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The usefulness of ecotoxicological tools to improve the assessment of water bodies in a climate change reality

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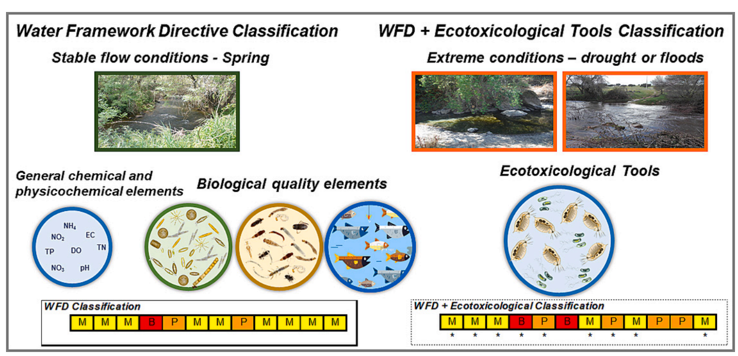
HIGHLIGHTS

- Development and testing of a classification system based on ecotoxicological endpoints
- Complementarity between ecotoxicological tools and WFD-based surface waters assessment
- Ecotoxicological tools for surface waters assessment under extreme conditions e.g., droughts and floods
- Ecotoxicological tools added value in the assessment of point-source pollution

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to analyse the added value of using ecotoxicological tools to complement and improve the assessment of natural water bodies status, in situations of climate change, with a higher frequency of extreme events as floods or droughts.

Four water bodies of streams in the Guadiana Basin (Álamos, Amieira, Lucefécit, Zebro) were studied in 2017 and 2018 and classified based on the Water Framework Directive (WFD) parameters: Biological Quality Element – Phytobenthos (diatoms), General chemical and physicochemical elements, Specific pollutants, and Priority Substances. Complementarily, bioassays (including lethal and sublethal parameters) were carried out with organisms of different trophic levels: (i) the bacteria *Aliivibrio fischeri;* (ii) the microalgae *Pseudokirchneriella subcapitata*; (iii) the crustaceans *Daphnia magna*, *Thamnocephalus platyurus* and *Heterocypris incongruens.* A classification system with 5 scores was developed, permitting to classify water bodies from non-toxic (EC_{50} > 100 %; growth and feeding rate > 80 %; blue) to highly toxic (EC_{50} < 10 %; growth and feeding rate < 10 %; red). The comparison between the classification based on the WFD parameters and on ecotoxicological endpoints showed similar results for 71 % of the samples, and significant positive Pearson correlations were detected between the diatom-based Specific Polluosensitivity Index (SPI) and $EC50_{V.fisheri}$, the algae growth rate and

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Shannon diversity index. These results indicate that when the biological quality elements cannot be used (namely under drought or flooding conditions) the application of ecotoxicological bioassays may be a good alternative. Further, when ecotoxicological parameters were included, an increase of worse classifications (Bad and Poor) was observed, revealing an improvement in the sensitivity of the classification, mainly in presence of specific and priority substances. So, the ecotoxicological analysis appears to provide useful information regarding the potential presence of both known and unknown contaminants at concentrations that cause biological effects (even within the WFD limits), in agreement with several authors that have already suggested its use in biomonitoring.

1. Introduction

Aware of the imperative need to stop, prevent and reverse the degradation of ecosystems, and to effectively restore aquatic ecosystems that have been degraded around the world, the United Nations (UN) has declared the period 2021 to 2030 as the Decade for Ecosystem Restoration (United Nations General Assembly, 2019). The European Green Deal is part of the European Commission's strategy to achieve the United Nations Sustainable Development Goals until 2030, providing an action plan to boost the efficient use of resources, restore biodiversity, reduce pollution, rebalance the aquatic ecosystems, and improve the well-being and health of citizens and future generations by providing clean water (European Commission, 2019).

The Water Framework Directive (WFD, Directive 2000/60/EC, 2000) is the central piece of water management legislation within Europe and its main aims are to prevent further deterioration of European water resources, to protect and enhance the status of the water bodies, in terms of their ecosystem structure and/or function (Martinez-Haro et al., 2022), and to contribute to mitigating the effects of floods and droughts (Directive 2000/60/EC, 2000). With the WFD, the water status assessment starts to include the concept of the 'Ecosystem Approach', reflecting the concern of European countries to preserve the ecological integrity of the aquatic ecosystems; as revealed in the Ecological status concept, defined in the WFD (Directive 2000/60/EC, 2000) as "an expression of the quality and functioning of aquatic ecosystems associated with surface waters, classified in accordance with Annex V". Hence, to follow the WFD strategy of inland surface, transition, coastal and groundwaters protection, it is necessary to implement monitoring programs and apply classification systems. These systems include the surface and groundwaters status classification, determined by the poorer of the ecological and chemical status for the surface and the quantitative and chemical status for the groundwaters. For surface waters (i.e., rivers, lakes, transitional waters, and coastal waters), according to the WFD Annex V, the ecological status classification is based on biological quality elements (BQEs), as well as hydromorphological, chemical and physicochemical elements supporting the BQEs.

To allow the comparability of the monitoring systems among Member States, the classification of the ecological status must be expressed as Ecological Quality Ratio (EQR, as a numerical value between 0 and 1), indicating the relationship between the values of the biological parameters for a given water body and those in reference conditions, with higher values representing higher ecological status (Directive 2000/60/EC, 2000). The ecological quality ratio scale is divided into five classes, to which a colour code is assigned: High (blue); Good (green); Moderate (yellow); Poor (orange); and Bad (red).

For rivers and lakes, the BQEs considered are: (i) the composition and abundance of aquatic flora (phytoplankton, macrophytes and phytobenthos); (ii) the composition and abundance of benthic invertebrate fauna; and (iii) the composition, abundance, and age structure of fish fauna (Directive 2000/60/EC, 2000). Most of the member states have considered the BQE "macrophytes and phytobenthos" as two separate sub-elements (Kelly, 2013), with diatoms already validated as proxies for phytobenthos in the ecological status assessment of standing waters (Kelly et al., 2008), and frequently representing phytobenthos in rivers (Kelly, 2013; Masouras et al., 2021). Therefore, diatoms have been used as ecological indicators during the last decades, with several studies supporting the use of diatom-based metrics in water quality monitoring, especially in lotic systems (Viso and Blanco, 2023). Several diatom indices have been developed, among which the weighted average-based Specific Polluosensitivity Index (SPI) stands out (Viso and Blanco, 2023).

The ecological and chemical assessment required by the WFD however, has some limitations as: (i) there is not an explicit requirement to establish cause-effect relationships in the assessment of quality status (Allan et al., 2006a, 2006b); (ii) overlooks ecosystem functioning and individual endpoints (Martinez-Haro et al., 2015; Palma et al., 2016); (iii) biotic indices lack preventive value, since they reflect the responses of the organisms after the ecosystem change/ damage (Rodrigues et al., 2021); (iv) isolated use of weighted average metrics risks overlooking other pressures than nutrient or organic pollution, e.g., acidification, as pointed out by Kelly (2013); (v) the SPI reflects the general water quality during the month before sampling (Viso and Blanco, 2023); (vi) diatom sampling for the ecological status assessment should be carried out in stable flow conditions (INAG I.P, 2008), avoiding zones of very slow current (European Committee For Standardization, 2003) and waiting four weeks before sampling after the occurrence of strong hydrological events as floods (AFNOR, 2007), which blocks the ecological status assessment during extreme flow conditions (dry periods and floods); (vii) furthermore, fish fauna are considered to be poor indicators in streams with temporary hydrological regimes (APA, 2021), as well as benthic invertebrates, that have high spatial (substrate-related) and temporal variability, related to insect hatching and water flow variation (European Commission. Directorate-General for the Environment, 2003).

All these limitations become more evident with climate change, which may be responsible for modulating the effects of stressors and playing a key role in defining the responses of aquatic ecosystems (Santos et al., 2021). Climate predictions include the increase in the frequency of extreme meteorological events such as floods, heat waves, severe droughts, and windstorms (Millennium Ecosystem Assessment, 2005). Changes in the hydrological cycle may also be expected (IPCC, 2018; Millennium Ecosystem Assessment, 2005), inducing a high variability of the level and flow of rivers, lakes, and oceans. Thus, it is essential to develop more comprehensive assessments, including new ecological perspectives, based on a holistic and multidisciplinary view, integrating multiple lines of evidence that allow an understanding of the exposure-response links between biological indicators, climatic factors, and anthropogenic stressors (Carvalho et al., 2019; Voulvoulis et al., 2017). So, with WFD as a driver, there is now an opportunity to start taking advantage of ecotoxicological research as rapid assessment tools, creating and integrating new cost-effective monitoring toolboxes, specifically in situations where biotic indices are not able to provide credible responses (Palma et al., 2016; Rodrigues et al., 2022). The ecotoxicological bioassays improve the ability to establish the reasons for a failing ecological status and whether pollutants are the cause for not reaching a 'good status', closing the gap between ecology and chemistry (Martinez-Haro et al., 2015; Santos et al., 2021).

Ecotoxicological bioassays aim to quantify the toxicity of individual chemicals or mixtures of known or unknown composition by exposing living organisms under standardised conditions, with the measurement of ecologically relevant responses (Rand, 1995), the so-called dose-response trials. Hence, to obtain pertinent outcomes at the ecosystem level, the assessed responses should have consequences on the biological health of the individual (e.g., mortality, growth, reproduction, feeding rates) (Palma et al., 2016; Pinto et al., 2021; Rodrigues et al., 2021).

