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Title: Unravelling the effects of multiple stressors on diatom and macroinvertebrate communities in European river basins using structural and functional approaches

Article Type: VSI: Multiple river stressors

Keywords: Biodiversity; biofilm; invertebrates; Toxic Units; traits; hydrology.

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Abstract: Rivers suffer from more severe decreases in species diversity compared to other aquatic and terrestrial ecosystems due to a variety of pressures related to human activities. Species provide different roles in the functioning of the ecosystem, and their loss may reduce the capacity of the ecosystems to respond to multiple stressors. The effects on diversity will differ based on the type, combination and severity of stressors, as well as on the characteristics of the community composition and tolerance. Multiple trait-based approaches (MTBAs) can help to unravel the effects of multiple stressors on communities, providing a mechanistic interpretation, and, thus, complementing traditional biodiversity assessments using community structure. We studied the relationships between diversity indexes and trait composition of macroinvertebrate and diatom communities, as well as environmental variables that described the hydrological and geomorphological alterations and toxic pollution (pesticides and pharmaceuticals) of three different European river basins: the Adige, the Sava, and the Evrotas. These river basins can be considered representative cases of different situations in European freshwater systems. Hydrological variables were the main drivers determining the community structure and function in the rivers, for both diatoms and macroinvertebrates. For diatom communities, pharmaceutical active compound (PhAC) toxic units were also identified as a very important driver of diversity changes, explaining up to 57% of the variance in taxonomic richness. For macroinvertebrates, river geomorphology was an important driver of structural changes, particularly affecting Plecoptera richness. In addition, PhAC and pesticide toxic units were also identified as stressors for macroinvertebrate communities. MTBA provided a detailed picture of the effects of the stressors on the communities and confirmed the importance of hydrological variables in shaping the functional attributes of the communities.

Response to Reviewers: REVIEWER RESPONSE

Reviewer #3: I appreciate the effort the authors have made to address the comments made by myself and the other reviewer. Especially the discussion has benefited a lot from the revision, as one can now easily follow the thoughts and lines of argumentation by the authors. In my opinion, the authors did a credible job at this. I believe the presented data are valuable and address an important topic.
My main concern remains with the exhaustiveness of the results section focusing on every little detail, while some numbers (p-values, % differences, absolute values) are missing. I understand that the study design lead to a vast amount of data that the authors want to present - as we all tend to do. However, I think that the results section could still be condensed down in order to facilitate the understanding. I will attempt to clarify my point in the comments below.

General comments:
L268-279: Here and in the following, I am still missing some relative numbers when river basins are compared. Also, the manuscript would greatly benefit from some specific absolute values, for instance in lines 276-279, when the exceed of sumTU threshold values are addressed. In the current form, the reader is wondering about such numbers (although they might be "hidden" in the SI). I am aware that I asked to condense the results section, but from my point of view, it would be critical for the clarity of the manuscript to add some important numbers here and in the following. At the same time, some less important results could be moved to the SI, given that the results section is still 8 pages long. Often results are only highlighted for specific basins or shown in every little detail, which can be confusing at times. With all these results shown, the reader can lose the overview quickly.

ANSWER: Result section has been reviewed and condensed. From 8 pages of result description in the previous manuscript version, we have reduced to 4,5 pages. We have eliminated detailed not essential information. Specific values have been added for TUs. Section 3.1 and 3.2. of the results have been shortened, and section 3.3 has been removed (these results are available in table S4). Section 3.4 has been restructured and shortened.
Tables 2 and 4, and 3 and 5 have been unified.

Specific comments:
L 15: Maybe refer to "more severe decreases" than "important decreases"
ANSWER: Done

L 18: Maybe refer to "multiple stressors" instead of "a stressor"
ANSWER: Done
L 26: What does "representatives of different real situations" refer to? I suggest to revise the sentence to avoid further confusion
ANSWER: It has been revised and modified.

L40-42: The sentence sounds odd, because it leads to the false conclusion that river biodiversity is strongly affected due to the human need for freshwater. I suggest to rewrite the sentence saying that the influx of chemicals and other substances due to anthropogenic land use leads to a strong decline in freshwater biodiversity.
ANSWER: It has been modified.

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SPEAR was due to the study of two different biological groups, namely inverts and diatoms. In the introduction you could now point out the advantage of MTBAs over other trait-based approaches, given that it can be used over a broader range of biological groups.
ANSWER: the sentence has been completed including their use in a broader range of biological groups (L 60-62).

L86 ff.: "The studied basins…" Until now, the reader has no idea that you studied several basins during the present study. I suggest to revise the sentence to avoid confusion. Furthermore, I suggest to list the major stressors in the individual basins already here.
ANSWER: It has been revised: L87: "We selected three river basins of different sizes that represented…"

L 97: Since you did not directly assess ecosystem functions in the present study, is suggest to alter the manuscript along the lines of "…translates into a loss of functional traits within a community…"
ANSWER: Done

L268-269: Compared to what sampling or sampling points was the toxicity of the chemicals higher in the mentioned basins? Please revise the sentence
ANSWER: The sentence has been completed and modified (L257-58).

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ANSWER: This part has been deleted (results available in SI). As said before, other parts of the results have been also deleted or condensed.

L300-307: Are these general results for all basins combined or do these results refer to a specific basin? Please check and revise the paragraph for clarity.
ANSWER: Yes, results of the correlation were for all basins combined. The sentence has been checked and corrected.

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ANSWER: Some p-values were deleted by mistake. They have been added. The forth corner p-values and r have been deleted because it was a lot of data, too long for the reader. They are available in two additional tables in SI (table S8 and S10).

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ANSWER: Conclusions have been re-written and significantly condensed.

Dear editor,

You will find attached our reviewed manuscript entitled **"Unravelling the effects of multiple stressors on diatom and macroinvertebrate communities in European river basins using structural and functional approaches",** which is a research article, for submission to the Virtual Special Issue "Global and regional perspectives of multiple stressor effects on river networks" of the journal Science of the Total Environment.

Thanks for considering our manuscript for publication in Stoten. First, we would like to thank the reviewer for his/her feedback on our manuscript. We have included all the recommendations and answered all the questions. Result section has been significantly reduced. We have eliminated not essential information. From the initial 8 pages in the previous version, we have reduced to 4,5 pages. Tables have been condensed in 3. Conclusions have been revised and reduced.

Thank you for your consideration.

Yours faithfully,

Isabel Muñoz
Núria De Castro Català
Faculty of Biology
Universitat de Barcelona
Avda. Diagonal, 643
08028 Barcelona

On behalf of all coauthors.
Barcelona, 24th June 2020

*Title Page

Unravelling the effects of multiple stressors on diatom and macroinvertebrate communities in European river basins using structural and functional approaches

Núria De Castro-Català[1*], Sylvain Dolédec[2], Elenni Kalogianni[3], Nikolaos Th. Skoulikidis[3], Momir Paunovic[4], Božica Vasiljević[4], Sergi Sabater[5, 6], Elisabet Tornés[5], and Isabel Muñoz[1]

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[2]Université Claude Bernard Lyon 1 (CNRS-LEHNA), Lyon, France

[3]Institute of Marine Biological Resources and Inland Waters, Hellenic Centre for Marine Research, Anavissos, Greece

[4]University of Belgrade, Institute for Biological Research Siniša Stanković (IBISS), Belgrade, Serbia

