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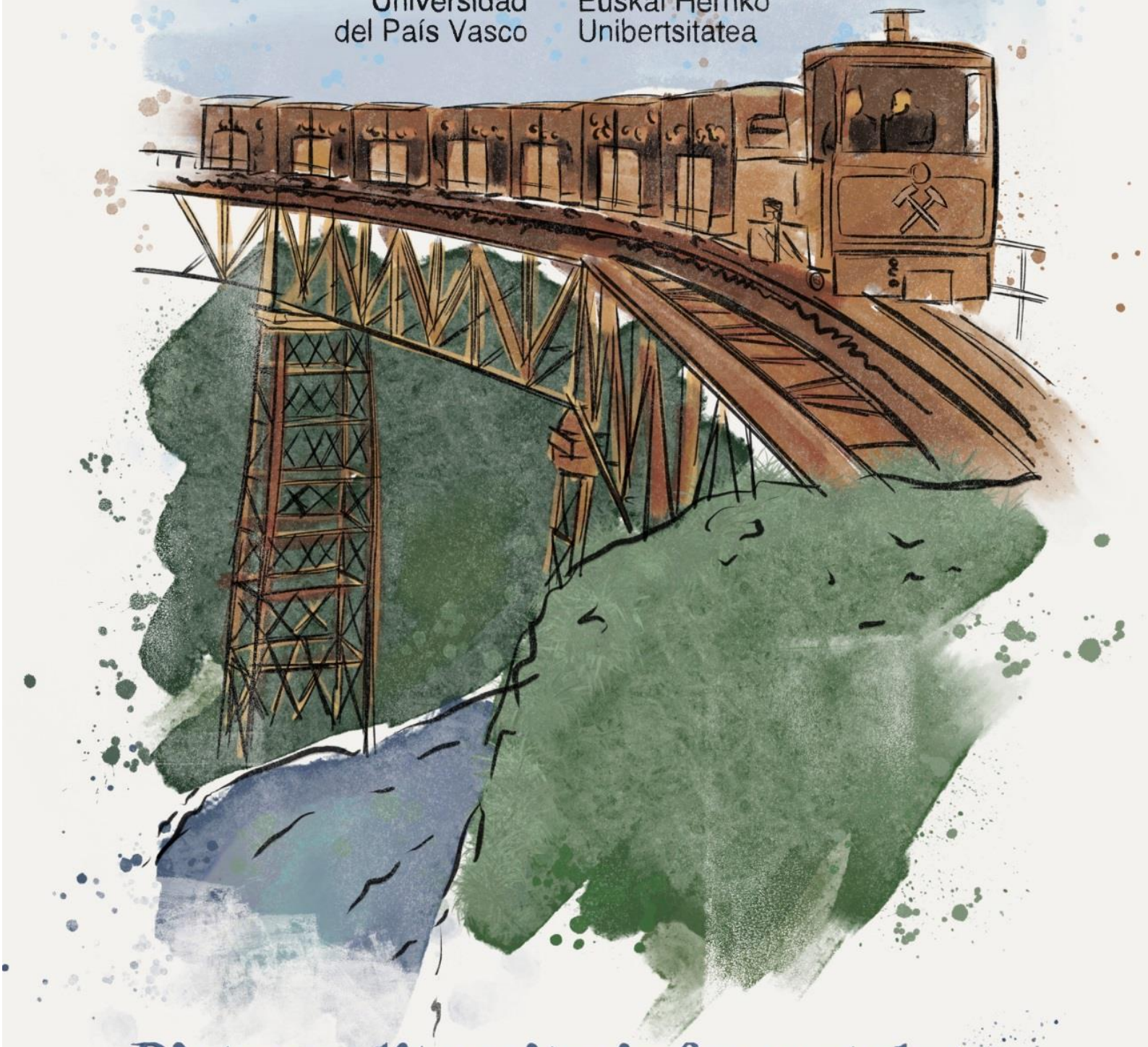

Biota quality criteria for metals to protect freshwater macroinvertebrate assemblages in the cantabrian region

Iñigo Moreno Ocio

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Supervisors: Pilar Rodríguez Rodríguez and Leire Méndez Fernández

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Resumen

Los metales y metaloides (en adelante, metales) están presentes de forma natural en el medioambiente y muchos de ellos son esenciales para la supervivencia de los seres vivos. A lo largo de la historia, los humanos han extraído los metales para su posterior uso, lo que ha conllevado el desarrollo de actividades mineras en zonas con litologías naturalmente ricas en estos elementos químicos. Dichas actividades han degradado significativamente el medioambiente, reduciendo la calidad de los ecosistemas. En España, la historia de la actividad minera se remonta a más de 4.000 años. Esta actividad se vio intensificada en el norte de España debido a la industrialización para la extracción de metales desde finales del siglo XVIII. La contaminación del medio acuático debido a la actividad minera es uno de los problemas ambientales de relevancia internacional que genera gran preocupación, ya que puede representar un riesgo significativo para la salud humana debido a la contaminación de alimentos y del agua potable. Además, la contaminación de las aguas es un problema ambiental para la conservación de la biodiversidad, ya que puede aumentar la biodisponibilidad de ciertos metales y causar toxicidad sobre los mismos.

Los sedimentos y la biota han sido identificados como matrices relevantes para monitorear los cambios a largo plazo en la calidad del agua de las masas de agua europeas. Sin embargo, sólo algunos estados miembros han desarrollado normas de calidad ambiental para los metales prioritarios en dichas matrices. En España, las concentraciones de las sustancias prioritarias en el sedimento y la biota se evalúan en base al principio *stand still*; es decir, su concentración no debe aumentar significativamente en el sedimento o la biota a largo plazo. Algunos metales traza pueden bioacumularse y presentan un riesgo de toxicidad importante para los macroinvertebrados bentónicos, y en ocasiones también para otras especies a lo largo de las cadenas tróficas, debido a procesos de biomagnificación. En la Comunidad Europea, el cadmio y el mercurio (y sus compuestos) se clasifican como *sustancias peligrosas prioritarias*, mientras que el plomo y el níquel (y sus compuestos) se clasifican como *sustancias prioritarias*. De acuerdo con la Directiva 2013/39/UE, deben desarrollarse normas de calidad ambiental para las sustancias prioritarias, de tal manera que puedan utilizarse en los planes de gestión de cuencas fluviales durante el período

2015-2021 para controlar su reducción a nivel *background natural* y la eliminación de su vertido a las aguas. Con la aplicación de este procedimiento, se esperaba alcanzar un buen estado químico de las aguas superficiales en relación a las sustancias prioritarias para finales de 2021, objetivo que no se ha cumplido y que ha sido recientemente prorrogado hasta el año 2027.

Con todo ello, la hipótesis general del presente trabajo es que la capacidad de los ecosistemas acuáticos para mantener una comunidad de macroinvertebrados saludable, medido en términos de composición, diversidad y organización funcional, puede verse perjudicada por la contaminación de metales en el sedimento y por la bioacumulación de los mismos en los organismos acuáticos. La evaluación del riesgo debido a la presencia de niveles peligrosos de metales en el sedimento y en la biota debe esclarecerse mediante una aproximación que proporcione una información sólida sobre los niveles ambientales esperados en condiciones ambientales inalteradas o de referencia.

La contribución al desarrollo de normas de calidad ambiental de metales para la biota es uno de los principales objetivos de este trabajo. Los niveles de los metales en tejido deseables se pueden derivar a partir de los datos obtenidos de las distintas especies de macroinvertebrados *in situ*, y sería esperable que dichos valores variasen según las características funcionales de las especies seleccionadas como biomonitores (nicho ecológico, tipo de alimentación, etc.). Por tanto, se requiere el estudio de varios taxones seleccionados, que pudieran ser representativos de varias características funcionales, de forma que aportasen un rango de concentraciones en tejido que permitiera esclarecer diferentes niveles de protección para la comunidad. En este sentido, se pueden definir dos niveles en el desarrollo de normas de calidad ambiental para la biota. Por un lado, niveles de referencia (o niveles base) por debajo de los cuales los efectos adversos en las comunidades de macroinvertebrados son poco probables. Por otro lado, niveles umbrales por encima de los cuales existen diferentes niveles de probabilidad de que se den efectos adversos en taxones seleccionados o en la comunidad. Dichos niveles umbrales pueden referirse a concentraciones de efectos bajos o moderados (umbral bajo) o elevados (umbral alto).

El desarrollo de criterios sólidos para la evaluación del riesgo de bioacumulación de metales en cuencas fluviales afectadas por actividades mineras es una herramienta necesaria para proteger a las comunidades acuáticas. El objetivo del **Capítulo I** de la tesis es proponer un índice integrador para residuos en tejido que sea adecuado para su uso en programas de vigilancia y monitoreo, que sea fácilmente interpretable en términos de evaluación de riesgo en ríos afectados por actividades mineras. Se determinó la concentración en tejido de siete metales y dos metaloides en diez taxones de macroinvertebrados de agua dulce de la cuenca del Nalón (Asturias, España), afectada por actividades mineras de Au, Cu y Hg. La evaluación del riesgo de bioacumulación en cada taxón se basa en el ratio promedio de las concentraciones en tejido de cada metal respecto a la correspondiente en lugares de referencia estudiados en la misma cuenca fluvial. Esta concentración de referencia se denomina concentración umbral ecológica en tejido (*ecological threshold tissue concentration*, ETTC) y al índice calculado como un ratio de cada metal con su valor de referencia, se le denomina TRT (*tissue residue ratio to threshold*). En comparación con los niveles de referencia, los taxones biomonitores de los distritos mineros de Au y Hg mostraron los niveles más altos de bioacumulación. Sin embargo, la bioacumulación en las localidades afectadas por minería histórica de Cu fue baja o no significativa. Análisis multivariantes (ANOSIM) realizados revelaron diferencias significativas en la bioacumulación entre los puntos de muestreo clasificados según su estado ecológico. La idoneidad de la selección de los biomonitores se evaluó utilizando modelos de regresión lineal ajustados a las relaciones entre los valores TRT y la contaminación del sedimento o los índices EQR de estado ecológico de cada punto de muestreo.

La evaluación del riesgo de bioacumulación en cada punto de muestreo se realizó tras una selección de los metales más relevantes en el sedimento del área de estudio (As, Cu y Hg) y de los biomonitores que presentan modelos de regresión mejor ajustados de la bioacumulación de estos metales con su concentración en sedimento y con el estado ecológico. Estos biomonitores son Baetidae, Hydropsychidae y los oligoquetos acuáticos Microdrili, que representan diferentes grupos funcionales, herbívoros, filtradores-colectores y sedimentívoros. La comparación de las evaluaciones de riesgo derivadas de la bioacumulación y del estado ecológico de las comunidades de

macroinverterbados, basadas en ambos casos en la desviación respecto a la condición de referencia, demuestra que los programas de monitoreo vigentes podrían optimizarse combinando ambos enfoques.

En el **Capítulo II** se trabajó con la base de datos de concentraciones de metales en sedimento y en tejido en puntos de muestreo de referencia y contaminados de la cuenca del Nalón. Los efectos se midieron en términos de alteración del estado ecológico de las comunidades de macroinvertebrados *in situ*, mediante dos aproximaciones (METI y NORTI) e índices de riqueza y abundancia de taxones sensibles (EPT: Ephemeroptera, Trichoptera y Plecoptera). Con los datos de bioacumulación de campo en diez taxones biomonitores se construyeron modelos de regresión no lineal relacionándolos con el estado ecológico de las comunidades de macroinvertebrados para derivar concentraciones umbrales de efecto en tejido (*effective tissue residues*, ERs). Dichos ERs fueron calculados para el valor oficial del límite de clases entre un estado ecológico bueno y moderado (ER_{GM}) y para la reducción de un 50% en las métricas de comunidad y de taxones sensibles (ER_{50}). Los metales que se analizaron mediante este procedimiento fueron As, Cu, Hg y Se y se calcularon ERs para cada uno de los diez taxones, que representaban diferentes grupos funcionales. Con los ERs calculados para As y Cu y para cada taxón se construyeron modelos de distribución de sensibilidad de especies y se calcularon las concentraciones peligrosas para la comunidad (*hazard concentracions,* HC_5 y HC_{50}). Los datos obtenidos para Hg y Se fueron insuficientes para construir dichos modelos, haciendo necesaria una mayor investigación para completar la base de datos de tal manera que sea posible en un futuro el cálculo de HCs para estos metales. La fiabilidad y las diferencias entre los distintos valores umbrales calculados se probó mediante la evaluación de riesgo de bioacumulación en el arroyo Cauxa, un afluente de la cuenca del Nalón afectado por actividades mineras para la extracción de oro, a través de una aproximación de concentraciones en tejido en organismos del campo. Dicha evaluación identificó la bioacumulación de As y el Cu como una de las causas más probables de la reducción del estado ecológico de las comunidades de macroinvertebrados. Este estudio indica que este tipo de aproximación puede ser útil para establecer futuras normas de calidad ambiental para la protección de la biota en agua dulce.

En el **Capítulo III** se estudió la influencia que puede tener el cambio de los valores límite que se establecen entre las clases de estado ecológico bueno y moderado en el cálculo de ERs de metales en tejido. Para ello, se recalcularon los ERs que se habían derivado en el capítulo anterior para nuevos puntos de corte (ER_{NB}) recientemente propuestos para METI y NORTI-EQRs. Utilizando los ERs de As, Cu, Hg y Se para 10 taxones como variable predictora, se volvieron a estimar las concentraciones de As, Cu y Hg peligrosas para la comunidad (HC) a partir de modelos de distribución de sensibilidad de especies (SSD). Los HC_5 y HC_{50} estimados fueron interpretados como concentraciones umbrales baja y alta en tejido, respectivamente, por encima de las cuales existe una probabilidad de alteración de las comunidades de macroinvertebrados, pasando de un estado ecológico bueno a moderado. Dichos umbrales tienen valores de concentración inferiores a los calculados con el corte oficial y, por lo tanto, pueden aportar una mayor protección para las comunidades de macroinverterbados. Sin embargo, para el caso estudiado en el río Cauxa, se concluyó que el efecto que puede derivarse de un cambio en los nuevos puntos de corte del estado ecológico no es determinante en la evaluación de riesgo, respecto a la efectuada con el punto de corte oficial. Una ventaja importante del los nuevos cortes propuestos es que han permitido estimar concentraciones umbrales HC para el Hg.

En el **Capítulo IV** se evaluaron dos distritos mineros de Pb/Zn de la región cantábrica (minas de Arditurri en la cuenca del Oiartzun y de Reocín en la cuenca del Saja-Besaya) siguiendo las siguientes líneas de evidencia (LOEs): la contaminación del sedimento, la toxicidad crónica del sedimento y la bioacumulación de metales en tejido de macroinvertebrados seleccionados como biomonitores en el campo. Dada la ausencia de normas de calidad ambiental de sedimento para la zona de estudio, para poder evaluar la contaminación del sedimento se estimaron valores de fondo y umbrales ecológicos de Cd, Pb y Zn a partir de una base de datos de sedimentos no contaminados y no tóxicos de localidades de referencia del norte de España. La toxicidad crónica del sedimento se evaluó mediante bioensayos de 28 días de exposición a sedimentos con la especie test *Tubifex tubifex* (Annelida, Clitellata). La bioacumulación en los macroinvertebrados de campo se evaluó a través del índice INTISS. Las tres LOEs se evaluaron en el marco comparativo con la condición de referencia, y mostraron un

gradiente de alteraciones debido a la contaminación del sedimento y la toxicidad crónica del sedimento en todos los puntos test. Únicamente un punto test presentó valores de bioacumulación en campo por debajo del umbral de peligro. En un marco integrador de evaluación de las tres LOEs, mediante el principio del peso de la evidencia (*weight of evidence*, WOE), se concluyó que sería necesario aplicar programas de remediación en ambas cuencas, y principalmente en la cuenca del río Oiartzun, para reducir la contaminación por Cd, Pb y Zn en dichos puntos. Por otro lado, se pudieron estimar ERs en tejido para *T. tubifex* a partir de los bioensayos de laboratorio, observándose su utilidad para evaluar el riesgo debido a la bioacumulación de Cd en oligoquetos.

En el **Capítulo V** se llevó a cabo un test de toxicidad aguda en agua con arsénico mediante la exposición de la especie test *T. tubifex* a diferentes concentraciones de As^{5+}, durante 96 h de exposición. Dicho test sirvió para evaluar *T. tubifex* como especie modelo para evaluar la toxicogenómica de respuesta al estrés debido al As en ambientes acuáticos. Para ello, y por primera vez, se ha reconstruido el transcriptoma de la especie y se han identificado y caracterizado varios biomarcadores tempranos de estrés en la especie relacionados con la toxicidad aguda. Se han estudiado un total de 11 genes en relación con la respuesta celular al estrés (*Hsp83*, *Hsp60*, *Hsp10*, *Hsc70*), desintoxicación y estrés oxidativo (*MnSOD*, *CAT*, *GSR*, *CoI*) y genes implicados en la homeostasis del organismo (*RPS13, CaM, UBE2*). La expresión diferencial mostrada por los genes del estudio confirmó su efectividad como potenciales biomarcadores a nivel molecular y su relación con parámetros letales. En este sentido, se estimaron varios parámetros toxicológicos relacionados con la mortalidad (LC_{50} y LR_{50}) y la autotomía de la cola como mecanismo de detoxificación de metales en la especie (EC_{50} y ER_{50}), tanto para el agua como para tejido, evidenciando su utilidad para evaluar el rango de concentraciones peligrosas de arsénico para el oligoqueto acuático *T. tubifex*.

En resumen, una evaluación de riesgo de los metales en zonas afectadas por actividades mineras debe estar basada en un marco general de comparación con la condición de referencia (*reference condition approach*), incluyendo varias Líneas de evidencia: una evaluación del riesgo de contaminación del sedimento a través de los umbrales ecológicos calculados para un área concreta, una evaluación de la toxicidad crónica de los sedimentos, una evaluación sobre el estado de la comunidad de

macroinvertebrados de campo y un estudio sobre los niveles de bioacumulación. El conocimiento y caracterización de los niveles de referencia y de niveles umbrales de efecto hacen posible una evaluación de riesgo realista y sólida de las comunidades de macroinvertebrados expuestas a los contaminantes. Además, resultaría apropiado incorporar en el futuro información acerca de la expresión génica de biomarcadores relacionados con rutas relevantes de respuesta al estrés en organismos de campo.

Finalmente, este estudio ha demostrado que la capacidad de los ecosistemas para mantener comunidades de macroinvertebrados saludables, en términos de composición, diversidad y organización funcional, ha sido alterada por la contaminación por metales en los sedimentos y por la bioacumulación de los mismos en los organismos del bentos. Dicha alteración se ha evidenciado mediante el uso de las concentraciones umbrales propuestas en este estudio, usando siempre una aproximación comparativa con la condición de referencia en zonas mineras del norte de España.

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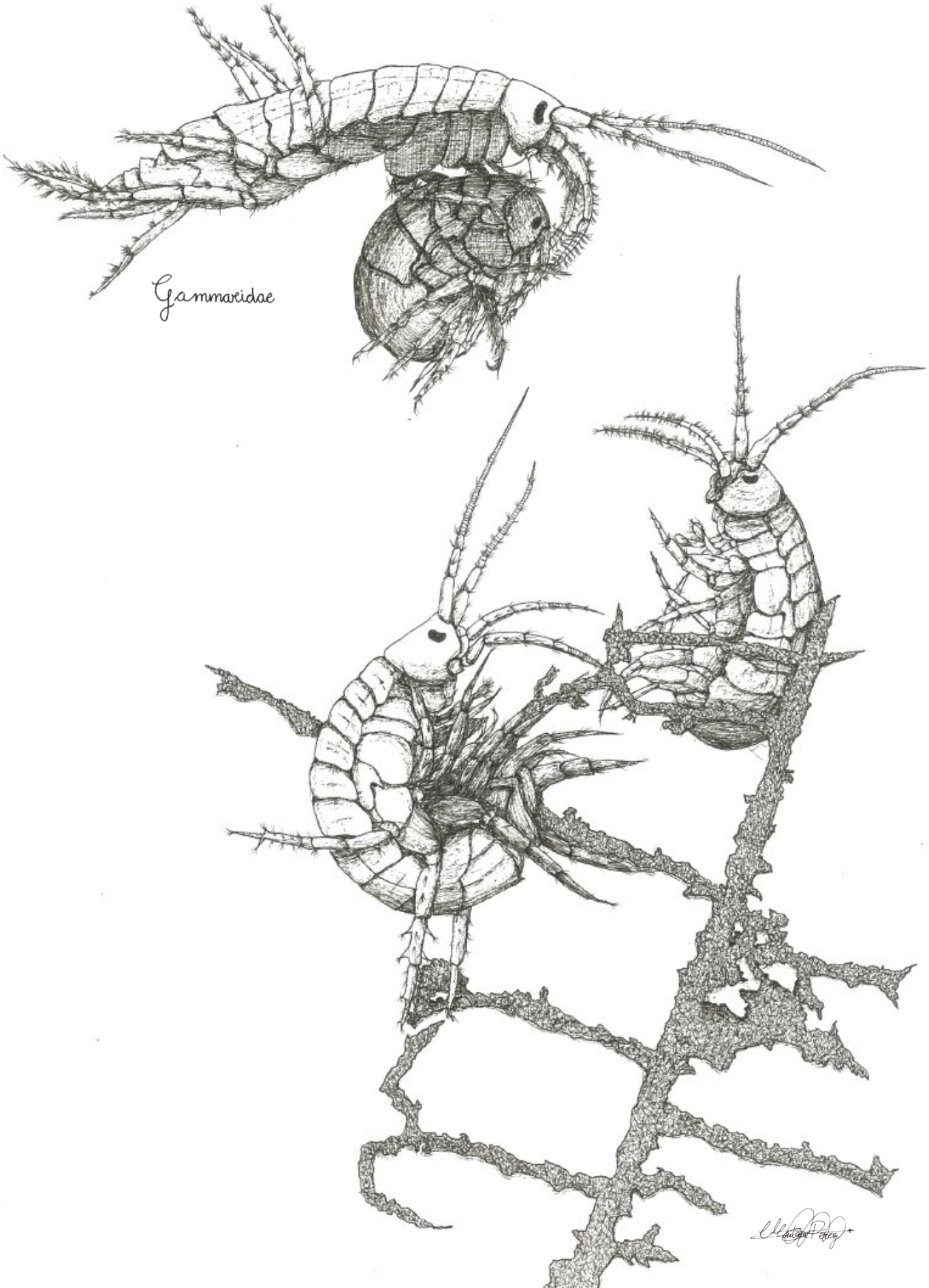
General introduction



General introduction

Metals are naturally present in the environment and many of them are essential to the survival of living organism. Humans have also extracted metals for their use throughout history, which had led to carry out mining activities in areas with naturally metal enriched lithologies. These activities have significantly altered the environment and the availability of metals, causing negative effects on environmental quality (Arnold *et al*., 2021). Spain has a long history of mining activities for metal extraction dating back more than 4,000 years. The northern Spain, specifically, has had an intense activity related to metal extraction from late 18th century (Rodríguez *et al*., 2006). The contamination of aquatic environments due to mining activities is an international environmental issue of great concern since it can pose a significant risk to human health through contamination of food or drinking water (Byrne *et al*., 2012) in addition to being an environmental problem for freshwater ecosystems (Luoma *et al*., 2010; Moreno-Ocio *et al*., 2022; Rodriguez *et al.*, 2021).

The risk assessment of metals and metalloids (hereinafter, metals) can be complicated due to bioaccumulation and essentiality. The bioaccumulation is metal specific, dynamic and a time-dependent process, and the interpretation may be difficult since some of them are essential for the metabolism of the organism and, therefore, their uptake and body concentration is actively regulated (Luoma & Rainbow, 2008). Sediments are an essential, integral and dynamic part of the aquatic ecosystems (Salomons & Brils, 2004) and their relevance for water quality assessment lies in their role as reservoir and source of contaminants to the water column and to the aquatic communities. Sediment and biota have been identified as relevant matrices to monitor long-term changes in the water quality of European water bodies (Carère *et al.,* 2012; EC, 2013), but only some State Members have developed the required environmental quality standards (EQS) of priority substances for some of these compartments (Bakke *et al*., 2010; Crommentuijn *et al*., 2000; de Deckere *et al*., 2011). In Spain, indeed, the chemical concentrations in the sediments and in the biota are evaluated based exclusively on the *stand still* principle, i.e. priority substances in the sediments or in the biota should not significantly increase their concentration in the long-term (Environmental Quality Standards Directive: EC, 2008). Priority

substances are prone to bioaccumulate and pose a significant toxicity risk to biota along the food chain. In the European Community, cadmium and mercury (and their compounds) are categorized as *Priority Hazardous substances* in the list of *Priority Substances* (Appendix A: EC, 2013), while lead and nickel (and their compounds) are *Priority substances*. According to EC (2013), Environmental Quality Ratios (EQR) for priority substances should be developed and used in river basin management plans during the 2015-2021 period. By applying this procedure, it was expected to achieve a good surface water chemical status regarding to priority substances by the end of 2021, an objective that has not been yet accomplished, and the terms of the management plans had been extended under a third cycle, till 2027 (Vermeulen *et al*., 2019).

Bioaccumulation is defined as the net uptake of chemicals from the environment by any or all the possible routes (i.e., respiration, diet, dermal) from any source in the aquatic environment where they are present (i.e., water, dissolved, colloidal or associated with particulate organic carbon, sediment, or other organisms) (Spacie *et al.*, 1995). It is important to understand that high metal tissue concentrations in macroinvertebrates do not necessarily imply that adverse biological effects are occurring; in fact, only those body concentrations that are related to alterations measurable at population or community levels can be regarded as potentially adverse (Rand *et al*., 1995). The relationship between tissue concentration and toxicity was described through the *Tissue Residue Approach* (McCarty *et al*., 2011; Meador *et al*., 2014). This approach aims to assess potential toxicity effects occurring when the chemical tissue concentration goes beyond a threshold level. However, defining when these concentrations are in excess has always been a problem for environmental assessment. There is a lack of official consensus benchmark or threshold for metals bioaccumulated by aquatic organisms, except for Hg (EC, 2013, Annex 1, Part A, EQS), a value that in fact is always on debate. With the intention of achieving a metal threshold proposal that could protect the aquatic macroinvertebrate assemblages in freshwater ecosystems, Rodriguez *et al*. (2018) proposed an innovative methodology based on the baseline tissue levels in the field organisms associated to the good ecological status of the macroinvertebrate assemblages. The derived baseline

tissue concentration was called the Ecological Threshold Tissue Concentration (ETTC), and was measured in selected taxa used as biomonitors that were representative of different functional traits (feeding group and habitat). The ETTC was defined as the concentration in a specific taxon below which the alteration of the benthic community is unlikely. The departure of the metal tissue residues from the baseline concentration in the Nalón River basin has been analyzed in **Chapter I,** as well as a methodology for the risk assessment due to metal bioaccumulation, which is in agreement with the risk assessment on the alteration of the macroinvertebrate assemblages.

The association of chemical tissue residues with effects of ecological relevance in aquatic organisms may help in developing environmental quality guidelines for ecological risk assessments (Sappington *et al*., 2011). However, this must be done with caution, since not all the tissue residues may be biologically active (Adams *et al*., 2011). But there is place for hope, since this approach has been demonstrated to be suitable to assess the metal toxicity in both *in situ* and laboratory test organisms, especially in areas affected by mining activities (Cain *et al*., 2004; Méndez-Fernández *et al*., 2015, 2017). The selection of suitable field biomonitors requires the evaluation of several criteria, such as density, ubiquity and bioavailability, the latter depending on several biological features like species behavior, feeding styles and animal physiology (De Jonge *et al.*, 2010; Luoma & Rainbow, 2005). In this sense, the selection of biomonitors carried out in the Nalón River basin has demonstrated to be useful for the assessment of areas where several metal tissue residues are higher than the reference levels (**Chapter I**).

The Water Framework Directive (EC, 2000) stated that an integrated assessment of the ecological status needs to determine the composition and structure of the field macroinvertebrate assemblage to detect the alterations that are occurring due to contaminants. The conservation status of the macroinvertebrate community assemblages is assessed by a variety of metrics, indexes and models, worldwide. Some of the most widely used indexes are the reduction in richness and abundance of the EPT (Ephemeroptera, Plecoptera, Trichoptera), regarded as sensitive taxa (Moskova *et al*., 2008; Rosenberg & Resh, 1993). In northern Spain, different classification systems have been simultaneously used during the last decade (Pardo *et al*., 2020). The IBMWP

(Alba-Tercedor *et al*., 2002) was originally developed in the United Kingdom for the assessment of organic pollution, and was later adapted to Spanish river types, and used by different Water Agencies as part of a integrative multimetric used for the river ecological status assessment. The biotic multimetric developed in Spain using the mandatory design established in the WFD is the river-type specific multimetric index, called METI (MAGRAMA, 2015; Pardo *et al*., 2010). In the same way, another classification system was developed for the north-northwest of Spain as a predictive model (NORTI: Pardo *et al.*, 2014). The METI- and NORTI-EQR are both calculated as the quotient between the observed/expected values of the score in an unimpaired, defined water body type (EC, 2000). For the classification of the ecological status of a specific site, the official boundary between good and moderate ecological status is 0.700 for both METI- and NORTI-EQR (MAGRAMA, 2015). The classification systems of ecological status with both the official METI and the non-official NORTI EQRs were successfully intercalibrated within the Central-Baltic River intercalibration groups (Bennett *et al*., 2011; Pardo *et al*., 2020). Recently, Pardo *et al*. (2020) have proposed new EQR boundaries for the ecological status change-point between good and moderate ecological status for both METI- and NORTI-EQR, being more protective for the benthic communities and providing a more sound interpretation of the degree of biodiversity loss in rivers from northern Spain.

In the last decade, there have been published different regression models of the relationships between macroinvertebrate tissue residues and ecologically relevant information on the conservation status of the macroinvertebrate assemblages. The models have allowed estimating effective tissue residues (ER) in different regions of the world (Belgium: Bervoets *et al*., 2016; De Jonge *et al*., 2013; Spain: Moreno-Ocio *et al*., 2022; USA: Schmidt *et al*., 2011). In **Chapter II**, non-linear regression models have been used on several biomonitors and metals to estimate ERs associated to the change-point of good/moderate ecological status of the macroinvertebrates in the Nalón River basin. However, the possibility of a future revision of the boundaries accepted nowadays for the good and moderate categories of the ecological status need to be evaluated when the tissue residues guidelines were established. Therefore, the proposal recently made by Pardo *et al*. (2020) of new change-points of

good/moderate ecological status has been used to evaluate the influence that the new EQR boundaries may have on the metal ERs (**Chapter III**).

When dealing with sediment pollution assessment, there are still very few EQS proposed for metals and, the guideline most widely used for freshwater sediment assessment is the one proposed by MacDonald *et al*. (2000) for the United States. It provides consensus-based sediment quality guidelines (SQG) for As, Cd, Cr, Cu, Pb, Hg, Ni and Zn that can be used as threshold values. There are two levels of protection based on the probability of toxicity: a low threshold concentration (the *Threshold Effect Concentration*, TEC) and a high threshold (the *Probable Effect Concentration*, PEC). The TEC provides a benchmark below which the absence of sediment toxicity can be predicted, while the PEC is a benchmark that indicates a high probability for sediment toxicity when it is exceeded. For other metals and metalloids, other sources are used in present study, e.g. the Canada benchmark value for Se (BC, 2014). In Europe, some other sediment guidelines are worth mentioning, e.g. those for Flanders region (Flemish Government, 2012) or The Netherlands (Crommentuijn *et al*., 1997). More recently, following the requirement of developing metal EQS for sediment in Europe (EC, 2008), Méndez-Fernández *et al*. (2019) derived a quality standard for total Hg in sediments, using ecological filed data and field sediment ecotoxicity bioassays. That study proposed a new methodological approach that can assist in developing future quality standards for other metals in freshwater sediments (**Chapter IV**).

In sediment pollution assessment, sediment chronic toxicity tests are fundamental tools for predicting a reliable risk of adverse effects for benthic organisms exposed to toxicants in the field sediments. Sediment chronic bioassays have the advantage of representing a more realistic exposure conditions than only-water toxicity tests (Chapman & Anderson, 2005; Ingersoll *et al*., 1995, 1997). The sediment toxicity assessment should not be done without considering the geographic or lithological context of the study sites. A reference condition approach (Reynoldson *et al*., 1997, 2002a) for the sediment toxicity evaluation compares the endpoint data (e.g. survival, reproduction, growth) obtained from the exposure of the test organisms to a test sediment with the expected conditions derived from their exposure to unimpaired

conditions (reference sites). That is, the reference sites anticipate the endpoint values of the no-toxic condition in the study area.

The aims of using sediment bioassays can be multiple: (a) to assess the implications of the bioavailability of contaminants in their toxic effects; (b) to determine the possible interactions between different chemicals; (c) to assess the environmental hazards of the contaminated sediments; (d) to focus on the spatial and temporal distribution of contaminants; (e) to prioritize areas that require management or remediation plans or to test the validity of these plans; and (f) to measure the toxicity of new substances licensing (ASTM, 2005). In ASTM (2005), eight sediment bioassays for different test species were included: the insects *Chironomus riparius*, *Chironomus dilutus* and *Hexagenia* spp; the crustaceans *Ceriodaphnia dubia*, *Daphnia magna*, *Diporeia* spp and *Hyalella Azteca*; and the aquatic oligochaete *Tubifex tubifex*.

In present work, the test species used for the sediment bioassays in the Zn/Pb mining districts was the worm *Tubifex tubifex* (Annelida, Clitellata) to assess bioavailability of relevant metals in the sediments and their toxicity (**Chapter IV**). *T. tubifex* is an infaunal, deposit-feeder species, whose entire life cycle takes place in the sediments of rivers, pond and lakes. The *T. tubifex* sediment bioassay was developed and standardized by Reynoldson *et al*. (1991) and was included in two editions of the American Society for Testing and Materials (ASTM, 1994, 2005) and in the OECD guidelines for testing chemicals for bioaccumulation (OECD, 2008). Chapman (2001) pinpointed the relevance of using aquatic oligochaetes in sediment toxicity and bioaccumulation assessment through environmental risk assessments (ERA) for both laboratory and field studies. The sediment bioassay for this species has been used in both North America (Bailey *et al*., 1995; Gillis *et al*., 2004a; Reynoldson *et al*., 1995) and Europe (Bervoets *et al*., 2016; Maestre *et al*., 2007; Martínez-Madrid *et al*., 1999; Méndez-Fernández *et al*., 2015; Pasteris *et al*., 2003). The main reason why aquatic oligochaetes are good biomonitors for sediment toxicity assessment is that they occupy the upper layers of the sediments and are deposit-feeders of the fine particles and detritus; therefore, their exposure to contaminants occurs through the body wall and the digestive system (Rodriguez & Reynoldson, 2011).

Several methods for integrating the information provided by the different lines of evidence have been proposed to assess the sediment quality and determine causation (Chapman, 2007; Reynoldson *et al*., 2002 a, b; Simpson & Batley, 2016). The classic lines of evidence that were originally included in this integrated approach following the Sediment Quality Triad philosophy (Chapman *et al*., 1987; Long & Chapman, 1985) were: the sediment chemistry, chronic toxicity and field benthic community alterations. Later, bioaccumulation and biomarkers were also considered as additional lines of evidence in the assessment in order to provide a more complete image of metal impact in aquatic organisms. There are many methodological choices in the integration and overall evaluation of several lines of evidence that may lead to considerable differences in the evaluation of the impairment (Reynoldson *et al*., 2002a). In present study, the main methodological issue in the decision-making process has been a reference condition approach to evaluate the sediment chemistry, the chronic toxicity and bioaccumulation. For sediment chemistry and bioaccumulation, the distance to the reference condition was measured as a ratio to the 90^{th} percentile, whereas for toxicity assessment was the distance of a test site to the multivariate reference ordination space built with the endpoint database obtained from reference sediment bioassays of northern Spain (**Chapter IV**).

Lastly, toxicogenomics is an interdisciplinary science that relates the molecular toxicology, functional adverse effects (or pathology) and functional genomics. The main goal of toxicogenomics studies is to detect changes in global gene expression (transcriptomics) due to the exposure of a test organism to hazardous chemicals and its relationship with toxicological endpoints, like histopathology, or other toxicological parameters (Uehara, 2013). The transcriptome is the total set of RNA transcripts produced in a cell under a specific developmental stage or physiological condition. Thus, transcriptomics is the study of the transcriptome and its study provides information about gene expression at the tissue level (Chavan-Gautam *et al*., 2017). Toxicogenomic studies contribute to improve the understanding of dose-response relationships of contaminants at low levels of exposure (Bardal *et al*., 2011). Thus, the development of the transcriptome of the test species used in toxicity bioassays is an important tool, but *T. tubifex* lacked such information. There have been described

different biomarkers for this species, for instance, metallothionein-like protein concentrations have been reported to increase with metal exposures (Gillis *et al*., 2002, 2004b; Mosleh *et al*., 2005a, b), and their genetic expression have also been related to metal detoxification and regulation processes in oligochaetes (Demuynck *et al*., 2007). Other authors also have reported effects related to Glutathione (GSH), Glutathione-S-transferase (GST), Glutathione-Reductase (GR), Catalase (CAT) and Superoxide dismutase (SOD) in *T. tubifex* (Mosleh *et al*., 2007; Widiastuti *et al*., 2019) in the presence of metal stress. In present study, arsenic acute responses (survival and autotomy) have been studied in a water-only 96-h toxicity test with *T. tubifex*, and its transcriptome has been developed and the expression of several genes was examined and characterized (**Chapter V**).

Hypothesis

The general hypothesis of the present work is that the capacity of aquatic ecosystems to maintain a healthy macroinvertebrate assemblage, measured in terms of composition, diversity and functional organization, can be hampered due to metal pollution in sediments and their bioaccumulation by aquatic organisms.

The environmental risk assessment due to the presence in sediment and organisms of hazardous levels of metals needs to be established by a reference condition approach, which provides reliable and faithful information on the expected environmental levels characteristic of unimpaired environmental conditions in the study region.

Environmental Quality Standards (EQS_{biota}) should preferably be derived on stream macroinvertebrate tissue residues, but the EQS_{biota} can vary depending on the choice of the biomonitor species. Thus, the EQS in the biota will be expressed as a concentration range of effect tissue residues for several benthic species, comprising different functional traits. In that sense, two levels of protection in the development of EQS_{biota} should be defined: baseline levels, below which adverse effects are unlikely; and different threshold levels, above which the probability of adverse effects in organisms or community could be low (low threshold) or high (high threshold).

Objectives

The specific objectives to be achieved in the present study are:

- To provide criteria to select a suitable set of relevant metals and macroinvertebrate taxa as biomonitors for the bioaccumulation risk assessment of a river basin affected by mining activities (**Chapter I**).

- To provide a methodological framework to develop an integrative tissue residue score (INTISS) for mixed-metal pollution situations, taking in consideration the baseline tissue residues measured for the biomonitors in the unimpaired sites of the region (**Chapter I**).

- To derive effective tissue residues (ERs) using nonlinear regression models relating taxa tissue residues against two general benchmarks: (1) the good/moderate boundary for macroinvertebrate ecological status and (2) the 50% reduction of the EQRs and EPT metrics used to assess biological integrity of the macroinvertebrate assemblages (**Chapter II**).

- To estimate the hazard concentrations of the relevant metals for the macroinvertebrates in the Nalón River basin, based on taxa-specific ERs, using species-sensitivity distribution (SSD) logistic models (**Chapter II**).

- To evaluate to what extent the change in official boundaries for the classification of the macroinvertebrate ecological status could affect the EQS_{biota} based on a tissue residue approach (**Chapter III**).

- To conduct an environmental risk assessment of sites influenced by Pb/Zn mining works, using sediment chemistry, *T. tubifex* chronic toxicity tests and bioaccumulation, in decision-making process (weight of evidence) based on the reference condition approach (**Chapter IV**).

- To develop *de novo* transcriptome of *T. tubifex* to identify and characterize genes related to cell stress response, detoxification and oxidative stress and homeostasis of organisms (**Chapter V**).

- To assess the genetic mechanisms of action of As in relation to the acute responses (autotomy and mortality) in *T. tubifex* exposed to a gradient concentrations in a water-only acute toxicity test (**Chapter V**).

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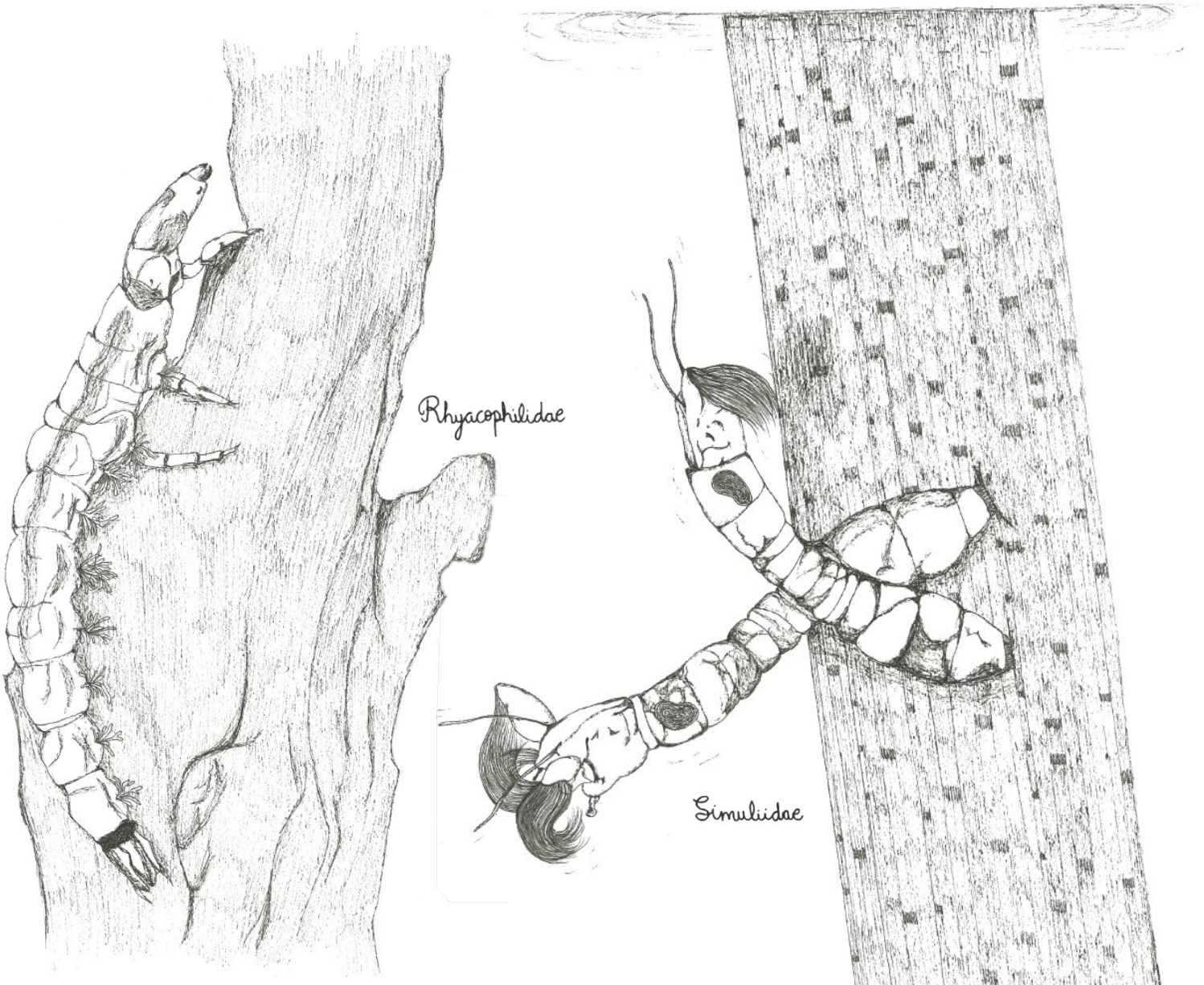
Chapter 1



Proposal of integrative scores and biomonitor selection for metal bioaccumulation risk assessment in mine-impacted rivers

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Abstract

Development of sound criteria for metal and metalloid bioaccumulation risk assessment in river basins affected by mining activities is a necessary tool to protect the aquatic communities. The aim of this study is to propose integrative scores for tissue residues that are suitable for surveillance programs and readily interpreted in terms of risk assessment in mining impacted rivers. Tissue residues of 7 trace metals and 2 metalloids were measured in ten macroinvertebrate taxa from the Nalón River basin (Spain), affected by Hg, Cu and Au mining activities. Compared with reference sites, biomonitor taxa from Hg and Au mining districts showed the highest bioaccumulation. However, low or non-significant bioaccumulation was found in sites influenced by historical Cu mining. Multivariate analyses (ANOSIM) performed on individual taxa revealed significant differences in tissue residues between sites classified according to their ecological status. The bioaccumulation risk assessment was based on the average ratio of the actual metal tissue residues in each macroinvertebrate taxon to the corresponding Ecological Threshold tissue concentration (Tissue residue Ratio to Threshold, TRT). The suitability of the biomonitors was evaluated using linear regression models fitted to the relationships between TRT scores and site sediment pollution or ecological status scores. Biomonitor selection also considered differences in invertebrate functional traits, which can influence metal and metalloid bioavailability. Site bioaccumulation risk was assessed on an Integrated Tissue concentration score (INTISS), calculated over a selection of the most relevant chemicals (As, Cu and Hg) and 3 biomonitor taxa (Baetidae, Hydropsychidae, Microdrile oligochaetes) comprising a set of feeding styles. Based on INTISS, it was possible to predict community alteration scores, using linear regression models. A comparison of site bioaccumulation and ecological status assessments based on the departure from reference conditions showed that operational monitoring programs in basins impaired by mining can be optimized by combining both approaches.

Keywords: tissue residues, ecological status, sediment pollution, arsenic, copper, mercury.

1. INTRODUCTION

The body concentration of a chemical is an integrated measure of its bioavailability, and includes the uptake, storage and elimination processes over the preceding time period. Biomonitor organisms has been shown to be a promising tool to identify and assess those sites having chemical tissue residues higher than background or reference levels (Adams *et al.*, 2011). Moreover, they provide an assessment of the relationships between tissue residues and benthic macroinvertebrate community's impairment over space and time in a particular area (Bervoets *et al*., 2016; Luoma *et al*., 2010).

In rivers affected by mining activities, metal body burden measured in selected macroinvertebrates provides evidence of metal bioavailability, and has been used to estimate critical levels to protect the aquatic invertebrate communities (De Jonge *et al*., 2013; Meador *et al*., 2014). To accomplish this goal, the relationships between the community-level effect of concern and the body metal concentration in biomonitors occurring across the range of metal exposure in a specified ecoregion must be modeled (Adams *et al*., 2011). These models can then be used to provide warning signals to protect freshwater biodiversity.

Several approaches used for predicting biological effects due to bioaccumulation were reviewed by Simpson & Batley (2007), e.g. the Biotic Ligand model (BLM) and the Simultaneously Extracted Metals / Acid-Volatile Sulfide (SEM/AVS) model. That review pointed out some limitations of the models used to predict alterations in field communities. Thus, the BLM assesses the bioavailability of dissolved metals, but it can be objected that diet is one of the main uptake routes for benthic macroinvertebrates (Luoma & Rainbow, 2005; Simpson & Batley, 2007). On the other hand, the SEM/AVS model does not always describe satisfactorily the metal availability from contaminated sediments (e.g. in oligochaetes and chironomids in polluted sediments: De Jonge *et al*., 2009; Méndez-Fernández *et al*., 2014). Moreover, the applicability of the SEM/AVS model to benthic organisms may be also compromised by the fact that this model is based on the chemistry of anoxic sediment (Casado-Martinez *et al*., 2010), a condition that rarely occurs in river (lotic) habitats. Additionally, because bioavailability of metals also depends on organism behavior, feeding selectivity and physiology (De Jonge *et al*.,

2010; Luoma & Rainbow, 2005), the assessment of bioaccumulation using a single species cannot provide the necessary information to evaluate risk at the community level. Moreover, differential bioaccumulation among metals and metalloids (hereinafter, referred to as metals) depends on metabolic essentiality, which in turn can explain differences among taxa in uptake rates, assimilation efficiencies, and detoxification mechanisms (Adams *et al*., 2011; Wang & Rainbow, 2008). Taking into consideration the aforementioned limitations, we approached the risk assessment of metal bioaccumulation in a different way, using several taxa and based on their departure from the baseline tissue residues previously measured in the mining region (Rodriguez *et al*., 2018).

In Europe, the core of the ecological status classification systems for water bodies under the Water Framework Directive (WFD: EC, 2000) is based on the selection of a spatial network of reference sites, and on the degree of deviation of biological variables from the reference values. The Nalón River basin has a long history of metal extraction, and its sediments can harbor high levels of certain metals due to the legacy of abandoned Hg and Cu mining activities. These metals have been shown to cause ecotoxicity and alterations to the macroinvertebrate community (Costas *et al*., 2018; Méndez-Fernández *et al*., 2015). In the absence of tissue quality standards for metals in the European regulations (except for Hg: EC, 2013), bioaccumulation risk assessment in the study region is addressed here using the ratio of the metal tissue residues in field organisms to the baseline value of the corresponding taxon (ETTC, Rodriguez *et al*., 2018). This ratio will provide information on the degree of departure of the metal bioaccumulation from background levels, and on the metal bioavailability gradients within the river basin. It was hypothesized that these gradients could also be related to the alterations measured in macroinvertebrate communities.

The present study measured the metal tissue concentration in several sites potentially affected by metal pollution, with the aim of performing a bioaccumulation risk assessment in the Nalón River basin through the integrative assessment of the most relevant metals in selected biomonitors. Therefore, the specific objectives of this study are: (1) to provide criteria to select a suitable set of relevant metals and macroinvertebrate taxa as biomonitors for the bioaccumulation risk assessment; (2) to

provide a methodological framework to assess metal bioaccumulation through integrative scores for mixed-metal pollution situations; and (3) to provide tools based on stream macroinvertebrate bioaccumulation that can assist in the decision making process in monitoring and restoration programs of water bodies in mining districts.

2. MATERIAL AND METHODS

2.1. Study area

This study was conducted in the Nalón River basin (Asturias, northern Spain), the largest basin within the Cantabrian water district (total area of 4907 km^2). A total of 29 sites were sampled for macroinvertebrate tissue residue analysis during summer, in 2014 and 2015. The sampling design included 14 reference sites and 15 test sites, whose environmental characteristics have been partially reported in previous publications (Costas *et al*., 2018; Méndez-Fernández *et al*., 2017; Rodriguez *et al*., 2018). These sites correspond to four Spanish river types (Types R-T21, R-T25, R-T28 and R-T31: BOE, 2015) (Table S1). Reference sites (REF group) were selected according to the European WFD criteria by the absence of significant anthropogenic pressures and validated following the procedure by Pardo *et al*. (2012). The test sites were selected as being potentially affected by different mining pressures and were identified based on previous data of the authors: 3 sites downstream from abandoned Cu mining activities (CU group); 8 sites downstream from abandoned Hg mining areas (HG group); and 4 sites close to an active gold mine (AU group: 1 site upstream, P1) and 3 sites downstream from the mine (Table S1). The ecological status at each river site was assessed by the EQR (Ecological Quality Ratio) score of the NORTI predictive model (NORThern Spain Indicators, Pardo *et al*., 2014), which measures the similarity of the faunal composition of a test sample to that of the reference community of the corresponding river-type predicted from a multivariate model.

2.2. Sampling strategy and metal analysis in sediment and tissue

At each site, 0.5 L of a composite sediment sample was collected with a stainless-steel spade from the upper 5–10 cm layer of fine sediment settled in submerged depositional areas, along an approximately 25-m river reach segment. Samples were

taken to the laboratory on ice and stored at 4°C, in the dark. Dissolved oxygen in the field was always > 8.5 mg L^{-1}, pH = 5.8–8.7 (mean = 7.8), conductivity = 31–1263 µS cm^{-1} (mean = 452), TOC% in sediment =0.4–6.3 (mean = 2.0), silt-clay% in sediment= 0.4–28.1 (mean = 5.7). Other physical and chemical water characteristics were published in Costas *et al*. (2018).

Macroinvertebrates were kick-sampled in a variety of lotic habitats and river margins, using a hand-net (maximum depth in sediment about 10 cm). Selected biomonitors belong to 10 taxa (8 insect families plus 2 oligochaete groups): Baetidae, Ephemerellidae, Ephemeridae, Heptageniidae, Hydropsychidae, Lumbricidae, Microdrile oligochaetes (mostly *Lumbriculidae*), Perlidae, Rhyacophilidae, and Simuliidae. These taxa can be regarded as representative of different metal exposure routes, based on their feeding styles (scrapers, filterers, deposit-feeders, generalist and predators), in addition to their general habits (epibenthic vs endobenthic); all are widespread within the study area.

Macroinvertebrate samples for bioaccumulation analyses were obtained at each site following a multi-habitat sampling scheme. For each taxon, 3 replicates consisting of 1–20 individuals were collected per site, depending on the individual size (see details in Rodriguez *et al*., 2018); when number or size of the sample was limited, individuals were pooled to obtain enough biomass for the metal analysis (see Table S2). In some occasions, the exceptionally large size of some taxa (e.g. Perlidae) required the analysis of only part of the body or the selection of medium-sized individuals. Organisms were sorted on site, held for 5–10 h in river water on ice and identified before being frozen. By then, they would have totally or largely emptied their guts (4-6 h clearing period in Cain *et al*., 1992). In aquatic oligochaetes, the gut content after the purging period represents a low fraction of the body weight. For example, after a 9-h purging period under 4°C, the remaining sediment in the gut of *Tubifex tubifex* represents only 11% of the total fecal production in 24 hours, and 5% of the mean worm's body (tissue) weight (unpublished data from authors).

All sampled taxa were analyzed (n = 117). Procedures related to the sediment and tissue acid digestion and measurement methods were described by Méndez-Fernández *et al*. (2017) and Rodriguez *et al*. (2018), and are briefly summarized here.

Nine trace metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Se and Zn) were analyzed in tissue and in the < 63 µm fraction of sediments. Macroinvertebrate samples were digested in nitric acid (70% Baker® Instra-Analyzed) and hydrogen peroxide (30% RP Suprapur Merck®). Sediment samples were digested using a microwave extraction method, in concentrated nitric acid and concentrated hydrochloric acid, following USEPA 3051 protocol (USEPA, 2007). All analytical samples included Mussel Tissue Standard Reference Material (NIST 2976), or Buffalo River sediment (RM8704, USA) and Sewage Sludge-3 (CRM 031-040, UK) as reference materials for quality control. All concentrations in present study are given on dry-weight (dw) basis.

To assess sediment pollution, we used the SedPoll score (Costas *et al*., 2018), an integrative sediment pollution score based on 6 metals (As, Cd, Cu, Hg, Pb and Se), selected as relevant for the macroinvertebrate community composition in the study area. This score is calculated for each site as the average of the ratios of each of the actual metal sediment concentrations to the corresponding 90th percentile of the REF group sediment concentration.