In this context, the present study aimed to assess whether the integration of ecotoxicological tools, reinforces (or not) the robustness of the assessment of the ecological status obtained through biotic indices, and can be an option when there are limitations in the use of biotic indices. This hypothesis was tested in streams of the Guadiana Basin (South of Portugal), some of them with intermittent hydrological regimes, including a period characterised by intense drought. The results can serve as a basis for the development of environmental management models that integrate biotic indices and ecotoxicological endpoints, especially in situations where biotic indices are insufficient for a relevant ecological analysis of the ecosystem.

2. Materials and methods

2.1. Site description and sampling procedures

In the framework of the project "Observation, prediction, and alert systems in atmosphere and water reservoirs of Alentejo" (ALOP, ALT20-03-0145-FEDER-000004), was carried out this study in four sampling sites in streams in the lower part of the Guadiana watershed, draining to the Alqueva reservoir: Zebro (Zb; 38°14,015.5" N, 7°19,040.32" W); Álamos (Al; 38°24,050.46" N, 7°2802.16" W); Amieira (Am; 38°16,058.80" N, 7°36,035.20" W), and Lucefécit (Lf; 38°36,059.19" N, 7°23,031.65" W) (Fig. 1). The sites were sampled bimonthly from January 2017 to November 2018 (in January (Jn), March (Mr), May (M), July (Jl), September (Sp), and November (Nv), for both years), in a total of 12 campaigns for the chemical and physicochemical elements supporting the biological elements and for the ecotoxicological assays. For Phytobenthos, sampling started in May 2017 and a total of 10 campaigns were carried out.

The year of 2017 was considered, according to IPMA (Instituto Português do Mar e Atmosfera), as in severe drought from April on, with the highest temperatures observed in the last 80 years, and with the lowest values of precipitation (IPMA, 2018), whereas the year of 2018 was classified as normal by IPMA regarding air temperature and rainfall, with the severe drought situation ending in March 2018. In addition, due to the marked difference in hydrological regimes throughout the year in the Alentejo region, a rainy season comprising the winter months, from October to April, and a dry season comprising the months of May to September were defined by the Agência Portuguesa do Ambiente (APA).

The sites have been thoroughly characterised by Palma et al. (2020a, 2020b). Briefly, they are small streams, classified as small rivers of Southern Portugal (S1 $\leq$ 100 km^2), according to the national WFD typology. Among the four streams, only Amieira integrates the Degebe sub-basin, whilst Álamos, Zebro and Lucefécit are located in the Guadiana sub-basin. Regarding the hydrological regimes, Amieira and Álamos are intermittent streams, forming a series of disconnected pools in summer campaigns (i.e., July and September); Zebro has an intermittent regime, with water all year long but without flow during some periods; Lucefécit is a perennial stream, with flowing water during the whole year.

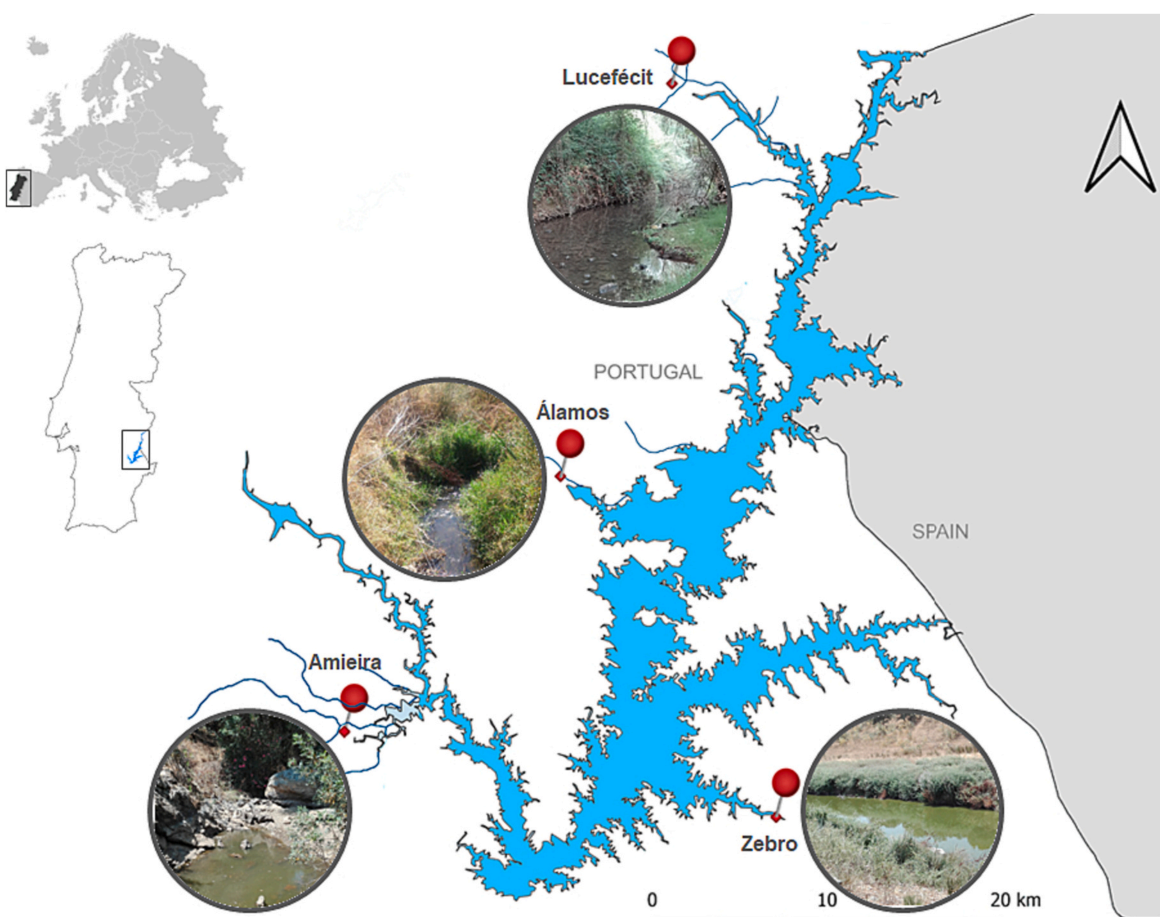

Fig. 1. Location of sampling sites in Alqueva reservoir.

2.2. Surface water status assessment – WFD-based

2.2.1. Ecological status assessment

The Biological Quality Element used for the ecological assessment was the Phytobenthos, with diatoms as representatives for the whole benthic algae, according to the standard national procedure (APA, 2016). Information on the other BQEs (macrophytes; the composition and abundance of benthic invertebrate fauna; and composition, abundance, and age structure of fish fauna) and the water bodies status classification was kindly provided by the Administration of the Hydrographic Region of Alentejo, a subdivision of the Portuguese Environment Agency, from the monitoring, carried out in Spring 2017. According to APA (2021), the sampling frequency, depicted in the 6-year planning cycle for biological quality elements, is 6 months for Phytoplankton, and every 3 years for the other aquatic flora (Phytobenthos and Macrophytes), benthic macroinvertebrates and fish.

2.2.1.1. Biological quality element - phytobenthos. Benthic diatoms were sampled following standard procedures during flowing conditions (INAG I.P., 2008; AFNOR, 2007; European Committee for Standardization, 2003), in brief, cobbles were collected in riffle areas not shaded, scrapped with a toothbrush, rinsed with stream water, and preserved with formaldehyde solution (4 %; *v*/v) immediately after sampling. However, the standards for sampling could not be applied in some situations, for instance, in the Amieira stream in July and September 2017 and 2018 and in the Álamos stream in July and September 2018 (dry phase), there were no flowing conditions and sampling was carried out in an isolated pool upstream of the regular sampling sites. In this case, the methodology presented by Novais et al. (2020) was applied. A minimum of 5 cobbles were collected at each pool and washed in the pool water to remove sand, sediment and dead diatoms that could have been deposited on the substrate, scraped with a toothbrush, rinsed with distilled water, and preserved with formaldehyde solution (4 %; v/v) immediately after sampling. In Lucefécit, sampling was not possible in the January 2018 campaign, due to flooding conditions.

A total of 39 diatom samples were oxidised with hot hydrogen peroxide (35 %) and diluted in hydrochloric acid (37 %), followed by rising and decantation (repeated at least 3 times) with distilled water to prepare a suspension of clean frustules (European Committee for Standardization, 2003; INAG I.P., 2008). Permanent slides were mounted using Naphrax, a high refractive index medium (Northern Biological Supplies, Ltd., UK, RI = 1.74). The identification was carried out to the possible lowest taxonomical level using light microscopy (LM) (Leica DMLB with 100× oil immersion objective, N.A. 1.40). The relative abundance of each taxon was determined, based on identifications, and counts of at least 400 valves per sample (INAG I.P., 2008). The identification was based on reference floras (e.g., Krammer and Lange-Bertalot, 1986, 1988, 1991a, 1991b; Blanco et al., 2010; Hofmann et al., 2011), as well as on recent bibliographic sources, including the series 'Diatoms of Europe', 'Iconographia Diatomologica', 'Bibliotheca Diatomologica' and relevant taxonomic papers, such as (Delgado et al., 2015, 2016; Novais et al., 2019, Novais et al., 2015; van de Vijver et al., 2011).

