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Title: Evaluating anthropogenic impacts on naturally stressed ecosystems: revisiting river classifications and biomonitoring metrics along salinity gradients

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Abstract: Naturally stressed ecosystems hold a unique fraction of biodiversity. However, they have been largely ignored in biomonitoring and conservation programs, such as the EU Water Framework Directive, while global change pressures are threatening their singular values. Here we present a framework to classify and evaluate the ecological quality of naturally stressed rivers along a water salinity gradient. We gathered datasets, including aquatic macroinvertebrate assemblages and environmental information, for 243 river locations across the western Mediterranean to: a) gauge the role of natural stressors (salinity) in driving aquatic community richness and composition; b) make river classifications by encompassing the wide range of environmental and biological variation exhibited by Mediterranean rivers; c) provide effective biomonitoring metrics of ecological quality for saline rivers. Our results showed that water salinity played a pivotal role in explaining the community richness and compositional changes in rivers, even when considering other key and commonly used descriptors, such as elevation, climate or lithology. Both environmental and biologically-based classifications included seven river types: three types of freshwater perennial rivers, one freshwater intermittent river type and three new saline river types. These new saline types were not included in previous classifications. Their validation by independent datasets showed that the saline and freshwater river types represented differentiable macroinvertebrate assemblages at family and species levels. Biomonitoring metrics based on the abundance of indicator taxa of each saline river type provided a much better assessment of the ecological quality of saline rivers than other widely used biological metrics and indices. Here we demonstrate that considering natural stressors, such as water salinity, is essential to design effective and accurate biomonitoring programmes for rivers and to preserve their unique biodiversity.

Response to Reviewers:
Barcelona, December 14th, 2018

Dear Prof. Sabater,
Attached you will find a revised version of the manuscript by Gutiérrez-Cánovas et al. entitled "Evaluating anthropogenic impacts on naturally stressed ecosystems: revisiting river classifications and biomonitoring metrics along salinity gradients" (ms. STOTEN-D-18-11709).
Thank you for your very helpful comments on our paper. I hope you can pass on our thanks to the referees for their constructive and positive views.

In your comments, you and the referees found our work interesting, novel, well-written and structured, but raised some minor concerns. Specifically, you showed concerns on the clarity and grammar of some sentences, along with other minor comments. In the reviewed version, we have amended all the issues and, additionally, we have sent the paper to an English native speaker corrector that reviewed the linguistic aspects.

We hope, therefore, that the changes and clarifications implemented across the text make our paper suitable for publication in the Science of the Total Environment.

We provide a detailed, point-by-point response in the attached letter, and look forward to hearing from you again in due course.

Sincerely,
Cayetano Gutiérrez-Cánovas (on behalf of all co-authors)

Research Data Related to this Submission

Title: Data for: Evaluating anthropogenic impacts on naturally stressed ecosystems: revisiting river classifications and biomonitoring metrics along salinity gradients
Repository: Mendeley Data
https://data.mendeley.com/datasets/nkzd5m67yg/draft?a=fcb4f993-f685-4720-9e0a-55776c8af891

Barcelona, 22 October 2018

Dear Professor Barceló,

Attached you will find the proposed *Full paper* entitled **'Evaluating anthropogenic impacts on naturally stressed ecosystems: revisiting river classifications and biomonitoring metrics along salinity gradients'** co-authored by C. Gutiérrez-Cánovas, P. Arribas; L. Naselli-Flores, N. Bennas, M. Finocchiaro, A. Millán and J. Velasco. We would appreciate that you consider this article for publication in *Science of the Total Environment.*

In this manuscript, we show the necessity of including naturally stressed rivers into biomonitoring and conservation programs for an effective preservation of biodiversity. Our results suggest that natural stressors, such as water salinity, are main drivers of river macroinvertebrate richness and composition, despite being largely ignored when defining major river types (e.g. Water Framework Directive). Our classifications show different types of river communities developing along broad gradients of environmental heterogeneity, where saline rivers are characterised by unique communities. Finally, we demonstrate that current biomonitoring metrics fail in detecting the degradation of saline rivers, and propose more effective metrics based on specialist taxon abundance. Considered together, these findings can foster a better understanding of ecosystem responses to stress and designing more effective biomonitoring and conservation programs to preserve aquatic biodiversity.

We believe that this manuscript touches on three spheres (*biosphere*, *hydrosphere*, *anthroposphere*) and fits in several journal's subject areas such as *Contaminant (bio)monitoring and assessment*, *Environmental management and policy*, *Stress ecology in marine, freshwater and terrestrial ecosystems*.

We affirm that this manuscript (with tables, figures, and supporting information) is our own original work, has not been published before, and is not being considered for publication elsewhere in its final version in either printed or electronic form. All authors agree with the content of the manuscript and approve of its submission to *Science of the Total Environment.*

We look forward to hearing from you in due course.

Sincerely,

Cayetano Gutiérrez-Cánovas (on behalf of all co-authors)

*Title Page

Title: Evaluating anthropogenic impacts on naturally stressed ecosystems: revisiting river classifications and biomonitoring metrics along salinity gradients

Authors: Cayetano Gutiérrez-Cánovas[1]; Paula Arribas[2]; Luigi Naselli-Flores[3]; Nard Bennas[4]; Marta Finocchiaro[5]; Andrés Millán[6] and Josefa Velasco[6]

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Barcelona, December 14th, 2018

Dear Prof. Sabater,

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Sincerely,

Cayetano Gutiérrez-Cánovas (on behalf of all co-authors)

Comments made by editor:

Dear Tano,

I have received the comments of two reviewers on your paper. The two are positive and stress the originality and interest of your work, though raise issues that you need to address before the paper can be considered for publication. Please respond to reviewer's queries in a one-by-one basis, and incorporate the changes into the revised manuscript. I look forward receiving the revised version

Sincerely yours- un abrazo,

Sergi Sabater

Associate Editor Science of the Total Environment

AR (Author's response): Many thanks for the positive comments. We are happy to see that you and the referees liked the manuscript. Please, find below the point-by-point responses to all the mentioned issues and comments.

Reviewer #1:

This is a novel and timely paper presenting new invertebrate-based biomonitoring metrics to incorporate saline rivers and streams as a new typology in water quality and ecological status assessment. These ecosystems have been largely neglected by water quality biomonitoring programmes (e.g. Water Framework Directive), and they need to be considered since they are home to a wide variety of organisms that need protection. Beyond is obvious interest for the assessment and protection of saline rivers and streams, the paper is relevant in the wider context of naturally stressed ecosystems, which are usually difficult to assess given their low alpha diversity. A very clear case of this are transitional waters (e.g. estuaries, coastal lagoons), which are lagging behind in the implementation of the Water Framework Directive due to the absence of robust biological metrics and the low number of reference (i.e. undisturbed) sites that can be found around Europe. Thus, this study falls within the aim and scope of STOTEN and presents relevant results that can be of interest for a wide readership. Moreover, the paper is well written and the methods are well designed and executed. taking all this into consideration, I recommend the paper to be published after some minor modifications that I detail below.

AR: We are very grateful for your positive comments and for your effort in improving the ms.

The use of the word "genuine" in the highlights and other sections of the text does not seem appropriate. All ecosystems are genuine. I understand what the authors are trying to say, but I think that a different term should be used.

AR: We have changed the word "genuine" by "singular" in L4 and L40. The first point in the highlights has changed, so it does not contain the word "genuine" any more.

Grammar should be revised (e.g. L40 Thread? do you mean threat?).

AR: Done, change by "threatened"

L50 What about other countries like Australia and the USA? It would be good to provide a wider perspective.

AR: We have broadened the geographical perspective of this sentence by saying that saline rivers have been neglected also in these two countries (L49-L52): "such as the EU Water Framework Directive (WFD, Directive 2000/60/EC) or the Australian or US river biomonitoring programmes (Nichols et al., 2017)"

Nichols, S.J., Barmuta, L.A., Chessman, B.C., Davies, P.E., Dyer, F.J., Harrison, E.T., Hawkins, C.P., Jones, I., Kefford, B.J., Linke, S., Marchant, R., Metzeling, L., Moon, K., Ogden, R., Peat, M., Reynoldson, T.B., Thompson, R.M., 2017. The imperative need for nationally coordinated bioassessment of rivers and streams. Mar. Freshw. Res. doi:10.1071/MF15329

L68 Substitute "rivers" by "ecosystems"?

AR: Done

L180 I think that if detailed flow metrics would have been included (e.g. Jaeger & Olden 2012) the hydrology could have been much more important in this study. Maybe this should be acknowledged.

AR: We completely agree with the referee. However, retrieving hydrological information for all the studied rivers was impractical considering 1) the large extent of territory covered and 2) the fact that the hydrology of most of the studied rivers is not tracked due to their small size (particularly, intermittent and perennial saline rivers). We have acknowledged this limitation in the Discussion (L418-L424).

L249-250 It is not clear how the metric was built. Did you assign a score to each taxa according to the IV? Does the metric rely only on presence/absence data or it also includes abundance?

AR: For each river type, we identified the indicator taxa for reference and disturbed types and for family, genus and species levels using the IndVal analysis (i.e. in total, six metrics per river type). For each sample, we calculated each metric values as the sum of the abundances of the organisms showing a significant Indicator Value for a certain combination of river type, reference or disturbance status, and taxonomic level. We have clarified this procedure in L255-L259.

L311 Maybe the mean and SD conductivities of each group could be reported here to make it easier to follow.

AR: We have added the range of conductivities (percentiles 10 - 90) for each classification and river type in L321-L327.

"The hyposaline type showed a conductivity range (Q10-Q90) of 8,323-24,567 µS cm-1 for the environmental classification, and one of 4,953-26,158 µS cm-1 for the biological classification. The mesosaline type gave a conductivity range of 60,480-110,100 µS cm-1 for the environmental classification and one of 24,133-98,200 µS cm-1 for the biological classification. The hypersaline type showed a conductivity range of 140,000 - 300,000 µS cm-1 for the environmental classification and one of 110,100 - 300,000 µS cm-1 for the biological classification."

L314 Shouldn't these be considered as different typologies? A permanent hyposaline river can be very different from an intermittent hyposaline river. It has been proved that the interaction between drought and salinity can be very relevant for aquatic invertebrates (e.g. Suárez et al. 2017).

AR: We understand that conductivity may have different effects depending on hydrology. However, it is necessary to assess the importance of the interactive effects of conductivity and hydrology relative to their single effects, and along which portion of the conductivity gradient the interactive effects become more relevant. Thus, our models showed that the interactive effects of conductivity and hydrology did not explain a large amount of variance (Fig. 1a) and that fitted values showed that hydrological effects were almost negligible at conductivities higher than 3,000 µS cm^{-1}(Fig. 2a). This is not surprising given that saline specialists may show co-tolerance to other drought-related stressors such as desiccation (Pallarés et al 2017). We have justified the use of saline river types that include both perennial and intermittent rivers within the same type in L410-L419.

Pallarés, S., Arribas, P., Bilton, D.T., Millán, A., Velasco, J., Ribera, I., 2017. The chicken or the egg? Adaptation to desiccation and salinity tolerance in a lineage of water beetles. Mol. Ecol. https://doi.org/10.1111/mec.14334

L318 According to these values, the CS was quite weak, right?

AR: CS values of our classifications are within the range (or even above) of the typical values for other environmental or biological based classifications (see Hawkins and Vinson, 2000 and Heino et al. 2006 for two relevant examples). We admit that these values are not particularly strong (CS values ranging 0.15-0.20, whereas ideally they should be close to 1), but this is common result in river classification approaches, which are the most common method employed by river managers, versus alternative modelling-then-predict approaches (e.g. RIVPACS).

Hawkins, C.P, Vison, M.R. 2000. Weak correspondence between landscape classifications and stream invertebrate assemblages: implications for bioassessment. Journal of the North American BenthologicalSociety, 19(3):501–517

Heino, J. et al. 2006. Assessing physical surrogates for biodiversity: Do tributary and stream type classifications reflect macroinvertebrate assemblage diversity in running waters? Biological Conservation, 129(3): 418-426

L327 The authors chose to focus on Coleoptera and Heteroptera, but other taxa (e.g. Chironomidae) could be very relevant since they respond to drought (Cañedo-Argüelles et al. 2016), salinity (Walker et al. 1995; Dickson et al. 2014) and pollution (Cañedo-Argüelles et al. 2012). In fact including Chironomidae at the family level does not make much sense, since they provide no relevant information (just "noise").

AR: We agree with the referee. It would have been great to include other widely-distributed taxa, such as Chironomidae, at genus or species level. Unfortunately, we only had suitable data covering the whole gradient of conductivity and the vast territory encompassed for Coleoptera. Besides, we believe that we should keep Chironomidae records despite their prevalence and even if they were identified at family level, because they provide relevant ecological information to discriminate among river classes for two reasons: 1) their abundances varied across sites depending on environmental settings and contribute to

community dissimilarities, and 2), they were absent from a relevant fraction of the sites (~15%), and particularly from those with greatest conductivity.

L322-335 This list of taxa might not mean much to the reader if she/he is not familiar with the auto-ecology of these taxa. Maybe a deeper analysis of the taxa from a functional perspective (i.e. functional traits) would be relevant?

AR: We feel that taxonomic information for indicator organisms is necessary to identify which families, genera and species might be useful to build future metrics for saline rivers. Additionally, we believe that the mentioned families and genera are relatively well known for a large extent of aquatic ecologists, even if they are not taxonomic specialists. Furthermore, although biomonitoring methods are often based on family and genus-level data, next generation sequencing methods are allowing the identification of finer taxonomic resolution, which will make species level information suitable for biomonitoring in a near future.

We decided not to use a trait-based approached because we would have needed very detailed physiological information of osmoregulation capacities to find significant differences between the species occurring at different river types. Unfortunately, these data are only available for a reduced set of genera and species (e.g. Arribas et al. 2014; Bradley, 2008; Carbonell et al. 2012; Pallarés et al. 2017). Besides, available trait-datasets (e.g. Tachet et al. 2002) describe biological attributes (e.g. body size, life-histories, respiration type, reproduction type) that do not allow to clearly discriminate the distribution of saline specialist with the detail we would need here (Picazo et al. 2012).

Arribas, P., Andújar, C., Abellán, P., Velasco, J., Millán, A., Ribera, I., 2014. Tempo and mode of the multiple origins of salinity tolerance in a water beetle lineage. Mol. Ecol. *https://doi.org/10.1111/mec.12605*

Bradley, T.J., 2008. Saline-water Insects: Ecology, Physiology and Evolution, in: Lancaster, J., Briers, R. (Eds.), Aquatic Insects: Challenges to Populations. CAB International, Oxford, United Kingdom.

Carbonell, J.A., Millán, A., Velasco, J., 2012. Concordance between realised and fundamental niches in three Iberian Sigara species (Hemiptera: Corixidae) along a gradient of salinity and anionic composition. FreshwaterBiology57, 2580–2590. *https://doi.org/10.1111/fwb.12029*

Pallarés, S., Arribas, P., Bilton, D.T., Millán, A., Velasco, J., Ribera, I., 2017. The chicken or the egg? Adaptation to desiccation and salinity tolerance in a lineage of water beetles. Mol. Ecol. *https://doi.org/10.1111/mec.14334*

Picazo, F., A. Millán, and S. Dolédec. 2012. Are patterns in the taxonomic, biological and ecological traits of water beetles congruent in Mediterranean ecosystems? Freshwater Biology 57:2192–2210.

L334-335 What is the geographical distribution of this species? Can it be widely used as bioindicator in hypersaline rivers?

AR: Some saline specialists (e.g. *Ochthebius glaber*, *Nebrioporus baeticus*) are endemics and can be used only at regional level. Fortunately, these taxa have sister species across different regions with very similar ecological requirements (Arribas et al., 2015). Therefore, species level indicators can be easily updated in other regions such as Morocco or Sicily. We have added this information in the Discussion L457-461.

L377 You might find this reference interesting: Gascon et al. 2016

AR: Thanks for the suggestion. We have added this reference.

L433-436 I would add that we need a complete and detailed inventory of saline rivers. For example, how many saline rivers are considered as water bodies (and therefore routinely monitored) within the Water Framework Directive?

AR: We agree with the referee and added a sentence in the discussion suggesting that environmental managers should better record and classify the naturally stressed rivers within each region (see L400-L404).

REFERENCES

Cañedo-Argüelles, M., Boix, D., Sánchez-Millaruelo, N., Sala, J., Caiola, N., Nebra, A., &Rieradevall, M. (2012). A rapid bioassessment tool for the evaluation of the water quality of transitional waters. Estuarine, Coastal and Shelf Science, 111, 129-138.

Cañedo-Argüelles, M., Bogan, M. T., Lytle, D. A., & Prat, N. (2016). Are Chironomidae (Diptera) good indicators of water scarcity? Dryland streams as a case study. Ecological indicators, 71, 155-162.

Dickson, T.R., Bos, D.G., Pellatt, M.G., Walker, I.R., 2014. A midge-salinity transfer function for inferring sea level change and landscape evolution in the Hudson Bay Lowlands, Manitoba, Canada. J. Paleolimnol. 51, 325-341. doi:10.1007/s10933-013-9714-x

Gascón, S., Arranz, I., Cañedo-Argüelles, M., Nebra, A., Ruhí, A., Rieradevall, M., ... &Boix, D. (2016). Environmental filtering determines metacommunity structure in wetland microcrustaceans. Oecologia, 181(1), 193-205.

Jaeger, K.L. & Olden, J.D. (2012) Electrical resistance sensor arrays as a means to quantify longitudinal connectivity of rivers. River Research and Applications, 28, 1843-1852.

Suárez, M. L., Sánchez-Montoya, M. M., Gómez, R., Arce, M. I., Del Campo, R., & Vidal-Abarca, M. R. (2017). Functional response of aquatic invertebrate communities along two natural stress gradients (water salinity and flow intermittence) in Mediterranean streams. Aquatic sciences, 79(1), 1-12.

Walker, I.R., Wilson, S.E., Smol, J.P., 1995. Chironomidae (Diptera): quantitative palaeosalinity indicators for lakes of western Canada. Canadan J. Fish. Aquat. Sci. 52, 950-960.

AR: Thanks for the suggestion. We have included some of these references (i.e. Cañedo-Argüelles et al. 2012; Gascón et al. 2016; Suárez et al. 2017)

Reviewer #3:

The manuscript examines the role of natural variation in salinity in Mediterranean rivers as a driver of community richness and composition and then presents analyses which show the importance of this relationship in the classification of rivers. In addition, the authors show that in these naturally saline systems, commonly used metrics for the determination of reference vs. disturbed sites are not adequate to discern between pristine and modified rivers. Furthermore, the authors provide an alternative approach to determining ecological status in naturally saline systems and demonstrate that this approach can detect disturbance where existing approaches do not. The manuscript is well organized, the subject matter will be of interest to readers of STOTEN, the conclusions are well supported by the data, and the study itself and the questions it addresses are sufficiently novel for publication in STOTEN. I believe the manuscript would make an important contributionto our understanding of naturally saline systems and more importantly the way in which these systems are classified and assessed. Prior to publication, the comments below should be addressed.

AR: We would like to thank you for your positive comments and for your effort in improving the ms.

General comments

1. An important part of the manuscript deals with rivers which are naturally saline. It would be useful to the reader if some additional context around these sites was provided. This would serve two purposes: 1. It would provide some important background in terms of why the study region has naturally saline rivers (e.g., is this mainly due to a concentration effect in semi-arid areas, naturally saline groundwater inputs etc.?); 2. It would allow for some additional discussion around the extent to which these types of systems occur outside the study area and in turn provide some additional information regarding the applicability of the manuscript outside of the study area. This additional context does not need to be extensive but a few sentences in the introduction and/or discussion might be useful.

AR: We have added a brief description about the original of the natural salinity in the study region (2.1. Dataset description, L93-95).

"In the study area, salinity tends to increase in rivers that drain basins with an arid climate and a soluble lithology (Millán et al., 2011). These rivers reach the highest mineralisation levels in areas dominated by evaporitic outcrops."

Also, we briefly mentioned other regions (outside of the study area) where naturally saline rivers may occur and to which degree our findings may apply (Discussion, L425-L430)

"Our findings might also be useful to biomonitoring naturally saline rivers in other regions outside the study area, such as Australia (Biggs et al., 2013), North and South America (Griffith, 2014; Orfeo, 1999), North Africa (Hamed and Dhahri, 2013) and Russia (Zinchenko et al., 2014). Specifically, although biogeographical differences may lead to very different taxonomic compositions, ecological responses to salinity might be similar and roughly close river types might be yielded depending on the available salinity gradient."

2. Although the manuscript is generally well written there are a number of minor grammatical issues throughout. I have highlighted a number of these in the specific comments section but given the large number I have come across I would suggest a thorough review and editing prior to re-submission. Issues are generally very minor and usually involve a single word needing to be inserted.

AR: We would like to thank you for carefully revising the linguistic aspects. We have amended all the highlighted issues and, additionally, the new version of our ms has been sent to a native corrector for a careful revision of the linguistics.

Specific comments

Graphical abstract: The relevance of the symbols given to demonstrate the salinity gradient were not clear to me. I would suggest replacing these with something that can be easily interpreted as representing a salinity gradient.

AR: We have unified the symbols of the graphical abstract to circles and used a gradient colour (from yellow to brown) to indicate the increasing salinity (either in the top bar and the central panel that illustrates the river classification along the salinity gradient). Hope that these new arrangements make the figure clearer.

Highlights: I think there should be at least one line describing what the study intended to achieve (i.e., what you did). I would suggest replacing the first line with something like: "Investigated role of natural salinity in community richness and composition"

AR: We have changed the first point to: "We examined the role of natural salinity in rivers to improve their classification and biomonitoring".

L41: "release the naturally stressed conditions". This is confusing

AR: Done

L67: insert "the" after "adapt

AR: Done

L74: replace "allows assessing" with "assesses"

AR: Done

L101: insert "our" after "combined"

AR: Done

L103: why use Appendices? Shouldn't this be listed as Supplementary Materials?

AR: As far as we are concerned, additional tables and figures should be placed in Appendices. Appendices should be labelled as A, B, C, etc. when there is more than one appendix.

L138: insert "to" after "according"

AR: Done

L161-L162: Did the models meet the assumptions and homoscedasticity? The sentence mentions that these were assessed graphically but I could not find mention elsewhere of whether these assumption were met

AR: Yes, models met the normality and homoscedasticity assumptions. We have modified the text to make it clearer in L165-L167: "All the models met the normality and homoscedasticity assumptions, which were validated by visually checking their residuals."

