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

Article Type: Research Paper

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

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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. Bennis, 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.

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2 **Title:** Evaluating anthropogenic impacts on naturally stressed ecosystems: revisiting river
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Barcelona, December 14th, 2018

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

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 $\mu\text{S cm}^{-1}$ for the environmental classification, and one of 4,953-26,158 $\mu\text{S cm}^{-1}$ for the biological classification. The mesosaline type gave a conductivity range of 60,480-110,100 $\mu\text{S cm}^{-1}$ for the environmental classification and one of 24,133-98,200 $\mu\text{S cm}^{-1}$ for the biological classification. The hypersaline type showed a conductivity range of 140,000 - 300,000 $\mu\text{S cm}^{-1}$ for the environmental classification and one of 110,100 - 300,000 $\mu\text{S cm}^{-1}$ 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 $\mu\text{S cm}^{-1}$ (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 Benthological Society, 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 approach 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*. *Freshwater Biology* 57, 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. *Canadian 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 contribution to 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

1 **Abstract**

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

25

26 **Keywords:** abiotic stress, macroinvertebrates, biomonitoring, global change, saline rivers,

27 Water Framework Directive

29 **1. Introduction**

30

31 Naturally stressed ecosystems are characterised by harbouring a set of natural conditions that
32 are persistently unsuitable for the vast majority of the regional species pool (Badyaev, 2005;
33 Parsons, 2005). Well-known examples of naturally stressed ecosystems are found in tundra,
34 deserts, volcanic springs, and in glacier, acid or saline inland waters (Cauvy-Fraunié et al.,
35 2016; Elliott and Quintino, 2007; Millán et al., 2011; Petrin et al., 2007). Their natural stressful
36 conditions reduce the local diversity of these systems ~~but, which is~~ also ~~constitutes~~ a powerful
37 driver of diversification (Parsons, 2005; Vetaas and Grytnes, 2002) ~~and so~~. So they usually
38 hold unique fractions of biodiversity characterised by high levels of specialisation and species
39 turnover (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013; Jacobsen et al., 2012).

40

41 Unfortunately, these ~~genuine~~ singular spots are ~~under threat~~ threatened by global change
42 pressures, which may ~~release~~ modify the naturally stressed conditions causing habitat loss ~~and~~
43 ~~reducing~~ reduce the community's singularity ~~of the community~~ (Finn et al., 2013; Gutiérrez-
44 Cánovas et al., 2013) or add new stressors, such as pesticides or microplastics (Beketov et al.,
45 2013; Windsor et al., 2019). This situation ~~is~~ has been strongly aggravated because many of
46 these naturally stressed ecosystems have been usually ignored ~~one~~ when cataloguing natural
47 heterogeneity, and have consequently been systematically excluded for biomonitoring and
48 conservation purposes (Millán et al., 2011; Stubbington et al., 2018).

49

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

58 | ~~there are~~ some generalised shortcomings ~~on the application of~~ come into when applying these
59 | procedures that ~~resulted~~ result in a deficient functionality for the assessment and protection of
60 | naturally stressed rivers. Firstly, main natural stressors, such as water salinity, are usually
61 | neglected or not ~~fully~~ completely considered in river classifications, ~~resulting and this results~~
62 | the exclusion or misclassification of naturally stressed rivers (Sánchez-Montoya et al., 2007).
63 | This occurs because the classification process tends to ~~be focused~~ focus on coarse environmental
64 | descriptors, which are weak proxies of ~~local~~ reach-scale stressors. Secondly, most ~~of the~~
65 | proposed biomonitoring metrics can result in equivocal evaluations of the ecological status of
66 | ~~the~~ naturally stressed rivers because they are ~~strongly~~ closely associated ~~to~~ with local
67 | richness/diversity and ~~so do~~ not ~~considering~~ consider naturally depauperate communities
68 | (Gutiérrez-Cánovas et al., 2008). Besides, other widely-used metrics are based on taxa or
69 | functional traits that are rare, or ~~not~~ even ~~not~~ present, in naturally stressed rivers, ~~such as; e.g.,~~
70 | the stress-sensitive orders Ephemeroptera, Plecoptera and Trichoptera (EPT) (Belmar et al.,
71 | 2013; Bonada et al., 2006; Millán et al., 2011). ~~Therefore, Hence~~ there is an urgent need to
72 | revisit and adapt the current vision and approaches to assess the ecological quality of naturally
73 | stressed river ecosystems, and ~~so~~ to improve their management and future preservation.

74 |

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

87

88 2. Material and methods

89

90 ~~2.1. Datasets description~~ Description of datasets

91

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

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

101

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

114

115 | ~~ClimaticClimate~~, geomorphologic, lithologic and land use variables at basin and reach scales
116 | were obtained from digital layers after delineating the river basins of each sampling site (see
117 | Appendix A, Table A1 for a complete list of the ~~used~~ variables~~—used~~). Water electrical
118 | conductivity was measured *in situ* on each sampling occasion as an osmotic stress indicator. To
119 | characterise flow intermittence, we categorised the hydrological regime of each site as perennial
120 | seasonal (typically flowing), intermittent (surface flow ceases during the dry season, pools
121 | remaining) or ephemeral (~~totallycompletely~~ dry during one season) ~~flowflows~~ from available
122 | hydrological information or field evidence (Belmar et al., 2013; Sánchez-Montoya et al., 2007).
123 | We categorised the sampling sites as *reference* when they were minimally disturbed (i.e.
124 | fulfilling ≥ 16 out of 20 of the Mediterranean Reference Criteria, MRC; Sánchez-Montoya et
125 | al., 2009) or disturbed when they were substantially impacted by anthropogenic activities (i.e.
126 | fulfilled < 16 MRC). In this study, we excluded large watercourses (mean basin area $\geq 1,000$
127 | km², ECOSTAT, type 3, see Table 1 and Sánchez-Montoya et al., 2007 for details) because of
128 | the paucity of reference sites (Sánchez-Montoya et al., 2009). We also excluded disturbed
129 | freshwater rivers, for which effective biomonitoring metrics can be found elsewhere (e.g. Birk
130 | et al., 2012; Bonada et al., 2006).

131

132 | 2.2. Data analysis

133

134 | Before performing the analyses, we applied a log-transformation to macroinvertebrate family
135 | richness and a square-root transformation to macroinvertebrate species richness. ~~Besides,~~
136 | ~~logitLogit~~-, log- or square-root-transformations were applied to the quantitative environmental
137 | variables to reduce their distribution skewness and to improve linearity, ~~whenwhenever~~
138 | necessary. ~~Moreover, allAll~~ the quantitative environmental variables were also standardised to
139 | mean=0 and SD=1 to facilitate model coefficient ~~comparisoncomparisons~~.

140

141 | 2.2.1. Ranking environmental variable importance

142

143 | To identify the main environmental factors ~~determining the~~that determine family and species
144 | richness, we used Random Forest (randomForestSRC R package, Ishwaran Ishwaran et al.,
145 | 2014) and Linear Mixed-effect Models (LMM, lme4 R packge, Bates et al., 2015). In these
146 | models, we utilised family-level datasets (i.e. ref_fam_ita, ref_fam_mor, ref_fam_spa, n=458)
147 | and species-level datasets (i.e. ref_spp_ita, ref_spp_mor, ref_spp_spa, n=211) from the
148 | reference sites (selected according to MRC; see above). Following Feld et al. (2016), we first
149 | ran Random Forest models to identify the most important predictors of family and species
150 | richness among the 24 potential candidates (Appendix A, Table A1) to be included into the
151 | LMM (see Appendix C for more details about exploratory analyses). After these exploratory
152 | analyses, we included basin area, mean basin altitude, mean basin slope, mean basin annual
153 | rainfall, evaporitic surface, flow intermittence, conductivity (single and quadratic terms), season
154 | and region as fixed factors in the LMM. As additional fixed factors, we also included the
155 | ~~pairwise~~pair-wise interactions of conductivity x flow intermittence, conductivity x season and
156 | conductivity x region. Site code was considered ~~as~~ a random factor to account for repeated
157 | measures in the same location. To rank the environmental predictor's importance on family and
158 | species richness, we adopted a multi-model inference approach (Grueber et al., 2011); using the
159 | *MuMIn* R package (Bartoń, 2016). This statistical technique ranks all the ~~models~~ generated
160 | models using all the possible ~~combination~~combinations of predictors ~~using~~based on Akaike's
161 | Information Criterion (AIC). Then, a set of top models ~~is~~was selected to produce an average
162 | model, but only if the model ranking first ~~is~~was ambiguously supported (model weight < 0.90).
163 | We chose top models ~~differing~~which differed in no more than two AIC units ($\Delta AIC \leq 2$) from the
164 | model ranked first (minimum AIC). We adopted a natural average method to conduct ~~the~~ model
165 | averaging, which consists in averaging predictors only over the models in which the predictor
166 | appears and in weighting predictor's ~~SES~~coefficients by the summed weights of these models
167 | (Burnham and Anderson, 2002). For each LMM model, two ~~measures of~~ goodness-of-fit
168 | measures were estimated (Nakagawa and Schielzeth, 2013): marginal goodness-of-fit (r^2_m)
169 | indicates the variance explained only by the fixed factors, while conditional goodness-of-fit (r^2_c)
170 | shows the variance accounted for by both fixed and random terms. We provide the mean
171 | ~~average~~ (based on model weights) of each goodness-of-fit measure for ~~each~~every averaged

172 | model. All ~~the~~ models met the normality and homoscedasticity assumptions, which were
173 | validated by visually checking their residuals ~~for normality and homoscedasticity.~~

174

175 | To identify the environmental drivers of community composition change, we used Multiple
176 | Regression models for distance Matrices (MRM; ecodist R package, Lichstein, 2007). This
177 | method is conceptually similar to traditional multiple regression, but with all variables being
178 | distance matrices instead of raw data and *P*-values being calculated ~~throughby~~ permutation tests
179 | (~~1000~~1,000 runs). To avoid lack of independence problems due to multiple samples belonging
180 | to the same site, we selected a reference subset of macroinvertebrate families (ref_fam_all,
181 | n=157 sites/samples) and species (ref_spp_all, n=76 sites/samples) occurrences with only one
182 | spring sample per site. We estimated ~~the~~ overall changes in community composition for each
183 | pair of sites of the family matrix ~~throughwith~~ the Sørensen dissimilarity index (β_{sor}) and ~~the~~
184 | ~~pairwise~~pair-wise dissimilarity due to turnover from the species matrix ~~usingwith~~ the Simpson
185 | index (β_{sim}). These calculations were made following ~~the~~ Baselga's (2010) framework for β -
186 | diversity partitioning using the *betapart* R package (Baselga and Orme, 2012). For each selected
187 | environmental predictor (basin area, mean basin altitude, mean basin slope, mean basin annual
188 | rainfall, evaporitic surface, flow intermittence, conductivity, geographic distance), we built a
189 | Euclidean distance matrix based on their transformed and standardised values. ~~Geographical~~The
190 | geographical distance between localities was based on a latitude and longitude original matrix
191 | ~~and, while~~ flow intermittence was based on ~~semiquantitative~~semi-quantitative values
192 | (perennial=0, intermittent=1, ephemeral=2).

193

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

197

198 | **2.2.2. Integrating saline rivers into biomonitoring typologies**

199

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

221

222 | To evaluate the performance of both classification procedures in a ~~wider~~broader geographical
223 | context, we estimated their classification strength based on ~~datasets of the~~ family and species
224 | ~~datasets~~ from reference sites ~~of in~~ Italy (ref_fam_ita, n=44; ref_spp_ita, n=31), Morocco
225 | (ref_fam_mor, n=28; ref_spp_mor, n=29) and Spain (ref_fam_spa, n=386; ref_spp_spa, n=151).
226 | We assigned environmental types to these new sites using the environmental classification
227 | criteria (Table 2). To assign biological types to the new sites, we built a Random Forest model
228 | ~~by~~ predicting biological types from environmental information. To develop the Random Forest

229 model (trees=2000, mtry=8), we used a subset of the ref_fam_spa dataset (n=258), while the
230 non-utilised samples (n=128) were ~~used~~employed along with the other independent subsets to
231 evaluate ~~the~~ classification performance. To estimate classification strength (CS), dissimilarity
232 matrices were converted ~~to~~into similarity matrices. CS was quantified as the difference between
233 the within-type mean similarity (W) and between-~~type~~type mean similarity (B) of the Bray-
234 Curtis ~~pairwise~~pair-wise similarity based on family abundances, and for the Simpson similarity
235 matrix based on species occurrences and turnover (CS=W-B) for the three regions. ~~CS~~The mean
236 CS values were calculated through a bootstrapping procedure, where we resampled 100 subsets
237 of n=28 from each Italian and Spanish macroinvertebrate ~~datasets~~dataset to make their CS
238 values comparable to those obtained for the Moroccan datasets, which had the ~~lowest number~~
239 ~~of~~fewest observations.