For each sample, taxa richness (S), Shannon diversity index (H′), and the Specific Pollution Sensitivity Index (SPI) (Coste, 1982) were determined using OMNIDIA v. 5.5 (Lecointe et al., 1993). SPI is a weighted average index based on the Zelinka and Marvan (1961) formula and consists in assigning pollution tolerance (S) and stenoecy degree (V) values to each taxon, with higher SPI values corresponding to lower pollution. The SPI was developed to assess the ecological quality of European rivers, is based on the autecology of almost all known taxa and has been recommended as a reference for Mainland Portugal (APA, 2016). The calculation formula is shown in Eq. (1).

$$SPI = \frac{\sum_{i=1}^{n} A_i s_i v_i}{\sum_{i=1}^{n} a_i v_i} \tag{1}$$

A_i = relative abundance of taxon j in the sample
si = pollution sensitivity of taxon j
vi = stenoecy degree /indicator value of taxon j

Specific Pollution Sensitivity Index values were then converted to EQR, considering the SPI reference value for the Small rivers of Southern Portugal (S1 ≤ 100 km^2) WFD type (16.35), and classified according to the thresholds defined for Mainland Portugal (APA, 2016): High ≥0.80; Good [0.60–0.80[; Moderate [0.40–0.60[; Poor [0.20–0.40[; Bad [0–0.20[.

2.2.1.2. General chemical and physicochemical elements (supporting biological elements). In Portugal the general chemical and physicochemical elements integrate seven parameters that allow the evaluation of the oxygenation, acidification and nutrient conditions (APA, 2016): dissolved oxygen (DO, $mgL^{-1}O_2$; oxygen saturation (O_2, %); Biochemical Oxygen Demand (BOD_5, mg O_2 L^{-1}); pH; ammoniacal nitrogen (NH_4, mg NH_4 L^{-1}); nitrate (NO_3, mg NO_3 L^{-1}); and total phosphorus (TP, mg P L^{-1}). The Boundary values for Good Status of these supporting elements, have been proposed for the Southern Grouping, which includes the Small rivers of Southern Portugal (S1 ≤ 100 km^2) type, to which the studied sites belong, and are as follows: DO ≥5 mg O_2 L^{-1}; O_2 saturation (%) between 60 % and 120 %; BOD_5 ≤ 6 mg O_2/L; pH between 6 and 9; NH_4 ≤ 1 mg NH_4 L^{-1}; NO_3 ≤ 25 mg NO_3 L^{-1}; and TP ≤ 0.13 mg P L^{-1} (APA, 2016).

A multiparametric YSI 6820 MPS probe® was used for the in situ measurements at each sampling site of the dissolved oxygen (DO; mg L^{-1} and %), and pH.

For the remaining general chemical and physicochemical elements, 2 L of surface water were collected in polyethylene (PET) bottles. The samples were transported to the laboratory at 4 °C, conserved and stored until the analysis following the requisites for each parameter (APHA, 2017). The BOD_5 was determined using the respirometric method, TP and NH_4 by molecular absorption spectrometry, and NO_3 by Ionic chromatography (APHA, 2017), as previously presented in Palma et al. (2020a).

2.2.1.3. Specific pollutants. Specific pollutants are chemical substances included in the Annex VII (Directive 2000/60/EC, 2000) that are not classified as Priority Substances. Among the 22 substances depicted in APA (2016), 2,4-Dichlorophenoxyacetic acid (2,4-D), bentazone, linuron, methylchlorophenoxypropionic acid (mecoprop or MCPP) and terbuthylazine were selected, taking into consideration that the most representative activity in the region is agriculture (Palma et al., 2020a).

For these specific pollutants analysis, water samples (250 mL) were collected in amber PET bottles, transported to the laboratory at 4 °C, and stored in the dark at −18 °C until analysis. The analysis was performed by online solid phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) as described in Köck-Schulmeyer et al. (2012, 2013).

The classification of the water bodies based on the Specific Pollutants includes two statuses: Good (all pollutants are below the directive limits) or Insufficient (at least one of the pollutants was quantified in concentrations above its limits) (APA, 2016). The boundaries for these Specific Pollutants are as follows: 2,4-D (0.30 μg L^{-1}), bentazone (80 μg L^{-1}), linuron (0.15 μg L^{-1}), MCPP (5.5 μg L^{-1}) and terbuthylazine (0.22 μg L^{-1}).

2.2.2. Chemical status

The chemical status of surface waterbodies was analysed in accordance with Directive 2013/39/EU of 12 August, which includes a group of 45 specific priority substances with their respective thresholds: Annual Average (AA) and Maximum Allowable Concentration (MAC); Annex I), classifying them as hazardous substances or priority hazardous substances. The chemical classification includes two statuses: Good (all pollutants are below the directive limits) or Insufficient (at least one of the pollutants was quantified in concentrations above its limits) (APA, 2016). For the Good status classification, the Annual Average (AA) of each pollutant or the Maximum Allowable Concentration (MAC) should be below the Environmental Quality Standard (APA, 2021). In the present study, were analysed 5 of those hazardous substances, namely: atrazine; alachlor; simazine; diuron; and chlorfenvinphos. The boundaries for these hazardous substances are as follows: atrazine (AA: 0.6 μg L^{-1}; MAC: 2.0 μg L^{-1}), alachlor (AA: 0.3 μg L^{-1}; MAC: 0.7 μg L^{-1}), simazine (AA: 1 μg L^{-1}; MAC: 4 μg L^{-1}), diuron (AA: 0.2 μg L^{-1}; MAC: 1.8 μg L^{-1}) and chlorfenvinphos (AA: 0.1 μg L^{-1}; MAC: 0.3 μg L^{-1}).

The water samples (250 mL), in a total of 48, were collected at 50 cm depth in amber PET bottles, transported to the laboratory at 4 °C, and stored in the dark at −18 °C until analysis. The analysis was performed by online solid phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) as described in Köck-Schulmeyer et al. (2012, 2013).

2.3. Ecotoxicological analysis

Ecotoxicological assays were performed using 2 L of water samples with a set of representative bioindicators from different trophic levels, and sensitivity to detect surface waters ecosystems unbalances/disruption, which can be promoted by different known or unknown hazardous substances. In addition, a set of rapid and easy-to-use screening bioassays were selected, that can provide information about different life cycle stages of organisms, such as growth, feeding rate, light inhibition, in a short period of time, as a complement to the biotic indices. The methodology was developed with the ecotoxicological assessment of water and sediments. Hence, the aquatic organisms and the respective responses analysed in the water were: (i) light inhibition of the bacterial *Aliivibrio fischeri*; (ii) growth inhibition of the green microalgae *Pseudokirchneriella subcapitata*; (iii) mortality of the crustacean *Thamnocephalus platyurus;* and (iv) feeding rate of the crustacean *Daphnia magna*.

The *Daphnia magna* culture was from the clone K6 (originally from Antwerp, Belgium), successfully cultured in laboratory conditions for more than 10 years. The microalgae culture of *P. subcapitata* is obtained from agar solid medium, bacteria free, for better conservation of the culture (*P. subcapitata*, Bacteria-Free, Living acquired to the company Carolina Biological Supply®) resuspended when necessary to renew the culture, which occurs every two years. *Thamnocephalus platyurus* shrimp larvae were obtained from cysts incubated when exposed to an incubation medium provided in the THAMNOTOXKIT FTM kit (Persoone, 1999). This species is not cultivated in the laboratory; the hatching of the larvae is carried out when necessary for the development of the tests.

The integration of the sediment compartment in the proposed classification was carried out with the results of the growth inhibition bioassay of the benthic species *Heterocypris incongruens*. This bioassay methodology and the analysis of respective toxicological results have already been published in Palma et al. (2023). Therefore, in this manuscript we use the results only to complement the classification system proposal, making it more robust and comprehensive.

For all bioassays, a test with a reference substance was performed, as a positive control. The sensitivity of the organisms was in accordance with the followed protocols. The control groups had a survival rate above 90 %.

For an integrative interpretation of both classifications (ecological and ecotoxicological), a toxicological classification system (TCS) was developed, adapted from Roig et al. (2015), with 5 scores that classified the waters from: class 1: non-toxic (EC_{50} > 100 %; and growth and feeding rate > 80 %; blue) – score 0; class 2: slightly toxic (61 % < EC_{50} < 100 %; and 50 % < growth and feeding rate ≤ 80 %; green) - score 1; class 3: Marginally toxic (21 % < EC_{50} < 60 %; and 20 % < growth and feeding rate ≤ 50 %; yellow) - score 2; class 4: Moderately toxic (10 % < EC_{50} < 20 %; and 10 % ≤ growth and feeding rate ≤ 20 %; orange) - score 3; class 5 Highly toxic (EC_{50} < 10 %, and 10 % > growth and feeding rate; red) - score 4.

2.3.1. *Aliivibrio fischeri* luminescence inhibition assay

Luminotox® was used to evaluate the luminescence inhibition of *A. fischeri* (NRRL B-11177), according to the protocol "DR LANGE luminescent bacteria test" following ISO 11348-2 (1998). Tests were carried out using water samples and their dilutions with a 2 % NaCl solution (50, 25, 12.5, 6.25 and 3.125 %, *v*/v). Two replicates per dilution were used. The light inhibition was measured against a nontoxic control (2 % NaCl solution), at a temperature of 15 ± 0.5 °C. For each sample, bioluminescence was measured before and after the incubation period of 30 min. The concentration (%; *v*/v), which reduced 50 % of the bacterial luminescence, was determined (30 min - EC_{50}).

