composition were "ALMO", "TDV2" and "CDRA" sampling sites.

4. Discussion

The findings of our study highlighted some relevant scenarios on the biological effects in invertebrates following exposure to REEs. Specifically, we demonstrated that (1) a teratogenic risk associated with GDA and its pharmacological formulation exists, (2) the teratogenic risk is greater under environmental exposure conditions than in Gd-contrast agent exposure conditions, and (3) a correlation of the investigated contaminants with the tentacle reactivity and the feeding assay exists.

4.1. Lab solutions

Comparing GDA and Omniscan®, no significant difference in teratogenic risk was found at equivalent concentrations. Solutions at or below 50 μg/L exhibited a low teratogenic risk (TRI value of 9), while concentrations exceeding 100 μg/L were associated with moderate teratogenic risk (TRI value of 6). This suggests that excipients in Omniscan® do not influence teratogenic risk. While the chelating agent in GDA may reduce acute toxicity on *H. vulgaris*, it still poses a moderate risk by altering cnidarian morphology. Differences between the pure active form and the drug formulation, particularly at 500 μg/l, indicate potentially more adverse effects from drug exposure, possibly due to excipients like sodium caldiamide or higher sodium content compared to the control solution.

The differences in teratogenic risk between GDA and Omniscan® have profound implications for environmental risk assessment and management strategies. Firstly, understanding these differences provides insight into the potential impact of gadolinium-based contrast agents (GBCAs) on aquatic ecosystems. The moderate teratogenic risk associated with both GDA and Omniscan® suggests that gadolinium, which can enter freshwater systems through wastewater treatment plants, may contribute to elevated teratogenic risks in rivers. This highlights the importance of monitoring and regulating the release of GBCAs into the environment to mitigate adverse effects on aquatic organisms and ecosystems. Moreover, elucidating the biological effects of GDA and Omniscan® allows for a better understanding of their mechanisms of action and potential hazards. Differences in biological effects may arise from variations in chemical composition, concentrations, or modes of action between the two compounds. By identifying these differences, researchers and regulators can prioritize the assessment and management of GBCAs based on their relative risks to environmental and human health. From a risk management perspective, these findings underscore the need for comprehensive environmental monitoring and risk assessment protocols for GBCAs. This includes monitoring GBCA concentrations in surface waters, evaluating their toxicity to aquatic organisms, and assessing their potential for bioaccumulation and biomagnification in food webs. Additionally, measures should be implemented to reduce GBCA inputs into wastewater treatment plants, such as promoting the use of alternative contrast agents with lower environmental impacts or implementing advanced treatment technologies to remove GBCAs from wastewater effluents.

Omniscan® was evaluated at a concentration of 783 mg/l, exceeding environmentally relevant levels. This concentration mirrors the potential blood level of GDA post-injection, allowing assessment of teratogenic risk, lethal concentration (LC) potential for *H. vulgaris*, and organism response under human-relevant concentrations. While direct comparisons between human and coelenterate cellular responses are impractical, the *Hydra* regeneration assay has effectively indicated teratogenicity across various substances. Notably, *H. vulgaris* has proven more reliable than in vitro mouse embryo testing for certain teratogens. Regarding GBCAs, controlled animal studies found no adverse outcomes in pregnant mice exposed to GBCAs. However, the safety of fetal gadolinium exposure in humans remains uncertain. While multiple case series have shown no harm at birth following gadolinium administration during pregnancy, these studies are limited by sample size and observational design. Notably, a large retrospective study by Ray et al. (2016) revealed a higher neonatal death rate in GBCA-exposed children, though with limitations including lack of MRI indications, low follow-up rates, and absence of trimester subset analysis or an ideal control group.

Table 2
Concentrations (μg/L) of Gadolinium (^{158}Gd), heavy metals (nickel – ^{60}Ni, copper – ^{65}Cu, zinc – ^{66}Zn, manganese – ^{55}Mn, iron – ^{56}Fe) and arsenic (^{75}As) in water samples collected from Tiber (CGB, TIB, TDV, CDRA), Almone (ALMO) and Sacco Rivers (COL), before and after (1, 2) a source of pollution.

