Association of toxic effects and the quality of surface water and wastewater: application under environmental conditions and literature overview by CiteSpace

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Abstract

Monitoring of wastewaters is crucial for the protection of surface waters and should contain quality and toxicity analyses to ensure safety of aquatic organisms. In this study, the impacts of industrial and municipal wastewaters were assessed by examining the responses of organisms from different trophic levels, *Pseudokirchneriella subcapitata* (microalgae) and *Daphnia magna* (crustaceans), together with physical and chemical water parameters and total metal(loid) concentrations, separately in wastewater lagoons and four nearby sites in the karst Krka River in spring, summer and autumn. The sites in close proximity to the inappropriately treated wastewaters exhibited diminished ecological status, especially regarding COD, nutrients, turbidity, mineral oils, and elevated concentrations of metals (Cd, Co, Cu, Fe, Na, Ni, and Zn). Toxicity effects were confirmed for surface river water near the municipal wastewater outlet (hazard class III) and for basins with industrial wastewater (hazard class IV). Although such approach enabled determination of the toxic hazard of complex mixtures in aquatic environments, literature overview by Cite Space showed that there is little data and that European countries dominate in this area of research. In addition, multivariate statistical analysis confirmed association of water quality data and toxic effects and the importance of microbiotests in assessment of ecologically relevant risks for aquatic organisms.

Keywords: physical and chemical parameters; metal(loid) exposure; ecotoxicity; microbiotests; scientometric analysis

1. INTRODUCTION

The karst environment is among the most fragile and vulnerable on Earth. In karst regions, water infiltrates rapidly through a highly interconnected network of conduits, caves, and sinkholes formed within soluble carbonate or evaporite bedrock (Parise et al., 2015; Ford and Williams, 2007). Even minor, diffuse inputs at the surface (such as agricultural runoff, septic effluent, or accidental spills) can quickly propagate throughout the entire system, making karst aquifers especially susceptible to contamination (White, 2002). However, many urban areas in karst regions face water quality degradation due to limited or no sewer systems and wastewater treatment plants, resulting in discharge of municipal and industrial wastewaters into karst streams without appropriate treatment (Ford and Williams, 2007; Hillebrand et al., 2012; Malá et al., 2022). Given that karst groundwater supplies up to 25% of the world's drinking water, preserving its quality is of critical importance (Goldscheider and Drew, 2007).

The methods for assessing the quality of water systems provided in the Water Framework Directive (WFD, Commission Directive 2013/39/ EU) mainly focus on single chemical analyses (Kortenkamp et al., 2019). However, this approach is often insufficient for quality control purposes, since chemical compounds occur as mixtures in the environment. Consequently, it is hard to predict physical, chemical and biological impact of pollutants, especially due to their antagonistic and synergistic effects in mixtures (Mendonça et al., 2013). To fully capture the integrated biological risks posed by these mixtures, there is growing recognition of the need for ecotoxicity testing in karst rivers. In the ecotoxicological testing of mixtures, the contributions and interactions of the individual mixture components are often investigated in the laboratory. However, it is strongly recommended to complement this approach with whole effluent toxicity testing, which characterizes the ecotoxicity of an entire mixture (including undetected and unmeasured substances) and provides an overall assessment of cumulative biological effects (Maloney et al., 2023; Menghini et al., 2023). Although whole effluent toxicity testing is applied in regulatory context, especially in the USA and Canada, linking toxic effects and environmental conditions is still challenging (Vosylienė, 2007). Increasingly, it is proposed to combine multiple bioassays, include organisms with different biological complexity and trophic roles, and expand the range of quantifiable endpoints (Menghini et al., 2023; Vosylienė, 2007).

Algae form the base of aquatic food webs and are widely used in phytotoxicity testing due to their sensitivity to chemical and biological changes in the environment (Burton et al., 2002). When compared to other species, algae are more sensitive to a diverse range of potential contaminants, including metals and organic pollutants, and to fluctuations in physical and chemical parameters (Tousova et al., 2018; Xin et al., 2021). Among aquatic invertebrates, daphnids are commonly used in zootoxicity assays due to their broad distribution in a wide range of habitats, sensitivity to numerous environmental contaminants, relatively small size, short life cycle and easy maintenance in the laboratory (Persoone et al., 2009). Essentially, the algae *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák, 1990 (initially named *Selenastrum capricornutum* Printz, 1914 and renamed *Raphidocelis subcapitata* (Korshikov) Nygaard, 1987) and the crustacean *Daphnia magna* Straus, 1820 are among the most frequently used and recommended species for freshwater toxicity testing, supported by standardized protocols and extensive data on their responses to a range of contaminants (Radix et al., 2000; Weyers et al., 2000; Moreira-Santos et al., 2004).

Although whole effluent toxicity assessment was developed in the 1950s, research combining tests on algae and daphnids appears to have begun in the 1990s, partially due to the development of “Toxkit” microbiotests. Unlike earlier bioassays, these commercially available tests contained dormant eggs of selected invertebrate species, immobilized microalgae, or ciliate protozoans and were maintenance-free, resulting in high popularity among researchers in many countries (Persoone et al., 2003). The majority of toxicity tests involving algae and daphnids were conducted to determine the toxicity of a specific chemical substance or compound (Garric et al., 2007; Hund-Rinke et al., 2018), while fewer studies were conducted using effluents or environmental water samples (Kusui et al., 2014; Luan et al., 2020).

In order to estimate possible relation between the toxic effects of contaminants and their concentrations and sources under the real environmental conditions, our study was performed in the karst Krka River. It is an ideal environment for conducting such research due to the pristine water at the river source and the direct influence of the mixtures of contaminants from industrial and municipal wastewaters downstream. As a result, ambient river samples and wastewater outlet samples differ in their physical, chemical and organic water parameters, as well as metal(loid) concentrations (Filipović Marijić et al., 2018; Sertić Perić et al., 2018; Mijošek et al., 2023). To assess rapid and ecologically relevant evaluation of toxicity in complex environmental samples, we applied standardized microbiotests using two aquatic species from different trophic levels: the green alga *Pseudokirchneriella subcapitata* (primary producer) and the freshwater crustacean *Daphnia magna* (primary consumer). The main objective was to compare experimental and environmental data, i.e. the results of toxicity tests on two species with physical and chemical parameters and metal concentrations in surface freshwater and in wastewaters. Possible relationship between the toxic effects and the effects of pollution on the living organisms in the complex and sensitive karst ecosystem was estimated, thus providing new data expressing the causal relationship between the mixture of pollutants and the toxic effects in environmental samples. To support and contextualize our findings, we also conducted a scientometric analysis using CiteSpace software. This analysis aimed to identify global research trends, major thematic clusters, and existing knowledge gaps in the field of ecotoxicological assessments of wastewaters and surface waters, particularly with respect to studies using multiple test organisms under environmentally relevant conditions. By integrating scientometric insights with original experimental data, we provide both new ecotoxicological information for the Krka River and a broader perspective on how this research contributes to the advancement of knowledge in karst aquatic ecosystems. Finally, assessed the potential and limitations of microbiotests for the detection and quantification of exposure to environmental pollutants and link toxic effects to their chemical causes.