240

241 2.2.3. Biomonitoring indicators for saline river types

242

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

258

259 To identify the families, genera and species ~~showing~~with a ~~greater~~higher affinity ~~for~~to each
260 reference and disturbed type, we ~~used~~ran an indicator species analysis (IndVal, Dufrêne and
261 Legendre, 1997). This analysis considers ~~the~~each taxon's percentage of occurrence and relative
262 abundance ~~of each taxon~~ for each type to obtain an indicator value (IV) and its significance
263 through Monte-Carlo permutations (1000 runs). We focused on the most frequent taxa, by
264 keeping taxa occurring in more than 10% of ~~the~~observations. From them, we selected those
265 taxa ~~showing~~which showed a significant Indicator Value ($P \leq 0.05$) as potential indicators for a
266 given type. From these results, we built the candidate metrics of reference and disturbed
267 conditions of the saline river types. To create ~~those~~these metrics, for each sample, we summed
268 the abundances of the indicator taxa of the assigned river type (ref_6, dis_6, ref_7, dis_7, ref_8)
269 for ~~the~~ family, genus, and species level (e.g. for ~~the~~ family level: ref_fam6, dis_fam6, ref_fam7,
270 dis_fam7, ref_fam8). In addition, for each sample, we estimated a set of widely-used
271 biomonitoring metrics (family richness, EPT family richness, IBMWP, IASPT) and multi-
272 metric ~~indexes~~indices (ICM-11a and IMMi-T) for Mediterranean rivers (Alba-Tercedor et al.,
273 2002; Munné and Prat, 2009). Finally, to evaluate the performance of these candidate metrics
274 against the widely-used biomonitoring metrics, we used LMM models ~~assessing~~to assess the
275 differences across all ~~the~~reference and disturbed types (levels= ref_6, dis_6, ref_7, dis_7, ref_8)
276 and Tukey-*t post-hoc* tests to evaluate differences between pairs of comparable reference and
277 disturbed types (i.e. ref_6 vs. dis_6, ref_7 vs. dis_7).

278

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

281

282 3. Results

283

284 3.1. Ranking environmental variable importance

285

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

304

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

314

315 3.2. Integrating saline rivers into biomonitoring typologies

316

317 ~~The nMDS ordinations of samples according~~According to their biological communities, the
318 nMDS ordinations of samples revealed that environmental and biological classification methods
319 produced roughly similar river types (Fig. 3 and Appendix G, Table G1, Figs. G2 and G3).
320 After ignoring large watercourses (type 3), both classifications included three types of
321 freshwater perennial rivers (types 1, 2 and 4), a type ~~mainly~~ comprised mainly of freshwater
322 intermittent and ephemeral rivers (type 5), and three types of saline rivers ~~of~~with increasing
323 conductivity (types 6, 7 and 8, see Appendix G, Table G1). ~~Freshwater~~The freshwater perennial
324 river types included headwater watercourses of very low conductivity ~~draining~~that drained
325 mountainous siliceous catchments (type 1), mid-mountain rivers of low conductivity
326 ~~draining~~that drained medium-~~size,~~sized calcareous catchments (type 2) and calcareous high
327 mountain headwaters of very low to low conductivity (type 4). Although both classifications
328 identified a type of temporary ~~rivers~~river (type 5), the type defined by the environmental
329 classification included a higher proportion of temporary rivers (68% of intermittent and 32% of
330 ephemeral watercourses) than the type defined by the biological classification (28% of
331 intermittent and 26% of ephemeral watercourses). The ~~new~~three new saline river types ~~(,~~
332 ~~hyposaline,~~ (type 6), mesosaline (type 7) and hypersaline ~~river types)~~ were ~~characterised by~~
333 ~~smaller basin areas, lower elevations, softer slopes, arid climates, and greater evaporitic surface~~
334 ~~and conductivity relative to the freshwater types (Table 2 and Appendix G, Table G1). Also,~~
335 ~~more than a half of the surveyed saline rivers were intermittent or ephemeral. The~~(type 8),
336 showed conductivity ranges that ~~define the saline types~~ were generally similar ~~in~~for both
337 classification procedures, but some minor discrepancies were also found (Appendix G, Table
338 G1). The hyposaline type showed a conductivity range (Q10-Q90) of 8,323-24,567 $\mu\text{S cm}^{-1}$ for
339 the environmental classification, and one of 4,953-26,158 $\mu\text{S cm}^{-1}$ for the biological
340 classification. The mesosaline type gave a conductivity range of 60,480-110,100 $\mu\text{S cm}^{-1}$ for the
341 environmental classification and one of 24,133-98,200 $\mu\text{S cm}^{-1}$ for the biological classification.
342 The hypersaline type showed a conductivity range of 140,000 - 300,000 $\mu\text{S cm}^{-1}$ for the
343 environmental classification and one of 110,100 - 300,000 $\mu\text{S cm}^{-1}$ for the biological

344 classification. All these types were characterised by smaller basin areas, lower elevations, softer
345 slopes, arid climates and greater evaporitic surfaces and conductivity in relation to the
346 freshwater types (Table 2 and Appendix G, Table G1). More than half the surveyed saline rivers
347 were also intermittent or ephemeral. Classification strength based on family abundances was
348 roughly similar between the environmental (CS=0.150±0.007) and biological
349 (CS=0.158±0.006) classification procedures (Appendix G, Fig. G1). However, environmental
350 classification (CS=0.203±0.008) ~~seems to be~~ seemed better ~~in~~ at representing species turnover
351 among types compared to the biological classification (CS=0.170±0.005).

352 **3.3. Biomonitoring indicators for saline river types**

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

367

368 For hyposaline rivers (type 6), the metrics based on the species ~~of~~ at reference sites (ref_spp6,
369 LMM $r^2_m=0.30$, Tukey t -test $p=0.001$) and the metrics based on the genera (dis_gen6, LMM
370 $r^2_m=0.23$, Tukey t -test $p=0.009$) and species (dis_spp6, LMM $r^2_m=0.19$, Tukey t -test $p=0.009$) of

371 ~~the~~ disturbed sites were the best indicators (Table 3). For mesosaline rivers (type 7), ~~the~~ metrics
372 based on ~~the~~ families (ref_fam7, $r^2_m=0.47$, Tukey t -test $p<0.001$), genera (ref_gen7, $r^2_m=0.50$,
373 Tukey t -test $p<0.001$) and species (ref_spp7, $r^2_m=0.45$, Tukey t -test $p<0.001$) of ~~the~~ reference
374 sites were the best indicators (Table 3). Contrarily, conventional biomonitoring metrics (family
375 richness, EPT family richness, IBMWP, IASPT) and multi-metric ~~indexes~~indices (ICM-11a and
376 IMMi-T) showed a null capacity ~~to discriminate~~for discriminating between ~~the~~ reference and
377 disturbed conditions for saline river types (Table 3).

378

379 4. Discussion

380 4.1. Water salinity as a driver of community richness and composition at regional- 381 continental scales

382

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

391

392 ~~Over~~All along a particular environmental gradient, the degree to which certain levels of
393 environmental filtering ~~could~~can be considered stressful or harmful depends on how well
394 adapted ~~is~~ the regional pool is (Badyaev, 2005; Taylor et al., 1990). Thus, the number of taxa
395 ~~able to cope over~~capable of coping with each portion of the stress gradient is linked to regional
396 and historical aspects, such as ~~the~~ long-term persistence and frequency of stressful conditions
397 (Taylor et al., 1990) and the evolutionary context of each lineage (Buchwalter et al., 2008). The
398 long-term persistence of the osmotic stress associated ~~to~~with Mediterranean saline rivers is

399 | expected to act as a strong driver of community assembly, but also as a source of ecological
400 | diversification in aquatic lineages. In naturally saline rivers, osmotic pressure imposes a chronic
401 | filter for organisms ~~trying that attempt~~ to colonise, thrive or reproduce (Velasco et al., 2019).
402 | Regarding insects, the ~~important major~~ drop in community richness at conductivities >larger
403 | than 3,000 $\mu\text{S cm}^{-1}$; is was strongly associated with the existence of few lineages ~~presenting that~~
404 | present specific mechanisms to maintain internal integrity once submerged under hyperosmotic
405 | media (Arribas et al., 2019; Bradley, 2008; Millán et al., 2011). These are mostly the taxa
406 | ~~belonging which belong~~ to the families Hydrophilidae, Dytiscidae, and Hydraenidae
407 | (Coleoptera), Corixidae (Hemiptera), and Culicidae, Ephyridae, Stratiomyidae, Chironomidae
408 | (Diptera) (Arribas et al., 2019; Bradley, 2008; Pallarés et al., 2017a); and they all ~~of them~~
409 | ~~comprising comprise~~ good biological indicators of the reference saline streams. Thus; our results
410 | reveal a clear differentiation in community composition, and; a particularly, ~~a~~ strong
411 | replacement of taxa along the conductivity gradient, which also ~~concordant coincides~~
412 | previous studies ~~enabout~~ this natural stress gradient (Gascón et al., 2016; Gutiérrez-Cánovas et
413 | al., 2013) ~~and on the, and with~~ high levels of habitat specificity associated ~~to the with~~
414 | stress (Carbonell et al., 2012).

415

416 | **4.2. Integrating saline types into river classifications**

417 | The main advantage of our approach is ~~the integration of that it integrates~~ the whole spectrum of
418 | environmental and biological variation into a single comprehensive classification, which is
419 | either environmentally or biologically based, ~~that and~~ allows a more accurate and simple
420 | classification of rivers in the Mediterranean region. This new integrated typology could help to
421 | better implement WFD ~~in the state members, whose legal criteria ignore or misclassify into~~
422 | Member States and to gather a comprehensive inventory of saline rivers; which are currently
423 | ignored or misclassified by current laws. For example, the ~~Spanish~~ official Spanish typology of
424 | rivers recognises three types of highly mineralised rivers (official types 7, 9 and 13). However,
425 | the mean conductivities of ~~this these~~ official types range is 448-545 $\mu\text{S cm}^{-1}$ (Spanish
426 | Government, 2009); which is significantly lower than the conductivities ~~showed shown~~ by the

427 saline rivers studied ~~here. Also, herein. Moreover~~ for the first time, our classifications implicitly
428 ~~recognises~~recognise the importance of considering the whole natural osmotic stress gradient, ~~by~~
429 providing a classification method that encompasses more biodiversity ~~that the~~than previous
430 individual attempts (e.g. Arribas et al., 2009; Sánchez-Montoya et al., 2007).

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

440 Taken together, these results advocate the use of river saline types that included both perennial
441 and intermittent rivers. However, future work using more precise hydrological data should
442 check the consistency of these saline river types along flow intermittency gradients.
443 Quantitative hydrological data offer a much more adequate indication of the drought stress that
444 affects biological communities (Belmar et al., 2013; Gallart et al., 2016; Jaeger and Olden,
445 2012). Unfortunately, these data are currently unavailable for most studied rivers, so simple
446 categorical descriptors of the hydrological regime were used instead.

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

4.3. Metrics to evaluate anthropogenic impacts on saline rivers

454

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

472

473 The abundance of specialist taxa seems to provide a much better indication of reference and
474 degraded conditions than diversity-based metrics ~~(Cañedo-Argüelles et al., 2012)~~. These
475 metrics can ~~be~~ also be used to monitor their populations, which are scattered across the territory
476 and threatened by human pressures (Arribas et al., 2015). Nonetheless, we admit that our
477 proposed metrics ~~are~~ is a first attempt to effectively showcase the type of biomonitoring tools
478 that would work in saline rivers, ~~so they should be cautiously taken~~. Therefore, they may
479 ~~require~~ benefit from further ~~refinement~~ refinements by gathering larger datasets of observational
480 data ~~and~~ combined with manipulative experiments, which both ~~covering~~ cover different types of
481 impacts (e.g. dilution, nutrient enrichment). Furthermore, some of the indicator species for the

482 saline types defined herein (e.g. *Ochthebius glaber*, *Nebrioporus baeticus*) are endemic of the
483 Iberian Peninsula. Fortunately, these taxa have sister species with very similar ecological
484 requirements in other biogeographic regions (Arribas et al., 2015), and could be used as
485 effective indicators of reference or disturbed conditions.