2.3. Integrated bioaccumulation scores

The Ecological Threshold Tissue Concentration (ETTC) for each metal and taxon (Rodriguez *et al*., 2018) was used as benchmark value to express the bioaccumulation in each site as a metal hazard quotient. Next, for each taxon, these quotients were averaged by the number of metals and expressed as Tissue-residue Ratio to Threshold (TRT) score TRT= $(\sum_i(TR_i/B_i))/n$, where i is the metal, TR_i the tissue concentration of each metal in the taxon, B_i the baseline or low threshold for each metal in that taxon, and n the number of metals. This index is based on the Cumulative Criterion Unit (Clements *et al*., 2000) and assumes metal mixture interactions as additive. Finally, for every site, an INtegrated TISSue score (INTISS) was calculated as the mean of the TRT scores of the selected taxa, and used as the base for the site classification.

Site bioaccumulation assessment used both the taxon TRT and the site INTISS scores, following a classification in four general categories: (1) Similar to reference, when bioaccumulation scores are ≤ 1.0 (boundary of 0 in log-scale); (2) Low bioaccumulation when scores are 1.1–2.0 (boundary of 0.3 in log-scale); (3) Medium

bioaccumulation when scores are 2.1–10.0 (boundary of 1.0 in log-scale); (4) High bioaccumulation when scores are > 10.

2.4. Statistical analyses

Regression analyses and univariate statistics were performed with IBM® SPSS® statistics 25 software. Metal concentration in the biota expressed in the form of integrative scores (taxon TRT and site INTISS) were related to the sediment pollution scores (SedPoll), and to the ecological status of benthic macroinvertebrate communities (EQR scores) using linear regression models. Metal tissue concentrations were not usually normally distributed (Shapiro-Wilk test), hence data were normalized using log-transformation. The standardized residuals were always within ± 3 standard deviations from zero.

Additionally, several multivariate analyses were performed for individual taxa by means of PRIMER 6 software (Clarke & Gorley, 2006). Using the Euclidean distance of the log-transformed and normalized tissue residue data, the one-way ANOSIM procedure (999 permutations) (Clarke, 1993) was applied to the tissue residue dissimilarity matrices to test the possible influence of the following site-grouping factors: (1) ecological status based on Good/Not Good classification of sites using a NORTI-EQR score boundary of 0.700; and (2) the anthropogenic pressures related to mining activities (REF, CU, HG and AU groups). Null hypotheses in ANOSIM were rejected when $p < 5\%$. The contribution of tissue metal residues in different taxa to the site group dissimilarities was analyzed through SIMilarity PERcentage analysis (SIMPER procedure) (Clarke & Gorley, 2006).

3. RESULTS

3.1. Biomonitors: first-level selection process

The selection of suitable biomonitor taxa in a reference condition approach depends primarily on their presence in both reference and polluted sites; thus, the selection of biomonitors should consider their tolerance to metals and other anthropogenic stressors. In this study, all the biomonitor taxa selected were present in

more than 70% of reference sites and in most test sites (in ≥ 70% sites, except for Ephemeridae and Perlidae in 67 and 47% of sites, respectively) (Table 1). The low presence of perlids suggests that this predator taxon might not be an appropriate biomonitor in the Nalón River basin, unless it appears to be suitable for trophic transfer studies. Hence, in the selection of biomonitors, it seemed necessary to choose several taxa relatively tolerant to anthropogenic pressures to prevent lack of information from sites with high mortality of most sensitive organisms, either due to pollution or to lack of suitable habitat. The next requirement is that the abundance and body size of potential biomonitors should be sufficient for tissue residue analysis (Table S2). Some of the taxa are very small, e.g. Baetidae and Simuliidae, and the collecting time required to get a minimum biomass was higher than for other taxa; however, these were usually very abundant. Finally, in an effort to represent the range of tissue concentrations at the sites, we also followed a complementary criterion: the selection of several biomonitor taxa with different functional traits (feeding styles and habits) as a strategy to represent the main metal uptake routes.

3.2. Metal tissue residues in macroinvertebrate taxa and their relationship to sediment metal concentrations

Sediment metal concentrations per site are shown in the supplementary Table S3. The highest values for As, Cu and Se were recorded in the AU group, with levels above the Probable Effect Concentration (PEC, MacDonald *et al*., 2000). Based on the Hg quality standards for sediments proposed by Méndez-Fernández *et al*. (2019), all reference sites (except N12) were classified as Good chemical status (< 0.49 µg Hg g^{-1} dw), while test sites in the HG group had Moderate to Bad chemical status, with particularly high levels at N9 and N15 (> 50 µg g^{-1} dw).

Overall, the sediment quality assessed by the SedPoll score (Figure 1) indicated that the REF group of sites was not polluted (scores ≤ 1). In the HG group, site N10 was assessed as moderately polluted (score 2.1 – 10), while sediments in N9 and N15 were highly polluted (scores 10.1 – 50). In the AU group, SedPoll scores indicated that sites P2 and P3 were highly polluted, and that there was a reduction to moderate pollution level at the downstream site, P4. The remaining test sites, including the CU group, were assessed as similar to the reference or with low metal pollution (scores ≤ 2).

Table 1. Presence (✓)/absence (x) of taxa selected as biomonitors in the study sites (identified by the potential mining influence on each site). Percentage of appearance at reference (REF) sites (n=14, in Rodriguez *et al*., 2018) and test sites (n=15) is indicated. **Abbreviations**: SC Scraper, CF Collector-Filterer, G Generalist, P Predator, DF Deposit Feeder, [Ep] epibenthic, [En] endobenthic.

Biomonitor Taxa	Functional Group	CU group			HG group								AU group				Percentage of Appearance	
		N4	N6	NAL 013	N9	N10	N11	N13	N14	N15	NAL 056a	NAL 056b	P1	P2	P3	P4	TEST Sites	REF Sites
Ephemeroptera																		
Baetidae	SC [Ep]	✓	✓	✓	✓	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	✓	93	100
Ephemerellidae	G [Ep]	✓	✓	✓	x	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	✓	87	86
Ephemeridae	CF [En]	✓	✓	x	x	✓	✓	✓	✓	x	✓	✓	✓	x	✓	x	67	86
Heptageniidae	SC [Ep]	✓	✓	✓	x	x	✓	✓	✓	x	✓	✓	✓	✓	✓	✓	80	100
Plecoptera																		
Perlidae	P [Ep]	x	✓	x	x	x	x	✓	✓	x	✓	x	✓	✓	✓	x	47	71
Trichoptera																		
Hydropsychidae	CF [Ep]	✓	✓	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	x	✓	✓	87	93
Rhyacophilidae	P [Ep]	✓	✓	✓	x	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	✓	87	93
Diptera																		
Simuliidae	CF [Ep]	✓	✓	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	x	x	x	73	79
Annelida Clitellata																		
Lumbricidae	DF [En]	✓	✓	✓	x	✓	x	✓	✓	✓	x	x	✓	✓	✓	✓	73	100
Microdrile oligochaetes	DF [En]	✓	✓	✓	✓	x	✓	✓	x	✓	✓	✓	✓	✓	✓	✓	87	86
Total taxa per site		9	10	8	3	7	8	9	9	4	9	8	10	7	9	7	-	-

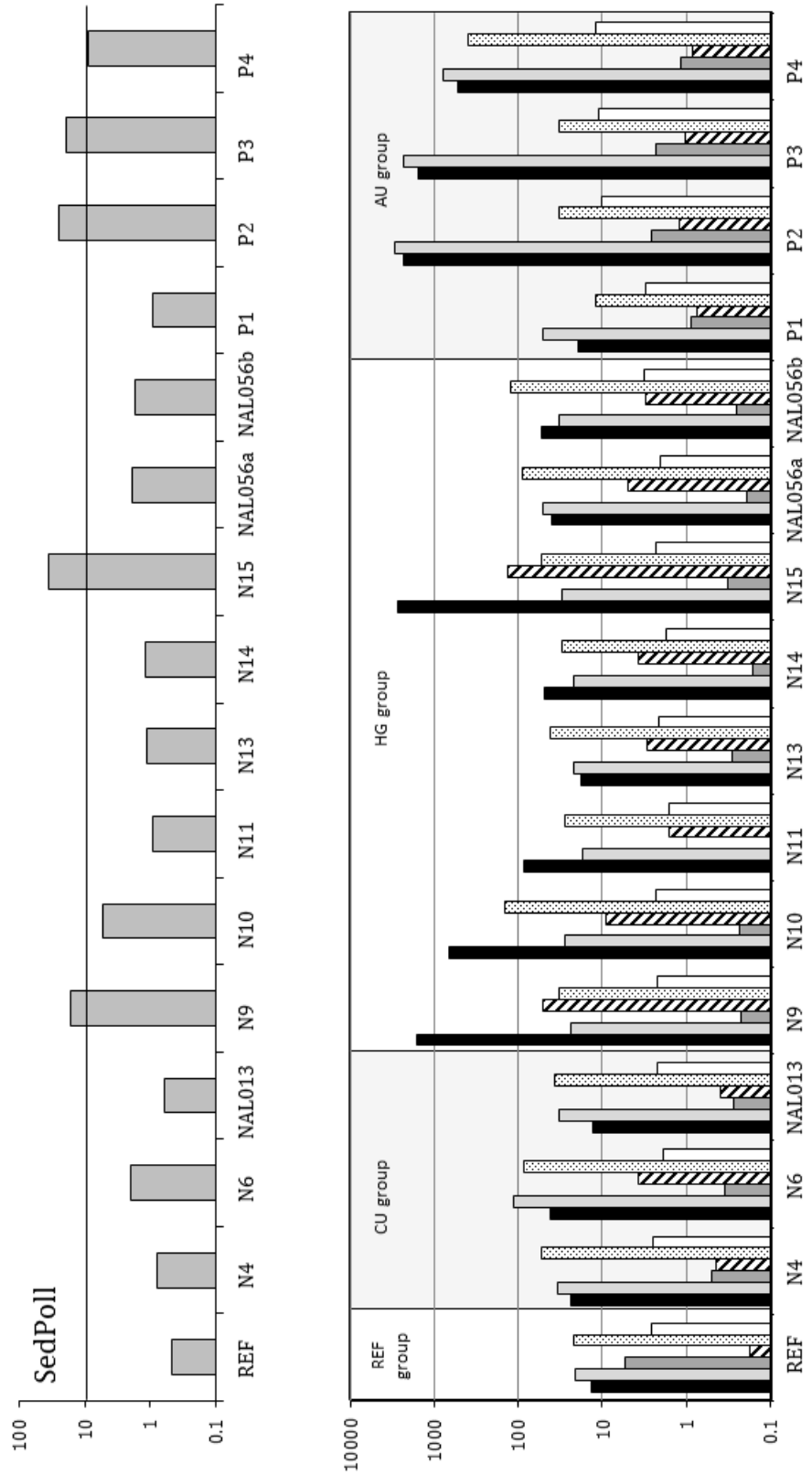

Figure 1. Sediment pollution score (SedPoll) (above) and sediment concentration of 6 metals (below) in the study sites. Metal codes (by order in the graph): As (black), Cu (light grey), Hg (striped), Pb (dotted) and Se (white). For the reference sites (n = 14), the median value is represented.

For each biomonitor taxon, metal availability from sediments was approached through linear regression models between the log-transformed metal concentration in sediment and tissue residues, including both reference and tests sites (Table 2). Linear regression analysis for As suggested that it is both highly bioavailable to all macroinvertebrate taxa in the Nalón River (r^2_{adj} > 0.700) and linearly dependent on sediment concentration (b = 0.7–1.2). Filterer taxa showed b > 1.0, suggesting that the increase in tissue residues can be the result of the ingestion of small, metal-rich sediment particles or a slow elimination pattern. In assessing As bioaccumulation, several taxa identified 4 sites in the HG group (N9, N10, N11, N15) as having Medium to High bioaccumulation and 3 sites (P2, P3 and P4) in the AU group (TR > 10 times the baseline ETTC, see Table S4). No single taxon discriminated the 7 sites, as each was absent from some of the sites. Nevertheless, Baetidae, Hydropsychidae and Microdrile oligochaetes could identify 5 or 6 out of the 7 aforementioned sites.

The fit of the linear models for Cu was better for the infaunal taxa that burrow into the sediment, i.e. Ephemeridae and oligochaetes, as well as for their potential predators (Rhyacophilidae) (r^2_{adj} > 0.700, Table 2). According to the slope values (b = 0.3–0.7), these models suggest a variable degree of regulation of the body concentration by these taxa when exposed to increasing Cu levels in the sediment. Regarding site assessment for Cu, the 3 sites in the AU group (P2 to P4) with the highest sediment loadings were evaluated as having Medium to High bioaccumulation by all the taxa, except for Ephemerellidae (see Table S4).

The fit of the linear regression models for Hg was low to moderate (r^2_{adj} = 0.224–0.579), but higher for the Lumbricidae (Table 2). According to the slope values (b = 0.2–0.6), these models suggest that Hg body concentration was kept at lower levels than expected when exposed to increasing Hg levels in the sediment, either due to body barriers or detoxification processes. However, the models are not easy to interpret due to the absence of many taxa from sites with higher Hg levels (e.g., N9 and N15), which presumably decreased both the slope and fit of the models. Medium to High Hg bioaccumulation was assessed for the 2 sites with the highest sediment values (N9 and N15) by Baetidae, Hydropsychidae, Simuliidae, Lumbricidae and Microdrile oligochaetes. Bioaccumulation assessments for N10, N11 and N13 were Low to Medium,

Table 2. Linear regression of the log-transformed metal tissue residues by taxon on the log metal concentration in the sediment. Only significant models are included (F test, $p < 0.05$).

Taxon	Metal	r^2_{adj}	b (SE)	a
Baetidae	As	0.801	0.801 (0.076)	-0.645
(n=28)	Cd	0.441	0.877 (0.186)	0.709
	Cu	0.430	0.327 (0.071)	0.992
	Hg	0.434	0.340 (0.073)	-0.639
	Zn	0.275	0.814 (0.243)	0.739
Ephemeridae	As	0.867	1.164 (0.099)	-1.140
(n=22)	Cd	0.333	1.223 (0.360)	0.436
	Cr	0.392	0.712 (0.187)	-0.697
	Cu	0.830	0.716 (0.070)	0.225
	Hg	0.338	0.407 (0.119)	-0.588
Ephemerellidae	As	0.789	0.710(0.072)	-0.340
(n=27)	Cd	0.222	0.596 (0.206)	0.785
	Cu	0.454	0.300 (0.063)	1.334
Heptageniidae	As	0.748	0.747 (0.086)	-0.530
(n=26)	Cd	0.508	0.833 (0.161)	0.095
	Cr	0.124	0.533 (0.250)	-0.575
	Cu	0.516	0.454 (0.086)	1.135
	Hg	0.363	0.326 (0.084)	-0.721
	Zn	0.465	0.793 (0.166)	0.749
Hydropsychidae	As	0.855	1.039 (0.085)	-1.252
(n=26)	Cd	0.145	0.485 (0.212)	-0.583
	Cu	0.567	0.319 (0.055)	0.743
	Hg	0.463	0.426 (0.090)	-0.548
	Se	0.143	0.440 (0.194)	0.129
Lumbricidae	As	0.718	0.797 (0.103)	-0.105
(n=24)	Cr	0.206	0.419 (0.159)	-0.253
	Cu	0.789	0.469 (0.050)	0.349
	Hg	0.579	0.577 (0.101)	-0.310
	Se	0.220	0.419 (0.153)	0.785
Microdrile	As	0.845	0.800 (0.070)	-0.037
oligochaetes	Cu	0.749	0.601 (0.071)	0.290
(n=25)	Hg	0.498	0.363 (0.073)	-0.190
	Se	0.223	0.493 (0.176)	0.451
Perlidae	As	0.891	0.877 (0.079)	-1.421
(n=16)	Cu	0.692	0.315 (0.053)	0.997
	Hg	0.433	0.317 (0.090)	-0.738
Rhyacophilidae	As	0.764	0.691 (0.076)	-1.140
(n=26)	Cd	0.383	0.876 (0.216)	-0.062
	Cr	0.182	0.811 (0.317)	-1.011
	Cu	0.787	0.276 (0.029)	0.840
	Hg	0.224	0.205 (0.072)	-0.604
	Ni	0.143	0.900 (0.396)	-1.681
	Se	0.178	0.440 (0.173)	0.254
	Zn	0.177	0.352 (0.139)	1.648
Simuliidae	As	0.917	1.177 (0.075)	-1.117
(n=23)	Cr	0.397	0.755 (0.196)	-0.552
	Hg	0.493	0.427 (0.090)	-0.288

depending on the taxa, and predator taxa showed Low or No bioaccumulation of Hg (≤ ETTC) in all the test sites (Table S4).

A moderate fit of Cd TR as a function of the sediment concentration was obtained in linear regression models for Heptageniidae (r^2_{adj} = 0.508) and Baetidae (r^2_{adj} = 0.441), and one of their potential predators, Rhyacophilidae (r^2_{adj} = 0.383) (Table 2). For Cr, Ni, Se and Zn, only a few taxa showed a significant (but mostly low) fit to the linear model. No relationship was found for Pb. These results can be partly due to the low concentration of these metals in sediments, which can also explain to some extent the low TR measured in many taxa. None of these metals reached the high bioaccumulation class (TR > 10 times the ETTC) in any of the biomonitor taxa (Table S4).

The fact that not all measured metals are equally available for bioaccumulation, or are likely to affect the macroinvertebrate community ecological status, led us to propose the use of integrative metal scores on a selection of relevant metals. For each biomonitor, the TRT scores were calculated using different number of metals (3 to 9 metals, TRT_3 to TRT_9). The metals selected for the combined index TRT_3 were As, Hg and Cu, based on the relationships of the tissue residues with sediment concentrations described above. The addition of metals to the TRT had an effect on the index similar to a dilution of the relevant metals (see Table S4 for comparison of TRT_3 and TRT_9). Log-transformed values of SedPoll and TRT scores for the different metal combinations were fitted to linear regression models for all taxa; in all instances, models were significant, showed positive slopes and moderate to good fit depending on the taxa and metal combinations (r^2_{adj} = 0.486–0.849, Table 3). In half of the cases, the highest determination coefficients were obtained for a three-metal combination (As, Cu, Hg = TRT_3). Only 3 cases were better adjusted for TRT_6 and 2 cases for TRT_9, but differences in model fit were not large. These models suggest, first, that the combination of metals considered in each case was highly bioavailable for the taxa. Additionally, those taxa with regression coefficient (b) closer to 1 allowed for a more straightforward prediction of bioaccumulation related to increased sediment pollution levels than those with low b values (e.g. Ephemerellidae and Heptageniidae), where either their feeding style, the regulation of the uptake or the active elimination processes of metals may make interpretation difficult.

Table 3. Linear regression of the log *SedPoll* (X) *vs* log *TRT* (Y) scores and log *TRT* (X) *vs NORTI-EQR* scores (Y), calculated for 3, 4, 6 and 9 metals (see text) over ten biomonitor taxa in the Nalón River basin. Highest determination coefficients (r^2_{adj}) for each taxon marked in bold. **Abbreviations**: SC scraper, F filterer, O opportunistic, DF deposit-feeder, P predator, ns non-significant.

	Metals	log *SedPoll* (X) vs log *TRT* (Y)			log *TRT* (X) vs *NORTI-EQR* (Y)		
		r^2_{adj}	b (SE)	a	r^2a_{dj}	b (SE)	a
Baetidae (SC)	As Cu Hg	**0.544**	0.712(0.175)	0.104	**0.712**	-0.234 (0.041)	0.758
	As Cd Cu Hg	0.539	0.688 (0.171)	0.036	0.685	-0.238 (0.044)	0.741
	As Cd Cu Hg Pb Se	0.508	0.620 (0.163)	-0.027	0.638	-0.249 (0.051)	0.722
	All 9 metals	0.446	0.536 (0.158)	-0.063	0.577	-0.260 (0.060)	0.706
Ephemeridae (F)	As Cu Hg	0.733	1.063 (0.210)	-0.010		ns	
	As Cd Cu Hg	0.722	1.029 (0.208)	-0.102		ns	
	As Cd Cu Hg Pb Se	0.754	0.949 (0.178)	-0.164		ns	
	All 9 metals	**0.761**	0.844 (0.155)	-0.199		ns	
Ephemerellidae (O)	As Cu Hg	**0.486**	0.487 (0.139)	-0.011	**0.539**	-0.191 (0.049)	0.725
	As Cd Cu Hg	0.468	0.422 (0.124)	-0.023	0.435	-0.198 (0.062)	0.719
	As Cd Cu Hg Pb Se	0.471	0.384 (0.112)	-0.119	0.425	-0.216 (0.069)	0.698
	All 9 metals	0.337	0.287 (0.018)	-0.131	0.248	-0.204 (0.092)	0.688
Heptageniidae (SC)	As Cu Hg	0.501	0.393 (0.113)	0.129		ns	
	As Cd Cu Hg	0.512	0.411 (0.116)	0.084		ns	
	As Cd Cu Hg Pb Se	**0.540**	0.399 (0.107)	-0.014		ns	
	All 9 metals	0.486	0.349 (0.103)	-0.037		ns	
Hydropsychidae (F)	As Cu Hg	**0.650**	1.014 (0.210)	0.019	**0.524**	-0.086 (0.023)	0.733
	As Cd Cu Hg	0.631	0.949 (0.205)	-0.020	0.488	-0.088 (0.025)	0.728
	As Cd Cu Hg Pb Se	0.626	0.874 (0.190)	-0.076	0.465	-0.094 (0.028)	0.722
	All 9 metals	0.598	0.793 (0.183)	-0.131	0.403	-0.095 (0.032)	0.714
Lumbricidae (DF)	As Cu Hg	**0.494**	0.816 (0.249)	0.094	**0.653**	-0.115 (0.026)	0.749
	As Cd Cu Hg	0.479	0.786 (0.246)	0.013	0.649	-0.118 (0.027)	0.739
	As Cd Cu Hg Pb Se	0.481	0.738 (0.230)	-0.058	0.628	-0.124 (0.029)	0.730
	All 9 metals	0.412	0.628 (0.222)	-0.073	0.549	-0.129 (0.036)	0.722
Microdrile oligochaetes (DF)	As Cu Hg	0.734	0.740 (0.127)	0.102	**0.470**	-0.192 (0.056)	0.749
	As Cd Cu Hg	0.737	0.681 (0.116)	0.063	0.452	-0.206 (0.062)	0.741
	As Cd Cu Hg Pb Se	**0.749**	0.644 (0.106)	-0.032	0.436	-0.216 (0.067)	0.721
	All 9 metals	0.748	0.562 (0.093)	-0.081	0.421	-0.244 (0.078)	0.707
Perlidae (P)	As Cu Hg	**0.849**	0.945 (0.160)	-0.081	**0.603**	-0.156 (0.049)	0.757
	As Cd Cu Hg	0.832	0.881 (0.159)	-0.118	0.559	-0.162 (0.055)	0.748
	As Cd Cu Hg Pb Se	0.815	0.826 (0158)	-0.211	0.554	-0.170 (0.059)	0.731
	All 9 metals	0.734	0.702 (0.0168)	-0.205	0.490	-0.184 (0.071)	0.723
Rhyacophilidae (P)	As Cu Hg	0.505	0.546 (0.150)	-0.012	**0.568**	-0.177 (0.043)	0.726
	As Cd Cu Hg	0.537	0.539 (0.1040)	-0.093	0.546	-0.181 (0.046)	0.712
	As Cd Cu Hg Pb Se	0.579	0.510 (0.122)	-0.179	0.515	-0.192 (0.052)	0.695
	All 9 metals	**0.620**	0.450 (0.099)	-0.212	0.488	-0.220 (0.062)	0.683
Simuliidae (F)	As Cu Hg	0.711	1.187 (0.235)	-0.015	**0.619**	-0.157 (0.038)	0.719
	As Cd Cu Hg	0.702	1.124 (0.227)	-0.061	0.598	-0.163 (0.041)	0.710
	As Cd Cu Hg Pb Se	**0.782**	1.023 (0.169)	-0.059	0.618	-0.190 (0.046)	0.713
	All 9 metals	0.748	0.934 (0.169)	-0.134	0.589	-0.201 (0.051)	0.695

3.3. Relationship between TRT and field community scores (EQR)

Linear regression analyses between the log-transformed TRT scores and EQR values were performed to evaluate the potential of the bioaccumulation scores to predict the ecological status of the macroinvertebrate communities. As expected, a reduction in the macroinvertebrate community EQR scores was associated with increasing metal bioaccumulation in most taxa (Figure 2). Linear regression models were significant for 8 of the 10 taxa assessed (none were significant for Ephemeridae and Heptageniidae) (Table 3). Models based on TRT_3 (As, Cu and Hg) provided the best fit, and the regression equations indicated that a doubling of the TRT_3 score can reduce the EQR by less than 0.1 units, but a 10-fold increase can reduce it by 0.1-0.2 units, depending on the taxon.

In Figure 2, test sites with no field community alteration (EQR ≥ 0.700) and no bioaccumulation fell in the upper left side of the graph, above the EQR line and to the left of $TRT_3 = 1$; sites with field alteration and high bioaccumulation fell in the right lower side, below the EQR line and to the right of $TRT_3 = 10$. In this way, only sites N9, N10, N15 (in HG group), and P2, P3 and P4 (in AU group) showed alterations attributable to moderate or high metal bioaccumulation for most taxa present at those sites. Finally, several sites (NAL056a and N4 and N11) appeared in a variable position, and often in the limit of the boundary lines, thus indicating a need for specific surveillance programs.

3.4. Integrated risk assessment of bioaccumulation using several biomonitor taxa in a mixed-metal pollution situation

Multivariate analyses using metal tissue residues (TR) for each taxon, both at reference and test sites, gave a good representation of the actual site dissimilarities (Table 4). One-way ANOSIM on metal TR, using two EQR classes (Good/Not Good) as a factor, resulted in significant differences between EQR classes for most taxa examined separately (Global $R = 0.317–0.632$, $p \leq 1\%$), except for Ephemeridae and Perlidae (Table 4). These analyses identified Lumbricidae, Microdrile oligochaetes, Hydropsychidae and Heptageniidae as the taxa with highest differences (ANOSIM Global $R > 0.500$), that is, tissue residues in these taxa were responsible for a greater dissimilarity between sites with Good and Not Good EQR values.

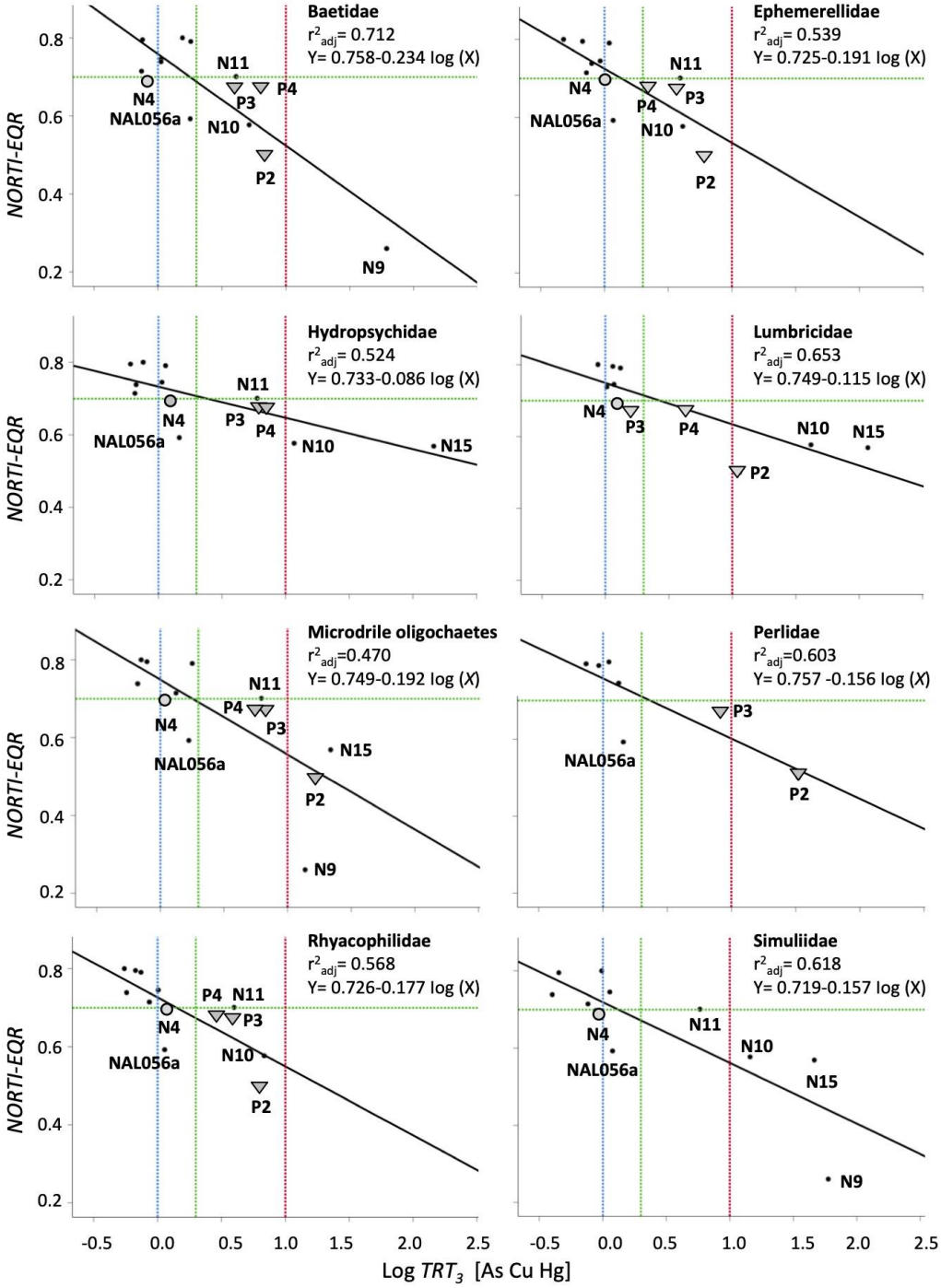

Figure 2. Linear regression models of the NORTI-EQR score on the log-transformed TRT score calculated for As, Cu and Hg tissue concentration in taxa from the Nalón River basin (regression non-significant for Ephemeridae and Heptageniidae). Horizontal green line: EQR boundary (=0.700) for Good/Moderate ecological status. Vertical overlaid lines: bioaccumulation boundaries of TRT scores: blue, No/Low bioaccumulation; green, Low/Medium; red, Medium/High. Mining area codes: black circle, mercury mines; gray circle, Cu mines; triangle, Au mines.

Table 4. Multivariate analyses of the metal tissue residues, separately for each taxon. One-way ANOSIM Global R values, using as factors two NORTI-EQR classes (Good and Not Good ecological status), and the main potential anthropogenic pressures (CU, HG and AU mining groups and REF, Reference sites). For each taxon, SIMPER procedure identified the metals (ordered by their % contribution) mainly contributing to dissimilarity between EQR site classes (Good and Not Good) and between anthropogenic pressure classes. ns: no significant, p ≥ 5%.

TAXA	ANOSIM (factor: EQR) Global R (p%)	SIMPER (factor: EQR) (cumulative %)	ANOSIM (factor: pressures) Global R (p%)	Pairwise R REF vs CU groups	Pairwise R (p%) REF vs HG groups	Pairwise R (p %) REF vs AU groups	SIMPER (factor: pressures) (cumulative %)
Baetidae	0.397 (0.6)	As Cu Hg (48%)	0.422 (0.1)	ns	R= 0.269 (2.0)	R= 0.972 (0.1)	REF *vs* HG: Hg As Cr (51%) REF *vs* AU: Cu Ni Cd (59%)
Ephemeridae	0.215 ns	Cu As Zn (53%)	0.269 (2.6)	ns	R= 0.246 (3.3)	R= 0.646 (2.2)	REF *vs* HG: As (52%) REF *vs* AU: As Cu (75%)
Ephemerellidae	0.317 (0.6)	As Cu Cd (48%)	0.455 (0.2)	ns	R= 0.305 (1.2)	R= 0.712 (0.1)	REF *vs* HG: As Pb (57%) REF *vs* AU: Pb Cu As (69%)
Heptageniidae	0.503 (0.4)	Cu As Cd (46%)	0.605 (0.1)	ns	R= 0.470 (0.4)	R= 0.996 (0.1)	REF *vs* HG: As Cu (63%) REF *vs* AU: As Pb (62%)
Hydropsychidae	0.542 (0.1)	Cu As Hg (51%)	0.382 (0.1)	ns	R= 0.315 (0.4)	R= 0.913 (0.2)	REF *vs* HG: As (65%) REF *vs* AU: As Pb (71%)
Lumbricidae	0.632 (0.1)	As Hg Cu (47%)	0.352 (0.9)	ns	R= 0.351 (3.9)	R= 0.692 (0.1)	REF *vs* HG: Hg As (48%) REF *vs* AU: Cu Ni Se (56%)
Microdriles	0.630 (0.1)	Cu As Hg (43%)	0.489 (0.1)	R= 0.319 (4.4)	R= 0.441 (0.3)	R= 0.821 (0.2)	REF *vs* HG: Hg As Zn (55%) REF *vs* AU: Cu Se Ni (53%)
Perlidae	0.328 ns	As Cu Ni (58%)	ns	–	–	–	REF *vs* HG: Pb Cr Ni (57%) REF *vs* AU: As Pb (51%)
Rhyacophilidae	0.498 (0.5)	Cu As Cd (44%)	0.308 (1.1)	ns	ns	R= 0.797 (0.4)	REF *vs* HG: Hg As Se (52%) REF *vs* AU: Cu Cd Ni (43%)
Simuliidae	0.462 (1.0)	As Hg (75%)	0.317 (1.4)	ns	R= 0.289 (1.3)	ns	REF *vs* HG: As (66%) REF *vs* AU: Ni Cu (65%)

One-way ANOSIM analysis using the anthropogenic pressures (HG, CU, AU mining and REF group) as factors resulted in Global R values of 0.308–0.605 ($p < 3\%$) for all taxa, except for Perlidae. In examining pairwise dissimilarities in metal TR, higher differences were observed between REF vs AU groups ($R = 0.646–0.972$, $p \leq 2.2\%$) for all examined taxa, compared with the lower differences between the REF vs HG groups ($R = 0.246–0.470$, $p < 5\%$). This may be due, in part, to absence of several taxa in sites of the HG group. No significant dissimilarity was observed between CU vs REF groups, except for Microdrile oligochaetes ($R = 0.319$, $p = 4.4\%$). Finally, the SIMPER procedure was applied for metal TR in each taxon across the anthropogenic pressure classes. The analysis showed that As was the main contributor to dissimilarities between REF vs HG groups, followed by Hg (37–66% contribution for As alone or As and Hg, depending on the taxon), except for Perlidae. The main metals contributing to dissimilarities between REF vs AU group were Cu, Ni and As (from higher to lower) (Table 4). Analyses of individual taxa using SIMPER dissimilarities between the two EQR classes showed that As, Cu, Hg and Cd were the main contributors (44–75%, depending on the taxon and metal combination) (Table 4).

These results, and others from previous sections, showed that a selection of 3 biomonitor taxa with demonstrated bioaccumulation potential can minimize sampling and analysis efforts in a bioaccumulation risk assessment, without loss of relevant information. In this regard, we explored various taxa combinations through INTISS scores (see section 2.3) calculated with TRT_3 (As, Cu and Hg). INTISS was calculated over the TRT_3 scores of all biomonitor taxa present at a site ($INTISS_{all}$) and also over a selection of 3 taxa representative of different feeding styles ($INTISS_3$: the scraper Baetidae, the deposit-feeder Microdrile oligochaetes and the filterer Hydropsychidae).

Linear regression models of the log $INTISS_3$ on the log SedPoll score showed an increase in INTISS in response to sediment pollution (Figure 3). Additionally, the regression model of the EQR scores on the log $INTISS_3$ showed a reduction in the ecological status with increasing INTISS values (Figure 3). The fit of the models built with the 3 selected taxa was similar to those with all the biomonitor taxa present at the site (log $INTISS_3$ and log $INTISS_{all}$ vs log SedPoll: r^2_{adj}= 0.720 and 0.732, respectively; NORTI-EQR vs log $INTISS_3$ and log $INTISS_{all}$: r^2_{adj} = 0.556 and 0.583, respectively).

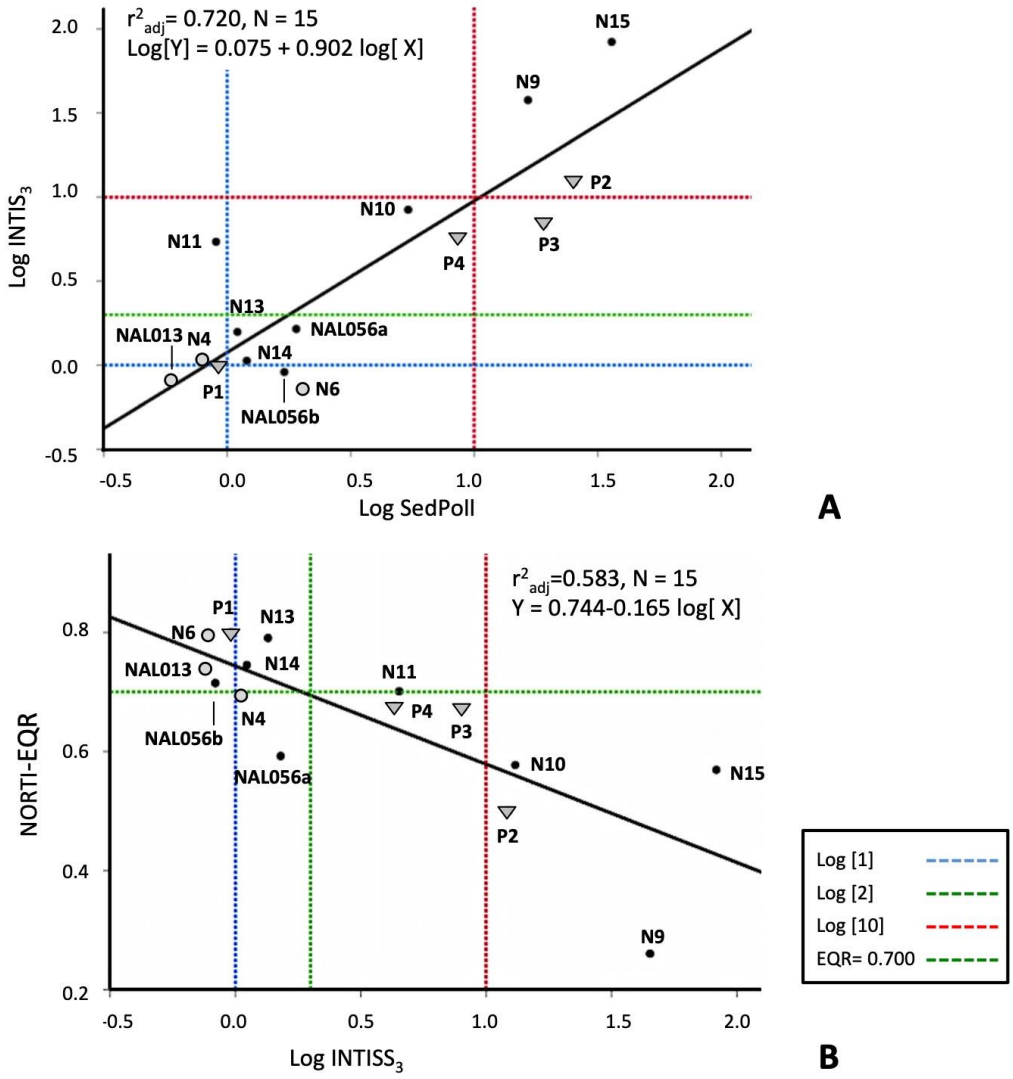

Figure 3. Linear regression models between (A) the log SedPoll score and the log $INTISS_3$ [As, Hg, Cu for 3 taxa; and (B) the log $INTISS_3$ and NORTI-EQR scores. Mining area codes: black circle, mercury mines; gray circle, Cu mines; triangle, Au mines.

The graphical classification of sites based on the regression model followed the same rationale as Figure 2. Thus, two sites in CU group were classified with No bioaccumulation and Good ecological status; several sites among the HG and AU groups showed Medium to High bioaccumulation, consistent with their Moderate to Poor ecological status; and sites N4 and N11 were at the boundary of Good/Not Good ecological status, though the latter fell within the Medium bioaccumulation class, requiring additional monitoring. The remaining sites were classified as Good ecological

status with No or Low bioaccumulation risk, except for NAL056a, which showed alterations in the field community above what was expected from its low TRT_3 score, probably due to reasons other than metal bioaccumulation.

The risk assessment of test sites due to metal bioaccumulation, using INTISS with 3 vs all taxa gave similar results for most sites (Table 5). In most instances, INTISS site assessment for bioaccumulation is consistent with that provided by the EQR scores on the ecological status.

4. DISCUSSION

Metal accumulation in tissues is not necessarily related to toxicity or trophic transfer (Chapman, 2008). Thus, environmental risk assessment derived from bioaccumulation has to be associated with the onset of adverse effects on the organisms. These are conceptually related to the metabolically available accumulated metal fraction, but at this time we cannot measure that concentration in a tissue (Rainbow, 2018). Moreover, exposure conditions as well as the multiple ecological, physiological and genetic factors that differentiate species are some of the obstacles to extrapolating tissue residue data from laboratory bioassays to field communities, and from one region to another. Therefore, it is not surprising that at the beginning of this century there was still a low emphasis on bioaccumulation in environmental regulations, and a limited knowledge of dose-effect relationships at the level of field populations and communities. More recently, there has been greater interest in investigating the range of tissue residues associated with adverse effects on field invertebrate communities (Adams *et al*., 2011). In his valuable review, McCarty *et al*. (2011) addressed the need for future efforts to enlarge the tissue residue-effect databases, to develop a new risk-based framework linking toxicology and ecology, and to improve regulatory guidance incorporating tissue residue-based approaches. We implemented this approach in this study by linking sediment levels of contaminants in the Nalón River basin to tissue concentrations in biomonitors, covering a variety of feeding styles. This procedure allowed us to model alterations in the community ecological status by using integrative bioaccumulation scores in mixed-metal site scenarios.

Table 5. Bioaccumulation risk assessment in 15 test sites of the Nalón River basin, in 3 mining areas (CU, HG and AU groups). For each taxon, the TRT scores were calculated for 3 relevant metals (As, Cu, Hg). The integrative bioaccumulation score (INTISS) for each site was calculated as the mean of the TRT_3 scores in a selection of 3 taxa (BAET, MICRO, HYDRO) and on all taxa present at the site. **Abbreviations**: BAET Baetidae, EPHE Ephemeridae, EPHELL Ephemerellidae, HEPTA Heptageniidae, HYDRO Hydropsychidae, LUMBR Lumbricidae, MICRO Microdrile oligochaetes, PERLI Perlidae, RHYA Rhyacophilidae, SIMU Simuliidae. *TRT* and *INTISS* color codes: No bioaccumulation < 1.0 (white); low 1.12.0 (green); moderate 2.1–10.0 (yellow); high > 10.0 (red). [(1)] from Costas *et al*., 2018).

		[As Cu Hg] TRT_3										$INTISS_3$	$INTISS_{all}$	*NORTI-*
	Sites	**BAET**	**EPHE**	**EPHELL**	**HEPTA**	**HYDRO**	**LUMBR**	**MICRO**	**PERLI**	**RHYA**	**SIMU**	**3 taxa**	**all taxa**	**EQR[(1)]**
CU group	**N4**	0.8	0.7	1.0	1.0	1.3	1.3	1.2	–	1.2	0.9	1.1	1.2	Moderate
	N6	0.8	1.0	0.7	1.0	0.6	1.1	0.8	0.7	0.7	0.5	0.7	0.7	Good
	NAL013	1.1		0.8	0.9	0.7	1.0	0.7	–	0.6	0.4	0.8	0.7	Good
HG group	**N9**	61.6		–	–	–	–	13.8	–	–	59.6	37.7	13.8	Poor
	N10	5.2	7.5	4.1	–	11.7	41.6	–	–	6.8	14.4	8.4	7.5	Moderate
	N11	4.1	3.2	3.9	2.8	6.0	–	6.2	–	4.0	5.8	5.4	5.0	Good
	N13	1.8	1.2	1.1	2.2	1.1	1.3	1.8	0.9	0.7	–	1.6	1.2	Good
	N14	1.1	1.3	0.9	1.0	1.1	1.2	–	1.3	1.0	1.1	1.1	1.0	Good
	N15			–	–	145.7	117.0	21.9	–	–	46.2	83.8	83.8	Moderate
	NAL056a	1.8	1.3	1.2	2.5	1.5	–	1.7	1.4	1.1	1.2	1.6	1.4	Moderate
	NAL056b	0.7	0.8	0.7	0.7	0.7	–	1.3	–	0.9	0.8	0.9	0.9	Good
AU group	**P1**	1.6	0.8	0.5	1.8	0.8	0.9	0.7	1.1	0.5	1.0	1.0	0.6	Good
	P2	6.8	–	6.0	4.7	–	11.4	17.7	33.2	6.0	–	12.2	9.9	Moderate
	P3	6.5	29.9	3.7	4.6	7.1	1.6	7.0	8.2	3.8	–	6.9	5.4	Moderate
	P4	4.2	–	2.2	3.8	6.6	4.5	6.2	–	2.8	–	5.6	4.5	Moderate

In rivers impacted by historical or active metal mining, differences in metal concentration among macroinvertebrate taxa from the same sites can be related to differences in exposure (infaunal vs epifaunal) and to the various uptake routes, reflecting differences in bioavailability (Luoma, 1989; Luoma & Rainbow, 2005). Thus, we were able to approach the bioaccumulation risk assessment using integrative scores applied to three taxa (Baetidae, Hydropsychidae and Microdrile oligochaetes) with different functional traits. The three invertebrate taxa selected for the Integrative scores (INTISS) have been also used as biomonitors by other authors (e.g. Luoma *et al*., 2010; Méndez-Fernandez *et al*., 2017, respectively). However, the selection in this study was not made *a priori*, but was the result of comparison with other possible biomonitors in the community, as described in section 3 (see also Figure 4). These taxa met the following criteria: they are high bioaccumulators of the most relevant metals and are well represented in both reference and test sites, allowing for the calculation of tissue residue ratios between both conditions. Taxa with higher tissue residues are potentially better biomonitors, as the higher bioavailability implies multiple uptake routes (Rainbow, 2018). These taxa were not only high bioaccumulators of the relevant metals, but their selection was supported by the good fit of linear regression models of the metal tissue concentration with sediment concentration and community ecological status.

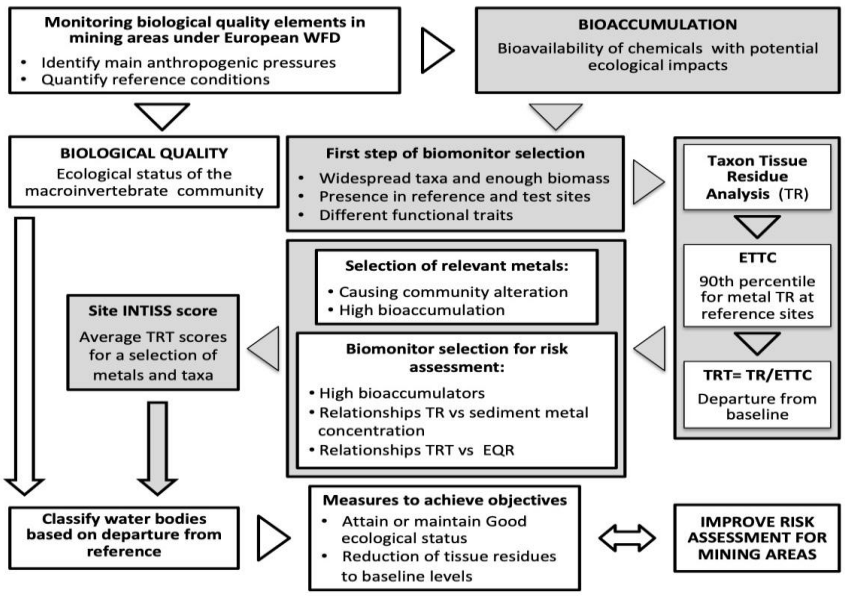

Figure 4. Schema of the study design proposed for bioaccumulation risk assessment using integrative scores for the protection of the aquatic community ecological status.

The methodological framework followed in the selection of biomonitors and relevant metals, and the calculation of Integrative scores (TRT and INTISS) for a bioaccumulation risk assessment in mining areas is summarized in Figure 4. This approach has also emphasized the objective to achieving good ecological status of macroinvertebrate assemblages by reducing the bioaccumulation of metals to baseline levels.

A key issue to be considered when assessing metal bioaccumulation is the difference in metabolic essentiality of some metals. Essential metals (e.g. Cu or Zn) are necessary for the normal growth, development and reproduction of organisms (Chapman & Wang, 2000), and in our results, some of these metals attained very high tissue concentrations, unlike the most toxic, non-essential metals (e.g., Cd, Hg) that are usually present at low levels. Some non-essential metals also do not show adverse effects at moderate tissue residues in field organisms, probably due to efficient detoxification processes (complexation, sequestration and/or storage as inactive substance) (McCarty *et al*., 2011), but also to genetic adaptation (Klerks & Bartholomew, 1991). Therefore, there is not an absolute definition of what is high or low tissue concentration of a particular metal across invertebrates (Rainbow & Luoma, 2011). Consequently, risk assessment applied in other river basins worldwide need to be adjusted at regional scale with a set of calibrated biomonitors, each with known baseline metal concentrations, for a reliable comparison.

Bioaccumulation risk assessment in the present study followed a reference condition approach based on the ratios of actual tissue residues in field organisms to the baseline thresholds (ETTC) set for each taxon from reference, unaltered or minimally disturbed sites of the same region (Figure 4). This procedure can help to interpret the causes of aquatic community alteration in other areas affected by historic or current mining activities. The ETTC can also provide reliable objectives for metal TR reduction in restoration programs of different Water Authorities. The levels of metals above the defined thresholds in the selected biomonitors can be used as a surrogate measure of ecotoxicologically significant effects on the community (Adams *et al*., 2011). The approach presented here can further refine these thresholds based on additional monitoring data.

Previous studies have demonstrated the relationships between metal bioaccumulation in particular biomonitors (Perlidae, Ephemerellidae, Hydropsychidae or Simuliidae) and some adverse effects measured in field macroinvertebrate communities, e.g. loss of taxa richness, loss of abundance of specific bioindicator groups (such as Heptageniidae) or reduction in community monitoring scores (Bervoets *et al*., 2016; De Jonge *et al*., 2013; Luoma *et al*., 2010; Schmidt *et al*., 2011). Here, we focused our analyses on integrative tissue residue scores (TRT and INTISS) after a critical selection of both relevant metals and biomonitors. These scores respond to increases in sediment pollution and to the reduction of the ecological status of the macroinvertebrate community, and these relationships were modelized. The site classification using boundaries of 2 and 10 times a baseline value will indicate the priorities for management actions in particular locations to reduce bioaccumulation, or to monitor the effectiveness of the measures applied in reducing the deviation from the Good class. The site INTISS scores in the Nalón River basin demonstrated that the atypically high tissue concentrations in sites of historic mercury mines and active gold mining could predict high risk of community alteration.

It is also important to acknowledge some of the limitations of the present approach. First, the lack of information about metal speciation could explain differences in metal tissue residues measured in the selected taxa, but it would require further research, focused on each metal and taxon. Second, although the calculation of TRT and INTISS scores is based on the assumption of additive effects of the most relevant chemicals in the basin, we are aware that in metal mixtures there can be also synergetic or antagonistic effects (Norwood *et al*., 2007). More complex mixing models for the presence of several metals could also improve the bioaccumulation risk assessment on the community ecological status in the future. Recently, some methods have been applied to assess field effects derived from the mixture of metals (the Chronic Criterion Accumulation Ratio: Schmidt *et al*., 2010; or the 90th quantile mixture regression model: De Jonge *et al*., 2013). Lastly, further improvement of the regional database is required to expand the range of tissue residue data for other metals, such as Cd, Ni, Pb or Zn, which are important in other mining districts in northern Spain.

The European strategy for water quality risk assessment using reference conditions as a baseline is a suitable starting point for the application of the proposed methodology. A comparison of site bioaccumulation and ecological status assessments, based on their departures from reference levels, showed that water quality monitoring programs can be optimized by combining the two approaches.

5. CONCLUSIONS

Our results show that bioaccumulation levels in field organisms from mining areas of the Nalón River can be expressed as gradients in metal bioavailability for As, Cu and Hg.

An integrative score (INTISS) based on a combination of macroinvertebrate biomonitors, representing different feeding styles and levels of metal bioaccumulation, should facilitate the identification of pollution stressors causing loss of ecological integrity in freshwater ecosystems. Specifically, tissue residues in Baetidae, Hydropsychidae and Microdrile oligochaetes have the potential to be used as early warning signals for metals that are likely to result in adverse effects for the macroinvertebrate community in the Nalón River.

This procedure for assessing metal bioaccumulation can also assist in the decision-making process of ongoing or specifically designed biomonitoring programs for rivers affected by mining activities. The methodology proposed includes criteria for sampling decisions, selection of biomonitor taxa and the calculation of integrative scores that can assist in environmental risk assessment in other mining areas, applying a reference condition approach.

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SUPPLEMENTARY MATERIAL

Table S1. Study sites in the Nalón River basin, location, coordinates, and river type (BOE, 2015) in the Cantabrian region. Ecological status classification based in the EQR of the site macroinvertebrate community to the type reference community of the NORTI predictive model (Pardo *et al*., 2014). Note: (1) Central-Baltic Intercallibrated river types: R-T21 = RC-1; R-T25 = RC-4; R-T28 = RC-5; R-T31= RC-6 (Pardo et al., 2012).

Reference Sites	River	Municipality	UTM_X H30	UTM_Y H30	River Type(1)	Sampling Year	NORTI EQR
NAL009	Huerna	Lena	262,769	4,767,937	R-T31	2014	Good
NAL011	Turón	Mieres	283,040	4,788,140	R-T25	2014	High
NAL029	Pomar	Cangas de Narcea	203,262	4,786,717	R-T31	2015	Good
NAL031	Arganza	Tineo	216,040	4,795,670	R-T28	2015	High
NAL038	Río del Coto	Cangas de Narcea	198,031	4,778,564	R-T31	2015	High
NAL042	Onón	Cangas de Narcea	219,588	4,790,741	R-T28	2015	Good
NAL043*	Genestaza	Tineo	227,060	4,795,591	R-T28	2014	Good
NAL047	Villabre	Yernes y Tameza	246,779	4,794,815	R-T25	2014	Good
NAL050	Teverga	Proaza	743,042	4,794,471	R-T21	2014	High
NAL055	Lena	Lena	270,698	4,777,131	R-T21	2014	Good
NO2259	Reguera	Riosa	264,220	4,789,220	R-T31	2015	Good
N12	Rubial	Lena	268,088	4,785,479	R-T31	2014	Good
R2	Lindes	Quirós	262,789	4,777,652	R-T25	2015	Good
R4	Mosa	Proaza	257,059	4,793,047	R-T28	2014	Good
			TEST SITES				
CU group							
N4	Reguero de Llamo	Riosa	265,613	4,785,980	R-T31	2015	Moderate
N6	Llamo	Riosa	265,907	4,789,095	R-T28	2015	Good
NAL013	Llamo	Mocín	267,410	4,794,605	R-T28	2015	Good
HG group							
N9	Reguero de La Soterraña	Lena	268,816	4,785,844	R-T31	2015	Poor
N10	Muñón	Lena	269,032	4,783,932	R-T28	2015	Moderate
N11	Muñón	Lena	269,135	4,783,836	R-T28	2015	Good
N13	Brañalemosa	Lena	267,849	4,783,599	R-T31	2015	Good
N14	San Tirso	Mieres	274,839	4,794,739	R-T28	2015	Good
N15	San Tirso	Mieres	274,519	4,794,130	R-T28	2015	Moderate
NAL056a	Caudal	Mieres	274,085	4,790,231	R-T21	2015	Moderate
NAL056b	Lena	Lena	270,794	4,784,390	R-T21	2015	Good
AU group							
P1	Cauxa	Belmonte de Miranda	718,641	4,794,352	R-T31	2015	Good
P2	Cauxa	Belmonte de Miranda	717,977	4,794,836	R-T31	2015	Moderate
P3	Cauxa	Belmonte de Miranda	717,421	4,795,742	R-T31	2015	Moderate
P4	Cauxa	Belmonte de Miranda	716,886	4,797,015	R-T28	2015	Moderate

Table S2. Mean, minimum and maximum number of individuals and biomass (in dry weight) analyzed per replicate for ten biomonitor taxa in reference (n=14) and test sites (n=15).