2. MATERIALS AND METHODS

2.1. Sampling procedures

Field research was conducted in the upper part of the Krka River, involving surface water at four localities along the river watercourse. Additionally, industrial wastewater from the basins of the nearby screw factory was directly sampled. Sampling was performed in spring (April 25-27), summer (July 20) and autumn (October 18-20) of 2021. Water samples and *in situ* measurements were taken at a depth of 0.1 m, approximately 1 m from the riverbank, involving the source of the Krka River, considered as a reference station without direct anthropogenic impact (KRS); Krka River watercourse downstream of the industrial wastewaters and along the municipal wastewater discharge of the Town of Knin (KRK); tributary Orašnica, which passes along the lagoons with industrial wastewater (TOR); tributary Butišnica, mainly influenced by agricultural runoff (TBU); lagoons with industrial wastewater from the screw factory (IWW) (Fig. S1). Air distance from KRS was 2.8 km for TOR, 2.9 km for IWW, 3.7 km for KRK and 4.1 km for TBU. Detailed data on ecological status, long term trends in physical and chemical water parameters and dissolved metal(loid) concentrations were recently described by Šariri et al. (2024) and Mijošek et al. (2023).

2.2. Analyses of the physical and chemical water parameters

Measurements of the pH and total dissolved solids (TDS) were measured *in situ* using portable field meters (Mettler Toledo), with exception of industrial wastewater, which was filtered in the laboratory prior to the measurements of pH and TDS due to its extremely poor quality. Other physical and chemical parameters (ammonia, nitrate, total nitrogen and phosphorus, turbidity, chemical oxygen demand (COD) and dissolved CO_2), were analyzed in the laboratory using the respective standardized methods (Hach Lange GmbH, 2013; APHA, 2018) as described by Šariri et al. (2024), total organic carbon (TOC) and dissolved organic carbon (DOC) according to HRN EN 1484 (Croatian Normative Document, 2002), while phenols and mineral oils according to ASTM D 4763-6 (2020). Surface river water data were compared with the values outlined in the Directive on water quality status issued by the Government of the Republic of Croatia (Official Gazette of the Republic of Croatia NN 96/2019, 2019), which defines the limit values for the ecological status of various types of water bodies, categorizing them as "very good", "good", or "below good". The industrial wastewater data were compared with the emission limit values defined by the Regulation on limit values for wastewater emissions (Official Gazette of the Republic of Croatia NN 26/2020, 2020). The aforementioned documents convey the EU Water Framework Directive (Official Gazette of the Republic of Croatia NN 96/2019, 2019; Official Gazette of the Republic of Croatia NN 26/2020, 2020).

2.3. Measurements of total metal(loid) concentrations in water

Mijošek et al. (2023) provided a detailed description of water sampling, as well as the procedure we utilized for the analysis of the total macro and trace elements in water samples using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx, Agilent Technologies, Tokyo, Japan). For the elements examined, the limits of detection (LODs) varied from 0.0005 $\mu g\ L^{-1}$ for Cs to 30 $\mu g\ L^{-1}$ for K. As part of quality control, three certified standard reference materials (NRCC SLRS-5, NIST SRM 1643e, and NIST SRM 1643f) were analyzed, and the recoveries ranged from 92% (Ni) to 109% (Cd) (Table S1).

2.4. Toxicity Tests

The toxicity of the samples to aquatic organisms was tested using the Microbiotests Toxkits supplied by MicroBioTestsInc. as Algaltoxkit F^{TM} (2004) and Daphtoxkit F^{TM} (2001), which follow the standardized methods and ensure reliable and comparable results (Vosylienė, 2007). Each water sample was collected in 1 L glass bottle, and then stored at 4 °C until the start of the experiments within 48 hours after the sampling. Based on estimation on water quality of the river water samples and according to the kit manufacturer instructions (ISO standard 8692, 2012; ISO standard 6341, 2012) water from KRS and TBU was not additionally diluted (used as 100% concentration), while other water samples were diluted with standard freshwater (used as algal culturing medium and hatching medium for crustaceans and provided with necessary nutrients). They were applied in the following concentration ranges: TOR as 100% and 50% concentrations, KRK as 100%, 50% and 25% concentrations and IWW as 100%, 50%, 25%, 12.5% and 6.25% concentrations.

2.4.1. Algal growth inhibition test

The green microalgae were de-immobilized from alginate beads for determination of algal growth inhibition after 72 h, according to ISO standard 8692 (2012). The test vials were incubated under controlled conditions in an incubator TC 135 S (Aqualytic, Germany) at 23±2 °C with continuous illumination of 10,000 lx for 24, 48 and 72 h. Optical density (OD) was measured at wavelength of 670 nm by UV-Vis spectrophotometer DR6000 (Hach, SAD), which was zero-calibrated with algal growth medium. The cell density of algal stock was adjusted to approximately $1*10^6$ cells/ml, according to OD/N regression, and initial cell density in each test vial was $1*10^4$ cells/ml. The growth and inhibition rate were calculated using the data from OD measurement for every volume of water sample and the control. Six replicates of controls (untreated) and three replicates of each test water concentration were prepared and applied, followed by triplicate measurements. Potassium dichromate ($K_2Cr_2O_7$) was used as positive control to ensure the validity of the test method. Since the industrial wastewaters were colored, a dilution series without algae was prepared for the IWW samples and used to zero-calibrate the spectrophotometer prior to the daily measurement of OD in the algae-IWW dilutions to minimize interference.