486

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

498

499 **5. Conclusions**

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

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

512

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529

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747

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 [haveobtain](#) an independent dataset to evaluate [the](#) 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
			Genus,								
ref_spp_spa_sal	Reference	Coleoptera	Species	Spain	Saline rivers	30	75				x

dis_fam_spa_sal	Disturbed	All	Family	Spain	Saline rivers	17	31	x
			Genus,					
dis_spp_spa_sal	Disturbed	Coleoptera	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 ($\mu\text{S cm}^{-1}$)
1	Small high-mid altitude rivers	<1,000	200-2,000	$\geq 40\%$ siliceous	Perennial flow	< 200
2	Small / medium lowland rivers	<1,000	<600	Mixed	Perennial flow	<5,000
3	Large lowland rivers	$\geq 1,000$ - 10,000	<600	Mixed	Perennial flow	<5,000
4	Small / medium mountain rivers	<1,000	600-1,500	$\geq 40\%$ 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

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

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_m^2 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.

		<i>Type 6 (hyposaline)</i>		<i>Type 7 (mesosaline)</i>	
	Metrics	r_m^2	P-value	r_m^2	P-value
<i>Widely-used</i>					
<i>metrics</i>	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
<i>Novel metrics</i>	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

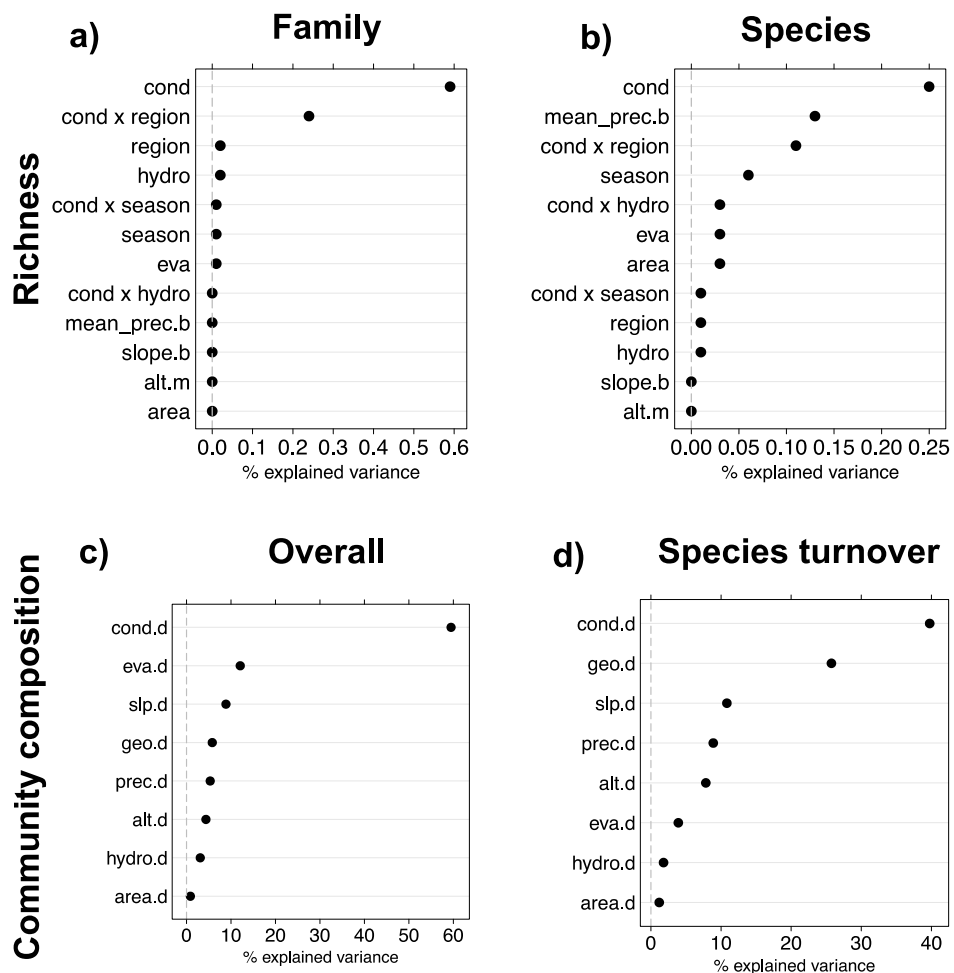


Fig. 2.

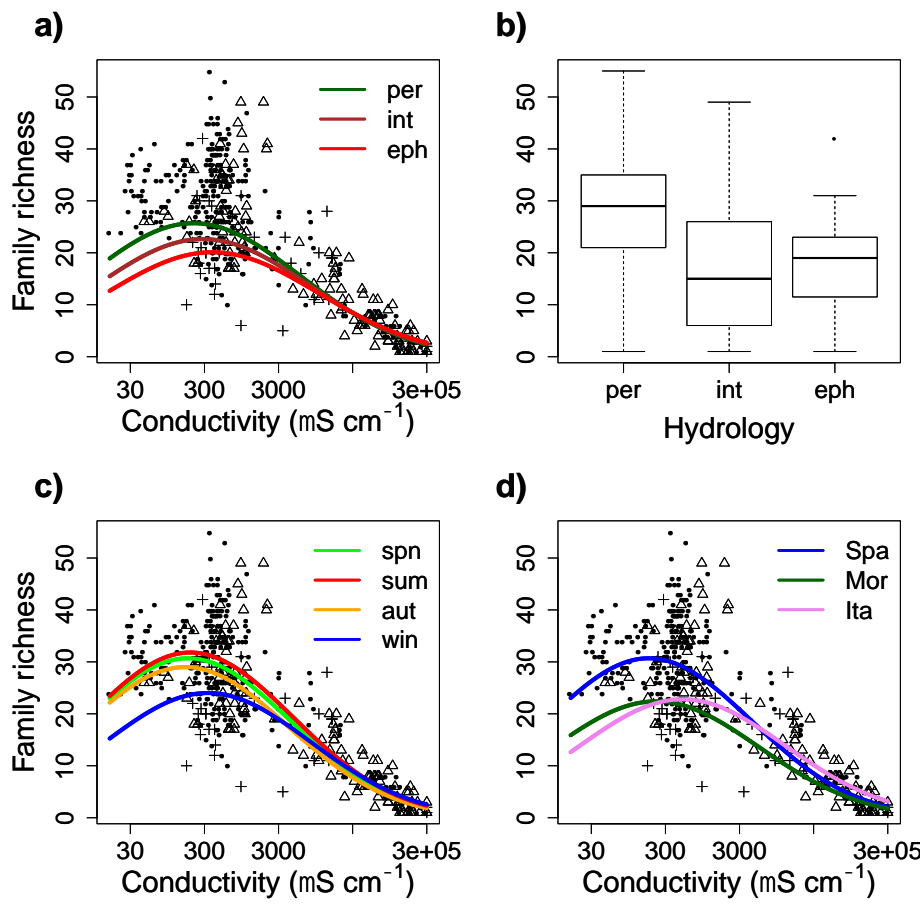
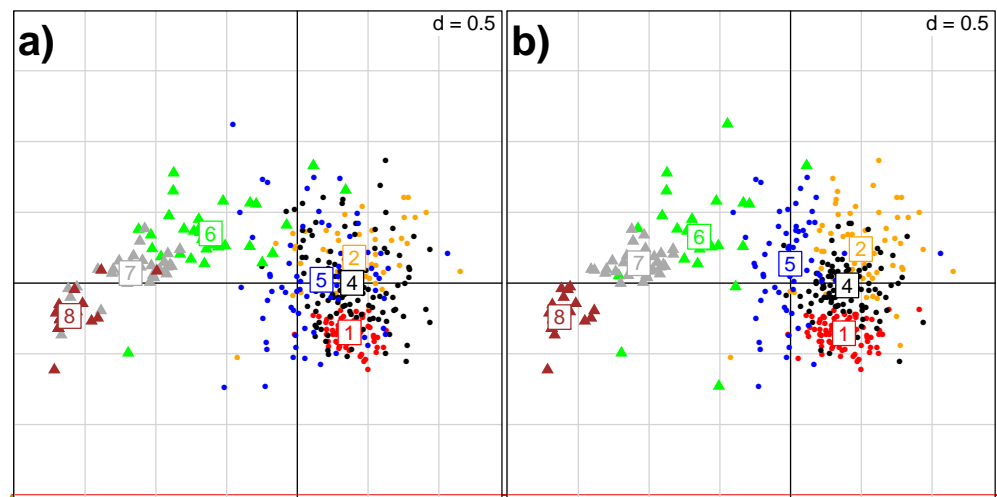
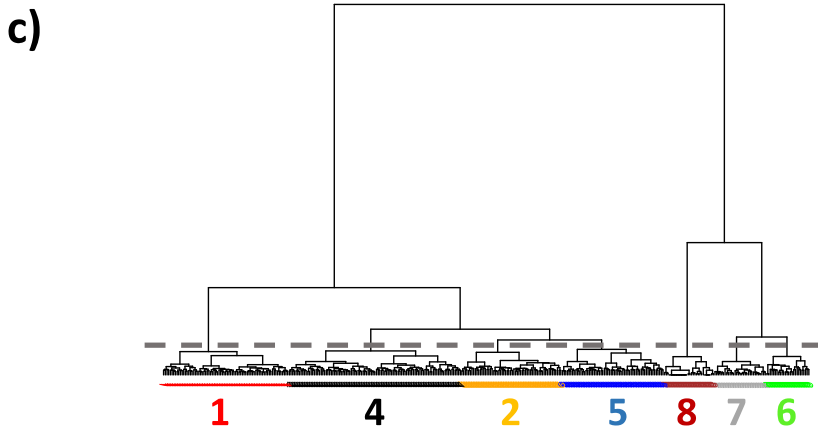
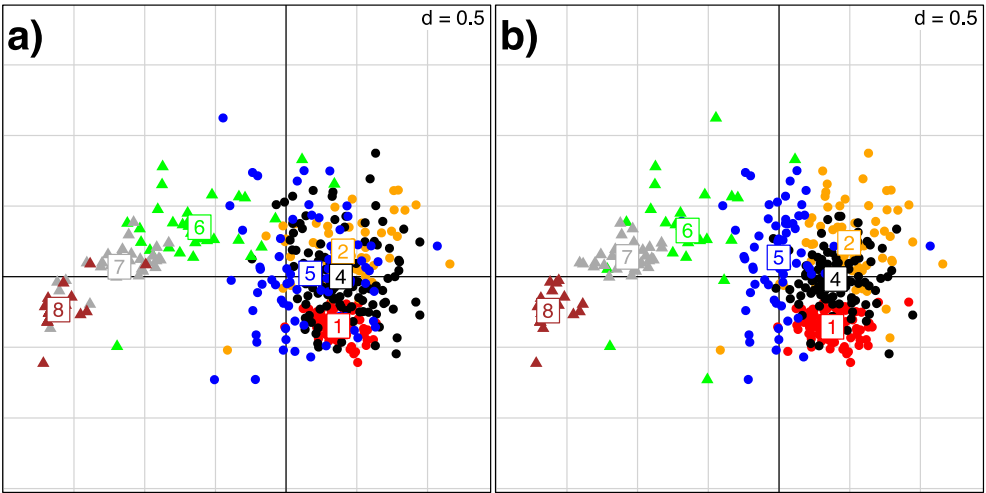


Fig. 3



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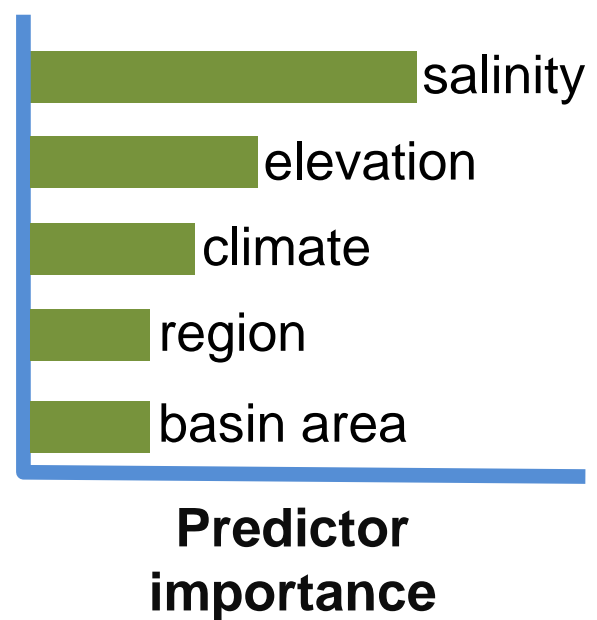