No. Individuals per replicate		Baetidae	Ephemeridae	Ephemerellidae	Heptageniidae	Hydropsychidae	Lumbricidae	Microdriles	Perlidae	Rhyacophilidae	Simuliidae
Reference sites	Mean	11	2	4	5	3	1	5	2	2	12
	Min-Max	6–20	1–3	1–6	2–11	2–6	1–2	2–8	1–6	1–5	6–21
Test sites	Mean	19	2	6	4	2	1	6	2	2	20
	Min–Max	11–30	1–2	2–9	1–6	1–4	1	1–17	1–2	1–3	1–30
Biomass per replicate (mg dw)											
Reference sites	Mean	0.7	15.2	1.8	4.4	6.8	15.7	0.7	18.3	5.1	0.3
	Min–Max	0.4–1.2	5.8–31.4	0.9–2.5	1.2–9.3	0.6–18.8	6.2–26.9	0.3–2.8	2.5–36.7	1.3–14.2	0.1–0.7
Test sites	Mean	0.4	11.6	1.7	4.0	9.6	18.0	0.8	16.1	8.1	0.3
	Min–Max	0.1–0.9	6.5–18.4	0.6–3.3	0.6–11.4	1.5–15.0	2.3–32.7	0.2–1.7	1.2–36.1	0.7–21.7	0.1–0.6

Table S3. Metal concentration (µg g^{-1}, dw) in sediments from sites of the Nalón River basin. *SedPoll* (Sediment Pollution score). Abbreviations: REF group, reference sites; CU, HG and AU groups, sites potentially affected by Cu, Hg and Au mining activities, respectively; PEC, Probable Effect Concentrations from MacDonald et al. (2000), except for those marked with * (i.e. Hg threshold from Méndez-Fernandez et al., 2019; Se threshold measured as the 90th percentile of the sediment concentration in reference sites of the basin). In bold, the concentrations exceeding the PEC.

	Site	As	Cd	Cr	Cu	Hg	Ni	Pb	Se	Zn	SedPoll
REF group	NAL009	17.9	0.16	30.2	21	0.07	32.6	25.4	**5.4**	81	0.52
	NAL011	15.5	0.37	35.6	33.9	0.28	47	35.7	2.8	135	0.64
	NAL029	10.1	0.19	106	10.5	0.25	**60.7**	25.5	2.1	58	0.38
	NAL031	**89.2**	0.94	82.4	25.4	0.15	**53.1**	23.7	2.8	111	0.99
	NAL038	14.3	0.33	77.0	13.3	0.19	**49.8**	22.0	3.7	79	0.47
	NAL042	14.9	0.29	85.5	25.8	0.28	**59.9**	16.4	3.2	100	0.50
	NAL043	8.06	0.18	20.5	17.6	0.07	16.7	22.3	2.3	51	0.36
	NAL047	10.3	0.23	29.9	16.8	0.06	27.3	19.2	5.1	69	0.45
	NAL050	12.5	0.22	19.2	20.7	0.09	24.1	20.6	1.8	83	0.38
	NAL055	16.6	0.31	38.3	34.4	0.16	39.7	35.0	5.3	145	0.68
	NO2259	9.7	0.23	23.7	18.2	0.20	33	18.6	2.0	83	0.38
	N12	16.1	0.19	23.1	21.8	**2.92**	34.8	20.8	1.9	95	0.92
	R2	12.9	0.17	22.5	27.7	0.20	37.6	18.5	1.5	101	0.41
	R4	10.7	0.34	21.7	12.9	0.04	26.5	14.0	1.7	79	0.33
CU group	N4	23.8	0.49	25.7	34.1	0.44	33.9	52.7	2.5	101	0.8
	N6	**41.2**	0.34	34.0	114	**3.75**	**53.3**	84.3	1.9	108	2.0
	NAL013	12.7	0.27	22.1	32.4	0.39	31.3	37.2	2.2	123	0.6
HG group	N9	**1595**	0.22	18.2	23.2	**51.4**	30.9	32.1	2.2	120	16.5
	N10	**669**	0.23	33.1	27.9	**9.03**	41.5	**146**	2.3	111	5.4
	N11	**86.1**	0.10	15.8	17.3	**1.60**	26.0	27.7	1.6	81	0.9
	N13	17.8	0.28	23.6	21.7	**2.95**	34.5	41.6	2.1	97	1.1
	N14	**48.1**	0.16	12.5	22.0	**3.74**	37.3	30.3	1.7	72	1.2
	N15	**2716**	0.32	30.4	29.8	**131**	**53.3**	53.4	2.3	106	36.0
	NAL056a	**40.2**	0.19	33.7	50.2	**4.99**	36.8	87.5	2.0	112	1.9
	NAL056b	**51.8**	0.25	35.5	32.0	**3.08**	43.1	123.0	3.1	141	1.7
AU group	P1	19.2	0.89	29.5	50.2	0.74	**77.9**	12.0	3.0	173.6	0.9
	P2	**2324**	2.62	26.2	**2912**	1.21	25.0	32.4	**10.0**	214.3	25.3
	P3	**1562**	2.27	30.0	**2315**	1.01	32.1	33.0	**11.2**	218.2	19.1
	P4	**513**	1.18	46.4	**775**	0.84	**62.7**	**392**	**11.7**	249.0	8.6
PEC		33	4.98	111	149	1.21*	48.6	128	5.3*	459	

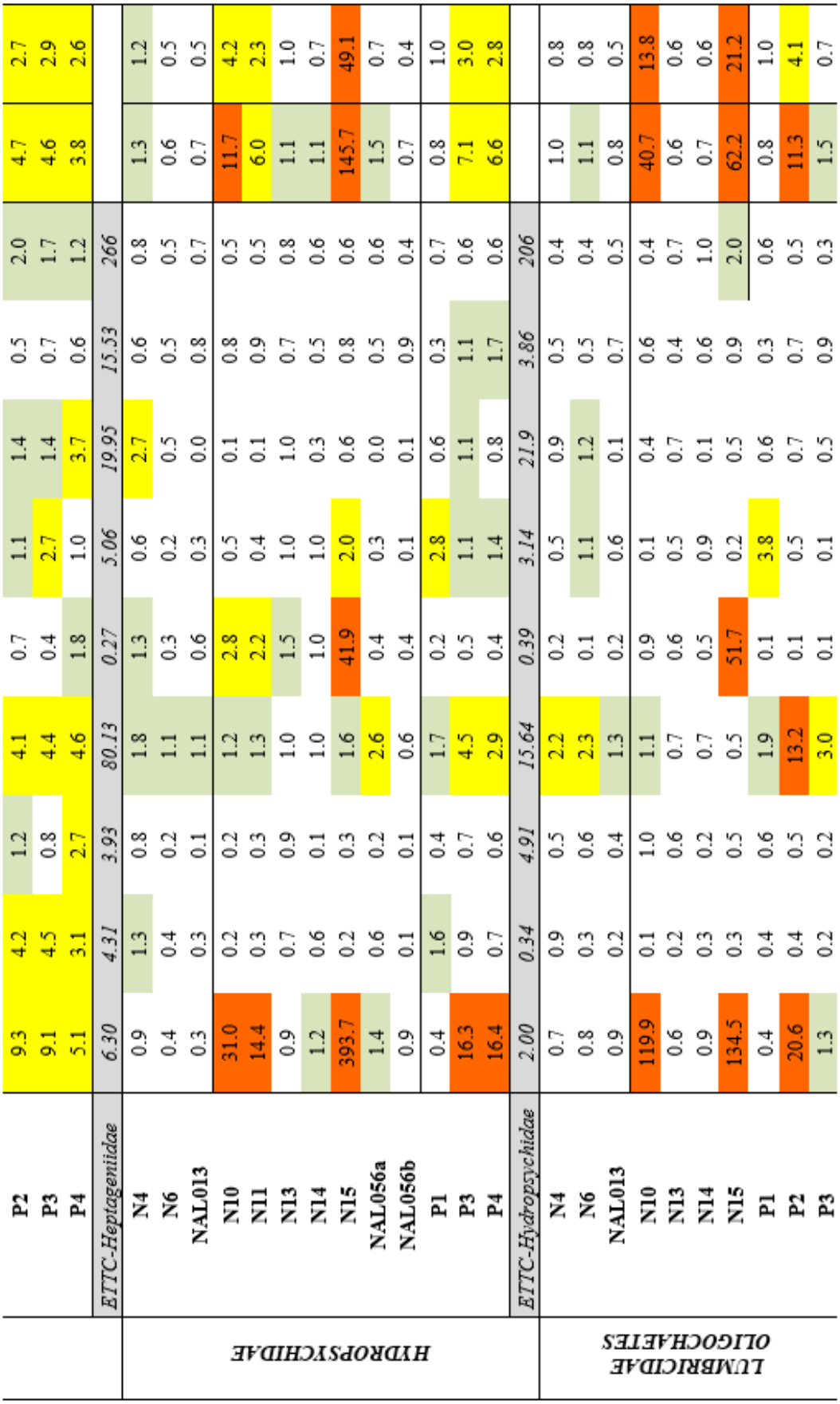
Table S4. Metal and metalloid tissue residues in taxa from test sites, expressed as a quotient to the Ecological Threshold Tissue Concentration (ETTC, from Rodriguez et al. 2018; * ETTC recalculated in 6 cases) for each of the selected biomonitor taxa measured in the reference sites. TRT scores calculated for 3 and 9 substances Color codes: <1.0 No bioaccumulation (white); 1.1-2.0 Low bioaccumulation (in green); 2.1-10.0 Medium bioaccumulation (in yellow); >10.0 High bioaccumulation (in red).

TAXA	Test sites	As	Cd	Cr	Cu	Hg	Ni	Pb	Se	Zn	*TRT* - 3 metals	*TRT* - 9 metals
BAETIDAE	**N4**	0.7	0.9	0.3	1.2	0.5	0.2	0.6	0.6	0.4	0.8	0.6
	N6	0.8	0.3	0.3	1.1	0.4	0.4	0.6	0.4	0.4	0.8	0.5
	NAL013	0.4	0.3	0.3	1.7	1.1	0.7	0.0	0.5	1.5	1.1	0.7
	N9	165.1	0.5	0.8	3.8	16.0	0.9	0.6	1.8	1.9	61.6	21.3
	N10	12.4	0.1	0.4	1.4	1.7	0.5	0.2	0.4	0.3	5.2	1.9
	N11	9.0	0.2	0.6	1.4	1.9	0.5	0.0	0.5	0.4	4.1	1.6
	N13	1.0	0.6	4.6	1.0	3.4	0.5	6.0	0.8	0.5	1.8	2.0
	N14	1.3	0.2	0.5	0.9	1.0	0.8	0.3	0.4	0.5	1.1	0.6
	NAL056a	1.3	0.3	0.3	3.4	0.7	0.6	0.1	0.6	0.6	1.8	0.9
	NAL056b	0.7	0.1	0.3	0.6	1.0	0.5	0.2	0.8	0.5	0.7	0.5
	P1	0.5	2.1	0.7	3.5	0.7	3.3	0.8	0.3	2.0	1.6	1.5
	P2	15.6	1.8	0.6	4.2	0.5	1.0	1.0	0.7	1.1	6.8	3.0
	P3	14.7	2.4	0.5	4.1	0.8	1.4	0.8	0.8	1.2	6.5	3.0
	P4	9.1	1.9	0.9	2.6	0.8	1.1	1.1	0.5	1.2	4.2	2.1
	ETTC-Baetidae	*3.11*	*4.97*	*5.05*	*29.23*	*0.26*	*3.17*	*23.21*	*11.98*	*397*		
EPHEMERELLIDAE	**N4**	0.7	1.4	0.6	1.8	0.5	0.1	0.8	0.7	0.4	1.0	0.8
	N6	0.5	0.4	0.2	1.3	0.3	0.2	0.3	0.4	0.3	0.7	0.4
	NAL013	0.4	0.4	0.9	1.5	0.5	0.9	0.0	0.4	1.2	0.8	0.7
	N10	10.7	0.2	0.3	0.3	1.3	0.5	0.1	0.6	0.2	4.1	1.6
	N11	9.4	0.4	0.7	0.7	1.6	0.8	0.1	1.1	0.5	3.9	1.7
	N13	1.4	1.0	1.0	0.6	1.3	0.6	0.1	0.6	0.5	1.1	0.8

Table S4. Continued.

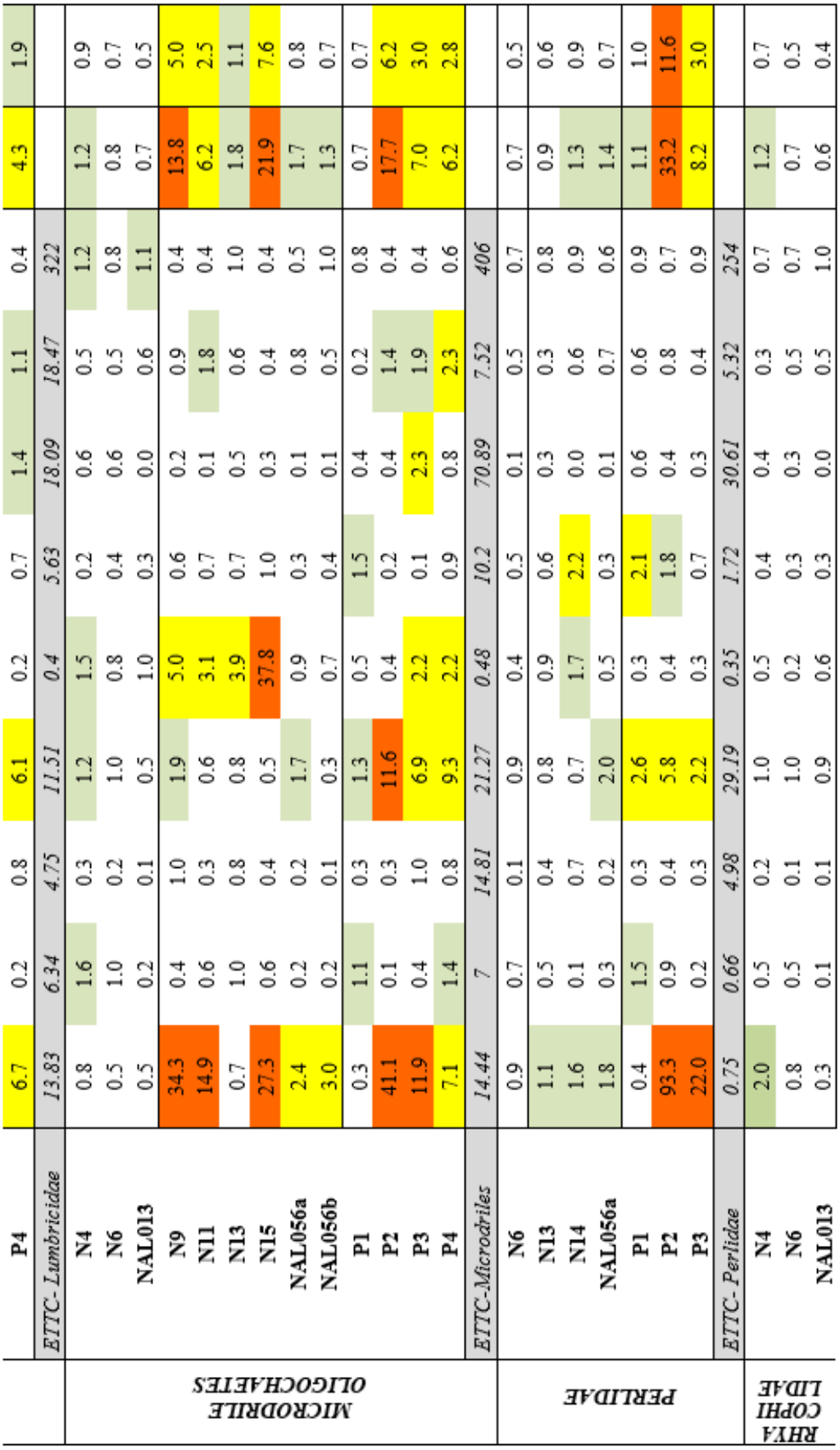
	N14	1.3	0.4	0.3	0.8	0.7	1.1	0.3	0.5	0.3	0.9	0.6							
	NAL056a	1.2	0.2	0.2	1.9	0.4	0.3	0.0	0.3	0.4	1.2	0.6							
	NAL056b	1.2	0.1	0.3	0.5	0.5	0.3	0.1	0.8	0.4	0.7	0.5							
	P1	0.2	2.3	0.3	1.0	0.2	3.8	0.3	0.2	1.7	0.5	1.1							
	P2	15.1	1.5	0.6	2.7	0.4	0.9	0.6	0.4	0.7	6.0	2.5							
	P3	8.8	1.3	0.5	1.7	0.5	1.3	0.6	0.5	0.5	3.7	1.8							
	P4	4.5	1.8	0.7	1.7	0.4	1.2	0.4	0.4	0.7	2.2	1.3							
	ETTC-Ephemerellidae	*6.48*	*6.83*	*4.28*	*90.91*	*0.36*	*4.8*	*30.49*	*9.92*	*977*									
EPHEMERIDAE	N4	0.2	0.1	0.2	1.6	0.4	0.2	0.3	0.5	0.4	0.7	0.4							
	N6	0.5	0.1	0.4	1.9	0.5	0.8	0.5	0.5	0.5	1.0	0.6							
	N10	18.7	0.0	0.3	1.8	1.9	0.3	0.1	0.7	0.4	7.5	2.7							
	N11	6.8	0.0	0.3	1.5	1.4	0.2	0.5	1.0	0.4	3.2	1.3							
	N13	0.6	0.1	0.4	0.7	2.2	0.8	0.2	0.6	0.8	1.2	0.7							
	N14	1.7	0.1	0.2	0.8	1.2	1.5	0.2	0.5	0.7	1.3	0.8							
	NAL056a	0.8	0.0	0.3	2.8	0.4	0.5	0.1	1.2	0.6	1.3	0.7							
	NAL056b	1.1	0.0	0.5	0.9	0.5	0.6	0.2	1.3	0.4	0.8	0.6							
	P1	0.2	1.0	0.3	2.0	0.2	1.6	0.6	0.4	1.0	0.8	0.8							
	P3	55.0	0.9	0.7	34.3	0.4	1.2	0.7	0.6	0.5	29.9	10.5							
	ETTC-Ephemeridae	*6.45*	*5.47*	*6.93*	*16.24*	*0.51*	*5.47*	*34.82*	*6.82*	*321*									
HEPTAGENIIDAE	N4	1.0	1.2	0.3	1.7	0.4	0.5	0.4	0.7	0.6	1.0	0.7							
	N6	0.8	0.7	0.3	1.7	0.5	0.6	0.3	0.6	0.5	1.0	0.7							
	NAL013	0.5	0.6	0.2	1.4	0.7	0.4	0.0	0.7	1.9	0.9	0.7							
	N11	5.4	0.5	0.4	1.0	1.8	0.7	0.0	0.6	0.7	2.8	1.3							
	N13	1.2	0.9	0.8	0.8	4.5	1.1	0.3	0.6	1.0	2.2	1.3							
	N14	1.2	0.4	0.2	0.8	1.2	1.1	0.1	0.4	0.7	1.0	0.7							
	NAL056a	1.5	0.5	0.4	5.2	0.6	1.0	0.1	0.5	1.0	2.5	1.2							
	NAL056b	1.1	0.2	0.2	0.4	0.7	0.7	0.2	0.7	0.9	0.7	0.6							
	P1	0.8	2.8	1.0	4.2	0.5	3.8	1.7	0.6	2.6	1.8	2.0							

Table S4. Continued.



	P2	9.3	4.2	1.2	4.1	0.7	1.1	1.4	0.5	2.0	4.7	2.7							
	P3	9.1	4.5	0.8	4.4	0.4	2.7	1.4	0.7	1.7	4.6	2.9							
	P4	5.1	3.1	2.7	4.6	1.8	1.0	3.7	0.6	1.2	3.8	2.6							
HYDROPSYCHIDAE	*ETTC-Heptageniidae*	*6.30*	*4.31*	*3.93*	*80.13*	*0.27*	*5.06*	*19.95*	*15.53*	*266*									
	N4	0.9	1.3	0.8	1.8	1.3	0.6	2.7	0.6	0.8	1.3	1.2							
	N6	0.4	0.4	0.2	1.1	0.3	0.2	0.5	0.5	0.5	0.6	0.5							
	NAL013	0.3	0.3	0.1	1.1	0.6	0.3	0.0	0.8	0.7	0.7	0.5							
	N10	31.0	0.2	0.2	1.2	2.8	0.5	0.1	0.8	0.5	11.7	4.2							
	N11	14.4	0.3	0.3	1.3	2.2	0.4	0.1	0.9	0.5	6.0	2.3							
	N13	0.9	0.7	0.9	1.0	1.5	1.0	1.0	0.7	0.8	1.1	1.0							
	N14	1.2	0.6	0.1	1.0	1.0	1.0	0.3	0.5	0.6	1.1	0.7							
	N15	393.7	0.2	0.3	1.6	41.9	2.0	0.6	0.8	0.6	145.7	49.1							
	NAL056a	1.4	0.6	0.2	2.6	0.4	0.3	0.0	0.5	0.6	1.5	0.7							
	NAL056b	0.9	0.1	0.1	0.6	0.4	0.1	0.1	0.9	0.4	0.7	0.4							
	P1	0.4	1.6	0.4	1.7	0.2	2.8	0.6	0.3	0.7	0.8	1.0							
	P3	16.3	0.9	0.7	4.5	0.5	1.1	1.1	1.1	0.6	7.1	3.0							
	P4	16.4	0.7	0.6	2.9	0.4	1.4	0.8	1.7	0.6	6.6	2.8							
LUMBRICIDAE OLIGOCHAETES	*ETTC-Hydropsychidae*	*2.00*	*0.34*	*4.91*	*15.64*	*0.39*	*3.14*	*21.9*	*3.86*	*206*									
	N4	0.7	0.9	0.5	2.2	0.2	0.5	0.9	0.5	0.4	1.0	0.8							
	N6	0.8	0.3	0.6	2.3	0.1	1.1	1.2	0.5	0.4	1.1	0.8							
	NAL013	0.9	0.2	0.4	1.3	0.2	0.6	0.1	0.7	0.5	0.8	0.5							
	N10	119.9	0.1	1.0	1.1	0.9	0.1	0.4	0.6	0.4	40.7	13.8							
	N13	0.6	0.2	0.6	0.7	0.6	0.5	0.7	0.4	0.7	0.6	0.6							
	N14	0.9	0.3	0.2	0.7	0.5	0.9	0.1	0.6	1.0	0.7	0.6							
	N15	134.5	0.3	0.5	0.5	51.7	0.2	0.5	0.9	2.0	62.2	21.2							
	P1	0.4	0.4	0.6	1.9	0.1	3.8	0.6	0.3	0.6	0.8	1.0							
	P2	20.6	0.4	0.5	13.2	0.1	0.5	0.7	0.7	0.5	11.3	4.1							
	P3	1.3	0.2	0.2	3.0	0.1	0.1	0.5	0.9	0.3	1.5	0.7							

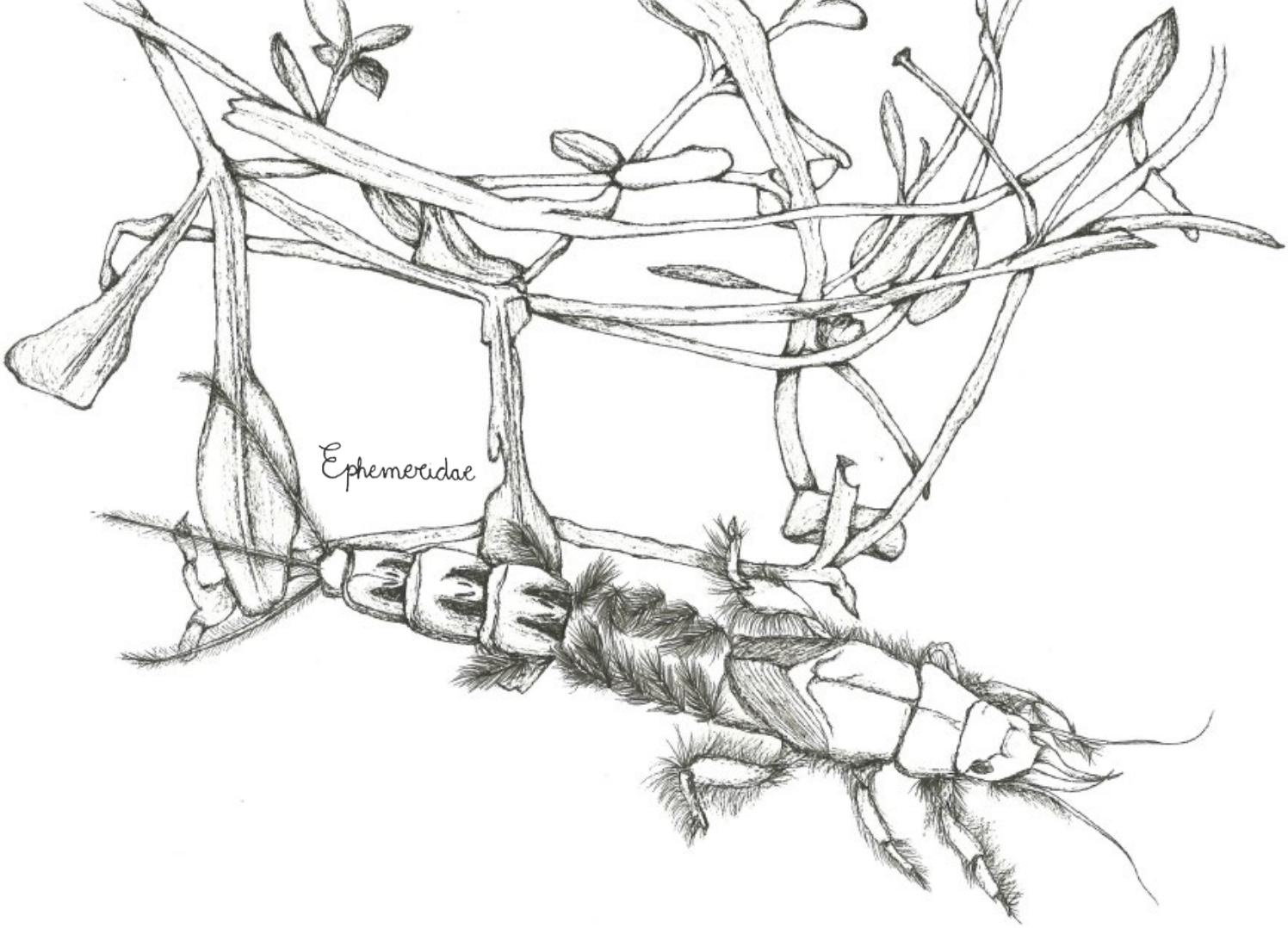
Table S4. Continued.



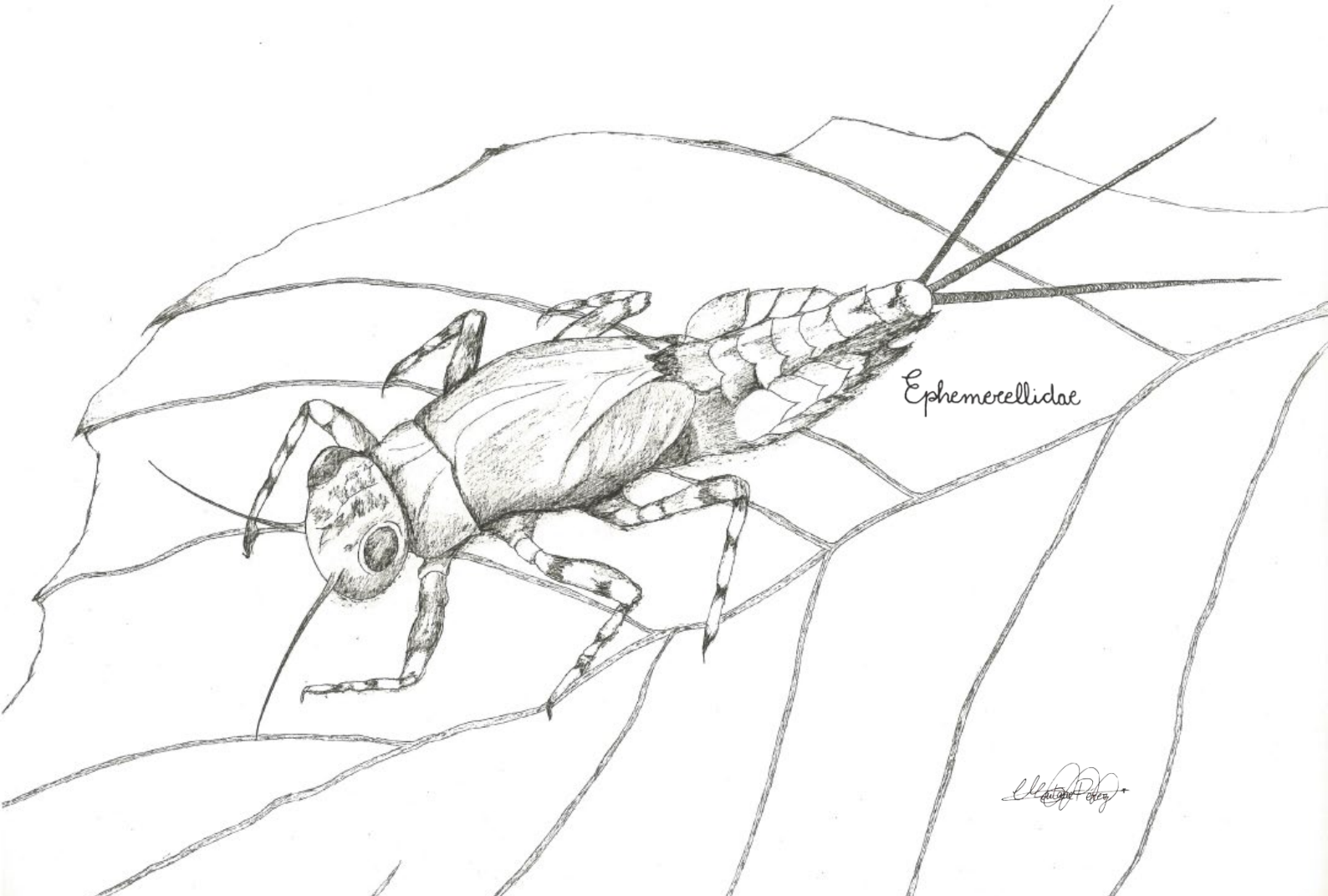
	P4	6.7	0.2	0.8	6.1	0.2	0.7	1.4	1.1	0.4	4.3	1.9
MICRODRILE OLIGOCHAETES	*ETTC- Lumbricidae*	*13.83*	*6.34*	*4.75*	*11.51*	*0.4*	*5.63*	*18.09*	*18.47*	*322*		
	N4	0.8	1.6	0.3	1.2	1.5	0.2	0.6	0.5	1.2	1.2	0.9
	N6	0.5	1.0	0.2	1.0	0.8	0.4	0.6	0.5	0.8	0.8	0.7
	NAL013	0.5	0.2	0.1	0.5	1.0	0.3	0.0	0.6	1.1	0.7	0.5
	N9	34.3	0.4	1.0	1.9	5.0	0.6	0.2	0.9	0.4	13.8	5.0
	N11	14.9	0.6	0.3	0.6	3.1	0.7	0.1	1.8	0.4	6.2	2.5
	N13	0.7	1.0	0.8	0.8	3.9	0.7	0.5	0.6	1.0	1.8	1.1
	N15	27.3	0.6	0.4	0.5	37.8	1.0	0.3	0.4	0.4	21.9	7.6
	NAL056a	2.4	0.2	0.2	1.7	0.9	0.3	0.1	0.8	0.5	1.7	0.8
	NAL056b	3.0	0.2	0.1	0.3	0.7	0.4	0.1	0.5	1.0	1.3	0.7
	P1	0.3	1.1	0.3	1.3	0.5	1.5	0.4	0.2	0.8	0.7	0.7
	P2	41.1	0.1	0.3	11.6	0.4	0.2	0.4	1.4	0.4	17.7	6.2
	P3	11.9	0.4	1.0	6.9	2.2	0.1	2.3	1.9	0.4	7.0	3.0
	P4	7.1	1.4	0.8	9.3	2.2	0.9	0.8	2.3	0.6	6.2	2.8
PERLIDAE	*ETTC-Microdriles*	*14.44*	*7*	*14.81*	*21.27*	*0.48*	*10.2*	*70.89*	*7.52*	*406*		
	N6	0.9	0.7	0.1	0.9	0.4	0.5	0.1	0.5	0.7	0.7	0.5
	N13	1.1	0.5	0.4	0.8	0.9	0.6	0.3	0.3	0.8	0.9	0.6
	N14	1.6	0.1	0.7	0.7	1.7	2.2	0.0	0.6	0.9	1.3	0.9
	NAL056a	1.8	0.3	0.2	2.0	0.5	0.3	0.1	0.7	0.6	1.4	0.7
	P1	0.4	1.5	0.3	2.6	0.3	2.1	0.6	0.6	0.9	1.1	1.0
	P2	93.3	0.9	0.4	5.8	0.4	1.8	0.4	0.8	0.7	33.2	11.6
	P3	22.0	0.2	0.3	2.2	0.3	0.7	0.3	0.4	0.9	8.2	3.0
RHYACOPHILIDAE	*ETTC- Perlidae*	*0.75*	*0.66*	*4.98*	*29.19*	*0.35*	*1.72*	*30.61*	*5.32*	*254*		
	N4	2.0	0.5	0.2	1.0	0.5	0.4	0.4	0.3	0.7	1.2	0.7
	N6	0.8	0.5	0.1	1.0	0.2	0.3	0.3	0.5	0.7	0.7	0.5
	NAL013	0.3	0.1	0.1	0.9	0.6	0.3	0.0	0.5	1.0	0.6	0.4

Table S4. Continued.

	N10	18.3	0.1	0.1	0.7	1.4	0.3	0.0	0.4	0.5	6.8	2.4							
	N11	9.8	0.1	0.2	0.9	1.2	0.1	0.1	0.6	0.5	4.0	1.5							
	N13	0.9	0.1	0.3	0.8	0.6	0.2	0.2	0.5	0.9	0.7	0.5							
	N14	1.9	0.5	0.1	0.6	0.5	0.8	0.1	0.2	0.5	1.0	0.6							
	NAL056a	1.1	0.2	0.1	2.0	0.3	0.5	0.0	0.4	0.8	1.1	0.6							
	NAL056b	1.2	0.0	0.2	0.8	0.6	0.3	0.1	0.8	0.8	0.9	0.5							
	P1	0.3	0.6	0.3	1.1	0.2	1.7	0.6	0.3	0.8	0.5	0.7							
	P2	13.5	2.0	1.3	3.4	1.2	0.6	1.3	0.5	1.6	6.0	2.8							
	P3	8.0	1.5	0.5	2.9	0.4	1.7	0.8	0.9	1.4	3.8	2.0							
	P4	5.7	0.4	1.3	2.2	0.7	1.1	1.5	1.1	0.7	2.8	1.6							
	ETTC- Rhyacophilidae	*0.82**	*1.48*	*7.08*	*19.75*	*0.50*	*1.20**	*40.64*	*6.85**	*304*									
SIMULIDAE	N4	0.6	0.9	0.6	0.6	1.4	0.0	0.3	0.5	1.3	0.9	0.7							
	N6	0.6	0.4	0.2	0.6	0.2	0.5	0.4	0.9	0.9	0.5	0.5							
	NAL013	0.4	0.2	0.1	0.4	0.4	0.5	0.0	0.4	0.9	0.4	0.4							
	N9	147.1	0.3	0.5	0.5	31.4	0.8	0.4	2.6	0.8	59.6	20.5							
	N10	41.0	0.3	0.2	0.3	2.1	0.9	0.1	0.6	0.8	14.4	5.1							
	N11	14.4	0.4	0.2	0.4	2.6	0.5	0.1	0.7	0.7	5.8	2.2							
	N14	2.0	0.5	0.2	0.3	1.0	1.1	0.3	0.6	0.8	1.1	0.8							
	N15	123.6	0.3	0.1	0.4	14.7	0.6	0.3	0.9	0.7	46.2	15.7							
	NAL056a	1.2	0.2	0.2	2.0	0.3	0.4	0.1	0.6	0.9	1.2	0.6							
	NAL056b	1.4	0.2	0.2	0.3	0.6	0.7	0.1	1.6	1.0	0.8	0.7							
	P1	0.6	1.7	0.3	2.0	0.3	3.6	0.3	0.5	1.0	0.8	1.1							
	ETTC- Simuliidae	*4.49*	*0.32**	*13.59*	*58.23*	*0.6*	*9.17*	*66.88*	*4.66**	*158**									



Chapter 2



Developing As and Cu tissue residue thresholds to attain the good ecological status of rivers in mining areas

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Abstract

The study was performed on residue-effects datasets from polluted and unpolluted sites in the Nalón River basin (northern Spain). The effects were measured in terms of alteration of field macroinvertebrate communities, and measured as ecological status scores, and number of families and abundance of Ephemeroptera, Plecoptera and Trichoptera (EPT). Non-linear regression models of the field-measured tissue residues in 10 taxa related to the ecological status of the macroinvertebrate communities were used to derive effective tissue residues (ERs). These were estimated for the good/moderate boundary defined by the ecological quality ratio (EQRs) score and for the 50% reduction of EQR and EPT metrics. As, Cu, Hg and Se ERs were calculated for several macroinvertebrate taxa with different feeding styles. The ER dataset allowed us to estimate As and Cu hazardous concentrations (HC), using species sensitivity distribution models, and were interpreted as community thresholds. Further studies for Hg and Se are needed to complete the database required for HC estimation. The reliability and differences of the several thresholds were tested in a risk assessment using a tissue-residue approach (TRA) conducted with field organisms from Cauxa Creek, a tributary from the same basin exposed to high levels of metals in the sediments due to gold mining activities. This risk assessment identified that As and Cu tissue residues satisfactorily explained the reduction in the ecological status of the macroinvertebrate assemblages. Our results indicate that TRA can help in setting future environmental quality standards for the protection of aquatic biota.

Keywords: metals, metalloids, macroinvertebrates, effective tissue residues, dose-response models.

1. INTRODUCTION

Areas having a high level of metals and metalloids (hereinafter, metals) due to their lithology have often, historically been exploited for metal extraction. Mining activities usually result in the disposal of soil tailings in the areas adjacent to the mine, which can leach metals to rivers and cause alterations to aquatic communities in the absence of adequate management (Loredo *et al*., 2010). Monitoring the levels of contaminants in water is a common strategy in European countries, but this may be not enough to achieve the desired level of protection of field communities (EC, 2008). For this reason, European policy also considers an important issue to set environmental quality standards in sediment and biota for some priority metals (Cd, Ni, Hg and Pb and their compounds) (EC, 2008, Annex II). The objective of this environmental policy includes monitoring the bioaccumulation potential and bioassessment of impacts and trends for certain chemicals, thus ensuring protection against secondary poisoning at the community level. The measurement of metal tissue residues reflects bioavailability, thus reducing uncertainty about the actual bioavailable fraction of chemicals based on external concentrations (Sappington *et al*., 2011). There is also a general consensus on the convenience of including bioaccumulation data measured in indigenous organisms for sediment risk assessment (Adams *et al*., 2011; Chapman, 2007) and for the integration of toxicological and ecological information toward regulatory applications using a tissue residue approach (McCarty *et al*., 2011). This integrative approach is especially suitable for assessing water quality in mining districts, where aquatic macroinvertebrates can accumulate high levels of metals (Cain *et al*., 2004; Méndez-Fernández *et al*., 2015; Solà *et al*., 2004) and can become a significant source of dietary uptake for their predators (Clements, 1991; EC, 2011). Furthermore, bioaccumulation can also be the cause of alterations in macroinvertebrate assemblages (Bervoets *et al*., 2016; De Jonge *et al*., 2013; Luoma *et al*., 2010).

The measurement of field alterations in the macroinvertebrate assemblage composition and structure is an essential component of the integrated assessment of ecological status, as established by the Water Framework Directive (EC, 2000). In that context, the relationships of metal tissue residues in selected biomonitors to adverse

effects on aquatic communities can be critical for developing reliable environmental quality criteria for the protection of biota. In a previous publication, the baseline metal concentration in 10 taxa was calculated from unaltered, field reference sites in the Nalón River basin (northern Spain) (Rodriguez *et al*., 2018), providing an estimate of the tissue concentration threshold for nine metals, below which alterations in the macroinvertebrate assemblages of the study region are unlikely (ETTC, ecological threshold tissue concentration). In the present study, we aimed to develop high-threshold tissue concentrations above which the impairment of macroinvertebrate assemblages is likely to occur. The impairment was measured through ecologically relevant metrics to protect field populations in the study region.

The tissue residue thresholds for benthic macroinvertebrates are valuable tools in water quality assessments in areas affected by mining activities since they can improve water and sediment quality standards; they can be used as screening benchmarks and provide necessary information in a weight-of-evidence approach using chemistry, toxicity and bioaccumulation data (Meador *et al*., 2014). We used two approaches to derive tissue residue thresholds: First, we derived the effective tissue residues (ERs) using nonlinear regression models relating taxa tissue residues against two general benchmarks: (1) the good/moderate boundary for macroinvertebrate ecological status, and (2) the 50% reduction of scores and metrics used to assess biological integrity of the macroinvertebrate assemblages. Second, using taxa-specific ERs estimated for As and Cu, we calculated the metal hazard concentrations (HCs) from a multitaxa risk assessment approach. Finally, the reliability of the proposed metal thresholds was tested in the Cauxa Creek risk assessment using a tissue-residue approach with field organisms.

2. MATERIAL AND METHODS

2.1. Study area and sampling

The Nalón River basin is located in northern Spain, and its catchment has experienced intense historical and present mining activity (Ordóñez *et al*., 2013). Samples of sediment and benthic macroinvertebrate taxa were collected and analyzed

in two sampling campaigns, 14 reference sites in July 2014 and September 2015 and 15 test sites in September 2015, in the Nalón River basin. Sites were located at four macroinvertebrate community-based river types in Spain (R-T21, R-T25, R-T28, R-T31: MAGRAMA, 2015). Detailed data on geographic information, water physicochemistry, and metal concentration in sediments and macroinvertebrate sampling strategy and composition from the study sites were published by Costas *et al*. (2018) (Supplementary Table S1). Cauxa Creek is a small branch of the Narcea River, the main tributary of the Nalón River, and it is subject to active gold mining (Figure S1). Cauxa Creek was resampled in July 2016 for a followup risk assessment. Four sites were scrutinized, one upstream (P1) and three downstream from the gold mining effluents (from P2, closer to the mining effluents, to P4).

At each site, a composite sample of the upper sediment layer was obtained from fine deposits in the riverbed to measure the sediment metal concentration. Sampling of macroinvertebrates to evaluate bioaccumulation followed a river transect or multihabitat schema to collect 10 biomonitor taxa grouped into general functional feeding groups: scrapers (Baetidae and Heptageniidae), filterers (Ephemeridae, Hydropsychidae and Simuliidae), generalists (Ephemerellidae), predators (Perlidae and Rhyacophilidae) and deposit feeders (Lumbricidae and Microdriles oligochaetes) at each site (Table S1). Three field replicates consisting of 1–20 individuals of the larger size class were taken for tissue residue analysis. Detailed information on macroinvertebrate sampling procedures can be found in Rodriguez *et al*. (2018) and Table S1.

2.2. Macroinvertebrate metrics and scores

The field community metrics were EPT richness (number of families of Ephemeroptera, Plecoptera and Trichoptera, EPT Fam), EPT abundance (number of individuals of EPT, EPT Ab), and the alteration in the ecological status of the macroinvertebrate assemblages, assessed through ecological quality ratios (EQRs), which are calculated as a quotient between the observed/reference value of a biological metric or score in a previously defined water body type (EC, 2000). The EQRs were calculated for the river-type specific multimetric index (METI: MAGRAMA, 2015) and for the scores derived from the NORThern Spain Indicators predictive model

(NORTI: Pardo *et al*., 2014), both used in the study region and called METI-EQR and NORTI-EQR, respectively. Data on the tissue residues and site ecological status from the sampling campaigns of 2014 and 2015 in the Nalón River basin were incorporated into the regression models, while data from 2016 in Cauxa Creek were analyzed separately for a tissue-residue risk assessment.

2.3. Metal analysis in sediments and macroinvertebrate tissue residues

A total of nine metals (As, Cd, Cr, Cu, Hg, Ni, Pb, Se and Zn) were measured in sediments and biota. Limits of quantitation (LOQ) for biota and sediment are shown in Table S2. Values below the LOQ were replaced with ½ of LOQ for statistical analysis (US EPA, 2000). In present study, we have assessed only four metals (As, Cu, Hg and Se) in biota that were relevant for the study area. All analytical methods can be examined in Costas *et al*. (2018) and Rodriguez *et al.* (2018) and are summarized in Table S1. All data in this study are reported in μg g^{-1} dw, and for data in the literature given on a ww basis, we used a conversion factor of 0.2 for all insect taxa (Meador, 2011) and 0.1 for oligochaetes (Méndez-Fernández *et al*., 2013). For each taxon and site, tissue residues are given as the mean of 3 field replicates (on a few occasions, a single pooled sample was measured because of the scarcity of specimens).

2.4. Data interpretation and statistical analyses

Sediment metal concentration was assessed using the probable effect concentration (PEC: MacDonald *et al*., 2000) and the sediment pollution score (SedPoll: Costas *et al*., 2018) calculated from As, Cd, Cu, Hg, Pb and Se sediment concentrations in the Nalón River basin. In our previous contributions we described the baseline concentrations (Ecological Tissue threshold concentration, ETTC of 9 metals in several biomonitors of the Nalón River basin, Rodriguez *et al*., 2018), and more recently a bioaccumulation risk assessment was done, using the number of times the baseline concentrations in selected biomonitor taxa were exceeded (Chapter I: Rodriguez *et al*., 2021). In the present contribution, ERs were estimated on the same dataset for each taxon and metal from nonlinear regression models of the tissue residues (abscissa) vs EQRs (ordinate). Using the official cutoff for good/moderate ecological status (EQR = 0.700, EQR-ER_{GM}: effective tissue residue above which the community status changes

from good to moderate), a boundary intercalibrated for METI-EQR by the Central/Baltic group for benthic macroinvertebrate fauna (EC, 2013a; MAGRAMA, 2015). The effect on ecological status was also measured as a 50% reduction in the maximum EQR values (EQR-ER_{50}: effective tissue residue above which a 50% reduction in the maximum EQR value occurs) in the model and a 50% reduction in the EPT richness and abundance metrics (EPT-ER_{50}: effective tissue residue above which a 50% reduction in EPT richness or abundance occurs) (Figure 1). In the regression models, ER_{GM} was calculated as the inverse function of the regression equations and solved numerically using Wolfram Mathematica 12 software from EQR = 0.700. The EQR-ER_{50} was estimated from the same selected models.

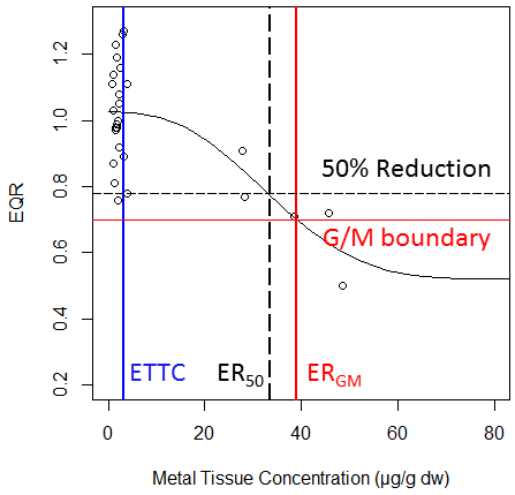

Figure 1. Scheme of the construction of the dose-response models between tissue concentration of the metal and the EQR (circles). The blue line is the ETTC value; the red lines are the EQR= 0.700 change point between Good and Moderate ecological status (G/M boundary) and the corresponding effective tissue concentration (ER_{GM}). Superimposed are the black lines that represent the effective tissue concentration (ER_{50}) related to the 50% reduction of the EPT indexes.

Nonlinear regression analyses were conducted using R software and the extension package drc (Ritz and Streibig, 2005). After a preliminary analysis, the best fitted models were selected from a set of 6 commonly used sigmoid models (log-logistic and Weibull) with 3 and 4 parameters:

Log-logistic (LL4): $y = c + (d - c)/(1 + \exp(b(Log\ x - Log\ e)))$

Weibull 1 (W1.4): $y = c + (d - c)\exp\left(-\exp\left(b(Log\ x - Log\ e)\right)\right)$

Weibull 2 (W2.4): $y = c + (d - c)\left(1 - \exp\left(-\exp\left(b(Log\ x - Log\ e)\right)\right)\right)$

The parameters *c* and *d* are the lower and upper asymptotes for the *y* variable, respectively, and they are in the same units as the *y* variable; parameter *e* is the inflection point of the dose-response curve and provides the ER_{50} value in the log-logistic models; and parameter *b* is proportional to the slope of the dose-response curve at dose *e* (Ritz, 2010). Three-parameter models were obtained from each model when *c* = 0.

The models with a difference in Akaike's Information Criteria (AIC) <2 were selected (Burnham & Anderson, 2002) and were validated when: (1) the *c* parameter was ≥0, the minimum value of the EQR and macroinvertebrate assemblage metrics; (2) the estimated ER was within the field range of tissue residue values; and (3) the standard error of the estimated ER was lower than the ER value. In the case of regression models built with the EQRs, we also considered for the validity of the model that the *d* parameter was ≤1.4, the maximum of the EQR value (Pardo *et al*., 2010). Graphical tests of the standardized and studentized residuals for the selected equations were examined, and studentized residuals were always <|3|.

For each taxon and metal, several EQR-ER_{GM}, EQR-ER_{50} and EPT-ER_{50} values were estimated from the validated models, and then averaged. The effective tissue residues (ER) estimated on the 50% reduction in the scores have the advantage over the ER_{GM} that we have been able to calculate the 95% confidence limits of the estimates. EQR-ER_{50} and ER_{GM} should be similar since the good/moderate boundary of 0.700 is half the maximum expected EQR. Using species sensitivity distribution (SSD) models (ETX v.2.1 program, Van Vlaardingen *et al*., 2004), the 5^{th} and 50^{th} percentile hazard concentrations (HC_5, HC_{50}) for the macroinvertebrate assemblages were calculated using the taxa ER_{GM}, EQR-ER_{50} and EPT-ER_{50} mean values.

Finally, the aforementioned threshold values were used in a risk assessment of Cauxa Creek, using the average ratios of the field tissue residues (TRs) to the EQR-ER_{GM} for all the biomonitor taxa present at each site. Four quality classes were considered for the risk assessment based on a tissue-residue approach: (1) Low risk for the community when TR/ER ≤1, (2) Moderate risk when TR/ER=1.1–2.0, (3) High risk when

TR/ER=2.1–10.0, and (4) Very High risk when TR/ER >10. The same classification was used to assess the ratios of TR to the HC_{50} values. No risk was expected only when the mean tissue residues were $<HC_5$ or the ETTC (ecological threshold tissue concentration).

3. RESULTS

3.1. Dose-response models and effective tissue residues for As, Cu, Hg and Se in macroinvertebrates

Dose-response models for the relationship of field taxa tissue residues to the macroinvertebrate assemblage metrics (METI- and NORTI-EQRs and EPT richness and abundance) were built when possible for 10 taxa. Tissue residues uploaded to the models from 15 potentially polluted sites from the Nalón River basin are shown in Table S3; data from 14 reference sites were reported by Rodriguez *et al*. (2018). In the study area, METI-EQR values ranged from 0.50–1.27, and NORTI-EQRs ranged from 0.26–1.27. The maximum EPT richness varied from 6 to 25 families, and the EPT abundance varied from 96 to 7333 individuals per site (2.5 m^2). A total of 254 (out of 960 calculated) dose-response models were validated following the criteria reported in Material and Methods section; 87 models for As, 75 for Cu, 52 for Se, and 40 for Hg. ER_{GM} was calculated from 128 models (64 models using METI-EQR data and 64 models using NORTI-EQRs), and the EQR-ER_{50} was calculated from 86 models (Table S4); EPT Fam-ER_{50} was estimated from 58 regression models, and EPT Ab-ER_{50} was estimated from 68 models (Table S5). Overall, ERs were calculated for all the study taxa from several selected models: Microdrile oligochaetes (42 models), Rhyacophilidae (33), Baetidae and Heptageniidae (28 each), Lumbricidae (26), Hydropsychidae (23), Ephemerellidae and Ephemeridae (22 each), Simuliidae (19) and Perlidae (11).

The mean ERs calculated for each metal and taxon relative to the good/moderate ecological status boundary (ER_{GM}) are shown in Table 1. The ER_{GM} concentrations estimated from NORTI-EQRs were generally 1–3 times lower than the ER_{GM} concentrations derived from the METI-EQRs. The highest As ER_{GM} values were for Simuliidae or Microdrile oligochaetes, while the lowest values were found in the

predators Rhyacophilidae and Perlidae. The Cu ER_{GM} was higher for Heptageniidae and Ephemerellidae, while the deposit feeders and filterers had lower values. Only a small number of models were validated for Hg and Se.

Regarding the ratios of the ER_{GM} to the baseline ETTC for each metal and taxon (Table 1), the METI-ER_{GM} values were usually 4–20 times the ETTC for As (but up to 60 for Simuliidae); these ratios ranged 2–7 times for Cu, 1–14 for Hg, and approximately 2 for Se. The ratios of NORTI-ER_{GM} to ETTC were generally lower, 2–13 for As, and varied typically from 1–2 for Cu, Hg and Se. In most instances, the EQR-ER_{50} values ranged between 0.4 and 7.1 times (mean = 1.7) the corresponding ER_{GM}.