[5]Catalan Institute for Water Research (ICRA), Girona, Spain

[6]Institute of Aquatic Ecology, Universitat de Girona, Girona, Spain

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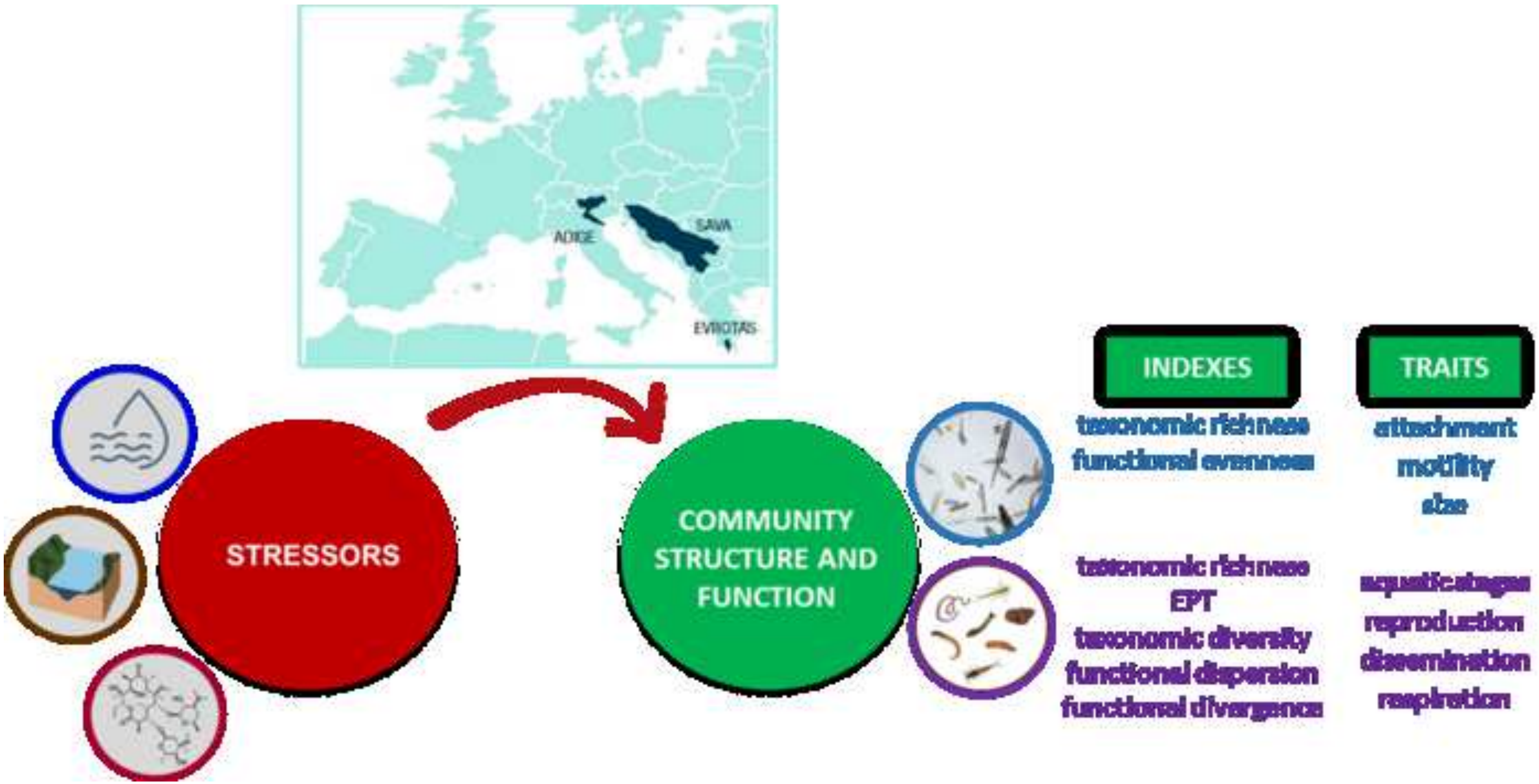
*Graphical Abstract



*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

Links between diversity and function of benthic communities, and multiple stressors were studied.

Structure-based approaches and multiple trait-based approaches allowed us to identify the causes of diversity impairment.

The main drivers for the diatom community structure and function were hydrological descriptors and PhAC toxicity.

Invertebrate community structure was affected by hydrology, geomorphology and pesticide toxicity.

The main drivers of the functional attributes of invertebrates were hydrological descriptors.

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Abstract

Rivers suffer from more severe decreases in species diversity compared to other aquatic and terrestrial ecosystems due to a variety of pressures related to human activities. Species provide different roles in the functioning of the ecosystem, and their loss may reduce the capacity of the ecosystems to respond to multiple stressors. The effects on diversity will differ based on the type, combination and severity of stressors, as well as on the characteristics of the community composition and tolerance. Multiple trait-based approaches (MTBAs) can help to unravel the effects of multiple stressors on communities, providing a mechanistic interpretation, and, thus, complementing traditional biodiversity assessments using community structure. We studied the relationships between diversity indexes and trait composition of macroinvertebrate and diatom communities, as well as environmental variables that described the hydrological and geomorphological alterations and toxic pollution (pesticides and pharmaceuticals) of three different European river basins: the Adige, the Sava, and the Evrotas. These river basins can be considered representative cases of different situations in European freshwater systems. Hydrological variables were the main drivers determining the community structure and function in the rivers, for both diatoms and macroinvertebrates. For diatom communities, pharmaceutical active compound (PhAC) toxic units were also identified as a very important driver of diversity changes, explaining up to 57% of the variance in taxonomic richness. For macroinvertebrates, river geomorphology was an important driver of structural changes, particularly affecting Plecoptera richness. In addition, PhAC and pesticide toxic units were also identified as stressors for macroinvertebrate communities. MTBA provided a detailed picture of the effects of the stressors on the communities and confirmed the importance of hydrological variables in shaping the functional attributes of the communities.

Keywords

Biodiversity; biofilm; invertebrates; Toxic Units; traits; hydrology.

1. Introduction

Freshwater ecosystems are among the most threatened ecosystems on Earth (Schinegger et al., 2016; C. J. Vörösmarty et al., 2010). Biological diversity is declining faster in freshwater ecosystems than in marine and terrestrial ecosystems, most likely because humans have settled for a long time besides rivers to use the freshwater resources as a provisioning service involving stream regulation and water abstraction. In addition, domestic, agricultural and industrial activities release mostly chemicals and other substances that strongly alter freshwater biodiversity (Dudgeon et al., 2006; Feld et al., 2016).

Biodiversity loss can compromise ecosystem functioning. The impacts of species loss on ecosystem functioning depend on the functional roles of individual species (i.e., the characteristics or components of an organism's phenotype that influence ecosystem functioning). Thus, the loss of species with key functional roles can lead to a reduction in ecosystem functions (Cardinale et al., 2012). Nonetheless, the link between ecosystem functioning and biodiversity is complex and depends on a multitude of factors, including the community structure, interactions among species, sequence of species loss, species traits and environmental context (e.g. Schmera et al., 2017;

Statzner and Moss, 2004; Vaughn, 2010). In recent years, multiple trait-based approaches (MTBAs) (e.g., Dolédec and Statzner, 2010; Menezes et al., 2010) have emerged as a useful complement to traditional biodiversity assessments using community structure and may represent a valuable tool for a more mechanistic understanding of the effects of environmental stressors on communities (Verberk et al., 2013). The main advantages of MTBAs are their applicability at a large scale (e.g., across ecoregions) because aquatic organisms worldwide can be described and compared on the same scale for a given trait (e.g., Bonada et al., 2006), their discrimination power for certain trait categories for different types of stressors (e.g., Doledec et al., 1999; Mondy et al., 2016; Stenger-Kovács et al., 2020), and their applicability to a broad range of biological groups (e.g., diatoms, macrophytes, invertebrates, vertebrates). However, the full potential of MTBAs as a complement or even an alternative to taxonomic-based methods has yet to be established.

Major stressors acting on freshwater communities include alteration of flow or increasing levels of agricultural, industrial and urban pollutants (Vörösmarty et al., 2010). The identification, characterization, and understanding of the effects of these stressors are important challenges for ecologists and managers (Hering et al., 2010), mostly because they frequently co-occur and their interaction can cause intricate effects in the communities. The development of cross-disciplinary research to successfully address multiple stressors is very important, and projects such as the GLOBAQUA project constitute excellent opportunities (Navarro-Ortega et al., 2015). In fact, the number of studies on freshwater ecosystems that adopt the multiple stressor perspective has progressively increased (Dolédec and Statzner, 2010; Kuzmanovic et al., 2017; Lemm and Feld, 2017; Meißner et al., 2019; Piggott et al., 2015; Rotter et al., 2013; Sabater et al., 2016; Schäfer et al., 2016; Statzner and Bêche, 2010; Wagenhoff et al., 2011). However, the mechanistic understanding of the impact of such stressors is far from clear, since co-occurring stressors in the environment and co-occurring traits in the species can potentially blur individual expected cause-effect relationships.

Thus, in this study, we used both taxonomic-based methods as well as MTBAs to disentangle the effects of multiple stressors on freshwater communities. We studied two different biological groups, diatoms and macroinvertebrates, to obtain a comprehensive assessment of the effects of the stressors. Diatoms and invertebrates are primary producers and consumers, respectively, with different sensitivities to perturbations. The use of these two biological groups is recommended for river quality assessments (Feio et al., 2007), since diatoms have relatively short generation times and react relatively quickly to changes in the environment such as eutrophication or short-term pollution events (Schmutz et al., 2006), while invertebrates are highly sensitive to morphological alterations of the channel, substratum composition, and habitat conditions (e.g., presence of dams or destruction of the riparian corridor) (Chen et al., 2019; Feio et al., 2007).