L200-L201: it would be useful to show these plots along with the cut off point for classification which was used

AR: We have added a plot showing the dendrogram of our classification into the Fig. 3 (panel c).

L232-L233: this sentence is confusing and should be reworded. Insert "of" after "its"; change "potential" to "potentially"

AR: We have reworded this sentence to make it clearer (L238-L240)

"For all these samples, we firstly assigned river types using the environmental classification thanks to its simplicity and potentially better performance compared to the biological classification (see the Results)."

L237: insert "a" after "As"

AR: Done

L352: I would suggest replacing "key" with "large" or something similar

AR: Done: "...large proportion of biological variation..."

L352-L357: I think this paragraph needs some clarification and relates to the issue of naturally vs. anthropogenically saline rivers. When you say that elevation, lithology and climate have previously been thought of as the main factors determining species richness etc., are you referring here to natural/pristine/undisturbed systems only? If so, this should be clarified. If you are however referring to drivers of richness across the complete disturbance gradient (i.e., from pristine through to impacted) then it is not necessarily surprising that local factors and in particular disturbances such as flow modification or contamination etc. play a key role. Furthermore, if you are referring here to only the drivers of richness etc. in natural conditions, then this raises two more questions: 1. When you say "local stressors such as salinity" what do you mean by "local" in terms of spatial scale? This could mean reach scale to some, sub-watershed, watershed or basin to others etc.

Secondly, if you are referring to localized natural salinity, wouldn't this be driven in large part by the previously mentioned factors (i.e., climate and lithology)? As such, isn't naturally high salinity simply a product of these drivers? Again, I think fleshing out some of the details around what is driving the natural salinity in the first place is warranted here.

AR: We thank the referee for this comment. We have included the suggested clarifications in L366-L372. In these lines we wanted to discuss the importance of salinity under natural conditions. In fact, to evaluate the role of salinity in determining river macroinvertebrate richness and composition, we utilised data from rivers unaffected by anthropogenic impacts.

Regarding local factors, we wanted to highlight that our data showed that "reach" factors (which vary at the small spatial scale) such as conductivity or hydrology are much more important for macroinvertebrate richness and composition, than other catchment-level attributes (basin area, climate, lithology).

Under natural conditions, we admit that river conductivity is generally influenced by catchment-scale attributes such as climate or lithology as partly showed here and elsewhere (Estévez et al. 2019). However, such catchment level attributes do not allow for an accurate prediction of conductivity and then show a weak capacity to predict macroinvertebrate communities (Heino et al. 2006). Alternatively, to better predict river conductivity, we need more precise hydro-geochemical models that account for water and salt balances and exchanges between ground and surface waters.

Estévez E, Rodríguez-Castillo T, González-Ferreras AM, Cañedo-Argüelles M, Barquín J. 2019 Drivers of spatio-temporal patterns of salinity in Spanish rivers: a nationwide assessment. Phil. Trans. R. Soc. B 374 : 20180022. http://dx.doi.org/10.1098/rstb.2018.0022

Heino, J. et al. 2006. Assessing physical surrogates for biodiversity: Do tributary and stream type classifications reflect macroinvertebrate assemblage diversity in running waters? Biological Conservation, 129(3): 418-426

L388: replace "this" with "these". Insert "from" before 448-545

AR: Done

L389: replace "showed" with "shown"

AR: Done

L388-L389: "Various authors, 2009". Please provide a more appropriate citation.

AR: Done. We have replaced by "Spanish Government, 2009"

L392: replace "that" with "than"

AR: Done

L396: "mean or sampled". What is the distinction here? Does "sampled" refer to a single sample vs. the mean of several samples?

AR: We have modified the sentence as suggested: "Thus, the prediction of their biological communities was fairly accurate based just on a single sample or the mean of several samples of conductivity."

L398: replace "in" with "on"

AR: Done

L411: replace "cause" with "indicates" or something similar

AR: Done

L418: insert "a" after "provide"

AR: Done

L419: switch "be" and "also"

AR: Done

L427: insert "a" after "posing"

AR: Done

L431: replace "endemism" with "endemic"

AR: Done

Abstract

Naturally stressed ecosystems hold a unique fraction of biodiversity. However, they have been largely ignored in biomonitoring and conservation programs, such as the EU Water Framework Directive, while global change pressures are threatening their ~~genuine~~singular values. Here~~,~~ we present a framework to classify and evaluate the ecological quality of naturally stressed rivers along a ~~gradient of~~ water salinity gradient. We gathered datasets, including aquatic macroinvertebrate assemblages and environmental information, for 243 river locations across the western Mediterranean to: a) gauge the role of natural stressors (salinity) in driving aquatic community richness and composition~~,~~; b) ~~develop~~make river classifications by encompassing the ~~broad~~wide range of environmental and biological variation exhibited by Mediterranean rivers ~~and~~; c) provide effective biomonitoring metrics of ecological quality for saline rivers. Our results showed that water salinity ~~had~~played a pivotal role in explaining the community richness and compositional changes in rivers, even when considering other key~~,~~ and commonly used descriptors, such as elevation, climate or lithology~~, are considered~~. Both environmental and biologically-based classifications included seven river types: three types of freshwater perennial rivers, one freshwater intermittent river type and three new saline river types~~, which~~. These new saline types were ~~absent~~not included in previous classifications. Their validation ~~using~~by independent datasets showed that the saline and freshwater river types represented differentiable macroinvertebrate assemblages at family and species levels. Biomonitoring metrics based on the abundance of indicator taxa of each saline river type provided a much better assessment of the ecological quality of saline rivers than other widely used biological metrics and ~~indexes~~indices. Here~~,~~ we demonstrate that considering natural stressors, such as water salinity, is essential to design effective and accurate biomonitoring ~~programs~~programmes for rivers and to preserve their unique biodiversity.

Keywords: abiotic stress, macroinvertebrates, biomonitoring, global change, saline rivers, Water Framework Directive

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1. Introduction

Naturally stressed ecosystems are characterised by harbouring a set of natural conditions that are persistently unsuitable for the vast majority of the regional species pool (Badyaev, 2005; Parsons, 2005). Well-known examples of naturally stressed ecosystems are found in tundra, deserts, volcanic springs, and in glacier, acid or saline inland waters (Cauvy-Fraunié et al., 2016; Elliott and Quintino, 2007; Millán et al., 2011; Petrin et al., 2007). Their natural stressful conditions reduce the local diversity of these systems~~ but~~, which is also~~ constitutes~~ a powerful driver of diversification (Parsons, 2005; Vetaas and Grytnes, 2002)~~, and so~~. So they usually hold unique fractions of biodiversity characterised by high levels of specialisation and species turnover (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013; Jacobsen et al., 2012).

Unfortunately, these ~~genuine~~singular spots are ~~under thread~~threatened by global change pressures, which may ~~release~~modify the naturally stressed conditions causing habitat loss~~ and reducing~~, reduce the community's singularity~~ of the community~~ (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013) or add new stressors, such as pesticides or microplastics (Beketov et al., 2013; Windsor et al., 2019). This situation ~~is~~has been strongly aggravated because many of these naturally stressed ecosystems have been usually ignored ~~once~~when cataloguing natural heterogeneity, and have consequently been systematically excluded for biomonitoring and conservation purposes (Millán et al., 2011; Stubbington et al., 2018).

This is particularly true ~~in~~for the case of naturally saline rivers (Millán et al., 2011), which have been mostly neglected by ~~the~~ large international efforts made to reverse ~~the~~ globalised river degradation, such as the EU Water Framework Directive (WFD, Directive 2000/60/EC) or the Australian or US river biomonitoring programmes (Nichols et al., 2017)~~).~~. These conservation frames are based fundamentally ~~based~~ on a two-step process ~~focused~~that focuses on identifying the~~ identification of~~ main river types over a particular region (river classification~~)~~), and the subsequent development and harmonisation of biomonitoring metrics ~~that~~to allow ~~assessing~~ the ecological status of ~~the~~ different river types to be assessed (Buffagni et al., 2007). However,

~~there are~~ some generalised shortcomings ~~on the application of~~come into when applying these procedures that ~~resulted~~result in a deficient functionality for the assessment and protection of naturally stressed rivers. Firstly, main natural stressors, such as water salinity, are usually neglected or not ~~fully~~completely considered in river classifications, ~~resulting~~and this results in the exclusion or misclassification of naturally stressed rivers (Sánchez-Montoya et al., 2007). This occurs because the classification process tends to ~~be focused~~focus on coarse environmental descriptors, which are weak proxies of ~~local~~reach-scale stressors. Secondly, most ~~of the~~ proposed biomonitoring metrics can result in equivocal evaluations of the ecological status of ~~the~~ naturally stressed rivers because they are ~~strongly~~closely associated ~~to~~with local richness/diversity and ~~so~~do not ~~considering~~consider naturally depauperate communities (Gutiérrez-Cánovas et al., 2008). Besides, other widely~~-~~ used metrics are based on taxa or functional traits that are rare, or not even ~~not~~ present, in naturally stressed rivers~~, such as~~; e.g., the stress-sensitive orders Ephemeroptera, Plecoptera and Trichoptera (EPT) (Belmar et al., 2013; Bonada et al., 2006; Millán et al., 2011). ~~Therefore,~~Hence there is an urgent need to revisit and adapt the current vision and approaches to assess the ecological quality of naturally stressed ~~rivers~~ecosystems, and ~~so~~ to improve their management and future preservation.

Here~~,~~ we benefit from a large compilation of datasets from rivers across the western Mediterranean and Moroccan Atlantic basins, which include almost the ~~complete~~whole natural salinity gradient (roughly, 30 to 300,000 $\mu S\ cm^{-1}$). On this comprehensive basis, we develop an integrated framework that ~~allows assessing~~assesses the ecological quality of naturally stressed rivers. ~~First, we~~We firstly rank the importance of natural stress (salinity) and other general descriptors commonly used for river classifications (e.g. elevation, river size, lithology, hydrology) in determining community richness and composition. ~~Second, we~~We secondly classify rivers ~~through~~according to their environmental characteristics and biological composition~~,~~ by considering specific types for saline rivers, and assessing their performance and concordance. Finally, we identify indicator taxa and metrics for naturally stressed rivers under reference and ~~anthropogenically~~anthropogenic disturbed conditions~~,~~ by testing their performance against other widely used river biomonitoring metrics and ~~indexes~~indices.

2. Material and methods

2.1. ~~Datasets description~~Description of datasets

The study was conducted across the western Mediterranean basin, including the watercourses from the eastern and southern Iberian Peninsula and the Balearic Islands (Spanish data), Sicily (Italian data) and from the Rif down to the Sahara Desert~~,~~ comprising the Rif and Moroccan Atlantic basins (Moroccan data) (Appendix A, Fig. A1). These regions were selected because they cover ~~a great~~wide environmental variability, including large gradients of elevation, climate, hydrology, lithology, salinity and anthropogenic impacts (Appendix A, Fig. A1 and Table A1). In the study area, salinity tends to increase in rivers that drain basins with an arid climate and a soluble lithology (Millán et al., 2011). These rivers reach the highest mineralisation levels in areas dominated by evaporitic outcrops.

We ~~have~~ used different subsets of environmental and aquatic macroinvertebrate assemblage data to address the study objectives ~~of the study~~ (Table 1). The description of each dataset includes anthropogenic disturbance ~~level~~levels (reference or disturbed sites), the macroinvertebrate groups used (all major orders or just aquatic Coleoptera) and their taxonomic resolution (family, genus, species levels), region (Italy, Morocco, Spain, all), the encompassed environmental gradient ~~encompassed~~ (all gradients or just saline rivers), the number of sampling sites, and the observations and ~~the~~ objectives for which each dataset was used. To compile this database of 243 river locations and 577 samples, we combined our own data with a large dataset from the Guadalmed ~~project~~Project (Prat, 2002). All the macroinvertebrate samples were collected following a multi-habitat ~~semiquantitative~~semi-quantitative kick-sample (Jáimez-Cuéllar et al., 2002). See Appendix A for more details about the datasets and Appendix B ~~to see~~for an extended description of the sampling procedure.

~~Climatic~~Climate, geomorphologic, lithologic and land use variables at basin and reach scales were obtained from digital layers after delineating the river basins of each sampling site (see Appendix A, Table A1 for a complete list of the used variables~~ used~~). Water electrical conductivity was measured *in situ* on each sampling occasion as an osmotic stress indicator. To characterise flow intermittence, we categorised the hydrological regime of each site as perennial seasonal (typically flowing), intermittent (surface flow ceases during the dry season, pools remaining) or ephemeral (~~totally~~completely dry during one season) ~~flow~~flows from available hydrological information or field evidence (Belmar et al., 2013; Sánchez-Montoya et al., 2007). We categorised the sampling sites as *reference* when they were minimally disturbed (i.e. fulfilling ≥ 16 out of 20 of the Mediterranean Reference Criteria, MRC; Sánchez-Montoya et al., 2009) or disturbed when they were substantially impacted by anthropogenic activities (i.e. fulfilled < 16 MRC). In this study, we excluded large watercourses (mean basin area ≥1,000 km^2, ECOSTAT, type 3, see Table 1 and Sánchez-Montoya et al., 2007 for details) because of the paucity of reference sites (Sánchez-Montoya et al., 2009). We also excluded disturbed freshwater rivers, for which effective biomonitoring metrics can be found elsewhere (e.g. Birk et al., 2012; Bonada et al., 2006).

2.2. Data analysis

Before performing the analyses, we applied a log-transformation to macroinvertebrate family richness and a square-root transformation to macroinvertebrate species richness. ~~Besides, logit~~Logit-, log- or square-root-transformations were applied to the quantitative environmental variables to reduce their distribution skewness and to improve linearity, ~~when~~whenever necessary. ~~Moreover, all~~All the quantitative environmental variables were also standardised to mean=0 and SD=1 to facilitate model coefficient ~~comparison~~comparisons.

2.2.1. Ranking environmental variable importance

To identify the main environmental factors ~~determining the~~that determine family and species richness, we used Random Forest (randomForestSRC R package, Ishwaran Ishwaran et al., 2014) and Linear Mixed-effect Models (LMM, lme4 R packge, Bates et al., 2015). In these models, we utilised family-level datasets (i.e. ref_fam_ita, ref_fam_mor, ref_fam_spa, n=458) and species-level datasets (i.e. ref_spp_ita, ref_spp_mor, ref_spp_spa, n=211) from the reference sites (selected according to MRC~~,~~; see above). Following Feld et al. (2016), we first ran Random Forest models to identify the most important predictors of family and species richness among the 24 potential candidates (Appendix A, Table A1) to be included ~~into~~in the LMM (see Appendix C for more details about exploratory analyses). After these exploratory analyses, we included basin area, mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence, conductivity (single and quadratic terms), season and region as fixed factors in the LMM. As additional fixed factors, we also included the ~~pairwise~~pair-wise interactions of conductivity x flow intermittence, conductivity x season and conductivity x region. Site code was considered ~~as~~ a random factor to account for repeated measures in the same location. To rank the environmental predictor's importance on family and species richness, we adopted a multi-model inference approach (Grueber et al., 2011)~~,~~ using the *MuMIM* R package (Bartoń, 2016). This statistical technique ranks all the ~~models~~ generated models using all the possible ~~combination~~combinations of predictors ~~using~~based on Akaike's Information Criterion (AIC). Then~~,~~ a set of top models ~~is~~was selected to produce an average model, but only if the model ranking first ~~is~~was ambiguously supported (model weight<0.90). We chose top models ~~differing~~which differed in no more than two AIC units (delta≤2) from the model ranked first (minimum AIC). We adopted a natural average method to conduct ~~the~~ model averaging, which consists in averaging predictors only over the models in which the predictor appears and in weighting predictor's ~~SES~~coefficients by the summed weights of these models (Burnham and Anderson, 2002). For each LMM model, two ~~measures of~~ goodness-of-fit measures were estimated (Nakagawa and Schielzeth, 2013): marginal goodness-of-fit (r^2_m) indicates the variance explained only by the fixed factors, while conditional goodness-of-fit (r^2_c) shows the variance accounted for by both fixed and random terms. We provide the mean ~~average~~ (based on model weights) of each goodness-of-fit measure for ~~each~~every averaged model. All the models met the normality and homoscedasticity assumptions, which were validated by visually checking their residuals~~ for normality and homoscedasticity~~.

To identify the environmental drivers of community composition change, we used Multiple Regression models for distance Matrices (MRM; ecodist R package, Lichstein, 2007). This method is conceptually similar to traditional multiple regression, but with all variables being distance matrices instead of raw data and *P*-values being calculated ~~through~~by permutation tests (~~1000~~1,000 runs). To avoid lack of independence problems due to multiple samples belonging to the same site, we selected a reference subset of macroinvertebrate families (ref_fam_all, n=157 sites/samples) and species (ref_spp_all, n=76 sites/samples) occurrences with only one spring sample per site. We estimated the overall changes in community composition for each pair of sites of the family matrix ~~through~~with the Sørensen dissimilarity index (β_{sor}) and ~~the pairwise~~pair-wise dissimilarity due to turnover from the species matrix ~~using~~with the Simpson index (β_{sim}). These calculations were made following~~ the~~ Baselga's (2010) framework for β-diversity partitioning using the *betapart* R package (Baselga and Orme, 2012). For each selected environmental predictor (basin area, mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence, conductivity, geographic distance), we built a Euclidean distance matrix based on their transformed and standardised values. ~~Geographical~~The geographical distance between localities was based on a latitude and longitude original matrix ~~and~~, while flow intermittence was based on ~~semiquantitative~~semi-quantitative values (perennial=0, intermittent=1, ephemeral=2).

Finally, we also performed ~~a~~ variance partitioning for the community richness and composition models~~,~~ using the *variancePartition* (Hoffman and Schadt, 2016) and *hierpart* (Walsh and MacNally, 2013) R packages.

2.2.2. Integrating saline rivers into biomonitoring typologies

To ~~develop a classification of~~classify rivers by encompassing the environmental and biological variability ~~occurring~~that occurs in the ~~studied~~study area, we used family abundances from reference Spanish sites (ref_fam_spa, n=386). We selected this dataset because it included more sites and samples, and it covered a broader environmental and biological spectrum ~~relative~~in relation to the Italian and Moroccan datasets (Appendix A, Table A1). ~~First, we~~We firstly classified sites into seven types according to their environmental variables (environmental classification) following an adaptation of the criteria suggested by the ECOSTAT intercalibration group for Mediterranean rivers using System A of the WFD (MedGIG European Commission, 2007)~~, which~~. This included mean conductivity, basin area, hydrology, site altitude and basin lithology (Table 2 and Appendix D). Secondly, in order to classify samples according to their biological communities (biological classification), we estimated a Bray-Curtis ~~pairwise~~pair-wise dissimilarity matrix, which derived from the abundance family matrix and produced a dendrogram based on the Bray-Curtis family dissimilarity matrix~~, using the following~~ Ward's clustering method. After making a visual inspection, we decided to prune the tree to produce seven biological types. For both classifications, we used the same ~~type numbers~~ previously utilised type numbers (European Commission, 2007; Sánchez-Montoya et al., 2007) for freshwater rivers and ~~the~~ numbers 6, 7 and 8 for the new saline river types. ~~The type~~Type 3 (large rivers) was not used because we excluded this river type ~~of rivers~~ from ~~the~~our analysis. We also performed~~ a~~ non-Metric Multidimensional Scaling (nMDS) ordination based on the Bray-Curtis family dissimilarity matrix to explore the concordance between the environmental and biological classifications.

To evaluate the performance of both classification procedures in a ~~wider~~broader geographical context, we estimated their classification strength based on ~~datasets of~~the family and species datasets from reference sites ~~of~~in Italy (ref_fam_ita, n=44; ref_spp_ita, n=31), Morocco (ref_fam_mor, n=28; ref_spp_mor, n=29) and Spain (ref_fam_spa, n=386; ref_spp_spa, n=151). We assigned environmental types to these new sites using the environmental classification criteria (Table 2). To assign biological types to the new sites, we built a Random Forest model by predicting biological types from environmental information. To develop the Random Forest model (trees=2000, mtry=8), we used a subset of the ref_fam_spa dataset (n=258), while the non-utilised samples (n=128) were ~~used~~employed along with the other independent subsets to evaluate ~~the~~ classification performance. To estimate classification strength (CS), dissimilarity matrices were converted ~~to~~into similarity matrices. CS was quantified as the difference between the within-type mean similarity (W) and between-~~types~~type mean similarity (B) of the Bray-Curtis ~~pairwise~~pair-wise similarity based on family abundances, and for the Simpson similarity matrix based on species occurrences and turnover (CS=W-B) for the three regions. ~~CS~~The mean CS values were calculated through a bootstrapping procedure, where we resampled 100 subsets of n=28 from each Italian and Spanish macroinvertebrate ~~datasets~~dataset to make their CS values comparable to those obtained for the Moroccan datasets, which had the ~~lowest number of~~fewest observations.

2.2.3. Biomonitoring indicators for saline river types

To develop metrics ~~indicating~~that indicate the ecological quality of saline river types and test their performance against widely used biomonitoring metrics, we ~~used~~resorted to a dataset ~~including~~that included the family and species-level data from Spanish reference (ref_fam_spa_sal, n=89; ref_spp_spa_sal, n=75) and disturbed (dis_fam_spa_sal, n=31; dis_spp_spa_sal, n=30) ~~Spanish~~ sites. ~~We first~~For all these samples, we firstly assigned river types ~~to all these samples~~ using the environmental classification ~~criteria because~~ thanks to its simplicity and ~~potential~~potentially better performance compared to the biological classification (see the Results). ~~Considering~~By considering that most ~~of the~~ disturbed sites ~~have~~had been affected by a drop in their natural conductivity levels as a result of freshwater inputs from agricultural ~~drainages~~drainage (Velasco et al., 2006), we compiled historical~~,~~ predisturbed conductivity information (Moreno et al., 1997; Vidal-Abarca, 1985) to correctly assign their river types. As a result, we obtained hyposaline, mesosaline and hypersaline river types ~~under~~according to the reference condition (ref_6, ref_7 and ref_8, respectively~~) and~~), as well as hyposaline and mesosaline river types ~~under~~according to the disturbed condition (dis_6, dis_7). For ~~the type~~Type 8, we did not find any disturbed site.