We found EPT Fam-ER_{50} for As (Table 2) to be lower for EPT abundance than for EPT richness. This is interpreted as As tissue residues causing a reduction in abundance of sensitive taxa before having an effect on the number of families. For Cu, the ratios of Cu EPT-ER_{50} to ETTC were 1–3 (except for Rhyacophilidae, with ratios <1), with similar values for richness and abundance. In the case of Hg and Se, the EPT-ER_{50} estimates were limited to a few taxa and, in most instances, <1 $\mu g\ g^{-1}$ for Hg, which resulted in ratios to ETTC ≤1. The EPT Ab-ER_{50} was 3 and 2 times higher than the baseline ETTC for As and Cu, respectively (Table 2; Figures 2 and 3, for Baetidae, Ephemerellidae, Lumbricidae and Rhyacophilidae). However, EPT Fam-ER_{50} values were much higher than ETTC for As (mean ratio 16.9) but only 2 times higher for Cu and equal or lower for Hg and Se (Table 2). The ratios for Se were very variable, although based on a limited number of data.

Last, using the SSD models based on the taxa ERs, we estimated hazard concentrations for the integrity of the whole community. The HC_5 and HC_{50} were estimated for As and Cu (Table 3) but not for Hg and Se due to the limited number of data points. The HC_5 derived from the EQR-ER_{GM} values for both As and Cu were similar to those estimated using EQR-ER_{50} or EPT-ER_{50} data (Table 3). The HC_{50} values estimated from the same SSD models were 11–25 times higher than the corresponding HC_5 for As and 4–5 times higher for Cu. The respective 90% confidence limits of HC_5 and HC_{50} did not overlap, which supports their use as low and high community thresholds, respectively.

Table 1. Mean effective tissue residues (ERs) (µg g^{-1} dw) calculated for As, Cu, Hg and Se from non-linear regression models of the tissue concentration and the EQR for each site and taxon. Their ranges are given when n >1. EQR-ER_{50} was calculated as the tissue residues corresponding to a 50% reduction in the EQR score. EQR-ER_{GM} was estimated from the models for the official EQR value used as the boundary between good and moderate ecological status of the macroinvertebrate assemblages. Ratios of the ER_{GM} to the baseline ETTC are shown. ETTC, ecological threshold tissue concentration; TR, tissue residues.

	Mean METI-EQR vs TR regression models			Mean NORTI-EQR vs TR regression models		
Taxon	**EQR-ER_{50} (range)**	**EQR- ER_{GM} (range)**	**Mean ER_{GM}/ETTC**	**EQR-ER_{50} (range)**	**EQR- ER_{GM} (range)**	**Mean ER_{GM}/ETTC**
As						
Baetidae	71.0 (32.1-186)	41.6 (37.3-52.2)	13.4	99.5 (51.5-123)	18.9 (16.3-21.7)	6.1
Ephemerellidae	-	67.7 (66.7-68.7)	10.5	-	35.5	5.5
Ephemeridae	-	-	-	-	44.3	6.9
Heptageniidae	-	48.6 (47.6-49.4)	7.7	7.8 (7.6-8.0)	9.0 (8.0-10.8)	2.3
Hydropsychidae	16.1 (2.6-29.5)	-	-	-	25.5 (23.1-27.8)	12.7
Lumbricidae	17.2	19.1	1.4	84.9 (83.5-86.2)	85.7 (84.5-86.9)	6.2
Microdrile	561	243.1 (230-256)	16.8	542.4 (509-570)	109.4 (79.0-126)	7.6
Rhyacophilidae	11.1 (10.7-11.7)	7.1 (6.9-7.4)	4.2	1.1	1.1	1.5
Perlidae	-	-	-	-	2.6	1.5
Simuliidae	120	269 (196-341)	59.8	-	17.8	4.0
Cu						
Baetidae	39.2	104.5	3.6	33.6 (32.8-34.6)	38.1 (36.6-39.6)	1.3
Ephemerellidae	237 (236-240)	194 (191-199)	6.5	-	134.4	1.5
Ephemeridae	37.0 (36.5-37.8)	-	-	19.1 (16.2-21.0)	22.9 (16.5-27.3)	1.4
Heptageniidae	220 (199-241)	409.7 (407-414)	5.1	98.1 (97-100)	158 (156-160)	2.0
Hydropsychidae	-	-	-	15.6 (15.3-15.9)	18.5 (17.9-19.1)	1.2
Lumbricidae	146 (145-147)	82.4 (77.7-86.8)	7.1	208	45.7	4.0
Microdrile	36.0 (35.7-36.4)	37.7 (37.3-38.1)	1.8	31.6 (30.1-33.0)	30.9 (29.6-32.2)	1.5
Perlidae	118 (105-131)	98.5 (97.7-99.3)	2.9	-	-	-
Rhyacophilidae	67.1	51.2 (49.5-52.5)	2.6	32.2 (31.9-32.5)	39.4 (32.9-44.7)	2.0
Simuliidae	-	-	-	15.8 (15.5-16.1)	37.2 (36.4-37.9)	0.7
Hg						
Baetidae	-	3.6	13.7	-	-	-
Ephemeridae	0.05	-	-	0.10	-	-
Heptageniidae	-	-	-	0.06 (0.06-0.07)	-	-
Perlidae	-	-	-	0.07 (0.06-0.07)	-	-
Rhyacophilidae	0.60	0.59	1.2	0.23 (0.21-0.24)	0.35 (0.34-0.36)	0.7
Simuliidae	-	7.9 (7.8-8.0)	13.1	-	0.97 (0.96-0.97)	1.6
Se						
Baetidae	-	20.1 (18.7-22.1)	1.8	15.2 (14.8-15.6)	8.7 (8.6-8.7)	0.7
Heptageniidae	-	-	-	3.4 (3.2-3.5)	-	-
Hydropsychidae	3.0	-	-	1.1 (0.9-1.1)	-	-
Microdrile	7.1 (6.6-7.6)	16.8	2.2	5.8	5.9	0.8

Table 2. Effective tissue residues (ER_{50}) of As, Cu, Hg and Se (µg g^{-1} dw) related to the 50% reduction in the EPT number of families (EPT Fam) and the EPT abundance (EPT Ab). ER_{50} are calculated as the mean ER_{50} values estimated per taxon from the validated regression models. Abbreviations: EPT, Ephemeroptera, Plecoptera and Trichoptera; ETTC, Ecological Threshold Tissue Concentration.

	As		Cu		Hg		Se	
Taxon	**EPT Fam-ER_{50}**	**EPT Ab-ER_{50}**	**EPT Fam-ER_{50}**	**EPT Ab-ER_{50}**	**EPT Fam-ER_{50}**	**EPT Ab-ER_{50}**	**EPT Fam-ER_{50}**	**EPT Ab-ER_{50}**
Baetidae	24.1	11.4	-	-	-	-	19.5	5.7
Ephemerellidae	92.1	8.4	237.2	192.0	0.06	-	-	-
Ephemeridae	20.5	-	38.0	-	0.14	0.61	-	-
Heptageniidae	8.1	18.1	-	-	-	-	-	4.8
Hydropsychidae	107.7	-	16.7	-	-	-	2.1	4.3
Lumbricidae	16.7	-	-	32.8	-	-	10.0	14.9
Microdrile oligochaetes	318.7	85.5	-	55.1	-	0.72	4.4	8.0
Perlidae	-	-	167.8	77.6	-	0.06	-	-
Rhyacophilidae	8.1	1.5	-	42.4	-	-	-	1.1
Simuliidae	171.5	8.4	-	-	-	0.72	-	-
ER_{50}/ETTC ratio	1.2–53.9	1.3–5.9	1.1–2.7	0.3–2.8	0.2–0.3	0.0–1.2	0.5–1.6	0.2–1.1
Mean	16.9	2.9	2.2	2.1	0.25	1.0	0.8	0.7

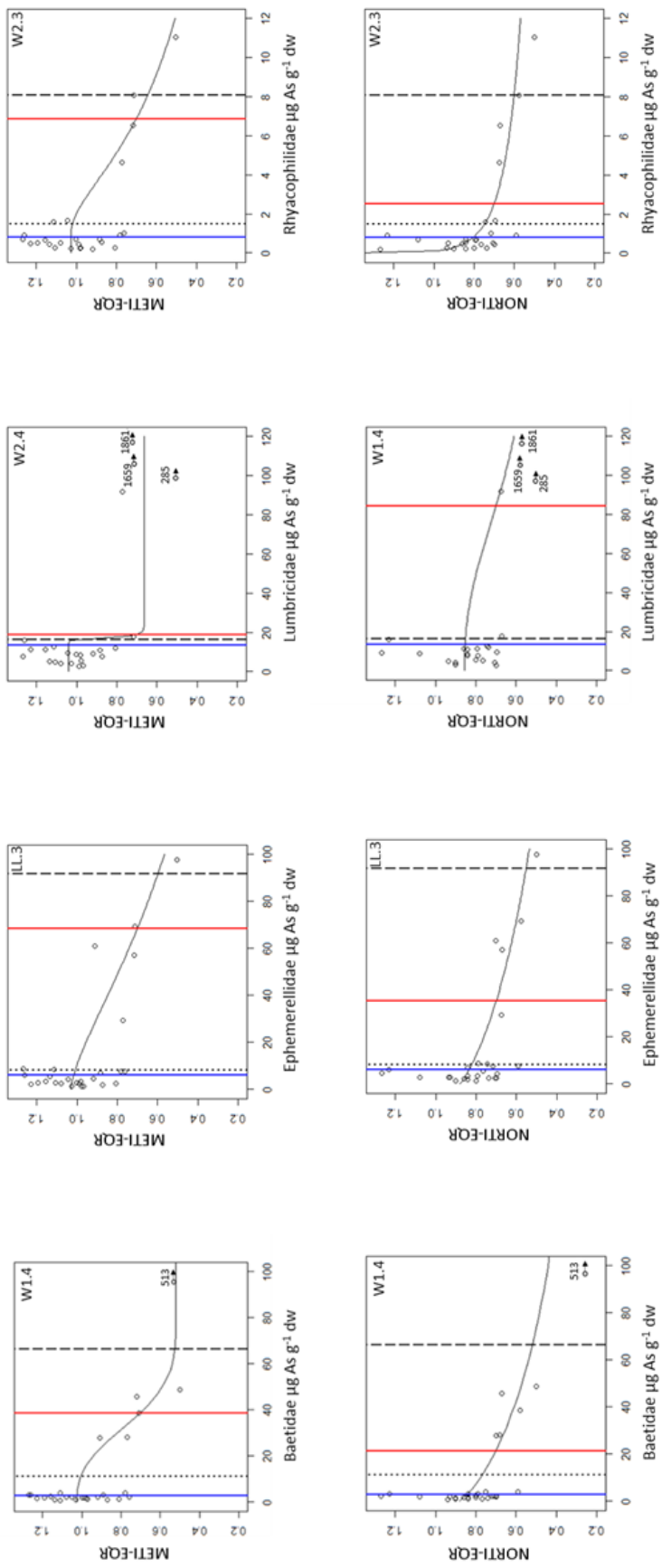

Figure 2. Arsenic dose-response models for METI- and NORTI-EQRs calculated for several taxa (Baetidae, Ephemerellidae, Lumbricidae and Rhyacophilidae) selected from sites in the Nalón River basin. Superimposed lines mark the low and high threshold values. The low threshold is in all instances the baseline concentration in the basin (ETTC), in blue. There are several parameters useful as high thresholds: METI- and NORTI-ER_{GM} in red. For comparison, the ER_{50} values calculated when possible for the 50% reduction of EPT number of families and abundances are also superimposed in black-dashed line and in black-dotted line, respectively. The represented regression models are indicated on the upper right corner of each plot.

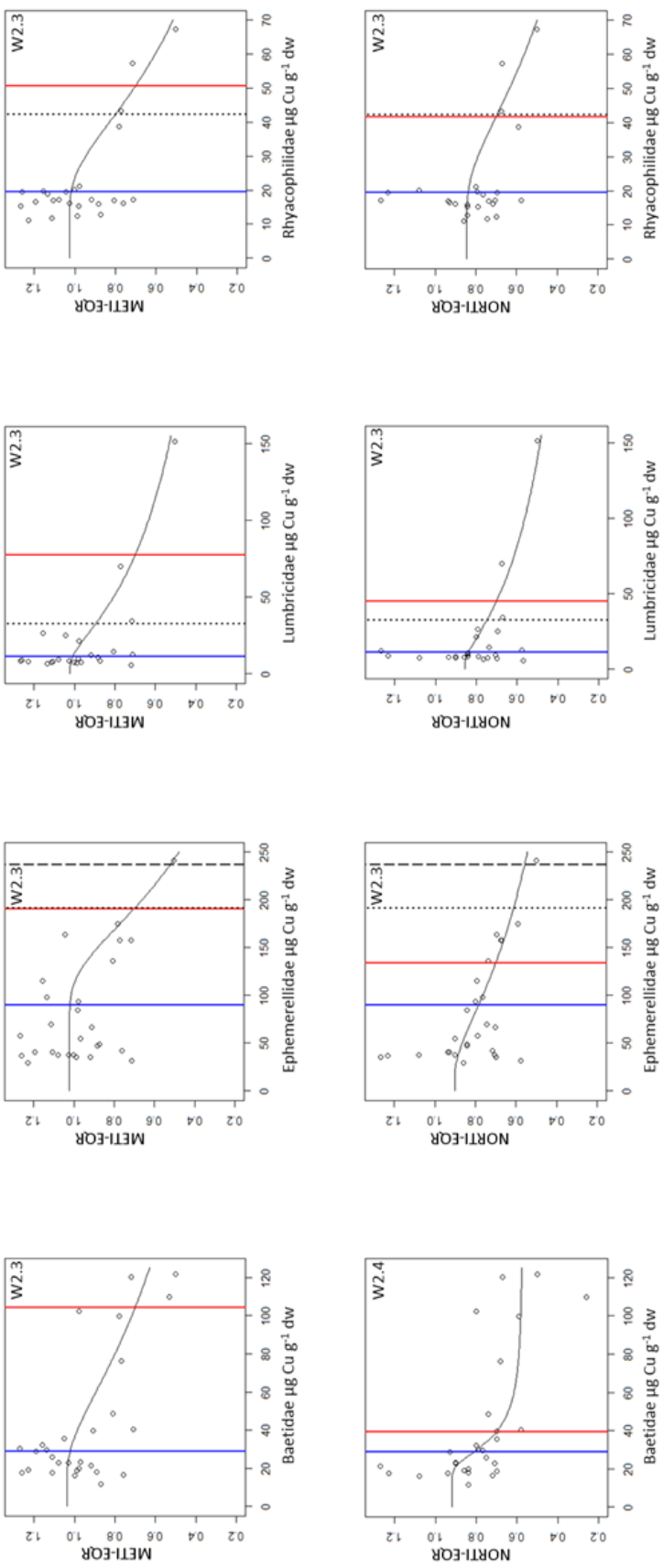

Figure 3. Copper dose-response models for METI- and NORTI-EQRs calculated for several taxa (Baetidae, Ephemerellidae, Lumbricidae and Rhyacophilidae) selected from sites in the Nalón River basin. Superimposed lines mark the low and high threshold values. The low threshold is in all instances the baseline concentration in the basin (ETTC), in blue. There are several parameters useful as high thresholds: METI- and NORTI-ER_{GM} in red. For comparison, the ER_{50} values calculated when possible for the 50% reduction of EPT number of families and abundances are also superimposec in black-dashed line and in black-dotted line, respectively. The represented regression models are indicated on the upper right corner of each plot.

Table 3. Hazard concentrations (HC_5, HC_{50}) for As and Cu and their 90% confidence limits (CL) derived from SSD models using several effective tissue concentrations (ER). EQR-ER_{GM}: Effective tissue residues related to the EQR boundary between Good and Moderate ecological status of the macroinvertebrate community; EQR-ER_{50}: Effective tissue residues related to the 50% reduction in the EQR; EPT-ER_{50}: Effective tissue residues related to the 50% reduction of the EPT metrics of abundance and richness. For comparison, the HC_{50} derived from the baseline concentrations of As and Cu in unpolluted reference sites is given (ETTC-HC_{50}, Rodriguez *et al*., 2018). n: number of data included in each of the models.

Chemical	HC	EQR-ER_{GM} (90% CL)	n	EQR-ER_{50} (90% CL)	n	EPT-ER_{50} (90% CL)	n
As	**HC_5**	2.41 (0.74 – 5.27)	17	1.67 (0.20 – 5.74)	11	2.12 (0.59 – 4.82)	15
	HC_{50}	27.98 (15.06 – 51.97)		41.05 (14.62 – 115.3)		24.13 (12.50 – 46.58)	
	As ETTC-HC_{50}	4.24 (2.46 – 7.30)					
Cu	**HC_5**	15.45 (7.52 – 24.70)	16	11.63 (5.25 – 19.51)	16	14.31 (4.32 – 27.48)	9
	HC_{50}	64.62 (44.47 – 93.89)		56.40 (37.34 – 85.20)		67.43 (38.44 – 118.3)	
	Cu ETTC-HC_{50}	29.16 (19.23 – 44.21)					

3.2. Cauxa Creek risk assessment: a tissue residue approach

The sediment metal concentration in the 2016 campaign in Cauxa Creek showed that 4 metals, As, Cu, Hg and Se, exceeded the PEC values in sites downstream of mine effluents (P2-P4) (Table S6). At P2, As was up to 48 times the PEC value, Cu 13 times and Se 4 times, while the Hg concentration barely exceeded the PEC. Additionally, sediment metal pollution assessed by the SedPoll index evaluated the upstream site, P1, as unpolluted or similar to the reference, while sites P2 to P4 were assessed as medium to highly polluted.

At site P1, ten biomonitors used in the Nalón River basin were found, but downstream (P2-P4), only four of them were present at the four study sites: Baetidae, Ephemerellidae, Lumbricidae and Rhyacophilidae. These taxa represent four different feeding styles: scraper, generalist, deposit feeder and predator. Bioaccumulation levels of As and Cu were high, up to a maximum of 49 times the ETTC (Baetidae) at P2, as expected from the high metal concentration in the sediment. However, the Hg and Se tissue residue to ETTC ratios were usually <1, with a maximum of 1.9 for Hg and 1.6 for Se (Lumbricidae) in P2 (Table S7).

The Cauxa Creek risk assessment based on the tissue residue approach was performed using the average ratios of tissue residues to their corresponding high thresholds (EQR-ER_{GM}) calculated for each taxon present (Table 4). These ratios were also averaged for each feeding style (Table S8). Site P1, upstream from the gold mine, showed in all cases low risk due to As, Cu, and Hg tissue residue ratios to EQR-ER_{GM}. In all cases, downstream sites were assessed as High Risk related to As bioaccumulation (Table 4). The Cu bioaccumulation risk assessment result was variable, depending on the EQR-ER_{GM} used. Se tissue residues downstream of the mine were assessed as Low or Moderate Risk. The Hg showed Low Risk related to the METI and NORTI assessments. The importance in this risk assessment of the high ratios obtained for predators for As and Se in downstream sites can be seen in Table S8.

Table 4. Cauxa River risk assessment based on the mean ratios of the tissue residues in biomonitor taxa to metal thresholds. The effective tissue residues (EQR-ER_{GM}) and the community hazard concentration (HC_{50}) were used as high thresholds. The ratios are classified within the following classes: Low Risk (≤1) in blue, Moderate Risk (1.1–2.0) in yellow, High Risk (2.1–10.0) in orange and Very High Risk (>10) in red. The ecological status of the benthic macroinvertebrate assemblages in the four sites of the Cauxa River assessed by the METI and NORTI EQRs are shown in blue (Good) and yellow (Moderate).

Ratios of tissue residues to effective tissue residues					
Threshold concentration	**Chemical**	**Site**			
		P1	**P2**	**P3**	**P4**
METI-EQR ER_{GM}	**As**	0.1	4.2	2.4	2.9
	Cu	0.5	1.4	1.0	1.3
	Hg	0.1	0.1	0.1	0.2
	Se	0.2	0.5	0.5	0.6
NORTI-EQR ER_{GM}	**As**	0.2	9.3	3.5	2.3
	Cu	0.9	3.5	3.5	1.9
	Hg	0.3	0.3	0.4	0.6
	Se	0.5	1.1	1.1	1.5
Hazard Concentrations					
EQR-ER_{GM} HC_{50}	**As**	0.1	3.3	2.9	2.4
	Cu	0.9	2.7	2.5	1.7
Ecological status					
METI-EQR		0.837	0.570	0.592	0.500
NORTI-EQR		0.779	0.621	0.665	0.582

Finally, the risk assessment for As and Cu tissue residues using their ratios to HC_{50} for the macroinvertebrate assemblage showed a consistent evaluation with the

EQR-ER_{GM} assessment (Table 4). The upstream site (P1) was at Low Risk, while sites downstream of the mine effluents (P2-P4) were assessed from High to Moderate Risk, in parallel with a decreasing concentration of pollutants in the sediment and with decreasing ecological status score values, both for METI and NORTI (<0.700, good/moderate boundary). When HC_5 was used as the benchmark, the four sites showed some degree of risk due to As and Cu bioaccumulation (tissue residue ratios to $HC_5 > 1$).

4. DISCUSSION

The main goal in water quality management is to attain or maintain the ecological integrity of aquatic communities, thus incorporating ecological realism into the regulatory framework (Kiffney & Clements, 2002). In Spain, the evaluation of exposures to contaminants in biota is based on the *standstill* principle, which states that priority substances in the sediments or biota should not significantly increase their concentration on a long-term basis (MAGRAMA, 2015). This evaluation is inadequate to prevent loss or to recover the good ecological status of aquatic communities, since bioaccumulation can be one of the causes of adverse effects, hindering the recovery of the good ecological status of the macroinvertebrate assemblages. In that context, the tissue residue approach provides a step in the evaluation of causal agents derived from contaminants (Meador *et al*., 2014) and is a necessary tool for developing tissue quality criteria that should improve risk assessment and remediation policies.

A comparison of our thresholds with others (calculated using different approaches, but also associated with a reduction in macroinvertebrate community metrics), showed that the similarity among values depends on the biomonitors selected. In other studies, adverse effects on aquatic organisms related to As bioaccumulation were similar to the As HC_{50} (28 µg g^{-1} dw) estimated in the present study: e.g. 1.3–5 µg As g^{-1} ww (≈ 6.5–25 µg g^{-1} dw) (Eisler, 2000), and 6.6 µg As g^{-1} ww (≈ 33 µg g^{-1} dw) (DEQ, 2007). Higher As thresholds were reported by Bervoets *et al*. (2016), but this probably was related to their selection of relatively tolerant biomonitors (Diptera: Chironomidae: 65–130 µg g^{-1} dw and tubificid oligochaetes: 85–

93 µg g^{-1} dw). In our study, the filterer Simuliidae (Diptera) and sediment-feeder Microdrile oligochaetes also showed higher As ERs (Table 1).

The Cu ER_{GM} in the present study attained high values (155.7–414.1 µg g^{-1} dw for Heptageniidae and 134.4–199.3 for Ephemerellidae, Table S4). However, the ER_{GM} HC_{50} (65 µg Cu g^{-1} dw, Table 3) was similar to that proposed by Bervoets *et al*. (2016) (57 µg g^{-1} dw). Higher Cu ERs have been reported for specific biomonitors. For example, the value for Hydropsychidae (> 170 µg g^{-1} dw) was associated with a reduction or absence of heptageniids and ephemerellid mayflies (Rainbow *et al*., 2012), and the values for Heptageniidae (165.2–349.5 µg g^{-1} dw) were associated with a 20–50% loss in macroinvertebrate richness (De Jonge *et al*., 2013).

The range of values estimated for Hg ER_{GM} in the present study is wide (0.35–7.9 µg g^{-1} dw), but the higher values estimated for Simuliidae and Baetidae (Table 1) should be viewed with caution, since they are very biased with respect to the median value (0.97 µg g^{-1} dw). The lower range is comparable to the Hg guidelines proposed for the biota (e.g. 0.12–1.68 µg g^{-1} dw: CCME, 2000; 20 ng g^{-1} ww ≈ 0.1 µg g^{-1} dw: EC, 2013b).

Only a small number of Se ER_{GM} could be estimated in our study, ranging from 5.9–20.1 µg g^{-1} dw, comparable to the tissue residues associated with sublethal toxic effects reported by DeBruyn & Chapman (2007) (1–30 µg g^{-1} dw), and dietary Se thresholds for fish (e.g. 3 to 11 µg g^{-1} dw, May *et al*., 2008).

In the selection of suitable bioaccumulation thresholds, it is desirable that there is a clear but not very large gap between low and high thresholds to reduce the probability of false positives or negatives in the risk assessment. This was the case for most selected taxa, which showed an ER_{GM}/ETTC ratios of >1–10 for As and Cu (Table 1). However, for Hg and Se these ratios were calculated in very few instances. In the case of Se, the low ratios are probably associated with its essential nature for metabolism, and to the fact that most species of aquatic macroinvertebrates are relatively insensitive to Se (Janz *et al*., 2014). The database for Hg and Se should be completed with supplementary sites to better understand these low ratios and provide a better risk assessment in the future.

The estimated metal thresholds (ERs) vary by one or two order of magnitude, depending on the selected biomonitors. However, the metal Hazard Concentrations (HC_5 and HC_{50}) calculated from different biological effective tissue residues are much more comparable to each other. The HC values estimated from SSD models using EQR-ER_{50} and ER_{GM} were very similar, and also were similar to the ER_{50} calculated from the 50% reduction of the abundance and richness of the EPT taxa. This suggests that thresholds estimated from the good/moderate boundary are a reliable measure of ecological status. The HC_5 90% confidence limits of As and Cu calculated from different ERs overlapped with each other and with the confidence limits of the baseline ETTC-HC_{50} (Table 3), thus making the HC_5 a reliable low threshold for risk assessment. Nevertheless, when tissue concentrations are close to HC_5, a comparison with the baseline concentrations of the biomonitor will improve the accuracy of the risk assessment. In addition, the accuracy of the HC thresholds would probably improve if the sensitivity of the biomonitors was within the HC confidence interval of the metal, avoiding false negatives or positives.

The risk assessment exercise in Cauxa Creek largely affected by gold mining clearly pointed toward the influence of As and Cu bioaccumulation by macroinvertebrates on the altered ecological status of sites downstream of the mine (P2-P4). The EQR-ER_{GM} thresholds are taxon specific, thus it is possible to get different assessments depending the biomonitors. In this case study, the average of the ratios of TR/ EQR-ER_{GM} from a selection of several biomonitor species comprise a wide range of sensitivity to the metals, which helps getting a weighted assessment of the risk. The risk assessment through NORTI-ER_{GM} was closer to the ecological status assessment than through METI-ER_{GM}. However, in Cauxa Creek, the ratios of the tissue residues to the community high thresholds (HC_{50}) resulted in a straightforward and consistent risk assessment, comparable to the ecological status assessment. The HC thresholds also have the advantage of being less dependent on the presence of certain biomonitor taxa at the study sites.

Despite the relevance of the interaction of several metals to evaluate the effects on the biota due to bioaccumulation, there are few studies that have addressed the effects of metal mixtures in field organisms (e.g., De Jonge *et al*., 2013). In the

present study, the interactions of Se with other bioaccumulated trace metals (e.g., As, Cu and Hg) must be analyzed in more detail, since interactions have been demonstrated in the literature. In particular, Se is recognized for its potential in reducing the toxicity of Hg compounds (Hamilton 2004), an issue that requires further research in Cauxa Creek. Specific thresholds for the protection of higher levels in the aquatic trophic chain should also be developed in the future for the satisfactory protection of aquatic communities. This problem is complex since the risk of metal transfer from macroinvertebrates to aquatic wildlife depends on the diet specificity, prey availability, accumulation pattern and ability of the organisms to depurate the metals (Rainbow, 2018).

5. CONCLUSIONS

This study is the first to derive the effective tissue concentration from the cutoff value of good/moderate ecological status of the macroinvertebrate assemblages using ten biomonitor taxa. The ecological status of the field community is regularly evaluated by the water authorities, following the European water directive; therefore, EQR can be useful to calculate environmental thresholds for macroinvertebrates derived through a tissue residue approach. The models provide a complementary tool not only to monitor environmental risk due to bioaccumulation, but also to predict alterations in the ecological status of field macroinvertebrate assemblages. The HC_5 and HC_{50} calculated for As and Cu are promising since they can be readily applicable as low and high thresholds in the mining districts of northern Spain. They can contribute to setting future environmental quality standards for the protection of aquatic biota. The same approach can be implemented in other European river basins to calculate threshold concentrations in the biota related to reductions in intercalibrated metrics of ecological status.

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SUPPLEMENTARY MATERIAL

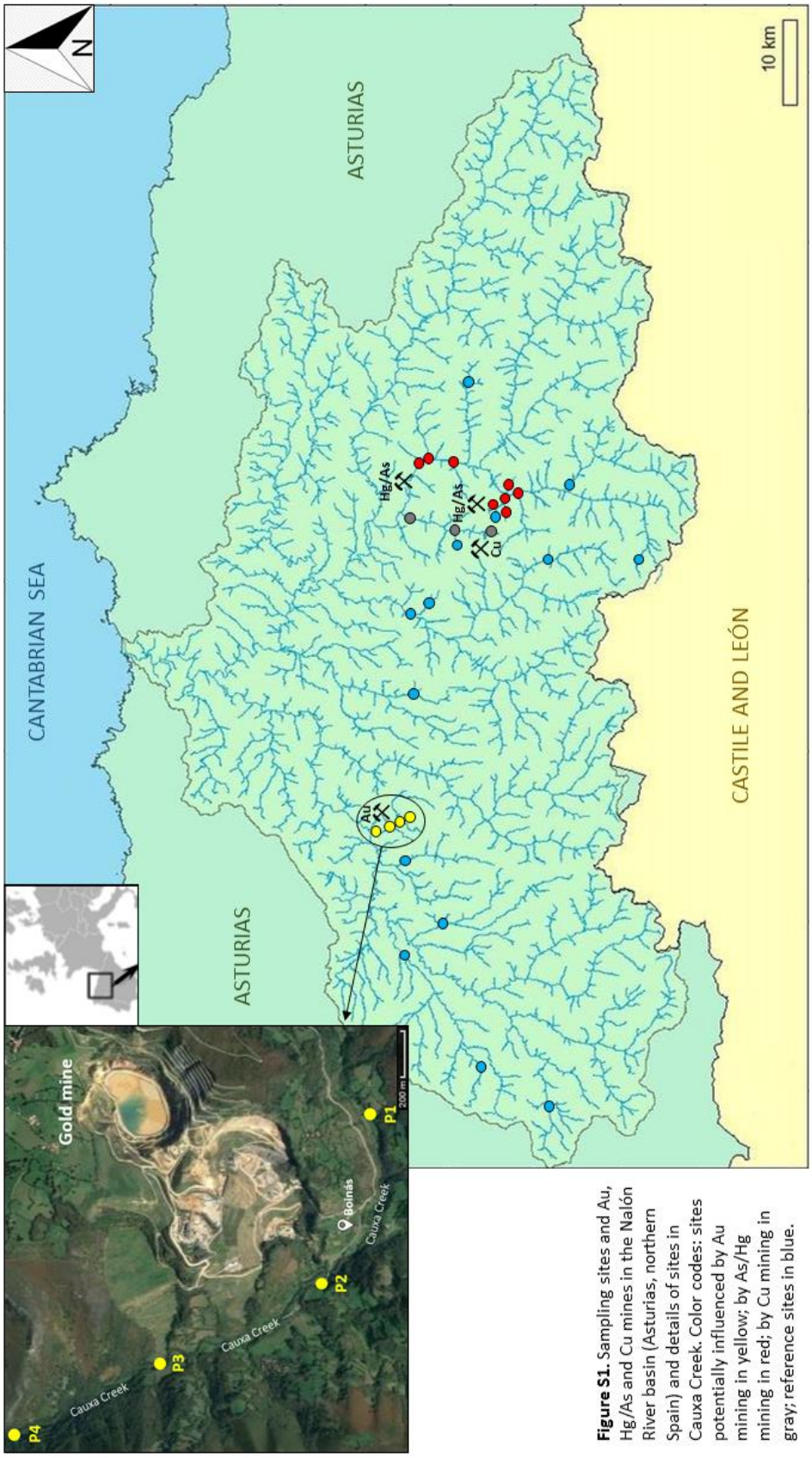

Figure S1. Sampling sites and Au, Hg/As and Cu mines in the Nalón River basin (Asturias, northern Spain) and details of sites in Cauxa Creek. Color codes: sites potentially influenced by Au mining in yellow; by As/Hg mining in red; by Cu mining in gray; reference sites in blue.

Table S1. Sampling design and methods for metal analysis of sediment and macroinvertebrates and the evaluation of the ecological status of macroinvertebrate assemblages in the Nalón River basin.

SAMPLING DESIGN	**METHODOLOGY**	**REFERENCES**
Sediment	In every site, a 0.5 L of a composite sediment sample was collected with a stainless-steel spade from the upper 5–10 cm layer of fine sediment settled along an approximately 25-m reach of the riverbanks. Samples were taken to the laboratory on ice and stored at 4 °C in the dark.	Méndez-Fernández *et al*. (2015)
Macroinvertebrate Ecological Status	A total sampled area of 2.5 m^2. At each study site, 20 'sample units' distributed proportionately in the main habitats existing along a 100 m reach were combined. Kick-net (500 µm mesh). Fixed and preserved in 70% ethanol.	Spanish official protocol ML-Rv-I (2013) Pardo *et al*. (2014)
Macroinvertebrate bioaccumulation	For each taxon, when possible 3 replicates consisting of 1–20 individuals were collected per site, placed in 15-ml tubes containing river water, and stored in ice. After 5-10 h, the macroinvertebrates were cleaned in dechlorinated water, identified and frozen at -20°C.	Rodriguez *et al*. (2018)
SAMPLE ANALYSIS	**METHODOLOGY**	**REFERENCES**
Sediment	After drying at room temperature, the sediment fraction < 63µm was digested using microwave extraction method, in concentrated nitric acid and concentrated hydrochloric acid (US EPA 3051 protocol). Metal analysis: ICP-MS (Agilent 7700X). Reference materials: Buffalo River sediment (RM8704, USA) and Sewage Sludge-3 (CRM 031-040, UK).	US EPA (2007)
Macroinvertebrate Ecological Status	Taxa richness and abundance were evaluated at the family level (except for aquatic oligochaetes and Hydracarina), under 57X magnification dissecting microscope (Olympus SZX9).	Costas *et al*. (2018)
Macroinvertebrate bioaccumulation	Samples were freeze-dried and weighted (Sartorius M3P balance). Acid digestion: 70% Nitric Acid Baker Instra-Analyzed+30% H_2O_2 Merck Suprapur. Metals analysis: ICP-MS (7500ce, Agilent Technologies). Reference material: NIST2976 mussel tissue.	Clements (1994) Rodriguez *et al*. (2018)

Funcional traits	**Families or higher taxa**	**Genera, spp**
Scraper (grazer)	Baetidae Heptageniidae	Mostly *Baetis* spp *Ecdyonurus, Heptagenia,* and *Epeorus* spp
Generalist	Ephemerellidae	*Serratella ignita*
Collector-filterer	Ephemeridae Hydropsychidae Simuliidae	*Ephemera* spp Mostly *Hydropsyche* spp undetermined
Collector-gatherer (deposit feeder)	Lumbricidae	*Eiseniella tetraedra*
	Microdrile oligochaetes	Mostly Lumbriculidae (mainly *Stylodrilus heringianus)*
Predator	Rhyacophilidae Perlidae	*Rhyacophila* spp *Perla* sp*, Dinocras cephalotes*

References Table S1

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Table S2. Limits of quantitation (LOQ) of As, Cd, Cu, Cr, Hg, Ni, Pb, Se and Zn for tissue residues and sediment (µg l^{-1}) from 2014, 2015 and 2016 sampling campaigns.

	Year	As	Cd	Cu	Cr	Hg	Ni	Pb	Se	Zn
Tissue	2014	0.009	0.014	0.060	0.040	0.014	0.073	0.040	0.567	0.833
	2015	0.010	0.010	0.097	0.049	0.031	0.171	0.079	0.586	1.443
	2016	0.050	0.008	0.197	0.040	0.008	0.196	0.070	0.520	0.740
Sediment	2016	0.010	0.010	0.100	0.030	0.010	0.030	0.030	0.400	50

Table S3. Tissue residues in taxa from 15 test sites in mining districts of the Nalón River basin (µg g^{-1} dw). Samples taken in 2014-2015. When the taxon was absent from a site, it was marked by a hyphen (-).

	Taxa	As	Cu	Hg	Se
Cu mine sites					
N4	Baetidae	2.32	35.62	0.14	7.77
	Ephemerellidae	4.30	163.34	0.20	6.50
	Ephemeridae	1.57	25.84	0.19	3.41
	Heptageniidae	6.36	135.87	0.11	10.15
	Hydropsychidae	1.75	27.54	0.49	2.40
	Lumbricidae	9.48	25.22	0.41	9.99
	Microdrile	10.83	26.18	0.71	3.74
	Perlidae	-	-	-	-
	Rhyacophilidae	1.67	19.41	0.25	2.15
	Simuliidae	2.80	37.29	0.81	2.31
N6	Baetidae	2.38	32.32	0.10	4.74
	Ephemerellidae	3.25	114.93	0.09	3.56
	Ephemeridae	2.92	30.64	0.28	3.45
	Heptageniidae	5.01	135.54	0.13	9.46
	Hydropsychidae	0.90	16.45	0.12	1.90
	Lumbricidae	11.15	26.37	0.14	8.67
	Microdrile	7.55	21.55	0.39	3.84
	Perlidae	0.69	26.23	0.14	2.41
	Rhyacophilidae	0.64	19.63	0.12	3.36
	Simuliidae	2.59	33.60	0.12	4.01
NAL013	Baetidae	1.34	48.49	0.28	6.15
	Ephemerellidae	2.51	135.93	0.18	4.28
	Ephemeridae	-	-	-	-
	Heptageniidae	2.96	112.06	0.19	10.57
	Hydropsychidae	0.69	16.91	0.23	3.06
	Lumbricidae	11.89	14.53	0.39	12.22
	Microdrile	7.42	10.74	0.47	4.48
	Perlidae	-	-	-	-
	Rhyacophilidae	0.25	17.08	0.28	3.36
	Simuliidae	1.81	21.36	0.26	1.91
Hg mine sites					
N9	Baetidae	513.38	109.76	4.16	22.04
	Ephemerellidae	-	-	-	-
	Ephemeridae	-	-	-	-
	Heptageniidae	-	-	-	-
	Hydropsychidae	-	-	-	-
	Lumbricidae	-	-	-	-
	Microdrile	495.31	41.04	2.42	6.72
	Perlidae	-	-	-	-
	Rhyacophilidae	-	-	-	-

	Simuliidae	660.48	26.49	18.82	12.02
N10	Baetidae	38.60	40.31	0.44	4.39
	Ephemerellidae	69.25	31.78	0.46	5.60
	Ephemeridae	120.34	30.00	0.97	4.54
	Heptageniidae	-	-	-	-
	Hydropsychidae	61.97	19.14	1.07	3.20
	Lumbricidae	1658.84	12.67	1.55	10.45
	Microdrile	-	-	-	-
	Perlidae	-	-	-	-
	Rhyacophilidae	15.02	14.79	0.72	2.69
	Simuliidae	183.92	18.34	1.24	2.71
N11	Baetidae	27.84	39.60	0.50	5.70
	Ephemerellidae	61.09	67.01	0.57	11.15
	Ephemeridae	43.61	24.70	0.74	6.51
	Heptageniidae	34.27	82.20	0.49	9.43
	Hydropsychidae	28.85	20.81	0.85	3.61
	Lumbricidae	-	-	-	-
	Microdrile	215.86	13.11	1.49	13.22
	Perlidae	-	-	-	-
	Rhyacophilidae	8.07	17.15	0.60	4.25
	Simuliidae	64.80	22.18	1.55	3.15
N13	Baetidae	3.12	30.26	0.88	9.78
	Ephemerellidae	8.79	57.53	0.46	5.93
	Ephemeridae	3.61	11.56	1.13	4.16
	Heptageniidae	7.70	67.71	1.20	9.65
	Hydropsychidae	1.74	16.21	0.59	2.86
	Lumbricidae	7.66	8.61	1.06	8.09
	Microdrile	10.76	16.24	1.85	4.25
	Perlidae	0.79	22.88	0.33	1.75
	Rhyacophilidae	0.71	15.38	0.29	3.60
	Simuliidae	-	-	-	-
N14	Baetidae	3.97	25.94	0.26	4.77
	Ephemerellidae	8.56	69.35	0.25	4.94
	Ephemeridae	11.18	13.65	0.63	3.27
	Heptageniidae	7.52	60.20	0.31	5.91
	Hydropsychidae	2.41	16.18	0.37	2.07
	Lumbricidae	12.53	7.54	0.78	11.59
	Microdrile	-	-	-	-
	Perlidae	1.18	19.37	0.61	3.23
	Rhyacophilidae	1.58	11.82	0.26	1.62
	Simuliidae	9.19	17.76	0.63	2.90
N15	Baetidae	-	-	-	-
	Ephemerellidae	-	-	-	-
	Ephemeridae	-	-	-	-
	Heptageniidae	-	-	-	-
	Hydropsychidae	787.45	24.42	16.33	3.00

	Lumbricidae	1860.81	5.55	86.37	17.19
	Microdrile	393.83	10.77	18.13	3.12
	Perlidae	-	-	-	-
	Rhyacophilidae	-	-	-	-
	Simuliidae	555.17	21.07	8.82	4.21
NAL056a	Baetidae	3.92	99.67	0.18	6.72
	Ephemerellidae	7.70	175.05	0.14	2.66
	Ephemeridae	5.33	45.00	0.20	8.03
	Heptageniidae	9.75	417.78	0.16	7.78
	Hydropsychidae	2.78	40.22	0.16	1.94
	Lumbricidae	-	-	-	-
	Microdrile	34.64	36.62	0.44	6.08
	Perlidae	1.36	57.21	0.19	3.64
	Rhyacophilidae	0.92	38.69	0.16	2.93
	Simuliidae	5.55	118.51	0.18	2.79
NAL056b	Baetidae	2.11	16.60	0.26	9.13
	Ephemerellidae	7.71	42.31	0.18	8.10
	Ephemeridae	6.82	15.12	0.28	8.97
	Heptageniidae	7.09	30.77	0.18	11.35
	Hydropsychidae	1.82	10.12	0.16	3.51
	Lumbricidae	-	-	-	-
	Microdrile	42.98	6.91	0.33	3.59
	Perlidae	-	-	-	-
	Rhyacophilidae	1.00	16.10	0.28	5.39
	Simuliidae	6.41	15.33	0.36	7.32
Au mine sites					
P1	Baetidae	1.47	102.17	0.18	4.05
	Ephemerellidae	1.29	93.33	0.07	2.32
	Ephemeridae	1.20	32.86	0.10	2.70
	Heptageniidae	5.13	333.17	0.14	8.74
	Hydropsychidae	0.73	26.54	0.09	1.30
	Lumbricidae	5.58	21.57	0.14	6.27
	Microdrile	4.54	27.67	0.24	1.67
	Perlidae	0.31	76.47	0.10	3.24
	Rhyacophilidae	0.28	21.16	0.12	2.29
	Simuliidae	2.86	114.75	0.19	2.15
P2	Baetidae	48.62	121.58	0.14	7.87
	Ephemerellidae	97.71	241.40	0.15	3.51
	Ephemeridae	-	-	-	-
	Heptageniidae	58.63	327.46	0.18	8.33
	Hydropsychidae	-	-	-	-
	Lumbricidae	284.50	151.48	0.16	12.86
	Microdrile	594.00	246.60	0.20	10.16
	Perlidae	69.94	169.89	0.13	4.02
	Rhyacophilidae	11.04	67.29	0.60	3.17
	Simuliidae	-	-	-	-

P3	Baetidae	45.67	120.30	0.20	9.24
	Ephemerellidae	57.10	157.68	0.18	4.62
	Ephemeridae	354.92	556.99	0.23	4.07
	Heptageniidae	57.40	353.86	0.11	10.77
	Hydropsychidae	32.63	69.94	0.19	4.18
	Lumbricidae	17.58	34.45	0.20	16.22
	Microdrile	171.85	146.28	1.08	14.30
	Perlidae	16.52	64.87	0.10	1.93
	Rhyacophilidae	6.53	57.33	0.21	6.21
	Simuliidae	-	-	-	-
P4	Baetidae	28.30	76.11	0.20	5.91
	Ephemerellidae	29.34	157.82	0.13	3.69
	Ephemeridae	-	-	-	-
	Heptageniidae	32.07	369.19	0.49	10.01
	Hydropsychidae	32.84	45.89	0.16	6.67
	Lumbricidae	91.97	69.97	0.27	20.43
	Microdrile	101.87	198.34	1.03	17.29
	Perlidae	-	-	-	-
	Rhyacophilidae	4.64	43.33	0.34	7.26
	Simuliidae	-	-	-	-
		As	Cu	Hg	Se
	min	0.25	5.55	0.07	1.30
	max	1860.81	556.99	86.37	22.04

Table S4. Parameters (b, c, d, e) of the nonlinear regression functions. Effective tissue residues for the 50% reduction of the EQR (EQR-ER_{50}) and for the EQR boundary of good/moderate ecological status (EQR-ER_{GM}). Models: W, Weibull; LL, log-logistic. SE, Standard Error.

Taxa	Metric	Model	b	c	d	e	EQR-ER_{50}	SE	EQR-ER_{GM}
Arsenic									
Baetidae	METI	W2.3	-0.339	0.000	1.068	62.910	185.7	126.9	52.2
Baetidae	METI	W2.4	-3.048	0.521	1.026	28.468	32.1	7.2	37.3
Baetidae	METI	LL.4	3.908	0.518	1.026	32.817	32.8	7.9	38.1
Baetidae	METI	W1.4	2.633	0.520	1.027	38.444	33.5	8.3	38.9
Baetidae	NORTI	W2.3	-0.435	0.000	0.873	49.372	114.7	65.7	16.3
Baetidae	NORTI	LL.3	0.669	0.000	0.905	123.080	123.1	76.6	19.6
Baetidae	NORTI	W1.4	0.828	0.253	0.880	80.202	51.5	40.4	21.7
Baetidae	NORTI	W1.3	0.453	0.000	0.953	239.811	108.8	81.5	18.0
Ephemerellidae	METI	W2.3	-0.615	0.000	1.029	82.532	-	-	66.7
Ephemerellidae	METI	LL.3	1.456	0.000	1.032	114.608	-	-	68.7
Ephemerellidae	METI	W1.3	0.133	0.000	1.033	141.067	-	-	-
Ephemerellidae	NORTI	LL.3	0.877	0.000	0.882	165.602	-	-	35.5
Ephemeridae	NORTI	W1.4	2.221	0.624	0.845	43.047	-	-	44.3
Heptageniidae	METI	W2.3	-0.764	0.000	1.030	56.383	-	-	47.6
Heptageniidae	METI	LL.3	1.713	0.000	1.035	74.889	-	-	48.7
Heptageniidae	METI	W1.3	1.514	0.000	1.038	91.330	-	-	49.4
Heptageniidae	NORTI	W2.4	-2.512	0.625	0.893	6.898	8.0	2.8	10.8
Heptageniidae	NORTI	LL.4	21.705	0.630	0.875	7.624	7.6	0.7	8.0
Heptageniidae	NORTI	W1.4	8.739	0.628	0.878	8.204	7.9	1.2	8.4
Hydropsychidae	METI	W2.4	-22.811	0.763	1.038	2.577	2.6	0.2	-
Hydropsychidae	METI	LL.4	22.435	0.716	1.031	29.528	29.5	2.8	-
Hydropsychidae	NORTI	W2.3	-0.083	0.000	1.335	0.630	-	-	23.1
Hydropsychidae	NORTI	W1.4	1.711	0.565	0.827	35.466	-	-	27.8
Lumbricidae	METI	W2.4	-19.051	0.664	1.042	16.871	17.2	0.8	19.1
Lumbricidae	NORTI	LL.4	5.483	0.548	0.856	86.245	86.2	47.5	86.9
Lumbricidae	NORTI	W1.4	2.298	0.549	0.857	97.940	83.5	55.2	84.5
Microdriles	METI	W2.3	-0.354	0.000	1.066	278.604	-	-	230.2
Microdriles	METI	W1.3	0.478	0.000	1.127	1205.979	560.6	261.7	255.9
Microdriles	NORTI	W2.3	-0.492	0.000	0.850	241.689	509.1	211.2	79.0
Microdriles	NORTI	LL.3	1.032	0.000	0.844	570.022	570.0	221.7	122.9
Microdriles	NORTI	W1.3	0.877	0.000	0.848	832.611	548.2	181.5	126.2
Perlidae	NORTI	LL.4	3.637	0.580	0.844	1.062	1.1	0.3	1.1
Rhyacophilidae	METI	W2.3	-0.934	0.000	1.029	7.933	11.7	4.3	6.9
Rhyacophilidae	METI	LL.3	1.745	0.000	1.035	10.932	10.9	2.7	7.2
Rhyacophilidae	METI	W1.3	1.494	0.000	1.038	13.702	10.7	2.3	7.4
Rhyacophilidae	NORTI	W2.3	-0.184	0.000	1.385	0.383	-	-	2.6
Simuliidae	METI	LL.3	0.760	0.000	1.009	1002.867	-	-	341.3
Simuliidae	METI	W1.4	1.670	0.622	0.999	149.276	119.9	82.6	195.8
Simuliidae	NORTI	W2.3	-0.155	0.000	1.338	2.583	-	-	17.8
Copper									

Taxa	Metric	Model	b	c	d	e	EQR-ER_{50}	SE	EQR-ER_{GM}
Baetidae	METI	W2.3	-1.025	0.000	1.039	116.764	-	-	104.5
Baetidae	METI	W2.4	-36.196	0.723	1.053	38.837	39.2	3.7	-
Baetidae	NORTI	W2.4	-2.961	0.572	0.918	30.529	34.6	9.6	39.6
Baetidae	NORTI	LL.4	5.134	0.587	0.928	33.340	33.3	6.8	38.2
Baetidae	NORTI	W1.4	4.943	0.602	0.924	35.346	32.8	5.5	36.6
Ephemerellidae	METI	W2.3	-2.239	0.000	1.023	203.799	240.1	40.2	191.3
Ephemerellidae	METI	W2.4	-2.338	0.062	1.023	198.249	-	-	191.0
Ephemerellidae	METI	LL.3	4.142	0.000	1.025	235.963	236.0	30.8	196.1
Ephemerellidae	METI	W1.3	3.488	0.000	1.027	262.386	236.2	26.5	199.3
Ephemerellidae	NORTI	W2.3	-0.774	0.000	0.903	225.649	-	-	134.4
Ephemeridae	METI	W2.4	-1.805	0.705	1.061	30.862	37.8	18.8	-
Ephemeridae	METI	LL.4	3.587	0.706	1.063	36.775	36.8	13.4	-
Ephemeridae	METI	W1.4	2.934	0.709	1.067	41.365	36.5	12.3	-
Ephemeridae	NORTI	W2.4	-5.097	0.672	0.882	18.722	20.1	13.7	27.3
Ephemeridae	NORTI	LL.4	188.769	0.688	0.889	16.238	16.2	0.2	16.5
Ephemeridae	NORTI	W1.4	7.984	0.688	0.882	21.953	21.0	10.0	25.0
Heptageniidae	METI	W2.3	-1.059	0.000	1.036	463.228	-	-	414.1
Heptageniidae	METI	W2.4	-5.676	0.743	1.034	186.742	199.2	133.7	-
Heptageniidae	METI	LL.3	2.292	0.000	1.040	558.892	-	-	407.9
Heptageniidae	METI	W1.4	7.647	0.751	1.034	252.503	240.7	152.7	-
Heptageniidae	METI	W1.3	2.032	0.000	1.041	641.735	-	-	407.2
Heptageniidae	NORTI	LL.4	2.959	0.644	0.908	99.620	99.6	52.5	155.7
Heptageniidae	NORTI	W1.4	1.765	0.648	0.928	118.837	96.6	62.7	159.9
Hydropsychidae	NORTI	W2.4	-5.971	0.664	0.998	13.315	15.3	1.8	19.1
Hydropsychidae	NORTI	LL.4	9.240	0.662	0.919	15.410	-	-	18.6
Hydropsychidae	NORTI	W1.4	8.437	0.663	0.912	16.594	15.9	1.7	17.9
Lumbricidae	METI	W2.3	-0.688	0.000	1.023	95.491	-	-	77.7
Lumbricidae	METI	LL.3	1.254	0.000	1.046	145.127	145.1	61.6	82.7
Lumbricidae	METI	W1.3	0.957	0.000	1.065	215.260	146.7	62.0	86.8
Lumbricidae	NORTI	W2.3	-0.590	0.000	0.857	111.989	208.4	184.5	45.7
Microdriles	METI	LL.4	56.913	0.629	1.009	36.372	36.4	1.5	37.3
Microdriles	METI	W1.4	13.870	0.630	1.010	36.653	35.7	3.6	38.1
Microdriles	NORTI	LL.4	17.926	0.538	0.825	30.067	30.1	7.3	29.6
Microdriles	NORTI	W1.4	9.644	0.530	0.824	34.305	33.0	5.5	32.2
Perlidae	METI	W2.3	-0.753	0.000	1.208	80.744	131.4	41.7	97.7
Perlidae	METI	LL.3	0.995	0.000	1.360	105.342	105.3	63.3	99.3
Rhyacophilidae	METI	W2.3	-1.534	0.000	1.027	55.527	-	-	50.9
Rhyacophilidae	METI	W2.4	-2.183	0.337	1.025	43.391	-	-	49.5
Rhyacophilidae	METI	LL.3	2.477	0.000	1.055	68.215	-	-	51.9
Rhyacophilidae	METI	W1.3	2.025	0.000	1.066	80.449	67.1	10.5	52.5
Rhyacophilidae	NORTI	W2.3	-1.319	0.000	0.846	64.347	-	-	41.9
Rhyacophilidae	NORTI	W2.4	-4.729	0.580	0.846	29.551	31.9	25.0	32.9
Rhyacophilidae	NORTI	LL.3	2.594	0.000	0.856	78.507	-	-	44.0
Rhyacophilidae	NORTI	W1.4	10.098	0.608	0.846	33.653	32.5	14.1	33.5
Rhyacophilidae	NORTI	W1.3	2.274	0.000	0.859	89.888	-	-	44.7

Simuliidae	NORTI	W2.4	-5.312	0.698	1.029	15.011	16.1	4.3	37.9
Simuliidae	NORTI	LL.4	6.346	0.698	1.080	15.525	15.5	6.8	36.4
Mercury									
Taxa	**Metric**	**Model**	**b**	**c**	**d**	**e**	**EQR-ER_{50}**	**SE**	**EQR-ER_{GM}**
Baetidae	METI	W1.3	0.326	0.000	1.173	27.110	-	-	3.6
Ephemeridae	METI	W2.4	-4.757	0.987	1.184	0.043	0.1	0.0	-
Ephemeridae	NORTI	W2.4	-1.717	0.731	1.079	0.076	0.1	0.1	-
Ephemeridae	NORTI	LL.4	3.888	0.730	1.080	0.096	0.1	0.0	-
Ephemeridae	NORTI	W1.4	2.425	0.731	1.073	0.117	0.1	0.0	-
Heptageniidae	NORTI	W2.4	-1.868	0.703	1.267	0.050	0.1	0.0	-
Heptageniidae	NORTI	LL.4	2.314	0.707	1.322	0.057	0.1	0.1	-
Heptageniidae	NORTI	W1.4	1.783	0.718	1.247	0.084	0.1	0.1	-
Perlidae	NORTI	W2.4	-6.631	0.752	1.233	0.067	0.1	0.0	-
Perlidae	NORTI	LL.4	8.210	0.753	1.233	0.070	0.1	0.0	-
Perlidae	NORTI	W1.4	2.959	0.752	1.272	0.071	0.1	0.0	-
Rhyacophilidae	METI	W2.3	-21.082	0.000	1.004	0.598	-	-	0.6
Rhyacophilidae	METI	LL.3	69.378	0.000	1.004	0.604	0.6	0.0	0.6
Rhyacophilidae	METI	W1.3	30.974	0.000	1.005	0.614	-	-	0.6
Rhyacophilidae	NORTI	W2.4	-1.834	0.601	0.949	0.199	0.2	0.2	0.4
Rhyacophilidae	NORTI	LL.4	3.364	0.647	0.959	0.214	0.2	0.1	0.3
Simuliidae	METI	W2.3	-0.186	0.000	1.274	2.366	-	-	8.0
Simuliidae	METI	LL.3	0.686	0.000	1.041	22.174	-	-	7.8
Simuliidae	NORTI	W2.3	-0.304	0.000	1.104	0.974	-	-	1.0
Simuliidae	NORTI	LL.3	0.360	0.000	1.348	1.204	-	-	1.0
Selenium									
Taxa	**Metric**	**Model**	**b**	**c**	**d**	**e**	**EQR-ER_{50}**	**S.E.**	**EQR-ER_{GM}**
Baetidae	METI	W2.3	-0.475	0.000	1.133	20.365	-	-	22.1
Baetidae	METI	LL.3	0.941	0.000	1.152	30.979	-	-	19.5
Baetidae	METI	W1.3	0.824	0.000	1.158	43.136	-	-	18.7
Baetidae	NORTI	LL.3	0.671	0.000	1.170	15.626	15.6	15.1	8.6
Baetidae	NORTI	W1.3	0.563	0.000	1.170	28.409	14.8	13.1	8.7
Heptageniidae	NORTI	W2.4	-4.083	0.739	1.088	3.183	3.5	0.6	-
Heptageniidae	NORTI	LL.4	4.596	0.739	1.111	3.478	3.5	0.8	-
Heptageniidae	NORTI	W1.4	2.029	0.739	1.179	3.870	3.2	1.8	-
Hydropsychidae	METI	W2.4	-72.389	0.813	1.080	2.934	3.0	0.1	-
Hydropsychidae	METI	W1.4	62.634	0.814	1.080	2.980	3.0	0.0	-
Hydropsychidae	NORTI	W2.4	-4.200	0.712	1.102	1.028	1.1	0.1	-
Hydropsychidae	NORTI	LL.4	5.038	0.714	1.143	1.090	1.1	0.2	-
Hydropsychidae	NORTI	W1.4	1.948	0.713	1.301	1.092	0.9	0.5	-
Microdriles	METI	W2.3	-0.777	0.000	1.012	20.695	-	-	16.8
Microdriles	METI	W2.4	-4.332	0.750	1.004	6.020	6.6	2.3	-
Microdriles	METI	LL.3	1.323	0.000	1.049	29.352	-	-	-
Microdriles	METI	LL.4	7.060	0.746	1.003	7.017	7.0	2.4	-
Microdriles	METI	W1.4	5.936	0.736	0.998	8.104	7.6	2.2	-
Microdriles	METI	W1.3	1.114	0.000	1.059	38.594	-	-	-

Microdriles	NORTI	LL.4	68.887	0.637	0.824	5.792	5.8	0.6	5.9
Microdriles	NORTI	W1.4	29.280	0.638	0.825	5.881	5.8	0.4	5.9

Table S5. As, Cu, Hg and Se effective tissue residues (EPT-ER_{50}) estimated for a 50% reduction in the number of EPT families (EPT Fam-ER_{50}) and EPT abundance (EPT Ab-ER_{50}) from nonlinear regression models (W, Weibull; LL, log-logistic). SE: Standard Error.