Sampling sites	^{158}Gd μg/L	^{60}Ni μg/ L	^{65}Cu μg/ L	^{66}Zn μg/ L	^{55}Mn μg/ L	^{56}Fe μg/ L	^{75}As μg/ L
CGB1	0.004	0.96	8.51	2.02	3.27	0.63	16.6
CGB2	0.011	1.12	0.94	1.30	10.32	4.38	8.9
TIB1	0.012	1.18	1.49	0.54	5.11	1.32	15.6
TIB2	0.015	1.35	0.79	1.72	14.08	1.81	20.0
TDV1	0.111	1.45	12.54	19.64	22.15	3.24	29.6
TDV2	0.007	1.02	0.71	0.40	6.80	1.47	8.0
CDRA	0.048	1.03	14.36	0.82	15.27	1.74	80.6
ALMO	0.100	1.03	1.70	6.28	47.46	13.77	19.2
COL1	0.035	0.40	1.21	1.48	9.26	11.54	12.8
COL2	0.068	0.40	1.26	1.05	18.37	8.31	6.2

4.2. Chemical analyses

The study emphasizes the challenge of directly attributing low gadolinium concentrations to human activities or assessing the risk posed by gadolinium spills to the ecosystem. This uncertainty arises from the inability to determine the chemical form and origin of gadolinium present in the samples, which may be influenced by natural or human-derived substances, such as chelating agents of gadolinium-based contrast agents (GBCAs). These considerations underscore the

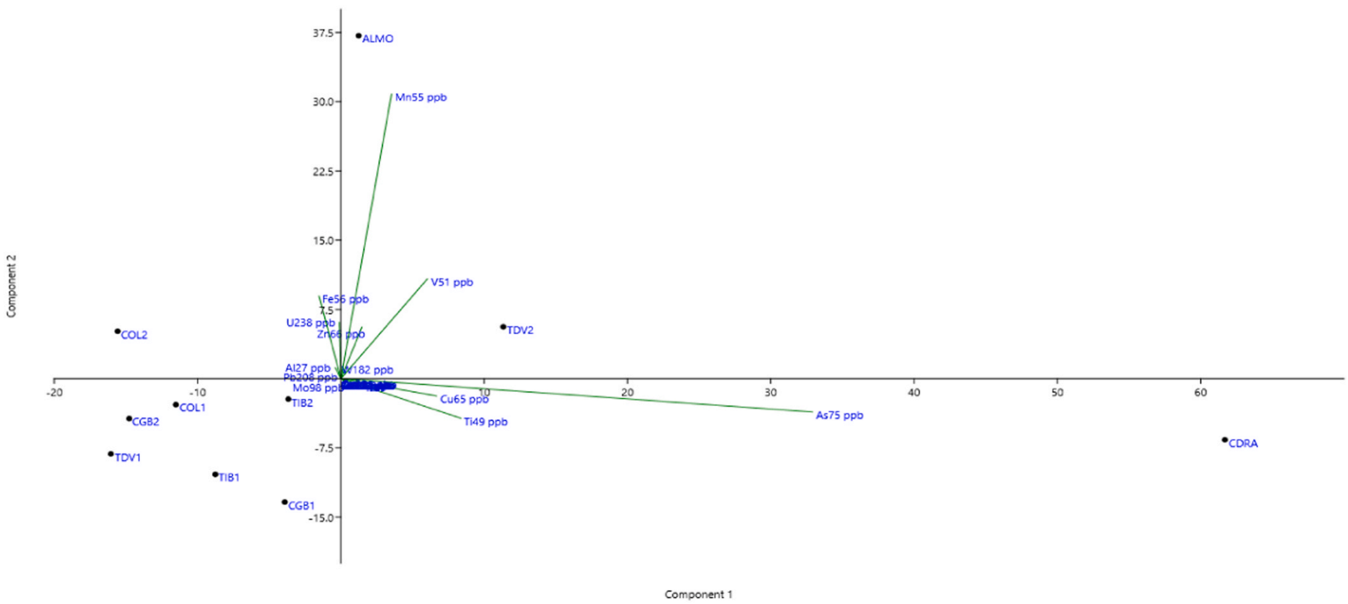

Fig. 6. Biplot of Principal Component Analysis to compare water samples collected from Tiber (CGB, TIB, TDV, CDRA), Almone (ALMO) and Sacco Rivers (COL), before and after (1, 2) a source of pollution, and the chemical composition of sampling sites in terms of heavy metals (nickel – ^{60}Ni, copper – ^{65}Cu, zinc – ^{66}Zn, manganese – ^{55}Mn, iron – ^{56}Fe), Gadolinium (^{158}Gd) and arsenic (^{75}As).