2.4.2. Daphnia immobilization test

The toxicity tests on *D. magna* were carried following ISO 6341 standard method (2012). *D. magna* were hatched from dormant eggs (ephippia) in three days under continuous illumination (6000 lx) at 20-22 °C. Neonates (younger than 24 h) were exposed to the undiluted and diluted samples for 24 h and 48 h at a temperature of 20 °C in darkness. Twenty neonates were used for each sample in a series of four wells; each well contained 10 mL of sample and 5 neonates. The toxicity of each sample was evaluated by the estimation of *D. magna* immobilization rates based on the number of dead or immobilized water fleas compared to the number of active organisms tested. Potassium dichromate ($K_2Cr_2O_7$) was used as positive control to ensure the validity of the test method.

2.4.3. Toxicity evaluation

Toxicity was evaluated by means of toxicity effect and the effective concentration. The toxicity effect was expressed as percentage of mortality/immobilization (*Daphnia* test) or inhibition (Algae test), depending on the effect criterion of the respective assay. The effective concentration (EC_{50}, % v/v), causing immobilization or inhibition in 50% of the tested population, was calculated using nonlinear regression analysis (ISO Standard 8692) and performed with software for automated data analysis of *Algaltoxkit* and *Daphtoxkit* results, provided by the kit manufacturer MicroBio Tests Inc. All results are presented as mean ± S.D. The toxicity data were also classified according to the hazard classification system as shown in Table S2 (Persoone et al., 2003), by using the equation TU = 100/EC_{50} to transform toxicity values (EC_{50}) into Toxic Units (TU).

2.5. Statistical and scientometric analysis

2.5.1. Statistics

Statistical analyses and calculations were done using SigmaPlot 11.0 (Systat Software, USA), and SPSS Statistics 20.0 (IBM). Total metal(loid) concentrations are presented as mean ± standard deviation (S.D.). Variability of total metal(loid) concentrations in water samples between the sites and seasons was performed using two-way ANOVA and Holm-Sidak test. Statistically significant differences between sites in each season are marked with different numbers: 0 – significantly different site from all other sites; 1-5 - significantly different site from the sites indicated by specific number as follows: 1- KRS; 2- TOR; 3- KRK; 4- TBU; 5- IWW; differences between seasons for each location are marked with different letters or asterisk: * - significantly different season from all other seasons; a-c - significantly different season from the seasons indicated by specific letter as follows: a – spring; b - summer; c - autumn. Reduction of dimensionality of a multivariate data set was performed by the Principal Component Analysis (PCA).

2.5.2. Scientometric analysis by CiteSpace

The scientometric analysis of the literature on testing the toxicity of effluents with algae and crustaceans was performed using CiteSpace 6.1.R6 (Basic). CiteSpace is a Java application for analyzing and visualizing scientific literature developed by Dr. Chaomei Chen and is increasingly used to identify trends and provide an objective overview of a state of the art. Based on a set of publications, it can create various types of visual analysis maps in which the objects of analysis (e.g., cited references, countries, keywords, etc.) are represented as nodes of a network and linked based on the relationships between them (co-citation, collaboration, co-occurrence, etc.). The importance of each node in a network is proportional to its Betweenness Centrality (BC) value, while Citation Burst (CB), representing an increase in the citation frequency of a node (i.e., a keyword) in a short period of time, can indicate an increase in interest in a particular topic within a research area (Chen, 2006). The burst strength, calculated using Kleinberg's algorithm, quantifies the intensity of this increase, helping to identify emerging trends or influential topics in a research field (Kleinberg 2002).

In this study, a dataset was created by searching the topics "alga" AND "daphnia" AND "effluents" AND "toxicity" in the Web of Science Core Collection (WoS), to collect scientific literature combining the whole effluent toxicity testing using both organisms. WoS was used because it proved to be the best data source for the CiteSpace analysis (Mostafaie et al., 2021). The document types and time of publications were not specified. A total of 125 publications from 1992-2022 were found and stored as complete records and cited references. To analyze the collaborations and identify the most important countries in this research domain, a network was created with node type = country, time slice = one year, scale factor k = 15, and node rendering mode = tree ring history. For the same dataset, keyword co-occurrence analysis, clustering, and burst detection were also used to identify the most active topics in the research domain. Accordingly, the node type was changed to keyword, and the other parameters remained the same. After clustering the network nodes, cluster labels were determined based on the title words using the log-likelihood ratio (LLR) algorithm.

3. RESULTS AND DISCUSSION

Karst catchments are vulnerable areas due to the porous nature of karst landscapes which can lead to the pollution of surface and groundwater sources, representing a threat to drinking water supplies. For this reason, the monitoring of water's physical and chemical factors holds great importance, as they serve as reliable indicators of the overall condition. However, it is also beneficial to involve organisms, as they can predict potential threats to aquatic species (Blanck et al., 1984).

3.1. Trends of the physical and chemical water properties

Detailed characterization on physical and chemical conditions of the Krka River and adjacent industrial wastewaters were previously published by Šariri et al. (2024), so in this study we focused on varying degrees of anthropogenic influence among four sampling locations of the Krka River and assessed the conditions of industrial wastewaters (Table 1). According to limit values for surface water bodies categorized in the Directive (Official Gazette of the Republic of Croatia NN 96/2019, 2019), only water from the reference site, KRS, could be regarded as water of "very good" quality in all seasons, except ammonium concentrations, which were in "good" quality range. When compared to other locations, KRS exhibited the highest level of clarity and the lowest concentrations of TDS, ammonium, nitrogen, nitrates and phosphorus in all seasons. Most parameters at TOR and KRK frequently exceeded the limit values and did not meet the desired quality standards, especially during summer and autumn, while TBU water was of "very good" or "good" quality according to the regulations. This confirmed deteriorated environmental conditions in tributaries which are directly influenced by wastewaters (Table 1). Based on the physico-chemical characterization, the ecological water status in the upper course of the Krka River generally adhered to the following order: KRS>TBU>TOR>KRK.

Physical and chemical properties of IWW exceeded emission limit values for many parameters set up for wastewaters (Official Gazette of the Republic of Croatia NN 26/2020, 2020). Organic parameters were the highest in IWW, but their higher levels were reflected in surface waters of KRK and TOR, which are located in the vicinity of municipal and industrial wastewater discharges, respectively. Therefore, contrary to physical and chemical parameters, concentrations of mineral oils, TOC, DOC and dissolved CO_2 tended to be higher at KRK than TOR, suggesting municipal wastewaters as the primary source of organic compounds in the Krka River (Table 1).