We selected three river basins of different sizes that represented common environmental situations of European freshwater systems impacted by multiple stressors. We hypothesized that the communities would be affected structurally and functionally by the different stressors. However, despite the particular synergies of each case study, we also expected to find general trends or links between the different measured biological descriptors (indexes) and the multiple stressors in the three river basins. We also expected that the MTBA would complement the results of the taxonomic-based analyses and provide specific information about the mechanistic effects of the stressors. Therefore, the aims of this study were (i) to compare the biodiversity response and functional patterns produced on two aquatic freshwater biota (macroinvertebrates and diatoms) by different co-occurring environmental stressors (flow intermittency, toxic pollution and geomorphological alteration) in three European watersheds; (ii) to examine the adequacy of using the MTBA to assess the effects of multiple stressors on biological communities; (iii) to study whether a loss of species translates into a loss of functional traits within a community; and (iv) to identify the community descriptors (structural and functional) that best respond to (particular or multiple) environmental stressors.

2. Methods

2.1. Study sites

The three case study basins comprised the Adige (Italy), the Evrotas (Greece) and Sava (Slovenia, Croatia and Serbia) river basins. The Adige River basin (12000 km^2) represents large rivers from mountainous regions subjected to different stressors associated with tourism and hydropower production. The Evrotas River basin (2418 km^2) was selected as a representative of Mediterranean rivers facing water scarcity problems, together with other problems, such as diffuse and point source pollution. The Sava River basin (97713 km^2) was chosen as a representative of continental large European rivers that flow through highly populated areas and are subjected to flow regulation and chemical pollution. A total of seven river sites were sampled in the Adige, eight sites were sampled in the Evrotas, and nine sites were sampled in the Sava (Fig. 1). Locations were selected according to the objectives of the GLOBAQUA project (Navarro-Ortega et al., 2015), targeting different sets of stressors to illustrate different management scenarios. The sites were sampled twice, once in 2014 and once in 2015 (Table S1). From now on, we will refer to them as the 1st and 2nd campaigns.

2.2. Diatoms

Benthic diatoms were sampled based on the standard EU protocol (EN 13946, 2003). In the laboratory, samples were digested using the hot hydrogen peroxide method to remove organic matter. Cleaned material was prepared (using Naphrax) in permanent slides for microscope examination. Up to 400 diatom valves were counted and identified at the species or subspecies level in each sample using light microscopy (Nikon Eclipse 80i, Tokyo, Japan) with Nomarski differential interference contrast optics at 1000× magnification.

2.3. Macroinvertebrates

Macroinvertebrates were collected with a kick net with a mesh of 500 µm according to the proportion of the main habitat types described in the STAR-AQEM methodology (AQEM Consortium, 2002). Ten to twenty subsamples were collected depending on the number of habitats and the size of the river. The sampling was carried out using a 25 cm × 25 cm square hand net with a 500-µm nytex mesh size. Subsamples were preserved in ethanol until transportation to the laboratory where they were sorted, and all individuals found were identified to genus level, where possible, using a stereomicroscope. Species abundance was reported as the number of individuals per m^2 in each site.

2.4. Environmental descriptors

Simultaneously, different environmental descriptors were measured at each site, including hydrological parameters, the concentrations of priority and emerging compounds associated with the land uses (agrochemicals and pharmaceutical active compounds, PhACs), nutrients (nitrates) and geomorphology (Table 1).

Geomorphological alteration was assessed through the geomorphological index (GMI). This index was developed based on the hydromorphological criteria established in the Water Framework Directive (EC, 2000). A questionnaire was completed by the different case-study leaders, who were acting as experts. This questionnaire included an evaluation at each site of the land use intensity, the typology and state of the riparian zone, the characteristics of the riverbed (e.g. diversity of habitats) and the morphological transformation of the riverbank. The scores for each item were weighted, and a final index was calculated. High values indicated high alteration (Table S2).

Composite water samples for the analysis of pesticides and PhACs were collected from surface waters on the left, centre and right sections of the river channel (20–30 cm below the water surface) and then mixed and transferred to 1 L polyethylene bottles for PhACs and 0.5 L amber glass bottles for pesticides. Samples were transported in refrigerated isothermal containers and stored at −20 °C until extraction (Giulivo et al., 2019; Mandaric et al., 2019). The PhAC analyses of water samples were conducted by using offline solid phase extraction (SPE) followed by ultra-high-performance liquid chromatography coupled to triple quadrupole linear ion trap tandem mass spectrometry (UHPLC-QqLIT-MS2) (Gros et al., 2012). The analyses of pesticides were performed using a method based on isotope dilution online solid phase extraction–liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) as described in (Palma et al., 2014). To assess the toxicological risk associated with the presence of these organic microcontaminants, toxic units (TUs) were calculated at each sampling site using the measured environmental concentrations of the compound of interest (MEC) and its respective acute toxicity data (EC50) for the green algae *Raphidocelis subcapitata* and for the crustacean *Daphnia* sp. (Table S3). These standard test species are phylogenetically closest to the diatom and invertebrate communities with available toxicity information, and the toxicity indexes represent a potential response of the community to toxic pollutants. Sums of TUs for each compound family or group (PhACs: antibiotics and non-antibiotics; pesticides: fungicides, herbicides and insecticides) were calculated to estimate the risk associated with different groups of toxicants (Backhaus and Karlsson, 2014; Schäfer et al., 2011) (Equation 1).

$$\sum TU_{i\,(algae,\ Daphnia\ sp.)} = \sum \frac{C_i}{EC50i} \qquad (1)$$

Nitrates were measured by ion-chromatography (761 Compact IC, Metrohm).

2.5. Taxonomic descriptors

Different biological descriptors were calculated for diatom and macroinvertebrate assemblages: total abundance (N), taxonomic richness (S), Margalef's index (d), Shannon–Wiener diversity index (H'), and Pielou's evenness (J').

For macroinvertebrates, three additional biological descriptors were calculated: the separate richness of Ephemeroptera, Plecoptera and Trichoptera (EPT), the EPT index (total richness of Ephemeroptera, Plecoptera, and Trichoptera) and the N EPT index, based on the total abundance of EPT. These three orders of macroinvertebrates include the most sensitive species and, thus, are commonly used as indicators of water quality (Lenat, 1993) (Table S4).

2.6. Biological traits

Diatoms

We used the available information on 22 trait categories distributed in 4 biological traits for European species published by (Rimet and Bouchez, 2012) (Table S5). The variables describing cell size (length, width, thickness, and biovolume) were processed by principal component analysis (PCA) to synthesize the information into one variable. The first PCA component explained 76% of the total variance and was split into four classes: very small, small, medium, and large (Fig. S1). The other traits included cell shape, colonialism, and type of attachment to the substrate.

In addition, we calculated the different components of functional diversity (functional divergence, functional richness and functional evenness; (Laliberté and Legendre, 2010; Villéger et al., 2008).

Invertebrates

We mainly used the available information on 11 biological traits for European macroinvertebrate genera (Schmidt-Kloiber and Hering, 2015; Statzner et al., 2007; Tachet et al., 2010), with some adaptations for the Mediterranean region when necessary (Bonada et al., 2007; Bonada and Dolédec, 2011). The biological traits describe the morphology, life history, feeding habits, resistance and resilience potential, and respiration types of macroinvertebrate genera by means of 59 trait categories (Table S6). In the corresponding databases, the affinity of each taxon for each trait category was quantified using fuzzy coding (Chevenet et al., 1994). Taxa trait profiles were further generated as frequency distributions of categories within each trait.

2.7. Statistical analyses

Environmental data (chemical, hydrological and geomorphological) were checked for normality and homoscedasticity, and log-transformed when necessary to meet these criteria. Statistical differences between basins were tested using one-way ANOVA.

We followed two different approaches, one using chemical information (i.e., the concentration of pollutants) and the other using toxicological information (i.e., TU calculation).

A first exploration of the relationships among environmental descriptors and biological indexes was carried out by means of Spearman's rank correlations.

Regression models (LM) were applied to test for the effects of the different environmental descriptors, considered as fixed effects, on the different biological descriptors. Residuals plotted against fitted values were visually examined for all the models to check the model assumptions.