To identify the families, genera and species ~~showing~~with a ~~greater~~higher affinity ~~for~~to each reference and disturbed type, we ~~used~~ran an indicator species analysis (IndVal, Dufrêne and Legendre, 1997). This analysis considers ~~the~~each taxon's percentage of occurrence and relative abundance~~ of each taxon~~ for each type to obtain an indicator value (IV) and its significance through Monte-Carlo permutations (1000 runs). We focused on the most frequent taxa~~,~~ by keeping taxa occurring in more than 10% of ~~the ~~observations. From them, we selected those taxa ~~showing~~which showed a significant Indicator Value ($P \leq 0.05$) as potential indicators for a given type. From these results, we built the candidate metrics of reference and disturbed conditions of the saline river types. To create ~~those~~these metrics, for each sample~~,~~ we summed the abundances of the indicator taxa of the assigned river type (ref_6, dis_6, ref_7, dis_7, ref_8) for the family, genus~~,~~ and species level (e.g. for the family level: ref_fam6, dis_fam6, ref_fam7, dis_fam7, ref_fam8). In addition~~,~~ for each sample, we estimated a set of widely-used biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-metric ~~indexes~~indices (ICM-11a and IMMi-T) for Mediterranean rivers (Alba-Tercedor et al., 2002; Munné and Prat, 2009). Finally, to evaluate the performance of these candidate metrics against the widely-used biomonitoring metrics, we used LMM models ~~assessing~~to assess the differences across all the reference and disturbed types (levels= ref_6, dis_6, ref_7, dis_7, ref_8) and Tukey-*t* *post*~~-~~ *hoc* tests to evaluate differences between pairs of comparable reference and disturbed types (i.e. ref_6 vs. dis_6, ref_7 vs. dis_7).

The code and functions used to run all these analyses are available in Appendix E, which were conducted using the R version 3.4.1 (R Core Team, 2016).

3. Results

3.1. Ranking environmental variable importance

Electrical conductivity was the most important variable ~~explaining~~to explain macroinvertebrate assemblage richness and composition (Fig. 1a,c, and Appendix F, Table F1). Family richness was explained primarily ~~explained~~ by conductivity (59%) and the interaction between conductivity and region (25%), ~~suggesting~~which suggests that conductivity had different regional effects (r^2_m=0.82). Generally~~, above~~ with conductivity values ~~of~~above 3,000 µS cm^{-1}, family richness responded only to conductivity changes. Family richness peaked at conductivities ~~ranging~~within the 300-1,000 µS cm^{-1}~~,~~ range, before declining progressively as conductivity ~~increases~~increased (Fig. 2a,c,d). Within this conductivity range, the family richness values showed the greatest dispersion, ~~indicating~~which indicates that other variables ~~had~~ also ~~a strong influence on~~influenced family richness. The interactive effects of conductivity with hydrology (Fig. 2a), season (Fig. 2c) and region (Fig. 2d) were evident only at freshwaters, and~~,~~ particularly~~,~~ within the conductivity range of 300-1,000 µS cm^{-1}, before becoming much weaker at conductivities ~~greater than~~over 3,000 µS cm^{-1}. ~~Rivers~~The rivers with a perennial flow tended to have a higher level of family richness than intermittent or ephemeral rivers (Fig. 2a,b), but as conductivity ~~increases~~increased, the effect of hydrology also became less important. Species richness showed roughly similar patterns in response to environmental variables~~,~~ where, once again, conductivity was~~ also~~ the most important predictor, but with higher contributions of mean basin precipitation and seasonality (Fig. 1b and Appendix F, Table F1).

Conductivity distance was also the most important variable ~~explaining~~to explain dissimilarity in family composition and species turnover (Fig. 1c,d). These results ~~indicate~~indicated that macroinvertebrate assemblages in rivers with different conductivity values tended to have a different family composition, and that these changes ~~seem~~seemed to arise through species replacement (and Appendix F, Table F2). Family composition was also significantly influenced by evaporitic surface, basin slope, geographic and hydrologic distances~~,~~ which, along with conductivity, explained community variance to a substantial extent ~~of the community variance~~ (r^2=63%). Changes due to species turnover were also linked to conductivity, ~~geographic~~geography, basin slope, ~~hydrologic~~hydrology and basin area distances (r^2=35%).

3.2. Integrating saline rivers into biomonitoring typologies

~~The nMDS ordinations of samples according~~According to their biological communities, the nMDS ordinations of samples revealed that environmental and biological classification methods produced roughly similar river types (Fig. 3 and Appendix G, Table G1, Figs. G2 and G3). After ignoring large watercourses (type 3), both classifications included three types of freshwater perennial rivers (types 1, 2 and 4), a type ~~mainly~~ comprised mainly of freshwater intermittent and ephemeral rivers (type 5~~)~~), and three types of saline rivers ~~of~~with increasing conductivity (types 6, 7 and 8, see Appendix G, Table G1). ~~Freshwater~~The freshwater perennial river types included headwater watercourses of very low conductivity ~~draining~~that drained mountainous siliceous catchments (type 1), mid-mountain rivers of low conductivity ~~draining~~that drained medium~~-size,~~-sized calcareous catchments (type 2) and calcareous high mountain headwaters of very low to low conductivity (type 4). Although both classifications identified a type of temporary ~~rivers~~river (type 5), the type defined by the environmental classification included a higher proportion of temporary rivers (68% of intermittent and 32% of ephemeral watercourses) than the type defined by the biological classification (28% of intermittent and 26% of ephemeral watercourses). The ~~new~~ three new saline river types~~ (~~, hyposaline~~,~~ (type 6), mesosaline (type 7) and hypersaline ~~river types) were characterised by smaller basin areas, lower elevations, softer slopes, arid climates, and greater evaporitic surface and conductivity relative to the freshwater types (Table 2 and Appendix G, Table G1). Also, more than a half of the surveyed saline rivers were intermittent or ephemeral. The~~(type 8), showed conductivity ranges that ~~define the saline types~~ were generally similar ~~in~~for both classification procedures, but some minor discrepancies were also found (Appendix G, Table G1). The hyposaline type showed a conductivity range (Q10-Q90) of 8,323-24,567 µS cm^{-1} for the environmental classification, and one of 4,953-26,158 µS cm^{-1} for the biological classification. The mesosaline type gave a conductivity range of 60,480-110,100 µS cm^{-1} for the environmental classification and one of 24,133-98,200 µS cm^{-1} for the biological classification. The hypersaline type showed a conductivity range of 140,000 - 300,000 µS cm^{-1} for the environmental classification and one of 110,100 - 300,000 µS cm^{-1} for the biological classification. All these types were characterised by smaller basin areas, lower elevations, softer slopes, arid climates and greater evaporitic surfaces and conductivity in relation to the freshwater types (Table 2 and Appendix G, Table G1). More than half the surveyed saline rivers were also intermittent or ephemeral. Classification strength based on family abundances was roughly similar between the environmental (CS=0.150±0.007) and biological (CS=0.158±0.006) classification procedures (Appendix G, Fig. G1). However, environmental classification (CS=0.203±0.008) ~~seems to be~~seemed better ~~in~~at representing species turnover among types compared to the biological classification (CS=0.170±0.005).

3.3. Biomonitoring indicators for saline river types

For hyposaline rivers (type 6) (Appendix H, Tables H1-H3), the best biological indicators of the reference conditions were the families Tabanidae, Libellulidae, Hydrometridae, Caenidae, Simuliidae, Nepidae, Gammaridae, Notonectidae and Dytiscidae, the genera *Yola*, *Laccobius* and *Enochrus*, and the species *Ochthebius delgadoi*, *Laccobius moraguesi* and *Enochrus politus*. The best indicators of the disturbed conditions for this type were the families Chironomidae, Baetidae, Corixidae, Naucoridae, Coenagrionidae, Hydrophilidae, Ceratopogonidae, Hydrobiidae, Culicidae and Aeshnidae, the genera *Berosus*, *Micronecta*, *Naucoris*, *Nepa*, *Hydroglyphus*, *Sigara* and *Notonecta* and the species *Micronecta scholtzi*, *Nepa cinerea*, *Naucoris maculatus* and *Sigara scripta*. For mesosaline rivers (type 7), the best indicators of reference condition were the families Hydraenidae and Stratiomyidae, the genera *Ochthebius* and *Nebrioporus* and the species *Nebrioporus baeticus*, *E. jesusarribasi* and *O. notabilis*. The indicators of the disturbed conditions for this type were the genus *Agabus* and the species *O. corrugatus*. For hypersaline rivers (type 8), *O. glaber* was the only indicator of the reference conditions.

For hyposaline rivers (type 6), the metrics based on the species ~~of~~at reference sites (ref_spp6, LMM r^2_m=0.30, Tukey *t*-test *p*=0.001) and the metrics based on the genera (dis_gen6, LMM r^2_m=0.23, Tukey *t*-test *p*=0.009) and species (dis_spp6, LMM r^2_m=0.19, Tukey *t*-test *p*=0.009) of the disturbed sites were the best indicators (Table 3). For mesosaline rivers (type 7), the metrics based on the families (ref_fam7, r^2_m=0.47, Tukey t-test p<0.001), genera (ref_gen7, r^2_m=0.50, Tukey t-test p<0.001) and species (ref_spp7, r^2_m=0.45, Tukey t-test p<0.001) of the reference sites were the best indicators (Table 3). Contrarily, conventional biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-metric ~~indexes~~indices (ICM-11a and IMMi-T) showed a null capacity ~~to discriminate~~for discriminating between the reference and disturbed conditions for saline river types (Table 3).

4. Discussion

4.1. Water salinity as a driver of community richness and composition at regional-continental scales

Our study shows that water salinity explains a ~~key~~large portion of the biological variation at regional and broad spatial scales. ~~Previously, elevation~~Elevation, lithology or climate have been previously used and considered ~~as~~the main factors ~~driving~~that drive richness and compositional patterns across river communities under reference conditions (e.g. Clarke et al., 2003; Poquet et al., 2009). However, our study suggests that ~~local~~reach-scale stressors, such as ~~waters~~water salinity or flow ~~intermittence~~intermittency, may play a pivotal role in shaping the structure of inland water communities in the absence of anthropogenic alterations (Diaz et al., 2008; Leigh and Datry, 2017; Suárez et al., 2017).

~~Over~~All along a particular environmental gradient, the degree to which certain levels of environmental filtering ~~could~~can be considered stressful or harmful depends on how well adapted ~~is~~ the regional pool is (Badyaev, 2005; Taylor et al., 1990). Thus~~,~~ the number of taxa ~~able to cope over~~capable of coping with each portion of the stress gradient is linked to regional and historical aspects, such as ~~the~~ long-term persistence and frequency of stressful conditions (Taylor et al., 1990) and the evolutionary context of each lineage (Buchwalter et al., 2008). The long-term persistence of the osmotic stress associated ~~to~~with Mediterranean saline rivers is expected to act as a strong driver of community assembly, but also as a source of ecological diversification in aquatic lineages. In naturally saline rivers, osmotic pressure imposes a chronic filter for organisms ~~trying~~that attempt to colonise, thrive or reproduce (Velasco et al., 2019). Regarding insects, the ~~important~~major drop in community richness at conductivities ~~>~~larger than 3,000 µS cm^{-1}~~, is~~ was strongly associated with the existence of few lineages ~~presenting~~that present specific mechanisms to maintain internal integrity once submerged under hyperosmotic media (Arribas et al., 2019; Bradley, 2008; Millán et al., 2011). These are mostly the taxa ~~belonging~~which belong to the families Hydrophilidae, Dytiscidae~~,~~ and Hydraenidae (Coleoptera), Corixidae (Hemiptera), and Culicidae, Ephydridae, Stratiomyidae, Chironomidae (Diptera) (Arribas et al., 2019; Bradley, 2008; Pallarés et al., 2017a)~~,~~, and they all ~~of them~~ ~~comprising~~comprise good biological indicators of the reference saline streams. Thus~~,~~ our results reveal a clear differentiation in community composition, and~~,~~ a particularly~~, a~~ strong replacement of taxa along the conductivity gradient, which also ~~concordant~~coincides with previous studies ~~on~~about this natural stress gradient (Gascón et al., 2016; Gutiérrez-Cánovas et al., 2013)~~ and on the~~, and with high levels of habitat specificity associated ~~to the~~with osmotic stress (Carbonell et al., 2012).

4.2. Integrating saline types into river classifications

The main advantage of our approach is ~~the integration of~~that it integrates the whole spectrum of environmental and biological variation into a single comprehensive classification, which is either environmentally or biologically based, ~~that~~and allows a more accurate and simple classification of rivers in the Mediterranean region. This new integrated typology could help to better implement WFD ~~in the state members, whose legal criteria ignore or misclassify~~into Member States and to gather a comprehensive inventory of saline rivers~~.~~, which are currently ignored or misclassified by current laws. For example, the ~~Spanish~~ official Spanish typology of rivers recognises three types of highly mineralised rivers (official types 7, 9 and 13). However, the mean conductivities of ~~this~~these official types range is 448-545 µS cm^{-1} (Spanish Government, 2009)~~,~~, which is significantly lower than the conductivities ~~showed~~shown by the saline rivers studied ~~here. Also,~~herein. Moreover for the first time, our classifications implicitly ~~recognises~~recognise the importance of considering the whole natural osmotic stress gradient~~,~~ by providing a classification method that encompasses more biodiversity ~~that the~~than previous individual attempts (e.g. Arribas et al., 2009; Sánchez-Montoya et al., 2007).

The definition of the three saline river types was relatively consistent for both environmental and biological classifications. Thus~~,~~ the prediction of their biological communities was fairly accurate, and was based ~~just~~ on only one single sample or the mean ~~or sampled~~of several samples of conductivity (Moreno et al., 1997), ~~as occurred~~similarly to which occurs in lentic systems (Gascón et al., 2016; Pinder et al., 2005; Williams, 1998). In fact~~,~~ our models identified how seasonal or hydrological variation had almost no effect ~~in~~on the biological communities occurring ~~at~~in highly mineralised rivers (>3,000 µS cm^{-1}). This is not surprising because saline specialists may show co-tolerance to other drought-related stressors such as desiccation (Pallarés et al., 2017b).

Taken together, these results advocate the use of river saline types that included both perennial and intermittent rivers. However, future work using more precise hydrological data should check the consistency of these saline river types along flow intermittency gradients. Quantitative hydrological data offer a much more adequate indication of the drought stress that affects biological communities (Belmar et al., 2013; Gallart et al., 2016; Jaeger and Olden, 2012). Unfortunately, these data are currently unavailable for most studied rivers, so simple categorical descriptors of the hydrological regime were used instead.

Our findings might also be useful to biomonitoring naturally saline rivers in other regions outside the study area, such as Australia (Biggs et al., 2013), North and South America (Griffith, 2014; Orfeo, 1999), North Africa (Hamed and Dhahri, 2013) and Russia (Zinchenko et al., 2014). Specifically, although biogeographical differences may lead to very different taxonomic compositions, ecological responses to salinity might be similar and roughly close river types might be yielded depending on the available salinity gradient.

4.3. Metrics to evaluate anthropogenic impacts on saline rivers

Generally, ~~the~~ UE ~~member states~~Member States are implementing WFD ~~through the classification of the~~by classifying water bodies and then ~~the development of~~developing appropriate biological indicators to evaluate their ecological status, rather than using model~~-~~-based methods (Birk et al., 2012). For these pragmatic reasons, we developed specific indicators for the obtained saline river types~~ obtained~~. Our results showed that the metrics based on ~~the~~taxon abundance~~ of taxa indicating~~, which either indicate reference or degraded conditions, were able to detect anthropogenic impacts on naturally saline rivers, while the metrics commonly used in freshwater rivers did not respond at all. ~~While~~Whereas conventional biomonitoring metrics, such as family or EPT richness, are good indicators of~~ the~~ ecosystem quality in freshwater rivers (Bonada et al., 2006),~~ the~~ intense abiotic filtering at naturally stressed rivers acts as a confounding factor for theses metrics. This fact ~~causes~~indicates that diversity-based indicators are inappropriate ~~to evaluate~~for evaluating saline watercourses, ~~being~~and are also potentially inaccurate for other naturally stressed systems (Cañedo-Argüelles et al., 2012; Elliott and Quintino, 2007). Previous studies have also demonstrated that conventional biomonitoring metrics ~~showed~~show substantial limitations ~~to evaluate~~when evaluating the ecological quality of naturally stressed ecosystems, such as intermittent rivers (Bruno et al., 2016; Wilding et al., 2018) or estuaries (Elliott and Quintino, 2007).

The abundance of specialist taxa seems to provide a much better indication of reference and degraded conditions than diversity-based metrics (Cañedo-Argüelles et al., 2012)~~.~~. These metrics can ~~be~~ also be used to monitor their populations, which are scattered across the territory and threatened by human pressures (Arribas et al., 2015). Nonetheless, we admit that our proposed metrics ~~are~~is a first attempt to effectively showcase the type of biomonitoring tools that would work in saline rivers~~.~~, so they should be cautiously taken. Therefore, they may ~~require~~benefit from further ~~refinement~~refinements by gathering larger datasets of observational data ~~and~~ combined with manipulative experiments, which both ~~covering~~cover different types of impacts (e.g. dilution, nutrient enrichment). Furthermore, some of the indicator species for the saline types defined herein (e.g. *Ochthebius glaber*, *Nebrioporus baeticus*) are endemic of the Iberian Peninsula. Fortunately, these taxa have sister species with very similar ecological requirements in other biogeographic regions (Arribas et al., 2015), and could be used as effective indicators of reference or disturbed conditions.

In some saline rivers, agriculture is diluting salt concentrations, ~~posing~~which poses a risk ~~to~~for their typical communities~~, which~~ that are confined to ~~such~~these peculiar environments (Carbonell et al., 2012; Gutiérrez-Cánovas et al., 2013; Pallarés et al., 2017a)~~, which~~, and leads to taxonomic homogenisation and loss of regional biodiversity~~ loss~~. Similarly~~,~~ in other naturally stressed systems, such as glacier-fed and alpine rivers, climate change is reducing the number of ~~endemism~~the endemic and specialist taxa~~, which~~ that typically inhabit those systems~~,~~ through ~~increases in~~increasing temperature and turbidity (Finn et al., 2013; Jacobsen et al., 2012). Consequently, we highlight the urgent need ~~of monitoring~~to catalogue and monitor naturally stressed rivers~~,~~ which, despite harbouring ~~a~~ reduced local diversity, contribute ~~genuinely~~substantially to regional and global biodiversity through their unique communities of stress~~-~~tolerant species (Finn et al., 2013, Millán et al., 2011).

5. Conclusions

Our study provides a better understanding of the environmental drivers that explain macroinvertebrate richness and composition along the broad heterogeneity exhibited by Mediterranean rivers, ~~emphasising~~and emphasises the role of natural stressors~~, such as~~ like water salinity. We also deliver classification approaches that encompass freshwater perennial and intermittent rivers along with three saline river types for the first time. Finally, we demonstrate that the conventional biomonitoring metrics and ~~indexes~~indices developed for freshwater rivers failed ~~in detecting~~to detect anthropogenic impacts on saline rivers~~ and so~~. So we provide new specific metrics based on the abundances of indicator taxa for these rivers ~~showing~~, which show better responses to degradation. Taken together, these new insights can improve ~~the~~our understanding of ~~the~~ ecological responses to natural and anthropogenic stressors

~~and~~, to foster the development of biomonitoring metrics for naturally saline rivers, ~~helping~~and to help preserve their unique biodiversity.

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Table 1. Dataset description including code used, disturbance level (reference or disturbed sites), taxa covered and their taxonomic resolution, region covered, environmental gradient encompassed, number of sites (Sites), number of observations (Obs.) and the paper objectives where each dataset was used. * This dataset was split to ~~have~~obtain an independent dataset to evaluate ~~the~~ classification performance (see the Materials and ~~Methods~~methods section for more details).

Code	Disturbance level	Taxa	Taxonomic resolution	Region	Environmental gradient	Sites	Obs.	Community models	Classification development	Classification evaluation	Biomonitoring metrics
ref_fam_ita	Reference	All	Family	Italy	All	18	44	x		x	
ref_fam_mor	Reference	All	Family	Morocco	All	28	28	x		x	
ref_fam_spa	Reference	All	Family	Spain	All	139	386	x	x	x*	
ref_spp_ita	Reference	Coleoptera	Species	Italy	All	19	31	x		x	
ref_spp_mor	Reference	Coleoptera	Species	Morocco	All	29	29	x		x	
ref_spp_spa	Reference	Coleoptera	Species	Spain	All	64	151	x		x	
ref_fam_all	Reference	All	Family	All	All	157	157	x			
ref_spp_all	Reference	Coleoptera	Species	All	All	76	76	x			
ref_fam_spa_sal	Reference	All	Family	Spain	Saline rivers	35	89				x
ref_spp_spa_sal	Reference	Coleoptera	Genus, Species	Spain	Saline rivers	30	75				x

dis_fam_spa_sal	Disturbed	All	Family	Spain	Saline rivers	17	31	x
dis_spp_spa_sal	Disturbed	Coleoptera	Genus, Species	Spain	Saline rivers	16	30	x
Overall						243	577	

Table 2. Description of the seven river types proposed for the environmental classification. Types 1 to 5 ~~were~~are defined in ECOSTAT (European Commission, 2007), and modified as specified in Appendix S2, whereas types 6, 7 and 8 ~~were~~are defined according to the conductivity thresholds used to classify the saline rivers reported in Arribas et al. (2009) and Millán et al. (2011).

Type	**Description**	**Basin area (km^2)**	**Altitude (m)**	**Lithology**	**Hydrology**	**Mean conductivity ($\mu S\ cm^{-1}$)**
1	Small high-mid altitude rivers	<1,000	200-2,000	≥ 40% siliceous	Perennial flow	< 200
2	Small / medium lowland rivers	<1,000	<600	Mixed	Perennial flow	<5,000
3	Large lowland rivers	≥1,000-10,000	<600	Mixed	Perennial flow	<5,000
4	Small / medium mountain rivers	<1,000	600-1,500	≥40% calcareous	Perennial flow	≥ 200-5,000
5	Small, lowland, temporary rivers	<1,000		Mixed	Intermittent or ephemeral flow	<5,000
6	Small medium-lowland hyposaline rivers	<1,000		Calcareous and evaporitic	Perennial, intermittent or ephemeral flow	5,000-32,000
7	Small medium-lowland mesosaline	<1,000		Calcareous and evaporitic	Perennial, intermittent or	32,000-130,000

	rivers			ephemeral flow	
8	Small medium-lowland hypersaline rivers	<1,000	Calcareous and evaporitic	Perennial, intermittent or ephemeral flow	>130,000

Table 3. Results of the models ~~evaluating~~that evaluated the differences in the biomonitoring metrics between the reference and disturbed saline rivers for types 6 and 7. Explained variance and significance are shown. r^2_m *accounts* for the variance explained by the fixed factors. Metric names - ref: reference condition, dis: disturbed condition; fam: family level, gen: genera level, spp: species level; 6 and 7 refer to the river type where the ~~metric~~metrics should be applied. ~~Metrics~~The metrics showing significant differences are in bold.