Table 1. Physical, chemical and organic parameters of surface waters of the Krka River (sampling sites 1 – 4) and of industrial wastewater (sampling site 5) in three sampling seasons (spring, summer and autumn) of 2021. According to the limit values for surface water bodies (sampling sites 1-4), those parameters which are categorized in the Directive (Official Gazette of the Republic of Croatia NN 96/2019, 2019) are presented as italic for "very good", underlined for „good" and bold for „bellow good" ecological status, while parameters above emission limit values set for wastewater (sampling site 5) emissions (Official Gazette of the Republic of Croatia NN 26/2020, 2020) are presented as bold/underlined.

Sampling	Spring (25.-27.4.2021)					Summer (20.7.2021)					Autumn (18.-20.10.2021)				
Parameters	1 KRS	2 KRK	3 TOR	4 TBU	5 IWW	1 KRS	2 KRK	3 TOR	4 TBU	5 IWW	1 KRS	2 KRK	3 TOR	4 TBU	5 IWW
Turbidity / FAU	0	4	8	3	2355	0	4	3	4	35	0	5	2	1	15
Total dissolved solids (TDS) / mg L^{-1}	193.9	215	564	440	1642	226	291	320	440	2750	216	291	403	478	5290
pH	*7.54*	*7.97*	*7.61*	*8.20*	7.32	*7.58*	*7.92*	*7.48*	*8.20*	7.75	*7.51*	*7.78*	*7.54*	*8.10*	7.89
Dissolved CO_2 / mg L^{-1}	4.7	31	2.7	1.1	14.01	4	4.1	1.7	1.6	8.8	5.7	3.6	2	4.9	9.1
Chemical oxygen demand (COD) /mg O_2 L^{-1}	*0.19*	**8.83**	**11**	**4.6**	**3030**	*0.72*	**165.5**	3.02	**4.41**	**2770**	*0.98*	**4.18**	*1.74*	3.11	101.7
Ammonium / mg N L^{-1}	0.02	0.04	**0.19**	0.02	7.88	*0.01*	**4.6**	0.02	0.05	**21**	0.02	**0.09**	**0.07**	0.03	2.4
Total nitrogen / mg N L^{-1}	*0.1*	0.8	**1.9**	*0.6*	**21.2**	*0.2*	**6.5**	*0.5*	0.8	**60**	*0.3*	**3**	**1.8**	0.8	**69**
Nitrate / mg N L^{-1}	*0.08*	*0.15*	*0.22*	*0.11*	**7.09**	*0.04*	*0.41*	*0.02*	0.1	**3.07**	*0.11*	*0.21*	*0.25*	*0.03*	**3.07**
Total phosphorus / mg P L^{-1}	*0.009*	**0.084**	0.053	*0.012*	**2.21**	*0.003*	**0.89**	**0.086**	*0.014*	**8.54**	*0.004*	**0.087**	*0.017*	0.037	1.3
Mineral oils / µg L^{-1}	< 5	< 5	56.7	< 5	**38177**	< 5	1863	380	< 5	**24902**	< 5	1787	540	< 5	**56811**
Phenols / µg L^{-1}	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	46.8	< 5	< 5	< 5	< 5	**182**
Total organic carbon (TOC) / mgL^{-1}	1.476	1.552	4.321	1.694	**100.0**	1.064	6.783	6.042	2.798	22.82	1.557	8.164	1.775	2.468	21.18
Dissolved organic carbon (DOC) / mgL^{-1}	1.381	1.496	3.519	1.272	76.36	0.876	5.483	2.517	1.272	11.97	0.268	7.794	1.535	1.021	20.36

3.2. Trends of the total metal(loid) concentrations in water

The average total concentrations of almost all elements were the highest in basins of IWW, even significantly higher compared to the other sites in the Krka River in all seasons (Table S3). Levels of Fe, Zn, Cs and Co were up to 1778 times, 1207 times, 450 times and 450 times higher at IWW than at TBU and KRS, respectively. Such trend is consistent with the "good" to "very good" ecological status of TBU and KRS (Table 1). Moreover, concentrations of several metal(loid)s at IWW were higher than at TOR (Cs: 91x, Zn: 65x, Cu: 63x, As: 30x), the site nearest to basins with industrial wastewater and characterized as having a "below good" ecological status in the Krka River (Table 1). Given the widespread industrial application of the aforementioned elements, particularly Fe and Cu in a screw production, it is evident that the IWW has a strong influence and presents a potential danger for aquatic organisms in the Krka River (Filipović Marijić et al., 2018; Mijošek et al., 2023).

The differences among the sampling sites in the Krka River, excluding IWW, mostly were not statistically significant and were still rather low and typical for karst ecosystems (Table S3), suggesting the efficiency of self-purification process (Filipović Marijić et al., 2018; Cukrov et al., 2008). Nevertheless, average concentrations of As, Cd, Co, Cu, Fe, Na, Ni, and Zn were clearly higher at TOR and KRK, influenced by wastewater discharges, than at KRS and TBU (Table S3).

Seasonal patterns for the majority of elements showed higher levels in summer than in other two seasons, probably as a consequence of low water levels during summer months. A similar trend was not seen in IWW, where metal(loid) concentrations (particularly As, Cu, Fe, and Ni) (Table S3) and organic matter (Table 1) peaked in spring, probably signifying the moment of wastewater discharge (Šariri et al., 2024). Similar patterns of increasing metal(loid) concentrations and higher TOC and DOC values were also noted at other locations, mostly in the summer (Table S3).

3.3. Toxicity evaluation

In addition to the analysis of physical, chemical and organic parameters and total metal(loid) concentrations, which provide an assessment of the present conditions and require long-term monitoring, biological analyses can give insight in potential toxicity and anthropogenic impact on the aquatic organisms within the sensitive karst ecosystem. Moreover, the inclusion of toxicity biotests has become increasingly important for potential hazards evaluation (Wolska et al., 2007).

3.3.1. Toxicity effects

The samples of surface Krka River water (sites KRK, TBU, TOR and KRK) mostly did not show toxic effect regarding *Daphnia* toxicity test (0-20% immobilization in all sites and seasons of 2021). Slightly higher toxic effect was observed at TOR and KRK than at other sites, probably confirming impact of industrial and municipal wastewaters at these locations (Fig. 1). Inhibition of algae growth varied more among sites and seasons than immobilization of daphnids, also indicating marginal effects in undiluted water samples from TOR (26.7% in autumn, Fig. 1c) and KRK (46.0% in summer and 82.7% in autumn, Fig. 1b, 1c). In spring, toxic effects on algae were less pronounced, but still detected at all locations (6.08-28.0%, Fig. 1a). Contrary, the wastewater at IWW exhibited high toxicity in both tests. During the spring, the toxic effect of undiluted and 50%-diluted IWW samples was confirmed by the 94.4% to 98.9% inhibition of phytoplankton growth and the 100% immobilization of *D. magna* (Fig. 1a). Industrial wastewaters showed lower toxic effects in summer than in spring (84.6% inhibition of algae growth; 20% immobilization of *D. magna,* Fig. 1b), which coincides to lower levels of metal(loid)s and some physical and chemical water parameters at that location (Table 1, Table S3).