complexity of assessing teratogenic risks associated with gadolinium exposure in freshwater environments. The significant increase in manganese levels at the "CGB2" sampling site, coupled with decreases in copper and zinc, suggests potential issues with the wastewater treatment plant in northern Rome, resulting in limited dilution of heavy metals before discharge. Similar trends were observed at sites before and after Tiber Island ("TIB1" and "TIB2"), as well as before and after the Tor di Valle wastewater treatment plant ("TDV1" and "TDV2"), indicating substantial differences in water chemistry despite short distances between sites. Despite these findings, it remains uncertain whether the low concentrations of gadolinium detected are linked to human activities or pose a risk to the ecosystem. This uncertainty stems from challenges in determining the chemical form and origin of gadolinium, influenced by various factors including natural and anthropogenic sources such as gadolinium-based contrast agents (GBCAs). It is estimated that during the period of the health emergency, the number of MRI scans performed dropped by 80 %, leading to a consequent reduction in the use of contrast media, and a lower release of gadolinium into water bodies (Brünjes and Hofmann, 2020). Therefore, both freshwater analysis and the assessment Gd presence could be essential to better understand the actual impact of GBCAs use in this post-pandemic period, focusing on 1) the increase in Gd concentration in aquatic ecosystems and 2) the accurate quantification of geogenic Gd (i.e., not attributable to anthropic activities) (Brünjes and Hofmann, 2020).

4.3. Field sample

The calculated TRI values indicate that 80 % of the analyzed sites exhibit a teratogenic risk. Specifically, 40 % of stations have a low risk, 20 % a moderate risk, 10 % a high risk, and 10 % a very high risk (Fig. 5b). This widespread teratogenic risk, with the potential to alter and endanger aquatic ecosystems, underscores the importance of early warning tools such as the regeneration assay of *H. vulgaris*. This assay can intercept the introduction of contaminants into rivers and identify any critical issues before biological communities and ecosystems suffer irreparable damaged (Cera et al., 2019). The most commonly observed aberrations in *H. vulgaris* include (i) tentacles arranged on different planes (often resulting in the occlusion of the mouth region due to misplaced tentacles), (ii) single tentacle that occludes the mouth, (iii) doubled tentacles, or even death (Fig. 4).

A new aberration, never previously described in the literature, has been observed: the regeneration of two heads instead of one (Fig. 4d). This newly discovered aberration had an incidence rate of less than 1 % and was observed in the CGB2 (with a TRI index of 9 and low teratogenic risk) and the CDRA station (with a TRI index of 6 and moderate teratogenic risk) stations. It is noteworthy that the CDRA station is characterized by the highest concentration of arsenic (Table 2), a semimetal known for its teratogenic power (Kaur et al., 2011).

Considering our findings, setting toxicity and mortality thresholds for metallic and semi-metallic elements becomes challenging due to the presence of a mixture containing a total of 23 chemical elements detected at varying concentrations. In general, results obtained from the *Hydra* regeneration assay and the application of the TRI appear to align with the findings of the PCA, which facilitated the categorization of sampling sites based on chemical composition. Sites exhibiting similar metal and semimetal concentrations showed comparable TRI values, except for the CGB1. In TDV2 and ALMO, mortality rates of 47 % and 100 % of individuals were observed within 96 h, respectively. These sites exhibit a total metal and semimetal content of 109 μg/l and 124.45 μg/l, respectively (including Gd).

Identifying one or more specific compounds possibly responsible for observed effects can be a complex task. However, this complexity underscores the usefulness of bioassays as early warning systems. Chemical analysis alone may not be sufficient to determine environmental quality, as it may fail to detect exceedances of toxicity thresholds for individual elements. Additionally, synergistic phenomena capable of generating detrimental effects at lower concentrations may go unnoticed. Indeed, although the environmental concentration of Gd was low, the *Hydra* regeneration assay showed greater effects downstream of the polluting source. Moving towards the mouth of the Tiber River, AF has increased (e.g., in TDV2 AF = 60 %), and often after the polluting source the RR is significantly reduced (e.g., in TDV2 RR = 2).