2.3.2. *Pseudokirchneriella subcapitata* growth inhibition assay

Microalgae were exposed to water samples for 72 h and algae growth was determined based on ISO 11348-2 (1998). At the beginning of the test, *P. subcapitata* (100 μL of inoculum with 3–5 × 10^4 cells mL^{-1}) was exposed to the respective diluted samples (12.5, 25, 50, 75, 100 %; v/v; 900 μL of total volume) performed with MBL medium (negative control). Test vials were incubated in an orbital shaker for 72 h at constant light (with an intensity of 60–120 μE m^{-2} s^{-1}, equivalent to 6000–10,000 lx) and a temperature of 21 ± 2 °C. The algal biomass was calculated by counting the number of cells using a Neubauer chamber.

The average growth rate for a specific period was determined from Eq. (2):

$$\mu_{i-j} = \left(ln\ B_j - ln\ B_i \right) / t_j - t_i \tag{2}$$

where: μ_{i-j} is the average specific growth rate from the time i to j; t_i is the time for the start of the exposure period; t_j is the time for the end of the exposure period, B_i is the biomass concentration at the time i, and B_j is the biomass concentration at time j.

The inhibition of algal growth was estimated from the Eq. (3):

$$\%I = [(\mu_c - \mu_t)/\mu_c]\ x\ 100 \tag{3}$$

where: % I is the mean percentage of inhibition for specific growth rate; μ_c is the mean value for the growth rate in the control, and μ_t is the mean value for the growth rate in the water samples.

2.3.3. *Thamnocephalus platyurus* mortality assay

The mortality assay with the crustacean *T. platyurus* was adapted from Persoone (1999). Larvae of the shrimp *T. platyurus* (<24 h, obtained from the hatching of cysts) were exposed to different dilutions of water samples (12.5, 25, 50, 75 and 100 %; *v*/v), during 24 h. Four replicates per dilution were performed. Organisms were not fed during the test. The number of dead organisms was used as an endpoint to determine the 24 h-EC_{50} (%; v/v) concentration that causes mortality of 50 % of the exposed organisms.

2.3.4. *Daphnia magna* feeding rate assay

The feeding rate assay with *D. magna* was adapted from the methodology reported by Mcwilliam and Baird (2002). Groups of five neonates (4 or 5 days old born between the 3rd to 5th broods) were exposed to the different water samples (100 mL of total volume). To each sample, a volume of algae, corresponding to a density of 3.0 × 10^5 cell mL^{-1} $Daphnia^{-1}$ (equivalent to 2.65 mg C mL^{-1}), was added. In all experiments, a comparative control with sample and algae at the density of 3.0 × 10^5 cells mL^{-1} $Daphnia^{-1}$, without daphnids, was added to control the growth of algae under the test conditions. Five replicates per sample and a negative control test (with ASTM) were carried out. At the start of the test, the number of algal cells was quantified using a Neubauer chamber, after which the organisms were added to each vessel. The test took place for 24 h at a temperature of 20 °C in the dark, to avoid algae growth. At the end of the test, the organisms were removed from each vessel, the solution was shaken to resuspend the algae in it, and the cells were counted. The Feeding rate was calculated according to Eq. (4), reported by Allen et al. (1995):

$$F = V \times (C_0 - C_{24})/t \quad (4)$$

where: F = feeding rate (cells*animal^{-1}*h^{-1}); V = volume of medium in the test vessel (mL); C_0 = the initial cell concentration (numbers*mL^{-1}); C_{24} = the final cell concentration (numbers*mL^{-1}); t = duration of the experiment (h).

2.4. Statistical analysis

General physicochemical elements (supporting biological elements), diatom indices (SPI, S, H′) and ecotoxicological essays endpoints were tested for normality (Shapiro-Wilk test), using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL).

Relatively to the ecotoxicological endpoints, the *A. fischeri* bioluminescence inhibition test, the EC_{50} (%) values were determined using LUMISsoft 4 Software™. The EC_{50} (%) values for the *T. platyurus* mortality were determined using the probit analysis (Finney, 1971). Data of sublethal endpoints (growth inhibition and feeding rate) were checked for homogeneity of variance by the Kolmogorov–Smirnov test and, when possible, subjected to one-way analysis of variance (ANOVA). Data that do not satisfy the assumption for ANOVA were analysed non-parametrically using Kruskal–Wallis ANOVA by ranks test. Whenever significant differences were found ($p < 0.05$), a post hoc *Dunnett's* test was used to compare treatments with the control, for a p value of 0.05 as the significant level (Zar, 1996).

Further, *Spearman* Rank Order correlations were applied between the general physicochemical elements, specific pollutants, diatom indices (SPI, S, H′) and ecotoxicological endpoints, since the general physicochemical elements and specific pollutants did not follow a normal distribution (Table S1 in Supplementary material). The diatom indices followed a normal distribution, and from the ecotoxicological parameters, the EC_{50} of *T. platyurus* and *A. fischeri*, and the *P. subcapitata* algae growth inhibition passed the normality test (Shapiro-Wilk test). Therefore, Pearson correlations were carried out between diatom indices and EC_{50} of *T. platyurus* and *A. fischeri*, and the *P. subcapitata* algae growth inhibition (Table S2 in Supplementary material); and *Spearman* Rank Order correlation was applied between *D. magna* feeding rate and the biotic indices, since the *D. magna* feeding rate did not follow a normal distribution (Table S1 in Supplementary material).

Differences in SPI values between water bodies were statistically tested by the One-Way ANOVA, after being tested for normality (Shapiro-Wilk test) and Equal Variance, followed by the Pairwise Multiple Comparison Procedures (Holm-Sidak method, with 0.05 overall significance level) to test which water bodies differed, using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL).

To test for differences between sampling years and periods (wet and dry), *t*-tests were applied, since data passed the normality test (Shapiro-Wilk) and the Equal Variance test, using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL).

All statistical analyses applied to ecotoxicological results were performed with the STATISTICA 7.0 (Software™ Inc., PA, USA, 2007).

3. Results and discussion

3.1. Surface water status assessment – WFD-based

3.1.1. Ecological status assessment

3.1.1.1. Biological quality element - phytobenthos. Lucefécit presented the highest taxa richness (S), reaching 60 taxa in September 2018 and a wider Shannon diversity range (H′) (1.81–4.81), followed by Amieira with 21–46 taxa and H′ between 2.53 and 4.25. On the other hand, Zebro had the lowest S (10–39 taxa, mean 18.10), and the lowest H′ (1.24–3.79), as seen in Table 1.

Specific Pollution Sensitivity Index (SPI) values ranged between 1.5 in Zebro in July 2017 and 14.8 in Amieira in January 2018, as seen in Table 1. The lowest values were detected in Zebro, followed by Lucefécit, Álamos and Amieira, as visible in the plot depicted in Fig. 2.

This information is in agreement with the diatom communities analysis, dominated (relative abundance >10 %) in Zebro by species tolerant to organic contamination and high nutrient content. These taxa range from eutrophentic to hypereutraphentic and α–mesosaprobic to polysaprobic and include: *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot, *Nitzschia amphibia* Grunow, *Nitzschia inconspicua* Grunow and a similar but broader species, *Nitzschia* cf. *inconspicua* Grunow, *Nitzschia palea* (Kützing) W. Smith, *Cyclotella meneghiniana* Kützing, *Eolimna subminuscula* (Manguin) Moser, Lange-Bertalot & Metzeltin, *Gomphonema saprophilum* (Lange-Bertalot & E.Reichardt) Abarca, R. Jahn, J.Zimmermann & Enke, *Navicula veneta* Kützing, *Halamphora veneta* (Kützing) Levkov and *Gomphonema parvulum* (Kützing) Kützing. These dominant taxa differed in relative abundance between campaigns, and in the accompanying species. The sensibility (s) values for these species ranged between 1.0 (*Halamphora veneta* and *Nitzschia palea*) and 3.4 (*Planothidium frequentissimum*), thus explaining the low SPI values.

In Lucefécit diatom communities were dominated by *Eolimna minima* (Grunow) Lange-Bertalot, *Gomphosphenia holmquistii* (Foged) Lange-Bertalot, *Amphora pediculus* (Kützing) Grunow, *Navicula tripunctata* (O. F.Müller) Bory, *Nitzschia inconspicua* and *Thalassiosira pseudonana* Hasle & Heimdal. These species are also eutraphentic to hypereutraphentic, β-mesosaprobic to α-meso-polysaprobic, with S values between 2.0 (*Gomphosphenia holmquistii*) to the maximum of 4.0 (*Amphora pediculus*).

Diatom communities in Álamos were dominated (relative abundance >10 %) by *Planothidium frequentissimum*, *Cocconeis euglypta* Ehrenberg, *Nitzschia inconspicua*, *Navicula catalanogermanica* Lange-Bertalot & Hofmann, *Amphora pediculus*, *Navicula recens* (Lange-Bertalot) Lange-Bertalot, *Navicula gregaria* Donkin, *Eolimna minima* and *Sellaphora seminulum* (Grunow) D.G.Mann. Most of these species are tolerant to organic loads and high nutrient content and were also present in Zebro and Lucefécit, however, the presence of more sensitive species as *Navicula catalanogermanica* (with a higher S value, of 4.8, and indicator value of 2.0) contributes to the highest SPI value in July 2017 campaign (12.4).