Differences between seasons were more pronounced in *Daphnia* test, where the toxic effect recorded for IWW in summer was 5 times lower than in spring (Figs. 1a, 1b). In autumn, the inhibition of algae growth was 98.3% and immobilization of *D. magna* 100% (Fig. 1c), which corresponded to the maximum levels of phenols, mineral oils, total nitrogen and TDS compared to spring and summer (Table 1). Overall, impact of physical and chemical disturbances and contaminant mixtures resulted in the highest toxicity observed at IWW, but also toxic effects were recorded at locations near wastewater outlets in the Krka River (Fig. 1). Comparison of the two testing organisms, primary producer and primary consumer, indicated that algae *P. subcapitata* was more sensitive to pollution than *D. magna*, with a higher percentage of toxic effect evident in all seasons (Fig. 1). In addition, water from KRK and TOR had a discernible effect solely on algae, with the exception of spring when minor effects were observed on both algae and daphnids (Fig. 1a). These findings are consistent with previous studies, reporting that among four toxicity tests to 16 chemicals algae were more sensitive compared to bacteria, rotifers and *D. magna* (Radix et al., 2000), and that *P. subcapitata* was shown as very sensitive to herbicides and fungicides (Yeh and Chen, 2006), both of which may be present in the area of the Town of Knin due to agricultural practices. Still, the proposed limitation of algae as primary producers is their inability to predict chronic toxicity to higher organisms, necessitating the further use of invertebrates like *D. magna*.

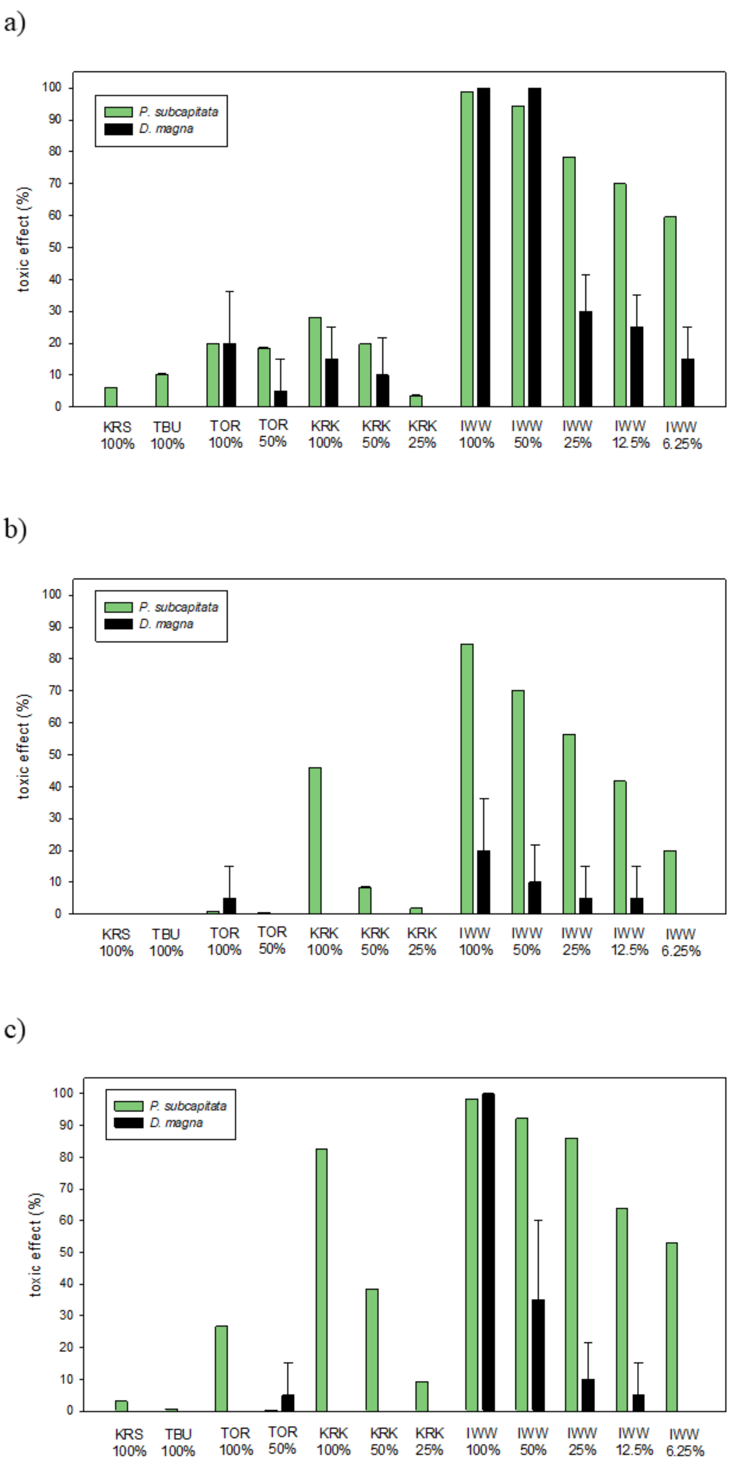

Fig. 1. Toxic effects (%) on *Pseudokirchneriella subcapitata* and *Daphnia magna* using dilution series of river water (sampling sites: KRS, TBU, TOR, KRK- 25; 50; 100%) and wastewater (sampling site: IWW- 6.25; 12.5; 25; 50; 100%) sampled in: a) spring; b) summer; c) autumn of 2021. The values of the standard deviations range from 0.02% to 0.18% for the growth inhibition of *P. subcapitata* and from 0.00% to 25.17% for the immobilization of *D. magna*.

3.3.2. Hazard toxicity classification

Based on hazard classification system (Table S2) (Persoone et al., 2003), only KRK water showed acute toxicity (TU = 2, hazard class III) for both tested species when surface river water was considered, indicating high sensitivity of the karst river water to the pressure of organic compounds. Industrial wastewater was classified as high acute toxicity (TU = 18 for daphnids and 32 for algae, hazard class IV) (Table 2). Generally, our results confirmed toxic effects of water from sites with direct influence of municipal (KRK) and industrial (IWW) wastewaters. Calculated EC_{50} (% v/v) for KRK river water was 50 for both testing organisms and for IWW 5.55 for *D. magna* and 3.125 for *P. subcapitata* (Table 2). In summary, the increase in toxicity of the analyzed river and wastewater samples follows the order: KRS ≤ TBU < TOR < KRK < IWW.