Arsenic				
Taxa	**Metric**	**Model**	**EPT-ER_{50}**	**SE**
Baetidae	Fam EPT	W2.3	106.7	71.8
Baetidae	Fam EPT	LL.3	88.1	62.9
Baetidae	Fam EPT	LL.4	35.5	11.0
Baetidae	Fam EPT	W1.4	36.2	11.6
Baetidae	Ab EPT	W2.3	11.4	9.7
Ephemerellidae	Fam EPT	W1.3	91.8	29.2
Ephemerellidae	Fam EPT	LL.3	92.5	35.6
Ephemerellidae	Ab EPT	W2.4	8.3	0.6
Ephemerellidae	Ab EPT	W1.4	8.4	0.9
Ephemeridae	Fam EPT	W2.4	11.3	2.3
Ephemeridae	Fam EPT	W1.4	29.6	26.8
Heptageniidae	Fam EPT	W2.4	7.9	1.1
Heptageniidae	Fam EPT	W1.4	8.2	1.8
Heptageniidae	Fam EPT	LL.4	8.1	1.3
Heptageniidae	Ab EPT	LL.3	18.8	13.8
Heptageniidae	Ab EPT	W1.3	19.3	14.0
Heptageniidae	Ab EPT	W2.3	16.7	11.3
Heptageniidae	Ab EPT	W1.4	17.7	15.9
Hydropsychidae	Fam EPT	W2.3	173.7	161.5
Hydropsychidae	Fam EPT	W1.4	41.8	15.7
Lumbricidae	Fam EPT	W2.3	693.2	547.3
Lumbricidae	Fam EPT	W1.4	167.8	105.2
Lumbricidae	Fam EPT	W2.4	136.2	88.8
Lumbricidae	Fam EPT	LL.4	146.7	101.1
Lumbricidae	Ab EPT	W2.4	16.4	4.6
Lumbricidae	Ab EPT	LL.4	17.0	1.0

Microdriles	Fam EPT	LL.3	356.2	120.1
Microdriles	Fam EPT	W1.3	361.4	117.6
Microdriles	Fam EPT	W2.3	371.1	160.8
Microdriles	Fam EPT	W1.4	186.1	140.4
Microdriles	Ab EPT	W2.3	81.3	35.8
Microdriles	Ab EPT	LL.3	89.2	39.1
Microdriles	Ab EPT	LL.4	86.0	58.9
Rhyacophilidae	Fam EPT	W2.3	9.6	2.5
Rhyacophilidae	Fam EPT	LL.3	9.8	2.4
Rhyacophilidae	Fam EPT	W1.3	9.9	2.2
Rhyacophilidae	Fam EPT	W1.4	6.5	1.2
Rhyacophilidae	Fam EPT	LL.4	6.5	1.4
Rhyacophilidae	Fam EPT	W2.4	6.4	2.1
Rhyacophilidae	Ab EPT	W2.3	1.5	0.3
Rhyacophilidae	Ab EPT	LL.3	1.5	0.2
Simuliidae	Fam EPT	W2.3	216.0	151.7
Simuliidae	Fam EPT	LL.3	181.5	138.5
Simuliidae	Fam EPT	W1.3	212.5	180.8
Simuliidae	Fam EPT	W1.4	76.0	71.4
Simuliidae	Ab EPT	W2.4	7.9	6.6
Simuliidae	Ab EPT	W1.4	8.8	2.6
Simuliidae	Ab EPT	LL.4	8.4	1.9
Copper				
Taxa	**Metric**	**Model**	**EPT-ER$_{50}$**	**SE**
Ephemerellidae	Fam EPT	W2.3	238.6	52.1
Ephemerellidae	Fam EPT	W1.3	236.9	36.1
Ephemerellidae	Fam EPT	LL.3	236.0	41.7
Ephemerellidae	Ab EPT	W2.3	182.9	42.0
Ephemerellidae	Ab EPT	W1.3	199.6	46.1
Ephemerellidae	Ab EPT	LL.3	193.6	46.7
Ephemeridae	Fam EPT	W1.4	41.3	12.7

Ephemeridae	Fam EPT	LL.4	37.3	18.7
Ephemeridae	Fam EPT	W2.4	35.4	23.7
Hydropsychidae	Fam EPT	W1.4	16.7	0.3
Hydropsychidae	Fam EPT	W2.4	16.7	0.3
Hydropsychidae	Fam EPT	LL.4	16.7	0.3
Lumbricidae	Ab EPT	W1.3	32.9	13.5
Lumbricidae	Ab EPT	LL.3	32.9	5.6
Lumbricidae	Ab EPT	W1.4	33.4	17.0
Lumbricidae	Ab EPT	LL.4	32.0	7.9
Microdriles	Ab EPT	W2.3	48.6	44.1
Microdriles	Ab EPT	LL.3	61.5	51.6
Perlidae	Fam EPT	W1.3	167.8	58.0
Perlidae	Ab EPT	W2.3	74.9	23.9
Perlidae	Ab EPT	LL.3	78.6	25.5
Perlidae	Ab EPT	W1.3	79.5	23.2
Rhyacophilidae	Ab EPT	W2.3	42.0	7.7
Rhyacophilidae	Ab EPT	W1.3	42.9	3.2
Rhyacophilidae	Ab EPT	LL.3	42.6	2.9
Rhyacophilidae	Ab EPT	W2.4	41.7	14.4
Rhyacophilidae	Ab EPT	W1.4	42.9	5.8
Rhyacophilidae	Ab EPT	LL.4	42.1	3.2
Mercury				
Taxa	**Metric**	**Model**	**EPT-ER_{50}**	**SE**
Ephemerellidae	Fam EPT	W1.4	0.1	0.0
Ephemerellidae	Fam EPT	LL4	0.1	0.0
Ephemerellidae	Fam EPT	W2.4	0.1	0.0
Ephemeridae	Fam EPT	W2.4	0.1	0.0
Ephemeridae	Fam EPT	LL4	0.1	0.1
Ephemeridae	Fam EPT	W1.4	0.1	0.1
Ephemeridae	Ab EPT	W2.3	0.6	0.6
Ephemeridae	Ab EPT	LL3	0.6	0.5

Ephemeridae	Ab EPT	W1.3	0.6	0.5
Microdriles	Ab EPT	W2.3	0.8	0.2
Microdriles	Ab EPT	LL3	0.7	0.1
Microdriles	Ab EPT	W1.3	0.8	0.2
Microdriles	Ab EPT	LL4	0.7	0.2
Microdriles	Ab EPT	W2.4	0.7	0.3
Microdriles	Ab EPT	W1.4	0.7	0.1
Perlidae	Ab EPT	W1.4	0.1	0.1
Simuliidae	Ab EPT	W2.3	0.8	0.2
Simuliidae	Ab EPT	LL3	0.9	0.3
Simuliidae	Ab EPT	W1.3	0.9	0.3
Selenium				
Taxa	**Metric**	**Model**	**EPT-ER_{50}**	**SE**
Baetidae	Fam EPT	W1.3	18.6	13.6
Baetidae	Fam EPT	LL3	20.3	16.0
Baetidae	Ab EPT	W2.3	9.1	6.6
Baetidae	Ab EPT	W2.4	2.3	0.8
Heptageniidae	Ab EPT	W2.4	4.3	3.3
Heptageniidae	Ab EPT	W1.4	5.3	1.8
Hydropsychidae	Fam EPT	W1.4	2.1	0.4
Hydropsychidae	Fam EPT	W2.4	2.0	0.3
Hydropsychidae	Fam EPT	LL4	2.1	0.3
Hydropsychidae	Ab EPT	W2.3	4.4	1.6
Hydropsychidae	Ab EPT	W1.3	4.3	1.5
Hydropsychidae	Ab EPT	LL3	4.3	1.4
Lumbricidae	Fam EPT	W1.4	10.1	0.5
Lumbricidae	Fam EPT	W2.4	10.0	0.1
Lumbricidae	Fam EPT	LL4	10.0	0.0
Lumbricidae	Ab EPT	W2.3	18.6	6.0
Lumbricidae	Ab EPT	LL3	18.7	5.0
Lumbricidae	Ab EPT	W1.3	18.7	4.5

Lumbricidae	Ab EPT	W2.4	9.4	6.1
Lumbricidae	Ab EPT	W1.4	12.2	6.0
Lumbricidae	Ab EPT	LL4	11.5	5.7
Microdriles	Fam EPT	W1.4	4.4	0.1
Microdriles	Fam EPT	W2.4	4.4	0.0
Microdriles	Fam EPT	LL4	4.4	0.1
Microdriles	Ab EPT	W2.3	8.3	2.0
Microdriles	Ab EPT	LL3	8.3	1.9
Microdriles	Ab EPT	W1.3	8.3	1.7
Microdriles	Ab EPT	W1.4	8.0	1.7
Microdriles	Ab EPT	W2.4	7.6	2.1
Microdriles	Ab EPT	LL4	7.8	1.9
Rhyacophilidae	Ab EPT	W2.4	1.1	0.1

Table S6. Sediment metal concentration (µg g^{-1} dw) from sites of the Cauxa River in the 2016 campaign. Values for probable effect concentrations (PECs) were obtained from McDonald *et al*. (2000), except for Hg (from Méndez-Fernández *et al*., 2019) and selenium (90^{th} percentile at reference site sediments, from Costas *et al*., 2018). In bold, metal levels > PEC. SedPoll index classes: Similar-to-reference (SimRef) ≤1.0, Low 1.1–2.0, Medium 2.1–10.0, High 10.1–50, Very High >50.

Site	As	Cd	Cr	Cu	Hg	Ni	Pb	Se	Zn	SedPoll	Class
P1	20	0.38	22	25	0.25	42.4	14	2.2	96	0.8	SimRef
P2	**1,593**	2.48	29	**1,891**	**1.35**	35.6	90	**18.8**	343	28.1	High
P3	**1,158**	1.75	32	**1,355**	1.16	41.0	65	**15.2**	282	20.4	High
P4	**414**	0.98	27	**583**	0.59	46.3	29	**8.7**	239	8.2	Medium
PEC	33	4.98	111	149	1.21	48.6	128	5.3	459		

Table S7. As, Cu, Hg and Se field tissue concentrations (µg g^{-1} dw) in ten biomonitor taxa from the Cauxa River (2016 sampling). In bold, the tissue residues > ETTC value (Rodriguez *et al.*, 2018). When the taxon was absent from a site, it was marked by a hyphen (–).

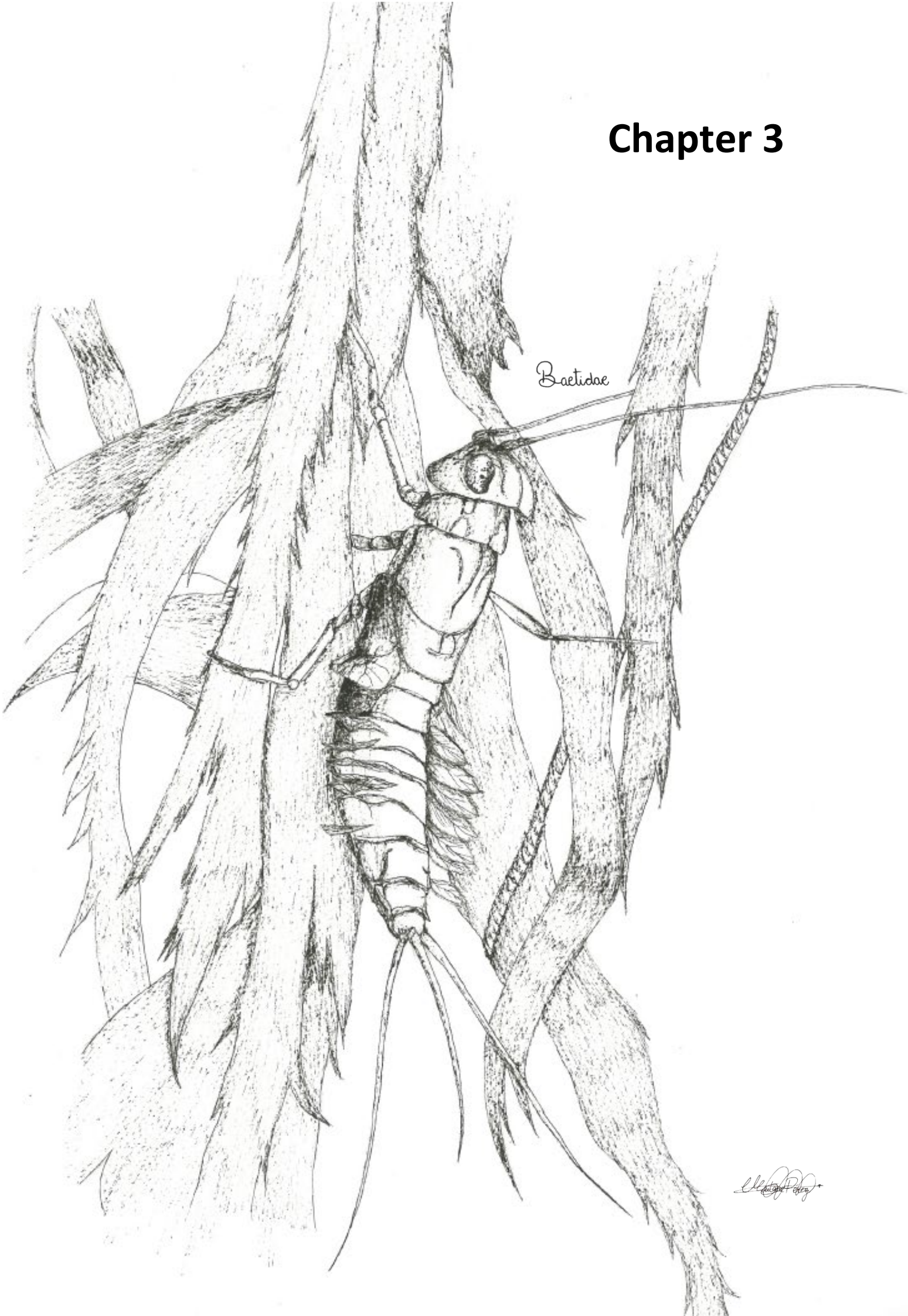
	Baetidae	Ephemerellidae	Ephemeridae	Heptageniidae	Hydropsychidae	Lumbricidae	Microdriles	Perlidae	Rhyacophilidae	Simuliidae
Arsenic										
P1	1.8	1.7	1.4	4.6	0.8	5.8	5.9	0.2	0.2	**12.4**
P2	**153.2**	**135.5**	**81.2**	**116.3**	**27.6**	**154.0**	–	**34.6**	**34.8**	–
P3	**72.7**	**57.1**	**145.6**	**52.9**	–	**147.5**	–	–	**5.2**	–
P4	**16.8**	**18.2**	–	–	–	**238.4**	**58.9**	–	**7.3**	–
As ETTC	3.11	6.48	6.45	6.30	2.00	13.83	14.44	0.75	0.82	4.49
Copper										
P1	**45.0**	**127.4**	**26.2**	**170.3**	**22.3**	**22.0**	**22.8**	**68.9**	**19.8**	36.9
P2	**174.6**	**327.9**	**159.4**	**380.9**	**52.5**	**141.3**	–	**75.0**	**75.3**	–
P3	**120.7**	**179.8**	**257.7**	**248.1**	–	**120.7**	–	–	**37.1**	–
P4	**63.0**	**298.4**	–	–	–	**38.5**	**111.1**	–	**50.0**	–
Cu ETTC	29.23	90.91	16.24	80.13	15.64	11.51	21.27	29.19	19.75	58.23
Mercury										
P1	0.08	0.08	0.09	0.09	0.10	0.17	0.17	0.12	0.12	0.21
P2	0.10	0.12	0.10	0.11	0.12	**0.75**	–	0.10	0.12	–
P3	0.11	0.10	0.22	0.17	–	0.37	–	–	0.13	–
P4	0.19	0.12	–	–	–	0.27	**0.81**	–	0.21	–
Hg ETTC	0.26	0.36	0.51	0.27	0.39	0.40	0.48	0.35	0.50	0.60
Selenium										
P1	4.7	2.7	3.7	8.3	2.2	13.5	3.1	3.7	2.6	3.8
P2	9.3	5.4	4.8	10.8	**4.3**	**30.2**	–	3.0	5.0	–
P3	9.7	5.3	6.1	11.4	–	18.0	–	–	**7.9**	–
P4	7.7	6.2	–	–	–	**27.9**	**12.9**	–	**10.2**	–
Se ETTC	11.98	9.92	6.82	15.53	3.86	18.47	7.52	5.32	6.85	4.66

Table S8. Cauxa River risk assessment based on the mean ratios of the tissue residues in biomonitor taxa to metal thresholds (EQR-ER_{GM} & EPT-ER_{50}) classified in 5 different feeding styles and the mean ratios of the community. The ratios are classified within the following classes: Low Risk (≤1) in blue, Moderate Risk (1.1–2.0) in yellow, High Risk (2.1–10.0) in orange and Very High Risk (>10) in red.

Cauxa River- Taxa tissue residue to ER ratios											
Threshold concentration	Metal	Feeding Style	Sites				Threshold concentration	Sites			
			P1	P2	P3	P4		P1	P2	P3	P4
METI-EQR ER_{GM}	As	Scraper	0.1	3.0	1.4	0.4	NORTI-EQR ER_{GM}	0.3	10.5	4.9	0.9
		Filterer	0.0	-	-	-		0.3	1.5	3.3	-
		Generalist	0.0	2.0	0.8	0.3		0.0	3.8	1.6	0.5
		Deposit-feeder	0.2	8.1	7.7	6.4		0.1	1.8	1.7	1.7
		Predator	0.0	4.9	0.7	1.0		0.1	22.5	4.7	6.6
		Mean	**0.1**	**4.2**	**2.4**	**2.9**		**0.2**	**9.3**	**3.5**	**2.3**
	Cu	Scraper	0.4	1.3	0.9	0.6		1.1	3.5	2.4	1.7
		Filterer	-	-	-	-		1.1	4.9	11.3	-
		Generalist	0.7	1.7	0.9	1.5		0.9	2.4	1.3	2.2
		Deposit-feeder	0.4	1.7	1.5	1.7		0.6	3.1	2.6	2.2
		Predator	0.5	1.1	0.7	1.0		0.5	1.9	0.9	1.3
		Mean	**0.5**	**1.4**	**1.0**	**1.3**		**0.9**	**3.5**	**3.5**	**1.9**
	Hg	Scraper	0.0	0.0	0.0	0.1		-	-	-	-
		Filterer	0.0	-	-	-		0.2	-	-	-
		Generalist	-	-	-	-		-	-	-	-
		Deposit-feeder	-	-	-	-		-	-	-	-
		Predator	0.2	0.2	0.2	0.4		0.3	0.3	0.4	0.6
		Mean	**0.1**	**0.1**	**0.1**	**0.2**		**0.3**	**0.3**	**0.4**	**0.6**
	Se	Scraper	0.2	0.5	0.5	0.4		0.5	1.1	1.1	0.9
		Filterer	-	-	-	-		-	-	-	-
		Generalist	-	-	-	-		-	-	-	-

		Deposit-feeder	0.2	-	-	0.8		0.5	-	-	2.2
		Predator	-	-	-	-		-	-	-	-
		Mean	**0.2**	**0.5**	**0.5**	**0.6**		**0.5**	**1.1**	**1.1**	**1.5**
EPT Fam-ER$_{50}$	**As**	Scraper	0.3	10.4	4.8	0.7	**EPT Ab-ER$_{50}$**	0.2	9.9	4.6	1.5
		Filterer	0.0	2.1	7.1	-		1.5	-	-	-
		Generalist	0.0	1.5	0.6	0.2		0.2	16.1	6.8	2.2
		Deposit-feeder	0.2	9.2	8.8	7.2		0.1	-	-	0.7
		Predator	0.0	4.3	0.6	0.9		0.1	23.2	3.5	4.9
		Mean	**0.1**	**5.7**	**4.5**	**3.3**		**0.4**	**14.8**	**4.9**	**2.3**
	Cu	Scraper	-	-	-	-		-	-	-	-
		Filterer	1.0	3.7	6.8	-		-	-	-	-
		Generalist	0.5	1.4	0.8	1.3		0.7	1.7	0.9	1.6
		Deposit-feeder	-	-	-	-		0.5	4.3	3.7	1.6
		Predator	0.4	0.4	-	-		0.7	1.4	0.9	1.2
		Mean	**0.7**	**2.3**	**3.8**	**1.3**		**0.6**	**2.2**	**1.8**	**1.5**
	Hg	Scraper	-	-	-	-		-	-	-	-
		Filterer	0.6	0.7	1.6	-		0.2	0.2	0.4	-
		Generalist	1.3	2.0	1.7	2.0		-	-	-	-
		Deposit-feeder	-	-	-	-		0.2	-	-	1.1
		Predator	-	-	-	-		2.0	1.7	-	-
		Mean	**1.0**	**1.4**	**1.6**	**2.0**		**0.7**	**0.9**	**0.4**	**1.1**
	Se	Scraper	0.2	0.5	0.5	0.4		1.3	1.9	2.0	1.4
		Filterer	1.0	2.0	-	-		0.5	1.0	-	-
		Generalist	-	-	-	-		-	-	-	-
		Deposit-feeder	1.0	3.0	1.8	2.9		0.6	2.0	1.2	1.7
		Predator	-	-	-	-		2.4	4.5	7.2	9.3
		Mean	**0.8**	**1.8**	**1.1**	**2.0**		**1.1**	**2.3**	**3.1**	**3.5**

Chapter 3



Influence of the definition of ecological status boundaries on metal biota quality standards: A case study in northern Spain

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Abstract

The effective tissue residues (ER) for of As, Cu, Hg and Se are calculated in a tissue residue approach using newly proposed ecological quality ratio (EQR) boundaries to protect the macroinvertebrate assemblages from the biodiversity loss in rivers of the northern Spain. Hazard concentrations (HC) of As, Cu and Hg chemicals were calculated from Species Sensitivity Distribution models, using the taxa ER as predictors. Tissue HC_5 and HC_{50} were interpreted as low- and high-threshold tissue concentrations, respectively, above which there is a probability of alteration of the macroinvertebrate assemblages from Good to Moderate Ecological Status. Differences between previous and newly proposed thresholds are evaluated.

Keywords: arsenic, copper, mercury, effective tissue residue, hazard concentration.

1. INTRODUCTION

Aquatic invertebrates can accumulate metals and metalloids (hereinafter, metals) when exposed to metal-contaminated or naturally metal-rich environments. This accumulation is the result of an uptake of metal ions in solution and from a variety of metal bioavailable fractions associated to the diet (detritus, organic and mineral particles, algae, or other animals). Toxic effects are likely if the rate of uptake is higher than the rates of excretion and detoxification (Beyer & Meador, 2011) and it is related to a threshold concentration of metabolically available metal fraction (Rainbow, 2002). In areas affected by mining activities, toxic effects due to metal bioaccumulation in bioassay organisms (Arambourou *et al*., 2020; Griffith *et al*., 2004; Méndez-Fernández *et al.*, 2015), or in the field community have been widely demonstrated (De Jonge *et al*., 2013; Luoma *et al*., 2010; Moreno-Ocio *et al*., 2022: Chapter II). Above a threshold level, trace metals are toxic, initially causing sublethal effects, but ultimately mortality at a higher exposure concentration or over a longer exposure period. These thresholds can vary between different taxa for the same metal, in part due to differences in the diet, but also due to different sensitivity to toxicants related to specific detoxification and elimination physiological processes (Rainbow, 2002, 2007). In the same way, thresholds of different metals for a particular taxon can vary depending, for instance, on the essentiality of the metal (Adams *et al*., 2011; Méndez-Fernández *et al*., 2013), the environmental conditions (e.g. dissolved oxygen or acid volatile sulfide concentrations, De Jonge *et al*., 2011, 2012) and genetic adaptations of field populations (Levinton *et al*., 2003). However, a new approach allowed for estimating effective tissue residues (ER) of metals relating metal bioaccumulation in selected biomonitors to the alteration of field macroinvertebrate metrics (Luoma *et al*., 2010). Specifically, the metal bioaccumulation in biomonitors was calibrated against the alteration of the abundance of sensitive taxa or of the metrics measuring the integrity of the macroinvertebrate assemblages (Bervoets *et al*., 2016; De Jonge *et al*., 2013; Moreno-Ocio *et al*., 2022: Chapter II; Rainbow *et al*., 2012). Those estimated thresholds open a new way to estimate Biota Quality Standards (BQS) as demanded by the Water Framework Directive (EC, 2013) in order to protect aquatic communities from long-term pollution.

In a previous publication, we calculated the ERs of As, Cu, Hg and Se for 10 taxa, using non-linear regression models of the metal tissue residues against the ecological quality ratio (EQR), which assesses the degree of alteration of the macroinvertebrate assemblages at a specific site and which determines the ecological status in water bodies according to the Water Framework Directive (EC, 2000) (Moreno-Ocio *et al*., 2022: Chapter II). The biota ER values were interpreted as taxon threshold concentrations above which there was a high probability of alteration of the macroinvertebrate assemblage ecological status. In Northern Spain two metrics are used to calculate the EQR for water quality assessment in rivers: the multimetric METI and the NORTI model (see below, in 2.2). In the present study, we recalculated the ERs for of As, Cu, Hg and Se, considering the new EQR boundary proposals by Pardo *et al.* (2020), since they were shown to provide a more robust interpretation of the biodiversity loss in rivers of the northern Spain, compared with previous boundaries. We also evaluated whether the use of new boundaries could have any relevant implication in the metal ERs calculated for the biota.

Thus, the general objective of this study is to contribute to the development of Biota Quality Standards (EC, 2013) suitable to protect the aquatic life, and specifically to be applied to the rivers in northern Spain, a region with a rich metal mining history. For that purpose, the level of protection for the aquatic biota was defined as the achievement or conservation of a Good ecological status for the macroinvertebrate assemblages. Consequently, the proposal of BQS for each chemical was based on the ERs calculated for a selection of taxa as the threshold concentrations related to a defined boundary between the Good and the Moderate ecological status of the biota. If so, our aim in present study was to evaluate to what extent the change in these boundaries could affect the BQSs based on a tissue residue approach.

2. MATERIAL AND METHODS

2.1. Study area, biomonitor taxa and metal analysis

This research is focused on an area from northern Spain affected by an intense historical and active mining activity, the Nalón River basin (Asturias). Data on

geographic coordinates of 29 study sites, water physico-chemistry, and metal bioaccumulation in ten selected biomonitors have been already published in previous contributions (Costas *et al*., 2018; Moreno-Ocio *et al*., 2022: Chapter II). The macroinvertebrate sampling and analysis of As, Cu, Hg and Se, as well as the ecological status assessment of the sites using the official EQR boundaries can be found in Rodriguez *et al*. (2018) and Costas *et al*. (2018), respectively. The macroinvertebrate taxa selected as biomonitors for the assessment of the metal bioaccumulation were: Baetidae, Ephemerellidae, Ephemeridae, Heptageniidae, Hydropsychidae, Lumbricidae, Microdrile oligochaetes, Perlidae, Rhyacophilidae and Simuliidae. In most sites, three field replicates per taxon were collected and analyzed separately, and for each taxon tissue residues were calculated as the mean of the field replicates in each site. In few occasions, the tissue residues were calculated on a single sample, with no replicates, due to the scarcity of individuals or low biomass. All taxa were frozen as whole organisms after emptying the gut, freeze-dried, weighed and stored at -20°C until acid digestion was conducted at room temperature (70% Nitric Acid Baker Instra-Analyzed + 30% H_2O_2 Merk Suprapur). For more details in sampling methods and As, Cu, Hg and Se analysis see the aforementioned publications and a summary can be found in Moreno-Ocio *et al.* (2022) (Chapter II).

2.2. Ecological status assessment using macroinverterbrate metrics

The alteration in the ecological status of the macroinvertebrate assemblages was assessed according to two indexes: the METI (índice Multimétrico Específico del Tipo de Invertebrados bentónicos en ríos, from the Spanish spelling), a Spanish river-type specific multimetric index (MAGRAMA, 2015), and the NORTI (NORThern Spain Indicators) predictive model (Pardo *et al*., 2014) that it is also used in the study area. The ecological quality ratios (EQRs) were calculated for each index as a quotient between the observed and reference values (EC, 2000). The official boundary between Good and Moderate Ecological Status classes is 0.700 (MAGRAMA, 2015), and was intercalibrated by the Central/Baltic group for benthic macroinvertebrate fauna (Bennett *et al*., 2011; EC, 2013). The new EQR boundaries proposed by Pardo *et al*. (2020) were also used in present study for ER estimations, since they provide a more

sound interpretation of the biodiversity loss in rivers of the region, namely 0.818 for METI and 0.760 for NORTI.

2.3. Statistical analyses

The estimations of the effective tissue residues (ER) were calculated for As, Cu, Hg and Se and each of the 10 selected biomonitor taxon, from non-linear regression models of the tissue residues (X) and the metric-EQR values (Y) at each sampling site, using the drc extension package in R software (Ritz & Streibig, 2005). The best-fitted models were selected using Akakike's Information Criteria (AIC: Burnham & Anderson, 2002) from a set of 6 log-logistic and Weibull models of 3 and 4 parameters (LL.3, LL.4, W1.3, W1.4, W2.3 and W2.4). The ERs were calculated as the inverse function of the selected regression equations, using Wolfram Mathematica 12 software, from an EQR change-point (Y) corresponding to the boundary between Good and Moderate Ecological Status classes. The ERs derived from the new boundaries proposed by Pardo *et al*. (2020) are named ER_{NB} (NB: New Boundary). By other side, Rodriguez *et al*. (2018) proposed the ecological threshold tissue concentration (ETTC) of the metals for 10 taxa, a baseline concentration below which adverse effects on the macroinvertebrate community are unlikely, since they are derived from tissue residues measured in taxa from reference, unpolluted sites (selected following the WFD: EC, 2000 and Pardo *et al*., 2012). The ER_{NB} was evaluated for each metal and taxa using the ratio ER_{NB}/ETTC, and compared with the ERs previously calculated using the official threshold of 0.700 (see Chapter II) to assess the possible influence in the change of boundaries. Differences in the mean values of taxa ER_{NB}/ETTC ratios where estimated for each chemical using the Wilcoxon signed-rank test (significance level, 0.05), with IBM-SPSS® v.25 software.

Finally, for each chemical, the ER_{NB} were used as predictors, to assess the Hazard Concentrations (HC) for the macroinvertebrate assemblages, that is, the metal tissue concentration at which there is a probability of alteration of the macroinvertebrate assemblages from Good to Moderate Ecological Status, using the Species Sensitivity Distribution (SSD) models (ETX v.2.1 program, Van Vlaardingen *et al*., 2004). The values estimated as HC_5 and HC_{50} were interpreted as community low- and high-threshold tissue concentrations, respectively; i.e. HC_5 is interpreted as the

concentration below which there is a low probability of ecological status alteration, and HC_{50} as the concentration above which there is high probability of alteration of the macroinvertebrate Ecological Status. The ER-HC_{50} for the macroinvertebrate assemblages were compared with the baseline ETTC-HC_{50}, which represents the median background tissue level for a pool of macroinvertebrate taxa representative of several feeding styles in reference condition (Rodriguez *et al*., 2018).

3. RESULTS

3.1. Effective tissue residues related to new boundaries (ER_{NB})

The ER_{NB} values for each taxon and metal estimated from several best-fitted regression models (see section 2.4) were averaged and in most cases, they were very similar, except in Hydropsychidae for As (Supplementary Table S1). A total of 23 non-linear models taxon-specific ER_{NB} were estimated from METI-EQR vs tissue residues (hereafter METI-ER_{NB}) and 28 non-linear models from NORTI-EQR vs tissue residues (NORTI-ER_{NB}). The METI-ER_{NB} for both As and Cu were calculated in 8 taxa, for Hg in 4 taxa, and in the case of Se, only in 3 taxa. The As NORTI-ER_{NB} was calculated in all the 10 taxa, in the case of Cu in 9 taxa, and for Hg and Se in 5 and 4 taxa, respectively. Baetidae and Microdrile oligochaetes were the only taxa that provided METI-ER_{NB} for the four metals. All the taxa METI-ER_{NB} values were higher than their respective ETTC (ratios >1), except for Hydropsychidae for Se (ratio = 0.8) (Table 1). The mean ratios of METI-ER_{NB} to ETTC calculated for each metal where generally higher for most toxic chemicals (9.1 for As, 4.3 for Hg) than for the essentials (2.6 for Cu, 1.0 for Se). These ratios were significantly higher for METI than for NORTI in the case of As (n=8) and Cu (n=7) (Wilcoxon signed-rank test, p = 0.050 and 0.018, respectively), but no differences existed for Hg (n=2) and Se (n=3), although the number of data in these cases was small.

In the case of NORTI-ER_{NB}, Heptageniidae was the only taxon that allowed for estimating ER_{NB} values for the four metals. NORTI-ER_{NB} values were lower than the METI-ER_{NB}, with only one exception (As-ER_{NB} in Lumbricidae) (Table 1). The NORTI-ER_{NB} values calculated for each taxon were usually higher than their respective ETTC for As

and Cu (except for Cu in Simuliidae); on the contrary, the NORTI-ER_{NB} values calculated for Hg and Se were lower than their respective ETTC values. The mean ratios for As (3.2) were higher than for the other metals (1.3 for Cu, 0.6 for Hg and 0.5 for Se).

Table 1. Mean effective tissue residues (ERs) (µg g^{-1} dw) for As, Cu, Hg and Se. Their ranges are given when the number of accepted models was >1. EQR-ER_{NB} was estimated for the new boundaries of the EQR values between the Good and Moderate Ecological Status of the macroinvertebrate assemblages. ETTC, Ecological Threshold Tissue Concentration; TR, Tissue Residues.

		Mean METI-EQR vs TR models		Mean NORTI-EQR vs TR models	
Taxon	Feeding Style	EQR- ER_{NB} (Range)	Mean ratio ER_{NB}/ETTC	EQR- ER_{NB} (Range)	Mean ratio ER_{NB}/ETTC
As					
Baetidae	Scraper	27.66 (20.92-30.21)	9	10.31 (9.08-12.35)	3
Heptageniidae	Scraper	33.63 (30.96-35.41)	5	7.77 (7.58-7.95)	1
Ephemerellidae	Generalist	42.36 (39.05-45.66)	7	20.48	3
Ephemeridae	Filterer	-	-	31.03	5
Hydropsychidae	Filterer	16.63 (2.75-30.51)	8	11.19 (5.06-17.31)	6
Simuliidae	Filterer	131.55 (115.66-147.43)	29	7.95	2
Lumbricidae	Deposit-feeder	17.46	1	69.40 (64.15-74.64)	5
Microdrile	Deposit-feeder	103.81 (95.81-111.81)	7	60.17 (46.78-67.25)	4
Perlidae	Predator	-	-	0.86	1
Rhyacophilidae	Predator	5.07 (4.85-5.24)	6	1.32	2
Cu					
Baetidae	Scraper	58.13 (40.01-76.24)	2	33.09 (32.98-33.15)	1
Heptageniidae	Scraper	287.11 (231.13-318.64)	4	110.77 (108.37-113.16)	1
Ephemerellidae	Generalist	167.62 (164.64-171.64)	2	102.30	1
Ephemeridae	Filterer	47.43 (43.90-52.61)	3	19.77 (16.29-21.93)	1
Hydropsychidae	Filterer	-	-	16.22 (15.95-16.48)	1
Simuliidae	Filterer	-	-	20.14 (20.12-20.16)	0.3
Lumbricidae	Deposit-feeder	51.23 (47.89-53.40)	5	29.90	3
Microdrile	Deposit-feeder	36.06 (35.73-36.38)	2	28.87 (28.07-29.67)	1
Perlidae	Predator	69.14 (68.61-69.66)	2	-	-
Rhyacophilidae	Predator	41.01 (39.92-41.74)	2	33.05 (28.80-35.68)	2
Hg					
Baetidae	Scraper	1.18	5	-	-
Heptageniidae	Scraper	-	-	0.15 (0.14-0.16)	0.6
Ephemeridae	Filterer	-	-	0.22 (0.17-0.31)	0.4
Simuliidae	Filterer	2.69 (2.05-3.32)	5	0.59	1
Microdrile	Deposit-feeder	3.46	7	-	-
Perlidae	Predator	-	-	0.12 (0.11-0.12)	0.3
Rhyacophilidae	Predator	0.58 (0.58-0.59)	1	0.26 (0.25-0.26)	0.5
Se					
Baetidae	Scraper	12.01 (11.95-12.13)	1	6.31 (6.24-6.37)	0.5
Heptageniidae	Scraper	-	-	6.46 (6.29-6.69)	0.4
Hydropsychidae	Filterer	3.08 (3.05-3.10)	0.8	1.23 (0.26-1.76)	0.3
Microdrile	Deposit-feeder	9.62 (7.87-11.45)	1	5.73 (5.71-5.74)	0.8

In general, the differences in ER_{NB} values between taxa within the same feeding style class were lower when estimated with the NORTI-EQR than with the METI-EQR (Table 1), providing more consistent values for each feeding style class. The predators showed the lowest ER_{NB} values for As and Hg, while the highest values were found for the deposit-feeders and Simuliidae. The filterers showed the lowest ER_{NB} values for Cu and Se, while the scrapers provided the highest values (Table 1).

The effective tissue residues estimated for the new Good/Moderate EQR boundaries (ER_{NB}) were generally below the ones estimated for the official boundary of 0.700 (ER_{GM}) (Moreno-Ocio *et al*., 2022: Chapter II), with only one exception for the Se NORTI-ER_{NB} in Hydropsychidae. This result was expected, on the one hand, because the dose-response models to assess the integrity of the macroinvertebrate assemblages show a reduction in the metal tissue residues when EQR values decrease. Therefore, for the same dataset, higher values of the cut-off value will be associated with lower tissue concentrations. The mean ratios of METI ER_{GM} values were 1.6 (1.1–2.3) times higher than the ER_{NB} for As, 1.4 (1.0–1.8) times for Cu, 2.3 (1.0–3.0) times for Hg and 1.6 (1.5–1.7) times for Se. In the case of NORTI, the mean ratios were 1.7 (1.2–2.3) times higher for As, 1.3 (1.1–1.8) for Cu, 1.5 (1.3–1.6) for Hg and 1.0 (0.5–1.4) for Se. Thus, the differences between both boundaries and metrics did not exceed 3 times, and were usually less than 2 times.

3.2. Hazard Concentrations (HC_5 and HC_{50}) for macroinvertebrate assemblages

The Figure 1 illustrates the species sensitivity distribution (SSD) models for As, Cu and Hg based on the effective tissue residues associated to a reduction of the ecological status from Good to Moderate using the new boundary (ER_{NB}). The models showed the relative sensitivity of the biomonitors related to the alteration boundary of the Good ecological status for the field communities. Predators (Rhyacophilidae and Perlidae) were the most sensitive taxa for As effects on ecological status, while the most tolerant taxa were the deposit-feeder oligochaetes and Simuliidae. In the case of Cu, the three filterers were the most sensitive taxa and Heptageniidae and Ephemerellidae the most tolerant. In the SSD model for Hg, Perlidae was the most sensitive while Microdrile oligochaetes and Simuliidae were the most tolerant taxa.

The low and high Hazard Concentrations, HC_5 and HC_{50}, were estimated for As, Cu and Hg through SSD models, with their 90% confidence intervals (Table 2). Selenium could not be modelized, due to limitation of data. HC values were compared with each other and with the baseline concentration (ETTC) specific for each metal and taxon in the Nalón River basin. Thus, the As-HC_5 was 10 times lower than the HC_{50}, 8 times lower in the case of Hg, but only 4 times lower for Cu. Compared with the baseline ETTC, the 90% confidence interval of the As-HC_5 overlaps with the lower range of the As-ETTC distribution values, while the As-HC_{50} is only slightly higher than the maximum ETTC value (Table 2). The Cu-HC_5 lies within the ETTC range of values and its confidence interval overlaps the lower range of the baseline ETTC distribution values. Likewise, the Cu-HC_{50} confidence interval falls within the range of the Cu ETTC. In the case of Hg, the HC_5 confidence interval is below the Hg-ETTC range of values and do not overlap, while the Hg-HC_{50} confidence interval encompasses the range of values of the baseline Hg ETTC.

Table 2. Hazard concentrations for As, Cu and Hg (HC_5, HC_{50}) and their 90% confidence limits (CL) derived from SSD models using several taxa effective tissue concentrations (ER_{NB}). ER_{NB}: Effective tissue residues related to the EQR new boundary between Good and Moderate Ecological Status of the macroinvertebrate community. For comparison, the ETTC-HC_{50}, and the ETTC range of values for each chemical is given (Rodriguez *et al*., 2018). n: number of data included in the models.

Metal	HC	ER_{NB} (90% CL)	n
As	**HC_5**	1.76 (0.61–3.58)	18
	HC_{50}	17.18 (9.83–30.02)	
	ETTC-HC_{50}	4.24 (2.46–7.30)	10
	ETTC range	0.75-14.44	
Cu	**HC_5**	13.11 (6.96–19.93)	17
	HC_{50}	48.66 (34.94–67.76)	
	ETTC-HC_{50}	29.16 (19.23–44.21)	10
	ETTC range	11.51-90.91	
Hg	**HC_5**	0.07 (0.01–0.16)	9
	HC_{50}	0.54 (0.25–1.15)	
	ETTC-HC_{50}	0.40 (0.34–0.47)	10
	ETTC range	0.26-0.60	

The comparison of the HC_5 with the baseline ETTC-HC_{50} showed that, in the case of As and Cu, the confidence intervals of the former overlapped with lower range

of values of the later, although only slightly. The Hg ER_{NB}-HC_5 and ETTC-HC_{50} confidence intervals did not overlap. The As-HC_{50} (and 90% CL) estimated from the ER_{NB} is well separated from the baseline ETTC-HC_{50} (Figure 1), which is an interesting issue if this parameter is used as community threshold or As tissue residues. On the other hand, there is a partial overlapping in the case of Cu-HC_{50} confidence intervals with the baseline ETTC-HC_{50}, and a complete overlapping for the Hg (Figure 1).

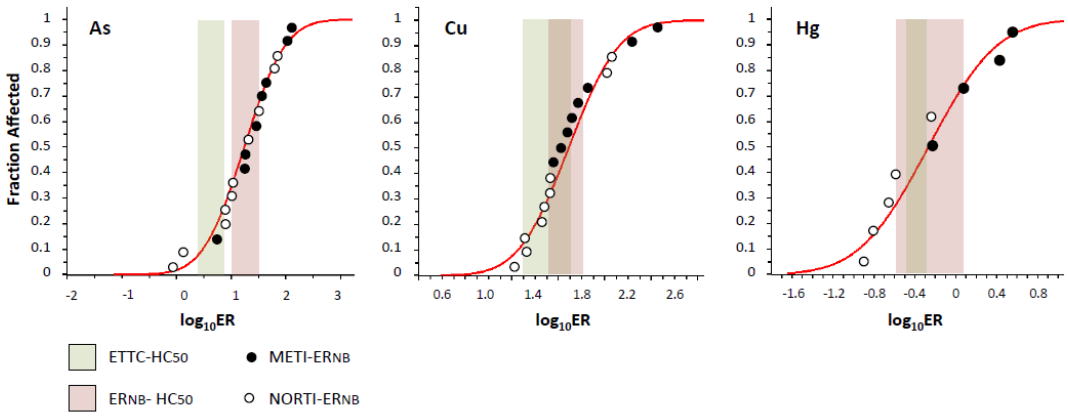

Figure 1. Species sensitivity distribution (SSD) models for As, Cu and Hg built with ER_{NB} data for each taxon derived from both METI- (black circles) and NORTI-EQR values (white circles) proposed by Pardo *et al*. (2020). The ranges of ER_{NB}-HC_{50} are represented in red and the ETTC-HC_{50} ranges are represented in green.

4. DISCUSSION

Areas with historical mining activities have diffuse input sources of metals to the river system through leachates and rainwater infiltration through surface tailings and mine landfills. The management and remediation programs for the river basins affected by mining activities need to consider not only the bulk concentration of metals in the sediments, but also the bioavailable fraction to the biota, as a potential cause of field toxicity. The environmental regulations in Europe developed from the Water Framework Directive (EC, 2000) required preserving or achieving of the Good Ecological Status for the aquatic communities in 2015, now postponed until 2027 (Vermeulen *et al*., 2019). There are multiple metrics used for the assessment of the ecological status in Europe, and class boundaries have been intercalibrated within member states (Bennett *et al*., 2011). However, in the next years we may see a

revision of such boundaries for a better protection of the aquatic communities. This study is the first known by the authors that evaluates the effect of changes in the Good/Moderate class boundary in the tissue concentration thresholds in macroinvertebrate assemblages in mining areas.

Relatively predictable changes in macroinvertebrate community structure as a result of pollution (e.g., decreased abundance and biodiversity, elimination of sensitive taxa) have led to the development of a number of biotic indices, but their performance success is not always satisfactory in mine water polluted sites, due to complicated interactions of the mine pollutants with water quality parameters, and natural tolerances and sensitivities of organisms (Byrne *et al*., 2012). Metal tissue thresholds or critical body concentrations have been proposed for the protection of aquatic communities and the wildlife (Table 3). The differences in the tissue residue thresholds reported in the literature for specific taxa are due, among other reasons, to the selection of the biomonitor taxa, their various feeding styles and habits, size, age or developmental stages (Eisler 2000; Mason *et al*., 2000; Rodriguez *et al*., 2018). However, the thresholds calculated for the community assemblages are not very different (Table 3), considering that different authors used different effect variables (taxa richness, abundance of a particular sensitive taxon, or a community metric) to assess a quantifiable effect and the differences in the environmental conditions (pH, oxygen, other metals, organic content) of the exposure (Penttinen *et al*., 2011).

The effective tissue residues estimated for the new EQR boundaries proposed by Pardo *et al*. (2020) (ER_{NB}) were about half the ERs estimated for the official boundary (ER_{GM}) (Moreno-Ocio *et al.*, 2022: Chapter II). It does not mean a big difference in the thresholds but the new boundaries are more protective for the macroinvertebrates assemblages. An additional advantage of the new boundaries is that they enable to estimate the Hg hazard concentrations (HC) for the community, in comparison with the official one (Moreno-Ocio *et al*., 2022: Chapter II), providing more information for the classification of metal bioaccumulation in an environmental risk assessment in mining areas.

Table 3. Threshold values for As, Cu, Hg and Se (μg g^{-1} dw) reported in the literature, in relation to the protection of the freshwater biota or of the wildlife. Abbreviations: HC: Hazard Concentration; CL, Confidence Limit; CBR, Critical Body Burden; CTL, Critical Tissue Level; LR, Lethal Body Residue; ER: Effective Body Residue; SE, standard error of the mean. Notes>: (1) transformed from ww to dw using the conversion factor 0.2 (Meador, 2011).

Community Thresholds	**As**	**Cu**	**Hg**	**Se**	**Source**
Community level HC_5 based on new boundaries	1.76	13.11	0.07	–	Present study
Community level HC_{50} based on new boundaries	17.18	48.66	0.54	–	Present study
Community level HC_5 based on official boundary	2.41	15.45	–	–	Moreno-Ocio *et al.*, 2022
Community level HC_{50} based on official boundary	27.98	64.62	–	–	Moreno-Ocio *et al.*, 2022
Macroinvertebrate community	6.5–25(1)	–	–	–	Eisler, 2000
Biota (Fish)	–	–	0.1(1)		EC, 2013
Sublethal effects fish	–	–	–	1.0–30	DeBruyn & Chapman, 2007
Dietary threshold for fish	–	–	–	3.0–11	May *et al.*, 2008
Wildlife	–	–	0.12–1.68	–	CCME, 2000
CTL Wildlife	33(1)	–	0.44(1)	0.12(1)	DEQ, 2007

The HC_5 values in present study were interpreted as low-thresholds for the macroinvertebrate assemblages, i.e. a tissue residue below which there is a low probability of loss of good ecological status for the macroinvertebrate community. The baseline ETTC-HC_{50} values calculated from reference sites (Rodriguez *et al.*, 2018), that proved to be useful as low threshold values in a risk assessment process (Rodriguez *et al*., 2021: Chapter I), were only slightly higher than the respective HC_5, with little overlapping of the confidence limits (Table 2). This is not necessarily something that could override the use of one or the other approaches. Recently, Lu *et al*. (2019) disagreed on the use of baseline concentrations, such as the ETTC, calculated from reference, unpolluted sites with good ecological status. These authors argued that ETTC values being higher than an estimated 5^{th} percentile derived statistically from logistic models is not the best approach for threshold proposals for wide areas. We have not contrasted these parameters in several regions, although in areas potentially affected by mining, and with metal-rich rocks, high metal concentrations exert a selective pressure in favor of best adapted populations, resulting in higher levels of bioaccumulation and tolerance to metals (Klerks & Levinton, 1989; Vidal & Horne, 2003). In this scenario, environmental risk assessment using a tissue residue approach should consider the baseline concentrations in the biota, until another appropriate threshold value can be used (Schneider, 2014). It is hard to prove that there is an environmental risk due to bioaccumulation when tissue residues in a study are lower or in the same level than those found in unpolluted sites of the study area. However, very toxic chemicals (e.g., Hg) may show overlapping between effect and baseline concentrations and a very narrow range from no-effect to effective concentrations. In these cases, the estimated HC_5 can be useful to define a theoretical, acceptable level. By other side, mining areas subject to remediation actions will progressively reduce the field non-point sources of pollution (e.g., atmospheric and from soil lixiviates) and the baseline tissue concentrations are also expected to decrease in several generations (Levinton *et al.*, 2003), and possibly become closer to the HC_5 values.

The SSD models might provide also some interesting clues in order to select the best biomonitors for specific chemicals. In fact, those taxa whose ER_{NB} falls between the HC_{50} confidence limits of the species sensitivity distribution, can reduce false

negatives derived from using less sensitive taxa as well as false positives from using the most sensitive taxa (Figure 1). Following this criterion, Baetidae and Hydropsychidae can be selected as suitable biomonitors for As, for both METI- and NORTI-ER_{NB}. In the case of Cu, there are five taxa (Baetidae, Ephemerellidae, Microdrile oligochaetes, Lumbricidae and Rhyacophilidae) with METI-ER_{NB} falling within the HC_{50} confidence interval; this group of taxa has the additional interest of including representatives of four different feeding styles; on the other hand, none of the study taxa was selected based on their NORTI-ER_{NB}. Interestingly, the predator Rhyacophilidae was selected as the most suitable biomonitor for Hg, for both METI- and NORTI-ER_{NB}.

5. CONCLUSIONS

There is not a significant influence of the definition of Ecological Status boundaries on metal effective tissue residues for the selected biomonitor taxa or the macroinvertebrate assemblages. The ERs estimated for the new boundaries (ER_{NB}) were about twice lower than the ones estimated for the official boundary. In an environmental risk assessment, the tissue residue thresholds calculated on the Good/Moderate boundary of the Ecological Quality of the macroinvertebrate fauna can provide information on cause-effect relationships based on metal bioavailability.

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SUPPLEMENTARY MATERIAL

Table S1. Parameters (b, c, d, e) of the nonlinear regression functions. Effective tissue residues for the EQR-new boundary between good and moderate ecological status (EQR-ER_{NB}). Models: W, Weibull; LL, log-logistic.