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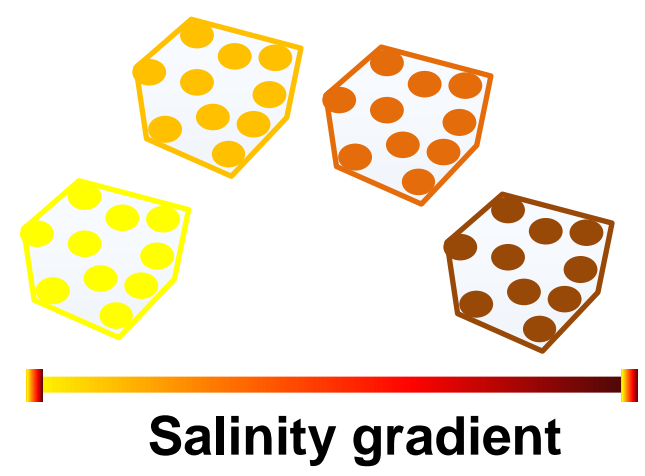
Salinity



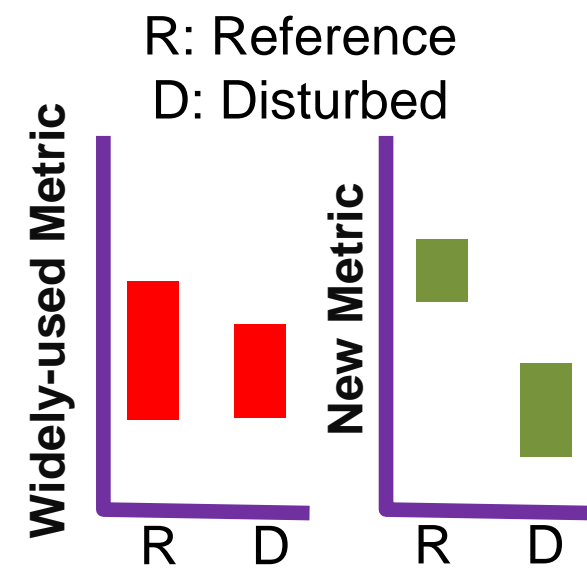
Drivers of biological richness and composition



Classifying rivers encompassing broad environmental gradients



Biomonitoring metrics for saline rivers



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

- 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

1 **Abstract**

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

24

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

27

28 **1. Introduction**

29

30 Naturally stressed ecosystems are characterised by harbouring a set of natural conditions that
31 are persistently unsuitable for the vast majority of the regional species pool (Badyaev, 2005;
32 Parsons, 2005). Well-known examples of naturally stressed ecosystems are found in tundra,
33 deserts, volcanic springs, and in glacier, acid or saline inland waters (Cauvy-Fraunié et al.,
34 2016; Elliott and Quintino, 2007; Millán et al., 2011; Petrin et al., 2007). Their natural stressful
35 conditions reduce the local diversity of these systems, which is also a powerful driver of
36 diversification (Parsons, 2005; Vetaas and Grytnes, 2002). So they usually hold unique fractions
37 of biodiversity characterised by high levels of specialisation and species turnover (Finn et al.,
38 2013; Gutiérrez-Cánovas et al., 2013; Jacobsen et al., 2012).

39

40 Unfortunately, these singular spots are threatened by global change pressures, which may
41 modify the naturally stressed conditions causing habitat loss, reduce the community's
42 singularity (Finn et al., 2013; Gutiérrez-Cánovas et al., 2013) or add new stressors, such as
43 pesticides or microplastics (Beketov et al., 2013; Windsor et al., 2019). This situation has been
44 strongly aggravated because many of these naturally stressed ecosystems have been usually
45 ignored when cataloguing natural heterogeneity, and have consequently been systematically
46 excluded for biomonitoring and conservation purposes (Millán et al., 2011; Stubbington et al.,
47 2018).

48

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

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

71

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

83

84 **2. Material and methods**

85

86 2.1. Description of datasets

87

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

97

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

109

110 Climate, geomorphologic, lithologic and land use variables at basin and reach scales were
111 obtained from digital layers after delineating the river basins of each sampling site (see
112 Appendix A, Table A1 for a complete list of the used variables). Water electrical conductivity
113 was measured *in situ* on each sampling occasion as an osmotic stress indicator. To characterise
114 flow intermittence, we categorised the hydrological regime of each site as perennial seasonal

115 (typically flowing), intermittent (surface flow ceases during the dry season, pools remaining) or
116 ephemeral (completely dry during one season) flows from available hydrological information or
117 field evidence (Belmar et al., 2013; Sánchez-Montoya et al., 2007). We categorised the
118 sampling sites as *reference* when they were minimally disturbed (i.e. fulfilling ≥ 16 out of 20 of
119 the Mediterranean Reference Criteria, MRC; Sánchez-Montoya et al., 2009) or disturbed when
120 they were substantially impacted by anthropogenic activities (i.e. fulfilled < 16 MRC). In this
121 study, we excluded large watercourses (mean basin area $\geq 1,000$ km², ECOSTAT, type 3, see
122 Table 1 and Sánchez-Montoya et al., 2007 for details) because of the paucity of reference sites
123 (Sánchez-Montoya et al., 2009). We also excluded disturbed freshwater rivers, for which
124 effective biomonitoring metrics can be found elsewhere (e.g. Birk et al., 2012; Bonada et al.,
125 2006).

126

127 **2.2. Data analysis**

128

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

135

136 **2.2.1. Ranking environmental variable importance**

137

138 To identify the main environmental factors that determine family and species richness, we used
139 Random Forest (randomForestSRC R package, Ishwaran Ishwaran et al., 2014) and Linear
140 Mixed-effect Models (LMM, lme4 R package, Bates et al., 2015). In these models, we utilised
141 family-level datasets (i.e. ref_fam_ita, ref_fam_mor, ref_fam_spa, n=458) and species-level
142 datasets (i.e. ref_spp_ita, ref_spp_mor, ref_spp_spa, n=211) from the reference sites (selected
143 according to MRC; see above). Following Feld et al. (2016), we first ran Random Forest models

144 to identify the most important predictors of family and species richness among the 24 potential
145 candidates (Appendix A, Table A1) to be included in the LMM (see Appendix C for more
146 details about exploratory analyses). After these exploratory analyses, we included basin area,
147 mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow
148 intermittence, conductivity (single and quadratic terms), season and region as fixed factors in
149 the LMM. As additional fixed factors, we also included the pair-wise interactions of
150 conductivity x flow intermittence, conductivity x season and conductivity x region. Site code
151 was considered a random factor to account for repeated measures in the same location. To rank
152 the environmental predictor's importance on family and species richness, we adopted a multi-
153 model inference approach (Grueber et al., 2011) using the *MuMIM* R package (Bartoń, 2016).
154 This statistical technique ranks all the generated models using all the possible combinations of
155 predictors based on Akaike's Information Criterion (AIC). Then a set of top models was
156 selected to produce an average model, but only if the model ranking first was ambiguously
157 supported (model weight < 0.90). We chose top models which differed in no more than two AIC
158 units ($\Delta \leq 2$) from the model ranked first (minimum AIC). We adopted a natural average
159 method to conduct model averaging, which consists in averaging predictors only over the
160 models in which the predictor appears and in weighting predictor's coefficients by the summed
161 weights of these models (Burnham and Anderson, 2002). For each LMM model, two goodness-
162 of-fit measures were estimated (Nakagawa and Schielzeth, 2013): marginal goodness-of-fit (r^2_m)
163 indicates the variance explained only by the fixed factors, while conditional goodness-of-fit (r^2_c)
164 shows the variance accounted for by both fixed and random terms. We provide the mean (based
165 on model weights) of each goodness-of-fit measure for every averaged model. All the models
166 met the normality and homoscedasticity assumptions, which were validated by visually
167 checking their residuals.

168

169 To identify the environmental drivers of community composition change, we used Multiple
170 Regression models for distance Matrices (MRM; *ecodist* R package, Lichstein, 2007). This
171 method is conceptually similar to traditional multiple regression, but with all variables being
172 distance matrices instead of raw data and *P*-values being calculated by permutation tests (1,000

173 runs). To avoid lack of independence problems due to multiple samples belonging to the same
174 site, we selected a reference subset of macroinvertebrate families (ref_fam_all, n=157
175 sites/samples) and species (ref_spp_all, n=76 sites/samples) occurrences with only one spring
176 sample per site. We estimated the overall changes in community composition for each pair of
177 sites of the family matrix with the Sørensen dissimilarity index (β_{sor}) and pair-wise dissimilarity
178 due to turnover from the species matrix with the Simpson index (β_{sim}). These calculations were
179 made following Baselga's (2010) framework for β -diversity partitioning using the *betapart* R
180 package (Baselga and Orme, 2012). For each selected environmental predictor (basin area,
181 mean basin altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow
182 intermittence, conductivity, geographic distance), we built a Euclidean distance matrix based on
183 their transformed and standardised values. The geographical distance between localities was
184 based on a latitude and longitude original matrix, while flow intermittence was based on semi-
185 quantitative values (perennial=0, intermittent=1, ephemeral=2).

186

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

190

191 **2.2.2. Integrating saline rivers into biomonitoring typologies**

192

193 To classify rivers by encompassing the environmental and biological variability that occurs in
194 the study area, we used family abundances from reference Spanish sites (ref_fam_spa, n=386).
195 We selected this dataset because it included more sites and samples, and it covered a broader
196 environmental and biological spectrum in relation to the Italian and Moroccan datasets
197 (Appendix A, Table A1). We firstly classified sites into seven types according to their
198 environmental variables (environmental classification) following an adaptation of the criteria
199 suggested by the ECOSTAT intercalibration group for Mediterranean rivers using System A of
200 the WFD (MedGIG European Commission, 2007). This included mean conductivity, basin area,
201 hydrology, site altitude and basin lithology (Table 2 and Appendix D). Secondly, in order to

202 classify samples according to their biological communities (biological classification), we
203 estimated a Bray-Curtis pair-wise dissimilarity matrix, which derived from the abundance
204 family matrix and produced a dendrogram based on the Bray-Curtis family dissimilarity matrix
205 following Ward's clustering method. After making a visual inspection, we decided to prune the
206 tree to produce seven biological types. For both classifications, we used the same previously
207 utilised type numbers (European Commission, 2007; Sánchez-Montoya et al., 2007) for
208 freshwater rivers and numbers 6, 7 and 8 for the new saline river types. Type 3 (large rivers)
209 was not used because we excluded this river type from our analysis. We also performed non-
210 Metric Multidimensional Scaling (nMDS) ordination based on the Bray-Curtis family
211 dissimilarity matrix to explore the concordance between the environmental and biological
212 classifications.

213

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

231

232 **2.2.3. Biomonitoring indicators for saline river types**

233

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

248

249 To identify the families, genera and species with a higher affinity to each reference and
250 disturbed type, we ran an indicator species analysis (IndVal, Dufrêne and Legendre, 1997). This
251 analysis considers each taxon's percentage of occurrence and relative abundance for each type
252 to obtain an indicator value (IV) and its significance through Monte-Carlo permutations (1000
253 runs). We focused on the most frequent taxa by keeping taxa occurring in more than 10% of
254 observations. From them, we selected those taxa which showed a significant Indicator Value
255 ($P \leq 0.05$) as potential indicators for a given type. From these results, we built the candidate
256 metrics of reference and disturbed conditions of the saline river types. To create these metrics,
257 for each sample we summed the abundances of the indicator taxa of the assigned river type
258 (ref_6, dis_6, ref_7, dis_7, ref_8) for the family, genus and species level (e.g. for the family
259 level: ref_fam6, dis_fam6, ref_fam7, dis_fam7, ref_fam8). In addition for each sample, we

260 estimated a set of widely-used biomonitoring metrics (family richness, EPT family richness,
261 IBMWP, IASPT) and multi-metric indices (ICM-11a and IMMi-T) for Mediterranean rivers
262 (Alba-Tercedor et al., 2002; Munné and Prat, 2009). Finally, to evaluate the performance of
263 these candidate metrics against the widely-used biomonitoring metrics, we used LMM models
264 to assess the differences across all the reference and disturbed types (levels= ref_6, dis_6, ref_7,
265 dis_7, ref_8) and Tukey-*t post hoc* tests to evaluate differences between pairs of comparable
266 reference and disturbed types (i.e. ref_6 vs. dis_6, ref_7 vs. dis_7).