		Type 6 (hyposaline)		***Type 7 (mesosaline)***	
	Metrics	r^2_m	***P*-value**	r^2_m	***P*-value**
Widely-used metrics	IBMWP	0.01	0.669	0.00	0.821
	Family richness	0.00	0.792	0.00	0.799
	EPT	0.00	0.991	-	-
	IASPT	0.03	0.300	0.00	0.844
	ICM11a	0.01	0.637	0.01	0.586
	IMMiT	0.01	0.699	0.01	0.622
Novel metrics	ref_fam6	0.00	0.984	0.07	0.141
	dis_fam6	0.10	0.062	0.05	0.237
	ref_gen6	0.03	0.400	0.12	0.074
	dis_gen6	**0.23**	**0.009**	0.10	0.094
	ref_spp6	**0.30**	**0.001**	0.03	0.418
	dis_spp6	**0.19**	**0.013**	0.01	0.543
	ref_fam7	0.02	0.423	**0.47**	**0.000**
	ref_gen7	0.06	0.248	**0.50**	**0.000**
	ref_spp7	0.03	0.419	**0.45**	**0.000**

Figure captions

Fig. 1. Variable importance for the models explaining family richness (a), species richness (b), overall community composition (c) and species turnover (d).

Fig. 2. Plots showing the family richness response to conductivity and hydrology (a), hydrology (b), conductivity and season (c) and conductivity and region (d). per: perennial seasonal flow (square), int: intermittent flow (triangle), eph: ephemeral flow (cross); spn: spring; sum: summer; aut: autumn, win: winter; Spa: Spain, Ita: Italy, Mor: Morocco.

Fig. 3. Multidimensional scaling plots showing the concordance between the ordination of the biological communities based on family abundances, and the environmental (a) and biological (b) classifications of the Spanish reference samples. Numbers represent the different river types (see Results and Table 2 for more information).

Fig. 1

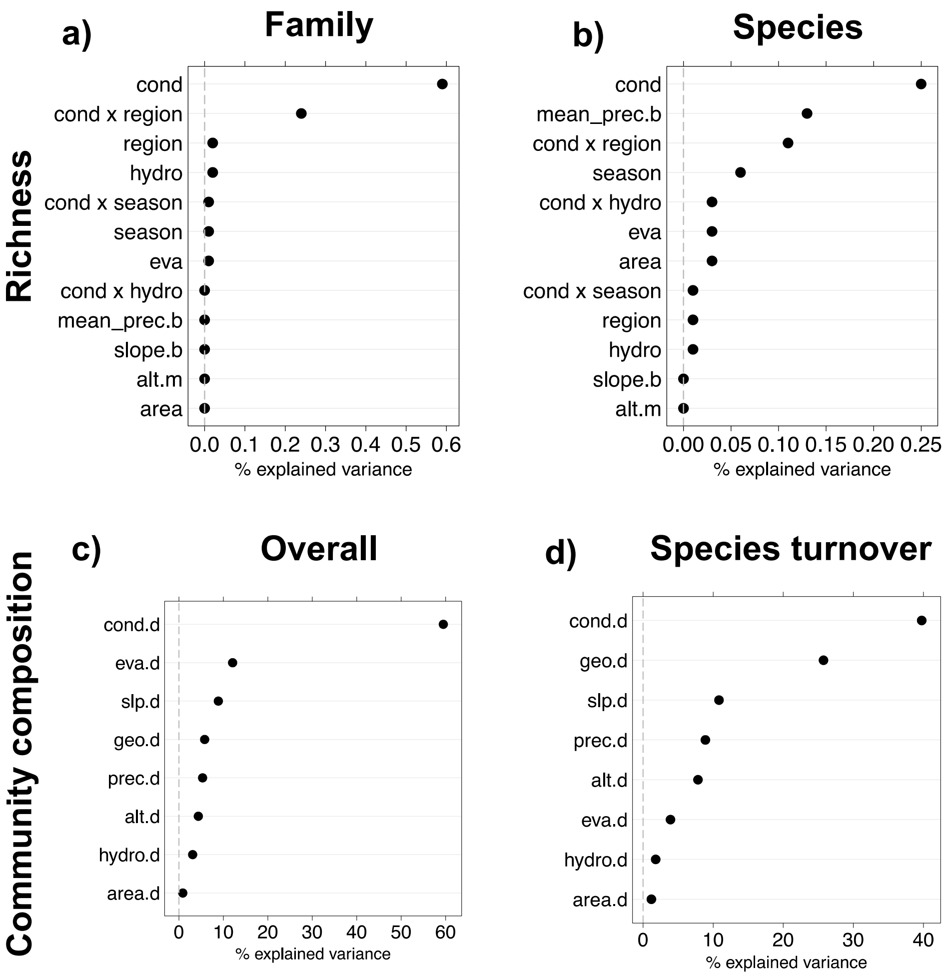
a)
Family
cond
cond x region
region
hydro
cond x season
season
eva
cond x hydro
mean_prec.b
slope.b
alt.m
area
0.0 0.1 0.2 0.3 0.4 0.5 0.6
% explained variance
b)
Species
cond
mean_prec.b
cond x region
season
cond x hydro
eva
area
cond x season
region
hydro
slope.b
alt.m
0.00 0.05 0.10 0.15 0.20 0.25
% explained variance
Richness
c)
Overall
cond.d
eva.d
slp.d
geo.d
prec.d
alt.d
hydro.d
area.d
0 10 20 30 40 50 60
% explained variance
d)
Species turnover
cond.d
geo.d
slp.d
prec.d
alt.d
eva.d
hydro.d
area.d
0 10 20 30 40
% explained variance
Community composition

Fig. 2.

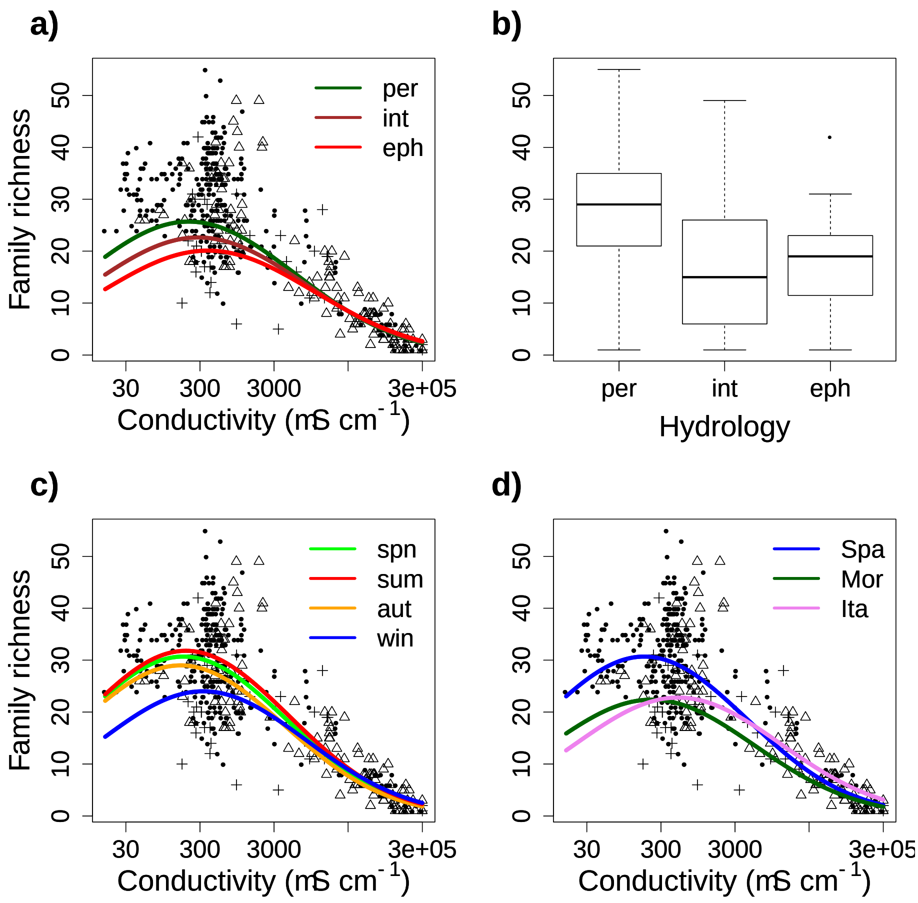

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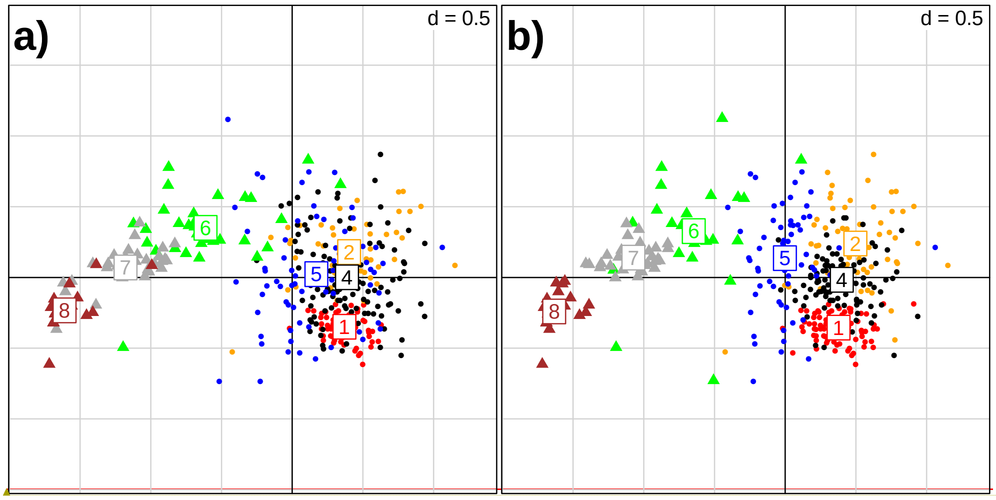
Fig. 3



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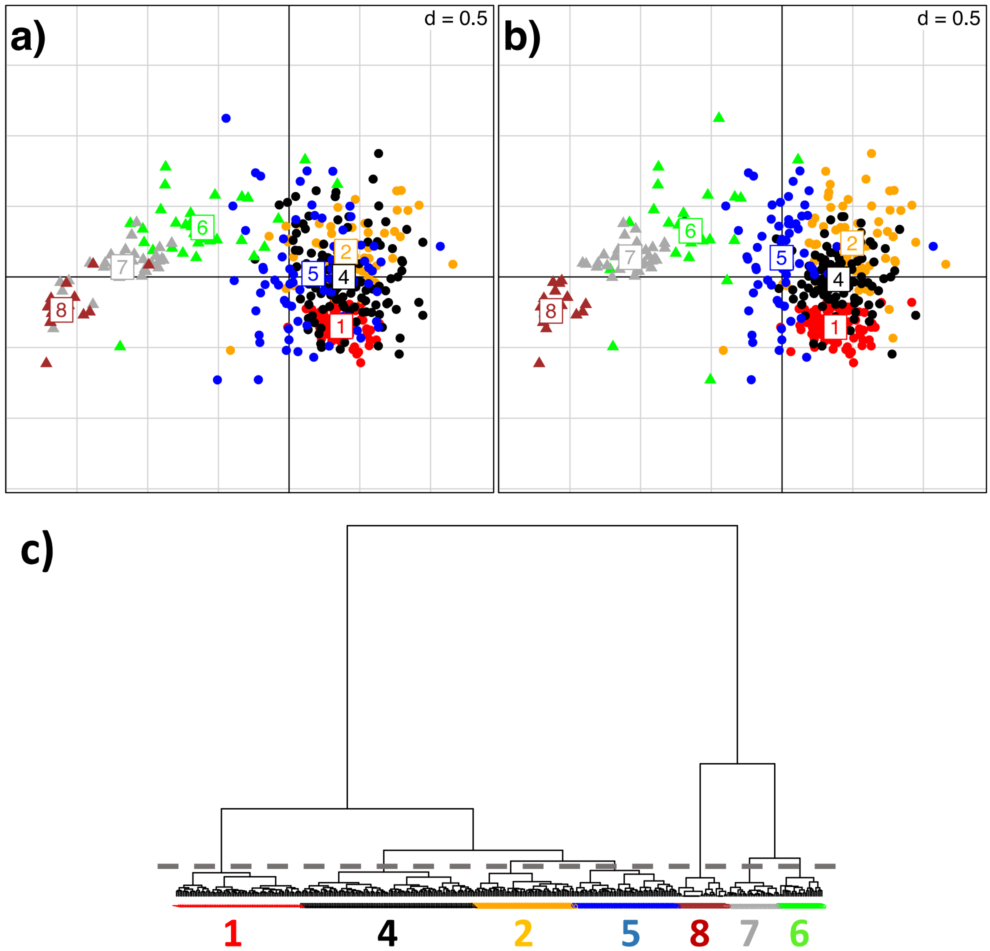

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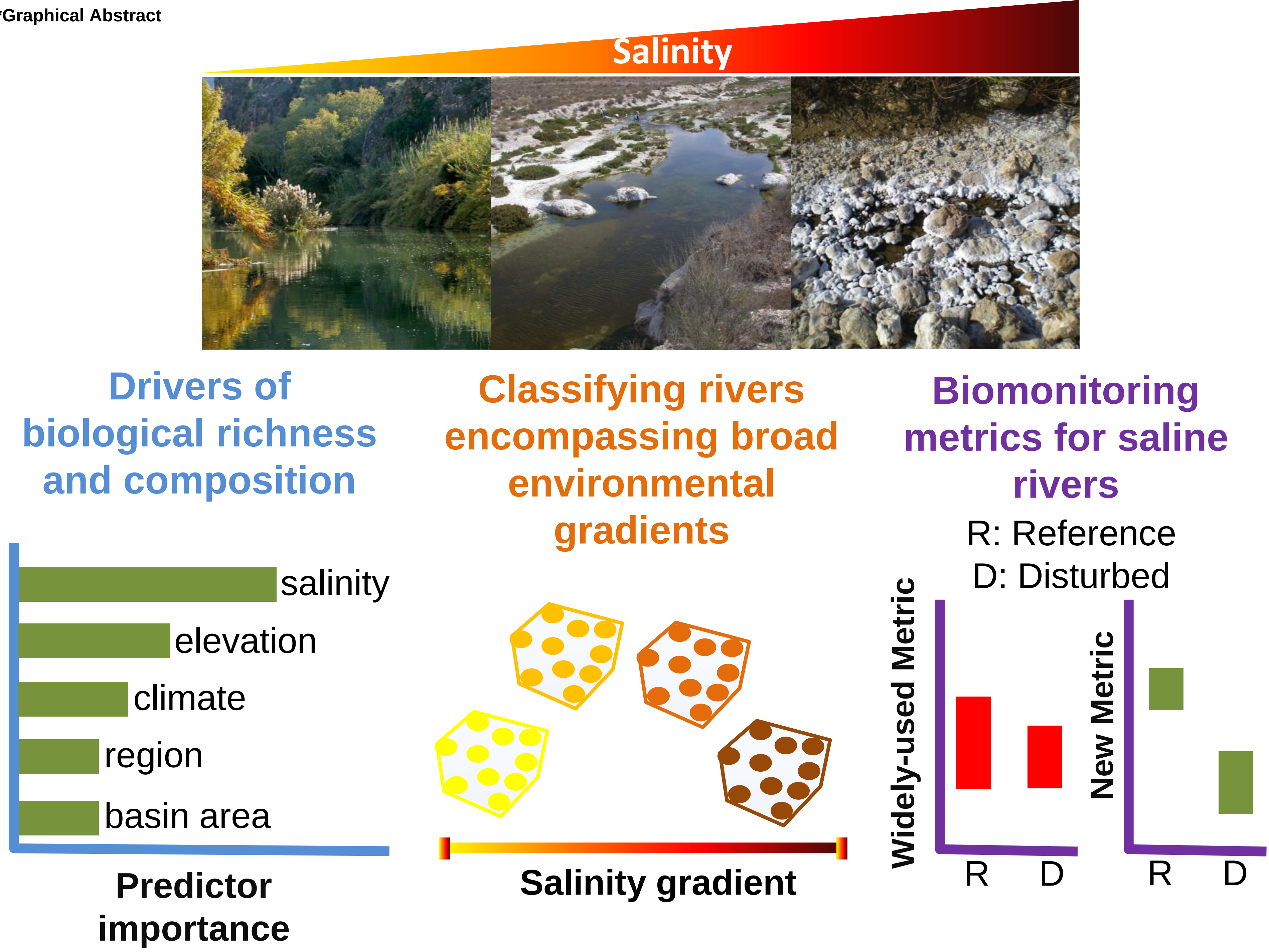
*Graphical Abstract



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

Highlights

- We examined the role of natural salinity in rivers to improve their classification and biomonitoring
- Salinity had a key role in explaining community richness and composition of rivers
- We found three new types of saline rivers that showed unique communities
- Our proposed metrics detected anthropogenic impacts better than widely used indexes
- We highlight the necessity of including saline rivers into biomonitoring programs

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

Highlights

- Naturally stressed ecosystems are genuine but unprotected by biomonitoring programs
- Salinity had a key role in explaining community richness and composition of rivers
- We found three new types of saline rivers that showed unique communities
- Our proposed metrics detected anthropogenic impacts better than widely used indexes
- We showed the necessity of including saline rivers into biomonitoring programs

Abstract

Naturally stressed ecosystems hold a unique fraction of biodiversity. However, they have been largely ignored in biomonitoring and conservation programs, such as the EU Water Framework Directive, while global change pressures are threatening their singular values. Here we present a framework to classify and evaluate the ecological quality of naturally stressed rivers along a water salinity gradient. We gathered datasets, including aquatic macroinvertebrate assemblages and environmental information, for 243 river locations across the western Mediterranean to: a) gauge the role of natural stressors (salinity) in driving aquatic community richness and composition; b) make river classifications by encompassing the wide range of environmental and biological variation exhibited by Mediterranean rivers; c) provide effective biomonitoring metrics of ecological quality for saline rivers. Our results showed that water salinity played a pivotal role in explaining the community richness and compositional changes in rivers, even when considering other key and commonly used descriptors, such as elevation, climate or lithology. Both environmental and biologically-based classifications included seven river types: three types of freshwater perennial rivers, one freshwater intermittent river type and three new saline river types. These new saline types were not included in previous classifications. Their validation by independent datasets showed that the saline and freshwater river types represented differentiable macroinvertebrate assemblages at family and species levels. Biomonitoring metrics based on the abundance of indicator taxa of each saline river type provided a much better assessment of the ecological quality of saline rivers than other widely used biological metrics and indices. Here we demonstrate that considering natural stressors, such as water salinity, is essential to design effective and accurate biomonitoring programmes for rivers and to preserve their unique biodiversity.

Keywords: abiotic stress, macroinvertebrates, biomonitoring, global change, saline rivers, Water Framework Directive

1. Introduction

Naturally stressed ecosystems are characterised by harbouring a set of natural conditions that are persistently unsuitable for the vast majority of the regional species pool (Badyaev, 2005; Parsons, 2005). Well-known examples of naturally stressed ecosystems are found in tundra, deserts, volcanic springs, and in glacier, acid or saline inland waters (Cauvy-Fraunié et al., 2016; Elliott and Quintino, 2007; Millán et al., 2011; Petrin et al., 2007). Their natural stressful conditions reduce the local diversity of these systems, which is also a powerful driver of diversification (Parsons, 2005; Vetaas and Grytnes, 2002). So they usually hold unique fractions of biodiversity characterised by high levels of specialisation and species turnover (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013; Jacobsen et al., 2012).

Unfortunately, these singular spots are threatened by global change pressures, which may modify the naturally stressed conditions causing habitat loss, reduce the community's singularity (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013) or add new stressors, such as pesticides or microplastics (Beketov et al., 2013; Windsor et al., 2019). This situation has been strongly aggravated because many of these naturally stressed ecosystems have been usually ignored when cataloguing natural heterogeneity, and have consequently been systematically excluded for biomonitoring and conservation purposes (Millán et al., 2011; Stubbington et al., 2018).

This is particularly true for the case of naturally saline rivers (Millán et al., 2011), which have been mostly neglected by large international efforts made to reverse globalised river degradation, such as the EU Water Framework Directive (WFD, Directive 2000/60/EC) or the Australian or US river biomonitoring programmes (Nichols et al., 2017). These conservation frames are based fundamentally on a two-step process that focuses on identifying the main river types over a particular region (river classification), and the subsequent development and harmonisation of biomonitoring metrics to allow the ecological status of different river types to be assessed (Buffagni et al., 2007). However, some generalised shortcomings come into when applying these procedures that result in a deficient functionality for the assessment and protection of naturally stressed rivers. Firstly, main natural stressors, such as water salinity, are usually neglected or not completely considered in river classifications, and this results in the exclusion or misclassification of naturally stressed rivers (Sánchez-Montoya et al., 2007). This occurs because the classification process tends to focus on coarse environmental descriptors, which are weak proxies of reach-scale stressors. Secondly, most proposed biomonitoring metrics can result in equivocal evaluations of the ecological status of naturally stressed rivers because they are closely associated with local richness/diversity and do not consider naturally depauperate communities (Gutiérrez-Cánovas et al., 2008). Besides, other widely used metrics are based on taxa or functional traits that are rare, or not even present, in naturally stressed rivers; e.g., the stress-sensitive orders Ephemeroptera, Plecoptera and Trichoptera (EPT) (Belmar et al., 2013; Bonada et al., 2006; Millán et al., 2011). Hence there is an urgent need to revisit and adapt the current vision and approaches to assess the ecological quality of naturally stressed ecosystems, and to improve their management and future preservation.