Constrained ordination analyses were applied to study the relationships between species composition (dependent variable) and the measured environmental descriptors (independent variables). To reduce the asymmetry of the species distributions (i.e., low or zero abundances), community data were transformed with the Hellinger transformation (Legendre and Gallagher, 2001). Ordination was performed using the vegan package (Oksanen et al., 2016). A detrended canonical correspondence analysis (DCA) was initially performed on the species distribution data to determine whether unimodal (canonical correspondence analysis, CCA) or linear ordination (redundancy analyses, RDA) was most appropriate (Lepš and Šmilauer, 2003). The correlation of environmental variables was evaluated to select those with a variance inflation factor (VIF) lower than 10. Forward and backward selection of significant variables was performed using the Monte Carlo permutations test (n=9999). The significance of the final models was tested using ANOVA. This procedure resulted in two different ordination analyses for each of the biological groups. Afterwards, and based on the ordination results, we decided to perform the multiple-trait based analyses using only the toxicological information (i.e., TUs).

The relationship between macroinvertebrate traits and environmental descriptors was investigated by RLQ (Dolédec et al., 1996) and fourth-corner analyses (Legendre et al., 1997). RLQ analysis combines the three separate ordinations of environmental descriptors, species distribution across samples, and trait affinity via co-inertia techniques to identify the primary relationship between environmental characteristics (table R) and functional traits (table Q), which are mediated by species abundances (table L) (Dray et al., 2003). Table L serves as a link between tables R and Q, and measures the intensity of the relationship between them. The overall significance (total variance of the RLQ analysis) of the relationship between environmental characteristics and traits was assessed by a global Monte Carlo test using 99999 random permutations of the array rows of R (sites; model 2) and of the array rows of Q (species; model 4) (Dray et al., 2003). In addition, fourth-corner analysis was used to assess for significant relationships between the combination of trait categories and environmental variables. The fourth-corner analysis tests the links between each environmental variable and the combination of trait categories (i.e., one environmental variable and one combination of trait categories at a time) and the significant relationships between each trait category and the combination of environmental variables (i.e., one trait category and one combination of environmental variables at a time) (Dray et al., 2014). In these two latter approaches, P-values were corrected using a false-discovery-rate (FDR) adjustment to limit bias due to multiple-test comparisons (Benjamini and Hochberg, 1995).

All analyses were performed using R software (R Core Team, 2016). Spearman rank correlations were computed using the Hmisc package (Farrell, 2012). Regression models were conducted using the lmer function in the lme4 package (Bates et al., 2018). The multivariate analyses were computed with the ade4 (Chessel et al., 2004; Dray et al., 2007; Dray and Dufour, 2007) and vegan packages (Oksanen et al., 2016). The functional diversity indexes were calculated with the FD package (Laliberté and Legendre, 2010).

3. Results

3.1. Environmental descriptors

The Sava River was the largest river with the highest mean discharge. The Evrotas River, which is a typical Mediterranean river, presented low flows and flow intermittency. The Adige had average flow in between these extremes (Table 1).

The highest concentrations of PhACs were detected in the Adige and the lowest in the Evrotas, with the Sava in between. Pesticides were always present at lower levels than PhACs. Nitrates were always below 5 mg/L in the three basins, with the exception of the Adige River in the first sampling campaign where values ~~reached~~ ranged between 21 mg/L and 100 mg/L according to river position.

The GMI tended to increase downstream of the rivers of the different basins. The impact was related to the homogenization of the habitats within the river, alteration of riversides, water abstraction, and loss of riparian vegetation.

3.2. Toxic units

The highest toxicity levels for green algae were detected in the Adige (upstream and in the first sampling), and in one site of the Evrotas during the second sampling campaign (Fig. S2). Antibiotics (mainly, clarithromycin) greatly contributed to the toxicity in both the Adige and Sava, whereas herbicides were prominent in the Evrotas.

The total toxicity of the chemicals for *Daphnia magna* was high in some sites of the Sava and Evrotas rivers, but was low in the Adige (Fig. S3). A threshold of 0.001 TUs (-3 using log-scale) has been indicated as the limit from which chemicals could exert chronic effects in invertebrates, and a threshold of 0.1 TUs (-1 using in log-scale) has been identified as the limit from which chemicals could cause lethal effects (Liess and Von Der Ohe, 2005; Schäfer et al., 2013). Both thresholds were reached in the Sava (-2.3 in S4, -1.1 in S2, and -1.9 in S3) and in the Evrotas (-1.5 in E1, and -1.6 in E2) rivers. The compounds responsible for these levels of toxicity were pesticides (mainly insecticides) (Table S7).

3.3. Links between structure and function

Combined all basins, functional richness was strongly correlated with taxonomic richness (S and Margalef's index) and taxonomic diversity (Shannon-Wiener index) (Fig. 2, data in Table S4). Functional dispersion was positively and strongly correlated with taxonomic diversity and Pielou's evenness (p-value<0.001) and, to a lesser extent, with taxonomic richness. Functional dispersion was also negatively correlated with the total abundance. Functional evenness was negatively correlated with taxonomic richness, taxonomic diversity and functional richness, and it was not correlated with Pielou's evenness. In general, the higher the richness and diversity of species, the lower the evenness in the trait distribution.

3.4. Response of communities to environmental constraints

3.4.1. Structure-environment relationships

3.4.1.1. Taxa-environment relationships

The first CCA using diatoms or macroinvertebrates explained 25.7% (11.9% for the first axis and 7.4% for second axis) and 20.9% (10% for the first axis and 4.9% for the second axis) of the total variance, respectively, and selected the mean discharge (Q), concentration of PhACs, GMI, and flow intermittency as significant drivers of the community composition ($p<0.05$) (Fig. 3A and BC). In both CCAs, the first axis clearly separated the Evrotas (low discharge and flow intermittency) from the Sava sites (higher discharge without flow intermittency). Along the second axis, PhACs pointed towards the Adige sites (Fig. 3A and 3C). The second CCAs using diatoms or macroinvertebrates and performed with the sum of TUs of the groups of contaminants instead of their concentrations explained 29.5% (11.9% for the first axis and 7.6% for the second axis) and 19.4% (10.1% for the first axis and 6.3% for the second axis) of the total variance, respectively. The selected drivers were partly different between the two taxonomic groups. The second CCA for the diatoms selected the same drivers as above but PhACs ($p<0.01$) and pesticide TUs ($p<0.05$) as chemical drivers (Fig. 3B). In addition to mean discharge and GMI, the second CCA for the macroinvertebrates selected the TUs of pesticides only (mainly, due to the toxicity of insecticides such as chlorfenvinphos or diazinon) ($p<0.05$) (Fig. 3D).

In all CCAs there was a clear distinction between the Sava, the Adige and the Evrotas sites along the first axis, which was mainly attributed to discharge. Along the second axis, flow intermittency and pesticide TUs distinguished the Evrotas from the Adige. Comparatively, the TUs of pesticides contributed slightly more than PhACs in the models (9% vs. 4%).

3.4.1.2. Taxonomy-based indices

Changes in diatom richness (S) and the diatom Shannon–Wiener diversity index (H) were significantly explained by the magnitude of the flow (34% and 29% of the variance respectively, Table 2). Half of the richness model variance was explained by basin and campaign differences (i.e., differences that were not explained by the environmental descriptors included as predictors in the model). When performing the richness models with only the Sava and Adige data, the richness variance equalled 32%, 57% and 46% for PhACs concentrations, PhACs TUs, and antibiotics TUs, respectively. In the three models, the higher the concentration and toxicity of the PhACs, the lower the species richness (Table 2). The Shannon–Wiener diversity index model relied solely on the effects of hydrology. The trend for both models was that the higher the mean flow, the higher the diatom diversity in the three basins.

Six taxonomy-based invertebrate indices were significantly related to either environmental variables (Table 2). Taxonomic richness (S) was negatively related to the flow magnitude and herbicide TUs. The Shannon–Wiener diversity index (H) was also related negatively to herbicide TUs and to flow intermittency. Taxonomic evenness (J) was only related to flow intermittency.

Flow also appeared as an important driver involving a decrease in the percentage (%EPT) and abundance (N EPT) of sensitive invertebrate taxa. In addition, the EPT abundance was explained by pesticides TUs and flow intermittency. Finally, Plecoptera richness was negatively related to the GMI.

3.4.2. Trait-environment relationships

3.4.2.1. RLQ and fourth corner approaches

For diatoms and macroinvertebrates, the global RLQ test revealed a significant relationship between taxa abundance and environmental variables (model 2, $p<0.001$ in both cases) as well as diatom taxa abundance and biological traits (model 4, $p<0.001$ and $p<0.005$ for diatoms and macroinvertebrates, respectively). In both RLQ analyses, the first and second diatom RLQ axis took most of the total variance of the environmental variables (~95%) into account and around half the total variance of the diatom traits (Table S8 and S10).

The two RLQ analyses clearly separated the Evrotas from the Adige and the Sava along the first two RLQ axes (Fig. 4A for diatoms, Fig. 5A for macroinvertebrates).