267

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

270

271 **3. Results**

272

273 **3.1. Ranking environmental variable importance**

274

275 Electrical conductivity was the most important variable to explain macroinvertebrate
276 assemblage richness and composition (Fig. 1a,c, and Appendix F, Table F1). Family richness
277 was explained primarily by conductivity (59%) and the interaction between conductivity and
278 region (25%), which suggests that conductivity had different regional effects ($r^2_m=0.82$).
279 Generally with conductivity values above 3,000 $\mu\text{S cm}^{-1}$, family richness responded only to
280 conductivity changes. Family richness peaked at conductivities within the 300-1,000 $\mu\text{S cm}^{-1}$
281 range, before declining progressively as conductivity increased (Fig. 2a,c,d). Within this
282 conductivity range, the family richness values showed the greatest dispersion, which indicates
283 that other variables also influenced family richness. The interactive effects of conductivity with
284 hydrology (Fig. 2a), season (Fig. 2c) and region (Fig. 2d) were evident only at freshwaters, and
285 particularly within the conductivity range of 300-1,000 $\mu\text{S cm}^{-1}$, before becoming much weaker
286 at conductivities over 3,000 $\mu\text{S cm}^{-1}$. The rivers with a perennial flow tended to have a higher
287 level of family richness than intermittent or ephemeral rivers (Fig. 2a,b), but as conductivity
288 increased, the effect of hydrology also became less important. Species richness showed roughly

289 similar patterns in response to environmental variables where, once again, conductivity was the
290 most important predictor, but with higher contributions of mean basin precipitation and
291 seasonality (Fig. 1b and Appendix F, Table F1).

292

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

302

303 **3.2. Integrating saline rivers into biomonitoring typologies**

304

305 According to their biological communities, the nMDS ordinations of samples revealed that
306 environmental and biological classification methods produced roughly similar river types (Fig.
307 3 and Appendix G, Table G1, Figs. G2 and G3). After ignoring large watercourses (type 3),
308 both classifications included three types of freshwater perennial rivers (types 1, 2 and 4), a type
309 comprised mainly of freshwater intermittent and ephemeral rivers (type 5), and three types of
310 saline rivers with increasing conductivity (types 6, 7 and 8, see Appendix G, Table G1). The
311 freshwater perennial river types included headwater watercourses of very low conductivity that
312 drained mountainous siliceous catchments (type 1), mid-mountain rivers of low conductivity
313 that drained medium-sized calcareous catchments (type 2) and calcareous high mountain
314 headwaters of very low to low conductivity (type 4). Although both classifications identified a
315 type of temporary river (type 5), the type defined by the environmental classification included a
316 higher proportion of temporary rivers (68% of intermittent and 32% of ephemeral watercourses)
317 than the type defined by the biological classification (28% of intermittent and 26% of ephemeral

318 watercourses). The three new saline river types, hyposaline (type 6), mesosaline (type 7) and
319 hypersaline (type 8), showed conductivity ranges that were generally similar for both
320 classification procedures, but some minor discrepancies were also found (Appendix G, Table
321 G1). The hyposaline type showed a conductivity range (Q10-Q90) of 8,323-24,567 $\mu\text{S cm}^{-1}$ for
322 the environmental classification, and one of 4,953-26,158 $\mu\text{S cm}^{-1}$ for the biological
323 classification. The mesosaline type gave a conductivity range of 60,480-110,100 $\mu\text{S cm}^{-1}$ for the
324 environmental classification and one of 24,133-98,200 $\mu\text{S cm}^{-1}$ for the biological classification.
325 The hypersaline type showed a conductivity range of 140,000 - 300,000 $\mu\text{S cm}^{-1}$ for the
326 environmental classification and one of 110,100 - 300,000 $\mu\text{S cm}^{-1}$ for the biological
327 classification. All these types were characterised by smaller basin areas, lower elevations, softer
328 slopes, arid climates and greater evaporitic surfaces and conductivity in relation to the
329 freshwater types (Table 2 and Appendix G, Table G1). More than half the surveyed saline rivers
330 were also intermittent or ephemeral. Classification strength based on family abundances was
331 roughly similar between the environmental (CS=0.150±0.007) and biological
332 (CS=0.158±0.006) classification procedures (Appendix G, Fig. G1). However, environmental
333 classification (CS=0.203±0.008) seemed better at representing species turnover among types
334 compared to the biological classification (CS=0.170±0.005).

335 **3.3. Biomonitoring indicators for saline river types**

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

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

350

351 For hyposaline rivers (type 6), the metrics based on the species at reference sites (ref_spp6,
352 LMM $r^2_m=0.30$, Tukey t -test $p=0.001$) and the metrics based on the genera (dis_gen6, LMM
353 $r^2_m=0.23$, Tukey t -test $p=0.009$) and species (dis_spp6, LMM $r^2_m=0.19$, Tukey t -test $p=0.009$) of
354 the disturbed sites were the best indicators (Table 3). For mesosaline rivers (type 7), the metrics
355 based on the families (ref_fam7, $r^2_m=0.47$, Tukey t -test $p<0.001$), genera (ref_gen7, $r^2_m=0.50$,
356 Tukey t -test $p<0.001$) and species (ref_spp7, $r^2_m=0.45$, Tukey t -test $p<0.001$) of the reference
357 sites were the best indicators (Table 3). Contrarily, conventional biomonitoring metrics (family
358 richness, EPT family richness, IBMWP, IASPT) and multi-metric indices (ICM-11a and IMMi-
359 T) showed a null capacity for discriminating between the reference and disturbed conditions for
360 saline river types (Table 3).

361

362 **4. Discussion**

363 **4.1. Water salinity as a driver of community richness and composition at regional-** 364 **continental scales**

365

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

373

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

395

396 **4.2. Integrating saline types into river classifications**

397 The main advantage of our approach is that it integrates the whole spectrum of environmental
398 and biological variation into a single comprehensive classification, which is either
399 environmentally or biologically based, and allows a more accurate and simple classification of
400 rivers in the Mediterranean region. This new integrated typology could help to better implement
401 WFD into Member States and to gather a comprehensive inventory of saline rivers, which are

402 currently ignored or misclassified by current laws. For example, the official Spanish typology of
403 rivers recognises three types of highly mineralised rivers (official types 7, 9 and 13). However,
404 the mean conductivities of these official types range is 448-545 $\mu\text{S cm}^{-1}$ (Spanish Government,
405 2009), which is significantly lower than the conductivities shown by the saline rivers studied
406 herein. Moreover for the first time, our classifications implicitly recognise the importance of
407 considering the whole natural osmotic stress gradient by providing a classification method that
408 encompasses more biodiversity than previous individual attempts (e.g. Arribas et al., 2009;
409 Sánchez-Montoya et al., 2007).

410 The definition of the three saline river types was relatively consistent for both environmental
411 and biological classifications. Thus the prediction of their biological communities was fairly
412 accurate, and was based on only one single sample or the mean of several samples of
413 conductivity (Moreno et al., 1997), similarly to which occurs in lentic systems (Gascón et al.,
414 2016; Pinder et al., 2005; Williams, 1998). In fact our models identified how seasonal or
415 hydrological variation had almost no effect on the biological communities occurring in highly
416 mineralised rivers ($>3,000 \mu\text{S cm}^{-1}$). This is not surprising because saline specialists may show
417 co-tolerance to other drought-related stressors such as desiccation (Pallarés et al., 2017b).

418 Taken together, these results advocate the use of river saline types that included both perennial
419 and intermittent rivers. However, future work using more precise hydrological data should
420 check the consistency of these saline river types along flow intermittency gradients.
421 Quantitative hydrological data offer a much more adequate indication of the drought stress that
422 affects biological communities (Belmar et al., 2013; Gallart et al., 2016; Jaeger and Olden,
423 2012). Unfortunately, these data are currently unavailable for most studied rivers, so simple
424 categorical descriptors of the hydrological regime were used instead.

425 Our findings might also be useful to biomonitoring naturally saline rivers in other regions
426 outside the study area, such as Australia (Biggs et al., 2013), North and South America
427 (Griffith, 2014; Orfeo, 1999), North Africa (Hamed and Dhahri, 2013) and Russia (Zinchenko
428 et al., 2014). Specifically, although biogeographical differences may lead to very different

429 taxonomic compositions, ecological responses to salinity might be similar and roughly close
430 river types might be yielded depending on the available salinity gradient.

431 **4.3. Metrics to evaluate anthropogenic impacts on saline rivers**

432

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

448

449 The abundance of specialist taxa seems to provide a much better indication of reference and
450 degraded conditions than diversity-based metrics (Cañedo-Argüelles et al., 2012). These metrics
451 can also be used to monitor their populations, which are scattered across the territory and
452 threatened by human pressures (Arribas et al., 2015). Nonetheless, we admit that our proposed
453 metrics is a first attempt to effectively showcase the type of biomonitoring tools that would
454 work in saline rivers, so they should be cautiously taken. Therefore, they may benefit from
455 further refinements by gathering larger datasets of observational data combined with
456 manipulative experiments, which both cover different types of impacts (e.g. dilution, nutrient

457 enrichment). Furthermore, some of the indicator species for the saline types defined herein (e.g.
458 *Ochthebius glaber*, *Nebrioporus baeticus*) are endemic of the Iberian Peninsula. Fortunately,
459 these taxa have sister species with very similar ecological requirements in other biogeographic
460 regions (Arribas et al., 2015), and could be used as effective indicators of reference or disturbed
461 conditions.

462

463 In some saline rivers, agriculture is diluting salt concentrations, which poses a risk for their
464 typical communities that are confined to these peculiar environments (Carbonell et al., 2012;
465 Gutiérrez-Cánovas et al., 2013; Pallarés et al., 2017a), and leads to taxonomic homogenisation
466 and loss of regional biodiversity. Similarly in other naturally stressed systems, such as glacier-
467 fed and alpine rivers, climate change is reducing the number of the endemic and specialist taxa
468 that typically inhabit those systems through increasing temperature and turbidity (Finn et al.,
469 2013; Jacobsen et al., 2012). Consequently, we highlight the urgent need to catalogue and
470 monitor naturally stressed rivers which, despite harbouring reduced local diversity, contribute
471 substantially to regional and global biodiversity through their unique communities of stress-
472 tolerant species (Finn et al., 2013, Millán et al., 2011).

473

474 **5. Conclusions**

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

484 natural and anthropogenic stressors, to foster the development of biomonitoring metrics for
485 naturally saline rivers, and to help preserve their unique biodiversity.