Here we benefit from a large compilation of datasets from rivers across the western Mediterranean and Moroccan Atlantic basins, which include almost the whole natural salinity gradient (roughly, 30 to 300,000 µS cm^{-1}). On this comprehensive basis, we develop an integrated framework that assesses the ecological quality of naturally stressed rivers. We firstly rank the importance of natural stress (salinity) and other general descriptors commonly used for river classifications (e.g. elevation, river size, lithology, hydrology) in determining community richness and composition. We secondly classify rivers according to their environmental characteristics and biological composition by considering specific types for saline rivers, and assessing their performance and concordance. Finally, we identify indicator taxa and metrics for naturally stressed rivers under reference and anthropogenic disturbed conditions by testing their performance against other widely used river biomonitoring metrics and indices.

2. Material and methods

2.1. Description of datasets

The study was conducted across the western Mediterranean basin, including the watercourses from the eastern and southern Iberian Peninsula and the Balearic Islands (Spanish data), Sicily (Italian data) and from the Rif down to the Sahara Desert comprising the Rif and Moroccan Atlantic basins (Moroccan data) (Appendix A, Fig. A1). These regions were selected because they cover wide environmental variability, including large gradients of elevation, climate, hydrology, lithology, salinity and anthropogenic impacts (Appendix A, Fig. A1 and Table A1). In the study area, salinity tends to increase in rivers that drain basins with an arid climate and a soluble lithology (Millán et al., 2011). These rivers reach the highest mineralisation levels in areas dominated by evaporitic outcrops.

We used different subsets of environmental and aquatic macroinvertebrate assemblage data to address the study objectives (Table 1). The description of each dataset includes anthropogenic disturbance levels (reference or disturbed sites), the macroinvertebrate groups used (all major orders or just aquatic Coleoptera) and their taxonomic resolution (family, genus, species levels), region (Italy, Morocco, Spain, all), the encompassed environmental gradient (all gradients or just saline rivers), the number of sampling sites, and the observations and objectives for which each dataset was used. To compile this database of 243 river locations and 577 samples, we combined our own data with a large dataset from the Guadalmed Project (Prat, 2002). All the macroinvertebrate samples were collected following a multi-habitat semi-quantitative kick-sample (Jáimez-Cuéllar et al., 2002). See Appendix A for more details about the datasets and Appendix B for an extended description of the sampling procedure.

Climate, geomorphologic, lithologic and land use variables at basin and reach scales were obtained from digital layers after delineating the river basins of each sampling site (see Appendix A, Table A1 for a complete list of the used variables). Water electrical conductivity was measured *in situ* on each sampling occasion as an osmotic stress indicator. To characterise flow intermittence, we categorised the hydrological regime of each site as perennial seasonal (typically flowing), intermittent (surface flow ceases during the dry season, pools remaining) or ephemeral (completely dry during one season) flows from available hydrological information or field evidence (Belmar et al., 2013; Sánchez-Montoya et al., 2007). We categorised the sampling sites as *reference* when they were minimally disturbed (i.e. fulfilling ≥ 16 out of 20 of the Mediterranean Reference Criteria, MRC; Sánchez-Montoya et al., 2009) or disturbed when they were substantially impacted by anthropogenic activities (i.e. fulfilled < 16 MRC). In this study, we excluded large watercourses (mean basin area ≥1,000 km^2, ECOSTAT, type 3, see Table 1 and Sánchez-Montoya et al., 2007 for details) because of the paucity of reference sites (Sánchez-Montoya et al., 2009). We also excluded disturbed freshwater rivers, for which effective biomonitoring metrics can be found elsewhere (e.g. Birk et al., 2012; Bonada et al., 2006).

2.2. Data analysis

Before performing the analyses, we applied a log-transformation to macroinvertebrate family richness and a square-root transformation to macroinvertebrate species richness. Logit-, log- or square-root-transformations were applied to the quantitative environmental variables to reduce their distribution skewness and to improve linearity, whenever necessary. All the quantitative environmental variables were also standardised to mean=0 and SD=1 to facilitate model coefficient comparisons.

2.2.1. Ranking environmental variable importance

To identify the main environmental factors that determine family and species richness, we used Random Forest (randomForestSRC R package, Ishwaran Ishwaran et al., 2014) and Linear Mixed-effect Models (LMM, lme4 R packge, Bates et al., 2015). In these models, we utilised family-level datasets (i.e. ref_fam_ita, ref_fam_mor, ref_fam_spa, n=458) and species-level datasets (i.e. ref_spp_ita, ref_spp_mor, ref_spp_spa, n=211) from the reference sites (selected according to MRC; see above). Following Feld et al. (2016), we first ran Random Forest models to identify the most important predictors of family and species richness among the 24 potential candidates (Appendix A, Table A1) to be included in the LMM (see Appendix C for more details about exploratory analyses). After these exploratory analyses, we included basin area, mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence, conductivity (single and quadratic terms), season and region as fixed factors in the LMM. As additional fixed factors, we also included the pair-wise interactions of conductivity x flow intermittence, conductivity x season and conductivity x region. Site code was considered a random factor to account for repeated measures in the same location. To rank the environmental predictor's importance on family and species richness, we adopted a multi-model inference approach (Grueber et al., 2011) using the *MuMIM* R package (Bartoń, 2016). This statistical technique ranks all the generated models using all the possible combinations of predictors based on Akaike's Information Criterion (AIC). Then a set of top models was selected to produce an average model, but only if the model ranking first was ambiguously supported (model weight<0.90). We chose top models which differed in no more than two AIC units (delta≤2) from the model ranked first (minimum AIC). We adopted a natural average method to conduct model averaging, which consists in averaging predictors only over the models in which the predictor appears and in weighting predictor's coefficients by the summed weights of these models (Burnham and Anderson, 2002). For each LMM model, two goodness-of-fit measures were estimated (Nakagawa and Schielzeth, 2013): marginal goodness-of-fit (r^2_m) indicates the variance explained only by the fixed factors, while conditional goodness-of-fit (r^2_c) shows the variance accounted for by both fixed and random terms. We provide the mean (based on model weights) of each goodness-of-fit measure for every averaged model. All the models met the normality and homoscedasticity assumptions, which were validated by visually checking their residuals.

To identify the environmental drivers of community composition change, we used Multiple Regression models for distance Matrices (MRM; ecodist R package, Lichstein, 2007). This method is conceptually similar to traditional multiple regression, but with all variables being distance matrices instead of raw data and *P*-values being calculated by permutation tests (1,000 runs). To avoid lack of independence problems due to multiple samples belonging to the same site, we selected a reference subset of macroinvertebrate families (ref_fam_all, n=157 sites/samples) and species (ref_spp_all, n=76 sites/samples) occurrences with only one spring sample per site. We estimated the overall changes in community composition for each pair of sites of the family matrix with the Sørensen dissimilarity index (β_{sor}) and pair-wise dissimilarity due to turnover from the species matrix with the Simpson index (β_{sim}). These calculations were made following Baselga's (2010) framework for β-diversity partitioning using the *betapart* R package (Baselga and Orme, 2012). For each selected environmental predictor (basin area, mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence, conductivity, geographic distance), we built a Euclidean distance matrix based on their transformed and standardised values. The geographical distance between localities was based on a latitude and longitude original matrix, while flow intermittence was based on semi-quantitative values (perennial=0, intermittent=1, ephemeral=2).

Finally, we also performed variance partitioning for the community richness and composition models using the *variancePartition* (Hoffman and Schadt, 2016) and *hierpart* (Walsh and MacNally, 2013) R packages.

2.2.2. Integrating saline rivers into biomonitoring typologies

To classify rivers by encompassing the environmental and biological variability that occurs in the study area, we used family abundances from reference Spanish sites (ref_fam_spa, n=386). We selected this dataset because it included more sites and samples, and it covered a broader environmental and biological spectrum in relation to the Italian and Moroccan datasets (Appendix A, Table A1). We firstly classified sites into seven types according to their environmental variables (environmental classification) following an adaptation of the criteria suggested by the ECOSTAT intercalibration group for Mediterranean rivers using System A of the WFD (MedGIG European Commission, 2007). This included mean conductivity, basin area, hydrology, site altitude and basin lithology (Table 2 and Appendix D). Secondly, in order to classify samples according to their biological communities (biological classification), we estimated a Bray-Curtis pair-wise dissimilarity matrix, which derived from the abundance family matrix and produced a dendrogram based on the Bray-Curtis family dissimilarity matrix following Ward's clustering method. After making a visual inspection, we decided to prune the tree to produce seven biological types. For both classifications, we used the same previously utilised type numbers (European Commission, 2007; Sánchez-Montoya et al., 2007) for freshwater rivers and numbers 6, 7 and 8 for the new saline river types. Type 3 (large rivers) was not used because we excluded this river type from our analysis. We also performed non-Metric Multidimensional Scaling (nMDS) ordination based on the Bray-Curtis family dissimilarity matrix to explore the concordance between the environmental and biological classifications.

To evaluate the performance of both classification procedures in a broader geographical context, we estimated their classification strength based on the family and species datasets from reference sites in Italy (ref_fam_ita, n=44; ref_spp_ita, n=31), Morocco (ref_fam_mor, n=28; ref_spp_mor, n=29) and Spain (ref_fam_spa, n=386; ref_spp_spa, n=151). We assigned environmental types to these new sites using the environmental classification criteria (Table 2). To assign biological types to the new sites, we built a Random Forest model by predicting biological types from environmental information. To develop the Random Forest model (trees=2000, mtry=8), we used a subset of the ref_fam_spa dataset (n=258), while the non-utilised samples (n=128) were employed along with the other independent subsets to evaluate classification performance. To estimate classification strength (CS), dissimilarity matrices were converted into similarity matrices. CS was quantified as the difference between the within-type mean similarity (W) and between-type mean similarity (B) of the Bray-Curtis pair-wise similarity based on family abundances, and for the Simpson similarity matrix based on species occurrences and turnover (CS=W-B) for the three regions. The mean CS values were calculated through a bootstrapping procedure, where we resampled 100 subsets of n=28 from each Italian and Spanish macroinvertebrate dataset to make their CS values comparable to those obtained for the Moroccan datasets, which had the fewest observations.

2.2.3. Biomonitoring indicators for saline river types

To develop metrics that indicate the ecological quality of saline river types and test their performance against widely used biomonitoring metrics, we resorted to a dataset that included the family and species-level data from Spanish reference (ref_fam_spa_sal, n=89; ref_spp_spa_sal, n=75) and disturbed (dis_fam_spa_sal, n=31; dis_spp_spa_sal, n=30) sites. For all these samples, we firstly assigned river types using the environmental classification thanks to its simplicity and potentially better performance compared to the biological classification (see the Results). By considering that most disturbed sites had been affected by a drop in their natural conductivity levels as a result of freshwater inputs from agricultural drainage (Velasco et al., 2006), we compiled historical predisturbed conductivity information (Moreno et al., 1997; Vidal-Abarca, 1985) to correctly assign their river types. As a result, we obtained hyposaline, mesosaline and hypersaline river types according to the reference condition (ref_6, ref_7 and ref_8, respectively), as well as hyposaline and mesosaline river types according to the disturbed condition (dis_6, dis_7). For Type 8, we did not find any disturbed site.

To identify the families, genera and species with a higher affinity to each reference and disturbed type, we ran an indicator species analysis (IndVal, Dufrêne and Legendre, 1997). This analysis considers each taxon's percentage of occurrence and relative abundance for each type to obtain an indicator value (IV) and its significance through Monte-Carlo permutations (1000 runs). We focused on the most frequent taxa by keeping taxa occurring in more than 10% of observations. From them, we selected those taxa which showed a significant Indicator Value ($P \leq 0.05$) as potential indicators for a given type. From these results, we built the candidate metrics of reference and disturbed conditions of the saline river types. To create these metrics, for each sample we summed the abundances of the indicator taxa of the assigned river type (ref_6, dis_6, ref_7, dis_7, ref_8) for the family, genus and species level (e.g. for the family level: ref_fam6, dis_fam6, ref_fam7, dis_fam7, ref_fam8). In addition for each sample, we estimated a set of widely-used biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-metric indices (ICM-11a and IMMi-T) for Mediterranean rivers (Alba-Tercedor et al., 2002; Munné and Prat, 2009). Finally, to evaluate the performance of these candidate metrics against the widely-used biomonitoring metrics, we used LMM models to assess the differences across all the reference and disturbed types (levels= ref_6, dis_6, ref_7, dis_7, ref_8) and Tukey-*t post hoc* tests to evaluate differences between pairs of comparable reference and disturbed types (i.e. ref_6 vs. dis_6, ref_7 vs. dis_7).

The code and functions used to run all these analyses are available in Appendix E, which were conducted using the R version 3.4.1 (R Core Team, 2016).

3. Results

3.1. Ranking environmental variable importance

Electrical conductivity was the most important variable to explain macroinvertebrate assemblage richness and composition (Fig. 1a,c, and Appendix F, Table F1). Family richness was explained primarily by conductivity (59%) and the interaction between conductivity and region (25%), which suggests that conductivity had different regional effects (r^2_m=0.82). Generally with conductivity values above 3,000 µS cm^{-1}, family richness responded only to conductivity changes. Family richness peaked at conductivities within the 300-1,000 µS cm^{-1} range, before declining progressively as conductivity increased (Fig. 2a,c,d). Within this conductivity range, the family richness values showed the greatest dispersion, which indicates that other variables also influenced family richness. The interactive effects of conductivity with hydrology (Fig. 2a), season (Fig. 2c) and region (Fig. 2d) were evident only at freshwaters, and particularly within the conductivity range of 300-1,000 µS cm^{-1}, before becoming much weaker at conductivities over 3,000 µS cm^{-1}. The rivers with a perennial flow tended to have a higher level of family richness than intermittent or ephemeral rivers (Fig. 2a,b), but as conductivity increased, the effect of hydrology also became less important. Species richness showed roughly similar patterns in response to environmental variables where, once again, conductivity was the most important predictor, but with higher contributions of mean basin precipitation and seasonality (Fig. 1b and Appendix F, Table F1).

Conductivity distance was also the most important variable to explain dissimilarity in family composition and species turnover (Fig. 1c,d). These results indicated that macroinvertebrate assemblages in rivers with different conductivity values tended to have a different family composition, and that these changes seemed to arise through species replacement (and Appendix F, Table F2). Family composition was also significantly influenced by evaporitic surface, basin slope, geographic and hydrologic distances which, along with conductivity, explained community variance to a substantial extent (r^2=63%). Changes due to species turnover were also linked to conductivity, geography, basin slope, hydrology and basin area distances (r^2=35%).

3.2. Integrating saline rivers into biomonitoring typologies

According to their biological communities, the nMDS ordinations of samples revealed that environmental and biological classification methods produced roughly similar river types (Fig. 3 and Appendix G, Table G1, Figs. G2 and G3). After ignoring large watercourses (type 3), both classifications included three types of freshwater perennial rivers (types 1, 2 and 4), a type comprised mainly of freshwater intermittent and ephemeral rivers (type 5), and three types of saline rivers with increasing conductivity (types 6, 7 and 8, see Appendix G, Table G1). The freshwater perennial river types included headwater watercourses of very low conductivity that drained mountainous siliceous catchments (type 1), mid-mountain rivers of low conductivity that drained medium-sized calcareous catchments (type 2) and calcareous high mountain headwaters of very low to low conductivity (type 4). Although both classifications identified a type of temporary river (type 5), the type defined by the environmental classification included a higher proportion of temporary rivers (68% of intermittent and 32% of ephemeral watercourses) than the type defined by the biological classification (28% of intermittent and 26% of ephemeral watercourses). The three new saline river types, hyposaline (type 6), mesosaline (type 7) and hypersaline (type 8), showed conductivity ranges that were generally similar for both classification procedures, but some minor discrepancies were also found (Appendix G, Table G1). The hyposaline type showed a conductivity range (Q10-Q90) of 8,323-24,567 µS cm^{-1} for the environmental classification, and one of 4,953-26,158 µS cm^{-1} for the biological classification. The mesosaline type gave a conductivity range of 60,480-110,100 µS cm^{-1} for the environmental classification and one of 24,133-98,200 µS cm^{-1} for the biological classification. The hypersaline type showed a conductivity range of 140,000 - 300,000 µS cm^{-1} for the environmental classification and one of 110,100 - 300,000 µS cm^{-1} for the biological classification. All these types were characterised by smaller basin areas, lower elevations, softer slopes, arid climates and greater evaporitic surfaces and conductivity in relation to the freshwater types (Table 2 and Appendix G, Table G1). More than half the surveyed saline rivers were also intermittent or ephemeral. Classification strength based on family abundances was roughly similar between the environmental (CS=0.150±0.007) and biological (CS=0.158±0.006) classification procedures (Appendix G, Fig. G1). However, environmental classification (CS=0.203±0.008) seemed better at representing species turnover among types compared to the biological classification (CS=0.170±0.005).

3.3. Biomonitoring indicators for saline river types

For hyposaline rivers (type 6) (Appendix H, Tables H1-H3), the best biological indicators of the reference conditions were the families Tabanidae, Libellulidae, Hydrometridae, Caenidae, Simuliidae, Nepidae, Gammaridae, Notonectidae and Dytiscidae, the genera *Yola*, *Laccobius* and *Enochrus*, and the species *Ochthebius delgadoi*, *Laccobius moraguesi* and *Enochrus politus*. The best indicators of the disturbed conditions for this type were the families Chironomidae, Baetidae, Corixidae, Naucoridae, Coenagrionidae, Hydrophilidae, Ceratopogonidae, Hydrobiidae, Culicidae and Aeshnidae, the genera *Berosus*, *Micronecta*, *Naucoris*, *Nepa*, *Hydroglyphus*, *Sigara* and *Notonecta* and the species *Micronecta scholtzi*, *Nepa cinerea*, *Naucoris maculatus* and *Sigara scripta*. For mesosaline rivers (type 7), the best indicators of reference condition were the families Hydraenidae and Stratiomyidae, the genera

Ochthebius and *Nebrioporus* and the species *Nebrioporus baeticus*, *E. jesusarribasi* and *O. notabilis*. The indicators of the disturbed conditions for this type were the genus *Agabus* and the species *O. corrugatus*. For hypersaline rivers (type 8), *O. glaber* was the only indicator of the reference conditions.

For hyposaline rivers (type 6), the metrics based on the species at reference sites (ref_spp6, LMM r^2_m=0.30, Tukey *t*-test p=0.001) and the metrics based on the genera (dis_gen6, LMM r^2_m=0.23, Tukey *t*-test p=0.009) and species (dis_spp6, LMM r^2_m=0.19, Tukey *t*-test p=0.009) of the disturbed sites were the best indicators (Table 3). For mesosaline rivers (type 7), the metrics based on the families (ref_fam7, r^2_m=0.47, Tukey *t*-test p<0.001), genera (ref_gen7, r^2_m=0.50, Tukey *t*-test p<0.001) and species (ref_spp7, r^2_m=0.45, Tukey *t*-test p<0.001) of the reference sites were the best indicators (Table 3). Contrarily, conventional biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-metric indices (ICM-11a and IMMi-T) showed a null capacity for discriminating between the reference and disturbed conditions for saline river types (Table 3).

4. Discussion

4.1. Water salinity as a driver of community richness and composition at regional-continental scales

Our study shows that water salinity explains a large portion of the biological variation at regional and broad spatial scales. Elevation, lithology or climate have been previously used and considered the main factors that drive richness and compositional patterns across river communities under reference conditions (e.g. Clarke et al., 2003; Poquet et al., 2009). However, our study suggests that reach-scale stressors, such as water salinity or flow intermittency, may play a pivotal role in shaping the structure of inland water communities in the absence of anthropogenic alterations (Diaz et al., 2008; Leigh and Datry, 2017; Suárez et al., 2017).

All along a particular environmental gradient, the degree to which certain levels of environmental filtering can be considered stressful or harmful depends on how well adapted the regional pool is (Badyaev, 2005; Taylor et al., 1990). Thus the number of taxa capable of coping with each portion of the stress gradient is linked to regional and historical aspects, such as long-term persistence and frequency of stressful conditions (Taylor et al., 1990) and the evolutionary context of each lineage (Buchwalter et al., 2008). The long-term persistence of the osmotic stress associated with Mediterranean saline rivers is expected to act as a strong driver of community assembly, but also as a source of ecological diversification in aquatic lineages. In naturally saline rivers, osmotic pressure imposes a chronic filter for organisms that attempt to colonise, thrive or reproduce (Velasco et al., 2019). Regarding insects, the major drop in community richness at conductivities larger than 3,000 µS cm^{-1} was strongly associated with the existence of few lineages that present specific mechanisms to maintain internal integrity once submerged under hyperosmotic media (Arribas et al., 2019; Bradley, 2008; Millán et al., 2011). These are mostly the taxa which belong to the families Hydrophilidae, Dytiscidae and Hydraenidae (Coleoptera), Corixidae (Hemiptera), and Culicidae, Ephydridae, Stratiomyidae, Chironomidae (Diptera) (Arribas et al., 2019; Bradley, 2008; Pallarés et al., 2017a), and they all comprise good biological indicators of the reference saline streams. Thus our results reveal a clear differentiation in community composition, and a particularly strong replacement of taxa along the conductivity gradient, which also coincides with previous studies about this natural stress gradient (Gascón et al., 2016; Gutiérrez-Cánovas et al., 2013), and with high levels of habitat specificity associated with osmotic stress (Carbonell et al., 2012).

4.2. Integrating saline types into river classifications

The main advantage of our approach is that it integrates the whole spectrum of environmental and biological variation into a single comprehensive classification, which is either environmentally or biologically based, and allows a more accurate and simple classification of rivers in the Mediterranean region. This new integrated typology could help to better implement WFD into Member States and to gather a comprehensive inventory of saline rivers, which are currently ignored or misclassified by current laws. For example, the official Spanish typology of rivers recognises three types of highly mineralised rivers (official types 7, 9 and 13). However, the mean conductivities of these official types range is 448-545 µS cm^{-1} (Spanish Government, 2009), which is significantly lower than the conductivities shown by the saline rivers studied herein. Moreover for the first time, our classifications implicitly recognise the importance of considering the whole natural osmotic stress gradient by providing a classification method that encompasses more biodiversity than previous individual attempts (e.g. Arribas et al., 2009; Sánchez-Montoya et al., 2007).