It's worth highlighting that the CGB1 site (TRI = 6), despite its moderate teratogenic risk, showed no significant differences from the control group in terms of tentacle reactivity or feeding behavior. This site presented a notably high regeneration value (RR) for *H. vulgaris*, while the moderate teratogenic risk is primarily attributed to the substantial presence of aberrant individuals, which, interestingly, exhibit normal reactivity and feeding capabilities. This site also differed in terms of chemical elements present, as it did not significantly diverge from sites with higher TRI values, even when considering their chemical composition. These findings appear to support the hypothesis that this site may be contaminated with organic substances and compounds, such as endocrine disruptors, to which *H. vulgaris* is highly sensitive (Pachura-Bouchet et al., 2006). These substances have the potential to modify cell proliferation without impacting differentiation, but affecting regeneration capacity, irrespective of aberration production, as demonstrated by previous studies (Cardenas et al., 2000; Ostroumova and Markova, 2002). Other sites with the same risk class (e.g., COL1 and COL2) were found to differ from the analysis of these two endpoints, highlighting the significance of taking into account behavioral endpoints beyond the morphology considered by the TRI.

The risk assessment of GBCA in freshwater ecosystems reveals temporal trends in teratogenic risk, particularly evident in the Tiber River. In 2017, the teratogenic risk in the stretch of the river near the Labaro-Castel Giubileo area was absent (Class I); however, the sample collected and analyzed today at site CGB1 shows a moderate risk level (Class III), indicating a two-class worsening of the risk (Traversetti et al., 2017; Cera et al., 2019). The estuarine area also shows an increase in the risk level, from low (Class II) in 2017 to moderate (Class III). However, the more significant deterioration that needs to be highlighted is the section of the Tiber River placed downstream of the Tor di Valle wastewater treatment plant. This site, in 2015, was identified as having no teratogenic risk (Class I) and was re-evaluated in 2017 as having low teratogenic risk (Class II) (Traversetti et al., 2017; Cera et al., 2019). However, it is currently classified as a high-risk site (Class IV). Instead, the teratogenic risk of the Sacco River has decreased and returned to the levels observed in 2015. The sampling site at Colleferro (COL2), which was classified as having low risk (Class II) in 2015, was re-evaluated two years later and found to have moderate risk (Class III) (Traversetti et al., 2017; Cera et al., 2019). However, this study now considers the area to be of low risk (Class II). It is important to note that the sampling was conducted during the COVID-19 health emergency period, and this situation may have resulted in a reduction in pollution related to the disposal of wastewater from industrial activities in this area.

5. Conclusions

Gadolinium, extensively used in medicine and technology, requires assessment of its ecological and human health impacts. Guidelines recommend cautious use of GBCA during pregnancy, but no limits exist for Gd release into the environment. Its potential teratogenic effects pose risks to aquatic organisms and human health, prompting laboratory analyses of specific GBCAs. Pure GDA and Omniscan® showed moderate teratogenic risks, urging further research into their effects on embryonic development in various organisms. The study marks the first attempt to quantify Gd presence in central Italy's riverine ecosystems, with concentrations below 0.1 μg/l, yet no clear cause-and-effect relationship was established. However, it is important to acknowledge some limitations of the study. Firstly, the research primarily focused on assessing the teratogenic risks of specific gadolinium-based compounds, thereby limiting the generalization of results to other chemical substances or environments. Additionally, the collection of environmental samples was conducted during a specific period, amidst the COVID-19 health emergency, which may have influenced pollution levels and the conditions of the sample sites. Lastly, the lack of long-term data and follow-up studies limits the comprehensive understanding of the long-term impacts of gadolinium on aquatic ecosystems and human health.

Ongoing monitoring of Gd effects and concentrations is crucial for understanding its impact on river ecosystems, allowing comparisons between pre-Gd conditions and anticipated future influences. Behavioral endpoints tested using *H. vulgaris* complement risk assessments, revealing differences between GBCAs in both laboratory and environmental samples. Incorporating such endpoints into risk indices enhances their comprehensiveness, enabling the identification of subtle abnormalities. Additionally, evaluating molecular markers in *H. vulgaris* could elucidate toxicity or teratogenic effects of Gd and its compounds.

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CRediT authorship contribution statement

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

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116442.

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