486

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502

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720

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
			Genus,						
dis_spp_spa_sal	Disturbed	Coleoptera	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 (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	<1,000		Calcareous and evaporitic	Perennial, intermittent or	32,000-130,000

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

		<i>Type 6 (hyposaline)</i>		<i>Type 7 (mesosaline)</i>	
	Metrics	r^2_m	P-value	r^2_m	P-value
<i>Widely-used</i>					
<i>metrics</i>	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
<i>Novel metrics</i>	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

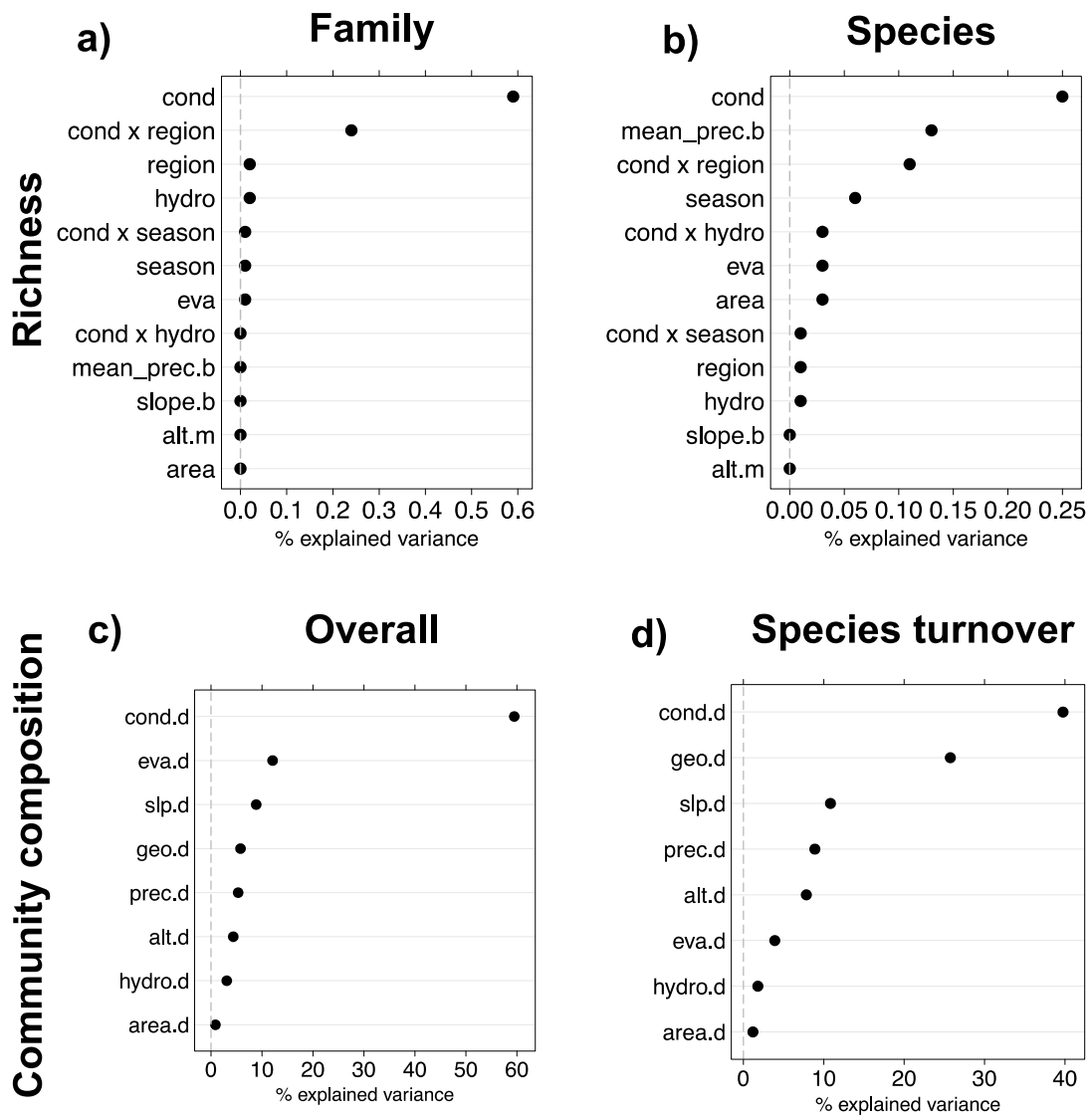


Fig. 2.

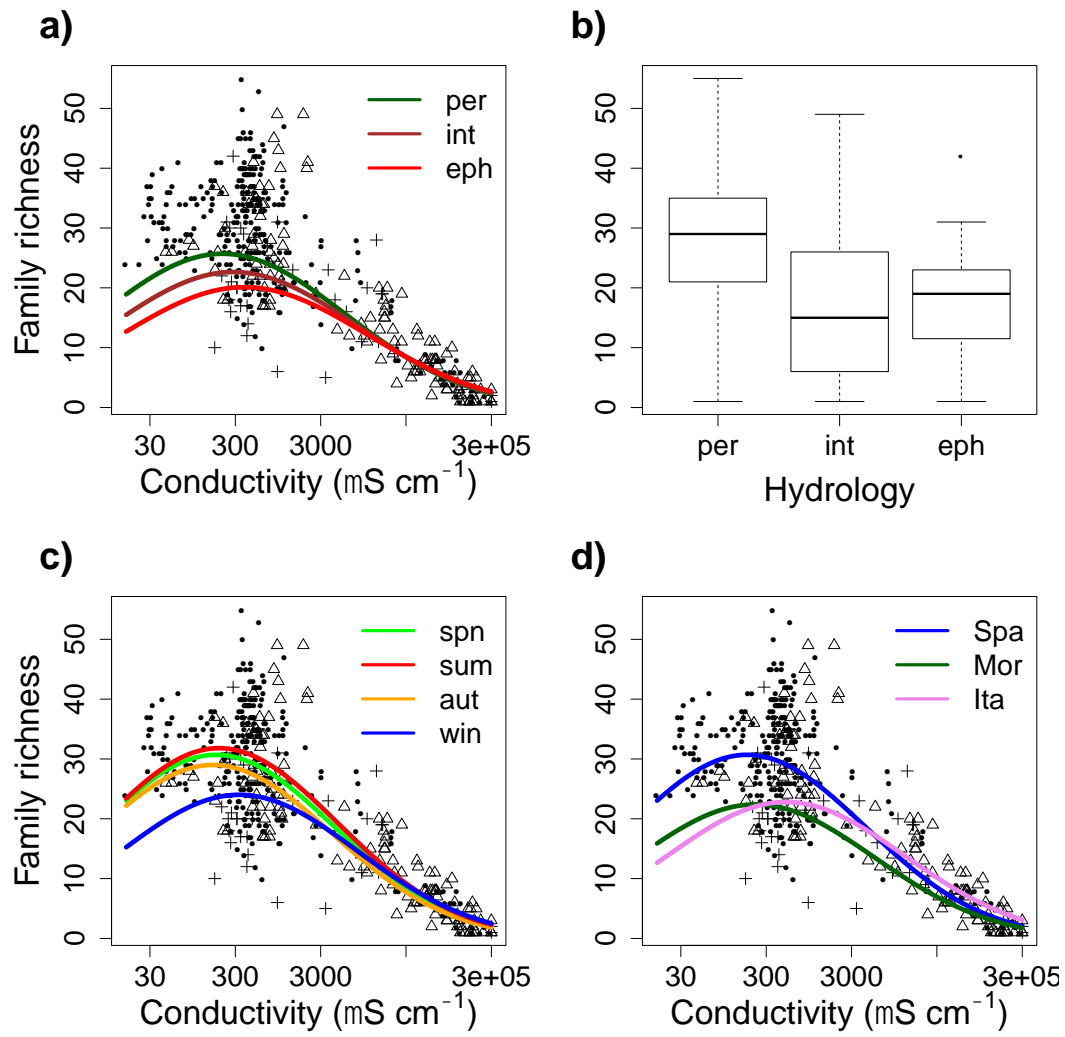
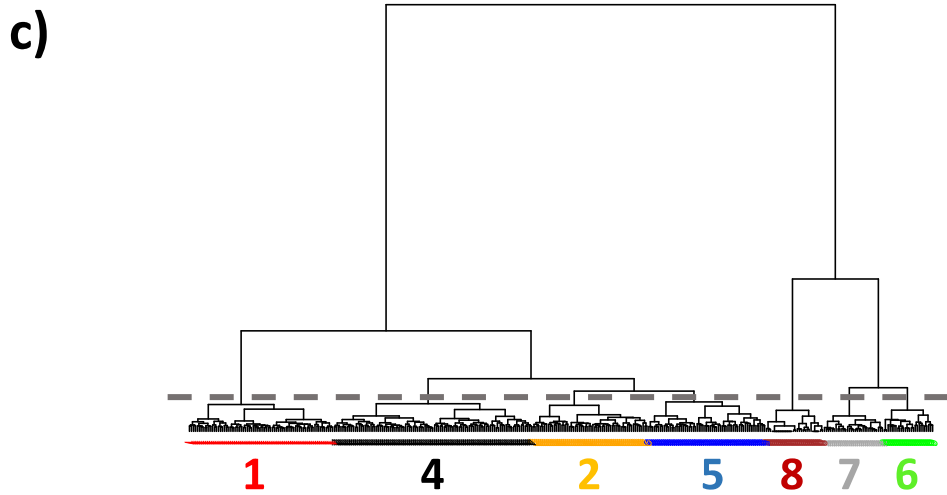
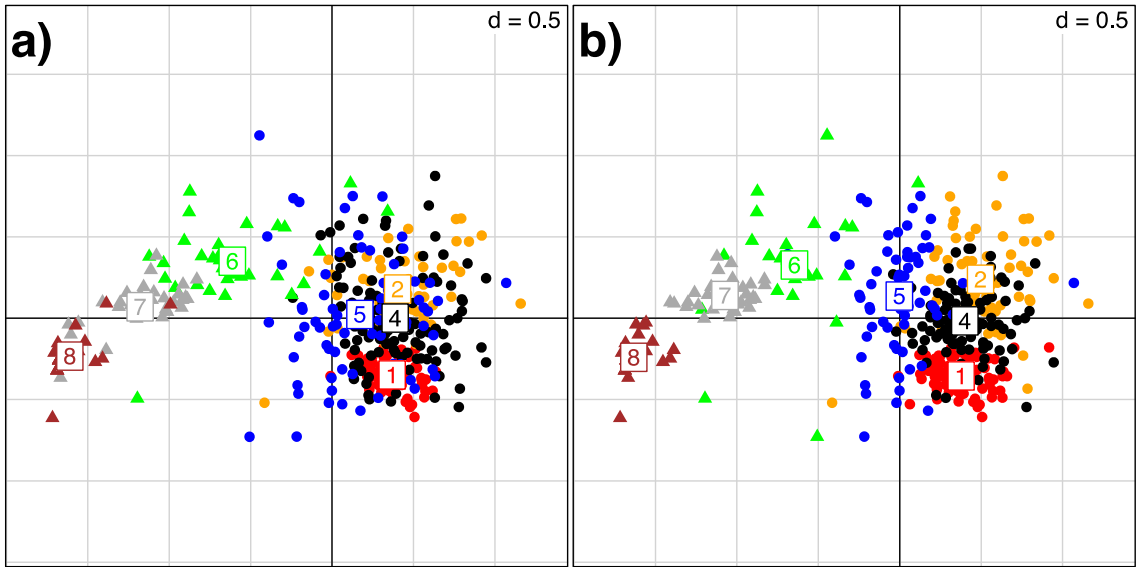


Fig. 3



1 **Abstract**

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

24

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

27

28 **1. Introduction**

29

30 Naturally stressed ecosystems are characterised by harbouring a set of natural conditions that
31 are persistently unsuitable for the vast majority of the regional species pool (Badyaev, 2005;
32 Parsons, 2005). Well-known examples of naturally stressed ecosystems are found in tundra,
33 deserts, volcanic springs, and glacier, acid or saline inland waters (Cauvy-Fraunié et al., 2016;
34 Elliott and Quintino, 2007; Millán et al., 2011; Petrin et al., 2007). Their natural stressful
35 conditions reduce the local diversity of these systems but also constitutes a powerful driver of
36 diversification (Parsons, 2005; Vetaas et al., 2002), and so they usually hold unique fractions of
37 biodiversity characterised by high levels of specialisation and species turnover (Finn et al.,
38 2013; Gutiérrez-Cánovas et al., 2013; Jacobsen et al., 2012).

39

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

47

48 This is particularly true in the case of naturally saline rivers (Millán et al., 2011), which have
49 been mostly neglected by the large international efforts to reverse the globalised river
50 degradation, such as the EU Water Framework Directive (WFD, Directive 2000/60/EC). These
51 conservation frames are fundamentally based on a two-step process focused on the identification
52 of main river types over a particular region (river classification) and the subsequent
53 development and harmonisation of biomonitoring metrics that allow assessing the ecological
54 status of the different river types (Buffagni et al., 2007). However, there are some generalised
55 shortcomings on the application of these procedures that resulted in a deficient functionality for
56 the assessment and protection of naturally stressed rivers. Firstly, main natural stressors such as

57 water salinity are usually neglected or not fully considered in river classifications, resulting in
58 the exclusion or misclassification of naturally stressed rivers (Sánchez-Montoya et al., 2007).
59 This occurs because the classification process tends to be focused on coarse environmental
60 descriptors, which are weak proxies of local stressors. Secondly, most of the proposed
61 biomonitoring metrics can result in equivocal evaluations of the ecological status of the
62 naturally stressed rivers because they are strongly associated to local richness/diversity and so
63 not considering naturally depauperate communities (Gutiérrez-Canovas et al., 2008). Besides,
64 other widely-used metrics are based on taxa or functional traits that are rare or even not present
65 in naturally stressed rivers, such as the stress-sensitive orders Ephemeroptera, Plecoptera and
66 Trichoptera (EPT) (Belmar et al., 2013; Bonada et al., 2006; Millán et al., 2011). Therefore,
67 there is an urgent need to revisit and adapt current vision and approaches to assess the
68 ecological quality of naturally stressed rivers, and so to improve their management and future
69 preservation.