The water body with the highest SPI values was Amieira, due to the community dominated by *Cocconeis euglypta*, *C. pseudolineata* (Geitler) Lange-Bertalot, *C. pediculus* Ehrenberg, *Eolimna minima*, *Amphora pediculus*, *Nitzschia inconspicua*, *Planothidium frequentissimum*, *Navicula gregaria* and *Eolimna subminuscula*. Most of these species were also present in the other three streams, however, in November 2017 the community was no longer dominated by tolerant species but was instead dominated by oligosaprobic to β-mesosaprobic, eutraphentic *Cocconeis* species, with S value between 3.6 (*C. euglypta*), 4.0 (*C. pediculus*) to 5.0 (*C. pseudolineata*), which contributed to the highest SPI value of 14.8.

In Fig. 2 it was possible to observe some differences between the four water bodies, further statistically validated by the One-Way ANOVA (F = 9.787, $p < 0.001$) since the data passed the normality Shapiro-Wilk test ($p = 0.235$) and the Equal Variance Test ($p = 0.253$). The Pairwise Multiple Comparison Procedures (Holm-Sidak method, with 0.05 overall significance level) revealed that Zebro was different from

Table 1
Range (minimum-maximum), mean and standard deviation (SD) values for SPI, S and H′ for the four water bodies (n=39).

Water body Index	Amieira (n = 10)		Zebro (n = 10)		Álamos (n = 10)		Lucefécit (n = 9)	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
SPI	8.20–14.80	11.08 ± 2.34	1.50–9.00	6.91 ± 2.35	8.40–12.40	10.69 ± 1.34	7.20–11.20	9.13 ± 1.25
S	21–46	32.20 ± 7.44	10–39	18.10–8.20	22–36	27.00 ± 4.81	17–60	37.22 ± 13.71
H′	2.53–4.25	3.44 ± 0.68	1.24–3.79	2.24–0.86	2.32–4.22	3.02 ± 0.54	1.81–4.81	3.43 ± 0.98

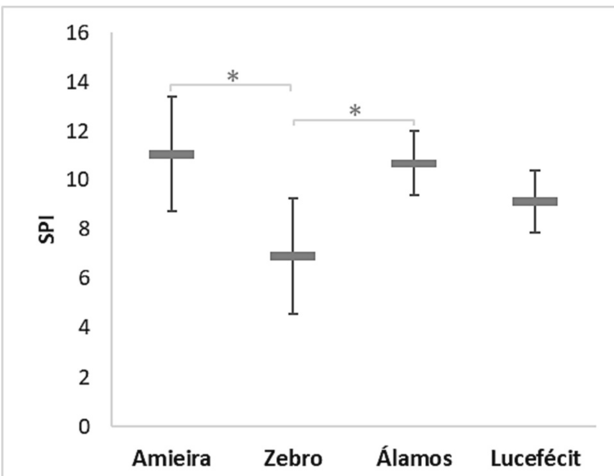

Fig. 2. Plot with the SPI mean values ± standard deviation, for the four water bodies. * Represents differences with $p < 0.05$.

Amieira and Álamos water bodies ($p < 0.05$), whilst no differences were detected between Lucefécit and Zebro, Amieira and Álamos, Lucefécit and Álamos nor Amieira.

The SPI temporal evolution (Fig. 3) revealed that Lucefécit presented the least variability, as seen in the lowest SD (1.25), whilst Zebro and Amieira presented the highest variability (SD in Zebro was 2.35 and in Amieira 2.34). However, this variability did not reflect the sampling year (2017 or 2018), as revealed in the t-test ($t = -0.766$ with 37 degrees of freedom, $p = 0.449$). Also, no differences were detected between the wet and the dry periods (t-test not significant, $t = -0.487$ with 37 degrees of freedom, $p = 0.629$).

Spearman Rank Order Correlations were further tested between SPI and General physicochemical elements, but no significant correlations were detected (Table S1). However, diatom richness (S) was negatively correlated ($p < 0.05$) with TP ($r = -0.41$) and NH_4^+ ($r = -0.63$). Also, Shannon diversity (H′) was negatively correlated ($p < 0.05$) with NH_4^+ ($r = -0.46$). When correlations between diatom indices and specific pollutants were tested, significant ($p < 0.05$) negative Spearman Rank Order correlations were obtained between S and 2.4-D ($r = -0.39$), between S and diuron ($r = -0.42$), between H′ and diuron ($r = -0.32$), and between SPI and 2.4-D ($r = -0.32$). This negative correlation between SPI and the 2.4-D is in accordance with Debenest et al. (2010), who also referred to an alteration in diatom communities when exposed to herbicides, with eutrophic species being more tolerant. These eutrophic species have lower pollution sensitivity values, therefore, their dominance in the community also decreases the SPI value. According to Debenest et al. (2010), the survival of species dominating the communities in the studied streams, such as *Planothidium frequentissimum*, *Gomphonema parvulum* or *Nitzschia palea* was favoured in streams with triazine herbicides (atrazine and irgarol) and *Eolimna minima* to isoproturon.

3.1.1.2. General physicochemical elements. The physicochemical results, considered for the WFD classification, are presented in Supplementary material (Table S3, Part 1 and 2), and were previously thoroughly analysed by Palma et al. (2020a, 2020b). In the present study, these results were further evaluated according to APA (2016) and with the WFD normative (Directive 2000/60/EC, 2000).

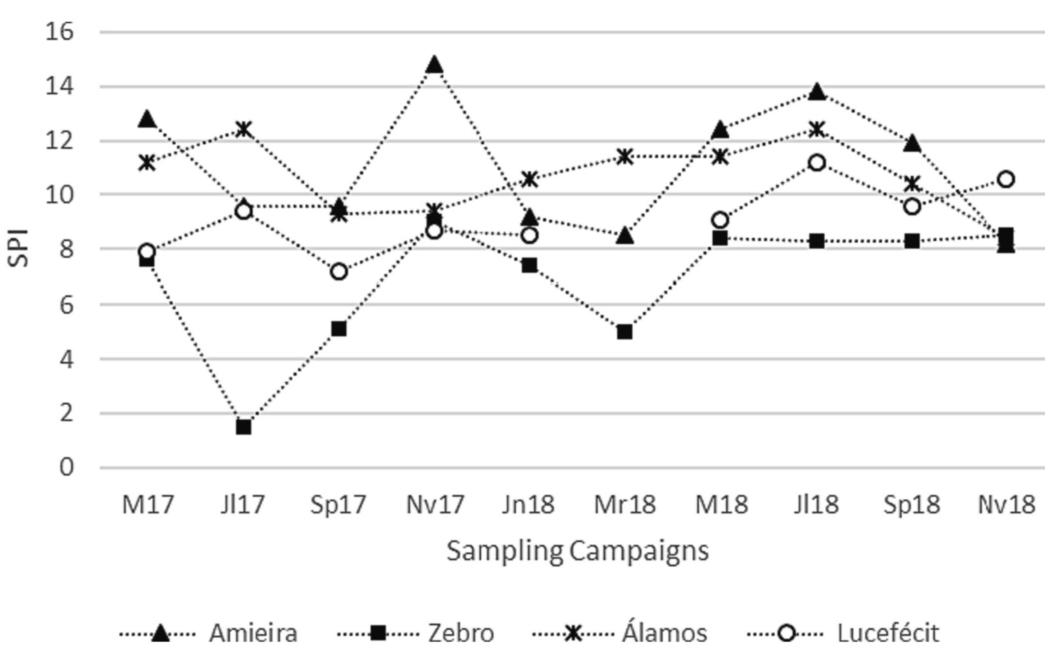

Fig. 3. SPI values over time for the studied water bodies. M17 = May 2017; Jl17 = July 2017; Nv17 = November 2017; Jn18 = January 2018; Mr18 = March 2018; M18 = May 2018; Jl18 = July 2018; Sp18 = September 2018; Nv18 = November 2018.

All the general physicochemical elements (DO, NO_3, NH_4, pH, TP and BOD_5) failed the normality test (Shapiro-Wilk), therefore, the results are presented as median and range (maximum-minimum) in Table 2.

Median DO levels were between the normal range (60 to 120 % of O_2 saturation), although in Amieira, Zebro and Álamos water bodies the maximum values were reached, and exceeded, in several campaigns. Lucefécit never surpassed the maximum value, nevertheless, in the last campaign (November 2018) the DO was below the low limit of 60 % of O_2 saturation. Minimum DO levels were registered in Zebro (Table 2). The median values of BOD_5 exceeded the limit of 6 mg O_2/L at Zebro and Álamos, as already reported by Palma et al. (2020a). In general, all the water bodies are slightly alkaline, with Amieira and Zebro presenting the highest pH values. Compared to the other water bodies, Zebro showed the highest median values of BOD_5, pH, NH_4 and NO_3, possibly mainly due to the Wastewater Treatment Plant (WTP) that discharges directly to this water body, as already reported in previous studies Palma et al. (2020a, 2020b).

Even though several physico-chemical parameters presented higher values during the dry phase in the intermittent streams, namely the BOD_5 in Zebro and Amieira; NH_4 in Amieira, Zebro and Álamos, as previously reported in Palma et al. (2020a), these differences were not visible in the classification, since TP exceeded the threshold for the Good status in most of the campaigns in Amieira, Zebro and Álamos, without a seasonal pattern.