Table 2. Toxicity classification for analyzed samples of the river water and wastewater from the Krka River catchment

Sampling sites	**Testing organism**	**EC_{50} % (vol/vol)**	**Toxicity unit (TU)**	**Hazard class**	**Toxicity**
KRS	*D. magna*	> 100	< 0,4	I	No acute toxicity
	P. subcapitata	> 100	< 0,4	I	No acute toxicity
TBU	*D. magna*	> 100	< 0,4	I	No acute toxicity
	P. subcapitata	> 100	< 0,4	I	No acute toxicity
TOR	*D. magna*	> 100	< 0,4	I	No acute toxicity
	P. subcapitata	> 100	< 0,4	I	No acute toxicity
KRK	*D. magna*	50	2	III	Acute toxicity
	P. subcapitata	50	2	III	Acute toxicity
IWW	*D. magna*	5.55	18	IV	High acute toxicity
	P. subcapitata	3.125	32	IV	High acute toxicity

3.4. Association of water quality and toxic effects on aquatic organisms

Our results pointed to the toxic effects recorded at site of the Krka River directly influenced by municipal wastewaters (KRK). Although situated in the vicinity and under the influence of IWW, site TOR was not classified as being acutely toxic (Table 2), indicating a higher sensitivity of the karst river water to the pressure of organic compounds, which are usually abundant in municipal wastewaters (KRK), rather than to the higher metal concentrations resulting from IWW. However, due to the complexity of the environmental conditions and physical and chemical matrix, it is extremely challenging, if not impossible, to establish a complete link with their respective degrees of toxicity. This is also in agreement with reports from similar studies on complex samples in which no clear relationship between the ecotoxicological findings and the results of the chemical analyses could be established (Persoone et al., 2003).

Accordingly, we performed Principal Component Analysis (PCA) to correlate results obtained by microbiotests (toxicity) with chemical analyses (metal(loid) levels and physical and chemical parameters), considering the sites with the highest hazard toxicity class, and one of them belonging to the Krka River watercourse (KRK) and the other to the basins with industrial wastewater (IWW). Two components, using Varimax rotation with Kaiser Normalization, were extracted explaining 66.7% of variance at KRK and 65.7% at IWW (Fig. 2). At both locations, association among different metal(loid)s was specific for the first component, while most of the organic parameters and data on toxic effects on algae were associated in the second component. Specifically, at KRK in the first component all metal(loid)s with COD, total N, total P and ammonium were extracted, while second component combined toxic effect on algae with TDS, turbidity, TOC, DOC and mineral oils. At IWW the first component combined metal(loid)s, dissolved CO_2, COD, TOC, DOC and nitrates, while in the second component toxic effects on algae were linked with TDS, mineral oils, phenols, but also Zn and Cs (Fig. 2). Such integrated approach confirmed municipal wastewaters as the primary source of organic compounds in the Krka River, with the highest influence on toxic impact on algae inhibition growth. Also, previous conclusion on seasonal variability of parameters at KRK showed the same patterns of toxic effects with TOC and DOC, mineral oils, nitrates, ammonium, nitrogen and TDS, but most of the metal(loid) concentrations did not show similar pattern among seasons (Table 1, Table S3), what was reflected in multivariate analysis. On the other hand, the relationship between physical and chemical variations and toxic effects was probably more specific in the basins into which wastewater is directly discharged (IWW), due to the lack of natural seasonality, circulation and self-purification, as well as the specific composition (metal(loid)s used in industry) and timing of wastewater discharge (Šariri et al., 2024).

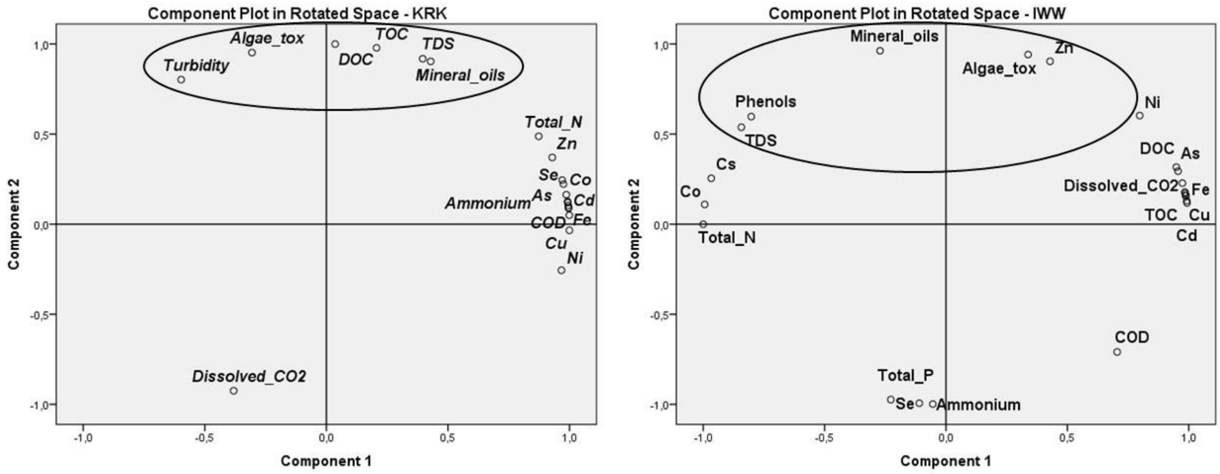

Fig. 2. Principal component plot using Varimax rotation for toxic impact observed on algae (Algae_tox) and physical and chemical parameters at site under the direct influence of municipal wastewaters (KRK) and industrial wastewater from the basins of the screw factory (IWW), showing association of parameters in two principal components (circled parameters are associated with toxic effects on algae).

3.5. CiteSpace analysis of the literature data

A comprehensive search of the WoS database revealed that 125 publications were published worldwide between 1992 and 2022 using a combination of the terms "alga," "daphnia", "effluents", and "toxicity", presenting a relatively small number. Nevertheless, the number of publications in this research domain is increasing, with the majority of publications from our dataset published in recent years (41% between 2016 and 2022), highlighting both the current relevance of the topic and the continued scarcity of experimental data, such as the toxicity assessment of the Krka River presented above (Fig. S2).