A total of thirteen out of 22 diatom trait categories whereas only six out of 59 macroinvertebrate trait categories were significantly related to environmental variables. Pedunculate and stalk attachment, very small size/biovolum, and free moving or floating diatoms decreased with discharge. Pedunculate and stalk attached diatoms were positively related to flow intermittency. Ribbon colonial, pedunculate and pad attached diatoms decreased with the GMI. Non-colonial, with pad diatoms increased with PhAC TUs whereas stalk attached, or ribbon colonial diatoms decreased (Table S9). For macroinvertebrates, imago as aquatic stage, ovoviviparity and passive aquatic dispersal were positively related to discharge whereas aerial active dispersal was negatively related to discharge. Spiracle respiration technique (aerial) and aerial active dispersal were positively related to flow intermittency (Table S11).

Diatoms trait combinations were significantly related to all environmental descriptors but pesticide TUs, either along the first RLQ axis (Discharge; flow intermittency; Fig. 4), or both axes (GMI; PhACs TUs; Fig. 4). In contrast, macroinvertebrate trait combinations were significantly related to all environmental descriptors either along the first RLQ axis (Discharge), the second RLQ axis (Pesticide TU; GMI), or both axes (flow intermittency; PhACs TUs).

3.4.2.2. Trait-based indices

The concentration of PhACs positively slightly affected the diatom functional evenness. For macroinvertebrates, functional dispersion and functional divergence were significantly but weakly related to flow intermittency, with a negative effect for the former and a positive effect for the latter (Table 3).

4. Discussion

Despite the different types and intensities of stressors in the studied basins, some general links between biological descriptors covering the structure and the functioning of two different groups of organisms (diatoms and macroinvertebrates) and different stressors of the Sava, Adige and Evrotas rivers were statistically validated.

The main drivers of the diatom community structure included the hydrology of the river (mean flow), and the toxicity level of pharmaceutical compounds. The main contributors to this toxicity were antibiotics: clarithromycin was detected in the three basins, and the highest toxicity levels occurred in the upstream sites of the Adige, and in most of the sites of the Sava, both in the first sampling campaign (Mandaric et al., 2019, 2017). Clarithromycin is highly toxic to the algae *R. subcapitata* (Watanabe et al., 2016) and diatoms (Minguez et al., 2016). Apart from clarithromycin, other antibiotics with high TUs that were found in the rivers comprised azithromycin (Evrotas) and sulfamethoxazole (Sava). Sites polluted with the most toxic PhACs (e.g., A2 and A5 in the Adige River) presented diatom taxa forming an attached mucilage pad (e.g., *Fragilaria*, *Diatoma*), which could play an important role as a protective barrier, delaying the diffusion of pollutants to the biofilm (Rimet and Bouchez, 2011; Val et al., 2016).

The regression models confirmed the influence of PhACs in the diatom diversity. The occurrence of these emerging pollutants was associated with a decrease in taxa richness, as well as an increase in the evenness of the taxa abundance distribution in the functional space. Thus, the functional traits associated with taxa disappearing because of chemical stressors were maintained by more tolerant diatom taxa. Similar results have been recently reported in fish (Teichert et al., 2018), but not in diatoms.

Evidence of the effects of pharmaceuticals (PhACs) on the algal community of the biofilm has been reported in several studies (e.g. Corcoll et al., 2015; Lawrence et al., 2005; Ponsatí et al., 2016). Diatoms have been found to be highly sensitive to PhACs, even more sensitive than green algae (Corcoll et al., 2015). This sensitivity can be particularly high after dry periods in intermittent rivers (Corcoll et al., 2015), which could explain the importance of this variable in shaping the diatom assemblage structure in the Evrotas River. The prominent diatom taxa in low flow sites, including sites with flow intermittency (Evrotas), were generally attached to the substrate (e.g., *Achnantidium* sp.), with a very small size compared to those taxa present in sites with high mean flow (mostly, sites located downstream of the Sava). In this latter case, most of the taxa were free moving (e.g. *Nitzschia sp*, *Navicula sp*, *Eolimna sp*). Large rivers such as the Sava River are more resilient to the hydrological dynamics (Wu et al., 2019) because they have a more stable habitat than small rivers (in our case, sites upstream of the Adige and Evrotas basins). The habitat instability of sites with low flow and flow intermittency may explain the presence of fixed non-colonial diatoms that grow firmly attached to the substratum and can sustain hydraulic changes (Graba et al., 2014; Tornés and Sabater, 2010; Watson et al., 2015). Lange et al., (2016) also found that the risk of drying out of streams increased the dominance of small, resilient taxa, which is consistent with our findings.

The main variables affecting macroinvertebrate community structure were river hydrology and geomorphology (in particular, the homogenization of the habitats within the river, alteration of riversides, and loss of the riparian corridor). Taxonomic richness and richness and abundance of EPT decreased with the increase in mean discharge. Apart from flow magnitude, Giulivo et al.

(2019) highlighted the role of hydrological variability (hydropeaking) in determining the presence and abundance of some invertebrates in the Adige River. Flow intermittency was a significant predictor of diversity and evenness (Pielou's index), and EPT abundance loss, meaning that specialist taxa were not very common or abundant in the intermittent sites. Decreased biodiversity and taxa richness in intermittent rivers with respect to perennial rivers has been reported in other studies (Datry et al., 2014; Soria et al., 2017).

Likewise, hydrology was related to changes in macroinvertebrate traits. The higher the magnitude of the flow (mean discharge), the higher the abundance of ovoviviparous taxa (embryos develop inside eggs that hatch within the uterus of the mother and the larva feeds on secretions produced by milk glands), with adult aquatic life stages (imago) and passive aquatic dispersal. These three traits correspond to Mollusca (e.g. *Lithogliphus* sp., *Corbicula* sp., *Esperiana* sp.) and Crustacean taxa (e.g. *Corophium* sp., *Gammarus* sp., *Dikerogammarus* sp.), which were very common in the Sava, especially downstream of the river with high mean flow. In this case, flow does not only directly affect traits, but the observed trends correspond to a longitudinal change from the macroinvertebrate assemblages typical of cold headwaters (e.g., family Capniidae and Nemouridae) to those of large lowland rivers, which tolerate high temperatures, are rather limnophilic (e.g. family Hydrobiidae), and in some cases are invasive species (e.g., *Corbicula fluminea*, *Dikerogammarus villosus*) (Lucić et al., 2015; Paunović et al., 2008).

Flow intermittency was related to the spiracle respiration technique and aerial active dispersal. A close look at the assemblages in sites with flow intermittency (Evrotas basin) revealed that the most abundant group of macroinvertebrates were insects, the majority of which presented aerial active dispersal (adult flight). This trait facilitates recolonization over large areas (Stubbington et al., 2017). Within the insects, some Coleopteran (e.g., *Laccophilus* sp., *Hydraena* sp.) and Dipteran (e.g., family Tipulidae, Anthomyidae, Dixidae) species breathe with spiracles. Both traits represent specialized strategies that confer resistance in the case of drying (Aspin et al., 2019, 2018). Aerial respiration (i.e., spiracles) is favoured over tegument respiration in the case of oxygen depletion in shrinking pools and during loss of water (Bonada et al., 2007), and the ability to disperse increases the resilience of the invertebrates to dry events.

In the macroinvertebrate trait-based regression models, flow intermittency appeared to be the only significant predictor for functional dispersion and functional divergence, with a negative influence for the former and a positive influence for the latter. Thus, putting it together, flow intermittency was linked with a decrease not only in taxonomic diversity but also in the abundance of EPT species and the volume of functional space occupied by the taxa. Many EPT taxa do not have resistant forms to withstand droughts and are therefore highly sensitive to desiccation (Sánchez-Montoya et al., 2018). The assemblages of the sites that presented flow intermittency (summer drought) were mainly composed of Dipteran and Coleopteran taxa that are functionally specialized to overcome dry periods (e.g., present aerial active dispersal and aerial respiration).

Pollutants were also significantly related to changes in macroinvertebrate structure, and the TU calculation revealed that pesticides (i.e., organophosphate insecticides, mainly chlorfenvinphos and diazinon) were the main drivers of these changes. Chlorfenvinphos and diazinon are frequently detected compounds in surface waters (Kuzmanović et al., 2015; Silva et al., 2015) and are hazardous for arthropods (Ashauer et al., 2010; Silva et al., 2015), a reason that lead to the declaration of chlorfenvinphos as a priority substance in the Water Framework Directive (Directive, 2012). Herbicide TUs appeared to be a significant predictor of diversity (richness and Shannon–Wiener index). The compound responsible for this potential effect on diversity was terbuthylazine, which is a persistent triazine that can potentiate the toxicity of organophosphates (e.g. chlorfenvinphos) (Pereira et al., 2017). Specific event controlled sampling would have helped confirm the potential effects of pesticides.