The definition of the three saline river types was relatively consistent for both environmental and biological classifications. Thus the prediction of their biological communities was fairly accurate, and was based on only one single sample or the mean of several samples of conductivity (Moreno et al., 1997), similarly to which occurs in lentic systems (Gascón et al., 2016; Pinder et al., 2005; Williams, 1998). In fact our models identified how seasonal or hydrological variation had almost no effect on the biological communities occurring in highly mineralised rivers (>3,000 µS cm^{-1}). This is not surprising because saline specialists may show co-tolerance to other drought-related stressors such as desiccation (Pallarés et al., 2017b).

Taken together, these results advocate the use of river saline types that included both perennial and intermittent rivers. However, future work using more precise hydrological data should check the consistency of these saline river types along flow intermittency gradients. Quantitative hydrological data offer a much more adequate indication of the drought stress that affects biological communities (Belmar et al., 2013; Gallart et al., 2016; Jaeger and Olden, 2012). Unfortunately, these data are currently unavailable for most studied rivers, so simple categorical descriptors of the hydrological regime were used instead.

Our findings might also be useful to biomonitoring naturally saline rivers in other regions outside the study area, such as Australia (Biggs et al., 2013), North and South America (Griffith, 2014; Orfeo, 1999), North Africa (Hamed and Dhahri, 2013) and Russia (Zinchenko et al., 2014). Specifically, although biogeographical differences may lead to very different taxonomic compositions, ecological responses to salinity might be similar and roughly close river types might be yielded depending on the available salinity gradient.

4.3. Metrics to evaluate anthropogenic impacts on saline rivers

Generally, UE Member States are implementing WFD by classifying water bodies and then developing appropriate biological indicators to evaluate their ecological status, rather than using model-based methods (Birk et al., 2012). For these pragmatic reasons, we developed specific indicators for the obtained saline river types. Our results showed that the metrics based on taxon abundance, which either indicate reference or degraded conditions, were able to detect anthropogenic impacts on naturally saline rivers, while the metrics commonly used in freshwater rivers did not respond at all. Whereas conventional biomonitoring metrics, such as family or EPT richness, are good indicators of ecosystem quality in freshwater rivers (Bonada et al., 2006), intense abiotic filtering at naturally stressed rivers acts as a confounding factor for theses metrics. This fact indicates that diversity-based indicators are inappropriate for evaluating saline watercourses, and are also potentially inaccurate for other naturally stressed systems (Cañedo-Argüelles et al., 2012; Elliott and Quintino, 2007). Previous studies have also demonstrated that conventional biomonitoring metrics show substantial limitations when evaluating the ecological quality of naturally stressed ecosystems, such as intermittent rivers (Bruno et al., 2016; Wilding et al., 2018) or estuaries (Elliott and Quintino, 2007).

The abundance of specialist taxa seems to provide a much better indication of reference and degraded conditions than diversity-based metrics (Cañedo-Argüelles et al., 2012). These metrics can also be used to monitor their populations, which are scattered across the territory and threatened by human pressures (Arribas et al., 2015). Nonetheless, we admit that our proposed metrics is a first attempt to effectively showcase the type of biomonitoring tools that would work in saline rivers, so they should be cautiously taken. Therefore, they may benefit from further refinements by gathering larger datasets of observational data combined with manipulative experiments, which both cover different types of impacts (e.g. dilution, nutrient enrichment). Furthermore, some of the indicator species for the saline types defined herein (e.g. *Ochthebius glaber*, *Nebrioporus baeticus*) are endemic of the Iberian Peninsula. Fortunately, these taxa have sister species with very similar ecological requirements in other biogeographic regions (Arribas et al., 2015), and could be used as effective indicators of reference or disturbed conditions.

In some saline rivers, agriculture is diluting salt concentrations, which poses a risk for their typical communities that are confined to these peculiar environments (Carbonell et al., 2012; Gutiérrez-Cánovas et al., 2013; Pallarés et al., 2017a), and leads to taxonomic homogenisation and loss of regional biodiversity. Similarly in other naturally stressed systems, such as glacier-fed and alpine rivers, climate change is reducing the number of the endemic and specialist taxa that typically inhabit those systems through increasing temperature and turbidity (Finn et al., 2013; Jacobsen et al., 2012). Consequently, we highlight the urgent need to catalogue and monitor naturally stressed rivers which, despite harbouring reduced local diversity, contribute substantially to regional and global biodiversity through their unique communities of stress-tolerant species (Finn et al., 2013, Millán et al., 2011).

5. Conclusions

Our study provides a better understanding of the environmental drivers that explain macroinvertebrate richness and composition along the broad heterogeneity exhibited by Mediterranean rivers, and emphasises the role of natural stressors like water salinity. We also deliver classification approaches that encompass freshwater perennial and intermittent rivers along with three saline river types for the first time. Finally, we demonstrate that the conventional biomonitoring metrics and indices developed for freshwater rivers failed to detect anthropogenic impacts on saline rivers. So we provide new specific metrics based on the abundances of indicator taxa for these rivers, which show better responses to degradation. Taken together, these new insights can improve our understanding of ecological responses to natural and anthropogenic stressors, to foster the development of biomonitoring metrics for naturally saline rivers, and to help preserve their unique biodiversity.

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Table 1. Dataset description including code used, disturbance level (reference or disturbed sites), taxa covered and their taxonomic resolution, region covered, environmental gradient encompassed, number of sites (Sites), number of observations (Obs.) and the paper objectives where each dataset was used. * This dataset was split to obtain an independent dataset to evaluate classification performance (see the Materials and methods section for more details).

Code	Disturbance level	Taxa	Taxonomic resolution	Region	Environmental gradient	Sites	Obs.	Community models	Classification development	Classification evaluation	Biomonitoring metrics
ref_fam_ita	Reference	All	Family	Italy	All	18	44	x		x	
ref_fam_mor	Reference	All	Family	Morocco	All	28	28	x		x	
ref_fam_spa	Reference	All	Family	Spain	All	139	386	x	x	x*	
ref_spp_ita	Reference	Coleoptera	Species	Italy	All	19	31	x		x	
ref_spp_mor	Reference	Coleoptera	Species	Morocco	All	29	29	x		x	
ref_spp_spa	Reference	Coleoptera	Species	Spain	All	64	151	x		x	
ref_fam_all	Reference	All	Family	All	All	157	157	x			
ref_spp_all	Reference	Coleoptera	Species	All	All	76	76	x			
ref_fam_spa_sal	Reference	All	Family	Spain	Saline rivers	35	89				x
ref_spp_spa_sal	Reference	Coleoptera	Genus, Species	Spain	Saline rivers	30	75				x

dis_fam_spa_sal	Disturbed	All	Family	Spain	Saline rivers	17	31	x
dis_spp_spa_sal	Disturbed	Coleoptera	Genus, Species	Spain	Saline rivers	16	30	x
Overall						243	577	

Table 2. Description of the seven river types proposed for the environmental classification. Types 1 to 5 are defined in ECOSTAT (European Commission, 2007), and modified as specified in Appendix S2, whereas types 6, 7 and 8 are defined according to the conductivity thresholds used to classify the saline rivers reported in Arribas et al. (2009) and Millán et al. (2011).

Type	**Description**	**Basin area (km^2)**	**Altitude (m)**	**Lithology**	**Hydrology**	**Mean conductivity ($\mu S\ cm^{-1}$)**
1	Small high-mid altitude rivers	<1,000	200-2,000	≥ 40% siliceous	Perennial flow	< 200
2	Small / medium lowland rivers	<1,000	<600	Mixed	Perennial flow	<5,000
3	Large lowland rivers	≥1,000-10,000	<600	Mixed	Perennial flow	<5,000
4	Small / medium mountain rivers	<1,000	600-1,500	≥40% calcareous	Perennial flow	≥ 200-5,000
5	Small, lowland, temporary rivers	<1,000		Mixed	Intermittent or ephemeral flow	<5,000
6	Small medium-lowland hyposaline rivers	<1,000		Calcareous and evaporitic	Perennial, intermittent or ephemeral flow	5,000-32,000
7	Small medium-lowland mesosaline	<1,000		Calcareous and evaporitic	Perennial, intermittent or	32,000-130,000

	rivers			ephemeral flow	
8	Small medium-lowland hypersaline rivers	<1,000	Calcareous and evaporitic	Perennial, intermittent or ephemeral flow	>130,000

Table 3. Results of the models that evaluated the differences in the biomonitoring metrics between the reference and disturbed saline rivers for types 6 and 7. Explained variance and significance are shown. r^2_m *accounts* for the variance explained by the fixed factors. Metric names - ref: reference condition, dis: disturbed condition; fam: family level, gen: genera level, spp: species level; 6 and 7 refer to the river type where the metrics should be applied. The metrics showing significant differences are in bold.

		Type 6 (hyposaline)		***Type 7 (mesosaline)***	
	Metrics	r^2_m	***P*-value**	r^2_m	***P*-value**
Widely-used metrics	IBMWP	0.01	0.669	0.00	0.821
	Family richness	0.00	0.792	0.00	0.799
	EPT	0.00	0.991	-	-
	IASPT	0.03	0.300	0.00	0.844
	ICM11a	0.01	0.637	0.01	0.586
	IMMiT	0.01	0.699	0.01	0.622
Novel metrics	ref_fam6	0.00	0.984	0.07	0.141
	dis_fam6	0.10	0.062	0.05	0.237
	ref_gen6	0.03	0.400	0.12	0.074
	dis_gen6	**0.23**	**0.009**	0.10	0.094
	ref_spp6	**0.30**	**0.001**	0.03	0.418
	dis_spp6	**0.19**	**0.013**	0.01	0.543
	ref_fam7	0.02	0.423	**0.47**	**0.000**
	ref_gen7	0.06	0.248	**0.50**	**0.000**
	ref_spp7	0.03	0.419	**0.45**	**0.000**

Figure captions

Fig. 1. Variable importance for the models explaining family richness (a), species richness (b), overall community composition (c) and species turnover (d).

Fig. 2. Plots showing the family richness response to conductivity and hydrology (a), hydrology (b), conductivity and season (c) and conductivity and region (d). per: perennial seasonal flow (square), int: intermittent flow (triangle), eph: ephemeral flow (cross); spn: spring; sum: summer; aut: autumn, win: winter; Spa: Spain, Ita: Italy, Mor: Morocco.

Fig. 3. Multidimensional scaling plots showing the concordance between the ordination of the biological communities based on family abundances, and the environmental (a) and biological (b) classifications of the Spanish reference samples. Numbers represent the different river types (see Results and Table 2 for more information).

Fig. 1

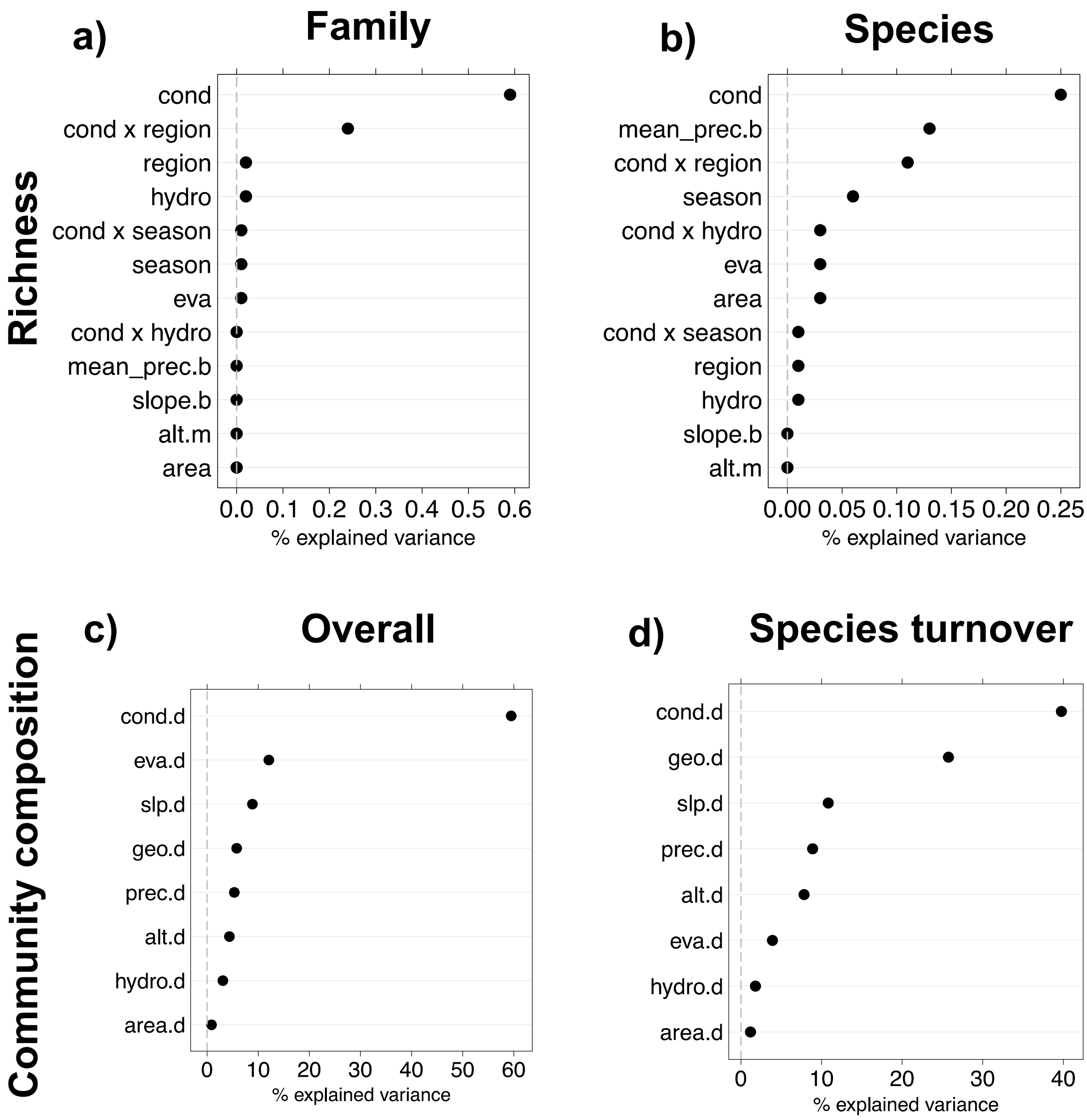

a)
Family
b)
Species
Richness
cond
cond x region
region
hydro
cond x season
season
eva
cond x hydro
mean_prec.b
slope.b
alt.m
area
0.0 0.1 0.2 0.3 0.4 0.5 0.6
% explained variance
cond
mean_prec.b
cond x region
season
cond x hydro
eva
area
cond x season
region
hydro
slope.b
alt.m
0.00 0.05 0.10 0.15 0.20 0.25
% explained variance
c)
Overall
d)
Species turnover
Community composition
cond.d
eva.d
slp.d
geo.d
prec.d
alt.d
hydro.d
area.d
0 10 20 30 40 50 60
% explained variance
cond.d
geo.d
slp.d
prec.d
alt.d
eva.d
hydro.d
area.d
0 10 20 30 40
% explained variance

Fig. 2.

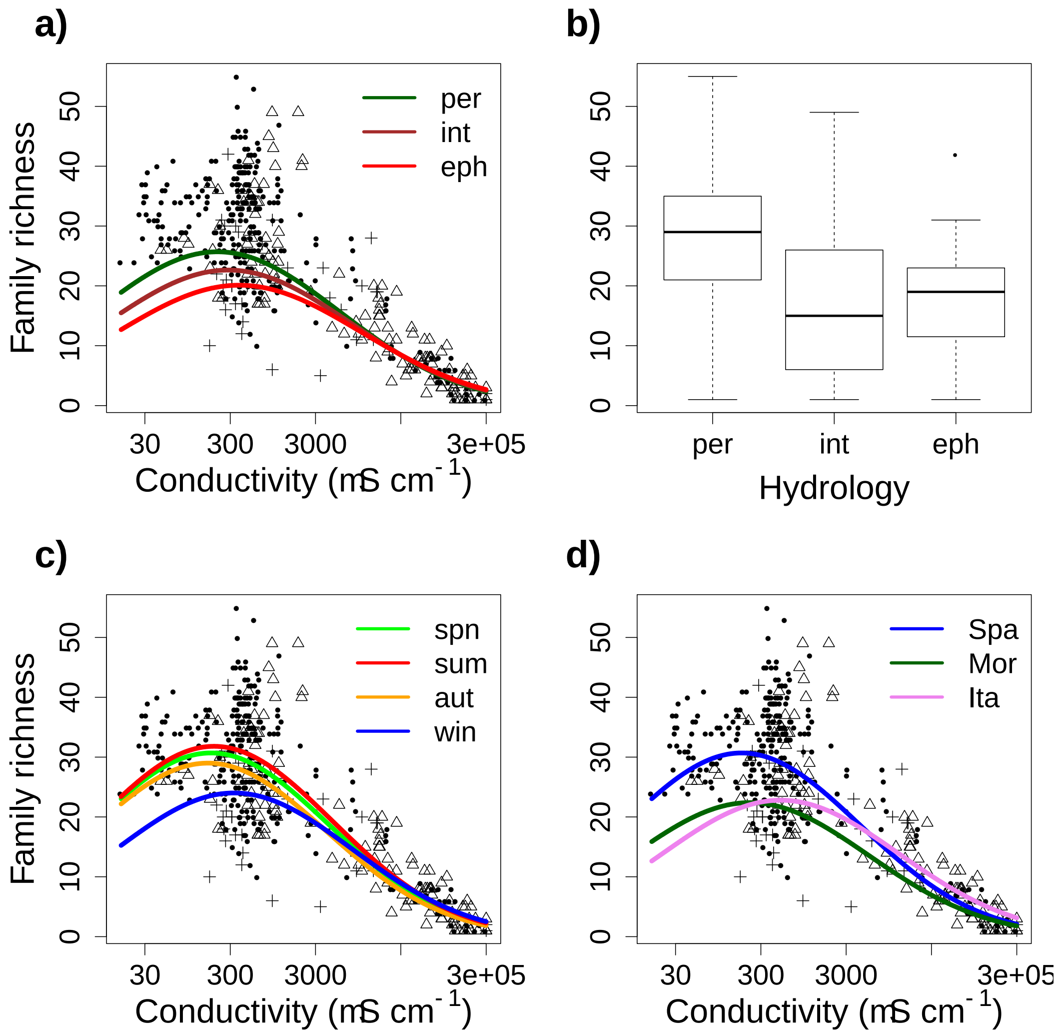
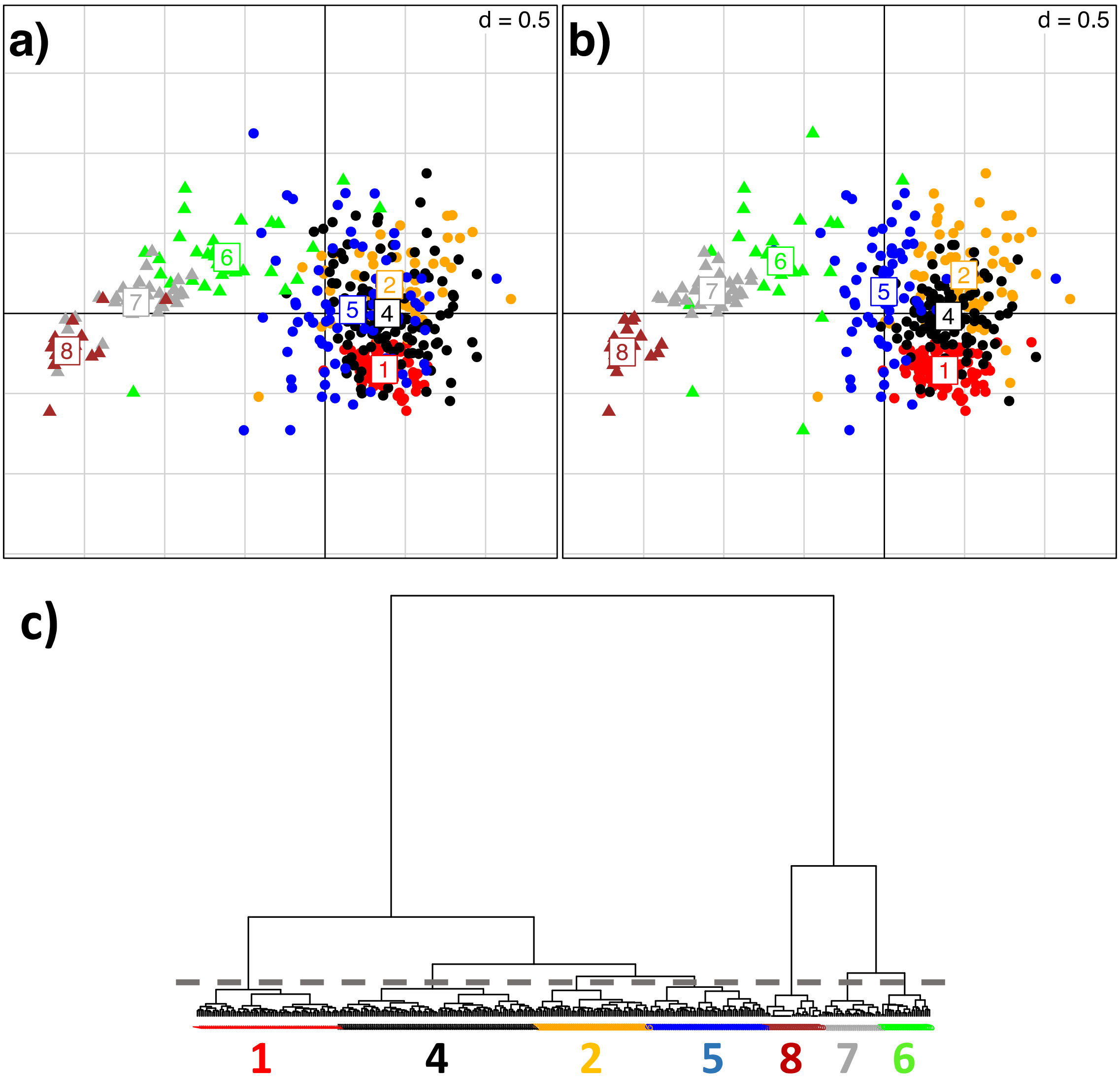

a)
b)
c)
d)
Family richness
Conductivity (mS cm-1)
Hydrology
per
int
eph
spn
sum
aut
win
Spa
Mor
Ita
0
10
20
30
40
50
30
300
3000
3e+05

Fig. 3


a)
d = 0.5
6
7
8
5
4
2
1
b)
d = 0.5
6
7
8
5
4
2
1
c)
1
4
2
5
8
7
6

Abstract

Naturally stressed ecosystems hold a unique fraction of biodiversity. However, they have been largely ignored in biomonitoring and conservation programs, such as the EU Water Framework Directive, while global change pressures are threatening their genuine values. Here, we present a framework to classify and evaluate the ecological quality of naturally stressed rivers along a gradient of water salinity. We gathered datasets including aquatic macroinvertebrate assemblages and environmental information for 243 river locations across the western Mediterranean to a) gauge the role of natural stressors (salinity) in driving aquatic community richness and composition, b) develop river classifications encompassing the broad range of environmental and biological variation exhibited by Mediterranean rivers and c) provide effective biomonitoring metrics of ecological quality for saline rivers. Our results showed that water salinity had a pivotal role in explaining the community richness and compositional changes in rivers, even when other key, commonly used descriptors, such as elevation, climate or lithology, are considered. Both environmental and biologically-based classifications included seven river types: three types of freshwater perennial rivers, one freshwater intermittent river type and three new saline river types, which were absent in previous classifications. Their validation using independent datasets showed that saline and freshwater river types represented differentiable macroinvertebrate assemblages at family and species levels. Biomonitoring metrics based on the abundance of indicator taxa of each saline river type provided a much better assessment of the ecological quality of saline rivers than other widely used biological metrics and indexes. Here, we demonstrate that considering natural stressors, such as water salinity, is essential to design effective and accurate biomonitoring programs for rivers and to preserve their unique biodiversity.