70

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

82

83 **2. Material and methods**

84

85 **2.1. Datasets description**

86

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

93

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

105

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

115 Mediterranean Reference Criteria, MRC; Sánchez-Montoya et al., 2009) or disturbed when they
116 were substantially impacted by anthropogenic activities (i.e. fulfilled < 16 MRC). In this study,
117 we excluded large watercourses (mean basin area ≥ 1000 km², ECOSTAT, type 3, see Table 1
118 and Sánchez-Montoya et al., 2007 for details) because of the paucity of reference sites
119 (Sánchez-Montoya et al., 2009). We also excluded disturbed freshwater rivers, for which
120 effective biomonitoring metrics can be found elsewhere (e.g. Birk et al., 2012; Bonada et al.,
121 2006).

122

123 **2.2. Data analysis**

124

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

131

132 **2.2.1. Ranking environmental variable importance**

133

134 To identify the main environmental factors determining the family and species richness, we
135 used Random Forest (randomForestSRC R package, Ishwaran Ishwaran et al., 2014) and Linear
136 Mixed Models (LMM, lme4 R package, Bates et al., 2015). In these models, we utilised family-
137 level datasets (i.e. ref_fam_ita, ref_fam_mor, ref_fam_spa, n=458) and species-level datasets
138 (i.e. ref_spp_ita, ref_spp_mor, ref_spp_spa, n=211) from reference sites (selected according
139 MRC, see above). Following Feld et al. (2016), we first ran Random Forest models to identify
140 the most important predictors of family and species richness among 24 potential candidates
141 (Appendix A, Table A1) to be included into the LMM (see Appendix C for more details about
142 exploratory analyses). After these exploratory analyses, we included basin area, mean basin
143 altitude, mean basin slope, mean basin annual rainfall, evaporitic surface, flow intermittence,

144 conductivity (single and quadratic terms), season and region as fixed factors in the LMM. As
145 additional fixed factors, we also included the pairwise interactions of conductivity x flow
146 intermittence, conductivity x season and conductivity x region. Site code was considered as a
147 random factor to account for repeated measures in the same location. To rank the environmental
148 predictor's importance on family and species richness, we adopted a multi-model inference
149 approach (Grueber et al., 2011), using the *MuMIn* R package (Bartoń, 2016). This statistical
150 technique ranks all the models generated using all the possible combination of predictors using
151 Akaike's Information Criterion (AIC). Then, a set of top models is selected to produce an
152 average model only if the model ranking first is ambiguously supported (model weight < 0.90).
153 We chose top models differing in no more than two AIC units ($\Delta AIC \leq 2$) from the model ranked
154 first (minimum AIC). We adopted a natural average method to conduct the model averaging,
155 which consists in averaging predictors only over models in which the predictor appears and
156 weighting predictor's SES by the summed weights of these models (Burnham and Anderson,
157 2002). For each LMM model, two measures of goodness-of-fit were estimated (Nakagawa and
158 Schielzeth, 2013): marginal goodness-of-fit (r^2_m) indicates the variance explained only by the
159 fixed factors, while conditional goodness-of-fit (r^2_c) shows the variance accounted for by both
160 fixed and random terms. We provide the mean average (based on model weights) of each
161 goodness-of-fit measure for each averaged model. All models were validated by visually
162 checking their residuals for normality and homoscedasticity.

163

164 To identify the environmental drivers of community composition change, we used Multiple
165 Regression models for distance Matrices (MRM; *ecodist* R package, Lichstein, 2007). This
166 method is conceptually similar to traditional multiple regression but with all variables being
167 distance matrices instead of raw data and *P*-values being calculated through permutation tests
168 (1000 runs). To avoid lack of independence problems due to multiple samples belonging to the
169 same site, we selected a reference subset of macroinvertebrate families (*ref_fam_all*, n=157
170 sites/samples) and species (*ref_spp_all*, n=76 sites/samples) occurrences with only one spring
171 sample per site. We estimated overall changes in community composition for each pair of sites
172 of the family matrix through the Sørensen dissimilarity index (β_{sor}) and the pairwise

173 dissimilarity due to turnover from the species matrix using the Simpson index (β_{sim}). These
174 calculations were made following the Baselga's (2010) framework for β -diversity partitioning
175 using the *betapart* R package (Baselga and Orme, 2012). For each selected environmental
176 predictor (basin area, mean basin altitude, mean basin slope, mean basin annual rainfall,
177 evaporitic surface, flow intermittence, conductivity, geographic distance), we built a Euclidean
178 distance matrix based on their transformed and standardised values. Geographical distance
179 between localities was based on a latitude and longitude original matrix and flow intermittence
180 was based on semiquantitative values (perennial=0, intermittent=1, ephemeral=2).

181

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

185

186 **2.2.2. Integrating saline rivers into biomonitoring typologies**

187

188 To develop a classification of rivers encompassing the environmental and biological variability
189 occurring in the studied area, we used family abundances from reference Spanish sites
190 (ref_fam_spa, n=386). We selected this dataset because it included more sites and samples and
191 covered a broader environmental and biological spectrum relative to the Italian and Moroccan
192 datasets (Appendix A, Table A1). First, we classified sites into seven types according to their
193 environmental variables (environmental classification) following an adaptation of the criteria
194 suggested by the ECOSTAT intercalibration group for Mediterranean rivers using System A of
195 the WFD (MedGIG European Commission, 2007), which included mean conductivity, basin
196 area, hydrology, site altitude and basin lithology (Table 2 and Appendix D). Secondly, to
197 classify samples according to their biological communities (biological classification), we
198 estimated a Bray-Curtis pairwise dissimilarity matrix derived from the abundance family matrix
199 and produced a dendrogram based on the Bray-Curtis family dissimilarity matrix, using the
200 Ward's clustering method. After visual inspection, we decided to prune the tree to produce
201 seven biological types. For both classifications, we used the same type numbers previously

202 utilised (European Commission, 2007; Sánchez-Montoya et al., 2007) for freshwater rivers and
203 the numbers 6, 7 and 8 for the new saline river types. The type 3 (large rivers) was not used
204 because we excluded this type of rivers from the analysis. We also performed a non-Metric
205 Multidimensional Scaling (nMDS) ordination based on the Bray-Curtis family dissimilarity
206 matrix to explore the concordance between the environmental and biological classifications.

207

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

225

226 **2.2.3. Biomonitoring indicators for saline river types**

227

228 To develop metrics indicating the ecological quality of saline river types and test their
229 performance against widely used biomonitoring metrics, we used a dataset including family and
230 species-level data from reference (ref_fam_spa_sal, n=89; ref_spp_spa_sal, n=75) and disturbed

231 (dis_fam_spa_sal, n=31; dis_spp_spa_sal, n=30) Spanish sites. We first assigned river types to
232 all these samples using the environmental classification criteria because its simplicity and
233 potential better performance compared to the biological classification (see Results). Considering
234 that most of the disturbed sites have been affected by a drop in their natural conductivity levels
235 as a result of freshwater inputs from agricultural drainages (Velasco et al., 2006), we compiled
236 historical, predisturbed conductivity information (Moreno et al., 1997; Vidal-Abarca, 1985) to
237 correctly assign their river types. As result, we obtained hyposaline, mesosaline and hypersaline
238 river types under reference condition (ref_6, ref_7 and ref_8, respectively) and hyposaline and
239 mesosaline river types under disturbed condition (dis_6, dis_7). For the type 8, we did not find
240 any disturbed site.

241

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

260

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

263

264 **3. Results**

265

266 **3.1. Ranking environmental variable importance**

267

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

285

286 Conductivity distance was also the most important variable explaining dissimilarity in family
287 composition and species turnover (Fig. 1c,d). These results indicate that macroinvertebrate
288 assemblages in rivers with different conductivity values tended to have different family

289 composition, and that these changes seem to arise through species replacement (and Appendix
290 F, Table F2). Family composition was also significantly influenced by evaporitic surface, basin
291 slope, geographic and hydrologic distances, which along with conductivity explained a
292 substantial extent of the community variance ($r^2=63\%$). Changes due to species turnover were
293 also linked to conductivity, geographic, basin slope, hydrologic and basin area distances
294 ($r^2=35\%$).

295

296 **3.2. Integrating saline rivers into biomonitoring typologies**

297

298 The nMDS ordinations of samples according to their biological communities revealed that
299 environmental and biological classification methods produced roughly similar river types (Fig.
300 3 and Appendix G, Table G1, Figs. G2 and G3). After ignoring large watercourses (type 3),
301 both classifications included three types of freshwater perennial rivers (types 1, 2 and 4), a type
302 mainly comprised of freshwater intermittent and ephemeral rivers (type 5) and three types of
303 saline rivers of increasing conductivity (types 6, 7 and 8, see Appendix G, Table G1).
304 Freshwater perennial river types included headwater watercourses of very low conductivity
305 draining mountainous siliceous catchments (type 1), mid-mountain rivers of low conductivity
306 draining medium size, calcareous catchments (type 2) and calcareous high mountain headwaters
307 of very low to low conductivity (type 4). Although both classifications identified a type of
308 temporary rivers (type 5), the environmental classification included a higher proportion of
309 temporary rivers (68% of intermittent and 32% of ephemeral watercourses) than the biological
310 classification (28% of intermittent and 26% of ephemeral watercourses). The new three saline
311 river types (hyposaline, mesosaline and hypersaline river types) were characterised by smaller
312 basin areas, lower elevations, softer slopes, arid climates, and greater evaporitic surface and
313 conductivity relative to the freshwater types (Table 2 and Appendix G, Table G1). Also, more
314 than a half of the surveyed saline rivers were intermittent or ephemeral. The conductivity ranges
315 that define the saline types were generally similar in both classification procedures, but some
316 minor discrepancies were also found (Appendix G, Table G1). Classification strength based on
317 family abundances was roughly similar between the environmental ($CS=0.150\pm 0.007$) and

318 biological (CS=0.158±0.006) classification procedures (Appendix G, Fig. G1). However,
319 environmental classification (CS=0.203±0.008) seems to be better in representing species
320 turnover among types compared to the biological classification (CS=0.170±0.005).

321 **3.3. Biomonitoring indicators for saline river types**

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

336

337 For hyposaline rivers (type 6), the metrics based on species of reference sites (ref_spp6, LMM
338 $r^2_m=0.30$, Tukey t -test $p=0.001$) and the metrics based on genera (dis_gen6, LMM $r^2_m=0.23$,
339 Tukey t -test $p=0.009$) and species (dis_spp6, LMM $r^2_m=0.19$, Tukey t -test $p=0.009$) of disturbed
340 sites were the best indicators (Table 3). For mesosaline rivers (type 7), metrics based on families
341 (ref_fam7, $r^2_m=0.47$, Tukey t -test $p<0.001$), genera (ref_gen7, $r^2_m=0.50$, Tukey t -test $p<0.001$)
342 and species (ref_spp7, $r^2_m=0.45$, Tukey t -test $p<0.001$) of reference sites were the best indicators
343 (Table 3). Contrarily, conventional biomonitoring metrics (family richness, EPT family
344 richness, IBMWP, IASPT) and multi-metric indexes (ICM-11a and IMMi-T) showed a null

345 capacity to discriminate between reference and disturbed conditions for saline river types (Table
346 3).

347

348 **4. Discussion**

349 **4.1. Water salinity as a driver of community richness and composition at regional-** 350 **continental scales**

351

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

358

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

373 (Hemiptera), and Culicidae, Ephydriidae, Stratiomyidae, Chironomidae (Diptera) (Arribas et al.,
374 2014; Bradley, 2008; Pallarés et al., 2017), all of them comprising good biological indicators of
375 reference saline streams. Thus, our results reveal a clear differentiation in community
376 composition and, particularly, a strong replacement of taxa along the conductivity gradient, also
377 concordant with previous studies on this natural stress gradient (Gutiérrez-Cánovas et al., 2013)
378 and on the high levels of habitat specificity associated to the osmotic stress (Carbonell et al.,
379 2012).