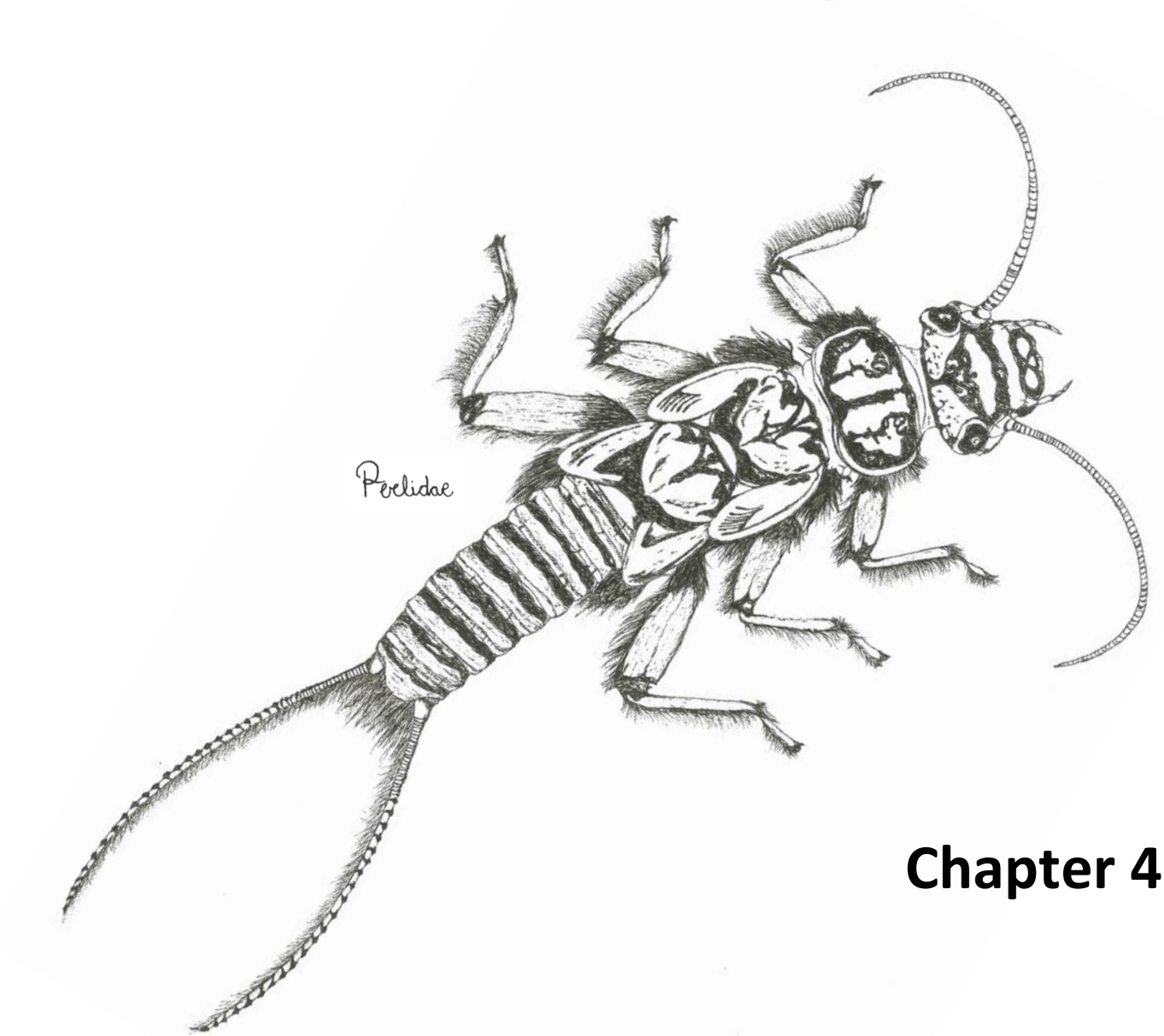
Arsenic							
Taxa	Metric	Model	b	c	d	e	ER_{NB}
Baetidae	METI	LL.4	3.908	0.518	1.026	32.817	29.9
Baetidae	METI	W1.4	2.633	0.520	1.027	38.444	30.2
Baetidae	METI	W2.3	-0.339	0.000	1.068	62.910	20.9
Baetidae	METI	W2.4	-3.048	0.521	1.026	28.468	29.6
Baetidae	NORTI	LL.3	0.669	0.000	0.905	123.080	10.3
Baetidae	NORTI	W1.3	0.453	0.000	0.953	239.811	9.1
Baetidae	NORTI	W1.4	0.828	0.253	0.880	80.202	12.4
Baetidae	NORTI	W2.3	-0.435	0.000	0.873	49.372	9.5
Ephemerellidae	METI	LL.3	1.456	0.000	1.032	114.608	45.7
Ephemerellidae	METI	W2.3	-0.615	0.000	1.029	82.532	39.1
Ephemerellidae	NORTI	LL.3	0.877	0.000	0.882	165.602	20.5
Ephemeridae	NORTI	W1.4	2.221	0.624	0.845	43.047	31.0
Heptageniidae	METI	LL.3	1.713	0.000	1.035	74.889	34.5
Heptageniidae	METI	W1.3	1.514	0.000	1.038	91.330	35.4
Heptageniidae	METI	W2.3	-0.764	0.000	1.030	56.383	31.0
Heptageniidae	NORTI	LL.4	21.705	0.630	0.875	7.624	7.6
Heptageniidae	NORTI	W1.4	8.739	0.628	0.878	8.204	7.8
Heptageniidae	NORTI	W2.4	-2.512	0.625	0.893	6.898	8.0
Hydropsychidae	METI	LL.4	22.435	0.716	1.031	29.528	30.5
Hydropsychidae	METI	W2.4	-22.811	0.763	1.038	2.577	2.8
Hydropsychidae	NORTI	W1.4	1.711	0.565	0.827	35.466	17.3
Hydropsychidae	NORTI	W2.3	-0.083	0.000	1.335	0.630	5.1
Lumbricidae	METI	W2.4	-19.051	0.664	1.042	16.871	17.5
Lumbricidae	NORTI	LL.4	5.483	0.548	0.856	86.245	74.6
Lumbricidae	NORTI	W1.4	2.298	0.549	0.857	97.940	64.2

Microdriles	METI	W1.3	0.478	0.000	1.127	1205.979	111.8
Microdriles	METI	W2.3	-0.354	0.000	1.066	278.604	95.8
Microdriles	NORTI	LL.3	1.032	0.000	0.844	570.022	67.3
Microdriles	NORTI	W1.3	0.877	0.000	0.848	832.611	66.5
Microdriles	NORTI	W2.3	-0.492	0.000	0.850	241.689	46.8
Perlidae	NORTI	LL.4	3.637	0.580	0.844	1.062	0.9
Rhyacophilidae	METI	LL.3	1.745	0.000	1.035	10.932	5.1
Rhyacophilidae	METI	W1.3	1.494	0.000	1.038	13.702	5.2
Rhyacophilidae	METI	W2.3	-0.934	0.000	1.029	7.933	4.9
Rhyacophilidae	NORTI	W2.3	-0.184	0.000	1.385	0.383	1.3
Simuliidae	METI	LL.3	0.760	0.000	1.009	1002.867	147.4
Simuliidae	METI	W1.4	1.670	0.622	0.999	149.276	115.7
Simuliidae	NORTI	W2.3	-0.155	0.000	1.338	2.583	8.0
Copper							
Taxa	**Metric**	**Model**	**b**	**c**	**d**	**e**	**ER_{NB}**
Baetidae	METI	W2.3	-1.025	0.000	1.039	116.764	76.2
Baetidae	METI	W2.4	-36.196	0.723	1.053	38.837	40.0
Baetidae	NORTI	LL.4	5.134	0.587	0.928	33.340	33.1
Baetidae	NORTI	W1.4	4.943	0.602	0.924	35.346	33.0
Baetidae	NORTI	W2.4	-2.961	0.572	0.918	30.529	33.2
Ephemerellidae	METI	LL.3	4.142	0.000	1.025	235.963	169.3
Ephemerellidae	METI	W1.3	3.488	0.000	1.027	262.386	171.6
Ephemerellidae	METI	W2.3	-2.239	0.000	1.023	203.799	164.9
Ephemerellidae	METI	W2.4	-2.338	0.062	1.023	198.249	164.5
Ephemerellidae	NORTI	W2.3	-0.774	0.000	0.903	225.649	102.3
Ephemeridae	METI	LL.4	3.587	0.706	1.063	36.775	45.8
Ephemeridae	METI	W1.4	2.934	0.709	1.067	41.365	43.9
Ephemeridae	METI	W2.4	-1.805	0.705	1.061	30.862	52.6
Ephemeridae	NORTI	LL.4	188.769	0.688	0.889	16.238	16.3
Ephemeridae	NORTI	W1.4	7.984	0.688	0.882	21.953	21.9

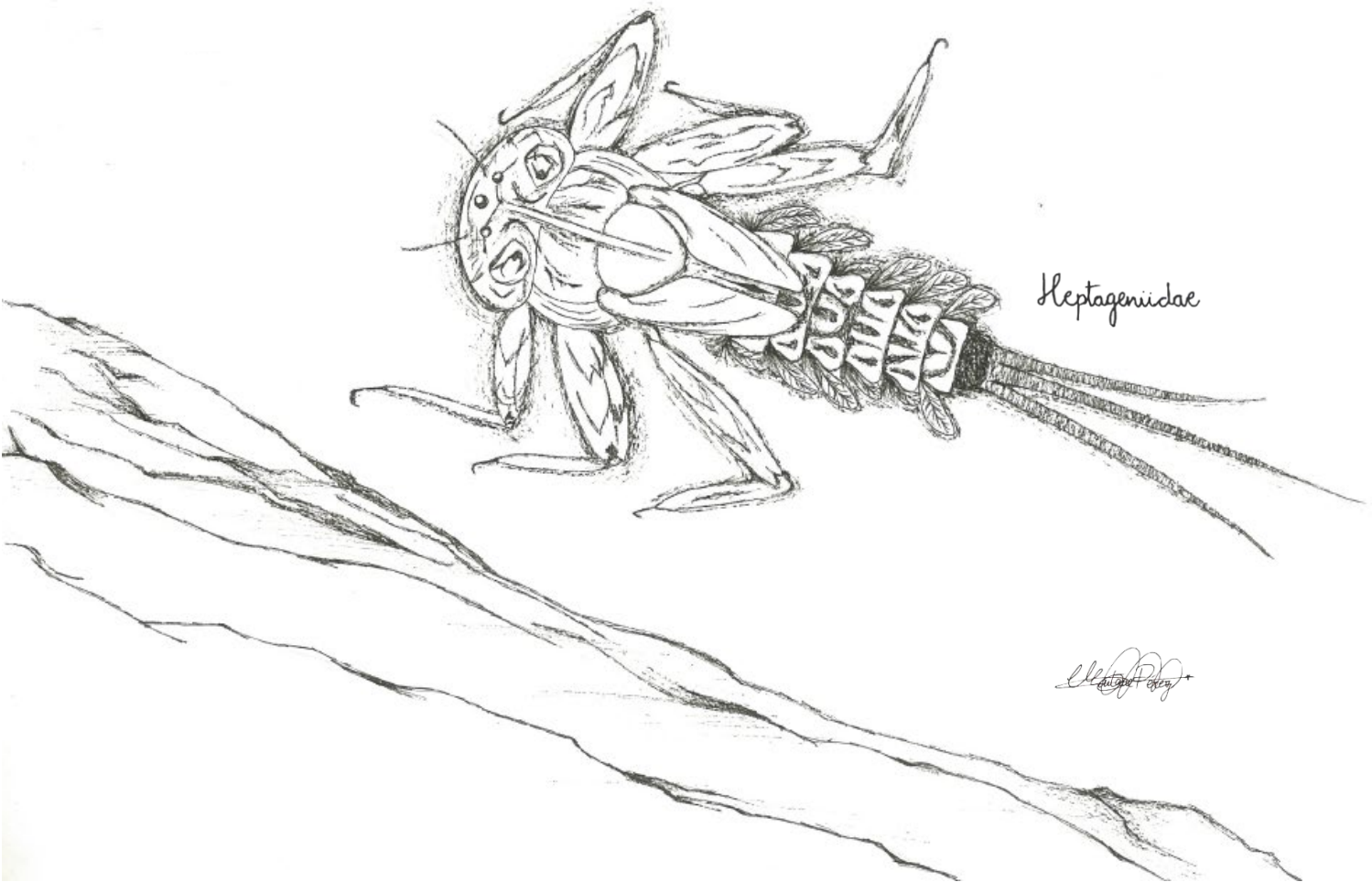
Ephemeridae	NORTI	W2.4	-5.097	0.672	0.882	18.722	21.1
Heptageniidae	METI	LL.3	2.292	0.000	1.040	558.892	316.4
Heptageniidae	METI	W1.3	2.032	0.000	1.041	641.735	318.6
Heptageniidae	METI	W1.4	7.647	0.751	1.034	252.503	264.7
Heptageniidae	METI	W2.3	-1.059	0.000	1.036	463.228	304.6
Heptageniidae	METI	W2.4	-5.676	0.743	1.034	186.742	231.1
Heptageniidae	NORTI	LL.4	2.959	0.644	0.908	99.620	108.4
Heptageniidae	NORTI	W1.4	1.765	0.648	0.928	118.837	113.2
Hydropsychidae	NORTI	LL.4	9.240	0.662	0.919	15.410	16.2
Hydropsychidae	NORTI	W1.4	8.437	0.663	0.912	16.594	16.5
Hydropsychidae	NORTI	W2.4	-5.971	0.664	0.998	13.315	16.0
Lumbricidae	METI	LL.3	1.254	0.000	1.046	145.127	52.4
Lumbricidae	METI	W1.3	0.957	0.000	1.065	215.260	53.4
Lumbricidae	METI	W2.3	-0.688	0.000	1.023	95.491	47.9
Lumbricidae	NORTI	W2.3	-0.590	0.000	0.857	111.989	29.9
Microdriles	METI	LL.4	56.913	0.629	1.009	36.372	36.4
Microdriles	METI	W1.4	13.870	0.630	1.010	36.653	35.7
Microdriles	NORTI	LL.4	17.926	0.538	0.825	30.067	28.1
Microdriles	NORTI	W1.4	9.644	0.530	0.824	34.305	29.7
Perlidae	METI	LL.3	0.995	0.000	1.360	105.342	69.7
Perlidae	METI	W2.3	-0.753	0.000	1.208	80.744	68.6
Rhyacophilidae	METI	LL.3	2.477	0.000	1.055	68.215	41.4
Rhyacophilidae	METI	W1.3	2.025	0.000	1.066	80.449	41.7
Rhyacophilidae	METI	W2.3	-1.534	0.000	1.027	55.527	41.0
Rhyacophilidae	METI	W2.4	-2.183	0.337	1.025	43.391	39.9
Rhyacophilidae	NORTI	LL.3	2.594	0.000	0.856	78.507	35.4
Rhyacophilidae	NORTI	W1.3	2.274	0.000	0.859	89.888	35.7
Rhyacophilidae	NORTI	W1.4	10.098	0.608	0.846	33.653	31.1
Rhyacophilidae	NORTI	W2.3	-1.319	0.000	0.846	64.347	34.3
Rhyacophilidae	NORTI	W2.4	-4.729	0.580	0.846	29.551	28.8

Simuliidae	NORTI	LL.4	6.346	0.698	1.080	15.525	20.1
Simuliidae	NORTI	W2.4	-5.312	0.698	1.029	15.011	20.2
Mercury							
Taxa	**Metric**	**Model**	**b**	**c**	**d**	**e**	**ER_{NB}**
Baetidae	METI	W1.3	0.326	0.000	1.173	27.110	1.2
Ephemeridae	NORTI	LL.4	3.888	0.730	1.080	0.096	0.2
Ephemeridae	NORTI	W1.4	2.425	0.731	1.073	0.117	0.2
Ephemeridae	NORTI	W2.4	-1.717	0.731	1.079	0.076	0.3
Heptageniidae	NORTI	LL.4	2.314	0.707	1.322	0.057	0.2
Heptageniidae	NORTI	W1.4	1.783	0.718	1.247	0.084	0.1
Heptageniidae	NORTI	W2.4	-1.868	0.703	1.267	0.050	0.2
Microdriles	METI	LL.4	0.261	0.311	1.337	3.155	3.5
Perlidae	NORTI	LL.4	8.210	0.753	1.233	0.070	0.1
Perlidae	NORTI	W1.4	2.959	0.752	1.272	0.071	0.1
Perlidae	NORTI	W2.4	-6.631	0.752	1.233	0.067	0.1
Rhyacophilidae	METI	LL.3	69.378	0.000	1.004	0.604	0.6
Rhyacophilidae	METI	W1.3	30.974	0.000	1.005	0.614	0.6
Rhyacophilidae	METI	W2.3	-21.082	0.000	1.004	0.598	0.6
Rhyacophilidae	NORTI	LL.4	3.364	0.647	0.959	0.214	0.3
Rhyacophilidae	NORTI	W2.4	-1.834	0.601	0.949	0.199	0.3
Simuliidae	METI	LL.3	0.686	0.000	1.041	22.174	3.3
Simuliidae	METI	W2.3	-0.186	0.000	1.274	2.366	2.1
Simuliidae	NORTI	LL.3	0.360	0.000	1.348	1.204	0.6
Simuliidae	NORTI	W2.3	-0.304	0.000	1.104	0.974	0.6
Selenium							
Taxa	**Metric**	**Model**	**b**	**c**	**d**	**e**	**ER_{NB}**
Baetidae	METI	LL.3	0.941	0.000	1.152	30.979	12.0
Baetidae	METI	W1.3	0.824	0.000	1.158	43.136	12.0
Baetidae	METI	W2.3	-0.475	0.000	1.133	20.365	12.1
Baetidae	NORTI	LL.3	0.671	0.000	1.170	15.626	6.2

Baetidae	NORTI	W1.3	0.563	0.000	1.170	28.409	6.4
Heptageniidae	NORTI	LL.4	4.596	0.739	1.111	3.478	6.4
Heptageniidae	NORTI	W1.4	2.029	0.739	1.179	3.870	6.7
Heptageniidae	NORTI	W2.4	-4.083	0.739	1.088	3.183	6.3
Hydropsychidae	METI	W1.4	62.634	0.814	1.080	2.980	3.1
Hydropsychidae	METI	W2.4	-72.389	0.813	1.080	2.934	3.1
Hydropsychidae	NORTI	LL.4	5.038	0.714	1.143	1.090	1.7
Hydropsychidae	NORTI	W1.4	1.948	0.713	1.301	1.092	1.8
Hydropsychidae	NORTI	W2.4	-4.200	0.712	1.102	1.028	1.7
Microdriles	METI	LL.3	1.323	0.000	1.049	29.352	11.2
Microdriles	METI	LL.4	7.060	0.746	1.003	7.017	8.0
Microdriles	METI	W1.3	1.114	0.000	1.059	38.594	11.5
Microdriles	METI	W1.4	5.936	0.736	0.998	8.104	8.3
Microdriles	METI	W2.3	-0.777	0.000	1.012	20.695	10.8
Microdriles	METI	W2.4	-4.332	0.750	1.004	6.020	7.9
Microdriles	NORTI	LL.4	68.887	0.637	0.824	5.792	5.7
Microdriles	NORTI	W1.4	29.280	0.638	0.825	5.881	5.7



Chapter 4



Sediment pollution, chronic toxicity and metal bioaccumulation in freshwater macroinvertebrates from Pb/Zn mining districts

Moreno-Ocio, I., Rodriguez, P., Martínez-Madrid, M., & Méndez-Fernández, L. Sediment toxicity and metal bioaccumulation in freshwater macroinvertebrates from Pb/Zn mining districts. *In preparation*.

Abstract

The assessment of two Pb/Zn mining districts in Cantabrian region (northern Spain) was carried out through the following lines of evidence: sediment chemistry, sediment chronic toxicity and metal bioaccumulation in field macroinvertebrate biomonitors. In the absence of sediment quality guidelines, ecological backgrounds and thresholds for Cd, Pb and Zn were estimated for the sediment pollution assessment. The sediment chronic bioassay was assessed through sediment *Tubifex tubifex* 28-day bioassay. The risk of metal bioaccumulation was assessed using the INTISS (Integrative Tissue concentration) score calculated by means of several *in situ* biomonitors. Using a reference condition approach, these three lines of evidence showed impairment due to sediment chemistry and chronic toxicity in all test sites, and only in one test site the hazardous levels of bioaccumulation were not attained. This assessment suggests applying management programs for reducing the source of metal pollution at these sites. Effective tissue residues were estimated through *T. tubifex* bioassay and their relevance to assess the field tissue residues is discussed.

Keywords: Cd, Pb, Zn, sediment thresholds; effective tissue residues; *Tubifex* bioassay.

1. INTRODUCTION

Mining activities for the extraction of metals have usually resulted in the disposal of soil tailings, which can leach metals to rivers and pose a significant risk for aquatic communities (Loredo *et al*., 2010). Sediment and biota have been recognized as suitable monitoring matrices for long-term changes in water quality of European water bodies (Carére *et al*., 2012; EC, 2010), but environmental quality standards, for both sediment and biota, have been developed only by some State Members. Sediment quality assessment is an important component of the environmental risk assessment (ERA) of water bodies and it usually includes sediment chemistry, chronic toxicity, bioaccumulation, and measures of the alteration of the field benthic communities. Toxicity tests are essential tools for assessing cause-effects relationships of the hazardous chemicals present in contaminated areas and the field alterations measured in the macroinvertebrate assemblages. Bulk sediment toxicity tests use more realistic exposure conditions than only-water toxicity tests (Chapman & Anderson, 2005; Ingersoll *et al*., 1995; Ingersoll *et al*., 1997) and, therefore, are preferred in ERA approaches. The effects on the exposed organisms are measured and evaluated by comparing the response to a test sediment using different toxicity endpoints (e.g., survival, growth and reproduction) with the response to a negative control, a condition where endpoint values provide the measure of the "no toxic" condition (ASTM, 2005). The negative control in present study is defined from several field-collected, natural sediments previously defined as unpolluted, reference sites. Such procedure used in sediment-quality assessment based on the comparison of endpoint values in test sites with the expected values derived from reference sites is known as the *reference condition approach* (Reynoldson *et al*., 1997). The criteria used for the selection of reference sites in Europe are defined by the European water Framework Directive (WFD: EC, 2000) for quality assessment of water bodies.

The test species used in present study to assess the sediment chronic toxicity was the aquatic oligochaete *Tubifex tubifex* (Annelida, Clitellata). The aquatic oligochaetes have been used in both laboratory (De Jonge *et al*., 2012; Lobo *et al*., 2021; Maestre *et al*., 2007) and field exposures (Bervoets *et al*., 2016; Méndez-Fernández *et al*., 2017; Protano *et al*., 2014) and their relevance in the evaluation of

sediment toxicity and bioaccumulation within ERA was supported by Chapman (2001). One of the reasons why aquatic oligochaetes are considered suitable test organisms and biomonitors is that most of them are detritivores, deposit-feeding invertebrates that occupy a variety of microhabitats in the sediments (Rodriguez & Reynoldson, 2011). In fact, their infaunal way of life explains why they are exposed to pollutants through various uptake routes, i.e. ingestion of contaminated particles and integumentary absorption of porewater and overlaying water (OECD, 2008). The burrowing activity of aquatic oligochaetes contributes to bioturbation processes in freshwater ecosystems, which triggers the exchange of pollutants from different matrices, such as the sediment and the water column (Ciutat *et al.*, 2005; Thibodeaux & Bierman, 2003).

A weight of evidence (WOE) framework helps integrating the information on the environmental risk derived from several biotic and abiotic components of the sediments that were assessed by independent lines of evidence (LOE), typically sediment chemistry, chronic toxicity and field data on *in situ* macroinvertebrates (Chapman, 2007a,b; Fernández *et al*., 2008; Grapentine *et al*., 2002).

Therefore, the general aim of the present work was to conduct an environmental risk assessment of sites influenced by historic Pb/Zn mining works, using a WOE approach. To accomplish this objective, the following specific goals were to be achieved: (1) to assess the sediment pollution in the rivers downstream abandoned Pb/Zn mines, (2) to evaluate sediment toxicity downstream Pb/Zn mines using the *T. tubifex* chronic sediment bioassay, (3) to measure metal bioaccumulation in field macroinvertebrates to assess field alteration, (4) to estimate effective tissue residues for Cd, Pb and Zn in *T. tubifex*. Finally, an overall WOE approach aimed to provide a screening-level of contaminated sediments (Chapman & McDonald, 2005) to judge possible ecological impacts from the alterations measured on the three investigated lines of evidence.

2. MATERIAL AND METHODS

2.1. Study area

Cantabrian Rivers (northern Spain) are typically short, with steep slopes, due to their flowing from relatively high Cantabrian Mountains and their proximity to the Cantabrian Sea. The region is characterized by oceanic climate, with abundant precipitation almost all along the year (mean annual precipitation: 1493 mm, with and oscillation between 1245 and 1996 mm: Basque Water Agency, 2019). The river catchments studied are located in the North-Pyrenean Belt, one of the Pb-Zn and Fe fold-belts limiting a unique metallic domain between Galicia and the Alps (Águeda Villar & Salvador González, 2008). Within this region, seven sites in two metal districts were studied, in July 2017 and 2018 (Figure 1, Table S1): Arditurri Pb/Zn mining district in Gipuzkoa (Basque Country, 5 sites) and Reocín Pb/Zn mining district (Cantabria, 2 sites). In Arditurri mining district, in the Oiartzun and Bidasoa River basins, two sites (END and OIA1) were selected as reference sites according to the water agency (Basque Water Agency, 2017: ENDARA-A and OIA044), whereas the other three sites (OIA2, ARD and OTS) where located downstream the historic mining activities. In Reocín mining district, the two study sites (SB013 and SB014) were located in the Besaya River, downstream the historic mining areas, as part of the surveillance networks of the water agency (CHC, no date).

Arditurri mining district is located in Aiako Harria Natural Park (Oiartzun), being the unique granitic outcrop in the Basque Country, formed between 300 and 250 million years ago. The galena was exploited through history to extract silver in roman times (Urteaga, 2002), and iron and lead during the Middle Ages. In the 1960s, open-air quarries were built, Santa Bárbara and Otsamantegi, for extraction of sphalerite and fluorite minerals, to obtain zinc in the former and hydrofluoric acid in the latter. Mines were definitively closed in 1984. Restoration on Arditurri mine village was conducted from 2002, and an interpretation center about Arditurri mines history was opened to the public in 2007.

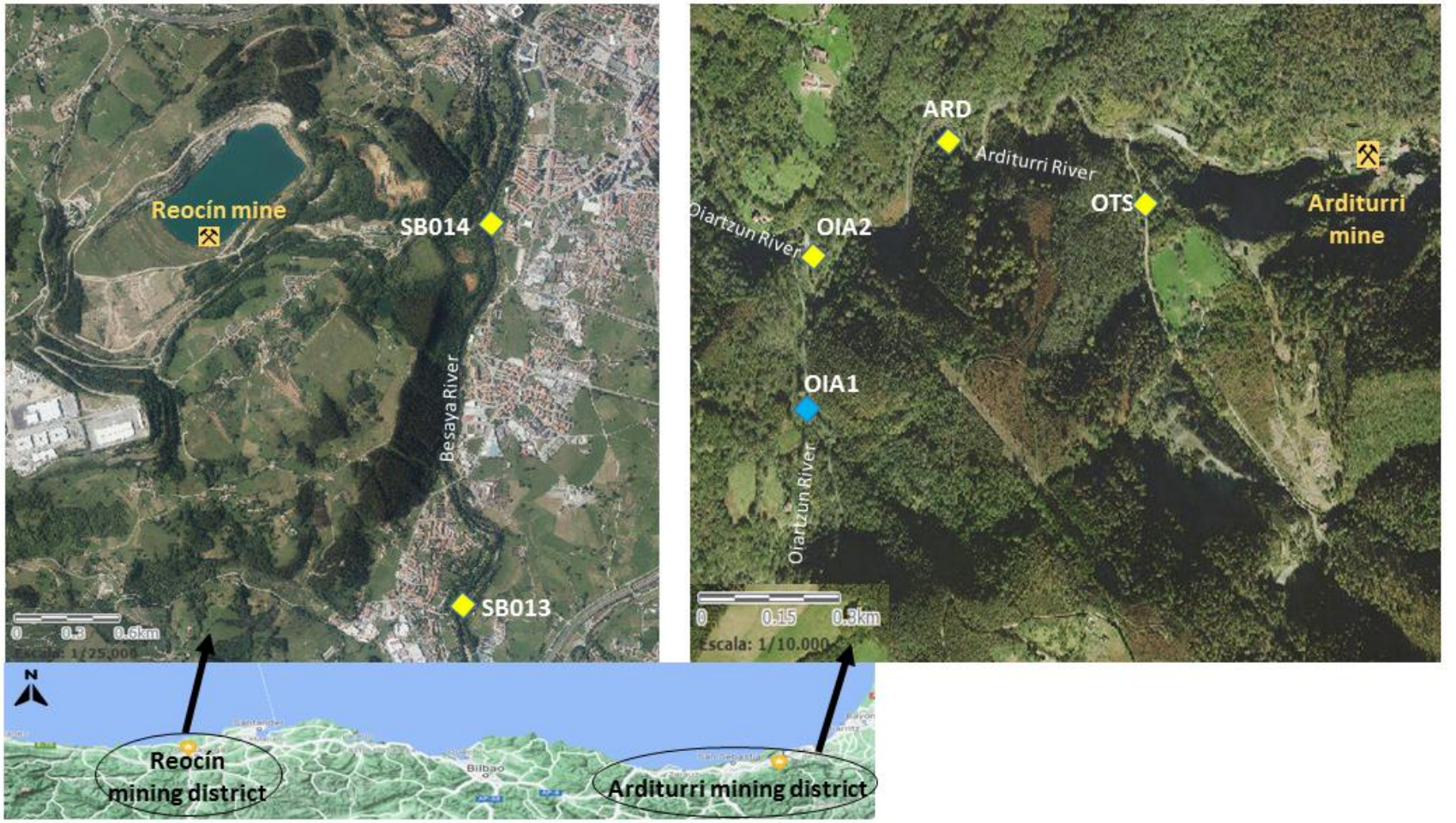


Figure 1. Arditurri and Reocín mining district location in northern Spain and detailed representation of each Pb/Zn mine and sampling sites. In blue, OIA1 reference site and in yellow, OIA2, ARD, OTS, SB013 and SB014 test sites (Photo from Google maps and CHC). END is not represented.

The Reocín mining district is located in Cantabria, and occupies a surface of 10 km^2 in the municipalities of Cartes and Torrelavega. Main mining activities started in the mid XIX century, for the extraction of zincblende, galena and pyrite, and there are some evidences back to the Roman times (Carballo, 1980). In Reocín mining district, the galena mineral appears scattered among the sphalerite and the dolomite (Águeda Villar & Salvador Gonzáliez, 2008; Castro *et al*., 2001). Between 1943 and 1965, mining was focused in the interior works, but a collapse caused the re-activation of the open-pit mining, called El Zanjón. Peak production was reached in 1990-95, the exhaustion of the deposit caused the closure of Reocín mine in 2003, and a restoration process of the area was started afterwards.

2.2. Sediment sampling and characterization

Sediment sampling was conducted under a low-flow regime, when most of the fine-grained suspended sediments become deposited on the riverbed (Mudroch & Azcue, 1995), and when worst conditions for toxicity and bioaccumulation for biota are expected to occur (AQEM, 2002). At each site, a composite sample of sediment was taken with a stainless steel spade from the upper 5–10 cm layer of fine sediment, settled along about 25-m reach of the river bank. The sediment for chronic bioassays was sieved in the field through 500-µm mesh size to eliminate coarse particles and indigenous fauna (Day *et al*., 1995). Samples were taken to the laboratory on ice and stored at 4°C, in the dark, during a maximum period of 6 months (as recommended by Reynoldson *et al*., 1991).

From the original composite sample, sediment subsamples for metal analyses were air-dried and sieved through a 63-µm mesh. Particle size distribution of the unsieved sediment was expressed as dry weight percentage, according to the Udden-Wenworth scale (Teruggi, 1982). Sediment TOC% was determined through the loss-on-ignition method, after calcination at 450°C, for 6 h, in a muffle furnace (Bryan *et al*., 1985; US EPA, 1990). Several water variables also were measured *in situ*: conductivity (Orion 3-Star, ThermoScientific), and dissolved oxygen, pH and temperature (Orion 5-star, ThermoScientific) (Table S1).

2.3. Chronic toxicity sediment bioassay

The 28-day *T. tubifex* chronic sediment bioassay (ASTM, 2005; Méndez-Fernández *et al*., 2013) was conducted with the field sediments. For a bioassay quality assurance, every bioassay included a control series with the sediment used for *T. tubifex* worms culture, collected in a mountain spring (Iturbatz, ITU) (for a detailed description of the site see Méndez-Fernández *et al*., 2013). At the laboratory, sediment from each site was homogenized and randomly distributed in five bakers, each with 100-ml sediment and covered by 100-ml dechlorinated tap water. At the beginning of each bioassay, four sexually mature worms with a well-developed clitellum were placed randomly into each test beaker. They were in their first reproductive period (6–7 week old) and had previously purged their gut content for 5 h, in dechlorinated tap water (see Table S2). After gut purging and before the introduction of the worms in test beakers, worms were individually weighed in an electrobalance (Sartorius M3P, detection limit, dl = 1 µg). The bioassay was run at 22±1°C, with gentle aeration and in the dark, during 28 days.

Sediment bioassays were run separately for each of the mining districts (Arditurri and Reocín), and each bioassay included one control series (ITU1 and ITU2). At the end of the 28-day sediment bioassay, surviving worms were purged for 5 h, frozen in liquid nitrogen and stored at -20°C (Table S2). Chronic toxicity endpoints were: survival (SUR%), number of total cocoons: TCC; number of empty cocoons: ECC; number of total young: TYG, and total growth rate (day^{-1}): TGR (Méndez-Fernández *et al*., 2013). Twice per week, the water temperature, dissolved oxygen, and pH (Orion 5-star) in the overlying water of the test beakers were controlled, while aeration was visually checked daily (Mon-Fri). Dissolved oxygen was maintained above 2.5 mg L^{-1} in the overlying water, pH values were between 7 and 9 and the conductivity between 249 and 566 µS cm^{-1}.

2.4. Macroinvertebrate sampling

At each site, a sample of the benthic macroinvertebrate for the study of metal bioaccumulation was taken with a hand-net, 200-µm mesh size, following a multi-habitat sampling scheme. Preliminary selection of macroinvertebrate to be used as

biomonitors of tissue residues included 10 taxa (8 insect families, 2 oligochaetes groups) that are representatives of different functional traits: Baetidae, Ephemerellidae, Ephemeridae, Heptageniidae, Hydropsychidae, Lumbricidae, Microdrile oligochaetes, Perlidae, Rhyacophilidae, and Simuliidae. For each taxon and site, 3 replicates consisting of 1–20 individuals were collected, the number depended on the individual size (see details in Rodriguez *et al.*, 2018). Organisms were sorted on site, held for 5–10 h in river water on ice, cleaned, and identified in the laboratory under a binocular microscope, before being frozen. The final selection of biomonitors for the risk assessment included only 4 taxa, which were the ones present in all the study sites. These 4 biomonitors were representative of 3 different feeding styles: Baetidae and Heptageniidae (herbivorous scrapers), Rhyacophilidae (predator) and Simuliidae (collector-filterer).

The ecological status of the macroinverterate community could be assessed in only three sites of the Arditurri district through ecological quality ratios (EQRs) using the official river-type specific multimetric index (METI: MAGRAMA, 2015). Original information to calculate the index was provided by the Gipuzkoa County Council and the Cantabrian Hydrological Confederation. The METI value can range from 0 to 1.4, a value of 0.700 indicating the threshold between Good and Moderate ecological status. METI-EQR values of the two reference sites indicated a Good ecological status (END: 0.823, OIA1: 0.878). The test site ARD also had a METI-EQR value (0.821) indicating Good ecological status. Other test sites in the Oiartzun River and the Besaya River basins were not assessed by the Water Authorities, so data on the macroinvertebrate ecological status were not available.

2.5. Metal analysis

Nine metals and metalloids were analysed in the sediment fraction <63 µm and Cd, Pb and Zn were identified as the most relevant (Table S1). Chemical concentrations in sediments were analyzed by SGIker Technical Services (UPV/EHU, Spain). Sediment samples were acid digested using microwave extraction method following EPA 3051 (US EPA, 2007). Digested sediment samples were measured by ICP-AES (Horiba Yobin Yvon Activa) and ICP-MS (7500ce, Agilent Technologies), and limit of quantification was 0.0009 µg g^{-1} for Cd, 0.009 µg g^{-1} for Pb, and 0.9 µg g^{-1} Zn. All batches included Buffalo

River sediment (RM8704, USA) and Sewage Sludge-3 (CRM 031-040, UK) as reference material for quality control and all metals recovery rates were within certified values (89-105%). All organisms (laboratory *T. tubifex* and field collected macroinvertebrates) were digested in trace element-free nitric acid (70% Baker Instra-Analyzed) for one week, and afterwards in H_2O_2 (30% R.P. Normapur Prolabo) in a ratio 10:1, at room temperature, for 24 h (Clements & Kiffney, 1994; Méndez-Fernández *et al*., 2013). Samples were stored at -20ºC until metal analysis was completed. Cd, Pb and Zn tissue residues were also measured by ICP-MS by SGIker Technical Services (UPV/EHU, Spain), and limits of quantification were 0.010 µg l^{-1} Cd, 0.060 µg l^{-1} Pb and 0.800 µg l^{-1} Zn. Every batch of tissue samples included 3 blanks and 3 replicates of a certified reference material (Mussel Tissue ERM-CE278, Belgium). Tissue reference material recovery rates were within the certified values for all metals (92.1–98.7%).

2.6. Statistical analyses and data processing

2.6.1. Estimation of background Cd, Pb, Zn sediment concentrations in northern Spain

The first step to estimate regional background levels was the appropriate selection of reference sites in northern Spain for the derivation process. For that purpose, we used the methodology proposed by Méndez-Fernández *et al*., (2019). The selection of sites was mainly based on the reference monitoring networks developed by the Water Authorities in Spain (Cantabrian Hydrographical Confederation and Ebro Hydrographical Confederation), according to the WFD criteria. We built a database of 31-55 sites as representative of the reference (unpolluted) condition for Cd, Pb and Zn, in sediments from northern Spain (Table S3). The metal concentrations at these sites were used to calculate the ecological background (mean concentrations) and ecological threshold (upper concentrations) associated with the condition of good ecological status of the macroinvertebrate communities and with non-toxic sediments. Based on the range of metal sediment concentrations, several methods were used to derive ecological background and threshold values: 1) the mean + 2 times the standard deviation (SD); 2) the Tukey inner fence (TIF); and 3) the 90^{th} bootstrapping percentile (P90). For a detailed description of this process, see Méndez-Fernández *et al*. (2019).

Descriptive statistics were calculated and bootstrap percentiles and confidence intervals at 95% were calculated (1000 re-samples), using IBM® SPSS software.

Sediment metal pollution was assessed with the SedPoll score (Costas *et al*., 2018), adapted for the 3 relevant metals in the area: Cd, Pb and Zn. At each site, every single metal concentration in the sediment was divided by the corresponding P90 value calculated in the whole database of the reference sites to get a metal quotient (P90-Q). The metal concentration in the sediment was then classified into five potential categories on the basis of the metal quotients as follows: I) Similar-to-reference sites where P90-Q ≤ 1.0; II) Low metal concentration where P90-Q = 1.1–2.0, III) Medium metal concentration where P90-Q = 2.1–10.0, IV) High metal concentration where P90-Q = 10.1–50.0, and V) Very high metal concentration where P90-Q ≥ 50.1. Next, at each site the 3 relevant metals P90-Q values were summed and averaged by the number of metals to get an average metal quotient, which is useful for describing the deviation in the metal concentration of each sediment with respect to the reference condition. This average metal quotient for the selected metals (Cd, Pb and Zn) was used as a Sediment Pollution score (SedPoll), an integrative sediment pollution index calculated for each study site, assuming that interactions among the metals were additive. The categories used for the SedPoll score assessment were the same as those mentioned above for the P90-Q classification.

2.6.2. Sediment toxicity assessment

Mortality frequencies in each sediment bioassay were first compared with the respective control using Fisher's Exact test. For the sublethal endpoints, parametric tests were conducted when possible, using ANOVA followed by Dunnett's t-test (ASTM, 2005). Normality of the data frequency distribution and homogeneity of the variances were tested with Shapiro-Wilk and Levene's test, respectively. When data were not normalized after transformation, Kruskal-Wallis and Dunn's tests were applied. Statistical analyses were done using IBM® SPSS® software. In all cases, the significance level was $\alpha = 0.05$, except for Shapiro-Wilk ($\alpha = 0.01$).

Sediment toxicity was evaluated with multivariate analysis, in a reference condition approach (Reynoldson *et al.*, 2002; Rodriguez *et al*., 2011), using the

probability ellipses of 80% and 95%, to evaluate the deviation of the toxic response in each site bioassay respect to the reference condition. The analysis was performed on a data matrix that included 58 reference sites in northern Spain (Méndez-Fernández, 2013; Rodriguez *et al*. 2011) plus the addition of the data derived from two reference sites in present study (END and OIA1) (Table S4). The reference sites were plotted in a multivariate ordination space, using nMDS analysis and Euclidean distance (PRIMER 6: Clarke & Gorley, 2006). Sediment toxicity assessment of the test-sediments was performed site by site, adding each site bioassay data to the nMDS space of the reference sites. Test sites were assessed as Non-Toxic (NT) when placed within the 80% probability ellipse and thus considered "similar to the reference condition"; sites were assessed as Potentially Toxic (PT) when placed within the 95% and 80% probability ellipses; finally, those sites placed outside the 95% probability ellipse were assessed as Toxic (T), being interpreted as "different from the reference condition".

2.6.3. Macroinvertebrate Tissue Residues

The bioaccumulation of the macroinvertebrate assemblages was assessed following the methodology proposed by Rodriguez *et al*. (2021) (Chapter I). For both the Oiartzun River (including the Endara tributary of the Bidasoa River basin, which runs close to the Oiartzun River) and the Saja-Besaya River basins, the metal Ecological Threshold Tissue Concentrations (ETTC) calculated in the Nalón River basin was used (Rodriguez *et al*., 2018). The metal ETTC values were used as benchmarks, and the metal bioaccumulation of Cd, Pb and Zn in each site was expressed as a metal hazard quotient (ETTC-Q). The next step consisted in calculating the metal *Tissue-residue Ratio to Threshold* (TRT), as the average of the ETTC-Qs calculated for each taxon. The TRT index assumes metal mixture interactions as additive, following the Cumulative Criterion Unit proposed by Clements *et al*. (2000). The last step consisted in the calculation for each site of the *INtegrated TISSue* score (INTISS) by averaging the TRT scores calculated for the selected taxa. The classification of the sites using the scores establishes four categories: (1) *Similar to reference*, when bioaccumulation scores are ≤ 1.0; (2) *Low bioaccumulation* when scores are 1.1–2.0; (3) *Medium bioaccumulation* when scores are 2.1–10.0; (4) *High bioaccumulation* when scores are > 10.

Additionally, toxicity responses and tissue residues were modelized by nonlinear regression analyses, conducted using R software and the extension package *drc* (Ritz & Streibig 2005). The models were applied to estimate effective residues (ER_{50}) from a selection of 9 log-logistic, log-normal and Weibull models: LL.2, LN.2, W2.2, LL.3, LL.4, W1.3, W1.4, W2.3 and W2.4. Model selection was carried out using Akaike´s information criterion (AIC < 2: Burnham & Anderson, 2002). Models were validated through graphical assessment and the ER value was accepted only when its standard error was lower than the corresponding ER. Among the selected models, the best fitted with the lowest ER standard error was chosen as most suitable. Potential outliers in the regression models were examined through the graphical test of the standardized and studentized residuals (Zuur *et al*., 2007). Goodness-of-fit was assessed by R^2 and the Neill's lack-of-fit test for replicates included in the *drc* package (Ritz & Streibig, 2005).

2.6.4. Weight of evidence framework

Three lines of evidence (LOEs) were examined to conduct a risk assessment using a weight of evidence (WOE) framework, integrating risk scores calculated from sediment chemistry, sediment chronic toxicity, and metal tissue residues in field organisms.

LOE 1: Sediment chemistry: Metal sediment concentration at each site was assessed using the SedPoll scores. A site with a SedPoll score category I was assessed as *unlikely to cause adverse effects* (○); a site within the SedPoll categories II and III was assessed as *may or may not cause adverse effects* (◉); a site within the SedPoll categories IV and V was assessed as *likely producing adverse effects* (●).

LOE 2: Sediment Chronic Toxicity: The sediment classified as *Non Toxic* was *similar to the reference* condition (into the ellipses of 80% probability) (○); *Potentially Toxic* when toxic response was between the probability ellipses of 95% and 80% (◉); and *Toxic* when toxic response was outside the ellipses of 95% (●)

LOE 3: Metal Tissue Residues: The integration of the metal tissue residues measured in several biomonitors and compared with the reference values, in one index was made using the INTISS score. The metal tissue residues in the

macroinvertebrate assemblages were assessed as *similar to reference* when INTISS ≤ 1.0 (○); *probably different to reference* when INTISS was between 1.1–10.0 (◉); *different to reference* when INTISS was > 10.0 (●). The higher the difference from the reference condition, the higher the probability of adverse effects in the ecological status of the field macroinvertebrate community.

The decision matrix developed for the WOE was made by ranking the three LOEs used. The next step consisted in the description of the environmental status of each site interpreting each LOE.

3. RESULTS

3.1. Sediment metal concentration

At the Arditurri mining district area, the maximum metal concentration in sediments were recorded downstream the mining area. The most relevant metals in the sediments were Cd, Pb and Zn, since they were the only metals that attained 2.5 times their PEC values in the study area (Table S1). The sediment metal concentration attained (in mg kg^{-1} dw) 27.8 for Cd at ARD; 4,600 for Pb and 12,974 for Zn at OTS (Table 1). In Reocín mining district, metal levels in sediments were well below the previous ones, and the most relevant metal was Pb ranging 72.6–119 mg kg^{-1} dw. Cd, Pb and Zn were the only metals that attained 2.5 times their PEC values in the study area. At control and reference sites, the lowest metal sediment concentrations were measured, ranging (in mg kg^{-1} dw) 0.5–1.11 for Cd, 16.2–96.6 for Pb and 41.0–336 for Zn.

The background concentration measured from field unpolluted and non-toxic reference sediments in northern Spain were used to approach the ecological thresholds relevant for sediment metal concentrations, as explained in section 2.6.1 (Table 2). Reference sediments had mean concentrations (in mg kg^{-1} dw) of 0.45 ± 0.50 for Cd, 24.3 ± 18.5 for Pb and 77.8 ± 59.4 for Zn. Comparing the Cd, Pb and Zn sediment concentrations with the ecological background values, they were more than 10 times higher at OIA2, ARD and OTS and more than 6 times higher at SB014 for Zn. In the reference sites (OIA1 and END), Pb and Zn sediment concentrations were some

Table 1. Sediment (Sed) metal concentration (mg kg^{-1} dw), ecotoxicity endpoints and metal tissue residues (TR) (µg g^{-1} dw) in *T. tubifex* sediment bioassay (28 days). Significant differences in comparison tests of each case with the respective ITU control are shown as *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Percentage of survival (SUR), total cocoons (TCC), empty cocoons (ECC), total young (TYG) and total growth rate (TGR).

	Sed Metals			Endpoints					TR Metals		
Site	Cd	Pb	Zn	SUR	TCC	ECC	TYG	TGR (day^{-1})	Cd	Pb	Zn
ITU 1	0.5	16.3	53.3	90.0 ± 13.7	33.8 ± 8.1	19.4 ± 6.5	82.4 ± 28.2	0.029 ± 0.007	0.4 ± 0.2	9.4 ± 2.5	447 ± 24
END	1.11	96.6	282	95.0 ± 11.2	45.8 ± 0.8**	27.8 ± 2.8*	116.0 ± 34.1	0.041 ± 0.004**	7.0 ± 5.5***	13.4 ± 4.4	723 ± 64**
OIA 1	0.97	85.8	336	95.0 ± 11.2	43.8 ± 4.4**	25.8 ± 4.1	130.8 ± 24.4	0.044 ± 0.003***	0.5 ± 0.3	17.3 ± 4.2**	626 ± 13
OIA 2	16.9	457	3,629	100.0 ± 0.0	50.4 ± 2.7***	29.8 ± 2.9**	182.0 ± 11.0**	0.058 ± 0.006***	0.8 ± 0.2	254 ± 68.2***	1.399 ± 148***
ARD	27.8	1,905	11,221	100.0 ± 0.0	44.0 ± 3.4**	26.8 ± 2.8*	146.0 ± 6.3**	0.047 ± 0.002***	1.4 ± 0.3**	320 ± 22.0***	2,423 ± 206***
OTS	16.2	4,600	12,974	100.0 ± 0.0	28.4 ± 4.8	22.2 ± 4.8	29.0 ± 5.6**	0.018 ± 0.003**	13.6 ± 2.4***	4,219 ± 576***	2,514 ± 71***
ITU 2	0.60	16.2	41.0	100.0 ± 0.0	31.6 ± 2.9	16.8 ± 2.2	103.8 ± 14.2	0.018 ± 0.006	0.17 ± 0.16	8.1 ± 2.0	371.1 ± 46.3
SB 013	0.63	72.6	250	95.0 ± 11.2	50.0 ± 4.2***	28.4 ± 2.6***	228.6 ±26.1***	0.050 ± 0.003***	0.05 ± 0.01**	12.9 ± 1.4**	340.8 ± 22.5
SB 014	1.24	119	921	100.0 ± 0.0	45.2 ± 6.1***	24.6 ± 5.6*	165.0 ± 62.1	0.060 ± 0.010***	3.23 ± 3.65	35.7 ± 10.2***	377.8 ± 32.4

higher than the respective ecological threshold. The ecological sediment thresholds (mean ± 2SD) were within the 95% confidence limits of the P90, except for the Zn that was slightly higher.

Table 2. Ecological background and thresholds estimated for sediment metal concentrations, in mg kg^{-1} dw. The threshold effect concentration (TEC) and the probable effect concentration (PEC) for sediments from MacDonald *et al*. (2000) are also shown, for comparison.

Ecological Backgrounds	**Cd**	**Pb**	**Zn**
Range	0.08-2.20	3.1-126	12-411
Mean (SD)	0.45 (0.50)	24.3 (18.5)	77.8 (59.4)
n	31	52	55
Ecological Thresholds	**Cd**	**Pb**	**Zn**
Mean + 2SD	1.44	61.3	197
TIF	0.87	44.4	171
P90 (95% CI)	1.33 (0.54-2.05)	39.1 (30.9-62.3)	136 (100-172)
TEC	0.99	35.8	121
PEC	4.98	128	459

The SedPoll scores calculated for Cd, Pb and Zn classified the sediments from reference sites and SB013 as *Low metal concentration* (SedPoll = 1.8, 1.8 and 1.4, respectively); SB014 was classified as *Medium metal concentration* (SedPoll = 3.6); OIA2 as *High metal concentration* (SedPoll = 17.0), and ARD and OTS as *Very High metal concentration* (SedPoll = 50.7 and 75.1, respectively) (Figure 2).

3.2. Chronic toxicity assessment and metal tissue residues in *T. tubifex* bioassay

Results from chronic bioassays are reported in Table 1. After 28-day exposure, the worms exhibited normal behaviour in both controls (ITU) and test conditions, i.e. with the head downwards in the sediment and the hind part of the body projecting upwards in the water and waving with a regular rhythm. Large amounts of faecal pellets were also observed at the sediment surface, which suggests normal burrowing activity, with no sediment avoidance. The results in the control batches were validated by the numbers and the variation coefficients (mortality <10%, Total cocoons: <25%, Total Young : <50%) (Méndez-Fernández *et al*., 2013) and were within the 15% of variation in the laboratory control charts (2010-2018 period).

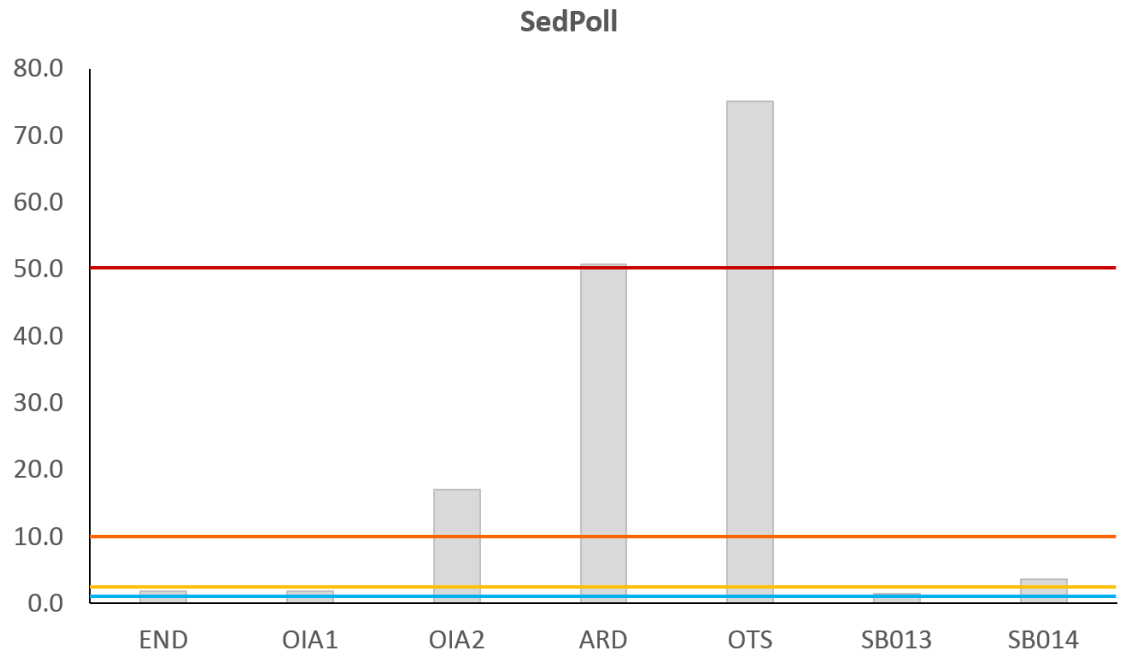


Figure 2. SedPoll scores for sediment metal concentration of all study sites. Blue: Class boundary I-II (low), in yellow Class boundary II-III (medium), in orange Class boundary III-IV (high) and in red Class boundary IV-V (very high).

At the end of the bioassay, most adults in ARD and OTS exhibited tail degradation and autotomy, although some of the worms had regenerated the pygidium, the posterior end of the annelids that contains the anus. At OTS, the cocoons showed a blue-green color that had never been seen before (Figure 3).