Keywords: abiotic stress, macroinvertebrates, biomonitoring, global change, saline rivers, Water Framework Directive

1. Introduction

Naturally stressed ecosystems are characterised by harbouring a set of natural conditions that are persistently unsuitable for the vast majority of the regional species pool (Badyaev, 2005; Parsons, 2005). Well-known examples of naturally stressed ecosystems are found in tundra, deserts, volcanic springs, and glacier, acid or saline inland waters (Cauvy-Fraunié et al., 2016; Elliott and Quintino, 2007; Millán et al., 2011; Petrin et al., 2007). Their natural stressful conditions reduce the local diversity of these systems but also constitutes a powerful driver of diversification (Parsons, 2005; Vetaas et al., 2002), and so they usually hold unique fractions of biodiversity characterised by high levels of specialisation and species turnover (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013; Jacobsen et al., 2012).

Unfortunately, these genuine spots are under thread by global change pressures, which may release the naturally stressed conditions causing habitat loss and reducing the singularity of the community (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013) or add new stressors, such as pesticides or microplastics (Beketov et al., 2013; Windsor et al., 2019). This situation is strongly aggravated because many of these naturally stressed ecosystems have been usually ignored once cataloguing natural heterogeneity and consequently systematically excluded for biomonitoring and conservation purposes (Millán et al., 2011; Stubbington et al., 2018).

This is particularly true in the case of naturally saline rivers (Millán et al., 2011), which have been mostly neglected by the large international efforts to reverse the globalised river degradation, such as the EU Water Framework Directive (WFD, Directive 2000/60/EC). These conservation frames are fundamentally based on a two-step process focused on the identification of main river types over a particular region (river classification) and the subsequent development and harmonisation of biomonitoring metrics that allow assessing the ecological status of the different river types (Buffagni et al., 2007). However, there are some generalised shortcomings on the application of these procedures that resulted in a deficient functionality for the assessment and protection of naturally stressed rivers. Firstly, main natural stressors such as water salinity are usually neglected or not fully considered in river classifications, resulting in the exclusion or misclassification of naturally stressed rivers (Sánchez-Montoya et al., 2007). This occurs because the classification process tends to be focused on coarse environmental descriptors, which are weak proxies of local stressors. Secondly, most of the proposed biomonitoring metrics can result in equivocal evaluations of the ecological status of the naturally stressed rivers because they are strongly associated to local richness/diversity and so not considering naturally depauperate communities (Gutiérrez-Canovas et al., 2008). Besides, other widely-used metrics are based on taxa or functional traits that are rare or even not present in naturally stressed rivers, such as the stress-sensitive orders Ephemeroptera, Plecoptera and Trichoptera (EPT) (Belmar et al., 2013; Bonada et al., 2006; Millán et al., 2011). Therefore, there is an urgent need to revisit and adapt current vision and approaches to assess the ecological quality of naturally stressed rivers, and so to improve their management and future preservation.

Here, we benefit from a large compilation of datasets from rivers across the western Mediterranean and Moroccan Atlantic basins, which include almost the complete natural salinity gradient (roughly, 30 to 300,000 µS cm^{-1}). On this comprehensive basis, we develop an integrated framework that allows assessing the ecological quality of naturally stressed rivers. First, we rank the importance of natural stress (salinity) and other general descriptors commonly used for river classifications (e.g. elevation, river size, lithology, hydrology) in determining community richness and composition. Second, we classify rivers through their environmental characteristics and biological composition, considering specific types for saline rivers and assessing their performance and concordance. Finally, we identify indicator taxa and metrics for naturally stressed rivers under reference and anthropogenically disturbed conditions, testing their performance against other widely used river biomonitoring metrics and indexes.

2. Material and methods

2.1. Datasets description

The study was conducted across the western Mediterranean basin, including watercourses from the eastern and southern Iberian Peninsula and the Balearic Islands (Spanish data), Sicily (Italian data) and from the Rif down to the Sahara Desert, comprising the Rif and Moroccan Atlantic basins (Moroccan data) (Appendix A, Fig. A1). These regions were selected because they cover a great environmental variability including large gradients of elevation, climate, hydrology, lithology, salinity and anthropogenic impacts (Appendix A, Fig. A1 and Table A1).

We have used different subsets of environmental and aquatic macroinvertebrate assemblage data to address the objectives of the study (Table 1). The description of each dataset includes anthropogenic disturbance level (reference or disturbed sites), macroinvertebrate groups used (all major orders or just aquatic Coleoptera) and their taxonomic resolution (family, genus, species levels), region (Italy, Morocco, Spain, all), environmental gradient encompassed (all gradients or just saline rivers), number of sampling sites and observations and the objectives for which each dataset was used. To compile this database of 243 river locations and 577 samples, we combined own data with a large dataset from the Guadalmed project (Prat, 2002). All macroinvertebrate samples were collected following a multi-habitat semiquantitative kick-sample (Jáimez-Cuéllar et al., 2002). See Appendix A for more details about datasets and Appendix B to see an extended description of the sampling procedure.

Climatic, geomorphologic, lithologic and land use variables at basin and reach scales were obtained from digital layers after delineating the river basins of each sampling site (see Appendix A, Table A1 for a complete list of the variables used). Water electrical conductivity was measured *in situ* on each sampling occasion as an osmotic stress indicator. To characterise flow intermittence, we categorised the hydrological regime of each site as perennial seasonal (typically flowing), intermittent (surface flow ceases during the dry season, pools remaining) or ephemeral (totally dry during one season) flow from available hydrological information or field evidence (Belmar et al., 2013; Sánchez-Montoya et al., 2007). We categorised sampling sites as *reference* when they were minimally disturbed (i.e. fulfilling $\geq$ 16 out of 20 of the

Mediterranean Reference Criteria, MRC; Sánchez-Montoya et al., 2009) or disturbed when they were substantially impacted by anthropogenic activities (i.e. fulfilled < 16 MRC). In this study, we excluded large watercourses (mean basin area ≥1000 km^2, ECOSTAT, type 3, see Table 1 and Sánchez-Montoya et al., 2007 for details) because of the paucity of reference sites (Sánchez-Montoya et al., 2009). We also excluded disturbed freshwater rivers, for which effective biomonitoring metrics can be found elsewhere (e.g. Birk et al., 2012; Bonada et al., 2006).

2.2. Data analysis

Before analyses, we applied a log-transformation to macroinvertebrate family richness and a square-root transformation to macroinvertebrate species richness. Besides, logit-, log- or square-root-transformations were applied to quantitative environmental variables to reduce their distribution skewness and improve linearity, when necessary. Moreover, all the quantitative environmental variables were also standardised to mean=0 and SD=1 to facilitate model coefficient comparison.

2.2.1. Ranking environmental variable importance

To identify the main environmental factors determining the family and species richness, we used Random Forest (randomForestSRC R package, Ishwaran Ishwaran et al., 2014) and Linear Mixed Models (LMM, lme4 R packge, Bates et al., 2015). In these models, we utilised family-level datasets (i.e. ref_fam_ita, ref_fam_mor, ref_fam_spa, n=458) and species-level datasets (i.e. ref_spp_ita, ref_spp_mor, ref_spp_spa, n=211) from reference sites (selected according MRC, see above). Following Feld et al. (2016), we first ran Random Forest models to identify the most important predictors of family and species richness among 24 potential candidates (Appendix A, Table A1) to be included into the LMM (see Appendix C for more details about exploratory analyses). After these exploratory analyses, we included basin area, mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence, conductivity (single and quadratic terms), season and region as fixed factors in the LMM. As additional fixed factors, we also included the pairwise interactions of conductivity x flow intermittence, conductivity x season and conductivity x region. Site code was considered as a random factor to account for repeated measures in the same location. To rank the environmental predictor's importance on family and species richness, we adopted a multi-model inference approach (Grueber et al., 2011), using the *MuMIM* R package (Bartoń, 2016). This statistical technique ranks all the models generated using all the possible combination of predictors using Akaike's Information Criterion (AIC). Then, a set of top models is selected to produce an average model only if the model ranking first is ambiguously supported (model weight<0.90). We chose top models differing in no more than two AIC units (delta≤2) from the model ranked first (minimum AIC). We adopted a natural average method to conduct the model averaging, which consists in averaging predictors only over models in which the predictor appears and weighting predictor's SES by the summed weights of these models (Burnham and Anderson, 2002). For each LMM model, two measures of goodness-of-fit were estimated (Nakagawa and Schielzeth, 2013): marginal goodness-of-fit (r^2_m) indicates the variance explained only by the fixed factors, while conditional goodness-of-fit (r^2_c) shows the variance accounted for by both fixed and random terms. We provide the mean average (based on model weights) of each goodness-of-fit measure for each averaged model. All models were validated by visually checking their residuals for normality and homoscedasticity.

To identify the environmental drivers of community composition change, we used Multiple Regression models for distance Matrices (MRM; ecodist R package, Lichstein, 2007). This method is conceptually similar to traditional multiple regression but with all variables being distance matrices instead of raw data and *P*-values being calculated through permutation tests (1000 runs). To avoid lack of independence problems due to multiple samples belonging to the same site, we selected a reference subset of macroinvertebrate families (ref_fam_all, n=157 sites/samples) and species (ref_spp_all, n=76 sites/samples) occurrences with only one spring sample per site. We estimated overall changes in community composition for each pair of sites of the family matrix through the Sørensen dissimilarity index (β_{sor}) and the pairwise dissimilarity due to turnover from the species matrix using the Simpson index (β_{sim}). These calculations were made following the Baselga's (2010) framework for β-diversity partitioning using the *betapart* R package (Baselga and Orme, 2012). For each selected environmental predictor (basin area, mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence, conductivity, geographic distance), we built a Euclidean distance matrix based on their transformed and standardised values. Geographical distance between localities was based on a latitude and longitude original matrix and flow intermittence was based on semiquantitative values (perennial=0, intermittent=1, ephemeral=2).

Finally, we also performed a variance partitioning for community richness and composition models, using the *variancePartition* (Hoffman and Schadt, 2016) and *hierpart* (Walsh and MacNally, 2013) R packages.

2.2.2. Integrating saline rivers into biomonitoring typologies

To develop a classification of rivers encompassing the environmental and biological variability occurring in the studied area, we used family abundances from reference Spanish sites (ref_fam_spa, n=386). We selected this dataset because it included more sites and samples and covered a broader environmental and biological spectrum relative to the Italian and Moroccan datasets (Appendix A, Table A1). First, we classified sites into seven types according to their environmental variables (environmental classification) following an adaptation of the criteria suggested by the ECOSTAT intercalibration group for Mediterranean rivers using System A of the WFD (MedGIG European Commission, 2007), which included mean conductivity, basin area, hydrology, site altitude and basin lithology (Table 2 and Appendix D). Secondly, to classify samples according to their biological communities (biological classification), we estimated a Bray-Curtis pairwise dissimilarity matrix derived from the abundance family matrix and produced a dendrogram based on the Bray-Curtis family dissimilarity matrix, using the Ward's clustering method. After visual inspection, we decided to prune the tree to produce seven biological types. For both classifications, we used the same type numbers previously utilised (European Commission, 2007; Sánchez-Montoya et al., 2007) for freshwater rivers and the numbers 6, 7 and 8 for the new saline river types. The type 3 (large rivers) was not used because we excluded this type of rivers from the analysis. We also performed a non-Metric Multidimensional Scaling (nMDS) ordination based on the Bray-Curtis family dissimilarity matrix to explore the concordance between the environmental and biological classifications.

To evaluate the performance of both classification procedures in a wider geographical context, we estimated their classification strength based on datasets of family and species from reference sites of Italy (ref_fam_ita, n=44; ref_spp_ita, n=31), Morocco (ref_fam_mor, n=28; ref_spp_mor, n=29) and Spain (ref_fam_spa, n=386; ref_spp_spa, n=151). We assigned environmental types to these new sites using the environmental classification criteria (Table 2). To assign biological types to the new sites, we built a Random Forest model predicting biological types from environmental information. To develop the Random Forest model (trees=2000, mtry=8), we used a subset of the ref_fam_spa dataset (n=258), while the non-utilised samples (n=128) were used along with the other independent subsets to evaluate the classification performance. To estimate classification strength (CS), dissimilarity matrices were converted to similarity matrices. CS was quantified as the difference between the within-type mean similarity (W) and between-types mean similarity (B) of the Bray-Curtis pairwise similarity based on family abundances and for the Simpson similarity matrix based on species occurrences and turnover (CS=W-B) for the three regions. CS mean values were calculated through a bootstrapping procedure, where we resampled 100 subsets of n=28 from each Italian and Spanish macroinvertebrate datasets to make their CS values comparable to those obtained for the Moroccan datasets, which had the lowest number of observations.

2.2.3. Biomonitoring indicators for saline river types

To develop metrics indicating the ecological quality of saline river types and test their performance against widely used biomonitoring metrics, we used a dataset including family and species-level data from reference (ref_fam_spa_sal, n=89; ref_spp_spa_sal, n=75) and disturbed (dis_fam_spa_sal, n=31; dis_spp_spa_sal, n=30) Spanish sites. We first assigned river types to all these samples using the environmental classification criteria because its simplicity and potential better performance compared to the biological classification (see Results). Considering that most of the disturbed sites have been affected by a drop in their natural conductivity levels as a result of freshwater inputs from agricultural drainages (Velasco et al., 2006), we compiled historical, predisturbed conductivity information (Moreno et al., 1997; Vidal-Abarca, 1985) to correctly assign their river types. As result, we obtained hyposaline, mesosaline and hypersaline river types under reference condition (ref_6, ref_7 and ref_8, respectively) and hyposaline and mesosaline river types under disturbed condition (dis_6, dis_7). For the type 8, we did not find any disturbed site.

To identify the families, genera and species showing a greater affinity for each reference and disturbed type, we used an indicator species analysis (IndVal, Dufrêne and Legendre, 1997). This analysis considers the percentage of occurrence and relative abundance of each taxon for each type to obtain an indicator value (IV) and its significance through Monte-Carlo permutations (1000 runs). We focused on the most frequent taxa, by keeping taxa occurring in more than 10% of the observations. From them, we selected those taxa showing significant Indicator Value ($P \leq 0.05$) as potential indicators for a given type. From these results, we built the candidate metrics of reference and disturbed conditions of the saline river types. To create those metrics, for each sample, we summed the abundances of the indicator taxa of the assigned river type (ref_6, dis_6, ref_7, dis_7, ref_8) for family, genus, and species level (e.g. for family level: ref_fam6, dis_fam6, ref_fam7, dis_fam7, ref_fam8). In addition, for each sample, we estimated a set of widely-used biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-metric indexes (ICM-11a and IMMi-T) for Mediterranean rivers (Alba-Tercedor et al., 2002; Munné and Prat, 2009). Finally, to evaluate the performance of these candidate metrics against the widely-used biomonitoring metrics, we used LMM models assessing differences across all reference and disturbed types (levels= ref_6, dis_6, ref_7, dis_7, ref_8) and Tukey-*t* post-hoc tests to evaluate differences between pairs of comparable reference and disturbed types (i.e. ref_6 vs. dis_6, ref_7 vs. dis_7).

The code and functions used to run all these analyses are available in Appendix E, which were conducted using the R version 3.4.1 (R Core Team, 2016).

3. Results

3.1. Ranking environmental variable importance

Electrical conductivity was the most important variable explaining macroinvertebrate assemblage richness and composition (Fig. 1a,c, and Appendix F, Table F1). Family richness was primarily explained by conductivity (59%) and the interaction between conductivity and region (25%), suggesting that conductivity had different regional effects (r^2_m=0.82). Generally, above conductivity values of 3,000 µS cm^{-1}, family richness responded only to conductivity changes. Family richness peaked at conductivities ranging 300-1,000 µS cm^{-1}, declining progressively as conductivity increases (Fig. 2a,c,d). Within this conductivity range, family richness values showed the greatest dispersion, indicating that other variables had also a strong influence on family richness. The interactive effects of conductivity with hydrology (Fig. 2a), season (Fig. 2c) and region (Fig. 2d) were evident only at freshwaters and, particularly, within the conductivity range of 300-1,000 µS cm^{-1}, becoming much weaker at conductivities greater than 3,000 µS cm^{-1}. Rivers with perennial flow tended to have higher family richness than intermittent or ephemeral rivers (Fig. 2a,b), but as conductivity increases, the effect of hydrology also became less important. Species richness showed roughly similar patterns in response to environmental variables, where conductivity was also the most important predictor, but with higher contributions of mean basin precipitation and seasonality (Fig. 1b and Appendix F, Table F1).

Conductivity distance was also the most important variable explaining dissimilarity in family composition and species turnover (Fig. 1c,d). These results indicate that macroinvertebrate assemblages in rivers with different conductivity values tended to have different family composition, and that these changes seem to arise through species replacement (and Appendix F, Table F2). Family composition was also significantly influenced by evaporitic surface, basin slope, geographic and hydrologic distances, which along with conductivity explained a substantial extent of the community variance (r^2=63%). Changes due to species turnover were also linked to conductivity, geographic, basin slope, hydrologic and basin area distances (r^2=35%).

3.2. Integrating saline rivers into biomonitoring typologies

The nMDS ordinations of samples according to their biological communities revealed that environmental and biological classification methods produced roughly similar river types (Fig. 3 and Appendix G, Table G1, Figs. G2 and G3). After ignoring large watercourses (type 3), both classifications included three types of freshwater perennial rivers (types 1, 2 and 4), a type mainly comprised of freshwater intermittent and ephemeral rivers (type 5) and three types of saline rivers of increasing conductivity (types 6, 7 and 8, see Appendix G, Table G1). Freshwater perennial river types included headwater watercourses of very low conductivity draining mountainous siliceous catchments (type 1), mid-mountain rivers of low conductivity draining medium size, calcareous catchments (type 2) and calcareous high mountain headwaters of very low to low conductivity (type 4). Although both classifications identified a type of temporary rivers (type 5), the environmental classification included a higher proportion of temporary rivers (68% of intermittent and 32% of ephemeral watercourses) than the biological classification (28% of intermittent and 26% of ephemeral watercourses). The new three saline river types (hyposaline, mesosaline and hypersaline river types) were characterised by smaller basin areas, lower elevations, softer slopes, arid climates, and greater evaporitic surface and conductivity relative to the freshwater types (Table 2 and Appendix G, Table G1). Also, more than a half of the surveyed saline rivers were intermittent or ephemeral. The conductivity ranges that define the saline types were generally similar in both classification procedures, but some minor discrepancies were also found (Appendix G, Table G1). Classification strength based on family abundances was roughly similar between the environmental (CS=0.150±0.007) and biological (CS=0.158±0.006) classification procedures (Appendix G, Fig. G1). However, environmental classification (CS=0.203±0.008) seems to be better in representing species turnover among types compared to the biological classification (CS=0.170±0.005).

3.3. Biomonitoring indicators for saline river types

For hyposaline rivers (type 6) (Appendix H, Tables H1-H3), the best biological indicators of reference conditions were the families Tabanidae, Libellulidae, Hydrometridae, Caenidae, Simuliidae, Nepidae, Gammaridae, Notonectidae and Dytiscidae, the genera *Yola*, *Laccobius* and *Enochrus*, and the species *Ochthebius delgadoi*, *Laccobius moraguesi* and *Enochrus politus*. The best indicators of disturbed conditions for this type were the families Chironomidae, Baetidae, Corixidae, Naucoridae, Coenagrionidae, Hydrophilidae, Ceratopogonidae, Hydrobiidae, Culicidae and Aeshnidae, the genera *Berosus*, *Micronecta*, *Naucoris*, *Nepa*, *Hydroglyphus*, *Sigara* and *Notonecta* and the species *Micronecta scholtzi*, *Nepa cinerea*, *Naucoris maculatus* and *Sigara scripta*. For mesosaline rivers (type 7), the best indicators of reference condition were the families Hydraenidae and Stratiomyidae, the genera *Ochthebius* and *Nebrioporus* and the species *Nebrioporus baeticus*, *E. jesusarribasi* and *O. notabilis*. The indicators of disturbed conditions for this type were the genus *Agabus* and the species *O. corrugatus*. For hypersaline rivers (type 8), *O. glaber* was the only indicator of reference conditions.

For hyposaline rivers (type 6), the metrics based on species of reference sites (ref_spp6, LMM r^2_m=0.30, Tukey *t*-test *p*=0.001) and the metrics based on genera (dis_gen6, LMM r^2_m=0.23, Tukey *t*-test *p*=0.009) and species (dis_spp6, LMM r^2_m=0.19, Tukey *t*-test *p*=0.009) of disturbed sites were the best indicators (Table 3). For mesosaline rivers (type 7), metrics based on families (ref_fam7, r^2_m=0.47, Tukey *t*-test *p*<0.001), genera (ref_gen7, r^2_m=0.50, Tukey *t*-test *p*<0.001) and species (ref_spp7, r^2_m=0.45, Tukey *t*-test *p*<0.001) of reference sites were the best indicators (Table 3). Contrarily, conventional biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-metric indexes (ICM-11a and IMMi-T) showed a null capacity to discriminate between reference and disturbed conditions for saline river types (Table 3).