380

381 **4.2. Integrating saline types into river classifications**

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

394 The definition of the three saline river types was relatively consistent for both environmental
395 and biological classifications. Thus, the prediction of their biological communities was fairly
396 accurate based just on mean or sampled conductivity (Moreno et al., 1997), as occurred in lentic
397 systems (Williams, 1998). In fact, our models identified how seasonal or hydrological variation
398 had almost no effect in the biological communities occurring at highly mineralised rivers
399 ($>3,000 \mu\text{S cm}^{-1}$).

400 **4.3. Metrics to evaluate anthropogenic impacts on saline rivers**

401

402 Generally, the UE member states are implementing WFD through the classification of the water
403 bodies and then the development of appropriate biological indicators to evaluate their ecological
404 status, rather than using model based methods (Birk et al., 2012). For these pragmatic reasons,
405 we developed specific indicators for the saline river types obtained. Our results showed that
406 metrics based on the abundance of taxa indicating either reference or degraded conditions were
407 able to detect anthropogenic impacts on naturally saline rivers, while metrics commonly used in
408 freshwater rivers did not respond at all. While conventional biomonitoring metrics, such as
409 family or EPT richness, are good indicators of the ecosystem quality in freshwater rivers
410 (Bonada et al., 2006), the intense abiotic filtering at naturally stressed rivers acts as a
411 confounding factor for these metrics. This fact causes that diversity-based indicators are
412 inappropriate to evaluate saline watercourses, being also potentially inaccurate for other
413 naturally stressed systems (Elliott and Quintino, 2007). Previous studies have also demonstrated
414 that conventional biomonitoring metrics showed substantial limitations to evaluate the
415 ecological quality of naturally stressed ecosystems, such as intermittent rivers (Bruno et al.,
416 2016; Wilding et al., 2018) or estuaries (Elliott and Quintino, 2007).

417

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

426

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

429 Gutiérrez-Cánovas et al., 2013; Pallarés et al., 2017), which leads to taxonomic homogenisation
430 and regional biodiversity loss. Similarly, in other naturally stressed systems, such as glacier-fed
431 and alpine rivers, climate change is reducing the number of endemism and specialist taxa, which
432 typically inhabit those systems, through increases in temperature and turbidity (Finn et al.,
433 2013; Jacobsen et al., 2012). Consequently, we highlight the urgent need of monitoring
434 naturally stressed rivers, which despite harbouring a reduced local diversity, contribute
435 genuinely to regional and global biodiversity through their unique communities of stress
436 tolerant species (Finn et al., 2013, Millán et al., 2011).

437

438 **5. Conclusions**

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

450

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464

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636

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
			Genus,						
dis_spp_spa_sal	Disturbed	Coleoptera	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 flow	32,000-130,000

				flow
--	--	--	--	------

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

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_m^2 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.

		<i>Type 6 (hyposaline)</i>		<i>Type 7 (mesosaline)</i>	
	Metric	r_m^2	P-value	r_m^2	P-value
<i>Widely-used</i>					
<i>metrics</i>	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
<i>Novel metrics</i>					
	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

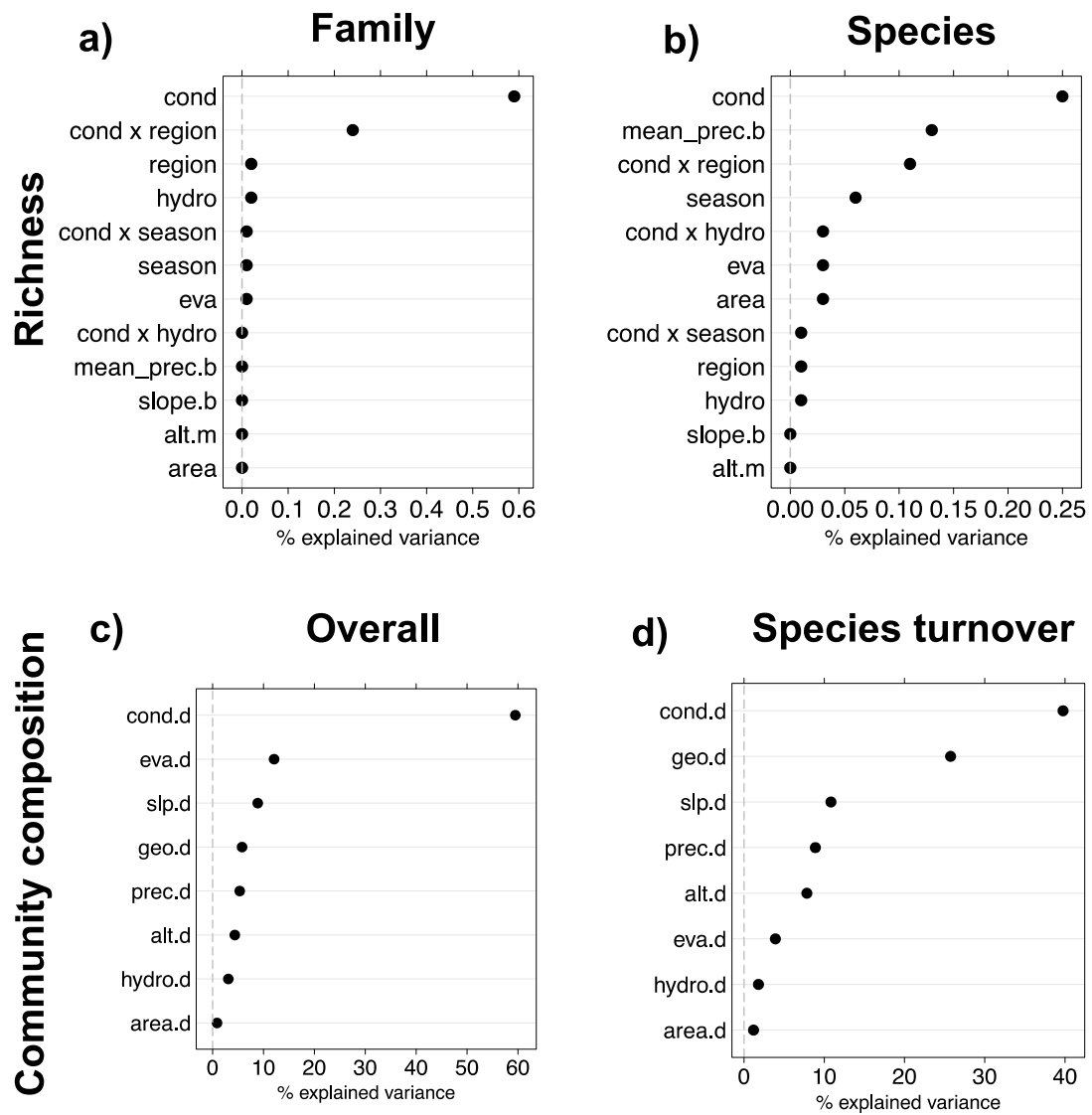


Fig. 2.

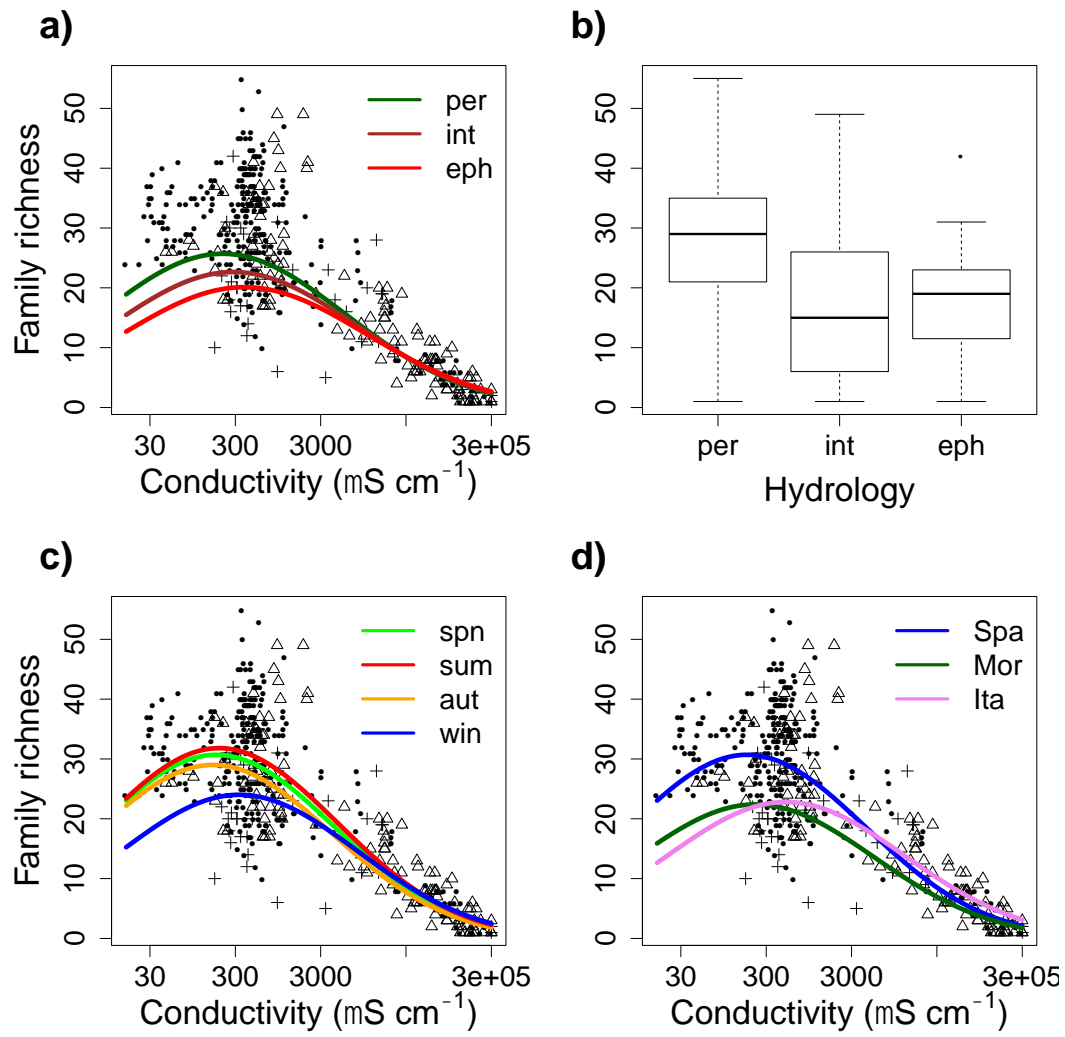
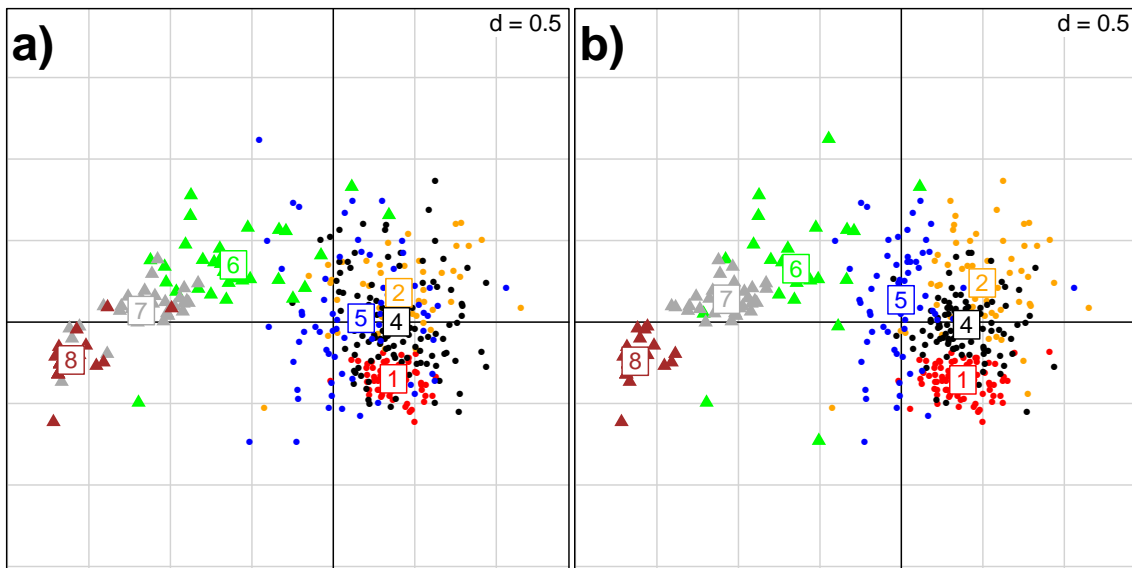


Fig. 3



Supplementary material for on-line publication only

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