3.5.1. The country collaboration analysis

Research connecting terms "alga," "daphnia", "effluents" and "toxicity" was conducted in a total of 37 countries, with most articles published by authors from Germany (13), Italy (13), the United States (13), England (12), and Portugal (12) (Fig. S3). However, it contained only 65 links between nodes and 57% of the included countries had a BC of 0.00, showing the significant lack of international collaborations in this research domain. Similar to the number of publications, the highest BC values were found in European countries, namely Germany (0.44), Belgium (0.27), England (0.19), Italy (0.17), and Portugal (0.13), probably due to common project funding opportunities and similar environmental standards and regulations related to biomonitoring and effluent toxicity control. Among non-European countries, the United States and Japan had the highest BC.

3.5.2. The keyword co-occurrence analysis

Apart from the basic keywords "toxicity" and "effluent", the top three keywords in terms of frequency and centrality were "*Daphnia magna*" (43 occurrences, centrality of 0.77), followed by "pharmaceutical" (15 occurrences, centrality of 0.18), and "acute toxicity" (12 occurrences, centrality of 0.19). Burst detection of keywords, as indicators of emerging trends, showed that the keywords "test" and "chemical" appeared more frequently in the papers published in the early 2000s, while more keywords started to burst since 2013 (Fig. 3). Although our dataset contained the papers published in 1992–2022 period, no significant trend keyword was observed until 2000. The keywords with the most recent bursts were "removal", "waste water", "pharmaceutical", "toxicity" and "risk assessment". Among them, "pharmaceutical", "toxicity" and "risk assessment" were used in the early 2000s but gained more attention after 2015, 2017 and 2020, respectively. On the other hand, "removal" and "waste water" appeared in publications for the first time in recent years and bursted shortly after. These are also two keywords with the highest burst strength (3.36 and 3.15). Although our experimental study does not fall under the currently prominent topic of contaminant removal, such as the elimination of pharmaceuticals from wastewater, keyword burst detection confirmed that toxicity testing and the environmental risk assessment of wastewater remain highly relevant. A distinctive contribution of our research lies in the assessment of surface waters within a karst ecosystem and the integration of toxicity results with environmental data—both of which are relatively understudied areas not reflected among the most frequently cited keywords.

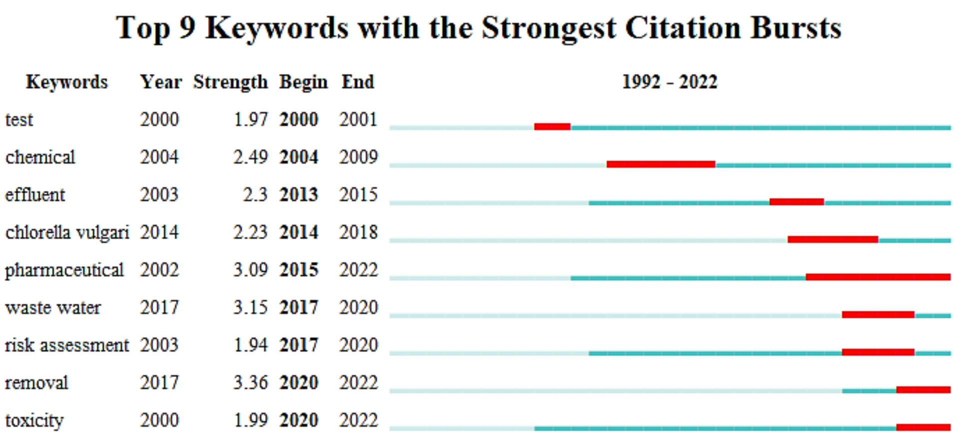

Fig. 3. Keywords indicating the major trends in research connecting topics "alga", "daphnia", "effluents" and "toxicity" from 1992 to 2022. The period in which each keyword occurs most frequently in the analyzed literature is highlighted in red.

The network of keywords was divided into 12 clusters (Fig. 4). The largest cluster, resulting from this analysis, labeled as “#0 emerging contaminant” (size 46, silhouette 0.752), contained many more members than other clusters. The size of the second largest cluster, labeled as “#1 hospital wastewater” (silhouette value 0.906), was 29. Both of them contained research papers that used a battery of toxicological tests, primarily to assess the toxicity of pharmaceuticals, but also disinfectants, illicit drugs, pesticides and persistent organic pollutants from wastewater and diffuse pollutant sources. However, cluster #1 contained much older publications (mean year 2005) than cluster #0 (mean year 2013).

Based on the mean years of publications in the obtained clusters, studies have previously been focused on assessing toxicity of environmental samples (#3 combination effect (2007)) and effluents from hospitals, industries and mines (#1 hospital wastewater (2005), #10 determination (2010), #6 abandoned uranium mine (2010)), but also of specific contaminants in environmental samples (#1 hospital wastewater (2005), #5 aquatic hazard assessment (2009), #9 true functional environmental genomics (2009)), shifting from acute to sublethal toxicity indicators and linking laboratory and field effects (#4 various acute sublethal (2005), #2 missing link (2008), #9 true functional environmental genomics (2009)). Research on effluent toxicity to algae and daphnids has increased over the past decade, and recent studies have focused on assessing the effects of exposure to emerging contaminants and metals using the battery of toxicity tests (#0 emerging contaminant (2013), #8 sensitive battery (2013)), the use of sublethal endpoints in toxicity tests (#0 emerging contaminant (2013), #7 behavioral responses (2014)), and the effects of water treatment methods prior to discharge to the environment on its toxicity (#0 emerging contaminant (2013), #7 behavioral responses (2014), #12 ballast water (2018)).