As we hypothesized, MTBA confirmed and complemented the results of the taxonomic-based analyses. The hydrological descriptors and the presence and toxicity of pharmaceuticals accounted for the changes in the diatom general growth types (attachment, colonialism), other traits (size), and the distribution of traits in the community (functional evenness). MTBA also indicated that the main drivers of the functional attributes of the invertebrate communities were hydrological descriptors. Trait combinations rather than particular traits were linked to the presence of toxic pollutants and the geomorphological impact. This constraint of trait metrics has already been shown in other studies (Berger et al., 2018; Kuzmanovic et al., 2017). The investment in adaptive traits (e.g., life-history strategies that confer resistance or resilience to flow intermittence) may hinder the investment in other adaptations due to trade-offs (e.g., investment in egg protection vs. number of eggs). In some cases, life-history strategies may favour resistance or resilience to other stressors (e.g., flying ability allows species to escape water contamination). Thus, traits may be adaptive only as part of a life-history strategy, but not independently from other traits. In this regard, Mondy et al., (2016) suggested further investigations of the trait associations that could help to identify stressor effects.

Some functional and structural indexes were significantly correlated for both invertebrates and diatoms. A loss in taxa richness was linked with a loss in functions (trait functional richness). Likewise, fewer species, and species that were less evenly distributed (i.e., less diversity) implied less functional dispersion (fewer distinct roles or traits in the community). Recent literature reviews have found that species richness is important to ecosystem functioning, contributing to ecosystem stability and production (Cardinale et al., 2012; Loreau et al., 2001). It has also been suggested that a large pool of species may be essential to sustain the assembly and multiple functions of ecosystems, especially in changing environments (Lefcheck et al., 2015; Loreau et al., 2001; Zavaleta et al., 2010). In this line, we found that the community descriptor that responded best to environmental pressures was precisely taxa richness, for both diatoms and macroinvertebrates. Our results thus confirm that taxonomic richness can be a useful index to efficiently measure the magnitude of community impairment at a large spatial scale. However, from a global perspective, the use of only one indicator for the assessment and management of aquatic ecosystems could lead to overlooking the important declines and even extinctions of species (Mantyka-Pringle et al., 2014). In addition, one drawback of traditional diversity indexes such as taxonomic richness is that, although they can be useful indicators of river impairment, they do not provide precise information about the nature of the impairment. If the river is affected by only one main stressor, traditional diversity indexes can be useful to identify the river sites or reaches that are imperilled, but in a multiple pressure context, stressor-specific indicators, such as specific traits (e.g., respiration technique in the case of macroinvertebrates, or the ability to grow attached to the substrate in the case of diatoms), are required to precisely diagnose the sources of impairment.

5. Conclusions

Structure-based indexes and MTBA allowed us to identify the main causes of diversity impairment in the different studied basins. Overall, hydrology was the main driver that determined community structure and function in the rivers, both for diatoms and macroinvertebrates. PhACs were identified as an additional driver of diversity changes for diatoms, and geomorphology for invertebrates, particularly affecting the Plecoptera taxa richness. Functional and structural indexes were significantly correlated; a loss in taxa richness was linked with a loss in functions and in the dispersion of the functions. The results in this article demonstrate that the use of integrative approaches that consider sets of environmental descriptors and their links with structural and functional biological patterns provide useful tools for disentangling, understanding and assessing the effects of multiple stressors in freshwater ecosystems.

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Table legends and figure captions

Table 1. Environmental descriptors measured in the three basins. Mean values and standard deviation (s.d.) for each basin are shown, including data from the 1st and 2nd sampling campaigns.

Table 2. Statistical test and coefficients of linear regression models for the relationship of structure/taxonomy-based indexes and environmental descriptors. S: taxonomic richness; H': Shannon-Wiener index.

Table 3. Statistical test and coefficients of linear regression models for the relationship of trait-based indexes and environmental descriptors.

Figure 1. Sampling sites at each river basin: (A) Adige (Italy); (B) Evrotas (Greece); and (C) Sava (Slovenia, Croatia, Bosnia and Herzegovina and Serbia).

Figure 2. Spearman rank correlations. Coloured squares represent significant rho values ($p<0.05$). FRic: functional richness; FDiv: functional diversity; FDis: functional dispersion; RaoQ: Rao's quadratic entropy; FEve: functional evenness; S: taxonomic richness; N: total abundance; d: Margalef's index; H: Shannon-Wiener index; and J: Pielou's evenness.

Figure 3. Results of the constrained ordination analysis performed on the three diatoms case study data sets. First row: diatom CCA biplot obtained using contaminant (A) water concentrations and (B) Toxic Units. Second row: macroinvertebrates CCA biplot obtained using contaminant (C) water concentrations and (D) Toxic Units. First sampling campaign (a), second sampling campaign (b).

Figure 4. Combined results of RLQ analysis and 4th corner test performed on the three-diatom case study data sets. A. Position of samples along the first-two RLQ axes grouped by basin and campaign (a: first sampling, b: second sampling) using ellipses. B. Contribution of environmental descriptors to the first-two RLQ axes. C. Contribution of trait categories to the first-two RLQ axes. In B and C significant associations (4th corner association after false discovery rate correction) are in blue along the first axis, orange along the second axis, and green along both axes.

Figure 5. Combined results of RLQ analysis and 4th corner test performed on the three macroinvertebrate case study data sets. A. Position of samples along the first-two RLQ axes grouped by basin and campaign (a: first sampling, b: second sampling) using ellipses. B. Contribution of environmental descriptors to the first-two RLQ axes. C. Contribution of trait categories to the first-two RLQ axes. In B and C significant associations (4th corner association after false discovery rate correction) are in blue along the first axis, orange along the second axis, and green along both axes.

Table1
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Table 1. Environmental descriptors measured in the three basins. Mean values and standard deviation (s.d.) for each basin are shown, including data from the 1st and 2nd sampling campaigns.

Pressure	Descriptor	Adige (mean±s.d.)	Sava (mean±s.d.)	Evrotas (mean±s.d.)	Differences among basins
Hydrological	Q (m^3/s)	57.3±82.1	612.6±521.5	1.3±1.4	***
	Flow intermittency (number of days dry)	0	0	18.8±31.8	***
Chemical	PhACs (ng/L)	800.7±1014.6	291.2±122.2	141.8±179.2	**
	Antibiotics	91.1±158.8	33.2±32	12.2±19.2	*
	Non-antibiotics	709.7±869.2	258.1±111.5	128.8±163.9	**
	Pesticides	6.6±6.5	60.9±124.3	73.6±173.5	*
	Herbicides	3.2±4.1	56.5±123.9	53±142.7	***
	Insecticides	3.4±5.4	4.4±12.7	14.7±44.2	n.s.
Geomorphological	GMI	3.5±2.8	4.3±1.3	4±1.5	n.s.
Nutrients	Nitrates (NO_3^-) (mg/L)	23.1±28.4	2.9±0.7	0.9±0.4	***

********p*<0.001; ***p*<0.01; **p*<0.05; n.s.: non-significant**

Table 2. Statistical test and coefficients of linear regression models for the relationship of structure/taxonomy-based indexes for the communities and environmental descriptors. S: taxonomic richness; H': Shannon-Wiener's index.

	Model	Adjusted R^2	F-stat	p-value of the model	Basins	Differences among basins	Differences among campaigns	Stepwise selection	ΔAIC	p-value	Coefficient estimate
Diatoms	**S**	33.9%	5.99	<0.001	S, A, E	Yes	Yes	**Flow (+)**	-7.1	0.005	7.33
	H'	28.7%	8.83	<0.001	S, A, E	No	No	**Flow (+)**	-9.7	0.01	0.16
								Days dry (-)	-2	0.05	-0.03
	S	32.1%	13.74	<0.005	S, A	No	No	**PhACs (-)**	-9.9	0.001	-10.12
	S	**56.8%**	11.51	0.01	S, A	No	No	**PhACs TUs (-)**	-6.7	0.01	-11.09
	S	46.3%	7.91	0.026	S, A	No	No	**antibiotics TUs (-)**	-4.7	0.026	-7.9
Macroinvertebrates	**S**	38.3%	7.83	<0.001	S, A, E	Yes	No	**Flow (-)**	-9.2	0.0005	-10.52
								Herbicides TUs (-)	-4.6	0.007	-1.22
								GMI (+)	-4.2	0.02	16.88
	H'	17.3%	4.91	<0.05	S, A, E	No	No	**Days dry (-)**	-3.4	0.02	-0.3
								Herbicides TUs (-)	-3.3	0.03	-0.04
	J'	19.4%	11.6	<0.001	S, A, E	No	No	**Days dry (-)**	-8.6	0.001	-0.15
	% EPT	**65.5%**	28.86	<0.001	S, A, E	Yes	No	**Flow (-)**	-14.8	<<0.001	-12.62
	N EPT	**48.6%**	15.25	<0.001	S, A, E	No	No	**Flow (-)**	-14	<<0.001	-14.5
								Pesticides TUs (-)	-8	0.005	-0.64
								Days dry (-)	-5	0.02	-17.03
	% Plecoptera	**68%**	32.12	<0.001	S, A, E	Yes	No	**GMI (-)**	-15.7	<<0.001	-11.87

Table 3
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Table 3. Statistical test and coefficients of linear regression models for the relationship of trait-based indexes for communities and environmental descriptors.