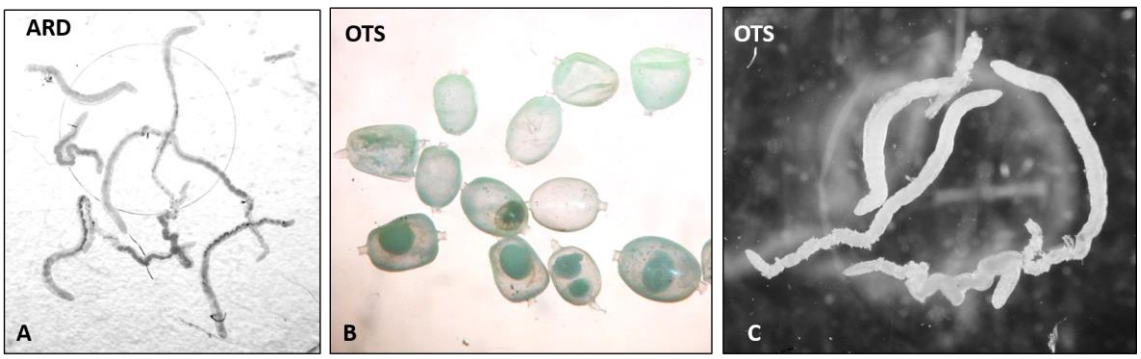


Figure 3. *Tubifex tubifex* and its cocoons exposed to ARD and OTS. A and C, worms presenting autotomy and tail degradation; B, cocoons.

After 28-day exposure, no significant mortality was observed in the test sites (< 5%). Reproductive impairment occurred at OTS, with significant reduction in the number of Total Cocoon (TTC) and Total Young (TYG) (Table 1). Total Growth Rate (TGR) was also significantly lower than in the controls at OTS; however, TYG and TGR increased compared with the control at all sites (40-120 % higher for TYG; 41–233% higher for TGR). In the reference sites END and OIA1, the increments of TYG and TGR respect to the bioassay control were small (40-58%), but the highest values were observed in the Reocín mining district sites for TGR (177-233% higher) and in OIA2 and SB013 for TYG (120% higher). Based on sediment metal concentrations and sublethal responses, it seems that the combination of high Pb and Zn sediment concentrations may cause growth and reproduction impairment at OTS, while a stimulatory response was produced where sediments showed only high Zn concentrations.

The reference sites database used for the toxicity classification (n = 60) showed maximum values of 100 % (mean = 94; $P75^{th}$ = 100) for %SUR; 45.6 (mean = 35.1; $P75^{th}$ = 39.2) for TCC; 26 (mean = 13; $P75^{th}$ = 16) for ECC; 296.6 (mean = 112.0; $P75^{th}$ = 144.5) for TYG; and 0.06 day^{-1} (mean = 0.03; $P75^{th}$ = 0.04) for TGR (Table S4). Site toxicity classification using probability ellipses in the nMDS multivariate space of the reference database resulted in 3 sites (ARD, OTS, SB014) classified as Potentially Toxic (PT) and 2 sites as Toxic (T) (OIA2 and SB013) (Figure 4).

In the *T. tubifex* bioassay, the highest Pb and Zn tissue residues were recorded at OTS (13.6, 4219, and 2514 µg g^{-1} dw, respectively), followed by ARD and OIA2, and all were significantly different compared to the bioassay control worms (Table 1). Cd tissue residues were highest in OTS (13.6 µg g^{-1} dw), but in END reference site the worms showed the second highest Cd tissue residues (7.0 µg g^{-1} dw), although no toxicity effects were observed at this site. As expected, *T. tubifex* in the control batches (ITU1 and ITU2) showed low and very similar metal tissue residues. It is noteworthy that in ARD *T. tubifex* kept Cd tissue residues well below 1.5 µg g^{-1} dw, although the Cd sediment concentration was the highest in present study. On the other hand, at OTS and OIA2 the Cd concentrations in the sediment were similar, but in the former the Cd tissue residues were more than 10 times higher than in the latter, suggesting an active elimination in some instances. Regarding Zn, *T. tubifex* tissue residues were

maintained around 2,500 µg g^{-1} dw at ARD and OTS, despite the sediment concentration were above 11,000 µg g^{-1} dw, suggesting the capability of internal regulation of these metal.

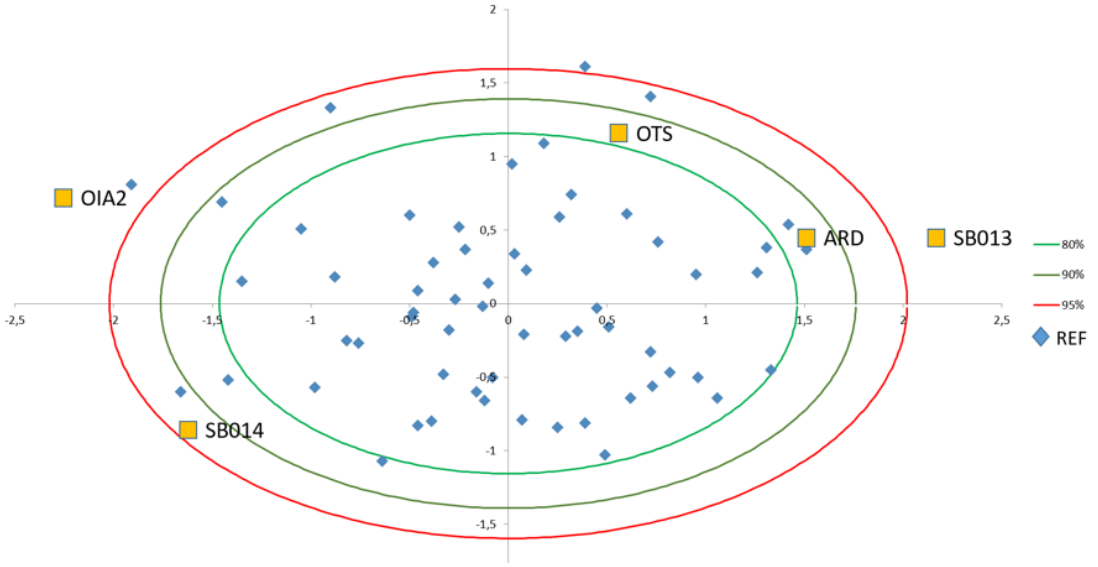


Figure 4. Sediment toxicity assessment through probability ellipses in the nMDS ordination space for reference sites (in blue). Test sites from Arditurri and Reocín mining districts are represented in yellow.

Nonlinear regression models of the toxicity endpoints values against Cd, Pb and Zn tissue residues were fitted using several nonlinear dose-response regression models, and effective tissue residues related to a reduction of 50% of a biological variable (ER_{50}) were estimated for each combination of metal tissue residue and toxicity endpoint (Figure 5). Reproduction and growth ER_{50} values were estimated from log-logistic models of 3 and 4 parameters. The only endpoints that allowed an estimation of ER_{50} values from validated models were Total Young (TYG) and Total Growth Rate (TGR). Effective tissue residues for TYG were Cd- ER_{50} (µg g^{-1} dw) = 8.61 ± 1.08 and Pb- ER_{50} = 2,064.4 ± 1,658. For TGR, Cd- ER_{50} (µg g^{-1} dw) = 11.07 ± 0.85 µg g^{-1} dw, Pb- ER_{50} = 3,170.4 ± 10.0 and Zn- ER_{50}= 2,496.3 ± 10.1. Hormetic models (BC.4, CRS.4a, CRS.4b and CRS.4c) of the metal tissue residues against toxicity endpoint relationships were also tested, but the data did not fit these models.

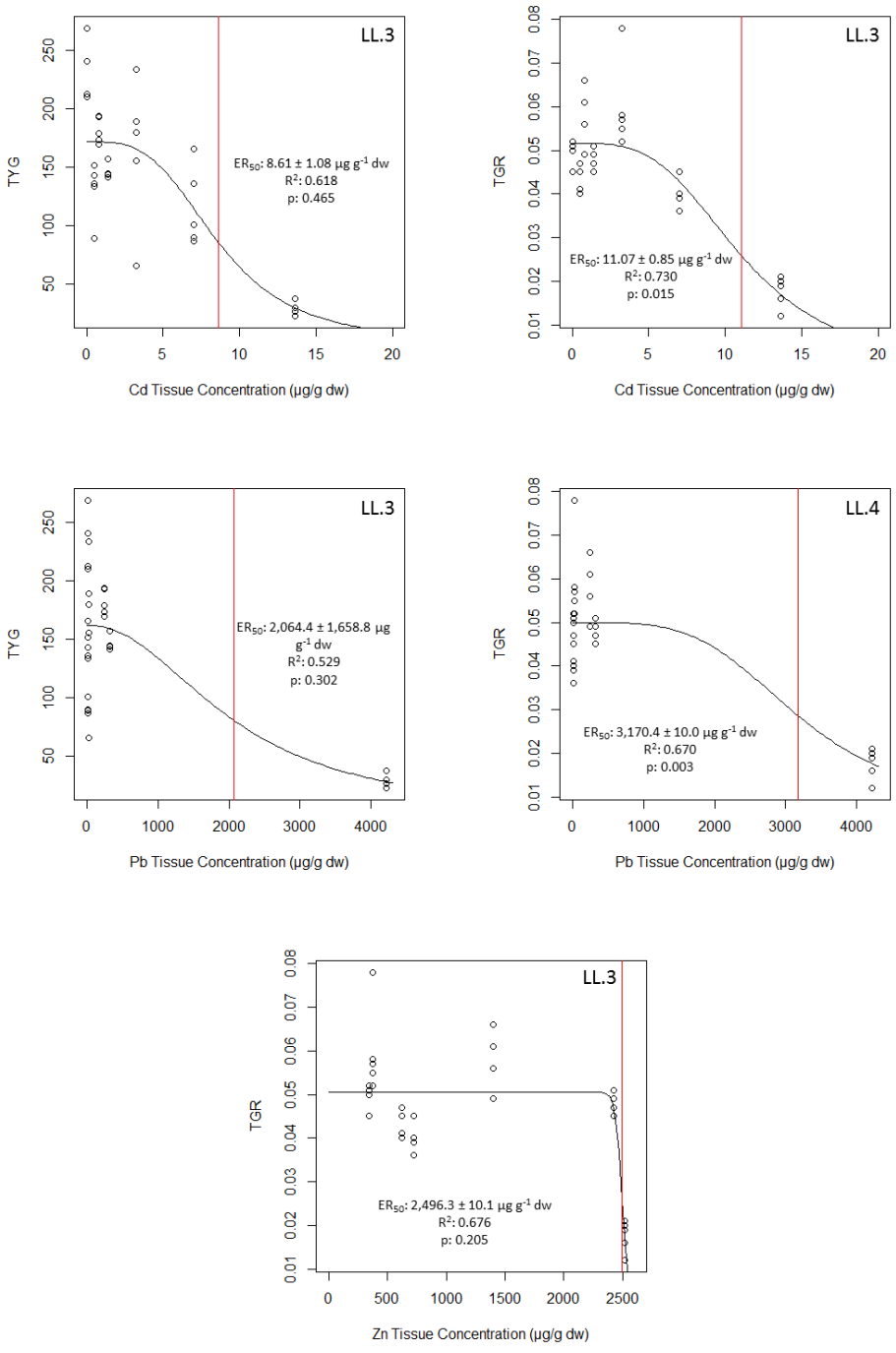


Figure 5. Dose-response models representing tissue residues vs toxicity endpoints, such as total young (TYG) and total growth (TGR). In red, ER_{50} is represented.

3.3. Field macroinvertebrate assessment through the INtegrated TISSue score (INTISS)

Tissue residues measured in 10 field macroinvertebrate taxa are reported in Table S5. All data are reported in μg g^{-1} dw. Maximum Cd tissue residues were found in Microdrile oligochaetes at OTS (66.70) followed by Heptageniidae at ARD (53.0). With regard to Pb, maximum tissue residues were shown in Simuliidae (1,870) followed by Microdrile oligochaetes (1,336.5), both at OTS. Very high Zn tissue residues were measured in Baetidae at ARD (20,402), OTS (14,601) and OIA2 (13,540), followed by Heptageniidae at OTS (14,377) and at ARD (11,054).

The macroinvertebrate bioaccumulation risk assessment performed through the INTISS was calculated over the total 10 taxa sampled (although many of them were missing from several sites) (Table 3). END and SB013 were assessed as *Similar to reference*, since their INTISS scores were < 1. Sites OIA1 and SB014 were assessed as *Low bioaccumulation* and OIA2 as *Medium bioaccumulation.* However, when the INTISS was calculated for a selection of four biomonitor taxa (Baetidae, Heptageniidae, Simuliidae and Rhyacophilidae) that were present in all the study sites, the risk assessment changed for OIA2 from *Medium* to *High bioaccumulation*. Sites ARD and OTS were assessed as *High bioaccumulation* using either of the INTISS scores. Therefore, the results indicate that there is a risk of alteration of the macroinvertebrate assemblages due to metal bioaccumulation in the Oiartzun River basin, specifically in OIA2, ARD and OTS. In Reocín mining district sites, the bioaccumulation in field macroinvertebrates shows no or low risk due to metal bioaccumulation.

Table 3. Results of the *Tissue-residue Ratio to Threshold* (TRT) score for Cd, Pb and Zn and of the *INtegrated TISSue* (INTISS) score for all taxa. The INTISS score was also calculated for the four taxa present in all the study sites: Baetidae, Heptageniidae, Simuliidae and Rhyacophilidae. TRT classes: *Similar to reference* (white) when bioaccumulation scores are ≤ 1.0; *Low bioaccumulation*, when scores are 1.1 - 2.0 (green); *Medium bioaccumulation* when scores are 2.1 - 10.0 (yellow); *High bioaccumulation* when scores are >10 (red).

Site	Baetidae	Heptageniidae	Hydropsychidae	Ephemeridae	Simuliidae	Rhyacophilidae	Perlidae	Ephemerellidae	Lumbricidae	Microdriles	*INTISS*	*INTISS*
					[Cd Pb Zn] *TRT*						*All taxa*	*4 taxa*
END	0.9	1.6	1.1	0.8	1.1	0.5	0.3	0.8	0.8	0.5	0.8	1.0
OIA1	1.5	2.6	0.9	1.3	0.9	1.4	0.7	2.3	1.0	0.8	1.3	1.6
OIA2	14.0	13.6	5.8	2.1	15.8	3.4	6.1	3.5	2.6	-	7.4	11.7
ARD	21.1	26.5	-	-	29.0	4.2	-	-	-	-	20.2	20.2
OTS	18.7	21.0	-	-	26.0	3.5	-	-	-	10.6	15.9	17.3
SB013	0.8	1.3	0.7	-	0.9	0.6	-	-	0.7	-	0.8	0.9
SB014	1.8	3.0	1.7	-	1.8	1.0	-	0.8	0.9	0.5	1.4	1.9

3.4. *Weight of Evidence* approach

Three lines of evidence (LOE) (sediment chemistry, sediment chronic toxicity and tissue residues) were used in this study for an integrative risk assessment at each site of the Pb/Zn mining districts (Table 4).

Table 4. Decision matrix for Weight of Evidence (WOE) categorizations of the study area based on three Lines of Evidence (LOE): sediment chemistry, sediment chronic toxicity, and metal bioaccumulation on four field biomonitors (Baetidae, Heptageniidae, Rhyacopohilidae and Simuliidae. ● Adverse effects are expected, ⦿ Adverse effects may occur, ○ Adverse effects are unlikely (see section 2.6.4 for details).

Site	Sediment Chemistry	Sediment Toxicity	Metal Bioaccumulation
OIA2	●	●	●
ARD	●	⦿	●
OTS	●	⦿	●
SB013	⦿	●	○
SB014	⦿	⦿	⦿

The test sites showed medium to high sediment contamination, higher in Arditurri mining district than in Reocín. In OIA2, the WOE assessment indicates maximum environmental risk with polluted sediments that caused chronic toxicity and high bioaccumulation. In the 3 sites located in the Arditurri mining district, high bioaccumulation in field organisms was observed, showing potential to high risk of alteration in the three LOEs. However, in Reocín mining district, the sediment pollution is medium, the sediments are potentially toxic or toxic, but the bioaccumulation in field organisms was *Similar to reference* in SB013 and *Low* in SB014. Therefore, there are enough evidences in all sites, except in SB013, for unacceptable environmental risk due to sediment contamination. These evidences point toward applying management programs for remediation. The lack of data on the ecological status of the field macroinvertebrate community does not allowed for an overall WOE.

4. DISCUSSION

Monitoring the levels of contaminants in the sediment and benthic macroinvertebrate tissue allows evaluating the cause-effects relationships of the pollutants on the aquatic organisms, and the risk probability posed to the benthic communities due to the exposure to toxic, hazardous chemicals. Biota and sediment have been recognized by the European directives as suitable matrices to monitor long-term changes in the water quality (EC, 2013a, b), but in practice, environmental quality standards (EQS) of metals for these compartments have been poorly developed. In Spain, contaminants in sediments and the biota are evaluated based on the *stand still* principle (EC, 2000), which says that priority substances in the sediments or biota should not significantly increase their concentration in long-term (EC, 2008). This principle results insufficient for the objective proposed by the WFD for attaining the good ecological status in rivers subject to historical contamination due to mining activities.

Regarding the sediment, some European countries have developed independent sediment quality guidelines (SQG) (Norway: Bakke *et al*., 2010; Belgium (Flanders): De Deckere *et al*., 2011), but the absence of SQGs in Spain limits the development of a sound ERA and water quality protection plans. In previous studies on sediment risk assessment of freshwater ecosystems in Spain (e.g., Méndez-Fernández *et al*., 2015), the threshold effect concentration (TEC) and the probable effect concentration (PEC) proposed for North American freshwater sediments (MacDonald *et al*., 2000) were used. In present study, the ecological thresholds of Cd, Pb and Zn based on the 90th percentile calculated from reference sediments were similar to the North American sediment guidelines and encompasses the TEC values within their confidence limits (Table 2). The use of these regional thresholds gives extra reliability to the risk assessment of the study sediments.

Table 5. Data from the literature on Cd, Pb and Zn tissue residues in field invertebrates. All data are given in µg g^{-1} dw.

Samples	Organisms	Data Type	Cd	Pb	Zn	References
Sediment bioassay	*T. tubifex*	TR vs TYG: ER_{50}	140.50			Méndez-Fernández *et al.*, 2013
		TR vs TGR: ER_{50}	130.63			
		TR vs TCC: ER_{50}	149.49			
		TR vs ECC: ER_{50}	142.75			
	T. tubifex	TR vs TCC: ER_{50}		6.6	2112	Méndez-Fernández *et al.*, 2015
		TR vs Survival: ER_{50}			2752	
		Minimum Tissue Residue	0.01	0.6		
	T. tubifex	Maximum Tissue Residue		188.6	298-2327	Gillis *et al.*, 2006
	T. tubifex	ER_{50}			>1805	Lobo *et al.*, 2021
Field	Microdrile oligochaetes	ETTC	7.00	70.9	406	Rodriguez *et al.*, 2018
	Tubificid oligochaetes	Field Critical Body Burden	28-71	79-98	930-1800	Bervoets *et al.*, 2016
	Freshwater invertebrates	Field Critical Body Burden		3253-6423	1981-20529	De Jonge *et al.*, 2013
	Freshwater invertebrates	Mean polluted sites	8.38			Poulton *et al.*, 1995
	Aquatic wildlife	Critical Tissue Level	0.75	0.6		DEQ, 2007

The estimated Cd-ER_{50} from the *T. tubifex* toxicity bioassay for growth and reproduction were similar to the ecological threshold tissue concentration (ETTC) proposed by Rodriguez *et al.* (2018) as the baseline value for field collected Microdrile oligochaetes in the Nalón River basin (7.00 µg g^{-1} dw), but lower than the Cd tissue residues threshold in Tubificidae proposed by Bervoets *et al*. (2016) (28–71 µg g^{-1} dw) causing alteration in the macroinvertebrate community (Table 5). However, the Pb-ER_{50} estimated in present study from the *T. tubifex* toxicity bioassay were 29-45 times higher than the ETTC, although very similar to the critical body burden estimated by De Jonge *et al.* (2013) for freshwater invertebrates (Table 5). Regarding the Zn-ER_{50} estimated from the *T. tubifex* toxicity bioassay related to a reduction of 50% on the growth rate (TGR-ER_{50}: 2,496 µg g^{-1} dw), it is within the range of ERs calculated by Méndez-Fernández *et al*. (2015) from the same bioassay for reproduction (TCC-ER_{50}= 2,112 µg g^{-1} dw) and survival (2,752 µg g^{-1} dw). The calculated Zn-ER_{50} is also in the low interval of the critical body burdens proposed for field freshwater invertebrates (1,981-20,529 µg g^{-1} dw) by De Jonge *et al*. (2013). Additionally, the Zn-ER_{50} estimated from the *T. tubifex* toxicity bioassay was 6 times higher than the ETTC.

In an attempt to compare field and laboratory approaches, the bioaccumulation of metals in Microdrile oligochaetes was assessed through the ERs estimated for *T. tubifex* in the sediment toxicity bioassay. Regarding to the Cd, both reference sites and SB014 were assessed at no risk, but OTS attained values up to 7.7 times the ERs proposed for this metal, showing a moderate risk due to the bioaccumulation of Cd. For Pb and Zn, all sites were below the proposed effective thresholds, which translates into no risk due to the bioaccumulation of these metals if ER_{50} values of *T. tubifex* are used. However, in OTS the Pb tissue residues in field Microdrile oligochaetes are 19 times higher than the ETTC and the Zn tissue residues 3 times higher. The assessment through ETTC in SB014 resulted in no risk due to Pb and Zn bioaccumulation for Microdrile oligochaetes. The absence of this taxon in other test sites did not allow a better assessment through this approach.

Although chronic bioassays with *T. tubifex* are suitable for an environmental risk assessment, since they inform on several biological effects (survival, growth, several reproductive endpoints, and bioaccumulation), other sediment bioassays

performed with several species, in a battery of tests, with different sensitivities and exposure routes can provide a wider information for the risk assessment of the macroinvertebrate community (Chapman *et al*., 2002; Rodriguez & Reynoldson, 1999). In that sense, it could be more interesting to include a variety of tests that can measure a different set of variables, rather than a variety of species measuring the same ones. Arambourou *et al*. (2020) exposed *Chironomus riparius* larvae to the same sediment batches sampled from two sites of the Oiartzun River basin (OIA1 and ARD) in present study. The life-cycle chronic toxicity test showed toxic effects in larvae exposed to ARD sediments regarding respiration, emergence rates and lipid composition (lipidomic analysis and transcriptional profile), while the reference site OIA1 showed no effects. In any case, usually there is not enough information or evidence from the laboratory toxicity test data for management and remediation programs. The reduction in response to toxicity and to physical characteristics of the sediment can confound the results.

Information on *in situ* benthic community ecological status is missing in the environmental risk assessment performed here, although it is also a necessary tool for a sounded and more reliable WOE approach (Chapman, 2007a, b) and Water Authorities should include more sites form these areas affected by historical Pb/Zn mining activities in their surveillance networks. However, the monitoring should also include bioaccumulation and toxicity data, since it is a known fact that field organisms can become acclimated to relatively high levels of metals. Acclimation to naturally high levels of metals can result in tolerant field population due to natural selection (Klerks & Levinton, 1989). Other interesting lines of evidence that should be including in future monitoring and restoration programs are the bioavailability of *in situ* contaminants and biomagnification of some chemicals.

This study aims to emphasize the value of a weight of evidence approach, combining laboratory and field data for an overall risk assessment. In particular, molecular and physiological endpoints are promising tools to be considered in future for environmental risk assessments, using a WOE approach, combined with information on benthic macroinvertebrate community status and the development of

sediment and tissue residues guidelines useful for an effective water quality conservation policy.

5. CONCLUSIONS

Sediment ecological thresholds proposed for Cd, Pb and Zn are comparable to other thresholds proposed in other geographic areas, and have been proved to be a useful tool for the pollution assessment in areas affected by historical Pb/Zn mining activities in northern Spain. Sediment chronic toxicity and tissue residues in field macroinvertebrates have demonstrated that the sediment pollution poses a risk for the benthic communities, due to the toxicity and the bioaccumulation of hazardous metals in most sites, related to the Arditurri and Reocín historical mining activities.

Tissue residues of Cd, Pb and Zn in field macroinvertebrates appear to be reliable tools for a bioaccumulation risk assessment in the Pb/Zn mining catchments of Arditurri and Reocín mining districts. In fact, the effective tissue residues (ER) estimated through chronic sediment bioassay resulted to be a useful tool for the assessment of Cd bioaccumulation. The other ERs were in line with data on literature but still need refinements.

The weight of evidence evaluation performed, using a reference condition approach evidenced the existence of moderate to high environmental risk. Quality surveillance nets of the Water Authorities in northern Spain should follow the ecological status of the *in situ* macroinvertebrate assemblages in the river reaches downstream the Pb/Zn mines to monitor their conservation status. Remediation plans in these basins, including chemistry, ecotoxicity, benthic community ecological status and bioaccumulation, provide the necessary information to attain a complete recovery of the ecological status in these water bodies.

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Table S1. Geographical data, general physical and chemical characteristics of study sites in the Pb/Zn mining districts. Abbreviations: SC, silt and clay (sediment fraction <63 µm), and TOC, Total Organic Carbon.

Sites				Geographical data			Water characteristics				
Site	River	Basin	Sampling year	UTM_X	UTM_Y	Altitude (m)	DO (mg/l)	% SAT	T (°C)	pH	Conduct (µs/cm)
END	Endara	Bidasoa	2017	603007	4794218	28	9.8	100.8	17.5	8.5	75.3
OIA1	Oiartzun	Oiartzun	2017	595738	4792511	95	9.1	91.4	15.2	7.8	82.7
OIA2	Oiartzun	Oiartzun	2017	595680	4792917	92	5.6	59.7	17.8	7.9	103.5
ARD	Arditurri	Oiartzun	2017	596001	4793075	109	8.8	98.0	20.0	7.9	137.1
OTS	Otsamantegi	Oiartzun	2017	596466	4792778	134	6.1	61.2	15.7	8.2	156.0
SB013	Besaya	Saja-Besaya	2018	413453	4797145	28	9.4	102.7	19.6	8.1	468.0
SB014	Besaya	Saja-Besaya	2018	413726	4799732	9	8.1	86.0	18.4	8.0	567.0

Sediment chemistry										Sediment characteristics	
Site	As	Cd	Cu	Cr	Hg	Ni	Pb	Se	Zn	TOC %	% SC
END	17.6	1.11	24.9	109	0.12	63.0	96.6	5.35	282	0.45	0.17
OIA1	8.39	0.97	24.9	48.4	0.18	25.7	85.8	4.82	336	1.86	1.85
OIA2	16.1	**16.9**	57.2	80.3	0.46	42.8	**457**	4.97	**3629**	1.24	0.76
ARD	53.0	**27.8**	165	40.7	1.87	45.7	**1905**	8.32	**11221**	4.18	1.01
OTS	**79.0**	**16.2**	116	39.0	1.05	57.2	**4600**	16.3	**12974**	1.45	0.75
SB013	8.11	0.63	18.4	16.4	0.61	11.8	72.6	0.85	251	2.11	6.62
SB014	11.9	1.24	45.0	27.8	0.28	23.6	120	1.71	**921**	0.77	1.81
TEC	9.79	0.99	31.6	43.4	0.18	22.7	35.8	-	121		
PEC	33.0	4.98	149	111	1.06	48.6	128	-	459		

Table S2. Gut-purging experiment with *Tubifex tubifex* under two different temperatures. Percentage of individuals with their gut emptied, weight of feces produced per individual, percentage of feces production compared to the total produced in 24 h, and percentage in weight of feces produced per worm.

Time (hours)	4 ºC	20 ºC	4 ºC	20 ºC	4 ºC	20 ºC	4 ºC	20 ºC
	Gut-purged worms (%)		Mean fecal production per individual (mg)		Feces production (%)		Feces/worm weight (%)	
5	54	96	0.241	0.326	74	94	26.5	32.7
9	79	100	0.048	0.022	89	100	5	1.4
24	96	-	0.037	-	100	-	3.7	-

Method: The gut-clearing period was calculated in a separate experiment, under two different temperatures (4°C and 20°C). For each temperature condition, 30 mature individuals randomly selected from the culture were disposed in Petri dishes with dechlorinated tap water, 6 individuals in each one (n=5 per condition) and left to empty the gut. All of the individuals had their gut full of sediment at the beginning of the experiment. The feces produced in each Petri dish were collected with a micropipette after 5, 9 and 24 hours from the beginning of the experiment, and put in a filter (Whitman, diameter), dried at 60°C and weighed in an electro-balance Sartorius M3P, detection limit =1 µg) . The guts were observed under a binocular microscope to know if the guts had been completely purged or only partially. Under 20°C, after 5 h of gut-clearing period 96% of the individuals had emptied their gut (100% after 9 hours). Under 4°C, only 54% of the individuals have emptied the gut (79% after 9 hours). The importance of the fecal material in terms of the worm weight is represented in the last columns.

Table S3. Database of sites represented as reference or unpolluted sites. Geographical data, sediment metal concentration for Cd, Pb and Zn (in mg kg^{-1} dw) and percentage of silt and clay (SC, <63 µm) and Total Organic Carbon (TOC) are shown (Rodriguez *et al.*, 2011, 2018; Méndez-Fernández, 2013).

SITE	CEDEX Typology	River	Basin	Province	UTMX	UTMY	Altitude (m)	Year	Cd	Pb	Zn	%SC	%TOC
ZBA088	11	Barrundia	Ebro	Araba	546046	4751571	606	2010	0.20	6.2	11.7	10.6	2.3
EGBI102	11	Izki	Ebro	Araba	545570	4727402	-	2005	**<LD**	14.5	40.6	7.6	0.9
RCVA178	11	Najerilla	Ebro	La Rioja	501228	4664283	-	2006	**<LD**	17.9	41.1	14.4	3.0
URS40	11	Nela	Ebro	La Rioja	501639	4659606	-	2006	**<LD**	20.4	95.3	5.1	3.9
URS65	11	Mayor	Ebro	La Rioja	524326	4661172	-	2006	**<LD**	16.9	40.2	7.8	1.4
URS66	11	Urbión	Ebro	La Rioja	510910	4662733	-	2006	**<LD**	8.8	21.1	22.3	2.3
URS67	11	Tirón	Ebro	Burgos	490020	4682917	-	2006	**<LD**	18.9	66.5	7.4	2.2
EG380	12	Ega	Ebro	Araba	553545	4724915	-	2004	0.35	4.1	27.4	8.6	2.2
41	12	Ega	Ebro	Navarra	569332	4722831	-	2006	**<LD**	15.2	49.6	20.4	3.5
D166	12	Jerea	Ebro	Burgos	470415	4737357	-	2006	**<LD**	8.2	56.9	17.7	2.0
OM244	12	Omecillo	Ebro	Araba	496050	4741605	-	2005	**<LD**	27.8	47.2	34.1	2.7
RCVA169	12	Oca	Ebro	Burgos	472535	4700115	-	2006	**<LD**	17.6	58.3	21.5	4.2
URS61	12	Rudrón	Ebro	Burgos	431779	4729853	-	2006	**<LD**	10.2	29.3	20.7	2.1
ZAI018	12	Ayuda	Ebro	Araba	533780	4733975	-	2005	**<LD**	16.8	33.9	22.9	2.0
NAL011	21	Turón	Nalón	Asturias	283040	4788140	423	2014	0.37	35.7	135.0	1.2	2.1
NAL047	21	Villabre	Nalón	Asturias	246779	4794815	609	2014	0.23	19.2	68.7	4.3	1.6
R2	21	Lindes	Nalón	Asturias	262789	4777652	234	2015	0.17	18.5	101.0	8.7	1.3
NAL042	21	Onón	Nalón	Asturias	219588	4790741	121	2015	0.29	16.4	100.0	1.1	1.2
NAL043	21	Genestaza de	Nalón	Asturias	227060	4795591	263	2014	0.18	22.3	50.9	1.1	0.6
NAL029	21	Pumar	Nalón	Asturias	203262	4786717	712	2015	0.19	25.5	58.0	0.4	0.4
NAL038	21	Coto de	Nalón	Asturias	198031	4778564	624	2015	0.33	22.0	79.0	0.6	1.0
LA001	22	Lamason	Nansa	Cantabria	379667	4792545	154	2008	0.08	19.6	44.9	4.5	0.8
SB002	22	Saja	Saja-Besaya	Cantabria	395193	4777339	363	2011	0.45	8.7	29.1	3.7	0.5
ART062	22	Artibai	Artibai	Bizkaia	538505	4789175	-	2002	1.4	30.0	81.9	-	-

M190	22	Barbadún	Barbadún	Bizkaia	490280	4796790	-	2004	0.69	37.1	106.0	7.5	3.6
L112	22	Lea	Lea	Bizkaia	537340	4795525	-	2002	1.56	31.0	97.0	-	-
L196	22	Lea	Lea	Bizkaia	540110	4799215	-	2004	0.41	38.3	96.5	3.4	2.2
BID1	23	Bidasoa	Bidasoa	Navarra	608247	4780209	-	2005	**<LD**	**<LD**	64.8	2.6	1.0
BID2	23	Bidasoa	Bidasoa	Navarra	612923	4777241	-	2005	**<LD**	**<LD**	137.0	4.4	0.7
BID3	23	Bidasoa	Bidasoa	Navarra	620904	4778550	-	2005	**<LD**	**<LD**	62.3	2.5	0.3
ON1	23	Onin	Bidasoa	Navarra	604036	4789210	-	2005	**<LD**	41.2	223.0	62.1	11.1
OIA044	23	Oiartzun	Oiartzun	Gipuzkoa	595819	4792944	-	2015	1.10	126.0	411.0	-	-
NAL009	25	Huerna	Nalón	Asturias	262769	4767937	1142	2014	0.16	25.4	80.8	5.5	2.0
NAN001	26	Nansa	Nansa	Cantabria	382612	4771787	956	2008	0.11	18.4	47.8	10.0	1.6
NAN002	26	Nansa	Nansa	Cantabria	384970	4773968	812	2008	0.12	19.9	60.1	2.4	1.1
PUR080	26	Purón	Ebro	Araba	481322	4744197	-	2002	2.20	47.0	56.6	-	-
OM080	26	Omecillo	Ebro	Araba	485750	4747045	-	2005	**<LD**	27.7	115.0	3.2	2.2
OMTU136	26	Tumecillo	Ebro	Araba	494540	4747042	-	2005	**<LD**	21.4	45.4	17.9	1.4
URS41	26	Erro	Ebro	Navarra	629520	4760509	-	2006	0.08	21.3	57.6	1.8	0.4
URS6	26	Esca	Ebro	Navarra	663198	4731389	-	2006	0.22	70.3	35.6	50.9	2.2
RCVA53	27	Subordan	Ebro	Huesca	684599	4734594	-	2006	**<LD**	18.7	60.8	30	2.7
URS42	27	Veral	Ebro	Huesca	679285	4748317	-	2006	0.56	3.11	144.7	25	1.0
OKM040	30	Mape	Oka	Bizkaia	523567	4801562	-	2005	**<LD**	30.9	91.9	12.4	1.7
NAL050	31	Teverga	Nalón	Asturias	743042	4794471	184	2014	0.22	20.6	82.8	1.2	1.4
NAL055	31	Lena	Nalón	Asturias	270698	4777131	353	2014	0.31	35.0	145.0	3.3	2.2
NAL031	31	Arganza	Nalón	Asturias	216040	4795670	112	2015	0.94	23.7	111.0	0.7	0.7
MIE002	32	Miera	Miera	Cantabria	443271	4793458	173	2008	0.14	12.7	51.6	1.8	0.5
SB003	32	Argonza	Saja-Besaya	Cantabria	406066	4774490	626	2008	**<LD**	19.9	42.8	13.8	8.1
SB017	32	Barranco	Saja-Besaya	Cantabria	416579	4790017	186	2008	**<LD**	17.6	38.6	2.4	1.0
SB022	32	Saja	Saja-Besaya	Cantabria	395300	4780409	366	2008	0.08	16.7	38.3	3.2	1.0
B062	32	Butrón	Butrón	Bizkaia	520475	4796745	-	2005	**<LD**	39.4	86.2	22.2	3.0
KAH100	32	Herrerías	Ibaizabal	Bizkaia	491610	4770875	-	2005	**<LD**	23.9	65.9	19.2	1.5

R4	-	Mosa	Nalón	Asturias	257059	4793047	365	2014	0.34	14.0	79.2	5.6	2.3
NO2259	-	Reguera de Brañanueva	Nalón	Asturias	264220	4789220	555	2014	0.23	18.6	83.0	6.6	1.4
N12	-	Rubial	Nalón	Asturias	268088	4785479	455	2015	0.19	20.8	94.8	3.9	2.1

Table S4. Reference database (n=60) endpoints measured in 28-day *T. tubifex* chronic toxicity bioassay. % SURV, percentage of survival; TCC, Total cocoons; ECC, Empty cocoons; TYG, Total number of Youngs; TGR, Total growth rate (day^{-1}). The maximum value, the 75th percentil and the mean value of each endpoint are included.

Site	% SURV	TCC	ECC	TYG	TGR
LA001	85.0	34.4	15.8	130.2	0.004
MIE002	90.0	36.4	16.0	164.0	0.024
NAL011	95.0	25.8	15.8	142.6	-0.005
NAL043	100.0	38.0	14.4	141.0	0.036
NAL047	95.0	37.6	17.8	142.0	0.035
NAL060	95.0	40.6	26.0	292.6	0.018
NAN001	100.0	42.6	24.8	204.8	0.014
NAN002	90.0	27.4	13.2	92.2	-0.007
SB002	100.0	37.0	20.0	144.2	0.016
SB003	90.0	41.2	20.2	172.0	0.025
SB017	95.0	34.2	13.2	120.4	0.022
SB022	80.0	34.2	18.0	137.6	0.019
ZBA088	95.0	39.0	15.8	160.2	0.027
41	100.0	37.4	7.2	71.8	0.047
141	100.0	36.2	10.8	43.0	0.027
ASON1	95.0	26.8	8.4	7.6	0.023
ASON2	100.0	40.0	13.2	156.4	0.061
B062	100.0	39.6	9.4	5.2	0.024
BID1	95.0	34.8	7.6	41.0	0.043
BID2	90.0	36.0	13.8	82.4	0.036
BID3	75.0	34.0	17.2	104.4	0.036
D166	100.0	40.4	10.2	80.4	0.046
EB1	100.0	37.0	13.0	104.0	0.047
EB2	90.0	31.2	8.2	58.2	0.042
EG380	100.0	33.6	5.6	22.2	0.036
EGBI102	100.0	24.4	16.4	256.0	0.041
IN235	100.0	37.4	14.6	144.6	0.029
KAH100	100.0	40.0	4.6	63.2	0.045
L196	100.0	39.2	16.4	153.6	0.033
MAL	95.0	29.4	7.2	55.8	0.021
M190	95.0	36.8	10.8	116.2	0.009

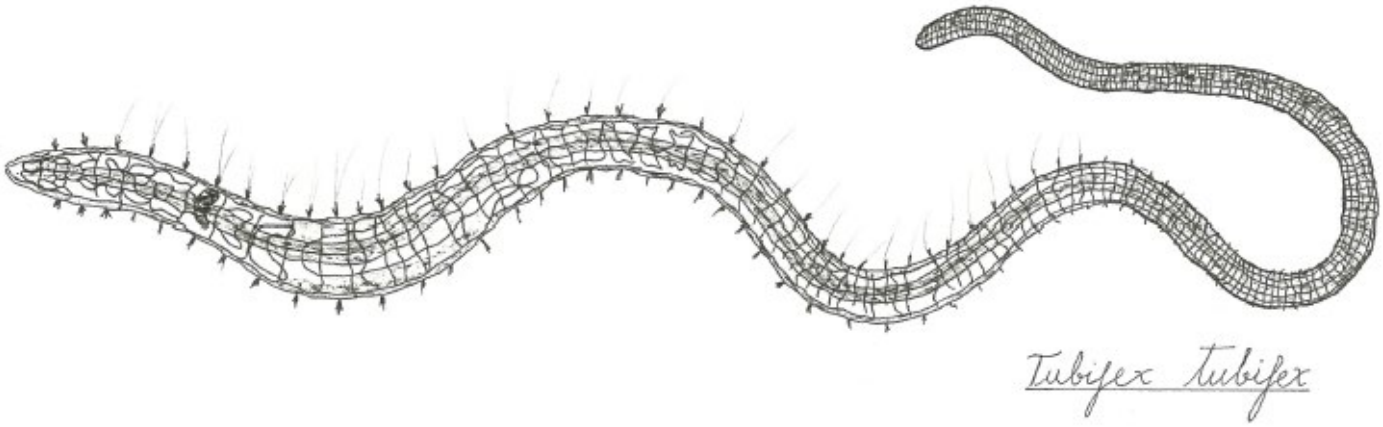
NEL1	95.0	39.2	18.8	176.0	0.054
NO3002	95.0	30.2	15.2	138.2	0.004
O262	100.0	30.2	11.8	77.0	0.012
OI102	95.0	40.6	6.2	93.2	0.041
OKM040	85.0	37.8	5.8	39.4	0.030
OM080	80.0	34.6	19.0	258.0	0.045
OM244	95.0	38.0	16.0	194.0	0.044
OMTU136	100.0	34.3	4.0	11.3	0.037
ON1	75.0	30.8	2.0	17.4	0.033
RCVA169	90.0	36.4	11.2	70.4	0.031
RCVA178	100.0	41.2	13.2	133.6	0.040
RCVA53	100.0	45.6	12.6	118.8	0.039
TRE1	95.0	30.8	14.2	57.8	0.039
U490	100.0	29.8	15.0	161.4	0.036
UGA1	90.0	29.8	14.6	135.8	0.031
URS25	100.0	23.4	8.6	34.0	0.027
URS40	95.0	41.0	10.2	120.0	0.042
URS41	65.0	28.4	11.0	62.6	0.019
URS42	95.0	35.6	16.0	61.0	0.009
URS57	100.0	21.4	9.6	31.2	0.025
URS6	100.0	21.2	7.4	43.8	0.032
URS61	93.8	37.3	14.5	121.0	0.023
URS65	75.0	37.2	9.8	75.4	0.027
URS66	95.0	42.4	11.0	115.4	0.037
URS67	95.0	43.4	12.0	144.0	0.048
ZAY018	100.0	32.8	20.0	236.8	0.035
ZBA088	95.0	37.4	19.6	160.2	0.046
END	95.0	45.8	27.8	116.0	0.041
OIA1	95.0	43.8	25.8	130.8	0.044
Maximum	100	45.6	26	296.6	0.06
75th percentil	100	39.2	16	144.5	0.04
Mean	94	35.1	13	112.0	0.03

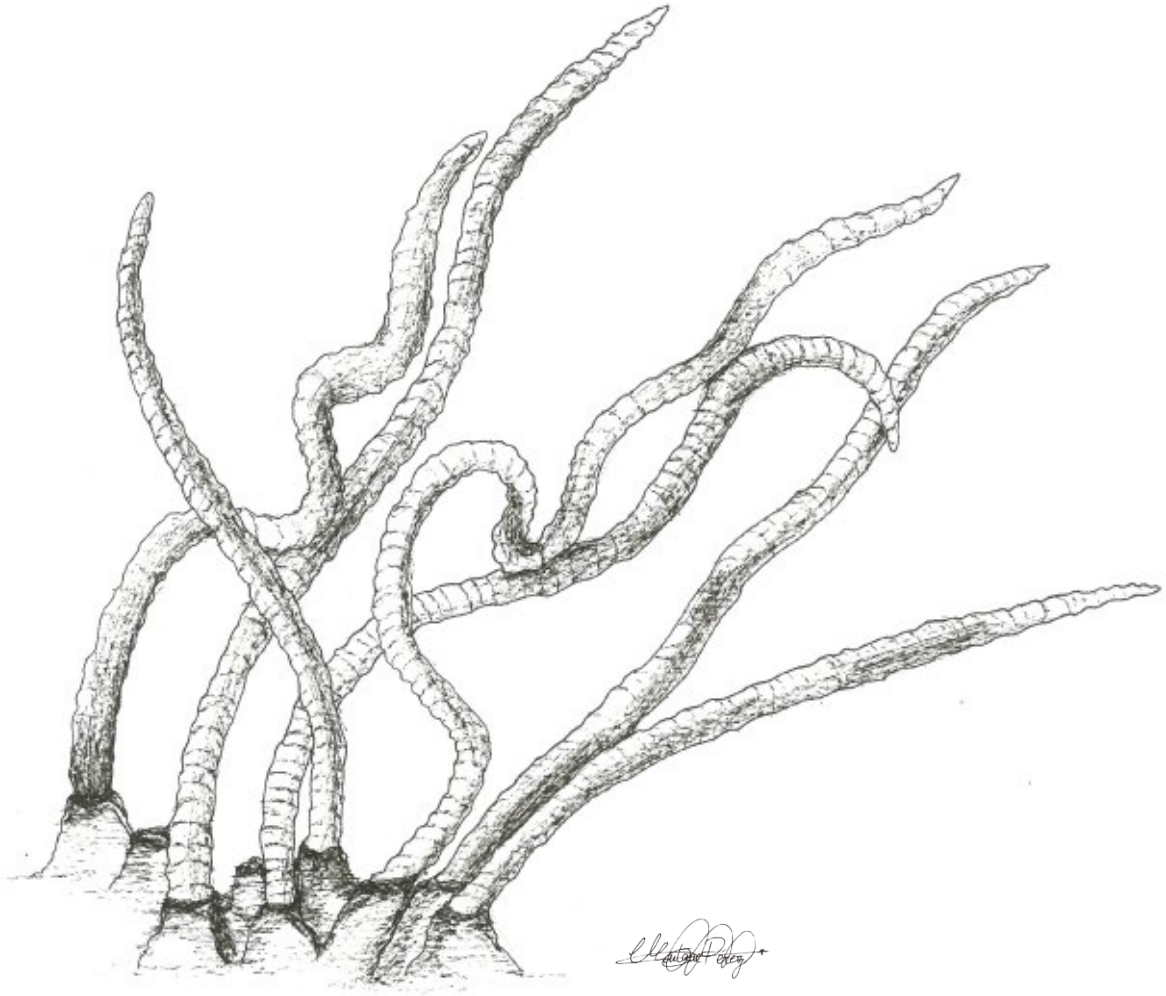
Table S5. Data on Cd, Pb and Zn tissue residues in 10 field macroinvertebrate taxa. Abbreviations: SC Scraper, CF Collector-Filterer, G Generalist, P Predator, DF Deposit Feeder, [Ep] epibenthic and [En] endobenthic.

		Metal		
Taxon	**Site**	**Cd**	**Pb**	**Zn**
Baetidae	**END**	7.43	1.3	398
SC [Ep]	**OIA1**	9.60	2.0	979
	OIA2	35.79	15.9	13540
	ARD	41.63	83.6	20402
	OTS	21.52	345.1	14601
	SB013	1.23	4.1	734
	SB014	1.58	4.3	1995
Ephemerellidae	**END**	10.99	2.4	716
G [Ep]	**OIA1**	20.44	1.4	3633
	OIA2	13.21	14.8	7992
	ARD	–	–	–
	OTS	–	–	–
	SB013	–	–	–
	SB014	2.68	7.7	1582
Ephemeridae	**END**	5.51	1.4	396
CF [En]	**OIA1**	7.34	7.2	748
	OIA2	12.88	9.1	1230
	ARD	–	–	–
	OTS	–	–	–
	SB013	–	–	–
	SB014	–	–	–
Heptageniidae	**END**	11.94	3.1	460
SC [Ep]	**OIA1**	13.37	15.4	1033
	OIA2	28.45	105.8	7657
	ARD	53.00	513.6	11054
	OTS	37.89	1.5	14377
	SB013	2.35	5.7	777
	SB014	3.61	5.9	2086
Hydropsychidae	**END**	0.67	4.6	200
CF [Ep]	**OIA1**	0.53	3.1	196
	OIA2	4.34	10.7	866
	ARD	–	–	–
	OTS	–	–	–

	SB013	0.37	6.7	156
	SB014	0.87	13.2	418
Lumbricidae	**END**	6.95	7.6	262
DF [En]	**OIA1**	5.21	18.9	340
	OIA2	16.38	31.8	1120
	ARD	–	–	–
	OTS	–	–	–
	SB013	1.75	16.0	274
	SB014	2.72	11.9	514
Microdrile oligochaetes	**END**	8.46	3.6	145
	OIA1	8.62	16.1	411
DF [En]	**OIA2**	–	–	–
	ARD	–	–	–
	OTS	66.70	1336.5	1337
	SB013	–	–	–
	SB014	4.62	21.3	247
Perlidae	**END**	0.33	0.8	123
P [Ep]	**OIA1**	0.72	1.6	223
	OIA2	10.32	1.5	683
	ARD	–	–	–
	OTS	–	–	–
	SB013	–	–	–
	SB014	–	–	–
Rhyacophilidae	**END**	0.78	0.3	300
P [Ep]	**OIA1**	3.72	1.6	484
	OIA2	9.30	< DL	1182
	ARD	9.96	17.8	1632
	OTS	3.71	128.7	1475
	SB013	0.52	1.9	390
	SB014	1.02	2.9	662
Simuliidae	**END**	0.60	8.1	220
CF [Ep]	**OIA1**	0.43	8.6	192
	OIA2	12.92	23.9	1052
	ARD	19.03	272.3	3691
	OTS	9.13	1870.0	3402
	SB013	0.37	14.9	192
	SB014	0.78	18.2	428

Chapter 5





Toxicogenomics in the aquatic oligochaete *Tubifex tubifex* in acute aqueous exposure to arsenic

Moreno-Ocio*, I., Llorente*, L., Méndez-Fernández, L., Aquilino, M., Martínez-Madrid, M., Rodriguez, P., & Planelló, R. Toxicogenomics in the aquatic oligochaete *Tubifex tubifex* in acute aqueous exposure to arsenic. *In preparation*. *Equal contribution to this paper.

Abstract

For the first time, *de novo* transcriptome of the aquatic oligochaete *Tubifex tubifex* (Annelida) was reconstructed, and genes related to cell stress response (*Hsp83, Hsp60, Hsp10, Hsc70*), oxidative stress and energy metabolism (*MnSOD, CAT, GSR, CoI*) and genes involved in homeostasis of organisms (*UBE2, CaM and RpS13*) were identified and characterized. A toxicogenomic approach was used for the risk assessment of arsenic in the aquatic environment, and gene expression was investigated after 96-h exposure in a water-only acute toxicity test with *Tubifex tubifex*. Several toxicological endpoints (survival and autotomy) of the oligochaetes and tissue residues were measured and dose-response models of gene expression data were studied. The potential of the genes identified for risk assessment in freshwater ecosystems as early biomarkers of arsenic toxicity is discussed.

Keywords: arsenic, *Tubifex tubifex*, gene expression, effect concentration, transcriptome.

1. INTRODUCTION

Arsenic is a common metalloid that occurs in the air, soil, water and all living tissues (Eisler, 2000). It is present in the environment from both natural and anthropogenic sources. One of the main sources of arsenic and other metal local contamination in freshwater ecosystems are the past and present mining activities (Loredo *et al*., 2006; Méndez-Fernández *et al*., 2015; Rainbow, 2018). In fact, arsenic appears in high concentrations in groundwater from geological sources, causing chronic health disorders (Bhattacharya *et al*., 2007). Arsenic pollution in sediments and bioaccumulation has been demonstrated as one of the main causes of the toxicity (Méndez-Fernández *et al.*, 2015) and also of the alteration of the ecological status in rivers affected by mining activities (Costas *et al*., 2018; Moreno-Ocio *et al*., 2022: Chapter II; Rodriguez *et al*., 2021: Chapter I).

In previous studies on As ecotoxicity, we have performed toxicity tests with the aquatic oligochaete *Tubifex tubifex* (Annelida) as test species, in both water-only and sediment exposures (Lobo *et al.*, 2016, 2021). This species is widely distributed, abundant in temperate regions, and easy to culture under laboratory conditions. It has been used in toxicity studies as test organism for a long time (Rodriguez & Reynoldson, 2011), its relevance have been pointed out by Chapman (2001), and there are standardized test methods useful for research on the effects of hazardous chemicals (ASTM, 2005; OECD, 2008).

The interdisciplinary science that relates the molecular toxicology, pathology and functional genomic is known as toxicogenomic. Its main goal is to detect changes in global gene expression (transcriptomics) due to the exposure of a test organism to hazardous chemicals and its relationship with toxicological endpoints (Uehara, 2013). Therefore, transcriptomics is the study of the transcriptome, the total set of RNA transcripts produced under specific circumstances in a cell, and its study provides information about gene expression at the tissue level (Chavan-Gautam *et al*., 2017). To date, there is scarce information available on this species or other aquatic oligochaetes in genomic databases, and most data are referred to studies with earthworms (e.g. Brulle *et al*., 2006 for exposure to Cadmium; Novo *et al*., 2020 for exposure to Zinc).

The aim of this study was: (1) to assess the genetic mechanisms of action of the arsenic in relation to exposures producing an acute toxic effect; (2) to evaluate the importance of *T. tubifex* as a model species for assessing to toxicogenomic of As in aquatic environments; (3) to study the gene expression related to the relevant routes related to the survival of the worms. In the present study, and for the first time in *T. tubifex*, we have identified and characterized genes related to cell stress response (*Hsp83*, *Hsp60*, *Hsp10*, *Hsc70*), detoxification and oxidative stress (*MnSOD*, *CAT*, *GSR*, *Col*) and genes involved in homeostasis of organisms (*UBE2, CaM* and *RpS13*). This work was conducted to describe potential biomarkers of toxicity and to assess their usefulness as early biomarkers of arsenic effect in this species.

2. MATERIAL AND METHODS

2.1. Water-only acute toxicity test for As^{5+}

An acute water-only toxicity test was run with Sodium arsenate (As^{5+}) dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$, Molecular Weight: 312.01 g mol^{-1}), using the oligochaete annelid *T. tubifex* from the laboratory culture stock as test organism. Arsenate (As^{5+}) is the chemical species that typically predominate in well-oxygenated waters and sediments (Chatterjee *et al*., 2017). The culture was kept at 22 ± 1°C, in the dark, with gentle aeration, and with about 3-cm sediment layer, sieved through a 250-µm mesh size, covered by dechlorinated tap water (more details in Méndez-Fernández *et al*., 2013).

The acute toxicity test was performed according to the methodology proposed by Maestre *et al.* (2009). The first step consisted in the preparation of a 1000 mg l^{-1} stock solution using an ACS (American Chemical Society) reagent metal salt ($Na_2HAsO_4 \cdot 7H_2O$) from which a serial dilution was made for the acute toxicity test. The test was performed into an incubator at 22.0 ± 1°C, in the dark, for 96 hours and without aeration or food addition. The toxicity test included six increasing nominal test concentrations of Na_2HAsO_4 (96 mg l^{-1}, 153 mg l^{-1}, 244 mg l^{-1}, 390 mg l^{-1}, 625 mg l^{-1} and 1000 mg l^{-1}) and a control series with only the dilution water (demineralized tap

water). Real concentrations are given in Table 1. Five replicates were used for each test concentration.

Table 1. Initial Na_2HAsO_4 nominal concentration and As real concentration in the water column used in the water-only *T. tubifex* toxicity test (mg l^{-1}).