3.1.1.3. Specific pollutants. The Specific pollutants results, considered for the WDF classification and analysed in the present study, are shown in Supplementary material (Table S3, Part 1 and 2).

Of the five substances analysed, three were detected (bentazone, terbuthylazine, 2,4-D). The concentrations of these specific pollutants were always below the limit reported in the WFD (Directive 2000/60/EC, 2000), generally allowing a classification of Good for this component of ecological status assessment. An exception was observed for 2,4-D in Zebro in July 2018, whose concentration of 3362 ng L^{-1} led the water body to be classified as insufficient in this specific month. Lucefécit was the water body with the highest concentration of the assessed specific pollutants.

3.1.2. Chemical status

Priority substances results, considered for the chemical status classification, are presented in Supplementary material (Table S3, Part 1 and 2). Among the five hazardous substances analysed, diuron and simazine were those quantified above the detection limit. In fact, the herbicide diuron was the substance that was most frequently detected in the four assessed water bodies, achieving the highest concentration in Zebro in September 2017. Despite that fact, the observed concentrations were always below the limits proposed in Directive 2013/39/EU of 12 August. According to these results, the water bodies presented a Good Chemical status.

3.2. Ecotoxicological analysis

The ecotoxicological results are displayed in Table S3 and Figs. 4 and 5. Relatively to the acute ecotoxicological bioassays, the most sensitive was the light inhibition of *A. fischeri*, detecting 33 % of the toxic samples, followed by the mortality test of the crustacean *T. platyurus*, with the identification of 23 % of the toxic samples (Table S3). The water bodies with the most toxic samples were Zebro and Álamos, in 2017. In the case of Zebro, of the 12 samples analysed, 6 presented a 30 min-EC_{50} lower than 61 % for *A. fischeri* and 5 of them with a 24 h-EC_{50} between 60 and 97 % for *T. platyurus*. Although the *A. fischeri* bioassay is mostly used to detect toxicity in effluents and samples contaminated with potentially toxic metals (Alvarenga et al., 2007; Bori et al., 2016, 2017), several studies have already reported its sensitivity in surface waters (Palma et al., 2016, 2018; Rodríguez Pérez et al., 2010). The results highlight the significant correlations observed between this bioassay and nutrients, mainly total phosphorus ($r = -0.78$), being a good option to detect eutrophic waters. Thus, it is currently considered a credible option to integrate Tier 1 of the Ecotoxicological Evidence Line (LoE), in alternative processes of ecological bioassessment, in relation to the current practice of compliance with the WFD (Santos et al., 2021).

The results with the green microalgae *P. subcapitata* showed a significant decrease in the growth of the algae for most samples ($p < 0.05$), being more pronounced in Zebro, during the drought year of 2017 (Fig. 4). Thus, the most toxic Zebro samples for the microalgae were those from March 2017, with a growth inhibition rate of 40 % (statistically validated by the One-Way ANOVA when comparing with the control MBL: $F_{4,24} = 209.47$; $p < 0.00005$), May 2017 (growth inhibition of 46 %; statistically validated by the One-Way ANOVA when comparing with the control MBL: $F_{4,21} = 87.87$; $p < 0.000001$), and July 2017 (growth inhibition of 50 %; statistically validated by the One-Way ANOVA when comparing with the control MBL: $F_{4,19} = 48.54$; $p < 0.00001$). The results of the feeding rate of the *D. magna* bioassay (Fig. 5) showed the high sensitivity of this test for assessing the quality of surface water. In fact, 62 % of the total samples analysed showed a reduction in the feeding rate greater than 80 %, with 12 % considered highly toxic, corresponding to the Zebro (November 2017) and Lucefécit (September 2017 and March 2018) samples. In this case, Lucefécit induced a high decrease in the feeding behaviour of *D. magna.* Contrary to the results observed for the other bioassays, Zebro was the water body that least disturbed the feeding behaviour of the crustacean, showing significant increases in relation to the control or slight decreases (<10 %) in 50 % of the analysed samples, mainly in spring-summer. Previous studies carried out in a Portuguese reservoir showed similar results, highlighting the loss of sensitivity of the bioassay with the increment of abiotic parameters such as light, temperature, nutrients and organic matter (Diogo

Table 2
Physicochemical parameters considered for the Ecological status assessment, with the APA (2016) limits for the Good status depicted. Median and range results, $n = 12$ per water body, are presented. Min – Minimum value; Max – Maximum value.

Parameter (Limits for the Good Status)	Amieira Median (Min - Max)	Zebro Median (Min - Max)	Álamos Median (Min - Max)	Lucefécit Median (Min - Max)
DO (% sat.)	91.65	84.36	69.46	73.09
(60 % - 120 % O_2 saturation)	(46.40–181.00)	(0.70–171.30)	(53.32–125.02)	(55.20–110.36)
BOD_5	2.33	15.00	6.83	3.00
(≤ 6 mg O_2 L^{-1})	(2.00–40.00)	(2.00–35.50)	(1.00–44.00)	(1.00–8.33)
pH (Sorensen scale)	7.93	8.07	7.79	7.60
(6–9)	(7.68–9.04)	(7.51–9.05)	(7.38–8.32)	(6.72–8.86)
Ammoniacal nitrogen	0.05	4.41	0.19	0.10
(≤ 1 mg NH_4 L^{-1})	(0.01–0.33)	(0.02–15.12)	(0.05–5.51)	(0.01–0.4)
Nitrate	0.67	1.51	0.85	9.70
(≤ 25 mg NO_3 L^{-1})	(0.32–18.86)	(0.67–7.12)	(0.67–33.19)	(0.67–23.46)
Total Phosphorus	0.24	1.75	0.45	0.07
(≤ 0.13 mg P L^{-1})	(0.10–3.29)	(0.10–6.10)	(0.02–3.07)	(0.01–2.00)

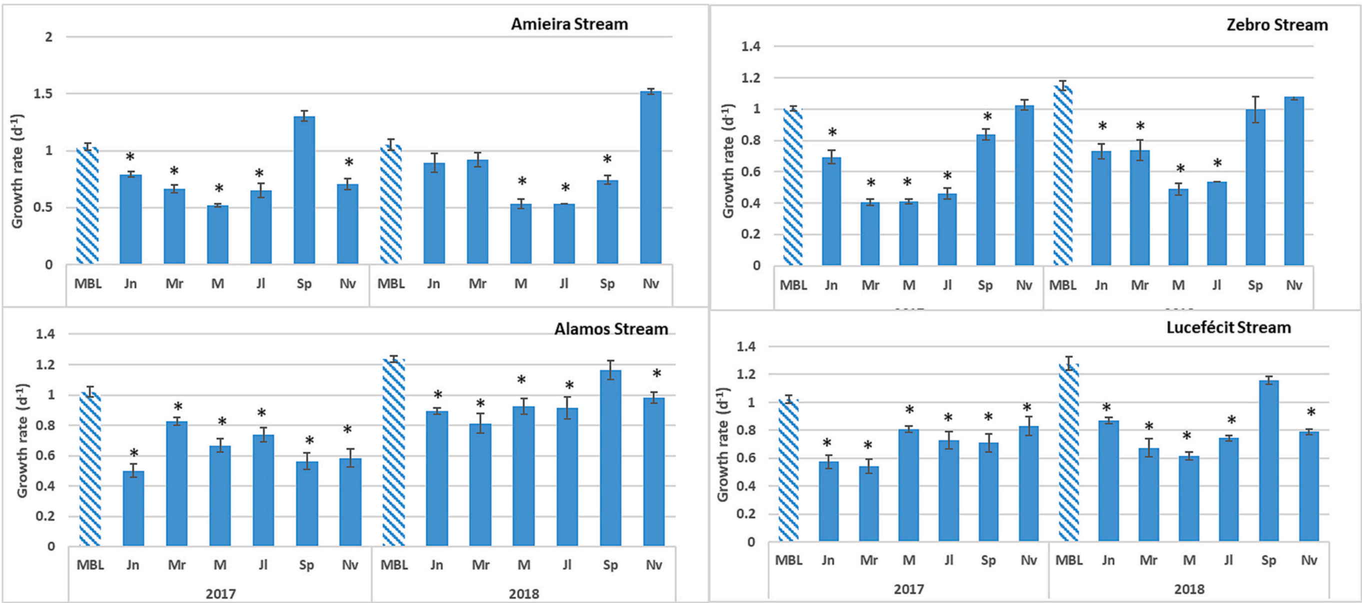

Fig. 4. Growth rate (d^{-1}) of algae *P. subcapitata* after 72-h exposure to water samples (undiluted sample), from Guadiana Basin streams, in January (Jn), March (Mr), May (M), July (Jl), September (Sp) and November (Nv) of 2017 and 2018. Mean ± standard deviation ($n = 6$), * $p < 0.05$, *Dunnett's* post hoc comparison test with the control MBL.