	Model	Adjusted R^2	F-stat	p-value of the model	Basins	Differences among basins	Differences among campaigns	Stepwise selection	ΔAIC	p-value	Coefficient estimate
Diatoms	Functional evenness	13.9%	7.3	0.01	S, A, E	No	No	PhACs (+)	-5	0.01	0.043
Macroinvertebrates	Functional dispersion	16.1%	9.43	0.004	S, A, E	No	No	Days dry (-)	-8.9	0.004	-0.15
	Functional divergence	20.9%	12.61	0.0009	S, A, E	No	No	Days dry (+)	-9.4	0.0009	0.11

Figure1

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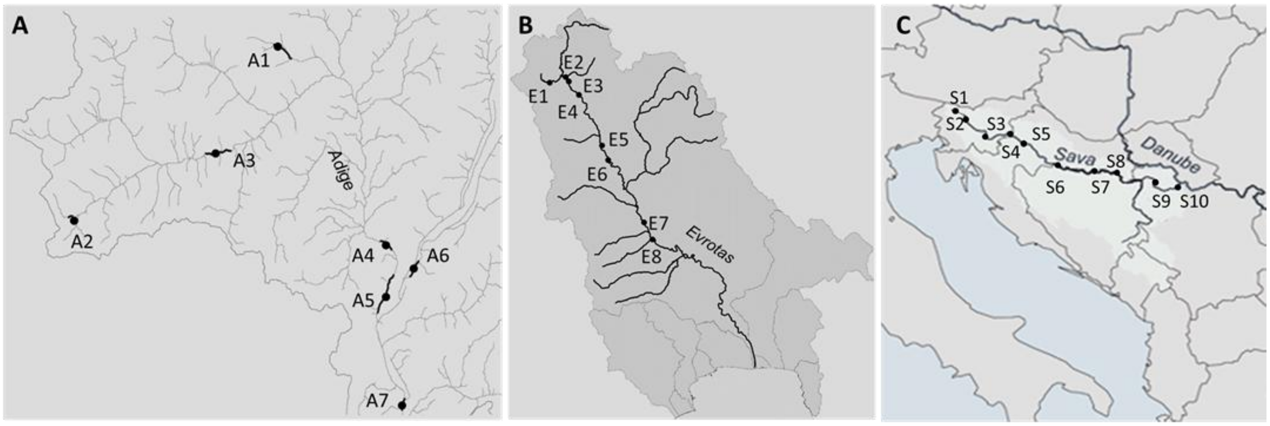

Figure 1. Sampling sites at each river basin: (A) Adige (Italy); (B) Evrotas (Greece); and (C) Sava (Slovenia, Croatia, Bosnia and Herzegovina and Serbia).

Figure2

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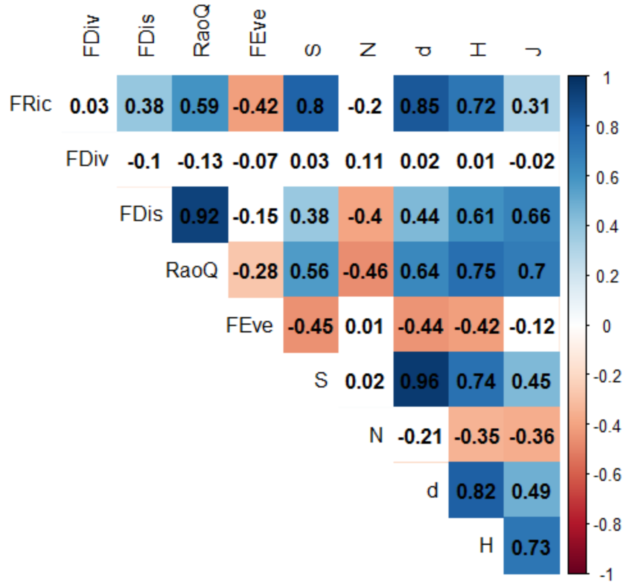

Figure 2. Spearman rank correlations. Coloured squares represent significant rho values (p<0.05). FRic: functional richness; FDiv: functional diversity; FDis: functional dispersion; RaoQ: Rao's quadratic entropy; FEve: functional evenness; S: taxonomic richness; N: total abundance; d: Margalef's index; H: Shannon-Wiener index; and J: Pielou's evenness.

Figure4

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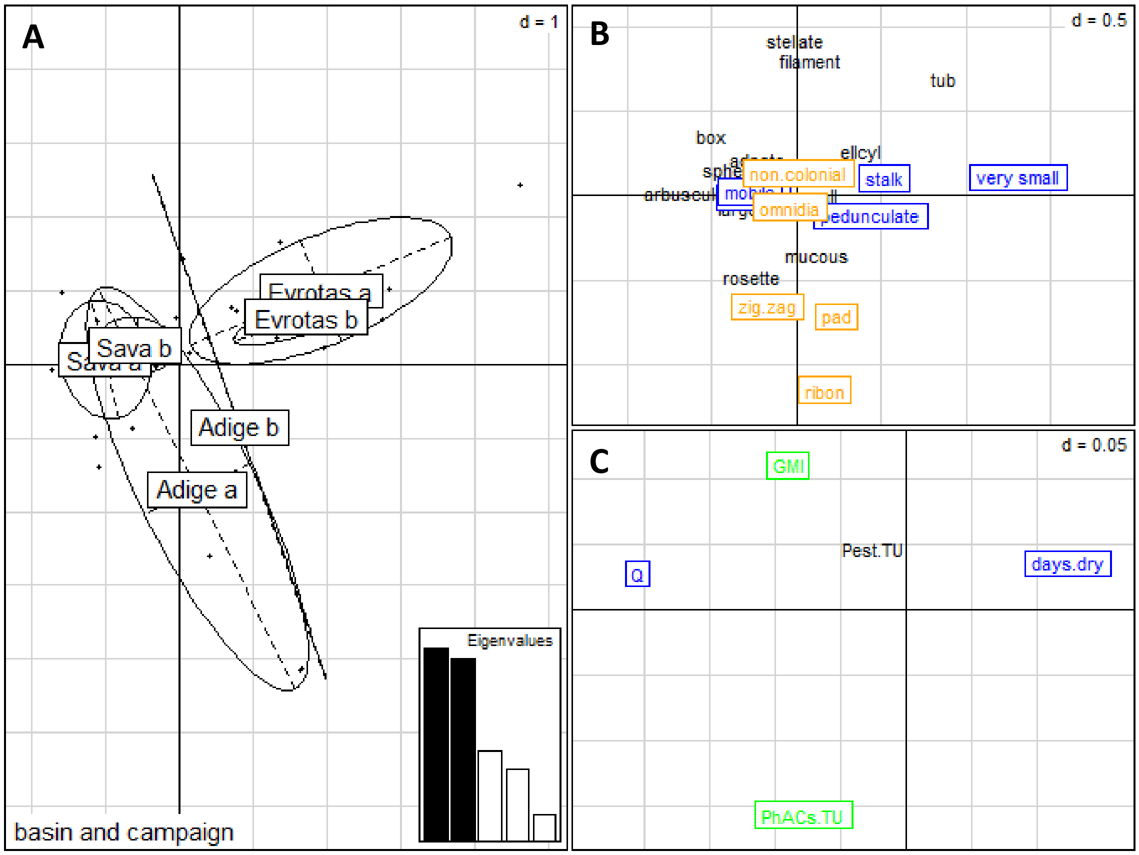

Figure 4. Combined results of RLQ analysis and 4th corner test performed on the three-diatom case study data sets. A. Position of samples along the first-two RLQ axes grouped by basin and campaign (a: first sampling, b: second sampling) using ellipses. B. Contribution of environmental descriptors to the first-two RLQ axes. C. Contribution of trait categories to the first-two RLQ axes. In B and C significant associations (4th corner association after false discovery rate correction) are in blue along the first axis, orange along the second axis, and green along both axes.