[Na_2HAsO_4] mg l^{-1}	Control	96	153	244	390	625	1000
[As] mg l^{-1}	0.00	38	63	100	159	250	407

The test was run in 250-ml glass beakers, pre-washed in acid solution (10% HNO_3), and with 100 ml of test solution in each beaker. Five individuals of *T. tubifex* were added per beaker. The worms used in this experiment were of 5-week-old immatures of similar size. Before placing the worms (a total of 175 individuals) into the beakers, they were distributed in Petri dishes in groups of 5 individuals with dechlorinated water to let them purge their guts during 4-5 hours at room temperature (Martínez-Madrid *et al.,* 1999). After that period, each group of worms was randomly distributed into the test beakers, and then covered with Parafilm® to prevent water loss. Before adding the oligochaetes to the beakers, a sample of 7 ml of each solution was taken and preserved with 20-µl nitric acid (70% Baker Instra-Analyzed) for chemical analysis by SGIKER laboratory (UPV/EHU). A subsample of 20 individuals was also separated from the same culture batch and purged for 5 hours to estimate the mean initial biomass of the test worms, in dry weight. These worms were dried at 50°C (for 24 h) to constant weight and, weighed individually in a Sartorius M3P electrobalance (detection limit, 1µg g^{-1}). The mean initial worm biomass was 0.767 ± 0.341 mg ww and 0.107 ± 0.049 mg dw.

The total exposure period of the toxicity test was 96 h, and every 24 h the mortality and the autotomy were recorded using a stereoscopic microscope. If a worm did not respond in 10 seconds after a disturbance with a bar, it was considered to be dead (APHA, 1989; Chapman *et al*., 1982). The water physical (pH, temperature) and chemical characteristics (dissolved oxygen, conductivity) were measured at the middle of the test (48 – 72 h). Mean values of the replicates for each concentration for pH ranged 7.76 – 8.47, the conductivity showed a gradient from the less to the highest concentration of 319 – 1377 µS cm^{-1} and the dissolved oxygen ranged 7.03 – 7.21 mg l^{-1} (% saturation: 84.0 – 86.7%).

After 24 h of exposure, one worm from each replicate, if still alive, was removed and was stored in 1-ml RNAlater™ Solution (Invitrogen by Thermo Fisher Scientific, Lithuania), at -80°C. At the end of the 96-h exposure, another worm from each replicate was stored in 1-ml RNAlater™ Solution, and the three remaining individuals from each replicate, if still alive, were frozen, freeze-dried, weighted (Sartorius M3P balance) and digested (70% Nitric Acid Baker Instra-Analyzed + 30% H_2O_2 Merck Suprapur) for tissue residue analysis (Clements & Kiffney, 1994). The metal analysis was conducted through ICP-MS (7500ce, Agilent Technologies. dl= 0.1 µg l^{-1} As). Reference material: NIST 2976 mussel tissue (TMDA 52.3 and NIST 1643e) at SGIker. All metal recoveries were within certified values. The remaining individuals in each replicate were fixed in 1-ml of RNAlater™ Solution (Invitrogen by Thermo Fisher Scientific, Lithuania), at -80 °C.

2.2. Gene expression study

2.2.1. Sequencing, *de novo* assembly and annotation of a reference transcriptome

A second water-only acute toxicity test with arsenic was performed using the same methodology as explained in 2.1, and after 96 h the surviving worms at each condition were stored in RNAlater™ (Invitrogen), for RNAseq. Three cDNA libraries were constructed by the Macrogen company following the Tru-Seq Stranded mRNA (Illumina, USA) protocol and sequenced on an Illumina Hi-Seq 4000 using a 100 cycles paired-ended protocol. For each sample, total RNA was extracted from pools of three worms using TRIzol Reagent (Invitrogen), following the manufacturer's instructions. RNA was then treated with DNase I (Invitrogen) and extracted with phenol:chloroform:isoamyl alcohol (Fluka, Germany) using 5PRIME Phase Lock Gel Light tubes (Quantabio, USA). Purified RNA was resuspended in nuclease-free water, quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Germany), and stored at −80 °C.

A reference transcriptome was assembled by integrating the RNA-seq reads from the three libraries obtained. Before assembly, the quality of the sequences was checked using FastQC v0.11.7 (Andrews, 2007), and reads with low-quality (Phred value < 33) and adaptor sequences from the raw data were removed using

Trimmomatic v0.32 (Bolger *et al*., 2014). The filtered reads were then de novo assembled into contigs with Trinity software (Grabherr *et al.*, 2011) using default parameters. All unigenes > 200 bp were searched using BLASTx against Metazoa with the following protein sequences databases: UNIPROT (v20170706), Kyoto Encyclopedia of Genes and Genomes (KEGG_v20170706) and GO (v20150407) (e-value < 10−5), to identify proteins with high sequence similarity, and to assign putative functional annotations. Subsequently, Gene Ontology (GO) annotations of the unigenes were obtained using Blast2GO (Conesa *et al*., 2005).

2.2.2 Gene characterization

Nucleotide sequences for relevant genes related to cell stress response, energy metabolism, oxidative stress response and homeostasis maintenance were obtained from our *T. tubifex de novo* transcriptome (data not published): *Hsp10* (heat shock protein 10), *Hsp60* (heat shock protein 60), *Hsc70-like* (heat shock cognate 70), *Hsp83* (heat shock protein 83), *CoI* (Cytochrome C oxidase), *MnSOD* (manganese superoxide dismutase), *CAT* (catalase), *GSR* (glutathione reductase), *CaM (Calmodulin), UBE2 (ubiquitin conjugating enzyme)* and *RpS13* (ribosomal protein S13). SnapGene ® (GSL Biotech), BLAST (Agarwala *et al*., 2018), and DOG 2.0 (Ren *et al*., 2009) software were used in the identification and characterization of these genes. Sequence alignments were performed with Clustal X Version 2 and MAFFT Version X. SnapGene (GSL Biotech LLC). The gene sequences are under registration process in the NCBI database and accession numbers will be available soon.

2.2.3. RNA extraction

The total RNA extraction from *T. tubifex* samples was carried out following the instructions of TRIzol® commercial kit (Invitrogen), consisting of a monophasic solution of phenol and guanidine isothiocyanate and suitable for isolating separate fractions of RNA, DNA and proteins from cells and tissues. The worms were extracted from the freezing vials (containing RNA Later) and transferred to 1.5-ml Eppendorf tubes, where they were homogenized in 200-µl TRIzol® and frozen in dry ice. Later, they were thawed in cold and centrifuged at 12000 rpm for 10 minutes, at 4°C, thus allowing the precipitation of cell membranes, polysaccharides, high molecular weight DNA and

extracellular material. The supernatant liquid was transferred to another 1.5-ml Eppendorf tube and 5 minutes were waited at room temperature. 40 µl of chloroform (200-µl chloroform / 1-ml TRIzol®) were added and the vials were shaken, letting them rest at room temperature for 3 minutes, after which they were centrifuged again at 12000 rpm for 15 minutes, at 4°C.

In this moment, the aqueous phase contained the RNA and the organic phase the DNA and proteins. The aqueous phase was transferred to another 1.5-ml Eppendorf tube, and there the genetic material was precipitated by adding 100 µl of isopropanol (0.5-ml isopropanol / 1-ml TRIzol®). Finally, the samples were washed 3 times by adding 200 µl of cold 70% ethanol (1-ml ethanol / 1-ml TRIzol®) and centrifuged at 10000 rpm for 5 minutes, at 4°C. The supernatant liquid (ethanol) was decanted and the precipitated RNA resuspended in 44.5 µl of Nuclease-free water (DEPC-Treated Water). The samples were place in the oven at 55–60°C, for 5 minutes.

To avoid the presence of DNA remains, the samples were treated with the enzyme DNase (RNase-free). To the 44.5 µl of sample, 0.5 µl of DNase and 5 µl of buffer were added to get a final volume of 50 µl. The mixture was incubated at 37°C, for 30 minutes. After the incubation period, 50 µl of phenol:chloroform:isoamyl were added to the samples, after which they were centrifuged at 10000 rpm for 15 minutes, at 4°C. The aqueous phase (100 µl) was transferred to a Phase Lock Gel column and 50 µl of chloroform were added. The tubes were shaken well by hand and centrifuged at 12000 rpm for 10 minutes, at 4°C. The supernatant liquid contained the RNA. It was transferred to another tube and stored at -80°C.

To determine the concentration of RNA in the samples, the absorption at 260 nm was measured in the spectrophotometer, and the RNA concentration measured in µg ml^{-1}. As indicators of good quality of the RNA samples, A260/280 optical density values between 1.80 and 2.00 and A260/230 values equal to or greater than 1.80 were considered. The integrity of the extracted RNA was checked by running 2 µl of RNA on a 1.5% agarose gel at 60 mV.

2.2.4. Gene expression analysis. Reverse transcription: obtaining cDNA

Five biological replicates with 1 worm per replicate were used for each experimental condition (n = 5 worm/condition) in the gene expression analyses. Aliquots containing 7 µg of isolated RNA were reverse-transcribed in an CFX96 Thermal Cycler (Bio-Rad, USA) using iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad), according to kit instructions. The obtained cDNA was conserved at -20 °C and used as a template for subsequent qPCR analyses.

The primers used for the amplification of the selected genes were designed based on the *de novo T. tubifex* transcriptome obtained in our laboratory and using Primer 3 version 0.4.0 software (Untergasser *et al*., 2012). The sequences of the primers and the size of the amplified fragments are shown in Table 2. The size of the PCR products was checked in a 1,5% agarose gel at 85 V for 2 h in 1x TBE buffer (Tris-borate-EDTA), stained with ethidium bromide and visualized with Chemigenius3 (Syngene, USA). The identities of the amplified fragments were verified by Sanger sequencing and BLAST (Altschul *et al*., 1990). Amplification efficiencies and correlation coefficients for each primer pair were calculated as described in the Real-Time PCR Applications Guide (Bio-Rad catalog #170–9799). For all genes the efficiencies were between 90 % and 105 % ($R^2 > 0.980$).

Real-time quantitative PCR (qPCR) was performed using a QuantStudio 1 Real-Time PCR System (Applied Biosystem, USA) with the Power SYBR™ Green PCR Master Mix (Applied Biosystem, USA), according to the manufacturer's protocol. Each qPCR was conducted in a 10 µl mixture containing 1 µl of sample cDNA, 0.6 µl of each primer (10 µM), 4,8 µl of nuclease-free water, and 5 µl of 2x Power SYBR™ Green PCR Master Mix. The qPCR cycling parameters were as follows: 30 s initial denaturation at 95ºC, followed by 40 cycles of 5 s denaturation at 95ºC and 30 s annealing at 58ºC, with a final 30 s for elongation at 60ºC.

Table 2. Primers used for cDNA sequencing and Real Time qPCR of genes studied in *T. tubifex*. Forward (F) and reverse (R) sequences, length of amplified fragments and origin of primers when corresponds.

Gene	Description	Primer sequence (5'-3')	Fragment size (bp)
Hsp83	83 kDa heat-shock protein	**F** GAGCAGATGGAGGACGGAGA **R** CGAATCTTGTCGAGGGCATC	152
Hsp60	60 kDa heat-shock protein	**F** GGTCCAAGACATCGCACACA **R** GGCTTCCATCACACCTCGTC	151
Hsp10	10 kDa heat-shock protein	**F** AAAGGTGCTCGAAGCGACAG **R** ACGACTGGAATTTGCCGAGA	191
Hsc70	70 kDa heat-shock cognate protein	**F** ATGGACAAGAGCAGCATACACG **R** GAGCGACAGGGGAGTGACAT	228
CoI	Cytochrome oxidase I	**F** CAGGCGTATGCTTAGCAAATTCA **R** CCGAATACTGCCCCCATTCT	103
MnSOD	Manganese superoxide dismutase	**F** TGCCGAAGCACAGGCTAAA **R** CTCAAGCGAACCAAAGTCACG	184
Cat	Catalase	**F** GTGCTGAACCGTAGCCCAAA **R** ACGAGAACAGACGACCCTGAAG	124
Gsr	Glutathione reductase	**F** ACCGTTGTGTTCAGCCATCC **R** TTCTTTTGTGTCATTGCGTGGT	137
UBE2	Ubiquitin-conjugating enzyme E2	**F** CGTCTGCTTGTGGTGGTGAC **R** TCGTTGTCCATCTCGTCGTAATAAT	118
CaM	Calmoduline	**F** AAGGAACTGGGGACCGTGAT **R** TCAGGAACTCGGGAAAGTCTATTG	121
RpS13	Ribosomal protein S13	**F** TTGGCGTTATTCTGCGTGACT **R** TTGTCCTTGCGGTTCCTCTC	178
gapdh	Glyceraldehyde 3-phosphate dehydrogenase	**F** GGTATTTCATTGAATGATCACTTTG **R** TAATCCTTGGATTGCATGTACTTG	103 (Herrero *et al.*, 2017)
26S	26S ribosomal ribonucleic acid	**F** TTCGCGACCTCAACTCATGT **R** CCGCATTCAAGCTGGACTTA	220 (Planelló *et al.*, 2011)

Genes encoding the 26S ribosomal subunit and GAPDH were used as endogenous reference genes. Fragments of these genes were amplified using the same pair of primers designed for ecotoxicity studies in *C. riparius* (Herrero *et al.*, 2017; Planelló *et al.*, 2011), and their identities were confirmed by Sanger sequencing

(STABvida company). The statistical validation of the stability of the reference genes was performed by means of real time PCR QuantStudio 1 (Applied Biosystems), using an iterative test for pairwise variation, according to Vandesompele *et al.* (2002). The $2^{-\Delta\Delta Ct}$ method was used to analyze relative changes in gene expression with Data and Analysis software (Thermofisher). For each experimental condition studied, five biological replicates were analysed and three technical replicates were carried out. Gene expression results were normalized to the control values for subsequent analysis.

2.3. Statistical Analyses

The NOEC (no-observed effect concentration) and the LOEC (lowest-observed effect concentration) were estimated through the Kruskal-Wallis non-parametric test and the Dunn's test, as post-hoc pairwise comparison test (using IBM®SPSS® Statistics 24 software).

Median effect concentrations and 95% confidence limits (LC_{50}, EC_{50}, LR_{50} and ER_{50}, 95% CL) were estimated for autotomy and mortality at 24, 48, 72 and 96 hours of exposure adjusting data to the Probit model (using IBM®SPSS® Statistics 24). In few cases (data from 72 h and 96 h of exposure), data were smoothed. When it was not possible to adjust data to the Probit model for calculating the LC_{50}, EC_{50}, LR_{50} and ER_{50}, the Trimmed Spearman-Karber method was used (Hamilton *et al*., 1977).

For gene expression study, the statistical analyses were performed using R. 3.4.3 software (R Core Team, 2018). Mean and median were calculated respectively as the average and the middle of a data set, while the standard deviation represented the square root of the variance. Regarding the survival studies, the Student t was used to check the statistically significant differences ($p < 0.05$) between the different experimental conditions. For transcriptional analyses, the normality and homoscedasticity of the data were checked with the Shapiro-Wilk and Levene tests, respectively. Normal and homoscedastic data were analyzed by ANOVA followed by Bonferroni's post hoc test. Otherwise, differences in transcript levels were evaluated using the nonparametric Kruskal-Wallis test followed by Mann-Whitney-Wilcoxon post hoc test. p-value < 0.05 and p-value < 0.1 were used as cutoff for statistical significance.

3. RESULTS

3.1. Water-only acute toxicity test for As^{5+}

The 96-h only-water toxicity test was validated based on the physico-chemical conditions which ranged within values indicated by ASTM (2005); in the overlying water pH values were 6 – 9, and dissolved oxygen concentration was always above 2.5 mg l^{-1} (Supplementary Table S1). The control batch showed 96% of survival and autotomy of the posterior body region was not observed. No other effects were recorded in control organisms.

The raw results of the acute toxicity test are shown in Supplementary Table S2. Regarding to the mortality of the test, after 24h mortality started in the three highest concentrations. At the end of the 96 h exposure, there was a gradient of mortality and autotomy, except in the 2 lowest concentrations. The concentration of As related to the 50% mortality and autotomy was given by the parameters LC_{50} and EC_{50} respectively (Table 3). The As LC_{50} was 136.87 mg l^{-1} at the end of 96h exposure, and As EC_{50} levels were slightly lower regarding the autotomy.

Table 3. NOEC, LOEC and EC_{50} in *T. tubifex* (in mg l^{-1}) with their 95% confidence interval (95% CI), related to mortality and autotomy after 24, 48, 72 and 96 hours of exposure to As in a water-only toxicity test. * Trimmed Spearman-Karber method. Abbreviations: p, test probability of rejecting H_0; ns, no significant

	Mortality				Autotomy			
	24 h	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
NOEC	250	-	159	159	-	159	159	159
LOEC	407	ns	250	250	ns	250	250	250
(p)	(0.041)	-	(0.019)	(0.028)	-	(0.013)	(0.011)	(0.015)
LC50/ EC_{50}	>407	260	163	137 *	387	149	126	120
95% CI	-	210–349	135–199	136–137	280–718	120–188	101–155	96–148

The Table 4 shows the As tissue concentrations related to the 50% of the maximum mortality (LR_{50}) and autotomy (ER_{50}). The mortality reached a 50% of its maximum at 1072 µg As g^{-1} dw in the first 24 hours of exposure (the maximum tissue residue attained in the experiment was 1083 µg g^{-1} dw). This concentration decreased in time until 578 µg g^{-1} dw at the end of the toxicity test. The tissue concentrations related to the autotomy were only a little smaller.

Table 4. Effective tissue residues (in µg g^{-1} dw) in *T. tubifex*, LR_{50} (mortality) and ER_{50} (autotomy) and their 95% confidence interval (95% CI) related to mortality and autotomy, after 24, 48, 72 and 96 hours of exposure to As in a water-only toxicity test,. * Trimmed Spearman-Karber method.

	Mortality				Autotomy			
	24 h	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
LR_{50} / ER_{50}	1072	-	679*	578*	1019	634*	555*	479*
95% CI	891–1502	-	671–687	574–582	795–1623	630–638	553–557	477–481

3.2. Gene characterization

An extensive transcriptome of *T. tubifex* was obtained from 5-week-old immature worms. All reads were deposited in the European Nucleotide Archive (ENA) project number PRJEB50858. A systematic search in *T. tubifex de novo* transcriptome rendered sequences with open reading frames (ORF) for their corresponding proteins. Seven sequences with the complete ORF (*Hsp10, Hsp60, CaM, CAT, MnSOD, GSR* and *RpS13*) and four incomplete sequences (*Hsp83, Hsc70-like, UBE2 and CoI*) were obtained. Table S3 shows the gene bank accession numbers, the lengths of ORFs, and the closest match in the database. Relevant domains of each ORF are presented in Figure 1.

Four nucleotide sequences encoding different heat shock proteins were identified, three ORFs were complete (Figure 1A-C) and one was incomplete (Figure 1D). The first HSP complete ORF was 101 aa in length corresponding to *Hsp10* which contains a chaperonin 10Kd subunit domain covering nearly all the protein (Figure 1A). The second complete ORF was a 1767 bp sequence encoding a protein with 588 residues and a highly conserved heat shock protein 60 domain; it corresponds to *Hsp60* gene (Figure 1B). The incomplete ORF for *Hsc70-like* was 1692 bp and encoded a 563 aa sequence corresponding to the 3´end of the protein (Figure 1C). Finally, the incomplete ORF had 840 pb and encoded a 280 aa sequence corresponding to the 5’ end of the protein *Hsp83* (Figure 1D).

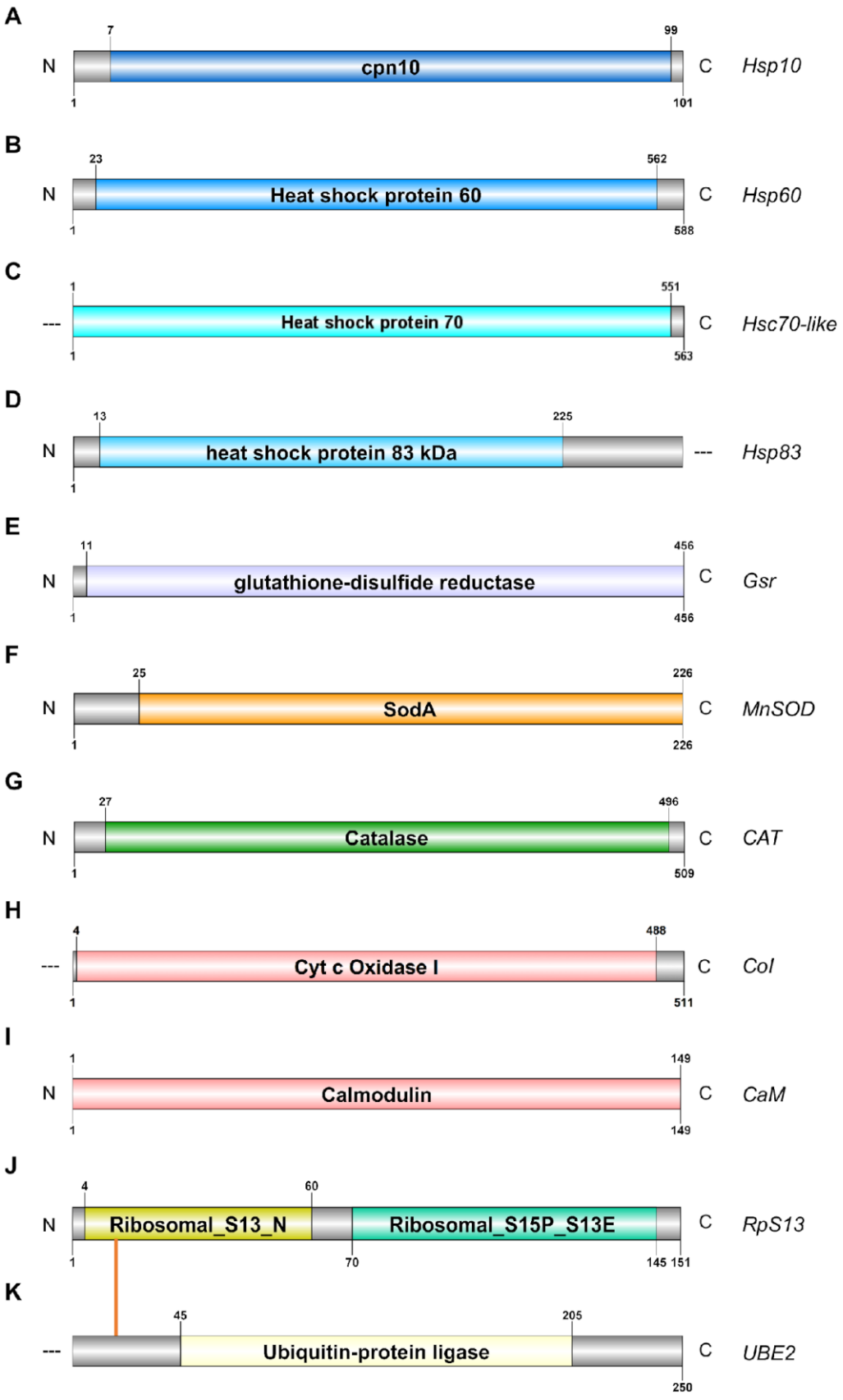


Figure 1. Characterization of proteins identified in the de novo transcriptome of *T. tubifex*. Diagram of the protein of *T. tubifex* identified as putative mRNAs and their conserved domains. Diagram designed with DOG V.2 software.

The complete ORF for *GSR* was 1371 bp that encoded a protein of 456 aa in length and contained a characteristic glutathione-disulfide reductase domain (Figure 1E). The complete ORF of *MnSOD* was coded by a DNA of 681 bp and it had a length of 226 aa (Figure 1F). The protein had a superoxide dismutase domain, and it shared a 69 % and 63 % of identity to the corresponding protein from *Eisenia Andrei* and *Perinereis nuntia* respectively (Table S3). The *CAT* ORF was 509 aa in length with a conserved catalase domain from residues 27 to 496 (Figure 1G). The protein shared more than 70% of identity to catalase gene from annelids species such as *Eisenia fetida* or *Perinereis aibuhitensis* (Table S3). The incomplete ORF for Cytochrome c oxidase I enzyme (COI) covered a region of 411 aa of the C-terminal and it had a cytochrome c oxidase I domain (Figure 1H).

The ORF for *CaM* gene was 450 bp encoding a protein with 149 aa corresponding entirely to a calmodulin domain (Figure 1I), it shared 100 % and 99 % identity with *Lumbricus rubellus* and *Hydroides elegans* respectively (Table S3). The ORF for *RPS13* was 456bp in length, encoding a protein with 151 aa residues (Figure 1J); it shared 91 % identity with *Arenicola marina* and 88 % with *Lumbricus rubellus* (Table S3). The last incomplete ORF encodes for ubiquitin conjugating enzyme (UBE2) and covered a region of 250 aa of the C-terminal and it had a ubiquitin-protein ligase domain (Figure 1K).

3.3. Gene expression study

The transcriptional profile of different genes of interest under the selected experimental conditions was analyzed through quantitative real time PCR. For this purpose, *T. tubifex* adults exposed to 38, 63, 100 and 159 mg l^{-1} As^{5+} in acute (after 96-h exposure) toxicity studies were used.

3.3.1. Cell stress response

From the four molecular cell stress-involved analyzed genes, the most striking differences compared to control were those related to *Hsp83* and *Hsp60*, as arsenic exposures led to a significant induction of transcriptional expression of both genes after the highest doses comparing to control. The upregulation was stronger for the *Hsp83*, with up to 7.3 and 6.7 folds after 100 mg l^{-1} and 159 mg l^{-1}, respectively ($p <$

0.05) (Figure 2A, B). Regarding the inducible mitochondrial HSP10, transcript levels of the *Hsp10* gene triggered a trend to increase, especially after 100 mg/L and 159 mg l^{-1} (up to 1.8 and 2.4 folds above control values, respectively), although with no statistical significance (Figure 2C). Finally, as expected, as the constitutive form of all the cell stress analyzed genes, *Hsc70-like* gene activity remained stable after arsenic exposures (Figure 2D).

3.3.2. Energy metabolism and oxidative stress biomarkers

The most acute statistically significant alterations were found for *CoI* and *MnSOD* transcript levels after 96 h in a different manner. The expression of *CoI* fell down 61%-45%, respectively, below control values (Figure 2E). In contrast, a clear time-dependent upregulation was observed for *MnSOD* with statistically significant changes after 96 h, after exposures to 100 and 159 mg l^{-1} As (Figure 2F). No significant changes were detected for the other two studied genes, *Cat* and *Gsr*. The same tendency was observed for both genes, with a slight induction at 38, 63 and 159 mg l^{-1}, and values of expression below control at 100 mg l^{-1} As (ns; $p > 0.05$) (Figure 2G, H).

3.3.3. Transcriptional alterations in homeostasis-related biomarkers

A dose-dependent repression of *UBE2* gene was observed after exposure to As, by 51% at 63 mg l^{-1}, 42 % at 100 mg l^{-1}, and 36 % at 159 mg l^{-1} ($p < 0.05$) (Figure 2I). Similar response was detected in *CaM* gene, whose expression fell down 29%, 40% and 23%, respectively, below control values (Figure 2J). Finally, although no significant, gene coding to small ribosomal protein subunit S13 was also dose-dependent reduced with values of about 33–40% below the control, under the highest concentration of arsenic tested (Figure 2K).

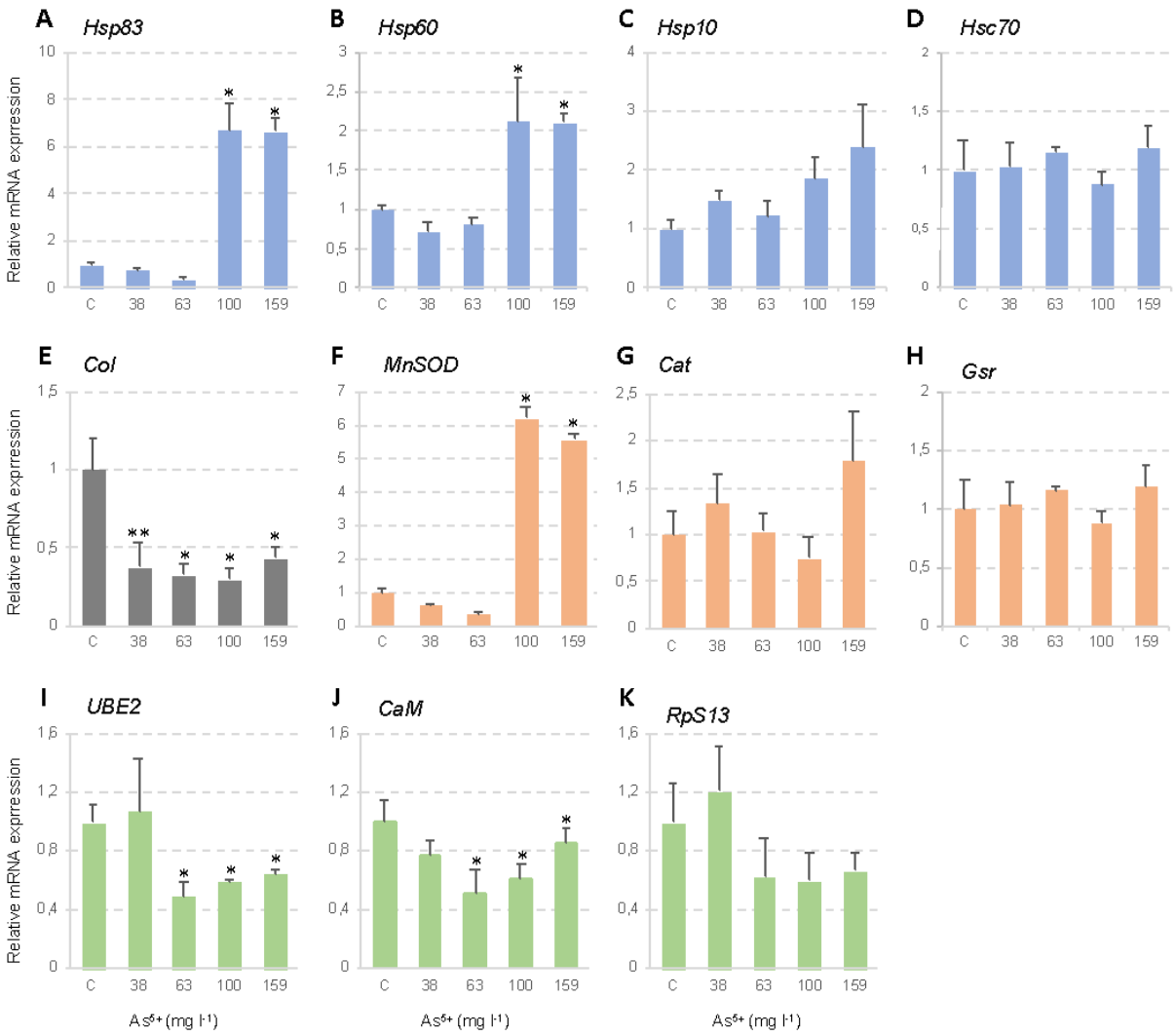


Figure 2. Transcriptional activity of genes related to cell stress response, oxidative stress response and homeostasis in *T. tubifex* exposed 96 h to 38 mg l^{-1}, 63 mg l^{-1}, 100 mg l^{-1} and 159 mg l^{-1} arsenic. Error bars indicate SE. Normalization was done using *gapdh* and *26S* as the reference genes. Bars represent the relative mRNA expression of *Hsp83* (A), *Hsp60* (B), *Hsp10* (C) and *Hsc70-like* (D), *Col* (E), *MnSOD* (F), *Cat* (G) and *Gsr* (H), *UBE2* (I), *CaM* (J) and *RpS13* (K) measured by real-time RT-PCR. Asterisks indicate significant differences with respect to control (C) values: $p < 0.05$ (*); $p < 0.1$ (**).

4. DISCUSSION

Soil and groundwater contamination with this metalloid, has become a major concern in areas where there are tailings and wastes from some mining activities. Arsenic (As) is recognized as a toxic metalloid and a severe threat to biodiversity due to its contamination in the freshwater ecosystems, such as death and malformations of toad embryos, growth inhibition of algae, or mortality of amphipods and gastropods (Eisler, 2004).

Previous studies on the water-only acute toxicity of *T. tubifex* exposed to As reported comparable results to the present study. Thus, 96 h-LC_{50} estimated by Fargasova (1994) and Lobo *et al.* (2016) were 127 mg l^{-1} and >118.18 mg l^{-1}, respectively. The EC_{50} estimated here for autotomy were very similar, although slightly lower, than those estimated from lethal effects. On the contrary, Khangarot (1991) proposed a LR_{50} that is much lower (8.9 mg l^{-1}), compared with the above-mentioned data. These discrepancies could be due to the sensitivity of the test organisms (e.g., age or culture characteristics), but also to the use of different test conditions (30°C). In fact, metal toxicity has been demonstrated to increase with temperature (Rathore & Khangarot, 2002; Wang, 1987).

There are no previous studies on As effective tissue residues in *T. tubifex* from acute toxicity tests for comparison. In present study, differences between estimated LR_{50} and ER_{50} for mortality and autotomy were greater through exposure time (95% confidence limits did not overlap at 72h and 96 h of exposure). It is interesting that the LR_{50} and ER_{50} are comparable with those estimated in *T. tubifex* from the 28-day sediment toxicity test (1,002 ± 55 µg g^{-1} dw: Lobo *et al*., 2021; 1,191.2 ± 151.3 µg g^{-1} dw: Méndez-Fernández *et al*., 2015). These results showed that the LR_{50} and ER_{50} calculated from acute water-only exposure can give a good approximation to the tissue residues after a sediment chronic exposure. The results are also comparable with a field approach for the estimation of tissue residues ER_{50} for aquatic Microdrile oligochaetes (ER_{50}= 509–570 µg g^{-1} dw: Moreno-Ocio *et al*., 2022: Chapter II) related to the 50% reduction of the river macroinvertebrate ecological status. Interestingly, that range of ER_{50} values from field aquatic oligochaete worms overlaps, or it is very close, the ER_{50} values estimated in present study for mortality and autotomy after 72 h and 96 h exposure, which suggests that the molecular information studied can be expected to be also useful in a more realistic scenario, in the field aquatic oligochaetes.

Analysis of genomic information enables identifying novel target genes to deep in the knowledges of effects of pollutants in exposed biota. This traditionally occurs through genomic, transcriptomic, and metabolomic analyses of model organisms; however, there is little genomic information available for many, including the aquatic oligochaeta *Tubifex tubifex*.

In this work, we have built a *de novo* transcriptome (Ref. PRJEB50858) of individual of *T. tubifex* from a laboratory culture (UPV, Bilbao, Spain). Besides, to assess their response as biomarkers of exposure to arsenic, we have identified and characterized for the first time in this species genes related to cell stress response (*Hsp83, Hsp60, Hsp10, Hsc70*), oxidative stress response (*MnSOD, CAT, GSR*), the energy metabolism (*CoI*) and genes involved in homeostasis of organisms (*UBE2*, *CaM*, *RpS13*). These data provide the scientific community new potential biomarkers that could help in the assessment of early toxic effects of environmental stressor.

Our bioassays proved the impact of arsenic in terms of transcriptional alterations in *T. tubifex*. The exposure to As resulted in the overexpression of cell stress response markers (i.e., *Hsp83, Hsp60*); the alteration of the oxidative stress response *(MnSOD*), the mitochondrial metabolism (*CoI*) also in relevant genes for the maintenance of homeostasis (*UBE2*, *CaM, RpS13*).

Two HSPs genes (Hsp83 and Hsp60) presented weak variations in the level of messenger after 96 h of exposure to 100 mg l^{-1} and 159 mg l^{-1} of As. An increase in the quantity of HSPs proteins after a metal exposure has been previously described in in *L. terrestris* and in *E. fetida* (Nadeau *et al*., 2001; Homa *et al*., 2005). Our results reinforce the idea of HSP as suitable biomarkers of metal exposure.

Cytochrome c oxidase is the terminal electron acceptor of the mitochondrial electron transport chain, catalysing the oxidation of ferrocytochrome c (Srinivasan & Avadhani, 2012). Cytochrome oxidase, which is recognized as an iron-containing enzyme, is necessary for oxidative phosphorylation and, therefore, aerobic energy generation. This enzyme acts in the mitochondrial electron transport chain, converting catabolic glucose products into adenosine triphosphate (ATP). Our results showed the dose-dependent repression of *CoI* transcriptional activity of up to 63%, 62% and 52% below values in control samples, after exposure to 63, 100 and 159 mg l^{-1} of As. The inhibition of *CoI* enzyme activity was described by other metals such as Cd^{2+} and Zn^{2+} in *Rhodobacter sphaeroides*, leading to a slow and less efficiency in proton pumping (Mills *et al*., 2002). The impact of the inhibition of *CoI* might include energy crisis due to lower ATP production, and increased formation of ROS in mitochondria, as described Srinivasan, & Avadhani (2012). Arsenite was also shown to diminish the

function of electron transport chain and, disrupt pyruvate metabolism in nematodes and human cells (Zhao *et al*., 2013; Luz *et al*., 2016). Our data suggest that arsenic could repress proton movement through a proton exit path, which can impair, in terms of regulation, the efficiency of energy transduction in mitochondria.

Antioxidant enzymes are explicit biomarkers of oxidative stress that can decimate ROS species and other cell pro-oxidative enzymes (Ajima *et al*., 2017). The oxidative stress enzyme that provides major defense against oxidative stress by converting reactive oxygen radicals to H_2O_2 by the process of dismutation is SOD (El Hajam *et al*., 2020; Kim *et al*., 2019). SOD helps to facilitate the transformation of superoxide anion radicals to hydrogen peroxide (H_2O_2). In the present study, SOD activity increased significantly by 6-folds and 5-folds after 96h in the concentration of As (100 mg l^{-1} and 159 mg l^{-1}, respectively) as compared to control. This spike in SOD activity could be due to superoxide ion induction, which activates biosynthesis of SOD, which bulwarks cells against oxidative damage, as described in *E. fetida* (Zhang *et al*., 2013). SOD activity levels also increased in *T. tubifex* worms exposed to $HgCl_2$ (Widiastuti *et al.*, 2019). The increased SOD activity in the *T.tubifex* might be a compensation mechanism against arsenic intoxication.

Calmodulin (*CaM*) is a versatile Ca(2+)-sensor/transducer protein that modulates hundreds of enzymes, channels, transport systems, transcription factors, adaptors and other structural proteins, controlling in this manner multiple cellular functions. Our work described a significant dose-dependent repression of *CaM* after exposure to 63, 100 and 159 mg l^{-1} of As. Ca2+/CaM is responsible of the activation of CaMKII, the most abundant multifunctional protein in neurons, which initiates the biochemical cascade, thereby facilitating synaptic transmission and synaptic plasticity. Arsenic in astrocytes might impair synaptic formation through disturbing astrocytic effects on neuronal signal transduction (Wang *et al*., 2013). Expression of CaMKII was decreased in mice exposed to arsenic, leading to the induction of damage in the structure of the hippocampus and to impairment of learning and memory (Wang *et al*., 2018). According to that, our data suggest that oxidative stress might perturb neuronal function via a decreased calmodulin levels and, consequently the ability to bind, activate, and regulate the interactions of CaMKII (Robison *et al*., 2007).

The ubiquitin-proteasome pathway (UPP) is the central protein degradation system in eukaryotic cells, playing a key role in homeostasis maintenance through proteolysis of regulatory as well as misfolded (potentially harmful) proteins. For its purpose, the UPP intersects many cellular events, such as cell-cycle, apoptosis, cell survival, and DNA repair (Della Sala *et al*., 2018). The inhibition of proteasomal activity causes inclusion formation in neuronal and non-neuronal cells related to neurodegenerative diseases (Ardley *et al*., 2003). Although the molecular mechanism is unclear, there is strong evidence that implicates abnormal processing of a variety of cellular proteins via the ubiquitin/26S proteasomal system in neurodegenerative diseases development. The UBE2 repression observed after arsenic exposures in *T. tubifex* suggest that this compound might impair the UPP normal activity and as a consequence, perturbing neuronal function.

To date, only a few studies in invertebrates have focused on the nucleolar region as a potential target of exposure to xenobiotics, and data are particularly scarce in invertebrates. In *C. riparius* larvae, cadmium exposures led to a significant reduction of the 32S and the 45S rRNA precursors (Planelló *et al*., 2007). Additionally, the down-regulation of *RpL15* mRNA was also reported in *C. riparius* exposed to cadmiun and silver nanoparticles (Nair & Choi, 2011), and repression of variations in the expression profile of ribosomal protein genes (*RpL4, RpL11, and RpL13*) after acute 24-h and 48-h exposures to a wide range of BBP and DEHP doses (Herrero *et al*., 2015, 2017). Although not significant, our work shows a dose-dependent trend towards *RpS13* repression. An in-depth study of the nucleolar region in *T. tubifex* and the effects that arsenic might have on it is still necessary, to decipher the potential effects of this heavy metal on the ribosomal machinery synthesis.

5. CONCLUSIONS

The estimation of toxicological endpoints and tissue residues of As in a short-term aqueous exposure provided useful benchmarks to assess the As environmental hazardous concentration range for the aquatic oligochaete *T. tubifex*. Autotomy and

mortality of individuals were found at similar As concentrations at the first hours of exposure, being the difference between both endpoints higher at the end of the test.

The transcriptome that we have characterized represents a valuable genomic resource for screening potential gene targets in *T. tubifex*. Eleven novel genes related to cell stress response, oxidative stress, energy production and cell homeostasis were *de novo* identified and showed differential expression in *T. tubifex* after exposure to different concentration of As, proving to be effective potential biomarkers at the molecular level to better understand the physiological response of these worms to this heavy metal in sediments. Arsenic resulted in the repression of genes related to mitochondrial energy metabolism, ribosome biosynthesis, cell homeostasis and ubiquitin-proteasome system. The induction of the response to oxidative stress and the cell stress response mediated by HSPs was also remarkable.

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SUPPLEMENTARY MATERIAL

Table S1. Mean values of the temperature, pH, conductivity and dissolved oxygen in the water column of toxicity test beakers.

As concentration mg l^{-1}	**Temperature (°C)**	**pH**	**Conductivity** (µS cm^{-1})	**Dissolved Oxygen** %SAT	mg l^{1}
0.00	23.6	7.76	319	85.8	7.18
38	23.5	8.12	415	85.2	7.12
63	23.5	8.25	477	85.4	7.09
100	23.7	8.28	577	86.1	7.19
159	23.5	8.31	733	84.0	7.03
250	23.6	8.40	979	85.2	7.10
407	23.7	8.47	1377	86.7	7.21

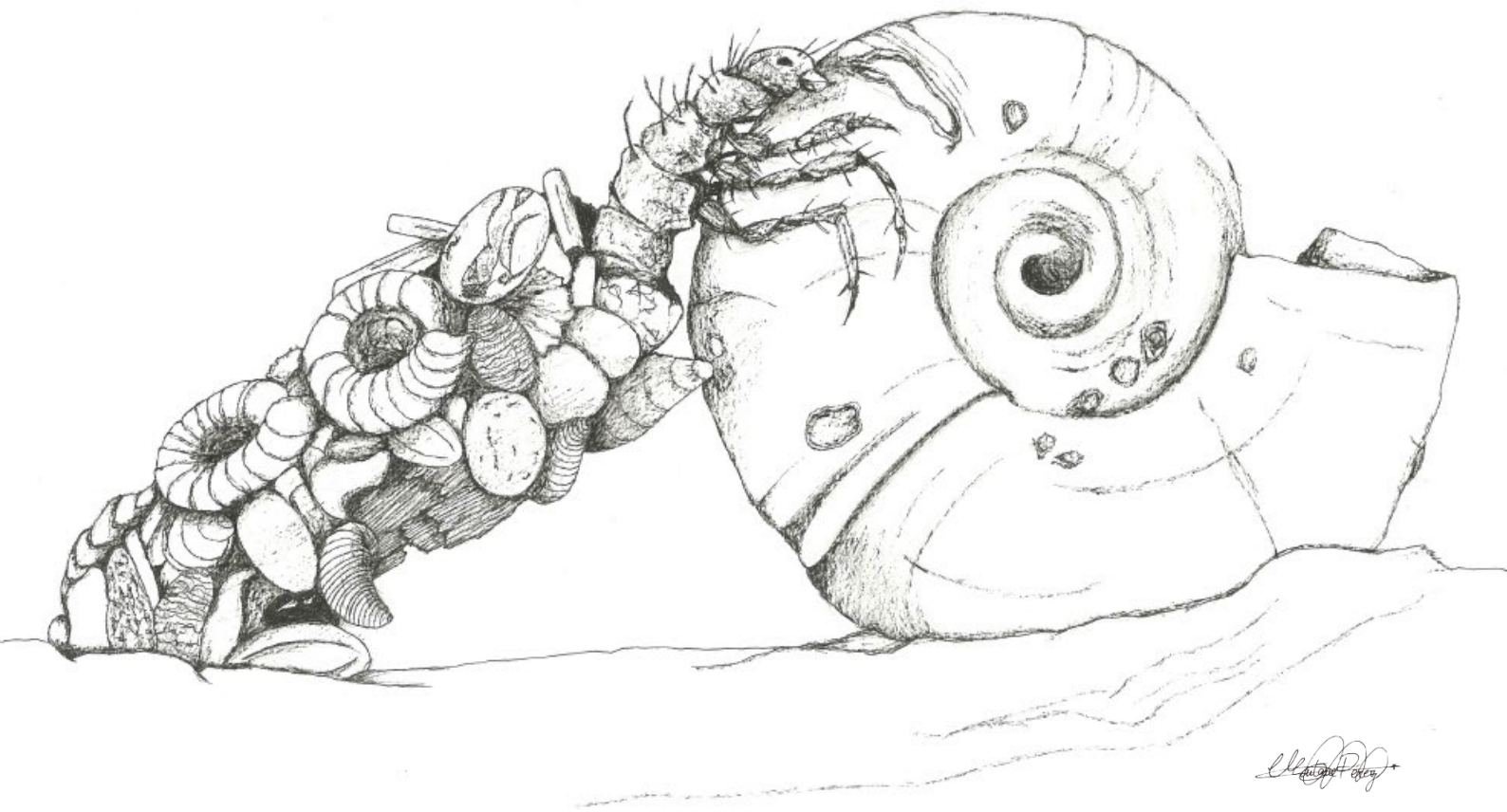
Table S2. Total mortality and autotomy data at 24, 48, 72 and 96 hours of As exposure, in the *T. tubifex water*-only toxicity test.

As concentration	**Mortality**				**Autotomy**			
mg l^{-1}	**24 h**	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
0.00	0	1	1	1	0	0	0	0
38	0	0	0	0	0	0	0	0
63	0	0	0	0	2	2	2	2
100	0	2	6	11	2	8	12	12
159	4	7	11	13	6	11	13	15
250	2	9	18	18	8	18	18	18
407	12	14	15	16	13	15	16	16

Table S3. *De novo* characterized *T. tubifex* genes related to cell stress response, detoxification, energy metabolism and homeostasis maintenance. Gene name, ORF and protein lengths of the *de novo* characterized *T. tubifex* genes as well as % of identity to closest species on databases.

Gene	Specie	Accession number	ORF length (aa)	Identity (%)
Hsp10	*Tubifex tubifex*	(under registration at BankIt)	101	-
	Crassostrea gigas	XP_011456448.1	101	76
	Argiope bruennichi	KAF8796450.1	101	75
Hsp60	*Tubifex tubifex*	(under registration at BankIt)	588	-
	Helobdella robusta	XP_009028388.1	584	78
	Biomphalaria glabrata	NP_001298236.1	571	75
Hsc70	*Tubifex tubifex*	(under registration at BankIt)	563 (incomplete)	-
	Urechis unicinctus	QAT77746.1	658	85
	Urechis caupo	AAA74394.1	658	85
Hsp83	*Tubifex tubifex*	(under registration at BankIt)	280 (Incomplete)	-
	Halyomorpha halys	XP_014284945.1	723	87
	Philodina roseola	ACC43981.1	739	80
GSR	*Tubifex tubifex*	(under registration at BankIt)	456	-
	Haliotis fulgens	AXR98615.1	452	65
	Pomacea canaliculata	XP_025078581.1	452	65
MnSOD	*Tubifex tubifex*	(under registration at BankIt)	226	-
	Eisenia andrei	AMZ80133.2	225	69
	Perinereis nuntia	AHE13889.1	224	63
CAT	*Tubifex tubifex*	(under registration at BankIt)	509	-
	Eisenia fetida	AEO50756.2	505	74
	Perinereis aibuhitensis	AHY86343.1	506	72
CoI	*Tubifex tubifex*	(under registration at BankIt)	511 (Incomplete)	-
	Limnodrilus hoffmeisteri	QWT71538.1	512	86
	Nais communis	QUT13265.1	512	81
CaM	*Tubifex tubifex*	(under registration at BankIt)	149	-
	Lumbricus rubellus	Q9GRJ1.3	149	100
	Hydroides elegans	AFI25239.1	149	99
RpS13	*Tubifex tubifex*	(under registration at BankIt)	151	-
	Arenicola marina	ABW23143.1	151	91
	Lumbricus rubellus	O77303.3	151	88
Ubiquitin	*Tubifex tubifex*	(under registration at BankIt)	250 (Incomplete)	-
	Branchiostoma floridae	XP_035678611.1	342	65
	Lingula anatina	XP_013421956.1	319	63

Conclusions



GENERAL CONCLUSIONS

1. The environmental risk assessment procedure in this research was based on a *reference condition approach* pointing toward the desired benchmarks concentrations in the field taxa nominated as representative of several functional traits in the benthic community. This step is crucial for estimating the deviation from the unimpaired, natural condition.

2. Criteria for the selection of a suitable set of relevant metals and macroinvertebrate taxa as biomonitors for the bioaccumulation risk assessment of a river basin are provided relying on the specific field conditions, considering relevant metals, wide distribution and functional traits of the taxa.

3. The assessment of different mixed-metal pollution situations was done through the provided methodological framework to develop an integrative tissue residue (INTISS) score, based on the selection of relevant metals and macroinvertebrate biomonitors, widely represented in the field at all levels of metal pollution situations. This score has assisted the identification of the pollution stressors potentially causing loss of ecological integrity in freshwater ecosystems. Tissue residues in the selected biomonitors have the potential to be used as early warning signals for metals that are likely to result in adverse effects for the macroinvertebrate community in the river basins of the Cantabrian region.

4. The proven cause-effect relationship between metal tissue residues and ecological status of the aquatic macroinvertebrate assemblages demonstrated the usefulness of the *tissue-residue approach* in the environmental risk assessment of the study area. Non-linear modeling of the environmental quality ratios (EQR) describing the macroinvertebrate ecological status conservation on a gradient of taxa tissue residues allowed for the calculation of environmental thresholds of metal bioaccumulation.

5. Environmental thresholds were calculated as the benchmarks to be used in a decision-making process of specifically designed biomonitoring and remediation programs for rivers affected by As, Au, Cu, Hg, Pb and Zn mining activities. Metal effective tissue residues were derived from dose-response models using the official cutoff of good/moderate ecological status of the macroinvertebrate assemblages (ER_{GM}). These tissue thresholds (ERs) were calculated for As, Cu, Hg and Se in ten biomonitor taxa and provided information on cause-effect relationships, based on metal bioavailability.

6. The taxon-specific metal thresholds estimated for 10 taxa in the Nalón River basin allowed for the estimation of the community hazard concentrations (HC_5 and HC_{50}) of As and Cu, based on species sensitivity distribution models. These hazard concentrations can be regarded as the low and high thresholds for the conservation of the macroinvertebrate assemblages at community level.

7. These thresholds were readily applicable in the gold mining district of the Cauxa River for a biota risk assessment. The same approach could be implemented in other European rivers to calculate threshold concentrations in the biota related to reductions in the metrics of ecological status. These thresholds aim to contribute to set future environmental quality standards for the protection of freshwater biota.

8. The proposal of new ecological status boundaries to protect the macroinvertebrate assemblages showed no significant changes on the taxa-specific effective tissue residues (ERs) or community hazard concentrations (HC_5 and HC_{50}). However, it is worth mentioning that the new tissue thresholds derived from the new ecological status boundaries will be more protective for the aquatic macroinvertebrate assemblages.

9. The decision-making process for the environmental risk assessment of rivers in Arditurri and Reocín mining districts involved three lines of evidence: sediment chemistry, sediment chronic toxicity and metal bioaccumulation in field macroinvertebrates. The sediment ecological thresholds proposed for Cd, Pb and Zn, calculated on a database of unpolluted and non-toxic sediments from northern Spain, have been proved a reliable tool for the sediment pollution assessment. Regarding these ecological thresholds, all the test sites in Arditurri and Reocín mining districts had medium to high pollution levels.

10. Ecotoxicity and bioaccumulation risk assessment in Arditurri and Reocín mining areas were evaluated by the effects measured in the *T. tubifex* sediment chronic bioassay and by the tissue residues measured in field macroinvertebrates. Data demonstrated that sediments, which were mainly polluted by Cd, Pb and Zn, can be hazardous for the benthic communities in a varying degree, due to ecotoxicity and bioaccumulation.

11. The effective tissue residues (ER) estimated through chronic bioassays performed with field sediments from Arditurri and Reocín mining districts in the laboratory were reliable for the Cd bioaccumulation risk assessment, since they were comparable to thresholds derived from field aquatic oligochaetes. However, the ERs calculated for Pb and Zn in the chronic bioassay were higher than the tissue residues ERs estimated from the field worms.

12. Combining the three lines of evidence to characterize risk in a weight of evidence framework, it is concluded that there is a significant deviation from the reference condition in the Arditurri and Reocín mining districts. Supplementary remediation plans should be implemented to reduce the alterations and attain a complete recovery of the ecological status in these water bodies. To monitor these plans, it will be desirable to include information on sediment chemistry and toxicity, the ecological status and the metal bioaccumulation of the *in situ* macroinvertebrates.

13. The *T. tubifex de novo* transcriptome was reconstructed and it represents a new valuable genomic resource for screening the potential of gene targets as stress biomarkers. Eleven genes related to cell stress response, oxidative stress, energy production and cell homeostasis were identified and showed differential expression in *T. tubifex* after a short exposure (96 hours) to different As concentrations.

14. Toxicogenomics could be used as early biomarkers at the molecular level to better understand the physiological responses of the worms to the stress, when exposed to As. The incorporation of differential gene expression in aquatic macroinvertebrates under different pollution scenarios in the field is a promising tool for future research on environmental risk assessment of heavy metals and metalloids.

THESIS

The capacity of aquatic ecosystems to maintain a healthy macroinvertebrate assemblage, measured in terms of composition, diversity and functional organization, was hampered due to pollution by metals and metalloids in the sediments and to their bioaccumulation by aquatic organisms.

Ecological thresholds for sediments proposed using a *reference condition approach* were reliable to assess pollution in mining areas of northern Spain. The integrative tissue residue (INTISS) scores and taxa-specific effective concentrations (ER) estimated for the selected metals were reliable in a risk assessment of metal bioaccumulation in mining areas of northern Spain. The benthic community hazard concentrations (HC) of As, Cu and Hg were useful tools as tissue concentration thresholds for the protection of the macroinvertebrate assemblages. Threshold concentrations derived from field-collected organisms or from ecotoxicity bioassays, and based on reliable effects are a necessary step to develop Environmental Quality Standards (EQS_{biota}), as required by the European water directives. The approach carried out in present study allows a definition of different threshold levels, above which the probability of adverse effects in specific taxa or at community level is low (low threshold) or high (high threshold).