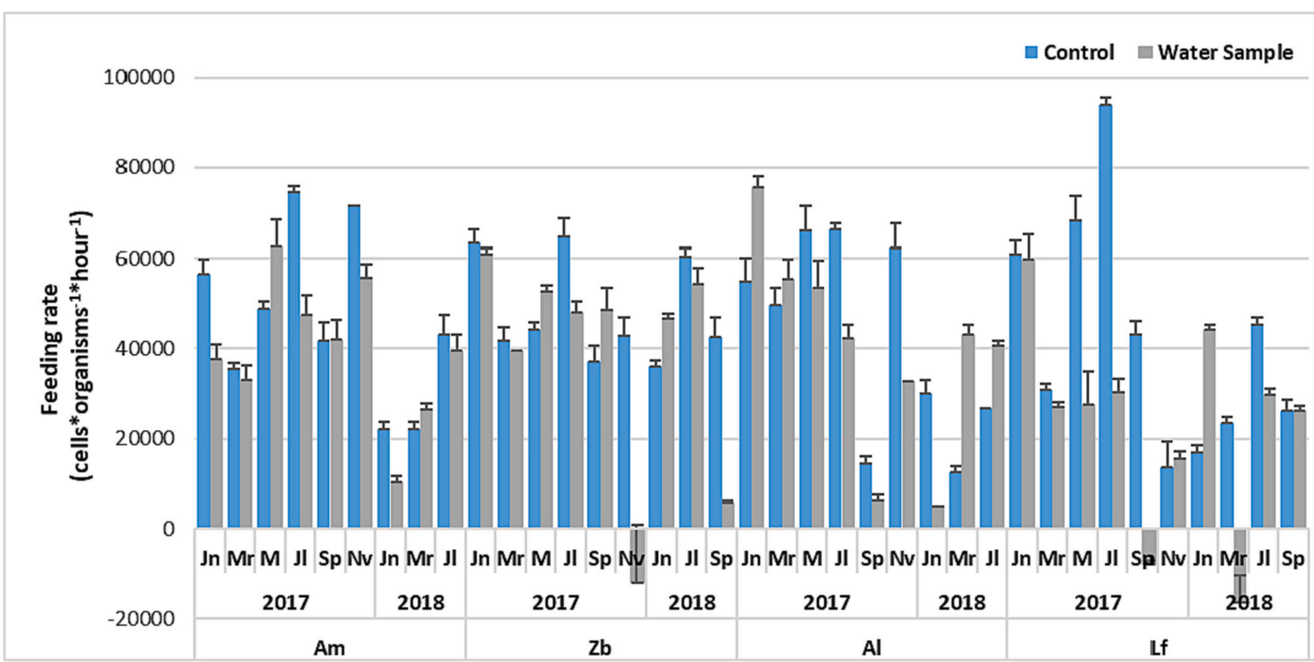

Fig. 5. Results of feeding rate of *D. magna,* after 24 h exposed to water samples from Guadiana Basin streams. Blue bars – control treatment; Grey bars: undiluted samples (100 %). January (Jn), March (Mr), May (M), July (Jl), September (Sp) and November (Nv) of 2017 and 2018. Am - Amieira, Zb - Zebro, Al - Álamos, Lf - Lucefécit. Mean ± standard deviation (n = 6), * $p < 0.05$, *Dunnett's* post hoc comparison test with the control MBL.

et al., 2022; Pinto et al., 2021). This behaviour can be correlated with the high concentrations of organic matter and total phosphorus, most of the time responsible for the moderate classification of the water body (Table 2 and Fig. 6).

Despite this, the results did not show significant correlations (Spearman rank order, since the general physicochemical elements, failed the normality test) between the feeding rate and the general physicochemical elements analysed (Table S1).

Only a significant correlation ($p < 0.05$) was obtained between algae growth inhibition and 2,4-D ($r = 0.31$), no other correlations were detected between the specific pollutants or hazardous substances and ecotoxicological assays.

3.3. *WFD surface water bodies status classification*

The result of the classification based on the BQE Phytobenthos-Diatoms varied between High (in November 2017 and in July 2018 in Amieira) and Bad (in July 2017 in Zebro). Álamos was classified as Good in six campaigns, followed by Amieira (with five campaigns as Good or above), whilst Lucefécit only had a Good status in two campaigns and

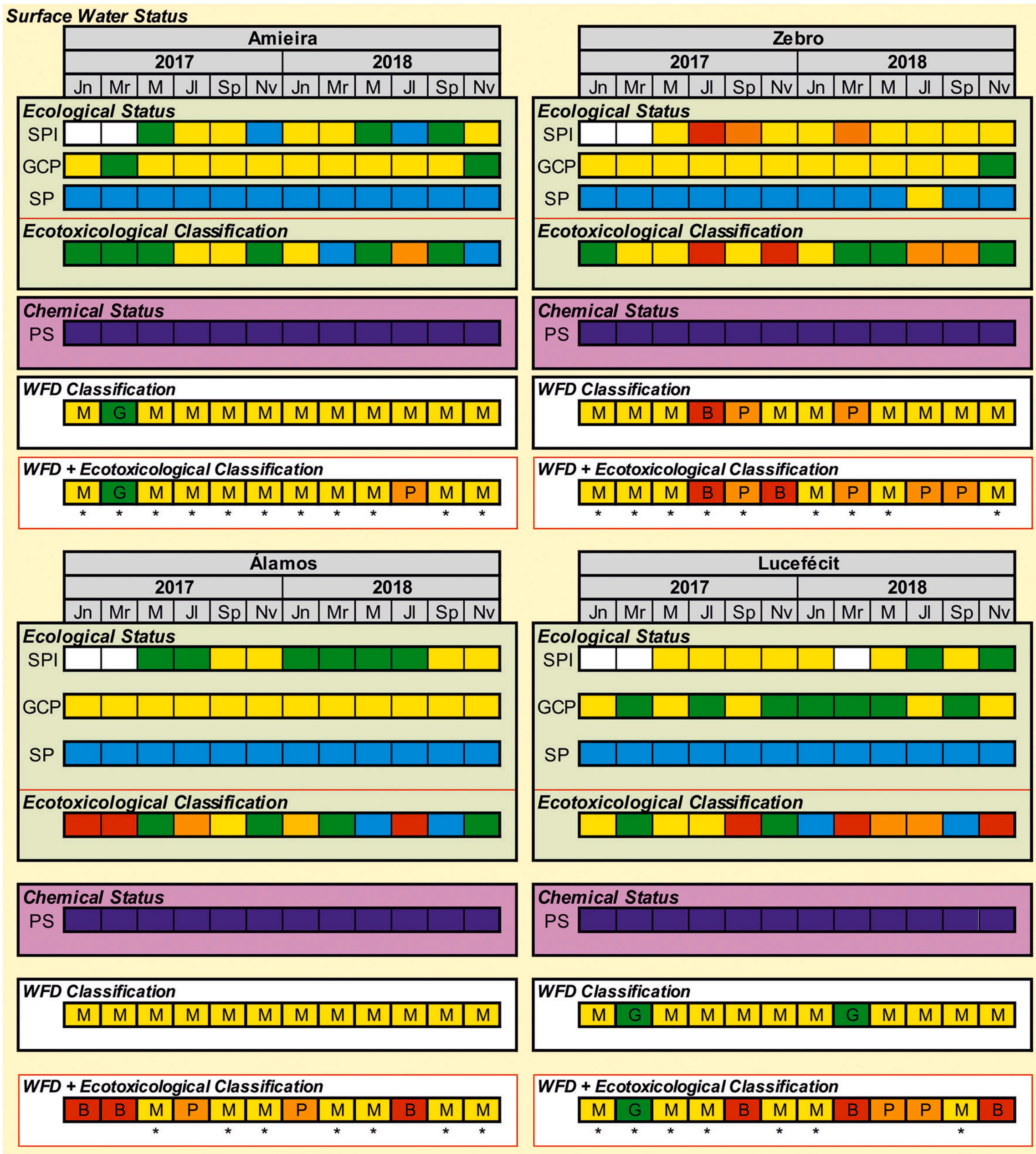

Fig. 6. Tables with the Surface water status classification based on the WFD parameters (including the Ecological and Chemical Status) and the Ecotoxicological Classification. SPI – Specific Polluosensitivity Index; GCP – General Chemical and physicochemical quality elements; SP – Specific pollutants; PS – Priority substances; WFD – Water Framework Directive; G – Good; M – Moderate; P – Poor; B – Bad. * - Cases where there is a correspondence between the WFD Classification and the WFD plus the Ecotoxicological assays.

Zebro was always classified below Good, with the classification ranging between Bad (July 2017), Poor (in September 2017 and March 2018) and Moderate in the remaining campaigns (as seen in Fig. 6). The High and Good classifications did not comply with the environmental standards for the supporting physicochemical conditions, especially the total phosphorus content, that exceeded the limit for the Good status in most of the analysed samples in Amieira, Zebro and Álamos. Amieira and Lucefécit are those water bodies where environmental standards for the general supporting physicochemical conditions were met during most of the studied period (see Table S3). In Álamos, the Phytobenthos-diatoms only slightly deviate from the reference condition values, however, the physicochemical conditions do not ensure the ecosystem functioning, exceeding the boundaries for the TP (except in January, March and September 2017, as previously reported), for NO_3 in November 2017, NH_4 in September 2017 and January, March and November 2018, and BOD_5 in January, March, May and September 2017 and January, March and September 2018. Almost all samples complied with the environmental standards for the selected specific pollutants, except for the 2,4-D, with a concentration of 3362 ng L^{-1} in the Zebro stream in July 2018, largely exceeding the 300 ng L^{-1} depicted in the environmental standards (APA, 2016).