4. Discussion

4.1. Water salinity as a driver of community richness and composition at regional-continental scales

Our study shows that water salinity explains a key portion of the biological variation at regional and broad spatial scales. Previously, elevation, lithology or climate have been used and considered as main factors driving richness and compositional patterns across river communities (e.g. Clarke et al., 2003; Poquet et al., 2009). However, our study suggests that local stressors, such as waters salinity or flow intermittence, may play a pivotal role in shaping the structure of inland water communities (Diaz et al., 2008; Leigh and Datry, 2017).

Over a particular environmental gradient, the degree to which certain levels of environmental filtering could be considered stressful or harmful depends on how well adapted is the regional pool (Badyaev, 2005; Taylor et al., 1990). Thus, the number of taxa able to cope over each portion of the stress gradient is linked to regional and historical aspects, such as the long-term persistence and frequency of stressful conditions (Taylor et al., 1990) and the evolutionary context of each lineage (Buchwalter et al., 2008). The long-term persistence of the osmotic stress associated to Mediterranean saline rivers is expected to act as a strong driver of community assembly but also a source of ecological diversification in aquatic lineages. In naturally saline rivers, osmotic pressure imposes a chronic filter for organisms trying to colonise, thrive or reproduce (Bradley, 2008). Regarding insects, the important drop in community richness at conductivities > 3,000 µS cm^{-1}, is strongly associated with the existence of few lineages presenting specific mechanisms to maintain internal integrity once submerged under hyperosmotic media (Bradley, 2008; Millán et al., 2011). These are mostly taxa belonging to the families Hydrophilidae, Dytiscidae, and Hydraenidae (Coleoptera), Corixidae

(Hemiptera), and Culicidae, Ephydridae, Stratiomyidae, Chironomidae (Diptera) (Arribas et al., 2014; Bradley, 2008; Pallarés et al., 2017), all of them comprising good biological indicators of reference saline streams. Thus, our results reveal a clear differentiation in community composition and, particularly, a strong replacement of taxa along the conductivity gradient, also concordant with previous studies on this natural stress gradient (Gutiérrez-Cánovas et al., 2013) and on the high levels of habitat specificity associated to the osmotic stress (Carbonell et al., 2012).

4.2. Integrating saline types into river classifications

The main advantage of our approach is the integration of the whole spectrum of environmental and biological variation into a single comprehensive classification, either environmentally or biologically based, that allows a more accurate and simple classification of rivers in the Mediterranean region. This new integrated typology could help to better implement WFD in the state members, whose legal criteria ignore or misclassify saline rivers. For example, the Spanish official typology of rivers recognises three types of highly mineralised rivers (official types 7, 9 and 13). However, the mean conductivities of this official types range 448-545 µS cm^{-1} (Various authors, 2009), significantly lower than the conductivities showed by the saline rivers studied here. Also, for the first time, our classifications implicitly recognises the importance of considering the whole natural osmotic stress gradient, providing a classification method that encompasses more biodiversity that the previous individual attempts (e.g. Arribas et al., 2009; Sánchez-Montoya et al., 2007)

The definition of the three saline river types was relatively consistent for both environmental and biological classifications. Thus, the prediction of their biological communities was fairly accurate based just on mean or sampled conductivity (Moreno et al., 1997), as occurred in lentic systems (Williams, 1998). In fact, our models identified how seasonal or hydrological variation had almost no effect in the biological communities occurring at highly mineralised rivers (>3,000 µS cm^{-1}).

4.3. Metrics to evaluate anthropogenic impacts on saline rivers

Generally, the UE member states are implementing WFD through the classification of the water bodies and then the development of appropriate biological indicators to evaluate their ecological status, rather than using model based methods (Birk et al., 2012). For these pragmatic reasons, we developed specific indicators for the saline river types obtained. Our results showed that metrics based on the abundance of taxa indicating either reference or degraded conditions were able to detect anthropogenic impacts on naturally saline rivers, while metrics commonly used in freshwater rivers did not respond at all. While conventional biomonitoring metrics, such as family or EPT richness, are good indicators of the ecosystem quality in freshwater rivers (Bonada et al., 2006), the intense abiotic filtering at naturally stressed rivers acts as a confounding factor for theses metrics. This fact causes that diversity-based indicators are inappropriate to evaluate saline watercourses, being also potentially inaccurate for other naturally stressed systems (Elliott and Quintino, 2007). Previous studies have also demonstrated that conventional biomonitoring metrics showed substantial limitations to evaluate the ecological quality of naturally stressed ecosystems, such as intermittent rivers (Bruno et al., 2016; Wilding et al., 2018) or estuaries (Elliott and Quintino, 2007).

The abundance of specialist taxa seems to provide much better indication of reference and degraded conditions than diversity-based metrics. These metrics can be also used to monitor their populations, which are scattered across the territory and threatened by human pressures (Arribas et al., 2015). Nonetheless, we admit that our proposed metrics are a first attempt to effectively showcase the type of biomonitoring tools that would work in saline rivers. Therefore, they may require further refinement by gathering larger datasets of observational data and combined with manipulative experiments, both covering different types of impacts (e.g. dilution, nutrient enrichment).

In some saline rivers, agriculture is diluting salt concentrations, posing risk to their typical communities, which are confined to such peculiar environments (Carbonell et al., 2012;

Gutiérrez-Cánovas et al., 2013; Pallarés et al., 2017), which leads to taxonomic homogenisation and regional biodiversity loss. Similarly, in other naturally stressed systems, such as glacier-fed and alpine rivers, climate change is reducing the number of endemism and specialist taxa, which typically inhabit those systems, through increases in temperature and turbidity (Finn et al., 2013; Jacobsen et al., 2012). Consequently, we highlight the urgent need of monitoring naturally stressed rivers, which despite harbouring a reduced local diversity, contribute genuinely to regional and global biodiversity through their unique communities of stress tolerant species (Finn et al., 2013, Millán et al., 2011).

5. Conclusions

Our study provides a better understanding of the environmental drivers that explain macroinvertebrate richness and composition along the broad heterogeneity exhibited by Mediterranean rivers, emphasising the role of natural stressors, such as water salinity. We also deliver classification approaches that encompass freshwater perennial and intermittent rivers along with three saline river types for the first time. Finally, we demonstrate that conventional biomonitoring metrics and indexes developed for freshwater rivers failed in detecting anthropogenic impacts on saline rivers and so we provide new specific metrics based on the abundances of indicator taxa for these rivers showing better responses to degradation. Taken together, these new insights can improve the understanding of the ecological responses to natural and anthropogenic stressors and foster the development of biomonitoring metrics for naturally saline rivers, helping to preserve their unique biodiversity.

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Table 1. Dataset description including code used, disturbance level (reference or disturbed sites), taxa covered and their taxonomic resolution, region covered, environmental gradient encompassed, number of sites (Sites), number of observations (Obs.) and the paper objectives where each dataset was used. * This dataset was split to have an independent dataset to evaluate the classification performance (see Materials and Methods section for more details).

Code	Disturbance level	Taxa	Taxonomic resolution	Region	Environmental gradient	Sites	Obs.	Community models	Classification development	Classification evaluation	Biomonitoring metrics
ref_fam_ita	Reference	All	Family	Italy	All	18	44	x		x	
ref_fam_mor	Reference	All	Family	Morocco	All	28	28	x		x	
ref_fam_spa	Reference	All	Family	Spain	All	139	386	x	x	x*	
ref_spp_ita	Reference	Coleoptera	Species	Italy	All	19	31	x		x	
ref_spp_mor	Reference	Coleoptera	Species	Morocco	All	29	29	x		x	
ref_spp_spa	Reference	Coleoptera	Species	Spain	All	64	151	x		x	
ref_fam_all	Reference	All	Family	All	All	157	157	x			
ref_spp_all	Reference	Coleoptera	Species	All	All	76	76	x			
ref_fam_spa_sal	Reference	All	Family	Spain	Saline rivers	35	89				x
ref_spp_spa_sal	Reference	Coleoptera	Genus, Species	Spain	Saline rivers	30	75				x

dis_fam_spa_sal	Disturbed	All	Family	Spain	Saline rivers	17	31	x
dis_spp_spa_sal	Disturbed	Coleoptera	Genus, Species	Spain	Saline rivers	16	30	x
Overall						243	577	

Table 2. Description of the seven river types proposed for the environmental classification. Types 1 to 5 were defined in ECOSTAT (European Commission, 2007), and modified as specified in Appendix S2, whereas types 6, 7 and 8 were defined according to the conductivity thresholds used to classify saline rivers reported in Arribas et al. (2009) and Millán et al. (2011)

Type	**Description**	**Basin area (km^2)**	**Altitude (m)**	**Lithology**	**Hydrology**	**Mean conductivity (µS cm^{-1})**
1	Small high-mid altitude rivers	<1,000	200-2,000	≥ 40% siliceous	Perennial flow	< 200
2	Small / medium lowland rivers	<1,000	<600	Mixed	Perennial flow	<5,000
3	Large lowland rivers	≥1,000-10,000	<600	Mixed	Perennial flow	<5,000
4	Small / medium mountain rivers	<1,000	600-1,500	≥40% calcareous	Perennial flow	≥ 200-5,000
5	Small, lowland, temporary rivers	<1,000		Mixed	Intermittent or ephemeral flow	<5,000
6	Small medium-lowland hyposaline rivers	<1,000		Calcareous and evaporitic	Perennial, intermittent or ephemeral flow	5,000-32,000
7	Small medium-lowland mesosaline rivers	<1,000		Calcareous and evaporitic	Perennial, intermittent or ephemeral	32,000-130,000

				flow	
8	Small medium-lowland hypersaline rivers	<1,000	Calcareous and evaporitic	Perennial, intermittent or ephemeral flow	>130,000

Table 3. Results of the models evaluating differences in biomonitoring metrics between reference and disturbed saline rivers for types 6 and 7. Explained variance and significance are shown. r^2_m *accounts* for the variance explained by the fixed factors. Metric names - ref: reference condition, dis: disturbed condition; fam: family level, gen: genera level, spp: species level; 6 and 7 refer to the river type where the metric should be applied. Metrics showing significant differences are in bold.

		Type 6 (hyposaline)		***Type 7 (mesosaline)***	
	Metric	r^2_m	***P*-value**	r^2_m	***P*-value**
Widely-used metrics	IBMWP	0.01	0.669	0.00	0.821
	Family richness	0.00	0.792	0.00	0.799
	EPT	0.00	0.991	-	-
	IASPT	0.03	0.300	0.00	0.844
	ICM11a	0.01	0.637	0.01	0.586
	IMMiT	0.01	0.699	0.01	0.622
Novel metrics	ref_fam6	0.00	0.984	0.07	0.141
	dis_fam6	0.10	0.062	0.05	0.237
	ref_gen6	0.03	0.400	0.12	0.074
	dis_gen6	**0.23**	**0.009**	0.10	0.094
	ref_spp6	**0.30**	**0.001**	0.03	0.418
	dis_spp6	**0.19**	**0.013**	0.01	0.543
	ref_fam7	0.02	0.423	**0.47**	**0.000**
	ref_gen7	0.06	0.248	**0.50**	**0.000**
	ref_spp7	0.03	0.419	**0.45**	**0.000**

Figure captions

Fig. 1. Variable importance for models explaining family richness (a), species richness (b), overall community composition (c) and species turnover (d).

Fig. 2. Plots showing the family richness response to conductivity and hydrology (a), hydrology (b), conductivity and season (c) and conductivity and region (d). per: perennial seasonal flow (square), int: intermittent flow (triangle), eph: ephemeral flow (cross); spn: spring; sum: summer; aut: autumn, win: winter; Spa: Spain, Ita: Italy, Mor: Morocco.

Fig. 3. Multidimensional scaling plots showing the concordance between the ordination of biological communities based on family abundances and the environmental (a) and biological (b) classifications of the Spanish reference samples.

Fig. 1

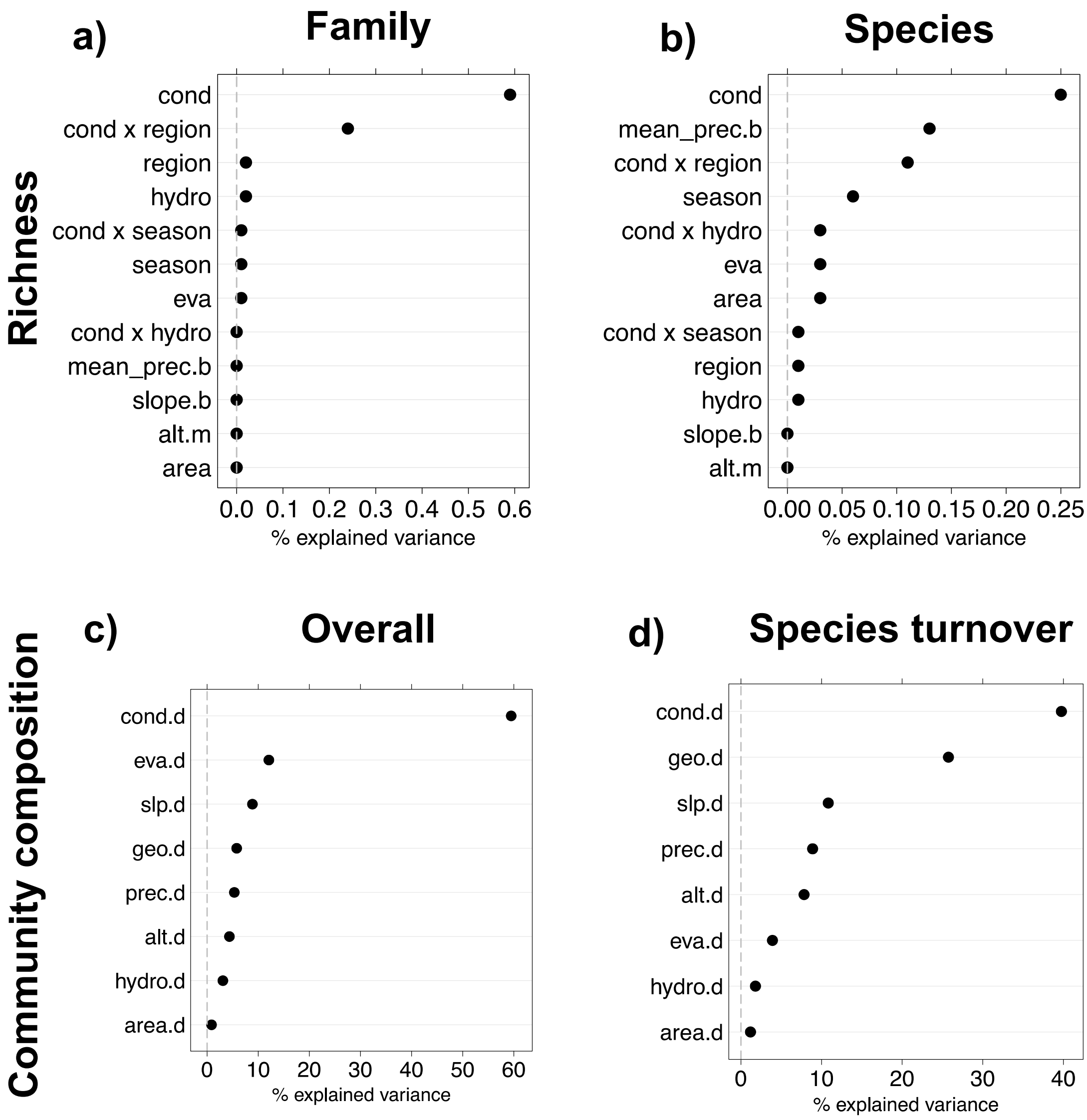
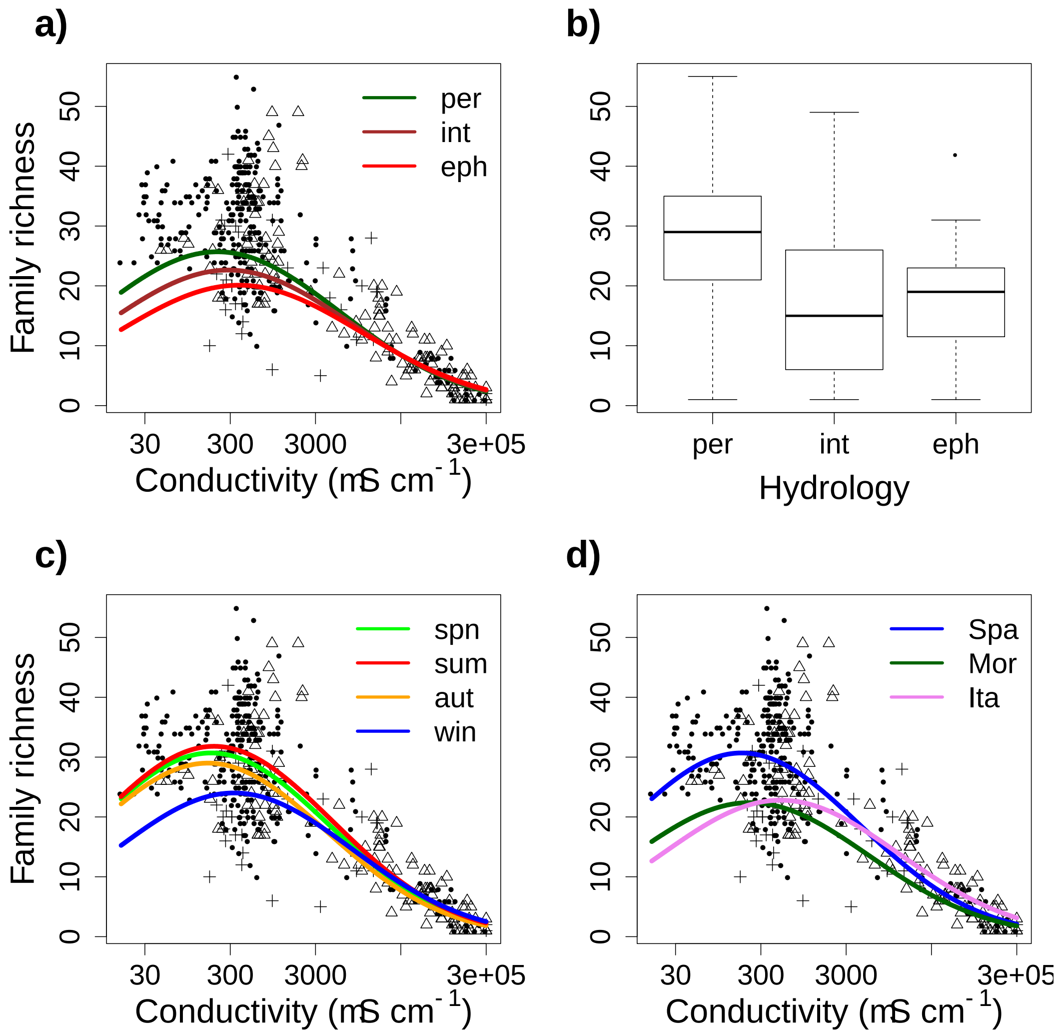
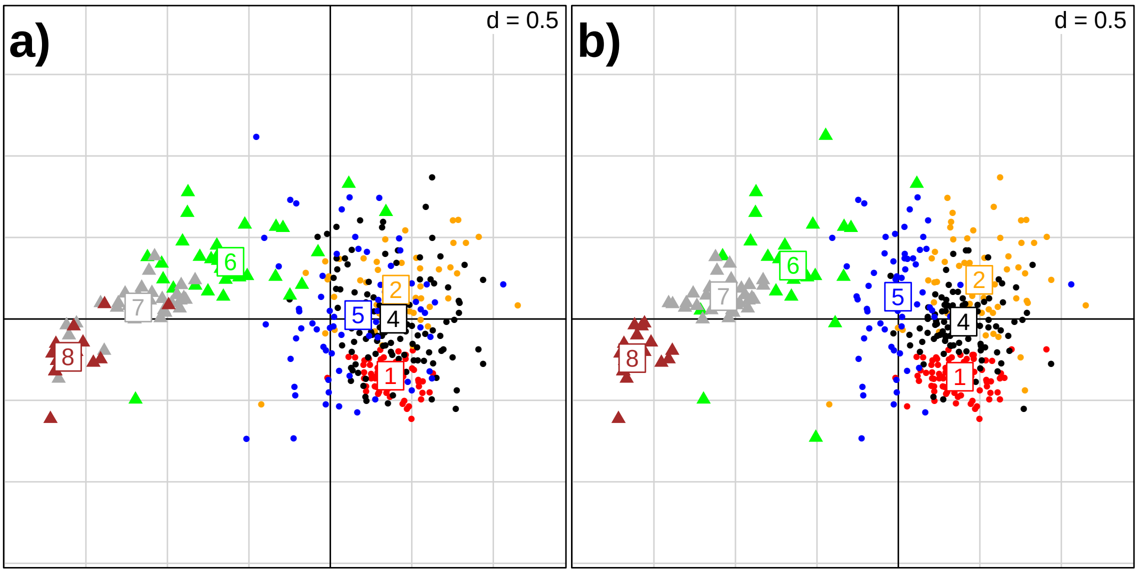

a)
Family
Richness
cond
cond x region
region
hydro
cond x season
season
eva
cond x hydro
mean_prec.b
slope.b
alt.m
area
0.0 0.1 0.2 0.3 0.4 0.5 0.6
% explained variance
b)
Species
cond
mean_prec.b
cond x region
season
cond x hydro
eva
area
cond x season
region
hydro
slope.b
alt.m
0.00 0.05 0.10 0.15 0.20 0.25
% explained variance
Community composition
c)
Overall
cond.d
eva.d
slp.d
geo.d
prec.d
alt.d
hydro.d
area.d
0 10 20 30 40 50 60
% explained variance
d)
Species turnover
cond.d
geo.d
slp.d
prec.d
alt.d
eva.d
hydro.d
area.d
0 10 20 30 40
% explained variance

Fig. 2.



a)
b)
c)
d)
Family richness
Conductivity (mS cm⁻¹)
Hydrology
per
int
eph
spn
sum
aut
win
Spa
Mor
Ita
30
300
3000
3e+05
0
10
20
30
40
50

Fig. 3



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