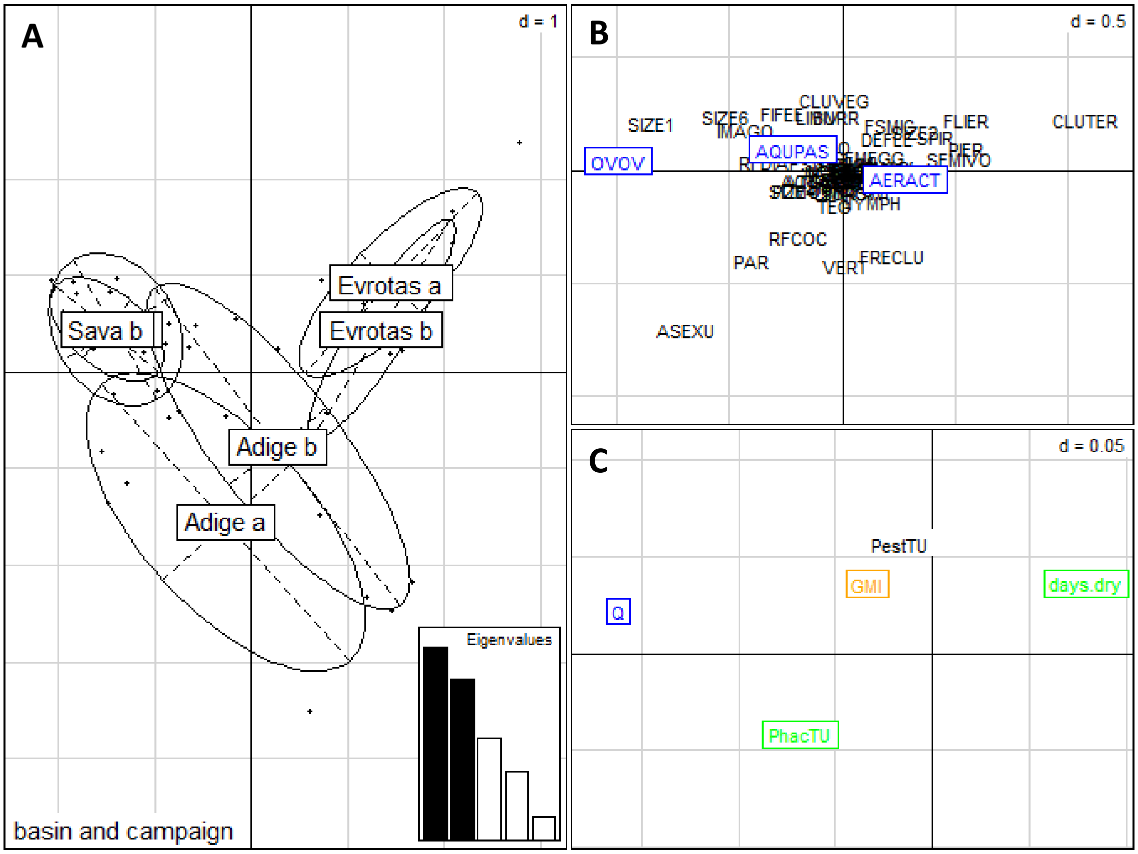

Figure 5. Combined results of RLQ analysis and 4th corner test performed on the three macroinvertebrate case study data sets. A. Position of samples along the first-two RLQ axes grouped by basin and campaign (a: first sampling, b: second sampling) using ellipses. B. Contribution of environmental descriptors to the first-two RLQ axes. C. Contribution of trait categories to the first-two RLQ axes. In B and C significant associations (4th corner association after false discovery rate correction) are in blue along the first axis, orange along the second axis, and green along both axes.

Figure3

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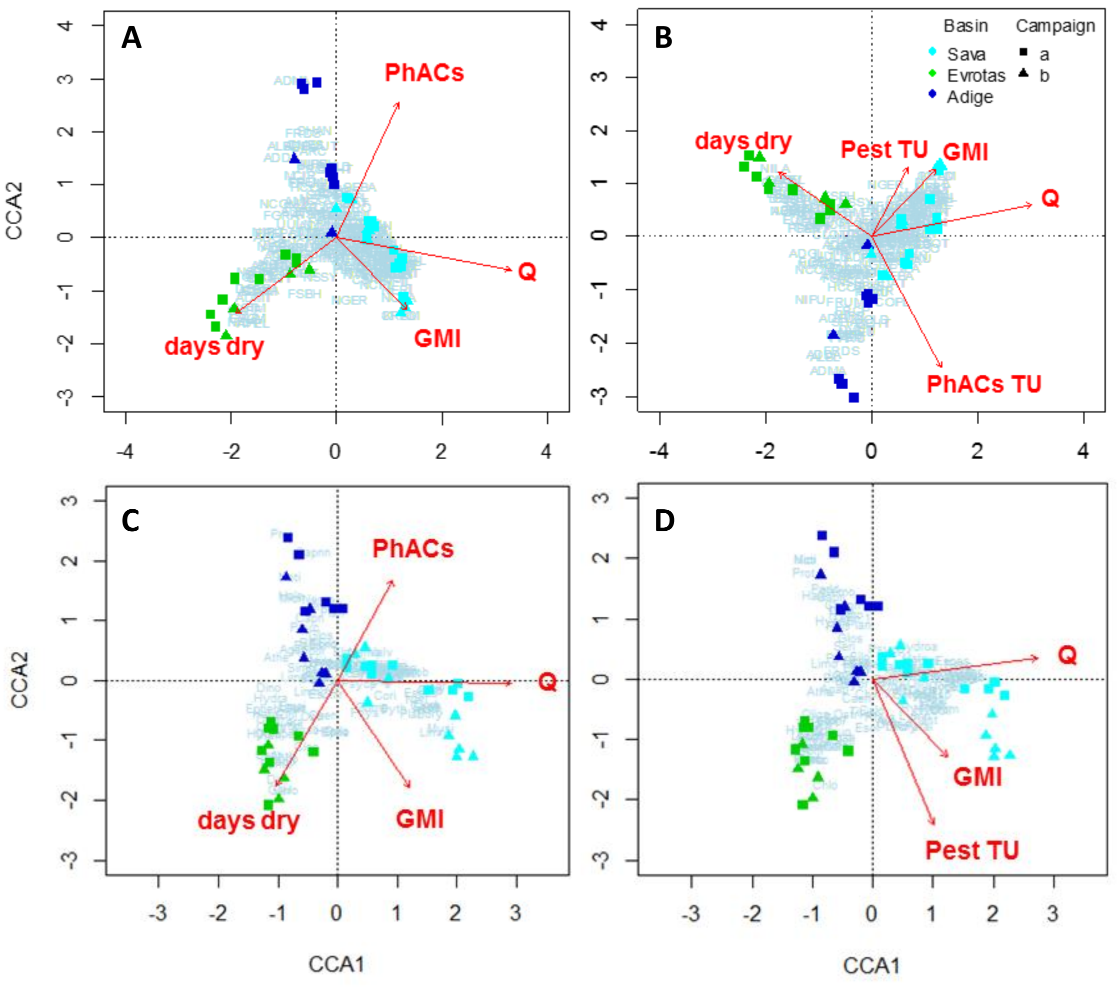

Figure 3. Results of the constrained ordination analysis performed on the three diatoms case study data sets.
First row: diatom CCA biplot obtained using contaminant (A) water concentrations and (B) Toxic Units.
Second row: macroinvertebrates CCA biplot obtained using contaminant (C) water concentrations and (D) Toxic Units.
First sampling campaign (a), second sampling campaign (b).

Supplementary material for on-line publication only
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***conflict of Interest Statement**

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

*Credit Author Statement

Author statement

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Sylvain Dolédec: methodology, data analyses, reviewing, funding acquisition.

Elenni Kalogianni: organized and performed the sampling, sample processing.

Nikolaos Th. Skoulikidis: organized and performed the sampling, sample processing, funding acquisition.

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Božica Vasiljević: organized and performed the sampling, sample processing.

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Elisabet Tornés: organized the sampling, sample processing

Isabel Muñoz: Methodology, organized and performed the sampling, sample processing, funding acquisition, writing, reviewing and editing.

All authors contributed critically to the drafts and gave final approval for publication.