Therefore, the Ecological status classification was mainly Moderate, with a few exceptions, such as the Bad and Poor classification of the Zebro in July 2017 (Bad) and in September 2017 and March 2018 (Poor). The Surface water status classification was only determined by the Ecological status, given that the analysed hazardous substances for the chemical status assessment were all within the thresholds depicted in Directive 2013/39/EU of 12 August (Annex I). In short, considering the classification carried out by the WFD, Zebro presented the worst ecological status, and the remaining water bodies showed an ecological status mostly Moderate, occasionally reaching the Good status.

The results presented are in line with those provided by the Administration of the Hydrographic Region of Alentejo, from spring 2017 (sampled in April and May 2017), confirming that the four water bodies were classified below the Good ecological status. In Lucefécit this was due to the fact that the macroinvertebrates deviate from the reference condition, the physicochemical conditions do not ensure ecosystem functioning, namely the BOD_5, PO_4^{3-}, TP and TSS (total suspended solids), and the environmental standards were not met for 2,4-D and dissolved Zn (ARH Alentejo, pers. comm.). In the remaining water bodies, the physicochemical conditions do not ensure the ecosystem functioning, namely the TP in the Amieira stream; the NH_4^+, TN, PO_4^3, TP and NO_2^- in the Zebro stream; and the NH_4^+, TN, PO_4^{3-}, TP and NO_2^-, NO_3^- in the Álamos stream (ARH Alentejo, pers. comm.).

The Portuguese Environmental Agency has recently prepared a document on Water Bodies Classification Criteria (APA, 2021), which was under public consultation until December 2022. This document includes the classification for all general conditions represented in Annex V of the WFD Directive (Directive 2000/60/EC, 2000), and the boundaries for the classes High, Good and Moderate, for the following parameters (underlined are those recently included): Total Phosphorus, <u>Phosphate (High: $\leq$ 0.20 mg PO_4/L; Good: $\leq$ 0.40 mg PO4/L)</u>, <u>Total Nitrogen (High: $\leq$ 1 mg N L^{-1}; Good: $\leq$ 4.5 mg N L^{-1})</u>, Ammoniacal Nitrogen, <u>Ammonia (Good: $\leq$ 0.025 mg NH_3 L^{-1})</u>, Nitrate, <u>Nitrite (High: $\leq$ 0.03 mg NO_2 L^{-1}; Good: $\leq$ 0.20 mg NO_2 L^{-1})</u>, <u>Total Suspended Solids (High: $\leq$ 12.5 mg L^{-1}; Good: $\leq$ 25 mg L^{-1})</u>, BOD_5, Dissolved Oxygen, Oxygen saturation, pH, <u>Temperature (Good: 10.0–27.0 °C)</u>, and <u>Conductivity (Good: $\leq$ 1000 μS/cm)</u>. Also, for some pre-existing parameters, stricter thresholds between the Good/Moderate classes were defined (APA, 2021), namely, for Ammoniacal Nitrogen (from 1 mg NH_4 L^{-1} to 0.5 mg NH_4 L^{-1}), for Nitrate (from 25 mg NO_3 L^{-1} to 10 mg NO_3 L^{-1}), for BOD_5 (from 6 mg O_2 L^{-1} to 5 mg O_2 L^{-1}). For oxygen saturation (%) the limits were slightly broadened (from 60 % - 120 % to 60 % - 125 %). Total Phosphorus was the only parameter with the boundary between the Good/Moderate status remaining unchanged. The results provided by the Administration of the Hydrographic Region of Alentejo for the Spring 2017, already used these last criteria of classification. The application of these stricter thresholds was further tested in the framework of this study, and five cases classified as Good according to APA (2016) are now classified below the Good status (APA, 2021), all in accordance with the Phytobenthos-Diatoms classification for these cases.

To increase the sensitivity of ecological status classification methodologies, the possible use of ecotoxicological parameters was assessed. Based on the toxicological classification system, Álamos and Lucefécit had 42 % of the samples classified as Moderately to Highly toxic (25 % highly toxic and 17 % classified as moderately toxic) (Table S3). Zebro was classified with 33 % of samples in a Moderately to Highly toxic status. Amieira was the least toxic stream, with 8 % of the samples classified as Moderately toxic. The comparison with the biotic indices showed similarities between the two approaches in 35 % of the samples (60 % in Amieira, 22 % in Lucefécit and 30 % in Álamos and Zebro). In fact, although bioassays do not replace the current methodologies (biotic indices) for the ecological status classification, it has been observed that the results of some assays are significantly correlated with those of biological indices, such as the positive Pearson correlations between SPI and $EC50_{A.fischeri}$ ($r = 0.80$, $p < 0.05$), and algae growth rate and Shannon diversity ($r = 0.34$, $p < 0.05$) (Table S2). This evidence may indicate that, in specific periods when some of the biotic indices cannot be used, the application of ecotoxicological bioassays may be a good alternative, as reported by other authors, using bioindicators and biomarkers in water and sediment compartments (Pinto et al., 2021; Rodrigues et al., 2021; Roig et al., 2015).

When we consider the ecotoxicological parameters, the results showed an increase in Bad and Poor classifications for the four streams (Fig. 6 – WFD + ecotoxicological endpoints). In Amieira, the use of ecotoxicological parameters maintained the 8 % of Good status (March 2017), decreasing the Moderate status from 92 % to 83 %, and in some periods of time from Moderate to Poor (8 %). The agreement between the two analysis strategies was 92 % (Fig. 6 – indicated with an asterisk *). In Zebro, the results of the integrative analysis pointed to a decrease in the Moderate classification (75 % to 50 %), with an increase in the Bad (8 % to 17 %) and Poor classification status (17 % to 33 %), and an accordance between the two methodologies of 75 %. In Álamos and Lucefécit, the addition of ecotoxicological status classification highlighted the increase in samples with Bad (0 % to 25 %) and Poor (0 % to 17 %) status. Lucefécit and Álamos were the sites where the combination of the ecotoxicological methodology for the classification of the ecological status showed more differences, with only 58 % of similarities between the two strategies. Overall, there was an agreement of 71 % in the classification based on the WFD parameters and on ecotoxicological endpoints. In fact, the addition of the ecotoxicological approach allows the increase in the sensitivity of the classification mainly in areas with greater number and quantity of specific and priority substances, as was observed in Lucefécit and Álamos, where the increase of Bad and Poor ecological conditions were of 42 %. Results consistent with those obtained in a previous study on the assessment of the environmental risk of pesticides in these streams, identifying Lucefécit and Álamos as the highest risk (Palma et al., 2021). Consequently, the ecotoxicological analysis appears to provide useful information regarding the potentially presence of both known and unknown contaminants at concentrations sufficient (even within the limit values proposed by the WFD) to cause biological effects, for which its use in biomonitoring has been suggested by several authors (Alvarenga et al., 2016; Martinez-Haro et al., 2015; Santos et al., 2021).

4. Conclusions

A classification system based on ecotoxicological endpoints was developed and tested with surface waters in Southern Portugal and can be further applied in regions with similar physicochemical conditions and pressures.

This study contributed to the validation of the hypothesis that the integration of ecotoxicological tools reinforces the robustness of the assessment of the water status based on the WFD elements. The integration of ecotoxicological endpoints increases the sensitivity of the assessment, allowing the detection of contaminants and their synergistic effects, even in conditions when these contaminants are not included in the legislation and therefore, are not included in the monitoring programs (e.g., most of the emerging contaminants, as newly synthesized molecules, or degradation products of existing ones). Further, these bioassays complement the information obtained using the WFD biological quality elements, since these do not detect the effects of punctual pollution events, which can be reflected only later in biological communities. Therefore, the integration of bioassays can fill in a temporal gap in the water status assessment, allowing the rapid detection of contamination and its effect on organisms of different trophic levels.

The integration of ecotoxicological endpoints in the water status assessment can also complement the current WFD elements when the legislated Biological Quality Elements are difficult to apply or give a mismatched result. This situation is particularly important under climate change, with the predictable increase in extreme events, such as floods and droughts, when the use of the current biological quality elements is difficult (visible in this study in the summer months in the temporary rivers, and in March 2018 in Lucefécit, when a flood occurred). In this case, the integration of sediments ecotoxicological analysis and biomarkers should be advised, to overcome the drawbacks that could arrive from the oversimplification of these methods. In this study, however, the integration of sediments in the proposed classification system did not change the results of the final classification, using both types of biological tools, probably because this type of strategy is being tested in streams where the toxicity of sediments for benthic species was very low. The importance of analysing this abiotic compartment in the proposed classification system could probably be more evident in reservoirs or rivers with more toxic sediments.

CRediT authorship contribution statement

Maria Helena Novais: Writing- Original draft preparation; Writing- Reviewing and Editing; Investigation; Visualization; Formal Analysis.

Alexandra Marchã Penha: Writing- Reviewing and Editing; Investigation; Visualization.

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Ana Lima: Investigation.

Manuela Morais: Writing- Reviewing and Editing.

Patrícia Palma: Conceptualization; Writing- Original draft preparation; Writing- Reviewing and Editing; Investigation; Supervision; Visualization; Formal Analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Novais M.H. reports financial support was provided by Foundation for Science and Technology. Novais M.H. reports was provided by Operational Program Competitiveness and Internationalization. Palma P. reports was provided by European Agricultural Fund for Rural Development. Alexandra Penha reports was provided by Foundation for Science and Technology. Morais M. reports financial support was provided by Foundation for Science and Technology. Palma P. reports was provided by Foundation for Science and Technology.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166392.

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