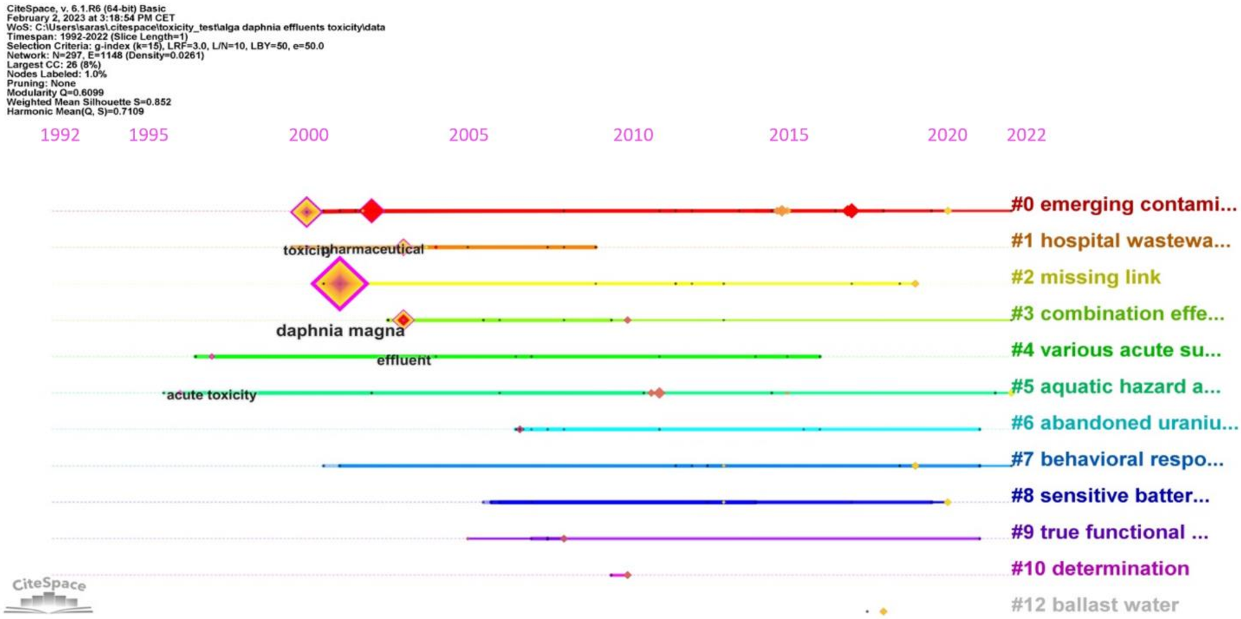

Fig. 4. Timeview of the clustered network of co-occurring keywords (diamonds) created from the literature linking the topics "alga", "daphnia", "effluents" and "toxicity".

Both the keyword burst detection and clustering results pointed to the same research trend over the last decade: the use of the battery of toxicity tests to evaluate the effects of mixtures of compounds present in wastewater effluents and, consequently, surface waters. This mainly included emerging contaminants, but also metals to a lesser extent. Since the results of toxicity tests of wastewaters were often highly variable due to their complex composition and different sensitivity of model organisms in bioassays, it was concluded that the use of a battery of toxicity tests with organisms of different trophic levels is a more effective approach (Liwarska-Bizukojc, 2022). In line with this current state of the art, our experimental study on the Krka River contributes novel data on a karst ecosystem by utilizing organisms from two distinct trophic levels, testing whole effluents and surface waters rather than isolated chemicals in laboratory settings, and linking observed toxic effects with metal contamination.

The clustering and burst detection results indicated that the toxicity of pharmaceuticals to aquatic ecosystems was recognized as an important research topic in early 2000s (leading to the discovery of other similarly hazardous compounds in wastewater effluents) and continues to be studied in recent years under the group term “emerging contaminants”. Emerging contaminants are diverse chemical compounds (pharmaceuticals, illicit drugs, personal care products, etc.) that often enter surface waters with wastewater, for which there are no environmental monitoring regulations, and which have recently been recognized as concerning aquatic pollutants due to their adverse effects on the endocrine system of humans and wildlife. Since only few of these compounds have been toxicologically evaluated and there are ongoing policy initiatives to standardize their research and propose regulations, it is understandable that research on effluent toxicity to algae and daphnids has prioritized "emerging contaminants" in recent years (Petrie et al., 2015). Although emerging contaminants were not included in the present toxicity study on the Krka River, we plan to address this in the future research by investigating microplastics—highly prevalent wastewater-related contaminants whose toxic effects remain largely unknown. This planned work will further enhance our understanding of pollution-related risks in karst aquatic ecosystems and contribute to more comprehensive environmental monitoring strategies.

4. CONCLUSIONS

Based on the physical and chemical thresholds set in the Croatian Regulation, poor water quality was found in the basins with wastewater from the screw factory (IWW) and downstream in the Krka River, especially at the site directly exposed to the municipal wastewater outlets (KRK). Higher concentrations of mineral oils, TOC, DOC and dissolved CO_2 at KRK indicated municipal wastewaters as the primary source of organic compounds, while concentrations of total metal(loid)s in water confirmed IWW as an important source of pollution in the Krka River, especially of metal(loid)s used in industry (Cr, Cu, Fe, or Ni).

Generally, toxic effects on *Pseudokirchneriella subcapitata* and *Daphnia magna* were consistent with the chemical data recorded in the Krka River, resulting in acute toxicity (TU = 2, hazard class III) at KRK, which is contaminated by organic compounds. Moreover, high acute toxicity was found at IWW (TU = 18 for daphnids and 32 for algae, hazard class IV), basins with industrial wastewater. Algae were shown to be more sensitive to wastewater pollution than crustaceans. Moreover, integral view on ecotoxicological effects and physical and chemical parameters based on multivariate PCA analysis confirmed stronger association of certain organic compounds with the toxic effects on algae than of metal(loid) concentrations.

CiteSpace, a scientometric analysis tool applied to global publications on effluent toxicity, has shown that research on algae and daphnids has increased over the past decade, focusing on emerging contaminants (mainly pharmaceuticals and metals) using the battery of toxicity tests. Based on the Betweenness Centrality, European countries play the key role in these scientific topics. These findings indicate that our study is among the few that presents a holistic assessment of the mixture of contaminants and their toxicity. While incorporating current recommendations and aligning with recent research trends identified through CiteSpace, our work provides a comprehensive and ecologically valuable evaluation of the effects of toxic compounds and the relationship between water quality parameters and toxicity in a complex and vulnerable karst river system, which is an ecosystem that remains underrepresented in toxicity studies.

Statements and Declarations

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Data availability: The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Author contributions: Sara Šariri - Formal analysis, Investigation, Writing – original draft, Writing – review & editing; Želimira Cvetković - Data curation, Formal analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing; Tatjana Mijošek - Formal Analysis, Investigation, Writing – original draft, Writing – review & editing; Zorana Kljaković-Gašpić - Formal Analysis, Investigation, Writing – review & editing; Damir Valić – Investigation; Tomislav Kralj - Investigation; Amalia Brkić - Formal Analysis, Writing – review & editing; Vlatka Filipović Marijić – Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. The first draft of the manuscript was written by Sara Šariri and all authors read and approved the final manuscript.

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